Improved Binding Affinity for Cyclophilin A by a Cyclosporin Derivative Singly Modified at Its Effector Domain

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The immunosuppressive drug cyclosporin A (CsA, Sandimmun, 1)[1] elicits most of its cellular effects through allosteric regulation of calcineurin (CaN), a Ca^{2+}/calmodulin-dependent Ser/Thr phosphatase. To this effect, cyclosporin first binds to its soluble intracellular receptor protein cyclophilin (CyP), and subsequently the resulting complex interacts with calcineurin and inhibits its phosphatase activity.[2] Among the various identified isoforms of CyP interacting with 1, cyclophilin A (CyP-A) and possibly cyclophilin B (CyP-B) are thought to be the intracellular target of cyclosporin A mediating immunosuppression.[3] The "CyP-A binding domain" of the latter has recently been determined based on detailed X-ray crystallographic analysis of the CsA/human recombinant CyP-A complex.[4] Residues MeBmt-1, Abu-2, Sar-3, MeLeu-9, MeLeu-10, and MeVal-11 of CsA are in contact with cyclophilin, whereas residues MeLeu-4, Val-5, MeLeu-6, Ala-7, and D-Ala-8 compose the "effector domain" thought to interact with CaN.

Although various studies aimed at cyclosporin derivatives with enhanced receptor affinity over CsA through derivation of the "binding domain" are well-documented,[5-7] only two compounds with higher affinity to CyP-A via modification of the "effector domain" were reported.[8,9] In this paper we disclose our own results obtained along these lines while probing the interaction of the CyP-A/CsA complex with CaN.[10]

On the basis of subtle modifications of the side chain of amino acid 4 of 1, we could demonstrate that CaN has a very "tight-binding" pocket for the side chain of amino acid 4 of CsA.[10] While pursuing our efforts to find a compound with increased immunosuppressive activity as compared to that of 1, we conceived and synthesized derivatives **2a** and **2b** in which CsA's *gem*-dimethyl moiety of MeLeu-4 is replaced by a *sec*-butyl one (Figure 1).

For the preparation of **2** from its precursor protected decapeptide H-Val-MeLeu-Ala-D-Ala-MeLeu-MeLeu-MeVal-MeBmt(Ac)-Abu-Sar-OMe, **10**,[11] 2-amino-*N*-(*tert*-butoxycarbonyl)-*N*-methyl-4-methylhexanoic acid (**9**) was needed. The synthesis of vinyl bromide **5**, the key intermediate to amino acid **9**, starting from acetamidomalonate and employing a biocatalytic kinetic resolution, was reported with 29% overall yield.[12] As depicted in Scheme 1, the very same compound **5** was efficiently obtained from chiral auxiliary **3** in 65% and >98% chemical and optical yield, respectively. Following Pd^0-catalyzed coupling with vinyltributyltin and subsequent hydrogenation of the resulting diene **6**, the fully protected amino acid **7** was obtained as a 1:1 diastereomeric mixture at position 4. Methylation of the urethane nitrogen gave **8** along with 6% of the 2-epimer,[13] which without further separation was hydrolyzed to give a diastereomeric mixture of methyl 2-amino-*N*-(*tert*-butoxycarbonyl)-*N*-methyl-4-methylhexanoates (**9**). The remainder of the synthesis was accomplished via the established four-stage procedure[10] involving coupling of the above amino acid with **10**, full deprotection of the resulting undecapeptide **11**, ring closure, and deacetylation to afford four diastereomeric cyclosporin derivatives **2**. The two minor (7.2%) diastereomers obtained by the incorporation of the (2*R*,4*S*)-**9** and (2*R*,4*R*)-**9** were easily separated chromatographically, but this was not the case for the diastereomers whose backbone had the natural (*S*) configuration. Indeed, besides the 1:1 mixture (**2b**) of the (2′*S*,4′*S*) and (2′*S*,4′*R*) diastereomers, only one could be isolated pure (**2a**) for which the absolute configuration of the chiral carbon of the side chain remained unknown.[14]

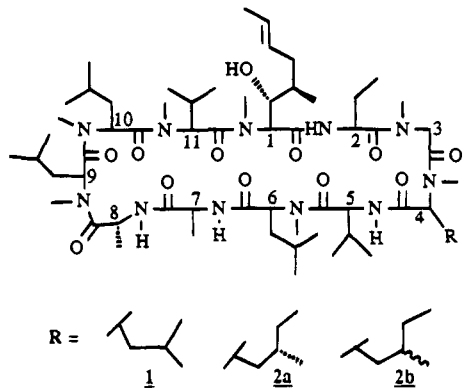

Figure 1. Structure of cyclosporin A, **1**, Me[(4*S*)-Me]norLeu-4 cyclosporin, **2a**, Me[(4*R*,4*S*)-Me]norLeu-4 cyclosporin, **2b**.

Compounds **2a** and **2b** were evaluated for their binding affinity to CyP-A[15] as well as for their immunosuppressive activity in the mouse mixed lymphocyte reaction (MLR–M) and the interleukin-2 reporter gene assay (IL2–RG)[16] (Table 1).

The analysis of the above results clearly indicated that **2a** *had reproducible affinity 4 times higher for CyP-A than the parent compound* 1. The corresponding immunosuppressive activity was 2–3 times lower, which is in line with the fact that CaN has a very tight binding pocket for MeLeu-4 of CsA.[10]

In view of these findings it was undertaken to co-crystallize **2a** with CyP-A in order to determine the 3D arrangement of the complex by X-ray crystallography at 1.86 Å resolution. This allowed the attribution of the absolute stereochemistry of Cγ as (*S*) of the introduced amino acid side chain at position 4 but it did not give any insight into conformational effects which led to the 4-fold increase in binding (Figure 2). Indeed, a comparison of the cyclosporin peptide backbone between bound CsA 1 and bound **2a** gave a root-mean-square (rms) difference of 0.09 Å for C, N, and C-α atoms with no significant variations as a function of the residue number. In addition, the rms difference between bound **2a** and bound CsA for all non-hydrogen atoms of the six residues interacting with CyP-A is 0.10 Å. Consequently, the conformation of **2a** in the **2a**/CyP-A complex can be considered as identical to that of CsA in the CsA/CyP-A complex (estimated error on

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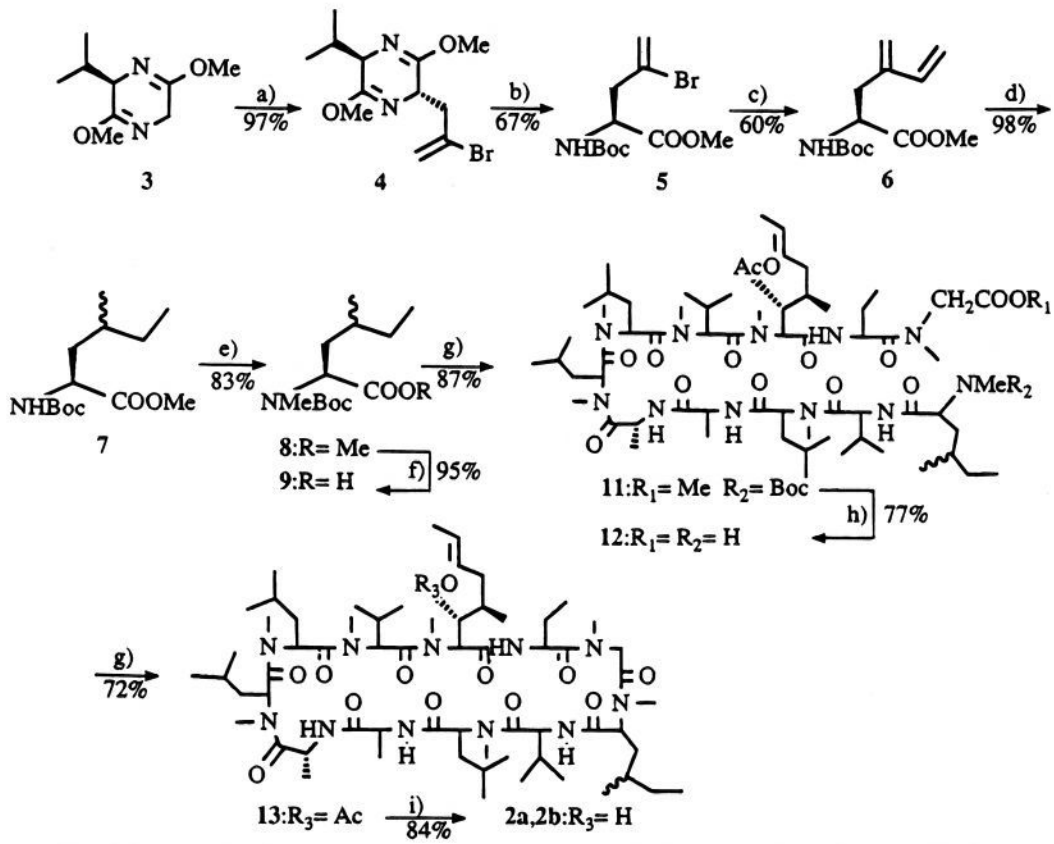
Scheme 1[a]



[a] (a) *n*-BuLi (1.05 equiv), THF, −78 °C, then bromoallyl bromide (1.3 equiv); (b) (i) 1 N HCl (excess), MeOH, (ii) di-*tert*-butyl carbonate (1.1 equiv), dioxane; (c) vinyltributyltin (1.01 equiv), $Pd(PPh_3)_4$ (5 mol %), toluene, Δ; (d) Pd/C, H_2, MeOH; (e) MeI (8 equiv), Ag_2O (4 equiv), DMF, 40 °C; (f) LiOH (1.1 equiv), THF/H_2O, 3/1; (g) **10** (0.9 equiv), (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium PF6 (2 equiv), DMAP (2 equiv), CH_2Cl_2; (h) (i) LiOH (1.05 equiv), THF/H_2O, 3/1, (ii) CF_3COOH (excess), 0 °C, 30 min; (i) NaOMe (100 eq), MeOH, room temperature.

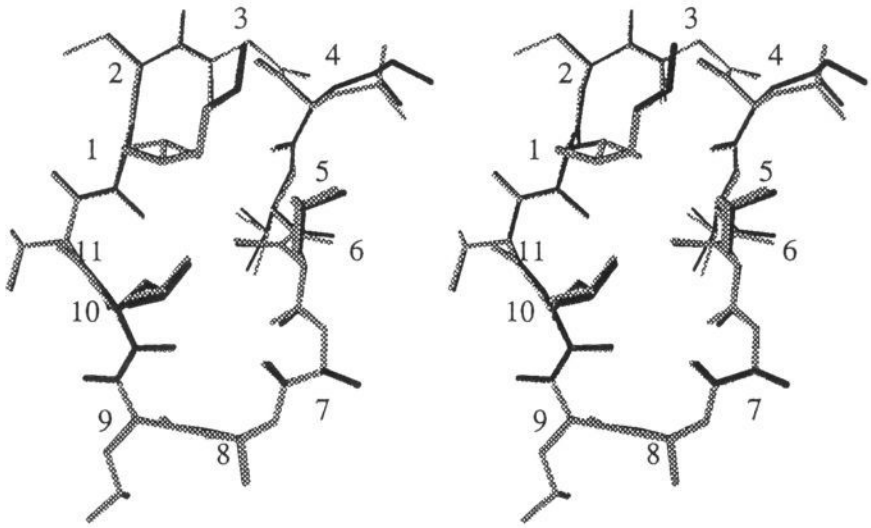


Figure 2. Stereoview of the superposition of CyP-A-bound CsA **1** (gray) onto CyP-A-bound **2a** (black) obtained by making a least-squares fit for all atoms of **1**.

the coordinates is 0.18 Å). Moreover, the conformation of the CyP-A binding site for **2a** as well as the network of interactions between CyP-A and **2a** can be regarded as identical to that of the CyP-A/CsA complex.[4] In analogy to the structure of the CsA/CyP-A complex, the newly introduced side chain at position 4 is not in contact with the protein. The rms difference for all cyclophilin atoms within 4 Å of **2a** and **1** is 0.15 Å.

The lack of significant conformational variations concerning both the CyP-A binding site and the cyclosporin backbone in **2a** and CsA **1** as well as the similarity of the ligand/receptor interactions clearly indicates that the *in vitro* obtained biological results cannot be explained by differences in the 3D architectures. Indeed, the analysis of a cyclosporin derivative having marginal affinity for CyP-A but still 30% of the

Table 1. In Vitro Biological Activities of the Compounds[a]

compound	CyP-A (rel IC_{50})	MLR_M (rel IC_{50})	IL2_RG (rel IC_{50})
CsA (1)	9.0 (1)	20 (1)	5.0 (1)
2a (*S*)	2.3 (0.25)	45 (2.3)	17 (3.4)
2b (*R*)	16.0 (1.8)	46 (2.3)	14 (2.8)

[a] Numbers indicate IC_{50} values in nM. The relative IC_{50} value is the ratio IC_{50} compound/IC_{50} CsA. Experiments were triplicated. The mean standard deviation for the binding experiments is ±0.5 nM.

immunosuppressive activity of CsA led to the assumption that conformer equilibria in aqueous solution govern binding.[17] Accordingly, the improved affinity of **2a** for cyclophilin most probably reflects a more favorable ratio between binding conformer population and nonbinding one present in water.[18] Thus, an effect on the distribution of this multiconformer equilibrium was obtained through substitution of the amino acid 4 in 1.

It thus appears that the introduced minor modification of the effector domain -Me(4-Me)norLeu instead of MeLeu- not only affected the inhibition of calcineurin as expected but also binding to CyP-A via stabilization of the bound conformation. Therefore, the conformational versatility of cyclosporin in solution, for which only empirical data is available,[19-22] should be one of the multiple factors to be taken into consideration when designing improved immunosuppressants.

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Supplementary Material Available: Detailed synthetic procedures for the preparation of the compounds as well as details concerning the crystallographic investigations (7 pages). Ordering information is given on any current masthead page.

References

(1) Borel, J.-F. In Vivo Pharmacological Properties of Cyclosporin. *Pharmacol. Rev.* **1989**, *41*, 259–373.
(2) Schreiber, S. L. Immunophilin-Sensitive Protein Phosphatase Action in Cell Signaling Pathways. *Cell* **1992**, *70*, 365–368.
(3) Bram, R. J.; Hung, D. T.; Martin, P. K.; Schreiber, S. L.; Crabtree, G. R. Identification of the Immunophilins Capable of Mediating Inhibition of Signal Transduction by Cyclosporin A and FK506: Roles of Calcineurin Binding and Cellular Location. *Mol. Cell. Biol.* **1993**, *13*, 4760–4769.
(4) Mikol, V.; Kallen, J.; Pfluegl, G.; Walkinshaw, M. D. X-ray Structure of a Monomeric Cyclophilin A-Cyclosporin A Crystal Complex at 2.1 Å Resolution. *J. Mol. Biol.* **1993**, *234*, 1119–1130.
(5) Sigal, N. H.; Dumont, F.; Durette, P.; Siekerka, J. J.; Peterson, L.; Rich, D.; Dunlap, B. E.; Staruch, M. J.; Melino, M. R.; Koprak, S. L.; Williams, D.; Witzel, B.; Pisano, J. M. Is Cyclophilin Involved in the Immunosuppressive and Nephrotoxic Mechanism of Action of Cyclosporin A? *J. Exp. Med.* **1991**, *173*, 619–628.
(6) Seebach, D.; Beck, A. K.; Bossler, H. G.; Gerber, C.; Ko, S. Y.; Murtiashaw, C. W.; Naef, F.; Shoda, S.; Thaler, A.; Krieger, M.; Wenger, R. Modification of Cyclosporin A: Generation of an Enolate at the Sarcosine Residue and Reaction with Electrophiles. *Helv. Chim. Acta* **1993**, *76*, 1564–1590.
(7) Papageorgiou, C.; Florineth, A.; French, R. R.; Haltiner, R.; Borer, X. Derivatives of Cyclosporin at Position 2 as Probes for Cyclophilin. *BioMed. Chem. Lett.* **1993**, *3*, 2559–2564.
(8) Alberg, D. G.; Schreiber, S. L. Structure-Based Design of a Cyclophilin-Calcineurin Bridging Ligand. *Science* **1993**, *262*, 248–250.
(9) Kofron, J. L.; Kuzmic, P.; Kishore, V.; Colon-Bonilla, E.; Rich, D. H. Determination of Kinetic Constants for Peptidyl Prolyl Cis-Trans Isomerases by an Improved Spectrophotometric Assay. *Biochemistry* **1991**, *30*, 6127–6134.
(10) Papageorgiou, C.; Borer, X.; French, R. R. Calcineurin has a Very Tight-Binding Pocket for the Side Chain of Residue 4 of Cyclosporin. *BioMed. Chem. Lett.* **1994**, *4*, 267–272.
(11) Liu Su, Z.; Wenger, R. Unpublished results.
(12) Leanna, M. R.; Morton, H. E. N-(Boc)-L-(2-Bromoallyl)-Glycine: A Versatile Intermediate for the Synthesis of Optically Active Unnatural Amino Acids. *Tetrahedron Lett.* **1993**, 4485–4488.
(13) The incorporation of racemic amino acids in position 4 of cyclosporin leads to diastereomers which can be easily separated by HPLC. The compound having the shortest t_R is invariably the (D) diastereomer.
(14) HPLC separation conditions: eluent CH_3CN/H_2O, 70/30; flow rate 1.7 mL/min; column RP18; oven temperature 70 °C; t_R of minor diastereomeric mixture (7.2%) 10.47 min; t_R of major diastereomeric mixture (88.8%) 13.77 min.
(15) Quesniaux, V.; Schreier, M. H.; Wenger, R.; Hiestand, P. C.; Harding, M. W.; Van Regenmortel, M. H. V. Cyclophilin binds to the region of cyclosporine involved in its immunosuppressive activity. *Eur. J. Immunol.* **1987**, *17*, 1359–1365.
(16) Baumann, G.; Zenke, G.; Wenger, R.; Hiestand, P. C.; Quesniaux, V.; Andersen, E.; Schreier, M. H. Molecular Mechanisms of Immunosuppression. *J. Autoimmun.* **1992**, *5* (Suppl. A), 67–72.
(17) Mikol, V.; Kallen, J.; Walkinshaw, M. D. The X-ray structure of (MeBm2t)[1]-cyclosporin complexed with cyclophilin A provides an explanation for its anomalously high immunosuppressive activity. *Protein Eng.* **1994**, *7*, 597–603.
(18) For an analysis of the effects of pre-binding equilibria on bioactivity, see: Wiley, R. A.; Rich, D. H. Peptidomimetics Derived from Natural Products. *Med. Res. Rev.* **1993**, *13*, 327–384.
(19) Wenger, R. M.; France, J.; Bovermann, G.; Walliser, L.; Widmer, A.; Widmer, H. The 3D structure of a cyclosporin analogue in water is nearly identical to the cyclophilin-bound cyclosporin conformation. *FEBS Lett.* **1994**, *340*, 255–259.
(20) Kofron, J. L.; Kuzmic, P.; Kishore, V.; Gemmecker, G.; Fesik, S. W.; Rich, D. H. Lithium Chloride Perturbation of Cis-Trans Peptide Bond Equilibria: Effect on Conformational Equilibria in Cyclosporin A and on Time-Dependent Inhibition of Cyclophilin. *J. Am. Chem. Soc.* **1992** *114*, 2670–2675.
(21) Altschuh, D.; Vix, O.; Rees, B.; Thierry, J.-C. A Conformation of Cyclosporin A in Aqueous Environment Revealed by the X-ray Structure of a Cyclosporin-Fab Complex. *Science* **1992**, *256*, 92–94.
(22) Rich, D. H. In Medicinal Chemistry 1992-Proceedings of the XIIth International Symposium on Medicinal Chemistry. **1993**, 15.