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# High-Affinity FKBP-12 Ligands Derived from (*R*)-(-)-Carvone. Synthesis and Evaluation of FK506 Pyranose Ring Replacements.

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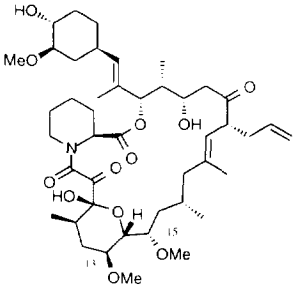
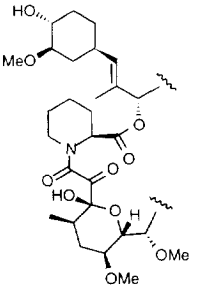
**Abstract:** The preparation and evaluation of potent small molecule inhibitors of FKBP-12 rotamase activity is described. These ligands contain many of the structural features of the FK506 pyranose ring region, yet are synthetically more accessible. The versatility of these FKBP-12 ligands is demonstrated with respect to effector domain exploration.

The macrocyclic natural product FK506 has drawn considerable attention over recent years due to its activity as a potent immunosuppressant.[1] Regulation of cellular events by FK506 is accomplished indirectly through its binding to the immunophilin FKBP-12 (FK506 binding protein).[2] The molecular association of FK506 and the ubiquitous protein FKBP-12 is well established.[3] The resulting bimolecular drug-protein complex has the ability to inhibit the protein phosphatase calcineurin (PP2B).[4] Inhibition of calcineurin results in the break down of distinct signal transduction pathways, ultimately disrupting essential T-cell activation events.[5]

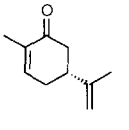
Structurally, FK506 is composed of an FKBP-12 binding domain and an effector domain which, together with essential FKBP-12 residues, interacts with calcineurin.[6] The search for synthetic immunosuppressants that inhibit calcineurin through an FKBP-12 complex has been the subject of many reports.[7,8] Generally, the identification of high-affinity FKBP-12 ligands that allow for elaboration into the effector domain has been the starting point for a number of research groups.[7,8] Moreover, utilization of X-ray crystal structure data of FKBP-ligand complexes has helped guide this unique endeavor.[3]




**FK506**




**FKBP Binding Domain**




**(*R*)-(-)-Carvone**

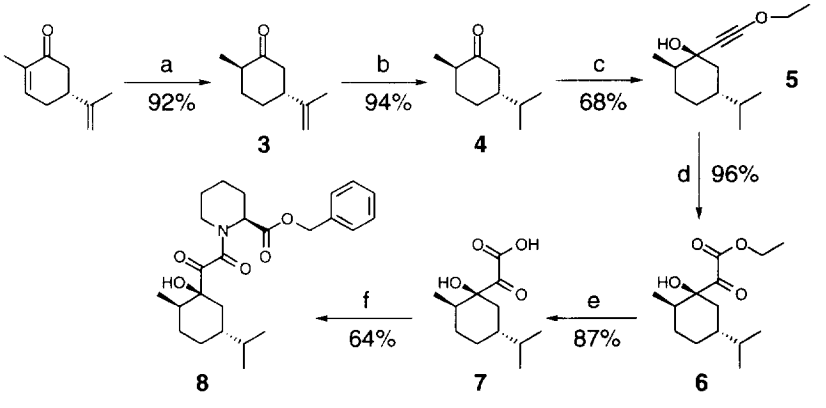
In the present communication, we wish to report the discovery of a series of molecules that bind tightly to FKBP-12, as determined by inhibition of FKBP-12 rotamase activity (Table 1).[9] These compounds serve as an efficient platform from which to position appropriate effector domain fragments. Our goal was to build synthetically accessible molecules possessing structural similarity to the FK506 binding domain.

With the pipecolinic acid portion of the binding domain held constant as the benzyl ester,[8] our attention turned toward the pyranose ring region.[10] (*R*)-(-)-Carvone was chosen as a synthetic starting point based on the

versatility of the isopropenyl functionality. It was envisaged that this group would serve as an excellent handle for effector region extension with appropriate vector positioning.[11] Additionally, the orientation of the isopropenyl group, relative to the other ring functionality, renders (*R*)-(-)-carvone well suited for manipulation into a pyranose ring replacement and mimic. A simple version was initially synthesized to establish chemistry and to test the basic replacement theory (Scheme 1).

(2*R*)-(5*R*)-(+)-Trans-dihydrocarvone (**3**) was obtained by a stereoselective 1,4-reduction of (*R*)-(-)-carvone using sodium hydrotelluride in ethanol.[12] Hydrogenation of the isopropenyl group gave (2*R*)-methyl-(5*R*)-isopropylcyclohexanone (**4**) in high yield. α-Keto-β-hydroxyester (**6**) was obtained by a novel ketone addition/alkyne oxidation sequence.[13] Lithio-ethoxyacetylene was added to ketone (**4**) yielding the chiral hydroxy alkynyl ether (**5**) after flash column separation of the minor diastereomer.[14] Alkynyl ether (**5**) was subsequently oxidized with potassium permanganate to provide α-ketoester (**6**). Hydrolysis of the α-ketoester, followed by a Mukaiyama coupling of the corresponding acid (**7**) with benzyl pipecolinate provided α-ketoamide (**8**),[15] which displayed potent FKBP-12 rotamase inhibition ($K_{i,app}$ = 210 nM). Having obtained this favorable initial result, further synthetic investigations were conducted to establish appropriate functionality for departure into the effector region (Scheme 2).

**Scheme 1.**

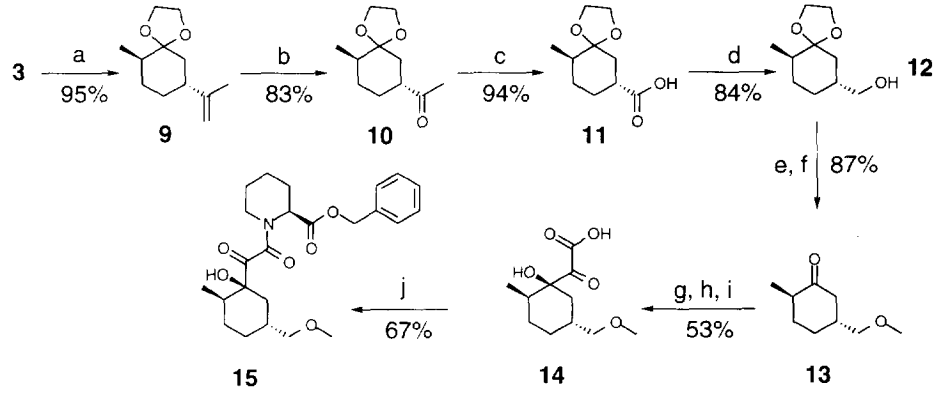



**Reagents and Conditions:** (a) NaHTe, EtOH (b) 10% Pd/C, $H_2$, EtOH (c) Ethyl Ethynyl Ether, *n*-BuLi, THF, -78 °C (d) $KMnO_4$, acetone, $H_2O$, pH 7 (e) LiOH, MeOH, $H_2O$ (f) Benzyl Pipecolinate, 2-Chloro-1-methylpyridinium iodide, $Et_3N$, $CH_3CN$.

Conversion of the isopropenyl group to a primary alcohol was investigated next. Dihydrocarvone (**3**) was protected as ketal (**9**) and ozonized to provide ketone (**10**) in excellent yield.[16] Carboxylic acid (**11**) was obtained by hypobromite oxidation of ketone (**10**).[17] The acid was reduced with borane•THF to give synthetic intermediate (**12**).[18] Alkylation of the primary alcohol, followed by ketone liberation afforded ether (**13**). This intermediate was manipulated into α-keto-β-hydroxy acid (**14**) using the addition / oxidation procedure described above. Coupling of the α-ketoacid to benzyl pipecolinate yielded α-ketoamide (**15**), possessing FKBP-12 rotamase inhibitory activity comparable to compound (**8**) ($K_{i,app}$ = 258 nM). Further investigations were conducted to discover molecules possessing more potent rotamase activity, while maintaining the same type of

synthetic versatility. This led to molecules containing functionality to mimic the C-15 methoxy of the FK506 macrocycle (Scheme 3).

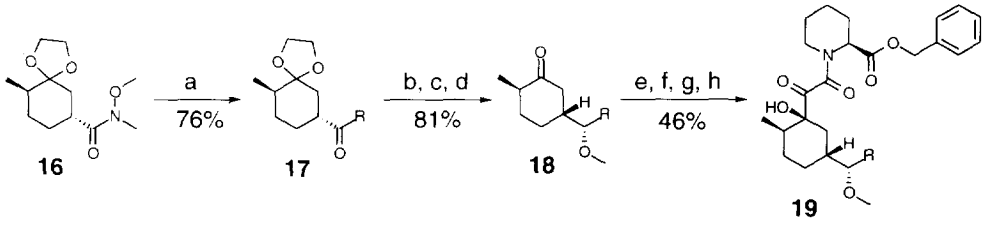
**Scheme 2.**




**Reagents and Conditions:** (a) Ethylene glycol, PPTS, Benzene (b) $O_3$, MeOH, -30 °C (c) NaOBr, 1,4-Dioxane, 10 °C (d) $BH_3$•THF, THF, 25 °C (e) $CH_3I$, NaH, THF, 25 °C (f) PPTS, Acetone, $H_2O$ (g) Ethyl Ethynyl Ether, *n*-BuLi, THF, -78 °C (h) $KMnO_4$, Acetone, $H_2O$, pH 7 (i) LiOH, MeOH, $H_2O$ (j) Benzyl Pipecolinate, 2-Chloro-1-methylpyridinium iodide, $Et_3N$, $CH_3CN$.

Carvone carboxylic acid (**11**) was converted to Weinreb amide (**16**) in excellent yield.[19] Treatment of amide (**16**) with a variety of Grignard reagents provided the corresponding substituted ketones (**17b-d**) (Table 1). During the course of subsequent ketone reductions several conditions were investigated. With α,β-unsaturated ketones (**17b and c**) in mind, it became necessary to find selective 1,2-reduction conditions. In addition, a reduction that would establish the desired (*S*) alcohol configuration was required. Ultimately, a cerium-mediated sodium borohydride reduction of ketones (**10**, **17b-d**) was successfully employed.[20] 1,2-Reduction was achieved, and exclusive formation of the desired carbinols was observed.[21,22] Methylation of the secondary alcohols, followed by ketone liberation, led to compounds (**18a-d**). These intermediates were converted to products (**19a-d**) by following the addition, oxidation and coupling procedures outlined above. Compounds (**19a-d**) displayed potent FKBP-12 rotamase inhibition (Table 1).

**Scheme 3.**




**Reagents and Conditions:** (a) RMgBr or RMgCl, THF, -30 °C (b) $CeCl_3$, $NaBH_4$, MeOH, THF (c) $CH_3I$, NaH, THF, 25 °C (d) PPTS, Acetone, $H_2O$ (e) Ethyl Ethynyl Ether, *n*-BuLi, THF, -78 °C (f) $KMnO_4$, Acetone, $H_2O$, pH 7 (g) LiOH, MeOH, $H_2O$ (h) Benzyl Pipecolinate, 2-Chloro-1-methylpyridinium iodide, $Et_3N$, $CH_3CN$.

**Table 1. Inhibition of FKBP-12 Rotamase Activity by Carvone-Derived Ligands**

| Entry | Compound | R | $K_{i,app}$ (nM) |
|---|---|---|---|
| 1 | 8 | — | 210 |
| 2 | 15 | — | 258 |
| 3 | 19 a | $CH_3$ | 81 |
| 4 | 19 b |  | 112 |
| 5 | 19 c |  | 68 |
| 6 | 19 d |  | 2.8 |

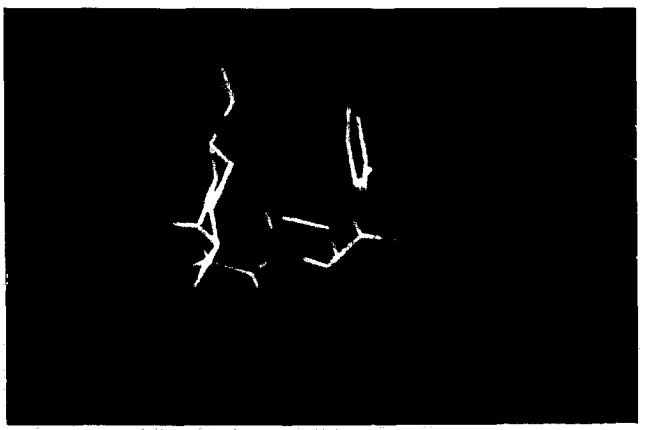


**Figure 1.** Superimposition of the FKBP12-FK506 binding region (green) and FKBP12-(**8**) (yellow) crystal structures.

The structure activity data generated in this investigation supports an important role of the C-15 methoxy. This functionality, in conjunction with C-15 alkyl substitution, contributed significantly to FKBP-12 binding, and in other reports has been shown to be important for calcineurin inhibition.[6a,23] Comparing compounds (**19c and d**) we observe a remarkable enhancement in rotamase inhibition with C-15 side chain saturation. The exact reason for this enhancement remains unclear at this point. One plausible explanation can be inferred from the crystal structure of compound (**8**) bound to FKBP-12 (Figure 1).[24] An interesting tendency for the molecule to undergo

a hydrophobic collapse was observed in the cocrystal structure. Maximization of intramolecular hydrophobic interactions between the benzyl ester and the cyclohexyl region results in a slight torsion of the cyclohexyl ring out of alignment with respect to the FK506 pyranose ring. This collapse may allow compound (**19d**) to adopt a more favorable preorganized binding conformation compared to compound (**19c**). The tendency for hydrophobic collapse has been observed, in other investigations, for a number of non-macrocyclic molecules.[3,25] This type of conformational change clearly affects binding to FKBP-12, as well as the ability of these molecules to successfully interact with calcineurin.[25] However, a constrained macrocyclic, or an appropriately constructed non-macrocyclic system could be employed to overcome these molecular forces. In fact, it has been reported that one such non-macrocyclic molecule has accomplished this task, and displayed appreciable calcineurin inhibition once bound to FKBP-12.[6a] The cyclohexyl region of the carvone-derived FKBP-12 inhibitor (**19b**) shares structural similarity with the corresponding portion of this non-macrocyclic molecule. This data certainly provides reinforcement of the concept that carvone derived FKBP-12 inhibitors could be used as a rational starting point for successful effector region exploration.

In conclusion, potent inhibitors of FKBP-12 rotamase activity were synthesized from highly versatile intermediates originating from (*R*)-(-)-carvone. These chiral pool-derived intermediates provide good FK506 pyranose ring mimics, and allow for relatively simple exploration of the effector region. Attempts to prepare dual domain inhibitors of calcineurin, using a carvone derived binding region, will be the subject of a future publication.

**Acknowledgment :** We thank Mr. M. Imbacuan and Mr. X. Chen for additional biochemical support, and Dr. Peter S. Dragovich for many insightful discussions.

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9. Human recombinant FKBP-12 was purified as described.[26] The peptidyl prolyl isomerase activity of FKBP (90 nM) was determined in a continuous spectrophotometric assay using 100 μM *cis*-succinyl-ala-leu-pro-phe-*p*-nitroanilide (Bachem) as substrate. Assays were performed at 10 °C as described[27] and initial velocities were determined by nonlinear least-squares fits of each progress curve to a single exponential. The kinetic parameters (kcat, Km) of purified hrFKBP were very similar to published values,[27] and active site titrations using FK-506 demonstrated that the protein was consistently ≥95% active. 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.[27] All data were analyzed using the software program KineTic (BioKin, Ltd., Madison, WI).
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24. Crystals of the FKBP-12 / (**8**) were grown by hanging drop vapor diffusion from 1.0M sodium citrate, 100mM cacodylate, pH6.5, using a 12mg/ml protein-2.5mM compound (**8**) solution. Crystals belong to space group $P2_12_12_1$ cell dimensions $a$=35.6, $b$=43.0, $c$=77.2Å with one molecule in the asymmetric unit. X-ray diffraction data were collected on one crystal (0.25x0.13x0.07mm$^3$) to 2.1Å resolution on an ADSC mutliwire area detector using a Rigaku RU200 copper rotating anode (50kV/150mA). A total of 18606 observations of 6993 unique reflections were measured with an $R_{sym}$ on intensities of 10.7% and 96% completeness. The structure was solved by molecular replacement using X- PLOR version 3.1 [Bruenger, A.T. (1992) X-PLOR v3.1 Manual (New Haven, Connecticut: Yale University Press)] and refined by conjugate gradient minimization to a current R-factor of 19.48% for 6411 reflections in the 10-2.1Å resolution shell (17.93% for 5640 reflections with $F>2\sigma_F$) with root mean square deviations from ideality of 0.017Å for bond distances and 2.967° on bond angles.
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