The Role of Water Molecules in the Structure-Based Design of (5-Hydroxynorvaline)-2-cyclosporin: Synthesis, Biological Activity, and Crystallographic Analysis with Cyclophilin A

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Received March 29, 1995[⊗]

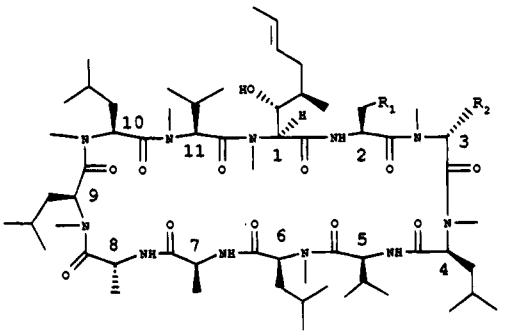
Analysis of the contact surface of the cyclophilin A (CypA)/cyclosporin A (CsA, **1**) crystal structure delineates a unique cavity between both molecules in the vicinity of the Abu-2 side chain atoms of **1** (Abu pocket). Therefore, (5-hydroxynorvaline)-2-cyclosporin (**2**) was designed and prepared as a CsA derivative which could mediate additional interactions within the pocket. The X-ray crystal structure of the CypA/2 complex at 1.76 Å resolution shows that **1** and **2** have identical backbone conformations and that the introduced hydroxypropyl chain makes indeed the expected supplemental interactions with CypA. However, **2** has 8–9-fold lower binding affinity than **1** for CypA. This results from a presumed unfavorable free energy change associated with the displacement of one of the tightly bound water molecules within the pocket and a change in prebinding equilibria. The role of the later was assessed by comparing the conformation distribution of **1** and **2** to that of norvaline-2-cyclosporin (**3**) and norvaline-2-(D-MeSer)-3-cyclosporin (**4**).

Introduction

The immunosuppressive drug cyclosporin A (CsA, Sandimmun, **1**; Figure 1) elicits most of its cellular effects by first binding to its soluble intracellular receptor protein cyclophilin (Cyp), forming a Cyp–CsA complex, which subsequently interacts with calcineurin and inhibits its phosphatase activity. Thus, the transcription of a family of early activation genes, including those of the principal T cell growth factor IL-2, is blocked.[1] It is still unclear whether cyclophilin A (CypA) or B (CypB) is the isoform mediating immunosuppression.[2,3] Consequently, the interaction between CypA and CypB with CsA has been investigated by X-ray analysis of the resulting complexes, and it has been shown that the sites recognized by the different Cyps on **1** are very similar.[4,5] No differences (rmsd < 0.15 Å) either in the hydrogen-bonding pattern or in the sequence and structure between the two complexes for any residues of the binding pocket within 5.0 Å of CsA could be observed. Moreover, water-mediated contacts as well as tightly bound water molecules are essentially the same. In both complexes, van der Waals contacts are made between Cyp and residues 9–11, 1, and 2 (binding domain) of **1**, providing a fit resembling a coin (CsA) partly inserted into a slot machine (Cyp). Four CsA residues, 4–7, none of which are in close contact with Cyp, protrude from the surface of the complex and delineate the effector domain which is thought to interact with calcineurin. In an attempt to obtain cyclosporin derivatives with increased binding affinity for its receptor, the 3D architecture of the mode of binding of **1** with CypA was analyzed.

Design Concept

The surface contact areas between CypA and CsA indicate that there is a snug fit for the side chain atoms of the residues of CsA's "binding domain" onto the surface of CypA (for instance, Val11 has an accessible surface area of 0.0 Å^2). The unique noteworthy exception concerns the side chain atoms of the Abu residue 2 which lie in a cavity (the Abu pocket) which is partly filled up with crystallographic water molecules[6] (Figure 2). At the bottom of the cavity, there are three very well ordered water molecules (W5–W7)[7] with an average temperature factor of 16.2 Å^2. This value is comparable with that of the protein atoms (13.0 Å^2). The average temperature factor of the 144 solvent molecules is 37.2 Å^2. In addition, a fourth water molecule, W133, is found at the side of the cavity and is less tightly bound as indicated by its temperature factor of 35.7 Å^2. In contrast to W5 and W7 which are buried as typified by an accessible surface area lower than 10 Å^2, W133 is in contact with the first hydration shell of CypA. The



$R_1 = CH_3$	$R_2 = H$	1
$R_1 = CH_2CH_2OH$	$R_2 = H$	2
$R_1 = CH_2CH_3$	$R_2 = H$	3
$R_1 = CH_2CH_3$	$R_2 = CH_2OH$	4

Figure 1. Structure of cyclosporin A, 1, (5-hydroxynorvaline)-2-cyclosporin, **2**, norvaline-2-cyclosporin (cyclosporin G, CsG), **3**, and (norvaline)-2-(D-MeSer)-3-cyclosporin, **4**.

[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1995.

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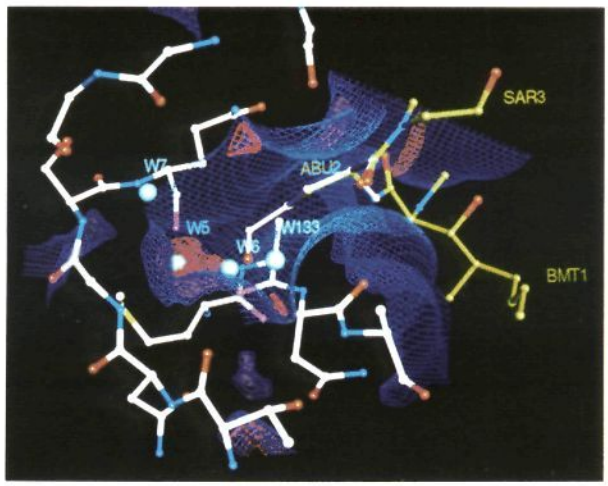

Figure 2. Abu pocket in the CypA/CsA crystal structure. Carbon, oxygen, and nitrogen atoms of **1** are displayed in green, red, and blue, respectively. The four water molecules which sit in the pocket are displayed as filled cyan circles. Carbon, oxygen, and nitrogen atoms of CypA which delineate the Abu pocket are represented in white, red, and blue, respectively. The red and blue surfaces represent the hydrophilic and the van der Waals surfaces, respectively, of the Abu pocket of the CypA molecule in the CypA/CsA crystal structure, with the crystallographic water molecules and CsA removed. The solvent molecules W5 and W6 appear to lie in strong hydrophilic areas of the Abu pocket. Surfaces were generated with the program PROBIS.[11]

remaining water molecule W6 has an accessible surface area of 14 Å^2 and can be considered as semiburied.[8] Moreover, W5−W7 and W133 are hydrogen bonded to three, one, two, and zero protein atoms, respectively. Interestingly, these four water molecules are also present in the CypB/CsA complex[5] as well as in all CypA/CsA derivative crystal complexes studied so far.[9,10] This suggests that they are part of the protein structure. Analysis of the hydrophilicity of the CypA molecule[11] clearly indicates that the three well-ordered water molecules bind to hydrophilic region of the Abu pocket, the rest of the pocket being rather hydrophobic. Thus (5-hydroxynorvaline)-2-cyclosporin, **2**, was designed as a compound capable of both mediating hydrogen bonding within the Abu pocket, either with water molecules or with backbone carbonyl oxygen atoms, as well as increasing the contact surface area with CypA. Furthermore the hydroxyl group is predicted either to displace or to replace one of the tightly bound water molecules.

Synthesis

For the synthesis of **2**, a suitably protected glutamic acid derivative was employed as a precursor for the desired hydroxypropyl moiety (Scheme 1). Thus, the decapeptide H-Sar-MeLeu-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-MeBmt-OMe, **6**,[12] was coupled with Fmoc-Glu(O-*t*-Bu)-OH to afford the fully protected undecapeptide **7** whose *tert*-butyl ester was hydrolyzed under acidic conditions leading to the glutamic acid derivative, **8**, in 76% overall yield. The acid moiety of the latter was selectively reduced to the corresponding alcohol **9** via its mixed anhydride in 89% yield. No cleavage of the Fmoc protecting group or reduction of the sensitive methyl ester was observed. The remainder of the synthesis was accomplished without protection

Scheme 1[a]

H-Sar-MeLeu-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeLeu-MeVal-MeBmt-OMe **6**

a) ↓ 92%

Fmoc-Glu(OtertBu)-Sar-MeLeu-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-MeBmt-OMe **7**

b) ↓ 83%

Fmoc-Glu-Sar-MeLeu-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-MeBmt-OMe **8**

c) ↓ 89%

Fmoc-(5-OH)Norval-Sar-MeLeu-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-MeBmt-OMe **9**

d) ↓ 76%

H-(5-OH)Norval-Sar-MeLeu-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-MeBmt-OMe **10**

e) ↓ 83%

H-(5-OH)Norval-Sar-MeLeu-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-MeBmt-OH **11**

f) ↓ 26%

2

[a] (a) DMAP (1.1 equiv), BOP (1.1 equiv), Fmoc-Glu(O-*t*-Bu)-OH; (b) CF_3CO_2H (excess), 0 °C, 1 h; (c) (i) Et_3N (1.1 equiv), $ClCO_2Et$ (1.1 equiv), −10 °C, (ii) $NaBH_4$ (2.2 equiv), 0 °C; (d) Et_2NH, Δ; (e) LiOH (1.1 equiv), THF−H_2O, 3:1; (f) DMAP (4 equiv), BOP (1.1 equiv), CH_2Cl_2.

of the hydroxypropyl moiety and consisted of the sequential deprotection of the N and C termini of **9** in 63% overall yield and the ring closure of the resulting undecapeptide **11** to give diastereomerically pure **2**.

Compound **4**, needed for conformational analysis and affinity measurements, was synthesized from the naturally occurring cyclosporin G (CsG, **3**).[13] In analogy to the synthesis of (D-MeSer)-3-CsA,[14] CsG was treated with an excess of lithium diisopropylamide (LDA), and the resulting hexalithio derivative was quenched with commercially available paraformaldehyde to yield (D-MeSer)-3-CsG (**4**) in 32% yield.

X-ray Crystallographic Studies of 2 Complexed with CypA

To gain insight into the ligand−receptor interaction, large single crystals of **2** complexed with CypA could be grown which allowed the high-resolution (1.76 Å) X-ray analysis of the structure to a crystallographic *R*-factor of 0.175 (Table 1). The root-mean-square (rms) difference between the CypA atoms found in the Abu pocket residues of this complex (all atoms within 5.0 Å of the Abu atoms) and of those found in the CypA/CsA complex is 0.16 Å, indicating similar geometry. A comparison of the cyclosporin peptide backbone between bound **1** and bound **2** gave a rms difference of 0.12 Å for C, N, and C^α atoms with no significant variations as a function of the residue number. The rms difference between bound **2** and bound CsA for all non-hydrogen atoms is 0.17 Å. Consequently, there are no significant conformational variations concerning the cyclosporin backbones of **1** and **2**. It is noteworthy that two out of the four water molecules found in the Abu pocket of the CypA/CsA crystal (W5−W7 and W133)[7] have been displaced upon binding of **2**. The solvent molecule W6 has moved slightly out of the pocket by 1.89 Å and is now both hydrogen bonded to the carbonyl oxygen atom

Table 1. Crystallographic Data of the CypA/**2** Complex

Crystal Parameters	
crystallization method	cross-seeding in hanging drop as previously described[32]
mother liquor	drop: 50 mM Tris/HCl, pH 8.2, 9.0% (w/v) PEG_{8000}, 2.5% (v/v) DMSO, 0.02% (w/v) NaN_3, [CypA:**2**] = 0.05 mM. reservoir: 100 mM Tris/HCl, pH 8.2, 18.0% (w/v) PEG_{8000}, 5.0% (v/v) DMSO, 0.02% (w/v) NaN_3.
space group, cell dimensions	$P2_12_12_1$, a = 36.44 Å, b = 61.32 Å, c = 72.57 Å, $\alpha = \beta = \gamma = 90°$
crystal size (mm)	0.6 × 0.4 × 0.3
Diffraction Data	
X-ray source, apparatus	rotating anode operating at 40 kV, 80 mA, Cu Kα, FAST television area detector
unique *hkl* (completeness)	*14 501 (87.2% complete to 1.76 Å)*
no. of measurements, R_{merge}	46 587, 4.3%
Phase Determination and Refinement	
method	molecular replacement[33]
model	monomeric complex without CsA[4]
refinement	X-PLOR[33,a]
Quality of the Structure	
resolution limits (Å)	8.0−1.76
final R-factor[b] (all data)	0.175
no. of cyclophilin non-H atoms	1265
no. of **2** non-H atoms	87
no. of solvent molecules	188
Ramachandran outliers	none
estimated error on the coordinaes (Å)	0.15
Weighted rms Deviations from Ideality	
bond length (Å)	0.011
bond angle (deg)	1.57

[a] The occupancies of the doubly disordered conformers were first set to 0.5 and then refined with the temperature factors with powell conjugate gradient minimization. [b] R-Factor = $\Sigma||F_o| - |F_c||/\Sigma|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively.

of Thr107 and bridged to the carbonyl oxygen atoms of Glu81 and Gly75 through W129. This has presumably induced the displacement of W133 by ca. 1.48 Å, which otherwise would have been in close contact with W6 (2.2 Å). The former (W133) is hydrogen bonded to the first hydration shell of the CypA molecule and has no hydrogen bond contact with the protein. The ligand/receptor interactions in the CypA/**1** and CypA/**2** complexes are similar with the exception of the hydroxypropyl chain in **2**. Indeed, this side chain lies in the Abu pocket as originally designed (Figure 3), and it adopts doubly disordered conformations. The B-factors for the hydroxyl group of the major and minor conformers are 16.0 and 17.7 Å^2, respectively and compare well with the average B-value for the atoms of **2** (16.9 Å^2) and for those of the protein (14.8 Å^2). The major conformer, with an occupancy of 0.62, leads to the formation of supplementary hydrogen bonds between the hydroxyl function and two water molecules of the Abu pocket (W6 and W7). The minor conformer, with an occupancy of 0.38, makes hydrogen bonds between the hydroxyl function and the carbonyl oxygen atom of Thr107 and the water molecule W6 (Figure 4, Table 2). Thus, the concept that supplementary H-bond interactions can be obtained through the introduction of polar moieties at Abu's ethyl side chain of CsA has been experimentally verified.[6] Furthermore, the contact surface area between **2** and CypA has been slightly increased up to 288 Å^2 when compared with bound CsA (270 Å^2) suggesting additional interactions.

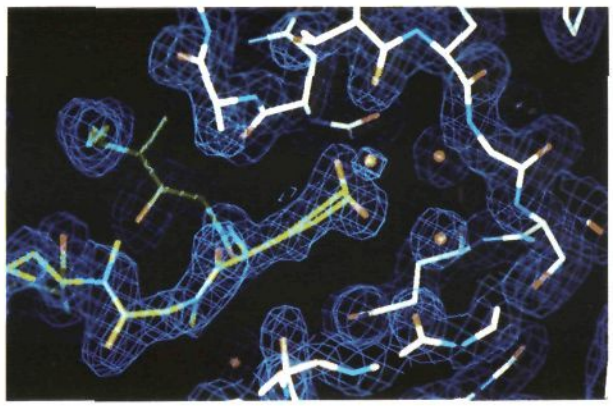

Figure 3. Electron density $2F_o - F_c$ map calculated at 1.76 Å and contoured at 1.0 σ of the two conformers of 2 bound to CypA in the Abu pocket. Carbon, oxygen, and nitrogen atoms of **2** are displayed in green, red, and blue, respectively. Water molecules are shown as filled red circles. CypA carbon, oxygen, and nitrogen atoms are displayed in white, red, and blue, respectively.

Table 2. Hydrogen Bonds and Water-Mediated Contacts between CypA and **2** Identified in the CypA/**2** Crystal Structure[a]

Hydrogen Bonds between **2** and CypA

2 atom	dist (Å)	CypA atom	**2** atom	dist (Å)	CypA atom
MeBmt1 O′	3.1	Gln63 N_ϵ2	MeLeu9 O′	2.9	Trp121 N_ϵ1
(OH)Nva2N	3.1	Asn102 O′	MeLeu10 O′	3.0	Arg55 N_η1
(OH)Nva2 O_ϵ^m	3.2	Thr107 O′	MeLeu10 O′	3.0	Arg55 N_η2

Water-Mediated Contacts between **2** and CypA

2 atom	dist (Å)	water molecule	dist (Å)	water molecule	dist (Å)	CypA atom
MeBmt1 O′	3.5	W18	2.8			His54 N_ϵ2
MeBmt1 O′	3.5	W18	3.3			Gln63 N,2
MeBmt1 O′	3.5	W18	2.6	W19	2.8	Asn71 O′
MeLeu6 O′	2.5	W87	2.5	W107	2.7	Arg55 N_η2
DAla8 O′	3.0	W87	2.5	W107	2.7	Arg55 N_η2
(OH)Nva2-O_ϵ^m	2.7	W6	2.9	W129	2.7	Glu81 O′
(OH)Nva2-O_ϵ^m	2.7	W6	2.9	W129	2.9	Glu81 O_ϵ2
(OH)Nva2-O_ϵ^m	2.7	W6	2.7			Thr107 O′
(OH)Nva2-O_ϵ^M	2.6	W6	2.9	W129	2.7	Glu81 O′
(OH)Nva2-O_ϵ^M	*2.6*	*W6*	*2.9*	*W129*	*2.9*	*Glu81 O_ϵ2*
(OH)Nva2-O_ϵ^M	2.6	W6	2.7			Thr107 O′
(OH)Nva2-O_ϵ^M	2.7	W7	3.0			Gly74 O′
(OH)Nva2-O_ϵ^M	2.6	W7	2.8			Ser110 N
(OH)Nva2-O_ϵ^M	2.6	W7	3.1			Gln111 N

[a] Superscripts M and m designate the major and minor conformer, respectively, observed for this side chain. (OH)Nva stands for 5-hydroxynorvaline.

Biological Results and Discussion

The *in vitro* biological activity of **2** concerning both its binding affinity to CypA as well as its immunosuppressive activity is summarized in the Table 3. Binding affinity was determined at 298 K under competitive ELISA systems[15] and by measuring the inhibition of the rotamase activity of cyclophilins,[16] whereas the immunosuppressive activity was determined in the mouse mixed lymphocyte reaction (MLR_M)[17] and the interleukin-2 reporter gene assay (IL2_RGA).[18]

These results clearly indicate that the compound has marginal immunosuppressive activity and approxi-

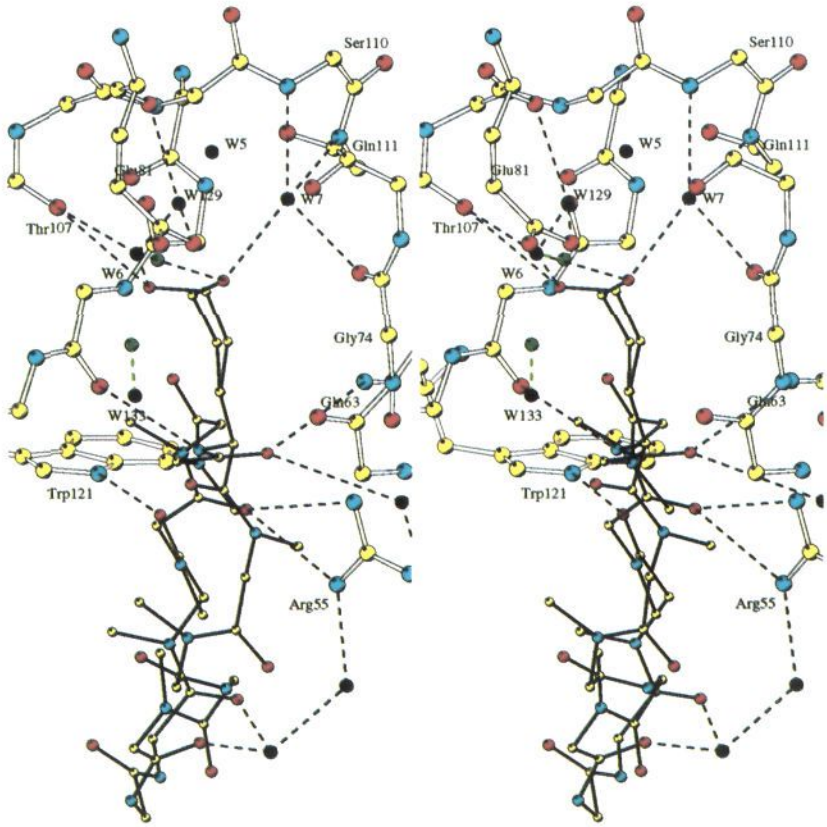

Figure 4. Hydrogen bond interactions of **2** with CypA. Stereopicture showing the peptide backbone conformation of **2** and the side chain atoms of hydroxynorvaline bound to CypA. Carbon, oxygen, and nitrogen atoms of **2** are displayed in yellow, red, and blue, respectively, and all covalent bonds of **2** are drawn with black lines. All CypA residues forming hydrogen bonds with bound **2** as observed in the CypA/**2** crystal structure are shown with their backbone atoms except Arg55, Gln63, Glu81, and Trp121 which make hydrogen bond interactions with their side chain atoms:carbon atoms (yellow), nitrogen atoms (blue), and oxygen atoms (red). Hydrogen bonds are drawn as dashed black lines. Water molecules are shown as filled black circles. The positions of the water molecules W6 and W133 observed in the CypA/**1** crystal structure[4] are represented by filled green circles. The displacement of these water molecules are represented by dashed green lines. Values of the distances between **2** and CypA and the water molecules are given in Table 2. The picture was prepared with the program MOLSCRIPT.[34]

Table 3. *In Vitro* Biological Activities of the Compounds[a]

compd	CypA[b] (rel IC_{50})	CypA[c] (rel IC_{50})	MLR (rel IC_{50})	IL2_RG (rel IC_{50})
1 (CsA)	62 (1)	27 (1)	20 (1)	5 (1)
2	450 (7.2)	256 (9.5)	140 (7)	70 (14)
3 (CsG)	310 (5)	130 (4.8)	46 (2.3)	13 (2.6)
4	93 (1.5)	48 (1.7)	56 (2.8)	26 (5.2)

[a] Numbers indicate the mean IC_{50} values in nM of three independent experiments. The relative IC_{50} value (rel IC_{50}) is the ratio of IC_{50}(compound)/IC_{50}(CsA). [b] Binding affinity determined by ELISA. Mean standard deviation is ±2.5 nM. [c] Binding affinity determined by the rotamase assay. Mean standard deviation is ±2.0 nM.

mately 8 times lower affinity than **1** for cyclophilin A. The immunosuppression being measured in cellular assays, the loss in activity is due to the polar character of **2** which prevents it from penetrating into the cell.[19] More surprising are the binding results. In thermodynamic terms, how can the difference in the free energy of binding between **1** and **2** ($\Delta\Delta G^{\circ 1\to 2}$) be explained?

The association of a ligand (L) with a protein receptor (R) in aqueous solution is described by the equilibrium L + R ⇌ LR, where R and L represent the initial state separated in solvent and LR is the final state in the solvent. The standard free energy change of this process (ΔG°) is related to the equilibrium constant K by the equation $\Delta G^\circ = -1.4 \log K$ (T = 298 K) and can be written[20] as $\Delta G^\circ = \Delta G_{int} + \Delta U + \delta G$, where ΔG_{int} is an organization term including all the contributions due to changes in the translational, rotational, and vibrational degrees of freedom of L and R, ΔU represents the direct interaction between L and R, and δG takes into account all the solvent effects (solvation energy). The latter corresponds to the free energy difference between the solvated ligand−receptor complex and the sum of the solvated protein receptor and solvated ligand when they are widely separated. For the comparison of the complex formation of CypA with **1** and **2**, the quantity of interest is the difference in binding free energy for the association of CypA with **1** and **2** ($\Delta\Delta G^{\circ 1\to 2}$) which, based on the measured binding constants, is ca. 1.3 kcal/mol at 298 K. Therefore, $\Delta\Delta G^{\circ 1\to 2} = \Delta\Delta G_{int}{}^{1\to 2} + \Delta\Delta U^{1\to 2} + \Delta\delta G^{1\to 2} = 1.3$ kcal/mol. The relative importance of each of the terms of the equation was estimated as follows.

(i) $\Delta\Delta G_{int}{}^{1\to 2}$. The crystallographic analysis shows that the structure of CypA when bound with **1** or **2** is virtually identical, and it suggests that the change in ΔG_{int} results exclusively from the differences between **1** and **2**. Indeed the modification of the side chain at position 2 of CsA could influence the conformational flexibility of the peptide backbone. Consequently, this could affect prebinding equilibria, leading to a lower population of Cyp-binding conformers.[9,21,22] Experimental evidence concerning prebinding equilibria was ob-

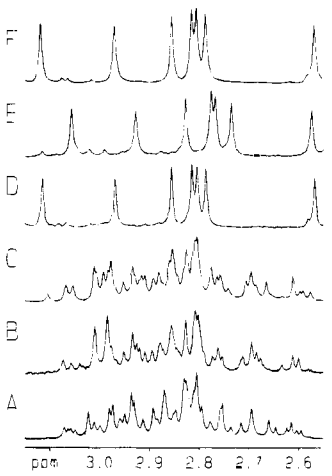

Figure 5. ^{1}H-NMR (DMSO-d_6) spectra of **1** (A), **2** (B), **3** (C), **4** (D), and **5** (F) and also that of **4** (E) in DMSO-d_6–D_2O (80:20). Only the regions corresponding to the *N*-methyl resonances are shown. One set of resonances, indicative of a unique conformation, is observed for **4** (D, E) and **5** (F)[23] as compared to multiple sets for **1**–**3** (A–C) confirming the stabilizing effect of D-MeSer in position 3.

tained on the basis of ^{1}H-NMR studies of compound **2**. The spectrum in polar solvents (D_2O or DMSO-d_6) of (D-MeSer)-3-CsA (**5**), a high-affinity CypA ligand, shows that the compound has one predominant conformation which is almost identical to the CypA-bound one.[23] The affinity of **5** is solely due to favorable binding equilibria since the X-ray crystallographic analysis of the CypA/**5** complex did not reveal additional receptor–ligand interactions as compared with the CypA/CsA complex.[24] The ^{1}H-NMR spectrum of **2** in DMSO-d_6 clearly showed a mixture of various conformations as indicated by the NMe signals (Figure 5). Therefore, the structure of **2** observed in the CypA/**2** complex might represent only a small percentage of the total population of conformers in solution. Despite the complexity of both spectrums for **1** and **2**, significant qualitative differences could be found in the number and intensities of the NMe signals between the NMR spectrum of **2** and the corresponding spectrum of **1**, suggesting different conformation distribution.

In order to assess the relative importance of the prebinding equilibria, the (D-MeSer)-3 derivative of **2** was conceived as a compound which might preferentially adopt the CypA-bound conformation in aqueous solution. Due to synthetic difficulties, this compound could not be prepared, and consequently, an indirect approach based on cyclosporin G, **3**, was explored. Molecular modeling of the CypA/CsG complex indicates that the norval-2 chain does not disturb the location of W5–W7 and W133 and still makes hydrophobic contacts similar to those observed with **2**. The ^{1}H-NMR (DMSO-d_6) spectrum of **3** shows multiple sets of resonances in the NMe area, but the corresponding spectrum of the (D-MeSer)-3-CsG derivative **4** shows predominantly one (Figure 5). Moreover, **4** has 3-fold higher affinity as compared to **3** which corresponds to a free energy change of ca. 0.7 kcal/mol. Since the additional hydroxymethyl moiety in position 3 of **4** is not in contact with the protein,[24] the calculated free energy variation is likely due to the conformational destabilization of **3** by the norvaline side chain. Hence, extrapolating for **2**, $\Delta\Delta G_{int}^{1\rightarrow 2}$ is positive and also ca. 0.7 kcal/mol.

(ii) $\Delta\Delta U^{1\rightarrow 2}$. The hydroxypropyl side chain of **2** leads to the formation of increased surface contact area, and it makes additional hydrogen bonds with the protein and solvent atoms when compared with CsA. Both the major and minor conformers of the hydroxynorvaline residue are bridged to CypA through water molecules, but the minor conformer makes a direct hydrogen bond with the carbonyl atom of Thr107 which, according to the distance (3.2 Å), can be considered as soft. It is difficult to assess the contribution of water-mediated hydrogen bonds, but it can be assumed that the term $\Delta\Delta U^{1\rightarrow 2}$ is negative and lies in the lower range of energy of one hydrogen bond formation[25] (ca. −0.5 kcal/mol).

(iii) $\Delta\delta G^{1\rightarrow 2}$. The difference in the desolvation free energy between **1** and **2** is due to the additional hydroxymethylene moiety. Its energetic contribution can be related to the desolvation of methanol (5 kcal/mol) corrected by geometrical factors involved in the extent of exposure of the CH_2OH group[26] and corresponds to a value of ca. 1 kcal/mol. Two water molecules (W133 and W6) have been displaced upon binding of **2** to CypA when compared with CsA. W133 was not bound to the protein but to the hydration shell of CypA, whereas W6 was relatively tightly bound to the carbonyl of Thr107. The solvent molecule W6 is still bound to this carbonyl atom and also to the hydroxyl group of the hydroxylpropyl side chain of each conformer. The free energy cost of the displacement of W6 is certainly less than that observed for a tightly bound water molecule in crystalline salt (at most 2 kcal/mol[27]). The term $\Delta\delta G^{1\rightarrow 2}$ which accounts both for the desolvation of **2**, a positive contribution, and for the displacement of W6 should therefore be lower than 2 kcal/mol.

In the CypA/**2** system, the favorable interaction term ($\Delta\Delta U^{1\rightarrow 2} \simeq -0.5$ kcal/mol) is presumably counterbalanced by the organization term ($0 \leq \Delta\Delta G_{int}^{1\rightarrow 2} \leq 0.7$ kcal/mol). The fact that the gain in hydrogen bond interactions arises from a supplemental soft direct hydrogen bond and additional water-mediated contacts which probably do not contribute significantly to the binding energy could explain this result. Therefore, the calculated value for $\Delta\Delta G^{\circ 1\rightarrow 2}$ of 1.3 kcal/mol is likely accounted for by the unfavorable desolvation energy ($\Delta\delta G^{1\rightarrow 2} \leq 2$ kcal/mol).

Conclusion

Although the Abu pocket of cyclophilin A appears to provide an ideal location for the structure-based design of cyclosporin derivatives with increased affinity for CypA, the attempts undertaken until now have remained unsuccessful.[6,12] In this paper, the limiting factors of such an approach have been semiquantitatively studied, illustrating some of the difficulties of structure-based drug design. (5-Hydroxynorvaline)-2-cyclosporin, **2**, was designed as capable of potentiating additional interactions with CypA in the Abu pocket. The compound was synthesized and showed by crystal structure analysis to indeed make supplementary interactions. However, the conformational rigidity of the peptide backbone was also affected. It results that the free energy gain due to additional interactions does not outweigh the unfavorable free energy associated with the conformational destabilization of the cyclosporin backbone and the presumed unfavorable desolvation free energy. This raises the general question in drug

design of the usefulness of incorporating the binding features of structural water molecules into an inhibitor. In the HIV protease field, a cyclic urea derivative capable of mimicking and displacing a structural water molecule had significantly increased potency as compared to the parent linear inhibitor.[28] In this latter case the nearly ideal geometry of the gained hydrogen bonds and the conversion of a flexible structure into a conformationally restricted one certainly contributed to the improved biological activity. It therefore appears that incorporation of binding features of structural water molecules into a ligand is likely to yield compounds with improved biological activity only if it can provide both conformational stabilization of the drug and strong direct hydrogen bonds with the receptor.[29,30]

Experimental Section

All compounds were characterized by 300 MHz proton nuclear magnetic resonance spectroscopy using a Bruker-300FT-NMR spectrometer. Chemical shifts are expressed as ppm downfield from tetramethylsilane; J values are reported in hertz (Hz). At room temperature, all peptidic sequences were mixtures of conformers. Fast atom bombardment mass spectroscopy (Xe, 8 keV) on a VG70-SE mass spectrometer was also used for the characterization of the reported compounds. C, H, and N analyses were curried out, and ±0.4% was acceptable. For the chromatographic purifications, the flash chromatography technique was applied using 230–400 mesh silica gel. The following abbreviation is used, (4*R*)-4-((*E*)-2-butenyl)-4,*N*-dimethyl-L-threonine (MeBmt).

Fmoc-Glu(O-*t*-Bu)-Sar-MeLeu-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-MeBmt-OCH$_3$ (7). To a solution of decapeptide[12] **6** (800 mg, 6.96 mmol) in dichloromethane (30 mL) was added sequentially (dimethylamino)pyridine (93.50 mg, 7.65 mmol), Fmoc-Glu(O-*t*-Bu)-OH (280 mg, 6.96 mmol), and (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluoroborate (BOP; 339 mg, 7.65 mmol). After stirring at room temperature for 3 h, water was added and the organic phase was collected, dried over $MgSO_4$, filtered, and concentrated to dryness. Purification on silica gel column chromatography (ethyl acetate–acetone, 9:1) gave **7** (1.0 g, 92%) as a white foam: $[\alpha]_D = +131.2°$ (c = 0.5, methanol); FAB-MS 1579 $(M + Na)^+$, 1341 (M − MeBmt-OMe), 1228 (1341 − MeVal), 1101 (1228 − MeLeu), 974 (1101 − MeLeu), 832 (974 − Ala-Ala), 606 (832 − MeLeu-Val), 479 (606 − MeLeu). Anal. ($C_{83}H_{133}N_{11}O_{17}$) C, H, N.

Fmoc-Glu-Sar-MeLeu-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-MeBmt-OCH$_3$ (8). A solution of **7** (945 mg, 6.07 mmol) in trifluoroacetic acid (15 mL) was maintained at 0 °C for 1 h and then evaporated to dryness (<40 °C). Aqueous saturated sodium bicarbonate (20 mL) and ethyl acetate (5 mL) were added. The two layers were separated, and the organic one was dried over $MgSO_4$, filtered, and concentrated to dryness. Purification on silica gel column chromatography (ethyl acetate–acetone, 9:1, then dichloromethane–methanol, 9:1) afforded **8** (760 mg, 83%) as a white foam: $[\alpha]_D = -126.0°$ (c = 0.5, methanol); FAB-MS 1538 $(M + K)^+$, 1522 $(M + Na)^+$, 1285 (M − MeBmt-OMe), 1045 (1285 − MeVal-MeLeu), 776 (1045 − MeLeu-Ala-Ala), 550 (776 − MeLeu-Val), 423 (550 − MeLeu). Anal. ($C_{79}H_{125}N_{11}O_{17}$) C, H, N.

Fmoc-(5-OH)Nv-Sar-MeLeu-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-MeBmt-OCH$_3$ (9). To a cold (−10 °C) solution of **8** (750 mg, 0.50 mmol) in THF (20 mL) was added triethylamine (0.074 mL, 0.55 mmol) and ethyl chloroformate (0.050 mL, 0.55 mmol). The solution was stirred at −10 °C for 30 min and the resulting precipitate filtered. To the filtrate were added at 0 °C 2 mL of water and $NaBH_4$ (42 mg, 1.10 mmol), and the solution was stirred at 0 °C for 2 h. Water was added, the undecapeptide was extracted with ethyl acetate, and the organic phase was dried over $MgSO_4$, filtered, and concentrated. The crude was chromatographed over silica gel (ethyl acetate-acetone, 9:1) to give **9** (665 mg, 89%) as a colorless oil: $[\alpha]_D = -131.0°$ (c = 0.5, methanol); ^{1}H-NMR (DMSO-d_6) δ 3.44 (t, J = 6.04, 1H, exchangeable with D_2O, (OH)Nva); FAB-MS 1508 $(M + Na)^+$, 1472 $(MH)^+$, 1271 (M − MeBmt-OMe), 1158 (1271 − MeVal), 1031 (1158 − MeLeu), 904 (1031 − MeLeu), 762 (904 − Ala-Ala), 536 (762 − MeLeu-Val), 409 (536 − MeLeu). Anal. ($C_{79}H_{127}N_{11}O_{16}$) C, H, N.

H-(5-OH)Nva-Sar-MeLeu-Val-MeLeu-Ala-(D)Ala-MeLeu-MeLeu-MeVal-MeBmt-OCH$_3$ (10). A solution of **9** (650 mg, 0.44 mmol) in dichloromethane (20 mL) and diethylamine (1 mL) was refluxed for 4 h. It was then evaporated to dryness and the crude purified on silica gel column chromatography (ethyl acetate–acetone, 9:1, then dichloromethane–methanol, 9:1) to give **10** (420 mg, 76%) as a white foam: $[\alpha]_D = -142.0°$ (c = 0.5, methanol); FAB-MS 1264 $(MH)^+$, 1049 (M − MeBmt-OMe), 936 (1049 − MeVal), 809 (936 − MeLeu), 682 (809 − MeLeu), 540 (682 − Ala-Ala). Anal. ($C_{64}H_{117}N_{11}O_{14}$) C, H, N.

[(5-OH)Nva]-2-cyclosporin (2). To a solution of **10** (310 mg, 0.24 mmol) in THF (8 mL) and water (2 mL) was added lithium hydroxide (11.3 mg, 0.27 mmol). After stirring for 4 h at room temperature, the solution was made acidic with HCl and extracted with dichloromethane. The organic layer was collected, dried over $MgSO_4$, filtered, and concentrated to dryness. Purification on silica gel chromatography (dichloromethane–methanol–ammonia, 90:10:1 → 60:40:1) afforded the amino acid **11** (255 mg, 83%, 81% purity according to ^{1}H-NMR) as a white foam: FAB-MS 1250 $(MH)^+$.

To a solution of the above amino acid (255 mg, 0.20 mmol) and (dimethylamino)pyridine (100 mg, 0.80 mmol) in dichloromethane (100 mL) was added BOP (100 mg, 0.22 mmol). After stirring overnight at room temperature, the solution was concentrated to dryness and chromatographed over silica gel (ethyl acetate–acetone, 3:2) to give cyclosporin **2** (67 mg, 26%): $[\alpha]_D = -158.0°$ (c = 0.5, methanol); HPLC t_R = 4.41 min (column RP-18, eluent water–acetonitrile, 30:70, oven temperature 70 °C, flow rate 1.7 mL/min); FAB-MS 1232 $(MH)^+$; ^{1}H-NMR ($CDCl_3$) δ 0.61–2.48 (m, 70H), 2.68 (s, 3H, NMe-10), 2.71 (s, 3H, NMe-11), 3.08 (s, 6H, NMe-9 and NMe-4), 3.24 (s, 3H, NMe-6), 3.38 (s, 3H, NMe-3), 3.47 (s, 3H, NMe-1), 3.52–3.64 (m, 2H, CH_2OH), 3.80 (br s, 1H, H-1β), 3.92 (br s, 1H, exchangeable with D_2O, OH), 4.52 (t, J = 7.4, 1H, H-7α), 4.63 (t, J = 9.0, 1H, H-5α), 4.72 (d, J = 13.1, 2H, H-3), 4.82 (t, J = 7.1, 1H, H-8α), 4.95 (ψq, J = 5.7, 1H, H-6α), 5.08 (ψt, J = 6.2, 1H, H-10α), 5.17 (d, J = 11.0, 1H, H-11α), 5.21 (ψq, J = 6.2, 1H, H-2α), 5.32-5.40 (m, 3H), 5.49 (d, J = 6.0, 1H, H-1α), 5.70 (dd, J_1 = 5.9, J_2 = 6.5, 1H, H-9α), 7.14 (d, J = 6.6, 1H, NH-8), 7.42 (d, J = 6.6, 1H, NH-5), 7.58 (d, J = 6.6, 1H, NH-7), 7.92 (d, J = 6.6, 1H, NH-2). Anal. ($C_{63}H_{113}N_{11}O_{13}$) C, H, N.

Nva-2-(D-MeSer)-3-cyclosporin (4). To a stirred and cold solution (−78 °C) of lithium diisopropylamide (11.50 mmol) in THF (50 mL) was added a solution of cyclosporin G (3; 2.0 g, 1.65 mmol) in THF (40 mL) dropwise. After stirring at −78 °C for 1 h, commercially available paraformaldehyde (4.0 g, 131.00 mmol; it does not have to be prepared as reported in ref 14) was added in one portion, and the mixture was allowed to reach room temperature while stirring. Water was added, and the solution was extracted with EtOAc. The organic phase was dried over $MgSO_4$, concentrated, and chromatographed over silica gel (ethyl acetate–acetone, 9:1) to give 4 (655 mg, 32%) as a white foam: $[\alpha]_D = -261.0°$ (c = 1.0, methanol); FAB-MS 1246 $(MH)^+$; ^{1}H-NMR (DMSO-d_6) δ 0.60–2.46 (m, 72H), 1.13 (d, J = 6.5, 3H, Me-8), 1.22 (d, J = 6.6, 3H, Me-7), 2.58 (s, 3H, NMe-10), 2.78 (s, 3H, NMe-11), 2.80 (s, 3H, NMe-9), 2.77 (s, 3H, NMe-4), 2.87 (s, 3H, NMe-6), 2.98 (s, 3H, NMe-3), 3.11 (s, 3H, NMe-1), 3.48–3.65 (m, 2H, CH_2OH), 3.95 (m, 1H), 4.17 (ψt, J = 6.0, 1H, H-7α), 4.39 (dd, J_1 = 6.6, J_2 = 6.9, 1H, H-5α), 4.48 (d, J = 4.8, 1H, OH), 4.57 (t, J = 6.0, 1H, OH), 4.72 (ψt, J = 8.4, 1H, H-8α), 4.80 (ψq, J = 6.6, 1H, H-2α), 5.00 (d, J = 12, 1H, H-11α), 5.05–5.60 (m, 3H), 7.60 (d, J = 6.6, 1H, NH-8), 8.15 (d, J = 6.8, 1H, NH-5), 8.22 (d, J = 6.8, 1H, NH-7), 8.38 (d, J = 6.7, 1H, NH-2). Anal. ($C_{64}H_{115}N_{11}O_{13}$) C, H, N.

Calculations of the Contact Surface. The contact surface areas for the CypA/**1** and CypA/**2** complexes were calculated using Connolly's program[31] implemented in the graphics package WITNOTP (A. Widmer, unpublished program). The radius of the spherical probe atom used to

represent a solvent molecule was set to 1.5 Å and the dot density to 25 dots/Å^2, and all hydrogen atoms of **1**, **2**, and the protein were included in the calculation in idealized positions. Crystallographic water molecules were excluded. The contact surface area of CypA/1 was estimated as the loss of accessible surface area of free **1** in the conformation observed in the CypA complex upon formation of the CypA/1 complex. The accessible surface areas of free 1 and free **2** in the conformation observed in the CypA complexes are 878 and 908 Å^2, respectively, and the accessible surface areas of **1** and **2** when bound to CypA are 608 and 620 Å^2, respectively. This therefore corresponds to contact surface areas of 270 Å^2 for the CypA/**1** complex and 288 Å^2 for the CypA/**2** complex.

Acknowledgment. We thank J. Causevic and Dr. M. Zurini for the purification of recombinant human cyclophilin A and Dr. A. Billich, Dr. V. Quesniaux, and Dr. G. Zenke for assessing the biological activity of the compounds. We also thank M. Ponelle and J. France for ^{1}H-NMR analysis and for kindly providing Figure 5 and Dr. M. D. Walkinshaw for critical assessment of the manuscript.

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JM950228A