SUPPORTING INFORMATION

Light-regulation of protein dimerization and kinase activity in living cells using photocaged rapamycin and engineered FKBP

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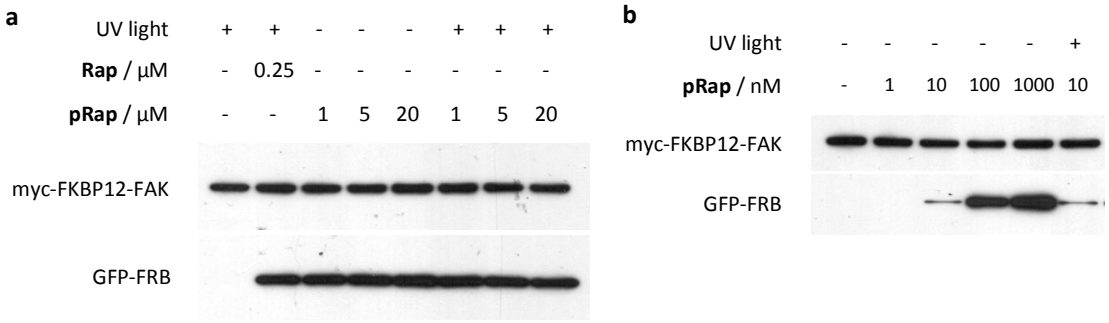
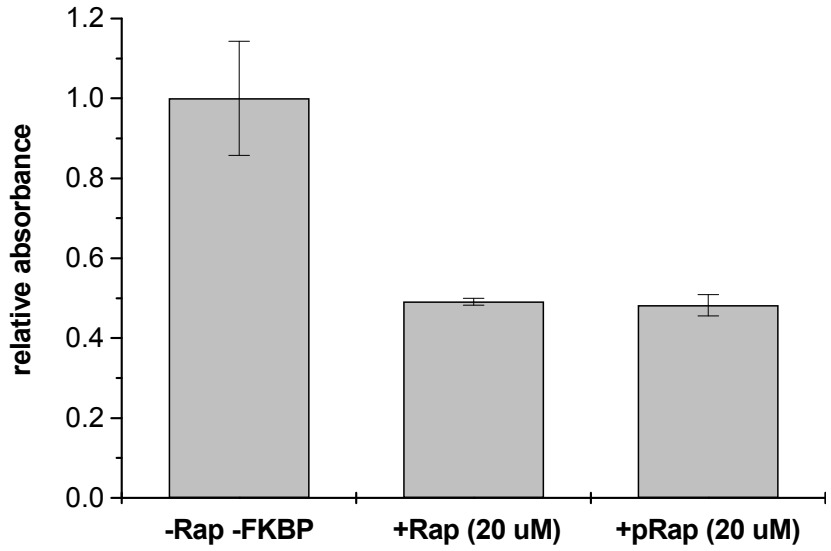
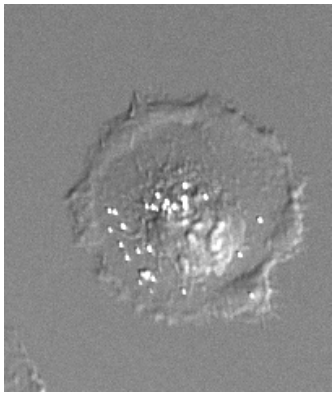
SUPPORTING FIGURES AND MOVIES



Supporting Figure 1. Light-regulated dimerization between FKBP12 and FRB. HEK293T cells co-transfected with myc-FKBP12-FAK and GFP-FRB constructs were treated with either the indicated amount of **pRap** or 0.5 μM of **Rap**. Ten minutes after small molecule addition, the cells were irradiated with UV light (365 nm) for 1 min (a) or 5 min (b) and incubated for 1 hour. Control cells were not irradiated. Myc-iFKBP-FAK was immunoprecipitated from cell lysates by using an anti-myc antibody and co-immunoprecipitation of GFP-FRB was detected by Western blot using an anti-GFP antibody.

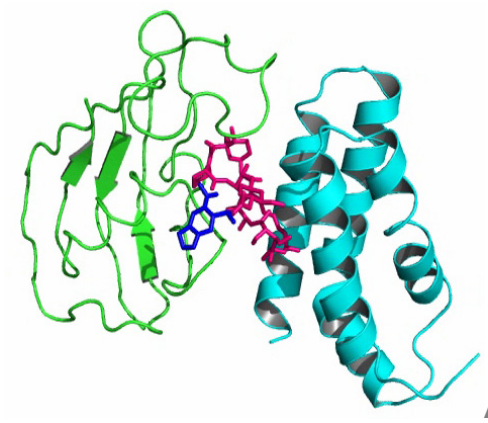


Supporting Figure 2. K-LISA™ mTor activity assay (EMD Biosciences) of rapamycin (**Rap**) and caged rapamycin (**pRap**). The K-LISA™ mTor Activity Kit is an ELISA-based activity assay that utilizes a p70S6K-GST fusion protein as a specific substrate of the kinase mTor. The mTor substrate is first bound to a glutathione-coated 96-well plate followed by incubation with ATP and mTor protein. Active mTOR phosphorylates p70S6K at Thr389 and the phosphorylated substrate is bound by an anti-p70S6K-T389 antibody, followed by detection with a horseradish peroxidase (HRP)-antibody conjugate through conversion of the substrate 3,3',5,5'-tetramethylbenzidine (TMB). Relative activity of mTor is determined by reading the absorbance at dual wavelengths of 450/540 nm or 450/595 nm. The formation of the ternary complex between FKBP, **Rap**, and mTor inhibits its activity. In the absence of **Rap** and FKBP, maximum mTor kinase activity is observed. The mTor activity is reduced to the same level in the presence of either **Rap** or **pRap**, indicating that introduction of the caging group at C-40 does not disrupt formation of the ternary complex between mTor, FKBP, and **pRap**. The assay was conducted according to the manufacturer's instructions and absorbance was measured on a SpectraMax 384 plus plate reader (Molecular Dynamics). The error bars represent standard deviations from two independent assays.



Available on the J. Am. Chem. Soc. website.

Supporting Movie 1. Light-induced activation of RapR stimulates formation of dorsal protrusions. HeLa cells co-transfected with GFP-RapR-FAK and mCherry-FRB were imaged 15 minutes before addition of **pRap** (1μM), 55 minutes after addition of **pRap** and before 365 nm UV light irradiation, and 170 min after UV irradiation (1 min, 365 nm). Images were taken every minute.



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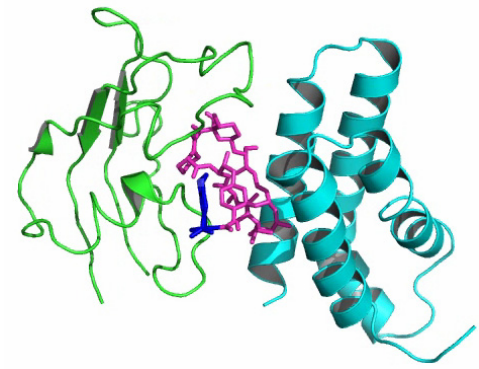
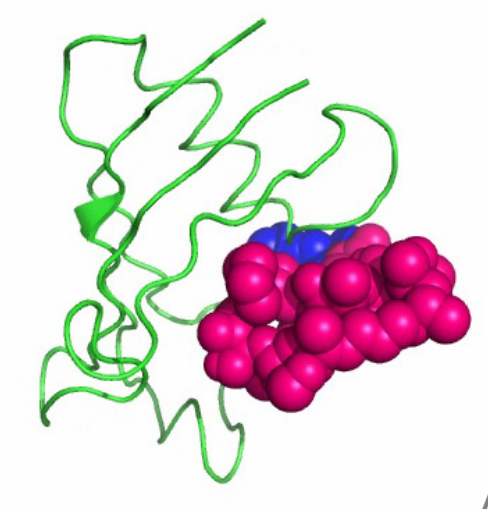
Supporting Movie 2. Dynamics of FKBP12+caged-RAP+FRB. FKBP is shown in green, FRB in cyan, and **pRap** in pink (with the caging group in blue). FKBP12 maintains its conformation and contact interface with FRB.



Available on the J. Am. Chem. Soc. website.

Supporting Movie 3. Dynamics of iFKBP+**pRap**+FRB. Color scheme is the same as in Movie Supporting Movie 1. iFKBP undergoes a large conformational change and looses its interface with FRB, but retains its contacts with **pRap**.



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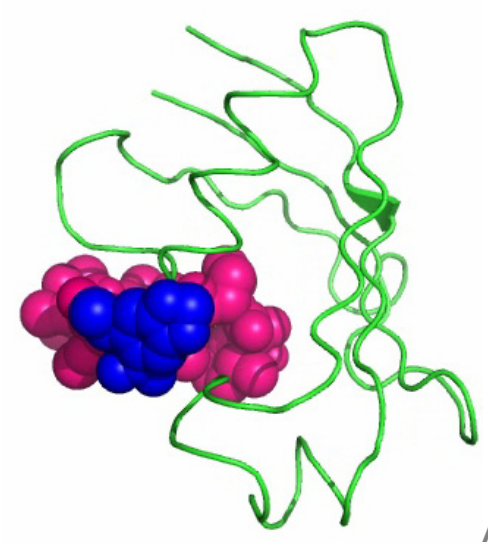
Supporting Movie 4. Dynamics of iFKBP (green ribbon) in complex with **pRap** (shown with spheres) in the absense of FRB. The **pRap** position is fixed for visual purposes. iFKBP undergoes a large conformational change to increase the area of the interface with the **pRap** molecule.



Available on the J. Am. Chem. Soc. website.

Supporting Movie 5. Sideview of movie 4.

EXPERIMENTAL PROTOCOLS

Synthesis of caged rapamycin (pRap):

α-Methyl-6-nitropiperonylol succinimidyl carbonate (MeNPOC-NHS). 1-(3,4-(Methylenedioxy)-6-nitrophenyl)ethanol was synthesized as reported (Lusic, H.; Deiters, A., *Synthesis* **2006**, 2147-2150) and 200 mg (0.947 mmol) were dissolved in 5 ml of dry CH_3CN. To the solution were added *N,N'*-disuccinimidyl carbonate (485 mg, 1.894 mmol) and TEA (0.396 ml, 2.840 mmol). The reaction was stirred at rt overnight. The solvent was removed under reduced pressure and the product was directly purified by column chromatography on SiO_2 (eluted with hexanes/Ethyl acetate 5:1), delivering 296 mg (89% yield) of **MeNPOC-NHS** as a light yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.49 (s, 1H), 7.09 (s, 1H), 6.39 (q, *J* = 6.4 Hz, 1H), 6.14-6.12 (m, 2H), 2.79 (s, 4H), 1.73 (d, *J* = 6.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.7, 153.0, 150.8, 148.0, 141.6, 133.2, 105.9, 105.6, 103.5, 76.5, 25.6, 22.3; HRMS calcd $C_{14}H_{12}N_2NaO_9$ 375.04405, found 375.0430.

MeNPOC-caged rapamycin (pRap). Under argon, rapamycin (**Rap**) (20.0 mg, 0.0219 mmol) was dissolved in dry DCM (0.6 ml). DMAP (5.4 mg, 0.0438 mmol) and **MeNPOC-NHS** (39.0 mg, 0.109 mmol) were added. The reaction mixture was stirred at rt for 24 h, the volatiles were evaporated, and the product was purified by column chromatography on SiO_2 (eluted with DCM/ethyl acetate 10:1, 5:1, 2:1, 1:1), delivering 9.1 mg (36% yield) of **pRap** as a light yellow solid. No attempts were made to separate the two generated diastereomers. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (s, 1H), 7.05 (s, 1H), 6.44-6.03 (m, 7H), 5.94-5.83 (m, 1H), 5.60-5.47 (m, 1H), 5.43 (m, 1H), 5.38-5.31 (m, 1H), 5.20-5.08 (m, 1H), 4.50-4.38 (m, 1H), 4.14-4.09 (m, 2H), 3.98-3.73 (m, 2H), 3.67-3.55 (m, 2H), 3.35-3.31 (m, 7H), 3.13-3.03 (m, 5H), 2.87-2.50 (m, 3H), 2.40-2.11 (m, 2H), 2.03-1.52 (m, 15H), 1.47-1.32 (m, 6H), 1.24-0.86 (m, 24H); HRMS calcd for $[M + Na]^+$ $C_{61}H_{86}N_2NaO_{19}$ 1173.5723, found 1173.5721.

Antibodies and reagents:

Anti-phospho-paxillin (Tyr31) and anti-GFP antibodies were purchased from Invitrogen. Anti-myc antibodies and IgG-coupled agarose beads were purchased from Millipore. Anti-paxillin antibodies were a gift from Dr. Michael Schaller. Rapamycin was purchased from Sigma.

Molecular biology:

The constructs for myc-tagged and GFP-tagged mouse FAK and FAK variants (FKBP12-FAK, iFKBP-FAK, FAK-iFKBP413 and RapR-FAK), GFP- and mCherry-tagged FRB were described presviously (Karginov, A.V., Ding, F., Kota, P., Dokholyan, N.V. & Hahn, K.M. Engineered allosteric activation of kinases in living cells. Nat. Biotechnol. (2010) in press). The construct expressing the GST-tagged N-terminal fragment of paxillin was a gift from Dr. Michael Schaller.

Immunoprecipitation and kinase assay:

HEK293T cells were co-transfected with myc-tagged FAK constructs and GFP-FRB. Transfected cells were treated with either rapamycin or **pRap** or equivalent volumes of

DMSO (solvent control). Ten minutes after addition of **pRap**, cells were irradiated with 365 nm light by placing them on a UVP LMW-20 transilluminator (8 W). After treatment, cells were incubated for 1 hour, washed with ice-cold PBS and lysed with Lysis Buffer (20 mM Hepes-KOH, pH 7.8, 50 mM KCl, 100mM NaCl, 1 mM EGTA, 1% NP40, 1 mM NaF, 0.1mM Na_3VO_4). Cleared lysates were incubated for 2 hours with IgG-linked agarose beads prebound with anti-myc antibody (4A6, Millipore). The beads were washed 2 times with Wash Buffer (20 mM Hepes-KOH, pH 7.8, 50 mM KCl, 100 mM NaCl, 1 mM EGTA, 1% NP40) and two times with Kinase Reaction Buffer (25 mM HEPES pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.5 mMEGTA, 0.005% BRIJ-35). 20 uL of bead suspension were used in kinase assays using the N-terminal fragment of paxillin as previously described (Cai, X. et al. Mol Cell Biol 28, 201-14 (2008)). For immunoprecipitation only experiments, the washing with kinase reaction buffer and the kinase reactions were omitted.

Cell imaging:

Cells were plated on fibronectin-coated coverslips (coated by incubation with 5 mg/L fibronectin for 13-16 hours) 2 hours prior to imaging, then transferred into L-15 imaging medium (Invitrogen) supplemented with 5% fetal bovine serum. Live cell imaging was performed in an open heated chamber (Warner Instruments) using an Olympus IX-81 microscope equipped with an objective-based total internal reflection fluorescence (TIRF) system and a PlanApo N 60x TIRFM objective (NA 1.45). All images were collected using a Photometrix CoolSnap ES2 CCD camera controlled by Metamorph software. The 468 nm line from an omnichrome series 43 Ar/Kr laser and the 598 nm line from a Cobolt Mambo continuous-wave diode-pumped solid-state laser were used for TIRF imaging. Epifluorescence images were taken using a high pressure mercury arc light source. Cells expressing GFP-RapR-FAK constructs and mCherry-FRB were selected using epifluorescence imaging. GFP-expressing cells were selected for quantification of the phenotype induced by RapR-FAK activation. Time-lapse movies were taken at 1 min time intervals. GFP-RapR-FAK expression level quantification and other image analyses were performed using Metamorph software. To uncage pRap, cells were irradiated on the stage with 365 nm light using a UVP UVGL-25 hand-held UV lamp (4 W) held 2-3 cm from the coverslip surface.

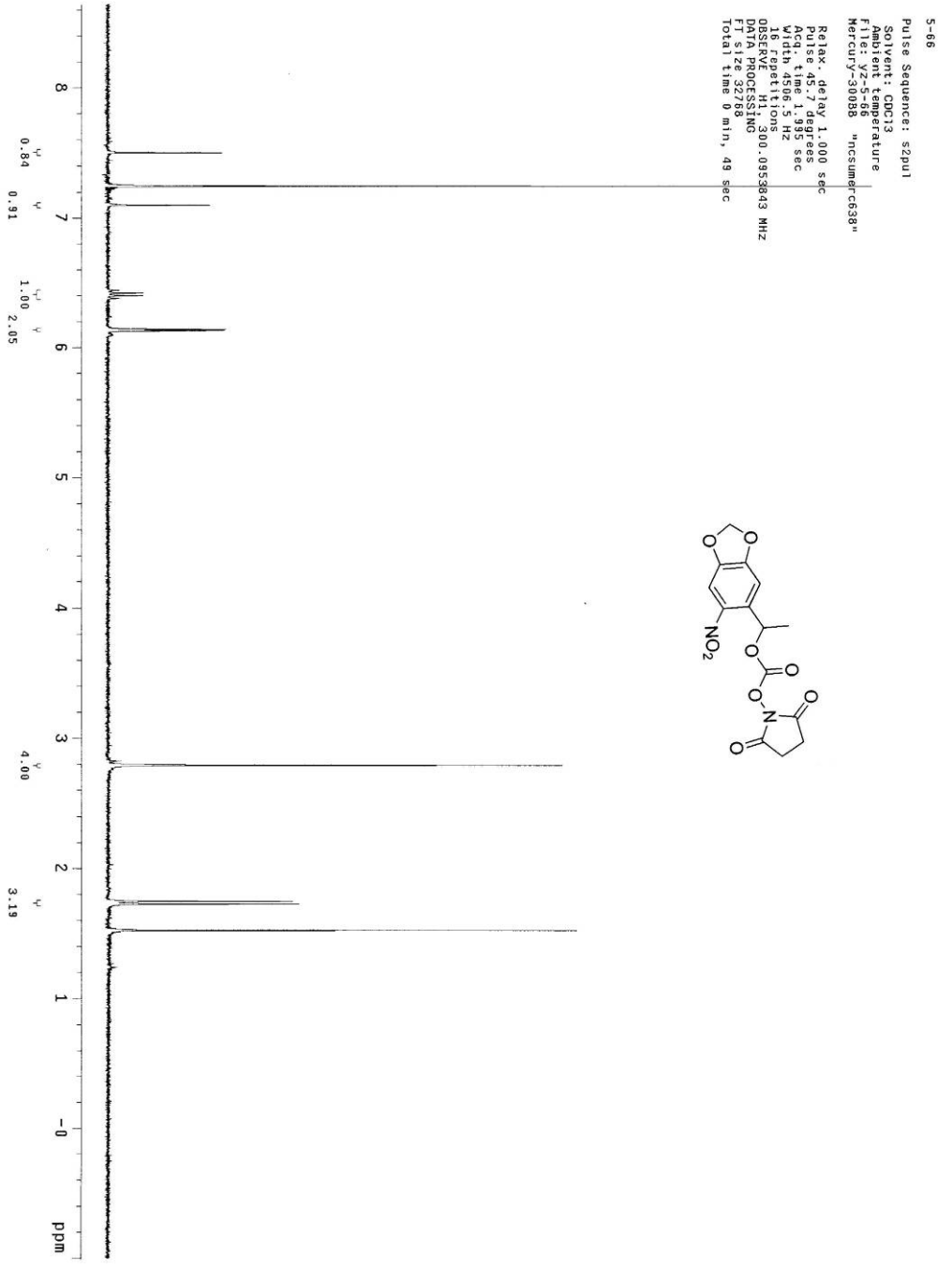
Molecular dynamics simulations:

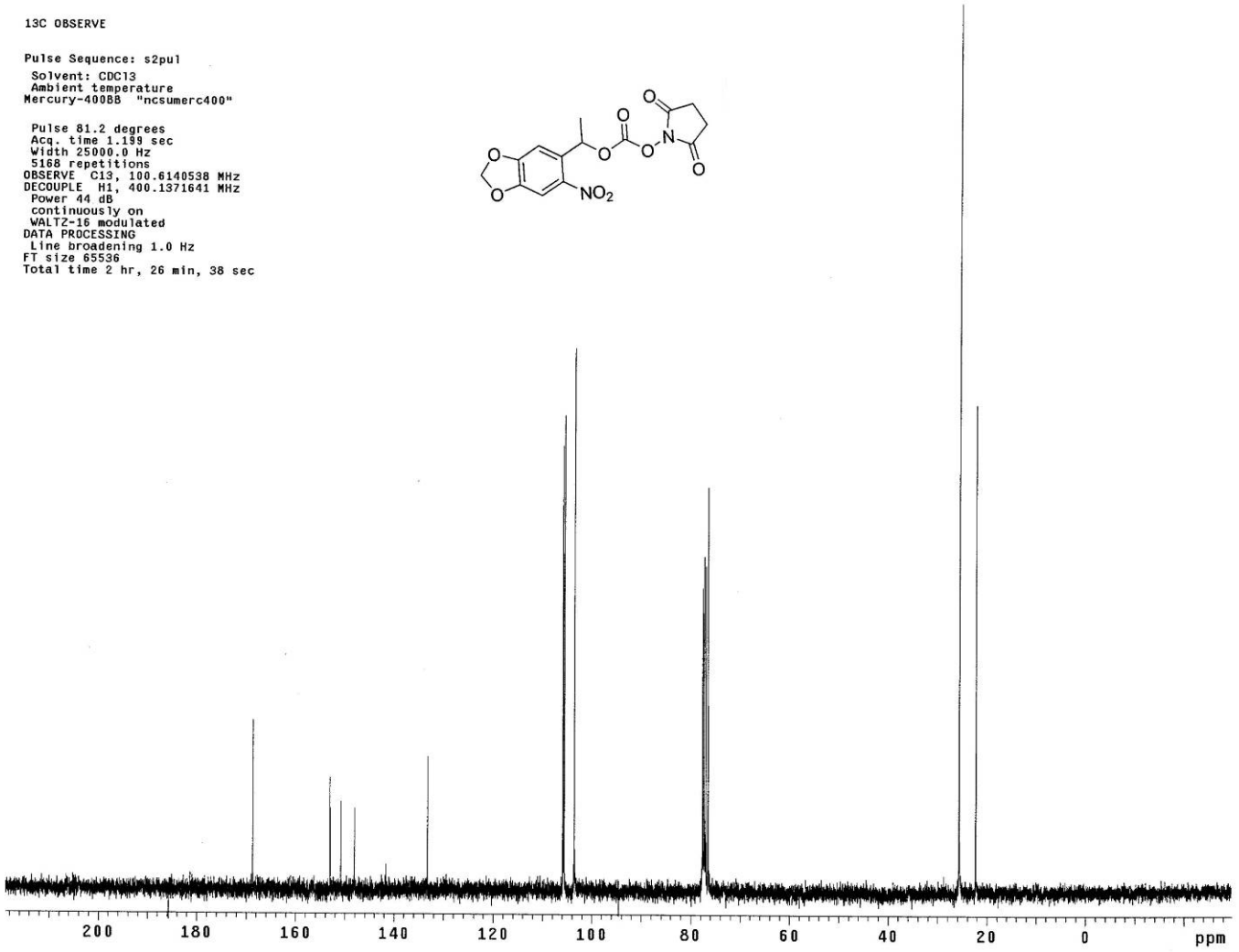
Discrete molecular dynamics (DMD) is allows effective conformational sampling and reach biologically-relevant time and length scales. DMD implements Medusa, a CHARMM based force field with implicit solvent, to treat atomic interactions [Ding and Dokholyan, *PLoS Comput. Biol.*, **e85**, (2006)]. We have applied and validated DMD in a number of studies using a range of model systems [Ding et al., *Structure*, **16,** 1010-1018, (2008); Ding et al, *Proteins: Structure, Function, and Genetics*, **53**, 220-228 (2003); Dokholyan et al. Fold Des **3**, 577-587 (1998)].

We performed DMD simulations of caged rapamycin-associated systems for 10^6 DMD time units, which correspond to ~50 μs of wall clock time. We use the crystal structure of FKBP12-rapamycin-FRB complex (PDB: 1FAP) as a starting configuration in our simulation

of this system. We constructed caged-Rapamycin by building the caging group onto rapamycin using PyMOL (http://pymol.sourceforge.net). In our simulations of (i)FKBP-(caged)rapamycin-FRB systems we imposed additional constraints on FRB backbone to preserve its secondary structure, in order to focus computational efforts on sampling conformations of (i)FKBP and (caged)RAP. To avoid clashes between the caging group and the rest of the complex, we performed side chain optimization of the (i)FKBP-(caged)RAP-FRB complexes using our Medusa toolkit [Ding and Dokholyan, *PLoS Comput. Biol.*, **e-85**, (2006)] before performing simulations.

NMR Spectra of NPOC-NHS and pRap:

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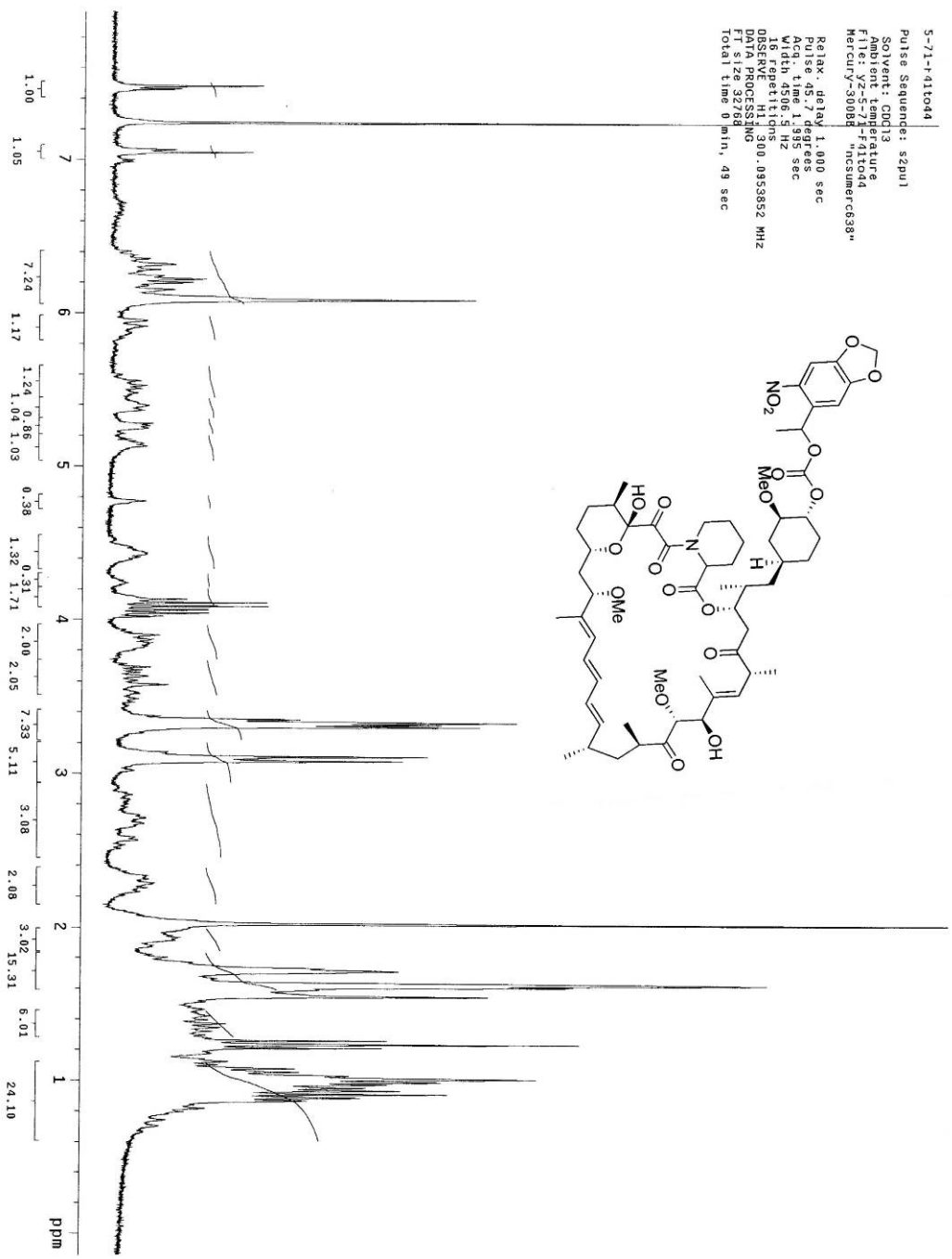

COMPLETE REFERENCE 15

Wagner, R.; Mollison, K. W.; Liu, L. P.; Henry, C. L.; Rosenberg, T. A.; Bamaung, N.; Tu, N.; Wiedeman, P. E.; Or, Y.; Luly, J. R.; Lane, B. C.; Trevillyan, J.; Chen, Y. W.; Fey, T.; Hsieh, G.; Marsh, K.; Nuss, M.; Jacobson, P. B.; Wilcox, D.; Carlson, R. P.; Carter, G. W.; Djuric, S. W., *Bioorg. Med. Chem. Lett.* **2005**, *15*, 5340.