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<Supplemental Information>

Development of a FRET-based High-Throughput Screening System for the Discovery of Hsp90 Inhibitors

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I. General Information about Experiments

All reagents in this synthetic procedure were purchase from Sigma-Aldrich [MO, USA] and TCI [Japan]. Gledanamycin was purchased from LC laboratories [MA, USA]. Radicicol was purchased from Sigma-Aldrich [MO, USA]. SNX-2112 was synthesized followed by the procedure previously described by Serenex Inc.[1] The progress of reaction was monitored using thin-layer chromatography (TLC) (silica gel 60 F_{254} 0.25 mm), and components were visualized by observation under UV light (254 and 365 nm) or by treating the TLC plates with ninhydrin staining solution followed by heating. Silica gel 60 (0.040-0.063 mm) used in flash column chromatography was purchased from Merck [Germany]. The optimized tetracysteine peptide for quality control FlAsH-EDT_2 was purchased from Bead-Tech Inc. [Seoul, Korea]. pGEX-4T-1 containing the full-length mouse Hsp90α was obtained from Ji-Sook Hahn in School of Chemical and Biological Engineering at Seoul National University. Oligonucleotides for PCR amplification were commercially synthesized by Bioneer [Daejeon, Korea]. All cell culture media and supplements were obtained from Gibco-BRL Life Technologies [MD, USA].

^{1}H and ^{13}C NMR spectra were recorded on a Varian Inova-500 [Varian Assoc., Palo Alto, USA], and chemical shifts were measured in ppm relative to internal tetramethylsilane (TMS) standard or specific solvent signal. Multiplicity was indicated as follows: s (singlet); d (doublet); t (triplet); m (multiplet); dd (doublet of doublet), etc. Coupling constants were reported in Hz. Routine mass analyses were performed on LC/MS system equipped with a reverse phase column (C-18, 50 × 2.1 mm, 5 μm) and photodiode array detector using electron spray ionization (ESI) or atmospheric pressure chemical ionization (APCI).

II. Synthetic Procedure and Compound Characterizations

***N*-(5-Aminopentyl)-4-[[4-(dimethylamino)phenyl]diazenyl]benzamide (1).** To a solution of dabcyl acid (0.20 g, 1.0 equiv.) in DMF (3 mL), EDC (0.21 g, 1.5 equiv.) and HOBt (0.20 g, 2.0 equiv.) were added and then completely dissolved. After complete dissolution, this mixture was added dropwise into 1,5-diaminopentane (0.43 mL, 5.0 equiv.) solution which was dissolved in DMF (2 mL) previously. It was stirred for 2 h at the room T. After the reaction completion monitored by TLC, the reaction mixture was diluted in EA (25 mL) and the washed with sat. $NaHCO_3$ and brine sequentially. The organic layer was dired over anhydrous $MgSO_4$, filtrated, and evaporated *in vacuo*. The resulting mixture was purified with silica-gel flash column chromatography (MeOH/MC = 1/5, 1% TFA) to provide desired product **1** (as TFA salt). Compound **1**: Yield = 96% (0.39 g); ^{1}H-NMR (500 MHz, CD_3OD): δ 7.94 (d, J = 8.5 Hz, 2H), 7.87 (d, J = 9.5 Hz, 2H), 7.83 (d, J = 8.5 Hz, 2H), 6.91 (d, J = 9.5 Hz, 2H), 3.43 (t, J = 7.0 Hz, 2H), 3.16 (s, 6H), 2.94 (t, J = 7.5 Hz, 2H), 1.76-1.66 (m, 4H), 1.51-1.45 (m, 2H); ^{13}C-NMR (125 MHz, CD_3OD): δ 169.6, 155.2, 154.9, 144.3, 135.7, 129.3, 127.4, 122.4, 113.7, 113.6, 40.9, 40.8, 40.6, 30.0, 28.2, 24.8.

GM-dabcyl (2). Geldanamycin (5 mg, 1.0 equiv.) and compound **1** (10 mg, 2.0 equiv.) were dissolved in DMF (0.5 mL). DIPEA (6 μL, 4 equiv.) was added in this mixture at 0 °C and then stirred for 10 h at room T. After reaction completion monitored by TLC, the reaction mixture was purified with preparatory HPLC (H_2O/ACN, gradient condition). GM-dabcyl (**2**): Yield = 95% (7 mg); ^{1}H-NMR (500 MHz, Acetone-d_6): δ 8.02 (d, J = 8.0 Hz, 2H), 7.87-7.83 (m, 4H), 7.27 (d, J = 11.5 Hz, 1H), 6.86 (J = 9.0 Hz, 2H), 6.63 (t, J = 6.5 Hz, 1H), 5.83 (t, J = 10.0 Hz, 1H), 5.79 (d, J = 9.5 Hz, 1H), 5.10 (s, 1H), 4.53 (d, J = 9.5 Hz, 1H), 3.64 (t, J = 6.5 Hz, 1H), 3.51 (dd, J = 6.0 and 2.0 Hz, 1H), 3.47-3.44 (m, 2H), 3.36-3.35 (m, 1H), 3.34 (s, 3H), 3.30 (s, 3H), 3.14 (s, 6H), 2.75-2.72 (m, 1H), 2.62 (dd, J = 8.5 and 4.0 Hz, 1H), 2.49-2.45 (m, 1H), 1.99 (s, 3H), 1.80-1.77 (m, 2H), 1.74 (d, J = 2.0 Hz, 3H), 1.57-1.52 (m, 2H), 1.00 (d, J = 7.0 Hz, 3H), 0.91 (d, J = 7.0 Hz, 3H), 0.89-0.86 (m, 1H); LRMS (ESI) *m/z* calculated for $C_{48}H_{62}N_7O_9$ $[M–H]^-$: 881.47; Found: 880.35.

III. Biological Procedure

Quality Control of FlAsH-EDT_2. The preparation and

the quality control of FlAsH-EDT_2 were based on the literature procedure previously described by Tsien and coworkers.[2] For the quality control of synthetic FlAsH-EDT_2, 25 μL of 1 M 2-mercaptoethanesulfonate (MES) in water and 2.5 μL of 10 mM EDT in DNSO were added into 2.5 μL of 100 mM aqueous solution of 4-morpholine-propanesulfonic acid (MOPS) buffer neutralized with NaOH to pH 7.2 to give final concentrations of 10 mM and 10 μM, respectively. Then, 2.5 μL of 1 mM FlAsH-EDT_2 in DMSO was added to give a final concentration of 1 μM. After 10 min, 10 μL of 10 mM tetracysteine peptide WDCCPGCCK (Ac-Trp-Asp-Cys-Cys-Pro-Gly-Cys-Cys-Lys-NH_2) in 50% (v/v) aqueous ACN containing 0.1% (v/v) TFA was added and mixed well. The emission at 530 nm with excitation at 508 nm was measured using fluorescence spectrophotometer (Varian Carey Eclipse, CA, USA).

UV Absorption of Dabcyl Group and Quenching of FlAsH Emission. The UV absorption of dabcyl acid (4-(4'-dimethylaminopheylazo)benzoic acid) was measured using UV spectrophotometer (Shimadzu UV-1650PC, Kyoto, Japan). To measure the fluorescence quenching ability of dabcyl group, the complex between FlAsH-EDT_2 and the tetracysteine peptide was prepared following the same procedure as described in the quality control. After the increased fluorescence had reached a plateau, 10 μL of 10 mM dabcyl acid was added to the reaction mixture to give final concentration of 40 μL. The emission at 530 nm with excitation at 508 nm was measured by fluorescence spectrophotometer.

Cloning, Expression, and Purification of the *N*-Terminal Domain of Hsp90. Cloning, expression, and purification of the Hsp90N were carried out based on a literature procedure previously described by Pearl and coworkers. PCR amplification of DNA encoding the *N*-terminal domain of Hsp90 (residues 1-240, Hsp90N) from the full-length mouse Hsp90α used the primers 5'-GGGTCGACTCATGCCTGAGGAAA-CCCAGACCC-3' (Primer 1) as the forward primer and 5'-ATGCGGCCGCTTACTCTTCCTTTTCTTCAGCCTCATC-3' (Primer 2) as the reverse primer. Then, the PCR product was cloned into pGEX-4T-1 using the *Sal*I and *Not*I (New England Biolabs, Ipswich, MA) restriction sites to produce an unmodified *N*-terminal domain of Hsp90 (Hsp90N-WT). To introduce a tetracysteine sequence (Cys-Cys-Pro-Gly-Cys-Cys, CCPGCC) into the Hsp90N, two different kinds of primers were designed. For the *N*-terminal CCPGCC tag (Hsp90N-N), PCR amplification used the primer 5'-GGGTCGACTC**TGTTGTCCTGGTTGTTGT**ATGCCTG AGGAAACCCAGACCC-3' (Primer 3, boldfaced for CCPGCC) as the forward primer and the Primer 2 as the reverse primer. For the *C*-terminal CCPGCC tag (Hsp90N-C), on the other hand, PCR amplification used the primer 5'-ATGCGGCCGCTTA**ACAACAACCAGGACAACA**CTC TTCCTTTTCTTCAGCCTCATC-3' (Primer 4, boldfaced for CCPGCC) as the reverse primer and the Primer 1 as the forward primer. The PCR products were also cloned into pGEX-4T-1 and produced the Hsp90N with *N*-terminally or *C*-terminally fused CCPGCC tags.

Expression was carried out in a BL21(DE3) strain by growth at 37 ºC in Luria broth (United States Biological Inc., Swampscott, MA) containing 100 μg/mL of ampicillin to an OD_{600} = 0.6-0.8, followed by induction with 1 mM IPTG for 12-16 h at 25 ºC. The culture was harvested by centrifugation at 6000 rpm for 30 min at 4 ºC, and the cells were resuspended in the binding buffer (25 mM Tris, 140 mM NaCl, 2.7 mM KCl, 10 nM EDTA, 5 mM DTT, pH 7.4, protease inhibitor cocktail). Cells were disrupted by sonication and clarified by centrifugation at 12,000 rpm for 20 min at 4 ºC. The supernatant, filtrated through a 0.45 μm filter, was added into Glutathione Sepharose 4B (GE Healthcare, UK) equilibrated in 1× TBS buffer (25 mM Tris, 140 mM NaCl, 2.7 mM KCl, pH 7.4), and incubated with gentle agitation at 4 ºC for 16 h. After washing three times with 1× TBS buffer, the bound of Hsp90N was eluted with 10 mM Glutathione in 1× TBS buffer containing 10 % glycerol and protease inhibitor. The purified Hsp90 was dialyzed against 20 mM Tris (pH 7.5) containing 1 mM DTT and protease inhibitor. After dialysis, the concentration of Hsp90N was measured by Bradford protein assay as recommended in the manufacturer's guide (Bio-rad, Hercules, CA), and then the mass of Hsp90N was confirmed by SDS-PAGE and MALDI-TOF.

Binding of FlAsH to the Hsp90N and quenching by GM-dabcyl. To 2.5 mL of 100 mM MOPS buffer neutralized to pH 7.2 in a fluorescence cuvette, 25 μL of 1 M 2-mercaptoethanesulfonate (MES) in water and 2.5 μL of 10 mM EDT in DMSO were added to give final concentrations of 10 mM and 10 μM, respectively. Then, 25 μL of 2 mM FlAsH-EDT_2 in DMSO was added to give a final concentration of 20 μM. After 10 min, 1 μM Hsp90N was added and mixed well. The emission at 530 nm with excitation at 508 nm was measured using fluorescence spectrophotometer.

When the complex between FlAsH and the Hsp90N became stable and the increased fluorescence had reached a plateau, 2.5 μL of 1 mM GM-Dabcyl in DMSO was added to give a final concentration of 1 μM. After incubation at the room temperature for 1 h, the emission at 530 nm with excitation at 508 nm was measured using fluorescence spectrophotometer, and the percent decrease of fluorescence by FRET quenching was calculated. For the comparison, 2.5 μL of 1 mM or 5 mM dabcyl acid was added to the FlAsH complex to give final concentrations of 1 μM and 5 μM, respectively, and the change in fluorescence was measured in the same manner.

Inhibition of FRET Quenching by Competitive Binding of GM. 10 to 20 μM of Hsp90N-N or Hsp90N-C was allowed to react with 200 μM of FlAsH-EDT_2 in the presence of MES and EDT for 4 h, and the unbound FlAsH-EDT_2 was removed by size exclusion chromatography. Size exclusion chromatography was conducted by PD-10 columns as recommended in the manufacture's manual (GE Healthcare). The concentrations of the eluted complex between FlAsH and the Hsp90N were measured by Bradford protein assay. Then, the eluted complex was diluted in 100 mM of MOPS buffer containing 1 mM TCEP to produce a 0.1 μM solution

of the Hsp90N complex.

After the Hsp90N complex was stabilized, serially diluted geldanamycin in DMSO was added to the complex, mixed well, and incubated at the room temperature. Finally, 0.5 μM of GM-dabcyl in DMSO was added and the emission at 530 nm with excitation at 508 nm was measured using fluorescence spectrophotometer.

Optimization of a FRET-based Assay in a 96-Well Plate. The complex between FlAsH and the Hsp90N in MOPS buffer was prepared following the same procedure as described. 100 μL of 0.1 μM solution of the Hsp90N complex was added to the each well of a 96-well clear bottom black plate (Corning Inc., Corning, NY). Serially diluted geldanamycin (GM), radicicol (RD), and SNX-2112 (SNX) were added to the each well, and mixed. After incubation at the room temperature for 2 h, 0.5 μM of GM-dabcyl in DMSO was added. The emission at 530 nm with excitation at 508 nm was measured using Synergy HT Microplate reader (BioTek Instruments, Inc., Winooski, VT) and the difference in fluorescence before and after the addition of GM-dabcyl was compared.

Screening of a Compound Library by the FRET-based Assay. The complex between FlAsH and the Hsp90N in MOPS buffer was prepared following the same procedure as described. 100 μL of 0.1 μM solution of the Hsp90N complex was added to the each well of a 96-well clear bottom black plate. Then, 0.2 μL of 5 mM compounds from the compound library in DMSO was transferred to the each well using a pin tool (V&P Scientific, Inc. San Diego, CA) to give a final concentration of 10 μM. Known inhibitors such as geldanamycin (GM), radicicol (RD), and SNX-2112 (SNX) as well as DMSO control were also added to the 12th column of the plate. After incubation at the room temperature for 2 h, 0.5 μM of GM-dabcyl in DMSO was added. The emission at 530 nm with excitation at 508 nm was measured by Synergy HT Microplate reader and the difference in fluorescence before and after the addition of GM-Dabcyl was compared.

Cell Proliferation Assay. T lymphocyte jurket cells were cultured in RPMI media 1640 containing 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution. Jurkat cells in a 96-well plate were incubated at 37 ºC for 24 h. The cells were treated with various concentration of each compound from in house library using pin tool. After 72 h

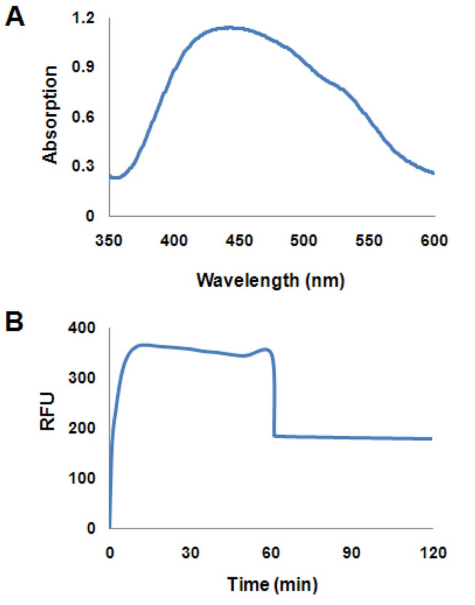


Figure S1. Dabcyl acid was chosen as a FRET acceptor and quencher in designed screening system. (A) UV absorption spectrum of dabcyl acid. (B) 40 μM of dabcyl acid was added to the fluorescent complex between FlAsH and tetracysteine peptide (WDCCPGCCK) at 60 min.

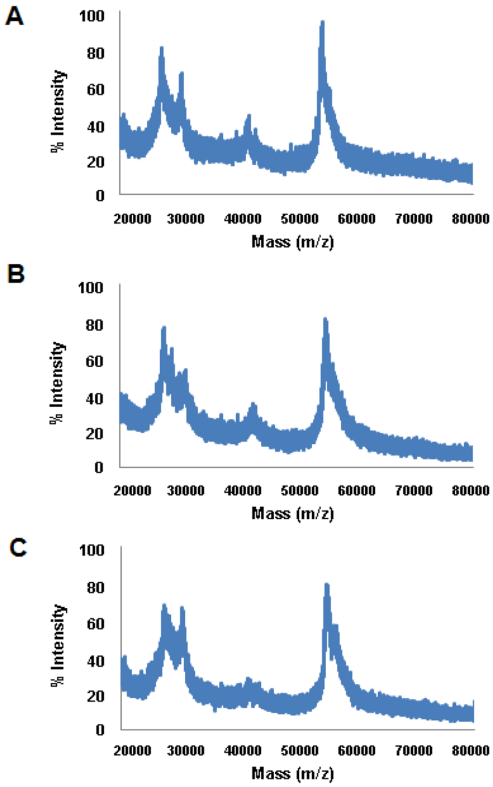


Figure S2. Mass confirmation of the Hsp90N by MALDI-TOF. MALDI-TOF data of (A) Hsp90N-WT, 54.36 kDa; (B) Hsp90N-N, 54.94 kDa; (C) Hsp90N-C, 54.98 kDa.

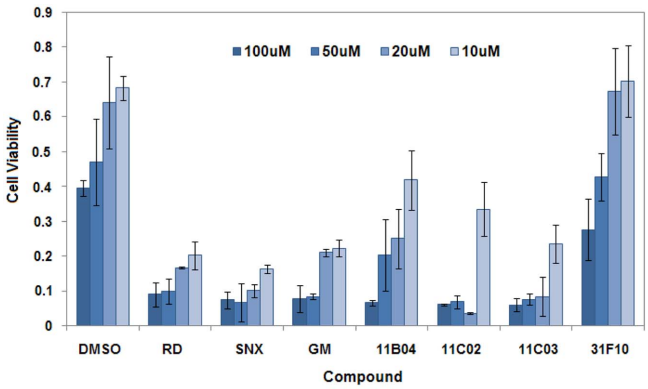
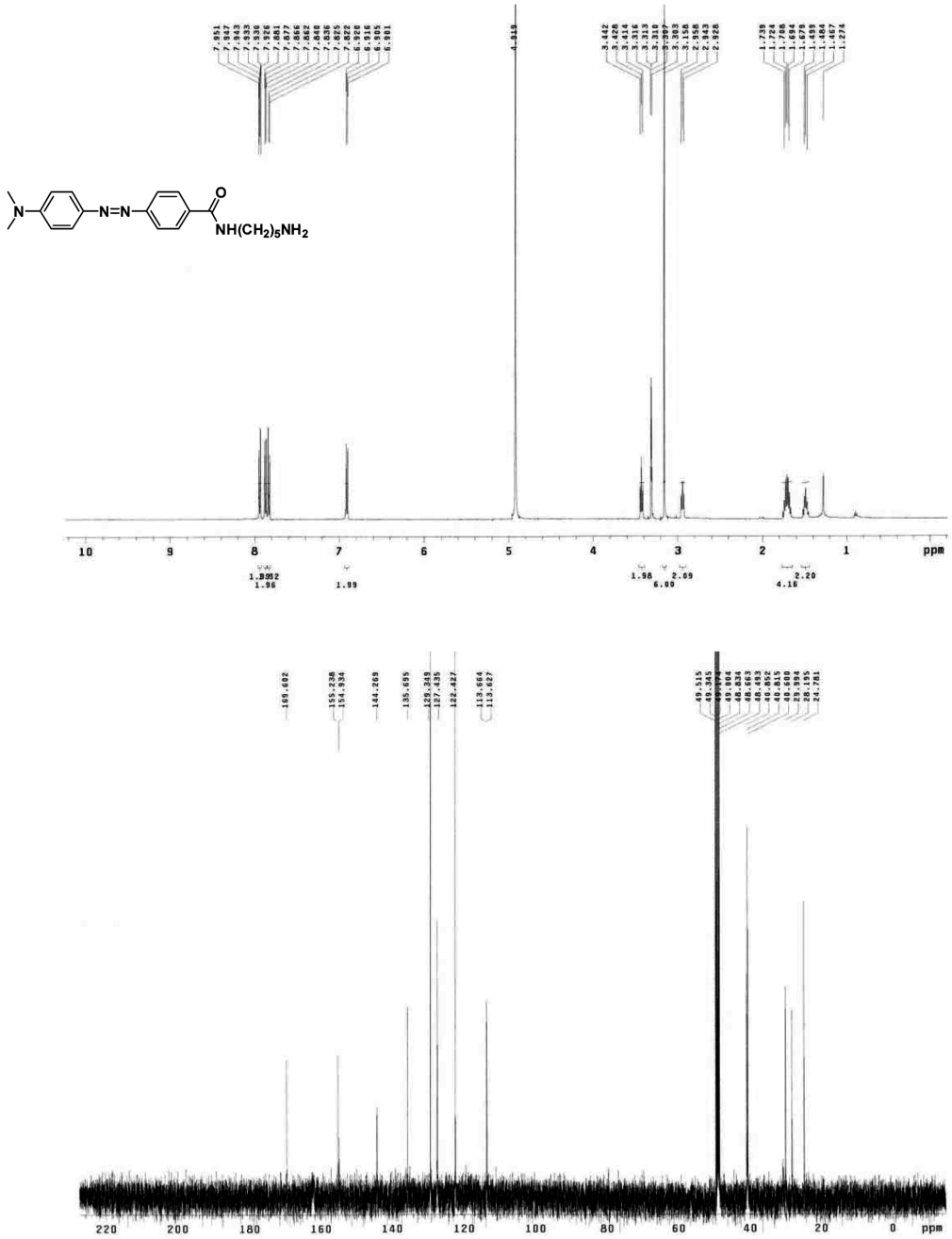


Figure S3. Cell proliferation and viability in T lymphocyte Jurkat cell lines monitored by mitochondrial activity using WST-1 assay at the varied concentration for 72 h incubation. 11B04, 11C02 and 11C03 are hit compounds in FRET-based HTS assay and 31F10 is the compound of negative control.

incubation, 10 μL of EZ-Cytox cell viability assay kit solution (Daeil lab service, Seoul, Korea) was added to the each well and re-incubated for another 1 h. The extent of the reduction of tetrazolium salts to formazan by metabolic activity within cells was quantified by measuring the absorbance at 455 nm using Synergy HT microplate reader.

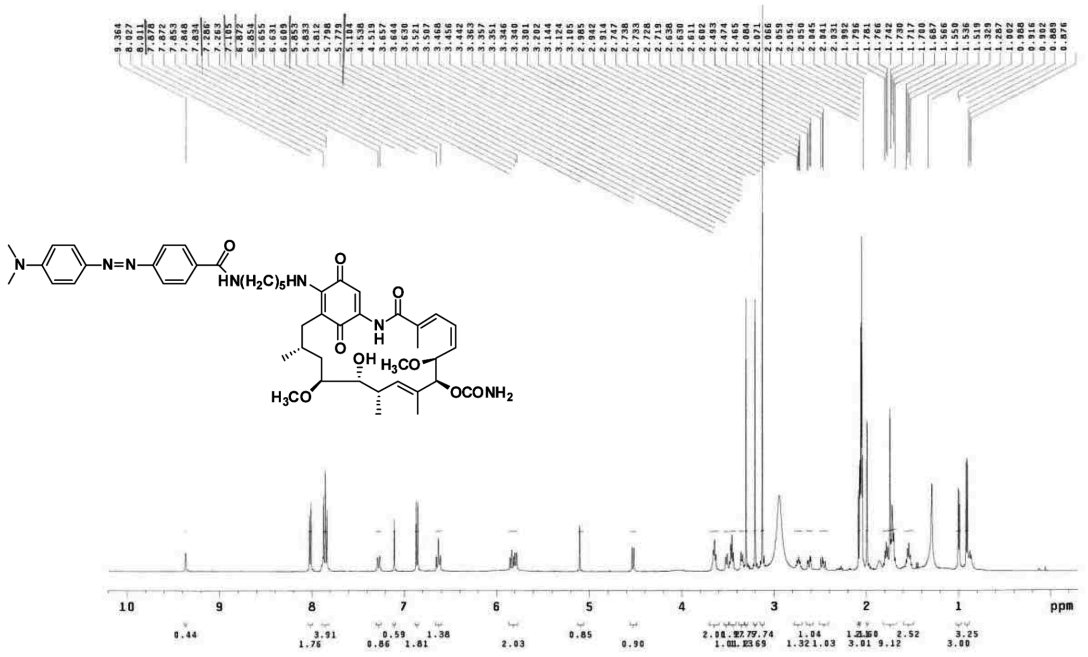
IV. Supporting Figures

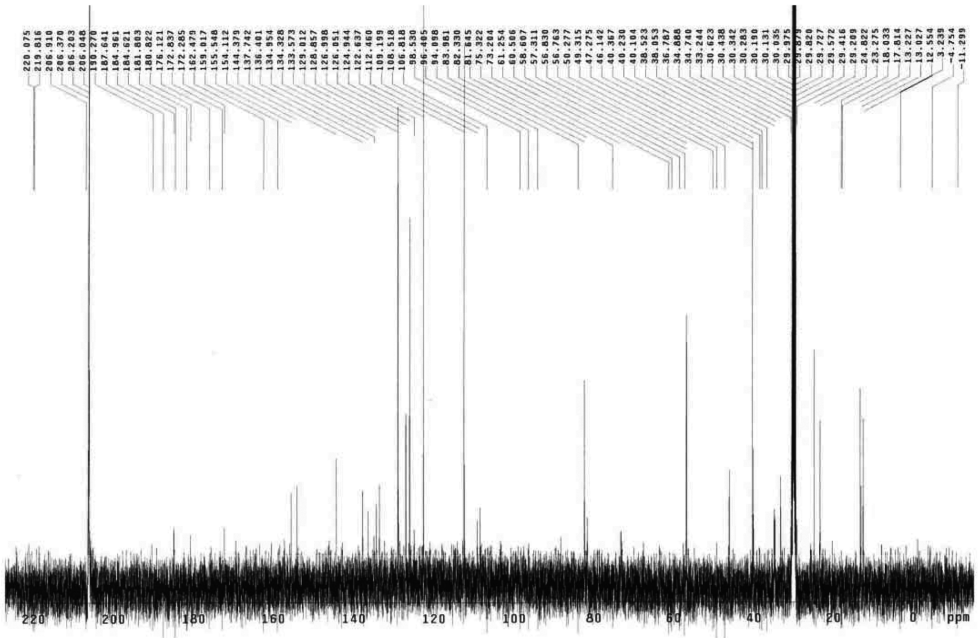
N-(5-Aminopentyl)-4-((4-(dimethylamino)phenyl)diazenyl)benzamide (1)



V. NMR Data

GM-dabcyl (2)





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