

Supplementary information for: A new perspective on how and why immunophilins ligands work

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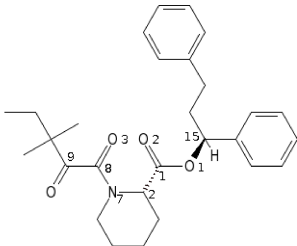
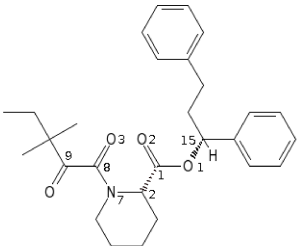
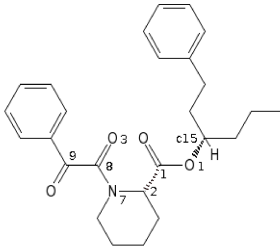
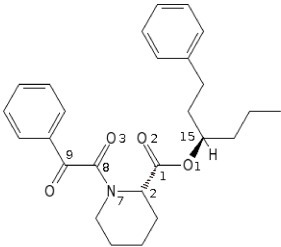
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1. Compounds

In Table 1 we report the chemical formulas of compound n. 10 of ref.¹ ((15S)-sb3), of compound n. 8. of ref.¹ ((15R)-sb3) of (15R)-Elte and of (15S)-Elte. In the following and throughout the paper we use the atom numbering of the (15R)-sb3-FKBP12 complex PDB file 1FKG. On the basis of the results reported on Ref.,² Elte421 was designed from sb3 by exchanging the phenyl moiety at C15 with 1-propyl 3,3 Dimethyl group at C9 (turned to a simple propyl group in Elte421). Carbon 15 labels the stereogenic center in Elte and in sb3 (C2 is fixed in the S configuration in both compounds). The relevant dihedral for optimal FKBP12 binding are ω (C9C8N7C2), ϕ

Table 1: Chemical formula of (1,3-Diphenyl-1-propyl-1-(3,3-Dimethyl-1,2-dioxopentyl)-2-piperidinecarboxylate ((15S)-sb3); (1,3-Diphenyl-1-propyl-1-(3,3-Dimethyl-1,2-dioxopentyl)-2-piperidinecarboxylate ((15R)-sb3); 1-phenylhexan-3-yl 1-(2-oxo-2-phenylacetyl)piperidine-2-carboxylate ((15S)-Elte); 1-phenylhexan-3-yl 1-(2-oxo-2-phenylacetyl)piperidine-2-carboxylate ((15R)-Elte)

(15S)-sb3	(15R)-sb3
	
(15S)-Elte	(15R)-Elte
	

(C9N7C2C1) and ψ (N7C2C1O1). In the complex (15R)-sb3-FKBP12 (PDB id: 1FKG) O2 and O3 are H-bonded to Ile56 and Tyr82, respectively.

2. Synthesis of Elte421

General

All the reactions are monitored by TLC on commercially available precoated plates (silica gel 60 F 254) and the products were visualized with acidic vanillin solution. Silica gel 60, 230-400 mesh, is used for column chromatography, unless otherwise stated. EtP refers to light petroleum, bp 40-60 °C and EtOAc to ethylacetate. ^1H and ^{13}C NMR spectra were recorded at 400 and 100, MHz on a Varian Mercury-400 instrument. FTIR spectra are recorded in CDCl_3 solutions. ESI-mass spectra are measured with a LCQ-FLEET, Thermo Scientific instrument. THF was distilled from sodium in the presence of the blue color of benzophenone ketyl, DCM was distilled from CaH_2 . Commercial available reagents, catalysts and ligands are used as obtained, unless otherwise stated, from freshly opened container without further purifications.

Synthesis of 1-phenylhexan-3-ol (1)¹

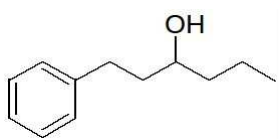


Figure 1: 1-phenylhexan-3-ol (1)

To a solution of 3-phenylpropionaldehyde (3.00 g, 22.36mmol) in 15 mL of dry THF is added dropwise a solution of N-propyl magnesium chloride 2M in ether at -78°C , under a nitrogen atmosphere.³ The reaction mixture is stirred at -50°C . After 4 hours the TLC analysis still shows the spot of the starting material so 20% of N-propyl magnesium chloride 2M in ether is added at -78°C . The reaction is stirred at -10°C for 17 hours, then poured onto 20 mL of saturated solution of

NH₄Cl and warmed at room temperature. The solution is extracted with diethyl ether (3x50 mL), the recollected organic layer is dried over Na₂SO₄, filtered and evaporated to obtain a light yellow oil (3.55 g). The crude is purified with flash chromatography with EtP:AcoEt=10:1 as eluent to obtain a colorless oil (1.56 g) as pure product in 40% yield.

Synthesis of t-butyl 1-phenylhexan-3-yl piperidine-1,2-dicarboxylate (2)

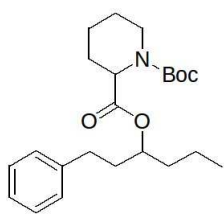


Figure 2: 2

To a solution of alcohol 1 (0.70 g, 3.89 mmol) in 7 mL of dry DCM, N-Boc pipercolic acid (1.78 g, 7.76 mmol), DMAP (0.05 g, 0.39 mmol) and diisopropylcarbodiimide (1.21 mL, 7.76 mmol) are added at 0°C, under a nitrogen atmosphere. A white precipitate is observed. The reaction mixture is stirred at r.t. till the complete consumption of alcohol 1 monitored by TLC to ensure the formation of a 1:1 mixture of diastereoisomeric esters (3 hours), then poured onto 30 mL of diethyl ether and washed with a saturated solution of NH₄Cl, until complete elimination of the urea byproducts, followed by a saturated solution of NaHCO₃ (2x70 mL). The recollected organic layer is dried over Na₂SO₄, filtered and evaporated to obtain a colorless oil (0.90 g) as pure product in 60% yield. ¹H NMR, 400 MHz, CDCl₃, δ: 0.91 (t, 3H, J=7.90 Hz, CH₃); 1.24-1.39 (m, 2H); 1.45 (s, 9H); 1.46-1.63 (m, 6H); 1.78-1.93 (m, 4H); 2.54-2.71 (m, 2H); 2.87-3.08 (m, 1H); 3.88-4.12 (m, 1H); 4.72-4.83 (m, 1H); 4.87-5.05 (m, 1H); 7.14-7.31 (m, 5H). ¹³C NMR, 100MHz, CDCl₃, δ (in the ¹³C NMR spectrum the total number of signals is due the partial superimposition of the carbon chemical shifts of the two rotamers for each diastereoisomer): 13.89; 18.55; 20.76; 24.67; 26.23; 28.33; 31.77; 36.06; 41.10; 42.08; 53.89; 54.87; 74.41; 79.74; 80.31; 125.93; 128.28; 128.40; 141.68; 155.84; 171.78. IR (CDCl₃), cm⁻¹: 1726.88 (m, C=O ester stretch.); 1683.87 (s, C=O carb. stretch.); 1365.59 (m, C-O stretch.); 1275.26 (m, N-CO-O stretch. as); 1043.01 (w,

N-CO-O stretch.).

Synthesis of 1-phenylhexan-3-yl piperidine-2-carboxylate (3)

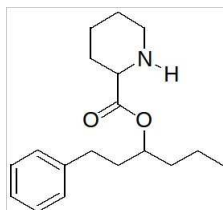


Figure 3: 3

To a solution of derivative 2 (0.12 g, 0.31 mmol) in 3 mL of dry DCM, TFA 99% (0.3 mL) is added dropwise at 0°C. The reaction mixture turns to yellow. The solution is stirred at r.t for 1 hour then poured onto 20 mL of a saturated solution of NaHCO₃ and extracted with DCM (3x40 mL). The organic layer is washed with a saturated solution of NaHCO₃, dried over Na₂SO₄, filtered and evaporated to obtain a yellowish oil (0.80 g) as pure product in 92% yield. ¹H NMR, 400MHz, CDCl₃, δ : 0.90 (t, 3H, J = 7.52 Hz); 1.24-1.39 (m, 2H); 1.42-1.63 (m, 6H); 1.78-1.93 (m, 5H); 2.54-2.71 (m, 3H); 3.01-3.13 (m, 1H); 3.29-3.36 (m, 1H); 4.97-5.05 (m, 1H); 7.14-7.30 (m, 5H). ¹³C NMR, 100MHz, CDCl₃, δ : 13.94; 18.48, 24.14, 25.88, 29.39, 31.74, 35.83, 36.30, 45.68; 58.72; 74.01; 125.86, 128.28, 128.36, 141.61; 173.33. IR (CDCl₃), cm⁻¹: 1722.58 (vs, C=O stretch.); 1602.15 (m, N-H bend.); 1197.84, 1124.73 (s, 2 C-O stretch.).

Synthesis of 1-phenylhexan-3-yl 1-(2-oxo-2-phenylacetyl)piperidine-2-carboxylate (Elte421)

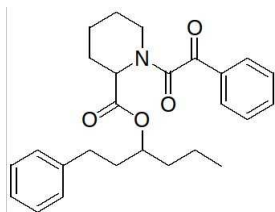


Figure 4: 1-phenylhexan-3-ol (1')

To a solution of amine 3 (0.085 g, 0.3 mmol) in 1 mL of dry DCM, TEA is added (85 μ L, 0.6 mmol) and the solution is cooled at -10°C, under a nitrogen atmosphere. A solution of phenylglyoxyl chloride (0.05 g, 0.3 mmol) is slowly added, dropwise at -10°C, then the reaction mixture is allowed to warm at room temperature and stirred for 4 hours. The solution is poured onto 20 mL of DCM and washed with Brine (3 x 30 mL). The recollected organic phase is dried over Na₂SO₄, filtered and evaporated to obtain a yellowish oil (0.109 g). The crude is purified with flash chromatography with EtP:AcoEt=4:1 as eluent to obtain a colorless oil (0.085 g) as pure product in 68% yield. In the ¹H NMR spectra certain signals indicate the presence of two diastereoisomers (d1 and d2 in 1:1 ratio) and two rotamers (r1 and r2 in 3:1) respectively ¹H NMR, 400MHz, DMSO d₆, δ : 0.76 (t, 3H, J = 8.5Hz, d1 r2); 0.79 (t, 3H, J = 8.5Hz, d2 r2); 0.869 (t, 3H, J = 7.5Hz, d1 r1); 0.874 (t, 3H, J = 7.5Hz, d2 r1); 0.99-1.17 (m, 2H); 1.21-1.40 (m, 2H); 1.39-1.49 (m, 2H); 1.55-1.64 (m, 2H); 1.68-1.80 (m, 3H); 1.84-1.92 (m, 2H); 2.16-2.29 (m, 1H); 2.38-2.45 (m, 2H); 2.51-2.86 (m, 2H); 2.86-2.96 (m, 1H); 3.07-3.17 (m, 1H); 3.33-3.40 (m, 1H); 4.32-4.37 (m, 1H); 4.38-4.44 (m, 1H, d1+d2 r2); 4.78-4.86 (m, 1H, d1+d2 r2); 4.93-5.02 (m, 1H, d1+d2 r1); 5.23 (at, J = 6.25 Hz, d1+d2 r1); 7.05-7.28 (m, 5H); 7.54-7.58 (m, 3H); 7.61-7.80 (m, 3H); 7.89-7.85 (m, 2H, d1+d2 r2); 7.94 (ad, 2H, J = 8.0 Hz, d1+d2 r1). ¹³C NMR, 100MHz, CDCl₃, δ (in the ¹³C NMR spectrum the total number of signals is due the partial superimposition of the carbon chemical shifts of the two rotamers for each diastereoisomer): 13.71;13.97; 18.43; 18.61; 21.21; 22.42; 24.45; 24.91; 26.37; 27.36; 31.75; 35.85; 36.28; 44.27; 51.72; 56.60; 75.39; 125.99; 128.21; 128.37; 129.03; 129.75; 133.19; 134.74; 141.16; 166.77; 167.35; 169.84; 170.10; 191.53; 191.73. IR (CDCl₃), cm⁻¹: 1726.88 (s, OC=O stretch.); 1679.56 (m, NC=O stretch.); 1640.86 (m, PhC=O stretch.). ESI-MS: *m/z* = 865 [2M+Na]⁺; 444 [M+Na]⁺.

¹H NMR spectrum in DMSO: discussion

In Figure 5 we report the ¹H NMR 400 Mhz spectrum of the 1:1 mixture of (15R)-Elte and (15S)-Elte in DMSO at 27° (see Table 1) in the region of the CH₃ group.

One can observe (see Figure 5) two doublets of triplets (corresponding to *trans* and *cis* ω

Figure 5: ^1H NMR 400 Mhz spectrum of Elte421 at 300 K in DMSO in the CH_3 region

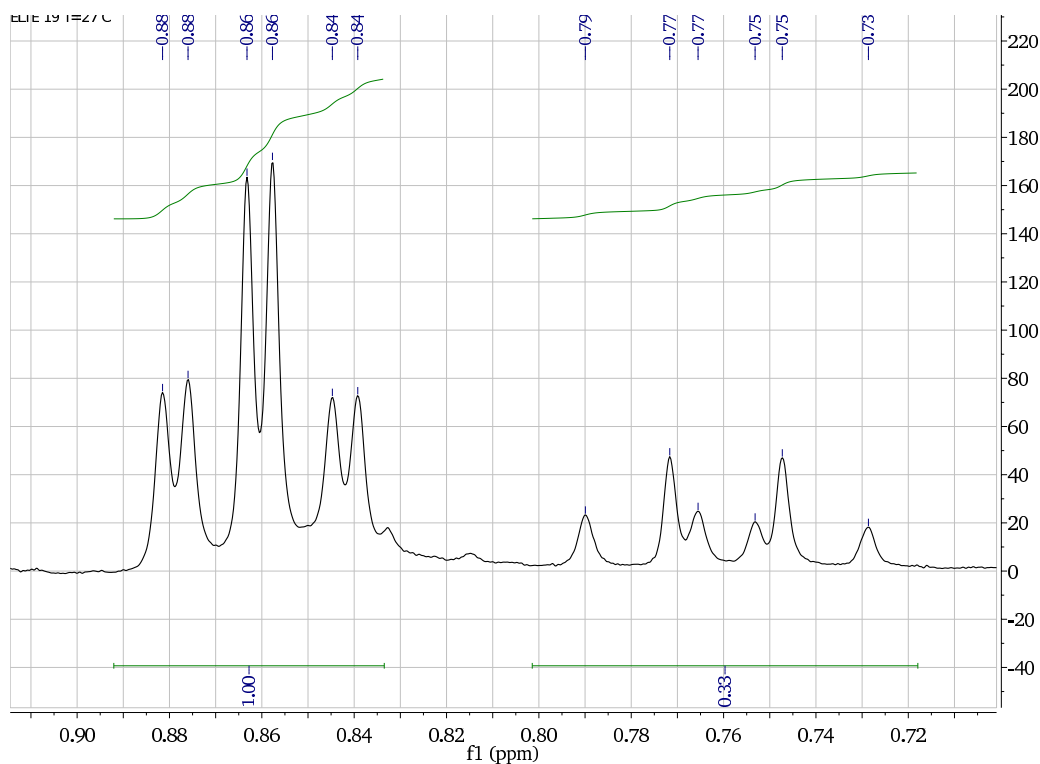
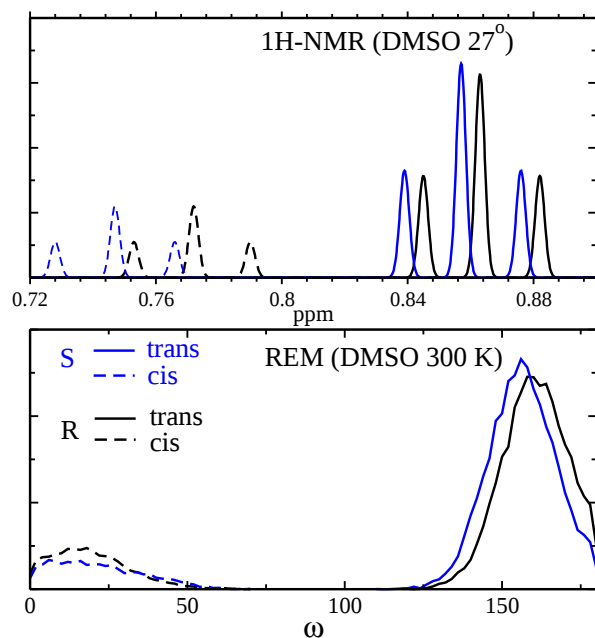


Figure 6: bottom panel: *trans-cis* probability distribution in DMSO at 27 and $P=1$ Atm calculated using REM simulation. Top panel: contributions to the full NMR spectrum in DMSO in the CH_3 region (see Figure 5) from each of the four species present in the mixture



rotamers of the mixture of the diastereoisomers) whose integrals yield the intensity ratio 3:1, indicating that the *trans/cis* ration in DMSO is identical for the diastereoisomers. The NMR spectrum of Elte421 in the CH₃ region can be fitted by combining the the NMR spectra of the four individual species present in the Elte mixture, i.e. the R-*cis* and L-*cis* rotamers and the R-*trans* and L-*trans* rotamers (see Figure 6). Due to the high barriers, the *cis-trans* isomerization in Elte421 follows, in standard conditions, a kinetics that is similar to that of the *cis-trans* isomerization in proline (i.e. *cis-trans* swaps in the order of seconds⁴ per molecule). In order to test the intrasolute force field,² the *cis-trans* equilibrium of (15R)-Elte and (15S)-Elte in DMSO has been also evaluated by REM simulations (methodological details in the next section). In Figure 2 of the Supporting information we show the probability distribution of the dihedral ω in (15R)-Elte and (15S)-Elte evaluated by REM simulations in DMSO yielding *trans/cis* ratio of the order of 4:1, in reasonable agreement with the 3:1 ratio of the experimental data.

3. Computational details of the Solute Tempering Replica Exchange (ST-REM) simulations of Elte421 in water solution and in DMSO solution

Force fields of the ligands

The force field for (15R)-Elte and (15S)-Elte is based on the AMBER03 parametrization.^{2,5} The atomic charges were determined using Density Functional Theory (DFT) with the B3LYP exchange-correlation functional⁶ and a 6-31Gd split valence basis set by evaluating on the optimized geometry a gridded electrostatic potential (ESP) according to the Merz-Singh-Kollman scheme.⁷ Equivalent charges (e.g. charges on the hydrogen atoms of methyl groups) have been symmetrized.

Simulations in bulk water

The ST-REM simulation for each ligand in bulk water was performed using the Hamiltonian REM approach as enforced in the program ORAC.^{2,8} Only the intraligand potential (torsional and non bonded) was scaled throughout the replica progression with a maximum and minimum scaling factors (1.0, 0.15) corresponding to a “temperature” of 300 K and of 2000 K, respectively. The potential energy for the k -th replica is then given by $V_k = V + (c_k - 1)V_T^{[\text{ligand}]}$ where V is the full potential energy of the system, $V_T^{[\text{ligand}]}$ is the intraligand potential and c_k is the corresponding scaling factor. Such a solute tempering approach,⁹ by reducing the degrees of freedom that are involved in the scaling in the generalized ensemble (i.e. only those affected by the proper, improper torsions and non bonded interactions *within* the ligand), allows to minimize, for a given replica exchange rate, the number of replicas needed in the given scaling range (300-2000 K) while maintaining at same time a high flexibility of the ligand in the “hot” replicas. Eight replicas were distributed in the given 300-2000 K range according to scheme given in Ref.¹⁰ The starting REM configuration for (15R)-Elte and (15S)-Elte in bulk water was prepared in a cubic box in the NPT ensemble as described in Ref.² The samples contained 499 and 501 TIP3 water molecules with a mean box length of 25.156 Å and 25.182 Å, for (15R)-Elte and (15S)-Elte respectively. The electrostatic interaction were treated using the Smooth Particle Mesh Ewald method,¹¹ with 20 grid points in each directions and a 4-th order B-spline interpolation. The equations of motion were integrated using a multiple time step r-RESPA algorithm¹² with a potential subdivision specifically tuned for biomolecular systems.^{13,14} For each ligand, the (Hamiltonian) REM simulations were conducted in the NPT ensemble with isotropic stress tensor¹³ at T=300 K and P=1 Atm, and lasted 18 ns for a total simulation of 144 ns in the generalized ensemble. The mean acceptance exchange ratio in both simulations resulted in the range 0.2-0.35.

Simulation of (15R)-Elte and (15S)-Elte in DMSO

REM simulations of (15R)-Elte and (15S)-Elte in DMSO were performed using an intrasolute scaling range of 1.0-0.2 (300-1500 K). The force field for DMSO was taken from ref.¹⁵ The cubic

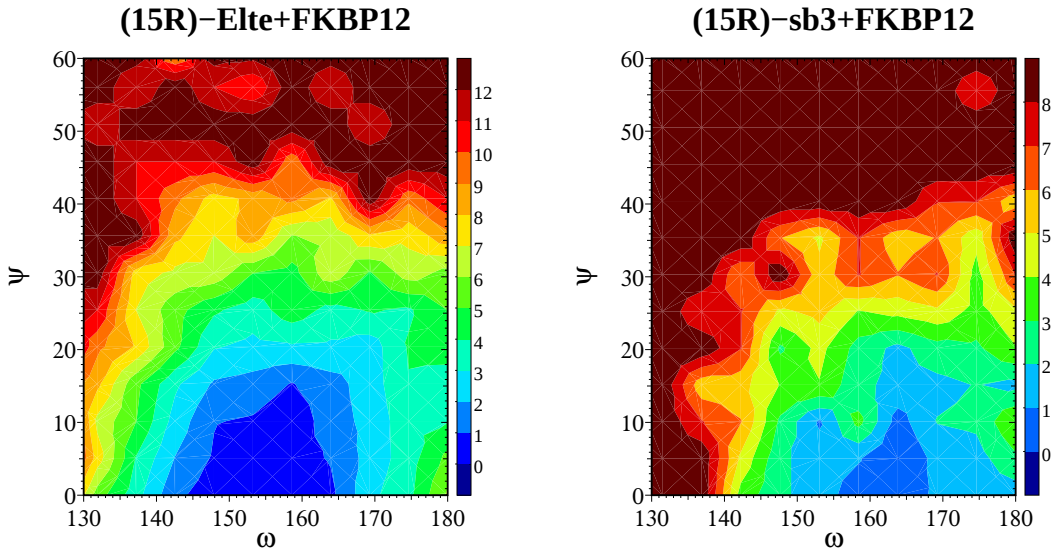
box contained in both cases 112 DMSO molecules with mean side length of 24.166 for (15S)-Elte and (15R)-Elte. The 18 ns REM simulations in DMSO (total time in the generalized ensemble of 144 ns) had a mean acceptance exchange ratio in the range 0.3-0.5.

Simulation of (15S)-Elte-FKBP12 and (15R)-sb3-FKBP2

In order to investigate the conformational distribution of the $\omega\psi$ angles of the binding domain of Elte421 and that of the parent compound sb3 when bound to the FKBP12 protein, we have simulated the complexes (15S)-Elte-FKBP12 and (15R)-sb3-FKBP12 by means of solute tempering replica exchange. The protein structure was taken from the protein data bank structure with PDB code 1FKG. The starting structure for the ligand in the complex was obtained by minimizing *in vacuo* the ligand only by conjugate gradient with the restraint involving the two H-bonds in the primary binding site, namely C8O3(ligand)-OH(Tyr82) and C1O2(ligand)-HN (Ile56). Explicit water was added in a triclinic box ($a=52.5$ Å, $b=53.7$ Å, $c=52.5$ Å, $\alpha=73.2^\circ$, $\beta=69.7^\circ$, $\gamma=80.1^\circ$) chosen so as to minimize the volume of the MD cell with the protein in the 1FKG orientation. The solute tempering protocol allows the scaling of the torsional and 14 non bonded potential for the ligand and for all atoms of the protein that are within a 7 Å distance from any atom of the ligand in the minimized starting configuration. The ligand is maintained in the primary site using a harmonic potential (force constant of 1 Kcal mol⁻¹ Å⁻²) involving the pair C8O3(ligand)-OH(Tyr82) and C1O2(ligand)-HN (Ile56). The electrostatic interaction were treated using the Smooth Particle Mesh Ewald method,¹¹ with 36 grid points in each directions and a 4-th order B-spline interpolation. The equations of motion were integrated using a 5 time r-RESPA algorithm described in Ref.¹³ Simulations were conducted in the NPT ensemble with isotropic stress tensor at T=300 K and P=1 Atm.¹³ 16 replicas were used for the runs with a torsional and 14 non bonded scaling factors ranging from 1 (the target replica) to 0.3. Replica exchanges were attempted every 0.25 ps obtaining an exchange rate in the range 30-40%. Each of the two simulations last for 4 ns per replica. In Figure 7 we show the free energy surface with respect to the ω and ψ coordinates (see definitions in Figure 1 of the paper) calculated at the target replica for the (15S)-Elte-FKBP12 and

(15R)-sb3-FKBP12 complexes. As expected, both ligands exhibit a comparable and rather limited

Figure 7: Free energy surface with respect to the dihedral angles (expressed in degrees) ω and ψ of (15S)-Elte and (15R)-sb3 in complex with FKBP12 at T=300 and P=1 Atm calculated via solute tempering REM. The energy scale (right vertical bar) is expressed in kJ mol^{-1}



$\omega - \psi$ conformational accessible space, with $130 < \omega \leq 180^\circ$ and $0 < \psi < 60^\circ$.

Clustering analysis

In order to analyze the conformational states of FKBP12 ligands we have classified the PDB structures according to the quality threshold (QT) clustering algorithm.¹⁶ For a N heavy atoms ligand, conformational clusters are grouped according to all possible interatomic $N(N - 1)/2$ distances. The distance between a point and a group of points is computed using complete linkage, i.e. as the maximum distance from the point to any member of the group. In the table below we collect the data obtained from QT clustering analysis for (15R)-Elte and (15S)-Elte in water and in DMSO. In table Table 2 we report the salient data obtained for the clustering analysis in R/E-Elte in water and DMSO.

Figure 8: Representative elements of the various structures in bulk water and DMSO solution of (15S)-Elte (p-r: propyl-ring; open: open structure; p-r c-r: propyl-ring piperidine-ring) and of (15R)-Elte (r-r(stacked) c-p: ring-ring piperidine-propyl; r-r: ring-ring(stacked))

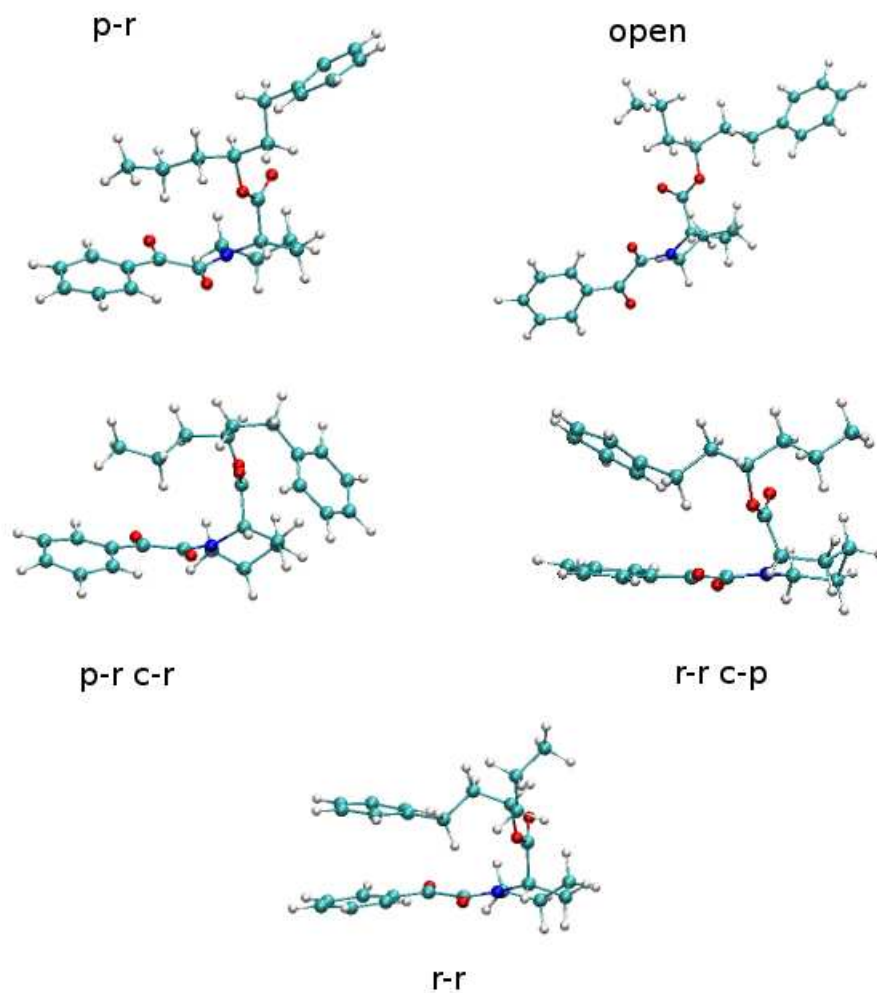


Table 2: Cluster analysis for (15R)/S-Elte in water and in DMSO at T=300 K and P=1 Atm, as obtained from the 18 ns of REM simulations. For each species we report the five more populated clusters (complete linkage distance threshold of 3 Å). In all cases these clusters account for more than 50% of all sampled structures. P_i indicates the cluster probability. CG indicates the mean conformational geometry of the cluster: *open* (open structures); *p-r* (propyl-phenyl interactions); *r-r* (phenyl- 3 propylphenyl interactions); *c-p* (piperidine ring-propyl interaction); *c-r* (piperidine ring-3 propylphenyl interaction)

	water				DMSO			
	(15S)-Elte		(15R)-Elte		(15S)-Elte		(15R)-Elte	
n.	P_i	CG	P_i	CG	P_i	CG	P_i	CG
1	0.16	p-r	0.27	r-r	0.47	open	0.42	open
2	0.13	open	0.10	r-r c-p	0.15	p-r	0.10	p-r
3	0.11	p-r c-r	0.08	p-r c-r	0.05	open	0.10	open
4	0.10	p-r c-r	0.07	p-r	0.05	open	0.05	p-r c-r
5	0.08	r-r c-p	0.06	r-r c-p	0.04	open	0.04	r-r c-p

In water compact structures are mainly found while in DMSO open structures with hydrophobic groups pointing away from each other prevail. In (15S)-Elte the prevailing intraligand interactions are of the propyl-phenyl type as in (15R)-sb3 (data not shown). In (15R)-Elte $\pi - \pi$ stacking is the most important intraligand interaction. (15R)-Elte has only a minor conformational population exhibiting the optimal binding configuration of the carbonyl units (see Figure 2 of the communication). The stacking between the two phenyl rings in (15S)-Elte does not therefore produce configuration with optimal ω and ψ angles as it does the propyl-phenyl interaction in (15S)-Elte. Elimination of the stereogenic carbon by eliminating the propyl moiety on the C15 carbon has been found to increase significantly the instability constant[US patent n. 5696135], showing that the propyl group in Elte is crucial for the FKBP12 affinity of the ligand. Starting from Elte, we can envisage an alternative elimination of the chirality in Elte by, e.g., *addition* of a propyl group at carbon 15.

4. Chemical equilibria

Dissociation constant for the *cis/trans* R/S mixture

cis/trans isomerism

The individual instability constant of the *cis/trans* isoform and the overall apparent instability constant of the *cis/trans* mixture of Elte in presence of the FKBP12 protein are given by

$$K_c = \frac{[E_c][P]}{[PE_c]}; \quad K_t = \frac{[E_t][P]}{[PE_t]}; \quad K_m = \frac{([E_c] + [E_t])[P]}{([PE_c] + [PE_t])} \quad (1)$$

with $[E_{c/t}]$, $[P]$ being the concentrations of free *cis* and *trans* rotamers and protein in solution. The *cis/trans* isomers obey the equilibrium $K = [E_t]/[E_c]$. Combining the above equations, the apparent instability constant of either (15S)-Elte or (15R)-Elte can be written as

$$K_m = \frac{K_t(1+K)}{K + K_t/K_c}. \quad (2)$$

In the assumption that $K \gg K_c$, $K \gg K_t$ and $K_t \ll K_c$, we obtain that

$$K_m = K_t \frac{1+K}{K}, \quad (3)$$

i.e. the observed K_m is essentially equal to the lowest instability constant K_t .

Effective dissociation constant of Elte421 (1:1 (15R)-Elte and (15S)-Elte mixture)

In this study we always work in condition of excess FKBP12 protein concentration ($1\mu\text{M}$). We therefore define for (15R)-Elte and (15S)-Elte the individual dissociation constants as

$$K_R = \frac{(C-x)C_p}{x} \quad K_S = \frac{(C-y)C_p}{y} \quad (4)$$

where x and y are the concentration of *free* R and S diastereoisomers, respectively, C_p is the protein excess concentration and C is the overall concentration of each of the diastereoisomers. In general, one of the two diastereoisomer will bind FKBP12 better than the other. With no loss of generality, we shall assume that $K_S > K_R$. In the 1:1 (15S)-Elte (15R)-Elte mixture, the effective dissociation constant is given by

$$K_{\text{eff}} = \frac{(2C - x - y)C_p}{x + y} = K_R \frac{x}{x + y} + K_S \frac{y}{x + y} \quad (5)$$

Using Eq. 4 to eliminate x and y from Eq. 5, we arrive at

$$K_{\text{eff}} = \frac{K_R(K_S + C_p) + K_S(K_R + C_p)}{K_S + K_R + 2C_p} \quad (6)$$

Defining $K_S = zK_R$ with $z > 1$, we finally obtain the effective dissociation constant against the ratio K_S/K_R , i.e.

$$\frac{K_{\text{eff}}}{K_R} = \frac{(2K_R + C_p)z + C_p}{K_R z + 2C_p + K_R} \quad (7)$$

If we assume that $C_p \gg zK_R$, we then find that $K_{\text{eff}} \simeq K_S/2$, i.e. the measured effective constant of the *1:1 mixture* of the two diastereoisomers is approximately equal to one half of the *larger* dissociation constant.

5. Fluorescence Emission and Excitation spectra

Experimental details

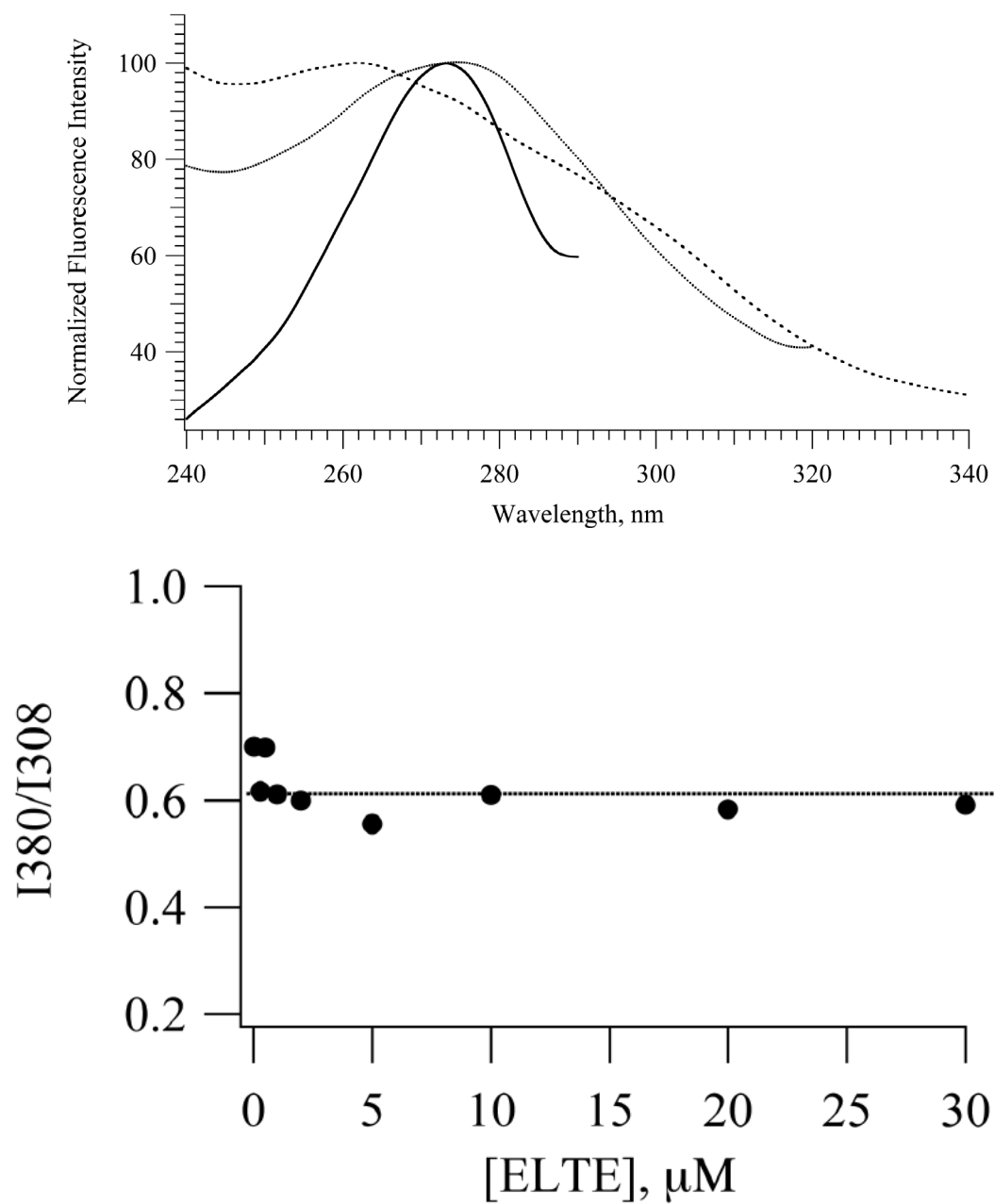
FKBP12 (expressed in *Escherichia coli*, M.W. 11000) and FK506 (M.W. 804.02) were supplied by Sigma-Aldrich (Italy). The purity of FKBP12 was greater than 90% determined by SDS electrophoresis; purity of FK506 was greater than 98% (obtained by HPLC). DMSO, NaCl, K_2HPO_4 and KH_2PO_4 were obtained from Sigma-Aldrich (Italy). Water (resistivity = 18 M Ω cm, pH = 5.6 at 20 °C) was obtained from a Milli-RO coupled with a Milli-Q set up (Millipore, Italy). Absorption spectra were recorded on a Lambda900 spectrophotometer (Perkin Elmer, Italy) with cuvette

cell holder connected to a thermostatic bath. Fluorescence spectra were recorded on a PerkinElmer LS 50B luminescence spectrometer, excitation and emission slits were set to 10 or 15 nm depending on the optical path length of cell employed. Emission measurements were performed using different excitation wavelengths in the range 270-310 nm, excitation spectra were run at $\lambda_{em} = 310$ nm, 350 and 380 nm. QS cell with 0.3 cm optical path length from Hellma (Hellma GmbH&Co. KG, Mtlheim, Germany) were used for quenching experiments, whereas 1 cm OP cuvettes were used for absorption measurements and single component solutions. Cuvettes were cleaned with piranha solution and carefully rinsed with water and ethanol. The cuvettes were dried by nitrogen flushing prior to each measurement. Fluorescence measurements were run at 15 °C, a temperature probe was inserted in the cuvette to ensure optimal temperature control. Stock FKBP12 concentration in PBS buffer (pH 7.4, 0.15 M NaCl) was determined by UV/Vis ($\epsilon_{280}=9970 \text{ M}^{-1}\text{cm}^{-1}$), aliquots of this solution were used to prepare adequate concentrations for quenching experiments by dilution with standard phosphate buffered saline. The molar extinction coefficient of Elte421 ligand in DMSO was obtained from a dilution series in the linear Lamber-Beer regime and resulted to be $\epsilon_{260}=16446 \text{ M}^{-1} \text{ cm}^{-1}$.

Binding assay

Fluorescence quenching assays were performed measuring the decrease of intrinsic tryptophan fluorescence at 330 nm as a function of ligand concentration. The final FKBP12 concentration used for all measurements was 1.09 μM unless otherwise specified in the text, the desired ligand concentration in the sample was obtained by appropriate dilution of ligand stock solution. Ligand concentrations in the range 10 nM to 400 nM were used, with a final DMSO concentration in the sample lower than 0.5%. Data points are the average of at least two separate experiments, emission spectra of the corresponding FKBP12-free samples were subtracted from each recording of fluorescence spectra. The apparent dissociation constant for ligands was calculated from the fluorescence intensity results, the quenching data were analyzed as previously described.¹⁷ Fluorescence measurements for low ligand concentrations, i.e. in a concentration range where the

Figure 9: Top: Excitation spectra of Elte421 in PBS at $\lambda_{em} = 310$ nm (solid line), $\lambda_{em} = 350$ nm (dotted line), $\lambda_{em} = 380$ nm (dashed line); Bottom: Intensities ratio I_{380}/I_{308} obtained from the emission spectra of Elte421 in PBS as a function of Elte421 concentration ($\lambda_{exc} = 270$ nm)



experimental errors are larger, were repeated three/four times whereas for higher concentration fluorescence results were run twice if identical readings were obtained. The measurements were repeated starting from different concentrations of the protein stock solution, moreover four different FKBP12 lots provided by the supplier were checked to account for different sample quality. In figure Figure 10 we show the relative fluorescence decrease at 330 nm of FKBP12 with FK506 and Elte421 along with their experimental errors.

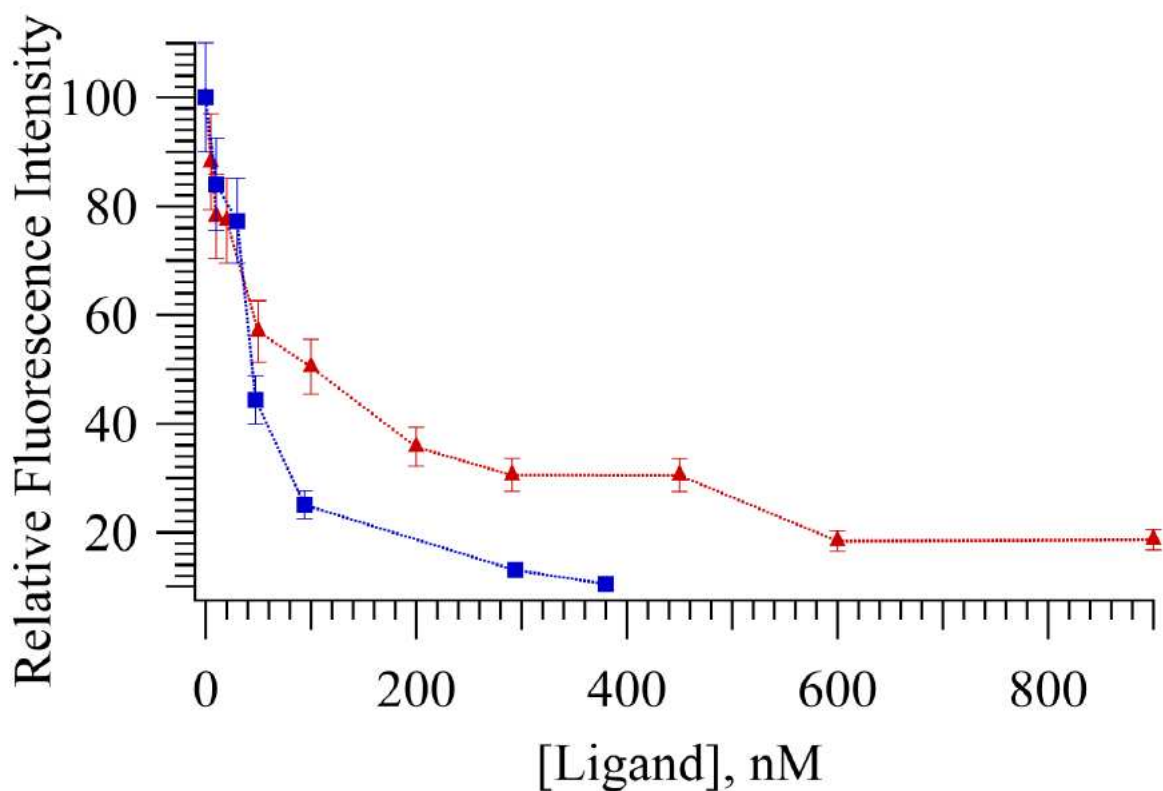


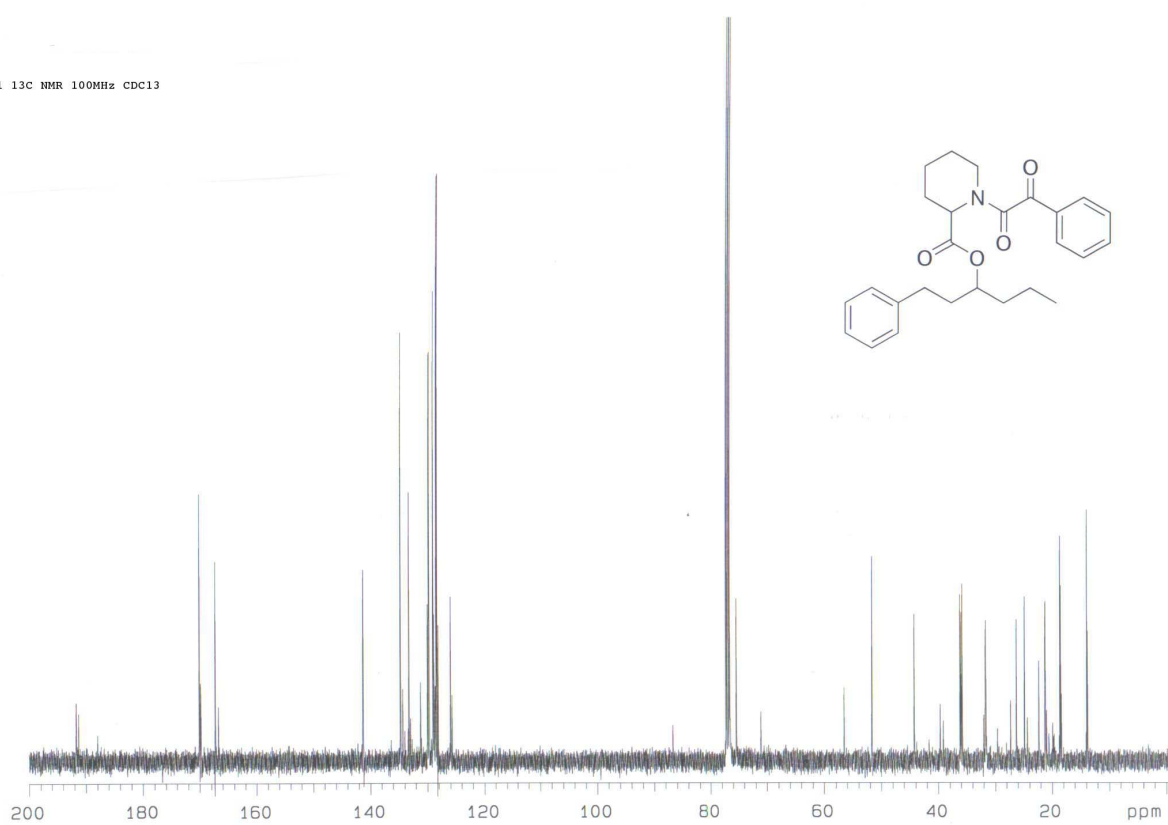
Figure 10: Relative fluorescence intensity decrease of FKBP12 as a function of ligand concentration: FK506 (squares), Elte421 (triangles). Errors bars for each concentration are also reported

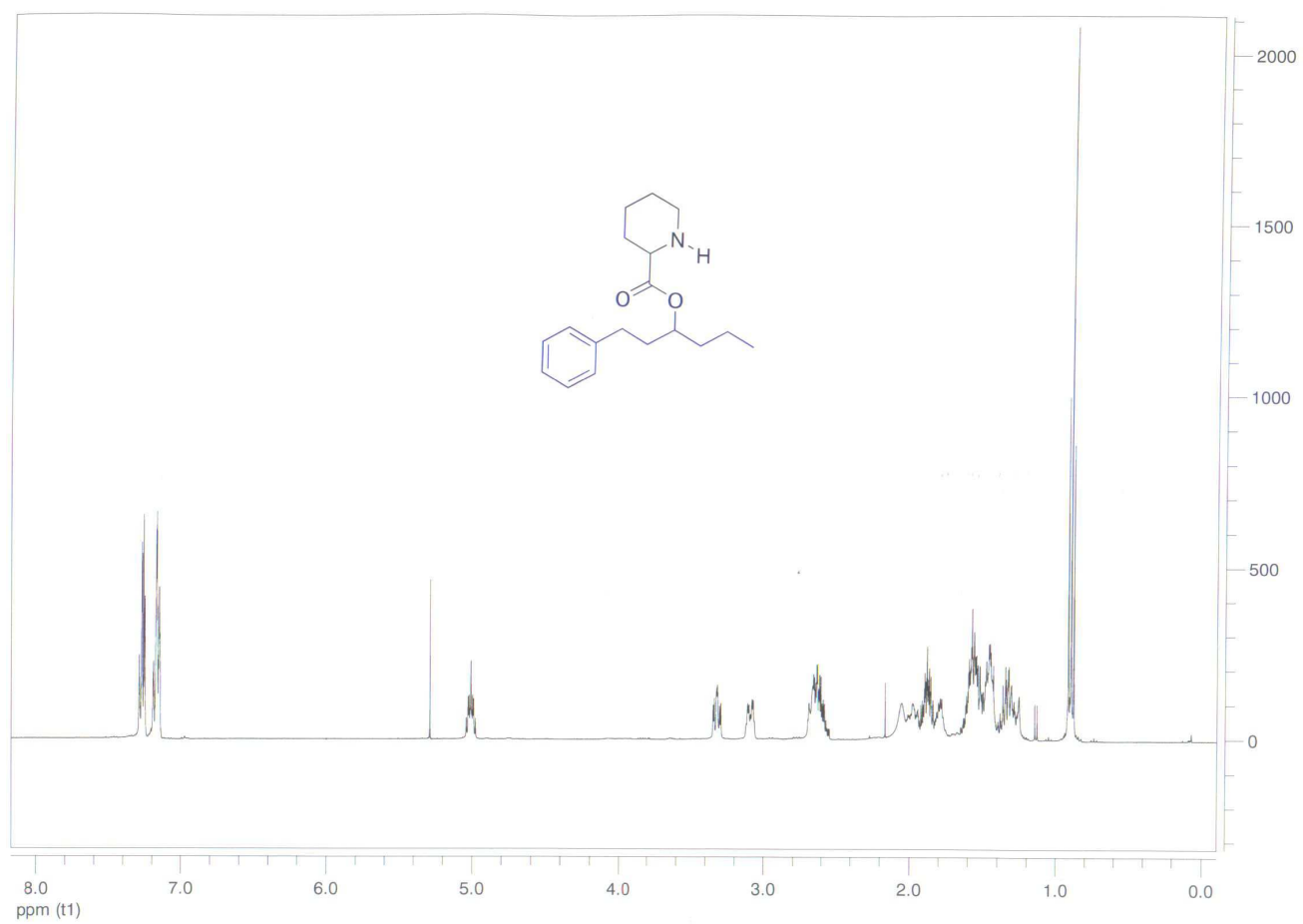
ELTE421 1H NMR 400 MHz DMSOd

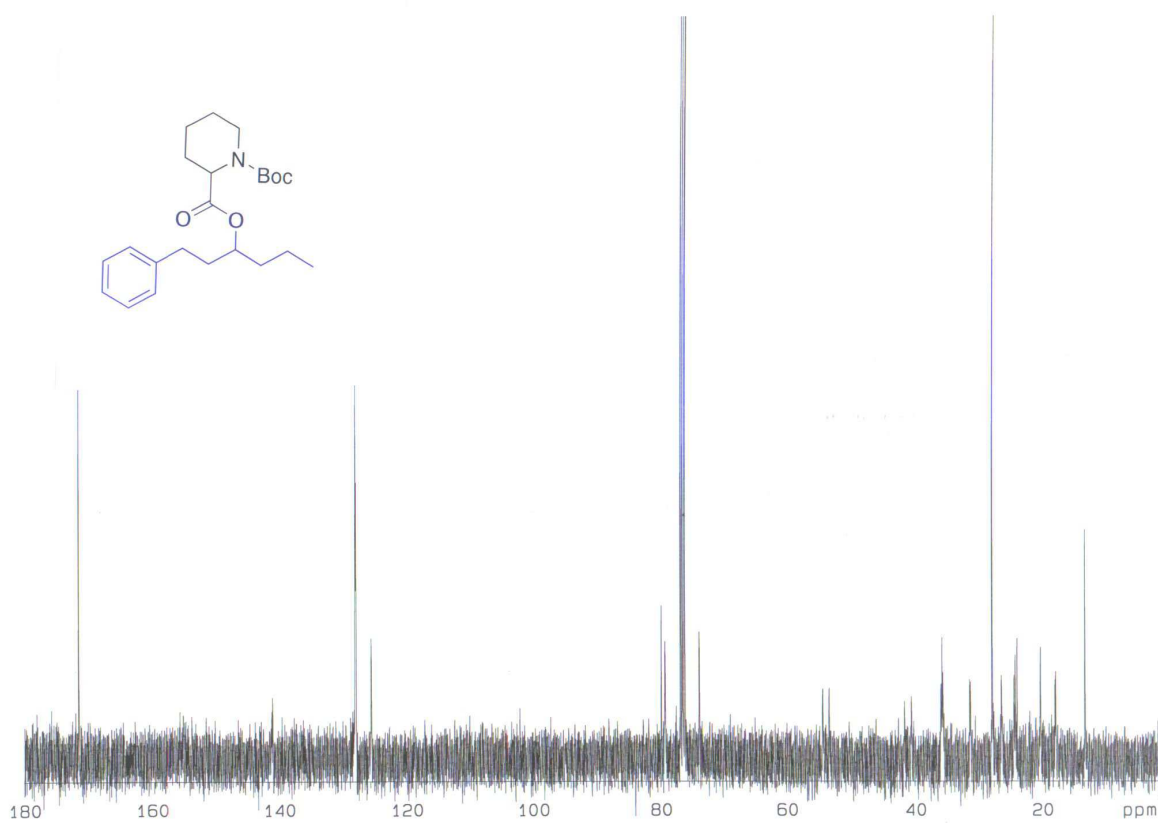
CCCC(=O)OC(=O)N1CCCCC1C(=O)OCCc2ccccc2

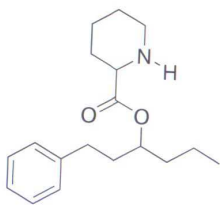
Chemical Shift (ppm)	Integration
7.75	1.00
7.65	1.00
7.55	1.00
7.45	1.00
7.35	1.00
7.25	1.00
5.45	1.00
5.25	1.00
5.15	1.00
4.95	1.00
4.85	1.00
4.75	1.00
4.65	1.00
4.55	1.00
4.45	1.00
4.35	1.00
4.25	1.00
4.15	1.00
4.05	1.00
3.95	1.00
3.85	1.00
3.75	1.00
3.65	1.00
3.55	1.00
3.45	1.00
3.35	1.00
3.25	1.00
3.15	1.00
3.05	1.00
2.95	1.00
2.85	1.00
2.75	1.00
2.65	1.00
2.55	1.00
2.45	1.00
2.35	1.00
2.25	1.00
2.15	1.00
2.05	1.00
1.95	1.00
1.85	1.00
1.75	1.00
1.65	1.00
1.55	1.00
1.45	1.00
1.35	1.00
1.25	1.00
1.15	1.00
1.05	1.00
0.95	1.00
0.85	1.00
0.75	1.00
0.65	1.00
0.55	1.00
0.45	1.00
0.35	1.00
0.25	1.00
0.15	1.00
0.05	1.00

ELTE421 13C NMR 100MHz CDC13









References

- (1) Holt, D. A.; Luengo, J. J. I.; Yamashita, D. S.; Oh, H. J.; Konialian, A. L.; Yen, H. K.; Rozamus, L. W.; Brandt, M.; Bossard, M. J.; Levy, M. A.; Eggleston, D. S.; Liang, J.; Schultz, L. W.; Stout, T. J.; Clardy, J. *J. Am. Chem. Soc.* **1993**, *115*, 9925–9938.
- (2) Bizzarri, M.; Marsili, S.; Procacci, P. *J. Phys. Chem. B* **2011**, *115*, 6193–6201.
- (3) Shi, L.; Tu, Y.-Q.; Wang, M.; Zhang, F.-M.; Fan, C.-A.; Zhao, Y.-M.; Xia, W.-J. *J. Am. Chem. Soc.* **2005**, *127*, 10836–10837.
- (4) Jacobson, J.; Melander, W.; Vaisnys, G.; Horvath, C. *J. Phys. Chem.* **1984**, *88*, 4536–4542.
- (5) Duan, Y.; Wu, C.; Chowdhury, S.; Lee, M. C.; Xiong, G.; Zhang, W.; Yang, R.; Cieplak, P.; Luo, R.; Lee, T.; Caldwell, J.; Wang, J.; Kollman, P. *J. Comp. Chem.* **2003**, *24*, 1999–2012.
- (6) Stephens, P. J.; Devlin, F. J.; Chablowski, C. F.; Frisch, M. *J. Phys. Chem.* **1994**, *98*, 11623–11627.
- (7) Singh, U. C.; Kollman, P. A. *J. Comput. Chem.* **1984**, *5*, 129–145.
- (8) Marsili, S.; Signorini, G. F.; Chelli, R.; Marchi, M.; Procacci, P. *J. Comp. Chem.* **2010**, *31*, 1106–11161.
- (9) Liu, P.; Kim, B.; Friesner, R. A.; Berne, B. J. *Proc. Acad. Sci.* **2005**, *102*, 13749–13754.
- (10) Fukunishi, H.; Watanabe, O.; Takada, S. *J. Chem. Phys.* **2002**, *116*, 9058–9067.
- (11) Essmann, U.; Perera, L.; Berkowitz, M. L.; Darden, T.; Lee, H.; Pedersen, L. G. *J. Chem. Phys.* **1995**, *101*, 8577–8593.
- (12) Tuckerman, M.; Berne, B. J.; Martyna, G. *J. Chem. Phys.* **1992**, *97*, 1990–2001.
- (13) Marchi, M.; Procacci, P. *J. Chem. Phys.* **1998**, *109*, 5194–5202.
- (14) Procacci, P.; Paci, E.; Darden, T.; Marchi, M. *J. Comp. Chemistry* **1997**, *18*, 1848–1862.

- (15) Gervasio, F. L.; Chelli, R.; Procacci, P.; Schettino, V. *J. Phys. Chem. A* **2002**, *106*, 2945.
- (16) Heyer, L. J.; Kruglyak, S.; Yooseph, S. *Genome Res.* **1999**, *9*, 1106–1115.
- (17) Roehrig, C. H.; Loch, C.; Guan, J.-Y.; Siegal, G.; Overhand, M. *Chem. Med. Chem.* **2007**, *2*, 1054–1070.