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STRUCTURE OF LIPID A FROM THE MARINE GRAM-NEGATIVE BACTERIUM *Pseudoalteromonas nigrifaciens* IAM 13010^T

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The structure of lipid A from the marine γ-proteobacterium Pseudoalteromonas nigrifaciens *IAM 13010^T that was prepared by hydrolysis of the corresponding lipopolysaccharide by acetic acid (1%) was determined by chemical analysis, ^{13}C NMR spectroscopy, and MALDI/TOF and LSIMS mass spectrometry. It was shown that lipid A is a β-1,6-bonded disaccharide of glucosamine that is substituted by two phosphoric acids (in the C1 and C4′ positions), two (R)-3-hydroxyalkanoic (normal and branched) acids with ester bonding (at the C3 and C3′-positions), and (R)-3-hydroxydodecanoic and (R)-3-dodecanoyloxydodecanoic acids (both with amide bonding at C2′ and C2, respectively). It was hypothesized that this type of structure is typical of lipid A from bacteria of the genus* Pseudoalteromonas *in general.*

Key words: marine gram-negative bacteria, *Pseudoalteromonas nigrifaciens*, LPS, lipid A, ^{13}C NMR spectroscopy, MALDI/TOF and LSIMS mass spectrometry.

Gram-negative bacteria contain an unusual glycophospholipid called lipid A in addition to classical lipids constructed from glycerine.

It is well-known that variations in the structure of lipid A are largely determined by the cultivation conditions of the microorganisms [1, 2]. Therefore, we supposed that marine bacteria, whose habitat is typically cold with high pressure and an elevated content of inorganic salts [3], could become a source of structural variants of lipid A that are interesting from a pharmacological viewpoint. However, information on the structure and biological activity of lipid A from marine gram-negative bacteria are available for a limited number of microorganisms [4-6].

We studied the structure of lipid A from a bacterium of the genus *Pseudoalteromonas*, *P. nigrifaciens* IAM 13010^T. This is an aerobic, heterotrophic, gram-negative bacterium [7] and widely distributed obligate marine microorganism that is easily isolated from littoral waters and from the open sea and requires seawater in the growth medium.

The most common method for preparing lipid A is hydrolysis by AcOH (1%) of lipopolysaccharide (LPS) [8], which is in turn obtained by extraction of defatted cells by hot aqueous phenol (45%) by the Westphal method [9]. Using this approach, lipid A was isolated from *P. nigrifaciens* and contained glucosamine (GlcN), phosphorus, and FA according to total chemical analysis.

The principal FA were 3-hydroxydodecanoic (3-OH-12:0, 36.1 mass %), a part of which (3.4%) occurred as the *iso*-form; dodecanoic (12:0, 12.8%); 3-hydroxyundecanoic (3-OH-11:0, 7.9%); 3-hydroxytetradecanoic (3-OH-14:0, 6.2%); 3-hydroxytridecanoic (3-OH-13:0, 5.0%); and 3-hydroxydecanoic (3-OH-10:0, 2.6%). Thus, lipid A from *P. nigrifaciens*, like lipid A from other bacteria of this genus [10], is highly heterogeneous with respect to the composition of 3-hydroxyalkanoic acids.

According to TLC, lipid A from *P. nigrifaciens* is a rather complex mixture of homologs with differing degrees of phosphorylation and acylation. This significantly complicates its further structural characterization. In order to decrease the heterogeneity of the studied preparation, lipid A was treated with HCl (0.1 M) in order to remove the glycoside-bound phosphoric acid, which is highly acid-labile [11].

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TABLE 1. MALDI/TOF and LSIMS Negative-Ion Mass Spectra of Lipids A from *P. nigrifaciens* IAM 13010^T, *P. haloplanktis* ATCC 14399^T, and *P. tetraodonis* IAM 14160^T

m/z	I_{rel}, %	Ion composition
Lipid A from *P. nigrifaciens* MALDI/TOF MS of diphosphorylated derivative		
1473.4	41	[M(2×GlcN+4×3-OH-12:0+1×12:0+2×H_3PO_4)-H]$^-$
1291.5	100	[M(2×GlcN+4×3-OH-12:0+2×H_3PO_4)-H]$^-$
1093.4	34	[M(2×GlcN+3×3-OH-12:0+2×H_3PO_4)-H]$^-$
MALDI/TOF MS of monophosphorylated derivative		
1393.3	57	[M(2×GlcN+4×3-OH-12:0+1×12:0+1×H_3PO_4)-H]$^-$
1195.6	100	[M(2×GlcN+3×3-OH-12:0+1×12:0+1×H_3PO_4)-H]$^-$
1013.4	70	[M(2×GlcN+3×3-OH-12:0+1×H_3PO_4)-H]$^-$
LSIMS MS of monophosphorylated derivative		
1195	25	[M(2×GlcN+3×3-OH-12:0+1×12:0+1×H_3PO_4)-H]$^-$
1013	58	[M(2×GlcN+3×3-OH-12:0+1×H_3PO_4)-H]$^-$
816	100	[M(2×GlcN+2×3-OH-12:0+1×H_3PO_4)-H]$^-$
654	63	[M(1×GlcN+2×3-OH-12:0+1×H_3PO_4)-H]$^-$
LSIMS MS of monophosphorylated derivative of lipid A from *P. haloplanktis*		
640.2	22	[M(1×GlcN+1×3-OH-12:0+1×3-OH-11:0+1×H_3PO_4)-H]$^-$
456.2	100	[M(1×GlcN+1×3-OH-12:0+1×H_3PO_4)-H]$^-$
LSIMS MS of monophosphorylated derivative of lipid A from *P. tetraodonis*		
815.3	20	[M(2×GlcN+2×3-OH-12:0+1×H_3PO_4)-H]$^-$
654.2	16	[M(1×GlcN+2×3-OH-12:0+1×H_3PO_4)-H]$^-$
456.3	100	[M(1×GlcN+1×3-OH-12:0+1×H_3PO_4)-H]$^-$

Fractionation of lipid A treated with HCl (0.1 M) by ion-exchange chromatography over a column of Servacel DEAE-52 cellulose produced two main fractions that differed from each other according to MALDI/TOF mass spectrometry (Table 1) by 80 amu and, therefore, were mono- and diphosphorylated derivatives. These data suggest that lipid A from *P. nigrifaciens* in general contains two phosphates, one of which is located on C1 of a glucosaminobiose.

Further structural investigations were carried out using the monophosphorylated derivative of lipid A. The ^{13}C NMR spectrum of this sample showed two resonances for anomeric C atoms of GlcN, which proved the disaccharide nature of lipid A. The chemical shifts (CS) (91.7 and 101.9 ppm) of these resonances indicated the α- and β-configurations of the reducing and nonreducing ends of the glucosaminobiose and the lack of a phosphate on C1 (the presence of phosphoric acid on C1 of GlcN is seen as a shift of the resonance of this C atom to weak field by up to ~94 ppm [12]).

The spectral region where C2 of GlcN resonates (two resonances with CS 52.6 and 54.4 ppm) confirmed the conclusion about the disaccharide nature of the compound and indicated the α- and β-configurations of the anomeric centers of the reducing and nonreducing GlcN. The strong-field shift of the resonances for both C2 atoms (compared with C2 resonances of 56.6 and 53.9 ppm, respectively) in the 6-diphenylphosphate of α-methyl-*O*-(2-deoxy-2-[(*R*,*S*)-3-hydroxytetradecanoylamino]-β,D-glucopyranosyl)-(1→6′)-2-(2-deoxy-2-[(*R*,*S*)-3-hydroxytetradecanoylamino]-D-glucopyranoside [13] leads to the conclusion that the C3 hydroxyls of both GlcN units are substituted.

The region of C6 resonances of GlcN units have one resonance (60.9 ppm). The resonance of the second C6 is shifted to 69 ppm, indicating a β-1,6′-glycoside bond in lipid A from *P. nigrifaciens*.

Thus, the carbohydrate skeleton of lipid A from *P. nigrifaciens* is a β-1,6′-bonded disaccharide of GlcN with two phosphates, one of which is located on C1. Both amino groups and the hydroxyls on C3 and C3′ of the glucosaminobiose have acyl substituents.

The region of carbonyl resonances in the ^{13}C NMR spectrum of lipid A from *P. nigrifaciens* contains five resonances of approximately equal strength. This is consistent with the presence of five FA, four of which are 3-hydroxyalkanoic (the

spectrum has four C2 resonances of 3-hydroxyalkanoic acids at 42-44 ppm [14, 15]). However, the region of C3 resonances of 3-hydroxyalkanoic acids (~68 ppm [14, 15]) contains three signals (68.7, 68.8, and 68.9 ppm), which suggests the presence of an acyl substituent on the hydroxyl of one of the four 3-hydroxyalkanoic acids in lipid A from *P. nigrifaciens*. The presence of a resonance with CS 35.1 ppm (region of C4 resonance of 3-acyloxyalkanoic acids [14, 15]) agrees well with this hypothesis.

Hydrolysates of lipid A from *P. nigrifaciens* did not contain (*E*)-2-unsaturated FA, dehydration products of 3-acyloxyalkanoic acids with ester bonds [16]. This can be taken as indirect proof that a 3-acyloxyalkanoic acid in this lipid A had an amide bond, which was mentioned above.

Direct proof of an amide-bonded 3-acyloxyalkanoic acid was obtained by analyzing the ^{13}C NMR spectrum in the region of C2 resonances of 3-hydroxy-(acyloxy-)alkanoic acids (39-44 ppm). It is known that C2 atoms of amide-bonded 3-hydroxyacids have CS of about 44 ppm [14, 15]. Substitution of the C3 hydroxyl of hydroxyacids with an amide bond shifts the C2 resonance to strong field by 41 ppm. The presence in the spectrum of lipid A from *P. nigrifaciens* of resonances with CS 43.8 and 41.5 ppm suggests that the amine of one GlcN is substituted by a 3-hydroxyalkanoic acid; the amine of the other, by a 3-acyloxyalkanoic acid (the lack of a color reaction on treatment of lipid A with ninhydrin indicates that free amines are absent). The other two 3-hydroxyalkanoic acids have ester bonds (the spectrum of lipid A has two resonances at 42.2 and 42.8 ppm, the region of C2 resonances of 3-hydroxyalkanoic acids [14, 15]) and are located on C3 and C3′ of the glucosaminobiose, the hydroxyls of which, as mentioned above, have acyl substituents.

The distribution of FA in the glucosaminobiose was determined more accurately by mass spectrometry. The negative-ion MALDI/TOF mass spectrum of the monophosphorylated derivative (Table 1) contained series of peaks (*m*/*z*) corresponding to penta- (1393.3), tetra- (1195.6 and 1209.5), and triacyl- (1013.4 and 1027.3) forms of lipid A. Peaks for ions within each series differed from each other by 14 amu, which confirmed the high heterogeneity of the studied lipid A with respect to 3-hydroxyalkanoic acids.

According to the mass spectra, partially deacylated derivatives are formed through successive loss of 3-OH-12:0, 12:0, and another 3-hydroxyalkanoic acid, which is consistent with the ester nature of the bonds of these acids to the disaccharide. [It is interesting that the diphosphorylated lipid A from *P. nigrifaciens* fragments differently (Table 1). Its tetra- and triacyl derivatives form through successive loss of 12:0 and 3-OH-12:0 acids.]

The negative-ion LSIMS mass spectrum of lipid A (Table 1) contained a peak at *m*/*z* 816, corresponding to a disaccharide derivative containing two 3-OH-12:0 acids with amide bonds because cleavage of an amide bond is improbable under LSIMS conditions.

Analysis of the fragment ion with *m*/*z* 654 provided important information about the structure of lipid A. It corresponded to a monosaccharide derivative substituted by two 3-OH-12:0 acids. The method of isolating the studied sample [treatment of starting diphosphorylated lipid A with HCl (0.1 M) to remove glycoside-bound phosphate] suggests that this ion should be the nonreducing end of the disaccharide, the phosphate group of which is more stable under acidic conditions. Thus, the nonreducing end of lipid A from *P. nigrifaciens* is GlcN phosphorylated at C4 (according to ^{13}C NMR spectroscopy C2 and C3 of this GlcN are substituted whereas C6 is unsubstituted) and has two FA.

The three other (of five) FA are located on the reducing end of the disaccharide. The lack of peaks containing 12:0 acid in the LSIMS mass spectrum indicates that this acid is also located on the reducing end of the disaccharide (which is not recorded under negative-ion mass spectrometry conditions) and acylates an amide-bonded 3-OH-12:0 acid (as shown above, the studied lipid A does not have acyloxyalkanoic acids with ester bonds).

Based on the results, it was concluded that lipid A from *P. nigrifaciens* is a β-1,6′-bonded disaccharide of D-glucosamine substituted by two phosphoric acids (at C1 and C4′), two ester-bonded *R*-3-hydroxyalkanoic acids (at C3 and C3′), and one *R*-3-OH-12:0 and one *R*-3-dodecanoyloxydodecanoic acid (both amide-bonded at C2′ and C2, respectively) and has the structure shown in Fig. 1a.

The structure of lipid A from a typical bacterium of the genus *Pseudoalteromonas, P. haloplanktis* ATCC 14399^T, has been reported [6]. It is similar in structure to lipid A from *P. nigrifaciens* and is the 1,4′-diphosphate of a β-1,6′-bonded disaccharide of D-GlcN substituted by five FA, three of which were ester-bonded and two of which (*R*-3-hydroxydodecanoic and *R*-3-dodecanoylhydroxydodecanoic) were amide-bonded. The positions of the amide-bonded FA (C2 or C2′ of the glucosaminobiose) were not established.

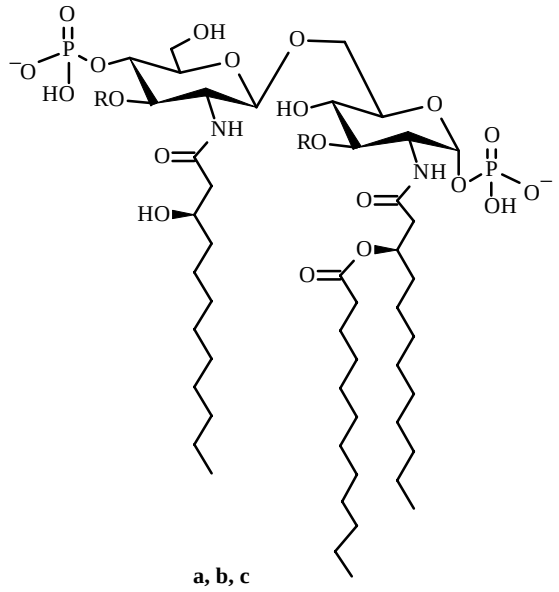

Fig. 1. Structure of lipids A from *P. nigrifaciens* IAM 13010^T (a), *P. haloplanktis* ATCC 14399^T (b), and *P. tetraodonis* IAM 14160^T (c), where R = 3-OH-10:0, 3-OH-11:0, 3-OH-12:0, 3-OH-13:0, 3-OH-14:0, 3-OH-*iso*-12:0 (a); R = 3-OH-11:0, 3-OH-12:0, 3-OH-13:0, 3-OH-*iso*-11:0, 3-OH-*iso*-12:0 (b); and R = 3-OH-10:0, 3-OH-11:0, 3-OH-12:0, 3-OH-13:0, 3-OH-*iso*-11:0, 3-OH-*iso*-12:0 (c).

In order to refine the structure of lipid A from *P. haloplanktis* ATCC 14399^T, it was treated with HCl (0.1 M). As mentioned above, such treatment led to partial dephosphorylation of lipid A through cleavage of the bond between the phosphate on C1 and the GlcN on which it was located. The negative-ion LSIMS mass spectrum of the resulting compound (Table 1) contained peaks (*m*/*z* 626, 640, 654, 668) differing from each other by 14 amu and corresponding to fragments consisting of one GlcN, one phosphoric acid, and two 3-hydroxyalkanoic acids.

One FA in all four derivatives was 3-OH-12:0. The other was one of the following FA: 3-OH-10:0 (*m*/*z* 626), 3-OH-11:0 (*m*/*z* 640), 3-OH-12:0 (*m*/*z* 654), 3-OH-13:0 (*m*/*z* 668). In addition to the diacyl derivatives, the LSIMS mass spectrum of the partially dephosphorylated lipid A contained a strong peak at *m*/*z* 456 that corresponded to a fragment ion consisting of one GlcN unit, one phosphoric acid, and one 3-OH-12:0 acid. A fragment ion with this *m*/*z* can be produced by loss of ester-bonded FA selected from 3-OH-10:0, 3-OH-11:0, and 3-OH-13:0. The 3-OH-12:0 acid is both ester-bonded (*m*/*z* 654) and amide-bonded (*m*/*z* 456).

As mentioned above, treatment of lipid A under acidic conditions removes a phosphate from the reducing end of the disaccharide. Therefore, peaks of phosphorylated fragment ions should belong to the nonreducing end of lipid A from *P. haloplanktis*, which, therefore, contains two of the five FA in it. The other three acyl substituents are located on the reducing end of the glucosaminobiose. Considering this information, we proposed a refined structure for lipid A from *P. haloplanktis* (Fig. 1b) that, by comparison with the data presented above, is identical to the structure of lipid A from *P. nigrifaciens* (Fig. 1a) and contains an amide-bonded 3-dodecanoyloxydodecanoic acid on the reducing end of the disaccharide.

The genus *Pseudoalteromonas* includes a rather large number of microorganisms [17]. In order to understand how often the lipid A structure described above is encountered in bacteria of this genus, we determined the FA distribution in lipid A from *P. tetraodonis* IAM 14160^T. Judging from the total chemical analysis of lipid A from *P. tetraodonis*, it consists of two GlcN, two phosphoric acids, and five FA. Like the aforementioned lipid A molecules, lipid A from this bacterium species is highly heterogeneous with respect to the 3-hydroxyalkanoic acids and contains 3-OH-10:0 (7.8%), 3-OH-11:0 (5.3%), 3-OH-12:0 (31.0%), 3-OH-13:0 (2.1%), 3-OH-*iso*-11:0 (2.7%), and 3-OH-*iso*-12:0 (5.7%) acids.

The negative-ion LSIMS mass spectrum of the monophosphorylated derivative (Table 1) produced by treatment of starting lipid A with HCl (0.1 M) was similar to that of lipid A from *P. nigrifaciens* and *P. haloplanktis* and contained peaks for ions corresponding to di- (*m*/*z* 815.3) and mono- (654.2 and 456.3) acyl derivatives. The presence of at least one 3-OH-12:0

acid in all fragment ions indicates that namely it acylates the amine of both GlcN units whereas the remaining FA, including the 12:0 acid, are ester-bonded.

The results in general lead to the conclusion that lipid A from *P. tetraodonis* has a structure (Fig. 1c) similar to those of lipid A from the other two bacterium species of the genus *Pseudoalteromonas* (Figs. 1a and 1b). Furthermore, it can be assumed that bacteria of the genus *Pseudoalteromonas* synthesize lipid A of similar structure based on the disaccharide of GlcN substituted by two phosphoric acids and five FA, two of which (3-hydroxydodecanoic and 3-dodecanoylhydroxydodecanoic) are amide-bonded (C2′ and C2, respectively).

EXPERIMENTAL

TLC of lipid A molecules was carried out on Sorbfil alumina plates using $CHCl_3$:CH_3OH:H_2O:NH_4OH (conc.) (5:3.125:0.5:0.25). ^{13}C NMR spectra were recorded on a Bruker Avance DRX-500 spectrometer relative to TMS (internal standard) using CD_3OD:$CDCl_3$:D_2O (3:2:1). Samples were prepared as before [18].

^{13}C NMR (FA): 14.29 (s, C-ω1), 23.11 (s, C-ω2), 25.63 (s, C-6), 30.13 (m, C-7—C-ω3), 32.40 (C-5), 35.06 (s, C-4, 3-acyloxy acid), 37.56 (m, C-4, 3-hydroxy acids), 41.49 (s, C-2, amide-bonded 3-acyloxy acid), 42.23, 42.79 (s, C-2, ester-bonded 3-hydroxy acids), 43.84 (s, C-2, amide-bonded 3-hydroxy acid), 68.71, 68.83, 68.95 (s, C-3, 3-hydroxy acids), 71.26 (s, C-3, 3-acyloxy acid), 172.09, 173.03, 173.59, 173.72, 174.93 (s, C=O); ^{13}C NMR (GlcN): 52.66 (s, C-2), 54.41 (s, C-2′), 60.94 (s, C-6′), 67.95 (s, C-4), 69.79 (s, C-6), 71.19 (s, C-5), 72.44 (s, C-3′), 73.17 (s, C-4′), 73.33 (s, C-3), 75.11 (s, C-5′), 91.79 (s, C-1), 102.09 (s, C-1′).

The total P content was determined by the molybdate method. Amino compounds prepared by hydrolysis of lipid A by HCl (6 M, 100°C, 24 h) were analyzed on an Alpha Plus LKB 4251 amino-acid analyzer; FA methyl esters prepared by alkaline hydrolysis of lipids A (6 M NaOH, 100°C, 4 h), using a GC [Shimadzu GC-9 chromatograph, capillary columns (30 m × 0.25 mm), Supelcowax 10 and SPB-5, 150→230°C, 5°C/min] and GC—MS [Hewlett—Packard Model 6890 chromatograph, HP 5 MS capillary column with 5% phenylmethylsiloxane (30 m × 250 μL × 0.25 μL), Hewlett—Packard Model 5973 mass spectrometer]. Analyzed samples were introduced by dividing flows in a 1:15 ratio. Ionizing electrons had 70 eV energy. The analysis was performed at 150→210°C (5°C/min) with He carrier gas. Spectra were recorded in the range 50-550 amu at 2.94 scans/s. An LKB-9000S GC—MS equipped with SE-30 (5%)) columns was used for GC—MS of polyol acetates.

LSIMS mass spectra were obtained in an AMD 604S high-resolution mass spectrometer with Cs^+ ions of 8 kV energy. Samples were dissolved in $CHCl_3$:CH_3OH (4:1) at 10 mg/mL. Sample (0.5 μL) was mixed with glycerine (0.5 μL). The resulting solution (0.5 μL) was placed on a metal sample holder and analyzed immediately after drying in a stream of air.

MALDI/TOF mass spectra were obtained in a Bruker Biflex III mass spectrometer with a nitrogen laser (337 nm). Samples (10 mg/mL) were dissolved in $CHCl_3$:CH_3OH (4:1). An aliquot (1 μL) was analyzed using α-cyano-4-hydroxycinnamic and/or 2,5-dihydroxybenzoic acid as the matrix. The absolute configuration of GlcN was determined by GC—MS of its acetylated 2-octylglycoside by the method of Leontein et al. [19]. The angle of rotation {$[\alpha]_D$ −8.2° (2.7, $CHCl_3$)} of the 3-hydroxyacids that were obtained using column chromatography of the free FAs over silica gel [hexane, hexane:diethylether (2:1 and 1:1), hexane:diethylether:AcOH (1:1:0.1)] was measured on a Perkin—Elmer 141 spectrophotopolarimeter.

Bacteria were grown in medium containing seawater at room temperature. LPS were obtained by the Westphal method of treatment with hot aqueous phenol [9] and hydrolyzed by acetic acid (1%) at 100°C for 3 h. Precipitates of lipids A produced during hydrolysis were separated by centrifugation, dissolved in $CHCl_3$, washed three times with distilled water, dried over anhydrous Na_2SO_4, and precipitated by acetone (five times the volume). Monophosphorylated derivatives were prepared by heating suspensions of lipids A in HCl (0.1 M) for 2 h. Ion-exchange chromatography was performed over a column of Servacel DEAE-52 cellulose [20] (3 mL, elution flow rate 1 mL/min, 3-mL fractions) using $CHCl_3$:CH_3OH:H_2O and $CHCl_3$:CH_3OH:NH_4Ac (60, 120, 240, 360, and 480 mM) (2:3:1).

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