# Promotion and Stabilization of $b_1$ ions in Peptide Phenythiocarbamoyl Derivatives: Analogies with Condensed-phase Chemistry

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**The preparation of the *N*-terminal phenylthiocarbamoyl (PTC) derivative is the first step in the condensed phase chemistry employed in the Edman method for peptide sequencing; subsequent treatment with anhydrous acid effects cleavage of the *N*-terminal peptide bond yielding a derivatized amino acid and a truncated peptide. Low-energy collisional activation of peptide PTC derivative $[M + 2H]^{2+}$ ions during electrospray tandem mass spectrometry results in highly favoured cleavage of the *N*-terminal peptide bond yielding complementary $b_1$ and $y_{n-1}$ fragments. The cleavage is evidently promoted by protonation of the peptide backbone. The apparently close mechanistic similarity between the gas-phase and condensed-phase processes may be readily understood in terms of current thinking concerning the mechanism of formation of b-type ions, which involves nucleophilic attack by an *N*-terminal carbonyl moiety on the carbonyl carbon of the first peptide bond. Collisionally activated decomposition of source-formed $b_1$ ions from a peptide PTC derivative is consistent with ion rearrangement similar to the PTC–phenylthiohydantoin isomerization observed in the condensed phase.** **© 1997 by John Wiley & Sons, Ltd.**

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## INTRODUCTION

Routine sequencing of peptides continues to rely heavily on the application of well understood Edman chemistry in which formation of an *N*-terminal thiocarbamoyl derivative is followed by acid-catalysed degradation to yield a thiazolone derivative of the *N*-terminal residue together with a truncated peptide.[1] Tandem mass spectrometry (MS/MS) represents an alternative approach to peptide sequence analysis that is particularly useful for the characterization of modified structures. The application of MS/MS to peptide sequencing is based on the presence of both *N*- and *C*-terminal fragment ions, the nomenclature of which is usually based on the suggestions of Biemann[2] and of Roepstorff and Fohlman.[3] Early studies of the formation of b ions assumed protonation of the amide nitrogen and inductive cleavage of the amide bond to yield an acylium ion structure.[4] This mechanism does not, however, explain observations such as the promotion of b ions at acidic residues[5,6] and the low yield of $b_n$ ions to the *C*-terminal side of proline.[7] Most critically, the mechanism does not account for the common lack of $b_1$ ions when higher members of the b series are abundant. Recent extensive studies by Harrison and co-workers[8,9] have provided a satisfactory rationale for these observations based on a proposed protonated oxazolone structure for b-type ions. The mechanistic proposals are essentially similar to those of Hunt and co-workers,[7] differing only in that the latter authors propose initial protonation of the amide oxygen rather than the amide nitrogen, as suggested by Harrison and co-workers.[8,9] Current thinking on the mechanism of formation of b and y ions is summarized in Fig. 1. It is apparent that these proposals readily explain the observation of relatively facile cleavage of the first peptide bond (from the *N*-terminus) after *N*-terminal acetylation, a process which introduces an amide function to mimic the presence of a new *N*-terminal amino acid residue. Similarly, the Harrison/Hunt mechanism may explain the promotion of peptide bond cleavage *C*-terminal to an acidic amino acid residue where nucleophilic attack is initiated from the side-chain. Stereochemical factors disfavor the formation of an oxazolone structure *C*-terminal to a proline residue.[7]

The proposed oxazolone structure of the b-type ion is analogous to the substituted thiazolone product of the degradation of a peptide *N*-terminal phenylthiocarbamoyl (PTC) derivative in anhydrous acid during the Edman sequencing procedure.[1] The work reported here explores this apparent parallel between gas- and condensed-phase chemistry by examining the collisionally activated decomposition of peptide *N*-terminal PTC derivatives.

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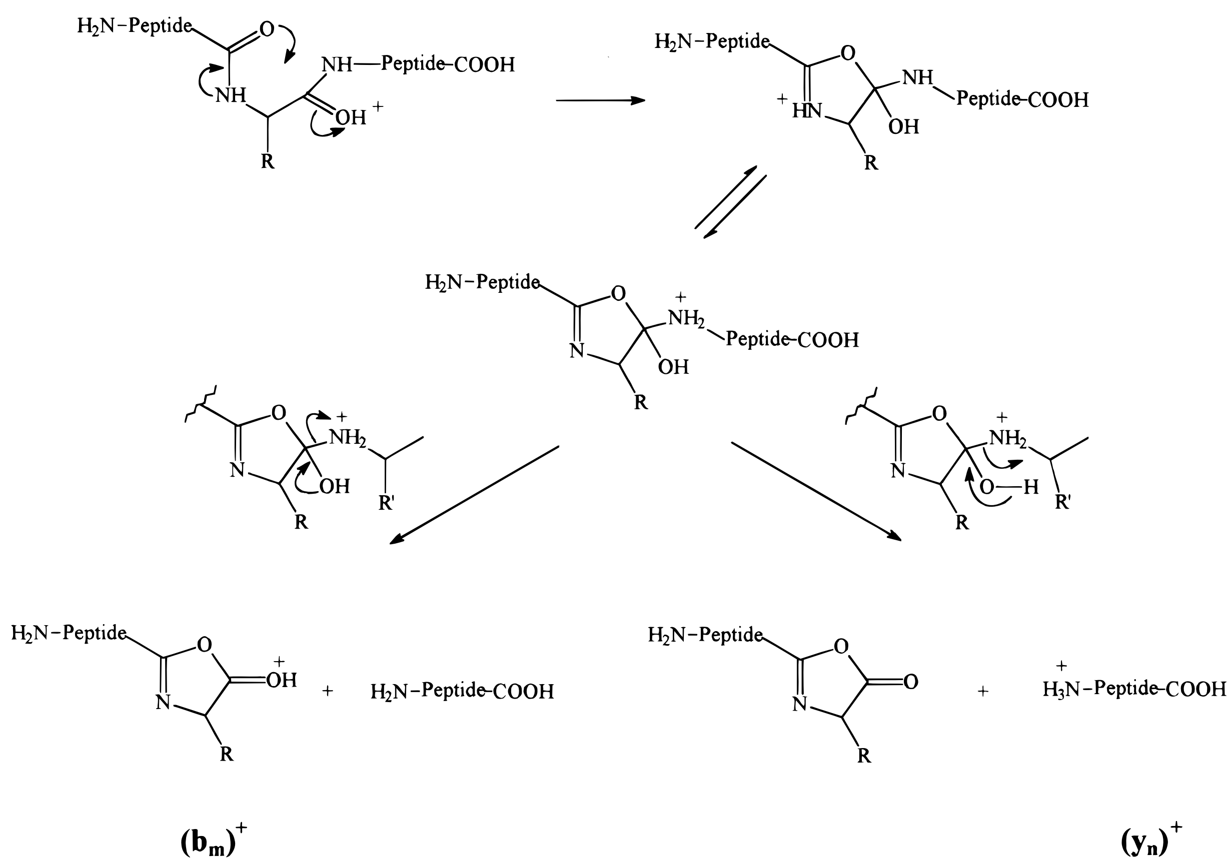


**Figure 1.** Mechanism of formation of peptide $b_n$ ions yielding a protonated oxazolone structure, as proposed by Harrison and co-workers[8,9] and Hunt and co-workers.[7]

## EXPERIMENTAL

### Sources of peptides

The 40-residue peptide urocortin was obtained from Peninsula Laboratories (Belmont, CA, USA). Other synthetic peptides and trypsin were purchased from Sigma (Poole, UK). Selective cleavage of urocortin was performed by digestion with trypsin in Tris buffer (0.1 M, pH 8.5) for 2 h at ambient temperature, using an enzyme-to-substrate ratio of 1:50. The reaction was quenched by addition of glacial acetic acid. The TLLELAR fragment was separated from the resultant mixture of peptides by reversed-phase high-performance liquid chromatography (HPLC) using a model 600 pump and controller (Waters, Milford, MA, USA) and a $C_{18}$ column (150 × 4.6 mm i.d.) (YMC., Morris Plains, NJ, USA). A linear gradient was formed from 70% water (0.1% trifluoroacetic acid)–30% acetonitrile (0.1% trifluoroacetic acid) to 10% water (0.1% trifluoroacetic acid)–90% acetonitrile (0.1% trifluoroacetic acid) in 30 min. The flow rate was 1.5 ml $min^{-1}$.

### Preparation of PTC derivatives[1]

Derivatization was performed by dissolving 100 μg of the dry peptide in 200 μl of a solution (5%, w/v) of phenyl isothiocyanate in ethanol–water–pyridine (1:1:1, v/v/v). Reaction was allowed to proceed for 30 min at 45 °C, after which the solvent was removed under a stream of nitrogen. The product was dissolved in 70% water–30% acetonitrile (200 μl) containing 0.1% trifluoroacetic acid and purified by reversed-phase HPLC using the conditions described above.

### Mass spectrometry

Low-energy collisionally activated dissociation (CAD) experiments were performed using a Quattro tandem quadrupole instrument (Micromass (formerly VG Organic), Manchester, UK) equipped with an electrospray interface and upgraded to Quattro II specifications. The peptide solution (10 pmol $\mu l^{-1}$ in 50:50 acetonitrile–water acidified by 0.1% formic acid) was introduced at a flow rate of 3–5 $\mu l\ min^{-1}$ using a syringe driver (Harvard Apparatus, South Natick, MA, USA). Collision cell voltages were 10–30 V; specific values are given at appropriate points in the text. Argon was the collision gas at a recorded pressure of (1–2) $\times 10^{-3}$ mbar (1 bar = $10^5$ Pa). Fragmentation was induced in the electrospray interface by increasing the cone potential to 50–60 V; these conditions were employed during MS/MS analyses of source-formed fragment ions. Data acquisition and processing were controlled via the MassLynx data system. The tandem mass spectra reported represent sums of 20–30 scans with a typical acquisition time of ~3–5 min.

## RESULTS AND DISCUSSION

Figure 2(a) shows the product ion spectrum recorded following low-energy collisonal activation of the

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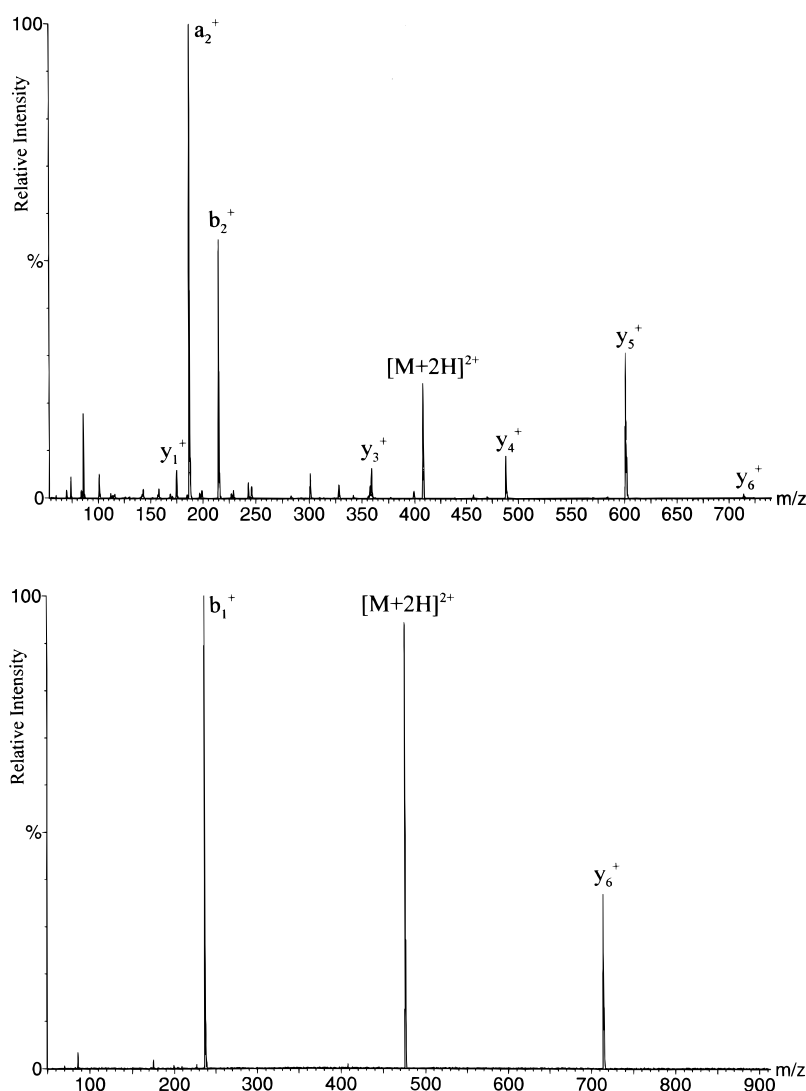


**Figure 2.** Product ion spectra recorded following low-energy CAD of $[M + 2H]^{2+}$ ions of (a) the peptide TLLELAR and (b) its *N*-terminal PTC derivative. (For clarity, the conventional nomenclature for the product ions is supplemented, in this and subsequent figures, by an indication of the charge state.)

$[M + 2H]^{2+}$ ion of the peptide TLLELAR. A high efficiency of decomposition is apparent with the formation of both *N*- and *C*-terminal ions. (Product ions are designated according to the nomenclature of Biemann.[2]) Both qualitative and quantitative features of the spectrum are readily explained in terms of the predicted locations of the ionizing protons in the $[M + 2H]^{2+}$ precursor ions.[10] Thus, the *C*-terminal arginine residue is expected to sequester a single proton with the second ionizing proton available to promote peptide backbone cleavage via charge-proximal fragmentations. Cleavages of peptide bonds may yield complementary b- and y-series fragments, the stabilities of which will in turn be determined by the extent of localization of the ionizing proton. Thus, for TLLELAR, the presence of the *C*-terminal arginine residue in y-series fragments leads to a strongly localized site of charge with consequent stability to further fragmentation. Accordingly, higher members of the y-series are abundant (Fig. 2(a)). In contrast, b-series ions will incorporate a 'mobile' proton and further fragmentation is expected; product ion current in the b-series is concentrated in the $b_2$ ion (Fig. 2(a)). As noted in the Introduction, detection of the $b_1$ ion is not expected, in view of the lack of a carbonyl group to initiate nucleophilic attack on the carbonyl carbon of the peptide bond between the first two amino acid residues.[7–9]

The product ion spectrum arising from low-energy collisional activation of the $[M + 2H]^{2+}$ ion of the PTC derivative of TLLELAR (Fig. 2(b)) is strikingly different from that of the underivatized analogue (Fig. 2(a)). A single fragmentation process is apparent, giving rise to complementary singly charged $b_1$ and $y_6$ product ions. During low-energy collisional activation over a range of laboratory collision energies (4–22 eV), fragmentation was observed to occur exclusively by cleavage of the *N*-terminal peptide bond. Furthermore, conditions promoting fragmentation in the electrospray interface also highly favoured the production of these two complementary ions.

Figure 3 shows the low-energy product ion spectrum of the $[M + H]^+$ ion of the PTC derivative of TLELLAR under collision conditions identical with those employed for the corresponding doubly charged

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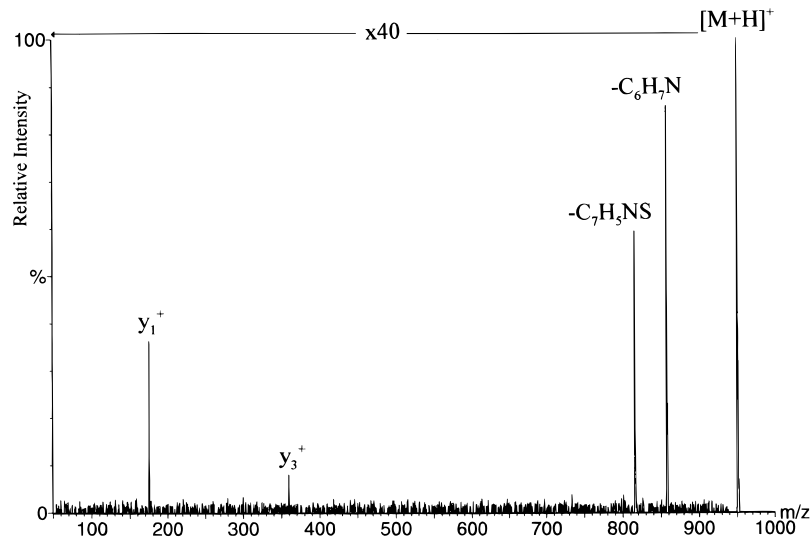


**Figure 3.** Product ion spectrum recorded following low-energy CAD of $[M+H]^+$ ions of the *N*-terminal PTC derivative of the peptide TLLELAR.

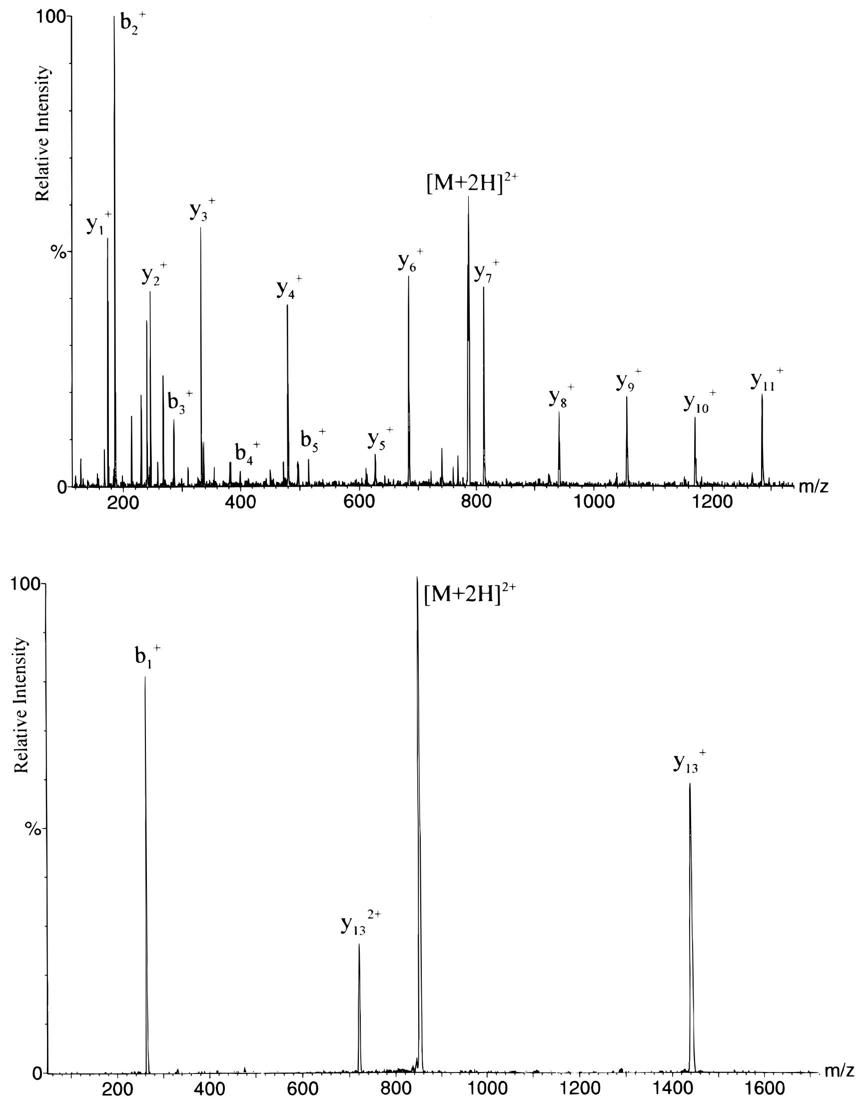


**Figure 4.** Product ion spectra recorded following low-energy CAD of $[M+2H]^{2+}$ ions of (a) the peptide EGVNDNEEGFFSAR and (b) its *N*-terminal PTC derivative.

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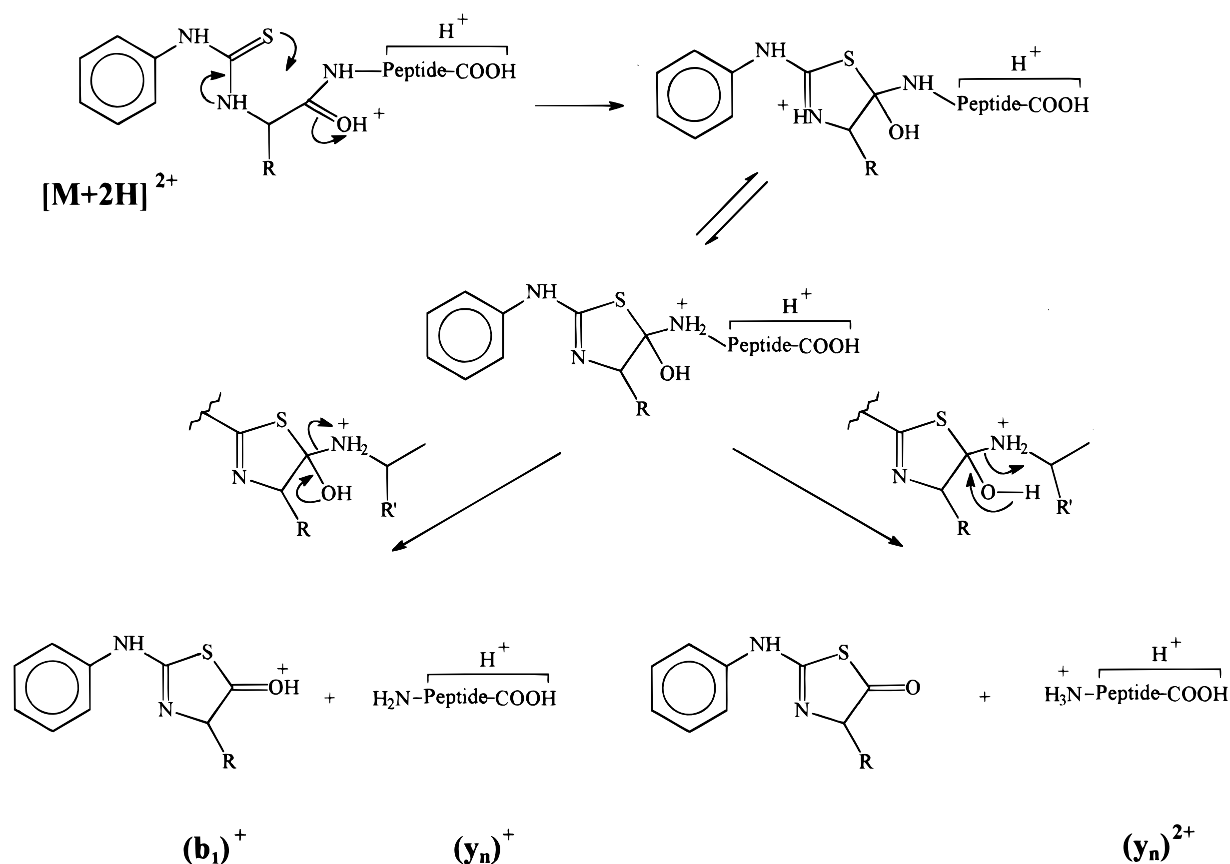


Figure 5. Proposed mechanism of fragmentation of $[M + 2H]^{2+}$ ions of peptide PTC derivatives to yield $b_1$ and $y_n$ product ions.

precursor ($E_{lab}$ = 8 eV). The decomposition efficiency of the singly charged ion is low; this is attributable to the preferential location of the single ionizing proton on the arginine side-chain. The product ions observed (albeit at low abundance) include $y_1$ and high-mass fragments corresponding to $[M + H - 93]^+$ and $[M + H - 135]^+$. The high-mass fragments may be attributed to loss of aniline ($C_6H_7N$) or phenyl isothiocyanate ($C_7H_5NS$) from the derivative group of the precursor ion. At higher collision energies further fragmentation channels are opened but without a significant yield of $b_1$ (data not shown). Thus, we conclude that promotion of the formation of the $b_1$ and complementary $y_8$ product ions from TLELLAR requires protonation of the peptide backbone.

The fragmentation properties of the $[M + 2H]^{2+}$ ion of the PTC derivative of TLLELAR are not peculiar to this peptide. As a further example, Fig. 4 compares the

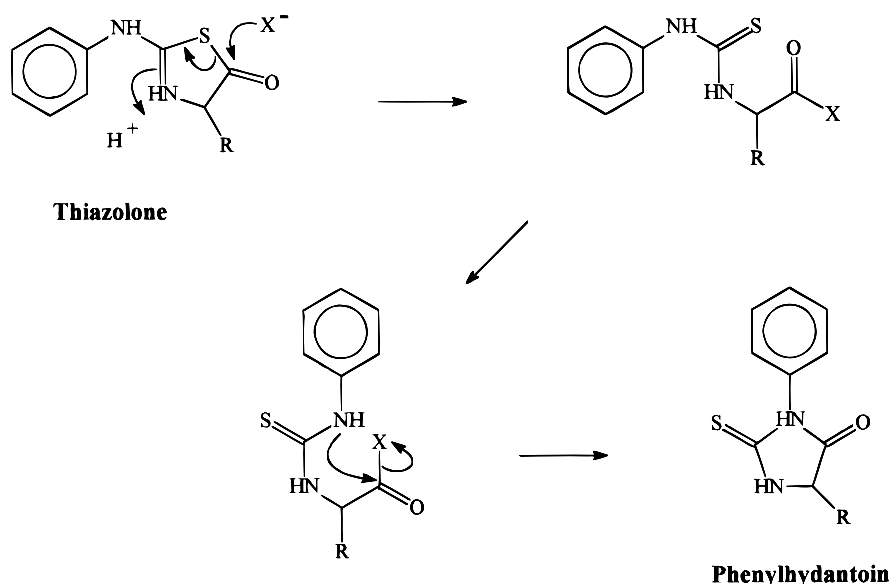


Figure 6. Condensed phase, acid-catalysed rearrangement of peptide PTC derivatives to yield phenylhydantoin structures.

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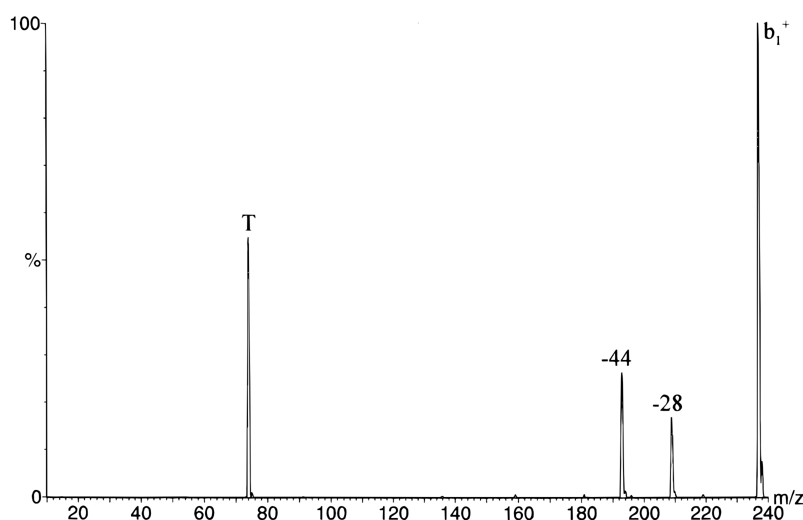


**Figure 7.** Product ion spectrum recorded following low-energy CAD (in the collision hexapole) of electrospray ion source-formed $b_1$ ions from the *N*-terminal PTC derivative of the peptide TLLELAR.

product ion spectra for the $[M + 2H]^{2+}$ ions of the native peptide and the PTC derivative of EGVNDNEEGFFSAR (Glu-fibrinopeptide). In the fragmentation of the underivatized peptide (Fig. 4(a)) the influence of the *C*-terminal arginine is again apparent in promoting a series of stable y ions. In contrast, only the lower members of the b-series are apparent, reflecting the secondary fragmentation of higher members due to the location of the ionizing proton on the peptide backbone. Once again, $b_2$ is prominent and $b_1$ is of negligible abundance. In marked contrast, the fragmentation of the PTC derivative (Fig. 4(b)) shows cleavage solely of the peptide bond between the first two residues. Concomitant $b_1$ and $y_{13}$ ions are abundant, together with the doubly charged $y_{13}$ ion (for which the concomitant is, of course, a neutral *N*-terminal fragment).

The profound influence of the PTC derivative group on the fragmentation of the doubly protonated peptide may be explained by a mechanism analogous to that now accepted for b-ion formation (Fig. 1). Thus, we propose that cleavage of the first peptide bond is promoted by nucleophilic attack of the thiocarbonyl

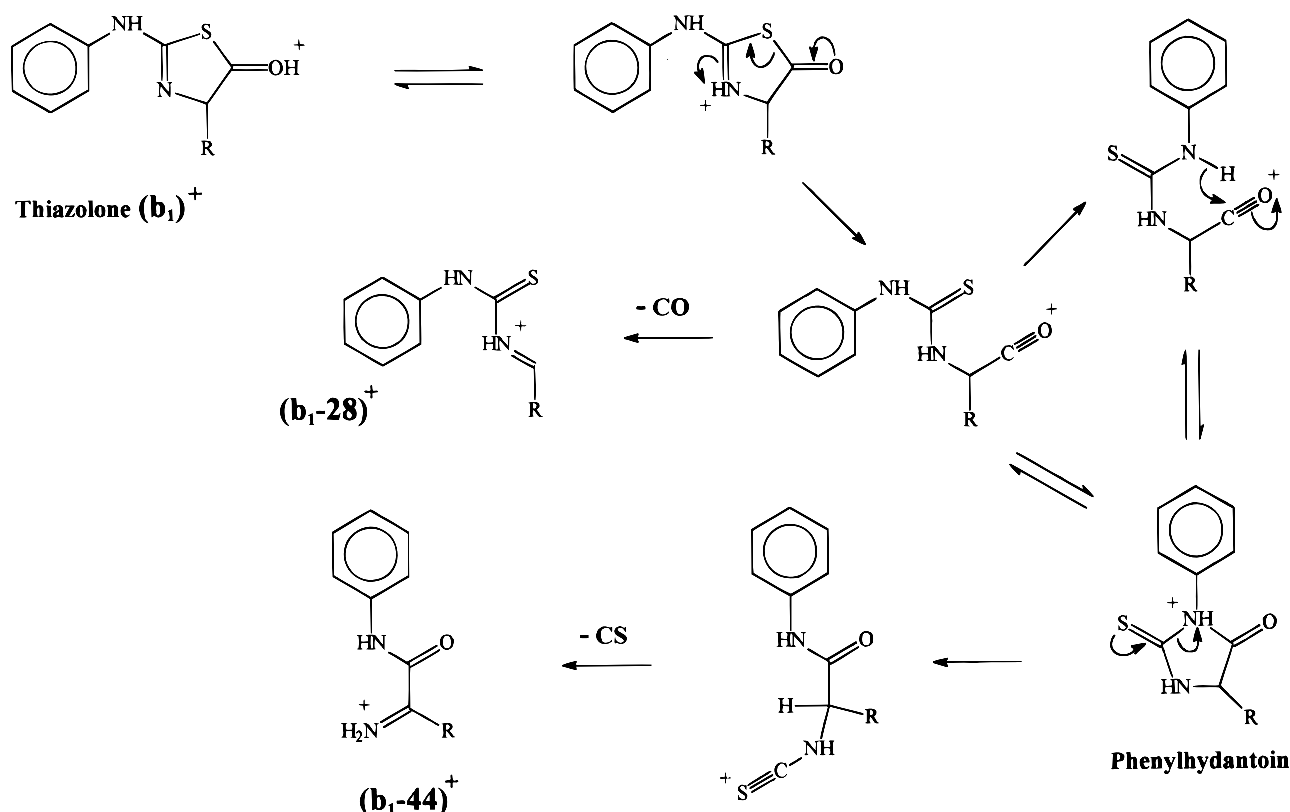


**Figure 8.** Proposed mechanism of fragmentation of $b_1$ ions derived from peptide PTC derivatives.

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moiety on the protonated carbonyl of the peptide bond (Fig. 5). The particular favouring of this fragmentation is attributable to the stronger nucleophilic character of the thiocarbonyl group, in comparison with carbonyl groups available *N*-terminal to other peptide bonds whose cleavage would give rise to higher members of the b-series. Evidently, nucleophilic attack of the thiocarbonyl group is limited to the closest peptide bond, forming a five-membered thiazolone structure. This finding parallels the conclusion of Harrison and coworkers[9] that the formation of $b_n$ ions, where $n > 2$, involves interactions of the charge site and the closest (on the *N*-terminal side) carbonyl group.

The proposed mechanism (Fig. 5) for formation of $b_1$ ions from $[M + 2H]^{2+}$ ions of peptide PTC derivatives closely resembles that accepted for the condensed phase cleavage, in anhydrous acid, of the *N*-terminal amino acid residue of a peptide PTC derivative during Edman sequencing, yielding a thiazolone product and the *N*-terminally truncated peptide.[1] In the condensed phase, subsequent acid-catalysed rearrangement of the thiazolone yields a phenylthiohydantoin derivative (Fig. 6). The parallels between the gas- and condensed-phase chemistries were therefore further investigated by recording the product ion spectrum following collisional activation of the $b_1$ ion formed in the electrospray source region (and promoted by suitable choice of potential difference between the cone and skimmer lenses) during analysis of TLLELAR. The spectrum (Fig. 7) shows three prominent fragmentation pathways, corresponding to losses of CO and CS, and formation of the threonine immonium ion. Loss of CS, in particular, may most readily be rationalized as occurring via formation of a protonated thiohydantoin structure (Fig. 8), suggesting that this form is present in the population of first generation product ions. The thermospray mass spectrum of the PTH derivative of threonine has been reported to include a fragment ion derived by loss of 44 Da from the $[M + H]^+$ ion.[11]

## CONCLUSIONS

Generally $b_1$ ions are not observed in the fragmentation of protonated underivatized peptides. Conversion of a peptide to the *N*-terminal acetyl derivative promotes cleavage of the bond between the first two amino acid residues, consistent with nucleophilic attack by the carbonyl moiety of the derivative group on the protonated backbone amide carbonyl. This work has demonstrated that conversion of peptides to the *N*-terminal phenyl thiocarbamoyl derivative introduces a marked propensity to formation of the derivatized $b_1$, and complementary ions, to the extent that other fragmentation pathways are not observed under the conditions of low-energy collisional activation. These findings are in keeping with the enhanced nucleophilic properties of the thiocarbonyl moiety (in comparison with carbonyl) and emphasize a close parallel with the condensed-phase chemistry employed in the Edman degradation procedure for *N*-terminal sequencing of peptides. Protonation of the peptide backbone is a prerequisite in both instances. The parallel has been extended by examination of the further decomposition of the derivatized $b_1$ ions, which may be rationalized, in part, by a rearrangement process that resembles the isomerization of PTC to phenylthiohydantoin derivatives observed under acid conditions in the condensed phase.

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## REFERENCES

1. G. Allen *Sequencing of Proteins and Peptides*, 2nd edn. Elsevier, Amsterdam (1996).
2. K. Biemann, *Biomed. Environ. Mass Spectrom.* **16**, 99 (1988).
3. P. Roepstorff and J. Fohlman, *Biomed. Mass Spectrom.* **11**, 601 (1984).
4. R. S. Johnson, S. A. Martin and K. Biemann, *Int. J. Mass Spectrom. Ion Processes* **86**, 137 (1988).
5. W. Yu, J. E. Vath, M. C. Huberty and S. A. Martin, *Anal. Chem.* **65**, 3015 (1993).
6. R. Bakhtiar, Q. Wu, S. A. Hofstadler and R. D. Smith, *Biol. Mass Spectrom.* **23**, 707 (1994).
7. D. Arnott, D. Kottmeier, N. Yates, J. Shabanowitz and D. F. Hunt, in *Proceedings of the 42nd ASMS Conference on Mass Spectrometry and Allied Topics*, Chicago, IL, 1994, p. 470.
8. T. Yalcin, C. Khouw, I. G. Csizmadia, M. R. Peterson and A. G. Harrison, *J. Am. Soc. Mass Spectrom.* **6**, 1165 (1995).
9. T. Yalcin, I. G. Csizmadia, M. R. Peterson and A. G. Harrison, *J. Am. Soc. Mass Spectrom.* **7**, 233 (1996).
10. K. A. Cox, S. J. Gaskell, M. Morris and A. Whiting, *J. Am. Soc. Mass Spectrom.* **7**, 522 (1996).
11. B. C. Pramanik, S. M. Hinton, D. S. Millington, T. A. Dourdeville and C. A. Slaughter, *Anal. Biochem.* **175**, 305 (1988).

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