Structure of the lipid A–inner core region and biological activity of *Plesiomonas shigelloides* O54 (strain CNCTC 113/92) lipopolysaccharide

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*Plesiomonas shigelloides* is a Gram-negative rod associated with episodes of intestinal infections and outbreaks of diarrhea in humans. The extraintestinal infections caused by this bacterium, for example, endophthalmitis, meningitidis, bacteremia, and septicemia, usually have gastrointestinal origin and serious course. The lipopolysaccharide (LPS, endotoxin) as virulence factor is important in enteropathogenicity of this bacterium. LPSs of *P. shigelloides* and especially their lipid A part, that is, the immunomodulatory center of LPS, have not been extensively investigated. The structure of *P. shigelloides* O54 lipid A was determined by chemical analysis combined with MALDI-TOF mass spectrometry, and the intact Kdo-containing core region was investigated by NMR spectroscopy on deacylated LPS. Products from alkaline deacylation of LPS, containing 4-substituted uronic acids, are usually very complex and difficult to separate. Since Kdo residues, like sialic acids, form complexes with serotonin, we used immobilized serotonin for one-step isolation of oligosaccharide containing the intact Kdo region from the reaction mixture by affinity chromatography. The major form of lipid A was built of β-D-GlcP4PEn-(1→6)-α-D-Glc(N1P) disaccharide substituted with 14:0(3-OH), 12:0(3-OH), 14:0(3-O-14:0), and 12:0(3-O-12:0) acyl groups at N-2, O-3, N-2′, and O-3′, respectively. This is a novel structure among known lipid A molecules. Analysis of intact Kdo-lipid A region, lipid A and its linkage with the core oligosaccharide completes the structural investigation of *P. shigelloides* O54 LPS, resolving the entire molecule. Biological activities and observed discrepancy between *in vitro* and *in vivo* activity of *P. shigelloides* and *Escherichia coli* LPS are discussed.

**Key words:** lipid A/lipopolysaccharide/MALDI-TOF mass spectrometry/NMR spectroscopy/Plesiomonas shigelloides

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Introduction

*Plesiomonas shigelloides* is a Gram-negative, facultatively anaerobic and flagellated rod, belonging to the *Enterobacteriaceae* family and is the only species in the genus *Plesiomonas* (Garrity *et al*., 2004). *P. shigelloides* is not a part of the normal human fecal flora. These bacteria are found in the aquatic environment in the tropical and subtropical regions, but also in cold climate. Bacteria occur as free-living cells in water or in water creatures, such as fish, crabs, prawns, mussels, and oysters. Thus water- and food-borne outbreaks of intestinal infections due to *P. shigelloides* have been reported. It was ranked third among etiological agents in outbreaks of travellers’ diarrhea in Japan and China (Stock, 2004). Various studies have reported on the invasive nature of the infection, which may cause a cholera-like illness (Wong *et al*., 2000).

*P. shigelloides* is also responsible for various extraintestinal infections of gastrointestinal origin, particularly in neonates and immunosuppressed adults or people with an underlying disease. The cases of cellulitis, cholecystitis, peritonitis, proctitis, pyosalpinx, septic arthritis, endophthalmitis, meningitidis in neonates, bacteremia, and septicemia were reported for *P. shigelloides* as an etiological agent (Wong *et al*., 2000; Stock, 2004). The outbreaks of *P. shigelloides*-related sepsis and meningitidis are associated with the serious course and high fatality rate (Stock, 2004). *P. shigelloides* adheres to and subsequently enters the human intestinal Caco-2 cells *in vitro* through phagocytic-like process. Moreover, it was found that live bacteria escape from cytoplasmic vacuoles (Theodoropoulos *et al*., 2001) and induce apoptotic cell death (Tsuigawa *et al*., 2005). To date several possible virulence factors of *P. shigelloides* have been discovered: cholera-like toxin (Gardner *et al*., 1987), thermostable and thermostable toxins (Matthews *et al*., 1988; Sears and Kaper, 1996), β-hemolysin (Janda and Abbott, 1993), and cytotoxin complex (Okawa *et al*., 2004). The cytotoxin having the potential role in enteropathogenicity of *P. shigelloides* is a heat-stable complex of lipopolysaccharide (LPS) and anticholera toxin-reactive proteins—LPS–ACRP complex (Okawa *et al*., 2004).

LPS (endotoxin), built of O-specific chain, core oligosaccharide, and lipid A, is the main constituent of the outer membrane of Gram-negative bacteria. LPS plays an important role for the effective barrier properties of the outer membrane (Nikaido and Nakae, 1979). It constitutes a ‘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. LPS plays a key role during severe Gram-negative infections, sepsis, and septic shock. Lipid A is an immunomodulatory center
of endotoxin and is recognized by different classes of receptors, including Toll-like receptors (Alexander and Zähringer, 2002).

Except for the detailed serotyping schemes of *P. shigelloides* (Aldova, 1992), the LPSs of these bacteria have not been extensively investigated (Linnerborg et al., 1995). Recently, we have presented the structure of the core oligosaccharide and the biological repeating unit of the O-antigen of *P. shigelloides* O54 LPS (strain CNCTC 113/92) (Czaja et al., 2000; Niedziela et al., 2002). The characteristic feature of the core decasaccharide isolated by mild acid hydrolysis of *P. shigelloides* O54 LPS is the lack of phosphate groups and the presence of GalA (Niedziela et al., 2002). The intact Kdo (3-deoxy-d-manno-oct-2ulosonic acid)-containing core region, the lipid A structure, and the linkage between them have not been investigated to date. Due to the role of LPS in *P. shigelloides* pathogenesis during intestinal and extraintestinal infections, it was important to determine the structure of its lipid A, completing the structural investigation of *P. shigelloides* O54 LPS.

The structural analysis of the intact Kdo-containing core region and its linkage with the lipid A requires de-N,O-acylation of LPS. The products formed by deacylation of smooth-type LPS, containing 4-substituted uronic acids, are usually complex mixtures of polysaccharides and oligosaccharides resulting from the β-elimination of the substituent at O-4 (Holst, 2000). It has been shown that sialic acids, Kdo residues, and Kdo-containing oligosaccharides derived from LPS form complexes with serotoninin (Sturgeon and Sturgeon, 1982; Rybka and Gamian, 2002). Previously, the immobilized serotinin was used to isolate free N-acetylated amino acid and oligosaccharides, polysaccharides, and glycoproteins, containing this sugar (Sturgeon and Sturgeon, 1982). We applied this technique for the one-step isolation of Kdo-containing oligosaccharides from the de-N,O-acylation products of LPS by affinity chromatography with immobilized serotoninin.

The structure of lipid A–inner core region of *P. shigelloides* O54 LPS was investigated by chemical analysis combined with NMR spectroscopy and MALDI-TOF mass spectrometry, using positive and negative ion modes. Thus this study complements the structural analysis of the *P. shigelloides* O54 lipopolysaccharide and reports on its biological *in vivo* and *in vitro* activity.

**Results**

**Isolation and NMR analysis of intact Kdo-containing core region of LPS**

LPS of *P. shigelloides* O54 (strain CNCTC 113/92) was isolated by phenol/water extraction and purified as previously reported (Petersson et al., 1997) (yield, 2% of dry bacterial mass).

LPS was de-N,O-acylated by hydrazinolysis and KOH treatment (Holst, 2000). As shown by NMR analysis, the deacylation of *P. shigelloides* O54 LPS yielded a mixture of incomplete core oligosaccharide linked to the lipid A carbohydrate backbone and fragments of core oligosaccharide substituted with different number of O-specific repeats (Figure 1A). The heterogeneity of the de-N,O-acylation products of LPS was a result of the β-elimination of the substituent at O-4 of α-D-GalPA, a constituent of the core oligosaccharide of *P. shigelloides* O54 LPS (Niedziela et al., 2002). The mixture (8 mg) was fractionated by affinity chromatography on a serotonin–Sepharose 4B column. Five fractions were eluted by a discontinuous ammonium acetate gradient and checked by NMR spectroscopy. The fraction eluted with 0.05 M ammonium acetate (3 mg) contained a decasaccharide built of incomplete core oligosaccharide with intact Kdo-containing region and lipid A carbohydrate backbone (Figure 1B). The sugar residues of the decasaccharide are notified by capital letters as shown in the structure (Figure 4). These letters refer to the corresponding residues through the entire text, tables, and figures, and the labeling is consistent with that used previously (Niedziela et al., 2002).

The affinity-purified decasaccharide was further investigated, and monosaccharides were identified on the basis of characteristic signals of deoxy protons of Kdo (residues A and Z), two signals of nitrogen-bearing carbons from aminosugars (residues X and Y), the characteristic resonance of H-4, C-4 (δ 5.79 and 109.1 ppm) belonging to the β-elimination product, 4-deoxy-β-L-threo-4-enopyranosyl (residue G), and the absence of signals of the O-specific chain constituents (Czaja et al., 2000) in the NMR spectra (Figures 1 and 2B and C). Chemical shifts of the identified residues (Table I) were compared with previously published NMR data for respective monosaccharides (Jansson et al., 1989). Chemical shift values of the →2,3,7-L-α-D-Hepp (D), →3,4-L-α-D-Hepp (B), terminal L-α-D-Hepp (F), terminal β-D-Glcp (E), and terminal β-D-Galp (C) were in agreement with those previously described as components of core oligosaccharide obtained by mild acid hydrolysis of *P. shigelloides* O54 LPS (Niedziela et al., 2002).

Residue G with the H-1/C-1 signals at δ 5.50/101.3 ppm, J_{H-1,H-2} < 2 Hz was assigned as the terminal 4-deoxy-β-L-threo-4-enopyranosyl (α-DGalpA), on the basis of the characteristic four-proton-spin system with the high chemical shift of the H-4 signal (δ 5.79 ppm) and the C-5 signal (δ 146.2 ppm).

Residue A was identified as the →4,5)-α-Kdo-(2→ on the basis of characteristic deoxy proton signals at δ 1.88 ppm (H-3ax) and δ 2.12 ppm (H-3eq) (Figure 3B), and a relatively high chemical shifts of the C-4 (δ 71.9 ppm) and C-5 (δ 70.4 ppm) signals.

Residue Z was identified as the terminal α-Kdo-(2→ on the basis of similar chemical shifts as those reported (Birnbaum et al., 1987; Müller-Loennies et al., 2002) (Figure 3B).

Residue X with the H-1/C-1 signals at δ 5.67/92.7 ppm, J_{H-1,H-2} < 3 Hz was assigned as the →6)-α-D-GlcnpN1P based on the typical chemical shifts of the H-1, C-1, and C-2 (δ 55.8 ppm) signals, the high chemical shift of the C-6 signal (δ 71.2 ppm), and the large vicinal couplings between H-2, H-3, H-4, and H-5 (J_{H-2,H-3}, J_{H-3,H-4}, J_{H-4,H-5} = 10 Hz). 1H, 31P-correlation experiment showed the connectivity between the phosphate monoester peak at δ 3.45 ppm and H-1 (δ 5.67 ppm) and H-2 (δ 3.42 ppm) of residue X (Figure 2E and F).

Residue Y with the H-1/C-1 signals at δ 4.81/100.7 ppm, J_{H-1,H-2} = 8 Hz was assigned as the →6)-β-D-GlcpN4P-(1→

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based on the low chemical shift of the C-2 signal (δ 57.0 ppm) and the large vicinal couplings between all ring protons. The chemical shift of the C-6 signal (δ 63.8 ppm) indicates the substitution by a Kdo residue (Bock et al., 1992; Oertelt et al., 2001). $^1$H,$^{31}$P-correlation experiment revealed the connectivity between the phosphate monoester peak at δ 1.77 ppm and H-4 (δ 3.80 ppm) of residue Y (Figure 2F), indicating the substitution of $\rightarrow 6$-β-D-GlcN-(1→ at O-4 by the phosphate group. The chemical shift values of residues X and Y were similar to these reported (Vinogradov and Bock, 1999).

Each disaccharide element in the decasaccharide was identified by HMBC (Figure 2A and D) and NOESY experiments (Table II), providing the sequence of sugar monomers in the decasaccharide. The HMBC spectrum showed cross-peaks between the anomeric proton and the carbon at the linkage position (Figure 2A) and between the anomeric carbon and the proton at the linkage position (Figure 2D), which confirmed the structure (Figure 4). The inter-residue NOEs were found between H-1 of E and H-2 of D, H-1 of G and H-3 of D, H-1 of F and H-7a,b of D, H-1 of D and H-3 of B, H-1 of B and H-5 of A, H-1 of C and H-4 of B, and H-1 of Y and H-6a,b of X (Table II). The inter-residue NOE signals between H-6 of residue Z and H-3ax (weak signal) and H-3eq (strong signal) of residue A further supported the disaccharide element: α-Kdo-(2→4)-α-Kdo (Birnbaum et al., 1987) (Figure 3A). These results suggest the following structure of the decasaccharide isolated from the LPS of P. shigelloides O54 containing the intact Kdo and lipid A carbohydrate backbone regions (Figure 4).

Isolation and compositional analysis of lipid A

The lipid A fraction was isolated by mild acid hydrolysis of P. shigelloides O54 LPS. Qualitative analysis of fatty acids was done separately for amide- and ester-bound fatty acids, using chemical analysis followed by GC-MS. The ($R$)-3-hydroxytetradecanoic acid (14:0(3-OH)) was identified as amide-linked fatty acid. Dodecanoic acid (12:0), ($R$)-3-hydroxydodecanoic acid (12:0(3-OH)), tetradecanoic acid (14:0), and trace amount of hexadecenoic acid (16:1) were detected as ester-bound fatty acids. Identification of the methoxy derivative—methyl ester of 3-methoxy-dodecanoic acid, among fatty acid methyl esters obtained by transesterification with sodium methanolate—showed also the presence of 12:0(3-OH), which was substituted by ‘secondary’ fatty acid in the native lipid A. The absolute configuration of GlcN residues in lipid A was determined as D on the deacylated, defosforylated lipid A fraction, using ($R$)-2-butanol as previously described (Gerwig et al., 1978, 1979).

MS analysis of lipid A

P. shigelloides O54 lipid A isolated by mild acid hydrolysis and native LPS were analyzed using MALDI-TOF MS (Figure 5; Table III).
The negative ion mode MALDI-TOF mass spectrum obtained for LPS contained three regions of ions (lipid A substituted with core oligosaccharide and one repeating unit of O-specific chain, lipid A substituted with core oligosaccharide and lipid A). Main ions, present in the lower mass range of the spectrum, corresponded to *Plesiomonas shigelloides* O54 lipid A (Figure 5A) and arose from fragmentation between polysaccharide and lipid A due to the acid lability of ketosidic bond in matrix solution. Observed ions reflected heterogeneity of the lipid A in native LPS. On the basis of the chemical composition, the most abundant ion at \( m/z \) 1741.8 [M–H] could be attributed to the hexaacylated lipid A, bisphosphorylated at O-1 and O-4′ and built up of two amide-bound 14:0(3-OH) and four ester-bound fatty acids: two 12:0(3-OH), one 12:0, and one 14:0, substituting the glucosamine backbone (Figure 5A, inset structure; Table III). Minor forms of lipid A were represented by peaks at \( m/z \) 1714.1 and \( m/z \) 1767.0. Peak at \( m/z \) 1714.1 originated from the molecule possessing a shorter (−28 Da) fatty acid. Peak at \( m/z \) 1767.0 was attributed to the presence in lipid A of a longer unsaturated (+26 Da) fatty acid (16:1) identified by chemical analysis. The ion at \( m/z \) 1864.4 corresponded to the lipid A molecule (major form) which was hexaacylated, bisphosphorylated, and additionally substituted with a phosphoethanolamine (PEtn). The ion at \( m/z \) 1889.7 represented minor form of *P. shigelloides* O54 lipid A, which was hexaacylated, bisphosphorylated, and substituted with PEtn.

The pattern of ions in negative ion MALDI-TOF mass spectrum of the free lipid A (Figure 5B) was more complex than that observed in the lower mass range of spectrum obtained for the native LPS (Figure 5A). Ions at \( m/z \) 1741.8 and 1714.1 corresponding to bisphosphorylated, hexaacylated forms of *P. shigelloides* O54 lipid A were also identified. The presence of peaks with lower \( m/z \) values (Table III) can be explained by partial degradation of lipid A during
work-up and lability of its acyl side chains and the phosphate group at O-1 during the acid hydrolysis (Wang and Cole, 1996). The ion at m/z 1661.4 corresponded to the monophosphorylated, hexaacylated lipid A molecule \([M–P–H]−\). Analysis of the complex pattern of this spectrum revealed several species, differing by the number of fatty acids. The peaks at lower m/z ratio represented monophosphorylated species lacking the 12:0 (m/z 1558.9) and the 12:0(3-O-12:0) (m/z 1280.3) at O-3′. The peak at m/z 682.8 arose from deprotonation, followed by simple cleavage of the glycosidic bond within the disaccharide backbone (Y1− ion), according to Domon and Costello nomenclature (Domon and Costello, 1988), 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 (Figure 5B, inset structure).

The substitution at O-4′ of distal GlcN (Y) by PEtn was deduced from the mass difference between ions at m/z 1784.9 and 1661.4 (Δ 123 Da). The ion at m/z 1661.4 corresponded to the lipid A monophosphorylated at O-4′ and devoid of phosphate group at O-1, which was lost during mild acid hydrolysis of the LPS (Table III). The presence of both α and β anomers of GlcN (residue X) was observed in the NMR spectra of the isolated lipid A (data not shown), confirming the lability of the phosphate group at O-1.

The structural information obtained from the negative ion mode MALDI-TOF MS was further supported by the positive ion mode (Figure 5C). MALDI-TOF MS analysis performed at high laser power settings in positive ion mode revealed the presence of sodium and potassium adducts of the monophosphorylated lipid A (data not shown) and

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<th>H-1/C-1</th>
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<th>H-3/C-3 (H-3ax/eq)</th>
<th>H-4/C-4</th>
<th>H-5/C-5</th>
<th>H-6a,b/C-6</th>
<th>H-7a,b/C-7</th>
<th>H-8a,b/C-8</th>
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<td>A</td>
<td>−4,5)-α-Kdo-(2→</td>
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<td>–</td>
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<td>4.11</td>
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<td>3.67</td>
<td>3.83</td>
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<td></td>
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<td>B</td>
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<td>5.25</td>
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<td>Y</td>
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<td>4.81</td>
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<td>100.7</td>
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<td>Z</td>
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<td>(1.75, 2.13)</td>
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<td>4.04</td>
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ax, axial position; eq, equatorial position; ND, not determined. Spectra were recorded for 2H2O solution at 30°C. Acetone (δH 2.225 and δC 31.05 ppm) was used as internal reference. The 1JC1,H1 values obtained from a non-decoupled HSQC experiment, confirmed the α-pyranosyl configuration for residues B, D, F, G, and X, and β-pyranosyl configuration for residues C, E, and Y.

*α-deoxy-β-L-threo-hex-4-enopyranosyl.

Table I. Li, 13C NMR chemical shifts (δ) of the decasaccharide isolated from Plesiomonas shigelloides OS4 LPS (strain CNCTC 113/92)

![Fig. 3. The fragments of NOESY (A) and HSQC-DEPT (B) spectra of the affinity-purified decasaccharide isolated from Plesiomonas shigelloides OS4 LPS (strain CNCTC 113/92). The cross-peaks are labeled as explained in the legend to Figures 1 and 2.](image-url)
oxonium ions (B₁⁺) (Figure 5C, inset structures). The B₁⁺ ions (m/z 1059.4, 831.6), arising from the cleavage of the glycosidic linkage between the two GlcN residues, were important for structure elucidation (Figure 5C). The oxonium ion at m/z 1059.4 corresponded to the phosphorylated, non-reducing tetraacylated GlcN (Y). On the basis of chemical analyses of fatty acids, this GlcN was substituted by 14:0(3-OH) at N-2′ and 12:0(3-OH) at O-3′, which could be further substituted by ‘secondary’ fatty acids: 14:0 and 12:0 (Figure 5C, inset structures). The ion at m/z 831.6 represented phosphorylated, non-reducing triacylated GlcN (Y) devoid of the 14:0 fatty acid (Δ 228 Da). The MS analyses of lipid A previously described by Sforza et al. (2004) demonstrated that the fatty acid at N-2′ was preferentially eliminated in the positive ion mode MS in comparison to the negative ion mode experiments. Thus, the presence of the ion at m/z 831.6 confirmed that 14:0 residue substituted the amide-bound 14:0(3-OH) at N-2′ of lipid A. This provided the evidence that the distal GlcN residue was substituted with 14:0(3-OH) and 12:0(3-OH) at positions N-2′ and O-3′, respectively. The B₁⁺ ion at m/z 1031.3 corresponded to one of the minor forms of the lipid A containing a shorter fatty acid (Δ 28 Da) in comparison with the major form. Such heterogeneity was not observed in the region of the ion at m/z 831.6. The mass difference between ions at m/z 1031.3 and 831.6 suggested that in the minor form of lipid A the 14:0 residue substituted the amide-bound 14:0(3-OH) at N-2′ of lipid A. This provided the evidence that the distal GlcN residue was substituted with 14:0(3-OH) and 12:0(3-OH) at positions N-2′ and O-3′, respectively. The B₁⁺ ion at m/z 1031.3 corresponded to one of the minor forms of the lipid A containing a shorter fatty acid (Δ 28 Da) in comparison with the major form. Such heterogeneity was not observed in the region of the ion at m/z 831.6. The mass difference between ions at m/z 1031.3 and 831.6 suggested that in the minor form of lipid A the 14:0(3-OH) at N-2′ was substituted with the secondary 12:0 instead of the 14:0 (major form of lipid A). On the basis of data obtained by chemical analysis and MALDI-TOF MS investigation, we conclude that the prevailing structure of
P. shigelloides O54 lipid A is built of the disaccharide $\beta$-D-GlcN-(1→6)$\alpha$-D-GlcN phosphorilated at O-1 and at O-4' and 14:0(3-OH), 12:0(3-OH), 14:0(3-O-14:0), and 12:0 (3-O-12:0) fatty acids at N-2, O-3, N-2', and O-3', respectively (Figure 5A, inset structure).

The complete structure of the P. shigelloides O54 LPS
The data of structural analysis of lipid A–inner core region were combined with those published for P. shigelloides O54 LPS core oligosaccharide (Niedziela et al., 2002) and biological repeating unit of the O-antigen (Czaja et al., 2000). The complete structure of LPS isolated from P. shigelloides O54 is presented here (Figure 6).
**Biological activity**

The cytokines (TNF-α and IL-6) and NO production by J774A.1 cells upon stimulation with LPS of *P. shigelloides* O54 and *Escherichia coli* O55 were compared. The LPS isolated from both bacteria demonstrated strong and dose-dependent stimulating activities on the J774A.1 cells (Figure 7). LPS of *P. shigelloides* O54 showed substantially stronger effect in vitro on the TNF-α production for the lowest dose (10 ng/ml) of LPS ($p < 0.05$). The differences in IL-6 production by J774A.1 cells upon stimulation with *E. coli* O55 and *P. shigelloides* O54 LPS were not statistically significant. The measurement of the total nitrite concentration demonstrated that J774A.1 cells responded similar to stimulation with both lipopolysaccharides, with a stronger effect for 1-μg dose of *P. shigelloides* O54 LPS ($p < 0.05$).

Endotoxicity of *P. shigelloides* O54 LPS in vivo was evaluated in actinomycin D-sensitized mice and was compared to that of LPS preparations of *E. coli* O55 and *E. coli* O1. The lethal effect of LPS administration was expressed as LD50 and was 10 times stronger in the case of *P. shigelloides* O54 LPS in comparison with the LPS of *E. coli* O55 and O1. The LD$_{50}$ values for LPS of *P. shigelloides* O54, *E. coli* O55, and *E. coli* O1 were 0.3, 3, and 3 ng per mouse, respectively.

**Discussion**

The pathogenicity of *P. shigelloides* is not yet fully understood. Endotoxin, the main surface antigen of Gram-negative bacteria, was found as a constituent of cytotoxin complex with anti-cholera toxin-reactive proteins exerted by *P. shigelloides*, thus being a potential virulence factor (Okawa *et al.*, 2004).

LPSs of *P. shigelloides* are poorly characterized, and until now, the structure of the *P. shigelloides* lipid A has not been determined. This prompted us to resolve the structure of entire LPS molecule by combining the data of structural analysis of the lipid A and its linkage with intact Kdo-containing core region to the previously published structures of the core oligosaccharide and O-specific biological repeating unit of *P. shigelloides* O54 LPS (strain CNCTC 113/92) (Czaja *et al.*, 2000; Niedziela *et al.*, 2002). These data showed that LPS of *P. shigelloides* O54 has the structure shown in Figure 6.

The typical core oligosaccharide of LPS contains a Kdo residue, which forms the linkage with lipid A via an acid-labile ketosidic bond. The presence of acid-labile constituents, such as diphosphate esters and one or two additional Kdo residues in the inner and outer core of LPS, was also reported (Holst, 2002). Resolving the complete structure of LPS requires examination of acid-labile Kdo-containing inner core region and its linkage with the lipid A carbohydrate backbone. The de-N,O-acylation of LPS, leading to the isolation of the intact Kdo-containing inner core region, is typically achieved using anhydrous hydrazine, followed by aqueous 4 M KOH treatment, to cleave ester- and amide-linked fatty acids with subsequent isolation of phosphorylated oligosaccharides by high-performance anion-exchange chromatography. This procedure, which is complementary to mild acid hydrolysis, is necessary to explore the intact Kdo-containing core region of LPS (Holst, 2000). Our preliminary structural studies of several *P. shigelloides* lipopolysaccharides (unpublished data) have shown a lack of phosphate groups and the presence of 4-substituted GalA as characteristic features of the core oligosaccharide of these bacteria. Thus NMR analysis of the intact Kdo-containing core region had to be preceded by isolation of...
groups limited to 12 or 14 carbons, the high cytokine-inducing activity of \textit{P. shigelloides} O54 LPS was expected and confirmed by the experimental data.

Among components of LPS (O-specific polysaccharide, core oligosaccharide, and lipid A), lipid A is known as the immunostimulatory center. It determines endotoxicity of LPS, which is modulated by core oligosaccharide and O-specific polysaccharide (Alexander and Zähringer, 2002). In contrast to the cytokine induction assay in vitro, there was significant difference between \textit{P. shigelloides} O54 LPS and both \textit{E. coli} O55 and \textit{E. coli} O1 LPS in their lethal effect evaluated in actinomycin D-sensitized mice. The compared lipopolysaccharides were smooth, that is, contained the core oligosaccharide substituted with O-specific polysaccharide and lipid A which allowed the full activity of those endotoxins (asymmetric, hexaacylated with fatty acids consisting of 12–14 carbons and bisphosphorylated lipid A [Alexander and Zähringer, 2002]). The difference between them in ‘general structure’ is the lack of phospho-ryl groups and the presence of GalA in the core oligosaccharide for \textit{P. shigelloides} O54. This difference did not affect the activity of \textit{P. shigelloides} O54 endotoxin in the \textit{in vitro} cytokine (TNFα and IL-6) and NO induction assays. As suggested by Mueller et al., (2004), the active units of endotoxins, amphiphilic molecules, are their aggregates. Thus the LPSs of \textit{E. coli} and \textit{P. shigelloides} O54 form aggregates which are biologically active and exhibit similar effects \textit{in vitro}. The lethal effect of such aggregates formed by \textit{P. shigelloides} O54 LPS, administered \textit{in vivo}, was 10 times stronger in comparison with that of \textit{E. coli} O55 and O1 LPS. The observed discrepancy could originate from differences in the complexity of \textit{in vivo} and \textit{in vitro} experiments. Injection of LPS \textit{in vivo} induces the production of various endogenous inflammatory mediators by effector cells and triggers the systemic regulatory mechanisms important to prevent endotoxin-induced pathogenic proinflammatory response and the onset of sepsis (Rietschel et al., 1996). Moreover, endotoxin clearance and neutralization mechanisms involve interaction between LPS and serum proteins (BPI and LBP) (Weiss, 2003; Hamann et al., 2005), antimicrobial peptides (Levy, 2000), and plasma lipoproteins (Brandenburg et al., 2002). Studies on the interaction between LPS and the integral outer membrane protein FhuA identified a conserved structural motif responsible for LPS recognition among LPS-binding proteins such as BPI, lactoferrin, lysozyme, and Limulus anti-LPS factor (Fergusson et al., 2000). In the case of FhuA–LPS and SMAP-29–LPS complexes (Tack et al., 2002), positively charged groups present in proteins interact with phosphorylated carbohydrate backbone of lipid A, Hep, Kdo, and negatively charged phosphate groups of the inner core oligosaccharide.

As most of the biophysical studies on the interactions of LPS with protein/peptide (Fergusson et al., 2000; Gutsmann et al., 2000; Gutsmann et al., 2001) and lipoprotein (Brandenburg et al., 2002) were limited to the isolated lipid A or R-forms of \textit{E. coli} LPS, the influence of non-typical structures of the core oligosaccharide on these interactions cannot be excluded. The presence of GalA and absence of phosphate residues in the core oligosaccharide for S-type \textit{P. shigelloides} O54 LPS could play role in dissagregation, less effective neutralization,
and clearance mechanisms resulting in higher toxicity of this LPS in vivo.

Materials and Methods

Bacteria

*P. shigelloides* serovar O54:H2 (strain CNCTC 113/92) was obtained from the Collection of the National Institute of Public Health, Prague, Czech Republic. Bacteria were grown and harvested as described previously (Petersson *et al.*, 1997). *E. coli* O1 was obtained from the Polish Collection of Microorganisms (PCM), Institute of Immunology and Experimental Therapy, Wroclaw, Poland.

Cell lines

The mouse macrophage-like cell line J774A.1 was obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). TNFα-sensitive WEHI 164.13 mouse fibrosarcoma cells were donated by Prof. M. Zimecki (Institute of Immunology and Experimental Therapy) and cultured as previously described (Lugowski *et al.*, 1996). The mouse IL-6-dependent B-cell hybridoma 7TD1 cell line, provided by Prof. S. Szymaniec (Institute of Immunology and Experimental Therapy), was cultured using Iscove’s medium containing 10% fetal calf serum, supplemented with 1.5 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 0.1 mM hypoxanthine, and 0.016 mM thymidine.

Animals

The BALB/c mice (females, 6–8 weeks of age) were housed at the animal facility of the Institute of Immunology and Experimental Therapy. The experiments were approved by the Local Ethical Commission for Animal Experimentation.

Lipopolysaccharide and lipid A isolation

LPS was isolated from lyophilized bacteria by the phenol/water extraction and purified as previously described (Westphal and Jann, 1965). Lipid A was obtained from LPS as a water-insoluble fraction by treatment with 1.5% acetic acid (45 min, at 100°C) followed by centrifugation (40,000 × g, 20 min).

De-N,O-acylation of LPS

LPS (200 mg) was de-N,O-acylated by mild hydrazinolysis followed by 4 M KOH treatment (Holst, 2000), and the reaction mixture was neutralized with HClO₃. The precipitated salt was removed by centrifugation for 30 min at 4°C (4000 × g), and the supernatant was further desalted using a Bio-Gel P-2 column (28 mg).

Purification of de-N,O-acylated LPS

The de-N,O-acylated LPS (8 mg) was purified using affinity chromatography on a serotonin–Sepharose 4B column. Briefly, Sepharose 4B was activated with BrCN (Cuatrecasas, 1970), and serotonin (200 mg) was coupled to the activated Sepharose 4B (10 ml) by incubating the mixture in 0.1 M sodium carbonate (pH 9.0) for 24 h at 4°C. The column (1.5 × 10 cm) was washed with 1 M ammonium acetate and equilibrated with water. De-N,O-acylated LPS fraction (8 mg) was dissolved in water and loaded on the column. Fractions (40 ml) were eluted by water and a discontinuous ammonium acetate gradient (0.025 M, 0.05 M, 0.1 M, 0.25 M, and 1 M), freeze-dried, and checked by NMR spectroscopy. The yields of the fractions eluted were 1.5 mg (water), 2.0 mg (0.025 M), 3.0 mg (0.05 M), 0.1 mg (0.1 M), 0.2 mg (0.25 M), and 0.8 mg (1 M).

Analytical procedures

To determine the absolute configuration of GlcN residues in the lipid A carbohydrate backbone, lipid A (5 mg) was hydrolyzed using 4 M HCl (12 h, 100°C). The solvent was evaporated, and the sample was subjected to chloroform/water extraction (1:2, v/v). The water-soluble part was hydrolyzed and then dephosphorylated with aqueous 48% HF (72 h, 4°C). The absolute configuration was determined as previously described (Gerwig *et al.*, 1978, 1979) using (+)-2-butanol for the formation of 2-butyl glycosides. The trimethylsilylated butyl glycosides were then identified by comparison with the standards produced from D-GlcN (Sigma, St. Louis, MO) and (+)-2-butanol and (-)-2-butanol (Fluka, Buchs, Switzerland) on GC-MS. Amide- and ester-bound fatty acids were analyzed separately (Wollenweber and Rietschel, 1990) by GC-MS performed with a Hewlett-Packard 5971A system, using an HP-1 fused-silica capillary column (0.2 mm × 12 m), the temperature program 100–270°C at 8°C/min. 3-Hydroxy fatty acids were converted to (S)-3-methoxy-phenylethylamide derivatives and analyzed by GC-MS using the HP-5 column (0.25 mm × 30 m) with a temperature program, 150–270°C at 8°C/min, to determine their absolute configurations (Gradowska and Larsson, 1994).

MALDI-TOF MS analysis

MALDI-TOF mass spectrometry, in positive and negative ion modes, was run on a Kratos Kompact-SEQ instrument. LPS was suspended in water (10 mg/ml) and desorbed with Dowex 50 Wx8 (H⁺). 2,5-Dihydroxybenzoic acid (10 mg/ml in 0.01 M citric acid) was used as matrix (Therisod *et al.*, 2001). Norharmane—9H-Pyrido[3,4-b]indole (1% in acetonitrile:water; 1:1, v/v)—was used as matrix for the analysis of lipid A in linear, negative ion mode. To obtain positive ion mass spectra of lipid A, sample was dissolved in CHCl₃/isopropyl alcohol/water (5:3:0.25, v/v/v) and desorbed as previously described (Gudlavalleti and Forsberg, 2003). 2,4,6-Trihydroxyacetophenone (25 mg/ml in acetonitrile:water; 1:1, v/v) was used as matrix. Lipid A was suspended in water and extracted with chloroform:methanol (3:1, v/v) prior to all MALDI-TOF MS analysis.

NMR spectroscopy

All spectra were obtained for ²H₂O solutions at 30°C on a Bruker DRX 600 instrument, using acetone (δH 2.225 ppm and δD 31.05 ppm) as internal reference and in the 31P NMR experiments 80% phosphoric acid (δp 0.0 ppm) as external reference. In the clean-TOCSY experiments, the mixing times used were 30, 60, and 100 ms. The delay time in HMBC was 60 ms, and the mixing time in NOESY was 200 ms.
**Conflicts of Interest Statement**

None declared.

**Abbreviations**

COSY, correlated spectroscopy; DEPT, distortionless enhancement by polarization transfer; GC, gas chromatography; GlcN, glucosamine; L-α-D-Hep, L-glycero-α-D-manno-heptose; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum coherence; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; IL-6: interleukin-6; LPS, lipopolysaccharide; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NO, nitric oxide; NOESY, nuclear Overhauser effect spectroscopy; P: phosphate; TNF-α, tumor necrosis factor alpha; TOCSY, total correlation spectroscopy.

**References**


Plesiomonas shigelloides O54 lipid A–inner core region


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