Conformational Heterogeneity about Pipecolic Acid Peptide Bonds: Conformational, Thermodynamic, and Kinetic Aspects

Wen-Jin Wu and Daniel P. Raleigh*,†

Department of Chemistry, State University of New York at Stony Brook, Stony Brook, New York 11794-3400

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Cis−trans isomerization about pipecolic peptide bonds has been studied using a set of designed tetrapeptides of the sequence acetyl-Gly-X-Pip-Gly-carboxamide (GXPipG), where X = A, F, Y, W, or cyclohexylalanine (Cha). The substitution of a pipecolic residue for a proline leads to (1) a significant increase of the population of the cis conformer, (2) a reduction in the Van't Hoff enthalpy for isomerism, and (3) acceleration of the rates of isomerization. GAPipG and GChaPipG are unstructured, and isomerization is controlled by local steric effects between the α and ϵ positions of the pipecolic ring and the α position of the preceding residue. The fraction of the cis isomer is the same for these two peptides, indicating that the bulk of the preceding amino acid does not play a significant role in influencing the equilibrium. In the trans form of the aromatic containing peptides, an i to $i + 2$ aromatic−amide interaction is observed, and in the cis form a stabilizing interaction between the aromatic residue and the succeeding pipecolic ring is observed. The sign of the Van't Hoff enthalpy indicates that the cis conformer is enthalpically favored for the aromatic containing peptides. The rate of isomerization for the aromatic containing peptides are reduced relative to GAPipG.

Introduction

The noncoded amino acid 2-piperidinecarboxylic acid (pipecolic acid, also known as homoproline) is a proline analogue which contains a six-membered hexahydropyridine ring. Pipecolic acid is found in several important natural products such as the immunosuppressants FK506[1] and rapamycin,[2] and in cyclic peptides which have antifungal activity.[3] It has been extensively used as a proline substitute in numerous syntheses of peptidomimetics. Recent selected examples include the conversion of a peptide substrate of HIV protease into a selective inhibitor by the substitution of proline with pipecolic acid at the scissile bond,[4] the design of synthetic MHC class II ligands as potential antirheumatoid arthritis drugs,[5] the development of small acyclic molecules which are designed to mimic the tricarbonyl region of FK506,[6] and the incorporation of D- and L-pipecolic acid into potent thrombin inhibitors.[7,8] Functionalized pipecolic acids are also used as constrained lysine analogues.[9] Pipecolic acid has also been incorporated into a variety of bioactive peptides including vasopressin, oxytocin, and angiotensin II.[10−12] Model peptide substrates containing pipecolic acid residues have been used in studies of the specificity of kinases and of cyclophilin.[13,14] Pipecolic acid has also been incorporated into proteins as a proline homologue in order to probe the role of ring size in structure and function.[15] It plays a central role in lysine metabolism, particularly in CNS tissue, and derivatives of pipecolic acid have been developed as inhibitors of the enzyme L-pipecolate oxidase which may offer potential as anticonvulsant drugs.[16] Pipecolic acid derivatives also find a role as β-turn mimics.[17,18]

Despite the obvious importance of pipecolic acid, surprisingly little is known about the thermodynamics and the kinetics of cis−trans isomerization about pipecolic acid peptide bonds. In contrast, there is a large body

† Graduate Program in Biophysics and Graduate Program in Molecular and Cellular Biology, State University of New York at Stony Brook. Telephone: 516-632-9547. Fax: 516-632-7960. E-mail: DRaleigh@ccmail.sunysb.edu.

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of literature on the cis−trans isomerism about proline peptide bonds. Understanding how the pipecolic ring modulates the thermodynamics and kinetics of peptide bond isomerism will prove useful in protein engineering experiments, in the design of peptidomimetics, and in the elucidation of the role of pipecolic acid residues in natural products. In this study we report a detailed investigation of the cis−trans isomerization about pipecolic peptide bonds in a set of five designed peptides. The peptides are of the general sequence of acetyl-Gly-X-Pip-Gly-NH_2 (abbreviated as GXPipG), where X = Ala, Phe, Tyr, Trp, or cyclohexylalanine (Cha) and Pip is pipecolic acid. These peptides are small enough so that there will be no significant tendency to form an ordered secondary structure, thus providing a good system for studying the local conformational tendencies of a pipecolic acid residue. Taken together, the data collected on this set of peptides allows the development of a detailed picture of the local effects of the substitution of a pipecolic acid residue for a proline. The results of this study also provide a detailed database with which to analyze the factors that control isomerism about pipecolic peptide bonds.

Results and Discussion

A set of tetrapeptides of the general sequence acetyl-Gly-X-Pip-Gly-carboxamide (GXPipG), where X = A, F, Y, W, or cyclohexylalanine (Cha) and where Pip is pipecolic acid, have been prepared. The two flanking glycine residues were introduced to avoid complications due to interactions between the two termini and the ends were acetylated and amidated to reduce potential stabilizing electrostatic interactions. Studies on proline-containing peptides have shown that the thermodynamics[19−24] and kinetics[22,24] of cis−trans isomerization are strongly influenced by the identity of the amino acid preceding the proline residue. The Ala- and Cha-containing peptides allow us to study the effect of varying the bulk of the preceding side chain while the Tyr-, Phe-, and Trp-containing peptides allow us to investigate the effect of any potential interaction between the aromatic and the hexahydropyridine rings. Previous studies of peptides in which an aromatic residue precedes a proline have shown that interactions between the aromatic side chain and the proline ring can profoundly modulate the kinetics[22,24] and thermodynamics[22−27] of cis−trans isomerization.

Conformational Analysis and the Effect of Pipecolic Acid on the Cis−Trans Equilibrium. The spectra of both the cis and the trans conformers of the peptides are well resolved and could be assigned via standard methods. The fingerprint region of the DQF−COSY spectrum of the peptide GWPipG in H_2O is shown in Figure 1A. The cis conformer was assigned on the basis of an intense ROE peak between the α proton of the pipecolic acid residue and the α proton of the preceding residue (Scheme 1). The trans conformer was identified by the intense ROE cross-peak between the downfield ϵ proton of the pipecolic acid residue and the α proton of the preceding residue. A portion of the ROESY spectrum which displays these connectivities is shown in Figure 1B. The N-terminal glycine was assigned by the ROE cross-peak between its amide proton and the N-terminal acetyl methyl protons and by the ROE peak between its α proton and the amide proton of the following residue. The C-terminal glycine was assigned on the basis of the ROE peak between its α proton and the C-terminal NH_2 protons and by the ROE peak between its amide proton and the α proton of the

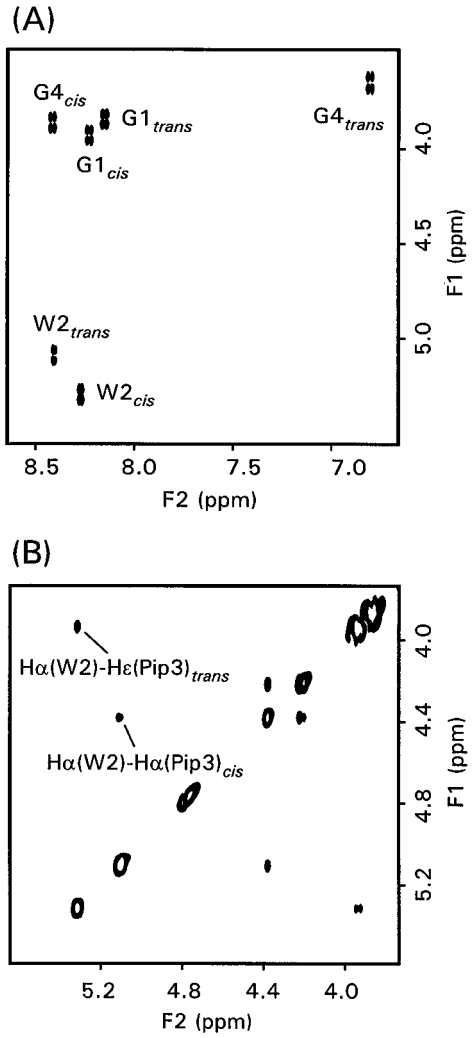
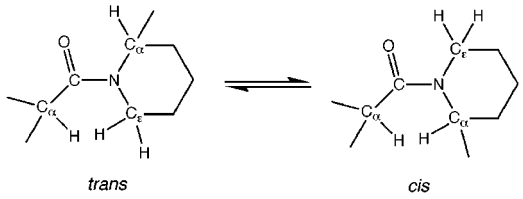


Figure 1. (A) Fingerprint region of the DQF−COSY spectrum of the peptide GWPipG in 90% H_2O/10% D_2O at 298 K, pH 4.3. (B) Portion of the ROESY spectrum of the peptide GWPipG in D_2O at 298 K, pD 7.4. The characteristic ROE cross-peaks for the trans conformer and for the cis conformer are shown.

Scheme 1



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preceding pipecolic acid residue. The observed pattern of *J* coupling constants between the ring protons indicates that, as expected, the pipecolic ring adopts a chair rather than a boat conformation (data not shown). The complete assignments for each of the peptides as well as the amide proton chemical shift temperature coefficients and the $^3J_{NH,\alpha}$ coupling constants for the second residue are included in the Supporting Information.

Proline residues are often found in turn conformations; however neither GAPipG or GChaPipG showed any evidence of adopting turn or hydrogen bond stabilized structures in either the cis or the trans form. Strong sequential α to amide rotating frame Overhauser effects (ROEs) characteristic of an extended conformation, were observed for all adjacent residues except, of course, for the X−Pip pair. No amide to amide ROEs were observed, and no specific ROEs indicative of a turn conformation were observed for either the trans or the cis isomers. The chemical shift temperature coefficients for the amide protons range from −5.8 to −9.5 ppb/K in both the cis and trans forms of GAPipG. These values lie within the range normally observed for amides which are not involved in hydrogen bonds. The $^3J_{NH,\alpha}$ coupling constant of the alanine residue in GAPipG is 6.7 Hz for the trans form and is 6.1 Hz for the cis form. These values are consistent with conformational averaging and are significantly larger than would be expected if a sizable fraction of the molecules adopted a well-defined turn. The pattern of ROEs and the measured *J* coupling constants (7.3 Hz in the trans form and 7.4 Hz for the cis form) indicate that the Cha-containing peptide is also unstructured.

Resonances from the cis and trans conformers are well resolved, and the fraction of molecules which contain a cis peptide bond can be reliably determined from peak integration. Substitution of a pipecolic acid residue for a proline residue leads to a significant increase in population of the cis conformer. Early NMR studies of the benzoyl derivatives of proline and pipecolic acid in $CDCl_3$ indicated that approximately 25% of the molecules adopt the cis conformer for the proline derivative while approximately 35% adopt the cis form for the pipecolic acid derivative.[28] The precision of these early measurements was not high because of peak overlap. The percentage of the cis conformer in GAPG is 7−9%,[24] while the percentage of cis GAPipG is 25%. The percentage of the cis isomer populated by the Cha-containing peptide (26%) is essentially the same as that for the GAPipG peptide (25%), indicating that the size of the side chain at the position immediately preceding the pipecolic acid reside has a negligible effect upon the position of the cis−trans equilibrium. The steric effects will be smaller if a glycine residue precedes the Pip residue. Our NMR studies clearly show that both peptides are unstructured in solution; therefore the higher population of cis peptide linkages observed for the peptides which contain a pipecolic acid is not due to formation of specific structure in the cis conformer. In proline-containing peptides the cis form is destabilized because of steric conflicts between the C_α substituents of the proline (the C_α proton and the carbonyl group of the proline) and the C_α substituent of the preceding residue.[29] There are also unfavorable interactions, albeit less so, between the C_δ protons of the proline and the preceding C_β protons and NH in the trans form. The larger size of the pipecolic acid ring leads to more unfavorable interactions in both the trans and in the cis form. Presumably the effect of the unfavorable steric interactions in the trans form is larger for a pipecolic acid residue than for a proline residue and thus shifts the cis−trans equilibrium toward the cis form, although the trans form still predominates.

Conformation Analysis of the Aromatic Containing Peptides. NMR experiments provide unequivocal evidence that the cis and the trans forms of GFPipG, GYPipG, and GWPipG have a propensity to populate some nonrandom structure. The trans isomers all form a small fraction of nonrandom structure due to an *i* to *i* + 2 aromatic−amide interaction between the aromatic ring and the Gly-4 amide proton. Similar interactions have been detected in small peptides[30,31] which do not contain proline or pipecolic acid residues and also in a set of tetrapeptides which do contain proline.[24] The major evidence for an aromatic−amide interaction is provided by the large perturbation experienced by the Gly-4 amide protons of the aromatic containing peptides. The Gly-4 amide resonance of the trans form experiences a significant upfield shift relative to the cis form. The Gly-4 amide peak of the trans form of the Ala- or Cha-containing peptides occurs at a normal frequency, indicating that the effect must be due to the presence of the aromatic ring at position 2. The amide proton chemical shift of Gly-4 in trans GWPipG is shifted to 6.81 ppm (Figure 1A), 1.44 ppm upfield of its value in GAPipG (8.25 ppm). The Gly-4 amide proton in trans GYPipG resonates at 7.41 ppm, 0.84 ppm upfield of its value in GAPipG. In GFPipG this resonance is found at 7.62 ppm, 0.63 ppm upfield of its value in GAPipG. No significant perturbations are observed for the Gly-4 resonance of the cis conformer of these peptides, indicating that the interaction is specific to the trans conformer.

The measured amide proton chemical shift temperature coefficients provide additional evidence for an aromatic−amide interaction. The Gly-4 NH of trans GWPipG has an anomalous chemical shift temperature coefficient, 2.0 ppb/K, which is opposite in sign to that typically observed. The sign of the chemical shift temperature coefficient of the Gly-4 NH (−2.7 ppb/K) in GYPipG is normal but the value of the coefficient is much smaller than the values for the other NH protons. The chemical shift temperature coefficients for all of the other amide protons lie between −5.8 to −10.3 ppb/K, within the range expected for unstructured peptides. The Gly-4 amide resonance of cis GFPipG has a normal amide proton chemical shift temperature coefficient (−8.4 ppb/K), suggesting that the aromatic−amide interaction is much weaker for the Phe-containing peptide. Aromatic−amide interactions are well documented in the X-ray structures of several proteins.[32−34] NMR studies of peptide fragments have provided evidence that these interactions can persist even in otherwise largely un-

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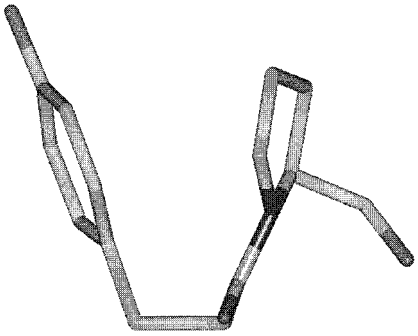
Scheme 2



structured peptides.[30,31] Particularly notable is the study of a peptide fragment from bovine pancreatic trypsin inhibitor by Creighton and co-workers.[31] In this work an interaction between the aromatic ring of Tyr-10 and the amide group of Gly-12 was observed which resulted in a significant shift of the amide resonance of Gly-12 to 6.7 ppm at 277 K with a temperature coefficient of 16.2 ppb/K. The interaction was shown to be present, although weaker, in tetrapeptides of the sequence ArXGY, where Ar is an aromatic amino acid, X is any residue except proline, and Y is any amino acid. It was suggested that such an interaction would not be possible if the residue following the aromatic residue was a proline because of steric considerations. Recently, we reported that aromatic−amide interactions are weaker but can, in fact, still take place in the trans form of the peptides GFPG, GYPG, and GWPG.[24] It appears, based upon the larger chemical shift perturbations and the values of the amide proton chemical shift temperature coefficients, that the *i* to *i* + 2 aromatic−amide interaction is stronger in pipecolic containing peptides than in proline-containing peptides. Although the chemical shift perturbations and anomalous amide chemical shift temperature coefficients provide excellent evidence for the aromatic−amide interaction, they do not provide sufficient structural constraints to uniquely define a structure.

It is well established that peptides which contain an aromatic residue immediately preceding a proline residue usually exhibit a higher percentage of the cis proline conformer.[22−24] The larger percentage of the cis isomer is due to specific interactions between the aromatic ring and the pyrrolidine ring.[24] A surveys of the protein database by MacArthur and Thornton has revealed that aromatic residues which immediately precede a cis proline often adopt a conformation in which the aromatic and proline rings are in a syn arrangement.[35] An example of this type of structure is depicted in Scheme 2 which shows the conformation of residues Tyr86 to Pro87 of the protein adenylate kinase (pdb code: 3adk). We and others have shown that some small peptides have a significant propensity to adopt a similar structure in solution.[23,24,36] The juxtaposition of the proline and aromatic rings has been shown to result in a stabilizing interaction which favors the cis conformer. A similar effect is observed for the pipecolic acid containing peptides studied in this work. The percentage of the cis conformer of each of the aromatic containing peptides is much higher than that of GAPipG or GChaPipG: 39%, 45%, and 50% for GFPipG, GYPipG, and GWPipG, respectively (Table 1). The similar percentage of the cis conformer observed for the Ala- and for the Cha-containing peptides indicates that the increase in the percentage of the cis pipecolic acid conformer is not due simply to the larger size of the aromatic side chain.

The difference in free energy between the cis and the trans conformers for the aromatic containing peptides and the GAPipG peptide ($\Delta\Delta G° = \Delta G°_{trans-cis}$ for the aromatic containing peptides minus $\Delta G°_{trans-cis}$ for GAPipG) are −0.36, −0.48, and −0.65 kcal/mol for GFPipG, GYPipG, and GWPipG, respectively. These values are approximately 0.2 kcal/mol smaller than what has been measured for proline-containing peptides suggesting that there may be competing interactions such as the aromatic−amide interaction which tend to favor the trans form and are stronger in the pipecolic acid containing peptides. Alternatively, the slightly smaller value of $\Delta\Delta G°$ may indicate that the interaction between the pipecolic acid and aromatic ring is weaker than the aromatic−proline interaction. Given the small magnitude of the differences it is difficult to distinguish between these two potential explanations.

Pipecolic acid can exist in two chair forms; in one case the carbonyl group is at an axial position and in the other case it is at an equatorial position. These two forms can be distinguished on the basis of the ROE intensities and the coupling constants between the C_α proton and each of the two C_β protons of the pipecolic acid ring. When the pipecolic carbonyl group is at the axial position, the α proton (at the equatorial position) is gauche to both of the β protons. The measured J coupling constants indicate that the pipecolic carbonyl group is at the axial position. For example, in the cis form of GFPipG, the coupling constant between the α proton and the upfield β proton is 5.5 Hz, and the coupling constant between the α proton and the downfield β proton is 3.4 Hz. This is the pattern which is expected if the α proton is at the equatorial position (that is, the pipecolic carbonyl group is at the axial position). The observed J coupling constants indicate that the upfield β proton is the axial proton and the downfield β proton is the equatorial proton. ROE intensities also support an axial orientation for the pipecolic carbonyl. In this configuration, the α proton is approximately equal distance from the two β protons. The measured ratio of the ROE intensities is 1.1 which is fully consistent with the carbonyl group being axial. Analysis of all of the other peptides shows that the carbonyl group is axial in both the trans and the cis conformations.

There are a number of lines of experimental evidence that indicate that there are specific interactions between the aromatic ring and the succeeding pipecolic ring in the cis conformers. Strong ring current induced upfield shifts are observed for the pipecolic acid ring protons in the cis conformer of each of the aromatic containing peptides, but not in the trans form. Small changes in chemical shift often arise from cis−trans isomerization in nonaromatic containing peptides but the ring current effects present in the cis conformer of the aromatic containing peptides dwarf the small shifts due to simple cis−trans isomerization. The chemical shift of the more upfield pipecolic β proton is especially strongly affected by the proximity of the aromatic ring. This resonance is due to the axial β proton. If the pipecolic and the aromatic rings adopt a conformation similar to that

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Table 1. Mole Fraction of the Cis Conformer and the Values of ΔΔ[a] for the Pipecolic Acid Protons in D_2O and in an 8 M Urea D_2O Solution

peptides	X_{cis}	$\Delta\Delta^{\alpha}$	$\Delta\Delta^{\beta 1}$, $\Delta\Delta^{\beta 2}$	$\Delta\Delta^{\gamma 1}$, $\Delta\Delta^{\gamma 2}$	$\Delta\Delta^{\delta 1}$, $\Delta\Delta^{\delta 2}$	$\Delta\Delta^{\epsilon 1}$, $\Delta\Delta^{\epsilon 2}$
			D_2O			
GAPipG	0.25	0.00	0.00, 0.00	0.00, 0.00	0.00, 0.00	0.00, 0.00
GFPipG	0.39	0.32	0.46, 1.30	−0.03, 0.19	0.10, 0.26	−0.10, 0.01
GYPipG	0.45	0.34	0.46, 1.30	−0.26, 0.17	0.09, 0.26	0.11, −0.01
GWPipG	0.50	0.54	0.75, 1.84	−0.12, 0.24	0.13, 0.76	0.21, −0.09
			Urea			
GAPipG	0.25	0.00	0.00, 0.00	0.00, 0.00	0.00, 0.00	0.00, 0.00
GFPipG	0.38	0.35	0.51, 1.39	0.03, 0.21	0.09, 0.28	0.11, 0.00
GYPipG	0.46	0.35	0.48, 1.33	−0.22, 0.17	0.09, 0.27	0.10, 0.01
GWPipG	0.48	0.50	0.75, 1.97	−0.04, 0.28	0.18, 0.79	0.23, −0.06

[a] $\Delta\Delta = (\delta_{trans} - \delta_{cis})$ for the aromatic containing peptide minus $(\delta_{trans} - \delta_{cis})$ for the GAPipG peptide, where δ_{trans} is the chemical shift of the trans pipecolic proton and δ_{cis} is the chemical shift of the corresponding cis pipecolic proton.

observed for aromatic−proline systems, then the axial β proton will be much closer to the aromatic ring than will the equatorial β proton. This proton is shifted to 0.34, 0.32, and −0.28 ppm in GFPipG, GYPipG, and GWPipG, respectively, while the chemical shift for the corresponding proton in the trans form is 1.71, 1.69, and 1.62 ppm. The other β resonance experiences much more modest shifts. A similar effect is observed with the δ protons; one of the two δ protons (the axial proton) is shifted significantly more than the other (the equatorial proton). Significant shifts are also observed for the α proton. The observed *J* coupling constants between the pipecolic ring protons and the pattern of ROEs shows that the protons which experience the largest ring current shifts are all on the same side of the pipecolic acid ring (data not shown). This indicates that, as expected, the aromatic ring interacts with only one face of the pipecolic ring.

There is a rough correlation between the magnitude of the ring current shift and the fraction of the cis conformer. To quantify these effects we calculate ΔΔ for each of the individual pipecolic ring protons. ΔΔ is defined as the difference in chemical shift between the trans and cis form of the aromatic containing peptide minus the difference in chemical shift between the trans and the cis form of the GAPipG peptide. This calculation should factor out the small general contribution to the observed shifts caused by cis−trans isomerization and should more accurately reveal the specific effects due to the aromatic amino acid side chain. To measure values for ΔΔ it is necessary to know which of the pipecolic protons in the cis form exchange with which protons in the trans form. The exchange partners were identified by two-dimensional exchange spectroscopy. The values of ΔΔ for GWPipG are bigger than those for GFPipG or GYPipG, and the fraction of the cis conformer for GWPipG is higher than that of GFPipG or GYPipG (Table 1). The correlation between ΔΔ and the amount of the cis conformer is not perfect since GFPipG and GYPipG have almost equal values of ΔΔ, but different fractions of the cis isomer.

The measured coupling constants and ROEs indicate that the aromatic side chain adopts a preferred rotamer in the cis conformer but not in the trans conformer. The two $^3J_{\alpha,\beta}$ coupling constants are unequal in the cis form. Using the method of Wagner and co-workers,[37] a combined analysis of the $^3J_{\alpha,\beta}$ coupling constants and the pattern of α to β and NH to β ROEs shows that the side chain of the aromatic acid residue preferentially adopts the rotamer with $\chi_1 = 180°$ in the cis conformer. The upfield β proton, denoted as β^B, which has a bigger coupling constant to the α proton is stereospecifically assigned to be the β_3 proton, and the downfield β proton, denoted as β^A, is assigned to be the β_2 proton. Analysis of the coupling constants using the method of Pachler[38] with the values for J_{gauche} and J_{anti} taken from the work by Wagner and co-workers[37] shows that the rotamer corresponding to $\chi_1 = 180°$ is populated 75% of the time for cis GWPipG, 73% of the time for cis GYPipG, and 66% of the time for cis GFPipG. In the trans form of all the aromatic containing peptides, the two $^3J_{\alpha,\beta}$ coupling constants are very similar and the two β protons have similar ROE intensities to the α proton and to the NH proton. These data indicate that in the trans form the aromatic side chain samples the three low-energy rotamers almost equally. The $^3J_{\alpha,\beta}$ coupling constants and the relative strength of the ROEs from each of the two β protons to the α proton and to the NH proton are included in the Supporting Information.

In the cis conformers, weak ROEs were observed from the α proton, from the most upfield β proton (the axial proton) and from the most upfield δ proton (the axial proton) of the pipecolic residue to the Tyr 2,6-ring protons for the peptide GYPipG and to the Trp 2H ring proton for the peptide GWPipG. In the trans conformers, no ROEs between the aromatic ring protons and the pipecolic ring protons were observed. No ROEs were observed between the aromatic protons and the proline ring protons in our previous studies of proline-containing tetrapeptides.[24]

In the cis form, each of the aromatic containing peptides appears to have some tendency to adopt a low fraction of nonrandom backbone structure, likely a type VI β-turn. Evidence for a type VI β-turn is provided by a reduced $^3J_{NH,\alpha}$ coupling constant for the aromatic residue and a weak cross turn $d_{\alpha,N}(2,4)$ NOE between the aromatic residue at position 2 and Gly-4.[23] The $^3J_{NH,\alpha}$ coupling constants for the aromatic residues are 1.4−1.6 Hz lower in the cis form than in the trans form. A weak ROE between $C_\alpha H$(Phe-2) and NH(Gly-4) in cis GFPipG and a weak ROE between $C_\alpha H$(Tyr-2) and NH(Gly-4) in cis GYPipG were observed. In cis GWPipG, the ROE between $C_\alpha H$(Trp-2) and NH(Gly-4) could not be positively identified due to overlap of the amide resonances of Trp-2 and Gly-4. It is interesting to note that there is no evidence for any formation of type VI β-turn in GAPipG or GChaPipG. This suggests that the small fraction of nonrandom backbone structure observed for

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the aromatic containing peptides is likely stabilized in part by the aromatic−pipecolic ring interaction. The values of the amide proton chemical shift temperature coefficients for the cis form of all the aromatic containing peptides lie between −6 and −10 ppb/K. These values indicate there is no significant intramolecular hydrogen bonding. There is a precedent for the stabilization of turns by side chain interactions involving proline and aromatic side chains. Dyson, Wright, and co-workers have observed that stacking interactions between a proline ring and two adjacent aromatic rings helped to stabilize a type VI turn structure in peptides of the sequence X−Ar−Pro-Ar−Hp, where X represents any amino acid, Ar an aromatic residue, and Hp a small hydrophilic residue.[23,36] It is very important to stress that the pipecolic acid containing peptides studied here have, as judged by NMR, only a slight tendency to adopt a nonrandom backbone structure. In particular there are not enough constraints to allow the meaningful calculation of a preferred backbone structure. This is to be contrasted to the proline-containing hexapeptides studied by Wright and co-workers. Some of those peptides have been shown to adopt highly populated turn structures. The different behavior of our peptides and those studied by Wright and co-workers should not be surprising. Their work was designed to probe the factors which stabilize turns and they necessarily choose to study peptides designed to have a high propensity to populate turn like structures. The goal of our work has been to provide a thorough characterization of the local effects of replacing a proline residue with a pipecolic acid residue, and we have deliberately designed a set of peptides which have a low tendency to populate stable turn like structures.

It is very interesting to note that the magnitude of ΔΔ of the pipecolic acid ring protons of the aromatic containing peptides as well as the mole fraction of the cis conformer are unaffected by 8 M urea (Table 1). The values of ΔΔ for the α and the most shifted β, γ, and ϵ protons all change by less than 10%. This result indicates that the interaction between the aromatic ring and the succeeding pipecolic ring is not attenuated by 8 M urea. A ROESY spectrum was recorded in 8 M urea for the GYPipG peptide. The same ROEs from the aromatic ring to the pipecolic ring that were detected in water were also observed in 8 M urea. The urea-induced denaturation of small helical peptides has been interpreted as resulting from the disruption of helix-stabilizing hydrogen bonds.[39] If this view of the effects of urea is correct, then our experiments offer further evidence that hydrogen bond mediated interactions do not play a significant role in stabilizing the aromatic−pipecolic interactions.

Thermodynamics of the Trans−Cis Isomerization. The Van't Hoff enthalpy of the trans−cis isomerization, $\Delta H°_{trans-cis}$, for each of the five peptides was measured in D_2O. Figure 2 shows the Van't Hoff plot. The thermodynamic parameters are listed in Table 2; also included are the previously measured thermodynamic parameters for the corresponding proline-containing peptides. The measured entropies of the trans−cis isomerization are small and negative for all of the peptides, indicating that the trans form is entropically more favored. For the peptide GAPipG, the effect of temperature on the value of equilibrium constant is minimal. It should be noted that the small amplitude of the change plus the uncertainty in measuring the ratio of cis to trans introduces some uncertainty in the measurement of $\Delta H°$. It is clearly simplistic to treat the equilibrium as a simple two-state process since both the cis and the trans pipecolic isomer likely sample a range of conformations. In particular, the cis form exhibits conformational heterogeneity since conformations with a syn and with an anti arrangement of the aromatic side chain and the pipecolic ring are both allowed. It is clear, however, based upon the *J* coupling constant analysis, observed ROEs, and chemical shifts that the syn arrangement of the rings is much more populated than the anti arrangement. In proline-containing peptides the trans conformation is normally enthalpically favored. In contrast, the measured enthalpy for GAPipG is near zero (0.01 kcal/mol). This value cannot be directly compared to the proline-containing GAPG peptide since it was not possible to measure the enthalpy of the trans to cis isomerization for this molecule due to spectral overlap. Raines and coworkers have reported a value of $\Delta H°_{trans-cis}$ of 1.27 kcal/mol in H_2O and in toluene for the model peptide *N*-acetylglycylproline methyl ester (Ac-GP-OMe).[40] The value of $\Delta H°_{trans-cis}$ for the GAPG peptide should be close to this value. The value of $\Delta H°_{trans-cis}$, 0.11 kcal/mol, is also near zero for the GChaPipG peptide. The very small

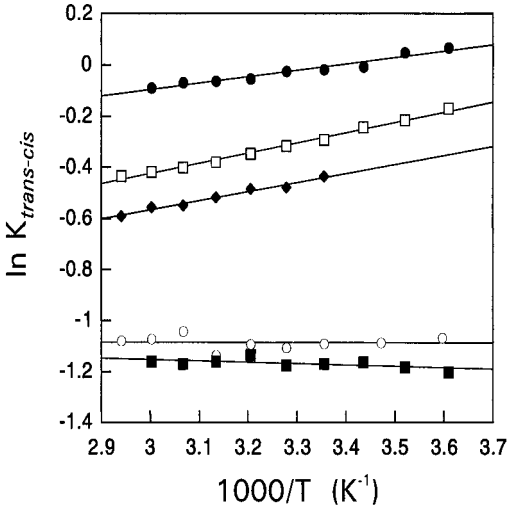

Figure 2. Van't Hoff plot for the trans to cis isomerization for the peptides. GWPipG (filled circles), GYPipG (open squares), GFPipG (diamonds), GAPipG (open circles), GChaPipG (filled squares). All measurements were made in D_2O at pD = 7.4

Table 2. Thermodynamics of the Trans−Cis Isomerization in D_2O

peptides	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/mol·K)	$\Delta G°$ (kcal/mol)[a]
GAPipG	0.01	−2.1	0.64
GChaPipG	0.11	−1.9	0.68
GFPipG	−0.71	−3.2	0.24
GYPipG	−0.80	−3.2	0.15
GWPipG	−0.50	−1.7	0.00
GFPG	0.53	−1.4	0.93
GYPG	0.65	−0.5	0.81
GWPG	0.75	0.3	0.66

[a] Measured at 298 K.

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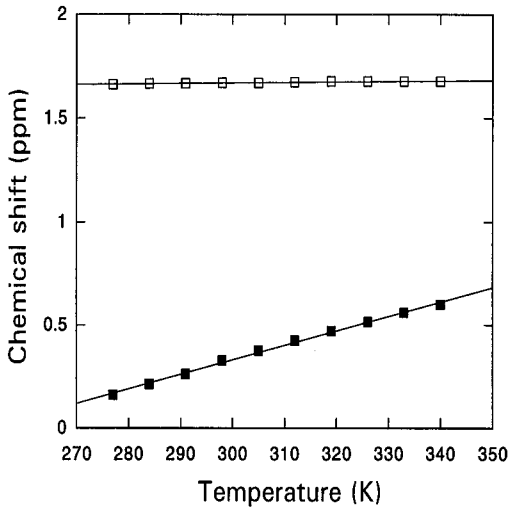

Figure 3. Temperature dependence of the chemical shift of the most upfield pipecolic β proton (the axial proton) in the cis form (closed squares) and in the trans form (open squares) of the peptide GYPipG.

values of $\Delta H°_{trans-cis}$ for GAPipG and GChaPipG relative to proline-containing peptides likely reflects an increase in unfavorable steric interactions in the trans form of the Pip-containing peptide. Since the value of $\Delta H°_{trans-cis}$ is independent of the bulk of the preceding side chain, interactions between the ϵ position of the pipecolic ring and the preceding α are the likely culprit.

The trans−cis equilibrium constants for all of the aromatic containing peptides decreased as the temperature increased. The measured $\Delta H°_{trans-cis}$ are −0.71, −0.80, and −0.5 kcal/mol for GFPipG, GYPipG, and GWPipG, respectively. The negative enthalpies for the trans−cis isomerization indicate that the cis conformer is enthalpically favored over the trans conformer for these peptides. This is the opposite of what has been measured for proline-containing peptides including those in which an aromatic residue precedes a proline. The negative values of $\Delta H°$ likely arises from two factors. First, the larger unfavorable steric interaction in the trans form of a pipecolic acid containing peptide decreases $\Delta H°$ to near zero, and second, the favorable aromatic−pipecolic acid interaction present only in the cis form causes $\Delta H°$ to change sign.

Temperature-dependent NMR studies provide good indirect evidence that the aromatic−pipecolic interaction is weakened at higher temperature. The decrease in $K_{trans-cis}$ with increasing temperature correlates nicely with the temperature-dependent changes in the chemical shifts of the pipecolic ring protons. The more upfield pipecolic β proton (the axial proton) of the cis form experiences the largest ring current shift, and hence its temperature dependence should most accurately reflect any changes in the aromatic−pipecolic interaction. The chemical shift of this proton is well separated from those of others, and it can be followed by a series of simple 1D ^{1}H NMR spectra. The chemical shift of this proton moves downfield with increasing temperature. The temperature dependence of the chemical shift of this β proton in the cis and in the trans form of GYPipG is shown in Figure 3. The temperature coefficient for this β proton is 6.1, 6.5, and 7.0 ppb/K for GFPipG, GYPipG, and GWPipG, respectively. In contrast, the chemical shift of the corresponding β proton in the trans form changed only slightly with temperature. The temperature coefficient for this proton is 0, 0.3, and 1.0 ppb/K for GFPipG, GYPipG, and GWPipG, respectively. The change in chemical shift of the cis β proton correlates well with the measured values of $K_{trans-cis}$ with correlation coefficients for the three peptides ranging from 0.97 to 0.99 for a linear fit, strongly suggesting a correlation between the strength of the aromatic pipecolic ring interaction, as measured by its effect on the chemical shifts, and the value of the equilibrium constant for isomerization. The slope of the chemical shift versus temperature plot is about two times larger for the ring current shifted pipecolic β proton than for the corresponding cis proline β proton, suggesting that the stabilizing interaction due to the aromatic residue is more sensitive to temperature for the pipecolic acid-containing peptides than for the proline-containing peptides.

Kinetics. The rates of cis−trans isomerization for the peptides in DMSO were measured using saturation transfer experiments. The rate of cis−trans isomerization in D_2O is too slow to be measured by these techniques. The rate is faster in DMSO-d_6, as judged by the more intense exchange cross-peaks between the trans peak and the corresponding cis peak in the ROESY spectra, and can be accurately measured. We have previously measured the rates of cis to trans and trans to cis isomerism for a related set of proline-containing peptides in DMSO.[24] Combining the two data sets allows us to probe the effect of a proline to pipecolic acid substitution upon the kinetics of the isomerism. It is important to point out that the same interactions that were detected in H_2O are also present in DMSO. The percentage of the cis conformer which is observed in DMSO (30%, 39%, 44%, and 46% for GAPipG, GFPipG, GYPipG, and GWPipG, respectively) is similar to that found in D_2O. The chemical shift of the Gly-4 amide proton experiences a significant upfield shift in the trans form of the aromatic containing peptides in DMSO, indicating that the aromatic−amide interaction is formed. In the cis isomer of these peptides the same pattern of ring current induced shifts is detected for the pipecolic protons. This provides evidence that the aromatic pipecolic ring interaction is still present in DMSO. The magnitude of the chemical shift perturbations is slightly smaller in DMSO. For example, the amide resonance of Gly-4 in the trans conformer of GWPipG is at 7.72 ppm in DMSO as opposed to 6.81 ppm in H_2O. In the cis form of this peptide, the most downfield shifted β proton resonance of the pipecolic ring is found at 0.00 ppm instead of −0.28 ppm. The slightly smaller perturbation of the chemical shifts in DMSO indicates that both the aromatic−amide interaction and the aromatic−pipecolic ring interaction are somewhat weaker in DMSO. This is consistent with the results of our previous study of a related set of proline-containing peptides.[24]

Selective saturation of the α proton of the cis pipecolic acid results in transfer to the corresponding α proton of the trans pipecolic acid. A representative saturation transfer curve from an experiment performed on the peptide GYPipG is shown in Figure 4 along with the α proton region of the difference spectrum. For comparison, the previously measured saturation transfer curve for the GYPG peptide is also plotted. It is clear that the rate of isomerization is faster for the pipecolic acid containing peptide.

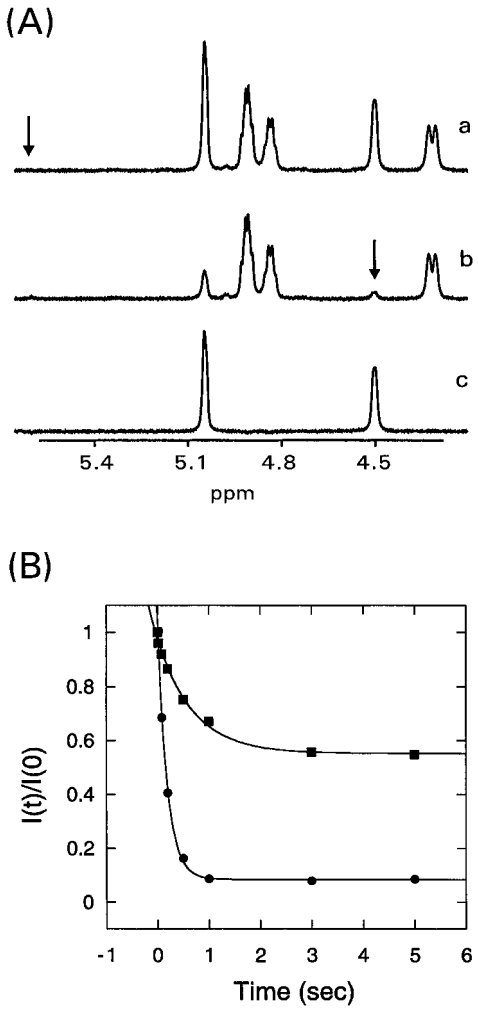

Figure 4. (A) Portion of the ^{1}H NMR spectrum of the peptide GYPipG in DMSO-d_6 at 323 K. The top spectrum (a) is a blank spectrum in which a presaturation pulse was applied at the position indicated by an arrow. The center spectrum (b) was recorded with a saturation pulse applied at the resonance of the α proton of the cis pipecolic residue as indicated by an arrow. The difference spectrum (a minus b) is displayed at the bottom (c). The presaturation time was 0.5 s. (B) Time development of the saturation transfer from the cis pipecolic α proton resonance to the α proton resonance of the trans pipecolic isomer of GYPipG (circles). The saturation curve for GYPG reported previously is also plotted (squares).

Table 3. Mole Fraction of the *Cis* Conformer (X_{cis}) and Rate Constants for the Isomerism Measured in DMSO-d_6 at 323 K

peptides	X_{cis}	$k_{cis-trans}$ (s^{-1})	$k_{trans-cis}$ (s^{-1})
GAPG	0.09	7.50	0.74
GAPipG	0.31	12.9	5.74
GFPG	0.17	2.60	0.44
GFPipG	0.36	8.26	4.65
GYPG	0.15	2.00	0.34
GYPipG	0.43	5.54	4.24
GWPG	0.18	1.10	0.24
GWPipG	0.44	4.76	3.77

The rate constants for the isomerization of the pipecolic-containing peptides along with the previously measured rate constants for the corresponding proline-containing peptides are summarized in Table 3. The value of $k_{cis-trans}$ is 1.7-fold larger for GAPipG than for GAPG, and $k_{trans-cis}$ is 7.8-fold larger for GAPipG than for GAPG. In all of the aromatic containing peptides the isomerization rates are faster for a pipecolic acid-containing peptide than for the corresponding proline-containing peptide. $k_{cis-trans}$ is 2.8−4.3-fold faster and $k_{trans-cis}$ is 10.6 to 15.7 times larger for the pipecolic-containing peptides. There are two plausible explanations for the increased rates. There should be more pronounced steric clashes in the ground state of the pipecolic-containing peptides than in the ground state of the proline-containing peptides due to the larger size of the pipecolic acid ring. Since the imide peptide bond in the transition state is 90° out of plane, any steric conflict in the transition state should be smaller than that in the two ground states. This effect should destabilize the cis and trans ground states relative to the transition state. The observation that the rate of trans to cis isomerism is enhanced more than the rate of cis to trans isomerism indicates that the trans conformer is destabilized to a greater extent than the cis conformer by the mutation of a proline to a pipecolic acid residue. This is fully consistent with the observed effects upon the cis−trans equilibrium. A second contributing factor is likely to be due to the greater pyramidalization of the pipecolic nitrogen atom. The increased pyramidalization will lower the barrier for isomerization.[41] X-ray structures of small molecules which contain pipecolic peptide bonds show that the imide nitrogen atom does not lie in the plane defined by its three covalently bound atoms. The deviation from planarity is larger than what is typically observed for small proline-containing peptides and can range up to as large as 0.36 Å.[42−46]

The rate of cis to trans proline isomerization is slower in peptides which contain an aromatic residue immediately prior to the proline compared to a proline-containing peptide with a nonaromatic residue preceding the proline.[24,26] Similar effects are observed for the corresponding pipecolic-containing peptides. The rate of trans to cis isomerization, $k_{trans-cis}$, and the rate of cis to trans isomerization, $k_{cis-trans}$, are slower for the aromatic containing peptides than for GAPipG. $k_{cis-trans}$ appears to be more affected by the presence of the aromatic residue than is $k_{trans-cis}$. $k_{trans-cis}$ is 1.2−1.5 times smaller for the aromatic containing peptides relative to GAPipG while $k_{cis-trans}$ is 1.6-fold to 2.7-fold smaller. Favorable intramolecular interactions present in the cis conformer and in the trans conformer will stabilize the ground states relative to the transition state and will slow the rates of isomerization relative to those measured for GAPipG. In the cis conformer the aromatic−pipecolic ring interaction will stabilize the ground state, and in the trans conformer the aromatic−amide interaction should stabilize the ground state. Neither of these interactions are expected to be present in the transition state.

Conclusions

There are a number of possible explanations for the stabilizing aromatic−pipecolic interactions. van der

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Waals interactions likely make a contribution, and hydrophobic interactions could also play a role, although the observation that the interactions persist in 8 M urea argues against hydrophobic interactions being the sole source of the stabilization. Electrostatic interactions between the aromatic ring and the imide nitrogen might also contribute.[47] Given the small (in energetic terms) size of the stabilizing interactions and the likelihood that several factors contribute, it is difficult to pinpoint the exact source of the stabilization.

The results of this study highlight the clear differences in the local conformational propensities of proline and pipecolic acid. These results indicate that substitution of a pipecolic acid residue for a proline can have significant structural, thermodynamic, and kinetic effects. Substitution of a pipecolic acid residue will significantly increase the tendency to adopt a cis peptide bond due largely to the destabilization of the trans form by an unfavorable steric interaction between the ϵ position of the pipecolic acid ring and the α position of the preceding residue. The rate of interconversion of the two isomers is faster for pipecolic acid containing peptides, and the Van't Hoff enthalpy is noticeably smaller. Particularly strong effects are observed when an aromatic residue precedes a pipecolic residue. In this case the propensity to adopt the cis conformer approaches 50% for the Trp-containing peptides, and the sign of the Van't Hoff enthalpy changes. This work should provide a useful database for predicting the effects of the substitution of an amino acid residue by a pipecolic acid residue in studies involving the rational modification of peptides and should aid in understanding the effect of the pipecolic ring system on the conformational equilbria.

Experimental Section

Peptide Synthesis and Characterization. All peptides were synthesized by solid-phase methods on a Perspective Model 9050 Plus peptide synthesizer using fluorenylmethyloxycarbonyl (Fmoc) protected free amino acids. The side chain of tyrosine was protected by a *tert*-butyl group. PAL resin substituted at ca. 0.38 mmol/g was used to afford carboxy terminal primary amides. Deprotection of Fmoc-protected amine groups was performed using a 6 min 20% piperidine/DMF wash. The reaction time for each coupling step was 60 min, and an extra coupling step was performed for the addition of the first amino acid to the resin. The peptides were N-acetylated on the resin using a solution of 5% pyridine, 5% acetic anhydride, and 90% DMF. Peptides were deprotected and cleaved from the resin by treatment with trifluoroacetic acid (TFA), thioanisole, ethanedithiol, and anisole (91:3:3:3) for 2 h. After filtration, the TFA solution was evaporated with N_2 to minimal volume. The resulting mixture was dried under vacuum and then dissolved in 5% acetic acid and lyophilized to dryness.

The peptides were purified by reversed phase high-pressure liquid chromatography (HPLC) with gradient elution using an A−B gradient (buffer A, 0.1% TFA in water; buffer B, 10% water and 0.1% TFA in acetonitrile). A gradient of 15−70% buffer B in 70 min with a flow rate of 10 mL/min was used for each peptide. The peptides were greater than 98% pure as judged by analytical HPLC. The peptides were characterized by ^{1}H NMR and by fast atomic bombardment mass spectrometry (University of Illinois Mass Spectrometry Center) [GAPipG expected 377.4, observed 378.2; GYPipG expected 447.4, observed 447.8; GWPipG expected 471.5, observed 471.5; GFPipG expected 431.4, observed 432.1; GChaPipG expected 437.4, observed 438.0].

NMR Spectroscopy. Samples for NMR experiments were between 5 and 20 mM. The pH value for samples in D_2O was 7.4 after correcting for isotope effects. The pH value for the peptides in 90% H_2O/10% D_2O was 4.3. The lower pH value was chosen for the H_2O experiments in order to reduce problems associated with cross saturation of the amide resonances. Both the chemical shifts of the side chain resonances and the percentage of the cis proline conformer are independent of pH over this range. Samples in DMSO were dissolved in H_2O, the pH value was adjusted to 7.4, and then the solution was lyophilized to dryness before dissolving in DMSO.

NMR spectra were acquired at the SUNY Stony Brook NMR center on a Bruker AMX-600 spectrometer and on Varian Inova 500 and 600 MHz spectrometers. Sequential assignments were achieved using double quantum filtered correlated spectroscopy (DQF−COSY) and rotating frame Overhauser effect (ROESY) experiments.[48] Quadrature detection was achieved by the use of time proportional phase incrementation (TPPI)[49] except where otherwise indicated. Data matrixes were typically 512 real by 4096 complex for the DQF−COSY experiments and 512 real by 2048 complex for ROESY experiments. The mixing time used for all ROESY experiments was 250 ms. To reduce TOCSY artifacts, two identical ROESY spectra with different carrier frequency positions were recorded. The spin-lock field strength used for ROESY experiments was 2.5 kHz. The spectral widths in both dimensions were 6666.7 Hz for the peptides GFPipG, GYPipG, GChaPipG, and GAPipG and 7812 Hz for the GWPipG peptide. Spectra were all internally referenced to 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt (TSP) at 0.0 ppm. The water signal was suppressed by weak presaturation during the 1.5 s relaxation delay. The fraction of cis pipecolic acid was determined by integrating well-resolved peaks in the one-dimensional ^{1}H NMR spectrum. $^3J_{NH,\alpha}$ coupling constants were measured from one-dimensional spectra collected at high digital resolution (0.1−0.2 Hz/point) except for GWPipG in which case the $^3J_{NH,\alpha}$ coupling constant was measured from a DQF−COSY spectrum acquired with 8192 data points in t2. $^3J_{\alpha,\beta}$ coupling constants were measured from E.COSY experiments acquired with a data matrix of 512 (complex) by 8192 using the method of States et al.[50] The exchange partner of each pipecolic proton was assigned by the exchange cross-peak between the proton in the trans form and the proton in the cis form in ROESY spectra recorded in DMSO. To measure the enthalpy of the isomerization, variable temperature experiments for each peptide were performed in D_2O from 277 to 340 K except for GFPipG which was followed over the range of 298 to 340 K. The temperature was changed in steps of 7 K. Amide proton chemical shift temperature coefficients were measured from variable temperatures experiments performed over the range of 278−313 K. Saturation transfer experiments were performed as described previously.[24]

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Supporting Information Available: A table of proton chemical shift assignments in 90% H_2O/10% D_2O at pH 4.3, 298 K for both the cis and the trans conformers of GAPipG, GChaPipG, GFPipG, GYPipG, and GWPipG. A second table

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listing the data used to stereospecifically assign the β protons of the aromatic residues and used to define the relative populations of the rotamers. This table lists the relative ROE intensities from the NH proton to the β protons and from the α proton to the β protons and the $^3J_{\alpha,\beta}$ coupling constants of the aromatic residues of GFPipG, GYPipG, and GWPipG (3 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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