Penultimate Proline in Neuropeptides

Matthew S. Glover,† Earl P. Bellinger,† Predrag Radivojac,‡ and David E. Clemmer*†

†Department of Chemistry, ‡Department of Computer Science and Informatics, Indiana University, Bloomington, Indiana 47405, United States

ABSTRACT: A recent ion mobility spectrometry-mass spectrometry (IMS-MS) study revealed that tryptic peptide ions containing a proline residue at the second position from the N-terminus (i.e., penultimate proline) frequently adopt multiple conformations, owing to the cis–trans isomerization of Xaa1–Pro2 peptide bonds [J. Am. Soc. Mass Spectrom. 2015, 26, 444]. Here, we present a statistical analysis of a neuropeptide database that illustrates penultimate proline residues are frequently found in neuropeptides. In order to probe the effect of penultimate proline on neuropeptide conformations, IMS-MS experiments were performed on two model peptides in which penultimate proline residues were known to be important for biological activity: the N-terminal region of human neuropeptide Y (NPY1−9, Tyr1-Pro2-Ser3-Lys4-Pro5-Asp6-Asn7-Pro8-Gly9-NH2) and a tachykinin-related peptide (CabTRP 1a, Ala1-Pro2-Ser3-Gly4-Phe5-Leu6-Gly7-Met8-Arg9-NH2). From these studies, it appears that penultimate prolines allow neuropeptides to populate multiple conformations arising from the cis–trans isomerization of Xaa1–Pro2 peptide bonds. Although it is commonly proposed that the role of penultimate proline residues is to protect peptides from enzymatic degradation, the present results indicate that penultimate proline residues also are an important means of increasing the conformational heterogeneity of neuropeptides.

Although the vast majority of peptide bonds adopt the trans configuration in polypeptides, Xaa–Pro bonds adopt both cis and trans isomers due to the unique structure of the proline side chain. Several studies have demonstrated that proline isomerization acts as a conformational switch in which the cis–trans isomerization of a single Xaa–Pro bond leads to global conformational changes, with the two forms having different functions. Proline residues and proline-rich regions of sequences are frequently found in dynamic regions of biomolecules, giving them a high degree of conformational heterogeneity. Such systems often do not adopt rigid secondary structures and are commonly described as unstructured or intrinsically disordered. It remains challenging to characterize the structure of dynamic proline-containing regions of biomolecules by traditional techniques such as nuclear magnetic resonance spectroscopy and X-ray crystallography, presumably because so many stable conformations are present simultaneously.

Several recent studies illustrate that ion mobility spectrometry-mass spectrometry (IMS-MS) provides structural insight into dynamic systems that populate multiple conformations, including proline-containing peptides. IMS separates ions based on differences in their shapes and charge states, making it an effective means of delineating differences in conformations. When coupled with electrospray ionization (ESI), it is possible to monitor populations of conformations emerging from solution. The evaporative cooling process of ESI is thought to aid in kinetically trapping solution populations.

In a recent study, we reported that peptides with a proline residue located at the second position from the N-terminus (i.e., penultimate proline) have a propensity to adopt multiple conformations from the cis–trans isomerization of Xaa1–Pro2 peptide bonds. We subsequently noticed that penultimate proline residues are frequently located in many bioactive peptides, prompting us to inspect the structural implications of penultimate proline residues. In this study, the role of penultimate proline residues in neuropeptide conformations is examined, as we found this class of neurochemical signaling molecules to frequently contain a proline residue in the N-terminal penultimate position. A statistical analysis of NeuroPedia, an online database of neuropeptide sequences, is performed to illustrate the frequency of proline position in neuropeptide sequences. We use IMS-MS studies to investigate two model neuropeptide systems, the N-terminal region of neuropeptide Y (NPY1−9, Tyr1-Pro2-Ser3-Lys4-Pro5-Asp6-Asn7-Pro8-Gly9-NH2) and a tachykinin-related peptide from Cancer borealis (CabTRP 1a, Ala1-Pro2-Ser3-Gly4-Phe5-Leu6-Gly7-Met8-Arg9-NH2), in detail. We selected these two systems because they both have penultimate proline residues that are proposed to be important for their biological activity.

Neuropeptide Y is one of the most abundant peptides in both the central and peripheral nervous systems and is involved in regulating a variety of biological processes such as food intake and blood pressure. Although the structure is debated, NMR and circular dichroism studies propose that the proline-rich N-terminal region lacks rigid secondary structure and has been described as unstructured. Several studies provide conflicting reports as to which specific Xaa–Pro peptide bonds in the N-terminal region adopt both cis and trans configurations.

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isomers.30−34 A study of Ala substituted analogues of NPY suggested that the Pro2 residue is important for binding the Y1 receptor.36 Furthermore, neuropeptide Y 3−36 is an endogenous peptide that is formed by cleaving Tyr1−Pro3 residues from the N-terminus and has a decreased affinity for an Y1-like receptor, supporting the idea that Pro3 is important for receptor-binding selectivity.37 In addition to serving as a model for penultimate proline isomerization, this system is chosen because it will provide insight into the type of structural information that can be gleaned from ion mobility studies of peptides that have been described as unstructured by traditional techniques.12,35

Tachykinin-related peptides (TRPs) are a large family of neuropeptides with five conserved C-terminal residues: Phe-Xaa-Gly-Xaa-Arg-NH2.36 TRPs are involved in a range of physiological processes in the nervous systems of invertebrates, such as excitation of the pyloric rhythm.28,36 We are interested in this family of peptides because the majority of known invertebrate TRPs have a penultimate proline.36,37 CabTRP Ia is of particular interest given the known biological significance of the Ala−Pro region of this molecule. CabTRP Ib is a truncated form of CabTRP Ia with the Ala1−Pro2 dipeptide cleaved from the N-terminus that was shown to have decreased bioactivity, suggesting that the Ala1−Pro2 motif is important for activity.

Below, we focus on understanding the structural implications of the frequent occurrence of penultimate proline residues in neuropeptide sequences through a series of IMS-MS experiments on two model neuropeptide systems. By analyzing N-terminally truncated and Pro → Ala substituted analogues, we demonstrate that penultimate proline residues are important for the conformational heterogeneity of NPY1−9 and CabTRP Ia. Molecular modeling simulations are performed to provide insight into the different intramolecular interactions that arise from the cis−trans isomerization of the Ala1−Pro2 peptide bond of CabTRP Ia. These simulations show that cis and trans configurations result in changes in the H-bonding pattern of the protonated N-terminus, leading to global conformational changes that may be important for biological activity.

## EXPERIMENTAL SECTION

### Ion Mobility Spectrometry-Mass Spectrometry

IMS-MS and IMS-IMS-MS experiments were conducted on a homemade instrument with a ~2 m drift tube coupled to a time-of-flight (TOF) mass spectrometer, previously described in detail.28,39 Ions are produced by electrospray ionization with a Triversa Nanomate (Advion Bioscience, Ithaca, NY, USA), stored in a Smith-geometry40 ion funnel, and periodically pulsed into the drift tube, which is filled with 3.0 ± 0.2 Torr He buffer gas and operated with an electric field of ~10 V/cm. Mobility separated ions are transferred through a differentially pumped region and analyzed with an orthogonal geometry TOF-MS in a nested geometry.41

IMS-IMS-MS experiments are performed by operating the drift tube as two independent drift regions separated by an ion funnel.28,39 Ions are mobility selected at the front of the mid funnel and collisionally activated by increasing the electric field across a 0.3 cm region at the end of the funnel. Conformational changes are monitored as a function of activation voltage by allowing annealed ions to mobility separate in the second drift region prior to mass analysis.

### Collision Cross-Section Distributions

Drift time (tD) distributions are measured and converted to collision cross-section (Ω) distributions according to

$$\Omega = \frac{(18\pi)^{1/2}}{16} \frac{ze}{(k_B T)^{1/2}} \left[ \frac{1}{M_1} + \frac{1}{M_2} \right]^{1/2} \frac{t_D E}{760/L P} \frac{T}{273.2 N}$$

where z is the charge of the ion, k_B is the Boltzmann constant, T is the temperature, E is the electric field, L is the length of the drift tube, P is the pressure, and N is the neutral number density at STP. M_1 and M_2 are the masses of the ion and buffer gas, respectively. Collision cross section distributions of Ala substituted peptide have been shifted by +2.5 Å² per Pro → Ala substitution to account for the intrinsic size difference between proline and alanine residues, as previously determined by intrinsic size parameters.43,44 The 2.5 Å² correction factor was effective for cis and trans isomer assignments of triply protonated bradykinin.13

### Peptide Synthesis

Peptides were synthesized by solid-phase Fmoc synthesis on an Apex 396 peptide synthesizer (AAPPTec, Louisville, KY, USA) similar to procedures previously described.13 Fmoc-Rink amide MBHA resins and Fmoc side chain protected amino acids were purchased from Midwest Biotech (Fischers, IN, USA). Briefly, 20% piperidine in dimethylformamide was used to perform Nα deprotections, and 3-(diethoxyphosphoryl)-1,2,3-benzotriazin-4(3H)-one was used as the coupling reagent. Peptides were cleaved from the resin with a solution of trifluoroacetic acid/triisopropylsilane/methanol at a 18:1:1 ratio with the addition of 5% 2,2'-ethylenedioxido)dianethanol for methionine-containing peptides. Peptides were precipitated and washed with diethyl ether and dried using a vacuum manifold. Peptides were reconstituted in H2O before dilution into the appropriate water/methanol ESI solution.

### Molecular Modeling

Molecular dynamics simulations were performed on doubly protonated CabTRP Ia ions with a simulated annealing procedure in the Insight II software suite (Accelrys, Inc., San Diego, CA, USA) similar to simulations on proline-containing peptides previously described.45 Simulations were performed on two initial structures of doubly protonated CabTRP Ia that differ by the configuration of the Ala1−Pro2 peptide bond. Charges were assigned to the N-terminus and Arg6 side chain. The Amber force field and a dielectric of 1 were used in the Discover 3 module of the Insight II software package. During simulated annealing cycles, ions are heated from 298 to 500 K over 5 ps, equilibrated at 500 K for 10 ps, cooled to 298 K over 5 ps, and equilibrated at 298 K for 30 ps. One-hundred trial geometries were saved, and collision cross-sections were calculated by the trajectory method in Mobcal.46

## RESULTS AND DISCUSSION

### Statistical Analysis of Penultimate Proline in Neuropeptide Sequences

We analyzed 847 neuropeptide sequences from humans and seven other animal species cataloged in the NeuroPedia database to assess the positioning of proline in neurobiological molecules. To report conservative significance values, we aligned these sequences using the Needleman–Wunsch algorithm with the BLOSUM62 substitution matrix and determined 181 distinct sequences sharing no more than 40% identity. While proline is present throughout human proteins with a frequency of 6.3%,49 proline occupies the penultimate position with a significantly
greater frequency than any other amino acid in the NeuroPedia database ($P = 0.0002$; binomial test with $p = 0.063$, $n = 181$, $x = 25$). Furthermore, proline occupies the penultimate position of these sequences significantly more often than any other of the first 20 positions ($P = 0.0003$; binomial test with $p = 0.05$, $n = 231$, $x = 25$). We permuted the data set and obtained the same result. Bar plots of proline’s occupational frequencies relative to other amino acids and other positions alike are shown in Figure 1. Proline occupies the penultimate position relative to other amino acids approximately 13.8% of the time. Proline occupies the penultimate position relative to the other positions approximately 10.8% of the time. In addition to database of sequences analyzed here, we note that several large families of peptides from organisms not included in NeuroPedia frequently possess penultimate proline residues, such as tachykinin-related peptides in invertebrates.36 These results suggest that proline’s occupation of the penultimate position in neuropeptide sequences has been conserved evolutionarily and may serve an important functional role in these molecules. This inspired us to examine the role of penultimate proline residues on the conformational dynamics of neuropeptides.

IMS-MS and IMS-IMS-MS of NPY$_{1-9}$. Collision cross-section distributions for doubly charged [NPY$_{1-9} + 2H$]$^{2+}$ ions produced by electrospray ionization from a range of water/methanol solutions are displayed in Figure 2. This study focuses on the doubly charged species, as this is the dominate charge state for all peptides presented here. The distribution of [NPY$_{1-9} + 2H$]$^{2+}$ ions is composed of at least four partially resolved peaks at 219, 224, 228, and 231 Å$^2$, referred to as conformers A, B, C, and D, respectively. It is important to note the four conformations are partially resolved and that cross-section distributions do not reach baseline for the entire range of conformations; thus, it is possible that additional conformations of relatively low abundance exist, making this a conservative estimate of the number of conformation types. Although the peaks are not fully resolved, it is interesting that we observe defined peaks in the mobility distribution given previous studies that report that this peptide is unstructured.30−34 CD and NMR studies of the N-terminal region on NPY suggest that it does not adopt a single well-defined structure; IMS-MS data suggests NPY$_{1-9}$ adopts an ensemble of defined conformer populations that are difficult to monitor in solution but are kinetically trapped during ESI and sampled by

![Figure 1](image1.png)  
*Figure 1.* Frequency of occupation for amino acids in the penultimate position of neuropeptide sequences from the NeuroPedia database (a). The frequency of proline occupation in the first 20 positions of neuropeptide sequences (b).

![Figure 2](image2.png)  
*Figure 2.* Collision cross-section distributions for [NPY$_{1-9} + 2H$]$^{2+}$ ions obtained from different water/methanol solutions. The distribution labeled activated is the quasi-equilibrium distribution obtained from collisional activation by IMS-IMS-MS experiments, as explained in the text.
IMS-MS. Although it is unlikely that the structures are identical to those from solution, this provides further evidence that IMS-MS can be used to monitor conformational changes in NPY1–9.23

Changes in conformer populations are observed as a function of the water/methanol ratio from which NPY1–9 is electrosprayed (Figure 2). From 100% H2O, conformer B is 80%, conformer A is 14%, and the unresolved conformers C and D are 6% of the collision cross-section distribution. However, as the methanol fraction is increased, conformer A increases in abundance and comprises 48% of the distribution from the 10:90 water/methanol solution. Although the combined abundance of conformers C and D remains constant at ~7% with increasing methanol content, conformer C increases relative to conformer D. The observation of changes in peptide conformation populations as a function of solution composition is similar to IMS-MS studies of triply protonated bradykinin (Arg1–Pro2–Pro3–Gly4–Phe5–Ser6–Pro7–Phe8–Arg9) electrosprayed from different water/methanol solutions.22 It is worth pointing out that bradykinin is similar to NPY1–9 in that both are proline-rich sequences, possess a penultimate proline, and lack rigid secondary structure in solution.12

Multidimensional IMS-IMS-MS experiments were conducted to further probe the role of solvent in the formation of specific NPY1–9 conformations. Upon collisional activation, we observe the formation of the compact conformation, A, regardless of the initial conformation selected (Figure 2). Previous studies describe collisionally activated distributions of gas-phase conformations obtained by IMS-IMS-MS experiments as the quasi-equilibrium distribution.50 A characteristic of the quasi-equilibrium distribution is that ions collisionally activated above the energetic barriers between states form the same distribution of annealed ions regardless of what conformation is selected and activated. This provides insight into the conformations that are preferred from solution versus those that are stable in the absence of solvent–molecule interactions. This agrees with the water/methanol solution scans that conformant B is favored in aqueous environments and conformation A is favored in nonaqueous environments such as the gas phase and high fractions of methanol.

IMS-MS of Pro → Ala Substituted and N-Terminally Truncated NPY. In order to provide insight into the specific proline residues that are important for the conformational heterogeneity of [NPY1–9 + 2H]+ ions, Pro → Ala substituted peptides were analyzed by IMS-MS. Pro → Ala substituted allow for the assignment of specific proline residues as either trans or cis because Xaa–Ala bonds are almost exclusively trans. Thus, we assign conformers that are present in both the natural and Ala substituted sequences as trans and those that are eliminated upon Pro → Ala substitution as cis. Furthermore, we monitor the number and relative populations of conformers upon substitution to provide insight into the specific residues important for conformational heterogeneity.

Figure 3 shows collision cross-section distributions for Pro2–→Ala, Pro5–→Ala, Pro8–→Ala, and Pro2,5,8–→Ala substituted analogues of [NPY1–9 + 2H]+ ions and N-terminally truncated [NPY9–9 + 2H]+ ions. Distributions are obtained from 50:50 water/methanol solutions. Ala substituted distributions are shown in red and overlaid on the dotted [NPY1–9 + 2H]+ distribution for ease of comparison. Distributions for Ala substituted peptides are shifted for size difference between Pro and Ala residues, as explained in the text.

Figure 3: Collision cross-section distributions for Pro2–→Ala, Pro5–→Ala, Pro8–→Ala, and Pro2,5,8–→Ala analogues of [NPY1–9 + 2H]+ ions and N-terminally truncated [NPY9–9 + 2H]+ ions. Distributions are obtained from 50:50 water/methanol solutions. Ala substituted distributions are shown in red and overlaid on the dotted [NPY1–9 + 2H]+ distribution for ease of comparison. Distributions for Ala substituted peptides are shifted for size difference between Pro and Ala residues, as explained in the text.

2H]+ ions. The distribution for the Pro3–→Ala is broad, with the most abundant cross-section at 224 Å2 and peak shoulders at ~220 and ~227 Å2. From this distribution, we assign Pro3 as trans for conformer B of NPY1–9, as this is the major peak from the Pro3–→Ala distribution. The Pro8–→Ala distribution has two conformations of similar abundance at 219 and 224 Å2 and a trailing shoulder of relatively low abundance at 228 Å2. This distribution is strikingly similar to that of [NPY1–9 + 2H]+ ions, suggesting that Pro8 is in the trans configuration for the two major conformers, A and B, observed for [NPY1–9 + 2H]+. Furthermore, this suggests that Pro8 contributes the least significantly to the conformational heterogeneity of NPY1–9.

The collision cross-section distribution for triply substituted Pro2,5,8–→Ala peptide has a single peak at 228 Å2 (Figure 3). This peptide allows for the all trans form of NPY1–9 to be assigned as conformer C. The presence of a single peak in the IMS distribution for triply substituted Pro2,5,8–→Ala peptide confirms that the conformational heterogeneity observed for [NPY1–9 + 2H]+ arises from the cis–trans isomerization of proline residues.

Considering all Pro → Ala substituted peptides, we assign conformer A as trans-Pro2, cis-Pro3, and trans-Pro8, as this peak dominates the mobility distribution for the Pro2–→Ala substitution and is in relatively high abundance for the Pro8–→Ala substitution. Conformer B is assigned as cis-Pro2, trans-Pro3, and trans-Pro8 because this peak is the most abundant.
conformation for Pro² → Ala and Pro⁸ → Ala substitutions and is not observed for the Pro⁵ → Ala or Pro⁷,⁹ → Ala peptides. As mentioned above, conformer C is assigned as trans-Pro⁵, trans-Pro⁹, and trans-Pro⁷ because the conformer is observed for the triply substituted peptide. We are unable to definitively assign the least abundant conformation, D, as we do not observe a substituted peptide with significant population near this peak.

In order to provide further evidence that the penultimate proline residue is important for the conformational heterogeneity of [NPY₁₋₉ + 2H]²⁺ ions, we have analyzed the peptide with the Tyr¹−Pro² residues truncated from the N-terminus (NPY₁₋₅). The collision cross-section distribution for [NPY₁₋₅ + 2H]²⁺ has a major peak at 181 Å² and a minor peak at 185 Å², with relative abundances of 92 and 8%, respectively (Figure 3). We do not observe a single peak that is >80% total abundance for any of the penultimate proline-containing peptides. However, both NPY₁₋₅ and the Pro² → Ala analogue have distributions that are dominated by a single conformation at >90% relative abundance, further evidence that penultimate proline is important for the conformational flexibility of the N-terminal region of neuropeptide Y. We point out that NPY₁₋₅ contains a proline that is penultimate from the C-terminus. However, the peptide bond that adopts both cis and trans configurations is N-terminal to Pro residues (e.g., Asn⁻−Pro⁹). The analogous peptide bond to N-terminal penultimate proline occurs when proline is located at the C-terminal position.

**IMS-MS of CabTRP Ia and Analogue Sequences.** Collision cross-section distributions for doubly protonated [M + 2H]²⁺ ions of CabTRP Ia (Ala¹-Pro²-Ser³-Gly⁴-Phe⁵-Leu⁶-Gly⁷-Met⁸-Arg⁹-NH₂), Pro² → Ala substituted CabTRP Ia, and CabTRP Ib (Ser³-Gly⁴-Phe⁵-Leu⁶-Gly⁷-Met⁸-Arg⁹-NH₂) electrosprayed from 50:50 water/methanol solutions are displayed in Figure 4. Two peaks are observed in the distribution for CabTRP Ia at 229 and 235 Å², with relative abundances of 86 and 14%, respectively. Unlike NPY₁₋₅, we do not observe major changes in the populations of CabTRP Ia when electrosprayed from different ratios of water/methanol. The contrast in solution dependence between NPY₁₋₅ and CabTRP Ia may be due to differences in amino acids surrounding the Xaa¹−Pro² peptide bond. Several studies have illustrated that both local and nonlocal sequence composition influence proline isomerization. For example, the residue N-terminally adjacent to proline is crucial for stabilizing different peptide bond configurations. The influence of solvent on specific intramolecular interactions is likely different for each unique peptide sequence.

A major change in the distribution of conformations upon collisional activation by IMS-IMS occurs with the conformation at 235 Å² comprising 100% of the activated quasi-equilibrium distribution (Figure 4). It is interesting that both peptides analyzed in this study populate multiple conformations from solution but prefer a single gas-phase conformation upon activation. This suggests that the configuration of Xaa¹−Pro² peptide bonds is sensitive to the local environment.

We analyzed the Pro² → Ala substituted analogue of CabTRP Ia by IMS-MS to assign the cis and trans isomers of [CabTRP Ia + 2H]²⁺ ions. The collision cross-section distribution has a single peak at 230 Å² (Figure 4). Therefore, we assign the peaks of [CabTRP Ia + 2H]²⁺ ions at 229 and 235 Å² as trans- and cis-Pro², respectively. This differs from the penultimate proline in NPY₁₋₅ that adopts the trans configuration in the quasi-equilibrium distribution. One potential explanation for this is differences in protonation sites. Both sequences have two basic sites: the N-terminus and Lys or Arg residue. However, the Lys⁴ residue of NPY₁₋₅ is located closer to the Pro² residue than the Arg⁹ residue of CabTRP Ia. This likely influences the intramolecular interactions that stabilize different cis and trans configurations.

We have analyzed the N-terminally truncated peptide CabTRP Ib by IMS-MS. The collision cross-section distribution for [CabTRP Ib + 2H]²⁺ has a single peak at 201 Å². This confirms that the cis-trans isomerization of Ala¹−Pro² is important for the multiple conformations observed for CabTRP Ia. From IMS-MS studies of CabTRP Ia and its analogues, we determined that CabTRP Ia samples two environment-dependent conformations that arise from the cis-trans isomerization of the penultimate proline residue and differ in population from solution versus after collisional activation, whereas the N-terminally truncated CabTRP Ib has a single conformation.

**Insight into Peptide Structure through Molecular Dynamics Simulations.** In order to gain insight into structural differences that arise from the rotation of the Ala¹−Pro² peptide bond, molecular modeling was performed on doubly protonated CabTRP Ia. CabTRP Ia was selected for simulations because cis and trans isomers for only a single proline residue need to be considered, making the simulations relatively straightforward. Figure 5 shows two representative low-energy structures for the trans and cis isomers of CabTRP Ia. Calculated collision cross-section values are within 1% of the experimental; the trans (Figure 5a) and cis (Figure 5b)
Studies of NPY1-9 and CabTRP Ia by IMS-MS demonstrated that these peptides adopt multiple conformations that arise from the cis–trans isomerization of Xaa–Pro2 peptide bonds, suggesting that penultimate proline residues are important for the conformational heterogeneity of neuropeptide sequences. Upon substitution or truncation of penultimate proline residues, we observe a decrease in the conformational heterogeneity of these systems. Furthermore, conformer populations related to different cis– and trans-Pro residues are sensitive to the local environment. That is, we observe changes in conformer populations as a function of solution composition or upon collisional activation (i.e., the gas-phase distribution). Molecular modeling simulations show that the configuration of the Ala1–Pro2 peptide bond of CabTRP Ia determines the network of intramolecular hydrogen bonds formed by the protonated N-terminus and therefore the orientation of the amino acid side chains and global conformation.

It is interesting to consider possible biological implications of the ability of proline-containing peptides to adopt multiple conformations. We propose that, in addition to the previously known role of protecting bioactive peptides from enzymatic degradation,55 penultimate proline residues may serve as a functional switch that allows bioactive peptides to sample multiple conformations that may have different functions or binding affinities for their receptors. The ability of proline residues and, specifically, penultimate prolines to allow neuropeptides to sample different conformer populations upon changes to the local environment is likely an important structural consideration as peptides experience similar environmental changes, such as when approaching or binding receptors.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: clemmer@indiana.edu.

Notes
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