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Enhanced Detection of Sphingoid Bases via Divalent Ruthenium Bipyridine Complex Derivatization and Electrospray Ionization Tandem Mass Spectrometry

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Abstract

Sphingoid bases, such as unsaturated sphingosine (So) and its corresponding dihydro-saturated species sphinganine (Sa), are present in cell samples in low abundance. This fact combined with their low-to-moderate electrospray ionization (ESI) potential, compared to other sphingolipids such as sphingomyelins, limits their detection and quantitation by liquid chromatography tandem mass spectrometry (LC-MS²). To enhance the ESI efficiency of sphingoid bases, a novel procedure to generate stably derivatized analytes that enhance the LC-MS² detection of sphingoid bases when analyzed using LC-MS² was developed. In this method, a ruthenium complex, [4-(N-succinimidylloxycarbonyl propyl)-4'-methoxy-2,2'-bipyridine] bis (2,2'-bipyridine) Ru(II) dihexafluorophosphate, is added directly to a cell extract. This complex reacts with and covalently binds to an amino group within the sphingoid bases. The dicationic nature of the ruthenium ion, enhances the compound's ionization efficiency resulting in increased LC-MS² signals for the derivatized sphingoid bases. Consequently, the detection and quantitation of sphingoid bases is greatly improved.

Sphingolipids, such as ceramides and sphingomyelins, are complex class of membrane biomolecules that provide structural frame work for plasma membrane organization, including membrane lipid rafts.¹ These biomolecules contribute to a number of biological functions including protein modification and trafficking, cellular homeostasis, angiogenesis, phagocytosis, differentiation in neurogenesis, growth morphogenesis, and apoptosis.^{2–8} The free sphingoid bases, ceramides, and their phosphorylated analogues are also important messengers for cell signaling and transduction events,^{9–13} while abnormally high levels of these compounds result in severe reproductive abnormalities and a decrease in lifespan.^{2, 3, 14,15} Sphingolipid abnormalities have also been implicated in the pathogenesis of other conditions such as atherosclerosis, diabetes, and Alzheimer's disease,¹⁶ underlying their roles in the proper functioning and development of tissues in eukaryotes.^{2, 3, 15, 16}

Mammals have a complex of sphingolipidome that is estimated to consist of over 400 head group variants.¹⁷ With increasing evidence of the importance of these compounds, the need to accurately quantitate sphingolipids and their related compounds becomes critical. While methods for the analysis of different sub-classes of sphingolipids, such as ceramides,

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sphingomyelins, sphingoid bases, and their phosphates have been developed based primarily on the methodology described by Merrill *et al.*¹⁸, the measurement of sphingoid bases such as sphingosine, and its dihydro analogue, sphinganine, using LC-MS remains challenging due to their low cellular concentrations and their weak SRM electrospray ionization (ESI) efficiencies.¹⁹ Although no single method can encompass the analysis of the entire lipidome, methods can be selectively built that target the measurement of certain classes of these analytes. It is well known that cells require treatment with fumonisin B₁ to disrupt sphingolipid biosynthesis resulting in increased levels of sphingoid-1-PO₄ and ceramide-1-PO₄ that can be measured using electrospray ionization mass spectrometry (ESI-MS). Sphingoid bases, which are present in low abundance within cells and also exhibit poor ESI-MS² detection,^{3, 19, 20} require modification with derivatizing agents such as o-phthalaldehyde,^{20, 21} to enable their quantitation using fluorescence spectroscopy. The sample preparation procedure for this method, however, is tedious, and samples have to be kept at low temperatures to prevent decomposition. Further, fluorescence spectroscopy has certain limitations including photo-decomposition, dependence on excitation and emission path lengths, instability of the light source, quenching due to aggregation of molecules, and the presence of interfering impurities in the sample. To overcome these difficulties, lifetime-assisted ratiometric sensing (LARS), which requires the combination of at least two fluorophores with widely differing lifetimes, has been developed to analyze the fatty acids.²²

In this manuscript we report the preliminary investigation for the analysis of sphingoid bases by LC-ESI-MS² that utilizes multiple reaction monitoring (MRM) for the detection of sphingoid bases using post-derivatization with a ruthenium chelating compound. In this work we focused on Sa d18:0, So-d18:1^{Δ4}, Sa d17:0, and So-d17:1^{Δ4} as model analytes. Derivatization of these compounds with the ruthenium chelating compound results in a dramatic enhancement of the ESI efficiency of the sphingoid bases, leading to an increase in the overall sensitivity for the measurement of these physiologically important biomolecules.

EXPERIMENTAL SECTION

Dimethylsulfoxide (DMSO), analytical grade reagents, sphingolipid standards D-erythro-dihydrosphingosine (Sa d18:0), and D-Erythrosphingosine (So-d18:1^{Δ4}), were purchased from Sigma-Aldrich (St. Louis, USA). D-Erythro-dihydrosphingosine (Sa d17:0), D-erythrosphingosine (So-d17:1^{Δ4}) and LM6002, which contains a mixture of ten sphingolipids each present at a concentration of 25 μM, were purchased from Avanti Polar Lipids Inc. [4-(N-succinimidylloxycarbonylpropyl)-4'-methy-2,2'-bipyridine] bis (2,2'-bipyridine) Ru(II) dihexafluorophosphate,²³ can be synthesized from 4-carboxypropyl-4'-methy-2,2'-bipyridine.²⁴ Tris-bipyridine-N-hydroxysuccinimide was a generous gift from Meso Scale Discovery (Gaithersburg, MD).

Derivatizing Procedure for Standard Reference Compounds

To 450 nmols of each of the individual standard compounds, Sa d18:0, So-d18:1^{Δ4}, Sa d17:0, and So-d17:1^{Δ4} dissolved in 100 μL of 1:1 (v/v) CH₃OH/DMSO, was added 500 nmols of ruthenium complex dissolved in 50 μL of DMSO. The reaction mixture was incubated at 37 °C with shaking for 4 hrs. The solution was then evaporated to a volume of approximately 25 μL using a SpeedVac concentrator. The sample was reconstituted in Reverse phase buffer A [(92/7/1 (v/v/v) H₂O:CH₃OH:HCOOH)] to provide a final sample concentration of 1 pmol/μL.

Preparation of Ruthenylated Sphingoid Bases from the Tissue

Lipid extracts were prepared according to the method described by Merrill *et al.*^{18, 19} In brief, to 100 μL of human embryonic kidney (HEK) 293E cells having a protein

concentration of approximately 2–3 mg/mL, were added 0.5 mL of CH₃OH, 0.25 mL of CHCl₃, 50–100 μ L of water, and 30 μ L of 25 μ M solution of LM6002 internal standard. The lipid aggregates in the mixture were dispersed by either sonicating four times using a Branson tip sonicator at an amplitude of 30% for a period of 10 seconds, or using a water bath sonicator for approximately 15–30 min. After sonication, the samples were incubated overnight at 48 °C with shaking. After cooling to ambient temperature, 75 μ L of 1 M methanolic KOH solution was added to the tubes followed by incubation at 37 °C for two hrs with shaking.

The sample solution was then evaporated to a volume of approximately 25 μ L using a Speed Vac Concentrator and reconstituted by adding 300–400 μ L of 1/1 (v/v) reverse phase solution A and reverse phase solution B [99:1 (v/v) CH₃OH: HCOOH]. After vortexing for 1 min and centrifugation using a desk-top centrifuge (Eppendorf Centrifuge 5415 D) for two minutes, the supernatant was collected. This supernatant was again evaporated using a SpeedVac concentrator to an estimated volume of 25–50 μ L. The samples were derivatized by adding excess (*ca.* 3 μ mol) of ruthenium complex as described above for the standards.

Liquid Chromatography Tandem Mass Spectrometry

Liquid chromatography-tandem mass spectrometry (LC-MS²) analyses were performed using a TSQ Discovery LC-MS triple quadrupole mass spectrometer (Thermo Electron Corp., San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. The mass spectrometer was coupled to an Agilent 1100 series HPLC system.

Sphingoid bases and their ruthenylated compounds were separated by reverse phase HPLC using a binary system and a Supelco 2.1 mm $\times$ 5 cm $\times$ 5 μ m Discovery C18 column operating at a flow rate of 250 μ L/min and maintained at 37 °C. Mobile phase A consisted of 92/7/1 (v/v/v) H₂O:CH₃OH:HCOOH, containing 5 mM HCOONH₄. Mobile phase B consisted of 99:1 (v/v) CH₃OH:HCOOH with 5 mM HCOONH₄. The LC-MS² experiments were performed by injecting 8 μ L of sample on the column using an auto-sampler. After sample injection, the column was eluted by increasing the initial gradient from 2 % to 95 % B buffer over a 12 min period. The gradient was then increased to 100 % B over the next 4 min and held there for 1 min, followed by drop to 2 % B over 0.1 min. The column was then equilibrated for 3.9 min. The LC run time for each analysis was 21 minutes.

The TSQ Quantum Discovery mass spectrometer was calibrated using a solution of polytyrosine-1,3,6 as per the manufacturer's recommendation (Thermo Electron Corp.). The tune file for the present analytes was obtained by optimizing ESI source conditions at a LC flow rate of 250 μ L/min and using standard sphingosine and sphinganine reference compounds, and derivatized ruthenylated sphingoid base compounds, respectively. The optimized source parameters were as follows: ionization mode, positive; sheath gas pressure, 20 psi; auxiliary gas pressure, 55 (arbitrary units); ion spray needle voltage, 4,000 V; capillary temperature, 350 °C; skimmer offset, 20 V. No source fragmentation was observed when direct infusion of reference compounds was made at this skimmer potential. For ruthenylated sphingoid bases, the collision energy and tube lens values were obtained through compound optimization. For sphingoid bases, tuned values for tube lens were used. Application of collision energy in the range of 24 to 27 V provided the best fragmentation of [M+H]⁺ ions to product ions at *m/z* 250.04, 251.94, 264.39 and 266.32, corresponding to doubly dehydrated product ion fragments for So-d17:1 Δ^4 , Sa d17:0, So-d18:1 Δ^4 , and Sa-d18:0, respectively.

Collision induce dissociation was performed using nitrogen gas within Q2, which was offset from Q1 by 10 V. Multiple reaction monitoring (MRM) scanning was accomplished by setting Q1 and Q3 to pass the precursor and product ions respectively. The acquisition

parameters used for all analytes were: scan width (m/z) 0.010; scan time, 0.10 sec for each transition; peak width (FWHM) 0.70 for both Q1 and Q3; and collision pressure 1.5 mTorr. Other acquisition parameters and chromatographic retention times are listed in the Table 1. Data acquisition and analysis were accomplished using Xcalibur software v.2.0.5 (Thermo Electron Corp.).

RESULTS AND DISCUSSION

Although sphingoid bases electrospray, their ionization efficiencies are comparatively smaller when compared to other sphingolipids such as SM and ceramides. In addition, sphingoid bases are present in low abundance in the cell, hampering their detection and quantitation using LC-MS², particularly, when the precursor to the double dehydrated sphingoid base product ion fragment, which is a very specific product ion for the sphingolipid class, is monitored using SRM.

In this manuscript a LC-MS² method that circumvents the problem of low ESI efficiency of sphingoid bases via chemical derivatization is presented. The sphingoid bases are reacted with an activated [(4-carbonylpropyl)-4'-methy-2,2'-bipyridine] bis (2,2'-bipyridine) Ru(II) dihexafluorophosphate compound to form a covalent amide linkage between an amino moiety of the sphingoid base and the carboxylic group of the bipyridine side chain moiety of the activated ruthenium complex (Fig. 1). The reference compounds are reacted directly in CH₃OH/DMSO solvent system. While this manuscript focuses on results obtained for the analysis of sphingoid bases, their phosphate analogues, and ceramide phosphoethanolamines² can also be modified by simply adding the activated ruthenium complex in DMSO solution to an aqueous methanolic solution of a cell extract, however, the other modified compounds from the cell solution will not interfere in the analysis of ruthenylated sphingoid bases due to the different m/z 's of the respective precursor and the product ions. No further bench-top purification steps are required as the samples are analyzed directly using LC-MS² operating in a MRM mode. Because of the divalent ruthenium ion, the derivatized molecule is doubly positively charged, facilitating fragmentation and enabling positive ionization MS to be used. As alluded to earlier, the presence of overall doubly positive charge on the molecule facilitates ion formation, and this is reflected in the increase of signal intensities of the analyte ions by almost two orders of magnitude, as indicated in Table 1. A full scan spectrum of Sa 18:0 before and after derivatization with a ruthenium compound is depicted in Fig. 2.

Ruthenium has seven naturally occurring isotopes. Therefore, its MS spectrum displays a cluster of seven isotopic peaks that will be separated from each other based on the ionization state of the metal ion. For a singly charge ion, each peak is separated by 1 unit, and by 0.5 units if the ion is doubly charged (as in the present case). Due to this isotopic characteristic, analysis of sphingoid-Ru analytes with overlapping m/z values is not possible by MS² through direct infusion or if they have identical chromatographic retention times. If the analytes have different chromatographic capacity (k), and/or selectivity (α) factors, however, they can be adequately separated and incontrovertibly quantitated. In the present study, ruthenium coupled to sphingosine So d17:1 Δ^4 and sphinganine Sa d17:0 have m/z ratios of 468.70 and 469.70, respectively for their $[M]^{+2}/2$ ions. The m/z ratios for these two ions clearly overlap as illustrated in Fig. 3. Likewise, the $[M]^{+2}/2$ ions for ruthenium coupled to sphingosine So d18:1 Δ^4 and sphinganine Sa d18:0 have overlapping m/z ratios of 475.71 and 476.71, respectively. Fortunately, these pairs of molecular ions can be separated using reverse phase LC. The LC-MS² (MRM mode) analysis shows that the Ru-So d17:1 Δ^4 and Ru-Sa d17:0 compounds elute with retention times of 5.9 and 6.1 min, respectively, while Ru-So d18:1 Δ^4 and Ru-So d18:0 compounds emerge at retention times of 6.1 and 6.2 min, respectively, (Fig. 4). Although the peaks from Ru-Sa d17:0 and Ru-So d18:1 Δ^4 co-elute

(i.e. retention time 6.1 min), different mass filters are used for each compound, hence quantitation can be achieved without any interference from the ruthenium isotopes. While we have attempted various modifications to the chromatography conditions, Ru-Sa d17:0 and Ru-So d18:1 Δ^4 consistently co-migrate.

It has been reported that free sphingoid bases are extremely susceptible to dehydration in the ion source, which adversely affects the ability to quantitate these compounds using MS.²⁵ Species containing a Δ^4 bond (a double bond on the fourth carbon atom starting from the terminal carbon containing an hydroxyl functional group), for example sphingosine compounds such as (So-d17:1 Δ^4 , So-d18:1 Δ^4), yield higher amounts of dehydration products compared to saturated dihydro species such as sphinganine (Sa d17:0, Sa d18:0), due to the formation of a stable conjugated carbocation due to the dehydration of water molecule from the allylic carbon atom present next to the double bond.²⁵ In our study, we have also found similar results, that sphingosine exhibited a relatively more intense peak than the corresponding dihydro-sphinganine at the same level of concentration. Nevertheless, the peak intensities for all the reference sphingoid bases were very low, as shown in Fig. 4. Covalent modification of these sphingoid bases with the ruthenium complex, however, resulted in an almost two-orders of magnitude increase in the peak intensities for both So and Sa ruthenylated molecular species at the same level of concentration, as illustrated in Fig. 4. In addition, the peak intensities for the ruthenylated Sa species were observed to be greater than those of the unsaturated So species. Close examination of the chromatographic peak areas show that the peak intensities of the saturated and unsaturated molecular ions are similar. This result indicates that the product ions formed from the different precursor ions, which are being monitored in the MRM transitions, are identical in all the So and Sa ruthenylated compounds. The moderate difference in intensities of the peaks from different compounds might be due to the variation in the degree of freedom of the sphingoid bases due to the differing carbon chain lengths of the precursor ions that resulted in dissipation of energy differently along the carbon chain during the MRM transitions.

A major disadvantage of derivatization procedures for sphingolipids is the low stability of the modified products,²⁰ which requires that samples be analyzed soon after they are derivatized. In the present case, however, re-analysis of the derivatized samples after being stored for two weeks at ambient temperature, resulted in no visible deterioration of the compounds as no additional peaks or change in peak intensity was observed. This result shows that the derivatized products are highly stable.

[4-(N-succinimidylloxycarbonylpropyl)-4'-methy-2,2'-bipyridine] bis (2,2'-bipyridine) Ru(11) dihexafluorophosphate exhibited an isotopic cluster with a peak maxima at m/z 912.05 corresponding to the singly charged $[M]^+$ ion (i.e., [4-(N-succinimidylloxycarbonylpropyl)-4'-methy-2,2'-bipyridine] bis (2,2'-bipyridine) Ru(11) monohexafluorophosphate). Product ion scans of the starting succinimidyl ruthenium complex and its ruthenium derivatized Sa and So molecular species were optimized by dissociating the compounds at different collision energies (25 to 50 V). Two major peaks at m/z 226.6 and 304.6 were observed for all the derivatized compounds, as shown in Fig. 5. The peak intensities for these two product ions were dependent upon the collision energy (CE) applied. As the CE is increased from 25 to 50 V, the intensity of the signal at m/z 226.6 increases compared to that of m/z 304.6. The intensity ratios for these two ion transitions were also constant when the MS² experiment was repeated three times, indicating these transitions are compound specific. The parent ruthenium complex dissociates to give totally different product ions than the derivatized Sa and So species, as m/z 226.6 or 304.6 were not observed even at high CE. Other evidence showing that m/z 226.6 and 304.6 originated from the derivatized analytes and have the same fragment structure is obtained when precursor ion scanning for the four combined ruthenylated analyte was performed. Two sets of tailing peaks, characteristic of precursor

ion scanning due to voltage delay between Q1 and Q3 quadrupoles, with m/z maxima displayed at 469.7 and 476.8 Da were observed, indicating the presence of Ru-Sa d17:0 and Ru-d18:0 ions, whilst the m/z from Ru-So-d17:1 Δ^4 and Ru-So-d18:1 Δ^4 were buried under the peaks of Ru-Sa d17:0 and Ru-d18:0. These peaks could not be identified, as alluded to earlier, due to the spread of isotopic peak pattern resulting from the ruthenium isotopes. Clearly, this result also demonstrates the limitation of precursor ion scanning for the analysis of same carbon So and Sa analytes due to the ruthenium isotopic effect. However, this isotopic envelope problem can be overcome if isotopic ruthenium derivatives are used. Based on these experiments, we propose the structure of these fragment ions are as shown in Fig. 6.

To determine if the enhanced signal detection observed using the sphingoid base standards could translate to cell measurements, we analyzed lipid extracts obtained from HEK239E cells using LC-MS² (MRM) with and without derivatization using the ruthenium compound. Analysis of the cell extracts without ruthenium derivatization did not produce any detectable signal. When the samples were derivatized with the ruthenium complex, all four ruthenylated sphingoid bases produced easily detectable signals when analyzed using MRM, as shown in Fig. 7.

Conclusions

Sphingolipids, such as sphingoid bases and sphingosines, play an important role in cell physiology and are being found to be involved in an increasing number of disease conditions. Unfortunately, due to their low ionization efficiencies and low abundance within cells, these compounds are difficult to quantitate using ESI-MS methods. In this manuscript, we present a method in which a ruthenium derivatizing reagent can be reacted directly with cell extracts providing stably modified sphingoid bases. Due to the dicationic nature of the ruthenium ion, ionization efficiency (and hence signal intensity) is increased for these compounds. This increased ionization efficiency might afford accurate detection and quantitation of sphingoid bases from within different type of cell extracts. We are presently working to streamline the methodology for the quantitation of all sphingoid base compounds and their phosphate analogues present within the cell extract by using commercially available ruthenylated compounds. We also anticipate that phosphorescence emission spectroscopy with ruthenium label can be used to study sphingoid bases in the future. If these ruthenylated sphingoid bases are to be used as luminescent labels for emission studies, however, they must first be purified either individually or as a class from the reacted cell samples. This need is because of the presence of many other reacting and interfering species present in the cell that contain with an amino group such as proteins, ceramide phosphorylethanolamines etc., which can react with an activated ruthenylated compound.

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Figure 1.

Structures for the 18 carbon atoms sphingoid bases: sphingosine, sphinganine, and the derivatized sphinganine as its dihexafluorophosphate. Notation: So-d18:1^{Δ4} is a 18 carbon chain skeleton with double bond on the fourth carbon atom.

Figure 2.
A full scan spectrum at arbitrary concentrations obtained by direct infusion of methanolic solution of Sa 18:0 before and after derivatization with ruthenium compound.

Figure 3.

An expanded region of the full scan profile spectrum showing the ruthenium(II) isotopic cluster. (A) [4-(N-succinimidyl-oxycarbonylpropyl)-4'-methoxy-2,2'-bipyridine] bis(2,2'-bipyridine) Ru(II). The peak at m/z 912.05 corresponds to the compound's monohexafluorophosphate ion. Peak separation of 0.5 m/z is displayed for the dicationic ruthenium derivatized compounds of (B) So d17:1 Δ^4 , (C) Sa d17:0 (D) So d18:1 Δ^4 , and (E) Sa d18:0. Panel F illustrates the peak overlap for So d17:1 Δ^4 and Sa d17:0 (left cluster) as well as So d18:1 Δ^4 and Sa d18:0 (right cluster).

Figure 4.

Extracted ion chromatogram of derivatized ruthenylated sphingoid bases reference compounds (Left hand panel) and their non-derivatized sphingoid bases (Right hand panel) acquired using reverse phase high performance liquid chromatography-tandem mass spectrometry (HPLC-MS²) operating in a selected reaction monitoring (MRM) mode. (A) Total ion chromatogram (TIC) at 1 pmol/μL level concentration of each analyte, with approximately 8 μL of solution injected on column. The TIC for (B) derivatized So d17:1 Δ^4 , (C) derivatized Sa d17:0, (D) derivatized So d18:1 Δ^4 , and (E) derivatized Sa d18:0 are also shown.

Figure 5.

Product ion ion spectrum for starting ruthenium complex and selected derivatized sphingoid bases acquired using different collision energies (A) Starting ruthenium reagent at 40 V, (B) derivatized Sa d17:0 at 25 V, (C) derivatized Sa d17:0 at 40 V, (D) derivatized Sa d17:0 at 50 V, (E) derivatized So d18:1 Δ^4 at 50 V, and (F) derivatized So d18:0 at 50 V.

Figure 6.
Proposed structure for the ions m/z 305.6 and 226.5 from the unsaturated sphingosine, and its dihydro-sphinganine compounds

Figure 7.

A reverse phase-HPLC-MS/MS (MRM mode) extracted ion chromatogram of the derivatized ruthenylated sphingoid bases for the cell sample. (A) Total ion chromatogram (TIC) obtained from injection of approximately 8 μ L of solution injected on column. The TIC of (B) derivatized So d17:1 Δ^4 , (C) derivatized Sa d17:0, (D) derivatized So d18:1 Δ^4 , and (E) derivatized Sa d18:0 are shown.

Table 1

Retention times and compound specific selected reaction monitoring (MRM) and multi reaction monitoring (MRM) parameters for some of the standard bases and their derivatized analogues analyzed in this study.

Analyte	Precursor [M+H] ⁺ or [M] ^{2/2} → Product ion(s)	Retention Time (min)	Collision Energy (V)	Tube Lens (V)	NL (Peak Area)*
SRM Transitions:					
So-d17:1^{A4}	286.270 → 250.040	6.25	27	Tuned	5.16 × 10 ⁴ (261761)
So-d18:1^{A4}	300.186 → 264.389	6.43	27	Tuned	1.41 × 10 ⁵ (796564)
Sa-d17:0	288.280 → 251.940	6.39	27	Tuned	2.30 × 10 ⁴ (117234)
Sa-d18:0	302.266 → 266.318	6.59	27	Tuned	2.87 × 10 ⁴ (144463)
MRM transitions:					
Ru-So-d17:1^{A4}	468.701 → 226.658 → 304.691	5.92	41 19	118 118	2.65 × 10 ⁶ (12760615)
Ru-So-d18:1^{A4}	475.708 → 226.758 → 304.582	6.07	40 26	118 118	4.09 × 10 ⁶ (19968440)
Ru-Sa-d17:0	469.701 → 226.758 → 304.663	6.06	36 30	117 117	5.49 × 10 ⁶ (25590767)
Ru-Sa-d18:0	476.709 → 226.720 → 304.879	6.22	37 24	111 111	1.03 × 10 ⁷ (49453802)

* The strength of the solution is 1 pmol/μl, and 8 μL was injected by auto-sampler for LC-ESI-MS/MS experiments. NL = Individual normalized peak level.