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Very-long-chain *iso* and *anteiso* branched fatty acids in *N*-acylphosphatidylethanolamines from a natural cyanobacterial mat of *Calothrix* sp.

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ABSTRACT

A combination of TLC, ESI–MS/MS and GC–MS was used to identify unusual molecular species of *N*-acylphosphatidylethanolamines containing very-long-chain *anteiso* branched fatty acids (VLCFAs) from *Calothrix* sp. collected in Antarctica and determine their component VLCFA up to 33-methyltetratriacontanoic acid as picolinyl ester derivatives using GC–MS.

1. Introduction

A large part of Antarctica is a frozen desert with little precipitations and very limited species diversity. As a result of the harsh climate, phototrophic life is limited to mostly mosses, liverworts, lichens, algae and cyanobacteria, which are able to tolerate various extreme environmental stresses. The conditions supporting growth generally occur for a few weeks in the summer.

In maritime Antarctica, ecologically delimited cyanobacterial mats containing only one or two species are common, especially in extreme microhabitats (Vincent, 2000; Komárek and Elster, 2008). James Ross Island is an irregularly shaped large island off the southeast side of Antarctic Peninsula, and 75 cyanobacterial morphotypes were recently determined in various habitats of its deglaciated part. Especially the heterocytous taxa, including the three identified *Calothrix* morphotypes, are currently unknown from any other ecosystem (Komárek et al., 2008). Although, *Calothrix* belongs to the most easily recognizable cyanobacterial genera, the phylogenetic analysis of the 16S rRNA gene has recently revealed large sequence diversity within this genus (Sihvonen et al., 2007).

The filamentous heterocytous genus *Calothrix* displays tapering filaments with clear or yellow to brown firm mucous sheaths. Heteropolar filaments are simple, single or in groups, but they are never unified into mucilaginous colonies. Ellipsoidal or spherical heterocysts are located at the base of the filaments. Ellipsoidal or cylindrical akinetes sometimes develop above the heterocysts from a vegetative part of the filament (Komárek et al., 2003).

N-acylphosphatidylethanolamine (*N*-acylPE) first identified as a minor constituent of wheat flour (Bomstein, 1965), is an unusual phospholipid that occurs in small amounts (about 1% of total phospholipids) in a wide range of organisms. Its molecule contains a third fatty acyl residue linked to the N atom of the ethanolamine head group by an amide bond. The biochemical properties of *N*-acylPE and its derivatives have been described by Chapman (2000).

N-acylPE has been identified among the lipids of the soil filamentous fungus *Absidia corymbifera* (Batrakov et al., 2001). It is also biosynthesized in cotyledons of cotton seedlings and was identified in dry cotton seeds (Sandoval et al., 1995). The major molecular species of *N*-acylPE were identified in both dry and soaked seeds by FAB–MS as 16:0–16:0/18:2-PE, 18:2–16:0/18:2-PE, and 16:0–18:2/18:2-PE. The biosynthesis of *N*-acylPE appears to occur by direct acylation of PE with free fatty acid (Chapman and Moore, 1993a,b; Chapman et al., 1995). Some seagrass species (*Zostera marina*, *Z. asiatica*, *Phyllospadix iwatensis*) of the subfamily

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Zosteroideae from the Sea of Japan (Khotimchenko, 1993) contain, besides major glycolipids, and phospholipids *N*-acylPE.

Methyl branched FA, particularly *iso* and/or *anteiso* (ω-2-methyl branched fatty acids, *ai*-FAs) are often found also in marine organisms and in natural waxes originating from plants or animals (Hamilton, 1995). *Anteiso*-fatty acids are usually odd-chain whereas *iso*-fatty acids can be both even and odd-chain as a result of their biosynthesis from different amino acids (Ile and Val or Leu, respectively). Although *ai*-FAs are found as minor lipid components in a wide range of living organisms and as major components in the lipids of diverse bacteria, little is known about the occurrence of very-long-chain *anteiso* acids (>C_{20}; *ai*-VLCFA) in the living organisms. The dominating bacterial *ai*-FAs in nature are *ai*-15:0 and *ai*-17:0 (Kaneda, 1991). *ai*-FAs play an important role in providing an appropriate degree of membrane fluidity at low temperatures, presumably through keeping up the fluid–liquid–crystalline state of membrane lipids (Lindstrom et al., 2006). If we do not consider animal waxes such as lanolin (Moldovan et al., 2002), only a few reports described the presence of *ai*-VLCFA from C_{20} to C_{30}, including odd and even ones, in cryptoendolithic microbial communities from Antarctica (Matsumoto et al., 1992, 2004) and from acidified freshwater lake Tazawa-ko sediments in Japan (Fukushima et al., 1996).

As a continuation of previous studies devoted to VLCFAs from different sources (Dembitsky et al., 1993a, b; Rezanka 1993; Rezanka et al., 1986; Rezanka and Podojil, 1984) we report here the discovery and ESI–MS/MS data of a series of very-long-chain *anteiso* alkanoic acids (*ai*-C20 to *ai*-C35) in N-acylPE of a mat-forming cyanobacterium (*Calothrix* sp.) collected from a lake in James Ross Island (Antarctica), and discuss their possible biological significance.

2. Results and discussion

Epilithic cyanobacterial mats formed by *Calothrix* sp. (Fig. 1) were collected on February 22, 2008 from the littoral zone of Green lake situated in the northern deglaciated part of James Ross Island, northwestern part of the Weddell Sea, Antarctica (63°54′12″S, 57°46′49″W). The sample was aseptically collected, transferred into laboratory in a thermos bottle, identified under light microscope and kept frozen until further analysis.

The mat was lyophilized (1.42 g) and then extracted according to Bligh and Dyer (1959). The extract yielded 173 mg crude lipids, which were fractionated by means of cartridges with aminopropyl silica-based polar bonded phase which were rinsed in a chloroform–methanol mixture. Phospholipids were further eluted by a mixture of chloroform–methanol–concentrated aqueous ammonia. Phospholipids (41% of total lipids, i.e. 70.9 mg) were further separated by two-dimensional thin-layer chromatography with chloroform–methanol-7 M ammonia in the first dimension and chloroform–methanol–acetic acid in the second dimension. UV-light detection after TLC showed a spot having R_f of 0.8–0.9 in both solvent systems. The spot gave positive responses to phosphate spray (Ryu and MacCoss, 1979), but a negative response to Dragendorff stain for choline, and the vicinal glycol detection spray (periodate-Schiff stain). This spot, denoted unknown lipid, was scraped from the plates and eluted, with a yield of 14% (9.93 mg) of total phospholipids. This unknown lipid gave a very faint spot migrating ahead of all known standards of phospholipids. This spot had a slightly higher mobility than *N*-palmitoyl-*O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)-ethanolamine (standard from Sigma), a feature which can be ascribed to the presence of acyl chains longer than palmitate.

Two-dimensional TLC was used to characterize also other complex glycerolipids (Fig. 2). Using specific agents and commercial standards we identified only phosphatidylglycerol (PG) and negligible amounts of bis-phosphatidic acid (bisPA), lyso-bis-phosphatidic acid (lysobisPA), phosphatidic acid (PA), and cardiolipin (CL) but no phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphatidylinositol (PI). The presence of PG and also CL is not surprising since both compounds have repeatedly been found in cyanobacteria (Reddy et al., 2003; Herrero et al., 2007). Based on these facts and on the careful inspection of the samples under light microscope, which revealed no additional organisms, the mat was concluded to be formed exclusively by *Calothrix* sp. We therefore assume that contamination by different microorganisms can be excluded.

^{1}H NMR data from Table 1 showed that the unknown lipid has signals in the regions typical for polar glycerolipids, i.e. long-chain hydrocarbons (acyls), and protons bound to double bond(s) in the interval ~5.3 ppm, and protons next to amino group (δ 3.4–5.2 ppm). The ^{13}C NMR spectrum featured signals belonging again to hydrocarbon chains (δ 14–35 ppm), carbons with double bond (δ

Fig. 1. Photograph of *Calothrix* sp., *bar* = 20 μm.

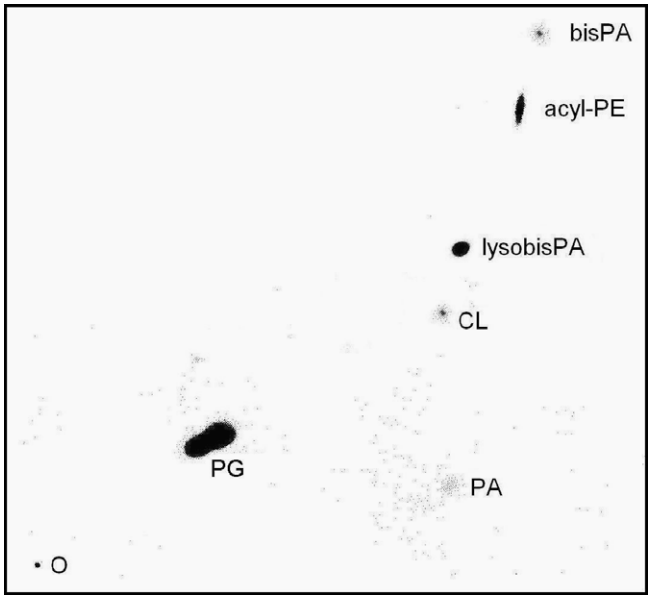

Fig. 2. 2D-TLC of phospholipids from *Calothrix* sp. sprayed by Dittmer-Lester reagent. Abbreviation: bis-phosphatidic acid (bisPA), *N*-acylphosphatidylethanolamine (acylPE), lyso-bis-phosphatidic acid (lysobisPA), cardiolipin (CL), phosphatidic acid (PA), phosphatidyl glycerol (PG).

Table 1
NMR chemical shifts of *N*-acylPE in $CDCl_3$–CD_3OD (1:1, v/v).

No.	^1H	^{13}C
ω	0.84	14.3
ω-1	1.20–1.40	23.2–23.5
ω-2	1.20–1.40	31.9–32.4
Methylenes	1.20–1.40	29.0–30.5
Allylic	1.98–2.02	27.4–27.6
Doubly allylic	2.70–2.75	25.9–26.2
Olefinic	5.28–5.32	128.5–130.4
1 Ester	–	174.1, 174.4
1 Amide	–	175.0
2 Ester	2.25–2.27	34.5–34.6
2 Amide	2.12	36.8
3	1.55–1.60	25.2–25.4
sn-1	4.14, 4.35	63.1
sn-2	5.17–5.20	70.8–70.9
sn-3	3.92–3.94	63.8–63.9
C1	3.85–3.86	64.6
C2	3.38	40.7

128–130 ppm), and carbonyls (δ 174–175 ppm). Three separate signals at δ 174.1, 174.4 and 175.0 ppm confirmed that the molecule of the unknown lipid contains three acyls. DEPT experiments showed that the unknown lipid molecule contains four methylene groups and one methine group attached to hydroxyls or amino groups. TOCSY, HMQC and COSY experiments revealed that the molecule contains two spin systems, the first belonging to diacylglycerophosphate and the second to disubstituted ethanolamine. This finding was confirmed by using a commercially available standard (see above) and comparing the values with those published earlier (Holmback et al., 2001; Batrakov et al., 2001). All these data imply that the unknown lipid could be *N*-acylPE. Acyl attached to an amide group shifts the signal downfield; this was confirmed by finding a difference (δ 0.25–0.35 ppm) between the unknown lipid and standard PE. Also the ^{13}C signal at C-1 is shifted downfield (~2.5 ppm) while, on the other hand, protons at the C-2 in acyl groups are more shielded in amides as compared to esters.

Negative ion ESI–MS of *N*-acylPE isolated from the cyanobacterium is shown in Fig. 3. A number of even mass ions corresponding to the $[M-H]^-$ ions of *N*-acylPE molecular species were evident in the high mass regions of the spectrum. Prominent peaks of fatty acid anions derived from the *O*-acyl groups were observed in the low-mass region (not shown) at *m/z* 255 (16:0), 253 (16:1), 283 (18:0), 281 (18:1), 279 (18:2), and 277 (18:3). No R_3COO^- ions, i.e. ions of the acid bound by an amidic bond, were found in keeping with previously published data (Sandoval et al., 1995; Holmback et al., 2001). Based on MS/MS analyses (Fig. 4) of isolated algal *N*-acylPEs, the main molecular species in the algal mixtures have been assigned as summarized in Table 2.

Full confirmation of the structure, i.e., 16:0–16:0/18:2 was performed according to (Holmback et al., 2001; Chapman and Moore, 1993b).

In another molecular isomer, an ion with the highest identifiable *m/z* value in ESI–MS of total *N*-acylPE was that at *m/z* 1218 $[M-H]^-$, which corresponds to the molecule containing 16:0, 18:2 and 35:0 acids (Fig. 5). The ion most abundant in the MS/MS spectrum is $[M-H]^-$ and acyl substituents derived from it, the more abundant ketenes ($[M-H-R'_xCH{=}C{=}O]^-$) predominating over acids $[M-H-RCOOH]^-$. Splitting off of ketene ion series $[M-H-R'CH{=}C{=}O]^-$ is confirmed by the presence of ions at *m/z* 956 and *m/z* 980; further cleavage yields highly abundant ions $RCOO^-$ at *m/z* 255 and *m/z* 279. In all these processes, sterically more favorable is the fragmentation at *sn*-2 than at *sn*-l, affording an ion at *m/z* 956 ion ($[M-H-R'_2CH{=}C{=}O^-$) which is more abundant than ion at *m/z* 980 ($[M-H-R'_1CH{=}C{=}O]^-$). The ions belonging to the polar head group, which can provide a basis for the identification of amidically bound fatty acid, are observed at *m/z* 644, 684 and 700, which implies the structure 35:0–16:0/18:2 (Fig. 6).

Since the mixture of *N*-acylPE was highly complex, we performed an enzymatic hydrolysis using phospholipases A_2 and D. Using phospholipase A_2 we demonstrated (see also Table 3), that only unsaturated FA are present in position *sn*-2. Acid 17:1, which

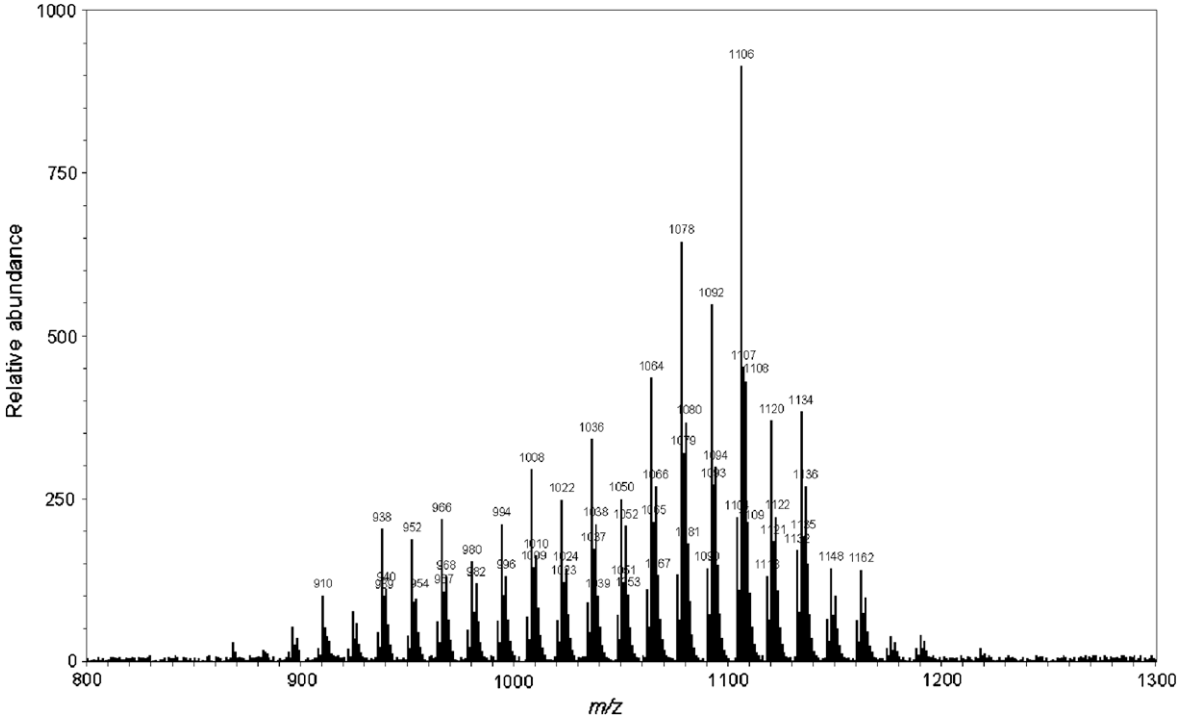

Fig. 3. Negative ion electrospray mass spectrum of deprotonated molecular ions of *N*-acylPE from *Calothrix*. The numbers above individual *m/z* peaks are explained in Table 2.

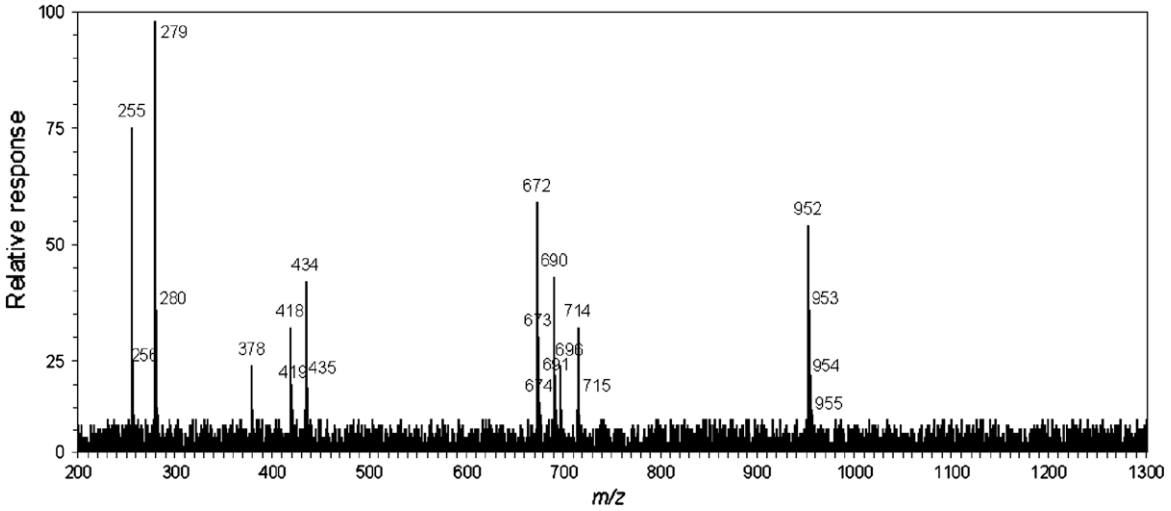

Fig. 4. The tandem quadrupole ESI product–ion spectra of the $[M-H]^-$ ion of 16:0–16:0/18:2 at *m/z* 952.

is present in a low amount (two orders of magnitude lower than 16:1 acid), could not be identified by ESI–MS, and identification was successful only when using GC–MS after hydrolysis by phospholipase A_2 from porcine pancreas. Following hydrolysis of monoacylglycerols obtained by two-step hydrolysis by phospholipases A_2 and D, only two acids, both saturated (16:0 and 18:0), were found in position *sn*-1, see Table 3.

The difficulty in identifying the VLCFA in the whole complex of fatty acids was eliminated by separating special class of phospholipid, i.e. *N*-acylPE from total lipids of the cyanobacterium. Although, the formation of higher homologues of VLCFA drops dramatically with proceeding chain elongation, the *ai*-35:0 is still detectable even though it constitutes only 0.15% of total *N*-acylPE fatty acids. The enrichment of the individual fractions with VLCFA enabled us to identify as yet unknown homologues. In the Table 4 are summarized FA obtained after enzymatic and basic hydrolysis of *N*-acylPE (from *N*-acylethanolamine). The fatty acids were saturated from C_{10} up to C_{35}, odd and even numbered with a straight- and branched- (*iso* and/or *anteiso*) chain.

The data in Table 4 were obtained from mass spectra of both FAME (fatty acid methyl ester) and the picolinyl esters. Picolinates appeared to be more suitable for identification – see the brief description of their splitting in mass spectrometer given below. The difference in the mass spectra of straight- and branched-chain of FAME is small; identification has to take into account the fact that order of elution from all GC phases is *i*-, *ai*-, straight-chain FAME (all FAME have of course also the same molecular formula). The $[M-15]^+$ may be larger in the spectrum of an *iso*-isomer than in the straight-chain analogue, and there is usually an ion for $[M-31]^+$ and absence of ion $[M-29]^+$. Any other significant ions are rather small (<1% of the base peak), and equivalent to $[M-65]^+$, $[M-55]^+$ and $[M-56]^+$, but these may not apply uniformly across the chain length range. The molecular ion of *ai*-FAME is clearly seen and the important distinguishing feature from that of the straight-chain analogue is that an ion at $[M-29]^+$ is more abundant than that equivalent to $[M-31]^+$. Also, an ion at $[M-61]^+$ is small but distinctive (sometimes with ion at $[M-79]^+$). As with normal saturated FAMEs, the McLafferty rearrangement ion at *m/z* = 74 is the base peak.

Picolinyl esters are better derivatives in this instance, especially for the *iso*- and/or *anteiso*-derivatives, which are often encountered in nature. It is typical in that it has prominent ions at *m/z* = 92, 108, 151 (the McLafferty ion) and 164, which are all fragments (in the neighbourhood) about the pyridine ring. The molecular ion $[M]^+$ is easily distinguished and it is always odd-numbered, because of the presence of the nitrogen atom, but most other ions are even numbered. Ions below *m/z* = 92 can usually be ignored. The spectra of both FAME (*iso* and/or *anteiso*) resemble that of a straight-chain saturated fatty acid except for the very obvious gap of 28 Da between $[M-15]^+$ and $[M-43]^+$ and $[M-29]^+$ and $[M-57]^+$ which represents the loss of penultimate and/or *ante*-penultimate carbon atom and its attached methyl group, respectively.

The content of fatty acids in the genus *Calothrix* has been determined several times. *Calothrix marchica*, which was cultivated as an axenic culture (Gugger et al., 2002), has FA content comparable with our analysis, which showed as a major compound (~50% proportion) 16:0 acid. Other identified acids included 16:1, 18:2, 18:3 and also branched *ai*-acids (*ai*-14:0 and *ai*-16:0).

A photosynthetic microbial mat containing many cyanobacterial species including those from the genus *Calothrix* (determined by 16S rRNA gene fragments) was investigated in a large pond of a Mediterranean saltern (Fourcans et al., 2004). Analysis of fatty acids showed that the sample contained both *iso* and *anteiso* acids C14-C17, as well as VLCFA up to C26.

The cyanobacterium *Calothrix* sp., isolated from a rice field, was able to produce the dominant 16:0 (23%), 18:1 (13%) and 18:2 (20%) acids (Olvera-Ramirez et al., 2000).

Based on the above data, as well as on previously published papers, the values given in Tables 3 and 4 imply that, except for the presence of VLCFA in our sample, the content of FA with chain lengths C16 and C18 is very similar.

As mentioned above, isolation and identification of *N*-acylPE containing specific FA was instrumental in identifying both the unusual molecular species of the phospholipid, and the broad interval of lengths of its component VLCFA. Similar unusual molecular species of different phospholipids were discovered in various strains of yeast. The presence of a novel C26-substituted PI (phosphatidylinositol) was described by Schneiter et al. (2004). This C26-PI accounts for ~1% of all the PI species, and is present in both the nuclear and the plasma membrane. Remarkably, this C26-PI is the only C26-containing glycerophospholipid that is detectable in wild type yeast. In contrast to PIs with normal long-chain fatty acids (C16 or C18), the C26-PI greatly reduced the bilayer to hexagonal phase transition of liposomes.

As described by Yokoyama et al. (2001), abnormal phospholipids, phosphatidylcholine and phosphatidylethanolamine, both of which contain a very-long-chain fatty acyl residue (1-melissoyl-2-oleolyl-*sn*-glycero-3-phosphocholine and 1-melissoyl-2-oleolyl-

Table 2
The main molecular species of *N*-acylPE from *Calothrix* sp.

[M–H]$^+$	%	*sn*-1	*sn*-2	*N*-acyl	Sum C:sum C=C	Abundance[a]
842	0.03	16:0	16:1	10	42:1	
856	0.04	16:0	16:1	11	43:1	
866	0.05	16:0	18:3	10	44:3	
868	0.27	16:0	18:2	10	44:2	
870	0.16	16:0	16:1	12	44:1	w
		18:0	16:1	10	44:1	w
		16:0	18:1	10	44:1	w
880	0.04	16:0	18:3	11	45:3	
882	0.26	16:0	18:2	11	45:2	
884	0.21	16:0	16:1	13	45:1	m
		18:0	16:1	11	45:1	w
		16:0	18:1	11	45:1	w
894	0.12	16:0	18:3	12	46:3	w
		18:0	18:3	10	46:3	w
896	0.50	16:0	18:2	12	46:2	m
		18:0	18:2	10	46:2	w
898	0.30	16:0	16:1	14	46:1	w
		18:0	16:1	12	46:1	w
		16:0	18:1	12	46:1	m
		18:0	18:1	10	46:1	w
908	0.18	16:0	18:3	13	47:3	m
		18:0	18:3	11	47:3	w
910	0.88	16:0	18:2	13	47:2	m
		18:0	18:2	11	47:2	w
912	0.57	16:0	16:1	15	47:1	m
		18:0	16:1	13	47:1	m
		16:0	18:1	13	47:1	m
		18:0	18:1	11	47:1	w
922	0.17	16:0	18:3	14	48:3	m
		18:0	18:3	12	48:3	w
924	0.68	16:0	18:2	14	48:2	m
		18:0	18:2	12	48:2	w
926	0.51	16:0	16:1	16	48:1	m
		18:0	16:1	14	48:1	w
		16:0	18:1	14	48:1	m
		18:0	18:1	12	48:1	w
936	0.40	16:0	18:3	15	49:3	m
		18:0	18:3	13	49:3	m
938	1.79	16:0	18:2	15	49:2	s
		18:0	18:2	13	49:2	w
940	0.98	16:0	16:1	17	49:1	m
		18:0	16:1	15	49:1	m
		16:0	18:1	15	49:1	m
		18:0	18:1	13	49:1	m
950	0.35	16:0	18:3	16	50:3	m
		18:0	18:3	14	50:3	w
952	1.60	16:0	18:2	16	50:2	s
		18:0	18:2	14	50:2	w
954	0.82	16:0	16:1	18	50:1	m
		18:0	16:1	16	50:1	m
		16:0	18:1	16	50:1	m
		18:0	18:1	14	50:1	m
964	0.53	16:0	18:3	17	51:3	m
		18:0	18:3	15	51:3	m
966	1.90	16:0	18:2	17	51:2	s
		18:0	18:2	15	51:2	m
968	1.12	16:0	16:1	19	51:1	m
		18:0	16:1	17	51:1	m
		16:0	18:1	17	51:1	m
		18:0	18:1	15	51:1	m
978	0.42	16:0	18:3	18	52:3	m
		18:0	18:3	16	52:3	m
980	1.33	16:0	18:2	18	52:2	s
		18:0	18:2	16	52:2	m
982	1.01	16:0	16:1	20	52:1	m
		18:0	16:1	18	52:1	m
		16:0	18:1	18	52:1	m
		18:0	18:1	16	52:1	m
992	0.52	16:0	18:3	19	53:3	m
		18:0	18:3	17	53:3	m
994	1.82	16:0	18:2	19	53:2	m
		18:0	18:2	17	53:2	m
996	1.14	16:0	16:1	21	53:1	m
		18:0	16:1	19	53:1	m
		16:0	18:1	19	53:1	m
		18:0	18:1	17	53:1	m

Table 2 (*continued*)

[M–H]$^+$	%	*sn*-1	*sn*-2	*N*-acyl	Sum C:sum C=C	Abundance[a]
1006	0.59	16:0	18:3	20	54:3	m
		18:0	18:3	18	54:3	m
1008	2.62	16:0	18:2	20	54:2	s
		18:0	18:2	18	54:2	m
1010	1.43	16:0	16:1	22	54:1	m
		18:0	16:1	20	54:1	m
		16:0	18:1	20	54:1	m
		18:0	18:1	18	54:1	m
1020	0.57	16:0	18:3	21	55:3	m
		18:0	18:3	19	55:3	m
1022	2.17	16:0	18:2	21	55:2	s
		18:0	18:2	19	55:2	m
1024	1.25	16:0	16:1	23	55:1	m
		18:0	16:1	21	55:1	m
		16:0	18:1	21	55:1	m
		18:0	18:1	19	55:1	m
1034	0.80	16:0	18:3	22	56:3	m
		18:0	18:3	20	56:3	m
1036	2.92	16:0	18:2	22	56:2	s
		18:0	18:2	20	56:2	m
1038	1.85	16:0	16:1	24	56:1	m
		18:0	16:1	22	56:1	m
		16:0	18:1	22	56:1	m
		18:0	18:1	20	56:1	m
1048	0.61	16:0	18:3	23	57:3	m
		18:0	18:3	21	57:3	m
1050	2.13	16:0	18:2	23	57:2	s
		18:0	18:2	21	57:2	m
1052	1.74	16:0	16:1	25	57:1	m
		18:0	16:1	23	57:1	m
		16:0	18:1	23	57:1	m
		18:0	18:1	21	57:1	m
1062	0.98	16:0	18:3	24	58:3	m
		18:0	18:3	22	58:3	m
1064	3.87	16:0	18:2	24	58:2	s
		18:0	18:2	22	58:2	m
1066	2.32	16:0	16:1	26	58:1	m
		18:0	16:1	24	58:1	m
		16:0	18:1	24	58:1	m
		18:0	18:1	22	58:1	m
1076	1.18	16:0	18:3	25	59:3	s
		18:0	18:3	23	59:3	m
1078	5.67	16:0	18:2	25	59:2	s
		18:0	18:2	23	59:2	m
1080	3.19	16:0	16:1	27	59:1	s
		18:0	16:1	25	59:1	m
		16:0	18:1	25	59:1	s
		18:0	18:1	23	59:1	m
1090	1.25	16:0	18:3	26	60:3	m
		18:0	18:3	24	60:3	m
1092	4.83	16:0	18:2	26	60:2	s
		18:0	18:2	24	60:2	m
1094	2.58	16:0	16:1	28	60:1	m
		18:0	16:1	26	60:1	m
		16:0	18:1	26	60:1	s
		18:0	18:1	24	60:1	m
1104	1.95	16:0	18:3	27	61:3	s
		18:0	18:3	25	61:3	m
1106	7.71	16:0	18:2	27	61:2	s
		18:0	18:2	25	61:2	m
1108	3.84	16:0	16:1	29	61:1	m
		18:0	16:1	27	61:1	m
		16:0	18:1	27	61:1	s
		18:0	18:1	25	61:1	s
1118	1.09	16:0	18:3	28	62:3	m
		18:0	18:3	26	62:3	
1120	3.22	16:0	18:2	28	62:2	s
		18:0	18:2	26	62:2	m
1122	1.90	16:0	16:1	30	62:1	m
		18:0	16:1	28	62:1	m
		16:0	18:1	28	62:1	m
		18:0	18:1	26	62:1	m
1132	1.45	16:0	18:3	29	63:3	m
		18:0	18:3	27	63:3	s
1134	3.41	16:0	18:2	29	63:2	s
		18:0	18:2	27	63:2	m

(*continued on next page*)

Table 2 (*continued*)

$[M-H]^+$	%	*sn*-1	*sn*-2	*N*-acyl	Sum C:sum C=C	Abundance[a]
1136	2.34	16:0	16:1	31	63:1	m
		18:0	16:1	29	63:1	m
		16:0	18:1	29	63:1	m
		18:0	18:1	27	63:1	m
1146	1.79	16:0	18:3	30	64:3	m
		18:0	18:3	28	64:3	m
1148	0.51	16:0	18:2	30	64:2	s
		18:0	18:2	28	64:2	m
1150	0.87	16:0	16:1	32	64:1	m
		18:0	16:1	30	64:1	m
		16:0	18:1	30	64:1	m
		18:0	18:1	28	64:1	m
1160	0.55	16:0	18:3	31	65:3	m
		18:0	18:3	29	65:3	m
1162	1.20	16:0	18:2	31	65:2	s
		18:0	18:2	29	65:2	m
1164	0.85	16:0	16:1	33	65:1	w
		18:0	16:1	31	65:1	m
		16:0	18:1	31	65:1	m
		18:0	18:1	29	65:1	m
1174	0.18	16:0	18:3	32	66:3	w
		18:0	18:3	30	66:3	m
1176	0.33	16:0	18:2	32	66:2	m
		18:0	18:2	30	66:2	w
1178	0.26	18:0	16:1	32	66:1	w
		16:0	18:1	32	66:1	w
		18:0	18:1	30	66:1	m
1188	0.18	16:0	18:3	33	67:3	w
		18:0	18:3	31	67:3	m
1190	0.35	16:0	18:2	33	67:2	m
		18:0	18:2	31	67:2	w
1192	0.28	16:0	16:1	35	67:1	w
		18:0	16:1	33	67:1	w
		16:0	18:1	33	67:1	w
		18:0	18:1	31	67:1	m
1202	0.03	18:0	18:3	32	68:3	
1204	0.02	18:0	18:2	32	68:2	
1206	0.04	18:0	18:1	32	68:1	
1216	0.06	16:0	18:3	35	69:3	w
		18:0	18:3	33	69:3	w
1218	0.17	16:0	18:2	35	69:2	m
		18:0	18:2	33	69:2	w
1220	0.10	18:0	16:1	35	69:1	w
		16:0	18:1	35	69:1	w
		18:0	18:1	33	69:1	w
1244	0.02	18:0	18:3	35	71:3	
1246	0.02	18:0	18:2	35	71:2	
1248	0.03	18:0	18:1	35	71:1	

[a] w, Weak; m, medium; s, strong.

sn-glycero-3-phosphoethanolamine), were accumulated in *fas2* temperature-sensitive strains. Accumulation of VLCFA-containing phospholipids may affect the construction of the membrane bilayer structure. A mutant strain of budding yeast deficient in acetyl-CoA carboxylase showed structural abnormality of the nuclear pores resulting from a decrease in cellular VLCFA. The dynamics and distribution of long-chain fatty acyl residues in lipids may control various cell functions, and their alteration may cause functional deficits.

Two papers by Matsumoto et al. (1992, 2004) stated that *ai*-VLCFA from C_{20} to C_{30}, including odd and even ones were identified in cryptoendolithic microbial communities from Antarctica. In this study we showed that branched FA are bound to *N*-acylPE as *N*-acyls. Based on all the above data we can presume that this minor phospholipid containing branched VLCFA plays a dominant role in the membranes of *Calothrix* sp., and represents another example of adaptation of lipid composition, enabling growth and survival of this mat-forming cyanobacterium under the extreme conditions of Antarctica.

3. Experimental

HPLC equipment consisted of a 1090 Win system, PV5 ternary pump and automatic injector (HP 1090 series, Hewlett Packard, USA) and Ascentis® Express HILIC HPLC column 2.7 μm particle size, $L \times$ I.D.: 15 cm × 2.1 mm (Supelco, Prague). This setup provided us with a high-efficiency column – approximately 107,000 plates/1 m. LC was performed at a flow rate of 300 μl/min with a linear gradient from mobile phase containing methanol/acetonitrile/aqueous 1 mM ammonium acetate (60:20:20, v/v/v) to methanol/acetonitrile/aqueous 1 mM ammonium acetate (20:60:20, v/v/v) for 40 min. The whole HPLC flow (0.37 ml/min) was introduced into the ESI source without any splitting.

The detector was an Applied Biosystems Sciex API 4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) using electrospray mass spectra. The ionization mode was negative, the nebulizing gas (N_2) pressure was 345 kPa and the drying-gas (N_2) flow and temperature were 9 l min^{-1} and 300 °C, respectively. The electrospray needle was at ground potential, whereas the capillary tension was held at 4000 V. The cone voltage was kept at 250 V. The mass resolution was 0.1 Da and the peak width was set to 6 s. For an analysis, total ion currents (full scan) were acquired from 200 to 1600 Da.

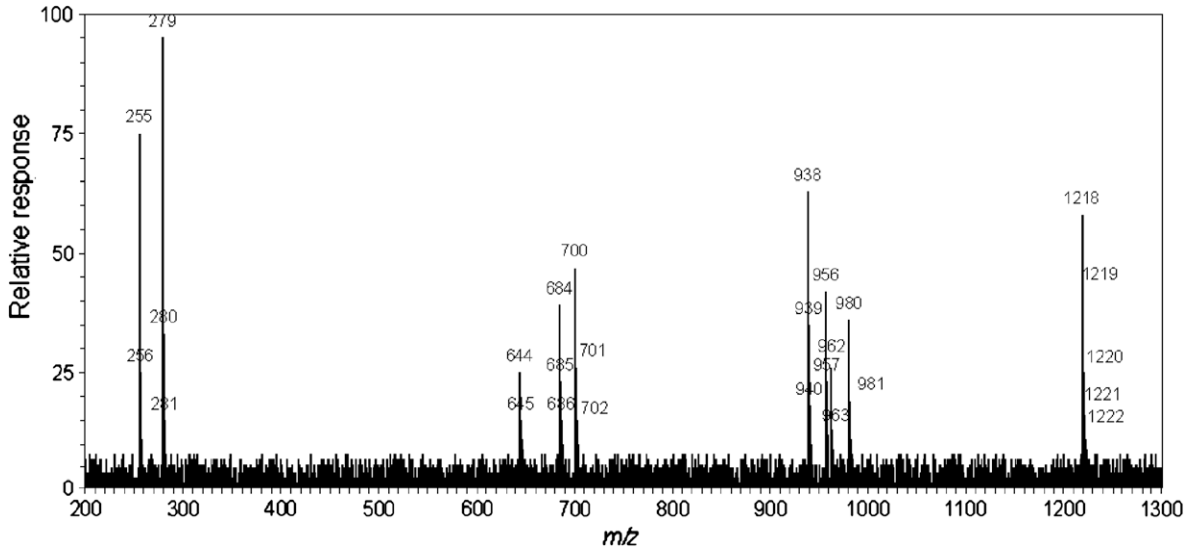

Fig. 5. The tandem quadrupole ESI product–ion spectra of the $[M-H]^-$ ion of 35:0–16:0/18:2-PE at *m/z* 1218.

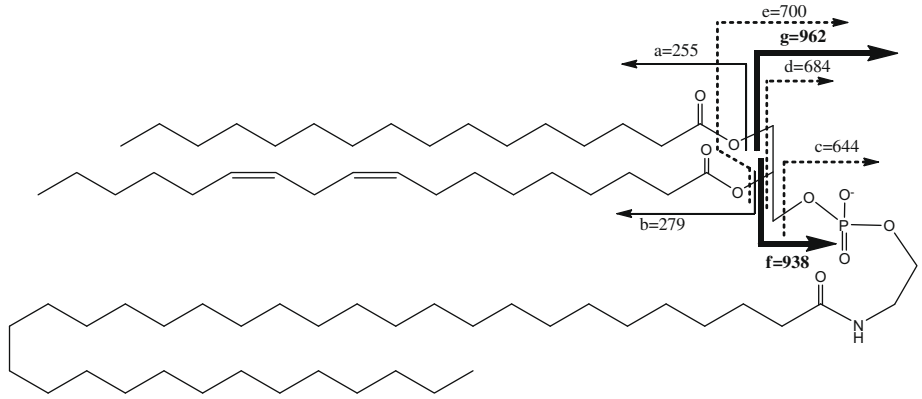

Fig. 6. Structure of *N*-35:0–16:0/18:2-PE. Suggested cleavage sites using negative ion electrospray with collision-induced dissociation.

CID ions mass spectra were acquired by colliding the Q1 selected precursor ions with Ar gas at a collision target gas and applying collision energy of 50 eV in Q2. Scanning range of Q3 was *m/z* 200–1100 with a step size of *m/z* 0.1 and a dwell time of 1 ms. A peak threshold of 0.3% intensity was applied to the mass spectra. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.4.1 software.

Gas chromatography–mass spectrometry of FAME was done on a GC–MS system consisting of Varian 450-GC, Varian 240-MS ion-trap detector with external ionization (EI), and CombiPal autosampler (CTC, USA). The sample was injected onto a 25 m × 0.25 mm × 0.1 μm Ultra-1 capillary column (Supelco, Czech Republic) under a temperature program of: 5 min at 50 °C, increasing at 10 °C min^{-1} to 320 °C and 15 min at 320 °C. Helium was the carrier gas at a flow of 0.52 ml min^{-1}. All spectra were scanned within the range *m/z* 50–600.

A fatty acid picolinyl ester mixture was done on an instrument, see above. Injection temperature (splitless injection) was 100 °C, and a fused-silica capillary column (Supelcowax 10; 60 m × 0.25 mm i.d., 0.25 μm film thickness; Supelco, PA) was used. The temperature program was as follows: 100 °C for 1 min, subsequently increasing at 20 °C min^{-1} to 180 °C and at 2 °C min^{-1} to 280 °C, which was maintained for 1 min. The carrier gas was helium at a linear velocity of 60 cm/s. All spectra were scanned within the range *m/z* 70–650.

NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^{1}H) and 125.7 MHz (^{13}C).

Epilithic cyanobacterial mats formed by *Calothrix* sp. were collected on February 22, 2008 from the littoral zone of Green lake situated in the northern deglaciated part of James Ross Island, northwestern part of the Weddell Sea, Antarctica. Green Lake (altitude 77 m a.s.l., 63°54′12″S, 57°46′49″W) is shallow (maximum depth ca 1.5 m), and its stony bottom is entirely covered by light green mats with a characteristic structure. The sample was aseptically collected, transferred into laboratory in a thermos bottle, identified under light microscope and kept frozen until further analysis. A subsample was fixed by formaldehyde.

Table 3
FA from *sn*-1 and *sn*-2 of *N*-acylPEs.

FA from *sn*-1	%	FA from *sn*-2	%
16:0	67.3	16:1	29.3
18:0	32.7	17:1[a]	0.2
		18:3	22.5
		18:2	30.4
		18:1	17.6

[a] Identified only by GC–MS after hydrolysis and not by ESI–MS by $RCOO^-$ ion.

The extraction procedure was based on the method of Bligh and Dyer (1959), except that 2-propanol was substituted for methanol, since *iso*-propanol does not serve as a substrate for phospholipase D (Kates, 1986). Briefly, the lyophilized cells (1.42 g) were added to hot iso-propanol (70 °C, 30 min) in the ratio of 1 part to 3 parts. The alcohol–water mixture was cooled and one part chloroform was added and the lipids were extracted for 30 min. Insoluble material was sedimented by centrifugation and the supernatant was separated into two phases. The aqueous phase was aspirated off and the chloroform phase was washed three times with two parts 1 M KCl each. The resulting chloroform phase was reduced to dryness under reduced pressure.

First, total lipid extracts (173 mg) were applied to Vac 35 cc (with 10 g of Silica; Waters, USA), and the cartridges were subse-

Table 4
FA from *N*-acyls of *N*-acylPEs.

FA	%	FA	%
i-10:0	0.12	21:0	0.94
ai-10:0	0.02	*i*-22:0	2.59
10:0	0.41	*ai*-22:0	0.35
i-11:0	0.06	22:0	2.61
ai-11:0	0.23	*i*-23:0	0.41
11:0	0.24	*ai*-23:0	2.52
i-12:0	0.23	23:0	1.08
ai-12:0	0.06	*i*-24:0	2.27
12:0	0.68	*ai*-24:0	0.94
i-13:0	0.08	24:0	4.26
ai-13:0	1.14	*i*-25:0	0.49
13:0	0.52	*ai*-25:0	9.39
i-14:0	0.76	25:0	1.46
ai-14:0	0.10	*i*-26:0	3.20
14:0	0.44	*ai*-26:0	1.56
i-15:0	1.02	26:0	4.56
ai-15:0	1.40	*i*-27:0	0.41
15:0	1.10	*ai*-27:0	12.83
i-16:0	1.97	27:0	1.64
ai-16:0	0.59	*i*-28:0	1.50
16:0	0.62	*ai*-28:0	0.56
i-17:0	0.79	28:0	3.78
ai-17:0	1.91	*i*-29:0	0.10
17:0	0.90	*ai*-29:0	5.31
i-18:0	1.32	29:0	0.30
ai-18:0	0.32	*i*-30:0	1.44
18:0	0.82	*ai*-30:0	0.14
i-19:0	0.24	30:0	0.44
ai-19:0	2.13	*i*-31:0	0.06
19:0	1.05	*ai*-31:0	1.80
i-20:0	3.58	31:0	0.10
ai-20:0	0.30	*i*-32:0	0.38
20:0	1.28	32:0	0.10
i-21:0	0.32	*ai*-33:0	0.53
ai-21:0	2.90	*ai*-35:0	0.30

quently washed with 10 ml of hexane, 10 ml of hexane/2-propanol (9:1, v/v), 40 ml of hexane/2-propanol (8:2), and 60 ml of acetone. A fraction of phospholipids was then eluted by 10 ml of chloroform/methanol (2:1) yielding 70.9 mg. The eluate was reduced in volume and subjected to two-dimensional TLC (PLC silica gel 60 F_{254} (0.5 mm × 20 × 20 cm or 1 mm × 20 × 20 cm or 2 mm × 20 × 20 cm)). The first solvent was chloroform–methanol–ammonium hydroxide (65:20:4, v/v/v); the second solvent was chloroform–methanol–acetic acid–water (85:12.5:12.5:3, v/v/v/v). Identification was made based upon cochromatography with commercial standards, spraying with specific reagents (phosphate stain as blue spots, specific for phospholipids; choline stain as orange spots, specific for PC; periodate-Schiff stain, as purple spots, specific for phosphatidyl glycerol; α-naphtol stain, as blue–purple spots, specific for glycolipids, all methods are described in Kates (1986), and lipids were also visualized under UV. The spot having R_f of 0.8–0.9 was scraped from the plates and eluted by diethylether, with a yield of 14% (9.93 mg) of total phospholipids.

The procedure for hydrolysis and identification of *N*-acylPEs was performed as described by Schmid et al. (1986). Briefly, 1 ml diethylether, 1 ml of 40 mM HEPES (pH 8.0), phospholipase A_2 from porcine pancreas (EC 3.1.1.4; ~200 units/mg), and 0.1 ml of a 100 mM solution of $CaCl_2$ were stirring at 32 °C for 2 h with approximately 5 mg *N*-acylPE. The ether was removed, 2 ml of chloroform/methanol (2:1) and 1 ml H_2O were added, and the lower layer was transferred to a second test tube. The upper layer was reextracted once with 2 ml of chloroform/methanol/water (86:13:1) and the combined lower phases (containing lyso *N*-acylPEs and free fatty acids) were taken to dryness under N_2. One ml diethylether, 0.2 ml of 40 mM MES, 200 units of phospholipase D from *Streptomyces chromofuscus* (EC 3.1.4.4; ⩾2000 units/mg), and 15 μl of 100 mM $CaCl_2$ were subsequently added and the mixtures were again stirred at 32 °C for 2–3 h. The products were extracted with chloroform/methanol and the *N*-acylethanolamine, monoacylglycerol, and free fatty acid, products of the combined reactions, were separated by TLC with hexane/diethyl ether/acetone/acetic acid (50:40:10:1) as mobile phase. The lipids were eluted from the adsorbent with chloroform/methanol (1:1). The fatty acids and monoacylglycerols were reacted separately with 3% conc. HCl in methanol at 80 °C for 1 h. *N*-acylethanolamines were converted to free acids by strong hydrolysis, as described by Rawyler and Braendle (2001). Briefly, 10% HCl was added to solution of *N*-acylethanolamines and mixture was heated by 1 h at 100 °C. Fatty acids were extracted with 2 ml of hexane, dried and methylated by diazomethane for GC–MS analysis.

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