ACS Medicinal Chemistry Letters

TECHNOLOGY NOTE

pubs.acs.org/acsmedchemlett

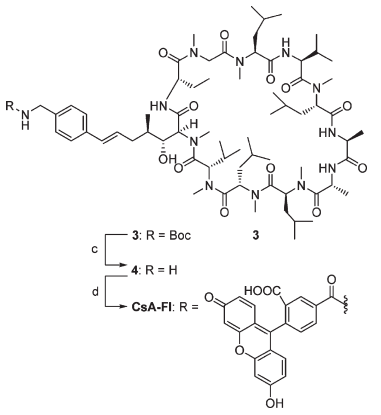
Facile Synthesis of a Fluorescent Cyclosporin A Analogue To Study Cyclophilin 40 and Cyclophilin 18 Ligands

Steffen Gaali,[†] Christian Kozany,[†] Bastiaan Hoogeland, Marielle Klein, and Felix Hausch*

AG Chemical Genomics, Max-Planck-Institute of Psychiatry, Kraepelinstrasse 2-10, 80804 München, Germany

ABSTRACT There are strong indications for the involvement of cyclophilin 40 in diseases caused by misregulation of steroid hormone receptors, like prostate or breast cancer. To identify novel inhibitors for this immunophilin, we developed a simplified fluorescence polarization assay based on the synthesis of a fluorescein-labeled tracer. This tracer was produced by a facile four-step synthesis involving Grubbs metathesis and standard amide bond coupling, to label cyclosporin A with fluorescein. We show the binding of this tracer to Cyp40 and Cyp18 with K_D values of 106 ± 13 or 12 ± 1 nM, respectively, by analyzing the anisotropy change and demonstrate its competition with cyclosporin A. Binding data obtained by fluorescence polarization were corroborated by an enzymatic activity assay. The described tracer allows for a robust assay in a high-throughput format to support the development of novel Cyp40 ligands.

KEYWORDS Cyclophilin, Cyp18, Cyp40, cyclosporin A, CsA, fluorescent analogue, fluorescence polarization assay



Cyclophilin 40 (Cyp40) was first identified as an interaction partner of the estrogen receptor.[1] Later, it was found to be involved in the assembly of numerous steroid hormone receptors (SHR), like the glucocorticoid receptor (GR),[2] the progesterone receptor (PR),[3] the aryl hydrocarbon receptor (AhR),[4] or the androgen receptor (AR).[5] The efficient hormone binding of these SHRs is regulated by their interaction with the chaperone Hsp90.[6] In addition to Cyp40, there are several other cochaperones known to be part of these Hsp90−SHR complexes, which are able to modulate the activity of SHRs, including the FK506-binding proteins 51 and 52 (FKBP51 and FKBP52),[7,8] the protein phosphatase 5 (PP5),[8] and Xap2/Ara9.[9] These cochaperones compete for the binding to Hsp90 via their tetratricopeptide repeat (TPR) motif.[6,8] Cyp40, FKBP51, and FKBP52 have an additional peptidyl-prolyl-isomerase (PPIase) domain that is also the binding site for the immunosuppressants cyclosporin A (CsA) and FK506 or rapamycin, respectively.[1,10,11]

As a modulator of SHRs, Cyp40 might be clinically relevant for endocrine disorders. For instance, Cyp40 was found to be overexpressed in breast[12] and prostate cancer cells.[5] Alterations of the normal balance of immunophilins could lead to the progression of ER-positive breast cancers to an estrogen-independent state.[13] Moreover, Cyp40 may influence the response to cancer treatment, as it was found to be up-regulated in nonresponders of radiotherapy in rectal cancer.[14] Cyp40 was further linked to prostate cancer by the observation that the Cyp40 ligand CsA was able to inhibit growth of several prostate cancer cell lines. Recently, the specific role of Cyp40 was confirmed and further defined by knockdown studies, which blocked AR responsiveness and CsA sensitivity in prostate cancer cells and reduced their proliferation.[15]

The human genome encodes at least 16 unique cyclophilins, all containing a highly conserved Cyp18-homology domain.[17] Many of them bind thightly to the pan-specific cyclophilin ligands CsA, sanglifehrin A,[18] or the CsA analogue NIM811 (in clinical trial for hepatitis C therapy).[16,19] To further define the pharmacology of cyclophilin ligands and the therapeutical potential of Cyp40, more selective inhibitors are needed. For the development of such ligands, fluorescence polarization assays can be applied using tracers targeting the PPIase domain of isolated, recombinant Cyp40. The selectivity of inhibitors could be examined by similar in vitro assays with purified Cyp18 or potentially other cyclophilins. A similar assay has been described for Cyp18, albeit using tracers that had to be laboriously synthesized in 11 steps[20] or requiring publically unavailable natural products.[21] Here, we describe a straightforward four-step synthesis of a fluorescent-labeled CsA analogue starting from commercially available CsA. This tracer can be used in fluorescence polarization assays targeting Cyp40.

CsA (Scheme 1) is a cyclic undecapeptide that is clinically used as an immunosuppressive agent.[22] It binds unspecifically to various cyclophilins in the nanomolar range, making it a promising starting point for the development of a fluorescent tracer.[16]

Received Date: June 2, 2010
Accepted Date: August 3, 2010
Published on Web Date: August 25, 2010

DOI: 10.1021/ml1001272 | *ACS Med. Chem. Lett.* **2010**, *1*, 536–539

ACS Medicinal Chemistry Letters

TECHNOLOGY NOTE

pubs.acs.org/acsmedchemlett

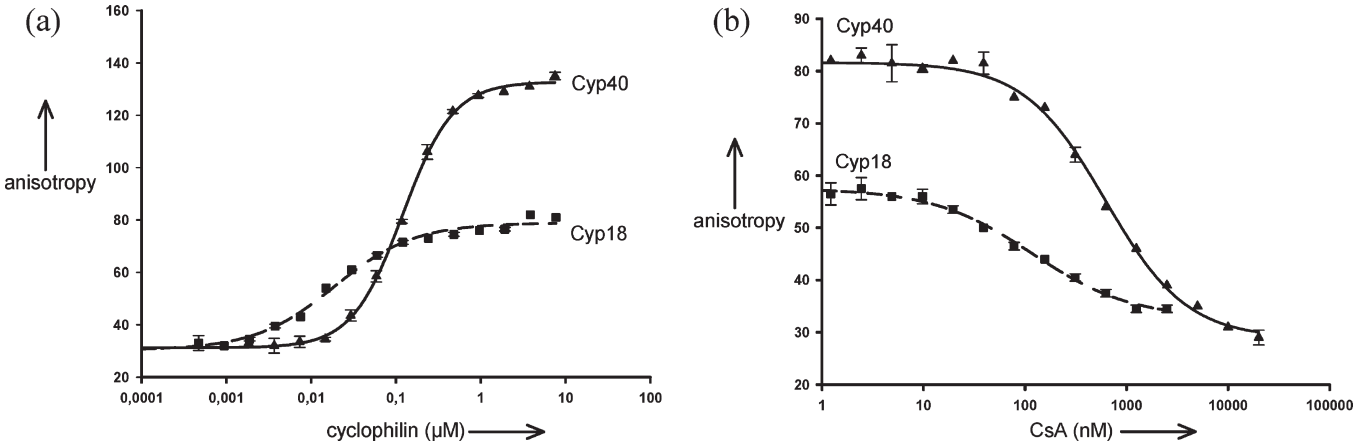
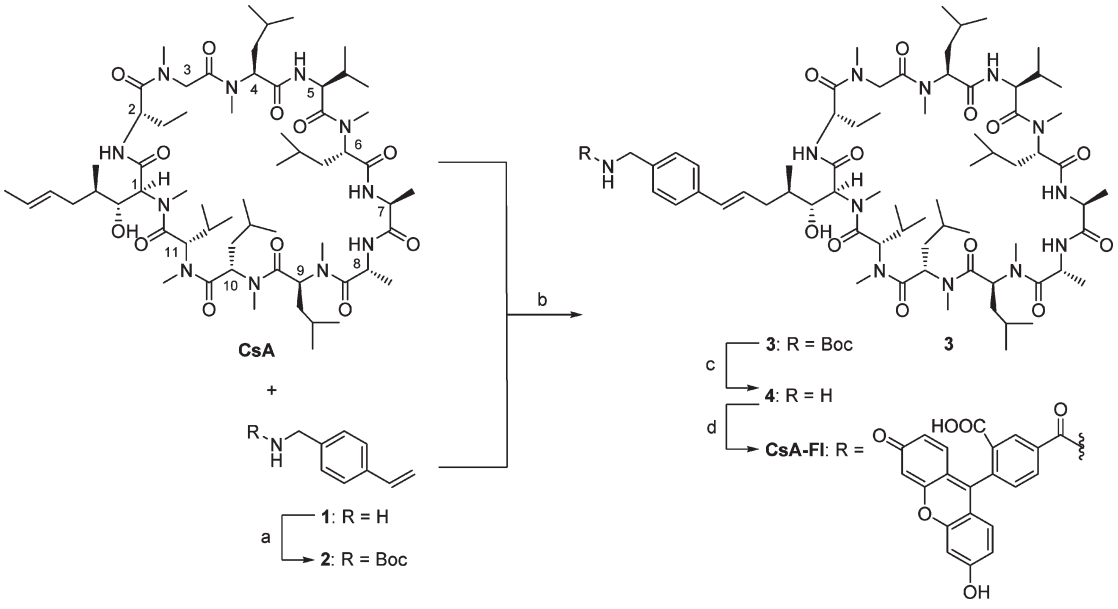


Figure 1. (a) Binding of CsA-Fl (10 nM) to Cyp18 (dashed squares) and Cyp40 (continuous triangles) measured by fluorescence polarization. (b) Competition of CsA with CsA-Fl (10 nM) for the binding to Cyp18 (10 nM) and Cyp40 (100 nM) measured by fluorescence polarization.

Scheme 1[a]



[a] Reagents and conditions: (a) Boc_2O, TEA, MeOH, room temperature. (b) Grubbs Cat. II. Gen, DCM, reflux. (c) 10% TFA, DCM, 0 °C. (d) NHS-fluorescein, TEA, DCM/THF 2:1, room temperature.

The cocrystal structure of Cyp18/CsA shows that the terminal *trans*-alkene methyl substituent of the unnatural amino acid butenyl-methyl-L-threonine (position 1 of CsA in Scheme 1) sticks out of the binding pocket and is solvent accessible.[23] Because the binding pocket of Cyp40 is highly conserved and structurally superimposable to that of Cyp18,[24] we decided to use this accessible alkene moiety for the introduction of a fluorescent label. Cross-metathesis using a second generation Grubbs catalyst was chosen, as it has proven its applicability to CsA and numerous different types of complex substrates throughout the literature.[25] We decided to attach an amine-containing linker to CsA that could be easily coupled to carboxy-containing fluorophores. Because metathesis catalysts are known to be sensitive to primary amines,[26] we first tried to couple Boc-allylamine or allylammoniumchloride to CsA, which resulted in poor yields probably because of the close distance between the amine and the double bond. We thought to overcome this problem by using a longer linker. Therefore, we first protected commercially availalable *para*-vinylaminobenzene **1** with Boc_2O to obtain **2** with quantitative yields. This was then coupled to CsA using second generation Grubbs catalyst to give **3** in good yields. The primary amine was liberated using 10% TFA to produce **4**, which was reacted with 5/6-carboxyfluorescein *N*-hydroxysuccinimide (NHS-fluorescein) to give the final compound CsA-Fl.

We then characterized the binding of CsA-Fl to Cyp40 and Cyp18 in a miniaturized microtiter plate-based fluorescence polarization assay.[27] N-Terminal His-tagged Cyp40 and Cyp18 were recombinantly expressed and purified by Ni-NTA

DOI: 10.1021/ml1001272 | *ACS Med. Chem. Lett.* **2010**, *1*, 536–539

ACS Medicinal Chemistry Letters

TECHNOLOGY NOTE

pubs.acs.org/acsmedchemlett

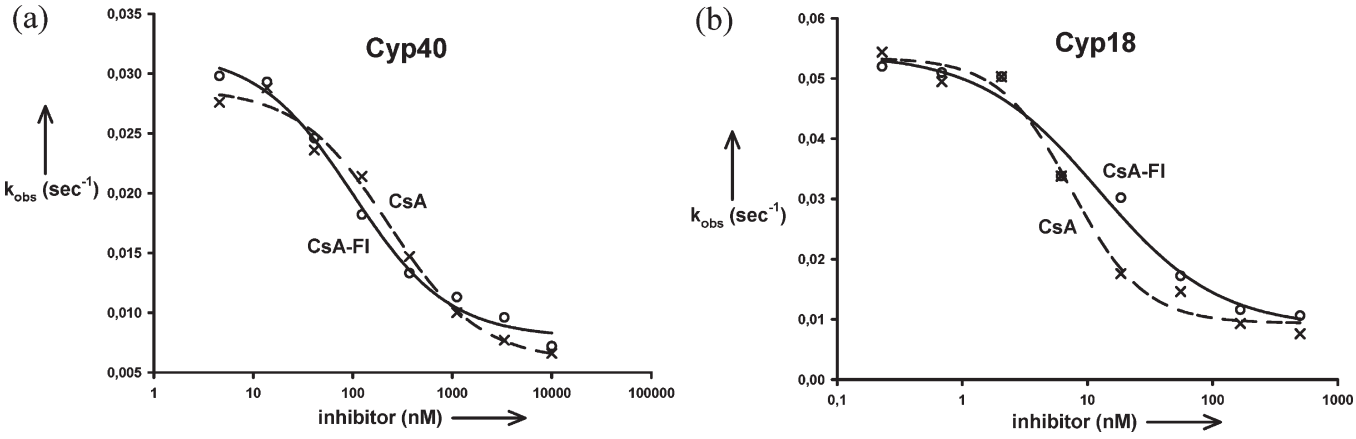


Figure 2. (a) Inhibition of the PPIase activity of Cyp40 (100 nM) by CsA (dashed, ×) and CsA-Fl (continuous, ○). (b) Inhibition of the PPIase activity of Cyp18 (10 nM) by CsA (dashed, ×) and CsA-Fl (continuous, ○).

Table 1. Binding and Inhibition Constants (nM) Measured by Fluorescence Polarization or Enzymatic PPIase Assay

	cyclophilin	FP assay (K_i/K_D)	PPIase assay (K_i)
CsA	Cyp40	227 ± 22	231 ± 55
	Cyp18	34 ± 6	7 ± 1
CsA-Fl	Cyp40	106 ± 13	101 ± 24
	Cyp18	12 ± 1	12 ± 4

affinity chromatography. The functionality of the purified proteins was verified by a coupled enzymatic assay measuring their PPIase activity. We determined K_d values of 106 ± 13 nM for Cyp40 and 12 ± 2 nM for Cyp18 (Figure 1a and Table 1). The absolute change in anisotropy was substantially larger for Cyp40 as compared to Cyp18, likely reflecting the bigger size of the former. To verify the binding affinity of tracer CsA-Fl to Cyp40 and Cyp18, a coupled enzymatic PPIase assay was performed (Figure 2).[28] The measured values of 101 ± 24 nM for Cyp40 and 12 ± 4 nM for Cyp18 match very well with the FP assay results shown above.

To demonstrate the use of the tracer CsA-Fl for the characterization of unlabeled ligands, we tested its performance in FP competition assays (Figure 1b). The prototypic ligand CsA could efficiently compete with CsA-Fl for the binding to Cyp40 and Cyp18. The measured K_d values were again corroborated with a PPIase assay (Figure 2 and Table 1). For Cyp40, there was an excellent match between FP and PPIase results, while for Cyp18, a slightly lower K_i was observed in the PPIase assay.

In general, CsA-Fl binds slightly more potently to both Cyp18 and Cyp40 than CsA likely due to additional contacts of the conjugated fluorescein. The affinities measured in this work are consistent with the literature values for Cyp40 as well as with the majority of reports for Cyp 18. For Cyp18, substantial discrepancies in CsA affinities have been reported. The consensus values, however, match very well with the results reported in this work.[29]

In summary, we describe the facile synthesis of the fluorescein-labeled tracer CsA-Fl, which shows high affinity binding to Cyp40 and Cyp18. CsA is able to compete with the tracer for the binding to Cyp40 and Cyp18. Therefore, CsA-Fl enables a simplified assay in high-throughput format, which can be used for screening and subsequent profiling of inhibitors of Cyp40 to identify structures for the development of potential new drugs against breast and prostate cancer.

SUPPORTING INFORMATION AVAILABLE

Synthetic procedures and characterization data, expression and purification of cyclophilins, inhibition of PPIase activity, and fluorescence polarization assay. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author: *To whom correspondence should be addressed. Tel: (+49)89-30622610. E-mail: hausch@mpipsykl.mpg.de.

Author Contributions: † These authors contributed equally.

Funding Sources: This work was supported by the CIPSM. We thank Professor Florian Holsboer for generous financial support.

ACKNOWLEDGMENT

We thank Claudia Dubler (LMU, Munich, Germany) and Elisabeth Weyher (MPI of Biochemistry, Martinsried, Germany) for NMR spectroscopy and MS measurements.

REFERENCES

(1) Kieffer, L. J.; Thalhammer, T.; Handschumacher, R. E. Isolation and characterization of a 40-kDa cyclophilin-related protein. *J. Biol. Chem.* **1992,** *267*, 5503–5507.

(2) Renoir, J. M.; Mercier-Bodard, C.; Hoffmann, K.; Le Bihan, S.; Ning, Y. M.; Sanchez, E. R.; Handschumacher, R. E.; Baulieu, E. E. Cyclosporin A potentiates the dexamethasone- induced mouse mammary tumor virus-chloramphenicol acetyltransferase activity in LMCAT cells: A possible role for different heat shock protein-binding immunophilins in glucocorticosteroid receptor-mediated gene expression. *Proc. Natl. Acad. Sci. U.S.A.* **1995,** *92*, 4977–4981.

(3) Milad, M.; Sullivan, W.; Diehl, E.; Altmann, M.; Nordeen, S.; Edwards, D. P.; Toft, D. O. Interaction of the progesterone receptor with binding proteins for FK506 and cyclosporin A. *Mol. Endocrinol.* **1995,** *9*, 838–847.

DOI: 10.1021/ml1001272 | *ACS Med. Chem. Lett.* **2010,** *1*, 536–539

(4) Shetty, P. V.; Wang, X.; Chan, W. K. CyP40, but not Hsp70, in rabbit reticulocyte lysate causes the aryl hydrocarbon receptor-DNA complex formation. *Arch. Biochem. Biophys.* **2004,** *429*, 42–49.

(5) Periyasamy, S.; Warrier, M.; Tillekeratne, M. P.; Shou, W.; Sanchez, E. R. The immunophilin ligands cyclosporin A and FK506 suppress prostate cancer cell growth by androgen receptor-dependent and -independent mechanisms. *Endocrinology* **2007,** *148*, 4716–4726.

(6) Pratt, W. B.; Morishima, Y.; Murphy, M.; Harrell, M. Chaperoning of glucocorticoid receptors. *Handb. Exp. Pharmacol.* **2006,** 111–138.

(7) Wochnik, G. M.; Ruegg, J.; Abel, G. A.; Schmidt, U.; Holsboer, F.; Rein, T. FK506- binding proteins 51 and 52 differentially regulate dynein interaction and nuclear translocation of the glucocorticoid receptor in mammalian cells. *J. Biol. Chem.* **2005,** *280*, 4609–4616.

(8) Ratajczak, T.; Ward, B. K.; Minchin, R. F. Immunophilin chaperones in steroid receptor signalling. *Curr. Top. Med. Chem.* **2003,** *3*, 1348–1357.

(9) Laenger, A.; Lang-Rollin, I.; Kozany, C.; Zschocke, J.; Zimmermann, N.; Ruegg, J.; Holsboer, F.; Hausch, F.; Rein, T. XAP2 inhibits glucocorticoid receptor activity in mammalian cells. *FEBS Lett.* **2009,** *583*, 1493–1498.

(10) Baughman, G.; Wiederrecht, G. J.; Campbell, N. F.; Martin, M. M.; Bourgeois, S. FKBP51, a novel T-cell-specific immunophilin capable of calcineurin inhibition. *Mol. Cell. Biol.* **1995,** *15*, 4395–4402.

(11) Peattie, D. A.; Harding, M. W.; Fleming, M. A.; DeCenzo, M. T.; Lippke, J. A.; Livingston, D. J.; Benasutti, M. Expression and characterization of human FKBP52, an immunophilin that associates with the 90-kDa heat shock protein and is a component of steroid receptor complexes. *Proc. Natl. Acad. Sci. U.S.A.* **1992,** *89*, 10974–10978.

(12) Kumar, P.; Mark, P. J.; Ward, B. K.; Minchin, R. F.; Ratajczak, T. Estradiol-regulated expression of the immunophilins cyclophilin 40 and FKBP52 in MCF-7 breast cancer cells. *Biochem. Biophys. Res. Commun.* **2001,** *284*, 219–225.

(13) Ward, B. K.; Kumar, P.; Turbett, G. R.; Edmondston, J. E.; Papadimitriou, J. M.; Laing, N. G.; Ingram, D. M.; Minchin, R. F.; Ratajczak, T. Allelic loss of cyclophilin 40, an estrogen receptor-associated immunophilin, in breast carcinomas. *J. Cancer Res. Clin. Oncol.* **2001,** *127*, 109–115.

(14) Watanabe, T.; Komuro, Y.; Kiyomatsu, T.; Kanazawa, T.; Kazama, Y.; Tanaka, J.; Tanaka, T.; Yamamoto, Y.; Shirane, M.; Muto, T.; Nagawa, H. Prediction of sensitivity of rectal cancer cells in response to preoperative radiotherapy by DNA microarray analysis of gene expression profiles. *Cancer Res.* **2006,** *66*, 3370–3374.

(15) Periyasamy, S.; Hinds, T., Jr.; Shemshedini, L.; Shou, W.; Sanchez, E. R. FKBP51 and Cyp40 are positive regulators of androgen-dependent prostate cancer cell growth and the targets of FK506 and cyclosporin A. *Oncogene* **2010,** *29*, 1691–1701.

(16) Gaither, L. A.; Borawski, J.; Anderson, L. J.; Balabanis, K. A.; Devay, P.; Joberty, G.; Rau, C.; Schirle, M.; Bouwmeester, T.; Mickanin, C.; Zhao, S.; Vickers, C.; Lee, L.; Deng, G.; Baryza, J.; Fujimoto, R. A.; Lin, K.; Compton, T.; Wiedmann, B. Multiple cyclophilins involved in different cellular pathways mediate HCV replication. *Virology* **2010,** *397*, 43–55.

(17) Galat, A. Function-dependent clustering of orthologues and paralogues of cyclophilins. *Proteins* **2004,** *56*, 808–820.

(18) Sanglier, J. J.; Quesniaux, V.; Fehr, T.; Hofmann, H.; Mahnke, M.; Memmert, K.; Schuler, W.; Zenke, G.; Gschwind, L.; Maurer, C.; Schilling, W. Sanglifehrins A, B, C and D, novel cyclophilin-binding compounds isolated from *Streptomyces* sp. A92- 308110. I. Taxonomy, fermentation, isolation and biological activity. *J. Antibiot.* (*Tokyo*) **1999,** *52*, 466–473.

(19) Goto, K.; Watashi, K.; Murata, T.; Hishiki, T.; Hijikata, M.; Shimotohno, K. Evaluation of the anti-hepatitis C virus effects of cyclophilin inhibitors, cyclosporin A, and NIM811. *Biochem. Biophys. Res. Commun.* **2006,** *343*, 879–884.

(20) Liu, Y.; Jiang, J.; Richardson, P. L.; Reddy, R. D.; Johnson, D. D.; Kati, W. M. A fluorescence polarization-based assay for peptidyl prolyl cis/trans isomerase cyclophilin A. *Anal. Biochem.* **2006,** *356*, 100–107.

(21) Zhang, Y.; Erdmann, F.; Baumgrass, R.; Schutkowski, M.; Fischer, G. Unexpected side chain effects at residue 8 of cyclosporin a derivatives allow photoswitching of immunosuppression. *J. Biol. Chem.* **2005,** *280*, 4842–4850.

(22) Ruegger, A.; Kuhn, M.; Lichti, H.; Loosli, H. R.; Huguenin, R.; Quiquerez, C.; von Wartburg, A. Cyclosporin A, a Peptide Metabolite from *Trichoderma polysporum* (Link ex Pers.) Rifai, with a remarkable immunosuppressive activity. *Helv. Chim. Acta* **1976,** *59*, 1075–1092.

(23) Ke, H.; Mayrose, D.; Belshaw, P. J.; Alberg, D. G.; Schreiber, S. L.; Chang, Z. Y.; Etzkorn, F. A.; Ho, S.; Walsh, C. T. Crystal structures of cyclophilin A complexed with cyclosporin A and N-methyl-4-[(E)-2-butenyl]-4,4-dimethylthreonine cyclosporin A. *Structure* **1994,** *2*, 33–44.

(24) Taylor, P.; Dornan, J.; Carrello, A.; Minchin, R. F.; Ratajczak, T.; Walkinshaw, M. D. Two structures of cyclophilin 40: Folding and fidelity in the TPR domains. *Structure* **2001,** *9*, 431–438.

(25) Binder, J. B.; Raines, R. T. Olefin metathesis for chemical biology. *Curr. Opin. Chem. Biol.* **2008,** *12*, 767–773.

(26) Philippe, C. Olefin Metathesis of Amine-Containing Systems: Beyond the Current onsensus. *Adv. Synth. Catal.* **2007,** *349*, 1829–1846.

(27) Kozany, C.; Marz, A.; Kress, C.; Hausch, F. Fluorescent probes to characterise FK506- binding proteins. *ChemBioChem* **2009,** *10*, 1402–1410.

(28) 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.

(29) Fanghanel, J.; Fischer, G. Thermodynamic characterization of the interaction of human cyclophilin 18 with cyclosporin A. *Biophys. Chem.* **2003,** *100*, 351–366.

DOI: 10.1021/ml1001272 | *ACS Med. Chem. Lett.* **2010,** *1*, 536–539