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Lipo-oligosaccharide of the *Campylobacter lari* type strain ATCC 35221. Structure of the liberated oligosaccharide and an associated extracellular polysaccharide

Gerald O. Aspinall [a,*], Mario A. Monteiro [a], Henrianna Pang [b]

[a] *Department of Chemistry, York University, North York, Toronto, Ontario M3J 1P3, Canada*
[b] *Carbohydrate Research Centre, Department of Molecular Biology and Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A5, Canada*

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Abstract

Lipo-oligosaccharide (LOS) from phenol–water extraction of cells of the *Campylobacter lari* type strain (ATCC 35221) 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 liberated oligosaccharide and the extracellular polysaccharide, variously using ^1H, ^{13}C, and ^{31}P NMR spectroscopy, linkage analysis, and fast atom bombardment-mass spectrometry of permethylated derivatives of the glycans and their products of chemical and enzymic degradation. The following structures are proposed for the highly branched region of the LOS:

$$\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow3)\text{-}\alpha\text{-D-Gal}p\text{NAc-}(1\rightarrow3)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow3)\text{-L-}\alpha\text{-D-man-Hep}p\text{-}(1\overset{2}{\underset{1}{|}}\alpha\text{-D-Glc}p$$

* Corresponding author.

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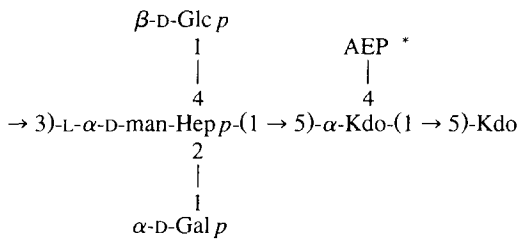


* AEP = 2-aminoethylphosphate

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

[- (PO_3^-) → 3)-β-D-Glc *p*NAc-(1 → 2)-6-d-α-L-*gul*-Hep *p*-(1 → 2)-3-d-β-D-*threo*-Pen *p*-(1 → 3)-6-d-α-L-*gul*-Hep *p*-]$_n$

Keywords: *Campylobacter lari* type strain (ATCC 35221); Lipo-oligosaccharide; Extracellular polysaccharide; Tetraglycosyl phosphate polymer

1. Introduction

Campylobacter species, especially *C. jejuni* and *C. coli*, are leading causes of human enteritis. Other species such as *C. lari* are of infrequent occurrence as human pathogens [1]. The discovery that *N*-acetylneuraminic acid (Neu5Ac) is a constituent of the core oligosaccharide regions of lipopolysaccharides (LPS) of low M_r of some of the more frequently encountered *C. jejuni* serotypes [2–6] had prompted an examination of LPS from other *Campylobacter* isolates for the presence or absence of this constituent as a possible virulence factor. One such isolate had been identified provisionally on the basis of biochemical tests as a strain (PC 637) of *C. lari*. As an aid to the confirmation of this strain's identity, parallel studies were undertaken of the LPS from this strain and from the *C. lari* type strain. Initial studies showed the absence of Neu5Ac as a constituent in both LPS preparations, but revealed the presence of unusual 6-deoxyheptose constituents which proved to be non-identical, but also different from the 6-deoxy-D-*altro*-heptose that had been characterized as a constituent of the O antigen chains from LPS of *C. jejuni* serotypes O:23 and O:36 [7]. The results of a structural investigation of the presumed LPS from the type strain showing the presence of a lipo-oligosaccharide and an associated polysaccharide are reported in this paper. The characterization of similar carbohydrate polymers, but with different structures, from the PC 637 strain is described in the preceding paper [8].

2. Experimental

Other than those listed below experimental methods were those described in the accompanying paper [8]. In addition, GLC separations were carried out on a capillary column with the following program: DB-23 (30 m × 0.25 mm) (F) isothermally at 150°C (10 min), 150–230°C (2°C/min), 230°C isothermally (20 min). 2D NMR experiments were performed with the following parameters: for $^1H-^{13}C$ HMQC–TOCSY

[512 × 1024 data matrix, mixing time of 35 ms, number of scans ranged from 64 to 128 per t_1 value]; for $^1H-^{13}C$ HMBC [512 × 1024 data matrix, 192 scans per $t_1$value]; for $^1H-^1H$ ROESY [1024 × 1024 data matrix, mixing time of 250 ms, phase sensitive using TPPI]. Digestion with exo-*N*-acetyl-β-D-glucosaminidase (Sigma) was carried out in 0.1 M sodium acetate buffer at pH 5.0 at 25°C for 12 h.

3. Results and discussion

Lipo-oligosaccharide (LOS) was isolated as a gel-like pellet by ultracentrifugation of the aqueous layer from the phenol–water extraction of bacterial cells. Mild acetic acid hydrolysis to cleave the ketosidic linkage to lipid A gave **OS1** which was fractionated on Bio-Gel P2, and compositional analysis gave Gal, Glc, LD-Hep [1], GlcNAc, and GalNAc in the approximate molar ratio of 2:2:1.4:1:1. As in other studies low values, as confirmed later, were obtained for Hep residues even though these carried no phosphate units. All sugars had the D-enantiomeric configuration as shown by the formation of acetylated chiral 2-butyl glycosides [9]. The presence of Kdo was detected through the formation of acetylated methyl ester methyl glycosides [10]. The strategy adopted in the structural characterization of the oligosaccharide region of the LOS and assigned structures for oligosaccharide (OS) derivatives are summarized in Fig. 1.

Composition, linkage, and sequence determination of core oligosaccharide.—The FAB-mass spectrum of permethylated **OS1** (Fig. 2) showed a pseudomolecular ion $[M + H]^+$ at *m/z* 2552 corresponding to a composition of $Hex_4Hep_2HexNAc_2Kdo_2$ and one *O*-(2-aminoethylphosphoryl) (AEP) substituent for the major component. A second pseudomolecular ion $[M + H]^+$ at *m/z* 2276 for a minor component corresponded to that of an oligosaccharide **OS1a** with only one Kdo residue but with an AEP substituent attached. The highly branched nature of the OS chain and the identities of the constituent units (other than for Kdo) were shown in the methylation linkage analysis (Table 1) with accompanying GLC-MS. Fragment ions in the FAB-mass spectrum established the sequence of residues in the OS backbone and allowed a structure to be proposed with partial assignment of linkages, but without specification of the three terminal hexopyranose residues or of the arrangement of the two Kdo residues, to one of which the AEP unit was attached (Fig. 3). ^1H NMR spectroscopy substantiated the presence of the two HexNAc residues from *N*-acetyl resonances at δ 1.97 and 1.98, and showed eight anomeric protons. The ^{13}C NMR spectrum (not shown) confirmed these features with seven resonances at δ_C 102.7, 102.5, 101.8, 100.2, 99.9, 99.2 (double intensity), and 96.3 for eight anomeric carbon resonances for two HexNAc residues at δ_C 54.4, 55.5 (C-2), 174.2, 174.1 (*C*O), and 22.5, 22.7 (CH_3), and for two Kdo residues at δ_C 173.0, 167.0 (*C*O), and 34.9, 34.5 (C-3). ^{31}P NMR showed a signal at δ 1.42 (pD 7.0) consistent with the presence of the AEP substituent as a phosphoric diester.

The anomeric protons (H-1) of each of the eight glycose residues served as the starting point in COSY, COSYRCT, and TOCSY experiments (Table 2) for the determination of connectivities and vicinal coupling constants in sufficient detail for

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

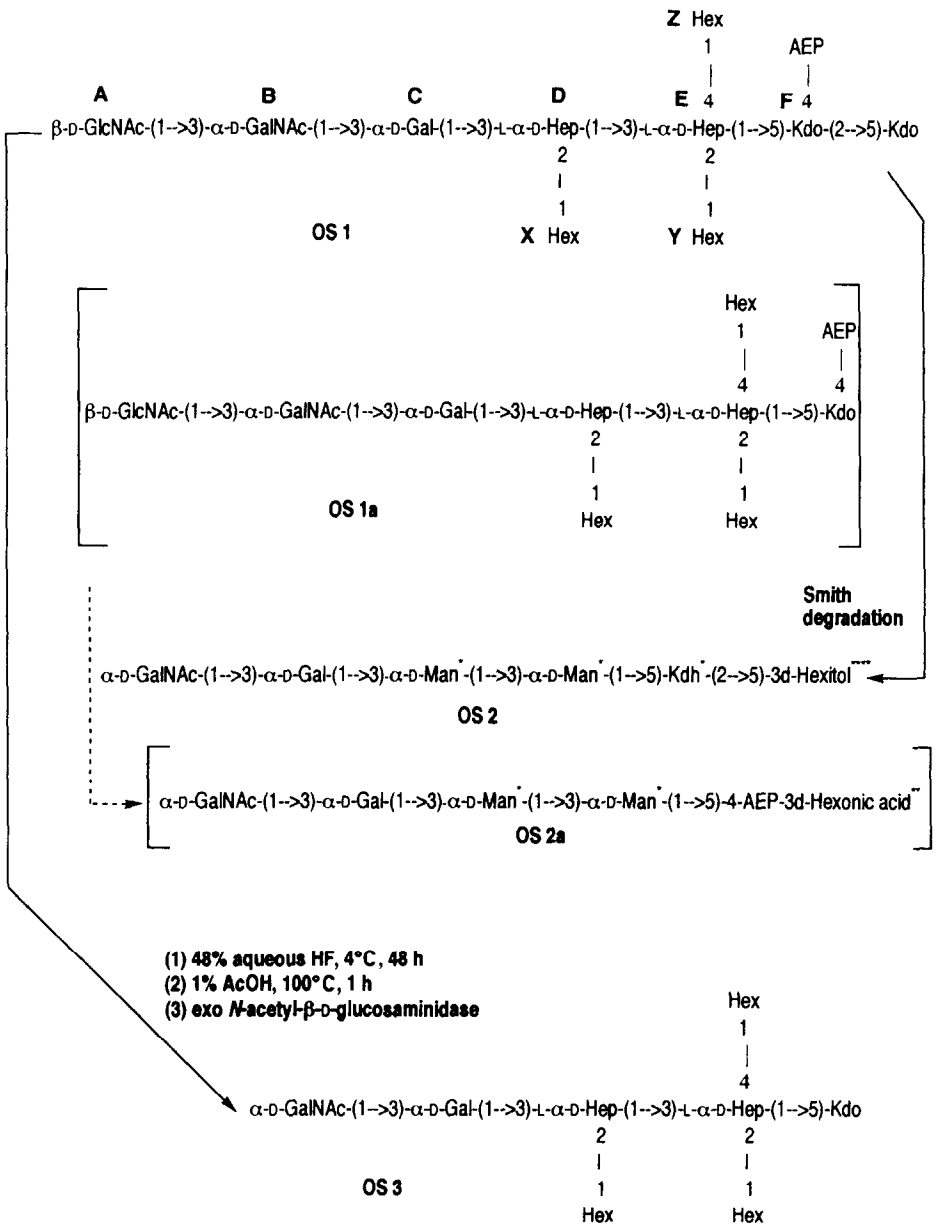


Fig. 1. Genesis of OS fractions from core **OS1** initially liberated from *C. lari* strain ATCC 35221 LPS. Kdh (cf. Kdo) = 3-deoxyheptulosonic acid.

assignments of ring configurations. The two anomeric resonances at δ 5.33 and 5.12 [bs = broad signal (unresolved d)] were characteristic of residues having the *α-manno* configuration and were assigned to the two LD-Hep residues. The anomeric resonances at δ 5.53 ($^3J_{H1,H2}$ 3.5 Hz), 5.15 ($^3J_{H1,H2}$ 3.7 Hz), and 5.01 ($^3J_{H1,H2}$ 3.8 Hz) were each

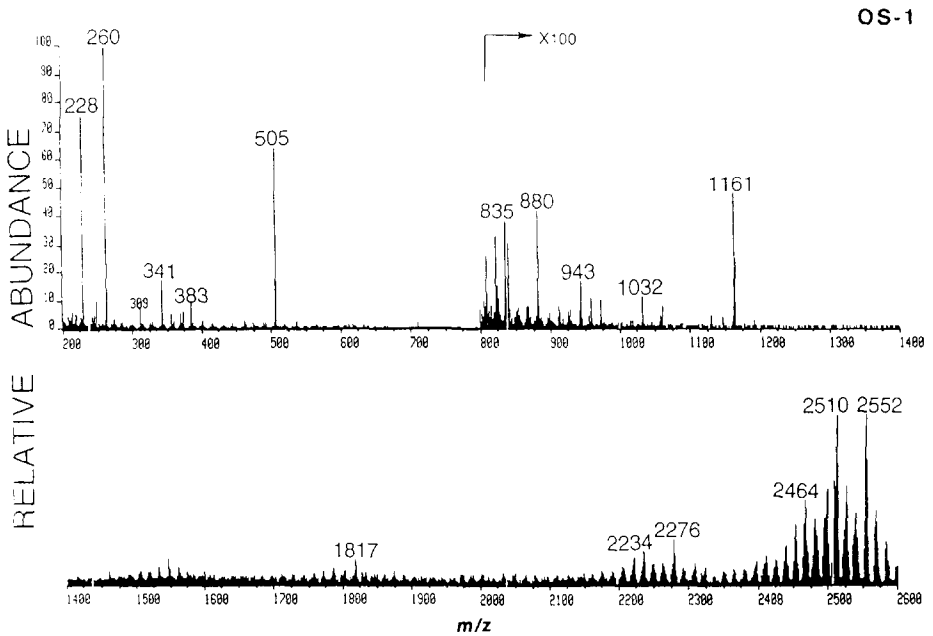


Fig. 2. Positive ion FABMS spectrum of permethylated derivative of **OS1** from the core oligosaccharide region of *C. lari* strain ATCC 35221 LPS.

assigned to residues with the *α-galacto* configuration on the basis of low $^{3}J_{H3,H4}$ values. The anomeric resonance at δ 5.46 ($^{3}J_{H1,H2}$ 3.8 Hz) in turn was assigned the *α-gluco* configuration on the basis of high $^{3}J_{H2,H3}$ and $^{3}J_{H3,H4}$ values. Similarly, resonances at δ 4.53 ($^{3}J_{H1,H2}$ 8.0 Hz) and 4.49 ($^{3}J_{H1,H2}$ 7.1 Hz) were assigned to residues with the

Table 1
Methylation linkage analysis of methylated core oligosaccharide derivatives [a] from the LPS of *C. lari* strain ATCC 35221

Methylated sugar (and linkage type)	Relative proportions in oligosaccharides [b]		
	OS1	**OS2**	**OS3**
2,3,4,6-Me_4Glc [Glc]	2		1
2,3,4,6-Me_4Gal [Gal]	1		2
3,4,6-Me_3N-MeGlcNAc [GlcNAc]	1		
3,4,6-Me_3N-MeGalNAc [GalNAc]		1	1
4,6-Me_2N-MeGalNAc [-3 GalNAc]	1		
2,4,6-Me_3Gal [-3 Gal]	1	1	1
2,4,6-Me_3Man* [c] [-3 Man*]		2	
4,6,7-Me_3Hep [branch]	1		1
6,7-Me_2Hep [d. branch]	1		1

[a] Core oligosaccharide derivatives are defined in Fig. 1.
[b] Unless otherwise stated in the text 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; derivatives of Kdo are not included.
[c] Man* = 6-[$^{2}H_1$]Man.

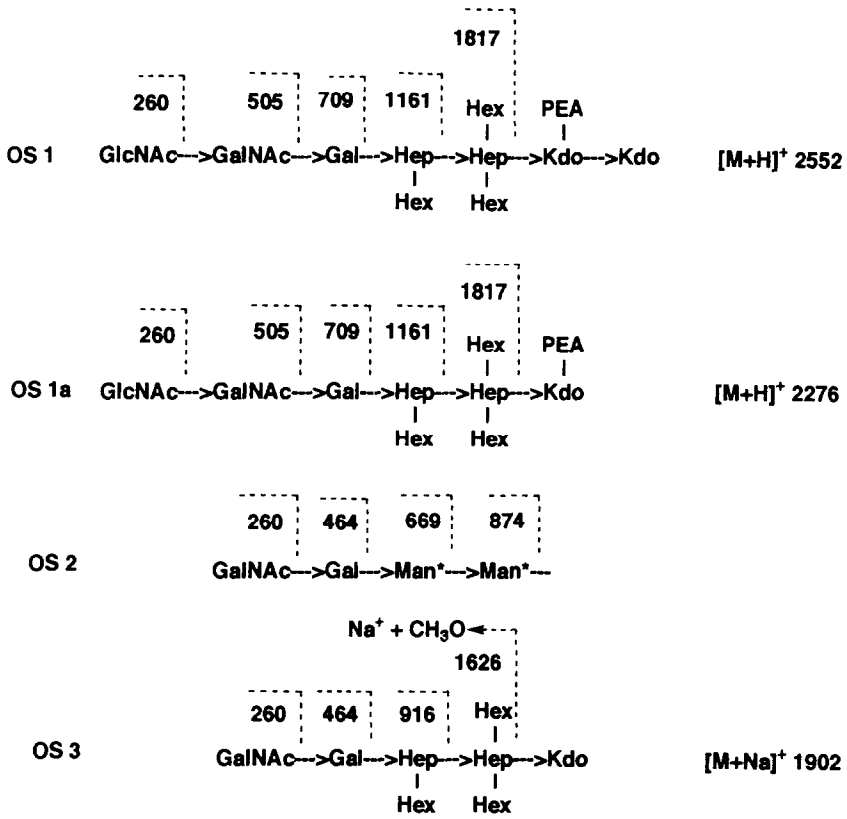


Fig. 3. Analysis of positive ion FABMS data for permethylated core OS derivatives from the core region of *C. lari* ATCC 35221 LPS. For the origins of derivatives see Fig. 1. 3-dHexitol = 3-deoxyhexitol. Asterisks show incorporation of the ^{2}H isotope.

β-gluco configuration. Assignments of proton resonances to individual residues in the partial structure for **OS1** (Fig. 3) required the generation of simpler oligosaccharides through specific degradations.

Smith degradation.—The sequence information for **OS1** from FABMS and methylation analysis data indicated that only the terminal residues would be vulnerable to endocyclic periodate oxidation and that the Smith degradation would be expected to yield an attenuated linear oligosaccharide. Core **OS1** was pre-reduced at the Kdo terminus with $NaBD_4$. The sequence of reactions of oxidation, reduction with $NaBD_4$, and controlled acid hydrolysis of acyclic acetals was followed by a further reduction ($NaBD_4$) of the modified Kdo residues able to undergo lactonization with O-4 [4,6]. The product [**OS2** with accompanying **OS2a**] was converted into the permethylated derivative for FABMS (Fig. 3) and linkage analysis (Table 1). These data established the sequence and linkage types for the surviving glycose residues in the outer tetrasaccharide segment as GalNAc-(1 → 3)-Gal (1 → 3)-Man*-(1 → 3)-Man*-(1-... with the last two ^{2}H-labelled residues (Man*) derived from attenuation of the LD-Hep residues. ^{1}H

Table 2
Selected ^{1}H NMR chemical shift (ppm) data and coupling constant ($J_{n,n+1}$) values [in parentheses (Hz)] for **OS1** from LPS of *C. lari* strain ATCC 35221

Residue [a]	Assigned ring configuration	H-1	H-2	H-3	H-4	H-5	H-6,6′
Y	α-Gal	5.53	3.91	3.93	4.18		
		(3.5)	(9.5)	(3.3)	(bs)		
X	α-Glc	5.46	3.86	3.63	3.42	4.15	3.80
		(3.8)	(9.2)	(10.0)			
E	α-Man [b] [of LD-Hep]	5.33	4.13	4.02	4.21		
		(bs)	(3.5)	(8.5)			
C	α-Gal [b]	5.15	4.50	4.05	4.17		
		(3.7)	(8.8)	(3.6)	(bs)		
D	α-Man [b] [of LD-Hep]	5.12	4.20	3.99	3.79		
		(bs)	(3.6)				
B	α-GalNAc [b]	5.01	4.29	4.07	4.20		
		(3.8)	(8.1)	(3.5)	(bs)		
A	β-GlcNAc [c]	4.53	3.64	3.49			
		(8.0)	(8.7)	(9.0)			
Z	β-Glc	4.49	3.67	3.46			
		(7.1)	(9.1)	(8.9)			
F	α-Kdo			1.93ax	4.17	4.24	
				2.07eq			

[a] Residues designated as in Fig. 1.
[b] Residues not undergoing endocyclic cleavage in the Smith degradation.
[c] Residue hydrolysed by exo-*N*-acetyl-β-D-glucosaminidase.
bs = broad signal (unresolved doublet).

NMR data showed anomeric protons at δ 5.33 and 5.12 (both bs), 5.11 ($^3J_{H1,H2}$ 3.8 Hz), and 4.92 ($^3J_{H1,H2}$ 4.0 Hz) for the four Hex and HexNAc residues with the α-D configuration. The FAB-mass spectrum failed to give satisfactory pseudomolecular ions from which to account for the fate of the Kdo residues during the Smith degradation. However, two other types of product were characterized in the linkage analysis and may be rationalized as originating from Kdo termini. A pair of diastereomeric 5-Ac-3-deoxy-1,2,4,6-Me_4-[$^2H_4$]Hexitols, which were characterized as described previously for the core OS termini in other *Campylobacter* strains [4,6], originated during the extended Smith degradation sequence from the 5-*O*-substituted reducing Kdo residue as the terminal unit in **OS1**. The mass spectral data for the second type of products were consistent with those of a pair of diastereomeric 1,4,5-Ac_3-3-deoxy-2,6-Me_2-[$^2H_4$]Hexitols, a pathway for whose formation is proposed (Fig. 4) from the reducing Kdo residue with an attached AEP substituent in the minor component **OS1a**. The AEP substituent may be expected to remain attached, possibly in modified form, through the Smith degradation so that lactonization of the 3-deoxyhexonic acid would not occur in the course of the mild acid hydrolysis of acyclic acetals. To account for the formation of this 3-dHexitol derivative, it is necessary to assume that the phosphate substituent is retained during permethylation, but is removed during subsequent hydrolysis to allow for lactonization, lactone reduction on treatment with $NaBD_4$, and acetylation in the standard procedure used in the conversion of sugar residues into partially methylated

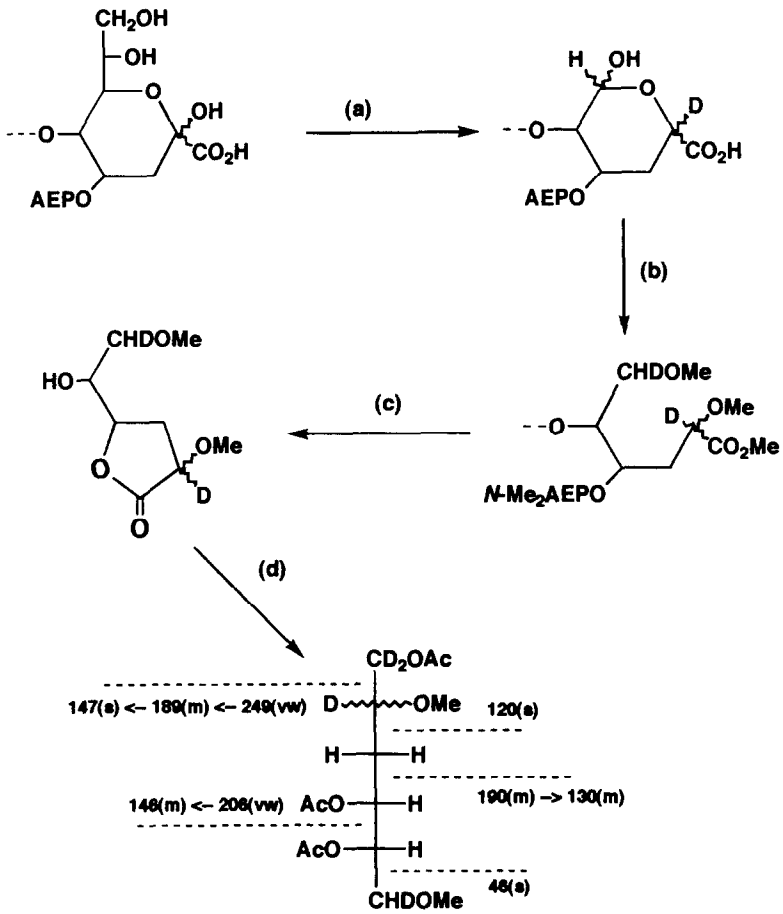


Fig. 4. Proposed pathway for modification of reducing Kdo terminus bearing a 4-*O*-(2-aminoethyl)-phosphoryl [AEP] substituent. (a) Reduction ($NaBD_4$), oxidation ($NaIO_4$); (b) reduction ($NaBD_4$) of 4-deoxyhexuronic acid (hemiacetal of 6-aldo-3-deoxyhexonic acid), methylation; (c) acid hydrolysis (of oligosaccharide) with concomitant de-*O*-phosphosphorylation and lactonization; (d) reduction ($NaBD_4$) of lactone and acetylation to give 1,4,5-Ac_3-3-deoxy-2,6-Me_2Hexitol from modified Kdo terminus with fragment ions in electron-impact MS. Relative intensities of primary and secondary fragment ions are shown as s (strong), m (medium), and vw (very weak).

alditol acetates. It is assumed in Fig. 1 that the distal phosphorylated Kdo residue in **OS1** undergoes exocyclic cleavage during the Smith degradation giving a postulated heptulosonic acid derivative (Kdh) in **OS2**.

Locations and assignments of individual anomeric configurations of hexopyranose end groups.—The Smith degradation resulted in the removal of four Hex and HexNAc end groups with one *α-gluco*, one *α-galacto*, and two *β-gluco* configurations. One of the residues with the *β-gluco* configuration was identified as that of the GlcNAc residue through the action of the corresponding exoglycosidase. Treatment of **OS1** with aqueous HF led to the removal the 2-aminoethylphosphate unit from the distal Kdo residue

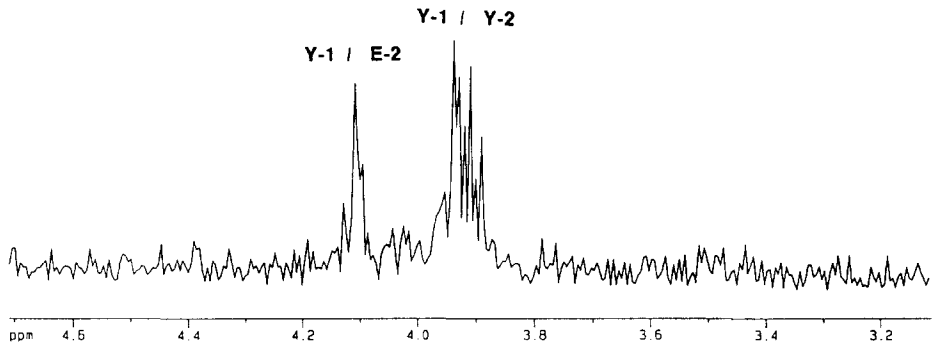


Fig. 5. 1D slice from the 400 MHz NOESY spectrum of core oligosaccharide **OS1** showing an interresidue connectivity from H-1 of α-Gal (**Y**) to H-2 of Hep (**E**), and an intraresidue NOE from H-1 to H-2 of Gal (**Y**) (mixing time 300 ms).

(disappearance of the ^{31}P resonance) and mild hydrolysis with acetic acid to cleave the innermost Kdo unit occurred with no other change in sugar composition (alditol acetate analysis). Digestion of the product with *N*-acetyl-β-D-glucosaminidase furnished **OS3** and FABMS of the permethylated derivative (Fig. 3) and linkage analysis (Table 1) showed that there was virtually complete removal of the non-reducing β-D-GlcNAc and the corresponding exposure of a GalNAc end group. The disappearance of the anomeric resonance at δ 4.53 showed that that at δ 4.49 could be assigned to the β-D-Glc residue. With the data from these experiments and from the Smith degradation the configurational assignments were established for residues **A**–**E** in the backbone of core **OS1** shown in Fig. 1 and Table 2. The differentiation of the sites of attachment of the three hexose residues **X**, **Y**, and **Z** was achieved through 2D NOESY and ROESY experiments. Interresidue NOE interactions were shown between H-1 (δ 5.46) of α-D-Glc and H-2 (δ 4.20) of the outer Hep residue (**D**), and between H-1 (δ 5.53) of α-D-Gal (**Y**) and H-2 (δ 4.13) of the inner Hep residue (**E**) (Fig. 5), thereby requiring attachment of the β-D-Glc to O-4 of Hep residue **E**. These experiments also showed for each of the α-D-glycose residues intraresidue interactions between H-1 and H-2. The absence of intraresidue NOE interactions between H-1 and H-3 and H-5 in both Hep residues were in accord with the assigned α-D-*manno* configurations. The results allowed a complete structure to be proposed for oligosaccharide **OS1** liberated from the LOS of *C. lari* ATCC 35221.

Composition of the extracellular polysaccharide ATCC 35221 and characterization of the repeating tetrasaccharide unit.—The glycan component ATCC 35221 **P1** was received as a freeze-dried solid from the aqueous layer from the phenol–water extraction of the bacterial cells. The ^1H NMR spectrum showed the presence of four anomeric protons, three at δ 4.90 ($^3J_{H1,H2}$ 3.0 Hz), 4.79 ($^3J_{H1,H2}$ 3.0 Hz), and 4.74 ($^3J_{H1,H2}$ 7.0 Hz), and a fourth at δ 5.18 with additional coupling indicative of a glycosyl phosphate ($^3J_{H1,H2}$ 3.0 Hz, and $^3J_{H1,P}$ 8 Hz), together with two groups of methylene protons at δ ~ 1.8 (2 × -CH_2-, later assigned to 6-deoxyheptose residues) and δ ~ 1.9 (1 × -CH_2-, later assigned to a 3-deoxypentose residue), and the three-proton signal of an *N*-acetyl group at δ 1.98. The ^{13}C NMR spectrum with support from a *J*-modulated ^1H–^{13}C

spin–echo experiment confirmed the following general features: (*i*) the presence of four anomeric carbon resonances at δ_C 102.3, 100.0 ($J_{C,P}$ 8 Hz), 99.5, and 93.1; (*ii*) two resonances for methylene carbons (CH_2) at δ_C 32.3 (intensity 2) and 31.5, two resonances assignable to oxygenated methylene groups at δ_C 63.8 (C-5 of a deoxypentose) and 58.5 (intensity 2, CH_2OH of deoxyheptoses); (*iii*) resonances at δ_C 55.5 (C-2), 174.9 (CO) and 22.4 (CH_3), and 60.0 (CH_2OH) characteristic of a HexNAc residue; (*iv*) a resonance at δ_C 74.8 ($J_{C,P} \sim 8$ Hz) indicating the other terminus of the single phosphoric diester linkage (δ 1.164). These data were consistent with the presence of a tetraglycosyl phosphate repeating unit.

On the assumption that the **P1** isolate might be a high M_r LPS component, it was heated with dilute acetic acid under conditions that would cleave the ketosidic linkage to lipid A, and the product was fractionated by GPC on Bio-Gel P-6. As with the corresponding glycan from *C. lari* PC 637 [8], the ^1H NMR spectrum showed that a change had occurred during the acetic acid treatment with the disappearance of one anomeric resonance at δ 5.18 and the appearance of new signals at δ 4.80 (major) and

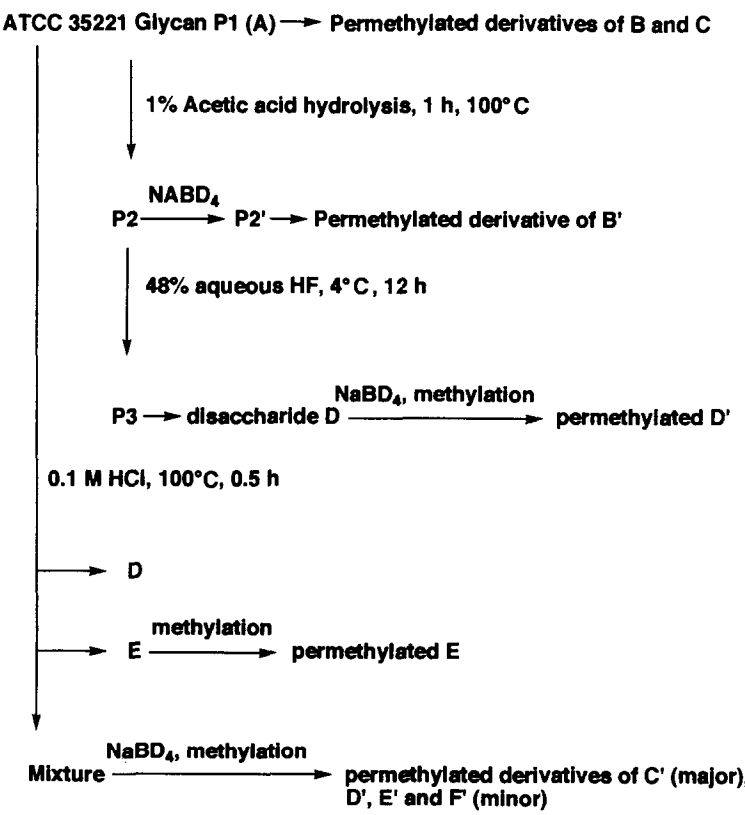
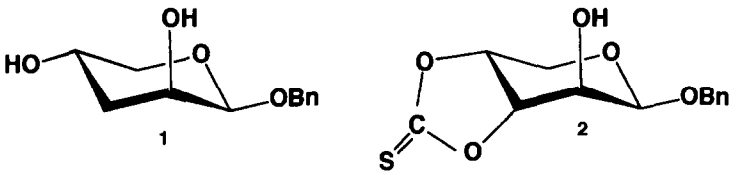


Fig. 6. Genesis of oligosaccharide fractions from poly(tetraglycosylphosphate) **P1** from *C. lari* strain ATCC 35221 with letter-designated oligosaccharides for whose permethylated derivatives shorthand structures are proposed.

4.93 (minor) corresponding to the anomeric resonances of a reducing sugar residue. Similarly the anomeric carbon resonance at δ_C 100.0 shifted to 98.5 with disappearance of $^{13}C-^{31}P$ coupling. This product was thus designated ATCC 35221 **P2** (see Fig. 6) and the disappearance of the new signals in the ^1H NMR spectrum on treatment of **P2** with $NaBD_4$ to give **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 of the alditol acetates showed the presence of derivatives of GlcNAc, a 6-deoxyheptose, and a 3-deoxypentose in the molar ratio of 1:2:0.6, together with a phosphate content of ca. 12% corresponding to one phosphate residue per tetrasaccharide repeating unit. The absence of sugar constituents found in the core region of the LPS indicated that this glycan was probably not an O antigen chain of a high M_r LPS.

In the alditol acetate analysis, approximately half of the 6-deoxyheptose component was detected as an anhydroalditol tetraacetate with ^2H incorporation from reduction with NaB^2H_4. The derivative had a pseudomolecular ion $[M + NH_4]$ at m/z 365, and a prominent fragment ion at m/z 129 was consistent with the compound being a 4,7-anhydrodeoxyheptitol. The 6-deoxyheptose as the 6-deoxyheptitol hexaacetate was distinguishable by GLC from the derivative from the 6-deoxy-L-*galacto*-heptose from *C. lari* PC 637 [8], and also from those of the previously characterized 6-deoxy-D-*altro*-heptose [7] and 6-deoxy-D-*talo*-heptose [11]. The identity of the *C. lari* ATCC 35221 constituent with synthetic 6-deoxy-L-*gulo*-heptose (courtesy of Professor A. Zamojski [12]) was established by GLC of the alditol acetate with substantiation of the L-enantiomeric configuration through the formation of chiral glycosides [9]. The 3-deoxypentitol tetraacetate was coincident with that formed from 3-deoxy-*threo*-pentose. The absolute configuration of this sugar was established as 3-deoxy-D-*threo*-pentose by the chiral glycoside procedure [9].

A sample of benzyl 3-deoxy-β-D-*threo*-pentopyranoside (**1**) was prepared in the following reactions with confirmation of the identities of all compounds by ^1H and/or ^{13}C NMR spectroscopy, although in non-optimized yields and without isolation of analytically pure samples. Reaction of benzyl β-D-arabinopyranoside with 5 M NaOH and carbon disulfide in dioxane [13] afforded the 3,4-thionocarbonate (**2**) (ν_{max} 1640 cm^{-1}). Treatment of **2** with tributyl stannane in toluene in the presence of radical initiator [14] furnished a mixture of benzyl 3-deoxy-β-D-*threo*-pentopyranoside (**1**) and the 4-deoxy isomer from which a sample of **1** was obtained by oxidation of the latter compound with periodate followed by column chromatography on silica gel. Hydrogenolysis of the benzyl glycoside (**1**) furnished the parent 3-deoxy-D-*threo*-pentose from which the diastereomeric sets of 2-(*S*)- and 2-(*R*)-butyl glycoside acetates were generated [9], each with one major component, the identities of which were confirmed by the detection of a glycosyloxonium ion at m/z 243 (GLC-EIMS), and a pseudomolecular ion $[M + H]^+$ at m/z 275 (FABMS). The diastereomeric glycoside acetates were separable by GLC (program F) with respective retention times for the (*R*) and (*S*) glycosides of 19.62 and 20.82 min, and comparison of the reference glycosides with the mixtures of chiral glycosides formed from ATCC 35221 **P1** showed that the natural sugar was the D-enantiomer.



Permethylation of the parent glycan ATCC 35211 **P1**, as for the phosphorylated glycan from *C. lari* PC637 [8], was accompanied by depolymerization and furnished derivatives for which FABMS (Figs. 7 and 8) showed ions of highest mass at m/z 952 and 858 (weak) corresponding to pseudomolecular ions $[M + H]^+$ of permethylated methyl glycosides of, respectively, phosphorylated (**B**) and non-phosphorylated (**C**) tetrasaccharides of composition GlcNAc 6-dHep$_2$ 3-dPen. Two series of glycosyloxocarbenium ions were also observed (Fig. 8) and these provided evidence for the sequence [P]–GlcNAc–6-dHep–3-dPen–6-dHep in the oligosaccharide moiety. The same derivatives were formed as the major products from methylation of the liberated tetrasaccharide fraction **P2**. Permethylation of the phosphorylated triglycosylalditol fraction **P2′** gave as the major component a derivative (**B′**) whose FABMS showed pseudomolecular ions $[M + H]^+$ and $[M + Na]^+$ at m/z 969 and 991, respectively, together with fragment ions (Fig. 8) that confirmed the sequence of residues. Less prominent pseudomolecular ions $[M + H]^+$ and $[M + Na]^+$ at m/z 875 and 897 were also observed for the corresponding non-phosphorylated derivative (**C′**). Linkage analysis of **P1** (Table 3) showed the formation of derivatives of 3,4,6-Me$_3$N-Me- and 4,6-Me$_2$N-Me-GlcNAc, 2,4,7-Me$_3$- and 3,4,7-Me$_3$-6-dHep, and 4-Me-3-dPen. In a parallel linkage analysis of **P2′** (Table 3) the characterization of 1,2,4,5,7-Me$_5$-6-dHep from methylated **P2′** defined the 3-linked 6-dHep residue as the reducing unit in **P2** and hence as one

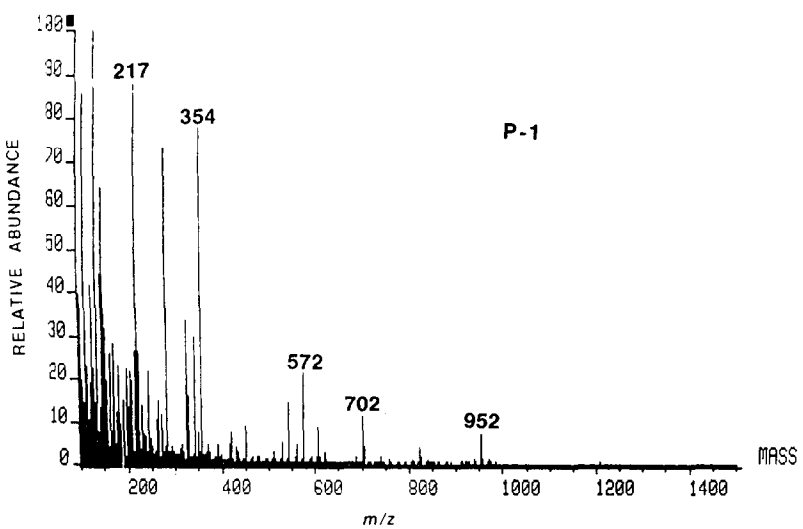


Fig. 7. Positive ion FABMS spectrum of permethylated derivatives formed from **P1**.

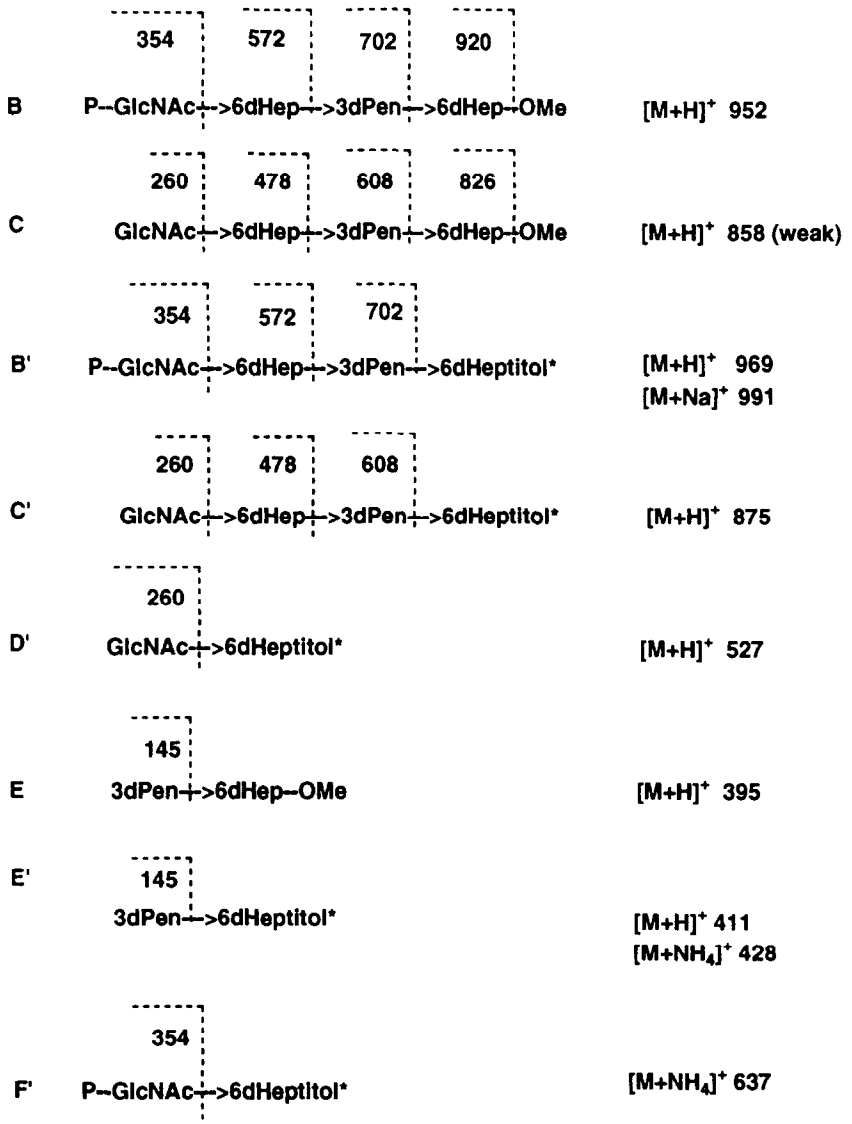


Fig. 8. Analysis of positive ion FABMS spectra of permethylated derivatives of methyl oligosaccharide glycosides and oligoglycosylalditols designated in Fig. 6.

terminus of the phosphoric diester linkage in the parent glycan **P1**. These combined sequence and linkage analysis data therefore located the differently linked 6-dHep residues within the tetrasaccharide unit and pointed to O-3 of the GlcNAc residue as the other terminus for the phosphoric diester linkage. The depolymerization of the glycan **P1** during mild hydrolysis to give the phosphorylated tetrasaccharide **P2** reflected the extreme susceptibility of the glycosyl phosphate linkage to acid cleavage. The linkage

Table 3
Linkage analysis of methylated derivatives **P1** and **P2′** from the glycan of *C. lari* strain ATCC 35221

Methylated sugar (and linkage type)	Relative proportions from derivatives	
	P1	**P2′**
4-Me3-dPen [-2 3dPen]	0.6	0.9
3,4,7-$Me_3$6-dHep [-2 6-dHep]	1.0	1.0
2,4,7-$Me_3$6-dHep [-3 6-dHep]	1.0	
1,2,4,5,7-$Me_5$6-dHep [-3 6-dHeptitol]		1.0
4,6-Me_2N-MeGlcNAc [-3 GlcNAc]	0.5	0.7
3,4,6-Me_3N-MeGlcNAc [GlcNAc]		0.2

analysis data for **P2′** also revealed the exposure of some 3-dPen and 6-dHep non-reducing end groups, and showed that the mild conditions resulted in detectable cleavage of glycosidic linkages within the tetrasaccharide unit.

Evidence for the placing of the differently linked 6-dHep residues in the tetrasaccharide unit was obtained from partial fragmentation. Treatment of glycan **P1** with aqueous 48% hydrogen fluoride effected dephosphorylation which was accompanied by depolymerization. The mixture of products **P3** was separated by GPC on Bio-Gel P-2 to give a single disaccharide **D** and a mixture of glycoses. The latter were identified as 3-deoxy-*threo*-pentose and 6-deoxy-L-*gulo*-heptose by reduction with $NaBD_4$ and GLC of the alditol acetates. The disaccharide contained GlcNAc and 6-dHep residues. ^{1}H NMR of the glycosylalditol **D′** from treatment with $NaBD_4$ showed a single anomeric proton at δ 4.64 ($^3J_{H1,H2}$ 8.4 Hz), and ^{13}C NMR showed signals for 15 carbon atoms including those for the GlcNAc residue at δ_C 22.4 and 174.5 (CH_3CO), and 56.0 (C-2), and for the 6dHeptitol residue at δ_C 34.9 (C-6) and 58.6 (C-7). FABMS of the methylated **D′** glycosylalditol showed a pseudomolecular ion $[M + H]^+$ at m/z 527 and linkage analysis gave inter alia 1-[$^2H_1$]-2-Ac-3,4,5,7-Me_4-6-dHep, thus establishing the structure of the disaccharide from **P3** as β-D-GlcNAc-(1 → 2)-6d-L-*gul*-Hep (**D**).

A deliberate partial hydrolysis of glycan **P1** with 0.1 M HCl at 100°C for 30 min gave a mixture of products (**P4**) and separation of the products by GPC on Bio-Gel P-2 led to the isolation of (*i*) 6-deoxy-L-*gulo*-heptose whose ^{1}H NMR spectrum (including the predominant anomeric proton [δ 4.88 ($^3J_{H1,H2}$ 3.3 Hz)] was identical to that of a synthetic sample, (*ii*) the previously characterized disaccharide **D**, (*iii*) a second disaccharide **E**, and (*iv*) a mixture of oligosaccharides. ^{1}H NMR of the second disaccharide showed the predominant anomeric proton for a reducing 6-dHep residue at δ 4.88 and that for the glycosidically linked 3-dPen residue at δ 4.84 ($^3J_{H1,H2}$ 3.3 Hz) and the respective methylene protons as multiplets at δ 1.60–1.95. FABMS of the methylated disaccharide (**E**) showed a pseudomolecular ion $[M + H]^+$ 395 with fragment ions at m/z 145 $[dPen]^+$ and 363 [3-dPen − 6-dHep]$^+$, and linkage analysis established the presence of terminal 3-dPen and 3-linked 6-dHep residues. Further evidence for the disaccharide structure was obtained from GLC-MS of the derived methylated [$^2H_1$]glycosylalditol (**E′**) which showed pseudomolecular ions (CIMS) at 411 $[M + H]^+$ and 428 $[M + NH_4]^+$ with fragment ions (EIMS) as shown (Fig. 8). This disaccharide was therefore defined as 3d-β-D-*threo*-Pen-(1 → 3)-6d-L-*gul*-Hep (**E**). The

Table 4
^{1}H chemical shift (ppm) data and coupling constant ($J_{n,n+1}$) values [in parentheses (Hz)] for polysaccharide **P1** from COSY, COSYRCT, and TOCSY experiments

Sugar residue	H-1	H-2	H-3	H-4	H-5	H-6,6′	H-7,7′
6-d-α-L-*gulo*-Hep*p*	5.18	3.80	4.19	3.63		1.8	3.1
	(3.0)	(3.5)	(1.8)	(3.1)		(m)	(m)
6-d-α-L-*gulo*-Hep	4.90	3.88	3.72			1.8	3.1
	(2.95)	(3.4)	(1.6)			(m)	(m)
3-d-β-D-*threo*-Pen*p*	4.79	4.00	1.90	3.65		H-5.5″	
	(3.0)	(m)	1.92	(m)		4.38	
			(m)			(m)	
β-D-GlcNAc*p*	4.74	3.85	4.02	3.51	3.68		
	(7.0)	(9.0)	(9.2)	(9.4)	(m)		

incompletely separated mixture of oligosaccharides was treated with $NaBH_4$ and converted into a mixture of methylated derivatives, which was shown by FABMS to include that formed from non-phosphorylated triglycosylalditol **C′** [GlcNAc–6-dHep–3-dPen–6-dHeptitol] as the major component, and by GLC-MS (CI and EI) glycosylalditols **D′**, **E′**, and **F′**.

*Two-dimensional NMR spectroscopy of ATCC **P1**, **P2**, and **P2′**, configurational assignments for glycose residues, and definition of termini of the phosphoric diester linkage.*—Proton resonances in the glycan **P1**, the liberated phosphorylated tetrasaccharide **P2**, and the derived triglycosylalditol **P2′** (Table 4) were assigned (partially) from 2D COSY and 2D COSYRCT, and completely from 1D TOCSY experiments. For each spin system the interpretations started from the anomeric protons previously assigned for the GlcNAc and 3-dPen residues in the respective disaccharides **D** and **E**, and those for the two 6-dHep residues were differentiated when one was changed in the liberation of **P2** and disappeared in the formation of **P2′**. Correlations of proton resonances were assisted by connectivities to the methylene groups at C-6 of 6-dHep and at C-3 of 3-dPen residues. Vicinal coupling constants were in accord with those expected for the β-D-GlcNAc in the $^4C_1$ conformation and both 6d-α-L-*gul*-Hep residues in the $^1C_4$ conformation. For the 6-dHep residues, assignments for the α-L glycosidic configurations were supported by the following intraresidue connectivities in the NOESY experiment: H-1 with H-2, H-2 with H-3, and H-4 with H-5. Carbon resonances were assigned directly for those characteristic nuclei mentioned above and for others as required by inverse ^1H–^{13}C chemical shift correlation experiments [HMQC and

Table 5
^{13}C NMR chemical shift (ppm) data for extracellular polysaccharide **P1** of *C. lari* strain ATCC

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6	C-7	CH_3	CO
3d-*threo*-Pen	93.1	77.2	31.5	70.9	63.8				
6d-*gulo*-Hep (2-linked)	99.5	78.3	65.0			32.3	58.5		
6d-*gulo*-Hep (3-linked)	100.0	68.5	78.1	65.4		32.3	58.5		
GlcNAc	102.3	55.5	74.8	64.8		60.0		22.4	174.9

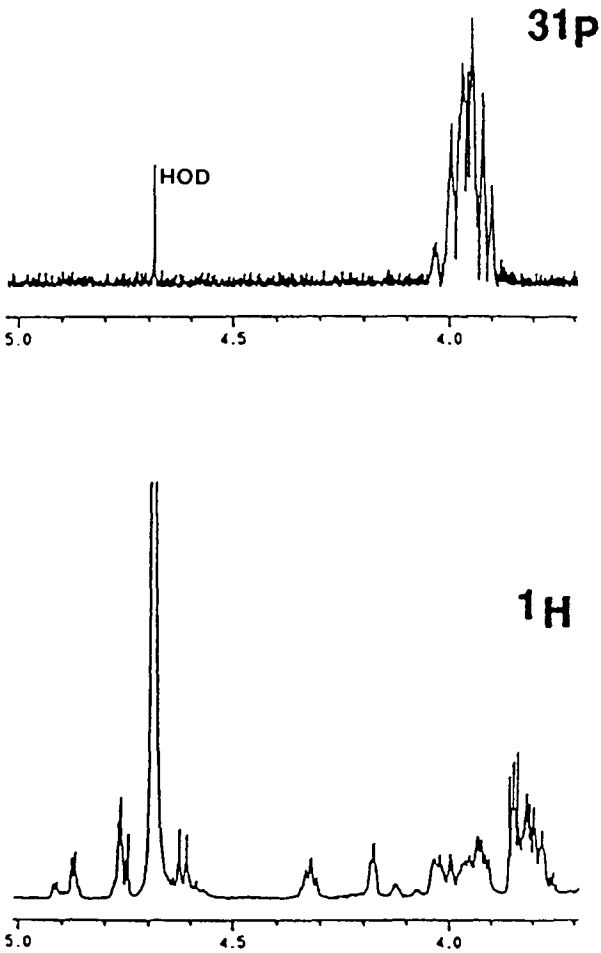


Fig. 9. 1D slices from a 2D $^{1}H-^{31}P$ HMBC spectra at 400 MHz of reducing tetrasaccharide **P2** showing connectivities from ^{31}P to H-1 of Gal.

HMQC–TOCSY]. Not all resonances were assigned unambiguously, but those required (e.g., for confirmation of linkage sites) showed downfield glycosylation shifts from regions of the spectrum with overlapping resonances (see Table 5).

Confirmatory evidence for the phosphoric diester termini was obtained from a long-range 2D $^{1}H-^{31}P$ HMBC experiment with **P1** (d_2 = 40 ms) (not shown) in which a strong three-bond interaction with H-1 (δ 5.18) of the 3-linked 6-dHep residue, but only a weak interaction with H-3 (δ 4.02) of GlcNAc, were observed. In a separate experiment with **P2** (d_2 = 60 ms) (Fig. 9) a strong three-bond connectivity was shown to H-3 of the GlcNAc residue. Assignments of carbon resonances showed that $^{13}C-^{31}P$ connectivities observed in the 1D ^{13}C spectrum were shown to involve a $^{2}J_{CP}$ coupling of 8 Hz of the anomeric carbon of the 3-linked 6-dHep and a $^{2}J_{CP}$ coupling of 8 Hz of C-3 of the GlcNAc residue. In confirmation of sugar residue sequences in the parent

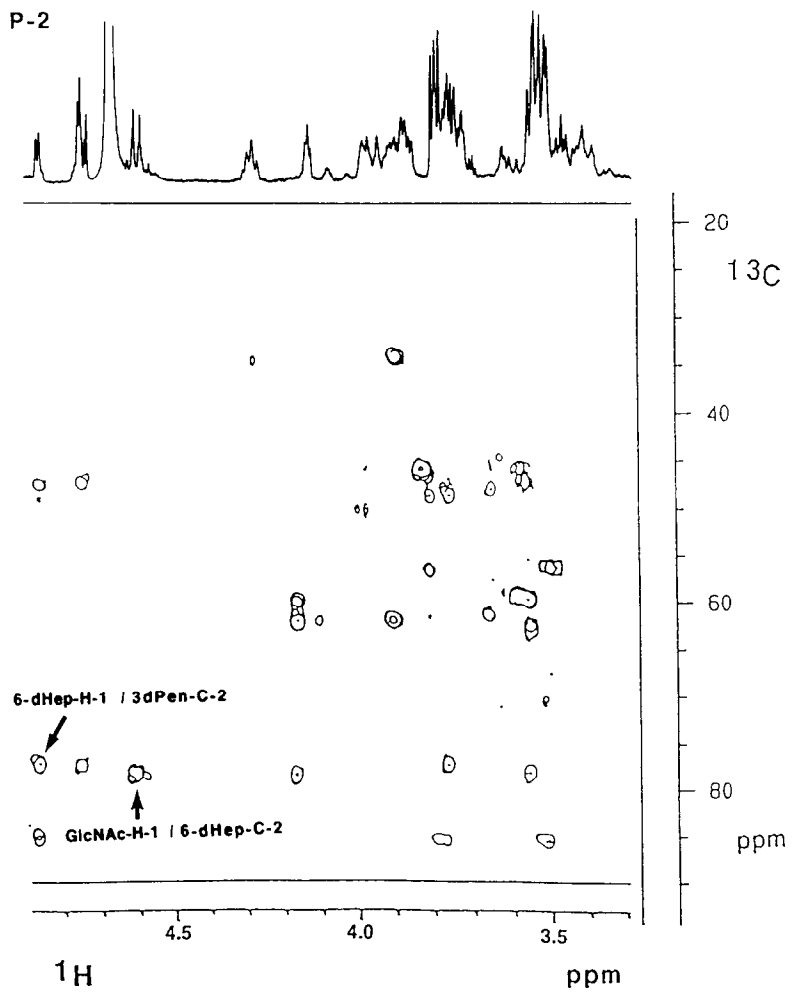


Fig. 10. $^{1}H-^{13}C$ HMBC spectrum of **P2** at 400 MHz showing long-range inverse detected interresidue connectivities between anomeric protons and linkage carbons in glycosidic linkages.

polysaccharide **P1** and the triglycosylalditol **P2**, $^{1}H-^{13}C$ HMBC experiments (Fig. 10) showed connectivities between H-1 of GlcNAc and C-2 of the distal 6-dHep residue (δ_C 78.3) and C-1 of GlcNAc and H-2 of 6-dHep, between H-1 of the distal 6-dHep and C-2 of 3-dPen (δ_C 77.2) and C-1 of 6-dHep and H-2 of 3-dPen, and between H-1 of 3-dPen and C-3 of the proximal 6-dHep residue (δ_C 78.1) and C-1 of 3-dPen and H-3 of 6-dHep.

The final question to be addressed by 2D NMR spectroscopy in conjunction with chemical experiments concerned the assignment of the anomeric configuration for the 3-deoxy-D-*threo*-pentose residue. With close similarities in the $J_{C,H}$ values of 163 Hz for this sugar residue in **P1** and **P2** and the synthetic benzyl glycoside **1**, the β-D

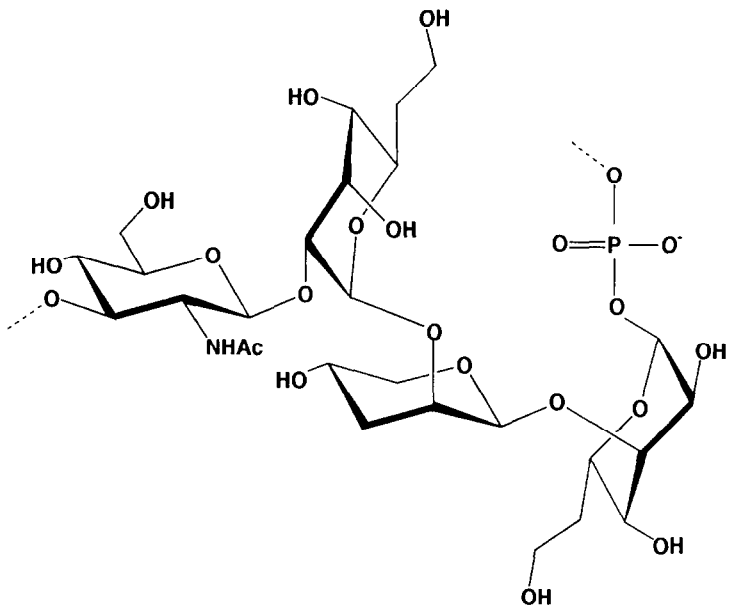


Fig. 11. Repeating unit of poly(tetraglycosylphosphate) **P1** from *C. lari* strain ATCC 35221.

configuration may be proposed for the sugar in the tetraglycosyl phosphate repeating unit of **P1** (Fig. 11).

Structural conclusions.—The *C. lari* type strain (ATCC 35221) has been shown to possess a LOS whose oligosaccharide component, like that of the PC 637 strain [8], bears little resemblance to the ganglioside-related outer regions of the more common and more extensively examined *C. jejuni* serotypes [2–6]. Neu5Ac residues are notably absent as are β-D-Gal residues at O-3 to which the former are often linked. The outer chains terminated by a HexNAc–HexNAc are closer in structure to the corresponding regions of low M_r LPS, now designated as LOS, from the related *C. lari* PC 637 [8], *C. coli* serotype O:30 [15], and other *Campylobacter* strains now under investigation. The outer chains do not carry the terminal α-D-Glc residue of the PC 637 strain, but the inner regions of both *C. lari* strains have in common the branched sequence:

$$\begin{array}{l} \qquad\qquad\quad \beta\text{-D-Glc} \\ \qquad\qquad\qquad\ \ 1 \\ \qquad\qquad\qquad\ \ | \\ \qquad\qquad\qquad\ \ 4 \\ \rightarrow 3)\text{-LD-Hep-}(1 \rightarrow 3)\text{-LD-Hep-}(1 \rightarrow 5)\text{-Kdo} \end{array}$$

Core **OS1** was isolated with 2 Kdo residues in linear sequence with the distal residue bearing an aminoethyl phosphate (AEP) substituent at O-4 which rendered its glycosidic linkage rather less susceptible to acid hydrolysis. No evidence was seen for the occurrence of a phosphoric monoester at O-4 of Kdo, the presence of which is often detected by the formation of anhydro Kdo derivatives on treatment with acid [16]. This is the first *Campylobacter* LPS studied to date that has been found to contain a

phosphoric ester substituent on a Kdo residue rather than on the adjacent LD-Hep residue, although neither residue is phosphorylated in the LPS from *C. lari* PC 637.

The polysaccharide that was extracted from the *C. lari* type strain in company with the LOS remained soluble in the aqueous supernatant liquid and, in the absence of core sugar components and of banding patterns on SDS–PAGE, would not appear to be the O antigen component of a high M_r LPS. This polysaccharide is of the same general type as that from *C. lari* PC 637 with a poly(tetraglycosyl phosphate) structure in which the glycosyl phosphate linkage is extremely susceptible to acid hydrolysis, and complete depolymerization occurred during the mild acetic acid treatment commonly used to cleave the Kdo–lipid A linkage in LPS structures. The phosphoric diester linkage is also susceptible to the base used for methylation linkage analysis so that this operation was also accompanied by extensive depolymerization. The most unusual features of this polysaccharide are the occurrence of two sugar components not previously found in polysaccharides, namely 6-deoxy-L-*gulo*-heptose and 3-deoxy-D-*threo*-pentose. The former provides the fourth example of a 6-deoxyheptose of unusual ring configuration found in carbohydrate polymers from *Campylobacter* species. As pointed out in the preceding paper [8] these sugars are found variously as components of true polysaccharides, teichoic acid-like polymers, and polyglycosylphosphates.

In summary, LOSs from the two *C. lari* strains are of the same general type, but significantly different from most of those from *C. jejuni* serotypes [2–8]. As with the LOS from *C. jejuni* serotypes, they differ sufficiently from each other in detailed chemical structure to account for their recognition as distinct serotypes. No evidence was obtained to indicate that the extracellular polysaccharides were the O antigen components of high M_r LPS. They are therefore more akin to capsular polysaccharides and it is quite possible that they might carry lipid termini such as di-*O*-acyl glycerol units [17,18] which were not detected.

Acknowledgements

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References

[1] M.B. Skirrow and M.J. Blaser, in I. Nachamkin, M.J. Blaser, and L.S. Tompkins (Eds.), Campylobacter jejuni. *Current Status and Future Trends*, Am. Soc. Microbiol., Washington, DC, 1992, pp 3–8.

[2] A.P. Moran, E.T. Rietschel, T.U. Kosunen, and U. Zähringer, *J. Bacteriol.*, 173 (1991) 618–626.

[3] 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.

[4] 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.

[5] 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.
[6] G.O. Aspinall, A.G. McDonald, H. Pang, L.A. Kurjanczyk, and J.L. Penner, *Biochemistry*, 33 (1994) 241–249.
[7] G.O. Aspinall, A.G. McDonald, and H. Pang, *Carbohydr. Res.*, 231 (1992) 13–30.
[8] G.O. Aspinall, M.A. Monteiro, H. Pang, L.A. Kurjanczyk, and J.L. Penner, *Carbohydr. Res.*, 279 (1995) 227–244.
[9] K. Leontein, B. Lindberg, and J. Lönngren, *Carbohydr. Res.*, 62 (1978) 359–362.
[10] A.K. Bhattacharjee and H.J. Jennings, *Carbohydr. Res.*, 51 (1976) 253–261.
[11] G.O. Aspinall, A.G. McDonald, H. Pang, L.A. Kurjanczyk, and J.L. Penner, *J. Biol. Chem.*, 268 (1993) 18321–18329.
[12] J.W. Krajewski, P. Gluzinski, Z. Pakulski, A. Zamojski, A. Mishnev, and A. Kemme, *Carbohydr. Res.*, 252 (1994) 315–325.
[13] B.S. Shashe, W.M. Doane, C.E. Russell, and C.E. Rist, *Carbohydr. Res.*, 3 (1966) 121–123.
[14] K. Kanemitsu, Y. Tsuda, M.E. Haque, K. Tsubono, and T. Kikuchi, *Chem. Pharm. Bull.*, 35 (1987) 3874–3879.
[15] G.O. Aspinall, A.G. McDonald, H. Pang, L.A. Kurjanczyk, and J.L. Penner, *J. Biol. Chem.*, 268 (1993) 6263–6268.
[16] S. Kondo, U. Zähringer, U. Seydel, V. Sinnwell, K. Hisatsune, and E.T. Rietschel, *Eur. J. Biochem.*, 200 (1991) 689–698.
[17] E.C. Gotschlich, B.A. Fraser, O. Nishimura, J.B. Robbins, and T.-Y. Liu, *J. Biol. Chem.*, 256 (1981) 8915–8921.
[18] M.A. Schmidt, B. Jann, and K. Jann, *FEMS Microbiol. Lett.*, 14 (1982) 69–72.