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THE ABU-POCKET PARADOX OF THE CYCLOPHILIN A / CYCLOSPORIN A INTERACTION

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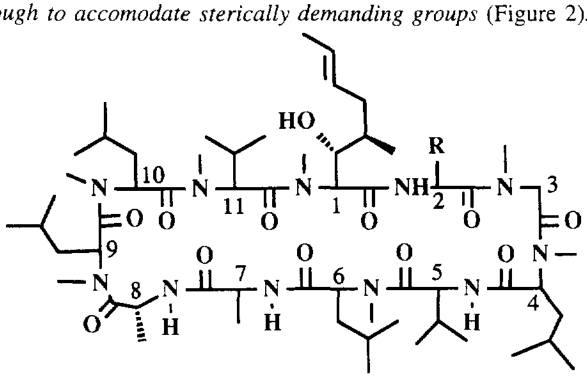
Abstract: A detailed analysis of the cyclosporin A (CsA) / cyclophilin A (CypA) interaction based on the ensemble of the available 3D information demonstrates that, although CypA's pocket binding the Abu-2 side chain of CsA seems to be large enough to accomodate bulky groups, in fact only unbranched chains bearing polar substituents are accepted due to the steric and physicochemical influence of a water molecule network.

In spite of the enormous knowledge accumulated in recent years concerning the various cyclophilin (Cyp) isoforms and their potential involvement in the mechanism of action of cyclosporin A (CsA) 1[1], the isoform responsible for mediating immunosuppression has not yet been unambiguously identified. It is generally considered that the primary enzymatic target of 1 is CypA or CypB[2]. Consequently, the interaction between CypA and CsA has been thoroughly investigated both in solution by 3D NMR[3] and in the crystal by X-ray crystallography[4,5,6]. In all available structures the architecture of the complex, including water mediated contacts,is conserved and only the side-chains of residues expected to be involved in binding to calcineurin, CsA's secondary target, show small differences. Thus, 1 binds with the side-chains of MeBmt-1, Abu-2, Sar-3, MeLeu-9, MeLeu-10 and MeVal-11 to a hydrophobic crevice defined by 13 residues of CypA that are within 4Å of the bound substrate (Figure 1). Moreover, five direct hydrogen bonds and a network of water-mediated contacts stabilize the complex. This mode of interaction explains the results obtained with cyclosporin derivatives modified at this "binding domain". For example, replacement of one of MeLeu-9 or MeLeu-10 by a MeAla yields compounds with marginal affinity to CypA[7]. The same holds for replacing MeVal-11 by MeAla[7] or (D)-MeVal (CsH)[8]. Nevertheless, *it appears that CypA's pocket fitting the small Abu-2 side chain of CsA 1 is large enough to accomodate sterically demanding groups* (Figure 2).



R =
CH_2CH_3 (CsA) 1
$CH(CH_3)CH_2Ph$ 2
$CH(OH)CH_2Ph$ 3
CH_2CH_2OH 4
CH_2COOH 15

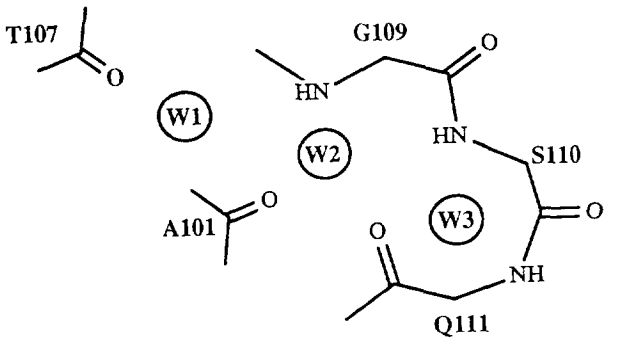
Figure 1

Figure 2: Detailed view of the Abu-2 residue (green) and the Abu-pocket in the CsA/CypA crystal structure. CypA's binding residues are shown in red ; the ones not involved in binding are shown as a Cα trace.

In order to rationally exploit the free space of the "Abu-pocket", two approaches were undertaken. For the first approach, LUDI, a program which docks fragments of known 3D arrangement into a defined binding pocket taking into account the possibility of H-bond formation and/or hydrophobic interactions was employed[9]. Using chemical synthesis feasibility criteria, compound **2**-(3S) was selected from the obtained hit list and was further modified to **3**-(3R) on the basis of established structure-activity relationships[10,11] (Figure 3). Indeed, a β-Me substituent on Abu-2 of **1** is detrimental for the affinity whereas a β-OH has no effect (Val-2 versus Thr-2 cyclosporin).

Figure 3: Close-up view of **2** docked into the Abu-pocket .The extension of the Abu-chain is shown in green.

The observation that in all CsA/CypA structures as well as in the CsA/CypB structure[12] there are three conserved water molecules forming a network of hydrogen bonds between themselves and residues defining the Abu-pocket provided the rationale for the second approach (Figure 4). The homoserin derivative **4** was thus conceived as a compound capable of potentiating H-bonding (Figure 5). Indeed, according to molecular modeling investigations[13], the O of the primary hydroxyl of **4** is 2.6Å and 3.0Å away from W1 and W2 respectively.



Water	CypA atom	Distance(Å)
W1	Thr107 O	2.93
W2	Gly109 N	2.92
W2	Gln111 O	3.06
W2	Ala101 O	2.77
W3	Gln111 N	3.18
W3	Ser110 N	2.81
	W1 to W2	3.30Å
	W2 to W3	3.15Å

Figure 4: Schematic representation and interatomic distances of the conserved water (W) network .

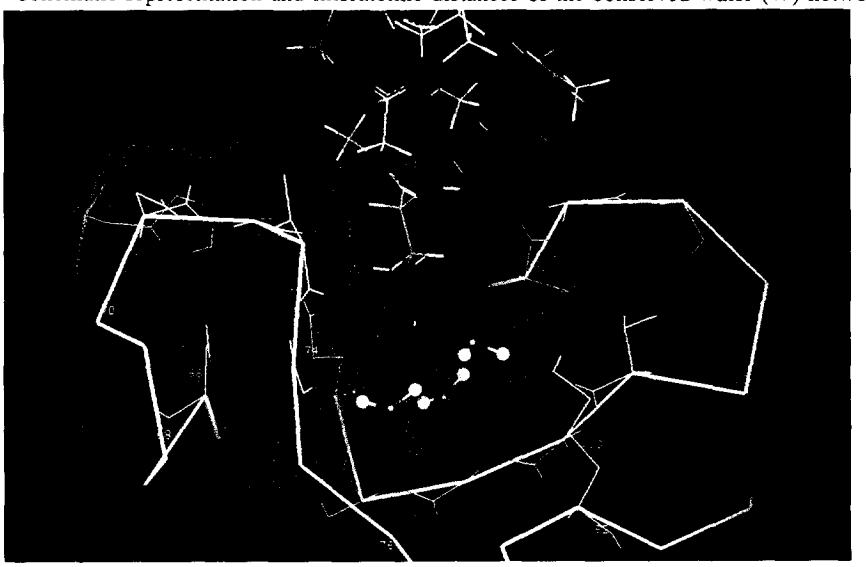
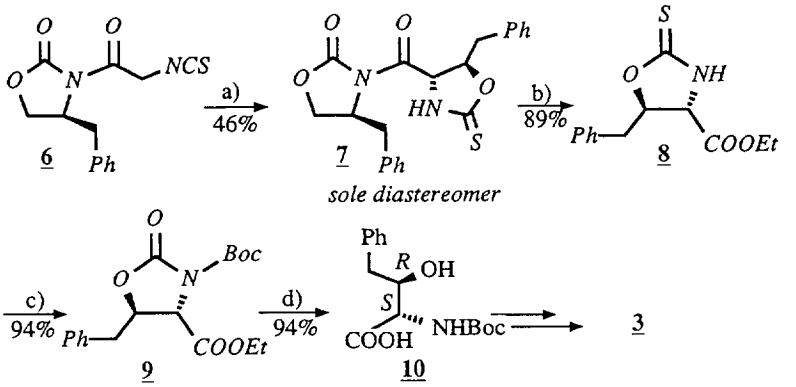


Figure 5: Close-up view of **4** docked into the Abu-pocket.The extension of the Abu-chain is shown in green.

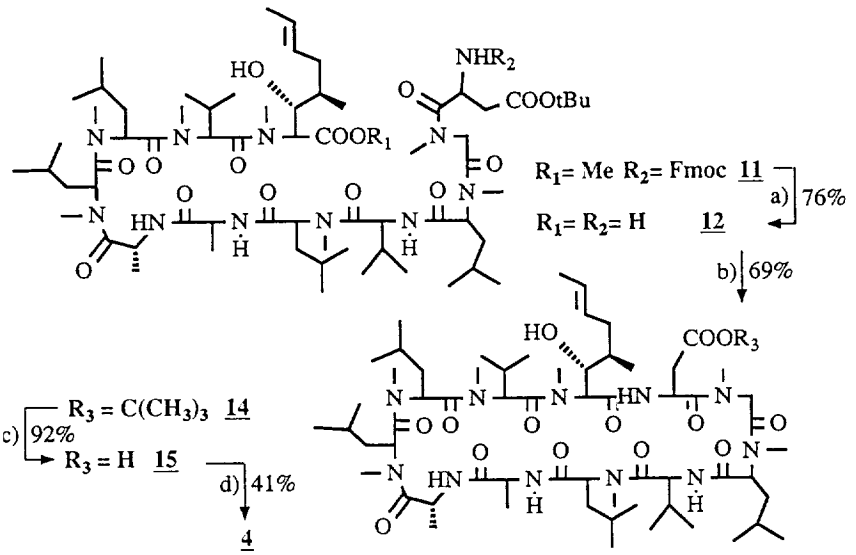
The preparation of the target compounds was based on the use of the decapeptide H-Sar-MeLeu-Val-MeLeu-Ala-(D)-Ala-MeLeu-MeLeu-MeVal-MeBmt-OMe **5** as key intermediate[10]. The synthesis was accomplished through coupling of the appropriate, N-protected amino acids **10** and **13** with **5** to afford the corresponding open chain cyclosporin derivatives followed by full deprotection and ring closure[14]. In analogy to related work[10,15],the

required β-hydroxy α-amino acid **10** was obtained in five steps starting from the chiral isothiocyanate **6**[15] in 36% overall yield (Scheme 1). For the synthesis of **4** (Scheme 2), Fmoc-Asp(OtBu)-OH **13** was coupled with **5** yielding the corresponding undecapeptide **11** (98%) which was transformed to the cyclosporin acid **15** by the standard methodology. Upon reduction of the mixed anhydride of the latter, the alcohol **4** was obtained in 41% yield.



Scheme 1

a)$Sn(OTf)_2$(1.1eq) , N-ethylpiperidine(1.3eq) , phenylacetaldehyde(1.1eq) , THF ,- 78°C b)$Mg(OEt)_2$(1.5eq) , EtOH , 0°C c)i)Boc_2O (1.1eq) , DMAP (1.1eq) ii)30% H_2O_2 , HCO_2H , 0°C to r.t d)2N LiOH (5eq) , dioxane



Scheme 2

a)i)Et_2NH(xs) ii)LiOH(1.1eq) , THF/H_2O:3/1 b)Benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium PF_6 (2.0eq) , DMAP(2.0eq) , CH_2Cl_2(1ml/mg) c)CF_3COOH(xs) , 0°C , 15min. d)i)$ClCO_2Et$(1.1eq) , Et_3N(1.1eq) , THF, 0°C ii)$NaBH_4$ (2.1eq) , -10°C

The compounds were evaluated *in vitro* for their affinity to CypA as well as for their immunosuppressive potential (Table). Competitive ELISA systems using protein-conjugated ligand CsA bound to a solid support, and biotinylated CypA as specific recognition structure were used for the binding assay[16]. The immunosuppressive activity was measured with the help of a reporter gene assay (IL2_RGA)[17] and a mouse mixed lymphocyte reaction (MLR_M)[18].

Table. *In vitro* biological activity of compounds 1, 3, 4, 15

Compound	CypA(rIC_{50})	ΔG(Kcal/mol)#	MLR_M(rIC_{50})	IL2_RG(rIC_{50})
CsA 1	45 (1)	-10.0	6 (1)	10 (1)
3	3600 (80)	-4.9	360 (60)	2000 (200)
4	140 (3)	-9.4	not determined	50 (5)
15	225 (5)	-9.1	not determined	860 (86)

Experiments were done in triplicate. Numbers indicate IC_{50} values in nM.
Relative IC_{50} values (rIC_{50}) express the ratio: IC_{50} Compound/IC_{50} CsA
#ΔG values calculated based on the equilibrium binding constant (IC_{50}) by the equation ΔG=-1.4 logK

The *in vitro* data obtained with 3 in combination with the results already reported while probing CypA with other cyclosporin derivatives[10] leads to the conclusion that the introduction of sterically demanding substituents at position 2 of CsA 1 is detrimental for the affinity to the receptor and, consequently, for the immunosuppression. This is related to the thermodynamically unfavorable equilibrium : [ligand] + [CypA·$3H_2O$] ⇌ [ligand·CypA] + 3[H_2O] for which the free energy (ΔG) cost for the removal of a firmly bound water from a protein is approximately 2 Kcal/mol[19]. Therefore, the ΔG value for lipophilic compounds, such as 3, should be at least -16Kcal/mol ($\Delta G_1+3\Delta G_{H2O}$) for such compounds to bind with equal affinity to CypA as CsA.

The free energy of the hydrophilic derivatives 4 and 15[20] is within <1Kcal/mol of that of CsA and reflects the difficulties inherent to fine-tuning based design of new CsA derivatives. Indeed, the energy loss due to even a small distortion of the Abu-pocket's water lattice induced by the substituent and/or the entropic cost of dissolvation of the polar groups could compensate the binding energy gain of 0.5-1.8 Kcal/mol which is expected for a hydrogen bond[21].

The ensemble of the results of the investigations with cyclosporin derivatives substituted at position 2 clearly shows that the network of the conserved water molecules found in the Abu-pocket reduces its volume and, therefore, large groups are not accepted both for steric and hydrophilicity reasons. Consequently, the potential of unbranched carbon chains bearing a terminal polar group as ligands for optimizing this interaction is emphasized[22].

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