

Supporting Information

Development of a facile antibody-drug conjugate platform for increased stability and homogeneity

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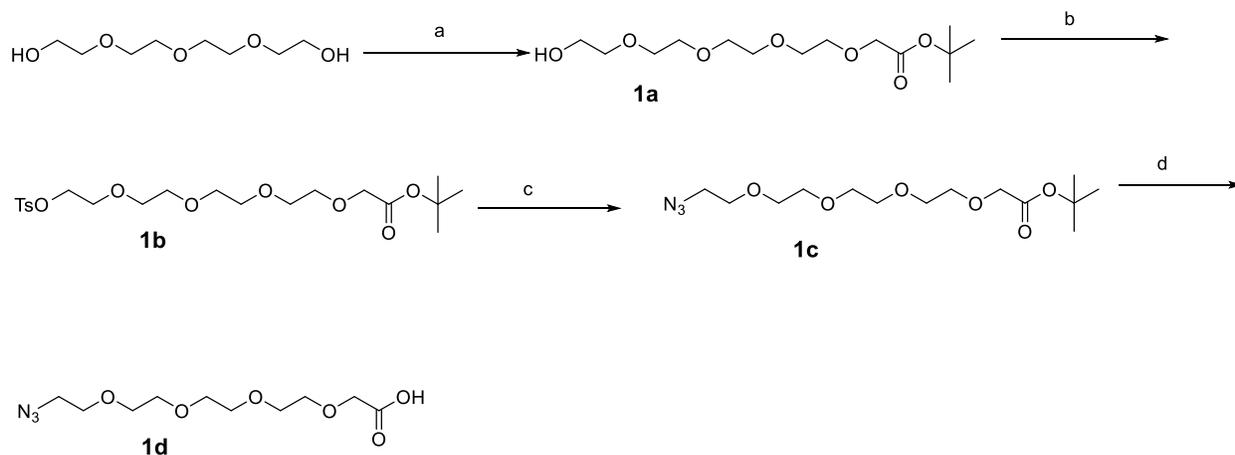
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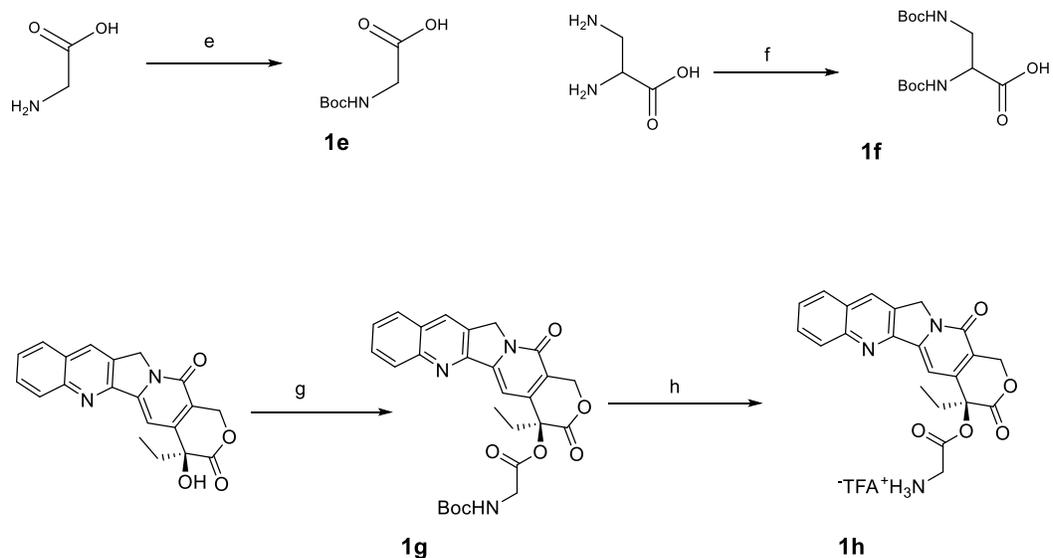
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Synthesis of 1 (Pt-PEG-CPT)

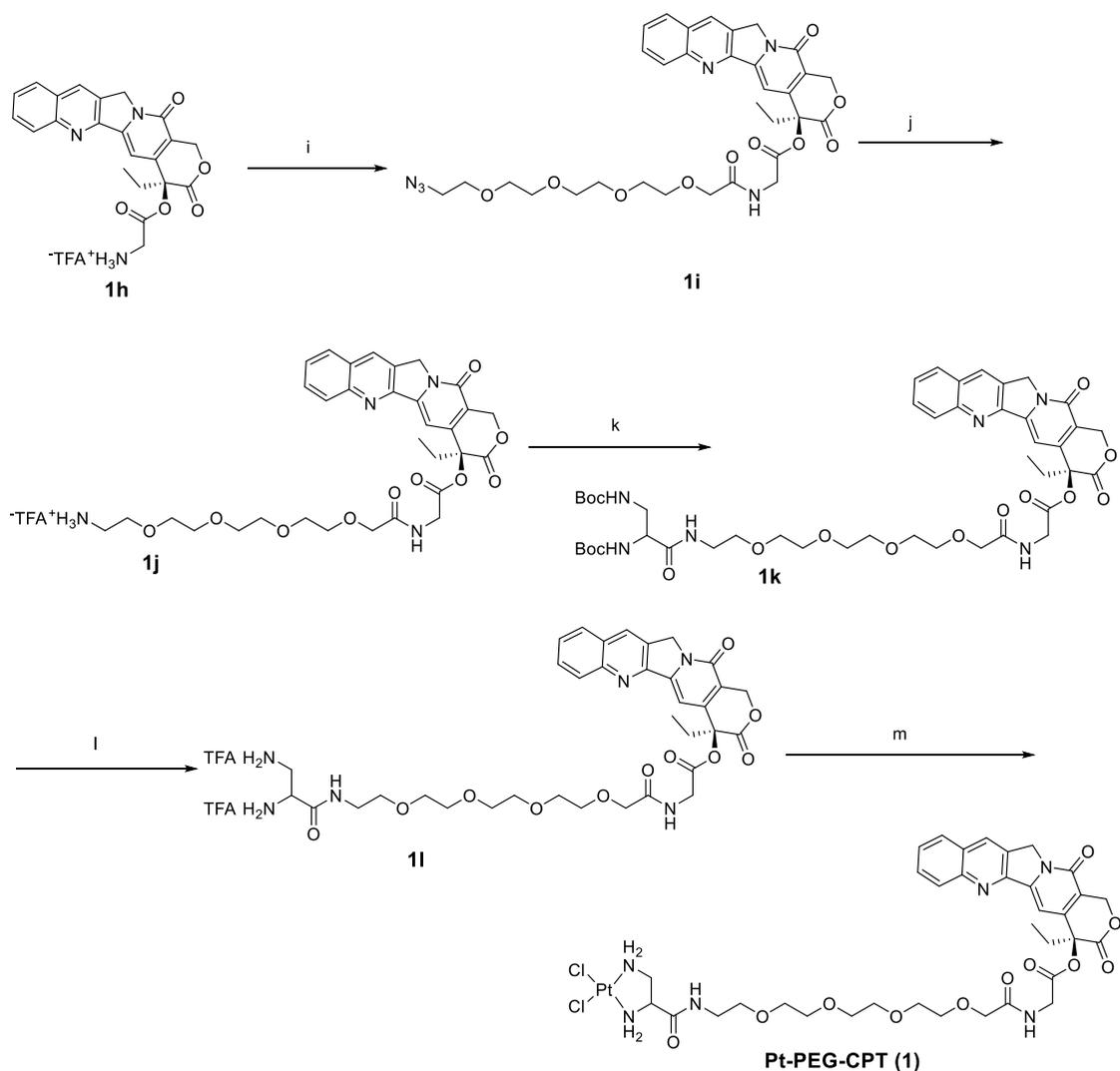
Synthesis of functionalized Tetraethyleneglycol fragment



Synthesis of camptothecin fragment

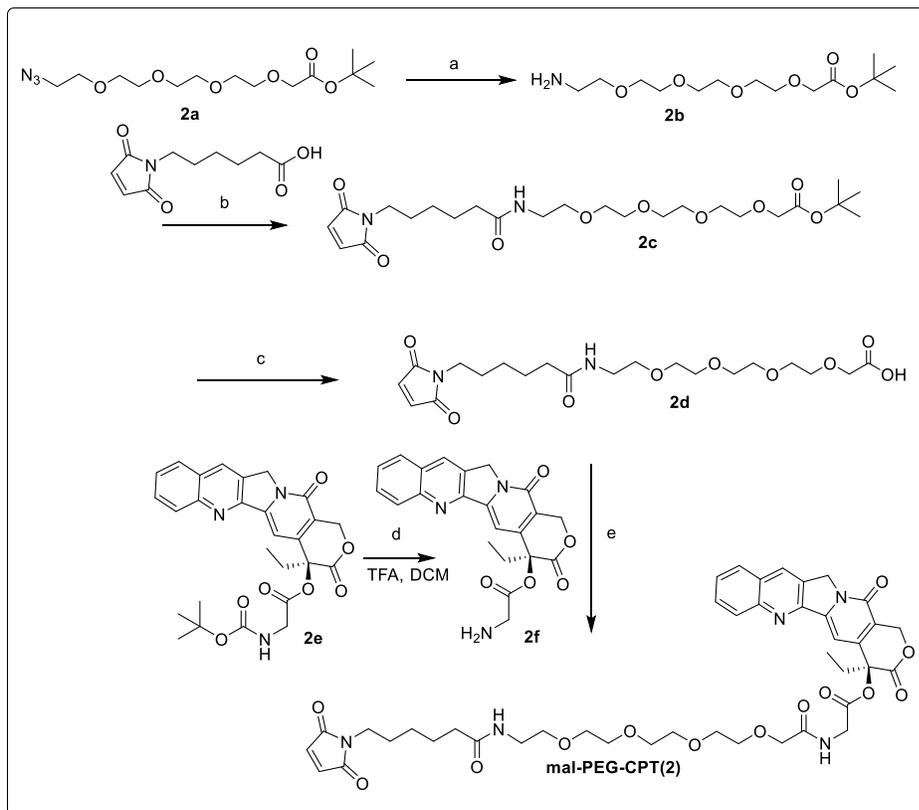


Synthetic Scheme for 1



Scheme 1. Synthetic scheme for Pt-PEG-CPT (1). Reagents and conditions: a) t-Butylbromoacetate, NaH, THF 0 °C-rt, 12 h; b) Tosylchloride, pyridine, CH₂Cl₂ 0 °C-rt, 12 h; c) NaN₃, cat. TBAB, DMF, rt 12 h; d) TFA, CH₂Cl₂ 0 °C-rt, 3 h; e) Boc₂O, NaOH, THF 0 °C-rt, 12 h; f) Boc₂O, NaHCO₃, Dioxane/water 0 °C-rt, 12 h; g) **1e**, DIPC, DMAP, DCM, 0 °C-rt, 12 h; h) TFA, CH₂Cl₂ 0 °C-rt, 3 h; i) **1d**, EDCI, HOBT, DCM 0 °C-rt, 3 h; j) Pd/C, TFA, Hydrogen gas, MeOH, rt, 2 h; k) **1e**, EDCI, HOBT, DCM 0 °C-rt, 3 h; l) TFA, CH₂Cl₂ 0 °C-rt, 3 h; m) K₂PtCl₄, Water rt, 12 h.

Synthetic Scheme for 2 (mal-PEG-CPT)



Scheme 2. Synthetic scheme for mal-PEG-CPT (**2**). Reagents and conditions: a) TPP, THF, 0 °C-rt, 6 h; b) 6-maleimidocaproic acid, EDCI, HOBT, DIPEA, DCM, 0 °C-rt, 12 h; c) TFA, DCM, rt, 12 h; d) TFA/DCM, 0 °C, 1 h; e) 2-chloro-1-methylpyridinium iodide, triethylamine, DCM, 2 h, 20 °C.

Experimental procedures

General

The key raw materials: diaminopropanoic acid, EDCI.HCl, Pd/C and K_2PtCl_4 were obtained from Sigma-Aldrich whereas camptothecin was obtained from Combi-Blocks Inc., USA and tetraethyleneglycol obtained from Alfa Aesar, USA. All materials were of analytical grade. Solvents were purified by standard procedures. Experiments were carried out under dry dinitrogen atmosphere. Melting points were recorded on Buchi B-540 melting point apparatus. 1H NMR spectra were recorded at 500 MHz using Brüker AVANCE 500 MHz and JEOL 400 MHz instruments at 278 K. Chemical shifts (δ) are reported in ppm downfield from tetramethylsilane with the solvent as the internal reference ($CDCl_3$: δ 77.26 ppm). 1H NMR data

is reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, br = broad, m = multiplet), coupling constants (Hz), and integration. ^{13}C NMR spectra were recorded on either a JEOL-400 (100 MHz) or a Bruker AVANCE 500 MHz (125 MHz) with complete proton decoupling. Mass spectra were recorded on a Bruker-Ultraflex TOF/TOF. Perkin Elmer FT-IR Spectrometer Spectrum RXI was used for recording IR spectra. Silica gel (100-200 mesh) was used for the purification of compounds. The known compounds were characterized by comparing their ^1H and ^{13}C NMR spectra to the reported data. Tetraethyleneglycol series of intermediates (**1a**¹, **1b**², **1c**³, **1d**⁴) were prepared using the literature protocols starting from tetraethyleneglycol. Camptothecin intermediates, **1g** and **1h**, were prepared using the reported protocols.⁵ Bocglycine (**1e**) and Dibocpropioncacid (**1f**) were prepared using reported protocols.^{6,7} **2** was synthesized following the procedure reported for an analogous camptothecin derivative.¹

Trastuzumab, cetuximab and rituximab were manufactured by Genentech Inc., USA, Merck KGaA, Germany and Roche Diagnostics GmbH, Germany respectively. Buffer components were purchased from Qualigens or Sigma. Gradient gels and molecular weight marker were purchased from Biorad. HPLC mobile phases were purchased from Merck or Fisher Scientific. For preparative HPLC, a Waters 1525EF system was used with a photodiode array (PDA) detector or a UV/visible detector. For analytical HPLC, a Waters e2695 system with PDA and fluorescence detector was used. For protein purification, a GE Akta Purifier 10 was employed. Pt-estimation by Graphite Tube Atomizer-Atomic Absorption Spectrometry (GTA-AAS) was carried out on PinAAcle 900Z (PerkinElmer).

Tumor cell lines (breast: MCF7, MDA-MB-231, MDA-MB-468, MDA-MB-453, SK-BR-3; ovarian: SK-OV-3; lung: A549 and colorectal: HT29, SW620) were obtained from American Type Culture Collection (ATCC). Cells were cultured in high glucose DMEM (HiMedia) or McCoy's media for SK-BR-3 (ATCC) supplemented with 10 % fetal bovine serum (Life Technologies) and 1 % Penicillin-Streptomycin solution (HiMedia). Cells were maintained at 37 °C in a humidified atmosphere of 5 % CO₂.

Synthesis of Pt-PEG-CPT (**1**)

[(2-(2-{2-[2-(2-Azido-ethoxy)-ethoxy]-ethoxy)-ethoxy]-acetylamino]-acetic acid 4-ethyl-3,13-dioxo-3,4,12,13-tetrahydro-1H-2-oxa-6,12a-diaza-dibenzo[b,h]fluoren-4-yl ester) [1i**]:** To intermediate **1d** (1.52 g, 5.24 mmol), 50 ml of DCM, EDCI (1.25 g, 6.55 mmol) and HOBt (1 g, 6.55 mmol) were added at 0 °C. The reaction mixture was stirred for 30 min and a solution of

1h (2.2 g, 4.37 mmol) in DCM (10 ml)/DIPEA (763 μ l) was added. The reaction mixture was stirred for 2 h under nitrogen atmosphere at room temperature. After completion of the reaction, 20 ml of saturated citric acid solution was added and stirred for 10 min and filtered through celite. The organic layer was washed with half-saturated citric acid twice (2 \times 50 ml). The organic layer was concentrated and precipitated with DCM/Ether (3 ml/30 ml), filtered and dried to yield 1.6 g of azide compound (**1i**) as a pale yellow solid (56 %). **MP**: 51 $^{\circ}$ C; **IR**: 3374, 2363, 2341, 2100, 1751, 1602, 1123, 760; **1 H NMR** (500 MHz, DMSO): δ 8.70 (s, 1H), 8.25 (t, J = 5.5 Hz, 1H), 8.15 (dd, J = 19.4, 8.2 Hz, 2H), 7.87 (t, J = 7.6 Hz, 1H), 7.72 (t, J = 7.4 Hz, 1H), 7.18 (s, 1H), 5.54 – 5.45 (m, 2H), 5.35 – 5.23 (m, 2H), 4.21 (dd, J = 18.0, 5.5 Hz, 1H), 4.08 (dd, J = 18.0, 5.9 Hz, 1H), 3.99 – 3.86 (m, 2H), 3.61 – 3.49 (m, 10H), 3.46 (m, 6H), 2.15 (m, 2H), 0.99 (d, J = 6.4 Hz, 2H), 0.90 (t, J = 7.1 Hz, 3H); **13 C NMR** (126 MHz, DMSO): δ 170.54, 169.38, 167.51, 156.93, 152.71, 148.25, 146.35, 145.55, 132.02, 130.91, 130.11, 129.31, 128.95, 128.36, 128.15, 119.37, 95.72, 76.73, 70.73, 70.19, 70.18, 70.11, 70.08, 69.87, 69.66, 66.75, 50.39, 41.13, 30.84, 23.75, 7.99; **MS**: 687.1 [M+Na] $^{+}$.

{2-[2-(2-[2-(2,3-Bis-tert-butoxycarbonylamino-propionylamino)-ethoxy]-ethoxy)-ethoxy]-acetylamino}-acetic acid 4-ethyl-3,13-dioxo-3,4,12,13-tetrahydro-1H-2-oxa-6,12a-diaza-dibenzo[b,h]fluoren-4-yl ester [1k]: The crude azide (1.6 g) was dissolved in methanol (30 ml) and 0.5 ml TFA, 200 mg of Pd/C was added. This solution was hydrogenated for 1 h, filtered and concentrated. The residue was precipitated with DCM/Ether (5 ml/25 ml), filtered and dried. The crude amine salt (**1j**) product was taken for the next step without further purification. To **1f** (820 g, 2.71 mmol), 25 ml of DCM, EDCI (647 mg, 2.5 mmol) and HOBt (518 mg, 2.5 mmol) were added at 0 $^{\circ}$ C. The reaction mixture was stirred for 30 min and a solution of **1j** (1 g, 1.35 mmol) in DCM (5 ml)/DIPEA (235 μ l) was added. The reaction mixture was stirred for 3 h under nitrogen atmosphere at room temperature. After completion of the reaction, 0.1 N HCl (20 ml) was added and stirred for 10 min and separated. The organic layer was again washed twice with 0.1 N HCl (2 \times 15 ml). The organic layer was concentrated and purified by column (1.5 % MeOH in CHCl₃). The fraction obtained from column was concentrated, the residue was precipitated with DCM/Ether (5 ml/25 ml), filtered and dried to yield 600 mg of coupling product **1k** (51 %). **MP**: 80 $^{\circ}$ C; **IR**: 3426, 2881, 2364, 2344, 1757, 1642, 1275, 1260, 749. **MS**: 947.10; **1 H NMR** (400 MHz, DMSO-D₆): δ 8.67 (s, 1H), 8.23 (t, J = 6.0 Hz, 1H), 8.14 (dd, J = 20.4, 8.3 Hz, 2H), 7.87 (t, J = 7.6 Hz, 1H), 7.71 (t, J = 7.5 Hz, 1H), 7.17 (s, 1H), 6.70 (t, J = 5.6 Hz, 1H), 6.64 (d, J = 8.0 Hz, 1H), 5.55 – 5.43 (m, 2H), 5.27 (m, 2H), 4.22 (dd, J = 17.8, 6.0 Hz, 1H), 4.08 (dd, J = 17.7, 6.1 Hz, 1H), 4.01 – 3.87 (m, 3H), 3.59 – 3.49 (m, 2H), 3.46 (m,

10H), 3.25 – 3.10 (m, 6H), 2.15 (m, 2H), 1.36 (s, 18H), 0.92 (t, $J = 7.3$ Hz, 1H); ^{13}C NMR (101 MHz, DMSO- D_6): δ 170.68, 170.50, 169.40, 167.52, 157.01, 156.21, 155.62, 152.85, 148.38, 146.45, 145.55, 132.09, 130.96, 130.25, 129.42, 129.05, 128.47, 128.23, 119.48, 95.76, 78.73, 78.41, 76.77, 70.79, 70.23, 70.17, 70.05, 69.93, 69.35, 66.82, 55.31, 50.71, 42.40, 39.02, 30.94, 28.64, 28.50, 8.02; **MS**: 947.1 $[\text{M}+\text{Na}]^+$.

Synthesis of Pt-PEG-CPT (1): TFA (1.5 ml) was added to diboc compound **1I** (200 mg, 0.21 mmol) in 4 ml of DCM and stirred for 1 h at 0 °C and then for 1 h at room temperature. TLC showed the completion of the reaction following which TFA was removed in vacuum. The residue was precipitated using DCM/Ether (1 ml/10 ml). To this, 8 ml of water was added and 150 mg (0.32 mmol) of K_2PtCl_4 in 1 ml of water was added drop-wise. Immediate precipitation was observed and the reaction was left at room temperature for 12 h. The reaction mixture was centrifuged and the solid obtained was washed with 5 ml of water. The crude product (**1**) obtained was purified by preparative HPLC using a Reprosil C18 column (Dr. Maisch, Germany; 250x20 mm, 5 μm) with 10 mM NaCl in water/acetonitrile gradient. **MP**: 112 °C; **IR**: 3367, 2934, 2369, 1673, 1521, 1166; ^1H NMR (500 MHz, MeOD): δ 8.66(s), 8.21(d), 8.09(d), 7.90(t) 7.73(t), 7.46(s), 5.67(s), 5.63(s), 5.51(s), 5.49(s), 5.48(s), 5.47(s), 5.37(s), 4.36-4.07 (m), 3.69 - 3.38(m), 2.27(q), 1.06(t); ^{13}C NMR (126 MHz, DMSO- d_6): δ 169.92, 168.80, 166.91, 166.39, 156.44, 152.25, 147.83, 145.89, 144.98, 131.53, 130.38, 129.64, 128.84, 128.47, 127.90, 127.65, 118.94, 95.16, 76.23, 70.25, 69.73, 69.66, 69.61, 69.50, 69.40, 68.61, 68.58, 66.28, 60.89, 60.77, 50.12, 49.58, 49.31, 30.44, 7.43; ^{195}Pt NMR (500 MHz, DMSO): - 2977; **MS**: 1029.24 $[\text{M}+\text{K}]^+$.

Synthesis of Mal-PEG-CPT (2)

Synthesis of 2b: Azide **2a** (5 g, 14.99 mmol) was taken in THF and TPP (7.8 g, 29.99 mmol) was added. During addition, the reaction mixture was kept at 0 °C under ice bath. The reaction was left stirring for 6 h and then 1 ml water was added. The reaction was left stirring overnight. The TLC was checked and after completion THF was removed. The residue was directly purified by column chromatography to yield **2b**. The column was carried out with MeOH/ CHCl_3 as mobile phase. The impurities were removed in 1-1.5 % MeOH/ CHCl_3 , the product started to elute from 3 % MeOH/ CHCl_3 and the column was run up to 5 % MeOH/ CHCl_3 to completely elute the product.

Synthesis of 2c: 6-maleimidocaproic acid (500 mg, 2.36 mmol), DCM (10 ml), EDCI and HOBt were mixed at 0 °C under nitrogen. The reaction mixture was stirred for 30 min and then **2b** was

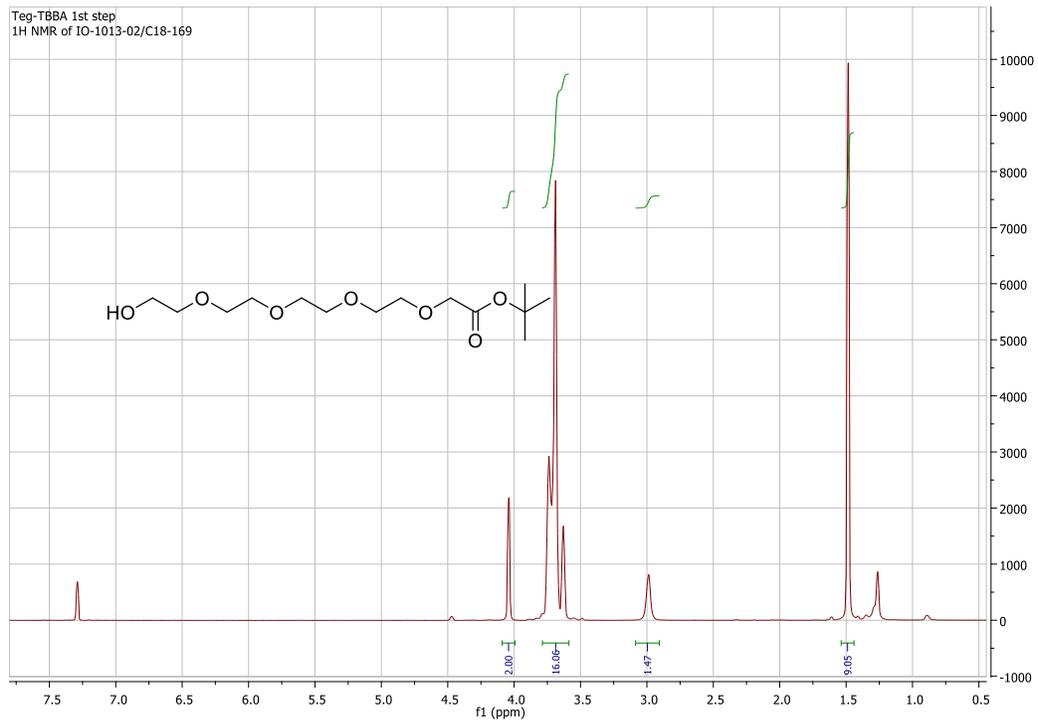
added to the reaction mixture. To the reaction mixture, few ml of DIPEA were added to keep the reaction slightly alkaline but care was taken to keep pH < 8. The reaction mixture was stirred overnight under nitrogen atmosphere at room temperature. After completion, the reaction was quenched with water and diluted with DCM. The organic layer was washed with 2x30 ml of 0.1 N HCl and 2x30 ml of water. Organic layer was concentrated and purified by column chromatography to obtain **2c**. The column was carried out with MeOH/CHCl₃ as mobile phase. The product eluted in 1 % MeOH/CHCl₃. **ESI-MS**: 501.22 (MH⁺); **¹H NMR** (CDCl₃): 6.66(s), 3.99(s), 3.39-3.68(m), 1.54-1.66(m), 1.45(s).

Synthesis of 2d: Intermediate **2c** (540 mg, 1.078 mmol) was taken in DCM and cooled to 0 °C under ice bath. To this cooled solution, TFA was added and left stirring for 2 h. The TLC was checked and after completion, the reaction mixture was concentrated under vacuum to remove TFA. The crude residue (**2d**) was used for next reaction.

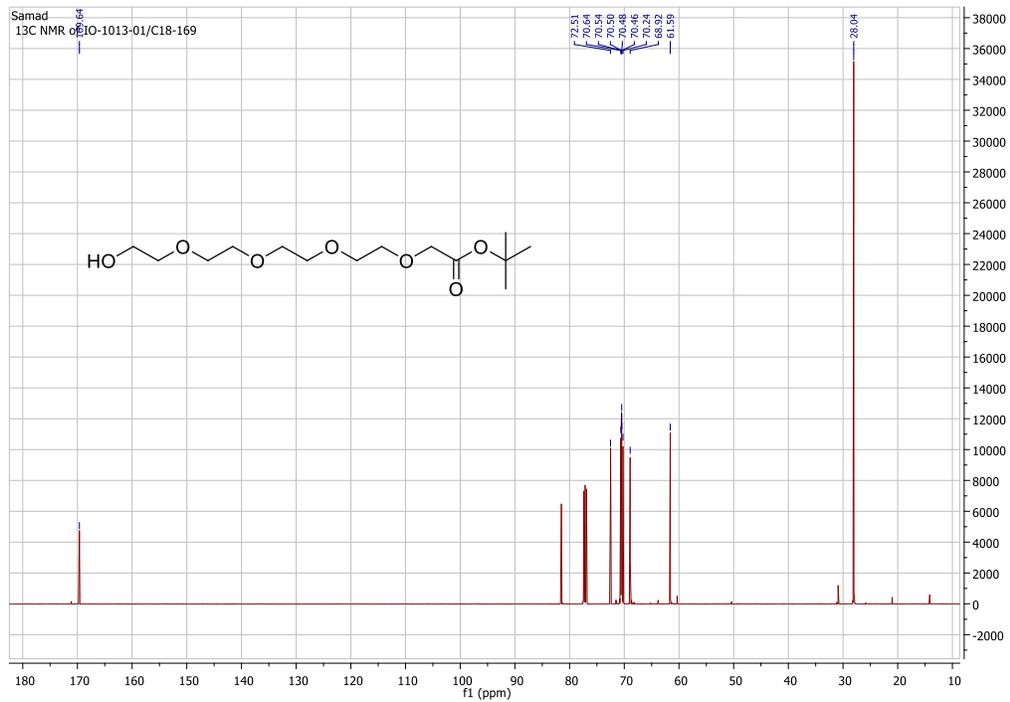
Synthesis of 2f: **2e** was dissolved in 10 ml dry DCM under nitrogen in cool conditions. To this solution, 6 ml TFA was added drop-wise and stirred for 1 h at the same temperature. Completion of reaction was checked by TLC. Reaction mixture was then concentrated on Rotaevaporator using NaOH as trap. Remaining residue was soaked with 1 ml DCM and excess of diethyl ether. The precipitate was centrifuged for 10 min and **2f** was obtained as residue.

Synthesis of mal-PEG-CPT (2): To a suspension of **2f** (290 mg, 0.71 mmol), **2d** (475 mg, 1.07 mmol) and triethylamine (0.3 ml, 2.13 mmol) in 20 ml of anhydrous CH₂Cl₂, 2-chloro-1-methylpyridinium iodide (Mukaiyama reagent) (270 mg, 1.07 mmol) was added as a finely ground solid. Stirring was continued for 2 h at room temperature during which time the initially cloudy mixture turned into a yellow solution. The mixture was diluted with 50 ml of CH₂Cl₂ and washed with 2x20 ml of 1 M HCl and 2x20 ml of water. The organic layer was dried over Na₂SO₄, and the solvent was removed in vacuum to obtain **2** which was further purified by preparative HPLC using a Reprosil C18 column (Dr. Maisch, Germany; 250x20 mm, 5 μm) with a water/acetonitrile gradient. **MP**: 68-70 °C; **ESI-MS**: 832.41 (MH⁺); **¹H NMR** (DMSO-d₆): 8.78(s), 8.31(t), 8.26(d), 8.22(d), 7.96(t), 7.89(m), 7.81(t), 7.26(s), 7.08(s), 5.59(s), 5.38(s), 4.1-4.4(m), 4.01(d), 3.22 – 3.66(m), 2.10 – 2.22(m), 1.51(m), 1.00(t); **¹³C NMR** (DMSO-d₆): 174.5, 172.5, 170.5, 170.2, 169.3, 167.5, 157.0, 152.8, 148.3, 146.4, 145.5, 132.0, 130.9, 130.2, 129.4, 129.0, 128.4, 128.2, 119.4, 96.7, 76.7, 70.7, 70.2, 70.1, 70.0, 69.9, 69.5, 68.5, 66.8, 51.7, 50.7, 38.8, 38.5, 35.5, 33.5, 30.9, 27.2, 26.3, 25.2, 20.9, 7.9.

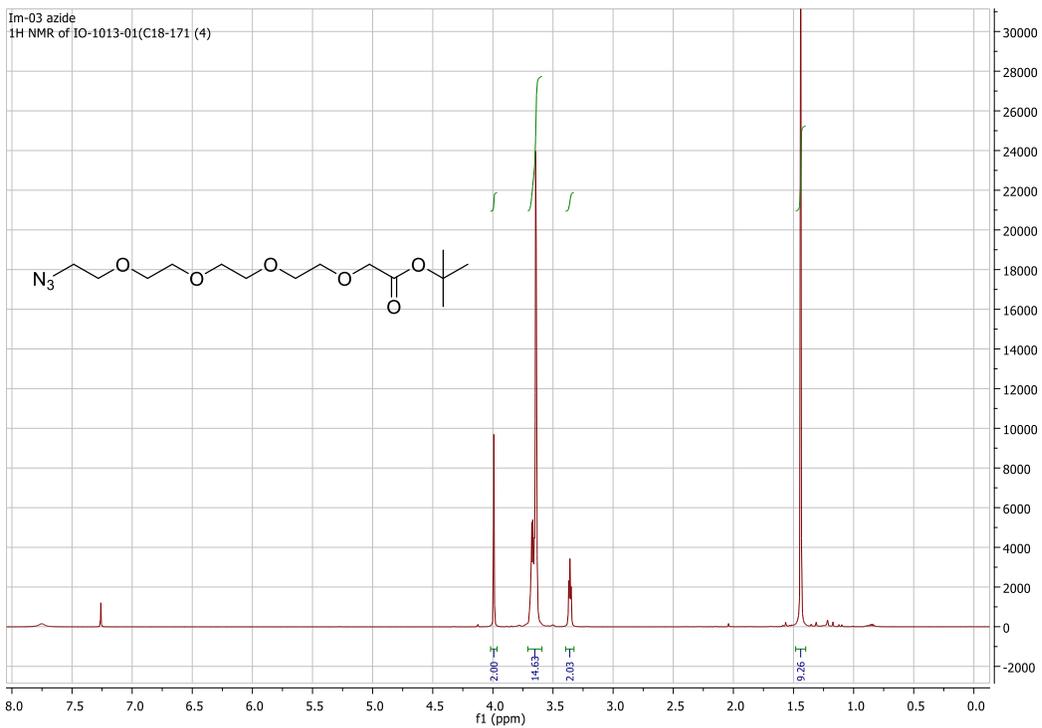
¹H NMR of 1a¹



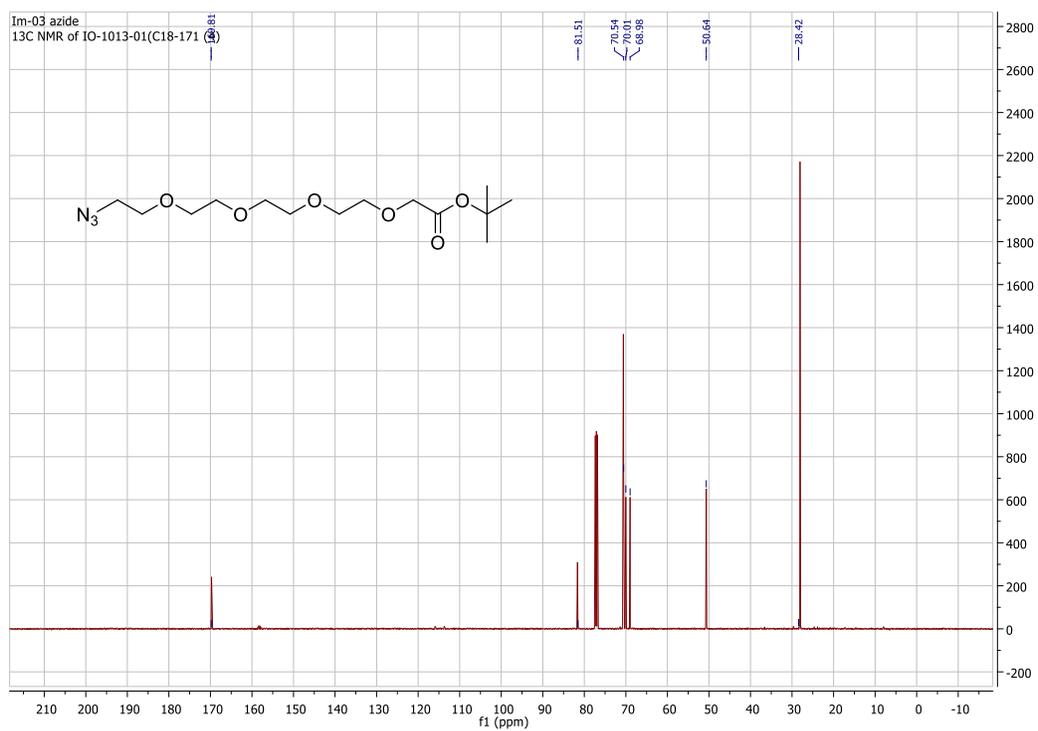
¹³C NMR of 1a



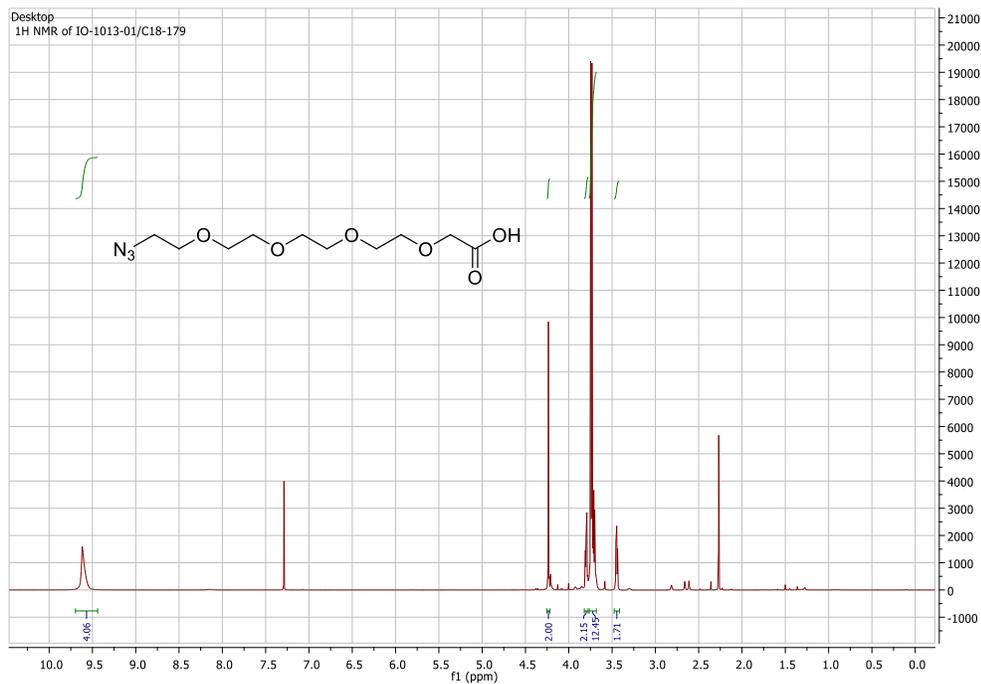
^1H NMR of 1c^3



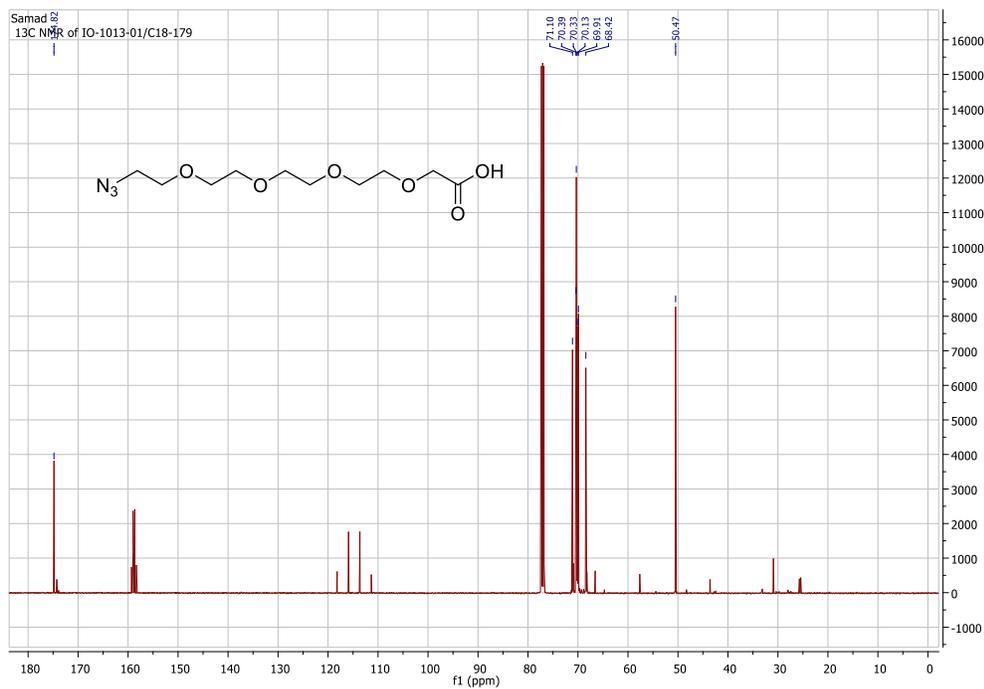
^{13}C NMR of 1c



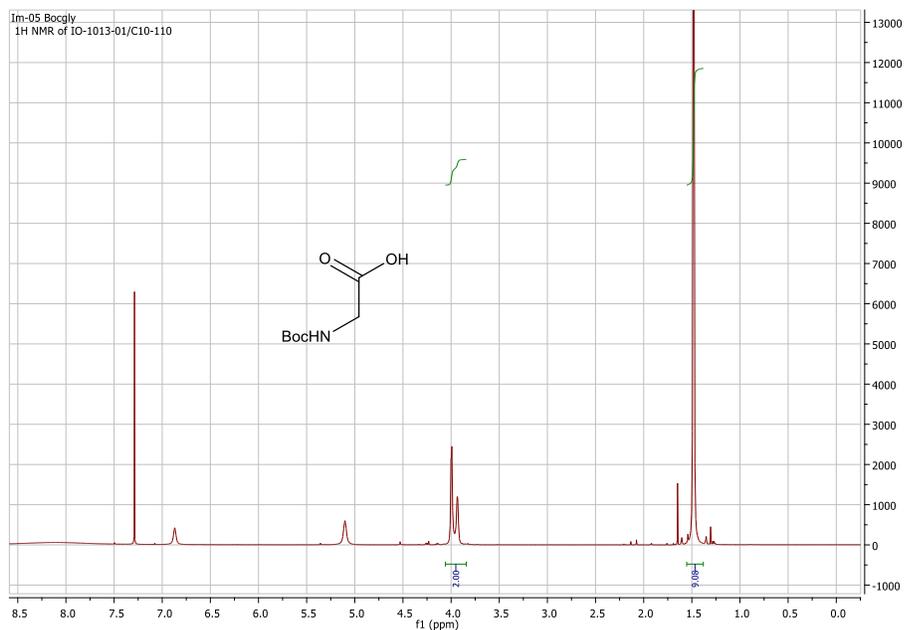
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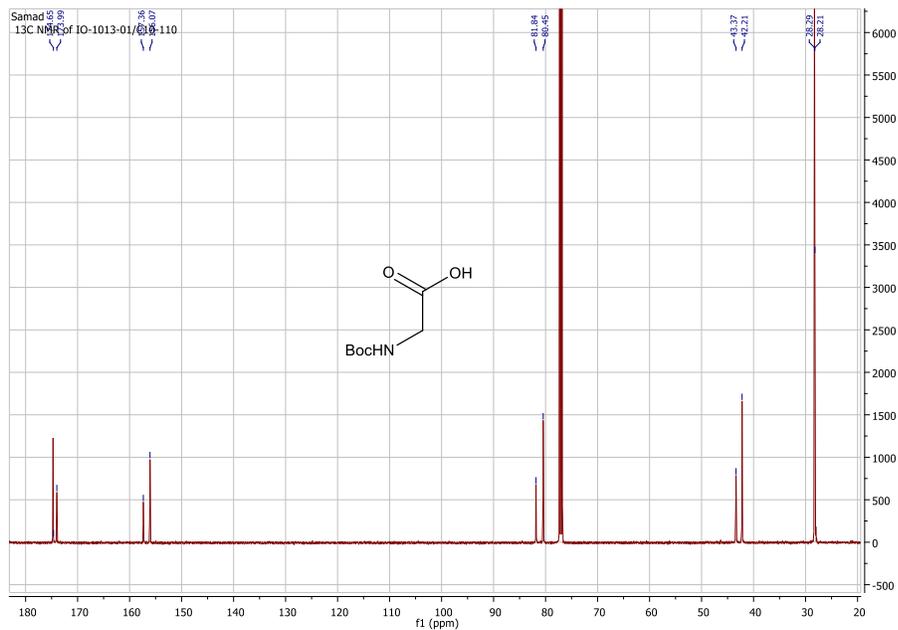
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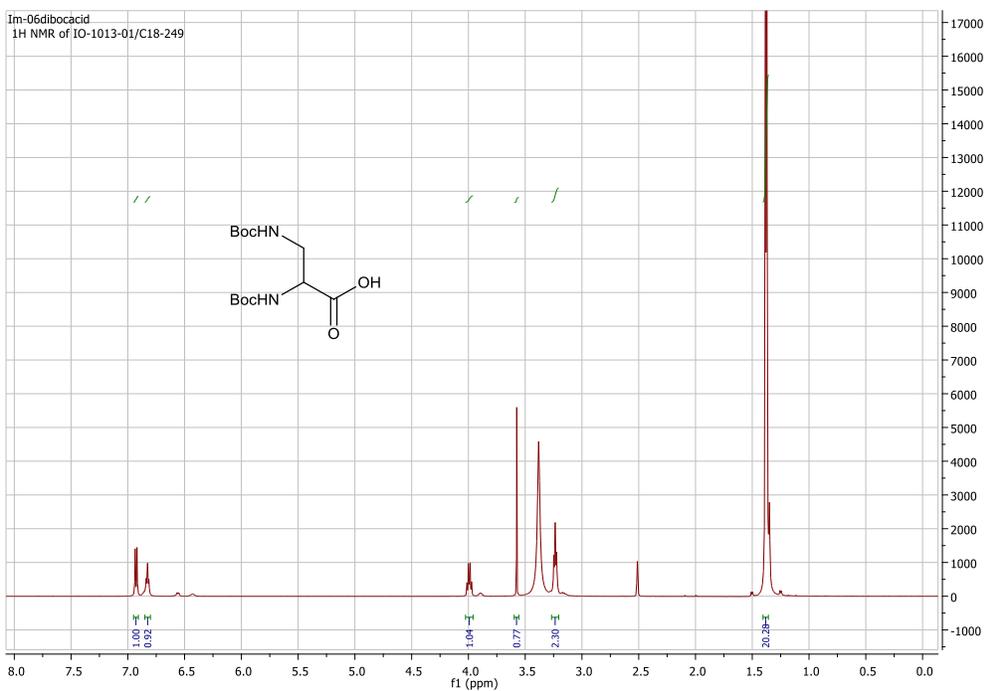
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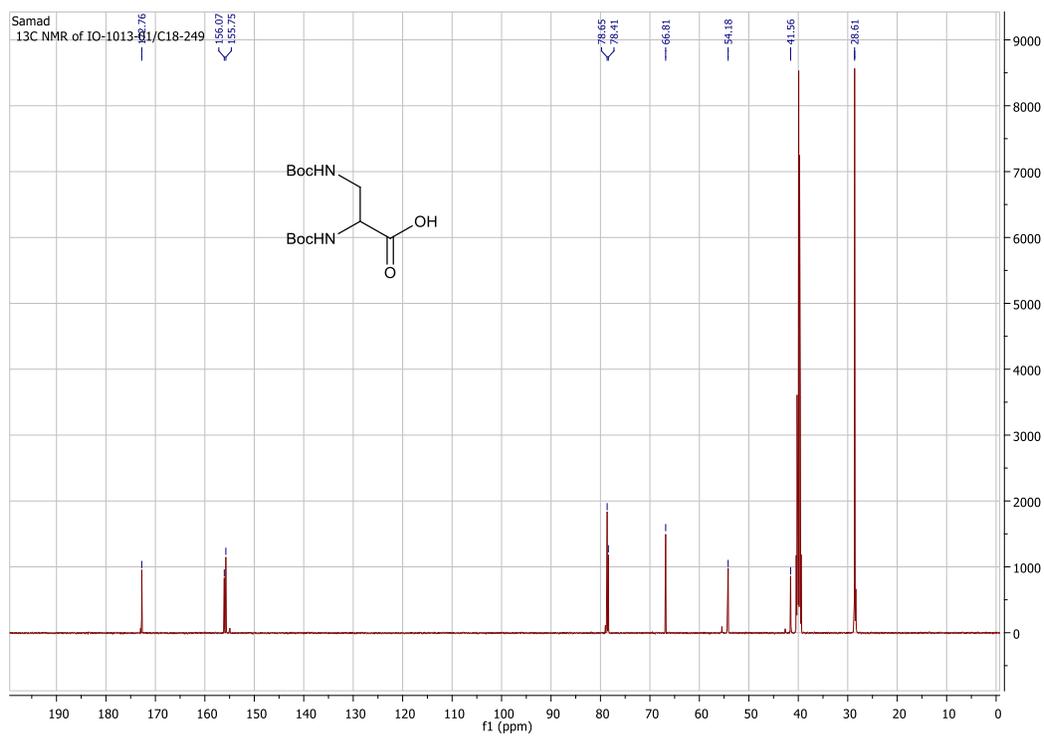
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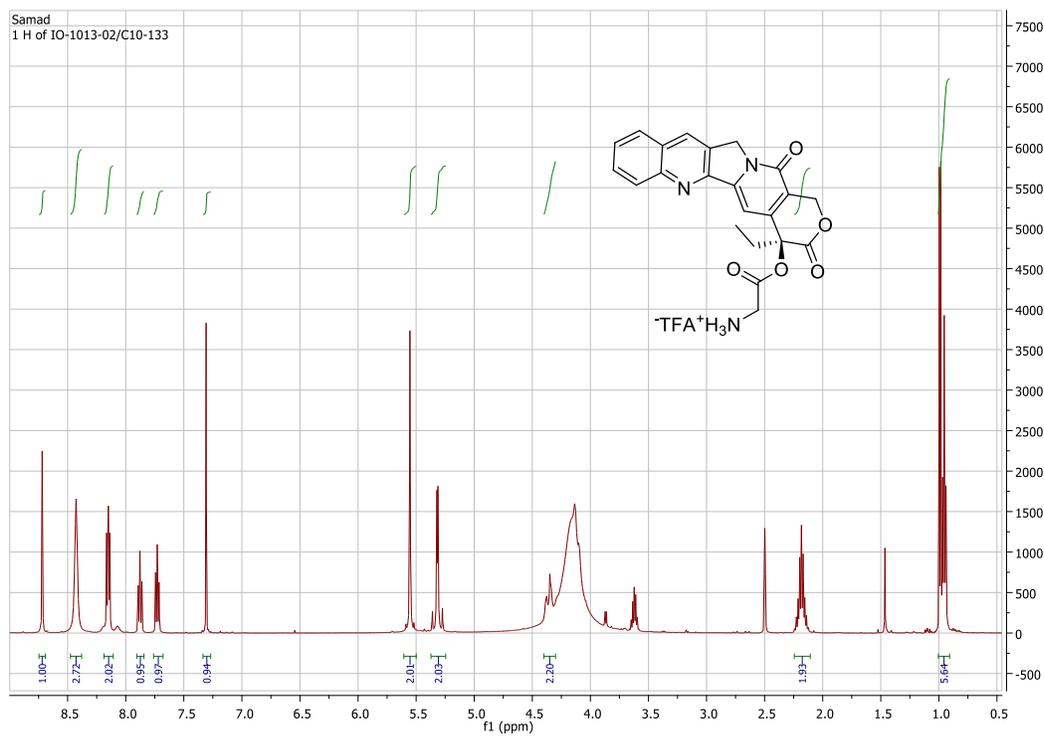
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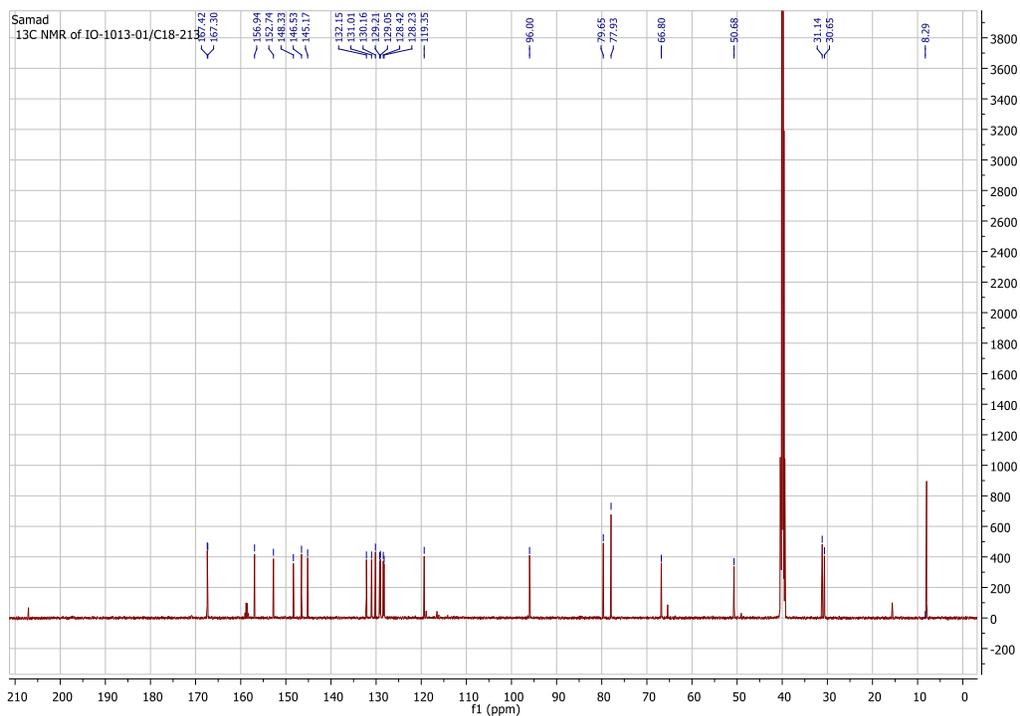
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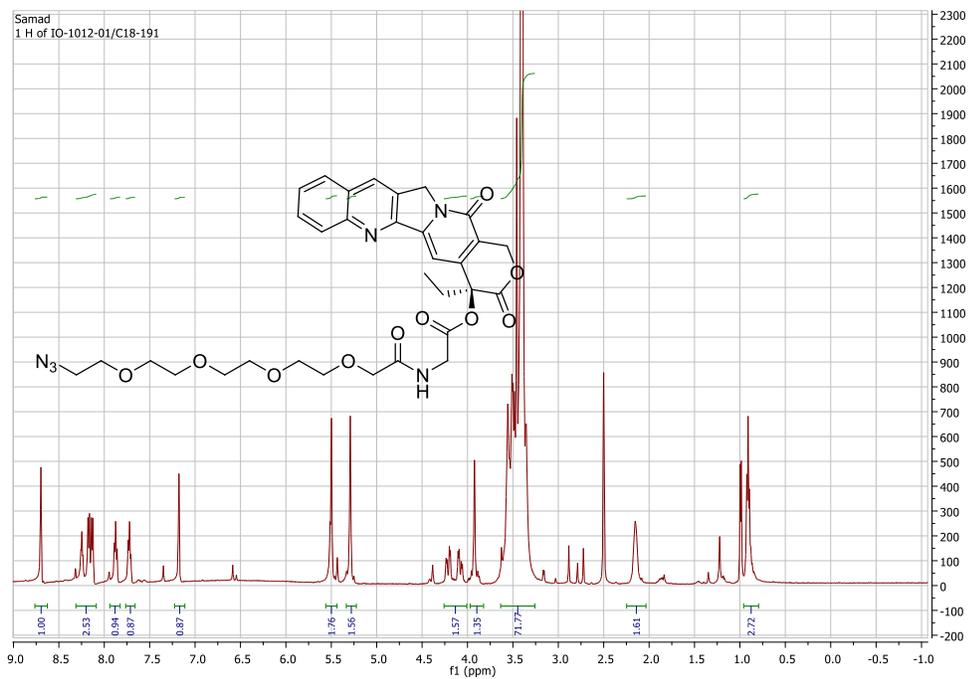
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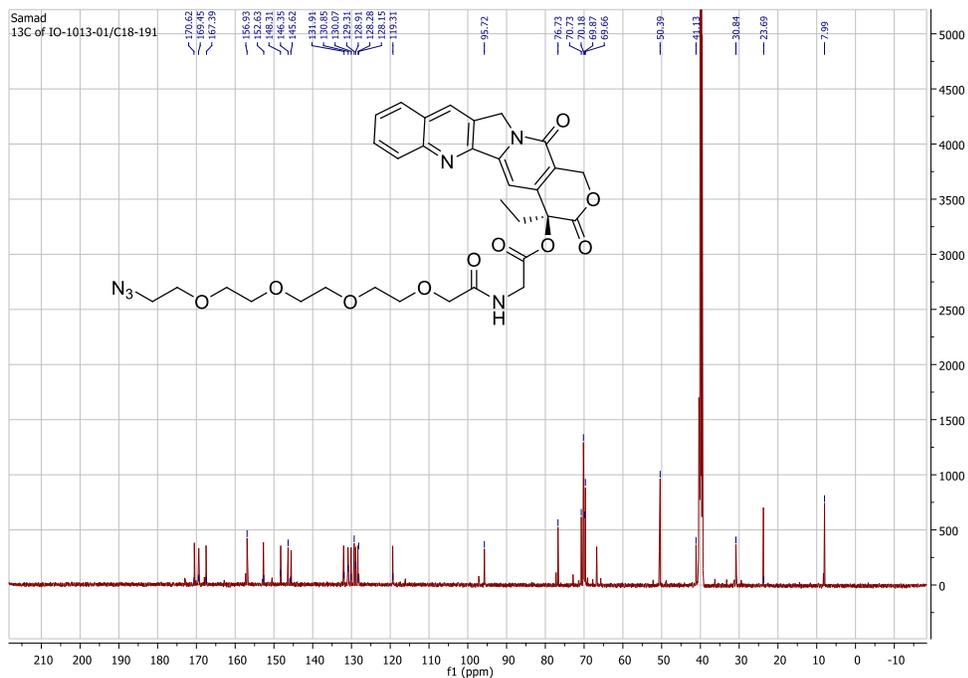
¹³C NMR of 1h



^1H NMR of 1i

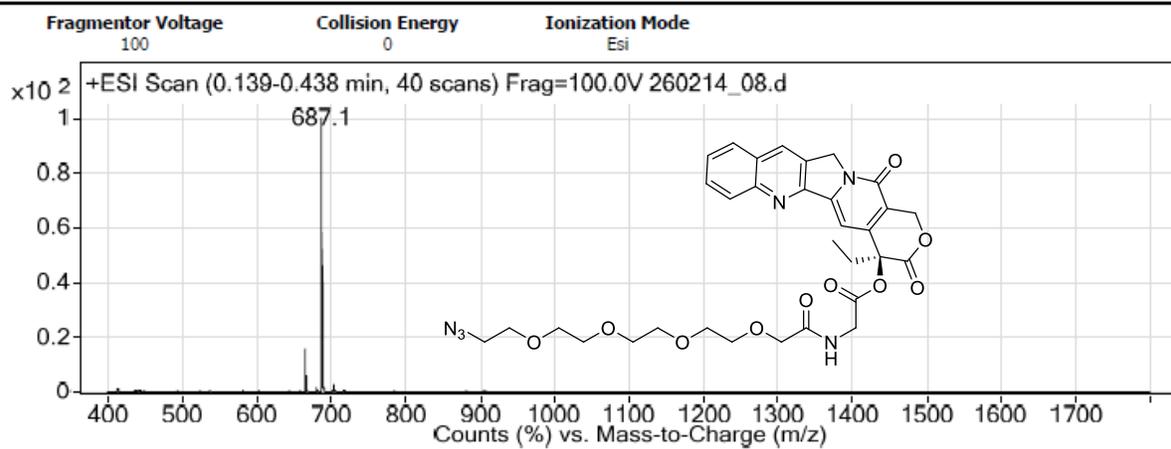


^{13}C NMR of 1i



MS of 1i

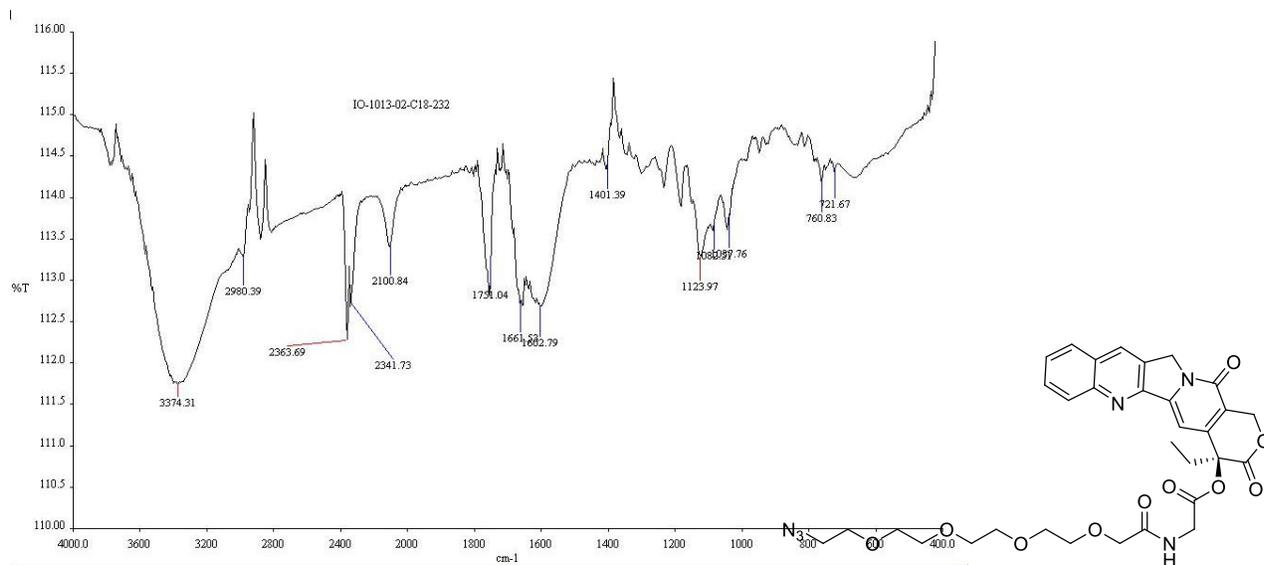
User Spectra



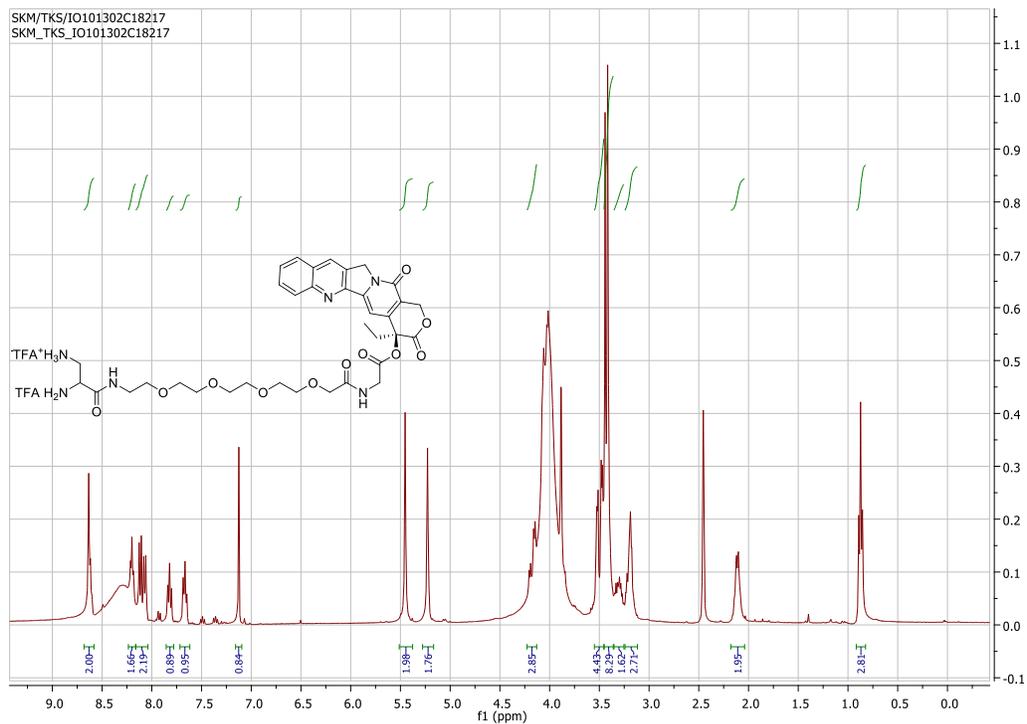
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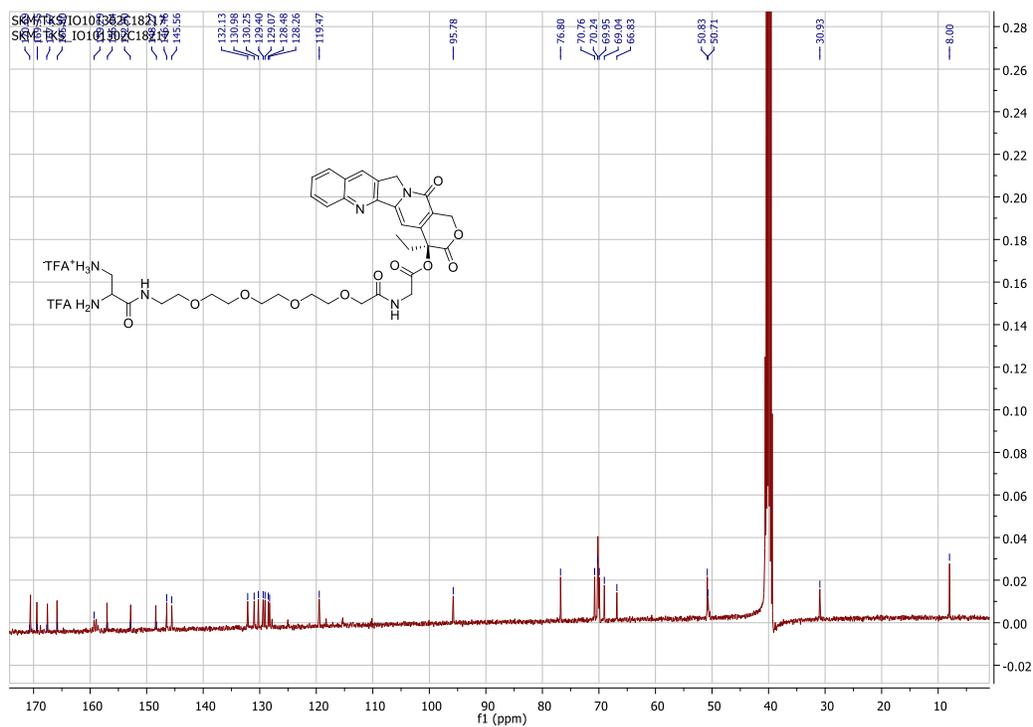
IR of 1i



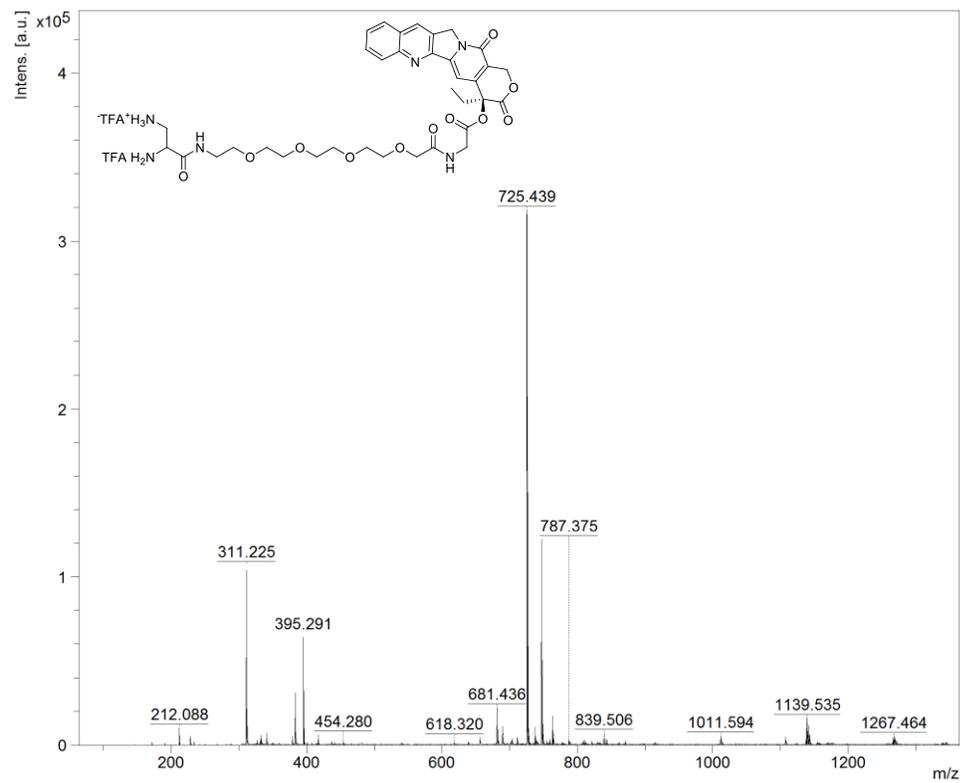
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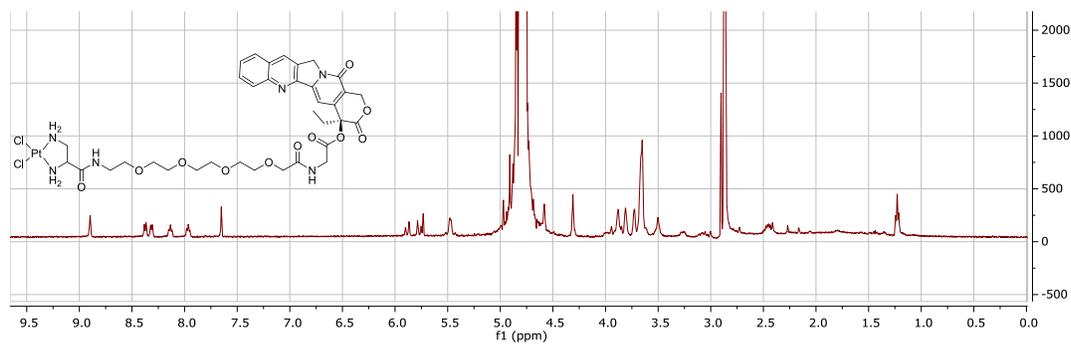
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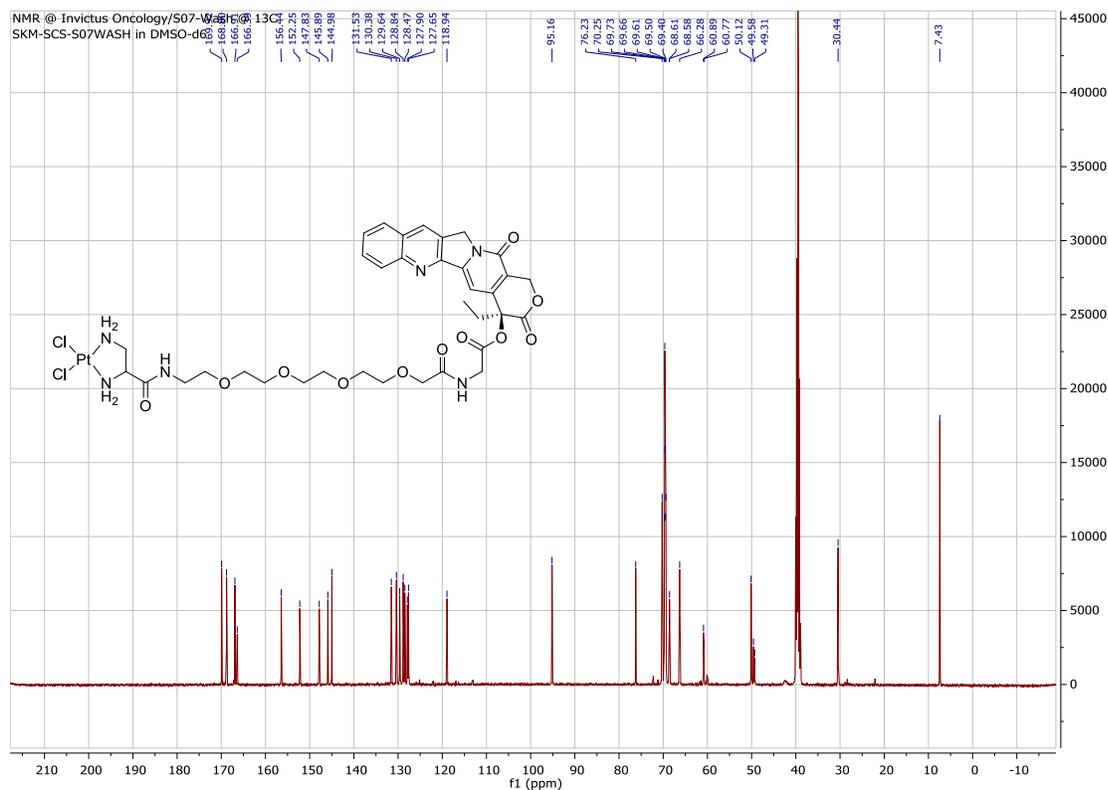
MS of 11



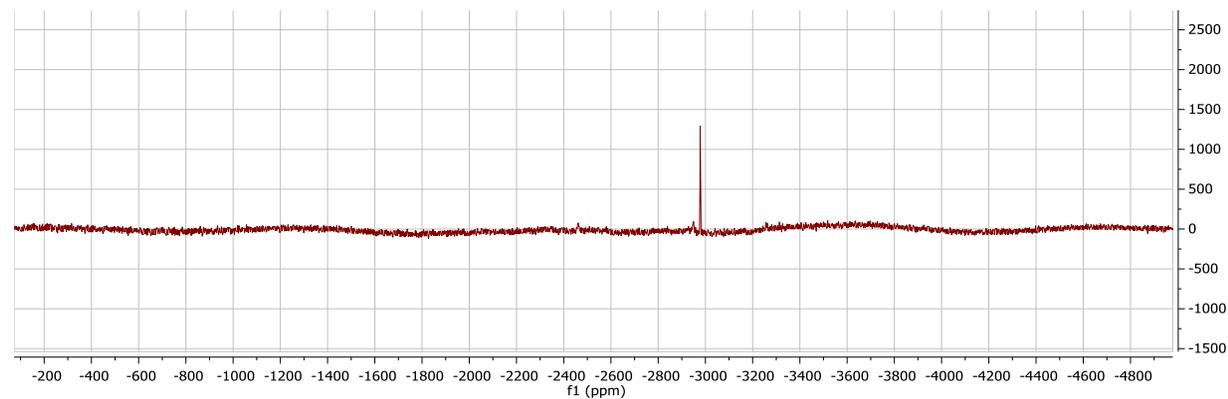
¹H NMR of Pt-PEG-CPT (1)



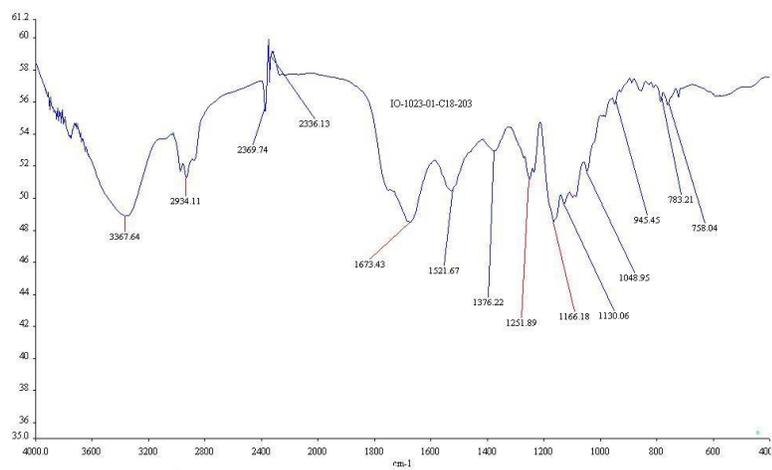
¹³C NMR of Pt-PEG-CPT (1)



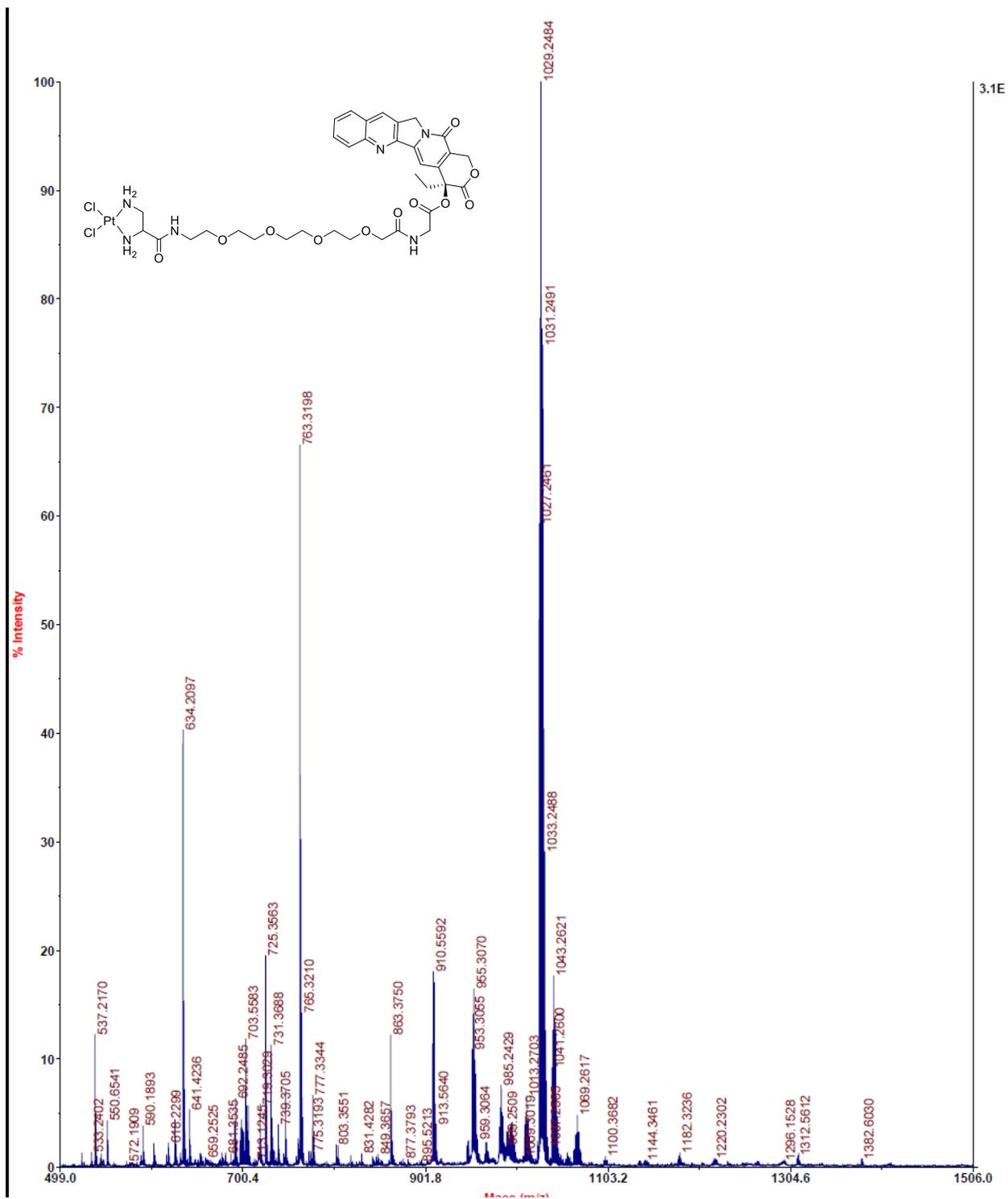
¹⁹⁵Pt NMR of Pt-PEG-CPT (1)



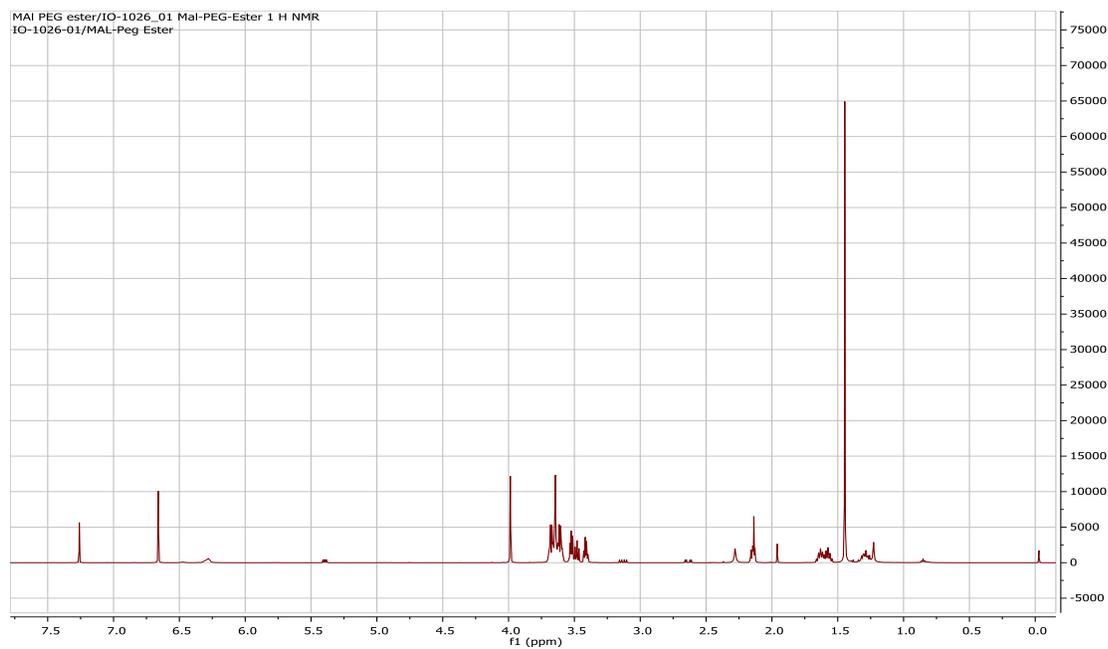
IR of Pt-PEG-CPT (1)



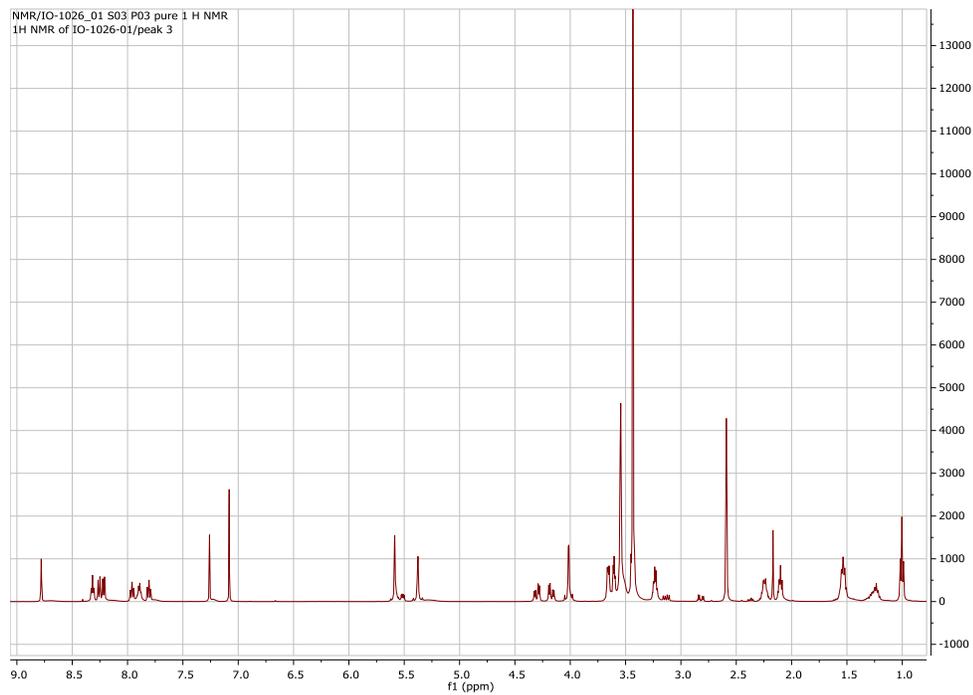
MS of Pt-PEG-CPT (1)



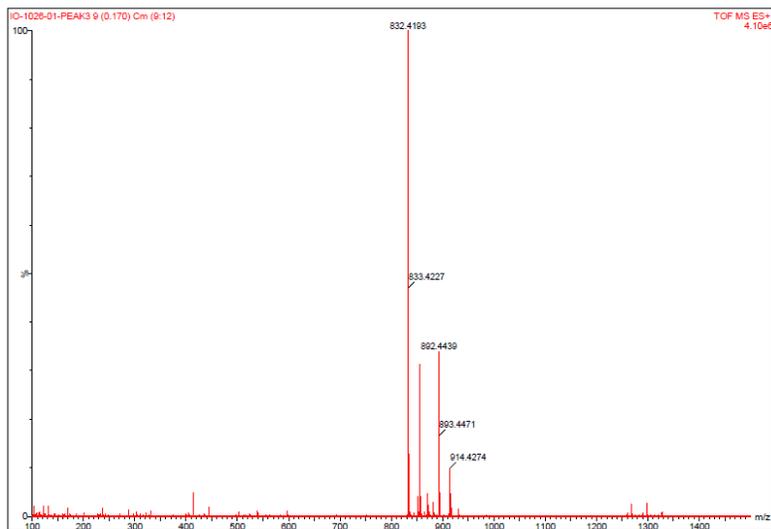
^1H NMR of 2c



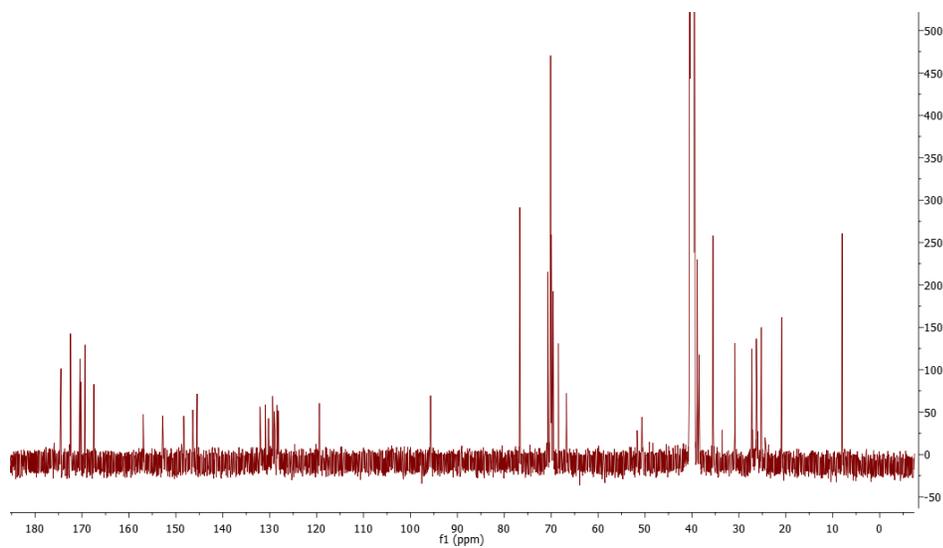
^1H NMR of mal-PEG-CPT (2)



Mass spec of mal-PEG-CPT (2)



¹³C NMR of mal-PEG-CPT (2)



Preparation of antibody-Pt-PEG-CPT and antibody-mal-PEG-CPT conjugates

Trastuzumab (Tra), cetuximab (Ctx) or rituximab (Rtx) was buffer-exchanged into 20 mM sodium phosphate, 150 mM sodium chloride, pH 6.5 (PBS, pH 6.5) buffer using either Zeba Spin Desalting Columns (Thermo Fisher Scientific; 7 K MWCO) or Amicon centrifugal filters (Millipore; 30 kDa). Antibody (30 μ M) was reduced completely using 10 mM TCEP for 1 h at 37 °C. TCEP concentration was reduced to less than 25 μ M using cold and nitrogen-flushed PBS, pH 6.5 by repeated buffer exchange using Amicon centrifugal filters (Millipore; 30 kDa). Using Ellman's assay, the presence of 8 thiols/antibody was confirmed. Pt-PEG-CPT was re-constituted in PBS, pH 6.5 at 1 mM concentration (for lower DARs) or in DMF at a concentration of 10 mM (for higher DARs). Excess of Pt-PEG-CPT was added to the reduced antibody and incubated at 37 °C for 1.5-2 h with shaking at 300 rpm. The mixture was centrifuged and filtered using 0.2 μ m filters to remove any precipitate. Unbound Pt-PEG-CPT was removed by repeated buffer exchange with PBS, pH 6.5 using Amicon centrifugal filters (Millipore; 30 kDa). The extinction coefficients of Pt-PEG-CPT and the antibody were calculated at 254 nm and 280 nm and the DAR of Tra-Pt-PEG-CPT, Ctx-Pt-PEG-CPT and Rtx-Pt-PEG-CPT was measured by UV spectroscopy.⁸

Antibody-mal-PEG-CPT conjugates were prepared similarly with mal-PEG-CPT dissolved in DMF at 10 mM concentration.

Pt estimation by atomic absorption spectroscopy (AAS)

Pt-estimation by Graphite Tube Atomizer-Atomic Absorption Spectrometry (GTA-AAS) was carried out on PinAAcle 900Z (PerkinElmer). Samples were digested in conc. nitric acid and diluted in 0.05 % hydrochloric acid. Pt concentration of the samples was calculated based on a calibration curve with standard stock solution.

Preparation of trastuzumab-fab and cetuximab-fab fragment

Trastuzumab at 21 mg/ml concentration was buffer-exchanged into 20 mM sodium phosphate, 10 mM EDTA, pH 7.0 buffer using Zeba Spin Desalting Columns (Thermo Fisher Scientific; 7 K MWCO). Cetuximab was buffer-exchanged and concentrated to obtain a 20 mg/ml solution using Amicon centrifugal filters (Millipore; 30 kDa). Immobilized Papain (Pierce) was activated using digestion buffer (20 mM sodium phosphate, 10 mM EDTA, 20 mM cysteine, pH 7.0). The antibody was diluted with digestion buffer and incubated with immobilized papain for 4 h at 37 °C with shaking at 300 rpm. The digest was recovered from the resin by centrifugation and 0.2 μ m filtration and the trastuzumab-fab fragment (Tra_{Fab}) or cetuximab-fab fragment (Ctx_{Fab}) was purified by MabSelect SuRe HiTrap column (GE Healthcare Life Sciences) on a GE Akta

Purifier 10 (GE Healthcare Life Sciences) chromatography system. The purified Fab fragment was concentrated and buffer-exchanged into PBS, pH 6.5 using Amicon centrifugal filters (Millipore; 10 kDa).

Preparation of Tra_{Fab}-Pt-PEG-CPT, Tra_{Fab}-mal-PEG-CPT, Ctx_{Fab}-Pt-PEG-CPT and Ctx_{Fab}-mal-PEG-CPT conjugates

Fab fragment (80 μ M) was reduced completely using 1 mM TCEP for 1 h at 37 °C. TCEP was brought to a concentration of <10 μ M using cold and nitrogen flushed PBS, pH 6.5 by repeated buffer exchange using Amicon centrifugal filters (Millipore; 10 kDa). Pt-PEG-CPT was dissolved in DMF at 10 mM concentration. Excess of Pt-PEG-CPT was added to the reduced Fab and incubated at 37 °C for 1.5-2 h with shaking at 300 rpm. The mixture was centrifuged and filtered using 0.2 μ m filters to remove any precipitate. Unbound Pt-PEG-CPT was removed by repeated buffer exchange with PBS, pH 6.5 using Amicon centrifugal filters (Millipore; 10 kDa). The extinction coefficients of Pt-PEG-CPT and the Fab fragment were calculated at 254 nm and 280 nm and the DAR was calculated by UV spectroscopy.⁸ mal-PEG-CPT conjugates was prepared similarly.

Size Exclusion-High Performance Liquid Chromatography (SE-HPLC)

Size-exclusion chromatography was carried out on a Waters e2695 analytical system with a PDA and fluorescence detector using Yarra SEC-3000 column (Phenomenex Inc.; 300 \times 4.6 mm, 3 μ m). PBS, pH 6.5, 10 % isopropanol, 0.025 % sodium azide was used as mobile phase and column temperature was set to 40 °C. Fluorescence was measured by excitation at 369 nm and emission at 430 nm.

Hydrophobic Interaction Chromatography (HIC) of Tra_{Fab}-Pt-PEG-CPT and Tra_{Fab}-mal-PEG-CPT

Analytical HIC was carried out on a Waters e2695 analytical system with a PDA and fluorescence detector using TSKgel Phenyl-5PW column (Tosoh Bioscience LLC; 2 \times 7.5 mm, 10 μ m). Analysis was performed with a gradient between 20 mM sodium phosphate, 600 mM sodium citrate, pH 6.5 and 20 mM sodium phosphate, 20 % isopropanol, pH 6.5. Peak area at 280 nm was used to assess the percentage distribution of the ADC species and to calculate DAR.

Preparative HIC was performed on GE Akta Purifier 10 (GE Healthcare Life Sciences) using a HiTrap Phenyl HP column (GE Healthcare Life Sciences; 1 ml). Separation was performed with a gradient between 20 mM sodium phosphate, 600 mM sodium citrate, pH 6.5 and 20 mM

sodium phosphate, 20 % isopropanol, pH 6.5. The collected fractions were buffer exchange into PBS, pH 6.5 by using Amicon centrifugal filters (Millipore; 10 kDa) and DAR was measured by UV spectroscopy as described above.

Enzyme-linked immunosorbent assay (ELISA)

Nunc maxisorp plates were coated overnight with EGFR antigen (Sino Biological Inc.) for cetuximab, HER2 antigen (Sino Biological Inc.) for trastuzumab and CD20 antigen (Elabscience Biotechnology Co., Ltd) for rituximab in PBS, pH 6.5 at a concentration of 2-2.5 µg/ml at 4 °C. The wells were washed with PBST, pH 6.5 (PBS, pH 6.5+0.1 % tween-20) and blocked using 2 % bovine serum albumin (BSA; Thermo) in PBST. After washing, the wells were incubated with various concentrations of antibody and ADC. The wells were further washed with PBST, followed by addition of horseradish peroxidase (HRP)-conjugated anti-human IgG1 antibody (Jackson ImmunoResearch Laboratories Inc.). Unbound secondary antibody was removed by washing and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), the colorimetric substrate for HRP, solution containing H₂O₂ was added. Reaction was stopped using 0.1 % sodium dodecyl sulfate (SDS) and the plate was read at 415 nm using a plate spectroscopic reader (Biorad). Absorbance at 415 nm versus concentration of antibody was plotted and fitted to one-site specific binding equation using GraphPad Prism software (GraphPad Software, Inc.).

In vitro cytotoxicity assay

The effects of trastuzumab, cetuximab and their conjugates on cell viability were tested using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Cells were plated in 96-well plates and allowed to adhere overnight. The compounds were diluted at varying concentrations in fresh 10 % DMEM or McCoy's media and added to cells the next day. Cells were incubated with the compounds for 48 hours (MCF7, MDA-MB-231, A549, HT29, SW620) or 72 hours (MDA-MB-468, MDA-MB-453, SK-OV-3, SK-BR-3) and then treated with MTT reagent (5 mg/ml) and incubated for further 3-4 hours. The formazan crystals formed were dissolved in methanol-DMSO solution (1:1) for 10-15 minutes at 37 °C and the signal was measured using a microplate reader (Biorad) at 550 nm with background correction at 655 nm. Dose response curves were generated using GraphPad Prism software (GraphPad Software, Inc.) with a log(inhibitor) vs. normalized response (variable slope) model.

Stability in presence of albumin

Ctx-mal-PEG-CPT, Ctx-Pt-PEG-CPT, Tra-mal-PEG-CPT and Tra-Pt-PEG-CPT conjugates were buffer exchanged into Dulbecco's phosphate buffer saline (DPBS; HiMedia Laboratories) using Zeba Spin Desalting Columns (Thermo Fisher Scientific; 7 K MWCO). The ADCs were

incubated at 37 °C with 20 mg/ml human serum albumin (HSA; Sigma) in DPBS at a final antibody concentration of 0.8-0.9 mg/ml. Aliquots were withdrawn at 0 h and 72 h and immediately stored protected from light at -80 °C until analysis. HSA was separated from the antibody using MabSelect SuRe HiTrap column (GE Healthcare Life Sciences) with PBS, pH 6.5 as elution buffer. Collected samples were concentrated and buffer-exchanged into PBS, 20 % isopropanol, pH 6.5 using Amicon centrifugal filters (Millipore; 30 kDa). The samples were injected on Yarra SEC-3000 column (Phenomenex Inc.; 300×4.6 mm, 3 µm) on a Waters e2695 analytical system with a PDA and fluorescence detector ($\lambda_{\text{ex}}=369$ nm; $\lambda_{\text{em}}=430$ nm). PBS, 20 % isopropanol, pH 6.5 was used as mobile phase. Baseline subtraction of the 72 h samples was performed using the respective 0 h samples to account for background fluorescence of HSA. Fluorescence peak area corresponding to the HSA peak was normalized using the 280 nm absorbance of the peak and fold-difference between 72 h samples of the two ADCs was computed.

Stability in Plasma

Human plasma (Sigma), adjusted to pH 7.2 using sodium phosphate monobasic, and mouse plasma, were spiked with 0.1 mg/ml of Ctx-Pt-PEG-CPT or Tra-Pt-PEG-CPT and incubated at 37 °C. Samples were withdrawn at various time points for a course of 15 days and extracted with acetonitrile, 0.1 % trifluoroacetic acid (TFA) solution. The suspension was centrifuged to remove the precipitate. The supernatant was filtered using 0.2 µm filter and stored protected from light at -20 °C until analysis. Samples were injected on a Reprosil C18 (Dr. Maisch, Germany; 250×4.6 mm, 5 µm) column with a water/acetonitrile (0.1 % TFA) gradient. Waters e2695 HPLC with in-line PDA and fluorescence detector ($\lambda_{\text{ex}}=369$ nm; $\lambda_{\text{em}}=430$ nm) was used for analysis. The fluorescence peak area was quantified using a camptothecin standard curve generated using similar procedure with correction for camptothecin recovery from plasma. Normalized response was fitted to a first-order exponential decay equation and half-life was computed using GraphPad Prism software (GraphPad Software, Inc.). Supernatant was also submitted for estimation of platinum content by Pt-AAS.

Determination of antitumor activity of Ctx-Pt-PEG-CPT in A549 model and TUNEL assay

Tumors were propagated in SCID Beige mice, by subcutaneous injection of 5×10^6 A549 (Non-small cell lung cancer) cells with Matrigel (1:1) in the flank region. Mice were examined for the presence of tumor and then randomized into treatment groups based on tumor volume (GR-I: untreated control, GR-II: Ctx-Pt-PEG-CPT, GR-III: cetuximab). Treatment was initiated with 20

mg/kg of test article, when tumor volumes reached between 80-120 mm³, with the animals being examined daily for signs of treatment-related toxicity or mortality. Tumor volume and body weight were measured twice weekly. Animals were taken off the study and sacrificed when their body weight loss was greater than 20 % compared to day 0 or when tumor volume reached 2000 mm³ or became necrotic.

Paraffin-embedded tumor sections were dewaxed and rehydrated, followed by incubation with trypsin for 1 h at 37 °C. Apoptotic cell death in sections was evaluated by TUNEL assay using the *In Situ* Cell Death Detection Kit, TMR red® (Roche, Germany), according to the manufacturer's protocol. Following washing, the sections were counterstained with Hoechst and the label incorporated at the damaged sites of DNA was visualized by fluorescence microscopy.

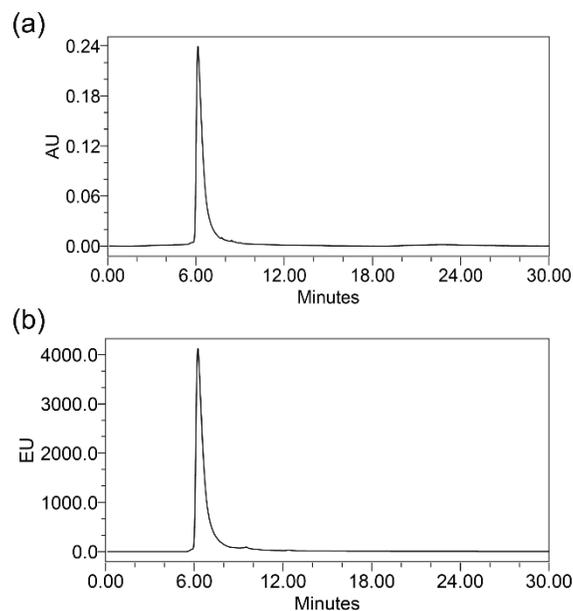


Figure S1. SE-HPLC chromatogram of Rtx-Pt-PEG-CPT (a) at 280 nm showing low presence of aggregates (< 1 %) and (b) fluorescence of CPT corresponding to the antibody peak with negligible presence of free camptothecin (< 1 %).

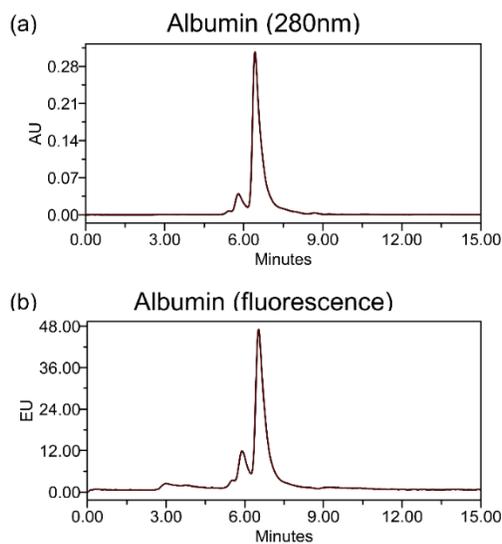


Figure S2. (a) UV chromatogram at 280 nm showing that albumin elutes at a retention time of 6.4 min on the SE-HPLC column. Presence of aggregates and lower molecular weight species can be visualized on the chromatogram (b) Fluorescence SE-HPLC chromatogram ($\lambda_{ex}=369$ nm; $\lambda_{em}=430$ nm) showing that albumin has an inherent fluorescence.

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