Alkyl 3-Position Substituents Retard the Isomerization of Prolyl and Hydroxyprolyl Amides in Water

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The influence of alkyl 3-position substituents on the rate of amide isomerization *N*-terminal to proline and hydroxyproline has been explored via the synthesis and analysis of (2*S*)-*N*-(acetyl)-proline *N*′-methylamide (**1**), (2*S*,4*R*)- and (2*S*,4*S*)-*N*-acetyl-4-hydroxyproline *N*′-methylamides **2** and **3**, and their respective 3,3-dimethyl analogues **4**−**6**. The relative populations of the amide *cis* and *trans* isomers as well as the rates for *cis*-to-*trans* and *trans*-to-*cis* isomerization of **1**−**6** in water were ascertained by NMR spectroscopy and magnetization transfer experiments. The relative populations of free *C*-terminal and hydrogen-bonded amides in the γ-turn conformation were also estimated by integrating the N−H stretch absorbances in the FT-IR spectra of **1** and **4** in $CHCl_3$. In addition, the structure of the amide *trans* isomer of (2*S*,4*S*)-*N*-acetyl-3,3-dimethyl-4-hydroxyproline *N*′-methylamide (**6**) was determined in the solid state by X-ray crystallographic analysis. In prolyl peptides **1**−**6**, the 3,3-dimethyl and hydroxyl substituents had little effect on the amide isomer equilibrium. A dramatic decrease in the rate of *cis*-to-*trans* amide isomerization was observed for *N*-acetyl-3,3-dimethylproline *N*′-methylamide (**4**), which exhibited a k_{ct} nearly 7-fold slower than that of **1**. Similar effects of the 3,3-dimethyl substituents were observed, albeit to a lesser degree, in the cases of the hydroxyprolyl peptides. The FT-IR data for **4** and X-ray data for **6** both demonstrated that the 3,3-dimethyl substituents restricted the proline ψ dihedral angle and prevented the formation of a γ-turn conformation, having a seven-membered hydrogen bond between the *C*-terminal amide NH and *N*-terminal amide carbonyl. Furthermore, restriction of the ψ dihedral angle by the methyl groups was observed in systematic computational conformational analyses of **1**−**6**, in which the ψ and ω dihedral angles were rotated at 30° intervals and the energies of the local minima were determined. Retardation of the rate of *cis*-to-*trans* amide isomerization in the dimethyl analogues may be attributed to steric interactions favoring a ψ dihedral angle at which the *C*-terminal amide carbonyl destabilizes the transition state through Coulomb repulsion of either the developing nitrogen lone pair or the carbonyl oxygen of the pyramidalized *N*-terminal amide. The consequences of 3-alkyl and 4-hydroxyl substituents on the rate of proline amide isomerization in water, which was observed to decrease in the order **1** ≈ **3** > **2** > **6** > **5** > **4**, may result from influences on the ψ dihedral angle geometry, inductive effects, and intramolecular hydrogen bonding.

Introduction

Prolyl residues can profoundly influence the conformation and reactivity of peptide structures.[1,2] Since amides *N*-terminal to proline possess energetically similar *cis* and *trans* isomers,[1] prolyl residues can act as junctions that alter peptide chain direction, creating multiple low-energy conformers. Consequently, prolyl amide isomerization can be a rate-limiting step in protein folding[3] and may induce functional changes in peptides and proteins.[4] Prolyl peptide *cis*−*trans* isomerases (PPIases),[5] as well as catalytic[6,7] and autocatalytic[8−10] mechanisms for prolyl amide isomerization, have thus been recognized as accelerators of protein folding that may serve important roles in various biological systems.

Alkylprolines that exhibit steric interactions on peptide geometry can serve as probes for exploring relationships between conformation and activity.[11−15] Alkylprolines can also be used to dissect conformational effects on

† Département de biochimie.

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(2) Reviewed in (a) Yaron, A.; Naider, F. *Crit. Rev. Biochem. Mol. Biol.* **1993**, *28*, 31. Discussed in (b) Williams, K. A.; Deber, C. M. *Biochemistry* **1991**, *30*, 8919.

(3) Reviewed in Fischer, G.; Schmid, F. X. *Biochemistry* **1990**, *29*, 2205.

(4) For leading references, see refs 2−5 in Beausoleil, E.; L'Archevêque, B.; Bélec, L.; Atfani, M.; Lubell, W. D. *J. Org. Chem.* **1996**, *61*, 9447.

(5) Reviewed in (a) Fischer, G. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 1415 and (b) Schmid, F. X.; Mayr, L. M.; Mücke, M.; Schönbrunner, E. R. *Adv. Protein Chem.* **1993**, *44*, 25.

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(7) For recent examples, see the following: (a) copper(II)-catalyzed isomerization: Cox, C.; Ferraris, D.; Murthy, N. N.; Lectka, T. *J. Am. Chem. Soc.* **1996**, *118*, 5332. (b) Catalytic antibody-catalyzed isomerization: Yli-Kauhaluoma, J. T.; Ashley, J. A.; Lo, C.-H. L.; Coakley, J.; Wirsching, P.; Janda, K. D. *J. Am. Chem. Soc.* **1996**, *118*, 5496.

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(10) Cox, C.; Young, V. G., Jr.; Lectka, T. *J. Am. Chem. Soc.* **1997**, *119*, 2307.

(11) Beausoleil, E.; Sharma, R.; Michnick, S.; Lubell, W. D. In *Peptides 1996 (Proceedings of the 24th European Peptide Symposium)*; Ramage, R., Epton, R., Ed.; ESCOM: Leiden, The Netherlands, 1997; pp 241−242.

(12) Beausoleil, E.; Lubell, W. D. *J. Am. Chem. Soc.* **1996**, *118*, 12902 and references therein.

(13) (a) Delaney, N. G.; Madison, V. *J. Am. Chem. Soc.* **1982**, *104*, 6635. (b) For a recent conformational analysis of 3-methylprolyl-proline dipeptide analogues, see Baures, P. W.; Ojala, W. H.; Gleason, W. B.; Johnson, R. L. *J. Peptide Res.* **1997**, *50*, 1.

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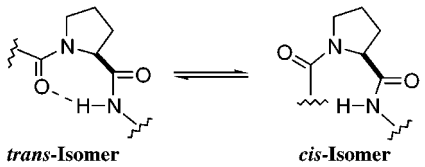

Figure 1. Amide isomer equilibrium *N*-terminal to proline derivatives.

prolyl isomerization.[11,12] For example, we have reported that 5-*tert*-butylprolines augment the amide *cis*-isomer population in prolyl peptides and may be used to construct type VI β-turn mimics.[12] In the case of the *cis* diastereomer of *N*-acetyl-5-*tert*-butylproline *N*′-methylamide, we observed that the 5-*tert*-butyl substituent reduced the barrier for amide isomerization by 3.7 kcal/mol compared to the barrier for *N*-(acetyl)proline *N*′-methylamide (**1**).[12] Acceleration of amide isomerization was attributed in part to ground-state destabilization resulting from the bulky 5-position substituent skewing the amide bond away from planarity.[16,17] In addition, the *tert*-butyl derivative adopted an energy minimum conformation with the ψ dihedral angle at $\psi \approx 0°$,[12] which has been suggested to stabilize the pyramidalized amide transition states[12] because the *C*-terminal NH group is able to interact with the nitrogen lone pair or the carbonyl oxygen of the rotating *N*-terminal amide.[8−10] Having demonstrated that steric interactions can lower the barrier for amide isomerization of 5-alkylprolyl peptides, we now report that 3-alkylprolines can create steric interactions that raise the barrier for amide isomerization. The results of the present study again draw attention to the importance of *C*-terminal amide interactions on prolyl peptide isomerization.

We have recently introduced methodology for efficiently synthesizing 3-alkylproline and 3-alkyl-4-hydroxyprolines.[15] Attachment of the side-chain functions of natural amino acids to the 3-position of the pyrrolidine ring provides proline−amino acid chimeras that can be used to explore the geometric relation of side-chain groups to the peptide backbone. Replacement of natural amino acids with such proline−amino acid chimeras has been used to study relationships between biological activity and conformation[15] because the 3-alkyl substituents can impose steric interactions that restrict the ψ dihedral angle to prevent formation of a γ-turn conformation ($\psi \approx 80°$) in different peptide structures.[11,13,14] The influence of the 3-position substituent is in part contingent on its relative stereochemistry. For example, the *trans* diastereomer was much less effective at perturbing the γ-turn conformation relative to the *cis* diastereomer in *N*-acetyl-3-methylproline *N*′-methylamides as demonstrated by circular dichroism (CD) and FT-IR spectroscopy.[13a] Although the consequence of a single 3-position substituent on prolyl geometry had been studied prior to our investigation, the influence of 3-position substituents on the rate of amide isomerization *N*-terminal to proline had not been reported. We have thus compared

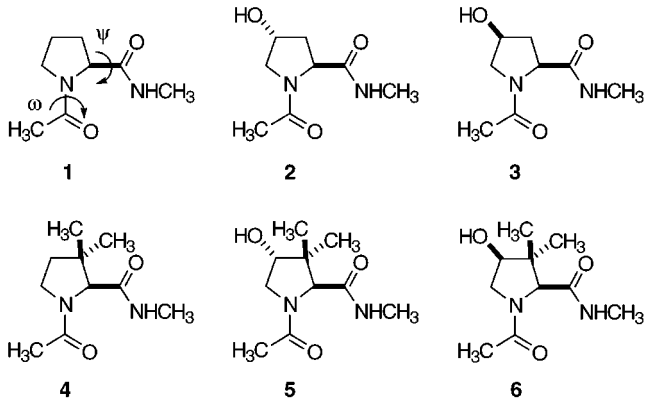

Figure 2. *N*-(Acetyl)proline *N*′-methylamides **1**−**6** (amide *trans* isomers shown).

N-(acetyl)proline *N*′-methylamide (**1**) with its 3,3-dimethylproline analogue **4** in order to ascertain the steric effects of two methyl substituents on the proline conformation and the rate of prolyl amide isomerization.

Electron-withdrawing groups at the 4-position of proline have also been suggested to accelerate amide isomerization by inductive effects that increase sp^3 pyramidalization of the prolyl nitrogen.[18] Such an effect has been observed in dioxane, where the *trans* diastereomer of *N*-acetyl-4-fluoroproline methyl ester exhibited a higher rate constant for amide isomerization relative to that of *N*-(acetyl)proline methyl ester.[18] On the other hand, the contrary was observed in water, when *cis*−*trans* isomerization of (2*S*,4*R*)-*N*-acetyl-4-hydroxyproline methyl ester was found to be slower than that of *N*-(acetyl)proline methyl ester.[18] To further examine this paradox, we have also investigated the consequence of a 4-position hydroxyl group on prolyl geometry and amide isomerization by studying (2*S*,4*R*)- and (2*S*,4*S*)-*N*-acetyl-4-hydroxyproline *N*′-methylamides (**2** and **3**) and their respective 3,3-dimethyl analogues **5** and **6**.

Results

Synthesis of *N*-(Acetyl)proline and *N*-Acetyl-4-hydroxyproline *N*′-Methylamides. Enantiopure (2*S*)-*N*-BOC-3,3-dimethylproline (**7a**) and (2*S*,4*R*)- and (2*S*,4*S*)-*N*-BOC-3,3-dimethyl-4-hydroxyprolines (**7b** and **7c**) were synthesized from (2*S*,4*R*)-hydroxyproline as described.[15] Regioselective enolization of (2*S*)-4-oxo-*N*-(9-phenylfluoren-9-yl)proline benzyl ester and bis-*C*-alkylation with iodomethane at the 3-position provided the 3,3-dimethyl analogue. Reduction of the ketone, followed by a one-pot hydrogenolysis of both the *N*- and *O*-protecting groups, and subsequent *N*-acylation with di-*tert*-butyl dicarbonate gave *N*-(BOC)amino acids **7a**−**c** that were suitable for peptide coupling.

(2*S*)-*N*-acetyl-3,3-dimethylproline *N*′-methylamide (**4**), (2*S*,4*R*)-*N*-acetyl-3,3-dimethyl-4-hydroxyproline *N*′-methylamide (**5**), and (2*S*,4*S*)-*N*-acetyl-3,3-dimethyl-4-hydroxyproline *N*′-methylamide (**6**) were all synthesized from their respective *N*-(BOC)amino acids **7a**−**c** (Scheme

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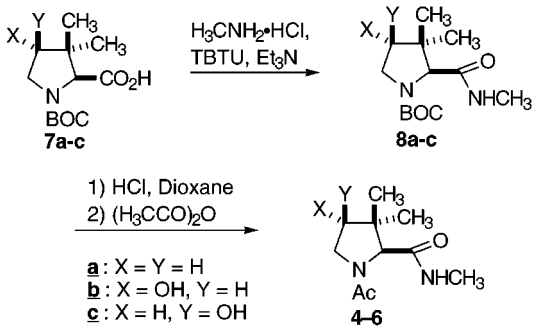
Scheme 1. Synthesis of *N*-Acetyl-*N′*-methylamides 4−6



1). Initially, *N*-(BOC)proline *N′*-methylamides **8a**−**c** were synthesized in good yields by coupling **7** to methylamine using benzotriazol-1-yl-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) in acetonitrile.[19] Solvolysis of the BOC group with HCl in dioxane and evaporation of the volatiles then gave the hydrochloride salts, which were acetylated in neat acetic anhydride. In the case of the hydroxyproline analogues, acetylation of the nitrogen was sometimes accompanied by *O*-acetylation. Selective hydrolysis of the *O*-acetyl group was then performed using potassium carbonate in methanol to furnish the desired *N*-acetyl-4-hydroxyproline *N′*-methylamides. Amide **1** was synthesized as previously described.[12] Amides **2** and **3** were prepared using the general procedure for the synthesis of **4** as described in the Experimental Section.[20]

Crystals of (2*S*,4*S*)-*N*-acetyl-3,3-dimethyl-4-hydroxyproline *N′*-methylamide (**6**) were grown from methanol and subjected to X-ray crystallographic analysis.[21] The amide *trans* isomer ($\omega = 173°$) was observed in the crystal structure of **6** (Figure 3). The values for the ψ and ϕ dihedral angles were respectively 145° and −78°, and the proline ring puckering was of C4-endo conformation.[22] An intramolecular hydrogen bond between the 4-hydroxyl group and the *C*-terminal amide carbonyl was also inferred from the X-ray analysis. The distance between the alcohol proton and the carbonyl oxygen was determined to be 1.91 Å, and the O−H−O angle was 166°.

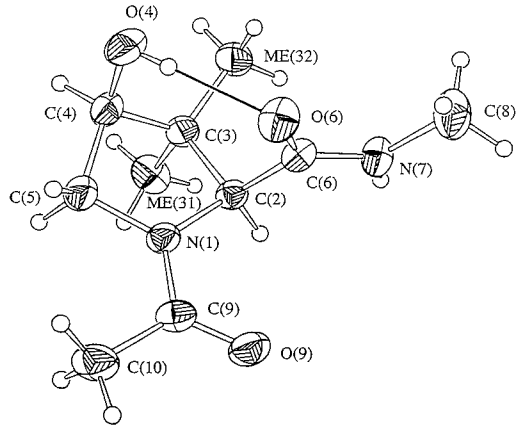


Figure 3. ORTEP drawing of (2*S*,4*S*)-*N*-acetyl-3,3-dimethyl-4-hydroxyproline *N′*-methylamide (**6**). Non-hydrogen atoms are represented by ellipsoids corresponding to 40% probability. Hydrogen atoms are represented by spheres of arbitrary size.

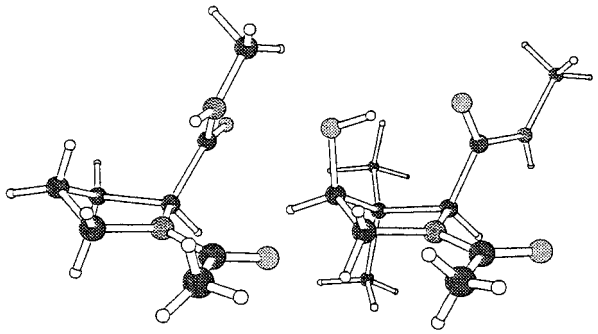


Figure 4. Structures of amides 1 and 6 from X-ray crystallography.[23,24a]

The conformation observed for **6** was similar to the crystal structure geometry of *N*-(acetyl)proline *N′*-methylamide (**1**)[23] with respect to its amide *trans* isomer ($\omega = 173°$), ϕ dihedral angle value ($\phi = -76°$), and C4-endo ring puckering. On the other hand, the ψ dihedral angle for **6** ($\psi = 145°$) was significantly different from that of **1** ($\psi = -16°$) in their respective crystal structures, as illustrated in Figure 4.[24a] In addition to crystal packing forces,[24b] the different orientations about the ψ dihedral angle in the structures of **1** and **6** may likely be due to a combination of steric interactions in the latter between the 3-position methyl groups and the *C*-terminal amide, as well as the hydrogen bond between the 4-position hydroxyl and *C*-terminal carbonyl groups.

FT-IR analysis of *N*-(acetyl)proline *N′*-methylamides 1 and 4 was performed in chloroform at 1×10^{-4} M concentration at 25 °C in order to examine for the presence of a γ-turn conformation in which a seven-membered-ring, intramolecular hydrogen bond exists between the *C*-terminal amide NH and the carbonyl of the *N*-terminal amide *trans* isomer.[25] In the spectrum of *N*-(acetyl)proline *N′*-methylamide (**1**) in chloroform,[12,13] the presence of the free N−H stretch band was observed at 3451 cm^{-1} for the non-hydrogen-bonded amide. The predominant N−H stretch band was observed at 3328

(19) (a) Dourtoglou, V.; Gross, B. *Synthesis* **1994**, 572. (b) Knorr, R.; Trzeciak, A.; Bannwarth, W.; Gillessen, D. *Tetrahedron Lett.* **1989**, *30*, 1927. (c) The crystal structure of HBTU, the corresponding PF_6 salt, substantiates a guanidinium-1-*N*-oxide salt for TBTU: Abdelmoty, I.; Albericio, F.; Carpino, L. A.; Foxman, B.; Kates, S. A. *Lett. Pept. Sci.* **1994**, *1*, 57.

(20) (2*S*,4*R*)-*N*-Acetyl-4-hydroxyproline *N′*-methylamide (**2**): $[\alpha]^{22}_D$ −62.1° (*c* 0.8, MeOH); ^{1}H NMR (CD_3OD) δ 1.9 (m, 1 H), 2.07 (s, 3 H), 2.14 (m, 1 H), 2.71 (s, 3 H), 3.5 (m, 1 H), 3.7 (m, 1 H), 4.4 (m, 2 H); ^{13}C NMR (CD_3OD, minor isomer is in parentheses) δ (21.8) 22.6, 26.6 (26.7), 39.7 (41.6), (56) 57.5, 60.5 (61.7), (69.6) 71, 172.8 (173.2), (175.1) 175.4; HRMS calcd for $C_8H_{15}N_2O_3$ (MH^+) 187.1083, found 187.1091. (2*S*,4*S*)-*N*-Acetyl-4-hydroxyproline *N′*-methylamide (**3**): $[\alpha]^{22}_D$ −55.6° (*c* 0.2, CH_3OH); ^{1}H NMR (CD_3OD) δ 2.0 (m, 1 H), 2.07 (s, 3 H), 2.34 (m, 1 H), 2.72 (s, 3 H), 3.52 (m, 1 H), 3.73 (m, 1 H), 4.38 (m, 2 H); ^{13}C NMR (minor isomer is in parentheses) δ (22) 22.3, 26.4 (26.5), 38.6 (40.5), (56.2) 57.5, 60.6 (62.1), (69.8) 71.2, 172.9 (173.1), (175.1) 175.3.

(21) The structure of **6** was solved and refined at l'Université de Montréal X-ray facility by the SHELX programs (SHELXS86 and SHELXL93): $C_{10}H_{18}N_2O_3$; $M_r = 214.26$; triangular prism, colorless crystal; space group $P2_1$; unit cell dimensions (Å) $a = 6.346(1)$, $b = 8.737(2)$, $c = 10.821(2)$; $\beta = 105.72(2)°$; volume of unit cell 577.5(2) Å^3; $Z = 2$; $R_1 = 0.0264$ for $I > 2\ \sigma(I)$, $wR_2 = 0.0737$ for all data; GOF = 1.060. The author has deposited the atomic coordinates for the structure of **6** 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.

(22) Haasnoot, C. A. G.; De Leeuw, F. A. A. M.; De Leeuw, H. P. M.; Altona, C. *Biopolymers* **1981**, *20*, 1211.

(23) Matsuzaki, T.; Iitaka, Y. *Acta Crystallogr.* **1971**, *B27*, 507.

(24) (a) The images were produced using the Chiron Program: Hanessian, S.; Franco, J.; Larouche, B. *Pure Appl. Chem.* **1990**, *62*, 1887. (b) Intermolecular hydrogen bonds between the *C*-terminal amide NH and the *N*-terminal amide carbonyl are observed in the crystal packing of the structures of both **1** and **6**.

Table 1. Amide Isomer Equilibrium and Isomerization Rates of 1−6 in Water

amide	$\delta(^{13}C^{\alpha})$ *cis*	$\delta(^{13}C^{\alpha})$ *trans*	$\delta(^{13}C^{\delta})$ *cis*	$\delta(^{13}C^{\delta})$ *trans*	*cis* rotamer % calcd[a]	*cis* rotamer % ± 3%[b]	k_{ct} (s^{-1})	k_{tc}[c] (s^{-1})
1	62.3	60.8	47.5	49.1	15	28 (29)	2.01 ± 0.09	0.82 ± 0.09
2	60.1	58.8	54.2	55.9	27	21 (24)	1.46 ± 0.13	0.47 ± 0.07
3	62.0	60.9	54.7	55.9	28	21 (29)	2.05 ± 0.50	0.82 ± 0.22
4	72.2	70.6	46.2	47.7	24	30 (30)	0.32 ± 0.07	0.12 ± 0.04
5	66.9	65.1	47.7	49.0	27	28 (25)	0.81 ± 0.19	0.27 ± 0.08
6	70.7	69.0	53.2	53.8	23	21 (25)	1.39 ± 0.16	0.47 ± 0.09

[a] Calculated with Macromodel 5.5x and the AMBER 94 force field. [b] Determined by 300 MHz NMR in D_2O at 25 °C (60 °C). [c] Calculated from k_{ct} and equilibrium at 60 °C as described in text.

cm^{-1} in the spectrum of **1** as the result of hydrogen bonding in the γ-turn conformation, and the ratio of the relative areas of the lower-versus higher-energy absorbance for the N−H stretches was 79:21. On the other hand, only a free N−H stretch band was observed at 3455 cm^{-1} for *N*-acetyl-3,3-dimethylproline *N*′-methylamide (**4**).[11] No second lower-energy N−H stretch band was observed for **4**, indicating the absence of a γ-turn conformation in chloroform. Similar FT-IR spectra to that of **4** have been reported with other 3-alkylproline analogues such as the *cis* diastereomer of *N*-acetyl-3-methylproline *N*′-methylamide[13] and BOC-Dtc-Ile-OMe[14] (where Dtc is 5,5-dimethylthiazolidine-4-carboxylate) in chloroform.

The amide *cis*- and *trans*-isomer populations and the rates for amide isomerization of *N*-(acetyl)-proline and hydroxyproline *N*′-methylamides 1−6 were determined by NMR analysis. Water (H_2O and D_2O) was chosen as solvent because of its physiological importance and for comparison with literature examples.[12,26] In comparison to reaction rates in nonprotic and nonpolar solvents, amide isomerization *N*-terminal to proline proceeds more slowly in water, which stabilizes the polar amide ground states relative to the less-polar transition state.[6,27,28] The assignments of the isomer geometry were made on the basis of the chemical shift values of the α- and δ-carbons in D_2O as previously described for **1** (Table 1).[12,29] The populations of the amide isomers were measured in the proton NMR spectra by integration of the *N*′-methyl and α-proton signals in D_2O at 25 and 60 °C. Amide isomer populations measured in water varied, to a limited extent, upon addition of methyl and hydroxyl group substituents. Dimethyl analogues **4**−**6** tended to exhibit 3−7% more *cis* isomer than the natural series **1**−**3** at 25 °C. The additional 4-hydroxyl group resulted generally in a 3−9% decrease of *cis*-isomer population (Table 1). These percentages were not statistically significant.

The rates of *cis*-to-*trans* isomerization of *N*-(acetyl)-proline *N*′-methylamides **1**−**6** were measured using ^{13}C NMR magnetization transfer experiments (Table 1). In these experiments, the signal of the α-carbon of the major amide isomer was selectively inverted and the rates of magnetization transfer between the major and minor isomer α-carbon signals were measured at different temperatures. Optimal magnetization transfer rates for **1**−**6** were observed at 60 °C, and we therefore used data collected at this temperature for model fitting. This experiment was used to determine the amide isomerization rate constant, k_{ct}. The *trans*-to-*cis* amide isomerization rate constant (k_{tc}) was calculated using the values for k_{ct} and the ratio of the *cis*- and *trans*-isomer populations at 60 °C. The amides displayed rate constants for *cis* to *trans* isomerization in water at 60 °C in the decreasing order **1** ≈ **3** > **2** > **6** > **5** > **4**.

The presence of two methyl substituents at the 3-position was found to significantly diminish the rate for prolyl amide isomerization. For example, the rate for *cis*-to-*trans* isomerization of dimethylproline amide **4** was nearly 7-fold slower than that of *N*-(acetyl)proline *N*′-methylamide (**1**). A nearly 2-fold reduction in the rate for amide isomerization was observed with (4*R*)-dimethyl-4-hydroxyproline amide **5** relative to that of (4*R*)-4-hydroxyproline amide **2**. In addition, the rate for isomerization of (4*S*)-dimethyl-4-hydroxyproline amide **6** was moderately slower than (4*S*)-4-hydroxyproline amide **3**.

In the case of the hydroxyproline amides, the isomerization rates were slower for the *trans* diastereomers relative to those for the *cis*-diastereomer counterparts: (4*R* < 4*S*), **2** < **3**, and **5** < **6**. In the natural series, a moderate reduction of the rate of amide isomerization was observed for (4*R*)-hydroxyproline amide **2** relative to that of proline amide **1**, and identical rates were observed for (4*S*)-hydroxyproline amide **3** and **1**. In contrast to the natural series, the dimethyl series exhibited a significant rate acceleration upon addition of a 4-position hydroxyl group. Relative to the case of dimethylproline amide **4**, rates for isomerization were observed to be 2.7-fold faster with (4*R*)-hydroxyproline amide **5** and 4.7-fold faster with (4*S*)-hydroxyproline amide **6** (Table 1).

Molecular Mechanics Calculations. The energy differences between the amide *cis* and *trans* isomers in **1**−**6** were compared, and the minimum for each isomer was calculated using the MacroModel 5.5x program and the AMBER 94 force field with the GB/SA solvent model for water.[30,31] Systematic analyses of **1**−**6** were then performed in which the ψ and ω dihedral angles were rotated at 30° intervals and the energy of the local minimum was determined for each conformation and plotted against the values for the ψ and ω dihedral angles.[12,30] The results of the systematic analyses illustrated that, relative to results for the natural analogues **1**−**3**, the conformational freedom about the ψ dihedral angle was more restricted in 3,3-dimethyl-

(25) The use of IR spectroscopy to study intramolecular hydrogen bonds in model peptides is presented in (a) Haque, T. S.; Little, J. C.; Gellman, S. H. *J. Am. Chem. Soc.* **1994**, *116*, 4105. (b) Maxfield, F. R.; Leach, S. J.; Stimson, E. R.; Powers, S. P.; Scheraga, H. A. *Biopolymers* **1979**, *18*, 2507. (c) Avignon, M.; Huong, P. V. *Biopolymers* **1970**, *9*, 427. (d) Tsuboi, M.; Shimanouchi, T.; Mizushima, S. *J. Am. Chem. Soc.* **1959**, *81*, 1406. (e) Rao, C. P.; Balaram, P.; Rao, C. N. R. *Biopolymers* **1983**, *22*, 2091.

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substituted analogues **4**−**6**. For example, the ideal γ-turn conformation at $\omega = 180°$ and $\psi = 80°$ was located at an energy minimum in the analyses of **1**−**3**[12] yet appeared at higher energy (~2 kcal/mol relative energy difference) in the analyses of **4**−**6**. Significant steric effects of the methyl substituents were observed as increases in energy at the ψ dihedral angle values centered at $\psi \approx 50°$ and $\psi \approx -130°$. These effects appeared to restrict the rotational liberty of the *C*-terminal carboxamide such that ψ dihedral angle values around $\psi \approx 150°$ were favored over values around $\psi \approx 0°$ for both the amide *cis* and *trans* isomers in the 3,3-dimethyl-substituted analogues **4**−**6**.

Except in the case of **2**, the ring puckering for the minima of the amide *cis* and *trans* isomers was consistent with a C4-endo conformation. The ring puckering for the energy minimum conformers of the *cis* and *trans* isomers of (4*R*)-4-hydroxyproline amide **2** both were of C4-exo conformation. The C4-exo ring puckering was the same as observed for (4*R*)-hydroxyproline inside collagen as determined by X-ray diffraction,[32] as well as inside collagen triple-helical peptides as shown by NMR[33] and X-ray crystallographic analysis.[34]

Discussion

We have examined the influences of 3-position methyl and 4-position hydroxyl substituents on the conformation of *N*-(acetyl)proline *N*′-methylamides **1**−**6** by a combination of X-ray crystallography, FT-IR spectroscopy, NMR techniques, and molecular mechanics. We have also used magnetization transfer experiments to determine the rates for *cis*-to-*trans* amide isomerization of these prolyl peptides, which decreased in the order **1** ≈ **3** > **2** > **6** > **5** > **4**. In light of proposed mechanisms for amide isomerization,[6,8] we may begin to interpret this order on the basis of the results of our conformational analysis. Previous studies have noted the importance of the ψ dihedral angle geometry and electron-withdrawing substituents on prolyl amide isomerization. For example, at a ψ dihedral angle around $\psi \approx 0°$, interactions between the *C*-terminal amide NH with the nitrogen lone pair of the rotating *N*-terminal amide have been suggested to stabilize the pyramidalized transition state and accelerate isomerization.[8−10] On the other hand, at ψ dihedral angles between 150° and 210° (−150°), an unfavorable Coulomb interaction between the *C*-terminal carbonyl oxygen and the developing lone pair on the pyramidalized nitrogen has been suggested to reduce the rate of prolyl amide isomerization.[8] The observed acceleration of isomerization on introduction of a fluoride at the proline 4-position has been implied to arise from inductive effects that facilitate sp^3 pyramidalization of the prolyl nitrogen.[18] Electron-withdrawing substituents have also been noted to increase the acidity of the *C*-terminal amide NH and thereby accelerate *N*-terminal amide isomerization in $CHCl_3$ by increasing the favorable interactions at $\psi \approx 0°$.[10]

Cis-to-*trans* isomerization rates for the dimethyl series **4**−**6** were always lower than those for the natural series **1**−**3**. This decrease in rate for the dimethyl analogues may arise from steric interactions between the 3-position methyl groups and the *C*-terminal amide that restrict the ψ dihedral angle to values away from $\psi \approx 0°$. For example, the ψ dihedral angle in dimethylhydroxyproline amide **6** was found to be 145° in the X-ray crystal structure. Methyl substituents at the 3-position prevented amide **4** from adopting a γ-turn ($\psi = 80°$), which was the preferred conformation for **1** in $CHCl_3$ as determined by examination of the N−H stretch region in their respective FT-IR spectra. In addition, conformational analyses of amides **1**−**6** revealed that the 3-position methyl substituents induced ψ dihedral angle values around 150° relative to values around $\psi \approx 0°$ for the parent series. The steric effects of the methyl substituents at the 3-position appear to favor ψ dihedral angle geometries that place the *C*-terminal carbonyl oxygen in a position which disfavors amide pyramidalization by Coulomb interactions. This geometry, which has been suggested to impede prolyl amide isomerization,[8] may account for the fact that *N*-acetyl-3,3-dimethylproline *N*′-methylamide (**4**) exhibits the slowest rate of isomerization relative to that of **1**.

Inductive effects of the 4-position hydroxyl group may be responsible for the increased isomerization rates of **5** and **6** relative to that of *N*-acetyl-3,3-dimethylproline *N*′-methylamide (**4**). The greater acceleration exhibited by (4*S*)-hydroxyproline amide **6** may be due to the added effect of the intramolecular hydrogen bond between the hydroxyl and *C*-terminal amide carbonyl groups, which should reduce Coulomb repulsion between the *C*-terminal carbonyl and the pyramidalized amide by diminishing the electronic density at the oxygen. The combination of steric effects from the methyl groups at the 3-position and inductive and hydrogen-bonding effects from the hydroxyl group at the 4-position may thus account for the observed order (**6** > **5** > **4**) of isomerization rate constants of the 3,3-dimethyl series.

Although the inductive effect of a 4-position electron-withdrawing group has been suggested to accelerate prolyl amide isomerization,[18] we did not observe such an effect on the rate constants in the natural series which decreased in the order of **1** ≈ **3** > **2**. As mentioned, *cis*−*trans* isomerization of (2*S*,4*R*)-*N*-acetyl-4-hydroxyproline methyl ester has also been found to be slower than that of *N*-(acetyl)proline methyl ester in water.[18] Amides **1**−**3** exhibit greater flexibility about the ψ dihedral angle relative to that of their dimethyl counterparts **4**−**6**. For example, studies of *N*-(acetyl)proline *N*′-methylamide (**1**) in water have indicated the presence of a γ-turn ($\psi = 80°$) on the basis of the pH dependencies of the *N*′-methyl proton resonances,[35] as well as α-helical ($\psi = -50°$) and polyproline II-like ($\psi = 150°$) conformations on the basis of CD and carbon NMR chemical shift data.[26] Since the potential for the *C*-terminal amide to either accelerate or retard isomerization about the *N*-terminal amide is

(30) The ψ, ϕ, and ω values for the calculated energy minima of the *trans* and *cis* isomers in **1**−**6** were as follows: **1** *trans* isomer, 128°, −57°, −179°, **1** *cis* isomer, 131°, −59°, 0°; **2** *trans* isomer, 162°, −57°, 177°; **2** *cis* isomer, 164°, −60°, −6°; **3** *trans* isomer, 152°, −72°, 180°; **3** *cis* isomer, 154°, −72°, −1°; **4** *trans* isomer, 154°, −71°, 180°; **4** *cis* isomer, 155°, −72°, −2°; **5** *trans* isomer, 151°, −72°, 180°; **5** *cis* isomer, 152°, −73°, −2°; **6** *trans* isomer, 141°, −71°, 180°; **6** *cis* isomer, 142°, −72°, −2°. Maps constructed for *N*-acetylproline *N*′-methylamides **1**−**6** by plotting the minimum energy value at each 30° interval against the values for the ψ and ω dihedral angles are included in the Supporting Information.

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regulated by the ψ dihedral angle, the inductive effects of the 4-position hydroxyl group in amides **2** and **3** should be considered with their influence on the *C*-terminal amide geometry. Rate acceleration may not be observed if the hydroxyl group influences the ψ dihedral angle to favor an orientation predisposed to Coulomb repulsion.[36] As in the case with dimethylhydroxyproline amides **5** and **6**, the faster rate of **3** relative to that of **2** may be due to the (4*S*)-hydroxyl group's ability to hydrogen bond with the *C*-terminal carbonyl oxygen and reduce the effects of Coulomb repulsion at $\psi \approx 150°$. Therefore, a combination of ψ dihedral angle geometry, inductive effects, and intramolecular hydrogen bonding may also be responsible for the measured decreasing order **1** $\approx$ **3** > **2** for the rate constants in the natural series.

Conclusion

Our studies have illustrated the influence of 3-alkyl and 4-hydroxyl substituents on the conformation and isomerization of prolyl amides. In particular, methyl substituents at the proline 3-position were shown to reduce the rate of prolyl amide isomerization in water, presumably by their steric effects on the ψ dihedral angle which may place the *C*-terminal carbonyl oxygen in position to repel the developing lone pair of the pyramidalized nitrogen by Coulomb interactions. Since these steric interactions should increase with larger *C*-terminal substituents, the replacement of proline by 3,3-dimethylproline is potentially a general method for reducing the rate of *N*-terminal amide isomerization in peptides. Our results with prolyl amides **1**−**6** may thus help guide future efforts involving the introduction of 3-alkylprolines into protein structures in order to explore the conformation and dynamics of prolyl residues in peptides.

Experimental Section

General. Unless otherwise noted, all reactions were run under a nitrogen atmosphere and distilled solvents were transferred by syringe. Dioxane, acetic anhydride, and CH_3CN were distilled from CaH_2; Et_3N was distilled from BaO; and MeOH was distilled from $Mg(OCH_3)_2$. Final reaction mixture solutions were dried over Na_2SO_4. Chromatography was on 230−400 mesh silica gel, and TLC was on aluminum-backed silica plates. Melting points are uncorrected. Mass spectral data, HRMS (EI), were obtained by the Université de Montréal mass spectrometry facility. ^{1}H NMR (300/400 MHz) and ^{13}C NMR (75/100 MHz) spectra were recorded in $CDCl_3$, except as specified. Chemical (δ) shifts are reported in ppm downfield of internal tetramethylsilane ($(CH_3)_4Si$). The chemical shifts for the carbons of the minor rotamer are reported in parentheses. FT-IR data collection and molecular mechanics calculations with the MacroModel program 5.5x were performed using the protocols summarized in the Experimental Section of ref 12.

Syntheses of (2*S*)-*N*-acetyl-3,3-dimethylproline and (2*S*,4*R*)- and (2*S*,4*S*)-*N*-acetyl-3,3-dimethyl-4-hydroxyproline *N*′-methylamides (4−6) were accomplished using the general protocol outlined below for the synthesis of (2*S*)-*N*-acetyl-3,3-dimethylproline *N*′-methylamide.

(2*S*)-*N*-BOC-3,3-Dimethylproline *N*′-Methylamide (8a). A suspension of (2*S*)-*N*-BOC-3,3-dimethylproline (**7a**, 24 mg, 0.1 mmol), methylamine hydrochloride (6.7 mg, 0.1 mmol), and triethylamine (0.04 mL, 0.3 mmol) in acetonitrile (2 mL) was treated with TBTU (35 mg, 0.11 mmol), stirred for 24 h, and evaporated to a residue that was partitioned between EtOAc (2 mL) and brine (2 mL). The aqueous layer was extracted with EtOAc (3 × 3 mL), and the combined organic layers were washed with water (3 × 2 mL), dried, and evaporated to a residue that was purified by silica gel chromatography using 2% MeOH in $CHCl_3$ as eluant. Evaporation of the collected fractions gave 23 mg (90%) of (2*S*)-*N*-BOC-3,3-dimethylproline *N*′-methylamide (**8a**): ^{1}H NMR δ 1.03 (s, 3 H), 1.16 (s, 3 H), 1.43 (s, 9 H), 1.63 (m, 1 H), 1.85 (m, 1 H), 2.8 (d, 3 H, J = 4.5 Hz), 3.55 (m, 2 H), 3.73 (s, 1 H), 5.83 (br s, 1 H); ^{13}C NMR δ 23.5, 25.8, 28.1, 28.3, 37.7, 42.1, 45.1, 70.9, 80.3, 154.8, 171.9; HRMS calcd for $C_{13}H_{25}N_2O_3$ (MH^+) 257.1865, found 257.1854.

(2*S*)-*N*-Acetyl-3,3-dimethylproline *N*′-Methylamide (4). (2*S*)-*N*-BOC-3,3-dimethylproline *N*′-methylamide (23 mg, 0.09 mmol) was dissolved in a 2.7 M solution of HCl in dioxane (0.6 mL), stirred for 2 h, and evaporated to provide 16 mg (98%) of the corresponding amino amide hydrochloride: $[\alpha]^{22}_D$ 20.6° (*c* 5.0, MeOH); ^{1}H NMR (CD_3OD) δ 1.0 (s, 3 H), 1.28 (s, 3 H), 1.96 (t, 2 H, J = 7.4 Hz), 2.81 (d, 3 H, J = 4.6 Hz), 3.43 (m, 2 H), 3.8 (s, 1 H); ^{13}C NMR δ 22.7, 26.5, 26.8, 39.7, 43.4, 44.8, 69.5, 168.1; HRMS calcd for $C_8H_{17}N_2O$ (MH^+) 157.1341, found 157.1350. The amino amide hydrochloride (16 mg, 0.09 mmol) was dissolved in acetic anhydride (1 mL) and stirred at room temperature (heated to 40 °C if necessary) until TLC showed complete consumption of the starting material. The volatiles were removed by evaporation under vacuum, and the resulting residue was chromatographed with 20% MeOH/EtOAc as eluant. Evaporation of the collected fractions gave 10 mg (60%) of (2*S*)-*N*-acetyl-3,3-dimethylproline *N*′-methylamide (**4**) as an oil: $[\alpha]^{22}_D$ −14.6° (*c* 0.1, CH_3OH); ^{1}H NMR (major isomer) δ 1.07 (s, 3 H), 1.08 (s, 3 H), 1.65 (m, 1 H), 2.08 (s, 3 H), 2.28 (m, 1 H), 2.79 (d, 3 H, J = 4.8 Hz), 3.54 (m, 1 H), 3.72 (m, 1 H), 3.93 (s, 1 H), 6.17 (br s, 1 H); ^{13}C NMR (major isomer) δ 22.1, 23.2, 26.0, 28.1, 37.6, 40.7, 46.7, 69.6, 170.2, 171.4; HRMS calcd for $C_{10}H_{19}N_2O_2$ (MH^+) 199.1447, found 199.1456.

(2*S*,4*R*)-*N*-Acetyl-3,3-dimethyl-4-hydroxyproline *N*′-Methylamide (5). Amide **5** was prepared from **7b** using the protocol described above. Acylation of methylamine gave (2*S*,4*R*)-*N*-BOC-3,3-dimethyl-4-hydroxyproline *N*′-methylamide (**8b**): 80%; oil; $[\alpha]^{22}_D$ 19.7° (*c* 0.7, CH_3OH); ^{1}H NMR δ 0.97 (s, 3 H), 1.16 (s, 3 H), 1.42 (s, 9 H), 2.56 (m, 1 H), 2.8 (s, 3 H), 3.45 (m, 1 H), 3.74 (dd, 1 H, J = 5.4, 11 Hz), 3.9 (s, 1 H); ^{13}C NMR δ 21, 25.8, 28.3, 38.5, 51.6, 69.5, 77.2, 80.3, 155, 171.6; HRMS calcd for $C_{13}H_{25}N_2O_4$ (MH^+) 273.1814, found 273.1803. Acid solvolysis removed the BOC group to give (2*S*,4*R*)-3,3-dimethyl-4-hydroxyproline *N*′-methylamide hydrochloride (90%): ^{1}H NMR δ (CD_3OD) 0.89 (s, 3 H), 1.25 (s, 3 H), 2.82 (s, 3 H), 3.2 (d, 1 H, J = 12.6 Hz), 3.7 (dd, 1 H, J = 3.3, 12.6 Hz), 4.0 (d, 1 H, J = 3.5 Hz), 4.05 (s, 1 H), 8.3 (br s, 1 H). Acetylation then provided **5** in 97% yield as an oil: $[\alpha]^{22}_D$ 34.6° (*c* 1.7, MeOH); ^{1}H NMR (CD_3OD) δ 0.96 (s, 2.6 H), 0.97 (s, 0.4 H), 1.07 (s, 2.6 H), 1.11 (s, 0.4 H), 1.9 (s, 0.4 H), 2.06 (s, 2.6 H), 2.71 (s, 2.6 H), 2.76 (s, 0.4 H), 3.35 (dd, 1 H, J = 6.5, 10.3), 3.79 (dd, 0.1 H, J = 6.7, 12 Hz), 3.89 (dd, 0.9 H, J = 6.7, 10.3 Hz), 4.01 (s, 1 H), 4.07 (t, 1 H, J = 6.6 Hz); ^{13}C NMR (CD_3OD) δ 21.4, 21.55 (21.62), 22.1 (22.3), 26.1 (26.2), 44.6 (46.6), (52.4) 53.6, 70.4 (72), (75.6) 76.6, 172.7 (172.9), (173) 173.3; HRMS calcd for $C_{10}H_{19}N_2O_3$ (MH^+) 215.1396, found 215.1386.

(2*S*,4*S*)-*N*-Acetyl-3,3-dimethyl-4-hydroxyproline *N*′-Methylamide (6). Amide **6** was synthesized using the above protocol on **7c**. Acylation of methylamine gave (2*S*,4*S*)-*N*-BOC-3,3-dimethyl-4-hydroxyproline *N*′-methylamide (**8c**): mp 199−200 °C; $[\alpha]^{22}_D$ 33.2° (*c* 1, CH_3OH); ^{1}H NMR δ 1.07 (s, 3 H), 1.11 (s, 3 H), 1.47 (s, 9 H), 2.81 (d, 3 H, J = 4.7 Hz), 3.63 (m, 2 H), 3.75 (dd, 1 H, J = 5, 12 Hz), 3.83 (s, 1 H), 4.4 (br s, 1 H), 7.0 (br s, 1 H); ^{13}C NMR δ 18.4, 26.2, 27.7, 28.7, 46.7, 54.8, 71.4, 78.5, 81.8, 156.2, 175.5; HRMS calcd for $C_{13}H_{25}N_2O_4$ (MH^+) 273.1814, found 273.1806. Solvolysis of the BOC group gave a 98% yield of (2*S*,4*S*)-3,3-dimethyl-4-hydroxyproline *N*′-methylamide hydrochloride: ^{1}H NMR δ (CD_3OD) 0.87 (s, 3 H), 1.23 (s, 3 H), 2.82 (s, 3 H), 3.14 (dd, 1 H, J = 8, 11 Hz), 3.55

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(36) It is interesting to note that the presence of a 4-position hydroxyl group increased the magnitude of the ψ dihedral angle from $\psi \approx 130°$ to $\psi \approx 153°$ to $\psi \approx 163°$ in the minimum energy conformations of the proline amides going from **1** to **3** to **2**.

(dd, 1 H, J = 7, 11 Hz), 3.91 (s, 1 H), 4.09 (t, 1 H, J = 7 Hz), 8.2 (br s, 1 H); ^{13}C NMR δ (CD_3OD) 16.1, 24.7, 26.7, 26.8, 45.4, 68, 77.5, 167.7. Acetylation then provided **6** in 83% yield as a white solid: mp 178 °C; $[\alpha]^{22}_D$ 41.4° (*c* 1.6, MeOH); ^{1}H NMR δ (major isomer) 1.1 (s, 3 H), 1.15 (s, 3 H), 2.17 (s, 3 H), 2.83 (d, 3 H, J = 4.9) Hz, 3.7 (d, 1 H, J = 11.3 Hz), 3.76 (d, 1 H, J = 4.7 Hz), 3.92 (dd, 1 H, J = 11.3, 4.7 Hz), 4.0 (s, 1 H), 6.83 (s, 1 H); ^{13}C NMR (CD_3OD) δ 18.4 (18.7), (22) 22.1, 26.3 (26.4), 27.7 (27.9), 45.6 (47.7), (55.5) 56.6, 70.8 (72.4), (78.1) 79.6, 173, (174.4) 174.6; HRMS calcd for $C_{10}H_{19}N_2O_3$ (MH^+) 215.1387, found 215.1396. In the acetylation step to synthesize hydroxyprolyl amides **2**, **3**, **5**, and **6**, some amounts of *O*-acetylation were detected and a selective hydrolysis was performed using potassium carbonate in methanol at room temperature to furnish the desired *N*-acetyl-4-hydroxyproline *N*′-methylamides after chromatography with MeOH in EtOAc as eluant.

NMR Experiments and Data Analysis. Samples were prepared by dissolving the compounds in distilled and deionized H_2O and/or D_2O at concentrations between 1×10^{-2} and 5×10^{-2} M and purged with nitrogen gas in NMR tubes used for the experiments. All experiments were carried out on either a Bruker AMX600 or DMX600 spectrometer, each equipped with selective excitation units and ^{1}H broad-band heteronuclear probes. Selective inversion of Cα *trans* or *cis* peaks was done with Gaussian pulses centered on resonance ± 25 Hz. Relaxation delays of 20 s and inversion−recovery delays of between 1 ms and 20 s were used. Data for each inversion recovery point were averaged over 32−128 points, depending on the concentration of the compound used. For each compound at 60 °C, inversion−recovery results were collected with selective inversion of the Cα *trans* peak. Data were fitted according to the protocol described in the Experimental Section of ref 12.

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Supporting Information Available: The ^{1}H, ^{13}C, and DEPT NMR spectra of **2**−**6** and **8**; plots of intensity versus mixing time for the magnetization transfer experiments on **1**−**6**; the FT-IR spectrum of the N−H stretch region of **4**; crystallographic data for **6**; and maps of energy versus conformation for **1**−**6** (29 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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