Electronic Supplementary Material (ESI) for ChemComm.

A new pH responsive crosslinker platform for antibody-drug conjugate (ADC) targeting delivery

Francesca Migliorini, Elena Cini, Elena Dreassi, Federica Finetti, Giovanni Ievoli, Giulia Macrì, Elena Petricci, Enrico Rango, Lorenza Trabalzini and Maurizio Taddei

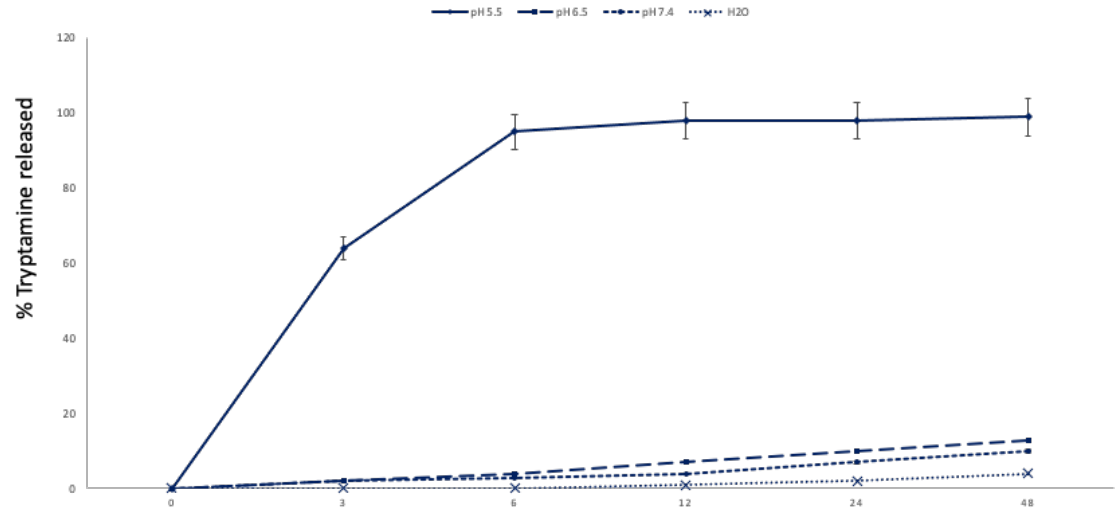
General experimental procedures, materiales and instruments

All reagents were used as purchased from commercial suppliers without further purification. The reactions were carried out in oven dried vessels. Solvents were dried and purified by conventional methods prior use or, if available, purchased in anhydrous form. Flash column chromatography was performed with Merck silica gel 60, 0.040-0.063 mm (230-400 mesh). MPLC Syncore® Büchi on highly resistant PP cartridges Normal Phase silica gel NP 40 – 63 μm particle size and 60 A pore size (Si60) withstand a maximum pressure of 10 bar (145 psi) column with petroleum ethet (eluent A) and Ethyl Acetate (Eluent B) as mobile phase. Merck aluminum backed plates pre-coated with silica gel 60 (UV254) were used for analytical thin layer chromatography and were visualized by staining with a $KMnO_4$ solution. NMR spectra were recorded at 25 °C or at 37 °C and 400 or 600 MHz for 1H and 101 or 151 MHz for 13C Brücker Advance NMR spectrometers. The solvent is specified for each spectrum. Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; bs, broad singlet. Chemical shifts (d) are given in ppm relative to the resonance of their respective residual solvent peaks. High and low resolution mass spectroscopy analyses were recorded by electrospray ionization with a mass spectrometer Q-exactive Plus. HPLC analysis were performed on a LC/MSD system InfinityLab LC/MSD iQ, Method: Column: InfinityLab PoroShell 120 EC-C18 2.1x50mmx2.7μm. Flow: 0.4 mL/min. Eluent A/B: H_2O/MeCN. Gradient: 5% B to 95% B in 10 minutes, 4 minutes at 95 % B and 3 minutes of re-equilibration. Detection: 254 nm and 210 nm.

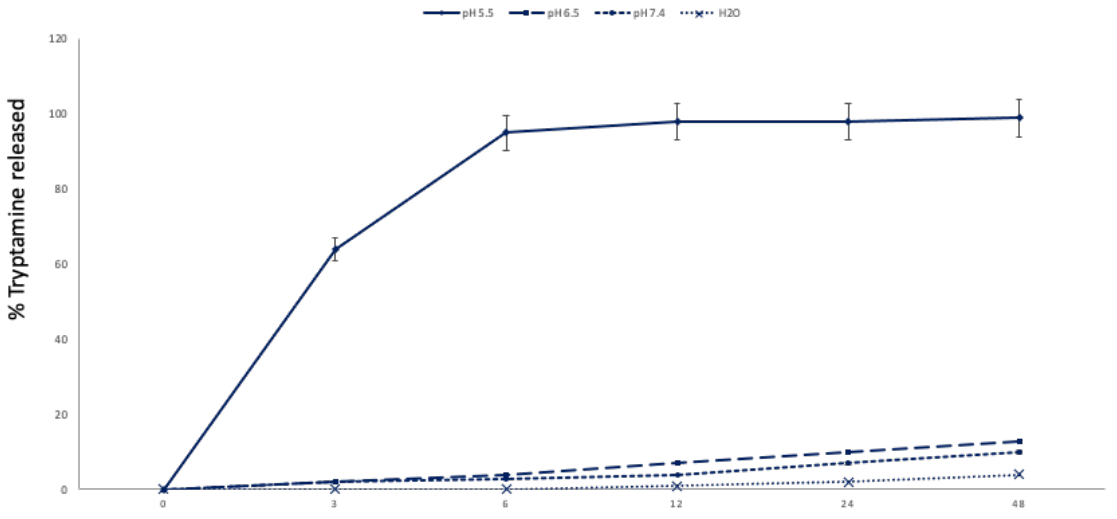
HPLC method for hydrolysis

Samples were prepared according to literature.[1]

A 10 mM solution of the desired compound (**8, 11, 15, 18**) was prepared in DMSO and diluted in H_2O or the corresponding buffer to obtain a 1 mM solution. For pH 7.4, 6.5, 5.5, phosphate buffers 0.1M were used (KH_2PO_4/K_2HPO_4). Each solution was analyzed by analytical HPLC (conditions mention above). Compounds **8, 11, 15, 18** were mixed with the buffers at 25 °C and further incubated at 37 °C. Injections were carried out every 3 h up to 48 h. All data were recorded in triplicate.



a



b

Figure S1: a HPLC hydrolysis kinetics of model system **8** in H_2O and at pH 5.5, 6.5, 7.4. **b** HPLC hydrolysis kinetics of model system **11** in H_2O and at pH 5.5, 6.5, 7.4

Disassembling mechanism for HMPO conjugates at pH 5.5 through ^{1}HNMR analysis.

The products ($4.8 \cdot 10^{-6}$ mol) were solubilized in acetonitrile-d_3 (160 µL) and added to a previously prepared deuterated buffer at pH 5.5 (50 mM, 640 µL). The NMR tubes were incubated at 37 °C, and the FID was recorded every 30 minutes.

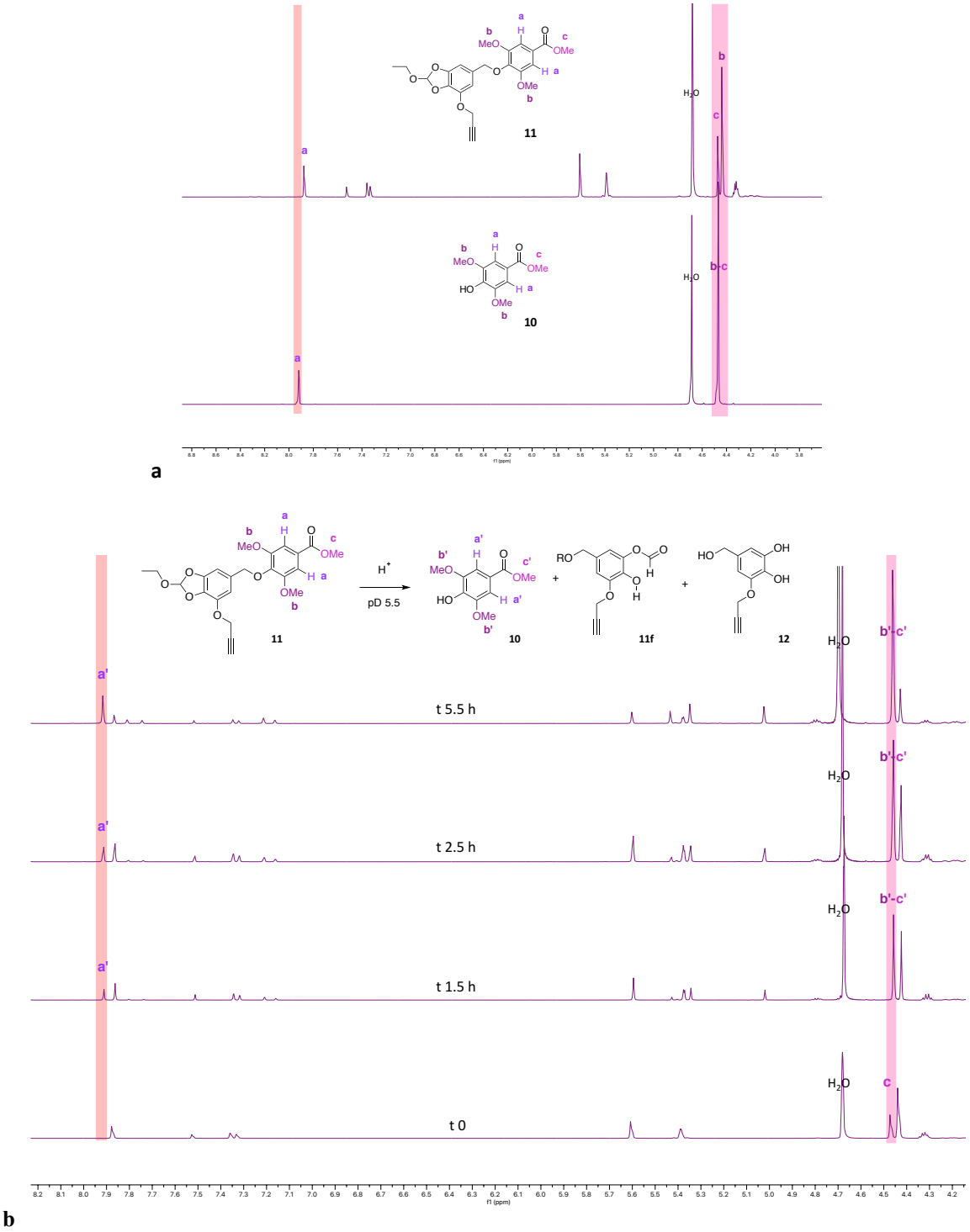


Figure S2. a) ^{1}HNMR spectra in D_2O and Acetonitrile-d_3 37 °C of the model system **11** and the phenol **10** that could be released at pH 5.5 at 37 °C. **b)** ^{1}H NMR spectra of **11** monitored during the hydrolysis process in acetic buffer D_2O (pH 5.5) at 37 °C. Underline in orange the aromatic –2H (a') and in pink the methoxy – 9H (b'-c') of the released phenol **10**.

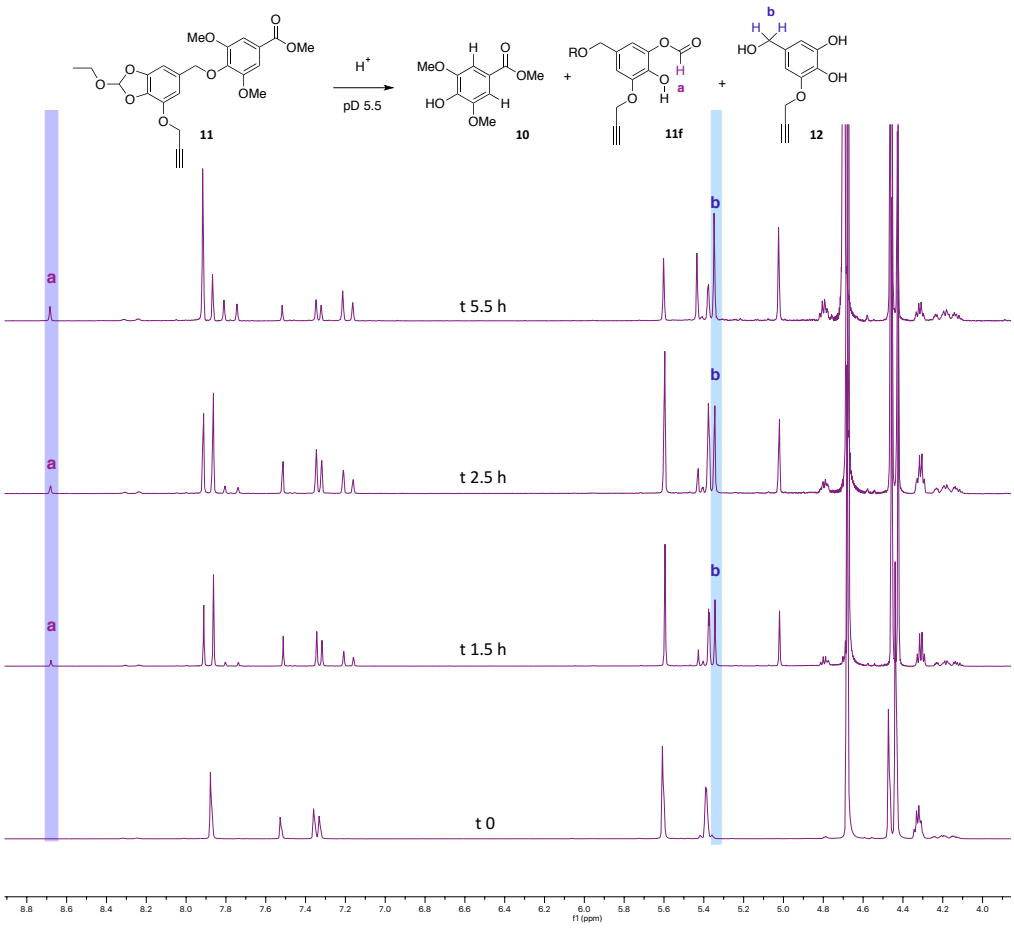


Figure S3. [1]HNMR spectra of **11** monitored during the hydrolysis process in acetic buffer D_2O (pH 5.5) at 37 °C. Underline in blue the transient formate **11f** –1H (a) and in light blue the benzylic – 2H (b) of the released 5-(hydroxymethyl)-3-(2-propynyloxy)pyrogallol **12**.

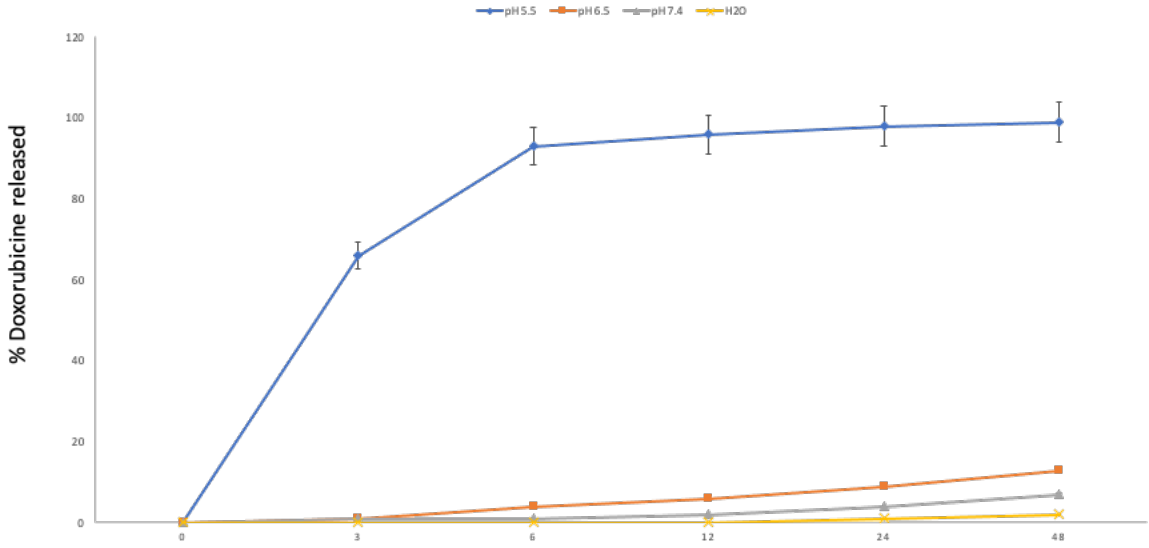


Figure S4: HPLC hydrolysis kinetics of compound **15** at pH 5.5, 6.5, 7.4 and in H_2O.

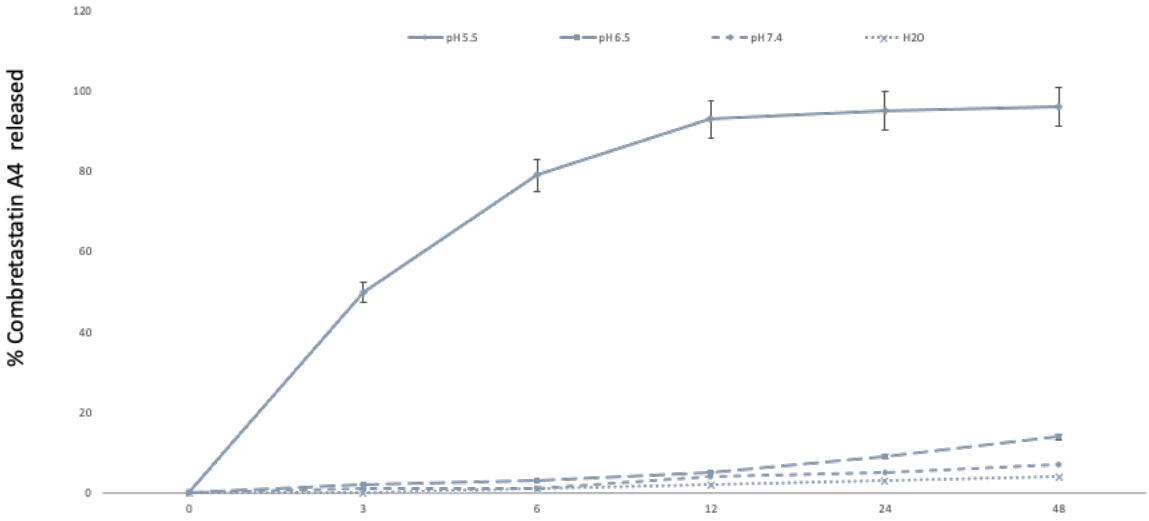


Figure S5: HPLC hydrolysis kinetics of model system **18** at pH 5.5, 6.5, 7.4 and in H_2O.

MALDI analysis of bioconjugates

The matrix solutions were prepared at two different concentrations, and both were used in parallel with the same sample: 20.0 mg or 25 mg of Super DHB were dissolved in a solution of 150 µl of MeCN, 350 µl of H_2O and 0.05 µl of TFA.

Usually, the matrix solutions were prepared the day before the deposition and conserved and room temperature. The stainless-steel target was placed in a termoblock setting to 39 °C and then 1.35 µl of the sample was deposited using a micropipette. After the evaporation of the solvent and when the sample was dried, 1.65 µl of matrix solution was deposited. The formation of the crystal, at this plate temperature, requires about 3-5 minutes. Once it was completely dried, it was possible remove the target from the termoblock.

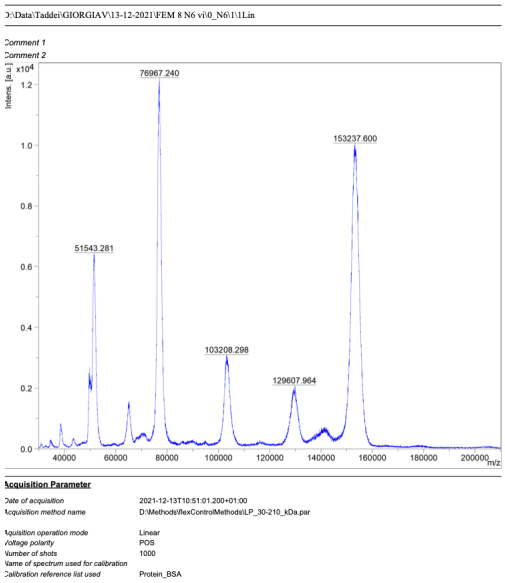
To acquire the sample spectra, the target plate was inserted into the MALDI-TOF instrument and appropriate instrumental parameters were chosen. In this case, the instrument was used in linear mode (not in reflector mode) and was set at 83% of laser intensity. The m/z range was from 30 kDa to 200 kDa.

For each sample spot, 10 shots were acquired, to improve the spectra quality and mass accuracy.

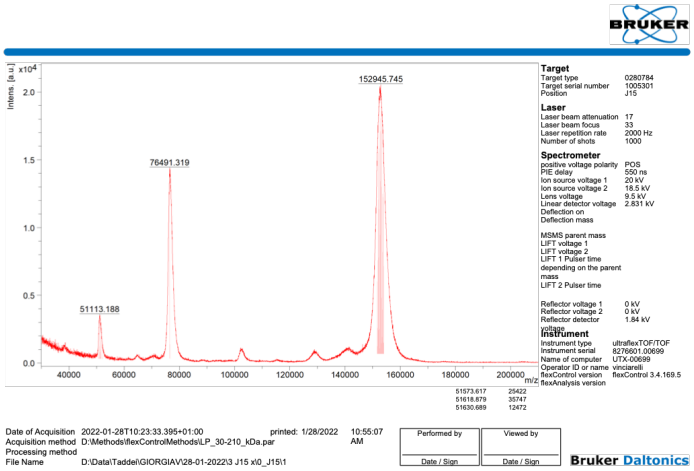
DAR was calculated dividing the difference between the ADC and the free antibody with the payload mass. Calculation was done on the mono-charged and double-charged peaks and the DAR expressed as medium value.

Table S1 Drug antibody ratio

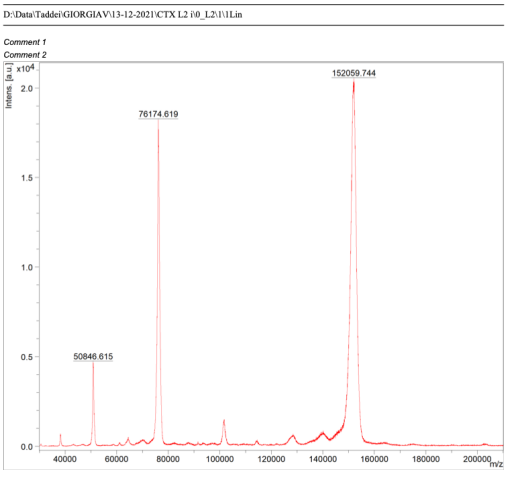
Antibody	Payload MW	MALDI monocharged peak M^+	MALDI bicharged peak M^{2+}	DAR (M^+)	DAR (M^{2+})	DAR
Ctx	-	152059	76174	-	-	-
ADC-21	974	153237	76969	1.3	1.6	1.5
Ctx	-	152059	76174	-	-	-
ADC-22	704	159945	76491	1.3	1. 2	1.25



a



b

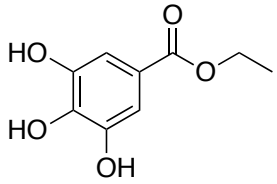


c

Figure S6. MALDI spectra of a) ADC **21**; b) ADC **22**; c) Cetuximab

Synthetic procedures

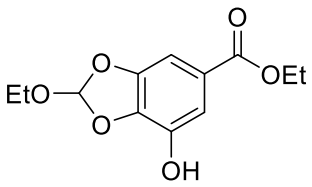
Ethyl 3,4,5-trihydroxybenzoate (2)



The product was prepared according to literature.[2]

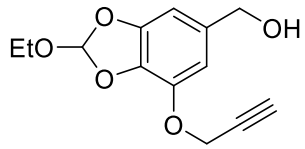
Gallic acid **1** (1 g, 5.88 mmol) was suspended in EtOH (40 mL) in a 250 mL round-bottomed flask. A catalytic amount of H_2SO_4 conc. was added at room temperature and was heated under reflux with continuous stirring for 16 h. The reaction mixture was cooled to rt and the solvent was evaporated under reduced pressure. The residue was dissolved in EtOAc (50 mL) and washed with a saturate solution of $NaHCO_3$ (x2, 100 mL) and NaCl (30 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum to get the esterified compound **2** (1.10 g, 5.59 mmol, 95% yield). mp. 153-155 °C (lit. mp 148–149)[2]. Spectral data coherent with literature.

Ethyl 2-ethoxy-7-hydroxybenzo[d][1,3]dioxole-5-carboxylate (3)[3]



Under N_2 atmosphere, Amberlyst® 15 (62 mg) was suspended in toluene dry (80 mL). After 30 minutes, ethyl gallate **2** (1 g, 5.046 mmol) and triethyl orthoformate (2.5 mL, 15.098 mmol) were added at rt and then the mixture was heated to reflux for 16 h. The reaction mixture was cooled to room temperature, filtered on Celite pad and toluene was evaporated under reduced pressure. The compound was purified by means of chromatography on silica gel with MPLC Syncore® Büchi eluting 0-30 % gradient of EtOAc in petroleum ether, as a white solid (1.06 g, 4.19 mmol, 83% of yield). **^{1}H NMR** (600 MHz, $CDCl_3$, δ ppm, *J* Hz): δ 7.43 (s, 1H), 7.15 (s, 1H), 6.92 (s, 1H), 6.46 (bs, 1H), 4.33 (q, J = 7.1 Hz, 2H), 3.72 (q, J = 7.3Hz, 2H), 1.35 (t, J = 7.1 Hz, 3H), 1.23 (t, J = 7.2 Hz, 3H).**^{13}C NMR** (151 MHz, $CDCl_3$, δ ppm): δ 166.84, 147.19, 139.08, 137.23, 124.21, 119.98, 114.11, 102.48, 61.47, 59.65, 14.74, 14.19. **ESI**: *m/z* 255 $[M+H]^+$; 277 $[M+Na]^+$.

(2-Ethoxy-7-(prop-2-yn-1-yloxy)benzo[d][1,3]dioxol-5-yl)methanol (5) HMPO



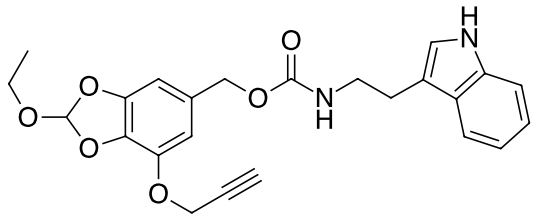
Under N_2, compound **3** (1.34 g, 5.27 mmol), K_2CO_3 (2.188 g, 15.83 mmol) and KI (875 mg, 5.27 mmol) in dry acetone (80 mL) were mixed together for 20 minutes. Then propargyl bromide **4** (1.41 mL, 15.83 mmol) was added and the reaction was carried out to reflux for 16 h. Acetone was evaporated and the crude was solubilized in EtOAc (50 mL), washed with H_2O (2 x 25 mL) and brine (25 mL) and the organic phases dried over anhydrous Na_2SO_4, filtered, and evaporated under reduce pressure. The alkylated product was purified by means of chromatography on silica gel with MPLC Syncore® Büchi eluting 0-20 % gradient of EtOAc in petroleum ether. (1.35 g, 4.64 mmol, 88% yield). **^{1}H NMR** (600 MHz, $CDCl_3$, δ ppm, *J* Hz): δ 7.46 (s, 1H), 7.29 (s, 1H), 6.96 (s, 1H), 4.84 (s, 2H), 4.34 (q, *J* = 7.1 Hz, 2H), 3.75 (q, *J* = 7.1 Hz, 2H), 2.54 (s, 1H), 1.37 (t, *J* = 7.1 Hz, 3H), 1.27 (t, *J* = 7.1 Hz, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$, δ ppm): δ 165.72, 147.33, 140.23, 138.48, 124.69, 120.12, 112.32, 104.19, 77.87, 76.30, 61.02, 59.59, 57.37, 14.77, 14.32. **ESI**: *m/z* 315 $[M+Na]^+$. Under N_2, the alkylated compound (1.07 g, 3.85 mmol) was solubilized in THF dry (60 mL) and mixture was cooled to 0 °C. Then a solution of $LiAlH_4$ 1M in THF (11.55 mL) was added slowly and mixture were carried out at room temperature for 1 h. Then HCl 0.5 N was added until pH 7-8 and the mixture was extracted with EtOAc (50 mL). The organic phase was separated and washed with H_2O (25 mL) and brine (25 mL) and dried over anhydrous Na_2SO_4, filtered, and evaporated under reduce pressure. Compound **5** was as a white solid in a very good yield (915 mg, 3.66 mmol, 95% yield). The analytical sample was crystallized from heptane. **^{1}H NMR** (600 MHz, $CDCl_3$, δ ppm, *J* Hz): δ 6.89 (s, 1H), 6.67 (s, 1H), 6.63 (s, 1H), 4.81 (s, 2H), 4.58 (s, 2H), 3.75 (q, *J* = 7.1 Hz, 2H), 2.52 (s, 1H), 1.26 (t, *J* = 7.1 Hz, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$, δ ppm): δ 147.68, 140.58, 135.42, 133.89, 119.50, 108.82, 101.75, 78.39, 75.94, 65.23, 59.37, 57.39, 14.82. **ESI**: *m/z* 273 $[M+Na]^+$. Elemental Analysis calcd. for $C_{12}H_{14}O_6$: C, 56.69; H, 5.55; found C, 56.82; H, 5.65.

General procedure for the synthesis of carbamates.

The activated linker (0.4 mmol) was solubilized in THF dry (5 mL) at r.t under N_2. At 0 ° C *p*-nitrophenyl chloroformate (89 mg, 0.44 mmol) and DMAP (98 mg, 0.8 mmol) were added, and the reaction was carried out for 30 minutes at 0 ° C. The formation of the activated compound was checked by TLC and the solution was dropped into a solution of the desired amine (0.6 mmol) and

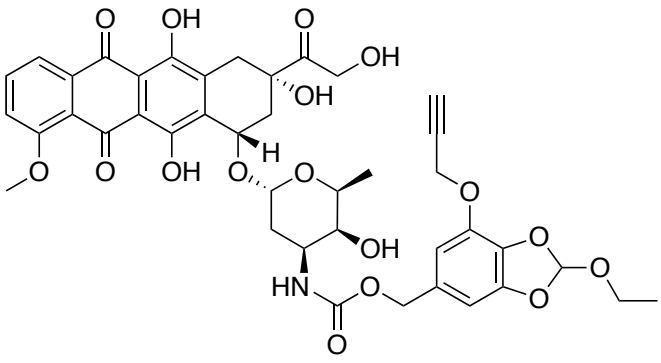
DIPEA (209 µL, 1.2 mmol) at 0 °C and the reaction was stirred at rt for 0.15 h- 16 h. The solvent was evaporated under reduced pressure. The residue was dissolved in EtOAc (10 mL) and washed with H_2O (5 mL) and brine (5 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated under vacuum.

(2-Ethoxy-7-(prop-2-yn-1-yloxy)benzo[d][1,3]dioxol-5-yl)methyl(2-(1H-indol-2-yl)ethyl)carbamate (8)



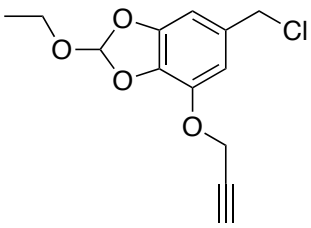
The compound **8** was purified by flash chromatography on silica gel with MPLC Syncore® Büchi eluting 0-40 % gradient of EtOAc in petroleum ether (139 mg, 0.32 mmol, 81% yield). **^{1}H NMR** (600 MHz, $CDCl_3$, δ ppm, *J* Hz): δ 8.16 (bs, 1H), 8.13 (d, J = 8.4 Hz, 1H), 7.60 (d, J = 7.9 Hz, 1H), 7.37 (d, J = 8.1 Hz, 1H), 7.21 (t, J = 7.7 Hz, 1H), 7.12 (t, J = 7.5 Hz, 1H), 6.98 (s, 1H), 6.89 (s, 1H), 6.65 (s, 1H), 6.62 (s, 1H), 5.00 (s, 2H), 4.88 (bs, 1H), 4.80 (s, 2H), 3.76 (q, J = 7.2 Hz, 2H), 3.54 (t, J = 6.6 Hz, 2H), 2.99 (t, J = 6.8 Hz, 2H), 2.51 (s, 1H), 1.27 (t, J = 7.0 Hz, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$, δ ppm): δ 162.65, 156.45, 147.60, 140.51, 136.44, 134.28, 130.92, 126.19, 122.23, 119.51, 118.71, 115.67, 112.66, 111.30, 110.26, 102.92, 76.08, 66.52, 59.58, 57.47, 41.29, 29.73, 25.65, 14.83. **ESI**: *m/z* 459 $[M+Na]^+$. HRMS (EI) calcd. For $C_{24}H_{24}N_2O_6Na$ $[M+Na]^+$ 459.1532, found 459.1529.

(2-Ethoxy-7-(prop-2-yn-1-yloxy)benzo[d][1,3]dioxol-5-yl)methyl ((2S,3S,4S,6R)-3-hydroxy-2-methyl-6-(((1S,3S)-3,5,12-trihydroxy-3-(2-hydroxyacetyl)-10-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydrotetracen-1-yl)oxy)tetrahydro-2H-pyran-4-yl)carbamate (15)



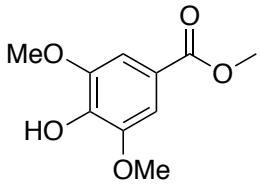
Compound **15** was purified with flash chromatography on silica gel eluting 0-10 % gradient of MeOH in CH_2Cl_2 (237 mg, 0.29 mmol, 73% yield). **^{1}H NMR** (600 MHz, $CDCl_3$, δ ppm, *J* Hz): δ 13.98 (s, 1H), 13.24 (s, 1H), 8.04 (d, J = 7.7 Hz, 1H), 7.79 (t, J = 8.0 Hz, 1H), 7.39 (d, J = 8.4 Hz, 1H), 6.87 (s, 1H), 6.62 (s, 1H), 6.58 (s, 1H), 5.51 (d, J = 4.2 Hz, 1H), 5.30 (s, 1H), 5.13 (dd, J = 5.7 Hz, 1H), 4.93 (s, 2H), 4.77 (s, 2H), 4.55 (s, 1H), 4.08 (s, 3H), 3.87 (bs, 1H), 3.77 – 3.60 (m, 3H), 3.15 (dd, J = 15.6, 8.4 Hz, 2H), 2.51 (s, 1H), 2.26 (dd, J = 6.8, 4.5 Hz, 2H), 1.98 – 1.74 (m, 4H), 1.62 (s, 2H), 1.35 – 1.11 (m, 6H). **^{13}C NMR** (151 MHz, $CDCl_3$, δ ppm): δ 213.86, 187.13, 186.73, 161.09, 156.19, 155.67, 155.42, 147.59, 140.44, 135.80, 135.53, 134.35, 133.60, 133.53, 130.60, 120.90, 119.88, 119.56, 118.47, 111.64, 111.46, 110.29, 102.95, 100.70, 76.65, 76.03, 69.70, 69.59, 67.27, 66.67, 65.56, 59.34, 57.41, 56.69, 47.03, 35.66, 34.04, 30.20, 29.71, 16.84, 14.78. **ESI**: *m/z* 842 $[M+Na]^+$. HRMS (EI) calcd. For $C_{41}H_{41}NO_{17}Na$ $[M+Na]^+$ 842.2273, found 842.2278.

6-(Chloromethyl)-2-ethoxy-4-(prop-2-yn-1-yloxy)benzo[d][1,3]dioxole (9)



Compound **5** (100 mg, 0.4 mmol) was solubilized in CH_2Cl_2 dry (6 mL) at 0 °C and $SOCl_2$ and Et_3N, both freshly distilled, were added carefully. The reaction was carried out at 0 ° C for 30 minutes. The mixture was concentered under N_2 and quickly filtrated on a pad of silica gel using PE:EtOAc 1:1. Filtrate was concentered under reduced pressure and was immediately used for the next step.

Methyl 4-hydroxy-3,5-dimethoxybenzoate (10)

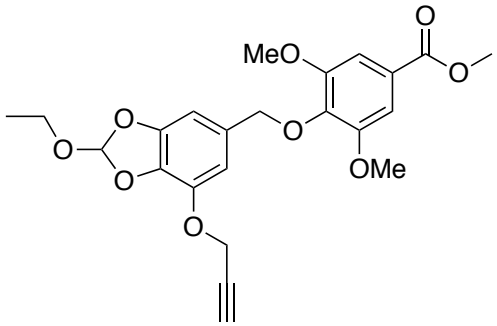


The product was prepared according to literature.[4]

Syringic acid (3 g, 15.14 mmol) was dissolved in MeOH (30 mL), and a catalytic amount of H_2SO_4 conc. was added. The reaction mixture was stirred at reflux for 4 h. The resulting mixture was concentrated under reduced pressure and the residue dissolved in EtOAc (50 mL). The solution was

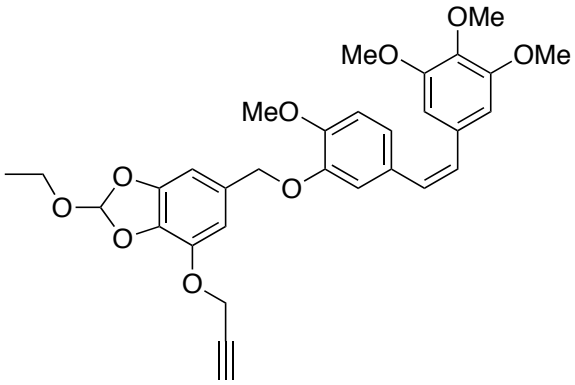
transferred into a separating funnel, washed with $NaHCO_3$ $_{SS}$ (100 mL) and NaCl $_{SS}$ (30 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated to give the methyl ester **10** in good yield (3.03 g, 14.38 mmol, 95% yield). **^{1}H NMR** (600 MHz, $CDCl_3$, δ ppm, *J* Hz): δ 7.30 (s, 2H), 3.91 (s, 6H), 3.88 (s, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$, δ ppm): δ 166.87, 146.67, 139.27, 121.05, 106.67, 56.40, 52.07. **ESI**: *m/z* 211 $[M-H]^+$.

Methyl 4-((2-ethoxy-7-(prop-2-yn-1-yloxy)benzo[d][1,3]dioxol-5-yl)methoxy)-3,5-dimethoxybenzoate (11)



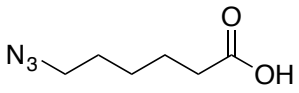
Compound **10** (100 mg, 0.47 mmol) was solubilized in dry acetone (10 mL) and K_2CO_3 an. (195 mg, 1.41 mmol) was added to the mixture at rt under Ar. After 10 minutes a solution of benzylchloride **9** (377 mg, 1.41 mmol) in dry acetone (10 mL) was added under Ar and the reaction was stirred for 30 min rt. The mixture was filtered and the filtrated concentrated under reduced pressure. EtOAc (20 mL) was added, washed with H_2O (10 mL) and brine (5 mL). The organic phases were dried over anhydrous Na_2SO_4, filtered and evaporated under reduce pressure. The product was purified by flash chromatography on silica gel eluting 0-40% gradient of EtOAc in petroleum ether (168 mg, 0.38 mmol, 81% yield). **^{1}H NMR** (600 MHz, $CDCl_3$, δ ppm, *J* Hz): δ 7.29 (s, 2H), 6.90 (s, 1H), 6.79 (s, 1H), 6.78 (s, 1H), 4.99 (s, 2H), 4.80 (s, 2H), 3.91 (s, 3H), 3.90 (s, 6H), 3.73 (q, J = 7.1 Hz, 2H), 2.51 (s, 1H), 1.25 (t, J = 7.0 Hz, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$, δ ppm): δ 166.74, 153.21, 147.41, 140.78, 140.25, 134.32, 131.79, 129.39, 125.38, 110.46, 110.13, 106.84, 105.44, 103.27, 75.84, 74.84, 59.13, 57.38, 56.23, 52.24, 29.71, 14.83. **ESI**: *m/z* 445 $[M+H]^+$; 467 $[M+Na]^+$. HRMS (EI) calcd. for $C_{23}H_{25}O_9$ $[M+H]^+$ 445.1499, found 445.1501.

(Z)-2-Ethoxy-6-((2-methoxy-5-(3,4,5-trimethoxystyryl)phenoxy)methyl)-4-(prop-2-yn-1-yloxy)benzo[d][1,3]dioxole (18)



Combretastatin A4 (100 mg, 0.32 mmol) was solubilized in DMF dry (3 mL) and was added into a DMF dry (2 mL) solution of previously washed NaH 60% (25 mg, 0.64 mmol) under Ar at 0 ° C. The mixture was stirred for 10 minutes and the activated linker **9** (257 mg, 0.96 mmol) solubilized in 3 mL of dry DMF was dropped slowly at 0 ° C. The reaction was carried out at rt for 16 h and at the end H_2O (5 mL) was added quenching NaH. The crude was extracted with EtOAc (10 mL), the organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated under reduce pressure. The product **18** was purified by means of chromatography on silica gel with MPLC Syncore® Büchi eluting 0-30 % gradient of EtOAc in petroleum ether (170 mg, 1.31 mmol, 98% yield). **^{1}H NMR** (600 MHz, $CDCl_3$, δ ppm, *J* Hz): δ 6.91 – 6.85 (m, 3H), 6.78 (d, J = 7.8 Hz, 1H), 6.64 (s, 1H), 6.61 (s, 1H), 6.51 (s, 2H), 6.49 – 6.39 (m, 2H), 4.81 (s, 2H), 4.79 (s, 2H), 3.86 (s, 3H), 3.83 (s, 3H), 3.77 – 3.68 (m, 8H), 2.49 (s, 1H), 1.26 (t, J = 6.7 Hz, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$, δ ppm): δ 153.01, 148.94, 147.68, 147.53, 140.51, 137.15, 134.13, 132.96, 131.29, 129.81, 129.62, 128.88, 122.60, 119.53, 114.68, 111.41, 109.39, 105.99, 102.16, 75.93, 70.81, 60.92, 59.29, 57.41, 55.97, 29.71, 14.82. **ESI**: *m/z* 571 $[M+Na]^+$. HRMS (EI) calcd. for $C_{31}H_{32}O_9Na$ $[M+Na]^+$ 571.1944, found 571.1946.

6-Azidohexanoic acid (16)



The product was prepared according to literature.[5]

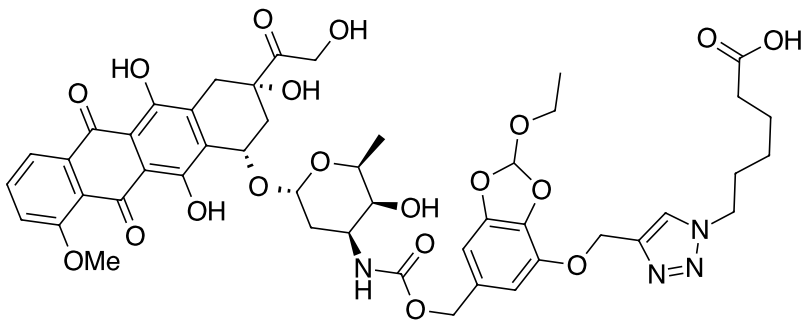
6-bromohexanoic acid (700 mg, 3.6 mmol) was solubilized in dry DMF (5 mL) and NaN_3 (1.17 g, 18 mmol) was added at the mixture at rt. The reaction was stirred for 16 h at 100 ° C. EtOAc (100 mL) was added at the crude faltered on Büchner and washed with $KHSO_4$ 1 M (2 x 50 mL), H_2O (2 x 30 mL) and $NaCl_{SS}$ (30 mL). The organic phase was dried over anhydrous Na_2SO_4, filtered, and evaporated under reduce pressure. The product **16** was obtained as yellow oil (289 mg, 1.84 mmol,

51% yield). **^{1}H NMR** (400 MHz, $CDCl_3$, δ ppm, *J* Hz): δ 10.93 (bs, 1H), 3.19 (t, J = 6.8 Hz, 2H), 2.28 (t, J = 7.4 Hz, 2H), 1.60-1.56 (m, 4H), 1.44 – 1.26 (m, 2H). NMR data are coherent with literature.[6]

General procedure for CuCAAC reaction.

The desired alkyne (0.07 mmol) and the compound 11 (9 mg, 0.056 mmol) were dissolved in DMF dry (2 mL) under Ar. The solution was degassed with three cycles of argon/vacuum. To this solution, a freshly prepared aqueous mixture (2 mL) of $Cu(OAc)_2$ (4 mg, 0.021 mmol) and Na ascorbate (8 mg, 0.042 mmol), previously degassed by argon/vacuum cycles, was added dropwise. The reaction mixture was degassed and left to stir under Ar. at rt for 16 h. The solvent was evaporated and the crude was purified by silica gel flash chromatography eluting 0-10 % gradient of MeOH in CH_2Cl_2 provide the desired compound.

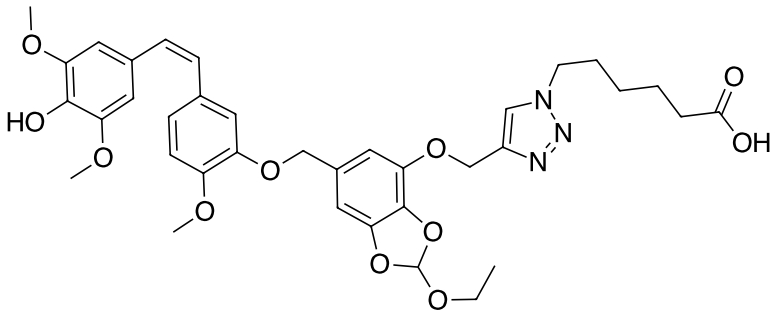
6-(5-(((2-Ethoxy-6-(((((2S,3S,4S,6R)-3-hydroxy-2-methyl-6-(((1S,3S)-3,5,12-trihydroxy-3-(2-hydroxyacetyl)-10-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydrotetracen-1-yl)oxy)tetrahydro-2H-pyran-4-yl)carbamoyl)oxy)methyl)benzo[d][1,3]dioxol-4-yl)oxy)methyl)-1H-1,2,3-triazol-1-yl)hexanoic acid (17)



Obtained 39 mg, 0.04 mmol, 64% yield. **^{1}H NMR** (600 MHz, dmso-d_6, δ ppm, *J* Hz): δ 14.02 (bs, 1H), 13.26 (bs, 1H), 11.83 (bs, 1H), 8.16 (s, 1H), 7.91 (t, J = 8.0 Hz, 1H), 7.64 (d, J = 7.5 Hz, 1H), 7.04 (s, 1H), 6.82 – 6.71 (m, 2H), 6.62 (s, 1H), 5.40 (s, 1H), 5.23 (s, 1H), 5.17 (s, 2H), 4.97 (s, 1H), 4.85 (s, 2H), 4.75 (bs, 1H), 4.56 (s, 2H), 4.32 (t, J = 7.1 Hz, 2H), 4.14 (d, J = 6.6 Hz, 1H), 3.98 (s, 3H), 3.75-3.70 (m, 1H), 3.64 (q, J = 6.9 Hz, 2H), 3.46 (s, 1H), 2.98 (s, 2H), 2.25 – 2.10 (m, 4H), 1.87-1.77 (m, 3H), 1.50 (p, J = 7.4 Hz, 3H), 1.23 (q, J = 7.8 Hz, 3H), 1.15-1.10 (m, 6H). **^{13}C NMR** (151 MHz, dmso-d_6, δ ppm): δ 187.13, 187.01, 174.74, 161.37, 156.60, 155.72, 155.02, 147.17, 142.68, 141.55, 136.69, 136.10, 135.28, 134.65, 133.59, 131.94, 124.98, 120.69, 120.33, 119.54, 111.38, 111.24, 109.81, 102.32, 100.72, 90.71, 80.65, 75.54, 70.37, 68.54, 67.22, 65.60, 64.12, 62.96, 60.07,

57.12, 49.73, 47.64, 37.26, 33.92, 32.68, 30.36, 29.86, 25.88, 24.32, 17.47, 15.22. **ESI**: *m/z* 999 $[M+Na]^+$. HRMS (EI) calcd. for $C_{47}H_{52}N_4O_{19}Na$ $[M+Na]^+$ 999.3124 (100%), 1000.3158 (51%) found 999.3127 (100%), 1000.3161 (51%).

(Z)-6-(5-(((2-Ethoxy-6-((2-methoxy-5-(3,4,5-trimethoxystyryl)phenoxy)methyl)benzo[d][1,3]-dioxol-4-yl)oxy)methyl)-1H-1,2,3-triazol-1-yl)hexanoic acid (19)



Obtained 21 mg, 0.03 mmol, 75% yield. **^{1}H NMR** (600 MHz, $CDCl_3$, δ ppm, *J* Hz): δ 7.65 (s, 1H), 6.90 – 6.86 (m, 3H), 6.79 (d, J = 8.1 Hz, 1H), 6.66 (s, 1H), 6.59 (s, 1H), 6.51 (s, 2H), 6.49 – 6.40 (m, 2H), 5.32 (s, 2H), 4.80 (s, 2H), 4.36 (t, J = 7.0 Hz, 2H), 3.86 (s, 3H), 3.83 (s, 3H), 3.76 – 3.70 (m, 8H), 2.35 (t, J = 7.2 Hz, 3H), 1.96-1.92 (m, J = 7.7 Hz, 2H), 1.69-1.65 (m, 2H), 1.40-1.35 (m, 2H), 1.26 (t, J = 6.7 Hz, 3H). **^{13}C NMR** (151 MHz, $CDCl_3$, δ ppm): δ 177.29, 152.99, 148.90, 147.56, 147.51, 141.34, 137.11, 133.87, 132.99, 131.46, 129.82, 129.62, 128.91, 122.59, 119.48, 114.65, 111.41, 109.08, 106.00, 101.88, 70.81, 63.52, 60.93, 59.55, 55.98, 50.08, 33.33, 29.86, 29.72, 25.80, 23.91, 14.84. **ESI**: *m/z* 707 $[M+H]^+$; 729 $[M+Na]^+$. HRMS (EI) calcd. for $C_{37}H_{44}N_3O_{11}$ $[M+H]^+$ 706.2976, found 706.2979.

General procedure for preparation of ADCs. 21 (Ctx-NH-17), 22 (Ctx-NH-19)

The proper carboxylic acid (28 μL, 10 mM in DMSO) was activated by adding S-NHS (5 μL, 100 mM in mQ water) and EDC (5 μL, 100 mM in mQ water). The reaction was left for 16 at rt PBS pH 7.4 (35 μL) and Cetuximab freshly dialyzed using a 10 kDa cutoff dialysis membrane (100 μL, 5 mg/mL in PBS pH 7.4) were added to the activated carboxylic acid solution. The reaction was incubated at rt and after 1 h quenched with a 20 mM glycine acqueous solution (27 μL). The final product (**20**, **21**) was purified using PD spintrap^{TM} G-25 column removing the unreacted excess of small molecules.

Stability in Human Plasma

Pooled human plasma (0.9 mL, 55.7 µg protein/mL),[7] hepes buffer (1.0 mL, 25 mM, NaCl 140 mM, pH 7.4) and tested compound dissolved in DMSO (100 µL, 2.0 mM) were mixed in a test tube that was incubated at 37 °C under continuous mechanical agitation. At set time points (0.0, 0.25, 0.50, 1.0, 3.0, 5.0, 8.0, and 24.0 h), samples of 100 µL were taken, mixed with 400 µL of cold acetonitrile and centrifuged at 5000 rpm for 15 min.[8] The supernatant was collected and analyzed by UV/LC-MS to monitor the amount of unmodified compound. For each compound, the determination was performed in three independent experiments.

UV/LC-MS methods

LC analyses of plasma stability tests were performed by using Agilent 1100 LC/MSD VL system (G1946C) (Agilent Technologies, Palo Alto, CA) constituted by a vacuum solvent degassing unit, a binary high-pressure gradient pump, an 1100 series UV detector, and an 1100 MSD model VL benchtop mass spectrometer. MSD single-quadrupole instrument was equipped with the orthogonal spray API-ES (Agilent Technologies, Palo Alto, CA). The pressure of the nebulizing gas and the flow of the drying gas (nitrogen used for both) were set at 40 psi, 9 L/min, respectively. The capillary voltage, the fragmentor voltage, and the vaporization temperature were 3000 V, 10 V, and 350 °C, respectively. MSD was used in the positive and negative ion mode. Spectra were acquired over the scan range m/z 100-2000 using a step size of 0.1. Chromatographic analyses were performed using a Phenomenex Kinetex EVO C18-100Å (150 x 4.6 mm, 5 µm particle size) at room temperature, at flow rate of 0.6 mL/min, and injection volume of 10 µL, operating with a gradient elution of A: water (H2O) and B: acetonitrile (ACN). Both solvents were acidified with 0.1% v/v of formic acid. UV detection was monitored at 254 nm. The analysis started with 0% of B, then B was increased to 80% (from t = 0 to t = 20 min), then kept at 80% (from t = 20 to t = 25 min) and finally return to 0% of eluent B in 5.0 min.

Cell culture

A431 epidermoid carcinoma cells and A549 lung carcinoma cells (ATCC, Rockville, MD, USA) were cultured in DMEM (Euroclone) supplemented with 10% fetal bovine serum (FBS, Euroclone), 100 U/ml penicillin/streptomycin (Euroclone), and 4 mM L-glutamine (Euroclone). All cell lines were grown at 37°C and 5% CO_2.

MTT assay

2.5 x 10^3 cells/well were seeded in 96-multiwell plates in medium 10% FBS. After adherence, cells were incubated in fresh medium with increasing concentrations of doxorubicin (0.05-50 μM), Combretastatin A4 (0.1-100 μM), Cetuximab (0.1-10 μg/ml), 21 (0.1-10 μg/ml) and 22 (0.1-10 μg/ml). At the indicated time (24, 48, 72 and 96 h) the medium was removed, and cells were incubated for 4h with fresh medium in the presence of 1.2 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich). The MTT solution was then removed and 50 μL of DMSO were added to each well to dissolve the blue formazan crystals. The absorbance of the formazan dye was measured at 570 nm with a microplate reader (EnVision, PerkinElmer, Waltham, MA, USA). Data were expressed as a percentage of the basal control.

Apoptosis assay with DAPI stain

A431 and A549 cell (3 x 10^3 cells/well) were seeded in 96-well plates. After 24 h, cells were treated with doxorubicin (10 μM), combretastatin A4 (10 μM), **21** (10 μg/mL), **22** (10 μg/mL), and cetuximab (10 μg/mL) for 48 h. Then cells were fixed with paraformaldehyde for 20 min and stained with DAPI (100 ng/mL) (Cell Signaling) for 5 min. Cell nuclei were photographed by a fluorescence microscope (Nikon, Eclipse Ts2).

Annnexin V-FITC/PI assay

A431 and A549 cell (8 x 10^4 cells/well) were seeded in 24-well plates with glass cover-slips for 24 h. Then cells were treated for 18 h with doxorubicin (10 μM), combretastatin A4 (10 μM), **21** (10 μg/mL), **22** (10 μg/mL) and cetuximab (10 μg/mL). Subsequently, apoptosis was monitored by a fluorescence microscope (Nikon, Eclipse Ts2), using an Annexin V-FITC Early Apoptosis Detection Kit (Cell Signaling).

Statistical analysis

Data were generated from three independent experiments and expressed as means ± standard deviation (SD). Statistical analysis was performed using Student's t test for unpaired data; $p<0.05$ was considered statistically significant.

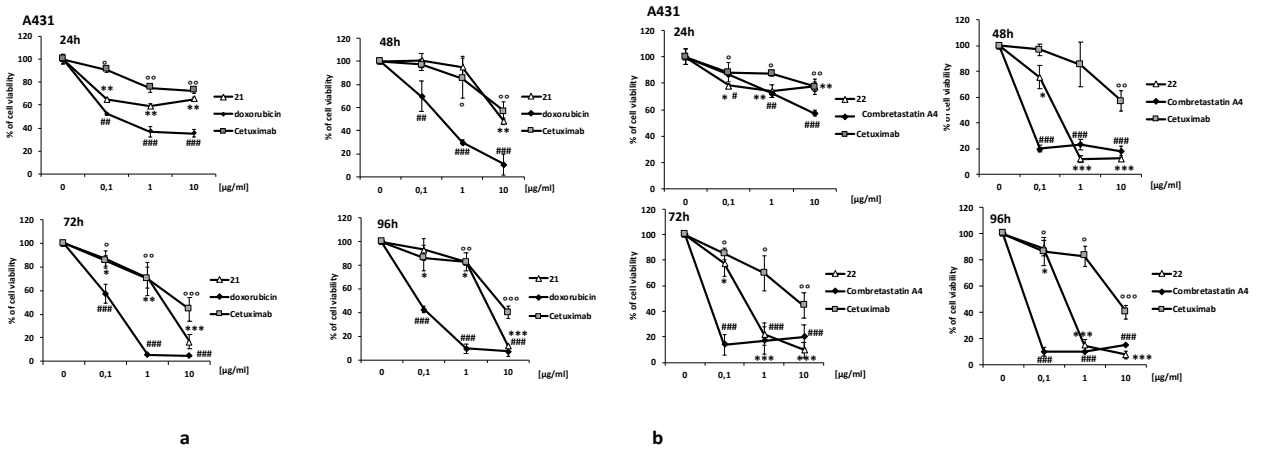


Figure S7: Cell viability. Cancer cell survival was evaluated by MTT test. Cells were exposed to increasing concentration of: **a**) conjugate **21**, doxorubicin and Cetuximab; **b**) conjugate **22**, Combretastatin A4 and Cetuximab. Data are expressed as percent of cell viability. $^{\#}$,*, ° $p<0.05$ vs basal; $^{\#\#}$,**, °° $p<0.01$ vs basal and $^{\#\#\#}$,***, °°° $p<0.001$ vs basal.

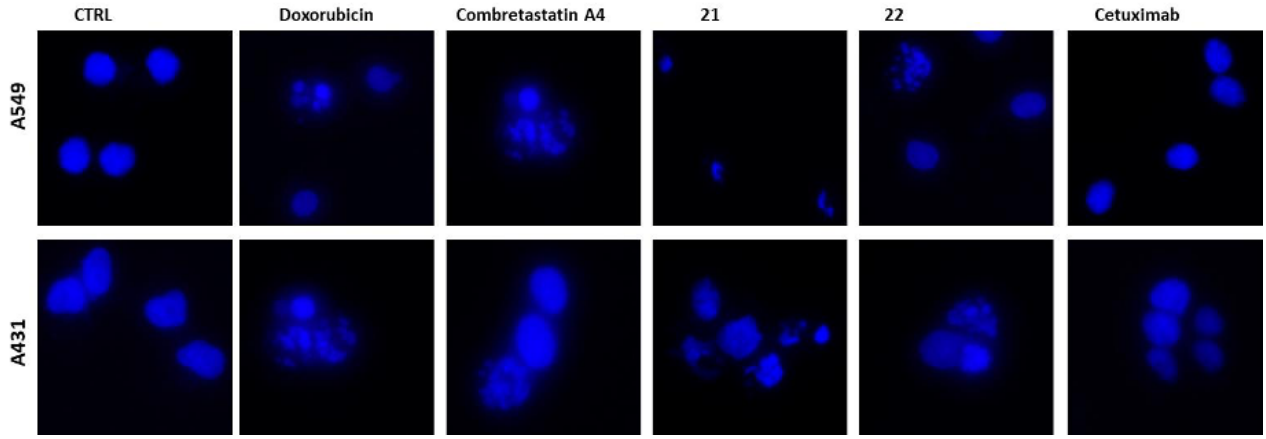


Figure S8. ADC **21** and **22** induce apoptosis in cancer cells. DAPI fluorescence images of apoptotic cancer cells indicating chromatin condensation in the cell nuclei.

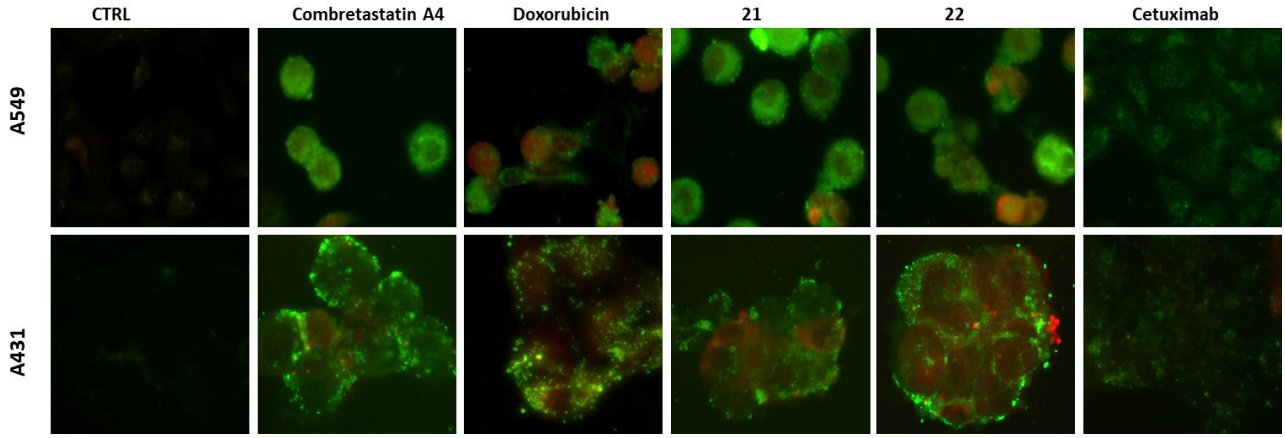


Figure S9**.** ADC **21** and **22** induce apoptosis in cancer cells. Apoptosis was analyzed with Annexin V-FITC and PI staining, and observed under a fluorescent microscope. Images are representative of three different experiments.

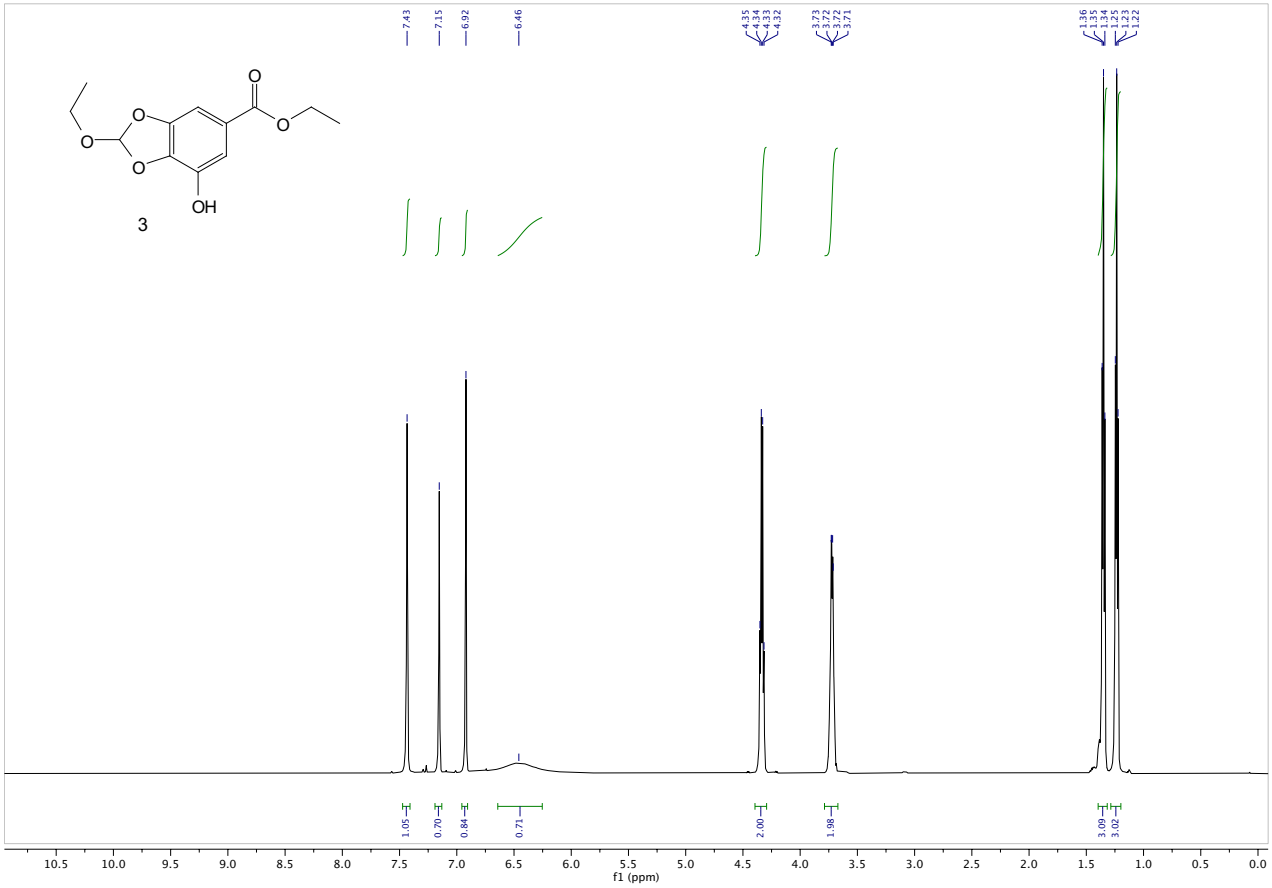
A549	% of cell viability			
19 µM	24h	48h	72h	96h
0	100	100	100	100
0.1	79,32	46,4	23,4	18,1
1	67,7	40,0	25,0	20,5
10	59,2	40,7	26,5	24,4

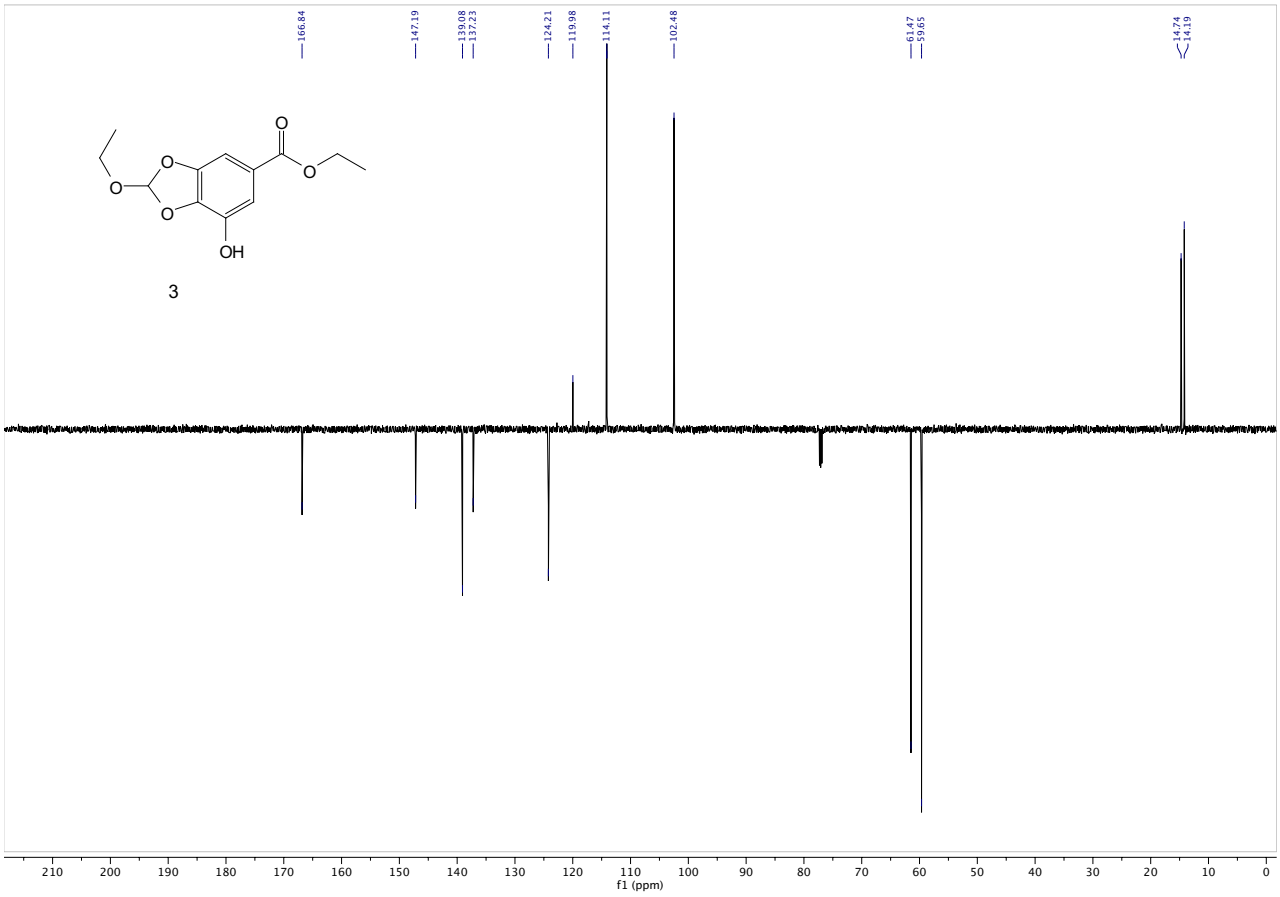
A549	% of cell viability			
5 µM	24h	48h	72h	96h
0	100	100	100	100
0.1	90,3	101,5	81,6	72,2
1	86,3	100,4	64,0	44,8
10	78,3	41,0	43,6	42,7

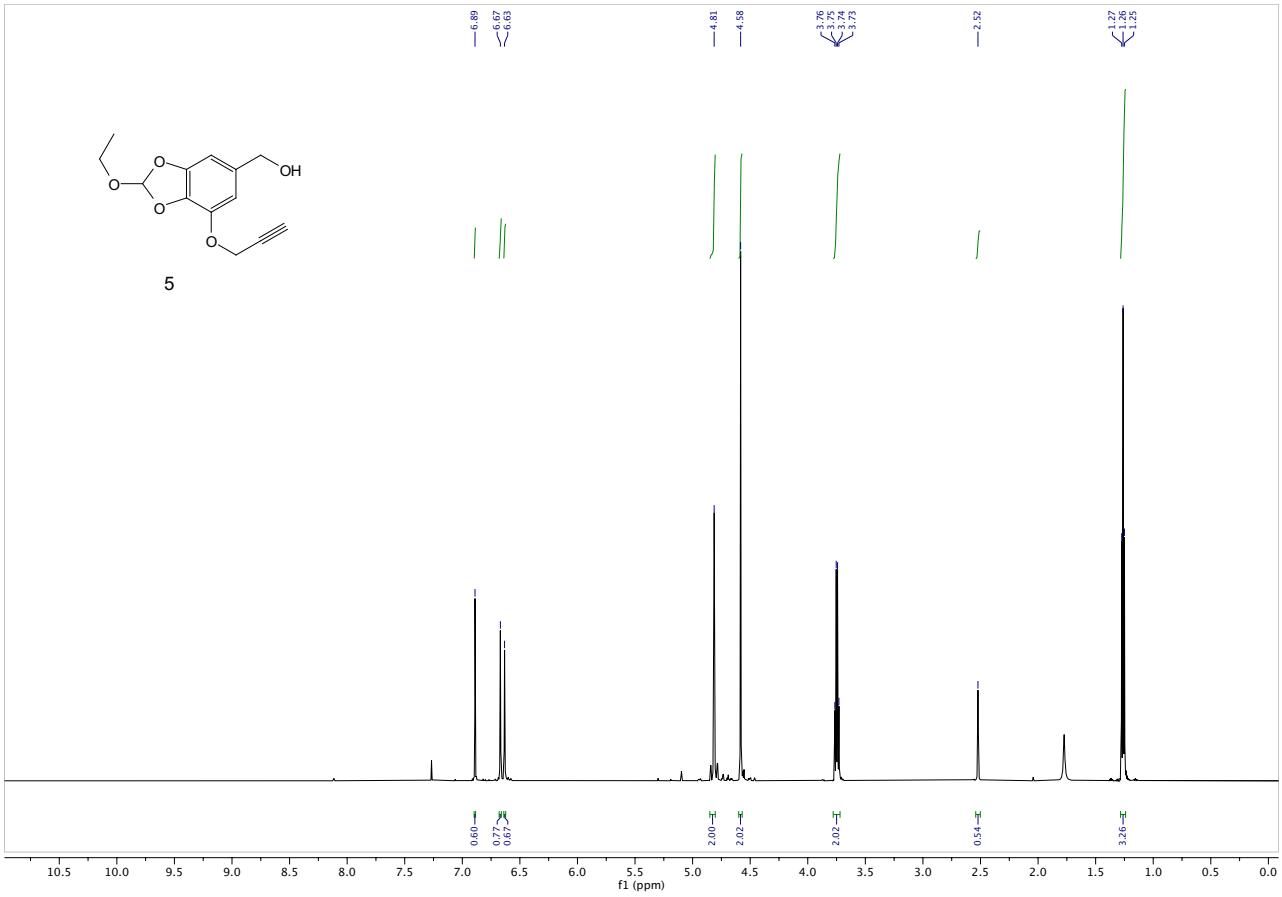
A431	% of cell viability			
19 µM	24h	48h	72h	96h
0	100	100	100	100
0.1	88,2	21,2	12,7	8,1
1	70,5	22,2	13,7	12,2
10	73,8	16,6	14,7	11,3

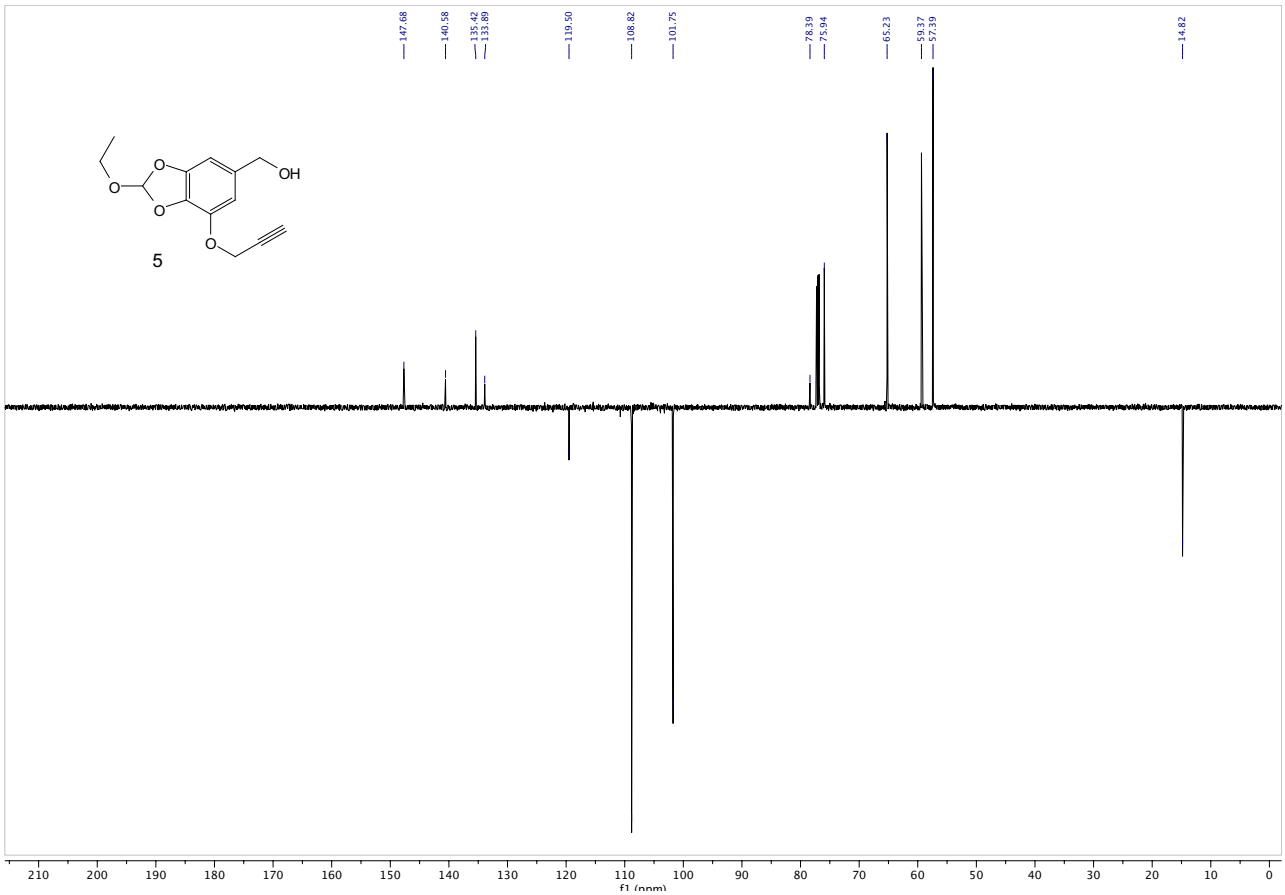
A431	% of cell viability			
5 µM	24h	48h	72h	96h
0	100	100	100	100
0.1	84,2	82,9	80,2	80,0
1	68,8	54,1	27,0	22,5
10	50,7	12,3	19,3	14,1

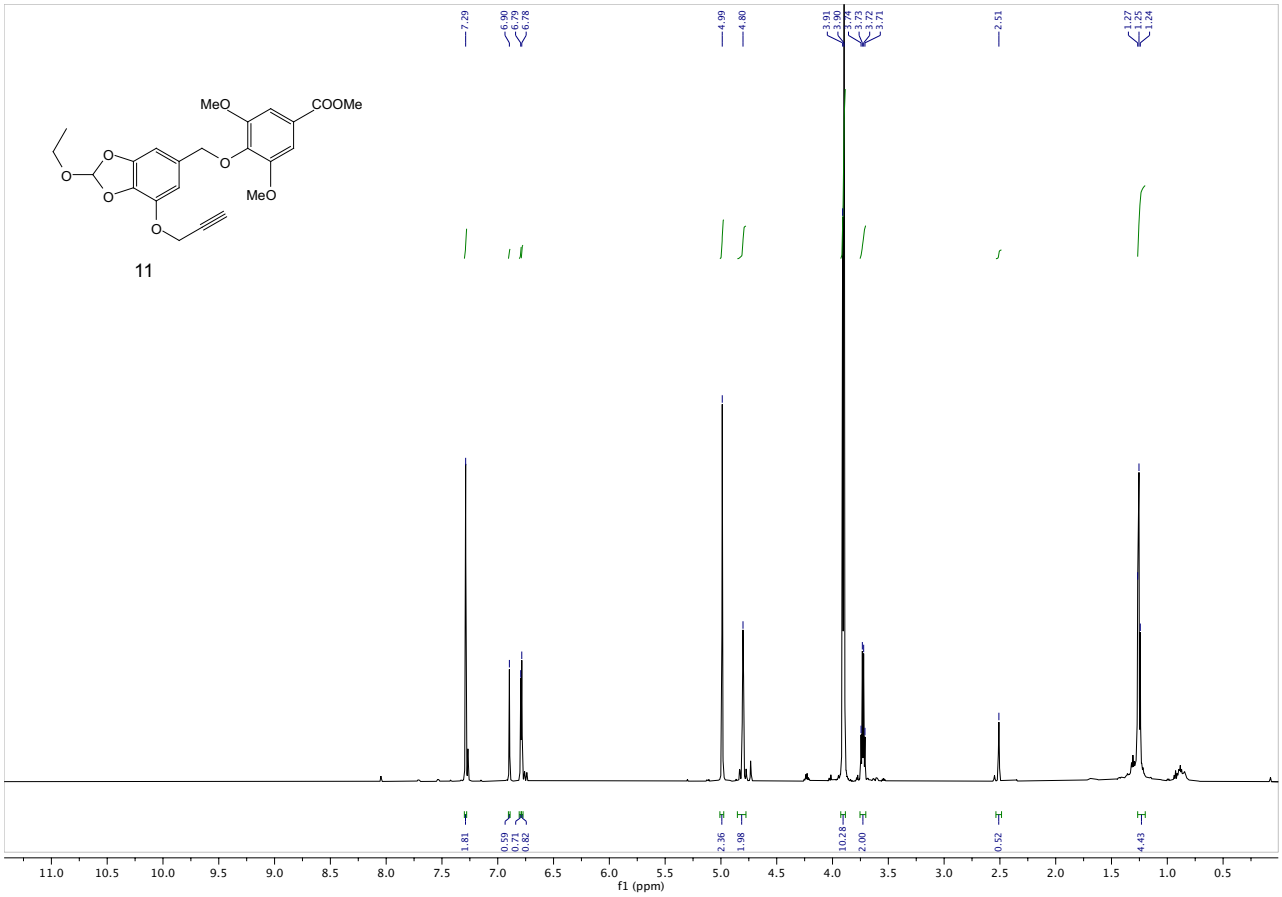
Figure S10**.** Comparison between citotoxicity of the linker payload conjugate **19** and the linker alone (**5**, HMPO).

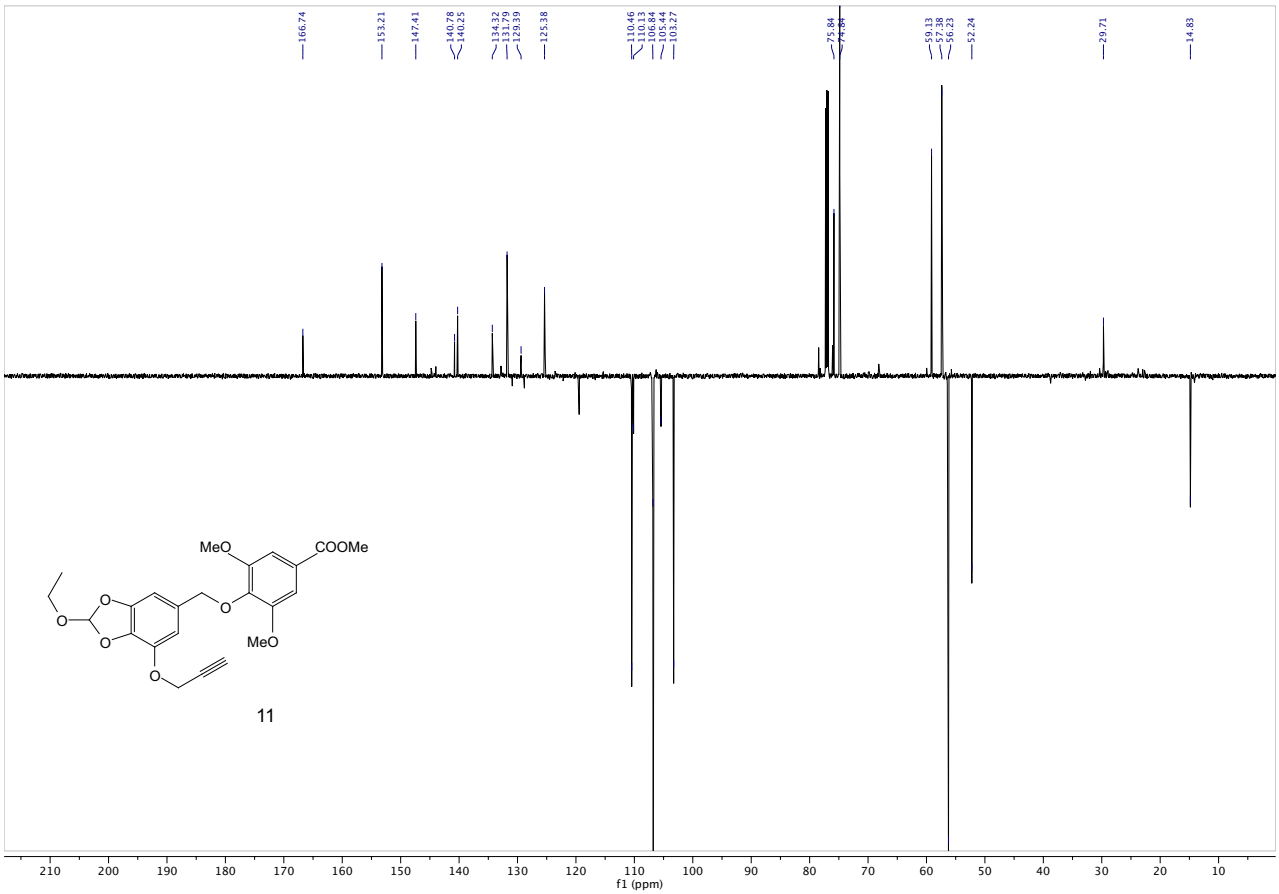

7.43
7.15
6.92
6.46
4.35
4.34
4.33
4.32
3.73
3.72
3.72
3.71
1.36
1.35
1.34
1.25
1.23
1.22
O
O
O
O
O
OH
3
1.05
0.70
0.84
0.71
2.00
1.98
3.09
3.02
f1 (ppm)

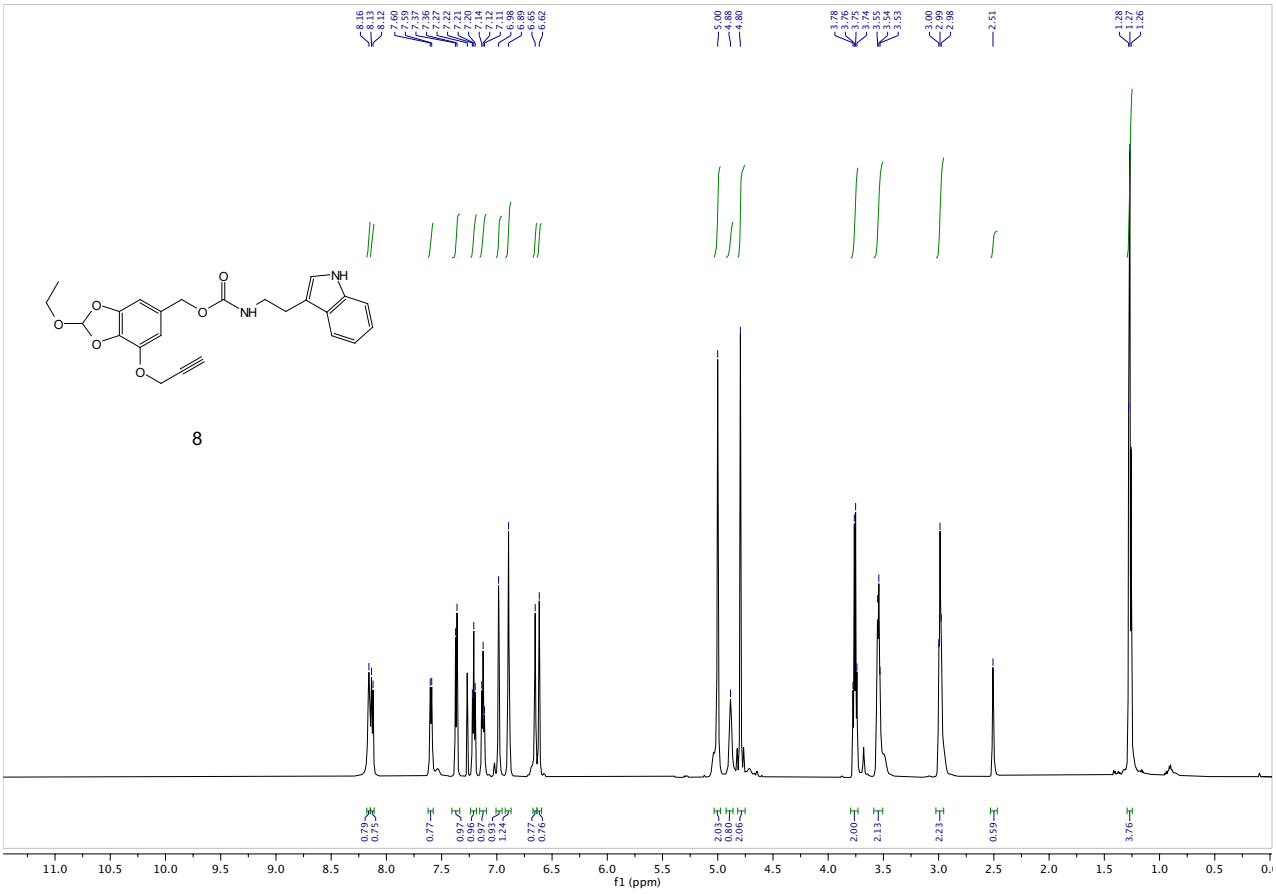

166.84
147.19
139.08
137.23
124.21
119.98
114.11
102.48
61.47
59.65
14.74
14.19
O
O
O
O
O
OH
3
f1 (ppm)

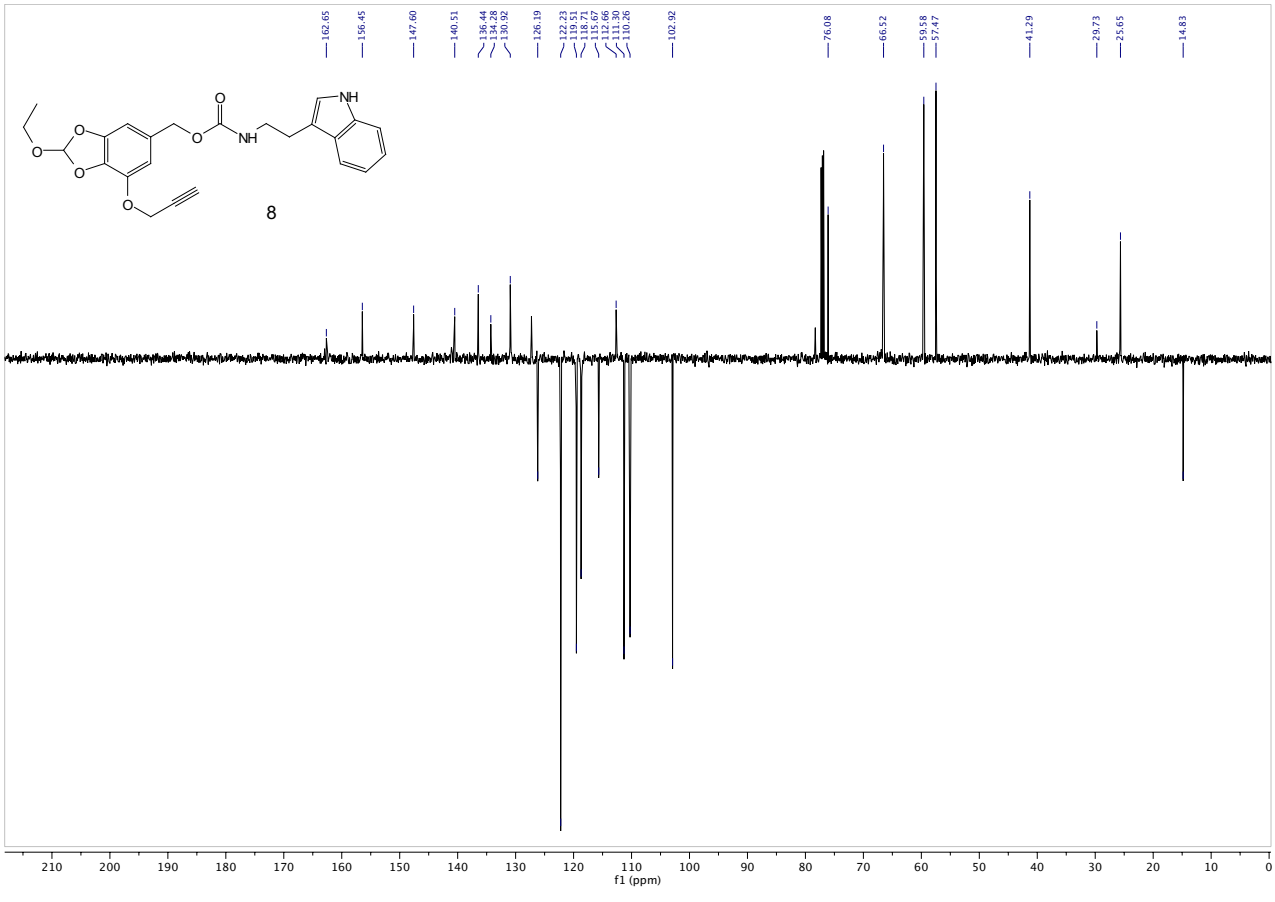

6.89
6.67
6.63
4.81
4.58
3.76
3.75
3.74
3.73
2.52
1.27
1.26
1.25
OH
O
O
O
O
5
0.60
0.77
0.67
2.00
2.02
2.02
0.54
3.26
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

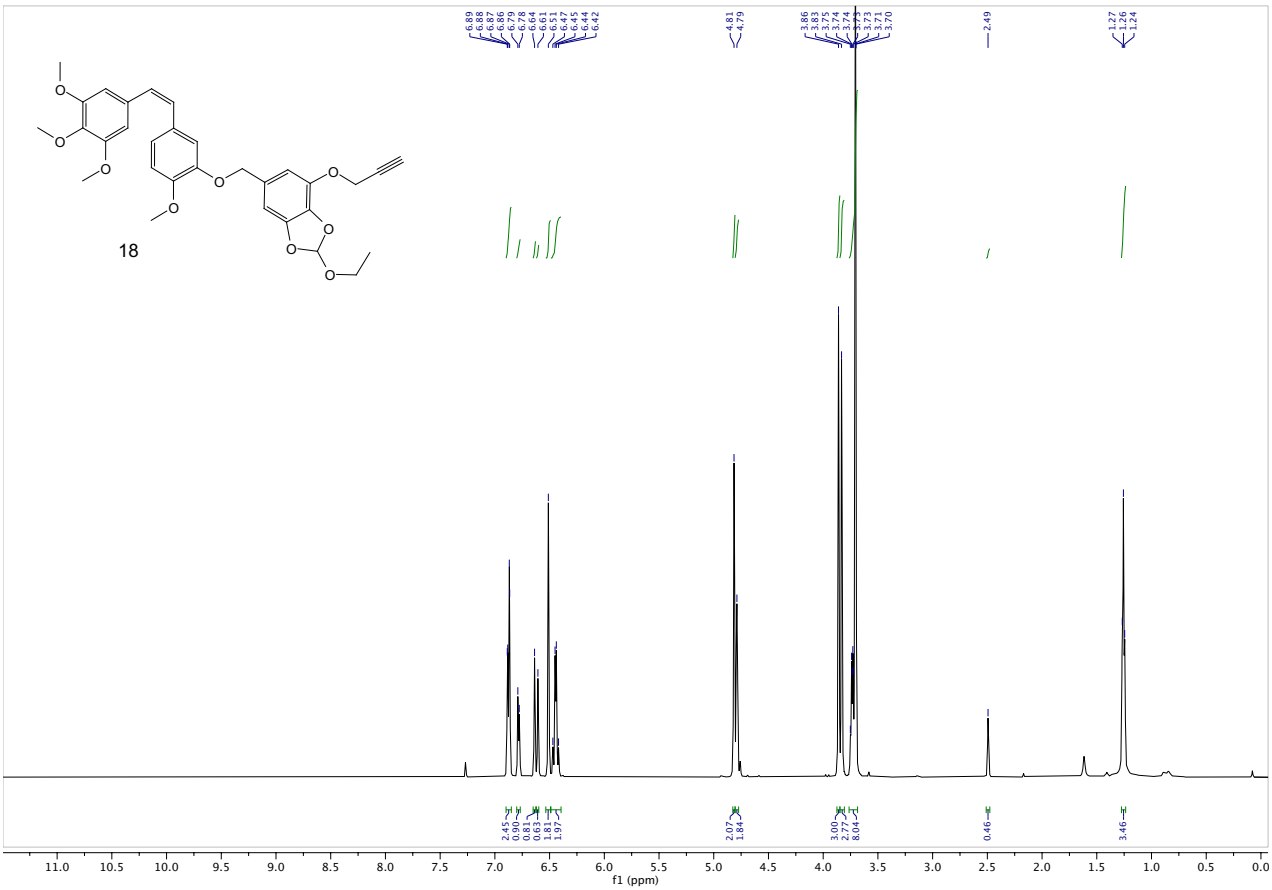

147.68
140.58
135.42
133.89
119.50
108.82
101.75
78.39
75.94
65.23
59.37
57.39
14.82
OH
O
O
O
O
5
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

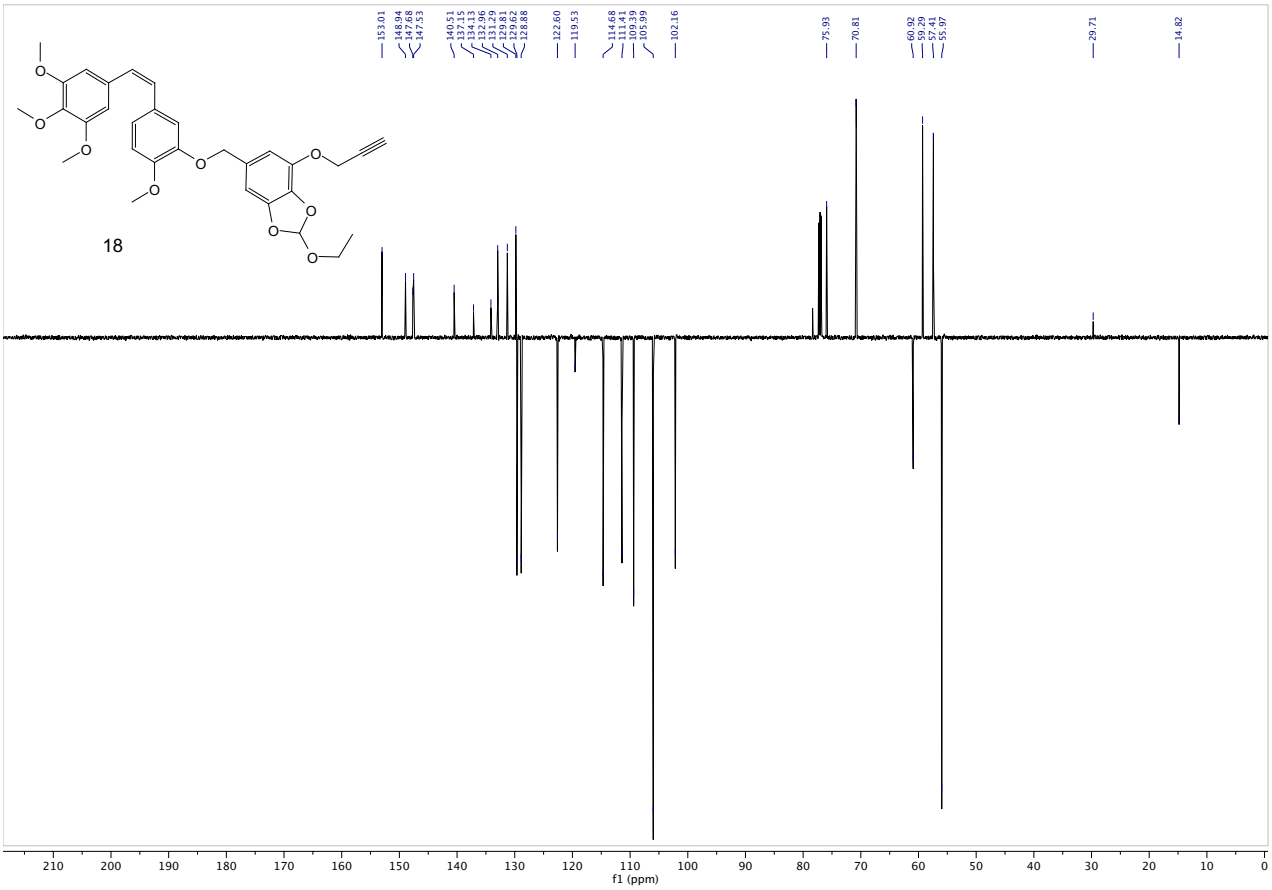

MeO
COOMe
O
O
O
O
MeO
O
11
7.29
6.90
6.79
6.78
4.99
4.80
3.91
3.90
3.74
3.73
3.72
3.71
2.51
1.27
1.25
1.24
1.81
0.59
0.71
0.82
2.36
1.98
10.28
2.00
0.52
4.43
f1 (ppm)

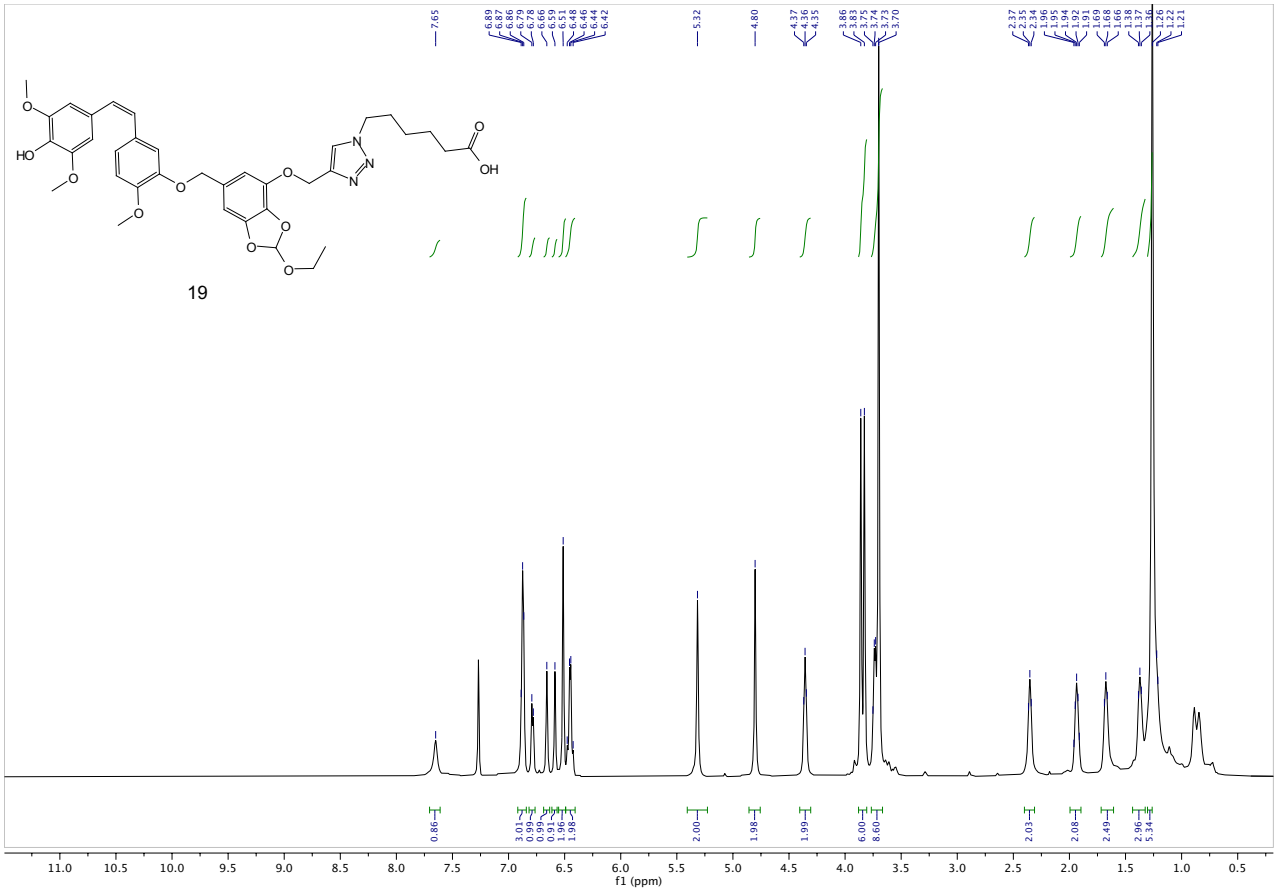

166.74
153.21
147.41
140.78
140.25
134.32
131.90
129.39
125.38
110.46
110.13
106.84
105.44
103.27
75.84
74.84
59.13
57.38
56.23
52.24
29.71
14.83
MeO
COOMe
O
O
O
O
MeO
O
11
f1 (ppm)


8.16
8.13
8.12
7.60
7.59
7.39
7.37
7.36
7.27
7.22
7.21
7.20
7.14
7.12
7.11
6.98
6.89
6.65
6.62
5.00
4.88
4.80
3.78
3.76
3.75
3.74
3.55
3.54
3.53
3.00
2.99
2.98
2.51
1.28
1.27
1.26
NH
O
O
O
O
O
O
NH
8
0.79
0.75
0.77
0.97
0.96
0.97
0.93
1.24
0.77
0.78
2.03
0.80
2.06
2.00
2.13
2.23
0.59
3.76
f1 (ppm)

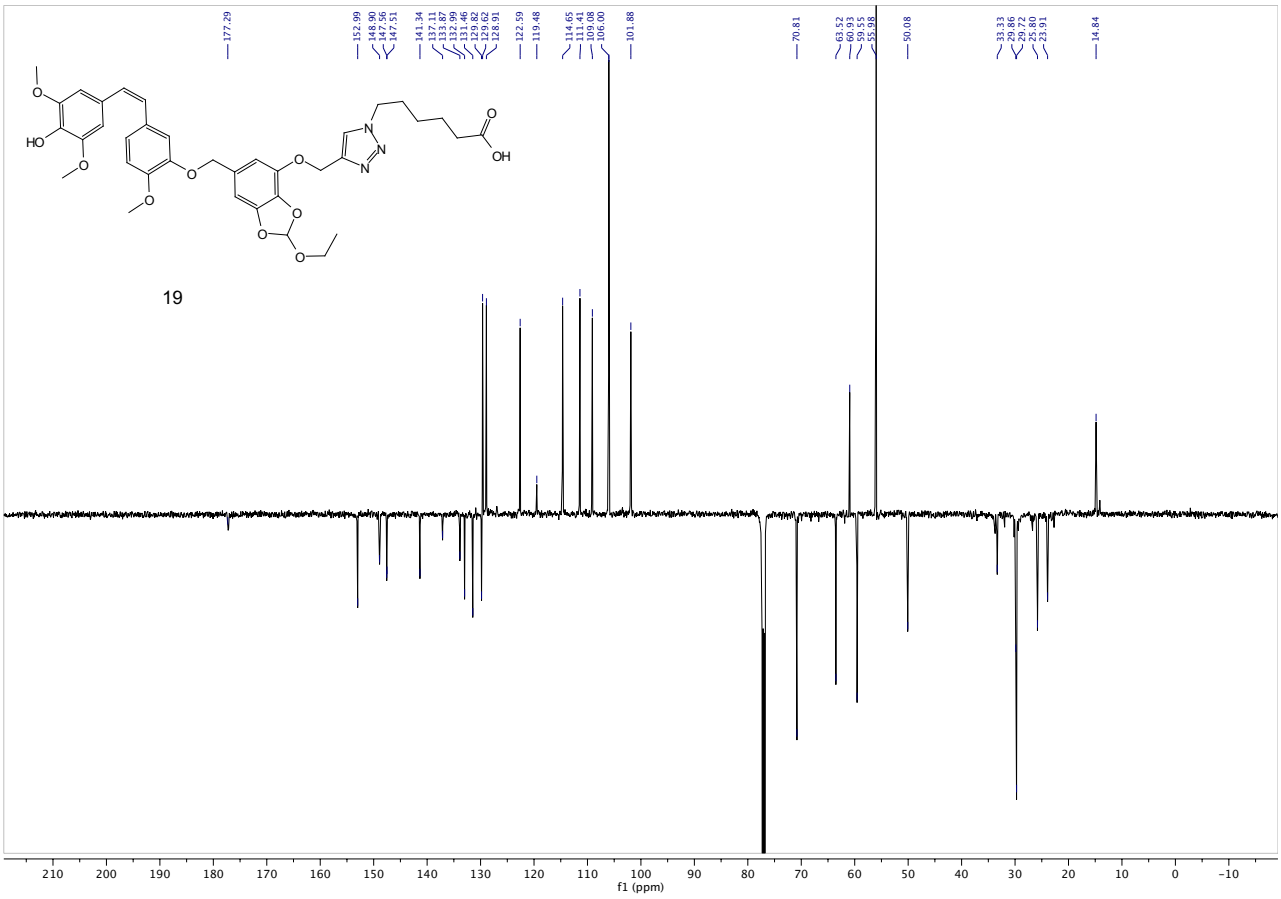

162.65
156.45
147.60
140.51
136.44
134.28
130.92
126.19
122.23
119.51
118.71
115.67
112.66
111.30
110.26
102.92
76.08
66.52
59.58
57.47
41.29
29.73
25.65
14.83
NH
O
O
O
O
O
O
NH
8
f1 (ppm)

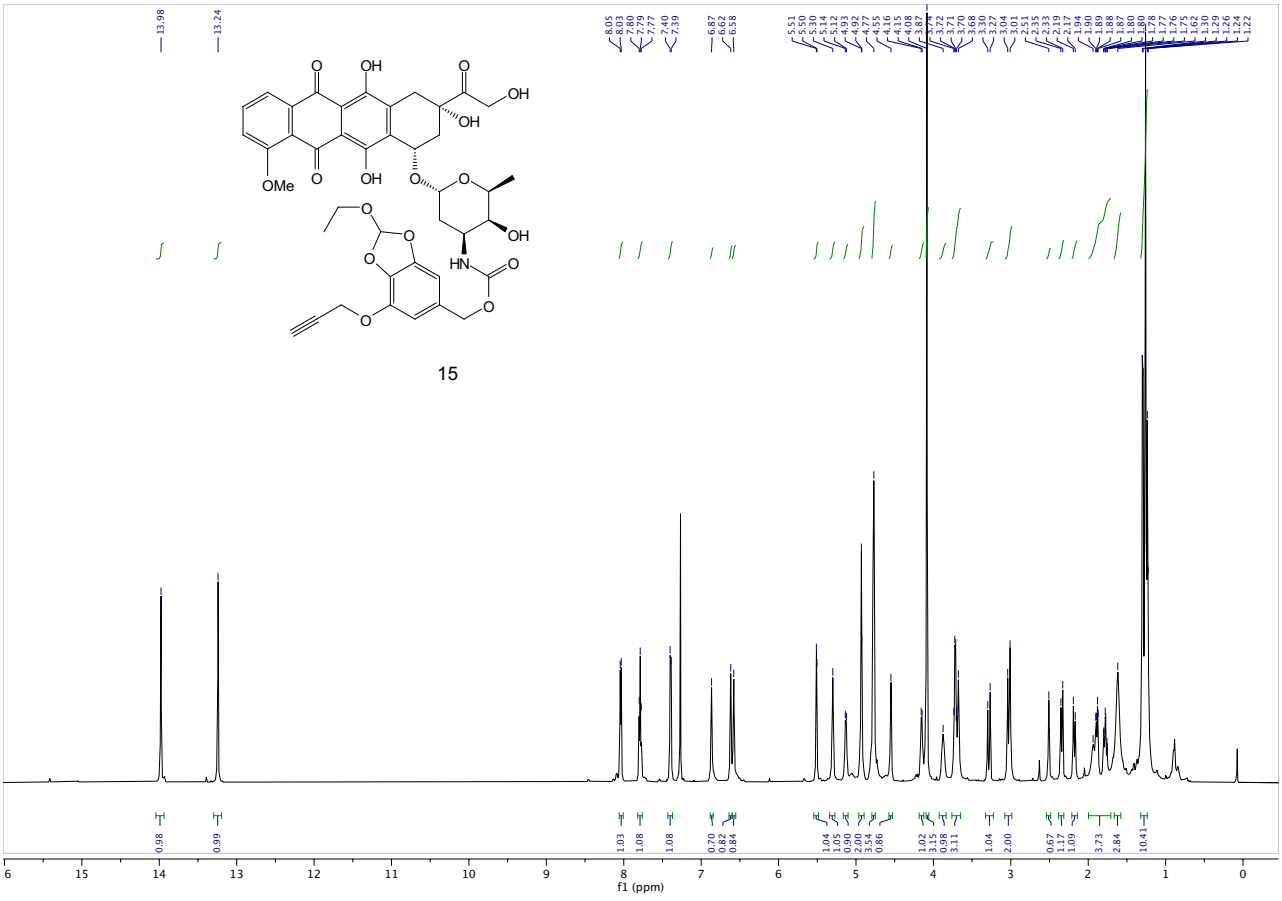

18
f1 (ppm)

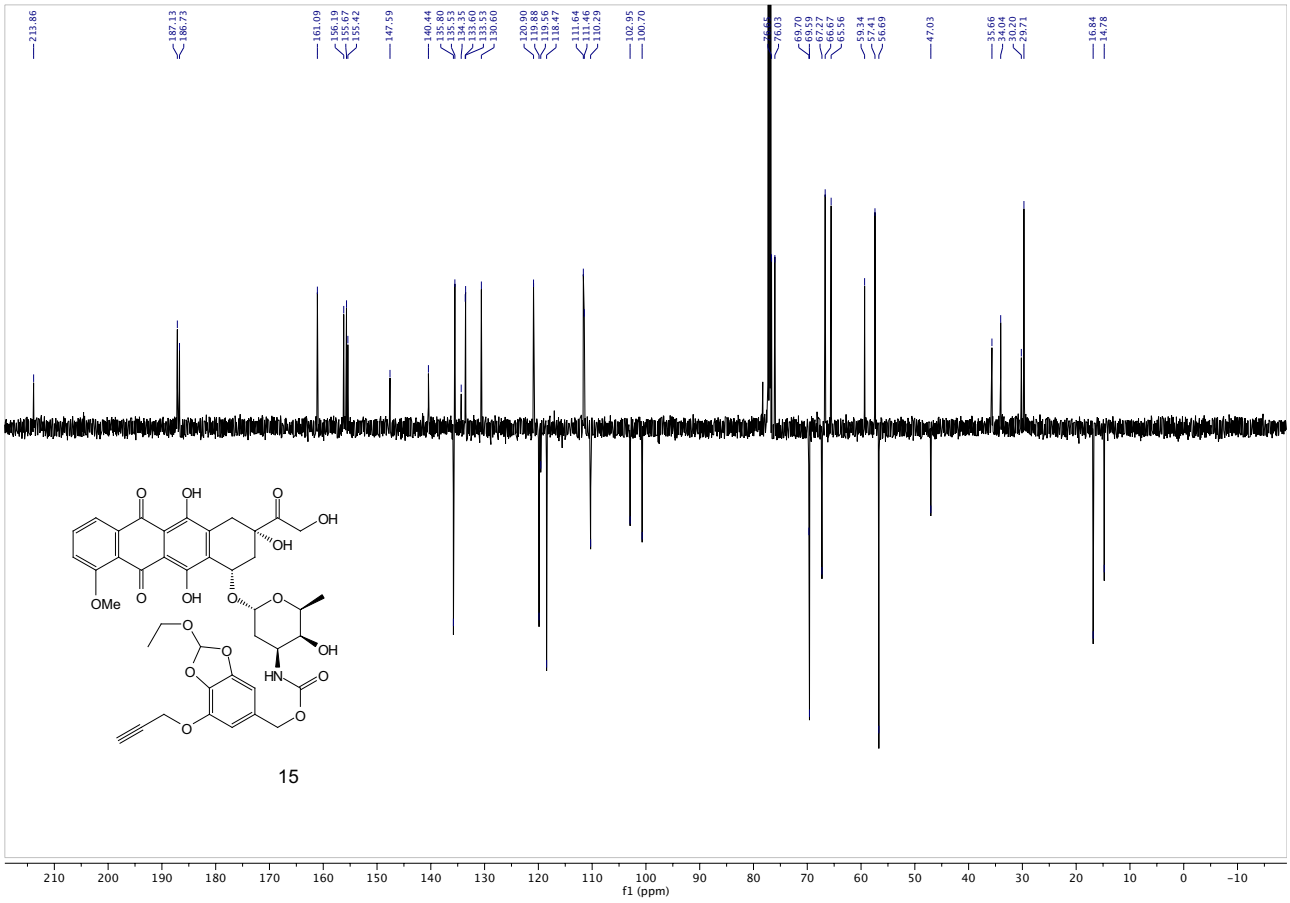

18
f1 (ppm)


7.65
6.89
6.87
6.86
6.79
6.78
6.66
6.59
6.51
6.48
6.46
6.44
6.42
5.32
4.80
4.37
4.36
4.35
3.86
3.83
3.75
3.74
3.73
3.70
2.37
2.35
2.34
1.96
1.95
1.94
1.92
1.91
1.69
1.68
1.66
1.38
1.37
1.36
1.26
1.22
1.21
0.86
3.01
0.99
0.99
0.91
1.96
1.98
2.00
1.98
1.99
6.00
8.60
2.03
2.08
2.49
2.96
5.34
11.0
10.5
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)

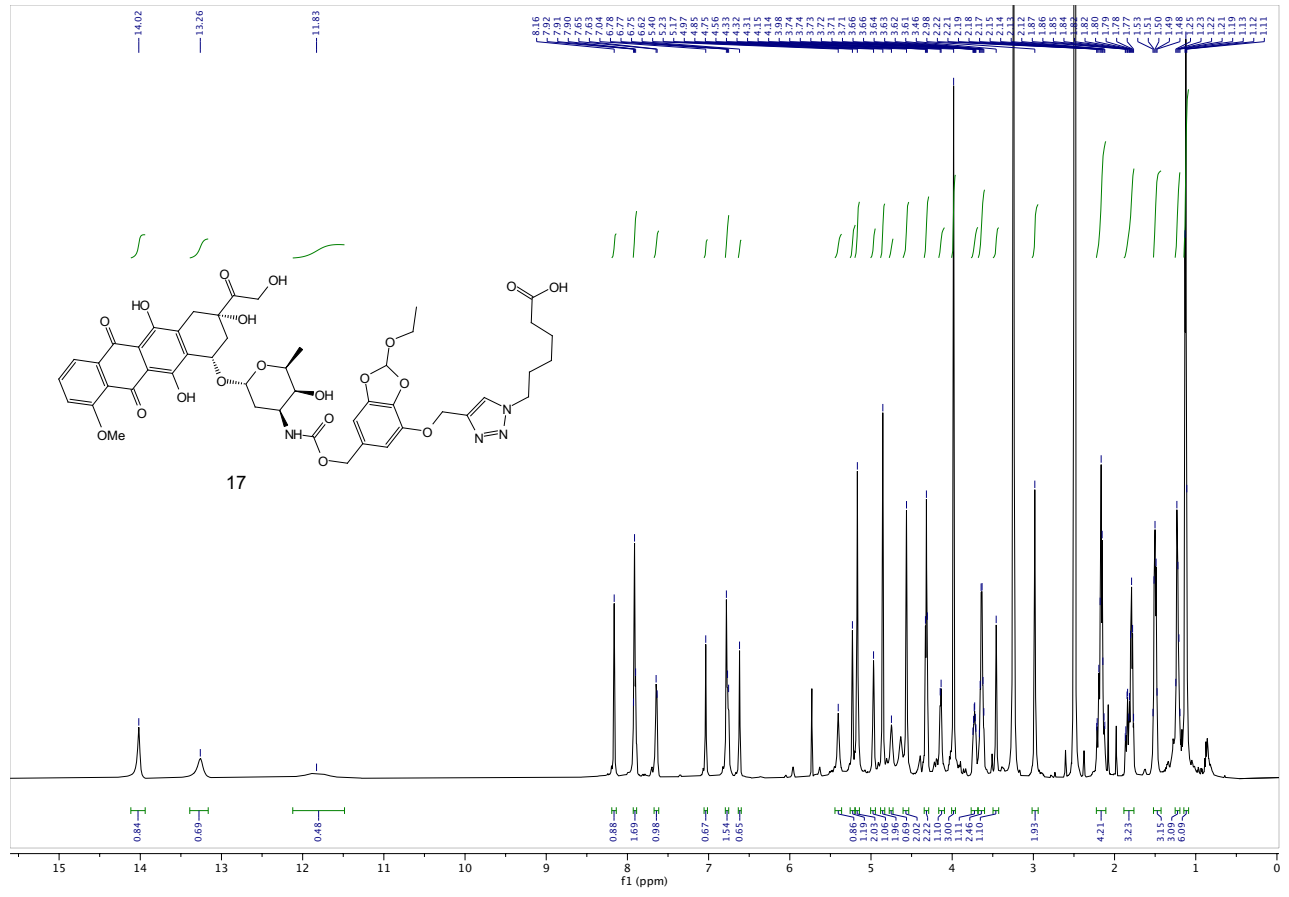
19


177.29
152.99
148.90
147.56
147.51
141.34
137.11
133.87
132.99
131.46
130.82
129.62
128.91
122.59
119.48
114.65
111.41
109.08
106.00
101.88
70.81
63.52
60.93
59.55
55.88
50.08
33.33
29.86
29.72
25.60
23.91
14.84
HO
O
OH
N
N
N
19
f1 (ppm)

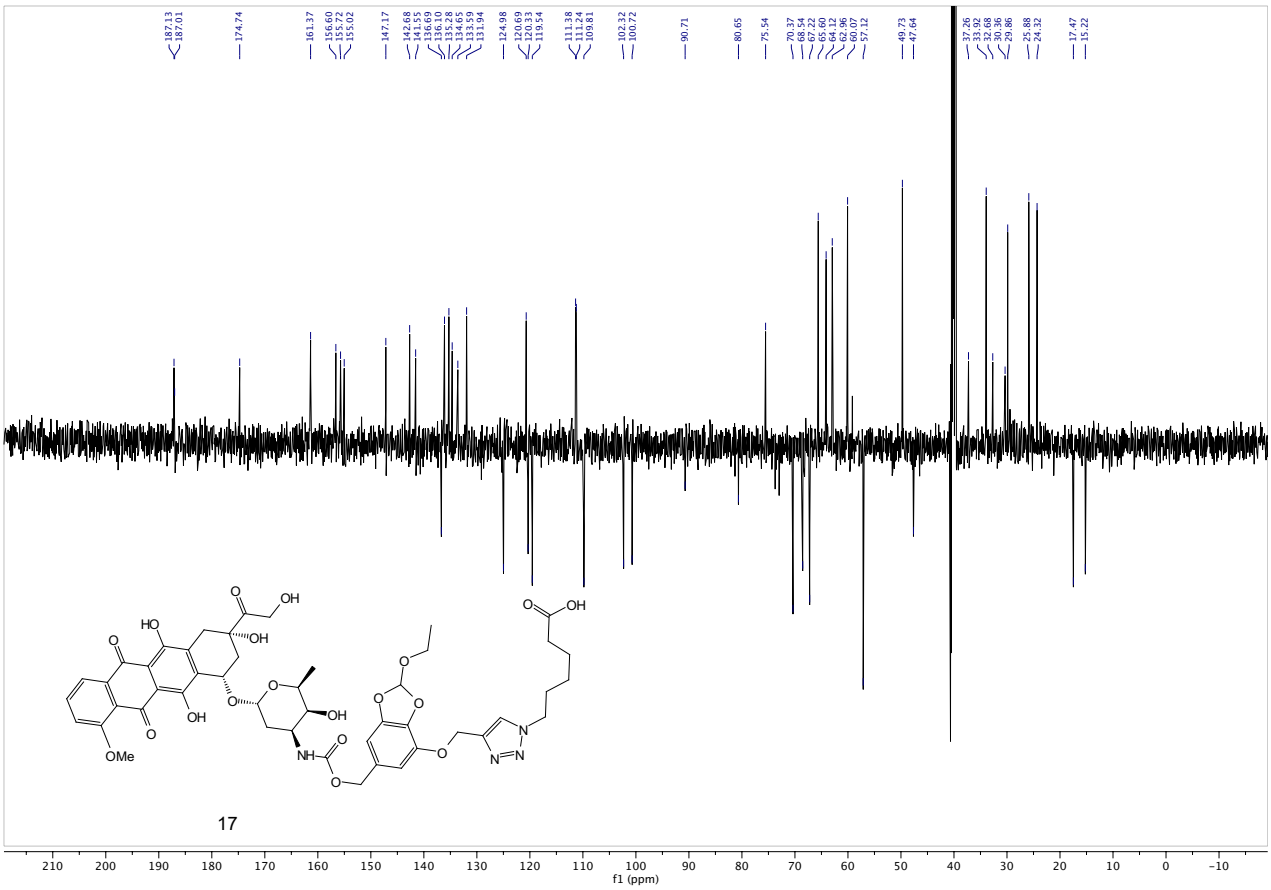

13.98
13.24
8.05
8.03
7.80
7.79
7.77
7.40
7.39
6.87
6.62
6.58
OMe
OH
HN
15
f1 (ppm)


213.86
187.13
186.73
161.09
156.19
155.67
155.42
147.59
140.44
135.80
135.55
134.35
133.60
133.53
130.60
120.90
120.38
119.56
118.47
111.64
111.46
110.29
102.95
100.70
76.03
69.70
69.59
67.27
66.67
65.56
59.34
57.41
56.69
47.03
35.66
34.04
30.70
29.71
16.84
14.78
f1 (ppm)

15


14.02
13.26
11.83
f1 (ppm)

17


187.13
187.01
174.74
161.37
156.60
155.72
155.02
147.17
142.68
141.55
136.60
136.10
135.28
134.65
133.59
131.94
124.98
120.69
120.33
119.54
111.38
111.24
109.81
102.32
100.72
90.71
80.65
75.54
70.37
68.54
67.22
65.60
64.21
62.96
60.07
57.12
49.73
47.64
37.26
33.92
32.68
30.36
29.86
25.88
24.32
17.47
15.22
f1 (ppm)

17

References

1 G. Leriche, M. Nothisen, N. Baumlin, C. D. Muller, D. Bagnard, J.-S. Remy, S. A. Jacques and A. Wagner, *Bioconjug. Chem.*, 2015, **26**, 1461–1465.

2 S. Takaoka, N. Takaoka, Y. Minoshima, J. M. Huang, M. Kubo, K. Harada, H. Hioki and Y. Fukuyama, *Tetrahedron*, 2009, **65**, 8354–8361.

3 P. H. J. Kouwer, C. J. Welch, G. McRobbie, B. J. Dodds, L. Priest and G. H. Mehl, *J. Mater. Chem.*, 2004, **14**, 1798–1803.

4 E. Orlowska, A. Roller, H. Wiesinger, M. Pignitter, F. Jirsa, R. Krachler, W. Kandioller and B. K. Keppler, *RSC Adv.*, 2016, **6**, 40238–40249.

5 B. E. Gryder, M. J. Akbashev, M. K. Rood, E. D. Raftery, W. M. Meyers, P. Dillard, S. Khan and A. K. Oyelere, *ACS Chem. Biol.*, 2013, **8**, 2550–2560.

6 R. Srinivasan, L. P. Tan, H. Wu, P.-Y. Yang, K. A. Kalesh and S. Q. Yao, *Org. Biomol. Chem.*, 2009, **7**, 1821–1828.

7 M. Bradford, *Anal. Biochem.* **1976**, *72*, 248–254.

(8) B. L. Barthel, D. L. Rudnicki, T. P. Kirby, S. M. Colvin, D. J. Burkhart, and T. H. Koch *J. Med. Chem.* **2012**, *55*, 6595–6607.