

# Infrared Multiple-Photon Dissociation Action Spectroscopy of the $b_2^+$ Ion from PPG: Evidence of Third Residue Affecting $b_2^+$ Fragment Structure

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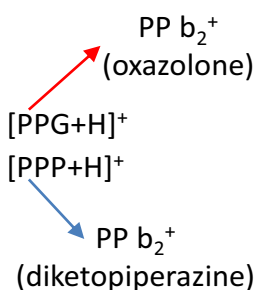
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**Abstract.** Infrared multiple-photon dissociation (IRMPD) action spectroscopy was performed on the  $b_2^+$  fragment ion from the protonated PPG tripeptide. Comparison of the experimental infrared spectrum with computed spectra for both oxazolone and diketopiperazine structures indicates that the majority of the fragment ion population has an oxazolone structure with the remainder having a diketopiperazine structure. This result is in contrast with a recent study of the IRMPD action spectrum of the PP  $b_2^+$  fragment ion from PPP, which was found to be nearly 100% diketopiperazine (Martens et al. *Int. J. Mass Spectrom.* 2015, **377**, 179). The diketopiperazine  $b_2^+$  ion is thermodynamically more stable than the oxazolone but normally requires a *trans/cis* peptide bond isomerization in the dissociating peptide. Martens et al. showed through

IRMPD action spectroscopy that the PPP precursor ion was in a conformation in which the first peptide bond is already in the *cis* conformation and thus it was energetically favorable to form the thermodynamically-favored diketopiperazine  $b_2^+$  ion. In the present case, solution-phase NMR spectroscopy and gas-phase IRMPD action spectroscopy show that the PPG precursor ion has its first amide bond in a *trans* configuration suggesting that the third residue is playing an important role in both the structure of the peptide and the associated ring-closure barriers for oxazolone and diketopiperazine formation.

**Keywords:** Peptide fragments, IRMPD spectroscopy,  $b_2$  ions

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## Introduction

With the reliance on mass spectrometry-based proteomics in the identification of proteins in biological research, the gas-phase fundamentals of peptide sequencing are of major

importance. Even with the development of increasingly more sophisticated software and instrumentation that has made data collection and processing simpler and more reliable, protein sequence coverage is still greatly affected by the presence or lack of specific amino acid residues or combinations of residues within a protein [1]. Much of the work that has been focused on improving algorithms has made use of specific amino acid sequence trends [2] that are still not fully understood. In order to further these pursuits, there must be explanations as to the underlying fundamentals of peptide fragmentation mechanisms and how specific residues may guide protein coverage challenges.

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Collision-induced dissociation produces fragments that form via cleavage at the C–N peptide bond along the peptide backbone, where charge can either be retained on the C-terminus to form a  $y$ -ion or N-terminally to produce a  $b$ -ion [3, 4]. One focus in peptide fragmentation and the underlying mechanisms has centered on the  $b_2^+$  ion, as its structure is directly tied to the conformation of the peptide backbone of the precursor peptide. Fragmentation may lead to either a five-membered oxazolone ring, which retains a *trans* conformation at the peptide bond, or a diketopiperazine six-membered ring structure, which can only form via *trans-cis* isomerization of the peptide bond [5, 6]. For most amino acid sequences, the most common structure formed for the  $b_2^+$  ion is the oxazolone despite the fact that the diketopiperazine is typically found to be more thermodynamically stable. The thermodynamic preference for *trans*-amide bonds in peptides is thought to be a contributing factor for this finding. The difference in ring closure barriers for the formation of oxazolone and diketopiperazine is also likely playing a role in the prevalence of the oxazolone [7, 8].

Proline is well known to significantly affect the secondary structure of peptides and proteins and the fragmentation of any peptide in which it resides, leading to the “proline effect” [9–11], which is preferential cleavage of the peptide bond N-terminal to the proline, resulting in enhanced intensity of the corresponding  $y$ -ion due in part to the increased proton affinity of the secondary amine proline [12, 13]. Consequently, proline has been part of many peptide fragmentation model studies. Proline-containing peptides have been shown to have a higher propensity for forming *cis* peptide bonds at the Pro residue as its barrier to *trans/cis* isomerization of the N-terminally adjacent peptide bond is  $\sim 55$  kJ/mol compared with the average  $\sim 80$  kJ/mol for the other 19 protein amino acids [14]. The difference in energy between the *trans* and *cis* isomers of proline-containing peptides is also 8 kJ/mol smaller than the difference for other residues [15]. Consequently, the diketopiperazine  $b_2^+$  ion is observed at greater frequency in model peptides that contain proline in the second position presumably because formation of the *cis*-peptide bond required to facilitate head-to-tail cyclization is significantly easier with proline in this position. In previous research, Gucinski et al. observed mixtures of oxazolone and diketopiperazine in the  $b_2^+$  ion structures of Val-Pro, Ala-Pro, and Ile-Pro, and only the diketopiperazine structure for His-Pro, which stressed the importance of the *trans/cis* isomerization barrier in the formation of the diketopiperazine and explained that for His-Pro there was a combined effect of the basic imidazole residue from the histidine in the first position and the second position proline contributing to the stability of the *cis*-form of the peptide [16].

Recent efforts have also dealt with the peptide fragments formed from proline at different positions within the peptide backbone and the ways that multiple proline residues within a peptide can affect their precursor and fragment structures [13, 17–20]. In analysis of precursor peptides containing proline, Masson et al., using both spectroscopy and ion mobility, confirmed that GPGG was comprised of a larger kinetically trapped *trans*-Pro population and a smaller *cis*-Pro population that was found to be more thermodynamically stable in the gas-phase [21].

While much of the work from previous studies has focused on the effect of the second position proline and the effect of single proline residues within a peptide, polyproline has also been an important biologically relevant sequence feature, most notably due to its presence in collagen and in structural motifs of proteins such as beta-helices [22–24]. Likewise, polyproline chains, which have been extensively studied both in solution and the gas phase for their structural effects, have been observed for their influence in the formation of  $b$ -ion structures [25]. Most recently, Martens et al. [26] have examined the fragment ions of small polyproline chains from the tripeptide to hexapeptide. IRMPD action spectroscopy was used to confirm ion mobility and modeling studies that found that small polyproline peptides adopt structures with the first amide bond in the *cis* configuration and the remaining amide bonds in either *cis* or *trans* configuration [22]. For the PPP tripeptide, this study found the resulting  $b_2^+$  ion to exist as exclusively a diketopiperazine with the IRMPD action spectrum of the precursor tripeptide most closely matching that of the *cis-cis* conformation for the two peptide bonds within the peptide.

In this work, we follow up the work from the polyproline PPP and compare the results to the tripeptide PPG, which is most relevantly found as part of the sequence of bradykinin, and serves to compare the influence of the third residue on the resulting  $b_2^+$  ion structure. As with the previous experiments performed on polyproline, ion structures were determined via IRMPD action spectroscopy and were compared to DFT calculations. Once again, the *cis/trans* nature of the amide bonds within the precursor tripeptide are of critical importance in addressing the nature of the  $b_2^+$  ion structure, and from this experiment direct comparisons can be made on the influence of the third residue.

## Experimental

Infrared multiphoton dissociation spectra were obtained at the Free Electron Laser for Infrared eXperiments (FELIX) [27, 28] facility in Nijmegen, the Netherlands and at the Centre Laser Infrarouge d’Orsay in Orsay, France [29, 30].

### FELIX

A dilute solution (0.1  $\mu$ M) of PPG in slightly acidified acetonitrile (0.1% formic acid) was directly infused into a modified Bruker Amazon ion trap mass spectrometer coupled to the FELIX beamline. The protonated peptide precursor ions  $[M + H]^+$  ( $m/z$  270) were generated from electrospray ionization, mass isolated, and allowed to undergo collision-induced dissociation (CID) with the background helium gas at a collision energy chosen to optimize the formation of the  $b_2^+$  product ion. A representative collision-induced dissociation spectrum for PPG +  $H^+$  is shown in Supplementary Figure S1 of Supporting Information. As shown in the spectrum, the major fragment is the “proline effect”  $y_2^+$  ion at  $m/z$  173, but there was sufficient intensity of the  $b_2^+$  ion at  $m/z$  195 for IRMPD action spectroscopy studies. The PP  $b_2^+$  fragment ions are re-isolated and then irradiated with infrared radiation from 900 to 2000  $\text{cm}^{-1}$  from the free-electron laser. The

infrared radiation comes in 5  $\mu\text{s}$  macro-pulses with an approximate energy of 30–40 mJ/pulse and a bandwidth of 0.4% of the center frequency. IRMPD action spectroscopy of the  $b_2^+$  ion gives an  $a_2^+$  fragment at  $m/z$  167 and three internal proline-derived ions at  $m/z$  126, 98, and 70. An IRMPD action spectrum for  $b_2^+$  is generated by plotting the dissociation yield for product ion formation  $\Sigma$  (product ions)/ $\Sigma$  (product ions + unreacted precursor) as a function of wavelength. The wavelength scale is calibrated using a grating spectrometer and the dissociation yield is linearly corrected for variations in laser power.

## CLIO

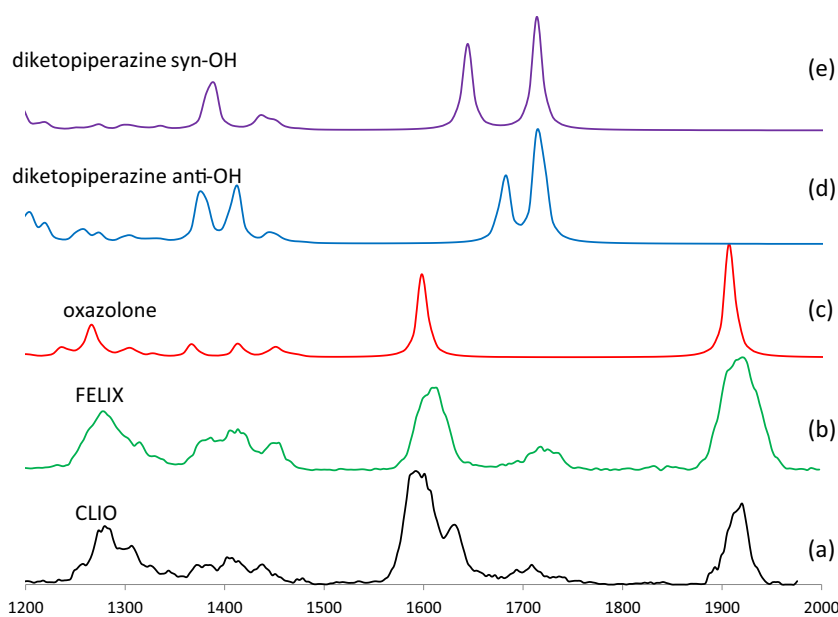
Similar procedures were employed to obtain IRMPD action spectra at the CLIO facility. A dilute solution (10  $\mu\text{M}$ ) of PPG in slightly acidified acetonitrile (0.1% formic acid) was directly infused into a modified Bruker Esquire ion trap mass spectrometer coupled to the CLIO free electron laser source. Parent ion isolation, collision-induced dissociation, and  $b_2^+$  product re-isolation procedures are the same as those in the FELIX laboratory. The collision-induced dissociation spectrum (not shown) for PPG +  $\text{H}^+$  was virtually identical to the spectrum obtained at FELIX, with a dominant  $y_2^+$  “proline effect” fragment ion and a minor  $b_2^+$  ion. The collision energy was varied in order to maximize the intensity of  $b_2^+$ . The FEL output consists of 8  $\mu\text{s}$  macropulses at 25 Hz. The macropulse energy is ca. 20 mJ. The laser wavelength is varied in ca. 6  $\text{cm}^{-1}$  steps and the laser bandwidth is about 0.3%–0.5% of the center wavelength. IRMPD action spectroscopy of the  $b_2^+$  ion gives the same four fragment ions as found in FELIX. The wavelength scale is calibrated by passing part of the beam through a polystyrene film and comparing peaks with the known IR spectrum in the region between 1000 and 2000  $\text{cm}^{-1}$ . The dissociation yield is linearly corrected for variations in laser power.

## Theoretical Methods

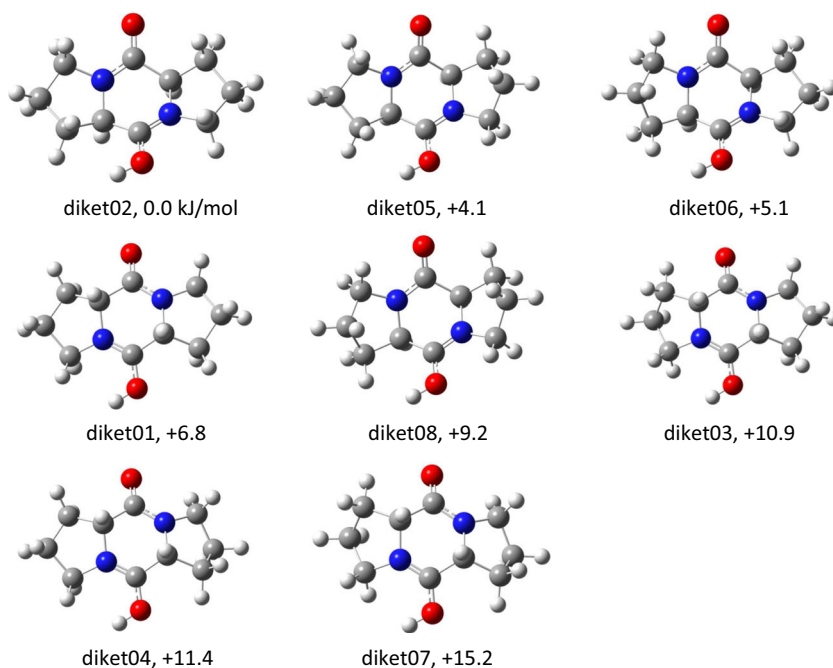
Low energy conformations for the PPG +  $\text{H}^+$  precursor, and both the protonated oxazolone and protonated diketopiperazine forms of PP  $b_2^+$ , were generated using the GMMX conformer searching routine in PCModel 9.3. All conformers within 60 kJ/mol of the global minimum structure were kept. Unique conformers from the GMMX search were used as starting structures for increasingly larger calculations. Geometries, zero-point energies, enthalpy- and free-energy-corrections, and harmonic vibrational frequencies were ultimately calculated at the B3LYP/6-31+G(d,p) level of theory. Single-point energies at the B3LYP/6-311++G(d,p) level of theory were carried out at the double zeta geometry. Harmonic vibrational frequencies for PP  $b_2^+$  were scaled by 0.965 and those for PPG +  $\text{H}^+$  were scaled by 0.970. Stick spectra were broadened by a 20  $\text{cm}^{-1}$  baseline-width Gaussian function.

## Results and Discussion

Collision-induced dissociation of the protonated peptide [PPG +  $\text{H}^+$ ] gives a peak with  $m/z$  195 that corresponds to  $b_2^+$ . This fragment ion was isolated and allowed to interact with tunable infrared radiation from the FEL. IRMPD action spectra for PP  $b_2^+$  ion from CLIO and FELIX are shown in Figure 1a and b, respectively, and clearly show an intense peak near 1925  $\text{cm}^{-1}$  that is diagnostic for an oxazolone structure [31, 32]. Computed infrared spectra for the lowest energy oxazolone and diketopiperazine conformers are also shown in Figure 1c and d, respectively. Additional peaks in the experimental spectrum near 1600, 1282, and 1100  $\text{cm}^{-1}$  also match the oxazolone spectrum. Smaller peaks near 1630 (CLIO), 1700, and 1400



**Figure 1.** Experimental IRMPD action spectrum for  $b_2^+$  ion from PPG obtained at (a) CLIO facility and (b) FELIX. Computed spectra for (c) lowest energy oxazolone conformer, (d) lowest energy diketopiperazine structure with *anti*-OH, and (e) lowest energy diketopiperazine structure with *syn*-OH



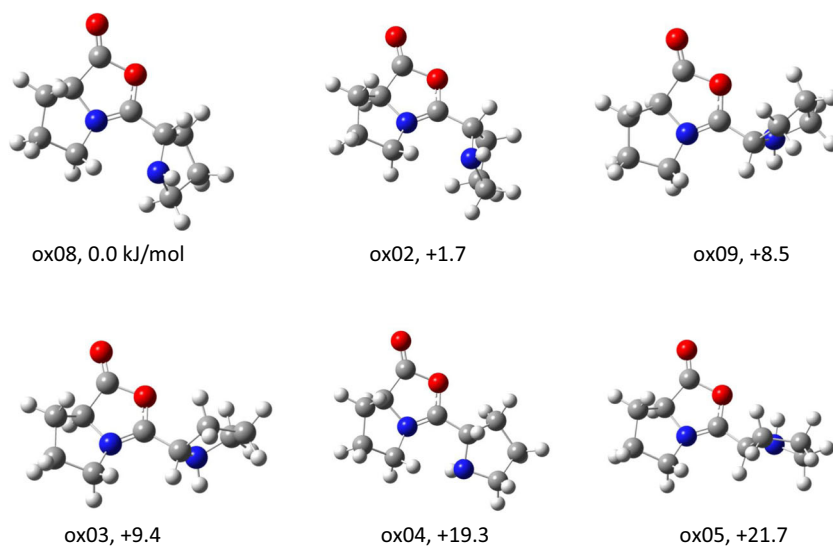
**Figure 2.** Low-energy conformers for diketopiperazine PP  $b_2^+$  ions. Numbers are  $\Delta G$  values in kJ/mol at 298 K relative to the lowest energy diketopiperazine conformer

$\text{cm}^{-1}$  indicate that the gas-phase ion population contains a small amount of the diketopiperazine isomer as well.

Supplementary Table S1 shows the relative energetics of the eight diketopiperazine structures and six oxazolone conformers found in the conformational search. The conformer with the lowest free-energy for PP  $b_2^+$  is a diketopiperazine and is shown in Figure 2. It has both proline rings puckered in the same direction and the ionizing proton *anti* to the ring nitrogen. Higher energy conformers arise from different ring puckering schemes and/or a *syn* arrangement for the ionizing proton with respect to the ring nitrogen atom (Figure 2). The different ring

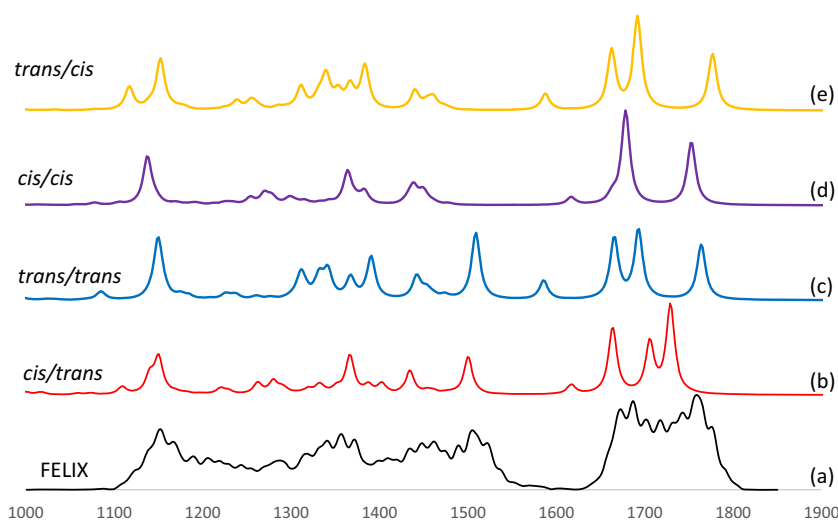
puckering schemes have a modest effect on the computed spectra in this region, whereas a *syn* arrangement for the ionizing proton with respect to the ring nitrogen atom shifts the peak near  $1682\text{ cm}^{-1}$  to the red. For comparison, the computed spectrum of the lowest-energy conformer with a *syn*-OH is also shown in Figure 1e. The four diketopiperazine conformers with the lowest free energies should account for >95% of the diketopiperazine population at 298 K.

The lowest energy protonated oxazolone structure lies ca. 70 kJ/mol higher in energy than the lowest energy diketopiperazine and is shown in Figure 3. Due to the cyclic



**Figure 3.** Low-energy conformers for oxazolone PP  $b_2^+$  ions. Numbers are  $\Delta G$  values in kJ/mol at 298 K relative to the lowest energy oxazolone conformer, refer to Supplementary Table S1 for  $\Delta G$  values relative to the lowest energy diketopiperazine conformer





**Figure 4.** Experimental IRMPD action spectrum for PPG +  $H^+$  precursor ion obtained at (a) FELIX. Computed spectra for the lowest energy (b) *cis/trans* isomer, (c) *trans/trans* isomer, (d) *cis/cis* isomer, and (e) *trans/cis* isomer of PPG +  $H^+$

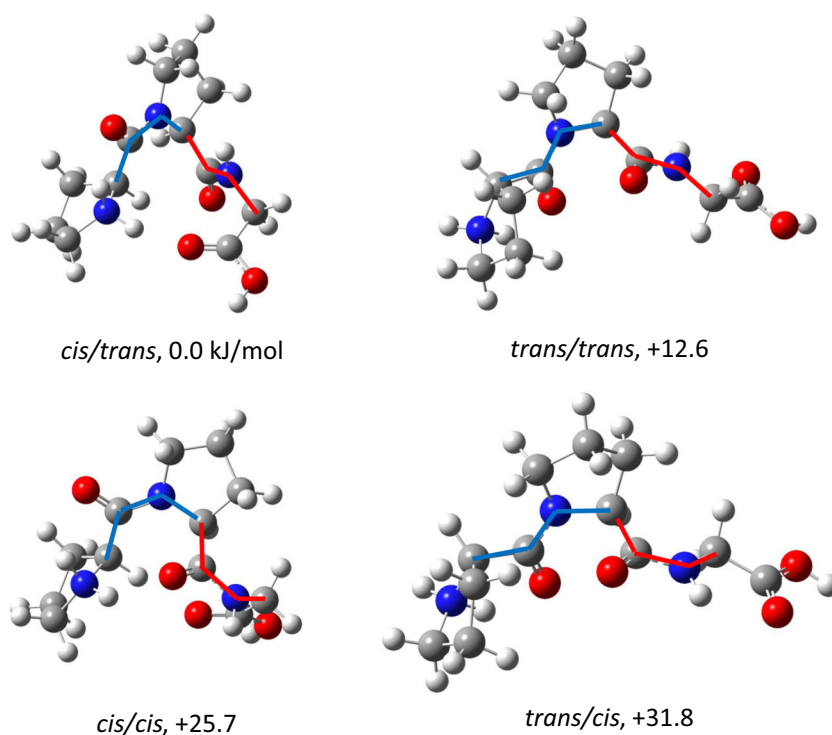
side chain of proline, the oxazolone structure is bicyclic and contains a formally positive nitrogen atom in the ring system. Higher energy conformers arise from different orientations of the external proline ring with respect to the oxazolone rings as well as from differences in the proline ring puckering. The conformers with the two lowest free energies should constitute >95% of the oxazolone Boltzmann population at 298 K. The diagnostic oxazolone peaks near 1925 and 1600  $cm^{-1}$  are in virtually the same position for both of these conformers.

It is clear from the experimental spectra (Figure 1a and b) that the majority of the conformers under both of our experimental conditions are oxazolones. This result is in stark contrast with previous results for the PP  $b_2^+$  fragment ion from PPP that was shown to be nearly 100% diketopiperazine [26]. In that study, the protonated [PPP +  $H^+$ ] precursor was found to be in the *cis/cis* configuration, which is primed for diketopiperazine formation. A collision-induced dissociation spectrum for PPP +  $H^+$  is shown in Supplementary Figure S2 of Supporting Information. As with PPG +  $H^+$ , the dominant fragment ion is the “proline” effect  $y_2^+$  ion. In this case, the  $b_2^+$  is more intense than in PPG +  $H^+$  and is of ample intensity for further IRMPD action spectroscopy studies.

In order to ascertain the conformation of the PPG +  $H^+$  precursor peptides, we obtained a solution-phase (50%  $D_2O$ :50%  $CD_3CN$ ) NMR spectrum of the neutral PPG peptide. The spectrum is shown in Supplementary Figure S3 of Supporting Information. Analysis of the  $C_\alpha$  hydrogens indicates that the neutral peptide is a mixture of isomers, with the dominant structure being the *trans/trans* isomer (ca. 5:1 of *trans/trans*:*trans:cis*). Peaks were assigned based on 1D-selective TOCSY experiments, and the conformations were established on the basis of 1D-selective NOESY experiments [33, 34]. We were also able to obtain the gas-phase IRMPD action spectrum of PPG +  $H^+$  precursor ion at the FELIX facility as shown in Figure 4. Also shown in Figure 4 are calculated scaled harmonic spectra at the B3LYP/6-31+G(d,p) level of theory for the lowest free energy

conformers of the four different isomers of PPG +  $H^+$  (*cis/trans*, *trans/trans*, *cis/cis*, and *trans/cis*). Figure 5 shows the lowest energy conformers for these isomers and Supplementary Table S2 gives relative 298 K free energy values at the B3LYP/6-311++G(d,p)//B3LYP/6-31+G(d) for all isomers found in our conformational search. The calculations predict that the lowest-energy isomer is *cis/trans*, with *trans/trans* isomers lying on the order of 13 kJ/mol higher in energy. The lowest energy *cis/cis* and *trans/cis* structures are on the order of 25 and 32 kJ/mol higher in energy at the B3LYP/6-31+G(d) level of theory.

An analysis of the IRMPD action spectrum shows that the experimental spectrum matches best with the *trans/trans* isomer, which means that the much of the gas-phase peptide population is being kinetically trapped in the favored solution-phase structure during the electrospray process. Given the intensity of the peak in the experimental spectrum near 1505  $cm^{-1}$ , we can conclude that the gas-phase sample does not contain significant amounts of *trans/cis* or *cis/cis* isomers as the calculated spectra have no significant peaks in this region of the spectrum. Thus the minor isomer in solution is absent from the spectrum. Additionally, the intensity in the experimental spectrum around 1730  $cm^{-1}$  indicates that the *cis/trans* isomer is present in the sample as at least a minor component. The fact that the  $b_2^+$  ion population contains mostly oxazolone with a small amount of diketopiperazine is consistent with a precursor mixture of mostly *trans/trans* (giving the oxazolone) with a sub-population of *cis/trans* PPG+ $H^+$  precursor (giving the diketopiperazine). An alternative explanation for the  $b_2^+$  structure distribution is that the barrier to *trans/cis* isomerization is below that of either of the ring-closure barriers for product formation and that the distribution arises from the Curtin-Hammett principle [35, 36]. This mechanism would be in agreement with calculations by Armentrout [8] on protonated diglycine that show that the rate limiting step is ring closure to the final product and with experimental data of Wysocki et al. [37] that show that the third residue of a peptide can



**Figure 5.** Lowest-energy conformers for the (a) *cis/trans* isomer, (b) *trans/trans* isomer, (c) *cis/cis* isomer, and (d) *trans/cis* isomer of PPG +  $H^+$ . Numbers are  $\Delta G$  values in kJ/mol at 298 K relative to the lowest energy conformer. Blue and red lines indicate the *cis/trans* configuration of the first and second peptide bonds

dramatically influence the  $b_2^+$  structure. A full computational study for these peptides and those containing dimethylproline, which locks peptide bonds into *cis* conformations, including the calculation of the relevant barrier heights for *trans-cis* isomerization and product formation, is underway.

## Conclusions

Collision-induced dissociation of protonated PPG tripeptide gives a mixture of  $b_2^+$  ions that are predominantly oxazolone. This is in contrast with the population of  $b_2^+$  ions from CID of protonated PPP. Clearly, the fragmentation mechanism for  $b_2^+$  ion formation from small peptides depends not only on the first two residues that make up the nascent  $b_2^+$  ion but also on the identity of trailing residues that affect the structure of the peptide and the relevant barriers for dissociation.

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