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Isomer differentiation via collision-induced dissociation: The case of protonated α-, β^2- and β^3-phenylalanines and their derivatives†

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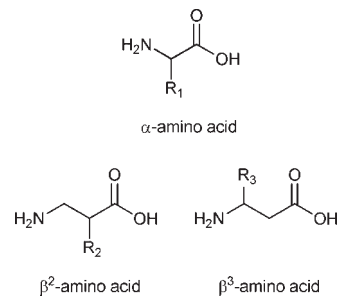
A combination of electrospray ionisation (ESI), multistage and high-resolution mass spectrometry experiments is used to examine the gas-phase fragmentation reactions of the three isomeric phenylalanine derivatives, α-phenylalanine, β^2-phenylalanine and β^3-phenylalanine. Under collision-induced dissociation (CID) conditions, each of the protonated phenylalanine isomers fragmented differently, allowing for differentiation. For example, protonated β^3-phenylalanine fragments almost exclusively via the loss of NH_3, only β^2-phenylalanine via the loss of H_2O, while α- and β^2-phenylalanine fragment mainly via the combined losses of $H_2O + CO$. Density functional theory (DFT) calculations were performed to examine the competition between NH_3 loss and the combined losses of H_2O and CO for each of the protonated phenylalanine isomers. Three potential NH_3 loss pathways were studied: (i) an aryl-assisted neighbouring group; (ii) 1,2 hydride migration; and (iii) neighbouring group participation by the carboxyl group. Finally, we have shown that isomer differentiation is also possible when CID is performed on the protonated methyl ester and methyl amide derivatives of α-, β^2- and β^3-phenylalanines. Copyright © 2010 John Wiley & Sons, Ltd.

The low-energy fragmentation reactions of protonated α-amino acids and their peptides are fairly well understood through the use of a range of tandem mass spectrometry (MS/MS) techniques and molecular modelling. This has led to the development of the concept of the mobile proton and its refinements.[1–7] In contrast, there has been considerably less work done on the gas-phase chemistry of the closely related β-amino acids, which are: involved in mammalian metabolism;[8–11] components of peptidic and non-peptidic natural products;[12] precursors in the biosynthesis of the cancer chemotherapy compound taxol.[13] With the discovery that β-amino acid containing peptides are ideal peptidomimetic candidates exhibiting a higher protease resistance than their α-amino acid analogues,[14] there has been growing interest in the biological use of various forms of β-amino acids. β-Amino acids differ from their α-counterparts in the presence of an extra methylene moiety between the amino and carboxyl termini, allowing for two types of β-amino acids: β^2 and β^3 (Scheme 1).

Apart from a number of studies focused on the gas-phase chemistry of β-alanine (**1**, Scheme 2) and its peptides,[15–19] only a few more complicated β-amino acids systems have been examined via MS-based methods. There have been reports on: the use of MS/MS to sequence synthetic β-peptides;[20] low-energy collision-induced dissociation (CID) of hybrid peptides;[21] interaction between various cations and β^3-amino acid containing peptides;[22] and differentiation of positional isomers containing α- and β^3-amino acids.[23–25] The differentiation of isomeric β^2- and β^3-amino acids via low-energy CID does not appear to have been addressed to date. Here we use a combination of multistage mass spectrometry experiments, CID, and density functional theory (DFT) calculations to compare the low-energy fragmentation reactions of β^2-phenylalanine (**2**) (R_2 = Phe) and β^3-phenylalanine (**3**) (R_3 = Phe); for structures, see Scheme 2. We also compare the fragmentation reactions of these β-amino acids with those of α-phenylalanine (**4**, Scheme 2), where we have previously shown that the main loss of the combined elements of water and CO proceeds via sequential bond-cleavage reactions involving ion-molecule complexes (path A of Scheme 3) and that the minor loss of ammonia proceeds via a neighbouring group reaction involving the phenyl ring to give a phenonium ion (path



Scheme 1. The two types of β-amino acids: β^2 and β^3.

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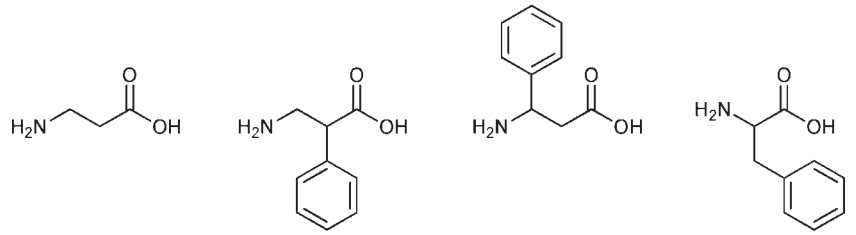
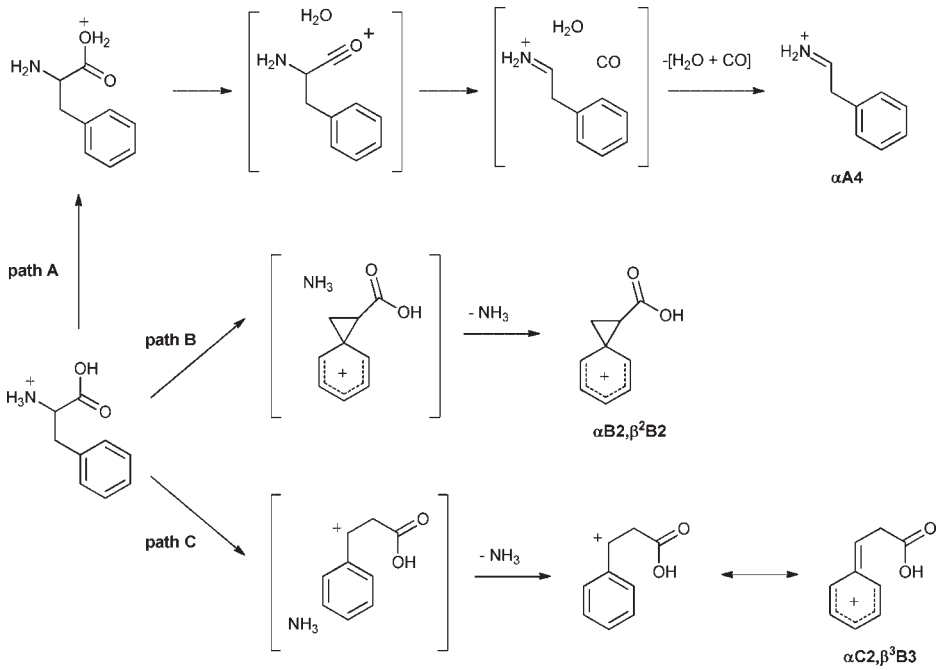


Scheme 2. Structures of β-alanine (**1**), β^2-phenylalanine (**2**) (R_2 = Phe), β^3-phenylalanine (**3**) (R_3 = Phe) and α-phenylalanine (**4**).



Scheme 3. Pathways for losses of $H_2O + CO$ and NH_3 from α-phenylalanine.

B of Scheme 3) rather than via a 1,2-hydride pathway to yield the benzyl cation (path C of Scheme 3).[26]

EXPERIMENTAL

Materials

All purchased materials were used without further purification: α-phenylalanine and β^3-phenylalanine were obtained from Sigma-Aldrich (St. Louis, MO, USA) and β^2-phenylalanine was purchased from Rare Chemicals GmbH (Kiel, Germany). Methanol (HPLC grade) and acetonitrile (HPLC grade) were obtained from Burdick & Jackson (Muskegon, MI, USA). Acetic acid (glacial) was obtained from BDH (Poole, UK).

Synthesis of N-terminal methyl ester derivatives

Lyophilised amino acids (10 mg) were dissolved in 1 mL of 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 lyophilisation and used without further purification.

Synthesis of N-terminal methyl amide derivatives

Following a variation of the method used by Feenstra *et al.*,[27] 2 mg of the unpurified amino acid methyl ester derivative 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.

Mass spectrometry experiments

All mass spectrometric experiments were conducted on a LTQ FT hybrid mass spectrometer (Thermo Scientific, Bremen, Germany) consisting of a linear ion trap (LTQ) coupled to a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. Methanolic samples were introduced into the electrospray ionisation (ESI) source of the mass spectrometer at a flow rate of 5 μL/min. The sheath gas flow rate, capillary voltage and temperature were adjusted to ca. 10 arbitrary units, 3.0 kV and 250°C, respectively. Low-energy CID studies using helium as the collision gas were performed within the LTQ using the standard procedure of mass selection of the desired precursor ion and subjection of these ions to collisional activation, in which the activation time for these experiments was 30 ms with the collision energy varied to ensure that 10–15% of the precursor ion remained.

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All high-resolution mass spectrometry (HRMS) experiments were conducted using the FT-ICR mass analyser component of the LTQ FT instrument. The ions of interest were mass-selected in the linear ion trap using standard procedures and subjected to CID. The resulting product ions were transferred to the FT-ICR cell to generate a high-resolution mass spectrum. Positive mode calibration was performed via the automatic calibration function using the recommended LTQ calibration solution, consisting of caffeine, the small tetrapeptide MRFA, and Ultramark 1621 solution.

Theoretical methods

Due to the large number of dihedral angles for these phenylalanine isomers, it was not feasible to systematically examine every possible conformer. Thus, a range of possible conformers for each isomer was constructed through rotation in 90° increments of the principle dihedrals of an initial conformer. Following geometry optimisation the lowest energy structure from this conformer search was used to construct the potential energy surfaces (PES) for the various fragmentation reactions.

Geometry optimisations and electronic energy calculations were performed using the Gaussian 03 molecular modelling package.[28] The structures of minima and transition states were optimised at the B3-LYP level of theory[29,30] with the 6-31 + G(d,p) basis set. All optimised structures were subjected to vibrational frequency analysis to ensure that they corresponded to either true minima (no imaginary frequencies) or transition states (one imaginary frequency). Intrinsic reaction coordinate (IRC) runs were performed on each transition state, followed by geometry optimisations to ensure that the states connected to the appropriate reactant and product(s). The final energies used to calculate the PES were corrected with the B3-LYP/6-31 + G(d,p) zero-point vibrational energies, (ZPVE) ($E_{reported} = E_{electronic} + E_{zpve}$). Complete structural details can be found in the Supporting Information. In cases where combined losses of molecules were modelled (i.e. loss of H_2O and CO) for both β²- and β³-phenylalanine, the initial departing neutral molecule was not removed from the intermediate ion-molecule complex as it was found to significantly lower the energies of both this intermediate and the subsequent transition-state structure(s). This is in line with the approach adopted in previous work.[26]

Due to the large number of possible structures involved in the PES of the three phenylalanine isomers, we have constructed a nomenclature for each structure based on its origin and role in the PES. All structures are prefixed with the identity of the phenylalanine isomer involved (i.e. α-, β²- and β³). This is followed by a set of letters denoting the role that the structure plays. Conformers of the phenylalanine isomers are denoted by use of a lower-case letter. Structures involved in a fragmentation reaction begin with an upper-case letter denoting the dissociation pathway, followed by the structure number. Finally, in cases where an individual ion can originate from two separate reactions by different phenylalanine isomers, two labels separated by a comma are used.

Examples of the nomenclature used in this text are summarised below:

β²a = Structure involved in the β²-phenylalanine PES, Protonated β²-phenylalanine, conformer **a**;

β³A1a = Structure involved in the β³-phenylalanine PES, Involved in dissociation pathway **A**, Structure number **1a**;

αC2,β³B3 = Structure involved in the α- and β³-phenylalanine PES, Involved in dissociation pathways **C** and **B**, respectively, Structure numbers **2** and **3**, respectively.

RESULTS AND DISCUSSION

Gas-phase fragmentation of α-, β²- and β³-phenylalanine

The MS/MS product ion spectra of protonated α-phenylalanine (**4**), β²-phenylalanine (**2**) and β³-phenylalanine (**3**) are shown in Figs. 1(A)–1(C). An examination of these spectra shows that the isomers can be distinguished from each other based on the different abundances of small molecule losses (e.g.

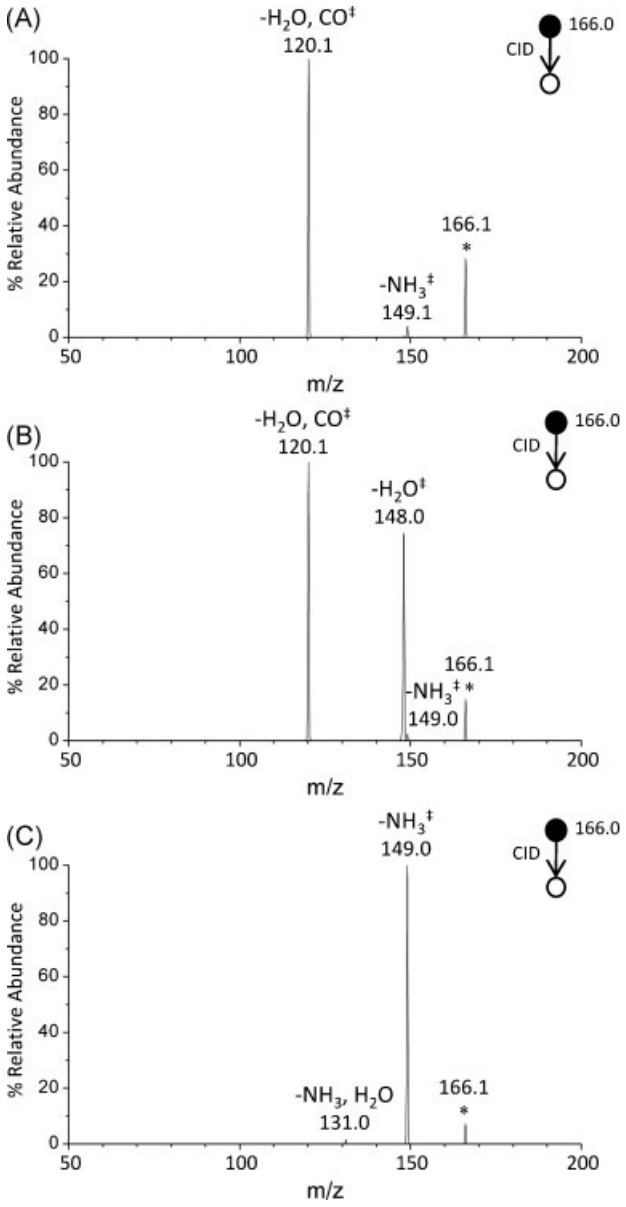

Figure 1. LTQ CID MS/MS spectra of the $[M + H]^+$ ions of: (A) α-phenylalanine; (B) β²-phenylalanine; and (C) β³-phenylalanine. An asterisk refers to the mass-selected precursor ion. The ‡ symbol indicates that the assignment of the molecular formula of the neutral(s) lost has been confirmed via HRMS.

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RCM

NH_3, H_2O and H_2O, CO). The fragmentation reactions of each of the three isomers will be discussed in further detail below.

α-Phenylalanine (Fig. 1(A))

Under low-energy CID conditions, the linear ion trap MS/MS spectrum for protonated α-phenylalanine (**4**) is almost identical to that observed previously in the 3D ion-trap.[26] Thus loss of $H_2O + CO$ (Eqn. (1)) to form the ion at m/z 120 (Fig. 1(A), path A of Scheme 3) dominates the spectrum, with a minor loss of NH_3 also being observed. The latter loss has been shown experimentally and via DFT calculations to occur via a neighbouring group process (Eqn. (2)) involving attack of the aryl ring onto the alpha carbon to yield a phenonium ion[26,31–33] (Scheme 3, path B) rather than via a 1,2-hydride migration mechanism (Scheme 3, path C).[34]

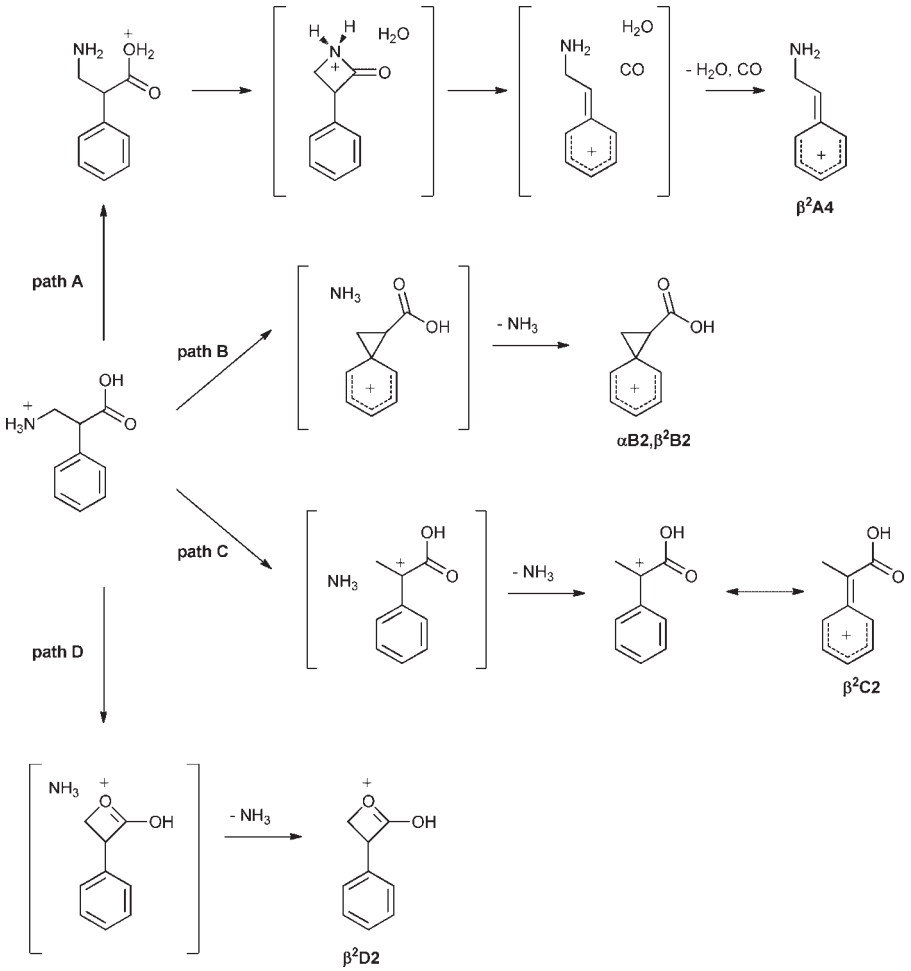
$$[H_2NCH(CH_2Ph)COOH + H]^+ \rightarrow [H_2NCH(CH_2Ph)]^+ + H_2O + CO \quad (1)$$

$$[H_2NCH(CH_2Ph)COOH + H]^+ \rightarrow [CH(CH_2Ph)COOH]^+ + NH_3 \quad (2)$$

β²-Phenylalanine (Fig. 1(B))

In a similar fashion to α-phenylalanine[26,31] (Fig. 1(A)), the main product ion for β²-phenylalanine (Fig. 1(B)) also arises via the loss of H_2O and CO to form m/z 120 (Eqn. (3)). Other fragmentation channels observed include an abundant loss of H_2O to yield the ion at m/z 148 (Eqn. (4)), together with a minor loss of NH_3 to form m/z 149 (Eqn. (5)). Water loss appears to be a diagnostic reaction channel that allows differentiation between the three isomers. It is worth noting that water loss has also been observed for β-alanine,[18,19] which contrasts with the virtual absence of water loss for α-alanine.[35] Possible mechanisms for these small molecule losses are shown in Scheme 4. By analogy with α-phenylalanine, the combined loss of H_2O and CO may involve stepwise bond cleavage (Scheme 4, path A). A difference is that cleavage of the C–O bond gives rise to a lactam, which is stable and can be observed at m/z 148 (Eqn. (4)). Loss of ammonia can proceed via three different mechanisms (Scheme 4, paths B–D). The first is a neighbouring group mechanism involving the phenyl group (Scheme 4, path B), thereby forming the same phenonium ion as for α-phenylalanine (Scheme 3, path B). The second is a 1,2-hydride shift to give a benzyl cation (Scheme 4, path C). The final mechanism involves the carboxyl group acting as a neighbouring group (Scheme 4, path D), to give a lactone. Each of these pathways was investigated via DFT calculations and they are discussed in detail below.

$$[H_2NCH_2CH(Ph)COOH + H]^+ \rightarrow [NH_2CH_2CH(Ph)]^+ + H_2O + CO \quad (3)$$



Scheme 4. Pathways for losses of H_2O, $H_2O + CO$ and NH_3 from β²-phenylalanine.

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$$[H_2NCH_2CH(Ph)COOH + H]^+ \rightarrow [NH_2CH_2CH(Ph)CO]^+ + H_2O \quad (4)$$

$$[H_2NCH_2CH(Ph)COOH + H]^+ \rightarrow [CH_2CH(Ph)COOH]^+ + NH_3 \quad (5)$$

β^3-Phenylalanine (Fig. 1(C))

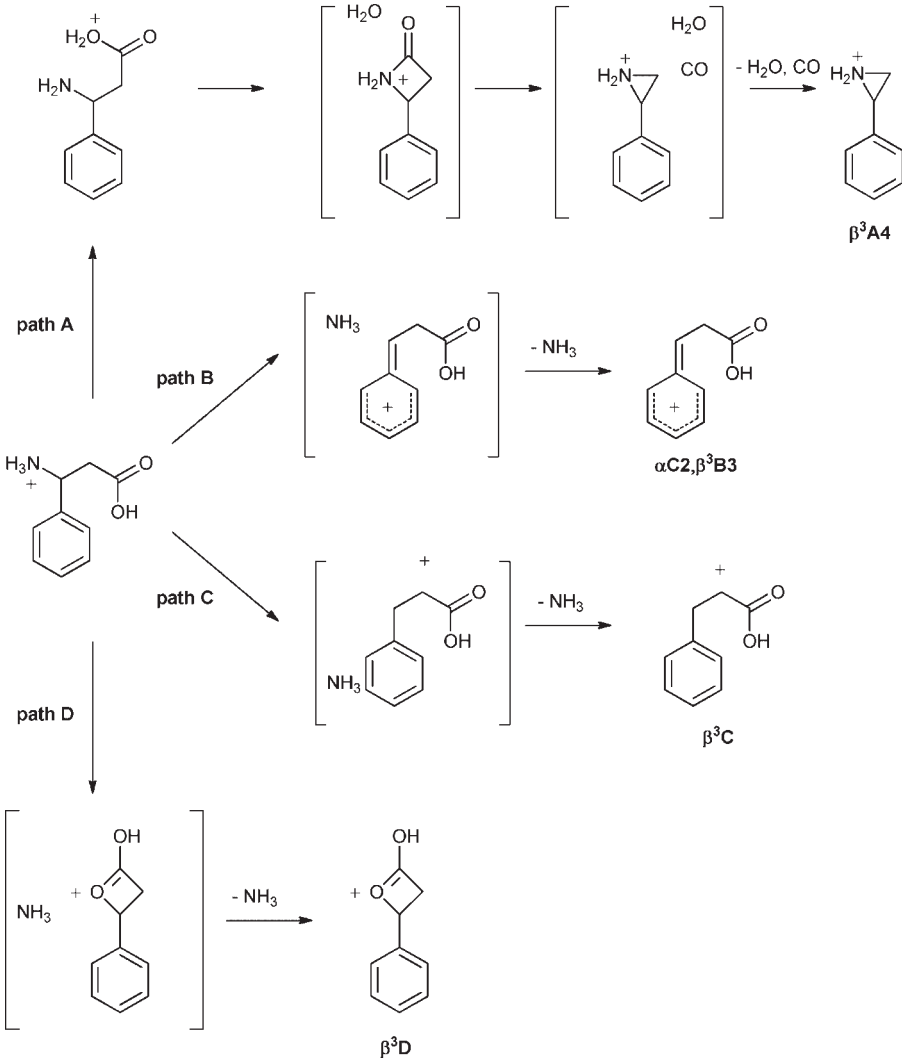
The low-energy CID spectrum of protonated β^3-phenylalanine (Fig. 1(C)) is quite different from those of α- and β^2-phenylalanine, which readily allows for isomer distinction. The dominant fragmentation channel is loss of NH_3 (to form m/z 149, Eqn. (6)), while a minor combined loss of NH_3 and H_2O is observed to yield a product ion at m/z 131 (Eqn. (7)). It is interesting to note that the ion arising via the combined loss of H_2O and CO (Scheme 5, path A) is absent for β^3-phenylalanine, which is in stark contrast to the CID spectra of the α- and β^2-phenylalanine isomers where it is the dominant reaction channel. Once again, there are three possible mechanisms for NH_3 loss (Scheme 5, paths B–D). In this case, however, the phenonium ion formed via a neighbouring group pathway for α- and β^2-phenylalanine isomers (Schemes 3 and 4, path B) is not possible. Instead, the phenyl group can promote loss of ammonia via formation of a benzyl cation (Scheme 5, path B, species **αC2,β^3B3**). NH_3 loss promoted via 1,2-hydride migration (Scheme 5, path C) and carboxyl neighbouring group participation (Scheme 5, path D) are also mechanistic possibilities.

$$[H_2NCH(Ph)CH_2COOH + H]^+ \rightarrow [CH(Ph)CH_2COOH]^+ + NH_3 \quad (6)$$

$$[H_2NCH(Ph)CH_2COOH + H]^+ \rightarrow [CH(Ph)CHCO]^+ + NH_3 + H_2O \quad (7)$$

DFT calculations of possible structures and relative energies of the product ion resulting from NH_3 loss ($C_9H_9O_2^+$)

As there are a variety of potential structures of the $C_9H_9O_2^+$ product ion that results from the loss of NH_3 from the MS/MS of protonated α-, β^2- and β^3-phenylalanine (Figs. 1(A)–1(C)), we have carried out a theoretical survey into all the possible structures that arise from the: (i) aryl-assisted neighbouring group (path B); (ii) 1,2 hydride migration (path C); and (iii) neighbouring group participation by the carboxyl group (path D). In total, nine different isomers were considered



Scheme 5. Pathways for losses of $H_2O + CO$ and NH_3 from β^3-phenylalanine.

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(Table 1), with the relative energies of each isomer expressed in two ways: (i) relative to the most stable $C_9H_9O_2^+$ isomer, **αC2,β³B3** (shown in italics); (ii) with respect to the specific isomer global minimum (shown in brackets).

The data shown in Table 1 demonstrates that 1,2-hydride migration (path C) results in the most stable products for both α-phenylalanine (**αC2,β³B3**, 26.8 kcal.mol^{-1}) and β²-phenylalanine (**β²C2**, 32.8 kcal.mol^{-1}). In contrast, the bicyclic ion from path B of these two amino acids, **αB2,β²B2**, is considerably less stable (>9 kcal.mol^{-1}). This difference may be attributed to the presence of the strained three-membered ring and loss of aromaticity from the phenyl group. Structure **αC2,β³B3** (path B) is the only stable product ion arising from the loss of NH_3 from β³-phenylalanine, with the ions resulting from paths C and D isomerising into **αC2,β³B3** following optimisation. A key finding is that loss of ammonia via path D is uncompetitive with the other possible mechanistic paths for all Phe isomers. Thus, product ions formed via path D are either the least stable $C_9H_9O_2^+$ isomer (**αD** and **β²D2**) or appear to be unstable as they isomerise into an ion formed via another pathway (**β³D**).

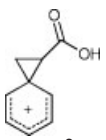
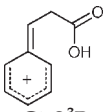
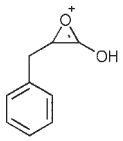
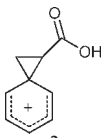
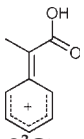
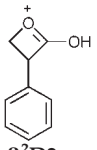
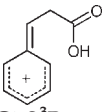
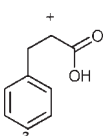
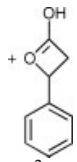
DFT calculations of potential mechanisms for the decomposition reactions of the amino acids of α-, β²- and β³-phenylalanine

Although the results of the DFT calculations shown in Table 1 allow us to predict the most stable $C_9H_9O_2^+$ isomer following loss of NH_3 from each phenylalanine species, they do not give any information about the relative barrier heights for the transition states associated with the various competing fragmentation reactions. Hence additional calculations were carried out on each of the three isomeric amino acids to determine the potential energy surfaces (PES) of each fragmentation pathway in order to establish the mechanisms by which they fragment under low-energy CID conditions. Of particular interest was the competition between NH_3 loss and the combined loss of H_2O and CO, which appear to be sensitive to the structure of the amino acid (Fig. 1). The possible pathways that we considered are those shown in Schemes 3–5. On a final note, the PES presented below all show the relative enthalpies for various processes at 0 K and thus are uncorrected for temperature and entropy. These relative entropies may underestimate processes where entropy is important. For example, the combined loss of H_2O and CO is expected to be entropically favoured over loss of water or ammonia alone.

Potential mechanisms for the fragmentation reactions of α-phenylalanine

Supplementary Fig. S1 (see Supporting Information) shows the PES for the loss $H_2O + CO$ (path A) and loss of NH_3 via an aryl-based neighbouring group attack (path B) and via hydride transfer (path C). The PES for paths A–C have been calculated in a previous study[26] and have been recalculated

Table 1. Potential isomers of formula $[C_9,H_9,O_2]^+$ corresponding to the ion arising from loss of NH_3 from the three isomeric phenylalanine derivatives, α-phenylalanine, β²-phenylalanine and β³-phenylalanine. The relative energies listed below each isomer are from DFT calculations carried out at the B3-LYP/6-31G(d,p) level of theory. The relative energies of each isomer (in kcal.mol^{-1}) are expressed in two ways: (i) relative to the most stable $C_9H_9O_2^+$ isomer (shown in italics); (ii) with respect to the specific isomer global minima (shown in brackets)

	Path		
	B	C	D
α-phenylalanine	 **αB2,β²B2** *12.4* (39.2)	 **αC2,β³B3** *0.0* (26.8)	 **αD** *43.5* (70.3)
β²-phenylalanine	 **αB2,β²B2** *15.8* (42.6)	 **β²C2** *6.0* (32.8)	 **β²D2** *22.9* (49.7)
β³-phenylalanine	 **αC2,β³B3** *6.7* (33.5)	 **β³C** (Isomerises into αC2,β³B3)	 **β³D** (Isomerises into αC2,β³B3)

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and included as a reference to the β-amino acids studied here. With the exception of slight differences in energies for the final products arising from paths B and C, the energies of the structures are identical to those published previously.[26] The PES corresponding to path D, involving a carbonyl-based neighbouring group attack, could not be found. However, as the final endothermicity of the product ion, **αD**, is considerably higher (Table 1) than both the energies of the transition states of the competing pathways and those of their final products, this pathway would not be expected to operate.

Loss of H_2O and CO from protonated α-phenylalanine

The loss of H_2O and CO to yield the ion **αA4** is predicted to occur stepwise beginning with the loss of H_2O via the transition state **TS(αAb-αA1)**, which has a barrier of 39.1 kcal.mol^{-1} above the local minima **αa**. Separation of the resultant ion-molecule complex **αA1** gives the ion **αA2** which is 49.2 kcal.mol^{-1} higher in energy than **αa**. The loss of CO from the complex **αA1** is proposed to occur via **TS(αA1-αA3)**, which lies 38.6 kcal.mol^{-1} higher in energy than **αa**. Separation of the resultant ion-molecule complex **αA3** yields the ion **αA4** with an overall endothermicity of 25.3 kcal.mol^{-1}.

Loss of NH_3 from protonated α-phenylalanine

As reported previously, the loss of NH_3 from protonated α-phenylalanine is predicted to occur via a neighbouring group attack by the aryl group to form a phenonium cation (path B) rather than by 1,2-hydride migration (path C).[26,31] Briefly, the thermodynamically less favoured hydride migration of path C is predicted to operate via **TS(αa-αC1)**, which lies 43.6 kcal.mol^{-1} higher in energy than **αa** to yield the product ion **αC2,β^{3}B3**. The more favourable neighbouring group path B is predicted to occur via **TS(αc-αB1)** which is 6.6 kcal.mol^{-1} lower in energy than the competing H_2O loss transition state **TS(αb-αA1)**. Optimisation of the ion that arises from the following separation of the ion-molecule complex **αB1** leads to the separated product ion **αB2,β^{2}B2** and NH_3, with an overall endothermicity of 39.2 kcal.mol^{-1}. Although the barrier against this pathway is smaller than the competing $H_2O + CO$ loss from path A, as noted previously[26] the minor abundance of the NH_3 loss ion compared with that of $H_2O + CO$ is because the $H_2O + CO$ pathway is kinetically favoured.

Potential mechanisms for the fragmentation reactions of β^2-phenylalanine

As shown in Fig. 1(B), the CID spectrum of protonated β^2-phenylalanine shows that it fragments mainly via the loss of H_2O and CO. The spectrum also shows the loss of NH_3 and the loss of H_2O. The latter loss is not observed in the CID spectra of the $[M + H]^+$ ions of the other isomers. The PES of all these losses are discussed below. In addition to the three possible paths for the loss of NH_3, two potential mechanistic pathways corresponding to the concerted and stepwise loss of $H_2O + CO$ were found.

Loss of H_2O and CO from protonated β^2-phenylalanine

The PES for the concerted loss of H_2O and CO via an aryl-assisted neighbouring group reaction is shown in Supplementary Fig. S2. Briefly the proposed reaction occurs via **β^{2}b**, which is 8.6 kcal.mol^{-1} higher in energy than **β^{2}a** (Supplementary Fig. S2, see Supporting Information). The dissociation occurs via a concerted mechanism in which the ionising proton is transferred from the N-terminal nitrogen onto the C-terminal hydroxyl group of the carboxylic acid, poised to lose H_2O and CO. This process occurs via transition state **TS(β^{2}b-β^{2}A3b)** which has been calculated to have a barrier of 49.9 kcal.mol^{-1} above **β^{2}a**. This yields the ion-molecule complex **β^{2}A3b**, which upon separation results in **β^{2}A4**.

The stepwise loss of $H_2O + CO$ shown in Fig. 2 (Eqn. (3), path A) from protonated β^2-phenylalanine occurs via a neighbouring group attack by the amino nitrogen to form the ion-molecule complex **β^{2}A1**. This pathway is predicted to occur via conformer **β^{2}b** which lies approximately 8.6 kcal.mol^{-1} above the local minimum, **β^{2}a**. The neighbouring group attack by the amino nitrogen onto the carbonyl carbon occurs via transition state **TS(β^{2}b-β^{2}A1)** with an energy of 27.6 kcal.mol^{-1} higher than **β2b**. Separation of the resultant ion-molecule complex, **β^{2}A1**, yields the lactam ion **β^{2}A2**, with an endothermicity of 40.2 kcal.mol^{-1}. The subsequent loss of CO from the ion-molecule complex **β^{2}A1** occurs via **TS(β^{2}A1-β^{2}A3a)**, which, following separation of the ion-molecule complex **β^{2}A3a**, yields the ion **β^{2}A4**. The barrier for this pathway is 45.6 kcal.mol^{-1} relative to the lowest energy structure **β^{2}a**. As the barrier against this stepwise pathway is considerably lower than that for the concerted mechanism H_2O, CO loss would be expected to occur via this pathway.

Loss of NH_3 from protonated β^2-phenylalanine

Each of the three mechanistic pathways for the loss of NH_3 from β^2-phenylalanine (Scheme 4) was modelled using DFT calculations and the pathways are shown in Supplementary Fig. S3 (see Supporting Information). From this figure it can be observed that path C which involves a 1,2-hydride migration via the key transition state, **TS(β^{2}a-β^{2}C1)**, has a barrier height of 49.8 kcal.mol^{-1} and is the highest energy pathway. Interestingly, the combined energy of the final products from this pathway also has the lowest endothermicity of all the products, with an endothermicity of 32.8 kcal.mol^{-1} (**β^{2}C2**). NH_3 loss via a neighbouring group reaction via the carbonyl oxygen (path D) occurs first via a gauche to anti conformational change, **TS(β^{2}a-β^{2}c)**, which has an activation energy of 9.0 kcal.mol^{-1}, followed by the energy required for the neighbouring group transition state, **TS(β^{2}c-β^{2}D1)**, of 41.8 kcal.mol^{-1}. Finally, the energetically favoured path B is predicted to arise via an aryl-assisted neighbouring group reaction, resulting in the formation of the phenonium ion **αB2,β^{2}B2**, which is identical to the product ion formed following loss of NH_3 from α-phenylalanine via the related phenyl neighbouring group processes (Supplementary Fig. S1, see Supporting Information). This process involves the transition state **TS(β^{2}b-β^{2}B1)** with an activation energy of 37.7 kcal.mol^{-1}. The low abundance of the ion for this pathway in B is due to this comparatively higher activation energy and the endothermicity of **αB2,β^{2}B2** (42.6 kcal.mol^{-1} relative to **β^{2}a**) than for the competing H_2O loss pathway (36.2 kcal.mol^{-1} relative to **β^{2}a**).

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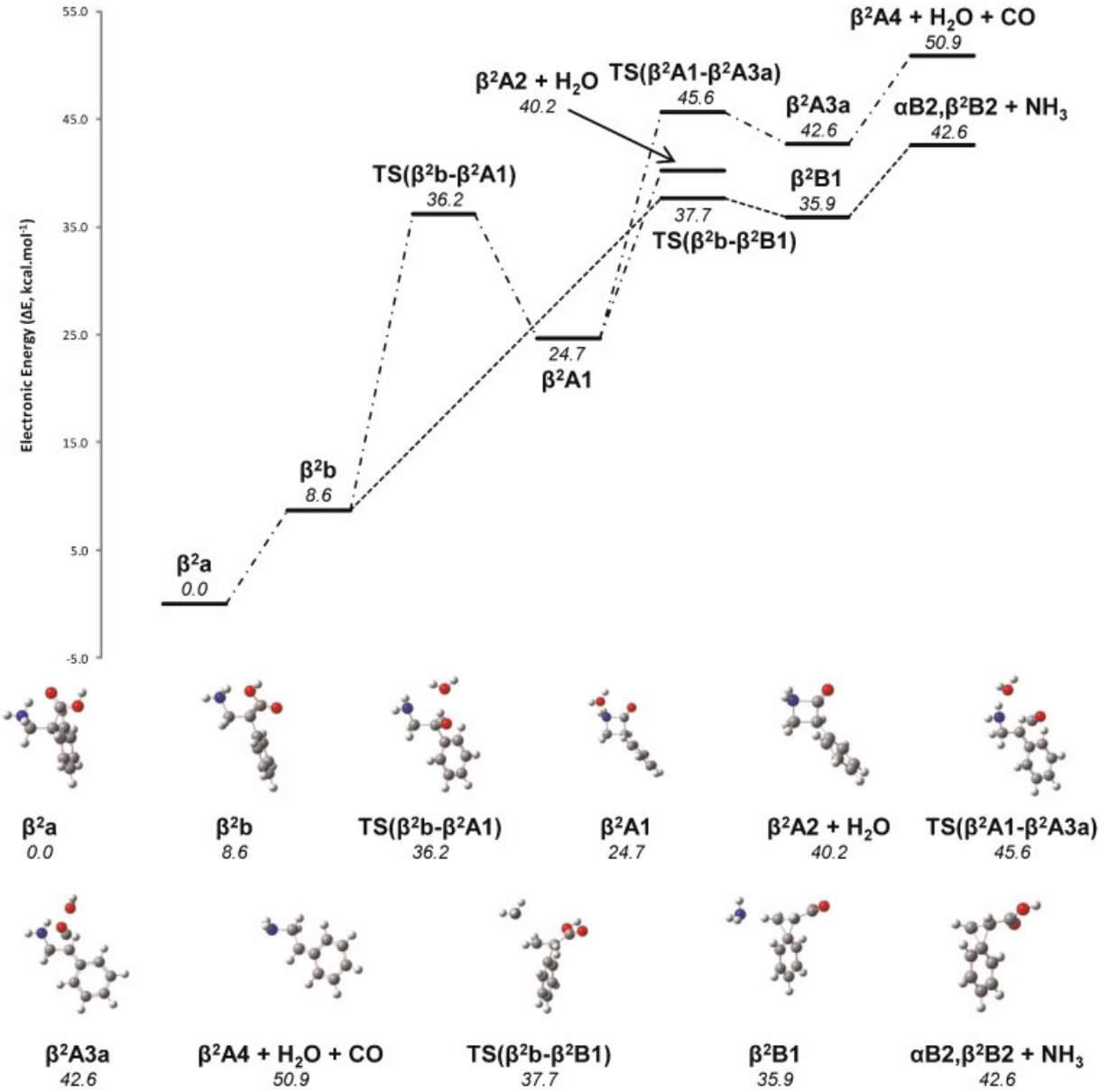

Figure 2. B3-LYP/6-31 + G(d,p) calculated reaction pathways and associated structures for key structures involved in the fragmentation of protonated β^2-phenylalanine: Pathway A: H_2O and CO loss (Major); and pathway B: NH_3 loss (Minor). All italicised numbers (in kcal.mol^{-1}) are relative to **β^2a**.

Potential mechanisms for the fragmentation reactions of β^3-phenylalanine

In contrast to α- and β^2-phenylalanine, CID of protonated β^3-phenylalanine almost exclusively involves the loss of NH_3, with virtually no loss of H_2O + CO being observed. The PES for this latter loss occurring via a concerted mechanism is discussed below. In the case of the loss of NH_3, although there are three possible paths as shown in Scheme 5, only the transition state corresponding to path B involving the aryl neighbouring group could be found, which is consistent with the final product instability of the ions from paths C and D (Table 1).

Loss of H_2O and CO from protonated β^3-phenylalanine

The neighbouring group reaction required for the loss of H_2O from β^3-phenylalanine occurs via **TS(β^3b-β^3A1a)** resulting in the **β^3A1a** complex (Fig. 3). This transition state has a barrier 34.6 kcal.mol^{-1} higher than the preceding ion, **β^3b**. This ion-molecule complex **β^3A1a** may separate to yield the ion **β^3A2** which has an endothermicity of 40.7 kcal.mol^{-1}. Further dissociation of the conformer **β^3A1b** via **TS(β^3A1b-β^3A3)** requires 49.6 kcal.mol^{-1} and results in an ion-molecule complex **β^3A4** which has an endothermicity of 48.4 kcal.mol^{-1} and consists of a protonated lactam surrounded by a CO and a H_2O molecule.

Loss of NH_3 from protonated β^3-phenylalanine

Formation of the benzyl cation (**αC2,β^3B3**) via NH_3 loss is predicted to first occur via a conformational change from an anti to a gauche conformation, **TS(β^3a-β^3c)**, resulting in **β^3c** (Fig. 3). This transition state has a barrier 10.3 kcal.mol^{-1} higher than the most stable

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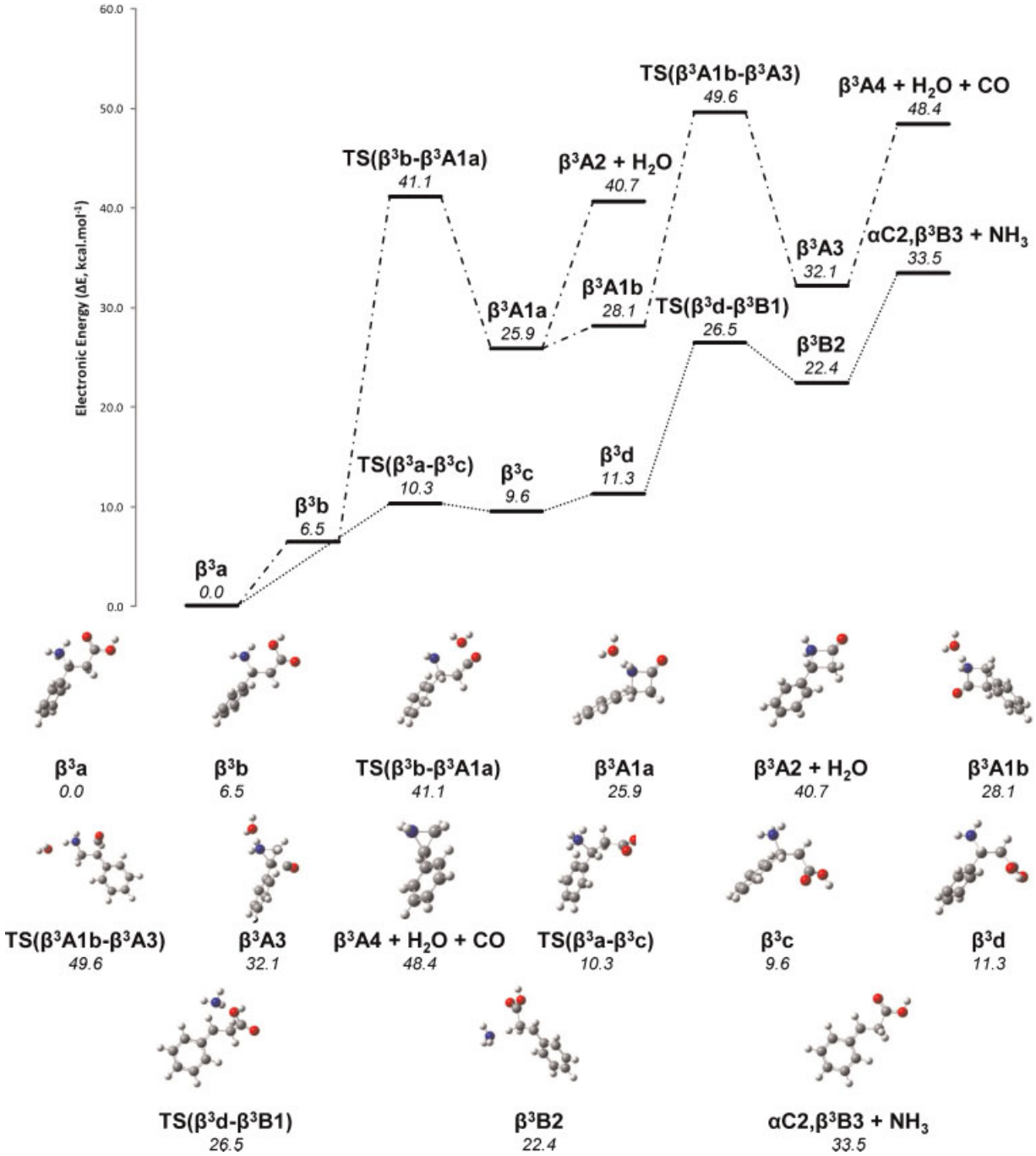

Figure 3. B3-LYP/6-31 + G(d,p) calculated reaction pathways and associated structures for key structures involved in the fragmentation of protonated β³-phenylalanine: Pathway A: H_2O and CO loss (Minor); and pathway B: NH_3 loss (Major). All italicised numbers (in kcal.mol^{-1}) are relative to **β³a**.

conformer of protonated β³-phenylalanine **β³a**. The transition state for the loss of NH_3, **TS(β³d-β³B1)**, involves an aryl-assisted neighbouring group attack, a process that lies 26.5 kcal.mol^{-1} higher in energy than **β³a**. The resultant ion-molecule complex separates to give the benzyl cation **αC2,β³B3** which has an endothermicity of 33.5 kcal.mol^{-1}. As the energy of **TS(β³d-β³B1)** is substantially lower than the barriers against the competing H_2O loss, **TS(β³b-β³A1a)**, 26.5 vs. 41.1 kcal.mol^{-1}, only NH_3 loss is observed in C. In addition this ion, **αC2,β³B3**, is the same ion as is formed following the loss of NH_3 from α-phenylalanine following 1,2-hydride migration (Scheme 3).

Key features and overall trends from the DFT calculations

To aid in the comparison of the PES of the three phenylalanine amino acids, the energies associated with the key barriers against the four pathways are summarised in Table 2. For path A, the loss of H_2O + CO, two columns corresponding to the barriers and endothermicities associated with H_2O and the subsequent CO losses have been included.

From the DFT-calculated PES values presented above, the following observations can be made:

(i) The endothermicity of the product ions does not give a good indication of the preferred mechanistic pathway.

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Table 2. Summary of the barriers for the key transition states involved in paths A–D. The numbers in parentheses corresponds to the final endothermicities for the products following separation of the ion-molecule complex. All numbers (in kcal.mol^{-1}) are relative to the most stable protonated conformer of the respective amino acid: **αa**, **β^{2}a** and **β^{3}a**

		Path			
	A (H_2O loss)	A (CO loss)	B	C	D
α-phenylalanine	39.1 (49.2)	38.6 (25.3)	32.5 (39.2)	43.6 (26.8)	(70.3)
β^2-phenylalanine	36.2 (40.2)	45.6 (50.9)	37.7 (42.6)	49.8 (32.8)	41.8 (49.7)
β^3-phenylalanine	41.1 (40.7)	49.6 (48.4)	26.5 (33.5)	(Isomerises into **αC2,β^{3}B3**)	

Examples of this are the product ions for α- and β^2-phenylalanine: although the final energies of the ions that result from 1,2-hydride migration (path C) are considerably lower than those obtained from the other products, 1,2-hydride migration is unlikely to occur due to the considerably higher activation energy than for the other competing pathways;

(ii) The almost exclusive loss of NH_3 from β^3-phenylalanine (Fig. 1(C)) can be rationalised by the low barrier of this loss (26.5 kcal.mol^{-1}) which is considerably lower than those of the competing H_2O (41.1 kcal.mol^{-1}) and CO losses (49.6 kcal.mol^{-1});

(iii) Comparison of paths B and D of β^2-phenylalanine gives us an indication of which neighbouring group is preferred. As the barrier for aryl neighbouring group participation is considerably lower than the corresponding carbonyl-based attack (37.7 vs. 41.8 kcal.mol^{-1}), this suggests that the phenyl group is the better neighbouring group. As far as we are aware,[36] this is the first comparison of the intrinsic neighbouring group abilities of Ph and COOH;

(iv) For all ions that contain a benzyl cation, the bond length between the benzylic carbon and the phenyl ring decreases, indicating charge delocalisation onto the ring. This is demonstrated by examining the ion that arises following the loss of CO from the protonated lactam **β^{2}A1** from β^2-phenylalanine. The position of the phenyl group allows for neighbouring group participation by the aryl group to form an α-aminomethyl-substituted benzyl cation (**β^{2}A4**). Due to the position of the phenyl group for β^3-phenylalanine, this pathway is not accessible and the aziridine ion **β^{3}A4** is formed instead.

Gas-phase fragmentation of C-terminal methyl ester and methyl amide derivatives of α-, β^2- and β^3-phenylalanine

The C-terminally protected methyl ester and methyl amide derivatives of each of the three phenylalanine isomers were subjected to positive ion mode ESI-MS and their $[M+H]^+$ ions were mass-selected and subjected to low-energy CID, resulting in the spectra shown in Fig. 4. A brief comparison of the spectra for the unmodified amino acids in Fig. 1 and their corresponding methyl esters and methyl amides in Fig. 4 indicates that introduction of a C-terminal protecting group can have a profound effect on the fragmentation chemistry that is observed for β-phenylalanine amino acids, whilst having little effect on their α-amino acid counterparts. A key example of this is the increased relative abundance of NH_3 loss with the introduction of the methyl ester and the amide nitrogen for β^2-phenylalanine (Figs. 1(B), 4(C), and 4(D)). Each of these amino acid derivatives is now discussed in further detail.

Gas-phase fragmentation of α-phenylalanine-OCH_3 and α-phenylalanine-$NHCH_3$

Collisional activation of the $[M+H]^+$ ion of α-phenylalanine-OCH_3 (Fig. 4(A)) results in a spectrum that is directly related to that observed for α-phenylalanine (Fig. 1(A)) with the a_1 ion (m/z 120) being the major product ion, and a minor loss of NH_3 also being observed. Likewise, the introduction of a methyl amide moiety (Fig. 4(B)) does not change the types of product ions formed, with the losses of $NH_2CH_3 + CO$ (to yield the ion at m/z 120) and NH_3 (to yield the ion at m/z 162) the only observable fragmentation pathways.

Gas-phase fragmentation of β^2-phenylalanine-OCH_3 and β^2-phenylalanine-$NHCH_3$

The low-energy CID of the methyl ester of β^2-phenylalanine shown in Fig. 4(C) yields a more complex spectrum than those observed for the parent amino acid (Fig. 1(B)). Although the major losses of CH_3OH and CO to form m/z 120 and CH_3OH (m/z 148) are analogous to the H_2O and CO, and H_2O losses in Fig. 1(B), the introduction of the methyl ester also promotes the fragmentation of β^2-phenylalanine via NH_3 loss (m/z 163). In addition, it introduces a variety of minor abundance ions not observed before, including a water adduct at m/z 166 and $NHCH_2$ loss (to yield the ion at m/z 151), probably occurring via a retro-Mannich mechanism discussed previously.[19,20,25] Collisional activation of protonated β^2-phenylalanine-$NHCH_3$ alters the preferred fragmentation pathways dramatically, with the dominant channel now being loss of NH_3 at m/z 162, with only a minor amount of $NHCH_2$ loss (m/z 150) being observed.

Gas-phase fragmentation of β^3-phenylalanine-OCH_3 and β^3-phenylalanine-$NHCH_3$

Figures 4(E) and 4(F) reveal that the CID spectra of β^3-phenylalanine-OCH_3 and β^3-phenylalanine-$NHCH_3$ are almost identical to that of the unprotected amino acid (Fig. 1(C)). Thus, each spectrum is dominated by NH_3 loss (to give the ions at m/z 163 and 162, respectively). Alteration of the protecting group appears to influence the minor peaks

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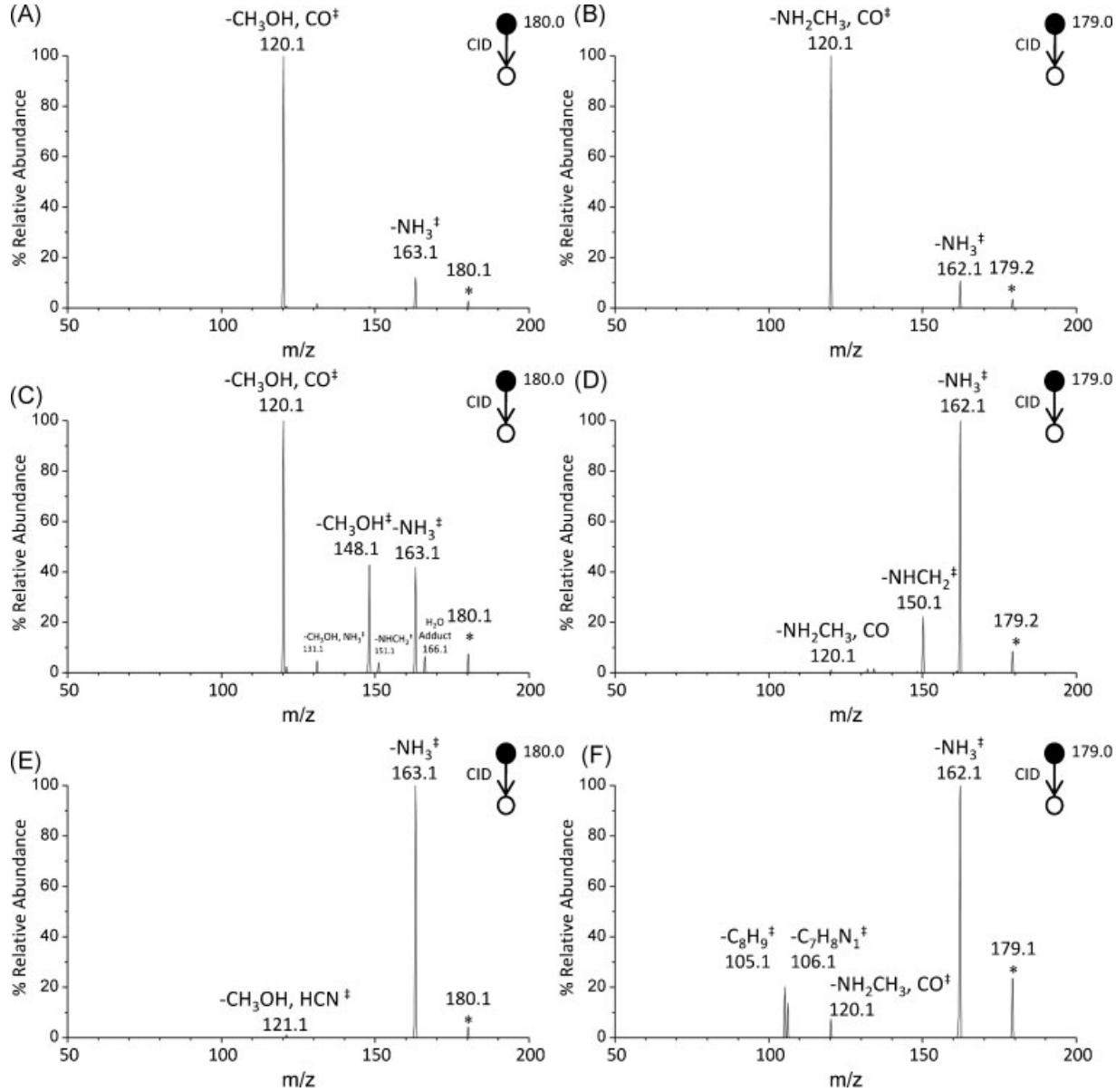

Figure 4. LTQ CID MS/MS spectra of the $[M+H]^+$ ions of the terminal methyl ester derivatives of: (A) α-phenylalanine; (C) β^2-phenylalanine; and (E) β^3-phenylalanine. MS/MS spectra of the $[M+H]^+$ ions of the terminal methyl amide derivatives of: (B) α-phenylalanine; (D) β^2-phenylalanine; and (F) β^3-phenylalanine. An asterisk refers to the mass-selected precursor ion. The ‡ symbol indicates that the assignment of the molecular formula of the neutral(s) lost has been confirmed via HRMS.

that are observed, with the amino acid (Fig. 1(C)) losing a combination of NH_3 and H_2O (to give an ion at m/z 131), while the methyl ester (Fig. 4(E)) loses CH_3OH and HCN (to give an ion at m/z 121). Finally, the methyl amide (Fig. 4(F)) has unique losses to form ions at m/z 105 and 106 that correspond to the losses of C_8H_9 and C_7H_8N, respectively. Finally, the minor ion at m/z 120 is proposed to arise via the combined loss of NH_2CH_3 and CO.

CONCLUSIONS

Previous work has highlighted a number of the key differences in the fragmentation reactions of α- versus β-amino acids. For example, extension of the carbon backbone of β-amino acids allows b_1 ions to be observed. In this study we have shown that the location of the phenyl group in isomeric phenylalanine residues can have a significant effect on the fragmentation chemistry that is observed. By moving the phenylalanine group from the α- to the β^2- or β^3-position, different types of fragmentation pathways become favoured. Thus, different types of small molecule losses dominate the low-energy CID spectra for α-, β^2- and β^3-phenylalanine, allowing for isomer distinction. Further work needs to be carried out to investigate the fragmentation chemistry of β-amino acids and their peptides that contain a wide range of different R groups at the β^2 and β^3 positions. It will be particularly interesting to examine the behaviour of side chains that are known to be reactive (e.g. basic, acidic and sulfhydryl) and play roles in promoting the fragmentation reactions of α-amino acids.

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Key questions that need to be addressed are: (i) how do these reactive side chains promote fragmentation of β-amino acids and their peptides and (ii) does placement of these substituents on either β^2 or β^3 influence the fragmentation chemistry?

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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