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Fluoresceinated FKBP12 Ligands for a High-Throughput Fluorescence Polarization Assay

Gene M. Dubowchik,* Jonathan L. Ditta, John J. Herbst, Sagarika Bollini and Alexander Vinitsky

Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, PO Box 5100, Wallingford, CT 06492-7660, USA

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Abstract—Several fluoresceinated FKBP12 ligands have been prepared for a high-throughput fluorescence polarization assay. K_is for FKBP12 rotamase inhibition by these ligands range from 1.3 μM to 32 nM, and their design is based on X-ray crystal structures of FKBP12 complexed with known immunophilin ligands.

FKBP12 is the most prominent member of a growing family of FK506 binding proteins (FKBPs) that are known to be involved in many cellular processes as chaperones and partners in multiprotein complexes.[1] Most well known is the ability of FKBP12 to cause immunosuppression by inhibition of calcineurin and FRAP (also known as RAFT or mTOR) following binding of the immunosuppressive drugs FK506 and rapamycin, respectively.[2] However, FKBP12 is also found in complex with intracellular calcium channels (RyR and IP_3R)[3] as well as members of the TGF-β family of receptors[4] where it most likely serves to modulate signaling. Recently, FKBP12 and related FKBPs have been associated with recovery from neuronal injury[5] and implicated as targets for neuroregeneration and neuroprotection.[6–8]

Screening for compounds that bind to FKBP12 has primarily depended on a fairly laborious assay that measures inhibition of the *cis-trans* peptidyl-prolyl isomerase (rotamase) activity of FKBPs.[9] In this assay, a variable existing fraction (usually $<50\%$) of the *cis*-isomer of an AA-Pro-containing fluorogenic tetrapeptide is converted to the *trans* isomer by the FKBP. The *trans* isomer, but not the *cis*, is a substrate for chymotrypsin, which releases the fluorophore, whose rate of appearance can be measured in the presence of an FKBP inhibitor. The assay suffers from high background signal due to the tendency of the peptide substrate to be in the energetically more favorable *trans* conformation, as well as a severe temperature sensitivity. As a result, it is not suitable for high-throughput screening of FKBP-binding compounds.

Recently, a scintillation proximity assay for FKBP12 binding has been described that utilizes radiolabeled FK506.[10] We sought an alternative screen that would avoid the expensive, hydrophobic ligand, and chose to develop a fluorescence polarization (FP) assay. This method relies on the decrease of a fluorescence signal generated by a small molecule fluorescent FKBP12 ligand bound to the protein measured in the presence of inhibitor. For this assay, we desired a ligand whose FKBP12 binding and solubility properties were compatible with reasonable screening concentrations of both protein and small molecule substrates.

For convenience, we used fluorescein as the reporter fluorophore since its optical properties are well known in this context.[11] Ligand design was based on previous non-immunosuppressive FKBP12-binding compounds.[12–14] These take advantage of a small accessory binding site that is connected to the primary pipecolate binding pocket by a shallow hydrophobic groove to increase affinity by linking an aromatic ring to the pipecolate/proline core via a short ester-alkyl chain (Fig. 1). Since available SAR suggests that this secondary site is quite promiscuous, we decided to design our

*Corresponding author. Tel.: +1-203-677-7539; fax: +1-203-677-7702; e-mail: gene.dubowchik@bms.com

Abbreviations: Boc, *t*-butyloxycarbonyl; DMAP, 4-dimethylaminopyridine; EDC, 3-ethyl-1-dimethylaminopropylcarbodiimide; FITC, fluorescein isothiocyante; FKBP, FK506 binding protein; FP, fluorescence polarization; FRAP, FKBP12-rapamycin-associated protein; IP_3R, inositol triphosphate receptor; MMT, monomethoxytrityl; mTOR, mammalian target of rapamycin; Phe, L-phenylalanine; Pro, L-proline; RAFT, rapamycin-associated FKBP12 target; RyR, ryanodine receptor; TFA, trifluoroacetic acid.

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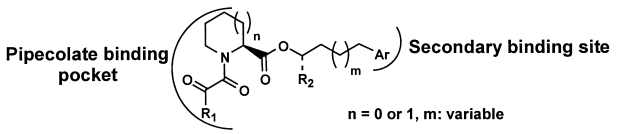


Figure 1. Schematic representation of binding mode of most non-immunosuppressive FKBP12 ligands (H-bonds exist between the amide CO and the Tyr82 OH, and between the ester CO and the Ile56 NH).

ligands such that the fluorescein moiety would be located there in the bound complexes.

Synthesis

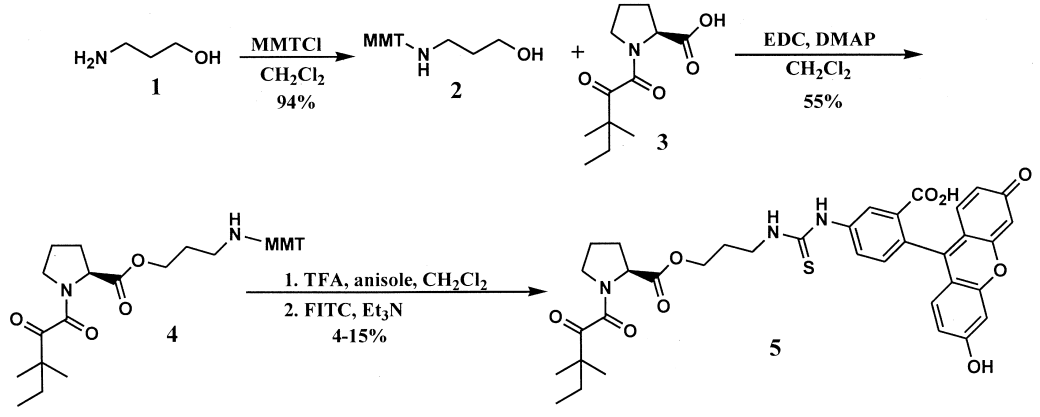
We initially prepared the proline-containing ligand **5** using monomethoxytrityl (MMT) amino protection as shown in Scheme 1. Selective tritylation of 3-aminopropanol **1** and esterification of **3** proceeded smoothly. Several attempts to carry out the MMT deprotection of **4** and FITC coupling without purification of the free amine led to low yields. In addition, co-elution of **5** and FITC in all normal and reverse-phase LC systems attempted required that the product be purified by iterative preparative TLC.

Believing that cleaner amino deprotection, with volatile side-products, would be advantageous in this instance we used a Boc protecting group in the preparation of three pipecolic acid-containing ligands. The branched side chain alcohol **8** was prepared in modest yield from *N*-Boc-aminopropanol **6** by Dess–Martin periodinane oxidation followed by Grignard addition of 3-phenylpropyl magnesium bromide to the resulting aldehyde **7** (Scheme 2).[15]

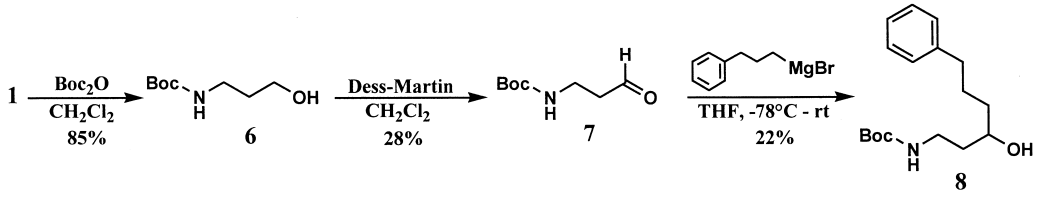
Three Boc-protected alcohols **9**–**11** were coupled to either or both of the pipecolic acid ketoamides **12** and **13** (Scheme 3) with the lowest yields obtained for the secondary alcohol **11**. Removal of Boc from the resulting esters **14** proceeded smoothly to give the corresponding amines which were immediately treated with 1 equiv of FITC (an excess was avoided to minimize the remaining FITC which continued to co-elute with the desired products). Yields of **15**–**17** were good-to-moderate, and reflect some product loss in mixed fractions after silica gel chromatography (1:1:0.01 CH_2Cl_2:EtOAc:AcOH).[16]

Rotamase Activities

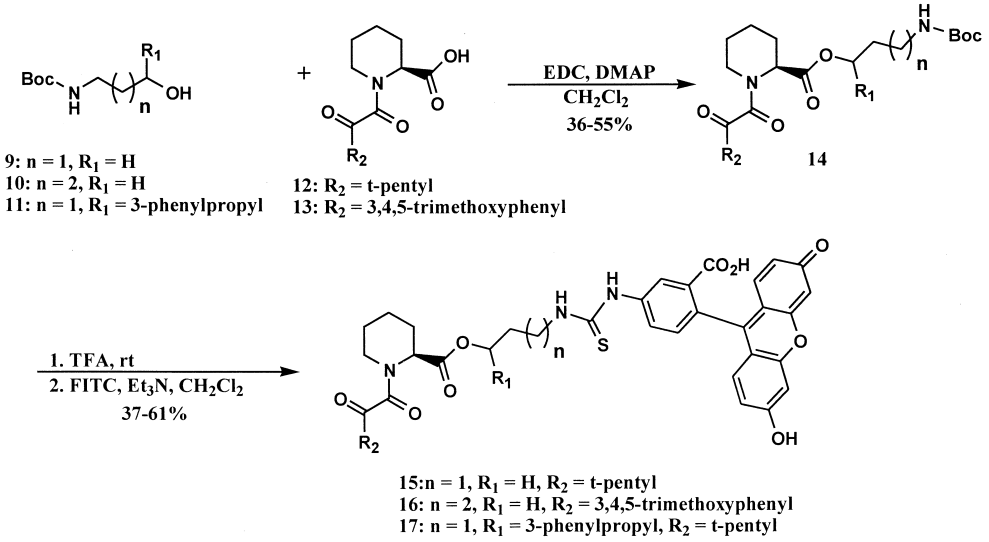
Inhibition of the rotamase activity of FKBP12 by the four fluorescent ligands was measured as described previously,[9] except that the assay was run at 10 °C instead of 0 °C. Results are shown in Table 1. The proline-containing substrate **5** showed the least affinity, in agreement with most, though not all,[17] literature studies that favor the six-membered over the five-membered core structure. The two pipecolate esters that contain an unbranched side chain, **15** and **16**, were 2–3-fold more potent than **5**. Because the fluorescence intensity of 3,4,5-trimethoxyphenylketoamide **16** was significantly attenuated in comparison with the *t*-pentylketoamides **5** and **15**, possibly due to intramolecular quenching, we chose to incorporate the alkyl ketone in the final ligand. Appropriately branched pipecolate esters have, in general, shown enhanced affinity for FKBP12, with the second branch providing additional hydrophobic interactions with Phe46 and the ketoamide side chain of the ligand itself.[12,14] In accord with these observations,



Scheme 1.



Scheme 2.

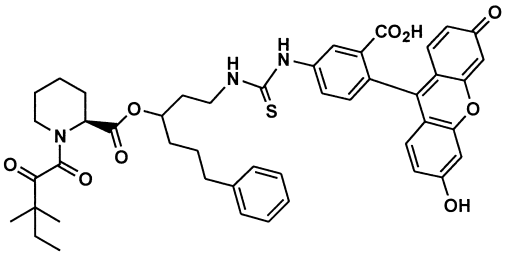


Scheme 3.

Table 1. FKBP12 binding properties of fluorescent ligands

Ligand	FKBP12 rotamase K_i (nM)	FP IC_{50} (μM)[a]
5	1300	1.65
15	482	0.98
16	526	0.41
17	32	0.25

[a]FKBP12 concentration required to produce a half-maximal FP signal at a ligand concentration of 100 nM.



17

Figure 3.

branched ester **17** was >10-fold more active than the corresponding unbranched ligand **15**.

Fluorescence Polarization Measurements

The FKBP12 binding assay was carried out in Costar black flat-bottomed plates in a final volume of 200 μL. The indicated concentration of FKBP12 was incubated for 15 min at room temperature with agitation in the presence of 100 nM of the fluorescent ligand in assay buffer (25 mM HEPES, 100 mM NaCl, pH 7.4). Following incubation, the plates were read in a BMG Fluostar Fluorescence Polarization Plate Reader. For purposes of clarity, the results presented in Figure 2 are normalized to a percent maximum polarization value achieved with each of the different ligands.

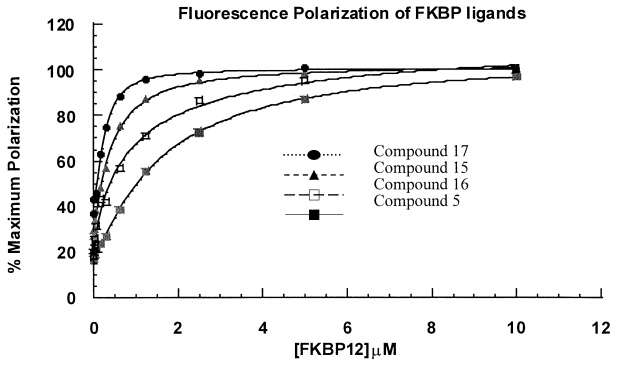


Figure 2.

The best results were achieved with the branched ester **17** (Fig. 3). This ligand produced the strongest FP signal at an FKBP12 concentration of 0.2 μM, a practical screening concentration in a high-throughput setting. Furthermore, its affinity for FKBP12 was not so strong that unreasonably high concentrations of competitive binding substrates, having affinities in the range expected for screening hits, were required to displace it. In this sense, ligand **17** represents a comfortable middle ground, and it has been successfully used for general screening. Results of those studies will appear in due course.

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15. It was subsequently found that the branched alcohol **8** could also be prepared more efficiently and in somewhat better overall yield as shown below (Scheme 4).
16. Analytical data for **17**: ^{1}H NMR (DMSO-d_6) δ 10.74 (br s, 1H), 9.95 (br s, 1H), 8.01 (s, 1H), 8.52 (br s, 1H), 7.75 (d, 1H), 7.28–7.16 (m, 7H), 6.64 (m, 2H), 6.61–6.58 (m, 5H), 5.12 (br s, 1H), 5.03 (br s, 1H), 3.69–3.18 (br m, 4H), 3.17–3.01 (br t, 1H), 2.59 (br s, 2H), 2.29–2.17 (br t, 2H), 1.98–1.69 (br m, 2H), 1.68–1.51 (br s, 6H), 1.41–1.28 (br s, 2H), 1.17 (m, 6H), 0.80 (t, 3H). HPLC–MS: (C-18, methanol:water:TFA linear gradient elution, 5 mL/min, 220 nm) single peak at 2.16 min. MS (ES$^+$) obsd $m/z = 820.33$.
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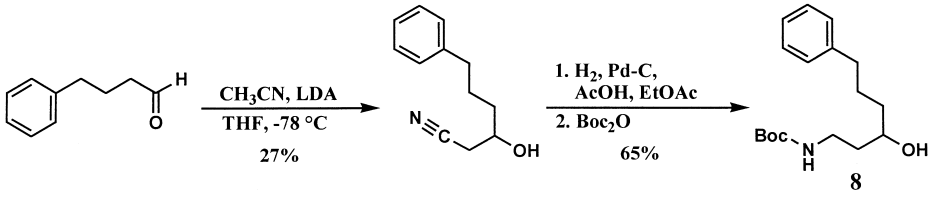

Scheme 4.