Supplementary Information

Fluoro arylsulfates: Kinetic analysis reveals new possibilities with sulfatase-cleavable linkers in ADCs

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General Experimental Details

Human and mouse plasma were purchased from Sigma-Aldrich. All starting materials, reagents and solvents were available in the laboratory or brought from commercial suppliers and used without further purification. Solvents used in reaction and for purification were of analytic grade purity (> 99.9%). Anhydrous THF, MeCN, DMF, DCM, EtOAc and Et_2O were obtained from a PureSolvTM MD-7 Solvent Purification System, Innovative Technology with Al_2O_3 as the stationary phase. When anhydrous solvents were used all glassware was dried either in a 150◦C oven for at least 2 hours or in vacuo overnight or flame dried just before use.

All reactions were monitored using thin-layer chromatography (TLC) and/or UPLC-MS. Analytical TLC was conducted on Merck Aluminum TLC plates, silica gel 60 coated with fluorescent indicator F254. TLC-plates developed using either UV-light (wavelength 10- 400 nm) or stained by dipping in a development agent followed by heating. $KMnO_4$ [3 g in water (300 mL) along with K_2CO_3 (20 g) and 5% aq. NaOH (5 mL)], ninhydrin [0.1 g in AcOH (0.5 mL) and acetone (100 mL)] or vanillin [15 g along with conc. H_2SO_4 (2.5 mL) in ethanol (250 mL)] were used as developing agents.

Flash chromatography was performed using glass columns of appropriate size packed with Merck Geduran 60 Angstrom silica gel (40-64 µm particles) as the stationary phase. Eluent systems for flash chromatography are specified for each step and are reported as volume ratios.

Removal of solvents were done under reduced pressure (*in vacuo*) at temperatures ranging from 25 to 90◦C. Traces of solvents were removed in vacuo with the use of a membrane pump. Analytic UPLC-MS analysis was run on a Waters AQUITY UPLC system equipped with PDA and SQD electrospray MS detector. Column: Kinetex 1.7 µm XB-C18, 2.1 x 50mm. Column temperature: 50◦C. Flowrate: 0.50 mL/min. Eluent A (0.1% HCOOH in H_2O) and B (0.1% HCOOH in MeCN) used in a linear gradient (5% B to 100% B) in 2.4 min or 4.8, hold 0.1 min, total run time 2.6 or 5.0 min. Alternatively, Eluent C (15 mM NH_4OAc in H_2O) and D (15 mM NH_4OAc in MeCN) used in a linear gradient (5% D to 100% D) in 2.4 min or 4.8, hold 0.1 min, total run time 2.6 or 5.0 min.

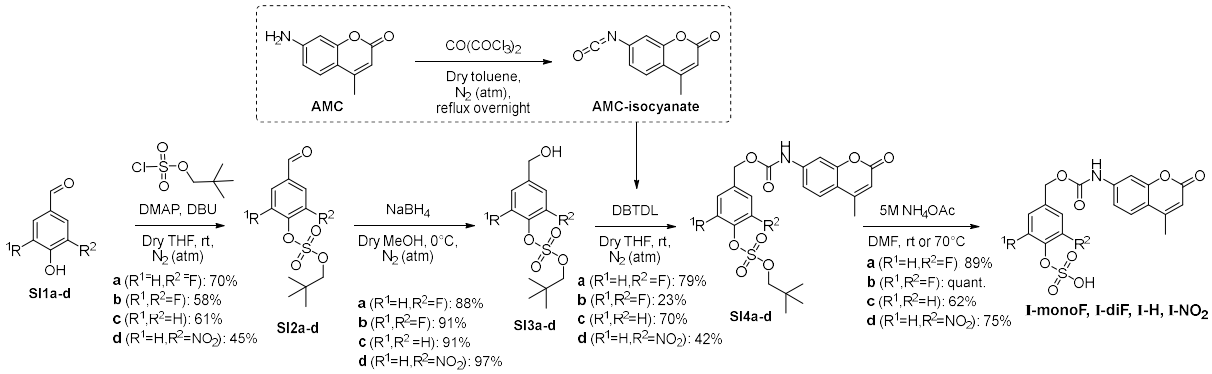
NMR spectra were recorded on Bruker Ascend spectrometer with a Prodigy cryoprobe operating at 400 or 800 MHz for ^{1}H-NMR, 101 MHz for ^{13}C-NMR and 377 MHz for ^{19}F-NMR using the residual non-deuterated solvent as a reference ($CDCl_3$, ^{1}H: 7.26 ppm, ^{13}C: 77.16 ppm; DMSO-d_6, ^{1}H: 2.50 ppm, ^{13}C: 39.52 ppm; MeOD, ^{1}H: 3.31, ^{13}C: 49.00 and D_2O, ^{1}H: 4.79 ppm). Chemical shifts (δ) are reported in ppm downfield from TMS (δ = 0); coupling constants (J) are reported in Hz and multiplicities are reported as singlet (s), doublet (d), doublet of doublets (d), doublet of triplets (dt), triplet, triplet of dublets (td), triplet of triplets (tt), quartet (q), pentet (p), septet (sep) and multiplet (m). All ^{1}H-NMR and ^{13}C-NMR spectra are found under “NMR spectra”.

Automated FCC was performed using Puriflash 5.250 system equipped with autosampler, UV/Vis and ELSD detectors (Interchim, Montluçon, France), with Puriflash® cartridges (silica gel 60 Å, 50µm, Interchim_PF-50SIHP). Automated reverse-phase liquid chromatography (RP-LC) was performed with a Pure Chromatography Instrument C-805 (Essential Flash Basic automated flash chromatography system with UV detection) using C18 30 µM spherical columns from Büchi. Flow-rate range from 0-250 mL/min with a max pressure of 50 bar. A deuterium light source and a diode array detector with available wavelengths at 200 - 400nm are used. Solvent A: 0.1% HCOOH in H_2O, Solvent B: 0.1% HCOOH in MeCN. Gradient 5% to 100%. Run time and gradient were determined for each specific purification.

Chemical synthesis

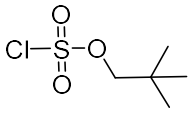
Sulfatase cleavable probes I-monoF, I-diF, I-H, and I-NO2

Synthesis of the fluoroarylsulfate-containing probes **I-monoF**, **I-diF**, **I-H**, and **I-NO$_2$** was achieved in 5 steps from commercially available 4-hydroxybenzaldehydes, see Scheme S1. First, neopentyl sulfurochloridate was coupled to a 4-hydroxybenzaldehyde (SI**1a-d**). Then the benzaldehydes were reduced to the corresponding benzylic alcohol (SI3a-d) and AMC attached through a carbamate moiety, providing SI**4a-d**. Ultimately, the neopentyl protecting group was removed under standard conditions exposing the free arylsulfates (**I-monoF**, **I-diF**, **I-H**, and **I-NO$_2$**).



Scheme S1: Synthesis of sulfatase-responsive probes I-monoF, I-diF, I-H, I-NO$_2$

Neopentyl sulfurochloridate



In a dry round-bottom flask, sulfuryl chloride (10.0 g, 6.15 mL, 74.1 mmol,1 equiv.) was dissolved in dry Et_2O (6 mL). This was cooled to -78°C using a dry-ice/acetone bath. In another dry round-bottom flask neopentyl alcohol (1.1 equiv., 7.18 g, 81.5 mmol) was added along with pyridine (1 equiv., 5.86 g, 6.0 mL, 74.1 mmol). This was dissolved in dry Et_2O (9 mL). When the mixture had dissolved completely it was added dropwise to the cold sulfuryl chloride solution. The reaction was then allowed to warm to room temperature and react for 2 hours before diluting with dry Et_2O (90 mL). The reaction was washed with 10% citric acid, then brine. The organic phase was dried over Na_2SO_4 before evaporating to dryness. The resulting product was a colorless oil isolated in a yield of 65%.

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.15 (s, 2H), 1.03 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 85.6, 32.0, 25.9

General procedure 1

The phenolic substrate (SI**1a**-**d**) (1.0 equiv.) was dissolved in dry THF (0.5 mmol/mL) under N_2, followed by the addition of DMAP (1.0 equiv.) and DBU (2.0 equiv.). The mixture was stirred until homogeneous, after which neopentyl sulfuryl chloridate (1.5 equiv.) was added dropwise at rt. The reaction was stirred at rt until completion (4-24 h, monitored by TLC or LC–MS), then quenched with water or brine and extracted with EtOAc. The combined organic layers are washed with 1 M HCl and brine, dried over

$MgSO_4$, filtered, and concentrated *in vacuo*. The crude material was purified by flash column chromatography using a heptane/EtOAc gradient (10:1 to 1:1) to afford the corresponding neopentyl sulfate ester (SI**2a**-**d**).

General procedure 2

The neopentyl sulfate aldehyde (**SI2a-d**) (1.0 equiv.) was dissolved in dry MeOH (0.2–0.3 mmol/mL) under N_2 and the solution was cooled to 0°C. $NaBH_4$ (2.0 equiv.) was added portion-wise, after which the reaction mixture was allowed to warm to rt and stirred until completion (typically 1–3 h, monitored by TLC or LC–MS). The reaction was quenched carefully with water, and the reaction mixture was diluted with EtOAc. The combined organic layers are washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo to obtain the corresponding benzyl alcohol (**SI3a-d**).

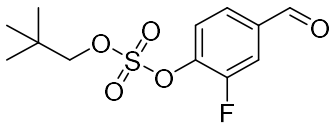
General procedure 3

7-Amino-4-methylcoumarin (1.2 equiv.) and triphosgene (0.5 equiv.) were suspended in dry toluene (30 mL/mmol coumarin) under N_2 and heated to reflux for 2–3 h to generate the corresponding isocyanate in situ. The solvent was removed under reduced pressure, and the crude activated coumarin was redissolved in dry THF. The neopentyl sulfate benzyl alcohol (**SI3a**-**d**) (1.0 equiv.) in dry THF and dibutyltin dilaurate (DBTDL, 0.1 equiv.) were added, and the reaction mixture was stirred at rt until completion (typically 24–72 h, monitored by TLC or LC–MS). The reaction was quenched with water and diluted with EtOAc. The organic layer was separated, washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. The crude product was purified by flash column chromatography using a heptane/EtOAc gradient (10:1 to 0:10) to afford the desired product (**SI4a-d**).

General procedure 4

The protected probe (**SI4a**-**d**) (1.0 equiv.) was dissolved in DMF, after which an equal volume of 2 M NH_4OAc (aq) was added under N_2. The reaction mixture was heated to 70 °C and stirred until complete (typically 2 h to overnight, monitored as needed to avoid side-product formation). The solvent was removed under a stream of N_2 or air. The crude material was either used directly or purified by preparative HPLC to afford the corresponding deprotected product (**I-monoF, I-diF, I-H, I-NO_2**).

2-Fluoro-4-formylphenyl neopentyl sulfate (SI2a)



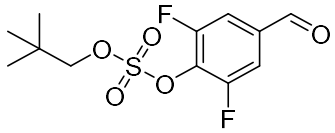
Compound **SI2a** was prepared using **general procedure 1**, affording the title compound as a yellow solid with a yield of 70%.

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.98 (d, J=2.0 Hz, 1H), 7.72-7.76 (m, 2H), 7.70-7.61 (m, 1H), 4.20 (s, 2H), 1.01 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 189.52, 155.71 (d, *J* = 255.5 Hz), 142.15 (d, *J* = 12.9 Hz), 136.31 (d, *J* = 5.4 Hz), 127.04 (d, *J* = 3.7 Hz), 124.63, 117.26 (d, *J* = 19.2 Hz), 84.75 (d, *J* = 1.7 Hz), 32.09, 25.97.

HRMS (ESI) m/z calculated for $[C_{12}H_{16}FO_5S]$ $[M+H]^+$ 291.0697, found 291.0704.

2,6-Difluoro-4-formylphenyl neopentyl sulfate (SI2b)



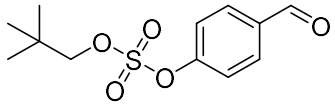
Compound **SI2b** was prepared using **general procedure 1**, affording the title compound as a yellow solid with a yield of 58%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.96 (t, J = 1.8 Hz, 1H), 7.95 (d, J = 7.7 Hz, 2H), 4.35 (s, 2H), 0.99 (s, 9H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 190.1, 155.0 (d, J = 255.3 Hz, 2C), 136.1, 129.9, 113.8 (d, J = 23.1 Hz, 2C), 85.2, 31.7, 25.3 .

HRMS (ESI) m/z calculated for $[C_{12}H_{15}F_2O_5S]$ $[M+H]^+$ 309.0603, found 309.0609.

4-Formylphenyl neopentyl sulfate (SI2c)



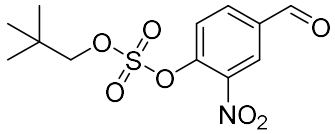
Compound **SI2c** was prepared using **general procedure 1**, affording the title compound as a yellow oil with a yield of 61%.

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.02, (s, 1H), 7.96 (d, J=8.6Hz, 2H), 7.48 (d, J=8.6Hz, 2H), 4.12 (s, 2H), 1.00 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 190.6, 154.5, 135.1, 131.7, 121.6, 84.2, 32.1, 26.0.

HRMS (ESI) m/z calculated for $[C_{12}H_{17}O_5S]$ $[M+H]^+$ 273.0791, found 273.0791

4-Formyl-2-nitrophenyl neopentyl sulfate (SI2d)



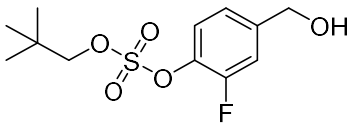
Compound **SI2d** was prepared using **general procedure 1**, affording the title compound as a yellow solid with a yield of 45%.

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.07 (s, 1H), 8.51 (d, J = 2.0 Hz, 1H), 8.20 (dd, J = 8.5, 2.0 Hz, 1H), 7.85 (d, J = 8.5 Hz, 1H), 4.26 (s, 2H), 1.03 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 187.89, 146.08, 134.96, 134.74, 127.11, 124.57, 85.84, 32.12, 25.93.

HRMS (ESI) *m/z* calculated for $[C_{12}H_{16}NO_7S]$ $[M+H]^+$ 318.0642, found 318.0647.

2-Fluoro-4-(hydroxymethyl)phenyl (neopentyl) sulfate (SI3a)



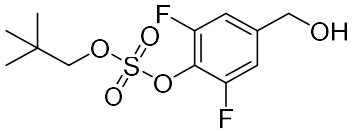
Compound **SI3a** was prepared using **general procedure 2**, affording the title compound as a white solid with a yield of 88%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.50 (t, *J* = 8.2 Hz, 1H), 7.44 – 7.35 (m, 1H), 7.29 – 7.22 (m, 1H), 4.52 (s, 2H), 4.26 (s, 2H), 0.96 (s, 9H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 153.17 (d, *J* = 249.8 Hz), 144.95 (d, *J* = 6.2 Hz), 134.98 (d, *J* = 12.8 Hz), 122.77 (d, *J* = 3.7 Hz), 114.86 (d, *J* = 18.3 Hz), 83.86, 61.62, 31.62, 25.40.

HRMS (ESI) m/z calculated for $[C_{12}H_{17}FNaO_5S]$ $[M+Na]^+$ 315.0673, found 315.0681.

2,6-Difluoro-4-(hydroxymethyl)phenyl neopentyl sulfate (SI3b)



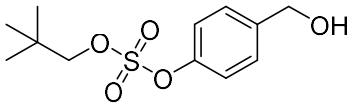
Compound **SI3b** was prepared using **general procedure 2**, affording the title compound as a white solid with a yield of 91%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.28 (d, J = 9.0 Hz, 2H), 4.52 (s, 2H), 4.29 (s, 2H), 0.97 (s, 9H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 154.3 (dd, J = 251.8, 3.5 Hz, 2C), 145.5 (t, J = 7.5 Hz, 1C), 123.9 (t, J = 16.1 Hz, 1C), 110.2 (d, J = 22.0 Hz, 2C), 84.5, 61.4, 31.6, 25.4 .

HRMS (ESI) m/z calculated for $[C_{12}H_{16}F_2NaO_5S]$ $[M+Na]^+$ 333.0579, found 333.0687.

4-(Hydroxymethyl)phenyl neopentyl sulfate (SI3c)



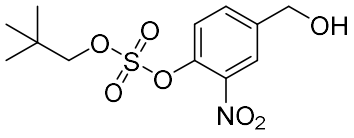
Compound **SI3c** was prepared using **general procedure 2**, affording the title compound as a white solid with a yield of 91%.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 (d, J=8.6Hz, 2H), 7.30 (d, J=8.6Hz, 2H), 4.70 (s, 2H), 4.09 (s, 2H), 0, 98 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 149.7, 140.2, 128.4 (2C), 121.3 (2C), 83.6, 64.5, 32.1, 26.1.

HRMS (ESI) m/z calculated for $[C_{12}H_{18}O_5S]$ $[M-H]^-$ 273.0802, found 273.0802

4-(Hydroxymethyl)-2-nitrophenyl neopentyl sulfate (SI3d)



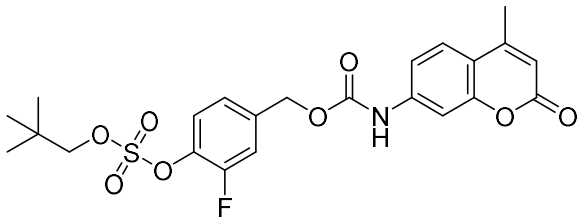
Compound **SI3d** was prepared using **general procedure 2**, followed by a short dry column chromatography step using heptane:EtOAc as eluent, affording the title compound as a yellow solid with a yield of 97%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.55 – 7.46 (m, 1H), 7.39 (dd, *J* = 11.6, 1.6 Hz, 1H), 7.29 – 7.22 (m, 1H), 4.52 (s, 2H), 4.30 (s, 2H), 0.96 (s, 9H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 144.3, 141.8, 139.3, 132.7, 123.9, 123.5, 84.5, 61.2, 31.7, 25.4.

HRMS (ESI) m/z calculated for [$C_{12}H_{17}NNaO_7S$] [M+Na]$^+$ 342.0618, found 342.0626.

2-Fluoro-4-((((4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl)oxy)methyl)phenyl neopentyl sulfate (SI4a)



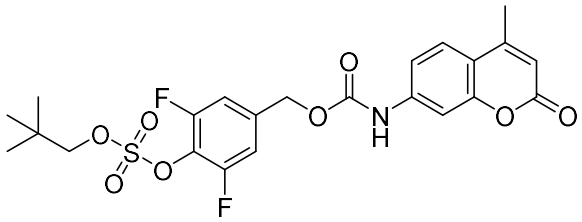
Compound **SI4a** was prepared using **general procedure 3**, affording the title compound as an off-white solid with a yield of 79%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.36 (s, 1H), 7.79-7.68 (m, 2H), 7.67-7.60 (m, 1H), 7.43 (m, 2H), 6.25 (s, 1H), 5.43 (s, 2H) 3.89, (s, 2H), 2.39 (s, 3H), 1.25 (s, 9H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 160.0, 159.1 (d, *J* = 250.2 Hz), 153.8, 153.2, 152.8, 150.1 (d, *J* = 11.4 Hz), 142.5, 132.39, 126.1, 123.10, 123.0 (d, *J* = 14.7 Hz), 117.7 (d, *J* = 3.7 Hz), 114.5, 114.3, 112.0, 109.9, 109.7, 104.5, 83.8, 59.8, 31.7, 25.4, 18.0.

UPLC-MS (ESI) m/z calculated for [$C_{23}H_{25}FNO_8S$] [M+H]$^+$ 494.1 found 494.5

2,6-Difluoro-4-((((4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl)oxy)methyl)phenyl neopentyl sulfate (SI4b)



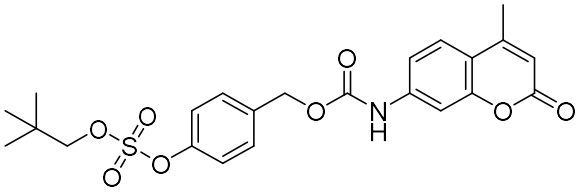
Compound **SI4b** was prepared using **general procedure 3**, affording the title compound as an off-white solid with a yield of 23%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.37 (s, 1H), 7.71 (d, J = 8.7 Hz, 1H), 7.56 (d, J = 2.1 Hz, 1H), 7.55 – 7.47 (m, 2H), 7.42 (dd, J = 8.6, 2.1 Hz, 1H), 6.25 (s, 1H), 5.23 (s, 2H), 4.31 (s, 2H), 2.39 (s, 3H), 0.98 (s, 9H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 160.0, 155.7, 153.8, 153.2, 152.9, 152.7, 142.4, 138.8, 126.1, 114.4 (d, J = 24.4 Hz), 112.3 (d, J = 22.0 Hz), 112.2, 112.0, 104.6, 84.7, 64.4, 31.6, 25.3 , 18.0.

HRMS (ESI) m/z calculated for [$C_{23}H_{23}F_2NO_8S$] [M+H]$^+$ 512.1185, found 512.1191

4-((((4-Methyl-2-oxo-2H-chromen-7-yl)carbamoyl)oxy)methyl)phenyl neopentyl sulfate (SI4c)

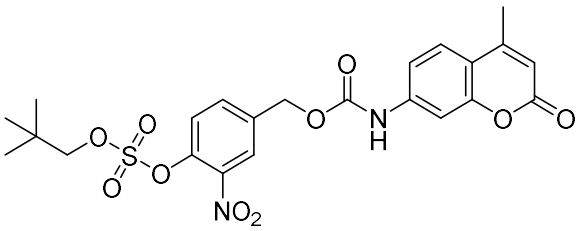


Compound **SI4c** was prepared using **general procedure 3**, affording the title compound as a white solid with a yield of 70%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.32 (s, 1H), 7.69 (d, J=8.7Hz, 1H), 7.62 (d, J=8.5Hz, 2H) 7.58-7.54 (m, 3H), 7.40 (dd, J=8.5, 2.1, 1H), 6.23 (s, 1H), 5.39 (s, 2H), 5.23 (s, 2H), 2.38 (s, 3H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 160.0, 153.8, 153.2, 153.0, 149.2, 142.6, 136.5 (2C), 130.1, 126.1, 121.7 (2C), 114.5, 114.3, 112.0, 104.5, 92.9, 80.1, 65.3, 26,1 , 18.0.

4-((((4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl)oxy)methyl)-2-nitrophenyl neopentyl sulfate (SI4d)



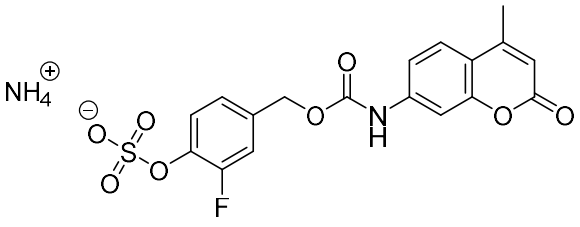
Compound **SI4d** was prepared using **general procedure 3**, affording the title compound as a yellow solid with a yield of 42%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.39 (s, 1H, NH), 8.29 (d, J = 2.2 Hz, 1H), 7.97 (dd, J = 8.5, 2.2 Hz, 1H), 7.78 (d, J = 8.5 Hz, 1H), 7.71 (d, J = 8.7 Hz, 1H), 7.56 (d, J = 2.1 Hz, 1H), 7.42 (dd, J = 8.7, 2.2 Hz, 1H), 6.25 (d, J = 1.3 Hz, 1H), 5.32 (s, 2H), 4.32 (s, 2H), 2.39 (d, J = 1.3 Hz, 3H), 0.96 (s, 9H)

^{13}C NMR (101 MHz, DMSO-d_6) δ 156.0, 153.8, 153.2, 152.8, 142.4, 141.9, 140.3, 137.9, 134.6, 126.1, 125.5, 124.3, 114.6, 114.3, 112.0, 104.6, 84.6, 64.3, 31.7, 25.4, 18.0.

HRMS (ESI) m/z calculated for $[C_{23}H_{25}N_2O_{10}S]$ $[M+H]^+$ 521.1224, found 521.1236

Ammonium 2-fluoro-4-((((4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl) oxy) methyl)phenyl sulfate (I-monoF)



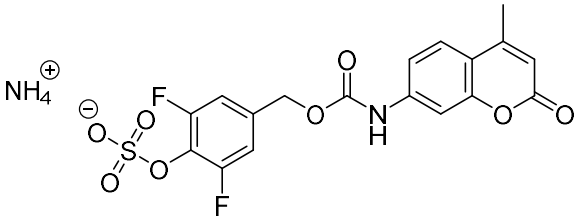
Compound **I-monoF** was prepared using **general procedure 4**, affording the title compound as a white solid following lyophilization with a yield of 89%.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.28 (s, 1H), 7.69 (d, J=8.8Hz, 1H), 7.55 (d, J=2.1 Hz, 1H), 7.51 (t, J=8.4Hz, 1H), 7.41 (dd, J= 8.4, 2.1Hz, 1H), 7.30 (dd, J=11.3, 2.1 Hz, 1H), 7.19 (dt, J=8.5, 1.3 Hz, 1H), 6.23 (d, J=1.4Hz, 1H) 5.12 (s, 2H), 2.38 (s, 3H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 161.2, 154.5, 153.8, 153.2 (d, J= 16.0Hz, 1C), 142.7, 132.1 (d, J=6.5Hz, 1C), 126.1, 124.2 (d, J=3.6Hz, 1C) 123.0, 116.1 (d, J=19.9Hz. 1C) 114.3 (d, =13.6Hz. 1C), 111.9, 104.4, 65.5, 18.0.

HRMS (ESI) m/z calculated for [$C_{18}H_{14}FNO_8S$] $[M+H]^+$ 424.0497, found 424.0496

Ammonium 2,6-difluoro-4-((((4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl)oxy) methyl) phenyl sulfate (I-diF)



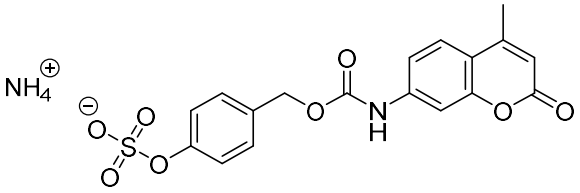
Compound **1-diF** was prepared using **general procedure 4**, affording the title compound as a white solid following lyophilization with in quant yield.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.33 (s, 1H), 7.70 (d, J = 8.7 Hz, 1H), 7.55 (d, J= 2.1 Hz, 1H), 7.41 (dd, J = 8.7, 2.1 Hz, 1H), 7.17 (d, J = 8.2 Hz, 2H), 6.24 (d, J = 1.3 Hz, 1H), 5.13 (s, 2H), 2.39 (d, J = 1.2 Hz, 3H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 160.0, 156.3 (d, J = 250.2 Hz, 2C), 153.8, 153.2, 152.9, 142.6, 133.3, 129.4 (d, J = 15.4 Hz, 1C), 126.1, 114.5, 114.3, 111.9, 111.8 (d, J = 34.5 Hz, 2C), 104.5, 65.0, 18.0.

HRMS (ESI) m/z calculated for [$C_{18}H_{14}F_2NO_8S$] $[M+H]^+$ 442.0403, found 442.0412

Ammonium 4-((((4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl)oxy)methyl)phenyl sulfate, (I-H)



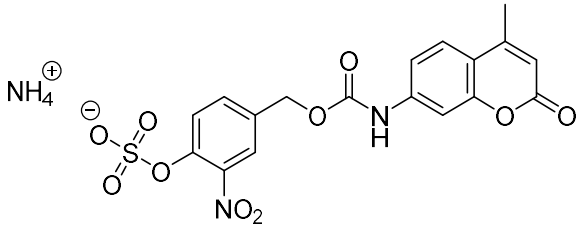
Compound **1-H** was prepared using **general procedure 4**, affording the title compound as a white solid following lyophilization with a yield of 62 %.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.26 (s,1H), 7.68 (d, J=8.7Hz, 1H), 7.55 (d, J=2.1Hz, 1H), 7.40 (dd, J=8.7, 2.1Hz, 1H), 7.36 (d, J=8.7Hz, 2H), 7.19 (d, J = 1.3 Hz, 1H), 6.23 (d, J = 1.3 Hz), 5.12 (s,2H), 2.38 (d, J = 1.2 Hz, 3H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 160.1, 153.9, 153.6, 153.2, 142.8, 130.6, 129.4 (2C), 127.0, 126.1, 120.4, 114.4, 114.3, 111.9, 104.4, 66.1, 18.0.

HRMS (ESI) m/z calculated for $[C_{18}H_{15}NO_8S]$ $[M-H]^-$ 404.0446, found 404.0440.

Ammonium 4-((((4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl)oxy)methyl)-2-nitrophenyl sulfate (I-NO_2)



Compound **1-NO_2** was prepared using **general procedure 4**, affording the title compound as a yellow solid following lyophilization with a yield of 75%.

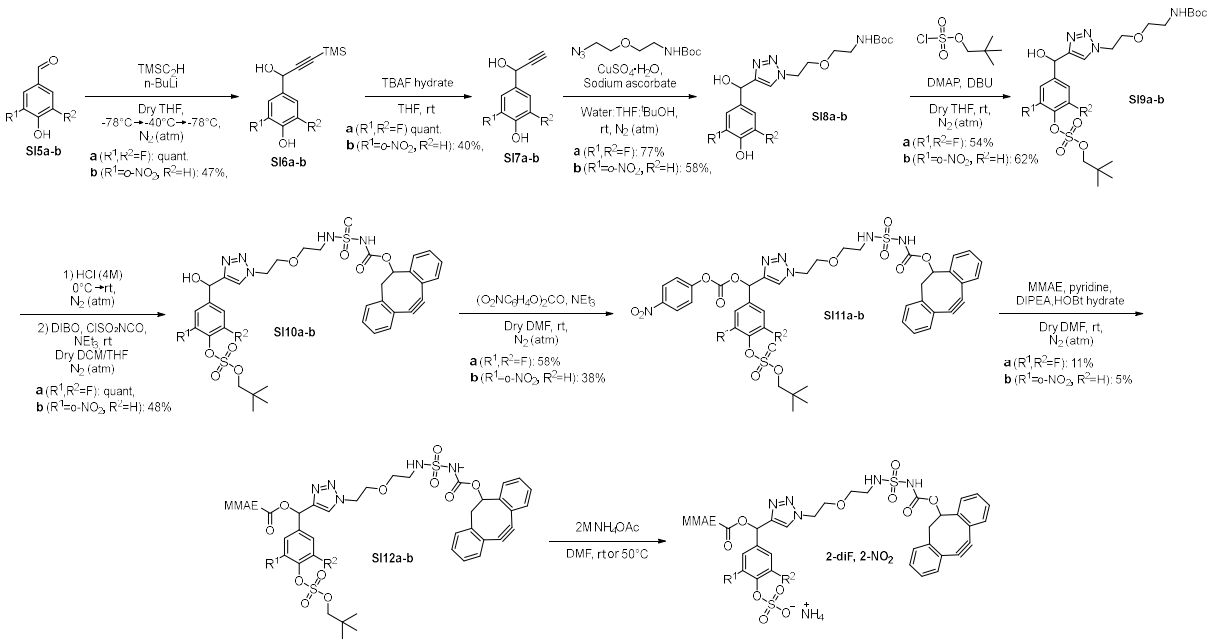
^{1}NMR (400 MHz, DMSO-d_6) δ 10.28 (s, 1H), 8.00 (d, J = 2.2 Hz, 1H), 7.69 (d, J = 8.8 Hz, 1H), 7.63 (dd, J = 8.6, 2.2 Hz, 1H), 7.54 (d, J = 2.1 Hz, 1H), 7.40 (dd, J = 8.7, 2.1 Hz, 1H), 7.17 (d, J = 8.6 Hz, 1H), 6.23 (d, J = 1.3 Hz, 1H), 5.15 (s, 2H), 2.38 (d, J = 1.3 Hz, 3H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 160.5, 154.3, 153.7, 153.5, 152.5, 143.1, 137.0, 136.0, 127.8, 126.5, 125.7, 119.7, 114.9, 114.7, 112.4, 104.9, 65.5, 18.5.

HRMS (ESI) m/z calculated for $[C_{18}H_{14}N_2O_{10}S]$ $[M-H]^-$ 449.0296, found 449.0305

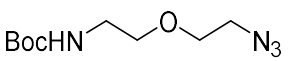
Sulfatase cleavable linker-payloads, 2-diF and 2-NO_2

Having confirmed the favourable properties of the difluoroarylsulfate **1-diF**, the linker motif was elaborated to include an antibody attachment handle and the cytotoxic agent MMAE for biological evaluation of the corresponding ADCs (structure **2-diF**). For comparison, also the corresponding payload based on the known nitroarylsulfate payload (**2-NO_2**) was prepared. Starting from the p-hydroxybenzaldehydes **SI5a-b**, a TMS-protected alkyne handle was installed, simultaneously introducing the benzylic alcohol for later MMAE attachment (structures **SI6a-b**). Removal of the alkyne TMS-protecting group exposed the terminal alkyne, which was used as handle to install the strained alkyne for later attachment to the antibody. A glycol spacer was incorporated to increase solubility and introduce distance between the antibody and the sulfatase recognition site. Selective installation of the neopentyl-protected sulfate on the phenol without touching the benzylic alcohol was achieved by reacting with neopentyl sulfurochloridate and DBU as base (**SI9a-b**). Finally, the MMAE was attached via a p-nitrophenol (pNP) activation (**SI12a-b**) and the neopentyl sulfate deprotected with aqueous NH_4OAc to afford linker-MMAEs **2-diF, 2-NO_2** in good overall yields, see Scheme S2 and supporting information.



Scheme S2: Synthesis of sulfatase-responsive payloads 2-diF, 2-NO_2

Tert-butyl (2-(2-azidoethoxy)ethyl)carbamate (S1)



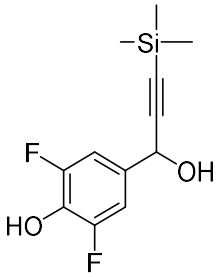
Tert-butyl (2-(2-hydroxyethoxy)ethyl)carbamate (7.00g, 34.1mmol, 1 equiv.) and TEA (4.75mL, 34.10mmol, 1 equiv.) were dissolved in 21mL dry toluene. The reaction was cooled to 0°C and MsCl (3.91g, 34.1mmol 1 equiv.) was added in portions. The reaction was allowed to reach rt and after 10 min, TBAI (12.60g, 34.10mmol, 1 equiv.) was added, followed by the addition of NaN_3 (2.22g, 34.1 mmol, 1 equiv.) in 11mL water. The reaction was heated to 70°C and stirred for 4h. The reaction was extracted

with EtOAc, the organic phase dried over Na_2SO_4 and the solvent removed in vacuo. The crude was purified by dry column chromatography, to afford the title compound **S1** in a quantitative yield.

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.91 (s, 1H), 3.64 (m, 2H), 3.54 (t, J = 5.2 Hz, 2H), 3.37 (m, 2H), 3.32 (q, J = 5.2 Hz, 2H), 1.44 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 156.0, 79.3, 70.3, 69.9, 50.7, 40.4, 28.4.

2,6-difluoro-4-(1-hydroxy-3-(trimethylsilyl)prop-2-yn-1-yl)phenol SI6a



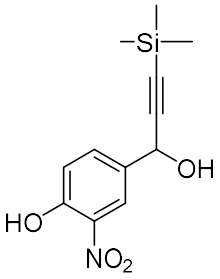
TMS-acetylene (4.10g, 41.73mmol, 2.2 equiv.) was dissolved in 100mL dry THF under inert atmosphere and the solution cooled to -78°C, followed by the slow addition of 2.2M n-BuLi in hexane (18mL, 39.84mmol, 2.1 equiv.). A solution of 3,5-difluoro-4-hydroxybenzaldehyde **SI5a** (3.00g, 18.97mmol, 1 equiv.) in 100mL dry THF was added dropwise. The reaction was stirred at - 78°C for 4h before being quenched by the addition of sat. aq. NH_4Cl. The reaction was extracted with Et_2O, the organic phase dried over Na_2SO_4 and the solvent removed *in vacuo*. The crude was purified by a short dry column chromatography step to afford the title compound **SI6a** in a quantitative yield.

^{1}H NMR (400 MHz, DMSO-d_6) δ 10.21 (s, 1H), 7.13 – 7.00 (m, 2H), 6.14 (d, J = 6.2 Hz, 1H), 5.30 (d, J = 6.2 Hz, 1H), 0.16 (s, 9H).

13C NMR (101 MHz, DMSO-d_6) δ 152.4 (dd, J = 242.0, 7.0 Hz, 2C), 151.2 (d, J = 7.1 Hz), 133.4, 133.2 (2C), 110.3 (d, J = 7.3 Hz), 110.1 (d, J = 7.3 Hz), 107.5, 89.5, 62.2, 0.3

UPLC-MS (ESI) m/z calculated for $[C_{12}H_{13}F_2O_2Si]$ $[M-H]^-$ 255.1, found 255.4

4-(1-Hydroxy-3-(trimethylsilyl)prop-2-yn-1-yl)-2-nitrophenol SI6b



TMS-acetylene (0.94mL, 6.58mmol, 2.20 equiv.) was dissolved in dry THF under inert atmosphere and the solution cooled to 0°C. 1.5M MeLi ·LiBr in Et2O (4.4mL, 6.58mmol, 2.20 equiv.) was added and the solution was stirred at rt for 4h. The reaction was again cooled to 0°C and 4-hydroxy-3-nitrobenzaldehyde **SI5b** (500mg, 2.99mmol, 1 equiv.) was added portion-wise. Upon completion (1h), the reaction was quenched by the addition of sat. aq. NH_4Cl and the reaction was extracted with EtOAc.

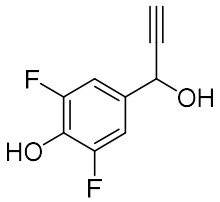
The organic phase was washed with brine, dried over $MgSO_4$, filtered, and the solvent removed *in vacuo*. The crude was purified by flash column chromatography, affording compound **SI6b** in a yield of 47%.

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.60 (s, 1H), 8.30 – 8.29 (m, 1H), 7.76 (ddt, J = 8.7, 2.2, 0.5 Hz, 1H), 7.17 (d, J = 8.7 Hz, 1H), 5.46 (bs, 1H), 0.21 (s, 9H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 155.0, 136.1, 133.2, 132.9, 123.2, 120.3, 103.7, 93.0, 63.5, -0.3

HRMS (ESI) m/z calculated for $[C_{12}H_{16}NO_4Si]$ $[M+H]^+$ 266.0843, found 266.0860

2,6-Difluoro-4-(1-hydroxyprop-2-yn-1-yl)phenol SI7a

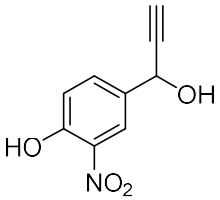


2,6-difluoro-4-(1-hydroxy-3-(trimethylsilyl)prop-2-yn-1-yl) phenol **SI6a** (4.86g, 18.97mmol, 1 equiv.) was dissolved in 7mL THF and a solution of TBAF hydrate (5.83g, 20.87mmol, 1.1 equiv.) in 21mL THF added. Upon completion (1h), the reaction was quenched by the addition of water, and the reaction was extracted with 3x EtOAc. The combined organic phase was dried over Na_2SO_4, filtered, and the solvent removed *in vacuo*, affording the title compound **SI7a** in a quantitative yield.

^{1}H NMR (400 MHz, DMSO) δ 7.05 – 7.01 (m, 2H), 5.26 – 5.25 (m, 1H), 3.51 (d, *J* = 2.2 Hz, 1H). (crude)

^{13}C NMR (101 MHz, DMSO-d_6) δ 152.7 (dd, J = 241.5, 7.5 Hz, 2C), 134.4 (d, J = 16.3 Hz), 132.2, 110.2 (t, J = 6.8 Hz, 2C), 85.4, 76.4, 61.7 (d, J = 2.1 Hz)

4-(1-Hydroxyprop-2-yn-1-yl)-2-nitrophenol SI7b

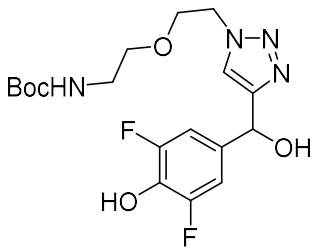


Compound **SI7b** was collected during purification of compound **SI6b** in a yield of 30%.

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.61 (s, 1H), 8.31 (dd, *J* = 2.3, 0.8 Hz, 1H), 7.78 (dd, *J* = 8.7, 2.3 Hz, 1H), 7.19 (d, *J* = 8.7 Hz, 1H), 5.50 – 5.43 (m, 1H), 2.74 (d, *J* = 2.2 Hz, 1H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 155.22, 136.09, 132.64, 123.25, 120.59, 82.52, 76.01, 63.07.

***Tert*-butyl (2-(2-(4-((3,5-difluoro-4-hydroxyphenyl)(hydroxy)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethyl)carbamate SI8a**



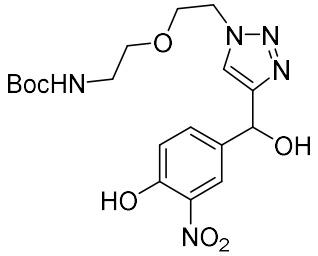
2,6-Difluoro-4-(1-hydroxyprop-2-yn-1-yl)phenol (**SI7a**) (3.49g, 18.95mmol, 1 equiv.) and tert-butyl (2-(2-azidoethoxy)ethyl)carbamate **S1** (50.02g, 21.97mmol, 1.15 eq.) were dissolved in 45mL water, 90mL THF and 90mL tBuOH and the reaction degassed with N_2 for 70min. A solution of $CuSO_4 \cdot 5H_2O$ (237mg, 0.95mmol. 0.05 equiv.) and sodium ascorbate (751mg, 3.79mmol, 0.2 equiv.) in 45mL water was degassed with N_2 for 70min. The solutions were mixed and upon completion (2h), EtOAc was added. The organic phase was washed with sat. aq. $NaHCO_3$ and water, before being dried over $MgSO_4$, filtered, and the solvent removed *in vacuo*. The crude was purified by a short dry column chromatography step, affording the title compound **SI8a** in a yield of 77%.

^{1}H NMR (400 MHz, CD_3OD) δ 7.87 (s, 1H), 7.06 – 6.94 (m, 2H), 5.84 (s, 1H), 4.56 (t, J = 5.1 Hz, 2H), 3.85 (t, J = 5.1 Hz, 2H), 3.47 (t, J = 5.6 Hz, 2H), 3.23 – 3.15 (m, 2H), 1.45 (s, 9H).

^{13}C NMR (101 MHz, CD_3OD) δ 157.1, 152.4 (dd, J = 242.3, 6.8 Hz, 2C), 150.8, 134.0 (t, J = 7.1 Hz), 133.1, 122.8, 109.4 (d, J = 7.4 Hz), 109.2 (2C), 78.8, 69.7, 68.8, 67.5, 50.0, 39.7, 27.3

HRMS (ESI) m/z calculated for $[C_{18}H_{25}F_2N_4O_5]$ $[M+H]^+$ 415.1788, found 415.1798

***Tert*-butyl (2-(2-(4-(hydroxy(4-hydroxy-3-nitrophenyl)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethyl)carbamate SI8b**



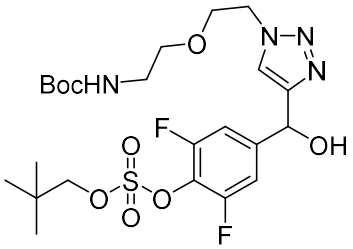
4-(1-Hydroxyprop-2-yn-1-yl)-2-nitrophenol **SI7b** (350mg, 1.81mmol, 1 equiv.), tert-butyl (2-(2-azidoethoxy)ethyl)carbamate **S1** (459mg, 1.99mmol, 1.1 equiv.) and $CuSO_4 \cdot 5H_2O$ (144mg, 0.91mmol, 0.5equiv.) were dissolved in 6mL H_2O:t-BuOH 1:1 and the solution degassed with N_2 for 10min, after which sodium ascorbate (160mg, 0.91mmol, 0.5 equiv.) was added. The reaction was stirred at rt under inert atmosphere overnight and the solvent removed in vacuo. The crude was purified by flash column chromatography, affording the title compound **SI8b** in a yield of 58%.

1H NMR (400 MHz, CD_3OD) δ 8.19 (dd, J = 2.2, 0.7 Hz, 1H), 7.93 (d, J = 0.6 Hz, 1H), 7.69 (ddd, J = 8.7, 2.3, 0.5 Hz, 1H), 7.16 (d, J = 8.7 Hz, 1H), 5.95 (d, J = 0.6 Hz, 1H), 4.58 (dd, J = 5.6, 4.6 Hz, 2H), 3.85 (dd, J = 5.5, 4.7 Hz, 2H), 3.47 (t, J = 5.7 Hz, 2H), 3.18 (t, J = 5.7 Hz, 2H), 1.44 (s, 9H).

13C NMR (101 MHz, CD_3OD) δ 153.5, 150.5, 135.4, 135.0, 134.0, 123.0, 122.4, 119.6, 78.7, 69.6, 68.7, 67.2, 50.1, 39.7, 27.3 .

HRMS (ESI) m/z calculated for $[C_{18}H_{26}F_2N_5O_7]$ $[M+H]^+$ 424.1827, found 424.1836

4-((1-(2-(2-((Tert-butoxycarbonyl)amino)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)(hydroxy)methyl)-2,6-difluorophenyl neopentyl sulfate (SI9a)



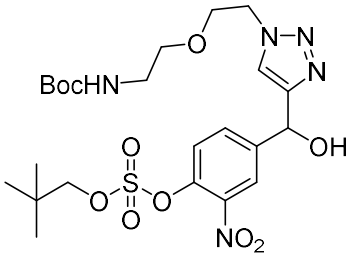
Tert-butyl (2-(2-(4-((3,5-difluoro-4-hydroxyphenyl)-(hydroxy)methyl) -1H-1,2,3-triazol-1-yl)ethoxy)ethyl) carbamate **SI8a** (0.99g, 2.39mmol, 1 equiv.) and DMAP (292mg, 2.39mmol, 1 equiv.) were dissolved in 24mL dry THF under inert atmosphere, followed by the dropwise addition of neopentyl sulfurochloridate (892mg, 4.78mmol, 2 equiv.) and DBU (364µL, 2.39mmol, 1 equiv.). The reaction was stirred for 75min at rt and quenched by the addition of sat. aq. $NaHCO_3$. The reaction was extracted with EtOAc, and the organic phase was washed with sat. aq. $NaHCO_3$, dried over $MgSO_4$, filtered, and the solvent removed *in vacuo*. The crude was purified by dry column chromatography, affording the title compound **SI9a** in a yield of 54%.

1H NMR (400 MHz, DMSO-d_6) δ 7.97 (s, 1H), 7.43 – 7.34 (m, 2H), 6.77 (d, J = 6.0 Hz, 1H), 5.89 (s, 1H), 4.48 (d, J = 5.3 Hz, 2H), 4.30 (s, 2H), 3.77 (t, J = 5.3 Hz, 2H), 3.38 (d, J = 6.0 Hz, 2H), 3.03 (d, J = 6.0 Hz, 2H), 1.37 (s, 9H), 0.98 (s, 9H).

^{13}C NMR (101 MHz, DMSO-d_6) δ 156.1, 153.4 (dd, J = 252.6, 3.3 Hz, 2C), 150.1, 147.0, 124.8, 123.3, 111.3 (2C), 85.1, 78.1, 69.5, 68.8, 67.0, 49.8, 32.1, 28.7, 25.8

HRMS (ESI) m/z calculated for $[C_{23}H_{35}F_2N_4O_8S]$ $[M+H]^+$ 565.2138, found 565.2147

4-((1-(2-(2-((Tert-butoxycarbonyl)amino)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)(hydroxy) methyl)-2-nitrophenyl neopentyl sulfate SI9b



Tert-butyl (2-(2-(4-(hydroxy(4-hydroxy-3-nitrophenyl) methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethyl) carbamate **SI8b** (140mg, 330µmol, 1 equiv.), DMAP (40mg, 330µmol, 1 equiv.) and TEA (96µL, 661µmol, 2 equiv.) were dissolved in 10mL dry THF under inert atmosphere. To the reaction neopentyl sulfurochloridate (123mg, 661µmol, 2 equiv.) was added dropwise and the reaction was stirred at rt for 2h. The reaction was then quenched by the addition of sat. aq. $NaHCO_3$. The reaction was extracted with EtOAc and the organic phase washed with brine. The organic phase was dried over $MgSO_4$and the

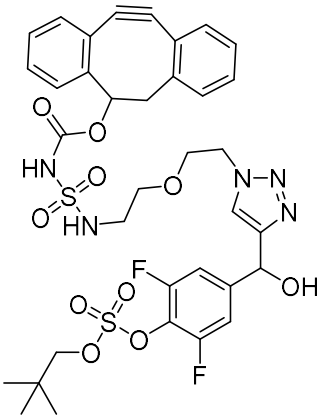
solvent removed in vacuo. The crude was purified by FCC, affording the title compound **SI9b** in a yield of 62%.

^{1}H NMR (400 MHz, CD_3OD) δ 8.20 (dd, J = 2.2, 0.7 Hz, 1H), 7.98 – 7.93 (m, 1H), 7.87 (ddd, J = 8.6, 2.2, 0.7 Hz, 1H), 7.65 (d, J = 8.5 Hz, 1H), 6.07 (d, J = 0.7 Hz, 1H), 5.51 (s, 2H), 4.57 (dd, J = 5.7, 4.5 Hz, 2H), 4.24 (s, 2H), 3.94 – 3.71 (m, 2H), 3.47 (t, J = 5.6 Hz, 2H), 3.18 (t, J = 5.7 Hz, 2H), 1.44 (s, 9H), 1.03 (s, 9H).

^{13}C NMR (101 MHz, CD_3OD) δ 157.1, 150.0, 144.3, 142.3, 140.7, 132.3, 123.7, 123.6, 123.1, 84.4, 78.8, 69.6, 68.7, 66.9, 53.4, 50.1, 39.7, 31.4, 27.4 , 24.7.

HRMS (ESI) m/z calculated for $[C_{23}H_{36}N_5O_{10}S]$ [M+H]+ 574.2177, found 574.2187

Compound SI10a



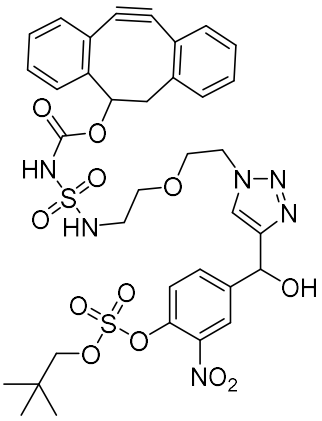
4-((1-(2-(2-((*Tert*-butoxycarbonyl)amino)ethoxy)ethyl)-1*H*-1,2,3-triazol-4-yl)(hydroxyl)methyl)-2,6-difluorophenyl neopentyl sulfate **SI9a** (500mg, 886µmol, 1 equiv.) was dissolved in cooled 4M HCl in dioxane and the reaction was stirred at rt for 30min, before removing the solvent in vacuo. In a separate flask, dibenzo[a,e]cycloocten-5-ol DIBO (205mg, 930µmol, 1.05 equiv.) and chlorosulfonyl isocyanide (132mg, 930µmol, 1 equiv.) were dissolved in 2.7mL dry DCM under inert atmosphere and the reaction stirred for 20min. at rt, before the dropwise addition of TEA (370µL, 2.66mmol, 3 equiv.). The reaction was stirred for 5min. before the addition of the Boc-deprotected linker in 1.7mL dry THF. Upon completion (1h), the solvent was removed in vacuo and the crude purified by flash column chromatography, affording the title compound **SI10a** in a quantitative yield.

1 H NMR (400 MHz, CD_3OD) δ 7.86 (d, J = 2.2 Hz, 1H), 7.60 (dq, J = 7.7, 1.0 Hz, 1H), 7.47 – 7.27 (m, 6H), 7.29 – 7.21 (m, 2H), 5.90 (s, 1H), 5.53 (t, J = 3.9, 2.1 Hz, 1H), 4.46 – 4.42 (m, 2H), 4.25 (s, 2H), 3.74 (t, J = 5.1 Hz, 2H), 3.54 (t, J = 5.3 Hz, 2H), 3.32 – 3.27 (m, 1H), 3.23 (td, J = 5.3, 1.7 Hz, 2H), 2.89 (dd, J = 15.2, 3.9 Hz, 1H), 1.03 (s, 9H).

^{13}C NMR (101 MHz, CD_3OD) δ 157.6, 155.1 (dd, J = 252.9, 3.3 Hz, 2C), 151.5, 150.9, 150.6, 149.7, 129.7, 128.0 (d, *J* = 2.6 Hz), 127.3, 127.1, 125.9, 125.6, 123.5, 123.5, 123.2 (d, J = 1.9 Hz), 121.0, 112.6, 110.4, 110.1 (d, J = 3.0 Hz), 109.4, 84.1(2C), 78.0, 68.9, 68.7, 66.9, 49.9, 45.4, 42.8, 31.3, 24.7.

HRMS (ESI) m/z calculated for $[C_{23}H_{38}F_2N_5O_{10}S_2]$ [M+H]+ 790.2023, found 790.2022

Compound SI10b



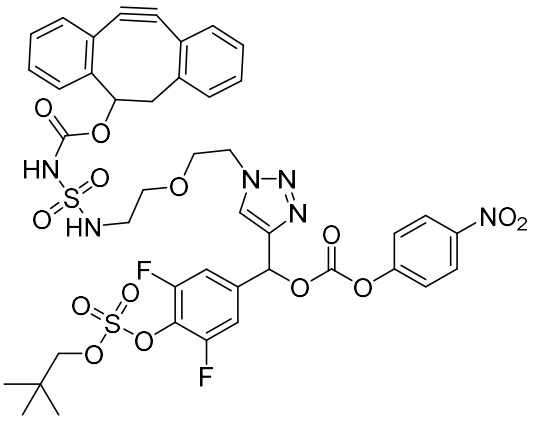
4-((1-(2-(2-((*Tert*-butoxycarbonyl) amino) ethoxy) ethyl)-1H-1,2,3-triazol-4- yl) (hydroxy) methyl)-2-nitrophenyl neopentyl sulfate (**SI9b**) (110mg, 192µmol, 1 equiv.) was dissolved in 4M HCl in dioxane and stirred at rt for 45min, before the removal of the solvent in vacuo. In a separate flask dibenzo[a,e]cycloocten-5-ol (DIBO) (42mg, 190µmol, 1 equiv.) and chlorosulfonyl isocyanide (27mg, 190µmol, 1 equiv.) were dissolved in 0.6mL dry DCM under inert atmosphere and the solution stirred at rt for 20min, whereupon the solvent was removed under a stream of N_2. The crude was dissolved in 1.2mL dry THF, followed by the addition of the Boc deprotected crude and TEA (55µL, 381µmol, 2 equiv.). The reaction was stirred at rt for 1h and upon completion, the solvent was removed in vacuo. The crude was purified by FCC, affording the title compound **SI10b** in a yield of 48%.

^{1}H NMR (400 MHz, CD_3OD) δ 8.17 (ddd, J = 3.0, 2.2, 0.7 Hz, 1H), 7.86 (bs, 1H), 7.79 (ddd, J = 8.6, 2.2, 0.7 Hz, 1H), 7.62 – 7.54 (m, 2H), 7.48 – 7.21 (m, 7H), 5.99 (s, 1H), 5.54 – 5.50 (m, 1H), 5.49 (s, 1H), 4.42 (dd, J = 6.8, 3.3 Hz, 2H), 4.21 (s, 2H), 3.75 – 3.69 (m, 2H), 3.66 (s, 1H), 3.55 – 3.47 (m, 3H), 3.32 (p, J = 1.7 Hz, 2H), 3.28 (ddd, J = 15.3, 2.4, 1.2 Hz, 1H), 3.21 (ddd, J = 6.9, 5.2, 2.0 Hz, 2H), 2.92 – 2.83 (m, 1H), 1.00 (s, 9H).

^{13}C NMR (101 MHz, CD_3OD) δ 151.5, 150.8, 150.6, 149.7, 144.1, 142.2, 140.7, 135.9, 132.4, 129.7, 128.1, 127.3, 127.1, 126.0, 123.7, 123.6, 123.5, 123.4, 123.3, 121.0, 112.6, 109.4, 84.4, 78.0, 68.9, 68.7, 66.7, 49.9, 45.3, 42.8, 31.4, 24.7.

UPLC-MS (ESI) m/z calculated for $[C_{35}H_{39}N_6O_{12}S_2]$ $[M+H]^+$ 799.2, found 799.1

Compound SI11a



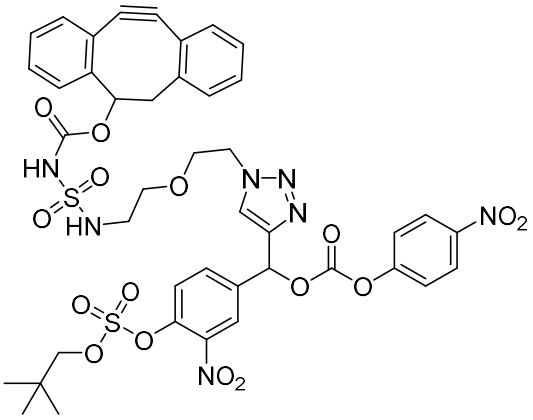
Compound **SI10a** (405mg, 513µmol, 1 equiv.) was dissolved in 17mL dry DMF under inert atmosphere and TEA (143µL, 1.03mmol, 2 equiv.) and bis(4- nitrophenyl) carbonate (468mg, 1.54mmol, 3 equiv.) were added. The reaction was stirred overnight at rt and upon completion, was acidified by the addition of 1% aq. HCl. The reaction was extracted with EtOAc and the organic phase then washed with sat. aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and the solvent removed in vacuo. The crude was purified by FCC, affording the title compound **SI11a** in a yield of 58%.

^{1}H NMR (400 MHz, CD_3OD) δ 8.31 – 8.22 (m, 2H), 8.00 (d, J = 4.3 Hz, 1H), 7.57 (t, J = 7.9 Hz, 1H), 7.51 – 7.24 (m, 11H), 6.90 (d, J = 6.3 Hz, 1H), 5.54 (dtd, J = 4.7, 3.0, 1.7 Hz, 1H), 4.47 (dt, J = 7.9, 4.8 Hz, 2H), 4.28 (d, J = 1.7 Hz, 2H), 3.81 – 3.70 (m, 2H), 3.60 – 3.51 (m, 2H), 3.34 – 3.27 (m, 1H), 3.29 – 3.19 (m, 2H), 2.92 – 2.82 (m, 1H), 2.05 (s, 2H), 1.04 (d, J = 1.0 Hz, 9H).

^{13}C NMR (101 MHz, CD_3OD) δ 157.3, 155.4 (d, J = 1.5 Hz), 155.3 (dd, J = 253.7, 3.3 Hz), 151.6, 151.5, 150.9, 150.6 145.6, 144.0, 129.7, 128.0, 127.3, 127.1, 126.0, 125.6 (d, J = 3.5 Hz), 125.1, 124.8, 123.5 (d, J = 2.7 Hz), 121.9, 121.0, 121.0, 112.6 (d, J = 1.6 Hz), 111.5, 111.3, 109.4, 84.3, 77.9, 77.9, 72.8, 68.8, 68.8, 68.6, 50.0, 45.3, 45.3, 42.9, 31.4, 24.7.

HRMS (ESI) m/z calculated for $[C_{42}H_{41}F_2N_6O_{14}S_2]$ $[M+H]^+$ 955.2085, found 955.2082

Compound SI11b



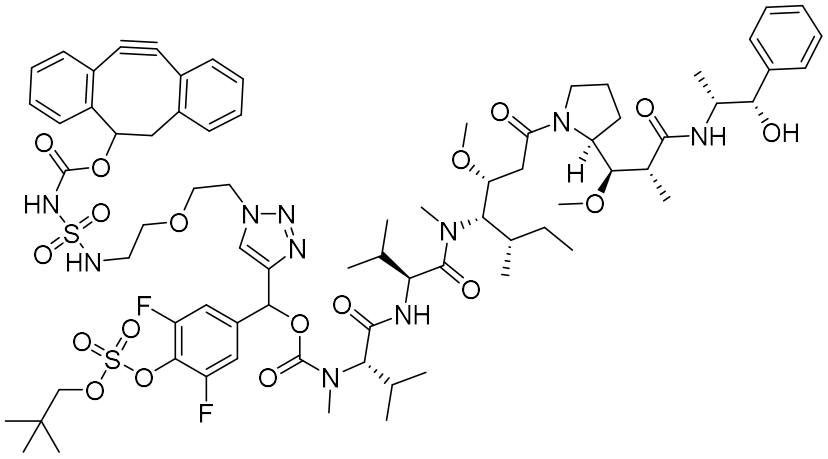
Compound **SI10b** (60mg, 75µmol, 1 equiv.) was dissolved in 1mL dry DMF under inert atmosphere and TEA (42µL, 300µmol, 4 equiv.) and bis(4- nitrophenyl) carbonate (69mg, 225µmol, 3 equiv.) were added.

The reaction was stirred overnight at rt and upon completion, was acidified by the addition of 1% aq. HCl. The reaction was extracted with EtOAc and the organic phase then washed with sat. aq. $NaHCO_3$ and brine, dried over $MgSO_4$ and the solvent removed in vacuo. The crude was purified by FCC, affording the title compound **SI11b** in a yield of 38%.

^{1}H NMR (400 MHz, CD_3OD) δ 8.25 (dt, J = 9.0, 1.8 Hz, 3H), 7.99 (s, 1H), 7.92 – 7.86 (m, 1H), 7.68 (s, 0H), 7.60 – 7.51 (m, 1H), 7.50 – 7.20 (m, 6H), 6.98 (d, J = 7.9 Hz, 1H), 5.58 – 5.48 (m, 1H), 4.46 (dq, J = 9.4, 4.6 Hz, 2H), 4.25 (d, J = 1.7 Hz, 2H), 3.81 – 3.63 (m, 2H), 3.54 (dq, J = 6.8, 5.2, 4.6 Hz, 2H), 3.28 (ddd, J = 15.3, 4.7, 2.2 Hz, 1H), 3.23 (td, J = 5.2, 1.7 Hz, 2H), 2.93 – 2.76 (m, 1H), 1.02 (dd, J = 3.9, 1.3 Hz, 9H).

^{13}C NMR (101 MHz, CD_3OD) δ 156.8, 153.2, 152.9, 152.3, 152.0, 147.0, 145.4, 143.0, 139.4, 134.7, 131.1, 129.4, 128.7, 128.5, 127.4, 127.1, 126.7, 126.2, 126.1, 125.5, 124.9 (2C), 123.3, 122.4 (2C), 116.5, 110.8 (2C), 86.0, 79.3, 79.2, 74.1, 70.1, 70.0, 51.5, 46.7, 44.3, 32.8, 26.1

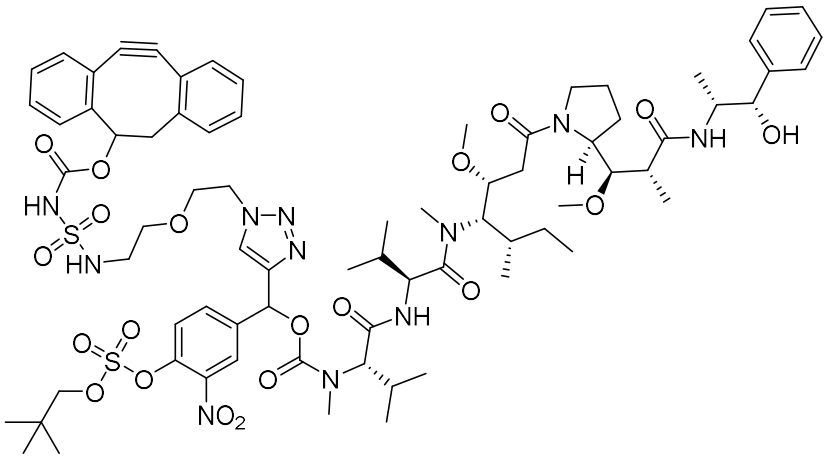
Compound SI12a



Compound **SI11a** (50mg, 52µmol, 1 equiv.), MMAE (56mg, 79µmol, 1.5 equiv.) and pyridine (148µL, 1.83mmol, 35 equiv.) were dissolved in 520µL dry DMF under inert atmosphere. To the reaction, were added DIPEA (9µL, 52µmol, 1 equiv.) and HOBt hydrate (8mg, 55µmol, 1.05 equiv.) and the reaction was stirred for 47h at rt. Upon completion, the reaction was purified by preparative HPLC, affording the title compound **SI12a** in a yield of 11%.

HRMS (ESI) m/z calculated for $[C_{75}H_{102}F_2N_{10}O_{18}S_2]$ $[M+H]^+$ 1533.6856, found 1533.6885.

Compound SI12b



Compound **SI11b** (10mg, 10µmol, 1 equiv.), MMAE (7mg, 10µmol, 1 equiv.) and pyridine (29µL, 363µmol, 35 equiv.) were dissolved in 300µL dry DMF under inert atmosphere. To the reaction, were added DIPEA (2µL, 10µmol, 1 equiv.) and HOBt hydrate (2mg, 13µmol, 1.25 equiv.) and the reaction was stirred for 72h at rt. The reaction was purified by preparative HPLC, affording the title compound **SI12b** in a yield of 1%.

HRMS (ESI) m/z calculated for $[C_{75}H_{103}N_{11}O_{20}S_2]$ $[M+H]^+$ 1542.6901, found 1542.6891

Sulfatase hydrolysis and stability

Before initiating any enzyme studies, a standard curve for AMC was generated to establish the relationship between fluorescence intensity and the concentration of AMC in solution. This standard curve was prepared using an AMC in DMSO stock and diluting with 0.1M NaOAc buffer at pH=5 to obtain concentrations between 0.02 to 12 µM, in a 0.1 M NaOAc buffer at pH = 5. The assay was performed in triplicate to reduce errors, and a blank containing only buffer solution was included to account for background noise. AMC was excited at λ_{ex} = 380 nm, and fluorescence emission was measured at λ_{em} = 460 nm with a 20 nm bandwidth. The resulting standard curve is shown in figure S1.

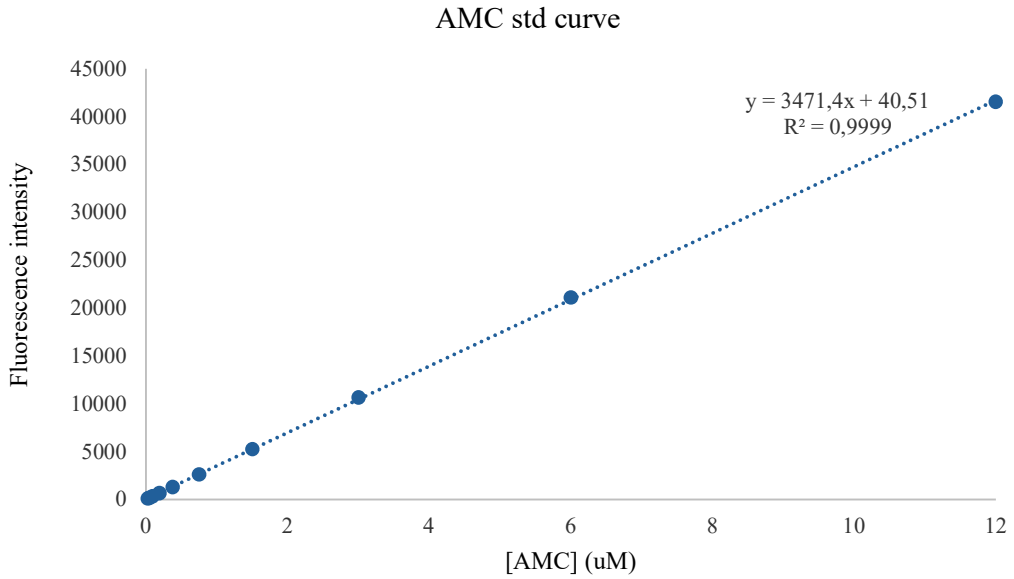


Figure S1: AMC Fluorescence standard curve.

The standard curve displayed excellent linearity, with a coefficient of determination, R^2, of 0.9999. The resulting equation, relating fluorescence intensity, F.I., to AMC concentration, C, was determined to be:

$$F.I. = 3471.4 \cdot C + 40.51$$

This standard curve was used in the following chapters to quantify fluorescence intensity during the incubation of probes with sulfatase enzymes.

Sulfatase from Helix Pomatia with I-monoF, I-diF, I-H, I-NO_2: Release profile

HP sulfatase activity: The activity of HP sulfatase was evaluated using *p*-nitrocatechol sulfate (NCS) as a substrate, following the procedure outlined by the vendor, R&D Systems.

A standard curve was generated using five different concentrations of *p*-nitrocatechol (NC) ranging from 0.05 mM to 0.6 mM. The assay was performed in triplicate to minimize errors, and a blank sample containing only buffer was included to account for potential background noise. The resulting standard curve is shown in figure S2, with an R^2 of 0.996.

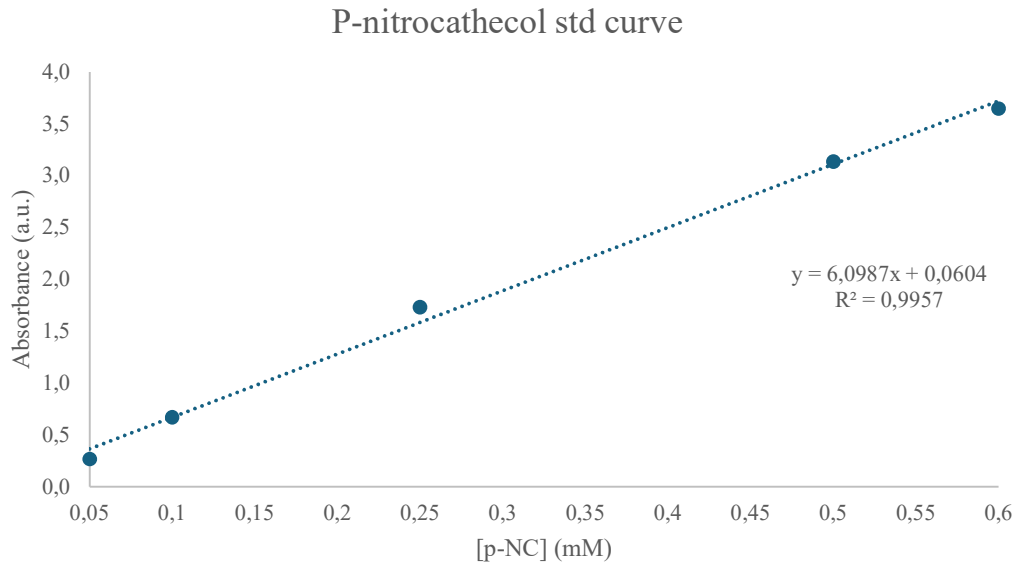


Figure S2: p-NC absorbance standard curve.

For the enzyme activity assay, a solution containing NCS and 13.55 µg mL HP sulfatase was incubated for exactly 30 minutes. This was performed in triplicate to reduce errors, and a blank containing only NCS and no enzyme was included to account for background noise. The reaction was quenched with 0.2M NaOH, and the absorbance measured at 510 nm: A_{510} = 0.286

The specific enzyme activity was calculated as described by R&D Systems:

$$\text{Specific activity } (\mu\text{mol min}^{-1}\mu\text{g}^{-1}) = \frac{\text{Absorbance (OD)} \cdot \text{Conversion factor } (\mu\text{mol/OD})}{\text{Incubation time (min)} \cdot \text{Amount of enzyme } (\mu\text{g})}$$

Using the measured absorbance and the conversion factor derived from the NC standard curve, the specific enzyme activity was calculated :

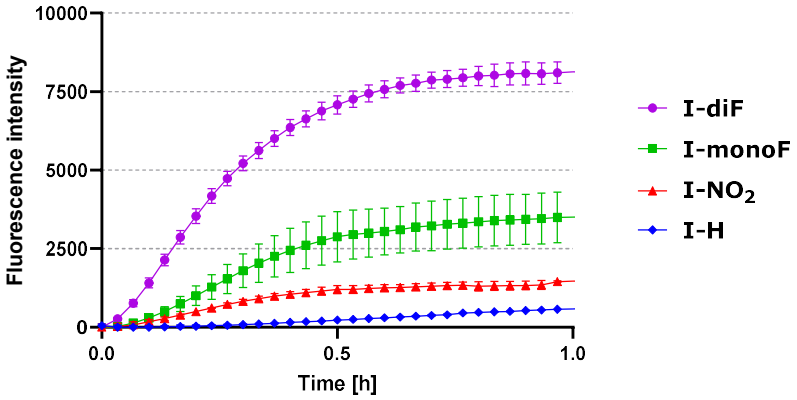
$$\text{Specific Activity} = 4.62 \cdot 10^{-4}\ \mu\text{mol min}^{-1}\ \mu\text{g}^{-1}$$

The enzyme activity unit, U, is defined as the ability of an enzyme to hydrolyze 1.0 µmol of NCS per hour at pH = 5 and 37°C. Therefore, the actual enzyme activity of HP sulfatase is:

$$\text{Specific Activity} = 0.02769\ \text{U/µg} = 27687\ \text{U/g}$$

Thus, one gram of enzyme catalyzes the conversion of 27687 µmol of *p*-nitrocatechol sulfate to p-nitrocatechol each hour.

Cleavage study: 100uL 10uM **I-monoF, I-diF, I-H, I-NO_2** in 10% DMSO and 0.1M NaOAc buffer (pH=5) was reacted with 100uL 0.1876U/mL *Helix Pomatia* in the same buffer. Blanks contained 100uL 10uM **I-monoF, I-diF, I-H, I-NO_2** in 10% DMSO and 0.1M NaOAc buffer (aq, pH=5) with 100uL buffer. Reacted for 1h at 37°C with reads every 2 minutes. Everything was done in triplicate to reduce errors. The listed values are means of technical duplicates with SEM errors.

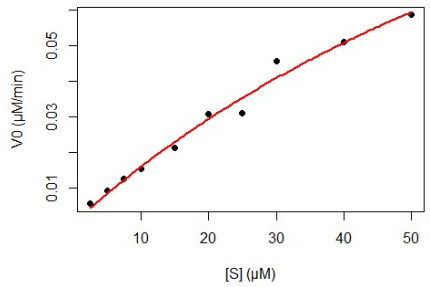
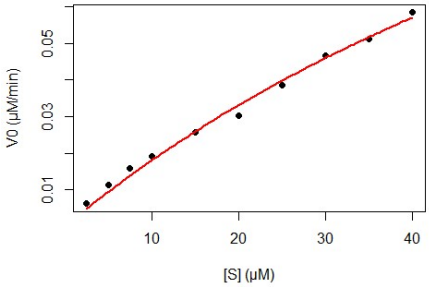
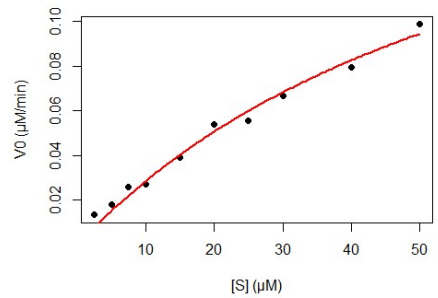
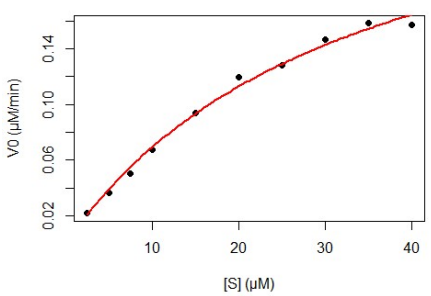


	AMC [µM]	%AMC release
I-diF	2.33	46.7
I-monoF	1.00	19.9
I-NO$_2$	0.41	8.2
I-H	0.16	3.1

Figure S3: Cleavage profiles of probe **I-monoF, I-diF, I-NO$_2$,** and **I-H** by HP Sulfatase.

Sulfatase from Helix Pomatia with I-monoF, I-diF, I-H, I-NO$_2$: Kinetic analysis

Michaelis Menten study: 100uL **I-monoF, I-diF, I-H, I-NO$_2$** of different concentrations (5uM-100uM) in 0.1M NaOAc buffer (pH=5) was reacted with 100uL 0.375U/mL *Helix Pomatia* in the same buffer. Blanks were included for each probe concentration. This reacted for 45 minutes at 37°C with reads every minute. Everything was done in triplicate to reduce error. The Michaelis Menten study was conducted twice and listed kinetic parameters are averages of technical duplicates.

Probe	First round	Second round
I-H	 Vmax = 0.19 ± 0.05 uM/min Km = 109 ± 36.0 uM	 Vmax = 0.21 ± 0.04 uM/min Km = 105 ± 29 uM
I-NO$_2$	 Vmax = 0.22 ± 0.04 uM/min Km = 68.0 ± 16.3 uM	 Vmax = 0.30 ± 0.02 uM/min Km = 33.6 ± 4.24 uM

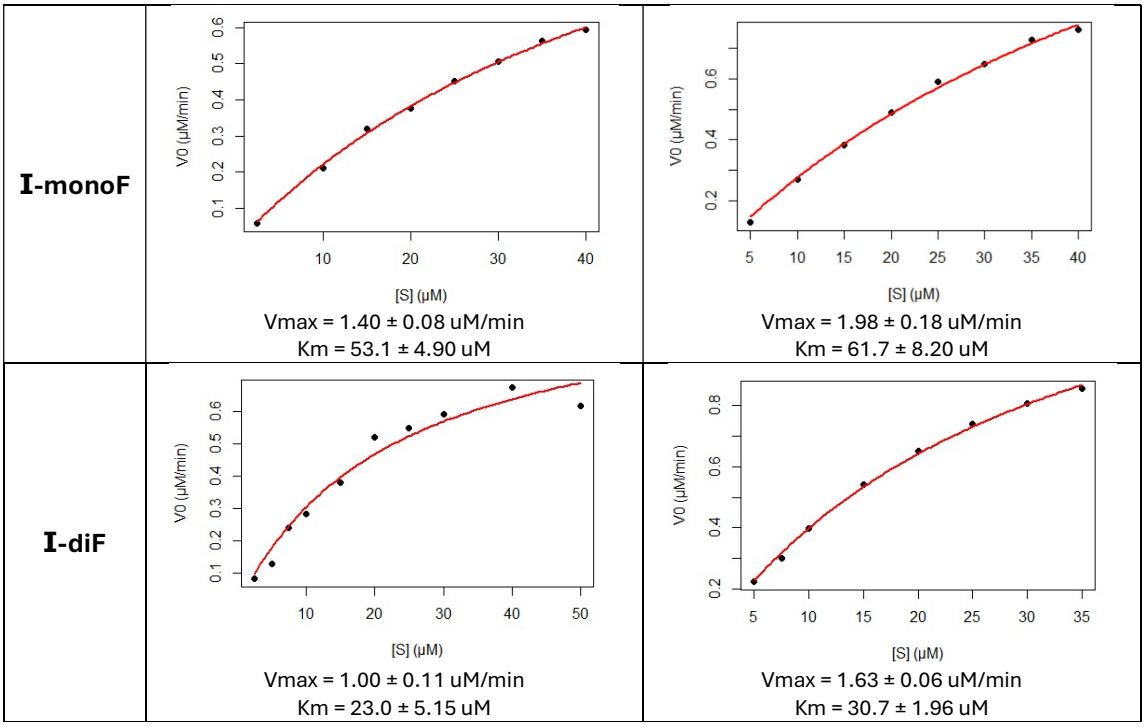


Figure S4: Individual Michaelis Menten plots for cleavage of probes **I-monoF, I-diF, I-H,** and **I-NO₂** by HP Sulfatase.

*Table S1.1 Summary table of kinetic parameters of **I-monoF, I-diF, I-H,** and **I-NO₂** with HP sulfatase. Averages of duplicates with SEM.*

	Vmax (uM/min)	Km (uM)	kcat (min^{-1})	kcat/Km ($uM^{-1}·min^{-1}$)
I-H	0.20 ± 0.01	107 ± 2.16	2.48 ± 0.11	0.023 ± 0.002
I-NO₂	0.26 ± 0.04	50.7 ± 17.1	3.29 ± 0.50	0.08 ± 0.04
I-monoF	1.69 ± 0.29	57.4 ± 4.27	21.2 ± 3.64	0.37 ± 0.04
I-diF	1.32 ± 0.31	26.9 ± 3.88	16.5 ± 3.89	0.61 ± 0.06

Arylsulfatase B (ARSB)

Enzyme activity: The activity of ARSB was evaluated using NCS as a positive control by incubating NCS and 1 µg/mL ARSB for exactly 30 mins before quenching with 0.2M NaOH (aq). The assay was performed in triplicate to reduce errors, and a blank containing only NCS and no enzyme was included to account for background noise. The resulting absorbance, adjusted for blank, was: A510 = 0.1786

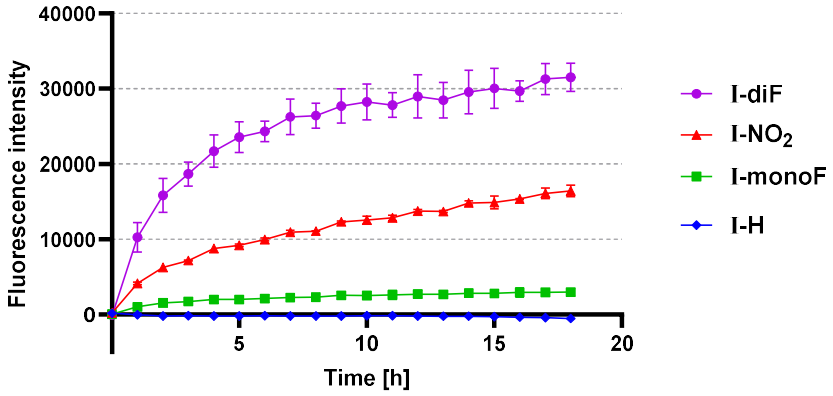
Using the equation given by R&D Systems as described for HP sulfatase**,** the specific activity was calculated:

Specific Activity = 234323.2 U/g

Arylsulfatase B (ARSB) with I-monoF, I-diF, I-H, I-NO₂: Release profile

Cleavage study: 100uL 100uM **I-monoF, I-diF, I-H, I-NO₂** in 10% DMSO and 0.1M NaOAc buffer (pH=5) was reacted with 100uL 1ug/mL ARSB enzyme in the same buffer. Blanks contained 100uL

buffer and no enzyme. Everything was done in triplicate to reduce errors, and the listed values are averages of two technical duplicates with SEM errors.



	AMC [µM]	%AMC release
I-diF	9.08	18.2
I-NO$_2$	4.72	9.4
I-monoF	0.90	1.7
I-H	0.05	0.1

Figure S5: Cleavage profiles of probe **I-monoF, I-diF, I-H,** and **I-NO$_2$** by ARSB.

Arylsulfatase B (ARSB) with I-diF and I-NO$_2$: Kinetic analysis

Michaelis Menten study: 100uL **I-diF** and **I-NO$_2$** of different concentrations (5uM-100uM) in 0.1M NaOAc buffer (pH=5) was reacted with 100uL 1ug/mL ARSB in the same buffer. Blanks were included for each probe concentration. This was reacted for 1h at 37°C with reads every two minutes. Everything was done in triplicate to reduce error. No technical duplicate due to lack of enzyme.

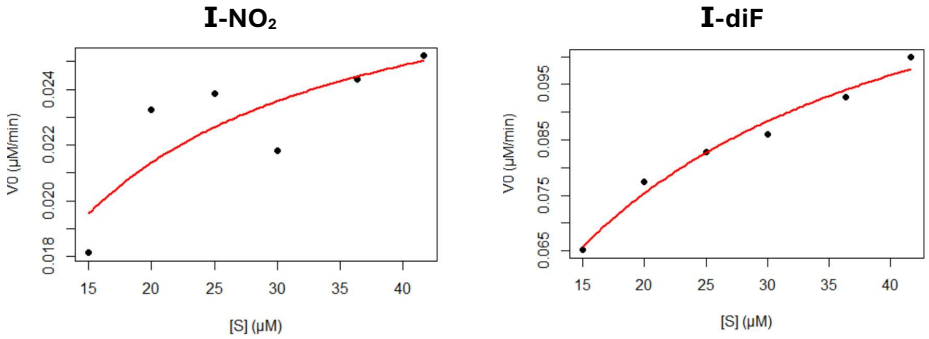


Figure S6: Individual Michaelis Menten plots for cleavage of probes **I-NO$_2$** and **I-diF** by ARSB.

	Vmax (uM/min)	Km (uM)	kcat (min^{-1})	kcat/Km (uM^{-1}·min^{-1})
I-NO$_2$	0.030	8.00	1.64	0.22
I-diF	0.135	15.75	7.44	0.47

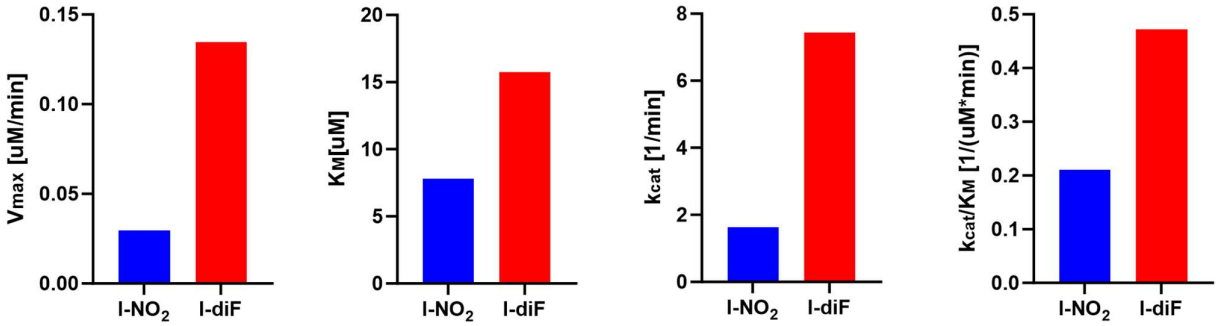


Figure S7: Michaelis Menten parameters for cleavage of probes **I-diF** + **I-NO₂** by ARSB.

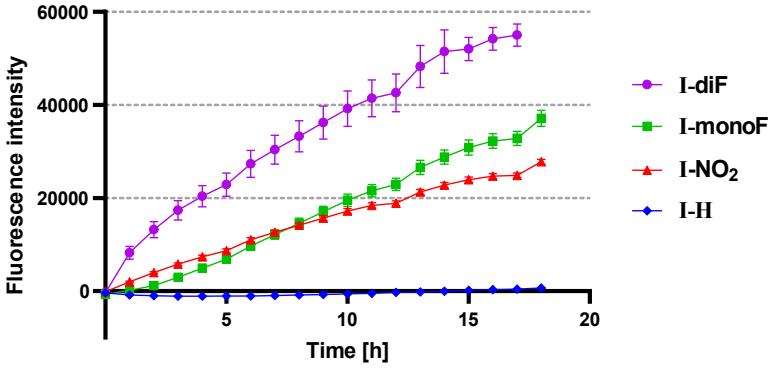
Arylsulfatase A (ARSA) with I-monoF, I-diF, I-H, and I-NO₂ : Release profile

Enzyme activity: *The* activity of ARSA was evaluated using NCS as a positive control by incubating NCS and 1 µg/mL ARSA for exactly 30 mins before quenching with 0.2M NaOH. The assay was performed in triplicate to reduce errors, and a blank containing only NCS and no enzyme was included to account for background noise. The resulting absorbance, adjusted for blank, was: A510 = 0.002067

Using the equation given by R&D Systems, see HP sulfatase**,** the specific activity was calculated:

Specific Activity = 2711.9 U/g

Cleavage study: 100uL 100uM **I-monoF, I-diF, I-H, I-NO₂** in 10% DMSO and 0.1M NaOAc buffer (pH=5) was reacted with 100uL 10ug/mL ARSA enzyme in the same buffer. Blanks contained 100uL buffer and no enzyme. Everything was done in triplicates to reduce errors, and listed values are averages of two technical duplicates with SEM errors.

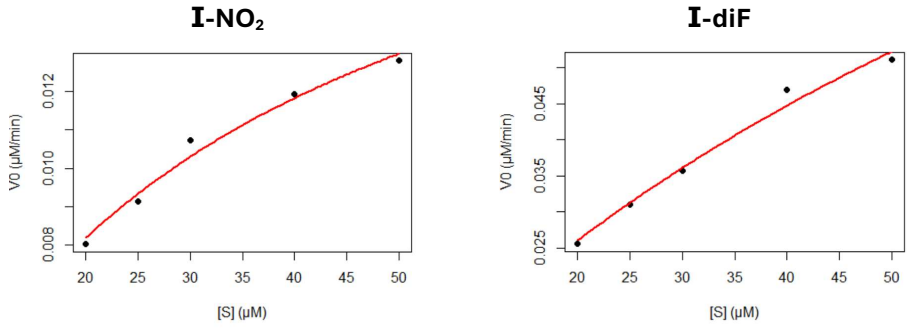


	AMC [µM]	%AMC release
I-diF	17.1	34.1
I-monoF	11.5	22.7
I-NO₂	8.4	16.8
I-H	0.28	0.6

Figure S8: Cleavage profiles of probe **I-monoF, I-diF, I-H,** and **I-NO₂** by ARSA.

Arylsulfatase A (ARSA) with I-monoF, I-diF, I-H, and I-NO₂: Kinetic analysis

Michaelis Menten study: 100uL **I-diF** and **I-NO₂** of different concentrations (5uM-100uM) in 0.1M NaOAc buffer (pH=5) was reacted with 100uL 10ug/mL ARSA in the same buffer. Blanks were included for each probe concentration. This reacted for 1h at 37°C with reads every two minutes. Everything was done in triplicate to reduce errors. No technical duplicate due to lack of enzyme.



Vmax = 0.021 ± 0.002 uM/min
Km = 32.0 ± 5.34 uM

Vmax = 0.158 ± 0.03 uM/min
Km = 101 ± 29.8 uM

Figure S9: Individual Michaelis-Menten plots for cleavage of probes **I-diF** and **I-NO₂** by ARSA.

	Vmax (uM/min)	Km (uM)	kcat (min^{-1})	kcat/Km ($uM^{-1}·min^{-1}$)
I-NO₂	0.021	32.0	0.113	0.00353
I-diF	0.158	101	0.838	0.00827

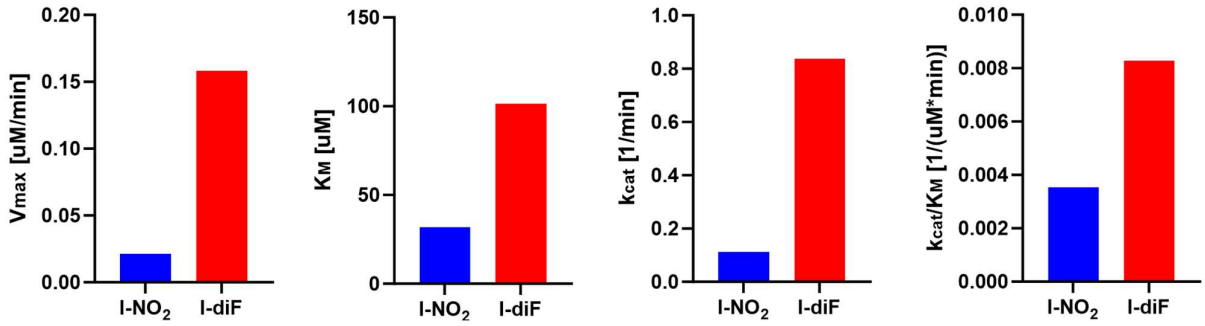


Figure S10: Michaelis Menten parameters for cleavage of probes **I-diF, I-NO₂** by ARSA

Plasma stability

Stability of probes **I-monoF** and **I-diF** was tested in human and mouse plasma. Each well contained 15uL 100uM probe, 75uL PBS (pH=7.43) and 60uL plasma (total well volume = 150uL). Blanks contained 15uL 100uL, 75uL PBS (pH=7.43) and 60uL H2O. In both human and mouse serum the probes are all stable over 12h. (Fig S11).

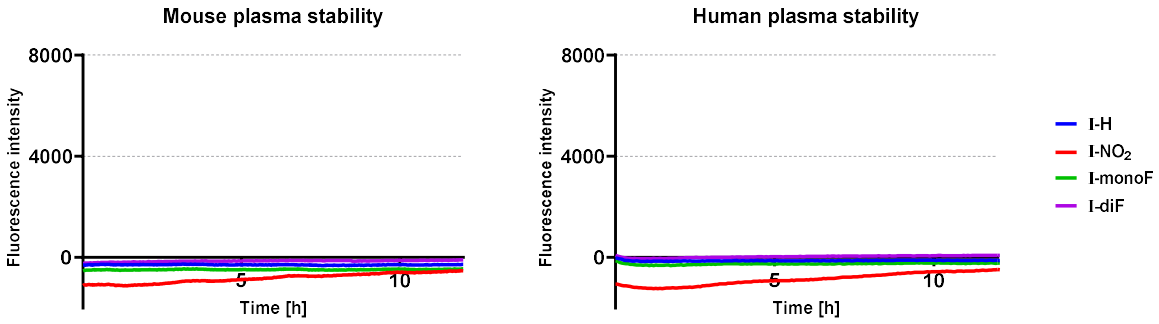


Figure S11: Incubation of probes **I-monoF, I-diF, I-H,** and **I-NO$_2$** in human plasma and mouse plasma, respectively, over 12 h.

Cytotoxicity of ADC in vitro cell viability assay

The viability assay procedure was inspired largely from a literature procedure.[4] Briefly, in a 96-well plate (Costar) cells were seeded out (1x10^3 cells/well) in a total volume of 90 uL culture medium. After 24 h, old medium was removed, and fresh media was added along with serially diluted ADB1 and ADC2 with a maximum final ADC concentration of 10 nM (10% PBS v/v). Cells were incubated for either 4 days or 7 days, at which point overall viability was evaluated by adding 15 µL CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) (Promega). The 96-well plate was incubated for an additional 60 min, and the plate read at 490 nm (background subtraction at 630 nm), and viability calculated as the percentage of internally untreated control cells.

HER2-positive and control cell lines

Cell lines positive for the HER2 receptor SKBR3 and BT474 were maintained in McCoy's 5A medium and in Dulbecco's Modified Eagle Medium (DMEM), respectively. The HER2 negative cell line MCF-7 was maintained in Eagle's Minimum Essential Medium (EMEM). All media were supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were incubated at 37°C/5% CO_2 and harvested at 50-75% confluency.

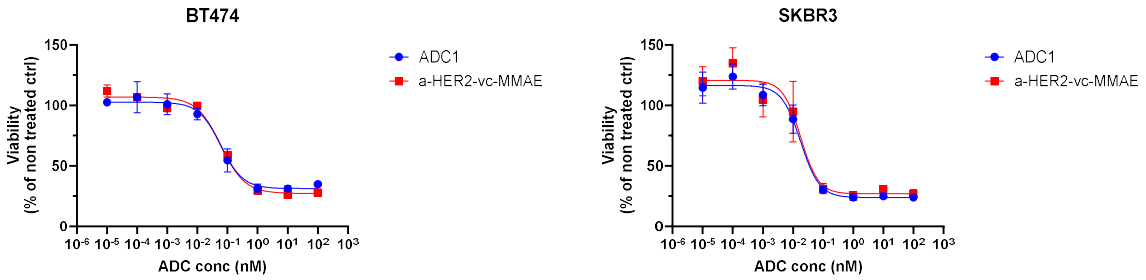
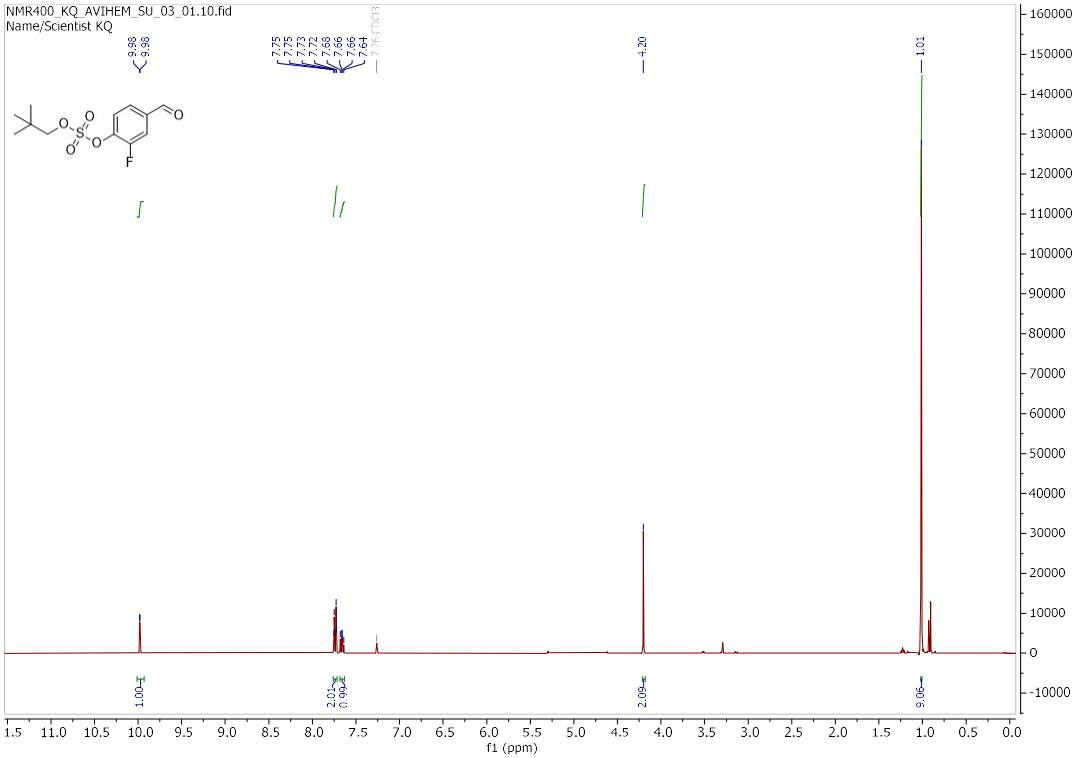
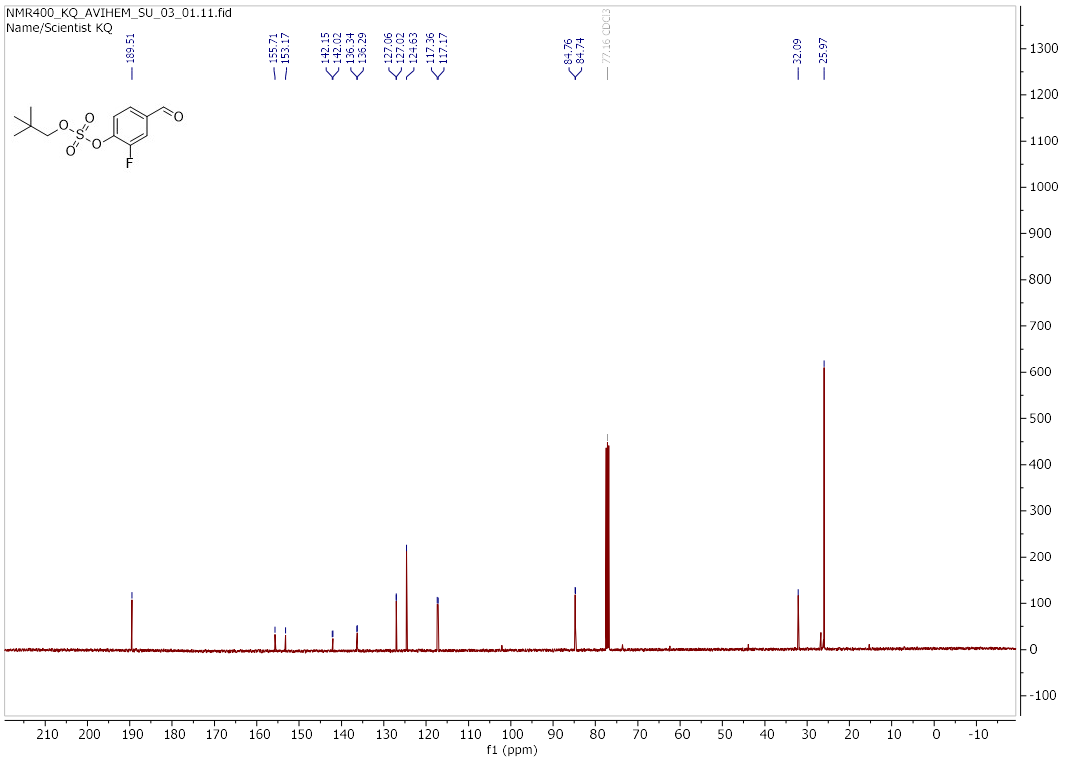


Figure S12: Anti-proliferative effect on HER2-positive SKBR3 and BT474 cells after 4 days incubation with ADC1 vs HER2-vc-MMAE.

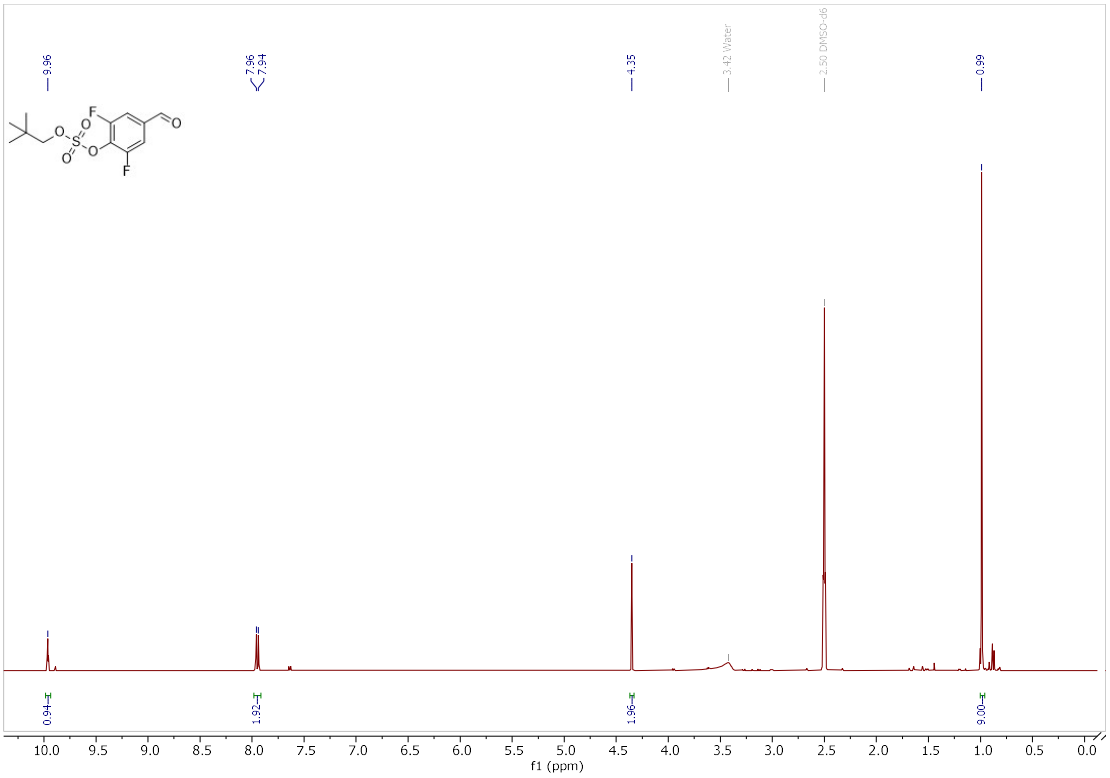
NMR Spectra

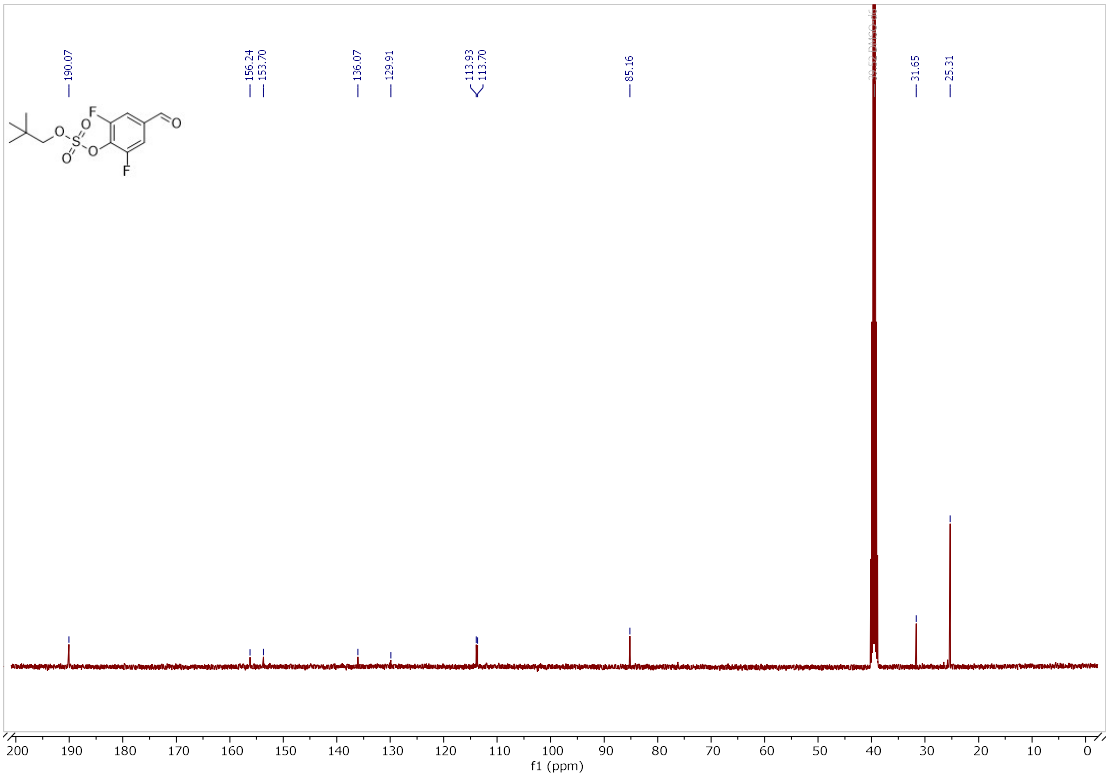
2-Fluoro-4-formylphenyl neopentyl sulfate SI2a



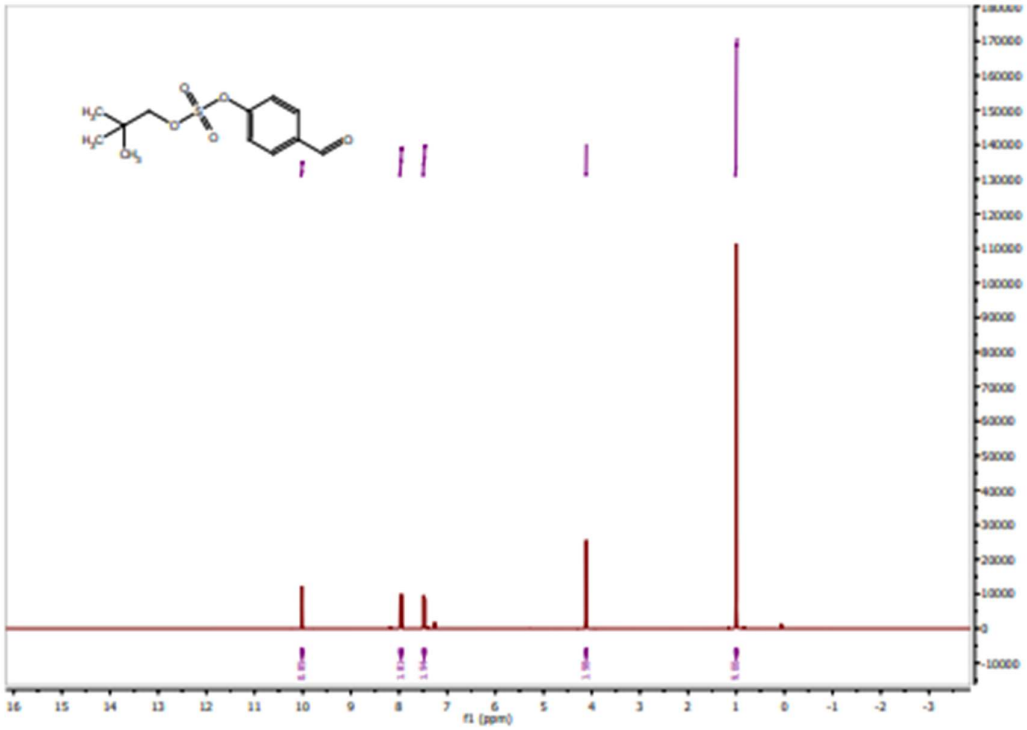


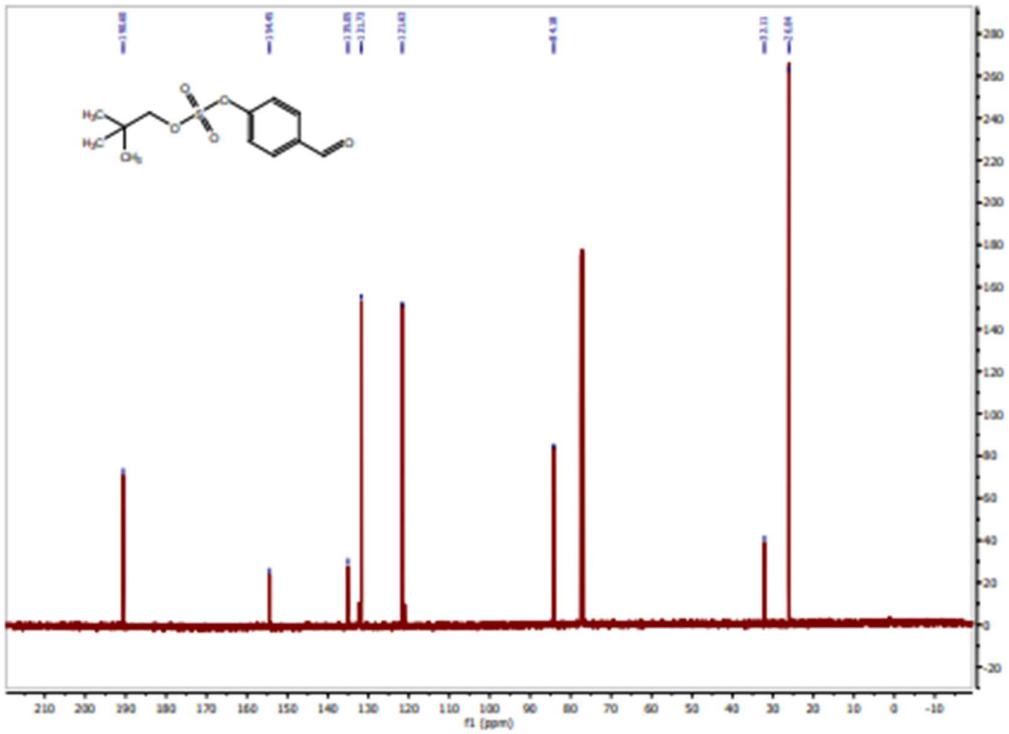
2,6-Difluoro-4-formylphenyl neopentyl sulfate (SI2b)



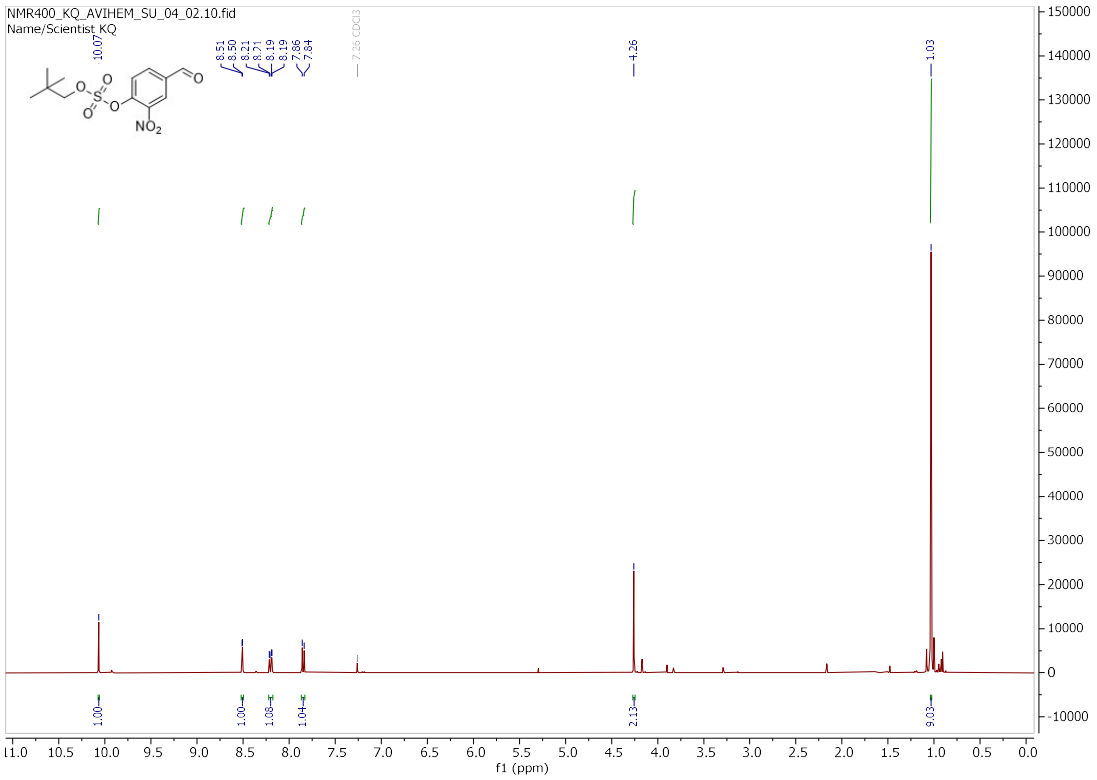


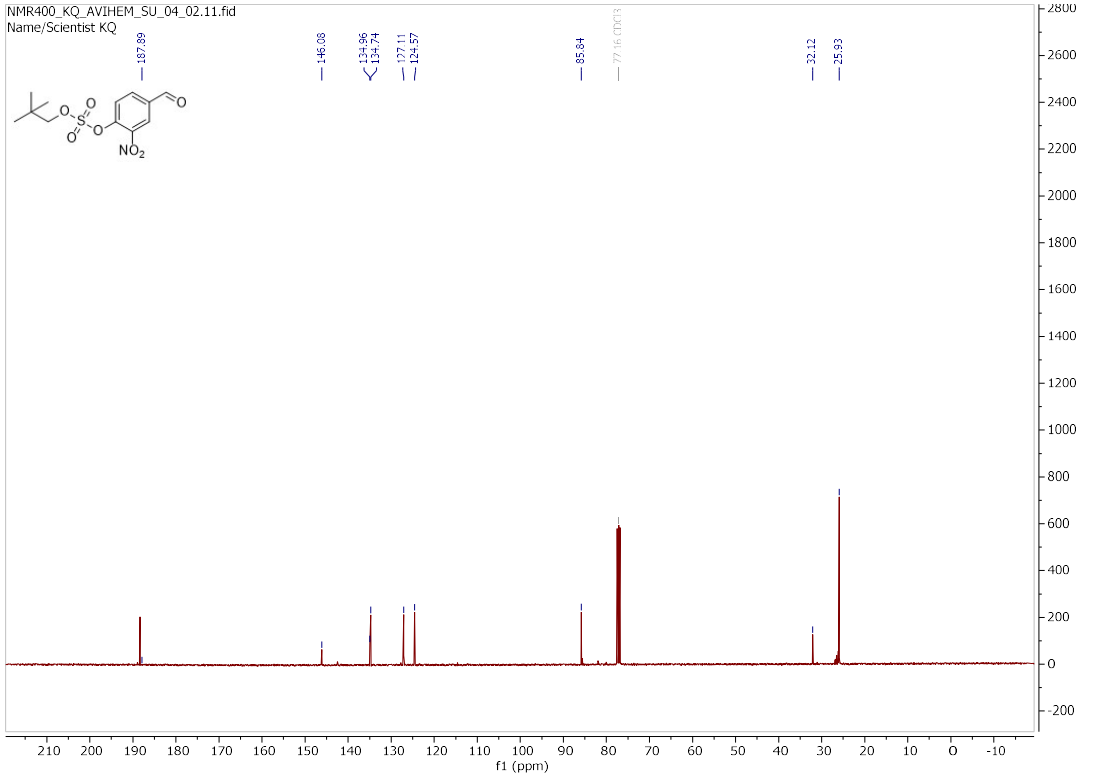
4-Formylphenyl neopentyl sulfate (SI2c)



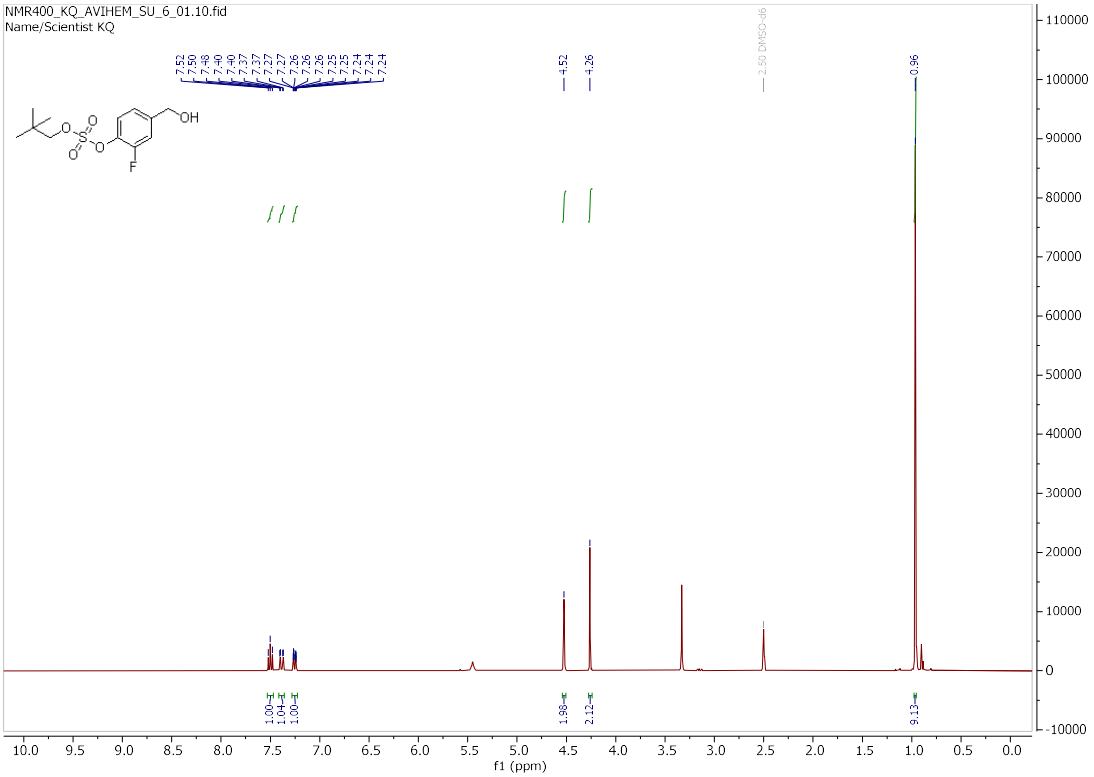


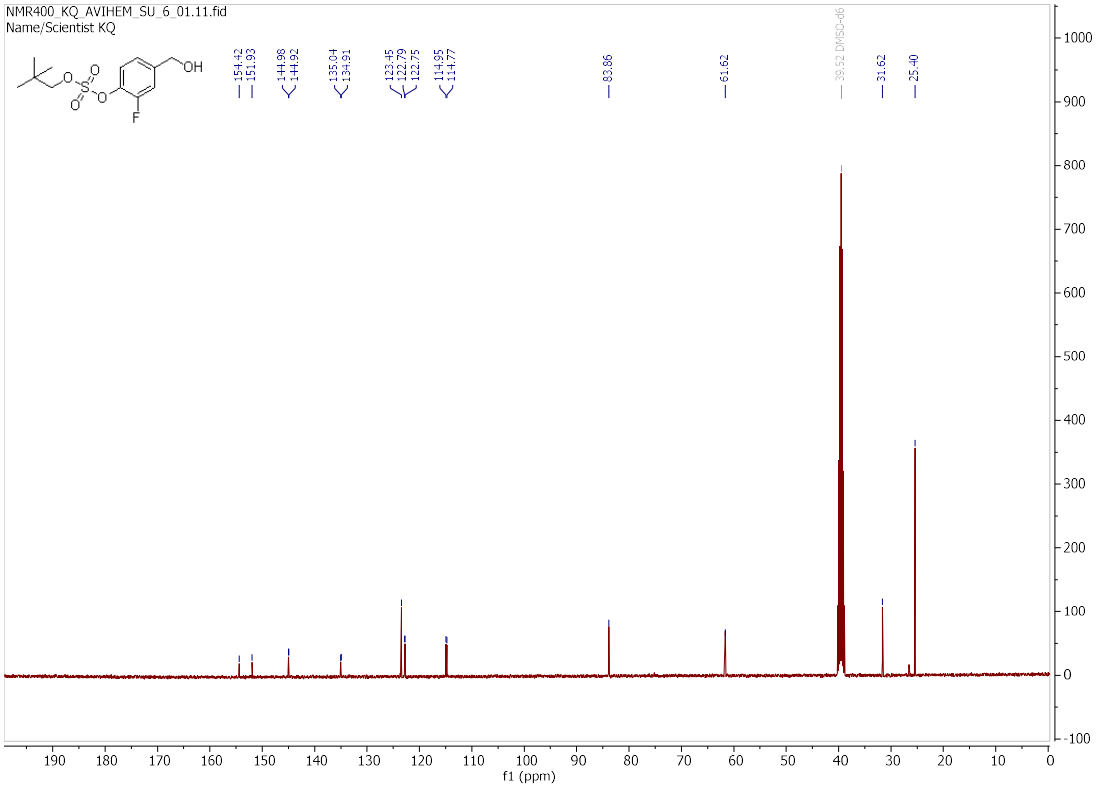
4-Formyl-2-nitrophenyl neopentyl sulfate (SI2d)



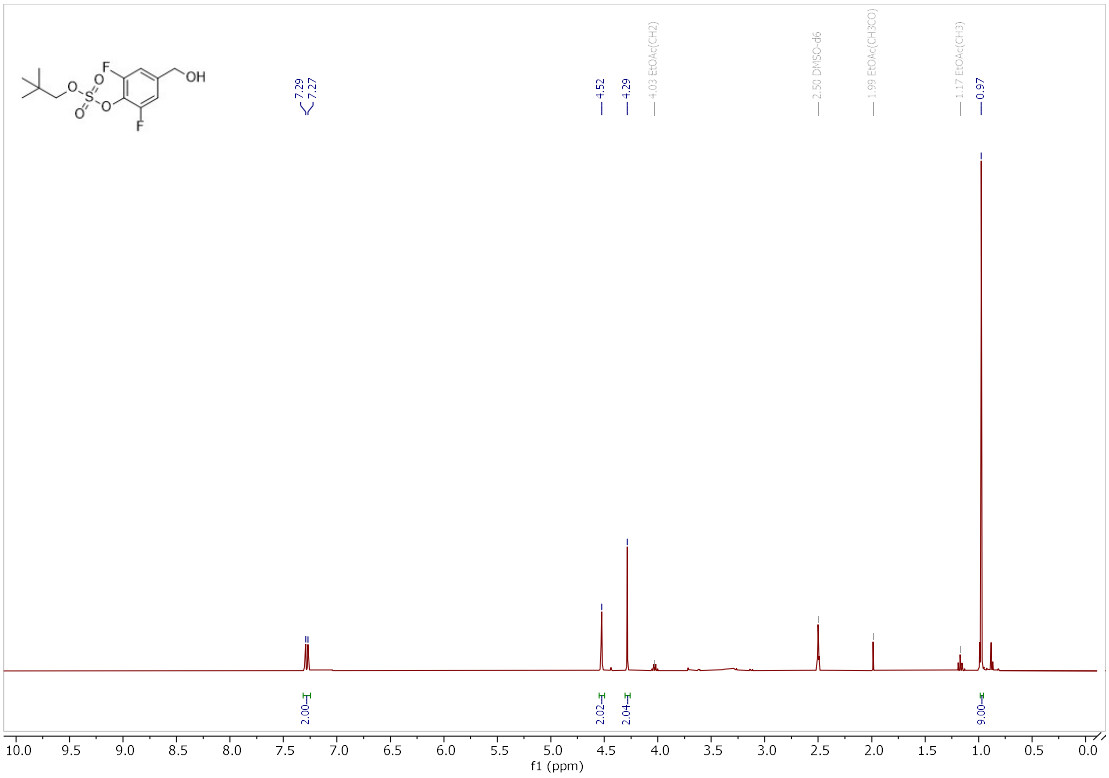


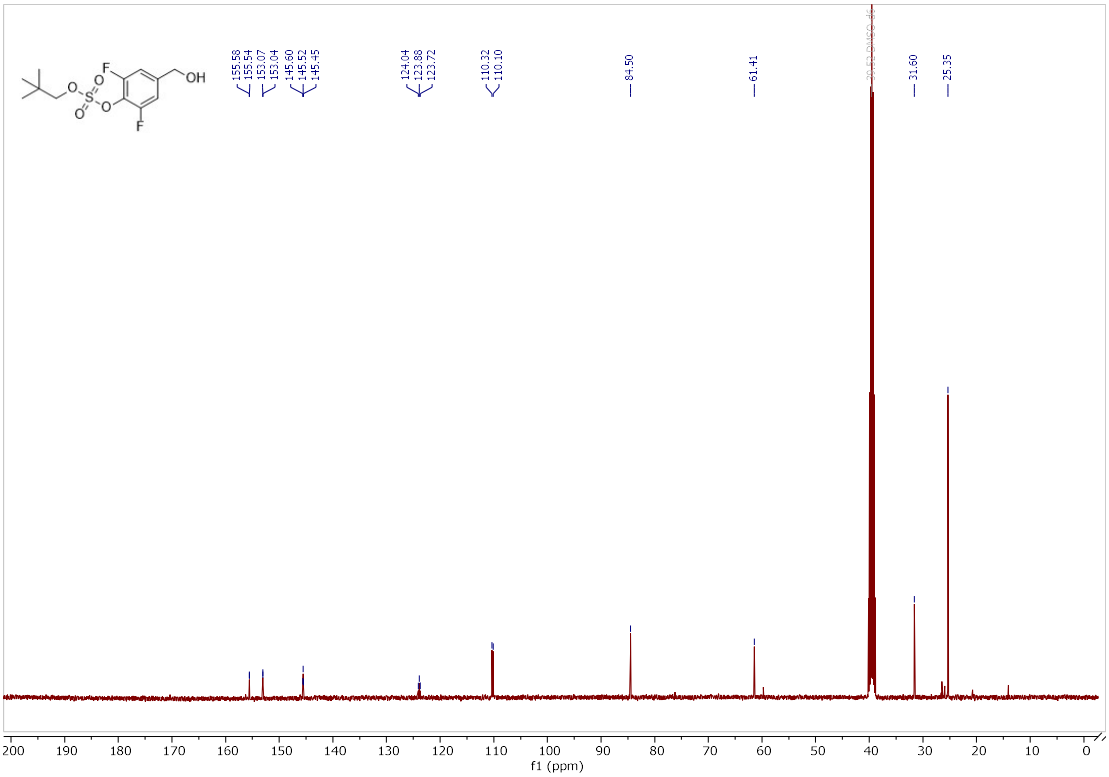
2-Fluoro-4-(hydroxymethyl)phenyl (neopentyl) sulfate SI3a



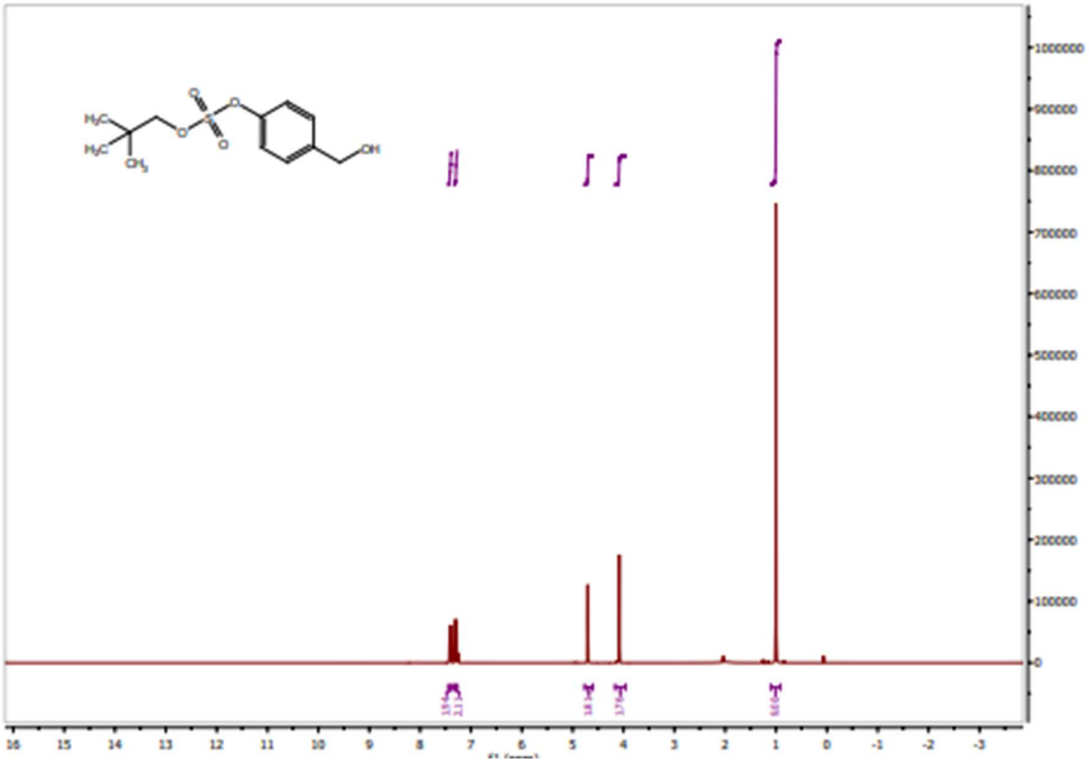


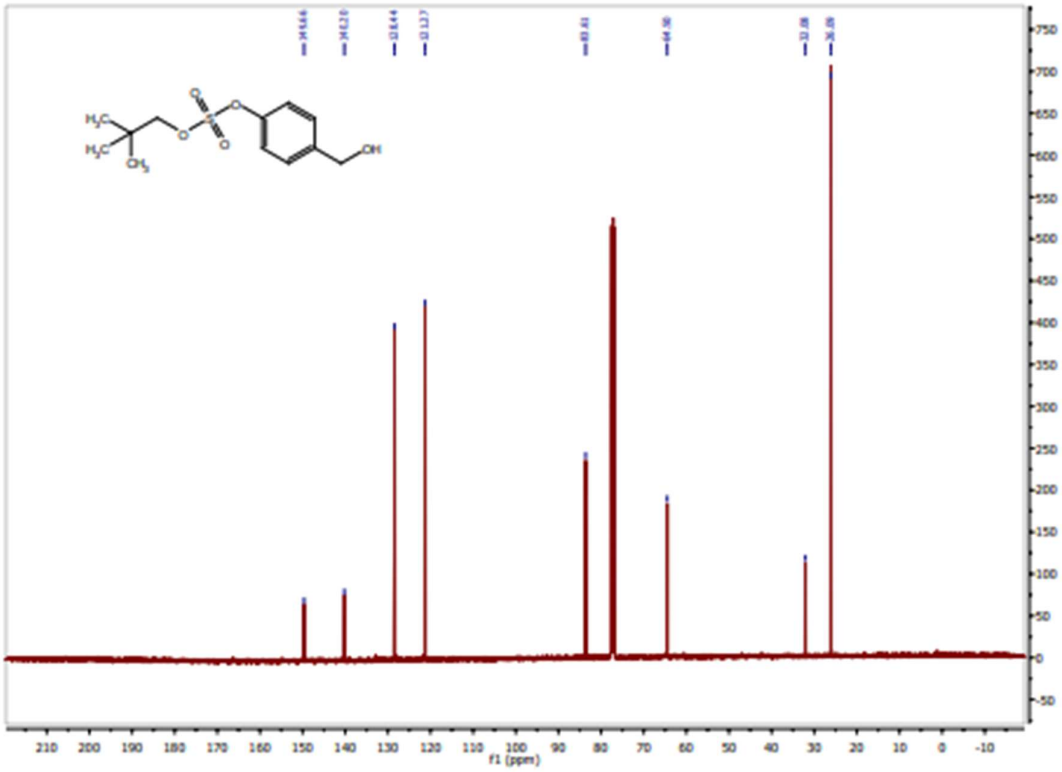
2,6-Difluoro-4-(hydroxymethyl)phenyl neopentyl sulfate SI3b



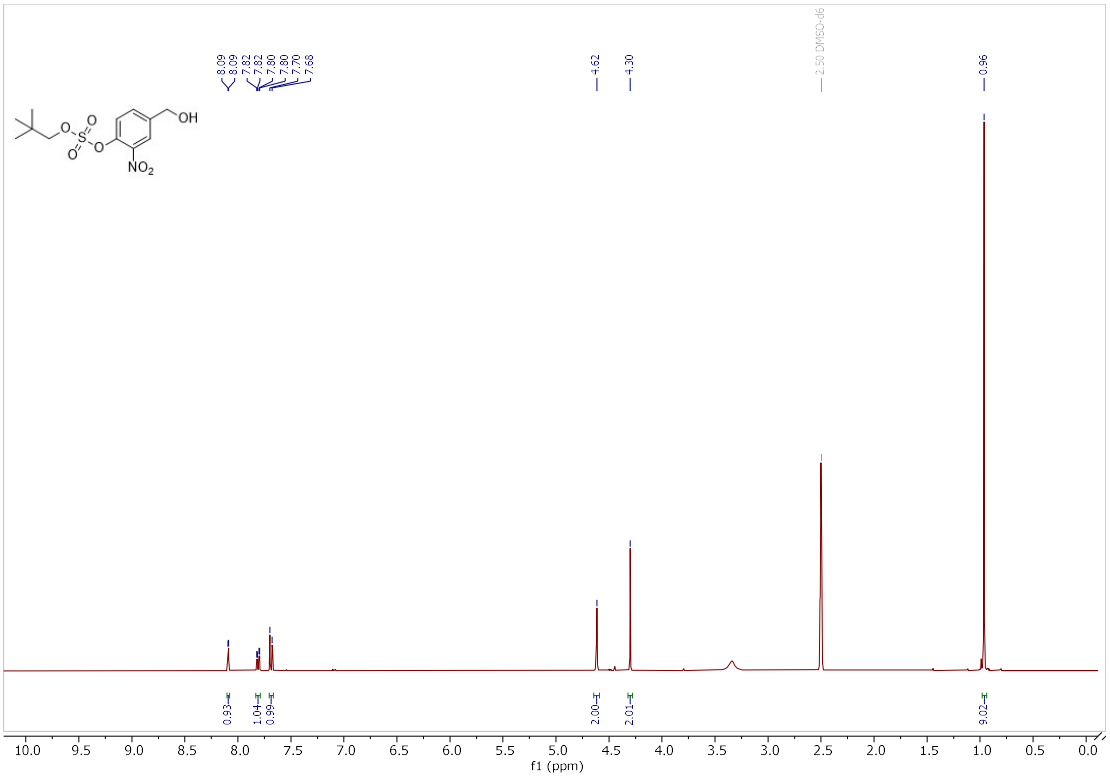


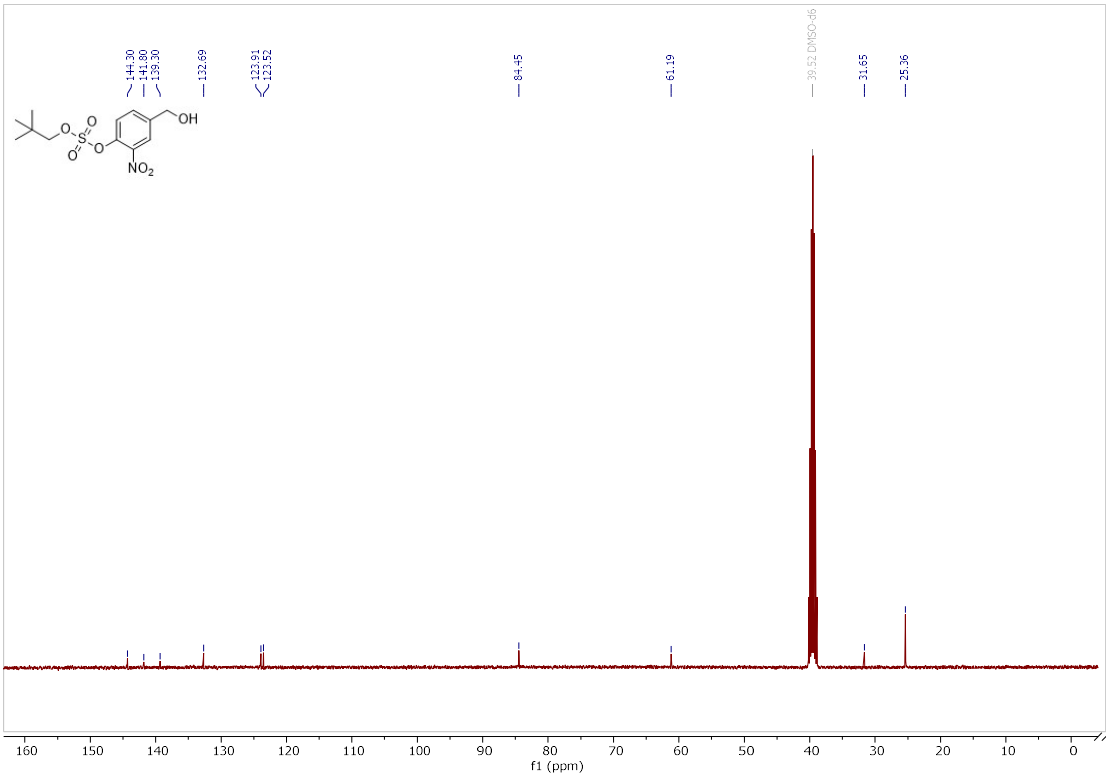
4-(Hydroxymethyl)phenyl neopentyl sulfate SI3c



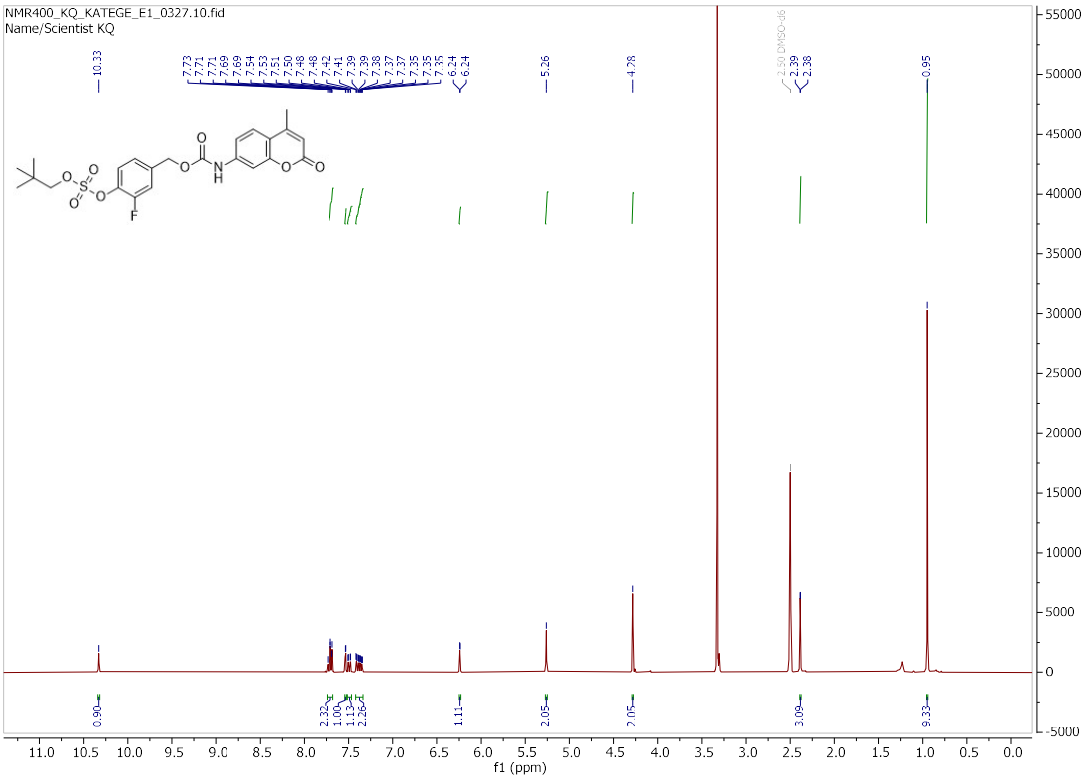


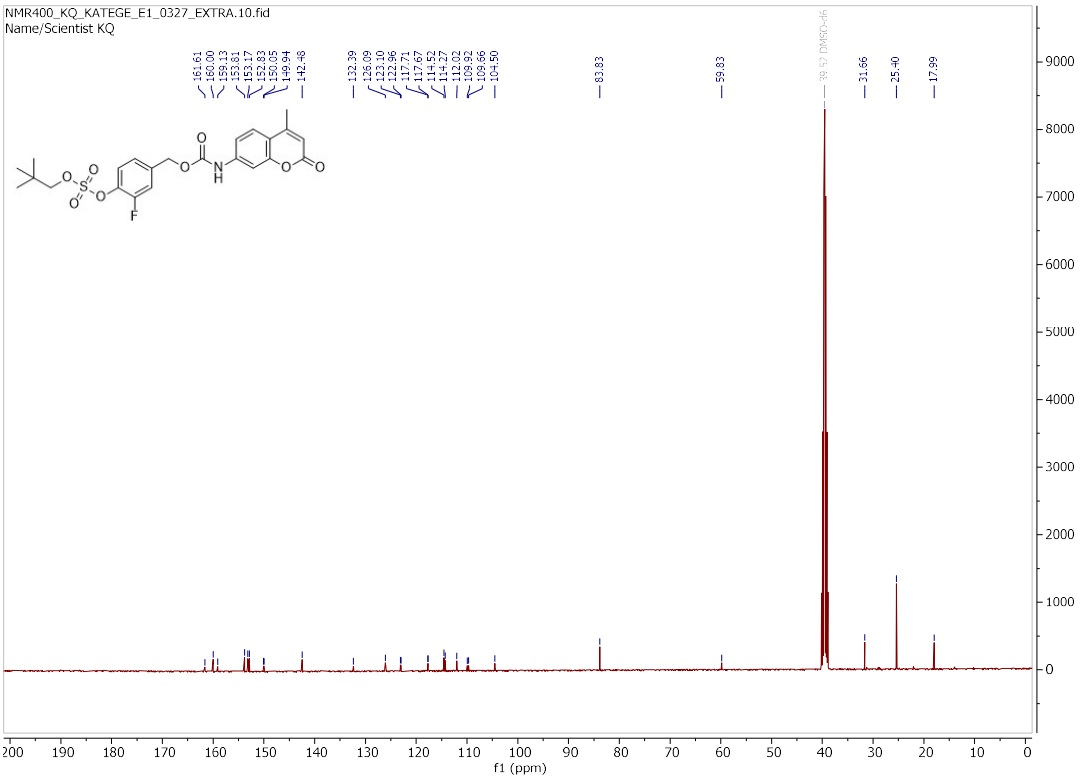
4-(Hydroxymethyl)-2-nitrophenyl neopentyl sulfate SI3d



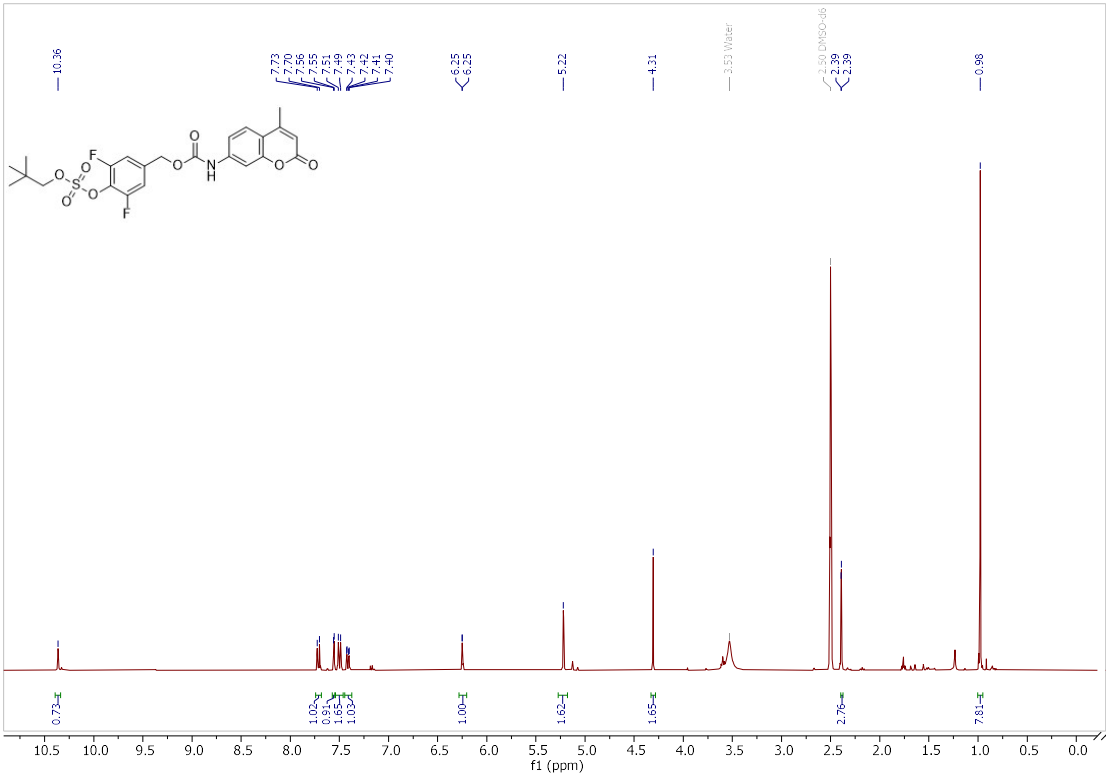


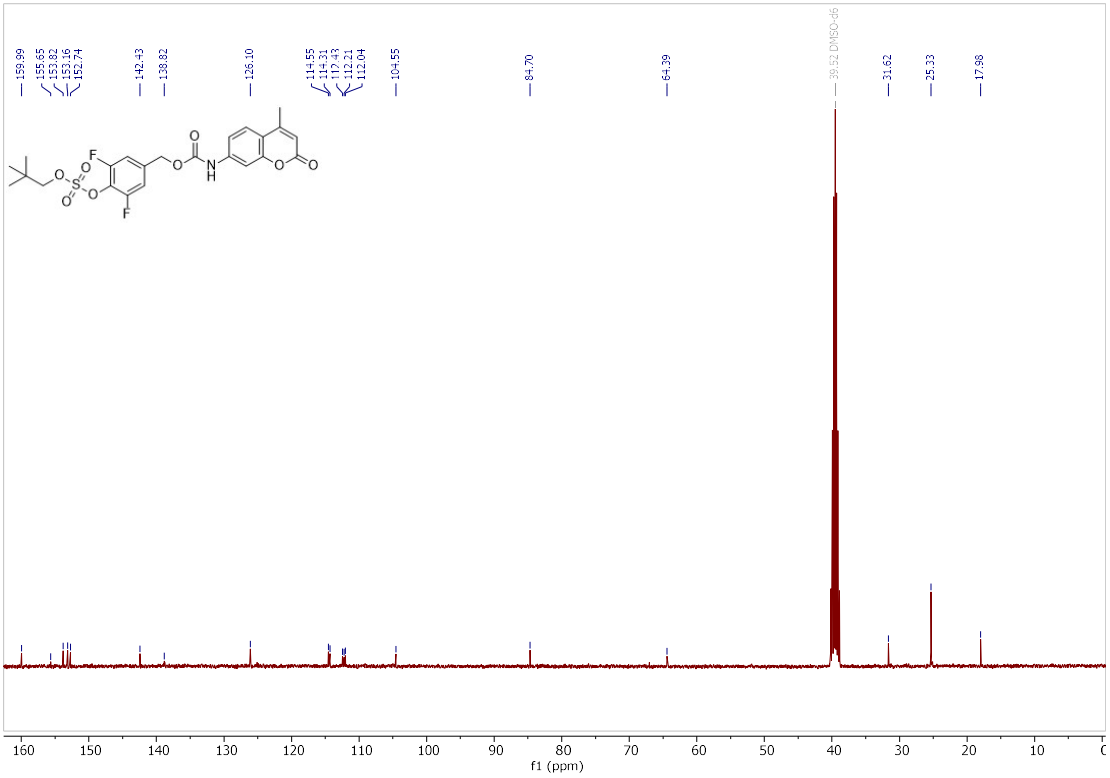
2-Fluoro-4-((((4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl)oxy)methyl)phenyl neopentyl sulfate SI4a



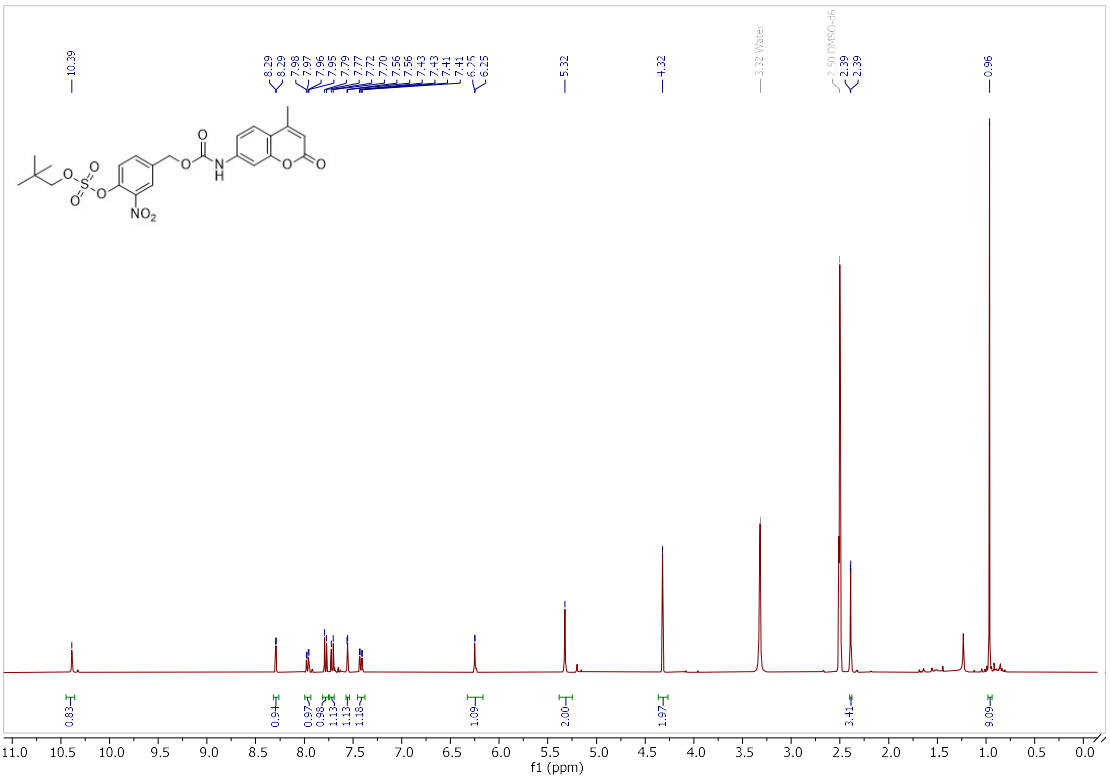


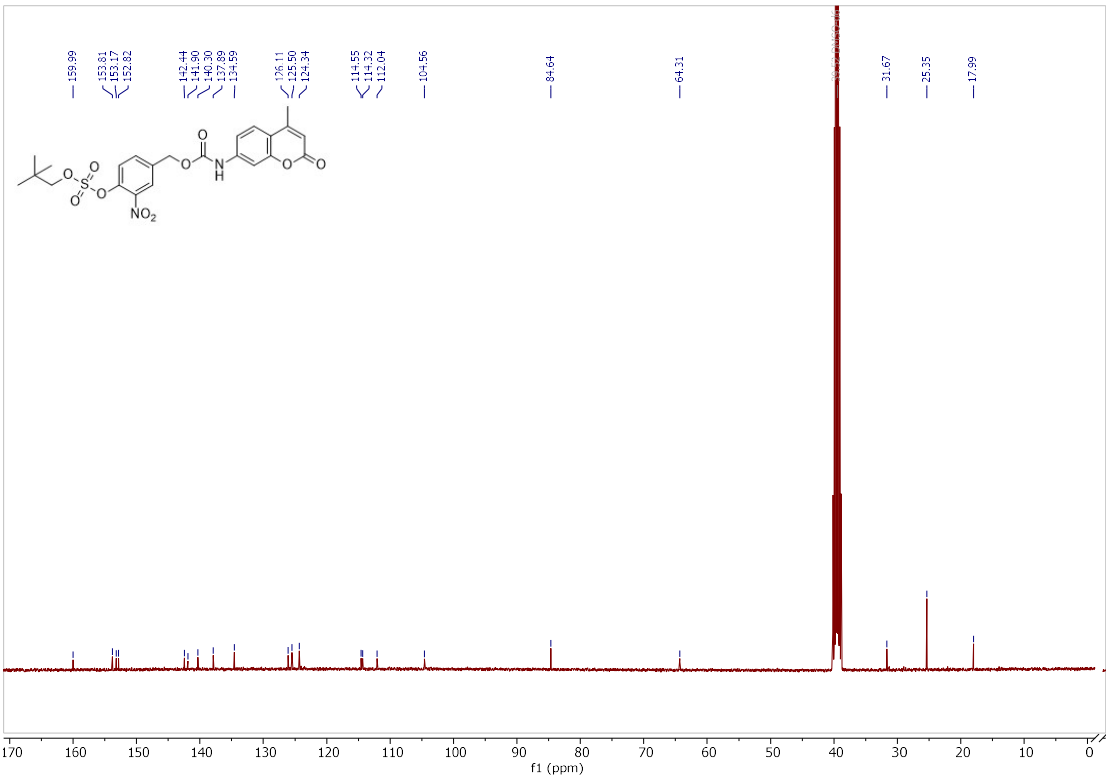
2,6-Difluoro-4-((((4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl)oxy)methyl)phenyl neopentyl sulfate (SI4b)



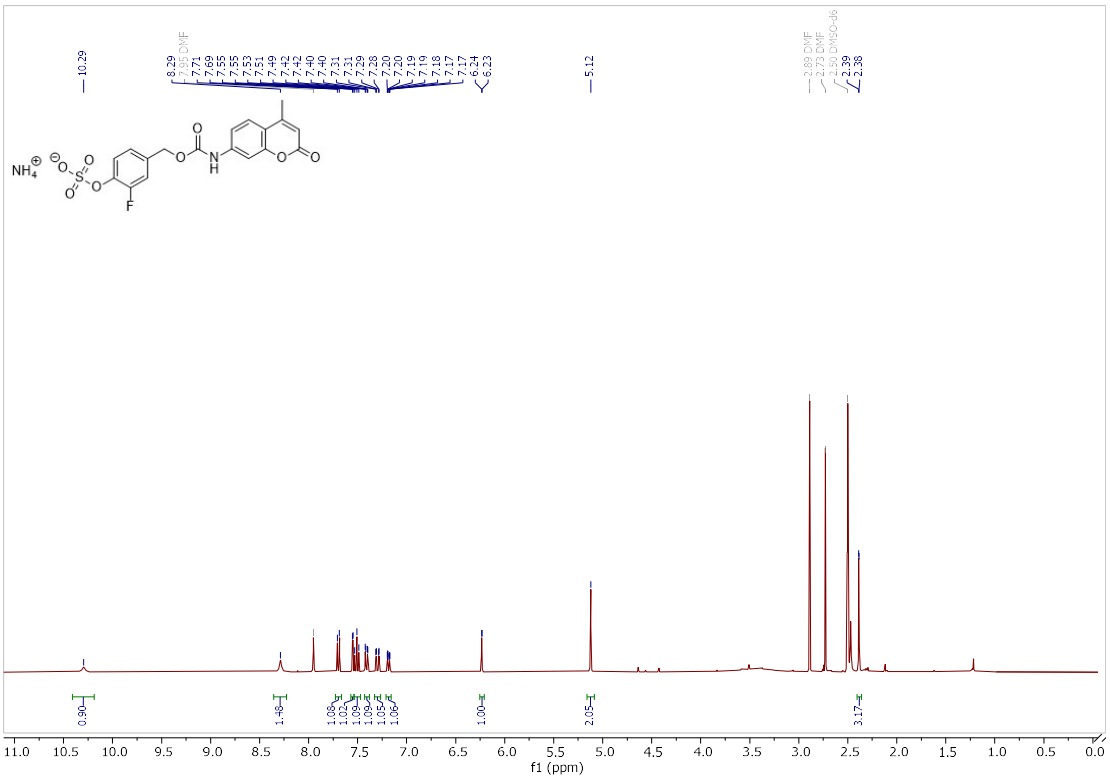


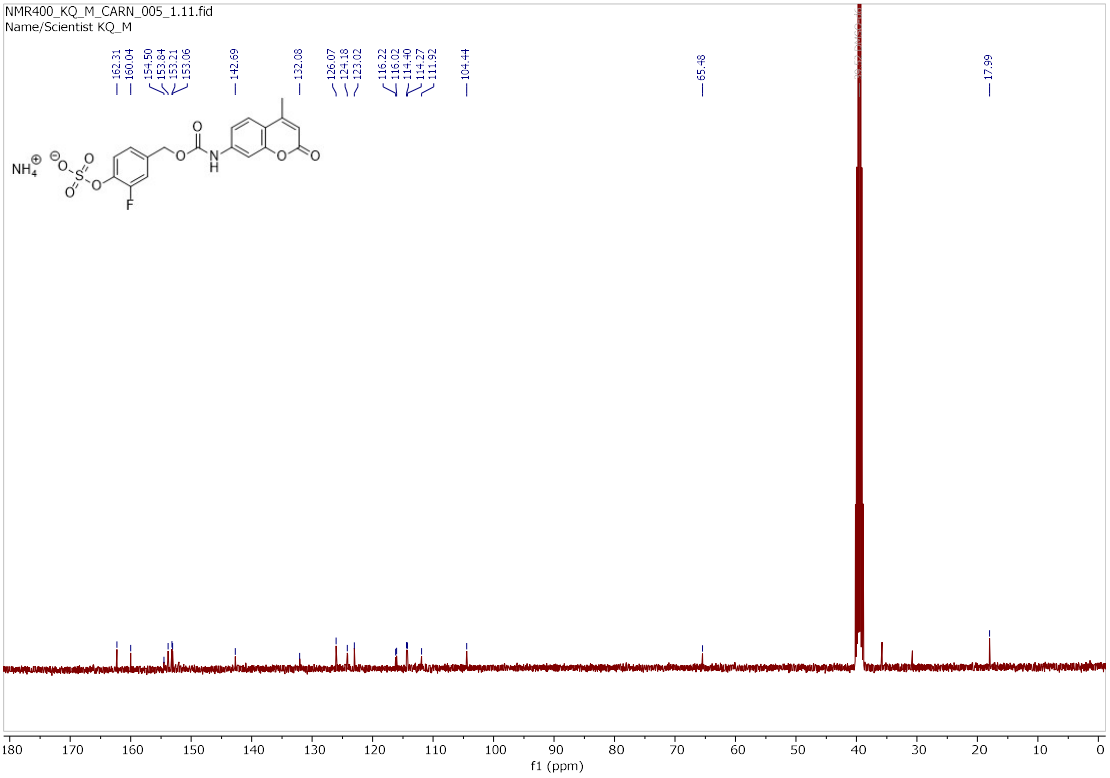
4-((((4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl)oxy)methyl)-2-nitrophenyl neopentyl sulfate (SI4d)



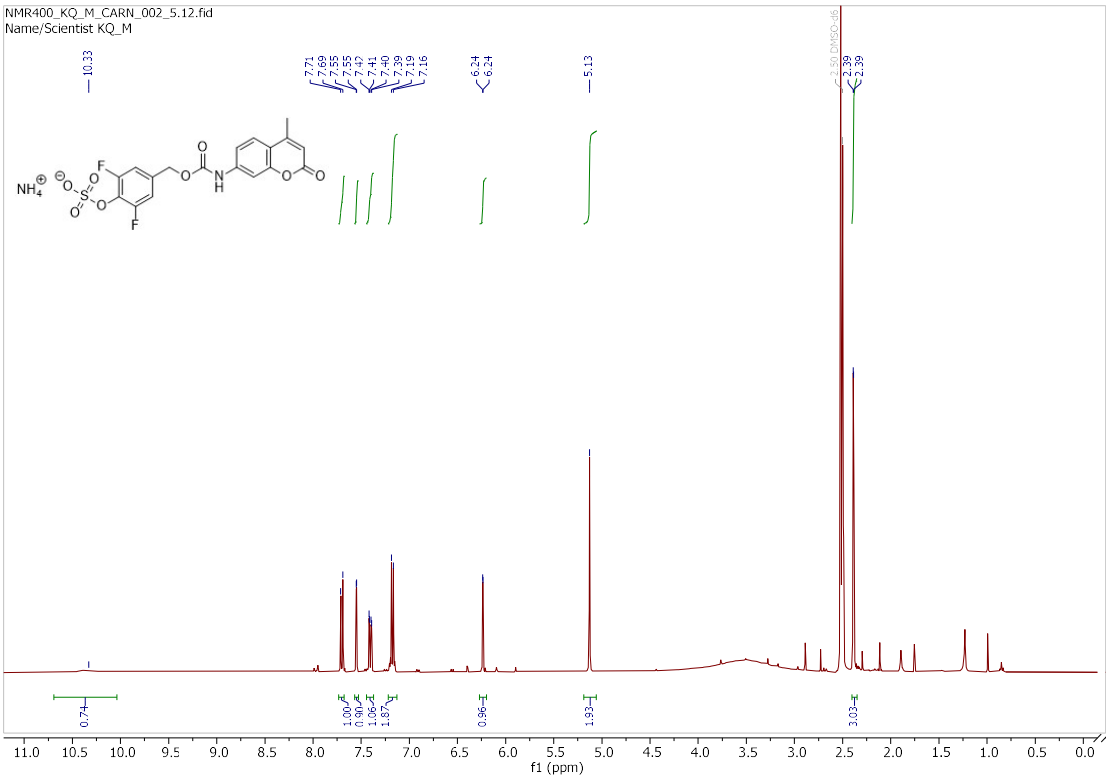


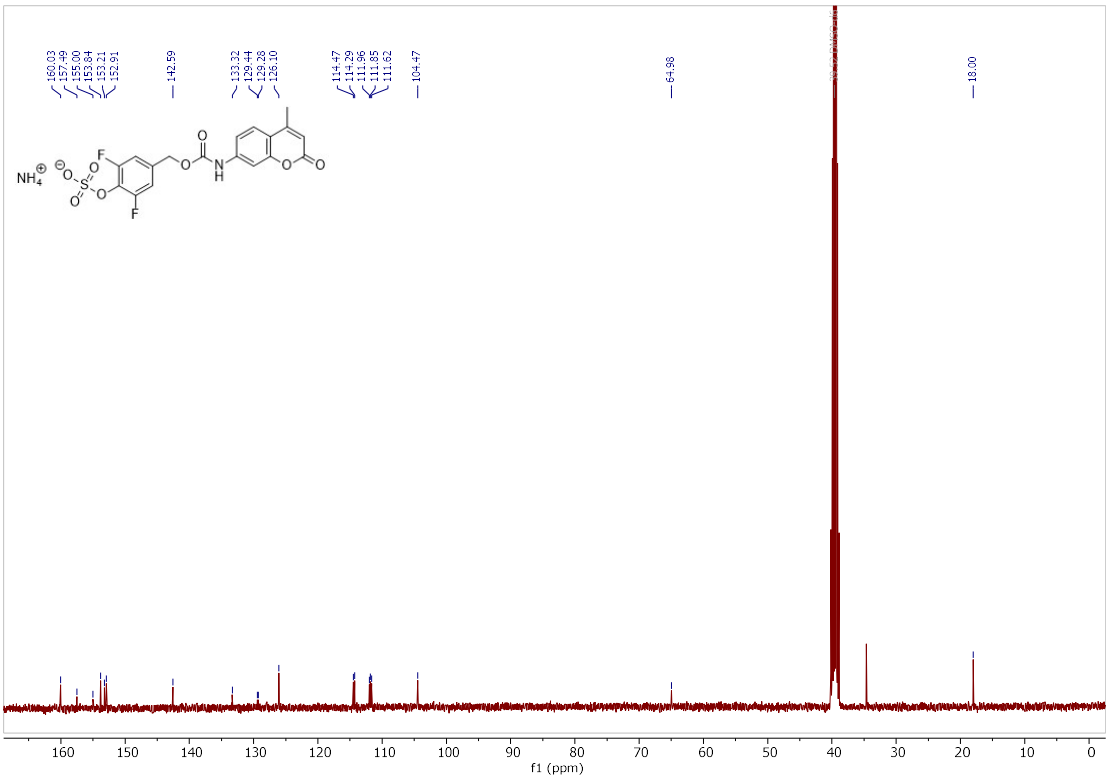
Ammonium 2-fluoro-4-((((4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl) oxy) methyl)phenyl sulfate (Ɪ-monoF)





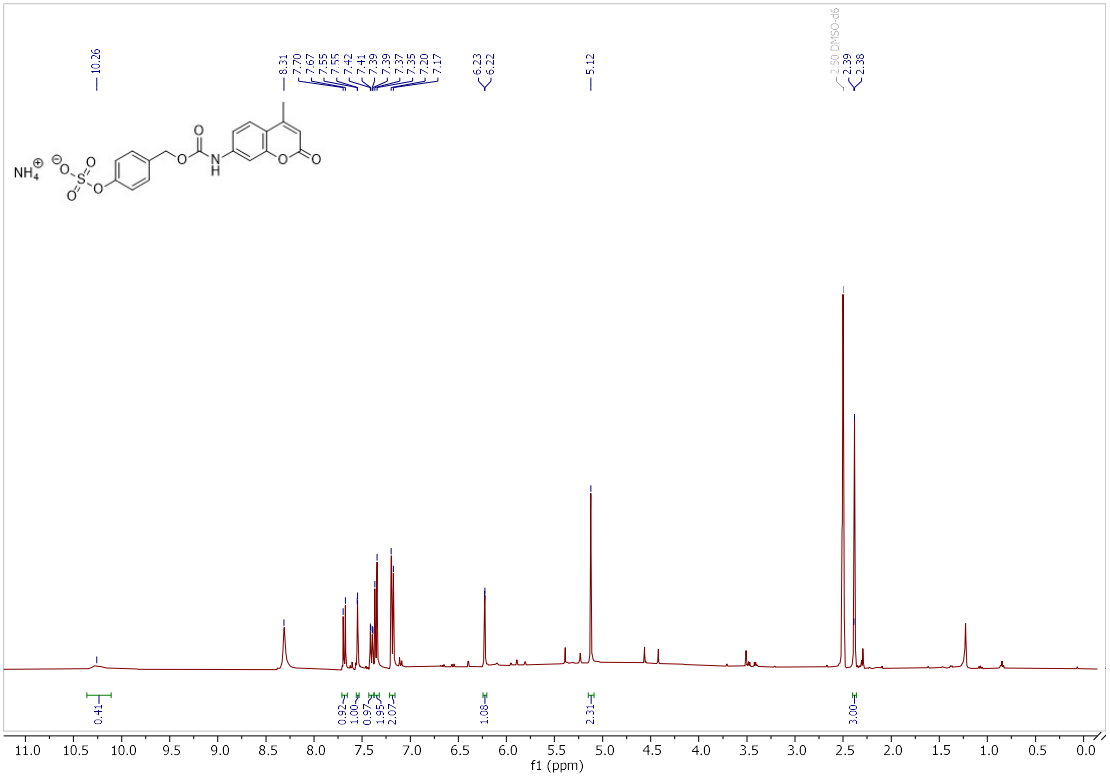
Ammonium 2,6-difluoro-4-((((4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl)oxy) methyl) phenyl sulfate 1-diFb

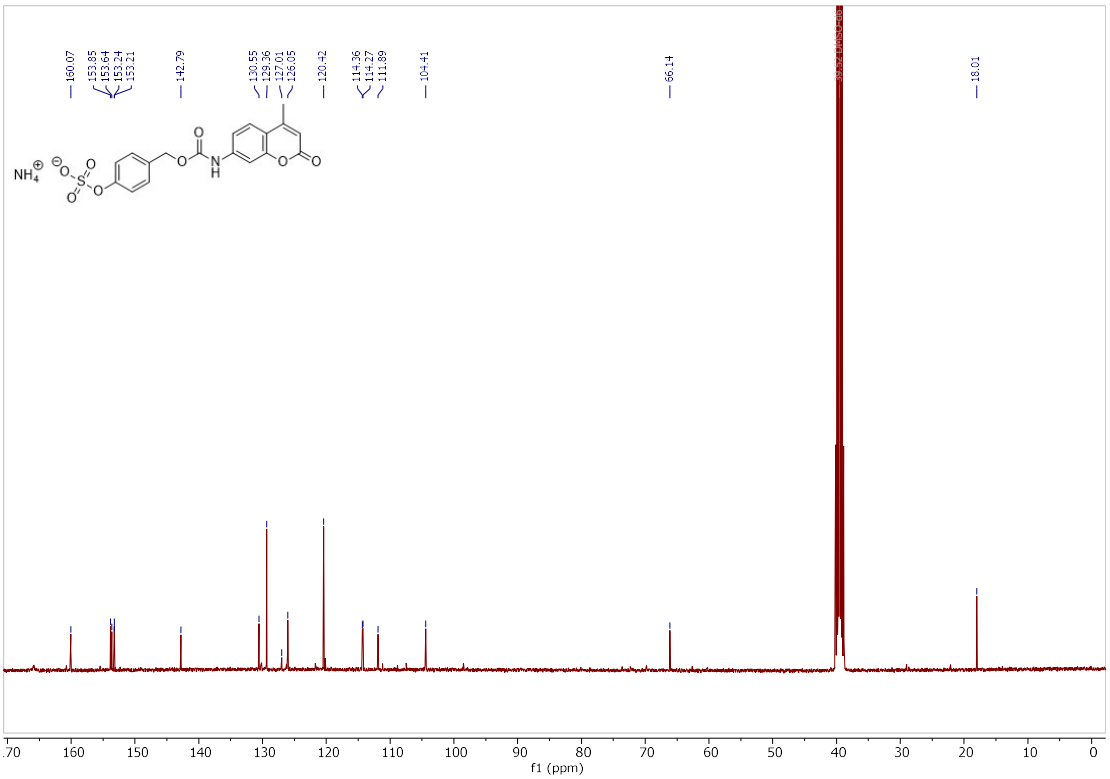




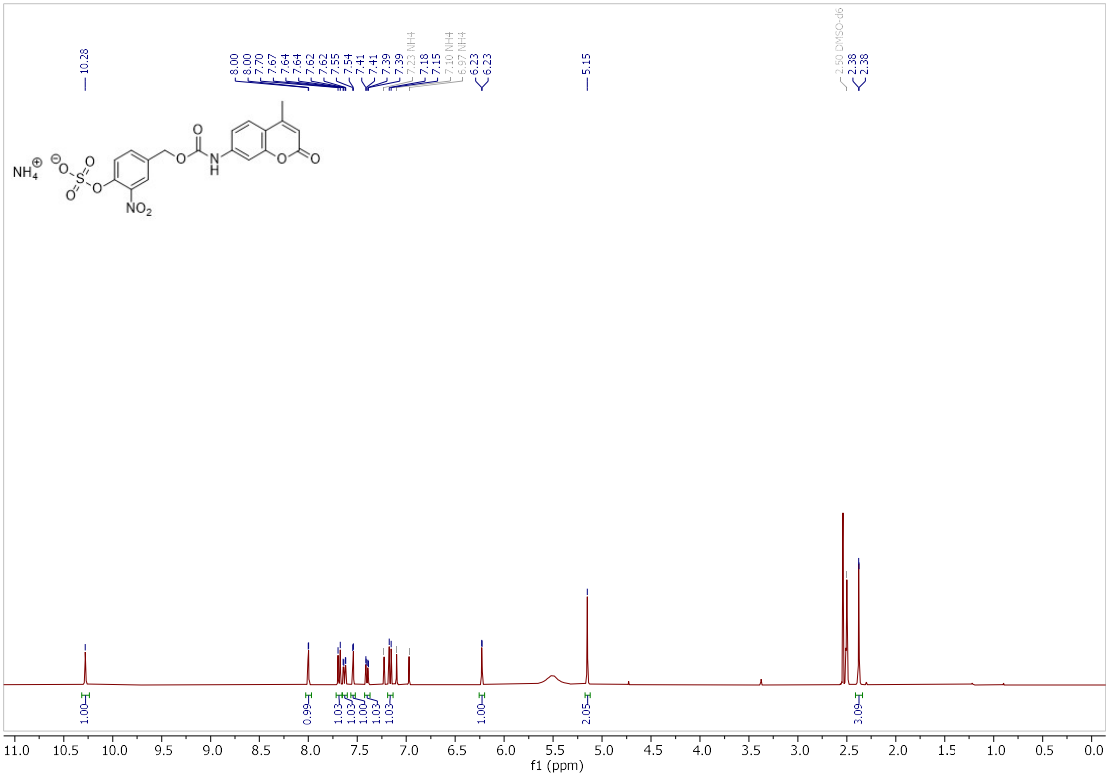
Ammonium 4-((((4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl)oxy)methyl)phenyl sulfate

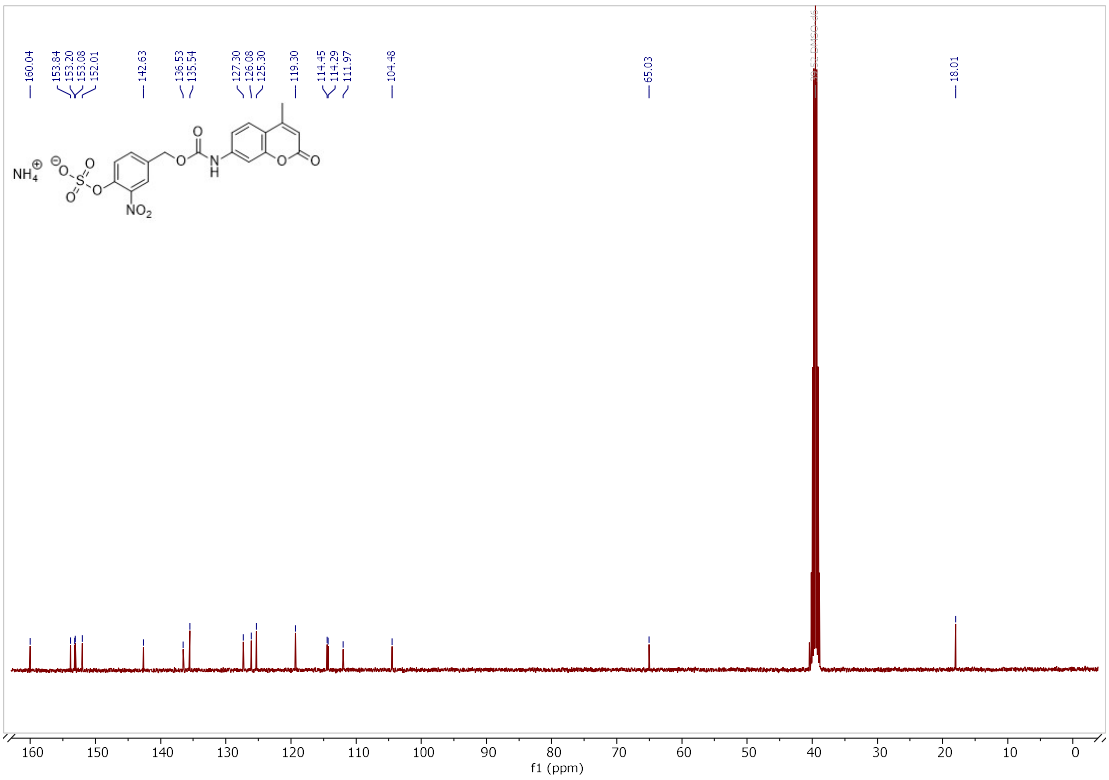
I-H



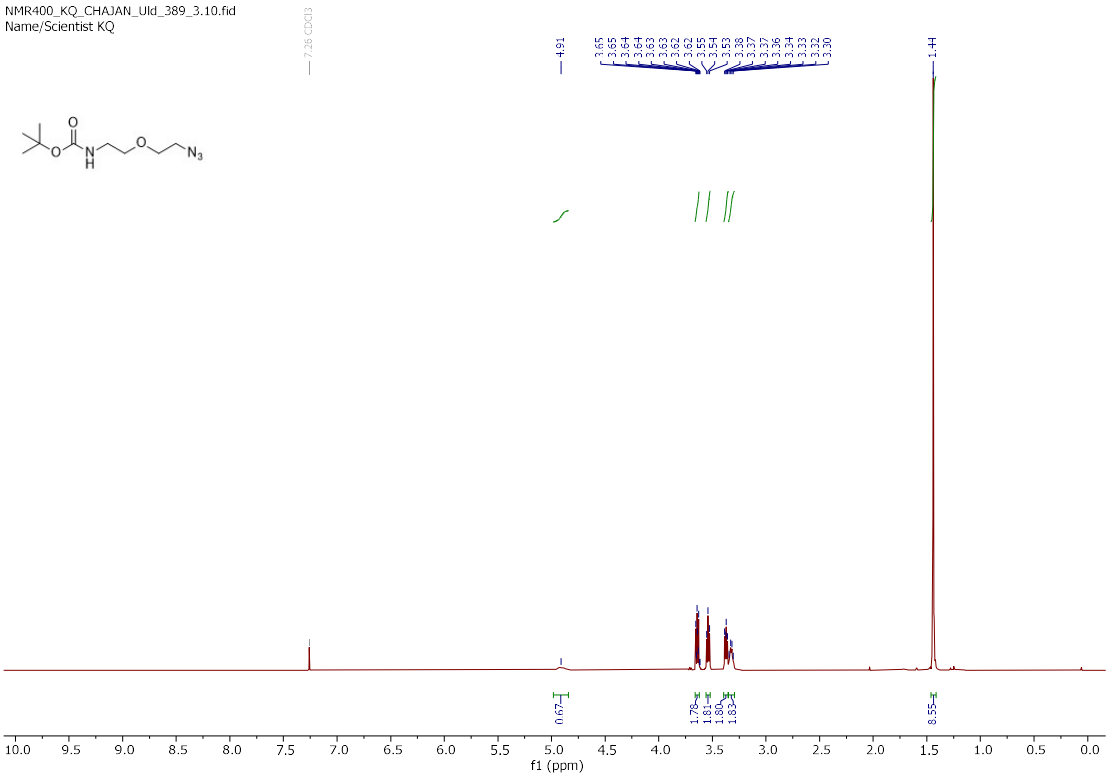


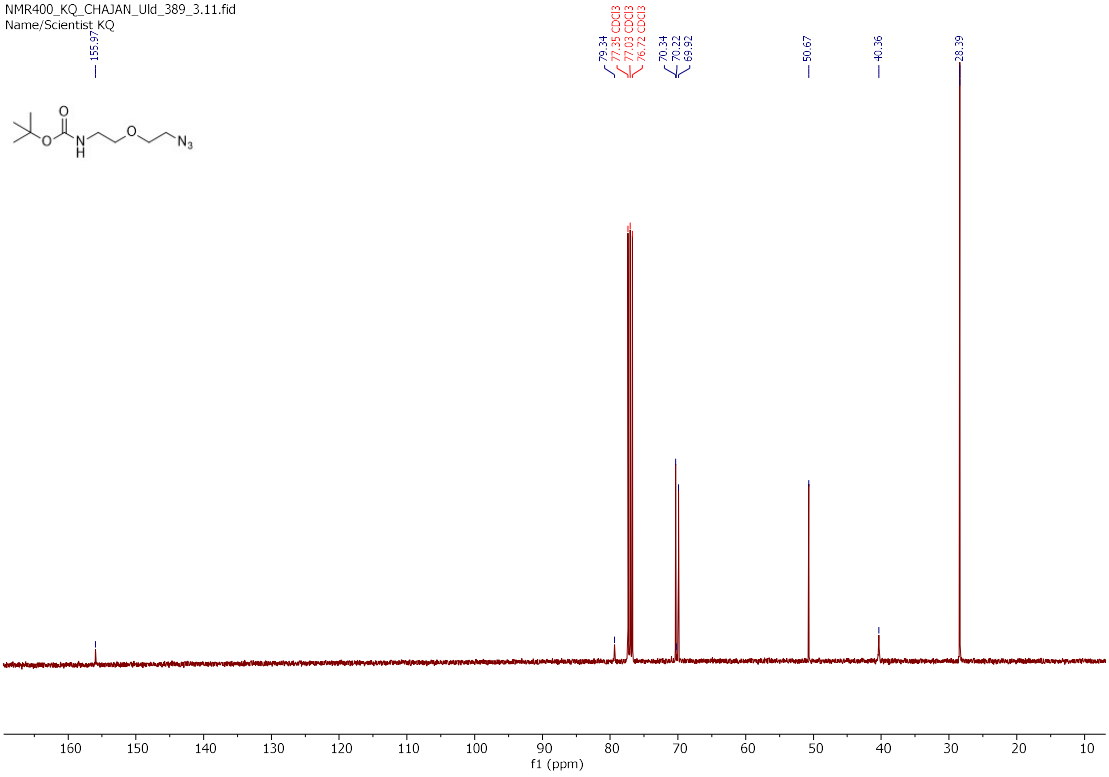
Ammonium 4-((((4-methyl-2-oxo-2H-chromen-7-yl)carbamoyl)oxy)methyl)-2-nitrophenyl sulfate 1-NO_2



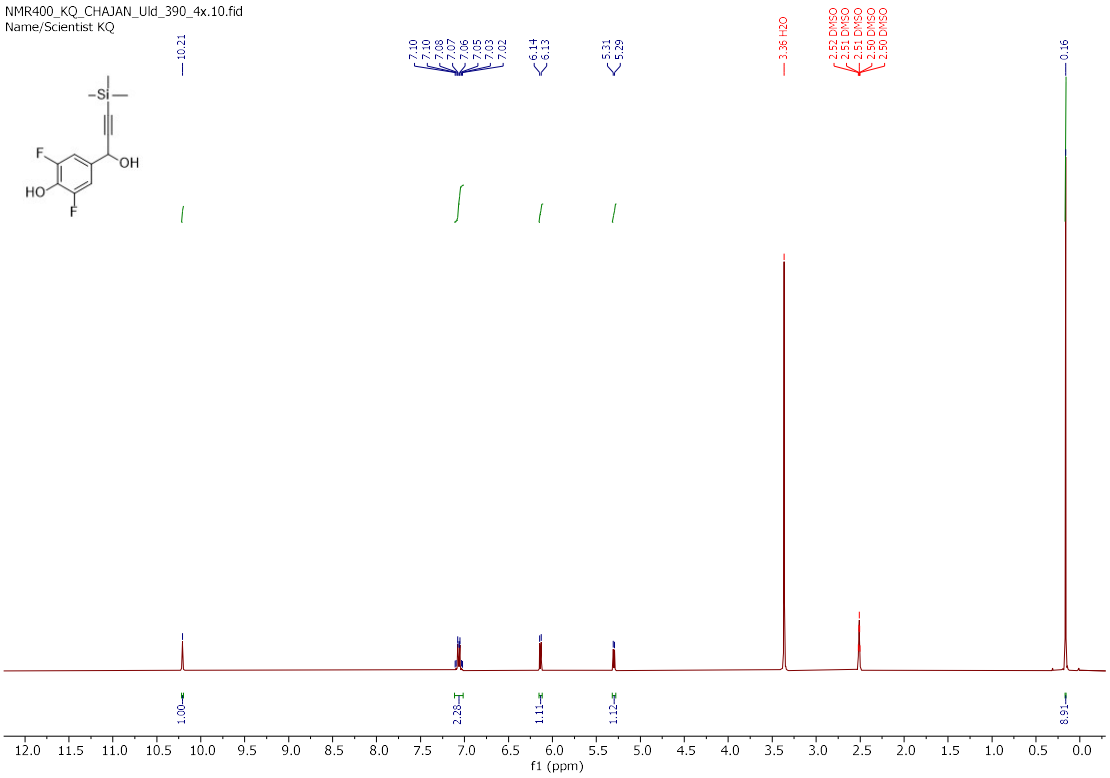


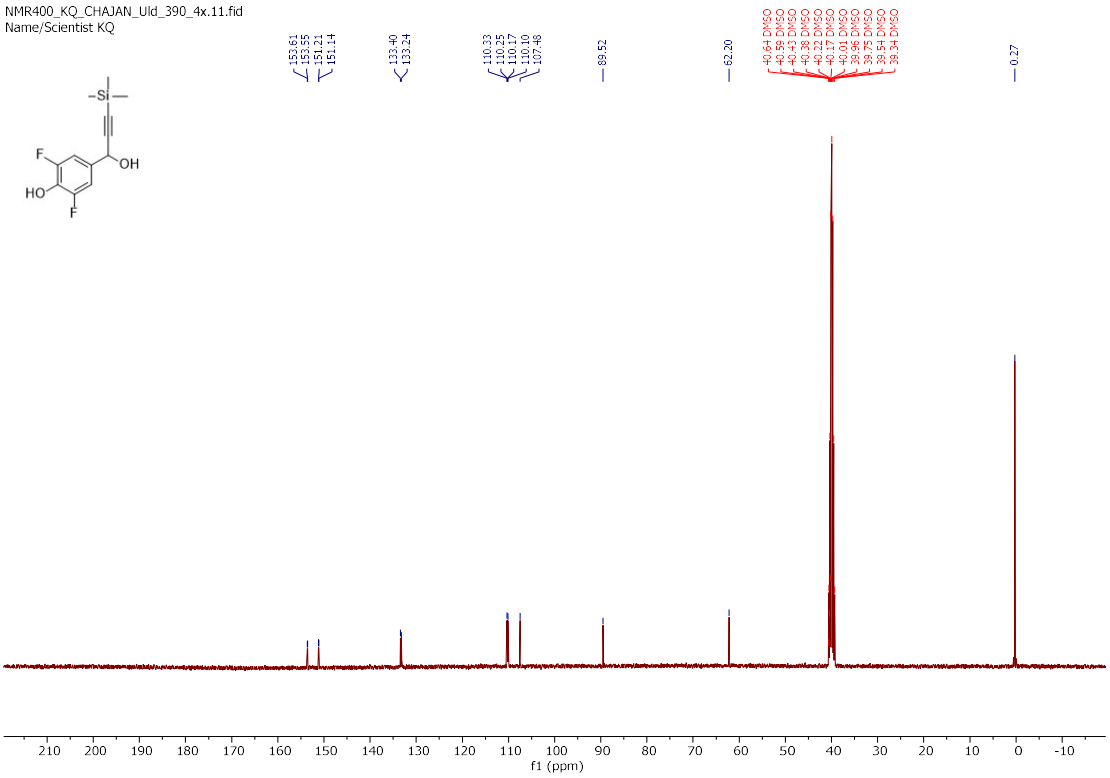
Tert-butyl (2-(2-azidoethoxy)ethyl)carbamate (S1)



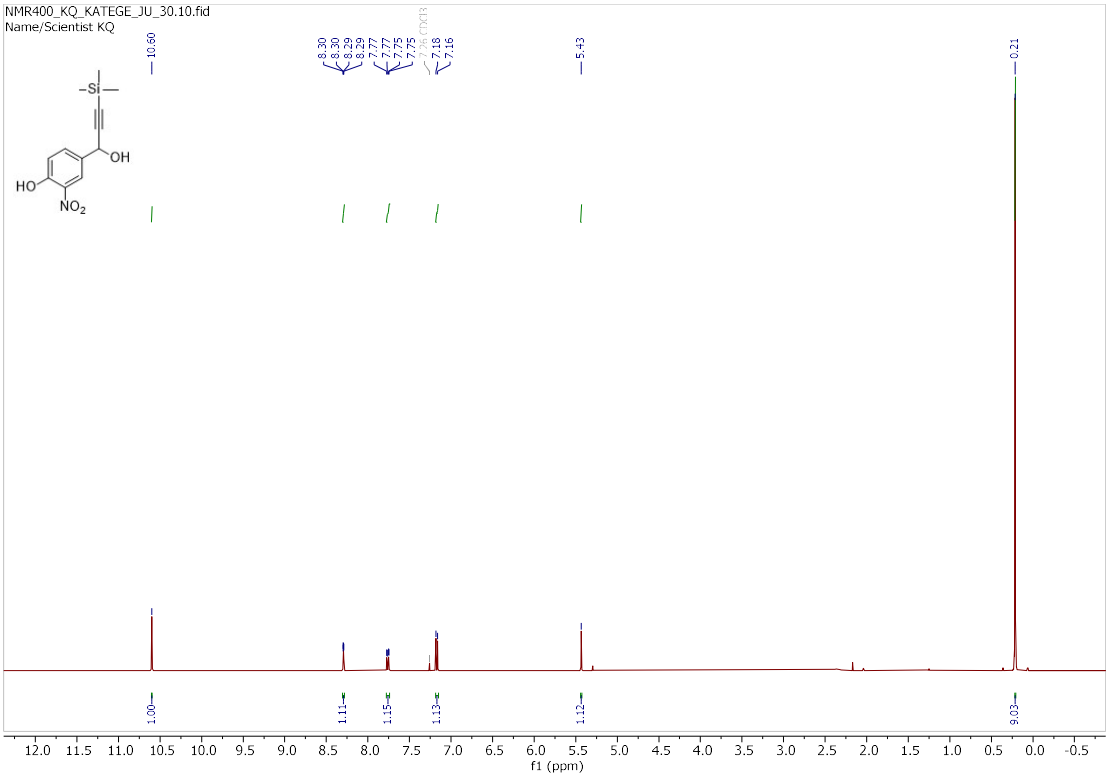


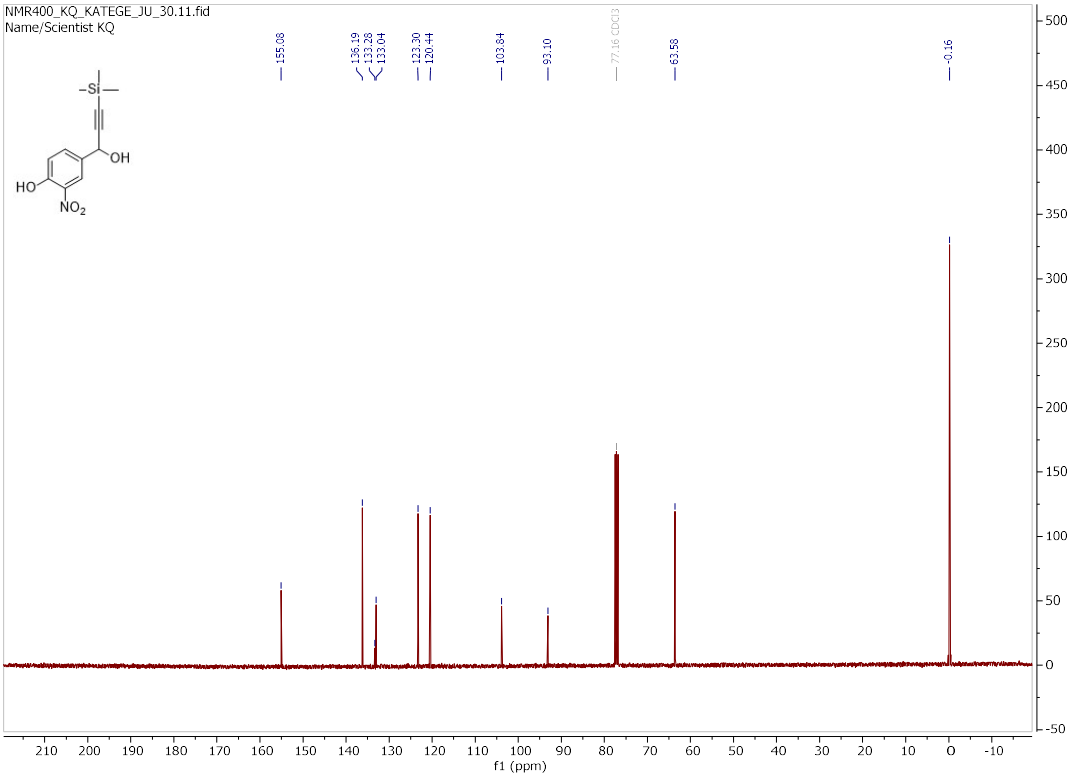
2,6-difluoro-4-(1-hydroxy-3-(trimethylsilyl)prop-2-yn-1-yl)phenol (SI6a)



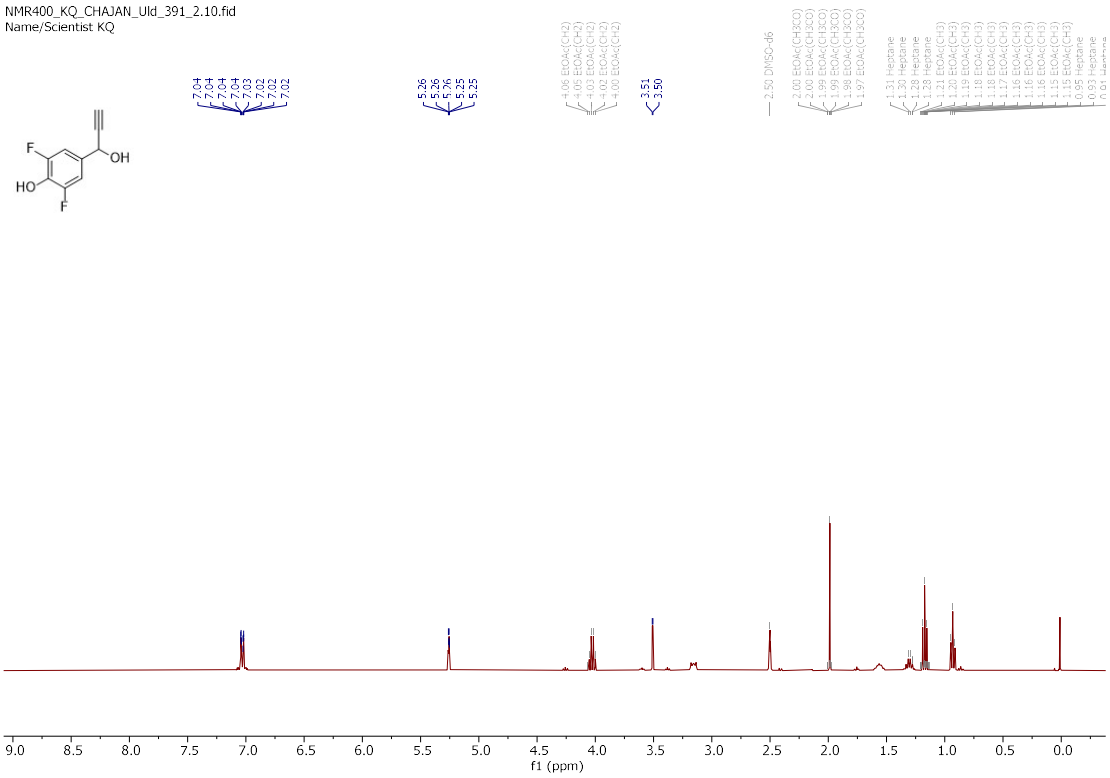


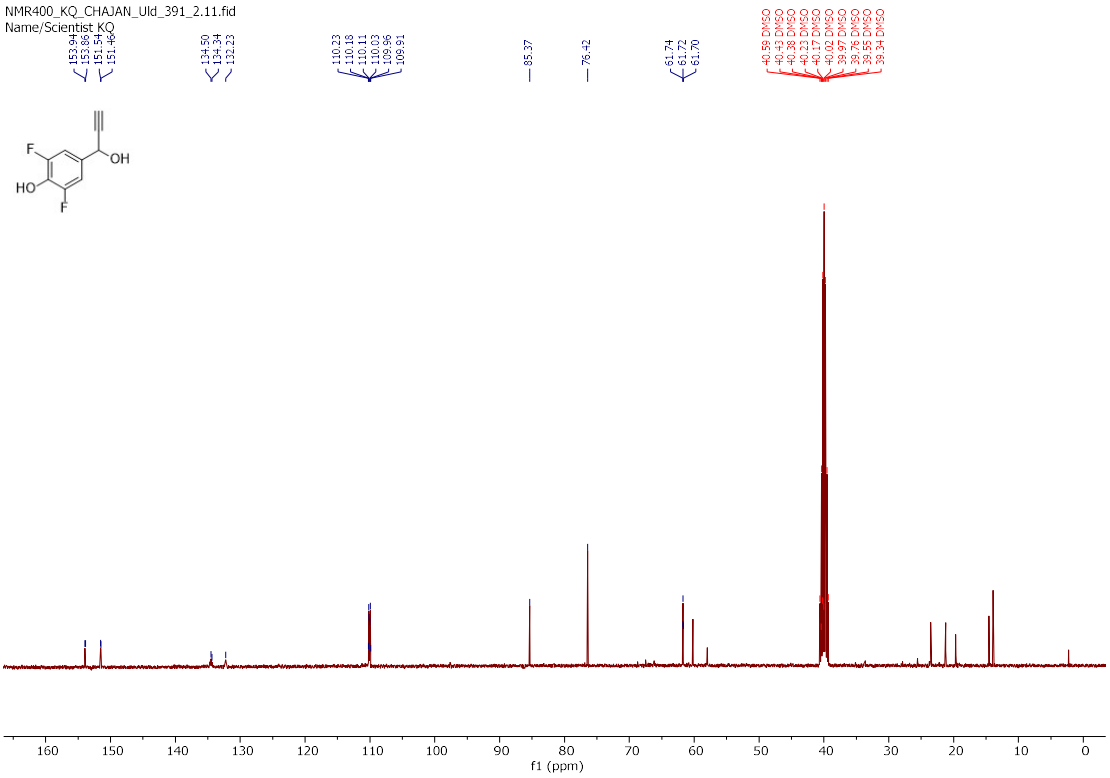
4-(1-Hydroxy-3-(trimethylsilyl)prop-2-yn-1-yl)-2-nitrophenol (SI6b)



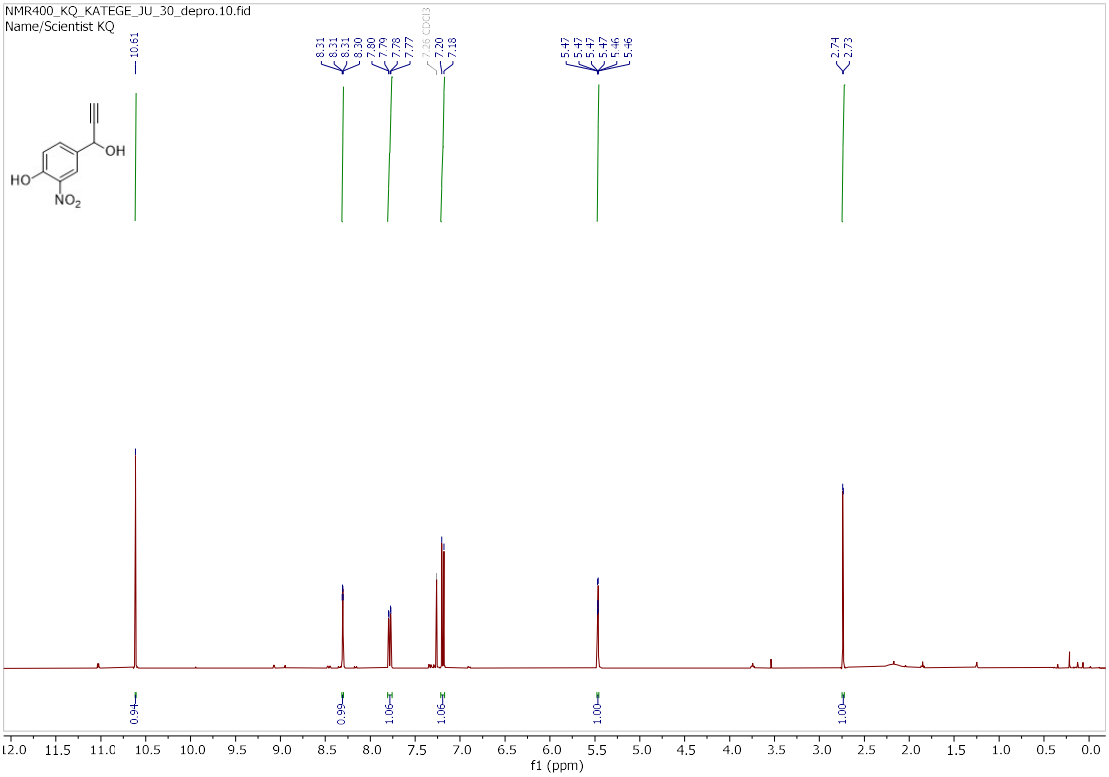


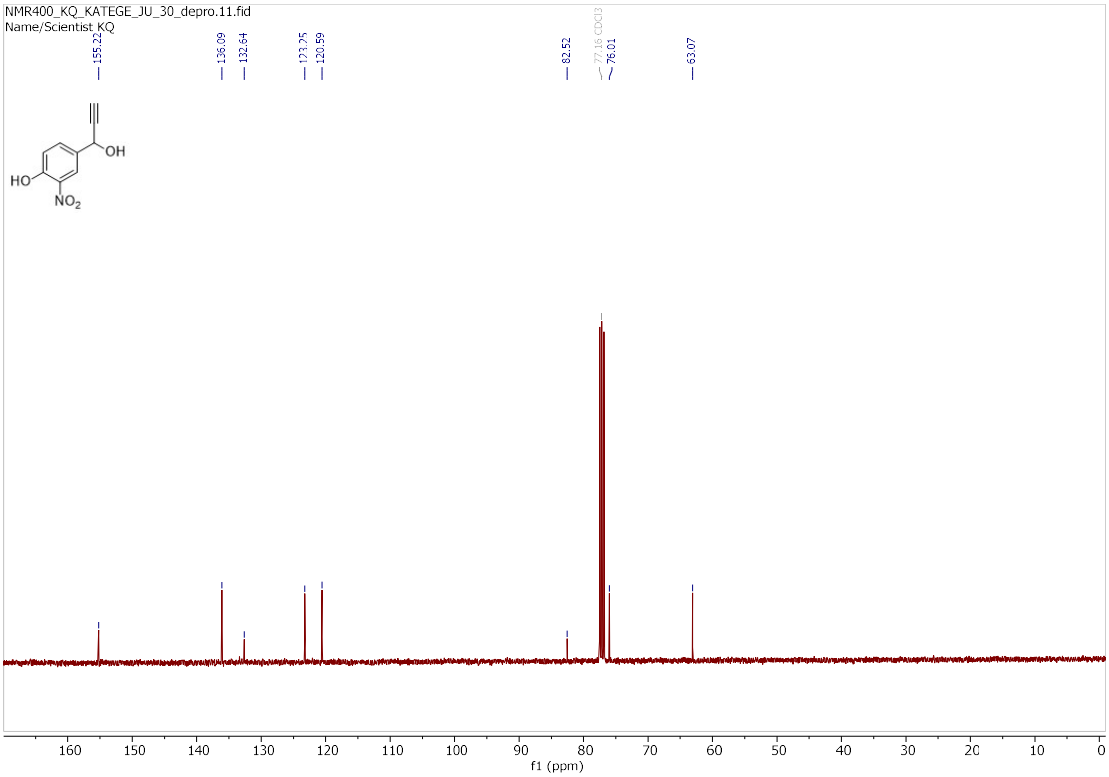
2,6-Difluoro-4-(1-hydroxyprop-2-yn-1-yl)phenol (SI7a)



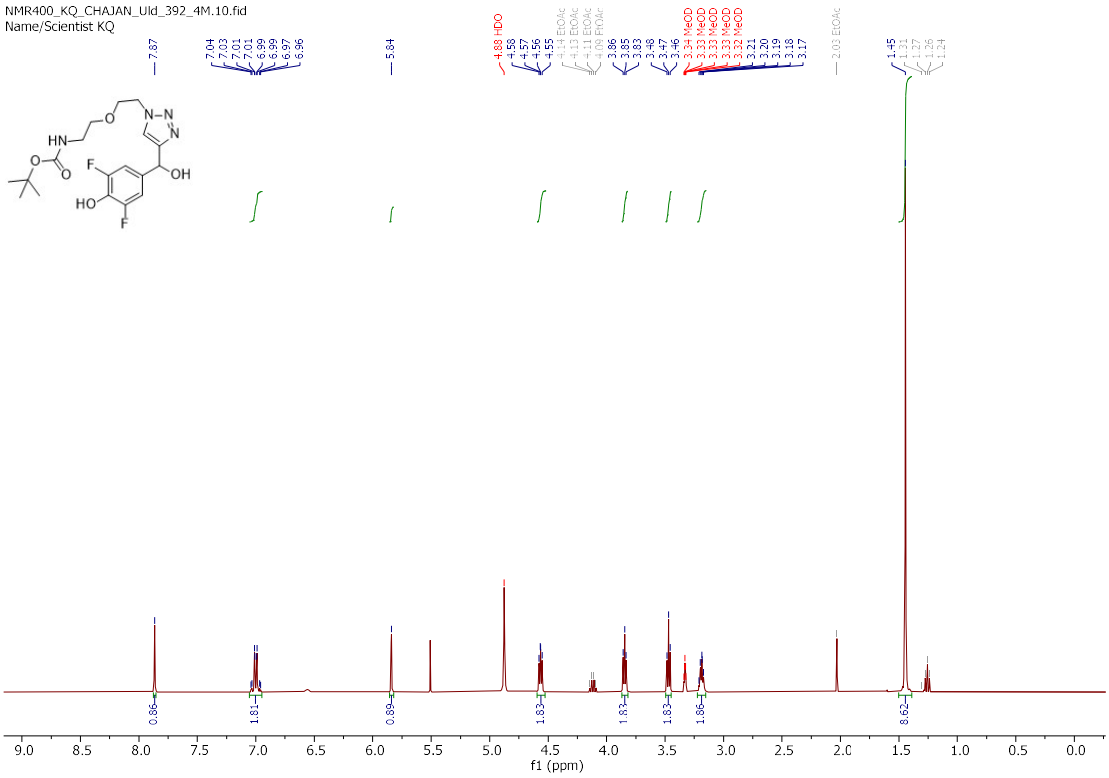


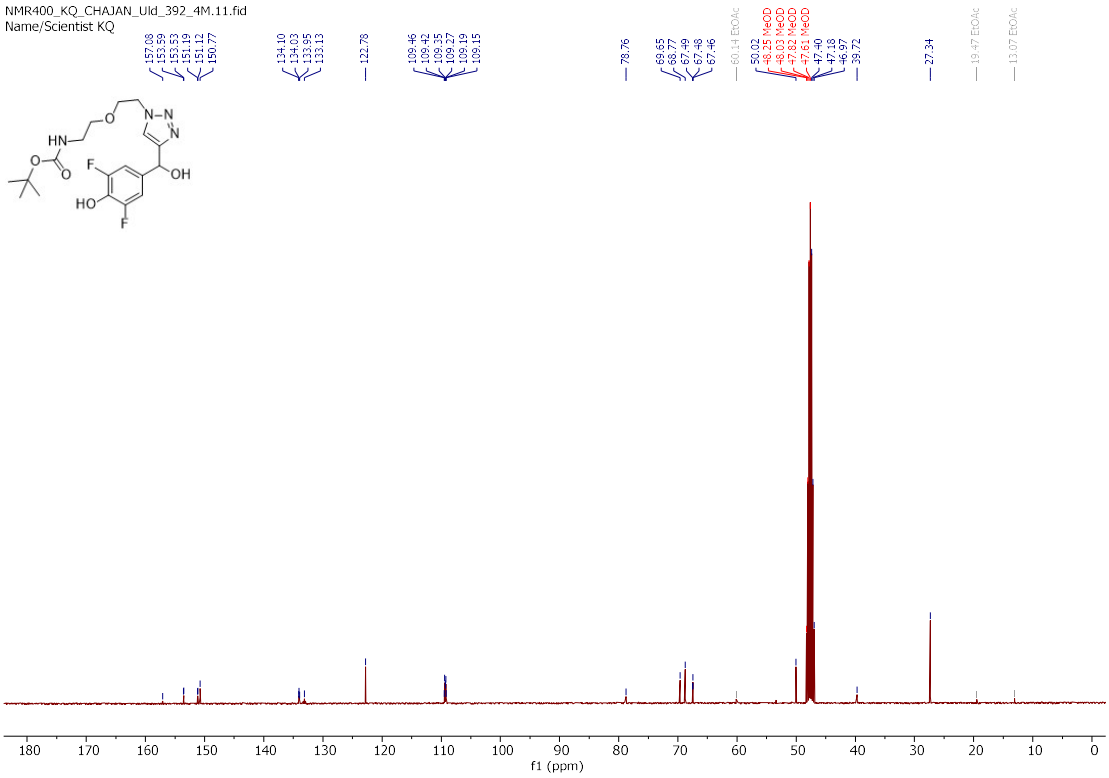
4-(1-Hydroxyprop-2-yn-1-yl)-2-nitrophenol (SI7b)



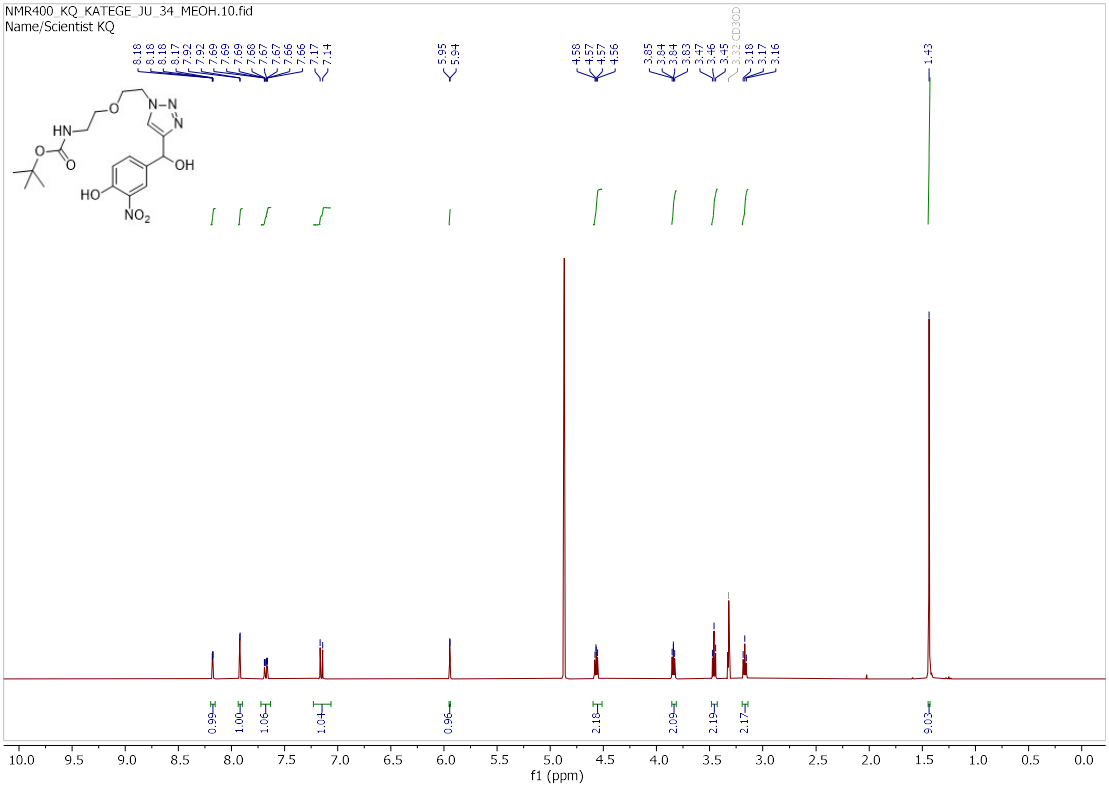


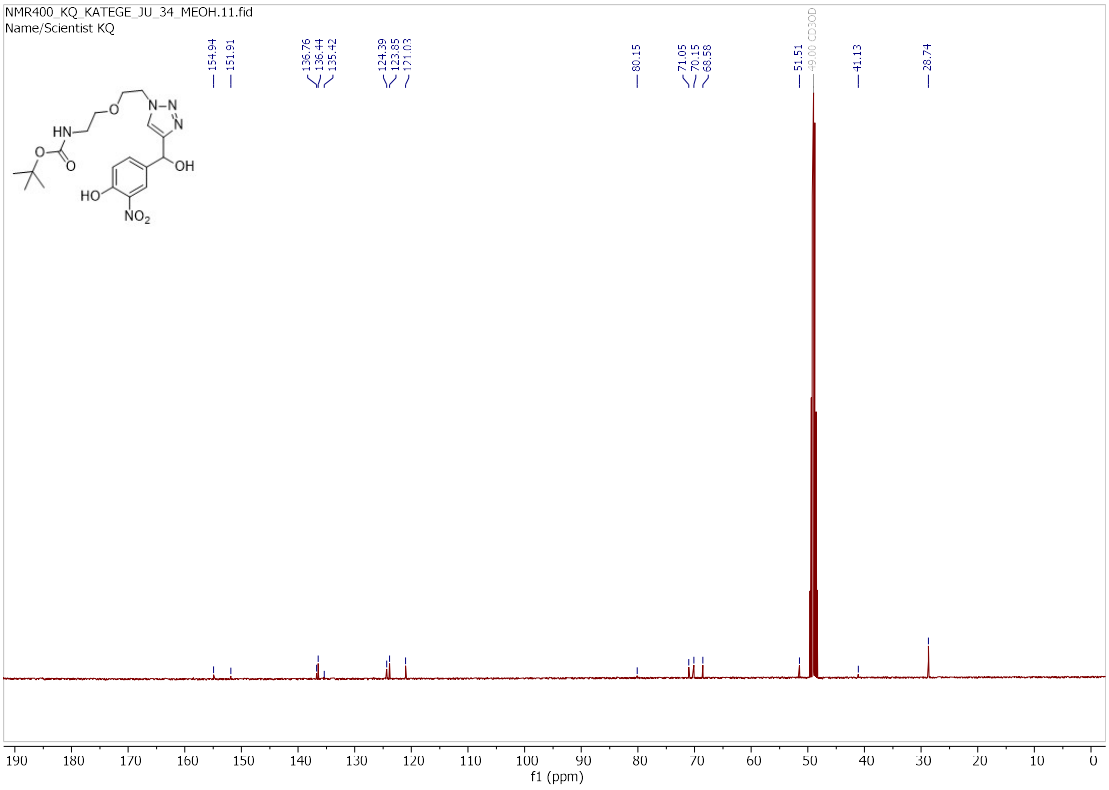
***Tert*-butyl (2-(2-(4-((3,5-difluoro-4-hydroxyphenyl)(hydroxy)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethyl)carbamate (SI8a)**



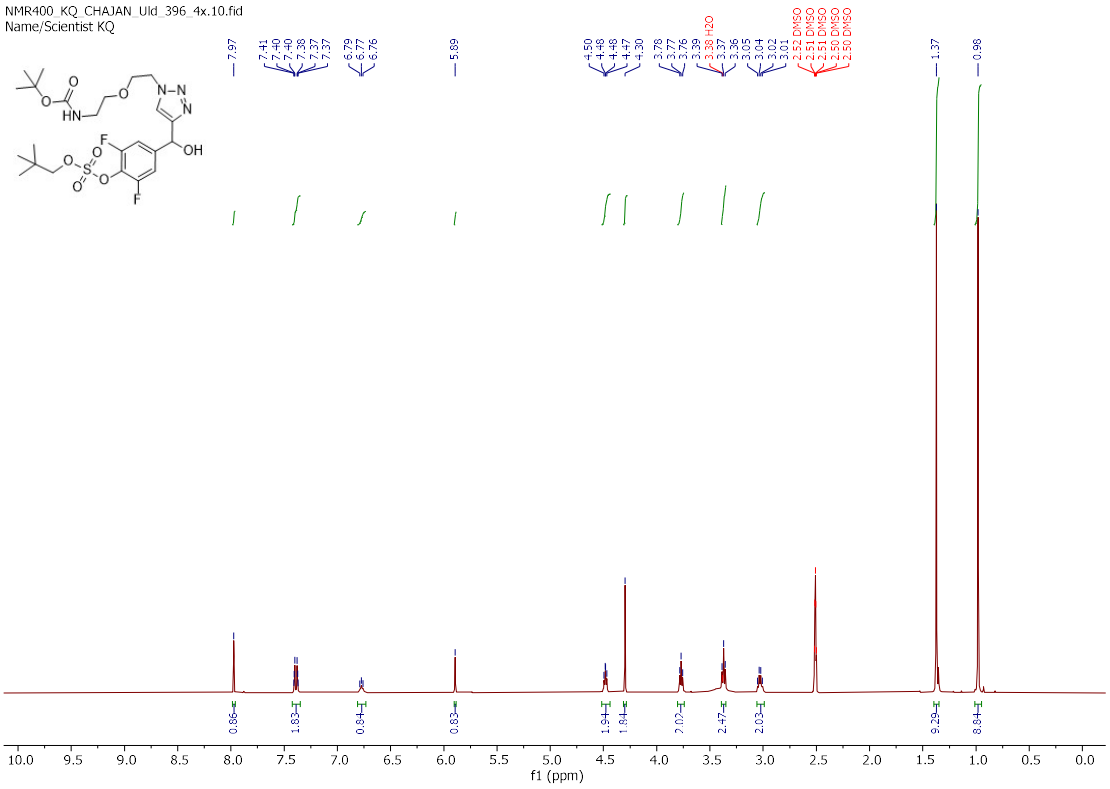


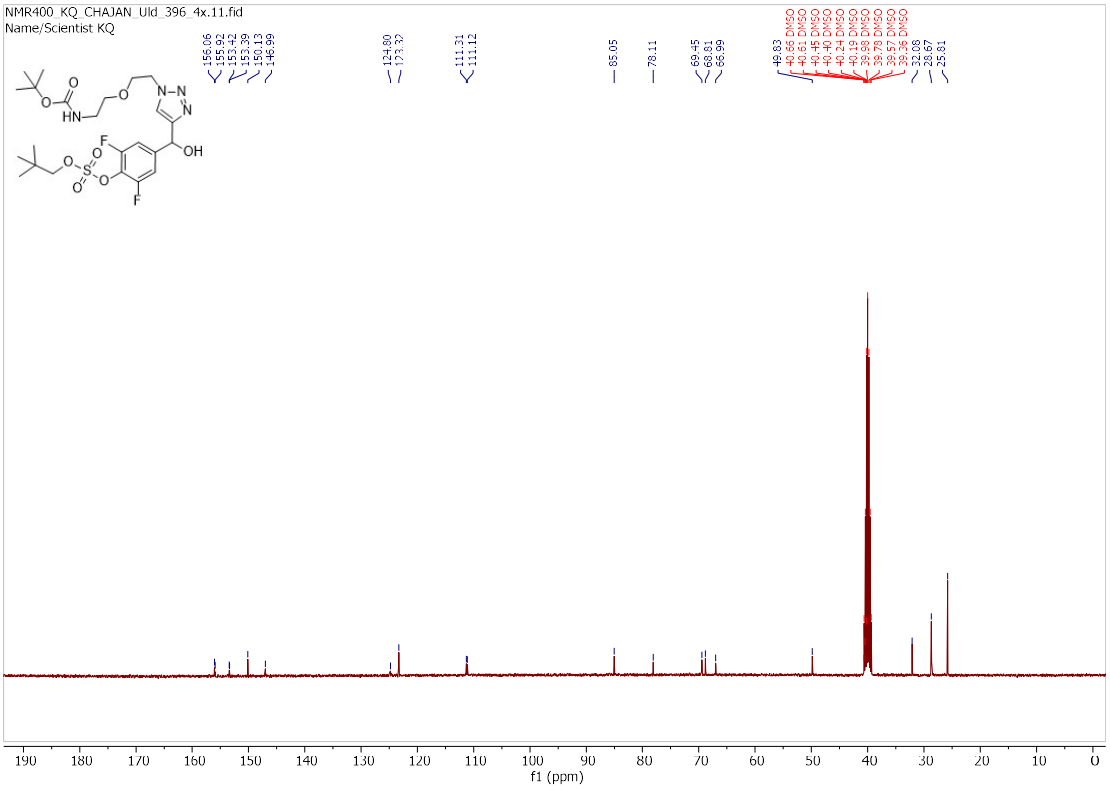
***Tert*-butyl (2-(2-(4-(hydroxy(4-hydroxy-3-nitrophenyl)methyl)-1H-1,2,3-triazol-1-yl)ethoxy)ethyl)carbamate SI8b**



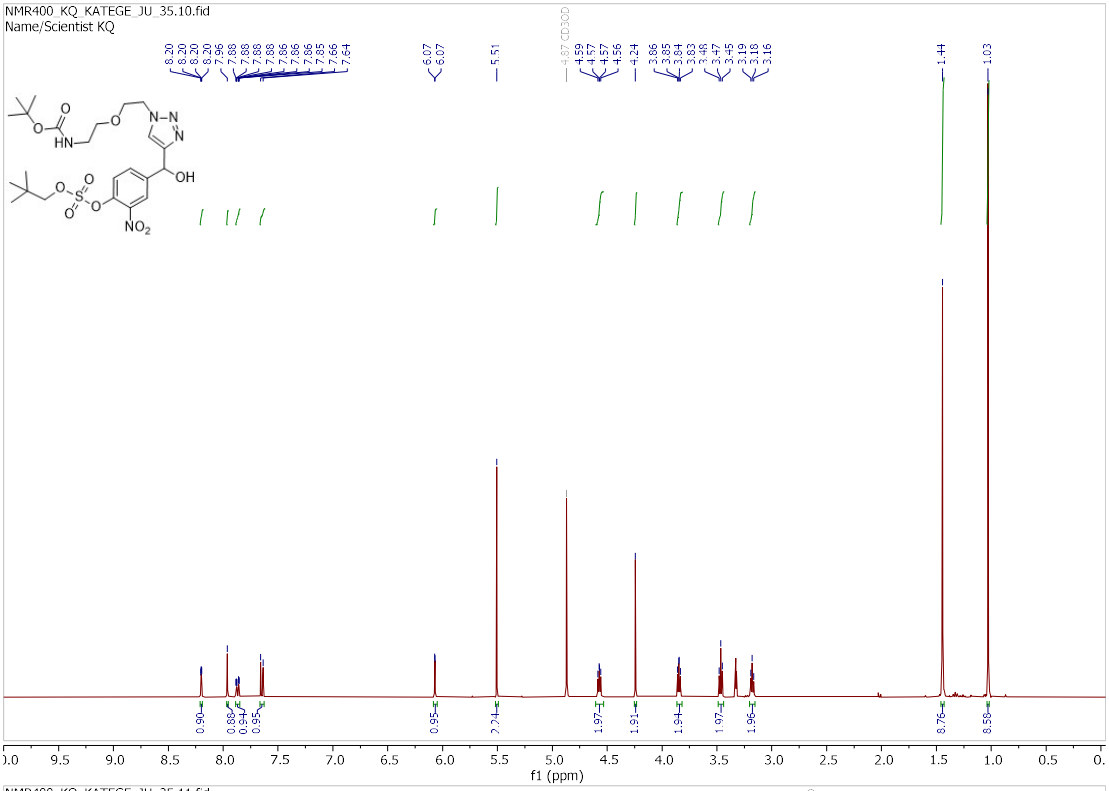


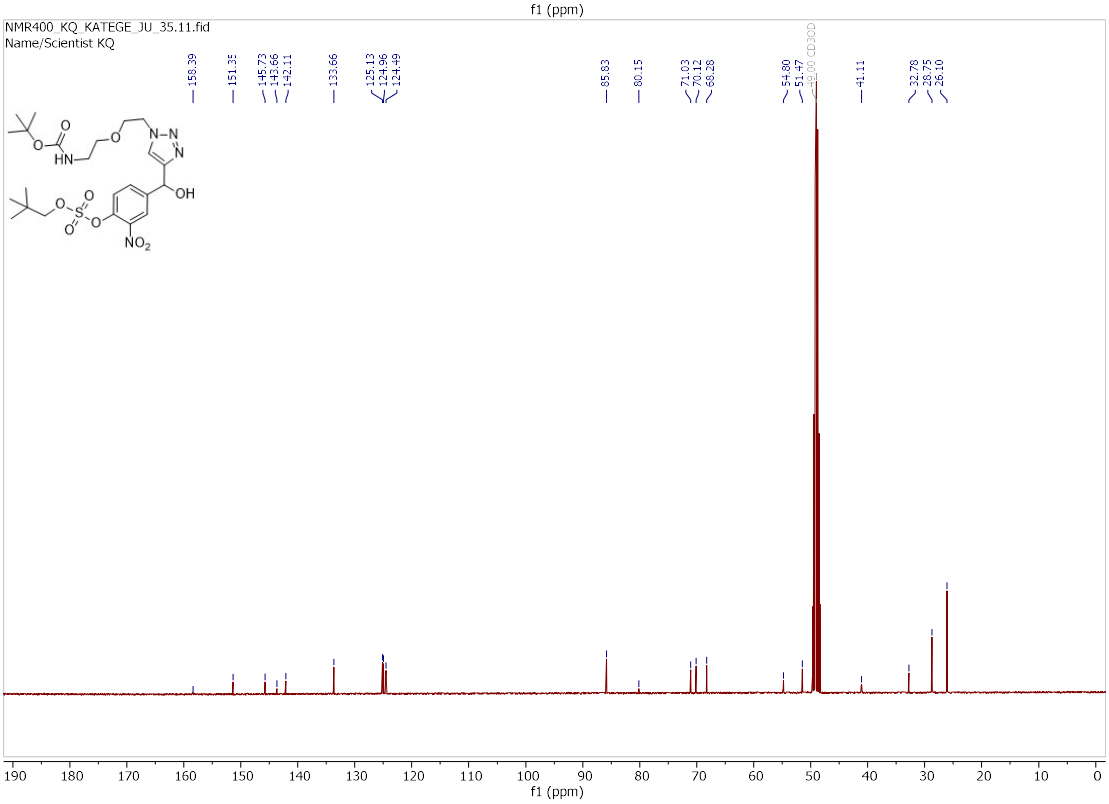
4-((1-(2-(2-((Tert-butoxycarbonyl)amino)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)(hydroxy)methyl)-2,6-difluorophenyl neopentyl sulfate (SI9a)



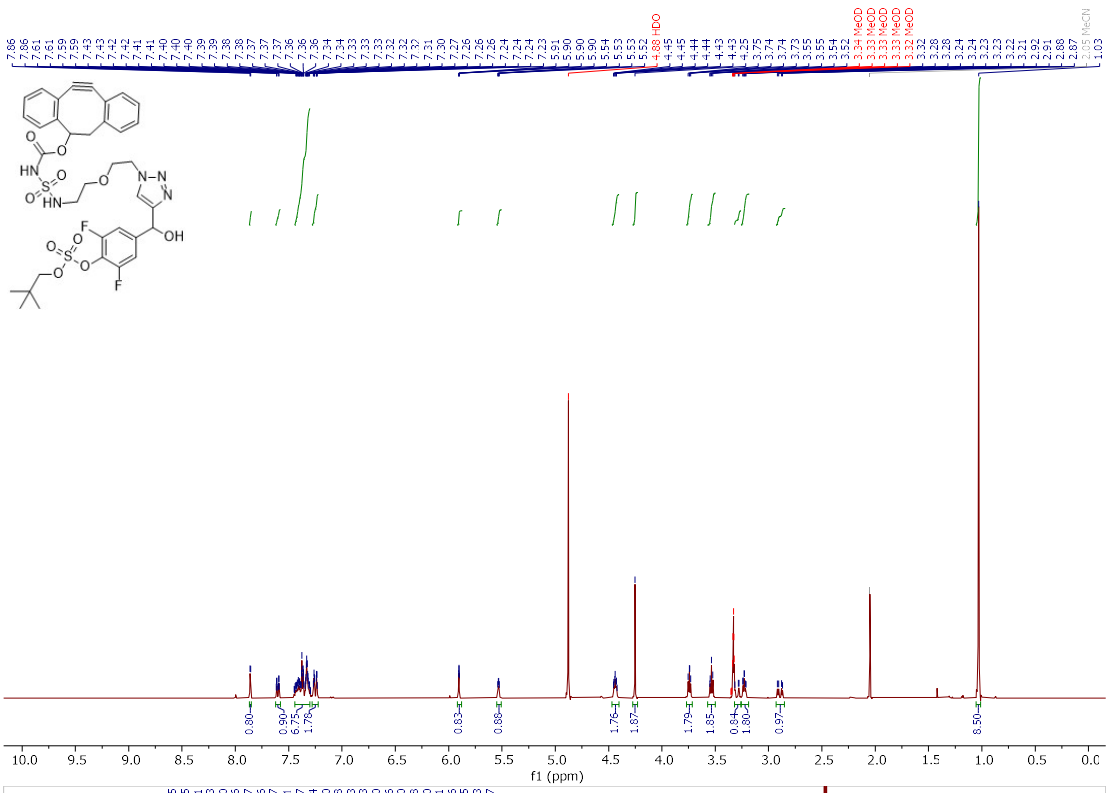


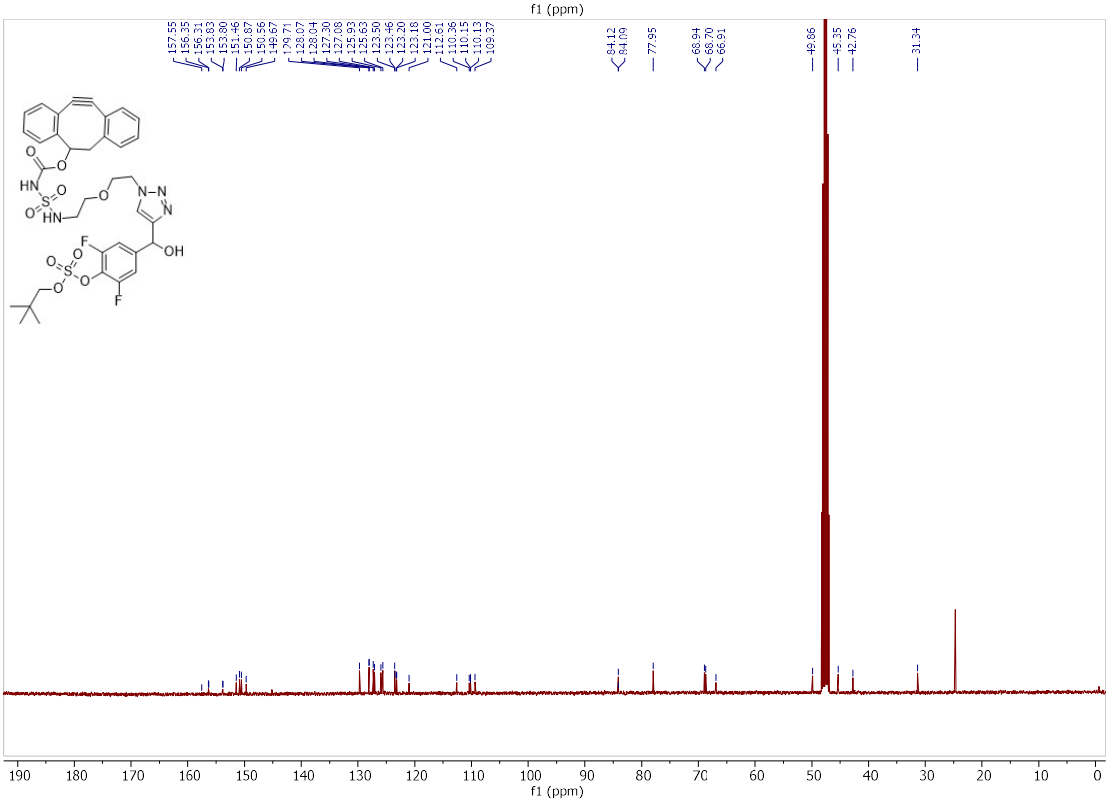
4-((1-(2-(2-((Tert-butoxycarbonyl)amino)ethoxy)ethyl)-1H-1,2,3-triazol-4-yl)(hydroxy) methyl)-2-nitrophenyl neopentyl sulfate SI9b



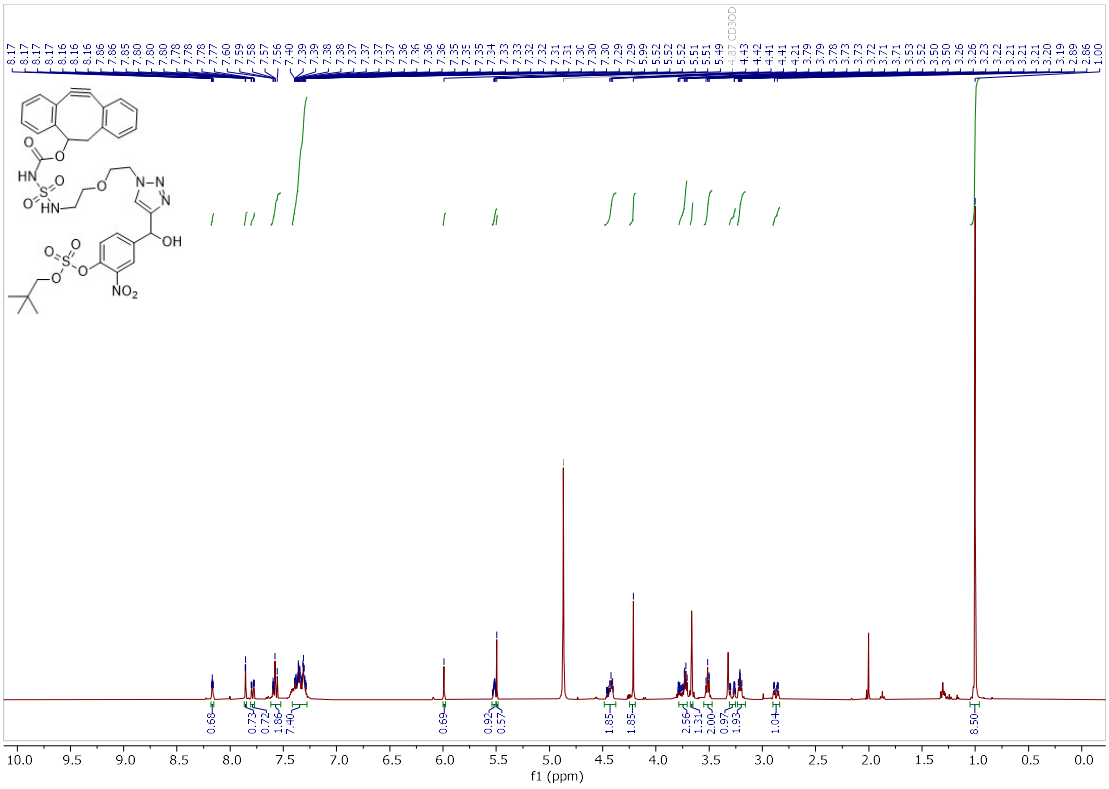


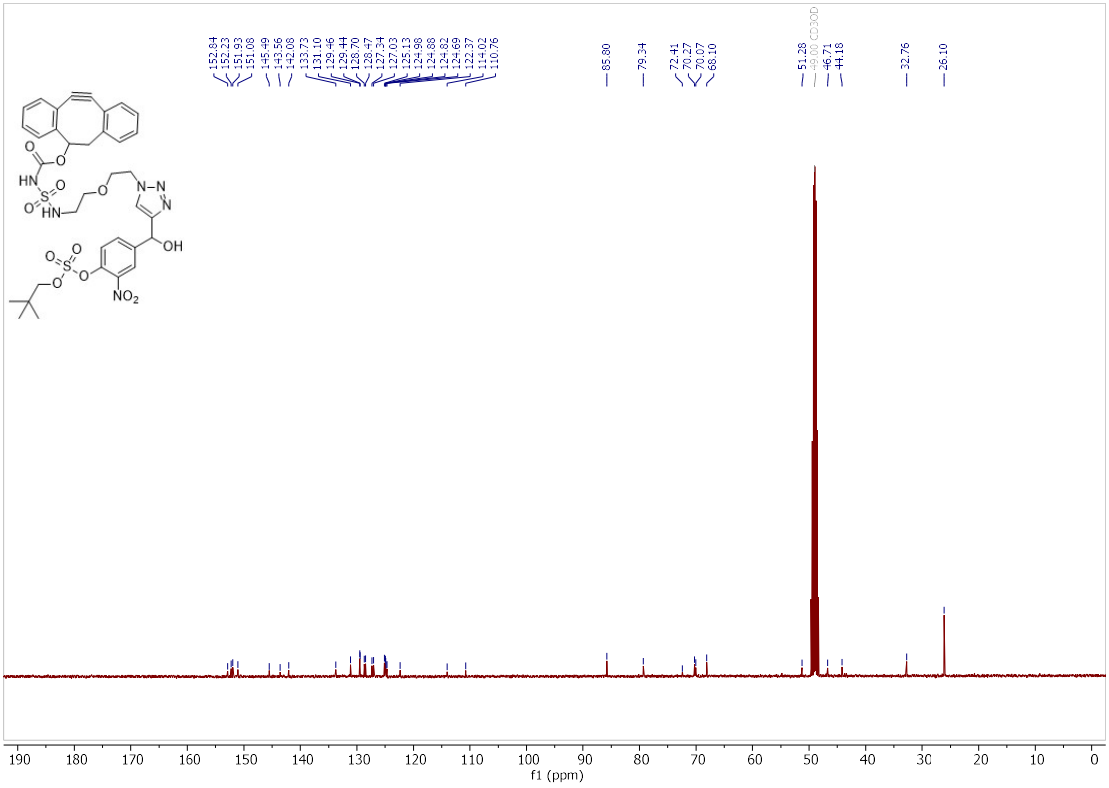
Compound SI10a



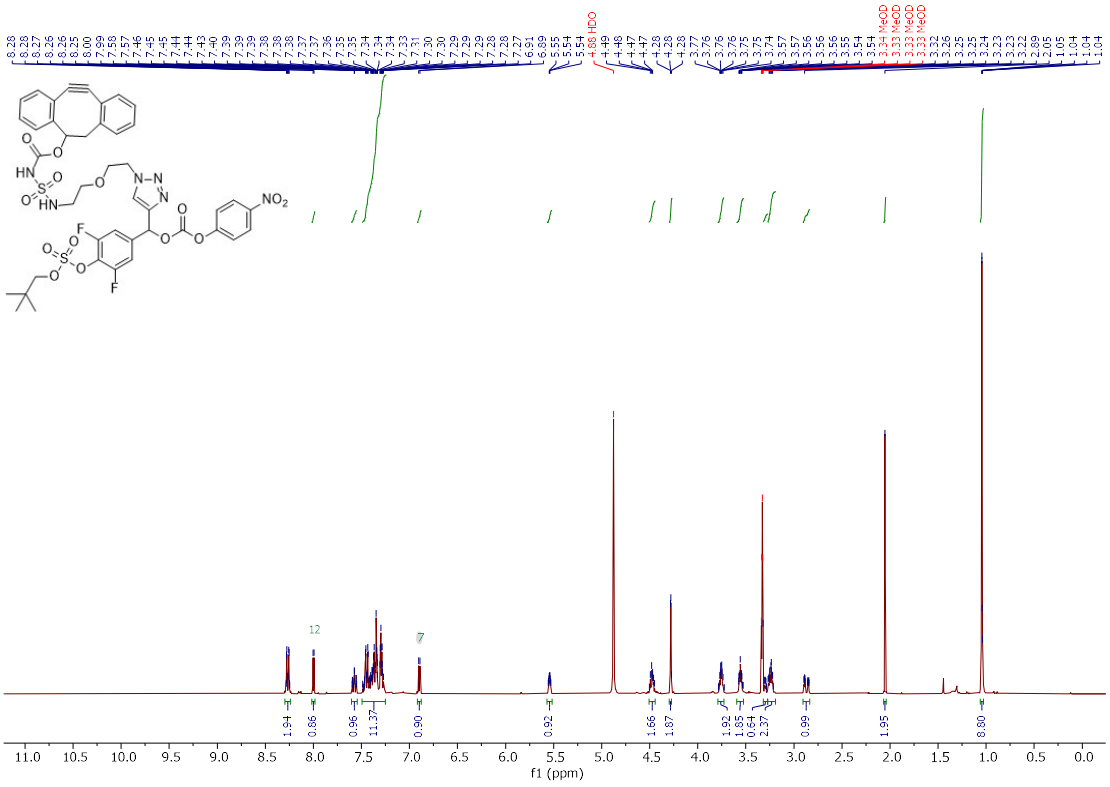


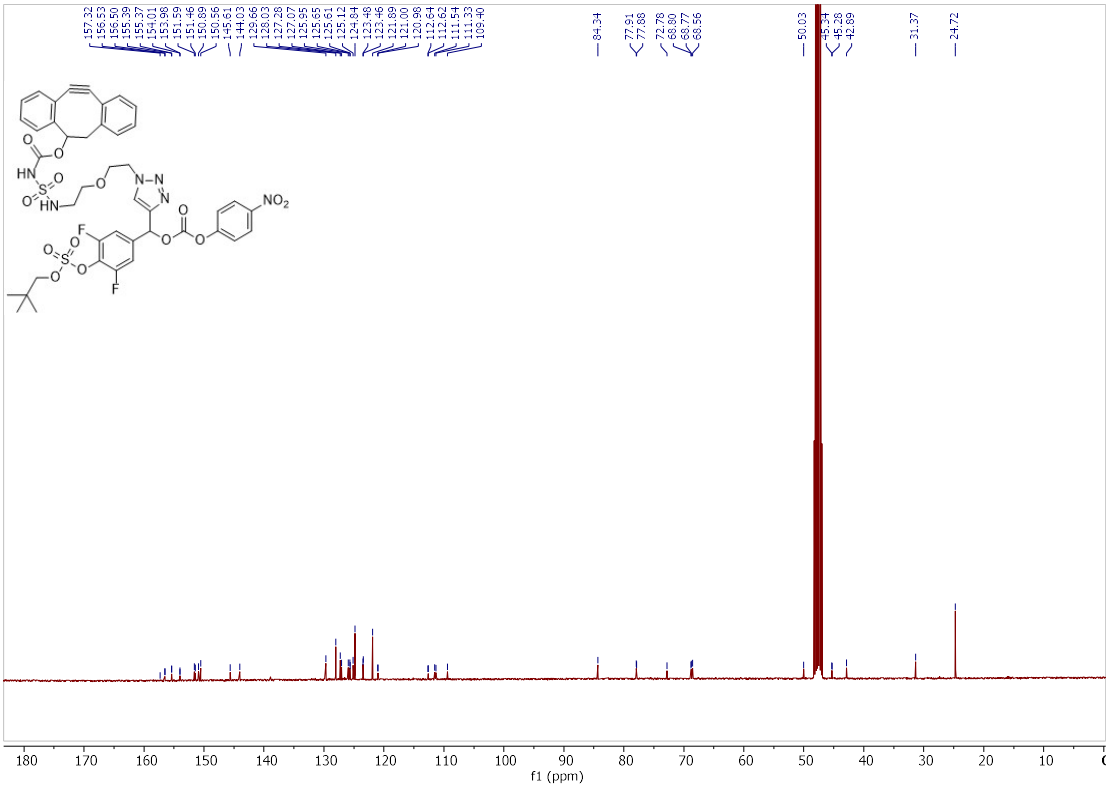
Compound SI10b



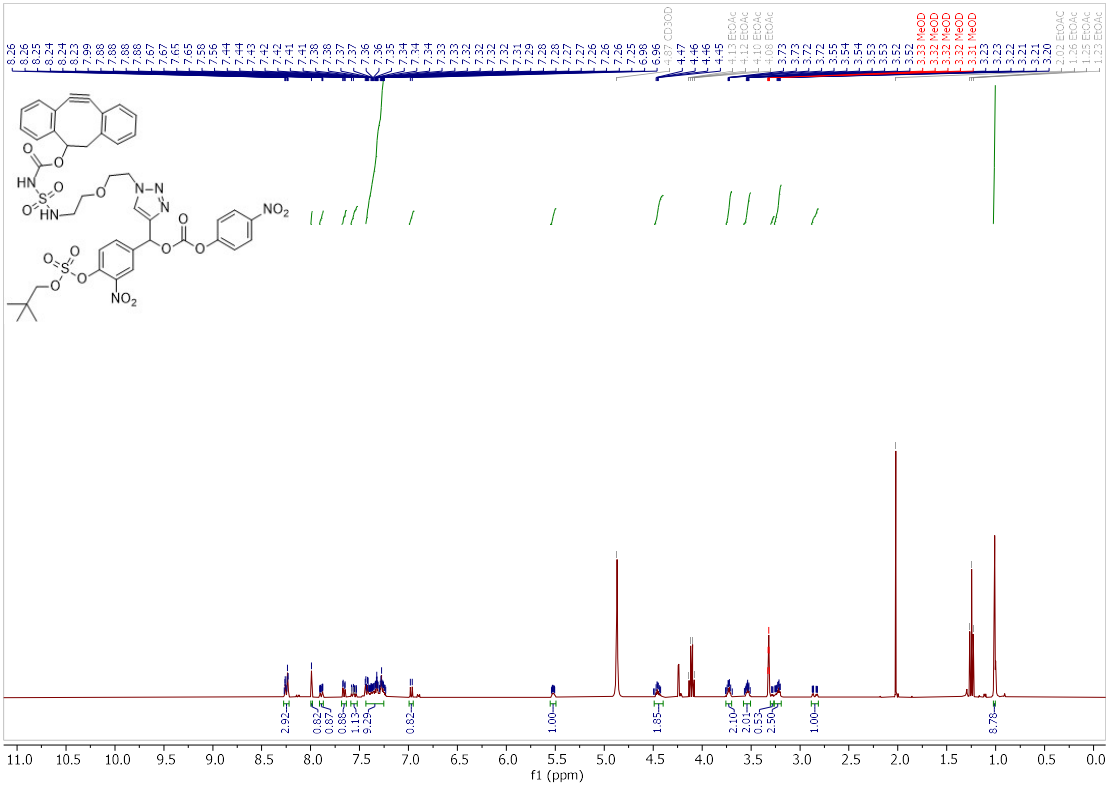


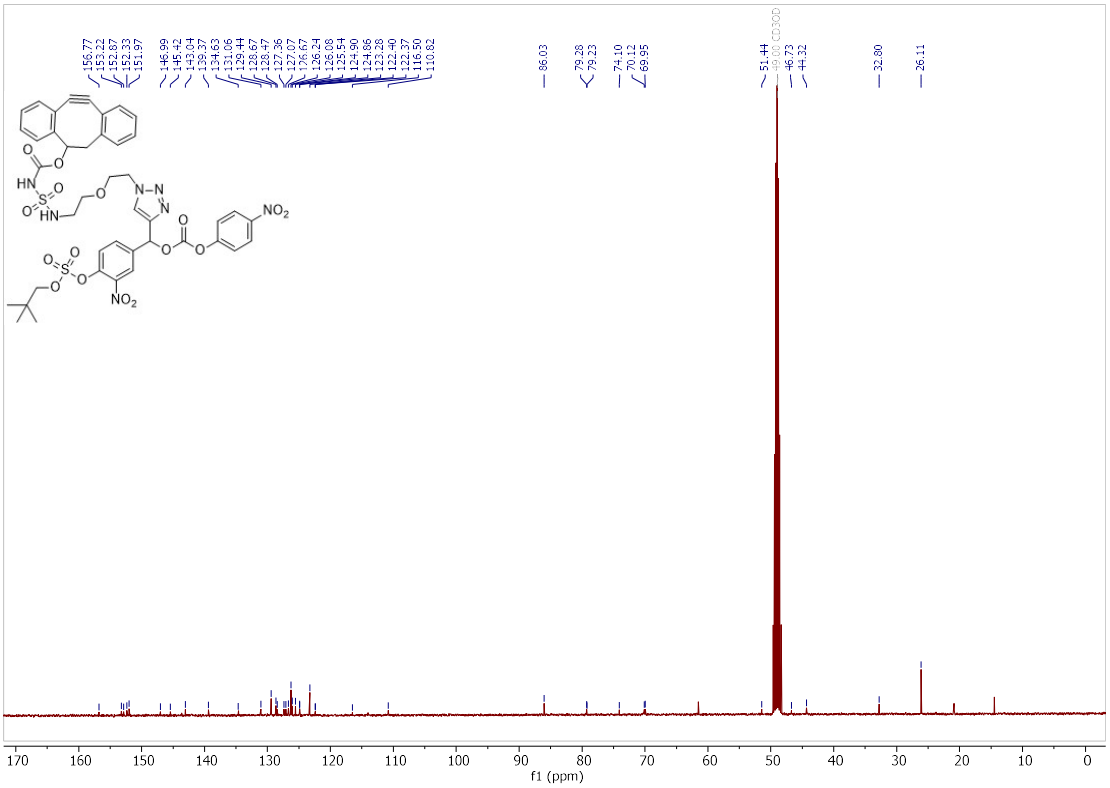
Compound SI11a





Compound SI11b

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CD3OD
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32.80
26.11
NO2
NO2
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130
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110
100
90
80
70
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50
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30
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f1 (ppm)