Charge-Remote Fragmentation of Lithiated Fatty Acids on a TOF-TOF Instrument Using Matrix-Ionization

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In numerous studies charge remote fragmentation (CRF) has been shown to be a powerful technique for determination of primary structure by allowing location of double bonds, various functional groups, and branching in a variety of compound types directly by mass spectrometry. Instrumentation and ionization methods traditionally used for CRF, however, are becoming rare, in large part because ESI and MALDI have to a significant extent replaced them. Here we demonstrate that by selecting a matrix that promotes rather than suppresses ionization of fatty acids (FA) by lithium ion adduction, and using a TOF-TOF mass spectrometer for high-energy collisional activation, CRF ions are produced that allow location of double-bond and branching positions. Further, we show that by using solvent-free MALDI sample preparation methods, thus eliminating the inherent segregation of the hydrophobic fatty acid from the hydrophilic LiCl that can occur during the evaporation of solvent, the desired $[FA-H+2Li]^+$ ions are greatly enhanced. Because FAs can be vaporized using laser desorption, matrix assistance in desorption of the fatty acid may occur, but is not necessary. However, the matrix plays a crucial role in enhancing or suppressing ionization. For example, matrix materials with acid (e.g., 2,5-dihydroxybenzoic acid) or hydroxy groups (e.g., dithranol) compete with the FA for Li^+ and because of the high ratio of matrix to analyte, FA lithium adduction is minimized. However, highly electron-deficient matrix materials (e.g., TCNQ) readily donate Li^+ to FAs because of the instability associated with being positively charged. (J Am Soc Mass Spectrom 2007, 18, 1967–1972) © 2007 American Society for Mass Spectrometry

Charge remote fragmentation (CRF) has been shown by Gross and others to have considerable analytical utility because fragmentation remote to the charge site occurs without carbon skeletal rearrangements [1–9]. Thus, features such as double-bond and branching positions of various functional groups can be determined directly by mass spectrometry (MS). CRF, however, requires ionization methods that provide fixed localized charge as well as high-energy collisional activation (CA). Most of the original studies of CRF involved soft ionization using fast atom bombardment (FAB) and high-energy MS/MS fragmentation afforded by magnetic sector instruments; various other ionization methods and instrumentation have been used for selected compound types [10]. However, the success of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) has resulted in instrumentation suitable for these ionization methods replacing newer ionization methods such as FAB as well as the instrumentation capable of high-energy CA, thus eliminating CRF capability in many laboratories.

Although there has been some controversy with respect to the energy regime of the CA process using MALDI-TOF instrumentation [10], it nevertheless offers the possibility of high-energy CA. Domingues and colleagues, for example, demonstrated CRF using MALDI post source decay and collisionally induced dissociation (CID) on fatty acids (FA) that were derivatized using a porphyrin macrocycle not only to aid desorption, but also to localize the charge site [11]. This work suggests that the internal energy of the collision activated ions is within the range of 1.4 to 2.9 eV reported to be necessary for CRF [3]. These authors also noted that MALDI is not amenable to ionization of FAs [11].

Because lipids and their FA constituents serve important biological functions [1] and the study of these materials is gaining in importance, we undertook to determine whether CRF amenable ions could be produced for MS/MS fragmentation in a TOF-TOF instrument. Although FA analysis is commonly accomplished using separation techniques such as gas chromatography to compare FA methyl esters to standards, these methods, although extremely useful when dealing with

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complex mixtures, are time intensive when dealing with high numbers of samples and are useful only for analyses in which standards are available [12]. Various mass spectrometric techniques have been used to determine positions of functionality [1], although none can compare to CRF and high-energy MS/MS fragmentation in the ability to instantly provide data for double-bond and branching location in FAs. CRF could become an important technique for high-throughput analyses.

The most straightforward analysis of FAs by CRF is fragmentation of the lithium ion adduct of the lithium salt, i.e., $[FA-H+2Li]^+$. This ion is readily produced using FAB ionization [2], demonstrating that the ion is stable to mass spectrometric analysis. Formation of this ion in MALDI should therefore also be possible. In fact, both the $[FA+Li]^+$ and the $[FA-H+2Li]^+$ ions are observed, although not with good sensitivity, by laser desorption of a mixture of stearic acid and LiCl placed on the MALDI target plate. We postulated that the $[FA-H+2Li]^+$ ion is not observed under solvent-based MALDI conditions, either because segregation of the hydrophobic FA from the lithium salt during the crystallization process reduces the opportunity for metal ion adduction [13] or ion suppression caused by charge competition for Li^+ from the matrix hinders ionization. The former problem can be addressed by solvent-free MALDI in which the grinding method increases the probability that the FA and salt are in intimate contact [13]. The latter can be addressed by selecting a matrix material that will not compete for the metal cation.

Solvent-free MALDI analysis readily ionizes compounds by metal adduction that are insufficiently basic to compete for a proton with common matrix materials used in MALDI analysis. This includes such compound types as polymers [14] and even peptides that contain no basic sites [15]. The recent multiplexing of the solvent-free MALDI method of sample preparation and simultaneous transfer of the matrix/salt/analyte powder to the MALDI target plate provide a path for high-throughput analyses and for reduced analyte requirements for solvent-free MALDI [16, 17]. The multiplex method was applied here to demonstrate that the combination of applying the solvent-free method for enhanced metal adduction and matrix selection based on eliminating matrix suppression of analyte ionization results in facile formation of the desired $[FA-H+2Li]^+$ ion. We also demonstrate that MS/MS on TOF-TOF instrumentation provides CRF ions that make it possible to determine the position of multiple double bonds in FAs as well as sites of branching.

Experimental

Material

2,5-Dihydroxybenzoic acid (DHB), dithranol, *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]-malononitrile (DCTB; Fluka grade) lithium chloride, and 7,7,8,8-tetracyanoquinodimethan (TCNQ) were obtained from Aldrich (Milwaukee, WI). The instrument used for MS was a Perseptives Biosystems (Framington, MA) Voyager-DE STR MALDI mass spectrometer and for MS/MS analyses was an ABI4800 MALDI TOF-TOF mass spectrometer (Applied Biosystems, Framington, MA).

Sample Preparation

A general protocol was used that homogenizes and transfers multiple dry matrix/salt/analyte samples directly to the MALDI plate [16, 17]. Briefly, matrix and LiCl salt were premixed in a 3:1 ratio by weight (molar ratio of about 1:1.5) and homogenized in a 2 mL vial using several 1.2 mm stainless steel balls (Biospec Products, Bartlesville, OK) for a period of 8 min on a vortex device (VWR Vortexer 2, VWR International, Gibbstown, NJ). The matrix/salt/bead mixture, after homogenization, was placed on weighing paper and using a small spatula sufficient material was shuffled into the 36 wells of a 96-well Nunc Bacti plate (Nunc, Roskilde, Denmark), which had been preloaded with 0.1 to 0.5 mg of various fatty acids to completely cover the bottom of the well and the fatty acid in the well. A 384-MALDI plate (Applied Biosystems), which fits exactly 96 wells of the 96-well Bacti plate, was tightly held in place over the filled wells using duct tape and vortexed for 10 min, flipping the device several times to ensure representative transfer of sample to the MALDI plate. The MALDI plate was detached from the Bacti plate and excess powder was removed using a stream of nitrogen gas before insertion into the MALDI ion source.

Nomenclature

In the present work we describe fragment ions that correspond to radical cations such as $[CO_2Li+Li]^{\cdot+}$ as $C_{1'}$ and refer to $[CH_2{=}CHCO_2Li+Li]^+$ as C_3.

Results and Discussion

Solvent-free sample preparation conditions were used with DHB and dithranol matrices that were pre-ground with LiCl followed by multiplexed vortexing with metal beads and stearic acid. Both $[RCO_2Li+Li]^+$ and $[RCO_2H+Li]^+$ ions, where $R = H(CH_2)_{17}$, were observed, but in low abundance relative to matrix and other background ions. Solvent-based sample preparation produced no lithium adducted stearic acid with DHB and a barely perceptible ion with dithranol. The solvent-free preparation method resulted in an increase in lithium adducted ions, but was still not satisfactory as a means of ionizing FAs or for making the desired $[FA-H+2Li]^+$ ions for CRF. Ion suppression of the analyte by the matrix was a possible explanation for the low lithium adducted FA salt observed using DHB or dithranol as matrices. Therefore, a matrix material was needed that promoted rather than suppressed lithium ion adduction to the FAs.

7,7,8,8-Tetracyanoquinodimethan (TCNQ) has previously been used as a matrix material for the MALDI analysis of such compounds as polycyclic aromatic hydrocarbons [14, 15, 18], dendrimers [14], synthetic polymers [19, 20], carbon pitches [21], and fullerenes [14, 22]; molecular ion species are often produced using significantly lower laser power [14, 20]. Because TCNQ is aprotic and very electron deficient, we reasoned that it would be an ideal candidate to transfer a Li^+ ion from a lithium salt to FAs. TCNQ is known to interact with a variety of salts, including lithium salts, by charge-transfer with the salt anion to form the TCNQ singly and doubly charged anions [23]. Mixing TCNQ with LiCl using the solvent-free preparation method resulted in the matrix color going from reddish-brown to green, upon transfer to the MALDI plate, indicative of formation of the TCNQ anions [23]. When irradiated using a 337 nm UV laser $[TCNQ]^+$, $[TCNQ+H]^+$, were produced as well as a series of lithium adducted ions ($[TCNQ+Li]^+$, $[TCNQ+2Li]^+$, $[TCNQ+3Li]^+$, $[2TCNQ+3Li]^+$, $[2TCNQ+4Li]^+$, $[2TCNQ+5Li]^+$). Interestingly, MS/MS of any of the TCNQ dimers produced the $[TCNQ+3Li]^+$ ions, suggesting that the cation of the TCNQ triply lithiated dianion produces a stable complex with TCNQ and neutral lithium salts of the TCNQ anion and dianion.

The electron deficiency of TCNQ is expected to make transfer of Li^+ from lithium cationized TCNQ and TCNQLi salts to a FA or FA lithium salt exothermic. This reaction was found to be so efficient that solvent-based MALDI using TCNQ as matrix with LiCl produced abundant $[FA+Li]^+$ and the desired $[FA-H+2Li]^+$ ions; isotopic distributions are similar to those described by Huysmans and colleagues [24], which are attributed to the production of ions with a high internal energy and fragment partially through loss of a H radical and H_2. However, using the solvent-free preparation method with this same mix of materials produced mass spectra of all FAs tested where the $[FA+Li]^+$ and the desired $[FA-H+2Li]^+$ ions dominate even the matrix ions (Figure 1). This is remarkable, especially in view of the expectation that MALDI is not suitable for producing the desired CRF ions from FAs [11]. These results demonstrate that solvent-free sample preparation is superior for this application and that taking into account ionization suppression of analyte by the matrix can lead to ionization of compound types thought not to be amenable to MALDI MS analysis.

Other electron-deficient matrix materials in combination with the solvent-free preparation method and a lithium salt might also be expected to produce lithium adducted FAs. Although not as efficient as TCNQ, DCTB—another aprotic electron-deficient compound—was shown to produce lithium adducted FAs. Other matrix materials that do not compete for the lithium cation might also be expected to allow metal adduction of FAs. The laser energy necessary for observation of Li^+ adducted FA ions is significantly lower than that required to observe these ions using laser desorption

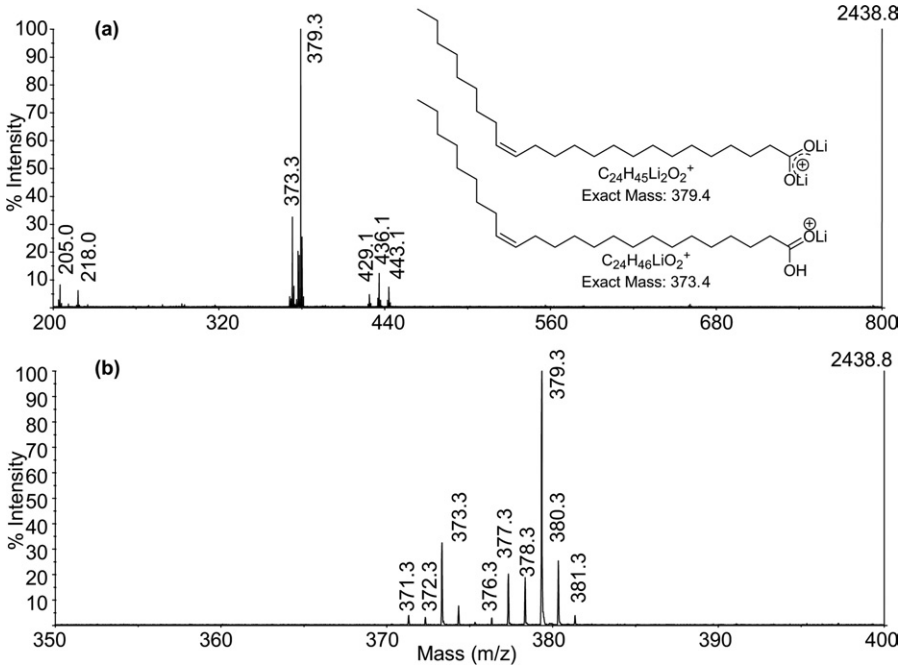

Figure 1. (**a**) Mass spectrum of nervenoic acid ($C_{24}H_{46}O_2$) prepared with solvent-free conditions using TCNQ mixed with LiCl as matrix and acquired using a relative laser power of 3500 on an ABI 4800 MALDI TOF-TOF mass spectrometer. The molecular ion species observed at *m/z* 205, 218, 429, 436, and 443 represent $[TCNQH]^+$, $[TCNQ+2Li]^+$, $[2TCNQ+3Li]^+$, $[2TCNQ+4Li]^+$, and $[2TCNQ+5Li]^+$, respectively. (**b**) The molecular ion species of nervenoic acid with *m/z* 373 representing $[M+Li]^+$ and *m/z* 379 $[M-H+2Li]^+$. Isotopes of the two lithiums contribute to the satellite peaks, as well as H radical and H_2 abstraction [24].

without the presence of a matrix, indicating that matrix assistance to desorption occurs. However, for FAs and other semivolatile compounds, matrix assistance for desorption might be desirable but unnecessary. This work suggests that for FAs and other volatile and semivolatile compounds, matrix ionization after laser

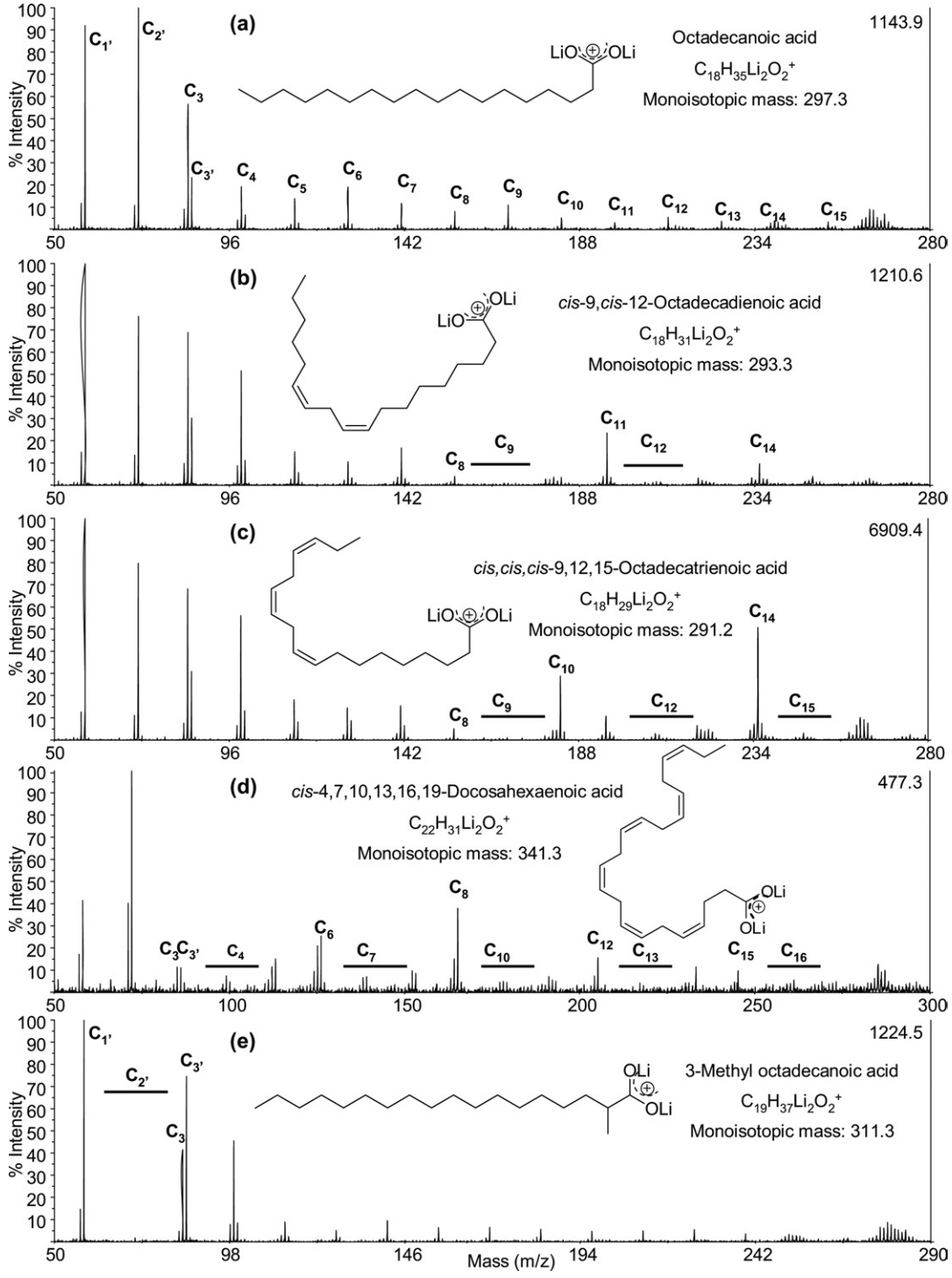

Figure 2. MS/MS spectra of the $[FA-H+2Li]^+$ product ions of (**a**) stearic acid ($C_{18}H_{36}O_2$), (**b**) *cis*,*cis*-9,12-octadecadienoic acid ($C_{18}H_{32}O_2$), (**c**) *cis*,*cis*,*cis*-9,12,15-octadecatrienoic acid ($C_{18}H_{30}O_2$), (**d**) *all cis*-4,7,10,13,16,19-docosahexaenoic acid ($C_{22}H_{32}O_2$), and (**e**) 3-methyl octadecanoic acid ($C_{19}H_{38}O_2$) obtained as in Figure 1 except with a higher relative laser power of 5400. The peaks labeled C_3 to C_{15} are CRF ions starting at the carboxy terminus (ions labeled $C_{1'}$–$C_{3'}$ are radical cleavage products). The molecular ion species is about fivefold more abundant than the most abundant product ions and is not shown to aid visualization of the CRF ions. Isotopes of the two lithiums contribute to the satellite peaks.

desorption is necessary, but matrix assistance for desorption may not be a requirement.

A TOF-TOF mass spectrometer was used to determine whether the $[FA-H+2Li]^+$ ions upon CA produced CRF. Mass selection of these ions from a number of unsaturated (mono and poly) and branched FAs upon CA all produced excellent CRF, thus allowing determination of the positions of unsaturation or branching. Deterding et al. [3] showed the internal energy necessary for production of CRF ions following CA to be between 1.4 and 2.9 eV. Obviously, the $[FA-H+2Li]^+$ product ions in the TOF-TOF instrument achieve the correct energy to produce CRF. Figure 2 shows the MS/MS fragmentation pattern of the $[FA-H+2Li]^+$ product ions from FAs containing 0, 2, 3, and 6 double bonds and a FA branched in the 2 position. Figure 2 does not show the molecular ion species of the spectrum to make the fragmentation more obvious. The MS/MS spectrum of the $[FA-H+2Li]^+$ mass selected molecular ion species of stearic acid is shown in Figure 2a. Because the alkyl chain is completely saturated, the product ions, denoted C_n, $n = 1$ to 3 and C_n with $n = 3$ to 15, increase in mass by 14 Daltons while decreasing in abundance in a reasonably even manner. On the other hand, Figure 2b–d shows a near absence of product ions in the positions of the double bonds, showing that little rearrangement of the carbon backbone occurs during decomposition. The positions of five of the six double bonds in *all cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) (Figure 2d) can be determined from the respective MS/MS fragmentation. The location of the double bond at position 19 has to be inferred from the product ion mass and the positions of the other double bonds. Figure 2e shows the MS/MS fragmentation pattern of the $[FA-H+2Li]^+$ product ions from a FA methyl branched in the 2-position. Determination of the point of branching is evident in the MS/MS spectrum because there is essentially no product ion present for $C_{2'}$.

Conclusion

Elimination of matrix induced ion suppression allows ionization of a class of compounds by lithium ion attachments that were thought not to be amenable by MALDI ionization. By using TCNQ, an aprotic strongly electron-deficient compound as matrix with LiCl, transfer of Li^+ to FAs and FA-lithium salts was facile, especially when solvent-free sample preparation methods are used. MS/MS fragmentation of the lithium adducted FA lithium salt $[FA-H+2Li]^+$ using TOF-TOF instrumentation produces almost exclusively CRF product ions that allow determination of the positions of unsaturation and branching. This work demonstrates that with the proper matrix and sample preparation methods, modern instrumentation can be used to successfully carry out CRF on underivatized FAs. Combining the recently described multisample solvent-free preparation method with TCNQ and LiCl salt as matrix is expected to provide a high-throughput method for analyzing FAs and other compound classes using CRF to probe structure.

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