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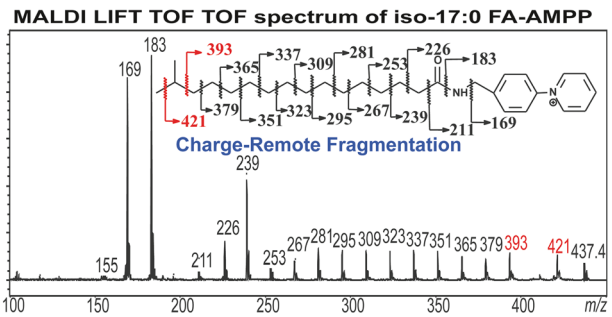
RESEARCH ARTICLE

Characterization of Long-Chain Fatty Acid as N-(4-Aminomethylphenyl) Pyridinium Derivative by MALDI LIFT-TOF/TOF Mass Spectrometry

Cheryl Frankfater,[1] Xuntian Jiang,[2] Fong-Fu Hsu[1]

[1]Mass Spectrometry Resource, Division of Endocrinology, Diabetes, Metabolism, and Lipid research, Washington University School of Medicine, Box 8127, 660 S Euclid, St. Louis, MO 63110, USA
[2]Diabetic Cardiovascular Disease Center, Department of Medicine, Washington University School of Medicine, St. Louis, MO 63110, USA



Abstract. Charge remote fragmentation (CRF) elimination of C_nH_{2n+2} residues along the aliphatic tail of long chain fatty acid is hall mark of keV high-energy CID fragmentation process. It is an important fragmentation pathway leading to structural characterization of biomolecules by CID tandem mass spectrometry. In this report, we describe MALDI LIFT TOF-TOF mass spectrometric approach to study a wide variety of fatty acids (FAs), which were derivatized to N-(4-aminomethylphenyl) pyridinium (AMPP) derivative, and desorbed as M^+ ions by laser with or without matrix. The high-energy MALDI LIFT TOF-TOF mass spectra of FA-AMPP contain fragment ions mainly deriving from CRF cleavages of C_nH_{2n+2} residues, as expected. These ions together with ions from specific cleavages of the bond(s) involving the functional group within the molecule provide more complete structural identification than those produced by low-energy CID/HCD using a linear ion-trap instrument. However, this LIFT TOF-TOF mass spectrometric approach inherits low sensitivity, a typical feature of high-energy CID tandem mass spectrometry. Because of the lack of unit mass precursor ion selection with sufficient sensitivity of the current LIFT TOF-TOF technology, product ion spectra from same chain length fatty acids with difference in one or two double bonds in a mixture are not distinguishable.

Keywords: HCD, Linear ion-trap, High-energy CID, Charge switch derivatization, Branched fatty acids, Microbial lipids, Lipidomics

AbbreviationsMALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; CID, collision-induced dissociation; MS, mass spectrometry; LIT, linear ion-trap; AMPP, N-(4-aminomethylphenyl) pyridinium

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Introduction

Characterization of long-chain fatty acids applying high-energy (HE) collision-induced dissociation (CID) combined with tandem mass spectrometry started in the FAB-tandem sector instrument era [1–3], soon after FAB ionization technique was introduced as a desorption method for biomolecules [4]. The follow-on advance in matrix-assisted laser desorption ionization (MALDI) technique [5, 6] coupled to TOF/TOF instruments rejuvenated the interest in application of HE CID tandem mass spectrometry for structural analysis of complex lipids. Several structural studies on lipids by MALDI TOF-TOF have been reported [7–12].

Fragment ions from post source decay (PSD) of precursor ions formed by MALDI-PSD are sum of all the fragmentation

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Correspondence to: Fong–Fu Hsu; *e-mail:* fhsu@im.wustl.edu

processes, mainly from high-energy CID, and less from metastable ion decay and low-energy CID [13, 14]. To detect the product ions with broad range of energies produced by PSD, a curved field reflectron time-of-flight/time of flight (MALDI TOF/ReTOF) mass spectrometer was constructed [14, 15]. The instrument has been applied in structural analysis of biomolecules, including a wide variety of lipids [7–9, 16]. More recently, a spiral MALDI-TOF/TOF instrument with 60 K resolving power was constructed [17, 18]. The instrument permits monoisotopic isolation of the precursor ions that can be further subjected to CID with 20 keV energy to obtain tandem mass spectrum. Applications in the characterization of lipid molecules also have been reported [11, 12, 19]. The MALDI TOF/TOF instrument developed by Bruker Daltonics used "LIFT" technology, which allows fragment ions formed by collision-induced dissociation (CID) with 8 keV energy to be detected in a single run [20], but the application of this technique in the study of lipid structures has not been reported.

Long-chain fatty acids were desorbed as cationized adduct ions or $[M–H]^-$ ions by FAB before subjecting to tandem MS for structure analysis, and the sensitivity is poor. FA samples prepared under solvent-based condition with the presence of LiCl also give very poor sensitivity in detection of $[FA+Li]^+$ and $[FA–H+2Li]^+$ ions by MALDI [10]. To overcome this issue, solvent-free sample preparation method applying electron deficient 7,7,8,8-tetracyanoquinodimethane (TCNQ) matrix mixed with LiCl has been used to improve detect FA as $[FA–H+2Li]^+$ ions [10]. Alternatively, very long chain mycolic acid has been derivatized to its methyl ester (FAME) derivative and analyzed as sodium adduct ions by MALDI-TOF [21, 22].

Recently, a charge switch derivatization strategy that converts FA to its N-(4-aminomethylphenyl) pyridinium (AMPP) derivative was used for quantitative and qualitative analysis. This FA-AMPP compound yields intense M^+ ions by ESI and $>10^2$ more sensitivity than that detected as $[M-H]^-$ ions has been reported [23–28]. This derivative is also readily detectable by MALDI-TOF with good sensitivity [25]. In this study, we exploit its superb sensitivity by MALDI and apply MALDI LIFT TOF-TOF method readily available in Bruker instrument to characterize a broad range of FAs derivatized to FA-AMPP. We evaluate the utility of this MALDI LIFT TOF-TOF technology in the structural determination of FAs, and the aspect of this technique in the lipid analysis is also discussed.

Materials and Methods

Materials

AMP+Mass Spectrometry Kit (50 test), containing N-(4-aminomethylphenyl) pyridinium (AMPP) derivatizing reagent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), n-butanol (HOBt), and acetonitrile/DMF solution, was purchased from Cayman Chemical Co. (Ann Arbor, MI). Lipid standards were purchased either from Avanti polar lipids Inc. (Alabaster, AL) or Matreya LLC (State college, PA). All other solvents (spectroscopic grade) and chemicals (ACS grade) were obtained from Sigma Chemical Co. (St. Louis, MO). Hydroxyphthioceranoic and phthioceranoic acids were prepared as described previously [25].

Preparation of AMPP Derivative

AMPP derivative was made with the AMP+Mass Spectrometry Kit, according to the manufacturer's instruction. Briefly, dried samples were resuspended in 20-μL ice-cold acetonitrile/DMF (4:1, *v*/*v*), and 20 μL of ice-cold 1 M EDCI (3-(dimethylamino)propyl)ethyl carbodiimide hydrochloride) in water was added. The vial was briefly mixed on a vortex mixer and placed on ice. To the vial, 10-μL solution of 5 mM *N*-hydroxybenzotriazole (HOAt) and 30-μL solution of 15 mM AMPP in distilled acetonitrile were added, mixed and heated at 65 °C for 30 min. After cooling to room temperature, 1 mL water and 1 mL n-butanol were added. The final solution was vortexed for 1 min, centrifuged at 1200×*g* for 3 min, and the organic layer was transferred to another vial.

Mass Spectrometry

Both MALDI-TOF and MALDI-TOF/TOF mass spectra were acquired using a Bruker Daltonics UltrafleXtreme TOF/TOF spectrometer (Bremen, Germany) equipped with a smartbeam-II™ laser, which is operated at repetition rate of 2 and 1 kHz for MS and MS/MS modes, respectively. The instrument consists of ion source 1 with a gridless MALDI ion source with delayed extraction (DE) electronics and a collision cell, and ion source 2 with a timed ion selector (TIS), and a "LIFT" accelerating cell. This is followed by a post-LIFT metastable suppressor (PLMS) device, which was served as a gridless space-angle and energy focusing reflector [20]. The ion family consisting of precursor ions and laser-induced fragment ions (induced by raising the laser intensity) traveling with the same velocity depart the MALDI plate and undergo 8 kV collisions with argon collision gas at a pressure of 1.7×10^{-4} Pa. The "ion family" reaches the timed ion selector (TIS) and was selected. The selected "ion family" passes the TIS and enters the LIFT device, which provides a 19 kV lift potential that allows ions to be post-accelerated to full speed, and then time-focused onto the FlashDetector™. The PLMS situated between the LIFT device and reflector was turned on to deflect the remaining precursor ions to prevent the formation of the undesired fragment ion after post-acceleration. FA-AMPP derivative in butanol was deposited on a MALDI target plate directly without matrix or with α-cyano-4-hydroxycinnamic acid (CHCA), 9-aminoacridine or with 2,5-dihydrobenzoic acid matrix, which was dissolved in 1:1 water/acetonitrile (*v*/*v*) containing 0.1% trifluoroacetic acid at a concentration of 10 mg/mL. Ultimately, CHCA was selected for acquiring LIFT MALDI-TOF/TOF spectra, as it gave the best sensitivity. Under no matrix condition, similar M^+ ions were also formed likely due to that FA-AMPP became the chromophore and can be desorbed by MALDI. However, the sensitivity is significantly lower (<

10). The CID/HCD tandem mass spectra were obtained with a Thermo Scientific (San Jose, CA) LTQ Orbitrap Velos mass spectrometer. The MS^2 spectra were acquired with an optimal normalized collision energy (http://tools.thermofisher.com/content/sfs/brochures/PSB104-Normalized-Collision-Energy-Technology-EN.pdf) ranging from 50 to 70% and with an activation q value at 0.25, and the activation time at 10 ms to leave a minimal residual abundance of precursor ion (around 20%). The mass selection window for the precursor ions was set at 1 Da wide to admit the monoisotopic ion to the ion-trap for collision-induced dissociation (CID) for unit resolution detection in the ion-trap or high-resolution accurate mass detection in the Orbitrap mass analyzer. Mass spectra were accumulated in the profile mode, typically for 1–5 min.

Results and Discussion

Charge Remote Fragmentation Under Low- and High-Energy CID

Figure 1 shows the tandem mass spectra of the ESI desorbed M^+ ions of $\Delta^9$18:1-AMPP at *m/z* 449 obtained with low-energy CID linear ion-trap (LIT) (panel a), LIT HCD (panel b), and of the corresponding MALDI M^+ ions obtained with LIFT TOF-TOF (panel c) instrument. All the spectra contained prominent ions at *m/z* 239, 226, 183, and 169, which are signature ions seen for FA-AMPP derivative [26]. Ions at *m/z* 226 and 169 are most likely distonic radical ions arising from homolytic cleavage of the C–C bonds, forming a stable resonance structure as shown in Scheme 1. The formation of these radical ions is further supported by accurate mass measurement of the ions and consistent with observation of *m/z* 240 (seen in the later spectrum of Fig. 5d) in the tandem mass spectrum of hydroxyphthioceranoic acid-AMPP, which contains an α-methyl group. Ions at *m/z* 433, 419, 395, 381, 377, and 365 arising from charge-remote losses of C_nH_{2n+2} residues are seen in Fig. 1c (inset), but these ions are of low abundance in the product ion spectra obtained with an orbitrap operating in higher-energy collision dissociation (HCD) mode (HCD is a term coined by Thermo Scientific applying < 1 kV collision energy in the HCD collision cell of orbitrap instrument that can generate a triple quadrupole-like product-ion spectrum), and nearly absent in the CID LIT MS^2 spectrum (panel a, inset). These differences may be mainly attributable to the notion that CRF losses of C_nH_{2n+2} residues in the dissociation of long-chain functionalized alkanes require high collision energy [2, 29–31], despite that the internal energy of the precursor ions may also play a role (ESI generated ions contain less internal energy) [30, 32–34].

In Fig. 1a, the ion at *m/z* 351 (loss of C_nH_{2n}; $n = 7$) is more abundant than the ion of *m/z* 349 (loss of C_nH_{2n+2}; $n = 7$), and the intensity ratio of *m/z* 351 to *m/z* 349 is close to 10, applying a normalized collision energy ranging from 50 to 90% (see supplemental material Figure s1a), while the abundance of these two ions is reversed (intensity ratio is 0.72) when obtained with an optimal HCD collision energy of 48% (Fig. 1b), and the ratio decreases as the collision energy increases (see supplemental material Figure s1b). The ion of *m/z* 351 may arise from β-cleavage (to the C=C bond) with γ-H shift (McLafferty rearrangement; Scheme 1), and the similar fragmentation process involving shift of the γ-H proximal to carbonyl group (i.e., γ′-H, Scheme 1) led to the formation of *m/z* 295. This is consistent with the notion that in an ion trap, resonance excitation dissociation is the major fragmentation process, and fragment ions often derive from cleavages of the weak bonds and from rearrangement reactions [30, 35–37]. This is also consistent with the notion that abundance ratio of the ions of *m/z* 351 to 349 is rather unchanged (close to 10) upon activation with different collision energies in an ion trap (Figure s1a) [38]. The *m/z* 349 ion is more abundant than *m/z* 351 as seen in the HCD (Fig. 1b), and LIFT TOF-TOF (Fig. 1c) spectra is also consistent with the notion that the ratio of products of C_nH_{2n} loss to the corresponding products of C_nH_{2n+2} loss decreases (for a given value of n) with increasing collision energy as described by Wysocki and Ross [30].

Differentiation of Positional Fatty Acid Isomers by MALDI LIFT TOF-TOF

(a) Monoenoic Fatty Acids The occurrence of fragment ions from CRF loss of C_nH_{2n+2} provides important structural information of the aliphatic tail (i.e., the $–CH_3$ terminus) of long chain fatty acids, resulting in assignment of the position of double bonds and distinction of positional fatty acid isomers. For example, the LIFT MALDI TOF-TOF mass spectrum of the M^+ ions of $\Delta^6$18:1-AMPP at *m/z* 449 (Fig. 1d) is featured by the ion pairs at *m/z* 253/307 that locate the C=C bond at C6, along with the ion series of *m/z* 433, 419, 405, 391, 377, 363, 349, 335, and 321 arising from CRF loss of C_nH_{2n+2} residues that define the aliphatic tail. Similarly, the LIFT MALDI TOF-TOF mass spectrum of the Δ^{11}18:1-AMPP (Fig. 1e) contained the analogous ion series at *m/z* 433, 419, 405, and 391 arising from cleavages C–C bond from the aliphatic tail along with the *m/z* 377/323 ion pair that give assignment of the double bond at C11. The differences among these LIFT MALDI TOF-TOF spectra (Fig. 1c, d, e) clearly demonstrate the utility of the approach in the structural characterization and distinction of FA positional isomers.

(b) Fatty Acids with Multiple Unsaturated Bonds MALDI LIFT TOF-TOF spectra of the AMPP derivative of fatty acids with multiple double bonds contain more complete structural information than their corresponding low-energy CID tandem mass spectra. For example, the LIFT TOF-TOF spectrum of the M^+ ions of $\Delta^{8,11,14}$20:3-AMPP at *m/z* 473 (Fig. 2a) contains ions at *m/z* 415, 375, and 335 arising from respective allylic cleavages of the C16-17, C13-C14, and C10-C11 bonds, along with ions at *m/z* 401, 363, 323 driving from vinylic cleavages of C15-16, C12-C13, and C9-C10 bonds, respectively [3]. These ions readily locate the double bonds at C8, C11, and C14, respectively. Similarly, the LIFT TOF-TOF spectrum of the

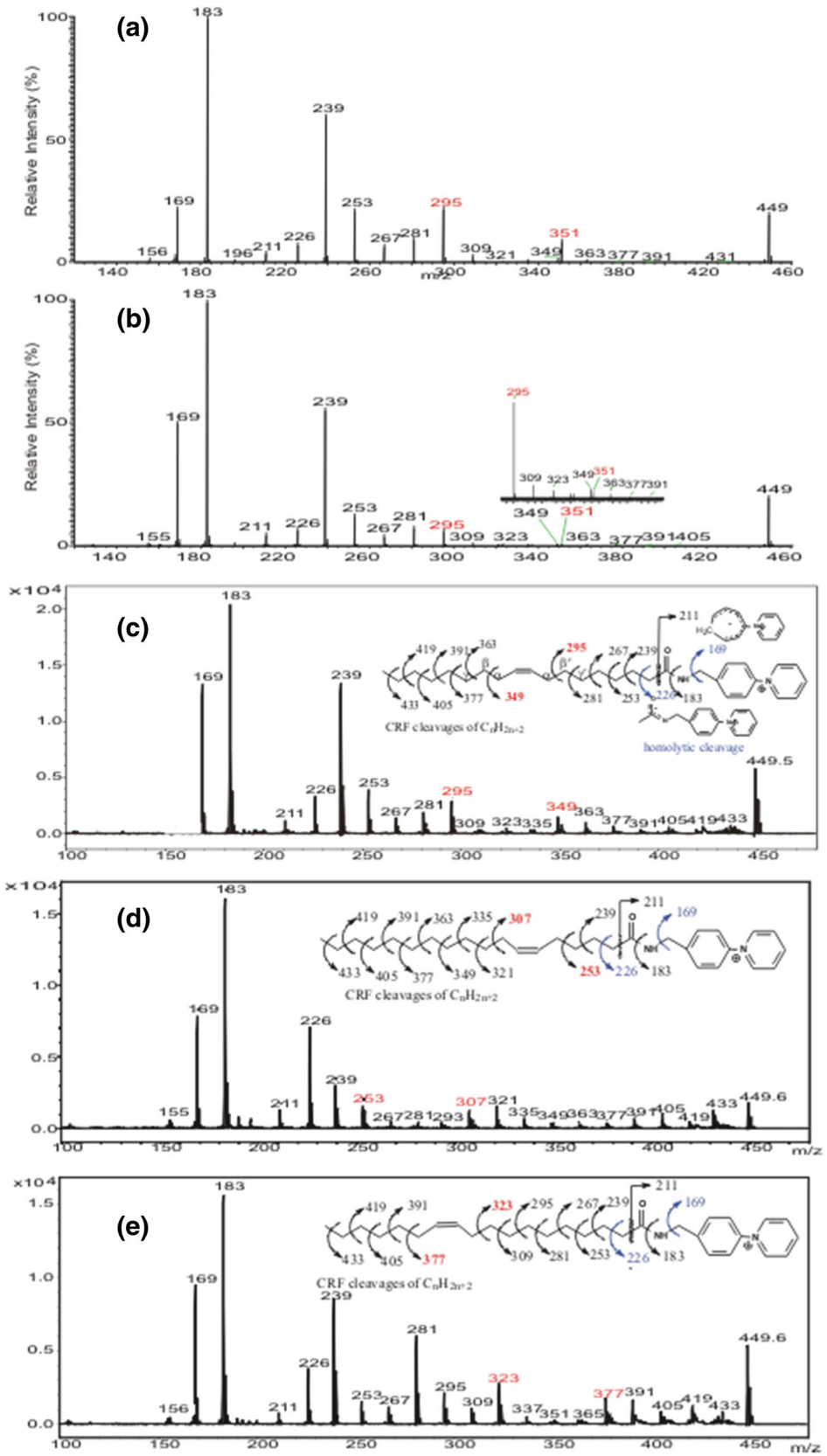
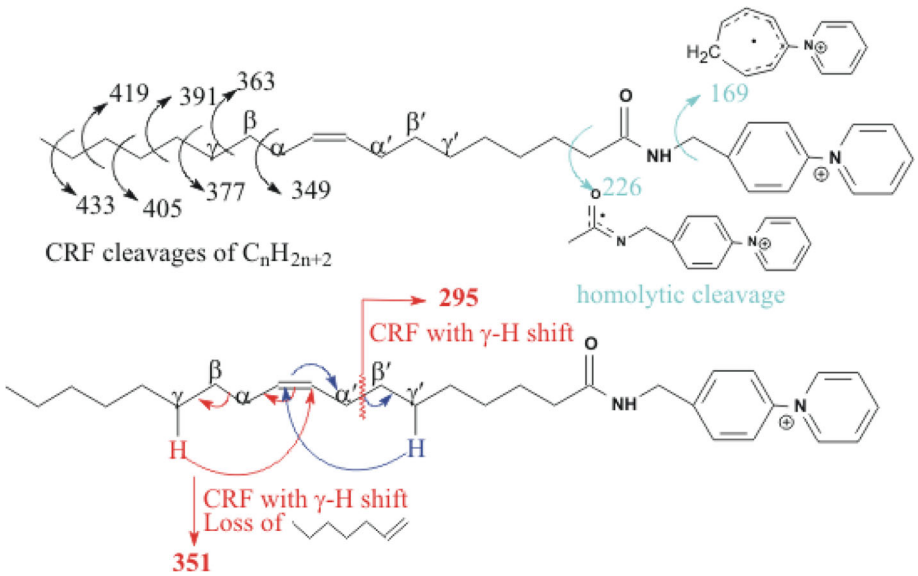


Figure 1. The MS^2 spectrum of the M^+ ions of $\Delta^9$18:1-AMPP at *m/z* 449 obtained with low energy CID (**a**), HCD (**b**) LIT, and with high-energy MALDI LIFT TOF-TOF (**c**). Panel d and panel e show the MALDI LIFT TOF-TOF spectra of $\Delta^6$18:1-AMPP (**d**) and Δ^{11}18:1-AMPP (**e**) isomers, respectively. High-energy CID TOF-TOF (panels **c**, **d**, and **e**) yielded fragment ions from CRF losses of C_nH_{2n+2} residues which are informative for complete structural characterization including double bond and the entire aliphatic tail



Scheme 1. The fragmentation pathways proposed for the M^+ ion of $\Delta^9$18:1-FA-AMPP at *m/z* 449

M^+ ions of $\Delta^{11,14,17}$20:3-AMPP at *m/z* 473 (Fig. 2b) contains ions at *m/z* 457, 417, and 377 arising from allylic cleavages of C19-20, C16-C17, and C13-C14 bonds, respectively, together with ions at *m/z* 443, 405, 365 arising from vinylic cleavages of C18-19, C15-C16, and C12-C13 bonds, respectively. These ions led to locate the double bonds at C11, C14, and C17.

Ions at *m/z* 375 and 323 arose from CRF fragmentation involving McLafferty rearrangements of the labile allylic hydrogens (Scheme 2) [3, 39]. These two ions are abundant in the LIT MS^2 spectrum of $\Delta^{8,11,14}$20:3-AMPP (Fig. 2c), but are of low abundance in the corresponding LIT HCD MS^2 spectrum (Fig. 2d), which is dominated by ions of *m/z* 239, 183, and 169, and the ion series of *m/z* 457, 443, 429, etc. arising from CRF loss of C_nH_{2n+2} residues. The low abundance of these ions arising from CRF losses of C_nH_{2n+2} residues is consistent with the fact that HCD is also a LE CID process with a higher collision energy than that in an ion trap, permitting CRF losses of C_nH_{2n+2} residues to occur with a lesser degree. The dominance of the ions of *m/z* 239, 183, and 169 is also consistent with the observation of low abundance of the ions of *m/z* 375 and 323, which undergo sequential dissociation to ions of 183 and 169 due to multiple collisions in an HCD cell. These sequential fragmentations are supported by the MS^3 spectra of *m/z* 375 (473 → 375) and 323 (473 → 323) (supplemental material, Figure s2a and s2b).

The above fragmentation processes are further realized by the tandem mass spectra of a very long chain fatty acid with multiple double bonds of $\Delta^{14,17,20,23,26,29}$32:6-AMPP (Fig. 2e), which contains ions at *m/z* 619 and 579 arising from CRF losses of CH_4 and C_2H_6, together with ions of *m/z* 539, 499, 459, and 419 from allylic cleavages, and ions at 565, 527, 487, 447, and 407 from vinylic cleavages via the similar fragmentation processes as described in Scheme 2 (see supplemental material Figure s3). The presence of these ions, again, readily locates the double bonds along the fatty acid chain. The LIFT TOF-TOF (Fig. 2e) and LE LIT CID MS^2 spectra of the ions at *m/z* 635 (Fig. 2f) are strikingly similar, despite that the low *m/z* ions such as *m/z* 183 in the latter (ion at *m/z* 169 is absent due to low mass cut-off of ion-trap) are less prominent. The prominence of the ions from CRF cleavages of the C–C bonds allylic (i.e., ions of *m/z* 539, 499, 459, and 419) and vinylic (i.e., ions of *m/z* 565, 527, 487, 447, and 407) to the C14, 17, 20, 23, 26 double bond, respectively (Fig. 2f), may be attributable to the notion that the activation energy to initiate radical formation for higher degree polyunsaturated fatty acids is lower than that of saturated fatty acids [40]. Thus, the similarity in possessing abundant product ions for the two spectra indicates that the structure of the molecules also play a very important role, in addition to the applied collision energy, in dictating the fragmentations [30, 33, 41]. The observation of abundant ions of low *m/z* (e.g., *m/z* 183) and of low abundance ions of *m/z* 539, 499, 459, and 419 (from allylic cleavages) and of *m/z* 565, 527, 487, 447, and 407 (from vinylic cleavages) in the corresponding LIT HCD MS^2 spectrum (Figure s3) is, again, attributable to the multiple collisions in a HCD cell that led to sequential fragmentations as described earlier.

(c) Fatty Acid with Cyclic Branch The MALDI LIFT TOF-TOF mass spectrum of dihydrosterculic acid (9,10-methyleneoctadecanoic acid)-AMPP (cPro^{9}19:0) is shown in Fig. 3a, which contained characteristic ion pairs of *m/z* 363 and 295, arising from β-cleavage of C–C bond to the cyclopropyl ring, and ions of *m/z* 349 and 309 arising from α-cleavages (see inset for fragmentation scheme), together with fragment ions of *m/z* 449, 433, 419, 405, 391, …,etc. arising from CRF cleavages of C-terminal C_nH_{2n+2} residues. In contrast, this ion series was absent in the low-energy HCD (Figure s4, supplemental material) and low-energy CID LIT spectra (not shown), and the structural information of the aliphatic tail is not available. The 40 Da interval between *m/z* 349 and 309 seen in the spectrum defines the cyclic chain, permitting its distinction from a double bond.

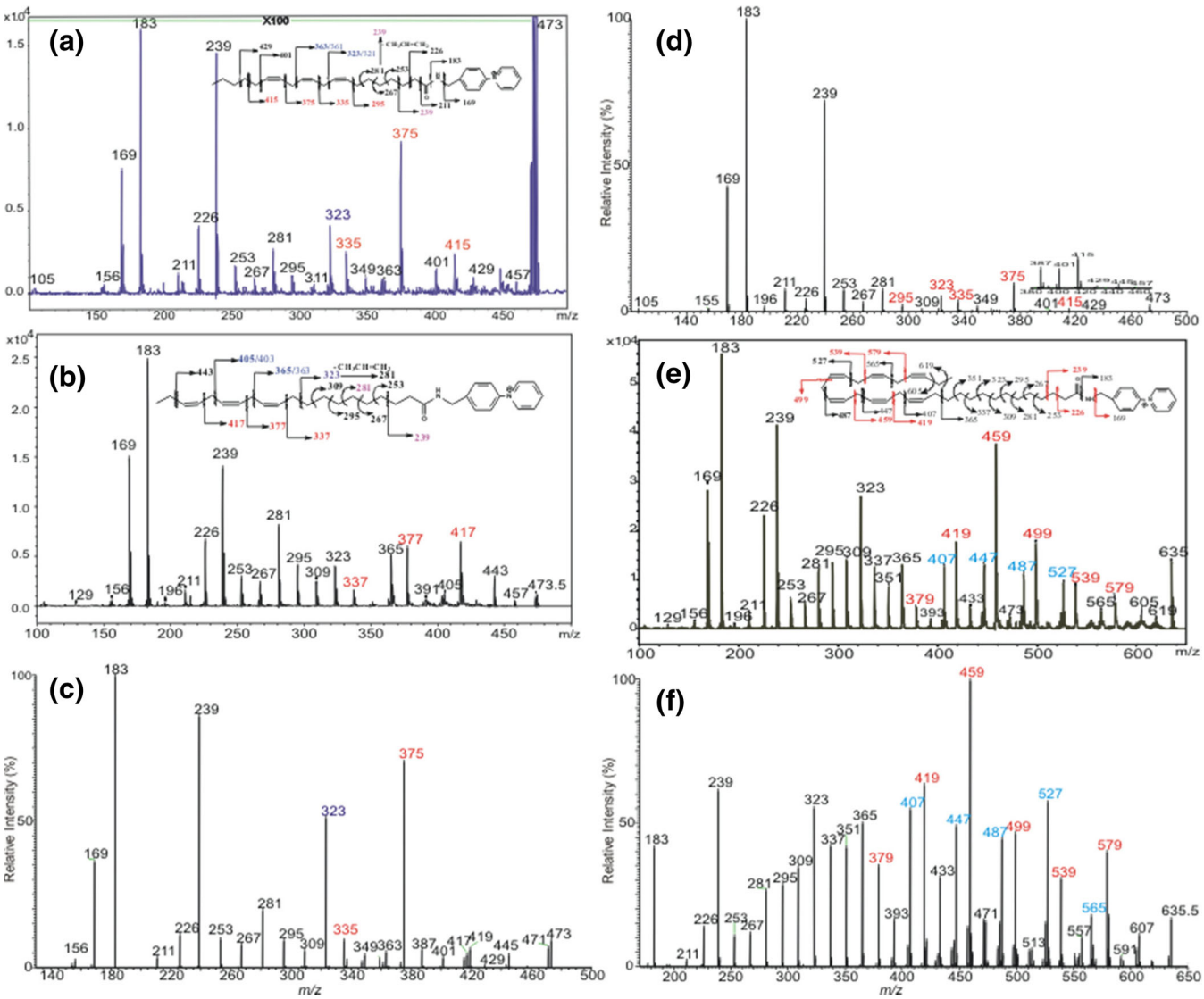
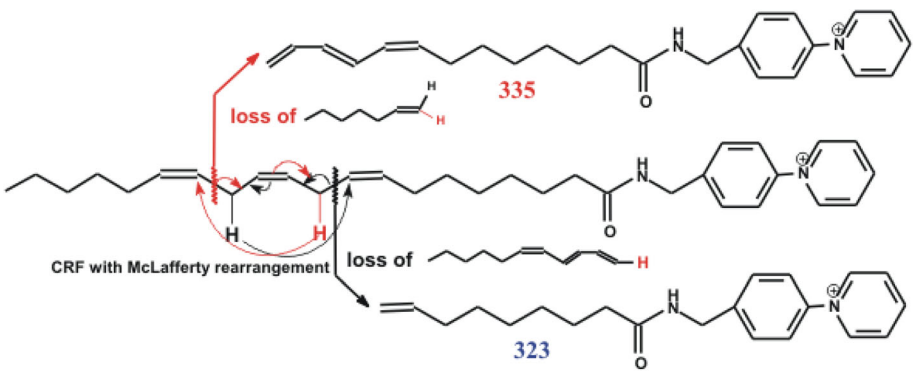


Figure 2. The MALDI LIFT TOF-TOF spectra of the M^+ ions of *m/z* 473 from $\Delta^{8,11,14}$20:3-AMPP (**a**), $\Delta^{11,14,17}$20:3-AMPP isomers (**b**), and of *m/z* 635 ions from $\Delta^{14,17,20,23,26,29}$32:6-AMPP (**c**). Panel d and panel e show the MS^2 spectra of $\Delta^{8,11,14}$20:3-AMPP obtained by a LIT instrument applying low-energy CID (optimized to 65% normalized CE) (**c**), and HCD (optimized to 50% normalized CE) (**d**). Panel f shows the low energy CID (60% normalized CE) LIT MS^2 spectrum of $\Delta^{14,17,20,23,26,29}$32:6-AMPP, and the spectrum is similar to that acquired by LIFT TOF-TOF (panel **c**)



Scheme 2. The CRF (with allylic-H shift) fragmentation pathways proposed for the formation of ions of *m/z* 323 and 335

(d) Fatty Acids with Methyl Branches FAB tandem mass spectrometry is useful for determination of iso- and anteiso-fatty acid isomers as $[M–H]^-$ ions [42]. Product ion spectra of AMPP derivative of iso- and anteiso-fatty acid obtained with low-energy CID with LIT instrument have also been previously used for differentiation of isomers, but the fragment ions indicative of the methyl side chain are of low abundance [28]. By contrast, the LIFT MALDI TOF-TOF mass spectrometric approach clearly locates the methyl branch. For example, the spectrum of 15-methyl-hexaoctanoic acid (iso-17:0) AMPP at *m/z* 437 (Fig. 3b) contains ions at *m/z* 421 from loss of CH_4, and the ions at *m/z* 393, 379, 365, 341,.. etc. arising from CRF loss of C_nH_{2n+2} residues. The 28 Da ($CHCH_3$) gap between *m/z* 421 and 393 in the series locates the methyl side chain at C15. Similarly, the LIFT MALDI TOF-TOF mass spectrum of 14-methyl-hexaoctanoic acid (anteiso-17:0)-AMPP at *m/z* 437 (Fig. 3c) is featured by the presence of ions of *m/z* 421 (loss of CH_4), 407 (loss of C_2H_6), together with ions of *m/z* 379, 365, 351, 337, 323, …etc. from CRF loss of C_nH_{2n+2} residues, pointing to the assignment of the methyl side chain at C14.

Figure 3d illustrates the MALDI LIFT TOF-TOF spectrum of phytanic acid (3,7,11,15-tetramethylhexadecanoic

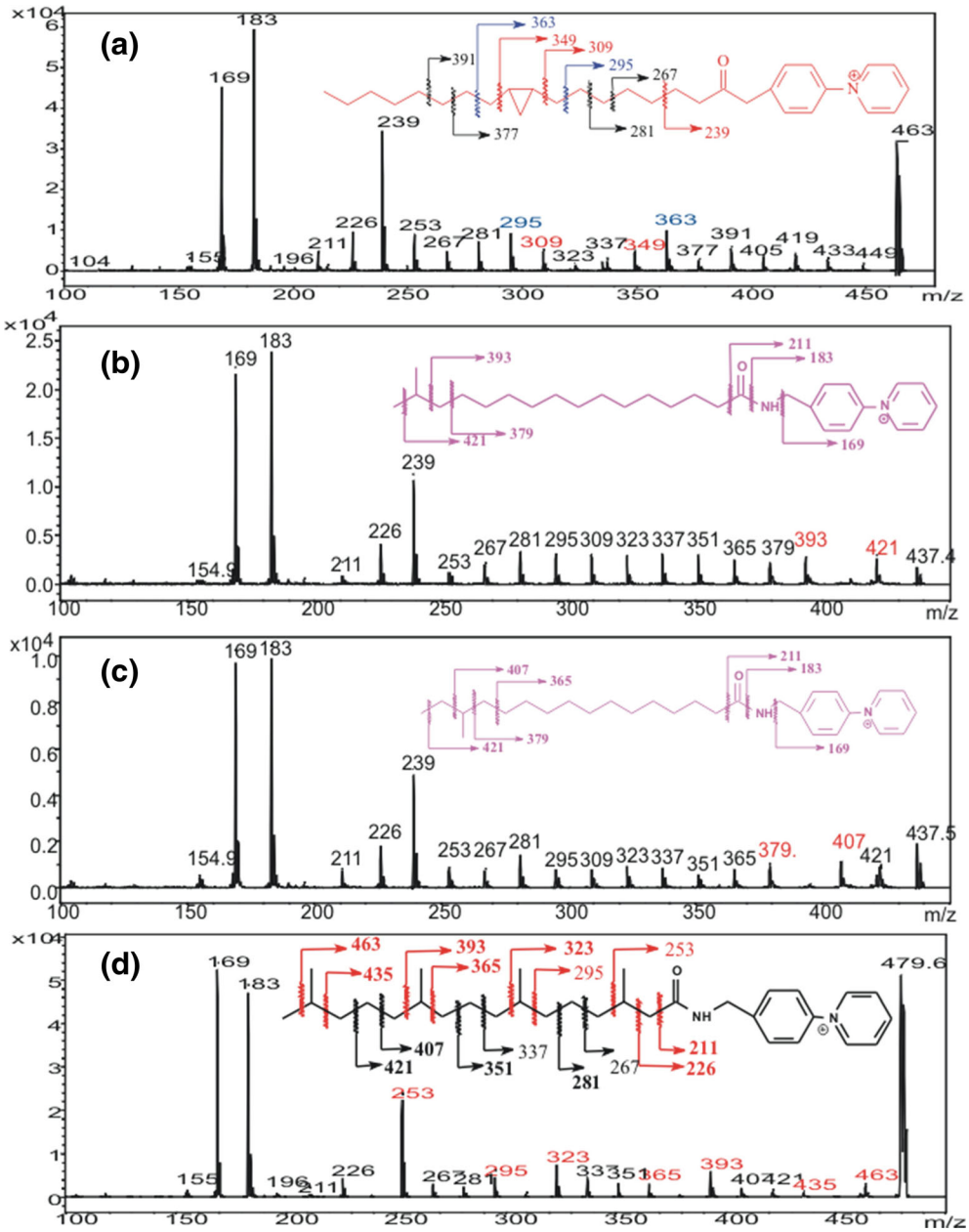


Figure 3. The MALDI LIFT TOF-TOF spectrum of the M^+ ions of (**a**) cPro(9)19:0-AMPP at *m/z* 463, (**b**) 14-methyl-hexadecanoic acid-AMPP (anteiso), and (**c**) 15-methyl-hexadecanoic acid-AMPP (iso) at *m/z* 437; and of 3,7,11,15tetramethyl hexadecanoic acid-AMPP (phytanic acid) at *m/z* 479. These TOF-TOF spectra easily locate the methyl side chain(s) along the long fatty acid chains

acid)-AMPP derivative, consisting of multiple methyl branches. The major fragment ions arose from cleavages of the C–C bond that is flanked to the carbon connected to the methyl branches. Thus, ions of *m/z* 253, 323, 393, and 463 arose from cleavages of the $C(4)H_2$-$C(3)H(CH_3)$, $C(8)H_2$-$C(7)H(CH_3)$, $C(12)H_2$-$C(11)H(CH_3)$, $C(16)H_2$-$C(15)H(CH_3)$ bonds, and ions of *m/z* 226, 295, 365, and 435, arose from cleavages of the $C(2)H_2$-$C(3)H(CH_3)$, $C(6)H_2$-$C(7)H(CH_3)$, $C(10)H_2$-$C(11)H(CH_3)$, $C(14)H_2$-$C(15)H(CH_3)$ bonds, respectively. Again, the intermittent 28 Da interval as seen in the spectrum (see inset for fragmentation scheme) indicates that the methyl branches are located at C3, C7, C11, and C15.

(e) Fatty Acids with Various Hydroxyl Group and Double Bond To characterize fatty acids with or without double bond with hydroxyl group at various locations using LIFT MALDI TOF-TOF is shown by the spectra of 3-hydroxy heptadecanoic acid (βh17:0)-AMPP at *m/z* 453 (Fig. 4a), 2-hydroxytetraeicosanoic acid (αh24:0)-AMPP at *m/z* 551 (Fig. 4b), and 12-hydroxy-9-cis-octadecenoic (ricinoleic) acid (h(12)$\Delta^9$18:1)-AMPP at *m/z* 465 (Fig. 4c). Figure 4a is dominated by the ion of *m/z* 227 arising from loss of $CH_3(CH_2)_{13}CHO$ residue via cleavage of C3(OH)–C2 bond. This loss of $CH_3(CH_2)_{13}CHO$ residue combined with an additional loss of CH_2=C=O led to *m/z* 185, departing it from the signature ion of *m/z* 183 commonly seen for FA-AMPP derivatives. The spectrum also contains the ion of *m/z* 255 arising from cleavage of C3(OH)–C4 bond. These *m/z* 227 and 255 ions arising from cleavages of the C–C bonds adjacent to the carbon bearing the hydroxyl side chain readily recognize the 3-hydroxyl group. The ion series of *m/z* 423, 409, 395, etc., with a CH_2 (14 Da) interval, also recognize the aliphatic alkyl chain, while the presence of *m/z* 435 arising from loss of H_2O is consistent with the presence of the hydroxyl group in the molecule.

In contrast, the TOF-TOF spectrum of 2-hydroxy-tetraeicos-15-enoic acid (h24:0)-fatty acid (Fig. 4b) contains ions of *m/z* 213 (loss of $C_{21}H_{43}CHO$), 185 (loss of [$C_{21}H_{43}CHO$+CO]), arising from cleavages of the C–C bond involving the participation of the 2-hydroxyl group (see inset for fragmentation scheme). The observation of the low abundances of the ion series arising from CRF elimination of C-terminal C_nH_{2n+2} residue (i.e., ions of 535, 521, …etc), again, supports the notion that the structure of the molecule (i.e., compound type), other than the applied collision energy, plays a pivotal role in the fragment ion formation as described earlier [30]. The spectrum also contained the ions of *m/z* 505, likely arising from a minor side reaction product of 2-N-(4-aminomethylphenyl) pyridinium tetraeisanoic acid isomer which gave rise to ions of *m/z* 505, by losses of (H_2+CO_2). The compound most likely arose from a reaction in which the AMPP reagent reacts with the α-OH group and gives rise to 2-N-(4-aminomethylphenyl) pyridinium tetraeisanoic acid (see supplemental material, Scheme s1).

The MALDI LIFT TOF-TOF spectrum of 12-hydroxy-9-cis-octadecenoic acid-AMPP at *m/z* 465 (Fig. 4c) is dominated by ions of *m/z* 351 arising from elimination of heptaldehyde ($C_6H_{13}CHO$) by cleavage of C11-C(12)HOH bond [43]. The presence of the ions of *m/z* 379 arising from cleavage of C(12)HOH–C(13) bond and of *m/z* 295 arising from allylic cleavage of C7–C8 bond is consistent with the assigned structure.

Again, the HCD MS^2 spectra of the 3-hydroxy heptadecanoic acid-AMPP at *m/z* 453 (Figure s5a), 2-hydroxytetraeicosanoic acid-AMPP at *m/z* 551 (Figure s5b), and ricinoleic acid-AMPP at *m/z* 465 (Figure s5c) are similar to their corresponding LIFT TOF-TOF spectra, respectively (Fig. 4a–c). However, ions from CRF losses of the C_nH_{2n+2} residues are either absent (Figure s5a and s5c) or of low abundance (Figure s5b), consistent with the notion that CRF cleavages of C_nH_{2n+2} of the alkyl tail require high collision energy (keV), as seen earlier.

Characterization of Very Long Chain Fatty Acid Substituents of Biological Origins

(a) Mycolic Acid from Mycobacterium tuberculosis (-M. tuberculosis) Current MALDI-TOF method for profiling mycolic acids from *M. tuberculosis* requires first conversion of mycolic acids to fatty acid methyl esters (FAME), followed by analysis of the derivatives as sodium adduct ions [21, 22], and there is no report on the structural characterization by MALDI TOF/TOF. To test the current method for mycolic acid analysis, *M. tuberculosis* mycolic acids were first converted into AMPP derivative, followed by MALDI-TOF analysis. The profile of the MALDI-TOF spectrum (Fig. 5a) is nearly identical to that of the native mycolic acids detected as the $[M–H]^-$ ions by ESI using an ion-trap instrument (Fig. 5b), but the sensitivity and resolution are significantly improved. The results clearly demonstrate the utility of the approach in the profiling of microbial mycolic acids.

To further investigate LIFT TOF/TOF in the structural identification of this lipid family, the product ion spectrum of the M^+ ions of *m/z* 1420.3, representing a C-86 methoxy-mycolic acid-AMPP was acquired (Fig. 5c). The spectrum is dominated by the ions of *m/z* 563, arising from elimination of the meromycolate chain via cleavage of the C2–C3(OH) bond to form the M^+ ions of 26:0-FA-AMPP, indicating the presence of α-tetraeicosyl (C_{24}) chain in the molecule. The spectrum also contains the ions of *m/z* 535, representing 24:0-FA-AMPP arising from loss of meromycolate residue with a C2 longer chain, indicating the presence of a minor isomer with α-docosanoyl (C_{22}) branch. The spectrum also contained the ions of *m/z* 169 and 185, signifying that the compound consists a β-OH residue as seen earlier (Fig. 4a).

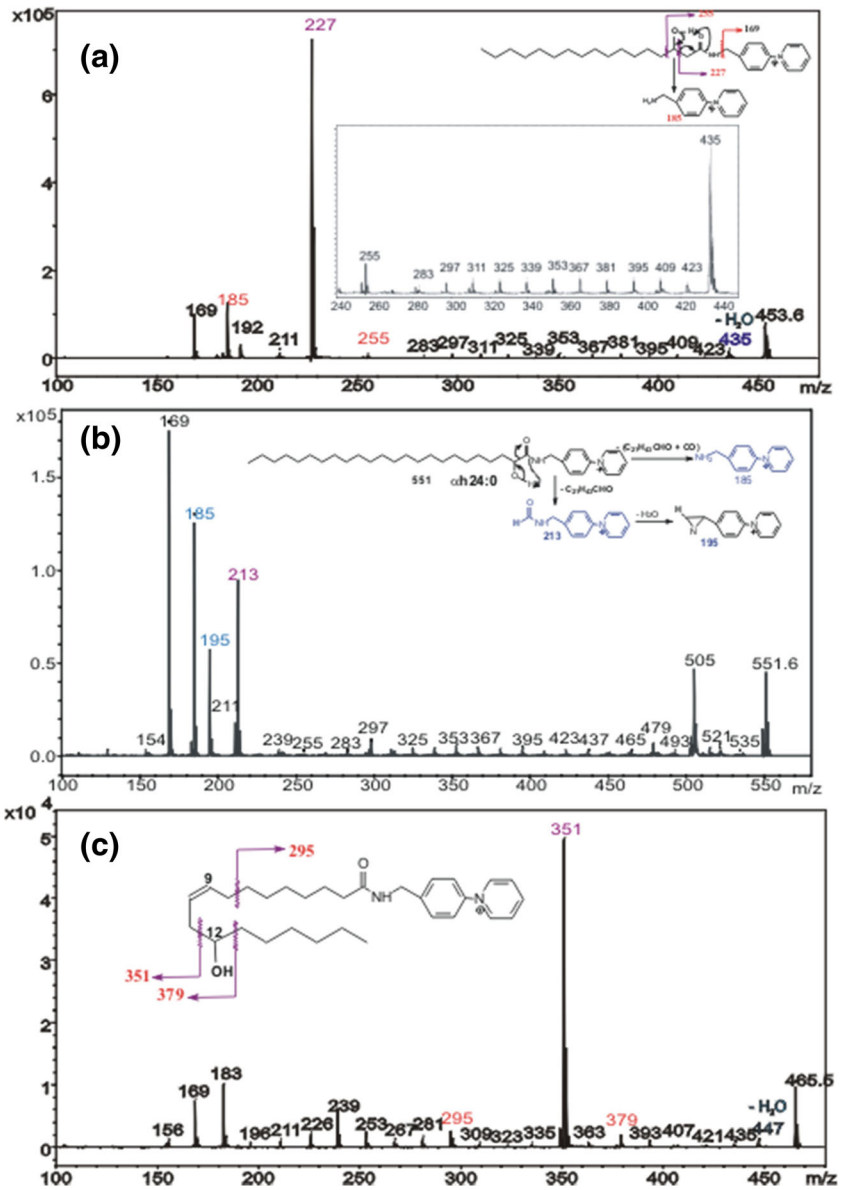


Figure 4. The MALDI LIFT TOF-TOF spectrum of the M^+ ions of (**a**) 3-hydroxy-heptadecanoic acid-AMPP at *m/z* 453, (**b**) 2-hydroxy-tetraeicosanoic acid-AMPP at *m/z* 551, and (**c**) 12-hydroxy-9-cis-octadecenoic acid-AMPP at *m/z* 465. The facile cleavage of the C–C(OH) bond distal to the aliphatic tail is reflected by the dominance of the ions as marked (in pink)

The structural information, such as the location of the methoxy branch and cyclopropyl chain on the meromycolate backbone of the molecule, is absent. This failure in characterization of the meromycolate chain may be attributable to the facile loss of the meromycolate residue as an aldehyde to form 26:0-FA-AMPP ions. This loss of meromycolate molecule deprives the CRF processes that may cleave the chain requiring a high (keV) collision energy. Thereby, the structural information of meromycolate chain is lost.

(b) Hydroxyphthioceranoic Acid in the Cell Envelope of M. tuberculosis (H37Rv Strain) In contrast, the MALDI LIFT TOF/TOF spectrum of the M^+ ions of 2,4,6,8,10,12,14,16-octamethyl-17-hydroxydotriacontanoic acid (hC_{40})-AMPP at *m/z* 775 (Fig. 5d) contains the abundant ion pairs at *m/z* 253/281, 295/323, 337/365, 379/407, 421/449, 463/491, and 505/533, arising from cleavages the C–C bonds flanked to the carbon bearing the methyl branches. The observation of these ions led to assignment of the multiple methyl side chains at C2, 4, 6, 8, 10, 12, 14, and 16. The spectrum also contained the ion at *m/z* 563, which is 30 Da (CH_2O) heavier than the ion of *m/z* 533, along with the ion series at *m/z* 577, 591, 605, 619, 633, 647… arising from CRF cleavages of C_nH_{2n+2} residues from the aliphatic tail. The results clearly locate the hydroxyl side chain at C-17 along the long C_{32}-fatty acid chain. By contrast, ions of *m/z* 577, 591, 605, 619, 633, 647… from CRF loss of C_nH_{2n+2} residues are of low abundance in the CID tandem mass spectra obtained with an ion-trap instrument, and the structural information of the alkyl

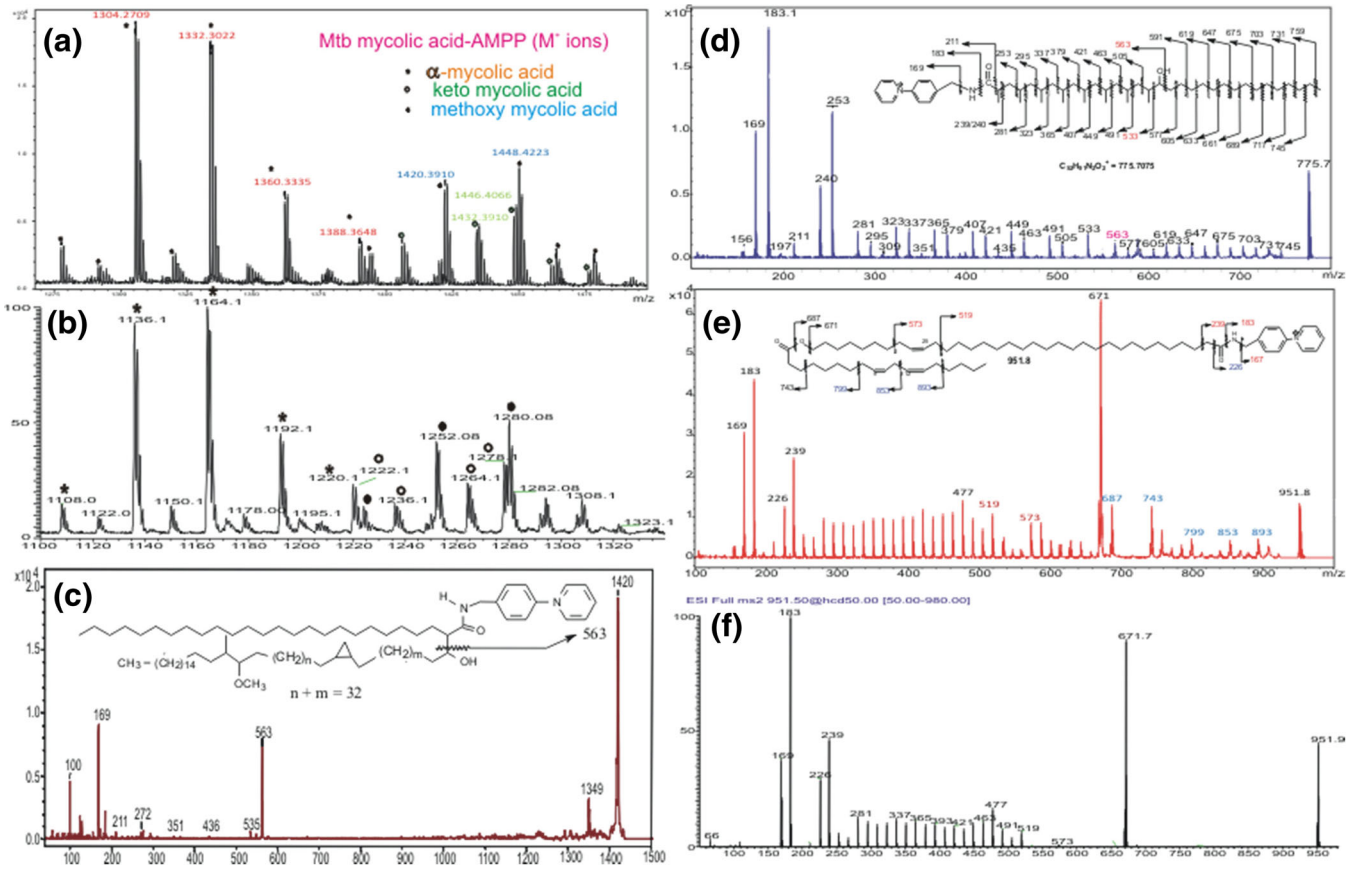


Figure 5. (**a**) The MALDI TOF (reflectron) spectrum of the M^+ ions of *M. tuberculosis* (bovine) mycolic acid-AMPP derivative and (b) the corresponding ESI mass spectrum seen as the $[M–H]^-$ ions of the underivatized mycolic acid obtained with a linear ion trap instrument. Panel c shows the MALDI LIFT TOF-TOF spectrum of a methoxy mycolic acid-AMPP species of *m/z* 1420, containing a α-tetraeicosanoyl (C24) chain. Panel d shows the LIFT TOF-TOF spectrum of the ions of C-40 HPA-AMPP at *m/z* 775, consisting of multiple methyl branches and hydroxyl side chain; and the fragmentation scheme (inset). Panel e and panel f show the MS^2 spectra of the M^+ ions of O-linoleoyl ω-hydroxytetratriacontenoic acid (O-$\Delta^{9,12}$-18:2-ωhΔ^{25} 34:1)-AMPP at *m/z* 951 obtained with LIFT TOF-TOF and LIT/HCD, respectively. The structural information of the $\Delta^{9,12}$-18:2 terminus is missing in Fig. 5f, due to absence of ions from CRF losses of C_nH_{2n+2} residues of the aliphatic tail that requires high collision energy. Please note that a cis configuration in inset is drawn; however, distinction between cis and trans isomers cannot be made by tandem mass spectrometry

chain terminus is lost [25]. These results, again, underscore the utility of LIFT TOF/TOF in the comprehensive structural analysis of complex long-chain fatty acids.

(c) O-Linoleoyl-ω-Hydroxy-Δ^{25} Tetratriacontenoic Acid (O-$\Delta^{9,12}$-18:2-ωhΔ^{25} 34:1) in Mouse Epidermis The MALDI LIFT TOF-TOF spectrum of O-$\Delta^{9,12}$-18:2-ωhΔ^{25} 34:1-AMPP is shown in Fig. 5e, which is dominated by the ion of *m/z* 671, arising from elimination of the terminal 18:2-fatty acid substituent. Ions from allylic cleavages of the ω-hydroxytetratriacontenoic acid (ωOH34:1) chain seen at *m/z* 573 and 519, together with the ion series of *m/z* 505, 491, 477, 463, 449, 435, 421, 407, 393, … etc. from CRF cleavage of C_nH_{2n+2} residues, locate the double bond at C25 of the ωOH34:1 FA chain. The ions from allylic cleavages of the 18:2-fatty acyl tail linked to the main ωh34:1-FA chain were seen at *m/z* 893, 853, and 799, which readily locate the double bond of the 18:2-FA moiety at C9 and C12. These results, therefore, led to a complete structural characterization of the molecule. In contrast, ions that are important for locating the unsaturated bonds are absent in the LIT HCD MS^2 spectrum (Fig. 5f), and complete structural analysis requires hydrolysis step to release the18:2-FA, followed by tandem mass spectrometry analysis (unpublished results).

Conclusions

The conversion of FA to FA-AMPP derivative permits its sensitive detection by MALDI TOF, and structural characterization by LIFT TOF-TOF with or without matrix becomes possible. Structural analysis of the $\Delta^9$18:1-AMPP (Figure s6a) and 24:0-AMPP (Figure s6b) (supplemental material) isolated from skin by LIFT TOF-TOF with no matrix showed clean spectra indicating that possible interference from matrix if present can be eliminated, but the

sensitivity is significantly lower. The present "LIFT" technology for collection and generation of fragment ions is a laser-induced dissociation (LID) process similar to PSD, and there is no significant difference in the product-ion spectra obtained with or without application of Ar collision gas, consistent with the previous findings [44]. This observation is also consistent with the earlier observation that MALDI-PSD is amenable to charge-remote fragmentation, and both MALDI-PSD and MALDI-CID spectra are similar to high-energy CID FAB mass spectra [45]. Because fragmentations in MALDI LIFT TOF/TOF are HE CID processes, ions from CRF losses of C_nH_{2n+2} residues along the aliphatic chain are more abundant and complete than those obtained by low-energy HCD/CID tandem mass spectrometry.

No study was carried out to measure the ion yields after LID, but the dissociation efficiency (precursor to product ion conversion) appears to be low (as an example shown in Fig. 2c, the fragment ion region is amplified by 100 times). This low efficiency is similar to that observed by HE CID tandem mass spectrometry with a sector instrument [46].

The unexpected artificial fragment ions are often present in the LIFT TOF-TOF spectra. The artifacts may arise from the "ion family" with same velocity entering the "LIFT" device after desorption with the elevated laser power for LID, but not filtered by the "timed ion selector" device. The artificial ions can be abundant in the product-ion spectra of lipid specimen in a mixture (e.g., biological samples in nature). These unpredictable artificial ions confound data interpretation, thus, hampering the application of this LIFT TOF-TOF method in the structural analysis of complex lipids. The application in lipid analysis is also limited by the precursor ion selection window of the instrument, which is designed for admission of all the isotopic ions, e.g., of peptide. Therefore, a fatty acid mixture consisting of two structures with a 2 Da difference in mass, for example, a sample consisting of 18:1-FA-AMPP and 18:0-AMPP that form ions of *m/z* 449 and *m/z* 451, respectively, are not separable, and their LIFT TOF-TOF product-ion spectra are indistinguishable. In this regard, MALDI-TOF/TOF instrument with monoisotopic precursor ion isolation feature becomes the most attractive and has shown promising results for lipid analysis [11, 12, 17, 18, 47].

Application of high-energy CID MALDI TOF-TOF for characterization of lipid structure as shown in this study deserves more attentions due to the many advantages of this technique over the other mass spectrometric approaches. Whether it has a niche in the characterization of complex lipid structures requires more evaluation. To explore the ever-expanding complex lipid structure, improvement in the techniques more suitable for the analysis is necessary.

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