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Pseudo-Prolines: Reversible Conformational Trap of Cyclosporin C as Novel Concept for Prodrug Design

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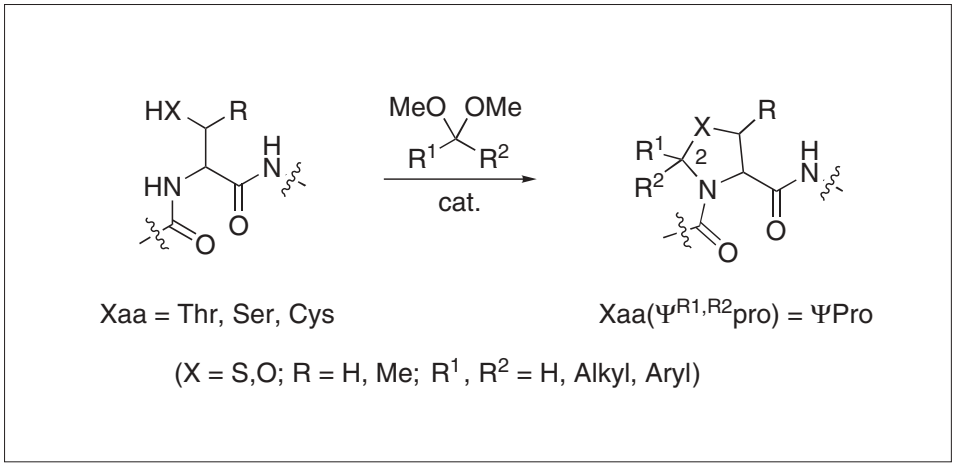
Abstract: The selective and reversible insertion of pseudo-proline (ΨPro) systems in cyclosporin C (CsC) featuring different C(2) substituents at the oxazolidine ring and its impact on the conformational and biological properties is described. The presence of a 5-membered ring exerts drastic effects upon the backbone conformation of CsC as demonstrated by NMR analysis. For example, the number of conformations, in particular in DMSO-d6, is strongly reduced and a *cis* 1-2 amide bond is induced when dialkylated at the C(2) position, resulting in a complete loss of the binding capacity to its receptor CypA. The reversibility of ΨPro insertion allows the temporary introduction of conformational constraints representing a new strategy in pro-drug design.

Keywords: Conformational constraints · Cyclosporin · Pro-drug · Pseudo-proline

Introduction

Our laboratory has developed a new class of proline mimics, referred to as pseudo-prolines (ΨPro), for the enhancement of the conformational effects of Pro (Scheme 1). A striking feature of C(2)-dialkylated pseudo-prolines is the induction of a *cis*-amide bond preceding the pseudo-proline unit [1]. As a consequence, the direction of the peptide chain containing such a building block is reversed, resulting in the disruption of secondary structures (notably of β-sheets) and consequently, in the enhancement of solvation effects during peptide synthesis due to the prevention of aggregation caused by hydrophobic interactions [2]. Furthermore, pseudo-prolines were used as a general tool for targeting molecular recognition processes [3]. We have recently demonstrated that ΨPro could be directly inserted into complex peptides such as cyclosporin C (CsC). So far we have shown that by reacting CsC with aromatic dimethylacetals, cyclocondensation occurs leading to oxazolidine (ΨPro) containing cyclosporin derivatives [4].



Scheme 1.

Based on these results, we describe in the present article the conformational and configurational aspects resulting from the reversible direct insertion of pseudo-proline (ΨPro) [1–3] into cyclosporin C. In particular, the structural impact of a ΨPro-induced 1-2 *cis*-amide bond on the overall structure of the cyclic peptide is evaluated by the insertion of various C(2) mono and dialkylated pseudo-proline systems [3a].

Product Synthesis and Characterization

Standard Procedure for the Synthesis of Thr(Ψ^{R1,R2}Pro)^{2}Cs

Dry CsC (100 mg, 0.082 mmol), $R^1R^2C(OMe)_2$ (4.11 mmol, 50 equiv.) and PPTS (6 mg, 0.024 mmol, 0.29 equiv.) in dry DMSO (8.2 ml) was heated to 100–120 °C. After 16 h, the cooled reaction mixture was poured into 500 ml of ethyl acetate/water 1:4 (v/v). The organic layer was washed with brine (5×200 ml) and dried over sodium sulfate. After concentration under reduced pressure, the crude material was pu-

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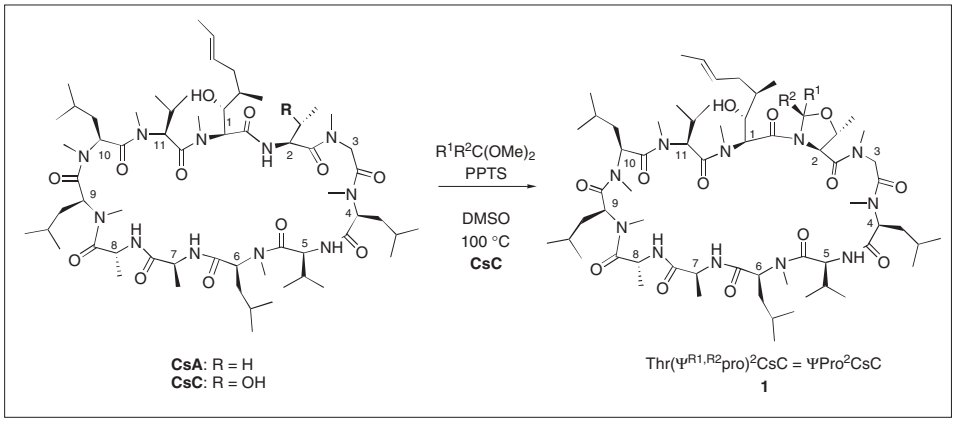
Table 1. Direct-insertion of ΨPro-systems into CsC (see Scheme 2)

	Compound **1**		Yield [%]
	R^1	R^2	
CsC	–		
1a	Me	H	33
1b	Et	H	34
1c	^{n}Pr	H	39
1d	^{i}Pr	H	19
1e	^{t}Bu	H	0
1f (S)	Ph	H	61
1f (R)	H	Ph	39
1i	Me	Me	26
1j	Et	Et	22
1k	^{n}Pr	^{n}Pr	12
1l	Ph	Ph	9
1m	^{i}Pr	^{i}Pr	0
1n	CF_3	CF_3	0



Scheme 2.

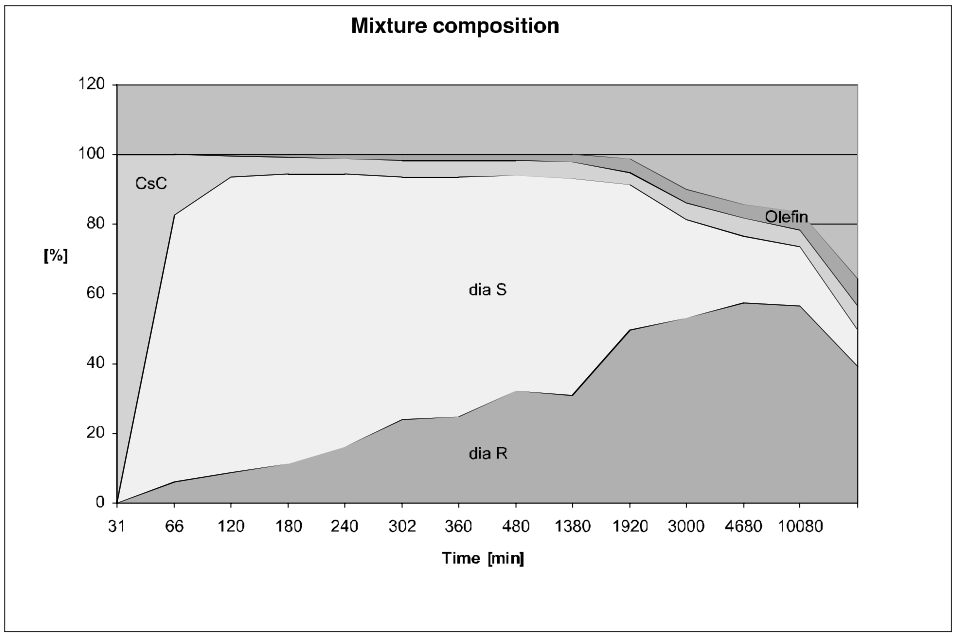


Fig. 1. Time-dependent evolution of the composition of the reaction mixture (**1f**). The kinetically favored (*S*)-diastereoisomer initially obtained is slowly converted to the (*R*)-diastereoisomer (thermodynamic product).

rified by chromatography on silica (acetone/hexane, 4:6) to yield Thr($\Psi^{R1,R2}$Pro)^2 CsC as a white powder.

All compounds were characterized by HPLC, mass spectroscopy, and ^{1}H NMR.

Results and Discussion

CsC (Scheme 2) differs from the well-known immunosuppressive analogue **CsA** by the presence of a trifunctional amino acid at position 2, *i.e.* a threonine (Thr^2) residue replacing 2-amino butyric acid (Abu^2) in CsA. While the biological and pharmacokinetic properties of CsC and CsA are very similar [5], the presence of the OH group of Thr^2 together with a non-methylated amide between residues 1 and 2 renders CsC a most attractive candidate for applying the ΨPro concept to study conformational changes [3a][4a].

Despite the steric constraints involved in the cyclocondensation of CsC with alkyl acetal derivatives (Scheme 2, Table 1, **1a–d**), the direct-insertion proceeds very selectively in one single step with acceptable yields (19–39%). Even strongly hindered oxazolidines derived from dimethylated, diethylated and di-n-propylated ketals were obtained in yields ranging from 12 to 26% (**1f–h**). The somewhat lower yields compared to the C(2)monosubstituted derivatives [3a] are indicative of the increased conformational constraints present in the target compounds **1f–i**. Furthermore, the synthesis of extremely sterically demanding oxazolidines such as C(2)-*tert*-butyl, -di(trifluoromethyl) and -diisopropyl derivatives *via* direct insertion of the corresponding substituted ketals proved to be impossible under the established experimental conditions.

In the case of C(2)-monosubstituted pseudo-prolines, alkyl derivatives yield exclusively the (*S*) epimer while in the case of aryl derivatives both epimers are observed. The ratio between the diastereoisomers (*S*) (kinetic derivative) and (*R*) (thermodynamic derivative) could be modulated by changing reaction time and temperature (Fig. 1).

The influence of the insertion of a ΨPro on the backbone conformation was investigated using 1D and 2D (TOCSY, ROESY, COSY-DQF, and HSQC) NMR spectroscopy. Because of the pronounced hydrophobicity and insolubility in D_2O of the target molecules, $CDCl_3$ and DMSO-d6 resembling physiological conditions to some extent [6], were used as solvent. Two categories of products have been investigated. Category one includes the C(2)-monosubstituted ΨPro derivatives **1a–h** which show only one conformation in $CDCl_3$ but multiple conformations in DMSO-d6. Category two includes C(2)-disubstituted ΨPro derivatives **1i–l** comprising a limited number

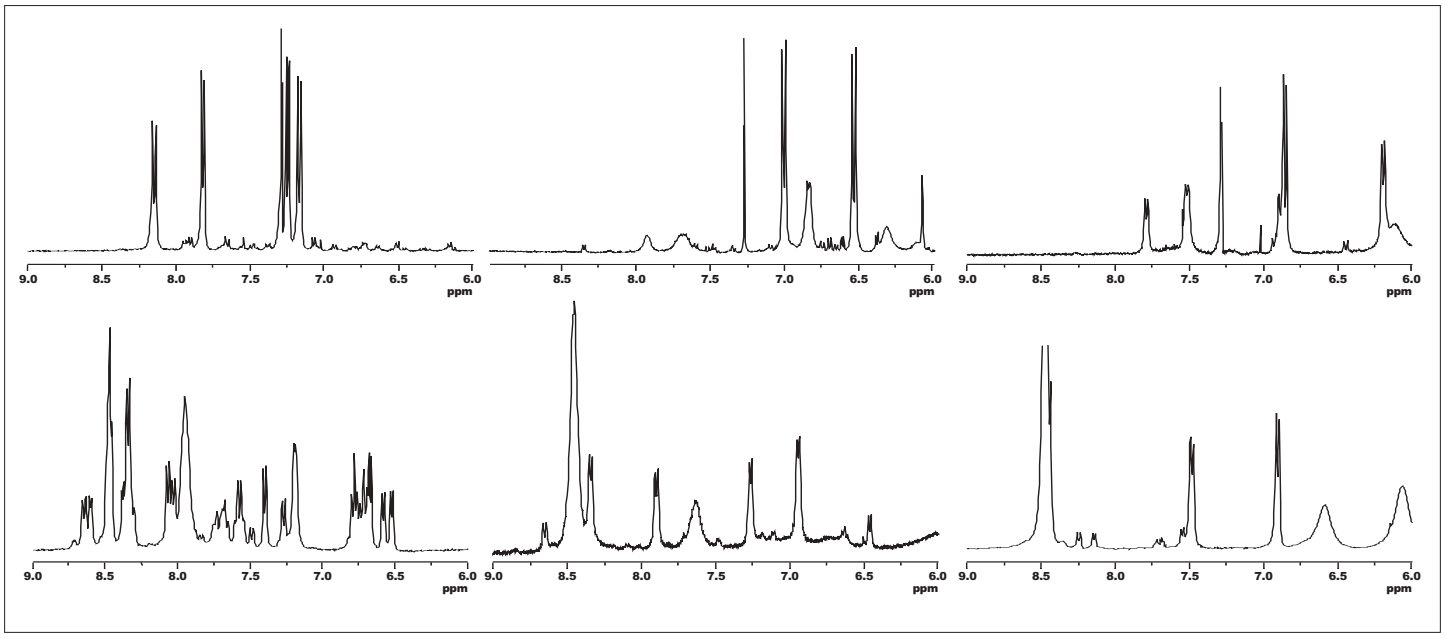


Fig. 2. ^{1}H NMR of the NH region of CsC (left), **1a** (middle) and **1i** (right) in $CDCl_3$ (top) and DMSO-d6 (bottom)

of conformations in $CDCl_3$ as well as in DMSO-d6 (Fig. 2). These results point to a striking similarity in the conformational properties of monosubstituted ΨPro-CsA derivatives and CsC. However, the presence of disubstituted ΨPro building blocks rigidifies the cyclosporin ring to such extent that only two or three conformations are accessible even in DMSO-d6.

The presence of *cis*/*trans* amide bonds was determined using ROESY experiments. A *cis*-amide bond is characterized by a strong correlation between two consecutive H_α. This is exemplified for derivative **1i** in DMSO-d6 showing that the two substituents at position C(2) of ΨPro induce a 1-2 *cis*-amide bond in both conformers (Fig. 3), in agreement with previous studies on model dipeptides of the type Xaa-ΨPro [7]. The main difference between the two conformers arises from amide bond 3-4, which was found to be *trans* in the major and *cis* in the minor conformer, whereas the remaining amide bonds exhibit exclusively the *trans* conformation. This result demonstrates that even in cyclic peptides the presence of a dimethylated ΨPro induces a *cis*-amide bond to more than 50%. More bulky alkyl chains at the C(2) position enhance this effect even further as shown for the di-n-propyl derivative **1i**.

In the case of C(2)-monosubstituted pseudo-proline derivatives, a new asymmetric center is generated during the reaction. The relative configuration was determined by 2D-NMR spectroscopy based on a ROESY spectrum. Here, a strong NOE effect was observed between $2H_\beta$ and $2H_{2\alpha}$ indicating an (*S*)-C(2) asymmetric carbon (Fig. 4). Interestingly, this observation is different from the conformational effects of ΨPro-systems in model dipeptides [8] and derivatives obtained by direct-insertion of aromatic ΨPro systems into CsC [3a][4a] where both epimers are present.

Stability of ΨPro

ΨPro-derivatives **1a–n** exhibit differential acid stability, depending on the character of the C(2)-substitution of the oxazolidine system. For the evaluation of the chemical stability of the ΨPro-ring, the compounds were subjected to conditions resembling the native environment. For example, in a mixture of fetal bovine serum/methanol 1:1 (v/v), **1i** proved to be completely stable (>1 month at 37 °C). In 1M HCl_{aq}/acetonitrile 1.1 (v/v) and in 0.1M HCl_{aq}/acetonitrile 1:1 (v/v), half-lives of around 83 min and 17 h, respectively, were determined as monitored by analytical HPLC (Fig. 5). Under the hydrolysis condi-

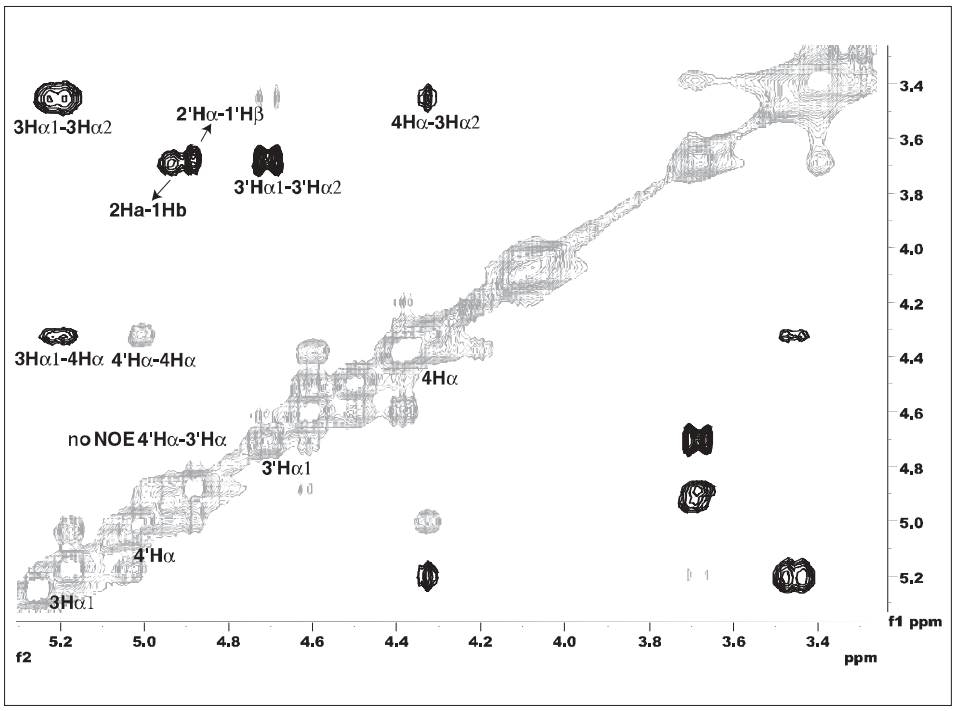


Fig. 3. ROESY of **1i** in DMSO-d6. The major conformer has 1-2 and 3-4 *cis*-amide bonds (NOE 2Hα-1Hβ and 3Hα-4Hα). The minor conformer shows only one *cis*-amide bond 1-2 (NOE 2'Hα-1'Hβ, no NOE between 3'Hα and 4'Hα).

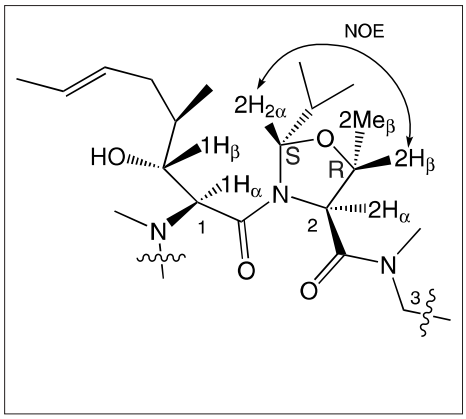


Fig. 4. ROESY experiment for the determination of the relative configuration of C(2) for the ^{i}Pr derivative **1d**.

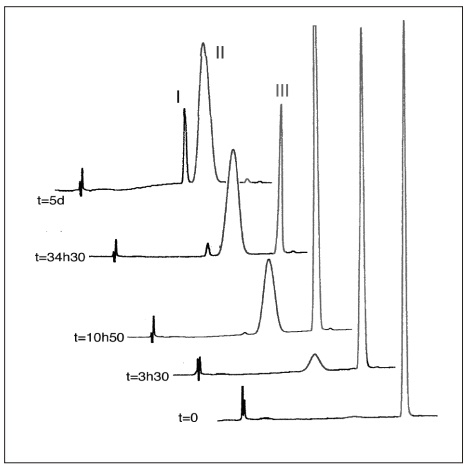


Fig. 5. Hydrolysis of **1i** in 0.1M HCl/acetonitrile 1:1 (v/v) (I: isoCsC, II: CsC, III: **1i**).

tions applied, the restored parent CsC slowly converted to isoCsC as described elsewhere [9].

At pH 6.6, all derivatives except **1g**(*S*) proved to be stable for several days. The stability of ΨPro-containing cyclosporins in water is strongly related to the capacity of the substituents to stabilize the cation at the C(2) position *i.e.* Me (**1a**) Ph (**1f**(*S*)) (Table 2). Most notably, the presence of a R^2 substituent increases the stability of the derivatives drastically (Table 2). Shielding of the oxygen and thus hampering protonation as the first step of the hydrolysis cascade might be a plausible explanation. In the case of R^2 substituted pseudo-prolines, the oxygen is surrounded by neighboring groups on both sides of the ring while in monosubstituted ΨPro one face of the oxygen can be freely approached by the proton.

The *in vitro* activity of cyclosporin derivatives was assessed by using the IL-2 reporter gene assay. The immunosuppressive activity of the derivatives is determined as substances interfering with the activation of the T cell signaling cascade inhibit IL-2 gene transcription and thus IL-2 production [10]. Furthermore, the binding affinity to CypA was assessed by using the improved spectrophotometric assay described by Rich and coworkers [11]. As expected from the observed conformational constraints induced by the insertion of ΨPro systems into CsC, derivatives **1a–n** proved to be trapped into a non-bioactive conformation thus providing a versatile target for prodrug design.

Table 2. Hydrolysis kinetics of representative pseudo-proline containing cyclosporins

	Compound **1**		Half-life time
	R^1	R^2	pH 1 [h]
1a	Me	H	7
1d	^{i}Pr	H	9.5
^{1}f(*S*)	Ph	H	1.7
^{1}f(*R*)	H	Ph	49
1i	Me	Me	17
1k	^{n}Pr	^{n}Pr	27
1l	Ph	Ph	27.5

Conclusions

In summary, the incorporation of ΨPro systems into CsC results in a pronounced reduction of the conformational space compared to the parent molecule. In tailoring the steric and electronic features of the C(2) substituents, differential acid stability of the ΨPro containing CsC derivatives is achieved, paralleled by a complete loss of their receptor binding capacity. Consequently, in applying polar C(2) substituents, the present concept gives access to a new class of water-soluble prodrugs ('soft prodrugs') of considerable therapeutic relevance.

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