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Synthesis and characterization of constrained cyclosporin A derivatives containing a pseudo-proline group

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Abstract—The chemical synthesis, conformational analysis and receptor binding studies of novel constrained cyclosporin A (CsA) analogues are described. The selective insertion of pseudo-proline (ΨPro) systems featuring different 2-C-substituents at the oxazolidine ring exerts dramatic effects upon the backbone conformation as demonstrated by NMR analysis. It is shown that the insertion of a Ψ^{MeMe}pro at position 5 (Thr^5CsA) maintains binding to cyclophilin A as well as to calcineurin and shows a 5-6 *cis* amide bond with all remaining amide bonds *trans*. The elaborated synthetic routes for generating ΨPro containing Cs derivatives pave the way for extended structure–activity relationship studies aiming at the design of potential pharmacologically active compounds with a selective activity profile.

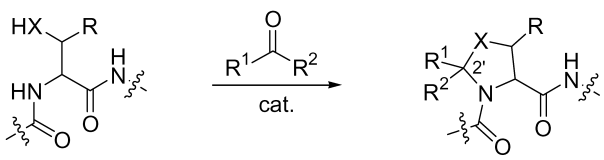
1. Introduction

Due to its cyclic nature, proline often occupies a key role in controlling the dynamic behavior of peptides and proteins. This property originates from the peptide bond prior to a proline that can adopt either the *trans* or the *cis* conformation.[1] In order to determine the bioactive conformation, the selective introduction of a *cis*-amide bond has become an established tool. The replacement of the key residues by constrained rigid structures like tetrazoles,[2] bicycles,[3] (*Z*)-alkenes[4] and alternatively, substitution of proline by modified amino acids, e.g. *N*-alkylated amino acids,[5] 5,5-dimethylated prolines[6] and 5-tert-butylproline[7] that induce a specific *cis* amide bond due to sterically demanding substituents while keeping the dynamic characteristics are two different approaches used currently.

Based on the intrinsic properties of proline we have developed a class of Pro-mimetics, referred to as pseudo-prolines (Ψro), with enhanced proline specific properties (Scheme 1). One striking feature of 2-C-dimethylated ΨPro is the temporary induction of a *cis* conformation of the amide bond preceding the ΨPro moiety.[8] As a consequence, the direction of the peptide chain is reversed resulting in a solubilizing effect of the building block due to prevention of aggregation caused by hydrophobic interactions.[9] Furthermore, pseudo-prolines for the tailor-made induction of *cis* conformations are versatile tools for targeting molecular recognition processes.[10]

More recently, we have demonstrated that ΨPro ring systems can even be introduced directly into complex, cyclic compounds like cyclosporin C (CsC). For this purpose, CsC is reacted with aromatic dimethylacetals resulting in oxazolidine (ΨPro) containing CsC derivatives obtained upon cyclocondensation. Most notably, all ΨPro-modified CsC compounds retained at least partial biological activity as assessed by determining the binding affinity to cyclophillin A, the natural binding protein of cyclosporins.[11]

CsA is widely used as clinical drug (Sandimmun™) to suppress immune reactions and thus to prevent graft rejection in patients.[12] The mechanism of immunosuppression has been



Xaa=Thr, Ser, Cys
(X=S,O; R=H,Me)

Xaa($\Psi^{R1,R2}$pro)

Scheme 1.

Keywords: cyclosporins; amino acids; pseudo-proline derivatives.

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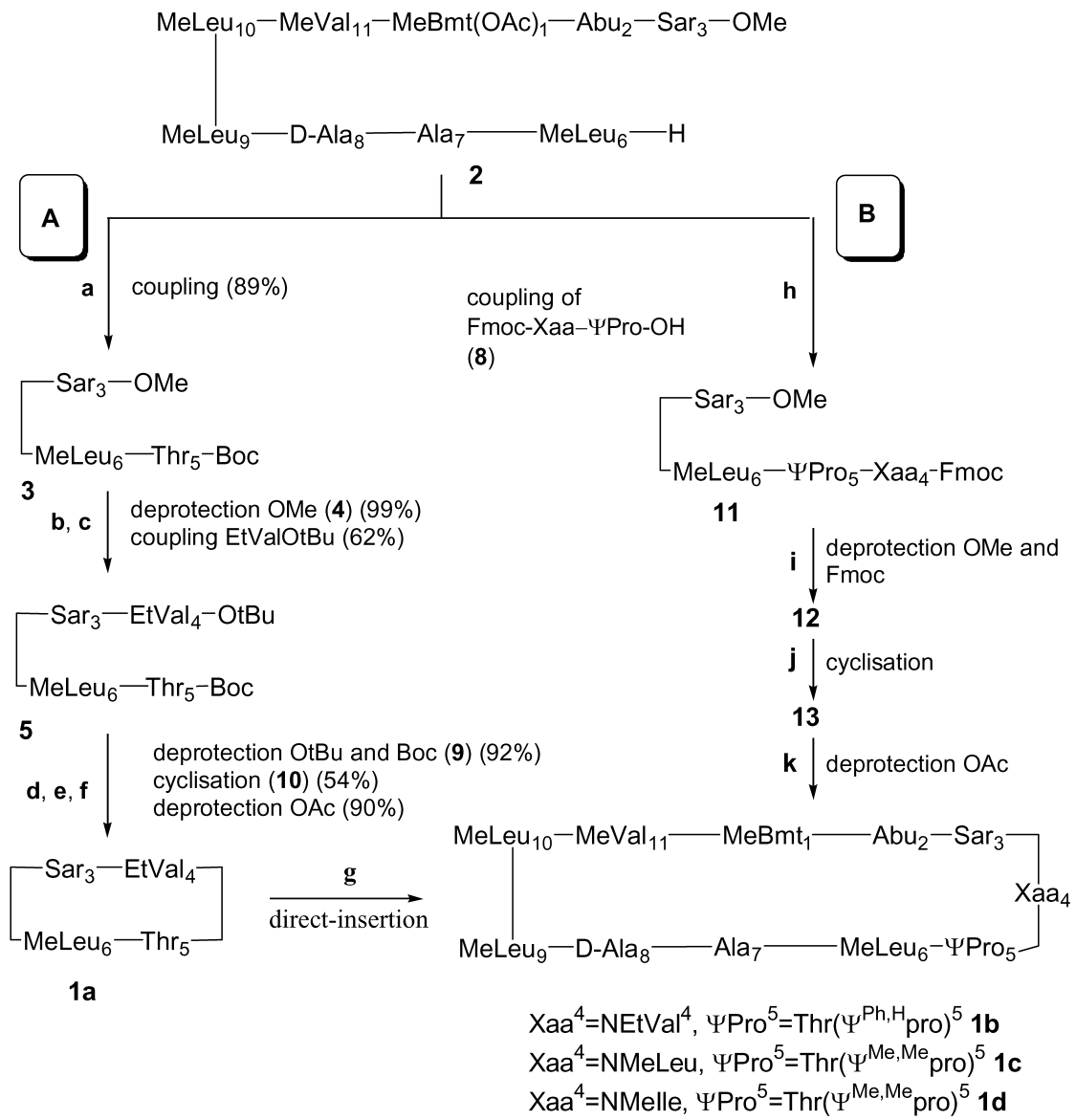

Scheme 2. (a) BocThrOH, DIPEA, HATU, DMF, 1 h, room temperature (89%), (b) LiOH, THF, 0°C (99%), (c) EtValOtBu, DIPEA, HATU, DCM, 5 h, room temperature (62%), (d) TFA, DCM, 1 h (92%), (e) TFFH, sym.collidine, DCM, 3 h (54%), (f) MeONa, MeOH, 26 h (90%), (g) PPTS, benzaldehyde dimethylacetal, DMSO, 100°C, 2 h (46%) (**1b**) (h) Fmoc-NMeLeu-Thr($\Psi^{Me,Me}$pro)-OH **8c** HATU, DIPEA, DCM, 24 h (65%) or Fmoc-NMeIle-Thr($\Psi^{Me,Me}$pro)-OH **8d** BOP-Cl, DIPEA, DCM, 5 h (73%), (i) DEA, acetonitrile, 12 h (73%), LiOH, THF/water, 30 min. (89%), (j) HATU, DCM, sym.collidine, 3 h (76%) (**13c**) or BOP-Cl, CH_3CN, DIPEA, 16 h (59%) (**13d**), (k) MeONa, MeOH, 24 h (92%) (**1c**) or MeONa, MeOH, 2.5 h (10%) (**1d**).

shown to depend on the inhibition of the calcium–calmodulin dependent phosphatase calcineurin (Cn) mediated by the CsA–cyclophilin A [CsA–CypA] complex.[13] On a molecular level, amino acid residues 3-8 of CsA are involved in Cn binding whereas residues at position 10, 11, and 1-3 interact with cyclophilin.[12] In contrast, the inhibition of human immunodeficiency virus type 1 replication by CsA occurs independently of calcineurin inhibition.[14] With the aim to address separately the two modes of action of CsA, increasing research is devoted to the synthesis of derivatives that bind with high selectivity to cyclophilin A but have drastically reduced affinity for calcineurin, i.e. to develop CsA-derived drugs antiviral but devoid of immunosuppressive activity.[14a,15]

In the context of structure-activity studies we extend in this article the use of the ΨPro concept[8–10] for elucidating the conformational aspects underlying the selective binding properties of cyclosporins to cyclophilin A with special emphasis on the evaluation of the structural and biological impact of ΨPro at position 5 of CsA. For this purpose, synthetic routes to a novel CsA analog, i.e. Thr^{5}CsA as prerequisite for the selective insertion of ΨPro building blocks are described.

2. Results and discussion

Synthesis. Recent structure activity studies revealed a drastic effect on the binding properties of Xaa^4-CsA analogs to CnA.[15] To test the hypothesis of a *cis* amide bond between residues 4 and 5 of CsA, Val at position 5 is replaced by either a Ser or Thr allowing for the direct introduction of ΨPro systems (Scheme 2).

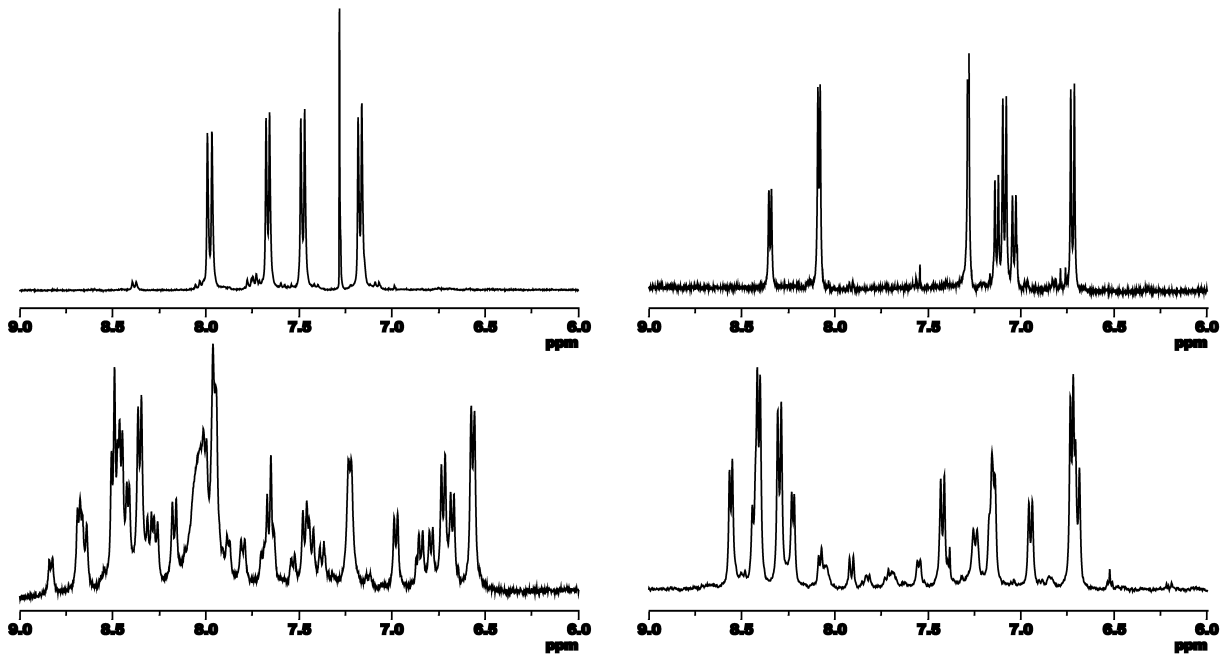

Figure 1. ^{1}H NMR of the NH region of CsA (left) and **1c** (right) in $CDCl_3$ (top) and DMSO-d_6 (bottom).

Based on previously reported procedures for ring opening and Edman degradation,[16] the linear nonapeptide **2** was used as a template for the incorporation of Thr[5]. As depicted in Scheme 2, two synthetic routes are feasible. Following procedure A, N^{α}-Boc protected Thr was coupled to **2** resulting in the protected decapeptide **3**. Deprotection of the C-terminus and subsequent coupling of NEtValOtBu resulted in the linear undecamer **5**. After removal of Boc and *t*Bu protection, cyclization and cleavage of the acetyl group, the novel CsA analog **1a** with a Thr at position 5 was obtained in overall yields of 25%. Compound **1a** served as starting material for the direct insertion of various pseudo-proline systems. For example, 2-C-monosubstituted derivative **1b** was obtained with 46% yield. However, applying similar experimental conditions, the synthesis of 2-C-dimethylated pseudo-proline derivative **1c** was not successful, possibly due to considerable conformational constraints imposed by the two 2-C substituents that are necessary for *cis* amide bond induction.

In order to bypass the critical step of direct insertion, an alternative strategy (Scheme 2, B) was developed where the ΨPro unit is introduced as a dipeptide building block. Starting from the linear precursor molecule **2**, the protected dipeptide Fmoc-NMeLeu-Thr($\Psi^{Me,Me}$pro)-OH **8** was coupled. After N^{α}-Fmoc deprotection and hydrolysis of the C-terminal methylester, cyclization was achieved by BOP-Cl activation restoring the 1-MeBmt-protected target peptide. Final deprotection of the MeBmt group with methanolate resulted in the ΨPro-modified CsA analog **1c**. Following strategy B and varying residue Xaa in the dipetide building block, the synthesis of a series of novel ΨPro-containing CsA analogs became available. For example, by using Fmoc-NMeIle-Thr($\Psi^{Me,Me}$pro)-OH as dipeptide unit, compound **1d** was prepared in good yields under identical reaction conditions.

2.1. NMR studies

All compounds were characterized by RP-HPLC, mass spectroscopy and ^{1}H NMR. The analogues **1a**–**c** were studied in more detail by 1D and 2D ^{1}H NMR spectroscopy (TOCSY, ROESY, COSY-DQF and HSQC) with focus on the impact of the insertion of a 2-C-disubstituted ΨPro (**1c**) on the backbone conformation. Due to the pronounced hydrophobicity of the target molecules, $CDCl_3$ and DMSO-d_6 mimicking to some extent physiological conditions,[17] were used as solvents. In $CDCl_3$ only a limited number of conformations are observed. While CsA shows one major conformation, two major conformations of compound **1c** at

Table 1. Results of NMR studies (see text)

Compound	$CDCl_3$[a]	DMSO-d_6[a]
CsA	1 Conformation, 9-10 *cis*	Numerous conformations
1a	1 Conformation, 9-10 *cis*	Numerous conformations
1b	1 Conformation, 11-1 *cis*, 5-6 *cis*	2 Conformations (81:19) Major: all-*trans* (3-4 undetermined)
1c	2 Conformations (67:33) Major: 5-6 *cis*, 11-1 *trans* Minor : 5-6 *cis*, 11-1 *cis*	4 Conformations (62:24:19:19) Major: all-*trans* (9-10 undetermined)

[a] Only conformations present as at least 25% of the major conformation are considered.

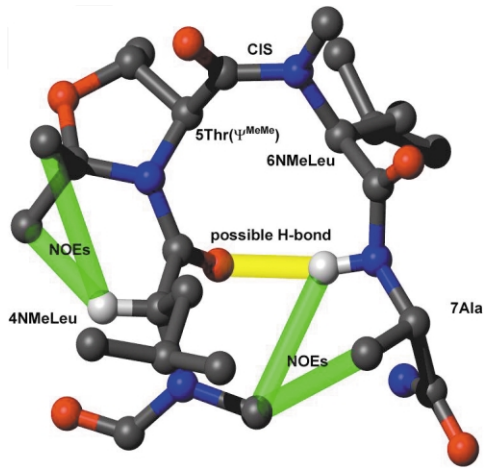

Figure 2. Model of a ΨPro containing β-turn type VI of analogue **1c**.

ratio 2:1 can be distinguished. Surprisingly, several conformers are present in DMSO-d_6 despite the structural constraint imposed by the ΨPro ring system (Fig. 1).

By comparing the number of conformations in $CDCl_3$ and DMSO-d_6 (Table 1) the strong influence of the solvent becomes apparent. It is well known that CsA adopts only one conformation in $CDCl_3$ but numerous conformations in DMSO-d_6.[17a] The presence of an oxazolidine ring reduces the number of conformations considerably. This effect is strongly dependent upon the nature of the substituents at the 2-C position as steric demands have important influence on the overall flexibility of the cyclosporin ring. For instance, mono-substitution at 2-C with a phenyl group results in a molecule with low flexibility in both solvents. Moreover, the spectra indicate the presence of an all-*trans* amide bond conformation, which seems to be a prerequisite for biological activity.[18]

As depicted in Figure 3, ROESY experiments of **1c** in $CDCl_3$ show that two substituents at 2-C of ΨPro induces a 5-6 *cis* amide bond in both conformers. This observation contrasts all previous studies on model dipeptides of type Xaa-ΨPro[19] and other ΨPro containing linear peptides,[10a] for which a *cis*-amide bond is always observed at the amide bond preceding the ΨPro. Careful analysis of the ROESY experiment shows surprising NOEs 7NH–4NMe and 7Hβ–4NMe suggesting a β-turn between residues 4-7 with the ΨPro residue at position $i+1$. This turn position is strikingly different from the established NMR structure of CsA in $CDCl_3$, which exhibits a β-turn between residues 2-5.[20] The formation of a type VI turn,[21] as depicted in Figure 2, featuring a *cis*-amide bond between $i+1$ (ΨPro^5) and $i+2$ (NMeLeu^6) and a hydrogen bond between i (NMeLeu^4) and $i+3$ (Ala^7) would be in harmony with the experimental data.

In contrast to CsA, both conformers exhibit a *trans* 9-10 amide bond. The difference between the two observed conformations originates from amide bond 11-1, which was found to be *trans* in the major and *cis* in the minor conformer whereas all remaining amide bonds are exclusively *trans*. The complete assignment of all signals obtained in DMSO-d_6 proved to be difficult due to numerous conformations present (Table 1). The structure of the main isomer was however resolved showing that the 4-5 and 5-6

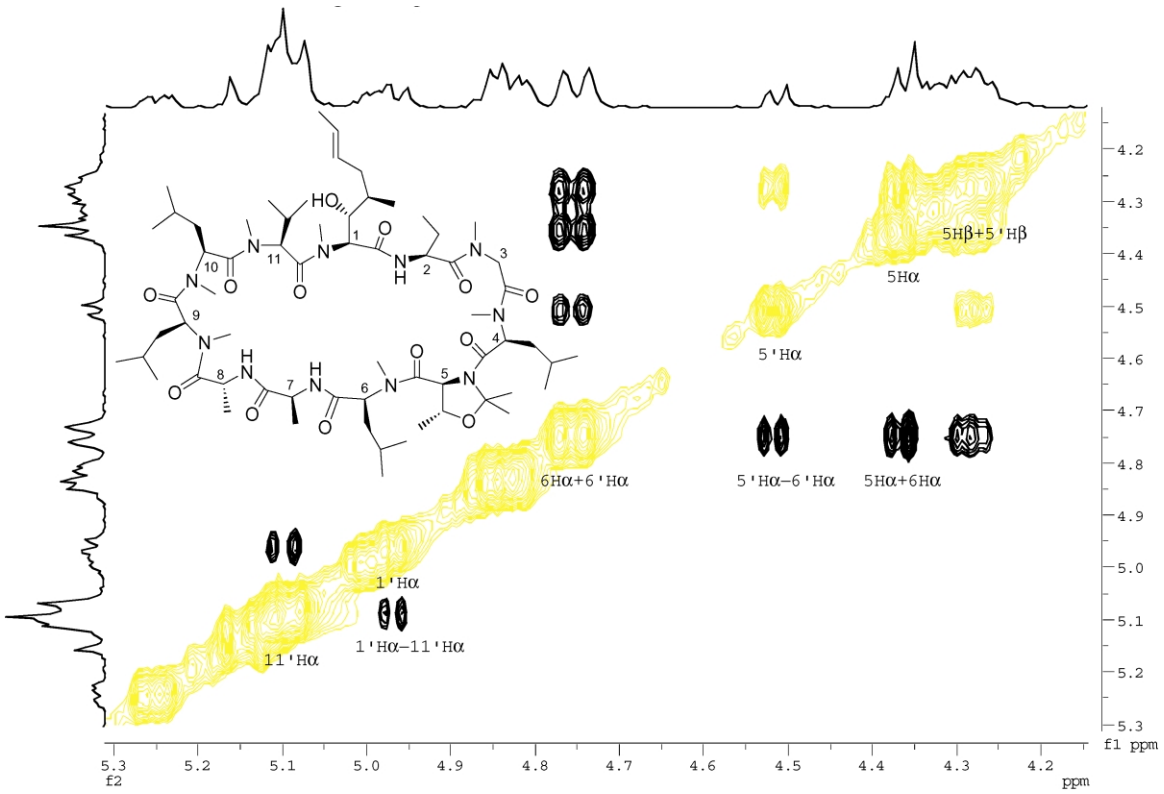

Figure 3. ROESY of **1c** in $CDCl_3$. The major conformer and minor isomer (′) has 5-6 *cis* amide bonds (NOE 5Hα-6Hα). The minor conformer shows 11-1 *cis* amide bond (NOE 1′Hα-11′Hα).

Table 2. In vitro biological activity of the novel CsA analogues

Products	CypA IC_{50} **1a–d**/IC_{50} CsA	IL2-RGA IC_{50} **1a–d**/IC_{50} CsA
CsA (cyclosporin)	1	1
Thr^5CsA[25]	5.0	3.0
$EtVal^4$CsA[15a]	0.67	>2500
$EtVal^4Thr^5$CsA **1a**	3.1	>2800
$EtVal^4Thr(\Psi^{Ph,H}pro)^5$CsA **1b**	5.2	903
$Thr(\Psi^{Me,Me}pro)^5$CsA **1c**	5.7	18
$MeIle^4Thr(\Psi^{Me,Me}pro)^5$CsA **1d**	4.7	n.d.

amide bonds adopt a *trans* conformation. This finding indicates that in highly constrained molecules such as CsA, a *trans* amide bond can exist even in the presence of a *cis*-inducing dimethyl-ΨPro. Indeed, the identical ΨPro binding block in the linear peptide sequence induces at least 80% *cis* conformation at the 4-5 amide bond.[8c] This points to the specific nature of CsA, which is sensitive to backbone modifications. For instance the presence of an α substituent at Sar^3 like in $DMeSer^3$CsA shows only one all-*trans* conformation in DMSO-d_6.[17a] In a similar way, N-5 alkylated derivatives also show an all-*trans* conformation pointing out to a 'remote-control' of the conformation of amide bonds[22] and the high susceptibility of CsA conformation to substitution (Fig. 3).

2.2. Receptor binding studies

In vitro activity of the cyclosporine derivatives were assessed using the IL-2 reporter gene assay (measuring immunosuppressive activity) and the binding affinity to CypA. The IL-2 reporter gene assay detects substances interfering with IL-2 gene activation along the T cell signaling pathway.[23] The binding to CypA was determined using the improved spectrophotometric assay described by Rich.[24]

The biological results observed for **1a** are similar to the results observed for $EtVal^4$CsA[15a] and show the impact of the β-substitution in position 4 (Table 2). Here the binding to cyclophilin is however reduced due to the presence of the hydroxyl group in position 5 as it was the case for the corresponding Thr^5CsA.[25] The presence of a pseudo-proline in position 5 (**1b–d**) has only a marginal effect on the binding to cyclophilin. More surprisingly, the presence of the bulky dimethylated pseudo-proline at position 5 (**1c**) has a smaller effect on the binding to cyclophilin than expected, showing that residue 5 is not crucial for binding. This result is in agreement with previous findings on the N^5-alkylated derivatives.[22] In contrast it has been shown that residue 4 has a large impact on the immunosuppressive activity[15] as exemplified in β-branched analogues which are devoid of immunosuppressive activity. Obviously, the simultaneous modification of position 4 and 5 results in negligible additive effects, position 4 largely dominating the overall effect on receptor binding as shown in **1a** and **1b**.

In summary, the synthesis and conformational analysis of new cyclosporin derivatives containing ΨPro at position 5 have been achieved. Despite the dramatic conformational changes induced by the selective introduction of ΨPro systems, all derivatives retained some cyclophilin A binding affinities, pointing to a stabilization of a bioactive conformation. Interestingly, the insertion of a ΨPro^5 has less impact on its calcineurin binding capacities compared to position 4. Further structure–activity studies along the lines described here are in progress.

3. Experimental

3.1. Materials and methods

Reagents and solvents were purchased from Fluka (Buchs, Switzerland) unless otherwise stated and were used without further purification. Calf thymus cyclophilin A and HEPES were purchased from Sigma (Steinheim, Germany), Suc-Ala-Ala-Pro-Phe-*p*NA from BACHEM (Bubendorf, Switzerland). Purifications were performed on a Waters HPLC using columns packed with Vydac Nucleosil 300 Å/5 μm C_4 particles. Analytical columns (250×4.6 mm) were operated at 1 mL/min and semi-preparative ones (250×21 mm) at 18 mL/min with UV monitoring at 214 nm. Solvent A was MilliQ (Millipore, Volketswil, Switzerland) purified water containing 0.09% TFA, and solvent B was acetonitrile/H_2O 9/1 HPLC-*R* (preparative) or HPLC-*S* (analytical; both purchased from Biosolve, Valkenswaard, Netherlands) containing 0.09% TFA. The solvent gradient is 50% of A in B to 100% of B in 30 min followed by 100% B during 5 min. All NMR spectra were measured on DRX400 Bruker spectrometer at 30°C. The products were characterized by two-dimensional ROESY experiments[26] (mixing time 200 ms), homonuclear Hartman–Hahn[27] (HOHAHA, TOCSY) experiments (spin-lock 100 ms), and COSY-DQF[28] in the phase sensitive mode using the time-proportional phase incrementation method.[29] A total of 2 K data points was collected in the F_2 dimension with a spectral width of 4000 Hz. In the F_1 dimension, 512 or 1 k points were measured. The data were processed using the SwaN-MR software.[30] A zero-filling in the F_1 dimension and a square sine-bell window shifted by 90° in both dimensions were applied prior to the two-dimensional Fourier transformation. In the case of COSY-DQF a square sine-bell window shifted by 0° was applied in F_1. Mass spectra were obtained by electron spray ionization (ESI-MS) on a Finnigan LC 710. UV absorption measurements were conducted on a Cary 50 spectrophotometer and the kinetics analyzed by the Cary 50 software package kinetics program. Abbreviations were used as follows: BOP-Cl=Bis(2-oxo-3-oxazolidinyl)phosphinic chloride, DCM=dichloromethane, DEA=diethylamine, DIPEA=diisopropylethylamine, DCC=dicyclohexylcarbodiimide, DMF=dimethylformamide, DMP=dimethoxypropane, HATU=O-(7-azabenzotrizol-1-yl)-1,1,3,3, tetra-methyl-uronium hexafluorophosphate, HEPES=*N*-2-hydroxyethyl-

piperazine-*N*′-2-ethanesulfonic acid, NMM=*N*-methylmorpholine, OSu=succinic ester, PPTS=pyridinium-*p*-toluene sulfonate, TFA=trifluoroacetic acid, THF=tetrahydrofuran.

3.2. Peptide synthesis, synthesis of the dipeptide precursors

Fmoc-NMeIle-OH **6d** ($C_{22}H_{25}NO_4$=367.4): H-*N*MeIle-OH (1 g, 7 mmol) was dissolved in a solution of sodium carbonate (10%, 8.5 mL) before addition of Fmoc-*O*Su (2 g, 7 mmol) in dioxane (14 mL). After 3 h reaction time, water (20 mL) was added and the aqueous phase extracted with ether (50 mL, 2×). The aqueous layer was acidified to pH 3 with hydrochloric acid (2N) and the precipitate was extracted with ethyl acetate (50 mL, 4×). The combined organic layers were dried over sodium sulfate and concentrated under reduced pressure. The crude product was purified on silicagel (eluent: ethyl acetate/MeOH=10:1) to obtain 1.15 g (45%) of **6d**. TLC analysis: R_f=0.4 (ethyl acetate/MeOH=10:1). MS-ESI (*m*/*z*): 368.2 $[M+H]^+$. HPLC t_R=21.20 min.

Fmoc-NMeLeu-Thr-OBz **7c** ($C_{33}H_{38}N_2O_6$=558.7): PyBOP (4.30 g, 7.17 mmol) is added to a solution of **6c** (2.63 g, 7.17 mmol), DIPEA (3.68 mL, 21.5 mmol) and H-Thr-OBzl (1.50 g, 7.17 mmol) in 500 mL of DCM. After 16 h, the solution is concentrated under reduced pressure and the residue is dissolved in ethyl acetate. The organic layer is washed with saturated $NaHCO_3$, citric acid 10% and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product is purified on silicagel (AcOEt/hexane 5:5, R_f=0.61) to yield **6c** (3.75 g, 94%) as a white foam. MS-ESI (*m*/*z*): 559.0 $[M+H]^+$, HPLC: t_R=22.15 min.

Fmoc-NMeIle-Thr-OBz **7d** ($C_{33}H_{38}N_2O_6$=558.7): pentafluorophenol (405 mg, 2.2 mmol) and dicyclohexyl carbodiimide (454 mg, 2.2 mmol) in DCM (20 mL) and THF (20 mL) were added to **6d** (735 mg, 2 mmol) and stirred for 16 h. The white suspension was filtered over Celite© and the solvent removed under reduced pressure. To this white solid, H-Thr-*O*Bz hemioxalate (837 mg, 2 equiv.) and NMM (463 μL, 2.1 equiv.) in DMF (10 mL) were added and stirred for 16 h. The solvent was evaporated and ethyl acetate (50 mL) was added. The organic layer was washed with citric acid (50 mL, 3×) and water (50 mL, 3×), dried over magnesium sulfate and concentrated under reduced pressure. The dipeptide was purified on silicagel (eluent: $CHCl_3$/MeOH/AcOH=100:10:1) to yield 436 mg (40%) of **7d**. MS-ESI (*m*/*z*): 559.7 $[M+H]^+$, HPLC: t_R=16.76 min.

Fmoc-NMeLeu-Thr($\Psi^{Me,Me}$*pro*)*-OH* **8c** ($C_{36}H_{42}N_2O_6$=509.4): **7c** (1.00 g, 1.79 mmol) dissolved 180 mL of THF containing DMP (2.21 mL, 17.9 mmol) and PPTS (130 mg, 0.53 mmol) was refluxed for 7 h. After concentration under reduced pressure, the crude product was purified on silicagel (AcOEt/hexane 3:7, R_f=0.62) to yield the protected dipeptide (330 mg, 31%) of a white foam. This product was dissolved in 50 mL of ethanol and hydrogenated in the presence of 0.03 g of Pd/C (5%) during 30 min. The solution was filtered on celite and concentrated under reduced pressure to yield 277 mg (98%) of **8c** as a colorless oil. HPLC t_R=23.52 min. ESI-MS (*m*/*z*)=509.7 $[M+H]^+$.

Fmoc-NMeIle-Thr($\Psi^{Me,Me}$*pro*)*-OH* **8d** ($C_{36}H_{42}N_2O_6$=509.4): DMP (122.3 μL, 5 equiv.) and PPTS (14 mg, 0.3 equiv.) were added to **7d** (112 mg, 0.2 mmol) dissolved in toluene (3 mL) and heated at 80°C for 16 h. The reaction mixture was allowed to cool to room temperature and ethyl acetate (10 mL) was added. The organic layer was washed with Na_2CO_3 10% (10 mL, 2×) and dried over magnesium sulfate. After filtration and removal of the solvent under reduced pressure, yellow oil was obtained which was taken up in methanol (5 mL). Addition of Pd/*C* (10 mg) and exposure under hydrogen atmosphere (H_2) during 2 h yielded **8d** as a yellowish solid that was purified on silicagel (eluent: $CHCl_3$/MeOH/AcOH=100:5:1) to yield 70 mg (68%) of **8d**. HPLC t_R=14.4 min. ESI-MS (*m*/*z*)=509.4 $[M+H]^+$.

3.3. Peptide synthesis, synthesis of the cyclosporin derivatives

Boc-Thr-MeLeu-Ala-D-Ala-MeLeu-MeLeu-MeVal-MeBmt-(OAc)-Abu-Sar-OMe **3** ($C_{62}H_{110}N_{10}O_{16}$=1251.6). To a solution of **2** (1.1 g, 1.05 mmol), DIPEA (0.90 mL, 5.25 mmol), Boc-Thr-OH (0.46 g, 2.10 mmol) in 13 mL of DMF was added HATU (0.80 g, 2.10 mmol). After 1 h, the DMF was evaporated and the residue dissolved in ethyl acetate. The organic phase was washed successively by a saturated solution of $NaHCO_3$, a 10% solution of citric acid and a saturated solution of NaCl, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified on silicagel (AcOEt/MeOH 98:2, R_f=0.47) to yield 1.16 g (89%) of **3** as a white foam. HPLC t_R=27.88 min. ESI-MS (*m*/*z*)=1251.8.

Boc-Thr-MeLeu-Ala-D-Ala-MeLeu-MeLeu-MeVal-MeBmt-(OAc)-Abu-Sar-OH **4** ($C_{61}H_{108}N_{10}O_{16}$=1237.6). An aqueous solution of $LiOH{\cdot}H_2O$ 0.19 M (12.5 mL, 2.50 mmol) is added to a solution of **3** in 40 mL of THF at 0°C. The reaction was stirred towards room temperature and after 80 min neutralized by 1 M solution of $NaHSO_4$. The solution was concentrated under reduced pressure and the residue placed in ethyl acetate, washed by a saturated solution of NaCl, dried over Na_2SO_4 and concentrated to yield 0.48 g (99%) of **4** as a white foam. HPLC t_R=26.32 min. ESI-MS (*m*/*z*)=1237.6.

Boc-Thr-MeLeu-Ala-D-Ala-MeLeu-MeLeu-MeVal-MeBmt-(OAc)-Abu-Sar-EtVal-OtBu **5** ($C_{72}H_{129}N_{11}O_{17}$=1420.9). A solution of H-NEtVal-O^{t}Bu[15a] (0.45 g, 2.24 mmol) in 5 mL of DCM is added dropwise to a solution of **4** (1.1 g, 0.90 mmol), DIPEA (0.85 mL, 4.93 mmol), HATU (0.68 g, 1.80 mmol) in 15 mL of DCM under inert atmosphere. The reaction was followed by HPLC and driven to completion by addition of 0.34 g of HATU. After 5 h, the solution was concentrated under reduced pressure and the compound dissolved in ethyl acetate. The organic phase is washed by $NaHCO_3$, citric acid 10% and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified on silicagel (AcOEt, R_f=0.50) to yield 0.68 g (62%) of **5** as a white foam. HPLC t_R=31.42 min. ESI-MS (*m*/*z*)=1421.0.

H-Thr-MeLeu-Ala-D-Ala-MeLeu-MeLeu-MeVal-MeBmt-(OAc)-Abu-Sar-EtVal-OH **9** ($C_{63}H_{113}N_{11}O_{15}$=1264.7). 3 mL of TFA was added to a solution of **5** (0.66 g, 0.46 mmol) in 15 mL of DCM. After 1 h, 3.62 g of $NaHCO_3$ are added slowly. The organic phase is washed by saturated $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated under reduced pressure to yield 0.54 g (92%) of **9** as a white foam. HPLC t_R=27.01 min. ESI-MS (*m/z*)=1264.5.

MeBmt(OAc)-EtVal^4*-Thr*^{5}*-CsA* **10** ($C_{63}H_{111}N_{11}O_{14}$=1246.6). A solution of **9** (0.50 g, 0.395 mmol), sym. collidine (0.58 mL, 4.35 mmol) in 50 mL of DCM was added dropwise to a solution of TFFH (0.31 g, 1.18 mmol) in 4 L of DCM under inert atmosphere. After 3 h, the reaction was hydrolysed by a 10% solution of Na_2CO_3 and the DCM was removed under reduced pressure. The residual was placed in ethyl acetate, washed by HCl 0.1 M and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified on silicagel (acetone/hexane 5:5, R_f=0.48) to yield 265 mg (54%) of **10** as a white solid. HPLC t_R=29.86 min. ESI-MS (*m/z*)=1246.9.

Fmoc-NMeLeu-Thr($\Psi^{Me,Me}$*pro*)*-MeLeu-Ala-D-Ala-MeLeu-MeLeu-MeVal-MeBmt(OAc)-Abu-Sar-OMe* **11c** ($C_{82}H_{129}N_{11}O_{17}$=1541.0): HATU (205 mg, 0.54 mmol) was added to a solution of **2** (293 mg, 0.28 mmol), DIPEA (0.24 mL, 1.40 mmol), **8c** (277 mg, 0.54 mmol) in 28 mL of DCM. After 24 h a second equivalent of HATU was added and the reaction stirred for another day. The solution was concentrated under reduced pressure and the residue dissolved in ethyl acetate, washed with $NaHCO_3$, citric acid 10% and brine and dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified on silicagel (AcOEt/hexane 9:1, R_f=0.52) to yield 280 mg (65%) of **11c** as a white foam. HPLC t_R=32.3 min. ESI-MS (*m/z*)=1541.2 $[M+H]^+$.

Fmoc-NMeIle-Thr($\Psi^{Me,Me}$*pro*)*-MeLeu-Ala-D-Ala-MeLeu-MeLeu-MeVal-MeBmt(OAc)-Abu-Sar-OMe* **11d** ($C_{82}H_{129}N_{11}O_{17}$=1541.0): 102 mg (97.2 μmol) of **2** (97.2 μmol) in DCM (2 mL), DIPEA (34 μL), BOP-Cl (25 mg, 97.2 μmol) and **8d** (45 mg, 88.3 μmol) were stirred at room temperature until all dipeptide had reacted (5 h). Solvent was evaporated and the residual was purified on silicagel ($CHCl_3$/MeOH/AcOH=100:7:1) to yield 101 mg (73.5%, 64.9 μmol) **11d**. HPLC t_R=26.16 min. ESI-MS: no mass could be detected.

H-NMeLeu-Thr($\Psi^{Me,Me}$*pro*)*-MeLeu-Ala-D-Ala-MeLeu-MeLeu-MeVal-MeBmt(OAc)-Abu-Sar-OH* **12c** ($C_{66}H_{117}N_{11}O_{15}$=1304.7). DEA (0.13 mL, 1.26 mmol) was added to a solution of **11c** in 12.6 of acetonitrile and stirred during 12 h. After concentration under reduced pressure, the crude product was purified on silicagel (DCM/MeOH 95:5, R_f=0.20) to yield 120 mg (73%) of a white foam. To this product in 3.7 mL of THF is added an aqueous solution of $LiOH{\cdot}H_2O$ (11 mg, 0.27 mmol) in 1.3 mL of water. After 30 min the mixture was cooled down to 0°C and neutralized by a solution of $KHSO_4$ 1 M. After concentration under reduced pressure, the crude product was dissolved in ethyl acetate, washed by an half saturated solution of NaCl, dried over Na_2SO_4 before concentrating under reduced pressure to yield 105 mg (89%) of **12c** that was used in the next step without further purification. HPLC t_R=30.05 min. ESI-MS (*m/z*)=1304.8 $[M+H]^+$.

MeBmt(OAc)^1*-Thr*($\Psi^{Me,Me}$*pro*)^5*-CsA* **13c** ($C_{66}H_{115}N_{11}O_{14}$=1286.7). A solution of HATU (87 mg, 0.23 mmol) in 760 mL of DCM was added dropwise under inert atmosphere to a solution of **12c** (100 mg, 0.076 mmol), sym.collidine (0.11 mL, 0.83 mmol) in 20 mL of DCM. After 3 h, the mixture was hydrolysed by a solution of Na_2CO_3 10% and concentrated under reduced pressure. The residual was dissolved in ethyl acetate, washed by citric acid 10% and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified on silicagel (acetone/hexane 5:5, R_f=0.62) to yield **13c** (74 mg, 76%) as a white solid. HPLC t_R=30.05. ESI-MS (*m/z*)=1286.7 $[M+H]^+$.

MeBmt(OAc)^1*-NMeIle*^{4}*-Thr*($\Psi^{Me,Me}$*pro*)^5*-CsA* **13d** ($C_{66}H_{115}N_{11}O_{14}$=1286.7). The fully protected linear undecapeptide **11d** was dissolved in ethanol (4.85 mL) and 0.2 M sodium hydroxide (975 μL) was added and left at −5°C/16 h in a precooled cryostat. The reaction was acidified with hydrochloric acid (0.1 M) to pH 5 and extracted with ethyl acetate (20 mL, 4×). The combined organic layers were dried over magnesium sulfate, concentrated under reduced pressure and purified by preparative HPLC using a gradient 50–100%, 20 min, C_{18}. Lyophilisation yielded 50 mg (59%) of peptide **12d**. LC-MS: t_R=11.21 min, gradient 50–100%, 20 min, C_{18}. *m/z*=1305.4 $[M+H]^+$).

Cyclization: 4.5 mg (3.45 μmol) of undecapeptide **12d** was dissolved with acetonitrile (4.5 mL) before adding BOP-Cl (1.75 mg, 2 equiv.) and DIPEA (1.2 μL, 2 equiv.) and stirred for 16 h to yield quantitatively **13d**. HPLC t_R=24.12. ESI-MS (*m/z*)=1287.1 $[M+H]^+$.

EtVal^4*-Thr*^{5}*-CsA* **1a** ($C_{61}H_{109}N_{11}O_{13}$=1204.6). A solution of sodium methanolate (0.080 mmol) in 0.88 mL of methanol was added to a solution of **10** (50 mg, 0.040 mmol) in 0.88 mL of methanol under inert atmosphere. After 26 h, the reaction was cooled to 0°C and hydrolyzed using 10% citric acid. After concentration under reduced pressure, the residue was placed in ethyl acetate, washed by saturated $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified on silicagel (acetone/hexane 5:5, R_f=0.45) to yield 43 mg (90%) of a white solid. HPLC t_R=27.20 min. ESI-MS (*m/z*)=1204.8. NMR ($CDCl_3$): 7.39 (7HN), 7.06 (8HN), 6.90 (2HN), 6.85 (5HN), 5.71 ($9H_\alpha$), 5.43 ($1H_\alpha$), 5.42 ($1H_\varepsilon$), 5.41 ($1H_\zeta$), 5.20 ($11H_\alpha$), 5.09 ($2H_\alpha$), 5.08 ($6H_\alpha$), 5.04 ($10H_\alpha$), 5.00 ($5H_\alpha$), 4.91 ($4H_\alpha$), 4.81 ($8H_\alpha$), 4.74 ($3H_{\alpha1}$), 4.51 ($7H_\alpha$), 4.44 (1OH), 4.16 ($1H_\beta$), 4.06 ($5H_\beta$), 3.76 ($4NH_{\alpha1}$), 3.54 (1NMe), 3.37 (3NMe), 3.30 ($4NH_{\alpha2}$), 3.29 ($3H_{\alpha2}$), 3.16 (6NMe), 3.07 (9NMe), 2.74 (11NMe), 2.73 (10NMe), 2.23 ($4H_\beta$), 2.17 ($11H_\beta$), 2.11 ($10H_{\beta1}$), 2.09 ($9H_{\beta1}$), 2.06 ($6H_{\beta1}$), 1.79 ($1H_{\delta1}$), 1.70 ($2H_{\beta1}$), 1.69 ($1H_{\delta2}$), 1.68 ($1H_\gamma$), 1.67 ($6H_\gamma$), 1.63 ($1H_\eta$), 1.57 ($2H_{\beta2}$), 1.47 ($6H_{\beta2}$), 1.40 ($7H_\beta$), 1.32 ($4NH_\beta$), 1.31 ($9H_{\beta2}$), 1.31 ($9H_\gamma$), 1.28 ($5H_\gamma$), 1.26 ($8H_\beta$), 1.23 ($10H_{\beta2}$), 1.23 ($10H_\gamma$), 1.05 ($4H_{\gamma1}$), 1.03 ($10H_\delta$), 1.03 ($11H_{\gamma1}$), 0.97 ($9H_{\delta1}$), 0.92 ($6H_{\delta1}$), 0.91 ($4H_{\gamma2}$), 0.90 ($9H_{\delta2}$), 0.88 ($6H_{\delta2}$), 0.85 ($11H_{\gamma2}$), 0.83 ($2H_\gamma$), 0.74 ($1Me_\gamma$). 9-10 *cis* bond.

EtVal ^{4}-*Thr*($\Psi^{Ph,H}$*pro*)^{5}-*CsA* **1b** ($C_{68}H_{113}N_{11}O_{17}$=1292.7). A solution of **1a** (43 mg, 0.038 mmol), PPTS (0.5 mg, 0.002 mmol) and benzaldehyde dimethylacetal (29 mg, 0.19 mmol) in 2 mL of dry DMSO was heated to 100°C during 2 h. The reaction was hydrolyzed by $NaHCO_3$ (1 M) and the aqueous phases extracted using ethyl acetate. The combined organic layers were washed by saturated $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was purified on silicagel (acetone/hexane 5:5, R_f=0.55) to yield 22 mg (46%) of a white solid. ESI-MS (*m*/*z*)=1292.7. ^{1}H NMR ($CDCl_3$): 8.73 (7HN), 7.70 (2HN), 7.39 ($5H_{m,o}$), 7.31 (8HN), 7.14 ($5H_p$), 6.33 ($5H_\delta$), 5.61 ($9H_\alpha$), 5.42 (1Hf), 5.42 ($10H_\alpha$), 5.38 ($1H_\alpha$), 5.30 ($1H_\varepsilon$), 4.99 ($11H_\alpha$), 4.94 ($2H_\alpha$), 4.81 ($6H_\alpha$), 4.79 ($8H_\alpha$), 4.67 ($5H_\alpha$), 4.47 ($5H_\beta$), 4.47 ($7H_\alpha$), 4.44 ($4H_\alpha$), 4.14 ($1H_\beta$), 3.89 ($3H_{\alpha2}$), 3.56 ($3H_{\alpha1}$), 3.49 ($4HN_{\alpha1}$), 3.24 ($4HN_{\alpha2}$), 3.23 (10NMe), 3.22 (11NMe), 3.03 (3NMe), 2.93 (1NMe), 2.84 (6NMe), 2.84 (9NMe), 2.63 ($10H_{\beta1}$), 2.57 ($10H_{\beta2}$), 2.46 ($6H_{\beta1}$), 2.41 ($11H_\beta$), 2.22 ($1H_{\delta1}$), 2.07 ($4H_\beta$), 2.01 ($1H_{\delta2}$), 1.84 ($6H_\gamma$), 1.71 ($2H_{\beta1}$), 1.71 ($9H_{\beta1}$), 1.66 ($1H_\gamma$), 1.54 ($1H_\eta$), 1.52 ($9H_{\beta2}$), 1.49 ($2H_{\beta2}$), 1.44 ($7H_\beta$), 1.40 ($5H_\gamma$), 1.37 ($9H_\gamma$), 1.35 ($10H_{\delta1}$), 1.34 ($8H_\beta$), 1.18 ($6H_{\beta2}$), 1.08 ($4HN_\beta$), 1.05 ($6H_{\delta1}$), 1.03 ($4H_{\gamma1}$), 1.03 ($6H_{\delta2}$), 1.02 ($10H_\gamma$), 1.01 ($1Me_\gamma$), 0.92 ($9H_\delta$), 0.88 ($11H_{\gamma1}$), 0.87 ($10H_{\delta2}$), 0.87 ($11H_{\gamma2}$), 0.75 ($2H_\gamma$), 0.63 ($4H_{\gamma2}$). ^{1}H NMR (DMSO-d_6): 2 conformations (81:19). Major isomer: 8.39 (7HN), 7.55 (8HN), 7.43 (H_{arom}), 7.37 (H_{arom}), 7.21 (H_{arom}), 7.20 (2HN), 6.36 (5H2a), 5.39 ($9H_\alpha$), 5.26 ($1H_\gamma$), 5.24 ($1H_\varepsilon$,H_ζ), 5.24 ($10H_\alpha$), 5.03 ($1H_\alpha$), 4.94 ($11H_\alpha$), 4.93 (1OH), 4.91 ($6H_\alpha$), 4.89 ($5H_\alpha$), 4.77 ($2H_\alpha$), 4.76 ($8H_\alpha$), 4.28 ($5H_\beta$), 4.15 ($7H_\alpha$), 4.14 ($3H_{\alpha1}$), 4.13 ($4H_\alpha$), 3.79 ($1H_\beta$), 3.24 ($3H_{\alpha2}$), 3.14 ($4NH_{\alpha1}$), 3.10 (10NMe), 3.05 ($4NH_{\alpha2}$), 3.02 (11NMe), 2.89 (3NMe), 2.83 (9NMe), 2.78 (1NMe), 2.67 (6NMe), 2.30 ($10H_{\beta1}$), 2.23 ($6H_{\beta1}$), 2.23 ($11H_\beta$), 2.17 ($1H_{\delta1}$), 2.15 ($4H_\beta$), 1.74 ($1H_{\delta2}$), 1.70 ($6H_\gamma$), 1.62 ($9H_{\beta1}$), 1.60 ($2H_{\beta1}$), 1.50 ($1H_\eta$), 1.39 ($2H_{\beta2}$), 1.37 ($9H_\beta2,H_\gamma$), 1.37 ($10H_\gamma$), 1.31 ($5Me_\beta$), 1.23 ($7H_\beta$), 1.15 ($6H_{\beta2}$), 1.15 ($8H_\beta$), 1.03 ($10H_{\beta2}$), 0.96 ($4H_{\gamma1}$), 0.88 ($4NH_\beta$), 0.86 ($1Me_\gamma$), 0.84 ($9H_\delta$), 0.82 ($11H_{\gamma1}$), 0.78 ($10H_\delta$), 0.68 ($2H_\gamma$), 0.68 ($6H_\delta$), 0.68 ($11H_{\gamma2}$), 0.54 ($4H_{\gamma2}$). All bond *trans* (undetermined 3-4).

Thr($\Psi^{Me,Me}$*pro*)^{5}-*CsA* **1c** ($C_{64}H_{113}N_{11}O_{13}$=1244.7). A solution of **13c** in 5.4 mL of methanol was added to a solution of MeONa (0.163 mmol) in 0.163 mL of methanol under inert atmosphere. After 24 h, the mixture was cooled to 0°C and hydrolyzed using 10% citric acid. After concentration under reduced pressure, the residue was dissolved in ethyl acetate, washed by 10% citric acid and brine, dried over Na_2SO_4 and concentrated. The crude product was purified on silicagel (acetone/hexane 4:6, R_f=0.33) to yield 62 mg (92%) of a white solid. HPLC t_R=29.22. ESI-MS (*m*/*z*)=1244.7 $[M+H]^+$. ^{1}H NMR ($CDCl_3$), 2 rotamers in a ratio 2:1. Major isomer: 8.08 (7HN), 7.08 (8HN), 6.72 (2HN), 5.67 ($10H_\alpha$), 5.55 ($4H_\alpha$), 5.41 ($1H_\varepsilon$,H_ζ), 5.14 ($3H_{\alpha1}$), 5.11 ($2H_\alpha$), 5.09 ($9H_\alpha$), 5.09 ($11H_\alpha$), 4.83 ($8H_\alpha$), 4.75 ($6H_\alpha$), 4.36 ($5H_\alpha$), 4.32 ($7H_\alpha$), 4.28 ($5H_\beta$), 4.01 ($1H_\beta$), 3.85 ($1H_\alpha$), 3.37 ($3H_{\alpha2}$), 3.24 (11NMe), 3.15 (9NMe), 3.09 (1NMe), 3.08 (4NMe), 3.05 (3NMe), 2.77 (6NMe), 2.51 ($1H_{\delta1}$), 2.50 ($6H_\beta$), 2.35 ($11H_\beta$), 1.98 ($2H_{\beta1}$), 1.97 ($5H_{2\beta1}$), 1.89 ($1H_{\delta2}$), 1.86 ($4H_\beta$), 1.82 ($5H_{2\beta2}$), 1.81 ($10H_{\beta1}$), 1.80 ($6H_\gamma$), 1.77 ($9H_{\beta1}$), 1.64 ($1H_\eta$), 1.57 ($10H_\chi$), 1.56 ($2H_{\beta2}$), 1.51 ($9H_\beta2,H_\gamma$), 1.49 ($10H_{\beta2}$), 1.47 ($1H_\gamma$), 1.45 ($4H_\gamma$), 1.37 ($7H_\beta$), 1.36 ($5Me_\beta$), 1.31 ($8H_\beta$), 1.02 ($6H_\delta$), 1.00 ($9H_{\delta1}$), 0.97 ($4H_\delta$),0.95 ($11H_{\gamma1}$), 0.84 ($2H_\gamma$), 0.84 ($9H_{\delta2}$), 0.82 ($1Me_\gamma$), 0.80 ($10H_{\delta1}$), 0.79 ($11H_{\gamma2}$), 0.75 ($10H_{\delta2}$); minor isomer: 8.34 (7HN), 7.12 (8HN), 7.03 (2HN), 5.66 ($10H_\alpha$), 5.51 ($1H_\varepsilon$,H_ζ), 5.50 ($4H_\alpha$), 5.25 ($9H_\alpha$), 5.09 ($11H_\alpha$), 4.99 ($2H_\alpha$), 4.96 ($1H_\alpha$), 4.83 ($3H_{\alpha1}$), 4.83 ($8H_\alpha$), 4.75 ($6H_\alpha$), 4.51 ($5H_\alpha$), 4.37 ($7H_\alpha$), 4.27 ($5H_\beta$), 4.05 ($1H_\beta$), 3.56 ($3H_{\alpha2}$), 3.15 (9NMe), 3.12 (11NMe), 3.10 (3NMe), 3.03 (10NMe), 3.02 (4NMe), 2.94 (1NMe), 2.77 (6NMe), 2.50 ($6H_\beta$), 2.45 ($11H_\beta$), 2.31 ($1H_{\delta1}$), 2.23 ($10H_\beta$), 2.05 ($1H_{\delta2}$), 1.93 ($5H_{2\beta1}$), 1.82 ($5H_{2\beta2}$), 1.81 ($2H_{\beta1}$), 1.80 ($6H_\gamma$), 1.77 ($4H_{\beta1}$), 1.72 ($1H_\gamma$), 1.66 ($9H_{\beta1}$), 1.65 ($1H_\eta$), 1.56 ($2H_{\beta2}$), 1.51 ($4H_{\beta2}$), 1.50 ($9H_\beta2,H_\gamma$), 1.41 ($4H_\gamma$), 1.40 ($7H_\beta$), 1.38 ($5Me_\beta$), 1.31 ($8H_\beta$), 1.11 ($10H_\gamma$), 1.06 ($1Me_\gamma$), 1.02 ($6H_\delta$), 0.95 ($4H_\delta$), 0.95 ($9H_{\delta1}$), 0.92 ($9H_{\delta2}$), 0.91 ($11H_{\gamma1}$), 0.87 ($10H_{\delta1}$), 0.80 ($11H_{\gamma2}$), 0.79 ($10H_{\delta2}$), 0.76 ($2H_\gamma$). Major isomer: amide 5-6 *cis*, minor isomer amide bonds 11-1 and 5-6 *cis*. NMR (DMSO-d_6), 4 rotamers in a ratio 62:24:19:19. Major isomer: 8.39 (8HN), 8.27 (2HN), 6.71 (7HN), 5.38 ($1H_\varepsilon$,H_ζ), 5.37 ($10H_\alpha$), 5.20 ($1H_\alpha$), 5.15 ($6H_\alpha$), 5.07 ($4H_\alpha$), 4.88 ($11H_\alpha$), 4.77 ($2H_\alpha$), 4.75 ($5H_\alpha$), 4.73 ($3H_{\alpha1}$), 4.49 ($8H_\alpha$), 4.29 ($7H_\alpha$), 3.97 ($5H_\beta$), 3.91 ($1H_\beta$), 3.46 ($3H_{\alpha2}$), 3.09 (3NMe), 2.89 (6NMe), 2.84 (4NMe), 2.81 (1NMe), 2.81 (11NMe), 2.75 (9NMe), 2.38 ($1H_{\delta1}$), 2.19 ($11H_\beta$), 1.84 ($10H_\beta$), 1.74 ($4H_{\beta1}$), 1.72 ($1H_{\delta2}$), 1.68 ($2H_{\beta1}$), 1.66 ($6H_\beta$), 1.65 ($5H2_{\beta1}$), 1.57 ($1H_\eta$), 1.54 ($2H_{\beta2}$), 1.44 ($6H_\gamma$), 1.41 ($5Me_\beta$), 1.38 ($5H2_{\beta2}$), 1.35 ($4H_\gamma$), 1.27 ($1H_\gamma$), 1.19 ($7H_\beta$), 1.19 ($10H_\gamma$), 1.17 ($8H_\beta$), 1.03 ($4H_{\beta2}$), 0.94 ($10H_{\delta1}$), 0.90 ($2H_\gamma$), 0.90 ($6H_{\delta1}$), 0.86 ($10H_{\delta2}$), 0.84 ($4H_{\delta1}$), 0.82 ($1Me_\gamma$), 0.82 ($11H_{\gamma1}$), 0.76 ($4H_{\delta2}$), 0.76 ($6H_{\delta2}$), 0.62 ($11H_{\gamma2}$). All *trans* bond (undetermined for 9-10). Minor isomer (24%): 8.55 (8NH), 7.16 (2NH), 6.69 (7NH), 5.01 (4Ha), 4.99 (6Ha), 4.83 ($3H\alpha_1$), 4.82 (2Ha), 4.44 (8Ha), 4.40 (7Ha), 3.74 ($3H\alpha_2$). 3-4 *cis* bond. (19%): 8.22 (8NH), 7.42 (7NH), 6.94 (2NH), 4.84 (2Ha), 4.76 (7Ha), 4.08 (8Ha). (19%): 8.44 (8NH), 7.21 (7NH), 7.15 (2NH), 4.57 (8Ha), 4.44 (2Ha), 4.31 (7Ha).

NMeIle ^{4}-*Thr*($\Psi^{Me,Me}$*pro*)^{5}-*CsA* **1d** ($C_{64}H_{113}N_{11}O_{13}$= 1244.7). 107.5 mL of a solution of sodium in methanol (111 mg in 11.3 mL) was added to 10 mg of **13d** in methanol (215 μL) and vigorously stirred. After 2.5 h, 20 mL of ethyl acetate was added and the solution was washed with citric acid (2×10 mL) and water (2×10 mL) dried over magnesium sulfate and concentrated under reduced pressure. The crude peptide was purified by semipreparative HPLC (gradient 50–100% in 20 min, C_{18}) to yield **1d** (10%). HPLC t_R=12.72 min. ESI-MS (*m*/*z*)=1245.3 $[M+H]^+$, 642.4 $[M+2H]^{2+}$.

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