Pseudoprolines (ΨPro) in Drug Design: Direct Insertion of ΨPro Systems into Cyclosporin C

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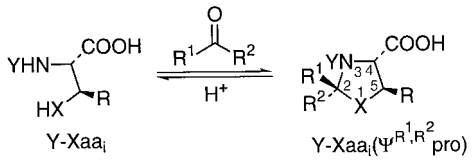
Abstract: The insertion of acetals that exhibit variable structural features into complex peptides such as cyclosporin C (CsC) results in oxazolidine derivatives (pseudoprolines, ΨPro) of tailored physico-chemical and biological properties. *N*,*O*-Acetalation of the 2-threonine hydroxyl group and the preceding amide nitrogen of CsC is achieved by treating the molecule with a number of both arylated and non-arylated dimethyl acetals. The ΨPro-containing CsC derivatives exhibit enhanced conformational backbone rigidity, as suggested by analytical HPLC, NMR spectroscopy and by kinetic measurements on binding with their receptor protein cyclophilin A (CypA) that were not time-dependent. IC_{50} values for calf-thymus CypA were obtained by kinetic evaluation of its *cis* → *trans* isomerase activity. The choice of the *para*-substituted aryl dimethyl acetals allows the inhibitory properties of the corresponding derivatives to be modulated to either prodrugs or moderately strongly binding cyclosporin C derivatives.

Keywords: amino acids · cyclosporin derivatives · drug research · isomerases · peptides · pseudoprolines

Introduction

Numerous chemical methods for the modulation or optimisation of the physico-chemical, pharmacological and biological properties of natural peptides or lead compounds have been developed and successfully applied in drug development. The recently introduced pseudoproline concept offers an elegant and versatile alternative to existing strategies for the chemical modification of bioactive peptides. According to this approach, serine-, threonine- and cysteine-containing peptides can readily be converted by intraresidual *N*,*O*- or *N*,*S*-acetalation into five-membered ring systems (oxazolidines or thiazolidines), referred to as pseudoprolines (ΨPro) due to their structural similarities to the naturally occurring amino acid proline (Scheme 1).[1] So far, pseudoprolines have found applications in providing solubilising, secondary-structure disrupting building blocks for the synthesis of difficult peptide sequences[1, 2] and for the reversible induction of *cis*-amide bonds into peptide backbones.[3] More recently, the ΨPro concept was extended to targeting *cis*-amide bonds in biologically relevant recognition processes.[4]



Scheme 1. Formation of pseudoprolines: Xaa$_i$ = Ser (X = O, R = H) or Thr (X = O, R = CH_3, Y = *N*-protected amino acid Xaa$_{i-1}$) give oxazolidine-containing dipeptide derivatives; Xaa$_i$ = Cys (X = S, R = H, Y = H) gives thiazolidines.

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Up to now, the incorporation of ΨPro systems into peptide backbones was achieved by preforming the corresponding ΨPro building blocks according to Scheme 1 and subsequently coupling them to the peptide chain. Here, we explore the direct incorporation of pseudoprolines into complex peptide structures using the example of cyclosporin C (CsC). This strategy allows the efficient preparation of a large variety of derivatives that exhibit differential structural and functional features.

Cyclosporins are a family of hydrophobic, cyclic undecapeptides with a remarkable variety of biological functions. Among these are immunosuppression,[5] promotion of nerve outgrowth in neurodegenerative diseases[6] and blockage of HIV-1 replication.[7] Today, some 30 members of the family have been isolated from natural sources.[5] The best known is cyclosporin A (CsA), which has been in clinical use since 1983 under the trade names Sandimmun® and Neoral® to prevent rejection of organ transplants. Since the discovery in 1986 that the activation of CD4+ cells is inhibited by CsA, research for new cyclosporin derivatives has intensified with the aim of finding a selective drug for immunosuppression and blockage of the HIV-1 pathway.[8] The mode of action of all cyclosporins and their derivatives is linked to their receptor proteins, the ubiquitously occurring peptidylprolyl *cis/trans* isomerase

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cyclophilin (Cyp).[9] The strong hydrophobic binding of the two loops protruding from the surface of the enzyme involves interactions between 13 residues of Cyp and residues 10(MeLeu), 11(MeVal), 1(MeBmt), 2(Abu) and 3(Sar) of the undecapeptide (Figure 1). The other half of the cyclic peptide remains exposed on the surface; this enables further interactions with a second protein, the cellular phosphatase calcineurin (Cn).[10] The complex [(CsA-Cyp)-Cn] has been proved to be responsible for immunosuppression by inhibiting the transcription of essential genes for cytokines, for example IL-2.

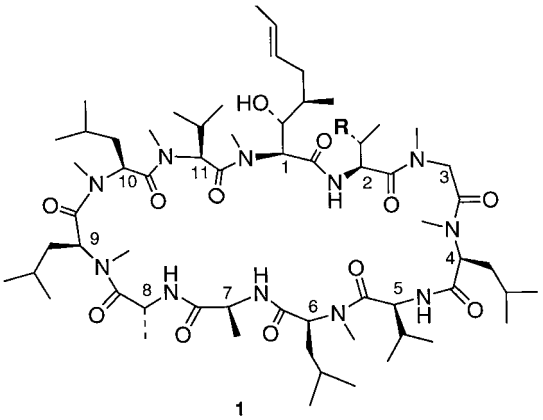
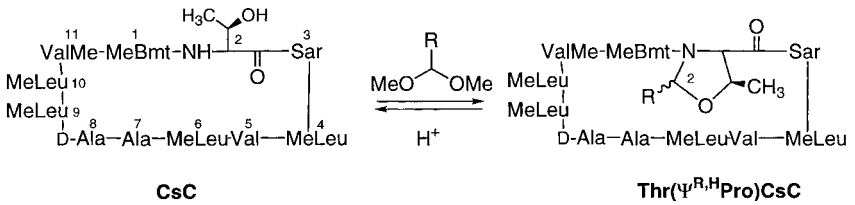


Figure 1. Molecular structure of cyclosporin A (CsA; R = H) and cyclosporin C (CsC; R = OH). The binding region for cyclophilin A comprises residues 1–3, 10 and 11.

CypA is also required for HIV-1 replication. The HIV-1 Gag polyprotein $Pr55^{gag}$ specifically binds CypA through a proline-rich conserved domain at the N-terminal region of the $p24^{gag}$ capsid protein.[11] In addition, host CypA is incorporated into HIV-1 virions and is a requirement for viral replication.[12] The presence of cyclosporin prevents these interactions and inhibits the replication of the virus. For this reason, a strong ligand for cyclophilin A which can interrupt the interaction between cyclophilin and $p24^{gag}$ would be a potent candidate for an HIV drug.[13]

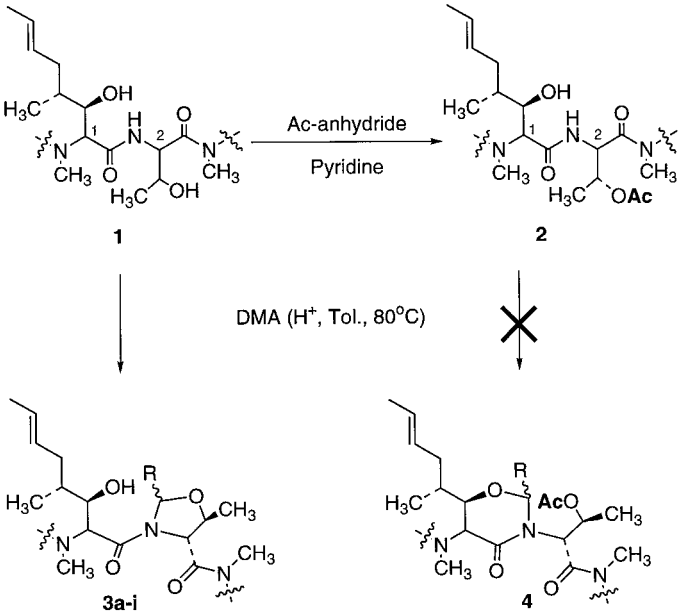
We focus here on the analogue cyclosporin C (CsC), which exhibits similar structural and functional properties to CsA.[14] Most notably, CsC differs from the commercial drug CsA only by the substitution of threonine for 2-aminobutyric acid (Abu) at residue 2 (Figure 1); this allows the application of a direct-insertion strategy for accessing novel ΨPro-containing derivatives. The structural modification in the binding region of cyclosporin with its receptor proteins[15] should afford new insights into the molecular recognition process of Cs receptor interactions. In addition, the temporary insertion of a C-2 substituent (R in Scheme 2) of variable structural and functional properties offers interesting perspectives in prodrug design.[16]



Scheme 2. Regioselective *N,O*-acetalation of cyclic undecapeptide CsC to 2-C-substituted ΨPro-derivatives of CsC.

Results and Discussion

Synthesis and characterisation of CsC derivatives 3a–i: During *N,O*-acetalation, the side chain OH functions of both the 1-Bmt and 2-Thr could potentially react to form a perhydro-oxazinone or an oxazolidine (ΨPro) system, respectively (Scheme 3). According to previous work by von Wartburg and co-workers,[17] a pronounced difference in the reactivities of the two hydroxyl groups results in a regioselective acetylation of the 2-L-threonine residue when treated



Scheme 3. Differential reactivity of the OH side chains of 1-Bmt and 2-Thr during *N,O*-acetalation of CsC. After selective acylation at 2-Thr to derivative **2**, no acetalation to **4** is observed. Direct insertion of a series of dimethyl acetals $RHC(OCH_3)_2$ (DMA) into **1** results in ΨPro-containing CsC derivatives (**3a–i** in Table 1)

with acetic anhydride in pyridine to yield **2**. In order to exclude the occurrence of a competing reaction that gives the perhydro-oxazinone (**4**), acetylation of CsC (**1**) was carried out and the isolated [2-Ac-Thr]CsC (**2**) was treated with a number of arylated dimethyl acetals under conditions typically applied in the formation of oxazolidines (ΨPro).

Even after extended reaction times at 80 °C, no detectable ΨPro insertion occurred. Therefore, it was concluded that the difference in reactivity of the two homologous side-chain functionalities is sufficiently high for a regioselective reaction of cyclosporin C with the corresponding dimethyl acetals. As shown in Table 1, a number of both arylated and non-arylated

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Table 1. ΨPro 2-C monosubstituted derivatives of CsC obtained by condensation of dimethyl acetals (DMA, $RHC(OCH_3)_2$) to CsC (see Schemes 2 and 3). IC_{50} values concern inhibition of calf-thymus cyclophilin A.

R	Compound	Reaction time [min]	Yield [%]	Mass calcd	Mass found m/z[a]	IC_{50}/IC_{50CsA}
Ph	**3a**[b]	45	74	1306.7	1306.7	6.0
p-Ph−C_6H_4	**3b**[b]	30	89	1382.8	1383.8	5.8
CH_2=CH	**3c**[b]	60	75	1256.7	1257.7	5.3
p-MeO_2C−C_6H_4	**3d**[b]	120	55	1364.7	1364.7	7.8
p-MeO−C_6H_4	**3e**[c]	60	90	1336.2	1337.2	52.1
	3f[c]	60	90	1336.2	1337.2	15.4
p-AllOOC−C_6H_4	**3g**	50	95	1390.7	1391	4.0
p-HOOC−C_6H_4	**3h**	50[d]	75	1350.7	1351	24.1
PEG	**3i**	240	20	≈1851	≈1851[e]	21.5

[a] ESI-MS. [b] Epimeric mixture [2-C(*R*) + (*S*)]. [c] Compounds **3e** and **3f** are separated epimers. The stereochemical assignment at the ΨPro 2-C position of epimers **3e** and **3f** was determined by ^{1}H NMR spectroscopy. [d] Obtained by allyl-deprotection of compound **3g**. All = allyl protection group. [e] Typical Gaussian distribution in ESI-MS due to polydisperse polymeric distribution.

dimethyl acetals react smoothly with cyclosporin C to give the corresponding oxazolidine derivatives **3a–i** (Scheme 3) in yields between 20 and 90%.

A striking feature of the HPLC analysis of compounds **3a–i** is the sharper shape of the peaks compared with CsC (Figure 2); this points to a narrower distribution of conformations[18] due to the conformational constraints induced by

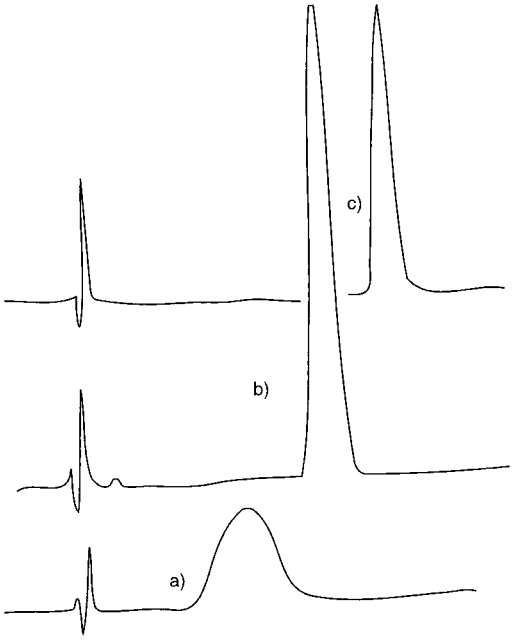

Figure 2. a) HPLC profile of CsC. b) HPLC profile of CsC ΨPro derivative **3e**. c) HPLC profile of CsC ΨPro derivative **3f**. Gradient: 50–100% acetonitrile in water, 50 min, C_{18}.

the oxazolidine system (Figures 2 and 4, shown below). This result is further confirmed by ^{1}H NMR analysis of the NH region. In the case of the pseudoproline derivative 2-L-Thr($\Psi^{Ph,H}$pro) (**3a**) only two major conformers are observed in DMSO, whilst in the case of the starting material CsC, a large number of conformers is present (Figure 3). This finding shows that the threonine–sarcosine region within cyclosporins is particularly involved in conformational stabilisation. As previously demonstrated by Wenger et al.,[19] the CsA derivative [D-MeSer^3]CsA exhibits nearly identical backbone conformations in solution and when bound to cyclophilin. This conformational stabilisation is not necessarily limited to residues in close sequential proximity to ΨPro and the biologically active all-*trans* form can be stabilised in solution through long-range interactions through the peptide backbone. It may therefore be suggested that, in the oxazolidine-containing derivatives of CsC, a backbone conformation similar to the bioactive conformation is stabilised. This hypothesis is supported by inhibitory measurements of derivatives **3a–i**. CsA and CsC are known to be slow-binding inhibitors, exhibiting maximum inhibition to cyclophilins only after 30 minutes from onset owing to major conformational changes. For the ΨPro-containing CsC derivatives, no such time-dependent kinetics could be detected; this suggests a preformed, bioactive-like backbone conformation for these peptides. In order to exclude traces of CsC under the conditions of the biological tests, a monoclonal antibody[20] raised against CsC was added. This revealed that the medium was devoid of detectable amounts of CsC.

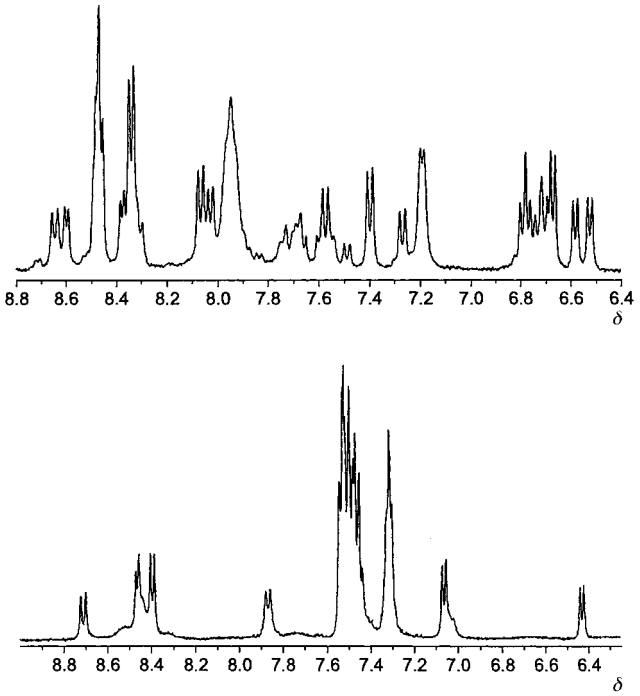

Figure 3. Top: ^{1}H NMR spectra of CsC. Bottom: ^{1}H NMR spectra of [2-L-Thr($\Psi^{Ph,H}$pro)]CsC **3a** in DMSO. The presence of the pseudoproline reduces the number of conformers.

The use of aldehydes to form intraresidual *N,O*-acetals results in a chiral centre at the 2-C position of the oxazolidine, that is, 2-C(*R*) and 2-C(*S*) (Scheme 3). In the case of the anisaldehydedimethyl acetal-derived epimers (**3e** and **3f**), we were able to separate the two products by reversed-phase HPLC. ^{1}H NMR analysis allowed for the stereochemistry of the pseudoproline unit to be attributed according to previously established techniques.[3a] The 2-C protons were identified by an NOE crosspeak to the *ortho* protons of the 2-C *p*-methoxyphenyl (pmp) substituent. In the case of the 2-C(*R*)

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epimer, the β-proton of the threonine forms a strong crosspeak to the *ortho*-pmp protons, which is missing for the 2-C(*S*) congener. Additionally, the α-Thr exhibits an NOE crosspeak to the 2-C(S) proton of the oxazolidine. In agreement with our findings for pseudoproline-containing dipeptides, the 2-C(*S*) epimer (**3e**) exhibits a double set of signals. This is most probably due to the higher transition-state barrier of *cis/trans* isomerisation along the ^{1}Bmt-^{2}Thr(ΨPro) peptide bond and results in separate signals for each conformer.[3b] The NMR analysis of the phenyl derivative (**3a**) in DMSO shows the presence of two conformers of a major product and, presumably, its epimer in a ratio of 50:30:20. This is in agreement with previous findings in ΨPro-containing dipeptide building blocks.[21]

The chemical stability of the cyclic peptides strongly depends on the substituent (R) at the *para* position of the aryl units, as revealed by a significant correlation between reaction time and stability of the oxazolidine systems.[21] Electron-withdrawing substituents stabilise the cyclic form against acidic hydrolysis, whilst electron-donating groups provide more acid-labile pseudoprolines. Under conditions similar to the digestive tract (HCl/THF at pH 1), cyclosporin C was reconstituted from derivatives **3a–i** ($t_{1/2}$ of about three days, see Figure 4); this suggests the potential use of these derivatives as prodrugs.

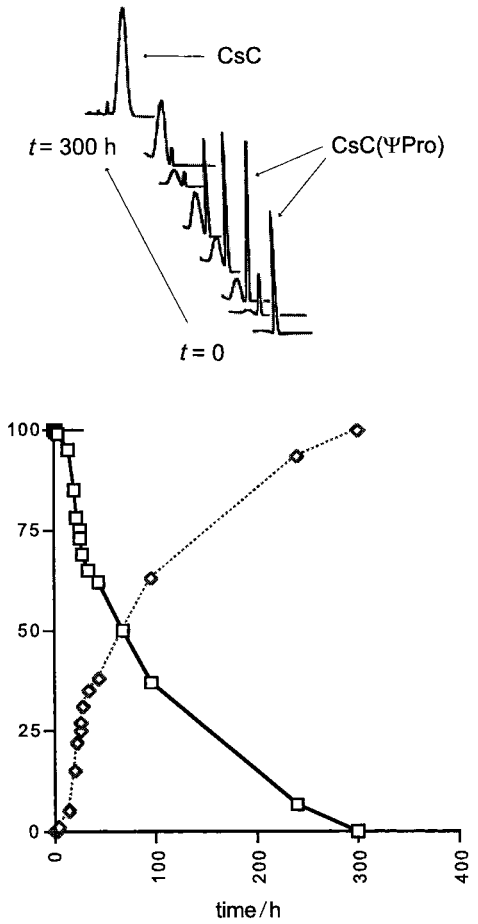

Figure 4. Hydrolysis experiments of **3e** at pH 1. Top: HPLC detection of the hydrolysis of **3e** to CsC. Gradient: 50–100% acetonitrile in water, 20 min, C_{18}. Bottom: Evolution (%) of CsC (◇) during hydrolysis of **3e** (□).

For example, the design of prodrugs that exhibit tailored physico-chemical and pharmaco-kinetic properties, such as solubility in water, appeared particularly appealing. For the temporary conversion of cyclosporin C into a water-soluble compound, polyethyleneglycol (PEG) aldehyde ($M_W \approx 850$) was transformed into the appropriate dimethyl acetal and treated with cyclosporin C to yield [2-L-Thr($\Psi^{PEG,H}$pro)]CsC (**3i** in Table 1) as prototype of this novel class of CsC derivatives.

Inhibitory effects of compounds 3a–i for calf-thymus cyclophilin A: The rather drastic chemical modification of cyclosporin C at the threonine residue implied in the binding to cyclophilin (the receptor protein of cyclosporins) suggests a significant impact on the biological activities of derivatives **3a–i**. An active-site-binding test for *cis*/*trans* isomerases developed by Fischer et al.[22] was used to probe the properties of the CsC derivatives on their binding to CypA.

For each measurement, the well-investigated CsA was taken as a reference. The difference between fully catalysed (noninhibited) and thermal isomerisation served as the end points of the scale. First-order rate constants of *cis* → *trans* isomerisation, measured as a function of different concentrations of pseudoproline-containing CsC derivatives, were plotted against the corresponding inhibitor concentrations, and experimental data were fitted into an exponential function. In the case of weak inhibition, such as for derivatives **3e**, **3h** and **3i**, no total inhibition was achieved at standard concentrations of the corresponding peptide and extrapolation was used to determine complete inhibition. Resolution to 50% inhibition of the corresponding equation gave the IC_{50} values (Table 1) for each compound.

Surprisingly, the oxazolidine-containing derivatives **3a–i** exhibit moderate to weak binding to cyclophilin A, the ubiquitous receptor protein of cyclosporins. Hydrophilic and polar derivatives, such as derivative **3h** which contains free carboxylic acid or the PEG-containing compound **3i**, show residual activities of $<5\%$ and are consequently candidates for prodrug design. Most notably, the introduction of functional groups, such as allyl or carboxyl, at the 2-C position of ΨPro (**3c**, **3h**) allows further modifications to be made. The separate investigation of the two epimers **3e** and **3f** indicates that the chirality of the 2-C position has a substantial impact on substrate recognition by CypA, with the two compounds differing in their inhibitory properties for the peptidylprolyl isomerase activity of cyclophilin A by a factor of about 3.5. Consequently, separated stereoisomers, such as **3e** and **3f**, allow for valuable insights into the conformational properties of these derivatives with regard to blocking the active site of CypA.

In conclusion, the regioselective insertion of ΨPro systems into complex peptides, such as CsC, has been achieved in one step in acceptable yields and results in a novel class of active-site inhibitors of cyclophilin A. By accessing CsC derivatives which exhibit tailored pharmacokinetic properties, the introduction of functional 2-C substituents into the ΨPro system in combination with strategies of combinatorial chemistry opens interesting perspectives for prodrug development. Work along these lines is in progress in our laboratory.

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Experimental Section

Material and methods: Reagents and solvents were purchased from Fluka (Buchs, Switzerland) unless otherwise stated and used without further purification. Calf-thymus cyclophilin A and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were purchased from Sigma (Steinheim, Germany). Suc-Ala-Ala-Pro-Phe-*p*NA came from BACHEM (Bubendorf, Switzerland). HPLC was performed on Waters equipment with columns packed with Vydac Nucleosil 300 Å/5μm C_{18} particles. Analytical columns (250×4.6 mm) were operated at 1 mL min^{-1} and preparative columns (250×21 mm) at 18 mL min^{-1} with UV monitoring at 214 nm. Solvent A was water purified on a Milli Q ion-exchange cartridge-containing 0.09% trifluoroacetic acid (TFA) and solvent B was either acetonitrile HPLC-*R* (preparative) or HPLC-*S* (analytical; both purchased from Biosolve, Valkenswaard, Netherlands) which contained 0.09% TFA. ^{1}H NMR experiments were run on a Bruker DPX 400. Mass spectra were obtained by electron spray ionisation (ESI-MS) on a Finnigan LC710. UV absorption measurements were conducted on a Cary 50 instrument and the kinetics analysed by the Cary 50 software package kinetics program. Abbreviations: NMM = *N*-methylmorpholine, DCM = dichloromethane, pNA = *para*-nitroaniline, PPTS = pyridinium-*p*-toluene sulfonic acid, DCC = dicyclohexylcarbodiimide, DMP = dimethoxypropane.

General synthetic procedure for 3a–i: Dry CsC (50 mg, 41 μmol), R^1,R^2-$C(OMe)_2$ (205 μmol, 5 equiv) and PPTS (4.0 mg, 0.4 equiv) in dry toluene (4 mL) were heated under reflux. After completion of the reaction (see Table 1), the organic layer was washed with Na_2CO_3 (10%, 2×5 mL) and water (2×5 mL), and dried over magnesium sulfate. The organic layer was concentrated under reduced pressure to give an oil. The crude material was dissolved in 2 mL of acetonitrile (solvent B)/water (solvent A) 1:1 (v/v) and purified by reversed phase HPLC (C_{18}, 60–100% solvent B, 40 min). Lyophilisation afforded [L-Thr(Ψ^{R^1,R^2}pro)]CsC as a white powder.

Determination of IC_{50} values for derivatives 3a–i: According to the chymotrypsin coupled assay,[22] the succinyl tetrapeptide Suc-Ala-Ala-Pro-Phe-pNA acts as a substrate for both cyclophilin and chymotrypsin, which specifically cleaves *trans*-Ala-Pro bond, leaving the *cis*-Ala-Pro bond in a disturbed equilibrium. This *cis*-Ala-Pro conformer of the tetrapeptide isomerises back to the *trans* form and is subsequently cleaved by chymotrypsin. UV monitoring at 390 nm allows the *cis* → *trans* isomerisation process to be measured as a first-order process. Addition of the peptidylprolyl *cis/trans* isomerase cyclophilin A accelerates the reaction of the thermal isomerisation at nanomolar concentrations by up to a factor of 20–25.[23] Upon addition of cyclosporin A and some other members of the cyclosporin family, cyclophilin is inhibited: this reverses the above accelerating effect of cyclophilin to the thermal isomerisation rate of Ala-Pro. X-ray crystallography, mainly carried out by the group of Walkinshaw,[24] has demonstrated that the cyclosporins bind to the active site of cyclophilins and exert an inhibitory effect on the *cis/trans* isomerase function of the enzyme. Cyclosporin A, which has been shown to inhibit cyclophilin A with a K_i of 2.5 nM[25] or 5.6 nM[23], was taken as a positive control. The acetylated CsC derivative **2** (Scheme 3) served as a negative control.

Suc-Ala-Ala-Pro-Phe-*p*NA was dissolved in TFE/LiCl (0.5 M) to a concentration of 10 mg mL^{-1}. For each experiment, 3 μL aliquots from this stock solution were added to a solution of chymotrypsin (25 μM) in HEPES (35 mM, 100 mM NaCl, pH 7.8) which contained 3.8 nM calf-thymus cyclophilin A. The cyclophilin A had been incubated with the corresponding inhibitor for at least 45 minutes for the experiments that were not time-dependent, and for intervals of 5, 10, 20 or 45 minutes for the time-dependent experiments. The UV absorption of liberated *para*-nitroaniline was monitored at 390 nm (ε for *p*NA = 11814 M^{-1} cm^{-1}) and the kinetic phases of the curves were analysed by first-order fit to give the corresponding pseudo-first-order rate constant for each inhibitor concentration. All experiments were carried out at 5 °C in triplicate.

Active-site titration of cyclophilin A: In order to determine the exact concentration of cyclophilin, active site titration with CsA was carried out as described by Kofron et al.[26] The mathematical model for competitive-binding inhibition as described by Williams and Morrison[27] was used to determine both the K_i of CsA and the concentration of CypA.[28] These stock solutions were used as standards for the determination of relative IC_{50} values of compounds **3a–i**.

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