ISSN 0026-2617, Microbiology, 2015, Vol. 84, No. 1, pp. 34–40. © Pleiades Publishing, Ltd., 2015.
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EXPERIMENTAL ARTICLES

Lipid and Fatty Acid Profiles of *Pseudomonas aurantiaca* DNA-Bound Lipids Determined by Mass Spectrometry

R. I. Zhdanov[a, b, 1], D. Kern[c], W. Lorenz[c], and M. Y. Ibragimova[a]

[a] *Kazan (Volga region) Federal University, Kazan, Russia*

[b] *Sholokhov Moscow State University for Humanities, Russian Institute for Advanced Study in Humanities and Technology, Moscow, 109240 Russia*

[c] *Martin Luther University of Halle-Wittenberg, Germany*

Received May 27, 2014

Abstract—An approach used on investigation of the lipid composition of loosely (fraction 1) and tightly (fraction 2) DNA-bound lipids of *Pseudomonas aurantiaca* cells by electrospray ionization using mass spectrometry (ESI-LC-MS) was used for determination of the lipidome of a prokaryotic cell. Free fatty acids $C_{16:0}$, $C_{18:1}$ (fraction 1), $C_{14:0}$, $C_{16:0}$, and $C_{18:2}$ (fraction 2) were detected. Both fractions of DNA-bound lipids were characterized by the presence of phosphatidylglycerol, phosphatidylserine, and lyso-phosphatidylinositol. The alcohol-soluble fraction 1 could also contain phosphatidylcholine and phosphatidylinositol, while fraction 2 probably contained triacylglycerides. Compared to gas chromatography, ESI-LC-MS provides new possibilities for investigation of the nucleoid lipidome, allowing for more detailed investigation of DNA-bound lipids in bacterial cells.

Keywords: *Pseudomonas aurantiaca*, DNA-bound lipids, fatty acids, mass spectrometry, electrospray ionization

DOI: 10.1134/S0026261714060228

Lipidomics occupies an important place among postgenome sciences [1–8]; however, in the presently applied protocols of lipidome investigation, a special group of lipids, namely, the lipids bound to DNA with a different degree of firmness, is missing [9, 10]. Such DNA-bound lipids are of great interest in connection with the data on the signaling role of lipids in prokaryotic and eukaryotic cells [11] and on the regulation of gene activity by lipids [12, 13]. Sequence-specificity of lipid binding to DNA was revealed in the experiments on titration with lipids of the DNA polynucleotides with different nucleotide sequences [14]. Fatty acids were found to interact with AT-rich sequences on the basis of recognition (one lipid molecule per one DNA coil = 10 bp); interaction with CG-rich DNA-sequences was based on saturation (up to the 1 lipid molecule per 2 bp) [14]. The complex phospholipid cardiolipin is able to interact with DNA polynucleotides irrespective of their nucleotide sequence [7, 15]. The cardiolipin content was shown to increase in active genomes of proliferating cells where the level of gene expression is high [16].

In our previous works, we investigated the fatty acid profile of two DNA-bound lipid fractions of the gram-negative bacterium *Pseudomonas aurantiaca* by gas chromatography after genomic DNA was isolated from the cells in the presence of detergents [17–19]. It was shown for the first time that the DNA-bound lipids could be detected in the genomic DNA preparations even under such tough isolation conditions and their profile was studied [18, 19]. We identified the fatty acids bound primarily to DNA, RNA, or proteins by treating the prokaryotic supramolecular complex of genomic DNA (high-molecular mass DNA, hmDNA), which contains four types of biomacromolecules (DNA, RNA, proteins, and lipids), with the enzymes DNase, RNase, and protease with subsequent gas chromatographic analysis of the fatty acid composition [20].

However, the data on the lipid profile of DNA-bound lipids and their functions in prokaryotic cells remain scarce and contradictory [9, 10, 21]. Using thin-layer chromatography, it was found that the DNA-bound lipids of *E coli* and *Shigella sonnei* (thermolabile strain) contained both neutral lipids (0.78 and 0.92 wt % of DCM, respectively) and phospholipids (0.11 and 0.14 wt % of DCM, respectively) [9, 21, 22]. The phospholipids of these bacteria were most probably represented by cardiolipin (CL) and phosphatidylethanolamine (PE) [21]. It was shown that the properties of *Pseudomonas aurantiaca* hmDNA (the molecular mass and elastoviscosity) and the fatty acid composition of DNA-bound lipids of these bacteria depended on the growth phase of their cultures attaining higher values during the stationary phase [23, 24].

[1] Corresponding author; e-mail: zrenad@gmail.com

Thus, lipids are likely to be involved in the supercoiling of the loops in prokaryotic genomic DNA. Their capacity for reception of alkylhydroxybenzenes, natural bacterial autoregulators, which take part in the control of the dissociative transfers determined by intragenomic restructurings, also depended on the physiological age (growth phase) of bacteria [23]. Based on the accumulated data on DNA-bound lipids, existence of the genomic DNA lipid code was hypothesized, suggesting that lipids are bound to DNA in a sequence-specific manner [25].

The goal of the present work was to determine the fatty acid and lipid composition of tightly and loosely DNA-bound lipids in *P. aurantiaca* cells (stationary phase) by mass spectrometry using electrospray ionization (ESI-LC-MS).

MATERIALS AND METHODS

All reagents were analytically pure (Merck, Sigma, Bohringer, Manheim GmbH); trimethylsulfonium hydroxide, TMSH (Macherey & Nagel, Germany) was used as a methylating agent. MilliQ double-distilled water was used for the preparation of all solutions. Organic solvents were distilled immediately before use.

Media and cultivation conditions. The study subject was the gram-negative bacterium *Pseudomonas aurantiaca* B-1558 (VKM) (All-Russian Collection of Microorganisms, Pushchino, Moscow oblast). The lipids were analyzed in the cells of the stationary growth phase culture (48 h of cultivation) [4, 16–21, 23, 24] grown in synthetic nutrient medium of the following composition (g/L): glucose, 2; $KH_2PO_4 \cdot 3H_2O$, 0.1; $(NH_4)_2SO_4$, 0.5; $K_2HPO_4 \cdot H_2O$, 1; $CaCl_2$, 0.2; $MgSO_4 \cdot 7H_2O$, 0.1; Difco yeast extract, 0.05; trace elements (mg/L): $FeSO_4 \cdot 7H_2O$, 20; $MnCl_2 \cdot 4H_2O$, 20; $ZnSO_4 \cdot 7H_2O$, 0.4; $B(OH)_3$, 0.5; $CuSO_4 \cdot 5H_2O$, 0.05; $Na_2MoO_4 \cdot 2H_2O$, 0.2; pH after sterilization 7.25. The bacteria were grown at 28°C in 2-L flasks (500 mL of the medium) on a shaker (140 rpm). The culture purity was tested by microscopy and by plating on petri dishes with agarized LB medium. Stationary-phase cells grown in the medium of the above-given composition were used as inoculum. The inoculum was introduced to obtain the initial optic density of 0.1 OD ($\lambda = 540$ nm, $l_{cuvette} = 10$ mm). The dry cell mass (DCM) was determined by drying to constant weight at 105°C.

Isolation of hmDNA. The high-molecular-mass DNA (hmDNA) preparations were isolated from bacterial cells by the soft phenol method [16–21]. An equal volume of 66% aqueous solution of freshly distilled phenol, pH 8.5, was added to the cell suspension in 0.14 M NaCl solution (pH 7.0) and extracted twice under careful stirring on an orbital shaker (60 rpm) for 1 h with the subsequent centrifugation for 20 min at 5000 *g* [21]. The upper DNA-containing phase was collected, supplemented with an equal volume of 66% phenol (pH 8.5), mixed for 20 min, and centrifuged for 1 h at 5000 *g*. The DNA solution was dialyzed against 0.14 M NaCl (pH 7.0) at 4°C for 72 h. The DNA and RNA contents were determined spectrophotometrically using Spirin's method; the hmDNA molecular mass and elastoviscosity were measured as described earlier [19, 21]; the hmDNA preparation obtained had the same characteristics as the preparation isolated in [24]: the DNA content 10.7 mg/g DCM, the molecular mass 19×10^4 kDa, elastoviscosity 770 ± 24 dL/g.

Isolation of DNA-bound lipid fractions. Two fractions of DNA-bound lipids from hmDNA were isolated as described earlier [18, 19]. The loosely bound (alcohol-soluble) lipid fraction (fraction 1) was extracted from hmDNA with 35% aqueous ethanol solution (24 h at 37°C). After incubation, the DNA was quickly precipitated by a double volume of cold 96% ethanol and washed three times with 70% ethanol; the ethanol extracts were then pooled. The solvents were evaporated at 40°C, and the residue was dissolved in the chloroform/methanol mixture (1 : 2 vol/vol) to obtain the fraction of loosely bound lipids (fraction 1). The ethanol-precipitated DNA was incubated with lipid-free DNase I (1 : 1 wt/wt) (Sigma) in 5 mL of 0.01 M magnesium chloride solution for 2 h at 37°C. The firmly bound lipids (fraction 2) were extracted from this mixture according to Bligh–Dyer's technique as described earlier [19].

Liquid chromatography and mass spectroscopy of DNA-bound lipid fractions. Mass spectra of the peaks of DNA-bound lipid fractions (fractions 1 and 2) were recorded using LC-ESI-MS (an API2000 mass spectrometer, Applied Biosystems, United States) (turbo spray) in the negative (negative mode, –Q1) or positive ionization mode (positive mode, +Q1); 350°C [26, 27]. The solvents from the lipid fractions were evaporated, and the residue was vacuum-dried. The fraction 1 or 2 lipid precipitates were dissolved in 500 μL of the chloroform/methanol mixture (2 : 1), and 5 μL of the solution was applied onto the RPC18 column of the high-performance chromatography device (HPLC, LC) module with the inverted phase acetonitrile : water = 9 : 1, pH 7, flow rate 200 μL/min. When the operation of the mass spectrometer was optimized, narasin ($C_{43}H_{72}O_{11}$, molecular mass of 765.05 g/mol) was used as the standard; in the positive mode, the device recorded a peak with m/z 765.

Statistical analysis. The results of measurement of elastoviscosity were processed by the methods for statistical analysis and given as the means ± standard deviation.

RESULTS AND DISCUSSION

The fatty acid profile of the DNA-bound lipid fractions was previously investigated by gas chromatography after hydrolysis of the DNA-bound lipid fractions

Table 1. Lipids detected in fraction 1 of *Pseudomonas aurantiaca* alcohol-soluble DNA-bound lipids*

Peak nos.	Peak *m/z*	Retention time, min	Electrical mode. Conditions	Identification	Theoretical ion mass, Da	Notes
1	255.3	8.380	Neg., pH 7	Anion $C_{16:0}$	255.4228	One peak
2a, 2b	255.4/281.5	8.606	Neg., pH 7	Anions $C_{16:0}/C_{18:1}$	255.4228 281.4608	Two peaks
2a, 2b	255.3/281.5	8.832	Neg., pH 7	Anions $C_{16:0}/C_{18:1}$	255.4228 281.4608	Two peaks
3a, 3b	621.4/623.2	29–30	Pos.	Lyso-PI	598.6687 (–2H) 600.6867	+23 (Na) Ion doublet
4	745.6	30	Neg.	PG ($C_{16:0}/C_{18:2}$)	747.0018	$-2H^+$
5	798.6/803.8	36.47	Pos.	PG ($C_{18:1}/C_{18:1}$)/PG ($C_{18:0}/C_{18:0}$)	775.0552/ 803.0910	+23 (Na)
6	747.8	37.7–39	Pos.	PG ($C_{16:1}$, $C_{18:1}$)	747.0018	16:0, 18:2
7	736.6	44	Pos.	PS ($C_{16:0}$)	733.9631	$+2H^+$
8	760.8	59.49–60.16	Pos.	PC ($C_{16:0}$, $C_{18:1}$)	760.0859	–
9	891.1	>60	Pos.	PI ($C_{18:0}/C_{18:0}$)	867.1513	+23 (Na)

* The following abbreviations are used in the table: Neg., negative mode; Pos., positive mode; PG, phosphatidylglycerol; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; Lyso-PI, lyso-phosphatidylinositol.

to fatty acid methyl ethers [17–19], and the phospholipid composition of both fractions was studied using the technique of thin-layer chromatography of residues [16, 21, 22]. To analyze the total lipids of fractions 1 and 2 of DNA-bound lipids, we applied high-performance liquid chromatography HPLC (LC) combined with mass spectrometry using electrospray ionization (ESI-LC-MS technique) (see, e.g., [26, 27]). With the use of HPLC with lipid elution by the acetonitrile : water mixture (9 : 1), they were separated on the RPC18 column according to their polarity, and mass spectrometry (ESI-MS) makes it possible to obtain mass spectra of the fraction components in different ionization modes (negative or positive mode).

Fatty acids. Tables 1 and 2 contain the *m/z* values and the molecular ion values of the main fatty acids, as well as those of (phospho)lipids of fractions 1 and 2 of *P. aurantiaca* DNA-bound lipids. HPCL separation of the total fraction 1 of DNA-bound lipids (ESI-LC-MS, negative mode, pH 7.0, acetonitrile/water, 9 : 1) revealed the peaks corresponding to the free fatty acid carboxylate-anions in the mass spectra of the components with a retention time of 8.38–8.83 min. These acids (in the C_{10}–C_{20} range) were identified as palmitic ($C_{16:0}$) (*m/z* 255.4; theoretical anion mass for $C_{16}H_{31}O_2$ = 255.4228) and oleic ($C_{18:1}$) (*m/z* 281.4; theoretical anion mass for $C_{18}H_{33}O_2$ = 281.4601) acids, which are basic for both total and DNA-bound *P. aurantiaca* lipids. Importantly, only palmitic acid was initially eluted, and after that both acids, $C_{16:0}$ and $C_{18:1}$, were eluted with the retention time 8.606–8.832 (Table 1, peaks 1 and 2a, 2b) in different ratios. In contrast to fraction 1, mass spectra of fraction 2 (retention times 5.419–6.423 min) of DNA-bound lipids revealed the peaks of the fatty acids $C_{14:0}$ (*m/z* 227.1; theoretical anion mass for $C_{14}H_{27}O_2$ = 227.3578), $C_{16:1}$ (*m/z* 253.3; theoretical anion mass for $C_{16}H_{29}O_2$ = 253.4068), and $C_{18:2}$ (*m/z* 279.3; theoretical anion mass for $C_{18}H_{31}O_2$ = 279.4428) (negative ionization) (Table 2, peaks 1 and 3). In the previous studies of the fatty acid profile of *P. aurantiaca* DNA-bound lipids

Table 2. Lipids detected in fraction 2 of *Pseudomonas aurantiaca* DNA-bound lipids*

Peak nos.	*m/z*	Retention time, min	Electrical mode. Conditions	Assignment	Theoretical ion mass, Da	Notes
1	227.1	5.4–6.4	Neg.	Anion $C_{14:0}$	227.3678	–
2	253.3	''	Neg.	Anion $C_{16:1}$	253.4068	–
3	279.3	''	Neg.	Anion $C_{18:2}$	279.4428	–
4 (14a, 14b, 14c)	621.4/623.4/625.6	22–24	Neg.	Lyso-PI ($C_{18:0}$)	598.6687 (–2H) 600.6867 602.7047(+2H)	+23 (Na) Peak triplet
5	745.6	29–30	Neg.	PG ($C_{16:0,}$ $C_{18:2}$)	747.0018	–2H
6	762.2	39.9–41.5	Neg.	PS ($C_{16:0}$, $C_{18:0}$)	762.0165	–
7	795.2	''	Neg.	PS or PG	775.0552	$+Na^+$
8	834.4	''	Neg.	TAG ($C_{16:0}$, $C_{16:0}$, $C_{18:0}$)	835.3831	–
9	805.5	''	Neg.	TAG ($C_{16:0}$)	807.3297	$+2H^+$
10	1468.4	>50	Pos.	CL (4–18:1) or PC dimer	1466.0585 or 2 × 734.0485	$+2H^+$

* The following abbreviations are used in the table: Neg., negative mode; Pos., positive mode; CL, cardiolipin; TAG, triacylglyceride; PG, phosphatidylglycerin; PS, phosphatidylserine.

[18, 19, 24], we also found linoleic acid $C_{18:2}$ in the cells of the exponential growth phase, although in trace amounts. This is unusual for two reasons: first, polyunsaturated acids are not typical of prokaryotes and were only revealed in their lipids under extreme conditions [19, 28]. Secondly, in this study, linoleic acid $C_{18:2}$ and the other fatty acids listed above were not found in the phospholipid composition, i.e., in the form of the corresponding methyl ethers, as is the case when the fatty acid composition is analyzed by gas chromatography [e.g., see 19]. With ESI-LC-MS analysis, fatty acids, including $C_{18:2}$, were revealed in the composition of the analyzed fractions without saponification to ethers, i.e., as free acids. It should be noted that the *trans, trans* conformation of linoleic acid $C_{18:2}$ facilitates its binding to the DNA minor groove in the form of a twisted boomerang [29]. This circumstance may explain the presence of free unsaturated fatty acids in the DNA-bound lipid fractions.

As could be expected, no peaks corresponding to fatty acids were recorded in the positive mode of recording the mass spectra of both fractions. Interestingly, stearic acid $C_{18:0}$, which is basic for the pool of the total cell lipids [24], was not found among the free fatty acids of both fractions of DNA-bound lipids. Neither did we reveal the peaks corresponding to the carboxylate ions of 3-hydroxy fatty acids, 3-OH-$C_{10:0}$ and 3-OH-$C_{12:0}$, in the ESI-MS mass spectra of DNA-bound lipids. This testifies to the fact that these lipid fractions contained no 3-hydroxy fatty acids, which are the markers for the total cell lipids of pseudomonads. Consequently, DNA-bound lipids are a separate lipid fraction differing in composition from the total lipids of *P. aurantiaca* cells [24]. Short-chain fatty acids (from C_4 to C_{10}) were not revealed either, which is not surprising, since such acids can only be detected with the use of special techniques [19].

Thus, the fatty acid profiles of *P. aurantiaca* DNA-bound lipid fractions determined by gas chromatography [16–20] (the basic fatty acids of complex lipids $C_{14:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$ in fractions 1 and 2) and by the LC-ESI-MS technique (the basic free fatty acids not entering into the composition of complex lipids: $C_{16:0}$ and $C_{18:1}$ in fraction 1 and the $C_{14:0}$, $C_{16:1}$, and $C_{18:2}$ in fraction 2) were different and supplemented each other, which may help to gain a keener insight into the role of DNA-bound fatty acids and lipids in chromatin functioning (Table 3).

Phospholipids. After chromatographic separation of fractions 1 and 2 of *P. aurantiaca* DNA-bound lipids, mass spectra of the main components with a certain retention time were obtained. Tables 1 and 2 contain the parameter values for the identified substances in the main peaks of the mass spectra of DNA-bound lipid fractions 1 and 2 obtained in the negative and positive ionization modes. In the mass spectra of fraction 1, the peaks with *m/z* 621.4 and 623.2 and retention time 29–30 min correlated with lyso-phosphatidylinositol (lyso-PI) (Table 1, peaks 3a, 3b). Note that the sodium ion mass was added to the theoretically calculated ion masses. The fraction 1 spectral peak (the negative ionization mode) with *m/z* 745.6 (Table 1, peak 4) may belong to the phosphatidylglycerol (PG $C_{16:1}/C_{18:1}$) anion. The following ion peaks were obtained in the positive mode: peak 5 with *m/z* 798.6 (Table 1) correlated with PG ($C_{18:1}/C_{18:1}$) with a sodium ion (theoretical mass 775.0552 Da plus a Na^+ ion mass of 23 Da); the peak with *m/z* 747.8 (Table 1, peak 6) also correlated with PG ($C_{16:1}/C_{18:1}$); the peak with *m/z* 736.6 correlated with phosphatidylserine (PS $C_{16:0}/C_{16:0}$) (Table 1, peak 7); the peak with *m/z* 760.8 correlated with phosphatidylcholine (PC $C_{16:0}/C_{18:1}$) (Table 1, peak 8). Mass spectra contained a peak with *m/z* 891.1 corresponding to phosphatidylinositol PI ($C_{18:0}/C_{18:0}$) (theoretical mass 867.1513 Da with a Na^+ ion 23 Da) (Table 1, peak 9).

Table 2 shows the data on the mass spectra of the fraction 2 components. Peaks 4a, 4b, and 4c with *m/z* 621.3/623.3/625.6 (retention time 22.3–23.8 min) (Table 2) may be identified as the lyso-PI derivative with a sodium ion (623.6867 Da). Peak 6 with *m/z* 762.2 correlated with PS ($C_{16:0}/C_{18:0}$); peak 8 with *m/z* 834.4 corresponded to triacylglyceride TAG ($C_{16:0}/C_{16:0}/C_{18:0}$) with a mass of 835.3831. Peak 9 with *m/z* 805.5 can be assigned to TAG ($C_{16:0}$). The fraction 2 mass spectrum also contained a low peak with retention transition time (50 min and more) with *m/z* 1468 (Table 2, peak 10), which may be due to the presence of cardiolipin CL or a phosphatidylcholine dimer.

The earlier published data on the lipid composition and content in the fractions of DNA-bound lipids of bacteria and eukaryotic cells were obtained by thin-layer chromatography; application of this technique requires certain experimental keenness [16, 21, 22]. At present, electrospray and other modern mass spectrometry techniques are used to study lipids and lipid metabolism [30]. In our previous works, we showed using thin-layer chromatography that the lipid fractions of eukaryotic firmly DNA-bound lipids contained (of the total amount of phospholipids) CL, 40–50%; PE, 25–30%; PC, 15–20%; PS, 5–7%; and PI, 3% [21, 31]. The fractions of firmly DNA-bound lipids (fraction 2) of prokaryotes (*E. coli B*) and phage T2 included CL, 60–70% and PE, 30–40% [21, 22]. It follows from these data that DNA-bound lipids have a specific composition differing from the composition of cell lipids in prokaryotes and from the lipid composition of the eukaryotic nuclear membrane, chromatin, and the nuclear matrix. The study of the DNA-bound lipids of the euchromatin (eDNA), heterochromatin (hDNA), and the nuclear matrix (nmDNA) of the rat thymus and liver demonstrated that eDNA contained loosely and firmly bound lipids differing in composition (fractions 1 and 2, respectively), whereas hDNA and nmDNA contained only fraction 2 of these lipids. The predominance of phospholipids over neutral lipids was characteristic of all the lipid fractions [32]. The eDNA and nmDNA fractions were active in replication and transcription and enriched with acidic PL (cardiolipin, phosphatidylserine, and phosphatidylinositol), as well as free fatty acids and diglycerides, as distinct from hDNA lipids [32]. An important role of cardiolipin in replication and of free fatty acids in DNA transcription was suggested. It was established in [33, 34] that acidic phospholipids (CL, PS) normally contributed to chromatin decondensation, while neutral ones, on the contrary, condensed it. This condensation was accompanied not only by dispersion of the chromatin fibrils but also by the transfer from the solenoid to nucleosome structure and by loss of histone H1 [34].

The study of prokaryotic cell chromatin lipidome based on the investigation of the lipid profile of DNA-bound lipids by electrospray ionization mass spectrometry (ESI-LC-MS) has been carried out in this work for the first time. Using this technique, we confirmed the data on the lipid profile of DNA-bound lipids obtained earlier and also revealed that both DNA-bound lipid fractions were characterized by the presence of phosphatidylglycerol, phosphatidylserine, and lyso-phosphatidylinositol. The alcohol-soluble fraction 1 of loosely DNA-bound lipids may also contain phosphatidylcholine and phosphatidylinositol; fraction 2 of firmly bound lipids may contain triacylglycerides and cardiolipin. The use of ESI-LC-MS and, subsequently, of LC-ESI-MS/MS extends the possibilities of studying the prokaryotic nucleoid lipidome in comparison to chromatography, which provided information concerning fatty acids in the lipid composition, and makes it possible to obtain information on both the free fatty acids and the (phospho)lipids of DNA-bound lipids and to look deeper into their func-

tion in the bacterial cell depending on its nature, physiological age, and the environmental conditions.

ACKNOWLEDGMENTS

We are grateful to Professors G.I. El'-Registan and A.L. Mulyukin (Winogradsky Institute of Microbiology, Russian Academy of Sciences, Moscow) for useful discussions, to Dr. A.S. Shmyrina and Dr. N.B. Strazhevskaya for help in the experiments, as well as to Dr. Yu. Schiller (Institute of Medical Physics and Biophysics, Medical Faculty, Leipzig University, Germany) for discussion of the indication of phospholipids. This work was supported by the Russian Foundation for Basic Research, project no. 12-03-97089rpovolzh'e, the Republic of Tatarstan Academy of Sciences, the Humboldt Foundation, project no. V-8121/RUS/1032332 (Bonn, Germany) (R. Zh.), the Upper Saxony Land grant, and the Martin Luther University, Halle-Wittenberg as an initiative project, as well as by the Russian Federation Ministry of Education and Science, project nos. KFU 12-26 ND02 and 13-64 for 2012–2014. The work was performed at the expense of the subsidy granted within the framework of the State support of the Kazan (Volga Region) Federal University in scientific activity and with the aim of increasing its competitiveness among the world's leading research and education centers.

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Translated by E. Babchenko