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Effect of Sequence on Peptide Geometry in 5-*tert*-Butylprolyl Type VI β-Turn Mimics

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Abstract: The influence of sequence on turn geometry was examined by incorporating (2*S*,5*R*)-5-*tert*-butylproline (5-^{t}BuPro) into a series of dipeptides and tetrapeptides. (2*S*,5*R*)-5-*tert*-Butylproline and proline were respectively introduced at the C-terminal residue of *N*-acetyl dipeptide *N*′-methylamides **1** and **2**. The conformational analysis of these analogues was performed using NMR and CD spectroscopy as well as X-ray diffraction to examine the factors that control the prolyl amide (in this text, the term "prolyl amide" refers to the tertiary amide composed of the pyrrolidine nitrogen of the prolyl residue and the carbonyl of the N-terminal residue) equilibrium and stabilize type VI β-turn conformation. The high cis-isomer population with aromatic residues N-terminal to proline was shown to result from a stacking interaction between the partial positive charged prolyl amide nitrogen and the aromatic π-system as seen in the crystal structure of **1c**. The effect of sequence on the prolyl amide equilibrium of 5-^{t}BuPro-tetrapeptides (Ac-Xaa-Yaa-5-^{t}-BuPro-Zaa-XMe, **13** and **14**) was studied by varying the amino acids at the Xaa, Yaa, and Zaa positions. High (>80%) cis-isomer populations were obtained with alkyl groups at the Xaa position, an aromatic residue at the Yaa position, and either an alanine or a lysine residue at the Zaa position of the 5-^{t}BuPro-tetrapeptide methyl esters in water. Tetrapeptides Ac-Ala-Phe-5-^{t}BuPro-Zaa-OMe (Zaa = Ala, Lys), **14d** and **14f**, with high cis-isomer content adopted type VIa β-turn conformations as shown by their NMR and CD spectra. Although a pattern of amide proton temperature coefficient values indicative of a hairpin geometry was observed in peptides **14d** and **14f**, the value magnitudes did not indicate strong hydrogen bonding in water.

Introduction

Reverse turns play important roles in protein folding.[1] Local sequence-specific interactions can initiate the folding process by enhancing turn structures that nucleate hairpins and thereby stabilize β-pleated sheets.[2] On the other hand, cis-trans isomerization about prolyl amide (in this text, the term "prolyl amide" refers to the tertiary amide composed of the pyrrolidine nitrogen of the prolyl residue and the carbonyl of the N-terminal residue) bonds in turn regions can be a rate-limiting step in the folding mechanism.[3] The factors that favor specific isomer geometry about prolyl amides can thus contribute significantly toward controlling peptide folding.

The type VI β-turn is a relatively rare secondary structure that features uniquely an amide cis isomer N-terminal to a proline residue situated at the $i + 2$ position of the peptide bend.[1,4] Type VI β-turns play important roles in protein folding.[5−7] They have been shown to be recognition sites for peptidyl prolyl isomerases (PPIases) which can accelerate protein folding by catalyzing the conversion of the cis isomer to its more thermodynamically stable trans conformation.[5,6] Type VI β-turns have also been implicated in other important recognition events of bioactive proteins. For example, a type VI β-turn conformation has been proposed for thrombin-catalyzed cleavage of the V_3 loop of HIV gp120, a prerequisite to viral infection.[7] In addition, in the X-ray structure of the ribonuclease S protein, a type VIa β-turn was located at the central position of a hairpin conformation.[4]

Type VI β-turns are classified into two sub-types based on the dihedral angle values of their central $i + 1$ and $i + 2$ residues.[4] In the type VIa β-turn, the proline ψ-dihedral angle is equal to 0°, and a 10-membered intramolecular hydrogen bond exists between the carbonyl oxygen of the *i* residue and the amide hydrogen of the $i + 3$ residue. This intramolecular hydrogen bond is not present in the type VIb β-turn in which the proline ψ-dihedral angle value is equal to 150°.[4]

As a minor isomer, the prolyl amide cis conformer is often difficult to observe in natural peptides.[8] Minor cis conformers may, however, exhibit significant effects on the transport, metabolism, and reactivity of biologically active peptides.[9] To

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enhance the cis-isomer population, several approaches have been tried to stabilize this geometry by the means of conformational constraint using structural links and steric interactions.[10−18] Alternatively, double bond isosteres have been employed to mimic the spatial orientation presented by the cis conformer.[19] These approaches have achieved effective replication of the backbone geometry of the type VI β-turn as well as analogues exhibiting inhibitory activity against PPIases.[20] Moreover, stabilization of a hairpin conformation has been achieved in a model linear tetrapeptide possessing an indolizidinone amino acid mimic of the central residues of type VIa β-turn as demonstrated by NMR in DMSO and IR spectroscopy in dichloromethane.[14d] These approaches have succeeded in replicating cis conformer geometry; however, an important aspect of prolyl amides that many such examples by design fail to mimic has been the conformational equilibrium exhibited by prolyl peptides.

We have synthesized and used (2*S*,5*R*)-5-*tert*-butylproline (5-^{t}BuPro) to explore both prolyl amide cis-isomer geometry as well as the amide isomer equilibrium N-terminal to proline in various peptides.[21−23] In Ac-Xaa-5-^{t}BuPro-NHMe, the 5-^{t}BuPro residue stabilized type VIa and VIb β-turn conformations contingent upon the stereochemistry of the N-terminal residue.[22] Dipeptides possessing Ala and Leu residues adopted type VIa and VIb β-turn conformations when the N-terminal amino acid possessed respectively L- and D-configuration as shown by NMR and CD spectroscopy as well as X-ray analysis.[22] Furthermore, the presence of phenylalanine at the N-terminal of 5-^{t}BuPro caused a remarkable increase in cis-isomer population (Ac−Phe-5-^{t}BuPro-NHMe exhibited >90% prolyl amide cis isomer in water).[22]

As well as its power to augment the cis-isomer population, the 5-*tert*-butyl substituent influences the barrier for amide isomerization.[21b] In the case of the (2*S*,5*R*)-diastereomer, the sterically bulky *tert*-butyl group interacts with the N-terminal residue such as to twist the amide bond away from planarity.[22] In *N*-acetylproline *N*′-methylamides, twisting of the prolyl amide was among factors that caused a reduction in the barrier for isomerization of 3.7 kcal/mol in the (2*S*,5*R*)-5-^{t}BuPro analogue relative to its proline counterpart.[21b] The influences of sequence and stereochemistry on the amide equilibrium become thus more apparent in (2*S*,5*R*)-5-^{t}BuPro peptides, because of the combination of the reduced isomerization barrier and the enhanced cis-isomer population.

We have synthesized a diverse array of 5-^{t}BuPro peptides by employing *N*,*N*′-bis(2-oxo-3-oxazolidinyl)phosphonic chloride (BOP−Cl)[24] as a coupling reagent to attach different amino acid electrophiles onto the sterically hindered prolyl residue. This synthesis achievement has allowed us to explore the influence of sequence on the equilibrium N-terminal to the prolyl residues. Study of the effect of sequence on isomer equilibrium in natural prolyl peptides has previously shown that aromatic residues adjacent to proline caused an augmentation in the cis-isomer population.[8,25−27a] Although aromatic residues N-terminal to proline have been shown to cause a 10-fold reduction in the cis to trans isomerization rate,[3a] to the best of our knowledge, little has been reported about the factors by which aromatic residues augment the cis-isomer population and increase the isomerization energy barrier. Amino acid residues possessing side chains with hydrogen-bond acceptor and donor moieties have been shown to stabilize turn conformations when adjacent to proline.[1a,27b] We report now the influence of hydrogen-bonding residues on the prolyl amide equilibrium and the cis-isomer population.

Examining the influence of sequence on turn geometry, we have introduced 5-^{t}BuPro into a series of dipeptide and tetrapeptide analogues possessing aromatic and hydrogen-donor and acceptor residues. By studying the conformations of these analogues using NMR and CD spectroscopy as well as X-ray diffraction, we have itemized factors that control the prolyl amide equilibrium and stabilize type VI β-turn geometry. The high cis-isomer populations and preponderance of type VIa β-turn conformation brought about by aromatic amino acid

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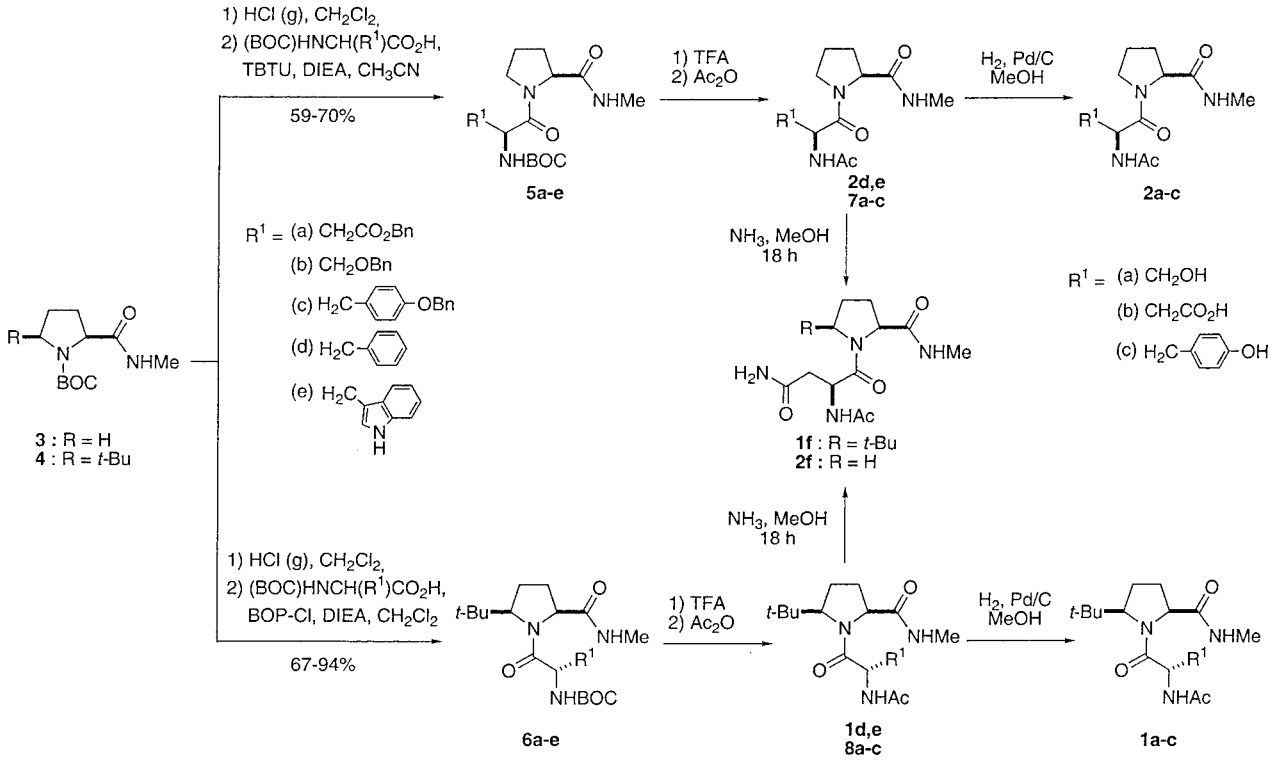
Scheme 1. Synthesis of *N*-(Acetyl)dipeptide *N*′-Methylamides **1a**−**f** and **2a**−**f**



residues has been shown to result from an interaction between the pyrrolidine ring and the aromatic π-system in the cis-amide as observed in the X-ray structure of Ac-Tyr-5-*t*BuPro-NHMe. Furthermore, hydrogen-bonding residues situated N-terminal to 5-*t*BuPro were found to destabilize the amide cis isomer. Studying tetrapeptide analogues, we have examined the propensity for type VIa β-turns to nucleate hairpins. A combination of aromatic residues at the N-terminal and small alkyl groups at the C-terminal of tetrapeptide esters has led to high populations of cis confomers that exhibited hydrogen-bonding characteristics of β-hairpins.

Results

Synthesis of Dipeptides 1 and 2. Dipeptides Ac-Xaa-5-*t*BuPro-NHMe (**1a**−**f**) were normally synthesized using Boc and benzyl (Bn) protecting groups for the respective protection of the amine and side-chain groups. The protected amino acids were coupled to the N-terminal of 5-*t*BuPro-NHMe[21a] using BOP−Cl and diisopropylethylamine (DIEA) in dichloromethane to furnish dipeptides **6** in 67−94% yield (Scheme 1). For comparison, dipeptides possessing natural proline, Ac-Xaa-Pro-NHMe (**2a**−**f**), were synthesized by coupling similarly protected amino acids to proline *N*′-methylamide using benzotriazol-1-yl-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and DIEA in acetonitrile which furnished dipeptides **5** in 59−70% yield. Removal of the Boc group with TFA in dichloromethane followed by acetylation of the amine with acetic anhydride (Ac_2O) and potassium carbonate in dichloromethane provided *N*-acetyl dipeptide *N*′-methylamides. The benzyl ester and ether groups of the *N*-acetyl *O*-benzyl dipeptide *N*′-methylamides **7a**−**c** and **8a**−**c** were then deprotected by hydrogenation using 1 atm of H_2 with Pd/C in methanol and afforded the *N*-acetyl dipeptide *N*′-methylamides **1a**−**c** and **2a**−**c** in 92−99% yield. Because of the low yields obtained in coupling reactions with *N*-Boc-asparagine, the 5-*t*BuPro and Pro dipeptides **1f** and **2f** were synthesized by converting their respective aspartic benzyl esters, **7a** and **8a**, to the corresponding amides, **1f** and **2f**, using liquid ammonia in methanol in 97−99% yield (Scheme 1).

Synthesis of Tetrapeptides. Longer peptide sequences have been synthesized with 5-*t*BuPro using a combination of solution and solid-phase chemistry.[21c,22c,23] Tetrapeptides **13**−**16** were prepared on oxime resin[28a] by employing a Boc-protected dipeptide possessing 5-*t*BuPro at the C-terminal. The first *N*-(Boc)amino acid was loaded onto oxime resin using *N*,*N*-dicyclohexylcarbodiimide (DCC) in dichloromethane for 18 h (Scheme 2).[28] The loading of the resin (mmol/g) was then determined by treatment of a precise amount of the resin with *n*-propylamine in chloroform[28b] and subsequent measurement of the weight and purity of the resulting *N*-(Boc)amino *N*′-propylamide as assessed by NMR spectroscopy. Sequential elongation involved deprotections using TFA in dichloromethane, couplings of *N*-(Boc)amino acids using TBTU and DIEA in DMF and acetylation using Ac_2O and DIEA in CH_2Cl_2.

The sterically hindered 5-*tert*-butylproline was introduced into the peptides as a dipeptide unit that was synthesized in solution (Scheme 2). Protection of *N*-Boc-5-*t*BuPro with benzyl bromide and DIEA in dichloromethane at reflux for 18 h gave ester **10** which was exposed to HCl (g) in dichloromethane to remove the Boc group and then coupled to *N*-(Boc)amino acids using BOP−Cl to provide the *N*-(Boc)dipeptide benzyl esters **11a**−**c**. The benzyl group was removed by hydrogenation to afford the *N*-(Boc)dipeptides **12a**−**c** that were coupled to the resin using TBTU and the solid-phase protocol described above.

The peptides were cleaved from the resin to afford the peptide *N*′-methylamides and methyl esters by using respectively

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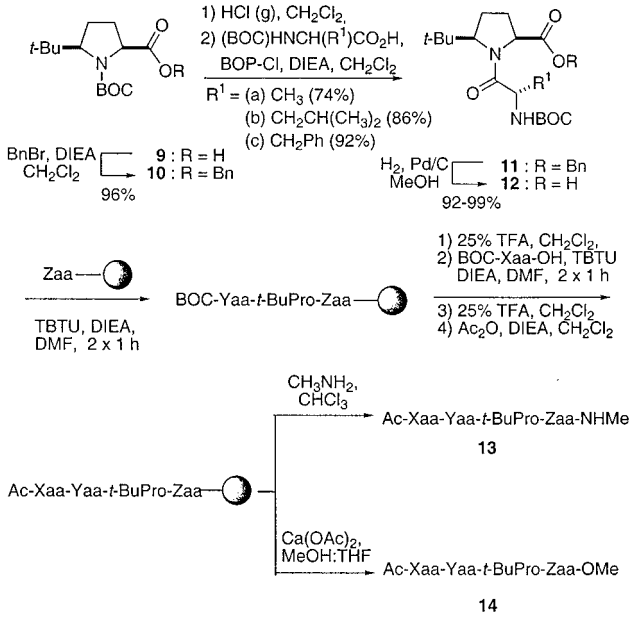
Scheme 2. Solid Phase of 5-*tert*-Butylprolyl Tetrapeptides **13** and **14**



methylamine in chloroform and calcium acetate in MeOH:THF at 40 °C.[29] The final peptides **13**−**16** were obtained in 21−61% overall yields after purification by C18 reverse-phase HPLC and lyophilization. The purity of the peptides **13**−**16** was examined by analytical HPLC and their compositions were verified by fast atom bombardment mass spectrometry.

Conformational Analysis of the Dipeptides

NMR Spectroscopy. The conformation of peptides **1** and **2** was analyzed by NMR spectroscopy in chloroform, DMSO, and water. The relative populations of the amide cis and trans isomers N-terminal to the prolyl residues were measured by integration of the isomeric *tert*-butyl singlets and *N*′-methyl doublets in the ^{1}H NMR spectra. As previously noted,[22] the *tert*-butyl singlet of the amide cis isomer appeared always upfield from that of the trans isomer in the 5-*tert*-butylprolyl peptides **1**. Nuclear Overhauser effect experiments were used to confirm the assignment of the cis isomer in dipeptides **1** and **2** on the basis of observation of a cross-peak between the N-terminal amino acid and the proline α-hydrogens in the NOESY and ROESY spectra.

In the prolyl dipeptides **2**, the amide trans isomer geometry N-terminal to the prolyl residue was the major conformer (Figure 1) in all three solvents, as observed in linear prolyl peptides.[7,26,27,30,31] Aromatic residues N-terminal to proline exhibited a 2−3-fold increase in the cis isomer population relative to their alanine counterpart. The largest amount of cis-amide (44%) was observed with Ac-L-Trp-Pro-NHMe among the prolyl peptides in water. On the contrary, the 5-*tert*-butylprolyl peptides **1** adopted the amide cis isomer in the major conformer (Figure 1). The presence of an aromatic amino acid N-terminal to 5-*tert*-butylproline caused a significant increase in the cis isomer population. The highest cis isomer population (96%) was observed for Ac-L-Trp-*t*BuPro-NHMe in water. Relative to their aliphatic and aromatic amino acid counterparts, hydrogen-bond donor and acceptor residues N-terminal to 5-*tert*-butylproline gave lower cis-isomer populations. An increase in cis-isomer population was observed when the aromatic residue was varied from phenylalanine to tyrosine to tryptophan in 5-*tert*-butylprolyl dipeptides, which has also been the trend seen in prolyl peptides.[26,27a] This tendency may be caused by the increase in electron density in the aromatic rings which interacts effectively with the partial positive charge of the prolyl nitrogen thus increasing the cis-isomer population.

In 5-*t*BuPro-peptides **1c**−**e** and **1g**−**j**, which have amino acids of L-configuration possessing aliphatic and aromatic side chains, the cis-isomer population was augmented on switching solvent from DMSO to water and from DMSO to chloroform. On the contrary, higher cis-isomer populations were observed in DMSO relative to water when the N-terminal residue was of L-configuration possessing a side-chain capable of forming hydrogen bonds (**1a**, **1b**, and **1f**), as well as when a D-amino acid residue was N-terminal to 5-*t*BuPro (**1d**′, **1h**′, and **1i**′). In the natural prolyl peptides which possess D-amino acid residues (**2d**′, **2h**′, and **2i**′), a large increase of cis isomer was also observed in DMSO relative to water and chloroform. However, in prolyl peptides **2** possessing a side-chain capable of forming hydrogen bonds (**2a**, **2b**, and **2f**), a greater amount of the cis isomer was found in water relative to DMSO.

The influence of solvent composition on the chemical shifts of the amide signals was used to identify amides engaged in intramolecular hydrogen bonds. In the major amide cis isomer of 5-*t*BuPro-peptides **1**, the signal for the *N*′-methylamide proton appeared always downfield relative to that for the acetamide proton in chloroform. The *N*′-methylamide proton signals appeared between 7.29 and 8.52 ppm whereas the acetamide proton signals came between 5.97 and 7.21 ppm for the cis isomer of peptides **1**. The downfield shifted amide proton signal suggested an intramolecular hydrogen bond between the *N*′-methylamide and acetamide carbonyl in a type VIa β-turn conformation. In contrast, the signals of the *N*′-methylamide and acetamide of the major trans conformer of prolyl peptides **2** showed no significant differences in their chemical shifts which appeared between 6.03 and 7.17 ppm. Solvent changes from chloroform to water and to DMSO caused little changes in the chemical shift of the *N*′-methylamide proton signal of peptide **1**, which moved 0.04−0.60 ppm and 0.17−1.12 ppm downfield, respectively. The signals for the acetamide proton of peptides **1** and **2** and the *N*′-methylamide proton of peptide **2** shifted significantly (0.40−2.55 ppm) with changes in solvent. In conclusion, the influence of solvent composition on the chemical shift of the amide signals demonstrated the *N*′-methylamide proton of the cis isomer of peptide **1** to be in an intramolecular hydrogen bond of a type VIa β-turn conformation.

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra of peptides **1a**−**f** and **2a**−**f** were measured in water and acetonitrile to study the influence of solvent composition on peptide conformation. The CD spectra of 5-*tert*-butylprolyl peptides possessing aromatic side chains **1c**−**e** exhibited a strong negative band at 190 ± 5 nm, a strong positive band at 205 ± 6 nm and a weak negative band at 230 ± 10 nm in water (Figure 2A). When changing the solvent from water to acetonitrile, the shape of the CD curves of peptides **1c**−**e** remained unchanged (Figure 2B). This type of CD curve has been identified as a class B CD spectrum which we have previously assigned to type VIa β-turn conformations.[22,32] Similar curve shapes were

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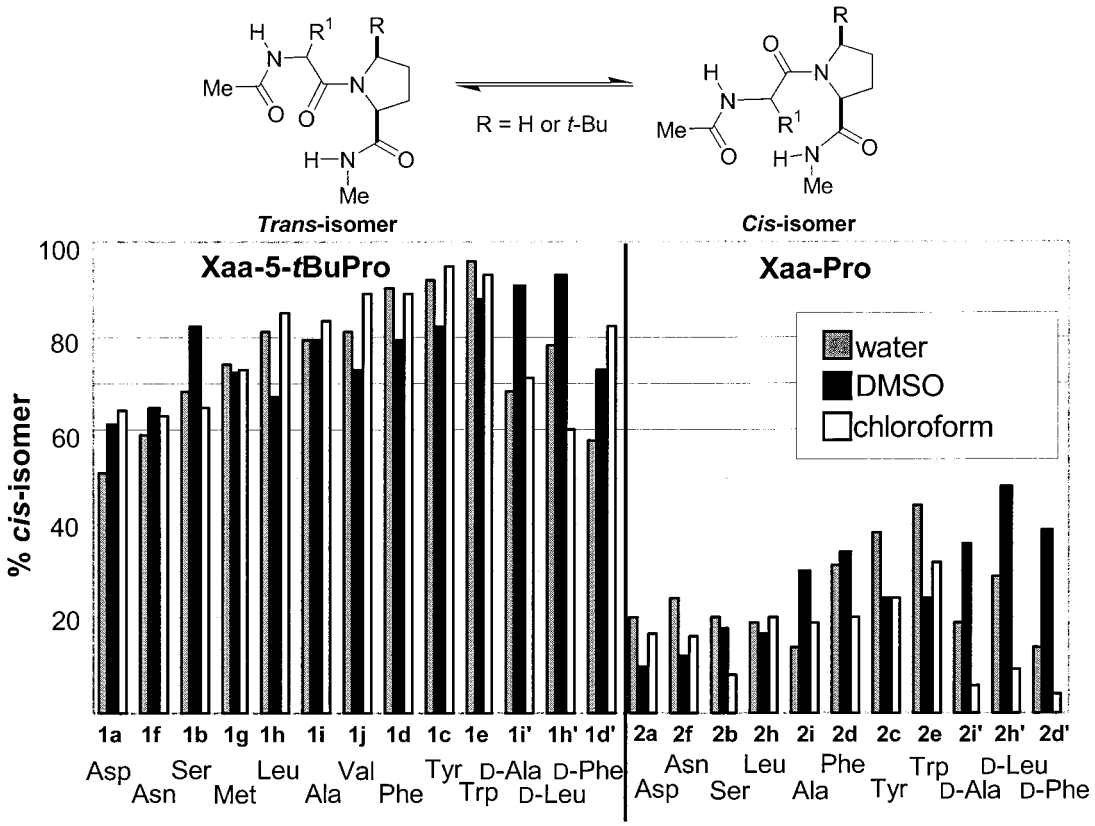

[a] The data for *N*-acetyl dipeptide *N*′-methylamides with alkyl and D-amino acid residues were obtained from Refs 22.

Figure 1. Amide equilibrium of *N*-acetyl dipeptide *N*′-methylamide.

obtained for 5-*tert*-butylprolyl peptides **1a**, **1b**, and **1f** which possessed hydrogen-bond donor and acceptor side chains (Figure 3, parts A and B).

The CD spectra of the prolyl peptides possessing aromatic side chains **2c**−**e** varied significantly with changes in solvent composition and exhibited no distinct CD curve shapes (Figure 2, parts C and D). On the other hand, the CD curves of prolyl peptides **2a** and **2f** which possessed Asp and Asn residues with hydrogen-bonding side chains were characterized by negative maxima at around 210 ± 2 and 220 ± 2 nm in acetonitrile that converged to a single band at around 205 nm in water (Figure 3, parts C and D). This type of CD curve has been assigned to a class C CD spectrum that has been reported for peptides adopting prolyl amide trans isomers in type II′ β-turn conformations.[22,32,33]

X-ray Crystallographic Analysis. Crystals of *N*-acetyl-L-tyrosyl-5-*tert*-butylproline *N*′-methylamide were obtained from a mixture of MeOH, EtOAc, and hexane. The analysis of the crystal structure by X-ray diffraction revealed an asymmetric unit with a molecule of water and two molecules of **1c** which adopted similar turn conformations.[34] The crystallographic analysis of **1c** demonstrated the presence of an amide cis isomer N-terminal to the 5-*tert*-butylprolyl residue with twisted dihedral angle values of $\omega = 8.5°$ and $\omega = 13.0°$ (Figure 4). As previously shown, the sterically bulky 5-*tert*-butyl substituent influenced the amide bond N-terminal to the 5-*tert*-butylprolyl residue and distorted it from planarity.[22a−b] Furthermore, an intramolecular hydrogen bond could be inferred to exist between the *N*′-methylamide proton and the acetamide carbonyl oxygen from measurement of an interatomic distance of 2.08 and 2.18 Å in the X-ray structure of **1c**. The dihedral angles of peptide **1c** ressembled those of the central $i + 1$ and $i + 2$ residues of an ideal type VIa β-turn. For comparison, the dihedral angles of peptide **1c**, the ideal values of type VIa and VIb β-turn conformations as well as those for Ac-L-Leu-^{t}BuPro-NHMe and Ac-D-Leu-^{t}BuPro-NHMe are listed in Table 1.[4,22] The 5-^{t}BuPro ψ-dihedral angle of dipeptide **1c** placed the *N*′-methylamide hydrogen at a 2.4 Å interatomic distance from the prolyl nitrogen, which has also been observed in the X-ray structure of Ac-L-Leu-^{t}BuPro-NHMe.[22a] The interaction between the prolyl amide nitrogen and the *N*′-methylamide hydrogen has been suggested to stabilize the pyramidalized amide in the transition state and thereby accelerate isomerization N-terminal to proline.[42]

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(34) The structure of **1c** was solved at l'Université de Montréal X-ray facility using direct methods (SHELXS96) and refined with NRCVAX and SHELXL96: $C_{42}H_{64}N_6O_9$; $M_r = 796.992$; orthorhombic, colorless crystal; space group $P2_12_12_1$; unit cell dimensions (Å) $a = 9.294$ (2), $b = 12.895$ (3), $c = 36.837$ (7); volume of unit cell (Å^3) 4415.12 (16); $Z = 4$; $R_1 = 0.0614$ for $I > 2\sigma(I)$, $wR_2 = 0.1569$ for all data; GOF = 1.054. The author has deposited the atomic coordinates for the structure of **1c** with the Cambridge Crystallographic Data Center. The coordinates can be obtained, on request, from the Cambridge Crystallographic Data Center, 12 Union Road, Cambridge, CB2 1EZ, U.K.

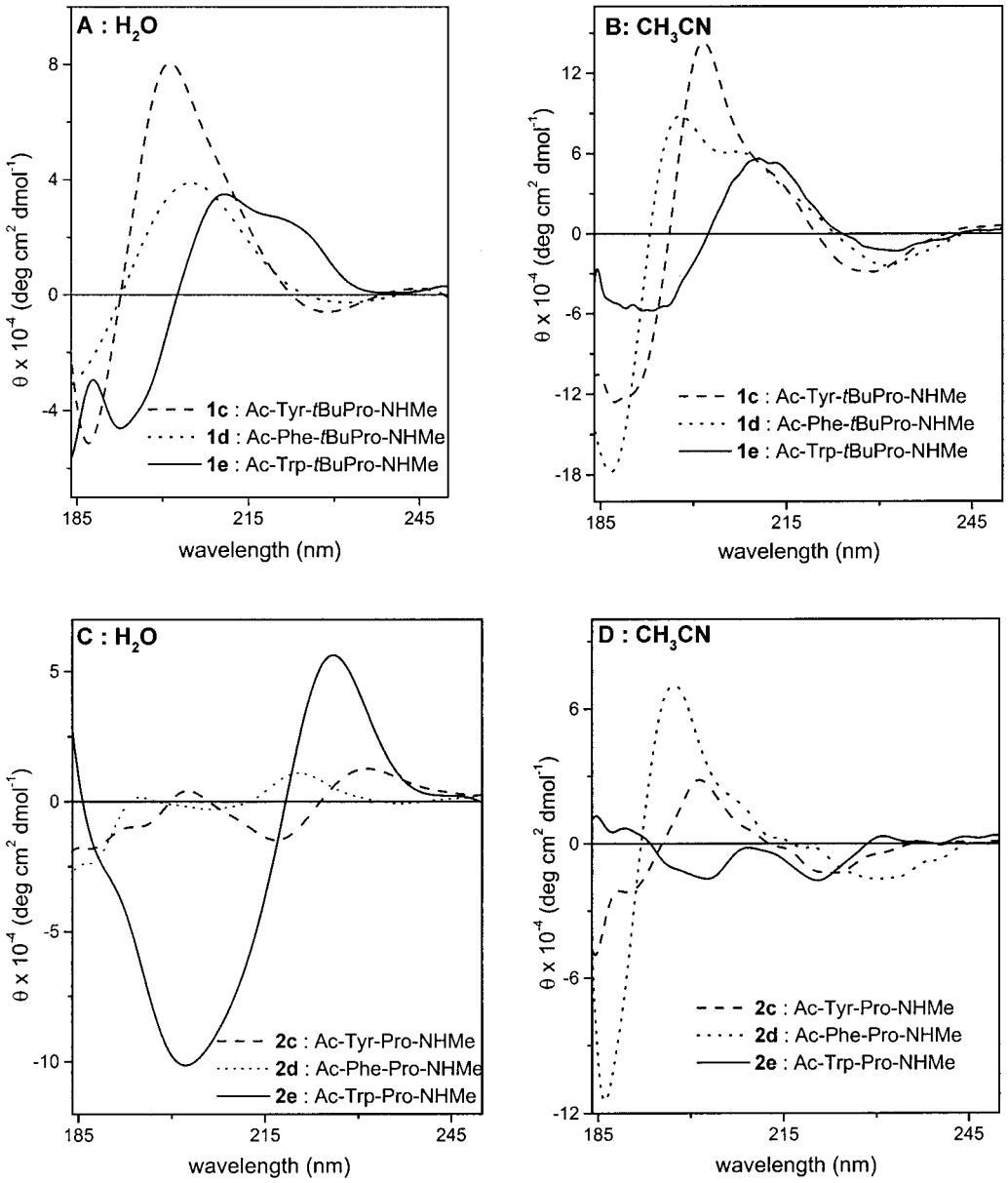

Figure 2. Circular dichroism spectra of dipeptides **1c**−**e** (A, in water, and B, in acetonnitrile) and **2c**−**e** (C, in water, and D, in acetonitrile) at 0.l mM.

In the structure of peptide **1c**, the Tyr side-chain adopted a χ_1 dihedral angle value of 172°. This trans conformer (Figure 5) positioned the tyrosyl aromatic ring beneath the proline ring (distance of 3.4−4.9 Å between the proline nitrogen and the tyrosyl phenyl group, Figure 4). Prior to our work, computational study had suggested that the amino−aromatic interactions occur between positively charged or $\delta(+)$ amine groups in side chains and the $\delta(-)$ π-electrons of the aromatic ring of Phe, Tyr, and Trp when they are separated by 3.4−6.0 Å.[35] Stacking interactions between the aromatic and pyrrolidine rings can be found in the crystal structures of proteins and cyclic peptides possessing aromatic residues N-terminal to proline.[8,36] For example, in the X-ray structure of the hexapeptide cyclo(L-Phe-L-Pro-D-Ala)$_2$, the L-Phe-L-Pro amide bonds are in the cis isomer with the χ_1 dihedral angle of the Phe residue in the trans conformer (Figure 5).[36a] Interactions between the aromatic and proline rings in the amide cis isomer of prolyl peptides containing aromatic residues have been observed by NMR spectra in which the chemical shift of the α-proton of proline was shifted upfield.[37]

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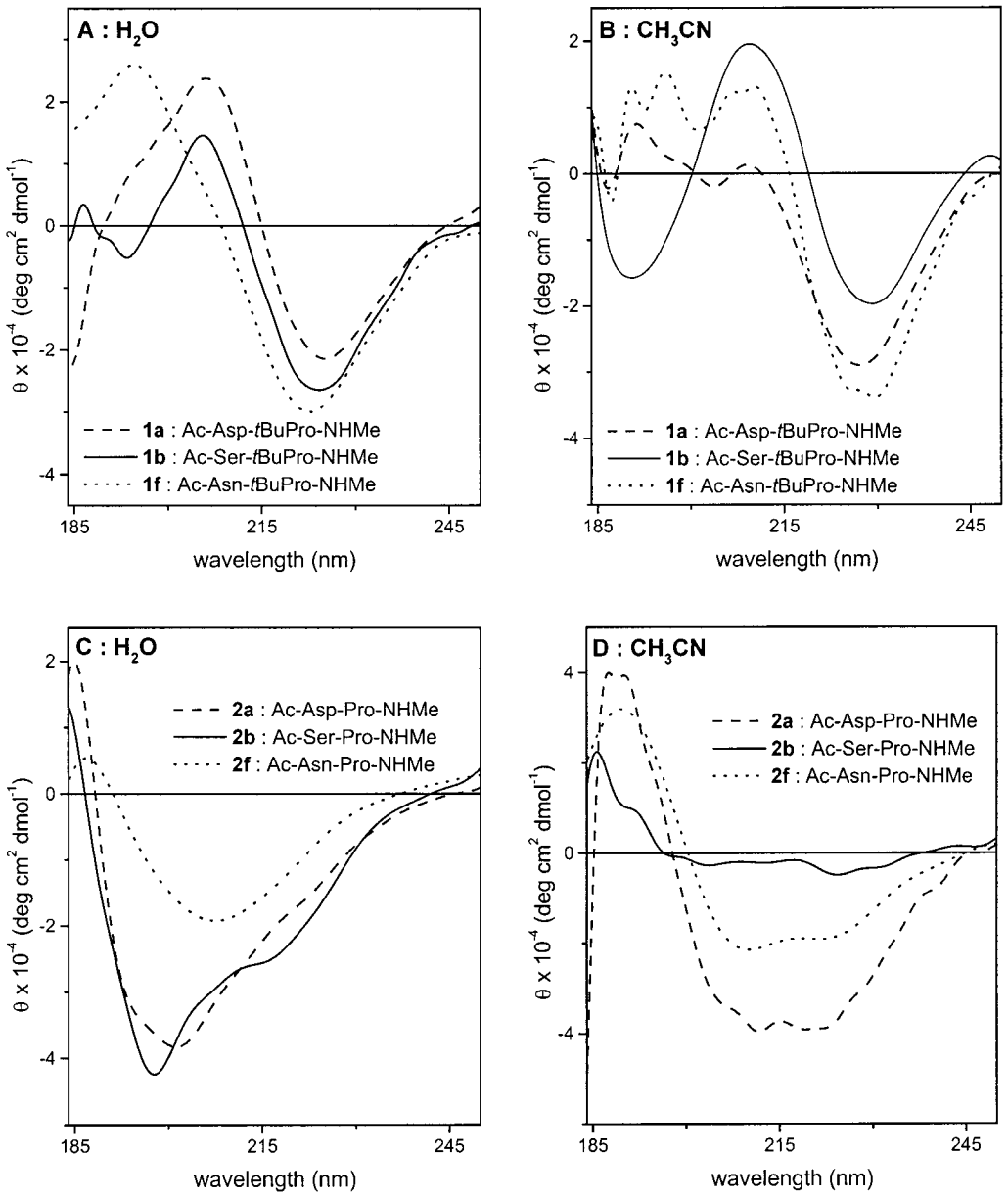

Figure 3. Circular dichroism spectra of dipeptides **1a**, **1b**, and **1f** (A, in water, and B, in acetonnitrile) and **2a**, **2b**, and **2f** (C, in water, and D, in acetonitrile) at 0.l mM.

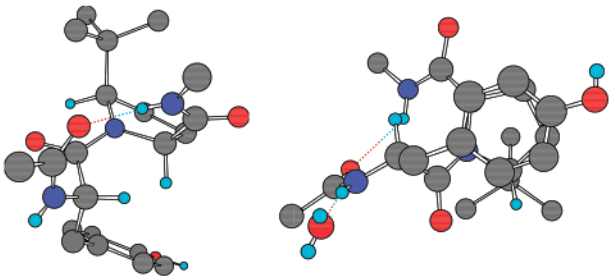

Figure 4. Structure of Ac-L-Tyr-5-tBuPro-NHMe **1c** from X-ray crystallography.[34] Hydrogens are shown on nitrogens and chiral carbons (C, gray; N, blue; O, red; H, turquoise).

Table 1. Comparison of the Dihedral Angles (deg) of Ideal Type VIa β-Turn and X-ray Structure of *N*-(Acetyl)tyrosyl-5-*tert*-butylproline *N*′-Methylamide (1c)

entry	ϕ_{i+1}	ψ_{i+1}	ω	ϕ_{i+2}	ψ_{i+2}
ideal type VIa β-turn[4]	−60	120	0	−90	0
Ac-L-Tyr-5-^{t}BuPro-NHMe (A)	−58	137	8	−101	26
Ac-L-Tyr-5-^{t}BuPro-NHMe (B)	−58	137	13	−94	11
Ac-L-Leu-5-^{t}BuPro-NHMe[22a]	−61	139	17	−95	19
ideal type VIb β-turn[4]	−120	120	0	−60	150
Ac-L-Leu-5-^{t}BuPro-NHMe[22b]	93	141	29	−81	157

Conformational Analysis of the Tetrapeptides

The conformations of tetrapeptides Ac-Xaa-Yaa-5-^{t}BuPro-Zaa-XMe **13** (X = NH) and **14** (X = O) and Ac-Xaa-Yaa-Pro-Zaa-XMe **15** (X = NH) and **16** (X = O) were studied by NMR spectroscopy in 9:1 H_2O/D_2O. The signals in the ^{1}H NMR were assigned using COSY, TOCSY and ROESY experiments. The relative populations of the prolyl amide cis and trans isomers of tetrapeptides **13**−**16** were measured by integration of the *tert*-butyl singlets and the *N*′-methylamide or methyl ester signals in the proton NMR spectra (Table 2).

The cis-isomer geometry was found to be stabilized by aliphatic residues such as alanine and valine relative to the

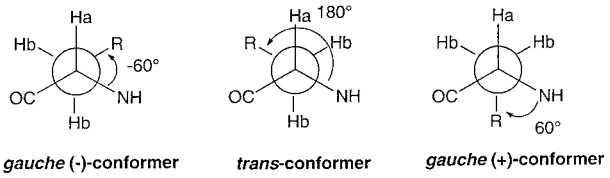

Figure 5. Rotamers around the $C_\alpha-C_\beta$ axis in amino acids.

Table 2. Yaa-5-^{t}BuPro Amide Isomer Equilibrium of Tetrapeptides Ac-Xaa-Yaa-5-^{t}BuPro-Zaa-XMe and Ac-Xaa-Yaa-Pro-Zaa-XMe in 10% D_2O/H_2O

entry	Xaa (*i*)	Yaa (*i*+1)	(*i*+2)	Zaa (*i*+1)	X	% cis isomer
13a	Ala	Ala	5-^{t}BuPro	Ala	NH	49
13b	Ala	Ala	5-^{t}BuPro	Leu	NH	44
13c	Ala	Ala	5-^{t}BuPro	Phe	NH	47
14a	Ala	Ala	5-^{t}BuPro	Phe	O	73
13d	Ala	Leu	5-^{t}BuPro	Leu	NH	50
13e	Ala	Leu	5-^{t}BuPro	Phe	NH	43
13f	Ala	Phe	5-^{t}BuPro	Phe	NH	62
12g	Ala	Phe	5-^{t}BuPro	Leu	NH	65
13h	Ala	Phe	5-^{t}BuPro	Val	NH	52
14b	Ala	Phe	5-^{t}BuPro	Val	O	68
14c	Ala	Phe	5-^{t}BuPro	Lys	O	79
13i	Ala	Phe	5-^{t}BuPro	Ala	NH	68
14d	Ala	Phe	5-^{t}BuPro	Ala	O	84
13j	Ser	Phe	5-^{t}BuPro	Ala	NH	70
14e	Ser	Phe	5-^{t}BuPro	Ala	O	73
14f	Val	Phe	5-^{t}BuPro	Ala	O	80
15	Ala	Phe	Pro	Ala	NH	22
16	Ala	Phe	Pro	Ala	O	9

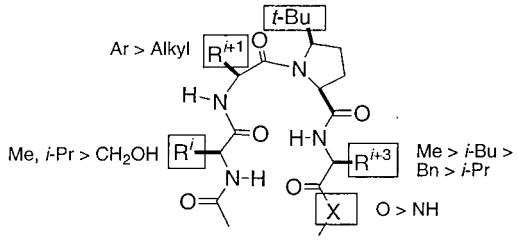

Figure 6. Influence of sequence on the amide equilibrium (% cis isomer) in 5-^{t}BuPro-tetrapeptides.

hydrogen-bonding residue serine at the *i* position (Figure 6). In the natural sequence Xaa-Tyr-Pro-Tyr-Asp-Val, an alanine residue (57%) gave rise to higher cis-isomer population than a serine residue (52%) at the *i* position.[26d] Protein X-ray analysis in contrast has shown the highest occurrence of serine at the *i* position in peptides possessing proline at the $i + 2$ position in the amide cis conformer.[38] As observed in the 5-^{t}BuPro-dipeptides, a significant augmentation of the cis-isomer population occurred when an aromatic residue was at the $i + 1$ position, N-terminal to 5-^{t}BuPro in the *N*-acetyl tetrapeptide *N*′-methylamides. When the 5-^{t}BuPro residue was replaced by proline, the cis-isomer population decreased from 68−84% to 9−22% (Table 2). Thus, the steric interactions between the *tert*-butyl substituent and the side-chain of the N-terminal residue disfavored the prolyl amide trans isomer and increased the cis-isomer population. At the $i + 3$ position of the tetrapeptides, alanine and lysine were found to give rise to higher amide cis-isomer populations relative to the β-branched alkyl residue valine. In protein X-ray data, alanine C-terminal to proline gave rise to the highest cis-conformer population.[38] Among the modifications to 5-^{t}BuPro-tetrapeptides, a significant increase in cis-isomer population was observed when the *N*′-methylamide was replaced by its methyl ester counterpart. For example, the cis-isomer population of tetrapeptide **13c** (47%) was augmented 26% on conversion to its methyl ester **14a** (73%, Table 2). This augmentation may be caused by the removal of competitive hydrogen-bonding conformations in the tetrapeptides *N*′-methylamides that favor the prolyl amide trans isomer.

The coupling constant values ($^3J_{NH-C\alpha H}$) for the $i + 1$ and $i + 2$ residues of tetrapeptides **14d** and **14f** exhibiting the highest cis-isomer populations were characteristic of turn structure.[39] The $^3J_{NH-C\alpha H}$ coupling constant value for the Phe residue in tetrapeptides **14d** and **14f** was 3.7 Hz and in good agreement with a 4 Hz coupling constant corresponding to the ϕ dihedral angle of −60° for the $i + 1$ residue in a type VIa β-turn conformation. The $^3J_{NH-C\alpha H}$ coupling constant values for the other residues in the tetrapeptides **14d** and **14f** were in the range of 6.2−8.4 Hz. Sequential HαN(i, i +1) NOEs were observed for all expected residues in the tetrapeptide **14d**. In the ROESY spectra of tetrapeptide **14d**, an additional HαN(i, i +2) NOE was observed between the α-hydrogen of Phe and amide proton of the C-terminal Ala characteristic of a β-turn conformation.

The temperature coefficient values ($\Delta\delta/\Delta T$) were measured for the amide protons in peptide **14d**, which possessed the highest cis-isomer population, its amide counterpart **13i**, and their respective prolyl analogues **16** and **15** in 10% D_2O/H_2O (Table 3). The amide protons of the alanine residues at the Xaa and Zaa positions of 5-^{t}BuPro-peptide **13i** and **14d** exhibited lower temperature coefficient values than the phenylalanine and *N*′-methylamide amide protons, which may indicate their participation in intramolecular hydrogen bonds. This tendency was not observed in the prolyl tetrapeptides **15** and **16** where the chemical shift variation with temperature for the amide protons were in the range of −6.1 to −10.4 ppb/K. The temperature coefficient values were measured in DMSO for the amide protons in 5-^{t}BuPro-tetrapeptides **14d** and **14f**, which possessed high cis-isomer populations. The amide proton of the alanine residue at the Zaa position of peptide **14d** exhibited similar temperature coefficient values in DMSO (−4.7 ppb/K) and water (−4.9 ppb/K). In DMSO, peptides **14d** and **14f** exhibited similar temperature coefficient values. Although temperature coefficient values greater than −3 ppb/K in DMSO have been suggested to indicate amide protons engaged in intramolecular hydrogen bonds, such values are normally measured in cyclic and larger peptides than those reported here.[40] The pattern of the temperature coefficient magnitudes in **13i**, **14d**, and **14f** did conform to a hairpinlike structure; however, the values themselves suggested solvent-exposed amides presumably due to the flexibility of the linear tetrapeptide.

The CD spectra of tetrapeptides **14d** and **14f** exhibited a negative maximum at 230 nm, a positive maximum at 215 nm, an intense negative maximum at 200 nm and a positive maximum at 190 nm in water (Figure 7). This type of CD curve has been classified as a class D CD spectrum which has been previously assigned to a β-turn conformation.[32]

Discussion

We have studied the influence of sequence and stereochemistry of the residue N-terminal to 5-^{t}BuPro by the synthesis and analysis of a series of dipeptide and tetrapeptide analogues (Ac-Xaa-5-^{t}BuPro-NHMe and Ac-Xaa-Yaa-5-^{t}BuPro-Zaa-XMe). All of the 5-^{t}BuPro-dipeptide analogues adopted predominant cis-isomer conformations about the prolyl amide bond. In the

Table 3. Influence of Temperature on the NH Chemical Shifts of the Major Isomer of the Tetrapeptides **13i**, **14d**, **14f**, **15**, and **16** in Water and in DMSO

	$-\Delta\delta/\Delta T$ (ppb/K)[a]			
peptides	NHXaa	NHPhe	NHZaa	NHMe
Ac-Ala-Phe-5-^{t}BuPro-Ala-NHMe (**13i**)	7.7	8.8	6.9	8.4
Ac-Ala-Phe-5-^{t}BuPro-Ala-OMe (**14d**)	6.9 (6.0)[b]	10.0 (6.9)[b]	4.9 (4.7)[b]	
Ac-Ala-Phe-Pro-Ala-NHMe (**15**)	7.2	7.8	10.4	7.1
Ac-Ala-Phe-Pro-Ala-OMe (**16**)	9.5	8.6	6.1	
Ac-Val-Phe-5-^{t}BuPro-Ala-OMe (**14f**)	6.0[b]	6.7[b]	4.5[b]	

[a] Determined by 600 MHz NMR in 10% D_2O/H_2O at 5 mM concentration. [b] Values are in DMSO.

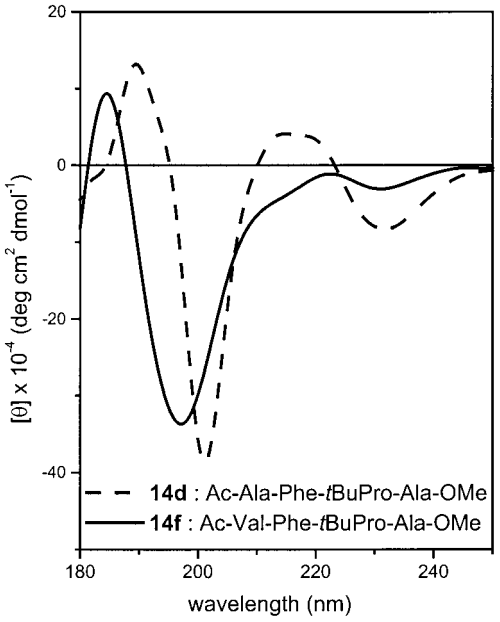

Figure 7. Circular dichroism spectra of tetrapeptides **14d** and **14f** in water at 0.1 mM.

dipeptide series, the turn type was contingent on the configuration of the amino acid N-terminal to 5-^{t}BuPro and adopted, respectively, type VIa and VIb β-turn conformations when the N-terminal amino acid was of L- and D-configurations.[22a,b]

In the *N*-acetyl dipeptide *N*′-methylamides, the cis-isomer population increased as the amino acid N-terminal to 5-^{t}BuPro was varied from a hydrogen-bonding residue to an alkyl residue to an aromatic residue. This pattern was also observed in the natural prolyl dipeptides where aromatic residues gave higher cis-isomer populations than alkyl and hydrogen-bonding residues. The augmentation of cis isomer with aromatic residues N-terminal to proline appears to be caused by the interaction between the $\delta(+)$ nitrogen of 5-^{t}BuPro and the aromatic ring of the tyrosyl side-chain which adopted a χ_1 value of 172° as observed in the X-ray structure of **1c**. This stacking interaction cannot be achieved in the trans-isomer geometry because the side-chain χ_1 dihedral angle adopts a gauche conformer (Figure 5).[37] In the dipeptides possessing hydrogen-bonding side chains (**1a**, **1b**, **2a**, and **2b**), a hydrogen-bond between the *N*′-methylamide hydrogen and the carbonyl oxygen of the side-chain in an Asx-turn may favor the trans isomer and therefore decrease the cis-isomer population.[41]

The influence of solvent composition on the cis-isomer equilibrium was contingent on the sequence of the dipeptide. Alkyl and aromatic residues in L-Xaa-5-^{t}BuPro dipeptides gave higher cis-isomer population in water than DMSO presumably due to favorable hydrophobic interactions between the proline ring and the N-terminal residue in the cis conformer. The downfield shift for the *N*′-methylamide proton signal of 5-^{t}BuPro-dipeptides **1** was indicative of an intramolecular hydrogen bond between the *N*′-methylamide proton and the acetamide carbonyl in a type VIa β-turn conformation. The presence of a type VIa β-turn geometry for 5-^{t}BuPro-dipeptides in solution, independent of solvent composition, was supported by the circular dichroism spectra of dipeptides **1a**−**f** in water and acetonitrile. In chloroform, the intramolecular hydrogen bond in the type VIa β-turn conformation may be stabilized, resulting in an increased cis-isomer population in 5-^{t}BuPro dipeptides with L-configuration possessing alkyl and aromatic residues; in DMSO, the cis-isomer population was reduced presumably by the disruption of this intramolecular hydrogen bond.[3b] In the prolyl peptides possessing hydrogen-bonding residue (**2a**, **2b**, and **2f**), higher cis populations were observed in water which may compete with the side-chain hydrogen donor and perturb intramolecular hydrogen bonding that can stabilize a trans conformer.

In the solid state, *N*-acetyl-L-tyrosyl-5-*tert*-butylproline *N*′-methylamide (**1c**) existed in a type VIa β-turn conformation as shown by X-ray diffraction. In addition, the aromatic and proline rings were stacked in the crystal structure in a way that may stabilize the cis-isomer geometry by an interaction between the partial positive charge of the amide nitrogen and the electron-rich aromatic π-system. The *tert*-butyl substituent distorted the prolyl amide from planarity which may result in lowering of the prolyl amide isomerization energy barrier. Stabilization of the transition state for prolyl isomerization may also occur from interaction between the lone pair of the pyramidalized prolyl amide nitrogen and the *N*′-methylamide hydrogen, which were inferred to be separated by 2.4 Å in the crystal structure of **1c**.[6a,42] A second stabilizing force in the transition state may arise from the aromatic side chain of tyrosine because as the prolyl amide carbonyl rotates, it may interact with the aromatic ring protons which from the crystal structure were inferred to be at a distance of 3.7−4.6 Å. Such a C−H···O interaction between the side chains of the aromatic residues in the binding pocket of the PPIase FKPB and the tertiary amide carbonyl of its substrate has previously been suggested to stabilize the transition state for enzyme-catalyzed isomerization.[6a,43] The increased cis-isomer population and relative increase in the prolyl amide isomerization energy barrier that have been observed in natural prolyl peptides possessing aromatic amino acids, both

(43) In a preliminary investigation, the tetrapeptide succinyl-Ala-Ala-5-^{t}BuPro-Phe-OMe (71% cis isomer in 10% D_2O/H_2O; t_R = 16.1; HRMS calcd for $C_{29}H_{43}N_4O_8$ (MH^+) 575.3081; HRMS found 575.3097) was synthesized, was tested, and exhibited no inhibition against the PPIase cyclophilin at 364.7 μM. We thank Professor Felicia Etzkorn and Dr. Scott, A. Hart for their analysis of the inhibitory activity of this tetrapeptide.

however, appear to be due to ground-state stabilization through interactions between the $\delta(+)$ nitrogen and π-aromatic system.

The effect of sequence on the prolyl amide equilibrium of 5-^{t}BuPro-tetrapeptides (Ac-Xaa-Yaa-5-^{t}BuPro-Zaa-XMe) was studied by varying the amino acids at the Xaa, Yaa, and Zaa positions. The highest cis-isomer populations were obtained with alkyl groups at the Xaa position, an aromatic residue at the Yaa position, and either an alanine or a lysine residue at the Zaa position of the 5-^{t}BuPro-tetrapeptide methyl esters. It has been previously illustrated that replacement of amide bonds with esters in peptides prevented the formation of undesired hydrogen-bonding conformations.[44] In natural prolyl peptides, high cis-isomer populations have been obtained by placing aromatic residues at both the N- and C-termini of proline.[26] In the 5-^{t}BuPro-tetrapeptide **13f**, a combination of Phe residues at the N- and C-termini to 5-^{t}BuPro did not produce the highest cis-isomer populations. On the contrary, amino acids with linear alkyl groups, such as alanine and lysine, at the C-terminal of 5-^{t}BuPro-tetrapeptide *N*′-methylamides **13g** and **13i** produced higher cis-isomer populations. Alkyl-branched residues at the C-terminal of 5-^{t}BuPro may not favor the cis isomer because of the steric interactions between the side-chain and *tert*-butyl subsituents.

Turn formation in peptides has been illustrated by the presence of a significant HαN(i, i +2) NOE connectivity.[26] A strong HαN(i, i +2) NOE was observed between the α-hydrogen of Phe and amide proton of the C-terminal Ala in tetrapeptide **14d**. The circular dichroism spectra of peptides **14d** and **14f** exhibited a similar curve shape that has been classified as a type D CD spectrum, assigned to β-turn conformations.[32] Thus, peptide **14d** was shown to adopt a type VIa β-turn conformation in water.

The temperature coefficient study of tetrapeptides **13i**, **14d**, and **14f** in water and DMSO did not provide values that corresponded to intramolecular hydrogen bonding amide protons. However, a pattern was observed where the amide protons of the residues at the Xaa and Zaa positions of 5-^{t}BuPro-peptides **13i**, **14d**, and **14f** exhibited lower temperature coefficient values than the phenylalanine and *N*′-methylamide amide protons. Polar solvents such as water and DMSO are known to perturb intramolecular hydrogen-bonds in peptides. Steric interactions of 5-*tert*-butylproline could not stabilize a hairpin conformation in a linear tetrapeptide relative to the stabilizing structural link in an indolizidinone amino acid type VIa β-turn mimic.[14d] Although a type VIa β-turn was located at the central position of a hairpin conformation about residues 92−94 in the X-ray structure of the ribonuclease S protein, the type VIa β-turn conformation has rarely been observed to stabilize a hairpin geometry in natural peptides.[4,45] Our data reflect the difficulty of stabilizing a hydrogen-bonded network in a small peptide possessing an amide cis isomer in polar solvents.

Protein folding implicates a set of conformational changes and multiple transition states to adopt finally the low energy native structure. Different interactions are involved in this dynamic process such as hydrophobic effects, solvation of polar groups and hydrogen bonding which involve both van der Waals and electrostatic interactions.[46] Prolyl amide isomerization in proteins can be a rate-limiting step in the folding mechanism. Although the trans-isomer geometry has been shown to be of lower energy, the cis isomer may be augmented by the hydrophobic stacking interaction of the N-terminal residue to proline with the pyrrolidine ring as observed in the X-ray structure of **1c**. The prolyl amide cis isomers in proteins may thus begin folding processes by a series of hydrophobic interactions prior to isomerization to the more stable trans isomer.

In conclusion, we have demonstrated that the prolyl amide equilibrium was influenced by the sequence in the 5-^{t}BuPro-dipeptides and tetrapeptides. X-ray analysis of Ac-L-Tyr-^{t}BuPro-NHMe has illustrated the stabilization of the prolyl amide cis isomer by a stacking interaction of the aromatic and pyrrolidine rings. By using steric constraints of a *tert*-butyl substituent, we were able to favor a type VIa β-turn conformation in *N*-acetyl tetrapeptide methylesters, possessing a prolyl amide cis isomer and a 10-membered intramolecular hydrogen bond. Incorporation of these type VI β-turn mimics into larger peptides is being pursued to provide more understanding of the impact of prolyl amide cis isomers and type VI β-turns on the folding of peptide structures.

Experimental Section

General Data. Solvents and reagents were purified as previously described. NMR and CD data were measured as described in ref 22. Mass spectral data, HRMS (EI and FAB), were obtained by the Université de Montréal Mass Spectroscopy facility.

General Procedure for Coupling to 5-*tert*-Butylproline *N*′-Methylamide. A solution of (2*S*,5*R*)-5-*tert*-butylproline *N*′-methylamide hydrochloride (0.37 mmol, prepared according to refs 21a and 22a), *N*-(Boc)amino acid (0.74 mmol), and DIEA (0.26 mL, 1.5 mmol) in CH_2Cl_2 (4 mL) was cooled to 0 °C, treated with BOP−Cl (188 mg, 0.74 mmol), stirred for 1 h, and allowed to warm to room temperature with stirring for 18 h. Brine was added, and the solution was extracted with EtOAc. The combined organic layers were washed with 0.1 M HCl (2 × 10 mL), 5% $NaHCO_3$ (2 × 10 mL), and brine (10 mL), dried, and evaporated to a residue that was purified by chromatography on silica gel using 35% EtOAc in hexane as eluant. Evaporation of the collected fractions furnished *N*-(Boc)dipeptide *N*′-methylamides.

General Procedure for Acetamide Synthesis. A solution of *N*-(Boc)dipeptide *N*′-methylamide (20.5 mg, 0.05 mmol) in 25% TFA in CH_2Cl_2 (1 mL) was stirred for 1 h, and the solvent was evaporated. The residue was dissolved in CH_2Cl_2 (1 mL), treated with K_2CO_3 (65.6 mg, 0.5 mmol) and acetic anhydride (45 μL, 0.5 mmol), and stirred for 18 h. The solution was filtered, washed with CH_2Cl_2 (2 × 5 mL), and evaporated to give the *N*-acetyl dipeptide *N*′-methylamide.

General Procedure for Benzyl Group Deprotection. A solution of *N*-acetyl-*O*-benzyl-dipeptide *N*′-methylamide **8a**−**c** (110.0 mg, 0.26 mmol) in MeOH (3 mL) was treated with Pd/C (26 mg, 10 wt %) under 1 atm of H_2 and stirred for 18 h at room temperature. The solution was filtered onto Celite and washed with MeOH, and the filtrate was evaporated to furnish *N*-acetyl dipeptide *N*′-methylamides **1a**−**c**.

General Procedure for Asparaginyl Dipeptide Synthesis. A solution of *N*-acetyl-*O*-benzyl-(2*S*)-aspartyldipeptide *N*′-methylamides **7a** and **8a** (100.0 mg, 0.23 mmol), respectively, in MeOH (2 mL) was treated with NH_3 (g) bubbles at 0 °C and stirred for 18 h at room temperature. The solution was evaporated and triturated with Et_2O to give *N*-acetyldipeptide *N*′-methylamides **1f** and **2f**, respectively.

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General Protocol for the Synthesis of *N*-Acetyl Dipeptide *N*′-Methylamides Possessing Natural Proline. A solution of *N*-(Boc)-amino acid (1.4 mmol) in CH_3CN (6 mL) was treated with DIEA (0.49 mL, 2.8 mmol), proline *N*′-methylamide hydrochloride (115 mg, 0.7 mmol, prepared according to ref 22a) and TBTU (0.45 g, 1.4 mmol), stirred at room temperature for 18 h, and partitioned between brine (10 mL) and EtOAc (10 mL). The organic phase was washed with 0.1 M HCl (2 × 8 mL), 5% $NaHCO_3$ (2 × 8 mL), and brine (10 mL), dried, and evaporated to a residue that was purified by chromatography on silica gel (35% EtOAc in hexane). The *N*-(Boc)dipeptide *N*′-methylamides **5a**−**e** were treated with 25% TFA in CH_2Cl_2 (10 mL) for 1 h and evaporated. The resulting dipeptide *N*′-methylamide trifluoroacetates were dissolved in CH_2Cl_2 and treated under the same acetylation and benzyl deprotection conditions as described above.

Peptide Synthesis. The synthesis of the tetrapeptides were carried out manually by a stepwise solid-phase procedure using oxime resin. Serine and lysine were introduced as Boc-L-Ser(Bn)-OH and Boc-L-Lys(Cbz)-OH. Couplings were performed with Boc-protected amino acids (200 mol %), TBTU (200 mol %), and DIEA (400 mol %) in DMF for 1 h. The resin was agitated with N_2 bubbles during the coupling, rinsing, and deprotection sequences. Coupling reactions were monitored by the Kaiser ninhydrin test.[47] In cases of incomplete couplings, the resin was resubmitted to the same coupling conditions. Deprotections were performed with 25% TFA in CH_2Cl_2 (2 × 30 min) and the resin was free-based with 10% DIEA in CH_2Cl_2 (2 × 5 min). N-Terminal acetylation of the peptides was accomplished by treating the resin with Ac_2O (1000 mol %) and DIEA (1000 mol %) in CH_2Cl_2 for 1 h. The tetrapeptide *N*′-methylamides were obtained by cleaving the peptides from the resin with 10% methylamine in $CHCl_3$ upon agitation with a mechanical shaker for 24 h. The tetrapeptide methyl-esters were obtained by treating the resin with $Ca(OAc)_2$ in MeOH: THF (1:4) at 40 °C for 48 h. The crude material was purified with semipreparative RP-HPLC (Higgins C18 column, 20 × 250 mm, particle size 5 μm) with solvent A, H_2O (0.05% TFA), and solvent B, 75% CH_3CN/H_2O (0.05% TFA). Analytical RP-HPLC was performed on a Higgins C18 (4.6 × 250 mm, particle size 5 μm) using a gradient of 0−90% eluant B (CH_3CN 0.003% TFA) in A (H_2O 0.03% TFA) over 30 min with a flow rate of 1.5 mL/min and the detector centered at 214 nm; retention times (t_R) are reported in minutes.

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Supporting Information Available: Text listing spectroscopic, chromatographic and analytical data for **1a**−**c**, **1e**, **1f**, **2a**−**f**, **6a**−**c**, **6e**, **8a**−**c**, **10**, **11a**−**c**, **12a**−**c**, **13a**−**j**, **14a**−**f**, **15**, and **16**, as well as details on purification of specific compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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