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International Journal of Mass Spectrometry 222 (2003) 229–242

International Journal of Mass Spectrometry

www.elsevier.com/locate/ijms

Involvement of salt bridges in a novel gas phase rearrangement of protonated arginine-containing dipeptides which precedes fragmentation☆

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Received 30 April 2002; accepted 24 July 2002

Dedicated to Professor Beauchamp on the occasion of his 60th birthday and in recognition of his many seminal contributions to the gas phase chemistry of ions.

Abstract

A novel gas phase rearrangement reaction has been discovered for $[M + H]^+$ ions of arginine-containing dipeptides. In the case of Gly-Arg and Arg-Gly, this leads to identical tandem mass spectra (MS/MS) thereby precluding their sequence assignment. Density functional theory (DFT) calculations and further multistage mass spectrometry experiments suggest a mechanism which involves the formation of salt bridges for Gly-Arg and Arg-Gly which then undergo ring closure followed by ring opening to form a mixed anhydride. Prevention of salt bridge formation switches off this reaction and yields different MS/MS spectra which allow sequence assignment. This can be achieved by preforming CID on the deprotonated dipeptides or their protonated methyl esters. (Int J Mass Spectrom 222 (2003) 229–242)

Keywords: Tandem mass spectrometry; Protonated peptides; Arginine residues; Sequencing; Salt bridges

1. Introduction

Recently there has been considerable interest in the formation of salt bridges [1] and their role in the gas phase bimolecular (e.g., H/D exchange reactions [2]) and unimolecular chemistry (e.g., modes of fragmentation [3–5]) of cationized peptides. Of all the 20 commonly occurring amino acids residues, arginine, which has the highest proton affinity appears to most readily form salt bridges in the gas phase [1]. Apart from the fundamental interest in salt bridges, it is important to gain an appreciation as to their potential role in sequencing applications. In particular, the successful sequencing of peptides via tandem mass spectrometry (MS/MS) hinges upon the formation of a complete set of sequence ions via random cleavage of each of the peptide bonds. Typically this is achieved via collision-induced dissociation (CID) of protonated peptides which yield the complementary b and y sequence ion series [6]. C-terminal arginine containing peptides, which are readily formed via tryptic digests of proteins, pose two main problems: (i) the high basicity of the arginine side chain means that the singly protonated peptides often fragment

☆ Part 32 of the series "Gas Phase Ion Chemistry of Biomolecules."

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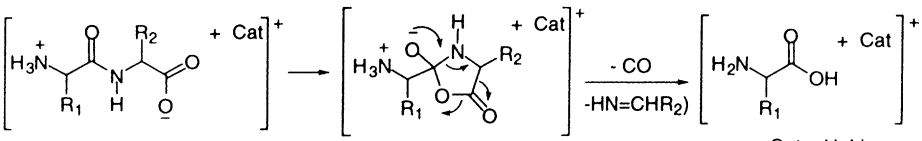
1387-3806/02/$ – see front matter
PII S1387-3806(02)00961-2

poorly since the proton is not readily mobilized [7] to the sites of peptide bond cleavage [8]; (ii) they often form $[b_n + H_2O]^+$ ions in the MS/MS spectra, which can be confused with y_n ions [3]. A mechanism which has been proposed for this rearrangement reaction involves an initial salt bridge structure forming a cyclic intermediate with subsequent fragmentation (Eq. (1)) [3]. The accompanying paper by Gronert and coworkers re-examines this mechanism for alkali metal ion complexes of peptides and finds an alternative pathway for decomposition of the cyclic intermediate shown in Eq. (1) [9]:



Cat = H, Li (1)

Herein we report on a novel rearrangement of protonated arginine-containing dipeptides Arg-Gly and Gly-Arg which results in essentially identical MS/MS spectra for both isomeric peptides [10]. A potential mechanism involving a salt bridge is examined through the use of multistage mass spectrometry and structural labeling experiments.

2. Experimental

All compounds were of reagent grade obtained commercially and were used without further purification. L-arginyl-glycine (Arg-Gly), glycyl-L-arginine (Gly-Arg), L-prolyl-glycine (Pro-Gly) and glycyl-L-proline (Gly-Pro) were obtained from Bachem (Bubendorf, Switzerland). Methyl esters were formed via a previously described method [11].

2.1. Mass spectrometry methods

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. Samples ($0.1\,mg\,mL^{-1}$) were introduced into the ESI source at a flow rate of $3.0\,\mu L\,min^{-1}$. 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 precursor ions using standard isolation and excitation techniques. All data collected were an average of 50 scans.

2.2. Computational methods

Several recent studies have examined various levels of theory for modeling salt bridges [12]. We have adopted the B3LYP/6-31+G* level for optimizations, frequency calculations and determining relative energies since Williams' study suggests that this level of theory offers an useful compromise between providing reasonable structures and energetics as well as not being computationally prohibitive [12a]. As in Gronert's paper, we have not done an exhaustive search of the potential energy surface. Instead we have carried out limited conformational searches to locate structures which maximize the number of hydrogen bonds at the B3LYP/6-31+G* level of theory. In most instances at least two conformations were examined at this level of theory. All calculations were carried out using GAUSSIAN 98 [13]. Zero point energy corrections to the theoretical energies used the vibrational frequencies calculated at the B3LYP/6-31+G* level and scaled by 0.9806 [13b].

3. Results and discussion

3.1. MS^n ($n = 2, 3$) studies on the $[M + H]^+$ and $[M–H]^-$ ions of glycyl-arginine and arginyl-glycine

The MS/MS spectra of protonated Arg-Gly and Gly-Arg are shown in Fig. 1(a) and (b). Both spectra

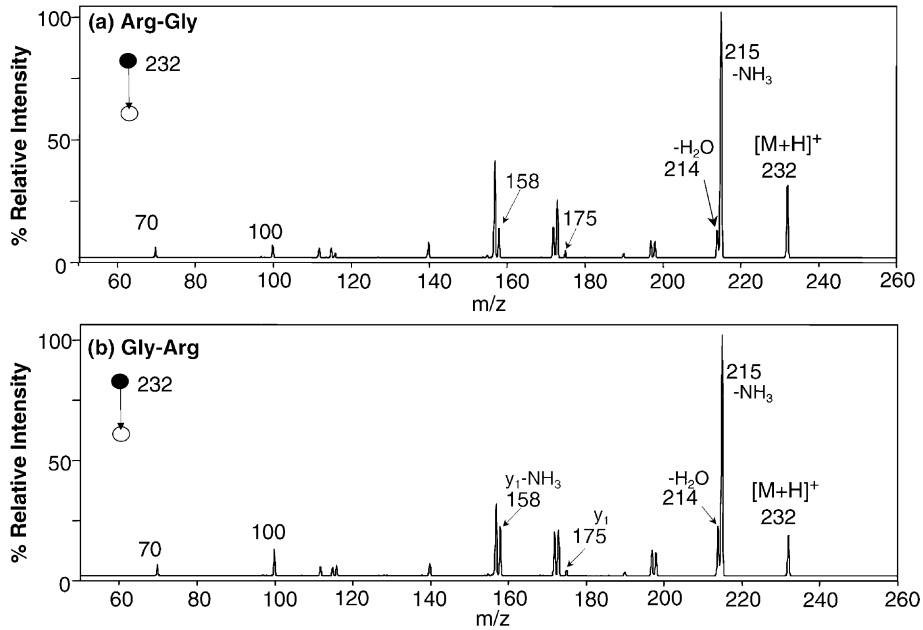


Fig. 1. MS/MS spectra of $[M + H]^+$ ions of: (a) Arg-Gly; (b) Gly-Arg.

are almost identical (the same peaks and very similar relative intensities) and show a series of non-sequence ions at *m/z* 215 ($–NH_3$), *m/z* 214 ($–H_2O$), *m/z* 198 ($–NH_3$, $–NH_3$), *m/z* 197 ($–H_2O$, $–NH_3$) and fragmentations of the arginine side chain at *m/z* 190 (–HN=C=NH) and *m/z* 173 ($–(NH)_2C=NH$, guanidine). Moreover, the b_1 sequence ion at *m/z* 157, which should only be observed for Arg-Gly, is also observed with nearly the same abundance for the isomeric peptide Gly-Arg. Other signals consistent with the Arg-Gly sequence but which are observed in *both* spectra, are *m/z* 140 ($b_1–NH_3$), *m/z* 112 ($a_1–NH_3$) and *m/z* 70 ($a_1–NH_3$, –HN=C=NH). A similar situation holds for the y_1 and ($y_1–NH_3$) sequence ions at *m/z* 175 and 158, which should only be observed for Gly-Arg, but are also present in the MS/MS spectrum of the isomeric peptide Arg-Gly. Thus, this MS/MS data suggests that either one or both of the $[M + H]^+$ ions isomerizes to the same intermediate which then undergoes fragmentation.

Further evidence for a rearrangement reaction was garnered by carrying out MS^3 experiments (data not shown, but available from the authors upon request) on most of the fragment ions (at *m/z* 112, 115, 116, 140, 157, 158, 172, 173, 175, 197, 198, 214 and 215). For example, MS^3 experiments on the $[M+H–NH_3]^+$ and $[M + H–(NH_2)_2C–NH]^+$ (see Section 3.4 for a more detailed discussion of this ion) ions of glycyl-arginine and arginyl-glycine once again yield identical spectra. CID on the fragment ions at *m/z* 157 (Arg-Gly b_1 ion), *m/z* 175 (Gly-Arg y_1 ion) and *m/z* 158 ($y_1–NH_3$ for Gly-Arg) give the same fragmentation ions and approximately the same relative intensities for both Arg-Gly and Gly-Arg samples. Thus, *all* sequence and non-sequence ions gave essentially identical MS^3 spectra.

In order to establish that this rearrangement occurs in the gas phase and not in solution, the CID spectra of the isomeric $[M–H]^-$ ions were examined (for CID on $[M–H]^-$ ions of isomeric arginine-containing dipeptides see) [14]. Fig. 2 shows that while there are some common fragments which arise from fragmentation of the arginine side chains (to yield the two non-sequence ions at *m/z* 213 ($–NH_3$) and *m/z* 188 (–HN=C=NH)), the key diagnostic sequence ions observed are at *m/z* 74 for Arg-Gly (Fig. 2a) and *m/z* 173 for Gly-Arg

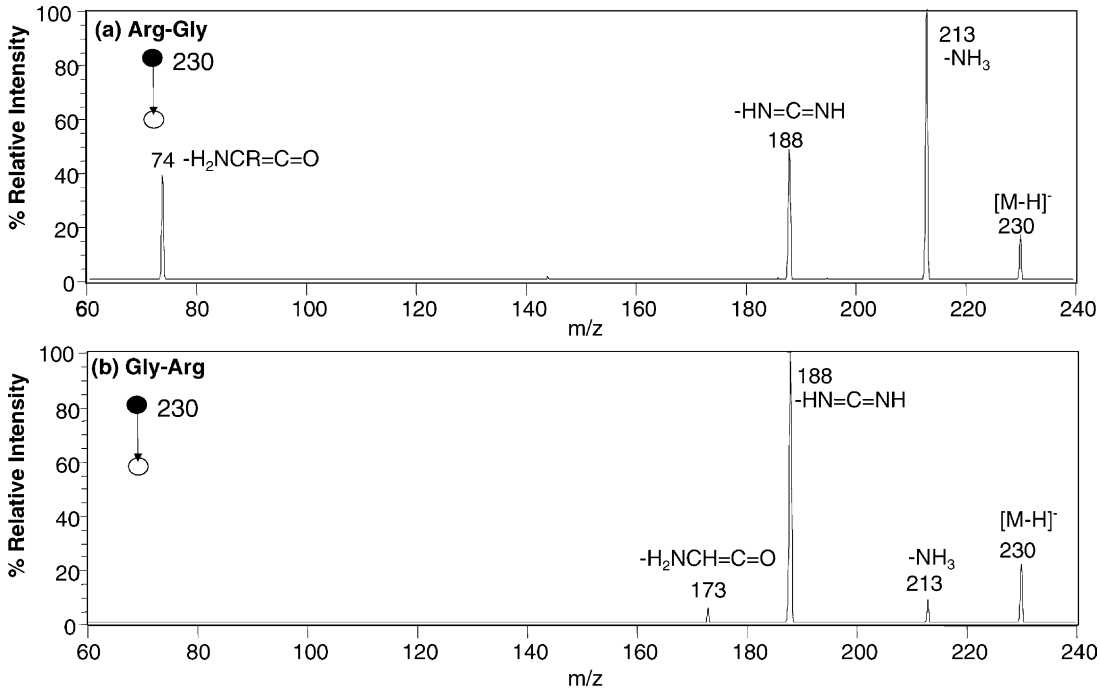
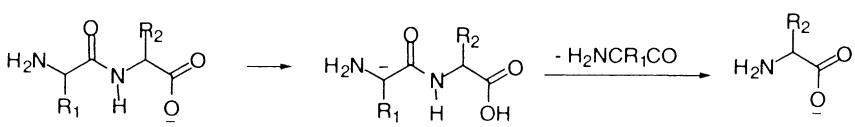


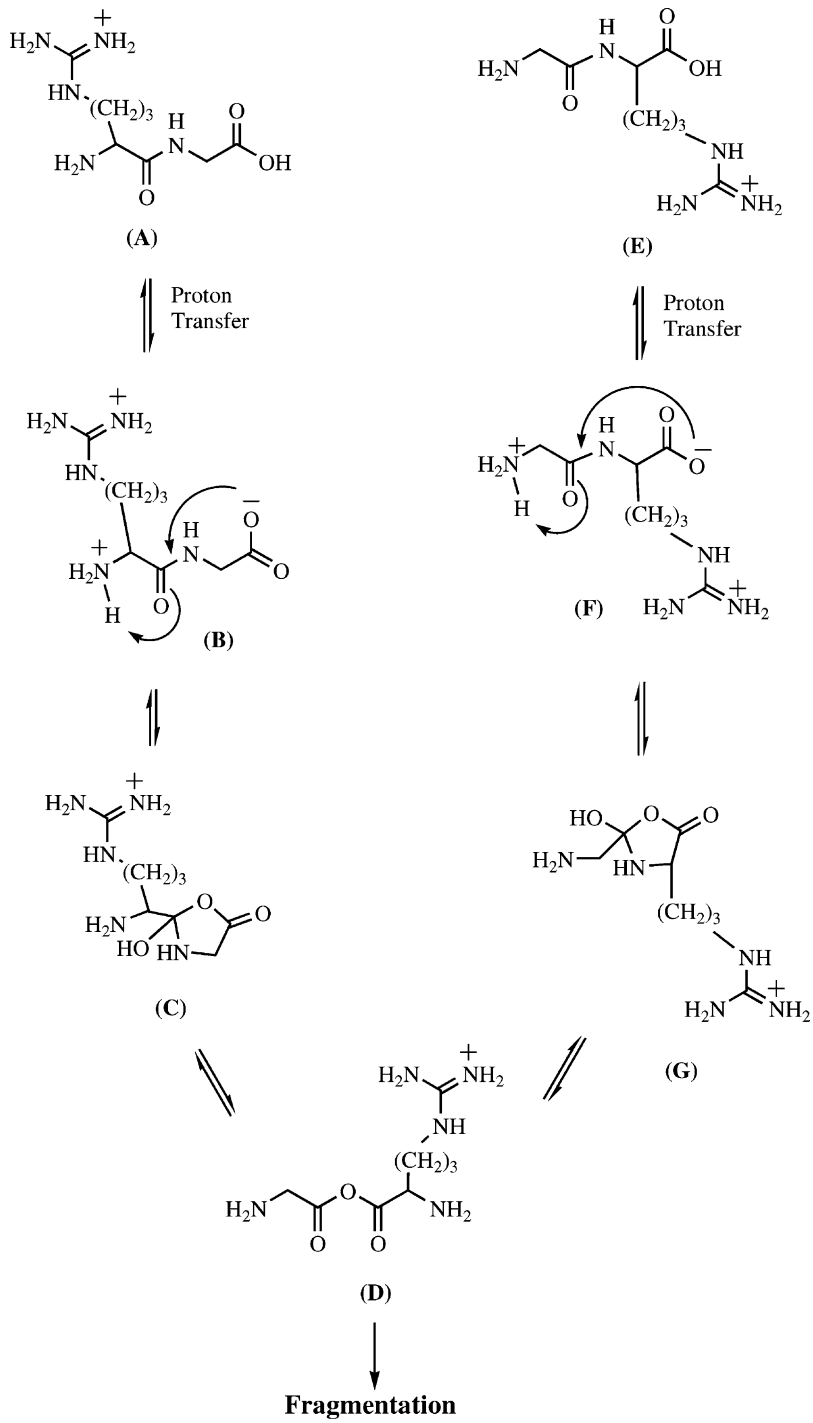
Fig. 2. MS/MS spectra of $[M–H]^-$ ions of: (a) Arg-Gly; (b) Gly-Arg.

(Fig. 2b). Note that the formation of these two sequence ions are consistent with the high energy CID of deprotonated Ala-Arg and Arg-Ala, which were studied by Eckersley et al. who proposed the sequence mechanism for dipeptides shown in Eq. (2) [14]. Furthermore, MS^3 experiments on the $[M–H–NH_3]^-$ and $[M–H–HN=C=NH]^-$ non-sequence ions of glycyl-arginine and arginyl-glycine yield different spectra (data not shown) indicating that the original $[M–H]^-$ ions have not undergone rearrangement:



(2)

Thus, the simple mechanism proposed in Eq. (1) cannot account for the essentially identical MS/MS and MS^3 spectra derived from the $[M+H]^+$ ions. Although caution should be exercised in directly relating condensed phase studies to the gas phase, it is interesting to note that solvolyses reactions of peptides [15] point to an alternative mechanism in which the salt bridge forms a cyclic intermediate which undergoes ring opening to a mixed acid anhydride. Thus, we propose the modified mechanism shown in Scheme 1 which hinges on the fact that arginine has the highest proton affinity of all the amino acids and that the proton will reside on the guanidine group. This allows the formation of the key salt bridge intermediates (**B**) and (**F**), which can cyclize to (**C**) and (**G**) and subsequently rearrange to form the mixed anhydride intermediate (**D**), analogous to the condensed phase mechanism formulated by Martin et al. [15]. Note that the cyclic intermediates (**C**) and (**G**) are related to that proposed by Thorne et al. for the formation of $[b_n + H_2O]^+$ ions in the gas phase cf. Eq. (1) [3a].



Scheme 1.

3.2. Does methyl ester formation "switch off" the rearrangement reactions of the $[M + H]^+$ ions of glycyl-arginine and arginyl-glycine?

In order to provide support for the involvement of salt bridges, Williams and coworkers have previously shown that methylation of the carboxylic acid group of the C-terminus of bradykinin profoundly influences its blackbody infrared radiative dissociation (BIRD) spectra, not only reducing the Arrhenius activation parameters but also dramatically changing the dissociation products observed [4]. If a salt bridge intermediate is involved in the rearrangement process (Scheme 1), then the methyl esters of glycyl-arginine and arginyl-glycine should give different MS/MS spectra [16]. To test this hypothesis, the methyl esters of Arg-Gly and Gly-Arg were synthesized and CID performed on the $[M + H]^+$ species of each (Fig. 3(a) and (b)). For the MS/MS $[M + H]^+$ of Arg-Gly-OMe we see the b_1 ion at *m/z* 157 representing the loss of glycine methyl ester. However, we do not see the y_1 ion of Gly-Arg-OMe, unlike the case for the parent peptide (Fig. 1(a)). The MS/MS $[M + H]^+$ of Gly-Arg-OMe does show the y_1 ion at *m/z* 189, however, the b_1 ion of Arg-Gly-OMe at *m/z* 157 is not observed.

Overall the CID spectra of the protonated methyl esters are significantly different, with other ions observed at *m/z* 214 (–MeOH), *m/z* 172 (y_1–NH_3), *m/z* 130 and *m/z* 100 for Gly-Arg-OMe, but not Arg-Gly-OMe, while the ions at *m/z* 140 (b_1–NH_3), *m/z* 115 and *m/z* 112 appear in the MS/MS of protonated Arg-Gly-OMe but not Gly-Arg-OMe. This is consistent with "switching off" the rearrangement mechanism shown in Scheme 1, since the methyl ester blocks salt bridge formation. Instead new fragmentation reactions, consistent with the sequence order, are observed.

3.3. Molecular modeling support for salt bridge-mixed anhydride mechanism

In order to find further support for the mechanism shown in Scheme 1, we have carried out DFT calculations at the B3LPY/6-31+G* level of theory. We recognize that the complete potential energy surfaces for these reactions are complex, with the possibility of multiple conformations, minima and transition states

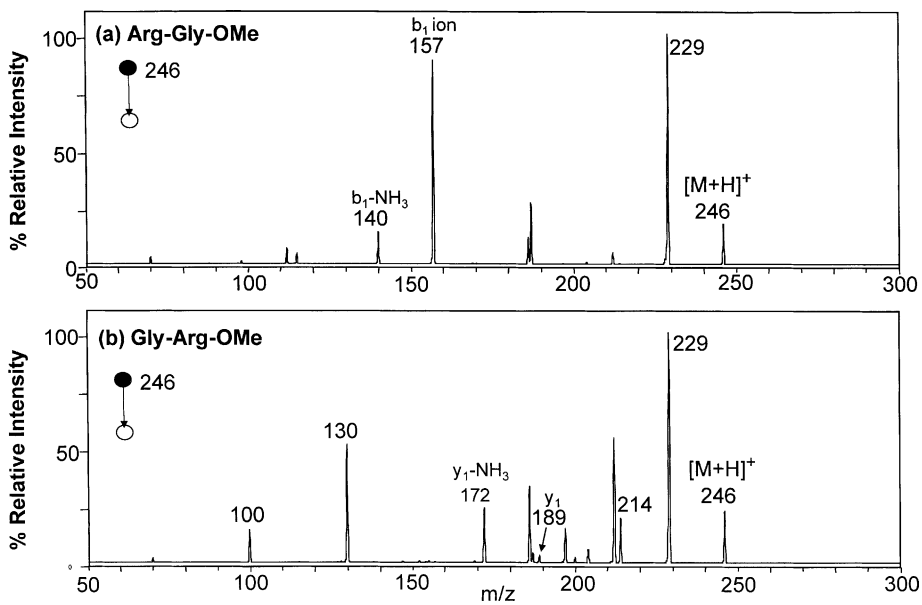
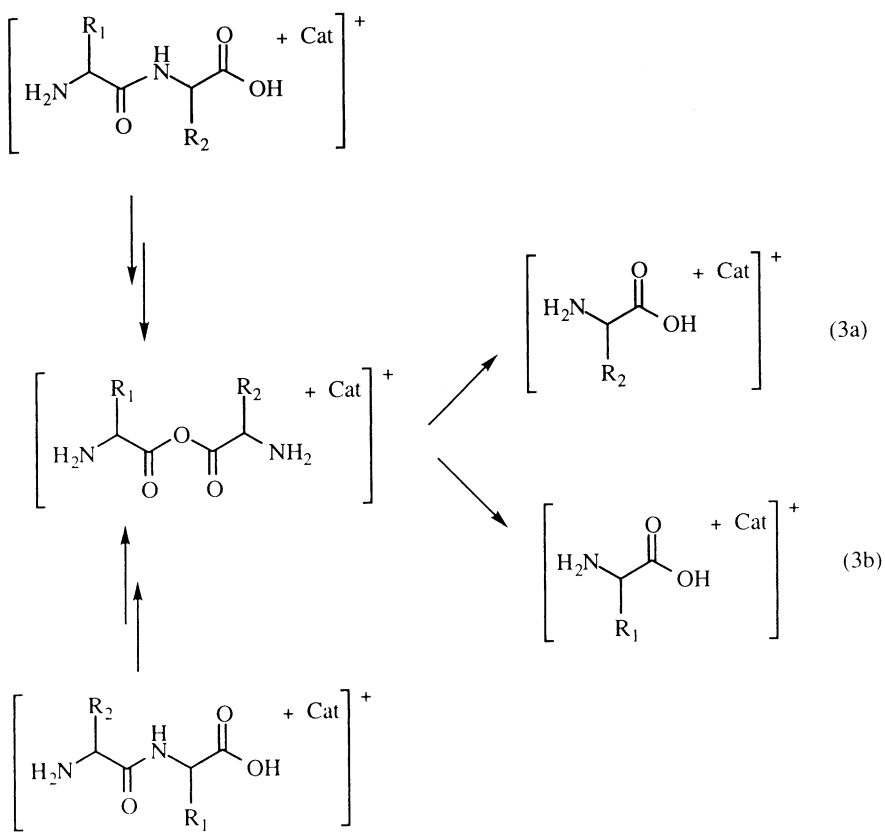


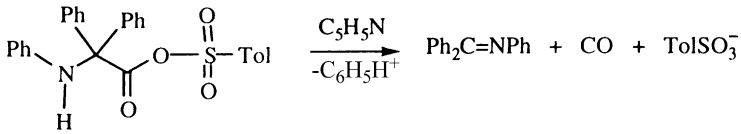
Fig. 3. MS/MS spectra of the $[M–H]^+$ ions of the methyl esters of: (a) Arg-Gly; (b) Gly-Arg.

[17]. Our main aim was to establish that the reactants and key intermediates (**A**)–(**G**) are all viable species in the gas phase and to gain some insights into their relative stabilities. In all cases, the fully optimized structures correspond to minima as shown by the frequency calculations (i.e., no imaginary frequencies). All the structures are shown in Fig. 4, while their energies are listed in Table 1. While we will not discuss the structures of (**A**)–(**G**) in any detail, it is worth noting that in all cases the arginine side chain is protonated and interacts with the peptide backbone to stabilize each of the structures via hydrogen bonding. In fact each of the structures benefits from at least two hydrogen bonds, which are indicated on each of the Fig. 4(a)–(g). The highest energy intermediate is the salt bridge (**B**) (+26.8 kcal mol^{-1} relative to Arg-Gly (**A**)). In both cases the salt bridge structures (**B**) and (**F**) are less stable than the conventional structures (**A**) and (**E**), consistent with the findings of Feng et al. [9]. Perhaps the most important finding is that the anhydride structure (**D**) is a stable structure, only 22.6 kcal mol^{-1} less stable than (**A**) and 15.2 kcal mol^{-1} less stable than (**E**).

3.4. MS^3 studies on the $[M + H–(NH_2)_2C{=}NH]^+$ ions of Arg-Gly and Gly-Arg

The next question is how does fragmentation occur after rearrangement? Several scenarios are possible including fragmentation solely via either protonated Gly-Arg, Arg-Gly or the mixed anhydride or any mixture of these species. Unfortunately it is not possible to synthesize the anhydride to examine the fragmentation reactions of its $[M + H]^+$ ion. In the accompanying paper Feng et al. suggest that the anhydride fragments to yield the $[b_1 + OH + Cat]^+$ and y_1 ions (Eqs. (3a) and (3b)) [9]. A search of the condensed phase literature reveals a similar mechanism for fragmentation of a related mixed anhydride (Eq. (4)) [18]:





(4)

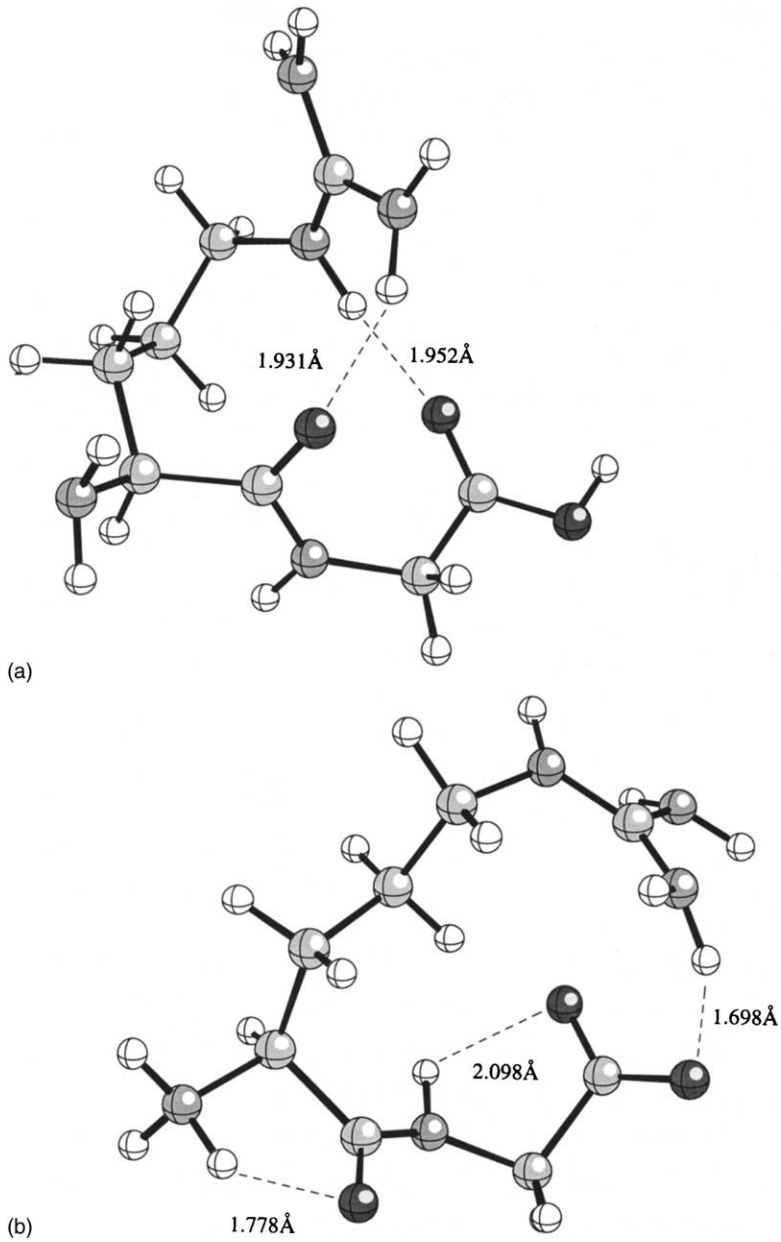


Fig. 4. B3LYP/6-31+G* optimized structures of key species in Scheme 1: (a) side chain protonated Arg-Gly (**A**); (b) salt bridge structure for Arg-Gly (**B**); (c) cyclic intermediate for Arg-Gly (**C**); (d) anhydride (**D**); (e) side chain protonated Gly-Arg (**E**); (f) salt bridge structure for Gly-Arg (**F**); (g) cyclic intermediate for Gly-Arg (**G**).

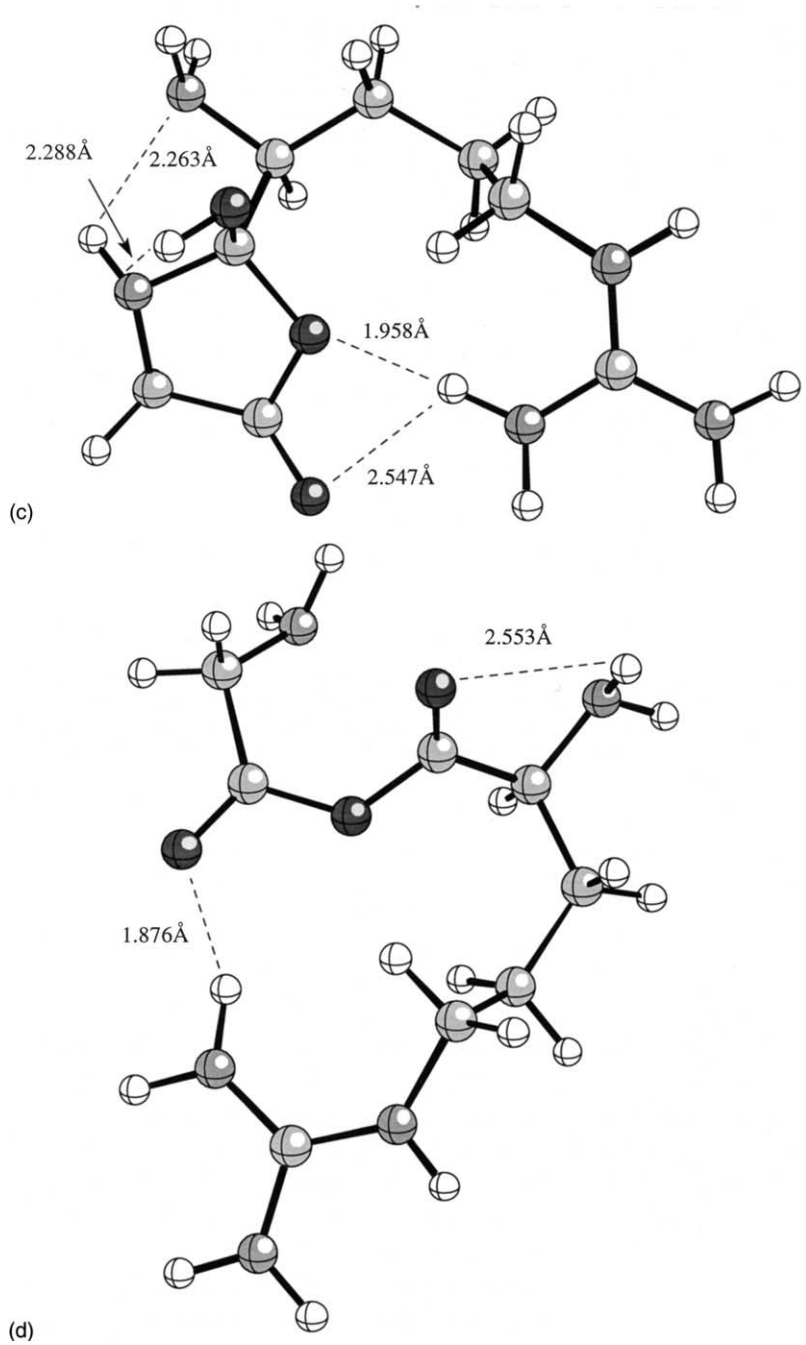


Fig. 4. (*Continued*).

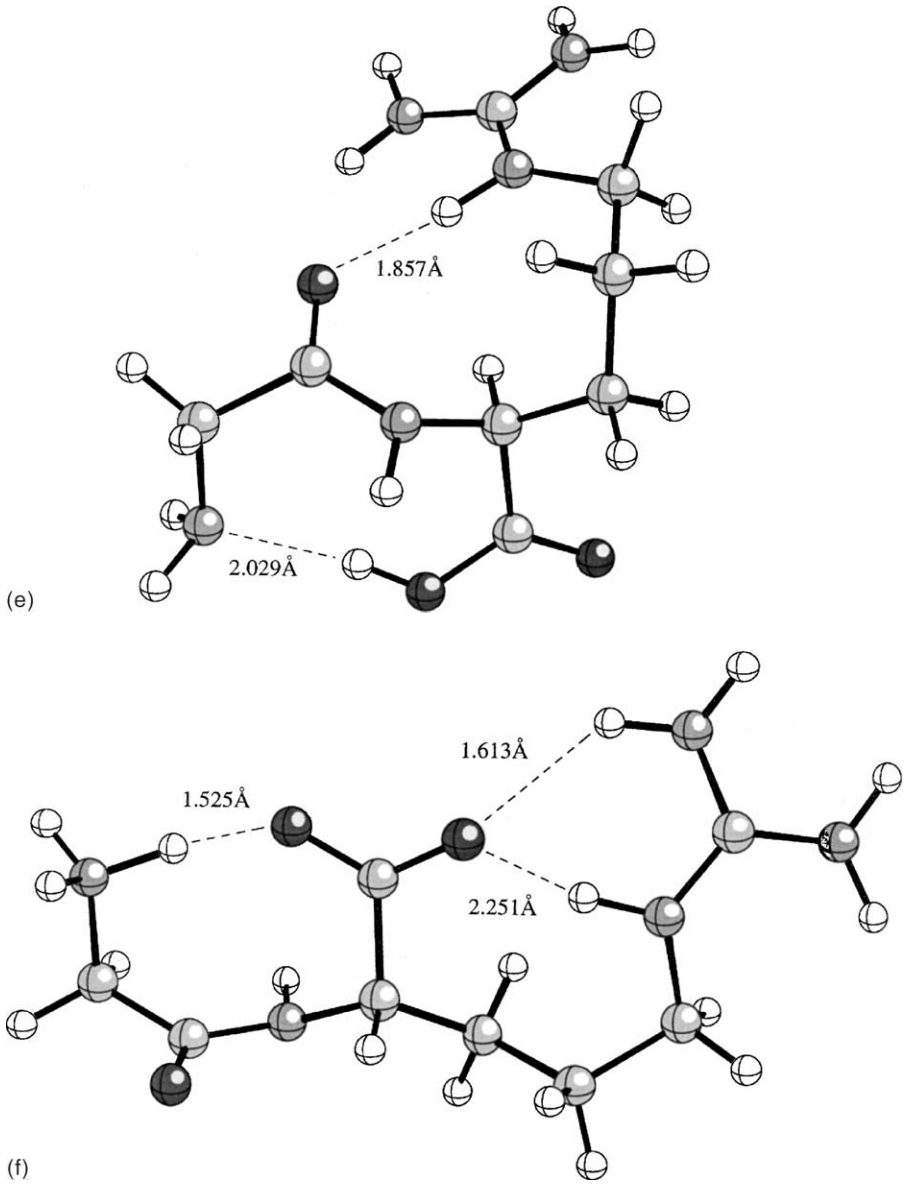


Fig. 4. (*Continued*).

We have chosen to address which of the species gives rise to the loss of guanidine from the side chain, since the related loss from protonated arginine has been suggested to occur to yield proline as shown in Eq. (5) [19]. Thus, we should be able to infer the structure of the $[M + H–(NH_2)_2C=NH]^+$ ions of Arg-Gly and Gly-Arg by comparing their CID spectra to those of protonated Pro-Gly and Gly-Pro. The results of these experiments are shown in Fig. 5. While the MS^3 spectra of the $[M + H–(NH_2)_2C=NH]^+$ ions of both Arg-Gly and Gly-Arg are essentially identical (Fig. 5(a) and (b)), they are different to the MS/MS spectra of Pro-Gly and Gly-Pro (Fig. 5(c) and (d)). This suggests that the $[M + H–(NH_2)_2C=NH]^+$ ions

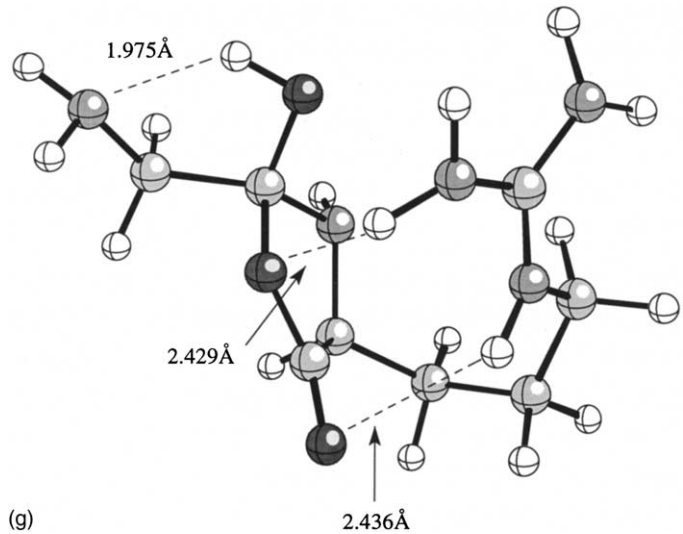


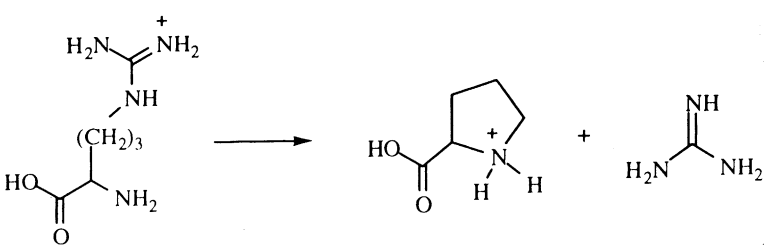
Fig. 4. (*Continued*).

of both Arg-Gly and Gly-Arg do not arise from either peptide, but rather from the anhydride. The presence of a base peak at *m/z* 70 is consistent with the formation of a proline residue from the anhydride as shown in Eq. (6). Given that *all* sequence and non-sequence ions derived from protonated Arg-Gly and Gly-Arg are essentially formed in the same abundance and yield virtually identical MS^3 spectra, it seems a reasonable proposition to assume that they are all formed directly from the anhydride intermediate:

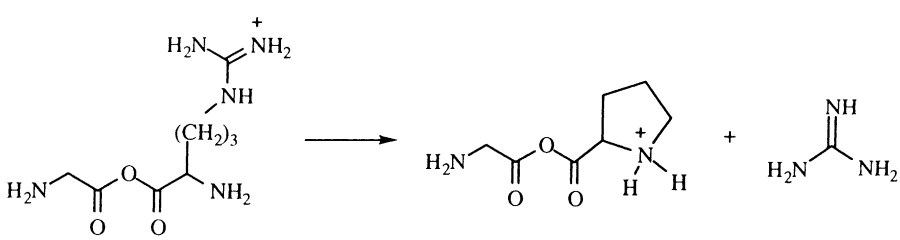
Table 1
Energies of DFT optimized structures shown in Fig. 4

Species	Energy (Hartrees)[a]	ZPVE (Hartrees)[b]	Relative energy (kcal mol^{-1})
(**A**)	−814.99681	0.29294	0
(**B**)	−814.95570	0.29452	26.8
(**C**)	−814.96634	0.29279	19.0
(**D**)	−814.95825	0.29031	22.6
(**E**)	−814.98501	0.29293	7.4
(**F**)	−814.98078	0.29306	10.1
(**G**)	−814.96191	0.29341	22.2

[a] B3LPY/6-31+G* optimization.
[b] Uncorrected.



(5)



(6)

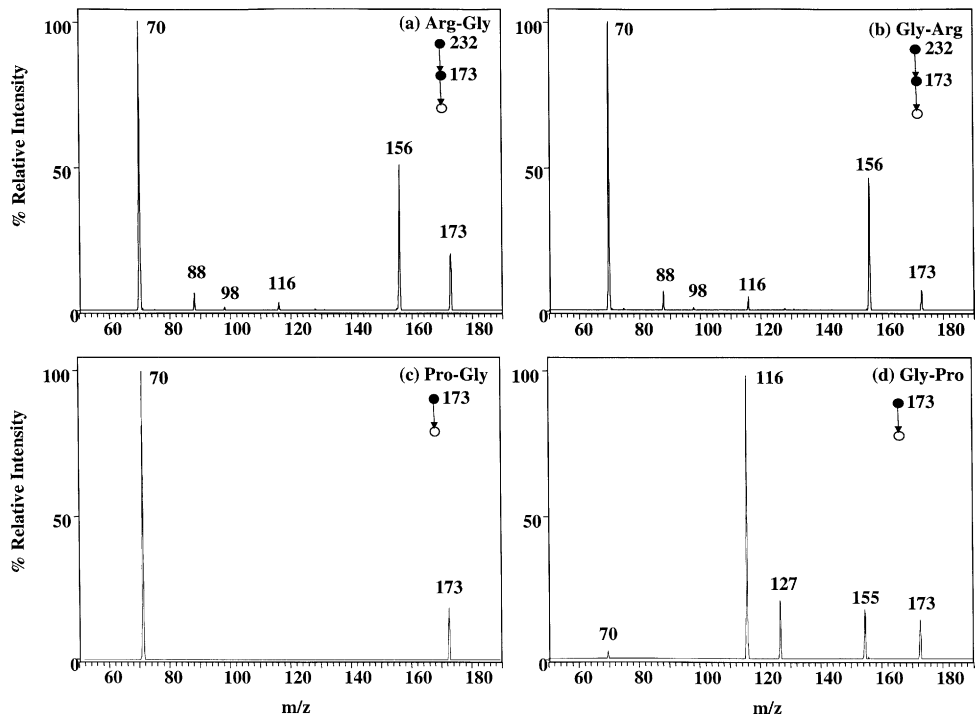
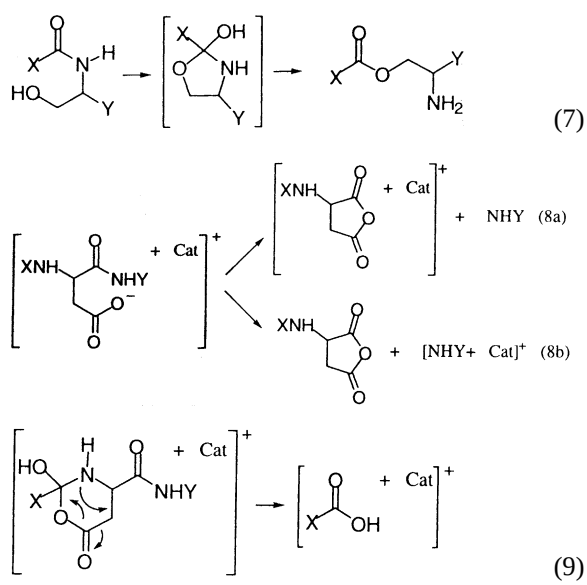
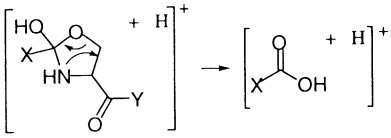


Fig. 5. Comparison of the MS^3 spectra of the $[M + H–(NH_2)_2C=NH]^+$ ions of: (a) Arg-Gly; (b) Gly-Arg with the MS/MS spectra of $[M + H]^+$ ions of: (c) Pro-Gly; (d) Gly-Pro.

3.5. Related fragmentations of peptide ions

It is worth noting that related mechanisms involving a neighboring group attack to form a five membered ring which undergoes subsequent fragmentation operate for: (a) the well known solution phase N–O serine shift (Eq. (7)) [20]; (b) the solution [21] and gas phase [5] aspartic acid C-terminal peptide bond cleavage (Eq. (8)). Furthermore, two recent gas phase rearrangement reactions involving $[b_n + OH + Cat]^+$ formation for serine [22] and α- and β-aspartic acid [23] have been reported. In both instances, concerted fragmentation of the ring intermediate was proposed (Eqs. (9) and (10)). Given the results presented here and in the accompanying paper by Gronert and coworkers [9], it is tempting to speculate that the ring intermediates decompose to form the ester (for serine) and the anhydride (for aspartic acid):





(10)

4. Conclusions

The results presented here highlight the important role that salt bridges play in the rearrangement reaction leading to $[b_n + H_2O]^+$ formation. Salt bridge formation is a result of insufficient "mobile protons" to facilitate bond cleavage, resulting in mobilization of the proton from the C-terminal carboxylic acid [8d]. This rearrangement can be prevented by blocking proton transfer via ester formation or by carrying out CID on the deprotonated peptide (yet again demonstrating the complementary value of MS/MS on negative ions [24]). Together with Gronert's results [9] they suggest that the original concerted mechanism [3] for ring opening is incorrect and that a stepwise mechanism proceeding via a mixed anhydride intermediate is preferred. Finally, these results further highlight the importance of neighboring group processes in peptide fragmentation reactions [8a] especially those which form five membered rings [8b].

Acknowledgements

R.A.J.O. thanks the Australian Research Council for financial support (grant #A29930202) and the University of Melbourne for funds to purchase the LCQ. We acknowledge the Melbourne Advanced Research Computing Centre (MARCC) for their generous allocation of computer time. We have benefited from discussions with Prof. Scott Gronert and thank him for sharing his data before publication. Finally we thank Thomas Taverner for carrying out some DFT calculations and Ana Vrkic for running some mass spectra.

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