STRUCTURAL FEATURES OF LIPID A OF *PISCIRICKETTSIA SALMONIS*, THE ETIOLOGICAL AGENT OF THE SALMONID RICKETTSIAL SEPTICEMIA

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Summary. – The composition and structure of lipid A isolated from the lipopolysaccharide (LPS) of *Piscirickettsia salmonis* were investigated by chemical analyses, gas chromatography/mass spectrometry (GC-MS), and electrospray ionization (ESI) combined with the tandem mass spectrometry (MS/MS). Our study revealed moderate compositional and structural heterogeneity of lipid A with respect to the content of phosphate groups and 4-amino-4-deoxy-L-arabinopyranose (Ara4N) residues and with regard to the degree of acylation. It appeared that at least two molecular species were present in lipid A. The major species represented the hexaacyl lipid A consisting of the ß-(1→ 6)-linked D-glucosamine (GlcN) disaccharide backbone carrying two phosphate groups. The first one at the glycosidic hydroxyl group of the reducing GlcN I and the second one at the O-4' position of the non-reducing GlcN II. The primary fatty acids consisted of three 3-hydroxytetradecanoic [C14:0(3-OH)] and one 3-hydroxyhexadecanoic [C16:0(3-OH)] acids. The latter was amide-linked to GlcN I and one C14:0(3-OH) was amide-linked to GlcN II. Two secondary fatty acids were represented by C14:0(3-OH) and were equally distributed between the O-2' and O-3' positions. The phosphate group at O-4' carried a non-stoichiometric substituent Ara4N. The minor lipid A species contained exclusively C14:0(3-OH) with an asymmetric distribution (4+2) at GlcN II and GlcN I, respectively. The *P. salmonis* lipid A resembles structurally strongly endotoxic enterobacterial lipid A. This could be one of the reasons for the observed high endotoxicity of *P. salmonis*.

Key words: Piscirickettsia salmonis; lipopolysaccharide; lipid A; chemical composition; structure

Introduction

P. salmonis is the etiological agent of the salmonid rickettsial septicemia or piscirickettsiosis. The bacterium was isolated in 1989 from a moribund coho salmon from a saltwater net pen site in the south of Chile. It was the first rickettsia-like organism recognized as a fish pathogen (Fryer *et al*., 1990). It is known that *P. salmonis* is geographically more widespread than it was initially suspected. It has been observed in Canada, Norway, and Ireland. However, mortalities in these countries have not been as high as those in Chile (Fryer and Hedrick, 2003). *P. salmonis* is a Gram-negative, predominantly coccoid bacterium, approximately 0.5–1.5 μm in diameter that stains dark blue with Giemsa reagent and it is an obligate intracellular pathogen of fish. The bacterium replicates within the membrane-bound cytoplasmatic vacuoles (Fryer *et al*., 1992) or in the cytosol of the infected host cells (Almendras *et al*., 1997). It can be grown *in vitro* in the fish cell lines (Fryer *et al*., 1990) and most recently in the insect cell lines (Birkbeck *et al*., 2004).

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Abbreviations: Ara4N = 4-amino-4-deoxy-L-arabinopyranose; [C14:0(3-OH)] = 3-hydroxytetradecanoic acid; [C16:0(3-OH)] = 3-hydroxyhexadecanoic acid; C14:1n3 = 3-tetradecenoic acid; ESI = electrospray ionization; GC = gas chromatography; GlcN = glucosamine; LPS = lipopolysaccharide; MS = mass spectrometry; MS/MS = tandem mass spectrometry; m/z = mass-to-charge ratio; TLC = thin layer chromatography

The phylogenetic analysis based on sequencing of the 16S rRNA gene placed *P. salmonis* in a new family *Piscirickettsiae* within the class of *γ-proteobacteria*, most closely related to *Coxiella*, *Francisella* and *Legionella* (Fryer *et al*., 1992). The bacterium produces a systemic infection in fish targeting predominantly kidney, liver, spleen, intestine, brain, ovary and gills. Fish begin to die 6–12 weeks after their transfer to seawater net pens in fall and spring.

The *P. salmonis* antigens of potential importance for development of vaccines have been identified by the use of polyclonal and monoclonal antibodies and by their reaction with convalescent salmon sera (Kuzyk *et al*., 1996). Rabbit antiserum generated against the *P. salmonis* whole-cells reacted with six predominant antigens**.** Four of them with Mr approx. 65, 60, 54, and 51 K had a protein composition, while remaining two (Mr approx. 16 and 11 K) had a carbohydrate composition. The carbohydrate antigen at about 11 K has been presumed to be a lipopolysaccharide (LPS) (Kuzyk *et al*., 1996), but nothing has been known about its composition and structure so far. Among various bacteria, lipid A is structurally the most conserved region of an LPS (Caroff and Karibian, 2003). It has been established that lipid A represents the endotoxic principle of LPS (Alexander and Rietschel, 2001). Thus, knowledge of the lipid A structure is a prerequisite for understanding the molecular mechanisms operating during endotoxemia (Zähringer *et al*., 1999). To our knowledge, there have been no detailed works published dealing with both structure and endotoxic activity of *P. salmonis* lipid A. Therefore, in our initial studies on the *P. salmonis* LPS we decided first to elucidate the structural features of its lipid A.

Materials and Methods

Isolation of LPS and lipid A of P. salmonis. Purified *P. salmonis* cells were a gift from PHARMAQ AS, 0213 Oslo, Norway. The lyophilized *P. salmonis* cells (1 g) were suspended in 100 ml of 50 mmol/l Tris-HCl buffer, pH 7.5 and treated simultaneously with RNase and DNase I, both from bovine pancreas (Boehringer) at 37°C for 16 hrs. The cells were then treated with trypsin (Serva) at 37°C for 90 mins followed by proteinase K from *Tritirachium album* (Sigma) at 37°C for 16 hrs. After enzyme treatment, the cell suspension was centrifuged at 14,000 x g, 10°C for 50 mins and the sediment was washed with acetone. The dried cells were extracted with chloroform-methanol (2:1, v/v) at 20°C overnight to remove phospholipids. The extraction was repeated with the fresh solvent mixture for 2 hrs. The cell suspension was centrifuged at 3,000 x g for 20 mins and the sediment was suspended in preheated distilled water (100 ml, 68°C) and extracted with an equal volume of aqueous 90% phenol as described (Škultéty *et al*., 1998). The yield of LPS was 30.2 mg (3.2 %) calculated on the weight of the lyophilized *P. salmonis* cells. From the LPS, lipid A was released by hydrolysis with aqueous 1% (v/v) acetic acid at 100°C for 2.5 hrs. The insoluble lipid A was separated from the hydrolysis mixture by centrifugation. The pellet was washed twice with distilled water followed by centrifugation to remove residual polysaccharide, dispersed in distilled water and lyophilized.

SDS-PAGE was performed in slabs containing 18% polyacrylamide and the gels were silver- stained for LPS as described (Škultéty and Toman, 1992). An SDS Molecular Weight Markers kit was purchased from Sigma.

Analysis of LPS and lipid A. The LPS was hydrolyzed with 2 mol/l trifluoroacetic acid at 120°C for 3 hrs and the neutral and amino sugars were analyzed as the corresponding alditol acetates (Škultéty *et al*., 1998) by gas chromatography (GC) and GC-mass spectometry (GC-MS). After hydrolysis of LPS and lipid A in 2 mol/l hydrochloric acid in dry methanol for 2 and 16 hrs, the released fatty acids were analyzed directly and after trimethylsilylation by GC and GC-MS (Hussein *et al*., 2001).

Thin-layer chromatography (TLC) of lipid A was accomplished on pre-coated Silica Gel 60 plates (Merck, Germany) with isobutyric acid-1 mol/l ammonium hydroxide (5:3, v/v). Compounds were visualized by charring after spraying with 10% sulphuric acid in ethanol.

GC and GC-MS. GC was performed with a Shimadzu Model 17A chromatograph equipped with flame ionization detector using helium as the carrier gas. Alditol acetates of neutral sugars were separated on an SP-2330 column (30 m x 0.25 mm, Supelco) using a temperature program of 80°C for 2 mins, 30°C/min to 180°C, 4°C/min to 245°C, and 18 mins at 245°C. Alditol acetates of amino sugars were separated on an HP-5 column (25 m x 0.32 mm, Hewlett Packard) using a temperature program of 80°C for 1 min, 12°C/min to 180°C, hold 1 min at 180°C, 3°C/min to 210°C, and 15 mins at 210°C. Fatty acid methyl esters and their trimethylsilylated derivatives were separated on a DB-1 column (60 m x 0.25 mm, Fison, UK) using a temperature program of 80°C for 2 mins, 20°C/min to 160°C, 4°C/min to 236°C, 2°C/min to 300°C, and 5 mins at 300°C. The identity of each fatty acid was established by comparison of its MS profile with that of the reference compound. GC-MS was performed on a Shimadzu model QP5000 instrument with helium as the carrier gas. Electron impact mass spectra were recorded at 70 eV and an ion-source temperature of 250°C. GC-MS was run with the columns and temperature programs already described.

ESI-MS. ESI mass spectra were acquired with a Q-Tof Premier instrument from Waters. The lipid A samples were extracted with chloroform-methanol (1:1, v/v) at a concentration of the sample 1 mg/ml. Samples were infused by a nanocapillary into ESI chamber at a flow rate of 1 µl/min. The nanospray pressure was 5 x 10^4 Pa and the cone gas was delivered at the rate of 20 l/hr. The source temperature was maintained at 30°C. Argon was used as the collision gas. MS/MS experiments were performed with the collision energies of 90–100 eV. Data were acquired from the mass-to-charge ratio (m/z) 100 to 2200. The spectra were averaged and smoothed using a Savitzky-Golay smoothing procedure (Micromass). Calculated monoisotopic masses were obtained from MolE web (Molecular Mass Calculator v2.0; http://library.med.utah.edu/masspec/mole.htm).

Results and Discussion

Characterization of LPS and lipid A

SDS-PAGE of the LPS gave a single band at about 11 K (Fig. 1) indicating a relative mass homogeneity of the investigated LPS. The sugar analysis revealed the presence of Glc, Gal, Man, L,D-Hep, and GlcN in a molar ratio 4.8:3:3:1:0.4, respectively. Analyses of fatty acids showed the presence of [C14:0(3-OH)] and [C16:0(3-OH)] in a molar ratio 11:1, respectively.

Lipid A released from the parent LPS was analyzed for the presence of fatty acids and amino sugars. Both their composition and molar ratio corresponded to those given above. The chloroform-methanol extract of lipid A was directly analyzed by ESI-MS.

ESI-MS of lipid A

The ESI-MS analysis in the negative ion mode gave three major ion signals at m/z 1805.0, 1885.1, and 1936.0 in the higher mass range of the spectrum (Fig. 2). The peak at m/z 1885.1 corresponds to two GlcN, two phosphates, five C14:0(3-OH), and one C16:0(3-OH) (Table 1, Scheme 1). The most intensive peak in this area at m/z 1805.0 corresponds to two GlcN, one phosphate, five C14:0(3-OH), and one C16:0(3-OH) (Table 1, Scheme 2). The peak at m/z 1936.0 was attributed to two GlcN, one phosphate, five C14:0(3-OH), one C16:0(3-OH), and a compound having the mass of 131. The latter corresponded to anhydro 4-amino-4-deoxy-arabinose (Ara4N), which was found to be present in various lipid A of enterobacterial LPS (Zähringer *et al*., 1999). It was reported (Lee *et al*., 2004) that a loss of phosphate group is more favorable than a loss of acyl groups. The phosphate group at O-1 is more labile than that at O-4', because the hemiacetal oxygen in the sugar moiety destabilizes the phosphate group at O-1. Therefore, it is likely that the primary product arises from removal of the phosphate group at O-1. However, it is also known (Zähringer *et al*., 1999) that a noticeable amount of glycosidically linked phosphate is cleaved upon mild acid hydrolysis of an LPS during the lipid A isolation. Thus, both effects might contribute to a preponderance of the monophosphorylated lipid A ions in the mass spectrum. In the higher mass range, a small peak at m/z 1857.0 was also detected. It might correspond to a molecular species with two GlcN, two phosphates and six C14:0(3-OH) (Table 1, Scheme 3). This peak could be attributed to a minor molecular species of lipid A, which differed from the major one seen at m/z 1885.1 by 28 (2 CH_2). Further evidence for the presence of this species substituted exclusively with C14:0(3-OH) is given below.

In the lower mass range, there were intensive peaks at m/z 879.5, 942.6, and 1008.1 (Fig. 2), which were assigned to

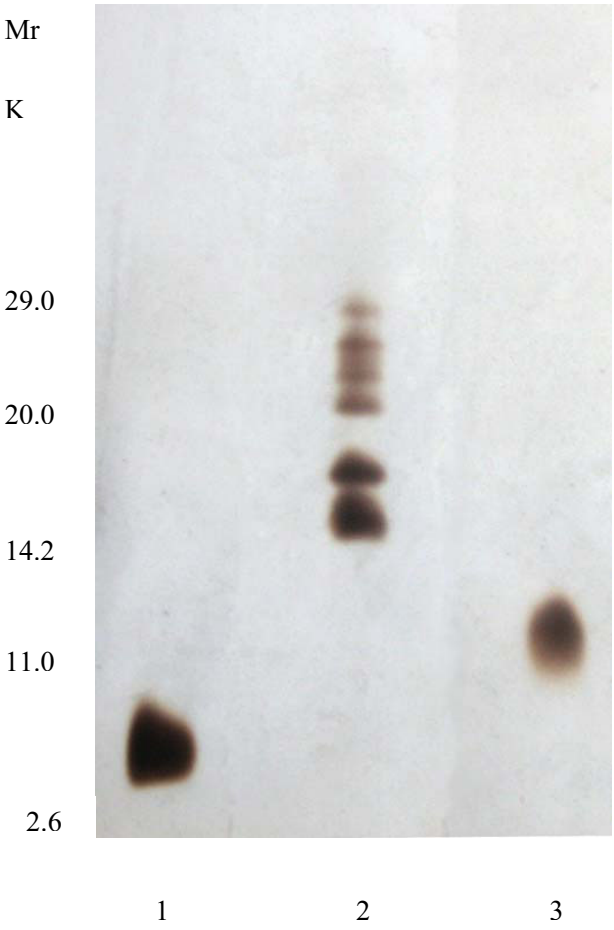


Fig. 1
SDS-PAGE of the LPSs from *C. burnetii* Nine Mile phase II (1), phase I (2), and *P. salmonis* (3)
Mr of the standards are on the left.

the doubly charged molecular species with m/z 1758.9, 1885.1, and 2016.1, respectively (Table 1). The peak at m/z 1758.9 could correspond to an anhydro derivative of the monophosphorylated hexaacylated molecular species consisting of two GlcN, one phosphate, and six C14:0(3-OH) (Scheme 3). A species with m/z 2016.1 was attributed to the diphosphorylated hexaacylated derivative consisting of two GlcN, two phosphates, five C14:0(3-OH), one C16:0(3-OH), and one Ara4N (Fig. 5).

Among smaller peaks present in the middle mass range of the spectrum (Fig. 2), only those found at m/z 1578.8, 1560.8, 1432.8, 1188.3, and 1062.4 could be interpreted (Table 1). The peak at m/z 1578.8 could arise from the monophosphorylated hexaacylated species at m/z 1805.0 with the loss of C14:0(3-OH) in a form of a ketene derivative (Scheme 2, pathway h). The peak at m/z 1560.8 also arises from the same species with the loss of free C14:0(3-OH)

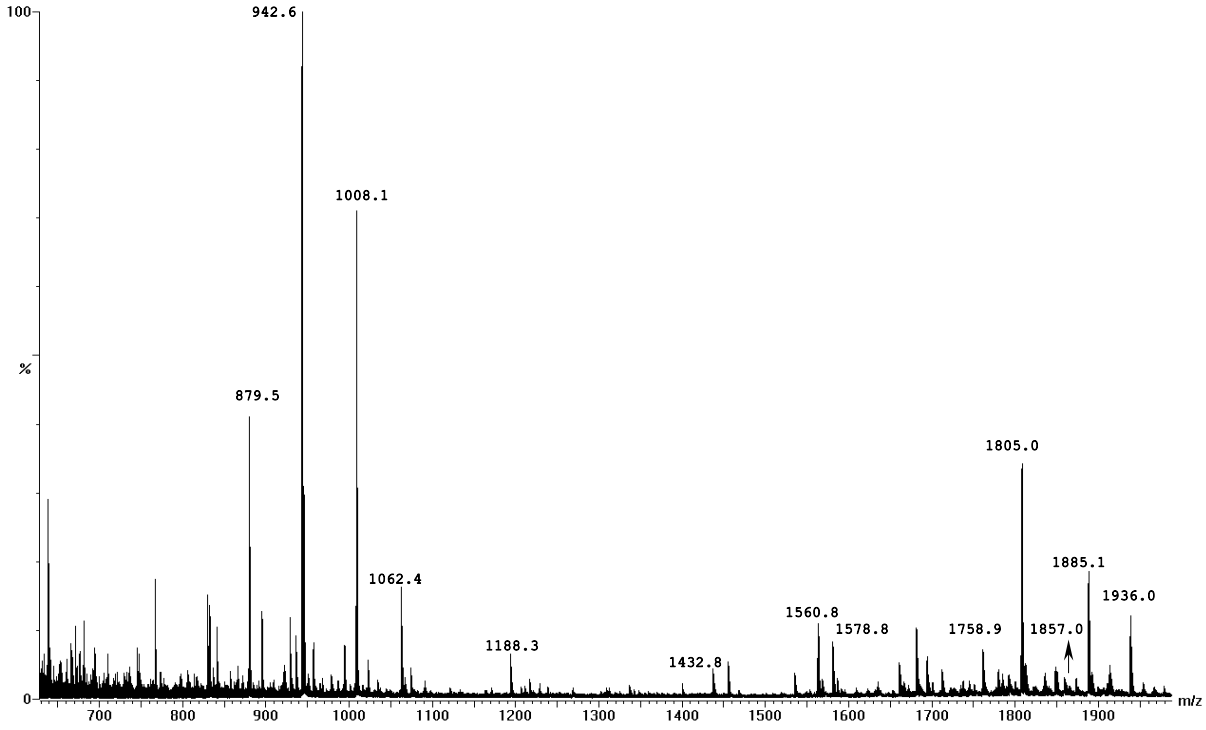


Fig. 2

ESI-MS spectrum (-ion mode) of the *P. salmonis* lipid A

x: mass to charge ratio; y: percentage of relative abundance.

(pathways f or i). Peak at m/z 1432.8 originates from the diphosphorylated hexaacylated form at m/z 1885.1 by the loss of an acyl-oxyacyl group C14:0[3-O(C14:0(3-OH))] in a form of a ketene derivative. The subsequent elimination of C14:0(3-OH) gave a fragment ion at m/z 1188.3 (Scheme 1, pathway e). Peak at m/z 1062.4 may originate from the diphosphorylated hexaacylated form of the minor molecular species at m/z 1857.0 with the loss of two free C14:0(3-OH) and one 3-tetradecenoic acid (C14:1n3) in a form of a ketene derivative (Scheme 3).

ESI-MS/MS of lipid A

The tandem mass spectrometry strategy was used to confirm the fatty acid substitution pattern in the *P. salmonis* lipid A species. MS/MS of the doubly charged ion at m/z 942.6 resulted in the most intensive fragment ions seen at m/z 1805.0, 1740.9, 1658.9 and a number of other ions (Fig. 3). Peak at m/z 1805.0 corresponds to the monophosphorylated hexaacylated molecular species already described. In contrast, the origin of the ion at m/z 1740.9 cannot be explained sufficiently at present, though it gives birth to the fragment ions at m/z 1496.7, 1270.5, and 1026.2 formed by the consecutive eliminations of C14:0(3-OH) (Table 1). The following peak at m/z 1658.9 arises from the diphosphorylated hexaacylated form from which one C14:0(3-OH) was eliminated in a form of ketene derivative (Scheme 1, pathway a). Further eliminations of this acid led to the subsequent formation of ions at m/z 1414.5 and 1170.2. However, the fragment ion at m/z 1170.2 could also arise through the less preferred fragmentation pathways c or d. It is not clear at present, whether the free fatty acid elimination takes place first at the acyl-oxyacyl group of O-3' or of O-3. Another type of the C14:0(3-OH) eliminations proceeds through the monophosphorylated hexaacylated species (m/z 1805.0) as given in the pathway b. A further fragmentation pathway e involved elimination of the acyl-oxyacyl group C14:0[3-O(C14:0(3-OH))] from the O-3' position of the diphosphorylated hexaacylated species as the ketene derivative giving birth to the fragment ion at m/z 1432.8, from which the ion at m/z 1188.3 was formed by elimination of free C14:0(3-OH).

MS/MS of the doubly charged ion at m/z 1008.1 led to the elimination of phosphate group from the C-1 position giving rise to the fragment ion at m/z 1936.0, which corresponded to the monophosphorylated hexaacylated molecular species with Ara4N attached to the phosphate located at O-4'. Other fragment ions observed represented the fatty acid eliminations.

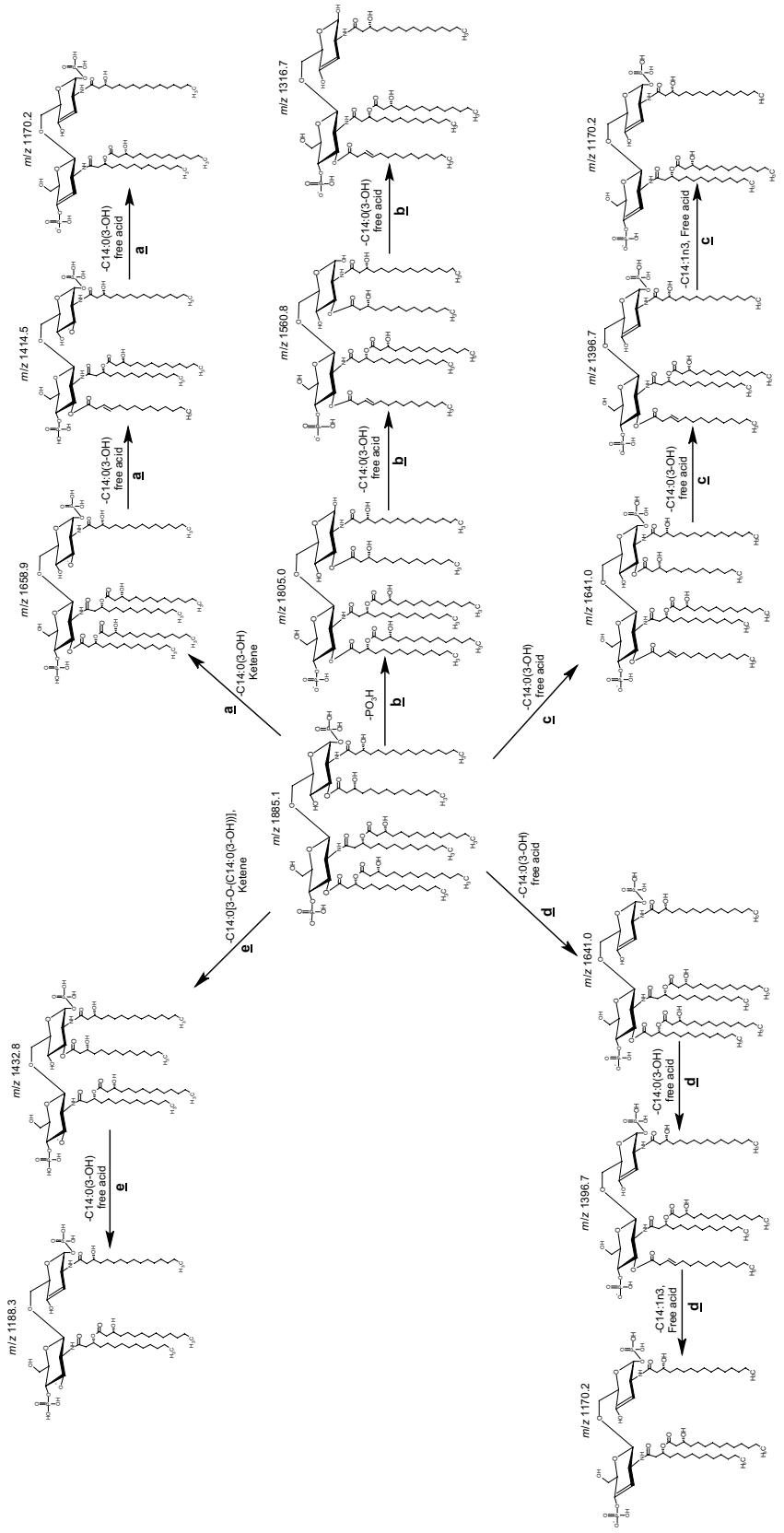
Table 1. Assignment of ions observed in ESI-MS and MS/MS spectra of *P. salmonis* lipid A

Molecular mass		Proposed interpretation
Observed	Calculated	
MS		
2016.1	2016.3	$[M_1\text{-H}]^-$, not seen in the spectrum
1936.0	1936.4	$[M_1\text{-}PO_3H\text{-H}]^-$
1885.1	1885.3	$[M_2\text{-H}]^-$
1857.0	1857.2	$[M_3\text{-H}]^-$
1805.0	1805.3	$[M_2\text{-}PO_3H\text{-H}]^-$
1758.9	1759.3	$[M_3\text{-}PO_4H_3\text{-H}]^-$
1578.8	1579.1	$[M_2\text{-}PO_3H\text{-H}]^-$ – C14:0(3-OH)[a]
1560.8	1561.1	$[M_2\text{-}PO_3H\text{-H}]^-$ – C14:0(3-OH) – H_2O
1432.8	1432.9	$[M_2\text{-H}]^-$ – C14:0[3-O(C14:0(3-OH))][a]
1188.3	1188.7	$[M_2\text{-H}]^-$ – C14:0[3-O(C14:0(3-OH))][a] – C14:0(3-OH) – H_2O
1062.4	1062.7	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – 2 C14:0(3-OH) – 2 H_2O – C14:1n3[a]
1008.1	1008.2	$[M_1\text{-2H}]^{2-}$
942.6	942.7	$[M_2\text{-2H}]^{2-}$
879.5	879.7	$[M_3\text{-2H-}PO_4H_3]^{2-}$
MS/MS of m/z 1008.1		
1936.0	1936.4	$[M_1\text{-}PO_3H\text{-H}]^-$
1692.0	1692.2	$[M_1\text{-}PO_3H\text{-H}]^-$ – C14:0(3-OH) – H_2O
1465.7	1466.0	$[M_1\text{-}PO_3H\text{-H}]^-$ – C14:0(3-OH) – H_2O – C14:1n3 – H_2O
MS/MS of m/z 942.6		
1805.0	1805.3	$[M_2\text{-}PO_3H\text{-H}]^-$
1740.9	1741.3	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – H_2O
1658.9	1659.1	$[M_2\text{-H}]^-$ – C14:0(3-OH)[a]
1641.0	1641.1	$[M_2\text{-H}]^-$ – C14:0(3-OH) – H_2O
1560.8	1561.1	$[M_2\text{-}PO_3H\text{-H}]^-$ – C14:0(3-OH) – H_2O
1514.9	1515.1	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – C14:0(3-OH) – H_2O
1496.7	1497.1	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – C14:0(3-OH) – 2 H_2O
1432.8	1432.9	$[M_2\text{-H}]^-$ – C14:0[3-O(C14:0(3-OH))][a]
1414.5	1414.9	$[M_2\text{-H}]^-$ – C14:0(3-OH)[a] – C14:0(3-OH) – H_2O
1396.7	1396.9	$[M_2\text{-H}]^-$ – 2 C14:0(3-OH) – 2 H_2O
1334.7	1334.9	$[M_2\text{-}PO_3H\text{-H}]^-$ – C14:0(3-OH)[a] – C14:0(3-OH) – H_2O
1316.7	1316.9	$[M_2\text{-}PO_3H\text{-H}]^-$ – 2 C14:0(3-OH) – 2 H_2O
1270.5	1270.9	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – 2 C14:0(3-OH) – 2 H_2O
1188.3	1188.7	$[M_2\text{-H}]^-$ – C14:0[3-O(C14:0(3-OH))][a] – C14:0(3-OH) – H_2O
1170.2	1170.7	$[M_2\text{-H}]^-$ – C14:0(3-OH)[a] – 2 C14:0(3-OH) – 2 H_2O
1170.2	1170.7	$[M_2\text{-H}]^-$ – 2 C14:0(3-OH) – 2 H_2O – C14:1n3 – H_2O
1044.4	1044.7	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – 2 C14:0(3-OH) – 2 H_2O – C14:1n3 – H_2O
1026.2	1026.7	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – 3 C14:0(3-OH) – 3 H_2O
MS/MS of m/z 879.5		
1514.9	1515.1	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – C14:0(3-OH) – H_2O
1270.5	1270.9	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – 2 C14:0(3-OH) – 2 H_2O
1062.4	1062.7	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – 2 C14:0(3-OH) – 2 H_2O – C14:1n3[a]
1044.4	1044.7	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – 2 C14:0(3-OH) – 2 H_2O – C14:1n3 – H_2O
1026.2	1026.7	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – 3 C14:0(3-OH) – 3 H_2O
818.2	818.5	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – 2 C14:0(3-OH) – 2 H_2O – 1 C14:1n3 – H_2O – C14:0(3-OH)[a]
800.1	800.5	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – 3 C14:0(3-OH) – 3 H_2O – C14:1n3 – H_2O
MS/MS of m/z 1805.0		
1560.8	1561.1	$[M_2\text{-}PO_3H\text{-H}]^-$ – C14:0(3-OH) – H_2O
1334.7	1334.9	$[M_2\text{-}PO_3H\text{-H}]^-$ – C14:0[3-O(C14:0(3-OH))] – H_2O
1334.7	1334.9	$[M_2\text{-}PO_3H\text{-H}]^-$ – C14:0(3-OH)[a] – C14:0(3-OH) – H_2O
1316.7	1316.9	$[M_2\text{-}PO_3H\text{-H}]^-$ – 2 C14:0(3-OH) – 2 H_2O
1026.2	1026.7	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – 3 C14:0(3-OH) – 3 H_2O
800.1	800.5	$[M_3\text{-}PO_4H_3\text{-H}]^-$ – 3 C14:0(3-OH) – 3 H_2O – C14:1n3 – H_2O
734.4	734.5	$^{0,4}A$[b] – C14:0[3-O(C14:0(3-OH))] – H_2O
716.4	716.6	$^{0,4}A$ – 2 C14:0(3-OH) – 2 H_2O
490.2	490.4	$^{0,4}A$ – 2 C14:0(3-OH) – 2 H_2O – C14:1n3 – H_2O

M_1 = 2 GlcN, 2 PO_3H, 5 C14:0(3-OH), 1 C16:0(3-OH), Ara4N; $M_2 = M_1$-Ara4N; M_3 = 2 GlcN, 2 PO_3H, 6 C14:0(3-OH).

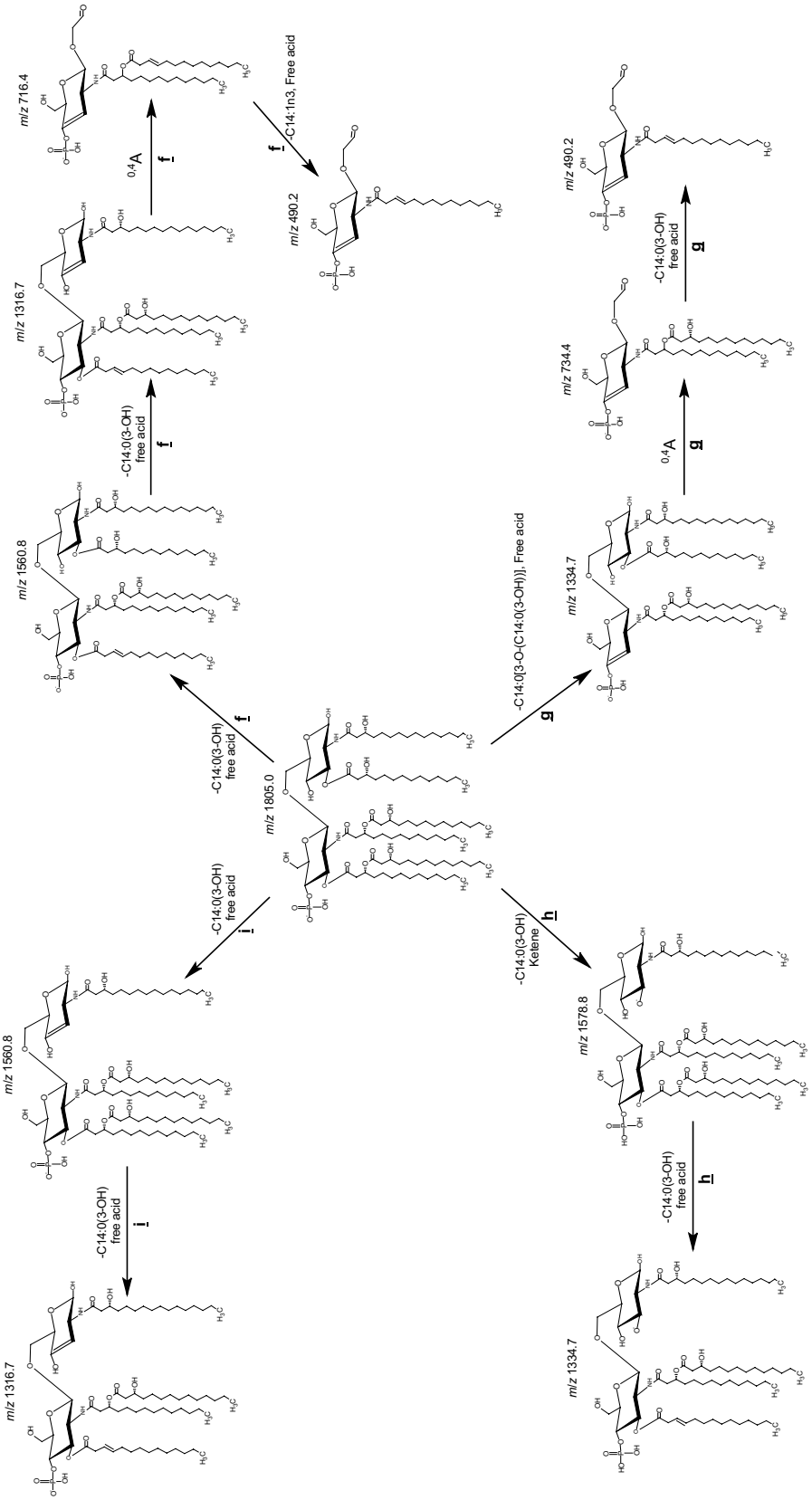
[a]Elimination of the fatty acid as a ketene derivative.

[b] $^{0,4}A$ – cross-ring fragments with cleavage in the sugar ring of GlcN I.



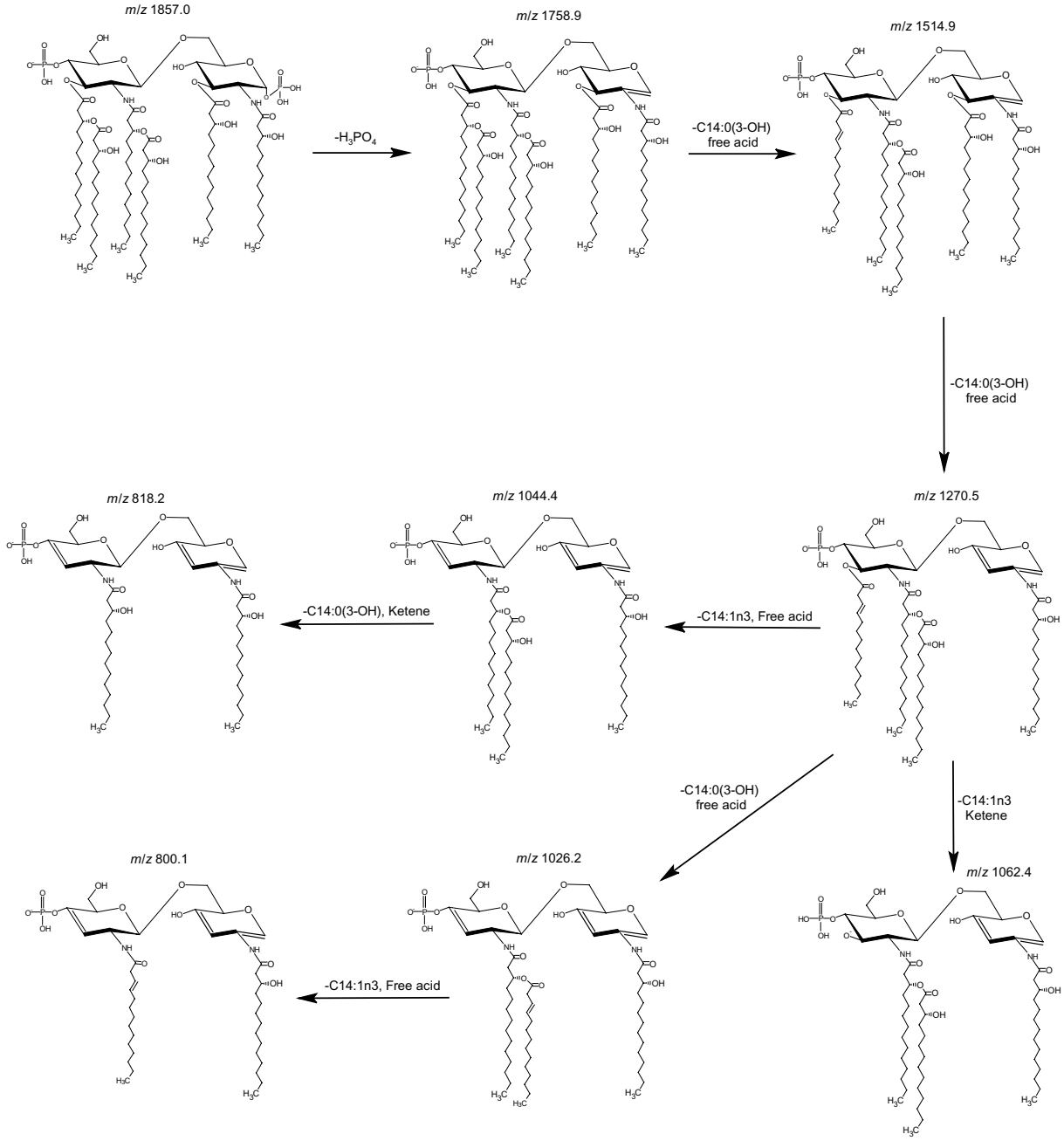
Scheme 1

Fragmentation pathways (a – e) of the lipid A precursor ion at *m/z* 1885.1



Scheme 2

Fragmentation pathways (f – i) of the lipid A precursor ion at *m/z* 1805.0



Scheme 3

Fragmentation pathways of the lipid A precursor ion at *m/z* 1857.0

MS/MS of the doubly charged ion at m/z 879.5 gave the fragment ions of the minor monophosphorylated molecular species of lipid A having six C14:0(3-OH) distributed along the diglucosamine disaccharide. The ions were formed by the consecutive eliminations of the acid as given in Table 1 and Scheme 3.

From the singly charged ions, the ion at m/z 1805.0 was investigated in detail. MS/MS of it (Fig. 4) resulted in the fragment ion at m/z 1560.8 corresponding to a loss of free C14:0(3-OH) either from the O-3' acyl-oxyacyl position or from O-3 (Scheme 2, pathways f and i). Subsequent eliminations of free C14:0(3-OH) proceeded in the already

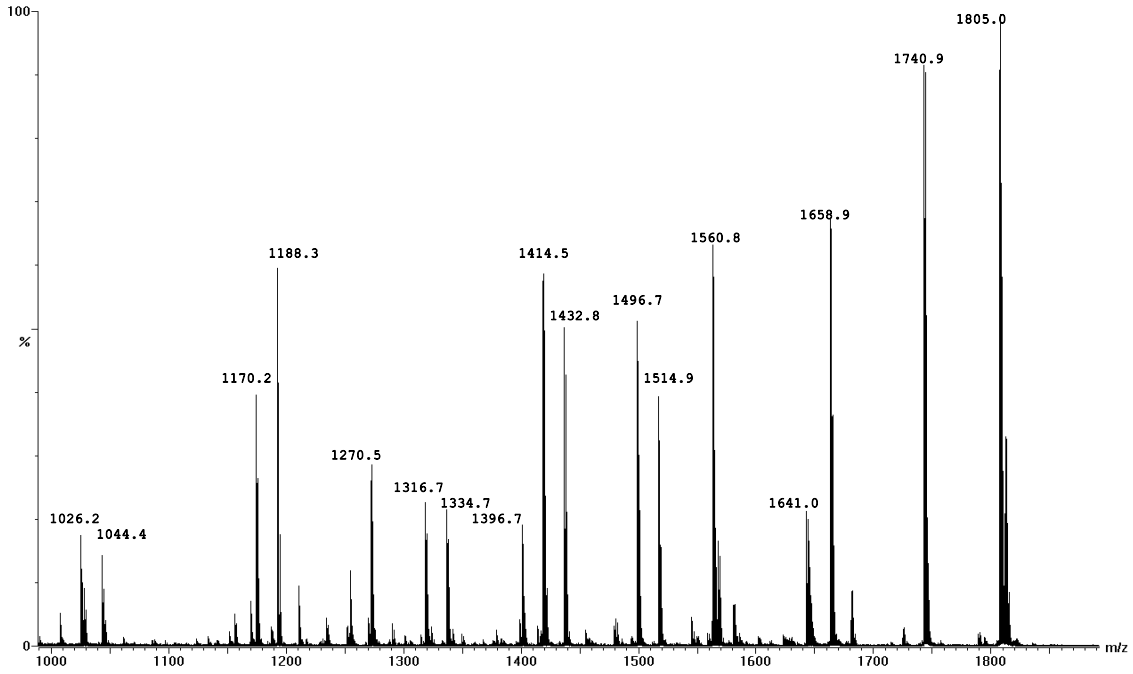


Fig. 3

ESI-MS/MS spectrum (-ion mode) of the doubly charged ion at *m/z* 942.6

x: mass to charge ratio; y: percentage of relative abundance.

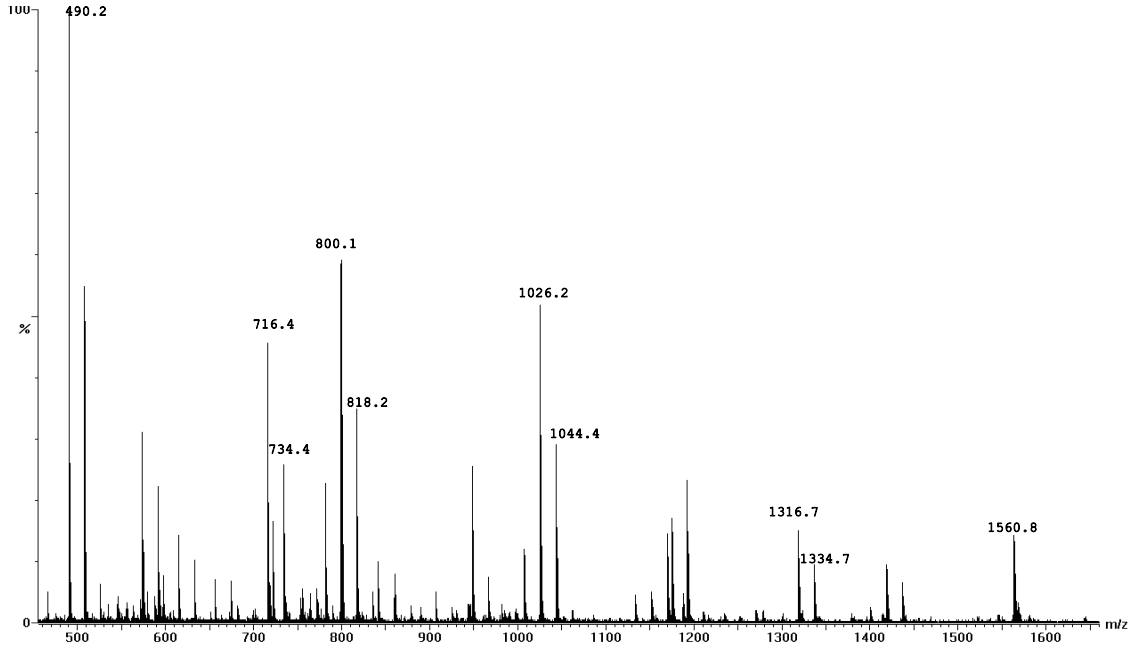


Fig. 4

ESI-MS/MS spectrum (-ion mode) of the singly charged ion at *m/z* 1805.0

x: mass to charge ratio; y: percentage of relative abundance.

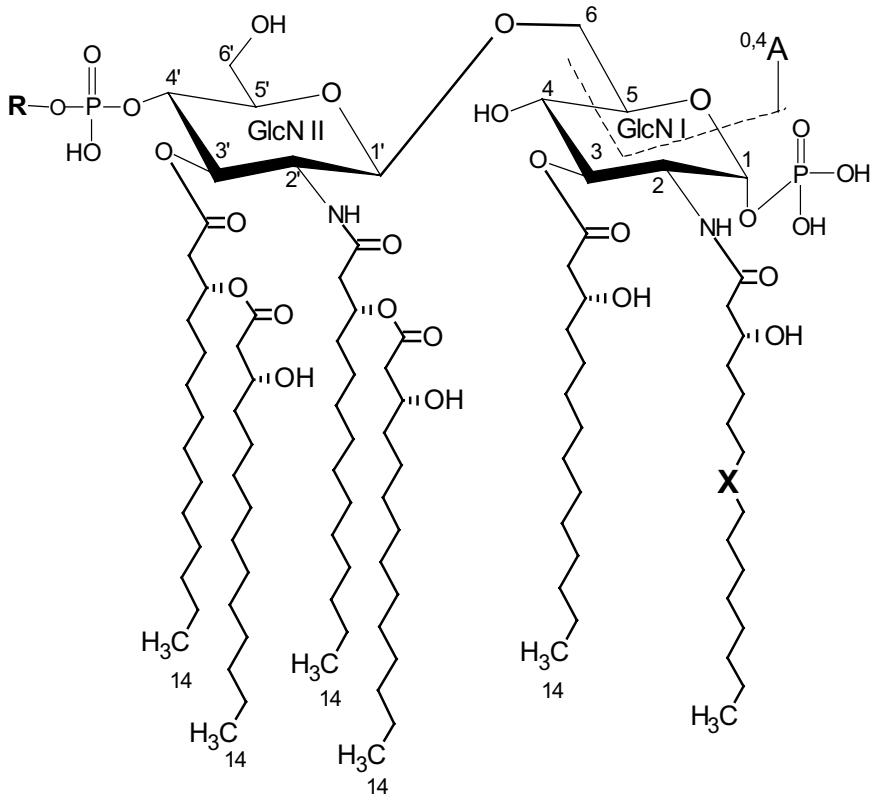


Fig. 5

Structural features of *P. salmonis* lipid A

X = C16:0(3-OH) or C14:0(3-OH) for the major or minor molecular species, respectively; R = H or nonstoichiometric Ara4N; ^{0,4}A = a ring fragmentation observed in ESI-MS/MS.

known way (Table 1). The ions at m/z 716.4 and 490.2 (pathway f) were assigned to the ^{0,4}A cross-ring fragments (Costello and Vath, 1990) arising of the ion at m/z 1316.7. From the precursor ion at m/z 1805.0, the ion at m/z 1334.7 was formed (pathway g) followed by two ^{0,4}A fragment ions at m/z 734.4 and 490.2. The origin of the ions at m/z 1026.2 and 800.1 (Fig. 4) could not be explained satisfactorily so far. They might be attributed to the minor monophosphorylated triacylated and diacylated molecular species, respectively (Table 1 and Scheme 3).

We conclude from our studies that the major *P. salmonis* lipid A species represents the hexaacyl form (Fig. 5) resembling classical lipid A found in the *Enterobacteriaceae* family. It consists of the ß-(1→6)-linked GlcN disaccharide backbone carrying two phosphate groups. One is linked to the glycosidic hydroxyl group of GlcN I and the other is ester linked to the O-4' position of GlcN II. The primary fatty acids consist of three C14:0(3-OH) and one C16:0(3-OH). The latter is amide-linked to GlcN I and one C14:0(3-OH) is amide-linked to GlcN II. Two secondary fatty acids are represented by C14:0(3-OH) and are equally distributed between the O-2' and O-3' positions. The phosphate group at O-4' carries a non-stoichiometric substituent Ara4N. The minor lipid A species contains exclusively C14:0(3-OH) with an asymmetric distribution (4+2) at GlcN II and GlcN I, respectively. Ions of this species were less intensive in the MS spectrum as those of the major molecular species and thus, we were unable to perform the corresponding MS/MS experiments on them.

Our studies revealed a moderate compositional and structural heterogeneity of the *P. salmonis* lipid A with respect to the content of phosphate groups and the Ara4N residues, and with regard to the degree of acylation. The observed heterogeneity was generated by an incomplete biosynthesis of lipid A in the living bacterium and by the degradation processes during its isolation from the parent LPS. It was reported in the past (Alexander and Rietschel, 2001) that the hexaacylated lipid A species consisting of two 3-hydroxyacyl and two 3-acyloxyacyl residues limited in length of individual acid chains to 12 or 14 carbons in

either *Escherichia coli* or *Neisseria meningitidis* type exhibit maximal endotoxic activities in numerous mammalian species. Structural features of the *P. salmonis* lipid A show a high degree of similarity with these classical forms of enterobacterial lipid A and this fact could be one of the reasons for a high endotoxic potency of the whole *P. salmonis* bacterium. However, more detailed structure/function relationship studies are needed to give an ultimate answer to these questions.

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