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Proline Editing: A Divergent Strategy for the Synthesis of Conformationally Diverse Peptides

Krista M. Thomas, Devan Naduthambi, Gasirat Tririya, and Neal J. Zondlo*

Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716

zondlo@udel.edu

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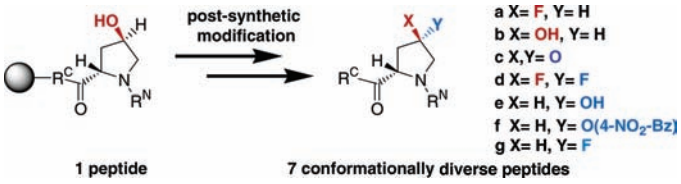
ABSTRACT



Strong conformational biases in peptides and proteins can be achieved with 4-substituted proline residues (cis-, trans-, or disubstituted fluoroproline or hydroxyproline). The practical, divergent synthesis of peptides containing these residues, via postsynthetic modification of a peptide containing an internal *trans*-hydroxyproline residue, is described. Significant differences in the conformations of the peptides Ac-TYXN-NH_2 were observed, including $K_{trans/cis}$ values, which varied from 1.5 (X = *cis*-fluoroproline) to 7.0 (X = *trans*-fluoroproline).

Protein function is tightly linked to protein conformation. Peptides are commonly used to study macromolecular interactions, but, due to significant conformational entropy, unstructured peptides often display only moderate biological activity. The limitations of unbiased peptides have stimulated the development of strategies to stabilize peptides in biologically active conformations.[1]

Proline performs numerous biological roles due to its unique status as a conformationally restricted amino acid.[2] 4-Substituted proline derivatives (Figure 1) exhibit strong and disparate conformational biases that are dependent on the stereochemistry and the electronics of the substitution.[3,4] Dramatically, collagen can be significantly stabilized or destabilized via appropriate site-specific incorporation of proline derivatives.[3,5] A general synthetic approach to peptides containing divergent 4-substituted proline residues would provide a new tool to investigate protein structure−function relationships.[6] Herein, we describe proline editing, a strategy for the preparation of a family of peptides that are individually conformationally restricted but collectively conformationally diverse.

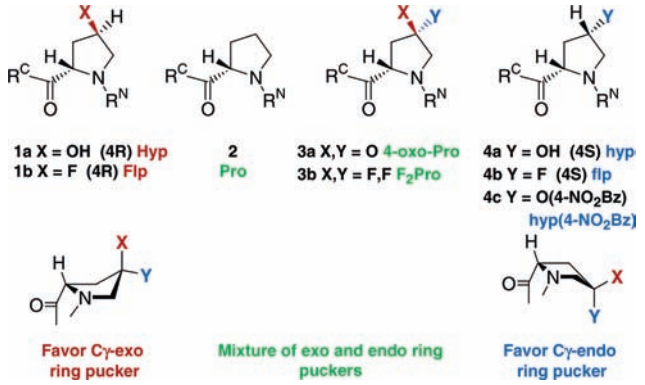


Figure 1. Proline and 4-substituted proline derivatives. (*R*)-Stereochemistry (trans substitution) at the proline 4-position is indicated by upper case letters in the name, whereas (*S*)-stereochemistry (cis substitution) is indicated by all-lower case letters in the name.

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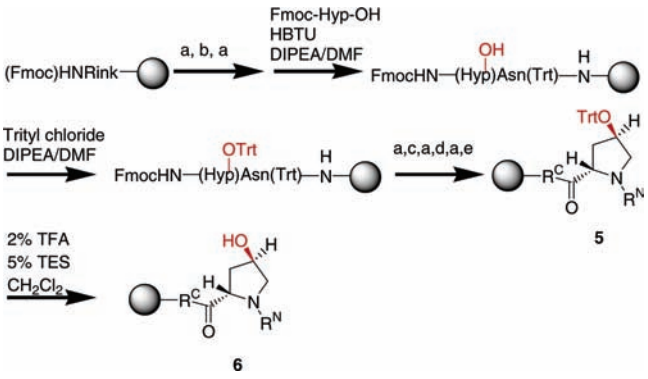
Proline derivatives with strong conformational biases are potent tools to elucidate the roles of secondary structure, turn geometry, and cis−trans isomerization in protein structure and function.[7] Trans-4-substituted prolines strongly favor trans amide bonds by promoting Cγ-*exo* ring pucker, and cis-4-substituted prolines favor Cγ-*endo* ring pucker and can promote cis amide bonds; 4,4-difluoroproline reduces the activation barrier of cis−trans isomerization, potentially a rate-limiting process in protein folding, protein misfolding and disease.[3,4,8]

Despite their utility, fluoroproline-containing peptides are infrequently reported. Although Fmoc-*trans*-fluoroproline (Flp) is commercially available, it is quite expensive. Fmoc-*cis*-fluoroproline (flp), Fmoc-4,4-difluoroproline (F_2Pro), Fmoc-*cis*-hydroxyproline (hyp), and Fmoc-4-oxoproline (4-oxo-Pro) are not commercially available. The most practical syntheses of these compounds require, for each Fmoc amino acid, 5−7 steps from commercially available *trans*-hydroxyproline methyl ester.[9] Moreover, using standard methodology, the synthesis of a series of *n* peptides, each containing a different proline derivative, would require the solution-phase synthesis of each Fmoc derivative, as well as the repetitive peptide synthesis of all *m* residues N-terminal to the proline derivative ($n \times m$ steps). Alternatively, an attractive divergent strategy would be to incorporate into a peptide sequence a single residue, which could be transformed, after peptide synthesis, into any member of a diverse series of residues.[10,11]

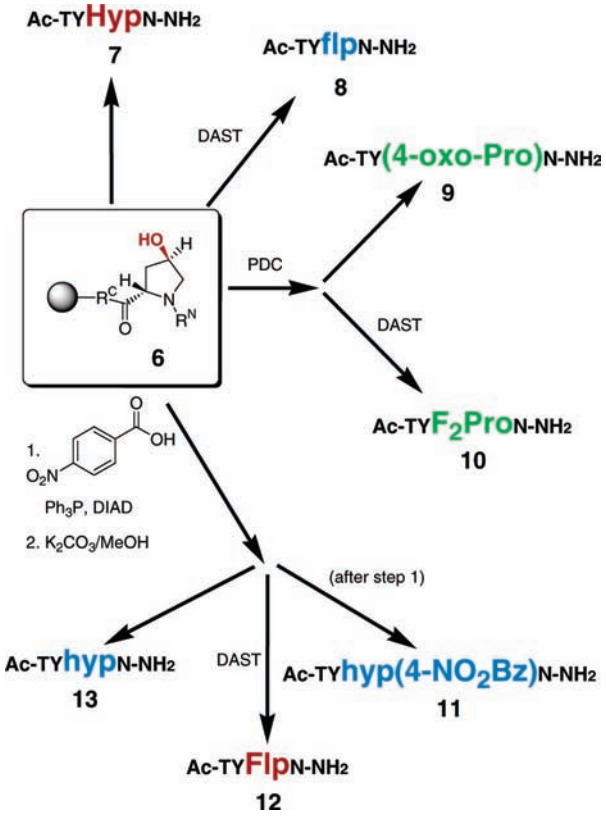
The overall approach is presented in Schemes 1 and 2. Commercially available and inexpensive Fmoc-*trans*-hydroxyproline (Hyp) was incorporated via standard solid-phase

Scheme 1. Solid-Phase Synthesis of Peptides Containing Tritryl-Protected *trans*-Hydroxyproline[a]



[a] Key: (a) 20% piperidine/DMF; (b) Fmoc-Asn(Trt)-OH, HBTU, DIPEA/DMF; (c) Fmoc-Tyr(O*t*Bu)-OH, HBTU, DIPEA/DMF; (d) Fmoc-Thr(O*t*Bu)-OH, HBTU, DIPEA/DMF; (e) 10% Ac_2O/pyridine.

Scheme 2. Proline Editing to Synthesize Conformationally Diverse Peptides[a]



[a] Each sequence was followed by standard acidic cleavage and deprotection of the peptide.

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peptide synthesis into a peptide chain containing a tyrosine-proline residue pair, which promotes cis amide bonds.[12,13] The coupling reaction with Hyp was immediately followed by a protection step, in which trityl chloride was allowed to react with the free hydroxyl group of the hydroxyproline. This protection step was conducted on the synthesizer in automated fashion in standard activator solution. The peptide synthesis was completed by standard Fmoc protocols and required no user intervention between the start of the peptide synthesis and N-acetylation of the peptide. The trityl group was then removed using standard selective deprotection conditions to reveal the free hydroxyl group on the hydroxyproline. The effectiveness of this reaction sequence was demonstrated by the absence of O-acetylated peptides in the crude HPLC chromatograms.[14]

The intermediate **6** was readily converted to 4-*cis*-fluoroproline-containing peptide **8** by reaction with DAST, followed by standard peptide cleavage/deprotection. The HPLC chromatogram (Figure 2) revealed nearly complete conversion to the fluoroproline-containing peptide in 4 h. This reaction was conducted at room temperature in disposable fritted polypropylene columns and required no special handling. Analysis of minor impurity peaks revealed no evidence of dehydration side product.

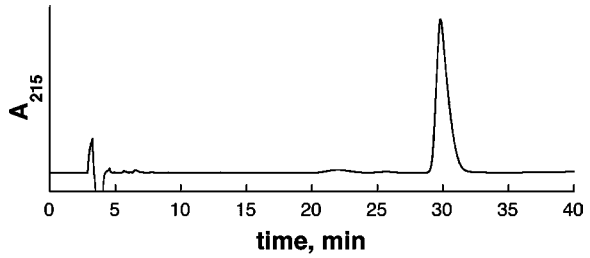

Figure 2. Crude HPLC chromatogram of **8**, indicating clean conversion to product. Crude HPLC chromatograms of other peptides all indicated high conversion to product (Supporting Information).

The intermediate **6** was readily converted to the 4,4-difluoroproline-containing peptide **10** by a two-step protocol. PDC oxidation of **6** generated the 4-oxoproline-containing peptide. Subjection to standard cleavage conditions yielded **9**, which contains an orthogonal ketone chemical handle.[15] Alternatively, reaction of the oxoproline-containing intermediate with DAST and subsequent standard cleavage/deprotection yielded **10**. The crude HPLC chromatograms revealed high conversion to the product in each reaction (Supporting Information).

The intermediate **6** was alternatively converted to the *cis*-hydroxyproline-containing peptide **13** via solid-phase Mitsunobu reaction with 4-nitrobenzoic acid.[3h,16] Reaction progress was followed by cleavage of **11** from resin and analytical HPLC, as the nitrobenzoate ester was stable to standard TFA cleavage conditions. The nitrobenzoate group was readily removed by treatment of the resin with K_2CO_3/MeOH. Cleavage/deprotection of this intermediate produced **13**. Alternatively, the intermediate was readily converted to *trans*-fluoroproline-containing **12** via DAST. Again, high conversion was observed for these transformations. The identities of compounds **13** and **12** were confirmed by ESI-MS and by comparison of the NMR spectrum to that of **7** or to that of **12** alternatively synthesized using Fmoc-Flp, respectively, indicating that the reactions proceeded stereospecifically, as expected.

Analysis of the peptides synthesized via proline editing revealed dramatic conformational differences (Figure 3 and Table 1). Previous data, in which proline derivatives were examined as esters of acetylated amino acids, revealed that

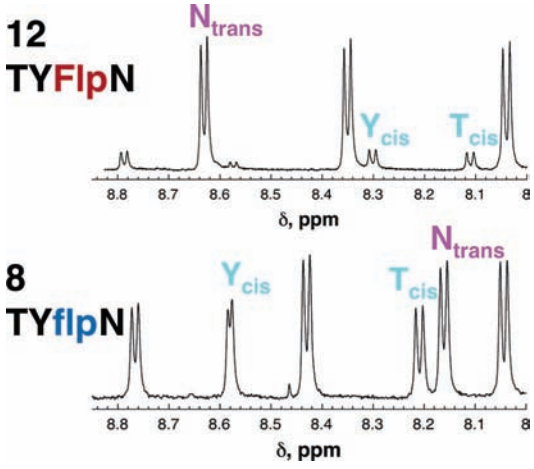

Figure 3. Amide region of the NMR spectra of peptides differing in the stereochemistry of a single side chain carbon: *cis*-fluoroproline-containing **8** and *trans*-fluoroproline-containing **12**. Key amide resonances from the cis (cyan) or trans (magenta) conformations are labeled. Other NMR spectra and full assignments can be found in Supporting Information.

(10) For example, the synthesis of a 20 residue peptide in which residue 11 is varied to incorporate each 4-substituted proline derivative described herein would require $7 \times 11 = 77$ amide coupling cycles (with each cycle including deprotection, coupling, and wash steps) after the resin split via standard methodology, versus 11 amide coupling cycles using proline editing, a significant reduction in required time and material resources. In addition, only seven manual on-resin reactions, in total, are required to prepare all seven derivatives (Scheme 2), versus 27 solution-phase synthetic steps to prepare the required Fmoc amino acids from *trans*-hydroxyproline methyl ester.

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(13) Peptide TYHypN was synthesized and Hyp transformed to other derivatives (Scheme 2) to determine the effect of 4-substituted proline derivatives on cis−trans isomerization within sequences that favor cis amide bonds. The sequence TYHypN was selected on the basis of model studies. TYPN exhibited the highest % cis of seven model tetrapeptides XYPZ, with X and Z chosen on the basis of high residue propensities at the *i* and $i + 3$ positions in type VI β-turns.[12d]

Table 1. NMR Characterization of the Conformational Properties of Peptides **7**−**14**[a]

peptide	$K_{trans/cis}$	ΔΔG	$^3J_{\alpha N}$	δH^N	δH^N
		kcal mol^{-1}	Tyr_{cis}	Tyr_{cis}	Asn_{trans}
TYFlpN (**12**)	7.0	-0.56	7.7	8.30	8.63
TYHypN (**7**)	5.6	-0.43	7.5	8.34	8.59
TY(4-oxo-Pro)N (**9**)	3.8	-0.20	5.7	8.56	8.72
TYhypN (**13**)	2.7	0.00	4.9	8.49	8.08
TYF_2ProN (**10**)	2.6	+0.02	n.d.	8.56	8.58
TYhyp(4-NO_2Bz)N (**11**)	1.8	+0.24	5.3	8.56	8.17
TYflpN (**8**)	1.5	+0.35	4.9	8.58	8.16
TYPN (**14**)	2.7	0.00	6.1	8.37	8.40

[a] ΔΔ*G* values are relative to TYPN (**14**). $^3J_{\alpha N}$ values are in hertz; δ values are in parts per million relative to TSP. $^3J_{\alpha N}$ is the backbone coupling constant between the amide and α protons and can be correlated via a parametrized Karplus equation to the backbone torsion angle ϕ.[17] $^3J_{\alpha N}$ was not determined for **10** because of spectral overlap.

substitution of a single Pro with Flp or flp resulted in a −0.22 to +0.37 kcal mol^{-1} change in $K_{trans/cis}$.[3e,4b,c] Our data, the first extensive analysis of 4-substituted proline derivatives in a non-collagen peptide environment, reveal a context dependence[5d] for the conformational effect of proline substitution, with Flp stabilizing the trans conformation by 0.56 kcal mol^{-1} relative to Pro and flp stabilizing the cis conformation by 0.35 kcal mol^{-1} relative to Pro. Interestingly, in this context, and in contrast to data from collagen and from model compounds, hyp had no effect on $K_{trans/cis}$ compared to Pro,[4d] although conformations differed significantly by NMR (see below). In contrast, hyp-4-nitrobenzoate significantly enhanced the cis preference of hyp, consistent with a strong gauche effect due to the electron-withdrawing nature of the nitrobenzoyl group, and indicating an alternative electronic approach to tuning peptide structure.[3i] Overall, a 4.7-fold change in trans/cis ratio was observed across the peptides, tunable by the choice of proline derivative.

Perhaps more dramatically, controlling the proline ring pucker fundamentally biased the main chain torsion angles of adjacent residues.[3] Most notably, peptides **8**, **11**, and **13** displayed significant ordering of the Tyr residue in the cis conformation, with $^3J_{\alpha N} = 4.9$ Hz for **8** and **13**, correlating with $\phi = -65°$ via analysis by a parametrized Karplus equation.[17] Interestingly, this value is similar to the expected ϕ value (−60°) for a type VI β-turn, in which a cis amide bond exists between the $i + 1$ and $i + 2$ residues. Similarly, in the trans conformation, these peptides displayed a significant upfield shift in the Asn amide resonance relative to the values observed in *trans*-**7** or *trans*-**12**, revealing conformational effects not only on $K_{trans/cis}$ but also on the nature of the individual trans or cis conformations.

Significant differences were also observed in the chemical shifts of all of the Thr_{cis} protons, indicating that changes in structure extended throughout the peptide.[3] Moreover, even among proline derivatives that minimally perturbed the macroscopic value $K_{trans/cis}$, significant microscopic conformational differences were observed. Peptides containing the less-biased residue Pro (**14**), the Cγ-*endo*-favoring residue hyp (**13**), and the isomerization activation barrier-lowering residue F_2Pro (**10**) exhibited essentially identical values of $K_{trans/cis}$. However, these peptides differed significantly in the nature of the trans and cis conformations (Table 1), as indicated by heterogeneity in coupling constants and chemical shifts, emphasizing the diversity engendered in 4-substituted proline residues. Each residue accessible by proline editing makes individual contributions to the peptide structure, providing a stronger conformational bias than is achieved by proline, which exhibits an average of the accessible conformations.

The conformational effect of proline editing was further examined by CD (Supporting Information). Comparison of the CD spectra of **7** and **8** indicated a significant difference in overall structure between these simple tetrapeptides, emphasizing the conformational diversity introduced via proline editing.

In summary, we have presented a strategy for the synthesis of conformationally biased peptides based on *postsynthetically* changing the identity of a residue within the peptide sequence. Proline editing is a practical approach to the synthesis of conformationally diverse peptides containing conformationally distinct proline derivatives. The synthesis uses inexpensive Fmoc-Hyp; involves manipulations performed on solid phase; can be readily conducted with no special equipment or handling; and requires no purification or separation beyond standard HPLC purification of the fully cleaved and deprotected peptide, providing ready and rapid access to peptides containing these useful amino acids. More generally, proline editing alters the side chain and backbone conformations in peptide structures, providing a new tool for the synthesis of strongly conformationally biased peptides and for diversity-oriented peptide synthesis.

Acknowledgment. We thank the University of Delaware, the American Heart Association, and the NIH (T32 GM 08550) for support of this work.

Supporting Information Available: Experimental procedures, characterization data, analytical HPLC traces of the crude cleavage mixtures, and NMR spectra of the amide regions for each peptide. This material is available free of charge via the Internet at http://pubs.acs.org.

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(14) Acetylation of the hydroxyl group was alternatively used as an effective orthogonal hydroxyl side chain protection strategy. See Supporting Information for details.

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