Can α- and β-Alanine Containing Peptides Be Distinguished Based on the CID Spectra of Their Protonated Ions?

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The fragmentation reactions of isomeric dipeptides containing α- and β-alanine residues (αAla-αAla, αAla-βAla, βAla-αAla, and βAla-βAla) were studied using a combination of low-energy and energy resolved collision induced dissociation (CID). Each dipeptide gave a series of different fragment ions, allowing for differentiation. For example, peptides containing an N-terminal β-Ala residue yield a diagnostic imine loss, while lactam ions at *m/z* 72 are unique to peptides containing β-Ala residues. In addition, MS^3 experiments were performed. Structure-specific fragmentation reactions were observed for y_1 ions, which help identify the C-terminal residue. The MS^3 spectra of the b_2 ions are different suggesting they are unique for each peptide. Density functional theory (DFT) calculations predict that b_2 ions formed via a neighboring group attack by the amide are thermodynamically favored over those formed via neighboring group attack by the N-terminal amine. Finally, to gain further insight into the unique fragmentation chemistry of the peptides containing an N-terminal β-alanine residue, the fragmentation reactions of protonated β-Ala-NHMe were examined using a combination of experiment and DFT calculations. The relative transition-state energies involved in the four competing losses (NH_3, H_2O, CH_3NH_2, and $CH_2{=}NH$) closely follow the relative abundances of these as determined via CID experiments. (J Am Soc Mass Spectrom 2008, 19, 1743–1754)

The gas-phase chemistry of protonated α-amino acids and their peptides has been studied extensively over the past two decades via a range of tandem mass spectrometry techniques and through the use of molecular modeling to the extent that the mechanisms for the formation of fragment ions are fairly well understood [1–7]. Key concepts include the "mobile proton" [5] and the idea that nucleophile-electrophile interactions can promote the fragmentation of bonds via neighboring group interactions [2]. In contrast, despite the growing interest in the use of β-amino acids in constructing peptides that are protease resistant [8] or that have unique architectures [9], the gas-phase chemistry of protonated β-amino acids and their peptides have received much less attention. Key exceptions include studies that have shown that β-alanine has a higher proton affinity than α-alanine [10]; protonated α- and β-alanine (1 and 2; see Scheme 1) fragment via different pathways [11]; the relative abundances of sequence ions in α- and β-alanine containing peptides can be different [12]; and β-alanine peptides can be identified via CID of the Boc-protected derivatives [13]. Furthermore, Seebach's group has shown that tandem mass spectrometry of protonated β-peptides can be used to confirm their structures via the formation of sequence ions [14]. They have modified the standard sequence ion nomenclature used for peptides containing the naturally occurring α-amino acids [15], and note that b and y ions are formed but that the ring sizes for b ions derived from β-alanine containing peptides is likely to be different from their α-alanine counterparts. Scheme **2** shows the nomenclature used for the formation of sequence ions and internal fragments for protonated peptides containing α- and β-amino acid residues. Ideally, isomeric peptides containing α- and β-alanine residues might be distinguished by unique fragmentation reactions much in the same way that methods have been sought to distinguish between peptides containing leucine and isoleucine residues [16]. Here we compare the fragmentation reactions of the $[M + H]^+$ of four isomeric peptides αAla-αAla, αAla-βAla, βAla-αAla, and βAla-βAla under CID con-

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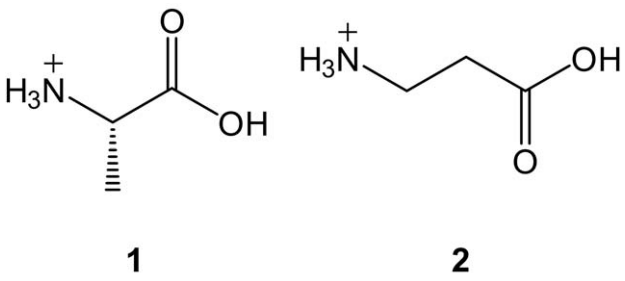


Scheme 1

ditions; examine the competition between a_1, b_1, and y_1 formation; probe the structures of the b_2 ions for all four peptides using a combination of CID experiments and DFT calculations; use experiments and DFT calculations to examine the gas-phase chemistry of β-Ala-NHMe as a model for peptides containing a β-alanine residue at the N-terminus.

Experimental

Materials

β-Alanine ($H_2NCH_2CH_2CO_2H$), Boc-Ala-OH ($CH_3CH[NHCO_2C(CH_3)_3]CO_2H$), αAla-αAla ($CH_3CH(NH_2)CONHCH(CH_3)CO_2H$), βAla-αAla ($H_2NCH_2CH_2CONHCH(CH_3)CO_2H$), *N,N′*-dicyclohexylcarbodiimide ($C_6H_{11}N{=}C{=}NC_6H_{11}$), 1-hydroxybenzotriazole hydrate ($C_6H_5N_3O{\cdot}xH_2O$), triethylamine ($[C_2H_5]_3N$), ethylamine ($C_2H_5NH_2$), and acetyl chloride were obtained from Aldrich (Milwaukee, WI). βla-OtBu·HCl ($H_2NCH_2CH_2CO_2C[CH_3]_3{\cdot}HCl$) and βAla-βAla ($H_2NCH_2CH_2CONHCH_2CH_2CO_2H$) were obtained from BAChem (Bubendorf, Switzerland). Methanol (HPLC grade) was purchased from Mallinkrodt (Melbourne, Australia). Methylamine (30% aqueous solution) and ammonia bicarbonate were purchased from Ajax Chemicals Pvt. Ltd. (NSW, Australia). Acetic anhydride was supplied from Fluka (Buchs, Switzerland). Dichloromethane (DCM) and trifluoroacetic acid (TFA) were obtained from BDH Chemicals Ltd. (Poole, England). All reagents were used without further purification.

Synthesis of Methyl Esters of β-Alanine and Dipeptides

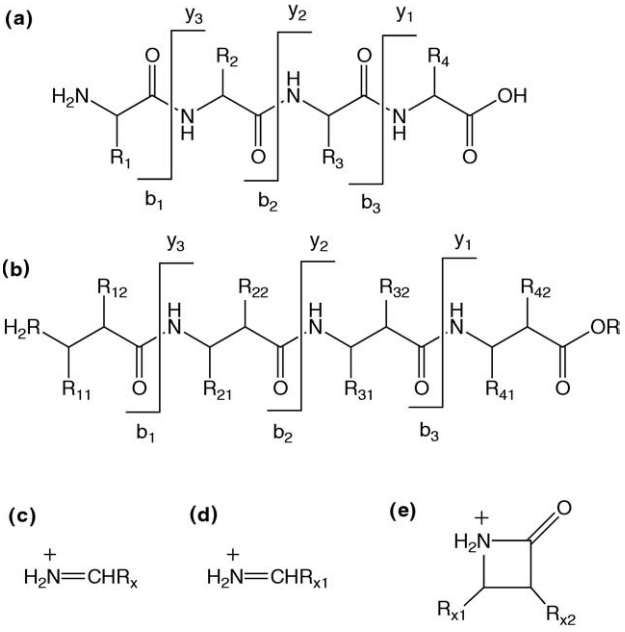
The lyophilized β-alanine or dipeptide (10 mg) was dissolved in 1 mL of the methyl esterification reagent (prepared by the dropwise addition of 800 μL of acetyl chloride to 5 mL of anhydrous methanol with stirring) and allowed to stand for 2 h at room temperature. The samples were dried by lyophilization and used without further purification.

Synthesis of Amides of β-Alanine

Following variation of the method used by Feenstra et al. [17], the β-alanine methyl ester derivative (2 mg) was dissolved in 1 mL of 30% CH_3NH_2/H_2O and allowed to stand for 30 min at room temperature. The methyl amide was then used without further purification. The ethyl amide was formed in a similar fashion by substituting methylamine with ethylamine.

Synthesis of the Dipeptide αAla-βAla

βAla-βAla was synthesized using a standard peptide coupling reaction [18]. Boc-L-alanine (1.01 g, 5.32 mmol), βAla-OtBu·HCl (1.00 g, 5.51 mmol), 1-hydroxybenzotriazole (7.26 mg, 5.38 mmol), and freshly distilled triethylamine (approximately 2 mL) was dissolved in dry CH_2Cl_3 (20 mL) and cooled to 0 °C. Following this *N,N′*-dicyclohexylcarbodiimide (1.12 g, 5.41 mmol) was added and the mixture stirred for a further 60 min at 0 °C, allowed to stir overnight at room temperature and then filtered through a pad of Celite. The resultant filtrate was concentrated in vacuo. The protecting groups were removed by the addition of 1:1 TFA and DCM as demonstrated by characterization of the deprotected peptide using high-resolution mass spectrometry (experimental: 161.09,192, theoretical: 161.09,207). The solution containing the deprotected peptide was not purified, but was diluted in 100% methanol to a concentration of 0.03 M, which was then used for subsequent MS/MS experiments.



Scheme 2. Nomenclature relevant to protonated peptide fragmentation. Sequence ion nomenclature relevant to amide bond cleavage for (**a**) α-amino acid residues (adapted from reference [15]); (**b**) β-amino acid residues (adapted from reference [14]). Internal fragment ion from residue x (**c**) immonium ion from α amino acid residue; (**d**) immonium ion from β-amino acid residue; (**e**) lactam ion from β-amino acid residue.

Mass Spectrometry Experiments

MS/MS and MS³ Experiments on a Finnigan LTQ-FT Hybrid Linear Ion Trap

Multistage mass spectrometry experiments were carried out using a Finnigan LTQ FT hybrid linear ion trap (Bremen, Germany). Samples were prepared by dissolving the peptide or amino acid in 100% methanol to a concentration of ~0.03 mM and introduced into the mass spectrometer via electrospray ionization using direct injection with a flow rate of 5 μL/min. The sheath gas, capillary voltage, and temperature were adjusted to ca. 3–25 (arbitrary units) ?, 3.0–8 kV, and 275 °C, respectively. The CID experiments were performed in the linear ion trap by using standard procedures of mass selecting the desired precursor ion, with an activation window of 2 *m/z*, and then subjecting it to CID using a corresponding normalized collision energy of 25% to 35% and an activation Q of 0.25 for a period of 30 ms. In some cases, the low mass function was used to identify immonium ions in MS^3 experiments.

High-Resolution Mass Spectrometry Experiments

All high-resolution mass spectrometry experiments were conducted using a commercially available hybrid linear ion trap and Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (Finnigan LTQ-FT hybrid linear ion trap, Bremen, Germany), which is equipped with ESI. The ions of interest were mass selected in the LTQ using standard procedures and were then analyzed in the FT-ICR MS to generate the high-resolution tandem mass spectrum. Positive mode calibration was done via the automatic calibration function using the suggested LTQ calibration solution, consisting of caffeine, MRFA, and Ultramark solution.

Energy Resolved CID Experiments

An Applied Biosystems 4000 QTRAP equipped with a TurboV ESI Source with Turbo-Ion Spray (MDS-SCIEX, Darmstadt, Germany) was used for all of the energy resolved CID experiments. Samples were prepared by dissolving the peptide or amino acid in 100% methanol to a concentration of ~0.03 mM and introduced into the mass spectrometer via direct injection through turbo ionization (TI) using a flow rate of 5 μL/min. The experiments were performed using the following conditions: curtain gas, 10.0 arb; collision gas, nitrogen; ion spray voltage (IS), 4500; temperature (TEM), 0.0; ion source gas 1 (GS1), 20.0; ion source gas 2 (GS2), 0.0; interface heater (ihe), On; declustering potential (DP), 60.0; entrance potential (EP), 10.0; collision cell exit, potential (CXP) 15.0; collar 2 (C2), 0.0; quadrupole 1 ion energy (IE1): 1.0; Q1 resolution: unit; quadrupole 3 ion energy (IE3): 2.0; Q3 resolution: unit; detector CEM (CEM), 2200. Energy-resolved CID experiments were performed by increasing the collision offset voltage of the second quadrupole from 5 to 35 or 50 V, depending on the sample, in 1 V increments. As each data point collected consisted only of one scan, 20 sets of data were collected, and the data averaged using Microsoft Excel 2007.

Theoretical Calculations

Due to the large number of dihedrals in most of the studied systems, it was not feasible to systematically examine every possible conformer. Thus, a range of possible conformers for each system was constructed through rotation in 90° increments of the principle dihedrals of an initial guess conformer. Following geometry optimization the lowest energy structure out of these possible structures was used in the PES.

Geometry optimizations for minima and transition states were optimized at the PM3 semi-empirical level of theory followed by optimization at the B3LYP level of theory with the standard 6-31G(d) basis set using the Gaussian 03 quantum mechanical program package [19]. The minima were connected to the transition-state using intrinsic reaction coordinate (IRC) calculations. All optimized structures were subjected to vibrational frequency analysis to determine the nature of the stationary points followed by single point energy calculations at the MP2/6-31G(d)//B3LYP/6-31G(d) level of theory and visualization using the computer package GaussView 3.0. All zero-point vibrations energies were scaled by 0.9806 [20].

Results and Discussion

Overview of the CID Spectra of the Isomeric Dipeptides αAla-αAla, αAla-βAla, βAla-αAla, and βAla-βAla

Each of the CID spectra of all four isomeric peptides shown in Figure 1 has a unique "fingerprint", and their assignments were confirmed by high-resolution mass measurements in the FT-ICR (data not shown). While all spectra show evidence for the formation of y_1 and b_2 ions, their relative ratios are different. Furthermore, each dipeptide gives a different series of fragment ions. In some cases, unique fragments are observed, which are indicative of structure. For example, an examination of the fragmentation reactions of the protonated methyl esters (Supplementary Figure S1, which can be found in the electronic version of this article) reveals unique water losses for peptides with an N-terminal β-Ala. These may involve loss of water from the backbone carbonyl groups [21]. In addition, both peptides containing an N-terminal β-Ala residue (Figure 1c and d) give a unique imine loss (eq 1) and loss of ammonia. Related losses of the N-terminal imine and ammonia have been observed by Seebach et al. for larger peptides containing N-terminal β-alanine residues [14]. Indeed, Seebach has termed the former reaction a retro-Mannich cleavage, Scheme **3** (for a review on the Mannich reaction see [22]); and the mechanisms of this reaction,

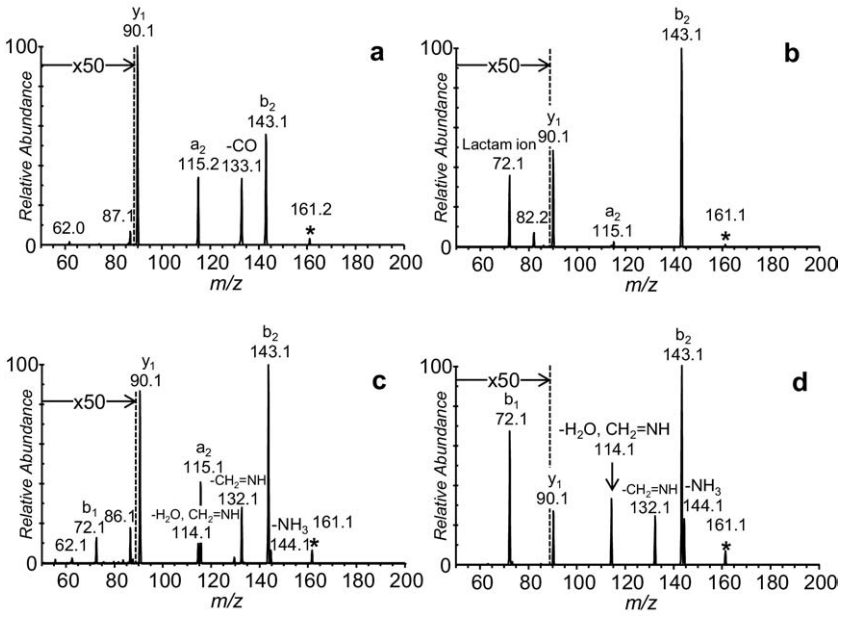
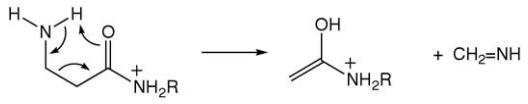


Figure 1. LTQ MS/MS CID spectra of protonated dipeptides containing α- and β-alanine (**a**) αAla-αAla; (**b**) αAla-βAla; (**c**) βAla-αAla; (**d**) βAla-βAla. An asterisk refers to the mass-selected precursor ion.

as well as those for NH_3 and H_2O loss, are explored in detail using a smaller model system, protonated βAla-NHMe. CO loss is only observed for protonated αAla-αAla and this most likely arises from the "a_1/y_n pathway" [23]. Unfortunately, due to the low mass cut-off, we are unable to observe the related formation of a_1 ions. Since the b_1 ions (and the related lactam internal ions) of aliphatic α-amino acids are unstable [24], the observation of a fragment ion at *m/z* 72 in all three spectra of peptides containing at least one β-alanine residue (Figures 1b–d) must be an indicator of the presence of at least one β-alanine residue in the dipeptide. For the two dipeptides containing an N-terminal β-Ala residue, the ion at *m/z* 72 corresponds to a b_1 ion, which can be formed from a "b_1/y_1 pathway", whereas for αAla-βAla, the ion at *m/z* 72 must correspond to a C-terminal lactam fragment ion, arising from a primary fragment ion. A most likely precursor candidate is the y_1 ion, which corresponds to protonated β-alanine. To further understand how the isomeric peptides gives rise to different types of sequence ions (y_1 and b_2) and immonium and lactam ions arising from backbone cleavages, in the next sections we: (1) describe the results of MS^3 experiments and energy resolved CID experiments relevant to immonium and lactam ion and y_1 formation; (2) examine the b_2 structures using a combination of experiment and DFT calculations; (3) examine the fragmentation reactions of a peptide model with an N-terminal β-Ala residue.

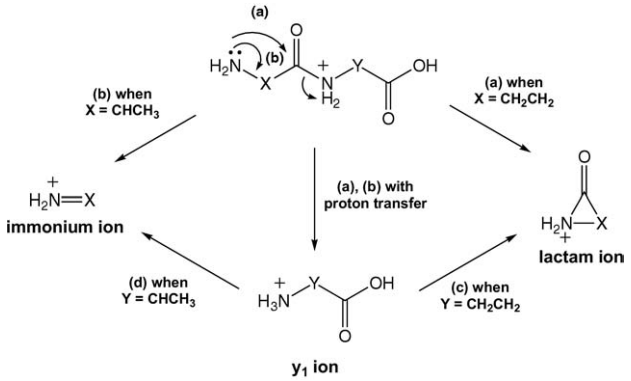


Scheme 3. Retro-Mannich loss of CH_2=NH indicative of an N-terminal β-alanine residue.

$$\left[H_2NCH_2CH_2COX + H\right]^+ \rightarrow \left[CH_2C(OH)X + H\right]^+ + CH_2{=}NH \quad (1)$$

Unraveling the Competition Between Immonium, Lactam, and y_1 Ion Formation in the Isomeric Dipeptides αAla-αAla, αAla-βAla, βAla-αAla, and βAla-βAla Via the Use of MS^3 and Energy Resolved CID Experiments

Scheme **4** illustrates how cleavage of the peptide bond can give rise to a_1, b_1, y_1 ions as primary products and



Scheme 4. Pathways for formation of immonium and lactam ions of isomeric dipeptides via initial cleavage of the peptide bond. Paths (**a**) and (**b**) are primary fragmentation channels, while paths (**c**) and (**d**) are secondary products arising from fragmentation of the y_1 ions.

immonium and lactam ions as secondary products arising from cleavage of the y_1 ion. The central hypothesis is that the nature of the residues (α-Ala versus β-Ala) present in the peptide dictates whether an immonium or a lactam ion is observed. Thus the primary "b_1/y_1 pathway" can only operate when there is a β-alanine residue at the N-terminus (path a of Scheme **4**) while the "a_1/y_1 pathway" can only operate when there is an α-Ala residue at the N-terminus (path b of Scheme 4). In a similar fashion, the secondary product immonium and lactam ions, which are indistinguishable from the primary a_1 and b_1 sequence ions are also indicators of the nature of the C-terminal residue. Thus, the lactam ion can only arise from a y_1 structure that corresponds to the amino acid β-Ala (path c of Scheme **4**) while the immonium ion can only arise from a y_1 structure that corresponds to the amino acid α-Ala (path d of Scheme **2**).

To confirm the origins of the immonium and lactam ions and the y_1 ions, further experiments were performed. The first set involved carrying out MS^3 experiments in the linear ion trap. Since all the y_1 ions have the same *m/z* values, we were interested in establishing their structure by comparison of the CID spectra with authentic samples of protonated α-alanine and β-alanine. Protonated α-alanine mainly fragments via the combined loss of H_2O and CO (eq 2, Supplementary Figure S2a) while β-alanine mainly fragments via the loss of water (eq 3, Supplementary Figure S2b). These results are consistent with previous studies on the fragmentation reactions of protonated α-alanine [24] and β-alanine [11]. As expected, the MS^3 spectra of the y_1 ions of αAla-αAla (Supplementary Figure S2c) and βAla-αAla (Supplementary Figure S2e) matched the MS/MS spectrum of protonated α-alanine (Supplementary Figure S2a), while the MS^3 spectra of the y_1 ions of αAla-βAla (Supplementary Figure S2d) and βAla-βAla (Supplementary Figure S2f) matched the MS/MS spectrum of protonated β-alanine (Supplementary Figure S2b). Similar MS^3 experiments allowed identification of the lactam ions at *m/z* 72 formed from the peptides. The authentic lactam ion derived from loss of water from the protonated amino acid β-alanine loses ketene (Supplementary Figure S3a). All the lactam ions derived from αAla-βAla, βAla-αAla, and βAla-βAla undergo an identical ketene loss (eq 4, Supplementary Figures S3b–d), thus identifying their origin from a β-alanine residue.

$$\left[H_2NCH(CH_3)CO_2H + H\right]^+ \rightarrow CH_3CH{=}NH_2^+ + H_2O + CO \quad (2)$$

$$\left[H_2NCH_2CH_2CO_2H + H\right]^+ \rightarrow \left[H_2NCH_2CH_2CO\right]^+ + H_2O \quad (3)$$

$$\left[H_2NCH_2CH_2CO\right]^+ \rightarrow CH_2{=}NH_2^+ + CH_2{=}C{=}O \quad (4)$$

We next examined the energy resolved CID spectra of each of the four isomeric dipeptides containing α- and β-alanine in the triple-quadrupole instrument. The complete energy resolved CID spectra are shown in the Supplementary material section (Figures S4a-d). In general, at low collision energies each of these spectra yields similar product ions to those observed for the CID spectra shown in Figure 1, although the triple quadrupole has an added benefit of readily detecting low mass ions (e.g., immonium ions). Figure S4a shows the breakdown graph for protonated αAla-αAla. At low collision energies the loss of CO via the a_1/y_1 pathway, which is unique to this dipeptide, is the most abundant product ion. At higher collision energies the precursor ion fragments to predominantly give y_1 ions. In addition the loss of H_2O to form the b_2 ion can be observed which further fragments to form an a_2 ion. Likewise at low collision energies, the breakdown graph of protonated αAla-βAla (Figure S4b) predominantly shows formation of the y_1 ion, whilst also yielding a b_2 ion that again fragments via elimination of CO to give the a_2 ion. The two N-terminal β-alanine containing dipeptides, βAla-αAla and βAla-βAla (Figures S4c, d), fragment via the diagnostic losses of NH_3 and $CH_2{=}NH$. Both of these peptides also result in primary fragment ions corresponding to the b_2 ion, however whilst the b_2 ion from βAla-αAla fragments further to primarily lose CO to give the a_2 ion, in the case of βAla-βAla it fragments to lose $CH_2{=}NH$.

To directly compare the formation of the immonium and lactam ions and y_1 ions from each of the isomeric dipeptides, Figure 2 shows the ion currents for each of these ions, extracted from the breakdown curves from Supplementary Figure S3. Noteworthy is that all peptides give rise to y_1 ions, and that the curves for their formation (Figure 2a) peak in the order: αAla-αAla (blue curve/circle markers, 18 V) < αAla-βAla (red curve/square markers 19 V) < βAla-αAla (purple curve/dash markers 21 V) < βAla-βAla (green curve/cross markers 22 V). The formation of immonium and lactam ions is also consistent with the MS/MS spectra from the linear ion trap. Thus only αAla-αAla, αAla-βAla, and βAla-αAla give rise to an immonium ion (Figure 2b), with the latter (purple curve/dash markers) having the latest appearance curve, consistent with it being a secondary product arising from fragmentation of the y_1 ion. Similarly, only βAla-βAla, βAla-αAla, and αAla-βAla give rise to a lactam ion (Figure 2c).

What are the b_2 Ion Structures Derived from the Isomeric Dipeptides αAla-αAla, αAla-βAla, βAla-αAla, and βAla-βAla?

Given that all the peptides give b_2 ions with the same *m/z* value and the renewed interest in b ion structures in general [25, 26], we wanted to establish whether each of these b_2 ions give different fragment ions under CID conditions in MS^3 experiments and examine potential structures for these b_2 ions using theoretical calculations. Just as the CID spectra of the protonated isomeric

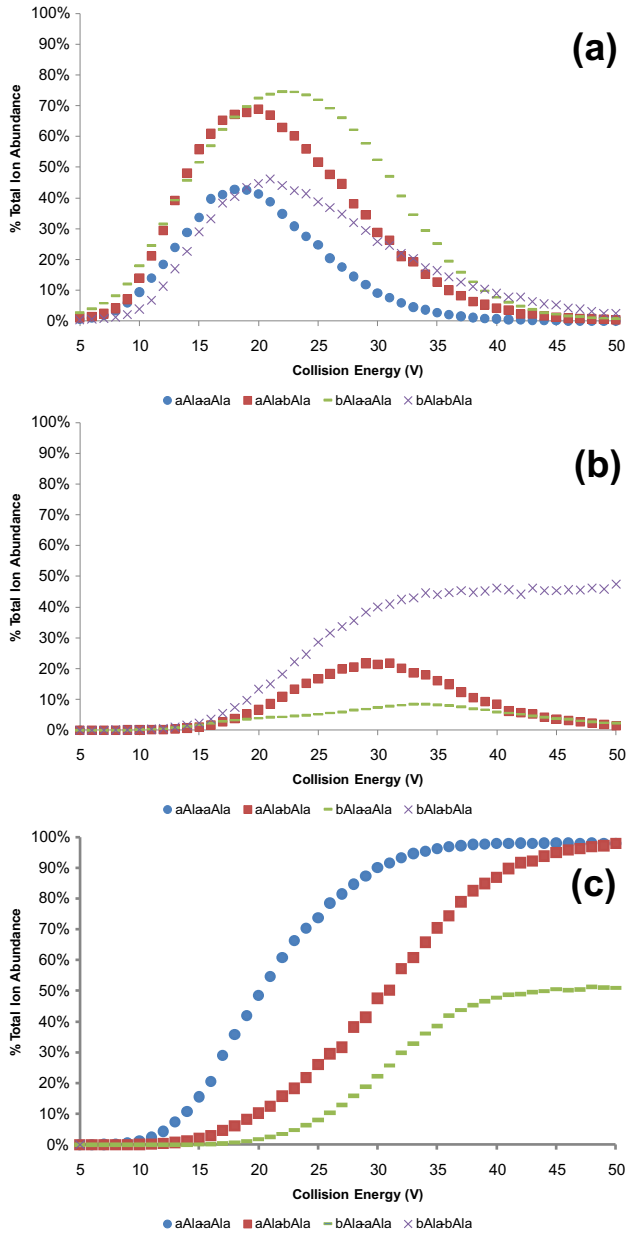
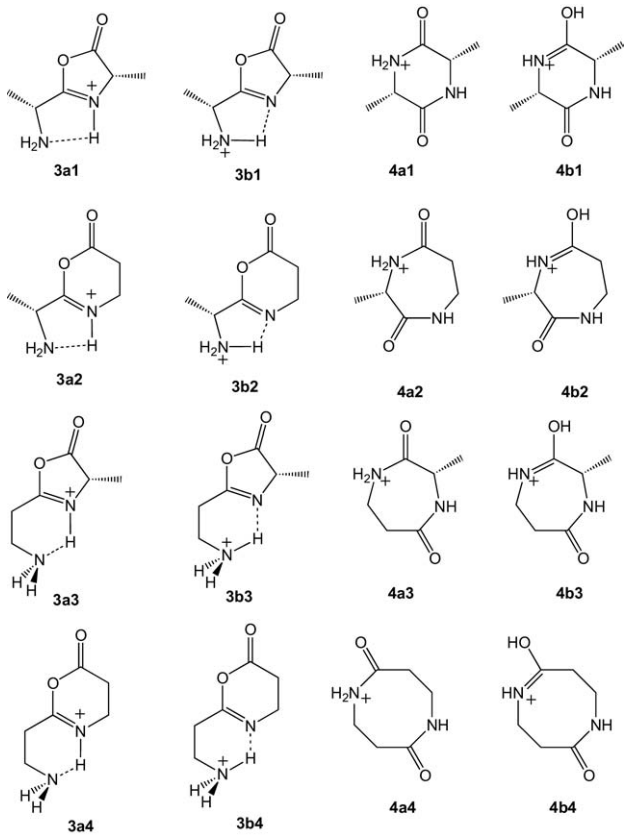


Figure 2. Ion appearance yields as a function of collision energy extracted from the energy resolved CID spectra of the dipeptides αAla-αAla, αAla-βAla, βAla-αAla and βAla-βAla. (**a**) Shows the formation of the y_1 sequence ions; (**b**) shows the formation of lactam ions; and (**c**) shows the formation of immonium ions. Note that all the original energy resolved CID spectra are given in Supplementary Figure S4.

dipeptides exhibit differences in both the types of ions formed and their relative abundances, so too are the MS^3 spectra of the b_2 ions different (see Supplementary material, Figure S5). The b_2 ion of α-Ala-αAla is dominated by CO loss (Figure S5a), while that of βAla-βAla is dominated by CH_2=NH loss (Figure S5d). The remaining two peptides both fragment via competitive CO and CH_2=NH losses. In addition, minor fragment ions are observed, some of which are unique to each dipeptide b_2 ion. These results suggest that the b_2 ion of each peptide is unique.

To gain insights into potential structures for these isomeric b_2 ions, we have prepared a theoretical survey of two classes of ion structures arising from neighboring group reactions involving the: (1) amide group to give ions of structure **3** (Scheme **5**); and (2) the N-terminal amine to give ions of structure **4** (Scheme **5**). In addition, since recent studies on b ion structures have shown that they can undergo intramolecular proton transfer [25], we have also considered ring structures in which the proton resides on two different sites. In total, 16 different isomers were considered (Scheme **5**), and calculations were carried out on several conformations of these isomers. The results of these theoretical studies are given in the Supplementary material (Supplementary Figure S6–S9) and the relative energies are given in Table 1.

While the theoretical survey presented here does not allow us to unequivocally establish the structure of each of the b_2 ions (which would require detailed calculations of the potential energy surfaces for various competing mechanisms), an examination of Table 1 reveals which of the b_2 ion neighboring group structures is *thermodynamically* favored is dependent on both the ring size and the capacity of the structure to help stabilize the charge via hydrogen bonding. Since the total electronic energies are being calculated, this allows the most stable b_2 ion of all the isomeric dipeptides to be determined as being structure **3b3** of Scheme **5**. This



Scheme 5. Potential b_2 ion structures of the isomeric dipeptides formed via neighboring group reactions involving amide to form ring structures 3; N-terminal amine to form ring structures 5.

Table 1. List of the 16 b_2 ion isomers derived from the isomeric dipeptides αAla-αAla, αAla-βAla, βAla-αAla, and βAla-βAla with their relative energies, in kcal mol^{-1}, compared to (a) other isomers of the same dipeptide and (b) all isomers

Isomer	(a) Relative energies of individual dipeptides (kcal mol^{-1})	(b) Relative energies between all isomeric dipeptides (kcal mol^{-1})
3a1	0.0	5.1
3b1	0.8	5.9
4a1	9.0	14.1
4b1	1.0	6.1
3a2	0.0	4.2
3b2	2.2	6.3
4a2	13.9	18.1
4b2	5.4	9.6
3a3	2.2	2.2
3b3	0.0	0.0
4a3	14.3	14.3
4b3	15.0	15.0
3a4	0.0	2.2
3b4	0.1	2.3
4a4	16.8	19.0
4b4	19.3	21.4

All structures were optimized at the MP2/6-31G(d)//B3LYP/6-31G(d) level of theory. The relative energies were calculated by correction for zero point energies and using single point energy calculations. All structures and absolute energies are given in the supplementary material Figures S6–S9.

structure, formed via neighboring group attack by the amide, is stabilized via hydrogen bonding to form a six-membered ring through hydrogen bonding of the ammonium group and the ester moiety (Supplementary Figure S8).

The data shown in Table 1 demonstrate that b_2 ion structures formed via the neighboring group attack by the amide to yield ring structures **3** are always more stable than the structures formed via neighboring group attack by the N-terminal amine to give the cyclic product ions **4**. The smallest difference is between the isomeric b_2 ion structures of αAla-αAla (**3a1**/**4b1**, 1.0 kcal mol^{-1}), followed by those of αAla-βAla (**3a2**/**4b2**, 5.4 kcal mol^{-1}). The differences in relative energies of the isomeric b_2 ions are largest for the N-terminal β-alanine dipeptides: βAla-αAla (**3b3**/**4a3**, 14.3 kcal mol^{-1}) and βAla-βAla (**3a4**/**4a4**, 16.8 kcal mol^{-1}). Interestingly, this is consistent with the CID results of the b_2 ions derived from each of these N-terminal β-alanine containing dipeptides (Figure S5c, d), whereby losses of $CH_2 = NH_2^+$ are observed, which are suggestive of structures **3a** that have a free primary amine capable of undergoing a retro-Mannich reaction (Scheme **6**).

Regarding **3a** and **3b**, since their energy differences are in the range of 0.1 to 2 kcal mol^{-1}, it is likely both isomers can interconvert. Indeed, the energy barriers for these proton transfers for αAla-αAla, αAla-βAla, βAla-αAla, and βAla-βAla are 6.6, 5.0, 2.3, and 0.6 kcal mol^{-1}, respectively (structures and energies in Supplementary material, Figure S10).

Protonated βAla-NHMe as a Model for a Peptide Containing an N-Terminal βAla Residue

To gain further insight into the unique fragmentation chemistry of the peptides containing an N-terminal β-alanine residue, the fragmentation reactions of the $[M + H]^+$ of the smaller model system β-Ala-NHMe were examined using a combination of experiment and theory. The low-energy CID spectrum of protonated β-Ala-NHMe (Supplementary Figure S11a) yields losses of NH_3 (eq 5) and CH_2=NH (eq 1), which were observed in the CID of N-terminal β-alanine containing dipeptides. The imine loss, previously noted by Tureček and coworkers [27], was confirmed to occur via the N-terminus through the observation of an identical loss from the CID of protonated β-Ala-NHEt (Supplementary Figure S11b). In addition, losses of H_2O (eq 6) and CH_3NH_2 (eq 7) are also observed. The most abundant peaks arise from loss of ammonia (eq 5) followed by water (eq 6). The losses of CH_2=NH (eq 1) and CH_3NH_2 (eq 7) are minor in comparison. The fragmentation reactions of protonated β-alanine-NHMe were further examined via energy resolved CID, yielding the break-down curve shown in Supplementary Figure S12a. Loss of NH_3 from the $[M + H]^+$ of β-alanine-NHMe dominates at low collision energies (< 10 V). As the collision energy is increased (≥ 15 V), higher energy fragmentation pathways corresponding to losses of H_2O, CH_2=NH, and CH_3NH_2 are observed (Figure S12b). The appearance energy of these fragment ions corresponds well to the relative abundances observed in the low-energy CID spectrum (Figure S11a for protonated β-alanine-NHMe). Minor formation of $CH_2{=}NH_2^+$ and $CH_3NH_3^+$ are observed at ≥ 15 V (Figure S12c) suggesting that these losses may also be primary fragmentation channels (eqs 8 and 9), which are not readily observed in the linear ion trap.

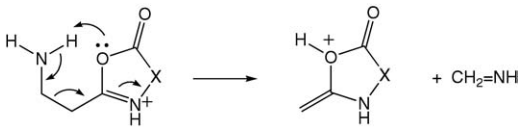
$$[H_2NCH_2CH_2C(O)NHCH_3 + H]^+ \rightarrow [CH_2CH_2CONHCH_3]^+ + NH_3 \quad (5)$$

$$\rightarrow [H_2NCH_2CH_2CNCH_3]^+ + H_2O \quad (6)$$

$$\rightarrow [H_2NCH_2CH_2CO]^+ + CH_3NH_2 \quad (7)$$

$$\rightarrow CH_2{=}NH_2^+ + CH_2C(OH)NHCH_3 \quad (8)$$

$$\rightarrow CH_3NH_3^+ + HNCH_2CH_2CO \quad (9)$$



Scheme 6. Retro-Mannich loss of CH_2=NH indicative of b_2 ion structures 3a3 (X=$CHCH_3$) and 3a4 (X=CH_2CH_2).

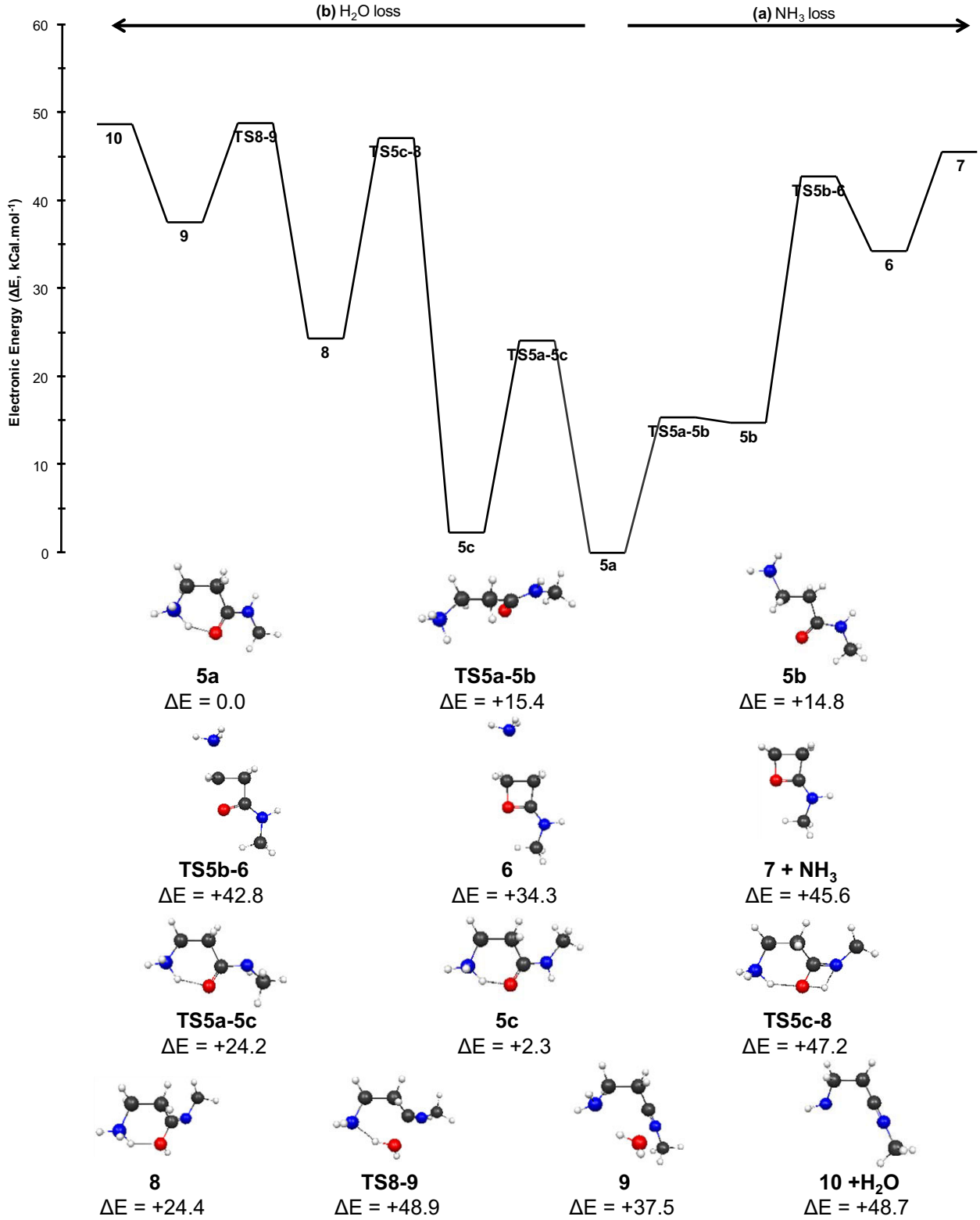


Figure 3. MP2/6-31(d)//B3LYP/6-31G(d) calculated PES and associated structures for key species involved in (**a**) ammonia loss and (**b**) water loss from protonated β-alanine-NHMe.

To gain insights into these competing fragmentation reactions, DFT calculations were used to investigate the potential energy surfaces (PES) associated with the losses of NH_3 (eq 5), H_2O (eq 6), CH_2=NH (eq 1), and CH_3NH_2 (eq 7), and the formation of CH_2=NH_2^+ (eq 8) and $CH_3NH_3^+$ (eq 9) from protonated β-alanine-NHMe. The final reaction endothermicities predicted for these pathways, which are 45.6, 48.7, 60.6, 70.1, 79.9, and 44.8 kcal

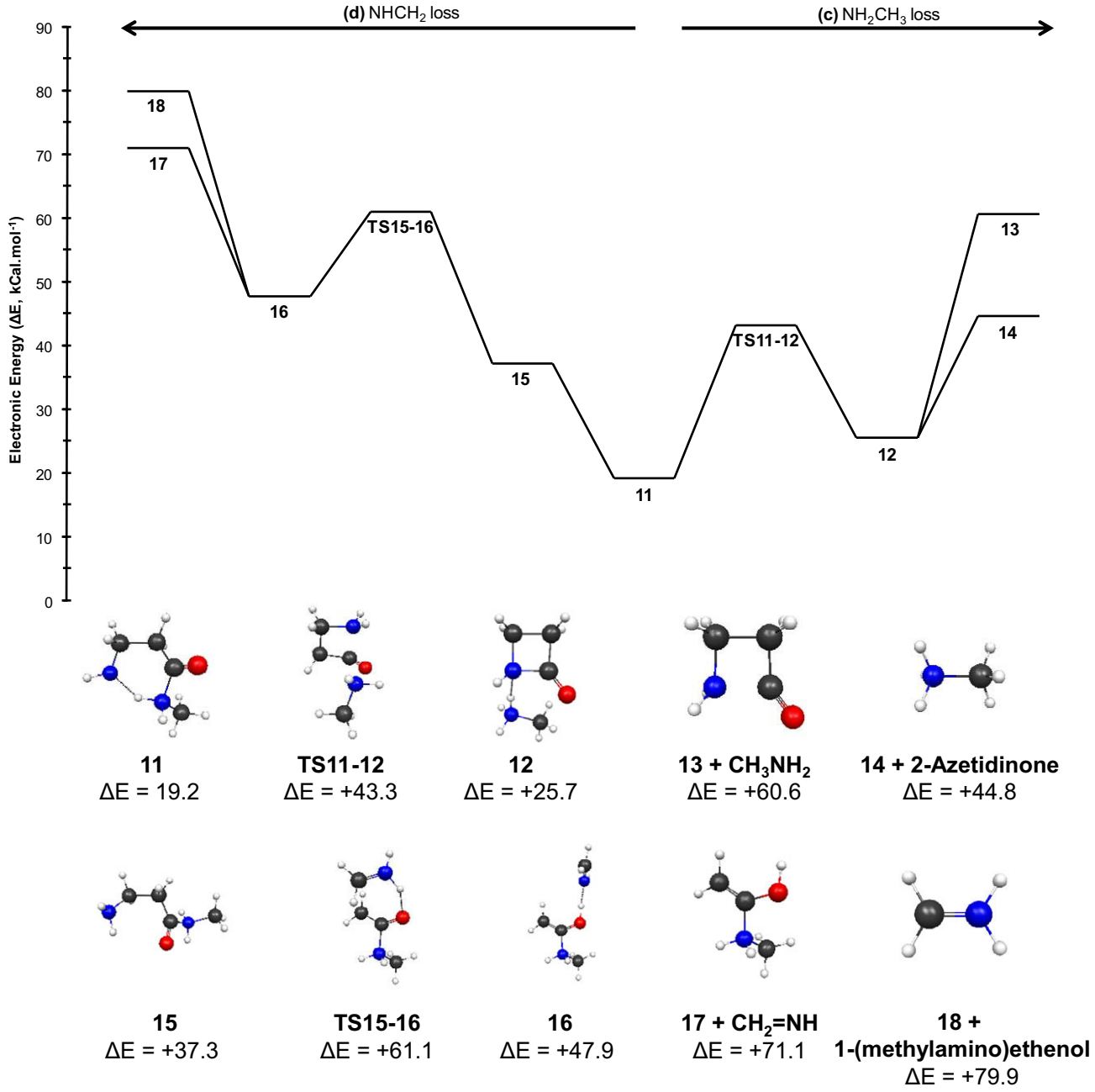


Figure 4. MP2/6-31(d)//B3LYP/6-31G(d) calculated PES and associated structures for key species involved in (**c**) CH_3NH_2 and $CH_3NH_3^+$ loss; and (**d**) CH_2 = NH and CH_2 = NH_2^+ loss from protonated β-alanine-NHMe.

mol^{-1}, respectively (Figures 3 and 4). Apart from the reaction endothermicity for formation of $CH_3NH_3^+$, there is reasonable agreement with the experimental results. Thus NH_3 loss (eq 5), which is the most abundant pathway is the least endothermic (45.6 kcal mol^{-1}); the second most abundant pathway, which involves H_2O loss (eq 6) is only slightly higher in energy (48.7 kcal mol^{-1}), whilst losses of CH_2=NH (eq 1) and CH_3NH_2 (eq 7) are considerably higher in energy, which is reflected in their low relative abundance in the CID spectrum (Figure S11a). The mechanism for each of these reactions is now described in further detailed.

Loss of NH_3 from Protonated β-Alanine-NHMe (eq 5, Figure 3)

The loss of NH_3 (eq 5) from the N-terminus of β-alanine-NHMe is likely to occur via a neighboring group reaction to yield a four-membered ring, as shown in Figure 3. The neighboring group reaction can be triggered by the lone pair on either the (1) carbonyl oxygen or the (2) amide nitrogen. DFT calculations (Figure 3 and Supplementary Figure S14) indicate that the oxygen participation is thermodynamically favored by around 10 kcal mol^{-1}. This pathway requires a *cis-trans*

conformational change from the most stable isomer of protonated β-alanine (**5a**). Owing to the drop in stabilization due to the breaking of the hydrogen bond, this step was found to be endothermic by 15.4 kcal mol^{-1}. The resultant conformer (**5b**) is primed for an exocyclic reaction by the oxygen nucleophile onto the β-backbone carbon, which involves a transition-state with an activation energy of 42.8 kcal mol^{-1}. The initially formed ion-molecule complex separates to give **7**.

Loss of H_2O from Protonated β-Alanine-NHMe (eq 6, Figure 3)

Formation of the imine ion (**10**) via water loss from the backbone of protonated β-alanine-NHMe is predicted to occur via a retro-Ritter type reaction, as shown in Figure 3 [21]. The first step involves isomerization of the amide bond via **TS5a-5c**, which lies 24.2 kcal mol^{-1} higher in energy than the most stable isomer (**5a**). This is followed by a tautomerization reaction, which proceeds via migration of the proton from the amide nitrogen onto the carbonyl oxygen, a process occurring via **TS5c-8**, which lies 47.2 kcal mol^{-1} higher in energy than **5a**. The final transition-state involves proton migration from the N-terminal nitrogen onto the oxygen of the iminol (**TS8-9**), a process that was calculated to be endothermic by ~48.9 kcal mol^{-1}. The resultant ion–molecule complex separates to give the imine (**10**). A very similar mechanism for water loss from protonated N-acridin-4-ylbenzylamide has been recently been reported based on DFT calculations [28].

Losses of CH_3NH_2 and $CH_3NH_3^+$ from Protonated β-Alanine-NHMe (eqs 7 and 9, Figure 4)

The losses of CH_3NH_2 and $CH_3NH_3^+$ (eqs 7 and 9) are predicted to arise from cleavage of the amide bond, which requires protonation of the amide nitrogen. Although we have not calculated the transition-state associated with this intramolecular proton transfer reaction, we note that the resultant isomer (**11**) lies ~19.2 kcal mol^{-1} higher in energy than the starting isomer (**5a**). Fragmentation of **11** via **TS11-12** requires 43.3 kcal mol^{-1} of energy, and results in an ion–molecule complex consisting of protonated methylamine and azetidin-2-one (**12**). Dissociation of this ion-molecule complex yields $CH_3NH_3^+$, while subsequent proton migration yields the fragmentation product (**13**) via loss of neutral methylamine. From the low-energy CID spectrum of β-alanine-NHMe (Supplementary Figure S11a) clearly the loss of CH_3NH_2 is not observed experimentally at high abundance. This is consistent with the reaction being endothermic by 60.6 kcal mol^{-1}, which is 15 and 11.9 kcal mol^{-1} more endothermic than the competing NH_3 and H_2O losses. Although we do not observe $CH_3NH_3^+$ due to the low mass cutoff of the linear ion trap, it is observed in the energy resolved spectrum (Supplementary Figure S12), albeit with a low abundance.

Losses of CH_2=NH and CH_2=NH_2^+ from Protonated β-Alanine-NHMe (eqs 1 and 8, Figure 4)

The losses of CH_2=NH and CH_2=NH_2^+ (eqs 1 and 8) are predicted to arise via a retro-Mannich cleavage reaction, involving a σ bond being converted into a π bond and migration of another σ bond, a process calculated to occur via the conformer **15**, which lies 37.3 kcal mol^{-1} higher in energy than **5**. The final products (**17** and **18**) occur via loss of CH_2=NH or from the ion-molecule complex (**16**) resulting from **TS15-16**. From the low-energy CID spectrum of β-alanine-NHMe (Supplementary Figure S11a) clearly the loss of CH_2=NH is not observed experimentally at high abundance. This is consistent with the reaction being endothermic by 71.1 kcal mol^{-1}, which is 25.5 and 22.2 kcal mol^{-1} more endothermic than the competing NH_3 and H_2O losses.

Conclusions

The simple act of moving the amino group from the α- to the β-position in an alanine residue in a peptide can have a profound effect on the fragmentation reactions of the protonated peptide. If the residue is at the N-terminus, b_1 ions are observed together with losses of NH_3 and CH_2=NH. The latter is a diagnostic loss and proceeds via a retro-Mannich reaction. When the residue is a C-terminal residue, b_2 and y_1 ions are observed, which are isomeric with those of α-alanine. These can, however, be distinguished via MS^3 experiments. Finally, while immonium ions are often used as indicators of the presence of specific residues in the peptide [29], b_1/lactam and y_1 ions derived from β-alanine ultimately fragment to form the immonium ion CH_2=NH_2^+, which could be misassigned as being the immonium ion derived from a glycine residue. Further work needs to carried out to: (1) see if the N-terminal fragmentation chemistry diagnostic for an N-terminal β-residue and the lactam fragments diagnostic of C-terminal or an internal β-residue are still important channels for larger peptides; (2 understand how substituents on β-alanine residues (i.e., Scheme **2**, where R_{x1} and/or $R_{x2} \neq H$), which can be placed on either the α-or the β-position, can further influence the fragmentation chemistry of protonated peptides. Such studies are underway and will be reported in due course.

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