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Charge Inversion of Phospholipids by Dimetal Complexes for Positive Ion-Mode Electrospray Ionization Mass Spectrometry Analysis

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Supporting Information

ABSTRACT: Phospholipids are vital constituents of living cells, as they are involved in signaling and membrane formation. Mass spectrometry analysis of many phospholipids is preferentially performed in the negative ion-mode because of their acidic nature. Here we have studied the potential of a digallium and dizinc complex to charge-invert a range of different types of phospholipids and measured their ion yield and fragmentation behavior in positive ion-mode tandem mass spectrometry. The dimetal complexes bind specifically the phosphate groups of phospholipids and add an excess of up to three positive charges per phosphate group. Three different phosphoinositide phosphates (mono-, di-, and triphosphorylated inositides), a phosphatidic acid, a phosphatidylcholine, a phosphatidylethanolamine, and a phosphatidylglycerol were investigated. The intensities obtained in positive ion-mode of phosphoinositide phosphates and phosphatidic acid bound to \( \{ \text{LGa}_2 \}^{5+} \) were between 2.5- and 116-fold higher than that of the unmodified lipids in the negative ion-mode. Native phosphoinositide ions yielded upon CID in the negative ion-mode predominantly product ions due to losses of \( \text{H}_3\text{PO}_4^−, \text{PO}_3^− \) and \( \text{H}_2\text{O} \). In comparison, CID spectra of \( \{ \text{LGa}_2 \}^{5+} \)-bound phosphoinositides generally resulted in fragment ions corresponding to loss of the full diglyceride chain as well as the remaining headgroup bound to \( \{ \text{LGa}_2 \}^{5+} \) as the most abundant peaks. A number of signature fragment ions of moderate abundance were observed that allowed for distinction between the three regioisomers of 1,2-di(9Z-octadecenoyl)-

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hospholipids are the major constituents of mammal, plant, and bacteria cells. Although phospholipids are known to be building blocks for membranes, they have been found to perform a variety of important functions, such as localization, cell signaling, determining protein activity, as well as being precursors for other molecules. The analysis of phospholipids has been greatly improved by the introduction of electrospray ionization mass spectrometry (ESI MS) with unprecedented sensitivity and the ability to ionize analytes without causing decomposition. ESI MS also allows for online high-performance liquid chromatography (HPLC MS) and various forms of tandem mass spectrometry (MS/MS) of isolated molecular ions to study the lipid structure. Some phospholipid classes such as phosphoinositides are very low abundant in cells and suffer from low ionization efficiencies in positive-ion mode due to their acidic functional groups. Other acidic phospholipids such as phosphatidic acid and lysophosphatidic acid can give rise to similar challenges. Chemical derivatization of lipid phosphate groups by methylation to improve positive-ion mode ionization has been demonstrated. Similar results have been obtained by ammonium adduct formation of both phosphoinositides and diacylglycerols. Improved ionization efficiency of diacylglycerols has also been achieved by esterification with \( \text{N,N}-\text{dimethylglycine} \). Additionally it has been shown that introduction of a fixed charge by methylation with diazomethane of the primary amine in phosphatidylserine, phosphatidylethanolamine, and other glycerophospholipids increases the intensity of ion signals in positive ion-mode ESI MS. And finally, derivatization of phosphatidylserine and phosphatidylethanolamine has also been demonstrated using a sulfonium reagent, \( \text{d}_{6}-\text{S,S}’-\text{dimethylthiobutanoxyhydroxysuccinimide ester} \). Here we report on a new technique of charge inversion of phospholipids to allow for analysis in positive ion-mode MS. This technique is based on the dimetallic complex \( \{ \text{LGa}_2 \}^{5+} \) (\( \text{L} = \text{2,6-bis(N,N’-bis(2-picolyl)amino)methyl}-4\text{-tertbutylphenolate} \)), which has been shown to be able to selectively recognize and bind phosphate groups in peptides with very high efficiency. The related complex \( \{ \text{LZn}_2 \}^{5+} \) has also been tested for binding phosphate diesters. The complexes are easily

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prepared as their solid perchlorate salts, \([\text{LGa}_2(\text{OH})_2(\text{H}_2\text{O})_2](\text{ClO}_4)_3\) and \([\text{LZn}_2(\text{HCOO})_2](\text{ClO}_4)\), with either labile water-derived or formate auxiliary ligands in the phosphate binding site. On dissolution in many common organic solvents (MeOH, MeCN, isopropanol, or acetone), the cations \([\text{LGa}_2(\text{OH})_2(\text{H}_2\text{O})_2]^{3+}\) and \([\text{LZn}_2(\text{HCOO})_2]^+\) form. These cations can then react with phosphate species by exchanging the labile auxiliary ligands with phosphate esters, thereby giving the negatively charged lipids a net positive charge (Figure 1).

Since fragmentation using different ion-modes and/or activation techniques is known to produce complementary information on most peptide ions, it is very attractive to investigate if similar improvement can be obtained for phospholipids. The metal conjugated lipids were analyzed in positive ion-mode MS and signal intensities were compared to the intensities obtained with unmodified phospholipids analyzed in negative ion-mode MS. Collisional induced dissociation (CID) experiments were carried out for all phospholipids with and without metal conjugation.

\section*{EXPERIMENTAL SECTION}

\textbf{Chemicals and Materials.} 1,2-Dimyristoyl-sn-glycero-3-phosphate (DMPA), 1,2-dioleoyl-sn-glycero-3-phospho-(1′-myo-inositol-4′-phosphate) (DOPI(4)P), 1,2-dihexanoyl-sn-glycero-3-phospho-(1′-myo-inositol-3′-phosphate) (DHPI(3)P), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DAPC), 1,2-di(9Z-octadecenoyl)-sn-glycero-3-[phosphoinositol-3,4-bisphosphate] (PI(3,4)P₂), 1,2-di(9Z-octadecenoyl)-sn-glycero-3-[phosphoinositol-3,5-bisphosphate] (PI(3,5)P₂), and 1,2-di(9Z-octadecenoyl)-sn-glycero-3-[phosphoinositol-4,5-bisphosphate] (PI(4,5)P₂) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). All other chemicals were purchased from Sigma and used without further purification. \([\text{LGa}_2(\text{OH})_2(\text{H}_2\text{O})_2](\text{ClO}_4)_3\) and \([\text{LZn}_2(\text{HCOO})_2](\text{ClO}_4)\) were synthesized as previously reported.\textsuperscript{25} Needles for offline ESI MS was obtained from ThermoFisher Scientific (Odense, Denmark). Offline ESI MS was done on an Orbitrap XL (ThermoFisher Scientific, USA).

\textbf{ESI MS and CID MS/MS.} Phospholipids were studied using two different solvent compositions (1:1 acetone:MeOH 95% or MeOH 95%) with addition of modifiers of either 0.1% NH₃ or 0.1% formic acid (FA). Reactions of phospholipids with dimetal complexes were done using 1 equiv of \([\text{LGa}_2(\text{OH})_2(\text{H}_2\text{O})_2](\text{ClO}_4)_3\) or \([\text{LZn}_2(\text{HCOO})_2](\text{ClO}_4)\) (final concentration 150 μM) for 1 h with \([\text{LGa}_2(\text{OH})_2(\text{H}_2\text{O})_2](\text{ClO}_4)_3\) and 3 h with \([\text{LZn}_2(\text{HCOO})_2](\text{ClO}_4)\) at rt prior to addition of modifiers. The final phospholipid concentration in MS experiments was 2 μM. All samples were prepared as triplicates and analyzed by electrospray ionization using a standard Ion Max Source (ThermoFisher Scientific, USA) in both negative and positive ion-modes. MS settings for negative ion-mode were: Source voltage = −4.0 kV, capillary voltage = −35 V, capillary temperature = 240 °C, tube lens voltage = −37 V, aux gas flow = 3, sheath gas flow = 8, FT resolution = 30,000 at m/z 400. Each mass spectrum was obtained averaging 30 acquisitions. MS settings for positive ion-mode were: Source voltage = +4.0 kV, capillary voltage = +41 V, capillary temperature = +240 °C, tube lens voltage = +37 V.

[Figure 1. Reaction of \([\text{LGa}_2(\text{OH})_2(\text{H}_2\text{O})_2](\text{ClO}_4)_3\) and \([\text{LZn}_2(\text{HCOO})_2](\text{ClO}_4)\) with phospholipids containing either phosphate mono- or diesters. Rₐ = diglyceride, Rₖ = CH₃CH₂NH₃⁺ or CH₃CH(OH)CH₃OH.]

[Figure 2. Structure of tested phospholipids and the dimetallic cationic complex core \([\text{LM}_2]^n⁺\) without auxiliary ligands.]

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=200 V, aux gas flow = 3, sheath gas flow = 8, FT resolution = 30,000 at m/z 400. Each spectrum was obtained by averaging 30 acquisitions. CID experiments were performed using offline nanoneedles. CID conditions were, negative ion-mode: source voltage = −1.5 kV, capillary voltage = −35 V, capillary temperature = 200 °C, tube lens voltage = −100 V, FT resolution = 30,000 at m/z 400. Each mass spectrum was obtained averaging 30–50 acquisitions. Positive ion-mode: source voltage = 1.1 kV, capillary voltage = 95 V, capillary temperature = 200 °C, tube lens voltage = 200 V, FT resolution = 30,000 at m/z 400. Each spectrum was obtained by averaging 30–70 acquisitions. All m/z values are quoted as their monoisotopic masses.

## RESULTS AND DISCUSSION

Three different phosphoinositide phosphates (mono-, di-, and triphosphorylated inositides), a phosphatidic acid, a phosphatidylycholine, a phosphatidylethanolamine, and a phosphatidyglycerol were investigated to determine if heptadentate phenolate-hinged dimetal complexes containing the cationic cores \{LGa\}$_{3}^{+}$ or \{LZn\}$_{3}^{+}$ can be utilized to invert the net charge of phospholipids in order to facilitate mass spectrometry studies in the positive ion-mode (Figure 2).

The selectivity of \{LGa$_{2}$(OH)$_{2}$(H$_{2}$O)$_{2}$\}(ClO$_{4}$)$_{3}$ and \{LZn$_{2}$(HCOO)$_{2}$\}(ClO$_{4}$)$_{3}$ in relation to different classes of phospholipids, with terminal phosphate groups (phosphate mono esters) or internal phosphate groups (phosphate di esters) was investigated. Due to the geometry of the binding site, the complex \{LGa$_{2}$(OH)$_{2}$(H$_{2}$O)$_{2}$\}(ClO$_{4}$)$_{3}$ exhibits a remarkable selectivity for lipids with ROPO$_{3}$ terminal phospho-head groups (phosphate monoesters). This is in line with previous observations of reactions with phosphopeptides. The complex was found to only bind lipids with ROPO$_{3}$-type head groups. Carboxylic acid head groups of fatty acids were also not bound, resulting in high selectivity. The affinity is likewise very good, showing no unreacted phospholipids present in ESI MS. The apparent association constant (K$_{a}$) for the reaction of \{LGa$_{2}$(OH)$_{2}$(H$_{2}$O)$_{2}$\}(ClO$_{4}$)$_{3}$ with Na$_{2}$HPO$_{4}$ was previously found to be (3.08 ± 0.31) × 10$^{6}$ M$^{-1}$ in 10 mM HEPES, pH 7.1.5

Since \{LGa$_{2}$(OH)$_{2}$(H$_{2}$O)$_{2}$\}(ClO$_{4}$)$_{3}$ did not react with phosphate diester groups, another complex was investigated to provide charge inversion of this kind of phospholipids. The complex \{LZn$_{2}$(HCOO)$_{2}$\}(ClO$_{4}$)$_{3}$ is less specific than the digallium complex and has been observed to bind carboxylates as well as other anions such as OH$^{-}$. If \{LZn$_{2}$(HCOO)$_{2}$\}(ClO$_{4}$)$_{3}$ mixed with equimolar amounts of ethyl- and diethylphosphate, almost exclusively signals assigned to binding of ethylphosphate were observed in ESI MS (Figure S1). This complex was found to only bind lipids with ROPO$_{3}$-type head groups. Carboxylic acid head groups of fatty acids were also not bound, resulting in high selectivity. The affinity is likewise very good, showing no unreacted phospholipids present in ESI MS. The apparent association constant (K$_{a}$) for the reaction of \{LGa$_{2}$(OH)$_{2}$(H$_{2}$O)$_{2}$\}(ClO$_{4}$)$_{3}$ with Na$_{2}$HPO$_{4}$ was previously found to be (3.08 ± 0.31) × 10$^{6}$ M$^{-1}$ in 10 mM HEPES, pH 7.1.5

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Figure 3 shows the mass spectra obtained when a solution of DOPI(4)P was obtained in negative/positive ion-mode and the comparative spectra obtained when a solution of [LGa$_{2}$(OH)$_{2}$(H$_{2}$O)$_{2}$]$_{3}^{+}$ with DOPI(4)P was used. The red peaks show the ions containing phosphoinositol. In Figure 3a, the ions [DOPI(4)P]$_{2}^{-}$ at m/z 470.2562, [DOPI(4)PH]$_{2}^{-}$ at m/z 941.5245, and [DOPI(4)PHNa]$^{-}$ at m/z 963.5010 were observed in negative ion-mode and no phospholipid was observed in positive ion-mode. When [LGa$_{2}$(OH)$_{2}$(H$_{2}$O)$_{2}$]$_{3}^{+}$ was present in the solution, the phospholipid ions formed (Figure 3b) were [LGa$_{2}$(DOPI(4)P)]$_{2}^{+}$ at m/z 824.3408 as well as several water and sodium adducts. No phospholipids were observed in negative ion-mode, indicating that all available phospholipid was bound by the cationic dimetal complex. The intensity increase when switching from negative to positive ion-mode and the isotope pattern provided by the gallium ions allows for rapid identification of peaks which contain the metal complex. On a general note, no sign of metal complex promoted hydrolysis of the phosphate was observed in any MS experiment. If dephosphorylation were to take place, it would be immediately obvious due to the appearance of an ion at m/z 402.0610 corresponding to [LGa$_{2}$(PO$_{4}$)$_{3}$]$_{3}^{+}$. Cleavage of the phosphoinositide group from the diglyceride was only observed in the case of the mono phosphorylated phosphoinositide DOPI(4)P as a peak of approximately 5% relative intensity at m/z 1045.1425 assigned to [LGa$_{3}$\{C$_{15}$H$_{25}$O$_{3}$P$_{3}$\}]$^{+}$. At this point...
it is not clear whether this cleavage is due to natural hydrolysis of the lipid or promoted by the metal complex. Figure 4 shows the intensities (summed isotope intensities) of lipids with and without \{LGa_2\}^5+ in both positive and negative ion-modes and in two different solvent systems (acetone:MeOH 1:1 95% or MeOH 95%). These solvent systems were chosen since they offer the best ability to dissolve both phospholipids and the dimetal complexes. Four classes of phospholipids which contain phosphate monoesters (phosphoinositid mono-, di-, and triphosphate and phosphatidic acid) were tested.

The intensities obtained in positive ion-mode of phosphoinositide phosphates and phosphatidic acid bound to \{LGa_2\}^5+ were between 2.5- and 116-fold higher than those for the unmodified lipids. The general observation was that the largest fold-change was obtained with 95% MeOH as solvent system. This may be caused by better dissolution of the lipids in the less polar acetone/MeOH mixed solvent system or better ionization. Additionally it was observed that the highest ionization efficiency was achieved using a combination of the dimetal complex and 0.1% FA. The gain in intensity means that less sample is needed for MS analysis and tandem MS/MS experiments can be more readily performed of low abundant phospholipids. The speciation of the phospholipid–metal complex ions is given in Table 1 along with their m/z-values (sodium/water adducts and clusters are not listed).

In contrast to the monophosphorylated phosphoinositide which binds one dimetal complex as expected, the di- and triphosphorylated phosphoinositides binds two metal complexes each. DHPI(3,4,5)P3 could not bind three gallium complexes which could be due to steric hindrance. For phosphatidic acid which has only one terminal phosphate and a lower overall negative charge than phosphoinositides \{LGa_2\}^5+ picks up a second anion to bring the charge to 2+. The second anion was either another molecule of DMPA or a residual hydroxy ion from the precursor complex. In formic acid solution formate was also observed as counteranion. As can be seen from Table 1 the reaction with \[LGa_2(OH)2(H_2O)2\]^{3+}
gives rise to a distribution of ionic species in the mass spectrum for some phospholipids. While this can complicate mass spectral interrogation obtained from more complex mixtures the unique isotopic pattern of \([LGa2]^{5+}\) offers an efficient mean of identifying any ion containing the metal complex.

**Charge Inversion of Phosphatidylethanolamine and Phosphatidylglycerol.** Three classes of phospholipids (phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylcholine) which contain phosphate diesters were tested. Figure 5 shows the intensities of phospholipids with and without \([LZn2]^{3+}\) in both positive and negative ion-modes and in two different solvent systems (acetone:MeOH 1:1 95% or MeOH 95%). Out of the three phospholipids tested, it was found that DAPC did not react with \([LZn2(HCOO)2]\)\(^{+}\), presumably due to the large and positively charged headgroup of the phosphatidylcholine lipid. The remaining two lipids are one neutral lipid (phosphatidylethanolamine) and one mono anionic (phosphatidylglycerol). In accordance with previous data, both lipids were found to ionize in the positive ion-mode without the conjugation of the dizinc complex.\(^{8,26}\) Consistently, little improvement in intensity was observed when mixing the neutral lipid with \([LZn2(HCOO)2]\)\(^{+}\), although the complex did bind to the phospholipids. This was not surprising, since the neutral lipid ionizes well in both ion-modes. However, a 5-fold gain in intensity was observed for the negatively charged phosphoglycerol, which ionizes less efficiently in positive ion-mode. In contrast to the experiments above, \([LZn2(HCOO)2]\)\(^{+}\) did not react fully with the phospholipids, even when present in 5-fold excess, as some unmodified lipid was still observed in the negative ion-mode. The speciations of the phospholipid–metal complex adduct ions are given in Table 2 along with their \(m/z\)-values (sodium/water adducts not listed).

### Table 1. Ions of Phospholipids Bound by \([LGa2]^{5+}\) Observed in Mass Spectra of Phospholipid/\([LGa2(OH)2(H2O)2]\)\(^{3+}\) Solutions

<table>
<thead>
<tr>
<th>Ions</th>
<th>(m/z)-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>([LGa2(DPPI(4)P)]^{2+})</td>
<td>824.3408</td>
</tr>
<tr>
<td>([LGa2(DHPI(3,5)P2)-2H]^{3+})</td>
<td>701.8153</td>
</tr>
<tr>
<td>([LGa2(DHPI(3,4,5)P3)]^{3+})</td>
<td>728.4670</td>
</tr>
<tr>
<td>([LGa2(DHPI(3,4,5)P3)-H]^{2+})</td>
<td>1092.2009</td>
</tr>
<tr>
<td>([LGa2(OH)(DMPA)]^{2+})</td>
<td>659.2862</td>
</tr>
<tr>
<td>(<a href="HCOO">LGa2(DMPA)</a>]</td>
<td>673.2842</td>
</tr>
<tr>
<td>(<a href="H">LGa2(DMPA)</a>]</td>
<td>946.4870</td>
</tr>
<tr>
<td>([LGa2(OH)(DMPA)]^{+})</td>
<td>1333.5725</td>
</tr>
</tbody>
</table>

Both phospholipids irrespective of their parent charge formed \(2^+\) ions with \([LZn2]^{3+}\) with no sign of hydrolysis of the phosphate groups observed. As a note, the low reaction efficiency and hence long reaction times needed for complexation with \([LZn2(HCOO)2]\)\(^{+}\) make this complex less suited for practical application in lipidomics.

### Table 2. Ions of Phospholipids Bound by \([LZn2]^{3+}\) Observed in Mass Spectra of Phospholipid/\([LZn2(HCOO)2]\)\(^{+}\) Solutions

<table>
<thead>
<tr>
<th>Ions</th>
<th>(m/z)-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>([LZn2(DPPE)-H]^{2+})</td>
<td>696.8454</td>
</tr>
<tr>
<td>([LZn2(DPPG)]^{2+})</td>
<td>712.3396</td>
</tr>
<tr>
<td>([LZn2(DPPG)-H]^{+})</td>
<td>1424.6806</td>
</tr>
</tbody>
</table>

**Comparison of Negative- and Positive-Ion Mode MS/MS of Phospholipids before and after Reaction with Dimetallic Complexes.** CID MS/MS experiments were done on phospholipid ions obtained in the negative ion-mode and compared to CID MS/MS spectra of the phospholipid–dimetal complex ions obtained in the positive ion-mode. The aim was to investigate if the phospholipids bound to dimetal complexes gave rise to fragments with complementary structural information. Distinguishing different isomers of phosphoinositides is a significant challenge due to their similar mass and fragmentation patterns\(^{14,27}\) and traditionally has been done either by radio-labeling or specialized LC-MS.\(^{28}\) No method for recognizing the different regioisomers based on MS/MS alone has yet been reported.\(^{29}\) When native phosphoinositide ions...
were fragmented by CID, the major products were due to losses of $\text{H}_3\text{PO}_4$, $\text{PO}_4^-$ and $\text{H}_2\text{O}$ (Figure 6). These losses are typical for CID fragmentation of phosphoinositides$^{30,31}$ and present one of the major challenges, as the analytical value of these losses is limited. Other structural fragments were limited to low abundance (<10% rel. int.) of one "leg" of the diglyceride tail. Furthermore, these losses were not observed for the short tail phosphoinositides (DHPi(3,5)P$_2$ and DHPi-diglyceride tail). MS/MS spectra of phosphoinositides obtained on an IRMPD instrument have been reported to produce more structurally informative fragments than those observed here for native phosphoinositide phosphates.$^{29}$

In comparison, CID spectra of phosphoinositides bound to one or more $\{\text{LGa}_2\}^{3+}$ generally showed fragment ions corresponding to loss of the full diglyceride chain as well as the remaining headgroup bound to $\{\text{LGa}_2\}^{3+}$ as the most intense peaks. Loss of the diglyceride chain as a charged species has been reported as particularly useful for quantitative measurements of phosphoinositides in lipidomics.$^{14}$

For the short tail lipids the fragments provide much more information after complexation with the digallium complex (the native lipids lose only phosphate on CID activation). The CID experiments show that complementary structural information can be gained from binding $\{\text{LGa}_2\}^{3+}$ to the di- and triphosphate phosphoinositides whereas, for monophosphate phosphoinositide and phosphatidic acid, no additional information was obtained. Phosphoglycerol and phosphoethanolamine can be ionized in both negative and positive ion-mode and fragmented in both ion-modes by CID (Figure S3a–f). Binding of $\{\text{LZn}_3\}^{3+}$ to these phospholipids did not produce complementary structural information, except it gave rise to positively charged intact diglyceride tails, which, as argued above, may hold analytical value.

**Distinguishing Phosphoinositide Bisphosphate Isomers by CID MS/MS.** To investigate if reaction with $\{\text{LGa}_2(\text{OH})_2(\text{H}_2\text{O})_2\](\text{ClO}_4)_3$ would allow the identification of different regioisomers of phosphoinositide bisphosphates, the three lipid isomers 1,2-di(9Z-octadecenoyl)-sn-glycero-3-[phosphoinositol-3,4-bisphosphate] (PI(3,4)P$_2$), 1,2-di(9Z-octadecenoyl)-sn-glycero-3-[phosphoinositol-3,5-bisphosphate] (PI(3,5)P$_2$), and 1,2-di(9Z-octadecenoyl)-sn-glycero-4,5-bisphosphate] (PI(4,5)P$_2$) were fragmented by CID in negative ion-mode. Subsequently the lipids were each reacted with 2 equiv of $[\text{LGa}_2(\text{OH})_2(\text{H}_2\text{O})_2](\text{ClO}_4)_3$ as before, and the resulting solutions were investigated in positive ion-mode. As expected, ions of the type $[\{\text{LGa}_2\}(\text{PIP}_2)]^{3+}$ (m/z 811.2594, 2.2 ppm dev.) were observed in the MS. These ions were isolated for each isomer and fragmented by CID.

Irrespective of what isomers were analyzed, the unmodified lipids analyzed in negative ion-mode gave rise in CID to one dominating fragment at m/z 941.51, corresponding to a loss of 79 Da ($\text{HPO}_4^-$). In addition, a minor ion at m/z 739.22 was observed due to loss of one of the hydrophobic legs ($\text{C}_{39}\text{H}_{71}\text{O}_4^-$). The complementary ion was observed at m/z 281.25. These losses do not give structural information about the PIP$_2$ headgroup. Absence of distinction of the PIP$_2$ isomers presents a major challenge in cell biology, since it is known that these isomers have different impacts on signaling in the cell.$^{32}$

The dimetal complex bound lipids yielded two or three major fragments and an abundant number of minor to moderate fragment ions. The most intense fragments resulted from cleavage of the C-OP bond linking the diglyceride and polar headgroup together, resulting in ions at m/z 603.5293 ($\text{C}_{39}\text{H}_{71}\text{O}_4^-$ - diglyceride tail), m/z 610.4097 ($[\{\text{LGa}_2\}_2(\text{C}_2\text{H}_4\text{O}_4\text{P}_3)]^{3+}$ - headgroup), and m/z 915.1127 ($[\{\text{LGa}_2\}_2(\text{C}_2\text{H}_6\text{O}_4\text{P}_3)]^{2+}$ - headgroup) (Figure 7d–e). While yielding more structural information than the negative ion-mode CID of the unmodified lipid, each of the three isomers shows these major fragments. Combined with several other ions which show significant variation in abundance across the three isomers, it should be possible to distinguish the isomers. Most pronounced was the observation of a unique fragment in 1,2-di(9Z-octadecenoyl)-sn-glycero-4,5-[phosphoinositol-4,5-bisphosphate] (PI(4,5)P$_2$) at m/z 1531.6688/766.3343 corre-

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**Figure 6.** Recorded fragment ions obtained for phospholipid ions and phospholipids bound to $\{\text{LGa}_2\}^{3+}$ ions and identified in MS and MS/MS. The red dotted lines show the bonds that were broken, the arrows indicate which fragment gains the charge, and the numbers represent the relative abundance of each fragment in the MS/MS spectra. RO$^-$ = L$^-$. 

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sponding to the loss of \([\text{LGa}_2(\text{PO}_4)_2\text{H}_3]^{2+}/[\text{LGa}_2(\text{PO}_4)_2\text{H}_2]^+\). These complementary ions were observed at \(m/z\) 451.0452 (2+) and at \(m/z\) 901.0856 (1+). We speculate that this loss indicates that in the PI(4,5)P2 isomer one digallium complex binds both of the terminal phosphates while the second molecule of the digallium complex is associated with the deprotonated hydroxyl groups on the sugar as well as the internal phosphate linking the sugar and the diglyceride tails, as this would explain the loss of mass equivalent to one digallium complex and two phosphate groups. In addition, the PI(4,5)P2 isomer has a number of fragments (\(m/z\) 568.3995, 769.2409) that were significantly more abundant than the similar losses in the CID spectra of the two other isomers. The criteria used to define a reliable ion signature for a PIP2 isomer was that the ion abundance should be at least 3 times more abundant than in the spectra of any of the alternative isomers. For (PI(3,4)P2) the fragment ion abundances of the complementary ions at \(m/z\) 687.5491 and 1060.6565 were also indicative for the presence of this isomer. The structures of these ions are unknown, but the isotope pattern reveals the presence of the digallium complex.

**CONCLUSION**

We have demonstrated a new technique to charge invert phosphoinositides allowing for ready MS analysis in the positive-ion mode without having to methylate the phosphate groups. It has been demonstrated that phosphoinositides and phosphatidic acid can undergo charge inversion on reaction with the dimetal complex \([\text{LGa}_2(\text{OH})_2(\text{H}_2\text{O})_2]\)(ClO4)3, allowing for analysis in positive ion-mode MS. This technique proved to be an efficient alternative to charge inversion of these species and resulted in up to a 100-fold increase in intensity relative to the unmodified phospholipids in negative ion-mode. CID experiments showed that the phosphoinositides when bound to \{LGa2\}5+ displayed fragmentation favoring cleavage of the phosphate ester bond linking the diglyceride to the headgroup, which yielded complementary (and in some instances superior) structural information to the CID obtained of unmodified phospholipids in negative ion-mode MS. Further research should reveal if reaction of phosphoinositides with...
[LGa₂(OH)₂(H₂O)₂](ClO₄)₃ can promote chromatographic separation of PIP and PIP₂ isomers. Another potential application of the metal complex would be aiding in extraction/dissolution of phosphoinositides from natural samples. Phosphoinositides are typically strongly bound to proteins in cells, which complicates their extraction. The highly positively charged metal complexes could promote dissociation of phosphoinositides from proteins by competing for their binding. This would potentially allow for avoiding the acidification step used in state-of-the-art extraction protocols which have been shown to promote acid hydrolysis. Further if the phosphoinositides are already bound to the highly positive metal complex would be aiding in separation of PIP and PIP₂ isomers. Another potential efficiency) while it was unable to react with phosphocholine, possibly due to a large and positively charged headgroup. Subsequent CID of phospholipids with [LGa₂]⁺ did not lead to any additional information, primarily because these less acidic lipids were able to ionize reasonably well in positive ion-mode, allowing for CID without reacting with the dimetal complex.

■ ASSOCIATED CONTENT

1 Supporting Information

Additional figures and tables as noted in text. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b01536.

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