Enhancing the Proline Effect: Pseudo-Prolines for Tailoring *Cis*/*Trans* Isomerization

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Abstract: *Cis*−*trans* isomerization of proline-like oxazolidines and thiazolidines, denoted pseudo-prolines, is investigated spectrophotometrically with a chymotrypsin coupled assay and by ^{1}H NMR. A series of peptides carrying substituents of varying stereochemistry at the 2-C position of ΨPro was prepared for evaluating kinetic and thermodynamic data pertaining to the isomerization of the imidic bond. ΨPro are shown to exhibit an enhanced proline effect, allowing the *cis* content along the imide bond to be tailored between 5 and about 100%. These Pro surrogates may serve as β-turn type VI mimetics and can be used to introduce specifically *cis*-imide bonds into peptides and proteins. ΨPro derived from Ser and Thr show a marked difference in the rate of isomerization about the imide bond compared to Cys derived thiazolidines. As with Pro itself, the *cis* content for the (*S*) epimers of oxazolidines and thiazolidines can be raised from about 40−60% using LiCl/TFE as a solvent, allowing more accurate measurements of the isomerization kinetics. In general, peptides containing ΨPro exhibit enhanced isomerization rate constants for *cis/trans* isomerization compared to their proline analogues depending on stereochemistry and degree of substitution at the 2-C ΨPro position. For example, the thermodynamic barrier of the isomerization process for 2-C dimethylated ΨPro is decreased by about 2−4 kcal/mol in comparison to Pro. In summary, ΨPro represent versatile Pro surrogates enhancing the conformational and structural effects of Pro and offer a wide range of applications in peptide design and engineering.

Introduction

A better understanding of the biological activity of peptide lead structures requires a detailed knowledge of their structure and conformation. Peptide chains exhibit a variety of conformations which are energetically competitive. Long-range cooperative effects stabilize specific conformations required for protein−protein or ligand−receptor interactions.[1] Oligopeptides lack these long-range effects, and covalent restrictions have been introduced to reduce their flexibility.[2] Conformationally constrained peptide analogues are a valuable tool in deciphering the receptor-bound conformation of biologically active peptides.[3] For example, alkylated amino acids have been proposed as a means of restricting the conformational flexibility of peptides targeted to a specific recognition site.[4] Being the only cyclic proteinogenic amino acid, Pro plays a particular role in determining the structural and conformational properties of peptides and proteins.[5] In particular, increased *cis* content along the imidic bond of Xaa-Pro is often observed. *trans*-Xaa-Pro peptide bonds introduce steric interactions between the pyrrolidine ring δ-position and the preceding residue.[6] This raises the free energy difference between *cis* and *trans* conformers relative to peptide bonds devoid of Pro. The energy difference between *cis* and *trans* isomers of a normal amide bond is thought to be due to steric conflicts between adjacent α-carbon substituents. This provides an explanation for the common occurrence of *cis* Xaa-Pro bonds among protein structures.[7] During the refolding process of denaturated proteins, slow *cis/trans* isomerization is often the rate-limiting step due to high rotational energy barriers of *cis/trans* interconversions.[8] For a better understanding of this biologically most relevant conformational change, model peptides containing Pro or Pro-surrogates represent a valuable tool to investigate the origin of isomerization. In this context, we recently introduced the pseudo-proline (ΨPro) concept as a structure disrupting protection technique in peptide chemistry and for modulating biological and pharmaco-kinetical properties of peptides.[9,10]

The straightforward synthetic access allows for the preparation of a variety of Pro surrogates tailored to the structural requirements of the desired target molecules. Most notably, the *cis* content along the imidic bond together with their differential

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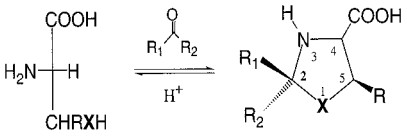

Figure 1. ΨProlines Xaa(Ψpro^{R1,R2}) are obtained by condensation reaction of aldehydes or ketones with Xaa = Ser, Thr, and Cys. X = O, R = CH_3: Thr-derived oxazolidines; X = O, R = H: Ser-derived oxazolidines; X = S, R = H: Cys-derived thiazolidines, R: see Table 1.

acid lability can be modulated within a wide range. Furthermore, structural studies by ^{1}H NMR are facilitated due to the heteroatom at the C1-position of the ring. The characteristic difference of the proton chemical shift of the 2-C protons of the *cis* and the *trans* conformers allows for the measurement of the *cis* content as well as the determination of the thermodynamic parameters $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $\Delta S^{\ddagger}$ and the rate constant of the isomerization of the imidic bond. To test the degree of tolerance of enzymes toward ΨPro as surrogates for Pro, the chymotrypsin coupled assay developed by *Fischer et al*.[12] was used to measure *cis*−*trans* isomerization of ΨPro containing substrates. This enzyme coupled assay exploits the isomer specificity of chymotrypsin mediated hydrolysis of an amide bond between the P2′-P3′ position.[13] Short model peptides of type **Suc-Val-Pro-Phe-*p*NA** are hydrolyzed by chymotrypsin between Phe and *p*NA only if the Val-Pro peptide bond is in the *trans* conformation.[14] After an initial Phe-*p*NA hydrolysis termed burst phase of the present *trans* isomers, only the *cis* isomers are left in solution. Subsequently, this temporary disturbance of the conformational equilibrium relaxes allowing for the kinetics and thermodynamics of the equilibrium to be analyzed by following the UV absorption of the liberated *p*NA. For our purpose, Pro was replaced by various ΨPro derivatives in order to investigate the influence of bulkyness and stereochemistry at 2-C upon the isomerization process. We focus especially on Ser and Cys-derived ΨPro so as to avoid further steric complications of oxazolidines derived from Thr.

Methods

Peptide Synthesis. The ΨPro containing peptides were synthesized according to methodologies described earlier.[9] Mono- and disubstituted oxazolidine containing peptides (**1** and **3**) were obtained by postinsertion[15] using dipeptide Fmoc-Val-Ser-OBzl. Mono- and disubstituted thiazolidine containing peptides (**2** and **4**) were synthesized by condensation of Cys with the corresponding aldehyde or ketone followed by coupling Fmoc-Val-F.[16] Peptide **5** was obtained by condensing aqueous formaldeyhde with Ser and subsequent trapping by Fmoc-Val-F. Suc-Val-Pro-Phe-*p*NA (**6**) was purchased from BACHEM (Bubendorf, Switzerland) and used without further purification.

The Chymotrypsin Coupled Assay. Typically, a stock solution of the substrate in DMSO (10 mg/mL) was prepared, of which 3 μL were pipetted to a solution of 50 μL of chymotrypsin (25 mg/mL, 1 mM HCl) and 1150 μL of buffer (HEPES 0.035 M, pH 7.8) at various temperatures between 3.6 and 16 °C. For each temperature, at least three measurements per compound were recorded until less than 1% deviation was reached. Measuring times varied between 400 and 600 s depending on the completion of isomerization. The absorption of the released *p*-nitroaniline (ϵ = 11 814 M^{-1} cm^{-1}) was monitored on a Hewlett-Packard 8452 A diode array spectrophotometer with a thermostated cuvette holder to give a final absorbance between 0.4 and 0.6 (390 nm). Constant temperature was maintained within the cell (d = 1 cm) by water circulated from a Cryostat Haake D8 (Haake Fisons, Germany). The Hewlett-Packard 89531 A MS-DOS−UV/vis operation software or Sigma Plot Scientific Graphing System Vers. 5.01 (Jandel Corp., U.S.A.) were used for data analysis.

NMR Spectroscopy. Spectra were recorded at 400 and 600 MHz using a Bruker ARX and AMX2 spectrometer, respectively. Samples of about 2−5 mg were dissolved in 0.4−0.5 mL of DMSO-d_6. Chemical shifts were calibrated with respect to the residual DMSO signal (^{1}H: 2.49 ppm; ^{13}C: 39.5 ppm). 2D experiments typically were acquired using 2K × 512 matrixes over a 4000 Hz sweep width in both dimensions. Quadrature detection in the indirect dimension was achieved by using the TPPI procedure.[17] Scalar connectivities were recovered from 2D double quantum filtration (DQF) COSY experiments.[18] Dipolar connectivities were obtained either by the conventional NOESY[19] or ROESY[20] sequence 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.[21] 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 result in square matrixes of 2K × 2K real point data. Proton resonance assignments of the two isomeric forms and assignment of the stereochemistry at the 2-C position were made using (DQF) COSY and ROESY experiments according to previously published protocols.[10] The determination of kinetic parameters was performed by NOESY spectra (600 MHz) recorded at variable temperature using values of 5 s for the recovery delay to ensure restoration of thermal equilibrium. This allowed for the measurement of the exchange ($I_{ct} = I_{tc}$) and diagonal peak (I_{cc} and I_{tt}) intensity. The kinetic constants k_{c-t} and k_{t-c} were calculated using formula (*I*) established under the assumptions of insignificant dipolar cross relaxation with neighboring protons as well as similar external relaxation rates between the exchanging spins.[11] These assumptions were satisfactorily verified within a short mixing time τ_m value (60 ms) selected from a series of NOESY (30, 40, 60 80, 120 ms) experiments showing absence of NOE and good signal-to-noise ratios for integration.

$$k_{t-c} = \frac{2x_c}{\tau_m} \operatorname{arctanh}\left(\frac{I_{ct}}{x_t I_{cc} + x_c I_{tt}}\right) \qquad (1)$$

X_t, X_c are the molar fraction, I_{ct}, I_{cc} the intensities of the cross-peaks in the NOESY spectrum, and k_{t-c} the rate constant for *trans* to *cis* isomerization.

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Cis/trans ratios were determined by integration of the characteristic 2-C protons in the case of **1***S*, **1***R*, **2***S*, **2***R* and for compounds **3** and **4** by integration of the NH-*p*NA protons.[22]

Results

Chymotrypsin Coupled Assay. Substrates **1**−**6** exhibiting different substituents (R_1, R_2) and stereochemistry at the 2-C position of the ΨPro ring were used to determine the kinetic and thermodynamic parameters of *cis/trans* isomerization about the Val-ΨPro imidic bond.

In a preliminary assay, the degree of tolerance of chymotrypsin for the ΨPro unit was tested. It was found that for the investigated ΨPro containing substrates **1**−**5** as well as for the Pro containing substrate **6**, no affection of neither the *cis* content nor the kinetic data could be observed by a 3-fold increase of the standard enzyme concentration ($[E_{st}] = 20\ \mu$M; substrate standard concentration $[S_{st}] = 40\ \mu$M). Consequently, comparable affinities of the enzyme for the artificial substrates as for the Pro substrate can be assumed.

(2-C)-Monosubstituted ΨPro Containing Substrates 1 and 2. Interestingly, even ΨPro with aromatic substituents at 2-C position were recognized by chymotrypsin as prolines enabling the rate constant and associated thermodynamic data for the *cis*−*trans* isomerization of each compound to be determined. They were found to be in good agreement with previously published data on similar systems.[14,34] 2-C (*S*)-configurated ΨPro generally show considerably higher *cis* contents (37%) than their 2-C (*R*)-configurated stereoisomers (5%). A pronounced influence of the heteroatom at position 1 of the ΨPro ring on the kinetics was observed. Oxazolidine derivative **1***S* isomerizes two times faster than its thiazolidine analogue **2***S* (Figure 3).

A severe limitation of the chymotrypsin coupled assay became apparent while measuring the kinetics of the 2-C (*R*)-epimers

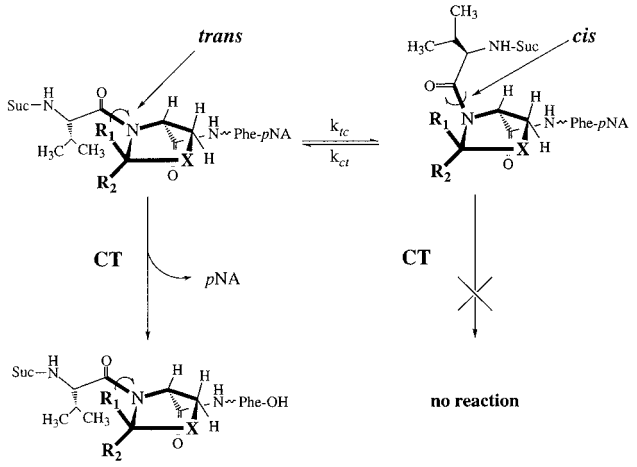

Figure 2. Isomer specific cleavage of the *trans*-Phe-*p*NA amide bond of ΨPro containing substrates by chymotrypsin. The *trans*-Val-ΨPro unit represents a nonnative cleavage site recognized by the serine protease chymotrypsin. Legend: CT = chymotrypsin, *p*NA = *p*-nitroaniline, Suc = succinate.

Table 1. Substrates Investigated in This Study (see Figure 2)[a]

substrate	Xaa	X	R_1	R_2
1*S*	Ser	O	pmp	H
1*R*	Ser	O	H	pmp
2*S*	Cys	S	pmp	H
2*R*	Cys	S	H	pmp
3	Ser	O	CH_3	CH_3
4	Cys	S	CH_3	CH_3
5	Ser	O	H	H
6	Pro	CH_2	H	H

[a] Legend: pmp = *p*-methoxyphenyl, X: heteroatom at position 1 of ΨPro unit. R_1, R_2: substituents at the 2-C position of ΨPro.

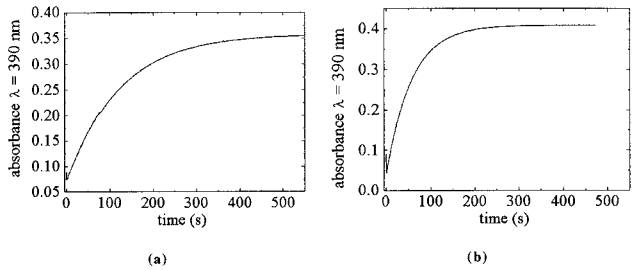

Figure 3. *Cis/trans* isomerization of (2-C)-dimethylated ΨPro containing substrates **3** (a) and **4** (b) as determined by the chymotrypsin coupled assay at $\lambda = 390$ nm.

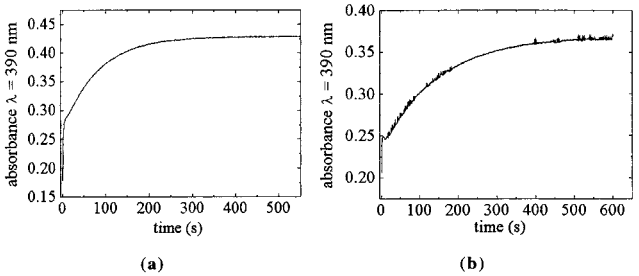

Figure 4. *Cis*−*trans* isomerization of monosubstituted ΨPro containing substrates 1*S* (a) and 2*S* (b) as determined by the chymotrypsin coupled assay at $\lambda = 390$ nm.

1*R* and **2***R* as well as the 2-C dihydrooxazolidine derivative **5**. Containing a vast excess of the *trans* form, the initial burst phase is too dominant to accurately measure the following isomerization kinetics. Since the content of *cis* isomers remains very low after the initial hydrolysis of *p*NA, a detection of the

(22) Integration of Cα protons affords the same *cis* content. Due to signal overlap in some cases, the NH-*p*NA was chosen for all compounds to determine their *cis/trans* ratios.

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(30) The *cis/trans* ratios of all compounds were determined by the integration of the 2-C protons which for each of the *cis* and the *trans* form give separate singlet-signals at around 6.1 and 6.2 ppm, respectively.

(31) The ^{1}H NMR spectrum in DMSO shows one predominant conformation. For certain protons such as the NH-*p*NA proton, traces of another conformation can be detected. However, due to a relative intensity of less than 3% with respect to the major conformation, an unambiguous assignment is impossible. It was yet detected by the CT coupled assay that very few percent of the *trans* form exist depending on temperature and solvent.

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Table 2. First-Order Rate Constants k_{ct} and Thermodynamic Values for the *Cis* to *Trans* Isomerization of Compounds **1**−**6** in 0.035 M HEPES (pH 7.8) as Determined by the Chymotrypsin Coupled Assay[12]

substrate	*cis* [%]	k_{ct}[a] × 10^{-3} s^{-1}	$\Delta G°$ [b] [kcal/mol]	$\Delta H^{‡}_{ct}$[c] [kcal/mol/K]	$\Delta S^{‡}_{ct}$[c] [cal/mol/K]	$\Delta G^{‡}_{ct}$[kcal/mol]	$\Delta G^{‡}_{tc}$[d] [kcal/mol]
1*S*	37 ± 1	17.3	−0.32	21.4 ± 0.9	9.3 ± 3.3	18.6	19
2*S*	37 ± 1	8.5	−0.32	21.8 ± 1	9.3 ± 3.7	19	19.4
3	85 ± 1	12.3	1	20.2 ± 0.3	7.9 ± 1.4	18.9	17.9
4	99 ± 1	37	2.7	19.9 ± 0.9	5.4 ± 3	18.3	15.5
6	5 ± 1[e]	4	−1.76	21.5 ± 0.8	6.8 ± 2.6	19.5	21.3

[a] Measured at 282.5 K at substrate concentrations of 40 μM. [b] From $\Delta G°_{ct} = -lnKRT$ [300 K]. [c] Determined from the Eyring equation $\ln(k/T) = [-\Delta H^{‡}/R][1/T] + \Delta S^{‡}/R + \ln(k_B/h)$. [d] Calculated from the Gibbs equation $\Delta G^{‡} = \Delta H^{‡} - T\Delta S^{‡}$ where R is the universal gas constant, k_B is the Boltzmann constant, and h is Planck's constant. [e] *Cis* content in HEPES (35 mM, pH 7.8) starting from the equilibrium in DMSO. The rate constant for this substrate was determined using LiCl/TFE as solvent.

Table 3. Effect of LiCl (0.5 M in TFE) on the *Cis* Content of ΨPro Containing Substrates (see Table 1) as Determined by the Chymotrypsin Coupled Assay[23]

	cis [%]	
substrate	DMSO	TFE (0.5 M LiCl)
1*S*	37	60
1*R*	5	5
2*S*	37	60
2*R*	5	5

subsequent isomerization becomes inaccurate due to low *cis/trans* ratios. Attempts to increase the *cis* content failed, thus restricting the investigation on the 2-C (*R*)-isomers **1***R* and **2***R* to dynamic ^{1}H NMR studies. For the compounds **1***S* and **2***S*, rate constants of 17.3 × 10^{-3} and 8.5 × 10^{-3} s^{-1} were found, respectively. Compared to the Pro containing substrate **6** (k_{ct} = 4 × 10^{-3} s^{-1}), all ΨPro containing substrates show remarkably increased isomerization rates.

Since this assay is severely limited in the case of substrates exhibiting low contents of *cis* conformation, considerable efforts have been devoted to increase the *cis* population of Xaa-Pro bonds. As reported by *Rich* et al.,[23] the Xaa-Pro amide bond in a solution of LiCl/TFE (or THF) exists predominantly in the *cis* conformation. When a LiCl/TFE solution of substrate is added to a biological buffer, the Xaa-Pro *cis/trans* conformational equilibrium is restored, and the enzymatic catalysis of this process can be monitored. The same holds for the 2-C (*S*)-stereoisomers for both thiazolidine and oxazolidine substrates. Solvation in 0.5 M LiCl/TFE increases the *cis* content to approximately 60% (Table 3). Surprisingly, no effect on the 2-C (*R*)-isomers is observed. Although no precise measurements on the 2-C (*R*)-isomers could be carried out over a broad range of temperature, preliminary assays indicate faster isomerization rates compared to their 2-C (*S*)-counterparts, as revealed by ^{1}H NMR measurements.[24]

(2-C)-Dimethylated ΨPro Containing Substrates 3 and 4. As depicted in Table 2, the *cis* content of the 2-C dimethylated thiazolidine ΨPro containing compound **4** is higher by about 15% compared to the corresponding oxazolidine derivative **3**. Surprisingly, the content of *cis* of compound **4** approaches up to >97%.

In contrast to the findings for the monosubstituted ΨPro substrates **1***S* and **2***S*, substrate **4** has an isomerization rate three times higher than its oxazolidine analogue **3** (Table 2). The transition state energies $\Delta G^{‡}_{ct}$ for **1**−**5** are typically around 1−2 kcal/mol lower than that of the Pro substrate **6**.

^{1}H NMR. The present kinetic and thermodynamic data obtained by the biological assay are in full agreement with the ^{1}H NMR data. However, due to the low solubility (≤1 mg/mL), the ^{1}H NMR measurements were limited to organic solvents which are known to accelerate *cis/trans* isomerization.[25]

(2-C)-Unsubstituted ΨPro Containing Substrate 5. ^{1}H NMR spectrum of compound **5** in DMSO shows broad signals for the *trans* form preventing adequate integration measurements. By applying lower temperatures (282 K) and a mixture of water/NaOH/DMSO (pH 8),[26] the *cis* content could be increased up to 14% while simultaneously reducing the rate of isomerization. The rate constant of 2.15 s^{-1} (Table 4) indicates a decrease of the free energy of activation $\Delta\Delta G^{‡}_{ct}$ (∼2.8 kcal/mol) compared to Pro containing peptide **6** (Table 2). As the steric interactions between the valine residue and the unsubstitued oxazolidine derivative **5** are similar to those found in the Pro containing peptide **6**, it is reasonable to assume that the *cis* ground states of **5** and **6** are comparable in energy. Consequently, the decrease of the free energy barriers $\Delta\Delta G^{‡}$ can be attributed to a stabilization of the transition state.

(2-C)-Monosubstituted ΨPro Containing Substrates 1 and 2. The ^{1}H NMR signals for the peptides **1***S* and **2***S* can be attributed to the two conformations *cis* and *trans* in slow exchange. The stereochemistry at position 2 for the two isomers was determined by using $^3J_{H\alpha-H\beta}$ as previously described.[10]

The substituent of the (*R*)-isomers *syn* to the ΨPro-CO induces mainly *trans*, whereas the (*S*)-epimer (*anti* to ΨPro-CO) adopts the *cis* form up to 50%. The assignment of the two epimers allowed for the determination of both rate constants and thermodynamic values in DMSO and $CDCl_3$ (Table 4). As reported earlier, hydrophobic solvents accelerate *cis/trans* isomerization due to the hydrophobic nature of the transition state.[28] Similar to Pro containing peptide **5**, the rate constants for the ΨPro containing compounds **1** and **2** were considerably faster in $CDCl_3$ and DMSO compared to water. As expected,

Table 4. First-Order Rate Constants and Thermodynamic Values for the *Cis* to *Trans* Isomerization of Compounds **1** and **2** at 300 K in DMSO and $CDCl_3$ as Determined by Dynamic ^{1}H NMR Measurements

substrate	$k_{cis-trans}$[a] [s^{-1}]	$\Delta H^{‡}_{ct}$[b] [kcal/mol]	$\Delta S^{‡}_{ct}$[b] [cal/mol/K]	$\Delta G^{‡}_{ct}$[c] [kcal/mol]
1*R*	6.2 (8.2)	18 ± 1 (18 ± 1)	4 ± 4 (7 ± 3)	16.5 ± 0.1 (16.3)
1*S*	0.4 (6)	20 ± 1 (20 ± 1)	6 ± 2 (10 ± 3)	18.3 ± 0.1 (16.6)
2*R*	2.2 (6)	20 ± 1 (22 ± 1)	10 ± 3 (19 ± 3)	17.2 ± 0.1 (16.6)
2*S*	0.7 (4.5)	16 ± 1 (17 ± 1)	−5 ± 3 (1 ± 3)	18 ± 0.1 (16.7)
5	2.15			16.7 ± 0.1[d]

[a−c] See Table 2. Values measured in $CDCl_3$ in brackets. Standard errors according to *Sandström* et al.:[38] $\Delta\Delta G^{‡}/\Delta G^{‡} = \{[\Delta T/T(\ln k_B T/h/k + 1)]^2 + (\Delta k/k)^2\}^{1/2}/(\ln k_B T/h/k)$; assumed errors ΔT = 0.5 K, k ± 20%. [d] Based on one single measurement at 282 K.

Table 5. Influence of the Polarity of Solvents on the *Cis* Content of Xaa-ΨPro

substrate	X	*cis* [%] H_2O^a	DMSO[b]	$CDCl_3$[b]
1*S*	O	37	41	55
2*S*	S	37	34	42
1*R*	O	5	24	11
2*R*	S	5	13	6
3	O	85	95	90
4	S	100	100	100
5	O	2−3	5	5

[a] Measured by the chymotrypsin coupled assay in HEPES (0.035 M, pH 7.8) starting from the equilibrium in DMSO. [b] Determined by ^{1}H NMR by integration of the *cis* and *trans* signals of the 2-C proton.

$CDCl_3$ being a more hydrophobic solvent further enhanced the rate constants compared to DMSO.

In particular, this method proved to be successful for measuring the rate constant values for 2-C (*R*)-derivatives which were not accurately measurable by the enzymatic assay. A clear decrease of the activation barrier for all compounds investigated outlines the influence of the solvent. DMSO as a more polar solvent is thought to stabilize the ground state, while $CDCl_3$ stabilizes the hydrophobic transition state. Such solvent assisted mechanisms for *cis/trans* isomerization has already been described for Pro and secondary amides.[29] While in the organic solvents $CDCl_3$ and DMSO, the *cis* content of all oxazolidine derivatives are generally higher compared to their thiazolidine analogues; no such differences are observed in DMSO/water systems (Table 5).[30]

(2-C)-Dimethylated ΨPro Containing Substrates 3 and 4. As previously demonstrated, 2-C dialkylated ΨPro induce predominantly the *cis* form of the Val-ΨPro imide bond (Table 2).[10] For the oxazolidine derivative **3**, a set of signals is observed pointing to the presence of about 10% *trans* as detected too by the enzymatic assay. Due to small chemical shift differences and overlaps of α protons, neither the assignment of that minor form nor the calculation of the kinetic constants could be performed in this case. Surprisingly, no clear evidence for the *trans* form of the thiazolidine derivative **4** was found by NMR. Even if small amounts of the *trans* isomer exist (1−2%) according to the enzymatic measurements, its small population disabled a detailed attribution; we can therefore not exclude that it might as well be traces of another conformation not due to *cis*−*trans* isomerism.[31]

The increased rate constant values as measured by the chymotrypsin coupled assay indicate that the equilibrium of the *cis/trans* isomerization is fast resulting in an averaging to one single set of signals. Temperature dependent measurements of the isomerization rates provided the corresponding values of $\Delta H^‡$ and $\Delta S^‡$ after fitting the Van't Hoff plot. The $\Delta H^‡$ values demonstrate that the rotational barrier is essentially enthalpic similar to Pro. The entropy contributions are estimated to be small; however, the limited temperature range of the measurements does not allow for quantitative evaluations of $\Delta S^‡$.

Discussion

Enhanced *cis/trans* rate constants and decreased transition state energies compared to Pro are characteristic for the Xaa-ΨPro moiety. The degree of tolerance of chymotrypsin toward the ΨPro unit suggests no direct interference of the sterically demanding substitutents at 2-C with the substrate binding site of the enzyme. The results show a clear correlation with those obtained in pure water by *Beausoleil* and co-workers[32] on 5-*tert*-butylproline systems and those of *Magaard* for δ-dimethylated Pro.[33] A quantitative assessment of the effect of the heteroatom of the ΨPro ring is difficult. Previous studies using 2-C-unsubstituted oxazolidines revealed considerably increased isomerization rates and a pronounced preference for *trans* preventing the application of the chymotrypsin coupled assay.[34] Decreased transition state energies and accelerated isomerization rates compared to Pro might be due to different ring puckerings induced by the heteroatom.[10,27] However, the effects of the ring conformation are difficult to evaluate, and with the present data an unambiguous attribution is impossible. Being 37% *cis*, the 2-C (*S*)-isomers prove to be ideal candidates for the chymotrypsin coupled assay for obtaining accurate kinetic and thermodynamic data. However, an unambiguous interpretation of the kinetic data cannot be given at this point. A number of parameters such as ring puckering and stereochemistry of the substituents appear to be important. As exemplified by compounds **1***S* and **2***S* showing transition state energies $\Delta G^‡$ of 18.6 kcal/mol (**1***S*) and 19 kcal/mol (**2***S*), the energy barriers for isomerization of ΨPro are lowered by about 1−2 kcal/mol compared to the natural Pro derivative **5**. The relatively large entropic contribution $\Delta S^‡$ of about 9 cal/mol/K suggests that the rate determining step is physical rather than chemical, i.e., the implicated imide bond in the transition state gains additional degrees of freedom compensating for the loss of amide resonance. Despite experimental difficulties due to the domination of the *trans* form, 2-C (*R*)-configurated ΨPro were found to exhibit increased isomerization rates compared to their 2-C (*S*)-counterparts. For 2-C dimethylated peptides **3** and **4**, a dramatic influence of the heteroatom on the isomerization kinetics is found. While for the oxygen containing oxazolidine a rate constant in the order of the monosubstituted substrates is observed, the sulfur containing thiazolidine derivative **4** shows a three times higher isomerization rate compared to compound **3**. The increased distance between 2-C and the sulfur atom compared to oxygen results in a shortened 2-C−N bond.[35] As a result, the 2-C dimethyl groups come closer to the isomerizing bond, which, as a consequence, may twist the amide bond and stabilize the transition state by the presence of the hydrophobic methyl groups.[36] The extraordinarily high *cis* content of 2-C dimethylated ΨPro **3** and **4** can be attributed to steric conflicts induced by the methyl groups in the *trans* form provoking a turn at this position of the peptide. Due to its nearly quantitative *cis* content, such a turn has striking similarities with β-turn type VI. Dimethylated ΨPro might thus find application as mimetics of β-turns type VI. The clearly lower entropic contribution to the free energy $\Delta G^‡$ compared to the monosubstituted substrates indicates a lower flexibility around the isomerizable amide bond imposing higher conformational constraints in the transition state. Theoretical studies by *Karplus* showed that autocatalysis might be an important factor in the catalytic pathway of the Pro dipeptide.[37] Twisting the ψ angle from 0° (characteristic for a type VIa β turn) to 38° leads to a decrease of the effective activation barrier by about 3.6 kcal/mol. Twisting the ψ angle out of plane has been shown to be an important factor in destabilizing the ground state. While water is thought to stabilize the imide bond against twisting, hydrophobic compounds such as organic solvents have been shown to destabilize and thus accelerate *cis/trans* isomerization.[25]

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Conclusion

Isomerization of the imidic bond of peptides containing ΨPro was studied applying a biological assay and dynamic ^{1}H NMR. It is shown that ΨPro are accepted by chymotrypsin as Pro and are isomer specifically hydrolyzed similar to their Pro containing analogues. The significant differences in transition state energies and kinetics of *cis*−*trans* isomerizations between Xaa-ΨPro and the Xaa-Pro systems are indicative for the conformational effects of the substituted five-membered rings. Moreover, ΨPro prove to be valuable tools for delineating the impact of hydrophobic substituents near the isomerizing imide bond on the isomerization barrier. The additional stereocenter at the 2-C ΨPro position allows for the investigation of both the (*R*)- and (*S*)-isomers which show pronounced differences in isomerization rates and *cis* contents. For the first time, it is shown that LiCl/TFE increases stereoselectively the *cis* contents of oxazolidine and thiazolidine imide bonds up to 60% *cis*.

The preferable adoption of the *cis* conformation of 2-C dimethylated ΨPro allows for their use as selective *cis*-Xaa-ΨPro bond inducer to chemically introduce constraint into peptides and proteins and to test the *cis*-imide bond as a structural requirement for the bioactive conformation.

Experimental Section

Materials. All protected amino acides were purchased from Calbiochem-Novabiochem AG (Läufelfingen, Switzerland). Phe-*p*NA was purchased at BACHEM (Bubendorf, 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. Analytical columns (250 × 4.6 mm) were operated at 1 mL/min and preparative columns (250 × 21 mm) at 18 mL/min, with UV monitoring at 214 nm. Solvent A is water (purified on a Milli Q Ion exchange cartridge) containing 0.09% TFA, and solvent B is acetonitrile HPLC−R (containing 10% water and 0.09% TFA), purchased from Biosolve, Valkenswaard, Netherlands. Mass spectra were obtained by electron spray ionization (ESI-MS) on a Finnigan LC 710. ^{1}H NMR spectra were obtained on a Bruker DPX-400 with trimethylsilane internal standard for intermediate compounds. Abbreviations used were as follows: NMM = *N*-methylmorpholine, THF = tetrahydrofurane, DCM = dichloromethane, DMSO-d_6 = dimethyl sulfoxide deuterated, DMF = dimethylformamide, DBU = 1,8-diazabicyclo[5.4.0]undec-7-ene(1,5−5), TFA = trifluoroacetic acid. For the syntheses of **1***R*, **1***S*, and **3**, see ref 10.

Suc-Val-Ser(Ψ^{H,H}pro)-Phe-*p*NA (5). Fmoc-Val-Ser(Ψ^{H,H}pro)-OH (0.35 g, 0.8 mmol) was dissolved in DCM (10 mL) followed by the addition of isobutyl chloroformate (120 μL, 1.05 equiv) and NMM (78 μL, 1.05 equiv) to give a white suspension which was stirred for 30 min under nitrogen at 10 °C. Phe-*p*NA (239 mg, 1.05 equiv) dissolved in DCM (2 mL) was added to give a clear solution which was stirred for 4 h to give Fmoc-Val-Ser(Ψ^{H,H}pro)-Phe-*p*NA in 60% (HPLC). All DCM was evaporated, and the remaining yellow solid taken up in DMF/morpholine (13 mL of a 5% solution). The solution was turning to yellow after stirring under nitrogen for 4 h. The DMF was removed and replaced by with ethyl acetate to precipitate. Cooling to 4 °C for 12 h completed the recrystallization. Two hundred ten milligrams (54%) of a white substance was separated by filtration, ESI-MS 484.5 [M + H]$^+$. Test of ninhydrin proved positive.

One hundrend milligrams of the above-described substance was dissolved with DMF (3 mL) before adding succinic anhydride (2 equiv, 42 mg) and NMM (2 equiv, 42 mg) and stirring for 12 h. The desired product, t_R = 9.59 min (60−100% B, 40 min, C_{18}, 68% purity) was separated from the impurities and secondary products by means of a C_{18} Sep-Pak column (isocratic, 30% A, 70% B) to give Suc-Val-Ser(Ψ^{H,H}pro)-Phe-*p*NA **5** with 88% purity (HPLC analytic). Purification to a single peak on HPLC analytic was done on a preparative HPLC (C_{18}, isocratic, 35% A, 65% B). Overall yield 60 mg (0.1 mmol, 13%). ^{1}H NMR (400 MHz, 303 K, DMSO-d_6, 10 mg/mL) 10.6 (*s*, 1H, NH-*p*NA), 8.46 (*d*, 1H, *J* = 8.44 Hz, NH−Phe), 8.21 (*d*, 2H, *J* = 9.08 Hz, *o*-*p*NA), 8.19 (*d*, 1H, *J* = 9.1 Hz, NH−Val), 7.8 (*d*, 2H, *J* = 9.16 Hz, *m*-*p*NA), 7.19−7.26 (*m*, 5H, aromatics-Phe), 5.29 (*d*, 1H, *J* = 3.36 Hz, 2-C ΨPro), 4.95 (*d*, 1H, *J* = 3.04 Hz, 2-C ΨPro), 4.61 (d × d, 1H, 6.76 Hz, α-Phe), 4.46 (*t*, 1H, *J* = 5.92 Hz, α-Ser), 4.17 (*t*, 1H, *J* = 7.36 Hz, *β*-Ser), 3.95 (*t*, 1H, *J* = 7.88 Hz, α-Val), 3.79 (d × d, 1H, 5.6 Hz, 5.12 Hz, *β*-Ser), 3.31 (*s*, H_20), 3.07 (d × d, 1H, *J* = 6.32 Hz, 5.76 Hz, *β*-Phe), 2.96 (d × d, 1H, *J* = 8.6 Hz, 7 Hz, *β*-Phe), 2.49 (*s*, DMSO) 2.3−2.49 (*m*, 4H, suc), 1.89 (*m*, 1H, *β*-Val), 0.86 (*d*, 3H, 3.16 Hz, *γ*-Val), 0.85 (*d*, 3H, 3.24 Hz, *γ*-Val). ESI-MS 584.2 [M]$^+$. HPLC single peak t_R = 9.51 min (60−100% B, 40 min, C_{18}).

Suc-Val-Cys(Ψ^{Me,Me}pro)-Phe-pNA (4). To Cys(Ψ^{Me,Me}pro)-OH·HCl (0.2 g, 1 mmol) in THF (10 mL) was added DIEA (210 mg, 2 mmol) and Fmoc-Val-F[16] (281 mg, 0.83 mmol) to stir for 12 h at room temperature. After removal of the solvent, the white solid was dissolved in methanol and passed through a silica column ($CHCl_3$/MeOH/HOAc = 100/10/1) to give 160 mg of pure Fmoc-Val-Cys(Ψ^{Me,Me}pro)-OH, ESI-MS 483.5 [M + H]$^+$. Activation of the dipeptide at −10 °C with isobutylchloroformate (54 mg, 1.2 equiv) in DCM (10 mL) using NMM (43 μL, 1.2 equiv) as a base followed by the addition of Phe-*p*NA (93 mg, 1.05 equiv) gave Fmoc-Val-Cys(Ψ^{Me,Me}pro)-Phe-*p*NA, which was deprotected without further workup using DBU (5% in DCM, 10 mL, 10 min). The yellow reaction mixture was evaporated to dryness and taken up in acetonitrile/water (5 mL, 1:1/v/v) before purifying on a HPLC preparative (C_{18}) using a gradient of 5−100% within 30 min. Forty milligrams (9.2%) of NH_2−Val-Cys(Ψ^{Me,Me}pro)-Phe-*p*NA was isolated after lyophilization, ESI-MS 528.5 [M + H]$^+$. Succinic anhydride (15 mg, 2 equiv) and NMM (17 μL, 2 equiv) in THF (5 mL) were added to obtain 24 mg (4.6%) of pure Suc-Val-Cys(Ψ^{Me,Me}pro)-Phe-*p*NA after purification on HPLC (50%-70%, 30 min, C_{18}). HPLC analytic: t_R = 11.29 min (60−100% B, 40 min, C_{18}), ESI-MS 628.5 [M + H]$^+$, ^{1}H NMR (400 MHz, DMSO-d_6, 14 mg/mL, 300 K) 10.68 (*s*, 1H, NH-*p*NA), 8.29 (*d*, 1H, d, NH, *J* = 6.64 Hz, Phe), 8.2 (*d*, 2H, *J* = 8.84 Hz, *o*-*p*NA), 7.87 (*d*, 1H, *J* = 6.72 Hz, NH−Val), 7.75 (*d*, 2H, *J* = 8.88 Hz, *m*-*p*NA), 7.27−7.16 (*m*, 5h, aromatics-Phe), 4.98 (*d*, 1H, *J* = 6.2 Hz, α-Cys), 4.68 (d × d, 1H, *J* = 4.2 Hz, α-Phe), 4.31 (*t*, 1H, *J* = 6.56 Hz, 6.72 Hz, α-Val), 3.35 (*d*, 1H, *J* = 10 Hz, *β*-Cys), 3.2 (*d*, 1H, *J* = 12.21 Hz, *β*-Cys), 3.12 (*t*, 2H, *J* = 6.43 Hz, 5.59 Hz, *β*-Phe), 2.5−2.2 (*m*, 4H, suc), 1.91 (d × d, 1H, *J* = 6.6 Hz, *β*-Val), 1.73 (*s*, 3H, *γ*-Val), 1.62 (*s*, 3H, *γ*-Val), 1.19 (*d*, 3H, *J* = 6.84 Hz, 2-C−CH_3), 0.9 (*d*, 3H, *J* = 6.52 Hz, 2-C−CH_3).

Suc-Val-Cys(Ψ^{pmp}pro)-Phe-pNA (2R and 2S). L-Cys hydrochloride monohydrate (3 g, 17.1 mmol) and potassium acetate (2.5 g, 18.4 mmol) were dissolved in water (26 mL) before the addition of *p*-methoxybenzaldehyde (2.86 g, 21 mmol). The reaction mixture was allowed to stand at room temperature for 3 h. A heavy white precipitate developed, which was filtrated and washed several times with cold ethanol before drying in vacuo to give dry (*R*)- and (*S*)-Cys(Ψ^{pmp}pro)-OH as a diastereomeric mixture. The above synthesized thiazolidine derivative (0.5 g, 2.09 mmol), NMM (212 mg, 2 equiv) in THF (30 mL), and Fmoc-Val-F (710 mg, 2.1 mmol) were stirred at room temperature to give Fmoc-Val-Cys(Ψ^{pmp}pro)-OH with 90% yield (HPLC). After passing the dipeptide through a silica column, 0.55 g (0.8 mmol, 47%) of the pure compound was obtained. Fmoc-Val-Cys(Ψ^{pmp}pro)-OH (137 mg, 0.24 mmol) was dissolved in DCM (10 mL) at −10 °C before adding NMM (49 mg, 0.48 mmol) and isobutylchloroformate (35 mg, 0.26 mmol), and stirring continued for 5 min. Phe-*p*NA (72 mg, 0.25 mmol, 1.05 equiv) in DCM (1 mL) was added, and the reaction mixture was allowed to slowly warm to roomtemperature. Without further workup, Fmoc-deprotection was carried out by the addition of DBU (105 μL, 2.75 equiv, 15 min). All liquid was evaporated, and the remaining yellow oil diluted in few acetonitrile/water (2 mL, 1:1 v/v). The desired compound NH_2−Val-Cys(Ψ^{pmp}pro)-Phe-*p*NA obtained as an epimeric mixture after purification on a HPLC preparative (C_{18}), using a gradient of 5−100% B within 30 min. Treating the epimers with succinic anhydride (40 mg, 2 equiv) in the presence of NMM (40 μL) in DCM (5 mL) gave Suc-Val-Cys-(Ψ^{pmp}pro)-Phe-*p*NA **2***S* and **2***R*. Separation of the stereoisomers was carried out by means of HPLC preparative using a gradient 55−65% B in 30 min (C_{18}). (*S*)-epimer (10%): Yield 24 mg (14%). HPLC analytic: t_R = 11.33 min (60−100% B, 40 min). HPLC analytic: (*R*)-

epimer (90%): Yield 8 mg (4.7%). HPLC analytic t_R = 12.03 min (60−100% B, 40 min). ESI-MS 706.3 $[M + H]^+$. ^{1}H NMR (400 MHz, 10 mg/mL, DMSO-d_6, 300 K).

(*R*)-Stereoisomer 2*R* (*all trans*): 10.5 (*s*, 1H), 8.66 (*d*, 1H, J = 7 Hz, NH−Phe), 8.24 (*d*, 1H, J = 8.7 Hz, NH−Val), 8.2 (2H, *d*, J = 9.2 Hz, *m*-*p*NA), 7.82 (*d*, 2H, J = 9.3 Hz, *o*-*p*NA), 7.68 (*d*, 2H, J = 8.8 Hz, *o*-pmp), 7.25−7.35 (m, 4H, *o*, *m*-Phe), 7.2 (*t*, 1H, *p*-Phe), 6.86 (*d*, 2H, 8.8 J = Hz, *m*-pmp), 6.71 (*s*, 1H, 2-C ΨPro), 4.67 (*m*, 1H, α−ΨPro), 4.65 (*m*, 1H, α-Phe), 3.97 (*t*, 1H, 9 Hz, α-Val), 3.71 (*s*, 3H, OMe), 3.46 (*m*, 1H, *β*−ΨPro), 3.28 (*m*, 1H, *β*−ΨPro), 3.0 (*m*, 2H, *β*-Phe), 2.43−2.34 (*m*, 4H, Suc), 1.77 (*m*, 1H, *β*-Val), 0.58 (*d*, 3H, J = 6.6 Hz, *γ*−Val), 0.33 (*d*, 3H, J = 6.7 Hz, *γ*-Val).

(*S*)-Stereoisomer 2*S* (*cis* 41% and *trans* 59%): 10.65 (*s*, 1H, NH-*p*NA *cis*), 10.58 (*s*, 1H, NH-*p*NA *trans*), 8.43 (*d*, 1H, J = 7.3 Hz, NH−Phe *cis*), 8.27 (*d*, 1H, J = 7.7 Hz, NH−Phe *trans*), 8.2 (*m*, 4H, *m*-*p*NA *cis* and *trans*), 7.81 (*d*, 2H, 9.3 Hz, *o*-*p*NA *trans*), 7.76 (*d*, 2H, J = 9.3 Hz, *o*-*p*NA *cis*), 7.65 (*d*, 1H, J = 8.6 Hz, NH−Val *cis*), 7.54 (*d*, 1H, 8.9 Hz, NH−Val *trans*), 7.3−7.25 (*m*, 10H, aromatics-Phe, *cis* and *trans*), 7.08 (*d*, 2H, J = 8.9 Hz, *o*-pmp *cis*), (*d*, 2H, J = 8.8 Hz, *o*-pmp *trans*), 6.82 (*d*, 2H, J = 9.3 Hz, *m*-pmp *trans*), 6.79 (*d*, 2H, J = 9.3 Hz, *m*-pmp *cis*), 6.37 (*s*, 1H, 2-CH *trans*), 6.12 (*s*, 1H, 2-CH *cis*), 5.73 (*s*, 2H, DCM impurity), 5.37 (*s*, 2H, COOH *cis* and *trans*) 5.2 (*d*, 1H, J = 6.51 Hz, α-ΨPro *cis*), 5.0 (*d*, 1H, J = 8.35 Hz, α-ΨPro *trans)*, 4.75 (d × d, 1H, J = 7.4 Hz, 14.6 Hz, α-Phe *cis*), (d × d, 1H J = 7.8 Hz, J = 14.0 Hz, α−Phe *trans*), 4.47 (d × d, 1H, J = 4.6 Hz, J = 8.8 Hz, α-Val *cis*), 4.47 (d × d, 1H, J = 6.2 Hz, 8.9 Hz, α-Val *trans*), 3.72 (*s*, 3H, OMe *trans*), 3.71 (*s*, 3H, OMe *cis*), 3.64 (d × d, 2H, J = 6.5 Hz, 12.3 Hz, *β*-ΨPro *cis*), 3.15 (d × d, 2H, J = 6.5 Hz, 12.3 Hz, *β*-ΨPro *trans*), 3.12 (*m*, 2H, *β*-Phe *cis*), 2.97 (*m*, 2H, *β*-Phe *trans*), 2.49 (*m*, 4H, suc *trans*), 2.44 (*m*, 4H, suc *cis*), 2.02 (*m*, 1H, *β*-Val *cis*), 1.99 (*m*, 1H, *β*-Val *trans*), 0.81 (*m*, 6H, *γ*-Val *cis* and *trans*), 0.73 (*m*, 3H, *γ*-Val *trans*), 0.62 (*d*, 3H, J = 6.74 Hz, *γ*-Val *cis*).

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Supporting Information Available: Tables comparing the presented data with those obtained by other groups and 400 MHz NMR spectrum of the 2-C region of compound 2S in correlation with the 400 MHz ROESY spectrum (3 pages, print/PDF). See any current masthead page for ordering and Web access instructions.

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