Structure−Activity Studies of Ground- and Transition-State Analogue Inhibitors of Cyclophilin

Harry C. Wang, Kyonghee Kim, Ray Bakhtiar,† and Juris P. Germanas*

Department of Chemistry, Southern Methodist University, Dallas, Texas 75275

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Peptidyl−prolyl isomerases (PPIases) are ubiquitous cellular enzymes that play roles in cellular signaling and protein folding. In addition, these proteins are the receptors for the widely used immunosuppressants cyclosporin A and FK506. We report the first structure−activity studies of de novo designed inhibitors of cyclophilin, the cellular target of cyclosporin A. Our mechanism-based inhibitors were modeled on the ground- and transition-state structures of proline-containing peptides, the natural substrates of the enzyme. Both ground-state analogues **1** and transition-state analogues **2** were prepared as single enantiomers from L-proline following a "self-reproduction of chirality" procedure. The binding affinities of the analogues for the active site of cyclophilin were measured by a fluorescence perturbation assay. While the transition-state analogues **2** did not display significant avidity for the active site (K_d = 77 μM for **2b**), several ground-state analogues bound to the enzyme with low micromolar affinity (K_d = 1.5 μM for **1e**). These results proclaim that properly designed small molecular weight molecules can form strong complexes with cyclophilin and may find use as probes in cell biology and as therapeutic agents.

Introduction

Peptidyl−proline isomerases (PPIases) are a class of enzymes that catalyze the interconversion of cis and trans rotamers of XaaPro peptide bonds.[1] These ubiquitous proteins have attracted attention not only because of their novel catalytic activity, but also because of their medical significance. The two best characterized members of the PPIase family, the cyclophilins and the FK506-binding proteins (FKBP's), are the cellular receptors for the clinically important immunosuppresants cyclosporin A (CsA)[2] and FK506,[3] respectively. These compounds bind to the active sites of their respective PPIases and inhibit their isomerase activities. Recently, PPIases have been shown to play critical roles in a variety of other biological processes, including protein folding,[4] HIV replication,[5] regulation of neuron growth,[6] and Ca^{2+}-mediated intracellular signaling.[7] The immunosuppressant function of CsA and FK506 has been shown to result not from the blocking of the catalytic activity of PPIases, but from inhibition of the phosphatase calcineurin by the PPIase−ligand complex.[8]

Early studies of the functional properties of the cyclophilins and FKBP's concentrated on the enzymatic activity of the native enzymes and select mutants. On the basis of the results of elegant experiments with native and isotopically labeled forms of peptide substrates, Stein and others proposed a "twisted amide" transition-state mechanism for cyclophilin-catalyzed peptide isomerization.[9] In this mechanism, the substrate adopts a transition-state conformation in which the carbonyl group and the nitrogen lone pair of the AlaPro amide bond are orthogonal to each other (Figure 1). This

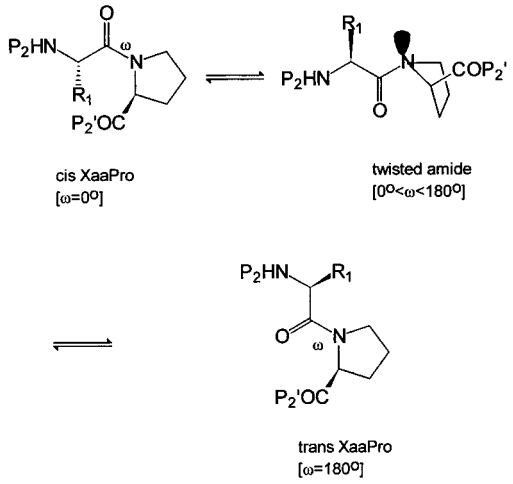

Figure 1. "Twisted amide" mechanism for cyclophilin-catalyzed peptide isomerization

hypothesis was bolstered by the finding that elimination of active site nucleophilic groups by site-directed mutagenesis had negligible effect on the isomerization activity of cyclophilin, which would have favored alternative catalytic mechanisms.[10] Support for the "twisted amide" transition state for FKBP catalysis was also seen in the structure of FK506. In the ground-state conformation of FK506, the ketone function of the critical α-ketoamide group assumed an orthogonal conformation to the adjacent amide function, suggesting that FK506 itself is a transition-state analogue of the peptide substrate.[11] Theoretical calculations have also favored "twisted amide" transition-states along the FKBP-mediated isomerization reaction coordinate.[12]

Other groups have attempted to define the catalytic mechanism of cyclophilin through structural studies of enzyme−substrate complexes. Using X-ray crystallography, Ke and co-workers defined the high-resolution

* Corresponding author. Tel: 214-768-2912. Fax: 214-768-4089. Email:germanas@mail.smu.edu.
† Merck Research Laboratories, Department of Drug Metabolism, Mail Stop: RY80L-109, Rahway, NJ 07065.

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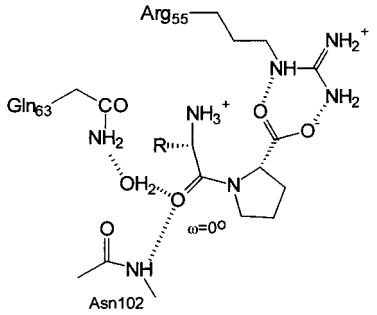


Figure 2. Active site interactions between substrate and enzyme in the crystal structure of the cyclophilin cis-AlaPro complex.[14] The side chain guanidium group of Arg55 is proposed to form a hydrogen bond to the proline nitrogen atom in the transition state.

structures of bimolecular complexes between human cyclophilin and several peptide substrates,[13] as well as select dipeptides XaaPro (Xaa = Gly, Ala, His, and Ser).[14,15] No distortion of the XaaPro peptide bond from planarity was observed in these structures. In all cases, the peptide bound to cyclophilin with the XaaPro peptide bond exclusively in the cis configuration (Figure 2). In contrast to the bound state, the trans conformer is 3- to 5-fold favored in the unbound state.

On the basis of the structural findings and theoretical calculations, a detailed model of the catalytic mechanism of cyclophilin and FKBP was proposed. After binding to the enzyme in the s-trans conformation, the substrate is distorted by the active site, and the amide bond is twisted out of planarity. Simultaneously, a hydrogen bond is formed between a donor the active site and the pyramidal Pro nitrogen.[15] The hydrogen bond donor in cyclophilin was proposed to be the side chain of the conserved active site residue Arg55, while the donor in the case of FKBP was the side-chain hydroxyl group of one of several active-site tyrosine residues (Figure 2). Support for this hypothesis was found in the drastically reduced catalytic activity of the Arg55Ala mutant of cyclophilin, which lacks the ability to form the postulated hydrogen bond.[16] Other support for the mechanism has emerged from model studies on amides with internal hydrogen bonding groups, which demonstrate accelerated amide rotamer interconversion.[17]

The wealth of structural and functional data on cyclophilin−peptide complexes has offered solid starting points for the rational design of inhibitors. Our group[18] as well as others[19−21] have described the design and synthesis of *cis*-proline peptidomimetics that resemble the cyclophilin-bound ground-state conformation of peptide substrates. These molecules indeed inhibited cyclophilin with micromolar affinity.[19,22] A third group reported that a *trans*-proline peptidomimetic inhibited FKBP-catalyzed peptide isomerization with a similar degree of affinity.[23] Since these compounds bore resemblance to the ground-state conformation of the substrate, however, the very tight binding desired from inhibitors and drugs could not be realized.

New inhibitors of PPIases would help define the catalytic mechanism of the enzymes, lead to a better understanding of their roles in biology, and potentially act as antiviral or immunomodulating agents. We report now the first extensive structure−function analysis of de novo designed ground-state analogue inhibitors of

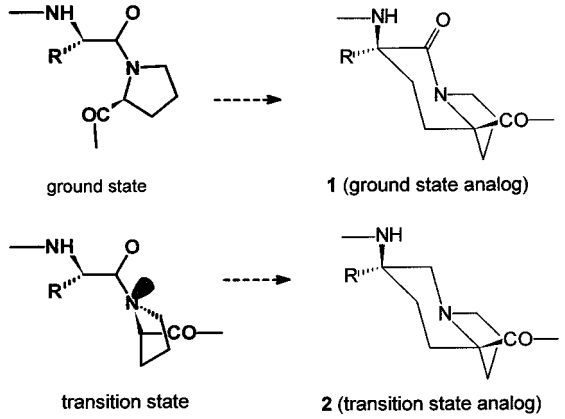


Figure 3. Ground- and transition-state analogue structures.

PPIases (**1** in Figure 3). Several of these compounds were found to bind to the active site of cyclophilin with high nanomolar affinity and could represent useful tools for probing the function of PPIases in cells. In addition, we report the design, synthesis, and evaluation of the first rationally designed transition-state analogue PPIase inhibitor (**2** in Figure 3), with resemblance to the proposed "twisted amide" transition state.

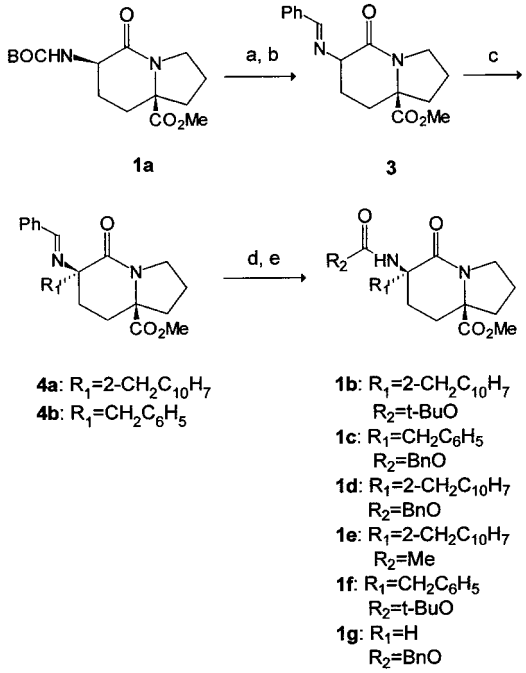
Chemistry

We previously described the de novo design of a bicyclic lactam (**1a**) that displayed high structural similarity to *cis*-GlyPro in the cyclophilin-bound conformation.[18] Using a fluorescence titration assay,[24] we demonstrated that this lactam bound to the active site of cyclophilin with a dissociation constant of approximately 200×10^{-6} M.[22] As a result of the unspectacular affinity, we sought to rationally modify the structure of the analogue to obtain second-generation analogues with increased binding avidity.

A unique feature of the structure of CsA is the large number of *N*-methyl amide groups and other hydrophobic functionalities. We reasoned, therefore, that elimination of ionizable groups from and introduction of nonpolar groups into the structure of the bicyclic lactam **1** would increase the affinity of these ground-state analogues toward cyclophilin. To this end, a series of derivatives of lactam **1** incorporating aryl groups adjacent to the lactam carbonyl, as well as various *N*-carboxamide functions, were chosen for synthesis and evaluation.

In addition to improving the affinity of the ground-state analogue inhibitor, we also resolved to design and synthesize the first transition-state analogue inhibitor of PPIases. This goal was based on the established concept that molecules that resemble the transition state bind more tightly to enzyme active sites than do molecules that resemble the ground state. Toward this end, inspection of the structure of the bicyclic lactam **1** revealed that the lactam nitrogen atom coincided with the substrate proline nitrogen atom that undergoes sp2-to-sp3 rehybridization in the course of isomerization. We therefore reasoned that reducing the lactam carbonyl group to an amine functionality, as in bicycle **2**, would provide a compound with not only high overall conformational similarity to the cyclophilin-bound substrates, but also with a tertiary amine nitrogen capable of interacting with the active site Arg55 residue.

Scheme 1[a]



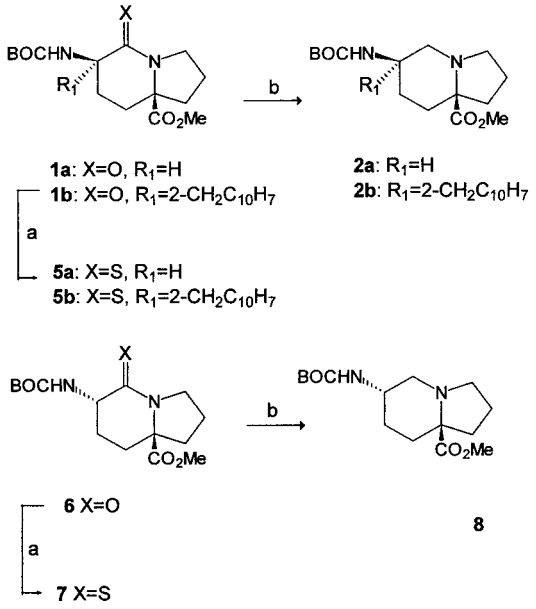
[a] Key: (a) TFA; (b) PhCHO, Et_3N; (c) LDA, R_1X, −78 °C; (d) MeOH, HCl; (e) R_2COX (overall yields: **1a**, 56%; **1b**, 49%; **1c**, 62%; **1d**, 67%; **1e**, 61% **1f**).

Synthesis of the ground-state analogue inhibitors **1** commenced with bicyclic lactam **1a**, which was obtained from L-proline following our improved procedure for preparing enantiomerically pure α-alkyl prolines (Scheme 1).[24] The corresponding benzaldehyde imine **3** was prepared,[21] and alkylation of the lithium enolate with benzyl or 2-bromomethylnaphthyl bromide furnished the α-aryl lactams **4**. Mild hydrolysis of the imine function of the crude alkylation products, followed by reaction of the resulting free amino group with select acylating agents, afforded the carboxamide ground-state analogues **1**.

Spectroscopic characterization of the products **1** revealed that only a single diastereomer was obtained from the alkylation reaction. The high diastereoselectivity of this reaction was consistent with our results from the alkylation of the dimethylformamidine analogue of imine **3**.[18] The stereochemistry at the carbon adjacent to the lactam carbonyl group of products **1** was assigned from the results of difference NOE spectra. In addition, the stereochemical assignment of product **4** could be supported by the similarity of its NMR spectroscopic properties to those of the analogous dimethylformamidine, whose structure was unequivocally established by X-ray crystallography.[22]

Preparation of the transition-state analogue was envisioned to proceed via chemoselective reduction of the lactam carbonyl group of bicycle **1** to amine **2** (Figure 2). After exploring several approaches, the successful strategy involved the selective reduction of the thioamide derivative of the amide (Scheme 2). Gratifyingly the thioamides **5** and **7** were obtained in good yield from the corresponding lactams **1** and **6**, respectively, by treatment with Lawesson's reagent in refluxing toluene.[26] Confirmation of the structures of thiolactams was established by the presence of characteristic ^{13}C NMR shifts for the thioamide carbonyl carbons and by the

Scheme 2[a]



[a] Key: (a) Lawesson's reagent, $PhCH_3$, 80 °C; (b) Raney nickel, H_2 (overall yields: **2a**, 70%; **2b**, 56%; **8**, 67%).

presence of correct molecular ions in the mass spectra. Interestingly, the presence of the quaternary carbon atom adjacent to the lactam carbonyl carbon did not hinder formation of the thio derivatives **5** and **7**. Additionally, we observed that the stereochemical integrity of the carbon adjacent to the lactam carbonyl was preserved in the thiolactam products.

Finally, treatment of the thioamides with Raney nickel in ethanol cleanly afforded the tertiary amines **2** and **8** (Scheme 2). Each amine displayed a correct molecular ion peak in the mass spectra and lacked lactam carbonyl carbons in the ^{13}C NMR spectra. Reduction of thioamides furnished single amine products, with no evidence of the diasteromeric amine. Thus, this procedure proceeded stereospecifically, in the presence of several sensitive functional groups and was not sterically inhibited by two adjacent quaternary centers. Few other methods are available to access such structures. This procedure may prove valuable to chemists attempting to prepare analogues of other biologically active bicyclic tertiary amines, such as coniceine and swainsonine.[28,29]

Results and Discussion

Stabilities of cyclophilin−ligand complexes have been assessed by a number of methods, including fluorescence spectroscopy,[2] enzyme kinetics,[26] and NMR line shape analysis.[27] In this study, stabilities of cyclophilin ground-state and transition-state analogues were evaluated by fluorescence titration experiments.

The fluorescence procedure monitors the change in the fluorescence spectrum of cyclophilin as a function of ligand concentration. Shifts in the fluorescence emission intensity and wavelength reflect perturbations of the environment surrounding the single tryptophan residue (W121) of the enzyme, which lies adjacent to the active site. Others have shown that binding of the immunosuppressant CsA to the active site resulted in a 2-fold increase in fluorescence intensity accompanied by a red shift of the emission wavelength.[2] This unusual

Table 1. Binding Affinities of Bicyclic Lactams and Bicyclic Amines to Cyclophilin ± Standard Deviation[a]

compound	K_d (M × 10^{-6})	λ_{max} (intensity enhancement)
1a	>200	349 (1.1)
1b	17 ± 8	349 (1.3))
1c	124 ± 30	348 (1.3)
1d	4.7 ± 2	349 (2.1)
1e	1.5 ± 0.05	350 (2.0)
1f	140 ± 32	348 (1.3)
1g	>200	348 (1.1)
2a	>200	348 (1.1)
2b	77 ± 18	350 (1.3)
6	>200	347 (1.1)
8	>200	346 (1.3)
cyclosporin	0.3 ± 0.15	349 (2.0)

[a] Data obtained from fluorescence titration experiments. λ_{max} represents the wavelength in nm for the emission maximum of Trp121. K_d represents the dissociation constant.

spectroscopic behavior was attributed to an increase in hydrophobicity of the environment of the tryptophan upon complex formation.

Complex formation between the ground-state and transition-state analogues and cyclophilin was indeed observed by fluorescence spectroscopy. Titration of cyclophilin with each of the analogues resulted in an increase in the intensity of the tryptophan fluorescence, which eventually reached a saturation value at higher analogue concentrations. The value of the dissociation constant K_d for each of the analogues was obtained by a nonlinear least squares regression fit (Table 1). Similar to that of CsA, the λ_{max} of the spectra of the analogue complexes also shifted to higher wavelengths. These results suggested that the analogues bound to the same site on the enzyme surface as CsA. Interestingly, the magnitude of the fluorescence intensity increase was much more substantial for the α-methylnaphthyl analogues **1d** and **1e** than for any of the other ligands.

Analysis of the binding affinities of the bicyclic amides **1** and amines **2** and **8** to cyclophilin revealed several distinct structure−activity trends. First, little information regarding the effect of stereochemistry at the position alpha to the lactam carbonyl on binding could be discerned due to the low affinity of the analogues **1a** and **6** and **2a** and **8** for the enzyme. One important trend, however, was the positive correlation between the magnitude of the dissociation constant and the size of the amino substituent R_2 in the bicyclic amides (Figure 4). As seen in the figure, with the same α-alkyl group, the affinity of the bicyclic amide increased as the size of the pendant alkylamide group decreased. For example, with a methylnaphthyl α-alkyl group, the dissociation constant decreased from 17 to 4.7 to 1.5 μM with *tert*-butoxycarbonyl, benzyloxycarbonyl, and acetyl alkylamide groups (row 3). Similarly, the dissociation constant decreased from 140 to 124 μM when the amide group was changed from *tert*-butoxycarbonyl to benzyloxycarbonyl with phenyl as the alkyl substituent (row 2).

The second trend was the inverse correlation between the magnitude of the dissociation constant and the size of the alkyl substituent R_1 adjacent to the carbonyl group. When the pendant alkylamide group was restricted to *tert*-butoxycarbonyl, the K_d values of the compounds decreased from over 200 to 140 to 17 μM when the alkyl group was varied from H to benzyl to

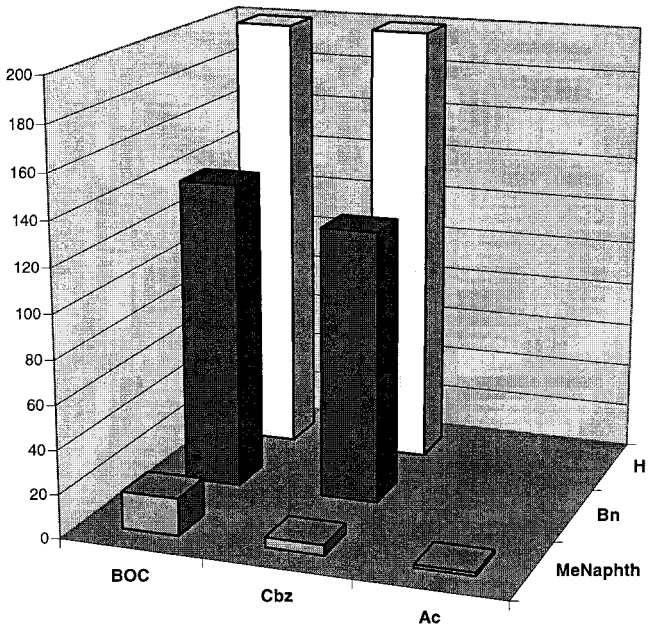


Figure 4. Three-dimensional plot of K_d as a function of *N*-alkylamide and α-alkyl substitutents. Dissociation constant on the *z* axis (M × 10^{-6}).

2-methylnaphthyl (column 1). A similar progression was observed when the pendant amide was set to benzyloxycarbonyl (column 2).

A structure−activity correlation of particular interest in this work was the trend between the functional group at the ring junction (i.e., amide vs amine) and the binding affinity. When the group adjacent to the ring carbonyl was set to H, no trend could be extracted because the binding affinities were too weak to be accurately measured. With the methylnaphthyl group adjacent to the carbonyl function, however, the affinity of the tertiary amine **2b** (77 μM) was in fact 4 times weaker than that of the corresponding lactam **1b** (17 μM). As a result, these data conflicted with the expected outcome that the proposed transition-state analogue would bind more strongly to the active site than a ground-state analogue.

The structure−activity correlations for the bicyclic lactams are consistent with the proposed binding model based on the structure of the dipeptide−cyclophilin complexes.[14,15] In this model, the substituent on the pendant amino group of amide **1** would locate itself within the groove that accommodates the *N*-terminus of the peptide substrate, while the alkyl group adjacent to the lactam carbonyl carbon would project into the hydrophobic pocket lined by the side chains of Ala101 and His126. Thus, bulky alkylamide groups, such as *tert*-butoxycarbonyl, would not be accommodated into the relatively narrow groove. On the other hand, larger and more hydrophobic groups, such as 2-methylnaphthyl, would form more favorable interactions with the hydrophobic pocket than would smaller groups. Hence, the highest-affinity analogue, compound **1e**, combined the smallest alkylamide substituent (*N*-acetyl) with the most hydrophobic α-alkyl group (2-methylnaphthyl). In fact, the affinity of this analogue was only 5 times lower that that of CsA, an undecapeptide.

Several causes may explain the lower affinity of the postulated transition-state analogue **2b** relative to the

ground-state analogue **1b**. One possibility is that the enzyme undergoes a slight conformational change upon binding of the bicyclic ligands, which positions the side chain of Arg55 away from the ligand binding site. This change could disfavor the formation of the tertiary amine−enzyme complex, which should gain stability from the presence of a hydrogen bond from the guanidium group to the tertiary nitrogen. A second possibility is that the carbonyl oxygen of the lactam group is important for the stability of the ligand−enzyme complexes. Our ligand−enzyme model includes a hydrogen bond from the peptide NH of Asn102 to the lactam carbonyl oxygen. Disruption of this interaction could lead to a decreased affinity for the tertiary amine. A third possibility is that cyclophilin utilizes a catalytic mechanism distinct from the twisted amide model that incorporates a hydrogen bond to the proline nitrogen in the transition state. The most likely possibility, however, is that the position of the nitrogen lone pair in transition-state analogue **2b** may not coincide with the position of the proline nitrogen lone pair on the reaction coordinate in cyclophilin-catalyzed isomerization. Thus, the pyrrolidine ring, α-alkyl group, and lone pair of the analogue **2b** may not all be geometrically properly aligned to form ideal noncovalent interactions with complementary groups in the enzyme's active site.

Conclusions

While PPIases, particularly cyclophilins, are widespread and abundant enzymes, their catalytic mechanism is poorly understood. In particular, the nature of the highest-energy transition state in the mechanism has not been experimentally confirmed. This work represents the first attempts to explore the structure of the transition state of PPIase-catalyzed reactions with rationally designed inhibitors. In addition, this work provides the most extensive study of structure−activity relationships of ground-state cyclophilin inhibitors to date.

We have demonstrated that properly designed small molecular weight compounds can form tight complexes with cyclophilin. Bicyclic lactam analogues **1** that mimic the ground state of the reaction vary in affinity with the identity of the pendant groups. Those with hydrophobic groups adjacent to the lactam carbonyl group bound with the greatest affinity, while those with large *N*-alkylamide substituents displayed the lowest affinity. A putative transition-state analogue, **2b**, with a tertiary amine group in place of a lactam amide group, bound less tightly than the corresponding lactam **1b**. The lower affinity of the transition-state analogue relative to that of the ground-state molecule could be due to several factors, including a nonoptimal geometric arrangement of functional groups. Nonetheless, these results indicate that relatively small molecules can bind tightly to cyclophilin, providing the possibility of PPIase inhibitors as orally available therapeutic agents or as probes of cellular biology.

Experimental Section

General. All starting materials and solvents were obtained from commercial suppliers and used without further purification. Acetonitrile was distilled from CaH_2 directly before use. Tetrahydrofuran (THF) was distilled just prior to use from sodium/benzophenone. Dimethylformamide (DMF) was distilled from CaH_2 under reduced pressure directly before use. Reactions were carried out under a dry nitrogen atmosphere in glassware that was flame- or oven-dried overnight (T > 100 °C).

^1H and ^{13}C NMR spectra were recorded at 300 MHz using $(CH_3)_4Si$ as an internal standard. ^{13}C NMR spectra displayed the required number of carbons except in cases of accidental degeneracy.

MS were obtained on a Finnigan TSQ 700 using an atmospheric pressure chemical ionization (APCI) source. The capillary and vaporizer temperatures were 160 and 380 °C, respectively. The sheath gas was set at 50 psi. The APCI corona discharge current was 900 V. Samples were dissolved in 80:20 $CHCl_3$/EtOH (5 mM ammonium acetate) and infused into the APCI source at a flow rate of 20 microliters/min. An HPLC system was used to deliver 0.98 mL/min of mobile phase composed of MeOH to a T-connector. A syringe pump was utilized to deliver the sample solution at a flow rate of 20 microliters/min to the T-connector. Subsequently, a net flow rate of 1.0 mL/min of solvent was introduced into the APCI source.

Column chromatography was performed on silica gel (Merck reagents silica gel 60, 230−400 mesh ASTM). Thin-layer chromatography (TLC) was carried out on Analtech Uniplate silica gel plates with a 0.25 mm coating containing fluorescent indicator. Spots were visualized using ninhydrin, iodine, or UV light.

(6*R*,8a*R*)-6-*N*,*N*-Monophenylimino-8a-carboxyindolizidin-5-one Methyl Ester (3). To a solution of ester **1a** (0.1 g, 0.32 mmol) in CH_2Cl_2 (10 mL) was added trifluoroacetic acid (0.19 mL, 2.44 mmol), and the solution was stirred at room temperature. After 2 h, CH_2Cl_2 and trifluoroacetic acid were removed under reduced pressure to give a yellow oil. The crude amine salt in CH_2Cl_2 (3 mL) was stirred with 4 Å molecular sieves (0.050 g), benzaldehyde (53 μL, 0.52 mmol), and triethylamine (72 μL, 0.52 mmol) at room temperature. After 1.5 h and again at 2.5 h, benzaldehyde (∼13 μL) was added. After 3 h, the mixture was filtered, evaporated, and dried in vacuo to afford **3** as green oil, spectroscopically comparable with the literature.[21] No purification was performed, and the compound was used directly in the next step.

General Procedure for Reaction of the Enolate of 3 with Electrophiles.[21] An ice-cold solution of LDA (1.5 equiv) in THF was added dropwise to a solution of methyl ester **3** (1.0 equiv) in THF at −78 °C. After 30 min, the electrophile was added, and the temperature was allowed to warm to −30 °C over a period of 2 h. After the reaction had reached completion according to TLC (R_f of 0.3 for **3** in 2:1 hexane/ethyl acetate), 0.02 M aqueous phosphate buffer (pH 7.0) was added to quench the reaction, and the mixture was extracted with Et_2O. The organic phase was dried with $MgSO_4$, and the solvent was removed in vacuo to afford the imine product, which was not further purified but used directly in the methanolysis step.

(6*R*,8a*R*)-6-Benzyl-6-*N*,*N*-monophenylimino-8a-carboxyindolizidin-5-one Methyl Ester (4a).[21] (88% crude yield) oil: ^1H NMR ($CDCl_3$) δ 7.26−7.17 (m, 10 H), 5.21 (s, 1H), 4.42 (s, 2H), 3.47 (s, 3H), 3.65−3.24 (m, 3H), 2.19−2.09 (m, 3H), 1.50−1.17 (m, 4H).

(6*R*,8a*R*)-6-(2-Methylnaphthyl)-6-*N*,*N*-monophenylimino-8a-carboxyindolizidin-5-one Methyl Ester (4b). (88% crude yield) oil: ^1H NMR ($CDCl_3$) δ 7.64−7.37 (m, 12H), 4.99 (s, 1H), 4.68 (s, 2H), 3.55 (s, 3H), 3.82−3.75 (m, 3H), 3.48−3.43 (m, 2H), 2.36−1.85 (m, 2H), 1.64−1.06 (m, 3H).

(6*R*,8a*R*)-6-(2-Methylnaphthyl)-*N*-*tert*-butoxycarbonyl-6-amino-8a-carboxyindolizidin-5-one Methyl Ester (1b). To a solution of methyl ester **4b** (0.12 g, 0.32 mmol) in dry methanol (10 mL) was added 1 N methanolic HCl (from acetyl chloride and methanol). The mixture was stirred at room temperature overnight under a nitrogen atmosphere. The solvent was removed under reduced pressure to afford the crude product as a brown oil. The crude product was then dissolved in 10 mL of methanol, and 3.5 equiv of $NaHCO_3$ (0.50 g, 6.0 mmol) was added. Di-*tert*-butyl dicarbonate (1.0 equiv,

0.4 mL) was added dropwise into the mixture. The reaction mixture was then stirred at room temperature for 3 h, filtered, and concentrated under reduced pressure. The resulting white solid was partitioned between 0.1 N HCl and dichloromethane. The remaining aqueous layer was back extracted with ethyl acetate. The combined organic layers were washed with a dilute $NaHCO_3$ solution, brine, dried over $MgSO_4$, and evaporated under reduced pressure to afford **1b** as clear oil that was subjected to purification by column chromatography with 3:1 hexane/ethyl acetate to give 0.081 g (56% from **1a**) of the product as an oil: ^{1}H NMR ($CDCl_3$) δ 7.69−7.16 (m, 7H), 5.46−5.36 (m, 2H), 3.72 (s, 3H), 3.61−3.55 (m, 2H), 3.49 (d, J = 12 Hz, 1H), 3.32−3.10 (m, 2H), 2.62−2.56 (m, 2H), 2.19−1.27 (m, 4H), 1.36 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 224.1, 186.4, 173.3, 167.1, 166.7, 159.3, 149.3, 143.1, 139.8, 125.9, 116.0, 110.1, 103.9, 99.0, 87.5, 66.6, 58.8, 50.7, 46.0, 41.6, 34.1, 29.7, 25.5, 20.9; MS (ES) m/z 453 (MH^+); (FAB) calcd $C_{26}H_{33}N_2O_5$ 453.2381, found 453.2368.

(6*R*,8a*R*)-6-Benzyl-*N*-benzyloxycarbonyl-6-amino-8a-carboxyindolizidin-5-one Methyl Ester (1c). To a solution of methyl ester **4a** (0.24 g, 0.60 mmol) in dry methanol was added 1 N methanolic HCl (from acetyl chloride and methanol). The mixture was stirred at room temperature overnight under a nitrogen atmosphere. The solvent was removed under reduced pressure to afford the crude product as a brown oil. The crude product was then dissolved in 10 mL of dry dioxane. Five equivalents of triethylamine were added (∼0.4 mL), and the reaction mixture was cooled to 0 °C before a solution of benzyl chloroformate (0.11 mL, 1.5 equiv) in dioxane (1 mL) was added dropwise into the mixture. The reaction mixture was then stirred at room temperature for 3 days. Triethylamine (0.24 mL) and benzyl chloroformate (0.076 mL, 1.0 equiv) were added together after 24 and 48 h. The reaction was quenched and partitioned between 1 N HCl (10 mL) and ethyl acetate (50 mL). The aqueous layer was extracted with ethyl acetate. The combined organic layer was washed with 1 N HCl and saturated $NaHCO_3$ and then dried over $MgSO_4$, filtered, and evaporated under reduced pressure. Purification by column chromatography using 3:1 hexane/ethyl acetate afforded the desired product as an oil (0.128 g, 57%): ^{1}H NMR ($CDCl_3$) δ 7.22−7.13 (m, 10H), 6.60 (br s, 1H), 5.35 (s, 2H), 3.91 (m, 2H), 3.69 (s, 3H), 3.61−3.56 (m, 2H), 3.31−3.24 (m, 2H), 2.89−2.84 (m, 2H), 2.23−1.84 (m, 2H), 1.62−1.25 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 205.9, 190.9, 186.7, 132.7, 131.4, 128.0, 107.4, 105.8, 101.8, 99.9, 66.8, 55.1, 47.8, 41.3, 40.1, 37.6, 34.4, 24.3, 19.4; HRMS (FAB) m/z calcd $C_{25}H_{29}N_2O_5$ (MH^+) 437.2069, found 437.2081.

(6*R*,8a*R*)-6-(2-Methylnaphthyl)-*N*-benzyloxycarbonyl-6-amino-8a-carboxyindolizidin-5-one Methyl Ester (1d). This compound was prepared according to the procedure for compound **1c**. Purification by column chromatography using 3:1 hexane/ethyl acetate afforded 0.12 g (62%) of the desired product as an oil: ^{1}H NMR ($CDCl_3$) δ 7,64−7.43 (m, 12H), 5.41 (s, 2H), 5.10 (s, 1H), 3.92−3.87 (m, 2H), 3.69 (s, 3H), 3.63−3.58 (m, 2H), 3.47−3.42 (m, 2H), 2.97−2.90 (m, 2H), 2.16−2.01 (m, 2H), 1.56−1.26 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 220.2, 180.2, 174.6, 151.9, 149.3, 146.6, 142.3, 141.2, 137.1, 130.7, 128.6, 125.9, 124.8, 119.9, 117.4, 114.5, 113.3, 61.5, 53.9, 38.6, 35.4, 25.2, 19.3; HRMS (FAB) m/z calcd $C_{29}H_{31}N_2O_5$ (MH^+) 487.2225, found 487.2235.

(6*R*,8a*R*)-6-(2-Methylnaphthyl)-*N*-acetyl-6-amino-8a-carboxyindolizidin-5-one Methyl Ester (1e). To a solution of methyl ester **4b** (0.17 g, 0.39 mmol) in dry methanol was added 1 N methanolic HCl (from acetyl chloride and methanol). The mixture was stirred at room temperature overnight under nitrogen atmosphere. The solvent was removed under reduced pressure to afford the crude product as a brown oil. The crude product was then dissolved in 10 mL of dry dioxane. Five equivalents of triethylamine were added (∼0.5 mL), and the reaction mixture was cooled to 0 °C before a solution of acetyl chloride (0.5 mL, 7.0 mmol) was added slowly into the reaction mixture. The reaction mixture was then stirred at room temperature for 24 h. The reaction was quenched and partitioned between 0.1 N HCl (10 mL) and ethyl acetate (50 mL). The aqueous layer was extracted with ethyl acetate. The combined organic layers were washed with 1 N HCl and saturated $NaHCO_3$ and then dried over $MgSO_4$, filtered, and evaporated under reduced pressure to afford crude product that was subjected to purification by column chromatography using 2:1 hexane/ethyl acetate as eluent to yield **1e** as an oil (0.107 g, 67%); ^{1}H NMR ($CDCl_3$) δ 7.77−7.30 (m, 7H), 5.75 (br s, 1H), 5.23 (s, 2H), 3.66 (s, 3H), 2.97−2.28 (m, 4H), 1.98 (s, 3H), 1.80−0.89 (m, 6H); ^{13}C NMR ($CDCl_3$) δ 215.0, 174.4, 165.9, 140.5, 128.2, 126.8, 117.2, 114.8, 106.4, 102.7, 95.5, 92.2, 74.0, 70.0, 64.6, 56.9, 47.3, 34.8, 31.7, 29.6, 22.6, 19.1; HRMS (FAB) m/z calcd $C_{23}H_{27}N_2O_5$ (MH^+) 411.1913, found 411.1902.

(6*R*,8a*R*)-6-Benzyl-6-*N*-*tert*-butoxycarbonylamino-8a-carboxyindolizidin-5-one Methyl Ester (1f). This compound was prepared according to the procedure for compound **1c**. Purification by column chromatography using 2:1 hexane/ethyl acetate as eluent yielded **1f** as an oil (61%); ^{1}H NMR ($CDCl_3$) δ 7.10−7.28 (m, 5H), 5.47 (br s, 1H), 3.73 (s, 3H), 3.58−3.68 (s, 2H), 3.29 (d, J = 13 Hz, 1H), 3.13 (d, J = 13 Hz, 1H), 2.61 (ddd, J = 1.5, 1.5, 10 Hz, 1H), 2.18−2.29 (m, 2H), 1.70−2.05 (m, 2H), 1.42 (s, 9H), 1.22−1.37 (m, 2H), 0.83 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 196.8, 173.8, 169.9, 154.7, 136.5, 130.0, 128.2, 127.0, 91.2, 69.0, 58.8, 52.8, 45.7, 45.0, 38.1, 30.7, 28.4, 20.6.

(6*R*,8a*R*)-*N*-tert-Butoxycarbonyl-6-amino-8a-carboxyindolizidin-5-thione Methyl Ester (5a). [2, 4-Bis(4-methoxyphenyl)-1, 3-dithia-2, 4-diphosphetane-2, 4-disulfide] (Lawesson's Reagent) (0.093 g, 0.23 mmol) was dissolved in dry toluene (5 mL) at 80 °C, and then a solution of **1a** (0.05 g, 0.1 mmol) in dry toluene (1 mL) was added dropwise. After 6 h, the reaction was stopped by first cooling the reaction mixture to room temperature, and then the toluene was removed in vacuo. The resulting crude product was subjected to purification by column chromatography using 2:1 hexane/ethyl acetate as the eluent to yield the product as an oil: ^{1}H NMR ($CDCl_3$) δ 5.68 (m, 1H), 4.15 (m, 1H), 3.90−4.01 (m, 2H), 3.75 (s, 3H), 2.50−2.61 (m, 3H), 1.80−2.03 (m, 4H), 1.48 (s, 9H), 1.41 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 197.8, 172.7, 156.3, 76.9, 72.4, 57.3, 54.0, 53.6, 38.0, 30.6, 28.7, 26.7, 20.7.

(6*R*,8a*R*)-6-(2-Methylnaphthyl)-*N*-*tert*-butoxycarbonyl-6-amino-8a-thiocarboxyindolizidin-5-one Methyl Ester (5b). This compound was prepared according to the procedure for compound **5a**. Purification by column chromatography with 2:1 hexane/ethyl acetate as eluent afforded the product as an oil: ^{1}H NMR ($CDCl_3$) δ 7.20−6.99 (m, 7H), 5.48 (s, 1H), 4.53 (m, 2H), 3.87 (s, 3H), 3.60−2.80 (m, 4H), 2.33−1.40 (m, 6H), 1.22 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 224.9, 207.0, 181.0, 172.3, 160.3, 133.9, 128.6, 114.1, 113.8, 112.6, 95.0, 91.7, 88.3, 74.5, 68.3, 55.4, 30.8, 29.5, 27.4, 25.7, 25.0, 21.9, 15.6; HRMS (FAB) m/z calcd $C_{26}H_{33}N_2O_4S$(MH^+) 469.2153, found 469.2167.

(6*S*,8a*R*)-6-*N*-*tert*-butoxycarbonylamino-8a-carboxyindolizidin-5-thione Methyl Ester (7). This compound was prepared according to the procedure for compound **5a**. Purification by column chromatography with 2:1 hexane/ethyl acetate as eluent afforded the product as an oil: ^{1}H NMR ($CDCl_3$) δ 6.34 (m, 1H), 3.94−4.00 (m, 3H), 3.77 (s, 3H), 2.70 (m, 1H), 2.52−2.60 (m, 2H), 1.65−2.00 (m, 5H), 1.44 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 197.7, 172.4, 155.4, 79.7, 70.1, 54.5, 54.2, 53.7, 40.5, 30.1, 28.6, 27.2, 21.9.

(6*R*,8a*R*)-6-*N*-*tert*-butoxycarbonylamino-8a-carboxyindolizidine Methyl Ester (2a). To a solution of **5a** (0.016 g, 0.05 mmol) in 20 mL of dry ethanol was added Raney nickel (0.10 g), and then hydrogen gas was bubbled through the solution for 10 min. The solution was stirred for 2 h under a hydrogen atmosphere before being filtered through Celite to remove the catalyst. The solvent was then removed under reduced pressure to give the crude product that was subjected to column chromatography using 3:1 hexane/ethyl acetate as the eluent to give 0.011 g of the product as a clear oil (73% from **1a**): ^{1}H NMR ($CDCl_3$) δ 4.03 (bd, J = 7 Hz, 1H), 3.83 (m, 1H), 3.30 (s, 3H), 3.14 (dd, J = 2,12 Hz, 1H), 3.02 (m, 1H), 2.81 (m, 1H), 2.63 (dd, J = 12, 12 Hz, 1H), 2.19 (m, 1H), 1.47−1.89 (m, 5H), 1.45 (s, 9H), 1.16 (ddd, J = 2, 2, 14 Hz, 1H), 0.86

(m, 1H); ^{13}C NMR ($CDCl_3$) δ 174.9, 155.2, 79.3, 66.9, 51.8, 50.7, 50.1, 44.2, 36.3, 30.3, 29.5, 28.3, 21.1.

(6*R*,8a*R*)-6-(2-Methylnaphthyl)-*N*-*tert*-butoxycarbonyl-6-amino-8a-indolizidine Methyl Ester (2b). This compound was prepared according to the procedure for compound **2a**. Column chromatography using 3:1 hexane/ethyl acetate gave 0.015 g of the product as a clear oil (56% from **1b**): ^{1}H NMR ($CDCl_3$) δ 7.71−7.64 (m, 3H), 7.55−7.29 (m, 4H), 5.67 (s, 1H), 4.33 (s, 3H), 3.90−3.15 (m, 6H), 2.56−1.45 (m, 8H), 1.28 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 178.6, 137.7, 134.2, 130.9, 128.8, 125.2, 115.0, 113.6, 74.7, 69.9, 65.6, 62.4, 57.7, 48.2, 45.7, 39.0, 30.6, 25.9, 23.8, 21.1, 19.2, 17.7, 13.7; HRMS (FAB) *m*/*z* calcd $C_{26}H_{35}N_2O_4$ (MH^+) 439.2588, found 439.2600.

(6*S*,8a*R*)-6-*N*-*tert*-butoxycarbonylamino-8a-carboxyindolizidine Methyl Ester (8). This compound was prepared according to the procedure for compound **2a**. Column chromatography using 3:1 hexane/ethyl acetate gave 0.021 g of the product as a clear oil (67% from **6**): ^{1}H NMR ($CDCl_3$) δ 5.31 (bd, J = 7 Hz, 1H), 3.76 (m, 1H), 3.66 (s, 3H), 3.15 (m, 1H), 2.96−3.05 (m, 2H), 2.69 (dd, J = 3, 12 Hz, 1H), 2.32 (m, 1H), 1.68−2.04 (m, 5H), 1.43 (s, 9H), 1.29−1.40 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 175.1, 155.2, 78.9, 67.7, 51.1, 50.6, 50.5, 44.8, 36.9, 29.5, 28.4, 27.1, 20.4.

Isolation of Cyclophilin A. Native cyclophilin A was obtained through expression in *Escherichia coli* (XA90).[16] A 2-liter culture of *E. coli* XA90/pHNJ was grown to 37 °C in LB medium containing ampicillin (100 mg/mL) to an A_{550} of 0.8; isopropyl β-D-thiogalactopyranoside (IPTG) was added to 2 mM, and the incubation was continued overnight. The cells were harvested and resuspended in 20 mM Tris-HCl (pH 7.8). This and subsequent steps were carried out at 4 °C. The cells were lysed by two passages through a French press at 20 000 psi. After separation of the cell debris by centrifugation (20 000*g*, 25 min), the nucleic acids were precipitated by protamine sulfate (0.4% final concentration). The crude cell extract was subjected to ammonium sulfate fractionation. The 40−60% (wt./vol.) ammonium sulfate pellet was collected, resuspended in 20 mM Tris-HCl (pH 7.8), and dialyzed against the same buffer overnight. The dialyzed crude cell extract was loaded onto a DEAE-Sepharose column (1.2 × 22 cm) equilibrated with the same buffer. The protein was identified based by molecular weight on a PAGE gel. The fractions collected from the column were combined and concentrated in an Amicon ultrafiltration cell (PM 10 membrane). The protein concentration was determined by UV absorbance at 280 nm (A_{280} = 0.44 at 1 mg/mL).

Determination of Dissociation Constants (K_d). Fluorescence measurements were performed on Shimadzu RF-1501 spectrofluorophotometer. Enhancement of intrinsic tryptophan fluorescence upon binding of cyclosporin A or mimics to cyclophilin A was monitored using the procedure developed by Handschumacher et al.[2] The experiments were performed at room temperature with a buffer solution of 50 mM Tris-HCl (pH 7.6) with 0.1 M NaCl. The protein concentration was varied in a range of 0−119 nM, and the ligand was in a range of 0−10 μM. The stock solutions of cyclosporin A or mimics were dissolved in *N*, *N*-dimethylformamide (DMF) due to low solubility in water. The dilutions of stock solutions were done using the buffer solution. The excitation wavelength was set at 290 nm, the emission range was set from 300 to 400 nm, and the bandwidth was set at 15 nm. Fluorescence readings were taken at the wavelength of maximum emission, generally 345−350 nm. The fluorescence intensity was plotted versus the total concentration of ligands and the K_d values were determined by the nonlinear least squares regression fits of the data to eq 1[10]

$$\Delta F_i/\Delta F_t = \frac{([E]_t + [S]_t + K_d) - (([E]_t + [S]_t + K_d)^2 - 4[E]_t[S]_t)^{1/2}}{2[E]_t} \quad (1)$$

where $[E]_t$ is the concentration of human cyclophilin A and $[S]_t$ is the total concentration of ligand.

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Supporting Information Available: Typical fluorescence plot and table of characterization of compounds **1b**−**f**, **2a**−**b**, **5a**−**b**, **7**, and **8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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