Supporting Information: Targeted Delivery of Alcohol-Containing Payloads with Antibody-Drug Conjugates

Contents

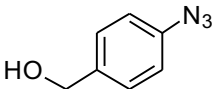
General experimental methods

Starting materials, reagents and solvents were purchased from commercial suppliers (Sigma-Aldrich, Combi-Blocks, FluoroChem) and were not analyzed or purified before use. All solvents were of HPLC quality. Anhydrous THF, CH_2Cl_2, ACN, DMSO and DMF were obtained from a PureSolvTM MD-7 Solvent Purification System, Innovative Technology stationary phase being Al_2O_3. Other dry solvents were purchased under septum and under inert atmosphere. Thin-layer Chromatography (TLC) analysis was performed using Merck aluminium sheets covered with silica gel C-60 F254 and developed by UV-light or stains ($KMnO_4$: 3.00g in 300mL water; ninhydrin: 0.10g in 0.5mL AcOH and 100mL acetone). Flash column chromatography was performed using glass columns packed with Merck Geduran 60 Angstrom silica gel (40-64 µm particles) as stationary phase. Solvent evaporation was done on a Heidolph laborota 4000 rotary evaporator, efficient under reduced pressure (*in vacuo*) at temperatures ranging from 20 to 90 °C. Solvent traces were removed *in vacuo* at 0.27 mbar by a membrane pump, operating via a Schlenk line. Inert atmosphere refers to a N_2 atmosphere dried over $CaCl_2$. Analytic UPLC-MS analyses were done on a Waters AQUITY UPLC system, equipped with PDA and SQD electrospray MS detectors. Column: Kinetex 1.7 µm XB-C18, 2.1 x 50mm. Column temperature: 50 °C. Flowrate: 0.6 mL/min. Solvent A: 0.1% HCOOH in water, Solvent B: 0.1% HCOOH in ACN. Gradient: 5% B to 100% B in 2.6min or 4.8min, hold 0.1 min, total run time 2.6min or 4.8min. Alternatively, solvent C: 15mM NH_4OAc in H_2O, Solvent D: 15mM NH_4OAc in ACN:H_2O 90:10. Gradient: 5% D to 100% D in 2.4min or 4.8min, hold 0.1 min, total run time 2.6min or 5.0min. Preparative HPLC was performed on a Waters 3767 Sample Manager equipped with a Waters 2545 Binary Gradient Module, a Waters UV Fraction Manager and C18 column for separation (flow: 20mL/min). Solvent A: 0.1% HCOOH in water, Solvent B: 0.1% HCOOH in ACN. Alternatively, solvent C: 15mM NH_4OAc in water, Solvent D: 15mM NH_4OAc in ACN:H_2O 90:10. Run time and gradient were determined for each specific purification. NMR spectra were recorded on a Bruker Ascend spectrometer with a Prodigy

cryoprobe operating at 400 MHz for ^{1}H-NMR and 101 MHz for ^{13}C-NMR. Chemical shifts (δ) are reported in ppm downfield from TMS (δ = 0) using solvent resonance as the internal standard: Chloroform-*d*, ^{1}H: 7.26 ppm, ^{13}C: 77.16 ppm; dimethylsulfoxide-d_6, ^{1}H: 2.50 ppm and 3.30 ppm (water), ^{13}C: 39.52 ppm; CD_3OD, ^{1}H: 3.31 ppm and 4.87 ppm (water), ^{13}C 49.00. Coupling constants (*J*) are reported in Hz. Multiplicities are reported as singlet (s), broad singlet (br. s), doublet (d), doublet of doublets (dd), doublet of triplets (dt), doublet of doublet of doublets (ddd), doublet of doublet of triplets (ddt), triplet (t), triplet of doublets (td), quartet (q), pentet (p), septet (sep) and multiplets (m).

Chemical Synthesis

(4-Azidophenyl)methanol (S1)



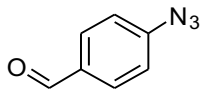
4-aminobenzylalcohol (1.00 g, 8.12 mmol, 1 equiv.) was dissolved in 8.1 mL 6 M HCl and was cooled to 0°C. A solution of $NaNO_2$ (0.84 g, 12.2 mmol, 1.5 equiv.) in 21 mL H_2O was cooled to 0 °C and was added dropwise to the solution of 4-aminobenzylalcohol. The reaction was stirred for 30 min at 0 °C before the dropwise addition of NaN_3 (2.10 g, 32.5 mmol, 4 equiv.) in 41 mL H_2O while keeping the temperature below 0°C. After 30 min at 0 °C, the reaction was neutralized by the addition of NaOAc until the reaction was at a basic pH and the reaction was allowed reach rt and was stirred for an additional 2 h. The reaction was extracted with 3x Et_2O dried over $MgSO_4$ and the solvent was removed *in vacuo*. The crude product was purified FCC affording the title compound **S1** in a yield of 99%.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.28 (d, J = 8.5 Hz, 2H), 6.97 (d, J = 8.5 Hz, 2H), 4.56 (s, 2H), 2.88 (s, 1H).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 139.27, 137.58, 128.52, 119.07, 64.44.

Data in accordance with literature[1].

4-Azidobenzaldehyde (1)



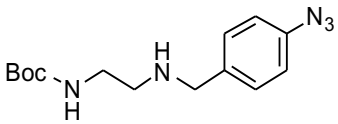
Compound **S1** (2.16 g, 14.48 mmol, 1 equiv.) was dissolved in 75 mL dry DCM followed by the addition of Dess-Martin Periodinane (9.21 g, 21.72 mmol, 1.5 equiv.) and the reaction was stirred for 18 h. the reaction was portioned between EtOAc and H_2O. The precipitate was filtered to ease the extraction and was washed with EtOAc. The resulting mixture was separated and the aqueous phase was extracted with DCM. The combined organic phase was washed with sat. aq. $Na_2S_2O_3$, sat. aq. $NaHCO_3$ and brine. The organic phase was dried over Na_2SO_4 and the solvent was removed *in vacuo*. The crude was FCC affording the title compound **1** in a yield of 92%.

^{1}H-NMR (400 MHz, $CDCl_3$) δ 9.94 (s, 1H), 7.88 (d, J = 8.5 Hz, 2H), 7.16 (d, J = 8.6 Hz, 2H).

^{13}C-NMR (101 MHz, $CDCl_3$) δ 190.69, 146.38, 133.35, 131.66, 119.59.

Data in accordance with literature[2].

***Tert*-butyl (2-((4-azidobenzyl)amino)ethyl)carbamate (2)**



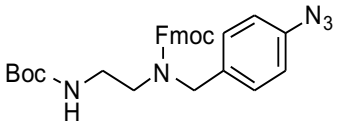
Compound **1** (2.00 g, 13.57 mmol, 1 equiv.) was dissolved in 24 mL dry MeOH under inert atmosphere and $MgSO_4$ (6.53 g, 54.29 mmol, 4 equiv.) was added. *N*-Boc-ethylenediamine (2.15 mL, 13.57mmol, 1 equiv.) was added and the reaction was stirred overnight at rt. The reaction was cooled to 0 °C and $NaBH_4$ (514 mg, 13.57 mmol, 1 equiv.) was added in portions. The reaction was stirred at 0 °C for 3.5 h before the addition of H_2O. The reaction was extracted with 3x EtOAc and DCM. The combined organic phase was washed with brine before being dried over $MgSO_4$ and the solvent was removed *in vacuo*. The crude was purified FCC affording the title compound **2** in a yield of 79%.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 – 7.29 (m, 2H), 7.00 – 6.96 (m, 2H), 4.99 (s, 1H), 3.77 (s, 2H), 3.24 (q, *J* = 5.8 Hz, 2H), 2.75 (t, *J* = 5.8 Hz, 2H), 2.55 (s, 2H), 1.44 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 156.28, 139.06, 136.46, 129.80, 119.19, 52.83, 48.54, 40.18, 28.55.

HRMS (ESI) *m/z* $[M+Na]^+$ 314.1588 $C_{14}H_{21}N_5NaO_2^+$ required 314.1587.

(9*H*-Fluoren-9-yl)methyl (4-azidobenzyl)(2-((*tert*-butoxycarbonyl)amino)ethyl) carbamate (3)



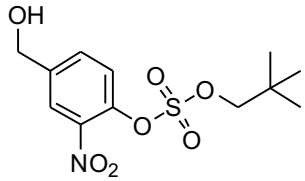
Compound **2** (2.96 g, 10.15 mmol, 1 equiv.) was dissolved in 7.5 mL 1,4-dioxane and 15 mL Na_2CO_3 (10 % w/w) and was cooled to 0 °C before the dropwise addition of 9-fluorenylmethyl chloroformate (3.94 g, 15.23 mmol, 1.5 equiv.) in 7.5 mL 1,4-dioxane. The reaction was stirred for 1 h at 0 °C and upon completion, was extracted with 3x DCM. The combined organic phase was washed with brine before being dried over $MgsO_4$ and the solvent was removed *in vacuo*. The crude was purified by FCC affording the title compound **3** in a yield of 97%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.93 – 7.82 (m, 2H), 7.72 – 7.62 (m, 1H), 7.53 (d, *J* = 7.5 Hz, 1H), 7.47 – 7.02 (m, 6H), 6.96 (q, *J* = 8.2 Hz, 2H), 6.81 (t, *J* = 5.8 Hz, 1H), 4.54 – 4.36 (m, 3H), 4.28 (dt, *J* = 20.3, 5.8 Hz, 1H), 4.16 (s, 1H), 3.17 – 3.07 (m, 2H), 2.97 (q, *J* = 6.6 Hz, 2H), 1.36 (s, 9H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 156.23, 156.07, 144.34, 141.30, 129.55, 129.35, 128.12, 127.64, 125.58, 125.23, 120.57, 119.67, 98.41, 78.16, 66.86, 49.79, 47.22, 46.54, 28.66.

HRMS (ESI) *m/z* found $[M+H]^+$ 514.2448 $C_{29}H_{32}N_5O_4^+$ required 514.2449

4-(hydroxymethyl)-2-nitrophenyl neopentyl sulfate (5)



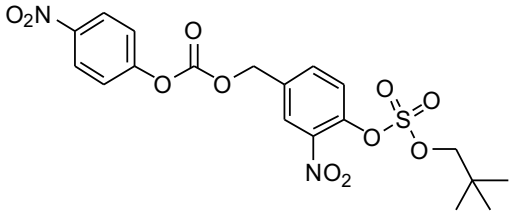
The 4-(hydroxymethyl)-2-nitrophenol **4** (350 mg, 2.07 mmol, 1 equiv.) was dissolved in dry THF (20 mL) under inert atmosphere and was added DMAP (506 mg, 4.14 mmol, 2 equiv.) and dropwise neopentyl sulfurochloridate (0.77 g, 4.14 mmol, 2 equiv.). The reaction was cooled to 0 °C and triethylamine (299 µL, 2.07 mmol, 1 equiv.) was added dropwise over 30 min. The reaction was stirred overnight at rt, before being quenched by the addition of DCM. The reaction was washed with water and brine, dried over $MgSO_4$ and the solvent was removed *in vacuo*. The crude was purified by flash chromatography affording compound **5** as a white powder in a yield of 479 mg (73%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (d, *J* = 2.1 Hz, 1H), 7.66 (dd, *J* = 8.4, 2.2 Hz, 1H), 7.58 (d, *J* = 8.6 Hz, 1H), 4.79 (s, 2H), 4.21 (s, 2H), 1.02 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 142.10, 141.56, 141.10, 132.31, 124.09, 123.99, 85.15, 63.30, 32.06, 25.97.

HRMS (ESI) *m/z* found $[M+Na]^+$ 342.0624 $C_{12}H_{17}NO_7SNa^+$ required 342.0618

Neopentyl (2-nitro-4-((((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl) sulfate (6)



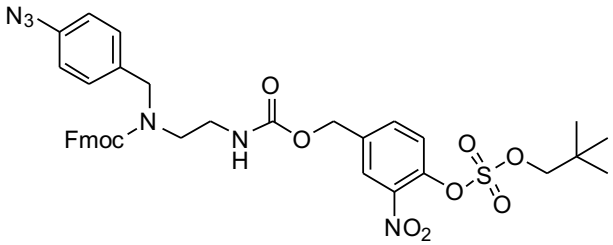
4-(hydroxymethyl)-2-nitrophenyl neo-pentyl sulfate **5** (679 mg, 2.12 mmol, 1 equiv.) was dissolved in 40 mL of dry DCM under inert atmosphere and was added para-nitrochlorofomate (977 mg, 4.85 mmol, 2.2 equiv.) and pyridine (444 µL, 5.51 mmol, 2.5 equiv.) and the reaction was stirred overnight. The reaction was partitioned between water and DCM and the organic phase was washed with 2x water, dried over $MgSO_4$ and solvent was removed *in vacuo*. The crude was purified by FCC, affording the title compound **6** in 66% yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.34 – 8.27 (m, 2H), 8.12 (d, *J* = 2.1 Hz, 1H), 7.76 (dd, *J* = 8.5, 2.2 Hz, 1H), 7.67 (d, *J* = 8.5 Hz, 1H), 7.40 (d, *J* = 9.1 Hz, 2H), 5.35 (s, 2H), 4.23 (s, 2H), 1.03 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 155.30, 145.67, 142.29, 134.83, 134.29, 126.31, 125.98, 125.50, 124.41, 121.85, 115.77, 85.42, 68.45, 32.05, 25.91.

HRMS (ESI) *m/z* found $[M+Na]^+$ 507.0684 $C_{19}H_{20}N_2O_{11}SNa^+$ required 507.0680

4-(7-(4-Azidobenzyl)-10-(9H-fluoren-9-yl)-3,8-dioxo-2,9-dioxa-4,7-diazadecyl)-2-nitrophenyl neopentyl sulfate (7)



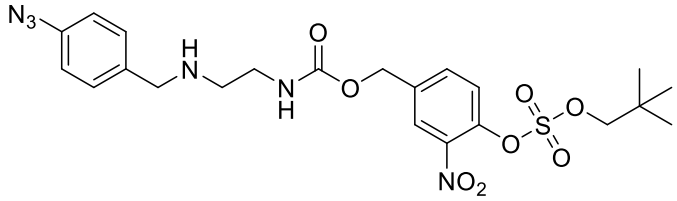
The (9H-fluoren-9-yl) methyl(4-azido-benzyl)(2-((tert butoxycarbonyl) amino)ethyl)carbamate **3** (576 mg, 1.12 mmol, 1 equiv.) was dissolved in 10 mL DCM and was added 1 mL TFA. The reaction was stirred for 1 h, whereupon THF was added to the reaction and the solvent was removed *in vacuo*. The crude was dissolved in THF together with neopentyl (2-nitro-4-((((4-nitrophenoxy) carbonyl)oxy) methyl) phenyl) sulfate **6** (543 mg, 1.12 mmol, 1 equiv.), before the addition of DIPEA (390 µL, 2.24 mmol, 2 equiv.). The reaction was stirred at rt overnight. The reaction was portioned between EtOAc and water and the organic phase was washed with 2x brine, dried over $MgSO_4$ and the solvent was removed *in vacuo*. The crude was purified by FCC, affording the title **7** in a yield of 78%.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.96 (s, 1H), 7.74 (dd, *J* = 14.1, 7.4 Hz, 3H), 7.58 (d, *J* = 6.4 Hz, 4H), 7.48 (d, *J* = 7.5 Hz, 1H), 7.40 (d, *J* = 7.8 Hz, 2H), 7.07 (d, *J* = 8.0 Hz, 1H), 6.94 – 6.86 (m, 1H), 5.35 (s, 1H), 5.07 (s, 2H), 4.72 (d, *J* = 4.5 Hz, 1H), 4.59 (d, *J* = 5.7 Hz, 1H), 4.22 (s, 6H), 3.36 (s, 1H), 3.29 (s, 1H), 2.90 (s, 1H), 2.78 (s, 1H), 1.03 (s, 11H)(broadening of peaks and rotamers observed).

^{13}C NMR (101 MHz, $CDCl_3$) δ 143.81, 141.54, 139.45, 137.31, 133.53, 129.42, 128.94, 127.90, 127.43, 127.22, 126.30, 125.17, 124.80, 124.20, 120.17, 120.05, 119.37, 115.70, 85.23, 67.49, 64.48, 50.41, 47.38, 46.45, 40.00, 32.06, 25.97.

HRMS (ESI) *m/z* found $[M+H]^+$ 759.2441 $C_{37}H_{39}N_6O_{10}S^+$ required 759.2443.

4-((((2-((4-azidobenzyl)amino)ethyl)carbamoyl)oxy)methyl)-2-nitrophenyl neopentyl sulfate (8)



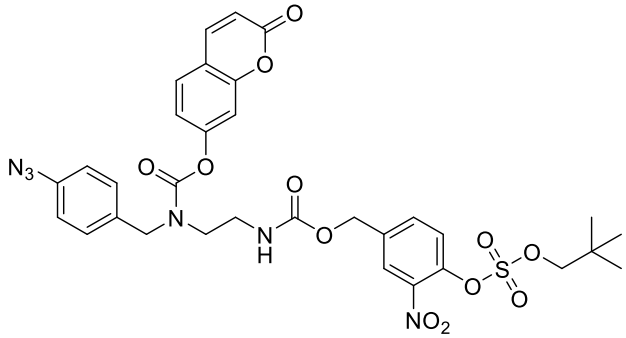
Compound **7** (146 mg, 193 µmol, 1 equiv.) was dissolved in 3 mL DCM under inert atmosphere and was added 1 mL diethylamine. Upon completion the solvent was removed, and the crude was purified by FCC affording compound **8** (86 mg, 83%)

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (s, 1H), 7.69 – 7.54 (m, 2H), 7.30 (d, *J* = 8.2 Hz, 2H), 6.98 (d, *J* = 8.3 Hz, 2H), 5.40 (t, *J* = 5.6 Hz, 1H), 5.14 (s, 2H), 4.22 (s, 2H), 3.78 (s, 2H), 3.32 (q, *J* = 5.8 Hz, 2H), 2.79 (t, *J* = 5.8 Hz, 2H), 1.03 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 155.64, 142.55, 141.61, 139.23, 137.49, 136.61, 133.64, 129.11, 127.19, 125.27, 122.86, 117.82, 113.86, 84.20, 65.01, 51.53, 47.59, 41.38, 32.53, 25.97.

HRMS (ESI) *m/z* found $[M+H]^+$ 537.1772 $C_{22}H_{29}N_6O_8S^+$ required 537.1762

4-((((2-((4-azidobenzyl)(((2-oxo-2H-chromen-7-yl)oxy)carbonyl)amino)ethyl)carbamoyl)oxy)methyl)-2-nitrophenyl neopentyl sulfate (S2)

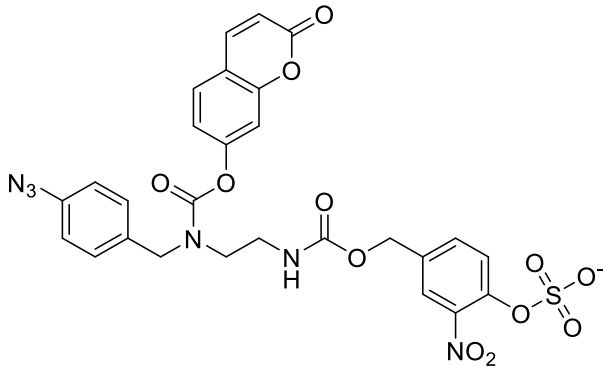


7-hydroxy-2H-chromen-2-one (76 mg, 466 µmol, 1 equiv.) and triphosgene (46 mg, 156 µmol, 0.34 equiv.) was dissolved in 5 mL dry THF and 5 mL dry DCM under inert atmosphere and triethylamine (170 µL, 932 µmol, 2 equiv.) was added dropwise. The reaction was stirred at rt for 1 h before 4-((((2-((4-azidobenzyl)amino)ethyl)carbamoyl)oxy)methyl)-2-nitrophenyl neopentyl sulfate **8**, (250 mg, 466 µmol, 1 equiv.) was added in 10 mL DCM. The reaction was stirred overnight and upon completion it was washed 2x with brine. The organic phase was dried over $MgSO_4$ and the solvent was removed in vacuo. The crude was purified by FCC affording compound **S2** in a yield of 30%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.13 (dd, *J* = 17.2, 2.1 Hz, 1H), 8.05 (t, *J* = 9.3 Hz, 1H), 7.84 (ddd, *J* = 18.5, 8.6, 2.1 Hz, 1H), 7.77 – 7.64 (m, 2H), 7.39 (dd, *J* = 12.0, 8.3 Hz, 2H), 7.30 – 7.06 (m, 4H), 6.46 (dd, *J* = 9.6, 3.3 Hz, 1H), 5.76 (s, 1H), 5.14 (d, *J* = 28.6 Hz, 2H), 4.55 (d, *J* = 44.9 Hz, 2H), 4.29 (d, *J* = 3.1 Hz, 2H), 3.43 (t, *J* = 6.0 Hz, 1H), 3.32 – 3.22 (m, 1H), 0.95 (d, *J* = 2.5 Hz, 9H). Rotamers observed for this compound.

HRMS (ESI) *m/z* found $[M+NH_4]^+$ 742.2136 [C32H36N7O12S+] required 742.2137

4-((((2-((4-azidobenzyl)(((2-oxo-2H-chromen-7-yl)oxy)carbonyl)amino)ethyl)carbamoyl)oxy)methyl)-2-nitrophenyl sulfate (10)



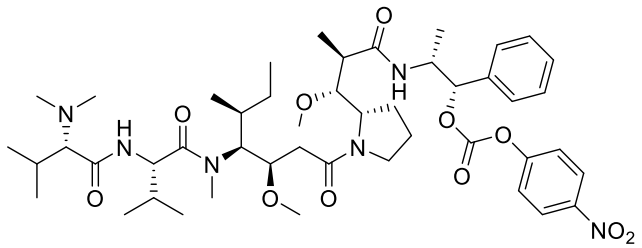
Carbamate **S2** (50.2 mg, 69.4 µmol) was dissolved in DMF (1.2 mL) and treated with 5 M NH_4OAc (aq) (0.8 mL) before stirring at 50 °C for 2 days. The cooled reaction was purified directly by reverse phase flash column chromatography (20-50% solvent B in solvent A. Solvent A: 0.1 M NH_4OH (aq). Solvent B: MeCN) and lyophilized to yield sulfate **10** (34.1 mg, 52.1 µmol, 75%) as a white solid.

^{1}H NMR (400 MHz, MeOD) δ 7.87 (dd, *J* = 9.6, 3.1 Hz, 1H), 7.82 – 7.67 (m, 1H), 7.65 – 7.41 (m, 2H), 7.31 (d, *J* = 8.4 Hz, 2H), 7.19 – 6.91 (m, 3H), 6.32 (dd, *J* = 9.5, 2.7 Hz, 1H), 5.03 (d, *J* = 35.3 Hz, 2H), 4.55 (d, *J* = 28.7 Hz, 2H), 3.53 – 3.38 (m, 2H), 3.35 (t, *J* = 5.7 Hz, 2H).

^{13}C NMR (101 MHz, MeOD) δ 162.62, 158.48, 156.05, 155.59, 155.30, 145.81, 145.35, 144.38, 140.74, 135.49, 135.27, 133.70, 130.67, 130.18, 125.32, 124.99, 120.28, 119.75, 117.84, 116.11, 111.01, 65.77, 51.34, 47.51, 39.96.

HRMS (ESI) *m/z* found $[M]^-$ 653.0950 $C_{27}H_{22}N_6O_{12}S^-$ required 653.0944

AuristatinE -pnp (11)

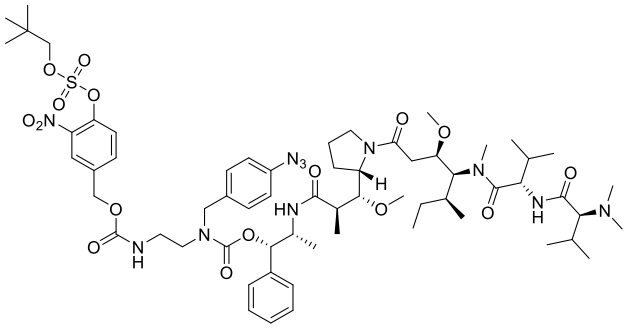


In a small vial, a solution of auristatin E (53 mg, 72 µmol) in anhydrous DMF (1 mL) was added triethylamine (40 µL, 0.29 mmol) and bis(4-nitrophenyl) carbonate (66 mg, 0.22 mmol) and the reaction was stirred over night at room temperature. The reaction was concentrated and subjected to flash chromatography (100% EtOAc to Teac/5% MeOH) to give **11** as a clear sticky oil (27 mg, 42%).

LCMS (ESI) *m/z* found $[M+H]^+$ 897.5 $C_{47}H_{73}N_6O_{11}$ required 897.5

Data in accordance with literature[3]

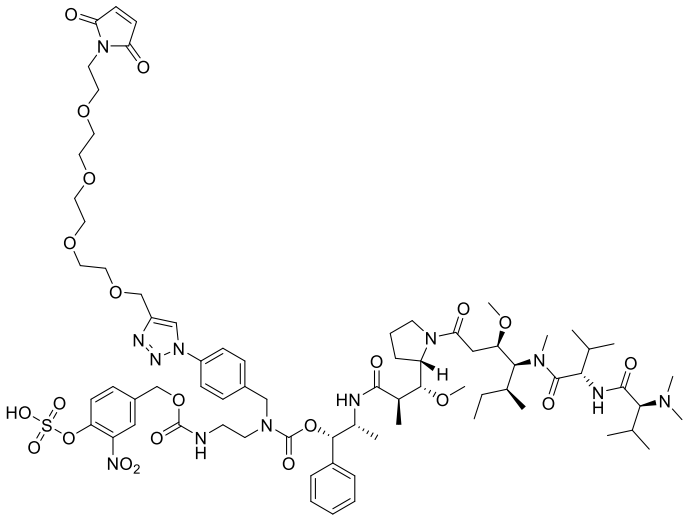
Neopentyl arylsulfate-linker-Auristatin E (12)



In a microwave vial, a solution of **AuristatinE-pnp 11** (27 mg, 30 µmol) in anhydrous CH_2Cl_2 (0.2 mL) was added triethylamine (13 uL, 75 µmol) followed by amine **8** (20 mg, 36 µmol), the vial was sealed and heated to 40 °C for 72 h. The reaction was concentrated and used without further purification in the next step.

HRMS (ESI) *m/z* found $[M+Na]^+$ 1316.6572 Calc 1316.6571

Neopentyl arylsulfate-linker-Auristatin E (13)



In a microwave vial, **12** (38 mg, 30 µmol) was dissolved in 300 uL DMF under inert atmosphere and 150 µL 1.5 M aqueous NH_4OAc was added dropwise. The reaction was stirred overnight at rt and was added 150 µL 1.5 M aqueous NH_4OAc the following day. The reaction was stirred overnight before being added 900 µL DMF and 200 µL 1.5 M aqueous NH_4OAc. The reaction was stirred for an additional 5 h before the solvent was removed under a stream of N_2. The crude was dissolved in *t*-BuOH/water (1:2, 0.17 mL) in a microwave vial, (36 mg, 30 µmol) and was added aqueous solutions of CuSO4.5H2O (50 mM, 30 µL) and sodium ascorbate (100 mM, 36 µL) followed by alkyne maleimide in DMSO (2.0 M, 5 µL). The reaction was degassed and stirred overnight at room temperature under protection of light. The reaction was filtered and subjected to preparative HPLC (MeCN/water) to give **13** as a white powder after lyophilization (5.7 mg, 12%).

HRMS (ESI) *m/*z found $[M]^-$ 1533.7189 $C_{73}H_{105}N_{12}O_{22}S^-$ 1533.7193.

Enzyme assay

For the enzyme assay, compound **10** was prepared in a 100 µM solutions in DMSO stock. Lyophilized sulfatase (*Helix pomatia*, EC 3.1.6.1, purchased from Sigma Aldrich) was prepared in a 3 U/mL NaOAc buffer (0.1 M, pH 5.0) stock. 10 µL substrate stock, 180 µL NaOAc buffer (0.1 M, pH 5.0) and 10 µL enzyme stock was added to black flat back microtiter plate with 96 wells. Control wells contained 190 µL NaOAc buffer (0.1 M, pH 5.0) and 10 µL substrate stock. The results were recorded at 37 °C in 5 min intervals of shaking over 20 h, λ_{ex} 330 nm and λ_{em} 460 nm. All assays were carried out in triplicate on an Infinite M200 PRO Tecan instrument plate reader and the plates were covered with a Thermo Scientific™ Nunc™ Sealing tape product nr 232701 to prevent solvent evaporation.

Serum stability of 10

The serum stability of **10** was determined by fluorescence spectroscopy. 10 µL of a 100 µM stock of **10** in DMSO was added to a black flat back microtiter plate with 96 wells and 190 µL human serum (from human male AB plasma, USA origin, sterile-filtered, Sigma Aldrich) was added. Control wells were added 190 µL demineralized water and 10 µL 100µM stock of **10** in DMSO. The results were recorded at 37 °C in 5 min intervals of shaking over 20 h, λ_{ex} 330 nm and λ_{em} 460 nm. All assays were carried out in triplicate on an Infinite M200 PRO Tecan instrument plate reader and the plates were

covered with a Thermo Scientific™ Nunc™ Sealing tape product nr 232701 to prevent solvent evaporation. For serum measurements, background blank serum measurements were subtracted.

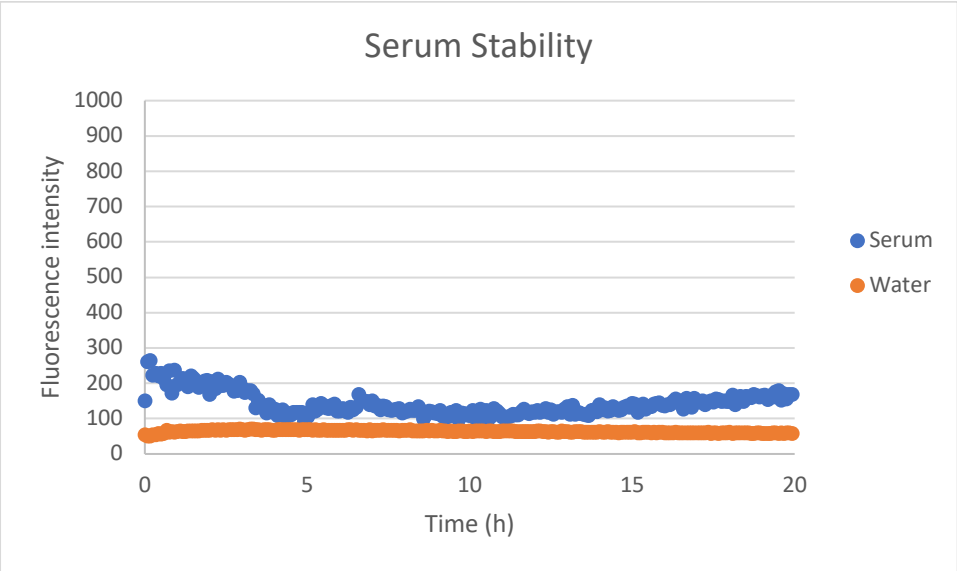


Figure S1: Serum stability assay

Bioconjugation

Conjugation of linker-payload **13** to Trazimera: To a solution of Trazimera (6.07 mg/mL) in reaction buffer (20 mM sodium phosphate, pH 7.5, 150 mM NaCl, 20 mM EDTA) TCEP was added (2 equivalents with respect to mAb). Antibody concentration was adjusted to 5.0 mg/mL with reaction buffer and the resulting mixture incubated at 40 °C for 1 h. Reduced Trazimera was cooled to room temperature. Linker-payload **13** was dissolved in DMF (6.5 mM) and the reagent (10 equivalents with respect to mAb, 10% DMF v/v) was added to the reduced Trazimera solution and the resulting conjugation reaction was carefully mixed and incubated at 22 °C for 1 h. The conjugation mixture was buffer-exchanged into PBS, pH 7.2 by gel filtration with a PD-10 column and the sample was concentrated using centrifugal concentrators (Vivaspin 20, 30,000 kDa, MWCO PES membrane). The sample was sterile filtered through 0.2 µm pore size syringe filter.

Size-Exclusion Chromatography

Analytical SEC was carried out using an ACQUITY UPLC BEH 200 SEC column (4.6 mm x 15 cm, 200 Å, 1.7 µm) equipped with a guard column (4.6 mm x 3 cm) and connected to a Dionex Ultimate 3000 HPLC system. The mobile phase was 0.2 M potassium phosphate buffer, pH 6.8, 0.2 M potassium chloride, 15% (v/v) isopropanol and the flow rate were kept at a constant 0.35 mL/min. The column was maintained at 30 °C throughout the analysis. The analysis was carried out in a 10 min isocratic elution with UV detection at 248 and 280 nm.

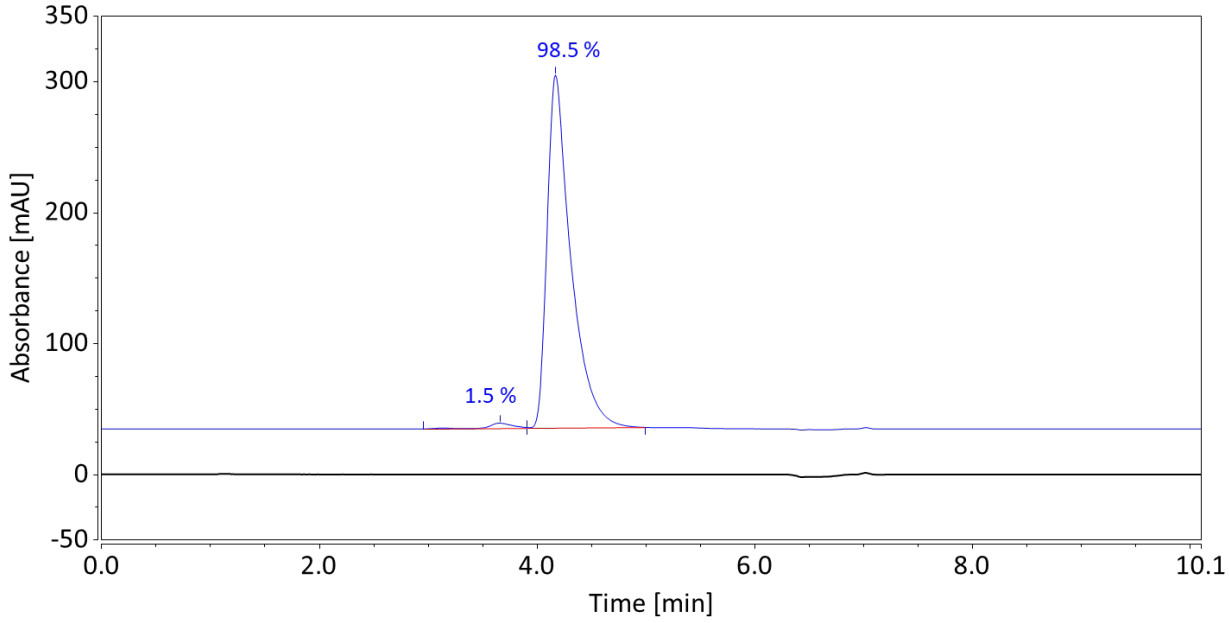


Figure S2: Size exclusion chromatogram at 280 nm of ADC1 (blue trace) and PBS blank (black trace)

Hydrophobic Interaction Chromatography (HIC)

Analytical HIC was carried out using a TOSOH, TSKgel Butyl-NPR column (4.6 mm x 3.5 cm, 2.5 µm) connected to a Dionex Ultimate 3000RS HPLC system. The method consisted of a linear gradient from 100% buffer A (1.5 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0) to 100% buffer B (20% isopropanol (v/v) in 50 mM sodium phosphate, pH 7.0) over 10.5 min at a flow rate of 1.35 mL/min. The column temperature was maintained at 30 °C throughout the analysis and UV detection was carried out at 280 nm. For each analysis an injection of 10 µg of antibody or ADC was carried out.

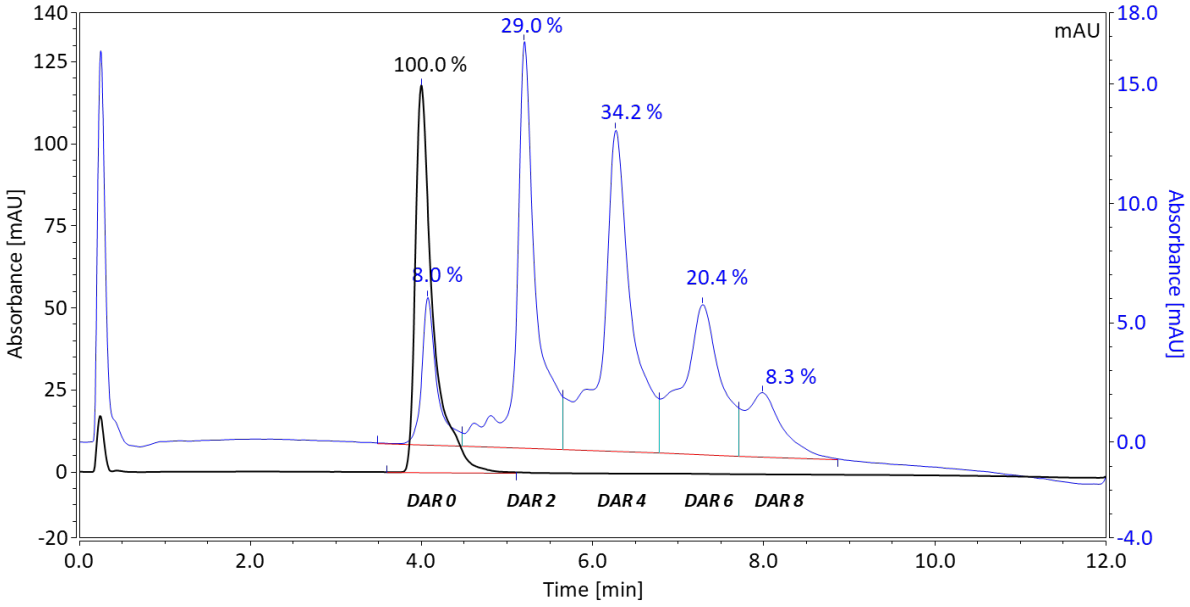


Figure S3: Hydrophobic interaction chromatograms at 280 nm of Trazimera (black trace) and ADC1 (blue trace)

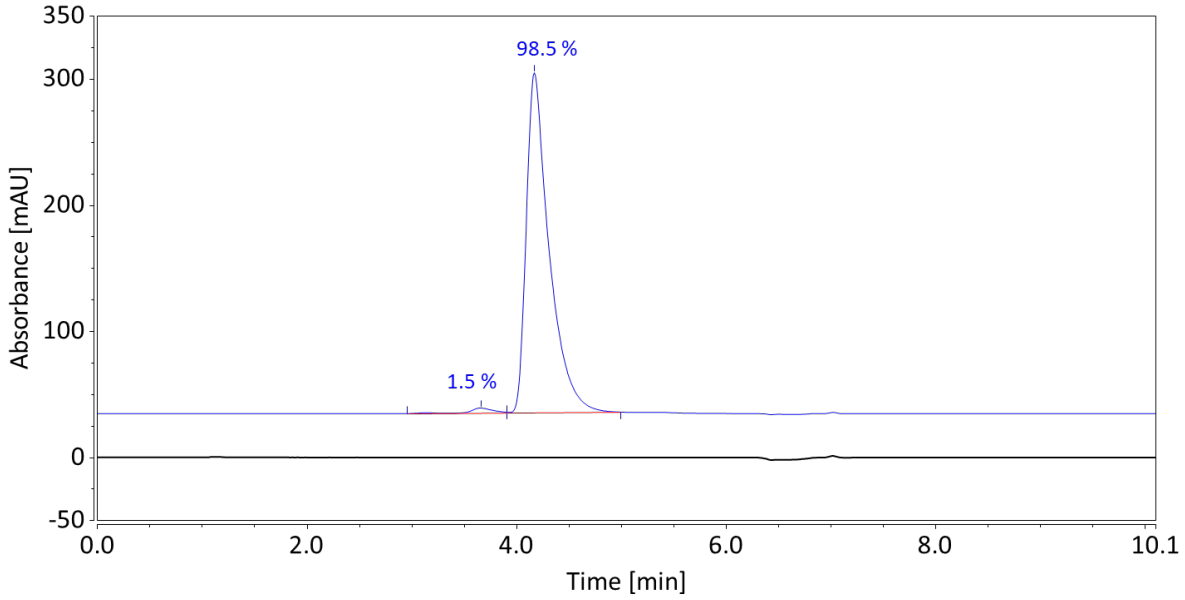


Figure S4: Size exclusion chromatogram at 280 nm of ADC1 (blue trace) and PBS blank (black trace)

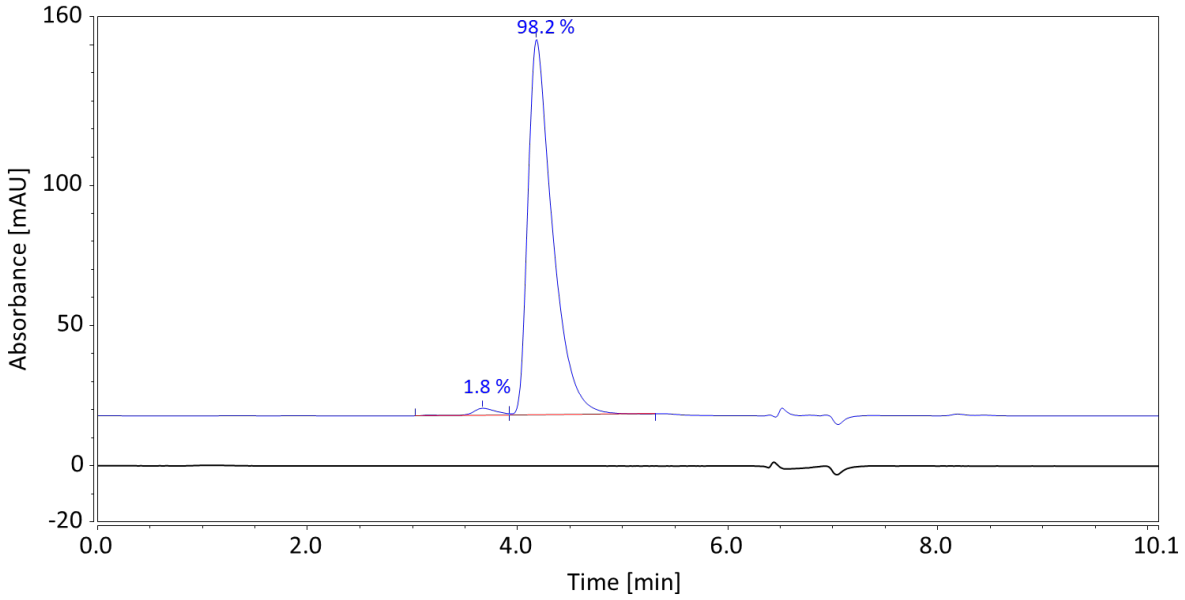


Figure S5: Size exclusion chromatogram at 248 nm of ADC1 (blue trace) and PBS blank (black trace)

Reverse phase chromatography electrospray ionization mass spectrometry (RP-HPLC-ESI-MS)

Protein RP-HPLC-ESI-MS was performed on a Xevo G2-S TOF mass spectrometer coupled to an Acquity UPLC system using an Acquity UPLC BEH300 C4 column (1.7 µm, 2.1 × 50 mm). H_2O with 0.1% formic acid (solvent A) and 95% MeCN and 5% H_2O with 0.1% formic acid (solvent B) were used as the mobile

phase at a flow rate of 0.2 mL/min. The gradient was programmed as follows: 95% A for 0.93 min, then a gradient to 100% B over 4.28 min, then 100% B for 1.04 minutes, then a gradient to 95% A over 1.04 min. The electrospray source was operated with a capillary voltage of 2.0 kV and a cone voltage of 190 V, 150V or 40 V. Nitrogen was used as the desolvation gas at a total flow rate of 850 L/h. Total mass spectra were reconstructed from the ion series using the MaxEnt 1 algorithm preinstalled on MassLynx 4.2 software according to the manufacturer's instructions. Trastuzumab samples were deglycosylated with PNGase F (New England Biolabs) prior to LC-MS analysis. Only the region of the total ion chromatogram (TIC) between 3.25-3.75 min was analyzed. Peaks outside of this range did not contain proteinogenic signals and were excluded. Analysis was conducted in the same way for all protein LC-MS traces.

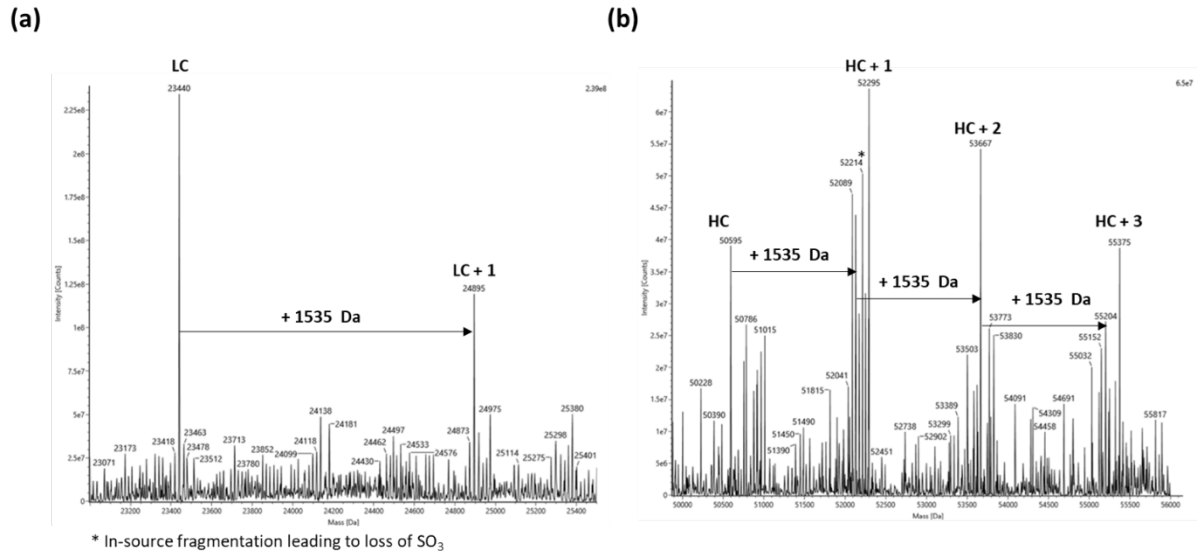


Figure S6: Deconvoluted LC-MS data for reduced ADC1: (a) light chain species; (b) heavy chain species

Ex vivo stability in mouse and human sera

The ADC1 conjugate was diluted into 50% IgG depleted serum from mouse (Molecular innovations) and human (BBI) in PBS, to a final concentration of 1 mg/mL. The mixtures were split into equal aliquots. Aliquots of 50% IgG depleted serum from mouse and human in PBS were also prepared as controls. For each type of serum, one aliquot of conjugate in serum and one serum control aliquot were immediately frozen to -80 °C. The remaining samples were transferred to an incubator and maintained at 37 °C. For each type of serum, aliquots of conjugate in serum and serum control aliquots were removed from the incubator and frozen to -80 °C after 24 h, 48 h and 96 h incubation. After the final time point, aliquots were directly analyzed by hydrophobic interaction chromatography using a TOSOH, TSKgel Butyl-NPR column (4.6 mm x 10 cm, 2.5 µm) connected to a Dionex Ultimate 3000RS HPLC system. The method consisted of a linear gradient from 90% buffer A (1.5 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0) to 100% buffer B (20% isopropanol (v/v) in 50 mM sodium phosphate, pH 7.0) over 18 min at a flow rate of 0.6 mL/min. The column temperature was maintained at 30 °C throughout the analysis and UV detection was carried out at 280 nm. For each analysis an injection of 10 µg of ADC was carried out. For mouse serum samples, each

conjugate containing serum sample chromatogram was corrected subtracting the chromatogram of the serum control at the corresponding timepoint.

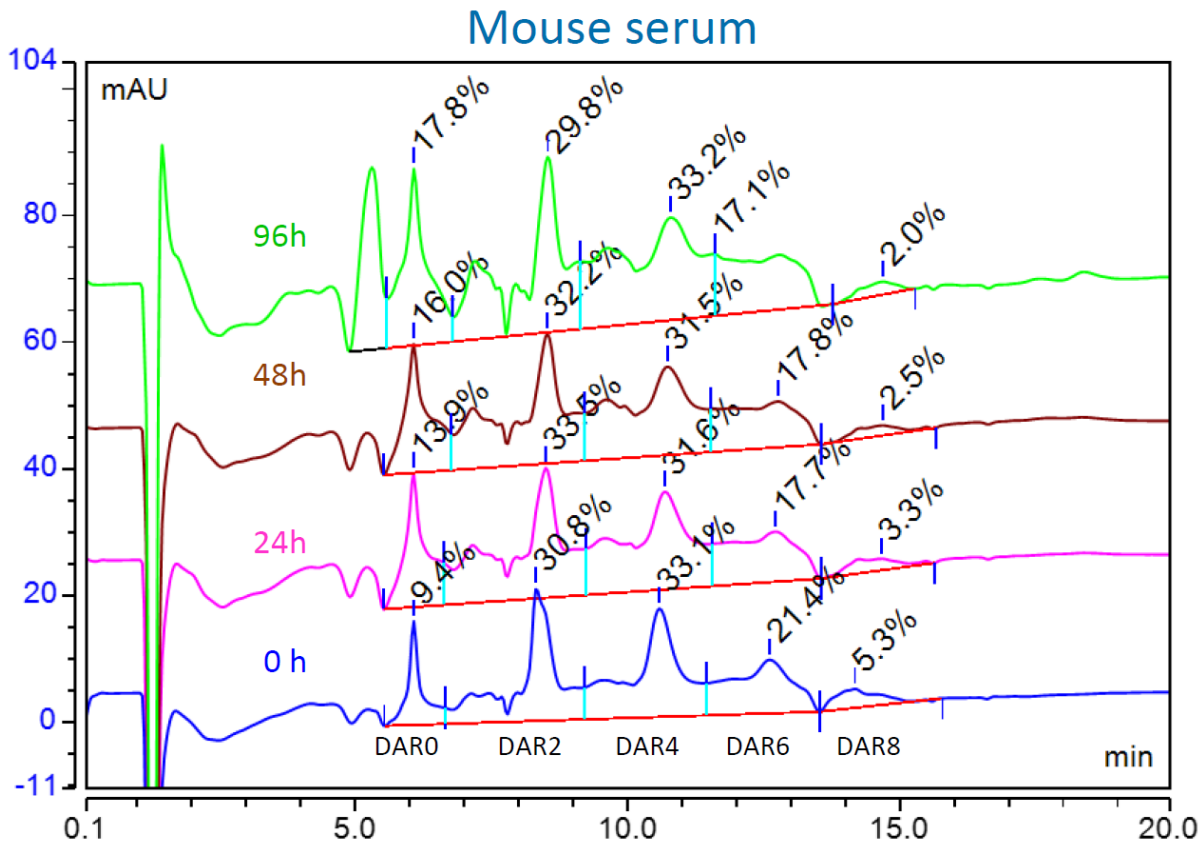


Figure S7: Hydrophobic interaction chromatograms at 280 nm of ADC1 incubated at 37 °C in IgG-depleted mouse serum for 0 h, 24 h, 48 h & 96 h.

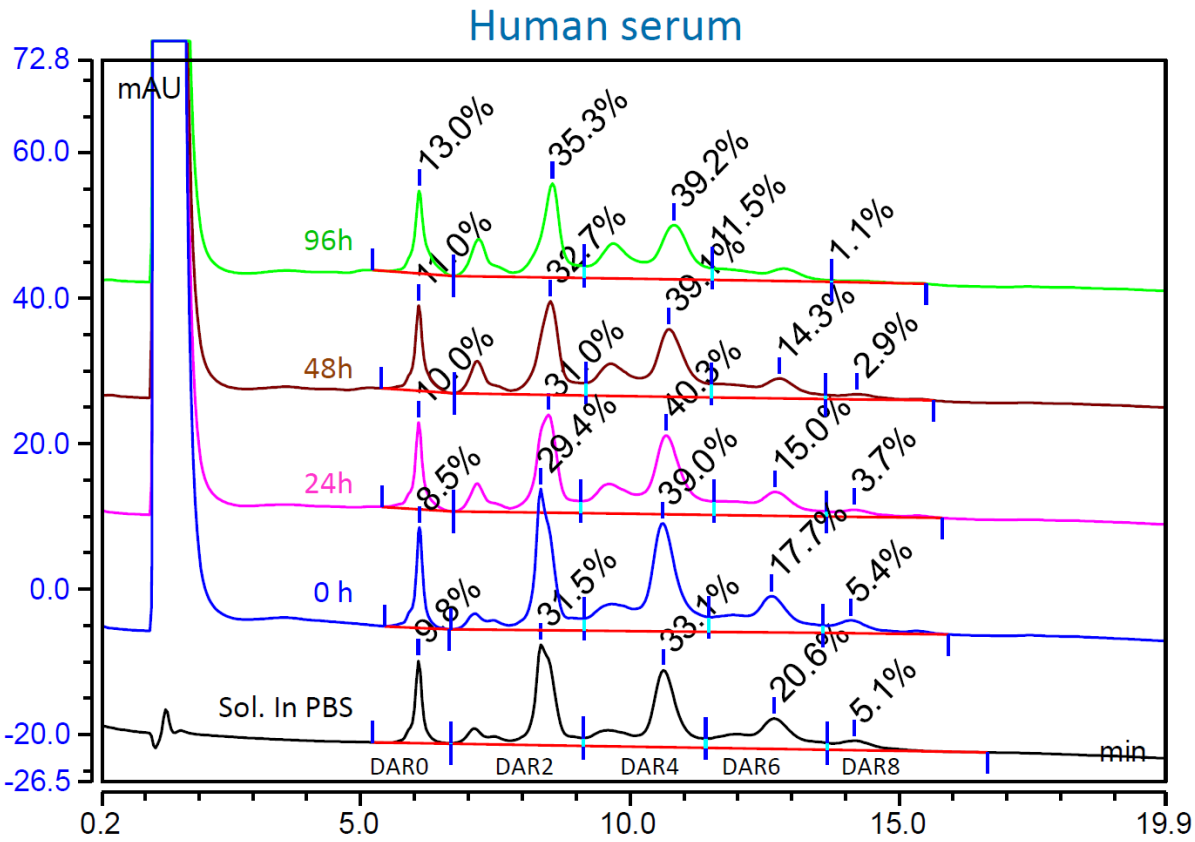


Figure S8: Hydrophobic interaction chromatograms at 280 nm of ADC1 incubated at 37 °C in IgG-depleted human serum for 0 h, 24 h, 48 h & 96 h.

In Vitro Cytotoxicity

The CellTiter-Glo® luminescence viability assay (Promega, Southampton, UK) was used to measure the inhibitory effect of the conjugates on cell growth. Any reduction in cell proliferation or metabolic activity is indicative of the cytotoxic and/or cytostatic properties of a compound.

SK-BR-3 cells (human breast adenocarcinoma, ATCC HTB-30) were cultured in McCoys 5A media (ThermoFisher Scientific, Loughborough, UK) supplemented with 200 U/mL penicillin, 200 µg/mL streptomycin and 20% heat-inactivated fetal bovine serum (Cytiva Hyclone™, ThermoFisher Scientific, Loughborough, UK). BT-474 cells (human ductal breast carcinoma,) were cultured in DMEM/F-12, HEPES media (ThermoFisher Scientific, Loughborough, UK) supplemented with 200 U/mL penicillin, 200 µg/mL streptomycin and 10 % heat-inactivated fetal bovine serum (Sigma-Aldrich, Poole, UK).

SK-BR-3 ($HER2^{HIGH}$), BT-474 ($HER2^{POS}$) cells were seeded in 384-well plates at a density of 1.25 x10^3 cells in 20 µL growth medium. 3x 384 well plates were prepared for each cell line to allow for 96h and 168h incubation timepoints. These were then incubated for 24 hours at 37 °C, 5% CO_2. After 24 hours, 20 µL 2 x serial dilutions of test samples (drug conjugate or free payload control) in growth medium was added. The optimized titration ranges are summarized in Table S1:

Sample	SK-BR-3	BT-474
ADC1	20 nM – 4x serial dilution	20 nM – 4x serial dilution

Kadcyla	20 nM – 4x serial dilution	100 nM – 4x serial dilution
Trazimera	20 nM – 4x serial dilution	20 nM – 4x serial dilution

Table S1: Optimized titration ranges

Viability was detected using the CellTiter-Glo® luminescence assay. Assay plates were equilibrated at room temperature for 20 minutes before addition of 40 µL CellTiter-Glo® reagent (prepared according to supplier's recommendation) per well. The plates were then mixed for 3 minutes at 300 rpm to assist cell lysis and incubated for a further 20 minutes at room temperature to stabilize the luminescence signal. Luminescence was recorded using a SpectraMax i3x plate reader (Molecular Devices, Wokingham, UK), with a default integration time of 0.5 s/well. Viability data was collected at 96 and 168-hour timepoints via the same procedure.

MCF-7 cells (human breast adenocarcinoma) were used to evaluated the anti-proliferation effect of the conjugate on HER2 negative cells. cultured in DMEM media (ThermoFisher Scientific, Loughborough, UK) supplemented with 200 U/mL penicillin, 200 µg/mL streptomycin and 10% heat-inactivated fetal bovine serum. For the assay, cells were seeded in 96-well plates at a density of 5 x10^3 cells per well in 80 µL of growth medium. 2x 96 well plates were prepared for each cell line to allow for 96h and 168h incubation timepoints. Cells were incubated for 24 hours at 37 °C, 5% CO_2. After 24 hours, 20 µL 5 x serial dilutions (100, 25, 6.25, 1.56, 0.4, 0.1, 0.025 nM) of test samples in growth medium were added (each sample was tested in triplicates, n=3). Cell viability was detected using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacture´s protocol. Briefly, 20µl of the CellTiter 96® AQueous One Solution Reagent was added to each well containing cells, and culture media was used as the background. Subsequently, the plates were incubated at 37°C for 2 hours in a humidified, 5% CO2 atmosphere. Following that, the absorbance at 490nm was recorded using a Tecan 96-well plate reader for calculating the cell viability with the equation below:

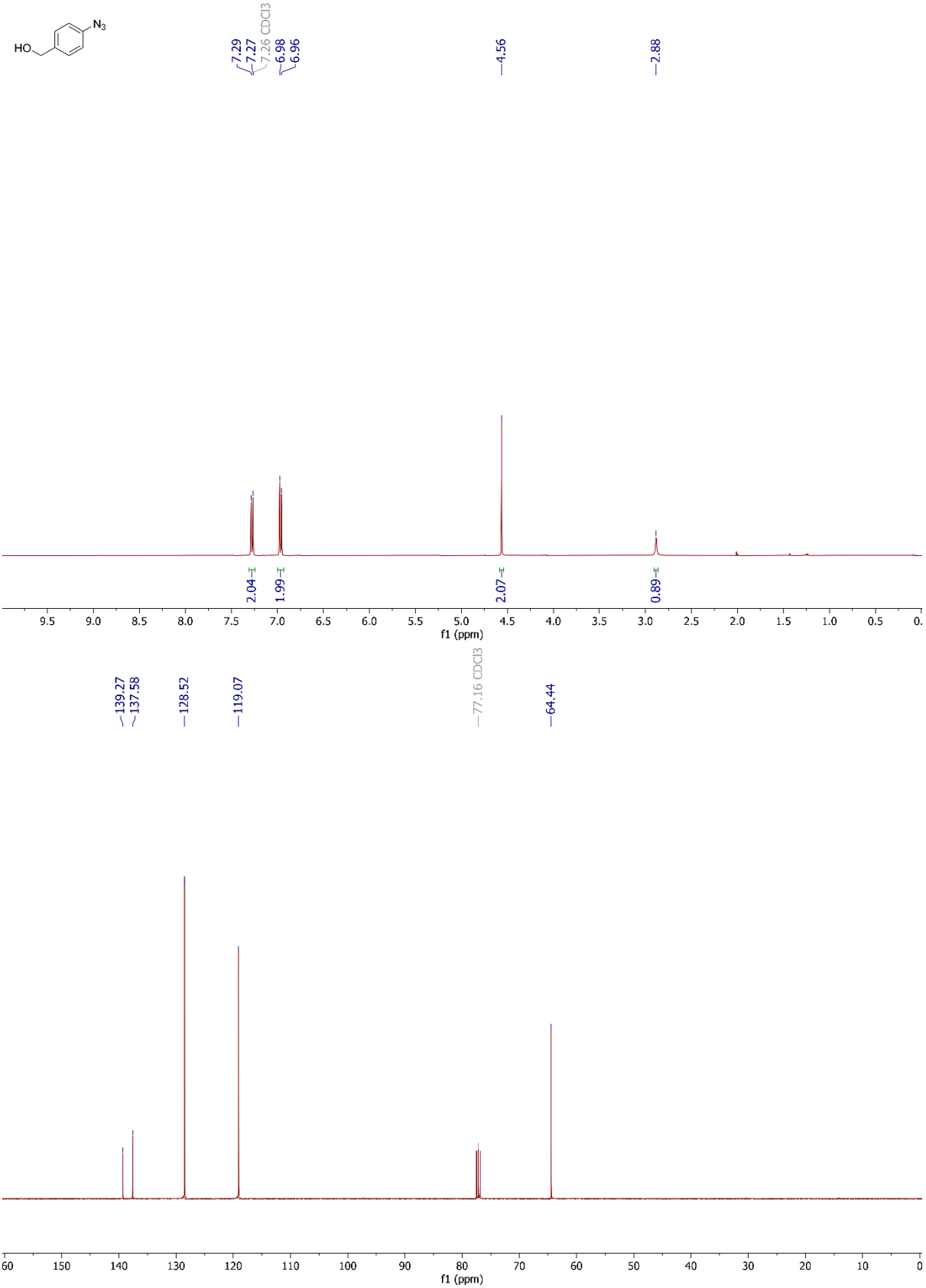
% viability =[(OD (sample) – OD (background) / OD (untreated) – OD (background)] x 100%

References

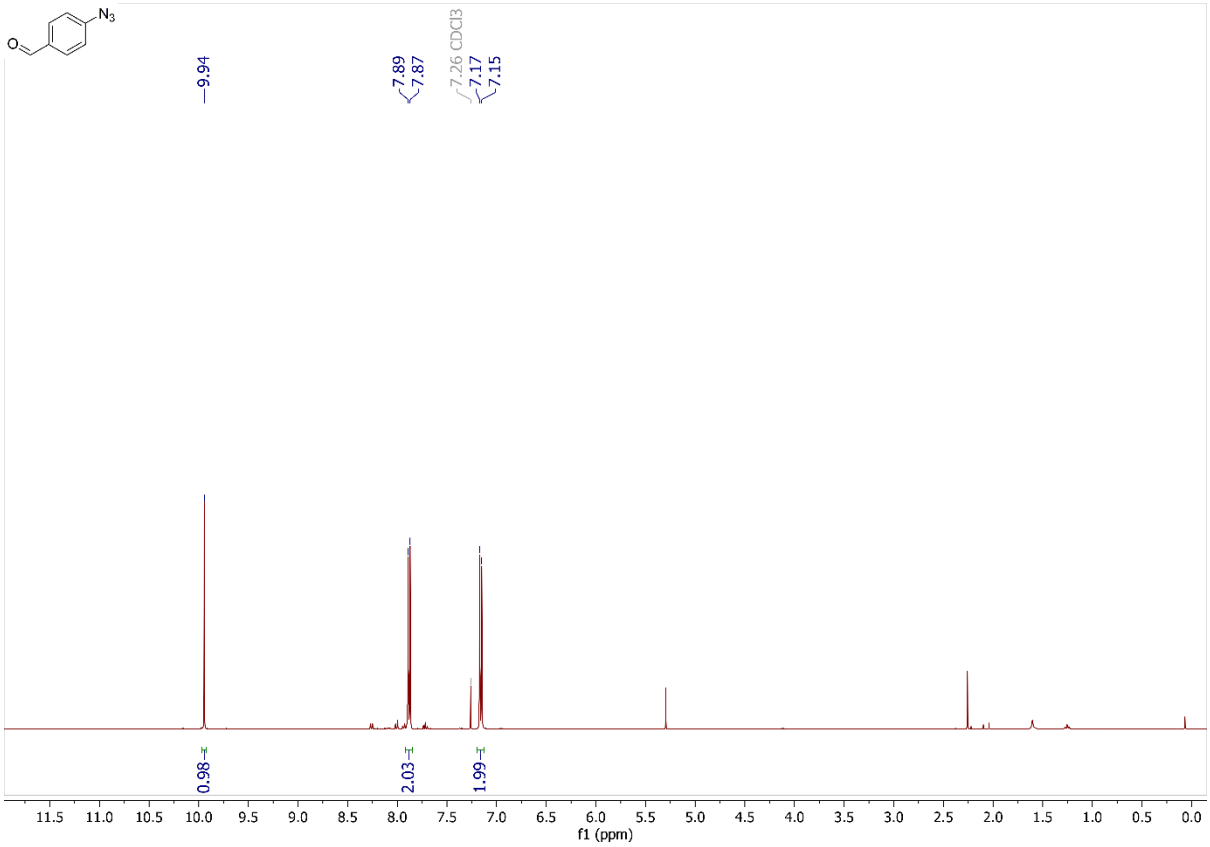
1. Griffin, R. J. *et al.* The 4-azidobenzyloxycarbonyl function; application as a novel protecting group and potential prodrug modification for amines. *J. Chem. SOC., Perkin Trans. 1* 1205–1211 (1996).
2. Chen, T. *et al.* A red emission fluorescent probe for hydrogen sulfide and its application in living cells imaging. *Tetrahedron Lett.* **54**, 2980–2982 (2013).
3. Han, N. *et al.* Derivatives of Dolastatin 10 and Uses Thereof. (2016).

NMR Spectra

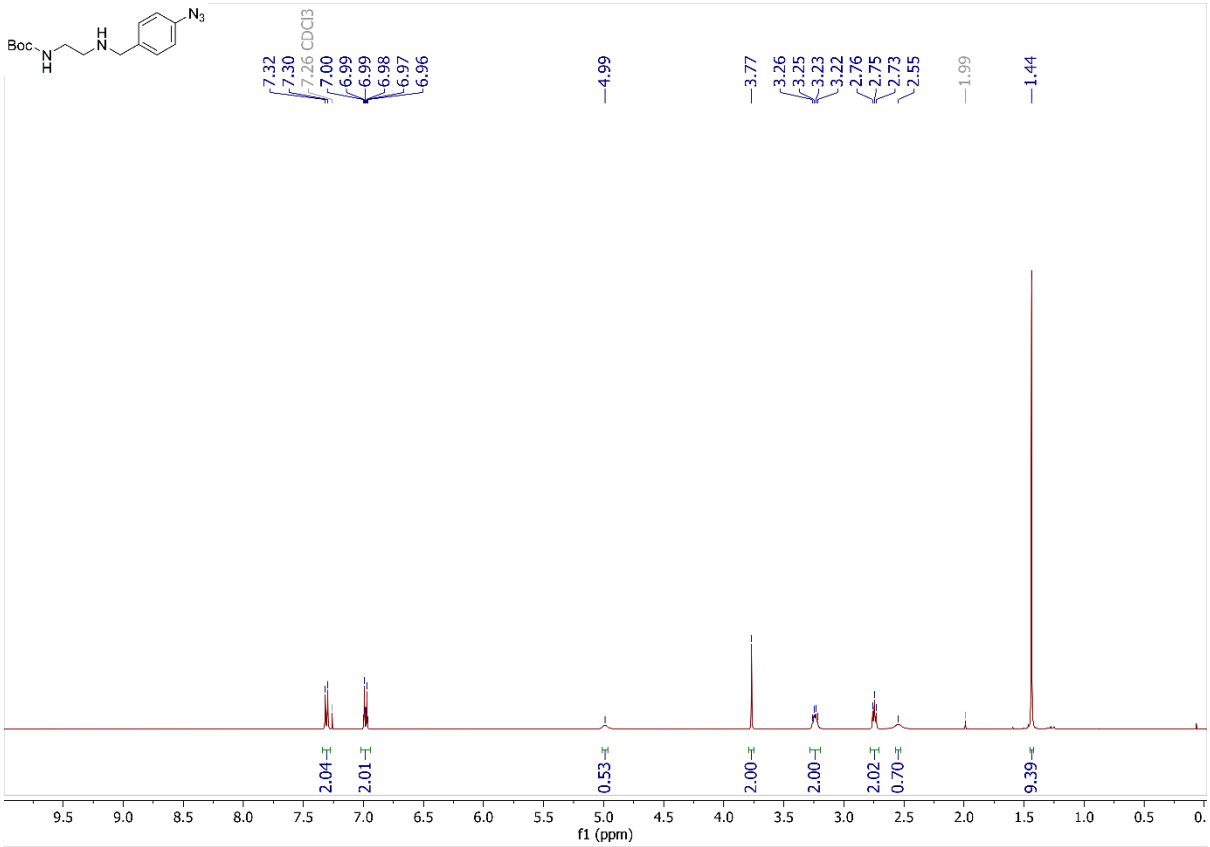
(4-Azidophenyl)methanol (S1)

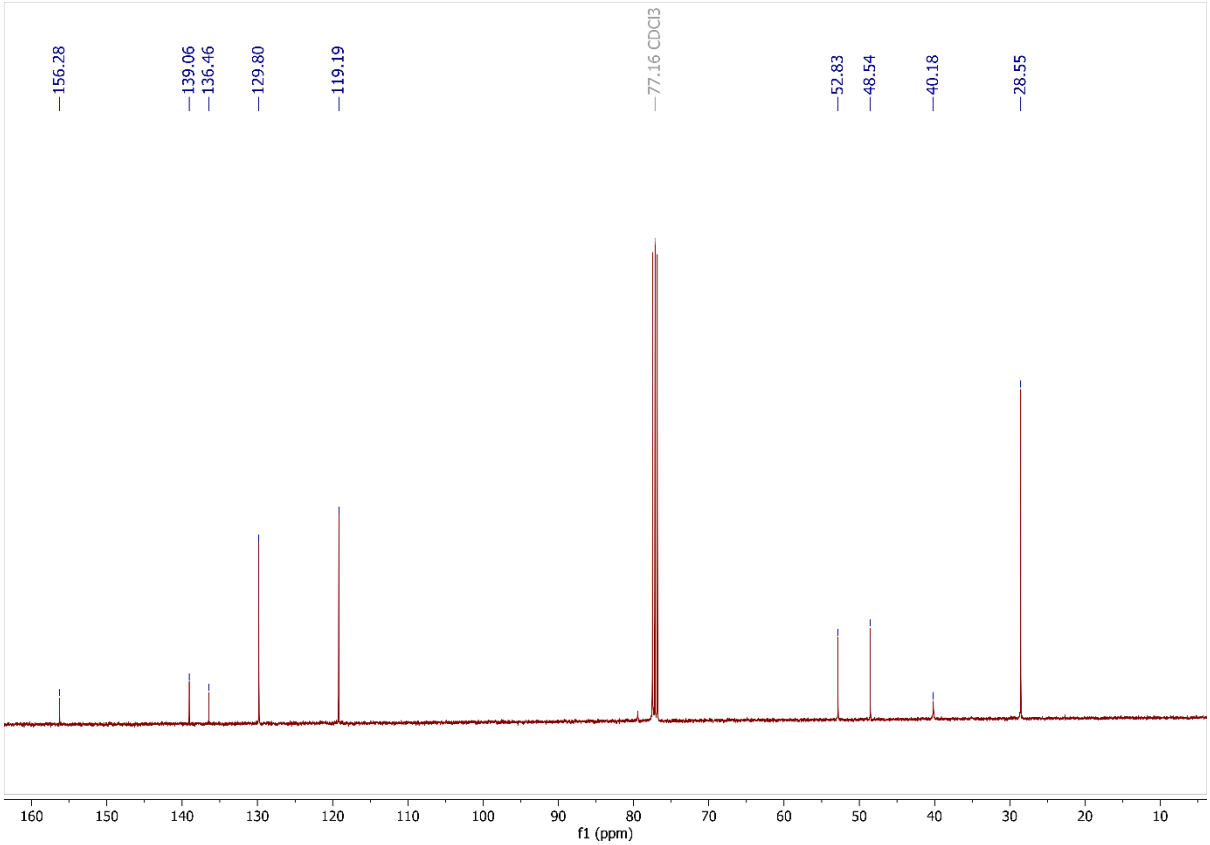


4-Azidobenzaldehyde (1)

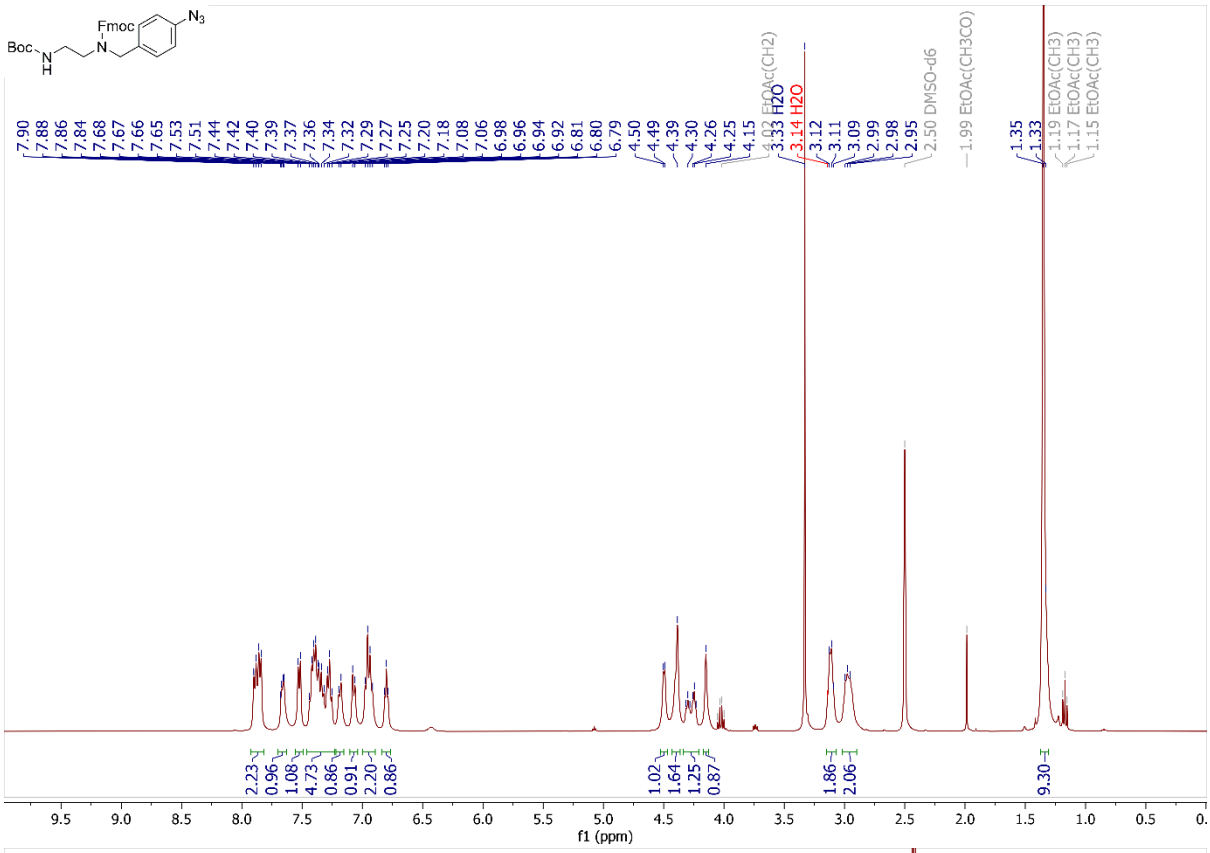


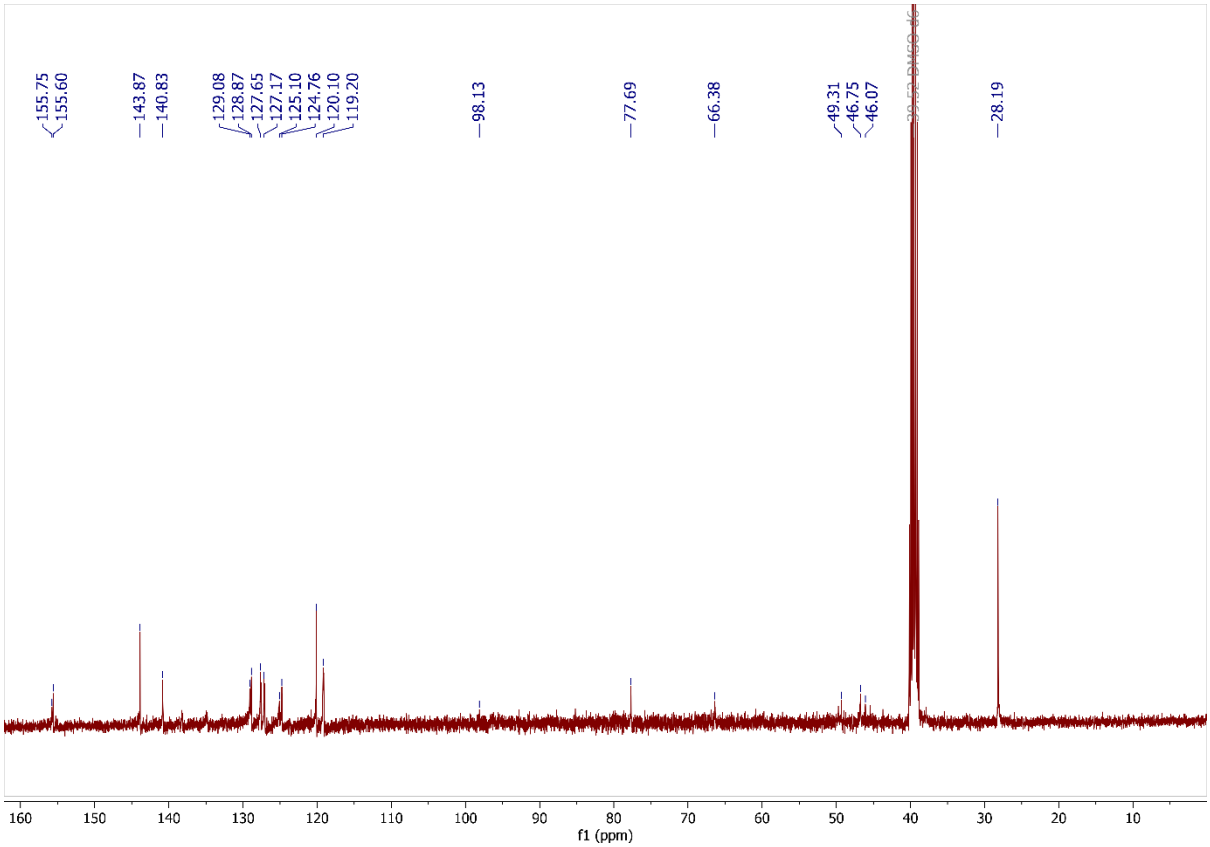
Tert-butyl (2-((4-azidobenzyl)amino)ethyl)carbamate (2)



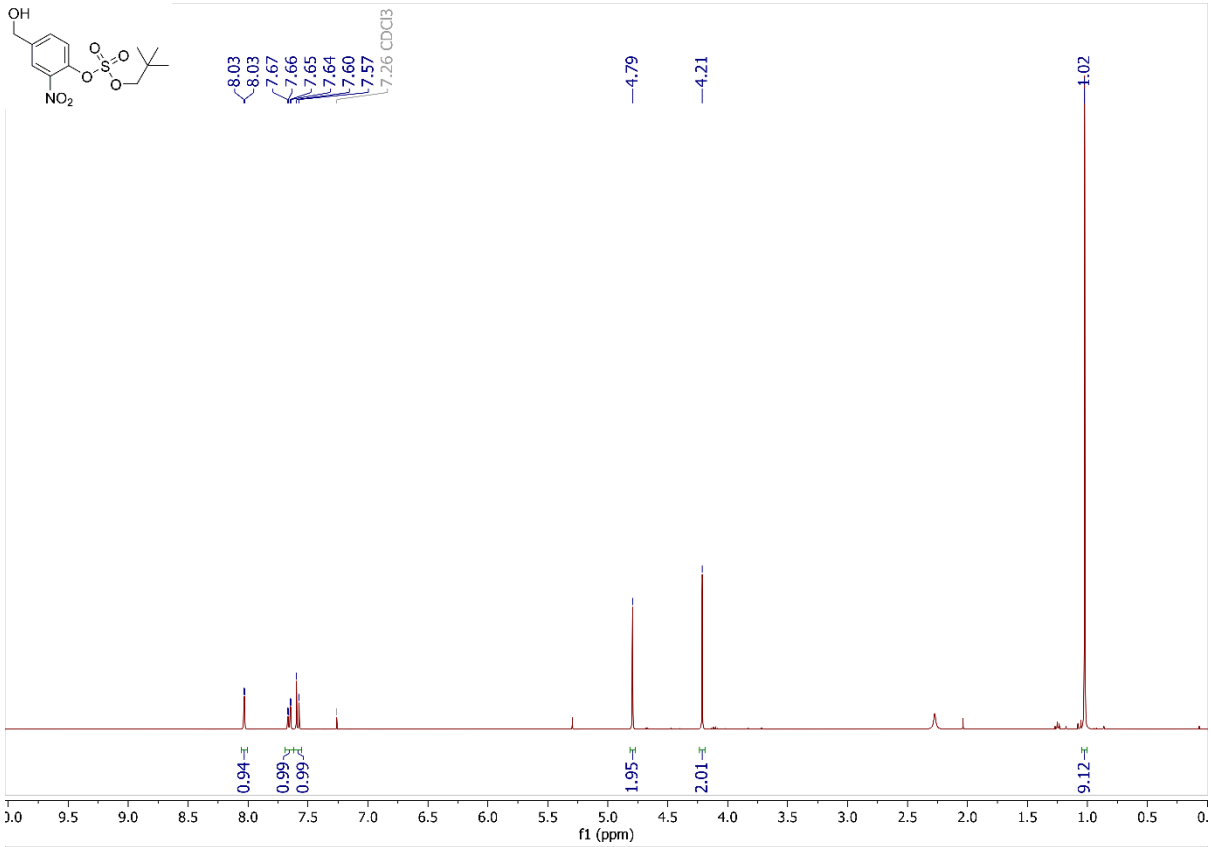


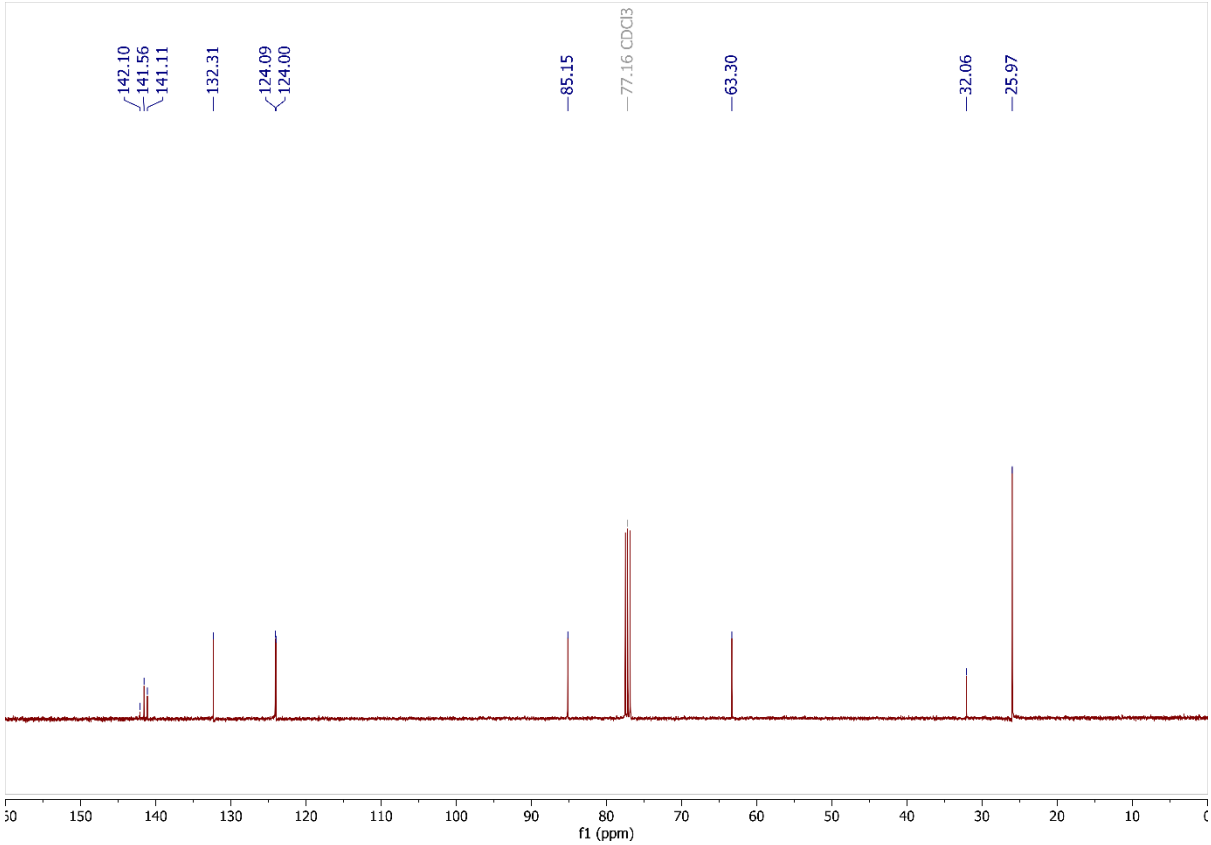
(9*H*-Fluoren-9-yl)methyl (4-azidobenzyl)(2-((*tert*-butoxycarbonyl)amino)ethyl) carbamate (3)



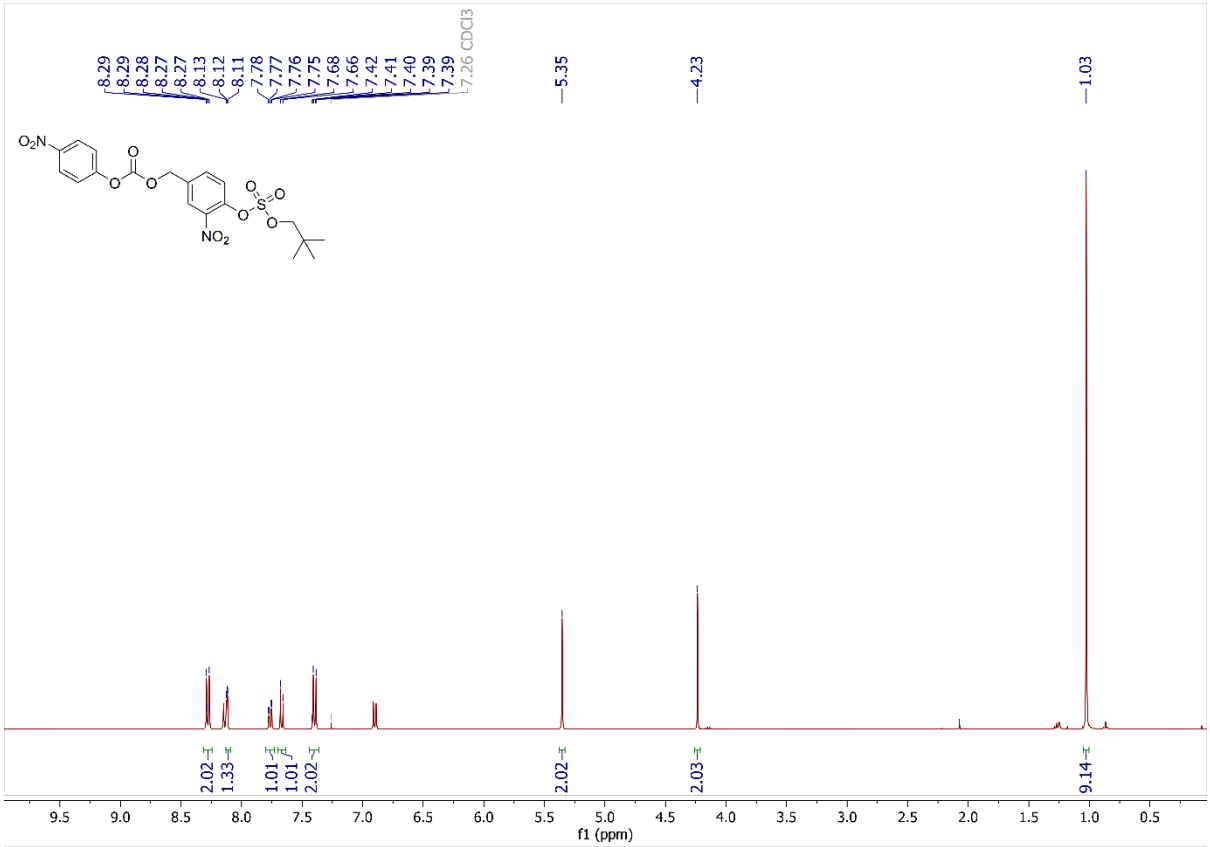


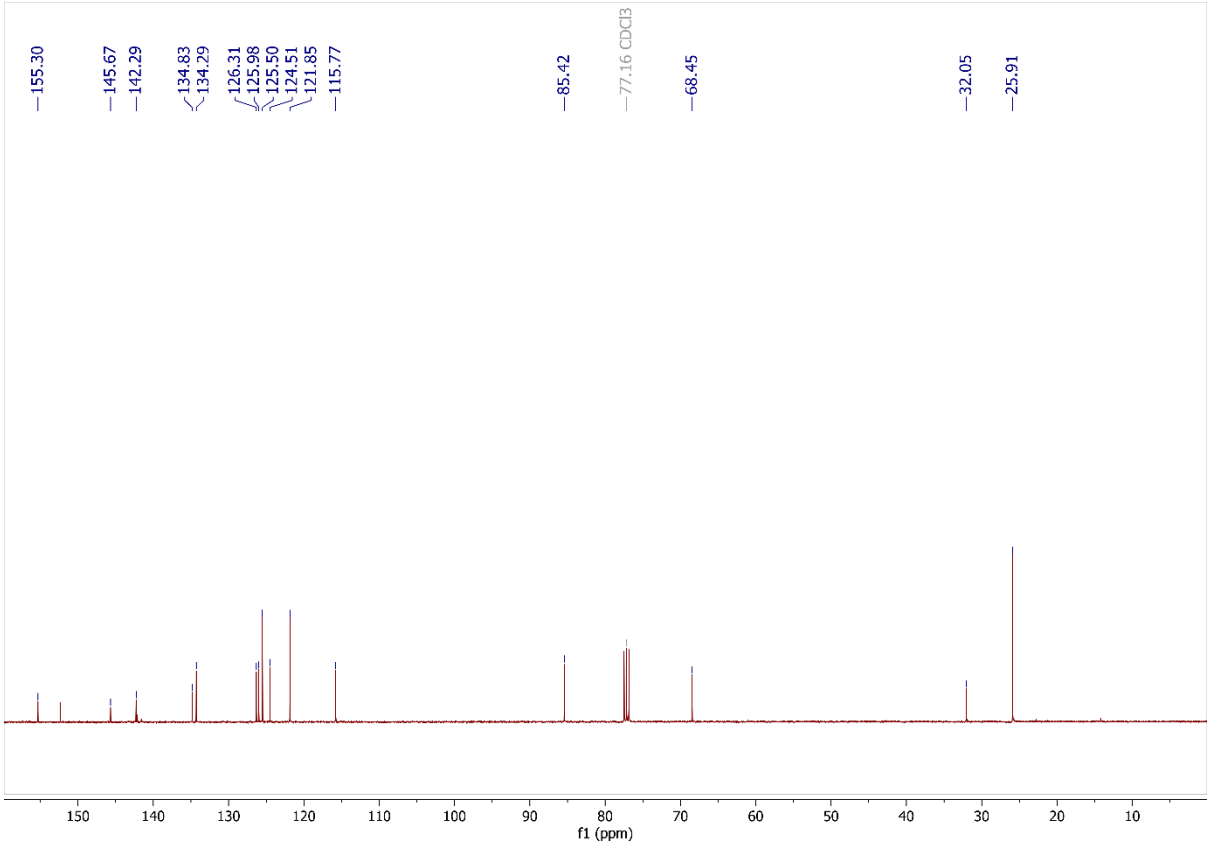
4-(hydroxymethyl)-2-nitrophenyl neopentyl sulfate (5)



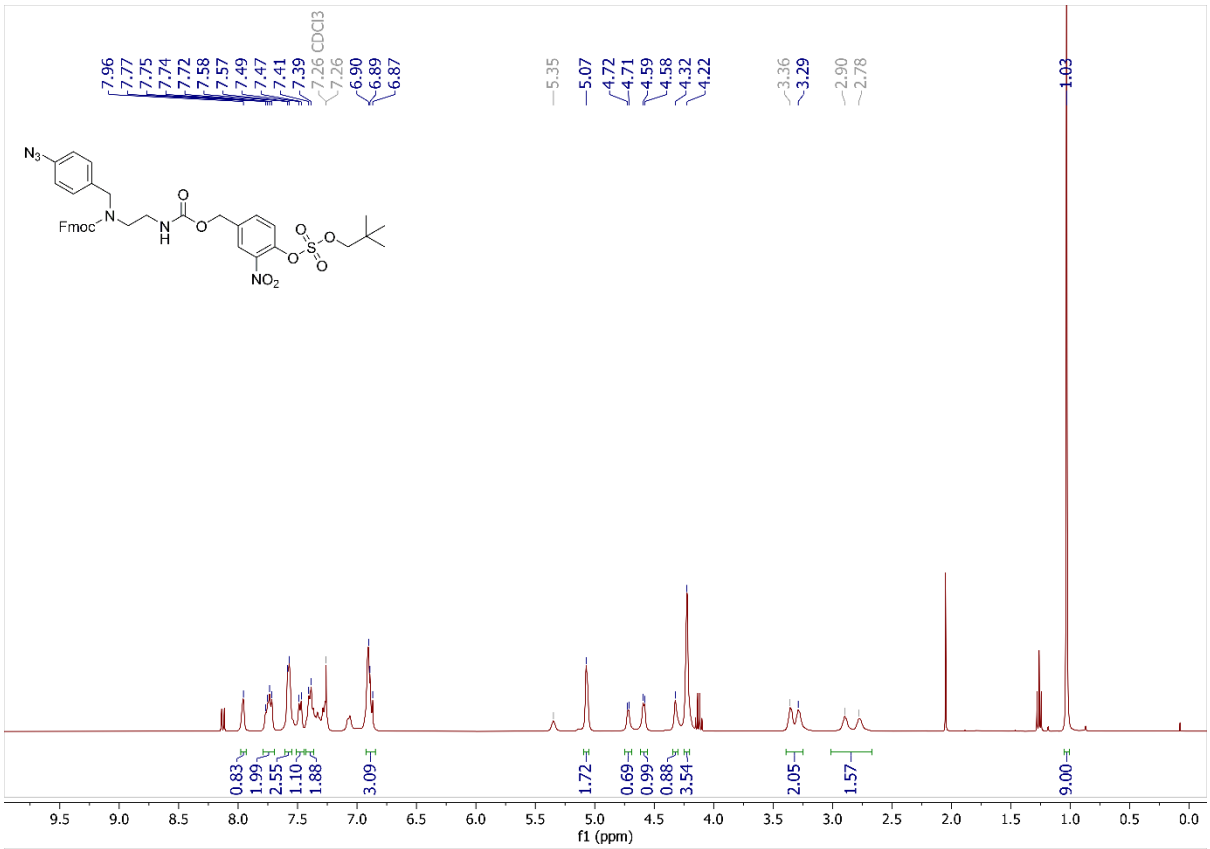


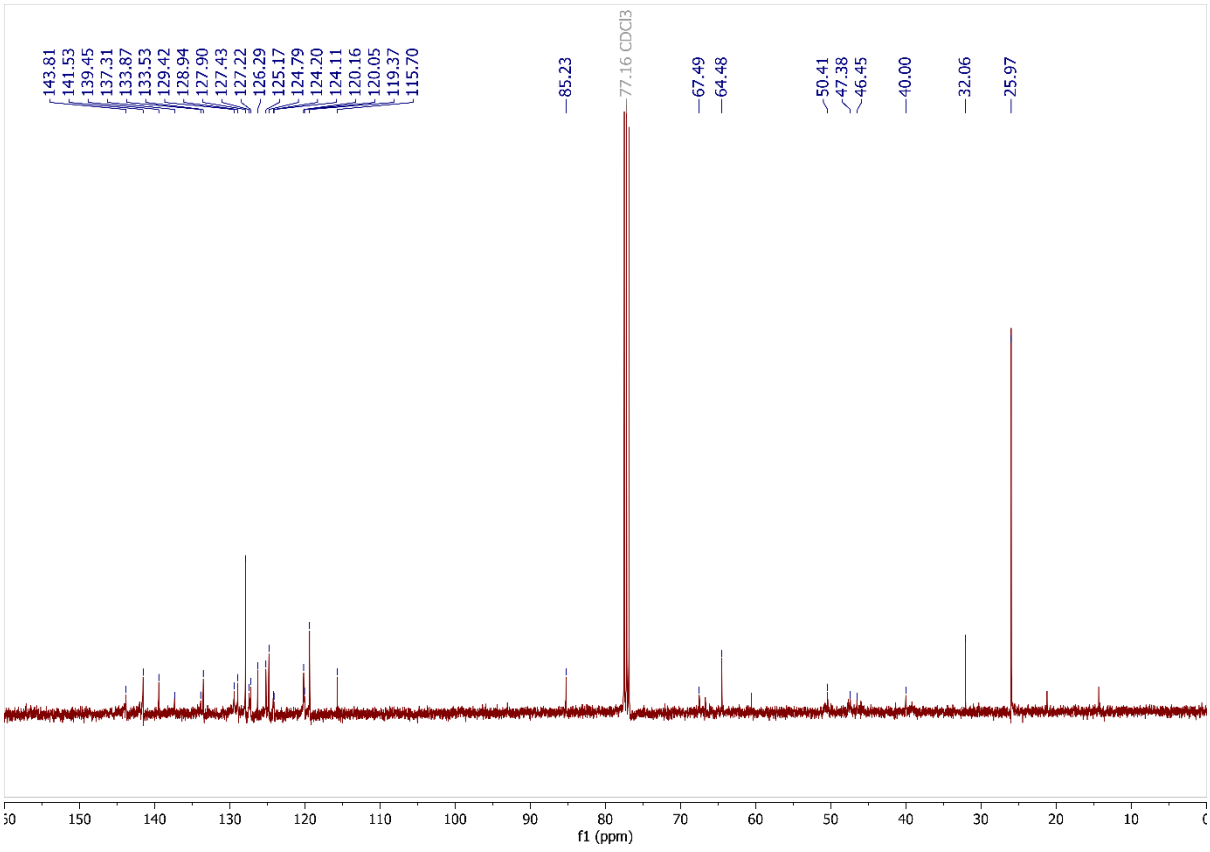
Neopentyl (2-nitro-4-((((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl) sulfate (6)



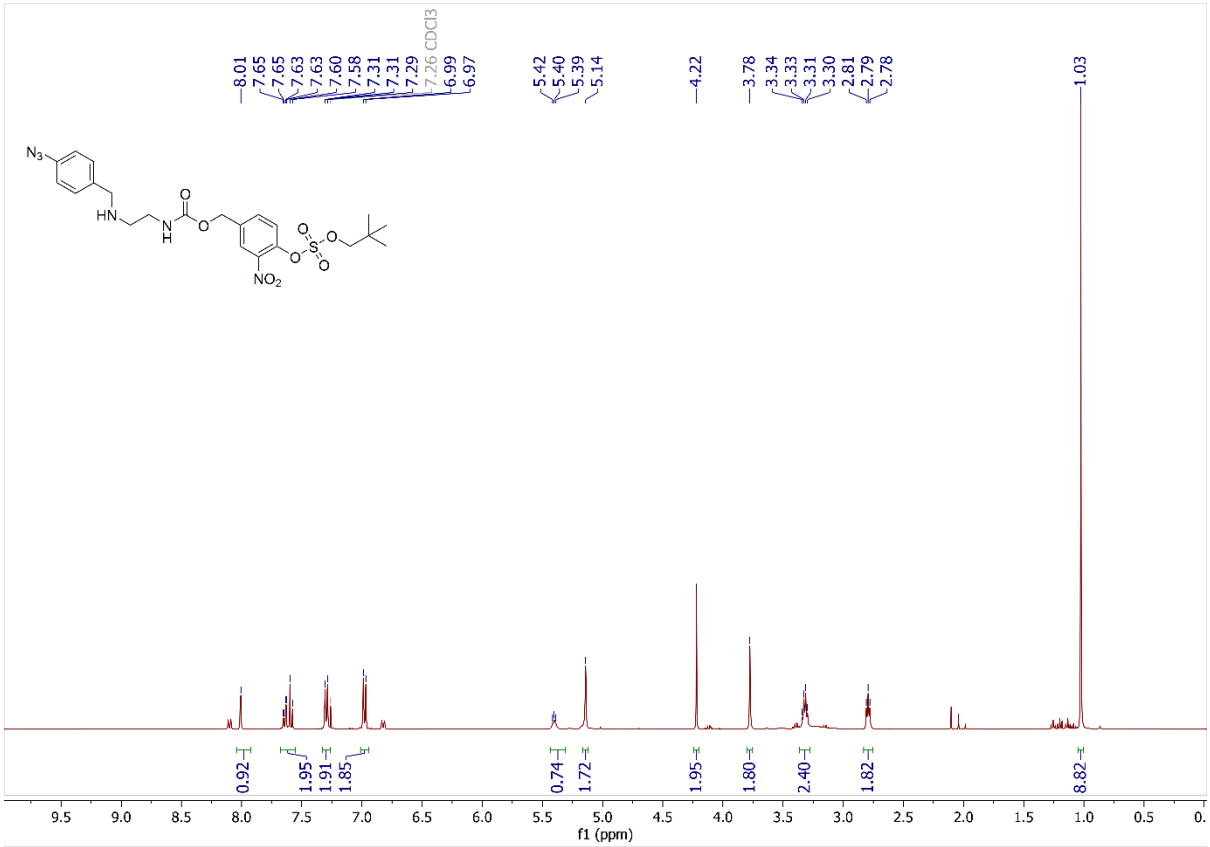


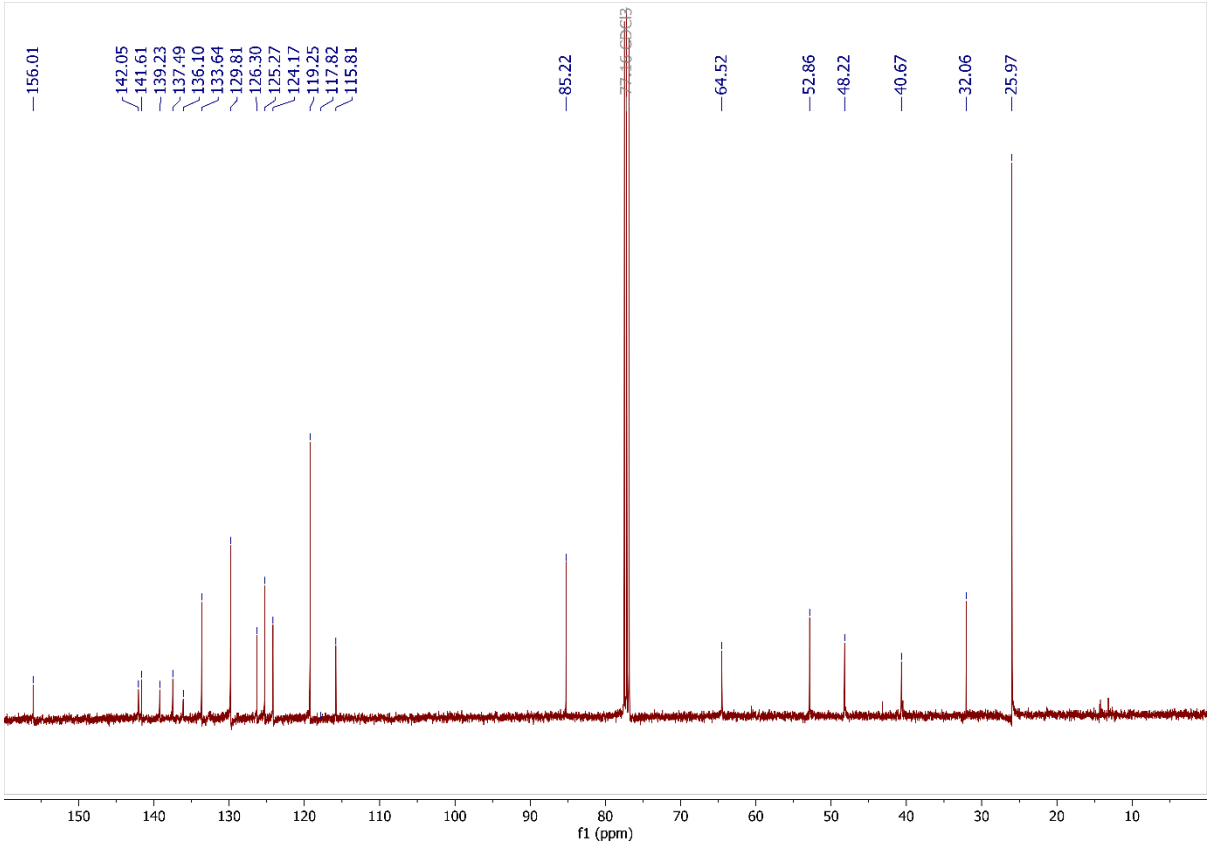
4-(7-(4-Azidobenzyl)-10-(9H-fluoren-9-yl)-3,8-dioxo-2,9-dioxa-4,7-diazadecyl)-2-nitrophenyl neopentyl sulfate (7)



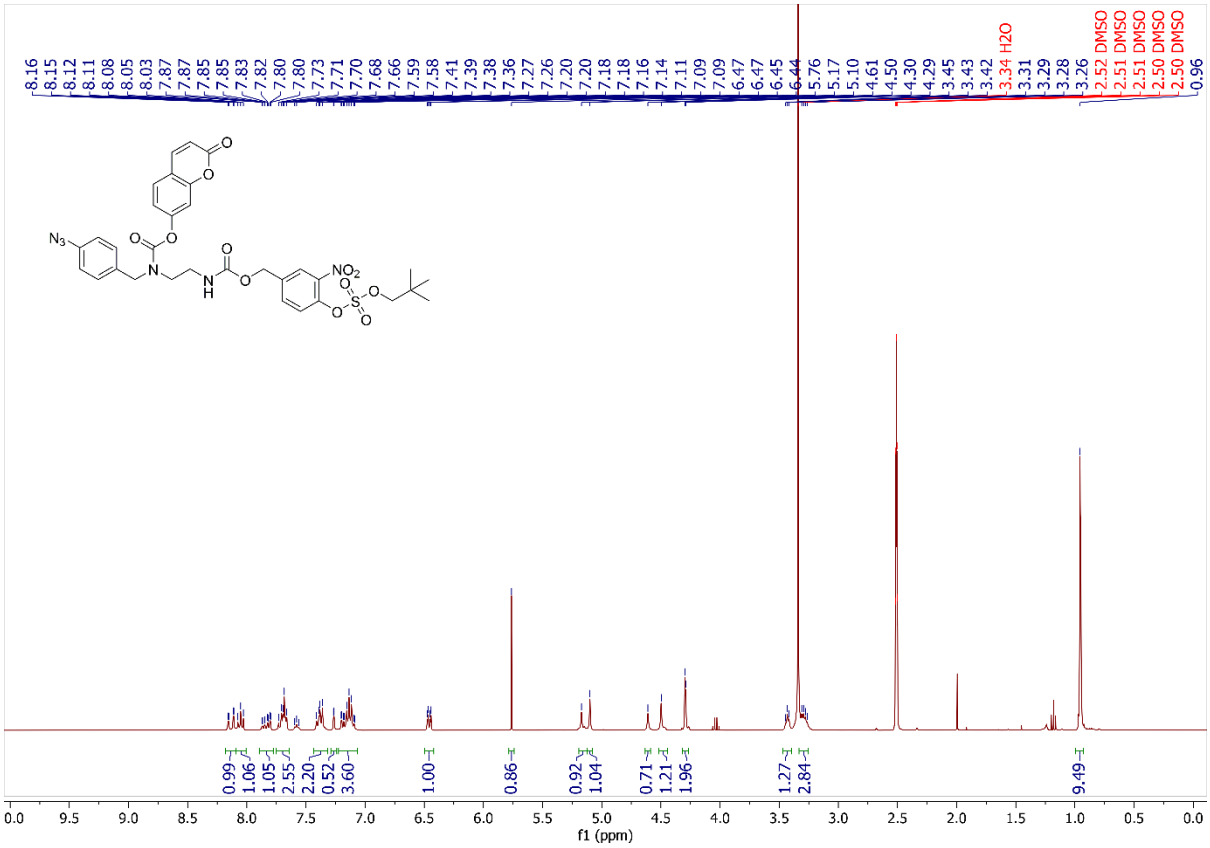


4-((((2-((4-azidobenzyl)amino)ethyl)carbamoyl)oxy)methyl)-2-nitrophenyl neopentyl sulfate (8)

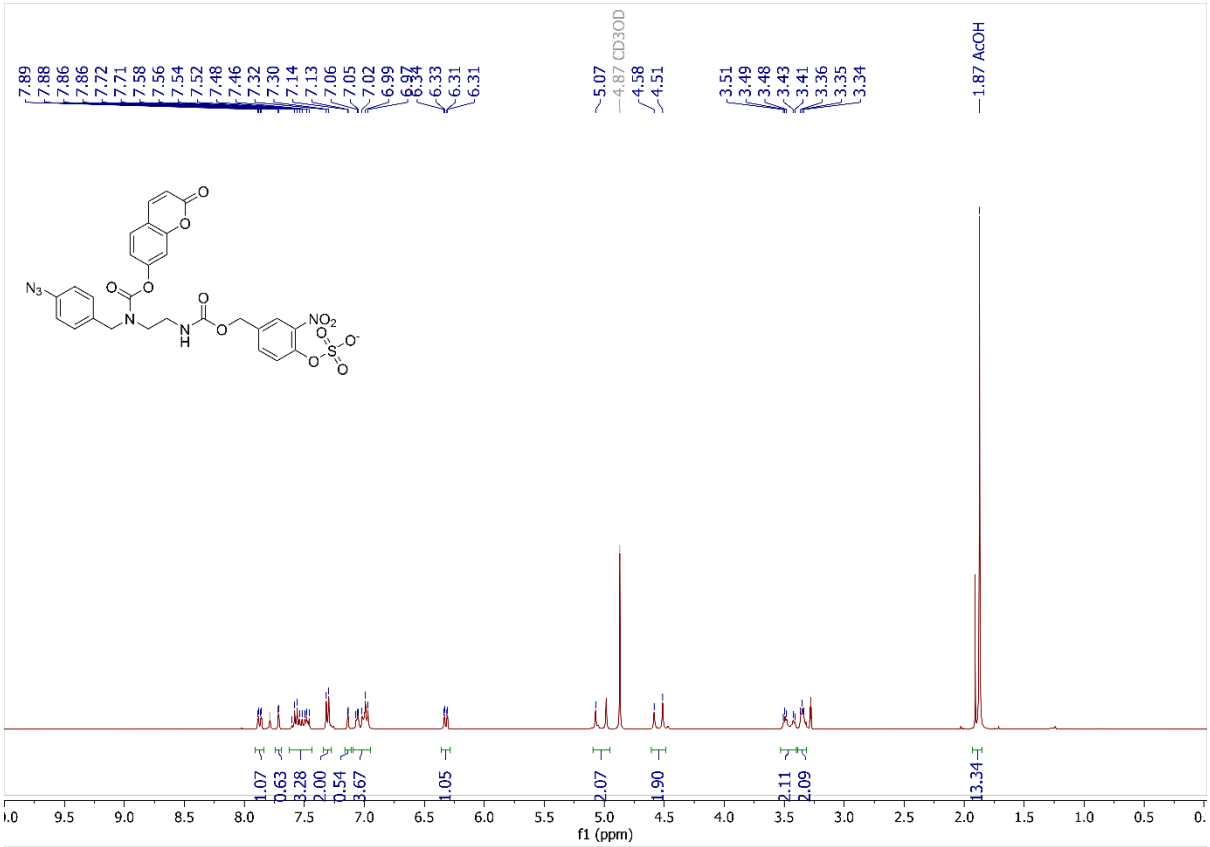


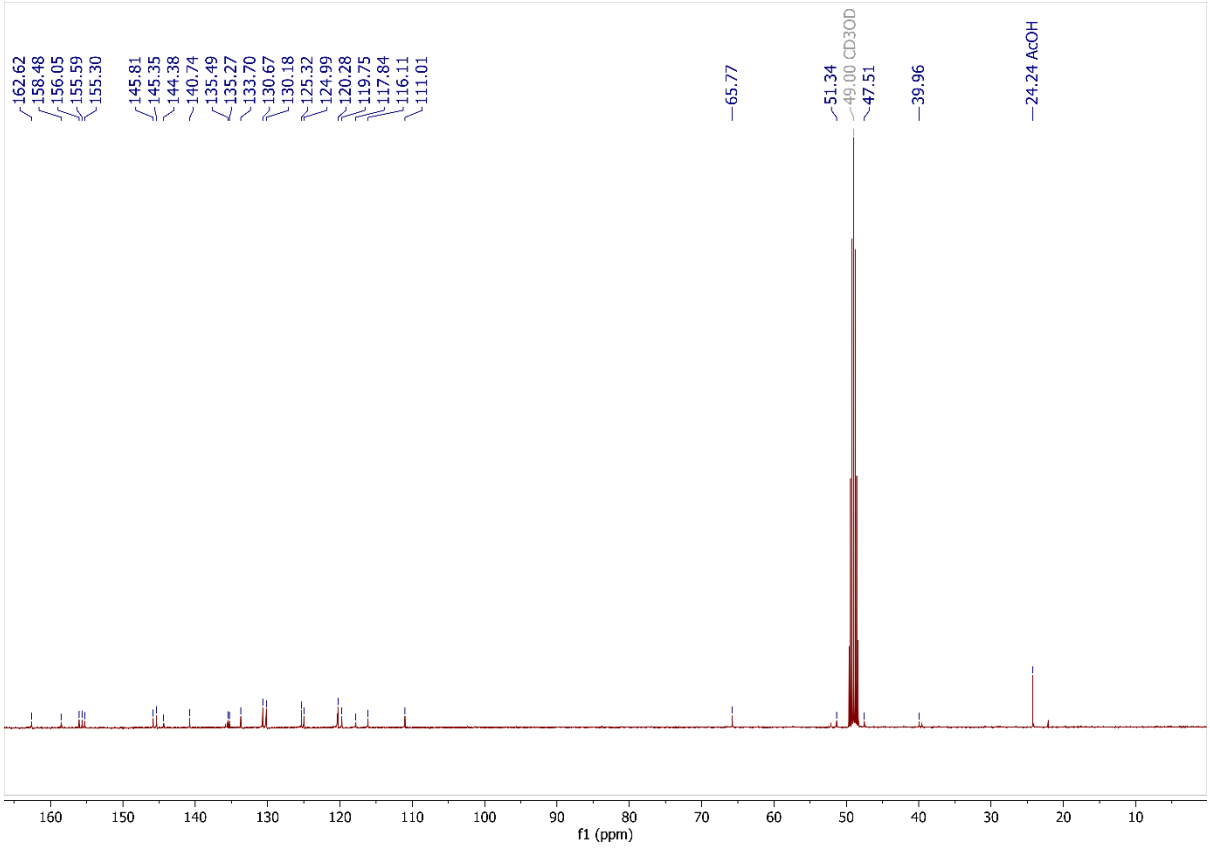


4-((((2-((4-azidobenzyl)(((2-oxo-2H-chromen-7-yl)oxy)carbonyl)amino)ethyl)carbamoyl)oxy)methyl)-2-nitrophenyl neopentyl sulfate (S2)



4-((((2-((4-azidobenzyl)(((2-oxo-2H-chromen-7-yl)oxy)carbonyl)amino)ethyl)carbamoyl)oxy)methyl)-2-nitrophenyl sulfate (10)





HPLC Chromatogram (Compound 13)