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# Fragmentation Pathway for Glutamine Identification: Loss of 73 Da from Dimethylformamide Glutamine Isobutyl Ester

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A fragmentation mechanism for the neutral loss of 73 Da from dimethylformamide glutamine isobutyl ester is investigated. Understanding this mechanism will allow to improve the identification and quantification of  $^{15}\text{N}$ -labeled and unlabeled glutamine and the distinguishing of glutamine and glutamic acid by electrospray ionization (ESI)-tandem mass spectrometry. Before mass spectrometry analysis, glutamine and glutamic acid are derivatized with dimethylformamide dimethyl acetal and isobutanol to form dimethylformamide isobutyl ester. Derivatization conditions are modified based on an existing method to ensure complete derivatization of glutamic acid and to prevent the hydrolysis of glutamine. The fragmentation mechanism of dimethylformamide glutamine isobutyl ester is studied and possible fragmentation pathways are proposed. Based on the fragmentation mechanism, a quantification method is developed to quantify both  $^{15}\text{N}$ -labeled and unlabeled glutamine and glutamic acid at a series of different neutral losses by performing multiple-reaction monitoring (MRM) scans in a triple-quadrupole mass spectrometer. Labeled glutamine includes  $^{15}\text{N}$ -amide labeled,  $^{15}\text{N}$ -amine labeled glutamine and glutamine  $^{15}\text{N}$ -labeled at both amide and amine positions. Deuterium labeled glutamine and glutamic acid are used as internal standards. Isotope effects are characterized for  $^{15}\text{N}$  labeled and deuterium labeled glutamine. It is found that the same method can be used to distinguish aspartic acid from asparagine. This study will improve the application of MS/MS for amino acid quantification and stable isotope labeling metabolism studies. (J Am Soc Mass Spectrom 2005, 16, 1192–1203) © 2005 American Society for Mass Spectrometry

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**A**mino acids (AA), as fundamental units of biological systems, fulfill numerous important functions. The identification and quantification of amino acids are of paramount importance for the diagnosis and monitoring of inherited diseases related to amino acid metabolism [1]. The characterization of amino acids is vital for assessing protein quality, predicting nutritional status, and also for the improvement of culture conditions for the production of amino acids in food industry [1–3]. The availability of rapid, sensitive and reliable methods for amino acid analysis is highly desirable.

Traditionally, the analytical methodologies applied to amino acid analysis include gas chromatography (GC) [4, 5], capillary electrophoresis (CE) [6] and high performance liquid chromatography

(HPLC) [6] coupled with either spectroscopy or mass spectrometry (MS) detectors. Laborious sample preparation or derivatization and long assay times are the major disadvantages of these methods. Moreover, traditional chromatography is not suitable for the study of isotope labeled amino acids due to its inability to distinguish labeled and unlabeled compounds. It was considered an important advancement when tandem mass spectrometry (MS/MS) alone was used for amino acid identification and quantification as a very sensitive, effective, comprehensive, rapid and reliable method [7]. One of the major applications of MS/MS in amino acid analysis has been neonatal blood screening [1]. It has been reported that tandem mass spectrometry, combined with simple chemical derivatization, has the ability to identify and quantify amino acids from one small spot of dry blood, and thus screen for 50 or more metabolic disorders, such as phenylketonuria [8, 9], homocystinuria [10], syrup urine disease [11], and tyrosinemia [1]. The newborn screening program

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Published online May 26, 2005

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employing this method is used for about 4 million babies in the United States each year [12].

Typical amino acid analysis by MS/MS includes two steps: 1) the amino acids are esterified as butyl esters with butanol-hydrogen chloride, and 2) the amino acid butyl esters then are subjected to collision-induced dissociation (CID), which results in the loss of butyl formate (102 Da) as a neutral molecule from most of derivatized amino acids, enabling sensitive analysis [1]. The quantification of amino acids can be achieved by adding deuterium labeled amino acids as internal standards. Johnson [13] improved the analysis sensitivity by derivatizing amino acids into formamidine butyl esters by heating (65 °C, 2 min) amino acids in dimethylformamide dimethyl acetal (DMF-DMA) followed by butanol esterification (65 °C, 15 min). Johnson's method [13] enhances analysis sensitivity by up to 20 fold by coupling the formamidine group to the amine nitrogen of amino acids and taking the advantage of high ionization efficiency of tertiary amines in ESI.

A notable deficiency of the above two derivatization methods, however, lies in their inability to distinguish glutamine and glutamic acid. This is because glutamine can be hydrolyzed to glutamic acid during the butanol derivatization. Moreover, the fragmentation of glutamine during CID gives a fragment peak at the same *m/z* value as pyroglutamic acid, making impossible the distinction of glutamine and pyroglutamic acid. This complicates the identification and the quantification of glutamine and makes inaccurate the quantification of glutamic acid and pyroglutamic acid.

The importance of glutamine and glutamate has been proven and investigated in numerous studies [14–17]. The important functions of glutamine and glutamate create the driving force for probing their roles in numerous processes by analytical identification and quantification techniques. Trinh et al. [18] modified Johnson's two-step derivatization method [13] to distinguish glutamine and glutamic acid. Trinh's butanol derivatization [18] was performed at ambient temperature and for a shorter time period (5 min) to minimize glutamine amide hydrolysis. Glutamine, glutamic acid and pyroglutamic acid can be distinguished by running the MS/MS in multiple-reaction monitoring (MRM) mode with a neutral loss (NL) of 73 Da for glutamine and 102 Da for glutamic and pyroglutamic acid [18]. Quantification of these three amino acids in stored blood samples was performed by adding corresponding deuterium labeled amino acid standards. However, the fragmentation mechanism for NL 73 Da was not clear.

Trinh's method has been modified in our laboratory to investigate the incorporation of <sup>15</sup>N label into several amino acids including glutamine and glutamic acid in mosquitoes. Quantification of <sup>15</sup>N-labeled and non-labeled amino acids was performed [19]. Because in biological studies glutamine could be labeled at either the side chain amide nitrogen or the backbone amine nitrogen, or both, it is necessary to understand the

mechanism for the NL 73 Da and to determine how the mass of the neutral loss changes as the <sup>15</sup>N labeling position changes.

This paper reports an investigation of the dimethylformamidine glutamine isobutyl ester (DMF-Gln-OBu), dimethylformamidine glutamine <sup>2</sup>H<sub>9</sub>-isobutyl ester (DMF-Gln-<sup>2</sup>H<sub>9</sub>-OBu), dimethylformamidine <sup>2</sup>H<sub>5</sub>-glutamine isobutyl ester (DMF-<sup>2</sup>H<sub>5</sub>-Gln-OBu) and dimethylformamidine <sup>15</sup>N-glutamine (side chain amide labeled) isobutyl ester (DMF-<sup>15</sup>N(amide)-Gln-OBu). The fragmentation mechanism involved in NL 73 Da was studied and the possible pathways for NL 73 Da are discussed. A quantification method for labeled and unlabeled glutamine was developed based on the fragmentation mechanism. It was also found that aspartic acid and asparagine can be distinguished by the same method.

Although deuterium labeled internal standards have been used for quantification of AAs, there is no reported study on the influence of the isotope effect of the labeled internal standard vs. unlabeled analyte. In our study, an unexpected isotope effect was observed for deuterium labeled glutamine. As the isotope effect is of paramount importance for the quantification, it was characterized in this study and taken into account for quantification.

## Experimental

### Chemicals

L-glutamine, L-glutamic acid, iso-butanol and thionyl chloride were purchased from Sigma-Aldrich (St. Louis, MO). (2,3,3,4,4)-<sup>2</sup>H<sub>5</sub>-L-glutamine and <sup>2</sup>H<sub>9</sub>-isobutanol were purchased from Cambridge Isotope Laboratories (Andover, MA). <sup>15</sup>N-(amide labeled)-L-glutamine was purchased from Sigma-Aldrich (Milwaukee, WI). Dimethylformamide dimethyl acetal (DMF-DMA) was purchased from Pierce (Rockford, IL). Trifluoroacetic acid was purchased from VWR (Brisbane, CA).

### Glutamine Derivatization

By using derivation methods reported by Trinh et al [18], we observed that incompletely derivatized glutamic acid overlaps in mass with <sup>15</sup>N-labeled dimethylformamidine glutamine isobutyl ester. To resolve this problem and to ensure complete derivatization of glutamic acid without hydrolyzing glutamine, we modified the derivatization conditions. The main modification made was to change the exposure time to isobutanol to 50 min to increase the extent of esterification of both side chain and carboxylic terminal of glutamic acid. In our laboratory the 5 min exposure suggested by Trinh et al. [18] resulted in incomplete derivatization of glutamic acid: only one carboxylic acid group is converted to isobutyl ester, this glutamic derivative overlaps with <sup>15</sup>N-glutamine in mass. Briefly: a 1mM glutamine stock solution was prepared. For each

derivatization, 30  $\mu\text{L}$  of the stock solution was dried using a vacuum centrifuge. Subsequently 240  $\mu\text{L}$  of DMF-DMA-acetonitrile-methanol (2:5:5 by volume) was added and left for 10 min at room temperature. Excess reagents were dried by  $\text{N}_2$  flow. The residue was treated with 200  $\mu\text{L}$  of isobutanol/3M-hydrogen chloride at room temperature for 50 min, and then blown to dryness by  $\text{N}_2$  flow.

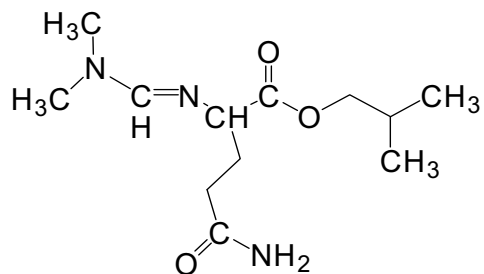
### Synthesis of Dimethylformamidine Glutamic Anhydride

Glutamic acid at 100 mg was mixed with 1.5 mL deionized water, and diisopropylethylamine (DIEA) was added in drops until the glutamic acid was dissolved completely. Subsequently, 1 mL DMF-DMA was added, and the reaction mixture was placed in a water bath for 30 min at 65  $^\circ\text{C}$ . Then the reaction mixture was dried by on a rotary evaporator at 40  $^\circ\text{C}$ . The reaction product was confirmed to be dimethylformamidine glutamic acid by the molecular weight obtained from mass spectrometry ( $\text{MH}^+$ : 200 Da). To obtain dimethylformamidine glutamic anhydride, 3 mL trifluoroacetic acid and 400  $\mu\text{L}$  thionyl chloride were added as reported in the literature [20].

### Low-Energy Collision-Induced Dissociation (CID)

**Ion trap and triple quadrupole mass spectrometer.** Low-energy CID experiments were performed in a Finnigan LCQ ion trap mass spectrometer or Finnigan MAT TSQ 700 (San Jose, CA) triple quadrupole mass spectrometer with an electrospray ion source operating in the positive mode. The glutamine derivatization products were dissolved in a solution of  $\text{H}_2\text{O}:\text{CH}_3\text{OH}$  (1:1; v/v) containing 1% acetic acid to produce a concentration of 20–30 pmol/ $\mu\text{L}$ . The solutions were then sprayed into the mass spectrometer at a rate of 3–5  $\mu\text{L}/\text{min}$ . The applied needle voltage used was 4.0 to 4.8 kV and the capillary temperature was maintained at 200  $^\circ\text{C}$  for all samples. Unit mass selection of the monoisotopic precursor ion was performed in order to avoid ambiguities from isotope contributions. For CID in the LCQ ion trap, helium was used as the collision gas, the excitation energy (indicated as % collision energy by the manufacturer and corresponding to the amplitude of a supplementary AC voltage) was incremented in small steps to selectively monitor low energy fragmentation processes for the precursor ions selected. For MS/MS/MS, desired fragment ions produced from the precursor (MS/MS) were selected and isolated in the trap and then excited. For CID in the TSQ 700, argon was used as the collision gas and laboratory collision energies of 15 to 20 eV were used.

**Fourier transform ion cyclotron resonance (FT-ICR).** An IonSpec (Lake Forest, CA) 4.7-Tesla Fourier transform mass spectrometer combined with an IonSpec electros-



**Figure 1.** Structure of dimethylformamidine glutamine isobutyl ester (DMF-Gln-OBu).

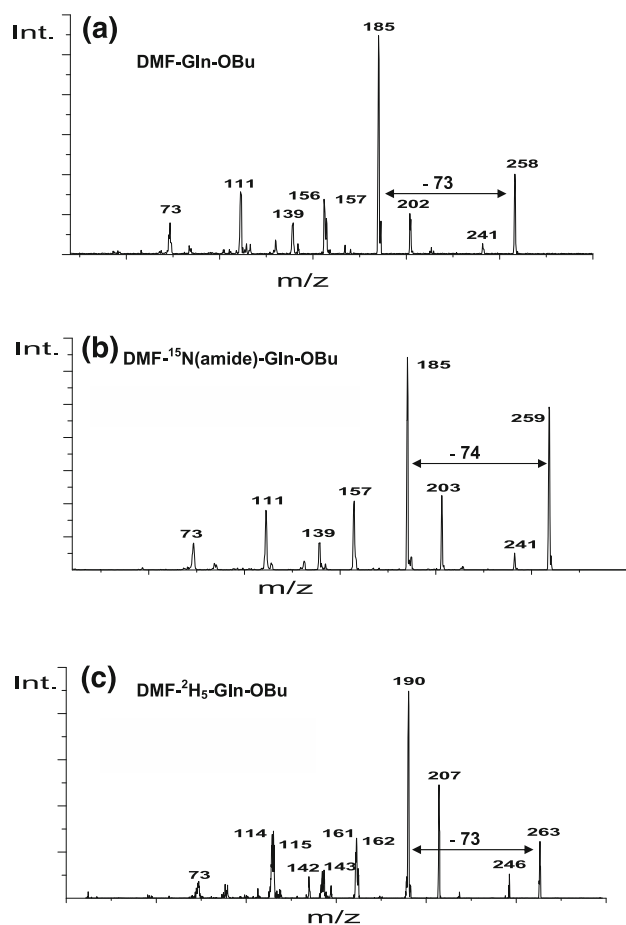
pray ionization source was also used for MS/MS studies. The solutions (10  $\mu\text{M}$ ) were electrosprayed (4 kV) into the vacuum system at a flow rate of 5  $\mu\text{L}/\text{min}$  using a Harvard apparatus syringe pump. Desolvation of the sample was achieved by heating the capillary with counter flow  $\text{N}_2$  gas at 200  $^\circ\text{C}$ . The CID activation method employed was sustained off-resonance irradiation (SORI). The collision gas pulsed into the ion cyclotron resonance (ICR) cell was  $\text{N}_2$ . For the SORI-CID experiments, the radio frequency (RF) irradiation was offset by 1000 Hz above or below the resonance frequency of the selected ions.

## Results and Discussion

### CID Fragmentation Spectra

The derivatization product from the reaction of glutamine with DMF-DMA and isobutanol is dimethylformamidine glutamine isobutyl ester (DMF-Gln-OBu) as shown in Figure 1. DMF-DMA forms a Schiff-base with the amino end of glutamine as reported [13]. The fragmentation spectra of labeled and unlabeled DMF-Gln-OBu are shown in Figure 2. Figure 2a shows the spectrum of unlabeled DMF-Gln-OBu. The peak at 258 represents the parent ion. The base peak at 185 is formed by loss of 73 Da. The peak at 156 results from the loss of 102 Da as butyl formate [21]. When butanol esterification is the only derivatization step for amino acid analysis, the neutral loss 102 Da occurs for all amino acids and is being used currently for the identification of amino acids in blood or plasma samples [1, 22]. In the case of DMF-Gln-OBu, however, neutral loss (NL) 73 Da, instead of 102 Da, produces the base peak, thus it is being used for glutamine identification and quantification as reported [18].

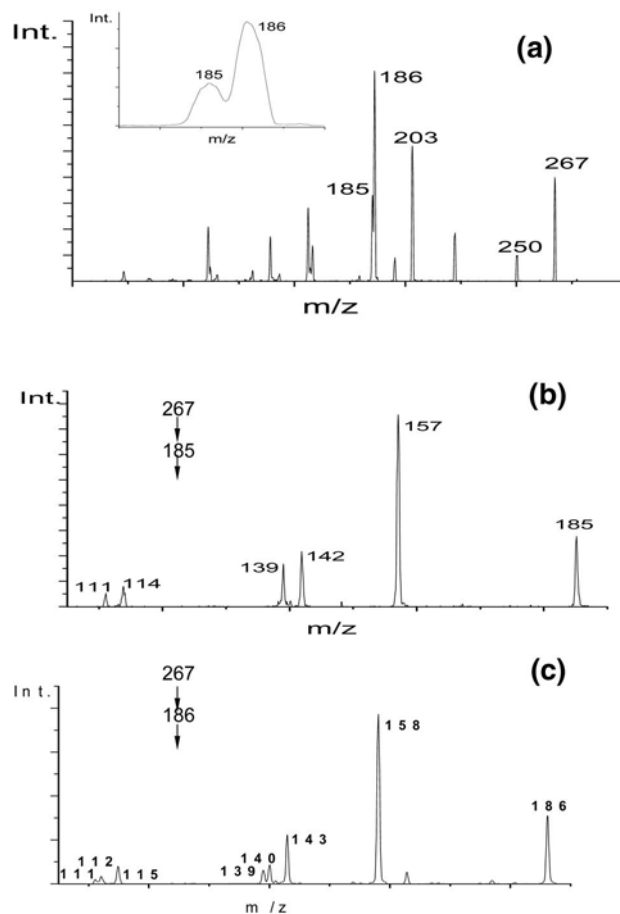
To investigate the fragmentation mechanism, the derivatization products of  $^{15}\text{N}$  (amide-labeled) glutamine and  $^2\text{H}_5$ -glutamine (i.e., DMF- $^{15}\text{N}$ (amide)-Gln-OBu, DMF- $^2\text{H}_5$ -Gln-OBu) were fragmented in the TSQ700 mass spectrometer. Figure 2b shows the fragmentation of the DMF- $^{15}\text{N}$ (amide)-Gln-OBu. The base peak still appears at 185 Da, but the neutral loss here is 74 Da, instead of 73 Da as in the case of unlabeled glutamine, indicating the loss of the side chain  $^{15}\text{N}$ . Fragmentation of the  $^2\text{H}_5$ -glutamine derivatization



**Figure 2.** ESI/low energy CID spectra of: (a) Dimethylformamidine glutamine sobutyl ester (DMF-Gln-OBu) (b) Dimethylformamidine  $^{15}\text{N}$ -(amide labeled) glutamine isobutyl ester (DMF- $^{15}\text{N}$ (amide)-Gln-OBu) (c) Dimethylformamidine  $^2\text{H}_5$ -glutamine isobutyl ester (DMF- $^2\text{H}_5$ -Gln-OBu); obtained with Finnigan TSQ700 using argon as the collision gas, the collision energy used is 20 eV.

product is shown in Figure 2c. The peak at  $m/z$  185 shifts to 190 with the neutral loss still 73 Da, indicating the deuterated side chain methylenes are not involved in the neutral loss of 73.

The fragmentation of dimethylformamidine glutamine  $^2\text{H}_9$ -isobutyl ester (DMF-Gln-O- $^2\text{H}_9$ -Bu,  $m/z$  267) was also investigated (Figure 3a). Two peaks appear at  $m/z$  185 and 186 corresponding to neutral losses of 82 (73+9) and 81 (73+8), with 185 at 40% of 186 (Figure 3a). Compared with neutral loss of 73, an additional 8 or 9 deuteriums are lost indicating loss of isobutyl group as part of neutral loss 73. Further fragmentation spectra of 185 and 186 from DMF-Gln-O- $^2\text{H}_9$ -Bu are shown in Figure 3b and 3c and indicates strong loss of 28 Da (CO). Moreover, the fragments of 185 from DMF-Gln-OBu and 190 from DMF- $^2\text{H}_5$ -Gln-OBu are listed in Table 1. The exact masses of fragments of 185 from DMF-Gln-OBu were obtained by FTICR-CID as shown in Table 2.



**Figure 3.** (a) ESI/low energy CID of dimethylformamidine glutamine  $^2\text{H}_9$ -isobutyl ester; the inset is an expanded view of the peaks at 185 and 186. (b)  $\text{MS}^3$  on 185 or (c)  $\text{MS}^3$  on 186.

### Possible Fragmentation Pathways

Two possible mechanisms for the neutral loss of 73 Da are shown in Schemes 1 and 2; an analysis of all acquired data is necessary to identify which mechanism actually happens. Dimethylformamidine glutamine isobutyl ester has four likely protonation sites: the side chain amide carbonyl oxygen, the ester carbonyl oxy-

**Table 1.** Fragments of 185 and its counterparts from  $^2\text{H}$ -labeled derivatization products

DMF-Gln-OBu	DMF- $^2\text{H}_5$ -Gln-OBu <sup>a</sup> ( $\Delta$ ) <sup>c</sup>	DMF-Gln-O- $^2\text{H}_9$ -Bu <sup>b</sup> ( $\Delta$ ) <sup>c</sup>
185	190 (5)	186 (1)
157	162 (5)	158 (1)
142	147 (5)	143 (1)
139	142 (3), 143 (4)	139 (0), 140 (1)
114	119 (5)	115 (1)
111	114 (3), 115 (4)	111 (0), 112 (1)
84	89 (5)	85 (1)

<sup>a</sup>DMF- $^2\text{H}_5$ -Gln-OBu:  $^2\text{H}_5$ -glutamine derivatized with DMF-DMA and isobutanol.

<sup>b</sup>DMF-Gln-O- $^2\text{H}_9$ -Bu: unlabeled glutamine derivatized with DMF-DMA and  $^2\text{H}_9$ -isobutanol

<sup>c</sup> $\Delta$  represents the number of mass shifts in Da compared to the fragments of DMF-Gln-OBu



**Table 2.** The exact mass of major fragments of A (Scheme 1,  $m/z$  185) by ESI-FTICR/SORI-CID

Proposed formula for MH <sup>+</sup>	Theoretical mass	Experimental mass	Delta (ppm)
C <sub>8</sub> H <sub>13</sub> O <sub>3</sub> N <sub>2</sub>	185.0926	185.0926	0
C <sub>7</sub> H <sub>13</sub> O <sub>2</sub> N <sub>2</sub>	157.0977	157.0972	3.1
C <sub>6</sub> H <sub>8</sub> O <sub>3</sub> N	142.0504	142.0498	4.2
C <sub>7</sub> H <sub>11</sub> ON <sub>2</sub>	139.0871	139.0866	3.5
C <sub>5</sub> H <sub>8</sub> O <sub>2</sub> N	114.0555	114.0550	4.3
C <sub>6</sub> H <sub>11</sub> N <sub>2</sub>	111.0922	111.0918	3.6

gen, and the geminal amine and imine nitrogens. The proton affinities of these sites can be compared to those of CH<sub>3</sub>CONH<sub>2</sub> (863.6 KJ/mol), CH<sub>3</sub>COOCH<sub>3</sub> (821.6 KJ/mol), and (CH<sub>3</sub>)<sub>2</sub>NCH=N-CH<sub>3</sub> (1002.5KJ/mol) respectively [23]. The amine and imine nitrogens have much higher proton affinities than the oxygens. The imine nitrogen could be the initial proton holder because more resonance structures result with the proton on the imine nitrogen than with the proton on the amine nitrogen. The -N-C=N- group is analog to a carboxyl group(-O-C=O-) with carbonyl oxygen as the more basic site. During collision induced dissociation (CID), the proton could be intramolecularly transferred to initiate cleavage.

Scheme 1 illustrates a two-step process for the loss of 73: i) the migration of a hydrogen from the butyl ester to the adjacent carbonyl oxygen via a six-membered ring structure which, together with the loss of isobutene (56 Da) leads to the dimethylformamidinium glutamine structure (202 Da). This hydrogen migration is analogous to the McLafferty rearrangement except that it does not involve a radical cation; thus in this paper, it is referred to as a McLafferty-type rearrangement. A similar rearrangement was also documented for metal adducts of peptide esters [24]. ii) the side chain carbonyl carbon could be attacked by either of the oxygens from the newly-formed carboxylic terminus followed by the loss of NH<sub>3</sub> (17 Da) from the side chain (56+17=73).

A cyclic-anhydride six-membered ring ( $m/z$  185, Scheme 1, A) is produced if the fragmentation mechanism for the neutral loss 73 Da follows Scheme 1. Upon losing an isobutene molecule, a proton-bridged structure is formed between two electronegative carbonyl oxygens. The free rotation of the sigma C-C bond between the carbonyl carbon and the  $\alpha$  C makes possible three inter-converting structures (Scheme 1). Either carboxylic acid oxygen can attack the side chain electropositive carbonyl carbon to produce a six-membered ring structure. A cyclic-anhydride forms after losing NH<sub>3</sub> as a neutral molecule. It is also possible that the NH<sub>3</sub> is the first step initiated by the attack of carboxylic ester oxygen on the side chain carbonyl carbon and followed by the loss of isobutene as the second step (mechanism not shown). The supporting information for this two-step process includes two peaks at 202 and 241, which are formed after losing an isobutene (CH<sub>2</sub>=CH(CH<sub>3</sub>)<sub>2</sub> and ammonia (NH<sub>3</sub>) respectively. Further fragmentation ions of  $m/z$  202 and 241 produces the peak 185 (data not shown), which is not direct

evidence of, but consistent with Scheme 1. Further fragmenting 185 generated from either 202 or 241 gives virtually identical spectra with the peaks at 157, 142, 139, 114, 111, indicating either the loss of isobutene or ammonia can occur first and produce the same product ions after the loss of 73 Da in total.

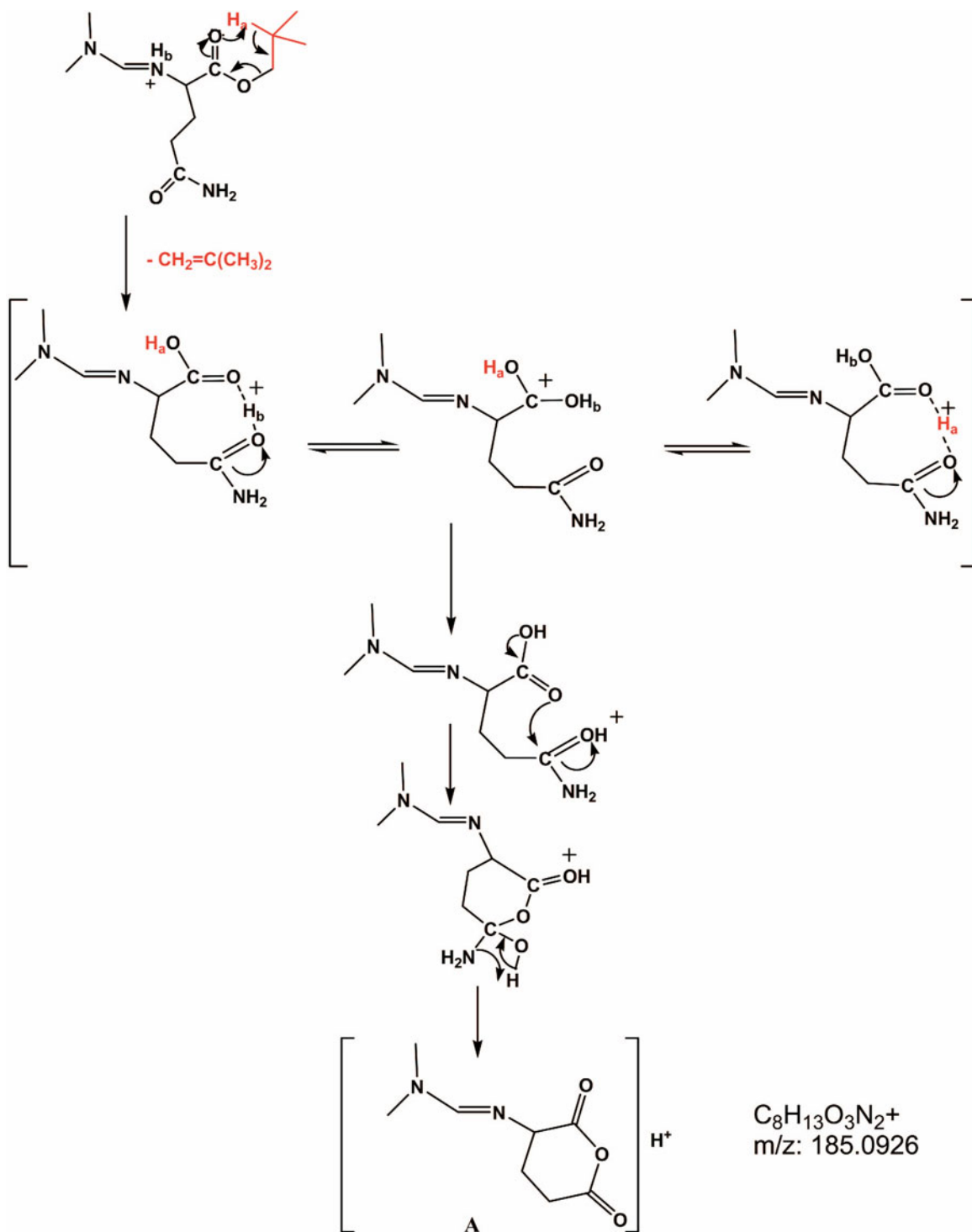
In Scheme 2, the butyl ester undergoes McLafferty-type rearrangement to lose a neutral isobutene (56 Da). Subsequently, a proton transfer from the imine nitrogen to the side chain amide oxygen promotes the nucleophilic attack by the imine nitrogen on the side chain amide carbonyl carbon by making the carbonyl carbon more electropositive. Similar mechanism was reported for the formation of lactam ring during peptide fragmentation [25,26]. The amine nitrogen provides anchimeric assistance for this attack. Upon losing NH<sub>3</sub>, the five-membered lactam ring ( $m/z$  185) is produced. Another possible pathway is the Alder-Ene-type reaction with intermediate structure 2a (Scheme 2) in which the proton is transferred from the amine nitrogen to the side chain carbonyl oxygen and the imine nitrogen attacks the side chain carbonyl carbon concurrently. Similar to Scheme 1, the loss of isobutene and NH<sub>3</sub> are two independent processes, either could happen first followed by the other one.

Both the proposed lactam ring and cyclic anhydride ring have the same elemental composition (C<sub>8</sub>H<sub>13</sub>O<sub>3</sub>N<sub>2</sub><sup>+</sup>) with a theoretical mass of 185.0926. SORI-CID has been performed in FTICR to obtain the accurate mass and confirm the elemental composition of the fragment at 185. The obtained accurate mass 185.0926 matches the theoretical mass well (Table 2).

### Experimental Evidence for Anhydride Ring Structure

To confirm whether the structure of 185 is a cyclic anhydride or a five-membered lactam ring or a mixture of both, and to determine which mechanism dominates, the fragmentation of a synthesized compound, dimethylformamidinium glutamic anhydride which should have the same structure as the proposed cyclic anhydride (Scheme 1, A), was investigated. A synthesized lactam ring was not obtained due to the absence of synthesis procedure from literature for such a compound.

MS/MS of the synthesized anhydride gives the same fragments as those of 185 from DMF-Gln-OBu as shown in Figure 4: <sup>157</sup>, <sup>142</sup>, <sup>139</sup>, <sup>114</sup>, <sup>111</sup> and <sup>72</sup>. This is direct

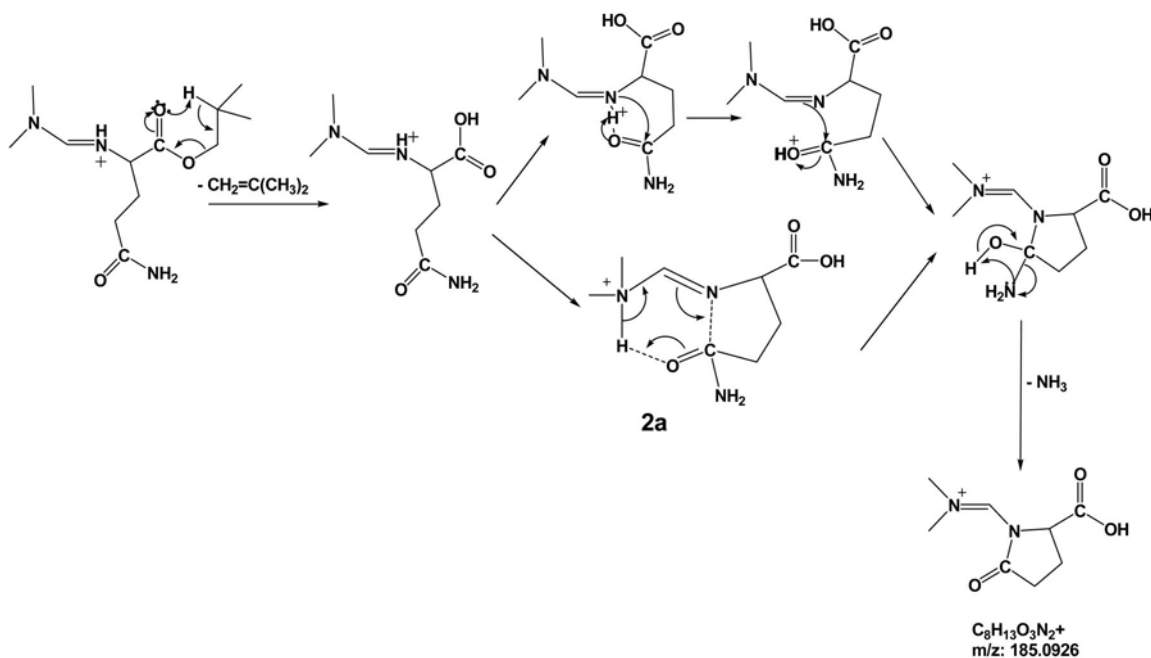


**Scheme 1.** Proposed fragmentation mechanism of DMF-Gln-OBu for the formation of a cyclic anhydride after neutral loss 73 Da.

evidence that the fragmentation of DMF-Gln-OBu follows Scheme 1 and that the fragment formed upon neutral loss of 73 ( $m/z$  185) has a cyclic anhydride structure.

To further illustrate the similarity in fragmentation behavior, fractional abundance curves for major fragments of both the synthesized product and the 185

(Scheme 1, A) from DMF-Gln-OBu were plotted versus different collision energies (by LCQ CID) as shown in Figure 5. The fractional abundance of a specific fragment was calculated as the intensity of that fragment over the total intensity of included peaks (185, 157, 142, 139, 114, 111). The fractional abundance curves for the fragments of 185 from



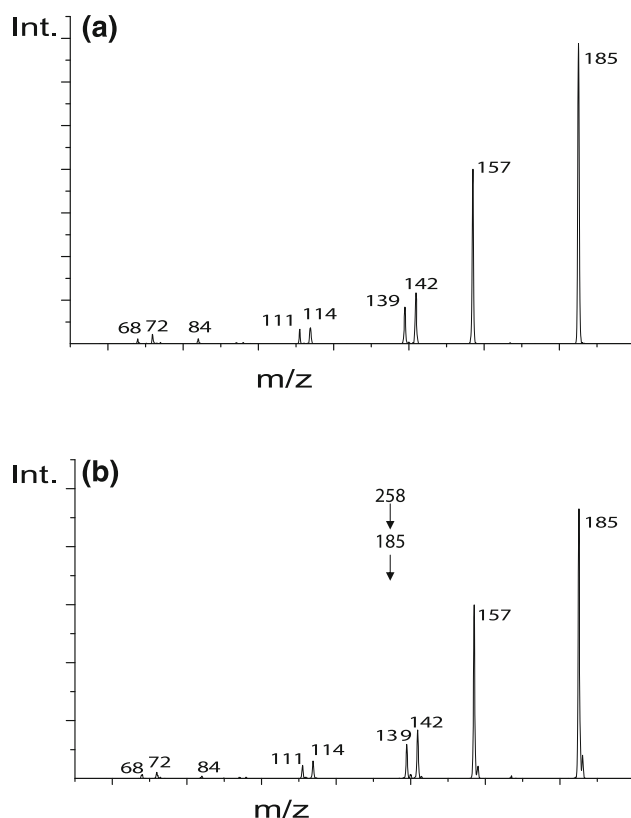
**Scheme 2.** Proposed fragmentation mechanism of DMF-Gln-OBu for the formation of cyclic lactam after neutral loss 73 Da.

DMF-Gln-OBu would be expected to be different from those of the synthesized cyclic anhydride if 185 is a mixture of both a cyclic anhydride and a cyclic lactam.

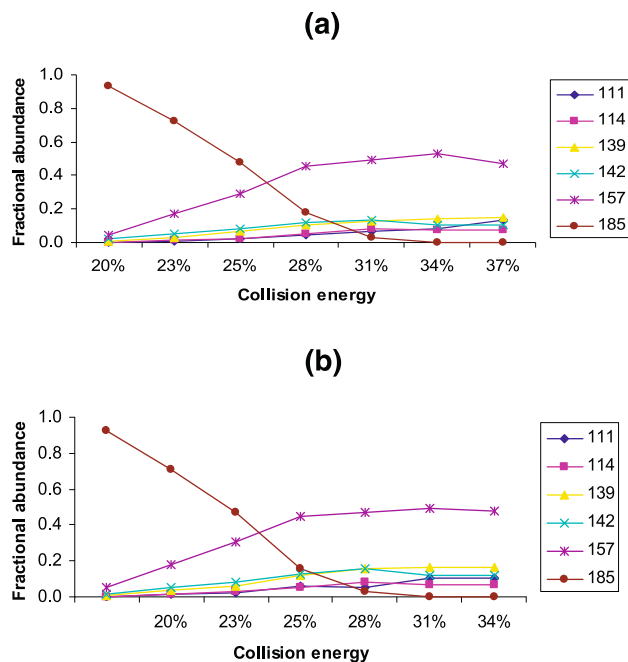
No obvious difference is observed for the two sets of fractional abundance curves as shown in Figure 5, indicating that the fragmentation follows Scheme 1, and the structure of 185 is a cyclic anhydride structure. Moreover, similar fractional abundance curves for the two compounds were also obtained from TSQ700 CID which deposits distributions of internal energy different from that deposited in an ion trap and allows for a different dissociation time frame (data not shown). Upon further fragmenting 157 in the LCQ, the most abundant fragment from 185, for both DMF-Gln-OBu and the synthesized product leads to same set of peaks: 73, 111, 139 (data not shown), which provides more supporting evidence for the cyclic anhydride structure.

#### Fragmentation of Dimethylformamidine Glutamine $^2\text{H}_9$ -isobutyl Ester

To confirm the McLafferty-type rearrangement, the fragmentation of the dimethylformamidine glutamine  $^2\text{H}_9$ -isobutyl ester ( $\text{MH}^+$ ,  $m/z$  267) was investigated. Compared with unlabeled DMF-Gln-OBu ( $\text{MH}^+$ ,  $m/z$  258), the peaks at 241 and 202 shift 9 units and 1 unit to 250 and 203 respectively (Figure 3a), consistent with the number of deuteriums in Scheme 1 after losing  $\text{NH}_3$  and  $^2\text{H}_8$ -isobutene. The mass shift of 202 to 203 and the appearance of 186 are direct evidence for the retention of one proton (or deuterium) from the isobutanol group upon losing isobutene. The peak at 185 splits into two



**Figure 4.** ESI/low energy CID of (a) a synthesized Compound A. (b)  $\text{MS}^3$  of DMF-Gln-OBu: 258 185 spectra obtained with a Finnigan ion trap spectrometer (LCQ) using helium as the collision gas. The “% relative collision energy” (defined by the instrument manufacturer) used for acquiring the spectra was 25%.



**Figure 5.** Fractional abundance (i.e., the abundance of a fragment vs. total abundance of all identified fragments) curves for fragments of (a) the synthesized Compound A; (b) ion at 185 from MS<sup>2</sup> of 258 (DMF-Gln-OBu). Fragmentation performed in a Finnigan LCQ using helium gas as the collision gas.

peaks at 185 and 186, which is consistent with the inter-converting structures in Scheme 1. The intensity ratio of 186 to 185 increases slightly with the increase of fragmentation energy, from 2 at 25eV to 3 at 45eV in the triple quadrupole mass spectrometer (data not shown), which is consistent with Scheme 1: at higher fragmentation energies, the faster process, attack and cleavage without prior proton transfer from the amide carbonyl oxygen to the carboxylic acid, should be favored. The formation of 186 does not require formation of a proton bridge and sigma C-C bond rotation according to Scheme 1. Thus at the higher fragmentation energy, a higher intensity ratio of 186 vs. 185 would be expected. Moreover, the same sets of peaks were detected upon

fragmenting 185 and 186 which is also consistent with Scheme 1°(Figure 3b°and°c).

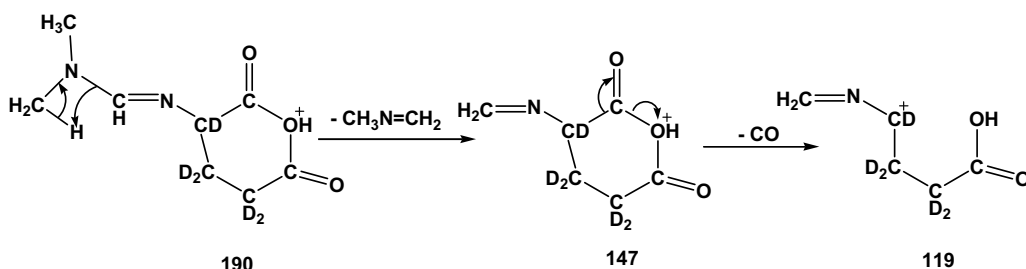
The McLafferty-type rearrangement is also consistent with 50% increase of ion intensity corresponding to neutral loss 73 if isobutanol is used for second step derivatization vs. n-butanol. In the case of isobutanol derivatization, a tertiary hydrogen is abstracted and a branched butene (isobutene) is lost as a neutral molecule, which is more favorable than abstraction of a secondary hydrogen and the loss of linear butene.

### MS/MS/MS of 185

In order to obtain further supporting evidence for the cyclic anhydride structure of 185, CID experiments were performed by LCQ to fragment 185 from DMF-Gln-OBu, 186 from DMF-Gln-O-<sup>2</sup>H<sub>9</sub>-Bu and 190 from DMF-<sup>2</sup>H<sub>5</sub>-Gln-OBu. The major fragments and mass shifts°for°deuteron°labeling°are°listed°in°Table°1.°Based on this information, a possible fragmentation mechanism for the cyclic anhydride ring was proposed as shown in Scheme 3.

Figure 4b°shows°all°the°fragments°of°185°with°the base peak at 157 and the other peaks at 111, 114, 139 and 142. Elemental compositions of all the fragments from the proposed mechanism (Scheme 3) match well with accurate°mass°measurements°by°FTICR°(Table 2).°Interestingly, the peak at 111, with the exact mass of 111.0918, appears as two peaks in the cases of both DMF-Gln-O-<sup>2</sup>H<sub>9</sub>-Bu (111 and 112) and DMF-<sup>2</sup>H<sub>5</sub>-Gln-OBu (114 and 115), as does the peak at 139 with the exact°mass°of°139.0866°(Table 1)°which°appears°as°two peaks for both DMF-Gln-O-<sup>2</sup>H<sub>9</sub>-Bu (139 and 140) and DMF-<sup>2</sup>H<sub>5</sub>-Gln-OBu (142 and 143). This indicates both peaks at 111 and 139 are formed by two different fragmentation pathways. The proposed mechanism accounts for this fact.

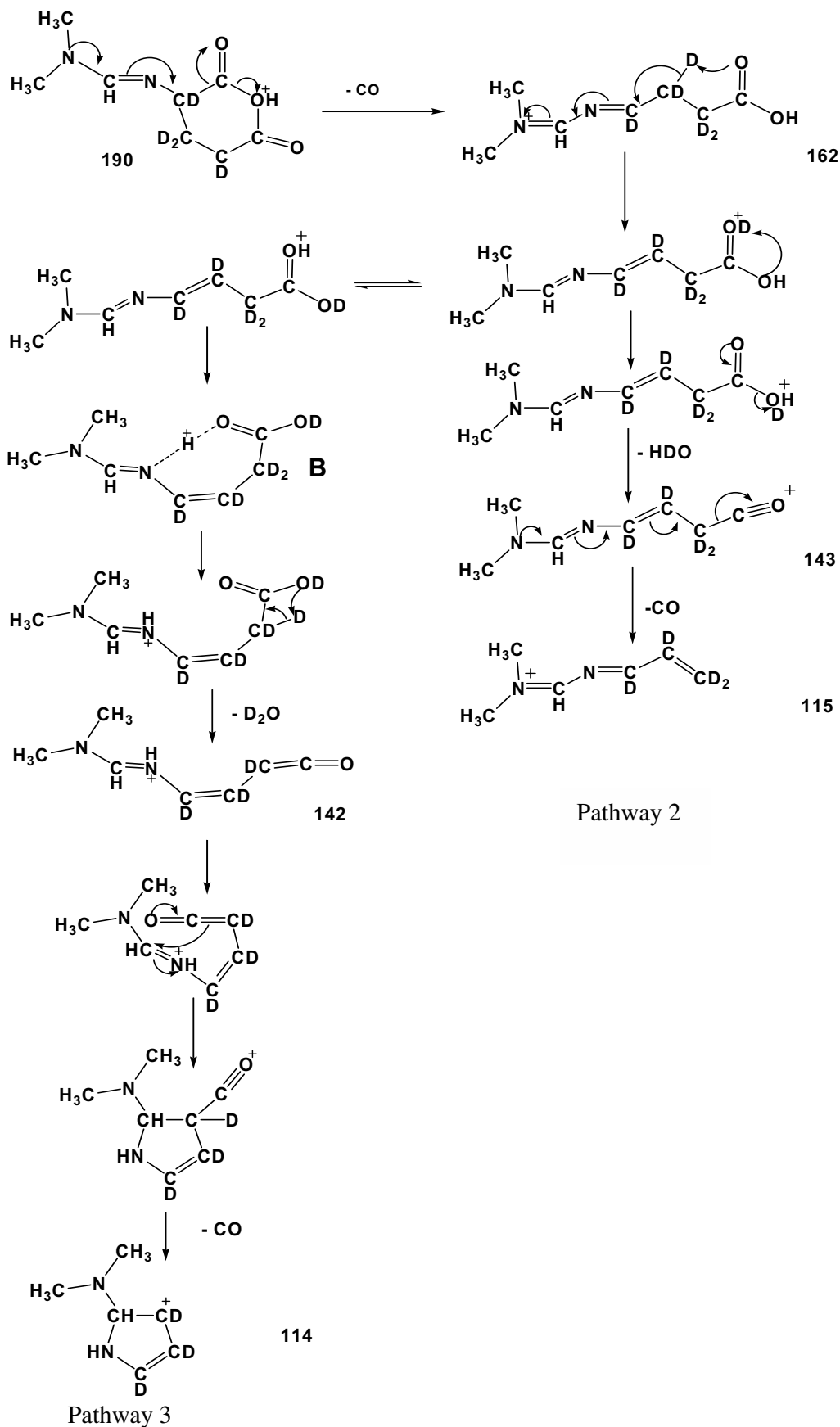
It can be envisioned that the isotopic splitting of peaks in the case of DMF-<sup>2</sup>H<sub>5</sub>-Gln-OBu and DMF-Gln-O-<sup>2</sup>H<sub>9</sub>-Bu are due to the proton transfer from the protonated carbonyl oxygen to the imine nitrogen, a site with higher proton affinity, via an intermediate struc-



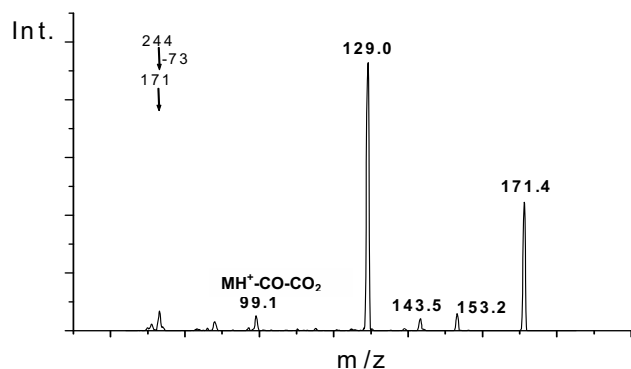
Pathway 1

**Scheme 3.** Fragmentation mechanism of A (185 from DMF-Gln-OBu) in three different pathways in the case of derivatized <sup>2</sup>H<sub>5</sub>-glutamine.





Scheme 3. (continued)



**Figure 6.** ESI/low energy CID MS<sup>3</sup> of dimethylformamidinium asparagine isobutyl ester with Finnigan Tsq700. Collision energy 15 eV.

ture with a proton solvated by both nitrogen and oxygen. For example, when <sup>2</sup>H<sub>5</sub>-glutamine is used for derivatization, the fragments formed upon NL 73 will appear at 190. Upon losing CO, a peak at 162 is produced. It is conceivable that the ionizing proton on the carbonyl oxygen can be transferred to the imine nitrogen via a seven-member ring proton bridging structure (Scheme 3, Pathway 3, B). Subsequently, the peaks at 142 and 114 are produced. If no proton transfer occurs, the peaks at 143 and 115 are produced (Scheme 3, Pathway 2). The same holds true for the fragmentation of DMF-Gln-O-<sup>2</sup>H<sub>5</sub>-Bu. The proton transfer here is supported by the mobile proton model, which has been proposed for peptide fragmentation in our previous studies [27].

Also consistent with the proposed cyclic anhydride structure from DMF-<sup>2</sup>H<sub>5</sub>-Gln-OBu, the fragments 147 and 119 are formed by the formal loss of CH<sub>3</sub>-N=CH<sub>2</sub> (43 Da) and then CO (28 Da) consecutively (supported by FTICR data), as shown in Pathway 1, Scheme 3.

#### Distinguishing Aspartic Acid from Asparagine

Aspartic acid and asparagine were subjected to the same two-step derivatization. The two derivatized amino acids can be distinguished with aspartic acid appearing at 301, and asparagine appeared at 244 (data not shown). As expected, a neutral loss of 73 was observed for the asparagine derivative: dimethylforma-

midine asparagines isobutyl ester (DMF-Asn-OBu; data not shown). It is expected that it will follow the same fragmentation mechanism as DMF-Gln-OBu to form cyclic anhydride, except that it should produce a five-membered ring instead of the six-membered ring.

The product from the neutral loss 73 from DMF-Asn-OBu (*m/z*: 171) is further fragmented. Interestingly, the peak at 129 is the base peak and the only dominant peak (Figure 6), which is probably due to the stable conjugated diene structure formed upon losing a ketene (Scheme 4).

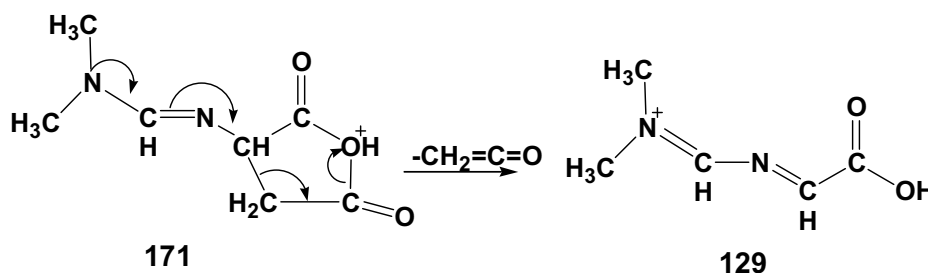
#### Isotopic Effect and Isobaric Interference

Although deuterium-labeled internal standards are commonly used for amino acid quantification [13, 18], no reported studies have characterized isotope effects. Isotope effects, if they exist, are very important for amino acid quantification. In the present study, the isotope effects of <sup>15</sup>N-amine labeled, <sup>15</sup>N-amide labeled and deuterium labeled glutamine were characterized for NL 73/74 at a collision energy of 20 eV (Table 3).

Although the <sup>15</sup>N-amine and <sup>15</sup>N-amide labeled glutamine demonstrated no obvious isotopic effect, a secondary isotope effect was demonstrated by <sup>2</sup>H<sub>5</sub>-Gln (*K<sub>D</sub>/K<sub>H</sub>* = 0.83). It has been previously documented that a normal secondary isotope effect (*K<sub>D</sub>/K<sub>H</sub>* < 1) can be caused by the hyperconjugative interaction of β deuteriums with orbital(s) of the carbon reacting center [28]. It can be envisioned that the isotope effect for <sup>2</sup>H<sub>5</sub>-Gln could be due to the hyperconjugative interaction of side chain deuteriums with the orbital(s) of the reactive carbonyl which is the site of attack for anhydride formation.

Although the neutral loss of 102 can not distinguish <sup>15</sup>N-amide and <sup>15</sup>N-amine labeled glutamine because the labeled atom is not involved in the neutral loss in either case, it is still possible to quantify the total amount of <sup>15</sup>N-amide and <sup>15</sup>N-amine labeled glutamine in one sample if they have the same or no isotope effect. Thus in the present study, the isotope effect was characterized for the neutral loss 102 for <sup>15</sup>N-amine labeled, <sup>15</sup>N-amide labeled and <sup>2</sup>H<sub>5</sub>-labeled glutamine at a collision energy of 15 eV (Table 3).

No obvious isotope effect is observed for <sup>2</sup>H<sub>5</sub>-Gln



**Scheme 4.** Mechanism for the formation of ion 129 by fragmenting the peak (171) after NL 73 for dimethylformamidinium asparagine isobutyl ester (DMF-Asn-OBu).

**Table 3.** The isotope effect for the neutral loss of 73 and 102<sup>a,b</sup>

	Gln	<sup>15</sup> N(amide)-Gln	<sup>15</sup> N(amine)-Gln	<sup>2</sup> H <sub>5</sub> -Gln
NL 73 (20eV)	1	0.998(±0.025)	0.96(±0.002)	0.83(+0.025)
NL 102 (15eV)	1	<sup>c</sup>	0.995(±0.003)	0.989(±0.05)

<sup>a</sup>Results obtained from Finnigan TSO700 using argon as the collision gas with collision cell pressure at 2.6 to 2.9 mtorr.

<sup>b</sup>Different methods were used for neutral loss 73 and 102 to derivatize labeled and unlabeled glutamine.

<sup>c</sup>Isotope effect can not be measured because of isobaric interference. A correction factor of 1.29 is needed.

and <sup>15</sup>N-amine labeled glutamine during neutral loss 102. However, an isobaric interference prevent the measurement of the isotope effect for <sup>15</sup>N-amide glutamine (Table 3), this is because the peak corresponding to neutral loss 102 (259—157) in the case of amide labeled glutamine (MH<sup>+</sup>: 259) overlaps with another pathway (259—185—157). A correction factor of 1.29 is needed for this isobaric effect at the laboratory collision energy used (i.e., 15 eV). Because this isobaric interference only happens for amide labeled glutamine but not for amine labeled glutamine, the total amount of <sup>15</sup>N-amide labeled and <sup>15</sup>N-amine labeled glutamine in one sample can not be quantified by the neutral loss of 102. Instead, neutral loss 73 can be used to quantify <sup>15</sup>N-amide and <sup>15</sup>N-amine labeled glutamine as discussed in this paper and is being used routinely in our laboratory.

## Conclusion

According to the literature, the neutral loss of 73 can be used for glutamine identification and quantification [18]. In the present study, the fragmentation mechanism involved in this neutral loss was proposed based on CID spectra of derivatized glutamine, <sup>15</sup>N-glutamine, <sup>2</sup>H<sub>5</sub>-glutamine and <sup>2</sup>H<sub>5</sub>-isobutanol derivatized glutamine. A two-step process leads to the neutral loss of 73Da: the loss of isobutene (56 Da) through a McLafferty-type rearrangement and the loss of NH<sub>3</sub> (17 Da) from the side chain amide. The final fragmentation product is a cyclic anhydride ring structure. The fragmentation of a compound synthesized to have the same anhydride structure was investigated. The cyclic anhydride ring structure was confirmed by identical fragments and very similar fractional abundance curves obtained from both the product ion and ionized synthesized compound. A hydrogen/deuterium isotope effect was discovered for NL 73 for dimethylformamidine <sup>2</sup>H<sub>5</sub>-glutamine isobutyl ester (K<sub>D</sub>/K<sub>H</sub>=0.83) and should be used for accurate quantification if <sup>2</sup>H<sub>5</sub>-glutamine is used as an internal standard.

## Acknowledgements

The authors thank Dr. Ron Wysocki at Chemical Synthesis Laboratory at Department of Chemistry in University of Arizona for his help with the synthesis of dimethylformamidine glutamic anhydride. The authors also thank Ms Kristin A. Herrmann for the accurate mass measurements by FTICR. This work was supported by NIH Grant AI 46541 awarded to Dr. Michael A. Wells and NIH grant GMR0151387 awarded to Dr Vicki H. Wysocki.

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