Chem Comm RSCPublishing

SUPPLEMENTARY INFORMATION

Self-Assembly of a 5-Fluorouracil-Dipeptide Hydrogel.

Yuan Sun, [a] Jonah A. Kaplan,[b] Aileen Shieh,[a] Hui-Lung Sun, [c] Carlo M. Croce,[c] Mark W. Grinstaff, [*b] and Jon R. Parquette [*a]

a. Department of Chemistry and Biochemistry, The Ohio State University, 100 W. 18th Ave. Columbus, Ohio 43210; Fax: 614 292-1685; E-mail: parquett@chemistry.ohio-state.edu
b. Departments of Biomedical Engineering and Chemistry, Boston University, 590 Commonwealth Ave. Boston, MA 02215; E-mail: mgrin@bu.edu
c. Department of Molecular Virology Immunology and Medical Genetics, The Ohio State University, 460 W. 12th Ave. Columbus, OH 43210

Received ooth February 2015,
Accepted ooth February 2015

DOI: 10.1039/x0xx00000x

www.rsc.org/

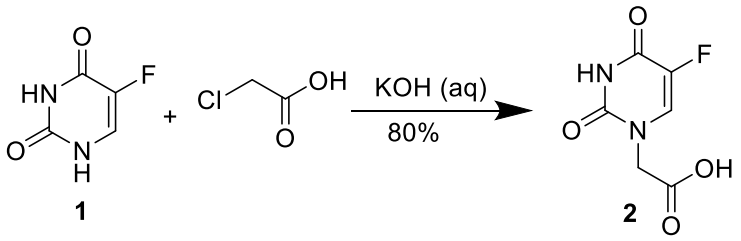
General Methods

Fourier transform-infrared (FT-IR) was performed on FT-IR spectrometer (Thermo Nicolet, Madison, WI). Transmission Electron Microscopy (TEM) was performed with Technai G2 Spirit instrument operating at 80 kV. All reactions were performed under an argon or nitrogen atmosphere. ^{1}H NMR was recorded at 400 MHz and ^{13}C NMR spectra at 100 MHz on a Bruker DPX-400 instrument. Water (pH 7.0), 2,2,2-trifluoroethanol (TFE), and methanol used for UV and TEM were of spectroscopic grade, and phosphate buffered saline (PBS, pH 7.4) was purchased from Gibco.

Synthesis of 5-Fluorouracil-1-acetic acid (2)

5-Fluorouracil (5-Fu, 500 mg, 3.85 mmol) was added into 100 mL round-bottom flask with 10 mL aqueous KOH solution (864 mg, 15.43 mmol) and stirred at room temperature for 20 minutes before chloroacetic acid (364 mg, 3.85 mmol) was added into reaction. The reaction mixture was stirred and heated at 60°C for 4 h, acidified with concentrated hydrochloric acid to a pH 2. The precipitate was collected as a crude product and recrystallized with water to afford 5-fluorouracil-1-acetic acid as white crystalline product[1] (560 mg, 80%). ^{1}H NMR (400 MHz;

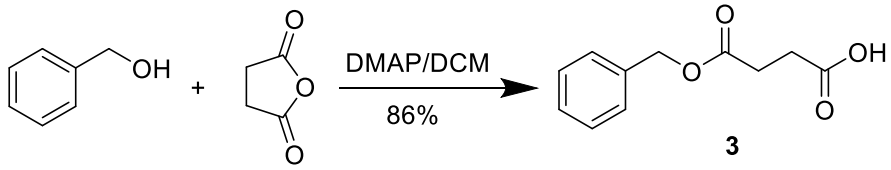
DMSO-d_6) δ 11.91 (1 H, d, *J*= 4 Hz), 8.06 (1 H, d, *J*= 6.4 Hz), 4.35 (2 H, s); ^{13}C NMR (100 MHz; DMSO-d_6) δ 169.22, 157.44, 149.61, 138.42, 130.5, 48.63; ESI-MS for $C_6H_5FN_2O_4$ $[M+H]^+$ calculated 189.0321; found 189.0185.



Scheme S1. Synthesis of of 5-fluorouracil-1-acetic acid (2-(5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)acetic acid)

Synthesis of Benzyl succinic acid (3)

Benzyl alcohol (12.67 g, 126.7 mmol) was added into 500 mL round-bottom flask with 100 mL dichloromethane (DCM) followed the addition of succinic anhydride (10.0 g, 92.4 mmol, 9.43 mL). 4-Dimethylaminopyridine (DMAP, 2.26 g, 18.5 mmol) was then added and the reaction mixture was stirred at room temperature for 12 h. After the completion of the reaction, saturated Na_2CO_3 solution was added to adjust the pH to 9. The aqueous layer was separated and reacidified with concentration hydrochloric acid to pH = 2. The organic layer was separated and dried with anhydrous $MgSO_4$, evaporation of the solvent afford the product as white solid without further purification[2] (16.53g, 86%). ^{1}H NMR (400 MHz; DMSO-d_6) δ 7.32-7.37 (5 H, m), 5.15 (2 H, s), 2.69-2.71 (4 H, m); ^{13}C NMR (100 MHz; DMSO-d_6) δ 176.82, 171.95, 135.69, 128.58, 128.31, 128.22, 66.67, 28.92, 28.71; ESI-MS for $C_{11}H_{12}O_4$ $[M+Na]^+$ calculated 231.0633; found 231.0509.

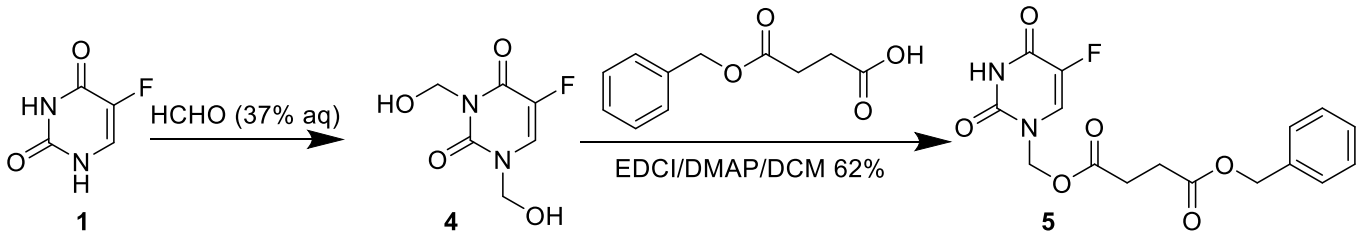


Scheme S2. Synthesis of of benzyl succinic acid (4-(benzyloxy)-4-oxobutanoic acid)

Synthesis of 5-fluorouracil-1-succinic benzyl ester (5)

5-Fluorouracil (1.3 g, 10 mmol) was added into the solution of 37% formaldehyde (1.64 mL, 22 mmol) and heated at 65°C for 45 minutes. A transparent oil was obtained and the trace amount of water was removed under vacuum with anhydrous acetonitrile. The remaining residue was redissolved in anhydrous acetonitrile and stirred under room temperature. Benzyl succinic acid (2.5 g, 12mmol) , N,N'-Dicyclohexylcarbodiimide (DCC) (2.48 g, 12 mmol) and 4-dimethylaminopyridine (DMAP) (60 mg, 0.5 mmol) was then added into reaction and stirred under room temperature for another 12 h. The precipitated dicyclohexylurea (DCU) was removed by filtration and washed with dichloromethane (DCM). The filtrate was concentrated under reduced pressure and taken up in 50 mL

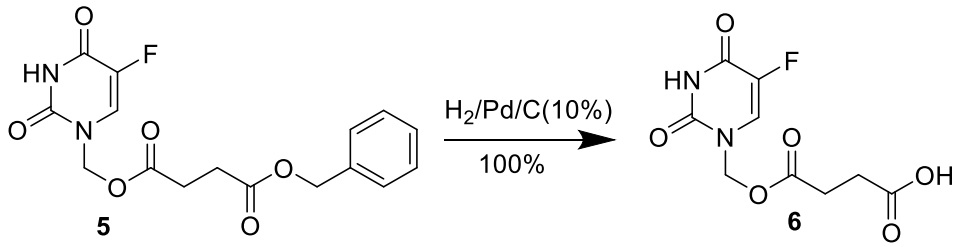
DCM before being washed with 1 M HCl (3 x 20 mL), 1 M $NaHCO_3$ (10 mL) , and brine (10 mL). The organic layer was dried by anhydrous $MgSO_4$ and purified by column (hexane: acetone = 2:1) to give the white powder product[1] (2.16 g, 62%). ^{1}H NMR (400 MHz; DMSO-d_6) δ 8.49 (1 H, br), 7.54 (2 H, d, *J*= 5.6 Hz), 7.32-7.36 (5 H, m), 5.62 (2 H, s), 5.12 (2 H, s), 2.70 (4 H, s); ^{13}C NMR (100 MHz; DMSO-d_6) δ 172.60, 171.75, 156.40, 148.94, 141.56, 139.01, 135.60, 128.68, 128.64, 128.48, 128.31, 69.81, 66.86, 28.92; ESI-MS for $C_{16}H_{15}FN_2O_6$ $[M+Na]^+$ calculated 373.0812; found 373.0625.



Scheme S3. Synthesis of 5-fluorouracil-1-succinic benzyl ester (benzyl ((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methyl) succinate).

Synthesis of 5-Fluorouracil-1-succinic acid (6)

10% Pd/C (60 mg) was added to a methanol solution 5-fluorouracil-1-succinic benzyl ester (400 mg, 1.14 mmol) and stirred under H_2 (1 atm.) at room temperature for 6 h. The Pd/C catalyst was removed by filtration and washed with methanol. The filtrate was concentrated under vacuum to afford white product without further purification[1] (295 mg, 100%). ^{1}H NMR (400 MHz; DMSO-d_6) δ 12.21 (1H, s), 11.98(1H, s), 8.11 (2 H, d, *J*= 6.4 Hz), 5.58 (2 H, s), 2.50(4 H, m); ^{13}C NMR (100 MHz; DMSO-d_6) δ 173.69, 172.44, 157.99, 149.67, 140.99, 130.12, 70.89, 29.24, 29.02 ; ESI-MS for $C_9H_9N_2O_6$ $[M+Na]^+$ calculated 283.0342; found 283.0177.



Scheme S4. Synthesis of 5-fluorouracil-1-succinic acid (4-((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methoxy)-4-oxobutanoic acid).

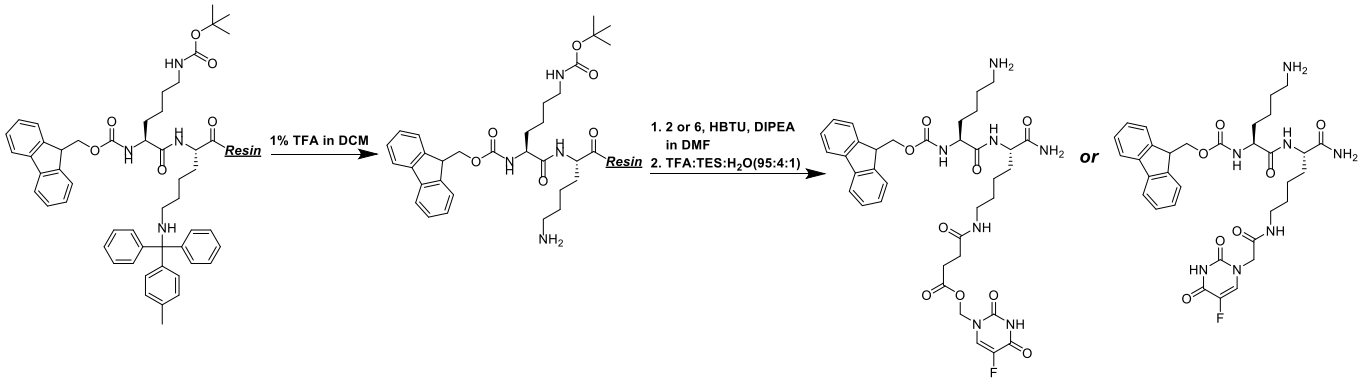
Synthesis of Fmoc-KK(5-Fu ester)-NH_2 and Fmoc-KK(5-Fu amide). (Compound A and B):

Dipeptides **A** and **B** were prepared *via* on-resin modification of the side chain (Scheme S5). The dilysine peptide, protected with Fmoc and Mtt groups, was manually prepared using Fmoc/t-Bu solid-phase peptide synthesis on rink amide resin (loading 0.80 mmol/g). Amide-coupling steps were accomplished with standard techniques for all amino acids: Fmoc-amino acid, 1,3-diisopropylcarbodiimide (DIC), and 1-hydroxybenzotriazole (HOBt) (300 mol% each relative to resin) in 1:1 DMF/DCM for 1.5 h. A solution of 20 % piperidine in DMF was

used for Fmoc removal and 1 % TFA in dichloromethane was used for Mtt group deprotection. A mixture of 5-Fluorouracil-1-acetic acid or 5-fluorouracil-1-succinic acid, HBTU, and DIPEA (200 mol% each relative to resin) in DMF was added to the resin. The reaction mixture was shaken for 24 h at room temperature and then filtered through a fritted syringe. The resin was washed thoroughly (3 x DMF, 3 x CH_2Cl_2) and the final 5-Fu-peptide conjugate was cleaved from the resin by the treatment with TFA/water/triethylsilane (95/1/4) at room temperature for 2 h. The crude peptides were precipitated with cold diethyl ether and purified by reversed-phased HPLC on preparative Varian Dynamax C18 column eluting with a linear gradient of CH_3CN/water containing 0.1 % TFA (10/90 to 100/0 over 30 minutes) and stored as lyophilized powers at 0°C. Peptide purity was assessed by analytical reverse-phase HPLC, and identity was confirmed using ESI-TOF mass spectrometry and NMR.

Fmoc-KK(5-Fu ester)-NH_2 (Compound A) ^{1}H NMR (400 MHz, DMSO-d^6) δ 11.97 (d, 1H, *J*= 5.2 Hz), 8.08 (d, 1H, *J*= 6.4 Hz), 7.90 (d, 2H, *J*= 7.6 Hz), 7.69-7.83 (m, 4H), 7.61 (s, 3H), 7.50 (d, 1H, *J*= 8.4 Hz), 7.40-7.44(t, 2H), 7.31-7.35 (t, 3H), 7.01 (s, 1H), 5.55 (s, 2H), 4.17-4.30 (m, 4H), 3.99-4.00 (m, 1H), 2.97 (d, 2H, *J*= 5.6 Hz), 2.76 (d, 2H, *J*= 5.2 Hz), 2.28-2.35 (m, 4H), 1.61-1.66 (m, 2H), 1.49-1.52 (m, 4H), 1.21-1.33 (m, 6H) ; ^{13}C NMR (400 MHz, DMSO-d^6) δ 173.50, 172.16, 171.55, 170.18, 157.51, 157.24, 155.95, 149.19, 143.87, 140.69, 129.62, 127.61, 127.06, 125.23, 120.08, 70.35, 65.58, 54.52, 52.11, 46.64, 38.67, 38.44, 31.79, 31.23, 29.99, 29.49, 28.77, 26.52, 22.61, 22.30; ESI-MS calculated for $C_{36}H_{45}FN_7O_9$ $[M+H]^+$ 738.3263, found 738.2744;

Fmoc-KK(5-Fu amide)-NH_2 (Compound B) ^{1}H NMR (400 MHz, DMSO-d^6) δ 11.80 (d, 1H, *J*= 5.2 Hz), 8.11 (m, 1H), 7.99 (d, 1H, *J*= 6.8 Hz), 7.89 (d, 2H, *J*= 7.6 Hz), 7.79 (d, 1H, *J*= 8 Hz), 7.72 (d, 2H, *J*= 8.4 Hz), 7.69 (m, 2H), 7.50 (d, 1H, *J*= 8 Hz), 7.40-7.44 (t, 2H), 7.31-7.36 (m, 3H), 7.01 (s, 1H), 4.17-4.30 (m, 6H), 3.99-4.00 (m, 1H), 3.02-3.04 (m, 2H), 2.75-2.76 (m, 2H), 1.64-1.66 (m, 2H), 1.48-1.52 (m, 4H), 1.27-1.38 (m, 6H) ; ^{13}C NMR (400 MHz, DMSO-d^6) δ 173.50, 171.58, 166.23, 157.49, 155.98, 149.65, 143.86, 143.68, 140.69, 138.09, 130.88, 127.62, 127.06, 125.21, 120.10, 65.58, 54.42, 53.36, 52.06, 49.56, 46.63, 38.69, 31.76, 31.18, 28.64, 26.52, 22.56, 22.31; ESI-MS calculated for $C_{33}H_{40}FN_7O_7$ $[M+H]^+$ 666.3051, found 666.2500;



Scheme S5. Synthesis of Fmoc-KK(5-Fu ester)-NH_2 and Fmoc-KK(5-Fu amide)-NH_2 (**A** and **B**)

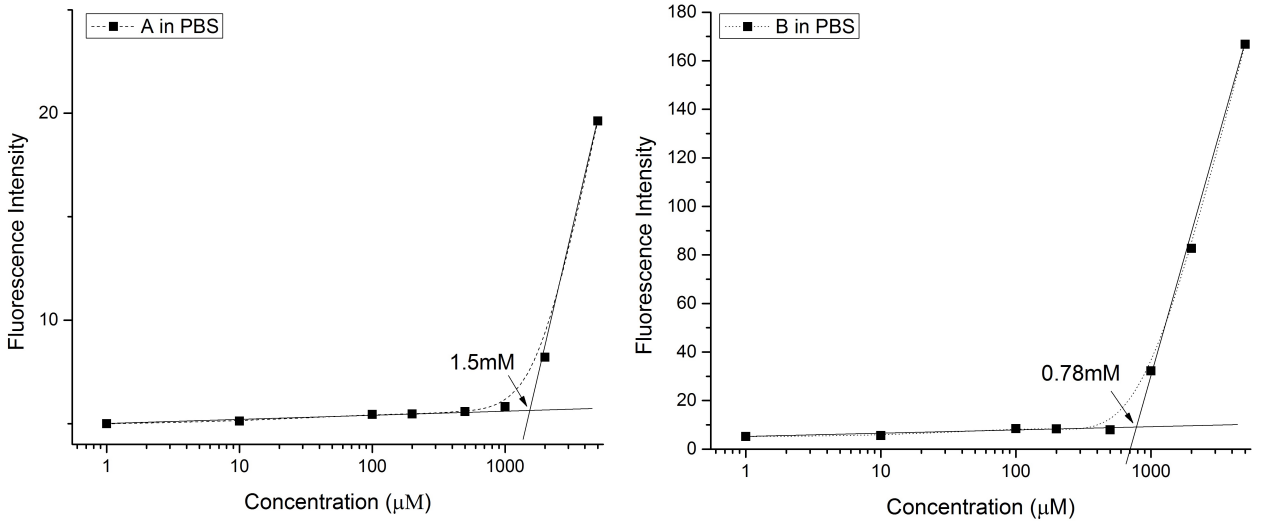


Figure S1. Plot of fluorescence intensity of Nile Red at 656 nm (Ex = 550 nm) versus the concentration (mM) of **A** and **B** in PBS buffer.

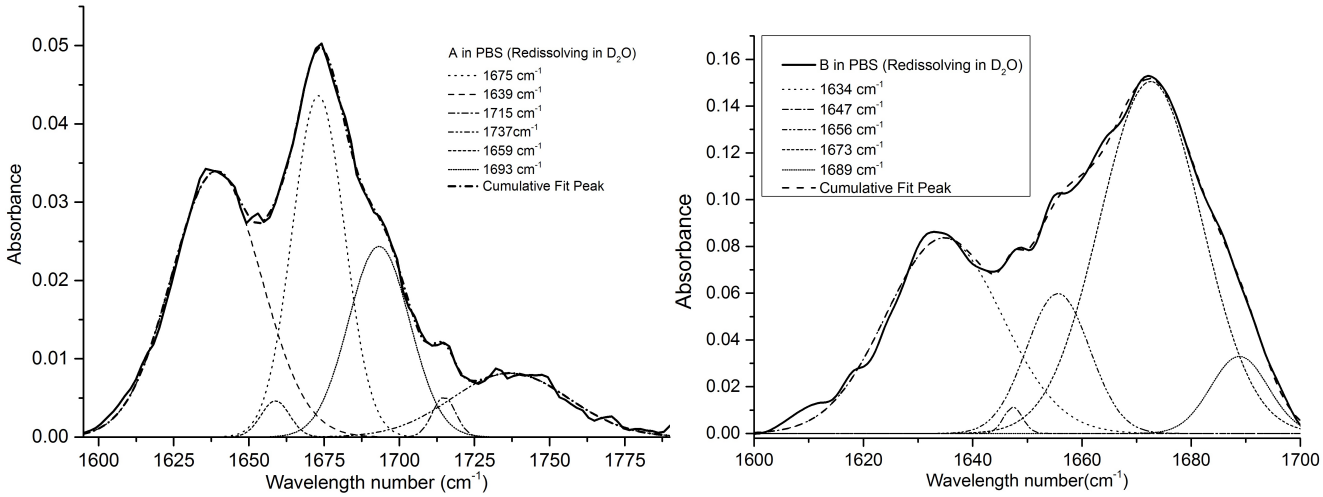


Figure S2. Deconvolution of FT-IR spectrum of **A** and **B**. The solution was prepared and aged in PBS (20 mM), lyophilized to eliminate the water, and redissolved in D_2O.

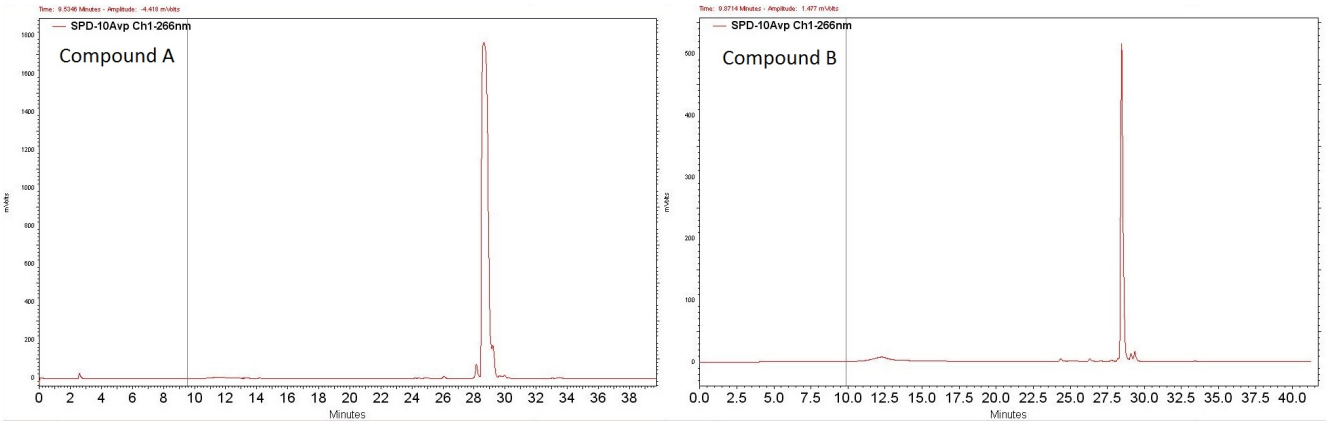


Figure S3. HPLC trace of purified **A** and **B** eluted with a linear gradient of CH_3CN/water containing 0.1% trifluoroacetic acid (TFA) in both H_2O and CH_3CN. The timeline was set to 0-60 min, 20-80% CH_3CN/Water.

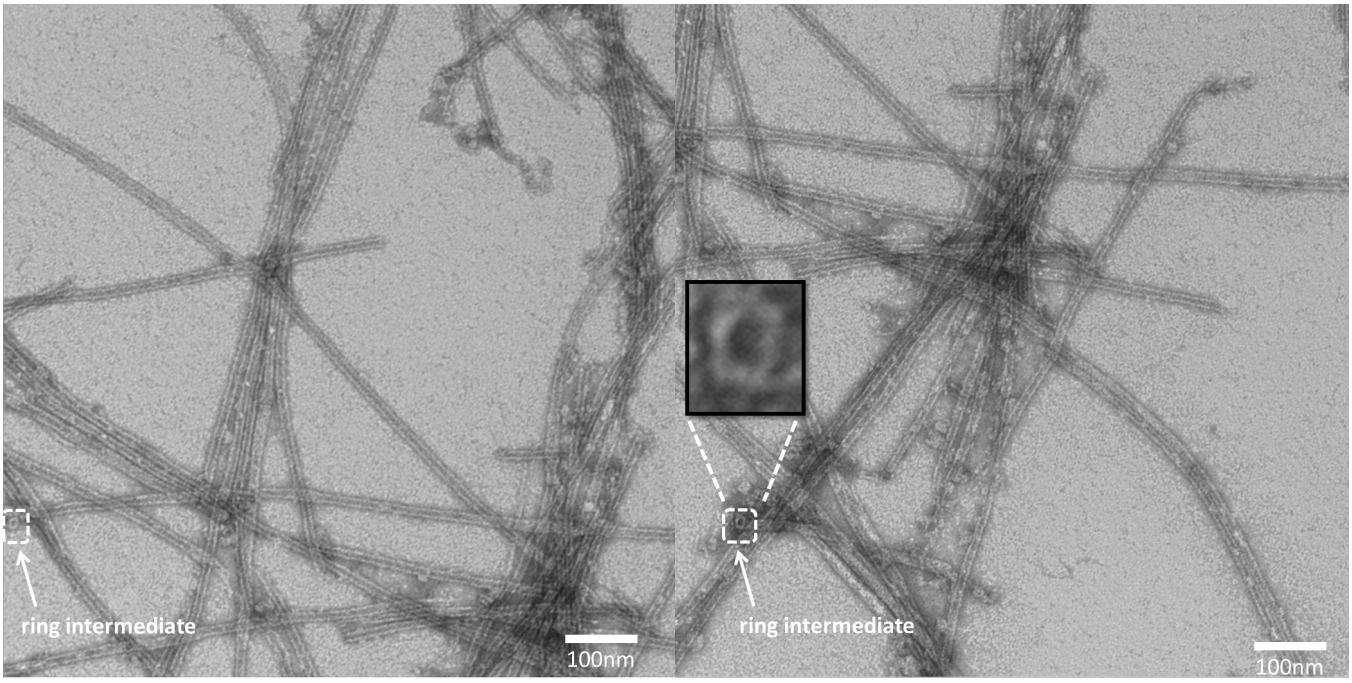


Figure S4. TEM images of **A** in PBS (pH 7.4) showing intermediate ring structure.

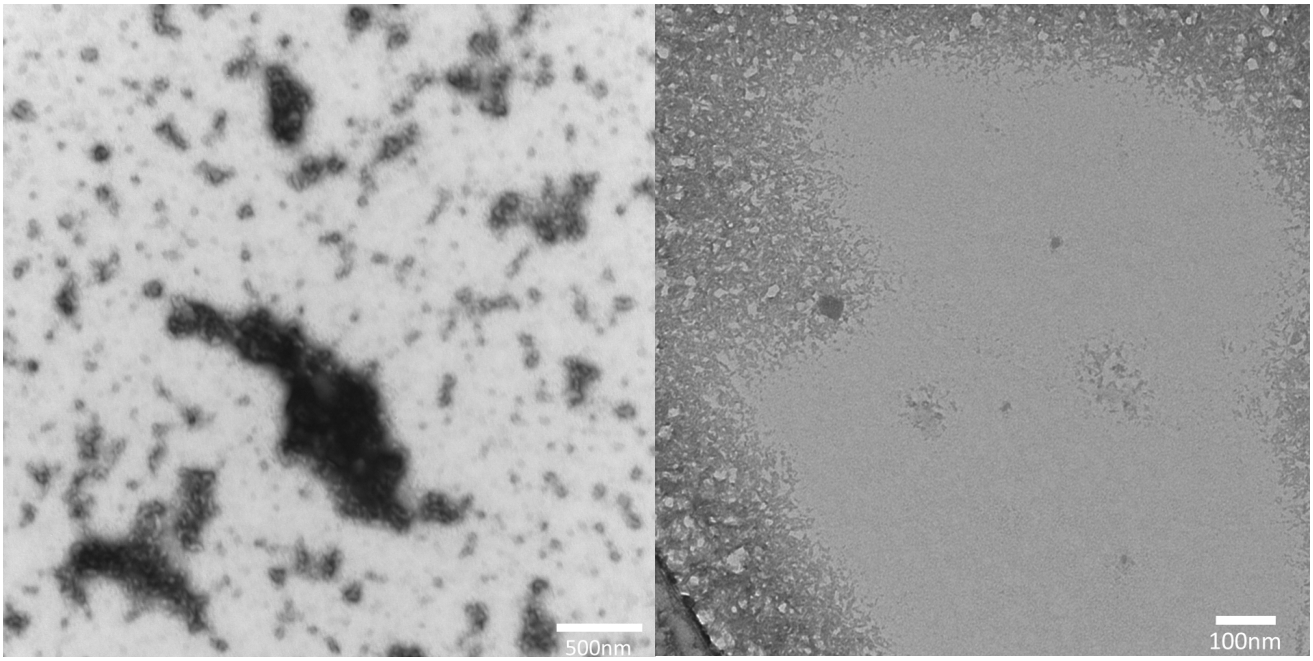


Figure S5. (Left) TEM image of **A** in PBS showing non-specific aggregates when aging at 0.25 mM; (Right) TEM image of **A** in TFE showing no self-assembly.

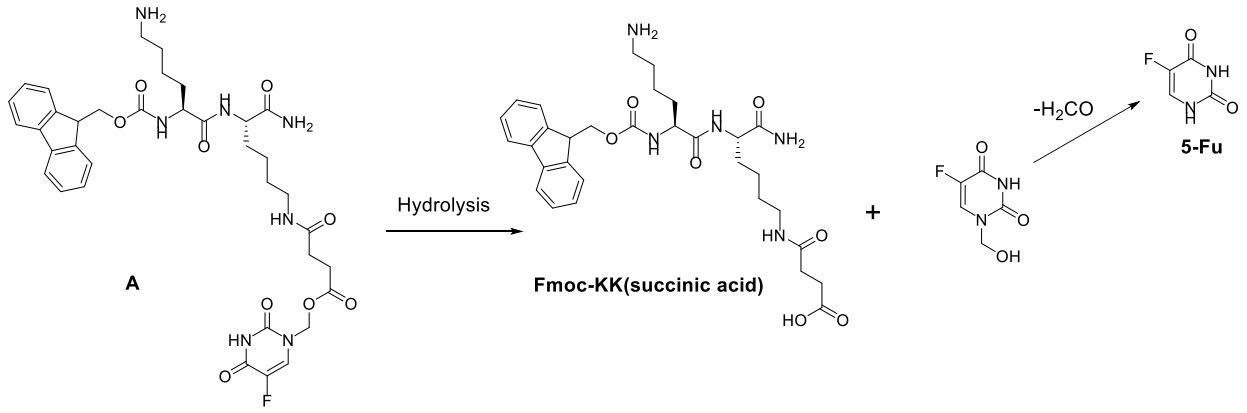


Figure S6. Proposed releasing mechanism of 5-Fu from **A** in PBS.

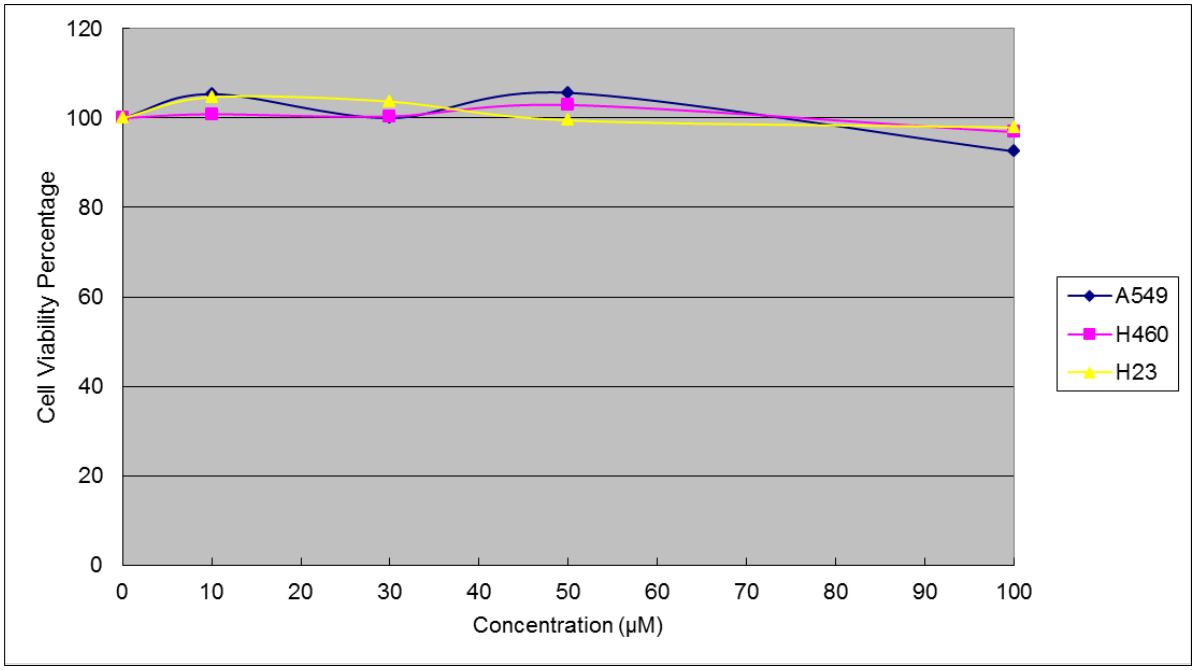


Figure S7. Cell viability of **Fmoc-KK(succinic acid)** against human non-small cell lung cancer cell lines A549, NCI-460, and NCI-H23.

UV-Vis Studies

Experiments were performed in a quartz cell with a 1 mm path length over the range of 190-800 nm. Samples were prepared from PBS (20 mM for A and 10mM for B) after 3 days incubation at room temperature and freshly diluted into 0.25 mM before the measurement.

Fourier Transform Infrared (FT-IR) Spectroscopy Measurement and Deconvolution Procedure

All FT-IR spectra were collected on a Nicolet FTIR spectrometer at ambient temperature. The instrument was continuously purged with CO_2-free dry air. Spectra were recorded between 1700 and 1600 cm^{-1} at a resolution of 4 cm^{-1}, and a total of 64 scans were averaged. Samples for FT-IR were first dissolved in PBS (20mM). A solution in PBS was freeze-dried to remove water and redissolved in D_2O. Spectra was analysed in a transmission cell having CaF_2 windows and a 0.025-μm path length. After subtracting the solvent spectra from the sample spectrum, the amide I band (1600-1700 cm^{-1}) of each spectrum was subjected to a fitting procedure using six Gaussian distribution (10 cm^{-1} full width at half maximum) centered at the frequencies based on Fourier self-deconvolution (FSD) spectra. During the fitting process, peak positions were constrained within limited interval, whereas the peak heights were not constrained. The fitting "module" of the Origin package was used for the fitting procedure. The contribution of each peak to the amide I band was quantified by the integrated areas of respective peaks.

Transmission Electron Microscopy Measurement – Negative Stain TEM

The 5-Fu-peptide sample in PBS (20 mM for **A** and 10 mM for **B**, pH 7.4) was prepared and aged for 1 day before the measurement. For the TEM microscopic studies, the samples were freshly diluted to 1 mM. Solutions (10 μL) in PBS and water were applied to carbon coated copper grid (Ted Pella, Inc.) for 2 min. After removing excess solution with filter paper, the grid was floated on 10 μL drops of 2 % wt uranyl acetate solution for negative staining for 1 min.

Scanning Electron Microscopy Measurement

Scanning Electron Microscopy (SEM) was performed with FEI Nova 400 Nano SEM instrument operating at 5.0 kV. The 5-Fu-peptide samples in PBS (20 mM for **A** and 10 mM for **B**, pH 7.4) was prepared and freshly diluted from to 1 mM before the measurement. A 20 μl aliquot of the resulting solution was dried at room temperature on a copper stubs and coated with gold.

Critical aggregation concentration (CAC) measurements

The critical aggregation concentration of **A** and **B** was characterized by fluorescence spectra using Nile Red as the fluorescence probe. The sample was prepared together with Nile Red at different concentrations in PBS with the same concentration of Nile Red (20 μM). Fluorescence spectra were then recorded at room temperature using a SHIMADZU RF-5301PC luminescence spectrometer. The excitation wavelength was set to 550 nm and the emission spectrum was recorded from 580 to 750 nm. Both excitation and emission slit widths were set to 5.0 nm. Upon the formation of micelle structures, the fluorescence intensity of Nile Red dramatically increased compared to non-encapsulated Nile Red, which would have a much lower fluorescence intensity. CAC values were calculated as the intersection of the tangents to the horizontal line of intensity ratio with relatively constant values and the diagonal line with rapidly increased intensity ratio.

5-Fluorouracil release experiment using analytical reversed-phase HPLC

The release of free 5-fluorouracil from **A** and **B** was measured by analytical reversed-phase HPLC (1 mL/min) under ambient temperature eluting with a linear gradient of CH_3CN/water containing 0.1% trifluoroacetic acid (TFA) in both H_2O and CH_3CN. For the release of 5-fluorouracil from hydrogel, compound **A** was prepared at 20 mM in PBS (without Ca^{2+} and Mg^{2+}) in a cylindrical vial (15 x 45 mm) and incubated at room temperature for 24 hours. The timeline was set to 0-30 min, 10-100% CH_3CN/Water 0.1%TFA. The presence of free 5-fluorouracil was detected by UV-Vis detector at 266 nm and confirmed by co-injecting 5-fluorouracil with **A** or **B**. The retention time (RT) of 5-FU is 4.10 min and the retention time of A and B was 24.81 min and 19.60 min.

Cell Culture and Reagent

Cytotoxicity assays was performed using human non-small cell lung cancer cell lines A549, NCI-H460, and NCI-H23, obtained from American Type Culture Collection (ATCC, Manassas, GA).

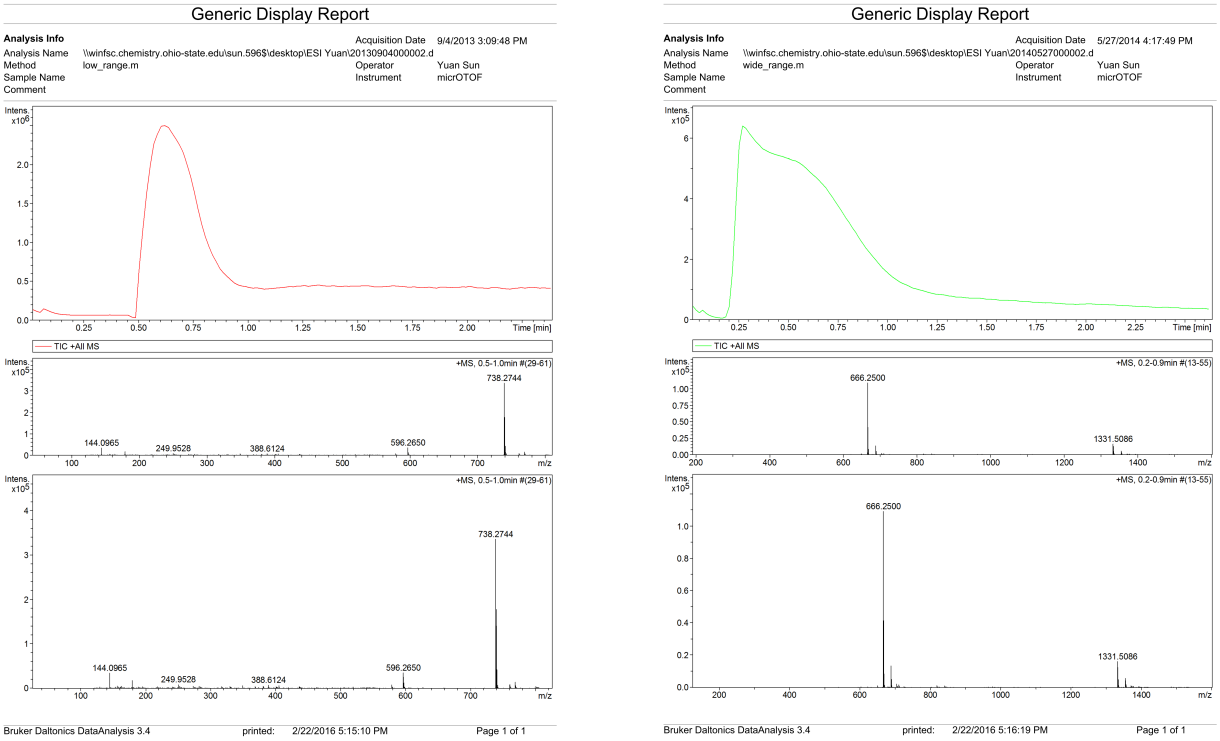
Human non-small cell lung cancer cell lines (A549, NCI-H460, and NCI-H23) were grown in RPMI-1640 medium (Sigma Aldrich) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and incubated in a humidified 37°C, 5% CO_2 environment. Compounds **A** and **B** were prepared at 10 mM in sterile PBS and allowed to age 3 days prior to experiments.

Cytotoxicity Assay

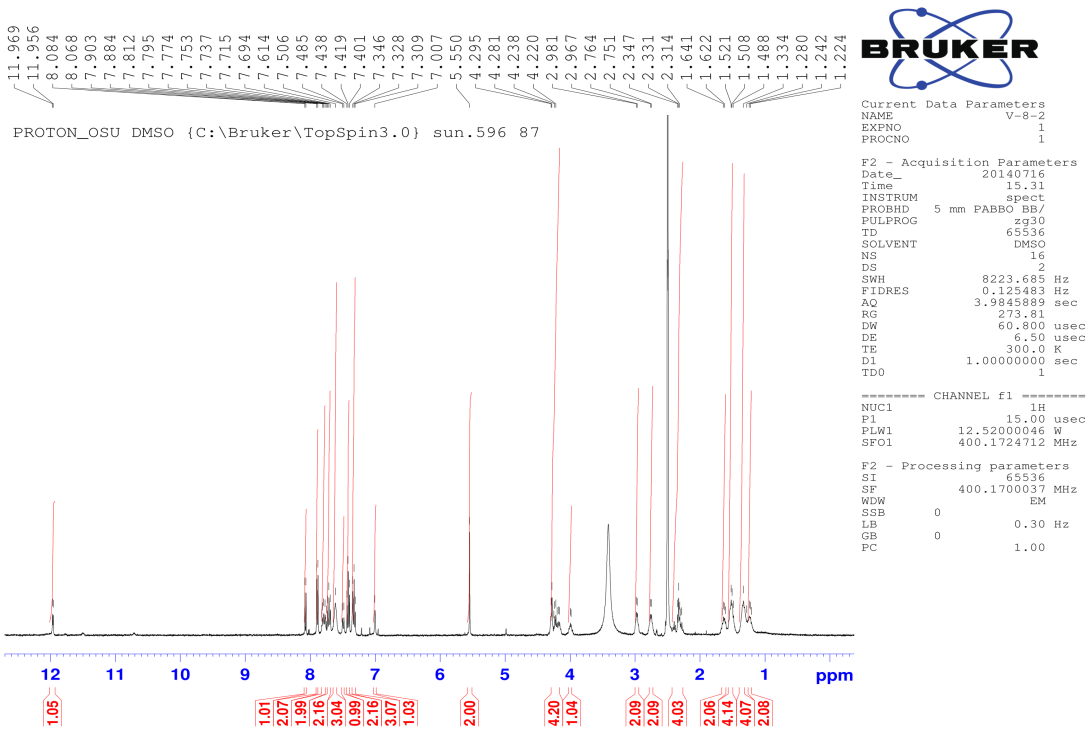
Human non-small cell lung cancer cell lines (A549, NCI-H460, and NCI-H23) cells were seeded in 48 well plates (1.0×10^4 cells/well) 24 h prior to the addition of drugs. Concentrations of drugs were 0, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 μM and cells were incubated at 37 °C under a humidified atmosphere of 5% CO_2 for 96 h prior to MTT viability analysis. Absorbance was measured with SpectraMax M2 Microplate Reader (Molecular Devices) and the effects of drug activity were evaluated by determining the 50% inhibition values (IC_{50}) as an average of three replicas. Each experiment was repeated at least 3 times.

Rheology Testing

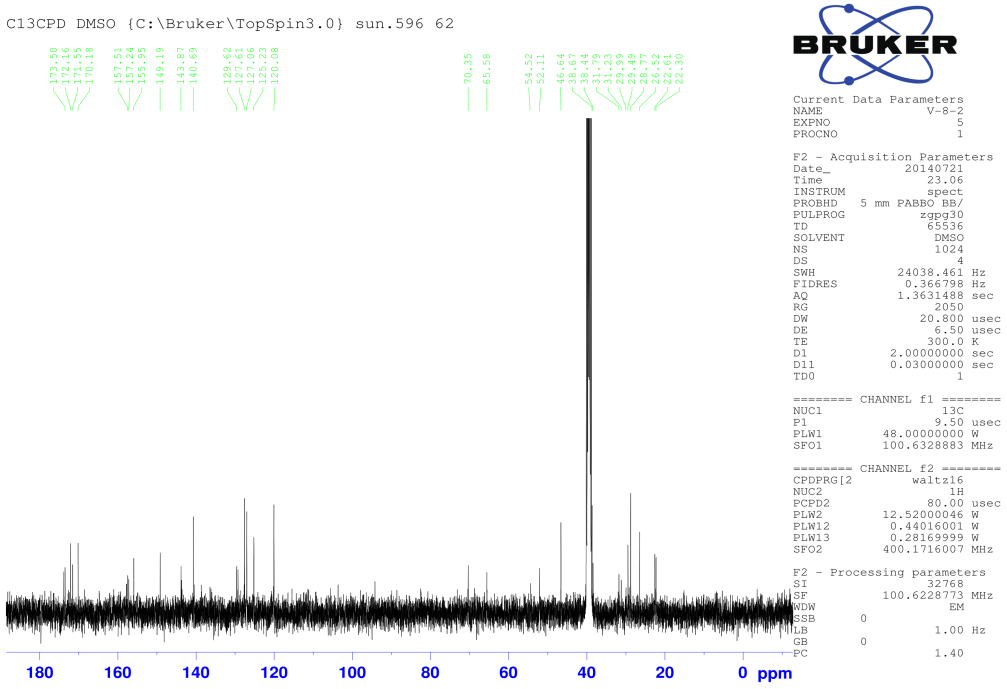
Compound **A** was prepared at 20 mM in PBS (without Ca^{2+} and Mg^{2+}) and incubated at room temperature for 24 hours. Compound **B** was prepared at 10 mM in PBS (without Ca^{2+} and Mg^{2+}) and incubated at room temperature for 24 h. Individual gel samples were removed from vials first using a scalpel blade, and then a curved spatula, to disengage them from the glass walls and bottom, respectively. The complex shear storage (G') and loss (G'') moduli of these self-assembled hydrogels were then measured as a function of oscillatory stress using a rheometer (AR1000, TA Instruments, Delaware) equipped with either 40-mm aluminum parallel plate (gels in pure water) or 12-mm stainless steel parallel plate geometries.



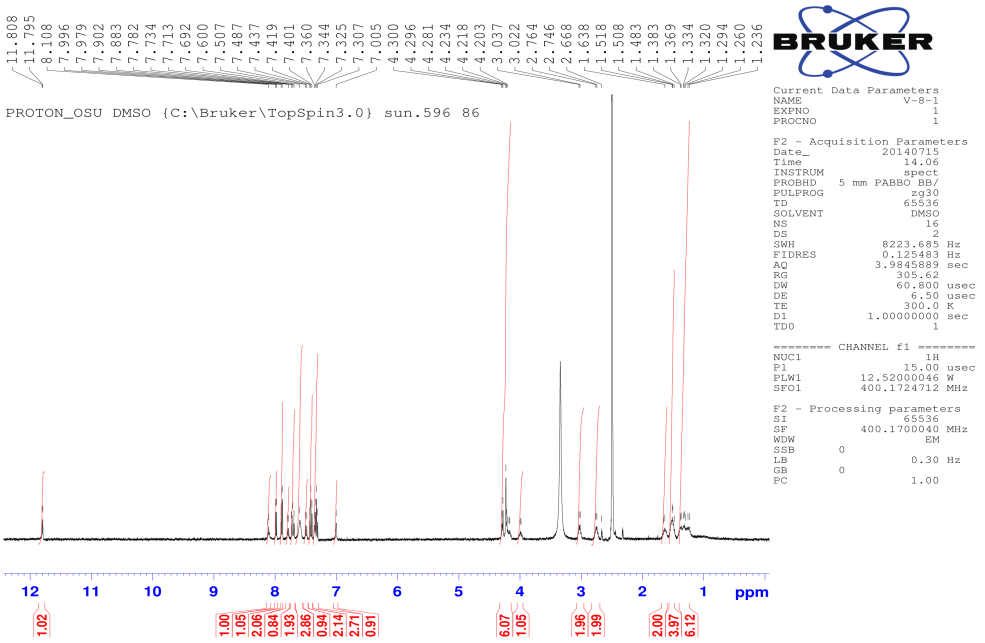
ESI-TOF-MASS spectrum of compound **A** (left) and **B** (right)



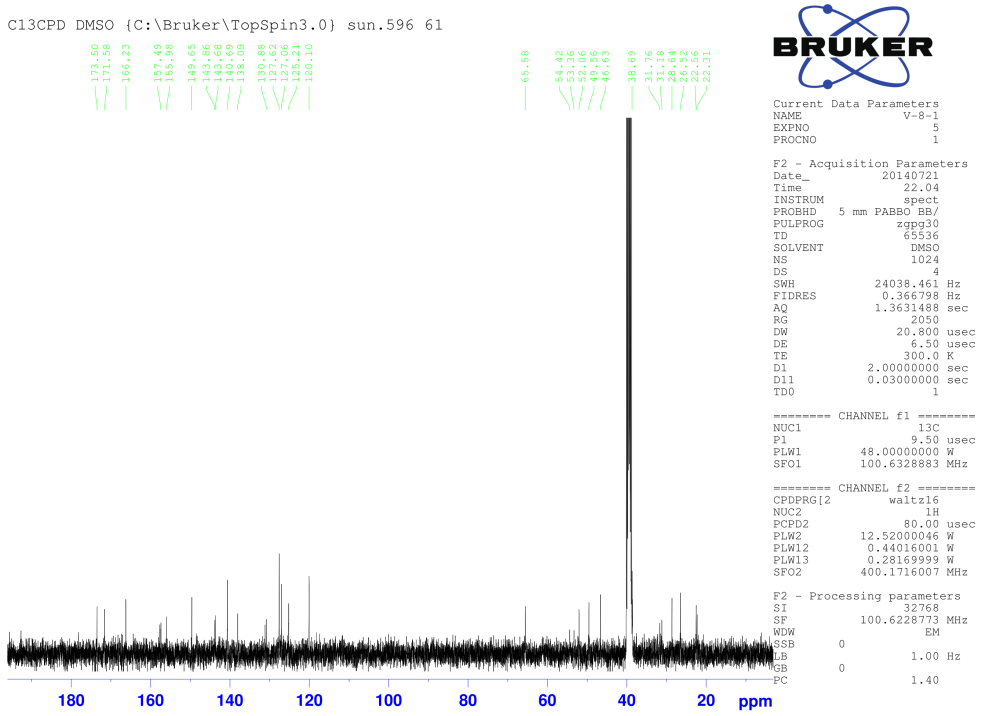
^{1}H NMR of Compound **A**



^{13}C NMR of Compound **A**



^{1}H NMR of Compound **B**



^{13}C NMR of Compound **B**

1. L. Ouyang, D. S. He, J. Zhang, G. He, B. Jiang, Q. Wang, Z. J. Chen, J. Z. Pan, Y. H. Li and L. Guo, *Bioorgan Med Chem*, 2011, **19**, 3750-3756.
2. A. Lundgren, Y. Hed, K. Oeberg, A. Sellborn, H. Fink, P. Lowenhielm, J. Kelly, M. Malkoch and M. Berglin, *Angew Chem Int Edit*, 2011, **50**, 3450-3453.