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Nucleation of β-Hairpin Structures with Cis Amide Bonds in *E*-Vinylogous Proline-Containing Peptides

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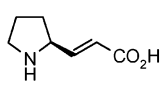
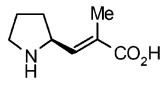
Abstract: Synthesis and conformational studies of peptides containing the *E*-vinylogous prolines **1** (VPro1) and **2** (VPro2), Boc-Ala-Val-VPro1-Xaa-Leu-OMe (**3**, Xaa = Gly; **4**, Xaa = Phe), Boc-Ala-Val-VPro2-Xaa-Leu-OMe (**5**, Xaa = Gly; **6**, Xaa = Phe), Boc-Leu-Ile-Val-VPro1-Xaa-Leu-OMe (**7**, Xaa = Gly; **8**, Xaa = Phe), and Boc-Leu-Ile-Val-VPro2-Xaa-Leu-OMe (**9**, Xaa = Gly; **10**, Xaa = Phe), were carried out. It has been shown that both VPro1 and VPro2 lead to the formation of 12-membered intramolecularly hydrogen bonded structures very similar to type VI β-turns with a cis Xaa−VPro amide bond in the major conformers in all the peptides **3**−**10**, resulting in the nucleation of β-hairpin type structures in these molecules in $CDCl_3$.

Proline plays a critical determinant role in protein folding and refolding, since it participates in nucleation of turns as well as in breaking of helices in proteins.[1] The only cyclic proteogenic natural α-amino acid, proline, can exhibit cis−trans rotamerization about the amide bond.[2] Invariably, the thermodynamically more stable trans rotamer predominates in solution, where the populations of the rotamers depend on the side chain of the preceding amino acid as well as the solvent.[1,2] The cis Xaa−Pro amide bond exists in several biologically active peptides, such as ribonuclease A,[3] BBI loop,[4] and aeruginosin EI461,[5] and in the V3 loop of HIV gp120, where it acts as a prerequisite to viral infection.[6] The cis amide bond permits a type VI β-turn in these peptides with the Pro unit occupying the $i + 2$ position of the turn.[7] It is often difficult to observe the prolyl amide cis conformers in natural peptides, where they exist as minor isomers. An increased cis content of the Xaa−Pro peptide bond is desired to carry out investigations on the structures and functions of biologically active peptides. The increased cis component also helps in studying the rates of cis−trans isomerization in prolyl amides, which is often the rate-limiting step in the refolding of unfolded proteins.[8] In view of the importance of the cis amide bond in prolyl amides, many attempts have been made to increase its population in solution by following various methods.[9] Herein, we report that *E*-vinylogous prolines **1** (VPro1) and **2** (VPro2) serve as nucleators of the cis amide bond preferentially, leading to the formation of structures resembling type VI β-turns in peptides **3**−**10**.[10]



1 (VPro1)



2 (VPro2)

Vinylogous amino acids in peptides have been studied before[10,11] as conformationally constrained γ-amino acid mimics. They adopt either an s-cis[11] or s-trans[10b] conformation in the resulting enamides with a preferred orientation of the γ-H atom lying in the plane of the enamide due to 1,3-allylic strain.[12] Peptides with *E*-vinylogous prolines in the N-terminal were reported earlier to have open conformations with no well-defined structure.[10b] However, terminally located residues seldom participate in turn structures, due to fraying at the ends. We envisaged that since *E*-vinylogous prolines favor a cis Xaa−VPro amide bond, they might lead to intramolecularly hydrogen-bonded structures when placed internally in the middle of a sequence, the choice of which was guided by the fact that similar sequences with proline in the middle, flanked on both sides with apolar residues, are known to adopt well-defined β-turn structures.[13] As expected, we also found the existence of 12-membered intramolecularly hydrogen bonded structures in all the peptides **3**−**10** that had centrally located

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TABLE 1. Chemical Shifts of the Intramolecularly Hydrogen Bonded Amide Protons of Peptides 3−10 in $CDCl_3$

amino acid	peptide	δ (ppm)	peptide	δ (ppm)
GlyNH	**3**	7.94	**5**	8.05
Leu(5)NH		7.33		7.01
PheNH	**4**	7.56	**6**	8.04
Leu(5)NH		7.48		6.92
GlyNH	**7**	8.13	**9**	8.21
IleNH		7.32		7.23
PheNH	**8**	7.95	**10**	8.30
IleNH		7.33		7.44

E-vinylogous prolines with cis Xaa−VPro bonds in their major conformers. This turn structure, similar to a type VI β-turn with vinylogous prolines in the $i + 2$ position,[7] induced further nucleation of a minimal hairpin conformation in hexapeptides **7**−**10**. In this paper, we describe the synthesis of peptides **3**−**10** and detailed studies on solution structures of some of these molecules using various NMR techniques.

The starting vinylogous prolines **1** and **2** were synthesized from Boc-prolinal by olefination using the stabilized ylides $Ph_3P{=}CHCO_2Et$ and $Ph_3P{=}C(Me)CO_2Et$, respectively, in dry benzene to furnish preferentially the *E* isomers of the protected vinylogous amino acids, Boc-Vpro1-OEt and Boc-Vpro2-OEt, respectively.[10] The general protocol followed for the synthesis of the peptides **3**−**10** is described in the Supporting Information.

Purified peptides were next subjected to conformational analysis by NMR. The assignments were carried out with the help of two-dimensional total correlation spectroscopy (TOCSY)[14] and were further confirmed by rotating frame nuclear Overhauser effect spectroscopy (ROESY) experiments,[14] which, in addition, provided information on the proximity of the protons. Detailed discussion on the structural studies will be restricted here to peptides **4** and **10**, representative peptides from vinylogous prolines VPro1 and VPro2, respectively, as the others in the family exhibited similar structures.

In a noncompetitive solvent such as $CDCl_3$, intramolecularly hydrogen bonded amide protons resonate downfield.[15] The ^{1}H NMR spectra of peptides **3**−**10** in $CDCl_3$ showed downfield chemical shifts for some of their amide protons, as shown in Table 1, indicating their participation in intramolecular hydrogen bonds. However, as addition of DMSO-d_6, a strong hydrogen-bonding solvent, to $CDCl_3$ solutions of these peptides caused shifts in their isomeric ratios, solvent titration studies were not useful.

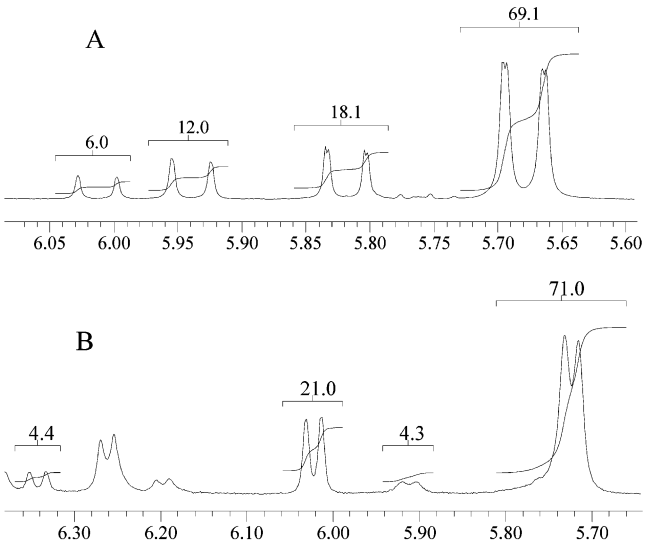

FIGURE 1. Sections of the ^{1}H NMR spectra of (A) **4** and (B) **10** in $CDCl_3$ showing the CαH signals of different isomers of the former and the CβH signals of the latter that were used to calculate the isomeric ratios of these peptides.

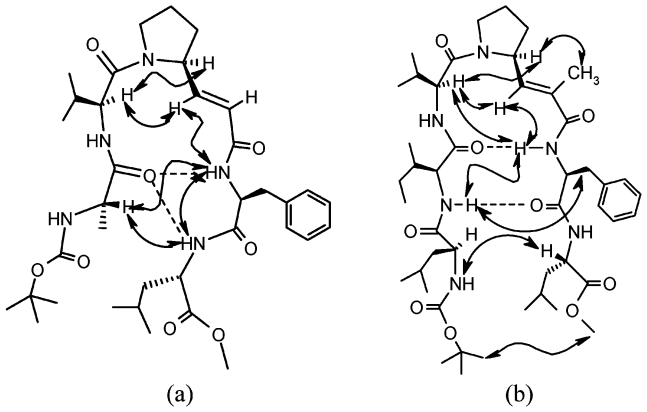

FIGURE 2. Schematic representation of the proposed structures of the major isomers of (a) **4** and (b) **10** with some of the prominent long-range ROE's seen in their ROESY spectra.

The ^{1}H NMR spectrum of **4** in $CDCl_3$ showed the presence of four species in a ratio of about 69:18:12:6. The signals of the olefin protons were used to calculate the ratios of the conformers, as shown in Figure 1A.

The olefin protons of the VPro1 residue showed only one ^3J value of 15.2−15.5 Hz in all the four isomers, indicating the presence of only an *E*-olefinic configuration. The ROE cross-peaks of the major isomer (Iso-I) seen in the ROESY spectrum of **4** are shown schematically in Figure 2a.

The appearance of a cross-peak for ValCαH ↔ VPro1CγH in the ROESY spectrum of **4** confirmed the presence of a cis amide bond between Val and VPro1 in the major isomer. Similarly, in the second most populated isomer (Iso-II), the cross-peak for VPro1CζH ↔ ValCαH confirms that the rotamer contains a trans amide bond

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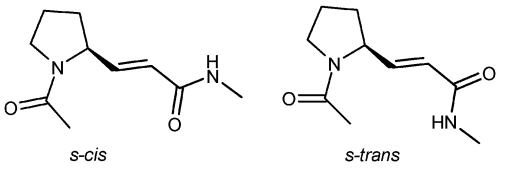

FIGURE 3. The two possible conformations of the enamide moiety.

TABLE 2. ^{1}H and ^{13}C Chemical Shifts (ppm) of the Ring Atoms of VPro Residues in 4 and 10 in $CDCl_3$, Assigned by GHSQC Experiments

	major isomer		minor isomer	
peptide	^{1}H	^{13}C	^{1}H	^{13}C
4	γ-H 4.62	γ-C 57.82	γ-H 4.76	γ-C 57.20
	δ-H 2.11	δ-C 31.51	δ-H 2.00	δ-C 30.03
	δ-H′ 1.97		δ-H′ 1.79	
	ϵ-H 1.83	ϵ-C 21.30	ϵ-H 1.94	ϵ-C 23.20
	ϵ-H′ 1.70		ϵ-H′	
	ζ-H 3.44	ζ-C 46.11	ζ-H 3.93	ζ-C 46.64
	ζ-H′ 3.40		ζ-H′ 3.52	
10	γ-H 4.51	γ-C 55.43	γ-H 4.75	γ-C 55.06
	δ-H 2.17	δ-C 33.37	δ-H 2.09	δ-C 30.95
	δ-H′ 1.71		δ-H′ 1.63	
	ϵ-H 1.84	ϵ-C 22.73	ϵ-H 2.02	ϵ-C 24.77
	ϵ-H′ 1.80		ϵ-H′ 1.93	
	ζ-H 3.58	ζ-C 46.62	ζ-H 3.79	ζ-C 47.10
	ζ-H′ 3.58		ζ-H′ 3.57	

preceding VPro1. The remaining two sets of resonances are probably due to the s-cis and s-trans isomers of the enamide moiety of the vinylogous amino acid, as shown in Figure 3. This was further supported by the observation of exchange cross-peaks in the ROESY spectrum and the strong ROE cross-peak for PheNH ↔ VPro1CβH, suggesting that VPro1 exists as the s-trans conformer in the predominant isomer. Thus, while the predominant isomer (Iso-I) has cis prolyl amide and s-trans enamide conformations, the second most populated isomer (Iso-II) has trans prolyl amide and s-trans enamide conformations. Due to the poor population of the other two isomers, we were unable to carry out their structural assignments.

Further proof for the cis prolyl amide bond in the major conformer of **4** was provided by the ^{13}C chemical shift difference of the δ and ϵ carbons of VPro1.[16] The ^{13}C chemical shift assignments of the ring carbons of the VPro1 residue in **4** were made by the gHSQC experiment[14] and are shown in Table 2. The difference between the ^{13}C chemical shifts of the δ and ϵ carbons of the major isomer of **4** is 10.21 ppm, while it is 6.83 ppm in the second most populated isomer, lending additional support in favor of the cis prolyl amide bond in the major conformer.[16]

The vicinal coupling constant ^3J of VPro1CγH (1.6 Hz), that of olefin protons (15.2 Hz), and the strong ROE peaks for PheNH ↔ VPro1CβH and VPro1CαH ↔ VPro1CγH strongly support the notion that VPro1 contains restricted backbone angles of $\varphi \approx 60°$, $\theta 1 \approx 180°$, $\theta 2 \approx 180°$, and $\psi \approx 0°$.[13c] These restricted backbone torsional angles led to the nucleation of a well-organized secondary structure.

The downfield appearances of amide chemical shifts of PheNH (7.56 ppm) and LeuNH (7.48 ppm) hint at their involvements in intramolecular hydrogen bonds. The

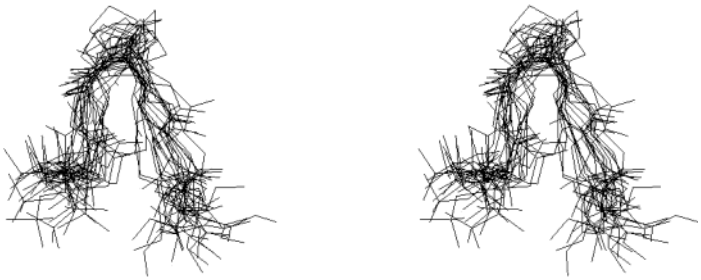

FIGURE 4. Stereoview of the 20 superimposed energy-minimized structures sampled during the 600 ps constrained MD simulations of peptide **4** following the Simulated Annealing protocol.

ROESY cross-peaks for PheNH ↔ LeuNH, PheNH ↔ VPro1CβH, VPro1CγH ↔ ValCαH, LeuNH ↔ VPro1CβH, VPro1CγH ↔ ValCαH, ValNH ↔ VPro1CβH, PheNH ↔ AlaCαH, LeuNH ↔ AlaCαH, and AlaNH ↔ ValNH, coupled with intramolecular hydrogen bonds involving PheNH and LeuNH, indicate the presence of a β-hairpin like structure with a three-center hydrogen-bonding network as shown in Figure 2a, which is consistent with the involvements of both PheNH and LeuNH in hydrogen bonds with a single AlaC═O.

The cross-peak intensities in the ROESY spectrum of **4** were used to obtain the restraints in constrained molecular dynamics (MD) simulation studies.[17] Several long-range (more than four bonds) distance constraints (see the Supporting Information) from the ROE's shown in Figure 2a were used in the energy calculations and MD studies. A 600 ps simulated annealing was run comprising 100 cycles each of 6 ps period. Each cycle involved a heating step to 700 K for 1 ps followed by cooling exponentially to a final temperature of 300 K in 5 ps. Structures were sampled after each 30 ps interval and energy-minimized. A superimposition of the backbone atoms of these 20 energy-minimized structures is displayed in Figure 4. A three-center intramolecular H-bonded conformation between PheNH and LeuNH to AlaC═O was found in 13 structures (65%). However, the other 7 structures did not differ very much from these, and the average pairwise heavy atom and backbone atom RMSD values were found to be 2.23 ± 0.52 and 0.72 ± 0.34 Å, respectively.

The proton spectrum of **10** in $CDCl_3$ showed the presence of four isomers in a ratio of about 71:21:4:4. Here also the olefin proton signals were used to calculate the ratios of the conformers, as shown in Figure 1B. Some of the important ROE cross-peaks of the major isomer of **10** seen in its ROESY spectrum in $CDCl_3$ are shown in Figure 5.

These ROE's are also schematically displayed in Figure 2b. For the most populated isomer (Iso-I), the appearance of an ROE cross-peak for VPro2CγH ↔ ValCαH implies the presence of a cis amide bond with the amino acid preceding VPro2. For the second most populated isomer (Iso-II), the presence of an ROE peak for ValCαH ↔

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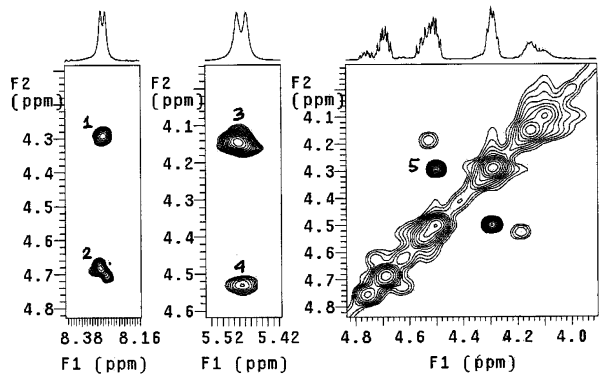

FIGURE 5. Expanded regions of the ROESY spectrum of **10** showing some of the distinctive ROE cross-peaks: (1) PheNH ↔ ValCαH; (2) PheNH ↔ PheCαH; (3) Leu(1)NH ↔ Leu(1)-CαH; (4) Leu(1)NH ↔ Leu(6)CαH; (5) VPro2CγH ↔ ValCαH. The last cross-peak supports the presence of a cis amide bond with the amino acid preceding VPro2.

VPro2CζH is in agreement with a trans amide bond preceding VPro2. The other two sets of resonances were due to other rotamers, probably the s-cis and s-trans isomers of the enamide. As seen in **4**, the ROESY spectrum of **10** also showed exchange peaks between the four isomers, which imply equilibrium between them. A strong ROE for PheNH ↔ VPro2CβH implies that VPro2 exists as the s-trans conformer in the major isomer. Once again, the predominant isomer (Iso-I) has cis prolyl amide and s-trans enamide conformations and the second most populated isomer (Iso-II) has trans prolyl amide and s-trans enamide conformations. The differences between the $^{13}C_\delta$ and $^{13}C_\epsilon$ chemical shifts (Table 2) in the major isomer ($\Delta_{\delta\epsilon} = 10.64$ ppm) and the second most populated isomer ($\Delta_{\delta\epsilon} = 6.18$ ppm) lend additional support in favor of the cis prolyl amide bond in the former and trans in the latter.[16]

The downfield appearances of PheNH (8.30 ppm) and IleNH (7.44 ppm) hint at their involvements in intramolecular hydrogen bonds. The ROE peaks for PheNH ↔ VPro2CβH, ValCαH ↔ VPro2CβH, VPro2CαCH$_3$ ↔ VPro2CγH, PheNH ↔ ValCαH, and ValCαH ↔ VPro2CγH, coupled with the participation of PheNH in the hydrogen bond, strongly support the presence of a well-defined 12-membered turn structure around Val-VPro2 residues. The unequivocal evidence for the nascent or minimal hairpin conformation in solution comes from the ROE cross-peaks for IleNH ↔ PheNH, Leu(1)NH ↔ Leu(6)CαH, Boc ↔ OMe and IleNH ↔ PheCβH (3.04 ppm), in addition to the participation of IleNH in an intramolecular hydrogen bond. The well-dispersed weak intraresidue ROE cross-peaks (NH_i ↔ αH_i) and strong inter-residue cross-peaks (αH_i ↔ NH_{i+1}) further support the backbone angles in the β-region of the Ramachandran plot. Though the presence of a minimal hairpin is established, the appearances of weak ROE cross-peaks for PheNH ↔ Leu(6)NH and IleNH ↔ Leu(1)NH imply that the strands are fraying at the terminals. Such fraying at the end also results in the cross-peak for Leu(1)NH ↔ Leu(6)CαH, instead of Leu(1)CαH ↔ Leu(6)CαH in the ROESY spectrum.

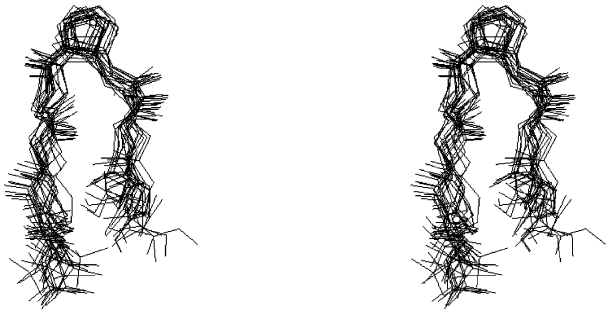

FIGURE 6. Stereoview of the 20 superimposed energy-minimized structures sampled during the 600 ps constrained MD simulations of peptide **10** following the Simulated Annealing protocol.

The ROE cross-peak intensities in the ROESY spectrum of **10** were used to obtain the restraints in constrained molecular dynamics (MD) simulation studies. By the same protocol as described above for **4**, 20 energy-minimized structures were generated, which were superimposed as shown in Figure 6. In these structures, a strong intramolecular H-bond between PheNH and IleC═O is distinctively visible, leading to a 12-membered-ring structure, somewhat reminiscent of a type VI β-turn, supported by the average φ, ψ angles of Val (−120, 118°) and Vpro2 (−76, −150°). This induces nucleation of an additional hydrogen bond, IleNH→PheC═O, resulting in the formation of a minimal hairpin structure with average pairwise heavy atom and backbone atom RMSD values of 1.69 ± 0.57 and 1.36 ± 0.57 Å, respectively.

In the other penta- and hexapeptides, the observation of similar structural signatures implied that they possess similar structures in solution. Further work is in progress.

In conclusion, the known propensity of *E*-vinylogous proline molecules to stabilize a cis amide bond with the preceding amino acid led to a stable 12-membered hydrogen-bonded ring structure that induced further nucleation of β-hairpin structures in short peptides. Although both VPro1 and VPro2 gave rise to the 12-membered hydrogen-bonded structures, these type VI β-turns induced by the latter with a trisubstituted double bond were more pronounced than those derived from the former. This will help in designing the mimics of the bioactive conformations of many peptides that contain cis proline residues in an $i + 2$ position, leading to the induction of relatively unfavorable type VI β-turns in these peptides.

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Supporting Information Available: Text, figures, and tables giving general experimental procedures, the physical data for **3**−**10**, ^{13}C NMR and ROESY spectra of **3**−**10**, expansions of the ROESY spectra of **4** and **10**, ROE cross-peaks in **4** and **10**, and statistics for the structures of **4** and **10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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