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Ozonolysis of Phospholipid Double Bonds during Electrospray Ionization: A New Tool for Structure Determination

Michael C. Thomas,[†] Todd W. Mitchell,[‡] and Stephen J. Blanksby*,[†]

Departments of Chemistry and Biomedical Science, University of Wollongong, Wollongong, New South Wales 2522, Australia

Received October 5, 2005; E-mail: blanksby@uow.edu.au

Phospholipids are the key structural component of cell membranes, and recent advances in electrospray ionization mass spectrometry provide for the fast and efficient analysis of these compounds in biological extracts.[1−3] The application of electrospray ionization tandem mass spectrometry (ESI-MS/MS) to phospholipid analysis has demonstrated several key advantages over the more traditional chromatographic methods, including speed and greater structural information.[4] For example, the ESI-MS/MS spectrum of a typical phospholipid—particularly in negative ion mode—readily identifies the carbon chain length and the degree of unsaturation of each of the fatty acids esterified to the parent molecule.[5] A critical limitation of conventional ESI-MS/MS analysis, however, is the inability to uniquely identify the position of double bonds within the fatty acid chains. This is especially problematic given the importance of double bond position in determining the biological function of lipid classes.[6]

Previous attempts to identify double bond position in intact phospholipids using mass spectrometry employ either MS^3 or off-line chemical derivatization.[7−11] The former method requires specialized instrumentation and is rarely applied, while the latter methods suffer from complications inherent in sample handling prior to analysis. In this communication we outline a novel on-line approach for the identification of double bond position in intact phospholipids. In our method, the double bond(s) present in unsaturated phospholipids are cleaved by ozonolysis within the ion source of a conventional ESI mass spectrometer to give two chemically induced fragment ions that may be used to unambiguously assign the position of the double bond. This is achieved by using oxygen as the electrospray nebulizing gas in combination with high electrospray voltages to initiate the formation of an ozone-producing plasma.

The oxidation of peptides during the electrospray ionization was originally reported by Mann and co-workers,[12] whereas subsequent studies by Maleknia and Downard demonstrated that oxidation can be enhanced by using oxygen as the nebulizing gas in conjunction with increases in the voltage carried by the electrospray capillary.[13−16] In the present study the negative ion ESI-MS spectrum of a methanolic solution of 2-oleoyl-1-paltmitoyl-*sn*-glycero-3-phosphate [GPA(16:0/9*Z*-18:1)] was obtained using oxygen as a nebulizing gas at an elevated electrospray voltage of −6 kV. Under these conditions a corona discharge at the tip of the electrospray capillary is observed, and the spectrum (Figure 1a) reveals production of fragment ions at *m*/*z* 563 and 611 in addition to the deprotonated precursor, $[GPA(16:0/9Z\text{-}18:1) - H]^-$, at *m*/*z* 673. In contrast, at lower spray voltages (e.g., −4 kV, Figure 1b) or with nitrogen as the nebulizing gas (Figure 1c,d) only the $[GPA(16:0/9Z\text{-}18:1) - H]^-$ anion is observed in the mass window shown. The production of ozone in atmospheric corona discharges is well-known: lightning strikes are the most famous example of this phenomenon! It has also been shown that discharges generated under negative polarity produce significantly more ozone than an analogous corona generated using a positive potential.[17] Indeed, in our experiments a stable corona discharge could not be obtained in positive ion mode. The role of ozone in the present study was examined by utilizing an ozone generator as the nebulizer gas supply under nondischarge conditions. The spectrum obtained using the authentic ozone supply (Figure 1e) is analogous to that obtained in the oxygen discharge (Figure 1a). The former approach produced greater abundances of chemically induced fragments and could be employed in both positive and negative ion modes.

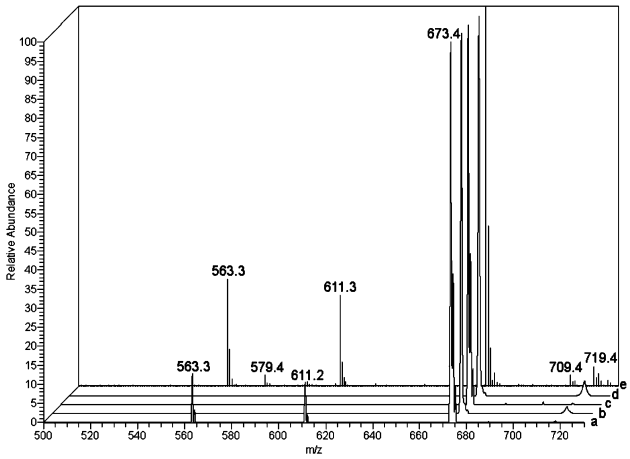

Figure 1. ESI-MS spectra of a 10 μM methanolic solution of GPA(16:0/9Z-18:1) (Avanti Polar Lipids, AL) obtained using a ThermoFinnigan LTQ ion-trap mass spectrometer at a flow rate of 10 μL min^{-1} and using (a) nebulizing gas = O_2, electrospray = −6 kV, (b) nebulizing gas = O_2, electrospray = −4 kV, (c) nebulizing gas = N_2, electrospray = −6 kV, (d) nebulizing gas = N_2, electrospray = −4 kV, (e) nebulizing gas = O_3/O_2 (produced by a NO_x analyzer, model 8840, Monitor Labs, CO), electrospray = −3 kV. Spectrum (e) was obtained using a Micromass QuattroMicro triple quadrupole mass spectrometer. Ions observed at *m*/*z* 709 and 719 are adducts of chloride and formate.

The fragment ions observed in Figure 1(a) and 1(e) can be rationalized via the reaction mechanism outlined in Scheme 1 where the products are consistent with the ozonolysis of alkenes in methanol.[18] Analogous products were also identified following the ozonolysis of phosphocholine vesicles in aqueous solution.[19] Ozonide intermediates, such as those observed by Murphy and co-workers[8,20] following ozonolysis of unsaturated phospholipids on a dry glass surface, were not detected in the present study. Their absence may be due to either the presence of solvent or solvent vapor in the ion source or simply the rapid gas-phase dissociation of the internally excited ozonide: addition of ozone to an alkene is exothermic by ca. 50 kcal mol^{-1}.[21] According to Criegee's mechanism,[22,23] dissociation of an asymmetric ozonide can occur by one of two competing dissociation channels, each of which produces a carbonyl oxide diradical and an aldehyde, but in this

[†] Department of Chemistry.
[‡] Department of Biomedical Science.

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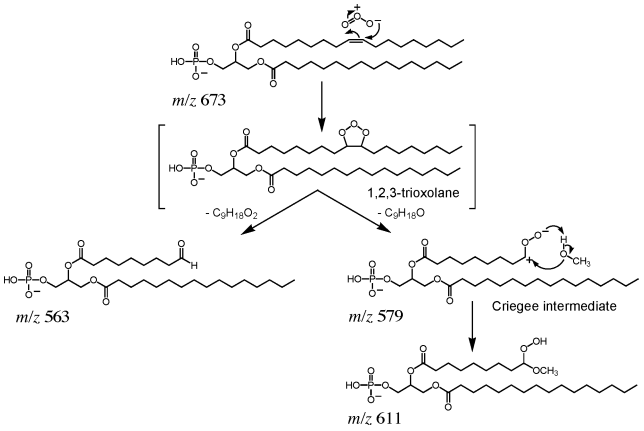
Scheme 1



instance only one species in each pair of products carries the charge and is thus observable in our experiment. While the charge-bearing aldehyde is detected at *m*/*z* 563, the reactive, charge-bearing Criegee intermediate adds methanol to form an α-methoxyhydroperoxide detected at *m*/*z* 611. The participation of the methanol was confirmed by repeating the corona discharge oxidation of [GPA(16:0/9*Z*-18:1) − H]$^-$ in both d_4-methanol and ethanol where the solvent-trapped diradicals were mass shifted by 5 and 14 Da, respectively. Interestingly, in the spectrum obtained using the ozone generator (Figure 1e) an ion is also observed at *m*/*z* 579, which may be tentatively assigned to the Criegee intermediate. Although no further structural information could be obtained, the observation of this intermediate would suggest that in-source ozonolysis is a gas-phase process.

The structure of the ozonolysis product ions was confirmed by the tandem mass spectra of source-formed *m*/*z* 563 and 611 ions obtained in an ion-trap mass spectrometer (see Supporting Information). Both spectra reveal the formation of charged fragment ions at *m*/*z* 255, indicating that the palmitic acid radyl is, as expected, unaltered by the oxidation in each case. In contrast, dissociation of *m*/*z* 563 shows a neutral loss of 172 Da and a fragment ion of *m*/*z* 171, indicative of 9-oxononanoic acid at the *sn-2* position, while *m*/*z* 611 reveals a significant water loss with subsequent loss of methanol consistent with a 9-hydroperoxy-9-methoxynonanoic acid radyl (cf. Scheme 1). Significantly, the ions at *m*/*z* 611 and 563 clearly identify the double bond position as between carbons 9 and 10, as expected for oleic acid.

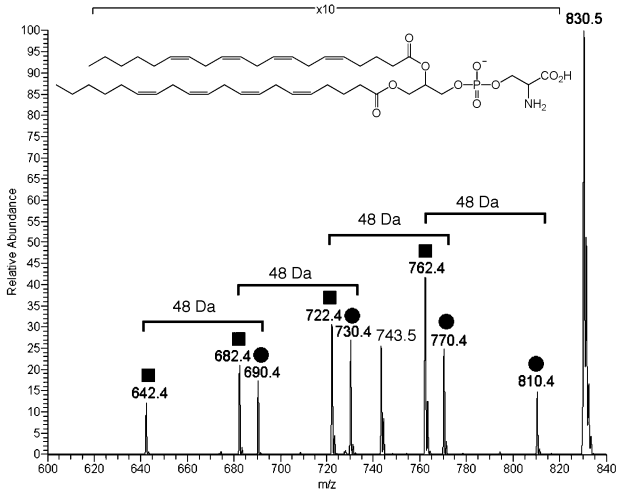


Figure 2. ESI-MS spectra of a 10 μM methanolic solution of 1,2-diarachidonoyl-*sn*-glycero-3-phosphoserine, obtained using the ion-trap mass spectrometer at a flow rate of 8 μL min^{-1} using nebulizing gas = O_2, electrospray = −5 kV. Pairs of ions corresponding to the ozonolysis at each double bond position are indicated with brackets and are separated by 48 Da (■ aldehydes, ● α-methoxyhydroperoxides). The ion observed at *m*/*z* 743.5 is due to in-source fragmentation of the serine headgroup.[2]

The example in Figure 2 shows the corresponding fragments from in-source ozonolysis of deprotonated 1,2-diarachidonoyl-*sn*-glycero-3-phosphoserine. The pairs of chemically induced fragment ions separated by 48 Da are readily identified, while the regular spacing of 40 Da between consecutive pairs can be assigned to the skip-conjugated arachidonic acid backbone with double bonds at the 5, 8, 11, and 14 positions.

We have successfully applied in-source corona discharge ozonolysis to a range of unsaturated phospholipids including GPA, GPSer, GPEtn, and cardiolipin. Although stable discharges could not be established in positive ion mode, the ozonolysis of GPCho was observed using an ozone generator. Selected examples are provided as Supporting Information. The simplicity of this method and its generality suggest that it is a significant step toward the full structural characterization of phospholipids by ESI-MS. Our current work aims to further develop gas-phase ozonolysis methods for the direct analysis of complex phospholipid mixtures. The in-source ozonolysis approach may also prove a valuable addition to the mass spectrometrist's tool-kit for general problems in structure elucidation.

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Supporting Information Available: Photographs of corona discharge; discharge ozonolysis spectra of GPA(16:0/*9Z*-18:1) in d_4-methanol and ethanol; MS/MS spectra of *m*/*z* 563 and 611 ions from Figure 1; further examples; experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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