What is the Structure of b_2 Ions Generated from Doubly Protonated Tryptic Peptides?

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A recent statistical study (Savitski, M. M.; Falth, M.; Eva Fung, Y. M.; Adams, C. M.; Zubarev, R. A. *J. Am. Soc. for Mass Spectrom.* doi: 10.1016/j.jasms.2008.08.003) of a large spectral database indicated that the product ion spectra of doubly protonated tryptic peptides fall into two distinct classes. The main factor distinguishing the two classes is the relative abundance of the y_{N-2} fragment: for Class I spectra y_{N-2} is the most abundant y fragment while for Class II other y ions dominate the corresponding spectra. To explain the dominance of y_{N-2} for Class I spectra formation of a nontraditional b_2 ion with a diketopiperazine (6-membered cyclic peptide) rather than an oxazolone structure was proposed. Here we present evidence from tandem mass spectrometry, hydrogen/deuterium exchange, and density functional calculations that do not support this proposal. Namely, that CID of doubly protonated YIGSR, YGGFLR, and YIYGSK produce Class I product ion spectra, yet the b_2 fragment is shown to have the traditional oxazolone structure. (*J Am Soc Mass Spectrom* 2009, 20, 618–624) © 2009 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Large-scale protein identification in proteomics is based on peptide sequencing by tandem mass spectrometry (MS/MS) [1–3]. Most commonly, proteins are digested with trypsin and the resulting peptides are introduced into the mass spectrometer by electrospray ionization (ESI) [4]. ESI typically produces multiply charged tryptic peptide ions. The product ion spectra of these ions are assigned by various peptide sequencing algorithms [3]. These currently work most reliably for doubly protonated tryptic peptides [1]. Consequently, the majority of identified proteins in large-scale proteomics projects are assigned on the basis of these spectra too, making the gas-phase fragmentation behavior of doubly protonated tryptic peptides one of the most important chemistries utilized in modern proteomics.

Tryptic peptides contain a basic amino acid residue at their C-terminus as a result of trypsin typically cleaving at the C-terminal of arginine (R) and lysine (K) residues [except when followed by proline (P)]. Notwithstanding the apparent practical importance, relatively little is known about the mechanistic, energetic, and kinetic details of the dissociation channels of the doubly charged ions of these peptides. In an effort to address this problem and build on the knowledge gained from earlier work [5–10] several recent statistical analyses [11–15] of large datasets of peptide mass spectra have been performed. A combined experimen-

tal and theoretical investigation of a model doubly protonated peptide, $[G_5R + 2H]^{2+}$ has also been undertaken recently [16].

Based on findings from principle component analysis of a database containing 15,000 validated MS/MS spectra of doubly protonated tryptic peptides, Zubarev and coworkers [15] recently showed that these spectra fall into two well separated classes. According to their analysis the main factor distinguishing the two classes was the relative abundance of the second peptide bond cleavage. This cleavage results in b_2 and y_{N-2} ions for peptides of N residues long. The product-ion spectra belonging to Class I are dominated by the corresponding y_{N-2} ions [15]. Conversely, product-ion spectra of Class II feature other predominantly y_{N-4} and y_{N-5} fragments as the most abundant. To explain their statistical analysis of y ion abundances the authors argued [15] that formation of a nontraditional b_2 ion for peptides producing Class I spectra was the determining factor producing the statistical anomaly, i.e., formation of protonated diketopiperazine [17, 18] (six-membered cyclic dipeptide) b_2 ions rather than the classical oxazolone [19–21] structure. Peptide ions producing Class II spectra were assumed to exclusively fragment to form b ions with oxazolone structures [15].

The fragmentation chemistries related to formation of the oxazolone and diketopiperazine b_2 isomers have been studied in detail [16, 22–27]. b_2 ions with the oxazolone structure are formed on the b_n - y_m pathways [22, 24]. The related chemistry involves mobilization of the backbone ionizing proton to the second amide bond. Cleavage of this bond is then initiated by nucleophilic attack of the N-terminal amide oxygen to form

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the oxazolone ring. On the other hand, formation of the diketopiperazine isomer b_2 ions on the *cyclic peptide* pathway requires *trans* to *cis* isomerization [23, 25, 26] of the N-terminal amide bond before cleavage of the second peptide bond (without this isomerization, the energetic barriers for the diketopiperazine b_2 ion formation are impassably high). Following this isomerization, a backbone ionizing proton is mobilized to the second amide nitrogen and amide bond cleavage is initiated by nucleophilic attack of the N-terminal amino group. While both pathways involve proton transfer and amide bond cleavage steps, the *trans* to *cis* isomerization step can be kinetically and/or thermodynamically controlled [25].

To assess the validity of Zubarev's "diketopiperazine" hypothesis on Class I spectra, we have investigated the dissociation chemistry of doubly protonated YIGSR, YGGFLR, and YIYGSFK using a combined experimental and computational strategy. Structures relevant to *trans* to *cis* isomerization of the N-terminal amide bond and to cleavage of the second amide bond have been calculated for doubly protonated YIGSR. Furthermore, we used the recently recommended SORI-hydrogen/deuterium exchange (SORI-HDX) technique [28] to probe the structure of b_2 ions formed by CID of the above peptides.

Materials

YIGSR, YIYGSFK, and YGGFLR were purchased from American Peptide Company (Sunnyvale, CA) and were used as received.

Mass Spectrometry

An IonSpec (Lake Forrest, CA) 4.7T FT-ICR instrument was used for the CID and HDX experiments. Sustained off-resonance irradiation-CID (SORI-CID) with Ar collision gas was used as the ion activation technique to induce fragmentation of the doubly protonated precursor ions. Singly and doubly protonated peptides were generated from ca. 10 μM MeOH:H₂O 1:1 solutions (containing 2% acetic acid) by an Analytica (Branford, CT) second-generation electrospray source. The capillary temperature was kept at 60 °C and a needle voltage of 3.8 kV was applied. The FT-ICR instrument was modified in-house for ion-molecule reaction studies by incorporating the pulsed-leak configuration described by Jiao et al. [29] Typical CD₃OD pressure was in the range of $3\text{--}5 \times 10^{-7}$ Torr, with a base pressure in the ICR analyzer of 7×10^{-11} Torr. The precursor *doubly* charged ions were selected monoisotopically but little "contamination" from the ¹³C isotope was detected. The H/D exchange reaction time and the gap between the SORI activation and beginning of H/D exchange were adjusted so that relative kinetic rate constants could be obtained. Note that there is no fragment ion selection after the fragmentation so that information on the relative H/D exchange kinetics was obtained under the *same* experimental conditions on *all* the fragments and the surviving precursor ion (if any) simul-

taneously. For further details and other examples for the usefulness of the SORI-HDX technique, see reference [28].

Calculations

A recently developed conformational search engine [16, 23–26] devised to deal with protonated peptides was used to scan the potential energy surface (PES) of doubly protonated YIGSR and its fragment ions. These calculations started with molecular dynamics simulations scanning various protonation sites using the Insight II program (Biosym Technologies, San Diego, CA) in conjunction with the AMBER force field [30] modified in-house to enable the study of oxygen and nitrogen protonated amide bonds, species with C-terminal oxazolone and imine moieties, and the b_n - y_m and *cyclic peptide* type transition structures [26].

During the dynamics calculations, we used simulated annealing techniques to produce candidate structures for further refinement, applying full geometry optimization using the AMBER force field. The AMBER optimized structures were analyzed by a conformer family search program developed in Heidelberg. This

Figure 1. (a) SORI-CID spectrum of doubly protonated YIGSR. (b) SORI-HDX spectrum of doubly protonated YIGSR. Fragments were exposed to CD₃OD for 10 s at 3.2×10^{-7} Torr pressure in the HDX experiment. Significant and characteristic D uptake is explicitly denoted by D_n .

program groups optimized structures into families for which the most important characteristic torsion angles of the molecule are similar. The most stable species in the families were then fully optimized at the PM3, HF/3-21G, B3LYP/6-31G(d), and finally at the B3LYP/6-31+G(d,p) levels, where the conformer families were regenerated at each level. Vibrational frequencies determined at the B3LYP/6-31G(d) level of theory were used to calculate zero-point energy corrections to the total energies obtained at B3LYP/6-31+G(d,p). Activation and reaction entropies were computed by partition functions obtained by the rigid rotor-harmonic oscillation

approach as implemented in the Gaussian suite of programs [31] using unscaled frequency calculations obtained at the 6-31G(d) level of theory.

Results and Discussion

CID of Doubly Protonated YIGSR, YGGFLR, and YIYGSFK

The SORI (Ar) MS/MS spectra of $[YIGSR + 2H]^{2+}$ (Figure 1a), $[YGGFLR + 2H]^{2+}$ (Figure S1a, Supporting Information, which can be found in the electronic version of this

Scheme 1. (a) Relay-type H/D exchange mechanism for (a) oxazolone and (b) diketopiperazine b_2 ions. The spectator amino acid side chains are denoted by R1 and R2.

Scheme 2. (a) Relay-type H/D exchange mechanism for linear a_2 ions (b). Cyclic a_2 ion structures do not undergo relay-type H/D exchange. The spectator amino acid side chains are denoted by R1 and R2.

article), and $[YYGSFK + 2H]^{2+}$ (Figure S2a, Supporting Information) display characteristic fragments that are usually observed in MS/MS spectra of doubly protonated tryptic peptides [6–9]. The high m/z region above the precursor ion m/z contains singly charged y ions [for example y_3 (m/z 329) for YIGSR in Figure 1a] while prominent a_2 , b_2 , a_1 and y_1 ions are present at lower m/z ratios. Additionally, several peaks resulting from small molecule losses are also observed. The presence of the dominating y_{N-2} peak in all three spectra shows that these product ion spectra belong to Class I as defined by Zubarev and coworkers [15], making them good model systems to test the “diketopiperazine” hypothesis. Accordingly, their proposal argues that the corresponding b_2 fragments will have the diketopiperazine structure.

Proton Mobility, *trans-cis* Isomerization and Amide Bond Cleavage Transition Structures of $[YIGSR + 2H]^{2+}$

Detailed scans of the potential energy surface of doubly protonated YIGSR were performed to probe whether the corresponding b_2 fragment is formed on the b_n - y_m or *cyclic peptide* pathways. In all calculations, we assumed that one of the extra protons is sequestered at the

Table 1. Relative energetics (E_{rel}) of selected $[YIGSR + 2H]^{2+}$ species calculated at the B3LYP/6-31 + G(d,p) level with zero-point energy (ZPVE) correction calculated at B3LYP/6-31G(d)

Position of the backbone ionizing proton	$E_{\text{rel}}/\text{kcal mol}^{-1}$
N-terminal amino ^a	0.0
Y(1)–I(2) amide oxygen ^a	5.8
I(2)–G(3) amide oxygen ^a	12.3
Y(1)–I(2) amide nitrogen ^a	21.5
I(2)–G(3) amide nitrogen ^a	19.8
N-terminal amino ^b	0.3
Y(1)–I(2) amide oxygen ^b	8.5

^aAll-*trans* configuration of peptide bonds in doubly protonated YIGSR.

^bThe Y(1)–I(2) amide bond is in *cis* configuration.

Table 2. Local proton affinities (PAs, kcal mol^{-1}) of oxazolone and diketopiperazine isomers for the YI and YG sequences. PAs are calculated at the B3LYP/6-31 + G(d,p) level with zero-point energy (ZPVE) correction determined at B3LYP/6-31G(d)

Protonation site	Proton affinity
YI oxazolone	
Amino nitrogen	223.3
Oxazolone nitrogen	226.6
YI diketopiperazine	
Y amide oxygen	215.2
Y amide nitrogen	199.2
I amide oxygen	211.9
I amide nitrogen	199.3
YG oxazolone	
Amino nitrogen	221.0
Oxazolone nitrogen	221.4
YG diketopiperazine	
Y amide oxygen	210.9
Y amide nitrogen	194.5
G amide oxygen	206.8
G amide nitrogen	193.3

C-terminal R residue due to its high basicity while the other extra proton occupies a backbone protonation site. Our data indicate (Table 1) that the global minimum of $[YIGSR + 2H]^{2+}$ is a species where the second backbone ionizing proton is located at the N-terminus. Amide oxygen protonated species are less favored and the relative energies of the Y(1)–I(2) and I(2)–G(3) amide O protonated structures are at 5.8 and 12.3 kcal mol^{-1} , respectively. As expected, transfer of the backbone ionizing proton to amide nitrogens is more demanding and requires $\sim 20 \text{ kcal mol}^{-1}$ relative energy.

To probe thermodynamic control on *trans-cis* isomerization of the N-terminal amide bond we determined N-terminal amino and Y(1)–I(2) amide oxygen protonated structures with *cis* N-terminal amide bonds. The amino protonated structure with *cis* Y(1)–I(2) amide bond (0.3 kcal mol^{-1} relative energy) is only slightly above the all-*trans* global minimum. This is in clear contrast to what we have found for doubly protonated G_5R [16] where the all-*trans* species were significantly more stable than those with N-terminal *cis* amide bond. This suggests that *trans-cis* isomerization of the N-terminal amide bond is not thermodynamically controlled for $[YIGSR + 2H]^{2+}$.

In the next step we attempted to find low-lying *trans-cis* isomerization transition structures for the first amide bond. These calculations did locate ways of isomerizing this bond, but at large energetic cost (i.e., required more energy than the oxazolone-forming b_2 - y_3 transition structure (TS), see below). This was also the case for $[G_5R + 2H]^{2+}$ previously [16]. This is strong evidence against the formation of diketopiperazine b_2 ions on the *cyclic peptide* pathway.

Next we determined transition structures that lead to the oxazolone and diketopiperazine isomers of b_2 ions. The oxazolone-forming b_2 - y_3 TS (Figure S5) has a low threshold energy at 31.7 kcal mol^{-1} and a positive entropy of activation (6.7 cal mol^{-1}). The *cyclic peptide*

TS (Figure S6) is far less energetically ($39.6 \text{ kcal mol}^{-1}$) and entropically ($-0.3 \text{ cal mol}^{-1}$) favorable than the corresponding oxazolone-forming b_2 - y_3 pathway, so based on the calculations, this is not a likely mechanism for the b_2 ion formation.

SORI-HDX Spectra of Doubly Protonated YIGSR, YGGFLR, and YIYGSFK

To gain further insight into the structures of b_2 and other fragment ions we used the recently introduced SORI-HDX technique [28]. The SORI-HDX spectra of $[\text{YIGSR} + 2\text{H}]^{2+}$, $[\text{YGGFLR} + 2\text{H}]^{2+}$, and $[\text{YIYGSFK} + 2\text{H}]^{2+}$ obtained after 10 s exchange with CD_3OD are shown in Figure 1b, Figures S1b and S2b (Supporting Information), respectively. The y fragments of these peptides were not found to undergo HDX under the applied experimental conditions. Conversely the b ions readily exchanged protons for deuteriums, for example b_2 of $[\text{YIGSR} + 2\text{H}]^{2+}$ exchanges up to 3 hydrogens in

10 s. It was also determined that none of the a_2 fragments formed from the corresponding b_2 fragments underwent HDX in these experiments.

The mechanism of HDX in the gas phase has been studied by Beauchamp and coworkers [32]. These authors found that relay-type H/D exchange with CD_3OD required two protonation sites with *similar* local proton affinities, which could be *spatially bounded* by the reagent molecule. Relay type HDX mechanisms are schematically illustrated for the oxazolone and diketopiperazine isomers of b_2 ions in Scheme 1. Here we assume that the amino acid side chains do not have a basic group that can take part in the HDX process. The oxazolone isomer has two likely protonation sites: the nitrogens of the N-terminal amino group and the oxazolone ring. For most of the oxazolones composed of two amino acid residues, these two sites have similar local proton affinities, the computed YI and YG values are summarized in Table 2. It is worth noting here that the differences between the two local PAs are small: 3.3

Figure 2. The a_2 - b_2 region of the product ion spectrum of doubly protonated YIGSR after (a) 0s, (b) 1s, (c) 10s, and (d) 30s HDX. Significant and characteristic D uptake is explicitly denoted by D_n .

and 0.4 kcal mol⁻¹ for the YI and YG sequences, respectively. Furthermore, the energetically most favored YI_{oxa} (Figure S7, Supporting Information) and YG_{oxa} structures are stabilized by strong H-bonds bridging these two protonation sites. This interaction keeps the two nitrogens in close proximity and therefore both the protonation energetics and the stabilizing H-bonding favor facile HDX for the oxazolone isomer of b_2 .

The diketopiperazine b_2 isomer also has two likely protonation sites: the two amide oxygens. It is not surprising that the local proton affinities of these sites are similar (Table 2). However, the two oxygens are kept far from each other by the rigid, planar diketopiperazine ring. This prevents the relay type HDX between these two sites from being possible (Reaction A in Scheme 1b). Another possibility for a relay type HDX involves the oxygen and nitrogen atoms of the same amide bond (Reaction B in Scheme 1b). This reaction is hindered by the large PA differences between amide oxygens and nitrogens in the 13–16 kcal mol⁻¹ range (Table 2). Consequently neither HDX possibility for diketopiperazine b_2 ions satisfies the prerequisites [32] of the relay mechanism so in theory, these ions should not undergo (or undergo very limited) HDX with CD₃OD.

As mentioned in the preceding, the SORI-HDX spectra of [YIGSR + 2H]²⁺, [YGGFLR + 2H]²⁺, and [YIYGSFK + 2H]²⁺ indicate facile HDX for the corresponding b_2 ions on the 10s time frame. Figure 2 summarizes further time-dependent data on the a_2 - b_2 region of the product ion spectrum of [YIGSR + 2H]²⁺. (Similar data are presented for [YGGFLR + 2H]²⁺ and [YIYGSFK + 2H]²⁺ in Figures S3 and S4 in the Supporting Information). Nearly complete exchange of the three labile protons is observed in 30 s. These spectra clearly suggest that the b_2 ions of these peptides are oxazolones and not diketopiperazines. As the product ion spectra of [YIGSR + 2H]²⁺, [YGGFLR + 2H]²⁺, and [YIYGSFK + 2H]²⁺ belong to Class I in Zubarev's classification, the finding of oxazolone b_2 ions means that some reason(s) other than the "diketopiperazine effect" must cause the bimodal distribution observed.

While not directly related to the major topic of this paper, our SORI-HDX experiments provide valuable information on the structure of a_2 fragments as well. These ions can have either linear or cyclic structures (Scheme 2 and Figure S7) [33–35], which can interconvert crossing a relatively low barrier. According to the mechanism of Scheme 2 and the local PA data presented in Table 3, the linear structure is expected to undergo facile HDX. On the other hand, the cyclic isomer does not have two spatially close protonation sites with similar local proton affinities. This clearly indicates that cyclic a_2 should undergo very limited HDX with CD₃OD. The SORI-HDX data indicate that a_2 is cyclic for the YI and YG sequences: no exchange is observed even on the 30 s time scale. This observation is in line with our theoretical data that shows that the

Table 3. Local proton affinities (PAs, kcal mol⁻¹) of the linear YI and YG imines and the corresponding cyclic forms. PAs are calculated at the B3LYP/6-31 + G(d,p) level with zero-point energy (ZPVE) correction determined at B3LYP/6-31G(d)

Protonation site	Proton affinity
YI linear imine	
Imine nitrogen	218.0
N-terminal nitrogen	216.5
YI cyclic form	
Cyclic Y nitrogen	225.3
Cyclic I oxygen	221.6
Cyclic I nitrogen	209.0
YG linear imine	
Imine nitrogen	200.6
N-terminal nitrogen	207.3
YG cyclic form	
Cyclic Y nitrogen	218.2
Cyclic G oxygen	216.3
Cyclic G nitrogen	198.6

cyclic a_2 structures are 6–7 kcal mol⁻¹ more stable than their linear counterparts and the TS necessary to form these cyclic species being less energetically demanding than the preceding b_2 - a_2 TS (Figure S7).

Conclusions

Our combined experimental and theoretical investigation yielded the following results on the fragmentation chemistry of doubly protonated YIGSR, YGGFLR, and YIYGSFK:

1. Low-energy SORI-CID of [YIGSR + 2H]²⁺, [YGGFLR + 2H]²⁺, and [YIYGSFK + 2H]²⁺ produces fragmentation patterns that are typical for doubly charged tryptic peptides. The corresponding product ion spectra belong to Class I according to Zubarev's classification [15].
2. Calculations indicate that *trans-cis* isomerization of the N-terminal amide bond of [YIGSR + 2H]²⁺ is kinetically controlled.
3. The b_2 - y_3 pathway has a low threshold energy clearly explaining the dominance of fragments originating from cleavage of the I(2)–G(3) amide bond. The *cyclic peptide* pathway could in principle produce the same ions, however the corresponding TS is both energetically and entropically less favored than the b_2 - y_3 TS.
4. Our SORI-HDX data indicate that b_2 ions formed from [YIGSR + 2H]²⁺, [YGGFLR + 2H]²⁺, and [YIYGSFK + 2H]²⁺ have a protonated oxazolone structure strongly suggesting that the *cyclic peptide* pathway is not active to any significant extent.
5. Our SORI-HDX data and calculations show that the a_2 ion is cyclic as had been proposed previously for some simpler a_2 ions [33].
6. The exact reason why a bimodal distribution is observed for the maximum intensity y fragments by Zubarev and coworkers [15] remains unresolved. Our combined experimental and computational

work here on model tryptic peptides suggests that this statistical observation cannot be explained solely by the “diketopiperazine” effect. Subsequent experimental and computational work is under way in our laboratories on further aspects of the fragmentation chemistry of doubly protonated tryptic peptides.

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References

- Aebersold, R.; Goodlett, D. R. Mass Spectrometry in Proteomics. *Chem. Rev.* **2001**, *101*, 269–295.
- Hunt, D. F.; Yates, John R. III; Shabanovitz, J.; Winston, S.; Hauer, C. R. Protein Sequencing by Mass Spectrometry. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 6233–6237.
- Steen, H.; Mann, M. The ABCs (and XYZs) of Peptide Sequencing. *Nat. Rev. Mol. Cell. Biol.* **2004**, *5*, 699–711.
- Fenn, J. B.; Mann, M.; Meng, C. K.; Wong, S. F.; Whitehouse, C. M. Electrospray Ionization for Mass-Spectrometry of Large Biomolecules. *Science* **1989**, *246*, 64–71.
- Vékey, K.; Candido, M.; Traldi, P. Use of Charge-Separation Reactions for Sequencing Peptides. *Rapid Commun. Mass Spectrom.* **1990**, *4*, 74–76.
- Tang, X.; Boyd, R. K. An Investigation of Fragmentation Mechanisms of Doubly Protonated Tryptic Peptides. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 651–657.
- Tang, X.-J.; Thibault, P.; Boyd, R. K. Fragmentation Reactions of Multiply-Protonated Peptides and Implications for Sequencing by Tandem Mass Spectrometry with Low-Energy Collision-Induced Dissociation. *Anal. Chem.* **1993**, *65*, 2824–2834.
- Burlet, O.; Orkiszewski, R. S.; Ballard, K. D.; Gaskell, S. J. Charge Promotion of Low-Energy Fragmentations of Peptide Ions. *Rapid Commun. Mass Spectrom.* **1992**, *6*, 658–662.
- Mann, M.; Wilm, M. Error-Tolerant Identification of Peptides in Sequence Databases by Peptide Sequence Tags. *Anal. Chem.* **1994**, *66*, 4390–4399.
- Adams, J.; Strobel, F. H.; Reiter, A.; Sullards, M. C. The Importance of Charge-Separation Reactions in Tandem Mass Spectrometry of Doubly Protonated Angiotensin II Formed by Electrospray Ionization: Experimental Considerations and Structural Implications. *J. Am. Soc. Mass Spectrom.* **1996**, *7*, 30–41.
- Kapp, E. A.; Schutz, F.; Reid, G. E.; Moritz, R. L.; O'Hair, R. A. J.; Simpson, R. J. Mining a Tandem Mass Spectrometry Database to Determine the Trends and Global Factors Influencing Peptide Fragmentation. *Anal. Chem.* **2003**, *75*, 6251–6264.
- Huang, Y. Y.; Triscari, J. M.; Tseng, G. C.; Pasa-Tolic, L.; Smith, R. D.; Wysocki, V. H. Statistical Characterization of the Charge State and Residue Dependence of Low-Energy CID Peptide Dissociation Patterns. *Anal. Chem.* **2005**, *77*, 5800–5813.
- Martin, D. B.; Eng, J. K.; Nesvizhskii, A. I.; Aebersold, R. Investigation of Neutral Loss During Collision-Induced Dissociation of Peptide Ions. *Anal. Chem.* **2005**, *77*, 4870–4882.
- Savitski, M. M.; Kjeldsen, F.; Nielsen, M. L.; Garbuzynskiy, S. O.; Galzitskaya, O. V.; Surin, A. K.; Zubarev, R. A. Backbone Carbonyl Group Basicities are Related to Gas-Phase Fragmentation of Peptides and Protein Folding. *Angew. Chem. Int. Ed.* **2007**, *46*, 1481–1484.
- Savitski, M. M.; Falth, M.; Eva, Fung Y. M.; Adams, C. M.; Zubarev, R. A. Bifurcating Fragmentation Behavior of Gas-Phase Tryptic Peptide Dications in Collisional Activation. *J. Am. Soc. Mass Spectrom.* **2008**, doi: 10.1016/j.jasms.2008.08.003.
- Bleilholder, C.; Suhai, S.; Somogyi, A.; Paizs, B. Fragmentation Pathways of Doubly Protonated Tryptic Peptides. *J. Am. Soc. Mass Spectrom.* unpublished.
- Cordero, M. M.; Houser, J. J.; Wesdemiotis, C. The Neutral Products Formed During Backbone Fragmentations of Protonated Peptides in Tandem Mass Spectrometry. *Anal. Chem.* **1993**, *65*, 1594–1601.
- Nold, M. J.; Wesdemiotis, C.; Yalcin, T.; Harrison, A. G. Amide Bond Dissociation in Protonated Peptides. Structures of the N-Terminal Ionic and Neutral Fragments. *Int. J. Mass Spectrom. Ion Processes.* **1997**, *164*, 137–153.
- Yalcin, T.; Csizmadia, I. G.; Peterson, M. R.; Harrison, A. G. The Structure and Fragmentation of $B - n$ ($n < 3$) Ions in Peptide Spectra. *J. Am. Soc. Mass Spectrom.* **1996**, *7*, 233–242.
- Yalcin, T.; Khouw, C.; Csizmadia, I. G.; Peterson, M. R.; Harrison, A. G. Why are b Ions Stable Species in Peptide Spectra? *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 1165–1174.
- Polfer, N. C.; Oomens, J.; Suhai, S.; Paizs, B. Spectroscopic and Theoretical Evidence for Oxazolone Ring Formation in Collision Induced Dissociation of Peptides. *J. Am. Chem. Soc.* **2005**, *127*, 17154–17155.
- Paizs, B.; Lendvay, G.; Vékey, K.; Suhai, S. Formation of b_{2+} Ions from Protonated Peptides: An Ab Initio Study. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 525–533.
- Paizs, B.; Suhai, S. Combined Quantum Chemical and RRKM Modeling of the Main Fragmentation Pathways of Protonated GGG. I. *Cis-trans* Isomerization Around Protonated Amide Bonds. *Rapid Commun. Mass Spectrom.* **2002**, *15*, 2307–2323.
- Paizs, B.; Suhai, S. Combined Quantum Chemical and RRKM Modeling of the Main Fragmentation Pathways of Protonated GGG. II. Formation of b_2 , y_1 , and y_2 ions. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 375–389.
- Paizs, B.; Suhai, S. Towards Understanding the Tandem Mass Spectra of Protonated Oligopeptides. 1: Mechanism of Amide Bond Cleavage. *J. Am. Soc. Mass Spectrom.* **2004**, *15*, 103–112.
- Paizs, B.; Suhai, S. Fragmentation Pathways of Protonated Peptides. *Mass Spectrom. Rev.* **2005**, *24*, 508–548.
- Farrugia, J. M.; O'Hair, R. A. J.; Reid, G. E. Do All b_2 Ions Have Oxazolone Structures? Multistage Mass Spectrometry and Ab Initio Studies on Protonated N-Acyl Amino Acid Methyl Ester Model Systems. *Int. J. Mass Spectrom.* **2001**, *210/211*, 71–87.
- Somogyi, A. Probing Peptide Fragment Ion Structures by Combining Sustained Off-Resonance Collision Induced Dissociation and Gas-Phase H/D Exchange (SORI-HDX) in Fourier Transform Ion-Cyclotron Resonance (FT-ICR) Instruments. *J. Am. Soc. Mass Spectrom.* doi:10.1016/j.jasms.2008.08.012.
- Jiao, C. Q.; Ranatunga, D. R. A.; Vaughn, W. E.; Freiser, B. S. A Pulsed-Leak Valve for Use with Ion Trapping Mass Spectrometers. *J. Am. Soc. Mass Spectrom.* **1996**, *7*, 118–122.
- Case, D. A.; Pearlman, D. A.; Caldwell, J. W.; Cheatham, T. E. III; Ross, W. S.; Simmerling, C. L.; Darden, T. A.; Merz, K. M.; Stanton, R. V.; Cheng, A. L.; Vincent, J. J.; Crowley, M.; Tsui, V.; Radmer, R. J.; Duan, Y.; Pitera, J.; Massova, I. G.; Seibel, G. L.; Singh, U. C.; Weiner, P. K.; Kollman, P. A. In: *AMBER 99*; University of California: San Francisco, 1999.
- Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A. Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. Gaussian 2004, Rev. C; Gaussian, Inc.: Wallingford, CT, 2004.
- Campbell, S.; Rodgers, M. T.; Marzluff, E. M.; Beauchamp, J. L. Structural and Energetic Constraints on Gas Phase Hydrogen/Deuterium Exchange Reactions of Protonated Peptides with D₂O, CD₃OD, CD₃CO₂D, and ND₃. *J. Am. Chem. Soc.* **1994**, *116*, 9765–9766.
- El Aribi, H.; Orlova, G.; Rodriguez, C. F.; Almeida, D. R. P.; Hopkinson, A. C.; Siu, K. W. M. Fragmentation Mechanisms of Product Ions from Protonated Tripeptides. *J. Phys. Chem. B* **2004**, *108*, 18743–18749.
- Harrison, A. G.; Young, A. B.; Schnoelzer, M.; Paizs, B. Formation of Iminium Ions by Fragmentation of a_2 Ions. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 1635–1640.
- Allen, J. M.; Black, D. M.; Johnson, J. S.; Bythell, B. J.; Paizs, B.; Glish, G. L. Why are a_3 Ions Rarely Observed? *J. Am. Soc. Mass Spectrom.* **2008**, *19*, 1764–1770.