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Direct ESI-MS analysis of *O*-acyl glycosylated cardiolipins from the thermophilic bacterium *Alicyclobacillus acidoterrestris*

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ABSTRACT

We used direct ESI-MS analysis to identify derivatives of cardiolipin molecular species (i.e. *O*-acyl glycosylated cardiolipins) from the thermophilic bacterium *Alicyclobacillus acidoterrestris*. We used triple-quadrupole type mass spectrometer for analysis of this complex lipid and enzymatic hydrolysis and ^{1}H and ^{13}C NMR for the identification of these cardiolipin derivatives. These techniques enabled us to identify and quantify the specific molecular species profiles of derivatives of cardiolipin directly from lipid extracts of the bacterium including the identification of the sugar moiety as α-D-mannose and all five acyls including their positional isomers.

1. Introduction

The genus *Alicyclobacillus* is non-pathogenic, thermoacidophilic, spore-forming, Gram-positive to Gram-variable bacterium (Chang and Kang, 2004). *Alicyclobacillus* species are distinctive from bacteria of the genus *Bacillus* due to the difference in 16S rDNA sequences, and the presence of the unique ω-alicyclic fatty acids in their membrane lipid bilayer that support their survival at low pH and high temperature (Wisotzkey et al., 1992).

The predominant anionic lipids in bacterial membranes are phosphatidylglycerol (PG) and cardiolipin (CL) (also called bisphosphatidylglycerol). Cardiolipin, an anionic tetraacylphospholipid, occurs in the cytoplasmic membrane of Gram-positive and Gram-negative bacteria (Hoch, 1992; O'Leary and Wilkinson, 1988; Wilkinson, 1988). The concentration in the membrane of Gram-positive bacteria varies from a few mole percent to 59 mol% in streptococci (Hoch, 1992). Since the backbone glycerol of cardiolipin is more rigid than the headgroups of other phospholipids (Allegrini et al., 1984), the conversion of phosphatidylglycerol to cardiolipin may increase the order and presumably the stability of the membrane. Due to the relatively small headgroup in comparison to the total hydrocarbon chain volume, cardiolipin is particularly prone to polymorphic phase behavior.

In Gram-positive organisms, several modified cardiolipin species have been detected, such as α-D-glucopyranosylcardiolipin in *Streptococcus* group B strains (Fischer, 1977), D-alanylcardiolipin in *Vagococcus fluvialis* (Fischer and Arneth-Seifert, 1998) and L-lysylcardiolipin in *Listeria* (Fischer and Leopold, 1999). Structural elements of different cardiolipins were determined by positive and negative ion high resolution fast atom bombardment mass spectrometry (FAB-MS) (Peter-Katalinic and Fischer, 1998). Homologues of D-glucopyranosylcardiolipin species purified from a whole cell lipid extract of *Geobacillus stearothermophilus* were identified by high resolution electro spray ionization-time-of-flight mass spectrometry (ESI-QTOF MS) (Beckedorf et al., 2002).

Relevant reviews (Schlame et al., 2000, 2005; Schlame, 2008) usually state that cardiolipins contain only one or two types of major fatty acids, which together account for 60–90% of the total fatty acid mass. This assertion is all the more surprising since a number of studies have described a large diversity of FA—for instance rat samples were found to contain tens of molecular species of CL (Teng and Smith, 1985), tens of cardiolipin molecular species were identified in lipid extracts of mouse heart (Han et al., 2006), 59 molecular species of CL occur in amounts of more than 0.2% of the total CL from *Streptomyces hygroscopicus* (Hoischen et al., 1997), or more than 50 molecular species were identified in *Escherichia coli* (Hsu and Turk, 2006a).

The complexities of the molecular species of cardiolipin stem not only from the diversity of different chain lengths and unsaturation of fatty acids, but also from permutations of the four fatty acyl chains that result in a large number of potential combinations. Thus, unraveling the structure of cardiolipin has been a very difficult task and has been previously approached by a rather complicated method that requires analysis of the total fatty acyl patterns and the positional distribution of acyl chains between *sn*-1 and *sn*-2

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positions, analysis of diacyl species of the 1′-phosphatidyl and 3′-phosphatidyl moieties and the resolution of tetraacyl species of derivatized cardiolipin. This approach in the analysis of CL has been mainly conducted by HPLC separation of its 1,3-bis-phosphatidyl-2-benzoyl-*sn*-glycerol dimethyl ester derivatives (Schlame et al., 1993; Schlame and Otten, 1991). ESI with tandem mass spectrometric methods including tandem quadrupole (Valianpour et al., 2002), Q-TOF (Beckedorf et al., 2002), and ion-trap (Lesnefsky et al., 2000) instruments have been employed for characterizing cardiolipin and glucosylcardiolipin, and resulted in identifying but not locating the fatty acyl substituents on the glycerol backbone. The subclasses of cardiolipin, i.e. glucosyl-, alanyl-, and lysylcardiolipins have also been previously characterized by FAB-sector mass spectrometric method, but the structural details including position of the fatty acyl substituents on the glycerol backbone could not be determined (Peter-Katalinic and Fischer, 1998).

Herein, we present tandem mass spectrometric methods using a triple-quadrupole mass spectrometer to characterize derivatives of cardiolipins isolated from *Alicyclobacillus acidoterrestris*. Structures of regio- and configuration isomers of cardiolipin molecules are revealed in detail by HPLC-MS/ESI and NMR spectra and by a series of enzymatic hydrolyses.

2. Results and discussion

A. acidoterrestris was cultivated in a 50 L fermentor and cells were harvested in log phase when the optical density of the culture was maximal. The time of cultivation was 24 h. The yield of lyophilized cells was 51.3 g.

The lipids were obtained by the Bligh–Dyer extraction (Bligh and Dyer, 1959) of lyophilized cells with a yield of 6.05 g (11.8%). A part of total lipids was fractionated by means of cartridges with aminopropyl silica-based polar bonded phase, which were rinsed in a chloroform–methanol mixture and phospholipids were further eluted by a mixture of chloroform–methanol–concentrated aqueous ammonia. Phospholipids (72% of total lipids, i.e. 297 mg) were further separated by two-dimensional thin layer chromatography with chloroform–methanol–water in the first dimension and n-propanol–water–glacial acetic acid in the second dimension. The major phospholipids were PG and CL; PA was present in minor amounts, PS, PC, and PE were not recovered.

UV-light detection after TLC showed a spot having R_f of 0.4–0.5 in both solvent systems. The spot gave negative responses to the ninhydrin spray for amine detection and Dragendorff stain for choline but a positive response to vicinal glycol detection spray (periodate-Schiff stain) and phosphate spray (Vaskovsky et al., 1975). This spot, denoted lipid X, was scraped off from the plates and eluted, with a yield of 9% (26.7 mg) of total phospholipids. Table 1 shows the composition of FAME of lipid X after GC–MS analysis. It should be noted that only fatty acids above 0.1% of total fatty acids are included in Table 1.

Table 1
Fatty acid composition in acyl-Man-CL.

Fatty acid	%[a]
14:0	0.7
i-15:0	3.2
ai-15:0	1.7
15:0	0.4
i-16:0	3.4
16:0	8.6
i17:0	2.7
ai-17:0	6.0
17:0	1.2
18:0	0.9
cy-15:0	1.8
cy-17:0	48.8
cy-18:0	0.4
cy-19:0	16.4
cy-20:0	0.2
cy-21:0	2.3
cy-23:0	1.2

[a] Minority fatty acids up to 0.1% of total fatty acids were omitted.

The ^{1}H NMR data of lipid X display a pattern of signals in the regions typical for polar glycerolipids, e.g., signals from the methyl, methylene and methine groups at δ 0.85–2.25 and from protons next to oxygen at δ 3.80–5.21 ppm. In the ^{13}C NMR spectrum signals from the acyl chain moiety appear in the δ 14.3–34.5 ppm region and carbonyls at δ 174.0–174.8 ppm.

The proton signal centered at about 4.95 ppm (J = 1.7 Hz) has chemical shift and coupling constants as an anomeric doublet of hexose (Rezanka, 2002; Rezanka and Dembitsky, 2002). This result indicates that the polar headgroup of the novel lipid X is composed of a monosaccharide (hexose).

In considering the phospholipid backbone, the high-field single proton of C-2 outer glycerols resonated typically at 5.17 ppm (^{1}H) and 70.5 ppm (^{13}C). The other glycerol protons of the outer and central glycerols could be assigned in the interval of 3.5–4.5 ppm. The AB system, i.e. the magnetically inequivalent methylene protons of the outer glycerols (C-1) showed *dd* at 4.12 and 4.32 ppm. The integral ratio of the proton signals was computed and it corresponded to the ratio typical for a CL. The HMQC spectrum of compound X allows the specific assignments of the nine protons from the two equivalent outer glycerols and from the central glycerol. The methylene protons from both C-3 outer glycerols at 64.6 ppm (^{13}C) appear as a multiplet at 4.00 ppm (^{1}H). Part of the 4.00 ppm resonances and the most shielded resonances around 3.93 ppm have been assigned to the central glycerol protons. All these resonances are nearly synchronous and show a complex multiplet from spin-spin interactions between phosphorus ^{31}P and vicinal and geminal protons. The signal at 66.6 ppm has been assigned to both phosphorylated C′-1 and C′-3 methylene groups, and δ 67.5 to central C′-2 that has a glycosylation shift (see also signal at 3.69 ppm). All these data lead to the conclusion that lipid X is an acyl-hexosyl-CL (acyl-Hex-CL).

The structure of acyl-hexosyl-CL was deduced on the basis of knowledge of negative mass spectra published previously by several authors (Hsu et al., 2005; Hsu and Turk, 2006a,b; Peter-Katalinic and Fischer, 1998; Beckedorf et al., 2002).

The major derivative of acyl-hexosyl-CL gives an $[M-H]^-$ ion at m/z 1812 and an $[M-2H]^{2-}$ ion at m/z 906.6. CAD of the $[M-2H]^{2-}$ ions at m/z 906 at a collision energy of 50 eV yield only one cy17:0-carboxylate anion at m/z 267, reflecting the uniform fatty acyl moiety of the molecule. The presence of cy17:0-fatty acyl substituent at the glycoside is consistent with the presence of the ions at m/z 1399 (1812–413), arising from losses (Fig. 1) of a acyl-hexosyl (i.e., hexose esterified with an cy17:0-fatty acyl group) residue, according to previously published papers describing similar compounds (Hsu et al., 2007a,b). As observed before in the detection of fragment ions due to the neutral loss of a hexose moiety, a fragmentation type was not detected in the MS/MS experiment using the $[M-2H+Na]^-$ as a precursor ion but only for $[M-H]^-$ (Beckedorf et al., 2002). The whole demonstration of structure of our complex lipid (acyl-hexosyl-CL) has thus in fact been translated into the simplified structure (CL) and analyzed as previously described (Hsu et al., 2005; Hsu and Turk, 2006a,b). The [M−H-acylhexose]$^-$ ion at m/z 1399 yields the m/z 1131 (1399–268) ion by elimination of a cy17:0-acid; this ion is one of the prominent ones. The spectrum also contains ions at m/z 671 (**a** and **b** ion), m/z 727 [(**b** + 56) and (**a** + 56)], and m/z 807 [(**a** + 136) and (**b** + 136)], which is consistent with the previously proposed mechanism (Hsu et al., 2005; Hsu and Turk, 2006a,b). The m/z 671 is structurally equivalent to a deprotonated di-cyclohexylundecanoyl phospha-

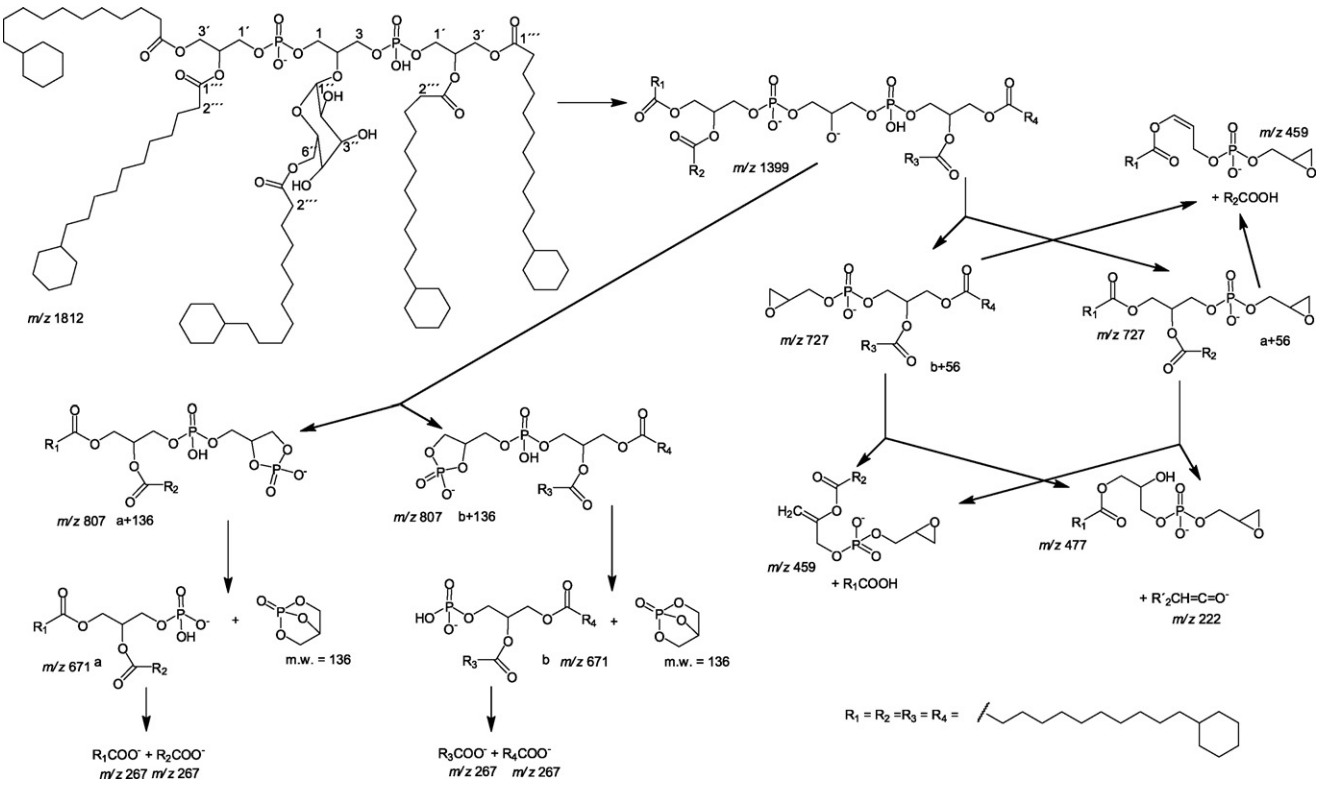

Fig. 1. The major fragmentation pathways proposed for formation of the ions from the $[M-H]^-$ ion of *O*-acyl mannosyl cardiolipin, including the numbering of atoms.

tidic acid (cy17:0/cy17:0-PA) anion, which arises from *m*/*z* 807 by neutral loss a glycerophosphate ester moiety of 136. The proposed structure of the *m*/*z* 727 ion [(**b** + 56) ion] resulting from the fragmentation process is also consistent with the hypothesis that the *m*/*z* 727 is a basic ion, which undergoes fragmentation processes similar to those observed for phosphatidylethanolamine (PE) and gives rise to a higher abundance of the *m*/*z* 477 ion (727–250) than of the *m*/*z* 459 ion (727–268) upon CAD due to the preferential loss of the cy17:0-fatty acyl as a ketene than as an acid. This confirms the assignment of the (cy17:0/cy17:0)cy17:0-Hex(cy17:0/cy17:0)-CL structure (Fig. 2).

Another compound gives an $[M-H]^-$ ion at *m*/*z* 1787. The presence of cy17:0-fatty acyl substituent at the glycoside is consistent with the presence of the ions at *m*/*z* 1375 (1787–412), arising from losses of an acyl-hexosyl residue.

The carboxylate anion reflecting the fatty acyl substituent at *sn*-1 (or *sn*-1′) is more abundant than the corresponding ion at *sn*-2 (or *sn*-2′) in the product-ion spectra of the $[M-H]^-$ ions (Hsu et

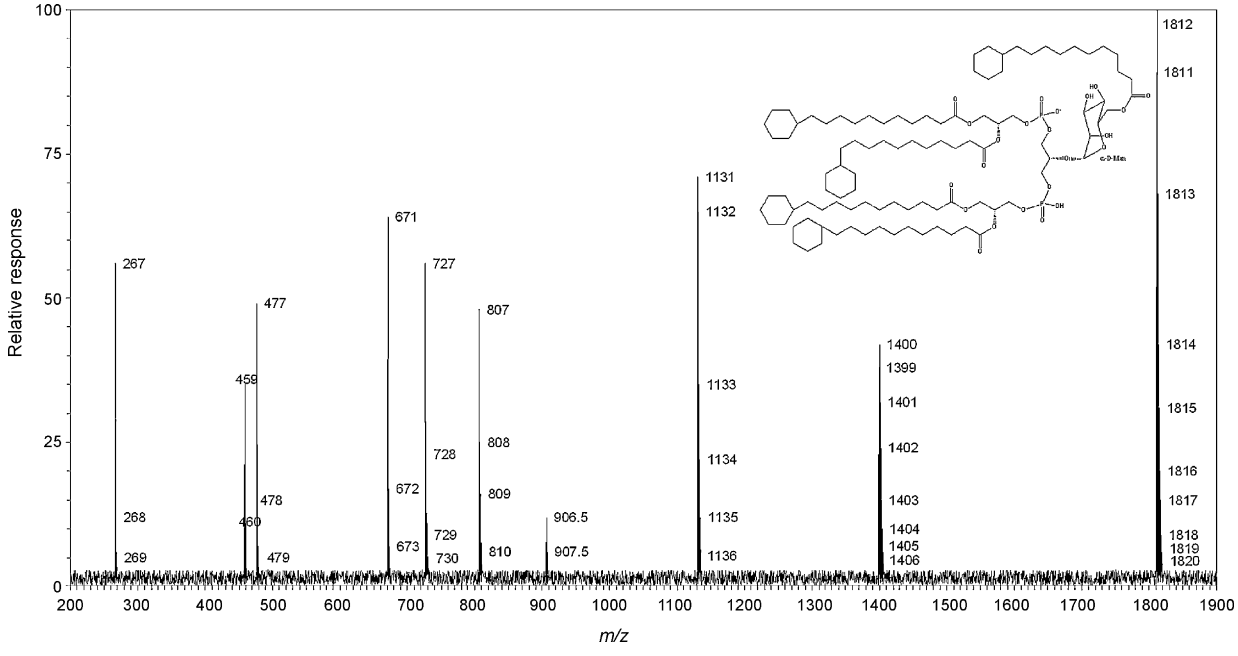

Fig. 2. The tandem quadrupole ESI product-ion spectrum of the $[M-H]^-$ ion of (cy17:0/cy17:0)cy17:0-Hex(cy17:0/cy17:0)-CL at *m*/*z* 1811.

al., 2005; Hsu and Turk, 2006a,b). In the product-ion spectrum of the (16:0/cy17:0)cy17:0-Hex(16:0/cy17:0)-CL ([M−H]$^-$ ion at *m/z* 1787) the $R_1CO_2^-$ (or $R_{1'}CO_2^-$) ion at *m/z* 255 is more abundant than the $R_2CO_2^-$ (or $R_{2'}CO_2^-$) ion at *m/z* 267.

The ions at *m/z* 1125 and 1107 are formed by the various losses of cy17:0-fatty acyl substituent as a ketene (M–H–$C_{15}H_{29}CHCO$, i.e. 1375–250) and free acid (M–H–$C_{16}H_{29}COOH$, i.e. 1375–268), respectively, more abundant than the ions at *m/z* 1137 and 1119, reflecting the various losses of 16:0-fatty acyl substituent. The results indicate that the cy17:0- and 16:0-fatty acyl substituents are located at *sn*-2 (or *sn*-2′) and *sn*-1 (or *sn*-1′) of the glycerols, respectively, and are consistent with the findings that the fragmentation process leading to loss of the fatty acyl substituents at *sn*-2 (or *sn*-2′) is more favorable than the corresponding loss at *sn*-1 (or *sn*-1′) (Hsu et al., 2005; Hsu and Turk, 2006a,b).

The [M−H]$^-$ ion results also in the formation of prominent (**a** + 136) and (**b** + 136) ions via cleavages of the C–O–P bonds, respectively. The spectrum is dominated by the ion at *m/z* 795, representing both an (**a** + 136) and a (**b** + 136) ions, which yield ions at *m/z* 659 (**a** and **b** ion) by the loss of a bicyclic glycerophosphate ester (136 Da). The pathway is consistent with that previously observed by Hsu (Hsu et al., 2005; Hsu and Turk, 2006a,b). Further identified ions include an ion at *m/z* 715 [(**a** + 56) and (**b** + 56)] that arises from the loss of a glycerophosphatidic acid of the [M−H]$^-$ ion according to the scheme proposed by Hsu (Hsu et al., 2005; Hsu and Turk, 2006a,b).

The CAD tandem mass spectrum of the *m/z* 715 ion for 1-hexadecanoyl-2-cyclohexyl undecanoyl moiety contains ions at *m/z* 477 and 465, corresponding to neutral loss of the fatty acyl group as a ketene at *sn*-1 ([715-$C_{14}H_{29}CHCO$]$^-$) and at *sn*-2 ([715-$C_{15}H_{29}CHCO$]$^-$), respectively. The latter ion is more abundant than the former, suggesting that the pathway leading to the ketene loss at *sn*-2 is likely sterically more favorable. This assumption is based on previously reported results (Hsu et al., 2005; Hsu and Turk, 2006a,b).

The spectrum of the *m/z* 659 ion contains major fragment ions at *m/z* 377 and 389 arising from the loss of the cy17:0-acid at *sn*-2 and loss of the palmitic acid (16:0) at *sn*-1, respectively. This confirms the assignment of the (16:0/cy17:0)(16:0/cy17:0)-CL partial structure and (16:0/cy17:0)cy17:0-Hex(16:0/cy17:0)-CL full structure.

The spectrum also contains a minor set of carboxylate anions at *m/z* 239 (15:0), 267 (cy17:0), and 269 (17:0), indicating the presence of an isobaric structure consisting of three fatty acyl substituents. The characterization of acyl-Hex-CL consisting of two various diacylglycerol moieties is illustrated by the product-ion spectra of the same *m/z* 1787 ion.

Like in an isobaric molecule, see above, also here the presence of cy17:0-fatty acyl substituent at the glycoside is consistent with the presence of the ions at *m/z* 1375 (1787–412), arising from losses of an acyl-hexosyl residue.

The spectrum contains the ion sets of *m/z* 645 (**a** ion), 701 (**a** + 56), 781 (**a** + 136) and of *m/z* 673 (**b**), 729 (**b** + 56), 809 (**b** + 136). In the spectrum the *m/z* 673 ion is very similar to that already described as a 16:0/cy17:0-PA moiety. In the spectrum outside the *m/z* 645 ion is dominated also by the *m/z* 375 ion, arising from a cy17:0-acid loss at *sn*-2′, and the less prominent ion at *m/z* 405 arising from the loss of the 15:0-acid at *sn*-1′, exhibiting a 15:0/cy17:0-PA structure. These results indicate that the *m/z* 1375 ion probably represents a (15:0/cy17:0)(17:0/cy17:0)-CL moiety. The *m/z* 645 (**a**) is more abundant than *m/z* 673 (**b** ion), which is consistent with the notion that CL contains two chemically distinct phosphatidyl moieties (Hsu et al., 2005; Hsu and Turk, 2006a,b). The observation of a greater abundance of the *m/z* 645 ion than the *m/z* 673 ion may indicate that the 15:0/cy17:0-glycero moiety is attached to the 1′ position of the central glycerol. This speculation is based on the literature (Hsu et al., 2004).

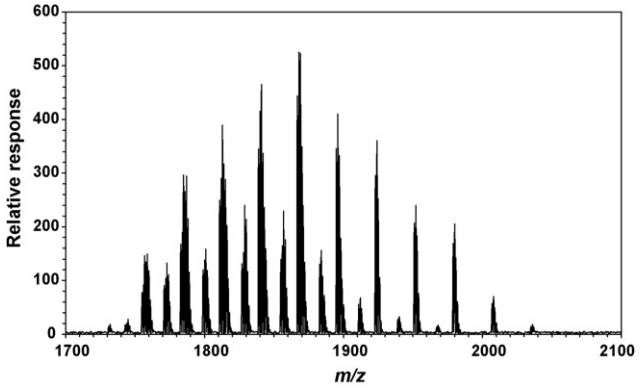

Fig. 3. ESI-mass spectrum of acyl-Man-cardiolipins from *Alicyclobacillus acidoterrestris* in negative ion mode.

The carboxylate anions were observed at *m/z* 239 (15:0), 267 (cy17:0), and 269 (17:0), and the abundance of *m/z* 267 ion is 5 times that of *m/z* 239 and of *m/z* 269 in the product-ion spectrum of the [M−H]$^-$ ion at *m/z* 1375. The results are also consistent with the fact that the molecule consists of two cyclohexylundecanoyl fatty acyl substituents, respectively residing at *sn*-2 and *sn*-2′, and one pentadecanoyl residing at *sn*-1 and one heptadecanoyl residing at *sn*-1′. The full structure of this molecular species is (15:0/cy17:0)cy17:0-Hex(17:0/cy17:0)-CL.

Structural characterization of acyl-Hex-CLs was often complicated by numerous isomers that consist of various homologues (Fig. 3). For example, the product-ion spectra of *m/z* 1979 ions contain minor ions at *m/z* 267, 295, 323, and 351 Da. Further, as shown above, the prominent ion set of *m/z* 671 (**a**), 727 (**a** + 56), and 807 (**a** + 136) points to a deprotonated di-cyclohexylundecanoyl phosphatidic acid (cy17:0/cy17:0-PA) anion. The set of **b** type ions was much more complicated. Multiple ion sets at different *m/z* were found. The ions were obtained from ion at *m/z* 1567 (M–H-acylHex; [M−H-412]$^-$) and included four sets of ions: the first set at *m/z* 839 (**b**), *m/z* 895 (**b** + 56), and *m/z* 975 (**b** + 136); second set *m/z* 811 (**b**), *m/z* 867 (**b** + 56), and *m/z* 947 (**b** + 136); third set *m/z* 783 (**b**), *m/z* 839 (**b** + 56), and

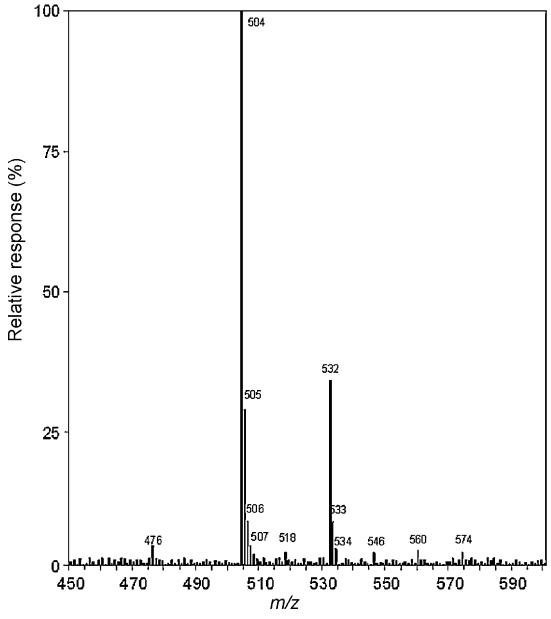

Fig. 4. ESI-mass spectrum of 6-*O*-acyl-Man-Gro in negative ion mode.

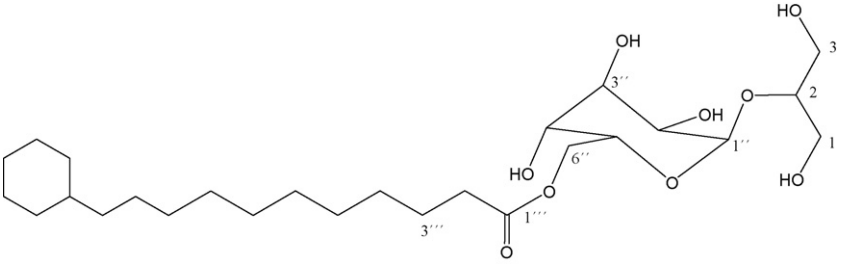

Fig. 5. The structure of compound acyl-Man-Gro whose NMR is described in Table 2.

m/*z* 919 (**b** + 136), and fourth set *m*/*z* 755 (**b**), *m*/*z* 811 (**b** + 56), and *m*/*z* 891 (**b** + 136). Based on these values we can conclude that the molecule contains these four phosphatidic acid moieties: cy23:0 + cy23:0, cy21:0 + cy23:0, cy21:0 + cy21:0, and cy17:0 + cy23:0, irrespective of the sequence at *sn*-1 or *sn*-2. Unfortunately, the acids in position *sn*-1 and *sn*-2 could not be distinguished because the ions $[\mathbf{b}–H–R_1COOH]^-$, $[\mathbf{b}–H–R_2COOH]^-$ as well as $([\mathbf{b}–H–R_{1'}CH{=}C{=}O]^-$ and also $[\mathbf{b}–H–R_{2'}CH{=}C{=}O]^-$) have the same *m*/*z* values (486–486, 486–458, 458–458, and 486–402) so that the acids in positions *sn*-1 and *sn*-2 cannot be determined from their abundances.

To elucidate the stereochemistry, i.e. the structure of the hexose and the glycosidic bond, acyl-Hex-CL was subjected to a series of enzymatic hydrolyses. In the first step the enzymatic hydrolysis of acyl-Hex-CL was done by using phospholipase D. Since hydrolysis with the generally available phospholipase D from cabbage was inefficient, we used another commercial enzyme, phospholipase D from *Streptomyces* sp. This enzyme hydrolyzed acyl-Hex-CL to acyl-Hex-PG and PA. This incomplete hydrolysis has also been described in the literature (Short and White, 1971). In the following step we then again used the phospholipase D from cabbage. The products of hydrolysis, i.e. PA and acyl-Hex-Gro (Gro = glycerol) from acyl-Hex-PG were isolated by TLC. The structure of compound acyl-Hex-Gro was identified by ^{1}H, ^{13}C NMR and ESI-MS. The negative ESI-MS displayed (Fig. 4) a series of homologues of $[M–H]^-$ ions differing by 14 Da (CH_2 group). After strong basic transesterification of acyl-Hex-Gro with methanol, the resulting FAMEs were analyzed by GC–MS (Table 1). The proportion of monoenoic FAs was found to be far below 0.1%, which implies that the acyl-Hex-CL molecule contains only saturated FAs and "unsaturation" is not due to double bonds but to cyclohexane ring (Fig. 5).

The ^{13}C NMR spectrum of the acyl-Hex-Gro showed eight intense signals in the region between 100 and 60 ppm, one of them corresponding to the anomeric carbon of mannose moiety (98.5 ppm), and a low intensity carboxyl signal at 174.5 ppm. The DEPT spectrum showed two negative signals at 63.1 and 64.2 ppm, corresponding to two unsubstituted primary carbons (CH_2OH). From the HMQC experiment, it was possible to correlate the C-1′ (98.5 ppm) with its geminal proton (H-1 at 4.88 ppm, $^1J_{H1,C1}$ 170.5 Hz). The complete assignment acyl-Hex-Gro is shown in Table 2.

The large vicinal coupling constants, $^3J_{3',4'} = 9.2$ Hz and $^3J_{4',5'} = 8.6$ Hz, and the NOE between H-3′ and H-5′ indicated that H-3′, H-4′ and H-5′ protons were located at axial position. The observation of NOE between H-4′ and 2′-OH proton suggested that 2′-OH was oriented at axial position. These data indicate that the monosaccharide is a mannopyranoside; α configuration was determined by a large coupling constant, $^1J_{C\text{-}1',H\text{-}1'} = 170.5$ Hz (>166 Hz) and small $^3J_{H\text{-}1'\text{-}H\text{-}2'} = 1.8$ Hz (Kasai et al., 1979).

Full confirmation of the structure of α-mannose including absolute configuration was performed after a hydrolysis of acyl-Man-Gro by commercially available α-D-mannoside mannohydrolase (EC 3.2.1.24) from *Canavalia ensiformis* (Jack bean).

Table 2
^{1}H and ^{13}C NMR data of acyl-Man-Gro in $CDCl_3$–CD_3OD (1:1, v/v).

No.	^{1}H	^{13}C
1	3.76 *m*	64.2
2	3.81 *m*	81.6
3	3.78 *m*	63.1
1″	4.84 (*d*, *J* = 1.8)	98.5
2″	4.01 (*dd*, *J* = 1.8, 3.5)	70.3
3″	3.93 (*dd*, *J* = 3.5, 9.3)	71.6
4″	3.61 (*dd*, *J* = 9.3, 9.5)	67.0
5″	3.72 (*m*)	72.1
6″	4.26 (*dd*, *J* = 11.5, 2.1)	64.0
	4.08 (*dd*, *J* = 11.5, 7.4)	
1‴		174.5
2‴	2.28 (*t*, *J* = 6.9)	32.5
3‴	1.70 *m*	33.4
4‴–19‴	1.05–1.60 *m*	25.1–38.9

The resulting mannose was identified by GC (as trimethylsilyl ether) by comparing the retention times with a D-mannose standard.

3. Conclusion

The present results demonstrate that multiple stage mass spectrometry with ESI is readily applicable for structural characterization of CL that was described earlier (Hsu et al., 2005; Hsu and Turk, 2006a,b). We show that the method can also be applied to acyl-Man-CL. Although it provides information applicable for establishing the linkage of the fatty acid substituent to the glycoside in acyl-Man-CL, the information obtained by further methods, i.e. NMR and enzymatic hydrolysis was necessary to provide complete structural information. In addition, though the structures of the molecular species that consist of various isomers can be revealed in detail, the implementation of an instrument with sufficient resolution for selecting the high *m*/*z* precursor ions, such as those from acyl-Man-CL, is necessary for their unambiguous structural assignment. The high number of different molecular species determined in the acyl-Man-CL is more likely to reflect the real situation in the bacterial cells than a low number calculated by presuming a minimal variation and combination among the fatty acyl chains. Even though phosphoglycolipids are presumed to be involved in the regulation of the membrane lipid composition in response to, e.g., high temperature and provide an appropriate packaging of phospholipid molecules in a stable lipid bilayer, the *in vivo* physiological relevance of this complex lipid in bacterial membranes is still unknown. The detection of these molecular species, some of which are present in substantial amounts, opens the way to make them available in larger quantities, also with the help of chemical synthesis, and to investigate specifically their role in the structural and functional organization of the bacterial membrane. In analogy to previous studies of bacterial derivatives of CL species (Hsu and Turk, 2006a), it is conceivable that acyl-Man-CL of *A. acidoterrestris* have predominantly odd numbered fatty acids distribution.

4. 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 × I.D. 15 cm × 2.1 mm (Supelco, Prague) was used. 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 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 2500 Da.

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 200–2500 with a step size of m/z 0.3 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 Combi-Pal 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: 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.

Saccharides were converted to *O*-trimethylsilyl ethers. Trimethysilylation was achieved by treating of the dry samples with 100 mL of *bis*(trimethylsilyl) trifluoroacetamide for 30 min at 60 °C. TMS-ethers were analyzed by GC HP 5890 using a flame-ionization detector (FID) under the following conditions: column HP 5 (30 m × 0.25 mm × 0.25 μm), mobile phase nitrogen, pressure 120 kPa (16 psi), temperature rate 150 °C (4 min) – 4 °C/min – 280 °C (2 min), injector temperature 250 °C, detector temperature 300 °C, injection volume 1 μL. Comparison of retention times of commercially obtained standards and sample was used for identification.

NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^1H) and 125.7 MHz (^{13}C). Optical rotations were measured with a Perkin-Elmer 243 B polarimeter.

A. acidoterrestris was cultivated in an alicyclobacillus medium containing (g L^{-1}): yeast extract 6.0, glucose 5.0, $CaCl_2 \cdot 2H_2O$ 0.25, $MgSO_4 \cdot 7H_2O$ 0.5, $(NH_4)_2SO_4$ 0.2, KH_2PO_4 3.0 and 1 mL L^{-1} of trace element solution ($ZnSO_4 \cdot 7H_2O$ 0.1 g L^{-1}, $MnCl_2 \cdot 4H_2O$ 0.03 g L^{-1}, H_3BO_3 0.3 g L^{-1}, $CuCl_2 \cdot 6H_2O$ 0.2 g L^{-1}, $CaCl_2 \cdot 2H_2O$ 0.01 g L^{-1}, $NiCl_2 \cdot 6H_2O$ 0.02 g L^{-1}, $Na_2MoO_4 \cdot 2H_2O$ 0.03 g L^{-1}), pH was adjusted to 4. A volume of 600 mL of inoculum was prepared in 500-mL round-bottom flasks on a temperature controlled shaker at 45 °C and 200 rpm. The biomass was cultivated in a 5 L mechanically stirred fermentor with the aim to gain inoculum for 50 L fermentor. Inoculum was cultivated for 12 h under aerobic conditions with aeration rate 0.8 VVM. The stirrer frequency was 300–400 rpm and the cultivation temperature was 45 °C.

The strain main cultivation was carried out in a 50 L mechanically stirred fermentor for 15 h, under aerobic conditions with aeration rate 1 VVM. The stirrer frequency was 350–400 rpm and the cultivation temperature was 45 °C. Cells were harvested in log phase when the optical density of the culture was maximal. The dry biomass was 1.49 g L^{-1} media.

The extraction procedure was based on the method of Bligh and Dyer (1959), except that 2-propanol was substituted for methanol, since isopropanol does not serve as a substrate for phospholipases (Kates, 1986). 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 evaporated to dryness under reduced pressure.

First, total lipid extracts were applied to Sep-Pak Cartridge Vac 35 cc (Waters; with 10 g of aminopropylsilica-based polar bonded phase), and from the cartridge were subsequently eluted nonacidic lipids with 40 mL of chloroform–methanol (7:3), and acidic lipids with 30 mL of chloroform–methanol–concentrated aqueous ammonia (70:30:2) containing 0.4% (w/v) ammonium acetate. The eluate was reduced in volume and subjected to two-dimensional preparative TLC (PLC silica gel 60 F_{254}, 2 mm × 20 × 20 cm). The first solvent was chloroform–methanol–7 M ammonia (12:7:1); the second solvent was chloroform–methanol–glacial acetic acid (65:25:8). Identification was made based upon cochromatography with commercial standards or by R_f described in the literature and a specific reagent, see below. In preparative mode, visualized spots under UV were scraped off and the lipid was eluted with 2 mL of chloroform–methanol (1:1, v/v). Specific reagents for phospholipids (7%, w/v, molybdophosphoric acid in ethanol), sugars (0.1%, w/v, orcinol in 40%, v/v, sulphuric acid), phosphorus (Dittmer–Lester spray) and amino groups (0.2%, w/v, ninhydrin in acetone) were used (Minnikin et al., 1977), together with authentic standards of CL, PG, PE, and PI.

The acyl-Man-CL (2 mg) was suspended in 0.1 mL of 2% (v/v) Triton X-100 solution by mixing on a Vortex mixer for 2 min. Water (0.15 mL) was added, the mixing was repeated, 0.5 mL of phospholipase D (from *Streptomyces*) was added, and the solution was mixed. Then 0.25 mL of 20 mm $MgCl_2$ in 100 mM phosphate buffer (pH 7.5) was added, bringing the final concentration of $MgCl_2$ to 10 mM, the phosphate buffer to 50 mM, and the Triton X-100 to 0.2%. After mixing and incubation at 37 °C for 1 h, the reaction was stopped by extraction with the Bligh and Dyer procedure. The PA and acyl-Man-PG were separated by chromatography on silica gel, the conditions see above. The acyl-Man-CL hydrolysis was 80 to 90% complete (Tucker and White, 1971). To a vial was added acyl-Man-PG (0.7 mg) in 1 mL CH_2Cl_2, 1 mL water and cabbage phospholipase D in 0.5 mL 0.2 M sodium acetate buffer, pH 5.5, containing 0.05 M $CaCl_2$. Samples were mixed at 25 °C (6 h) and after hydrolysis the sample was partitioned according to Bligh and Dyer, see above (Piazza and Marmer, 2007). The PA and acyl-Man-Gro were again separated by chromatography on silica gel. A solution of glycoside (0.2 mg) in acetate buffer (pH 4.4, 1 mL) was treated with α-D-mannosidase for 48 h at 37 °C. The reaction solution was evaporated to dryness, and the residue was dissolved in pyridine and BSA. *N*,*O*-Bis(trimethylsilyl)acetamide was added to this solution which was left to stand for 30 min. The derivatives were then chromatographed under these conditions: the column SPB-5 (30 m × 0.25 mm × 0.25 μm film thickness) was held at 100 °C for 2 min and after that the temperature was programmed from 100 to 280 °C at 10 °C/min (inlet: 250 °C; splitless injection/purge time: 1.0 min; initial temperature: 100 °C; initial time: 2.0 min), gas: helium (1 mL/min), solvent delay: 4 min.

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