Characterization of Acylphosphatidylglycerols from *Salmonella typhimurium* by Tandem Mass Spectrometry with Electrospray Ionization

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Acylphosphatidylglycerol (Acyl-PG), a polar lipid class containing three fatty acyl groups, was isolated from *Salmonella* bacteria and characterized by tandem quadrupole and quadrupole ion-trap mass spectrometric methods with electrospray ionization. The structural characterization of the acyl-PG with various acyl groups (A-B/C-PG, where $A \neq B \neq C$) is based on the findings that the carboxylate anions ($R_x\text{CO}_2^-$) arising from *sn*-2 ($R_2\text{CO}_2^-$) is more abundant than that arising from *sn*-3' ($R_3\text{CO}_2^-$), which is much more abundant than that arising from *sn*-1 ($R_1\text{CO}_2^-$). This information provides a simple method for determination of the fatty acyl moieties and their positions in the molecule. The structural identification of the molecule can also be achieved by the findings that the fragment ion reflecting the ketene loss at *sn*-2 is more prominent than that reflecting the acid loss (i.e., $[M - H - R'_2\text{CH}=\text{CO}]^- > [M - H - R_2\text{CO}_2\text{H}]^-$), while the ion arising from acid loss at *sn*-1 or *sn*-3' is, respectively, more abundant than the corresponding ketene loss (i.e., $[M - H - R_1\text{CO}_2\text{H}]^- > [M - H - R'_1\text{CH}=\text{CO}]^-$; $[M - H - R_3\text{CO}_2\text{H}]^- > [M - H - R'_3\text{CH}=\text{CO}]^-$). The identity of the acyl moiety at *sn*-3' can be confirmed by an acyl-glycerophosphate anion observed in the product-ion spectrum obtained with a triple-stage quadrupole (TSQ) instrument, but not in that obtained with an ion-trap mass spectrometer (ITMS). However, the MS^2 -spectrum obtained with an ITMS is featured by the ion series that abundances of $[M - H - R'_2\text{CH}=\text{CO} - R_3\text{CO}_2\text{H} - 74]^- > [M - H - R'_2\text{CH}=\text{CO} - R_1\text{CO}_2\text{H} - 74]^- \gg [M - H - R'_{1(\text{or } 3')}\text{CH}=\text{CO} - R_{3'(\text{or } 1)}\text{CO}_2\text{H} - 74]^-$. This information also facilitates structural elucidation of the acyl-PG subclass that contains various acyl substituents. Structural identifications of molecular species having two identical fatty acyl substituents at *sn*-1, *sn*-2, or *sn*-3' or consisting of more than one isomeric structures are also demonstrated. The identities of the minor isomeric species in the molecules can be revealed by the aforementioned structural information arising from the various ion series combined. (J Am Soc Mass Spectrom 2004, 15, 1–11) © 2004 American Society for Mass Spectrometry

Acylphosphatidylglycerol (1,2-diacyl-*sn*-glycero-3-phospho-(3'-acyl)-1'-*sn*-glycerol) (I) (Acyl-PG), a polar lipid containing three fatty acyl substituents, has been identified in mycoplasma [1], bacteria [2–5], mold [6], and trichomonads [7]. Small quantities of acyl-PG are also present in certain animal tissue [8–10] and cereals [11].

Structural determination of acyl-PG in oat oil was recently described by Holmback et al. [11], who used

one- and two-dimensional ^1H - and ^{13}C -NMR, mass spectrometric methods including HPLC-tandem quadrupole mass spectrometry with electrospray ionization to analyze intact acyl-PG molecules, and GC/MS with electron impact ionization to analyze the fatty acid hydrolysates derivatized to picolinyl esters. Although the methods give information including the positions of the double bonds in the fatty acid substituents, structural details such as positions of the various fatty acyl substituents in the molecules consisting of multiple isomeric configurations were not available. Collisional-activated dissociation (CAD) tandem sector mass spectrometric methods with FAB were also used to identify acyl-PGs isolated from *Corynebacterium* spp. [12] and trichomonads [13].

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However, a systematic approach towards the structural characterization of acyl-PGs using low-energy or high-energy CAD tandem mass spectrometry with electrospray ionization has not been described.

1,2-diacyl-*sn*-glycero-3-phospho-(3'-acyl)-1'-*sn*-glycerol (I)

We previously reported tandem mass spectrometric method in the determination of phosphatidylglycerol (PG). Structural information including the identities and the positions of the fatty acyl substituents in the glycerol backbone can be easily determined [14]. Herein, we present tandem mass spectrometric methods using both a triple quadrupole and a quadrupole ion-trap mass spectrometers to characterize acyl-PG. Structures of regio- and configuration isomers of acyl-PG molecules are revealed in details.

Materials and Methods

Bacterial Strain and Medium

Salmonella enterica serovar typhimurium strain used in this study is wild-type 14028s. N-minimal medium pH 7.4 supplemented with 0.1% casamino acids and 38 mM glycerol, and 10 μ M or 10 mM of MgCl_2 were prepared as described [15].

Isolation of Inner and Outer Membranes

Bacteria were inoculated into N minimal medium at pH 7.4 containing 10 mM MgCl_2 for overnight growth at 37 $^\circ\text{C}$ with aeration. The cultures were harvested and washed three times with the N minimal medium (pH 7.4, with 10 μ M MgCl_2). Cells were diluted to 1:100 with the same N minimal medium. Bacteria were incubated for 4 h at 37 $^\circ\text{C}$ with aeration. Cells were harvested from 300 ml culture and washed once with 50 ml phosphate buffered saline (PBS). Pellet was suspended in 4 ml of 10 mM Tris-HCl (pH 8.0) containing 20% sucrose, and sonicated for 5 min. Cell debris was removed by centrifugation at 6500 rpm for 10 min at 4 $^\circ\text{C}$. Inner and outer membranes were isolated by one step sucrose gradient procedure as previously described [16] (Hancock, <http://www.cmdr.ubc.ca/bobh/methodsall.html>). Briefly, the supernatant was loaded on a 60% and 70% sucrose discontinuous gradient centrifuge tube and spun at 38,000 rpm at 4 $^\circ\text{C}$ for 18 h. Upper reddish band near top of 60% sucrose that represents inner membrane portion and a band on the top of 70% sucrose containing outer membrane were collected

and dialyzed against PBS (pH 7.4) for overnight with stirring. The membrane preparations were stored at -80°C .

Extraction of Phospholipids from Membrane Preparations

The procedure was as described [17]. To 1 ml inner or outer membrane sample, 1.25 ml chloroform and 2.5 ml methanol were added. After vortexing for 2 min, 1.25 ml chloroform was added, and vortexing continued for another 30 s. This is followed by the addition of 1.25 ml H_2O , and vortexing for another 30 s. The mixture was centrifuged for 5 min at 3000 rpm. Lower phase containing phospholipids was collected for ESI/MS analysis.

Mass Spectrometry

Low-energy CAD tandem mass spectrometry experiments were conducted on a Finnigan (San Jose, CA) TSQ 7000 mass spectrometer equipped with ICIS data system or on a LCQ DECA ion-trap mass spectrometer with X-caliber operation system. Extracts containing acyl-PG in methanol/chloroform solution (vol/vol, 1/1) were continuously infused into the ESI source with a Harvard syringe pump at a flow rate of 1 $\mu\text{L}/\text{min}$. The skimmer was at ground potential and the electrospray needle was set at 4.5 kV. The temperature of the heated capillary was 260 $^\circ\text{C}$. For product-ion spectra obtained with a triple stage quadrupole (TSQ) instrument, the $[\text{M} - \text{H}]^-$ ions were selected in the first quadrupole (Q1), collided with Ar (2.3 mTorr) in the rf-only second quadrupole (Q2) using a collision energy of 34 eV and analyzed in the third quadrupole (Q3). Both Q1 and Q3 were tuned to unit mass resolution and scanned at a rate of 3 s/scan. The mass spectra were accumulated in the profile mode, typically for 5–10 min for a tandem mass spectrum. For CAD tandem mass spectra obtained with a quadrupole ion-trap instrument, the automatic gain control of the ion trap was set to 5×10^7 , with a maximum injection time of 400 ms. Helium was used as the buffer and collision gas at a pressure of 1×10^{-3} mbar. MS^n experiments were carried out with a relative collision energy ranged from 30–35% and with an activation q value, with which the mass range for the product-ion scan can be set from above the precursor ion mass to the lowest trapped mass that covers the m/z value of the expected carboxylate anions. The activation time was set at 100 ms. The mass resolution was 0.6 Da at half peak height.

Results and Discussion

ESI Mass Spectrometry of the Acylphosphatidylglycerols Isolated from *Salmonella typhimurium*

When subjected to ESI in the negative-ion mode, the lipid extract yielded abundant $[\text{M} - \text{H}]^-$ ions, that

Figure 1. The negative-ion ESI mass spectra of acyl-PG extracts from (a) inner membrane, (b) outer membrane of *Salmonella typhimurium*.

could be attributed to acylphosphatidylglycerols (acyl-PG). However, in the positive-ion mode, ions reflecting the molecular species of acyl-PG were not observed. The ESI mass spectra of the acyl-PG acquired by a triple quadrupole instrument for the extracts isolated from inner (Figure 1a) and outer membranes (Figure 1b) of *Salmonella typhimurium* cells contain homologous $[M - H]^-$ ions ranging from m/z 889 to 1011, and are identical to that obtained by an ITMS (data not shown). More than one isomer was observed in most of the molecular species, as revealed by their tandem mass spectra (Table 1). The structural characterization of the molecules is described below.

Characterization of Acylphosphatidylglycerols by CAD Tandem Quadrupole and MS^2 -Ion-Trap Mass Spectrometry

We previously reported tandem quadrupole mass spectrometric method in the characterization of phosphatidylglycerols (PG) [14]. The structural identification was based on the findings that: (1) Ions reflecting the losses of the fatty acyl substituent as a free acid ($[M - H - R_2CO_2H]^-$) and as a ketene ($[M - H - R'_2CH=C=O]^-$) at sn -2 are respectively more abundant than the analogous losses at sn -1 (i.e., $[M - H - R_1CO_2H]^-$ and $[M - H - R'_1CH=C=O]^-$ ions). (2) The ion arising from a com-

Table 1. Acylphosphatidylglycerol compositions in *Salmonella typhimurium* determined by tandem mass spectrometry

$[M-H]^{-2}$	Inner membrane	Outer membrane	Fatty acid		
	Relative Intensity (%)		sn -1	sn -2	sn -3'
899	6	0 ³	14:0	16:1	14:1
			16:0*	16:1	12:1
			18:1*	14:1	12:0
901	15	14	16:0	16:1	12:0
			16:0	14:0	14:1
925	7	0 ³	12:0	18:1	14:0
927	54	30	16:1	16:1	14:1
			16:0	16:1	14:1
929	47	32	18:1	16:1	12:0
			16:0	16:1	14:0
953	20	24	16:0	18:1	12:0
			16:1	16:1	16:1
			18:1	16:1	14:1
955	100	58	16:0	16:1	16:1
			16:1	18:1	14:1
957	45	100	16:0	18:1	14:0
			16:0	16:1	16:0
969 ¹	6	32	16:0	17:1	16:1
			16:0	17:1	16:0
971	0 ³	30	16:0	18:1	15:0
			16:0	16:1	17:0
981	35	18	18:1	16:1	16:1
			16:1	18:1	14:1
983	53	0 ³	16:0	18:1	16:1
			18:1	16:1	16:0
983 ²	0 ³	65	16:1	18:1	16:0
			16:0	18:1	16:0
985	17	48	16:0	18:1	16:0
997	0 ³	18	18:1	17:1	16:0
			17:0	18:1	16:1
999	0 ³	15	16:0	19:1	16:0
			17:0	18:1	16:0
1009	10	0 ³	18:1	18:1	16:1
1011	7	18	18:1	18:1	16:0

*Structure not conclusive.

¹Structure not conclusive, minor species contains 14:1-, 14:0-, 15:0-, 18:1-, 19:1-fatty acyl

²Species observed in the outer membrane

³Abundance close to background

bined losses of the sn -2 fatty acid substituent and polar head group ($[M - H - R_2CO_2H - 74]^-$) is more abundant than the ion arising from the corresponding losses at sn -1 ($[M - H - R_1CO_2H - 74]^-$). (3) The $[M - H - R'_2CH=C=O]^-$ ion is more abundant than the $[M - H - R_2CO_2H]^-$ ion, whereas the $[M - H - R_1CO_2H]^-$ ion is more abundant than the $[M - H - R'_1CH=C=O]^-$ ion. (4) The carboxylate anion arising from sn -2 ($R_2CO_2^-$) is more abundant than that arising from sn -1 ($R_1CO_2^-$). As shown in Figure 2, the product-ion spectrum of the $[M - H]^-$ ion of 16:0/16:1-PG standard at m/z 719 (Panel a) is dominated by the carboxylate anions at m/z 253 ($R_2CO_2^-$) and 255 ($R_1CO_2^-$), and the former ion is more abundant than the latter. The m/z 483 ($[M - H - R'_2CH=C=O]^-$) and m/z 465 ($[M - H - R_2CO_2H]^-$) ions are respectively more abundant than m/z 481 ($[M - H -$

Figure 2. The tandem quadrupole ESI product-ion spectra of the $[M - H]^-$ ions of (a) 16:0/16:1-PG at m/z 719, and (b) 16:0/18:1-PG at m/z 747.

$R'_1CH=C=O]^-$ and 463 ($[M - H - R_1CO_2H]^-$). The m/z 483 ($[M - H - R'_2CH=C=O]^-$) ion is more abundant than m/z 465 ($[M - H - R_2CO_2H]^-$), whereas the m/z 481 ($[M - H - R'_1CH=C=O]^-$) is slightly less abundant than m/z 463 ($[M - H - R_1CO_2H]^-$). The spectrum is also featured by the much greater abundance of the m/z 391 ion ($[M - H - R_2CO_2H - 74]^-$) (465 - 74) than m/z 389 ($[M - H - R_1CO_2H - 74]^-$) (463 - 74). These spectrum features were also observed in the product-ion spectrum of 16:0/18:1-PG standard at m/z 747 (Panel b) [14].

When subjected to low-energy CAD, the $[M - H]^-$ ions of acyl-PG yielded major ion series similar to those observed for PG. The product-ion spectrum contains the ion series that correspond to neutral loss of free fatty acid ($[M - H - R_xCO_2H]^-$), neutral loss of ketene ($[M - H - R'_xCH=C=O]^-$), along with the fatty carboxylate anions ($[R_xCO_2]^-$). The spectrum also contains the ion series that arises from further losses of the fatty acid moiety at *sn*-1 (or *sn*-3') and a dehydrated glycerol from $[M - H - R'_2CH=C=O]^-$ ($[M - H - R'_2CH=C=O - R_xCO_2H - 74]^-$, where $x = 1$ or $3'$).

The $[M - H - R_xCO_2H]^-$ and $[M - H - R'_xCH=C=O]^-$ Ions

As shown in Figure 3, the product-ion spectrum of the $[M - H]^-$ ion at m/z 927 (Panel a) obtained with a TSQMS is dominated by the carboxylate anions at m/z

Figure 3. The tandem quadrupole ESI product-ion spectra of the $[M - H]^-$ ions of (a) 14:1-16:0/16:1-PG at m/z 927, (b) 14:0-16:0/16:1-PG at m/z 929.

255 ($C_{15}H_{31}CO_2^-$), 253 ($C_{15}H_{29}CO_2^-$) and 225 ($C_{13}H_{27}CO_2^-$), reflecting the palmitoyl (16:0), palmitoleoyl (16:1), and myristoleoyl (14:1) substituents of the molecule, respectively. This is consistent with the observation of the ions at m/z 701 ($[M - H - C_{13}H_{25}CO_2H]^-$), 673 ($[M - H - C_{15}H_{29}CO_2H]^-$) and 671 ($[M - H - C_{15}H_{31}CO_2H]^-$), arising from losses of the 14:1, 16:1 and 16:0- fatty acid, respectively. The fatty acyl substituents are also reflected by the fragment ions at m/z 719 ($[M - H - C_{12}H_{23}CHC=O]^-$), 691 ($[M - H - C_{14}H_{29}CHC=O]^-$) and 689 ($[M - H - C_{14}H_{31}CHC=O]^-$), arising from the losses of the 14:1, 16:1, and 16:0-fatty acyl ketene from m/z 927, respectively. The m/z 691 ion is more abundant than the m/z 673 ion, and the ion pairs are the most prominent among the ion series (Panel a, subset). In contrast, the abundances of the m/z 701 and m/z 671 ions are respectively greater than those of the m/z 719 and m/z 689 ions. The preferential loss of 16:1-ketene to the m/z 691 ion over the loss of 16:1-acid to m/z 673 ion, indicates that the 16:1-fatty acyl substituent probably resides at *sn*-2 of the glycerol backbone, whereas the 16:0-fatty acyl group resides at *sn*-1, similar to that observed for PG (Figure 2). The remaining 14:1-fatty acyl substituent is attached to the 3' position

Scheme 1

of the glycerol head group. This speculation is based on the findings that ions at m/z 719 and 701 arising from a 14:1- ketene and acid losses, respectively, are more abundant than the ions at m/z 689 and 671, arising from the analogous losses from a 16:0 fatty acid. The production spectrum of the $[M - H]^-$ ion at m/z 929 (Panel b) contains a similar ion profile that reflects the presence of a 14:0-, 16:0-, and 16:1-fatty acyl moieties and represents a 14:0-16:0/16:1-PG structure. The identities of the fatty acyl moieties and their positions in the glycerol backbone or in the glycerol head group were further confirmed by the $[M - H - R'_2CH=C=O - R_xCO_2H - 74]^-$ and $R_xCO_2^-$ ion series, and by the MS^2 and MS^3 product-ion spectra obtained with an ITMS as described later.

The $[M - H - R'_2CH=C=O - R_xCO_2H - 74]^-$ Ions

In addition to the above ions readily available for structural assignment, the product-ion spectrum of m/z 927 (Figure 3a) contains ions at m/z 391 ($[M - H - R'_2CH=C=O - 74 - R_3CO_2H]^-$) and 361 ($[M - H - R'_2CH=C=O - 74 - R_1CO_2H]^-$), probably arising from an initial loss of the 16:1-fatty acyl substituent at *sn*-2 as a ketene to m/z 691, which dissociates to m/z 465 and 435 by losses of the fatty acid substituents at *sn*-3' and *sn*-1, respectively. This is followed by an additional loss of a dehydrated glycerol (74 Da) to yield ions at m/z 391 and 361, respectively. This fragmentation process results in probably a five-member ring ion, comprising a 16:0- or 14:1-acyl moiety, respectively (Scheme 1a). The above fragmentation pathways are further supported by the MS^3 -product-ion spectrum of m/z 691 (Figure 5c). In

contrast, the m/z 389 ion, comprising a 16:1 fatty acyl that resides at *sn*-2 of the glycerol backbone is of low-abundance, similar to that observed for 16:0/16:1-PG (Figure 2a) [14]. A unique ion at m/z 379, corresponding to a 3'-myristoleoyl glycerophosphate anion (Scheme 1b) is also observed. An analogous ion at m/z 381 is also present in the product-ion spectrum of 14:0-16:0/16:1-PG at m/z 929 (Panel b). The results are consistent with the notion that a 14:1- and 14:0-fatty acyl moieties are respectively positioned at the *sn*-3' of the glycerol head group of the two molecules.

The Carboxylate Anions ($R_xCO_2^-$)

Both the $[M - H]^-$ ions of PG and acyl-PG share a structure, in which the anionic charge site probably resides at the phosphate. As previously described by us [14, 18, 19], the preferential formation of the $R_2CO_2^-$ ion over the $R_1CO_2^-$ ion for PG is due to the fact that nucleophilic attack by the anionic phosphate charge site on the C-2 of the glycerol backbone to yield a $R_2CO_2^-$ ion is sterically more favorable than that on C-1 that results in $R_1CO_2^-$. This is consistent with the notion that the m/z 253 ion arising from a 16:1-fatty acyl at *sn*-2 is the most prominent in the spectrum of m/z 927 (Figure 3a), whereas the less prominent carboxylate anions at m/z 255 and 225, arising from a 16:0- and 14:1-fatty acid moieties, respectively, are likely located at *sn*-1 and *sn*-3', respectively. This assumption is also based on the findings that the abundance ratios of the m/z 255 ion to m/z 253 observed for m/z 927 (Figure 3a) is similar to that observed for the 16:0/16:1-PG standard (Figure 2a).

Figure 4. The ESI ITMS MS² spectra of the [M - H]⁻ ions of (a) 14:1-16:0/16:1-PG at *m/z* 927, and (b) 14:0-16:0/16:1-PG at *m/z* 929.

The observation of the abundances of: $R_2CO_2^- > R_3CO_2^- > R_1CO_2^-$ in the product-ion spectrum of acyl-PG provides the simplest method to locate and identify the fatty acyl moieties of the molecule.

Characterization of Acylphosphatidylglycerols by the MS² Spectrum Obtained with ITMS

The MS² spectrum of an acyl-PG obtained with an ITMS contains fragment ions similar to those obtained with a TSQ and is readily applicable for structural characterization. However, the spectrum is dominated by [M - H - R_xCO₂H]⁻, [M - H - R_xCHC=O]⁻, [M - H - R'₂CH=C=O - 74 - R₃CO₂H]⁻ and [M - H - R'₂CH=C=O - 74 - R₁CO₂H]⁻ series ions, and the R_xCO₂⁻ ions are of low abundance. As shown in Figure 4, the structural characterization of the 14:1-16:0/16:1-PG at *m/z* 927 (Panel a) can be easily achieved by the findings that the ion prominence follows the pattern: *m/z* 691 ([M - H - C₁₄H₂₇CH=CO]⁻) > 673 ([M - H - C₁₅H₂₉CO₂H]⁻); 719 ([M - H - C₁₂H₂₃CH=CO]⁻) < 701 ([M - H - C₁₃H₂₅CO₂H]⁻); *m/z* 689 ([M - H - C₁₄H₂₉CH=CO]⁻) < *m/z* 671 ([M - H - C₁₅H₃₁CO₂H]⁻). The *m/z* 253 (R₂CO₂⁻) is more abundant than *m/z* 225 (R₃CO₂⁻), which is more abundant than *m/z* 255 (R₁CO₂⁻), consistent with that observed with a TSQ and readily for structural determination. Similar results were also observed for the [M - H]⁻ ion of 14:0-16:0/16:1-PG at *m/z* 929 (Panel b).

One of the most interesting feature in the spectrum is that the *m/z* 391 ion ([M - H - R'₂CH=CO - 74 - R₃CO₂H]⁻) appears to be the most prominent and is slightly more abundant than *m/z* 361 ([M - H - R'_{2(or 1)}CH=CO - 74 - R_{1(or 2)}CO₂H]⁻), which is much more abundant than *m/z* 389 ([M - H - R'₃CH=C=O - 74 - R₂CO₂H]⁻). Compared to the 16:0/16:1-PG standard (Figure 5a) obtained under the same condition, the striking similarity in the profile of the *m/z* 391 and 389 ions suggests that the *m/z* 927 ion likely possesses a 16:0- and 16:1-acyl at *sn*-1 and *sn*-2, respectively. Since the ion series is the most prominent in the spectrum, the observation of the abundances of *m/z* 391 > *m/z* 361 >> *m/z* 389 provides an unambiguous method with which 16:0-, 16:1-, and 14:1-fatty acyl substituents can be easily assigned at *sn*-1, *sn*-2 and *sn*-3', respectively.

The prominence of the *m/z* 361 ion may also suggest that the 14:1-acyl moiety resides at *sn*-3' rather than at *sn*-2' of the glycerol head group. Probably, the pathways for the ketene or acid loss at *sn*-3' are similar to those leading to the analogous loss at *sn*-1, and different from those accountable for the losses at *sn*-2.

Structural Confirmation of Acylphosphatidylglycerols by MS³ Ion-Trap Tandem Mass Spectrometry

As described earlier, the acyl moiety at *sn*-3' is reflected by the presence of the ions at *m/z* 719 and 701 arising from losses of 14:1- ketene and of 14:1-acid, respectively. This is further confirmed by the MS³ spectrum of the *m/z* 719 ion (Figure 5b), arising from *m/z* 927. The spectrum is nearly identical to the MS² spectrum of 16:0/16:1-PG at *m/z* 719 (Figure 5a), indicating that probably a gaseous ion of *m/z* 719, similar to a [M - H]⁻ ion of 16:0/16:1-PG, has been formed from *m/z* 927 by elimination of the 14:1-ketene at *sn*-3'. In contrast, the MS³ spectrum of the *m/z* 691 ion (Figure 5c), originated from a 16:1-ketene loss at *sn*-2 to become a 3'-myristoleoyl 1-palmitoyl 2-lyso phosphatidylglycerol (14:1-16:0/lyso2-PG), is dominated by ions at *m/z* 465 (691 - C₁₃H₂₅CO₂H), 435 (691 - C₁₅H₃₁CO₂H), 391 (465 - 74), and 361 (435 - 74), and can readily be differentiated from a product-ion spectrum of PG. The ion profile of the MS³ spectrum of the *m/z* 689 ion (data not shown), originated from a 16:0-ketene loss at *sn*-1 to a 14:1-lyso1/16:1-PG ion, is also similar to that of a diacyl-PG. This result is consistent with the notion that the 14:1-fatty acyl substituent resides at *sn*-3' rather than at *sn*-2' as described earlier.

The MS³ spectrum of the [M - H - R₃CO₂H]⁻ ion of *m/z* 701 ion (Figure 5d) from a primary loss of 14:1-fatty acid from *m/z* 927 contains major ions involving further losses of the 16:1-ketene at *sn*-2 or the 16:0-ketene at *sn*-1. These fragmentation processes lead to *m/z* 465 (701 - C₁₄H₂₇CH=CO) and 391 (701 - C₁₄H₂₇CH=CO - 74), involving loss of a 16:1-ketene moiety, and ions at 463 (701 - C₁₄H₂₉CH=CO) and 389 (701 - C₁₄H₂₉CH=CO -

Figure 5. (a) The ITMS MS² spectrum of 16:0/16:1-PG at m/z 719. (b–d) The ITMS MS³ spectra of m/z 719 ($[927 - R'_3CH=CO]^-$) (b), m/z 691 ($[927 - R'_2CH=CO]^-$) (c), and m/z 701 ($[927 - R'_3CO_2H]^-$) (d), from MS² of m/z 927 (14:1-16:0/16:1-PG, Figure 4a).

74), involving a 16:0-ketene loss. The m/z 465 is more abundant than m/z 463, reaffirming that the 16:1-fatty acid is located at *sn*-2 of the glycerol backbone. The m/z 465 and 463 ions are also more abundant than the m/z 447 ($701 - C_{14}H_{27}CO_2H$) and 445 ($701 - C_{14}H_{29}CO_2H$), respectively. This is consistent with the idea that m/z 701 ion resulting from an initial loss of acid from m/z 927 becomes more basic (or less acidic) and undergoes preferential ketene than acid loss [14, 18, 19]. The ion profile of the MS³ spectrum of the $[M - H - R_1CO_2H]^-$ ion of m/z 671 ion (data not shown) is similar to that arising from m/z 701 and is readily differentiable from that arising from the $[M - H - R_2CO_2H]^-$ ion of m/z 673 (data not shown). This is consistent with the idea that the pathways for the ketene or acid loss at *sn*-3' and *sn*-1 are similar but different from that at *sn*-2.

Differentiation of Positional Isomers of Acyl-PG

Positional isomers of acyl-PG were also observed in the extracts from inner and outer membranes (Table 1). As shown in Figure 6a, the product-ion spectrum of m/z 983 in the extract isolated from inner membrane is dominated by the carboxylate anions at m/z 281 (18:1),

255 (16:0), and 253 (16:1). Since the m/z 281 ion is more abundant than m/z 253, which is much more abundant than m/z 255, the structure of the molecule can be easily assigned as 16:1-16:0/18:1-PG. This assignment is further supported by the observation of the abundances of m/z 719 ($[M - H - C_{16}H_{31}CH=CO]^-$) > m/z 701 ($[M - H - C_{17}H_{33}CO_2H]^-$), signifying that the 18:1-acyl moiety resides at *sn*-2. The assignment is also consistent with the fact that the m/z 719 and 701 ion pairs are the most prominent among the ion series arising from acid or ketene loss. The m/z 745 ($[M - H - C_{14}H_{29}CH=CO]^-$) ion is less abundant than m/z 727 ($[M - H - C_{15}H_{31}CO_2H]^-$) and the ion pairs are the least prominent in the series. The results suggest that the 16:0-acyl moiety positions at *sn*-1, whereas the 16:1-acyl is located at *sn*-3' and are in accord with the observation of the 16:1-acyl glycerophosphate anion at m/z 407.

The abundance of the $R_2CO_2^-$ ion at m/z 253 appears to be higher than that expected for a 16:1-16:0/18:1-PG, attributable to the fact that a minor 16:0-18:1/16:1-PG isomer is also present. This assumption is based on the findings that the m/z 747 ($[M - H - C_{14}H_{27}CH=CO]^-$) ion is slightly more abundant than m/z 729 ($[M - H - C_{15}H_{29}CO_2H]^-$), indicating the presence of a 16:1-acyl at

Figure 6. The tandem quadrupole product-ion spectra of the m/z 983 ions isolated from (a) inner membrane, and (b) outer membrane. The profiles of the two spectra are different substantially, indicating that they are positional isomers. The two compounds also contain minor positional isomers, which are marked with an asterisk.

sn-2. The structural assignment of this latter isomer is further confirmed by the observation of the 16:0-acyl glycerophosphate ion at m/z 409, which signifies a 16:0-acyl at *sn*-3'. The abundance of m/z 417 ion is also higher than that anticipated for a 16:1-16:0/18:1-PG and consistent with the notion of the presence of a second 16:0-18:1/16:1-PG isomer that results in the rise of the m/z 417 ion.

The abundance of m/z 253 ion is greater than that of m/z 255, which is more abundant than m/z 281 in the product-ion spectrum of m/z 983 arising from the extract of outer membrane (Figure 6b). Hence the species can be easily identified as 16:0-18:1/16:1-PG. The assignment is further supported by the observation of m/z 747 ($[M - H - C_{14}H_{27}CH=CO]^-$) > m/z 729 ($[M - H - C_{15}H_{29}CO_2H]^-$), confirming a 16:1-acyl at *sn*-2, along with the presence of m/z 745 ($[M - H - C_{14}H_{29}CH=CO]^-$) < m/z 727 ($[M - H - C_{15}H_{31}CO_2H]^-$), signifying the existence of a 16:0-acyl at *sn*-3' or *sn*-1. The assignment of the 16:0-acyl at *sn*-3' is determined by the presence of m/z 391 together with m/z 409, a 16:0-acyl glycerophosphate ion that involves the *sn*-3' fatty acyl substituent.

The abundances of the m/z 255 and 281 ions are higher than those predicted for a 16:0-18:1/16:1-PG, suggesting the presence of an additional isomer. This is also based on the findings that the abundance of m/z 719 ($[M - H - C_{16}H_{31}CH=CO]^-$) is slightly greater than m/z 701 ($[M - H - C_{17}H_{33}CO_2H]^-$), indicating the presence of a 18:1-acyl at *sn*-2. The 16:0-acyl glycerophosphate at

m/z 409 that signifies a 16:0-acyl at *sn*-3' is the exclusive ion of the ion series in the spectrum, indicating that both the isomers possess a 16:0-acyl at *sn*-3'. Therefore, the minor isomer with a structure of 16:0-16:1/18:1-PG can be deduced.

Structural Characterization of the Molecular Species Containing Two Identical Fatty Acyl Moieties

Although the observation of abundances of $R_2CO_2^- > R_3CO_2^- \gg R_1CO_2^-$ provides a simple method leading to the assignment of the structure of an acyl-PG, this strategy is not applicable for molecules having two identical fatty acyl substituents at two different positions, or comprising more than one isomeric structures. This is attributable to the fact that the abundances of the carboxylate anions are complicated by the contribution of the identical fatty acyl groups at different positions or by the contribution of the fatty acyl groups from various isomers. For example, the product-ion spectrum of the $[M - H]^-$ ion at m/z 1009 (Figure 7a) contains $R_xCO_2^-$ ions at m/z 253 and 281, indicating the presence of both 16:1- and 18:1-fatty acyl moieties, but their position in the molecule based on their abundances is not applicable. However, the identity of the 18:1-fatty acyl substituent at *sn*-2 can be easily recognized by the observation of the greater abundance of the m/z 745 ion, arising from loss of 18:1-ketene, than

Figure 7. The tandem quadrupole product-ion spectra of $[M - H]^-$ ions of (a) m/z 1009, (b) m/z 981, (c) m/z 985, and (d) m/z 957. The structural characterizations are complicated by the presence of two identical fatty acyl substituents in the molecules however, the identifications can be achieved by the combined information arising from the various ion series.

m/z 727, arising from loss of 18:1-acid. The m/z 773 ion arising from loss of 16:1-ketene is less abundant than m/z 755, arising from loss of 16:1-acid. This information leads to the assignment of the 16:1-acyl residing at either *sn*-1 or *sn*-3'. A 16:1-acyl glycerophosphate anion at m/z 407 along with m/z 389, rather than a 18:1-acyl glycerophosphate anion at m/z 453, is also observed in the spectrum. This confirms the assignment of 16:1-fatty acyl at *sn*-3', whereas the remaining 18:1-fatty acyl is located at *sn*-2.

Similar results were also observed for the $[M - H]^-$ ion at m/z 981 (Figure 7b), which comprises a 18:1-16:1/16:1-PG structure, along with a minor 14:1-18:1/18:1-PG isomer. The structure of 14:1-18:1/18:1-PG is identified by the observation of the abundances of m/z 773 < m/z 755, arising from a respective 14:1-ketene and 14:1-acid loss, along with ions at m/z 361 and 379 that signify the presence of a 14:1-acyl residing at *sn*-3'. The presence of 14:1-acyl is also consistent with the observation of a carboxylate anion at m/z 225.

Structural identification of acyl-PGs with identical fatty acyl substituents at *sn*-1 and *sn*-3' (a-b/c, where a = b) is exemplified by the product-ion spectrum of the $[M$

$- H]^-$ ion at m/z 985 (Figure 7c), which contains carboxylate anions at m/z 255 and 281, reflecting the 16:0- and 18:1-acyl constituents. The structural identification is achieved by the observation of the greater abundance of m/z 721 ($[M - H - C_{16}H_{31}CH=CO]^-$) than m/z 703 ($[M - H - C_{17}H_{33}CO_2H]^-$), signifying a 18:1-acyl residing at *sn*-2, along with the observation of the preferential formation of m/z 729 ($[M - H - C_{15}H_{31}CO_2H]^-$) over m/z 747 ($[M - H - C_{14}H_{29}CH=CO]^-$), signifying the presence of a 16:0-fatty acyl at *sn*-1 and/or *sn*-3'. Since a prominent ion at m/z 391 together with m/z 409 is also present in the spectrum, the assignment of 16:0-acyl at *sn*-3' can be easily confirmed.

The product-ion spectrum of m/z 957 (Figure 6d) contains a similar ion profile, which leads to identification of a 16:0-16:0/16:1-PG structure. However, carboxylate anions at m/z 281 and 227 were also observed in the spectrum, suggesting that another isomer comprising a 18:1- and/or 14:1-acyl moiety may also exist. This is further revealed by observation of the greater abundance of the m/z 693 ($[M - H - C_{16}H_{31}CH=CO]^-$) ion than m/z 675 ($[M - H - C_{17}H_{33}CO_2H]^-$), which arise

Figure 8. The tandem quadrupole product-ion spectra of the $[M - H]^-$ ions of (a) m/z 953, and (b) m/z 901. The molecular species consist of two (m/z 953, Panel a) and three (m/z 901, Panel b) configuration isomers, respectively.

from losses of 18:1-acyl moiety residing at *sn*-2 as a ketene and acid, respectively. The spectrum also contains ions at m/z 747 ($[M - H - C_{12}H_{25}CH=CO]^-$), which is less abundant than m/z 729 ($[M - H - C_{13}H_{27}CO_2H]^-$), indicating the presence of a 14:0 at *sn*-1 or *sn*-3'. The 14:0-acyl at *sn*-3' is confirmed by the presence of the ions at m/z 363 and 381. Therefore, a minor isomer with a 14:0-16:0/18:1-PG structure can be determined.

Structural Determination of Acyl-PG Molecular Species with Multiple Isobaric Isomers

As shown in Figure 8a, the product-ion spectrum of m/z 953 is dominated by the $R_xCO_2^-$ ions at m/z 281 (18:1), 253 (16:1), and 225 (14:1). Because the abundances of the ions are in the order: m/z 253 > m/z 225 > m/z 281, the structure can be easily assigned as 14:1-18:1/16:1-PG. This configuration is consistent with the observation of the abundances of m/z 717 ($[M - H - C_{14}H_{29}CH=CO]^-$) > m/z 699 ($[M - H - C_{15}H_{31}CO_2H]^-$); m/z 745 ($[M - H - C_{12}H_{23}CH=CO]^-$) < m/z 727 ($[M - H - C_{13}H_{25}CO_2H]^-$); m/z 689 ($[M - H - C_{16}H_{33}CH=CO]^-$) ≤ m/z 671 ($[M - H - C_{17}H_{35}CO_2H]^-$). However, the abundance of $R_2CO_2^-$ ion at m/z 253 is higher than that predicted for 14:1-18:1/16:1-PG that consists of various acyl moieties. A 16:1-acyl glycerophosphate ion at m/z

407 that signifies a 16:1-acyl at *sn*-3' is also observed in the spectrum, suggesting the presence of a 16:1-16:1/16:1-PG isomer. This is further evidenced by the rise of the m/z 717 and 699 ions, which are higher than the abundances anticipated from a 14:1-18:1/16:1-PG, along with a prominent m/z 389 ion, which arises from the 16:1-16:1/16:1-PG isomer.

Several isomers were identified for the ions at m/z 901, 955 and 971 (Table 1). The complexities of the structures of the molecules are demonstrated by the product-ion spectrum of m/z 901 (Figure 8b), which contains $R_xCO_2^-$ ions at m/z 281 (18:1), 255 (16:1), 227 (14:0), 225 (14:1), and 199 (12:0). Three sets of ions featured by abundances of $[M - H - R'_xCH=CO]^- > [M - H - R_xCO_2H]^-$, that reflects the acyl substituents at *sn*-2 position were observed at: m/z 665 ($[M - H - C_{14}H_{27}CH=CO]^-$) > m/z 647 ($[M - H - C_{15}H_{29}CO_2H]^-$); m/z 691 ($[M - H - C_{12}H_{25}CH=CO]^-$) > m/z 673 ($[M - H - C_{13}H_{27}CO_2H]^-$); and m/z 637 ($[M - H - C_{16}H_{33}CH=CO]^-$) > m/z 619 ($[M - H - C_{17}H_{35}CO_2H]^-$). These results suggest that the $[M - H]^-$ ion of m/z 901 represents three isomeric structures possessing 16:1-, 14:0-, and 18:1-acyl substituents at the *sn*-2 position, respectively. The acyl-PG with a 16:1-acyl at *sn*-2 is the major species, because the m/z 665 and 647 ion pairs are the most prominent in the series. The ion-pairs with abundances of $[M - H - R'_xCH=CO]^- < [M - H - R_xCO_2H]^-$ were observed at: m/z 719 ($[M - H - C_{10}H_{21}CH=CO]^-$) < m/z 701 ($[M - H - C_{11}H_{23}CO_2H]^-$); m/z 663 ($[M - H - C_{14}H_{29}CH=CO]^-$) < m/z 645 ($[M - H - C_{15}H_{31}CO_2H]^-$); m/z 693 ($[M - H - C_{12}H_{23}CH=CO]^-$) < m/z 675 ($[M - H - C_{13}H_{25}CO_2H]^-$). These results lead to identify the fatty acyl moieties of 12:0-, 16:0-, and 14:1 at *sn*-1 or *sn*-3'. The ion set of m/z 719 and 701 is the most prominent among the ion series, indicating that probably an acyl-PG possessing a 16:1-acyl at *sn*-2, a 12:0-acyl moiety at *sn*-1 or *sn*-3', together with a 16:0-acyl is the major species. The determination of the fatty acyl substituent at *sn*-3' is achieved by observation of the acyl glycerophosphate ions at m/z 353, 379, and 381, reflecting a 12:0-, 14:1-, and 14:0-acyl moieties, respectively. Therefore, the major species with a 12:0-16:0/16:1-PG structure can be determined. The identification of the two minor species with a respective 14:0- and 18:1-acyl at *sn*-2, along with a 14:1- or 14:0-acyl group at *sn*-3' are described below.

The sum of the number of the unsaturated bonds of the fatty acyl substituents in the molecule is one, as observed for the major 12:0-16:0/16:1-PG species. Hence, the species with a 18:1-acyl at *sn*-2 most likely contains a 14:0-acyl at *sn*-3' to constitute a 14:0-12:0/18:1-PG, while the species with a 14:0-acyl at *sn*-2 may be a 14:1-16:0/14:0-PG. This assumption is also based on the findings that the 14:0-12:0/18:1-PG is the least prominent, as observed by the abundance of the carboxylate anion at m/z 281, and the abundances of the ions at m/z 637 and 619. In contrast, carboxylate anions at m/z 225 and 227, ions at m/z 361, 363, and 379, along with the ion sets of m/z 693 and m/z 675, and of m/z 691

and m/z 673 are all in moderate abundance and are likely arising from the 14:1-16:0/14:0-PG.

Conclusions

Product-ion spectra of acyl-PG obtained with TSQ or ITMS contain rich structural information, with which structural characterization can be achieved. The product-ion spectra acquired by TSQ are dominated by carboxylate anions, whereas structurally informative fragment ions observed by ITMS are more evenly distributed. The mass spectrometric methods are applicable to acyl-PG mixtures, and structural details, including the identities and the positions of the fatty acyl substituents, can be determined. In contrast, determination of the structure of acyl-PG as described by Holmback et al. [11] required several analytical methods including NMR, LC/MS/MS, and GC/MS and structural information such as the positions of the fatty acyl moieties of complex molecules are not available. Characterization of acyl-PG molecules of biological origins is complicated by the presence of various isomers, including configuration and positional isomers. However, a complete structural elucidation can be achieved by the fragment ion information arising from the various ion series as described in this study.

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