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# DESIGN, SYNTHESIS AND X-RAY CRYSTALLOGRAPHIC STUDIES OF [7.3.1] AND [8.3.1] MACROCYCLIC FKBP-12 LIGANDS

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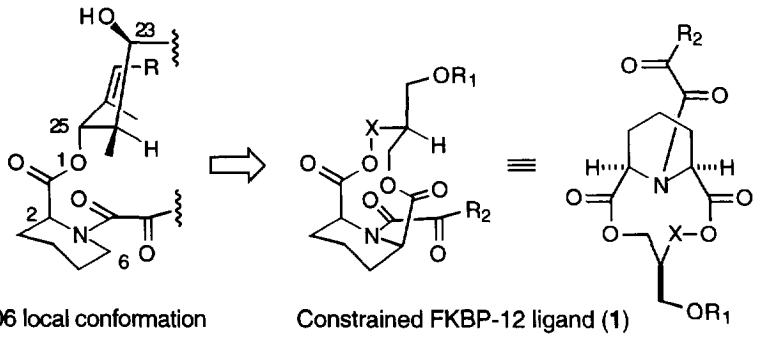
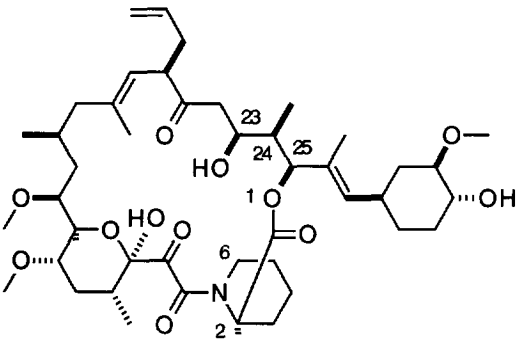
**Abstract:** The design, synthesis, and structure activity relationships of a series of novel macrocyclic FKBP-12 ligands **1** are reported. The crystal structure of the complex between **7** and human FKBP-12 is reported and some SAR results are discussed based on this complex.

The immunosuppressive natural product FK506 is an amazing molecule. It has evolved the ability to promote complex formation between FKBP-12 and the serine/threonine phosphatase calcineurin.[1] The formation of this complex inhibits the phosphatase activity of calcineurin and blocks important signal transduction pathways in a number of cells.[2] Examination of the structure of FK506 reveals a 21-membered macrocyclic ring with several methyl and methoxy groups plus an allyl group in 1,3 relationships to each other.[3] The crystal structure[4] of the complex between FK506 and FKBP-12 shows that these structural features are acting as local conformational locks[5] and, in concert with binding to FKBP-12, generate a rigid protein-ligand complex that then inhibits calcineurin. One local conformational feature of FK506 bound to FKBP-12 is the presence of a 180° dihedral between O1-C25-C24-C23 (PDB entry 1FKF numbering). We wanted to enforce this local conformational feature in new synthetic FKBP-12 ligands. The constrained macrocyclic ligands **1** (in which C2 and C6 are tied back in a 10 or 11 membered ring) are potentially able to achieve this objective. We now describe the synthesis of a number of these compounds related to **1**, and their ability to inhibit FKBP-12 rotamase activity. In addition, we report a cocrystal structure of one member of this series with FKBP-12.

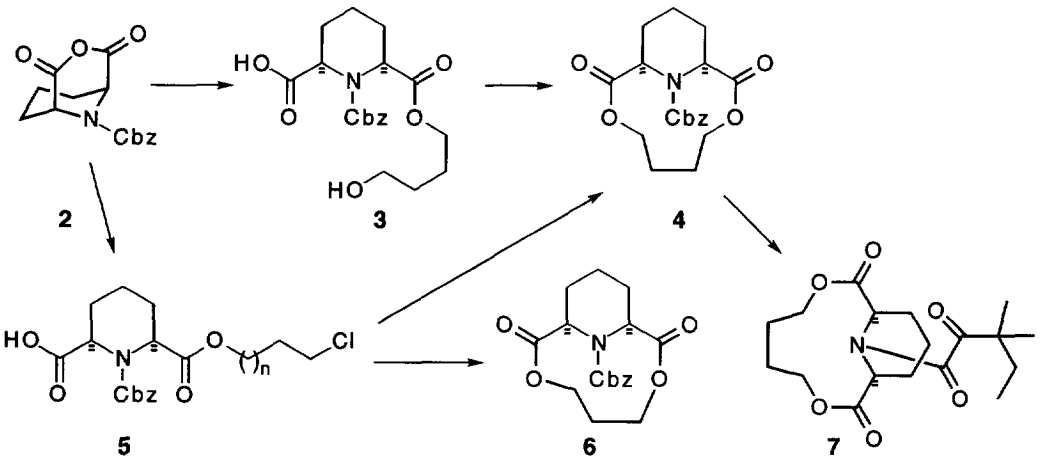



FK506 local conformation  Constrained FKBP-12 ligand (**1**)



**FK506**

The synthetic chemistry leading to the formation of compounds of structure **1** proved to be quite challenging. Meso anhydride **2**[6] was converted in high yield into hydroxy acid **3** upon treatment with 1,4-butanediol in methylene chloride for 12 h. Several attempts to form the [8.3.1]macrocycle using active ester approaches did not produce **4** as product. We then investigated Mitsunobu[7] type approaches; under optimal conditions ($Bu_3P$, DEAD, THF, 72 h.) a 25% yield of **4** was obtained. Since $S_N2$ type reactions resulted in the formation of the desired product we chose to explore other related procedures. Our optimized conditions are the cyclization of chloro acid **5** (n = 2), prepared from **2** and 4-chlorobutanol, to give **4** in 70% yield upon treatment with cesium carbonate in acetonitrile[8] for 7 days. The related [7.3.1] macrocycle **6** was obtained from **5** (n = 1) in 75% yield under the same conditions. The conversion of **4** into **7** in 60% yield was achieved by hydrogenolysis of the Cbz group followed by treatment of the resulting amine with the appropriate alpha-ketoacid in the presence of DCC, HOBT and DMAP in methylene chloride. In a similar manner, **4** was converted into **8**, and **6** was converted into **9** and **10**.

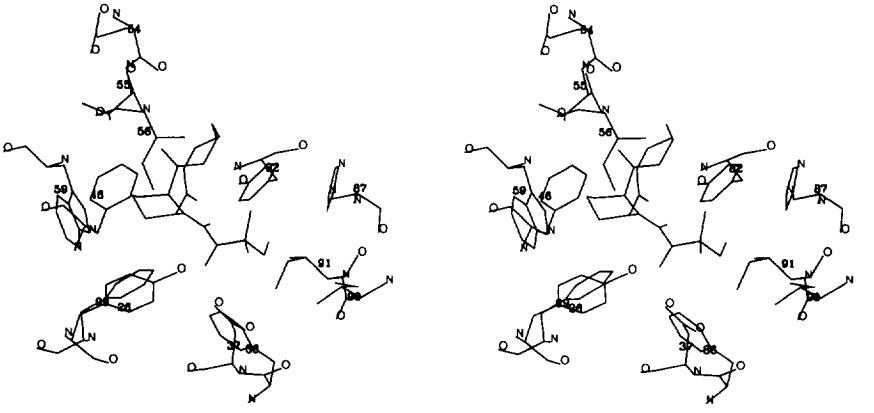


Compound **7** inhibits the rotamase[9-11] activity of human FKBP-12 with a $Ki_{app}$ of 8.3 μM. On the basis of model building experiments there were two families of conformations considered possible for the macrocyclic

ring in its complex with FKBP-12. In both families the S-carbonyl group (the two ester groups are designated R or S based on the absolute configuration of the stereogenic atom they are attached to, see **1A**) is expected to make a hydrogen bond to the backbone NH of Ile-56. In one family the two carbonyl groups are roughly parallel and point in the same direction, whereas in the other they are parallel but point in opposite directions. Functionalization of the macrocycle to mimic an FK506 local conformation requires knowledge of the direction that each CH bond vector[12] of the macrocycle points. The direction of these vectors is in turn dependent upon the ring conformation. Therefore, further elaboration of this system was dependent upon obtaining a cocrystal structure between **7** and FKBP-12.

A 1.9 Å crystal structure was obtained[13] for the complex between **7** and human FKBP-12. Figure 1 shows a "relaxed" stereo view of ligand **7** and the protein residues in close proximity to the ligand. As expected, this structure showed a hydrogen bond between one of the ester carbonyl groups (the S-carbonyl group) and the backbone NH of Ile-56, a hydrogen bond between the amide carbonyl and Tyr-82, and the ketone carbonyl filling the small hydrophobic, electropositive cavity formed by Tyr-26, Phe-36 and Phe-99.[4] The high resolution of the structure allows for determination of the conformation of the macrocyclic ring. The two carbonyl groups are roughly parallel and point in opposite directions. The observed ring conformation is presumably more stable because the alternative conformation, having the two carbonyl groups pointing in same direction, would bury the R-carbonyl group in the protein, without hydrogen bond formation, resulting in a desolvation penalty.

**Figure 1** "Relaxed" stereo view of ligand **7** and the protein residues in close contact to the ligand.



On the basis of the experimentally determined macrocycle conformation of bound **7** and a model built conformation of the related **8** we felt our original hypothesis of using a [7.3.1] or [8.3.1] macrocycle as a conformational lock was reasonable. Since synthetic intermediate **2** is a meso compound, treatment with chiral chloroalcohols (or diols), followed by macrocyclization leads to a mixture of diastereomers. Based on these synthetic and stereochemical considerations we decided to focus our attention on a three carbon linker (i.e., **1** where X = $CH_2$). Key intermediate **13** was prepared from **11** ($NaOCH_2Ph$, THF) via **12** (1. $BH_3$-THF; 2.

$NaBO_3 \cdot 4H_2O$[14]). Coupling of **13** with **2**, as described for the preparation of **4** and **6**, provided the late stage intermediate **14** as an inseparable mixture of diastereomeric meso compounds. Controlled hydrogenation of **14** led either to selective removal of the Cbz group or removal of both the Cbz group and the benzyl ether. Thus, **14** was converted into **15-20** by standard transformations.

Table 1 shows the ability of these compounds to act as inhibitors of human FKBP-12 rotamase activity.[9] Comparison of compounds **7** and **9** shows that there is a negligible difference in activity between the [8.3.1] and [7.3.1] ring systems. Compound **8** with a trimethoxyphenyl group[15] as a mimic of the FK506 pyran group shows the most potent rotamase inhibition (277 nM). Compound **10** with the carvone derived[16] group **CD1** has activity equivalent to **9** with a dimethylethyl group.[17]

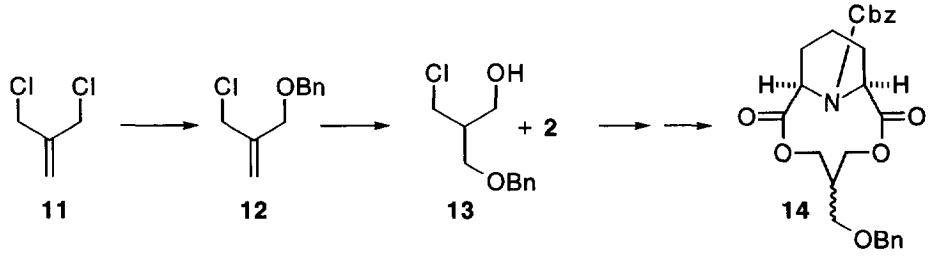
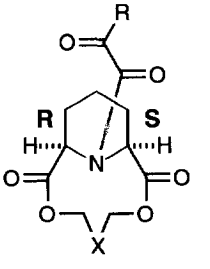


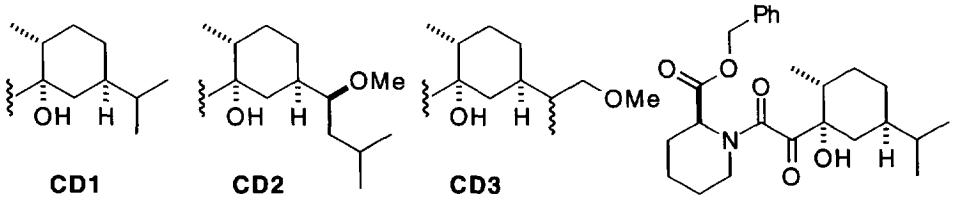

**Table 1.** Ability of compounds to inhibit the rotamase[9] activity of human FKBP-12.

| Compound | X | R | Ki,app (μM) |
|---|---|---|---|
| **7** | $CH_2CH_2$ | $CMe_2Et$ | 8.3 |
| **8** | $CH_2CH_2$ | (3,4,5-OMe)Ph | 0.28 |
| **9** | $CH_2$ | $CMe_2Et$ | 10.0 |
| **10** | $CH_2$ | CD1 | 8.1 |
| **15** | $CHCH_2OBn$ | (3,4,5-OMe)Ph | 1.2 |
| **16** | $CHCH_2OTBS$ | (3,4,5-OMe)Ph | 1.5 |
| **17** | $CHCH_2OH$ | CD2 | NI |
| **18** | $CHCH_2OBn$ | CD2 | NI |
| **19** | $CHCH_2OBn$ | CD3 | NI |
| **20** | $CHCH_2OH$ | CD3 | >50. |

NI = no inhibition




**1A**




**CD1** **CD2** **CD3**

**21**

In the absence of the structure of calcineurin (CaN), we began to explore the FKBP-12 binding of new compounds containing simplified CaN effector domains by modification of the X and R groups of **1A**.

Compounds **15** and **16** with a trimethoxyphenyl R group and hydrophobic X groups retain the ability to bind to FKBP-12. Compounds containing elaborated carvone derived groups and simple X groups (**17-20**) lost their affinity for FKBP-12. None of the compounds in Table 1 are inhibitors of calcineurin.[18-19]

One important SAR feature is that **10** shows a 50-fold reduction in activity compared to **21** ($Ki_{app}$ = 210 nM).[16] Insight into this difference in binding affinity was achieved by superimposing the cocrystal structure of **7** on the cocrystal structure of **21**.[16] This superpositioning showed that the R-carbonyl of **7** and the isopropyl group of **21** are close in space (2.2 Å for =O - Me and 2.9 Å for =O - CH); thus if both moieties were within the same molecule either the R-carbonyl group or the **CD1** group would have to assume an alternative bound geometry. The lack of inhibitory activity of **17-20** is consistent with this analysis. This information suggests that further improvement within the carvone derived series would require the removal of the R-ester group.

The recently solved structure[20] of the complex between FK506, FKBP-12, calcineurin A, and calcineurin B now allows us to re-evaluate this design. When the structure of the FK506-FKBP12-CaNA-CaNB complex[20b] was superimposed on the structure between **7** and FKBP-12, several features become apparent. The macrocyclic framework indeed has potential as a conformational lock to introduce groups into the hydrophobic pocket formed by Trp-352 and Phe-356 of CaNA and Met-119 of CaNB (the site where the allyl group of FK506 binds). To make interactions with Trp-352 of CaNA, in a manner similar to FK506, a novel R group in **1A** would need to be designed. The presence of the R-ester group could possibly be detrimental to calcineurin binding.

We have described a new class of FKBP-12 ligands. The SAR and structural data reported in this paper, in conjunction with the calcineurin structure, provides a new starting point for the rational design of novel "molecular glue" inhibitors of calcineurin.

**Acknowledgement:** We wish to thank the fermentation team at Agouron Pharmaceuticals for production of *E. coli* expressing recombinant FKBP-12.

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6. Anhydride **2** was prepared in 3 steps (75% yield) from 2,6-pyridinedicarboxylic acid (1. $H_2$, Rh/Al, 1N NaOH, 50 psi, 16 hrs. 2. Cbz-Cl, 1N NaOH, 16 hrs. 3. $Ac_2O$, reflux, 3 hrs.)

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9. The affinities of compounds for FKBP-12 were determined by their ability to competitively inhibit the prolyl isomerase activity of human recombinant FKBP-12 (purified as described[10]). Compounds were assayed for inhibition of rotamase activity of FKBP-12 in the presence of 50 mM HEPES (pH 8.0), 100 mM NaCl, 100 μM 2-mercaptoethanol, 1% trifluoroethanol, 2.5 mM LiCl, 1% DMSO, 90 nM FKBP-12, 6 mg/mL α-chymotrypsin coupling enzyme and 100 μM *cis*-succinyl-ala-leu-pro-phe-*p*-nitroanilide (Bachem) as substrate. The percentage of *cis* succinyl-ala-leu-pro-phe-*p*-nitroanilide present in the equilibrium mixture was usually 45%, allowing significant portions of the reaction progress curves to be obtained. Assays were performed at 10°C as described[11] and initial velocities were determined by nonlinear least-squares fits of each progress curve to a single exponential. Apparent Ki's were determined by analyzing the concentration-dependence of each inhibitor on prolyl isomerase activity and fitting the data to an equation for competitive tight-binding inhibition with a correction for uncatalyzed isomerization.[11] Compounds were tested up to concentrations slightly less than their solubility limit under the experimental conditions. In some cases, the poor solubility of the compound prevented determination of

an accurate $Ki_{app}$ (reported as "$Ki_{app}$ > 50 μM"). All data were analyzed using the software program KineTic (BioKin, Ltd., Madison, WI).

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13. Crystals of the complex between FKBP-12 and **7** were grown by vapor diffusion from 1.8 M sodium/potassium phosphate, 100 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.0, using a 12 mg/mL protein-2.5 mM compound (**7**) solution. Crystals grew in space group R3 with one molecule in the asymmetric unit of a cell with dimensions a=b=84.3, c=38.8Å. X-ray diffraction data were collected on one crystal (0.2 mm x 0.1 mm x 0.1 mm) to 1.9Å resolution on an ADSC multiwire area detector using graphite monochromatized $CuK_{\alpha}$ radiation from a Rigaku RU200 rotation anode operating at 50kV/150mA (500 micron focus). A total of 18467 observations of 7952 unique reflections were measured with an overall agreement ($R_{sym}$) on intensities of 5.9% and completeness of 96%. The structure was solved by difference Fourier techniques using protein coordinates from a previous structure solved in this space group by molecular replacement [X-PLOR version 3.1, Bruenger,, A. T. (1992) X-PLOR v 3.1 Manual (Yale University Press, New Haven, Connecticut)]. The structure was refined by conjugate gradient minimization to a current R-factor of 17.0% for 7431 reflections in the 10-1.9Å resolution shell (16.1% for 6883 reflections with $F>2\sigma_F$) for a model containing 832 protein, 25 ligand and 90 water atoms, with root mean square deviations from ideality of 0.015Å for bond distances and 2.86° on bond angles.

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18. The phosphopeptide phosphatase activity of calcineurin was assayed at 30 °C using a continuous coupled spectrophotometric assay[19] and the phosphorylated 19-mer peptide substrate derived from the regulatory subunit ($R_{II}$) of cAMP-dependent protein kinase. The assay mixture contained 50 mM MOPS (pH 7.5), 0.1 M NaCl, 6 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin, 0.5 mM dithiothreitol, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 20 μM phosphorylated $R_{II}$ peptide, 20 nM human recombinant calcineurin, 40 nM calmodulin, 10 μg/ml purine ribonucleoside phosphorylase and 200 μM methylthioguanosine as described[19], plus 1% dimethyl sulfoxide as cosolvent and 100 μM FKBP. Compounds were tested for FKBP-dependent inhibition of calcineurin at their maximum solubility. Under these conditions, the apparent inhibition constant for inhibition of human recombinant calcineurin by FKBP-FK506 was 43 nM.

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