Pseudo-Prolines as a Molecular Hinge: Reversible Induction of *cis* Amide Bonds into Peptide Backbones

Pascal Dumy,* Michael Keller, Declan E. Ryan, Barbara Rohwedder, Torsten Wöhr, and Manfred Mutter

Contribution from the Institute of Organic Chemistry, University of Lausanne, BCH-Dorigny, CH-1015, Lausanne, Switzerland

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Abstract: Serine, threonine-derived (4*S*)-oxazolidine-4-carboxylic acid, and cysteine-derived (4*R*)-thiazolidinecarboxylic acid, denoted pseudo-proline (Xaa[Ψ^{R_1,R_2}pro]), serve as structure disrupting, solubilizing building blocks in peptide synthesis. Variation of the 2-C substituents within the heterocyclic system results in different physicochemical and conformational properties. NMR studies of a series of pseudo-proline (ΨPro)-containing peptides reveal a pronounced effect of the 2-C substituents upon the *cis* to *trans* ratio of the adjacent amide bond in solution. 2-C unsubstituted systems show a preference similar to that of the proline residue for the *trans* form, whereas 2,2-dimethylated derivatives adopt the *cis* amide conformation in high content. For 2-monosubstituted ΨPro, the *cis*−*trans* distribution depends on the 2-C chirality. For the 2-(*S*)-diastereoisomer, both forms are similarly populated in solution, whereas the 2-(*R*)-epimer adopts preferentially the *trans* form. The results are supported by conformational energy calculations and suggest that, by tailoring the degree of substitution, pseudo-prolines may serve as a temporary proline mimetic or as a hinge in peptide backbones.

Introduction

The proline residue plays a peculiar role in peptide and protein secondary structure formation[1,2] and thereby represents an important means in nature to switch and direct peptide chains into favorable topologies. The cyclic nature of the proline residue confers unique conformational properties to the peptide or protein backbone when compared to the common proteinogenic amino acids. For instance, on the one hand, the proline ring serves to intrinsically restrict its Φ dihedral angle around $-60° \pm 15$, while on the other hand, the imidic bond formed with the preceding residue (Xaa-Pro) is readily subject to *cis*−*trans* isomerization. Accordingly, proline residues are usually encountered in loop or turn[1,2] motifs with the utmost preference at the $i + 1$ position for β-turn type I or type II when the Xaa−Pro imide bond is *trans* ($\omega_i = 180°$) or at the $i + 2$ position of turn type VI ($\omega_{i+1} = 0°$) in the *cis* form.[1,3,4] In addition, the characteristic low activation barrier for isomerization combined with the small free energy difference[5] between the two Xaa−Pro peptide bond isomers provides a rationale for the putative role of proline in the limiting steps of the protein folding pathway.[6] This is supported by the discovery of the ubiquitously occurring family of prolyl-peptidyl *cis*−*trans* isomerases such as cyclophilins[7] and FKBPs[8] which catalyze the *cis*−*trans* isomerization of peptidyl−prolyl bonds *in vivo*[9] and *in vitro*.[7,8] The prevalence of proline residues in biological processes such as protein folding and protein recognition[6,7,8] has led to the development of numerous mimetics[10,11] and substituted-proline analogues[12,13] intended to constrain and control the peptide backbone in reverse turn motifs or to alter the imide *cis*−*trans* ratio.[14] For example, substitution with β-alkyl proline[12] results in a Ψ angle constrained by the *syn*-β substituent around 0° while (*S*)-α-methyl-substituted proline[12] (P^{Me}) induces preferentially the *trans* form of the Xaa−Pro bond due to unfavorable steric interaction between the methyl group and the α-proton of Xaa in the *cis* form.

Recently we reported that Ser- or Thr-derived 2-substituted oxazolidine-4(*S*)-carboxylic acid and Cys-derived 2-substituted thiazolidine-4(*R*)-carboxylic acid, denoted as pseudo-prolines (ΨPro's) (Xaa[Ψ^{R_1,R_2}pro], Figure 1), exert a pronounced effect upon the peptide backbone, preventing peptide aggregation and self-association during solid phase conditions, thus improving the efficacy of peptide synthesis.[15−18] Such building blocks are readily accessible by cyclocondensation of Ser, Thr, or Cys with aldehydes or ketones[18] (Figure 1) and represent useful protecting techniques for the parent amino acids. Furthermore, variation of the substituents R_1 and R_2 at position 2 affects the ring stability, resulting in pseudo-proline (ΨPro) of differential chemical stability.[17]

In this paper, we describe the imide *cis*−*trans* distribution upon variation of the 2-C substituents (R_1, R_2) by NMR

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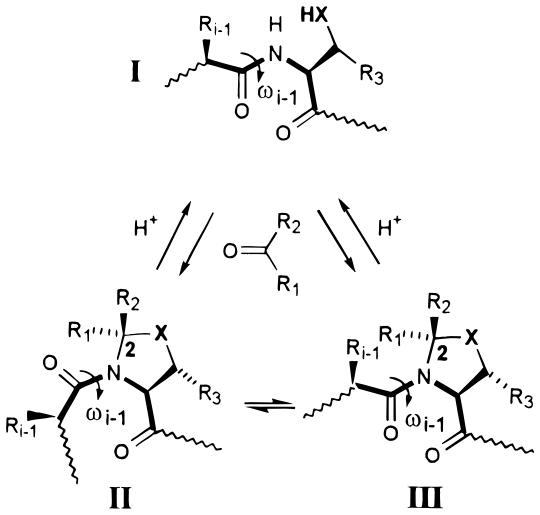

Figure 1. Reaction of Ser, Thr, or Cys (Xaa_i)-containing peptide **I** with carbonyl compounds resulting in the formation of pseudo-prolines ($Xaa[\Psi^{R_1,R_2}pro]_i$) exhibiting a *cis* ($\omega_{i-1} = 0°$, **II**) or trans ($\omega_{i-1} = 180°$, **III**) amide bond, depending on the nature of R_1 and R_2. Cleavage of the cyclic system with acid (H^+) transforms the constrained peptide **I** backbone to a regular *all-trans* amide conformation.

spectroscopy. This distribution is essentially driven by the number and steric requirements of the 2-C substituents which destabilize one of the two rotamers. We demonstrate that variation of these substituents at the position 2 of the ring system provides an efficient synthetic tool to tailor the *cis* to *trans* ratio of the $Xaa_{i-1}-Xaa_i[\Psi^{R_1,R_2}pro]$ amide bond in peptides.

Methods

Peptides Synthesis. The ΨPro-containing peptides were synthesized according to methodologies[17,18] described earlier. Unsubstituted ΨPro's **4** and **7** (Figure 1) were obtained by cyclocondensation with formaldehyde on the corresponding free amino acid. 2-Alkylated ΨPro's were obtained by cyclocondensation with the corresponding ketal (2,2-dimethoxypropane for 2,2-dimethyl-ΨPro compounds **1**−**3** and **8**, 2,2-dimethoxyanisaldehyde for compounds **9** and **10**) at the dipeptide stage according the post-insertion method[18] and subsequent peptide chain prolongation.

NMR Spectroscopy. Spectra were recorded at 400 MHz using a Bruker ARX spectrometer at 300 K. Samples of around 25 mg were dissolved in 0.4−0.5 mL of DMSO-d_6. Chemical shifts were calibrated with reference to the residual DMSO signal (^{1}H, 2.49 ppm; ^{13}C, 39.5 ppm). 2D experiments were typically acquired using 2K × 512 matrices over a 4000 Hz sweep width in both dimensions. Quadrature detection in the indirect dimension was achieved by using the TPPI procedure.[19] Scalar connectivities were recovered from 2D double quantum filtration (DQF) COSY experiments.[20] Dipolar connectivities were obtained by either the conventional NOESY sequence[21] or the ROESY sequence[22] with mixing times from 150 to 200 ms. A randomization of the mixing length (±5%) was introduced in the NOESY experiments in order to minimize coherence transfer. The spin lock mixing interval of the ROESY sequence was applied by coherent CW irradiation at $\gamma B_2/2\pi = 2$ KHz. Experimental data processing was performed using the Felix software package.[23] The standard sinebell squared routine was employed for apodization with a shift range of 60−90° and zero filling in both dimensions before 2D transformations were applied to end up with square matrices of 2K × 2K real point data. Complete proton resonance assignments were made using (DQF) COSY experiments, HMQC was used to assign unambiguously the carbon resonances. *J* coupling constants were directly measured from high-resolution ^{1}H−^{1}D spectra (±0.1 Hz) or simulated for complex or overlapped systems. Prochiral assignments for the ΨPro β and Me protons were achieved, in some cases, on the basis of relative interproton ROE intensities and *J* coupling constant values.

Table 1. *cis* to *trans* Ratio of Peptides Ac-Ala-Xaa[Ψ^{R_1,R_2}pro]-NH-Me **1**−**6** and succinyl-Val-Xaa[Ψ^{R_1,R_2}pro]-Phe-NH-pNA **7**−**10**

compd	R_1	R_2	Xaa	X	*cis* (%)[a]
1	Me	Me	Ser	O	∼100
2	Me	Me	Thr	O	∼100
3	Me	Me	Cys	S	∼100
4	H	H	Ser	O	∼33
5			Pro	CH_2	∼18
6	biphenyl	H	Ser	O	∼63
7	H	H	Ser	O	∼15
8	Me	Me	Ser	O	> 95
9	H	PMP[b]	Ser	O	∼10
10	PMP	H	Ser	O	∼50

[a] Determined by ^{1}H NMR in DMSO at 300 K. [b] PMP: *p*-methoxyphenyl.

Computational Methods. All the simulations were carried out *in vacuo* using the CVFF[24] force field as implemented in Discover.[23] The calculations used a value of 1 for the macroscopic dielectric constant and an infinite cutoff for nonbonded interactions. Structure minimizations for each isomer were carried out using the Newton−Raphson algorithm until the gradient was less than 0.001 kcal/mol. Ramachandran plots[25] were obtained by mapping the energy of the structure upon incrementation of the Φ/Ψ dihedral angles of alanine. Contour levels are plotted with an interval of 10 kcal/mol.

Results

In order to evaluate the effects exerted by the substituents in position 2 within the heterocycle of ΨPro on the *cis*−*trans* isomerization of the peptide bond, we prepared a number of peptide derivatives (**1**−**6**, Table 1) of the types Ac-Ala_{i-1}-$Xaa_i[\Psi^{R_1,R_2}pro]$-NHMe(Figure1)andsuccinyl-Val_{i-1}-$Xaa_i[\Psi^{R_1,R_2}$-pro]-Phe-NH-pNA (compounds **7**−**10**). Both N and C peptide termini were capped to minimize intramolecular electronic interactions, which have been shown to alter the *cis*−*trans* ratio of the Xaa−Pro bond.[14] As relevant spectral parameters on thiazolidine are available in literature, we focused mainly on the 2-substituted oxazolidine-4(*S*)-carboxylic acid derivatives.

The conformational preference of the $Xaa_{i-1}-Xaa_i[\Psi^{R_1,R_2}$-pro] imide bond was investigated by ^{1}H NMR and ^{13}C NMR spectroscopy. Examination of the 1D spectra in DMSO of model compounds **1**−**10** immediately reveals two spectroscopic characteristics: 2,2-dimethyl-ΨPro derivatives (**1**−**3** and **8**) exhibit a predominant set of resonances for the *cis* conformer, while for the monosubstitued compounds **6**, **9**, **10**, the unsubstituted compounds **4** and **7**, and the proline containing compounds **5**, two sets of resonances were obtained due to a comparable amount of *cis* and *trans* conformers in solution. Table 1 summarizes the *cis*−*trans* ratios of the Xaa−ΨPro bond measured by ^{1}H NMR for different 2-C substituents of the five-membered ring. For the proline analogue **5**, the occurrence of both isomers in solution is readily confirmed by ^{13}C NMR on the basis of the well-documented shift differences observed for γ and β carbons.[26,27]

2-C Disubstituted ΨPro-Containing Peptides. The observation of a highly predominant set of resonances in the ^{1}H NMR

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Table 2. ΨPro Relevant Spectral Parameters for Compounds **1**−**10** in DMSO-d_6[a]

entry	compd	α (^3J αH-βH)[b]	β (^2J βH-βH)[b]	2-H(Me) ($^2J_{HH}$)
1	Ser($\Psi^{Me,Me}$pro)	4.44 (6.1$^{pro\text{-}R}$/−)	4.17/4.05$^{pro\text{-}R}$ (8.8)	1.52/1.43$^{pro\text{-}S}$
2	Thr($\Psi^{Me,Me}$pro)	4.03 (5.7)	4.12/1.29	1.52/1.50$^{pro\text{-}S}$
3	Cys($\Psi^{Me,Me}$pro)	5.34 (6.4$^{pro\text{-}R}$/−)	3.62 $^{pro\text{-}R}$/3.20 (11.8)	1.93/1.85$^{pro\text{-}S}$
4	Ser($\Psi^{H,H}$pro)	4.31	4.09/3.83	5.17/4.96 (3)
4 (*cis*)	Ser($\Psi^{H,H}$pro)	4.48	4.18/4.03	4.88
7	Ser($\Psi^{H,H}$pro)	4.485 (7.3 $^{pro\text{-}R}$/5.0)	4.174 $^{pro\text{-}R}$/3.818 (8.7)	5.289 (3.7)/4.95
8	Ser($\Psi^{Me,Me}$pro)	4.64 (6.5 $^{pro\text{-}R}$/1.9)	4.102 $^{pro\text{-}R}$/4.00 (9.1)	1.43
9 (*trans*)	2-(*R*)-Ser($\Psi^{H,PMP}$pro)	4.65 (7.4 $^{pro\text{-}R}$/5.6)	4.122 $^{pro\text{-}R}$/4.005 (8.9)	6.396
10 (*trans*)	2-(*S*)-Ser($\Psi^{PMP,H}$pro)	4.704 (7.2 $^{pro\text{-}R}$/3.1)	3.979 $^{pro\text{-}R}$/3.864 (9.3)	6.32
10 (*cis*)	2-(*S*)-Ser($\Psi^{PMP,H}$pro)	4.853 (6.3 $^{pro\text{-}R}$/1.9)	4.29 $^{pro\text{-}R}$/4.02 (8.9)	6.17

[a] Chemical shifts (δ in ppm; ±0.01). [b] α stands for the 4-H, β for the 5-H, J (in Hz ± 0.2).

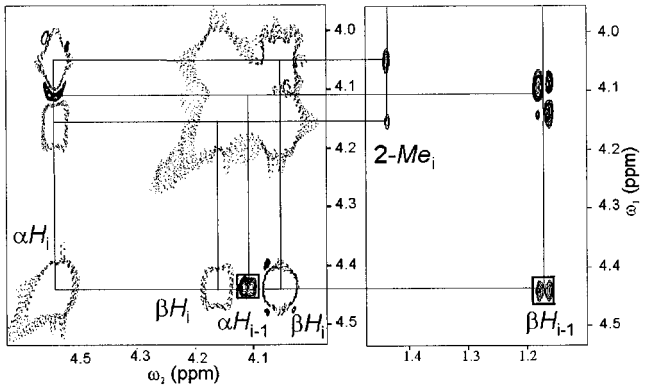

Figure 2. Expanded region of the ^{2}D ^{1}H NMR ROESY spectrum at 400 MHz (τ_m = 200 ms) of **1**, in DMSO at 300 K. The boxed peaks indicate the $\alpha H_{i-1}-\alpha H_i$ and $\beta H_{i-1}-\alpha H_i$ (right) connectivities between Ala$_{i-1}$ and Xaa$_i$[$\Psi^{Me,Me}$pro] typically observed for the *cis* conformer.

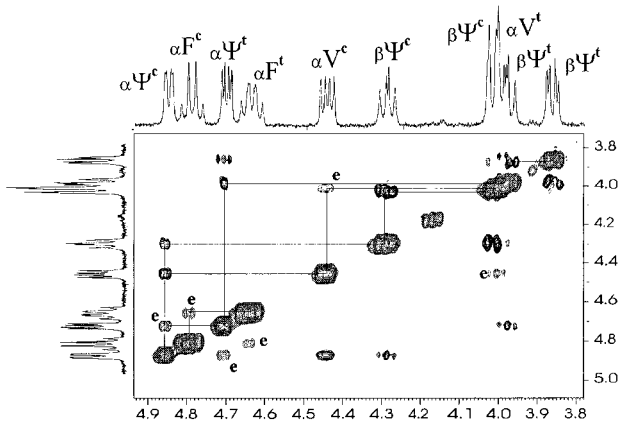

Figure 3. Expanded region of the ^{2}D ^{1}H NMR ROESY spectrum of **10** showing the assignment of the *cis* and *trans* isomer (400 MHz, τ_m = 200 ms, in DMSO at 300 K). The *cis* and *trans* isomers are denoted by a small superscript letter (c or t) in the ^{1}D NMR spectrum; exchange ROE cross peaks are labeled with an e on the map (same phase as diagonal).

and ^{13}C NMR spectra of peptides **1**−**3** upon varying both solvent (H_2O/D_2O, $CDCl_3$, or DMSO-d_6) and temperature is consistent with the presence of a major conformation in solution. From 2D ^{1}H NMR ROESY experiments we observe the typical pattern[28] expected for a *cis* amide bond (Figure 2, boxed peak), i.e. $\alpha H_{i-1}-\alpha H_i$ and $\beta H_{i-1}-\alpha H_i$ ROE cross-peaks between Ala$_{i-1}$ or Val$_{i-1}$ and Xaa$_i$[$\Psi^{Me,Me}$pro] reflecting the spatial proximity of the corresponding protons in the *cis* form. For peptide **8**, a set of minor resonances is also detected (less than 5% as estimated by 1D integrals) and is assigned to a minor conformation in slow exchange with the major one on NMR time scale. However, neither chemical shifts criteria (*vide supra*) nor ROE measurements allow for the assignment of the latter minor species to the *trans* form (*vide supra*). Stereospecific assignment is realized for the ΨPro β-protons on the basis of their relative ROE cross-peak intensities with the ΨPro α proton and their ^3J αH-βH values (Table 2). The *pro-R* β-protons (*anti* to the carboxyl group, Figure 1, R_3 = H) are shifted downfield (except for **1**) and exhibit a greater coupling constant (Table 2) with the α-proton compared to that of the *pro-S* β-proton (*syn* to the carboxyl group). On the basis of the latter assignment, the upfield 2-methyl group (*syn* to the carboxyl, Figure 1, R_2 = H) is assigned to *pro-R* by the presence of a ROE cross-peak with the *pro-S* β-proton. Interestingly, upon substitution of Ala for Gly or D-Ala in peptide **2**, no significant effect on the backbone conformation is observed by NMR (data not shown), indicating a pronounced preference of the *cis* conformer in the 2,2-dimethyl-ΨPro-containing peptides **1**−**3**.

2-C Unsubstituted ΨPro-Containing Peptides. For peptides **4** and **7**, the occurrence of exchange cross peaks in ROESY experiments between each observed set of resonances and their coalescence upon warming points to the presence of *cis* and *trans* conformers in slow exchange is similar to that of proline-containing peptides[26,27] and thiazolidine derivatives.[29−31] The more abundant *trans* isomer is characterized by a $\alpha H_{i-1}-2\text{-}H_i$ and $\beta H_{i-1}-2\text{-}H_i$ ROE cross-peak between Ala$_{i-1}$ or Val$_{i-1}$ and 2-H$_i$ in analogy to the short $\alpha H_{i-1}-\delta H_i$ distance typically observed in *trans*-proline peptides.[28] The *cis* isomer is only characterized by an $\alpha_i-\alpha_i$ exchange cross-peak, the α- and β-protons being broadened and deshielded compared to those of the *trans* form, while the protons at position 2 are found upfield in agreement with previous reports on related thiazolidine derivatives.[31]

2-C Monosubstituted ΨPro-Containing Peptides. The presence of the two conformers in solution is confirmed by the same type of ROE cross peaks (Figure 3) as mentioned for the 2,2-dimethyl and unsubstituted compounds (*vide infra*).

A striking difference between the two diastereoisomers with respect to the *cis*−*trans* population is observed in the 1D spectra. For the 2-(*S*) epimer (*vide supra*), the distribution of the 2-H signals (*cis* and *trans*) is nearly identical, while in the second case, a ratio of 90:10 is measured (Figure 4).

Stereospecific assignments of the ΨPro β-protons (Table 2) were performed as exemplified above for the 2-dimethyl

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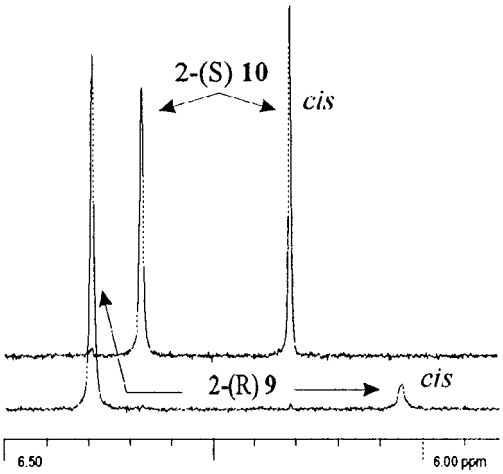

Figure 4. Expanded region of the ^{1}D NMR of **9** (bottom) and **10** showing the 2-H signal of the *trans* (left downfield) and *cis* (right upfield).

Table 3. Amide Geometry and *cis*−*trans* Energy Calculated for Compounds **1**, **4**, **5**, and **6** (*R*/*S*)

peptide	energy (*trans*/*cis*) (kcal/mol)
1	−44.9/−48
4	20.1/19.9
5	27.5/29.9
6 (*S*)	124.7/126.6
6 (*R*)	120.4/123.6

compounds. The stereochemistry of the 2-C position inferred for **9** and **10** is on the basis of ROE cross peaks between the 2-C substituents, namely the *ortho* protons of the *p*-methoxyphenyl (PMP) group or the 2-H, with the β-protons of the ΨPro. For instance, the 2-(*S*) epimer (Figure 1, R_1 = PMP, R_2 = H) is set by the ROE cross peaks between the 2-H and the *pro-S* β-proton (*cis* relationship) while a ROE between the PMP *ortho* protons and the *pro-S* β-proton is of diagnostic value for the 2-(*R*) epimer. For compound **6**, the 2-(*S*) center can also be assigned on the basis of the 2-H and the β-(*pro-S*)-proton ROE cross peak, in harmony with the X-ray data of the compound.[32] Figure 3 shows the assignment of all the *cis* and *trans* protons of compound **10**. The chemical shift values show that α- and β-protons are shifted downfield while the 2-H proton is upfield in the *cis* form, supporting the corresponding observations for the unsubstituted compounds **4** and **7**. Differences in the ^3J αH-βH values are also indicative: the *pro-S* β-protons range from 2 Hz in the *cis* to 3−6 Hz in the *trans* form, whereas for the *pro-R* β-protons a constant value of 6−7 Hz regardless of the nature of the isomer is observed.

Conformational Energy Calculations. Table 3 summarizes the conformational energies found after minimization of compounds **1**, **4**, **5**, and **6** (both 2-C epimers) in the *cis* or *trans* form. While in general these values do not perfectly match the experimental data found in solution, they still reflect the general tendencies of the *cis* and *trans* forms.

Although the same dihedral angle values are found qualitatively along the contour lines in the Ramachandran map,[25] the analysis of the energy difference corresponding to a given couple of (Φ, Ψ) for $\omega_{i-1} = 0$ and $\omega_{i-1} = 180°$ is instructive. For compound **1**, the preference for *cis* amounted to a value up to 15 kcal/mol, whereas for proline-containing peptide **4** and for the unsubstituted derivative **5**, the preference for one conformer (*trans* in that case) is much less pronounced. Interestingly, for compound **6**, the two rotameric forms have nearby the same energy values along the contour plot for the (*S*)-epimer (Figure 5, top) while differing by up to 3 kcal/mol for the (*R*)-form (Figure 5, bottom).

(32) Evaluation of the rate constant and thermodynamic parameters of the *cis*−*trans* equilibrium in compounds **4**−**7**, **9**, and **10** as well as the solid state conformation for the peptides **4**−**6** will be subject of a separate publication.

Discussion

In general, steric interactions between the C(δ) pyrrolidine and the preceding residue provide some rational for the *cis*−*trans* ratio found in proline-containing peptides and proteins.[14] The *trans* peptide bond introduces steric interactions between the δ-CH_2 in Pro and the preceding residue resulting in an increase of the net free energy difference between the *cis* and *trans* conformers. In ΨPro-containing model peptides, the substituents at the 2-C_i of the cyclic system interact severely with the side chain R_{i-1} or the peptide backbone of the preceding residue (Figure 1, **III**) in the *trans* form, whereas in the *cis* conformer, the corresponding interactions stem from the carbonyl group of residue $i - 1$. Consequently, in compounds **1**−**3** and **8**, the two 2-C methyl groups (*pro-R* and *pro-S*) result in more severe steric restriction in the *trans* conformer compared to the *cis*. These findings are confirmed by comparing the energy differences between the two rotamers along the Ramachandran plot. Starting from a given value of the *cis* (Φ, Ψ) angles, rotation of 180° around ω_{i-1} results in the *trans* conformer with up to 15 kcal/mol higher in energy. This is consistent with the substitution of Ala_{i-1} for Gly or for D-Ala which did not result in detectable amounts of the *trans* conformer. Furthermore, substitution of Ala for Gly also indicates that not only the side chain but also the peptide backbone accounts for steric interactions with the 2-C substituents of ΨPro. Despite the strong thermodynamic preference for the *cis* in 2,2-dimethyl-containing ΨPro derivatives, the presence of a small amount of *trans* conformer cannot be ruled out by NMR measurements (*vide infra*). Preliminary experiments based on the chymotrypsin-coupled assays developed by Fischer[7] show that in compound **8** about 8% of the *trans* isomer is present in aqueous solution. A similar preference for *cis* has been observed for a compound based on δ-dimethyl-substitued proline.[33] Notably, strong preference for the *cis* conformation in solution has been reported for N-acetylated 2,2-methylthiazolidine on the basis of chemical shift criteria and 1D-NOE analysis.[34] Crystallographic data on the protected dipeptide Fmoc-Ala-Cys(Ψ^{MeMe}pro)-OH reveal also the *cis* geometry[35] for the peptide bond. Most notably, an inversion of that *cis*−*trans* preference has been reported for *N*-formyl-2,2-methylthiazolidine,[36] supporting the findings that steric hindrance is the major driving force for adapting a high *cis* content.

Beside a significant amount of the *cis* conformer in solution (Table 1), the unsubstituted peptide **4** adopts a preference for the *trans* conformation with a value of about 0.5 kcal/mol for the free energy difference of the *cis*−*trans* equilibrium similar to that of the proline-containing peptide **5**. Similarly, unsubstituted 2-thiazolidine-4-carboxylic acid analogues also display a strong preference for the *trans* form and have been used as a proline surrogate into biological peptides[37] and polymers.[29] The structural similarity of compound **4** with proline is also observed in the solid state as delineated by their X-ray structures.[32]

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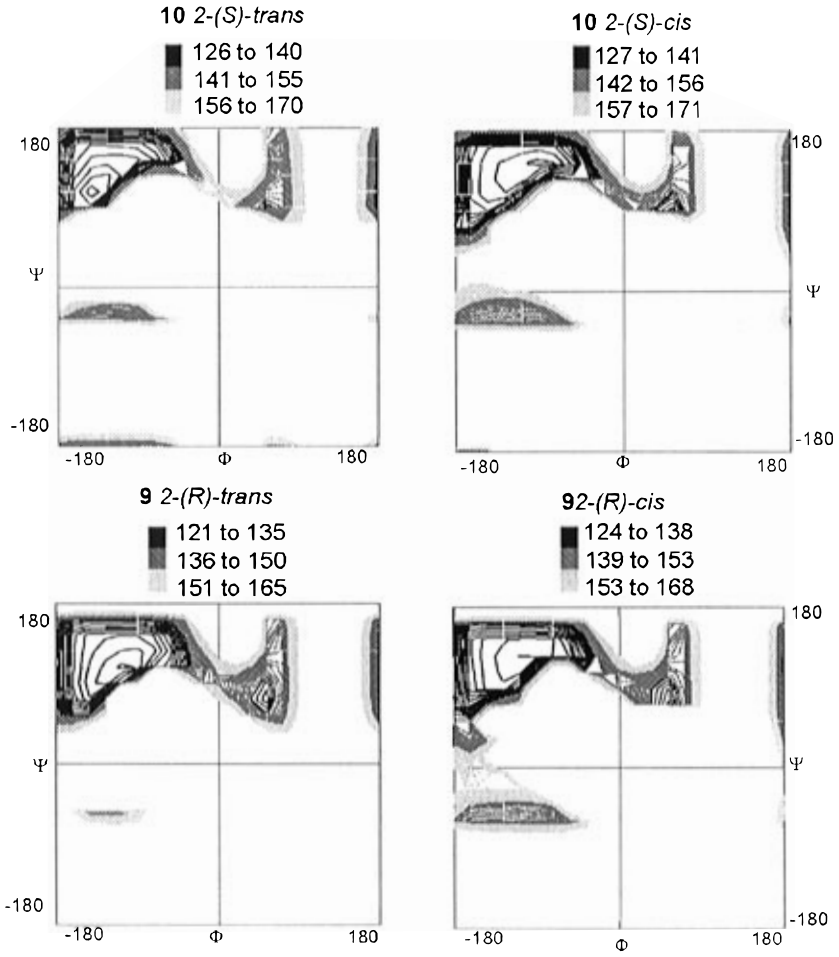

Figure 5. Ramachandran plot of compounds **9** (bottom) and **10** calculated for each rotameric form.

The presence of a single substituent leads to *cis*−*trans* ratios depending on the 2-C chirality. As for the 2,2-dimethyl-ΨPro, the contour levels in the Ramachandran plot (Figure 5) reveal that steric factors determine the preferential geometry of the imide bond. When the substituent is *anti* to the carboxyl group (2-(*S*), Figure 1, R_2 = H), the two forms have nearly the same free energy ($\Delta G°_{t-c} \approx 0$), whereas a difference in energy of about 1.3 kcal/mol is found for the 2-(*R*) epimer by NMR. The *anti* and *syn* diastereoisomers of *N*-acetyl-5-methylproline *N*′-methylamide are reported[38] to display a similar *cis* to *trans* distribution in solution which again can be rationalized invoking steric effect of the 5-substituents. Such a dependency on the chirality at the 2-C position is reported in the literature for thiazolidine congeners, but no unequivocal assignments of the *cis* or the *trans* form have been provided so far.[39] It is important to note that the chirality is usually inferred by the sum of the coupling constants between the α- and *β*-protons of the thiazolidine ring. This sum is calculated for temperatures above the coalescence of the two isomers on the basis of the ^{1}D NMR spectrum in order to simplify the corresponding spectra and to overcome the problems of resonance overlapping.[39,40] A sum less than 10 Hz is of diagnostic value for the (*S*)-epimeric center, whereas for values exceeding 10 Hz, the (*R*)-form is inferred. The isomer weighted sum results in 9.2 Hz for compound **10** and 13 Hz for compound **9** (Table 2), resulting according to the above rule in the (*S*) and (*R*) stereochemistry assignments for the 2-C center in agreement with our NMR assignments. Thus, the 2-C chirality of the ΨPro compounds can be determined directly by ^{1}H NMR using the coupling constant values and the *cis* to *trans* distribution. The *cis* and *trans* isomer can be easily assigned by ^{1}D NMR on the basis of their chemical shift differences and coupling constant.

The differences in coupling constant values in both epimers also provide information on the puckering of the heterocycle.[32] Table 2 shows that the ^3J αH−*β*H values are conserved within each isomeric form irrespective of the nature of substitution. In the *cis* conformation, the sum of the ^3J αH−*β*H coupling constants is always less than 9 Hz (Table 2), corresponding to a staggered conformation along the Cα−C*β* bond. A greater sum is only consistent with a conformation where the α-proton is *gauche* to the *pro-R* *β*-proton while the *pro-S* *β*-proton is pseudo-axial. On the basis of X-ray data[30,35] and similar conclusions[31] established for thiazolidine, these arrangements lead to the *cis* form with a preferential *β*-exo envelope conformation. The fact that one arrangement of the substituent[31] corresponds to one isomer form may reflect an influence of the puckering for the *cis*−*trans* ratio. Although the puckering contribution alone cannot be easily decoupled and evaluated from the steric effects,[36] it still may explain the observed difference between **9** and **10**. In the 2-(*S*) diastereoisomer, steric hindrance between PMP and the preceding Val does not destabilize the pseudo-equatorial (*cis*) form relative to the pseudo-axial position (*trans)* form whereas the PMP group in a pseudo-equatorial position destabilizes the *cis* in the 2-(*R*)

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isomer. In the same vein, the spatial arrangement of the substituents should also have a subtle effect in 2-monosubstituted compound on the *anti* and *syn* transition[41] state by decreasing one state relative to the other as found for 5-methyl-substitued proline derivatives.[38] The determination of the corresponding kinetic parameters are currently under investigation in our laboratory.[32]

Due to the described structural properties, the incorporation of a ΨPro residue into peptide backbone provides also a powerful means to overcome some fundamental problems of peptide synthesis.[17] For example, ΨPro residues have been shown to disrupt β-sheet structure formation during stepwise peptide synthesis, resulting in increased solvation of the peptide chain. The *cis*−*trans* isomerization process described here provides a rational for these experimental findings. The *cis*−*trans* isomerization induced by ΨPro results in a kink conformation of the peptide backbone, thus preventing the onset of peptide self-aggregation. ΨPro therefore may act as a molecular hinge within the peptide chain.

Conclusion

Incorporation of a pseudo-proline residue into the peptide backbone results in variable *cis*−*trans* ratios of the preceding amide bond depending on the nature of the 2-C substituents of the ring system. The conformational preference is essentially driven by the number and steric requirements of the 2-C substituents which destabilize one of the two conformers. Due to the comparable structural properties with proline and their high chemical stabilities, unsubstituted Ser-, Thr-, and Cys-derived ΨPro's represent useful proline surrogates. In contrast, the unusually high *cis* amide content of 2-C disubstituted ΨPro offers a simple and attractive means to constrain selectively peptide bonds, *i.e.* to induce a *cis* amide in the peptide backbone especially for the temporary insertion of β-turns into polypeptide chains. Most notably, type VI β-turns with 2,2-methyl-ΨPro residue at position $i + 2$ can be induced.[42] Ring opening under acidic conditions allows to reconstitute the regular *all-trans* peptide. More subtle effects on the imide bond geometry are observed for 2-C monosubstituted ΨPro. In this context, ΨPro represent a versatile tool for investigating the influence of ring substitution upon the isomerization of the imide bond. On the basis of these results and their ready synthetic access, ΨPro can be used as a molecular hinge to explore and control the peptide backbone conformation. Thus, pseudo-prolines offer a wide range of applications as in peptide-based drug and prodrug design, molecular recognition studies, or protein folding and self-aggregation processes.

Experimental Section

Materials. All protected amino acids were purchased from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland); reagents and solvents were purchased from Fluka (Buchs, Switzerland) and used without further purification. HPLC was performed on Waters equipment using columns packed with Vydac Nucleosil 300 Å 5 μm C_{18} particles unless otherwise stated. The analytical column (250 × 4.6 mm) was operated at 1 mL/min and the preparative column (250 × 21 mm) at 18 mL/min, with UV monitoring at 214 nm. Solvent A consisted of 0.09% TFA and solvent B of 0.09% TFA in 90% acetonitrile. TLC was performed on silica gel plates Merck 60 *F254*, visualized by UV or 5% vanillin in concentrated sulfuric acid. Flash chromatography was performed on Merck silica gel 60 (40−63 mesh). Mass spectra were obtained by electron spray ionization (ESI-MS) on a Finnigan LC710 or by chemical ionization (CI-MS) on a Nermag R10-10C. ^{1}H-NMR spectra were obtained on a Bruker-WH250 or Bruker DPX-400 with trimethylsilane as the internal standard for intermediate compounds. Infrared spectra were recorded on a Perkin-Elmer 1430 spectrometer using the KBr disc method. Elemental analysis (±0.4%, C, H, N) has been performed at the Mikroanalytisches Laboratorium (Kronach, Germany). Melting points are uncorrected values measured with a Büchni 510 point apparatus.

General Procedure for the Preparation of ΨPro-Containing Dipeptide. Fmoc-Ala-ΨPro-OH was obtained by reacting Ser, Thr, or Cys with formaldehyde to give the unsubstituted compounds or by reacting Ser- or Thr-containing dipeptides with the corresponding ketal according to published procedures of the post-insertion method.[17,18] In the latter case, the reaction was carried out in the presence of catalytic amounts of pyridyltoluene-4-sulfonate (PPTS) and 2,2-dimethoxypropane (DMP) in THF.

Ac-Ala-Ser($\Psi^{Me,Me}$pro)-NHMe (1). Z-Ala-Ser($\Psi^{Me,Me}$pro)-OH[17] (1.35 g, 4.0 mmol) was dissolved in 30 mL of THF/DCM (1:1; v/v) and reacted with 1.14 g (5.54 mmol) of DCC and 1.05 g (6.65 mmol) of HOBt at room temperature. After the suspension was stirred for 20 min, 1.0 mL of a 8.02 M solution of methylamine in ethanol was added. The mixture was stirred for 10 min at 0 °C and further 30 min at room temperature before filtering off the unsoluble solid and evaporating the solvent. The resulting material was taken up in 100 mL of ethyl acetate, washed [with a 5% aqueous sodium carbonate solution (3 × 50 mL), a 5% aqueous solution of citric acid (3 × 50 mL), and brine (3 × 50 mL)], and dried over magnesium sulfate. The solvent was evaporated and the residue purified on silica gel (flash, eluent $CHCl_3$/MeOH, 100:4), resulting in 1.29 g (89%) of Z-Ala-Ser($\Psi^{Me,Me}$pro)-NHMe as a white powder. $C_{18}H_{25}N_3O_5$ (363.41). Mp 134−136 °C. R_f ($CHCl_3$/MeOH, 20:1) 0.29. t_R = 17.5 min (20−80% B, 30 min, C_{18}). CI-MS (NH_3): 364 (1, $[M + 1]^+$), 305 (23, $[M -$ acetone$]^+$), 206 (8), 143 (5), 127 (11), 91 (100). Pd (98 mg) on charcoal was added to a solution of 980 mg of Z-Ala-Ser($\Psi^{Me,Me}$pro)-NHMe in 30 mL of methanol under H_2 atmosphere. After complete disappearance of the starting material, the mixture was filtered through Celite and the solvents were removed under reduced pressure. The acetylation was performed in 10 mL of acetic anhydride/water (1:1; v/v) within 2 h at room temperature. The solvents were removed under reduced pressure, and the crude product was purified by column chromatography (eluent EtOAc/MeOH, 10:1) to give 630 mg (98%) of Ac-Ala-Ser($\Psi^{Me,Me}$pro)-NHMe as a colorless solid. $C_{12}H_{21}N_3O_4$ (271.32). Mp 60−70 °C. t_R = 14.1 min (5−50% B, 30 min, C_{18}). CI-MS (NH_3): 272 (2, $[M + 1]^+$), 214 (23, $[M -$ acetone$]^+$), 185 (8), 156 (1), 143 (16), 127 (20), 114 (43, $[AcAla]^+$), 91 (100), 86 (60). IR (KBr): 3274 (m), 3059 (w), 2988 (w), 2942 (w), 1641 (vs), 1552 (s), 1424 (s), 1383 (m), 1340 (m), 1306 (m), 1253 (m), 1232 (m), 1203 (w), 1145 (s), 1093 (w), 1057 (s), 1008 (w), 954 (w). Anal. ($C_{12}H_{21}N_3O_4$) C, H, N.

Ac-Ala-Thr($\Psi^{Me,Me}$pro)-NHMe (2). The compound was synthesized starting from Z-Ala-OSu and L-threonine by following the above procedure for Ac-Ala-Ser($\Psi^{Me,Me}$pro)-NHMe: $C_{13}H_{23}N_3O_4$ (285.34). Mp 47−50 °C. t_R = 15.6 min (5−50% B, 30 min, C_{18}). CI-MS (NH_3): 286 (86, $[M + 1]^+$), 228 (70, $[M -$ acetone$]^+$), 199 (10), 141 (11), 114 (100), 99 (13), 86 (30). IR (KBr): 3312 (m), 3084 (w), 2985 (w), 2939 (w), 1640 (vs), 1551 (s), 1420 (s), 1376 (s), 1250 (m), 1216 (w), 1170 (s), 1113 (m), 1094 (m), 989 (m), 954 (m). Anal. ($C_{13}H_{23}N_3O_4$) C, H, N.

Ac-Ala-Cys($\Psi^{Me,Me}$pro)-NHMe (3). Fmoc-Ala-Cys($\Psi^{Me,Me}$pro)-OH[17] (100 mg, 220 μmol) was reacted with a solution of dimethylformamide (DMF) containing 30% diethylamine for 60 min. After the completeness of the reaction (TLC), the solvent was removed and the residue treated with $CHCl_3$ and then filtered to afford 32 mg (63%) of a white solid. Subsequent acetylation was performed with Ac_2O (12.3 μL) in DMF in the presence of *N*,*N*-diisopropylethylamine (24 μL) to provide 35 mg of the desired acetylated compound. Subsequently, the compound was reacted with 80 mg of pyBOP in a saturated solution of methylamine in DMF (3 mL) for 1 h. The solvents were removed under reduced pressure, and the crude product was purified by column chromatography (eluent $CHCl_3$/MeOH/AcOH, 92:6:2) to give 30 mg (98%) of Ac-Ala-Cys($\Psi^{Me,Me}$pro)-NHMe as a colorless solid.

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Ac-Ala-Ser($\Psi^{H,H}$pro)-NHMe (4). To a solution of 5.0 g (12.2 mmol) of Fmoc-Ala-Ser($\Psi^{H,H}$pro)-OH,[17] were added 1.44 g (12.2 mmol) of *N*-hydroxysuccinimide, 70 mL of dioxane/ethyl acetate (6:1, v/v), and 2.76 g (13.4 mmol) of DCC at 0 °C. The suspension was stirred overnight at room temperature. After filtration the solvent was evaporated. The residue was dissolved in 150 mL of ethyl acetate and washed with a 5% solution of sodium carbonate (2 × 80 mL) and brine (2 × 80 mL). The combined organic phases were dried over magnesium sulfate and evaporated. The crude product was purified by precipitation from dichloromethane (DCM)/hexane to yield 4.76 g (9.37 mmol, 78%) of Fmoc-Ala-Ser($\Psi^{H,H}$pro)-OSu as a white powder. Fmoc-Ala-Ser($\Psi^{H,H}$pro)-OSu: $C_{26}H_{25}N_3O_8$ (507.50). Mp 98−100 °C. R_f ($CHCl_3$/MeOH/AcOH, 100:1:1) 0.18. t_R = 14.6 min (40−100% B, 30 min, C_{18}). ^{1}H-NMR (250 MHz, DMSO-d_6, 49 mM): 7.90−7.29 (m, 8 arom. H), 7.83 (*d*, ^3J = 7.1, HN), 5.13 (br., 2 H, H_2-C(2)), 5.00 (dd, ^3J = 3.4, ^3J = 7.3, H-C(4)), 4.38−4.11 (m, 6 H, H-C_{Fmoc}, H_2-C_{Fmoc}, H-C^{α}_{Ala}, H_2-C(5)), 2.80 (s, 4 H, H_2-C_{Su}), 1.20 (d, J = 7.0, H_3-C^{β}_{Ala}). CI-MS (NH_3): 508 (0.7, $[M + 1]^+$), 392 (1, $[M + HOSu]^+$), 279 (3), 178 (100). A 0.80 M solution of methylamine in ethanol (14.7 mL, 9.31 mmol) was added dropwise over a period of 45 min at 0 °C to a solution of 4.50 g (8.87 mmol) of Fmoc-Ala-Ser($\Psi^{H,H}$Pro)-OSu in 30 mL of chloroform. The mixture was stirred for another 20 min at room temperature, diluted with 90 mL of chloroform and washed with 60 mL of a 5% solution of sodium carbonate, 60 mL of a 0.1 M of hydrochloric acid, and brine (3 × 60 mL). The combined organic phases were dried over magnesium sulfate, and the solvent was evaporated. The resulting material was purified by column chromatography on silica gel (eluent EtOAc/AcOH, 50:1) to give 3.27 g (87%) of pure Fmoc-Ala-Ser($\Psi^{H,H}$pro)-NHMe: $C_{23}H_{25}N_3O_5$ (423.47). Mp 122 °C. R_f (EtOAc/AcOH, 50:1) 0.21. t_R = 10.7 min (40−100% B, 30 min, C_{18}). ^{1}H-NMR (250 MHz, DMSO-d_6, 59 mM, two conformers: *major (75%)*, minor (25%)): 7.89−7.28 (m, 10 H, 8 arom. H, 2 HN), 5.19/4.88 (br., AB, 2-H), *5.12/4.93* (AB, J = 3.4, $\Delta\nu$ = 46, 2-H), 4.50−4.07 (m, 6 H, H-C_{Fmoc}, H_2-C_{Fmoc}, H-C(4), H_2-C(5)), 3.82 (quint., J = 4.3, H-C^{α}_{Ala}), 2.57 (d, J = 4.6, H_3-CNH), *1.20* (*d*, J = 6.9, H_3-C^{β}_{Ala}), 1.13 (br., H_3-C^{β}_{Ala}). CI-MS (NH_3): 424 (3, $[M + 1]^+$), 279 (3), 202 (2, $[M - Fmoc]^+$), 178 (100). Purified Fmoc-Ala-Ser($\Psi^{H,H}$-pro)-NHMe (2.09 g, 4.94 mmol) was dissolved in 50 mL of piperidine/DMF, 1:4 (v/v), and stirred for 15 min at room temperature. After evaporation of the solvent, the yellow residue was purified by column chromatography on silica gel (eluent $CHCl_3$/MeOH, 10:1, and $CHCl_3$/MeOH/triethylamine, 50:50:1) to yield 915 mg (92%) of crude H-Ala-Ser($\Psi^{H,H}$pro)-NHMe. Quantitative acetylation was performed by treatment with acetic anhydride/water (1:1, v/v, 12 mL) for 2 h at room temperature. The solvent was evaporated and the crude product purified by chromatography on silica gel (eluent $CHCl_3$/MeOH, 10:1) and crystallized from DCM/cyclohexane to afford 863 mg (78%) of crystalline Ac-Ala-Ser($\Psi^{H,H}$pro)-NHMe. $C_{10}H_{17}N_3O_4$ (243.26). Mp 148−150 °C. R_f ($CHCl_3$/MeOH, 10:1) 0.26. t_R = 17.8 min (0−15% B, 30 min, C_{18}). CI-MS (NH_3): 244 (18, $[M + 1]^+$), 185 (3, $[M - HNCOMe]^+$), 159 (8), 131 (42), 114 (51), 86 (100), 72 (32). Anal. ($C_{10}H_{17}N_3O_4$) C, H, N. The carbon combustion analysis was found to be higher than the expected value; this is supported by the X-ray data which show that the above compound cocrystallized with molecules of CH_2Cl_2 in the solid state.

Ac-Ala-Pro-NHMe (5). L-Proline (9.43 g, 81.9 mmol) was added to 10 mL of an aqueous solution and the pH adjusted to 9 by adding ∼0.1 g of Na_2CO_3. A 1 M solution of Fmoc-Ala-Cl (4.50 g, 13.7 mmol) in acetone was added dropwise over a period of 90 min while periodically adjusting the pH to 8−9 with sodium carbonate (∼2 g). The reaction mixture was then cooled to 0 °C and 3 N hydrochloric acid (30 mL) added to adjust the pH to 2. The aqueous suspension was extracted with ethyl acetate (3 × 100 mL), and the organic phases were combined, washed with brine (2 × 50 mL), and dried over magnesium sulfate. Purification by column chromatography on silica gel (eluent EtOAc/MeOH/AcOH, 100:2:2) resulted in 4.11 g (74%) of pure Fmoc-Ala-Pro-OH. $C_{23}H_{24}N_2O_5$ (408.45). Mp 102−105 °C (lyophilized material). R_f ($CHCl_3$/MeOH/AcOH, 100:5:1) 0.29. t_R = 11.3 min (40−100% B, 30 min, C_{18}). ^{1}H-NMR (250 MHz, DMSO-d_6, 65 mM): 7.89−7.28 (m, 8 arom. H), 7.61 (d, J = 7.5, HN), 4.35−4.16 (m, 5 H, H-C^{α}_{Ala}, H-C^{α}_{Pro}, H-C_{Fmoc}, H_2-C_{Fmoc}), 3.62−3.46 (m, H_2-C^{δ}_{Pro}), 2.10 (m, H-C^{β}_{Pro}), 1.94−1.78 (m, 3 H, H-C^{β}_{Pro}, H_2-C^{γ}_{Pro}), 1.19 (d, J = 7.0, H_3-C^{β}_{Ala}). CI-MS (NH_3): 326 (0.2), 266 (0.5),178 (100). IR (KBr): 3312 (m), 2980 (m), 1718 (vs), 1639 (vs), 1528 (m), 1450 (s), 1247 (s), 1074 (m), 741 (s). The transformation of 3.20 g (7.83 mmol) of Fmoc-Ala-Pro-OH to Ac-Ala-Pro-NHMe was performed by following the procedure for the synthesis of Ac-Ala-Ser($\Psi^{H,H}$Pro)-NHMe. The final product was crystallized from DCM/cyclohexane to yield 840 mg (3.48 mmol) of pure Ac-Ala-Pro-NHMe. $C_{11}H_{19}N_3O_3$ (241.29). Mp 170−172 °C. R_f ($CHCl_3$/MeOH, 85:15) 0.44. t_R = 20.5 min (0−15% B, 30 min, C_{18}). CI-MS (NH_3): 242 (2, $[M + 1]^+$), 210 (1), 183 (3,$[M - HNCOMe]^+$), 155 (2), 129 (19), 114 (51), 86 (9), 72 (100). IR (KBr): 3329 (vs), 2987 (s), 1653 (vs), 1544 (vs), 1418 (s), 1309 (s), 1256 (m), 1199 (m), 972 (m). Anal. ($C_{11}H_{19}N_3O_3$) C, H, N.

Ac-Ala-Ser($\Psi^{(2S)Biphe,H}$pro)-NHMe (6). The compound was synthesized starting from Z-Ala-OSu and L-serine by following the above procedure for Ac-Ala-Ser($\Psi^{Me,Me}$pro)-NHMe and using biphenyl-4-carboxaldehyde dimethyl acetal instead of 2,2-methoxypropane (DMP) in the acid-catalyzed acetalization step. After chromatography purification and crystallization, the pure (*S*)-isomer was obtained. The 2-C chirality is inferred on the basis of X-ray data obtained on the precursor Z-Ala-Ser($\Psi^{(2S)Biphe,H}$pro)-OH.[32] $C_{22}H_{25}N_3O_4$ (395.46). Mp > 180 °C (dec). R_f (EtOAc/MeOH 17:3) 0.33. t_R = 17.9 min (20−80% B, 30 min, C_{18}). CI-MS (NH_3): 396 (1, $[M + 1]^+$), 214 (27, $[M -$ biphenylcarboxaldehyde$]^+$), 310 (28), 281 (35), 224 (31), 152 (13).

Synthesis of Suc-Val-Ser($\Psi^{H,H}$pro)-Phe-pNA (7). Fmoc-Val-Ser($\Psi^{H,H}$Pro)-OH (0.35 g, 0.8 mmol) was dissolved in DCM (10 mL) and isobutyl chloroformate (1.05 equiv, 120 μL) and NEM (1.05 equiv, 78 μL) added to give a white suspension which was stirred for 30 min under nitrogen at −10 °C. H-Phe-pNA (1.05 equiv, 239 mg) dissolved in DCM (2 mL) was added to give a clear solution which was stirred for 4 h to give Fmoc-Val-Ser($\Psi^{H,H}$pro)-Phe-pNA in 60% yield. DCM was evaporated and the residual substance taken up in DMF/morpholine (13 mL of a 5.4% solution). The already slightly yellow solution was stirred under nitrogen for 16 h to give a deep yellow solution. DMF was removed and replaced by ethyl acetate to give a white precipitate, 210 mg (54%) of the white substance (H-Val-Ser($\Psi^{H,H}$pro)-Phe-pNA: ESI-MS 484.5 $[M + 1]^+$). The free amino compound (100 mg) was dissolved with DMF (3 mL) before adding succinic anhydride (2 equiv, 42 mg) and *N*-ethylmorpholine (NEM) (2 equiv, 42 mg) and stirred for 12 h. The desired product was then purified by a preparative C_{18} Sep-Pak column (isocratic 30% A) to give Suc-Val-Ser($\Psi^{H,H}$pro)-Phe-pNA. Mp 124−125 °C. ESI-MS: 584.2 $[M + 1]^+$. t_R = 9.59 min (40−100% B, 15 min, C_{18}). Anal ($C_{28}H_{33}N_5O_9$) C, H, N.

Synthesis of Suc-Val-Ser($\Psi^{Me,Me}$pro)-Phe-pNA (8). Fmoc-Val-Ser-OBzl (0.7 g, 1.36 mmol), PPTS (100 mg, 0.3 equiv), and DMP in toluene/DMF (46 mL/4 mL) under nitrogen atmosphere were heated under reflux during 15 h to give a slightly yellow solution. After evaporation of the solvent, the remaining yellow oil was taken up in $CHCl_3$/MeOH/HOAc (100:3:1, 3 mL) and passed through a silica column using the same eluent to give 370 mg (49%) of Fmoc-Val-Ser($\Psi^{Me,Me}$pro)-OBzl. ESI-MS 557.7 $[M + 1]^+$. t_R = 20.43 min (40−100% B, 15 min). The dipeptide was dissolved in methanol (20 mL) and hydrogenated with Pd/C during 2 h to give Fmoc-Ser($\Psi^{Me,Me}$pro)-OH in quantitative yield. The product was purified by preparative HPLC (C_{18}) to give 260 mg (0.56 mmol) of the expected compound. The activation of the carboxyl group was achieved with cyanuric fluoride (600 mg) in DCM (10 mL) in the presence of pyridine (44 mg, 0.56 mmol) by heating the solution under reflux for 2 h. To the yellowish solution was added water (10 mL) dropwise while keeping the temperature at 0 °C. The organic layer was dried over $MgSO_4$ and the solvent filled up to 10 mL with DCM. H-Phe-pNA (160 mg, 1 equiv), and DIEA (145 mg, 1 equiv) was added and stirred for 1 h. The solvent was removed and replaced by with a solution of DBU in DCM (20%) to immediately turn to a deep yellow solution which was stirred for 30 min. After removal of the solvent, the brown oil was purified by preparative HPLC to give 219.3 mg (76%) of H-Val-Ser-($\Psi^{Me,Me}$pro)-Phe-pNA (ESI-MS: 512 $[M + 1]^+$). The free amine containing product (88 mg), succinic anhydride (18.1 mg, 1.05 equiv) and NMM (18 mg, 19 μL, 1 equiv) were stirred for 30 min to give Suc-Val-Ser($\Psi^{Me,Me}$pro)-Phe-pNA quantitatively. The product was purified by preparative HPLC (20−100% B, 30 min) with 20% yield (68 mg). Mp 186−189 °C. ESI-MS: 612.5 $[M + 1]^+$. t_R = 11.71 min (40−100% B, 15 min, C_{18}). Anal. ($C_{30}H_{37}N_5O_9$) C, H, N.

Synthesis of Suc-Val-Ser($\Psi^{(2R)H,PMP}$pro)-Phe-pNA (9) and Suc-Val-Ser($\Psi^{(2S)PMP,H}$pro)-Phe-pNA (10). Fmoc-Val-Ser-OBzl (0.5 g, 0.968 mmol), PPTS (73 mg, 0.3 eq), and anisaldehyde dimethyl acetal (880 mg, 5 equiv) in THF (dry, 30 mL) were heated under reflux for 2 h. The THF was evaporated and replaced with methanol (30 mL) before a stream of hydrogen (H_2) was bubbled through the solution in the presence of Pd/C catalyst (10%) until full deprotection of the carboxyl was reached (2 h). The black suspension was filtered on Celite and the solvent evaporated (ESI-MS 515.3 $[M + 1]^+$). The coupling with phenylalanine-4-nitroanilide (H-Phe-pNA; 276 mg, 1.05 equiv) was carried out in DCM (25 mL) according the mixed anhydride method (−10 °C, 30 min) using *tert*-butylchloroformate (126 μL) in the presence of *N*-methylmorpholine (NMM) (106 μL) to give Fmoc-Val-Ser($\Psi^{PMP,H}$pro)-Phe-pNA (89%). Deprotection of the Fmoc protection group was achieved without further workup using DBU (200 μL) in DCM (10 mL) within 30 min. After evaporation of the solvent and the DBU, the yellow oil was taken up in 5 mL of acetonitrile/water (1:1) and purified by preparative HPLC(C_{18}) to give the two epimers of H-Val-Ser-($\Psi^{PMP,H}$pro)-Phe-pNA. Acetylation of the mixture with succinic anhydride (100 mg, 1.1 equiv) in DCM (10 mL) in the presence of NMM (35 μL, 1.1 equiv) gave the desired Suc-Val-Ser-($\Psi^{PMP,H}$-pro)-Phe-pNA as an epimeric mixture. Purification by preparative HPLC (20−100% B, 30 min) gave the minor (2*R*) (compound **9**) and the major (2*S*) (compound **10**) epimers separately with 30% and 70% yield respectively. Mp 214−216 °C. ESI-MS: 690.7 $[M + 1]^+$. t_R = 12.77 min (compound **10**) and t_R = 13.3 min (compound **9**) (40−100% B, 15 min, C_{18}). Anal. ($C_{35}H_{39}N_5O_{10}$) C, H, N.

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Supporting Information Available: ^{1}H and ^{13}C NMR tables and Ramachandran maps of the pseudo-proline-containing peptides (13 pages). See any current masthead page for ordering and Internet access instructions.

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