

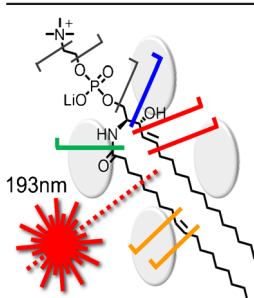
Detailed Structural Characterization of Sphingolipids via 193 nm Ultraviolet Photodissociation and Ultra High Resolution Tandem Mass Spectrometry

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Abstract. Sphingolipids serve not only as components of cellular membranes but also as bioactive mediators of numerous cellular functions. As the biological activities of these lipids are dependent on their structures, and due to the limitations of conventional ion activation methods employed during tandem mass spectrometry (MS/MS), there is a recognized need for the development of improved structure-specific methods for their comprehensive identification and characterization. Here, positive-ionization mode 193 nm ultraviolet photodissociation (UVPD)-MS/MS has been implemented for the detailed structural characterization of lipid species from a range of sphingolipid classes introduced to the mass spectrometer via electrospray ionization as their lithiated or protonated adducts. These include sphingosine

d18:1(4E), dihydrosphingosine (sphinganine) d18:0, sphingadiene d18:2(4E,11Z), the isomeric sphingolipids ceramide d18:1(4E)/18:0 and dihydroceramide d18:0/18:1(9Z), ceramide-1-phosphate d18:1(4Z)/16:0, sphingomyelin d18:1(4E)/18:1(9Z) the glycosphingolipids galactosyl ceramide d18:1(4E)/24:1(15Z) and lactosyl ceramide d18:1(4E)/24:0, and several endogenous lipids present within a porcine brain total lipid extract. In addition to the product ions formed by higher energy collision dissociation (HCD), UVPD is shown to yield a series of novel structurally diagnostic product ions resulting from cleavage of both sphingosine carbon–carbon and acyl chain carbon–carbon double bonds for direct localization of site(s) of unsaturation, as well as via diagnostic cleavages of the sphingosine backbone and N–C amide bond linkages. With activation timescales and dissociation efficiencies similar to those found in conventional MS/MS strategies, this approach is therefore a promising new tool in the arsenal of ion activation techniques toward providing complete structural elucidation in automated, high-throughput lipid analysis workflows.

Keywords: Lipidomics, Photodissociation, Unsaturation, High resolution mass spectrometry, Sphingolipid

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Introduction

Sphingolipids represent one of the eight major structurally distinct categories of lipids [1]. Sphingolipids are ubiquitous in all eukaryotic cells and play important structural roles in regulation of the fluidity and subdomain structure of lipid bilayers [2], and as bioactive regulators of cellular proliferation,

differentiation, cell migration, cell signalling, autophagy, and cell death [3]. The involvement of sphingolipids in a diverse range of cellular processes has implications for the understanding of cancer biology, arthritis, inflammation, diabetes, immune function, and neurodegenerative disorders [3–5]. Not surprisingly, therefore, there is a recognised need for effective methods that enable qualitative and quantitative profiling of the diverse range of sphingolipids that may be present within complex lipid matrices, i.e., ‘sphingolipidomics’, under different physiological or pathological conditions. Importantly, the physiochemical properties and biological functions of sphingolipids are dependent on their individual structures, whereby seemingly simple alterations involving the absence,

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presence, or site-specific location of a double bond within the sphingoid backbone or acyl chain components can have a profound impact [6]. Thus, sphingolipidomic studies that aim to comprehensively determine the functional role of sphingolipids must endeavour to ultimately characterize the entire ensemble of ‘structurally defined molecular lipid’ [7] species that are present, including (1) the identity and chain length of the sphingoid base, i.e., sphingosine versus dihydrosphingosine (sphinganine), (2) the chain length of any amide-linked acyl groups, (3) the number, locations, and diastereomeric configurations of any sites of unsaturation within the acyl and/or sphingoid chains, and (4) the identity of any head groups that are present [8].

Electrospray ionization-mass spectrometry (ESI-MS), coupled with tandem mass spectrometry (MS/MS) for structural elucidation, has emerged as the key enabling platform technology for lipidomics [9–11]. However, the sphingolipidome can potentially be comprised of tens of thousands of discrete molecular species, many of which are isomeric, thereby making their comprehensive identification and characterization a significant analytical challenge [8, 12]. Conventional MS/MS-based structural elucidation methods employing low-energy collision-induced dissociation (CID) or higher collision-energy dissociation (HCD) can provide information about the overall length and total number of double bonds that comprise the sphingoid base and the amide-linked acyl chain, as well as the identity of the head group [10–14]. However, molecular species-specific information to confirm the identity of the sphingoid base (i.e., sphingosine versus an unsaturated dihydrosphingosine), and the site-specific locations and diastereomeric configurations of any sites of unsaturation within the sphingoid base or amide-linked acyl chains, are typically not obtained. Indeed, this is a general limitation for all classes of lipids when using low energy collisional activation MS/MS methods.

Several strategies have been developed and applied to overcome some of these deficiencies. These include the use of multistage tandem mass spectrometry (i.e., CID-MS³) on the lithiated adducts of unsaturated glycerophospholipids and triacylglyceride ions [15, 16], or by performing low energy CID-MS/MS on unsaturated fatty acid ions subjected to ‘charge switch’ derivatization [17], that each yield product ions indicative of the location of C=C double bonds within these species. More recently, the initial products formed by photochemical Paternò-Büchi reaction occurring within a nanoelectrospray ionization (nanoESI) plume (i.e., a [2 + 2] cycloaddition reaction that results in highly specific modification of the C=C double bonds within unsaturated lipids, where acetone is used as the reagent under 254-nm UV irradiation conditions) have been shown to subsequently dissociate under low energy CID-MS/MS conditions to yield abundant carbon-carbon double bond specific product ions for locating C=C isomers within unsaturated fatty acids and glycerophospholipid species [18]. This approach has subsequently been applied to reveal significant changes in the ratios of these isomers between normal and cancerous tissues [19]. Similarly, in a technique termed radical-

directed dissociation (RDD), radical product ions are formed by 266 nm irradiation of a mass selected unsaturated lipid precursor ion complexed covalently or noncovalently with a molecule containing a photocaged radical initiator, via selective cleavage of a carbon-iodine bond. Upon further mass selection, these products undergo subsequent low energy CID-MS/MS to yield characteristic fragments that are diagnostic for the double bond position and the positions of chain-branching in glycerophospholipids, sphingomyelins, and triacylglycerols [20, 21]. This approach has also been recently applied to the differentiation of glucosyl versus galactosyl epimers of various glycosphingolipid ions [22].

The elucidation of double bond positions in unsaturated lipids can also be achieved directly via a gas-phase ion-molecule reaction between mass selected unsaturated lipid precursor ions and ozone (i.e., the well-established ozone-induced dissociation (OzID) technique) [23, 24], the unstable initial product of which decomposes to yield a pair of product ions that are diagnostic for the location of each carbon-carbon double bond that is present within the molecule. Importantly, by performing sequential CID and OzID, whereby the initial product ions arising from CID are isolated and then subjected to ozonolysis, additional information regarding the *sn*-positions of acyl substituents on the glycerol backbone of phospholipid ions can also be obtained [25].

As an alternative to collisional activation, electron-induced dissociation (EID) [26] and electron impact excitation of ions from organics (EIEIO) [27] have both been recently applied toward the identification of lipid class, and localization of acyl chains and double-bond positions within glycerophosphatidylcholine lipids, as well as for sphingolipid molecular species [28]. Finally, Penning ionization of the singly charged precursor ions of phospholipid cations to their respective radical dications, using He-metastable atom-activated dissociation (He-MAD), has been reported to induce fragmentations consistent with those formed via high-energy collision induced dissociation, including product ions corresponding to cleavage at or near the double bond position [29].

Each of the techniques described above have its own strength, but depending on the technique can potentially be limited by its necessity for specialized instrumentation, added mixture complexity due to incomplete reaction of non-mass selected precursor ions, or relatively poor signal-to-noise levels within the product ion spectrum due to limited dissociation efficiencies or ‘dilution’ of the spectrum when the double bond is localized by the absence of a fragmentation within a series of nonspecific fragmentations along the acyl chain.

Over the past decade, there has been increasing interest in the potential for ultraviolet photodissociation (UVPD) as an ion activation method that is independent or complementary to CID for direct dissociation, characterization, and isomer differentiation of a range of biomolecular species, including peptides, proteins, and lipids [30]. To date, 157 nm [31] and 193 nm [32–34] wavelengths have been reported for UVPD-MS/MS structural characterization of lipids. For example, it was demonstrated that isomeric leukotrienes i.e., bioactive inflammatory lipid mediators produced by the enzymatic

oxidation of arachidonic acid (AA) and eicosapentaenoic acid (EPA), can be differentiated by using 157 nm UVPD, which results in the formation of specific diagnostic radical ions [31]. Brodbelt and colleagues have demonstrated the benefits of negative ionization mode 193 nm UVPD-MS/MS for the characterization of lipid A (typically composed of a bisphosphorylated diglucosamine with a variable number of amide and ester-linked fatty acid chains) structures, including assignment of acyl chain C–O, C–N, and C–C bond cleavages and glycosidic C–O and cross-ring cleavages [32–34], as well as other rough-type lipopolysaccharides [35]. O'Brien and Brodbelt also reported the novel use of 193 nm UVPD for the improved structural characterization of various glycosphingolipids and gangliosides in negative-ionization mode, including the observation of several types of diagnostic fragment ions, including glycosidic cross-ring cleavages and C–C and C–N bond cleavages at the sphingosine and fatty acid moieties, which are not observed using conventional collisional activation MS/MS methods [36].

To date, there has been only one report of 193 nm UVPD-MS/MS for lipid structural analysis in positive-ionization mode [37]. In this recent report, Klein and Brodbelt demonstrated that UVPD of protonated glycerophosphatidylcholine (PC) lipid ions resulted in cleavage of the carbon–carbon bonds adjacent to double bonds located within their acyl chains, providing a diagnostic mass difference of 24 Da, and thereby enabling localization of the double bond positions and differentiation of double-bond positional isomers. Herein, to further explore the photodissociation behavior of lipid ions in the gas phase, we report the first study of positive-ionization mode 193 nm UVPD-MS/MS combined with ultra-high resolution mass spectrometry for the structural characterization of several major

classes of sphingolipids (Scheme 1), including sphingosine, dihydrosphingosine (sphinganine), sphingadiene, ceramide, ceramide-1-phosphate, sphingomyelin, galactosyl ceramide, and lactosyl ceramide, as well as several endogenous sphingolipids present within a total lipid extract of porcine brain.

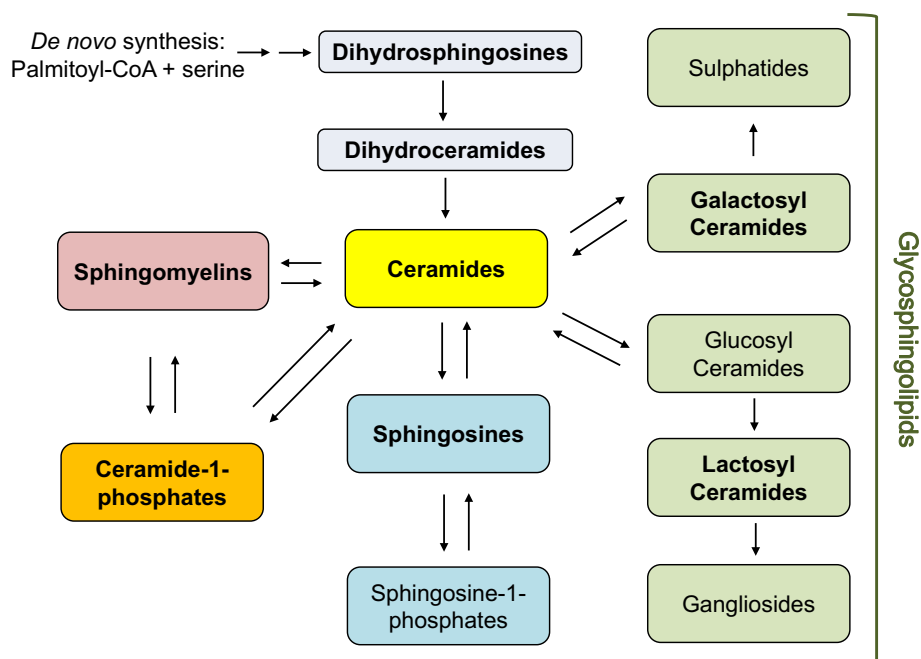
Experimental

Materials and Methods

Sphingosine d18:1(4E), dihydrosphingosine d18:0, sphingadiene d18:2(4E,11Z), ceramide d18:1(4E)/18:0, dihydroceramide d18:0/18:1(9Z), ceramide-1-phosphate d18:1(4E)/16:0, sphingomyelin d18:1(4E)/18:1(9Z), galactosyl ceramide d18:1(4E)/24:1(15Z), lactosyl ceramide d18:1(4E)/24:0, and porcine brain total lipid extract were purchased as powders from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Isopropanol, methanol, and chloroform (high performance liquid chromatography grade) were from Fisher Scientific (Waltham, MA, USA). Lithium acetate was from Sigma Aldrich (St. Louis, MO, USA).

Sample Preparation

Solutions of individual lipid standards (10 μ M) were prepared by placing 10 μ L of 100 μ M individual lipid standards dissolved in chloroform into the wells of an Eppendorf twin-tec 96-well PCR plate, dried down under nitrogen, then resuspended in 100 μ L isopropanol:methanol:chloroform (4:2:1, v:v:v) containing 2 mM lithium acetate (i.e., a final concentration of 10 μ M). The porcine brain total lipid extract was prepared at a



Scheme 1. Overview of the major lipid classes involved in sphingolipid metabolism. Representative lipid species from sphingolipid classes or subclasses labeled in bold text were examined in this study

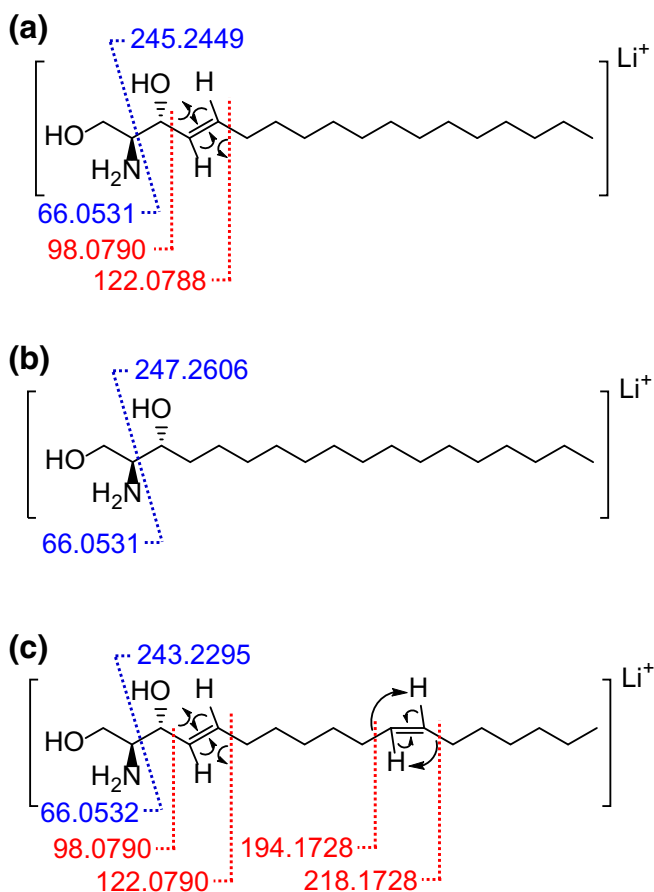
2 eV. For HCD-MS/MS, the collision energies were individually optimized to provide similar dissociation efficiencies to those observed for each UVPD experiment. Spectra shown are the average of 100 scans in order to provide high signal-to-noise ratios for the initial structural assignments. However, the average of only 3–5 scans was found to provide sufficient signal-to-noise for practical applications.

Results and Discussion

193 nm UVPD- and HCD-MS/MS of Sphingoid Bases

Sphingoid bases or long-chain bases (LCB) are the fundamental building blocks of all sphingolipids, consisting of 2-amino-1,3-dihydroxy-alkanes or alkenes with variable alkyl chain lengths. Over 60 different species of LCBs have been reported, with alkyl chain lengths containing 14 to 22 carbons, and with the possibility of containing double bonds in addition to that at the C4 position within sphingosine lipids [12, 39, 40]. However, in mammals, the main sphingoid bases are the C18 sphingosine and dihydrosphingosine (sphinganine) derivatives (Scheme 1).

Initially, to compare the photodissociation and collisional activation fragmentation behavior of these simplest of sphingolipids, 193 nm UVPD and HCD-MS/MS spectra were acquired for the $[M + Li]^+$ adducts of sphingosine d18:1(4E) (m/z 306.2976) (Figure 1a and b, respectively), and dihydrosphingosine d18:0 (m/z 308.3130) (Supplementary Figure S1a and S1b, respectively). For the sphingosine ion, UVPD was observed to generally yield the same products as HCD, i.e., cleavage of the alkyl chain at the C2–C3 position to form m/z 245.2499 and m/z 66.0531 – see Scheme 2a, as well as losses of various low molecular weight structurally uninformative neutrals [e.g., NH_3 , H_2O , and $-(NH_3 + H_2O)$], albeit with different ratios. However, several unique UVPD-MS/MS product ions were also observed. For example, the abundant ion at m/z 96.0634, assigned as $C_3H_7NO_2Li^+$, is proposed to be formed via a cis1,2 elimination reaction at the C3–C4 position of the alkyl chain (Supplementary Scheme S1). Notably, a pair of unique product ions were also observed at m/z 98.0790 and 122.0788 (losses of $C_{15}H_{28}$ and $C_{13}H_{28}$, respectively) (Δ 23.9998 Da). These ions were assigned as resulting from cis1,2 elimination at the C3–C4 and C5–C6 positions, respectively (i.e., either side of the sphingosine C4–C5 double bond), from the photoexcited species resulting from absorption of 193 nm photons into the sphingosine double bond (Scheme 2a). These ions, analogous to those recently reported by Klein and Brodbelt [37] for assigning the location of double bonds within the acyl chains of protonated PC lipids, therefore provide confirmation of the position of the sphingosine double bond and allow this lipid to be readily distinguished from possible isomeric unsaturated dihydrosphingosine species (i.e., where the double bond would be located at other sites along the alkyl chain). Further evidence for this photo absorption-induced double bond selective fragmentation behavior was obtained by examination of the spectra obtained from UVPD of the saturated sphingoid base, dihydrosphingosine



Scheme 2. Summary of the 193 nm UVPD-MS/MS dissociation behavior of the $[M + Li]^+$ precursor ions of (a) sphingosine d18:1(4E), (b) dihydrosphingosine d18:0, and (c) sphingadiene d18:2(4E,11Z). Structurally diagnostic UVPD specific product ions corresponding to cleavage of the sphingosine C=C (panels a and c) and acyl chain C=C (panel c) double bonds are indicated using red text, whereas structurally diagnostic sphingosine backbone cleavage product ions common to both HCD and UVPD are indicated in blue text. Arrows show the proposed mechanisms for formation of the sphingosine C=C and alkyl chain C=C double bond specific product ions

d18:0 (Supplementary Figure S1a and Scheme 2b). In this spectrum, essentially no product ions were observed, presumably due to the low molar absorptivity of the saturated lipid [41, 42].

UVPD- and HCD-MS/MS spectra acquired for the $[M + Li]^+$ precursor ion of sphingadiene d18:1(4E,11Z), containing C=C double bonds at both the C4 and C11 positions along the sphingoid base alkyl chain, are shown in Figure 2a and b, respectively. Similar to that described above for sphingosine, UVPD- yielded a pair of unique product ions differing by 24.0000 mass units (m/z 98.0790 and 122.0790), thereby confirming the presence of the sphingosine C4–C5 double bond (Scheme 2c). In addition, a second pair of product ions differing by 24.0000 mass units was also observed at m/z 194.1728 and 218.1728, corresponding to loss of C_8H_{14} and C_8H_{14} via cleavage of the C10–C11 and C12–C13 bonds, respectively, along the sphingoid chain. These ions therefore confirm the location of the C11–C12 unsaturated second

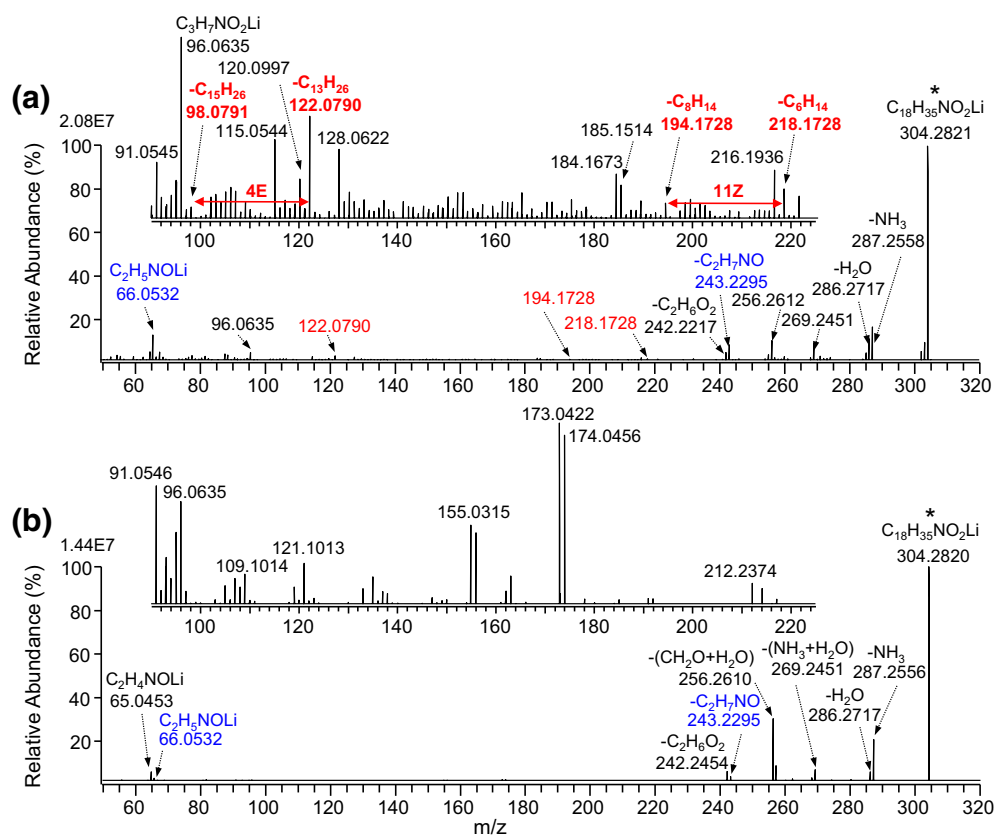


Figure 2. (a) 193nm UVPD- and (b) HCD-MS/MS of the $[M + Li]^+$ precursor ion of sphingadiene d18:2(4E,11Z). Structurally diagnostic UVPD specific product ions corresponding to cleavage of the sphingosine C=C and alkyl chain C=C double bonds (panel a) are indicated using red text. Structurally diagnostic sphingosine backbone product ions common to both HCD and UVPD are indicated in blue text. See Scheme 2c for structural information

double bond within the sphingadiene d18:1(4E,11Z) alkyl chain (Scheme 2c), similar to that reported by Klein and Brodbelt for unsaturated PC lipids [37], and demonstrates the ability of the UVPD technique to simultaneously localize multiple double bonds within a single alkyl chain.

Finally, similar information to that described above was also provided by UVPD- and HCD-MS/MS of the protonated (i.e., $[M + H]^+$) precursor ion of sphingadiene d18:1(4E,11Z) (Supplementary Figure S2a and S2b, respectively, and Supplementary Scheme 2). However, presumably due to the more limited ‘mobility’ of the Li adduct compared with a proton, we have observed that Li adduction generally promotes more effective charge remote fragmentation upon 193nm UVPD-MS/MS, resulting in the formation of more abundant double bond diagnostic product ions (e.g., compare the abundance of the sphingosine C4–C5 double bond diagnostic product ions at m/z 98.0791 and 122.0790 in Figure 2a with m/z 92.0711 and 116.0710 in Supplementary Figure S2a).

193 nm UVPD- and HCD-MS/MS of Ceramide Lipids

Ceramides consist of a fatty acyl chain of variable length linked via an amide bond to a sphingoid base (typically sphingosine or dihydrosphingosine). The amide-linked acyl chains are

generally saturated or monounsaturated in mammalian cells, with chain lengths containing 16 to 24 carbons [43]. To further examine the 193 nm UVPD induced formation of sphingosine C4–C5 double bond specific product ions in a more complex sphingolipid structure, UVPD-MS/MS was performed on the $[M + Li]^+$ precursor ion of ceramide d18:1(4E)/18:0, containing a sphingosine base and a saturated C18 amide-linked acyl chain, and is shown in Figure 3a. The corresponding HCD-MS/MS spectrum is shown in Supplementary Figure S3a. In both the UVPD- and HCD-MS/MS spectra, structurally diagnostic product ions are observed at m/z 245.2448 and 332.3129, corresponding to cleavage of the sphingosine backbone (Scheme 3a), along with various structurally uninformative product ions formed via low molecular weight neutral losses. However, similar to that observed for the sphingosine and sphingadiene lipids in Figures 1a and 2a, a unique pair of diagnostic product ions at m/z 364.3391 and 388.3390 were observed in the UVPD spectrum, confirming the presence of the sphingosine C4–C5 double bond, while a second unique UVPD induced product ion at m/z 304.2818 (formed via cleavage of the amide bond, Scheme 3a) provided confirmation of the identity of the C18:0 saturated amide-linked acyl chain. UVPD- and HCD-MS/MS of the protonated (i.e., $[M + H]^+$) precursor ion of ceramide d18:0/18:1(9Z) (Supplementary Figure S4a and S4b, respectively, and Supplementary Scheme 3)

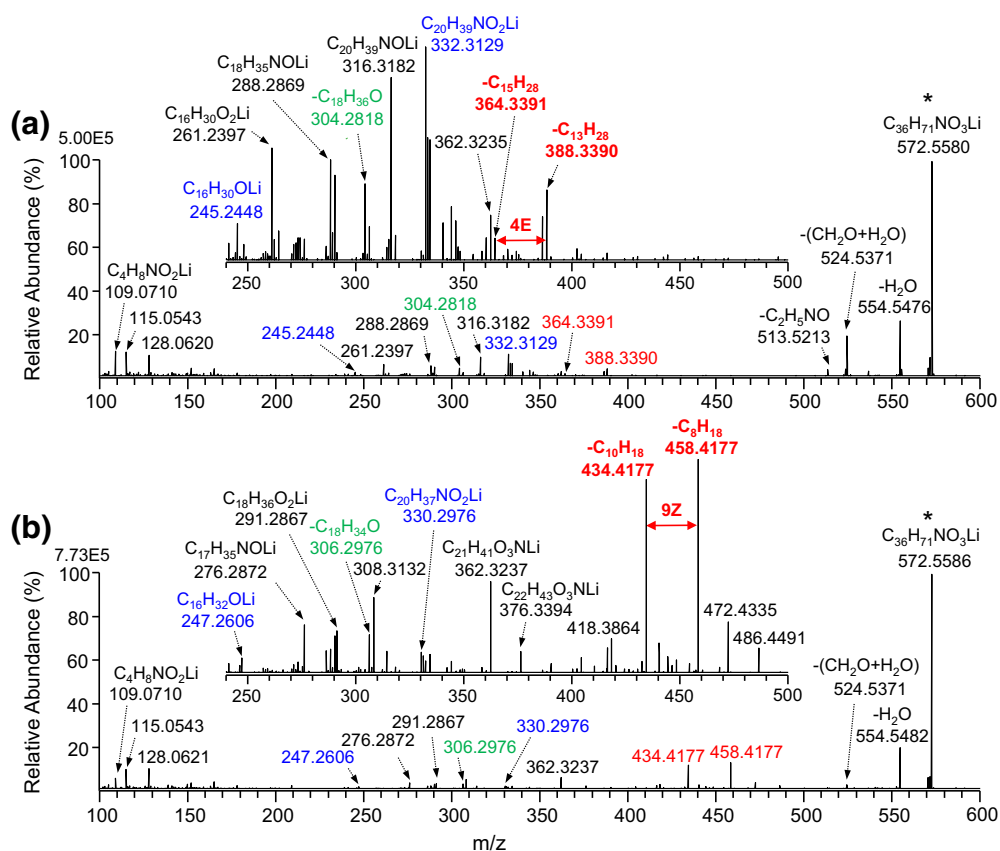


Figure 3. 193 nm UVPD-MS/MS of the $[M + Li]^+$ precursor ions of the isomeric lipid species (a) ceramide d18:1(4E)/18:0 and (b) dihydroceramide d18:0/18:1(9Z). Structurally diagnostic UVPD specific product ions corresponding to cleavage of the sphingosine C=C (panel a) or acyl chain C=C (panel b) double bonds are indicated using red text, whereas those corresponding to cleavage of the N–C amide bond are indicated using green text (panels a and b). Structurally diagnostic sphingosine backbone product ions common to both HCD and UVPD are indicated in blue text. See Scheme 3 for structural information

was observed to fragment more efficiently compared with the lithiated adduct described above. However, the resultant UVPD fragmentation behavior was found to be more similar to those formed by collisional activation (i.e., HCD) [10], and the desired diagnostic fragments were generally found to be lower in relative abundance.

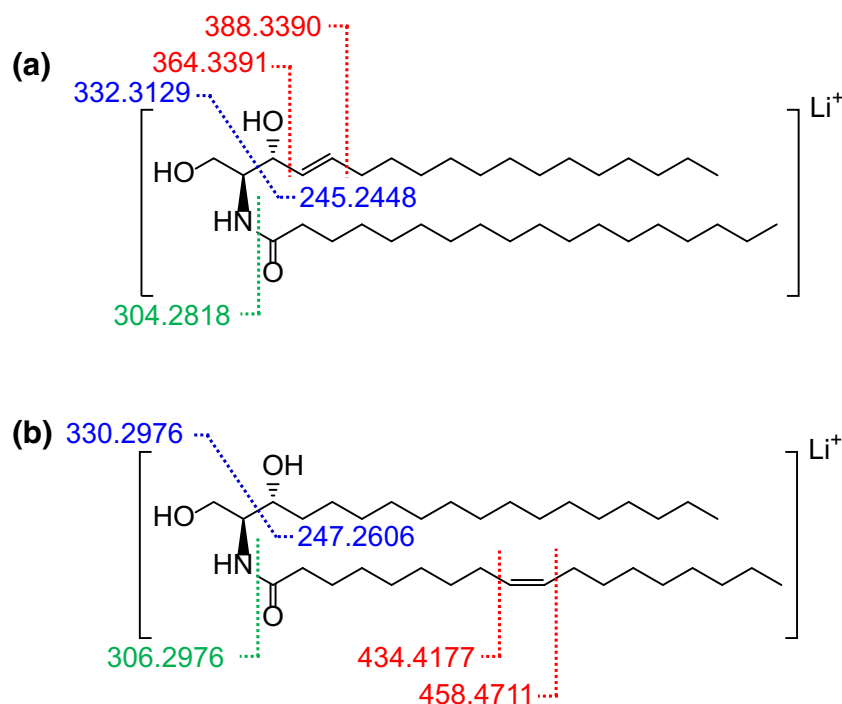
The UVPD-MS/MS spectrum for the $[M + Li]^+$ precursor ion of ceramide d18:0/18:1(9Z) containing a dihydro sphingosine base and an unsaturated fatty acid, i.e., a structural isomer of the ceramide d18:1(4E)/18:0 lipid in Figure 3a, is shown in Figure 3b. The corresponding HCD-MS/MS spectrum is shown in Supplementary Figure S3b. Analogous to the fragmentation behavior described above for the sphingosine containing ceramide, UVPD- and HCD-MS/MS of the dihydro sphingosine containing ceramide d18:0/18:1(9Z) lipid resulted in the observation of characteristic product ions indicative of the dihydro sphingosine backbone (m/z 247.2606 and 330.2976, via cleavage at the C2–C3 position of the sphingoid base, Scheme 3b). The UVPD spectrum also contained a unique diagnostic amide bond cleavage product at m/z 306.2976, analogous to that observed in Figure 3a, confirming the presence of the monounsaturated C18:1 amide linked acyl chain. Notably, similar to the diagnostic ions described above for the sphingadiene C11–C12 double bond in Figure 2a above, and reported by Klein and

Brodbeck for unsaturated PC lipids [37], a unique pair of abundant product ions were also observed at m/z 434.4177 and 458.4177, corresponding to the loss of $C_{10}H_{18}$ and C_8H_{18} via cleavage of the C8–C9 and C10–C11 bonds along the fatty acyl chain, respectively. These ions therefore confirm the location of the C9–C10 unsaturated double bond within the C18:1 amide linked acyl chain (Scheme 3b).

Collectively, therefore, positive ionization mode 193 nm UVPD-MS/MS of the $[M + Li]^+$ ions readily enables the differentiation and ‘near-complete’ structural characterization of these two structural isomeric species (note that the differentiation of *cis/trans* stereoisomers in both the C4 or C10 double bonds of these lipids is not possible using UVPD due to immediate photoisomerization of the double bond upon photon absorption, consistent with that previously described for the photochemistry of alkenes in solution – data not shown) [44].

193 nm UVPD- and HCD-MS/MS of Complex Sphingolipids

Ceramide-1-Phosphate Having established the improved dissociation behavior of 193 nm UVPD compared with HCD for simple sphingolipids, we next examined the effect of adding



Scheme 3. Summary of the 193 nm UVPD-MS/MS dissociation behavior of the $[M + Li]^+$ precursor ions of the isomeric lipid species (a) ceramide d18:1(4E)/18:0 and (b) dihydroceramide d18:0/18:1(9Z). Structurally diagnostic UVPD specific product ions corresponding to cleavage of the sphingosine C=C (panel a) or acyl chain C=C (panel b) double bonds are indicated using red text, whereas those corresponding to cleavage of the N-C amide bond are indicated using green text (panels a and b). Structurally diagnostic sphingosine backbone cleavage product ions common to both HCD and UVPD are indicated in blue text

various head groups to ceramide (i.e., complex sphingolipid structures) on formation of the structurally diagnostic product ions described above. The simplest of the complex sphingolipids is ceramide-1-phosphate. Ceramide-1-phosphate is a bioactive sphingolipid synthesized intracellularly from direct phosphorylation of ceramide by ceramide kinase, and has been reported to be an important mediator of cell growth, inflammatory response, anti-apoptotic signalling, and cell migration [45]. As a representative example of this class of complex sphingolipid, UVPD- and HCD-MS/MS spectra were acquired for the $[M - H + 2Li]^+$ adduct of ceramide-1-phosphate d18:1(4E)/16:0 (Figure 4a and b, respectively). Similar to that described above for the simple sphingolipids, in addition to the relatively structurally uninformative product ions observed by HCD involving dominant losses of the phosphate head group, UVPD also yielded an array of product ions that readily allowed confirmation of the sphingosine d18:1 backbone identity (m/z 390.2552) and the location of its C4-C5 double bond (m/z 422.2815 and 426.2812) (Scheme 4). For this lipid, the amide bond cleavage product ion corresponding to loss of the C16:0 saturated fatty acid chain (m/z 390.2567, $-C_{16}H_{32}O$) was isomeric with the C2-C3 sphingosine cleavage, so could not be independently confirmed.

Sphingomyelin Sphingomyelin, containing a phosphocholine head group attached to the 1-hydroxy group of a ceramide lipid, is the dominant class of sphingolipid enriched in the outer

leaflet of plasma membranes within mammalian cells. In addition to being a major source of ceramides and other bioactive sphingolipids via the action of sphingomyelinases, sphingomyelins are also involved in various cellular functions, including regulation of the activity of a number of membrane-bound proteins, and have been reported to be an independent risk factor in the development of cardiovascular disease [4, 6]. To examine the fragmentation of this class of sphingolipid, UVPD- and HCD-MS/MS spectra were acquired for the $[M + Li]^+$ precursor ion of sphingomyelin d18:1(4E)/18:1(9Z) (Scheme 5), containing a C18 sphingosine base and a mono-unsaturated C18:1 amide linked acyl chain (Figure 5a and b, respectively). Similar to sphingosine (Figure 2a), the appearance of the diagnostic UVPD ion at m/z 495.3542, and the pair of ions at m/z 527.3804 and 551.3806, confirm the presence and identity of the sphingosine backbone, whereas the identity and structure of the C18:1 acyl chain is confirmed by the observation of the amide bond cleavage product ion at m/z 496.3386, and the pair of diagnostic double bond product ions at m/z 597.4593 and 621.4590 (Scheme 4). Thus, unlike HCD, 193 nm UVPD can be employed for the detailed structural elucidation of complex sphingolipid structures in positive ionization mode, including those containing multiple sites of unsaturation in different alkyl chains.

Glycosphingolipids Glycosphingolipids are the most structurally diverse class of complex sphingolipids, containing one

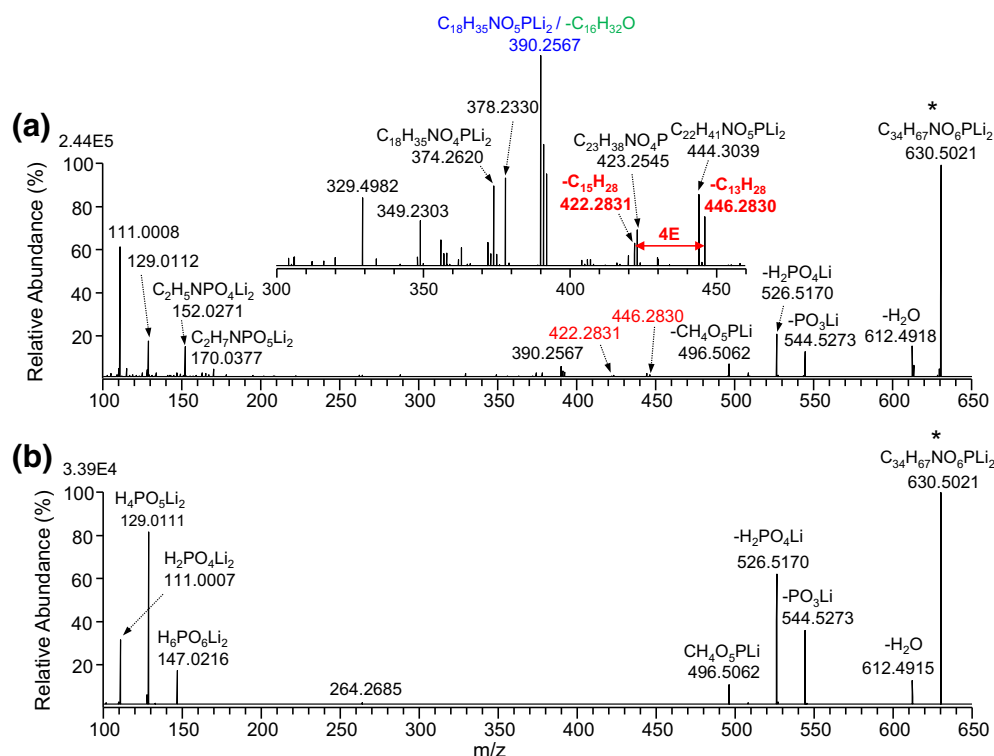
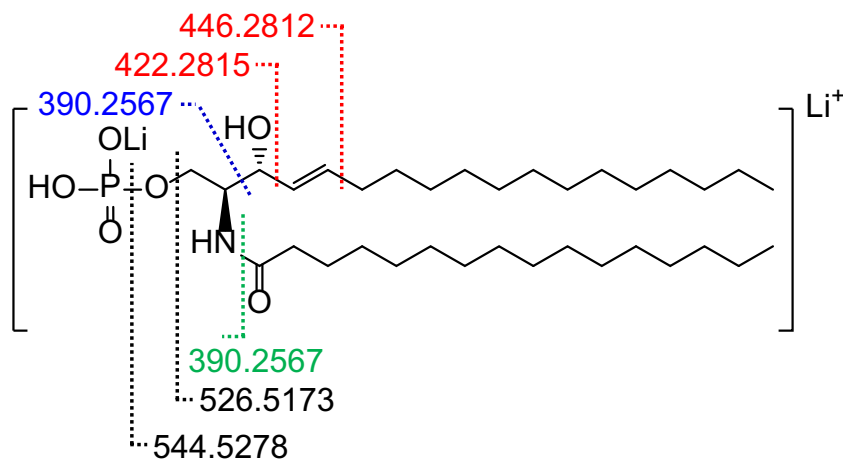


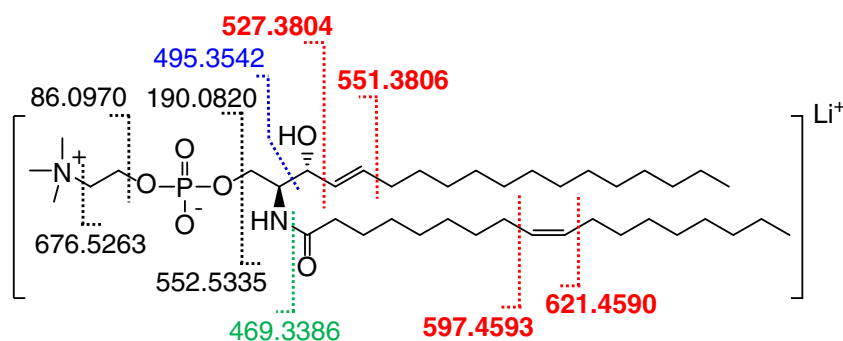
Figure 4. (a) 193 nm UVPD- and (b) HCD-MS/MS of the $[M - H + 2Li]^+$ precursor ion of ceramide 1-phosphate d18:1(4E)/16:0. Structurally diagnostic UVPD specific product ions corresponding to cleavage of the sphingosine C=C double bond are indicated using red text, whereas those corresponding to cleavage of the N-C amide bond or sphingosine backbone are indicated using green and blue text, respectively. See Scheme 4 for structural information

(e.g., glucosyl- and galactosyl-ceramides, and sulfatides) or more (e.g., lactosyl ceramides and gangliosides) sugar residues linked to ceramide via a β -glycosidic linkage [46, 47]. Glycosphingolipids are involved in the mediation of biological functions as diverse as proliferation, differentiation, and apoptosis, and as specific membrane receptors of cellular recognition, adhesion, cell-cell communication, and membrane microdomain-mediated signal transduction [47–50].

As an example of the utility of 193 nm UVPD for glycosphingolipid characterization, the product ion spectra acquired for the $[M + Li]^+$ adduct of galactosylceramide d18:1(4E)/24:1(15Z) is shown in Figure 6a (also see Scheme 6a). Despite the added head group complexity of this lipid, diagnostic sphingosine and fatty acyl C=C double bond location specific products (i.e., m/z 632.4704 and 608.4704, and m/z 702.5487 and 678.5487, respectively) as well as sphingosine and amide bond-specific cleavages



Scheme 4. Summary of the 193 nm UVPD-MS/MS dissociation behavior of the $[M - H + 2Li]^+$ precursor ion of ceramide 1-phosphate d18:1(4E)/16:0. Structurally diagnostic UVPD specific product ions corresponding to cleavage of the sphingosine C=C double bond are indicated using red text, whereas those corresponding to cleavage of the N-C amide bond or sphingosine backbone are indicated using green and blue text, respectively



Scheme 5. Summary of the 193 nm UVPD-MS/MS dissociation behavior of the $[M + Li]^+$ precursor ions of sphingomyelin d18:1(4E)/18:1(9Z). Structurally diagnostic UVPD specific product ions corresponding to cleavage of the sphingosine C=C and acyl chain C=C double bonds are indicated using red text, whereas those corresponding to cleavage of the N-C amide bond and sphingosine backbone are indicated using green and blue text, respectively

(m/z 576.4440 and 466.3346, respectively) were each observed. In addition, a series of galactose head group cleavage products were also observed, similar to those formed by HCD (Figure 6b) and CID (previously reported by Hsu and Turk [13]), thereby enabling the near complete structural characterization of this lipid. Although the 193 nm UVPD product ion spectrum of the glucosylceramide d18:1(4E)/24:1(15Z) epimer was found to be essentially identical to the galactosylceramide d18:1(4E)/24:1(15Z) lipid (data not shown), selective phenyl boronic acid complexation of the galactosylceramide *cis*1,2 diol moiety could potentially be used to differentiate between these epimers prior to UVPD, analogous to that described by Pham and Julian [22].

Finally, UVPD- and HCD-MS/MS spectra for the $[M + Li]^+$ adduct of another glycosphingolipid, lactosylceramide d18:1(4E)/24:0, were also acquired (see Figure 7a and b, respectively) (also see Scheme 6b), while the HCD spectrum was dominated by glycosidic cleavages similar to those previously reported for CID by Merrill et al. [12]. UVPD afforded dominant diagnostic products allowing unambiguous assignment of the sphingosine (m/z 740.5147) and amide-linked (m/z 628.3894) acyl chains, as well as a diagnostic pair of product ions enabling localization of the sphingosine C4-C5 double bond (m/z 796.5410 and 772.5406). Interestingly, while the majority of these cleavages were also reported by O'Brien and

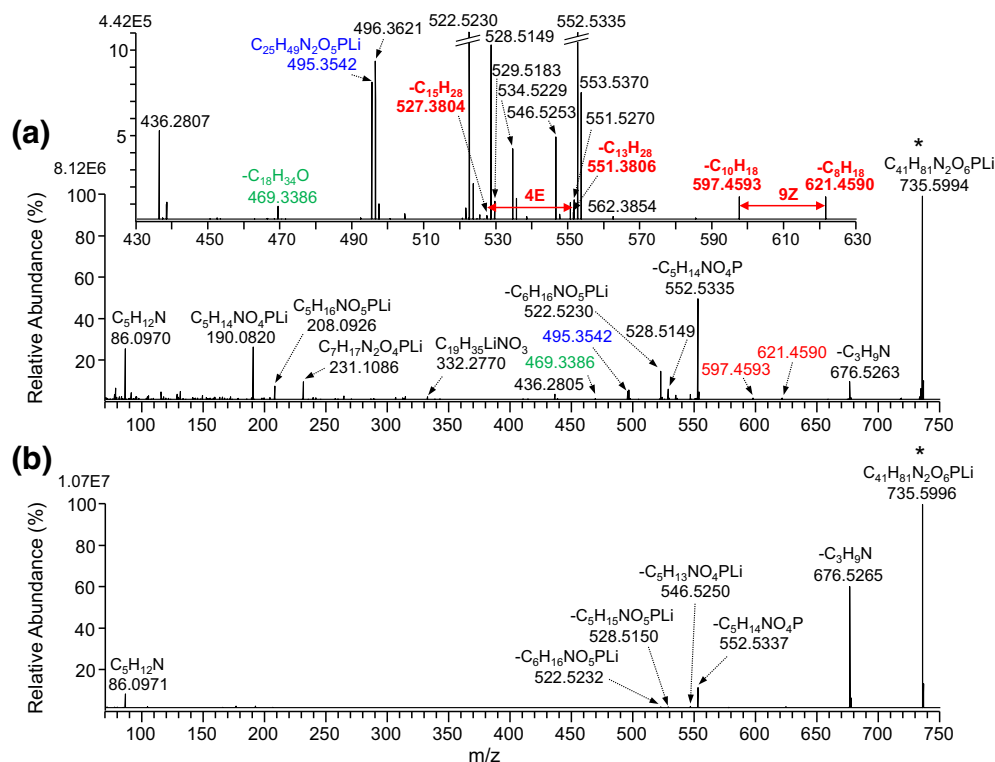


Figure 5. (a) 193 nm UVPD- and (b) HCD-MS/MS of the $[M + Li]^+$ precursor ion of sphingomyelin d18:1(4E)/18:1(9Z). Structurally diagnostic UVPD specific product ions corresponding to cleavage of the sphingosine C=C and acyl chain C=C double bonds are indicated using red text, whereas those corresponding to cleavage of the N-C amide bond and sphingosine backbone are indicated using green and blue text, respectively. See Scheme 5 for structural information

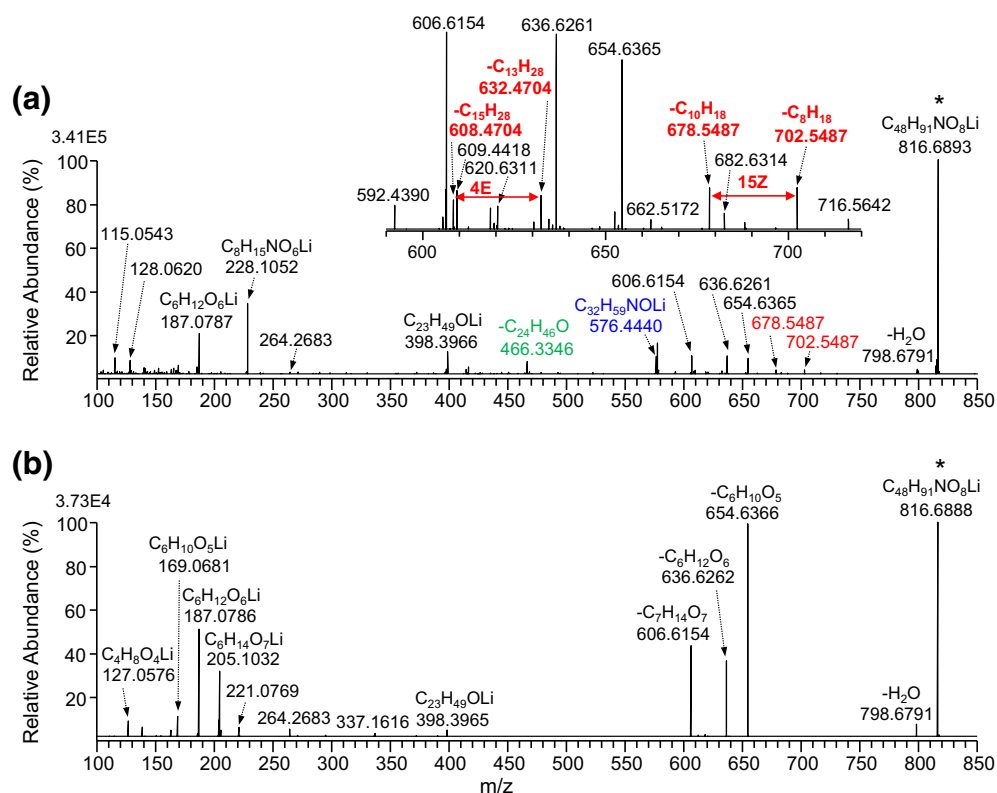
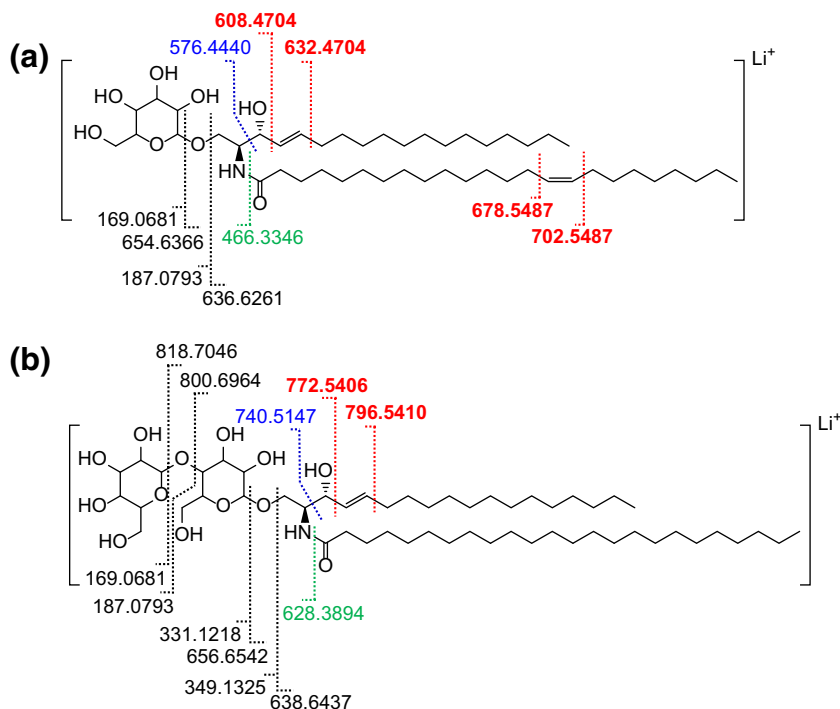


Figure 6. (a) 193 nm UVPD- and (b) HCD-MS/MS of the $[M + Li]^+$ precursor ion of galactosyl ceramide d18:1(4E)/24:1(15Z). Structurally diagnostic UVPD specific product ions corresponding to cleavage of the sphingosine C=C and acyl chain C=C double bonds are indicated using red text, whereas those corresponding to cleavage of the N-C amide bond and sphingosine backbone are indicated using green and blue text, respectively. See Scheme 6a for structural information



Scheme 6. Summary of the 193 nm UVPD-MS/MS dissociation behavior of the $[M + Li]^+$ precursor ion of (a) galactosyl ceramide d18:1(4E)/24:1(15Z) and (b) lactosyl ceramide d18:1(4E)/24:0. Structurally diagnostic UVPD specific product ions corresponding to cleavage of the acyl chain C=C and/or sphingosine C=C double bonds are indicated using red text, whereas those corresponding to cleavage of the N-C amide bond and sphingosine backbone are indicated using green and blue text, respectively

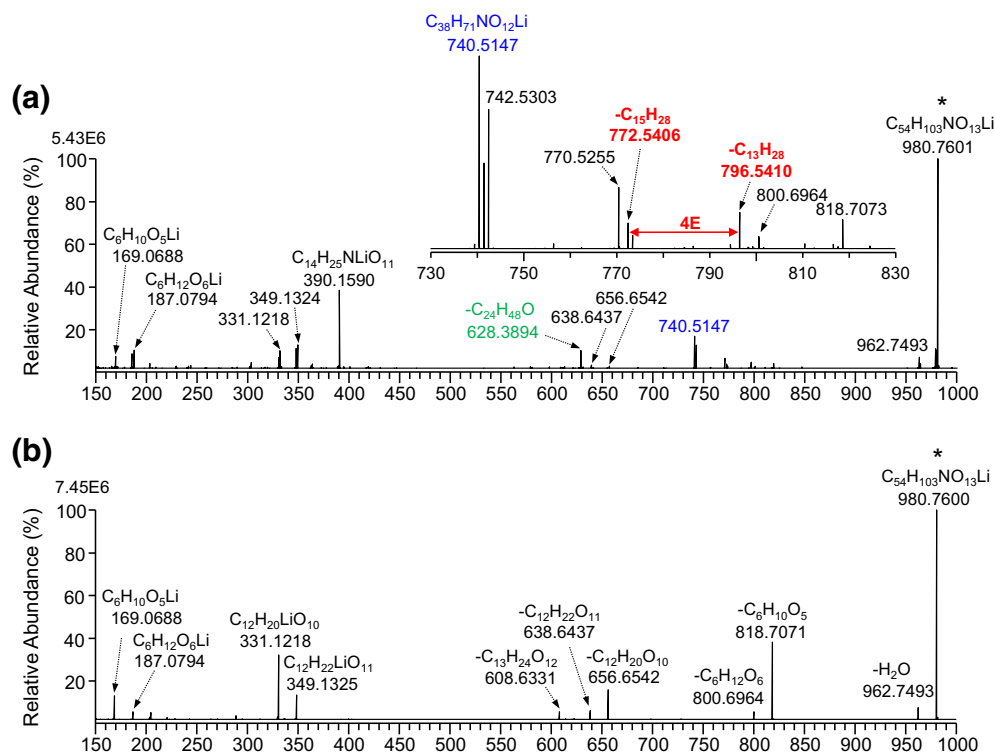


Figure 7. (a) 193 nm UVPD- and (b) HCD-MS/MS of the $[M + Li]^+$ precursor ion of lactosyl ceramide d18:1(4E)/24:0. Structurally diagnostic UVPD specific product ions corresponding to cleavage of the sphingosine C=C double bond are indicated using red text, whereas those corresponding to cleavage of the N-C amide bond and sphingosine backbone are indicated using green and blue text, respectively. See Scheme 6b for structural information

Brodbelt [36] for 193 nm UVPD of the singly deprotonated lactosylceramide d18:1/12:0, cleavages indicative of the sphingosine C=C double bond, or indicative of the sites of unsaturation within amide-linked acyl chains, were not reported in negative ionization mode.

Finally, as an initial demonstration of the practical analytical utility of 193 nm UVPD-MS/MS for the characterization of endogenous lipids found within complex tissue extracts, two low or moderately abundant sphingolipid lipid species, namely hexosyl ceramide d18:1(4E/Z)/24:1(15Z/E) (Supplementary Figure S5a) and ceramide d18:1(4E/Z)/18:0 (Supplemental Figure S5b) were identified from their $[M + Li]^+$ precursor ions within a total lipid extract of porcine brain following its introduction to the mass spectrometer at a concentration of 50 $\mu\text{g}/\text{mL}$ via nanoESI. Notably, the resultant spectra for these endogenous lipids, obtained with only 30 s of spectral averaging, were essentially identical to those obtained from the corresponding standards described above in Figure 6a and Figure 3a, respectively). Furthermore, based on observation of the UVPD-specific and structurally diagnostic sphingosine C=C and acyl chain C=C double bond cleavage product ions, and those formed by diagnostic cleavages of the N-C amide bond and sphingosine backbone, these spectra readily allowed the unambiguous differentiation of these lipid species from other potential isomeric lipids that could have been present. Note that as UVPD (or HCD)-MS/MS cannot differentiate between galactosyl and glucosyl ceramide head groups, the endogenous

lipid was assigned simply as a hexosyl ceramide. Also, as mentioned above, due to photoisomerization of *cis/trans* double bonds upon photon absorption, the locations of unsaturation within the endogenous lipids have been assigned ambiguously as E and/or Z (i.e., E/Z).

Conclusions

In this study, we have demonstrated the utility of 193 nm UVPD in positive ionization mode as an alternate ion activation method that enables the detailed structural characterization of a variety of different sphingolipid classes, including sphingoid bases, ceramides, and complex sphingolipids. Importantly, UVPD was found to result in the formation of unique pairs of structurally diagnostic product ions indicative of the presence and site-specific locations of both sphingosine and acyl chain C=C double bonds, as well as diagnostic ions enabling assignment of the sphingoid base and fatty acid identities, and head group structures. Although further studies are required in order to evaluate and extend this mode of ion activation to other lipid categories, as well as to explore the potential benefits of combining sequential HCD and UVPD, or other noncovalent adduction, to provide additional structural information such as *sn*-linkage positions within diacyl-containing lipid species, this approach clearly has great

promise for improving the capabilities of tandem mass spectrometry methods for 'top down' shotgun lipidomics applications, and/or to reduce the burden of chromatographic fractionation methods that are commonly employed for the quantitative identification and quantification of complex lipid mixtures.

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