General Strategy for Structural Analysis of the Oligosaccharide Region of Lipooligosaccharides. Structure of the Oligosaccharide Component of *Pseudomonas aeruginosa* IATS Serotype 06 Mutant R5 Rough-Type Lipopolysaccharide†

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ABSTRACT: A general NMR-based strategy for the structural analysis of rough-type lipopolysaccharides, i.e., lipooligosaccharides, is introduced that involves initial deacylation of the glycolipids. The approach is illustrated here with the lipooligosaccharide (LOS) of the *Pseudomonas aeruginosa* serotype 06 rough-type mutant R5, which consists of a single major low molecular weight component. The LOS was isolated by using a modified phenol/chloroform/petroleum ether extraction method. Chemical analysis of the core oligosaccharide obtained from this LOS indicated that it was composed of D-glucose (D-Glc), 2-amino-2-deoxy-D-galactose (D-GalN), L-*glycero*-D-*manno*-heptose (L,D-Hep), 3-deoxy-D-*manno*-octulosonic acid (KDO), L-alanine (Ala), and phosphate. The glycan structure of the LOS was elucidated by employing a novel strategy that involved the use of one- and two-dimensional nuclear magnetic resonance techniques and mass spectrometric based methods on the backbone oligosaccharide obtained from the LOS by deacylation, dephosphorylation, and reduction of the terminal glucosamine. The location of phosphomonoester groups was unambiguously established by a 2D ^{1}H–^{31}P chemical shift correlation experiments on an *O*-deacylated sample of the LOS (LOS-OH). The LOS-OH carries amide-linked 3-hydroxydodecanoic acid groups and Ala on the two D-glucosamine residues and the D-galactosamine residue, respectively.

Lipopolysaccharide (LPS[1]) is a major component of the outer membrane of Gram-negative bacteria (Rietschel et al., 1988; Mayer et al., 1989). The LPS molecule carries *O*-antigen and endotoxin activities and participates in several distinct membrane functions (Rietschel et al., 1988; Lüderitz et al.; 1982, Nikaido, 1970). The structures of the LPSs of enteric bacteria have been investigated extensively [see Rietschel et al. (1988)]. They exhibit a common molecular architecture consisting of three distinct regions: a high molecular weight O-specific polysaccharide composed of repeating oligosaccharide units, covalently linked to a low molecular weight core oligosaccharide, which in turn is attached to a hydrophobic lipid A moiety (Raetz et al., 1990).

LPS comprises a heterogeneous mixture of molecules in which the *O*-polysaccharide chains differ in the degree of polymerization of their oligosaccharide units (S-type LPS). In addition, a population of molecules may be present that is completely devoid of *O*-polysaccharide (R-type LPS), consisting only of core oligosaccharide and lipid A components (Lüderitz et al., 1982). Moreover, some Gram-negative bacteria, including *Haemophilus* and *Neisseria* spp., produce only R-type LPS that contains mixtures of low molecular weight but structurally diverse oligosaccharide components (Holst & Brade, 1992). R-type LPS is frequently referred to as lipooligosaccharide (LOS). Both the core oligosaccharide and *O*-polysaccharide region contain antigenic determinants to which specific immune responses are directed. A detailed description of the molecular structural features of the antigenic determinants is essential for understanding the role of these surface-exposed virulence factors in host pathogen interactions.

The conventional approach to LPS structural analysis involves taking advantage of the acid lability of the KDO ketosidic linkage that joins the carbohydrate component to the lipid A moiety (Lüderitz et al., 1971). Structural analysis of the liberated carbohydrate components can then be determined by chemical and NMR- and MS-based methods. This approach has been very successful for the analysis of high molecular weight *O*-polysaccharides (Kenne & Lindberg, 1983; Perry et al., 1990). Another approach for obtaining oligosaccharide components suitable for structural analysis of R-type LPS (i.e., LOS) involves increasing the water solubility of the glycolipids by partial or complete removal of the fatty acyl groups from the lipid A region. An effective deacylation procedure was reported recently by Holst et al. (1991). The general method involves the initial release of lipid A ester-bound fatty acids by treatment with anhydrous

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[1] Abbreviations: KDO, 3-deoxy-D-*manno*-octulosonic acid; LPS, lipopolysaccharide; LOS, lipooligosaccharide; LOS-OH, *O*-deacylated LOS; lipid A-OH, *O*-deacylated lipid A; OS, oligosaccharide; PEA, phosphoethanolamine; PPEA, pyrophosphoethanolamine; DOC, deoxycholate; PAGE, polyacrylamide gel electrophoresis; GLC–MS, gas–liquid chromatography–mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; COSY, correlated spectroscopy; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond correlation; HPAEC, high-performance anion exchange chromatography; EDTA, ethylenediaminetetraacetic acid.

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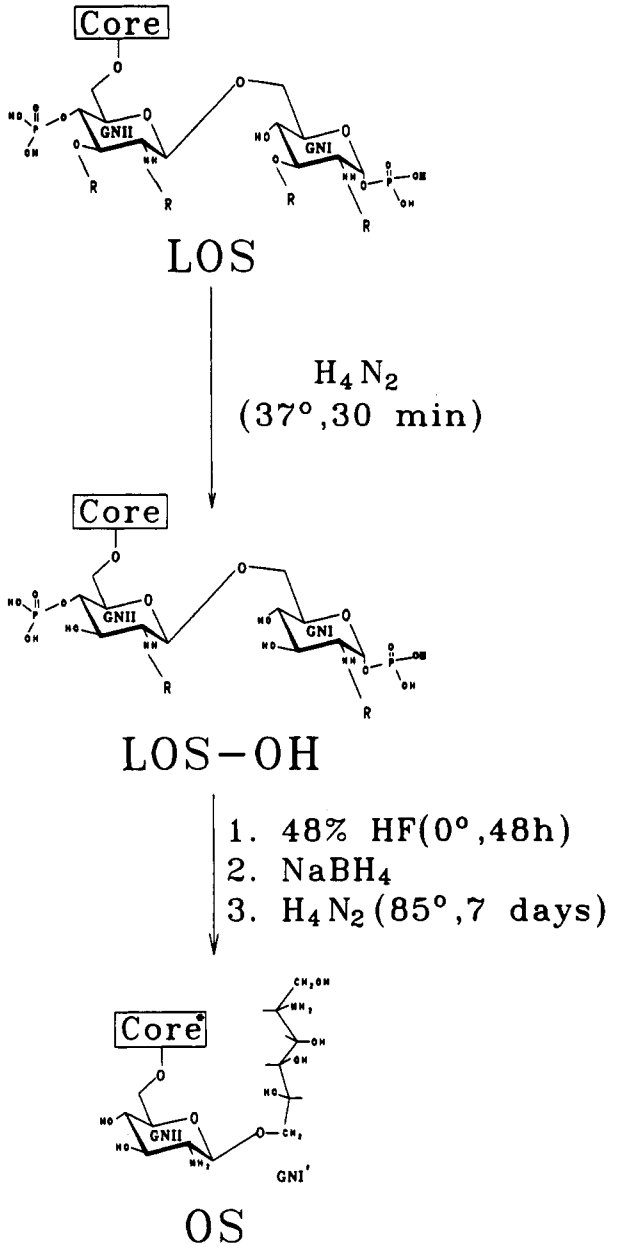
Scheme 1: General Procedure for the Preparation of Backbone Oligosaccharides from LOS



hydrazine under relatively mild conditions (37 °C for 30 min) to give an *O*-deacylated LOS (LOS-OH). Subsequent treatment of the LOS-OH with aqueous HF followed by $NaBH_4$ reduction of the reducing terminus glucosamine residue and then N-deacylation with anhydrous hydrazine under more vigorous conditions affords completely deacylated and dephosphorylated backbone oligosaccharide (OS) components suitable for sequence determination by NMR methods (Scheme 1). A significant advantage of this approach is that the derived oligosaccharides are representative of the intact backbone of the LOS.

Recently, we reported the application of a NMR- and MS-based strategy for probing the structures of the lipid A region of LPS from *Pseudomonas aeruginosa* (Karunaratne et al., 1992) and *Moraxella catarrhalis* (Masoud et al., 1994b). This approach involved the analysis of O-deacylated lipid A and LOS samples by two-dimensional multinuclear NMR spectroscopy and fast atom bombardment (FAB) mass spectrometry. A similar approach has also been used for determining the structure of an *Escherichia coli* lipid A (Holst et al., 1993), and Gibson and co-workers have reported a novel application of electrospray ionization MS methods for probing the structural heterogeneity of LOS-OH produced by *Haemophilus* and *Neisseria* species (Gibson et al., 1993; Philips et al., 1993).

In the present investigation, we describe a general analytical strategy for determination of the structure of the carbohydrate components of LOS. The method makes use of hydrazinolysis procedures to generate samples of the *O*-deacylated LOS and fully deacylated, dephosphorylated, and reduced LOS (Scheme 1) for structural analysis by chemical and NMR-based methods. The general procedure is illustrated here for an O-chain-deficient LPS mutant derived from *P. aeruginosa* IATS serotype 06, providing the first definitive report of a common inner basal structure for the *P. aeruginosa* core oligosaccharide region.

MATERIALS AND METHODS

Isolation of Lipooligosaccharide from P. aeruginosa Serotype 06 Mutant R5. *P. aeruginosa* IATS serotype 06 mutant R5 was cultivated aerobically using trypticase soy broth–BBL medium in 25- and 65-L fermentors (New Brunswick Scientific) at 37 °C. Cultures were harvested in the stationary phase, and cell pellets were washed successively, once with ethanol, twice with acetone, and twice with petroleum ether (35–60 °C). The LOS was then extracted from the dried cell pellets by means of the modified phenol/chloroform/light petroleum ether method (Brade & Galanos, 1982) and purified by successive ultracentrifugation (105000*g*, 4 °C, 4 h).

Isolation of Core Oligosaccharide. LOS (100 mg) was hydrolyzed in 1% aqueous acetic acid (20 mL) for 2.5 h at 100 °C, the solution was cooled (4 °C), and precipitated lipid A was removed by centrifugation (5000 rpm, 30 min). The supernatant solution was lyophilized to afford the core oligosaccharide, which was purified by gel filtration chromatography on a Bio-Gel P-2 column (2.6 × 140 cm, 200–400 mesh, Bio-Rad) eluted with pyridinium acetate (0.05 M, pH 4.5). Column eluants were continuously monitored for changes in refractive index by a Waters R403 differential refractometer, and collected fractions (4.5 mL) were assayed colorimetrically for neutral glycoses (Dubois et al., 1956). Core oligosaccharide (10 mg) was dephosphorylated with 48% aqueous HF (1.0 mL) in a Teflon vial at 4 °C for 48 h, followed by evaporation under a stream of N_2.

Isolation and Configurational Analysis of KDO. LOS (44 mg) was hydrolyzed in 0.5 M trifluoroacetic acid (TFA) (80 mL) for 40 min at 95 °C, the solution was cooled, and precipitated lipid A was removed by centrifugation. KDO was isolated from the supernatant solution by preparative thin-layer chromatography on silica gel (E. Merck) with *n*-propanol/concentrated ammonia/water (6:3:1 (v/v)) and purified as its ammonium salt (Williams & Perry, 1976). The absolute configuration of the KDO sample was determined from the specific optical rotation of the ammonium salt: $[\alpha]_D$ +33° (*c* 0.028, water). An authentic sample of ammonium 3-deoxy-D-*manno*-octulosonate (Williams & Perry, 1976) had $[\alpha]_D$ +38°.

O-Deacylation of LOS. LOS was *O*-deacylated according to the procedure of Holst et al. (1991). Samples (200 mg) were treated with anhydrous hydrazine (10 mL) at 37 °C for 30 min, the reaction mixtures were cooled, and cold acetone (3 vol, −70 °C) was added to precipitate the product. LOS-OH was then isolated by low-speed centrifugation (5000 rpm,

10 min) and purified on the aforementioned Bio-Gel P-2 gel filtration system (yield, 150 mg).

Preparation of LOS Backbone OS. LOS-OH was dephosphorylated by treatment of a sample (150 mg) with 48% aqueous HF (15 mL) at 0 °C for 48 h. The reaction mixture was evaporated under a stream of N_2, and the product was dissolved in water, dialyzed, and lyophilized. The dephosphorylated LOS-OH was dissolved in water (10 mL) and reduced with $NaBH_4$ (100 mg) (1 h, 22 °C). Following acidification with glacial acetic acid, the mixture was evaporated to dryness under a stream of N_2. The residue was evaporated from methanol containing 10% acetic acid to remove borate, dissolved in water, dialyzed, and then lyophilized. The product was dried *in vacuo* over P_2O_5 and *N*-deacylated by treatment with anhydrous hydrazine (15 mL) at 85 °C for 7 days (Holst et al., 1991). After the removal of excess hydrazine *in vacuo* over concentrated H_2SO_4, OS product was obtained by anion exchange chromatography on a poly(ethylene imine) cellulose column (2 × 8 cm, medium mesh, 1.06 mequiv/g, Sigma) eluted with water (75 mL). The LOS backbone OS was obtained by lyophilization and purified by gel filtration on Bio-Gel P-2, as described above (yield, 30 mg).

Analytical Methods. Glycoses were determined by GLC as their alditol acetate derivatives. Samples (0.2-0.5 mg) were hydrolyzed with 2 M TFA for 90 min at 125 °C and evaporated to dryness under a stream of nitrogen. The liberated glycoses were reduced ($NaBH_4$) and acetylated (Ac_2O) as previously described (York et al., 1985b). Peracetylated heptitol derivatives were found to have the L-*glycero*-D-*manno* (or D-*glycero*-L-*manno*) configuration by comparison of their GLC retention times with that of an authentic standard. The L-*glycero*-D-*manno* absolute stereochemistry is assumed on biosynthetic grounds (Coleman, 1983). The hexose residues were determined to have the D-configurations by GLC of their acetylated (*R*)-2-butylglycoside derivatives (Gerwig et al., 1979), while the L-configuration of alanine was confirmed by comparison of the GLC retention time of the *N*-acetyl-(*R*)-2-octyl ester with that of an authentic sample.

GLC analysis was performed with a Hewlett-Packard Model 5710A gas chromatograph fitted with a hydrogen-flame ionizer using a fused-silica capillary column (0.3 mm × 25 m) containing 3% OV 17; an initial column temperature of 180 °C was held for 2 min, followed by an increase to 240 °C at 4 °C/min.

High-performance anion exchange chromatography (HPAEC) was performed using a Dionex Bio-LC gradient system fitted with a CarboPac PA1 pellicular anion exchange column (4.6 × 250 mm) and a CarboPac PA1 guard column (10 × 32 mm) with pulsed amperometric detection (PAD). Prior to injection, the column was equilibrated with a 95:5 mixture of 150 mM NaOH (eluant A) and 1 M sodium acetate in 150 mM NaOH (eluant B). The backbone OS was eluted using the following gradient: 95:5 equilibration mixture of eluants A and B for 5 min, followed by an increase in eluant B at a rate of 0.42%/min over 60 min. The flow rate was 1.0 mL/min. The following pulse potentials and durations were used: $E_1 = 0.05$ V ($t_1 = 360$ ms); $E_2 = 0.60$ V ($t_2 = 120$ ms); $E_3 = -0.80$ V ($t_3 = 300$ ms). The response time of the PAD was set to 3 s for a pulse duration range of 2.

Methylation Analysis. OS samples (2–5 mg) were methylated with iodomethane in dimethyl sulfoxide containing an excess of potassium (methylsulfinyl)methanide (Hakomori, 1964). Excess iodomethane was evaporated under a stream of nitrogen, water (3 mL) was added, and the methylated products were extracted into chloroform (2 × 5 mL). The chloroform extracts were evaporated under a stream of nitrogen, and methylated OS was purified on a Sep-Pak C-18 cartridge as previously described (Mort et al., 1983). For linkage analysis of the KDO residues, LOS-OH (≈10 mg) was methylated and purified as described earlier. The KDO carboxymethyl ester groups so obtained were reduced by treatment of the methylated product with 1 M lithium triethylborodeuteride in tetrahydrofuran (super deuteride, Aldrich) (0.5 mL) for 2 h at 22 °C. Excess reagent was destroyed by the addition of glacial acetic acid, and the reaction mixture was evaporated under a stream of N_2, followed by evaporation from methanol containing 10% acetic acid (twice) and from methanol. The methylated and reduced product was desalted on a small ion exchange column containing Dowex 50 (H^+ form) eluted with water/methanol (1:1), and the lyophilized product was remethylated as described earlier.

Purified methylated oligosaccharides were hydrolyzed with either 2 M TFA at 100 °C for 1 h or with 0.25 M H_2SO_4 in 95% acetic acid at 85 °C overnight, reduced ($NaBD_4$), and acetylated according to the acetolysis procedure of Stellner et al. (1973). Partially methylated alditol acetates were separated by GLC and identified by EI-MS on a Hewlett-Packard 5958B GLC–MS system fitted with a DB-17 fused-silica capillary column (0.25 mm × 25 m), utilizing an ionization potential of 70 eV and the temperature program, 180 °C for 2 min followed by an increase to 320 °C at 5 °C/min.

Mass Spectrometry. Fast atom bombardment (FAB) mass spectra were acquired on a JEOL AX505H double-focusing mass spectrometer operating at an accelerating voltage of 3 kV. Samples were analyzed in the positive and negative ion modes with a mass resolution of 1500, using thioglycerol and dithiothreitol/dithioerythritol (5:1) as the supporting matrices, respectively. Samples were dissolved in water or methylene chloride and dried onto the stainless steel probe tip, followed by the addition of matrix prior to FAB analysis. A Xe atom beam of 6 kV was used to sputter and ionize samples, and spectra were calibrated with an Ultramark 1621.

NMR Spectroscopy. NMR spectra were obtained on a Bruker AMX 500 spectrometer using standard Bruker software. Measurements were made on solutions at 27 °C at concentrations of ~2 mg in 0.5 mL of D_2O, subsequent to several lyophilizations with D_2O.

Proton NMR spectra were measured at 500 MHz using a spectral width of 6.0 kHz and a 90° pulse. Broadband proton-decoupled ^{13}C NMR spectra were obtained at 125 MHz using a spectral width of 33 kHz, a 90° pulse, and WALTZ decoupling (Shaka et al., 1983). Acetone was used as the internal standard, and chemical shifts were referenced to the methyl resonance (δ_H, 2.225 ppm; δ_C, 31.07 ppm). Two-dimensional homonuclear proton correlation experiments (COSY) (Bax et al., 1981) were measured over a spectral width of 2.38 or 1.59 kHz, using data sets ($t_1 \times t_2$) of 256 × 2048 or 512 × 2048 points, and 16 or 32 scans were acquired, respectively. Spectra were processed in the magnitude mode with symmetrization about the diagonal. Two-dimensional nuclear Overhauser effect experiments (NOESY) (Kumar et al., 1980) were performed over a spectral width of 2.51 kHz, using a data set of 256 × 2048 points and 64 scans. A mixing time of 200 ms was employed for the NOESY experiment.

Heteronuclear 2D ^{13}C–^1H chemical shift correlations were measured in the ^1H-detected mode via multiple quantum coherence (HMQC) (Bax et al., 1983) with proton decoupling in the ^{13}C domain, using data sets of 2048 × 256 points and

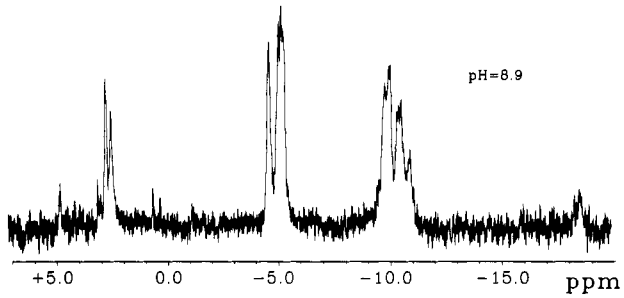


FIGURE 1: ^{31}P NMR spectrum of complete LOS from *P. aeruginosa* IATS serotype 06 mutant R5. The spectrum was measured in the presence of 5 mM EDTA and 2% DOC in 0.5 mL of D_2O, with chemical shifts referenced to an external standard of 85% phosphoric acid (δ_P 0.00) at 27 °C.

spectral widths of 4.5 and 13.9 kHz for ^1H and ^{13}C domains, respectively. Thirty-two scans were acquired for each t_1 value. Heteronuclear multiple bond correlated (HMBC) experiments were carried out in the ^1H-detected mode using the pulse sequence described by Bax and Summer (1986).

Phosphorus-31 spectra were measured at 202 MHz by employing spectral widths of 10-12 kHz and a 90° pulse, and phosphoric acid (85%) was used as the external standard (δ_P, 0.0 ppm). ^1H–^{13}P correlations (HMQC) were made in the ^1H-detected mode by using a data matrix of 16 × 1024 points, sweep widths of 10 kHz for ^{31}P and 1.3 kHz for ^1H, and a mixing time of 60 ms.

RESULTS

P. aeruginosa IATS serotype 06 derived mutant R5 was generated by means of phage infection (Dasgupta et al., 1994). LOS was isolated from the mutant strain by using a modified phenol/chloroform/petroleum ether extraction method (Brade & Galanos, 1982) in a yield of ca. 5% from the dried bacterial cells. Polyacrylamide gel electrophoresis (PAGE) of the mutant strain R5 LOS indicated that it was completely devoid of A- and B-band polysaccharides, consistent with its being a rough-type LPS composed of a lipid A moiety and a low molecular weight oligosaccharide component (Dasgupta et al., 1994; Masoud et al., 1992). The LOS showed a single band in the deoxycholate PAGE, which was faster migrating than that of the complete core oligosaccharide species of the wild-type parent strain IATS serotype 06 LPS, indicating several sugar deletions in the core region (Masoud et al., 1992).

Partial acid hydrolysis of the LOS with dilute aqueous acetic acid afforded an insoluble lipid A and a core oligosaccharide fraction. Core oligosaccharide was purified by size exclusion chromatography on a Bio-Gel P-2 system. Glycose analysis indicated the core oligosaccharide to be composed of D-glucose (D-Glc), 2-amino-2-deoxy-D-galactose (D-GalN), and L-*glycero*-D-*manno*-heptose (L,D-Hep) residues, which were identified by GLC–MS of the corresponding alditol acetate and (*R*)-2-butylglycoside derivatives. In addition, colorimetric analyses of the intact LOS indicated the presence of phosphate (7.7%), KDO (5.0%), and alanine (which was detected on an amino acid analyzer) (Dasgupta et al., 1994).

The ^{31}P NMR spectrum of the LOS [in D_2O containing 5 mM EDTA and 2% DOC (Batley et al., 1985)] showed mainly three distinct groups of resonances, indicating the presence of different phosphate-containing substituents (Figure 1): two resonances in the 2.0–3.0 ppm region due to phosphate monoesters; a group of resonances centered at ca. −5 ppm from pyrophosphate monoesters; and a group of resonances centered at −10 ppm from pyrophosphate diesters (Rosner et al., 1979). The integrity of the pyrophosphate groups was

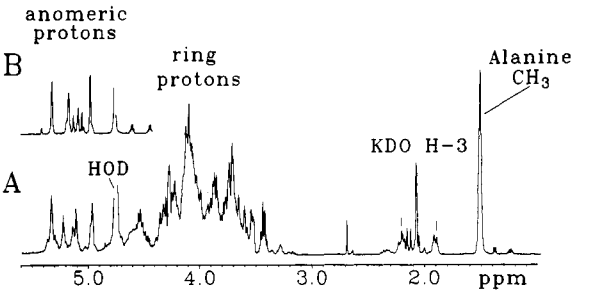


FIGURE 2: ^1H NMR spectra for core oligosaccharide (A) and the anomeric region of dephosphorylated oligosaccharide (B) from LOS of *P. aeruginosa* IATS serotype 06 mutant R5. The spectra were recorded in D_2O at 27 °C. The anomeric protons, ring protons, KDO methylene protons, and L-alanine methyl protons are indicated.

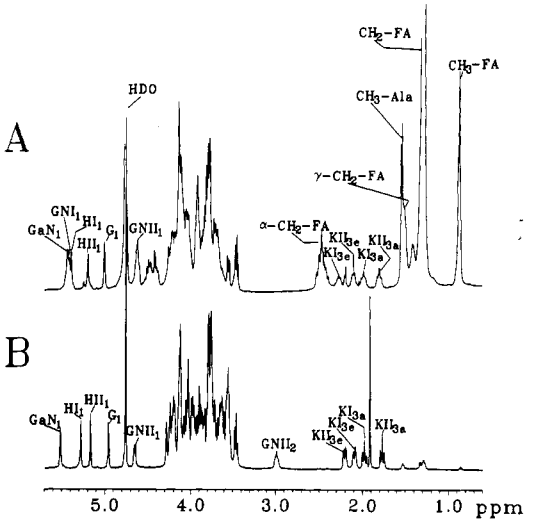


FIGURE 3: ^1H NMR spectra for LOS-OH (A) and LOS backbone OS (B) of *P. aeruginosa* IATS serotype 06 mutant R5, recorded in D_2O at pH 7.4 and 27 °C. The equatorial (e) and axial (a) protons from H-3 of the KDO residues, the protons from 3-hydroxydodecanoyl groups, and methyl protons of L-alanine are indicated.

sensitive to dilute acid or alkaline treatment, even under extremely mild conditions: resonances from pyrophosphates were absent from the ^{31}P NMR spectra of the core oligosaccharide (obtained from the LOS by treatment with 1% acetic acid at 100 °C for 2 h) as well as an O-deacylated sample of the LOS (LOS-OH, obtained by anhydrous hydrazine treatment at 37 °C for 30 min). Ethanolamine and phosphoethanolamine (PEA) were detected in the LOS mild acid hydrolysis products (0.05 M HCl, 100 °C, 2 h) (Mühlradt et al., 1977), pointing to the occurrence of pyrophosphoethanolamine (PPEA) in the intact LOS. The highly phosphorylated nature of *P. aeruginosa* LPS has been reported previously (Nikaido & Hancock, 1986).

In the ^1H NMR spectrum of the R5 core oligosaccharide (Figure 2A), signals at 1.50 ppm and at 1.90 and 2.20 ppm could be attributed to the methyl protons from the L-alanine moiety and the two methylene protons of the KDO residue (Carlson et al., 1988) at the reducing terminus, respectively. The anomeric region of the spectrum (5.5–4.4 ppm) showed a complex pattern of signals, indicating considerable structural heterogeneity in the sample. The observed structural heterogeneity was still apparent, even after dephosphorylation with aqueous HF (Figure 2B), and was thought to arise from modifications to the terminal KDO residue that had occurred during mild acid hydrolysis.

Examination of the ^1H NMR spectrum of an *O*-deacylated LOS that was obtained from native material by treatment with anhydrous hydrazine under relatively mild conditions

Table 1: Proton Chemical Shifts (ppm) and Coupling Constants (Hz) of Backbone OS from *P. aeruginosa* 06 (Mutant R5)[a]

residue	glycose unit	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3a[b] ($J_{3a,4}$)	H-3e[b] ($J_{3e,4}$, $J_{3e,3a}$)	H-4 ($J_{4,5}$)	H-5	H-6 ($J_{5,6}$)	H-6′ ($J_{5,6'}$, $J_{6,6'}$)	H-7 ($J_{6,7}$)	H-7′ ($J_{6,7'}$, $J_{7,7'}$)	H-8 ($J_{7,8}$)	H-8′ ($J_{7,8'}$, $J_{8,8'}$)
G	α-D-Glc*p*-(1→	4.96	3.56	3.74		3.46	4.13	3.78					
		(4.6)	(10.2)	(9.6)		(9.6)							
GaN	→4)-α-D-Gal*p*N-(1→	5.52	3.56	4.21		4.12	4.19	3.85	3.94				
		(4.8)	(10.4)	(3.6)		(≈1.0)		(3.8)	(7.7, 11.5)				
HI	→3)-L-α-D-Hep*p*-(1→	5.28	4.13	4.03		3.95	3.89	4.03		3.75	3.75		
		(≈1.0)	(4.2)	(9.6)		(9.6)		(2.9)		(≈1.0)	(≈1.0, 5.8)		
HII	→3)-L-α-D-Hep*p*-(1→	5.17	4.28	4.13		4.07	3.82	4.06		3.76	3.69		
		(≈1.0)	(3.8)	(9.6)		(9.6)		(≈1.9)		(5.8)	(8.6, 10.5)		
KI	→5)-α-KDO*p*-(2→			1.97	2.09	4.23	4.24	3.64		3.85		3.85	3.62
	4			(12.5)	(3.6, 12.5)	(2.9)		(<1.0)		(9.6)		(4.8)	(6.3, 10.5)
	↑												
KII	α-KDO*p*-(2→			1.77	2.21	4.09	4.03	3.67		3.97		3.99	3.77
				(12.5)	(4.5, 12.5)	(4.8)		(≈1.0)		(8.6)		(≈1.9)	(7.6, 11.5)
GNII	→6)-β-D-Glc*p*N-(1→	4.65	2.98	3.55		3.46	3.64	3.62	3.54				
		(7.6)	(9.6)	(9.6)		(9.6)		(≈1.9)	(5.80, 9.6)				
GNI′	→6)-D-GlcNol[c]	3.89	3.56	4.12		3.72	3.97	4.19	3.76				
		(3.8)	(4.8)	(≈1.0)		(8.6)		(≈1.0)	(7.7, 10.5)				

[a] Measured at 27 °C in D_2O (pH 7.4). [b] Letters e and a indicate the equatorial and axial H-3's of KDO, respectively. [c] Proton chemical shift for H-1′ = 3.77 ppm, and coupling constants $J_{1,1'}$ = 12.5 Hz and $J_{1',2}$ = 6.8 Hz.

(37 °C, 30 min) revealed considerably less heterogeneity (Figure 3A). Although slightly broadened signal line widths were observed, particularly for those resonances associated with inner core residues, subsequent 2D NMR and MS analyses confirmed that the sample consisted of a single molecular species. Moreover, dephosphorylation, reducing terminus reduction, and *N*-deacylation of the LOS-OH afforded a single oligosaccharide (Scheme 1; OS), as evidenced by the occurrence of a single peak on high-performance anion exchange chromatography (HPAEC) and by its NMR characteristics (Figure 3B).

The line-broadening effect observed in the ^{1}H NMR spectrum of the LOS-OH most likely arises from the partial aggregation of LOS molecules due to the presence of the amide-linked fatty acyl groups in the putative lipid A region. The structure of the lipid A-OH region of *P. aeruginosa* LPS was recently elucidated by NMR and FAB-MS techniques (Karunaratne et al., 1992), and it was found to consist of a β-1,6-linked D-glucosamine disaccharide backbone that carries *N*-linked 3-hydroxydodecanoic acid chains at C-2 and C-2′ and phosphomonoester groups at C-1 and C-4′. In the present investigation, the positive ion FAB-MS of the LOS-OH showed abundant fragment ions at m/z 897 and 799 from the lipid A-OH moiety and a dephosphorylated fragment ion, respectively, confirming the presence of 3-hydroxydodecanoyl groups in the lipid A region of the intact LOS (Karunaratne et al., 1992).[2] As expected, an intense envelope of resonances between 1.2 and 1.3 ppm and a sharp resonance at 0.8 ppm together with a multiplet centered at 2.5 ppm from the methylene, methyl, and hydroxylated methine protons of two amide-linked 3-hydroxy fatty acyl groups are prominent features in the ^{1}H NMR spectrum of the LOS-OH sample (Figure 3A). Moreover, in the high-field region of this spectrum, four resonances between 1.8 and 2.3 ppm point to the presence of two KDO residues, while the doublet at 1.54 ppm (J = 6.5 Hz) can be attributed to the methyl protons from the L-alanine group.

[2] The preponderance of lipid A-OH derived fragment ions pointed to the lability of the KDO linkage under the FAB-MS conditions employed; no detectable pseudomolecular ions representative of the parent LOS-OH species were detectable by FAB-MS. By contrast, a permethylated sample of the backbone OS showed an abundant pseudomolecular ion at m/z 1963 (M + K), together with structurally significant fragment ions at m/z 1674 (M – HexNol), 1658 (M – Hex – CH_3OH), 1486 (M – Hex – HexN), and 1471 (M – HexN – HexNol).

^{1}H and ^{13}C NMR analyses of LOS-OH and the backbone OS indicated that both oligosaccharides contain eight sugar residues. The spectra were completely assigned by 2D homo- and heteronuclear chemical shift correlation techniques, and the data are recorded in Tables 1 and 2.

In the 1D ^{1}H NMR spectrum of the backbone OS, discrete resonances of equal signal areas at 5.52 (d, $J_{1,2}$ = 4.8 Hz), 5.28 (s), 5.17 (s), 4.96 (d, $J_{1,2}$ = 4.6 Hz), and 4.65 ppm (d, $J_{1,2}$ = 7.6 Hz) were observed for the anomeric protons from five glycose residues (Figure 3B). These resonances served as the starting point for 2D spectral analysis, from which subspectra corresponding to the ring systems from each of the five glycopyranosyl residues were identified in a COSY contour map (Figure 4). The proton subspectra corresponding to the component monosaccharides were identified on the basis of the connectivity pathways delineated in the COSY contour map, the respective chemical shift values (Bock & Thøgersen, 1982), and the magnitude of the vicinal proton coupling constants (Altona & Haasnoot, 1980). In addition, signals for the H-3 equatorial (2.21 and 2.09 ppm) and axial (1.97 and 1.77 ppm) protons from two α-linked KDO pyranosyl residues were identified (Carlson et al., 1988; York et al., 1985a) in the high-field region of the spectrum (Figure 3B).

Seven anomeric ^{13}C resonances were observed between 97 and 103 ppm in the 1D ^{13}C NMR spectrum of the backbone OS (Table 3). Five of these resonances could be directly correlated with the corresponding anomeric proton resonances from an HMQC experiment. The HMQC experiment also served to identify the quaternary ketosidic (C-2) ^{13}C resonances (100.53 and 100.84 ppm) from the two KDO residues due to the absence of any ^{1}H/^{13}C connectivities. These latter assignments were confirmed from an HMBC experiment (van Halbeek, 1990), which showed three bond connectivities between the C-2 and H-3 resonances within each of the KDO ring systems. Three ^{13}C resonances in the region (50–60 ppm) characteristic of amino-substituted carbons (Bock & Pedersen, 1983) indicated the presence of three amino sugars in the backbone OS. Signals at 56.15 and 56.84 ppm were assigned via ^{13}C/^{1}H correlations to the H-2 resonances of the α-D-GalN residue and the putative lipid A-OH β-D-GlcN, respectively, while a ^{13}C/^{1}H connectivity from a signal at 52.06 ppm provided access via H-2 to the COSY ^{1}H spin system from the terminal D-glucosaminitol residue. Part of the ^{1}H/^{13}C contour plot is shown in Figure 5. Assignments

Table 2: Proton Chemical Shifts (ppm) and Coupling Constants (Hz) of *O*-Deacylated LOS from *P. aeruginosa* 06[a] (Mutant R5)

residue	glycose unit	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3a[b] ($J_{3a,4}$)	H-3e[b] ($J_{3e,4}$, $J_{3e,3a}$)	H-4 ($J_{4,5}$)	H-5	H-6 ($J_{5,6}$)	H-6′ ($J_{5,6'}$, $J_{6,6'}$)	H-7 ($J_{6,7}$)	H-7′ ($J_{6,7'}$, $J_{7,7'}$)	H-8 ($J_{7,8}$)	H-8′ ($J_{7,8'}$, $J_{8,8'}$)
G	α-D-Glc*p*-(1→	5.01 (4.4)	3.54 (10.3)	3.83 (9.3)		3.44 (10.3)	4.13	4.77 (4.1)					
GaN	→4)-α-D-Gal*p*N-(1→	5.44 (4.7)	4.22 (10.3)	4.13 (3.7)		4.12 (≈1.0)	4.19	3.84 (5.6)	3.92 (6.5, 11.2)				
	↓ 4												
HI	→3-L-α-D-Hep*p*-(1→	5.40 (<2.0)	4.59 (3.2) (9.6)[c]	4.22 (7.3)		4.49 (8.8) (10.2)[c]	4.38	4.13 (≈2.0)		3.79			
	2 ↑												
HII	→3-L-α-D-Hep*p*-(1→	5.20 (≈2.9)	4.42 (3.7)	4.11 (9.3)		4.06	≈4.05	4.00		3.76			
KI	→5)-α-KDO*p*-(2→			1.99 (12.1)	2.27 (–,12.1)	4.09	4.26	3.71		3.80		3.92	3.65
	4 ↑												
KII	α-KDO*p*-(2→			1.81 (12.1)	2.11 (–,12.1)	4.16	4.10	3.71		4.02		3.78	3.93
GNI	→6)-α-D-Glc*p*N-(1→	5.42 (4.7) (8.1)[c]	3.92 (11.2)	3.79		3.61	4.05	4.13	≈3.82				
GNII	→6)-β-D-Glc*p*N-(1→	4.62 (7.3)	≈3.81	≈3.79		4.63	3.90	4.63 (4.6)	4.47 (8.1, 10.2)				
L-Ala		4.1	1.54										

[a] Measured at 27 °C in D_2O (pH 7.0). [b] Letters e and a indicate equatorial and axial H-3's of KDO, respectively. [c] $J_{P,H}$ in hertz.

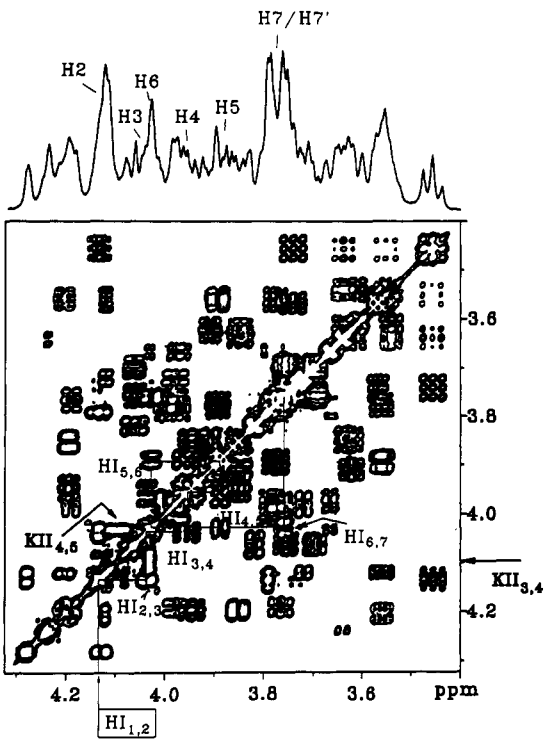


FIGURE 4: Contour plot of the 2D COSY for the ring proton region (4.3–3.4 ppm) of LOS backbone OS. The spectrum was measured using a narrow sweep width (1.59 kHz) and a data set of 512 × 1024 points. The connectivity pathway for heptose (HI) is indicated.

of the ^{13}C resonances, effected by correlation with the attached proton resonances, are presented in Tables 3 and 4 for the backbone OS and the LOS-OH, respectively.

In conjunction with the results from compositional analysis, 2D subspectral analysis clearly established the backbone OS to be composed of the following components: α-D-Gal*p*N (GaN), two L-α-D-Hep*p* (HI and HII), α-D-Glc*p* (G), β-D-Glc*p*N (GNII), two KDO*p* (KI and KII), and D-glucosaminitol (GNI′). The two heptose residues were identified in the COSY by the observed small $J_{1,2}$ (<1 Hz) and $J_{2,3}$ (~3 Hz) values, which pointed to *manno*-pyranosyl ring systems, and by the fact that eight ^1H resonances could be associated with each subspectrum. The connectivity pathway identified for heptose HI is shown in Figure 4. The α-configuration was evident for both of these residues from the 170 Hz value measured for $^1J_{C\text{-}1,H\text{-}1}$ (Bock & Pedersen, 1974) and the occurrence of a single intraresidue NOE between the H-1 and H-2 resonances (Richards & Perry, 1988). Analogously, the magnitude of the vicinal couplings led to the identification of α-linked ($J_{1,2} \leq 4$ Hz) and β-linked ($J_{1,2} \approx 8$ Hz) *gluco*-pyranosyl ring systems ($J_{2,3}$, $J_{3,4}$, and $J_{4,5} \approx 10$ Hz) and one α-linked *galacto*-pyranosyl unit ($J_{1,2}$, $J_{3,4}$, and $J_{4,5} < 4$ Hz; $J_{2,3} \approx 10$ Hz). Chemical shift values (Bock & Thøgersen, 1982) indicated that the first ring system corresponded to an α-D-Glc*p* residue, while the latter two spin systems were attributed to the β-D-Glc*p*N and α-D-Gal*p*N residues, respectively, since the respective H-2 resonances could be mapped directly via $^1J_{C,H}$ connectivities (in the HMQC) into the amino-substituted region of the ^{13}C spectrum (50–60 ppm) (Tables 1 and 3).

Apart from the resonances associated with the β-1,6-linked D-glucosamine disaccharide moiety of the lipid A-OH (GNI and GNII) present in the LOS-OH, significant chemical shift differences (>0.1 ppm) were observed only for ^1H resonances associated with the heptose HI residue and GalN H-2 (Tables 1 and 2). The latter resonance showed a substantial downfield shift (0.65 ppm) in the LOS-OH, which could be attributed to substitution at the N-2 position.

The H-2, H-4, and H-5 resonances of HI showed substantial downfield shifts of ca. 0.5 ppm. Furthermore, a comparison of the ^{13}C NMR data from backbone OS and the LOS-OH (Tables 3 and 4) suggested that the heptose HI residue in the LOS-OH is substituted at the O-2 and O-4 positions. This was readily apparent from the observed downfield-shifted values (ca. 4 ppm) of C-2 and C-4 resonances with concomitant upfield-shifted values (4–5 ppm) for the adjacent C-1 and C-3 resonances in the LOS-OH (Bock & Pedersen, 1983). Methylation analysis also pointed to substitution at these positions (see below). These positions were subsequently identified as the sites of phosphate substitution from a ^{31}P–^1H correlation experiment. In contrast, in the spectrum of the LOS-OH, the heptose HII connectivity pathway was difficult to follow beyond H-4; even so, the chemical shifts of H-1 to

Table 3: ^{13}C Chemical Shifts (ppm) of Backbone OS from *P. aeruginosa* 06 LOS[a] (Mutant R5)

residue	glycose unit	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
G	α-D-Glc*p*-(1→	100.84	72.42	73.38	70.08	72.75	61.02		
GaN	→4)-α-D-Gal*p*N-(1→	98.79	56.15	67.38	77.98	73.08	61.31		
HI	→3)-L-α-D-Hep*p*-(1→	101.38	70.96	79.29	66.47	73.38	69.89	64.68	
HII	→3)-L-α-D-Hep*p*-(1→	102.91	70.81	79.29	66.14	72.75	69.89	63.56	
KI	→5)-α-KDO*p*-(2→ 4 ↑	175.23[b]	100.53	35.06	70.67	73.38	72.87	70.08	64.27
KII	α-KDO*p*-(2→	175.74[b]	100.84	35.06	66.89	66.89	72.87	70.21	64.02
GNII	→6)-β-D-Glc*p*N-(1→	101.70	56.84	74.42	70.21	75.46	62.45		
GNI′	→6)-D-GlcNol	59.71	52.06	66.89	71.64	70.67	72.87		

[a] Measured at 27 °C in D_2O (pH 7.4). [b] Assignments may be reversed.

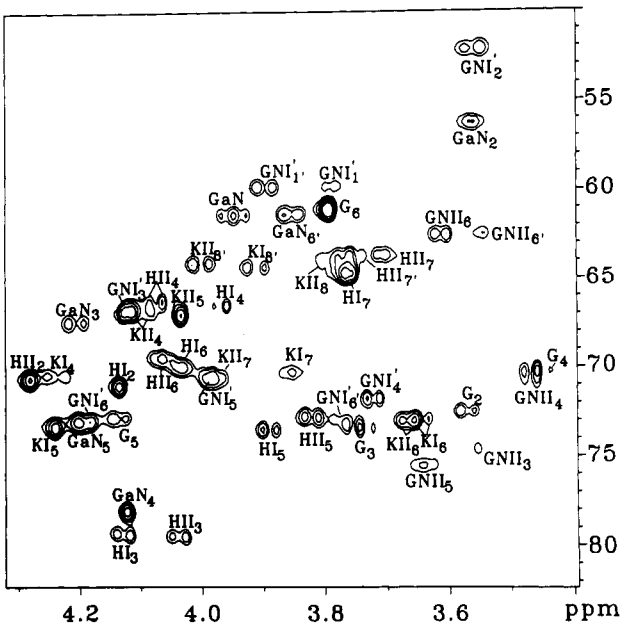


FIGURE 5: Heteronuclear 2D ^{13}C–^1H chemical shift correlation map of the ring region (4.3–3.4 ppm) for the LOS backbone OS. Assignments are indicated.

H-4 showed little difference from those of the HII residue in the backbone OS, which served to establish its identity.

The ^{31}P NMR spectrum of the LOS-OH showed four major phosphomonoester peaks at 3.21, 2.58, 0.50, and 0.02 ppm. As expected, strong connectivities were observed in the 2D ^{31}P–^1H HMQC correlation map (Figure 6) between the ^{31}P signals at 0.02 and 3.21 ppm and the ^1H resonances for GNI H-1 and GNII H-4, respectively, in the lipid A-OH region of the molecule. In addition, $^{31}P/^1H$ connectivities were observed

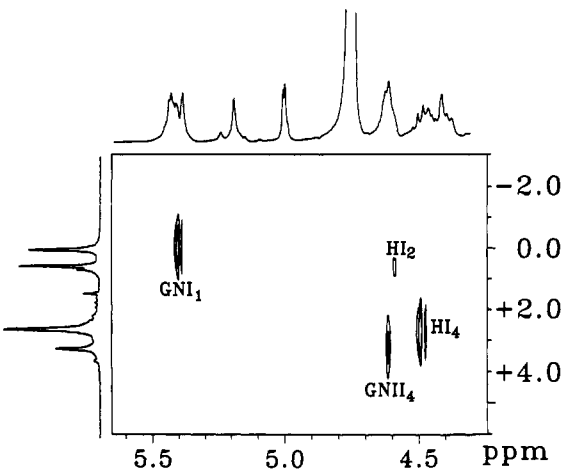


FIGURE 6: Heteronuclear ^{31}P–^1H chemical shift correlation map of the LOS-OH recorded in D_2O at pH 7.0 and 27 °C. The assignments are indicated.

between the signal at 0.50 ppm and HI H-2 and between the signal at 2.58 ppm and HI H-4, unequivocally demonstrating phosphate substitution at both the C-2 and C-4 positions of this heptose residue.

The sequence of the glycoses within the outer core region of the LOS oligosaccharide was established from the occurrence of transglycosidic proton NOE connectivities between anomeric and aglyconic protons on adjacent glycosidically linked residues in the backbone OS. NOEs were measured in the 2D mode, and part of the NOESY contour plot is shown in Figure 7. Transglycosidic NOEs were observed for G H-1/GaN H-4, GaN H-1/HII H-3, HII H-1/HI H-3, and HI H-1/KI H-5 resonance pairs, which established the linear sequence of glycoses in the core region of the LOS as α-D-

Table 4: ^{13}C Chemical Shifts (ppm) of *O*-Deacylated LOS from *P. aeruginosa* 06[a] (Mutant R5)

residue	glycose unit	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
G	α-D-Glc*p*-(1→	100.25	72.37	72.83	69.50	72.17	60.07		
GaN	→4)-α-D-Gal*p*N-(1→	98.43	50.83	67.07	77.96	71.90	62.23		
	↓ 4								
HI	→3)-L-α-D-Hep*p*-(1→	97.35	74.50	74.40	70.51	72.67	69.19	63.20	
	2 ↑								
HII	→3)-L-α-D-Hep*p*-(1→	102.33	69.41	76.59	68.75				
KI	→5)-α-KDO*p*-(2→			34.17	66.15	67.71	≈72.37	69.70	63.84
	4 ↑								
KII	α-D-Kdo*p*-(2→			35.36	65.95	66.90	≈72.37	70.83	62.73
GNII	→6)-β-D-Glc*p*N-(1→	101.51	55.33	70.76	69.30	70.97	63.84		
GNI	→6)-α-D-Glc*p*N-(1→	93.53	53.92	74.30	69.50	72.01	68.20		
L-Ala		49.35	17.02						

[a] Measured at 27 °C in D_2O (pH 7.0).

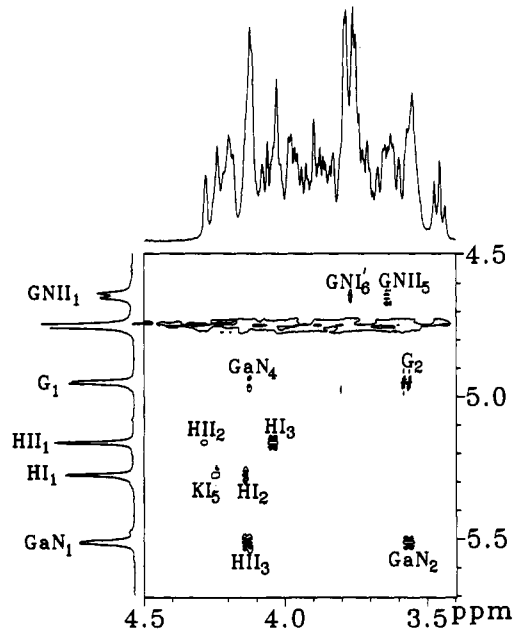


FIGURE 7: 2D NOESY spectrum of the LOS backbone OS showing NOE connectivities relating anomeric proton resonances of the five glycopyranosyl residues.

Glc*p*-(1→4)-α-D-Gal*p*N-(1→3)-L-α-D-Hep*p*-(1→3)-L-α-D-Hep*p*-(1→5)-α-KDO*p*. The NOE connectivities are shown in Figure 8. Intraresidue NOEs were observed between the H-1 and H-2 of each of the four glycose residues (Figure 7), confirming the assigned α-configurations of each of these residues. In addition, a transglycosidic NOE was also observed between H-1 of the GNII and the H-6 protons of the D-glucosaminitol (GNI′) (Figure 7), confirming the presence of the β-1,6-linkage in the D-glucosamine disaccharide moiety (Figure 8).

Results from methylation analysis on samples of the core oligosaccharide obtained from the LOS by mild acid hydrolysis and its dephosphorylated analogue confirmed the positions of the glycosidic linkages (Table 5). The methylated and dephosphorylated core oligosaccharide, upon hydrolysis with TFA, did not lead to quantitative release of the methylated GalN and Hep derivatives, indicating the relative resistance of particularly the GalN glycosidic linkage. This was confirmed from positive FAB-MS analysis of the acid hydrolysate, following $NaBD_4$ reduction and acetylation, which showed abundant ions at m/z 712 and 960 arising from the respective derivatized GalN(Ala)–heptitol and GalN-(Ala)–Hep–heptitol derivatives (Figure 9). This result also served to confirm the partial sequences of these residues in the oligosaccharide and was consistent with substitution of the GalN residue by the alanine moiety.

Table 5: Methylation Analysis of the Core Oligosaccharide and the Derived Dephosphorylated Analogue from *P. aeruginosa* 06 (Mutant R5)

	relative detector response[a]	
methylated product	core oligosaccharide	dephosphorylated core oligosaccharide
2,3,4,6-Me_4-Glc	1.0	1.0
2,3,6-Me_3-GalN	0.59	0.30
2,4,6,7-Me_4-Hep	0.27	1.70
4,6,7-Me_3-Hep	0.21	
6,7-Me_2-Hep	0.20	
1,2,4,6,7,8-Me_6-KDO	0.23	

[a] Relative to 2,3,4,6-tetramethylglucose = 1.0.

Methylation analysis of the LOS-OH by employing a modified procedure, involving carboxyl reduction of the KDO methyl esters, subsequent methylation, and hydrolysis (Masoud et al., 1994a), afforded 1,4,5,7,8-penta-*O*-methyl and 1,7,8-tri-*O*-methyl derivatives of KDO, which unambiguously established the presence of the terminal KDO residue and the substitution sites on the branching KDO residue (i.e., KI). Correspondingly, a comparison of the ^{13}C chemical shift values of C-4 and C-5 for the two KDO residues in the backbone OS (Table 3) shows that these resonances are downfield shifted by 4–6 ppm in KI, consistent with substitution at the corresponding positions. As discussed above, the proton NOE results indicate that the O-5 position in KI serves as the point of attachment for the core oligosaccharide chain (Figure 8). A three-bond $^{13}C/^{1}H$ connectivity between KII C-2 (100.8

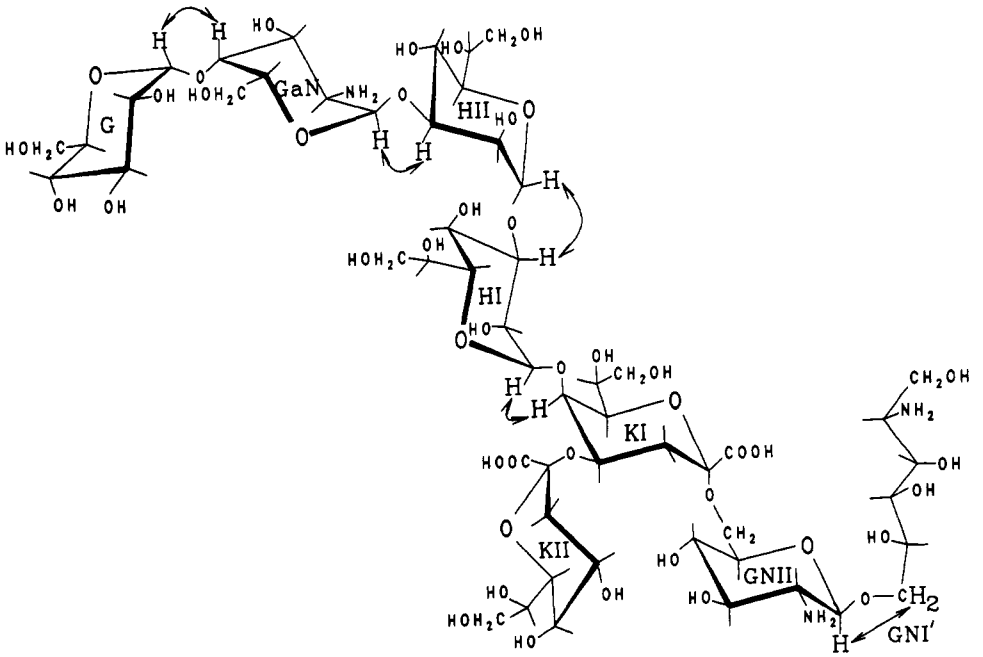


FIGURE 8: Structure of the backbone OS from *P. aeruginosa* IATS serotype 06 mutant R5 LOS, illustrating the network of observed transglycosidic NOE connectivities used to establish the sequence of the glycopyranosyl residues.

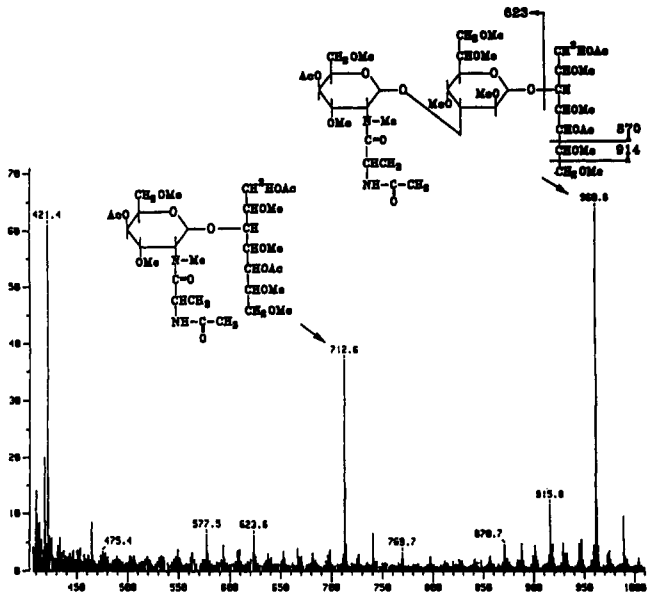


FIGURE 9: Positive ion FAB mass spectrum of the methylated, partially hydrolyzed, reduced ($NaBD_4$), and acetylated OS from *P. aeruginosa* IATS serotype 06 mutant R5 LOS.

ppm) and KI H-4 (4.24 ppm) in the HMBC experiment clearly established the ketosidic linkage between KII and KI. Unambiguous assignment of the KII C-2 resonance was made possible by the occurrence of intraresidue two-bond $^{13}C/^{1}H$ connectivities between the ^{13}C signal at 100.8 ppm and the KII H-3 protons at 2.21 and 1.77 ppm. Long-range $^{13}C/^{1}H$ correlations from KI C-2 (100.5 ppm) to KI H-3 protons provided further evidence for assignment of the ketosidic carbon resonances; however, the expected KI C-2/GNII H-6 transglycosidic connectivity was not detectable. The absence of this $^{13}C/^{1}H$ connectivity may result from the conformation about the KI C-2–GNII C-6 glycosidic linkage, as perhaps is reflected by the unusually high-field value of the GNII C-6 resonance both in the backbone OS (62.45 ppm) and the LOS-OH (63.84 ppm) (Strain et al., 1985). FAB-MS of the permethylated backbone OS showed an abundant M + K ion (m/z 1963; M_r = 1924), providing firm evidence for an intact OS containing both the core and β-D-Glc*p*N-(1→6)-glucosaminitol moieties. On biosynthetic grounds (Raetz, 1990), one theoretically would expect that the core oligosaccharide is linked to the lipid A region via a ketosidic linkage from the first KDO to the O-6 position of the β-linked D-glucosamine residue (GNII). The backbone OS structure is shown in Figure 8.

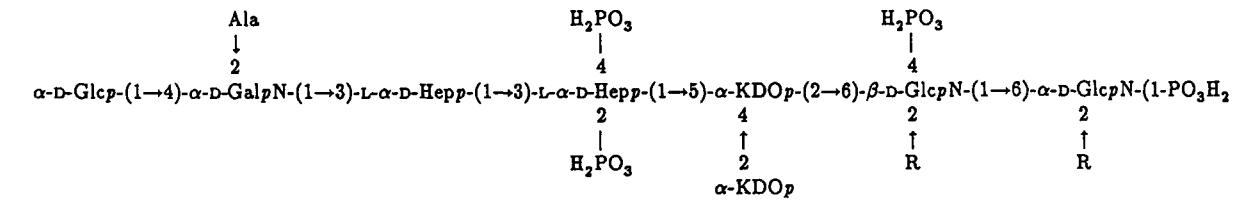
DISCUSSION

LPS of *P. aeruginosa* possesses the same general architecture as that of *Enterobacteriaceae*, consisting of an O-specific polysaccharide that is linked to a lipid A via a core oligosaccharide (Wilkinson, 1983). Currently, 20 serotypes are recognized (Liu & Wang, 1990) on the basis of differences in the nature of their O-specific polysaccharides (Knirel, 1990). The chemical structures of the oligosaccharide repeating units of the *O*-polysaccharides of 17 *P. aeruginosa* serotypes have been reported (Knirel, 1990). In contrast, investigations of the core region of *P. aeruginosa* LPS have provided only limited structural details. Compositional analysis has indicated that Glc, Rha, KDO, Hep, GalN, Ala, and phosphate are the major components (Drewry et al., 1975; Rowe & Meadow, 1983), and Rowe and Meadow (1983) have reported a partial structure for the core oligosaccharide of a serotype 03 strain (PAC1R) and its LPS defective mutants. Immunochemical investigations of O-chain-deficient and core defective mutants have indicated that the core oligosaccharide regions from different serotypes may differ in structure, although recent studies (de Kievit & Lam, 1993) point to the presence of a conserved inner core region. *P. aeruginosa* serotype 06 is the most frequently encountered strain from clinical sources and was therefore selected for the OS structural studies. Western immunoblots of wild-type and mutant LPSs with a series of core-specific monoclonal antibodies indicated a common basal structure. One of these mutants, mutant R5 derived from serotype O6, showed a single LOS band migrating faster than that of the complete core region of wild-type LPS (Dasgupta et al., 1994; Masoud et al., 1992), indicating several sugar deletions. This observation was well supported by compositional analysis, which showed the absence of L-Rha and significantly less D-Glc in the core region of the mutant R5 LOS.

In most of the chemical studies reported to date, the analytical strategy for LPS structural analysis has taken advantage of the acid liability of the KDO ketosidic linkage for obtaining polysaccharide and/or oligosaccharide components (Lüderitz, 1971). This has proved to be a very successful approach for the structural analysis of the O-specific polysaccharides, but has led to only limited structural information in the core oligosaccharide region (Knirel, 1990). In the case of R-type LPS, mild acid hydrolysis often affords mixtures of oligosaccharides arising from acid-mediated modifications of the KDO residue at the reducing terminus (McNicholas et al., 1987; Phillips et al., 1990; Auzanneau et al., 1991; Melaugh et al., 1992), which generally are difficult to purify. This has been observed previously by us (Lacroix et al., 1993) and by others (Philips et al., 1992), and it also proved to be the case with the R5 LOS, as evidenced by the apparent heterogeneity in the anomeric proton region of the ^{1}H NMR spectrum of the core oligosaccharide and its aqueous HF derived dephosphorylated analogue. With high molecular weight LPS, acid-catalyzed modifications of KDO residues do not pose a problem since the reducing terminus KDO is only a minor component of the polymeric material. A further deficiency of this approach is the loss of structural information for the inner core region since all KDO residues are cleaved during mild acid hydrolysis.

By employing a deacylation strategy, oligosaccharides are obtained that are attached to a putative lipid A moiety and thereby are representative of the complete backbone LOS structure. Thus, complete deacylation and dephosphorylation

Chart 1



of the R5 LOS followed by reduction of the reducing terminus glucosamine residue gave the backbone oligosaccharide, which could be analyzed directly by ^1H and ^{13}C NMR methods. During this sequence of reactions, the KDO region of the molecule remained intact, providing for the first time a definitive structural model for the core–lipid A region of *P. aeruginosa* LPS. Furthermore, examination of *O*-deacylated LOS by ^1H and ^{31}P NMR led to the identification of the substitution pattern of phosphate groups in the inner core region of the LOS. Substitution of inner core sugar residues by mono- and pyrophosphoesters is known to be important in maintaining the integrity of the *Pseudomonas* outer membrane and may play a role in membrane–antibiotic interactions (Moore & Hancock, 1986; Cox & Wilkinson, 1991). The O-deacylated LOS of the mutant strain R5 derived from *P. aeruginosa* IATS serotype O6 has the structure given in Chart 1.

On the basis of immunochemical (Dasgupta et al., 1994; de Kievit & Lam, 1993) and structural studies (Altman et al., 1993) performed in our laboratories, we have established that the oligosaccharide from the mutant R5 LOS is a common basal structural feature expressed by most *P. aeruginosa* LPS serotypes. Therefore, the R5 core oligosaccharide structure provides a molecular template for further chain extensions.

The NMR-based analytical strategy presented here for the structural determination of LOS is of general applicability. This procedure has been utilized for probing the complete LPS core structures of *P. aeruginosa* IATS serotypes O6 (Masoud et al., 1992) and 05 (Altman et al., 1993), as well as the highly branched heptose-deficient LOS of a serotype A reference strain of *Moraxella catarrhalis* (Masoud et al., 1994a), leading to detailed structural models. A major requirement for the success of this approach is the availability of sufficient amounts of material for 2D NMR analysis (practical lower limit of 0.5-1 mg of purified backbone OS sample). The hydrazinolysis approach for obtaining oligosaccharide samples that are representative of the intact LOS backbone structure can also be used for structural studies of LOS that express complex, heterogeneous oligosaccharide mixtures. Currently, we are applying this approach in conjunction with separation techniques, such as HPAEC and capillary electrophoresis, for defining the detailed structures of wild-type and mutant strains of *H. influenzae* type b.

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