Engineering Cyclic Tetrapeptides Containing Chimeric Amino Acids as Preferred Reverse-Turn Scaffolds

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Four residues making almost a complete 180° turn in the direction of the peptide chain define a reverse turn, a common motif and recognition site in proteins. Cyclization between residues *i* and *i* + 3 and incorporation of heterochiral dipeptides (such as D-Pro-L-Pro) in the *i* + 1 and *i* + 2 positions are used to constrain a peptide to a reverse-turn conformation. A combined approach, cyclic tetrapeptides (CTPs) based on heterochiral dipeptides of chimeric amino acids, is evaluated as minimalist scaffolds for reverse-turn conformations. *Cyclo*-(D-Pro-L-Pro-D-Pro-L-Pro) has been studied with density functional theory (DFT) calculations and molecular dynamics simulations. The all-trans amide conformer was the most stable in vacuo, while the cis-trans-cis-trans (ctct) or trans-cis-trans-cis (tctc) amide conformer was more favored in water due to its large dipole moment. Different conformations could be selectively stabilized by different substitutions on the proline rings. Due to the small 12-membered ring and exocyclic constraints, conformational interconversions could only occur at high temperature. The presence of seven hydrogens on each ring that could be functionalized offers an overwhelming diversity to design molecules to probe receptors. The spatial relationships of $C_\alpha-C_\beta$ vectors of reverse turns in proteins were subjected to principal component analysis for determination of the relative orientation of the $C_\alpha-C_\beta$ vectors. Most reverse-turn structures could be mimicked effectively with a subset of CTP scaffolds with an root-mean-square displacement (RMSD) of approximately 0.5 Å. Structural diversity of CTP scaffolds could be enhanced by the incorporation of proline analogues, such as azaproline (azPro) or pipecolic (Pip), azapipecolic (azPip), nipecotic (Nip), and isonipecotic (Inp) acids.

Introduction

Rational design of pharmaceuticals derived from naturally occurring peptides has been enhanced recently by two major breakthroughs. First, peptide and peptidomimetic libraries have been instrumental in producing hundreds of thousands of different compounds for biological screening. Second, cloning and expression of potential therapeutic targets have become routine to provide reagents for screening and possible structural determination, a prerequisite for structure-based drug design. The main obstacle for rational drug design for many biological systems, however, is the lack of information about the three-dimensional structure of the peptide in the receptor−ligand complex. This is often due to the limited information regarding the three-dimensional structure of the receptor, or other therapeutic target, despite the rapid progress in structural determination by crystallography and NMR spectroscopy. Studies of the isolated ligand or therapeutic target alone can be misleading since the bound structure of the ligand−receptor complex is often different from the combined structures of the unbound ligand and receptor.[1,2] It would be extremely useful, therefore, to develop a variety of "conformational templates",[3,4] that is, model ligands, which should satisfy at least three requirements: (1) they should possess only one or a few well-determined 3D structures, (2) they should be readily accessible synthetically, and (3) they should be able to uniquely orient peptide side chains ($\alpha-\beta$, $\beta-\gamma$, and $\gamma-\delta$ bonds when present) that are believed to transfer most of the information during peptide−receptor interaction. A conformationally diverse library of constrained compounds would provide an efficient way to deduce/test alternative hypotheses concerning the receptor-bound conformation from biological testing.

Reverse turns are common motifs and recognition sites in proteins.[5] Receptor recognition, substrate specificity, and catalytic function generally reside in these loop regions that often connect residues of the α-helices and β-strands contributing to the structural stability of proteins. β-turns, the most common type of reverse turn comprised of four residues, are characterized by the Φ and Ψ torsion angles of the *i* + 1 and *i* + 2 residues. The classical β-turn is stabilized by an intramolecular hydrogen bond between the carbonyl oxygen of residue *i* and the amide hydrogen of residue *i* + 3, although this is not an essential feature of four-residue reverse turns common in proteins. Reverse turns are ideal sites for receptor recognition because they present side chains in a solvent-accessible arrangement around a compact folding of the peptide backbone.[1,6] Examples of turns as recognition motifs can be found in many crystal structures of protein complexes. Thus, development of reverse-turn mimics has become an important aspect of elucidating the receptor-bound conformation of a biologically active peptide.

Numerous compounds have been developed to stabilize the reverse turn in a peptide chain. The dipeptide lactam,[7] the bicyclic dipeptide BTD[8] and similar proline derivatives,[9,10] spirolactam bicyclic and tricyclic systems based on proline,[11−14] substitution by α,α-dialkylamino acids,[15−17] *N*-aminoproline,[18] functionalized dibenzofurans,[19−21] and substitution by dehydroamino acids[22−25] are all examples that enhance reverse-turn propensity.[26,27] Other efforts have focused on stabilizing a type VI β-turn with a cis amide bond between residue *i* + 1 and *i* + 2 through using the 1,5-disubstituted tetrazole ring,[28−30] 1,2,5-triazole ring,[31] or 1,2,4-triazole[32] as cis amide bond surrogates,

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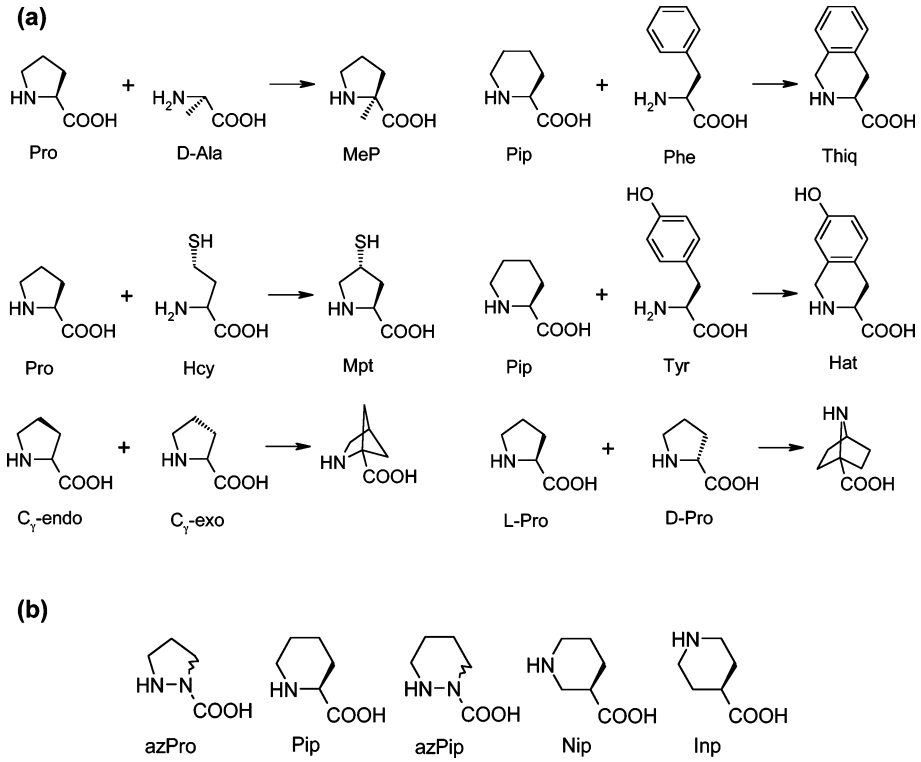


Figure 1. (a) Chimeric amino acids of proline and pipecolic acid and (b) constrained proline analogues.

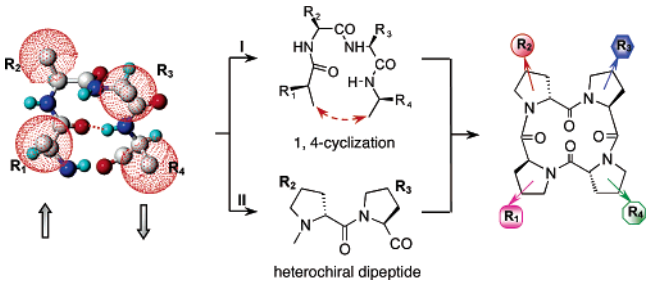
incorporating vicinal disulfide bonds,[33,34] bicyclic dipeptide analogues,[35−37] and certain sequences into cyclic peptides.[38] This can also be accomplished by incorporation various *cis*-Pro analogues at residue $i + 2$, including 5-alkylproline,[39−41] oxazolidine or thiazolidine-derived proline analogues,[42−44] and the amino acid analogue azaproline.[45,46] Benzodiazepines,[47−49] sugars,[50] and metal complexes of linear peptides[51] or chiral pentaazacrowns[52,53] have also been explored as conformational templates for β-turn recognition by appending desired substituents at selected positions. However, many of the reverse-turn mimics described above, particularly polycyclic systems, require multiple-step syntheses that severely limit the practical possibilities for selective side-chain incorporation. The availability of other rigid conformational templates that are synthetically accessible for chiral side-chain placement to constrain the conformation of peptides in various conformational states would be extremely useful.

One method is to introduce a covalent linker between residues i and $i + 3$, such as head-to-tail cyclization, while retaining the reverse-turn conformation. Many natural peptides with different kinds of biological activities, such as hormones, antibiotics, ion-transport regulators, and toxins, are cyclic peptides.[54] They have been reported to bind multiple unrelated classes of receptors with high affinity. Cyclic peptides are thus considered to be "privileged structures" capable of providing useful ligands for more than one receptor due to their high content of reverse-turn motifs. For example, cyclic tetrapeptides (CTPs), the minimalist reverse-turn mimetics, have been characterized as potent and highly selective molecules in a diverse range of therapeutic areas. Typical examples are the cytotoxic and antimitogenic agents HC-toxin[55] and chlamydocin,[55] the antitumor agent trapoxin,[56] the antimalarial apicidins,[57] and the tyrosinase inhibitor *cyclo*-(L-Pro-L-Tyr-L-Pro-L-Val).[58] In comparison to linear peptides, cyclic peptides are more stable to peptidases, are more bioavailable, and possess entropic advantages in molecular recognition.

The other method is to incorporate heterochiral dipeptides at residues $i + 1$ and $i + 2$. Marshall and co-workers[26,27] described theoretical calculations that suggest D-Pro-L-Pro, L-Pro-D-Pro, L-Pro-D-Pip, D-Pro-L-Pip, D-Pro-NMe-AA, and Pro-D-NMe-AA (Pip = pipecolic acid, AA = amino acid other than glycine) offer relatively rigid scaffolds on which to orient side chains for interaction with receptors that recognize reverse-turn conformations. Gellman and his colleagues[59,60] suggested that heterochiral dinipecotic acid (Nip) segments, (*R*)-Nip-(*S*)-Nip and (*S*)-Nip-(*R*)-Nip, could also promote reverse-turn formation. Experimental confirmations of the predicted reverse-turn nucleation properties of D-Pro-L-Pro[61] and (*R*)-Nip-(*S*)-Nip[59,60] are available from two different systems, one using α-amino acids and the other using β-amino acids, respectively. The availability of chimeric amino acid analogues[62−73] of proline, azaproline (azPro), pipecolic acid, azapipecolic acid (azPip), nipecotic acid, and isonipecotic acid (Inp) (Figure 1) where side chain functional groups have been stereoselectively attached to the rings suggest the preparation of chimeric analogues of D-Pro-L-Pro and (*R*)-Nip-(*S*)-Nip and other heterochiral dipeptide analogues that contain side-chain functional groups appended at all four positions in the turn.

This study evaluates the potential of a combined approach, CTPs based on heterochiral dipeptides of chimeric amino acids as novel conformational templates (Scheme 1), for instance, *cyclo*-(D-Pro-L-Pro-D-Pro-L-Pro), because synthetic routes to chimeric prolines containing 2-, 3-, 4-, or 5-substitutents are abundant. From our point of view, heterochiral dipeptide-based CTPs are excellent candidates for conformational templates because they possess both the required limited conformational flexibility of their peptide backbone and a great diversity of rigidified spatial combinations of side chains. First, they are expected to be relatively conformationally rigid due to the small 12-membered ring and exocyclic constraints. There are only two mirror images of cycloenantiomeric backbone (cis-trans-cis-trans (ctct) or trans-cis-trans-cis (tctc)) conformations ob-

Scheme 1. CTP Scaffolds Based on Heterochiral Dipeptides of Chimeric Amino Acids



served experimentally for *cyclo*-(D-Pro-L-Pro-D-Pro-L-Pro).[74] Interconversion between the two isomers was only detectable at 80 °C in H_2O and 45 °C in DMF.[75] In the case of *cyclo*-(D-Pro-L-Pro(4-OH)-D-Pro-L-Pro(4-OH)), only one (tctc) was produced upon cyclization of the linear tetrapeptide.[76] The substitution of the two chiral hydroxyl groups renders the two conformations (ctct or tctc) of the cyclic compound diastereomeric, which provides a basis for the single conformer seen in solution. Interconversion to the other cycloenantiomer was not observed upon heating. Second, derivatives can be prepared readily by solid-phase synthesis[74] or by a convergent solution route[76] with a yield of 85% during the final cyclization. Third, the presence of four potentially functionalized and stereochemically controlled centers (α, β, γ, and δ carbons) on each proline ring offers seven hydrogens for side-chain replacement, providing an overwhelming diversity to custom design molecules to generate and test a pharmacophore model. In addition to proline, similar constrained amino acids, including azaproline and pipecolic, azapipecolic, nipecotic, and isonipecotic acids, etc., can further tailor structural and functional diversity. An additional advantage of these CTP scaffolds is their stability to proteolytic cleavage due to their cyclic constraint and exocyclic rings (most proteolytic enzymes do not cleave adjacent to proline residues or require access to linear segments of the peptide backbone[77]) offering potential as oral therapeutics. The main goal of this study is to examine the conformational preference of CTP scaffolds and to explore the structural and functional diversity by engineering heterochiral dipeptides and appending desired substituents at selected positions. Molecular libraries based on CTP scaffolds can be used to design novel compounds that are able to align the amino acid side chains in diverse orientations. These geometrical probes of the recognition motif can then be used to determine the three-dimensional requirements for molecular recognition.

Methods

Structural Analysis of Cyclic Peptides. A total of 274 cyclic peptides containing two to ten residues were comprehensively analyzed from the structural data retrieved from the Cambridge Structure Database (CSD version 5.24, November 2002) for analyzing the distribution of cis/trans amide bonds and Φ and Ψ backbone torsional angles.

Conformational Analysis. The potential surfaces of CTP scaffolds were first explored using a normal-mode local-search method,[78] followed by a Monte Carlo multiple-minimum (MCMM)[79] search to ensure that the whole conformational space was covered. Three different all-atom force fields (AMBER, CHARMM, and OPLS-AA) were used in this study for comparison purposes. The GB/SA implicit-solvation models for water and chloroform were applied for solution-phase studies. The normal-mode local searches were performed with TINKER, version 3.9, and the MCMM searches with MacroModel, version 7.2.

The geometrical requirement of CTP scaffolds for nonplanar amide bonds requires a change in the hybridization of the amide

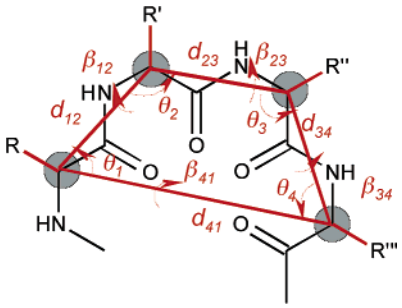


Figure 2. Schematic of a tetrapeptide showing the distances and the angles between C_α-atoms and the torsions between $C_\alpha-C_\beta$ vectors used to characterize side-chain orientations.

and suggests the use of quantum chemistry rather than molecular mechanics parametrized for planar amide systems. Molecular geometries of different backbone conformations were optimized using density functional theory (DFT) at the B3LYP/6-31G* level of theory with Gaussian03. The solvation effects on conformational preferences were estimated implicitly with the polarizable continuum model (PCM) in chloroform and water.

Probable interconversion pathways between different backbone isomers were addressed with a high-temperature molecular dynamics (MD) simulation and a variant of Elber's Lagrangian multiplier-based reaction path following algorithm.[80] The MD simulation was carried out at 1000 K in chloroform with the AMBER all-atom force field. It was based on the assumption that the conversion pathway for such constrained molecules without intramolecular hydrogen bonds should be similar regardless of the temperature and the solvent, which might accelerate the conformational transition rate. The simulation was performed with a time step of 0.5 fs for 10 ns, with samples taken at 0.1 ps intervals yielding 10 000 conformations for analysis. The Elber's path following calculation was used to locate a series of structures equally spaced along a conformational pathway connecting two initial input structures. A series of constrained optimizations orthogonal to the path was done via Lagrangian multipliers. Both calculations were performed using TINKER (http://dasher.wustl.edu/, version 3.9).

Structural Diversity. To quantify structural diversity and information content of CTP scaffolds, the spatial relationships (distances, angles, and virtual torsion angles, Figure 2) of $C_\alpha-C_\beta$ vectors of all tetrapeptide sequences found in a representative list of crystal structures of proteins (PDB_SELECT,[81] December 2003, with no protein chain pair of more than 25% sequence identity) were subjected to statistical analysis (PCA). The PCA model was based on approximately 100 000 tetrapeptide sequences extracted from the protein structures in PDB_SELECT,[81] December 2003. A similar analysis was carried out on CTP scaffolds with different patterns of substitutions as new sets of virtual $C_\alpha-C_\beta$ vectors besides those existing C−C or C−N bonds on the proline ring. The PCA analysis offers an easy way to visualize the structural diversity and to compare CTP scaffolds with protein structural epitopes. The structural diversity analysis was performed with SHAPES, molecular-design software based on the "privileged-structure hypothesis" − *any organic scaffolds mimicking protein surface* structures are potential privileged structures as protein-complex antagonists.[82]

Results and Discussion

Structural Analysis of Cyclic Peptides. The distribution of cis/trans amide bonds and $\Phi-\Psi$ backbone torsions are shown in Figures 3 and 4. A list of crystal and solution structures of representative CTPs is summarized in Table 1.

Analysis of crystal structures of cyclic peptides available in CSD revealed that cis amide bonds are mandatory for cyclization of both di- and tripeptides, while trans amide bonds are predominately found in cyclic pentapeptides or longer. CTPs often contain cis amide bonds, but they are not mandatory. This

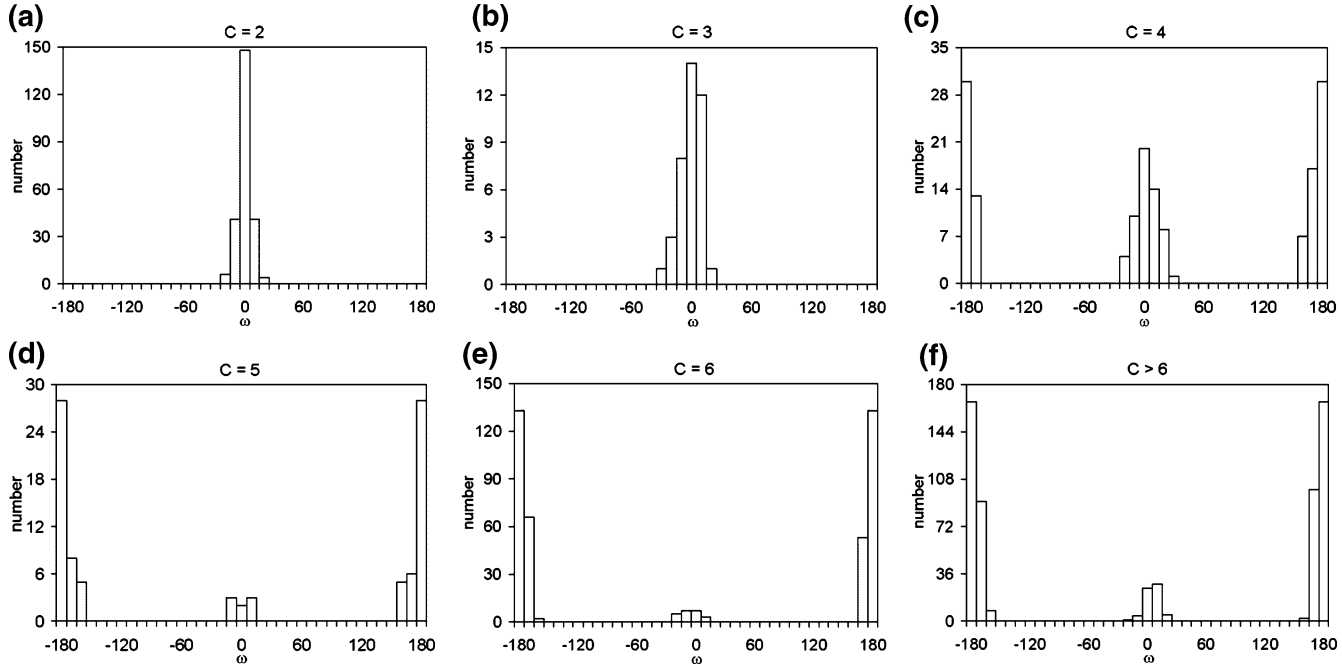


Figure 3. The distribution of cis/trans amide bonds for cyclic peptides retrieved from CSD (C = number of residues in a cyclic peptide): (a) 120 cyclic dipeptides with exclusively 240 cis amide bonds (100%); (b) 14 cyclic tripeptides with exclusively 42 cis amide bonds (100%); (c) 31 cyclic tetrapeptides with 57 cis amide bonds (46%) and 67 trans amide bonds (54%); (d) 12 cyclic pentapeptides with 8 cis amide bonds (13%) and 52 trans amide bonds (87%); (e) 46 cyclic hexapeptides with 22 cis amide bonds (8%) and 254 trans amide bonds (92%); (f) 51 longer cyclic peptides with 63 cis amide bonds (14%) and 379 trans amide bonds (86%).

observation corroborates early computational studies on cyclic tripeptides[83] requiring all cis amide bonds, and ones that showed that CTPs could be formed with all trans amide bonds only by slight violation of rigid-valence geometry for planar amide bonds.[84,85] In fact, the presence of any trans amide bonds in CTPs, readily observed in CSD, allows CTPs to be employed for designing rigid scaffolds of systems where molecular recognition is associated with normal reverse-turn motifs. For any CTP, it is possible to define six types of backbone conformers: all-trans amides (tttt); three trans, one cis amides (tttc, etc.); two trans amides and two cis amides in alternative order (ctct or tctc); two trans amides and two cis amides in sequential order (ttcc, etc.); one trans, three cis amides (tccc, etc.); all-cis amides (cccc). Except the ttcc-amide conformer, all five other types have been observed in crystal structures or in solutions. Clearly, compared to other types of cyclic peptide, CTP possesses more diverse backbone conformations in terms of amide-bond conformations. The exact conformation of its amide bonds can be affected by many factors, including the nature of amino acids (steric bulk of side chains, L- or D-configuration, etc.), the presence of N-methylation and intramolecular hydrogen bonds, solvent conditions, etc. Obviously, CTPs are much more flexible in terms of possible regions of torsion angles, Φ and Ψ, than di- and tripeptides where geometrical constraints require all cis amide bonds. The observed distribution of Φ and Ψ in CTPs is closer to those seen with linear peptides or proteins. In addition, our earlier theoretical and experimental studies[4] have demonstrated a considerable degree of conformational averaging in NMR studies of cyclic pentapeptides advocated as receptor probes. By inclusion of additional exocyclic rings and control of possible intramolecular hydrogen bonds by N-substitution, as seen in *cyclo*-(D-Pro-L-Pro-D-Pro-L-Pro), conformational averaging of CTPs is reduced to a manageable level with significant activation barriers between different conformers.

Conformational Analysis of *cyclo*-(D-Pro-L-Pro-D-Pro-L-Pro). Molecular modeling of any system requires the availability of appropriate parameters for the force field of choice and validation of the calculations by comparison with known thermodynamic measurements. The initial studies by Chalmers and Marshall[26] of heterochiral proline dipeptides used MacroModel with the GB/SA solvation model and a number of different force fields. As a control for that study, the relative stabilities of low-energy conformers of *cyclo*-(D-Pro-L-Pro-D-Pro-L-Pro) were determined. The original AMBER* parameter found a conformer containing ttcc amide bonds, not observed in any crystal or solution structures of CTPs, which was at least 4.9 kcal/mol more stable than the conformer ctct or tctc observed experimentally. This observation provoked McDonald and Still[99] to reparametrize the secondary amide parameters in MacroModel, and an excellent study showed the ability of this new force field to reproduce a variety of experimental studies.

Here, the entire potential surface was explored using the normal-model local search and the MCMM protocol with three different force fields. Generally speaking, except the ttcc amide conformer, all other five types of backbone conformations, tttt, tttc, tctc, tccc, and cccc, were found within 20 kcal/mol of the global minimum. The small size of rigid CTP scaffolds and the nonideal planar amide bonds suggest the use of DFT for more accurate geometries and energies rather than force fields normally parametrized for planar amides. All methods agreed that the ctct and tctc amide conformers, observed in crystal structures, were the most stable in water due to a large molecular dipole moment, while the all-trans amide conformer with a minimal dipole was significantly more stable in vacuo, though the magnitudes were different depending upon the methods (Table 2). DFT-optimized structures of *cyclo*-(D-Pro-L-Pro-D-Pro-L-Pro), including tttt, tttc, tctc, tccc, and cccc amide conformations, are illustrated in Figure 5, and backbone geometries and dipole moments are summarized in Table 3.

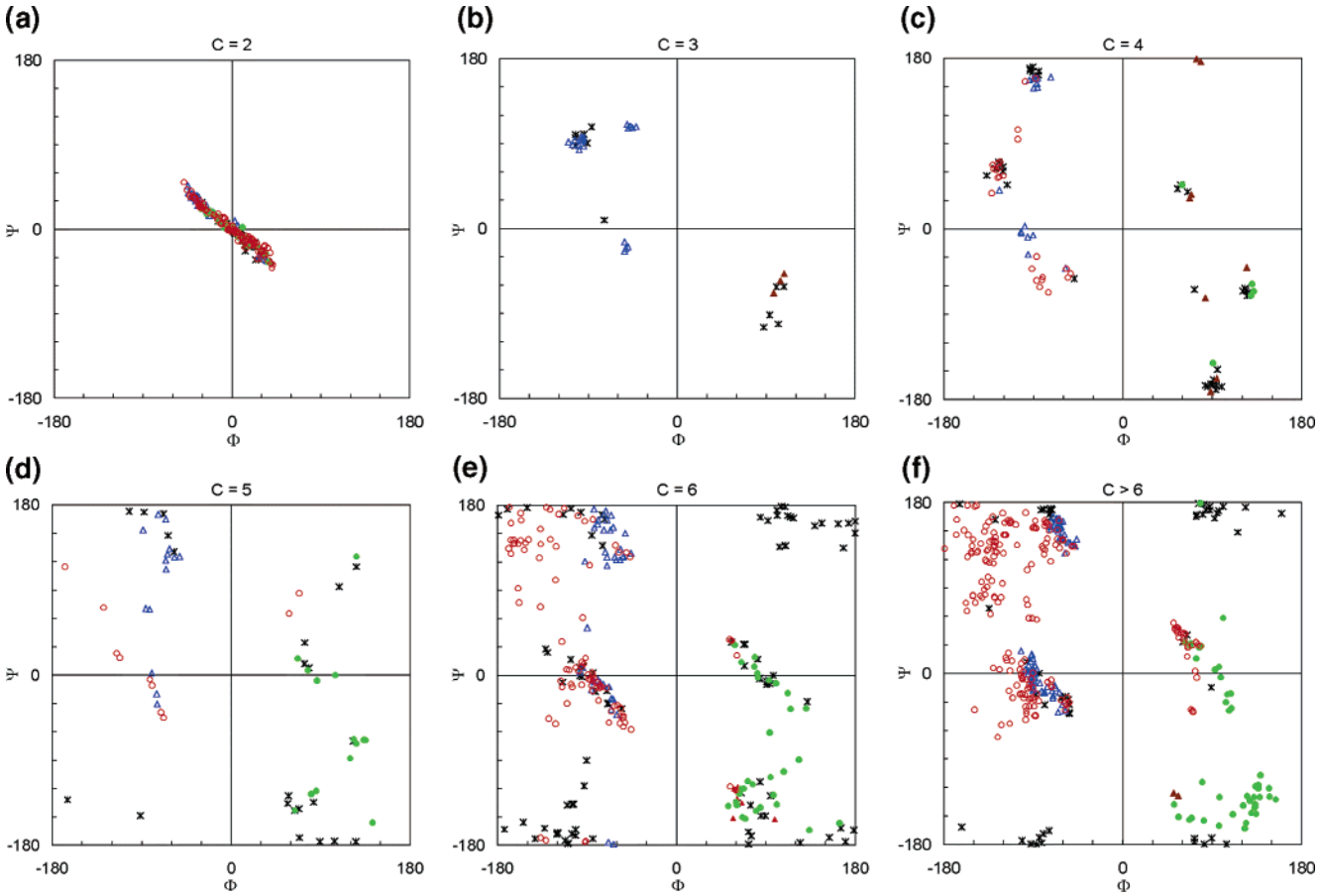


Figure 4. The distribution of Φ and Ψ torsion angles for cyclic peptides retrieved from CSD (*C* = number of residues in a cyclic peptide): (a) 120 cyclic dipeptides; (b) 14 cyclic tripeptides; (c) 31 cyclic tetrapeptides; (d) 12 cyclic pentapeptides; (e) 46 cyclic hexapeptides; (f) 51 longer cyclic peptides; (∗) glycine and other residues without C_α chiral center; (△) L-proline and other L-type N-methylated amino acid derivatives; (▲) D-proline and other D-type N-methylated amino acid derivatives; (○) other L-type residues; (●) other D-type residues.

Table 1. Crystal and Solution Structures of Representative CTPs

CTP[a]	exptl methods	ω
c(D-Pro-L-Pro-D-Pro-L-Pro)[74]	X-ray	ctct/tctc, 50:50
c(D-Pro-D-Pro-L-Pro-L-Pro)[86]	X-ray	ctct
	NMR in TFE	ctct/ccct, 86:14
c(L-Pro-Gly-L-Pro-Gly)[87]	X-ray	tctc
c(L-Pro-Sar-L-Pro-Sar)[88]	X-ray	all-cis
c(L-Pro-L-Val-L-Pro-L-Val)[89,90]	X-ray	tctc
	NMR in $CDCl_3$	tctc
c(L-Pro-D-Val-L-Pro-D-Val)[90]	NMR in DMSO	tctc
	NMR in TFE	all-trans
c(L-Pro-D-Phe-L-Pro-D-Phe)[91]	NMR in $CDCl_3$	all-trans
c(L-Pro-L-Phe-L-Pro-L-Ala)[92]	X-ray	tctc
c(L-Pro-D-Phe-L-Pro-L-Ala)[92]	X-ray	tctc
c(Gly-L-Phe-D-Pro-L-Ala)[91]	NMR in $CDCl_3$	all-trans
c(Aib-L-Phe-D-Pro-L-Aoe)[93]	X-ray	all-trans
c(D-Pro-L-Ala-D-Ala-L-Aoe)[94]	NMR in $CDCl_3$	all-trans
c(L-Phe-L-Phe-D-Pip-L-Aoe)[95]	X-ray	tctt
c(Gly-Gly-Gly-Gly)[96]	NMR in DMSO or TFA	all-trans
c(L-Ala-L-Ala-L-Ala-L-Ala)[97]	NMR in $CDCl_3$	(ctct + tctc)/tttt, 76:24
c(Sar-Sar-Sar-Sar)[98]	X-ray	ctct and tctc

[a] Aib, α-aminoisobutyric acid; Aoe, 2-amino-8-oxo-9,10-epoxydecanoic acid; Sar, Sarcosine (*N*-methyl glycine).

Table 2. Energy Differences (kcal/mol) Compared to the All-Trans Amide Conformer, of *cyclo*-(D-Pro-L-Pro-D-Pro-L-Pro) in Vacuo, in Chloroform, and in Water Using DFT and Three Different Force Fields

	B3LYP/6-31G*	AMBER	CHARMM	OPLS-AA
		In Vacuo		
ΔE(cttt − tttt)	8.68	5.44	8.96	9.03
ΔE(ctct − tttt)	7.67	6.04	10.98	9.47
ΔE(tccc − tttt)	11.39	14.19	17.39	26.38
ΔE(cccc − tttt)	9.20	7.47	12.03	23.19
		Chloroform ($\epsilon = 4.9$)		
ΔE(cttt − tttt)	4.01	2.83		7.23
ΔE(ctct − tttt)	−0.33	0.00		5.51
ΔE(tccc − tttt)	5.89	12.02		25.06
ΔE(cccc − tttt)	4.68	5.89		22.61
		Water ($\epsilon = 78.39$)		
ΔE(cttt − tttt)	1.34	−0.17	5.05	5.12
ΔE(ctct − tttt)	−5.33	−8.45	−1.19	−0.73
ΔE(tccc − tttt)	3.53	9.33	16.80	23.52
ΔE(cccc − tttt)	2.66	0.42	12.55	18.24

The DFT results for the ctct and tctc amide conformers were in excellent accord with the experimental values from X-ray crystallography. The root-mean-square displacement (RMSD) of all atoms between the crystal structure and the DFT result was only 0.037 Å. Several conformational characteristics of particular interest are briefly discussed in the following. (1) The all-trans amide conformer is the most stable in vacuo, 7.67 kcal/mol more favorable than either the ctct or tctc amide conformer. The all-trans isomer adopts a highly symmetrical (S_4) conformation and is mainly stabilized by intramolecular dipole interactions, which replace the intramolecular hydrogen bonds formed in the γ-turns of similar cyclic peptides, such as those seen in the crystal structure of chlamydocin (*cyclo*-(Aib-L-Phe-D-Pro-L-Aoe)) derivatives (Aib = α-aminoisobutyric acid; Aoe = 2-amino-8-oxo-9,10-epoxydecanoic acid)[93] and solution structures of *cyclo*-(L-Pro-D-Val-L-Pro-D-Val)[89] and *cyclo*-(L-Pro-D-

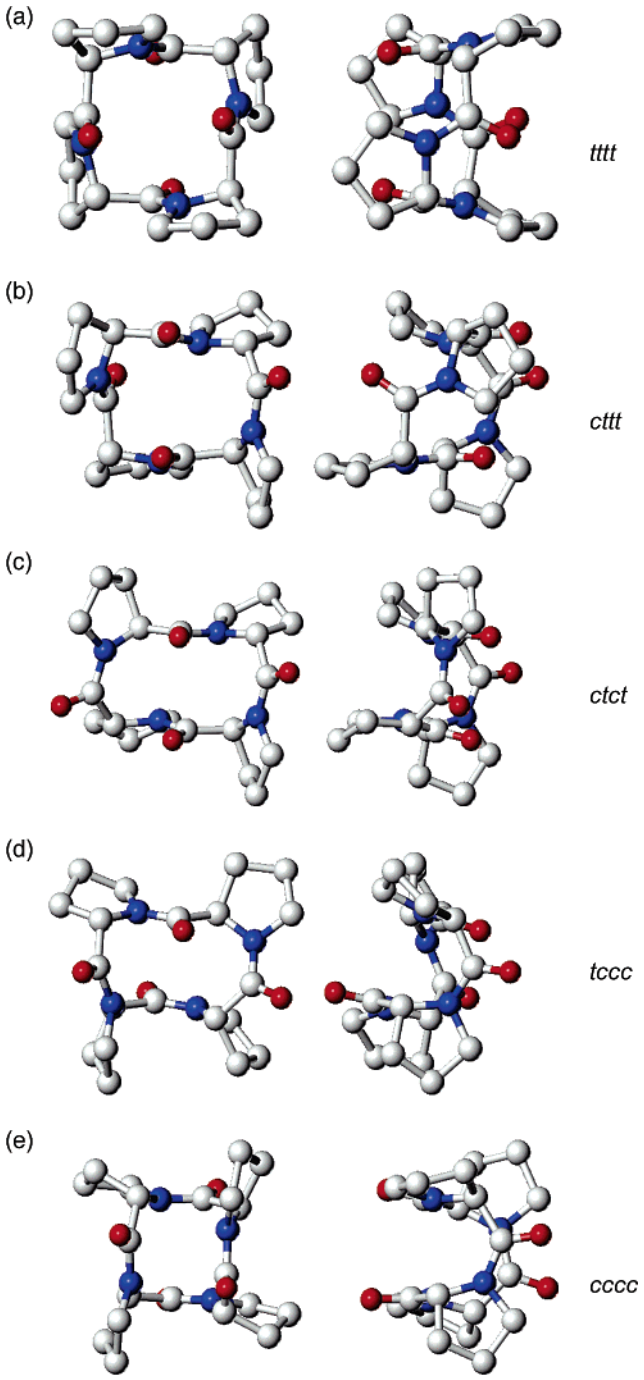


Figure 5. Orthogonal views of DFT-optimized structures of cyclo-(D-Pro-L-Pro-D-Pro-L-Pro): (a) all-trans, (b) cttt, (c) ctct, (d) tccc, and (e) all-cis amide conformations. Hydrogen atoms are omitted for clarity.

Phe-L-Pro-D-Phe).[90] In the all-trans amide conformer, the peptide carbonyl groups are arranged up−down−up−down with regard to the mean plane of the macrocycle ring with minimal total dipole moment but greater intramolecular dipole−dipole interactions. All four amide bonds are nonplanar ($\omega \approx 160°$) due to the constrained cyclic tetrapeptide geometry. (2) The nonsuperimposable mirror-image conformers, ctct and tctc, are strongly stabilized in water, 5.33 kcal/mol more favorable than the all-trans conformer. Even in chloroform, a low-dielectric solvent ($\epsilon = 4.9$), the ctct or tctc amide conformer is still slightly favored by 0.33 kcal/mol. This solvent-dependent conformational preference is largely due to the significant difference in dipole moments between the all-trans and the ctct or tctc amide conformations. In the cis−trans amide alternating conformation, two proline rings opposite the mean plane of the macrocycle ring are parallel and nearly perpendicular to the plane of the 12-membered macrocycle. The other two proline rings are approximately parallel to the plane of the macrocycle. Comparing the two nonsuperimposable cycloenantiomers, ctct and tctc, one can see that the proline rings change their relative orientations with amide bond isomerization (Figure 6a). In either amide conformer, all four peptide carbonyls are approximately perpendicular to the plane of the macrocycle and face the same direction, which results in a large dipole moment, $\mu > 10$ D. The four proline rings are aligned on the other face of the hydrophobic surface, where functional substitutions will be placed. It may be noted that among all possible amide conformers for CTPs, ctct or tctc is the most abundant conformation. However, our results suggested that such conformational preference was mainly due to the orientation of amide bonds instead of the ring strain of the 12-membered macrocycle. (3) The all-cis amide conformer is much less stable compared to the all-trans or cis−trans alternating conformation either in vacuo or in water. One notable result from the DFT calculations is that the energy difference between cccc and tttt amide conformers is largely reduced from 9.20 kcal/mol in vacuo to 2.66 kcal/mol in water, even though both conformers have an S_4 symmetry with minimal dipole moment. This may partly reflect the fact that the cccc amide conformer has a smaller macrocycle ring, in which neighboring peptide carbonyl groups move further way in the opposite direction resulting a larger charge separation. Such a conformation is usually more favored in water. The possibility of an all-cis amide conformation in CTPs was predicted as early as 1952 by Pauling and Corey[100] from an analysis of molecular models and later had been observed in the crystal structure of *cyclo*-(L-Pro-Sar-L-Pro-Sar) (Sar = sarcosine (*N*-methyl glycine)).[88] Similar to the tttt conformer, all four amide bonds in the cccc amide isomer deviate from planar geometry ($\omega \approx 18°$). (4) The cttt amide conformer has a moderate dipole moment. It is only slightly favored compared to the cccc conformer, either in vacuo or in water. This backbone conformation is rare in crystal structures. So far, it has been only seen in the crystal structure of *cyclo*-(L-Phe-L-Phe-D-Pip-L-Aoe), with a cis amide bond before the pipecolic acid residue. The cttt amide isomer has been observed more often within solvent-dependent equilibria between tttt and ctct conformers of DLDL-configured sequences.[91,101] The tccc amide conformer is even less stable. It has never been observed in crystal structures and so far only been estimated to contribute 14% population of *cyclo*-(D-Pro-D-Pro-L-Pro-L-Pro).[86] The cctt amide conformer has never been confirmed experimentally, neither in crystal nor in solution structures. (5) The results from force fields are all in reasonable agreement that the tttt amide conformer is the most stable in vacuo and the ctct or tctc isomer is more favored in water. Overall, the DFT results favor AMBER as having the more compatible substituted-amide parameters. This led us to use AMBER for qualitative evaluation of conformational conversion between ctct and tctc.

One notable experimental discovery was that *cyclo*-(D-Pro-L-Pro-D-Pro-L-Pro) did not crystallize in the racemic form but as enantiomerically pure crystals that could be separated.[74] This allowed the interconversion between ctct and tctc amide conformers to be studied by optical rotatory dispersion (ORD) and circular dichroism (CD) with interconversion only detectable at 80 °C in water and 45 °C in DMF.[75] A probable transition path with a series of four cis−trans amide isomerizations, ctct → cttt → tttt → tctt → tctc, was proposed based on Elber's reaction path algorithm, as shown in Figure 6b, which is similar

Table 3. Backbone Geometries (Angles, deg) and Dipole Moments (D) for Different Conformations of *cyclo*-(D-Pro-L-Pro-D-Pro-L-Pro)

	Φ_1	Ψ_1	ω_{12}	Φ_2	Ψ_2	ω_{23}	Φ_3	Ψ_3	ω_{34}	Φ_4	Ψ_4	ω_{41}	μ
						Crystal							
tctc	90	−153	−174	−67	−32	−3	87	−149	−177	−68	−36	−4	
						DFT (B3LYP/6-31G*)							
tttt	82	−97	161	−82	97	−161	82	−97	161	−82	97	−161	0.0
cttt	73	48	1	−88	161	−162	76	−94	152	−91	76	178	6.3
ctct	68	34	−5	−86	149	176	68	34	−5	−86	149	176	10.2
tctc	86	−149	−176	−68	−34	5	86	−149	−176	−68	−34	5	10.2
tccc	39	−153	177	−58	−33	−28	103	24	−8	−124	23	30	5.1
cccc	89	16	18	−89	−16	−18	89	16	18	−89	−16	−18	0.0

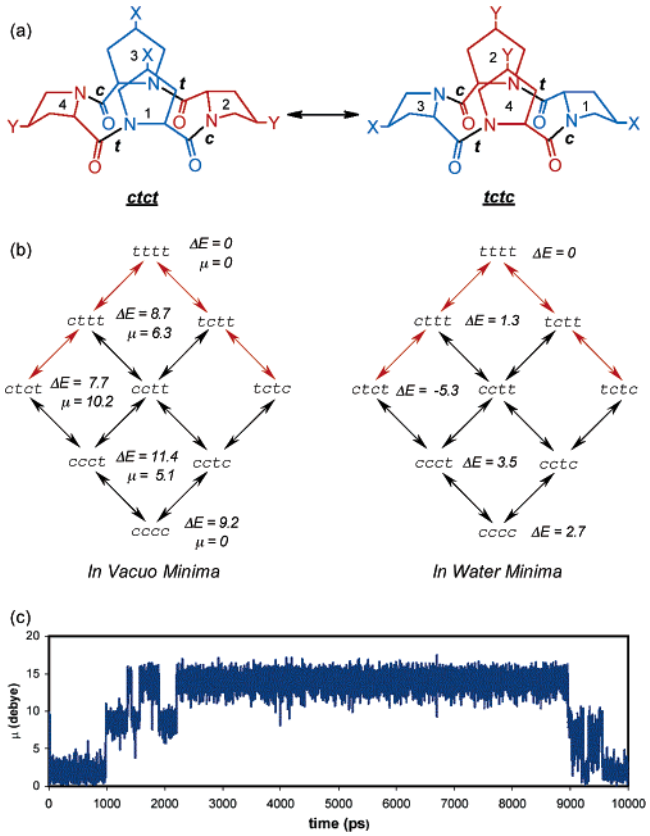


Figure 6. Conformational interconversion of *cyclo*-(D-Pro-L-Pro-D-Pro-L-Pro): (a) the interconversion between the ctct and tctc amide conformers; (b) DFT energies and dipole moments for different amide-bond conformers (red lines indicate probable transition paths based on Elber's path algorithm); (c) the MD trajectory simulated at 1000 K using the AMBER force field with the GB/SA solvation model for chloroform.

to a previous study[75] based on the adiabatic mapping method. The interconversion pathway was confirmed by MD simulation, which was performed in chloroform at $T = 1000$ K. Since the conformational energies of the tttt and ctct amide conformers are similar in chloroform with the AMBER force field, the simulation can sample the two conformations more equally. The conversion paths should be similar to those happening in water because the molecule has a constrained structure without any intramolecular hydrogen bond that might compete for hydrogen bonding with water molecules. Due to the large differences in dipole moments among different conformers, the dipole moment can be used to monitor conformational transitions (Figure 6c). According to the magnitude of dipole moments, the conformations can be divided into three groups: (a) the tttt and cccc amide conformers with minimal dipole moments ($\mu < 5$ D), (b) the ctct and tctc amide conformers with large total dipole moments ($\mu > 10$ D), and (c) the cttt and tccc amide conformers with intermediate dipole moments ($5 < \mu < 10$ D). The simulation trajectory clearly indicated that the transition between the tttt and ctct amide conformers is via a structure with an intermediate dipole moment, cttt in this case due to its relatively lower energy compared to that of tccc. Another notable observation from the simulation was that during the amide isomerization, the peptide carbonyl groups move up on the outside, not through the center, of the molecule to be positioned on the other face of the molecules. This observation is contrary to the previous hypothesis[76] that the peptide carbonyl groups move up through the center of the molecules during the amide isomerization, which likely requires more energy due to increased steric interactions.

Substitutions on Proline Rings. Chiral substitutions on proline residues can exert a selective effect between the two cycloenantiomers ctct and tctc in water. Previously, the Gilbertson group determined the solution conformation of *cyclo*-(D-Pro-L-Pro(4-OH)-D-Pro-L-Pro(4-OH)); only one cycloenantiomer (tctc) was produced upon cyclization of the linear tetrapeptide.[76] Here, we tried to deconvolute the relative contributions of steric and electrostatic factors through similar DFT calculations on single and double substitutions on proline residues. Substitutions of methyl groups on different positions of proline residues provide the basis for elucidating steric factors, and substitutions with hydroxyl groups can be used to evaluate the contribution of electrostatic factors, such as dipolar interactions with hydroxyl groups, impact on solvation, etc. The energy differences between those ctct and tctc amide conformers of single substitutions of either methyl groups or hydroxyl groups, as in *cyclo*-(D-Pro(X)-L-Pro-D-Pro-L-Pro), are summarized in Figure 7.

The influence of methyl substitutions on amide-bond conformations can be explained from the impact of steric effects on the cis−trans isomerism of the preceding amide bond. Because the steric properties of proline due to disubstitution on the α-nitrogen are quite similar in either cis or trans conformation, molecules that contain prolines are known to exist in a conformational equilibrium due to the cis−trans amide isomerism. The incorporation of a methyl substituent has a direct influence on this equilibrium due to the steric effect exerted by the extra methyl group, particularly with the 2- and 5-substitutions. The incorporation of 2-methyl proline shifts the equilibrium to the trans conformation, and the resulting scaffold strongly favors the ctct conformer, which is 9.73 kcal/mol more stable than the corresponding tctc amide isomer. In contrast, the introduction of a single substituent at the proline 5-position augments the populations of the cis amide bond, and this favors the scaffold in the tctc amide conformation by 3.8−4.8 kcal/mol. Monosubstitution at the proline 3- or 4-position has small direct effects on the cis−trans amide isomerism. The conformational preference of the scaffold depends on whether the substituent is axial or equatorial to the 12-membered macrocycle. The calculations have shown that the amide conformer with

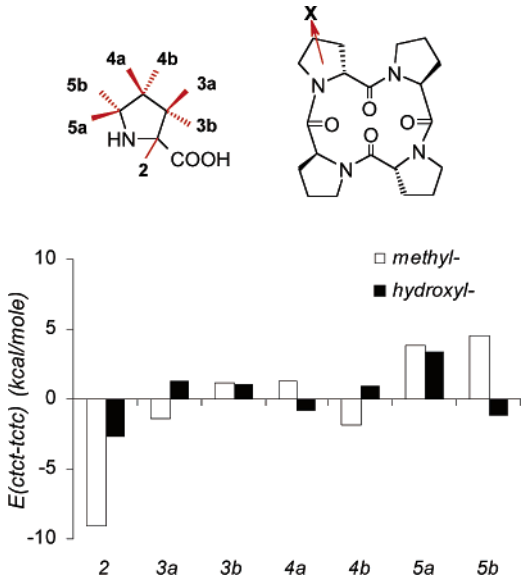


Figure 7. Energy differences between ctct and tctc amide conformers of single substitutions for *cyclo*-(D-Pro(X)-L-Pro-D-Pro-L-Pro) calculated using DFT (B3LYP/6-31G*) with the PCM water model.

the methyl group in an equatorial position is more favored. So substitution at the proline 3a- and 4b-positions favors the ctct, and 3b- and 4a-substitutions prefer the tctc conformer.

The hydroxyl-substituent exerts less steric effect compared to the methyl group due to a smaller size. For instance, 2-hydroxyl proline stabilizes the ctct amide conformer by only 2.8 kcal/mol. On the other hand, the substituent on the axial site can augment the total dipole moment, and the equatorial substituent may reduce it. This effect reverses the steric impact on the conformational preference of the scaffold. For example, the hydroxyl substitution at the proline 4a-position increases the dipole moment to 11.2 D for the ctct amide conformer and reduces it to 9.1 D for the tctc isomer. This allows the ctct amide conformer to be more favored in water. Among the seven hydroxyl substitutions, four of them reverse the preference compared to those of methyl substitutions.

We reexamined the conformational preference of *cyclo*-(D-Pro-L-Pro(4-OH)-D-Pro-L-Pro(4-OH)) in water using DFT calculations. The results indicated that the substitution of the two chiral side-chain hydroxyl groups both at the 4*R*-position could further augment the dipole moment to 12.2 D for the tctc amide conformer, which was 1.8 kcal/mol more stable than the ctct amide isomer with a dipole moment of only 7.8 D. These results are consistent with the experimental observation by Gilbertson and Pawlick[76] that only the tctc amide conformer of *cyclo*-(D-Pro-L-Pro(4-OH)-D-Pro-L-Pro(4-OH)) was found in solution. Similarly, substitution of two chiral hydroxyl side-chain groups at the proline 4*S*-position would be expected to favor the ctct amide conformer instead of the tctc isomer.

Structural Diversity. Molecular diversity of proteins and peptides can be described in terms of structural diversity (relative orientation of the side chains) and functional diversity (types of side chains). These orientations are defined by the $C_\alpha-C_\beta$ bonds that link the side chain to the backbone. This split of molecular diversity can also be applied to conformational templates, which are merely there to control relative orientation of the functional substitutions. Different patterns of substitutions on CTP scaffolds offer an overwhelming diversity of side-chain orientations, since there are 28 hydrogen sites on the cyclic tetraproline scaffold that could be chirality substituted. Considering only single substitutions of each proline ring, $7^4 = 2401$ unique compounds are possible, all with similar molecular properties due to the same molecular weight and same type of functional groups but with different orientations of the substituents. This gives the chemist plenty of scope to custom design molecules to fit a pharmacophoric model and leads to rapid identification of geometrical requirements from compounds active in screening.

Table 4. Proportion of Variance Explained by the PCA Model for Tetrapeptides in Protein Structures[a] and Coefficients and Loadings of Fields to the PCA Model

(a) Proportion of Variance Explained

comp 1	comp 2	comp 3	comp 4	comp 5	comp 6
0.602	0.103	0.077	0.071	0.069	0.023
comp 7	comp 8	comp 9	comp 10	comp 11	comp 12
0.020	0.017	0.012	0.007	0.001	0.001

(b) Coefficients and Loadings of Fields

	comp 1		comp 2	
	norm coeff	loading	norm coeff	loading
d_{12}	0.067	−0.072	0.453	0.613
d_{23}	0.075	−0.086	0.491	0.669
d_{34}	0.075	−0.067	0.484	0.664
d_{41}	0.141	0.976	−0.006	−0.163
θ_1	−0.137	−0.959	0.010	0.163
θ_2	0.124	0.903	−0.039	−0.193
θ_3	0.123	0.890	−0.035	−0.195
θ_4	−0.137	−0.952	0.010	0.166
β_{12}	0.144	0.844	0.126	−0.007
β_{23}	0.127	0.903	−0.012	−0.170
β_{34}	0.133	0.894	0.017	−0.128
β_{41}	−0.108	−0.496	−0.204	−0.120

[a] The optimum number of principal components is two, based on the Kaiser criterion.[102]

To quantify structural diversity and estimate the information content of conformational templates, factor (principal component) analysis, PCA, was used to analyze the spatial relationships of $C_\alpha-C_\beta$ vectors of tetrapeptide sequences. The PCA model, which is based on approximately 100 000 tetrapeptide sequences, is summarized in Table 4. The scorings of the first two principal components, which account for 70% of the total variation in data, are plotted in Figure 8. The figure also illustrates the comparison of the scaffold with the diverse tetrapeptide set above. This analysis gives a general protocol to assess how well a conformational template can mimic various protein conformations.

The PCA analysis yielded two optimal principal components, according to a conservative statistical rule of thumb that attributes significance only to those components having eigenvalues greater than 1.0, that is, those explaining at least as much of the overall data variance as did one of the original variables (Kaiser criterion[102]). The first component, having an eigenvalue of 7.22, will reproduce 60.2% of the original variance. Its combination with the second component, eigenvalue = 1.24, will reproduce 70.5% of the original variance. The following three components with eigenvalues (0.83−0.92) slightly less than one, together span 21.7% of the overall original variance. The first component is highly correlated with the distance between $C_{\alpha1}$ and $C_{\alpha4}$, the angles around $C_{\alpha2}$ and $C_{\alpha3}$, the virtual torsions between adjacent $C_\alpha-C_\beta$ vectors (large positive loadings, >0.90), and the angles around $C_{\alpha1}$ and $C_{\alpha4}$ (large negative loadings, <-0.95). Instead, the second component is more correlated with the distances between adjacent α-carbons. This result suggests to us that the first component describes the compactness of tetrapeptide motifs, because the α-helices (the most compact secondary structure) clustered to the left with

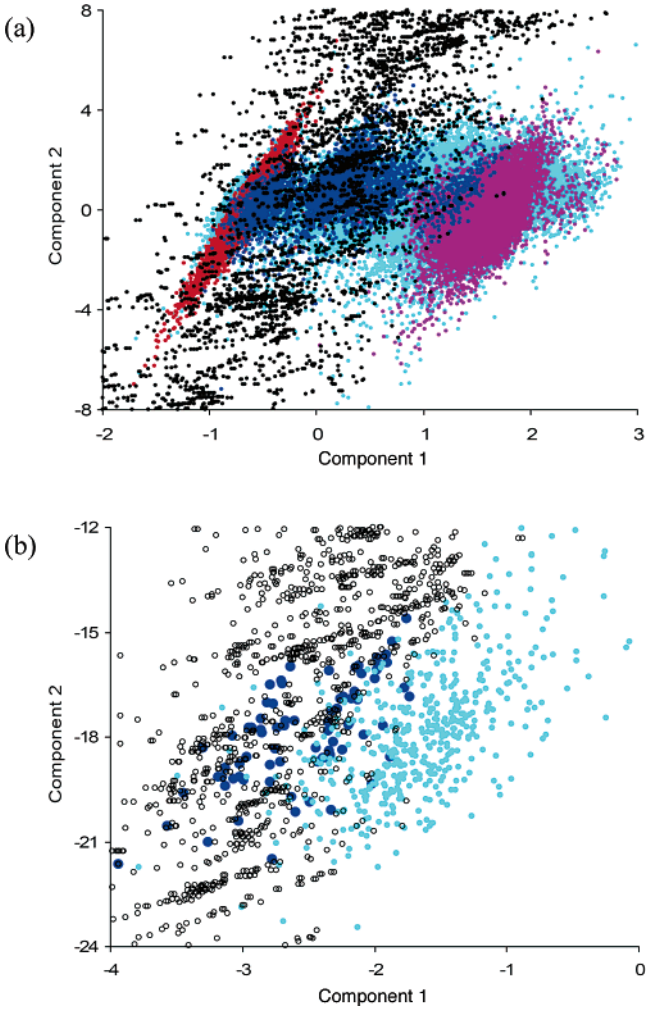


Figure 8. Comparison of structural diversity accessible through peptide backbone or cyclic tetraprolines. Each point represents a set of four sequential $C_\alpha-C_\beta$ vectors in peptides or those vectors derived from appending substituents to a scaffold (α-helix in red, β-sheet in magenta, turn in blue, nonregular motif in turquoise, and *cyc*Pro_4 scaffolds in black). The window a, $-2 <$ comp1 < 3 and $-8 <$ comp2 < 8, covers mainly all-trans amide tetrapeptides, and the window b, $-4 <$ comp1 < 0 and $-24 <$ comp2 < -12, includes primarily tetrapeptide sequences with at least one cis amide bond, such as type VI β-turn.

smaller scorings of the first component, the β-sheets (the most extended secondary structure) clustered to the right, and the turns in the middle. On the other hand, the second component describes the local geometry of tetrapeptides, such as cis or trans amide bonds. For example, in the scatter plot, the type VI β-turns with large negative scorings of the second component were clearly separated from other motifs. The PCA analysis also revealed another noticeable observation. Unlike the regular structural motifs, α-helices or β-sheets, which can be grouped into single clusters, reverse turns are distributed among several sets due to their nonrepetitive nature in backbone torsional angles, which results in several subtypes. This also suggests that reverse turns can be classified based solely on side-chain orientations, which is more useful for the development and application of β-turn mimetics, rather than the traditional backbone dihedral angles. Furthermore, this also reflects the different roles of secondary structural motifs in mediating protein−protein recognitions.[82] The α-helix is the most abundant regular conformation and has often been used as a common motif to recognize protein-interaction "hubs". For example, actin is a "hub" for protein−protein interaction, and it participates in more protein interactions than any other known protein. Complex structures of actin with its binding proteins have revealed that the vast majority of actin-binding proteins share a common helical motif, providing a mechanism whereby actin-binding proteins compete for a common binding site. When the α-helical structure serves as the recognition motif for many homologous protein complexes, such as those seen in two-component systems in bacteria, the "sequential variation" is often used to accomplish the binding specificity. The reverse turn, on the other hand, not only uses the "sequential variation" scheme but also often applies the "structural variation" strategy to achieve such binding specificity. For instance, many adhesive proteins present in extracellular matrices contain the common motif Arg-Gly-Asp as their cell recognition site. Adhesive recognition involves a number of specific cell-surface receptors, and each of them only recognizes the RGD sequence in a certain conformation. The importance of such different characteristics of structural motifs in mediating protein−protein recognition will be further discussed elsewhere.

A similar geometrical analysis was carried out on *cyc*Pro_4 scaffolds (tttt, ctct, tctc, and cccc conformers are included) and their scorings were also drawn in Figure 8. The comparison indicated that most reverse-turn conformations can be mimicked effectively with a subset of substitution patterns on the scaffold. In addition, the scaffold also covered partially the diversity space of α-helices but overlaps little with those of β-sheets. The enormous substitution patterns on the scaffold were evenly distributed in the structural diversity space, which is particularly useful to custom design molecules for a specific reverse-turn conformation. Examples for mimicking the most common β-turns, types I and II, and their mirror images (of the backbone, but not the side chains), types I′ and II′, with only the ctct amide conformer of the scaffold are illustrated in Figure 9. For example, a type I β-turn sequence, Asn-Lys-Asp-Lys, can be mimicked with substitutions at the proline 3-, 3-, 2-, and 3-positions, respectively. The root-mean-square displacement between the $C_\alpha-C_\beta$ vectors in the type I β-turn and the substitution vectors ($C_\alpha-C_\beta$, $C_\alpha-C_\beta$, $C_\alpha-H$, and $C_\beta-H$, respectively) in the scaffold is 0.468 Å. Very importantly, this substitution pattern not only preorganizes all four χ_1 values, the torsional angle associated with the $C_\alpha-C_\beta$ bonds, but also fixes orientations of two $C_\beta-C_\gamma$ bonds. These preorganized compounds are particularly useful for determination of the precise spatial requirements for molecular recognition. Similarly, a type II β-turn can be mimicked with substitutions at the proline 2-, 3-, 2-, and 3-positions, respectively. The $C_\alpha-C_\beta$ vectors were superimposed with $C_\alpha-H$, $C_\alpha-C_\beta$, $C_\alpha-H$, and $C_\beta-H$ bonds, respectively, with the RMSD of 0.471 Å. A type I′ β-turn can be mimicked with substitutions at the proline 2-, 5-, 2-, and 5-positions, respectively. The $C_\alpha-C_\beta$ vectors were aligned with $C_\alpha-H$, $N-C_\delta$, $C_\alpha-H$, and $C_\delta-H$ bonds, respectively, with the RMSD of 0.572 Å. A type II′ β-turn can be mimicked with substitutions at the proline 2-, 3-, 2-, and 4-positions, respectively. The $C_\alpha-C_\beta$ vectors were superimposed with $C_\alpha-H$, $C_\alpha-C_\beta$, $C_\alpha-H$, and $C_\beta-C_\gamma$ bonds, respectively, with the RMSD value of 0.545 Å. It should be noted that the effects of all these substitution patterns on the amide-backbone conformation were consistent with our DFT calculations, that is, they further stabilized the ctct amide isomer.

Scaffold Variations. The structural diversity of CTP scaffolds can be considerably increased by the incorporation other constrained proline analogues, such as *N*-methyl alanine, azaproline, pipecolic acid, azapipecolic acid, and nipecotic acid. Similar DFT calculations and diversity analysis were carried

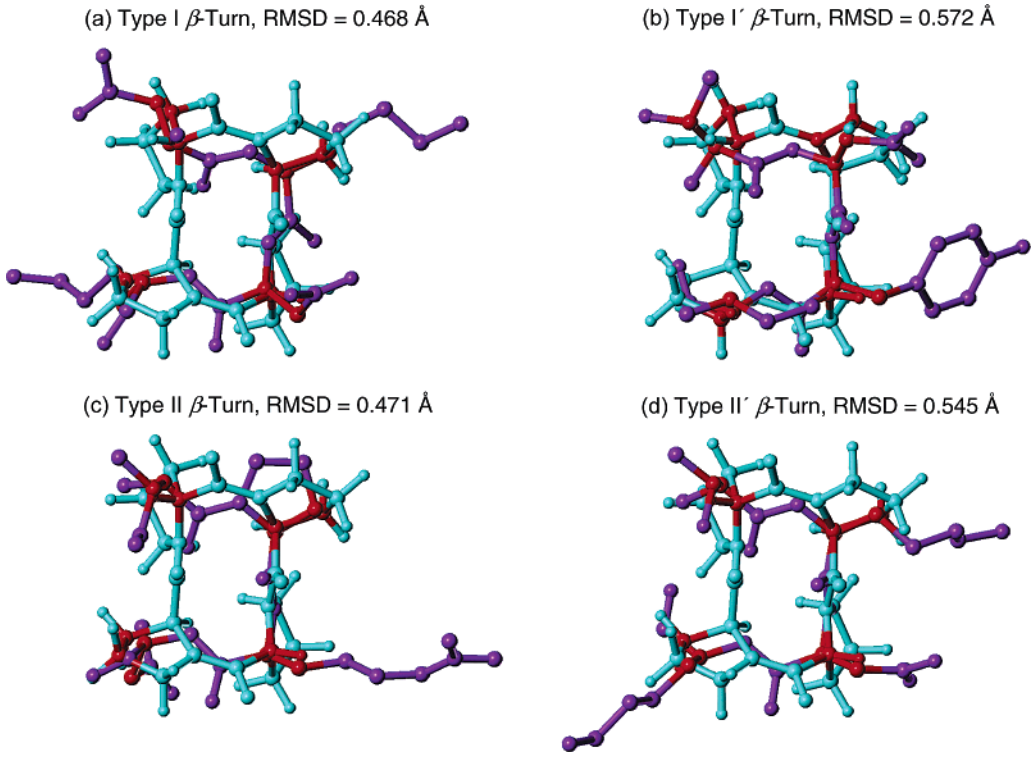


Figure 9. The ctct amide conformer of *cyclo*-(D-Pro-L-Pro-D-Pro-L-Pro) aligned with different types of β-turn structures: (a) type I β-turn (Asn^{27}-Lys^{28}-Asp^{29}-Lys^{30}, PDB 1regX); (b) type I′ β-turn (Thr^{69}-Asp^{70}-Tyr^{71}-Ile^{72}, PDB 1g13A); (c) type II β-turn (Ile^{61}-Pro^{62}-Arg^{63}-Asn^{64}, PDB 1ogoX); (d) type II′ β-turn (Thr^{229}-Lys^{230}-Asp^{231}-Lys^{232}, PDB 1rypA). The cyclic tetraproline is colored in cyan, and the β-turn structure is colored in violet. The superimposed $C_\alpha - C_\beta$ vectors are colored in red, and the root-mean-square displacement between them is indicated above the figures.

Table 5. Number of β-Turn Hits for Each CTP Scaffold

	−1 < comp1 < 1 and −4 < comp2 < 4				−4 < comp1 < 2 and −20 < comp2 < −15			
CTPs	tttt	ctct	tctc	cccc	tttt	ctct	tctc	cccc
c(D-Pro-L-Pro-D-Pro-L-Pro)	139	463	423	557	12	112	90	181
c(D-NMe-Ala-L-Pro-D-Pro-L-Pro)	141	223	340		15	44	51	
c(D-Pro-L-NMe-Ala-D-Pro-L-Pro)	187	362	264		16	46	129	
c(azPro-L-Pro-D-Pro-L-Pro)	156	218	355	367	34	29	83	151
c(D-Pro-azPro-D-Pro-L-Pro)	152	331	192	444	44	106	73	192
c(azPro-L-Pro-azPro-L-Pro)	143	189	275	393	19	28	70	77
c(D-Pro-azPro-D-Pro-azPro)	154	291	163	427	54	106	57	169
c(azPro-azPro-azPro-azPro)	153	192	188	272	28	39	44	84
c(D-Pip-L-Pro-D-Pro-L-Pro)	131	322	535	605	21	55	99	196
c(D-Pro-L-Pip-L-Pro-L-Pro)	188	447	242	477	17	48	107	155
c(azPip-L-Pro-D-Pro-L-Pro)	127	334	483	428	29	91	106	168
c(D-Pro-azPip-D-Pro-L-Pro)	185	309	232	-	19	108	103	-
c((*S*)-Nip-L-Pro-D-Pro-L-Pro)	132	371	331	359	8	96	88	105
c(D-Pro-(*R*)-Nip-D-Pro-L-Pro)	158	374	362	334	6	118	64	48

out on CTP scaffolds containing these constrained analogues, and the results are summarized in Figure 10 and Table 5, respectively.

The variations in scaffold composition provide another means to stabilize a particular amide-backbone conformation, particularly when the proline analogue affects cis−trans amide isomerism. For example, the analogue azaproline, which contains a nitrogen atom in the place of the C_α of proline, strongly stabilizes the cis conformation of the preceding amide bond through the unfavorable lone-pair/lone-pair repulsion between the carbonyl oxygen and the α-nitrogen in the trans amide conformer.[46] The incorporation of two azPro residues in the opposite position in the CTP scaffold would strongly stabilize the cccc amide conformer, which is one of the most unstable conformations for the *cyclo*-Pro_4 scaffold. For the *cyclo*-$azPro_4$, such stabilization is even more dramatically for the cccc amide conformation, which is 10.3 kcal/mol more favorable than the second lowest conformation, ctct or tctc, in chloroform and 6.9 kcal/mol in water. The whole energy profile and the conformational interconversion pathways are turned "upside down" compared to that of *cyclo*-Pro4 (Figure 11). For the other CTP scaffolds, the ctct or tctc amide conformer is still the preferred conformation in water. But other conformations, such as the tttt amide conformer, can be strongly stabilized in chloroform, providing a mechanism to synthetically prepare alternative conformational templates.

We developed simple criteria, based on the scorings of the first two principal components, to quantitatively compare structural diversity for different conformational templates. If a substitution pattern on a certain CTP scaffold had a value of the first scoring (comp1) between −1 and +1 and a value of the second scoring (comp2) between −4 and +4, we called it a hit for β-turn structures with a trans amide bond between residues $i + 1$ and $i + 2$; if the comp1 value was between −4 and +2 and the comp2 value was between −20 and −15, we called the substitution pattern a hit for type VI β-turn (Table

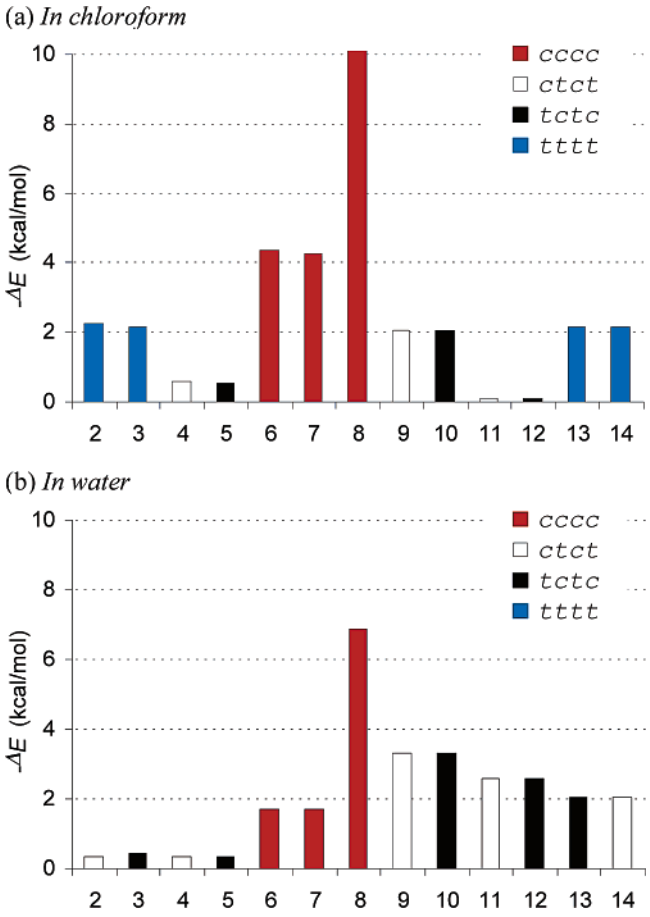


Figure 10. DFT energy differences between the lowest conformation (ctct, white; tctc, black; cccc, red; tttt, blue) and the second lowest conformation of CTP scaffolds (a) in chloroform and (b) in water.

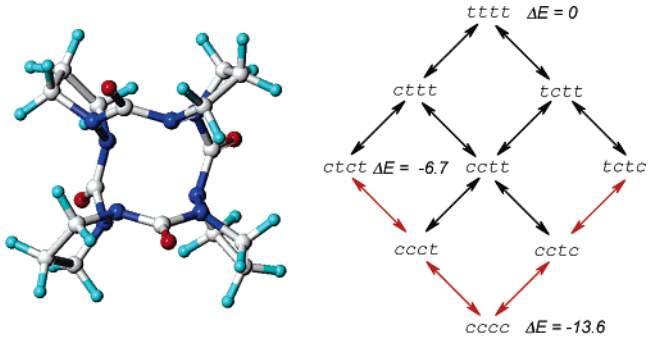


Figure 11. The DFT potential surface of *cyclo*-azPro$_4$: (a) the structure of the cccc amide conformer; (b) the probable transition path (red) between ctct and tctc, which is through the cccc amide isomer, is "upside down" compared to that of *cyclo*-(D-Pro-L-Pro-D-Pro-L-Pro).

5). For example, for the *cyclo*-Pro$_4$ scaffold, there were about 2000 hits for all four amide-bond conformations, in which the ctct amide conformer had 463 hits for common β-turns. In some cases, these numbers became higher for substitutions in six-membered exocyclic rings, such as Pip. For instance, the tctc conformer of *cyclo*-(D-Pip-L-Pro-D-Pro-L-Pro) had 535 hits. Among the possible conformations, the cccc amide conformer often had more hits than the others. This observation suggested the incorporation of azPro should be particularly useful. The small hit number for the *cyclo*-azPro$_4$ scaffold only reflects the fact that there is no α-proton in the scaffold, which reduces the total possible hydrogen-substitution sites from 28 to 24. More importantly, visual inspection indicated that the results were evenly distributed in diversity space and different scaffolds complemented each other.

Conclusions

Developing small molecules that modulate protein−protein or protein−peptide interactions continues to be of great interest in drug discovery.[82] A long-standing goal is to rationally design novel compounds based on knowledge of a protein's three-dimensional structures. For inhibiting protein−protein interaction, general methods for mimicking protein surfaces represent a significant advance. Turns are the most common type of nonrepetitive structure in proteins and have frequently been implicated as protein-recognition sites. This study evaluates the potential of CTPs based on heterochiral dipeptides as novel turn mimetics. Structural database analysis and DFT calculations have demonstrated that (a) CTPs possess diverse backbone conformations in terms of amide bonds, (b) CTPs are relatively rigid due to the small 12-membered macrocycle and the inclusion of exocyclic rings, and (c) chiral substitutions on exocyclic rings can stabilize preferred amide-backbone conformations. Structural comparisons have further indicated that the potential side-chain orientations of CTPs are in close agreement with those of many reverse turns. The modest changes in side-chain orientation with different constrained proline analogues offer a tremendous opportunity for optimization of molecular recognition. Preparation of chemical libraries based on these CTP scaffolds is currently underway. The use of CTPs containing chimeric amino acids should facilitate the determination of the receptor-bound conformation in combination with the variety of other peptidomimetic scaffolds currently in use.

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