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Structural studies on archaeal phytanyl-ether lipids isolated from membranes of extreme halophiles by linear ion-trap multiple-stage tandem mass spectrometry with electrospray ionization

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Abstract

The structures of archaeal glycerophospholipids and glycolipids are unique in that they consist of phytanyl substituents ether linked to the glycerol backbone, imparting stability to the molecules. In this contribution, we described multiple-stage linear ion-trap combined with high resolution mass spectrometry toward structural characterization of this lipid family desorbed as lithiated adduct ions or as the $[M - H]^-$ and $[M - 2H]^{2-}$ ions by ESI. MS^n on various forms of the lithiated adduct ions yielded rich structurally informative ions leading to complete structure identification of this lipid family, including the location of the methyl branches of the phytanyl chain. By contrast, structural information deriving from MS^n on the $[M - H]^-$ and $[M - 2H]^{2-}$ ions is not complete. The fragmentation pathways in an ion-trap, including unusual internal loss of glycerol moiety and internal loss of hexose found for this lipid family were proposed. This mass spectrometric approach provides a simple tool to facilitate confident characterization of this unique lipid family.

Keywords

Archaea; archaeal phospholipids; archaeal glycolipids; cardiolipin; Isoprenoid chains; Halobacteriaceae

1. Introduction

Most of Archaea are extremophiles that can live in harsh environments, such as hot springs, rift vents in deep sea, extremely alkaline and acid waters, and salt lakes. The biochemistry of Archaea is quite different from that of other forms of life. One of the most peculiar aspects of the biochemistry of this third domain of phylogenetic tree is represented by the unique

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Supplementary materials are available.

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structures of cell membrane lipids, which consist of diphytanylglycerol diether lipids [1–5], in contrast with Eukarya and Bacteria that contain largely diacylglycerol- and some monoacyl-monoalkylglycerol-derived lipids. The saturated isoprenoid hydrocarbon chains impart stability to peroxidation degradation; and the ether linkages are resistant to chemical hydrolysis over a wide range of pH. These properties appear to be advantageous for microorganisms growing over a pH range of 5–10, exposed to air and sunlight at temperature even higher than 100 °C [4,6].

The diether core lipid that forms the basis for most polar lipid structures present in archaeal microorganisms is 2,3-di-O-phytanyl *sn*-glycerol (C20, C20), also called archaeol. Thus, these archaeal diether phospholipids are 2,3-di-O-alkyl *sn*-glycerol-1-phosphates (S configuration), which are mirror image of the analogous 1,2-diacyl *sn*-glycerol-3-phosphate isomers (R configuration) typically found in bacterial and eukaryal phospholipids. Examples of phospholipids present in the archaeal microorganisms are archaeol analogues of cardiolipin, phosphatidylglycerol, methyl ester of phosphatidylglycerophosphate, phosphatidylglycerosulfate and phosphatidic acid (PA), in which PA is normally detected as minor components [7]. While the chemical physical properties and aggregation states of membrane lipids of Bacteria and Eukarya are well known, the knowledge of the physical properties of archaeal lipids is relatively limited. Nevertheless, it is clear that archaeal lipids are well adapted as membrane components in extreme environmental conditions.

The presence of cardiolipin analogues in the Archaea domain was first reported by Corcelli et al [1], who established the structure of archaeal bisphosphatidylglycerol (BPG) as *sn*-2,3-di-O-phytanyl-1-phosphoglycerol-3-phospho-*sn*-di-O-phytanylglycerol, which is present in the extremely halophilic archaeon, namely, *Halobacterium salinarum*. Interestingly, the various species of archaeal extreme halophilic microorganisms all carry BPG bearing four identical C20 phytanyl chains [8], while BPGs of some extreme haloalkaliphiles have both C20 and C25 phytanyl chains [7,9]. In addition, complex dimeric phosphosulfoglycolipids (i.e. glycosylated cardiolipin) are also present in the membranes of Archaea [9–11].

Mass spectrometry, in particular, multiple-stage ion-trap mass spectrometry has played important roles in the determination of cardiolipin structures, including the chain length of fatty acyl substituents and their location on the glycerol backbone, as well as the position of the unsaturated bonds, leading to reveal various isobaric isomers [12–15]. Structural studies on archaeal lipids using ESI with tandem quadrupole and with ion-trap instruments coupled with HPLC have been reported [9,16–20]. Among them, Yoshinaga et al conducted a systematic study on a variety of archaeal lipids ionized as the $[M + H]^+$ and $[M + NH_4]^+$ ions by ESI, unveiling the fragmentation patterns useful for characterization of novel lipid structures [17]. Herein, we describe multiple-stage linear ion-trap mass spectrometric approaches in combination with high resolution mass spectrometry toward structural studies on the multiple lithiated adduct ions of the various archaeal ether lipids, including the substituted cardiolipins constituted by sulfoglycolipids esterified to the phosphate group of phosphatidic acids, to reveal the structural details, including the location of the methyl branches of the phytanyl chain, and the mechanism(s) underlying the ion formation of this lipid family.

2. Materials and Methods

2.1. Materials

All organic solvents used were commercially distilled and of the highest available purity (Sigma-Aldrich). Plates for thin layer chromatography (TLC) (Silica gel 60A), obtained from Merck, were washed twice with chloroform/methanol (1:1, v/v) and activated at 120°C before use.

2.2. Microorganism cultures

The archaeal microorganisms used through this study were the *Halobacterium salinarum* (NRC-1 strain) and an isolate closely related to *Halorubrum trapanicum* sp. (MdS1 strain). Both the cultures were grown in light at 37°C in liquid growth medium containing neutralized peptone (L34, Oxoid), prepared as previously described [11,21]. Cells at stationary growth phase were harvested by centrifugation and immediately frozen.

2.3. Lipid extraction and isolation

Total lipids were extracted from whole cells and/or cellular membranes using the Bligh and Dyer method, as modified for extreme halophiles [22]. Total lipid extracts were analyzed by TLC in solvent A (chloroform/methanol/90% acetic acid, 65:4:35, by vol) and all lipids detected by spraying with 5% sulfuric acid, followed by charring at 120°C [22]. The isolation and purification of individual lipid components were performed by scraping the corresponding silica band from preparative TLC plates and extracting from the silica, as previously described [1]. The individual lipids were carefully dried under N₂ before weighing and then dissolved in chloroform. In particular phosphatidylglycerophosphate methyl ester (PGP-Me), 3'-HSO₃-Gal β 1-6Man α 1-2Glc α 1-1-[*sn*-2,3-di-*O*-phytanylglycerol] (S-TGD-1) and 3'-HSO₃-Gal β 1-6Man α 1-2Glc α 1-1-[*sn*-2,3-di-*O*-phytanylglycerol]-6-[phospho-*sn*-2,3-di-*O*-phytanylglycerol] (S-TGD-1-PA) were isolated from *Halobacterium salinarum*, while archaeal cardiolipin (BPG), 2'-HSO₃-Man α 1-2Glc α 1-1-[*sn*-2,3-di-*O*-phytanylglycerol] (S-DGD) and 2'-HSO₃-Man α 1-2Glc α 1-1-[*sn*-2,3-di-*O*-phytanylglycerol]-6-[phospho-*sn*-2,3-di-*O*-phytanylglycerol] (S-DGD-5-PA) from *Halorubrum trapanicum* sp..

2.4. Mass spectrometry

Both high-resolution (R=100,000 at *m/z* 400) and low-energy CAD tandem mass spectrometry experiments were conducted on a Thermo Scientific (San Jose, CA) LTQ Orbitrap Velos mass spectrometer (MS) with Xcalibur operating system. Lipids in chloroform/methanol (1/2) were infused (1.5 μ Lmin⁻¹) to the ESI source, where the skimmer was set at ground potential, the electrospray needle was set at 4.0 kV, and temperature of the heated capillary was 300°C. The automatic gain control of the ion trap was set to 5x10⁴, with a maximum injection time of 100 ms. Helium was used as the buffer and collision gas at a pressure of 1x10⁻³ mbar (0.75 mTorr). The MSⁿ experiments were carried out with an optimized relative collision energy ranging from 35–45% and with an activation *q* value at 0.25, and the activation time at 10 ms to leave a minimal residual abundance of precursor ion (around 20%). The mass selection window for the precursor ions was set at 1 Da wide to admit the monoisotopic ion to the ion-trap for collision-induced dissociation (CID) for unit resolution detection in the ion-trap or high resolution accurate mass detection in the Orbitrap mass analyzer. Mass spectra were accumulated in the profile mode, typically for 3–10 min for MSⁿ spectra (n=2,3,4).

3. Nomenclature

To simplify data interpretation, we incorporated the previously defined abbreviations to designate the compounds included in this study [1]. Archaeal cardiolipin (CL) of bis-(*sn*-2,3-di-*O*-phytanyl-1-phosphoryl)-1',3'-*sn*-glycerol is abbreviated as (a20:0/a20:0)₂-CL to signify that the C-20-phytanyl (alkoxide) groups are alkyl ether-linkage to the *sn*-2, *sn*-3 (glycerol A), and *sn*-2', *sn*-3' (glycerol B) of the glycerol backbones, respectively. The fragment ions observed in the CAD tandem mass spectra of CLs are designated as previously described [12–15]. The phosphatidylglycerophosphate methyl ester (PGP-Me), such as *sn*-2,3-di-*O*-phytanyl-1-phosphoglycerophosphate methyl ester, is abbreviated as a20:0/a20:0-PGP-Me. The diglycosyldiether S-DGD, 2,3-di-*O*-phytanyl-1-*O*-[β -D-

glucopyranosyl-(1 → 2)-*O*- α -D-galactopyranosyl]-*sn*-glycerol 2-sulfate is abbreviated as *a20:0/a20:0*-S-DGD. Sulfo-triglycosyl-diether (S-TGD-1), (3'-sulfo)Gal β 1-6Man α 1-2Glc α 1-1-[*sn*-2,3-di-*O*-phytanylglycerol] is abbreviated as *a20:0/a20:0*-S-TGD-1; while glycardiolipin (GlyC) of 3'-HSO₃-Gal β 1-6Man α 1-2Glc α 1-1-[*sn*-2,3-di-*O*-phytanylglycerol]-6-[phospho-*sn*-2,3-di-*O*-phytanylglycerol] (S-TGD-1-PA) is abbreviated as (*a20:0/a20:0*)₂-S-TGD-1-PA.

4. Results and Discussion

In addition to the presence of phytanyl group linkage to the glycerol backbone, the uniqueness of the structure of archaeal glycerophospholipids also lies on the presence of multiple phosphate groups attached to the glycerol backbone and in some lipid species, on the presence of an additional sulfate group attached to the sugar moiety that links to the glycerol. These structure features facilitate ion formation when subjected to ESI, resulting in various molecular ion species. In the negative-ion mode, abundant [M - H]⁻ and [M - 2H]²⁻ ions, as well as ions of [M - 2H + Alk]⁻ and [M - 3H + 2Li]⁻ were observed if alkali metal ions (Alk = Li⁺, Na⁺) are present in the solution; and in the positive-ion mode, ions in the fashions of [M - (n-1)H + nAlk]⁺ (where n = 1, 2, 3) were readily formed dependent on the concentration of Alk⁺. These various molecular ion forms are all applicable for structural determination and the structure assignment can be easily confirmed. The LIT MSⁿ combined with high-resolution mass spectrometric approaches leading to characterization of these archaeal lipids are described below.

4.1. Characterization of archaeal cardiolipin (CL)

We first applied high resolution (*R* = 100,000 at *m/z* 400) mass spectrometry to deduce the elemental compositions of the molecular species in various forms (Table S1), which are consistent with the structure of *sn*-2,3-di-*O*-phytanyl-1-phosphoglycerol-3-phospho-*sn*-di-*O*-phytanylglycerol (elemental composition: C₈₉H₁₈₂O₁₃P₂) previously reported [1].

MS² on the [M - H]⁻ ions of *m/z* 1520.3 (Fig. 1a) yielded the major fragment ions at *m/z* 731 (a + b), together with ions at *m/z* 787 [(a+56) + (b+56)] and 867 [(a+136) + (b+136)] analogous to the ions previous defined for non-archaeal CL [15]. The results are consistent with the presence of the four identical phytanyl residues attached to the glycerol backbone. Further dissociation of the ion of *m/z* 731 (1520 → 731; Fig. 1b) gave rise to ions at *m/z* 451 and 433 arising from loss of a phytanyl residue as an alkene (280 Da) and as an alcohol (3,7,11,15- tetramethyl-1-hexadecanol; 298 Da), respectively. These losses of the alkene and phytanol residues were also supported by high resolution mass measurements (data not shown). The above spectra are simple, reflecting the unique simple structure of archaeal CL.

In contrast, the MS² spectrum of the [M - 2H + Li]⁻ ion at *m/z* 1526.3 (Fig. 1c) is dominated by the ion at *m/z* 873 [(a+136) & (a+136)] from cleavage of the P-O bond (Scheme 1). Further dissociation of the ion of *m/z* 873 (1526 → 873; Fig. 1d) gave rise to fragment ions at *m/z* 593 and 575 by elimination of phytanylene and phytanol, respectively, and ions at *m/z* 817 and 519 arising from loss of the central glycerol as a C₃H₄O (56 Da) residue from ions of 873 and 575, respectively. This internal loss of glycerol residue may involve a rearrangement process that yielded an intermediate bearing a terminal glycerol residue (Scheme 1). Similar loss of internal glycerol has been reported previously [23]. The spectrum also contained a series of ions at *m/z* 787, 759, 745, 731, 717, 689, 675, 661, 647 and 619 arising from cleavages of the linear C-C bond of the phytanyl chain (Scheme 1), together with low abundance ions at *m/z* 773, 703, and 633 probably from further loss of the methyl side chain. The results are consistent with the presence of the methyl branches at C3, C7, C11, and C15 reported for the phytanyl group.

The structural characterization was also conducted on the corresponding $[M - H + 2Li]^+$ ion at m/z 1534.3, which gave rise to the predominant ion at m/z 881 ($a + 136$) (Fig. 2a). The MS^3 spectrum of the ion of m/z 881 ($1534 \rightarrow 881$; Fig. 2b) contained ions at m/z 659 arising from elimination of the terminal glycerol diphosphate residue (222 Da), and at m/z 745 arising from loss of a bicyclo trimethylene phosphate ($C_3H_5O_3PO$; 136 Da) [24] to a dilithiated diphytanyl phosphatidic acid ion, which further eliminates a phytanol residue (298 Da) to m/z 447 (Scheme 2). This latter fragmentation process was supported by MS^3 on the ion of m/z 745 (data not shown).

Further dissociation of the ion of m/z 659 ($1534 \rightarrow 881 \rightarrow 659$; Fig. 2c) gave rise to ions at m/z 379 and 361, arising from elimination of the phytanyl group as an alkene and alcohol, respectively, together with the ion series at m/z 405, 433, 447, 462, 475, 489, 509, 517, 531, 545, 559, 573, 587, 601, 615, 629, 643 (Fig. 2c, inset) analogous to those seen in Fig. 1d, leading to locate the methyl branches of the phytanyl chain (Scheme 2). The MS^5 spectrum of the ion of m/z 379 ($1534 \rightarrow 881 \rightarrow 659 \rightarrow 379$; Fig. 2d) contained the ion at m/z 305 arising from loss of glycerol (loss as dehydrated glycerol) and the prominent ion at m/z 361 ($379 - H_2O$), which gave rise to the ion of m/z 331 by loss of $HCHO$ (30 Da). The spectrum also contained the ion series at m/z 293, 265, 251, 237, 223, 195, 181, and 153 (inset) that are analogous to those seen in Fig. 1d and 2c, further supporting the assignment of the methyl branches on the phytanyl chain (Scheme 2).

Similarly, MS^2 on the $[M - 2H + 3Li]^+$ ion at m/z 1540.3 (Fig. 3a) gave rise to the prominent $[(a + 136) \& (b + 136)]$ ions at m/z 887, along with the ion at m/z 659, representing a lithiated diphytanylglycerol ion (Scheme 3) as seen earlier. Further dissociation of the ion of m/z 887 (Fig. 3b; $1540 \rightarrow 887$) yielded abundant ion at m/z 831 arising from loss of the central glycerol residue (loss as 56 Da), along with ions at m/z 751 arising from losses of $C_3H_5O_3PO$ (136 Da), and at m/z 665 (dilithiated diphytanylglycerol). The formation of the ion of m/z 831 may again, involve a similar rearrangement process (Scheme 3) as described in Scheme 1. This fragmentation pathway is consistent with the observation of the ions at m/z 745 (loss of PO_3Li), 551 (loss of phytanylene) and 533 (loss of phytanol), together with the ions at m/z 659 and 641 arising from further losses of PO_3Li and of H_2LiPO_4 residues from m/z 745, respectively, as well as the prominent ion at m/z 447 deriving from additional loss of $LiPO_3$ residue from m/z 533, in the MS^4 spectrum of the ion of m/z 831 ($1540 \rightarrow 887 \rightarrow 831$; Fig. 3c) and MS^3 spectrum of the ion of m/z 887.

The ions at m/z 745, 659 and 447 (Fig. 3b – 3c) are identical to those seen in the MS^n spectra of the $[M - H + 2Li]^+$ ion of m/z 1534 (Fig. 2a–2b). The MS^3 spectra of m/z 659 ($1540 \rightarrow 659$) and m/z 745 ($1540 \rightarrow 745$) (data not shown) and the MS^4 spectrum of m/z 447 ($1540 \rightarrow 745 \rightarrow 447$; Fig. 3d) are also identical to those seen earlier (e.g., Fig. 2c–2d), providing structural information toward complete characterization of the molecule.

4.2. Characterization of Phosphatidylglycerophosphate methyl ester (PGP-Me)

High resolution mass measurements on the various molecular species (Table S1) readily supported the structure of $a20:0/a20:0$ -PGP-Me as previously reported. The structural characterization of the molecule as the $[M - H]^-$ and $[M - 2H + 3Li]^+$ ions is described below.

As shown in Fig. 4a, the MS^2 spectrum of the $[M - H]^-$ ion at m/z 899.7 contained the ion at m/z 867 arising from loss of CH_3OH , and the ions at m/z 731 and 787 arising from further losses of $C_3H_5O_3PO$ (136 Da) and PO_3H , respectively, supporting the notion that the molecule consists of a methyl group attached to the 3'-phosphate head group. The presence of methyl group is further confirmed by MS^2 on the $[M - 2H]^{2-}$ ion of m/z 449.3 (Fig. 4b), which yielded the prominent ion at m/z 433 ($449 - 32/2$) deriving from loss of CH_3OH , and

the ion at m/z 787 arising from cleavage of methylphosphate anion ($[\text{OP}=\text{O}(\text{OH})(\text{OCH}_3)]^-$). The spectrum also contained the ion at m/z 297 (Fig. 4b), representing a phytanoxide anion, which further dissociates to m/z 295 by loss of H_2 . These fragmentation processes, including the loss of CH_3OH residue were supported by high resolution mass measurement (data not shown).

The above spectra do not provide the structural information for assignment of the methyl branches of the phytanyl group. However, MS^2 on the $[\text{M} - 2\text{H} + 3\text{Li}]^+$ ion of m/z 919.7 (Fig. 4c) yielded predominant ion at m/z 659 as seen earlier, together with the ion at m/z 887 ($919 - \text{CH}_3\text{OH}$) from elimination of the methyl group attached to the terminal phosphate. The MS^3 spectrum of the ion of m/z 659 ($919 \rightarrow 659$; data not shown) is identical to that shown in Fig. 2d, consistent with the notion that presence of the PGP-Me contains the identical phytanyl chain.

The MS^3 spectrum of the ion of m/z 887 ($919 \rightarrow 887$; not shown) and the MS^4 spectrum of the ion of m/z 831 ($919 \rightarrow 887 \rightarrow 831$; data not shown) are identical to those shown in Fig. 3b and Fig. 3c, respectively. The results indicate that the ion of m/z 887 represents the $[\text{a} + 136]$ (or $[\text{b} + 136]$) ion (Scheme S1) via cleavage of the methanol residue and undergoes the similar fragmentation processes as described for CL (Scheme 3; § 4.1).

4.3.Characterization of sulfodiglycosyldiether S-DGD and sulfodiglycosyldiether S-DGD phospho-sn-2,3-di-O-phytanylglycerol (S-DGD-5-PA)

The difference between the structures of S-DGD and S-DGD-5-PA is that the latter compound contains an additional diphytanylphosphatidic acid residue attached to the 6-position of the galactoside. This structural difference leads to the drastic differences in their tandem mass spectra.

As shown in Fig. 5a, the MS^2 spectrum of the $[\text{M} - \text{H}]^-$ ion of the major archaeal S-DGD at m/z 1055.7 contained the ions at m/z 1037 and 1019 arising from consecutive losses of H_2O , and the ion at m/z 403 representing a Glc-Gal-sulfate anion, together with the prominent ions at m/z 935, arising from cleavage of C1-O and C2-C3 bonds across the glucose ring and at m/z 893, arising from loss of a hexose residue (Scheme 4). This loss of hexose is supported by accurate mass measurement which showed the loss of a $\text{C}_6\text{H}_{10}\text{O}_5$ residue (Table 1). This unusual loss of an internal hexose residue may require rearrangement of the sulfate residue to the inner hexose to form an intermediate containing a terminal hexose, followed by further dissociation to release a hexose (Scheme 4). The MS^3 spectrum of the ion of m/z 893 ($1055 \rightarrow 893$; Fig. 5b) contained the ions at m/z 803, and 773, arising from cleavages of the galactose ring, and at m/z 731 arising from another internal loss of hexose, along with ions at m/z 613 and 595 arising from elimination of the phytanylene and phytanol, respectively, consistent with the proposed structure of m/z 893 deriving from m/z 1055 by hexose loss. Internal loss of hexose residue has been reported previously [25–29].

The above spectra do not provide the structural information about the location of the methyl branches on the phytanyl chain and the presence of sulfate group. However, in positive-ion mode, MS^2 on the corresponding $[\text{M} - \text{H} + 2\text{Li}]^+$ ion at m/z 1069.8 (Fig. 5c) yielded ions at m/z 989, arising from loss of SO_3 group, together with ions at m/z 827 and 821, representing a dilithiated and monolithiated 1,2-diphytanyl-3-glactosyl glycerol, respectively (Scheme 5). Further dissociation of the ion of m/z 821 ($1069 \rightarrow 821$; Fig. 5d) yielded ion of m/z 659 (loss of hexose), which gave MS^4 spectrum ($1069 \rightarrow 821 \rightarrow 659$; data not shown) identical to that seen in Fig. 2c. The MS^5 spectrum of the ion of m/z 379 ($1069 \rightarrow 821 \rightarrow 659 \rightarrow 379$) (data not shown) are also identical to that shown earlier (Fig. 2d) and readily gave complete structural assignment, including the position of the methyl side chain on the phytanyl group.

In contrast, the MS² spectrum of the [M – H][–] ion of *a*20:0/*a*20:0-S-DGD 5-PA at *m/z* 1770.4 (Fig. 6a) is dominated by the ion of 1690.5 from loss of SO₃, and the ion corresponding to loss of a hexose residue as seen for the [M – H][–] ion of *a*20:0/*a*20:0-DGD-S is absent. This drastic difference in the MS² spectrum of *a*20:0/*a*20:0-S-DGD 5-PA from that of *a*20:0/*a*20:0-S-DGD indicates that *a*20:0/*a*20:0-S-DGD 5-PA may undergo different fragmentation processes, attributable to the fact that the negative charge of the precursor ion may reside at phosphate rather than sulfate group and charge-remote fragmentation to eliminate SO₃ residue is the major fragmentation pathway (Scheme S1); while the charge site of the *a*20:0/*a*20:0-DGD-S ion at *m/z* 1055 is located at the sulfate. Charge-remote fragmentations may also account for the formation of *m/z* 989 by loss of SO₃ seen in the MS² spectrum of the [M – H + 2Li]⁺ ion at *m/z* 1069, in which the Li⁺ may attach to the glycoside.

In Fig. 6a, the ion at *m/z* 1528.5 from additional loss of hexose, and the ion at *m/z* 1117 arising from loss of a diphytanylglycerol residue, together with the ions at *m/z* 1055 and 1037 were also observed. The ion at *m/z* 1055 gave rise to the identical MSⁿ spectra to those arising from the [M – H][–] ion of *a*20:0/*a*20:0-S-DGD (data not shown), indicating that the charge site of the ion of *m/z* 1770 can also reside at sulfate (Scheme S1), resulted in the formation of the identical ion of *m/z* 1055 by neutral loss of the diphytanylphosphatidic acid residue (loss as dehydrated diphytanylphosphatidic acid).

The MS² spectrum of the corresponding [M – 2H + 3Li]⁺ ions of *a*20:0/*a*20:0-S-DGD-5-PA at *m/z* 1790.4 (Fig. 6b) contained prominent ions at *m/z* 1542 arising from loss of lithium glucosylsulfate residue, and at *m/z* 1686 arising from loss of LiHSO₄, together with ions at *m/z* 1710 (loss of SO₃), 1137 (loss of diphytanylglycerol), 895 (1137 – hexosylsulfate), 751 (trilithiated diphytanyl phosphatidic acid cation) that are consistent with the structure of the molecule (Scheme S2). Further dissociation of the ion of *m/z* 1542 (1790 → 1542; Fig. 6c) yielded ions at *m/z* 1262, 889, 871, 853, 745 (ion assignments see Scheme S2), together with ions at *m/z* 665 and 659 as seen earlier. The ion of *m/z* 659 represent a lithiated diphytanylglycerol and gave rise to MS⁴ spectrum (1790 → 1542 → 659; data not shown) identical to that shown in Fig. 2d, providing important structural information for assignment of the phytanyl substituent.

4.4. Characterization of Sulfo-triglycosyldiacylglycerol-sn-2,3-di-O-phytanylglycerol (S-TGD-1) and glycardiolipin (GlyC) of 3'-HSO₃-Galpβ1-6Manpα1-2Glcα1-1-[sn-2,3-di-O-phytanylglycerol]-6-[phospho-sn-2,3-di-O-phytanylglycerol] (S-TGD-1-PA)

The structural difference between S-TGD-1 and S-TGD-1-PA pairs is that the latter contains an additional diphytanylphosphatidic acid residue attached to the 6-position of the glucoside, similar to the differences observed for the DGD-S and S-DGD-5-PA pairs as described earlier.

MS² on the [M – H][–] ion of the major S-TGD-1 species of (3' - sulfo)Galpβ1-6Manpα1-2Glcα1-1-[sn-2,3-di-O-phytanylglycerol] (*a*20:0/*a*20:0-S-TGD-1) at *m/z* 1217.8 (Fig. 7a) yielded predominate ion of *m/z* 1055 arising from internal loss of a hexose residue, as supported by high resolution mass measurements (Table 2). MS³ on the ion of *m/z* 1055 (1217 → 1055; Fig. 7b) gave rise to the prominent ion at *m/z* 893 arising from another loss of a hexose residue, along with ions at *m/z* 935, 875 (893 – H₂O), and 403 that were present in Fig. 5a observed for (*a*20:0/*a*20:0)-DGD-S. However, the profiles of the two spectra (i.e., Fig. 7b and Fig. 5a) are readily distinguishable. This variation in the profiles maybe attributable to the fact that the ion of *m/z* 1055 originated from *m/z* 1217 (Fig. 7a) contains a sulfo-6Manpα1-2Glcα1-1-[sn-2,3-di-O-phytanylglycerol] structure following internal loss of the galactose residue (Scheme 6), while (*a*20:0/*a*20:0)-DGD-S (Fig. 5a) consists of a galactosylglucose residue (Scheme 4). The MS⁴ spectrum of the ion of

m/z 893 ($1217 \rightarrow 1055 \rightarrow 893$; Fig. 7c) also contained the fragment ions similar to those seen in the MS^3 spectrum of the ion of m/z 893 ($1055 \rightarrow 893$) observed for (a20:0/a20:0)-DGD-S as shown in Fig. 5b, but the two profiles are readily distinguishable. The results indicate that these two ions represent two isomeric structures.

In the positive-ion mode, MS^2 on the corresponding $[M - H + 2Li]^+$ ion of S-TGD-1 at m/z 1231.8 (Fig. 8a) gave rise to abundant ions at m/z 1151 and 1127 arising from loss of SO_3 and $HLiSO_4$, respectively, and at m/z 983 from elimination of a sulfo-sugar residue (Scheme S3); together with ions at m/z 827 and 821, respectively representing the dilithiated and monolithiated 1,2-diphytanyl-3-hexose as seen earlier (Fig. 5c). Again, the MS^3 spectrum of the ion of m/z 821 ($1069 \rightarrow 821$), the MS^4 spectrum of the ion of m/z 659 ($1069 \rightarrow 821 \rightarrow 659$) and the MS^5 spectrum of the ion of 379 ($1069 \rightarrow 821 \rightarrow 659 \rightarrow 379$) (data not shown) are identical to those shown earlier (Fig. 2d and 2e) and readily give assignment of the position of the methyl side chain of the phytanyl groups.

In contrast, the MS^2 spectra of the $[M - H]^-$ ions of (a20:0/a20:0)-S-TGD-1-PA at m/z 1932.4 (Fig. 8b) is dominated by the ion of m/z 1852 arising from loss of SO_3 , indicating that the charge site of the m/z 1932 precursor ion is located at the phosphate group; and charge-remote fragmentation process that eliminates the SO_3 group is the major fragmentation pathway. This loss of SO_3 residue was supported by high resolution mass measurements (not shown). MS^3 on the ion of m/z 1852 ($1932 \rightarrow 1852$; Fig. 8c) gave rise to ions at m/z 1690, 1528, arising from consecutive losses of hexose, together with ions at m/z 1217 (loss of diphytanylglycerol - H_2O), 1199 (loss of diphytanylglycerol), 1037 (1199 - hexose), 875 (1037 - hexose) and 731 (diphytanylphosphatidic acid anion) (Scheme S4), reflecting the presence of the sugar moiety of the molecule.

The above spectra also do not provide the structural information that characterizes the phytanyl chain. In contrast, MS^2 on the corresponding $[M - 2H + 3Li]^+$ ion of a20:0/a20:0-S-TGD-1-PA at m/z 1952.8 (Fig. 8d) yielded ions at m/z 1872 and 1848 from cleavages of SO_3 and $LiHSO_4$ residues, respectively, via charge-remote fragmentation processes, suggesting that the Li^+ charge site is attached to the sugar moiety. The spectrum also contained the ion of m/z 1704 arising from elimination of the terminal lithium glycosylsulfate (248 Da), and the ion of m/z 1542 from further loss of a hexose residue; while the ion at m/z 1300 arose from elimination of the diphytanylglycerol residue as seen earlier. The MS^3 spectrum of the ion of m/z 1704 ($1952 \rightarrow 1704$; Fig. 8e) contained the ions at m/z 1052 arising from loss of diphytanylglycerol residue, consistent with the observation of the ion of m/z 659 representing a lithiated diphytanylglycerol cation, which gave rise to MS^4 spectrum ($1952 \rightarrow 1704 \rightarrow 659$; data not shown) identical to that shown in Fig. 2d that located the methyl branches on the phytanyl chain. The loss of a hexose residue from m/z 1052.1 gave rise to m/z 889.9, which also yielded m/z 659 (Scheme S5).

5. Conclusions

The MS^2 mass spectra of the various precursor ions of this lipid family are often simple and do not provide complete structure information. However, fragment ions resulting from further dissociation (MS^n ; $n \geq 3$) yielded rich structurally informative ions that are readily applicable for confident structural assignment of this unique lipid family including the location of the methyl branches of the phytanyl group that has not been reported previously using low energy CAD tandem mass spectrometry. The understanding of the mechanisms underlying the losses of internal glycerol and hexose is also important. Thus, misinterpretation of data can be avoided and structure can be accurately assigned.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ESI-MS	electrospray ionization-MS
HRMS	high resolution mass spectrometry
LIT	linear ion-trap
diphosphatidylglycerol	DPG

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Highlights

- Characterization of archaeal phytanyl ether lipids of extreme halophiles was described.
- Multiple stage (MS^n) linear ion-trap high resolution mass spectrometry was used.
- Structurally informative ions defined phytanyl ether chain were observed.
- Unique losses of internal hexose and of glycerol were observed.
- Fragmentation processes leading to the ion formation were proposed.

Fig. 1. The MS² spectrum of the $[M - H]^-$ ions of $(a20:0/a20:0)_2$ -CL at m/z 1520.3 (a), its MS³ spectrum of m/z 721 ($1520 \rightarrow 731$) (b); and the MS² spectrum of the corresponding $[M - 2H + Li]^+$ ions at m/z 1526.3 (c) and its MS³ spectrum of 873 ($1526 \rightarrow 873$) (d).

Fig. 2. The MS² spectrum of the $[M - H + 2Li]^+$ ions of $(a20:0/a20:0)_2$ -CL at m/z 1534.3 (a), its MS³ spectrum of m/z 881 ($1520 \rightarrow 881$) (b), MS⁴ spectrum of m/z 659 ($1520 \rightarrow 881 \rightarrow 659$) (c) and its MS⁵ spectrum of 379 ($1520 \rightarrow 881 \rightarrow 659 \rightarrow 379$) (d).

Fig. 3. The MS² spectrum of the $[M - 2H + 3Li]^+$ ions of $(a20:0/a20:0)_2$ -CL at m/z 1540.3 (a), its MS³ spectrum of m/z 887 ($1540 \rightarrow 887$) (b), and MS⁴ spectra of the ions at m/z 831 ($1540 \rightarrow 887 \rightarrow 831$) (c) and at m/z 447 ($1540 \rightarrow 745 \rightarrow 447$) (d).

Fig. 4. The MS² spectra of the [M - H]⁻ ion of *a*20:0/*a*20:0-PGP-Me at *m/z* 899 (a), of the [M - 2H]²⁻ ion at *m/z* 449.7 (b), and of the [M - 2H + 3Li]⁺ ion at *m/z* 919 (c).

Fig. 5.

The MS² spectrum of the $[M-H]^-$ ion of the major archaeal (*a20:0/a20:0*)-S-DGD at m/z 1055.7 (a), its MS³ spectrum of the ion of m/z 893 (1055 $\rightarrow$ 893)(b); and the MS² spectrum of the corresponding $[M-H+2Li]^+$ ion at m/z 1069.8 (c), and its MS³ spectrum of m/z 821 (1069 $\rightarrow$ 821) (d).

Fig. 6. The MS² spectra of the [M - H]⁻ ion of a20:0/a20:0-S-DGD 5-PA at m/z 1770.3 (a), of the corresponding [M - 2H + 3Li]⁺ ions at m/z 1790 (b), and its MS³ spectrum of the ion at m/z 1542 (1790 → 1542) (c).

Fig. 7. The MS² spectrum of the $[M - H]^-$ ion of the major *a*20:0/*a*20:0-S-TGD-1 at m/z 1217 (a), its MS³ spectrum of the ion at m/z 1055 (1217 $\rightarrow$ 1055) (b), and MS⁴ spectrum of the ion at m/z 893 (1217 $\rightarrow$ 1055 $\rightarrow$ 893)(c).

Fig. 8.

The MS² spectrum of the $[M - H + 2Li]^+$ ion of $a20:0/a20:0$ -S-TGD-1 at m/z 1231.8 (a), and the MS² spectrum of the $[M - H]^-$ ions of $(a20:0/a20:0)$ -S-TGD-1-PA at m/z 1932.4 (b), its MS³ spectrum of the ion at m/z 1852 ($1932 \rightarrow 1852$) (c); and the MS² spectrum of the corresponding $[M - 2H + 3Li]^+$ ion at m/z 1952.8 (d), and its MS³ spectrum of the ion of m/z 1704 ($1952 \rightarrow 1704$) (e).

Scheme 1.

The fragmentation processes proposed for the $[M - 2H + Li]^-$ ion of $(a20:0/a20)_2$ -CL at m/z 1526.3

Scheme 3.

The fragmentation processes proposed for the $(M - 2H + 3Li)^+$ ion at m/z 1540.3

Scheme 4.

The fragmentation processes proposed for the $[M - H]^-$ ion of $a_{20:0}/a_{20:0}$ -DGD-S at m/z 1055

Scheme 5.

The fragmentation processes proposed for the $[M - H]^-$ ion of *a20:0/a20:0*-S-TGD-1 at m/z 1217

Table 1

High resolution MS² spectrum of the [M - H]⁻ ion of a20:0/a20:0-S-DGD at m/z 1055

Measured m/z	Rel. Intensity (%)	Calc. Mass (Da)	Deviation (mmu)	Elemental Composition	Ion assignments
403.0554	16.32	403.0552	0.19	C12 H19 O13 S	Glc-Gal-OSO ₃ ⁻
731.6231	4.78	731.6229	0.19	C43 H87 O6 S	[893 - C ₆ H ₁₀ O ₅] ⁻
757.4048	0.85	757.4050	-0.14	C35 H65 O15 S	[M - H - Phytanol] ⁻
775.4157	424	775.4155	0.13	C35 H67 O16 S	[M - H - Phytanylene] ⁻
875.6652	5.55	875.6651	0.07	C49 H95 O10 S	[M - H - C ₆ H ₁₂ O ₆] ⁻
893.6756	55.18	893.6757	-0.11	C49 H97 O11 S	[M - H - C ₆ H ₁₀ O ₅] ⁻
935.6862	100	935.6863	-0.04	C51 H99 O12 S	[M - H - (CH ₂ O) ₄] ⁻
1037.7181	3.17	1037.7180	0.14	C55 H105 O15 S	[M - H - H ₂ O] ⁻
1055.7284	5.93	1055.7285	-0.15	C55 H107 O16 S	[M - H] ⁻

Table 2

High resolution MS² spectrum of the [M - H]⁻ ion of a20:0/a20:0-S-TGD-1 at m/z 1217.8

measured m/z	Rel. Intensity (%)	Calc. Mass (Da)	Deviation (mmu)	Elemental Composition	Ion assignments
385.0450	1.71	385.0446	0.41	C12 H17 O12 S	Man-Gal-OSO ₃ ⁻ - H ₂ O
403.0557	100	403.0552	0.5	C12 H19 O13 S	Man-Gal-OSO ₃ ⁻
565.1086	10.53	565.1080	0.58	C18 H29 O18 S	Glc-Man-Gal-OSO ₃ ⁻
731.6232	0.44	731.6229	0.28	C43 H87 O6 S	[893 - C ₆ H ₁₂ O ₆] ⁻
893.6761	5.15	893.6757	0.43	C49 H97 O11 S	[M - H - C ₆ H ₁₂ O ₆] ⁻
919.4588	0.45	919.4578	1.06	C41 H75 O20 S	[M - H - Phytanol] ⁻
935.6867	1.44	935.6863	0.42	C51 H99 O12 S	[1097 - C ₆ H ₁₀ O ₅] ⁻
937.4689	3.73	937.4684	0.55	C41 H77 O21 S	[M - H - Phytanylene] ⁻
1055.7287	59.34	1055.7285	0.16	C55H107 O16 S	[M - H - C ₆ H ₁₀ O ₃] ⁻
1097.7394	7.62	1097.7391	0.27	C57 H109 O17 S	[M - H - (CH ₂ O) ₄] ⁻
1199.7705	1.42	1199.7708	-0.32	C61 H115 O20 S	[M - H - H ₂ O] ⁻
1217.7817	45.37	1217.7814	0.36	C61 H117 O21 S	[M - H] ⁻