c[D-pro-Pro-D-pro-N-Methyl-Ala] Adopts a Rigid Conformation That Serves As a Scaffold to Mimic Reverse-Turns

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INTRODUCTION

Two common problems in developing therapeutics are determining an accurate binding mode of ligands being mimicked and finding rigid structures that accurately mimic the geometry of molecular recognition. Both of these problems can be addressed by developing libraries of privileged scaffolds that mimic the

ABSTRACT:
Naturally occurring cyclic tetrapeptides (CTPs) such as tentoxin (Halloin et al., Plant Physiol 1970, 45, 310–314; Saad, Phytopathology 1970, 60, 415–418), ampicidin (Darkin-Rattray, Proc Natl Acad Sci USA 1996, 93, 13143–13147), HC-toxin (Walton, Proc Natl Acad Sci USA 1987, 84, 8444–8447), and trapoxin (Yoshida and Sugita, Jpn J Cancer Res 1992, 83, 324–328; Itazaki et al., J Antibiot (Tokyo) 1990, 43, 1524–1532) have a wide range of biological activity and potential use ranging from herbicides (Walton, Proc Natl Acad Sci USA 1987, 84, 8444–8447; Judson, J Agric Food Chem 1987, 35, 451–456) to therapeutics (Loiseau, Biopolymers 2003, 69, 363–385) for malaria (Darkin-Rattray, Proc Natl Acad Sci USA 1996, 93, 13143–13147) and cancer (Yoshida and Sugita, Jpn J Cancer Res 1992, 83, 324–328). To elucidate scaffolds that have few low-energy conformations and could serve as semirigid reverse-turn mimetics, the flexibility of CTPs was determined computationally. Four analogs of cyclic tetraproline c[Pro-pro-Pro-pro] with alternating L- and D-prolines, namely c[pro-Pro-pro-NMe-Ala], c[pip-Pro-pip-Pro], c[Pro-Pip-pro-Pro], and c[Ala-Pro-pip-Pro] were synthesized and characterized by NOESY NMR. Both molecular mechanics and Density Functional Theory quantum calculations found these head-to-tail CTPs to be constrained to one or two relatively stable conformations. NMR structures, while not always yielding the same lowest energy conformation as expected by in silico predictions, confirmed only one or two highly populated solution conformations for all four peptides examined. c[pro-Pro-pro-NMe-Ala] was shown to have a single all trans-amide bond conformation by overlapping four Cα-Cβ bonds with those for ~ 6.5% (Tran, J Comput Aided Mol Des 2005, 19, 551–566) of reverse-turns in the Protein Data Bank PDB with a RMSD of 0.57 Å. © 2007 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 90: 384–393, 2008.

Keywords: cyclic tetrapeptide; privileged scaffold; conformation; proline; pipecolic acid

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well defined tertiary structure in macromolecules. Screening of these privileged scaffolds libraries would help determine how ligand side chains are stabilized upon binding as well as establish lead compounds for therapeutic optimization. Reverse turns, which have been found to make up one-fourth of the residues in high-resolution protein structures in the Protein Data Bank (PDB) are often viewed as targets for therapeutic design because they are generally found on the exterior of proteins and often involved in macromolecule interactions. Reverse turns are characterized by a four-residue sequence that creates a \( \sim 180^\circ \) turn in the polypeptide chain, with the first and fourth C-atom within 7 Å. As a subclass of reverse turns, classical \( \beta \)-turns, contain a hydrogen bond between the first residue’s carbonyl oxygen and the fourth residue’s amide hydrogen. Rigid compounds that have modifiable bonds overlapping Cα-Cβ bonds in reverse turns, such as benzodiazapenes, can be considered privileged mimetic scaffolds; they can bind to molecular recognition sites for reverse turns with the potential for high affinity due to their preorganization.

Two methods used to nucleate reverse-turn structures incorporate a sequence of either pro-Pro or Pro-pro at the i+1 and i+2 positions of the turn, or use small cyclic peptides either cyclized through side chains or head-to-tail. Cyclic peptides are widely investigated for therapeutic use due to their rigid scaffold and bioavailability. Cyclic peptides, such as \( \theta \)-Defensins, naturally occur in many organisms and have a broad range of activity, acting as antibotics or antivirals for HIV-1, herpes simplex, or influenza A viruses. Cyclic tetrapeptides (CTPs), ampicidin, HC-toxin, and trapoxin all have been found to be inhibitors of histone deacetylase which control the expression of genes. These CTPs are therefore thought to be possibly useful as cancer therapeutics. CTPs seem particularly apt at mimicking reverse turns in that they inherently have a main chain that turns \( \sim 180^\circ \) four consecutive times in four residues.

Combining two approaches by incorporating pro-Pro or Pro-pro sequences into a CTP should yield conformationally constrained reverse-turn mimetics. Because of their macrocyclic ring constraint, the conformation of head-to-tail CTPs can largely be described by the cis/trans-conformation of their backbone amide bonds. Unlike cyclic tripeptides and pentapeptides which contain either only cis-, or largely trans-amide bonds, respectively, CTPs contain a mixture of both cis- and trans-peptide bonds. This “sweet spot” of CTPs seems ideal for fine tuning of various rigid structures since the positions of cis/trans-amide bonds largely determines the conformation of the mimetic scaffold. The CTP \( \text{c[Pro-pro-Pro-pro-N-Methyl-Ala]} \) is a combination of these two reverse-turn mimetic approaches shown to exist with stable cis-trans-cis-trans and trans-cis-trans-cis amide bond conformers at room temperature in water. In this study, we modified the CTP c[Pro-pro-Pro-pro] by substituting L- or D-proline with alanine, N-methyl-alanine (NMe-Ala), or L- or D-pipecolic acid (Pip) to stabilize other sets of amide conformations such as trans-trans-trans-trans and trans-trans-cis-trans. Different sequences of amide-bond torsions, which dictates the backbone conformations, changes the relative orientation of the proline and Pip rings enabling them to act as mimics for multiple turn types. CTPs macrocycles tend to either a boar or chair conformation, in a similar fashion to cyclohexane.

To use CTPs as effective turn mimics, this boar or chair conformation must be known. In addition the various macrocycles have different dipoles that might impact binding to different receptors. Cyclic peptide reverse-turn mimics which have a proline or pipecolic residue have the added benefit that the side-chain exocyclic ring can be used to rigidify bonds (\( z_1, z_2, \) etc.) distal to the Cα-Cβ of the synthetic side chain. For these reasons, it would be useful to have multiple CTPs representing each turn type. Screening a large library of such geometrically constrained molecular probes should assist in identification of the relative orientation of the side chains of the bioactive peptides involved in molecular recognition of a reverse turn at a given receptor.

**METHODS**

**Computational Conformational Searches**

The conformational effects of three noncoded d- and l-amino acids (N-methyl-alanine (NMe-Ala), pipecolic acid (Pip), and aminoisobutyric acid (Aib)) on CTPs (Table I) were examined through a Monte-Carlo Multiple Minimum (MCMM) conformational search. Searches were done in both vacuum and water using the Onsanger model. Results are shown in Table I.

**Quantum Minimizations**

Because the 12-membered macrocycle of CTPs are highly constrained, their amide bonds can be distorted significantly from the ideal \( 180^\circ \) (trans) or \( 0^\circ \) (cis) torsions. Density Functional Theory (DFT) minimizations were performed both in vacuo and water on the lowest energy and second-lowest energy conformers found from the MCMM search (Table I). DFT calculations were done using Gaussian98 at the B3LYP/6-31G* level and all aqueous estimations were done using the OPLS2001 Force Field and charges.
Peptide Synthesis, Purification, and Characterization

All peptides were synthesized by solid phase peptide synthesis. 2-Chlorotrityl resin (GL Biochemistry) was used and substituted with 2M equivalents Fmoc-Pro and 5M equivalents DIEA in 8 mL N,N-dimethylformamide (DMF)/g resin and shaken for 30 min, followed by a DCM/DIEA/MeOH 16:3:1 wash to cap the unreacted trityl groups. This yielded a 0.5 M loaded Fmoc-Pro-trityl-resin. Coupling of amino acid residues was done in the presence of 3M equivalents. Fmoc-protected amino acids, benzotriazol-1-yloxytris(dimethylamino) phosphonium hexafluorophosphate (BOP), 1-hydroxybenzotriazole (HOBt), and 6M equivalents of DIEA were preixed in a minimum of DMF on ice for 5 min and then added to resin bound peptide. After 2–3 h on a shaker for each coupling, removal of the Fmoc protecting group was done with 22% piperidine in DMF for 2 × 10 min. Coupling was monitored by the chloranil test26 for secondary amines (Pro, Pip, NMe-Ala) and the ninhydrin test27 for primary amines and a second recoupling performed if required. Peptides were cleaved from the trityl resin with 2% trifluoroacetic acid (TFA) in DCM for 5 min. Purification of all peptides was done on a Rainin XPHL HPLC system with a Dynamax UV lamp, using a Vydac 218TP1022 (C18, 10 μm, 2.2 cm ID × 25 cm L) RP-HPLC column. All peptides were purified in a A = 100% H2O and B = 90% ACN/10% H2O solvent system at a speed of at 10 mL/min. Linear and cyclic peptides were purified in 0.025% HCl and 0.025% TFA solvents, respectively. Cyclizations were carried out in presence of 3M equivalents of BOP, HOBt, and 6M equivalent DIEA at a 0.5 mg/mL (~0.1 mM) dilution in DMF and shaken for 3 days. Cyclization was followed by TLC, and if incomplete, PyBOP was added for one additional day which greatly facilitated some cyclizations. The solution was then dried by rotary evaporation and dissolved in 1:1 H2O/ACN for RP-HPLC purification. MS was done on a Finnigan LCQ Classic LC/MS with ion trap and liquid infusion. Because of the lack of ionizable atoms, the cyclic peptides mass spectra mainly appeared as a mixture of cyclic monomers with Na and ionic dimers with Na. The monomer became more apparent, and the ionic dimer vanished in the mass spectra when the CTP solution was greatly diluted (Supplementary Material).

c\[\text{[Pro-Pro-NMe-Ala]}\]. The linear peptide NH2-pro-NMe-Ala-pro-COOH was synthesized by standard SPPS as described above by coupling Fmoc-\(\text{\footnotesize{D}}\)-pro, Fmoc-N-Me-Ala, and Fmoc-\(\text{\footnotesize{L}}\)-pro consecutively to \(\text{\footnotesize{L}}\)-Pro-trityl-resin. After removal of the N-terminal Fmoc group, the linear tetrapeptide was released from the resin support by treatment with 2% TFA in DCM followed by purification on RP-HPLC. After a 3-day cyclization with HOBt, 3M equivalents of PyBOP and DIEA were added for one additional day. The linear and cyclic peptide were purified in a 10–90% B gradient over 15 min and eluted at 9.0 and 15.2 min, respectively, with a 11% overall yield for the pure CTP. Linear NH2-pro-NMe-Ala-pro-COOH: ESI-TOF/MS: (\(m/z\)) Calcd for C19H30N4O5H [M + H]+, 395.3, 417.4, respectively. c\[\text{[pro-Pro-pro-NMe-Ala]}\] was analyzed by 1H, COSY, TOCSY, NOESY NMR (500 MHz, CDCl3, −20°C) Table II; ESI-TOF/MS: (\(m/z\)) Calcd for 2(C19H30N4O5)H [(D + H)+], 2(C19H30N4O5)Na [(D + Na)+]: 753.91, 775.90, respectively. Obsd 753.6, 775.7, respectively.

c\[\text{[Pro-Pip-Pro-Pro]}\]. The linear peptide NH2-pro-Pip-Pro-Pro-COOH was synthesized by standard SPPS as described above by coupling Fmoc-\(\text{\footnotesize{D}}\)-pro, Fmoc-\(\text{\footnotesize{L}}\)-Pip, and Fmoc-\(\text{\footnotesize{D}}\)-pro consecutively to \(\text{\footnotesize{L}}\)-Pro-trityl-resin. After removal of the N-terminal Fmoc group, the linear tetrapeptide was released by treatment with 2% TFA in DCM from the resin support followed by purification on RP-HPLC. The linear and cyclic peptides were purified with a 10–50% B and 10–90% B gradient, respectively, over 15 min. The peptides eluted at 9.8 and 12.5 min, respectively, with a 12% overall yield for the pure CTP. Linear NH2-pro-Pip-Pro-Pro-COOH: ESI-TOF/MS: (\(m/z\)) Calcd for C21H32N2O4H [(M + H)+], C21H32N2O4Na [(M + Na)+]: 421.52 and 443.50, respectively. Obsd 421.35, 443.43, respectively.

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c[π-pro-Pro-π-pro-N-Methyl-Ala]

Table II  Cyclic Tetrapeptides – NMR Chemical Shifts

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Proton Chemical Shifts (ppm) of c[pip-Pro-pip-Pro], c[pro-Pro-pro-NMe-Ala], c[Ala-Pro-pip-Pro], c[pro-Pip-pro-Pro] in CDCl3 (at 25°C except for c[pro-Pro-pro-NMe-Ala] which was measured at –20°C). For c[pro-Pro-pro-Pro] kiloHertz are for the major conformation observed. NMR verified amide bond conformations listed (i, trans; c, cis, first letter refers to amide bond between first and second amino acid in first column).

c[π-pro-Pro-pro-Pro]. The linear peptide NH₂-pip-Pro-pip-Pro-COOH was synthesized by standard SPPS as described above by coupling Fmoc-π-pip, Fmoc-π-Pro, and Fmoc-π-Pro consecutively to l-Pro-trityl-resin. After removal of the N-terminal Fmoc group, the linear tetrapeptide was released by treatment with 2% TFA in DCM from the resin support followed by purification on RP-HPLC. After a 3-day cyclization with HOBT, 3 equivalents of PyBOP and DIEA were added for one additional day. The linear and cyclic peptide were purified in a 10–90% B gradient over 15 min and eluted at 10.0 and 15.0 min, respectively, with an 18% overall yield for the pure CTP. Linear NH₂-Ala-Pro-pip-Pro-COOH ESI-TOF/MS: (m/z) calcd. for C₁₉H₃₀N₄O₅H [(M + H)+], C₁₉H₃₀N₄O₅Na [(M + Na)+], 2(C₁₉H₃₀N₄O₅)H [(D + H)+], 2(C₁₉H₃₀N₄O₅)Na [(D + Na)+]: 377.46, 753.93, 775.36, respectively. c[Ala-Pro-pip-Pro] was analyzed by ¹H, COSY, TOCSY, NOESY NMR data were collected on c[pro-Pip-pro-Pro] ppms are for the major conformation observed. NMR verified amide bond conformations listed (i, trans; c, cis, first letter refers to amide bond between first and second amino acid in first column).

NMR Structural Analysis
¹H, COSY, TOCSY, and NOESY NMR data were collected on c[pro-Pro-pro-NMe-Ala], c[pro-Pip-pro-Pro], c[pip-Pro-pro-Pro], and c[Ala-Pro-pip-Pro]. NMR spectra were recorded with a Varian Inova-600 (Varian, Palo Alto, CA) spectrometer and the data processed with VNMRS software. The total correlation (TOCSY) spectra were recorded using an MLEV-17 mixing sequence of 120 ms flanked by two 2 ms trim pulses. Phase-sensitive 2D spectra were obtained by employing the hypercomplex method. A total of 2 × 256 × 2048 data matrices with 16 scans per t1 increment were col-
lected. Gaussian and sine-bell apodization functions were used in weighting the t2 and t1 dimensions, respectively. After two-dimensional Fourier transformation, the 2048 × 2048 frequency domain representation was phase and baseline corrected in both dimensions.

The NOESY spectrum resulted a 2 × 256 × 2049 data matrix with 32 scans per t1 increment. Spectra were recorded with 250 and 450 msec mixing time. The use of a 450 msec mixing time afforded a better result. The hypercomplex method was used to yield phase-sensitive spectra. The time-domain data were zero filled to yield a 2048 × 2048 data matrix and were processed in a similar way as the 2D TOCSY spectrum described above. Chemical shift data for the four cyclic peptides in CDCl3 at −20°C is detailed in Table II. ROESY NMR spectra were obtained on all four peptides to see if more through-space cross peaks could be observed, possibly absent in NOESY spectra due to the effect of molecular mass on correlation time, but no new peaks were observed (data not shown).

A sodium-dimer model of c[Pro-Pro-Pro-NMe-Ala] was developed to explain an NOE peak between the N-methyl hydrogens and the proceeding Dpro-Cβ hydrogen. Two all-trans amide bond DFT structures were used to sandwich a Na atom between them. All NOE constraints were imposed with a 100 kJ/mol·Å2 penalty if more than 4 Å apart. This locked all amide bonds in the trans configuration and set a constraint between the N-methyl, to D-pro/γ-1. Spring constants of 100 kJ/mol·Å2 were also imposed between the sodium and Cz atoms if more than 5 Å apart. A conformational search was done using the OPLS-2001 force field in vacuum taking 1000 Monte-Carlo steps. The lowest energy structure satisfied all NOE peaks was found and is shown in Figure 2b.

Overlap of Structures With Reverse-Turn Classes From PDB

Recently Tran et al. elucidated nine sets of four vectors which represent almost all (~90%) of the relative orientations of the four Cx-Cβ bonds found in reverse turns in the PDB.27 The ability of our CTPs NMR conformation to mimic reverse turns was established by how well the NMR experimental structure had bonds overlapping the nine sets of four vectors established by Tran et al.27 as representative of reverse-turn Cx-Cβ bonds. It was found that overlapping a Cx-Cβ vector of a Tran et al. cluster with a C—H bond on a CTP gave an undesired biased overlap compared to overlapping a C—C bond in the CTP. Even if the C—H bond was oriented in the correct direction, the difference in bond length from a C—H and C—C bond increased the total RMSD of the overlap. Therefore, each Tran cluster of four vectors was converted into its own subcluster by changing the bond lengths combinatorially to represent C—C and C—H bonds (1.52 Å and 1.10 Å, respectively) keeping the four Cz atoms in place. There were 16 sets of 4 vectors for each initial set, and a total of 144 sets of 4 vectors. This enabled us to directly overlap possible combinations of C—H and C—C bonds on our CTPs more accurately. These four vector structures were overlapped with the all trans NMR structure c[pro-pro-pro-NMe-Ala] to find rigid structures that could serve as privileged scaffolds. The program FOUNDATION was used for overlapping using a clique-finding algorithm to exhaustively overlap bonds in order.38 Searches were done which looked for 3- and 4-bond overlaps with RMSD of all 6, or 8 atoms, respectively of less than 0.3 Å, 0.5 Å, and 0.7 Å.

RESULTS AND DISCUSSION

Conformational Search of CTPs

To screen CTPs as reverse-turn mimics to act as probes for molecular recognition or as potential therapeutics, computational studies were used to find CTPs that had similar stable conformations in both vacuum and water. The objective was reverse-turn mimics whose unique conformers were retained in both the hydrophilic bulk water and while being bound in a hydrophobic environment, such as a receptor recognition site. Single and double amino acid substitutions of the parent peptide c[Pro-pro-Pro-pro]25 were simulated to determine how the noncoded amino acids NMe-Ala,12 Pip, and Aib would affect the cis/trans/cis/trans conformation of amide bonds in CTPs. This resulted in 9 CTPs which were initially investigated by performing Monte-Carlo conformational searches (MCCS) in both vacuum and water (Table I). All 12 bonds in the cyclic peptide-chain macrocycle were rotated, including the four amide bonds. In addition the proline and Pip side chains were allowed to sample different side-chain ring puckers. Eight of the nine CTPs were found to have the same low-energy conformation in both vacuum and water by MCCS (Table I). c[Pro-pro-NMe-Ala-NMe-Ala] was the only CTP with different conformers in vacuo and in water, and was not studied further. As a control for adequate sampling, all compounds had their mirror-image counterparts conformationally searched in the same manner. An identical set of low-energy mirror-image conformers were found as expected suggesting adequate sampling. In addition, for all CTPs, MCCS runs found all 16 possible cis-trans-amide bond conformers. The expected puckering of the proline and pipocyclic rings, up and down, or boat and chair, respectively, was also observed (data not shown). All these observations suggested that the conformational hyperspace available to these CTPs was adequately sampled.

CTPs have a strained macrocyclic ring that can cause their amide bonds to be significantly divergent from the ideal 180° or 0° α-torsion values. Therefore, a quantum treatment of this system was more appropriate than molecular mechanical, which is inadequately parameterized for nonide al amide-bond torsions. For this reason, the lowest and second-lowest energy conformations for these eight structures from the MCMC calculations were minimized with DFT in both vacuum and H2O.

In both the MCCS and DFT calculations, all the CTPs with Pip (c[Pro-Pro-Pro-pip], c[pip-Pro-pip-Pro], c[Pro-pro-pip-pip]) adopted all trans-amide bond conformations (as did their enantiomers) (Table I). The ΔΔG between the lowest energy and second-lowest energy conformation for these three CTPs was significantly greater (5–9 kcal) in vacuo than in water because their all trans-amide conformation has a net dipole of ~0, which is favored in vacuum, while the

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second-lowest energy conformers with a higher dipole paid an energetic cost of solvation in vacuum compared to water. The peptide c[pro-Pro-Pro-NMe-Ala] was also shown to adopt an all \(trans\)-amide conformation by both MCCS and DFT in both vacuum and water. CTPs that adopt this all \(trans\)-amide bond conformation in water could be expected to rigidify even further when bound in a nonpolar binding site in a receptor. All four of these CTPs low-energy all \(trans\) conformations was more than 5 kcal/mol more stable than their second-lowest energy conformation in both water and vacuum as determined by DFT (Table I). The fact that all four of these structures were found computationally to have all \(trans\)-amide bonds is not surprising given that most crystal structures of CTPs are in either the all \(trans\) or an alternating \(cis\)/\(trans\)/\(cis\)/\(trans\) conformation (Cambridge Structural Database 2007 analysis). None of the other CTPs were shown by DFT to have as stable a structure, and were not further experimentally explored.

The three rigid CTPs c[pro-Pro-Pro-NMe-Ala], c[pro-Pip-pro-Pro], and c[pip-Pro-pip-Pro] all have tertiary amides for each amino acid and, therefore, have no NH’s available for intramolecular hydrogen bonding. After NMR structural analysis, the peptide c[pip-Pro-pip-Pro] was found to have a \(trans\)-\(cis\)-\(trans\)-\(cis\) amide bond conformation and c[Ala-Pro-pip-Pro] was synthesized by SPPS as described earlier in an attempt to incorporate a hydrogen bond between the alanine NH and either the preceding or subsequent proline carbonyl to further stabilize this \(trans\)-\(trans\)-\(cis\)-\(trans\) structure.

**Synthesis and NMR Characterization**

The linear peptides NH\(_2\)-pro-Pip-pro-Pro-COOH, NH\(_2\)-pip-Pro-pip-Pro-COOH, NH\(_2\)-Ala-pro-Pro-COOH, and NH\(_2\)-Ala-pip-Pro-pip-Pro-COOH were synthesized by standard solid phase peptide synthesis (SPPS), purified by RP-HPLC, cyclized in a dilute 0.1 m\(\text{H}_2\text{O}\) solution to prevent dimerization, and again purified by RP-HPLC. The total yield for the cyclic peptides ranged between 11–22%. All four CTPs c[pro-Pro-Pro-NMe-Ala], c[pro-Pip-pro-Pro], c[pip-Pro-pip-Pro], and c[pro-\(\text{P}^\text{pro}\)-pro-Pro] were characterized by \(^1\text{H}\), COSY, TOCSY, and NOESY NMR (Table II) and mass spectra. The NOESY data was used to determine the \(cis\)/\(trans\) conformation of the peptide bonds. In a \(\text{Pro}\)-pro or \(\text{pro}\)-\(\text{N}\)-methylated dipeptide sequence, a NOE peak between succeeding \(\text{CzH-CzH}\) and \(\text{CzH-CzH}\) indicates a \(cis\)-\(trans\)-amide bond, respectively. This holds true for Pro-pip and pro-Pip as well, except the \(trans\) NOE observed is between a Cz and the succeeding Cz.

Proton chemical shifts of c [Ala-Pro-pip-Pro] were assigned completely by analysis of TOCSY (Figure 1d) and COSY spectra. The Ala residue was identified first by the spin propagation from NH through \(\beta\)-CH\(_3\). The correlated proton resonances at 3.0–3.8 ppm were unambiguously assigned to \(\delta\) and \(\varepsilon\) protons of the Pro and pip residues (Figure 1e).

Sequential assignment of c[Ala\(_1\)-pro\(_1\)-pip\(_2\)-pro\(_3\)-pro\(_4\)] was obtained by using the NH(i)–NCH3(i–1), \(\delta\)H(i)–\(\delta\)H(i–1) fingerprint region of the NOESY spectrum (Figure 1c). Observed NHA\(_1\)(i)–\(\delta\)H(Pro\(_2\)), \(\delta\)H(pip\(_2\))–\(\varepsilon\)H(Pro\(_2\)) and \(\delta\)H(Pro\(_4\))–\(\delta\)H(Ala\(_1\)) NOE’s indicated the \(trans\) conformation of the peptide bonds between these adjacent residues. However, a strong \(\varepsilon\)H(Pro\(_4\))–\(\varepsilon\)H(pip\(_3\)) peak revealed a \(cis\)-amide bond conformation at the Pro\(_4\)-pip\(_3\) segment, hence a \(trans\)-\(trans\)-\(cis\)-\(trans\) conformation. NOESY spectrum of c[pro\(_1\)-pro\(_2\)-pip\(_3\)-pro\(_4\)] also suggested a \(trans\)-\(trans\)-\(cis\)-\(trans\) amide conformation as evident by the observed \(\varepsilon\)H(Pro\(_2\))–\(\varepsilon\)H(pip\(_3\)), \(\delta\)H(pip\(_3\))–\(\varepsilon\)H(Pro\(_2\)), \(\delta\)H(Pro\(_2\))–\(\delta\)H(Pro\(_3\)) and \(\delta\)H(pip\(_3\))–\(\delta\)H(Pro\(_3\)) NOE’s in Figure 1b. Observed \(\delta\)(i)–\(\varepsilon\)(i–1) cross peaks in addition to the NCH3(Ala\(_4\))–\(\varepsilon\)H(Pro\(_3\)) NOE peak in Figure 1a indicated an all \(trans\)-amide bond conformation appeared in c[pro\(_1\)-pro\(_2\)-pip\(_3\)-pro\(_4\)]NOE’s. NOESY spectrum for c[pro\(_1\)-Pip\(_2\)-pro\(_3\)-pro\(_4\)] revealed two conformations, the major conformation (80%) in CDCl\(_3\) at 25°C only reveals two \(trans\)-amide bonds at pro\(_1\)-Pip\(_2\) and pro\(_3\)-pro\(_4\). Neither a typical \(trans\)-\(cis\)-\(trans\)-\(cis\) bond was seen between the Pip\(_2\)-pro\(_3\) and Pro\(_1\)-Pro\(_2\) (Supplementary Material). The minor conformation revealed trans bonds between pro\(_1\)-Pip\(_2\), Pip\(_2\)-pro\(_3\), and Pro\(_1\)-Pro\(_2\) but neither a typical \(trans\)-\(cis\)-\(trans\)-\(cis\) bond was seen between the Pip\(_2\)-pro\(_3\).

Complete assignments for each residue of c[Ala\(_1\)-pro\(_2\)-pip\(_3\)-pro\(_4\)], c [pip\(_1\)-pro\(_2\)-pip\(_3\)-pro\(_4\)], c[pro\(_1\)-pro\(_2\)-NMeAla\(_1\)], and c[pro\(_1\)-pip\(_3\)-pro\(_3\)-pro\(_4\)] are shown in Table II. The temperature dependences of the Ala\(_1\) amide proton shows evidence of intramolecular hydrogen bonds in c[Ala\(_1\)-pro\(_2\)-pip\(_3\)-pro\(_4\)]. The \(\Delta\delta/\Delta T\) values of 2.2 \(\times\) 10\(^{-3}\) ppm/K indicated that amide protons were somewhat shielded from solvent (Table II). Interestingly, it was found that the chemical shift differences for the two \(\varepsilon\)-pip\(_3\) protons were quite large (\(\Delta\delta = 0.63\) ppm). The intramolecular hydrogen bond may involve the carbonyl group of the pip\(_3\)-pro\(_4\) amide bond due to the \(cis\)-amide bond conformation. In such a case, one of the \(\varepsilon\)-pip\(_3\) proton was fixed in the shielding zone of peptide bonds due to the difference in orientation of these two \(\varepsilon\) protons with respect to the carboxyl group of neighboring residues, which leads to a strong nonequivalence in chemical shift.

c[pro-Pro-Pro-NMe-Ala] was the only compound with enough NOESY NMR data to validate a single low-energy rigid conformation supported by computational conformational searches (Tables I and II, Figure 1a) and was, therefore, the best prospect to screen as a reverse-turn privileged scaffold. A conformational search of c[pro-Pro-Pro-NMe-Ala] was performed with the 8 NMR constraints pro\(_1\)-pro\(_2\)-pro\(_3\)-pro\(_4\),
A sodium ionic dimer model of c[pro-Pro-pro-NMe-Ala], as seen by Mass Spec, was developed to explain a NOE peak between the N-methyl and preceding D-pro C\textsubscript{i}+1 hydrogens. A conformational search restricting a Na\textsuperscript{+} atom between two cyclic monomers with NOE constraints resulted in a low-energy structure that confirmed the N-methyl to D-pro C\textsubscript{i}+1 NOE (Figure 2b). The model also predicted a NOE between the pro\textsubscript{3}\textsubscript{d}-Pro2\textsubscript{γ} which we then also confirmed in the NOE spectra. We then proceeded to screen the all-trans c[pro-Pro-pro-NMe-Ala] monomer as a reverse-turn mimic.

**Reverse-Turn Mimetic Screen**

Historically, reverse turns have been classified by the 4 torsions of the i+1 and i+2 residues, i.e., the ϕ and ψ of the second and third residues of the reverse turn. However, there...
are two torsions, $\psi$ of the first residue and $\varphi$ of the fourth residue that affect orientation of the first and fourth side chains that were not taken into account in this traditional reverse-turn nomenclature. It has been shown that reverse turns in the same classical-turn type can differ in the orientation of their side chains.\(^{37}\) Because reverse turns are usually recognized in biological systems by their relative side-chain orientation and not direct interactions with their backbones, the classical classification method is not optimal. Recently, Tran et al.\(^{37}\) have elucidated nine classes of orientations of the four consecutive Cα-Cβ bonds of a reverse turn that represent the vast majority (\(\sim 90\%\)) of reverse turns found in the PDB. These nine classes were used to screen our semi-rigid CTPs as reverse-turn mimics.

The DFT-minimized all trans conformer for c[pro-Pro-pro-NMe-Ala] was tested for overlap with the nine reverse-turn clusters of Tran et al.\(^{37}\) (Table III). An excellent overlap was found for all four Cα-Cβ bonds of cluster 6 of Tran et al.'s with a deviation of 0.57 Å RMSD (Figure 2a). Cluster 6 is largely made up of classical Type I $\beta$-turns.\(^{37}\) The Cα-Cβ bonds of the N-Me-Ala, the succeeding D-pro, and the succeeding Pro overlap the i, i+1, and i+2 Cα-Cβ bonds of cluster 6, while one of the Cγ-H bonds on the first residue, Pro, overlaps that last Cα-Cβ+1 bond. If used as a mimic, the side-chain substituents mimicked by the 3 Cα-Cβ bonds of c[pro-Pro-pro-NMe-Ala] would also have at least one of their later side-chain torsion angles locked in place. Therefore, this CTP would likely not work to mimic a glycine or alanine at one of these positions due to the extra steric bulk of the proline exocyclic ring. However, if a larger side chain branched off the c[pro-Pro-pro-NMe-Ala] structure at one of the two Cβ-H bonds, this would be equivalent to locking not only the Cα-Cβ bond, but also the Cβ-Cγ of the side chain. If the correct side-chain conformation followed the ring up to Cβ-Cγ, and then branched off one of the two Cγ-H bonds of the proline ring, this would in essence rigidify the “side chains” Cα, Cβ, Cγ, and Cδ atoms. As mentioned earlier, this could probe the receptor with a very well-determined side-chain conformation to assist in elucidating an unknown bound conformation of the bioactive parent peptide.

A short 1 ps dynamics run was done on c[pro-Pro-pro-NMe-Ala] using the Amber force field to estimate vibrational flexibility of the CTP at room temperature. The limited set of 200 conformers sampled effectively adopt the same ring conformation (Figure 3a). Trans $\beta$-turn clusters 2, 3, 4, 6, 7, and 8 (which are represented largely by classical $\beta$-turns types II, I and IV, II and IV, I, II and IV, and VIII, respectively) all had 3 bonds overlapped by a snapshot of the c[pro-Pro-pro-NMe-Ala] dynamics run with a RMSD of \(<0.3\) Å (Figure 3b–g, Table III). This exemplifies the point of how well suited CTPs are at mimicking reverse turns. They inherently present a 180° reverse turn of consecutive four residues no matter which residues you pick as the first residue. In essence, therefore, every CTP has 4 “chances” of rotating and interacting with a receptor as a new reverse-turn mimic.

**CONCLUSIONS**

Having a diverse set of CTPs incorporating D and L proline that mimic reverse turns enables both useful probes for determining the optimal side-chain geometry and for reverse-turn recognition. c[Pro-pro-NMe-Ala-pro] was shown to adopt an all trans amide-bond conformation that could mimic all the Cα-Cβ bonds in the classical type I

**FIGURE 2** Overlap of CTP with Cα-Cβ bonds of Cluster 6. The all trans c[pro-Pro-pro-NMe-Ala] is overlapped (0.57 Å RMSD) by all 4 Cα-Cβ bonds in Tran et al.'s cluster 6 which mainly represents classical $\beta$-turn Type I. The Cα and Cβ atoms are in black and yellow respectively (a). A dimer model of 2c[pro-Pro-pro-NMe-Ala]●Na that has the N-methyl hydrogen within 3 Å of the proceeding pro$_1$ hydrogen of the other monomer justifying an observed NOE peak (b).
\( \beta \)-turns (Figure 2a), with the ability to lock out \( \text{C}\beta-\text{C}1 \) and possibly even \( \text{C}1-\text{C}6 \) torsions of three of the four side chains. In addition, \( \text{c[Pro-pip-NMe-Ala-pro]} \) could mimic 3 out of 4 of the \( \text{C}x-\text{C}\beta \) bonds of Type I, II, IV, and VIII extremely well (Figure 3, Table III). CTPs can inherently mimic reverse \( \beta \)-turns well as exemplified by how many different turn types for which \( \text{c[pro-Pro-pro-NMe-Ala]} \) could mimic 3 of the 4 side chains. However, to mimic all four \( \text{C}x-\text{C}\beta \) side chains of all the reverse turns, a library of many CTPs with varying \( \text{cis/ trans-amide bond torsions} \) will be needed. Future work will include determination of the impact of methyl group for proton substitution on the CTP to disrupt or stabilize the conformations determined to be most stable by these studies.

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