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Dissociation of Protonated Phenylthiohydantoin-Amino Acids and Phenylthiocarbamoyl-Dipeptides

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The *N*-terminal phenylthiocarbamoyl (PTC) derivatives of peptides and the phenylthiohydantoin (PTH) derivatives of amino acids are the two major types of products generated in the Edman protein sequencing method. Understanding the fragmentation pathways of these species should facilitate structural elucidation and chemical identification based on the fragment ion mass spectra, particularly when mass spectrometry is combined with the Edman sequencer for the analysis of non-standard and modified amino acids. In this study, dissociation of the protonated PTH-X (where X = Thr, Ser, Trp and Tyr), PTH-Gly, PTC-X-Leu and PTC-Gly-Leu in electrospray ionization mass spectrometry was examined to investigate whether there is any isomerization of PTC to PTH derivatives in the gas phase during the fragmentation. It is shown that dissociation of the protonated PTH-X proceeds via hydrogen transfer from the side-chain of the amino acid to the PTH moiety with the elimination of the side-chain as a neutral species. The ions at *m*/*z* 193 formed from the source fragmentation of the protonated PTH-X are found to have the same structure and fragmentation pathways. The presence of this *m*/*z* 193 ion and its collisionally induced dissociation (CID) spectrum are unique for the PTH derivatives and they can be used to detect the presence of the PTH ions. It is shown that there is no isomerization of the thiazolone ions to the PTH ions during the dissociation of PTC-X-Leu (in this case, the b_1 ions from PTC-X-Leu are believed to have the protonated thiazolone structure). In addition, comparative studies of CID spectra of PTH-X and PTH-Gly or PTC-X-Leu and PTC-Gly-Leu are presented. The proposed fragmentation mechanisms for the protonated PTH and PTC derivatives and the *m*/*z* 193 ions are given. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: ion dissociation; phenylthiohydantoin amino acids; phenylthiocarbamoyl peptides; electrospray ionization; tandem mass spectrometry

INTRODUCTION

In Edman sequencing of a peptide, the reagent phenyl isothiocyanate (PITC) reacts with the peptide's *N*-terminal residue. The modified thiazolone amino acid can be cleaved, leaving the rest of the peptide intact. Before detection, the thiazolone amino acid is converted into its isomeric form, phenylthiohydantoin (PTH) amino acid. PTH derivatives are most commonly identified by high-performance liquid chromatography (HPLC) with UV absorbance detection. Mass spectrometry (MS) has the potential to become an alternative method of analysis for the PTH derivatives from an Edman sequencer. The advantages of the MS approach include improved speed and sensitivity and unsurpassed specificity for possible identification of non-standard or modified amino acids. Indeed, MS has previously been used for the identification of thiohydantoin,[1–3] methyl[4] and phenylthiohydantoin[5–9] amino acid derivatives. With the advance of liquid chromatography/electrospray ionization (ESI) MS, sensitive analysis of PTH derivatives has now become possible. For example, we have recently demonstrated that ESI-MS using an ion trap/time-of-flight mass spectrometer can provide detection of all 20 standard PTH-amino acids at the 100 fmol level.[10] Structural information can be obtained based on the fragment ion spectra of PTH derivatives produced by source fragmentation. Shortly after the submission of this paper, Zhou *et al.* reported an ESI-MS study of PTH-amino acids in an ion-trap mass spectrometer and demonstrated the utility of ion fragmentation in the trap for the structural analysis of these compounds.[11]

It is clear that studies of the fragmentation pathways of PTH derivatives have significance not only in understanding the fundamentals of ion chemistry but also in practical applications, particularly for the structural characterization of non-standard or modified PTH-amino acids. For PTH-amino acids, major fragment ions observed in chemical ionization[5] and thermospray ionization[6] MS have been reported; but no detailed studies of the fragmentation mechanisms were reported. Recently, Gaskell and co-workers[9] examined several

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PTC derivatives and presented a study of fragmentation pathways of major product ions by using ESI with low-energy collisional activation. In this paper, we present an in-depth investigation of the collision-induced dissociation (CID) reactions of *N*-terminal phenylthiohydantoin derivatives and phenylthiocarbamoyl (PTC) derivatives of several amino acids and dipeptides. Several common fragment ions were observed in ESI mass spectra from source fragmentation. These source-formed ions were subjected to CID and their product ions were recorded. The proposed fragmentation mechanisms of these ions are presented.

EXPERIMENTAL

All high-energy CID experiments were carried out by using the ZabSpec (ZAB) orthogonal acceleration (OA) time-of-flight (TOF) instrument from Micromass (Manchester, UK). The ESI method was used for creating gas-phase ions. The precursor ions were accelerated by a voltage of 4 kV and mass selected with the EBE mass spectrometer. The ions were introduced into the collision cell floated at 3600 V. Thus, for high-energy CID, the laboratory kinetic energy of a singly charged precursor ion is 400 eV. Argon was used as the collision gas and, on average, the abundance of the precursor ions was attenuated by ~30%. The ions exiting the collision cell were guided into the OA-TOF spectrometer and pulsed into the flight tube for mass separation. Ions were detected with a microchannel plate detector.

The dipeptides (Gly-Leu, Ser-Leu, Thr-Leu, Trp-Leu and Tyr-Leu), PITC and PTH derivatives were purchased from Sigma Chemical (St Louis, MO, USA) and used without purification. HPLC-grade acetonitrile, HPLC-grade water and certified reagent-grade ammonium acetate were purchased from Fisher Scientific (Nepean, ON, Canada). PTC derivatives of dipeptides were synthesized by dissolving a dipeptide (30 μmol) in 500 μl of ethanol–pyridine–water (1:1:1, v/v/v) and by adding a sevenfold molar excess of PITC. The solvent was removed under a stream of nitrogen. The product was analyzed without further purification. Solutions for ESI-MS analysis were prepared by dissolving a sample compound in a mixture of 80% acetonitrile and 20% 0.25 mM ammonium acetate in water (by volume). For continuous infusion experiments, the sample solution (100 μM) was delivered directly from a syringe to the electrospray source at a flow-rate of 6 μl min^{-1}.

Low-energy CID experiments were performed using a Quattro tandem quadrupole instrument (VG Biotech, Winsford, Cheshire, UK) equipped with an electrospray interface. The voltage of the collision cell was 20 V. The laboratory kinetic energy of a singly charged precursor ion for low-energy CID was about 20 eV. Argon was used as the collision gas and, on average, the abundance of the precursor ions was attenuated by ~30%. The tandem mass spectra reported represent sums of 20–30 scans.

RESULTS AND DISCUSSION

Electrospray ionization mass spectra of several *N*-terminal PTC derivatives of dipeptides and the underivatized dipeptides were recorded. The b_1 or thiazolone ion was detected in the ESI spectra of the PTC derivatives of dipeptides, PTC-X-Leu (where X = Thr, Ser, Trp and Tyr) and PTC-Gly-Leu. The underivatized dipeptides did not display the b_1 ion peaks in the ESI spectra. These findings are consistent with the results obtained by others.[9,12] The thiazolone ions formed from PTC-X-Leu were of sufficient abundance in the ESI mass spectra that they could be mass selected to study the CID fragmentation reactions. Figure 1(A) shows the product ion spectrum of the thiazolone-Thr ion from PTC-Thr-Leu obtained using low-energy collisional activation in a triple-quadrupole mass spectrometer. The low-energy product ion spectrum of the protonated PTH-Thr is shown in Fig. 1(B) for comparison. The spectrum shown in Fig. 1(A) is in good agreement with that obtained by fragmentation of the b_1 ion originated from PTC-TLLELA peptide.[9] In Fig. 1(A), four major fragmentation products are observed. These are the ions at *m/z* 209 and 193 generated from the *m/z* 237 ion with the loss of 28 and 44 u, respectively, the threonine immonium ion at *m/z* 74 and the phenyl isothiocyanate ion at *m/z* 136. The product ion spectrum of the protonated PTH-Thr ion shows one prominent fragmentation product at *m/z* 193 corresponding to the loss of 44 u, and several minor fragment ions corresponding to the immonium ion of the threonine at *m/z*

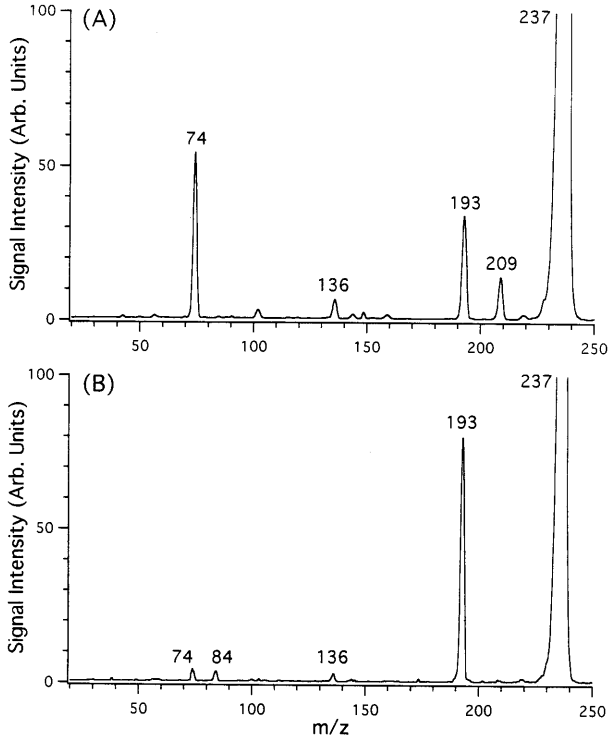


Figure 1. Low-energy CID mass spectra of the *m/z* 237 ion derived from (A) PTC-Thr-Leu and (B) PTH-Thr.

74, the *m/z* 84 ion and the phenyl isothiocyanate ion at *m/z* 136.

Figure 1 clearly shows that the product ion spectrum of the *m/z* 237 ion derived from PTC-Thr-Leu is different from that of PTH-Thr. This indicates that the fragmentation pathways of the b_1 ion derived from PTC-Thr-Leu are significantly different from those of the phenylthiohydantoin ion. One of the common product ions observed in Fig. 1(A) and (B) is the ion at *m/z* 193. In the previous study,[9] it was proposed that this ion is produced by the loss of CS after isomerization of the thiazolone ion (the b_1 ion) to the phenylthiohydantoin ion. In order to explore further the gas-phase ion chemistry for both isomers, we carried out more detailed studies on PTH derivatives and PTC derivatives by using CID in a sector/OA-TOF system.

Figure 2 shows the product ion spectra of the protonated PTH-X (where X = Thr, Ser, Trp and Tyr) obtained by using the high-energy CID technique. To our surprise, the ion peak expected to be from the elimination of CS from the protonated PTH-amino acids is absent for PTH-Ser, PTH-Trp and PTH-Tyr [Fig. 2(B), (C), and (D)]. The most intense peaks are from the immonium ion (i.e. *m/z* 74 for PTH-Thr, 60 for PTH-Ser, 130 for PTH-Trp and 136 for PTH-Tyr, which overlaps with phenyl isothiocyanate), phenyl isothiocyanate at *m/z* 136, the phenyl ion at *m/z* 77 and the ion at *m/z* 193. The *m/z* 193 ion, a common fragment ion product of PTH-X, appears to be from side-chain (R) elimination with hydrogen migration from the R-group to the ion at *m/z* 193 (see below). Note that the loss of the side-chain neutral species (CH_3CHO) produces a product ion with a mass of 44 u less than the mass of the protonated PTH-Thr ion.

The CID spectra of the thiazolone ions derived from PTC-X-Leu are shown in Fig. 3. The fragment ions observed include the immonium ion, phenyl isothiocyanate at *m/z* 136, the phenyl ion at *m/z* 77 and a product ion corresponding to CO elimination from the molecular ion. Only the thiazolone ions derived from PTC-Thr-Leu and PTC-Trp-Leu produce the ion at *m/z* 193 by a loss of 44 and 129 u from its respective precursor ion.

Since fragment ions at *m/z* 193 are observed in both PTH and PTC derivatives, understanding the origins of these ions and their fragmentation pathways should provide additional information on the ion chemistry. We therefore focused on studying the product ion spectra of the *m/z* 193 ion from different sources. Figure 4 shows the product ion mass spectra of the *m/z* 193 ions derived from PTH-X. Similar spectra were obtained, indicative of similar fragmentation reaction

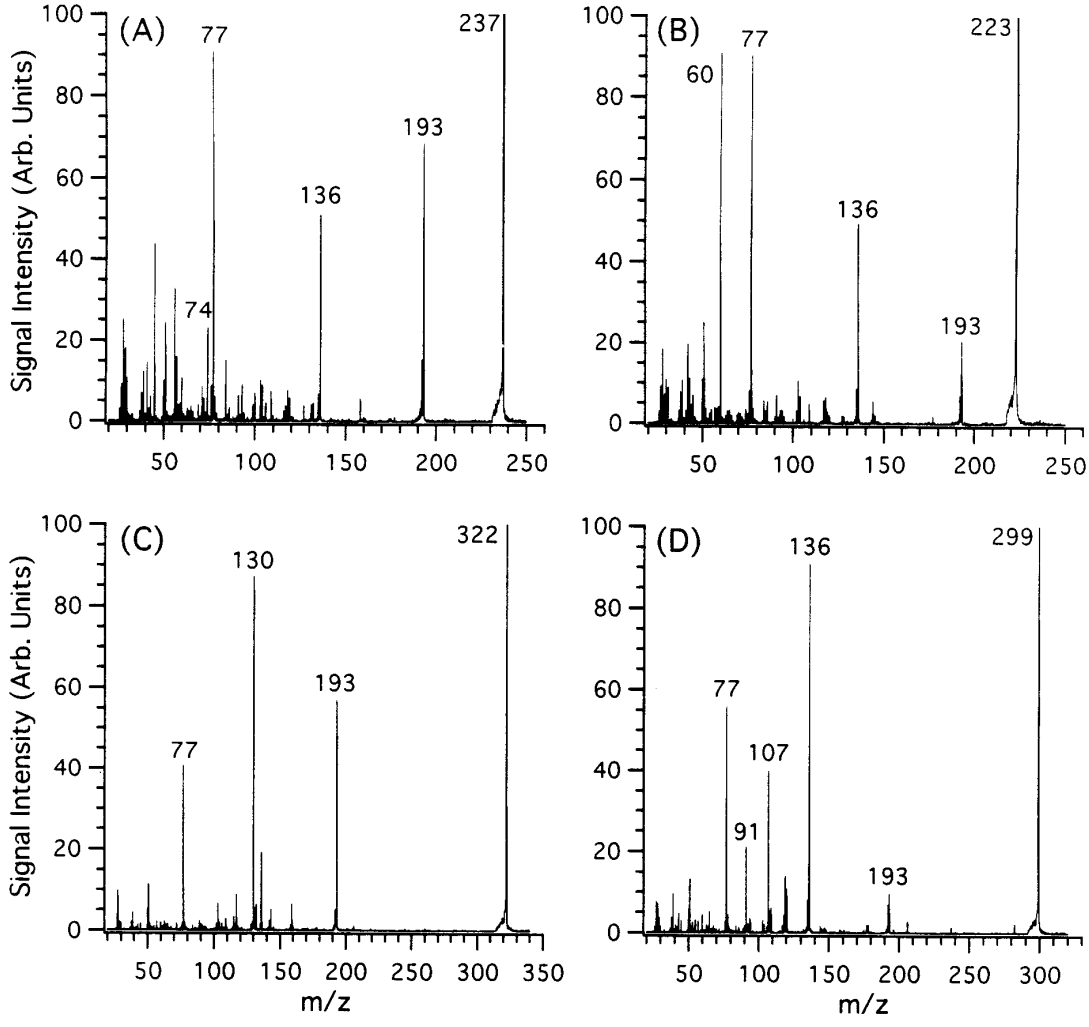


Figure 2. High-energy CID mass spectra of the protonated PTH-amino acids: (A) PTH-Thr; (B) PTH-Ser; (C) PTH-Trp; (D) PTH-Tyr.

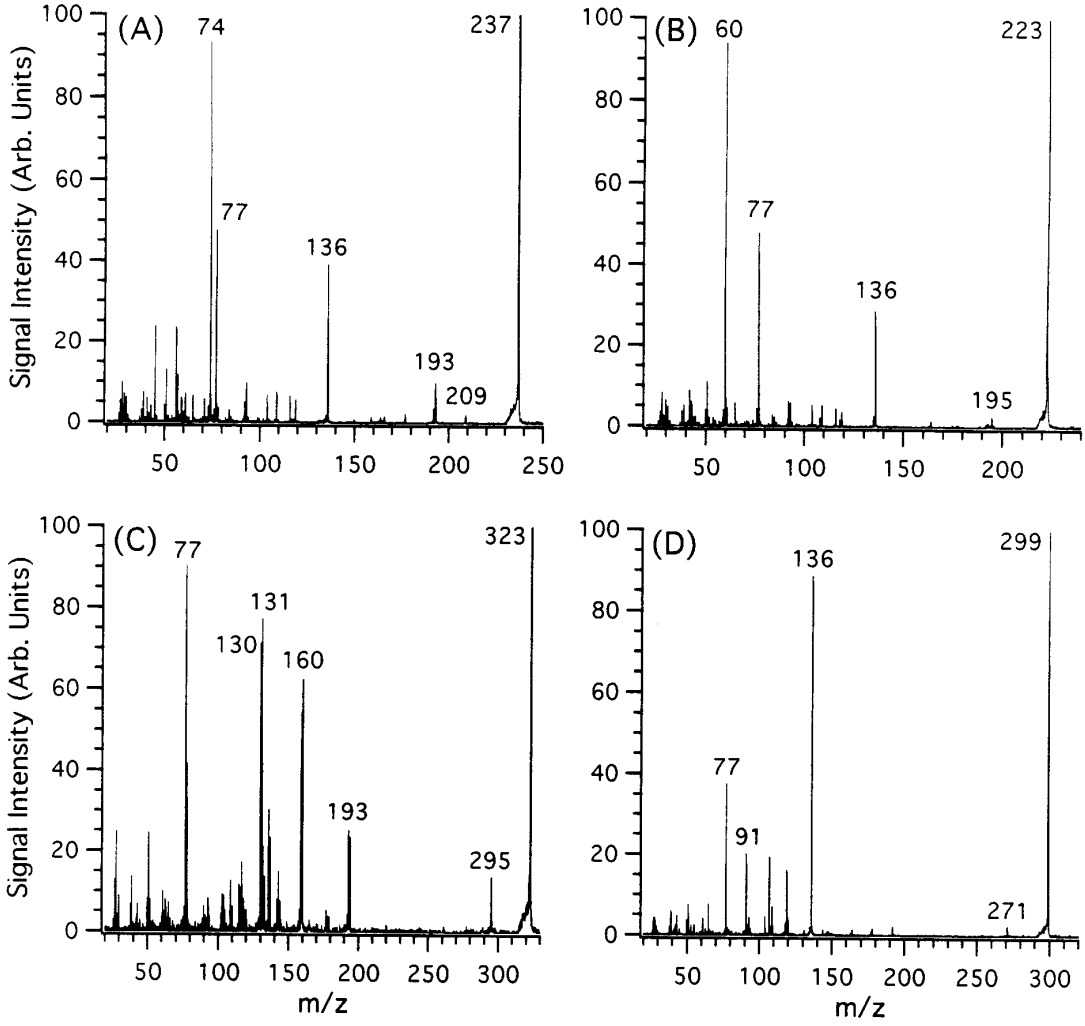
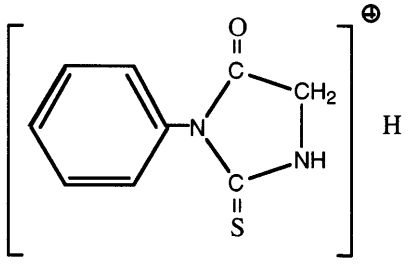


Figure 3. High-energy CID mass spectra of the thiazalone ions derived from (A) PTC-Thr-Leu, (B) PTC-Ser-Leu, (C) PTC-Trp-Leu and (D) PTC-Tyr-Leu.

pathways of these ions. If the loss of CS from the phenylhydantoin ion derived from PTH-Thr took place, it would result in an ion with significantly different structure from that produced by the side-chain elimination from the phenylhydantoin moiety. From the results shown in Fig. 4, we can conclude that the $[b_1 - 44]^+$ peak from PTH-Thr is not from the loss of CS from the phenylhydantoin ion.

As was indicated above, there is hydrogen migration from the R group to the charge-retaining moiety, yielding the *m/z* 193 ion, with the rest of the R group leaving the ion as a neutral species. At first, we thought the structure of the ion at *m/z* 193 derived from PTH-X is identical with that of the ion of the protonated PTH-Gly, i.e.



Indeed, this structure of the *m/z* 193 ion was proposed previously by others in the analysis of PTH derivatives by chemical ionization.[5] If this is the case, the CID product ion spectrum of the ion at *m/z* 193 derived from PTH-X should be the same as the CID spectrum of PTH-Gly. Figure 5 shows high-energy CID mass spectrum of the protonated PTH-Gly. This spectrum is different from those shown in Fig. 4. The spectrum of protonated PTH-Gly shows three prominent fragmentation ions, namely phenyl isothiocyanate at *m/z* 136, phenyl ion at *m/z* 77 and glycine immonium ion at *m/z* 30. By contrast, the spectrum of the *m/z* 193 ion derived from PTH-X shows an intense *m/z* 28 ion peak with a much less intense glycine immonium ion peak. Another major spectral difference is that, in Fig. 4, two product ions at *m/z* 175 and 160 are observed whereas the spectrum shown in Fig. 5 from the protonated PTH-Gly does not display these peaks. It is obvious that the fragmentation pathways of the ions at *m/z* 193 derived from PTH-X is not entirely the same as that of PTH-Gly.

We propose that the fragmentation of the *m/z* 193 ion derived from PTH-X follows the scheme shown in Fig. 6 (PTH-Ser is shown as an example). In the process of generating the *m/z* 193 ion from PTH-X, the elimination of the neutral species from the side-chain group is accompanied by hydrogen transfer from the R group to

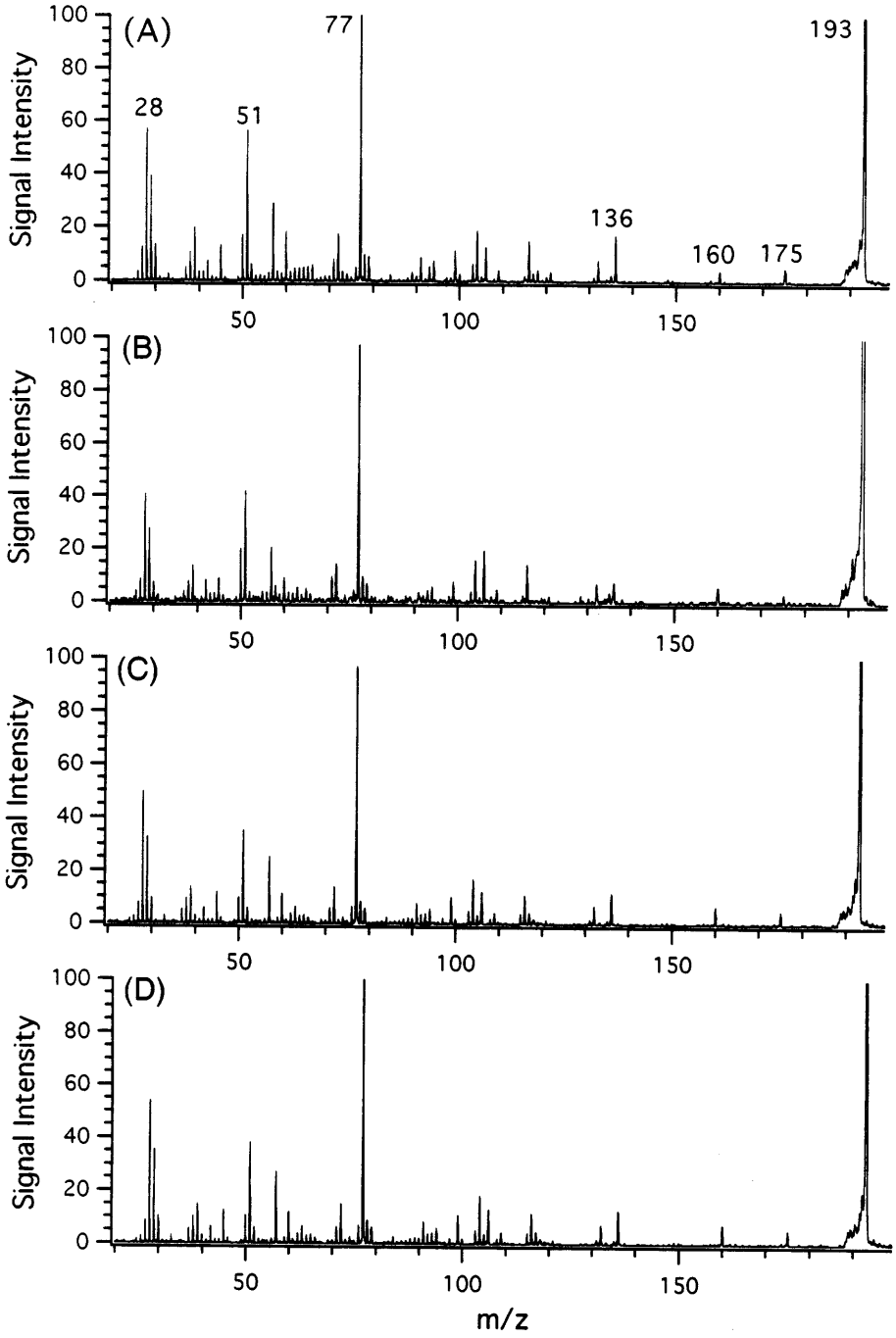


Figure 4. High-energy CID mass spectra of the *m/z* 193 ions derived from (A) PTH-Thr, (B) PTH-Ser, (C) PTH-Trp and (D) PTH-Tyr.

the charge-retaining moiety, yielding the *m/z* 193 ion. The proposed structures of the neutral species eliminated from PTH-X are shown in Fig. 7. We propose that hydrogen transfer takes place via hydrogen bonding with the carbonyl oxygen in the phenylhydantoin moiety. As shown in Fig. 6, water is subsequently eliminated to form the ion at *m/z* 175. This process is driven by the migration of one of the labile hydrogens to the hydroxy group in the *m/z* 193 ion. The fragmentation pathway for the generation of the *m/z* 160 ion is unclear. It may be formed by the elimination of SH from the *m/z* 193 ion.

Figure 8 shows the proposed fragmentation mechanism for the *m/z* 193 ion corresponding to protonated PTH-Gly. Depending on the location of the proton in the phenylhydantoin moiety, two dissociation paths are proposed. If the protonation occurs at phenylated amide nitrogen, the reaction follows path 1. If the protonation occurs at the other amide nitrogen, the reaction follows path 2. This scheme accounts for the production of the major ions at *m/z* 136 and 30. In the case of the protonated PTH-Gly, the dissociation path of water elimination shown in Fig. 6 for PTH-X is not present. Hence this fragment ion was not detected in Fig. 5.

The formation of the common ion at *m/z* 193 from PTH-X along with the product ion spectrum of this ion (Fig. 4) provides an ideal diagnostic tool on the pres-

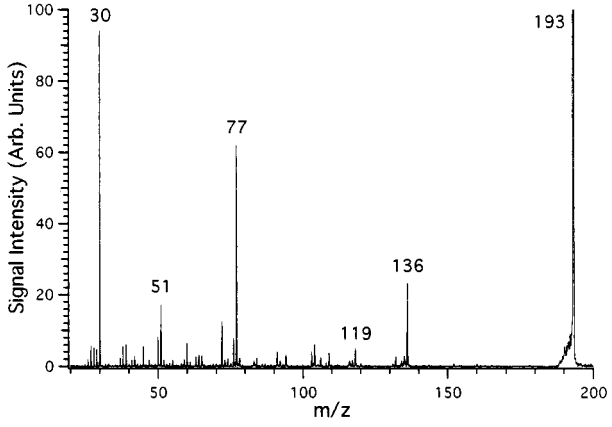


Figure 5. High-energy CID mass spectra of the *m/z* 193 ion derived from PTH-Gly.

ence of the protonated PTH derivatives in the gas phase. Examining the spectra shown in Fig. 3 from PTC-X-Leu, it can be seen that no ion peak at *m/z* 193 is detected in the product ion spectra of the b_1 ions derived from PTC-Tyr-Leu and PTC-Ser-Leu. Hence there is no isomerization of thiazolone to phenylhydantoin for the b_1 ions of these two PTC derivatives. In the cases of PTC-Thr-Leu and PTC-Trp-Leu, an ion peak at *m/z* 193 is observed. We examined the fragmentation patterns of the *m/z* 193 ions originating from these thiazolone ions. Figure 9 shows the product ion spectra of the *m/z* 193 ions derived from PTC-Thr-Leu, PTC-Trp-Leu and PTC-Gly-Leu. As Fig. 9(A) and (B) illustrate, common product ions are observed for the fragmentation of the thiazolone ions derived from PTC-Thr-Leu and PTC-Trp-Leu. These ions include the phenyl isothiocyanate ion at *m/z* 136 and the phenyl ion at *m/z* 77. However, these spectra are different from those shown in Fig. 4. In Fig. 9(A) and (B), peaks corresponding to the ions from H_2O elimination are not observed. In contrast, H_2O elimination was observed for the *m/z* 193 ion originating from PTH-X (Fig. 4). This implies that the fragmentation pathways of the *m/z* 193 ions originating from PTC-Thr-Leu and PTC-Trp-Leu are different from those of the *m/z* 193 ions originating from PTH derivatives. Hence isomerization of thiazolone to phenylhydantoin for the b_1 ions of these two PTC derivatives is also not detected.

The spectrum shown in Fig. 9(C) from PTC-Gly-Leu is different from those shown in Fig. 9(A) and (B). It resembles the spectrum shown in Fig. 5 for the *m/z* 193 ion from PTH-Gly with the exception that the product ion spectrum from PTC-Gly-Leu contains the *m/z* 165 peak which is probably from CO elimination of the *m/z* 193 ion.

By analogy with the proposed mechanism of fragmentation for PTH-X, Fig. 10 shows the formation and fragmentation mechanism of the *m/z* 193 ion derived from PTC-Thr-Leu and PTC-Trp-Leu. We propose that a thiazolone ion/neutral complex is formed,[13] followed by the *C*-terminal group leaving the complex as a neutral species, resulting in a cyclic ion like the b ions.[12] A hydrogen migration from the side-chain group takes place during the formation of the *m/z* 193 ion. Figure 11 shows the proposed structure and fragmentation mechanism of the *m/z* 193 ion derived from PTC-Gly-Leu.

CONCLUSIONS

The fragmentation pathways of the protonated PTH-X (where X = Thr, Ser, Trp and Tyr), PTH-Gly, PTC-X-Leu and PTC-Gly-Leu generated by ESI have been examined. The collisionally activated product ion spectra of the *m/z* 193 ions from PTH-X are found to be the same, suggesting they have the same structure. However, the product ion spectrum of the *m/z* 193 ion from PTH-Gly is different from those of PTH-X. It is shown that the source-formed ion at *m/z* 193 derived from PTH-X is from the side-chain elimination of the R group with a hydrogen migration from the R group to the phenylhydantoin moiety. The hydrogen migration is proposed to be via a hydrogen bond with the carbonyl group in the phenylhydantoin moiety. Hence the structure of the *m/z* 193 ions from PTH-X is different from that from PTH-Gly. This gives the rationale for the observed differences in product ion spectra of the *m/z* 193 ions from PTH-X and PTH-Gly. We did not observe CS elimination from the thiazolone ion. The $[b_1 - 44]^+$ peak in the product ion spectrum of the protonated PTH-Thr is from the loss of a neutral species (CH_3CHO) by side-chain elimination, rather than CS elimination. Since a common ion at *m/z* 193 is generated from PTH-X, it is argued that this ion along with its CID spectrum can be used to examine whether there is any phenylthiohydantoin ion present in the gas phase. For PTC-X-Leu, only PTC-Thr-Leu and PTC-Trp-Leu give rise a peak at *m/z* 193 in the source-formed fragment ion spectra. Examination of the CID spectra of the *m/z* 193 ions derived from these two derivatives reveals that these ions have different fragmentation pathways from that of the *m/z* 193 ion from PTH-X. This illustrates that there is no isomerization of thiazolone and phenylthiohydantoin in the gas phase. Furthermore, the fragmentation pathways of these ions are different from that of the *m/z* 193 ion derived from PTC-Gly-Leu. The proposed fragmentation mechanisms of these PTH and PTC derivatives and the source-formed *m/z* 193 ions from these derivatives are given.

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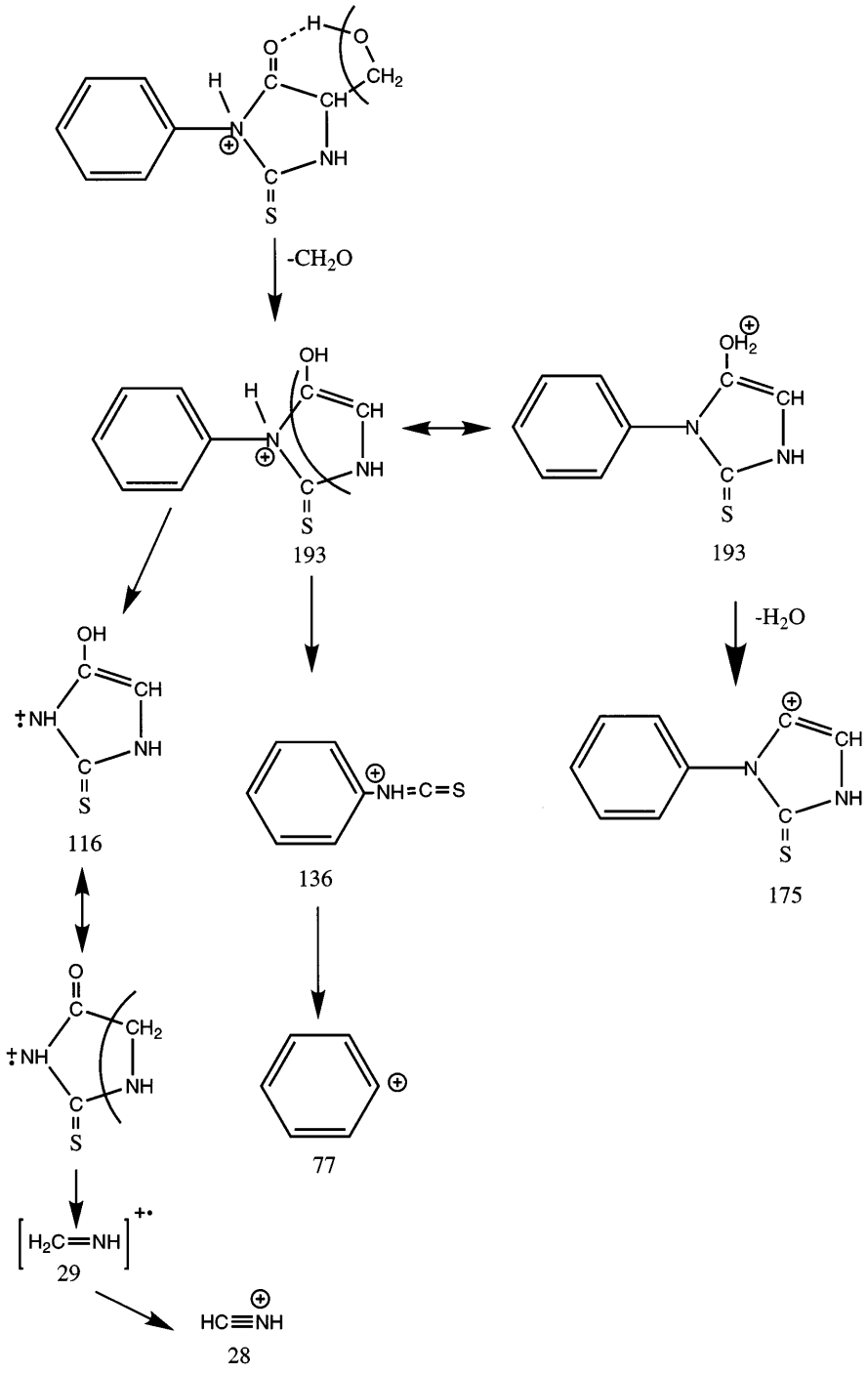

Figure 6. Proposed mechanism of fragmentation of the *m/z* 193 ions derived from PTH-X using PTH-Ser as an example.

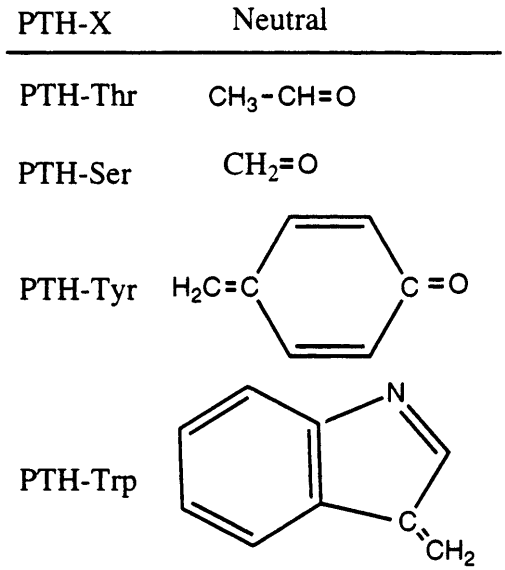

Figure 7. Structures of neutral species eliminated from the side-chains of X during the formation of the *m/z* 193 ion from PTH-X.

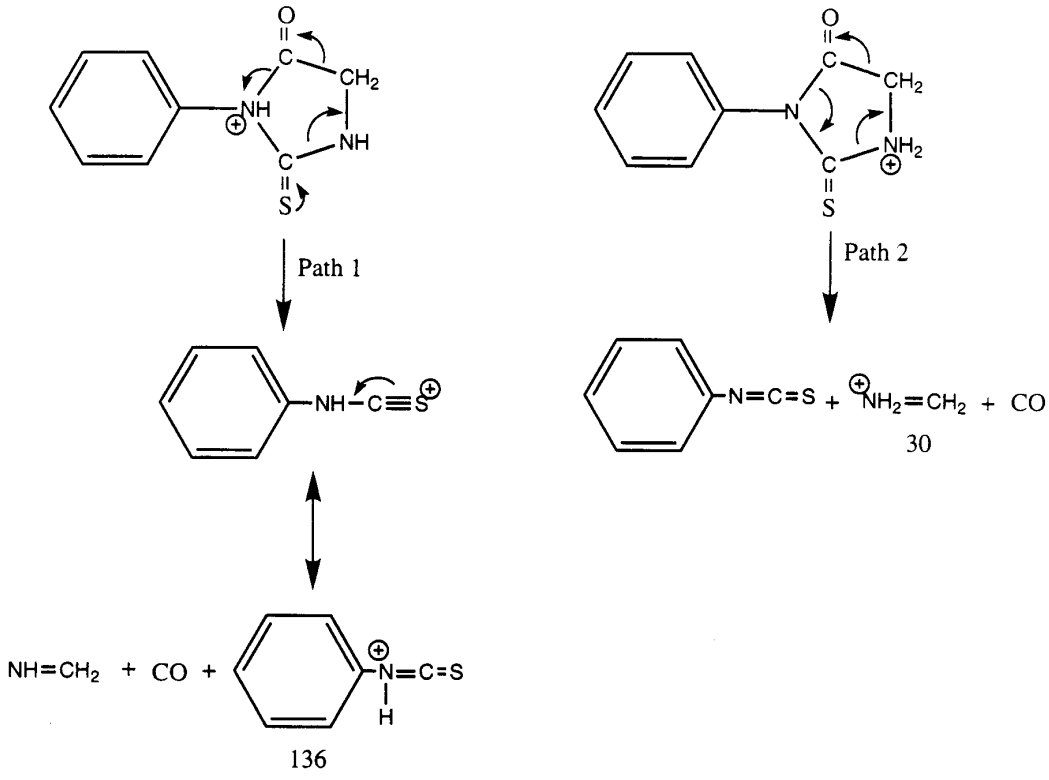

Figure 8. Proposed mechanism of fragmentation of the *m/z* 193 ion derived from PTH-Gly.

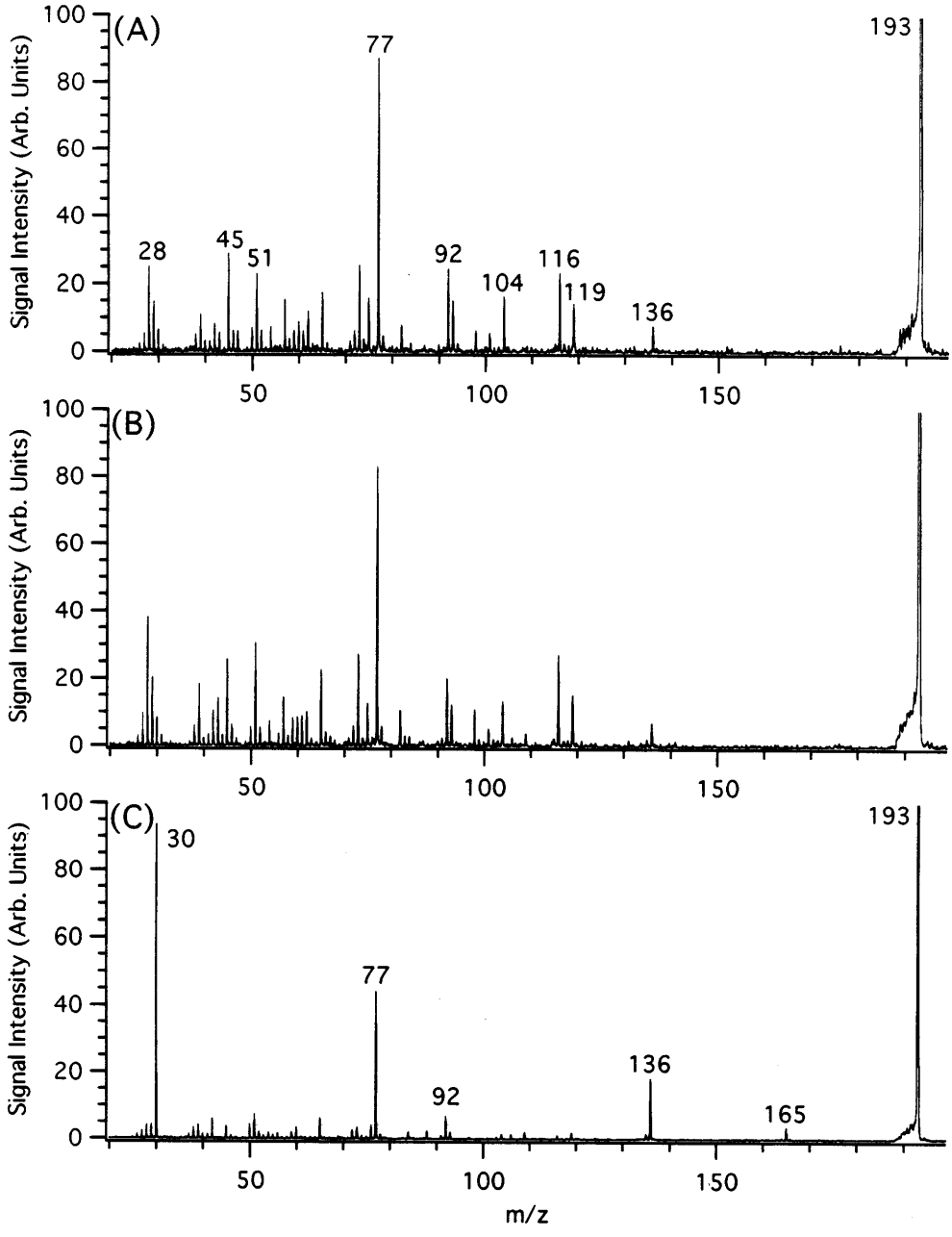

Figure 9. High-energy CID mass spectra of the *m/z* 193 ions derived from (A) PTC-Thr-Leu, (B) PTC-Trp-Leu and (C) PTC-Gly-Leu.

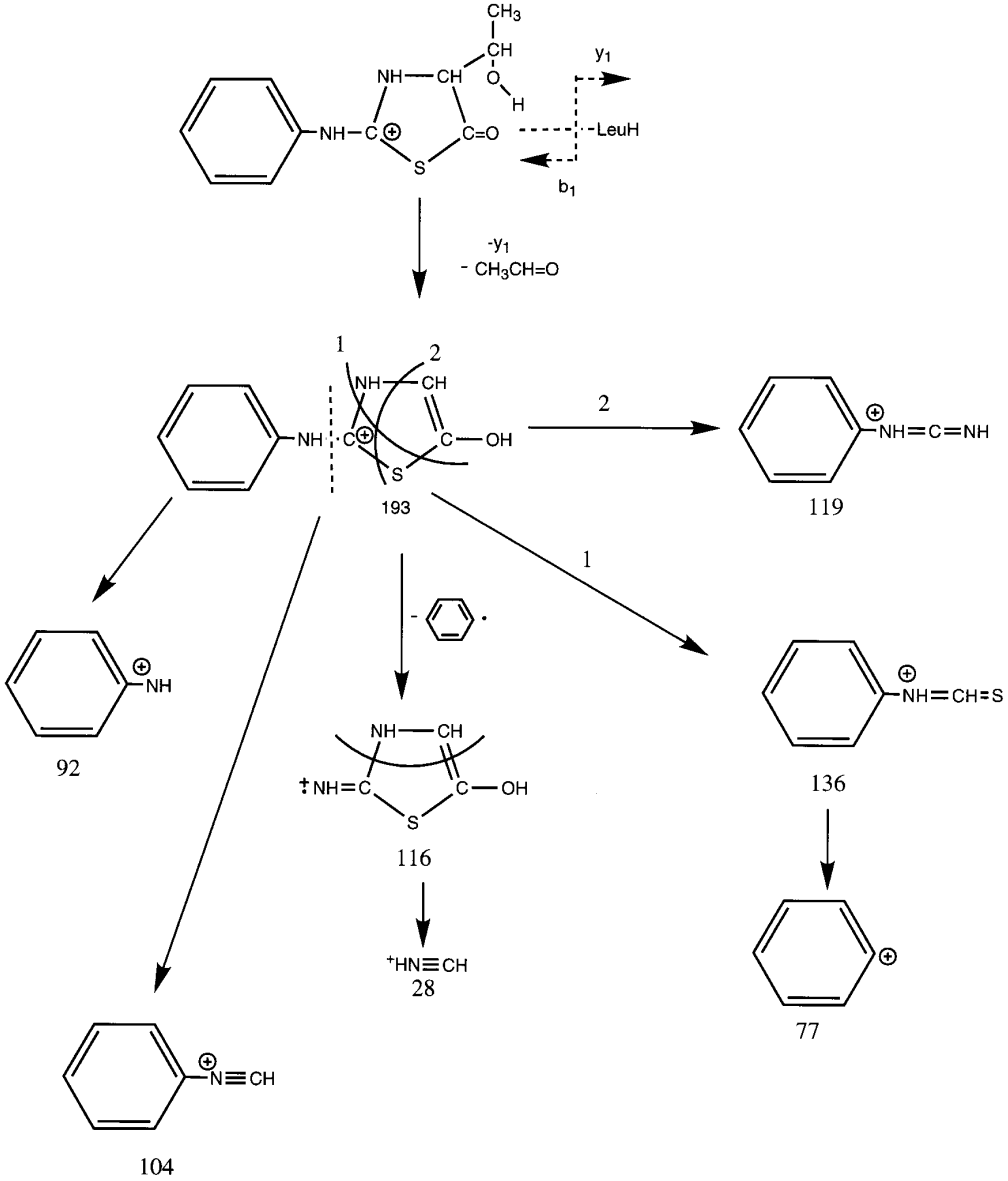

Figure 10. Proposed mechanism of fragmentation of the *m/z* 193 ions derived from PTC-Thr-Leu and PTC-Trp-Leu using PTC-Thr-Leu as an example.

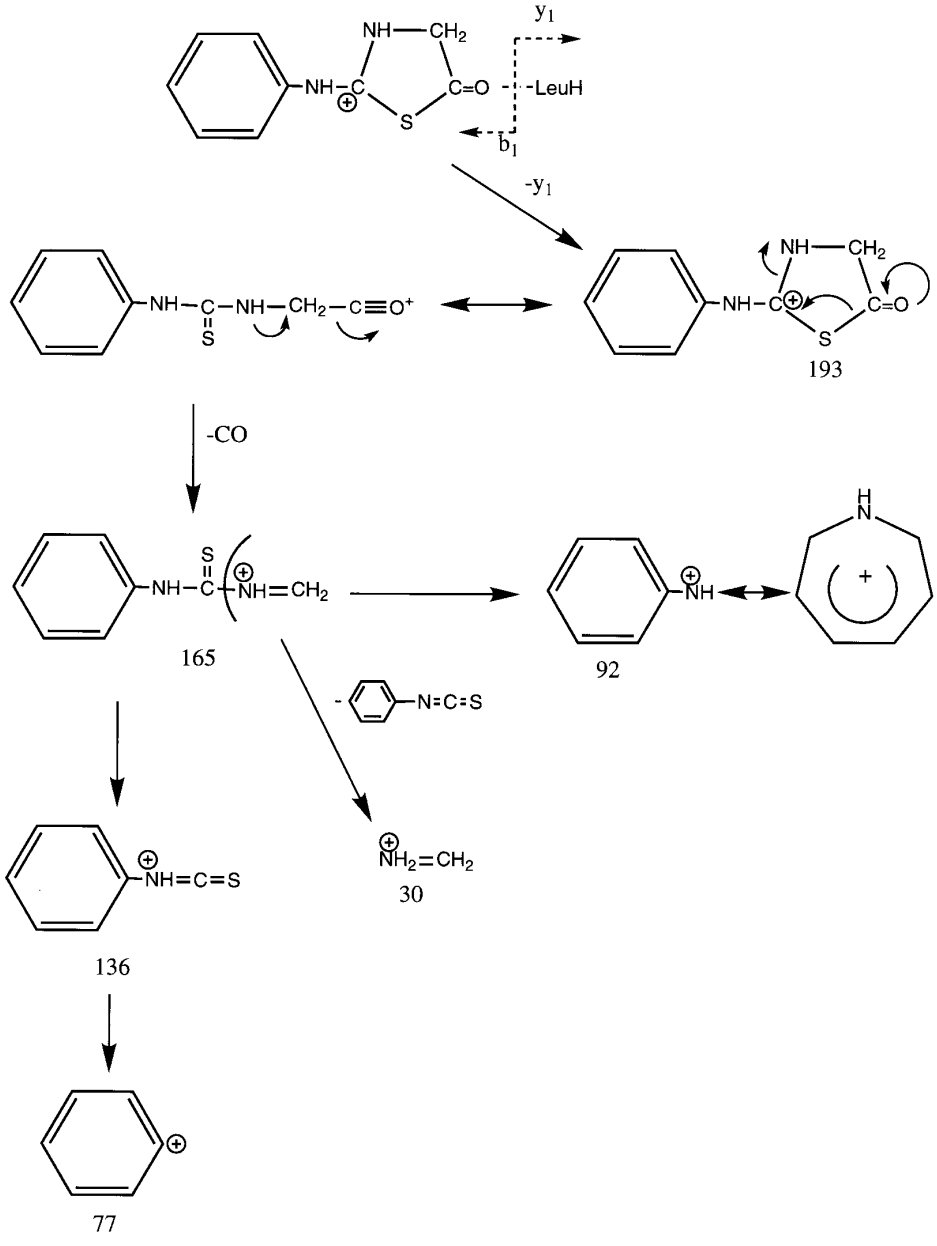

Figure 11. Proposed mechanism of fragmentation of the *m/z* 193 ion derived from PTC-Gly-Leu.

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