Characterization of Ethyl Chloroformate Derivative of β-Methylamino-L-alanine

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β-Methylamino-L-alanine (BMAA) is a neurotoxic amino acid that can be produced by cyanobacteria in aqueous environments. To analyze this compound by gas chromatography/mass spectrometry (GC/MS), BMAA must be derivatized to a nonpolar, volatile compound. This can be accomplished by reacting BMAA with ethyl chloroformate. While carrying out electron ionization (EI) mass spectrometric analysis on the ^{13}C-labeled derivative, it was discovered that the formation of an ion with a peak at *m/z* 245.12 is the result of [$CH_3CH_2O\cdot$] loss from the amino groups resulting from α-cleavage. This differs from previous reports that attributed this peak to α-cleavage of the carboxylic ester portion of the BMAA derivative. This finding is important for understanding BMAA derivative mass spectrometric fragmentation patterns and ultimately to properly identifying and quantifying BMAA. Fragmentation pathways for the formation of other major peaks observed in the EI mass spectra are also proposed. (J Am Soc Mass Spectrom 2007, 18, 817–825) © 2007 American Society for Mass Spectrometry

An early breakthrough in quantitative amino acids analysis using GC/MS was the one-step derivatization of the amino acids with chloroformates in an aqueous medium of water–ethanol–pyridine (Scheme **1**) [1].

This derivatization procedure enables the conversion of a polar amino acid into a nonpolar volatile product by simultaneous blocking of both the amino and carboxylic acid groups, which is not readily attainable by other derivatization methods. The reaction product, *N*-ethoxycarbonyl amino acid ethyl ester (ECEE), shown in Scheme **1**, exhibits an excellent peak resolution on a nonpolar GC capillary column [1–3]. An interesting finding for the reaction of chloroformate with amino acids is the pyridine-catalyzed exchange reaction of the carboxylic–carbonic anhydride side group of the derivative, RCO_2CO_2R', with the alcohol in the aqueous reaction medium (Scheme **1**) [2]. This finding indicates that the ester moieties of derivatives are determined by the alcohol in the aqueous reaction medium (Scheme **1**).

Mass spectra of ethyl esters of amino acids were investigated by Biemann et al. [4]. Moini et al. reported the mass spectrometric studies of ECEE derivatives, treated with trifluoroethanol–ethanol–chloroformate, in both positive and negative chemical ionization modes [5]. The results showed that the derivatives have a characteristic loss of $NH_2CO_2CH_2CH_3$ (*m/z* 89). Electron ionization fragmentation patterns of ECEEs were thoroughly studied by Huang et al. [6]. The derivatives of basic amino acids—ornithine, lysine, and such—demonstrate a similar fragmentation pattern: formation of cyclic immonium through the consecutive losses of [$CH_2CH_3O_2C\cdot$] and $NH_2CO_2CH_2CH_3$ [6].

This study was initiated because of the need to analyze the amino acid β-methylamino-L-alanine (BMAA), a neurotoxic compound manufactured by cyanobacteria (blue-green algae). It has been suggested that BMAA plays a role in neurological diseases, such as Alzheimer's and amyotrophic lateral sclerosis [7–9]. Thus, there is increased interest in measuring the levels of BMAA in the environment (including measuring BMAA in different levels of the food chain, which would indicate whether biomagnification is occurring) [10].

Using the ethyl chloroformate derivatization procedure (Scheme **2**), Moini et al. first reported analysis of BMAA in aqueous samples [11].

The mass spectra of the ethyl derivative show several characteristic peaks at *m/z* 291, *m/z* 245, and *m/z* 217, which have been interpreted as representing the molecular ion, and fragments resulting from [$CH_2CH_3O\cdot$] loss and [$CH_3CH_2O_2C\cdot$] loss, respectively (Scheme **2**) [11]. A closer examination of the structure of the ethyl chloroformate derivative of BMAA, however, casts doubt on the earlier assumption [11] about the fragmentation pathway responsible for the formation of the ion represented by the peak at *m/z* 245. This is because there are three possible channels leading to the formation of the ion at *m/z* 245: one involving the α-cleavage of the carboxylic ester bond and two involving an ethoxy-modified amino group.

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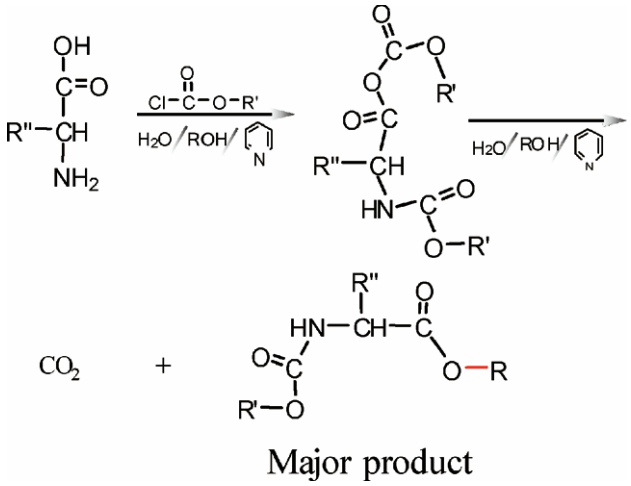


Scheme 1

In this study, we report an alternative fragmentation path. Given the growing interest in BMAA analysis, as well as the more general interest in the fragmentation channels of organic molecules with oxygen- and nitrogen-containing poly-functional groups, understanding the fragmentation channel of ECEE derivatives of BMAA under EI conditions is important.

Experimental

Chemicals

BMAA was purchased from Sigma–Aldrich (St. Louis, MO, USA). Methyl and ethyl chloroformates (≥98%), methanol (≥99.8%), ethanol (≥99.5%), and pyridine (≥99.8%) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). Ethyl-1-^{13}C-alcohol (98 atom % ^{13}C) was purchased from Sigma–Aldrich (Milwaukee, WI, USA). HPLC grade dichloromethane was purchased from Burdick and Jackson (Muskegon, MI, USA). Mass spectrometry standard, high-boiling-point perfluorokerosene (PFK), was purchased from Fluka (Glossop, UK). Microreaction vessels (2-mL) were purchased from Supelco (Bellefonte, PA, USA).

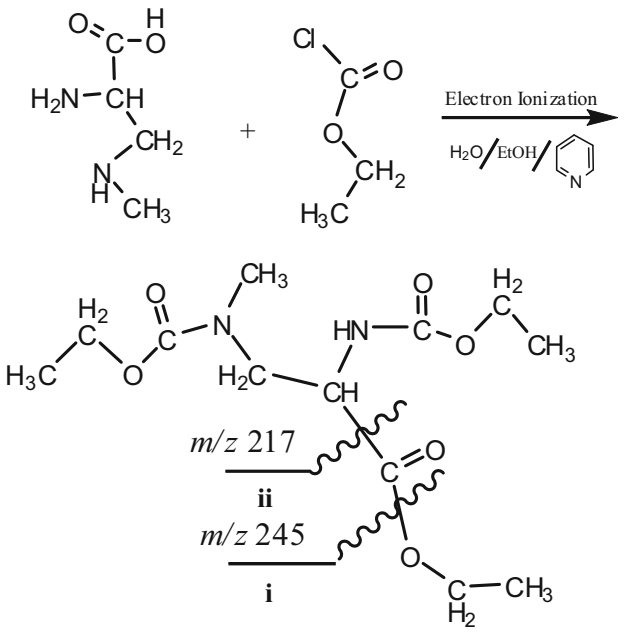
Derivatization and Extraction Procedures

Details about the chloroformate derivatization conditions were previously reported by Hušek [3]. In this study, several chloroformate reagents including methyl, ethyl, and butyl chloroformate, and 2,2,2-trichloroethyl chloroformate were used as reagents to study the derivatization conditions. The effect of alcohol concentration on the reaction yield was also studied. Results show that the reaction of BMAA with ethyl chloroformate offers the highest yield.

BMAA derivatives were prepared by treatment of 250 μL of BMAA solution (10 ng/μL) with a mixture of 100 μL ethanol and 80 μL pyridine (65:25:10 by volume). Ethanol was substituted by ethyl-1-^{13}C-alcohol in the isotopically labeled experiments. A 15-μL aliquot of ethyl chloroformate was added and the mixture was shaken vigorously for about 30 s until the evolution of CO_2 gas was complete [10]. The derivatives were extracted with 100 μL of dichloromethane, with vigorous shaking. The mixture solution was centrifuged at 3500 rpm for 10 min. The extraction was performed twice. The organic layer was dried under N_2 gas and reconstituted by adding 100 μL of dichloromethane. The dichloromethane solution was then transferred to a 500-μL insert in a 2-mL GC autosampler vial by a 50-μL gastight Hamilton syringe. A 1-μL aliquot of extract was injected into the GC injection port running in a solvent vent mode.

Instrumentation

All GC-MS analyses were carried out with an Agilent GC 6890N (Waldbronn, Germany), equipped with a PAL autosampler (CTC Analytics, Zwingen, Switzerland) and interfaced with a ThermoFinnigan MAT 95XP doubling-focusing mass spectrometer (Bremen, Germany). All mass spectra were recorded by an Xcalibur data system (Thermo Instruments, Norfolk, UK). To achieve sensitive analysis, the mass spectrometer was operated in the electron ionization (EI) mode for all low-resolution and high-resolution measurements. The EI source temperature was 200 °C. The electron energy was 70 eV. A resolving power of 1000 (10% valley definition) was used for low-resolution measurements. All low-resolution mass spectra were recorded in the magnetic scan mode, scanning from 40 to 350 (*m/z*) at a scan duration of 0.6 s.



Scheme 2

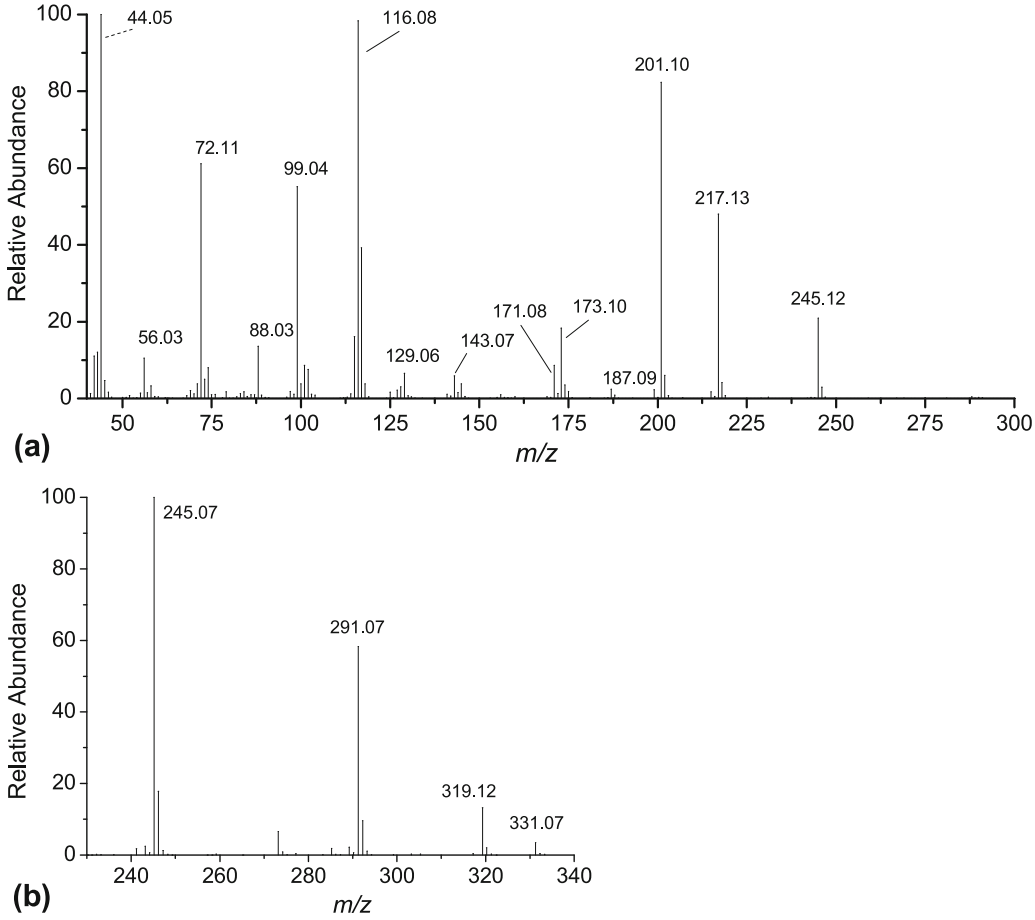


Figure 1. (**a**) EI spectra; (**b**) CI spectra of ethyl chloroformate derivatization of BMAA.

Chemical ionization (CI) experiments were performed for the purpose of confirmation of the molecular weight of the derivatives. Methane was used as the CI reagent gas. The source pressure was 2.4×10^{-4} mbar. The CI source temperature was 180 °C.

GC Conditions

To preserve the integrity of the BMAA derivatives in the GC injection port, a solvent vent mode was used to transfer the derivatives from the injection port into the GC capillary column. The initial temperature of the injection port was held at 70 °C for 1.45 min and then ramped to 250 °C at a rate of 150 °C/min. The final temperature of 300 °C was reached in 0.5 min. A 30-m Zebron nonpolar ZB-5MS column with a 5-m guard column (O.D.: 0.25 mm; I.D.: 0.25 μm) was used to separate the derivatives. The carrier gas was helium at a flow rate of 1.0 mL/s. The oven temperature program started at 60 °C for 4 min, and rose to 300 °C at a rate of 20 °C/min. The final holding time was 1 min. The GC transfer line temperature was 240 °C. Under these conditions, the retention time of the BMAA derivative was 12.80 min.

Exact Mass Measurement

The mass spectrometer was operated in the electronic scan mode for all exact mass measurements. The resolution (10% valley definition) was set at 11,000. The electronic scan range covered 10 to 30 mass units across the unknown peak to minimize higher mass signal diminishment resulting from accelerating voltage jumping. The scan duration was 0.6 s. PFK was used as the internal standard to calibrate all of the mass spectra. Mass spectra of exact masses were processed by Xcalibur high-resolution MS Version 1.4: CMASS and List. For exact mass calculations, the following numbers (in parentheses) of atoms of various elements were considered: carbon (1–14), nitrogen (0–4), hydrogen (1–26), and oxygen (0–8).

Results and Discussion

An EI mass spectrum of BMAA derivatives with ethanol is shown in Figure 1a. Only peaks with a relative intensity $> 0.5\%$ are plotted. The molecular ion peak, *m/z* 290, is absent. For the purpose of molecular weight confirmation, a CI mass spectrum is also shown in Figure 1b, where the *m/z* 291 peak can be assigned to the

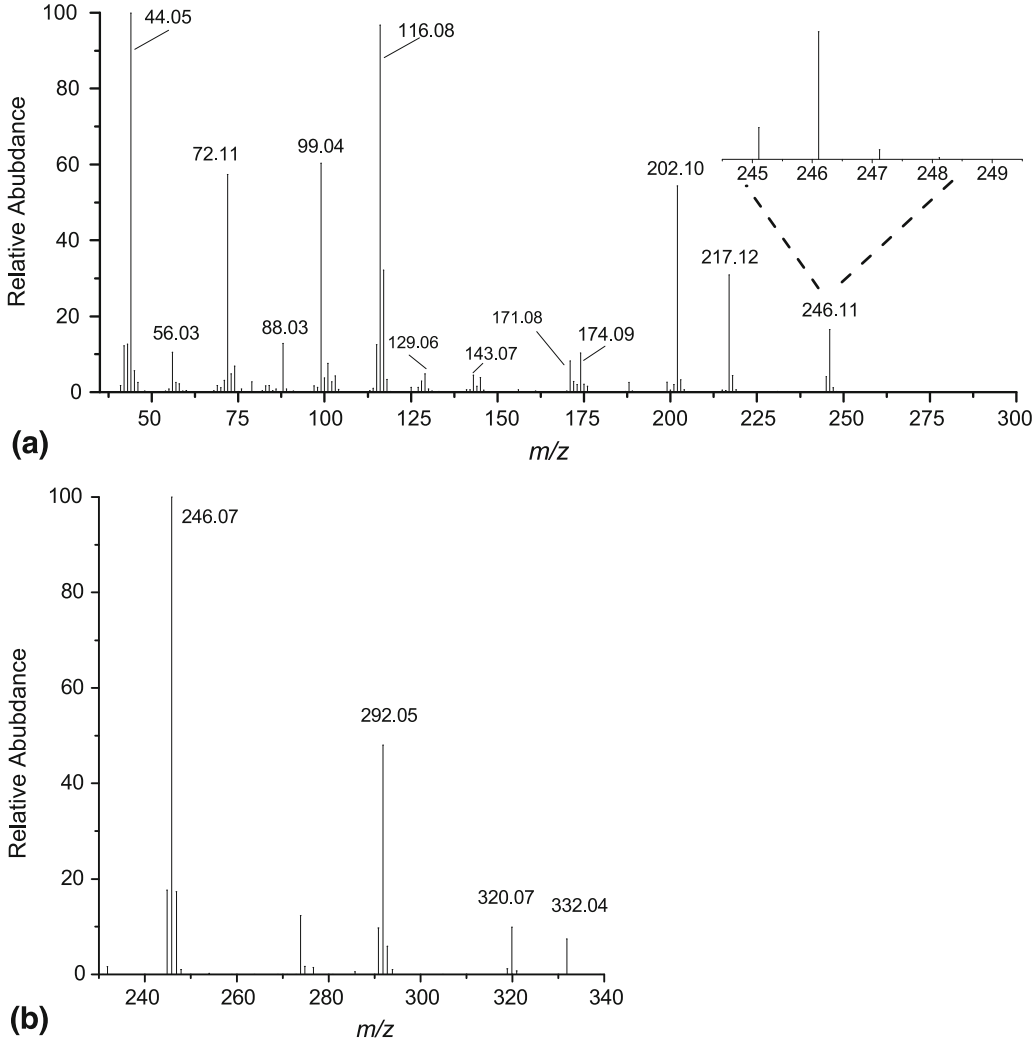
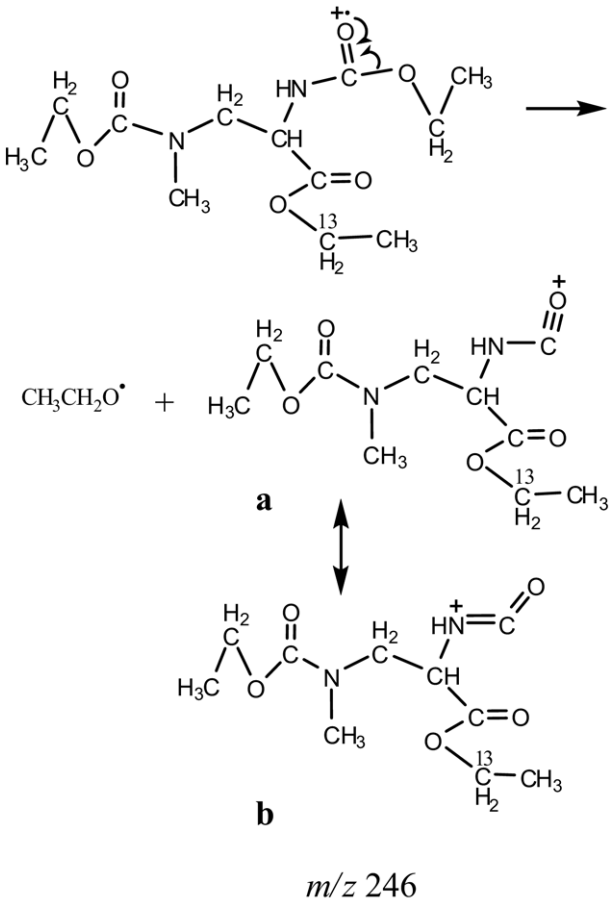


Figure 2. (**a**) EI spectra; (**b**) CI spectra of ^{13}C-labeled ethyl chloroformate derivatization of BMAA.

ion of $[M+H]^+$. The EI spectrum in Figure 1a shows the base peak at *m/z* 116.08 and the peak at the highest *m/z* value of 245.12. The peak at *m/z* 245.12 has been interpreted as the loss of $[CH_3CH_2O\cdot]$ resulting from the α-cleavage of the ECEE carboxylic ester group in the earlier report (Scheme **2**, path **i**) [11]. However, a closer look at the structure of the BMAA derivative (Scheme **2**) reveals a possible competition of $[CH_3CH_2O\cdot]$ loss between the carboxylic ester site and the remote amino groups. The α-cleavage taking place at either site can yield the peak at *m/z* 245.12 with the same composition and exact mass. This fact brings up the question of whether this is the only pathway or whether there is an alternative fragmentation route responsible for formation of the peak at *m/z* 245.12. An easy way to answer this question is to substitute the $[CH_3CH_2O\cdot]$ group with a ^{13}C-labeled alcohol group. Because the moiety of this ester is dependent on the alcohol in the aqueous medium [2], the ^{13}C-labeled derivative can be synthesized by treatment of BMAA with ethyl-1-^{13}C-alcohol and pyridine under the same reaction conditions.

Figure 2a shows the EI mass spectrum of the ^{13}C-labeled ECEE BMAA derivative. The molecular ion of ^{13}C-labeled ECEE (*m/z*, 292.07) is shown in the CI mass spectrum (Figure 2b). Compared with the nonlabeled EI mass spectrum in Figure 1a, the highest *m/z* value, 246.11, in Figure 2a is one mass unit greater than the nonlabeled ECEE one at *m/z* 245.12. This interesting result can most probably be given the interpretation that the formation of the ion at *m/z* 245.12 is attributed to the loss of $[CH_3CH_2O\cdot]$ from the amino group (Scheme **3**).

Considering the nitrogen atom as an unusual electron-donating agent, the ion corresponding to **a** most likely isomerizes to the ion **b**, shown in Scheme **3**. Another possible pathway for the formation of the ion at *m/z* 246.11 is a cyclic one, shown in Scheme **4**. The elimination of radical $[CH_3CH_2O\cdot]$ from the molecular ion yields a stable five-member cyclic species **c** containing an even number of electrons and nitrogen atoms. Thus this five-member cyclic ring structure represents the second pathway for the formation of the fragment ion at *m/z* 246.11 in Figure 2a.



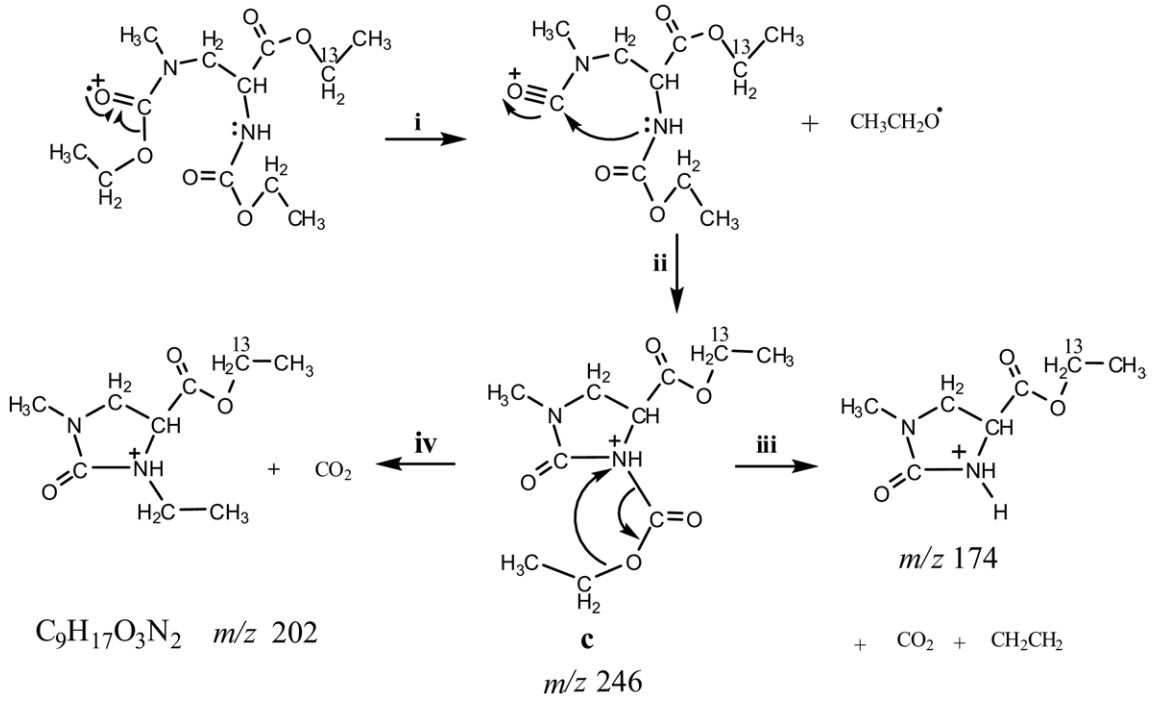
Scheme 3

One should note that a closer examination of the expanded spectrum at *m/z* 245, inserted in the spectrum in Figure 2a, reveals that a peak at *m/z* 245.12 is also present. Observation of the peak at *m/z* 245 in Figure 2a can be explained as (1) the yield of the ^{13}C-labeled ECEE derivative being <100% or (2) a small percentage of the ^{13}C-labeled ECEE derivative undergoing the loss of [$CH_3{}^{13}CH_2O\cdot$] from the ester group in the EI source.

Another earlier reported fragmentation path of the ECEE derivative of basic amino acids is the ejection of CH_3CH_2OH through a McLafferty rearrangement in which a stable odd electron immonium cyclic ion $[M—HOCH_2CH_3]\cdot^+$ *m/z* 244 is formed [6]. However, this process is not observed in our results, which is likely explained by either the lack of an available H at the tertiary amide moiety or a possible steric effect.

Examination of Figure 2a reveals another noteworthy peak at *m/z* 202.10. This peak was not interpreted in the earlier report [11]. However, the formation of this fragment ion is of particular interest because of the shift of *m/z* value between the ^{13}C-labeled and the non-^{13}C-labeled mass spectra. This fact is indicative of a conservation of the ester side carboxylic acid group during the process of dissociation. Exact mass measurement suggests that the composition of this fragment ion is $C_9H_{15}O_4N_1$ (Table 1). The formation of this ion may be rationalized in terms of a neutral loss of $NH{=}COHOCH_2CH_3$ through a γ-H rearrangement. A possible fragment route is shown in Scheme **5**.

The McLafferty rearrangement of molecular ions plays a role during the process of ejection of the neutral loss of $NH{=}COHOCH_2CH_3$ from the molecular ion. The γ-H rearrangement generates an open chain immonium



Scheme 4

Table 1. Exact mass measurements of fragmentation ions

Mass	Theoretical mass	Delta (ppm)	Delta (mmu)	Composition
44.0495	44.0495	1.4	0.1	$C_2H_6N_1$
56.04974	56.0495	4.7	0.3	$C_3H_6N_1$
72.0811	72.0808	4.6	0.3	$C_4H_{10}N_1$
88.0396	88.0393	3.5	0.3	$C_3H_6O_2N_1$
99.0552	99.0553	−0.9	−0.1	$C_4H_7O_1N_2$
116.0704	116.0706	−1.8	−0.2	$C_5H_{10}O_2N_1$
201.0992	201.0996	−1.6	−0.3	$C_9H_{15}O_4N_1$
217.1186	217.1183	1.6	0.3	$C_9H_{17}O_4N_2$

ion (Scheme **5**) and the subsequent dissociation of the neutral moiety from the molecular ion yields a distonic ion [12–15] containing an odd number of electrons and nitrogen atoms (Scheme **5**.). Usually, the distonic ions are predicted to be thermodynamically more stable than their counterpart ions [14, 15]. Considering the competition of dissociation channels of the molecular ions, another possible fragmentation route responsible for the formation of this ion is shown in Scheme **4**. In this fragmentation route, a five-member cyclic species **c** is formed through the ejection of the radical [CH_3CH_2O·]. Two possible fragmentation pathways can subsequently take place from this fragment ion: path **iii** is a γ-H rearrangement accompanied by the expulsion of CO_2 and CH_2CH_2 from the ion, which leads to the formation of an ion represented by a peak at *m/z* 174.10 (Figure 2a); path **iv** represents another possible fragmentation pathway for the formation of an ion at *m/z* 202 through the ejection of CO_2. An exact mass measurement, however, excludes the possibility of the formation of the ion at *m/z* 202 through path **iv**.

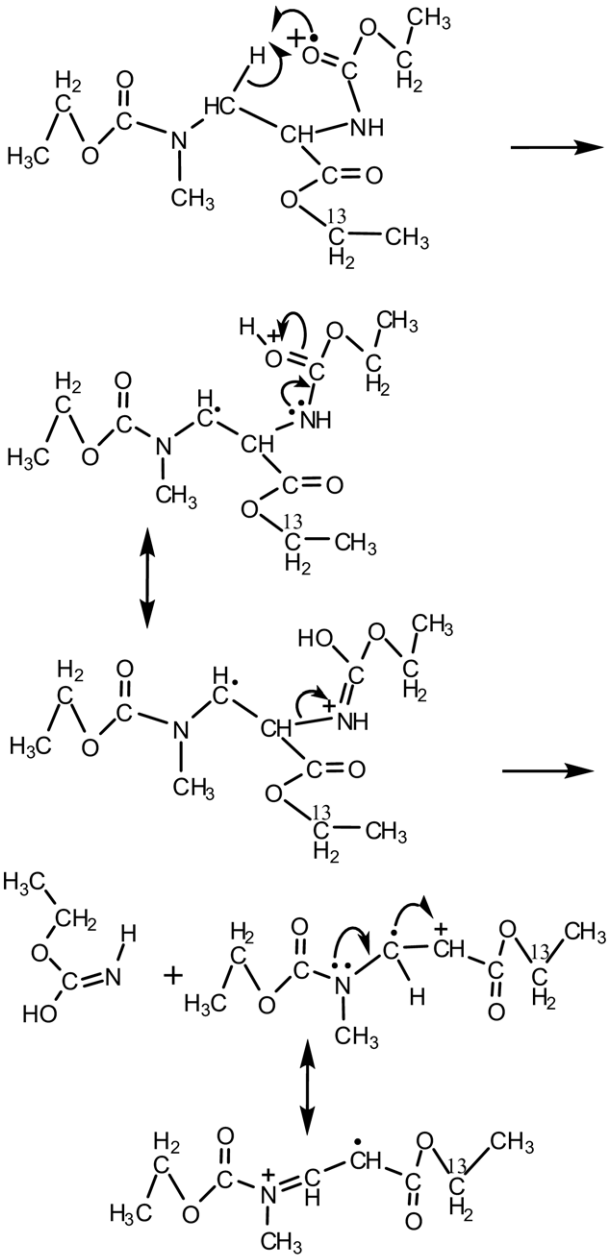
It is obvious that the majority of peaks in Figure 2a do not present a shift in the *m/z* value. This is highly indicative of an ejection of the carboxylic acid ester group [$CH_3{}^{13}CH_2O$·] from the fragments. The formation of the ion at *m/z* 217 may be explained in terms of the α-cleavage of the carboxylic acid ester group from the molecular ion. The possible two-step fragmentation pathway is shown in Scheme **6**.

First, the α-cleavage of the carboxylic acid ester ejects the radical [CH_3CH_2O·]; second, elimination of CO caused by the second α-cleavage gives rise to the peak at *m/z* 217. An isomerization between ions **d** and **e** would occur as a result of the unusual positive charge-retention ability of nitrogen, shown in Scheme **6**. This fragment ion is characterized by an even number of electrons and nitrogen atoms. The composition of this species is confirmed by the exact mass measurement (Table 1).

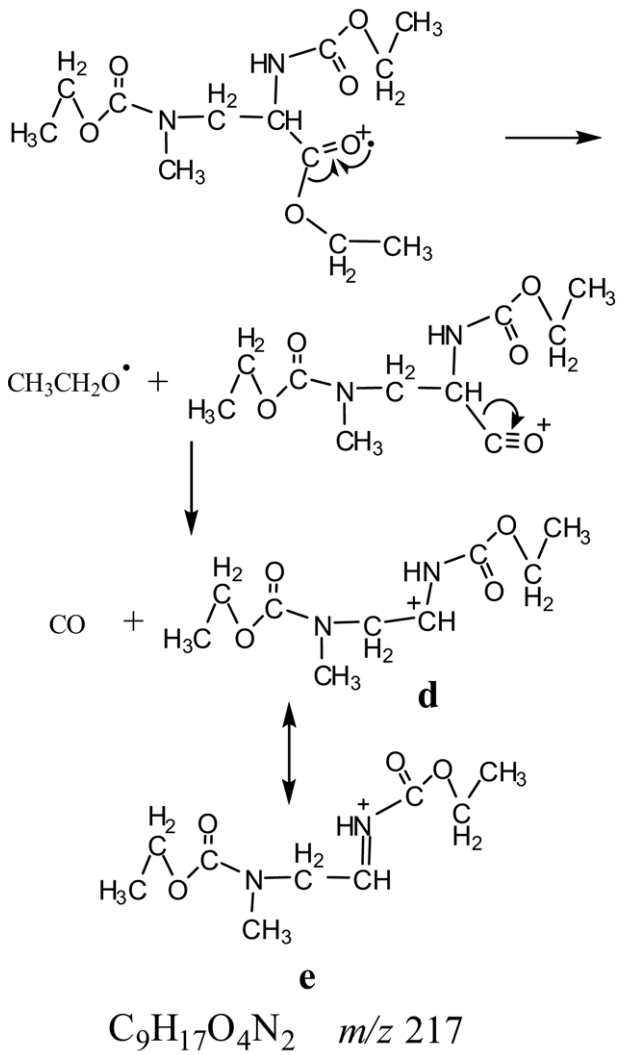
The molecular ion peak of the BMAA derivative is absent in both EI mass spectra (Figures 1a and 2a). Peaks at *m/z* 116.08, 44.05, and 72.11 dominate the low-mass range and deserve our attention. The formation of the ion at *m/z* 116 may well be explained in terms of the amine α-cleavage. The possible fragmentation route is shown in Scheme **7**.

This fragment ion contains an even number of electrons and an odd number of nitrogen atoms. Two other peaks that show no shift between the ^{13}C-labeled and unlabeled mass spectra are at *m/z* 44.05 and 72.11. The formation of these two peaks can be interpreted in terms of the successive expulsion of CO_2 and CH_2CH_2 from the precursor ion at *m/z* 116, shown in Scheme **8**, **i** and **ii**.

The ejection of CO_2 proceeds through a [$CH_3CH_2^{\cdot}$] transfer, which gives rise to the peak at *m/z* 72.11 (Scheme **8i**). The succeeding expulsion of ethylene through the β-H migration produces the complex ion,



Scheme 5



Scheme 6

in which the incipient imine is coordinated with the putative ethane [16]. The subsequent dissociation leads to the formation of immonium ion at *m/z* 44 as a result of the greater proton affinity of imines (Scheme **8ii**). Another fragment ion at *m/z* 88 may be explained as a result of the expulsion of CH_2CH_2 from the ion at *m/z* 116 (Scheme **8iii**). It is evident, by examining the relative abundances of ions $[116\text{-}CH_2CH_2]^+$ and $[116\text{-}CO_2]^+$ in the mass spectra, that CO_2 loss through the transfer of CH_2CH_2 is favored over direct expulsion of CH_2CH_2. The elemental compositions of these fragments are confirmed by exact mass measurements (Table 1).

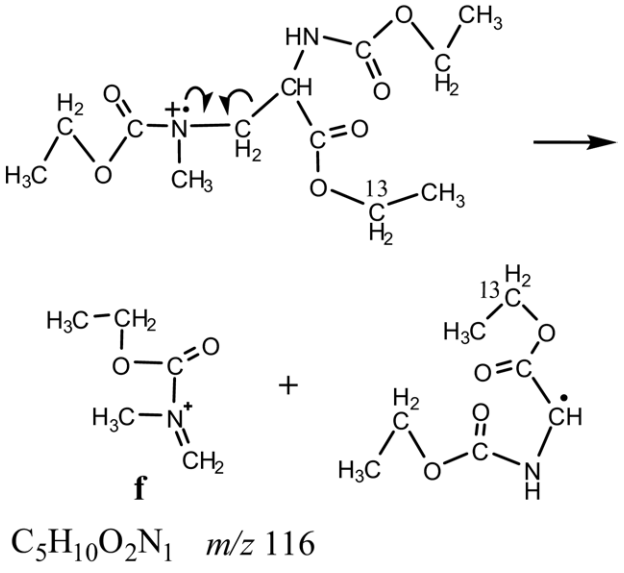
Another peak with an odd-numbered *m/z* value in Figures 1a and 2a is the one at *m/z* 99. Observation of a constant *m/z* value in the spectra indicates a $[CH_3{}^{13}CH_2O\cdot]$ loss during the dissociation process. An exact mass measurement suggests that the composition of this peak is $C_4H_7ON_2$. The formation of the ion at *m/z* 99 may be explained by a cyclic mechanism. The stepwise fragmentation process is illustrated in Scheme **9**.

A subsequent cleavage at the carboxylic ester side group from the molecular ion gives rise to the peak at *m/z* 217 (Scheme **6**). The succeeding ejection of ethanol from $[M—COOCH_2CH_3]^+$ through the [H·] transfer generates a five-member cyclic intermediate at *m/z* 171. The ejection of CH_3CH_2OH is assisted by the polarity of the C—O bond and the amine group. The final product, a stable five-member cyclic immonium ion, is formed as a result of [H·] rearrangement through the ejection of CO_2 and CH_2CH_2 from the intermediate (Scheme **9**). Another possible pathway for the formation of ion *m/z* 99 is the expulsion of $[CH_3CH_2O_2C\cdot]$ from the cyclic immonium ion *m/z* 246 (Scheme **10**). Both pathways lead to the formation of the cyclic structure ion; this cyclic mechanism can be rationalized by the SNi (Substitution Nucleophilic internal) reaction [17, 18]. Both fragmentation processes yield a fragment ion with an even number of electrons and nitrogen atoms.

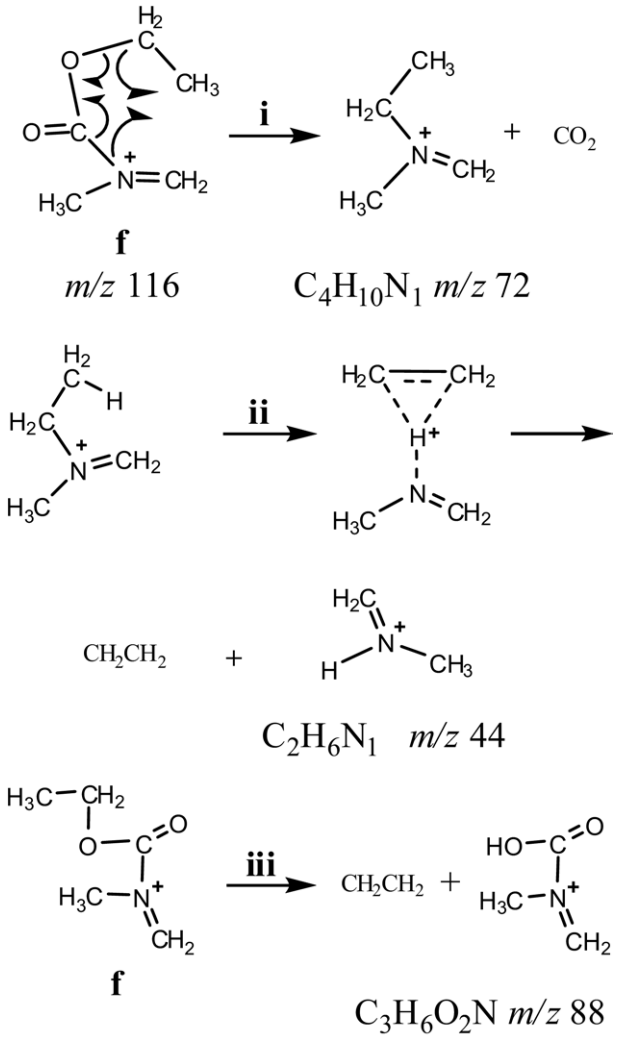
A plausible two-step process for the formation of ions at *m/z* 56 is shown in Scheme **11**. First, rearrangement of ion **d** leads to the formation of a three-member cyclic immonium ion. Second, expulsion of radicals $[CH_3CH_2O_2C\cdot]$ and $[CH_3CH_2O_2CNH\cdot]$ gives rise to the three-member cyclic immonium ion *m/z* 56.

Conclusion

It is shown that the formation of the ion at *m/z* 245.12 arises from $[CH_3CH_2O\cdot]$ loss. There are three dissociation channels that can be assigned to $[CH_3CH_2O\cdot]$ loss. It has been shown, based on the ^{13}C-isotope labeling results, that the α-cleavage from the amino side group is responsible for the formation of the peak at *m/z* 245.12. Considering oxygen as a weak charge-stabilizing agent, it is not surprising that the acylium ion at the carboxylic group side would preferably undergo further CO loss (28 u), forming the stable carbenium ion at *m/z* 217



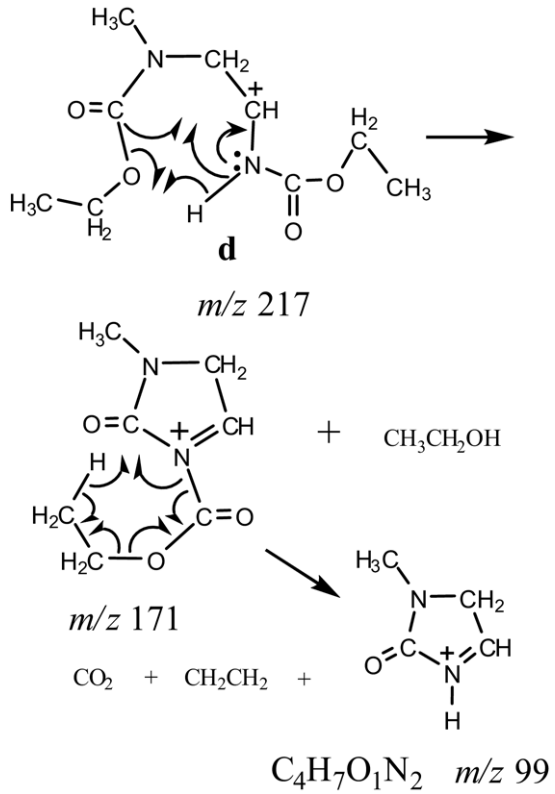
Scheme 7



Scheme 8

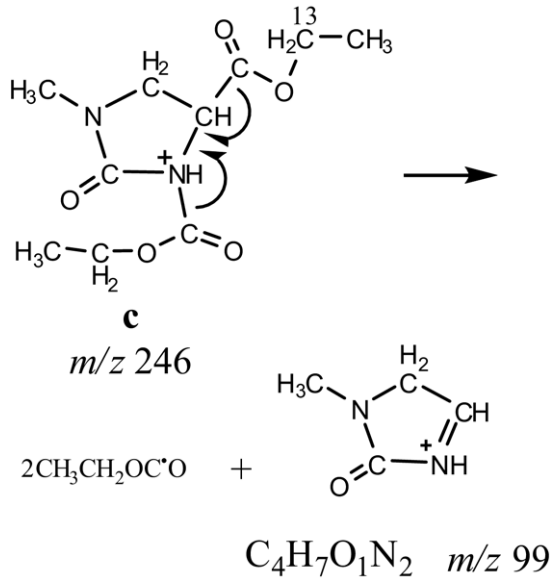
rather than the ion at *m/z* 245. Whereas nitrogen is a somewhat stronger charge stabilizer than oxygen, the fragment ion (*m/z* 245.12) formed at the amino side can be stabilized by the positive charge distribution through the nitrogen atom. We suggest that the peak at *m/z* 202 be assigned to a distonic ion. The exact mass measurement excludes the possibility of the cyclic ring structure. The γ-H rearrangement shown in Scheme **5** results in a charge migration and a loss of stable neutral molecule, NH=COHOCH$_2$CH$_3$. During the process of dissociation, a competition between the separating groups for the charge would result in a stable neutral loss and a fragment with a lower ionization energy carrying the charge. The thermodynamic stability of the open-chain distonic ion *m/z* 202 and the unusual charge-stabilizing ability of the nitrogen atom would account for the formation of this open-chain structure ion at *m/z* 202; the partial driving force could be the loss of the stable neutral species NH=COHOCH$_2$CH$_3$.

The ion at *m/z* 217 can be assigned to a two-stepwise α-cleavage of the carboxylic acid ester group from the

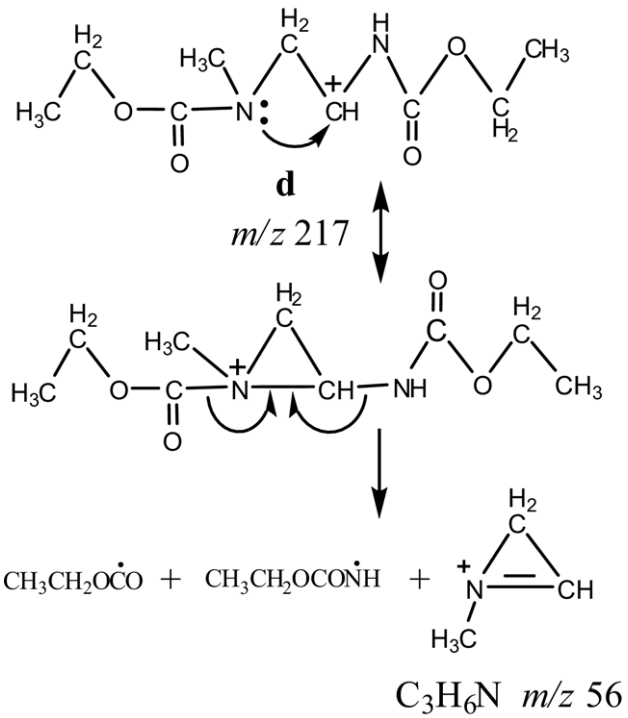


Scheme 9

molecular ion. Another odd-numbered peak at *m/z* 99 could be interpreted as a five-member cyclic immonium ion, which is the daughter ion of the peak at *m/z* 217 or 245. The cyclic mechanism can be rationalized by considering the SNi reaction. The peaks at *m/z* 88, 72, and 44 can be explained as the fission of the precursor amine ion *m/z* 116 by ejection of CO_2 and CH_2CH_2.



Scheme 10



Scheme 11

Acknowledgments

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