Complete Lipopolysaccharide of *Plesiomonas shigelloides* O74:H5 (Strain CNCTC 144/92). 2. Lipid A, Its Structural Variability, the Linkage to the Core Oligosaccharide, and the Biological Activity of the Lipopolysaccharide[†,‡]

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ABSTRACT: *Plesiomonas shigelloides* is a Gram-negative bacterium associated with waterborne infections, which is common in tropical and subtropical habitats. Contrary to the unified antigenic classification of *P. shigelloides*, data concerning the structure and activity of their lipopolysaccharides (LPS and endotoxin) are limited. This study completes the structural investigation of phenol- and water-soluble fractions of *P. shigelloides* O74 (strain CNCTC 144/92) LPS with the emphasis on lipid A heterogeneity, describing the entire molecule and some of its biological in vitro activities. Structures of the lipid A and the affinity-purified decasaccharide obtained by de-N,O-acylation of *P. shigelloides* O74 LPS were elucidated by chemical analysis combined with electrospray ionization multiple-stage mass spectrometry (ESI-MS^n), MALDI-TOF MS, and NMR spectroscopy. Lipid A of *P. shigelloides* O74 is heterogeneous, and three major forms have been identified. They all were asymmetric, phosphorylated, and hexaacylated, showing different acylation patterns. The β-Glc*p*N4P-(1→6)-α-Glc*p*N1P disaccharide was substituted with the primary fatty acids: (*R*)-3-hydroxytetradecanoic acid [14:0(3-OH)] at N-2 and N-2′ and (*R*)-3-hydroxydodecanoic acid [12:0(3-OH)] at O-3 and O-3′. The heterogeneity among the three forms (I−III) of *P. shigelloides* O74 lipid A was attributed to the substitution of the acyl residues at N-2′ and O-3′ with the secondary acyls: (I) *cis*-9-hexadecenoic acid (9*c*-16:1) at N-2′ and 12:0 at O-3′, (II) 14:0 at N-2′ and 12:0 at O-3′, and (III) 12:0 at N-2′ and 12:0 at O-3′. The pro-inflammatory cytokine-inducing activities of *P. shigelloides* O74 LPS were similar to those of *Escherichia coli* O55 LPS.

Plesiomonas shigelloides is a Gram-negative, facultatively anaerobic, flagellated rod, belonging to the family Enterobacteriaceae (*1*). Although *P. shigelloides* is particularly common in tropical and subtropical habitats (*2*), it can be found in the aquatic environment of cold climates as a cause of waterborne infections (*3*). It was reported as the third most common microbial etiological agent in outbreaks of travellers' diarrhea in Japan and China (*2*). *P. shigelloides* is also responsible for a variety of extraintestinal infections, such as cholecystitis, proctitis, pyosalpinx, septic arthritis, endopthalmitis, menningitidis, and bacteraemia, which usually have an gastrointestinal origin (*2*, *4*). Sepsis and meningitis are the major and the most serious forms of extraintestinal disease caused by *P. shigelloides* and are associated with a high mortality rate, despite the appropriate antibiotic therapy and intensive care (*2*).

The pathogenicity of *P. shigelloides* is not yet fully understood. These bacteria release several toxins to the media, e.g., cholera-like toxin (*5*), thermostable and thermolabile toxins (*6*, *7*), β-hemolysin (*8*), and cytotoxin complex (*9*). One of the potential virulence factors of *P. shigelloides* is endotoxin (LPS).[1] It is the major component of the outer membrane and the main surface antigen (O-antigen). LPS was also identified as the constituent of cytotoxin complex, formed with anti-cholera toxin-reactive proteins released by *P. shigelloides* (*9*). LPS plays a key role during severe Gram-negative infections, sepsis, and septic shock. It is one of the most powerful natural activators of the innate immune system and provides a "pathogen-associated molecular pattern" recognized by Toll-like receptors (*10*).

Chemically, LPS is composed of O-specific polysaccharide, core oligosaccharide, and lipid A. The biological activity

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[1] Abbreviations: LPS, lipopolysaccharide; ESI, electrospray ionization; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; MS^n, multiple-stage mass spectrometry; PBS, phosphate-buffered saline; GC, gas chromatography; COSY, correlated spectroscopy; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; ROESY, rotating frame nuclear Overhauser effect spectroscopy; HMBC, heteronuclear multiple-bond correlation; HMQC, heteronuclear multiple-quantum coherence; HSQC, heteronuclear single-quantum coherence; DEPT, distortionless enhancement by polarization transfer; L-α-D-Hep, α-L-*glycero*-D-*manno*-heptose; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid.

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of LPS depends mainly on the chemical structure of lipid A and is only modulated by core oligosaccharide and O-specific polysaccharide. Lipid A, upon recognition by eukaryotic cells, initiates the inflammatory response and activates defense mechanisms of the host (*11*).

Lipid A is an unusual phosphoglycolipid and structurally the most conserved segment of LPS. All endotoxically active lipid A forms investigated to date share a common architecture. They are built of the D-*gluco*-configured disaccharide of pyranosidic hexosamines, which are substituted with ester-linked and amide-linked (*R*)-3-hydroxy fatty acids, with two of them being further acylated. The fine structure of this basic template varies among bacteria of different origin. The structural diversity of lipid A depends mainly on the type of hexosamine residues, the presence of phosphates and other negatively charged groups, and especially the type, chain length, and location of the acyls (*12*).

Contrary to the unified antigenic classification of *P. shigelloides* (*13*−*16*), data concerning the structure and activity of *P. shigelloides* LPS are limited. To date, only structures of the O-specific polysaccharides from strains 22074, 12254 (*17*), and CNCTC 113/92 (O54) (*18*) and the core oligosaccharide of strain CNCTC 113/92 (*19*) were elucidated.

Recently, we have elucidated for the first time the structure of *P. shigelloides* lipid A isolated from strain CNCTC 113/92 (*20*), which was identified as a novel structure among known lipid A molecules. In our ongoing studies on the endotoxins of *P. shigelloides*, LPS from strain CNCTC 144/92 (O74) has been chosen due to the lack of serological cross-reactivity with the known core oligosaccharide of *P. shigelloides* O54 LPS and unusual physicochemical properties of this LPS. In ref *60*, we present structural studies of *P. shigelloides* O74 (strain CNCTC 144/92) LPS, comprising the O-specific polysaccharide, the core oligosaccharide, and the linkage between them.

In contrast to *P. shigelloides* O54 LPS, *P. shigelloides* O74 LPS partitioned between phenol and water phases during the workup procedures and the majority of this LPS was recovered from the phenol phase. As demonstrated in ref *60*, both phenol- and water-soluble preparations represented smooth forms of the LPS. The lipophilic nature of phenol-soluble LPS is caused by O-acetylation of O-repeats. HR-MAS NMR of the O-antigens directly on bacterial cells demonstrated that *P. shigelloides* O74 LPS is present predominantly in the O-acetylated form. Preliminary MALDI-TOF comparative analysis of lipid A fractions isolated from *P. shigelloides* O74 and O54 LPS suggested, despite some similarities, higher heterogeneity and the presence of a novel form characteristic of lipid A of *P. shigelloides* O74.

It is known that some pathogenic Gram-negative bacteria synthesize naturally heterogeneous lipid A as a result of different conditions of microbial growth; therefore, a number of lipid A species can be found in a single bacterial population. However, even small deviations from the basic lipid A structure can lead to different responses of the host cells in vitro (*11*). To gain further insight into the lipid A expressed by *P. shigelloides*, it was important to determine the structure of the lipid A part of *P. shigelloides* O74 LPS with the emphasis on its heterogeneity.

Here we report on the structure of the lipid A isolated from phenol- and water-soluble *P. shigelloides* O74 LPS and its linkage to the core oligosaccharide. We have characterized its detailed structure, including the location, isomerism of identified fatty acids, and heterogeneity of lipid A forms derived from both water- and phenol-soluble fractions of LPS.

Thus, this study completes the structural investigation of *P. shigelloides* O74 LPS, resolving the entire molecule and describing some of its biological activities in vitro.

EXPERIMENTAL PROCEDURES

Bacteria. *P. shigelloides* strain CNCTC 144/92, classified as serovar O74 according to Aldova's antigenic scheme (*14*−*16*, *21*, *22*), was obtained from the Institute of Hygiene and Epidemiology (Prague, Czech Republic). The bacteria were grown and harvested as previously described (*18*, *23*).

Cell Lines. The mouse macrophage-like J-774A.1 cell line was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). J-774A.1 cells were grown in Dulbecco's medium supplemented with 10% fetal calf serum (FCS) (Gibco, Biocult, Glasgow, U.K.).

Lipopolysaccharide and Lipid A. LPS was extracted from bacterial cells by the hot phenol/water method (*24*) and purified as previously reported (*23*). LPS was collected from both phenol and water phases as described in ref *60*. Lipid A was isolated from the LPS (200 mg) by mild acidic hydrolysis with 1.5% acetic acid, at 100 °C, for 2 h. The reaction mixture was cooled and centrifuged. The sediment was resuspended in water and freeze-dried. The yields of lipid A preparations were 11 and 16% of the LPS extracted into water and phenol phases, respectively.

De-N,O-Acylation of LPS. De-O-acylation of LPS was achieved by mild hydrazinolysis (*25*). Briefly, LPS (200 mg) was dissolved in anhydrous hydrazine (5 mL), and the reaction was carried out at 37 °C for 30 min. The mixture was cooled and added to cold acetone (−20 °C) to convert hydrazine to acetone hydrazone. The precipitate of the de-O-acylated LPS was collected by centrifugation (4000*g* at −20 °C for 30 min), dissolved in water, and freeze-dried. The de-O-acylated LPS was dissolved in aqueous 4 M KOH (10 mL) and hydrolyzed in a sealed tube under nitrogen (120 °C for 16 h). The reaction mixture was neutralized with $HClO_4$. Most of the insoluble $KClO_4$ sediment was removed by centrifugation and further desalted using a Bio-Gel P-2 column. Products from the de-N,O-acylated LPS were separated by affinity chromatography on a serotonin−Sepharose 4B column (*20*). De-N,O-acylated LPS (10 mg) was dissolved in water (1 mL) and loaded on the column. Fractions (40 mL) eluted by a discontinuous ammonium acetate gradient (0.025, 0.05, 0.1, 0.25, and 1 M) were collected, freeze-dried, and checked by ^{1}H NMR spectroscopy. The fraction (3.5 mg) containing core oligosaccharide and lipid A disaccharide backbone, devoid of the O-specific polysaccharide, was further analyzed by NMR spectroscopy.

Analytical Procedures. To determine the absolute configuration of GlcN residues of the lipid A carbohydrate backbone, lipid A (5 mg) was hydrolyzed in 4 N HCl at 100 °C for 12 h. The solution was evaporated to dryness with a stream of compressed air. The sample was subjected to chloroform/water extraction (v/v, 1:2). The water-soluble part was lyophilized and transferred to a HF reaction vessel. Dephosphorylation was performed with aqueous 48% HF

(1 mL) at 4 °C for 72 h, and then HF was evaporated to dryness with a stream of compressed air. The dried sample was dissolved in water and lyophilized. The absolute configuration of GlcN was determined as described by Gerwig et al. using (*R*)-2-butanol for the formation of 2-butyl glycosides (*26, 27*). The trimethylsilylated butyl glycosides were then identified by comparison with authentic samples produced from GlcN (Sigma, St. Louis, MO) and (*R*/*S*)-2-butanol (Fluka, Buchs, Switzerland) on GC−MS. GC−MS was carried out with a Hewlett-Packard 5971A system using an HP-1 fused-silica capillary column (0.2 mm × 12 m) and a temperature program (from 150 to 270 °C at 8 °C/min). Qualitative analysis of lipid A was done separately for amide- and ester-bound fatty acids using chemical analysis followed by GC−MS (*28*). Ester-bound fatty acids were liberated from intact LPS by treatment with CH_3ONa in methanol, and the amide-linked fatty acids were released from de-O-acylated LPS using aqueous 4 M KOH at 120 °C for 16 h. Prior to analysis, LPS was additionally purified by extraction with a chloroform/methanol solution (2:1, v/v) to remove membrane phospholipids. The location of the double bond was determined by conversion of the methyl esters of the monoenoic fatty acid to dimethyldisulfide (DMDS) derivatives and GC−MS analysis (*29*). Cis−trans isomerism of the double bond was assessed using silver ion TLC (*30, 31*) with modification. Briefly, TLC plates (Merck, Darmstadt, Germany) were impregnated in a TLC tank that contained 15% $AgNO_3$ in acetonitrile. Shortly before being used, the plates were activated by being dried at 110 °C for 1 h. Development was carried out at −20 °C with toluene, and spots were located by thoroughly spraying with 10% H_2SO_4 in ethanol and visualized by heating at 200 °C. The absolute configurations of 3-hydroxy fatty acids in the LPS were identified by GC−MS (HP-5 S.M. column, 0.25 mm × 30 m; temperature program from 150 to 270 °C at 8 °C/min) using (*S*)-phenylethylamide 3-methoxy derivatives (*32*).

Mass Spectrometry. Matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry was carried out on a Kratos Kompact-SEQ instrument. 9*H*-Pyrido[3,4-*b*]indole [1% in a 1:1 acetonitrile/water mixture (v/v)] and 2,4,6-trihydroxyacetophenone [25 mg/mL in a 1:1 acetonitrile/water mixture (v/v)] were used as matrices for the analysis of lipid A in negative and positive ion mode, respectively. To obtain positive-ion mass spectra of lipid A, the sample was dissolved in a chloroform/isopropyl alcohol/water mixture (5:3:0.25, v/v/v) and desodiated as described by Gudlavalleti et al. (*33*). Lipid A was extracted with a chloroform/methanol mixture prior to all MS analyses. Negative-ion electrospray mass spectra (ESI-MS^n) were recorded using an ESQUIRE-LC (Bruker Daltonics, Bremen, Germany) ion trap mass spectrometer. Prior to the analyses, lipid A samples were desalted by extraction with a chloroform/water mixture (1:1, v/v) and dissolved in a methanol/chloroform solution (1:1, v/v, 1 mg/mL). The samples were continuously infused through the capillary head at 4 kV into the ion source, using a linear syringe pump at a rate of 2 μL/min. Spectra were scanned in the range of *m*/*z* 200−2000. The mass isolation window for the precursor ion selection was set to 4 Da in all the MS^n analyses.

NMR Spectroscopy. All NMR spectra were obtained on Bruker DRX 400 and DRX 600 spectrometers. NMR spectra of isolated poly- and oligosaccharide fractions were obtained for ^2H_2O solutions at 30 °C using acetone (δ_H 2.225, δ_C 31.05) as an internal reference. In ^{31}P NMR spectroscopy experiments, 80% phosphoric acid was used as an external reference. The poly- and oligosaccharides were repeatedly exchanged with ^2H_2O (99%) with intermediate lyophilization. The data were acquired and processed using standard Bruker software. The processed spectra were assigned with the help of SPARKY (*34*). The signals were assigned by one- and two-dimensional experiments (COSY, clean-TOCSY, NOESY, ROESY, HMBC, HSQC-DEPT, and HSQC with and without carbon decoupling). In the clean-TOCSY experiments, mixing times of 30, 60, and 100 ms were used. The delay time for HMBC was 60 ms, and the mixing time for NOESY was 200 ms.

Stimulation of TNFα, IL-6 Release, and NO Production in J-774A.1 Cells. J-774A.1 cells were plated in 24-well tissue culture plates (Nunc, Roskilde, Denmark) at a density of 10^6 cells/well in 1 mL of Dulbecco's medium supplemented with 10% FCS (v/v) and incubated in a humidified atmosphere at 37 °C with 5% CO_2 for 16 h. Prior to the stimulation with the appropriate LPS, the cells were washed twice with the serum-free medium. The cells were stimulated with LPS (1 μg, 100 ng, or 10 ng) in 1 mL of medium. The supernatants were collected after 4, 24, and 48 h for determination of TNFα, IL-6, and NO concentrations, respectively, and stored at −80 °C.

IL-6 and TNFα Assays. Concentrations of IL-6 and TNFα in the supernatants were determined using the OptEIA kits (Pharmingen, San Jose, CA). Tests were performed using polystyrene MaxiSorb microtiter plates (Nunc) according to the provided instructions. A TMB (3,3′,5,5′-tetramethylbenzidine) reagent set, provided by Pharmingen, was used as a substrate for horseradish peroxidase. The optical density was measured at 450 nm using a Behring EL311s microplate reader.

Determination of the Amount of NO. The amount of NO in the supernatants collected from stimulated J-774A.1 cells was measured spectrophotometrically as the total nitrite (*35*) using the colorimetric assay with Griess reagent as previously described (*36*).

Statistical Analysis. Assays were performed using triplicate cultures. Data are expressed as means ± the standard deviation (SD), and the significance of differences between them was analyzed using a one-way analysis of variance.

RESULTS

Isolation and Chemical Analysis of Lipid A. LPS of *P. shigelloides* O74 was extracted from bacterial cells by the hot phenol/water method (*24*). Both phenol and water phases were collected, and LPS preparations were purified as described in ref *60*. Mild acidic hydrolysis was used to isolate lipid A from the polysaccharide part of the LPS. The yields of lipid A preparations were 11 and 16% of LPS extracted with the water and phenol phase, respectively. MALDI-TOF mass spectra obtained for both preparations exhibited heterogeneity of lipid A. Ions at the same *m*/*z* values but with different intensities were observed in spectra (Figure 1A,B). Mono- and bisphosphorylated hexaacylated lipid A forms and those additionally substituted with phosphoethanolamine (PEtn) were identified on the basis of mass differences (80 and 123 Da) observed between related ions

Table 1: MALDI-TOF and ESI-MS Analyses of *P. shigelloides* O74 Lipid A[a]

observed ion (*m*/*z*) (MALDI-TOF/ESI-MS)	calculated mass of ion (AVERAGE/MONOISOTOPIC)	interpretation
LA_I [M_I = 1 × 14:0(3-OH), 1 × 12:0(3-OH), 1 × 14:0(3-*O*-9*c*-16:1), 1 × 12:0(3-*O*-12:0), 2 × P, PEtn]		
1890.0/−	1890.3/1889.2	$[M_I - H]^-$
1809.8/1809.3	1810.3/1809.2	$[M_I - P - H]^-$
1767.2/1766.0	1767.3/1766.2	$[M_I - PEtn - H]^-$
1686.8/1686.5	1687.3/1686.2	$[M_I - P - PEtn - H]^-$
−/1504.5	1505.0/1504.0	$[M_I - P - PEtn - 12:0 - H]^-$
1487.6/1487.3	1489.0/1488.0	$[M_I - P - PEtn - 12:0(3\text{-}OH) - H]^-$
−/1450.5	1450.9/1450.0	$[M_I - P - PEtn - 16:1 - H]^-$
−/1269.9	1270.7/1269.9	$[M_I - P - PEtn - 12:0^b - 12:0(3\text{-}OH)^b - H]^-$
682.9/−	682.8/682.4	Y_1^-
LA_{II} [M_{II} = 1 × 14:0(3-OH), 1 × 12:0(3-OH), 1 × 14:0(3-*O*-14:0), 1 × 12:0(3-*O*-12:0), 2 × P, PEtn]		
1864.1/1863.3	1864.3/1863.2	$[M_{II} - H]^-$
1784.1/1783.7	1784.3/1783.2	$[M_{II} - P - H]^-$
1741.1/1739.5	1741.3/1740.1	$[M_{II} - PEtn - H]^-$
1661.1/1660.5	1661.3/1660.2	$[M_{II} - P - PEtn - H]^-$
1558.2/1557.3	1559.0/1558.0	$[M_{II} - PEtn - 12:0 - H]^-$
682.9/−	682.8/682.4	Y_1^-
LA_{III} [M_{III} = 1 × 14:0(3-OH), 1 × 12:0(3-OH), 1 × 14:0(3-*O*-12:0), 1 × 12:0(3-*O*-12:0), P, PEtn]		
1756.1/−	1756.3/1755.2	$[M_{III} - P - H]^-$
1713.3/1712.3	1713.2/1712.1	$[M_{III} - PEtn - H]^-$
1632.7/1632.5	1633.2/1632.2	$[M_{III} - P - PEtn - H]^-$
682.9/−	682.8/682.4	Y_1^-

[a] Average and monoisotopic masses were calculated for different forms of lipid A (LA_I−LA_{III}) and compared with the detected *m*/*z* values. [b] Elimination as a free fatty acid.

(Table 1). Identified forms were substituted with acyls varying in length (mass differences of 28 Da) and in length and saturation (mass differences of 26 Da). The lipid A fraction isolated from the phenol-soluble LPS was used for structural analysis. Determination of the absolute configuration of GlcN constituting the lipid A carbohydrate backbone showed a D-configuration, detected by GC−MS analysis of the trimethylsilylated methyl glycosides. Qualitative analyses of the acyl components of lipid A were carried out separately for amide- and ester-bound fatty acids by using chemical methods and analyzed as methyl esters by GC−MS. The (*R*)-3-hydroxytetradecanoic acid [14:0(3-OH)] was identified as the only amide-linked fatty acid. Dodecanoic (12:0), (*R*)-3-hydroxydodecanoic [12:0(3-OH)], tetradecanoic (14:0), and unsaturated *cis*-9-hexadecenoic (9*c*-16:1) acids were identified as ester-linked fatty acids. Identification of the methoxy derivative, the methyl ester of 3-methoxydodecanoic acid, among fatty acid methyl esters obtained by transesterification with sodium methanolate, showed also the presence of 12:0(3-OH), which was substituted with the "secondary" fatty acid in the native lipid A.

ESI-MS^n Analysis of the Fatty Acid Distribution in Lipid A. The ESI mass spectrum recorded in the negative ion mode showed the heterogeneity of the *P. shigelloides* O74 lipid A isolated from phenol phase LPS (Figure 1C). The most abundant ions at *m*/*z* 1686.5, 1660.5, and 1632.5 belonged to three groups of related ions and represented three populations of lipid A, recognized as the monophosphorylated and hexaacylated molecules. Mass differences between them (26 and 28 Da) showed that molecules represented by these ions differed in length (28 Da) and length and saturation (26 Da) of fatty acids.

To determine the distribution of the fatty acids within the three forms of *P. shigelloides* O74 lipid A, multiple-stage ESI mass spectrometry (ESI-MS^n) analysis was performed for isolated ions at *m*/*z* 1686.5, 1660.5, and 1632.5. The fragmentation rules previously described in the published ESI-MS studies on glycolipids, phospholipids, and lipid A were applied during interpretation of mass spectra (*37*−*39*). Ions arising from the elimination of acyl groups at N-2 and N-2′ were not detected or exhibited very low intensities, due to the higher stability of the acyl amides (*40*). The most abundant ions detected during ESI-MS^n analysis were attributed to the loss of primary or secondary ester-bound fatty acids at O-3 and O-3′. It is known that "charge-remote" and "charge-driven" processes of fatty acid elimination compete during ESI-MS^n (*41*). The charge-remote process dominates in ESI-MS^n analysis, yielding the elimination of free fatty acids and generating a double bond at the substitution position (*39*). The phosphate present at O-4′ of the distal GlcN induced also elimination of the acyl or acyloxyacyl group as neutral ketene derivatives by a charge-driven mechanism, leaving the hydroxyl group at O-3′ of GlcN at the nonreducing end.

ESI-MS^n Analysis of the Lipid A Form Represented by the Ion at m/z 1686.5. To determine the structure corresponding to the ion at *m*/*z* 1686.5, this ion was isolated and fragmented via MS^2 (Figure 2A). On the basis of chemical analysis, this ion could be attributed to monophosphorylated, hexaacylated lipid A substituted with two 14:0(3-OH) fatty acids, two 12:0(3-OH) fatty acids, one 12:0, and one 9*c*-16:1 (Figure 2, outlined structure). The ion at *m*/*z* 1587.8 arose from elimination of the phosphoric acid at O-4 of distal GlcN. Peaks at *m*/*z* 1486.1 and 1470.1 represented neutral loss due to the elimination of ester-linked 12:0 and 12:0(3-OH), respectively. Since elimination of the acyloxyacyl group from O-3 of the distal GlcN could occur by both charge-driven (elimination as ketene) and charge-remote processes (elimination as free fatty acid), the pair of ions at *m*/*z* 1305.9 and 1287.7 were diagnostic for the substitution of 12:0(3-*O*-12:0) at O-3′ (Figure 2, outlined structure). The mass difference between parent and daughter ions corresponded to the calculated mass of 12:0(3-*O*-12:0), which was eliminated as a free acyloxyacyl residue (398.3 Da) and as a ketene

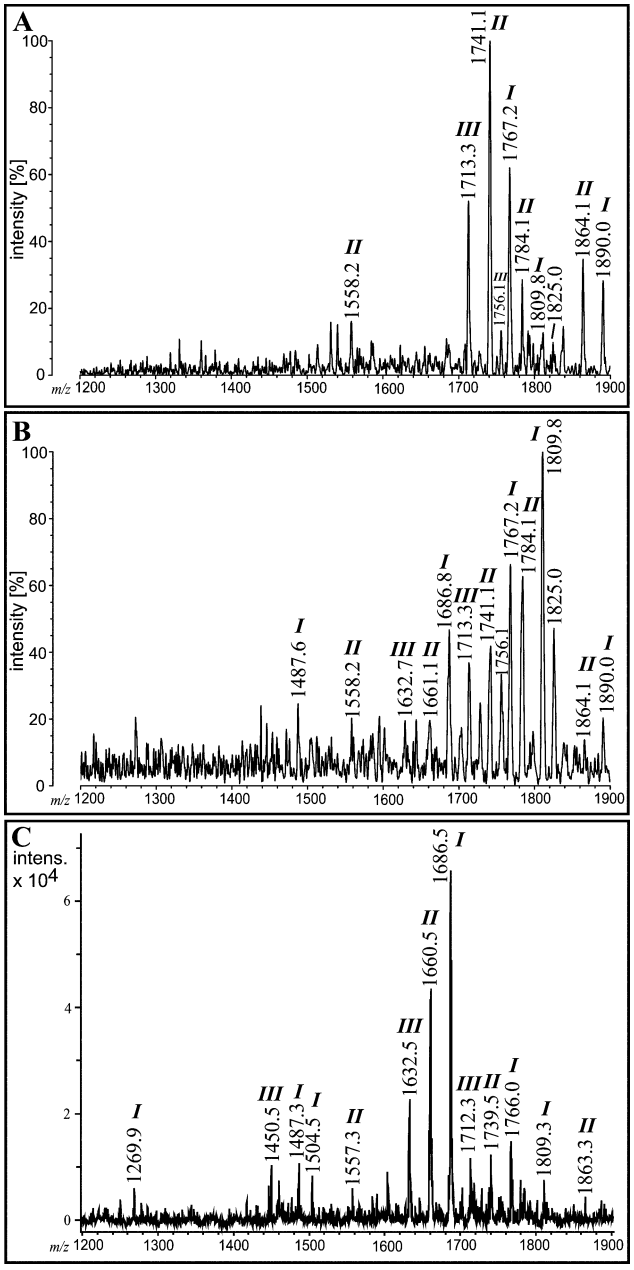


FIGURE 1: Negative ion MALDI-TOF mass spectra obtained for lipid A isolated from *P. shigelloides* O74 lipopolysaccharide: water phase (A), phenol phase (B), and negative ion ESI mass spectrum obtained for lipid A isolated from phenol-soluble LPS (C). The spectra were recorded in the negative, reflectron mode with 9*H*-pyrido[3,4-*b*]indole as a matrix in MALDI-TOF MS analysis. The proposed interpretation of the observed ions is summarized in Table 1. Ions representing different forms of *P. shigelloides* O74 lipid A (LA_I−LA_{III}) are denoted as ***I***−***III***, respectively.

derivative (380.3 Da). The most intense ion at *m*/*z* 1269.9 was formed from the parent ion by the separate elimination of 12:0 and 12:0(3-OH), generating double bonds at the substitution positions. Both fatty acids were identified as ester-linked fatty acids by chemical analysis. Considering the higher stability of acyl amides, we conclude that the reducing GlcN is substituted with 12:0(3-OH) at O-3 and nonreducing GlcN with 12:0(3-*O*-12:0) at O-3. Ions with lower intensities correspond to fragments arising from the inter-residual, intraring fragmentation and loss of water, according to the nomenclature of Domon and Costello (*42*). Such fragments were identified only for the daughter ions (*m*/*z* 1486.1, 1269.9, and 1089.9) and not for the isolated parent ion. Fragments $^{0,4}A_2$ (*m*/*z* 942.6) and $^{0,2}A_2$ (*m*/*z* 1200.8) of the ion at *m*/*z* 1486.1 were detected. The mass difference between ions $^{0,4}A_2$ and $^{0,2}A_2$ clearly showed that reducing GlcN was substituted with 14:0(3-OH) at N-2 and 12:0(3-OH) at O-3. Thus, the nonreducing GlcN, phosphorylated at O-4, was substituted with 14:0(3-*O*-9*c*-16:1) and 12:1 Δ^3 at N-2 and O-3, respectively. The double bond of 12:1 Δ^3 was formed as a result of the previous elimination of 12:0 from acyloxyacyl residue 12:0(3-*O*-12:0) at O-3′ upon MS^2 analysis (Figure 2A).

The most intense ion at *m*/*z* 1269.9 from the MS^2 stage was selected for the MS^3 stage to determine the distribution of the remaining fatty acids (Figure 2B). The mass of the isolated ion was in agreement with the calculated mass of two GlcN molecules, one phosphate, and four fatty acids: 14:0(3-OH), 14:0(3-*O*-9*c*-16:1), and 12:1 Δ^3. The double bond of 12:1 Δ^3 was a result of the elimination of 12:0 from 12:0(3-*O*-12:0) in the MS^2 stage. The reducing GlcN possesses a double bond between C-3 and C-4 as a consequence of previous elimination of 12:0(3-OH) at O-3 in the MS^2 stage. Peaks at *m*/*z* 1251.9 and 1171.9 arose from the elimination of water (−18 Da) and phosphoric acid from O-4′ (−98 Da), respectively. Subsequent elimination of an acyl from O-3′ yielded a characteristic most abundant pair of ions at *m*/*z* 1089.9 and 1071.7. Ions at *m*/*z* 1089.9 and 1071.7 corresponded to the loss of a 12:1 Δ^3 as a ketene (−180 Da) and a free fatty acid (−198 Da), respectively. The elimination of this acyl residue by both charge-driven and charge-remote processes suggested its location at O-3′. The ion at *m*/*z* 942.6 represented a $^{0,4}A_2$ fragment of the parent ion. These peaks indicated that in the precursor ion the reducing GlcN was substituted with 14:0(3-OH) at N-2 and the nonreducing GlcN was phosphorylated at O-4 and substituted with 14:0(3-*O*-9*c*-16:1) at N-2 and 12:1 Δ^3 at O-3.

For the most intense daughter ions (*m*/*z* 1089.9 and 1071.7), the $^{0,4}A_2$, C_1, and B_1 fragments were detected (Figure 2B). These ions corresponded to the in-source intra- and inter-ring fragmentation and confirmed the distribution of fatty acids. The ions at *m*/*z* 762.4 and 744.4 represented $^{0,4}A_2$ and originated from the ions at *m*/*z* 1089.9 and 1071.7, respectively. These ions, together with the observed pairs of C_1 (*m*/*z* 720.4 and 702.4), B_1 (*m*/*z* 702.4 and 684.3), and B_2 (*m*/*z* 1053.8) (Figure 2B), confirmed the location of the amide-linked acyloxyacyl group [14:0(3-*O*-9*c*-16:1)] at N-2 of the nonreducing GlcN. The $^{0,2}A_2$ fragments were not detected, for neither parent nor daughter ions, and that could be explained by the formation of the double bond between C-3 and C-4 of the reducing GlcN, which made such fragmentation impossible (*37*). Ions corresponding to the loss of the amide-bound acyloxyacyl [14:0(3-*O*-9*c*-16:1)] or acyl residues [14:0(3-OH)] were also detected but, due to the greater stability of acyl amides, exhibited very low intensities. Two low-intensity ions at *m*/*z* 835.7 and 817.6 were the result of the elimination of the 9*c*-16:1 chain as a free fatty acid (−254 Da) from ions at *m*/*z* 1089.9 and 1071.7, respectively. In summary, the fragments detected during ESI-MS^n analysis indicated that the lipid A form of *P. shigelloides* represented by the ion at *m*/*z* 1686.5 (LA_I form) was built of the glucosamine backbone, phosphorylated at O-4′ and substi-

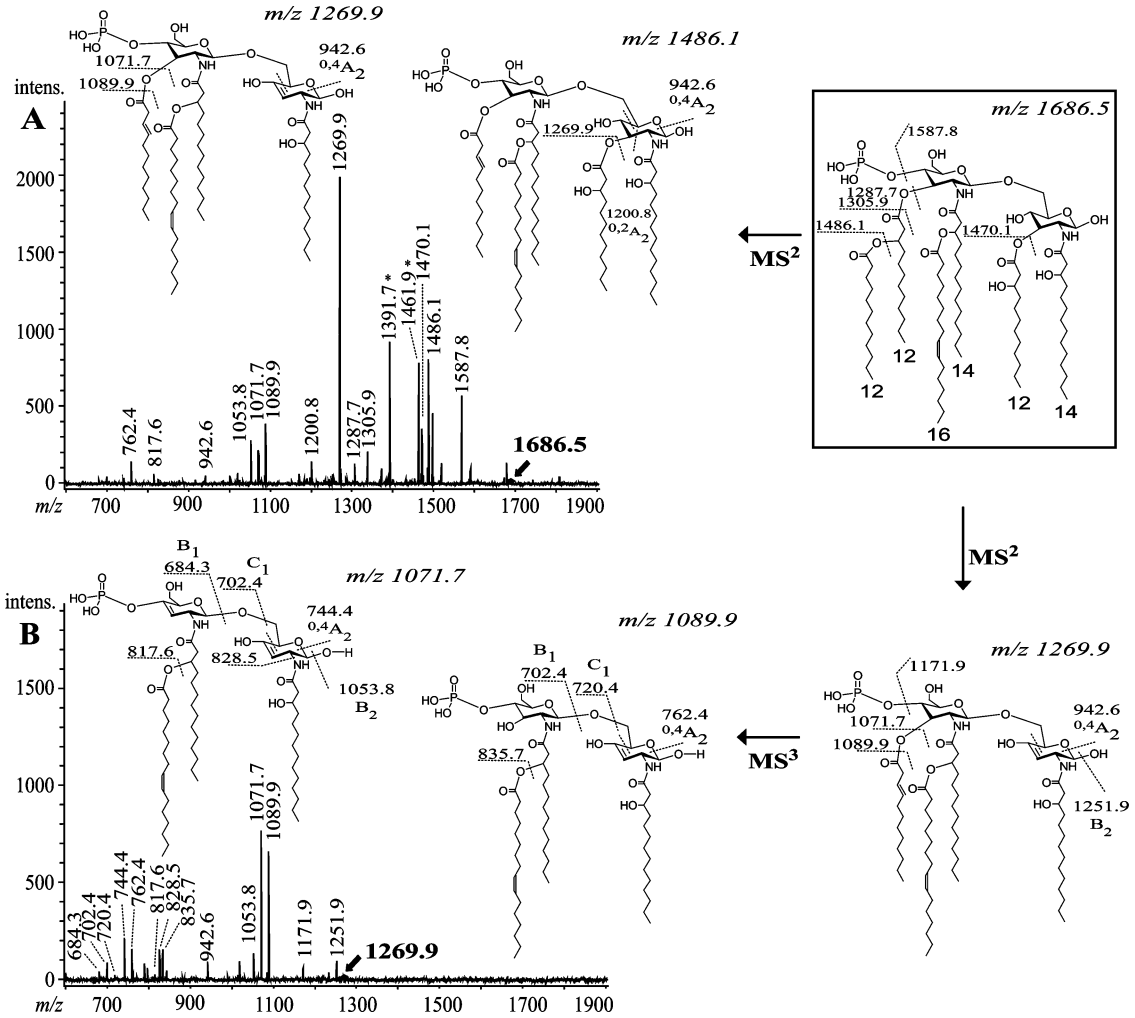


FIGURE 2: Negative ion mode ESI-MS^n of the LA$_1$ form of lipid A isolated from phenol-soluble *P. shigelloides* O74 LPS represented by the ion at *m*/*z* 1686.5 (outlined structure). (A) MS^2 of the ion at *m*/*z* 1686.5. (B) MS^3 of the ion at *m*/*z* 1269.9. The interpretation of observed fragment ions is presented in inset structures. A_2, $^{0,2}A_2$, $^{0,4}A_2$, B_1, B_2, and C_1 stand for ions described by the nomenclature of Domon and Costello (*42*). Ions marked with asterisks could not be interpreted. The number of carbons in fatty acids is indicated by 12, 14, 16, etc.

tuted with 14:0(3-OH) at N-2, 12:0(3-OH) at O-3, 14:0(3-*O*-9*c*-16:1) at N-2′, and 12:0(3-*O*-12:0) at O-3′.

ESI-MS^n Analysis of the Lipid A Form of P. shigelloides O74 Represented by the Ion at m/z 1660.5. The ion at *m*/*z* 1660.5 could represent the monophosphorylated, hexaacylated lipid A substituted with two 14:0(3-OH) fatty acids, two 12:0(3-OH) fatty acids, one 12:0, and one 14:0 (Figure 3, outlined structure). The ion at *m*/*z* 1660.5 was isolated and subjected to MS^2 (Figure 3B). The obtained spectrum exhibited peaks, which mainly reflected the neutral loss of fatty acids: 12:0 (*m*/*z* 1460.1) and 12:0(3-OH) (*m*/*z* 1444.2) resulting from charge-remote elimination, generating a double bond in place of substitution. As in the fragmentation observed during MS^2 on the ion at *m*/*z* 1686.5 (described in the previous section), the most intense ion at *m*/*z* 1243.9 was formed from the parent ion by separate elimination of 12:0(3-OH) and 12:0, generating a double bond at the substitution position. Considering the higher stability of acyl amides, these results showed that the reducing GlcN is substituted with 12:0(3-OH) at O-3 and the nonreducing GlcN with 12:0(3-*O*-12:0) at O-3. Ions with lower intensities corresponded to fragments arising from the inter-residual and intraring fragmentation. Such fragments were identified only for the daughter ions (*m*/*z* 1460.1, 1243.9, and 1045.7) and not for the isolated parent ion, and most of them are described during interpretation of the MS^3 spectrum of ions at *m*/*z* 1243.9 and 1045.7.

The most abundant ion at *m*/*z* 1243.9 was selected for MS^3 fragmentation (Figure 3A). Ions at *m*/*z* 1225.9 and 1144.9 corresponded to the loss of water and elimination of phosphoric acid at O-4′, respectively. The loss of 12:1 Δ^3 at O-3′ during MS^3, by both charge-driven and charge-remote processes, yielding a characteristic pair of ions at *m*/*z* 1063.7 and 1045.7, which confirmed the substitution at O-3′ and the presence of a phosphoric group at O-4′. The ^{0,4}A intraring fragment at *m*/*z* 916.6 supported the location of 14:0(3-OH) at N-2 of the reducing GlcN. Other fragments arising from the inter-residual and intraring fragmentation were identified only for the daughter ions at *m*/*z* 1063.7 and 1045.7 and not for the isolated parent ion. Ions at *m*/*z* 736.2 and 718.4 were identified as the ^{0,4}A fragments of ions at *m*/*z* 1063.7 and 1045.7, respectively.

Structures represented by ions at *m*/*z* 1063.7 and 1045.7 were built of a glucosamine backbone substituted with a phosphate group at O-4′, 14:0(3-OH) at N-2, and 14:0(3-*O*-14:0) at N-2′ and corresponded to the loss of a 12:1 Δ^3 as a ketene (*m*/*z* 1063.7) or free fatty acid (*m*/*z* 1045.7) at O-3′.

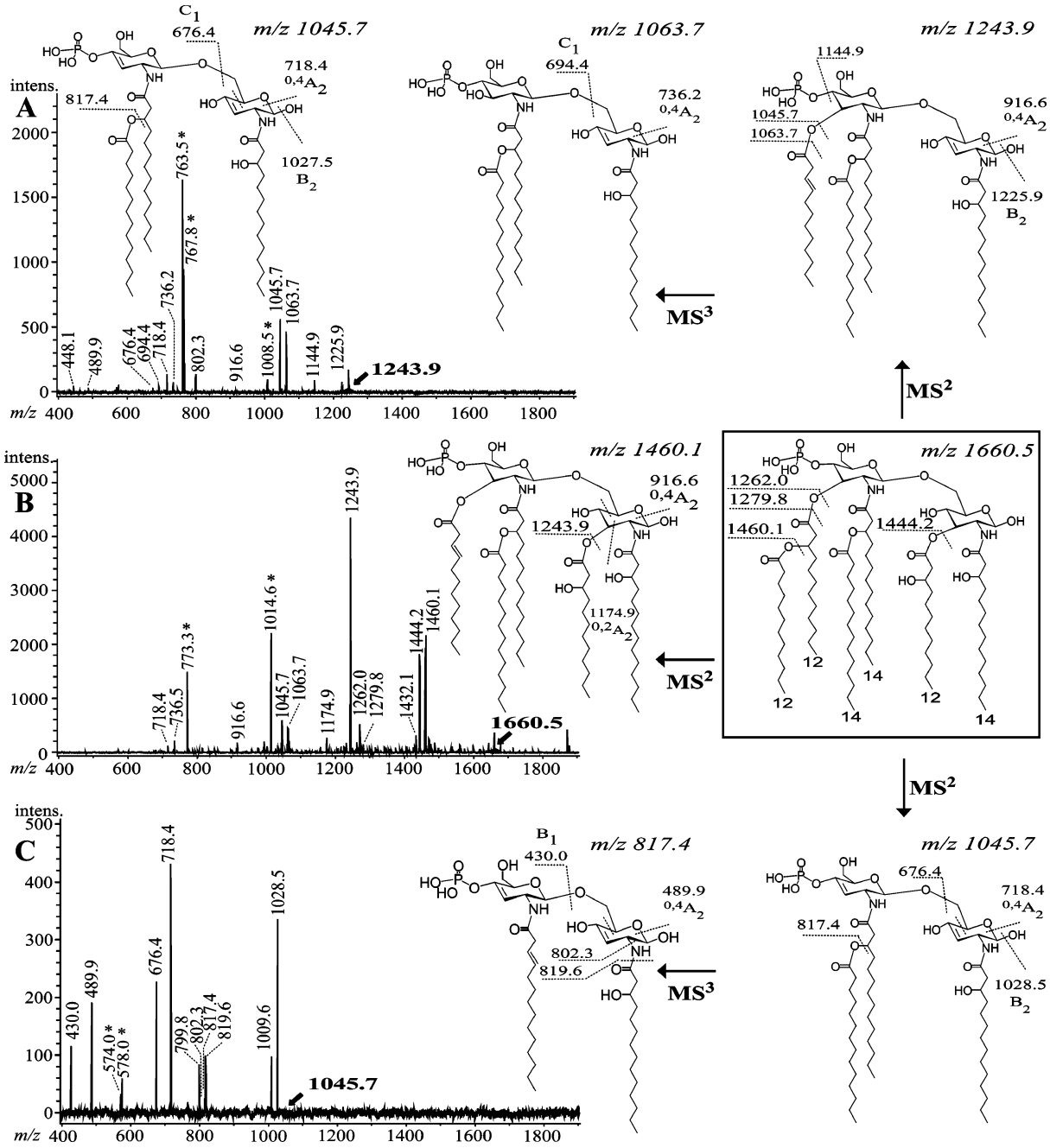


FIGURE 3: Negative ion mode ESI-MS^n of the LA$_{II}$ form of lipid A isolated from phenol-soluble *P. shigelloides* O74 LPS represented by the ion at *m*/*z* 1660.5 (outlined structure). (A) MS^3 of the ion at *m*/*z* 1243.9. (B) MS^2 of the ion at *m*/*z* 1660.5. (C) MS^3 of the ion at *m*/*z* 1045.7. The interpretation of observed fragment ions is presented and marked using inset structures. The interpretation of the observed ions is described as explained in the legend of Figure 2.

The structure corresponding to the ion at *m*/*z* 1045.7 was further confirmed by MS^3 of this ion (Figure 3C). The ions with high intensity corresponded to the elimination of water (*m*/*z* 1028.5 and 1009.6) and 14:0 (*m*/*z* 817.4). The $^{0,4}A_2$ (*m*/*z* 718.4) and C_1 (*m*/*z* 676.4) fragments of the isolated ion were detected as well. The $^{0,2}A_2$ inter-ring fragment was not detected during MS^3 analysis. This could be explained by the presence of a double bond between C-3 and C-4 of the reducing GlcN as a consequence of the previous elimination of 12:0(3-OH) from O-3 in the MS^2 stage, making such fragmentation impossible. Ions at *m*/*z* 489.9 and 430.0 were attributed to the $^{0,4}A_2$ and B_1 fragments of daughter ion at *m*/*z* 817.4, respectively. Thus, combined data on the fragments detected during ESI-MS^n analyses indicated that the lipid A form of *P. shigelloides* represented by the ion at *m*/*z* 1660.5 (LA$_{II}$ form) was built of the glucosamine backbone phosphorylated at O-4′ and substituted with 14:0(3-OH) at N-2, 12:0(3-OH) at O-3, 14:0(3-*O*-14:0) at N-2′, and 12:0(3-*O*-12:0) at O-3′.

ESI-MS^n Analysis of the Lipid A Form of P. shigelloides O74 Represented by the Ion at m/z 1632.5. This ion could represent the monophosphorylated, hexaacylated form of lipid A. The mass difference between the ion at *m*/*z* 1632.5 and the ion at *m*/*z* 1660.5 (form LA$_{II}$) was 28 Da (C_2H_4 fragment). The observed mass difference and results of chemical analysis of fatty acids suggested that in comparison with the LA$_{II}$ form the ion at *m*/*z* 1632.5 could represent the monophosphorylated lipid A substituted with two 14:0(3-

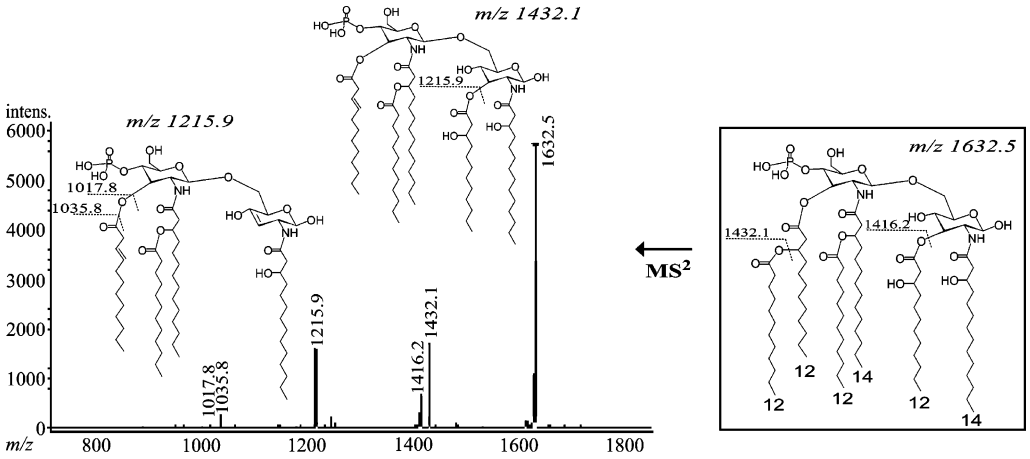


FIGURE 4: Negative ion mode ESI-MS^2 of the LA$_{III}$ form of lipid A isolated from phenol-soluble *P. shigelloides* O74 LPS represented by the ion at *m*/*z* 1632.5 (outlined structure). The interpretation of observed ions is described as explained in the legend of Figure 2.

OH), two 12:0(3-OH), and two 12:0 fatty acids (Figure 4, outlined structure). The distribution of these acyl residues was determined on the basis of MS^2 analysis of this ion (Figure 4) and MALDI-TOF MS. Due to the greater stability of acyl amides, the obtained spectrum exhibited peaks which mainly reflected the loss of fatty acids, 12:0 (*m*/*z* 1432.1) and 12:0(3-OH) (*m*/*z* 1416.2), as a consequence of the charge-remote elimination, generating a double bond in place of a substitution. The ion at *m*/*z* 1215.9 was formed from the parent ion by separate elimination of 12:0(3-OH) and 12:0. The characteristic pair of ions at *m*/*z* 1035.8 and 1017.8 resulted from the charge-remote elimination of 12:0(3-OH) at O-3 and by the charge-driven and charge-remote elimination of 12:0(3-*O*-12:0) at O-3′. The mass differences between the similar types of fragment ions present in the MS^2 spectra obtained for the ions at *m*/*z* 1660.5 and 1632.5 were also 28 Da. Thus, considering the greater stability of acyl amides and the presence of 14:0(3-OH), which was the only amide-linked fatty acid identified by chemical analysis, the mass difference is related to the substitution of 14:0(3-OH) at N-2′ with 12:0 as a secondary linked fatty acid instead of 14:0 (LA$_{II}$ form). Due to the low intensity of fragment ions, their MS^3 analysis was not performed. Fragments detected during ESI-MS^n analysis and the presence of characteristic Y_1^- ion and oxonium ions (B_1^+) in MALDI-TOF mass spectra (data not shown, described below) obtained for lipid A of LPS *P. shigelloides* O74 indicated that the lipid A form of *P. shigelloides* represented by the ion at *m*/*z* 1632.5 (LA$_{III}$ form) was built of the glucosamine backbone phosphorylated at O-4′ and substituted with 14:0(3-OH) at N-2, 12:0(3-OH) at O-3, 14:0(3-*O*-12:0) at N-2′, and 12:0(3-*O*-12:0) at O-3′.

MALDI-TOF MS Analysis of P. shigelloides O74 Lipid A. MALDI-TOF mass spectra recorded in the negative ion mode showed the heterogeneity of the lipid A isolated from the phenol (Figure 1B) and water phase (Figure 1A) of *P. shigelloides* O74 LPS (Table 1). The ions corresponding to those observed in ESI-MS^n analysis were detected. Three of the observed ions (*m*/*z* 1686.8, 1661.1, and 1632.7) were characterized previously by ESI-MS^n analysis and represented forms of *P. shigelloides* O74 lipid A (LA$_I$, LA$_{II}$, and LA$_{III}$), differing in fatty acid composition. Besides these ions, bisphosphorylated hexaacylated, pentaacylated lipid A forms and those additionally substituted with PEtn were identified on the basis of mass differences (80 and 123 Da) observed between related ions. Ions attributed to monophosphorylated molecules and not substituted with PEtn could be products of the mild acidic hydrolysis of LPS.

In a region of low *m*/*z* values, the peak at *m*/*z* 682.9 (Y_1^- ion) was observed (data not shown, Table 1). This ion arose from deprotonation, followed by simple cleavage of the glycosidic bond within the disaccharide backbone of lipid A, according to the nomenclature of Domon and Costello (*42*), and represented the proximal GlcN residue phosphorylated at O-1 and acylated by 14:0(3-OH) at N-2 and 12:0(3-OH) at O-3. The presence of such ions confirmed that alkene heterogeneity among three forms of *P. shigelloides* O74 lipid A is due to those substitutions of the nonreducing GlcN. Such heterogeneity was not observed in the region of the Y_1^- ion.

The positive ion mode MALDI-TOF MS analysis of lipid A performed at high laser power settings revealed the presence of the group of oxonium ions (B_1^+) at *m*/*z* 1059.6 and 1031.4 (data not shown). These ions arose from the cleavage of the glycosidic linkage between two GlcN residues in LA$_{II}$ (*m*/*z* 1059.6) and LA$_{III}$ (*m*/*z* 1031.4) and corresponded to the nonreducing tetraacylated GlcN (**Y**), phosphorylated at O-4. The ion at *m*/*z* 831.6 represented phosphorylated, nonreducing triacylated GlcN (**Y**) devoid of the 14:0 fatty acid (−228 Da) or 12:0 (−200 Da) in the case of LA$_{II}$ and LA$_{III}$, respectively. It was previously shown that the preferential elimination of the fatty acid at N-2′ was observed via positive ion mode MS (*38*). Thus, the presence of the ion at *m*/*z* 831.6 confirmed that either 14:0 (form LA$_{II}$) or 12:0 (form LA$_{III}$) is linked to the amide-bound 14:0(3-OH) at N-2′ of lipid A as a secondary linked fatty acid.

On the basis of mass differences (80 and 123 Da) observed between ions, groups of related ions were identified for each form of the *P. shigelloides* O74 lipid A represented by ions at *m*/*z* 1686.5, 1660.5, and 1632.5 in MALDI-TOF mass spectra (Figure 1A,B and Table 1). ESI-MS^n analyses were not performed for bisphosphorylated hexaacylated forms and those additionally substituted with PEtn molecules due to the low intensity of these ions. Thus, identification of structures of the lipid A molecules corresponding to these ions is tentative.

The group of ions (Table 1) related to that at *m*/*z* 1686.5 ([M$_I$ − P − PEtn − H]) corresponded to monophosphorylated, hexaacylated lipid A substituted with 14:0(3-OH) at

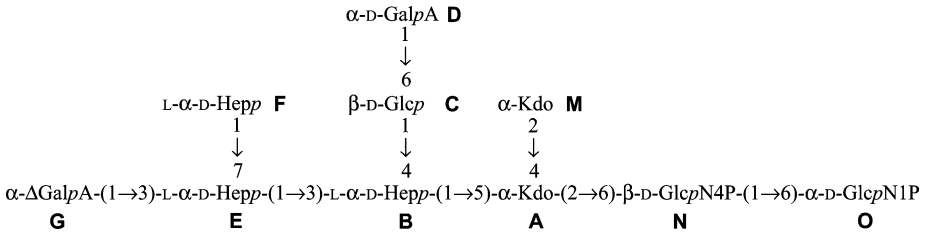


FIGURE 5: Structure of the decasaccharide isolated by de-N,O-acylation of *P. shigelloides* O74 LPS. De-N,O-acylation of LPS was achieved by mild hydrazinolysis followed by KOH treatment. The mixture of products from deacylation of LPS was fractionated by affinity chromatography on a serotonin−Sepharose 4B column and eluted with a discontinuous ammonium acetate gradient. Decasaccharide was eluted with 0.05 M $CH_3COO(NH_4)$. α-ΔGal*p*A is 4-deoxy-*β*-L-*threo*-hex-4-enopyranosyl.

N-2, 12:0(3-OH) at O-3, 14:0(3-*O*-9*c*-16:1) at N-2′, and 12:0(3-*O*-12:0) at O-3′, and a phosphate residue at O-4′ is identified as ***I*** in Figure 1. The peak at *m*/*z* 1809.8 ($[M_I - P - H]$) corresponded to the hexaacylated lipid A substituted with the phosphate group and PEtn. The ion at *m*/*z* 1890.0 ($[M_I - H]^-$) represented the hexaacylated LA_I form of lipid A substituted with phosphate groups at O-1 and O-4′ and PEtn. The peak at *m*/*z* 1767.2 ($[M_I - PEtn - H]^-$) could be explained by the M_I structure devoid of the PEtn. The ions at lower *m*/*z* ratios could be attributed to a pentaacylated (*m*/*z* 1487.6) and a tetraacylated LA_I form of lipid A.

The group of ions related to the ion at *m*/*z* 1660.5 corresponded to the monophosphorylated, hexaacylated form of *P. shigelloides* O74 lipid A which was substituted with 14:0(3-OH) at N-2, 12:0(3-OH) at O-3, 14:0(3-*O*-14:0) at N-2′, and 12:0(3-*O*-12:0) at O-3′ and with a phosphate residue at O-4′ is identified as ***II*** at Figure 1. In comparison with LA_I, the 14:0 fatty acid replaced 9*c*-16:1 as the secondary acyl residue at N-2′ in the case of the LA_{II} form of *P. shigelloides* O74 lipid A. Peaks corresponding to the LA_{II} form of lipid A which were bisphosphorylated (*m*/*z* 1741.7) and substituted with PEtn (*m*/*z* 1864.1), monophosphorylated and substituted with PEtn (*m*/*z* 1784.1), and bisphosphorylated and pentaacylated (*m*/*z* 1558.2) were also identified (Table 1).

The group of ions related to the ion at *m*/*z* 1632.5 corresponded to the monophosphorylated, hexaacylated form of *P. shigelloides* O74 lipid A which is substituted with 14:0(3-OH) at N-2, 12:0(3-OH) at O-3, 14:0(3-*O*-12:0) at N-2′, and 12:0(3-*O*-12:0) at O-3′ and with a phosphate group at O-4′ is identified as ***III*** at Figure 1. The MALDI-TOF mass spectrum showed ions corresponding to the bisphosphorylated LA_{II} (*m*/*z* 1713.3) and monophosphorylated LA_{II} substituted with PEtn (*m*/*z* 1756.1) of lipid A (Table 1).

MALDI-TOF MS analysis of phenol phase lipid A showed an additional ion at *m*/*z* 1825.0, which could represent the fourth form of *P. shigelloides* O74 lipid A. The mass difference (16 Da) between that ion and the ion at *m*/*z* 1810.7 (form LA_I) indicated the presence of an additional hydroxyl group. However, this ion was not detected during ESI-MS analysis of phenol phase lipid A. Thus, it could not be isolated for MS^n to determine the fatty acid distribution.

NMR Analysis of the Carbohydrate Region of Lipid A and Its Linkage to the Core of P. shigelloides O74 LPS. LPS of *P. shigelloides* O74, obtained from the water phase, was de-N,O-acylated by hydrazinolysis with subsequent KOH treatment (*25*) to obtain the oligosaccharide consisting of the lipid A carbohydrate backbone and the intact Kdo-containing core region. Deacylation of 4-substituted galacturonic acid-containing LPS yielded a mixture of incomplete core oligosaccharides linked to the lipid A backbone and the O-specific polysaccharide components, as a result of the alkaline *β*-elimination of the substituent at O-4 of GalA in the core oligosaccharide (*43*). The mixture (10 mg) was fractionated on the serotonin−Sepharose 4B column using ammonium acetate solutions with different ionic strengths for elution (*20*). Five fractions were eluted and checked by one- and two-dimensional NMR spectroscopy.

The ^1H NMR spectrum of the fraction eluted with 0.05 M ammonium acetate (3.5 mg) showed the presence of the signals originating from the core oligosaccharide constituents and was devoid of the CH_3 signal of deoxyhexose residues, constituents of the O-specific polysaccharide (*60*). The datailed NMR analysis revealed signals for eight anomeric protons and carbons, two signals of nitrogen-bearing carbons, and in addition two Kdo (3-deoxy-D-*manno*-oct-2-ulosonic acid) spin systems and a characteristic spin system of 4-deoxy-*β*-L-*threo*-hex-4-enopyranosyl (α-ΔGalA) (Table S1 of the Supporting Information). The ^1H and ^{13}C NMR spectra of the affinity-purified decasaccharide were assigned using a previously described procedure (*19*) (Table S2 of the Supporting Information). The residues are identified with uppercase letters as shown in the structure below (Figure 5), and these letters refer to the corresponding sugars throughout the entire manuscript and are in agreement with those used in ref *60*.

Residue **A** was identified as the 4,5-disubstituted α-Kdo on the basis of characteristic deoxy proton signals at *δ* 1.93 ($H\text{-}3_{ax}$) and *δ* 2.12 ($H\text{-}3_{eq}$), and a large chemical shift of the C-4 (*δ* 70.8) and C-5 (*δ* 70.2) signals.

Residue **G** with the H-1/C-1 signals at *δ* 5.13/98.9 ($J_{H-1,H-2} < 2$ Hz) was recognized as the terminal α-ΔGalA, on the basis of the characteristic four-proton spin system with the large chemical shift of the H-4 signal (*δ* 5.81) and the C-5 signal at δ_C 145.5 (*44*).

Residue **M** was identified as the terminal α-Kdo on the basis of characteristic deoxy proton signals at *δ* 1.77 ($H\text{-}3_{ax}$) and *δ* 2.17 ($H\text{-}3_{eq}$). The $H\text{-}3_{ax}$ and $H\text{-}3_{eq}$ signals, observed in the region of *δ* 1.7−2.38, and the chemical shift values of the H-5 proton indicated the α-pyranosidic configuration of this residue (*43*, *45*).

Residue **N** with the H-1/C-1 signals at *δ* 4.85/99.7 ($J_{H-1,H-2} \sim 8$ Hz) was assigned as the 6-substituted *β*-D-Glc*p*N4P residue, on the basis of the typical chemical shifts of the H-1, C-1, and C-2 (*δ* 55.9) signals and the large vicinal couplings between all ring protons. The chemical shift of the C-6 signal (*δ* 62.8) indicated the substitution with a Kdo residue (*46*, *47*).

Residue **O** with the H-1/C-1 signals at *δ* 5.71/91.6 ($J_{H-1,H-2} < 3$ Hz) was recognized as the 6-substituted α-D-

Glc*p*N1P residue on the basis of the small chemical shift of the C-2 signal (δ 54.7), the large chemical shift of the C-6 signal (δ 70.1), and the large vicinal couplings among H-2, H-3, H-4, and H-5 ($J_{H-2,H-3}$, $J_{H-3,H-4}$, and $J_{H-4,H-5} \sim 10$ Hz).

The residues →3,7)-L-α-D-Hep*p*-(1→ (**E**), terminal L-α-D-Hep*p*-(1→ (**F**), →3,4)-L-α-D-Hep*p*-(1→ (**B**), →6)-β-D-Glc*p*-(1→ (**C**), and terminal α-D-Gal*p*A-(1→ (**D**) exhibited chemical shift values very similar to those obtained for the corresponding residues in the core oligosaccharide of *P. shigelloides* O74 LPS (*60*).

The one-dimensional ^{31}P NMR spectrum obtained for the decasaccharide showed two signals at δ_P 1.58 and 1.76 characteristic of the phosphate monoesters. The location of phosphate residues in the decasaccharide was unequivocally determined by ^{1}H−^{31}P HMBC (Figure S1 of the Supporting Information). Cross-peaks between the signal at δ_P 1.58 (top trace) and H-1 (δ 5.69) and H-2 (δ 3.44) of residue **O** and that of the signal at δ_P 1.76 (bottom trace) and H-4 (δ 3.82) of residue **N** were identified.

The disaccharide elements in the decasaccharide were identified by HMBC and NOESY experiments (Tables S1 and S2 and Figure S2 of the Supporting Information) showing inter-residue connectivities between adjacent sugar residues and thus providing the sequence of sugar monomers in Figure 4. The observed inter-residual NOEs between H-3$_{ax}$ (weak signal) and H-3$_{eq}$ (strong signal) of residue **A** and H-6 of residue **M** confirmed the presence of the disaccharide element α-Kdo-(2→4)-α-Kdo (*43*).

These results suggest the following basic structure of the decasaccharide product from de-N,O-acylation of the LPS *P. shigelloides* O74 (Figure 5). Two residues, **N** and **O**, linked by a β-(1→6)-glycosidic linkage and substituted with phosphate groups at O-1 and O-4′ represented the carbohydrate backbone of lipid A.

Complete Structure of the P. shigelloides O74 LPS. The analysis of the main LPS segments of *P. shigelloides* O74 allowed the determination of its complete structure. The data on the core oligosaccharide, the biological repeating unit of the O-antigen, and the linkage between them appear in ref *60*. In comparison to the core oligosaccharide fraction obtained by mild acidic hydrolysis, the oligosaccharide obtained by de-N,O-acylation of LPS revealed the presence of 4,5-substituted α-Kdo (**A**) instead of 5-substituted α-Kdo and an additional terminal α-Kdo (**M**) in the innermost core region and two 6-substituted D-Glc*p*N residues (**N** and **O**), constituting the disaccharide backbone of lipid A. Like the core oligosaccharide of *P. shigelloides* O54, the core region of *P. shigelloides* O74 LPS is devoid of phosphate groups. The complete heteropolysaccharide, including the O-specific polysaccharide and the core oligosaccharide, was bound to the lipid A moiety by the ketosidic linkage between →4,5)-α-Kdo (residue **A**) and →6)-β-D-Glc*p*N4P-(1→ (residue **N**).

Lipid A, isolated from both phenol-soluble and water-soluble LPS, consisted of β-D-Glc*p*N4P-(1→6)-α-D-Glc*p*N1P disaccharide. Lipid A of *P. shigelloides* O74 was heterogeneous, comprising three asymmetric, bisphosphorylated, and hexaacylated forms with different acylation patterns. The disaccharide backbone was substituted with the primary (*R*)-3-hydroxy fatty acids: 14:0(3-OH) at N-2 and N-2′ and 12:0(3-OH) at O-3 and O-3′. Three identified forms of *P. shigelloides* O74 lipid A were substituted with the secondary fatty acids: 9*c*-16:1 at N-2′ and 12:0 at O-3′ (form LA$_I$), 14:0 at N-2′ and 12:0 at O-3′ (LA$_{II}$), and 12:0 at N-2′ and 12:0 at O-3′ (LA$_{III}$). Forms of lipid A additionally substituted with PEtn were also identified by MS analyses. Thus, the combined results suggest the following structure of the complete LPS of *P. shigelloides* O74 (Figure 6).

Biological Activity of P. shigelloides O74 LPS. Production of cytokines and NO by J-774A.1 cells stimulated with water- and phenol-soluble *P. shigelloides* O74 LPS was examined and compared to that following stimulation with *Escherichia coli* O55 LPS (Figure 7). All LPS exhibited strong and dose-dependent activity. *E. coli* O55 LPS and water-soluble LPS isolated from *P. shigelloides* O74 had similar effects in vitro on the TNFα and IL-6 production for all doses. The measurement of the total nitrite concentration demonstrated that J-774A.1 cells responded more weakly for a 1 μg dose ($P < 0.005$) and similarly for 100 and 10 ng doses of water-soluble O74 LPS in comparison to *E. coli* O55 LPS.

The phenol-soluble *P. shigelloides* O74 LPS exhibited an effect in vitro on TNFα production by J-774A.1 cells similar to that of water-soluble LPS O74.

There were significant differences between these two preparations of *P. shigelloides* O74 LPS with respect to the IL-6 and NO production by macrophages. Phenol-soluble LPS of *P. shigelloides* O74 had a weaker effect on the IL-6 production for all doses of LPS and on NO production for the 1 μg dose.

DISCUSSION

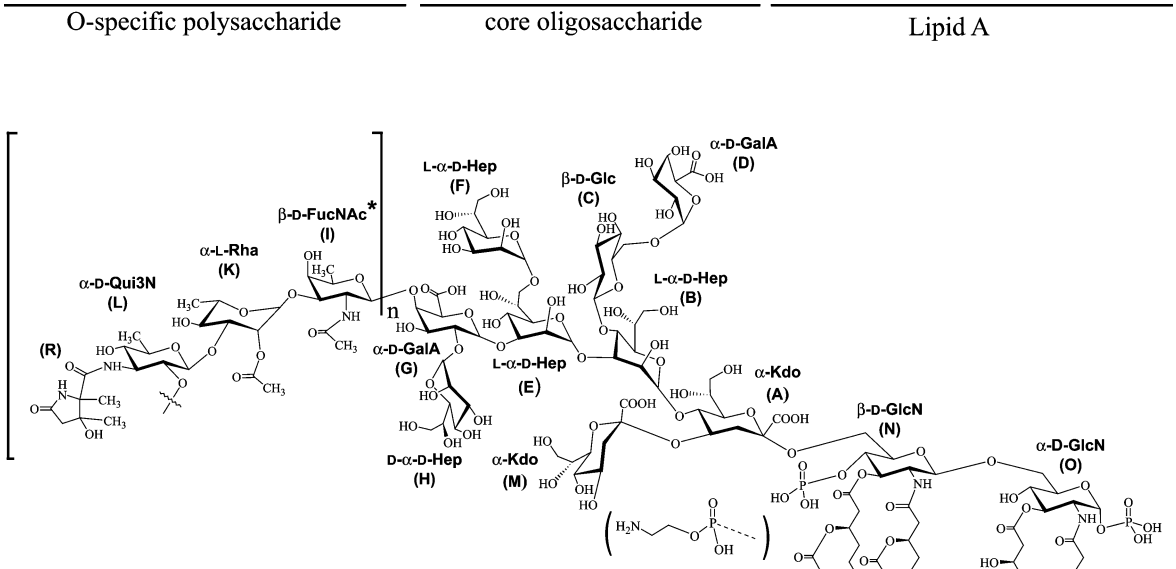
Lipopolysaccharide constitutes one of the elements of the pathogen-associated molecular pattern for host infection by Gram-negative bacteria and is one of the most powerful natural activators of the innate immune system. Lipid A is the endotoxic center of LPS recognized by different classes of receptors, including Toll-like receptors (*10*).

Chemically, lipid A is a unique phosphoglycolipid exhibiting similar basic structure throughout all Gram-negative bacteria. However, differences with regard to structural features within both hydrophilic and hydrophobic segments contribute to variations of its endotoxic activities (*48*).

The most stimulatory lipid A structures that activate the innate immunity in humans and other mammalian hosts are represented by the hexaacylated disaccharide backbone of D-*gluco*-configured hexosamines that are phosphorylated at positions 1 and 4′. The length of the fatty acids is typically limited to 12 or 14 carbons. Both asymmetrical (*E. coli*-type) and symmetrical (*Neisseria*-type) lipid A molecules can be found as constituents of active LPS (*10*).

We present here the structure of lipid A of *P. shigelloides* O74 and its linkage to the core oligosaccharide. This study complements the structural investigation of *P. shigelloides* O74 LPS (*60*), providing structural details for the entire molecule and some of its biological activities in vitro.

As shown in ref *60*, the phenol/water extraction of *P. shigelloides* O74 yielded two distinct LPS fractions retained in the phenol (2%) and water phase (0.8%). Both LPS preparations exhibited typical S-type behavior in SDS−PAGE but different degrees of polymerization of the O-repeats. Chemical analysis revealed the difference between preparations concerning the O-specific polysaccharide and lipid A parts. We found that most of the O-specific repeats



Forms of lipid A	Position of acyl/acyloxyacyl Glc*p*N (**N**) O-3	N-2	Glc*p*N (**O**) O-3	N-2
LA*I*	12:0(3-*O*-12:0)	14:0(3-*O*-9*c*-16:1)	12:0(3-OH)	14:0(3-OH)
LA*II*	12:0(3-*O*-12:0)	14:0(3-*O*-14:0)	12:0(3-OH)	14:0(3-OH)
LA*III*	12:0(3-*O*-12:0)	14:0(3-*O*-12:0)	12:0(3-OH)	14:0(3-OH)

FIGURE 6: Complete structure of the *P. shigelloides* O74 lipopolysaccharide, containing the LA_I form of lipid A. The inset table gives the fatty acid distribution in forms LA_I−LA_{III} identified for *P. shigelloides* O74 lipid A. The uppercase letters refer to carbohydrate residues. Residues **A**−**H** and **X** constitute the core oligosaccharide. R is 3-hydroxy-2,3-dimethyl-5-oxopyrrolidine-2-carboxylic acid (3-hydroxy-2,3-dimethyl-5-oxoproline). The asterisk denotes that the anomeric configuration of Fuc*p*NAc is α in the subsequent O-repeats (*60*).

of the phenol phase were O-acetylated (84%) compared to the average O-acetylation of water phase LPS (56%). Moreover, the lipid A obtained from phenol-soluble LPS was more heterogeneous.

In structural analysis of the lipid A isolated from *P. shigelloides* O74, we used the strategy based on ESI-MS^n. Previously, this ESI-MS^n technique was successfully used to identify the fatty acids and their position in the heterogeneous lipid A structures (*37*, *40*, *49*−*51*). Ions corresponding to monophosphorylated molecules were isolated, and their structures were unambiguously determined by a series of MS^n experiments. The high-molecular weight ions of bisphosphorylated lipid A molecules and these substituted with PEtn appeared with much lower intensities in the ESI-MS spectrum than in the MALDI-TOF experiment. This could be explained not only by less effective ionization of these molecules but also by the hydrolysis at the anomeric phosphate following the exposure to a chloroform/methanol solvent (*52*, *53*).

The lipid A isolated both from phenol- and water-soluble *P. shigelloides* O74 LPS is heterogeneous and is represented by three forms differing in their acylation patterns (Figure 6). All forms contained *β*-Glc*p*N4P-(1→6)-α-Glc*p*N1P disaccharide, substituted with four primary fatty acids [two ester-linked 12:0(3-OH) and two amide-linked 14:(3-OH) fatty acids]. Two of these forms have been recently identified in *P. shigelloides* O54 lipid A (*20*). The main population of lipid A isolated from phenol-soluble LPS contained an unsaturated fatty acid, 9*c*-16:1, seldom found in this type of molecule. Previously, we identified a trace amount of this acyl among ester-linked fatty acids identified for *P. shigelloides* O54 LPS via GC−MS (*20*). As this fatty acid is also one of the common constituents of *P. shigelloides* membranes (*54*), the phenol-soluble LPS preparation was additionally purified by extraction with chloroform and methanol to remove any possible phospholipid contaminations. ESI-MS^n analysis showed that 9*c*-16:1 is an integral part of the major form of *P. shigelloides* O74 lipid A.

It is a rather uncommon constituent of lipid A molecules, and to date, it was found in only lipopolysaccharides isolated from pathogenic *Yersinia* species grown at low temperatures (*55*, *56*). Palmitoleate is also present in lipid A of *E. coli* and *Salmonella typhimurium* cells subjected to low temperatures. This unsaturated fatty acid was not present in lipid A of *E. coli* grown at 37 °C. It was found that palmitoleoyl transferase was induced upon cold shock (*57*). *P. shigelloides* O74 cells cultured at 37 °C produced lipid A containing 9*c*-16:1. Therefore, it suggests that this specific palmitoleoyl transferase is active in these bacteria also at elevated temperatures.

The primary fatty acid composition of lipid A of *P. shigelloides* O74 is similar to that identified in lipid A of *Neisseria* spp. (*10*, *58*). However, it differs from the neisserial lipid A in the presence of asymmetrically distributed fatty acids. Such a distribution resembles closely that of lipid A of the *E. coli* type. Thus, lipid A isolated from *P. shigelloides* O74 LPS represents a novel structure among previously identified lipid A molecules.

The structure of the Kdo-containing region of the core oligosaccharide and its linkage with the lipid A was

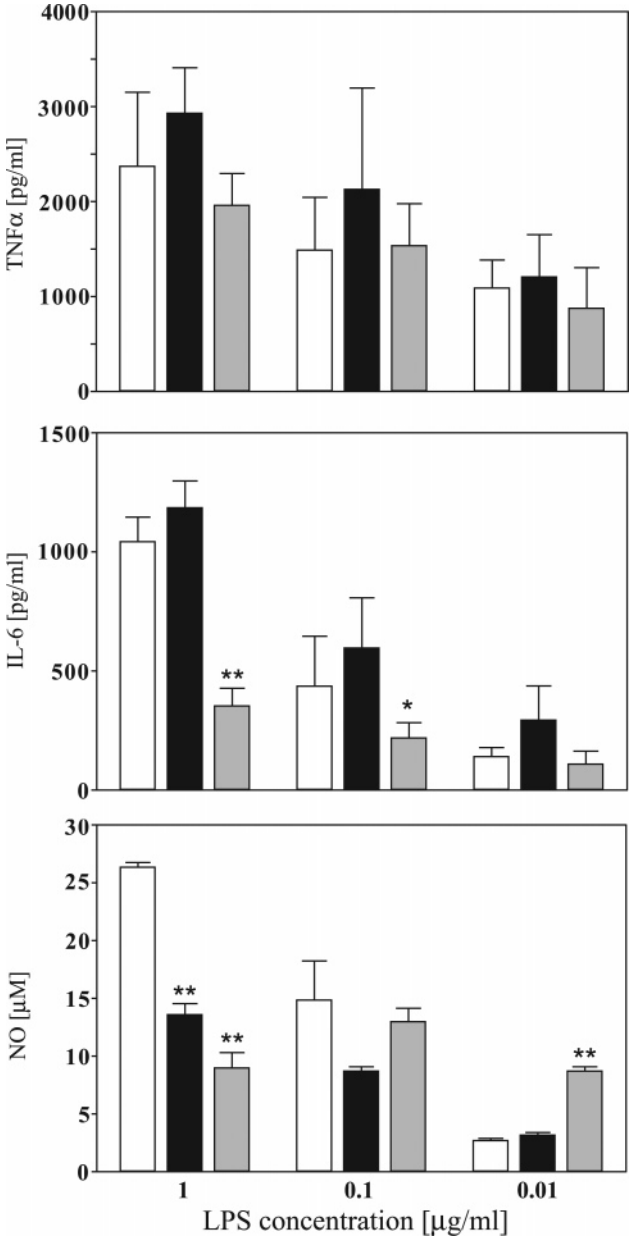


FIGURE 7: Stimulatory effect of *P. shigelloides* O74 LPS on the production of TNFα, IL-6, and NO by J-774A.1 cells. The effects of stimulation of J-774A.1 cells with *E. coli* O55 (□) and *P. shigelloides* O74 LPS [(■) water phase and (gray bars) phenol phase] were compared. The doses of LPS were 1.0, 0.1, and 0.01 μg per 1×10^6 cells. The supernatants were collected after 4, 24, and 48 h for determination of TNFα, IL-6, and NO concentrations, respectively. Concentrations of IL-6 and TNFα in the supernatants were determined using the OptEIA kits (Pharmingen, San Jose, CA). Griess reaction was performed to assay for total nitrite production. Assays were performed using triplicate cultures. Data are expressed as means ± the standard deviation (SD). Differences within groups receiving (i) water-soluble O74 LPS and *E. coli* O55 LPS or (ii) phenol-soluble and water-soluble O74 LPS were analyzed using a one-way analysis of variance. Differences significant at $P < 0.005$ and $P < 0.05$ are marked with one and two asterisks, respectively.

determined on the oligosaccharide obtained by de-N,O-acylation of LPS. Alkaline hydrolysis of LPS, followed by affinity chromatography on a serotonin−Sepharose 4B column, was used to isolate the oligosaccharide with the intact Kdo region, retaining all acid labile linkages, e.g., ketosidic bonds. Structural analysis of the isolated decasaccharide allowed the identification of a characteristic structural motif of enterobacterial LPS, →5)-[α-Kdo-(2→4)]-α-Kdo-(2→6)-β-D-GlcN4P-(1→6)-α-D-GlcN1P.

These results have been combined with data on the O-specific polysaccharide and the core oligosaccharide (*60*), allowing the presentation of the complete structure of LPS (Figure 6).

The biological activity of *P. shigelloides* O74 LPS, expressed as its ability to stimulate macrophage-like cells for cytokine (TNFα and IL-6) and NO production, was compared to that of *E. coli* LPS. We have examined production of cytokines and NO by J-774A.1 cells after stimulation with water- and phenol-soluble *P. shigelloides* O74 LPS.

E. coli O55 LPS and water-soluble LPS isolated from *P. shigelloides* O74 had similar effects in vitro. Both LPS isolated from smooth bacteria were found in the water phase during phenol/water extraction. Despite different structural features in lipid A and the core oligosaccharide of *P. shigelloides* O74, i.e., the presence of a long, unsaturated fatty acid (9*c*-16:1) in the major form of lipid A and the lack of phosphates in the core oligosaccharide, no significant differences were noted between the biological activities in vitro of endotoxins isolated from this bacterium and *E. coli*. The phenol-soluble *P. shigelloides* O74 LPS also stimulated J-774A.1 cells with respect to TNFα, IL-6, and NO production in vitro. There were significant differences between phenol- and water-soluble *P. shigelloides* O74 LPS in stimulation of macrophages with respect to the IL-6 and NO production. However, the observed differences were not systematic and difficult to explain. Endotoxins as amphiphilic molecules form aggregates in water in a concentration-dependent process. It was shown that this type of aggregate depends on the LPS structure and correlates with its ability to activate host cells (*59*). The lipophilic nature of phenol-soluble *P. shigelloides* O74 LPS, caused by O-acetylation of O-repeats, could influence the type of aggregates formed in an aqueous environment and its biological activity.

SUPPORTING INFORMATION AVAILABLE

Two extensive tables describing ^{1}H and ^{13}C NMR chemical shifts and selected inter-residue NOE and $^3J_{H,C}$ connectivities of the affinity-purified decasaccharide isolated from the de-N,O-acylated LPS of *P. shigelloides* O74. This material is available free of charge via the Internet at http://pubs.acs.org.

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