RESEARCH ARTICLE

Electrospray MS-based characterization of β-carbolines – mutagenic constituents of thermally processed meat

Antônio Eduardo Miller Crotti[1,2], Paul J. Gates[3], João Luis Callegari Lopes[4] and Norberto Peporine Lopes[4]

[1] Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto–USP, Ribeirão Preto, SP, Brazil
[2] Núcleo de Pesquisas em Ciências Exatas e Tecnológicas, Universidade de Franca, Franca, SP, Brazil
[3] School of Chemistry, University of Bristol, Cantock's Close, Bristol, UK
[4] Departamento de Ciências Farmacêuticas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto–USP, Ribeirão Preto, SP, Brazil

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The β-carbolines 1-methyl-9*H*-pyrido [3,4-*b*]indole and 9*H*-pyrido[3,4*b*]indole have been implicated as having causative roles in a number of human diseases, such as Parkinson's disease and cancer. As they can be formed during the heating of protein-rich food, a number of analytical methodologies have been proposed for their detection and quantification in foodstuff. For this purpose, LC-MS and LC-MS/MS have emerged as the most specific analytical methods, and the quantification is based on the occurrence of unusual ions, such as $[M+H-(H^{\bullet})]^+$ and $[M+H-2H]^+$. In this study, we have investigated the formation of these ions by accurate-mass electrospray MS/MS and demonstrated that these ions are formed from gas-phase ion-molecule reactions between water vapor present in the collision cell and the protonated molecule of 1-methyl-9*H*-pyrido [3,4-*b*]indole and 9*H*-pyrido[3,4*b*]indole. Although this reaction has been previously described for heterocyclic amine ions, it has been overlooked in the most of recent LC-MS and LC-MS/MS studies, and no complete data of the fragmentation are reported. Our results demonstrate that additional attention should be given with respect to eliminating water vapor residues in the mass spectrometer when analysis of β-carbolines is performed, as this residue may affect the reliability in the results of quantification.

Keywords:
β-Carbolines / Food mutagens / Fragmentation reactions / LC-MS / MS

1 Introduction

The β-carbolines harman (1-methyl-9*H*-pyrido [3,4-*b*]indole) and norharman (9*H*-pyrido[3,4*b*]indole) (Fig. 1) are aromatic heterocyclic amines that can be formed during the heating of protein-rich food [1–3]. The probable main sources of these compounds are the industrial processing of meat and fish; however, a number of external sources have been identified, such as alcoholic drinks, tobacco smoke, brewed coffee and medicinal plants [1, 4].

Harman and norharman have been implicated in a number of human diseases including Parkinson's disease [5] and cancer [6]. They have also been shown to be endogenous mutagens in the presence of other aromatic amines, such as aniline and *o*-toluidine [7]. For these reasons, they must be present only in the human diet below particular safety levels [1, 4]. Therefore, the legislative demands have led to an increasing number of studies and publications relating to their selective and sensitive detection, especially in foodstuffs [8–16].

Correspondence: Professor Norberto Peporine Lopes, Departamento de Física e Química, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo. Av. Café s/n, CEP 14040-903, Ribeirão Preto-SP, Brazil
E-mail: npelopes@fcfrp.usp.br
Fax: +55-16-633-2960

Abbreviations: Harman, 1-methyl-9*H*-pyrido [3,4-*b*]indole; **Norharman,** 9*H*-pyrido[3,4*b*]indole

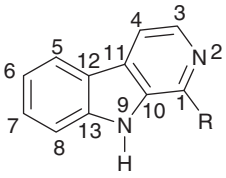

Figure 1. Structure of harman (1) and norharman (2).

LC-MS has emerged as the most specific analytical method for qualitative and quantitative analysis of harman and norharman and other β-carbolines in complex samples without derivatization [14, 16]. Several LC-MS methodologies have been published using atmospheric pressure chemical ionization [8, 17] and ESI [18–22] in the positive ionization mode. The quantification of harman and norharman in foodstuff uses the ions $[M+H-(CH_3^{\bullet})]^+$ and $[M+H-(H^{\bullet})]^+$, respectively. The formation of these ions from their protonated molecules violates the ''Even-Electron Rule'' [23]. Other ions that are used have only been tentatively assigned as $[M+H+16]^+$ and $[M+H-2H]^+$ based on low-resolution MS.

Previous studies using Fourier-Transform ion cyclotron resonance MS investigated the reactivity of protonated heterocyclic amines in the gas-phase [24]. Using ultra-high resolution accurate-mass analysis of the CID reactions, it was demonstrated that these compounds are highly reactive with water vapor or other nucleophilic contaminants in the mass analyzer. The occurrence of ion-molecule reactions of the aromatic nucleophilic substitution type reactions leads to the formation of the, apparently unexpected, ions in the mass spectra, especially in ion-trap systems. However, some more recent LC-MS methodologies for the identification and quantification of harman and norharman in foodstuffs were still being based on the uncommon ions $[M+H-(CH_3^{\bullet})]^+$, and $[M+H-(H^{\bullet})]^+$, $[M+H+16]^+$ and $[M+H-2H]^+$ with little regard for the occurrence of any ion–molecule reactions and the ions that can originated from them [12, 13, 22]. Furthermore, the scientific literature seems to contain no detailed information on the formation of these ions.

Therefore, the aim of this study is to investigate the fragmentation pathways of the protonated β-carbolines 1 ($[M+H]^+$ *m/z* 183) and 2 ($[M+H]^+$ *m/z* 169) using ultra-high resolution accurate-mass ESI-MS/MS. These two molecules differ only in the presence (or not) of a methyl substituent at C(1) (Fig. 1). It will be demonstrated that this small difference causes some significant variation in the appearance of product ion spectra of harman and norharman.

2 Materials and methods

HPLC grade acetonitrile was obtained from Aldrich (Gillingham, Dorset, UK). Deionised water was used throughout the study. Harman and norharman were obtained from Sigma (Poole, Dorset, UK). Stock solutions of each compound (10 mg/mL) were prepared in methanol and stored at 4°C. Dilute standards were prepared prior to each analysis in methanol–water 8:2 v/v with 0.25 mg/mL as the final concentration. Deuteration was performed by dilution of the stock solution in CD_3OD:D_2O (1:1 v/v). The solution was thoroughly mixed to ensure all exchangeable protons were deuterated.

MS analyses were performed on a BioApex II (4.7 Tesla) Fourier-Transform ion cyclotron resonance instrument (Bruker Daltonics, Billerica, MA, USA). Solutions were infused by syringe pump through the Analytica ESI source at 100 μL/h operating in the positive mode. Fragmentation analyses were performed on the isolated precursor ions by sustained off-resonance irradiation-CID using CO_2 collision gas at 40 eV. Sustained off-resonance irradiation-CID allows more collisions to occur between the analyte ions and collision gas, thus increasing the efficiency at lower energy. Precursor ions were selectively isolated through correlated sweep isolation. The cell conditions were tuned to fragment at least 50% of the precursor ion. Accurate masses were obtained within 5 ppm for all fragment ions.

3 Results and discussion

3.1 Analysis of the MS/MS data of protonated 1 and 2

The product ion spectrum of protonated 1 (*m/z* 183) and 2 (*m/z* 169) are shown in Figs. 2A and B, respectively. Data from these spectra are also summarized in Table 1. Peaks at higher *m/z* than the corresponding precursor ions (for harman at *m/z* 185 and *m/z* 199 and norharman at *m/z* 185) are the result of the aromatic nucleophilic substitution reactions at positions 3 and 1, respectively, as reported previously [24]. Analysis of the accurate-mass data reveals that the ions at *m/z* 182 (for 1) and *m/z* 168 (for 1 and 2) derived from a loss of ammonia from *m/z* 199 and *m/z* 185, instead of being formed from the protonated molecule by homolytic cleavage of the C(1)–R bond, as previously proposed based on low-resolution data only [8, 17–22].

The product ions at *m/z* 115 are the result of consecutive losses of acetonitrile and HCN (for harman) and two consecutive losses of HCN (for norharman), as described by Toribio *et al.* [8, 17]. In addition, the previously unreported product ions at *m/z* 166 ($[M+H-(HCN)]^+$) and 129 ($[M+H-(2HCN)]^+$) were observed in the spectrum of 1.

Comparison between data from the MS/MS spectra of protonated harman and norharman strongly suggests that the methyl substituent group of harman is lost as acetonitrile followed by a further loss of HCN. In contrast, norharman, which is unsubstituted at position 1, showed two losses of HCN. The loss of two HCN molecules from the protonated molecule of harman, which arise from the previously unreported ion at *m/z* 156, is evidence for two

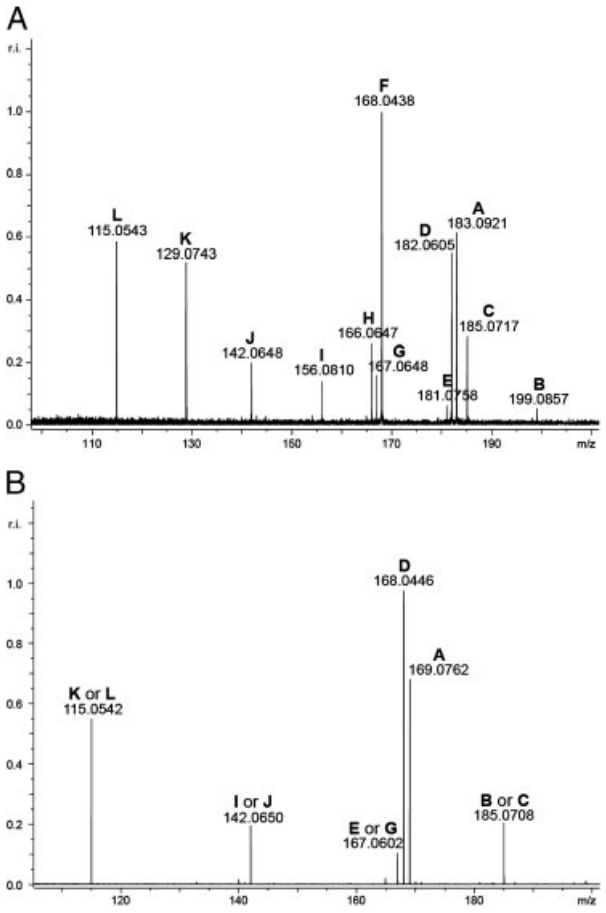

Figure 2. Product ion spectrum of protonated 1 (A) and 2 (B). Protonated molecules were obtained by ESI (positive mode) and their corresponding product ion spectra were obtained by CID using CO_2 as collision gas at 40 eV.

alternative fragmentation routes for the protonated 1 and 2, as discussed above.

3.2 Formation of $[M+H-(HCN)]^+$, $[M+H-(RCN)]^+$ $[M+H-(2HCN)]^+$ and $[M+H-(CH_3CN)-(HCN)]^+$

The additional energy content that is transferred to the protonated molecule of 1 (*m/z* 183) and 2 (*m/z* 169) due to the activation with the collision gas may overcome the critical barrier for proton migrations between the nitrogen atoms, as well as for the hydrogen migration from N(9) to the α-carbon C(13) of the indole ring [25], with consequent loss of aromaticity (Fig. 3). An equilibrium between the protonated forms A1 and A2 is made easier due to the change in the hybridization of N(9), which results in the intermediate ion A2. Opening of the five-membered ring is driven by the re-aromatization of the benzene ring, and results in the open-chain ion A3, which is a putative intermediate for the subsequent fragmentation (Fig. 3).

Elimination of RCN from A3 (pathway I, Fig. 4) requires the nucleophilic attack of the π-cloud of the benzene ring to C(3) to produce a five-membered ring. The resulting product ion J (of *m/z* 156 for harman and *m/z* 142 for norharman) can isomerize to J1 by means of a 1,3-hydrogen rearrangement so that the aromaticity of the benzene ring is re-established. Further elimination of HCN produces L (*m/z* 115), which is resonantly stabilized. (Fig. 4).

Similarly, pathway II involves the attack of the π-cloud of the benzene ring to C(1), which is made easier due to its spatial proximity, as shown in Fig. 4. The resulting product ion A4 can isomerize to produce A5 by means of a sigmatropic 1,3-hydrogen rearrangement. Elimination of HCN from A5 can occur following two competitive pathways: (i) a ring contraction (pathway IIa) and (ii) heterolytic cleavage of the C(10)–C(11) to produce I1, whose structure is identical to J1 (pathway IIb). Further elimination of HCN by a mechanism similar to that operating in the formation of I1 from A5 results in the product ion K (*m/z* 129 for harman, and *m/z* 115 for norharman), which also is resonantly stabilized.

3.3 Formation of $[M+H+16]^+$, $[M+H-1]^+$ and $[M+H-2H]^+$

The unusual product ion $[M+H+16]^+$ (*m/z* 199 for harman and *m/z* 185 for norharman) is produced from the protonated molecule as a result of the nucleophilic attack of residual water vapor in the collision cell to C(1) or C(3) (Fig. 5), followed by RH or H_2 elimination, respectively, as previously demonstrated by Lopes *et al.* [24] (Fig. 5).

The most intense product ion in the MS/MS spectra of both of the compounds is ion D (*m/z* 168), which arises from C ($[M+H-(RH)]^+$) by means of an elimination of ammonia. Ammonia elimination was also observed to occur from protonated 1 (*m/z* 183) to produce *m/z* 166, whereas the corresponding ion for 2 (*m/z* 152) was not observed. This indicates that the hydrogen of the substituent at positions 1 or 3 (a hydroxyl, for the substitution product of both of the compounds, or a methyl, for protonated 1) plays a key role in the elimination of ammonia. In order to confirm the influence of the hydrogen at position 1 on the mechanism, the analysis was repeated with norhaman in deuterated solvents. This results in exchange of the proton to a deuteron, thus allowing the corresponding mass shifts to be followed throughout the fragmentation mechanism. Figure 6 shows the product ion spectrum of deuterated norharman $[M+2D]^+$ (*m/z* 187). It is clear that both the deuterons remain in the structure after the ammonia elimination as the product ion resulting from the loss of ammonia is observed at *m/z* 170 – a loss of NH_3 – *i.e.* no deuterons are lost.

Figure 5 shows the formation of the product ions from C [24]. The mechanism that leads to the formation of the intermediate ion C3 (Fig. 7) is similar to that for the formation of A3 (Fig. 4), but in this case, R = OH. Pathway

Table 1. Ions observed in the MS/MS spectra of harman and norharman in the positive mode

Fragment	Formula	Relative intensity (%)	Observed mass	Mass error (ppm)
Harman		5		
B (A+H_2O–H_2)[a)]	$C_{12}H_{11}N_2O^+$	30	199.0857	−4.52
C (A+H_2O–CH_4)[a)]	$C_{11}H_{19}N_2O^+$	60	185.0717	+4.32
A	$C_{12}H_{11}N_2^+$	25	183.0921	+2.18
D (B–NH_3)[a)]	$C_{12}H_8NO^+$	55	182.0605	+2.75
E (B-H_2O)[a)]	$C_{12}H_9N_2^+$	5	181.0758	−2.76
F (C–NH_3)[a)]	$C_{11}H_6NO^+$	100	168.0438	−3.57
G (C–H_2O)[a)]	$C_{11}H_7N_2^+$	20	167.0648	+1.79
H (A–NH_3)	$C_{12}H_8N^+$	30	166.0647	−2.41
I (A–HCN)	$C_{11}H_{10}N^+$	15	156.0810	+1.28
J (A–CH_3CN)	$C_{10}H_8N^+$	20	142.0648	−2.11
K (G–HCN)	$C_{10}H_9^+$	55	129.0703	+3.10
L (H–HCN)	$C_9H_7^+$	60	115.0543	+0.87
Norharman				
B or C (A+H_2O–H_2)[a)]	$C_{11}H_9N_2O^+$	20	185.0708	−0.54
A	$C_{11}H_9N_2^+$	70	169.0762	+1.18
D (B or C–NH_3)[a)]	$C_{11}H_6NO^+$	100	168.0446	+1.19
E (B–H_2O) or G (C–H_2O)[a)]	$C_{11}H_7N_2^+$	10	167.0602	−1.19
G or H (A–HCN)	$C_{10}H_8N^+$	20	142.0650	−0.70
I (G–HCN)/J (H–HCN)	$C_9H_7^+$	55	115.0542	0.00

a) Indicates the product ions arising from the aromatic nucleophilic substitution of AHA ions.

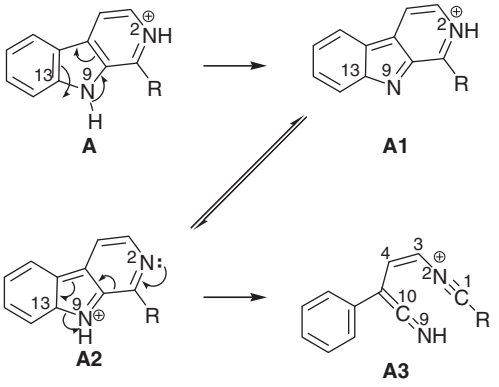

Figure 3. The proposed pyridine and pirrole ring opening of protonated 1 and 2.

IV is similar to pathway I (Fig. 4) and results in the product ions J and L. Pathway III is initiated by the nucleophilic attack of the π cloud of the aromatic ring to C(1), resulting in the formation of the intermediate ion C1. This step is facilitated by the spatial proximity of the π cloud to C(1), which is easily visualized by inspection of a molecular model. The intermediate ion C1 can re-aromatize to produce C2 (pathway IIIb) and C7 (pathway IIIa). Further water elimination from C5 produces G (*m/z* 167), which was erroneously assigned as ([M+H-$CH_3^{\bullet}$-$H^{\bullet}$]$^+$) for harman and ([M+H-2H]$^+$) for norharman in some articles [8, 17]. Pathway IIIb involves C2 isomerization to produce C3. Further, lone-pair-assisted ring contraction from a seven- to a six-membered ring converts C3 into C4, which exhibits a primary amine function. Previous studies undertaken with other β-carbolines reported that the ammonia elimination easily occurs from compounds exhibiting primary amines in their structures [17]. Thus, it would be expected that the ammonia elimination from C4 involves the participation of the hydrogen at N(9). However, experiments with deuterated solvents (Fig. 6) showed loss of NH_3 (17 mass units) instead of NH_2D (18 mass units). This strongly suggests the ammonia elimination is preceded by a rearrangement, which is proposed to occur by abstraction of the hydrogen at C(3) by the lone-pair electrons of N(2). The resulting intermediate ion C4 can rearrange to produce C6, which can easily eliminate NH_3 to result in the product ion D (*m/z* 168) which is highly stabilized by resonance. In the case of harman, the elimination of NH_3 and H_2O from B (Fig. 5), which leads to the formation of the unusual product ions [M+H-H]$^+$ (D, *m/z* 182) and [M+H-2H]$^+$ (E, *m/z* 181), as well the product ion [M+H-(NH_3)]$^+$ (H, *m/z* 166) proceed by a similar mechanism to that shown in Fig. 7.

4 Concluding remarks

In summary, we have concluded from ultra-high resolution accurate-mass data that the apparently uncommon product ions [M+H-2H]$^+$ and [M+H-H]$^+$ in the MS/MS spectrum of harman and norharman are respectively formed by water and ammonia elimination from the product of ion-molecule reaction ([M+H+16]$^+$) between water vapor in the collision

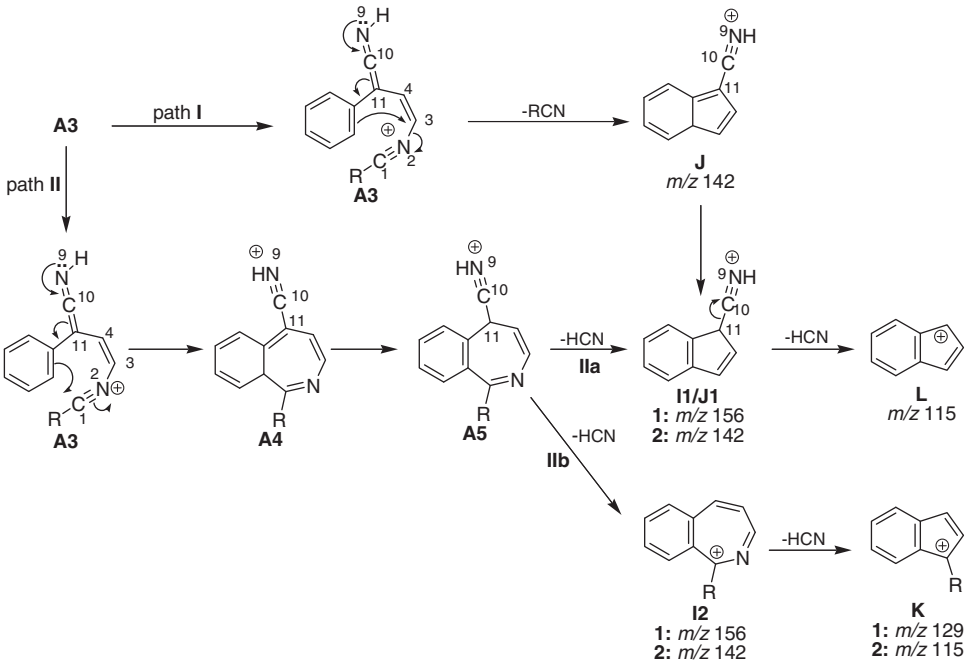

Figure 4. The proposed formation of the product ions I, J, K and L.

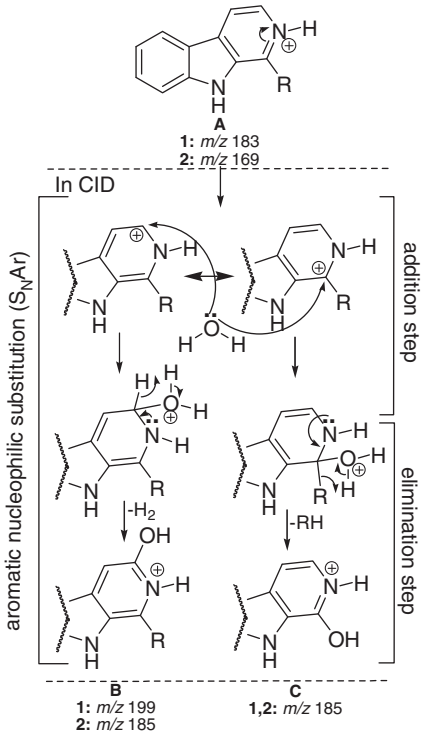

Figure 5. The formation of the product ions B and C [24].

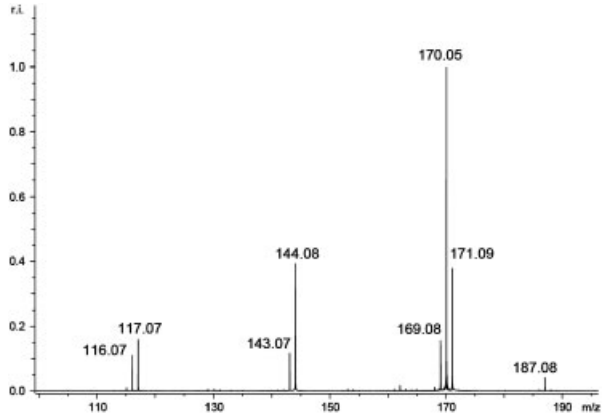

Figure 6. The product ion spectrum of deuterated norharman $[M+2D]^+$ (*m/z* 187) obtained in positive mode (collision gas at 40 eV).

cell and the highly reactive protonated molecules of these compounds. Several previous articles have erroneously assigned the ion $[M+H-H]^+$ of harman and norharman, which has being formed by radical elimination of methyl and hydrogen, respectively. Our results demonstrate that careful attention must be given with respect to the presence of water residues in the collision cell when β-carbolines are analyzed. The residual water may affect the reliability of the results of quantification of harman and norharman in foodstuff by LC-MS and LC-MS/MS or at least lead to difficulties in the reproduction of methodologies between different laboratories. We also must emphasize the importance of using dry collision gases when performing LC-MS/MS experiments, especially for the analysis of foodstuffs where it is essential to determine that safety levels of potential toxins are not being breached.

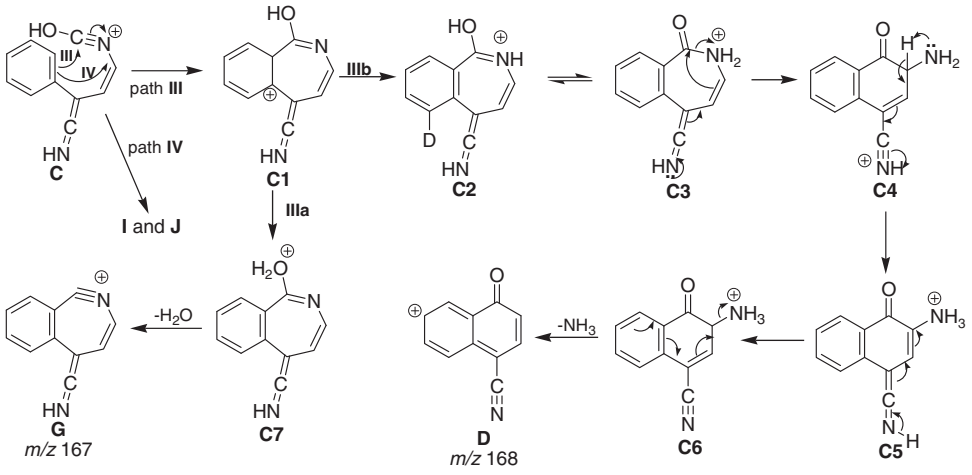

Figure 7. The proposed formation of the product ions D, G, I and J.

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The authors have declared no conflict of interest.

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