Electronic Supporting Information for:

Linker-Determined Drug Release Mechanism of Free Camptothecin from Self-Assembling Drug Amphiphiles

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Contents

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S1 Synthesis and molecular characterization

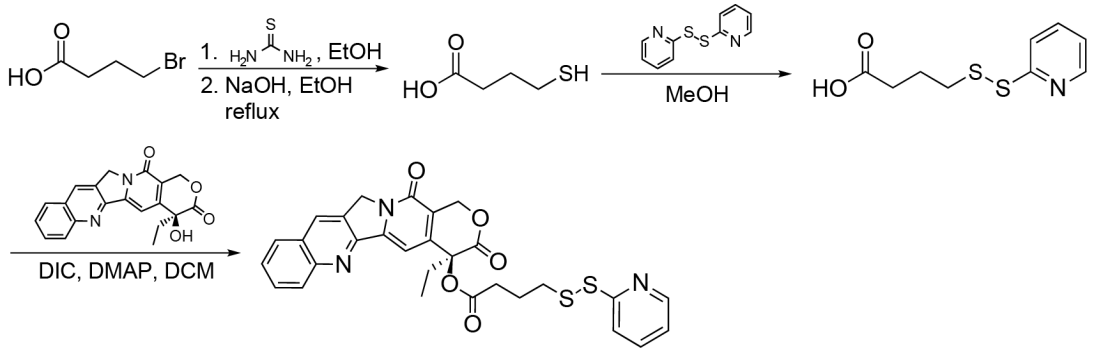
S1.1 Materials and methods

Reagents. Fmoc amino acids (unless otherwise stated) and coupling reagents (HBTU or HATU) were purchased from Advanced Automated Peptide Protein Technologies (AAPPTEC, Louisville, KY). Rink Amide MBHA resin and Fmoc-Lys(Fmoc)-OH were obtained from Novabiochem (San Diego, CA). Camptothecin was purchased from AvaChem Scientific (San Antonio, TX) and all other reagents were sourced from Sigma-Aldrich (St. Louis, MO) or VWR (Radnor, PA) and used as supplied.

Instruments and Methods. RP-HPLC was performed on a Varian ProStar Model 325 HPLC (Agilent Technologies, Santa Clara, CA) equipped with a fraction collector. Preparative separations utilized a Varian PLRP-S column (100 Å, 10 µm, 150 × 25 mm), whilst analytical HPLC used a Varian Pursuit XRs C_{18} column (5 µm, 150 × 4.6 mm). Water and acetonitrile containing 0.1% *v*/*v* TFA were used as the mobile phase. Purified molecules were lyophilized using a FreeZone –105 °C 4.5 L freeze dryer (Labconco, Kansas City, MO). Mass spectrometric data for characterization was acquired using either a Finnigan LCQ ion trap mass spectrometer (Thermo-Finnigan, Waltham, MA) for ESI-MS or Autoflex III Smartbeam (Bruker, Billerica, MA) for MALDI-Tof MS using α-cyano-4-hydroxycinnamic acid as the matrix. LC-MS analysis was performed using a Thermo-Finnigan Surveyor LC-MS system equipped with a PDA spectroscopic detector and an LCQ Fleet Ion Trap Mass spectrometer. Chromatographic separation was carried out using an Agilent Eclipse Plus C18 column (50 mm × 2.1 mm, 1.8 µm), eluting with a water-acetonitrile gradient containing 0.1% formic acid. Data was processed using Thermo XCaliber software. Bruker Avance 300 or 400 MHz FT-NMR spectrometers were used for the acquisition of ^{1}H and ^{13}C NMR spectra.

S1.2 Synthesis and characterization of the CPT-linker molecules

Scheme S1. Synthesis of the CPT-linker molecule, **CPT-buSS-Pyr**.



4-(Pyridin-2-yldisulfanyl)butanoic acid (HO_2C-BuSS-Pyr). This was synthesized using a modification of a previously published method.[8] Briefly, 4-bromobutyric acid (2.0 g, 12.0 mmol) and thiourea (1.06 g, 14.0 mmol) were refluxed in EtOH (50 ml) for 4 h. NaOH (4.85 g, 121 mmol) in EtOH (50 ml) was added and reflux was continued for 16 h. After cooling to room temperature and concentration *in vacuo*, the residue was diluted to 50 ml with water that was extracted twice with Et_2O. The aqueous portion was then acidified to pH 5 with 4 M HCl, giving a cloudy solution that was extracted with Et_2O. The organic extracts were dried over anhydrous Na_2SO_4 and concentrated to give 4-sulfanylbutyric acid as a clear oil (802 mg, 56%) that was used without further purification. 4-Sulfanylbutyric acid (802 mg, 6.7 mmol) was dissolved in MeOH (5 ml) and added dropwise to a solution of 2-aldrithiol (3.03 g, 13.8 mmol) in MeOH (5 ml), which developed a yellow color. After 3 h, the mixture was purified by RP-HPLC, collecting the major peak and removing the solvents *in vacuo*. The resultant

oil was dissolved in $CHCl_3$, dried over Na_2SO_4 and solvent removed to give **HO_2C-BuSS-Pyr** as a pale yellow viscous oil (1.02 g, 67%). δ_H (400 MHz, $CDCl_3$, Me_4Si) 8.59 (1 H, d, $J_{1,3}$ 4.6), 7.81–7.91 (2 H, m), 7.25–7.30 (1 H, m), 2.88 (2 H, t, $J_{1,3}$ 7.1), 2.50 (2 H, t, $J_{1,3}$ 7.2), 2.00–2.09 (2 H, m).

Camptothecin-4-(pyridin-2-yldisulfanyl)butanoate (CPT-buSS-Pyr). Camptothecin (200 mg, 574 μmol) was suspended in DCM (32 ml) and dimethylaminopyridine (44 mg, 360 μmol), **HO_2C-BuSS-Pyr** (280 mg, 1.22 mmol) and diisopropylcarbodiimide (436 μl, 2.80 mmol) were added. The mixture was stirred for 36 h, with TLC (3% MeOH in $CHCl_3$) showing complete consumption. The solution was then filtered, diluted with $CHCl_3$ (60 ml), extracted with sat. $NaHCO_3$ (50 ml), brine (50 ml), dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by flash chromatography using EtOAc (500 ml) then 0.5% MeOH in EtOAc (250 ml). Product fractions were identified by TLC, combined and solvent removed *in vacuo* to give **CPT-buSS-Pyr** as a pale yellow solid (195 mg, 61%); δ_H (400 MHz, $CDCl_3$, Me_4Si) 0.97 (3 H, t, $J_{1,3}$ 7.5), 2.03–2.31 (2 H, m), 2.57–2.75 (2 H, m), 2.86 (2 H, t, $J_{1,3}$ 7.1), 5.29 (2 H, s), 5.40 (1 H, d, $J_{1,2}$ 17.2), 5.67 (1 H, d, $J_{1,2}$ 17.3), 7.04 (1 H, m), 7.20 (1 H, s), 7.60 (1 H, m), 7.65–7.70 (2 H, m), 7.80–7.90 (1 H, m), 7.94 (1 H, $J_{1,3}$ 8.2), 8.23 (1 H, d, $J_{1,3}$ 8.6), 8.40 (1 H, s), 8.43 (1 H, d, $J_{1,3}$ 4.2); δ_C (100 MHz, $CDCl_3$, Me_4Si) 7.9, 24.0, 31.2, 32.0, 32.4, 37.7, 50.2, 67.4, 76.2, 96.1, 120.4, 128.3, 128.4, 128.62, 128.63, 129.9, 130.9, 131.4, 131.5, 137.3, 146.1, 146.6, 149.1, 149.9, 152.6, 157.6, 160.3, 167.7, 172.1; MS (MALDI-TOF): 560.065 $[M+H]^+$.

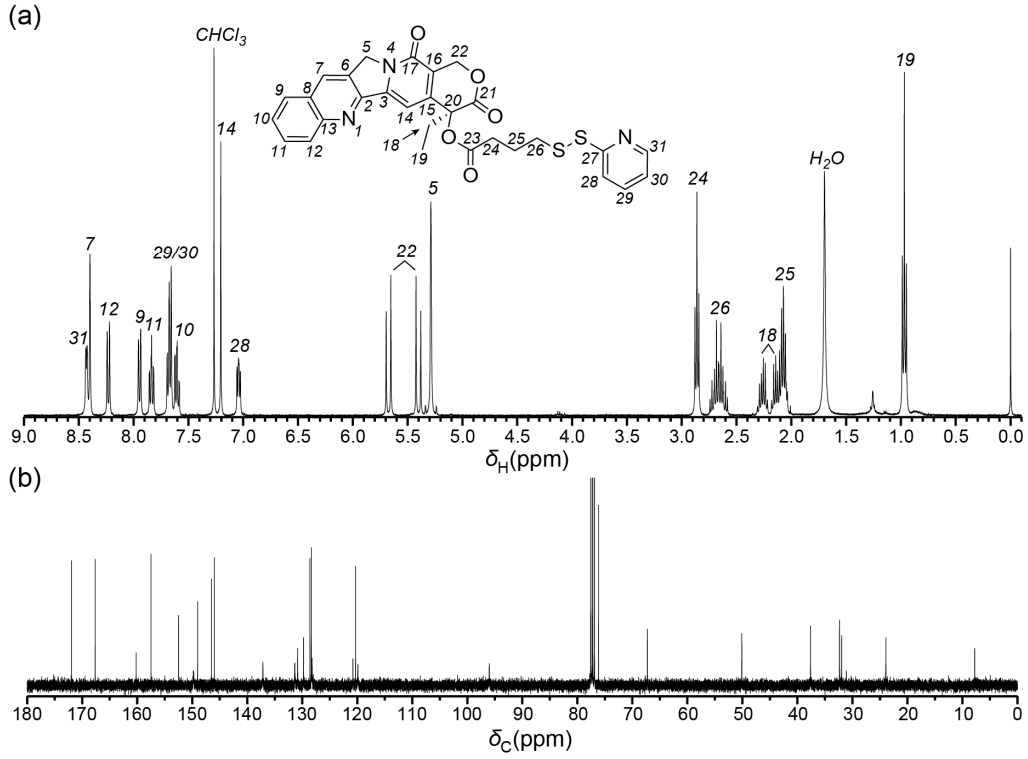
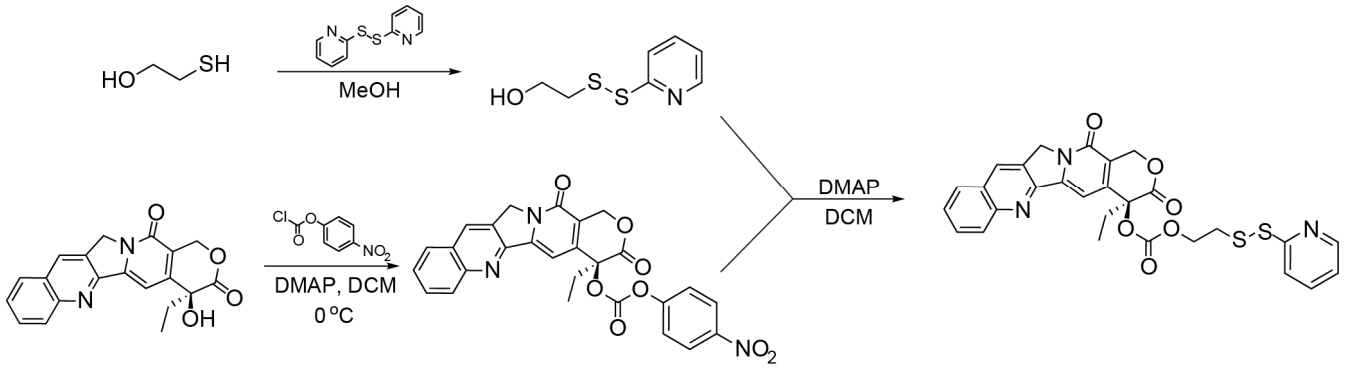


Figure S1. (a) ^{1}H NMR (400 MHz, $CDCl_3$) and (b) ^{13}C NMR (100 MHz, $CDCl_3$) of **CPT-buSS-Pyr**.

Scheme S2. Synthesis of CPT-linker molecule, **CPT-etcSS-Pyr**.



2-(Pyridyl-disulfanyl)ethanol. The synthesis of this precursor was adapted from a previously reported procedure for the formation of activated disulfides.[8] 2-Aldrithiol (1.29 g, 5.86 mmol) was dissolved in MeOH (3.5 ml) and 2-mercaptoethanol (300 μl, 334 mg, 4.28 mmol) was added dropwise over 5 min, the solution turning a yellow color. After 3 h, the solution was diluted with 0.1% aq. TFA (4.5 ml) and purified by RP-HPLC. Product fractions were combined and solvents removed in vacuo. A solution of sat. $NaHCO_3$ (15 ml) was added to neutralize the TFA, allowing to stand for 30 min before extracting into DCM. The organic extract was dried over Na_2SO_4 and solvents removed to give **2-(pyridyl-disulfanyl)ethanol** as pale yellow oil (561 mg, 70 %). δ_H (300 MHz, $CDCl_3$, Me_4Si) 2.91–2.99 (2 H, m), 3.80 (2 H, br s), 7.15 (1 H, m), 7.41 (1 H, dt, $J_{1,3}$ 8.0, 1.0), 7.54–7.63 (1 H, m), 8.47–8.53 (1 H, m).

Camptothecin-4-nitrophenyl carbonate. Camptothecin (100 mg, 287 μmol) and nitrophenylchloroformate (203 mg, 1.00 mmole) were dissolved/suspended in dry DCM (15 ml) at 0 °C. Dimethylaminopyridine (DMAP, 210 mg, 1.72 mmol) was added, turning the solution yellow. After 3 h, the yellow-brown solution was filtered, washed with 1 N HCl (20 ml), dried over Na_2SO_4 and concentrated in vacuo. Purification by flash chromatography—DCM (50 ml), 1:1 DCM/EtOAc (100 ml), EtOAc (200 ml), then 1% MeOH in EtOAc (100 ml)—gave camptothecin-4-nitrophenyl carbonate as a pale yellow solid (75 mg, 51%). δ_H (400 MHz, $CDCl_3$, Me_4Si) 1.01–1.12 (3 H, m) 2.16–2.45 (2 H, m) 5.24–5.38 (2 H, m) 5.42 (1 H, d, $J_{1,2}$ 17.3) 5.72 (1 H, d, $J_{1,2}$ 17.2) 7.36–7.44 (3 H, m) 7.70 (1 H, ddd, $J_{1,3}$ 8.2, 6.9, 1.2) 7.86 (1 H, ddd, $J_{1,3}$ 8.5, 7.0, 1.5) 7.96 (1 H, dd, $J_{1,3}$ 8.2, 1.3) 8.17–8.28 (3 H, m), 8.43 (1 H, s).

Camptothecin-(4-pyridyldisulfanyl)ethyl carbonate (CPT-etcSS-Pyr). Camptothecin-4-nitrophenyl carbonate (70 mg, 136 μmol) and 2-(pyridyl-disulfanyl)ethanol (42 mg, 225 μmol) were dissolved in dry DCM (15 ml), and DMAP (31 mg, 254 μmol) was added and the mixture was refluxed (55 °C) overnight. After cooling, the mixture was washed with 1 M $NaHCO_3$ (3 × 15 ml) till colorless, dried over Na_2SO_4 and concentrated in vacuo. Purification by flash chromatography—DCM (50 ml), 1:1 DCM/EtOAc (300 ml), 1:3 DCM/EtOAc (100 ml), EtOAc (200 ml), 1% MeOH in EtOAc (100 ml), then 2% MeOH in EtOAc (100 ml)—to give **CPT-etcSS-Pyr** as a pale yellow solid (57 mg, 75%). δ_H (400 MHz, $CDCl_3$, Me_4Si) 1.01 (3 H, t, $J_{1,3}$ 7.5), 2.10–2.21 (1 H, m), 2.24–2.34 (1 H, m), 3.06 (2 H, t, $J_{1,3}$ 6.6), 4.30–4.42 (2 H, m), 5.27–5.30 (2 H, m), 5.39 (1 H, d, $J_{1,2}$ 17.2), 5.69 (1 H, d, $J_{1,2}$ 17.2), 7.03 (1 H, td, $J_{1,3}$ 5.0, 3.4), 7.34 (1 H, s), 7.62 (1 H, d, $J_{1,3}$ 1.4), 7.63–7.64 (1 H, m), 7.65–7.70 (1 H, m), 7.83 (1 H, m), 7.94 (1 H, dd, $J_{1,3}$ 8.2, 1.3), 8.22 (1 H, d, $J_{1,3}$ 8.7), 8.39 (1 H, s), 8.42 (1 H, dt, $J_{1,3}$ 4.8, 1.4); δ_C (100 MHz, $CDCl_3$, Me_4Si) 7.6, 31.9, 36.9, 50.0, 66.4, 67.1, 78.0, 96.0, 119.9, 120.3, 120.9, 128.1, 128.2, 128.4, 129.7, 130.7, 131.2, 137.2, 145.5, 146.5, 148.9, 149.7, 152.3, 153.4, 157.3, 159.3, 167.3.

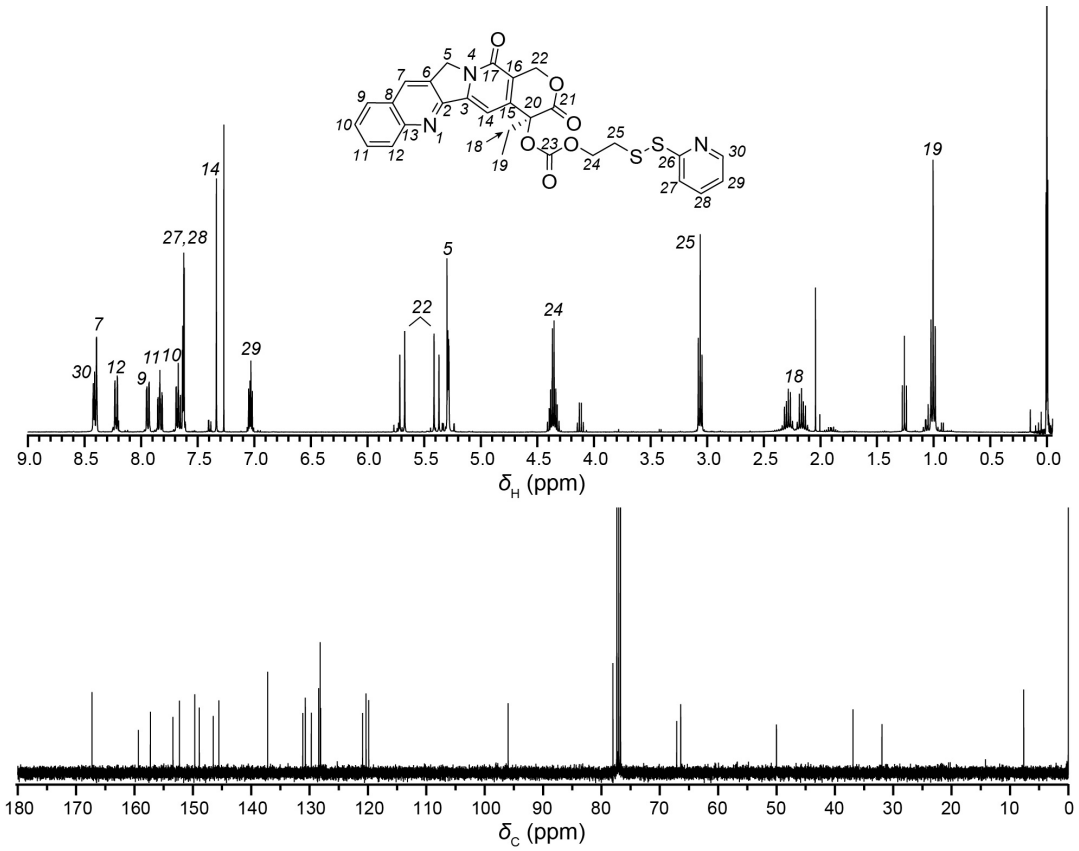


Figure S2 (a) ^{1}H NMR (400 MHz, $CDCl_3$) and (b) ^{13}C NMR (100 MHz, $CDCl_3$) of **CPT-etcSS-Pyr.**

S1.3 Synthesis and characterization of Cys-Tau

The peptide Ac-CGVQIVYKK-NH_2, **Cys-Tau**, was synthesized on a Focus XC automated peptide synthesizer (AAPPTEC, Louisville, KY) using standard Fmoc-solid phase synthesis techniques, using 20% 4-methylpiperidine in DMF for Fmoc deprotections and amino acid/HBTU/DIEA (4:4:6 relative to the resin) in DMF for couplings (with 2 min activation time and 1 h reaction time). Acetylation was carried out manually using 20% acetic anhydride in DMF after *N*-terminal Fmoc deprotection.

The peptide was cleaved from the resin using the standard cleavage solution of TFA/TIS/H_2O (95:2.5:2.5) for 2 h and isolated by trituration into cold diethyl ether, followed by filtration and drying under suction. The crude peptide was then purified to >95% homogeneity by preparative RP-HPLC, initially dissolving in 1 ml AcOH and then diluting with 0.1% aqueous TFA to 20 ml. Peptide identity and purity were confirmed by MALDI-Tof (Figure S3).

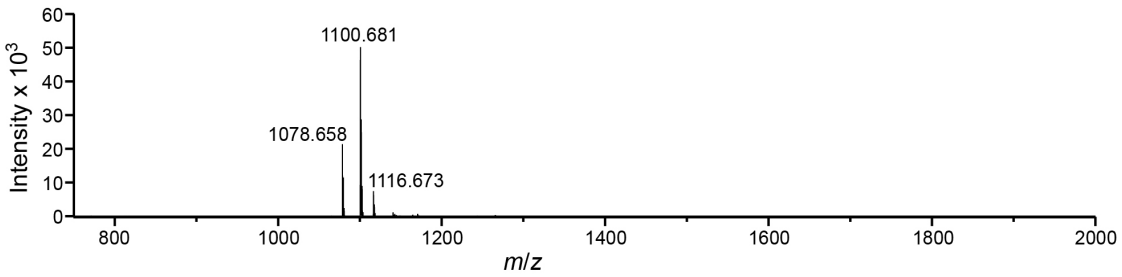


Figure S3. MALDI-Tof characterization of **Cys-Tau**.

S1.4 Synthesis and characterization of the drug amphiphiles

S1.4.1 CPT-buSS-Tau, 1

Cys-Tau (14.6 mg, 13.5 μmol) was dissolved in an N_2-purged DMSO solution **CPT-buSS-Pyr** (10 mg in 1.50 ml, 17.8 μmol) and shaken overnight. The reaction was diluted to 30 ml with 0.1% aqueous TFA, giving a slightly viscous solution that was then purified by RP-HPLC. Product fractions were combined and immediately lyophilized. The pale yellow solid obtained was dissolved in 25 ml 1:1 H_2O/MeCN and the product concentration was determined by TCEP calibration (see S1.4) to be 233 μM (8.9 mg, 43%). The solution was then aliquotted into cryo-vials, lyophilized and stored at −30 °C.

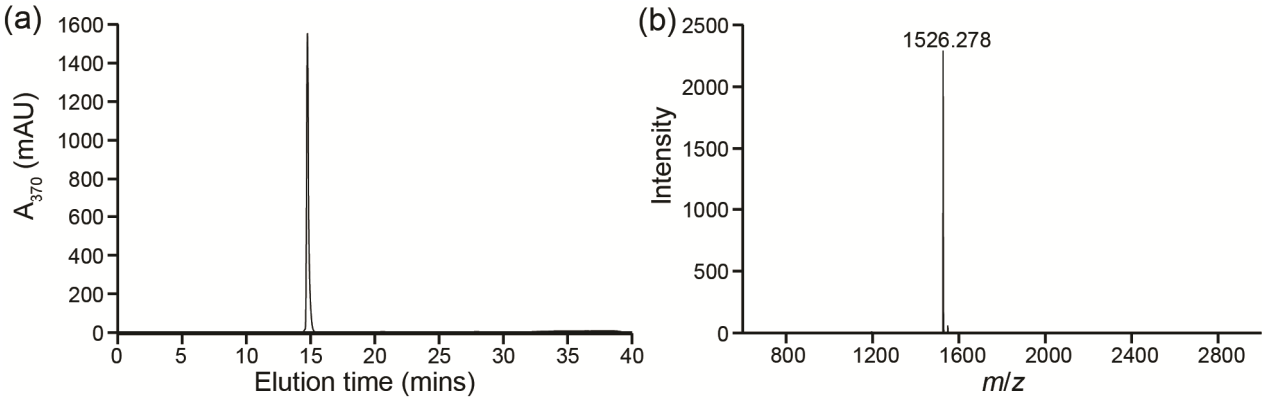


Figure S4. RP-HPLC (a) and MALDI-Tof MS (b) characterization of **1**.

S1.4.2 CPT-etcSS-Tau, 2

Cys-Tau (22.8 mg, 21.2 μmol) was dissolved in an N_2-purged DMSO solution of **CPT-etcSS-Pyr** (15.4 mg, 27.5 μmol) and allowed to react overnight. The solution was diluted to 9 ml with 0.1% aqueous TFA and purified by RP-HPLC. Product fractions were combined and immediately lyophilized. The pale yellow solid obtained was dissolved in 1:1 H_2O/MeCN (10 ml) and the product concentration determined by TCEP calibration (see S1.4) to be 1.61 mM (24.6 mg, 76%). The solution was aliquotted into cryo-vials, lyophilized and stored at −30 °C.

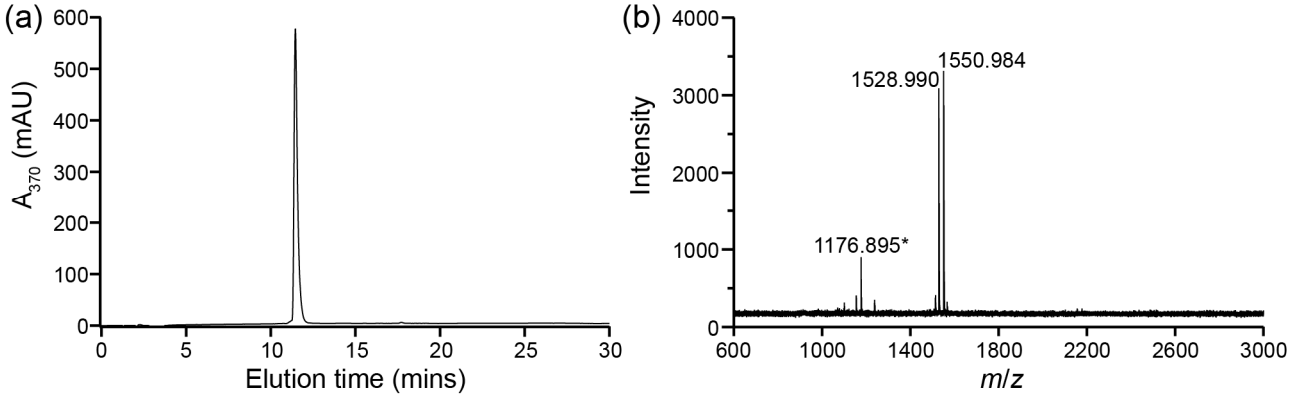


Figure S5 RP-HPLC (a) and MALDI-Tof MS (b) characterization of **2**. In-source fragmentation was observed corresponding to the loss of one CPT–O–C(=O)– moiety (indicated by *).

S1.5 Synthesis and characterization of the CPT-buSS-Tau degradation intermediates

S1.5.1 CPT-buSH, 3

CPT-buSS-Pyr (10.0 mg, 17.9 μmol) was dissolved in a 1:1 mixture of water/acetonitrile containing 0.1% TFA (10 ml), to which an excess of tris-carboxyethylphosphine (TCEP, 89.6 mg, 358 μmol) was added. After 30 min, the mixture was purified by HPLC and the product fractions were combined and concentrated to give a solution of sulfide **3**. Calibration of this solution using the HPLC protocol from the TCEP reduction assay gave a final concentration of 1.33 mM.

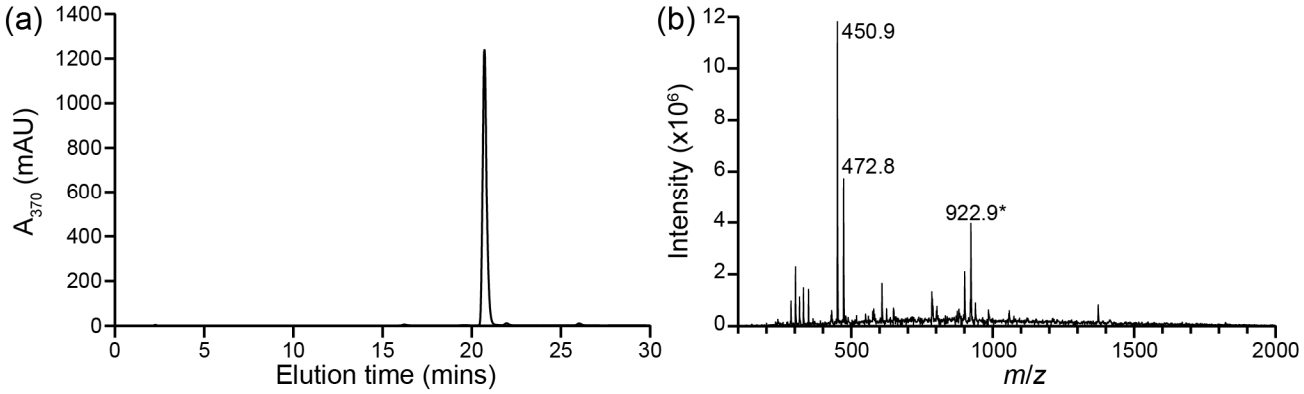


Figure S6 RP-HPLC (a) and ESI-MS (b) characterization of **3**. Formation of a dimeric species could be observed under the MS analysis conditions (indicated by *), the mass of which is 2 mass units greater than the disulphide dimer **4**, indicating this species is a dimeric ion formed by two molecules of **3** that are not covalently linked by a disulphide bond, e.g. $[\mathbf{3}_2+Na]^+$.

S1.5.1 CPT-buSSbu-CPT, 4

CPT-buSS-Pyr (2 mg, 3.6 μmol) and **3** (1.8 mg, 4.0 μmol) were dissolved in DMSO (3 ml), followed by the addition of DIEA (10 μl, 57.4 μmol) and allowed to react for 12 h. The mixture was purified by preparative HPLC, and the product fractions were combined and concentrated to give a solution of disulfide **4**. Calibration of this solution using the TCEP reduction assay gave a final concentration of 273 μM.

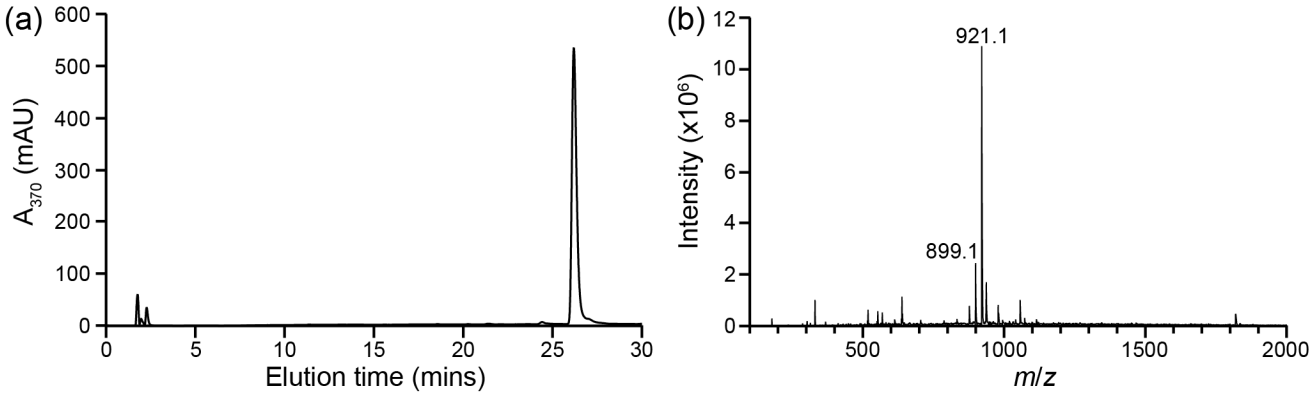


Figure S7 RP-HPLC (a) and ESI-MS (b) characterization of **4**.

S1.6 Drug amphiphile calibration assay

To determine the CPT concentration of the purified drug amphiphiles, TCEP reduction assay was developed. Briefly, 5 μl of the stock solution of **1** in (see synthetic procedure) was diluted by the addition of 1:1 H_2O/MeOH (15 ml). 20 μl of freshly prepared 1 M aqueous TCEP was then added allowed to stand for at least 30 mins with periodic vortexing. The whole sample was then injected onto the HPLC column (so as to completely fill the 20 μl sample loop), measuring the area of the peak due to **CPT-buSH**. The CPT concentration of the analyzed solution in μM is given by (peak area)/0.2964 (as determined by a calibration curve study of TCEP-reduced **CPT-buSS-Pyr**, Figure S6). The conjugate concentration of the original solution was calculated based on the applied dilutions and number of CPT molecules the conjugate possesses. The same protocol could be used for the calibration of the carbonate-based drug amphiphile **2** as the acidic conditions prevented intramolecular cyclization of the intermediate, **CPT-etcSH**.

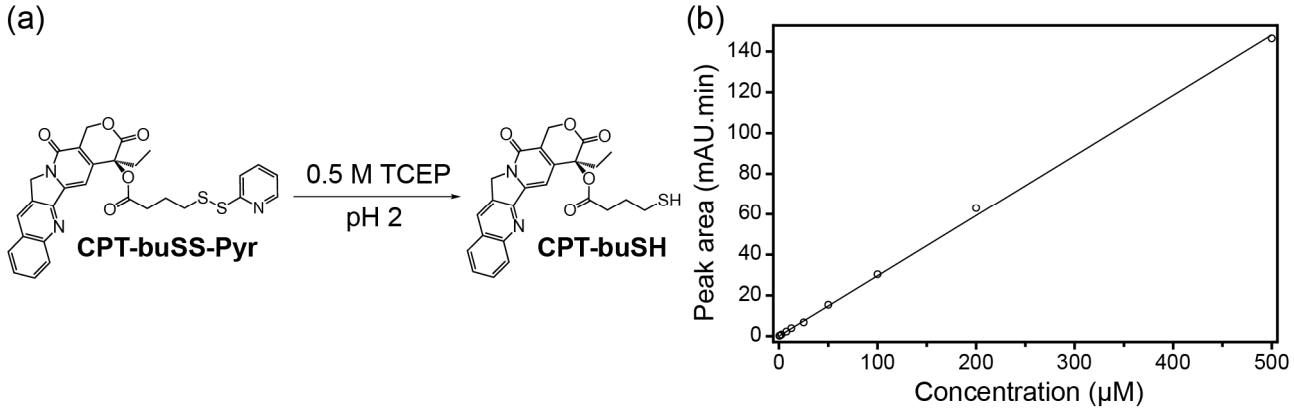


Figure S8. Drug amphiphile concentration assay. (a) TCEP reduction of **CPT-buSS-Pyr** to **CPT-buSH** and (b) RP-HPLC-derived calibration curve of **CPT-buSH,** showing a linear increase in peak area with concentration.

S2 Self-Assembly Characterization Protocols

Transmission electron microscopy (TEM) imaging protocol: 1 mM stock solutions of **1** and **2** in water were prepared by direct dissolution of the respective lyophilized powders, and allowed to age overnight. A 100 μM aqueous solution of each conjugate was then prepared immediately prior to TEM sample preparation. A sample for imaging was prepared by depositing 7 μL of the solution onto a carbon-coated copper grid (Electron Microscopy Services, Hatfield, PA, USA), wicking away the excess solution with a small piece of filter paper. Next, 7 μL of a 2 wt % aqueous uranyl acetate solution was deposited and the excess solution was carefully removed as above to leave a very thin layer. The sample grid was then allowed to dry at room temperature prior to imaging. Bright-field TEM imaging was performed on a FEI Tecnai 12 TWIN Transmission Electron Microscope operated at an acceleration voltage of 100 kV. TEM images were recorded by a SIS Megaview III wide-angle CCD camera.

Circular dichroism (CD) protocol: The CD spectra of **1** and **2** were recorded on a Jasco J-710 spectropolarimeter (JASCO, Easton, MD, USA) using a 1 mm path length quartz UV-Vis absorption cell (Thermo Fisher Scientific, Pittsburgh, PA, USA). The 100 μM samples prepared above were used in these measurements. A background spectrum of the solvent was acquired and subtracted from the sample spectrum. Collected data was normalized with respect to sample concentration and β-sheet forming residues.

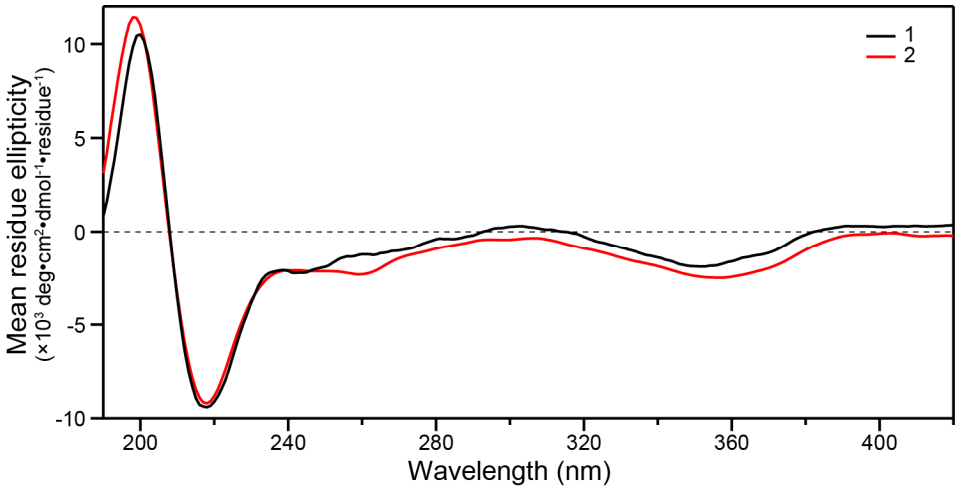


Figure S9. Circular dichroism (CD) spectra of 100 µM aqueous solutions of **1** (black) and **2** (red), indicating the b-sheet secondary structure adopted by these drug amphiphiles and the presence of signals due to the CPT molecules being in a chiral environment.

S3 Release mechanism study

S3.1 Release protocol

To 650 μl of a 10 or 100 μM aqueous solution of **1** or a 100 μM aqueous solution of **2** (both conjugate solutions were prepared from a 1 mM stock solution in H_2O that has been equilibrated for several days) was added an equal volume of 20 mM glutathione in 20 mM sodium phosphate (adjusted to pH 7.4 with 1 M NaOH), giving final conjugate concentrations of 5 or 50 μM (as appropriate). The mixture was incubated at 37 °C in a water bath, withdrawing 200 μl aliquots at the desired time points. Aliquots were treated with 200 μl of 0.1% aqueous TFA to quench the reaction, flash frozen and lyophilized. The residues were then dissolved in 40 μl water and analyzed by analytical RP-HPLC (whole loop injection). The samples were found to have retained their acidity after this treatment (pH 3). The relative proportions of the reaction products were determined by measuring the peak areas of the respective signals at 362 nm.

S3.2 Validation of the analysis method

In order to verify that the method used to evaluate the release products gave an accurate representation of the sample solution content, a comparison between the lyophilized sample and direct injection of the study solution was carried out (Figure S10). We found that there was little difference in the product distribution of both samples, and any minor difference can be attributed to the slight delay between the samples being taken and experimental error. The method utilized was therefore deemed to provide an accurate representation of the reaction products.

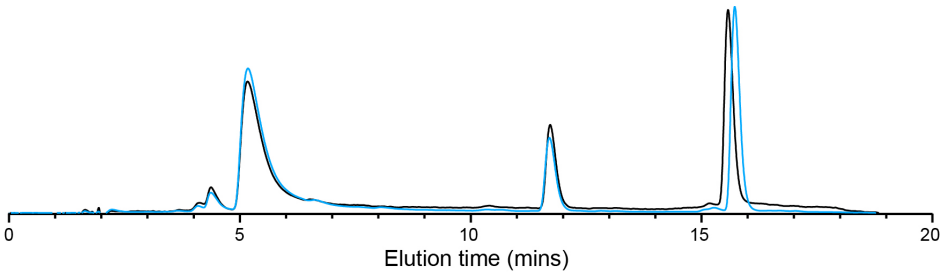


Figure S10. Comparison of the HPLC chromatograms obtained by direct injection (black) or after lyophilization treatment (blue) of the study solution. The 25 μM sample of **CPT-buSS-Tau** was treated for 1 h with 10 mM GSH at 37 °C in 10 mM sodium phosphate. The traces have been scaled with respect to the most intense peak.

S3.3 LC-MS analysis of the reaction products

To establish the identity of the reaction products (Table S1), we carried out LC-MS analysis of a 50 μM solution of **mCPT-buSS-Tau** that had been incubated with 0.5 mM GSH in 10 mM sodium phosphate (pH 7.4) at 37 °C (Figure S11). A lower GSH concentration was used to prevent contamination of the LC-MS system. The analysis solution was withdrawn after 90 min and filtered through a 0.2 μm PVDF filter, in accordance with the MS facility's sample preparation requirements. Filtering led to significant loss of material due to the presence of nanofilaments, and as such any product distribution observed in the LC-MS chromatograms does not represent the actual solution distribution and is therefore suitable for identification of the compounds present only. Excess GSH and sodium phosphate were eluted directly to waste to prevent contamination of the mass spectrometer. The reduction product **3** could not be identified by this method and was instead isolated by the collection and lyophilization of the appropriate fractions from HPLC analysis. An LCQ-Deca ion trap mass spectrometer (Thermo-Finnigan) was used to obtain confirmation of its identity (Figure S11d).

Table S1. Exact masses of the expected reaction products during incubation of **mCPT-buSS-Tau** with GSH.

Compound	1	CPT	3	4	CPT-GSH	Tau	Tau-GSH
Exact Mass (Da)	1525.71	348.11	450.12	898.23	754.20	1077.60	1381.67

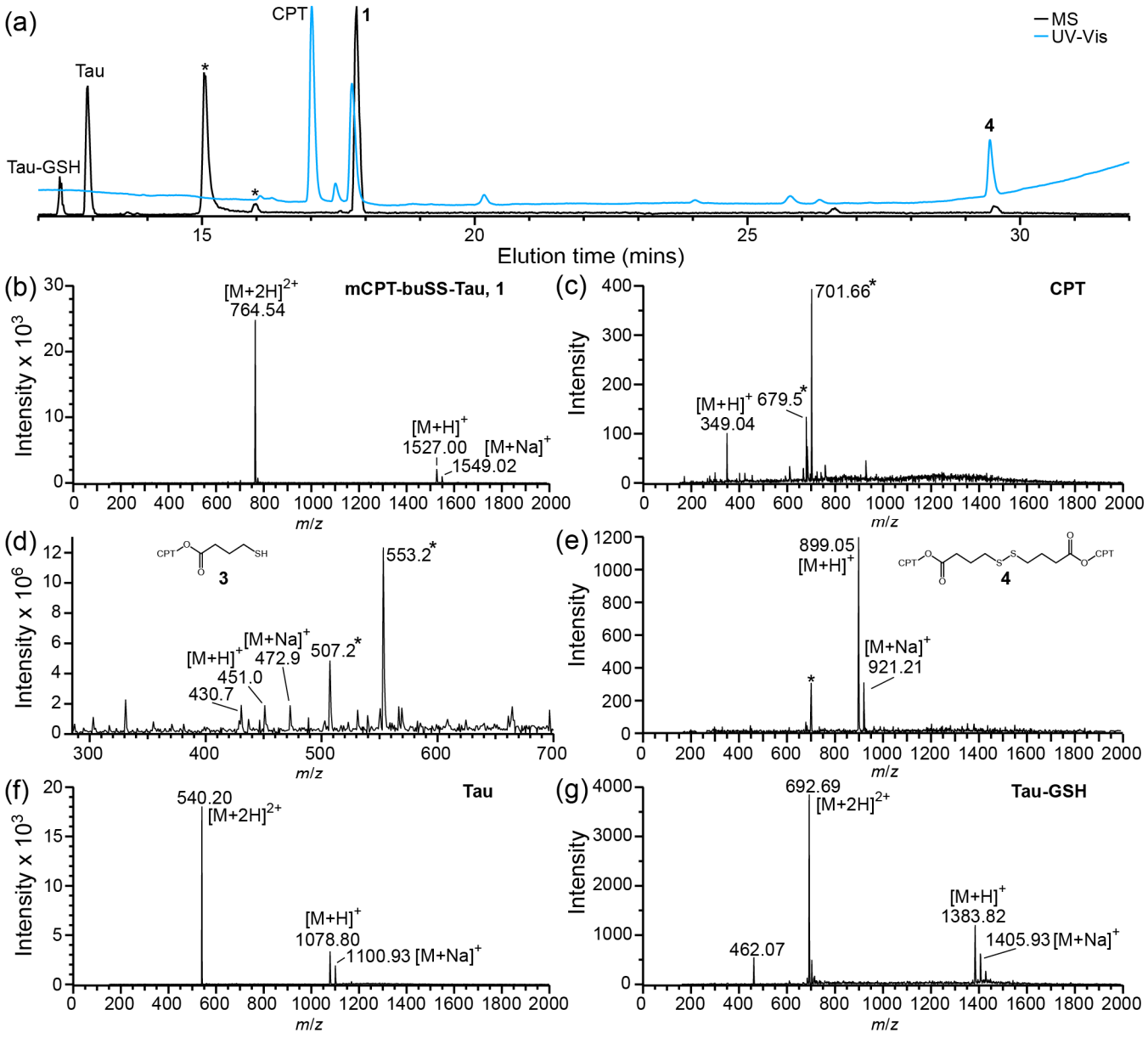


Figure S11. LC-MS analysis of a 50 µM solution of **mCPT-buSS-Tau**, **1**, after 90 min incubation with 0.5 mM GSH in 10 mM sodium phosphate (pH 7.4) at 37 °C. ESI-MS (extracted base peak chromatogram (BPC), 300–1550 Da) and UV-Vis (extracted 350–400 nm) chromatograms (* indicates system impurities) (a) and the ESI spectra of the reaction species: **1** (b), CPT (c), **4** (d), **4** (e), Tau (f) and Tau-GSH (g). Note that **3** could not be identified directly from the LC-MS analysis and was later confirmed by collecting fractions from HPLC analysis of a reaction solution. **CPT-GSH** could not be found using either method, though its identity and elution time was inferred from the reaction of **CPT-buSS-Pyr** with GSH.

S3.5 Reduction of CPT-buSS-Pyr and comparison with 1

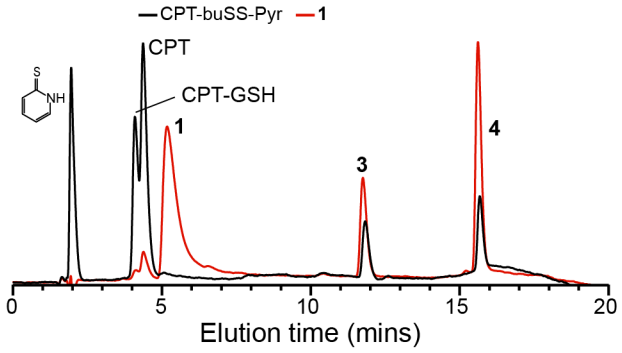


Figure S12. HPLC chromatograms (monitoring wavelength 362 nm) comparing the reaction products after 1 h treatment of 25 μM solutions of **CPT-buSS-Pyr** (black) and **1** (red) with 10 mM GSH at 37 °C in 10 mM sodium phosphate. The chromatograms have been normalized with respect to the most intense peak.

S4 Cytotoxicity protocol

The relative cytotoxicity of CPT, **1, 2**, **3**, **4** and ethylene sulphide was evaluated on the MCF-7 human breast cancer cell line. Briefly, cells were initially seeded into 96-well plates at 5000 cells/well and incubated in the medium—DMEM containing 10% FBS and 1% of an antibiotics solution (penicillin and streptomycin)—for 12 hours at 37 °C in a 5% CO_2 atmosphere (Oasis CO_2 incubator, Caron, Marietta, OH). The medium was then replaced with freshly prepared medium containing varying concentrations of conjugates or free camptothecin, and incubated for a further 48 hours. Cell viability was determined using the SRB assay (Sigma-Aldrich, USA) according to the manufacturer's instructions and expressed as a percentage of the untreated control cells. Data was fitted using the Hill equation curve analysis function within IGOR Pro (Wavemetrics Inc., Lake Oswego, Or).