Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supporting Information

Self-Assembly of a 5-Fluorouracil and Camptothecin Dual Drug Dipeptide Conjugate

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Experimental Section.

General Methods. Atomic force microscopy (AFM) was conducted on a Bruker AXS Dimension Icon Atomic Force Microscope. Transmission Electron Microscopy (TEM) was carried out with Technai G2 Spirit instrument operating at 80 keV. ^{1}H and ^{13}C NMR were recorded at 400 MHz on a Bruker Avance III instrument. ESI mass spectra were recorded on a Bruker MicrOTOF coupled with HPLC. Circular dichroism were performed in a JASCO J-815 CD Spectrometer under nitrogen atmosphere in a 1 mm path length quartz cuvette at 25°C. Fluorescence excitation and emission experiments were performed on a Shimadzu RF-5301PC Spectrofluorophotometer in 3mm path length quartz cuvette. UV-vis experiments were performed on a Shimadzu UV-2450 Spectrophotometer in a 1 mm cuvette. FTIR spectra were obtained from a SHIMADZU IR Affinity-1 fourier transform infrared spectrophotometer and deconvoluted using Origin 8 software.

Atomic Force Microscopy (AFM): AFM images were collected on Bruker AXS Dimension Icon Atomic Force Microscope in ScanAsyst mode using the SAA-HPI-SS probe from Bruker with a 1 nm tip end radius and a 0.25 N/m spring constant. Samples (1 mM) were deposited onto freshly cleaved mica for 5 min, rinsed with water for 10 s, then dried with nitrogen gas. Images were analyzed with Bruker's software.

Transmission Electron Microscopy (TEM): Samples (1 mM) were dropped on carbon-coated copper grids (Ted Pella, Inc.) for 1 min. After removal of excess solution, the sample grid was negatively stained with 2% (w/w) uranyl acetate solution for 30 s and rinsed with water. The dried specimen was observed with Technai G2 Spirit TEM instrument operating at 80 keV. Images were analyzed with FIJI imaging software.

Critical micellar concentration (CMC) measurements

Critical micelle concentrations were characterized by fluorescence using Nile Red as the fluorescence probe. The assembled compound **1** (aged at 20 mM in PBS for 72 h) was diluted to concentrations of ranging from 1 mM to 0.1 μM, then 50 μL of 20 μM Nile Red in acetone was added to each sample. After 5 minutes, fluorescence spectra were recorded at room temperature using a SHIMADZU RF-5301PC spectrometer. The excitation wavelength was set to 550 nm and the emission spectrum was recorded at 656 nm. Both excitation and emission slit widths were set to 1.5 nm.

Critical aggregation concentration (CAC) measurements

Critical aggregation concentrations were characterized by recording CPT fluorescence at 425 nm upon excitation at 360 nm. Both compounds **1** and **2** were assembled at 10 mM in PBS for 72 h and diluted to concentration ranging from 1 mM to 0.1 μM, samples incubated for 12 h at each concentration before measurements were taken. Fluorescence spectra were taken at room temperature using a SHIMADZU RF-5301PC spectrometer, both excitation and emission slit widths were set to 1.5 nm. The fluorescence value at 425 nm was corrected for by concentration and plotted to determine the CAC.

Drug Release Measurements in PBS by HPLC

The release profile of 5-Fu and CPT from self-assembled nanotubes of **1** were measured by analytical reversed-phase HPLC (1.6 mL/min) under ambient temperature eluting with a gradient of KH_2PO_4/water (pH 5.0). The timeline was set to 0-50 min, starting with 10 minutes of 99%

KH_2PO_4, followed by a linear gradient to 80% H_2O, using detection wavelengths of 266 nm, 254 nm, and 365 nm. An internal standard of benzophenone was used to measure the concentrations of **1**, **2**, CPT, and 5-FU. Peptide **1** was dissolved in PBS at 20 mM and aged 72 h to form the assembled nanotubes Aliquots were taken and diluted with PBS to the desired concentration at 37°C then monitored for 5 days by analytical reversed-phase HPLC. Drug release was measured in human serum by diluting pre-assembled samples in PBS in human serum then incubating at 37 °C. A 10 μL aliquot from each sample was withdrawn, diluted with 120 μL cold methanol to precipitate the protein, and centrifuged at 5000 rpm for 5 minutes. Forty microliters of supernatant were then analyzed by HPLC for lactone stability in human serum each day for 5 days.

Cell Culture and Reagents

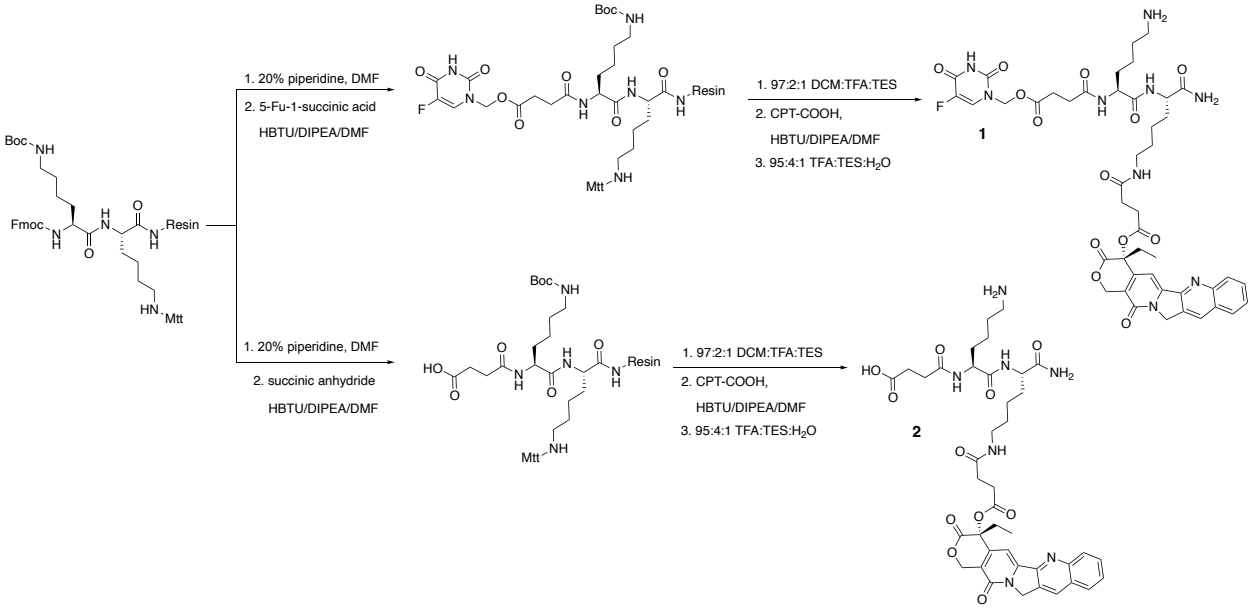
Cytotoxicity assays were performed using human non-small cell lung cancer cell lines (H116 and SW620). Peptide **1** was prepared at 20 mM in sterile PBS and allowed to age 3 days prior to experiments. Immediately prior to experiments, *S*-camptothecin (95%, Sigma-Aldrich) was dissolved in dimethyl sulfoxide. Concentrations 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. Absorbances were measured with a SpectraMax M2 Microplate Reader (Molecular Devices) and the effects of compound 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.

Peptide Synthesis The peptides **1** and **2** were manually prepared using Fmoc/*t*-Bu solid-phase peptide synthesis on rink amide resin (loading 0.8 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 DMF for 6-24 h. A solution of 20% piperidine in DMF was used for Fmoc removal and 2% TFA in DCM with triethylsilane (TES) (DCM:TFA:TES=97:2:1) was used for Mtt group deprotection. The coupling of CPT and 5-FU was achieved by adding a mixture of CPT-CO_2H ((R)-4-((4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1H-pyrano[3',4':6,7]indolizino[1,2-b]quinolin-4-yl)oxy)-4-oxobutanoic)[1] and 5-fluorouracil-1-succinic acid (4-((5-fluoro-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)methoxy)-4-oxobutanoic acid)[2] with HBTU, and DIPEA (300 mol% each relative to resin) in minimal amount of DMF to resin and shake for 12-24 h under room temperature. The resin was washed thoroughly (2 x DCM, 2 x DMF, then 2 x DCM) after each coupling. The final dual drug peptide was cleaved from the resin by the treatment with TFA/water/triethylsilane (95/1/4) at room temperature for 4 h. The final dual drug peptide was cleaved from the resin by the treatment with TFA/water/triethylsilane (95/1/4) at room temperature for 4 h. The crude product was precipitated with cold diethyl ether and purified by reversed-phased HPLC on XBridge Prep C8 column eluting with a linear gradient of CH_3CN/water containing 0.1% TFA (10/90 to 95/5 over 40 minutes) and stored as lyophilized powers at 0°C. Compound purity was assessed by analytical reverse-phase HPLC, and the identity was confirmed using ESI-TOF mass spectrometry and NMR. Peptide **1**: ^{1}H NMR (400 MHz, DMSO-d^6) δ 0.92 (t, *J*= 7.4 Hz, 3H, C$\underline{H}_3$CH$_2$-), 1.25-1.75 (m, 12H, (-C$\underline{H}_2$-C$\underline{H}_2$-C$\underline{H}_2$-CH$_2$-NH$_2$) x 2), 2.1 (m, 2 H, CH$_3$-C$\underline{H}_2$-), 2.25-2.51 (m, 6H, succinate linker (C$\underline{H}_2$-C$\underline{H}_2$) x 2), 2.6-2.71 (m, 4H, succinate C$\underline{H}_2$ + C$\underline{H}_2$-NH$_2$), 3.0 (m, 2H, C$\underline{H}_2$-NH$_2$), 4.1 (m, 1H, HN-C$\underline{H}$(CH$_3$)-C(O)), 4.2 (m, 1H, HN-C$\underline{H}$(CH$_3$)-C(O)), 5.3 (ABq, *Jab* = 17 Hz, 2H, N-C$\underline{H}_2$-C=C (CPT)), 5.4 (ABq, *Jab* = 19 Hz, 2H, O-C$\underline{H}_2$-C=C, CPT), 5.5 (s, 2H, N-C$\underline{H}_2$-O, 5-FU linkage), 6.95 (bs, 1H, exchangable) 7.10 (s, 1H arom C-$\underline{H}$, CPT), 7.25 (s, 1H,

exchangable), 7.60 (bs, 2H, N<u>H</u>$_2$), 7.75 (m, 2H, arom C-<u>H</u>, N<u>H</u>), 7.80 (m, 2H, arom C-<u>H</u>, N<u>H</u>), 8.05 (m, 2H, 5-FU, N<u>H</u>), 8.1-8.2 (m, 2H, arom C-<u>H</u>,), 8.7 (s, 1 H, arom C-<u>H</u>), 11.99 (bs, 0.5 H, N<u>H</u> (5-FU)). ^{13}C NMR (400 MHz, DMSO-d^6) 8.03, 22.6, 23.2, 27.0, 29.3, 29.4, 30.1, 30.8, 34.0 (~3 isochronous peaks), 39.0, 39.2 (1 overlapping, under DMSO peak), 50.7, 51.8, 52.6, 52.9, 66.7, 76.3, 95.7, 119.3 128.2, 128.4, 129.0, 129.4, 129.7, 130.3, 130.9, 132.0, 145.9, 146.4, 148.4, 149.7, 152.9, 157.0, 158.3, 158.6, 167.7, 170.4, 171.8, 172.0, 172.6 173.4, 174.0. ESI-MS calculated for $C_{45}H_{53}FN_9O_{13}$ $[M+H]^+$ 946.3747, found 946.3650.

Peptide **2**: ^{1}H NMR (DMSO d^6, 400 MHz): δ0.84 (3H, t, *J* = 7.4 Hz, C<u>H</u>$_3$CH$_2$-), 1.07-1.65 (m, 12H, (-C<u>H</u>$_2$-C<u>H</u>$_2$-C<u>H</u>$_2$-CH$_2$-NH$_2$) x 2), 2.06 (m, 2H, CH$_3$-C<u>H</u>$_2$-), 2.25-2.38 (m, 6H, succinate linker (C<u>H</u>$_2$-C<u>H</u>$_2$) x 2), 2.55-2.75 (m, 4H, succinate C<u>H</u>$_2$ + C<u>H</u>$_2$-NH$_2$), 2.89-2.98 (m, 2H, C<u>H</u>$_2$-NH$_2$), 4.12 (m, 1H, HN-C<u>H</u>(CH$_3$)-C(O)), 4.00 (m, 1H, HN-C<u>H</u>(CH$_3$)-C(O)), 5.23 (s, 2H, N-C<u>H</u>$_2$-C=C (CPT)), 5.42 (s, 2H, O-C<u>H</u>$_2$-C=C, CPT), 6.89 (s, 1H, exchangable NH), 7.07 (s, 1H, arom C-H, CPT), 7.05 (s, 1H, exchangable NH), 7.55 (bs, 2H, N<u>H</u>$_2$), 7.65 (m, 2H, arom C-H, NH), 7.8 (m, 2H, arom C-H, CPT, NH), 7.95 (d, *J* = 7.7, 1H, NH), 8.05 (d, *J* = 7.8 Hz, 1H, arom C-H), 8.10 (d, *J* = 7.8 Hz, 1H, arom C-H), 8.65 (s, 1H, arom C-H, CPT), 11.75 (bs, 1H, CO$_2$<u>H</u>). ^{13}C NMR (151 MHz, DMSO) δ 8.0, 22.6, 23.3, 27.0, 27.2, 29.3, 29.4, 30.1, 30.8, 31.7, 32.0, 39.0, 39.2, 39.6, 50.7, 52.7, 52.9, 66.7, 76.3, 95.7, 119.3, 128.2, 128.5, 129.0, 129.4, 130.3, 131.0, 132.1, 146.4, 148.4, 152.9, 157.1, 158.3, 158.5, 167.8, 170.5, 171.9, 172.0, 174.0,174.4. $C_{40}H_{50}N_7O_{11}$ $[M+H]^+$ 804.3568, found 804.3558.



Scheme S1: Solid-phase peptide synthesis of **1** and **2** on rink amide resin.

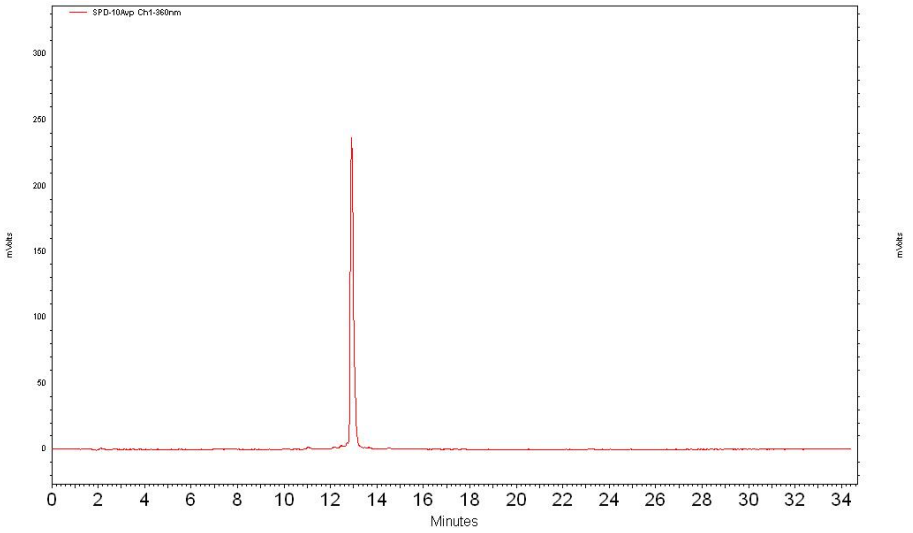


Figure S1. Analytical HPLC trace (1 mL/min, ramped from 10-70% CH_3CN in water with 0.1 %TFA) of **1** after purification via preparatory HPLC.

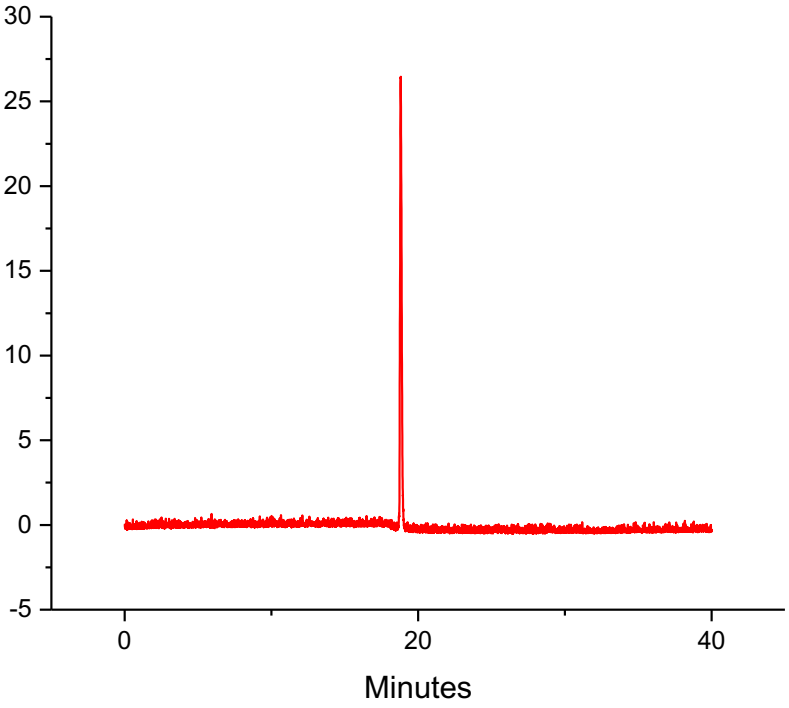


Figure S2. Analytical HPLC trace (1 mL/min, ramped from 15-80% CH_3CN in water, spiked with 0.1% TFA) of **2** after purification via preparatory HPLC.

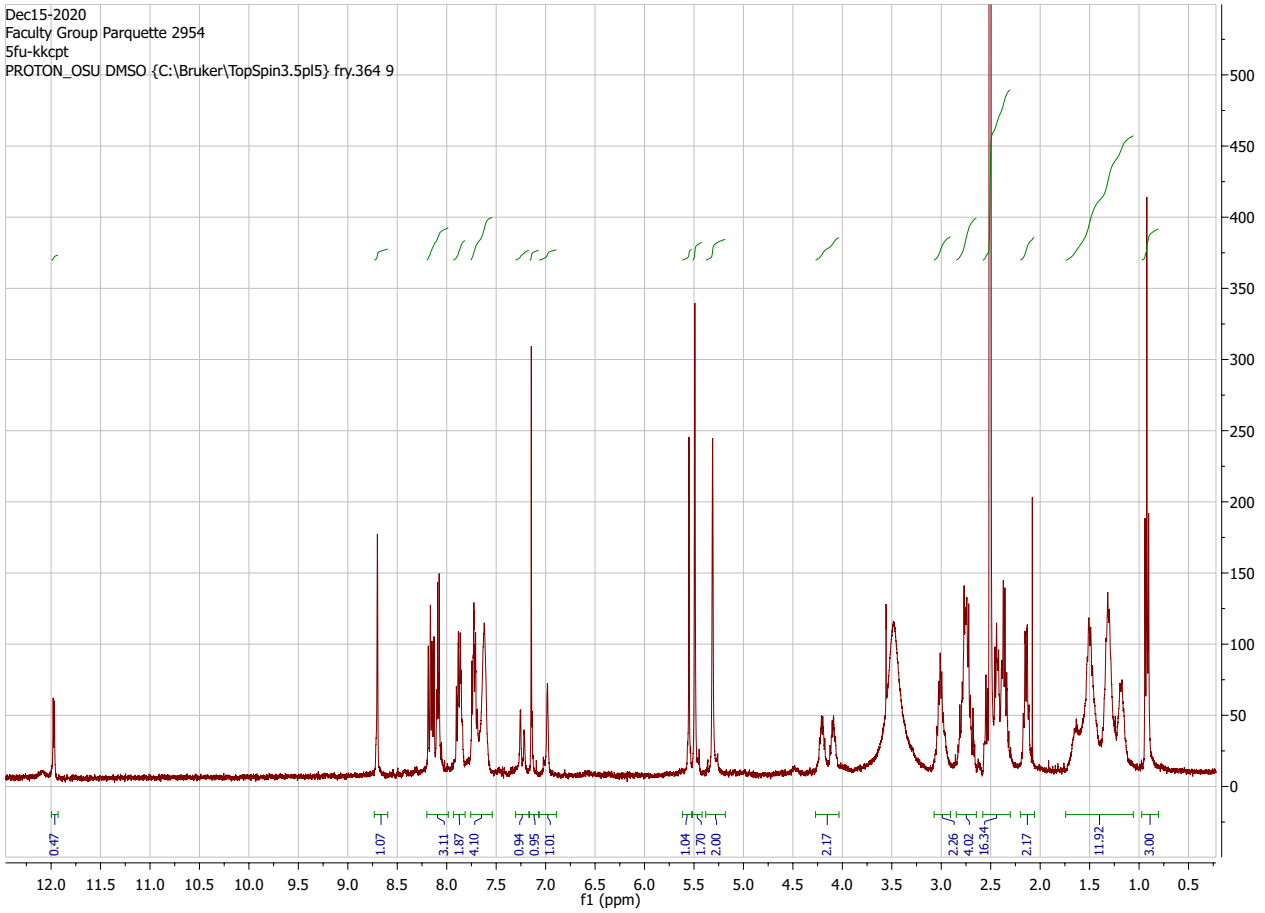


Figure S3: ^{1}H-NMR spectra of **1** in DMSO-d^{6}

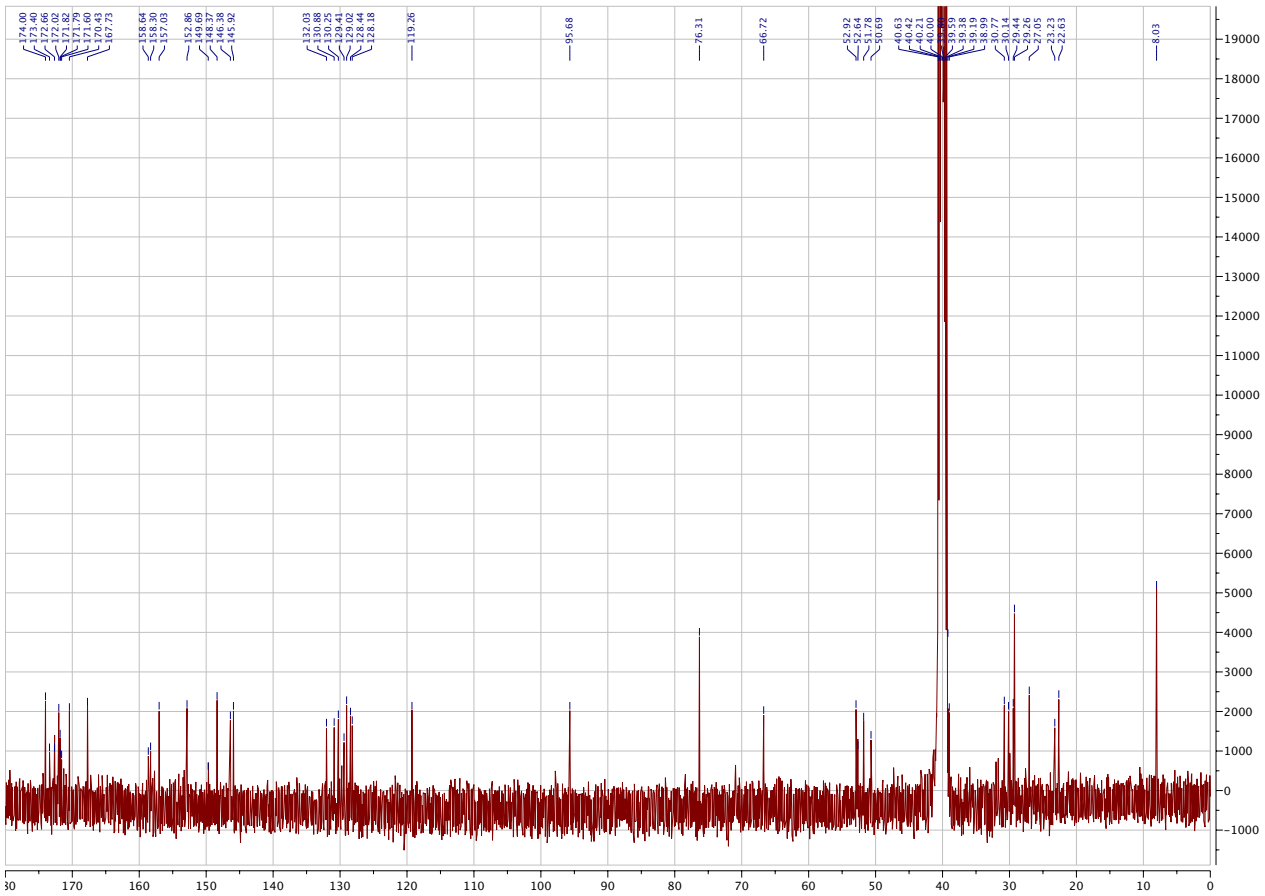


Figure S4: ^{13}C-NMR spectra of **1** in DMSO-d^{6}

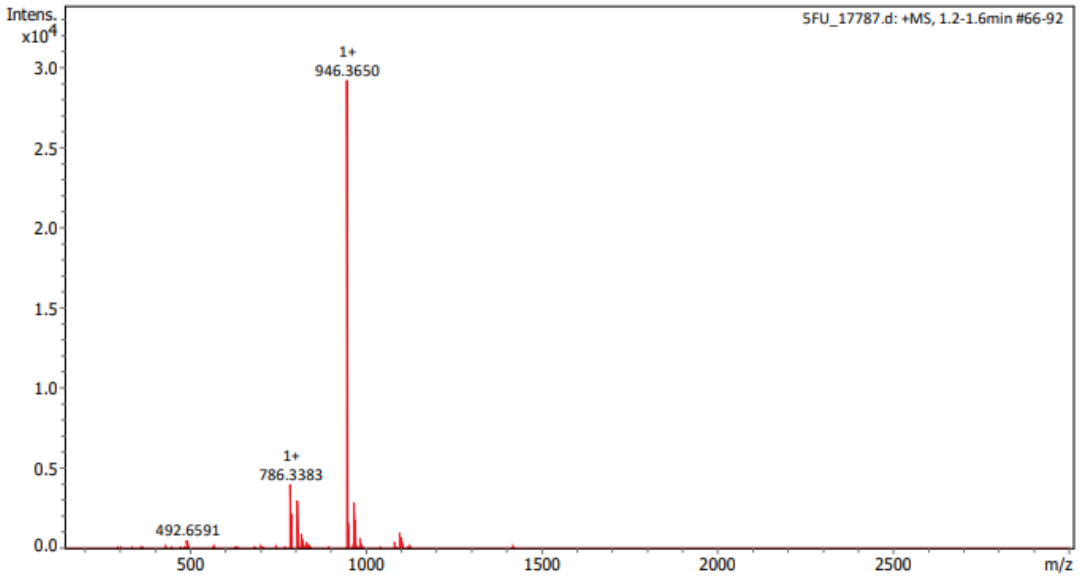


Figure S5: ESI High-resolution mass spectrum of **1,** obtained in positive mode with 80/20 MeCN/water.

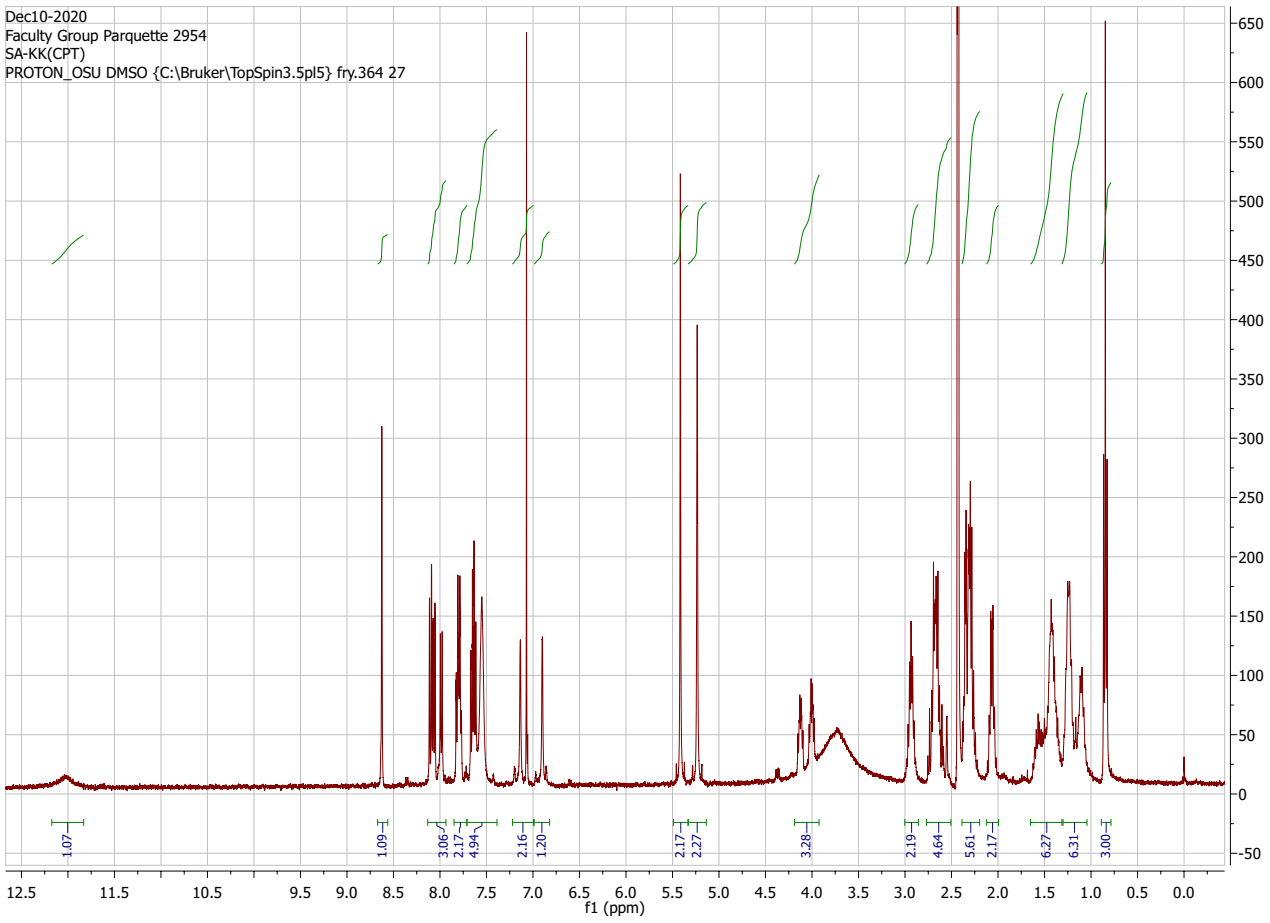


Figure S6: ^{1}H-NMR spectra of **2** in DMSO-d^{6}.

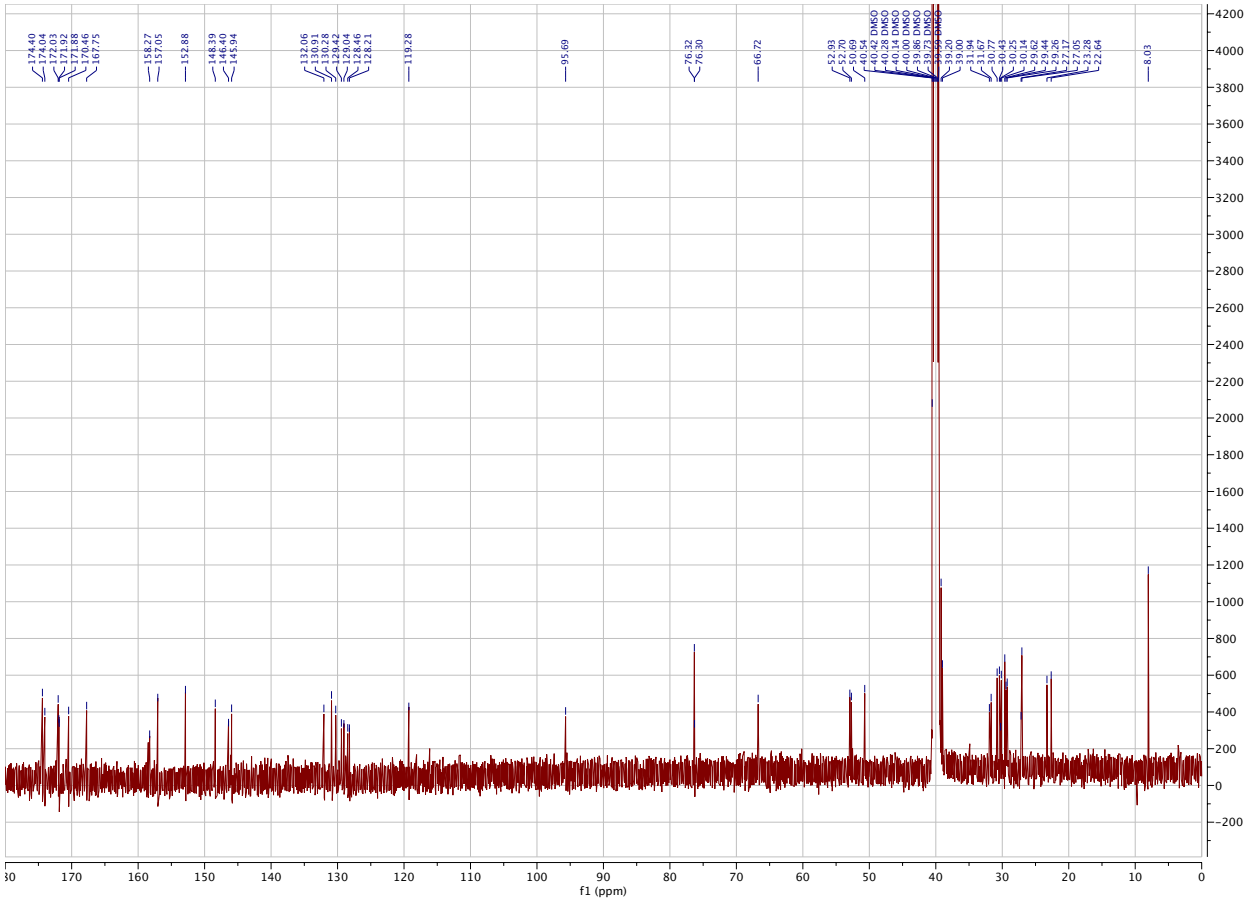


Figure S7: ^{13}C-NMR spectra of **2** in DMSO-d^{6}. Significant aggregation at the concentration needed for 13C NMR produced additional peaks in the spectrum.

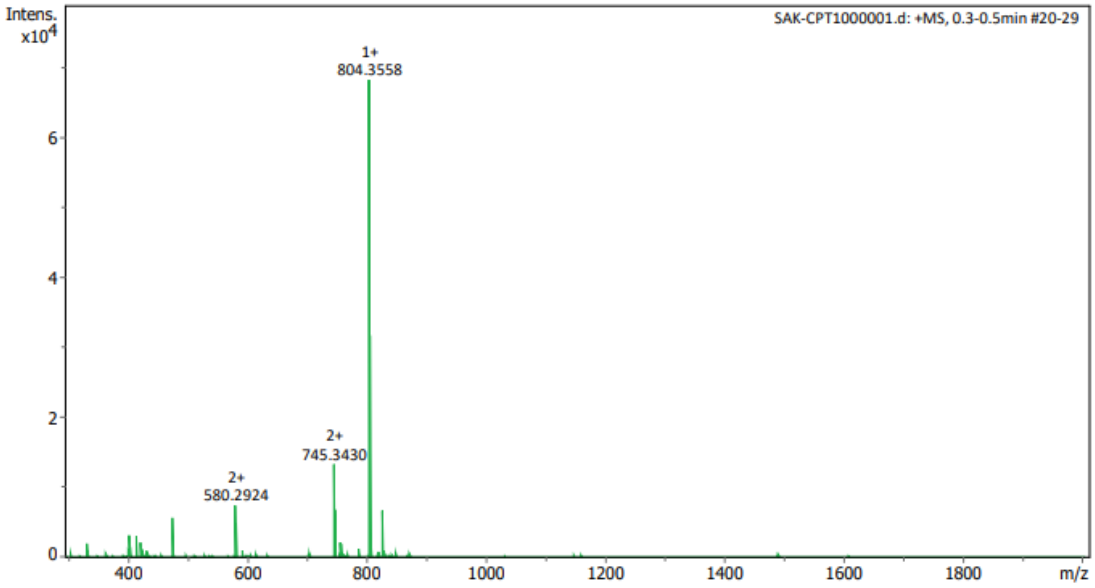


Figure S8: ESI High-resolution mass spectrum of **2,** obtained in positive mode with 80/20 MeCN/water.

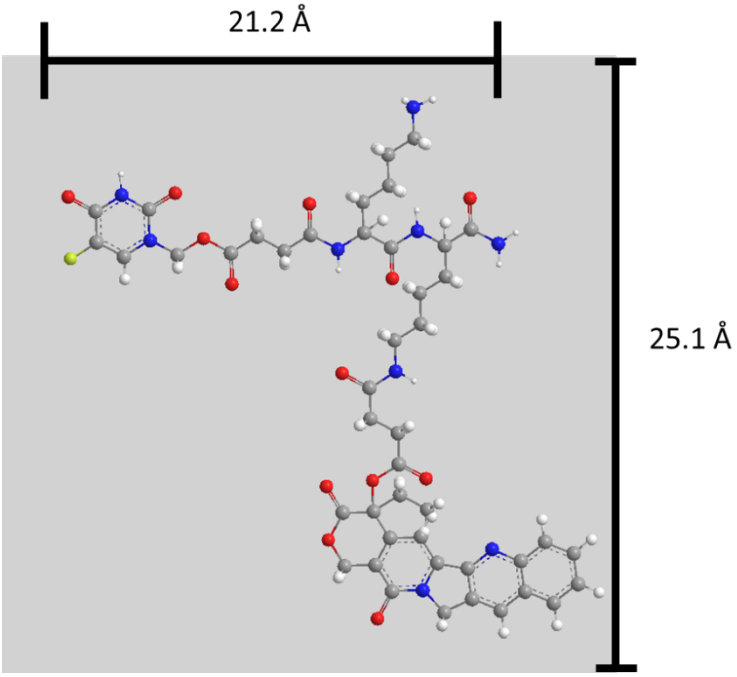


Figure S9: Rendering of the extended conformation of monomer **1**, with measurements.

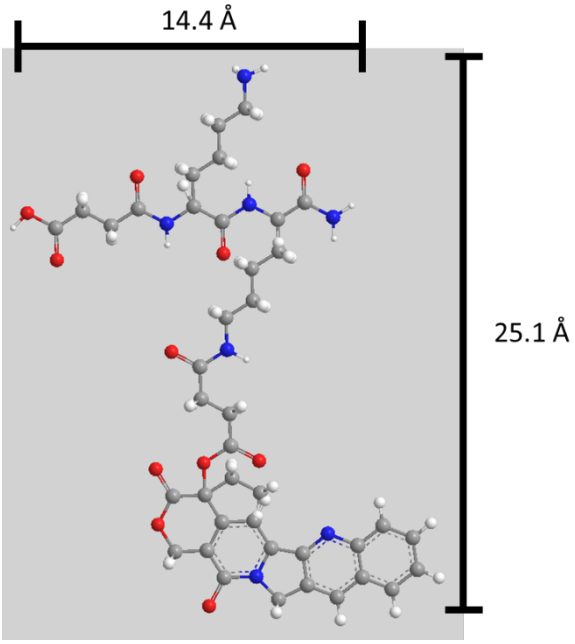


Figure S10: Rendering of the extended conformation of monomer **2**, with measurements.

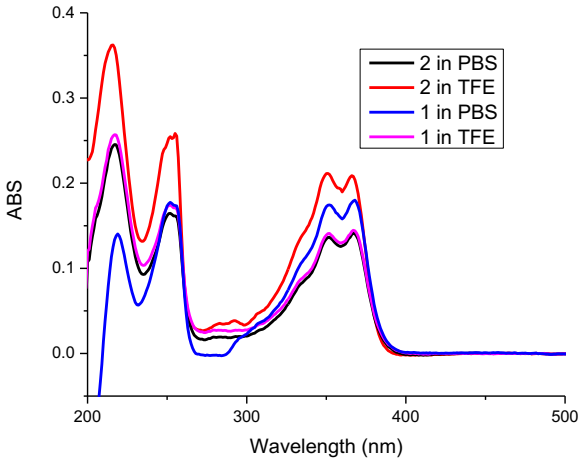


Figure S11: UV-Vis absorption spectra of peptides **1** and **2** in PBS and TFE. Each sample was aged in TFE or PBS at 10 mM for 72 h, then were diluted to 0.1 mM to record the spectra.

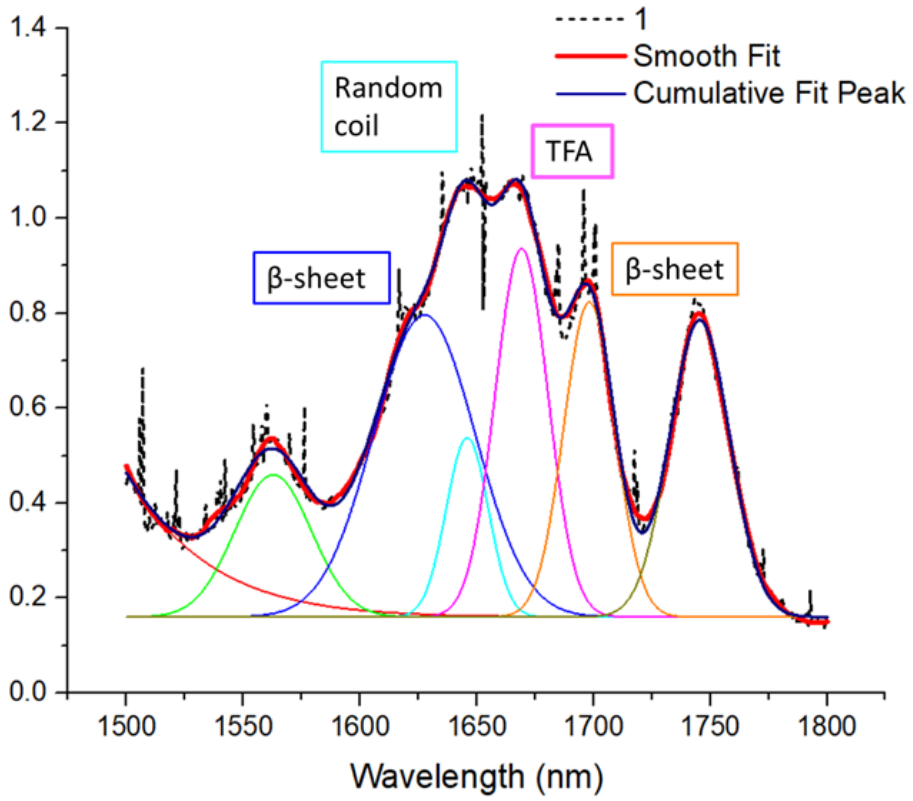


Figure S12: Deconvoluted FT-IR spectrum of **1** in (PBS, D_2O). Sample was prepared by assembling at 20 mM in PBS for 72 h followed by two cycles of lyophilization and resuspension in D_2O. The deconvoluted spectrum indicates the presence of β sheet and random coil (~86% β character and 14% random coil).

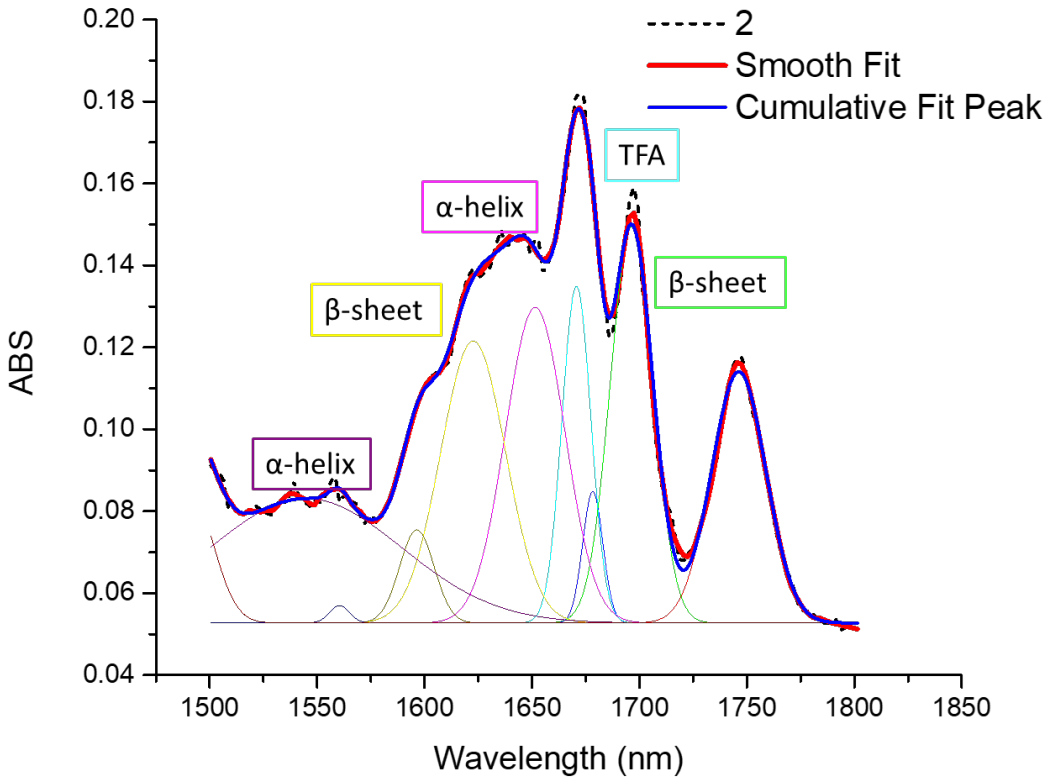


Figure S13: Deconvoluted FT-IR spectrum of **2** in (PBS, D_2O). Sample was prepared by assembling at 10 mM in PBS for 72 h followed by two cycles of lyophilization and resuspension in D_2O. The deconvoluted spectrum indicates the presence of β sheet and random coil (~53% β character and 47% α-helix).

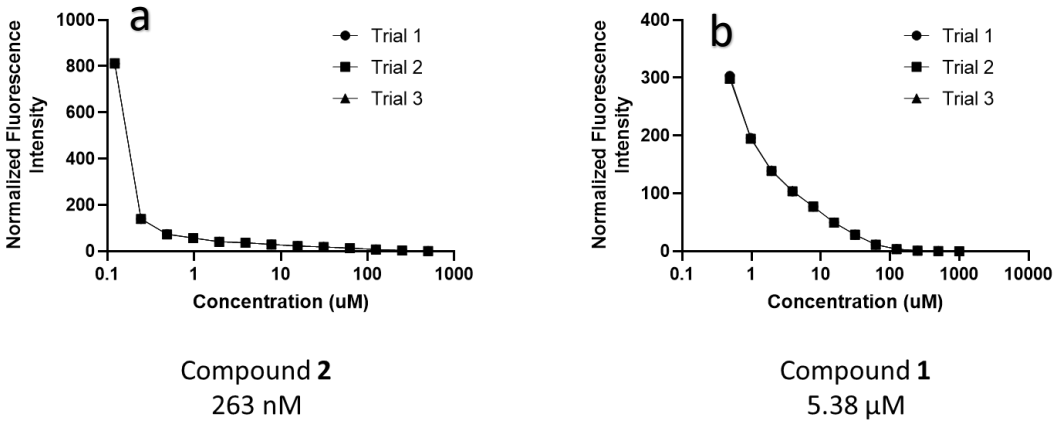


Figure S14: CAC plots for the determination of the CAC for compound **2** (a) and **1** (b) using CPT florescence. Samples of both **1** and **2** were aged at 10 mM in PBS (pH 7.4) for 72 h before dilution to concentrations for analysis. The CAC of compound **1** was found to be 5.38±0.01 µM. The CAC of compound **2** was found to be 263 nM.

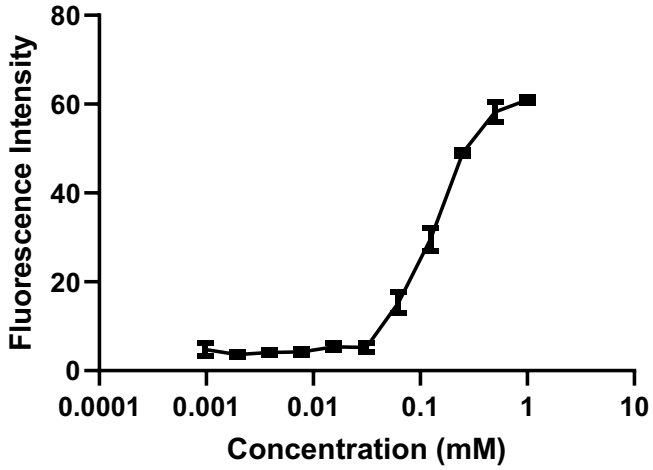


Figure S15: CMC plot for compound **1** using Nile red (50 µL of 20 µM Nile Red in acetone was added to each sample) as a florescent probe. Compound **1** was aged at 10 mM in PBS (pH 7.4) for 72 h before dilution to concentrations for analysis. The CMC of compound **1** was found to be 38 ± 3 µM.

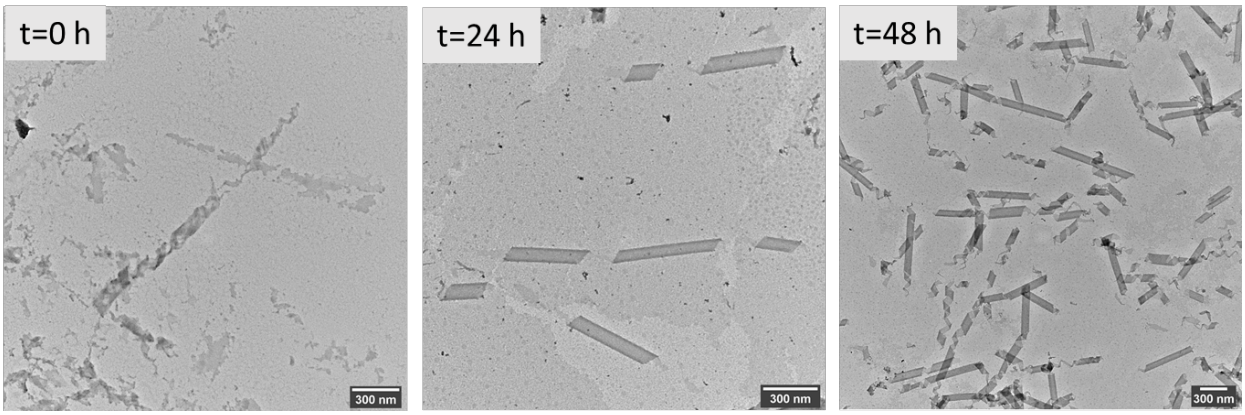


Figure S16: TEM images of **1** during the assembly process. Samples were aged for 72 h at 20 mM in PBS (pH 7.4). Aliquots were removed for imaging at various time points after dissolution in PBS. Samples were diluted to 1 mM prior to imaging.

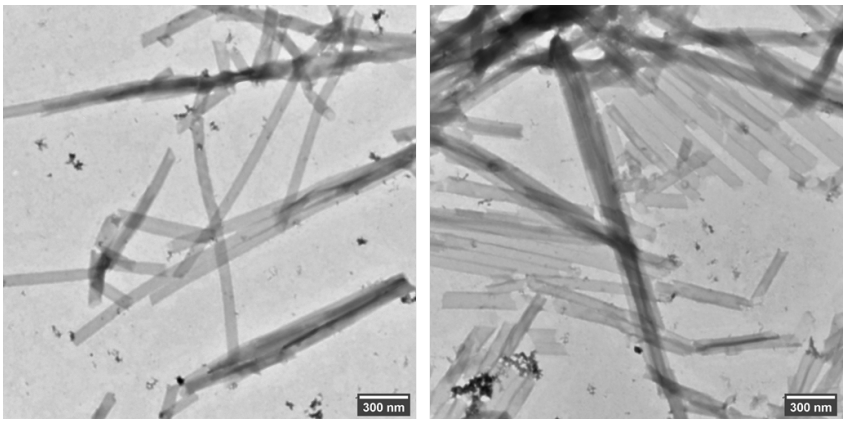


Figure S17: TEM images of **2** after assembling in PBS at 10 mM for 72 h. Samples were diluted to 1 mM prior to imaging.

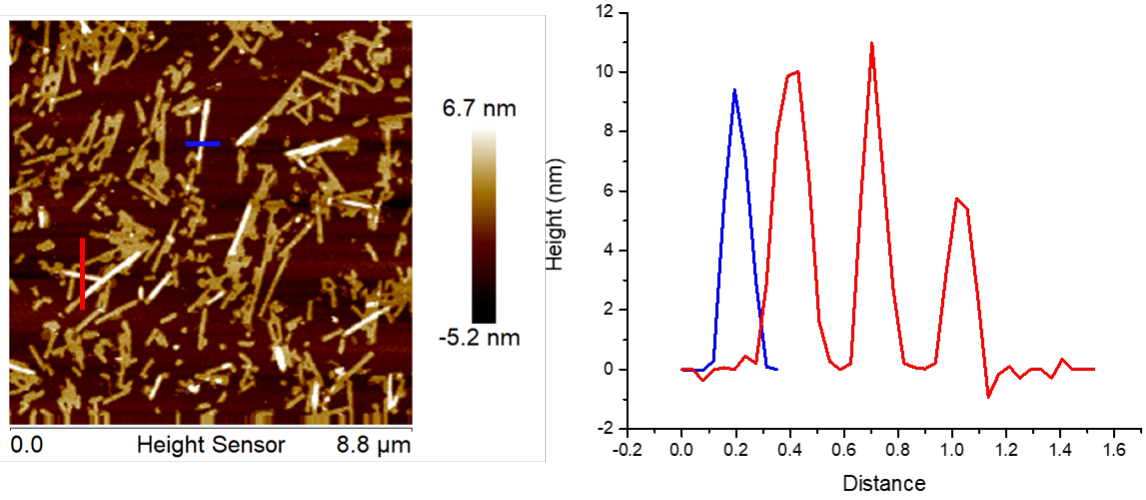


Figure S18: AFM image of compound **1** Samples were aged for 72 h at 20 mM in PBS (pH 7.4), then diluted to 1.0 mM and deposited onto freshly cleaved mica for 5 min, rinsed with water for 10 s, then dried with nitrogen gas. Images were analyzed with Bruker's software. Cross-sectional information was gathered using Bruker's software and plotted with origin.

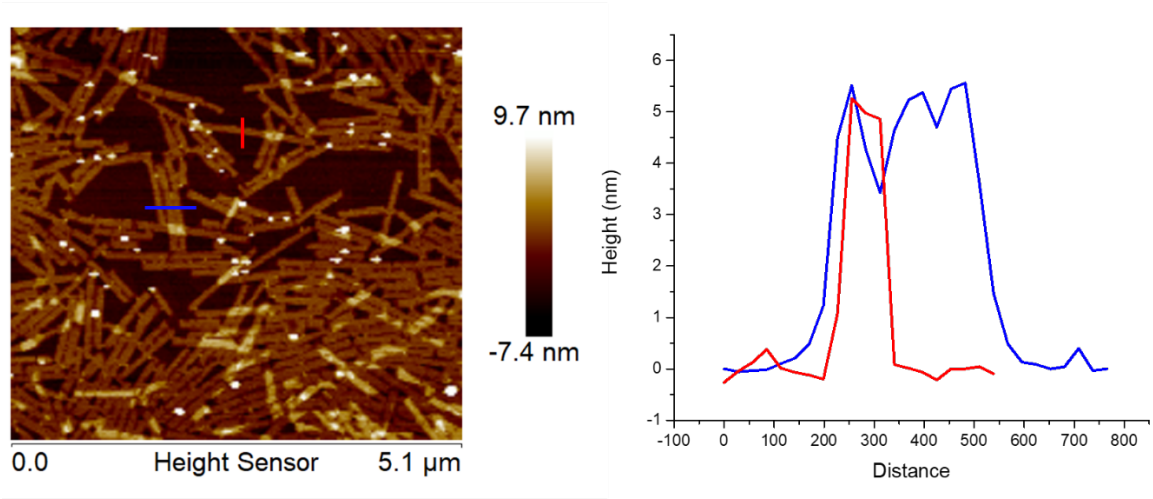


Figure S19: AFM image of compound **2.** Samples were aged for 72 h at 10 mM in PBS (pH 7.4), then diluted to 1.0 mM and deposited onto freshly cleaved mica for 5 min, rinsed with water for 10 s, then dried with nitrogen gas. Images were analyzed with Bruker software. Cross-sectional information was gathered using Bruker's software and plotted with origin.

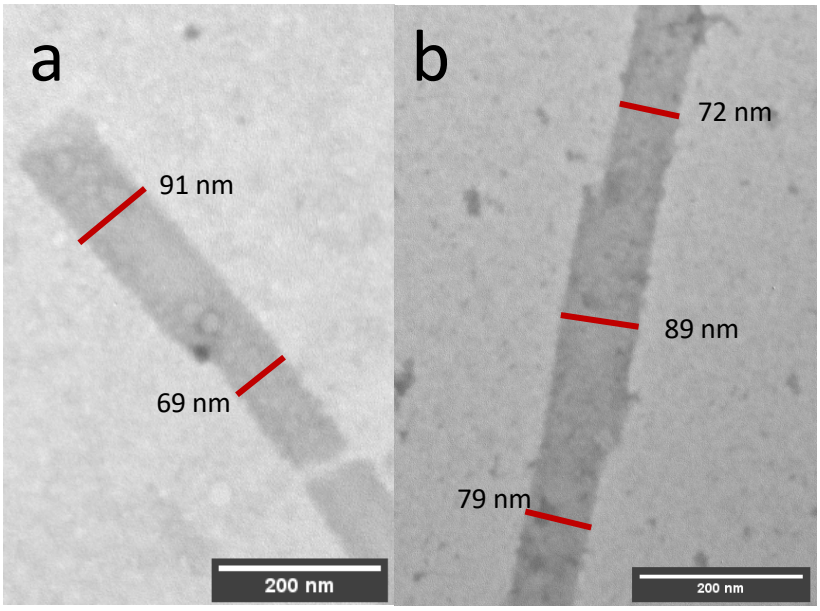


Figure S20: TEM images of **1**, assembled in PBS (pH 7.4) for 72 h at 10 mM, then diluted to 0.1 mM and heated to 37°C for (a) 24 h and (b) 72 h to induce drug release. Image shows regions of the nanotube with smaller diameters, consistent with hydrolytic release of 5-FU and the formation of smaller nanotubes from **2**.

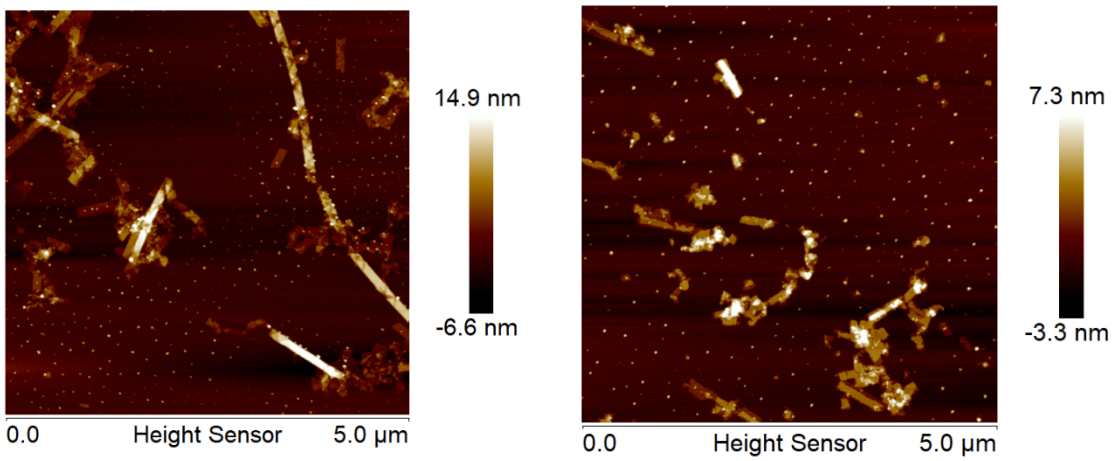


Figure S21: AFM images of drug release from compound **1.** Samples were aged for 72 h at 10 mM in PBS (pH 7.4), then diluted to 1.0 mM and heated to 37°C for 48 h. Samples (1.0 mM) were deposited onto freshly cleaved mica for 5 min, rinsed with water for 10 s, then dried with nitrogen gas. Images were analyzed with Bruker's software.

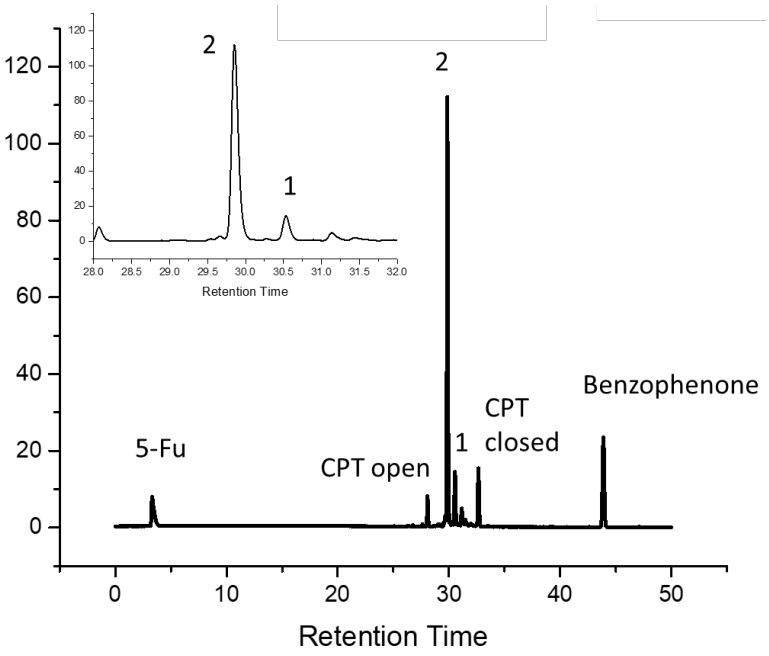


Figure S22: HPLC trace of **1**, assembled in PBS (pH 7.4) for 72 h at 10 mM, then diluted to 1 mM and heated to 37°C for 48 h to induce partial release of 5Fu. Trace plotted was measured at 254 nm, and all peaks were verified by co-injection of the individual components.

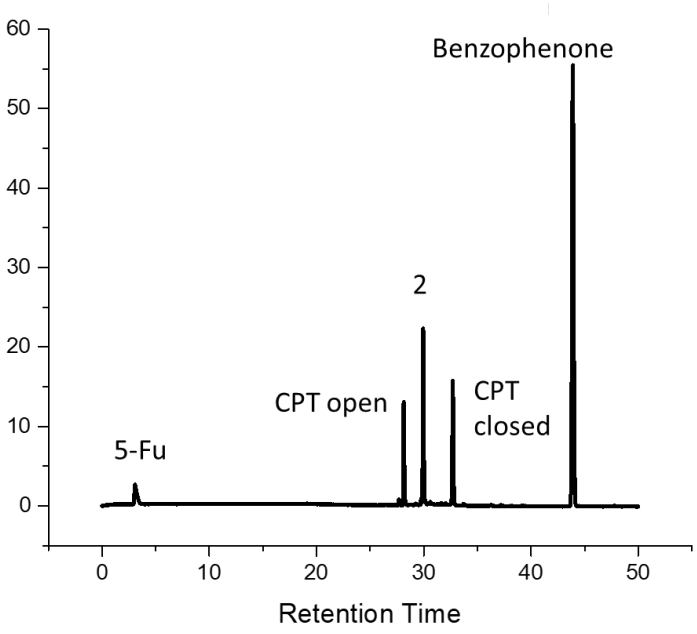


Figure S23: HPLC trace of **1**, assembled in PBS (pH 7.4) for 72 h at 10 mM, then diluted to 0.1 mM and heated to 37°C for 96 h to induce partial release of 5-FU and CPT. Trace plotted was measured at 254 nm, and all peaks were verified by co-injection of the individual components.

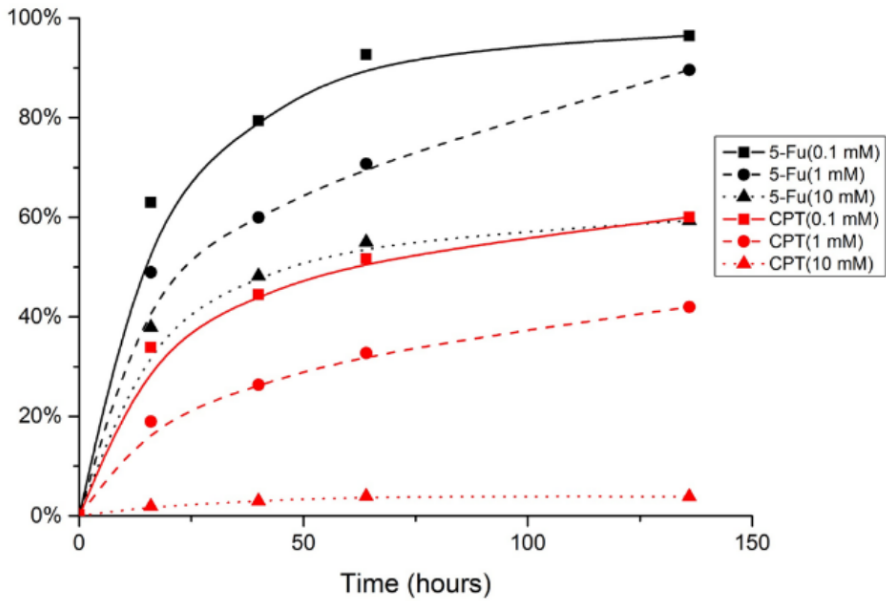


Figure S24: Release of 5-FU and CPT from compound **1** in human serum at 37°C over time as a function of concentration.

References

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