Negative Ion Electrospray Tandem Mass Spectrometric Structural Characterization of Leukotriene B_4 (LTB_4) and LTB_4-Derived Metabolites

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The low energy collision induced dissociation (CID) of the carboxylate anions generated by electrospray ionization of leukotriene B_4 (LTB_4) and 16 of its metabolites was studied in a tandem quadrupole mass spectrometer. LTB_4 is a biologically active lipid mediator whose activity is terminated by metabolism into a wide variety of structural variants. The collision-induced dissociation spectra of the carboxylate anions revealed structurally informative ions whose formation was determined by the position of hydroxyl substituents and double bonds present in the LTB_4 metabolite. Major ions resulted from charge remote α-hydroxy fragmentation or charge directed α-hydroxy fragmentation. The conjugated triene moiety present in some metabolites was proposed to undergo cyclization to a 1,3-cyclohexadiene structure prior to charge remote or charge driven α-hydroxy fragmentation. The mechanisms responsible for all major ions observed in the CID spectra were studied using stable isotope labeled analogs of the LTB_4 metabolites. In general, the collision-induced decomposition of carboxylate anions produced unique spectra for all LTB_4 derived metabolites. The observed decomposition product ions from the carboxylate anion could be useful in developing assays for these molecules in biological fluids. *(J Am Soc Mass Spectrom 1996, 7, 129–139)*

Leukotriene B_4 [5(*S*),12(*R*)-dihydroxy-6(*Z*),8(*E*),10(*E*),14(*Z*)-eicosatetraenoic acid (LTB_4)] is a potent neutrophil chemotactic agent produced from arachidonic acid that follows activation of 5-lipoxygenase with the intermediate formation of leukotriene A_4 and hydrolysis catalyzed by LTA_4 hydrolase [1, 2]. The bioactivity of this important lipid mediator is terminated rapidly by metabolism that involves several pathways unique to LTB_4 and also by pathways of normal fatty acid metabolism. Elucidation of these metabolic processes has resulted from the identification of numerous LTB_4-derived metabolites.

The first LTB_4 metabolic pathway identified was ω-oxidation in human polymorphonuclear leukocytes that resulted in formation of 20-hydroxy-LTB_4 (20-OH-LTB_4) [3] and involved the activity of a specific cytochrome $P\text{-}450_{LTB\omega}$ [4]. This ω-oxidized product retains substantial bioactivity and is further metabolized to 20-carboxy-LTB_4 (20-COOH-LTB_4) [5, 6]. Formation of these metabolites also has been identified in isolated rat hepatocytes [7] and rat hepatic cell preparations [8, 9]. Further metabolism of 20-COOH-LTB_4 by β-oxidation results in formation of 18-COOH-LTB_4 and 16-COOH-LTB_3 [7, 10].

Metabolism of LTB_4 by reduction of the 10,11 double bond of the conjugated triene system has been reported in several cell types that do not metabolize LTB_4 by ω-oxidation [11, 12]. This metabolism involves the intermediate formation of 12-oxo-LTB_4 and 10,11-dihydro-12-oxo-LTB_4 [13]. Reduction of the 10,11 double bond also has been observed in conjunction with reduction of the 14,15 double bond that results in formation of 10,11,14,15-tetrahydro-12-oxo-LTB_4 [14] and 10,11,14,15-tetrahydro-LTB_4 (5,12-dihydroxy-6,8-eicosadienoic acid; unpublished results from LTB_4 metabolism in perisinusoidal stellate cells). Alternatively, reduction of the 14,15 double bond may occur without reduction of the 10,11 double bond that results in formation of 14,15-dihydro-LTB_4 (unpublished results from LTB_4 metabolism in RAW 264 cells, a macrophage cell line).

Metabolism of LTB_4 by carboxy-terminus β-oxidation to form 3-OH-LTB_4 (3,5,12-trihydroxy-6,8,10,14-eicosatetraenoic acid) has been reported in isolated rat hepatocytes in the presence of ethanol [15] and in human-derived hepatoma Hep G2 cells [16]. Further β-oxidation at the carboxy terminus has been suggested to result in the chain-shortened metabolite 10-hydroxy-4,6,8,12-octadecatetraenoic acid [16, 17]. Com-

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1044-0305/96/$15.00
SSDI 1044-0305(95)00629-X

Received July 3, 1995
Revised September 8, 1995
Accepted September 8, 1995

bination of metabolism by double bond reduction and carboxy-terminus β-oxidation may result in the reduced metabolites 10,11-dihydro-3-OH-LTB_4 and 10-hydroxy-4,6,12-octadecatrienoic acid [16, 18].

Finally, LTB_4 metabolism also may result in the formation of glutathione conjugates [18]. In this pathway, 12-oxo-LTB_4 is the likely substrate for a Michael-type addition of the tripeptide moiety that yields a metabolite that is a peptidolipid.

Identification of these metabolites has required initial purification from sample matrix, usually by solid phase extraction and reverse phase high-performance liquid chromatography (HPLC) analysis. Structural characterization of purified metabolites has been achieved by a variety of mass spectrometric methods that include gas chromatography–mass spectrometry (GC/MS) analysis in both positive and negative ion modes [11–18], which require prior derivatization, and by high energy and low energy fast-atom bombardment tandem mass spectrometry (FAB-MS/MS) analysis [18, 19]. For complete structural characterization of some metabolites, additional chemical modifications were necessary. These modifications include hydrogenation followed by GC/MS analysis to determine location of hydroxy substituents and ozonolysis followed by GC/MS analysis, which established double bond location in metabolites of the 10,11-reductase pathway. Presently, no single analytical tool that identifies all LTB_4 metabolites has been used.

The present study examines the use of negative ion electrospray ionization (ESI) tandem mass spectrometry for the complete structural characterization of the many LTB_4 metabolites. Formation of product ions after collision-induced decomposition of the $[M - H]^-$ ion provided unique spectra for each metabolite. Decomposition mechanisms also have been studied by the use of stable isotope-labeled analogs and generally can be described by mechanisms previously proposed for the decomposition of monohydroxy unsaturated fatty acids [20].

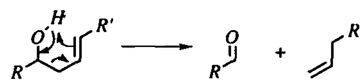
Methods

Leukotriene B_4 was obtained from Biomol Research Laboratories (Plymouth, PA). 5-Trans-LTB_4, 12-epi-6-trans-LTB_4 and 5,12-diHETE were obtained from Cayman Chemical (Ann Arbor, MI). All LTB_4-derived and 6-*trans*-LTB_4-derived metabolites were obtained from metabolism studies with Hep G2 cells, Ito cells, human keratinocytes, and rat hepatocytes. These metabolites were purified and structurally characterized as previously described [18]. Metabolites were dissolved in methanol:water (1:1) at 1–4 ng/μL for analysis by negative ion electrospray ionization mass spectrometry (ESI/MS). Samples (1–5 μL) were introduced into a Sciex API III$^+$ triple quadrupole mass spectrometer (PE-Sciex, Thornhill, Ontario, Canada) by flow injection through a 0.5-m × 50-μm fused silica capillary with methanol:water (1:1) as the mobile phase at a flow rate of 10 μL/min. Spectra were acquired at 3 s/scan over the mass range m/z 50 to 500 for most metabolites; the mass range was extended to m/z 700 for glutathione-containing metabolites. A spray voltage of −2400 V was used with an orifice voltage of −60 V and a collisional offset potential of 20 eV. Collision-induced dissociation (CID) spectra were obtained with a pressure of argon in the second quadrupole equivalent to 200×10^{12} molecules/cm^2. This resulted in a 40–80% reduction in the precursor ion for most metabolites. Deuterium exchange of labile hydroxy and carboxy protons was performed by dissolving the sample in 100-μL MeOD, evaporating under N_2, and redissolving the sample in MEOD:D_2O (1 : 1). These samples were analyzed by using MeOD:D_2O (1:1) as the mobile phase. Exchange of carboxylate oxygens with ^{18}O was performed by treatment of the dried samples with 50 units of porcine liver esterase in 100-μL $H_2{}^{18}O$ for 2 h at 37°C [21]. Samples were acidified with formic acid and extracted twice with ethyl acetate. The combined ethyl acetate extracts were dried under N_2 and dissolved in methanol:water (1:1) for ESI analysis.

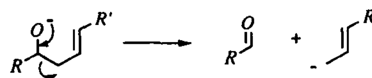
Results and Discussion

Negative ion ESI/MS of all LTB_4-derived metabolites produced abundant carboxylate anions. Generally, the most abundant ion produced by collision-induced dissociation of the $[M - H]^-$ anions resulted from α-hydroxy fragmentation. Fragmentation mechanisms previously described for CID processes that involve monohydroxy unsaturated fatty acid anions described α-hydroxy fragmentation by either charge-remote or charge-directed mechanisms [20]. The charge-remote process requires a double bond two carbons removed from the hydroxy-substituted carbon with fragmentation that results in formation of an aldehyde moiety and a terminal alkene after hydroxy proton transfer (Scheme I).

This type of fragmentation for compounds that contain a double bond one carbon removed from the hydroxy-substituted carbon (an apparent vinylic fragmentation) also was observed and suggested to occur after double bond rearrangement. The charge-directed process also requires a double bond two carbons removed from the hydroxy-substituted carbon, or rearrangement of double bonds to this position, with fragmentation that results in formation of a terminal



Scheme I. Charge-remote α-hydroxy fragmentation.



Scheme II. Charge-directed α-hydroxy fragmentation.

aldehyde and a charge-stabilized allylic anion (Scheme II). These mechanisms also may describe the fragmentation processes after CID of the LTB_4-derived metabolites. However competing mechanisms that do not involve α-hydroxy fragmentation also were observed.

LTB_4 (Figure 1A)

Collision-induced dissociation of the $[M - H]^-$ ion of LTB_4 produced ions that resulted from loss of H_2O (m/z 317) and loss of $2H_2O$ (m/z 299), with additional losses of CO_2 from the dehydrated ions to produce ions at m/z 273 and 255. An abundant ion at m/z 195, which was shifted to m/z 197 in the CID spectrum of d_3-OD-LTB_4 and to m/z 199 in the CID spectrum of $[^{18}O_2]$-LTB_4, was consistent with α-hydroxy charge-directed fragmentation of the C-11,C-12 bond—an apparent vinylic position. A possible mechanism for the formation of this ion involves initial rearrangement of the conjugated triene system to a cyclohexadiene structure followed by C-12 alkoxide abstraction of the C-5 proton. This results in an ion structure that is a tautomer of a 5-oxo structure (Scheme III, Structure 1) and has the charge site delocalized by resonance over seven carbon atoms.

Charge-directed fragmentation initiated by the C-12 alkoxide ion then results in loss of a neutral terminal aldehyde and the observed ion. Thermal rearrangement of the conjugated triene moiety to a cyclohexadiene structure was observed previously in GC/MS analysis of derivatized 5,12-diHETE and required the specific trans, cis, trans double bond geometry [22, 23].

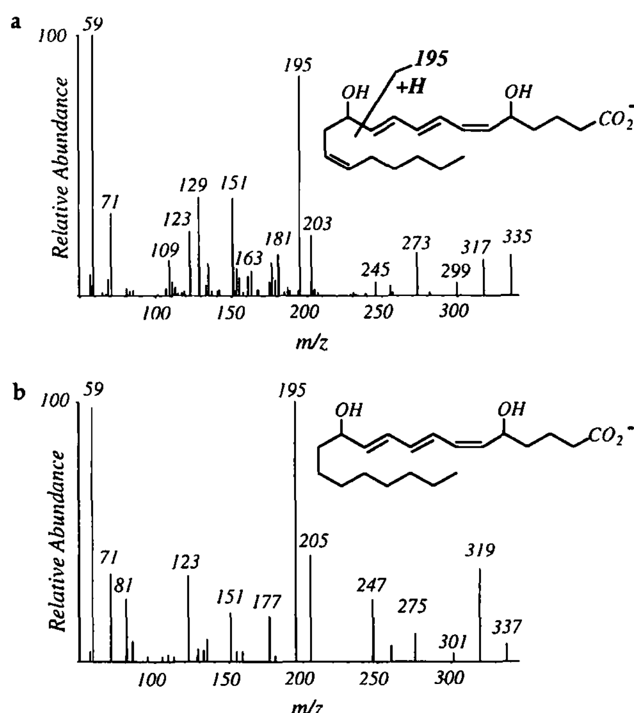
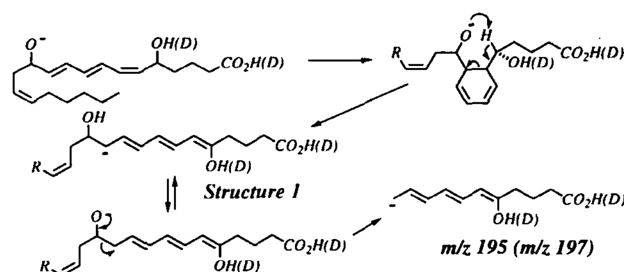


Figure 1. Negative ion ESI tandem mass spectrometry product ion spectra of (A) Leukotriene B_4 ($[M - H]^-$, m/z 335) and (B) 14,15-dihydro-LTB_4 ($[M - H]^-$, m/z 337).

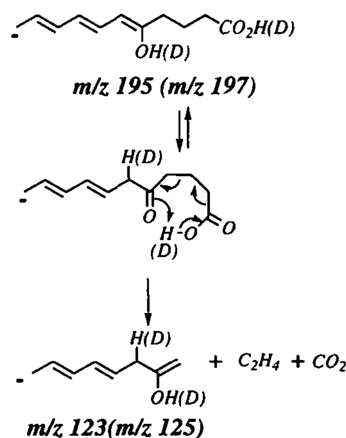


Scheme III

In the present study, analysis of 5,12-diHETE, 6-trans-LTB_4, and 12-epi-6-trans-LTB_4 produced spectra identical to that of LTB_4 (data not shown), which suggests that neither initial double bond stereochemistry nor chirality affect fragmentation. Decarboxylation of m/z 195 (loss of 44 u) would produce the ion at m/z 151, which also retained two deuteriums in the CID spectrum of d_3-OD-LTB_4 (m/z 153), but was unchanged in the CID spectrum of $[^{18}O_2]$-LTB_4 consistent with loss of CO_2. Loss of CO_2 and ethylene (28 u) from m/z 195 as the 5-oxo structure would result in the ion observed at m/z 123. This ion was observed at m/z 125 in the CID spectrum of d_3-OD-LTB_4 and was unchanged in the CID spectrum of $[^{18}O_2]$-LTB_4. A possible charge-remote mechanism for the formation of this ion with retention of two deuterium atoms is shown in Scheme IV; however, other charge-directed mechanisms are possible also.

Formation of m/z 195 also could be described by a charge-remote mechanism that involves transfer of the C-12 hydroxy proton to the cyclohexadiene structure. However, the extended conjugation that involves the C-5 enolate structure produced a more stabilized anion compared to localization of the charge site at the carboxy terminus. In addition, formation of a C-5 oxo or enolate structure more readily accounted for formation of the lesser abundant ions.

With charge localized as the carboxylate anion in the cyclohexadiene structure, dehydration that involves the C-12 hydroxy substituent and the C-5 pro-

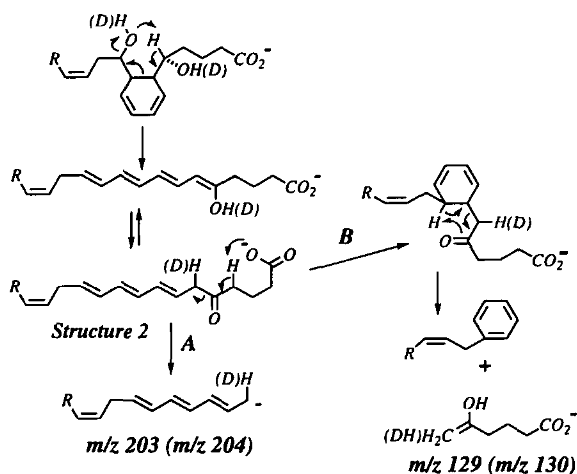


Scheme IV

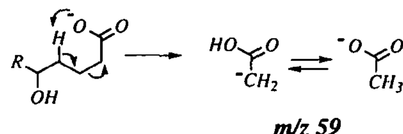
ton would result in an extended conjugated enolate structure that also could tautomerize to a 5-oxo structure (Scheme V, Structure 2). Fragmentation initiated by proton abstraction at the C-4 position by the carboxylate ion would result in loss of a neutral ketene moiety and formation of the ion at m/z 203, which is stabilized by the conjugated triene structure (Scheme V, pathway A). Consistent with this mechanism, this ion was observed at m/z 204 in the CID spectrum of the deuterated analog and was unchanged in the spectrum of [$^{18}O_2$]-LTB_4. Alternatively, rearrangement of the conjugated triene moiety in Structure 2 to a cyclohexadiene structure followed by transfer of the C-12 proton to the 5-oxo substituent would result in loss of a neutral-substituted benzene and formation of the ion at m/z 129 (Scheme V, pathway B). This ion was shifted to m/z 130 in the CID spectrum of d_3-OD-LTB_4 and was observed at m/z 133 in the CID spectrum of [$^{18}O_2$]-LTB_4 consistent with retention of the carboxylate moiety. Formation of m/z 129 by the mechanism shown in Scheme V, pathway B, was suggested previously to account for the observation of this ion in the CID spectrum of the $[M - H]^-$ ion of 5-oxo-eicosatetraenoic acid [24].

The ion at m/z 245 was shifted to m/z 247 in the CID spectrum of d_3-OD-LTB_4 and was unchanged in the CID spectrum of [$^{18}O_2$]-LTB_4. This was consistent with C-3,C-4 fragmentation via a mechanism analogous to formation of m/z 123 (Scheme IV) from the enolate form of Structure 1 after dehydration of the C-12 hydroxy substituent to form a double bond at C-12,C-13.

Low mass fragment ions at m/z 59 and 71 were not shifted in the CID spectrum of d_3-OD-LTB_4, but both were shifted by 4 u to m/z 63 and 75, respectively, in the CID spectrum of [$^{18}O_2$]-LTB_4. A portable mechanism that leads to the formation of m/z 59 would involve C-4 proton abstraction by the carboxylate moiety and subsequent loss of a neutral terminal alkene and formation of acetic acid enolate anion (Scheme VI). This anion may rearrange to the isomeric acetate anion



Scheme V



Scheme VI

[25, 26]. Formation of m/z 71 may involve dehydration of the C-5 hydroxy substituent by proton transfer from C-2 with loss of a neutral terminal alkene and formation of ionized 2-propenoic acid (Scheme VII).

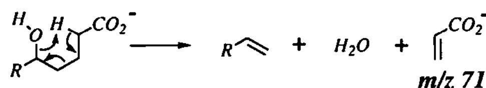
14,15-Dihydro-LTB_4 (Figure 1B)

Collision-induced dissociation of the carboxylate ion of 14,15-dihydro-LTB_4 resulted in ions formed due to loss of H_2O (m/z 319), loss of H_2O and CO_2 (m/z 275), and loss of $2H_2O$ (m/z 301). Fragmentation of the carbon skeleton resulted in several ions identical to the fragment ions observed for LTB_4 and were consistent with the lack of involvement of the C-14 double bond in fragmentation. Fragment ions that contain the methyl terminus at m/z 205 and 247 were shifted appropriately by 2 u from those observed in LTB_4. The ion at m/z 177 also was observed as a low abundance ion in the CID spectrum of LTB_4, where it was observed at m/z 178 in the deuterated analog and at m/z 179 in the $^{18}O_2$ analog. This was consistent with loss of water from m/z 195 at the carboxylic acid moiety, which accounts for loss of one ^{18}O, with formation of a ketene structure.

20-OH-LTB_4, 20-COOH-LTB_4, and 16-COOH-LTB_3 (Figure 2)

Modification by the addition of the hydroxy substituent at C-20 in 20-OH-LTB_4 had no affect on fragmentation pathways in the CID analysis of this metabolite with major fragment ions observed as identical to those in LTB_4. Ions at m/z 219 and 261, analogous to m/z 203 and 245 in LTB_4, reflect the presence of the additional oxygen in the methyl terminus.

Ions that resulted from fragmentations identical to those of LTB_4 also were observed in the CID spectrum of 20-COOH-LTB_4 (Figure 2B). In addition, abundant ions were observed that resulted from localization of the charge site as the C-20 carboxylate ion. The ion at m/z 141 likely is formed by charge-remote fragmentation that involves transfer of the C-12 hydroxy proton to C-15 followed by loss of the neutral aldehyde (C-1 through C-12) and formation of the observed terminal



Scheme VII

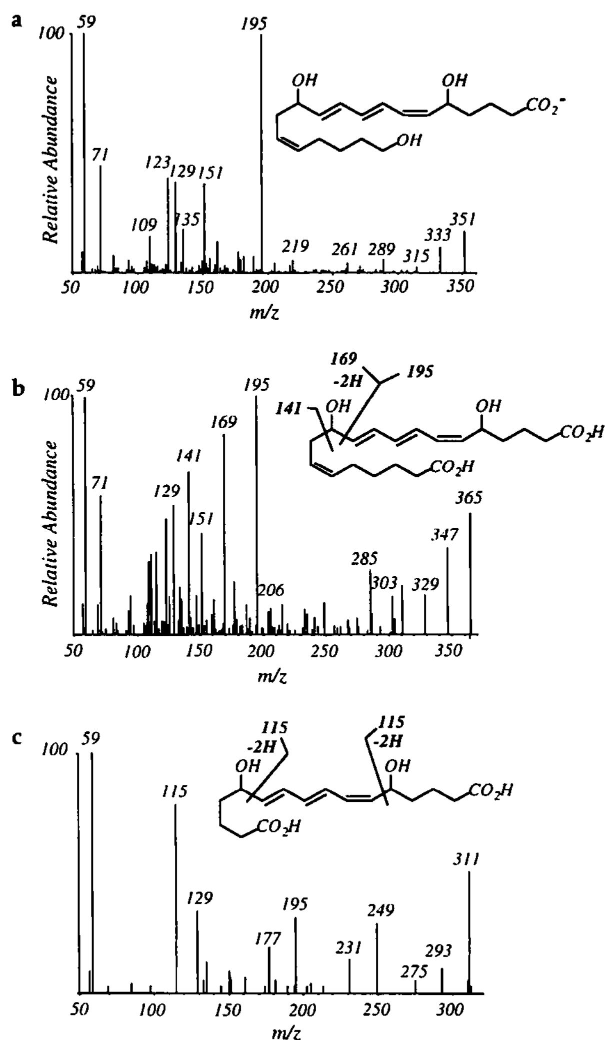


Figure 2. Negative ion ESI tandem mass spectrometry product ion spectra of (A) 20-hydroxy-LTB$_4$ ([M − H]$^-$, m/z 351), (B) 20-carboxy-LTB$_4$ ([M − H]$^-$, m/z 365), and (C) 16-carboxy-LTB$_3$ ([M − H]$^-$, m/z 311).

alkene C-20 carboxylate ion. The ion at m/z 169 also may be formed by a charge-remote fragmentation that involves C-12 hydroxy proton transfer to C-9 in the rearranged cyclohexadiene structure. Fragmentation of the C-11,C-12 bond then results in the observed terminal aldehyde that contains the C-20 carboxylate anion.

CID of the carboxylate ion of 16-COOH-LTB$_3$ (m/z 311) resulted in prominent fragment ions at m/z 195, 177, and 129 identical to LTB$_4$ (Figure 2C). Except for initial double bond stereochemistry, this is a palindromic compound, and the fragmentation mechanisms described for LTB$_4$ would result in identical ions with the charge site localized as either the C-1 carboxylate ion or the C-16 carboxylate ion. An abundant ion at m/z 115 was observed that corresponds to fragmentation of the C-5,C-6 bond with charge at C-1 or the C-11,C-12 bond with charge at C-16. Formation of this ion may involve the intermediate formation of the cyclohexadiene structure followed by hydroxy proton transfer in a charge-remote process that results in formation of a terminal aldehyde that contains the carboxylate moiety.

10,11-Dihydro-LTB$_4$, 6,7-Dihydro-LTB$_4$, and 10,11,14,15-Tetrahydro-LTB$_4$ (Figure 3)

The major fragment ion observed in the CID spectrum of 10,11-dihydro-LTB$_4$ (5,12-dihydroxy-6,8,14-eicosatrienoic acid; m/z 115; Figure 3A), was consistent with charge-remote fragmentation of the C-5,C-6 bond that involved a proton transfer. This bond is at an apparent vinylic position and fragmentation may involve rearrangement of the conjugated diene by a hydrogen transfer to form a C-7,C-9 conjugated diene (Scheme VIII) as has been proposed for the rearrangement of the conjugated diene in monohydroxy

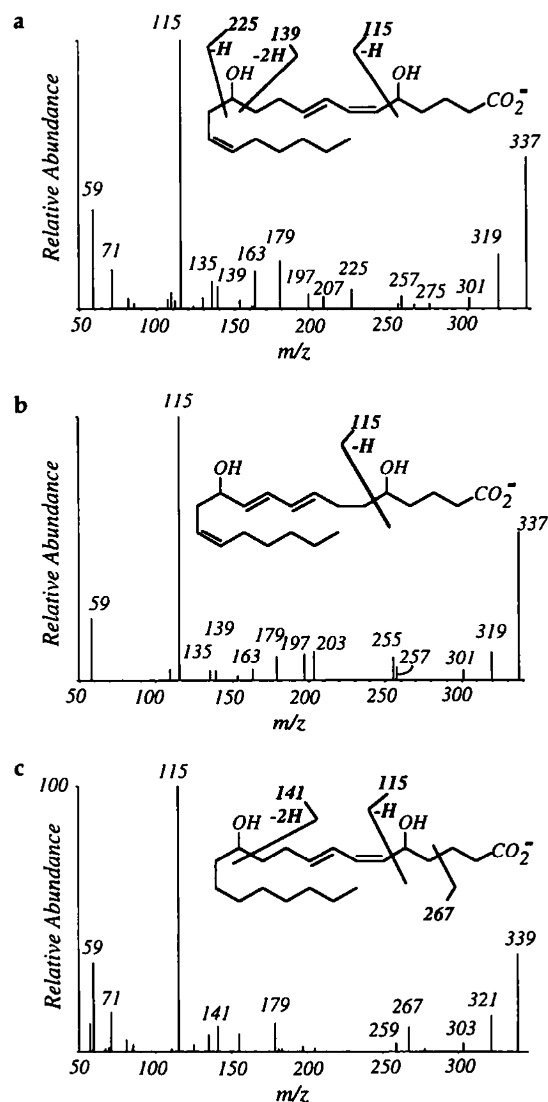
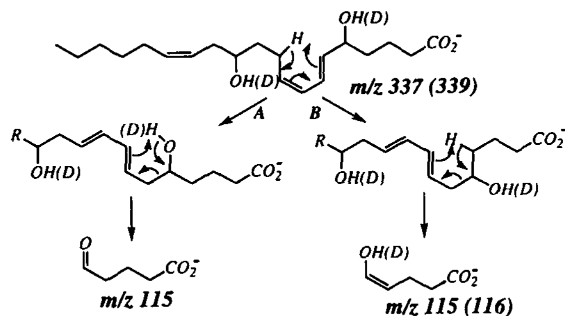
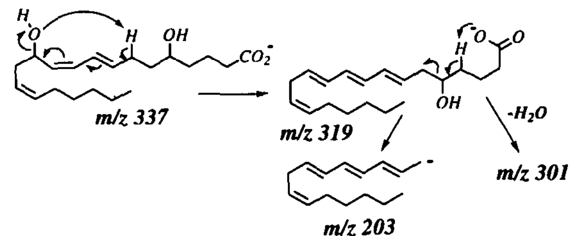


Figure 3. Negative ion ESI tandem mass spectrometry product ion spectra of (A) 10,11-dihydro-LTB$_4$ ([M − H]$^-$, m/z 337), (B) 6,7-dihydro-LTB$_4$ ([M − H]$^-$, m/z 337), and (C) 10,11,14,15-tetrahydro-LTB$_4$ ([M − H]$^-$, m/z 339).



Scheme VIII

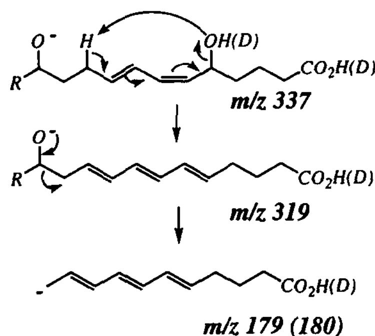
eicosatetraenoic acids [20]. Charge-remote fragmentation with transfer of the C-5 hydroxy proton to C-8 would result in the C-5 terminal aldehyde that contains the C-1 carboxylate anion at m/z 115 (Scheme VIII, pathway A). Consistent with this mechanism, this ion was also the base peak in the CID spectrum of d_3-OD-10,11-dihydro-LTB_4 and was observed at m/z 119 in the CID spectrum of [$^{18}O_2$]-10,11-dihydro-LTB_4. A less abundant ion at m/z 116 (15%) in the CID spectrum of d_3-OD-10,11-dihydro-LTB_4 may imply transfer of the C-4 proton to C-8 with fragmentation that results in a terminal enolized aldehyde, which would retain one deuterium (Scheme VIII, pathway B). Charge-remote fragmentation of the C-12,C-13 bond with a proton transfer to C-15 resulted in an observed ion at m/z 225 that was shifted to m/z 229 when [$^{18}O_2$]-10,11-dihydro-LTB_4 was analyzed. This ion was shifted to both m/z 226 (4%) and m/z 227 (8% in the CID spectrum of d_3-OD-10,11-dihydro-LTB_4, which implies competitive mechanisms of proton transfers that involve either the C-12 hydroxy proton or a proton at C-11, analogous to Scheme VIII. The ion observed at m/z 179, which shifted to m/z 183 for [$^{18}O_2$]-10,11-dihydro-LTB_4, may result from initial dehydration of the C-5 hydroxy substituent that involves abstraction of the C-10 proton and formation of a conjugated triene system (Scheme IX). Charge-directed fragmentation of the C-11,C-12 bond would result in loss of a neutral terminal aldehyde with formation of the observed stabilized ion, which retained one deuterium in the CID spectrum of d_3-OD-10,11-dihydro-LTB_4.



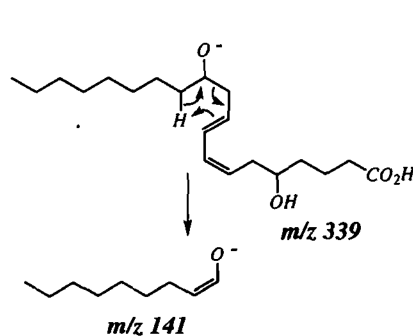
Scheme X

Due to rearrangement of the conjugated diene system, an almost identical spectrum was obtained from CID of the carboxylate ion of 6,7-dihydro-LTB_4 (Figure 3B), a compound which has been identified as a metabolite of 6-trans-LTB_4 [27]. One distinguishing ion was observed at m/z 203. This ion may be formed by dehydration of the C-12 hydroxy substituent (m/z 319) followed by charge-directed fragmentation of the C-5,C-6 bond (Scheme X), analogous to formation of m/z 179 from 10,11-dihydro-LTB_4. An apparent competitive reaction of this ion (m/z 319) would be further loss of water to form a conjugated tetraene carboxylate anion at m/z 301.

Collisional activation of the carboxylate ion of 10,11,14,15-tetrahydro-LTB_4 (m/z 339) resulted in observed ions at m/z 115 and 179 (Figure 3C), which would be consistent with fragmentations identical to that of 10,11-dihydro-LTB_4. Fragmentation of the C-12,C-13 bond, which resulted in an observed ion at m/z 225 for 10,11-dihydro-LTB_4, was not observed for the tetrahydro analog, consistent with involvement of the C-14 double bond in dihydro-LTB_4 in this fragmentation. The ion observed at m/z 141 likely involved C-11,C-12 bond fragmentation and a proton transfer with charge localized as the C-12 alkoxide ion. The mechanism for this fragmentation would require prior double bond rearrangement to a C-7,C-9 conjugated diene followed by charge-remote C-13 proton transfer to C-9 and cleavage of C-11,C-12 that resulted in a stabilized alkoxide ion (Scheme XI). The corresponding fragmentation also was observed for 10,11-dihydro-LTB_4. An observed ion at m/z 139 reflects the additional unsaturation at C-14 and was not shifted in the CID spectrum of d_3-OD-10,11-dihydro-LTB_4 or



Scheme IX



Scheme XI

[$^{18}O_2$]-10,11-dihydro-LTB$_4$. The ion at m/z 267 was consistent with C-3,C-4 bond fragmentation and was observed at low abundance for 10,11-dihydro-LTB$_4$ at m/z 265.

12-Oxo-LTB$_4$, 10,11-Dihydro-12-Oxo-LTB$_4$, and 10,11,14,15-Tetrahydro-12-Oxo-LTB$_4$ (Figure 4)

CID of the carboxylate ion of the 12-oxo metabolites resulted in loss of H_2O (18 u) and loss of H_2O and CO_2 (loss of 62 u) for each metabolite. Product ions that follow CID of the carboxylate ion of 12-oxo-LTB$_4$ (Figure 4A), which did not contain the carboxy terminus as evidenced by analysis of [$^{18}O_2$]-12-oxo-LTB$_4$, were observed at m/z 219, 179, and 153. These ions may be formed by initial oxidation of the C-5 hydroxy substituent via intermediate formation of the cyclohexadiene structure (Scheme XII). This would result in enolate structures at both C-5 and C-12.

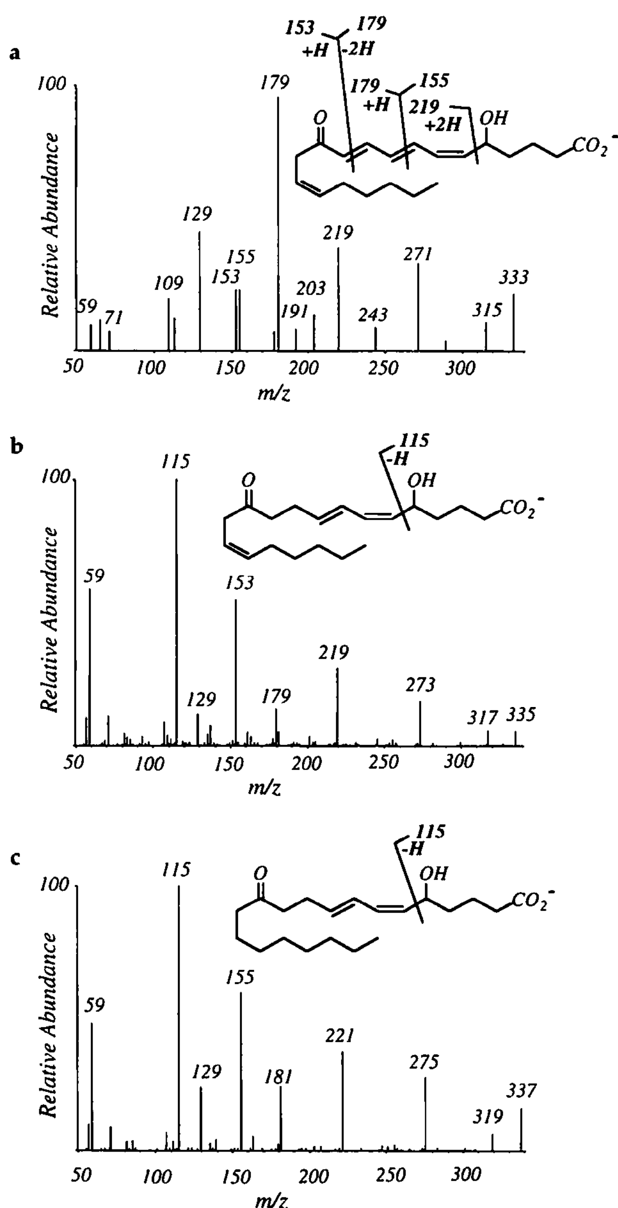
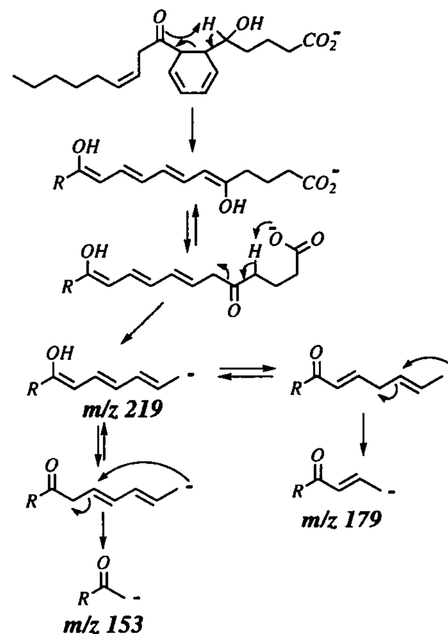
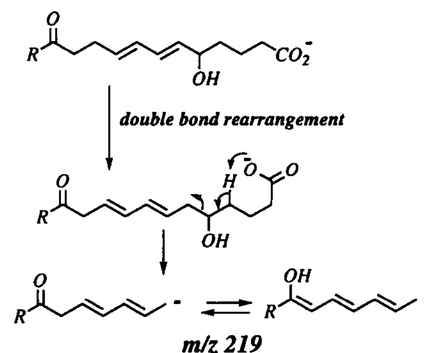


Figure 4. Negative ion ESI tandem mass spectrometry product ion spectra of (A) 12-oxo-LTB$_4$ ([M − H]$^-$, m/z 333), (B) 10,11-dihydro-12-oxo-LTB$_4$ ([M − H]$^-$, m/z 335), and (C) 10,11,14,15-tetrahydro-12-oxo-LTB$_4$ ([M − H]$^-$, m/z 337).



Scheme XII

Abstraction of the C-4 proton by the carboxylate ion would result in loss of a neutral ketene and formation of the stabilized ion at m/z 219. Further losses of cyclopropene or cyclopentadiene would result in ions at m/z 179 and 153, respectively. These reactions are plausible but have not been proven. Similar rearrangement reactions that involve cyclization due to nucleophilic attack by a carbanion on unsaturated systems have been reported [28]. These ions also were present in the CID spectrum of 10,11-dihydro-12-oxo-LTB$_4$ (Figure 4B), where they may be formed by initial double bond rearrangement followed by loss of a neutral enolate rather than loss of ketene to form m/z 219 (Scheme XIII). These ions are shifted by 2 u in the CID



Scheme XIII

spectrum of 10,11,14,15-tetrahydro-12-oxo-LTB_4, consistent with saturation of the C-14 double bond (Figure 4C).

The ion at m/z 179 in the spectrum of 12-oxo-LTB_4 (Figure 4A) was observed at both m/z 179 (83%), consistent with the preceding mechanism, and at m/z 183 (100%) in the CID spectrum of $[^{18}O_2]$-12-oxo-LTB_4. This was not the case for the dihydro analogs where m/z 179 (10,11-dihydro-12-oxo-LTB_4) and the corresponding ion in the tetrahydro-12-oxo metabolite (m/z 181) showed no contribution from the carboxylate moiety in the CID spectra of the $[^{18}O_2]$ analogs. Formation of the ion that contains the carboxy terminus is consistent with fragmentation of the C-10,C-11 double bond and transfer of two hydrogens. This may involve intermediate formation of enolate structures at both C-5 and C-12.

Other ions unique to 12-oxo-LTB_4 include m/z 203, which may have the structure shown in Scheme V and was not changed in $[^{18}O_2]$-12-oxo-LTB_4, and m/z 155, which was shifted to m/z 159 in the $^{18}O_2$ analog, which suggests fragmentation of the C-8,C-9 double bond. Mechanisms for the formation of both of these ions also may involve intermediate formation of a 5-oxo structure. The ion at m/z 129, which was shifted to m/z 133 in the CID spectrum of $[^{18}O_2]$-12-oxo-LTB_4, also was observed in the CID spectra of both dihydro-12-oxo-LTB_4 and tetrahydro-12-oxo-LTB_4. Formation of this ion also may involve the intermediate formation of a 5-oxo structure as shown in Scheme V for LTB_4.

A distinguishing fragment ion of 10,11-dihydro-12-oxo-LTB_4 at m/z 115, which was shifted to m/z 119 in analysis of $[^{18}O_2]$-10,11-dihydro-12-oxo-LTB_4, likely is formed by fragmentation of the C-5,C-6 bond after double bond rearrangement as observed for 10,11-dihydro-LTB_4 (Scheme VIII). This ion also was observed in 10,11,14,15-tetrahydro-12-oxo-LTB_4 (Figure 4C).

3-OH-10,11-Dihydro-LTB_4 and 3-OH-LTB_4 (Figure 5)

Introduction of an additional hydroxy substituent at C-3 resulted in a change in the fragmentation pattern for CID of 3-OH-10,11-dihydro-LTB_4 and 3-OH-LTB_4 away from α-hydroxy fragmentation, which resulted in ions at m/z 115 or 195, to α-hydroxy fragmentation, which resulted in loss of the first four carbons. The only significant high mass ion observed in the CID spectrum of 3-OH-10,11-dihydro-LTB_4 anion (m/z 249; Figure 5A) was consistent with charge-remote fragmentation of the C-4,C-5 bond with charge localized as the C-12 alkoxide ion. Transfer of the C-5 hydroxy proton to the 3-OH substituent and subsequent loss of H_2O and 3-butenoic acid (104 u) would result in formation of the observed ion at m/z 249 (Scheme XIV). The CID spectrum of 3-OH-LTB_4 (Figure 5B) shows an additional fragmentation after loss of 104 u. This metabolite contains the 3-OH-1,5-diene moiety required for an oxy-Cope rearrangement (C-10 through C-14). This rearrangement previously was proposed to account for the formation of odd electron ions that followed CID of monohydroxy fatty acids [20]. An oxy-Cope rearrangement of the proposed structure that corresponds to [(M − 1) − 104] for 3-OH-LTB_4 followed by homolytic bond fragmentation would result in the odd electron ion at m/z 136 (Scheme XV). Consistent with this mechanism, m/z 136 was not shifted in the CID spectrum of d_4-OD-3-OH-LTB_4. An abundant ion at m/z 271 may result from loss of $2H_2O$ and CO_2, which results in an ion stabilized by extended conjugation. This is not as favorable a process for the 10,11-dihydro analog due to interruption of the conjugated system.

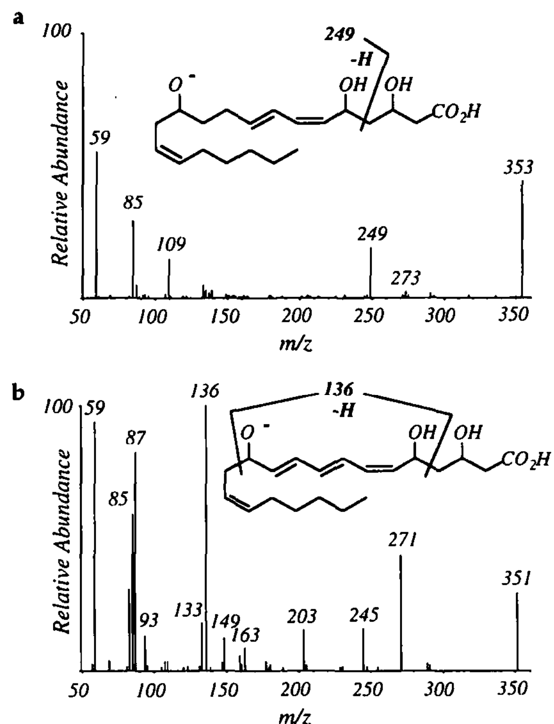
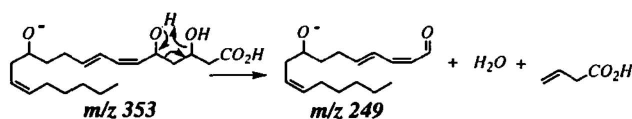


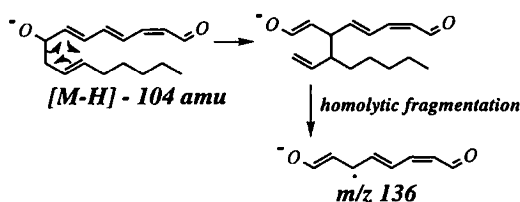
Figure 5. Negative ion ESI tandem mass spectrometry product ion spectra of (**A**) 3-hydroxy-10,11-dihydro-LTB_4 ($[M - H]^-$, m/z 353) and (**B**) 3-hydroxy-LTB_4 ($[M - H]^-$, m/z 351).

10-OH-4,6,8,12-Octadecatetraenoic Acid (10-HOTE) and 10-OH-4,6,12-Octadecatrienoic Acid (10-HOTrE) (Figure 6)

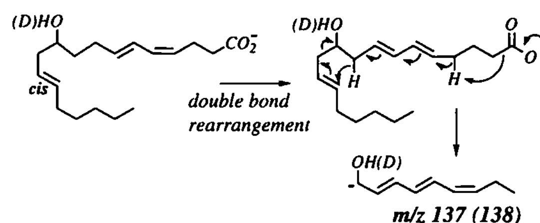
Collisional activation of the carboxylate ion derived from 10-HOTE (m/z 291) resulted in formation of an abundant ion at m/z 135, and CID of the carboxylate



Scheme XIV



Scheme XV



Scheme XVI

ion of 10-HOTrE (m/z 293) resulted in an ion at m/z 137 (Figure 6A and B, respectively). These fragment ions, which differ by 2 u, are consistent with fragmentation of the C-10,C-11 bond in both compounds with loss of 45 u. The loss of 45 u involved loss of the carboxylate moiety because these ions were not shifted in the CID spectra of [$^{18}O_2$]-10-HOTE and [$^{18}O_2$]-10-HOTrE. Surprisingly, the additional loss of 1 u did not involve loss of the C-10 hydroxy proton via a charge-remote fragmentation (Scheme I) with C-10 proton transfer to C-13 because these ions were shifted to m/z 136 and 138 in the CID spectra of the deuterated analogs. Possible mechanisms that lead to the formation of these ions must account for the loss of both carboxylate oxygen atoms and retention of one exchangeable deuterium atom and may involve prior rearrangement of the double bonds and transfer of a methylene hydrogen with fragmentation of the C-10,C-11 bond. One proposed but unproven mechanism consistent with these requirements is shown in Scheme XVI for 10-HOTrE. The abundant ion at m/z 229 in the CID spectrum of 10-HOTE may be due to loss of H_2O and CO_2 and was not shifted in the CID spectrum of either [$^{18}O_2$]10-HOTE or the deuterated analog. Loss of H_2O may involve the C-10 hydroxy substituent and a proton at C-3, which results in formation of an extended tetraene system that starts at C-3. This would stabilize a C-2 anion formed by loss of CO_2. Notably, the low mass ion at m/z 59 observed in the CID spectra of other metabolites was not observed in the CID spectra of these chain-shortened compounds. This was consistent with the location of the double bond at C-4 (see Scheme VI).

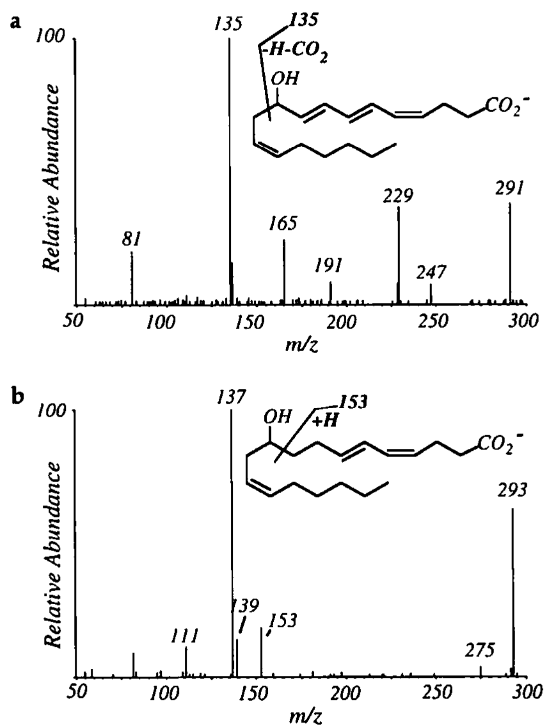


Figure 6. Negative ion ESI tandem mass spectrometry product ion spectra of (**A**) 10-hydroxy-4,6,8,12-octadecatetraenoic acid (10-HOTE) ([M − H]$^-$, m/z 291) and (**B**) 10-hydroxy-4,6,12-octadecatrienoic acid (10-HOTrE) ([M − H]$^-$, m/z 293).

5,12-Dihydroxy-6-Glutathionyl-7,9,14-Eicosatrienoic Acid (c-LTB$_3$) and 5,12-Dihydroxy-6-Cysteinylglycyl-7,9,14-Eicosatrienoic Acid (d-LTB$_3$) (Figure 7)

The CID spectrum of c-LTB$_3$ was dominated by ions that resulted from fragmentation of the peptide moiety rather than α-hydroxy-type fragmentations. The abundant ions at m/z 272 and 254 (loss of H_2O from m/z 272) also were observed in the FAB-MS/MS spectrum of LTC$_4$ [29] and result from fragmentation at the sulfur atom with charge located at one of the carboxylate positions in the peptide. The ion at m/z 143, which also was present in the CID spectrum of d-LTB$_3$, could result from additional loss at the glutamic acid moiety. The CID spectrum of d-LTB$_3$ revealed an abundant ion (m/z 229) that resulted from α-hydroxy fragmentation of the carbon–carbon backbone. After loss of the peptide group, this ion could result from either a charge-remote fragmentation of the C-10,C-11 bond with hydroxy proton transfer to C-9 and charge located at the carboxy terminus or a charge-driven fragmentation of the same bond initiated by a C-12 alkoxide ion.

Conclusion

Negative ion ESI tandem mass spectrometry produced unique spectra for all LTB$_4$-derived metabolites via routine use of 1–5 ng of sample. Sensitivity levels were not optimized in the present study and the use of precolumn concentration and HPLC introduction of samples undoubtedly would provide greater sensitivity for tandem mass spectrometry analysis. This may be particularly useful for identification of glutathione conjugates of LTB$_4$ which cannot be analyzed by

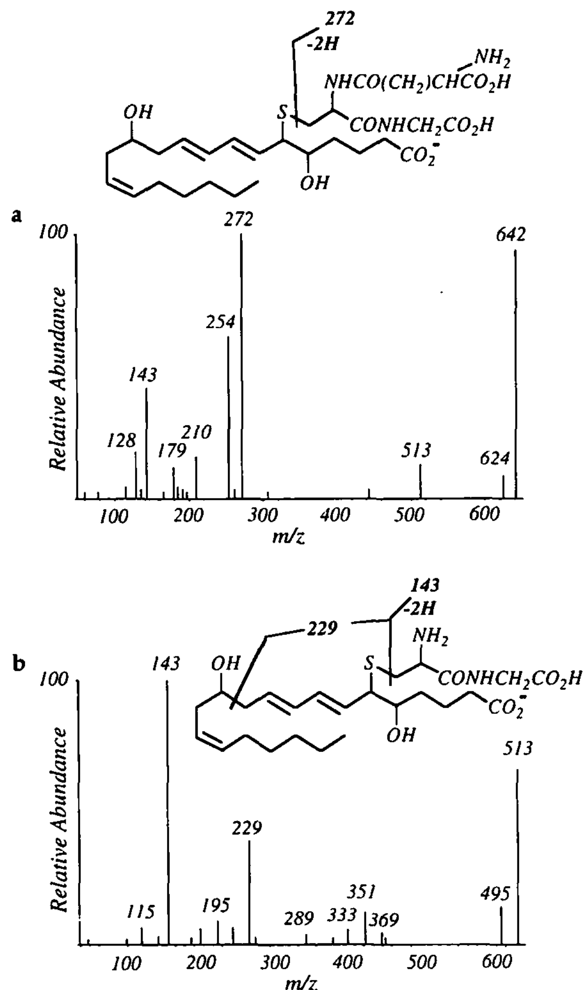


Figure 7. Negative ion ESI tandem mass spectrometry product ion spectra of (A) 5,12-dihydroxy-6-glutathionyl-7,9,14-eicosatrienoic acid (*c*-LTB_3) ($[M - H]^-$, *m/z* 642) and (B) 5,12-dihydroxy-6-cysteinylglycyl-7,9,14-eicosatrienoic acid (*d*-LTB_3) ($[M - H]^-$, *m/z* 513).

derivatization and GC/MS analysis. These metabolites previously could be analyzed only by FAB-MS/MS, which requires 40–100 ng of sample [18]. Also, analysis of other LTB_4-derived metabolites by derivatization and GC/MS analysis required 10–50 ng for full structural characterization via both negative and positive ion detection.

Knowledge of the fragmentation processes described in the present study also may allow use of tandem mass spectrometry protocols such as parent ion scanning for the detection of LTB_4-derived metabolites. This would be expected to result in an even greater gain in sensitivity. For example, a characteristic product ion at *m/z* 195 was indicative of LTB_4 and all metabolites of LTB_4 that were modified by methyl terminus metabolism. Also, reduction of one of the double bonds of the conjugated triene system resulted in an abundant product ion at *m/z* 115. CID mechanisms that result in the formation of these ions generally are described by charge-remote or charge-directed processes that involve the hydroxy substituent and a double bond two carbon atoms removed from the hydroxy-substituted carbon. For most of the fragmentations observed in the LTB_4-derived metabolites, this requires double bond rearrangement prior to fragmentation. Rearrangement of the conjugated triene system to a cyclohexadiene structure previously has been suggested [22, 23] and is consistent with the gas chromatography behavior of these compounds. In the present study, this rearrangement apparently does not differentiate double bond stereochemistry as was observed in the thermal rearrangement. Rearrangement of a conjugated diene also was suggested in GC/MS analysis [20]. This rearrangement was supported in the present study by the nearly identical spectra obtained for 6,7-dihydro-LTB_4 and 10,11-dihydro-LTB_4. For these structures, the use of less abundant ions may be necessary for structural characterization.

Acknowledgment

This work was supported by a grant from the National Institutes of Health (HL25785).

References

1. Samuelsson, B.; Funk, C. D. *J. Biol. Chem.* **1989**, *264*, 19469.
2. Ford-Hutchinson, A. W.; Bray, M. A.; Doig, M. V.; Shipley, M. E.; Smith, M. J. H. *Nature* **1980**, *286*, 264.
3. Shak, S.; Goldstein, I. M. *J. Biol. Chem.* **1984**, *259*, 10181.
4. Shak, S.; Goldstein, I. M. *J. Clin. Invest.* **1985**, *76*, 1218.
5. Soberman, R. J.; Sutyak, J. P.; Okita, R. T.; Wendelborn, D. F.; Roberts, L. J., II; Austin, K. F. *J. Biol. Chem.* **1988**, *263*, 7996.
6. Sumimoto, H.; Minakami, S. *J. Biol. Chem.* **1990**, *265*, 4348.
7. Shirley, M.; Murphy, R. C. *J. Biol. Chem.* **1990**, *265*, 16288.
8. Romano, M. C.; Eckardt, R. D.; Bender, P. E.; Leonard, T. B.; Straub, K. M.; Newton, J. F. *J. Biol. Chem.* **1987**, *262*, 1590.
9. Gotoh, Y.; Sumimoto, H.; Takeshige, K.; Minakami, S. *Biochim. Biophys. Acta* **1988**, *960*, 342.
10. Jedlitschky, G.; Huber, M.; Volkl, A.; Muller, M.; Leier, I.; Muller, J.; Lehman, W.-D.; Fahim, H.; Keppler, D. *J. Biol. Chem.* **1991**, *266*, 24763.
11. Powell, W. S.; Gravelle, F. *J. Biol. Chem.* **1989**, *264*, 5364.
12. Kaever, V.; Martin, M.; Fauler, J.; Marx, K.; Resch, K. *Biochim. Biophys. Acta* **1987**, *922*, 337.
13. Wainwright, S. L.; Powell, W. S. *J. Biol. Chem.* **1991**, *266*, 20899.
14. Yokomizo, T.; Izumi, T.; Takahashi, T.; Kasama, T.; Kobayashi, Y.; Sato, F.; Taketani, Y.; Shimizu, T. *J. Biol. Chem.* **1993**, *268*, 18128.
15. Shirley, M.; Murphy, R. C. *J. Am. Soc. Mass Spectrom.* **1992**, *3*, 762.
16. Wheelan, P.; Murphy, R. C. *Arch. Biochem. Biophys.* **1995**, *321*, 381.
17. Wheelan, P.; Travers, J. B.; Morelli, J.; Murphy, R. C. In *Lipid Mediators in Health Disease*; Zur, U., Ed.; Freund Publishing House, Ltd.: London, 1994; p 151.
18. Wheelan, P.; Zirrolli, J. A.; Morelli, J. G.; Murphy, R. C. *J. Biol. Chem.* **1993**, *268*, 25439.
19. Deterding, L. J.; Curtis, J. F.; Tomer, K. B. *Biol. Mass Spectrom.* **1992**, *21*, 597.
20. Wheelan, P.; Zirrolli, J. A.; Murphy, R. C. *Biol. Mass Spectrom.* **1993**, *22*, 465.

21. Westcott, J. Y.; Clay, K. L.; Murphy, R. C. *Biomed. Mass Spectrom.* **1985**, *12*, 714.
22. Wheelan, P.; Zirrolli, J. A.; Murphy, R. C. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 40.
23. Borgeat, P.; Pilote, S. *Prostaglandins* **1988**, *35*, 723.
24. MacMillan, D. K.; Murphy, R. C. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 1190–1201.
25. O'Hair, R. A. J.; Gronert, S.; DePuy, C. H.; Bowie, J. H. *J. Am. Chem. Soc.* **1989**, *111*, 3105.
26. Grabowski, J. J.; Cheng, X. *J. Am. Chem. Soc.* **1989**, *111*, 3106.
27. Wheelan, P.; Murphy, R. C. *J. Biol. Chem.* **1995**, *270*, 19845.
28. Bowie, J. H. *Mass Spectrom. Rev.* **1990**, *9*, 349.
29. Raftery, M. J.; Thorne, G. C.; Orkiszewski, R. S.; Gaskell, S. J. *Biomed. Environ. Mass Spectrom.* **1990**, *19*, 465.