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Side-chain involvement in the fragmentation reactions of the protonated methyl esters of histidine and its peptides†

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Abstract

The gas phase fragmentation reactions of $[M+H]^+$ ions of the methyl esters of histidine and histidine containing di- and tripeptides were examined by electrospray ionization (ESI) multistage mass spectrometry (MS^n) experiments using a quadrupole ion trap mass spectrometer. The MS/MS spectra tend to be dominated by b_n sequence ions, whose structures were probed via MS^3 experiments and ab initio calculations at the MP2(FC)/6-31G*//HF6-31G* level of theory (for b_n ions where $n = 1$ and 2). The ab initio calculations suggest a structure for the b_1 ion that is stabilized by the formation of a bicyclic ring via involvement of the side-chain imidazole ring. In contrast, MS^3 experiments reveal that the b_2 ion derived from the sequences HG-Y and GH-Y yield identical spectra to the MS/MS spectrum of the protonated diketopiperazine of (GH). These experimental results are consistent with ab initio calculations that reveal the side-chain protonated diketopiperazine of (GH) to be thermodynamically favored over all other b_2 isomeric structures. Thus, the histidine side chain appears to exert both a direct and an indirect (through base catalysis) role in the formation of b_n sequence ions from protonated peptides. (Int J Mass Spectrom 209 (2001) 99–112) © 2001 Elsevier Science B.V.

Keywords: Histidine; Protonated peptide fragmentation; Multistage mass spectrometry; Ab initio calculations

1. Introduction

A recent group of four articles and an editorial [1–5] have been devoted to defining the "state of the art" in determining the gas phase fragmentation mechanisms of peptide ions. A resurgence of interest in this area of research has been fueled by the proteomics revolution [6]. Two tandem mass spectrometry approaches to uncovering the fundamental gas phase chemistry of peptide ions have been described: Using various physical organic tools to examine the fragmentation reactions of a series of small peptides in which the types of residues and the size of the peptide are systematically varied [2], and "data mining," in which the fragment ions of a set of tryptic peptides are statistically examined to uncover preferred cleavage reactions at specific residue sites [5]. Using the first approach, several studies on protonated peptides have revealed that their low-energy fragmentation reactions are often dominated by neighboring group pathways [2]. Furthermore, these gas phase pathways often have solution phase analogues [2].

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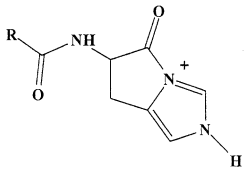


Structure A.

As part of our ongoing research, which has uncovered several neighboring group fragmentation pathways for protonated serine [9], cysteine [10,11], and methionine [12] containing peptides, we now turn our attention to the role of histidine residues. Histidine residues are known to play important catalytic roles in condensed phase intramolecular [13,14] and intermolecular [15,16] acyl bond-cleavage reactions. These reactions can occur via general acid–base mechanisms [13,14] or via transacylation reactions [15,16], both of which involve the side-chain imidazole moiety.

The gas phase basicities (GB) of histidine and its dipeptides and tripeptides have been measured and compared to their glycine analogs [17,18]. In each case, the GB of the histidine system was higher than its glycine analog, with the largest difference being observed in the amino acids. This difference has been attributed to protonation on the basic side-chain imidazole ring of the histidine residue. Despite the thermodynamically favored site of protonation being the imidazole ring, under collision-induced dissociation (CID) conditions, the proton has been proposed to be mobilized [19] to facilitate loss of the combined elements of H_2O and CO from protonated histidine [20,21]. Furthermore, it has been suggested that mobilization of the proton from the side-chain imidazole moiety facilitates cleavage of the peptide bond C terminal to the histidine residue via the formation of the bicyclic species (Structure A) [4,22–24]. Other types of gas phase fragmentation reactions also appear to be influenced by the histidine side chain. For example, water loss from the $[M+H]^+$ ion of the methyl ester of the tetrapeptide HGHG-OMe [25] may involve the imidazole moiety, while Turecek *et al.* [26] have used both N-1 and N-3 methylated histidine to show how the nonequivalent imidazole nitrogen atoms can influence the fragmentation mechanism of histidine. (We use the standard single-letter code to designate an amino acid residue within a peptide. For example, the dipeptide L-histidyl-glycine is designated as HG, while its methyl ester is designated as HG-OMe.) Here we examine the role of the histidine side chain on the fragmentation reactions of protonated methyl esters of histidine and the peptides HG; GH, HGG; GHG; GGH. In particular, the formation and structure of sequence ions, particularly b_n ions, are evaluated using multistaged mass spectrometry (MS) and ab initio calculations (where $n = 1$ and 2).

2. Experimental

2.1. Materials

L-Histidine methyl ester (H-OMe), L-histidyl-glycine (HG), glycyl-L-histidine (GH), L-histidyl-glycyl-glycine (HGG), glycyl-L-histidyl-glycine (GHG), and glycyl-glycyl-L-histidine (GGH) were all purchased from Bachem (Bubendorf, Switzerland). All reagents were used without further purification.

2.2. General procedure for the methyl esterification of dipeptides

Dipeptide methyl esters were synthesized according to the method of Reid et al. [10]. Acetyl chloride, 800 μL, was added to 5 mL of anhydrous methanol with stirring at 25 °C to yield 2N HCl in methanol. After 5 min, 100 μL of this solution was added to 1 mg of the dipeptide, and the reaction was allowed to proceed for 2 hours at room temperature. The resultant solution was lyophilized by freeze drying and used without further purification.

2.3. Mass spectrometry

All MS experiments were performed on a quadrupole ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA) equipped with an electrospray ionization (ESI) source. Samples were dissolved in

methanol : water (1 : 1) containing 1% acetic acid to a concentration of 0.1 mg mL^{-1} and were introduced into the ESI source at a flow rate of 3.0 μL min^{-1}. Nitrogen sheath gas (35 psi), a heated capillary temperature of 200 °C, and a spray potential of −5.50 kV were used.

CID MS experiments were performed by mass selecting target ions using standard isolation and excitation techniques. All data collected were an average of 50 scans.

2.4. Computational methods

The structures of ions were optimized via standard ab initio methods at the Hartree-Fock (HF) level of theory on Gaussian 98 molecular modeling software [27]. The standard 6-31G* basis set was utilized [28–30]. All optimized structures were then subjected to a single-point energy calculation of the correlated energy at the MP2(frozen core)/6-31G* level of theory. Energies were corrected for zero-point vibrational energy (ZPVE), which was scaled by a factor of 0.9135 [31]. Complete structural details and lists of vibrational frequencies for each HF/6-31G* optimized structure are available from the authors on request.

3. Results and discussion

3.1. MS/MS spectra of the protonated methyl esters of histidine and its dipeptides and tripeptides

The MS/MS spectra of the $[M+H]^+$ ions of the methyl esters of histidine and histidine di- and tripeptides are shown in Fig. 1. The simplest spectrum is for histidine, which yields the a_1 ion as the major fragment ion, with a minor amount of b_1 ion formed. Although the structure of this b_1 ion is discussed in further detail in section 3.2., we note that the simple acyl cation b_1 ion structures are inherently unstable [32] and thus, this ion is likely to be stabilized via cyclization to yield a bicyclic structure related to Structure A. In all systems where histidine is N terminal, both abundant b_1 and a_1 ions are observed. To further examine the role that the position of the histidine residue within a peptide has on the fragmentation reactions of that peptide, we compare the relative yields (expressed as a percentage of the sum of all fragmentation channels) of the b_1, b_2, y_1, and water-loss channels for the methyl esters of the peptides HG, GH, HGG, GHG, and GGH to those of GG [11] and GGG [33]. From the chart shown in Fig. 2, a number of differences emerge. For example, the y_1 ions dominate for GG-OMe and GGG-OMe, but with the exception of GGH-OMe, the b_n ions are often the major fragment ions in the remaining histidine peptides. N-terminal histidine facilitates b_1 formation, but the b_2 ion channel still remains competitive for HG-OMe and HGG-OMe. Perhaps the most dramatic difference is for the water-loss channel, which appears for all the histidine containing peptides but is only a minor channel for GG-OMe and is virtually nonexistent for GGG-OMe. Clearly, the histidine residue plays an important role in facilitating this reaction.

To gain further insights into the structures of the product ions, in the next sections we describe the results of MS^3 and ab initio studies.

3.2. MS^3 and ab initio studies on the b and y sequence ions

The MS^3 spectra of the y_1, b_1, and b_3 ions derived from the $[M+H]^+$ ions of the methyl esters of histidine and histidine di- and tripeptides are listed in Table 1. The y_1 ions derived from the $[M+H]^+$ ions of GH-OMe and GGH-OMe give identical spectra (Table 1) to the MS/MS spectrum of protonated H-OMe (Fig. 1a), thereby identifying this sequence ion as the protonated amino acid. This result is entirely consistent with previous studies that have shown that y_n ions are truncated peptides or amino acids (when $n = 1$) [33]. As higher y_n ions (i.e., $n > 1$) are virtually nonexistent in the MS/MS spectra of the histidine-containing tripeptides, we now turn our attention to potential structures of the b_n ions.

3.2.1. b_1 ion: structure and formation

All b_1 ions fragment via the sole loss of CO (Table 1). Although this loss is consistent with, but

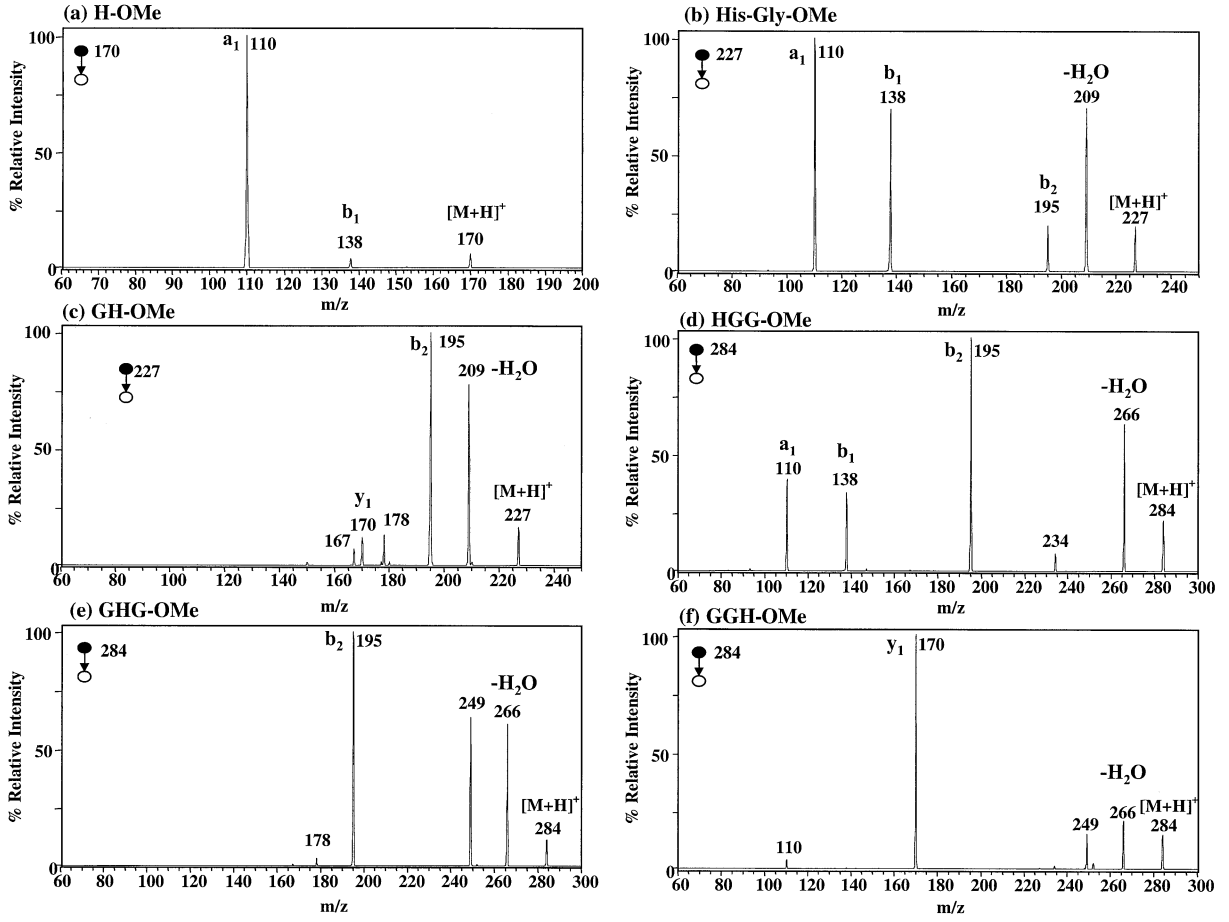


Fig. 1. MS/MS $[M+H]^+$ of the methyl esters of (a) H, (b) HG, (c) GH, (d) HGG, (e) GHG, and (f) GGH.

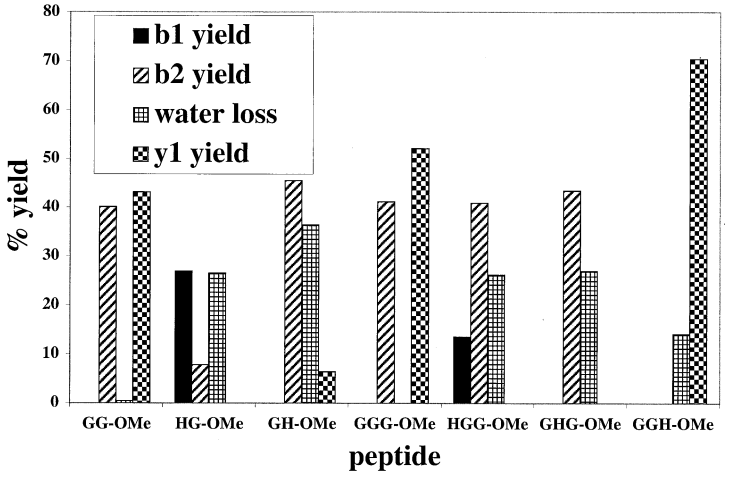


Fig. 2. Yields of fragmentation reactions. Comparison of the percentage yields of b and y ions derived from the methyl esters of GG, GH, HG, GGG, HGG, GHG, and GGH.

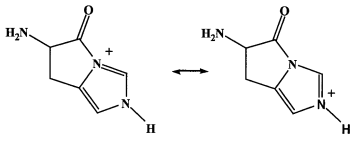
Table 1
CID MS^3 spectra of selected b and y ions derived from the $[M+H]^+$ ions of methyl esters of histidine peptides

MS/MS precursor (m/z)[a]	MS/MS product sequence ion (m/z)	MS^3 fragment ions; m/z, (neutral species loss), abundance %[b]
GH-OMe (227)	y_1 (170)	138(CH_3OH)5, 110(CH_3OH, CO)100
GGH-OMe (284)	y_1 (170)	138(CH_3OH)5, 110(CH_3OH, CO)100
H-OMe (170)	b_1 (138)	110(CO)100
HG-OMe (227)	b_1 (138)	110(CO)100
HGG-OMe (284)	b_1 (138)	110(CO)100
GGH-OMe (284)	b_3 (252)	234(H_2O)100, 224(CO)3, 138($NHCH_2CONHCH_2CO$)26, 110($NHCH_2CONHCH_2CO$, CO)39
GGGG-OMe (247)[c]	b_3 (172)	144(CO)55, 127(CO,NH_3)100; 115(HNCHCO)9

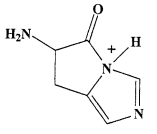
[a]Formed via electrospray ionization mass spectrometry.
[b]Only ions with a relative abundance greater than 1% shown.
[c]From PhD thesis of Gavin E. Reid, University of Melbourne, Australia, 2000.

not necessarily unique to, a structure related to Scheme A, we once again note that the b_1 ions are major signals in both the fragmentations of HG-OMe and HGG-OMe (Fig. 1b and 1d). As b_1 ions are unstable for most simple aliphatic systems, the histidine side chain must have a role in stabilizing the b_1 ion. Ab initio calculations on two tautomeric structures, Structure B1 and Structure B2 of the potential b_1 ion, were performed, and their optimized structures are shown in Fig. 3, while their energies are listed in Table 2. The tautomer in Scheme B1 is more stable than that of Structure B2 by 54.4 kcal $mole^{-1}$ (at the MP2(FC)/6-31G*//HF/6-31G* level of theory, Table 2), which is consistent with a conservation of the aromaticity of the imidazole ring in Structure B1.

On the basis of the results of the MS/MS and MS^3 experiments as well as the ab initio calculations, two potential mechanisms can operate for the formation of the b_1 ion from all systems with an N-terminal histidine residue. These, as well as the other reactions that result in the formation of the a_1 ions, are shown in Scheme 1. Initially, the side-chain protonated histidine moiety (species [**C**] in Scheme 1) acts as an acid to transfer a proton to the adjacent C-terminal acyl bond. Protonation on the heteroatom Y (where Y is either an ester oxygen atom or an amide nitrogen atom) yields species (**D1**), while protonation on the acyl oxygen (which is the stronger base [35]) yields species (**D2**). Species (**D1**) can undergo two reactions: loss of the combined elements of YH and CO (reaction (**1**) in Scheme 1) and loss of YH (reaction (**2**) in Scheme 1). The latter reaction is an S_N2-like intramolecular transacylation reaction, which has precedence in the gas phase [36–38]. In contrast: if species (**D2**) fragments via a tetrahedral intermediate, then it can only undergo loss of YH (reaction (**3**) in Scheme 1). The final reaction involves loss of CO from species (**B1**) to give the a_1 ion (reaction (**4**) in Scheme 1). Note that unequivocally determining whether (**D1**) or (**D2**) are the sole intermediates in acyl bond cleavage, or whether both are involved, is impossible. This point is further discussed for the fragmentation of peptide bonds in $[M+H]^+$ ions in references [1,2,3]. In subsequent sections, we will draw structures related to (**D1**) for convenience but do not imply that this is the decomposing species.



Structure B1.



Structure B2.

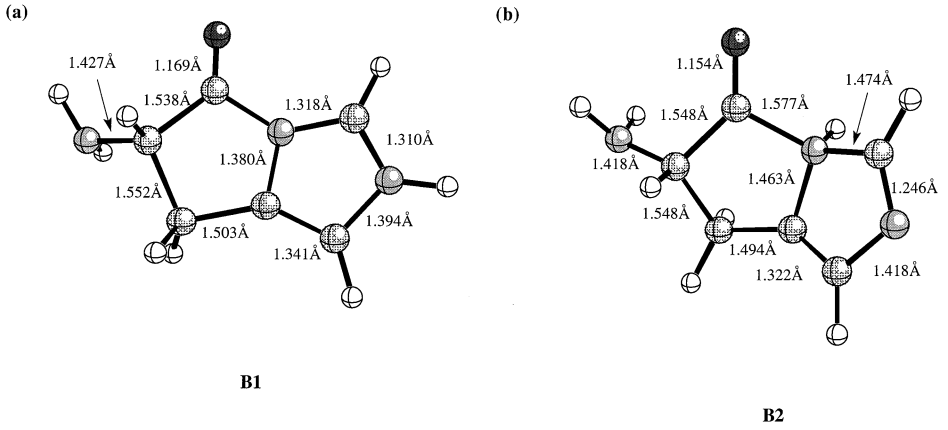


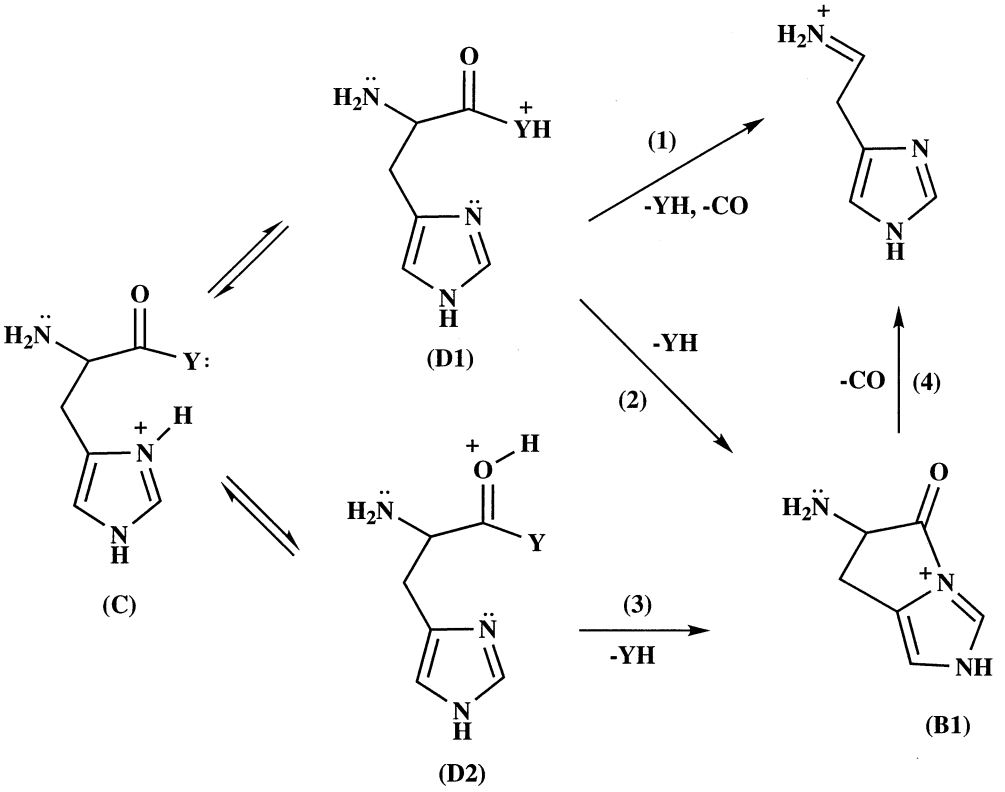
Fig. 3. Comparison of two tautomeric structures of potential histidine b_1 ions optimized at the HF 6-31G* level of theory.

3.2.2. b_2 ion formation: direct involvement of the histidine side chain via nucleophilic attack?

The formation of the b_2 ions from a range of dipeptides and tripeptides of the sequence GH-Y and HG-Y (where Y = OMe and G-OMe) provides a unique opportunity to examine their structures via MS^3 experiments and ab initio calculations and, thereby, to establish which mechanism(s) is (are) responsible for their formation. In principle, there are four types of structures for b_2 ions formed from the sequence GH-Y (Scheme 2), and three types of structures for b_2 ions formed from the sequence HG-Y (Scheme 3). Reaction (**5**) requires direct involvement of the imidazole ring in the sequence GH-Y as a neighboring group to yield an ion (**E**), which is related to the ions shown in Structures A and B1, discussed previously for other b_n ions. Such a reaction is not possible in the formation of a b_2 ion for the sequence HG-Y because the imidazole ring is not directly adjacent to the breaking acyl bond. Alternative reactions in which the histidine plays no role in acyl bond cleavage would result in the b_2 oxazolone structures (**F**) (reaction (**6**) in Scheme 2) and (**I**) (reaction (**9**) in Scheme 3), typically found for aliphatic peptides [39–41]. Related deprotonated b_2 oxazolone structures require involvement of the histidine residue via base catalysis to form ions of structure (**G**) (reaction (**7**) in Scheme 2) and (**J**) (reaction (**10**) in Scheme 3). The final type of reaction involves the histidine residue acting as a base to catalyze attack of the

Table 2
Energies of various isomeric b_1 and b_2 ions of histidine calculated at the MP2/6-31G*//HF/6-31G* level of theory

Isomer	HF/6-31G* energies in hartrees	MP2/6-31G* single-point energies in hartrees	ZPVE in hartrees	Relative energies (MP2 + ZPVE) in kcal mol^{-1}	Figure in which structure is shown
B1	−469.86231	−471.26961	0.15903	0	3a
B2	−469.77136	−471.18098	0.15686	54.4	3b
E	−676.67838	−678.66917	0.21910	0	5a
F	−676.68075	−678.67794	0.21904	−5.0	5b
G	−676.68259	−678.67931	0.22029	−5.7	5c
H1	−676.71449	−678.71226	0.22160	−25.6	5d
H2	−676.67962	−678.67770	0.22038	−4.1	5e
I1	−676.67745	−678.67606	0.21973	−4.0	5f
I2	−676.67429	−678.67425	0.21942	−3.0	5g
J	−676.67238	−678.67035	0.21994	−0.3	5h



Scheme 1.

N-terminal amino group to facilitate the formation of the side-chain-protonated diketopiperazine (**H**) shown in reaction (**8**) in Scheme 2 and reaction (**11**) in Scheme 3. Of all the reactions shown for the sequences GH-Y and HG-Y, only reactions (**8**) and (**11**) result in the formation of the same product ion. Thus, the MS^3 spectra of the b_2 ions of all sequences should be the same for structure (**H**), and these should, in turn, be identical to the MS/MS spectrum of the $[M+H]^+$ ion of an authentic example of the diketopiperazine of GH. In contrast, if isomeric b_2 ion structures are formed for the sequences GH-Y and HG-Y, then they should yield different MS^3 spectra. An examination of Fig. 4 reveals that the MS^3 spectra of the b_2 ions derived from HG-OMe, GH-OMe, HGG-OMe, and GHG-OMe are not only identical to each other but are also identical to that of the MS/MS spectrum of the $[M+H]^+$ ion of the diketopiperazine of GH. Despite not being able to independently synthesize "authentic" oxazolone structures such as (**F**), (**G**), (**I**), and (**J**) to prove that they fragment differently than the b_2 ions of GH-Y and HG-Y peptides as well as the $[M+H]^+$ ion the diketopiperazine of GH, the data from Fig. 4 are strongly suggestive that the base catalyzed diketopiperazine reactions operate for all systems (i.e., reactions (**8**) and (**11**)). Although this appears to be the first evidence for a b_2 ion with a diketopiperazine structure in the gas phase, an almost identical condensed phase reaction involving acyl bond cleavage in the peptide esters HPF-OR, to give the diketopiperazine of HP and the ester of the amino acid phenylalanine, was reported in 1963 [15].

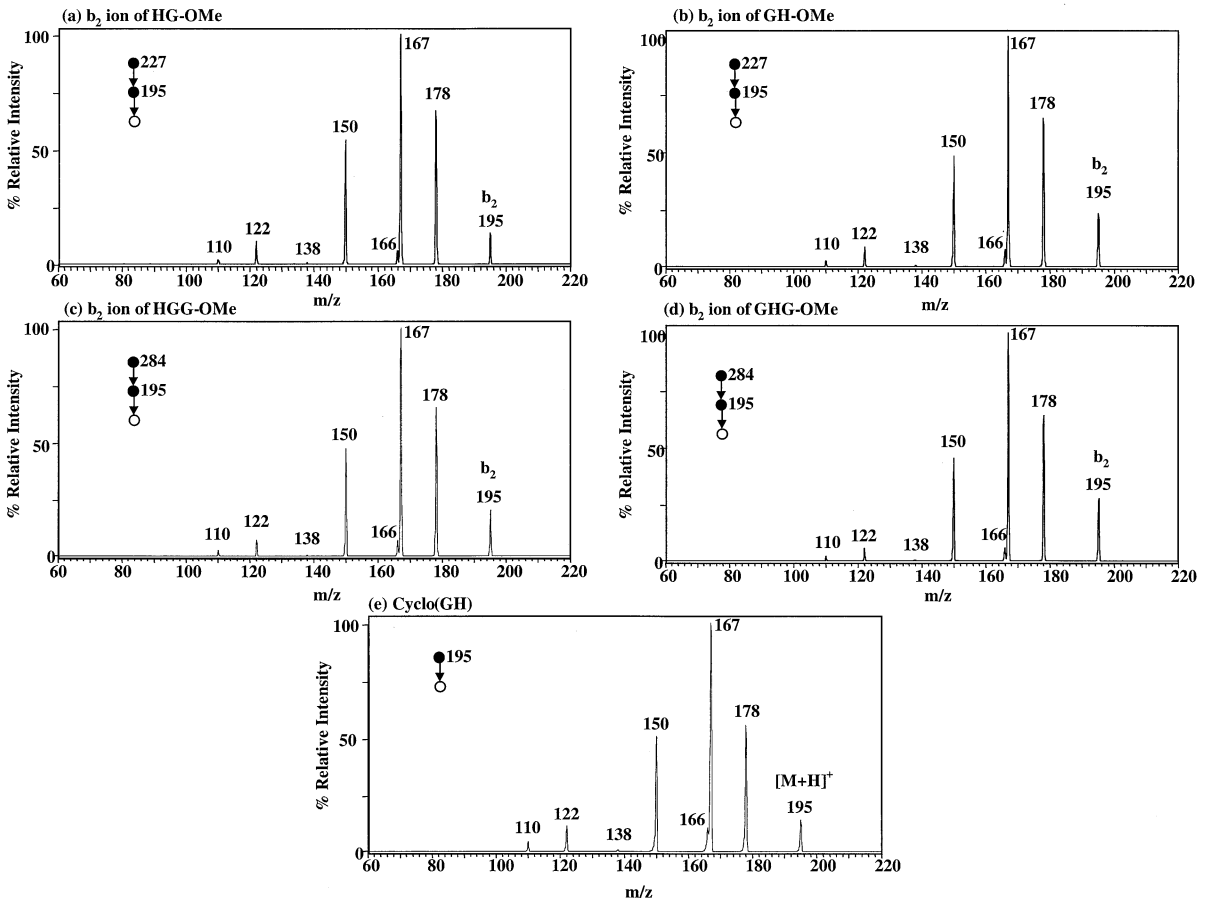
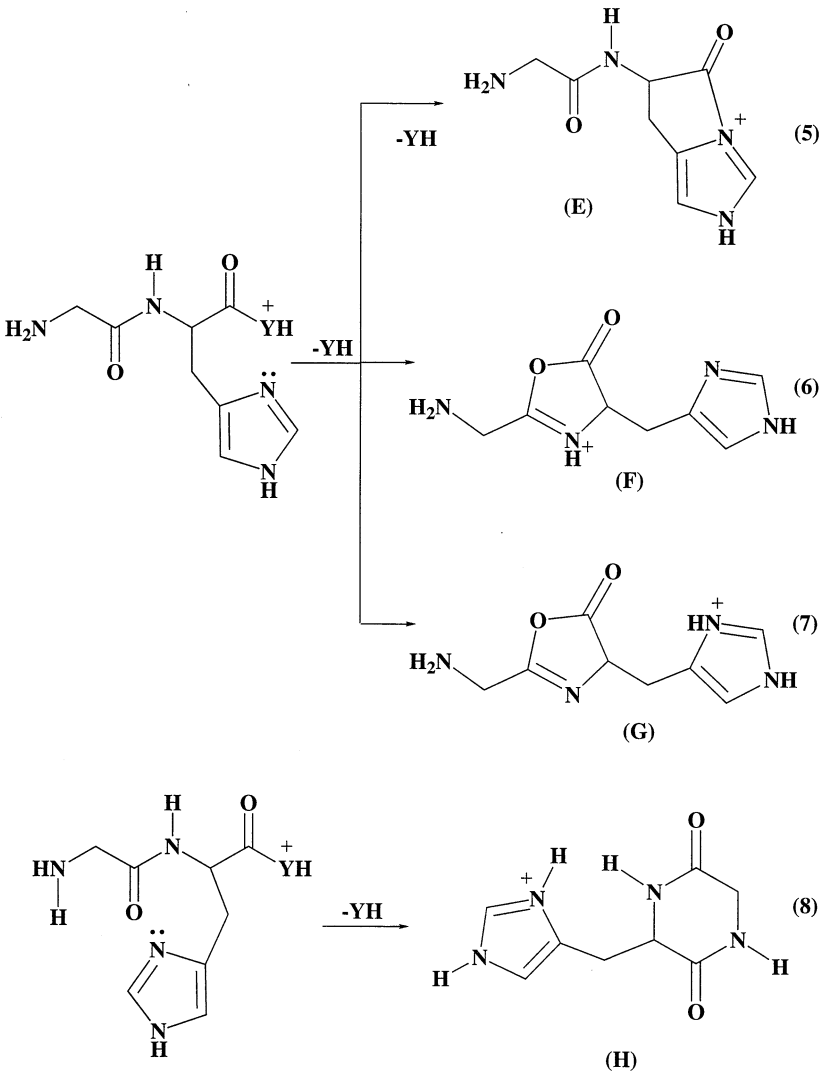


Fig. 4. Comparison of the MS^3 spectra of the b_2 ions of the methyl esters of (a) HG, (b) GH, (c) HGG, and (d) GHG, with (e) the MS/MS spectrum of the $[M+H]^+$ of cyclo(GH).

To gain further insights into the formation of the diketopiperazine structure (**H**), we have examined the structures and relative energies of all the b_2 ion structures shown in Schemes 2 and 3 using ab initio calculations at the MP2(fc)/6-31G*//HF/6-31G* level of theory. The results of this study are shown in Fig. 5 and Table 2. The diketopiperazine isomer in which protonation has occurred at the imidazole side (**H1**) is not only more stable than isomer (**H2**), in which protonation has occurred on the diketopiperazine ring, but is the most stable of all the isomeric structures examined. Thus, not only is this the kinetically favored product (from the experimental results), but it appears to be the thermodynamically favored product (from the ab initio results).

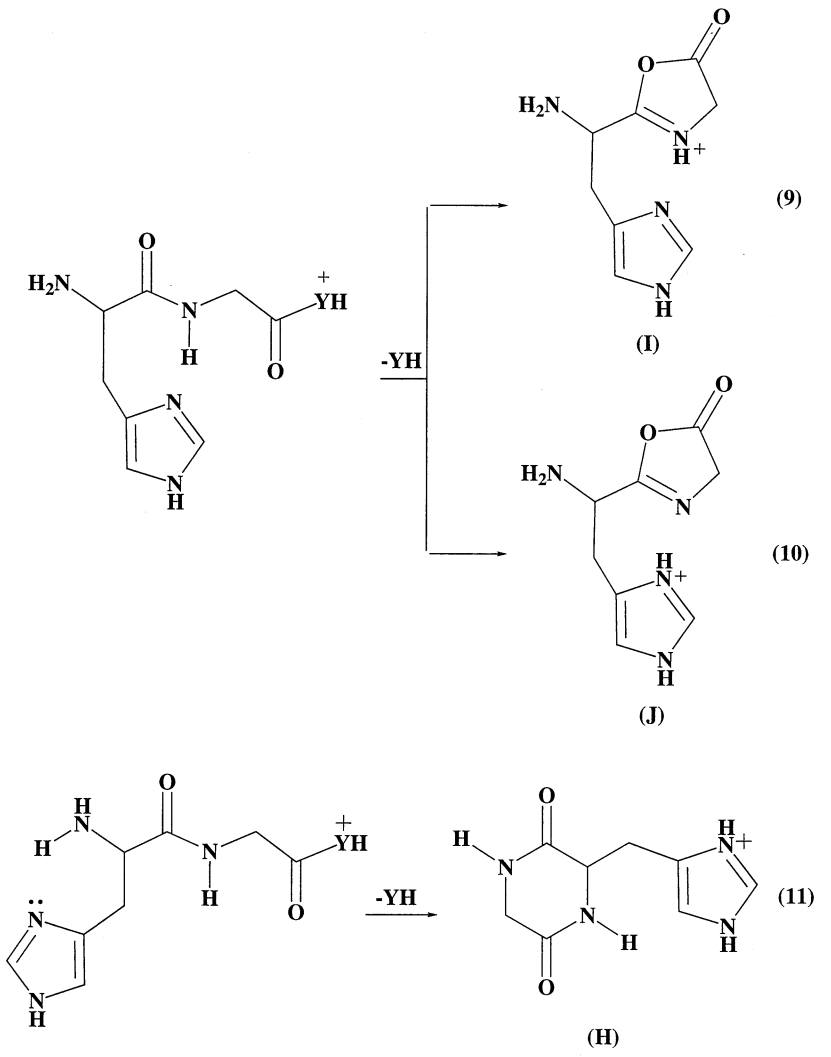
3.2.3. b_n ion formation where the histidine residue is at the n*th position: direct involvement of the histidine side chain via neighboring group attack or indirect involvement of the histidine side chain via base catalysis?*

Given that the b_2 ion of GH-Y showed a surprise deviation from the expected (**E**) ion structure, this raises the question of whether the general (**A**) ion structure previously proposed for b_n ion formation where the histidine residue is at the *n*th position occurs for $n > 1$. (Note that a diketopiperazine structure is no longer possible when $n > 2$.) Although we cannot directly answer this by comparing the MS^3 spectrum of a b_n ion to the MS/MS spectrum of an authentic (**A**) ion structure, let us consider two points. First, an examination of



Scheme 2.

the ab initio results for the b_2 ion isomers (**E**)–(**G**) reveals that the bicyclic structure (**E**) is not the thermodynamically favored of the three and is, in fact, very similar in energy to the oxazolone structures (**F**) and (**G**). Although we must be cautious in extrapolating this result to larger b_n ions, it does suggest that there may not be a thermodynamic preference for the formation of ions of structure (**A**). Second, a comparison of the MS^3 spectrum of the b_3 ion of GGH-OMe with that of the b_3 ion of GGGG in Table 1 reveals quite different behavior. Several groups have examined the fragmentation behavior of b_n-type ions [41–48] and have found that CO loss occurs to yield a_n-type immonium ions ($b_n \rightarrow a_n$). Whereas the previously accepted mechanism involved b_n ions of oxazolone structures undergoing ring opening to a transient acylium ion intermediate with spontaneous CO loss [45], a more recent ab initio study has revealed a more energetically favorable concerted process [48].



Scheme 3.

Under favorable conditions, depending on the nature of the adjacent amino acids, competing ($b_n \rightarrow a_{n-1}$) [41] or ($b_n \rightarrow b_{n-1}$) fragmentations [46] may also occur directly from the b_n ion precursor. It is noteworthy that whereas the b_3 ion of GGGG fragments to form three product ions (i.e., a_3, a_3-NH_3, and b_2 ions) indicative of the oxazolone structure, these reaction channels are suppressed for the b_3 ion of GGH-OMe. Instead, products arising from loss of water and from the formation of internal fragment ions are observed. A possible mechanism for the formation of the internal fragment ion (corresponding to the b_1 ion of histidine) is shown in Scheme 4.

3.2.4. Water loss induced via side-chain base catalysis

The histidine side chain has so far been shown to facilitate sequence ion formation. We next turn our attention to whether the histidine side chain can

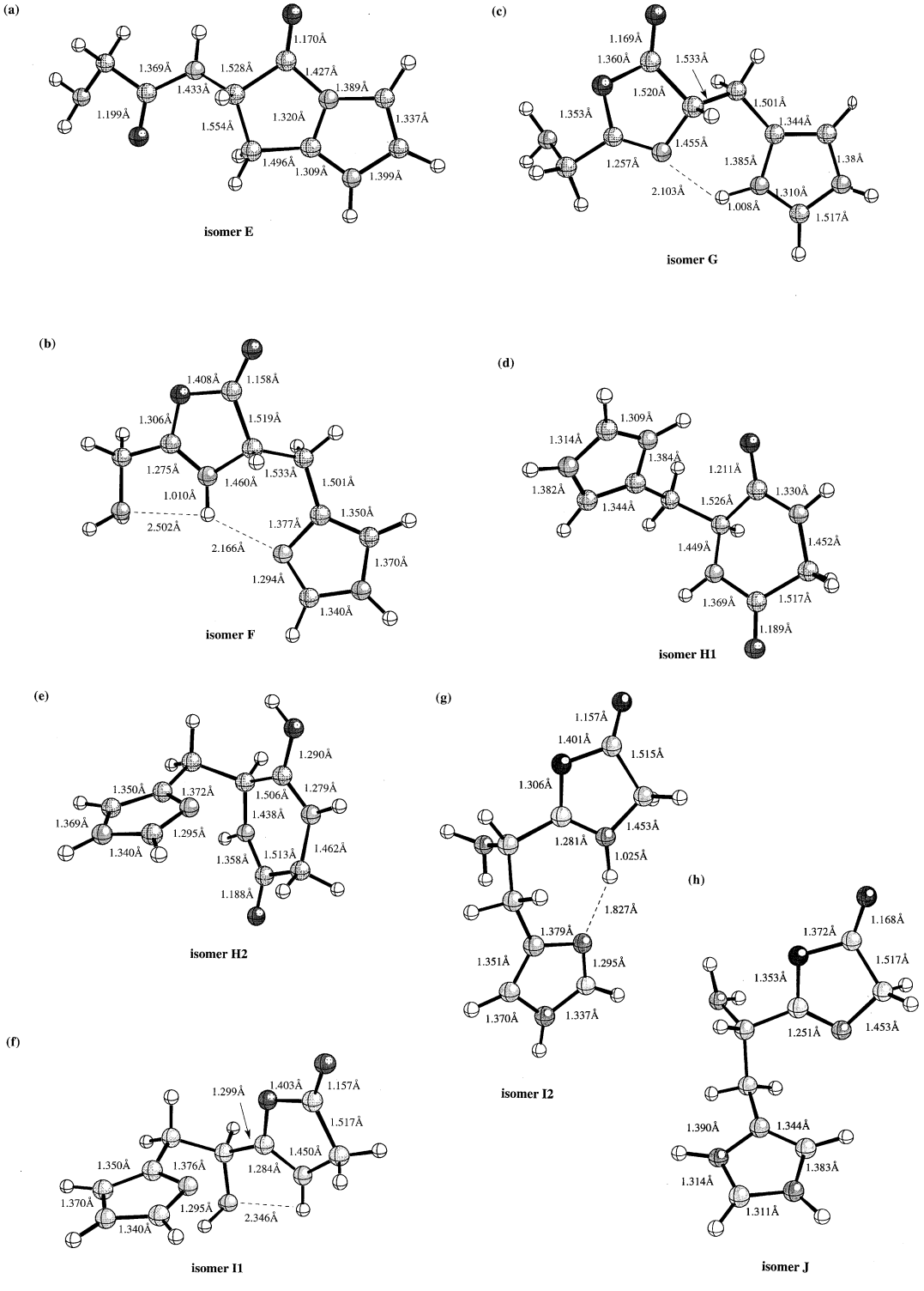
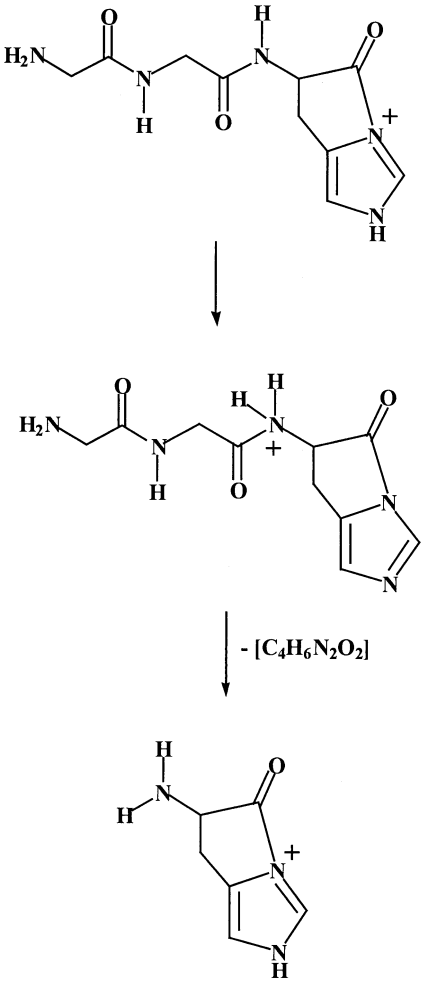


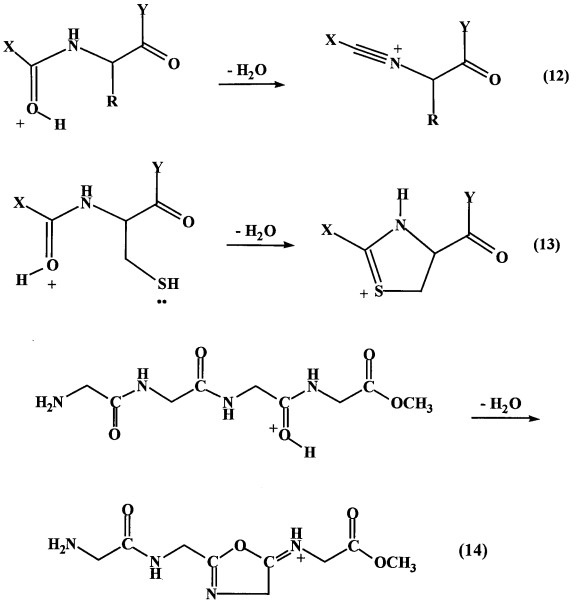
Fig. 5. Comparison of various structures of potential histidine b_2 ions optimized at the HF 6-31G* level of theory.

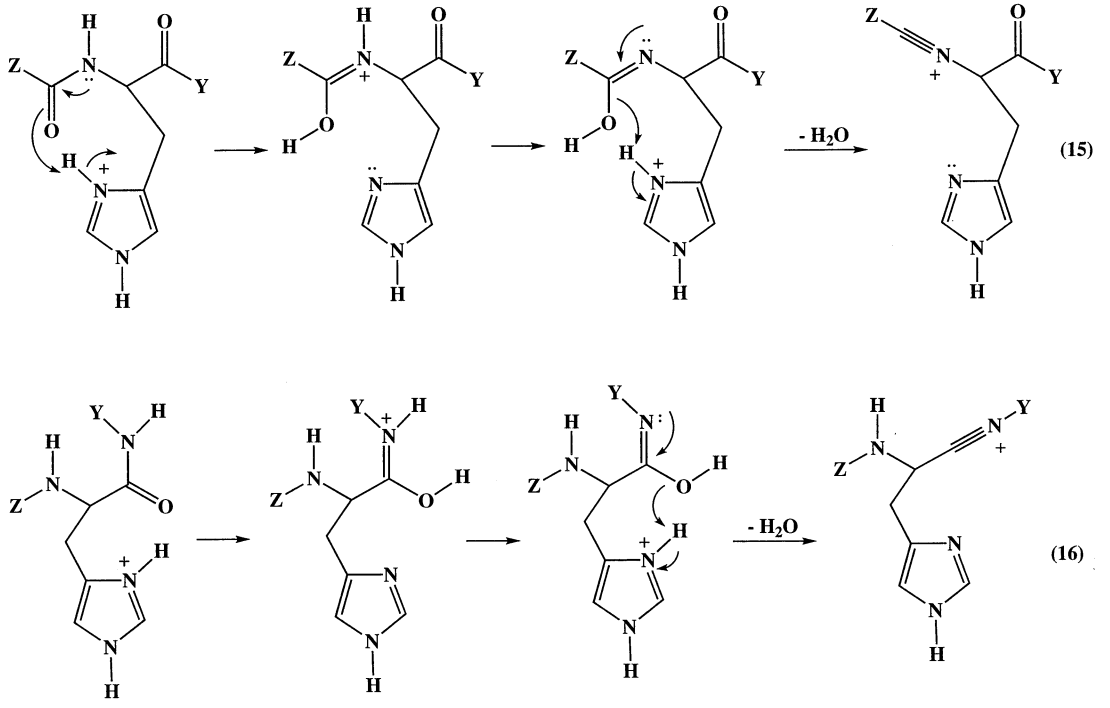


Scheme 4.

facilitate the formation of nonsequence ions. An examination of Fig. 2 reveals that the loss of H_2O is a significant reaction channel for many of the methyl esters of the histidine di- and tripeptides examined. In contrast, this water loss is minute for GG-OMe and essentially nonexistent for GGG-OMe. What role does the histidine residue play in this water-loss channel? We have discussed previously three potential dehydration mechanisms involving loss of a carbonyl oxygen atom from an amide bond within the $[M+H]^+$ ions of methyl esters derived from peptides [10,11,33]. These involve: a "retro-Ritter" reaction that results in the formation of a nitrilium ion (Eq. [12]) [10]; a neighboring group pathway with direct attack by a nucleophilic side chain of an amino acid residue to the C-terminal acyl position, as illustrated for the cysteine residue in Eq. (13) [10,11]; a neighboring group pathway involving attack by neutral peptide acyl oxygen atom on an adjacent O-protonated peptide bond, as illustrated for the tetrapeptide GGGG-OMe in Eq. (14) [33].

Reaction (13) is sequence dependent occurring for GC-Y peptides (where Y=OMe and G-OMe) but not for CG-Y Y peptides (where Y=OMe and G-OMe) [11]. The fact that the histidine-containing peptides do not exhibit such a sequence dependence suggests that a related water mechanism does not operate for these systems. Instead, we favor an acid–base catalyzed retro-Ritter mechanism that can involve either the N-terminal (Scheme 5; Eq. [15]) or the C-terminal (Scheme 5; Eq. [16]) peptide bond adjacent to the histidine residue. Unfortunately, MS^3 experiments on the $[M+H-H_2O]^+$ are not structurally revealing, with small molecule losses (e.g., H_2O, CO, NH_3, and MeOH) dominating the spectra.





Scheme 5.

4. Conclusions

Histidine residues appear to play an unique role in promoting the gas phase fragmentation reactions of protonated histidine-containing peptides. While it is likely that the side chain is initially protonated, on CID, the side chain acts as an acid to transfer a proton to the peptide backbone. Its role does not finish there, with the resultant side chain conjugate base being able to induce cleavage at neighboring peptide bonds through either a direct neighboring group reaction mechanism (as shown in the b_1 ion case) or via base catalysis mechanisms (as shown in the b_2 ion case). Given the diverse range of gas phase chemistry discovered for peptides with different reactive side chains [2], we are currently focusing our efforts on establishing the role that arginine residues play on the fragmentation reactions of peptides and will report our findings in due course.

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