

## Supporting information

# Synthesis and study of novel pH-independent fluorescent mitochondrial labels based on Rhodamine B

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## General Information

Solvents and chemicals were purchased from Aldrich (Milwaukee, IL, [www.sigmaaldrich.com](http://www.sigmaaldrich.com)). The Wang resin (100-200 mesh, 1% DVB, 1.0 mmol/g) was obtained from AAPPTec (Louisville, KY, [www.aapptec.com](http://www.aapptec.com)). Synthesis was carried out on Domino Blocks in disposable polypropylene reaction vessels (Torviq, Niles, MI, [www.torviq.com](http://www.torviq.com)).

All reactions were carried out at ambient temperature (21 °C) unless stated otherwise. The volume of wash solvent was 10 mL per 1 g of resin. For washing, resin slurry was shaken with the fresh solvent for at least 1 min before changing the solvent. After adding a reagent solution, the resin slurry was manually vigorously shaken to break any potential resin clumps. Resin-bound intermediates were dried by a stream of nitrogen for prolonged storage and/or quantitative analysis.

For the LC/MS analysis a sample of resin (~5 mg) was treated by TFA in DCM (50% v/v), the cleavage cocktail was evaporated by a stream of nitrogen, and cleaved compounds extracted into 1 mL of MeOH.

The LC/MS analyses were carried out on two machines: UHPLC-MS system consisting of UHPLC chromatograph Accela with photodiode array detector and triple quadrupole mass spectrometer TSQ Quantum Access (both Thermo Scientific, CA, USA), using Nucleodur Gravity C18 column at 30°C and flow rate of 800  $\mu$ L/min (Macherey-Nagel, 1.8  $\mu$ m, 2.1 x 50 mm, Germany). The APCI source operated at discharge current of 5  $\mu$ A, vaporizer temperature of 400°C and capillary temperature of 200 °C. UHPLC-MS system consisting of UHPLC chromatograph Acquity with photodiode array detector and single quadrupole mass spectrometer (Waters), using X-Select C18 column at 30 °C and flow rate of 600  $\mu$ L/min. The ESI I source operated at discharge current of 5  $\mu$ A, vaporizer temperature of 350 °C and capillary temperature of 200 °C. Mobile phase was (A) 0.01 M ammonium acetate in water, and (B) acetonitrile, linearly programmed from 10% to 80% B over 2.5 min, kept for 1.5 min. The column was preequilibrated with 10% of solution B for 1 min.

Purification was carried out on C18 column 19 x 100 mm, 5  $\mu$ m particles; gradient was formed from 10 mM aqueous ammonium acetate and acetonitrile, flow rate 15 mL/min.

NMR  $^1\text{H}/^{13}\text{C}$  spectra were obtained on a Varian UnityPlus (299.89 MHz,  $^1\text{H}$ ) instrument, Jeol ECX-500SS (500.16 MHz,  $^1\text{H}$ ) or Jeol ECX-500SS (400 MHz,  $^1\text{H}$ ). NMR spectra were recorded at ambient temperature (21 °C), unless stated otherwise, in DMSO- $d_6$  solutions and referenced to the resonance signal of DMSO. Chemical shifts  $\delta$  are reported in ppm and coupling constants  $J$  in Hz. Acetate salts exhibited singlet at 1.7 – 1.9 ppm in the  $^1\text{H}$  NMR spectrum and two resonances at 173 and 23 ppm in  $^{13}\text{C}$  spectrum.

HRMS analysis was performed using an Orbitrap Elite high-resolution mass spectrometer (Thermo Fischer Scientific, MA, USA) operating at positive full scan mode (120 000 FWHM)

in the range of 200–1000 m/z. The settings for electrospray ionization were as follows: oven temperature of 300 °C, sheath gas of 8 arb. units and source voltage of 1.5 kV. The acquired data were internally calibrated with diisooctyl phthalate as a contaminant in methanol (m/z 391.2843). Samples were diluted to a final concentration of 20 µmol/l with 0.1% formic acid in water and methanol (50:50, v/v). The samples were injected by direct infusion into the mass spectrometer.

### **Analytical data of individual compounds**

Note:

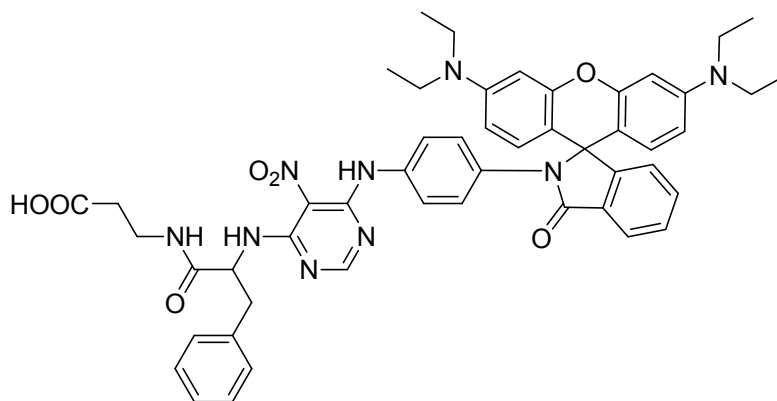
a) In the NMR spectra the isomery for some derivatives was observed (see the NMR studies in publication). For better spectra interpretation some compounds were measured under higher temperature what caused more or less fusion of the signals for most of the compounds. Only exception was derivative **11(1,2,3)**, which kept the complete splitting of the signals even under elevated temperature.

b) Although derivative **11(2,1,2)** was observed as a one isomer (according to <sup>1</sup>H NMR spectrum), the intensity of the signals in <sup>13</sup>C spectrum was too low. Thus the measurement was performed under elevated temperature what increased the intensity and the number of identified carbon signals.

c) Generally, because the carbon signals of derivatives **6(R<sup>1</sup>,R<sup>2</sup>,R<sup>3</sup>)** and **11(R<sup>1</sup>,R<sup>2</sup>,R<sup>3</sup>)** are very often overlapped due to structure complexity and total high number of carbons, the number of identified signals is lower than expected in many cases and change of temperature and/or acquisition time did not afford expected number of signals.

#### **6(1,1,1)**

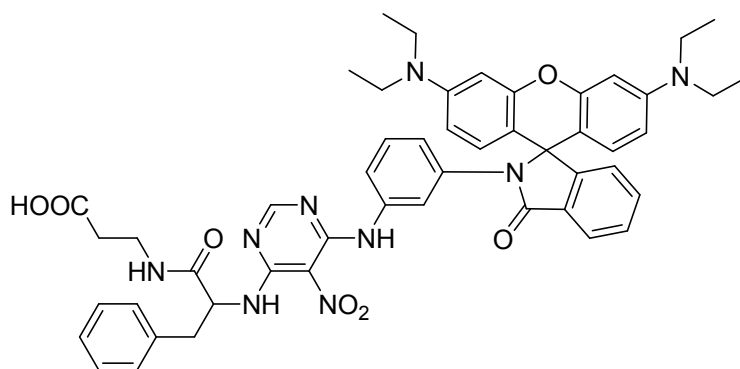
3-(2-(((6-((4-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid



Yield 56.5 mg (61%) of pink amorphous powder.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  10.76 (s, 1 H), 9.41 (d,  $J = 7.1$  Hz, 1 H), 8.36 (t,  $J = 5.2$  Hz, 1 H), 8.04 (s, 1 H), 7.82 - 7.93 (m, 1 H), 7.48 - 7.61 (m, 2 H), 7.38 (d,  $J = 8.8$  Hz, 2 H), 7.09 - 7.30 (m, 5 H), 6.99 - 7.08 (m, 1 H), 6.86 (d,  $J = 8.8$  Hz, 2 H), 6.58 (d,  $J = 8.8$  Hz, 2 H), 6.38 (dd,  $J = 8.9, 2.1$  Hz, 2 H), 6.22 - 6.33 (m, 2 H), 5.01 (q,  $J = 6.3$  Hz, 1 H), 3.23 - 3.34 (m, 8 H) 2.95 - 3.23 (m, 4 H) 2.37 (t,  $J = 6.8$  Hz, 2 H) 1.06 (t,  $J = 6.9$  Hz, 12 H).  $^{13}\text{C}$  NMR (75 MHz,  $\text{DMSO-}d_6$ )  $\delta$  173.4, 170.2, 167.5, 159.7, 156.4, 155.6, 154.2, 152.7, 148.9, 137.1, 135.8, 134.6, 133.9, 129.9, 129.6, 129.0, 128.8, 127.2, 126.6, 124.9, 124.3, 123.4, 113.1, 108.7, 106.0, 97.8, 67.0, 56.1, 44.2, 38.5, 35.5, 34.1, 12.9. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{50}\text{H}_{52}\text{N}_9\text{O}_7$   $[\text{M} + \text{H}]^+$  890.3984, found 890.3976.

### 6(1,1,2)

3-(2-(((3-(2-(6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl)benzamido)phenyl)amino)-5-nitropyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid

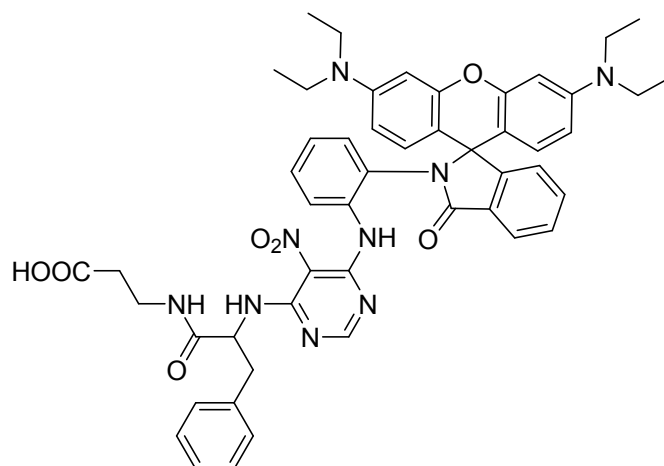


Yield 67.0 mg (86%) of pink amorphous powder.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  10.70 (br. s., 1 H), 9.40 (d,  $J = 7.1$  Hz, 1 H), 8.38 (t,  $J = 4.8$  Hz, 1 H), 7.96 (s, 1 H), 7.89 (dd,  $J = 5.6, 1.9$  Hz, 1 H), 7.48 - 7.64 (m, 2 H), 7.12 - 7.33 (m, 8 H), 7.07 (d,  $J = 6.2$  Hz, 1 H), 6.81 (d,  $J = 7.7$  Hz, 1 H), 6.55 (m,  $J = 9.0$  Hz, 2 H), 6.36 (m,  $J = 9.0$  Hz, 2 H), 6.23 (br. s., 2 H), 4.95 - 5.08 (m, 1 H), 3.05 - 3.27 (m, 12 H), 2.36 (t,  $J = 6.6$  Hz, 2 H), 0.90 - 1.06 (m, 12 H).  $^{13}\text{C}$  NMR (75 MHz,

DMSO- $d_6$ )  $\delta$  173.6, 170.1, 167.1, 159.5, 156.3, 155.2, 153.9, 152.9, 152.8, 152.7, 150.5, 148.8, 137.8, 137.6, 137.5, 137.2, 137.1, 133.9, 130.2, 129.9, 129.8, 129.0, 128.9, 128.8, 128.7, 128.6, 127.2, 124.3, 124.1, 123.4, 122.0, 121.9, 113.1, 108.7, 106.0, 97.7, 67.1, 56.3, 44.1, 38.5, 35.7, 34.5, 12.9, 12.8. HRMS (ESI)  $m/z$  calcd for  $C_{50}H_{52}N_9O_7$   $[M + H]^+$  890.3984, found 890.3978.

### 6(1,1,3)

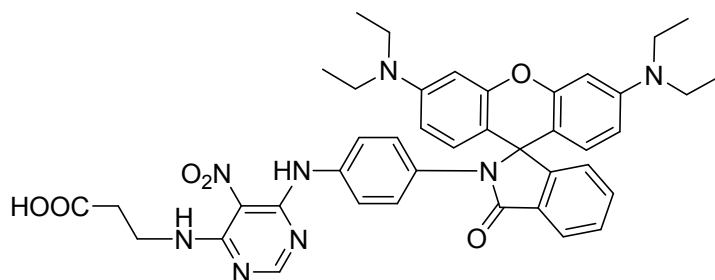
3-(2-((6-((2-(2-(6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl)benzamido)phenyl)amino)-5-nitropyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid



Yield 63.9 mg (83%) of pink amorphous powder.  $^1H$  NMR (400MHz, DMSO- $d_6$ )  $\delta$  10.59 (d,  $J$  = 11.7 Hz, 1 H), 9.39 (d,  $J$  = 7.4 Hz, 1 H), 8.39-8.26 (m, 1 H), 8.04 - 7.95 (m, 1 H), 7.82-7.77 (m, 1H), 7.67-7.65 (m, 3 H), 7.33 - 7.10 (m, 7 H), 6.95 (t,  $J$  = 7.8 Hz, 1 H), 6.63 - 6.47 (m, 2 H), 6.30 (br. s., 1 H), 6.11 (d,  $J$  = 7.8 Hz, 1 H), 6.02 (t,  $J$  = 9.6 Hz, 2 H), 5.59-5.49 (m, 1 H), 5.06 - 4.88 (m, 1 H), 3.32 (4 H, overlapped with water), 3.28 - 3.17 (m, 2 H), 3.17 - 3.07 (m, 2 H), 3.07 - 2.93 (m, 4 H), 2.43 - 2.29 (m, 2 H), 1.10 (t,  $J$  = 6.5 Hz, 6 H), 0.96 - 0.80 (m, 6 H).  $^{13}C$  NMR (75MHz, DMSO- $d_6$ )  $\delta$  173.3, 170.1, 166.6, 159.4, 156.3, 156.2, 155.4, 154.2, 153.3, 152.7, 149.3, 148.5, 137.0, 136.9, 135.4, 133.9, 131.2, 130.0, 129.8, 129.7, 129.5, 129.2, 128.8, 128.7, 128.4, 128.2, 128.1, 127.3, 126.2, 124.8, 123.7, 113.4, 109.1, 107.2, 106.9, 106.3, 104.9, 97.9, 97.6, 97.4, 68.1, 56.5, 55.8, 44.3, 43.8, 43.7, 38.6, 35.6, 34.2, 12.8. HRMS (ESI)  $m/z$  calcd for  $C_{50}H_{52}N_9O_7$   $[M + H]^+$  890.3984, found 890.3979.

### 6(2,1,1)

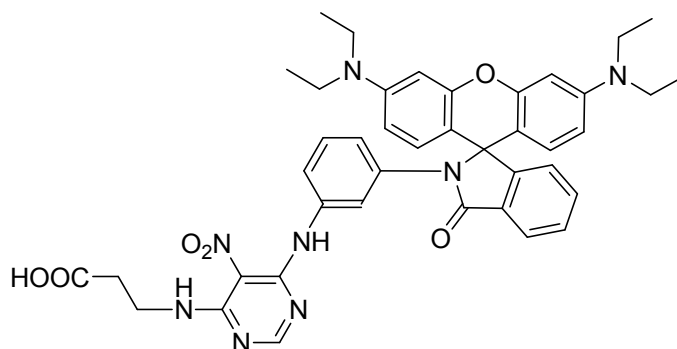
3-((6-((4-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)propanoic acid



Yield 33 mg (25%) of orange amorphous powder.  $^1\text{H}$  NMR (500 MHz,  $\text{DMSO-}d_6$ )  $\delta$  10.77 (s, 1 H), 9.52 (t,  $J = 5.7$  Hz, 1 H), 8.06 (d,  $J = 4.5$  Hz, 1 H), 7.90 – 7.80 (m, 1 H), 7.58 – 7.46 (m, 2 H), 7.37 (d,  $J = 8.9$  Hz, 2 H), 7.01 (d,  $J = 6.8$  Hz, 1 H), 6.82 (d,  $J = 8.9$  Hz, 2 H), 6.54 (d,  $J = 8.8$  Hz, 2 H), 6.35 (dd,  $J = 8.9, 2.4$  Hz, 2 H), 6.26 (d,  $J = 2.5$  Hz, 2 H), 3.72 (dd,  $J = 12.7, 6.5$  Hz, 2 H), 3.31 – 3.20 (m, 8 H), 2.52 (t,  $J = 6.6$  Hz, 2 H), 1.03 (t,  $J = 7.0$  Hz, 12 H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{DMSO-}d_6$ )  $\delta$  173.8, 167.4, 159.8, 157.0, 155.8, 154.2, 152.7, 148.9, 135.9, 134.4, 133.9, 130.0, 129.0, 126.6, 124.7, 124.3, 123.4, 112.9, 108.7, 106.0, 97.8, 66.9, 44.2, 37.7, 34.1, 12.9. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{41}\text{H}_{43}\text{N}_8\text{O}_6$   $[\text{M} + \text{H}]^+$  743.3300, found 743.3300.

### 6(2,1,2)

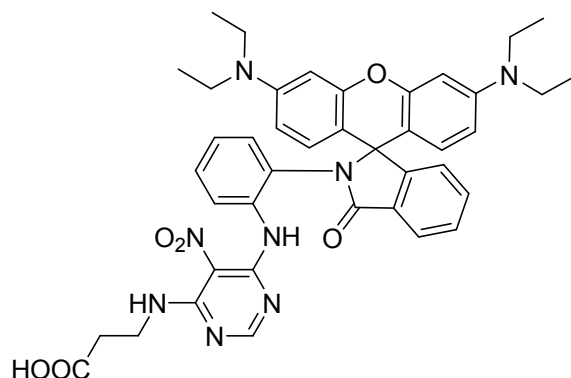
3-((6-((3-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)propanoic acid



Yield 64.6 mg (37%) of orange amorphous powder.  $^1\text{H}$  NMR (500 MHz,  $\text{DMSO-}d_6$ )  $\delta$  10.71 (s, 1 H), 9.49 (t,  $J = 5.8$  Hz, 1 H), 7.97 (s, 1 H), 7.90 – 7.77 (m, 1 H), 7.61 – 7.44 (m, 2 H), 7.21 (d,  $J = 8.1$  Hz, 1 H), 7.17 (s, 1 H), 7.13 (t,  $J = 8.0$  Hz, 1 H), 7.03 (d,  $J = 7.0$  Hz, 1 H), 6.79 (d,  $J = 8.3$  Hz, 1 H), 6.52 (d,  $J = 8.9$  Hz, 2 H), 6.34 (dd,  $J = 9.0, 2.4$  Hz, 2 H), 6.22 (d,  $J = 2.4$  Hz, 2 H), 3.74 (dd,  $J = 12.8, 6.6$  Hz, 2 H), 3.28 – 3.17 (m, 8 H), 2.56 (t,  $J = 6.8$  Hz, 2 H), 0.98 (t,  $J = 7.0$  Hz, 12 H).  $^{13}\text{C}$  NMR (126 MHz, )  $\delta$  173.7, 167.2, 159.6, 156.9, 155.4, 153.9, 152.8, 148.9, 137.9, 137.5, 133.9, 130.1, 129.0, 128.9, 124.3, 123.9, 123.4, 121.8, 121.7, 112.9, 108.7, 106.0, 97.8, 67.1, 44.1, 37.6, 33.9, 12.8. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{41}\text{H}_{43}\text{N}_8\text{O}_6$   $[\text{M} + \text{H}]^+$  743.3300, found 743.3300.

### 6(2,1,3)

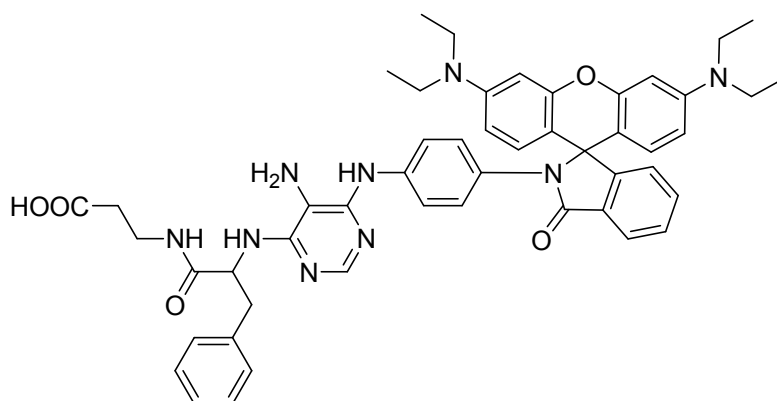
3-((6-((2-(2-(6-(diethylamino)-3-(diethyliminio)-3H-xanthen-9-yl)benzamido)phenyl)amino)-5-nitropyrimidin-4-yl)amino)propanoic acid



Yield 58 mg (50%) of orange amorphous powder.  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  10.60 (s, 1 H), 9.49 (t,  $J = 5.7$  Hz, 1 H), 7.94 - 8.00 (m, 1 H), 7.81 (s, 1 H), 7.69 (d,  $J = 7.5$  Hz, 1 H), 7.62 - 7.67 (m, 2 H), 7.25 (t,  $J = 7.2$  Hz, 1 H), 7.15 - 7.21 (m, 1 H), 6.93 (t,  $J = 7.2$  Hz, 1 H), 6.47 - 6.59 (m, 2 H), 6.28 (br. s., 1 H), 6.08 (d,  $J = 7.9$  Hz, 1 H), 5.97 - 6.05 (m, 2 H), 5.61 (d,  $J = 7.9$  Hz, 1 H), 3.70 - 3.76 (m, 2 H), 3.25 - 3.41 (4 H, overlapped with water), 3.04 - 3.1 (m, 4 H), 2.56 (t,  $J = 6.8$  Hz, 2 H), 1.09 (t,  $J = 6.6$  Hz, 6 H), 0.92 (t,  $J = 6.6$  Hz, 6 H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )  $\delta$  173.5, 166.4, 159.3, 156.7, 155.4, 154.1, 153.2, 153.1, 152.5, 149.2, 148.9, 148.4, 135.4, 133.8, 131.2, 130.7, 129.4, 129.3, 129.1, 128.7, 128.3, 128.2, 128.1, 127.9, 126.0, 124.7, 123.5, 113.1, 108.9, 108.6, 106.9, 106.2, 104.9, 97.8, 97.7, 97.3, 68.0, 44.2, 43.8, 37.4, 33.7, 12.8, 12.8. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{41}\text{H}_{43}\text{N}_8\text{O}_6$   $[\text{M} + \text{H}]^+$  743.3300, found 743.3298.

### 6(1,2,1)

3-(2-((5-amino-6-((4-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid

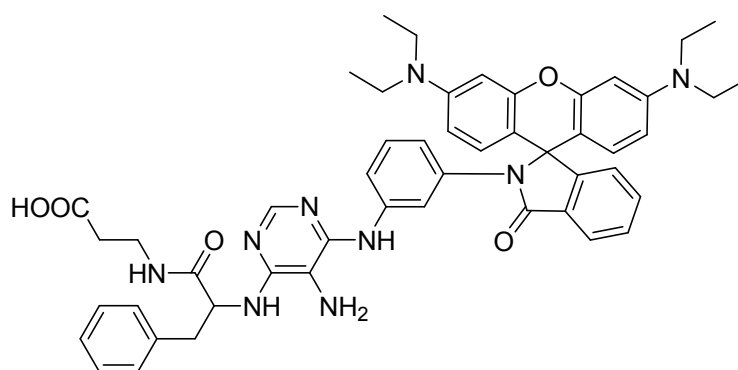


Yield 33.0 mg (36%) of pink amorphous powder.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  8.08 (t,  $J = 5.5$  Hz, 1 H), 7.78 - 7.89 (m, 2 H), 7.65 (s, 1 H), 7.49 - 7.60 (m, 2 H), 7.01 - 7.35 (m, 9 H),

6.54 (dd,  $J = 8.9, 2.7$  Hz, 4 H), 6.39 (dd,  $J = 8.9, 2.5$  Hz, 2 H), 6.20 - 6.29 (m, 3 H), 4.65 - 4.78 (m, 1 H), 3.24 - 3.35 (m, 8 H), 3.02 (dd,  $J = 13.6, 4.7$  Hz, 2 H), 2.85 - 2.92 (m, 2 H), 2.30 (t,  $J = 7.0$  Hz, 2 H), 1.06 (t,  $J = 7.0$  Hz, 12 H).  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ )  $\delta$  173.4, 172.9, 167.1, 153.8, 152.9, 151.5, 148.8, 146.9, 146.7, 140.6, 139.0, 133.6, 130.8, 129.7, 129.5, 129.2, 128.9, 128.5, 127.5, 126.6, 124.4, 123.2, 118.9, 110.1, 108.6, 106.2, 97.7, 66.9, 56.3, 44.2, 38.7, 35.3, 34.3, 12.9. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{50}\text{H}_{54}\text{N}_9\text{O}_5$   $[\text{M} + \text{H}]^+$  860.4242, found 860.4237.

### 6(1,2,2)

3-(2-((5-amino-6-((3-(2-(6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl)benzamido)phenyl)amino)pyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid

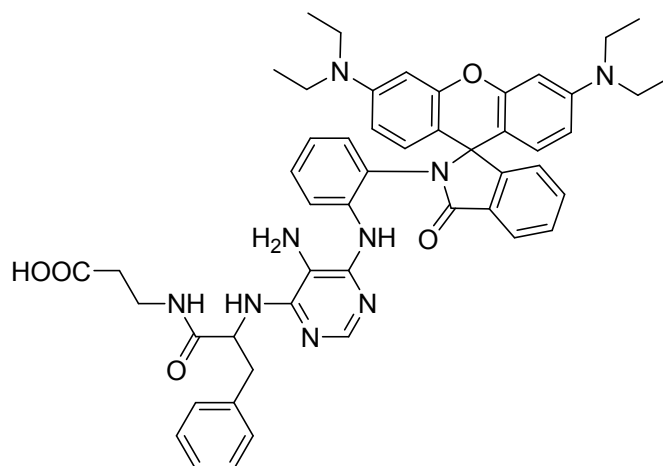


Yield 45.4 mg (72%) of red/pink amorphous powder.  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  8.09 - 8.03 (m, 1 H), 7.89 - 7.84 (m, 1 H), 7.72 (s, 1 H), 7.58 (s, 1 H), 7.57 - 7.49 (m, 2 H), 7.36 (br. s., 1 H), 7.32 - 7.19 (m, 4 H), 7.19 - 7.13 (m, 2 H), 7.06 - 7.00 (m, 1 H), 7.00 - 6.92 (m, 1 H), 6.55 - 6.48 (m, 2 H), 6.42 - 6.31 (m, 3 H), 6.27 - 6.20 (m, 3 H), 6.17 (d,  $J = 8.2$  Hz, 1 H), 4.72 (dt,  $J = 5.1, 8.6$  Hz, 1 H), 3.34 - 3.13 (m, 8 H), 3.09 - 2.98 (m, 2 H), 2.95 - 2.83 (m, 2 H), 2.33 - 2.26 (m, 2 H), 1.08 - 0.95 (m, 12 H).  $^{13}\text{C}$  NMR (101 MHz, DMSO- $d_6$ )  $\delta$  173.5, 172.8, 167.2, 167.1, 154.0, 152.7, 151.4, 151.2, 150.2, 148.7, 146.8, 146.5, 144.6, 142.1, 140.9, 139.0, 137.3, 136.8, 133.6, 130.4, 130.2, 130.0, 129.6, 128.8, 128.4, 128.3, 126.9, 126.5, 124.2, 123.2, 118.7, 118.2, 117.9, 117.7, 117.3, 109.6, 108.5, 106.1, 106.0, 97.7, 66.9, 56.5, 56.3, 44.0, 38.7, 35.3, 34.5, 12.8. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{50}\text{H}_{54}\text{N}_9\text{O}_5$   $[\text{M} + \text{H}]^+$  860.4242, found 860.4237.

### 6(1,2,3)

3-(2-(((5-amino-6-((2-(2-(6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl)benzamido)phenyl)amino)pyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid



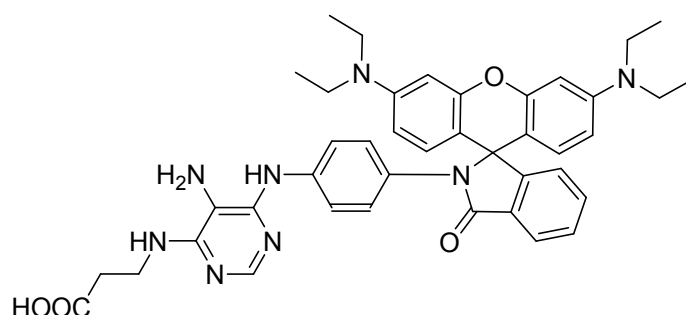


Yield 44.7 mg (70 %) of red/pink amorphous powder.  $^1\text{H}$  NMR (400MHz,  $\text{DMSO-}d_6$ )  $\delta$  7.98 - 7.93 (m, 1 H), 7.75 - 7.69 (m, 1 H), 7.67 (s, 1 H), 7.66 - 7.60 (m, 3 H), 7.25 - 7.23 (m, 2 H), 7.19 (t,  $J = 7.0$  Hz, 2 H), 7.14 - 7.12 (m, 2H), 7.10 - 7.06 (m, 2 H), 6.65 (t,  $J = 7.8$  Hz, 1 H), 6.51 (t,  $J = 8.4$  Hz, 2 H), 6.18 - 6.16 (m, 2 H), 6.10 (d,  $J = 7.8$  Hz, 2 H), 4.80 - 4.72 (m, 1 H), 3.30 - 3.16 (m, 8 H), 3.07 - 3.02 (m, 2 H), 2.95 - 2.89 (m, 2 H), 2.30 (t,  $J = 7.0$  Hz, 2 H), 1.09 - 0.97 (m, 12 H).  $^{13}\text{C}$  NMR (101MHz,  $\text{DMSO-}d_6$ )  $\delta$   $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )  $\delta$  173.1, 172.6, 166.8, 154.6, 153.6, 153.4, 151.6, 149.8, 149.3, 138.9, 138.7, 133.7, 131.4, 129.6, 129.2, 129.0, 128.5, 128.1, 127.7, 126.8, 126.6, 124.7, 123.4, 123.3, 121.6, 108.8, 107.9, 98.1, 68.4, 56.3, 44.1, 38.9, 35.4, 34.4, 12.8. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{50}\text{H}_{54}\text{N}_9\text{O}_5$   $[\text{M} + \text{H}]^+$  860.4242, found 860.4245.

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed at 70°C.

### 6(2,2,1)

3-((5-amino-6-((4-(3',6'-bis(diethylamino)-3-oxospiro[isoinoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)propanoic acid



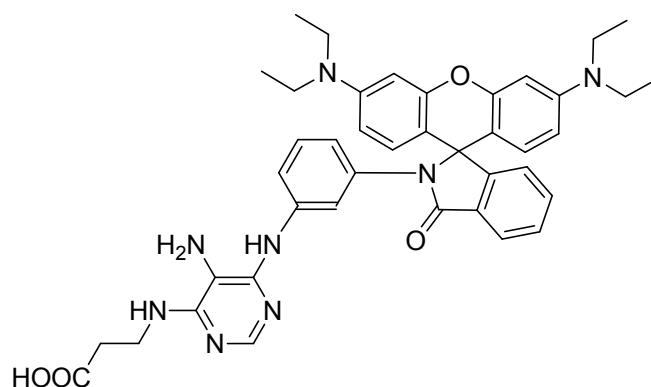
Yield 38.3 mg (32%) of red/pink amorphous powder.  $^1\text{H}$  NMR (500 MHz,  $\text{DMSO-}d_6$ )  $\delta$  7.82 (dt,  $J = 14.0, 7.5$  Hz, 2 H), 7.78 (s, 1 H), 7.70 (s, 1 H), 7.56 - 7.48 (m, 2 H), 7.29 (d,  $J = 9.1$  Hz, 2 H), 7.03 (dd,  $J = 6.2, 1.7$  Hz, 1 H), 6.51 (dd,  $J = 8.9, 4.6$  Hz, 4 H), 6.36 (dd,  $J = 9.0, 2.6$  Hz, 2 H), 6.22 (d,  $J = 2.5$  Hz, 2 H), 3.48 (t,  $J = 6.9$  Hz, 2 H), 3.26 (q,  $J = 7.3$  Hz, 8 H), 2.38 (t,

$J = 6.9$  Hz, 2 H), 1.59 – 1.53 (m, 2 H), 1.03 (t,  $J = 7.0$  Hz, 12 H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{DMSO-}d_6$ )  $\delta$  174.7, 167.1, 153.8, 152.9, 148.8, 147.4, 146.4, 140.8, 133.6, 130.8, 129.3, 129.2, 128.9, 127.6, 123.2, 121.9, 120.5, 118.7, 109.7, 108.6, 106.2, 97.7, 66.9, 44.2, 38.0, 36.0, 12.9. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{41}\text{H}_{45}\text{N}_8\text{O}_4$   $[\text{M} + \text{H}]^+$  713.3558, found 713.3557.

Note: the signals of the residual piperidine are present in the NMR spectra (multiplets at 2.88 and 1.50 in the  $^1\text{H}$  spectrum and signals at 22.4, 22.8 and 44.3 in the  $^{13}\text{C}$  spectrum).

### 6(2,2,2)

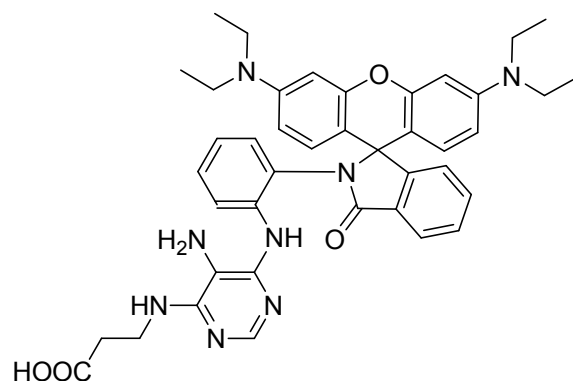
3-((5-amino-6-((3-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)propanoic acid



Yield 43.4 mg (23%) of red/pink amorphous powder.  $^1\text{H}$  NMR (500 MHz,  $\text{DMSO-}d_6$ )  $\delta$  7.84 (dd,  $J = 6.3, 1.4$  Hz, 1 H), 7.70 (s, 1 H), 7.64 (s, 1 H), 7.50 (pd,  $J = 7.3, 1.2$  Hz, 2 H), 7.37 (t,  $J = 1.9$  Hz, 1 H), 7.20 (dd,  $J = 8.2, 1.2$  Hz, 1 H), 6.99 (dd,  $J = 6.3, 1.4$  Hz, 1 H), 6.93 (t,  $J = 8.1$  Hz, 1 H), 6.50 (d,  $J = 8.9$  Hz, 2 H), 6.33 (dd,  $J = 9.0, 2.5$  Hz, 2 H), 6.26 (d,  $J = 8.5$  Hz, 1 H), 6.21 (d,  $J = 2.5$  Hz, 2 H), 3.50 (t,  $J = 6.8$  Hz, 2 H), 3.28 – 3.17 (m, 8 H), 2.47 (t,  $J = 1.5$  Hz, 2 H), 1.00 (t,  $J = 7.0$  Hz, 12 H).  $^{13}\text{C}$  NMR (126 MHz,  $\text{DMSO-}d_6$ )  $\delta$  174.3, 167.3, 154.3, 152.8, 152.3, 148.8, 147.3, 146.4, 142.3, 137.4, 133.7, 130.2, 128.9, 128.4, 124.3, 123.3, 117.7, 117.2, 109.4, 108.6, 106.2, 97.9, 97.8, 67.0, 44.1, 37.6, 35.4, 12.9. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{41}\text{H}_{45}\text{N}_8\text{O}_4$   $[\text{M} + \text{H}]^+$  713.3558, found 713.3557.

### 6(2,2,3)

3-((5-amino-6-((2-(2-(6-(diethylamino)-3-(diethyliminio)-3H-xanthen-9-yl)benzamido)phenyl)amino)pyrimidin-4-yl)amino)propanoic acid

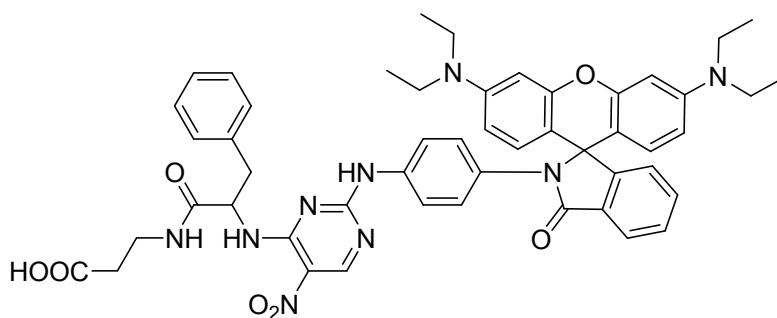


Yield 18.3 mg (11%) of red/orange amorphous powder.  $^1\text{H}$  NMR (500 MHz,  $\text{DMSO-d}_6$ )  $\delta$  7.94 – 7.92 (m, 1 H), 7.67 (s, 1 H), 7.65 (s, 1 H), 7.63 – 7.60 (m, 2 H), 7.14 – 7.12 (m, 1 H), 7.08 (t,  $J = 7.5$  Hz, 1 H), 6.98 (s, 1 H), 6.59 (t,  $J = 7.5$  Hz, 1 H), 6.50 (d,  $J = 8.8$  Hz, 2 H), 6.18 (s, 2 H), 6.10 (d,  $J = 7.5$  Hz, 2 H), 3.55 (t,  $J = 6.6$  Hz, 2 H), 3.27 – 3.21 (m, 8 H), 2.50 (2 H, overlapped with DMSO), 1.04 (t,  $J = 7.0$  Hz, 12 H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-d}_6$ )  $\delta$  173.7, 166.9, 155.2, 153.6, 153.4, 151.1, 149.9, 149.2, 138.9, 133.6, 131.4, 129.1, 129.0, 128.1, 127.9, 126.7, 124.7, 123.4, 123.3, 121.4, 108.7, 107.8, 98.0, 68.3, 44.2, 37.5, 35.3, 12.9. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{41}\text{H}_{45}\text{N}_8\text{O}_4$   $[\text{M} + \text{H}]^+$  713.3558, found 713.3557.

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed at  $70^\circ\text{C}$ .

### 11(1,1,1)

3-(2-((2-((4-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid



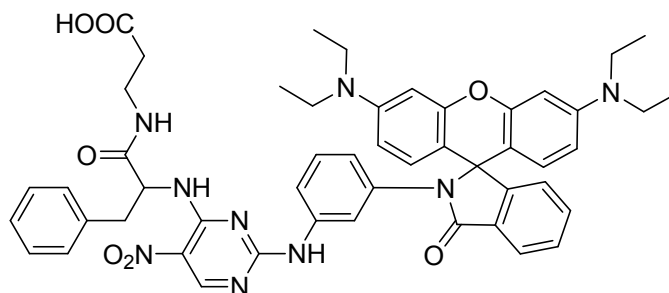
Yield 65.7 mg (70 %) of pink amorphous powder.  $^1\text{H}$  NMR (400MHz,  $\text{DMSO-d}_6$ )  $\delta$  9.92 (br. s., 1 H), 8.91 (s, 1 H), 8.78 (d,  $J = 7.8$  Hz, 1 H), 8.06 (br. s., 1 H), 7.90 (dd,  $J = 2.2, 6.1$  Hz, 1 H), 7.62 - 7.52 (m, 2 H), 7.46 (d,  $J = 9.0$  Hz, 2 H), 7.21 - 7.10 (m, 5 H), 7.08 (dd,  $J = 2.0, 6.3$  Hz, 1 H), 6.82 (d,  $J = 9.0$  Hz, 2 H), 6.59 (dd,  $J = 3.3, 8.8$  Hz, 2 H), 6.44 - 6.40 (m, 2 H), 6.31 (s, 2 H), 4.96 - 4.88 (m, 1 H), 3.30 (qd,  $J = 6.9, 13.3$  Hz, 8 H), 3.20 - 2.95 (m, 4 H), 2.38 - 2.31 (m, 2 H), 1.13 - 1.03 (m, 12 H).  $^{13}\text{C}$  NMR (101MHz,  $\text{DMSO-d}_6$ )  $\delta$  172.0, 169.1, 166.2, 158.9, 156.6, 154.5, 152.8, 152.1, 148.3, 136.4, 136.1, 132.6, 131.8, 129.8, 128.8, 127.9, 127.7,

126.2, 126.0, 123.3, 122.3, 119.7, 108.1, 105.8, 97.4, 97.3, 66.3, 54.9, 43.2, 37.6, 34.7, 33.3, 11.9. HRMS (ESI)  $m/z$  calcd for  $C_{50}H_{52}N_9O_7$   $[M + H]^+$  890.3984, found 890.3978.

The  $^1H$  and  $^{13}C$  NMR experiments were performed at 80°C.

### 11(1,1,2)

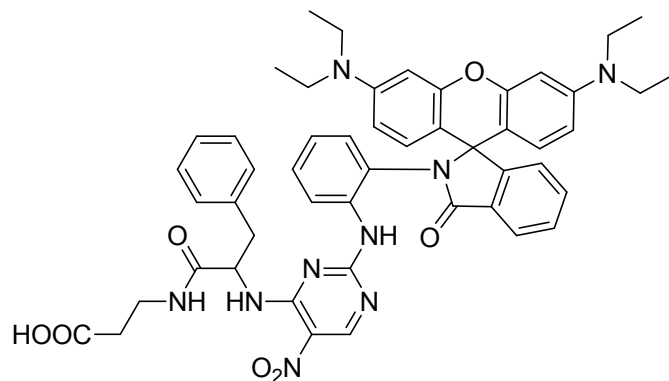
3-(2-((2-((3-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid



Yield 86.5 mg (92%) of pink amorphous powder.  $^1H$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  12.22 (br. s., 1 H), 10.26 (br. s., 1 H), 8.85 (s, 1 H), 8.69 (br. s., 1 H), 8.16 (t,  $J = 5.7$  Hz, 1 H), 7.90 (br. s., 1 H), 7.58 - 7.45 (m, 3 H), 7.26 (br. s., 1 H), 7.16 - 6.95 (m, 7 H), 6.57 (br. s., 1 H), 6.43 (d,  $J = 8.6$  Hz, 1 H), 6.31 (dd,  $J = 2.2, 8.8$  Hz, 1 H), 6.123 - 6.16 (m, 3 H), 5.03 (br. s., 1 H), 3.23 - 3.18 (m, 8 H), 3.05 - 2.89 (m, 4 H), 2.26 (t,  $J = 6.5$  Hz, 2 H), 1.02 - 0.89 (m, 12 H).  $^{13}C$  NMR (75 MHz, DMSO- $d_6$ )  $\delta$  173.4, 170.2, 167.7, 159.8, 157.8, 155.2, 154.0, 152.9, 152.8, 148.9, 148.8, 139.3, 137.5, 137.0, 134.0, 130.2, 129.9, 129.1, 129.0, 128.8, 128.7, 127.2, 124.3, 123.5, 119.5, 108.7, 108.6, 106.1, 106.0, 97.8, 67.5, 55.4, 44.2, 38.6, 35.6, 34.2, 12.9, 12.8. HRMS (ESI)  $m/z$  calcd for  $C_{50}H_{52}N_9O_7$   $[M + H]^+$  890.3984, found 890.3979.

### 11(1,1,3)

3-(2-((2-((2-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid

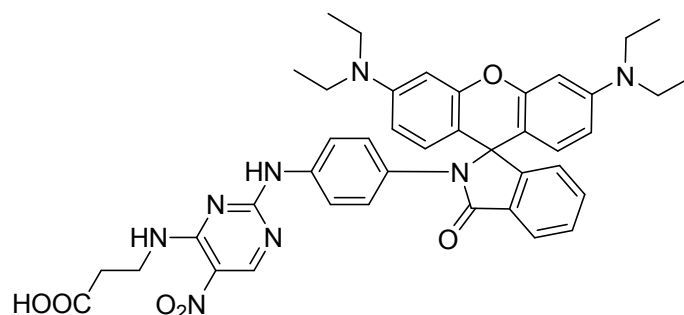


Yield 50.1 mg (76 %) of red amorphous powder.  $^1H$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.89 (s, 1 H), 8.79 (s, 1 H), 8.68 (d,  $J = 6.7$  Hz, 1 H), 8.02 - 7.89 (m, 2 H), 7.74 - 7.61 (m, 3 H), 7.57 (br.

s., 1 H), 7.24 (t,  $J = 7.8$  Hz, 1 H), 7.21 - 7.09 (m, 4 H), 7.03 - 6.94 (m, 2 H), 6.89 (t,  $J = 7.8$  Hz, 1 H), 6.52 - 6.32 (m, 3 H), 6.23 - 6.12 (m, 2 H), 6.10 (br. s., 1 H), 4.64 (br. s., 1 H), 3.34 - 3.18 (m, 8 H), 3.03 - 2.91 (m, 4 H), 2.99 - 2.85 (m, 1 H), 2.32 - 2.29 (m, 2 H), 1.11 - 0.88 (m, 12 H).  $^{13}\text{C}$  NMR (101MHz,  $\text{DMSO-}d_6$ )  $\delta$  172.8, 170.0, 167.0, 159.9, 157.7, 154.9, 153.7, 152.7, 149.3, 136.9, 135.9, 133.9, 131.2, 129.7, 129.3, 128.9, 128.8, 128.6, 128.5, 128.3, 126.9, 125.0, 124.7, 123.4, 122.1, 110.0, 98.2, 98.1, 68.7, 55.7, 44.0, 38.6, 35.5, 34.1, 12.7. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{50}\text{H}_{52}\text{N}_9\text{O}_7$   $[\text{M} + \text{H}]^+$  890.3984, found 890.3979. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed at  $80^\circ\text{C}$ .

### 11(2,1,1)

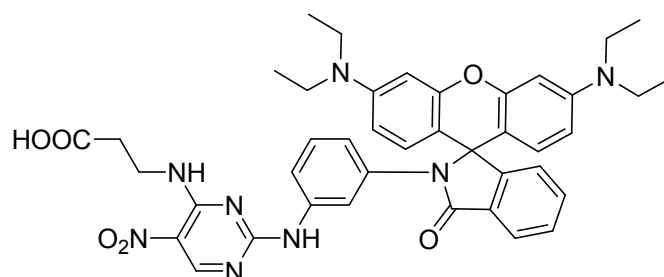
3-((2-((4-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)propanoic acid



Yield 25 mg (23 %) of pink amorphous powder.  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  10.32 (br. s., 1 H), 8.93 (s, 1 H), 7.86 (d,  $J = 6.6$  Hz, 1 H), 7.47 - 7.59 (m, 4 H), 7.06 (d,  $J = 7.0$  Hz, 1 H), 6.70 (d,  $J = 8.8$  Hz, 2 H), 6.53 (m, 2 H), 6.38 (dd,  $J = 9.0, 2.4$  Hz, 2 H), 6.25 (d,  $J = 2.2$  Hz, 2 H), 3.67 - 3.72 (m, 2 H), 2.53 - 2.57 (m, 2 H), 1.05 (t,  $J = 7.0$  Hz, 12 H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )  $\delta$  173.5, 167.1, 157.9, 155.7, 153.8, 152.7, 148.8, 137.6, 133.8, 132.3, 130.3, 129.0, 128.9, 127.2, 126.8, 124.3, 123.5, 123.2, 122.8, 120.3, 108.6, 105.9, 97.6, 66.9, 44.1, 37.1, 33.6, 12.8. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{41}\text{H}_{43}\text{N}_8\text{O}_6$   $[\text{M} + \text{H}]^+$  743.3300, found 743.3296.

### 11(2,1,2)

3-((2-((3-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)propanoic acid

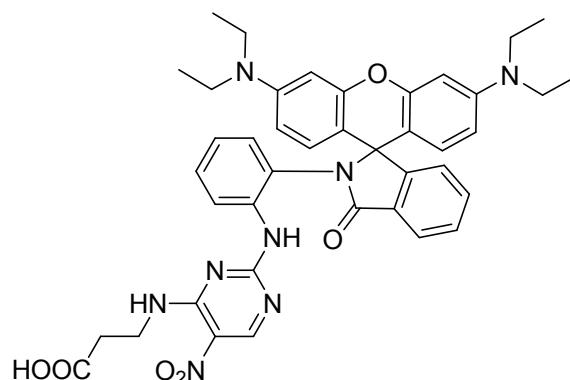


Yield 17 mg (15 %) of red amorphous powder.  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  12.33 (br. s., 1 H), 10.31 (br. s., 1 H), 8.89 (br. s., 1 H), 7.88 (d,  $J = 6.1$  Hz, 1 H), 7.51 - 7.60 (m, 3 H), 7.01 - 7.09 (m, 2 H), 6.51 (d,  $J = 9.2$  Hz, 2 H), 6.36 (dd,  $J = 9.2, 2.2$  Hz, 2 H), 6.23 (d,  $J = 2.2$  Hz, 2 H), 3.70 - 3.78 (m, 2 H), 3.20 - 3.29 (m, 8 H), 2.52 - 2.60 (m, 2 H), 1.01 (t,  $J = 6.8$  Hz, 12 H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )  $\delta$  173.5, 167.1, 157.9, 155.7, 153.8, 152.7, 148.8, 137.6, 133.7, 132.3, 130.3, 129.0, 128.9, 127.2, 126.9, 124.3, 123.5, 123.2, 122.8, 120.4, 108.6, 105.9, 97.6, 66.9, 44.1, 37.1, 33.6, 12.8. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{41}\text{H}_{43}\text{N}_8\text{O}_6$   $[\text{M} + \text{H}]^+$  743.3300, found 743.3296.

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed at  $80^\circ\text{C}$ .

### 11(2,1,3)

3-((2-((2-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)propanoic acid

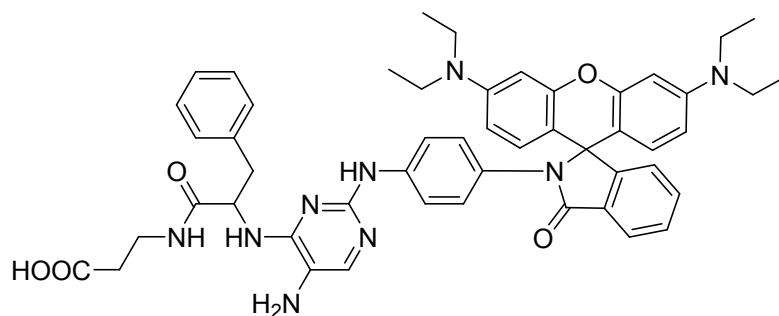


Yield 14 mg (13 %) of pink/orange amorphous powder.  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  ppm 8.85 (s, 1 H), 8.69 (t,  $J = 5.2$  Hz, 1 H), 7.93 - 8.05 (m, 1 H), 7.75 (d,  $J = 8.3$  Hz, 1 H), 7.70 - 7.64 (m, 2 H), 7.60 (br. s., 1 H), 7.17 - 7.25 (m, 2 H), 6.86 (t,  $J = 7.8$  Hz, 1 H), 6.42 (br. s., 2 H), 6.10 - 6.23 (m, 4 H), 3.53 - 3.51 (m, 2 H), 3.28 - 3.21 (8 H, overlapped with water), 2.44 (t,  $J = 6.7$  Hz, 2 H), 1.05 - 1.01 (m, 12 H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )  $\delta$  173.2, 167.1, 159.9, 157.9, 155.6, 153.8, 152.7, 149.3, 136.0, 134.0, 131.3, 129.4, 128.8, 128.7, 128.4, 128.1, 125.8, 125.1, 124.8, 123.5, 122.0, 108.6, 105.9, 98.1, 68.8, 44.1, 36.9, 33.6, 12.8. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{41}\text{H}_{43}\text{N}_8\text{O}_6$   $[\text{M} + \text{H}]^+$  743.3300, found 743.3298.

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed at  $70^\circ\text{C}$ .

### 11(1,2,1)

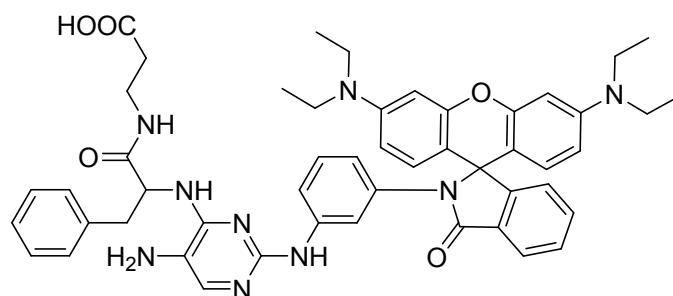
3-(2-((5-amino-2-((4-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid



Yield 21.0 mg (23 %) of pink amorphous powder.  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  7.87 (d,  $J$  = 5.9 Hz, 2 H), 7.61 - 7.50 (m, 3 H), 7.30 - 7.00 (m, 9 H), 6.64 - 6.50 (m, 4 H), 6.41 - 6.39 (m, 2 H), 6.32 - 6.20 (m, 3 H), 4.73 - 4.67 (m, 1 H), 3.32 - 3.13 (m, 8 H), 3.11 - 2.89 (m, 4 H), 2.31 (t,  $J$  = 6.8 Hz, 2 H), 1.11 - 0.97 (m, 12 H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )  $\delta$  174.3, 171.8, 167.1, 154.6, 153.9, 153.5, 153.4, 152.9, 151.7, 148.8, 141.3, 140.2, 138.9, 138.4, 133.6, 133.5, 130.9, 130.8, 129.7, 129.2, 129.1, 128.9, 128.8, 128.7, 128.6, 128.1, 127.6, 126.9, 126.6, 124.4, 123.2, 123.3, 121.2, 118.0, 117.1, 115.6, 108.6, 108.5, 106.2, 106.1, 97.7, 66.9, 66.8, 58.0, 44.2, 38.1, 36.1, 35.8, 14.0, 12.9. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{50}\text{H}_{54}\text{N}_9\text{O}_5$   $[\text{M} + \text{H}]^+$  860.4242, found 860.4236.

### 11(1,2,2)

3-(2-((5-amino-2-((3-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid

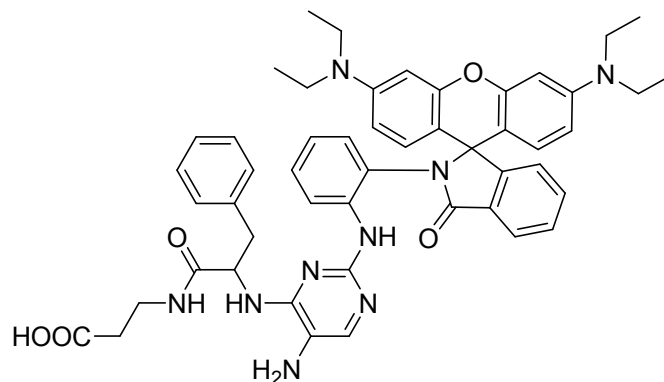


Yield 11.9 mg (14 %) of pink amorphous powder.  $^1\text{H}$  NMR (300 MHz,  $\text{DMSO-}d_6$ )  $\delta$  8.34 (s, 1 H), 8.01 (t,  $J$  = 5.6 Hz, 1 H), 7.96 - 7.88 (m, 1 H), 7.62 - 7.50 (m, 2 H), 7.46 - 7.40 (m, 1 H), 7.31 (s, 1 H), 7.29 - 7.19 (m, 3 H), 7.19 - 7.09 (m, 3 H), 7.02 - 7.08 (m, 2 H), 6.88 (t,  $J$  = 8.1 Hz, 1 H), 6.58 (d,  $J$  = 8.8 Hz, 1 H), 6.49 - 6.33 (m, 3 H), 6.28 - 6.16 (m, 3 H), 6.02 (dd,  $J$  = 7.8, 0.8 Hz, 1 H), 4.75 - 4.63 (m, 1 H), 3.34 - 3.14 (m, 8 H), 3.13 - 2.88 (m, 4 H), 2.22 (t,  $J$  = 7.1 Hz, 2 H), 1.03 (m, 12 H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )  $\delta$  173.0, 171.8, 166.8, 153.7, 152.8, 152.3, 152.1, 148.3, 148.2, 142.1, 138.3, 136.6, 133.2, 131.4, 129.8, 129.2, 128.5, 128.4, 128.0, 127.9, 126.2, 123.8, 122.8, 120.6, 116.7, 116.4, 115.6, 108.0, 107.9, 105.7, 105.4,

97.2, 97.1, 66.7, 55.6, 43.6, 43.5, 40.4, 37.8, 34.9, 33.9, 12.4, 12.3. HRMS (ESI)  $m/z$  calcd for  $C_{50}H_{54}N_9O_5$   $[M + H]^+$  860.4242, found 860.4236.

### 11(1,2,3)

3-(2-((5-amino-2-((2-(3',6'-bis(diethylamino)-3-oxospiro[isoinoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid

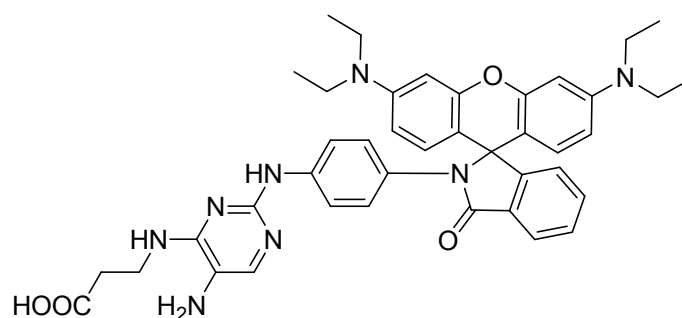


Yield 42.6 mg (67 %) of pink amorphous powder.  $^1H$  NMR (400 MHz,  $DMSO-d_6$ )  $\delta$  8.05 (d,  $J$  = 8.2 Hz, 1 H), 8.00 - 7.95 (m, 1 H), 7.73 - 7.62 (m, 3 H), 7.31 (d,  $J$  = 2.0 Hz, 1 H), 7.22 - 7.05 (m, 7 H), 6.57 - 6.49 (m, 3 H), 6.41 (s, 1 H), 6.19 (br. s., 2 H), 6.13 (d,  $J$  = 7.4 Hz, 2 H), 6.06 (dd,  $J$  = 1.6, 7.8 Hz, 1 H), 4.67 - 4.59 (m, 1 H), 3.37 - 3.04 (8 H, overlapped with water), 3.03 - 2.85 (m, 4 H), 2.31 - 2.22 (m, 2 H), 1.03 (d,  $J$  = 6.3 Hz, 12 H).  $^{13}C$  NMR (101 MHz,  $DMSO-d_6$ )  $\delta$  173.1, 171.8, 166.7, 154.0, 153.7, 152.9, 152.7, 149.2, 139.6, 139.4, 138.5, 133.7, 131.5, 129.6, 129.2, 129.1, 129.0, 128.4, 128.3, 126.6, 124.9, 124.8, 123.4, 121.3, 121.2, 120.2, 109.1, 108.9, 106.2, 98.2, 98.1, 68.3, 56.0, 44.3, 44.2, 41.3, 38.7, 35.4, 34.5, 12.8. HRMS (ESI)  $m/z$  calcd for  $C_{50}H_{54}N_9O_5$   $[M + H]^+$  860.4242, found 860.4236.

The  $^1H$  and  $^{13}C$  NMR experiments were performed at 70°C.

### 11(2,2,1)

3-((5-amino-2-((4-(3',6'-bis(diethylamino)-3-oxospiro[isoinoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)propanoic acid



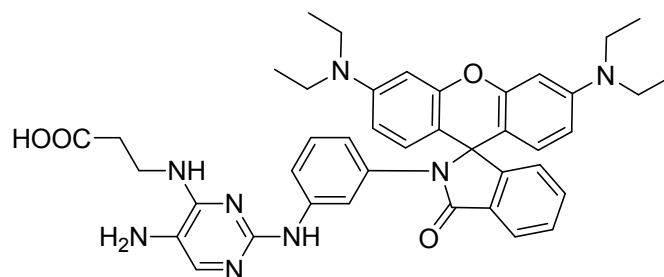


Yield 13 mg (12 %) of pink amorphous powder. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 8.50 (s, 1 H), 7.85 (dd, *J* = 6.1, 2.6 Hz, 1 H), 7.51 - 7.61 (m, 2 H), 7.45 (d, *J* = 8.8 Hz, 1 H), 7.33 (s, 1 H), 7.07 (dd, *J* = 6.6, 2.2 Hz, 1 H), 6.51 - 6.55 (m, 2 H), 6.45 (d, *J* = 9.2 Hz, 2 H), 6.40 (dd, *J* = 9.0, 2.4 Hz, 2 H), 6.21 - 6.27 (m, 2 H), 3.51 - 3.56 (m, 2 H), 3.23 - 3.43 (8 H, overlapped with water), 1.07 (t, *J* = 6.8 Hz, 12 H). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 173.6, 166.9, 154.0, 153.6, 153.1, 152.8, 148.7, 141.3, 137.9, 133.4, 130.9, 129.1, 128.8, 128.0, 127.5, 124.3, 123.1, 121.0, 117.0, 108.5, 106.1, 97.6, 66.8, 44.1, 36.9, 34.2, 12.8. HRMS (ESI) *m/z* calcd for C<sub>41</sub>H<sub>45</sub>N<sub>8</sub>O<sub>4</sub> [M + H]<sup>+</sup> 743.3300, found 743.3298.

Note: the signals of the residual piperidine are present in the NMR spectra (multiplets at 2.88 and 1.50 in the <sup>1</sup>H spectrum and signals at 22.4, 22.8 and 44.3 in the <sup>13</sup>C spectrum).

### 11(2,2,2)

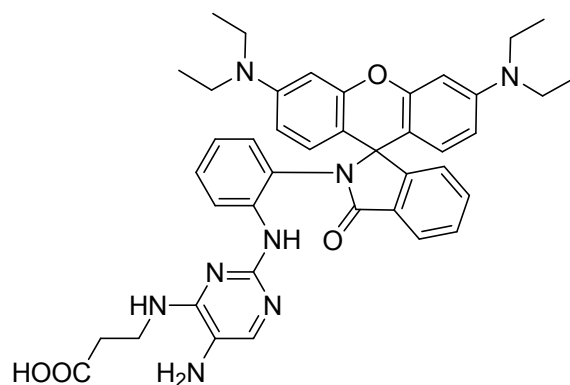
3-((5-amino-2-((3-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)propanoic acid



Yield 16 mg (15 %) of pink amorphous powder. <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 8.39 (s, 1 H), 7.85 (d, *J* = 6.1 Hz, 1 H), 7.50 - 7.57 (m, 3 H), 7.34 (d, *J* = 8.8 Hz, 1 H), 7.29 (s, 1 H), 7.02 (d, *J* = 6.6 Hz, 1 H), 6.85 (t, *J* = 8.3 Hz, 1 H), 6.49 - 6.53 (m, 2 H), 6.32 - 6.43 (m, 3 H), 6.21 - 6.26 (d, *J* = 2.2 Hz, 2 H), 6.01 (d, *J* = 7.9 Hz, 1 H), 3.50 - 3.59 (m, 2 H), 3.22 - 3.31 (m, 8 H), 2.51 (2H, overlapped with DMSO), 1.03 (t, *J* = 7.0 Hz, 12 H). <sup>13</sup>C NMR (101 MHz, DMSO-*d*<sub>6</sub>) δ 173.8, 167.0, 154.1, 154.0, 153.0, 152.7, 148.6, 142.8, 138.0, 137.3, 133.6, 130.4, 128.9, 128.8, 128.2, 124.2, 123.1, 120.8, 116.9, 115.7, 108.4, 106.2, 97.7, 66.8, 44.1, 36.9, 34.3, 12.8. HRMS (ESI) *m/z* calcd for C<sub>41</sub>H<sub>45</sub>N<sub>8</sub>O<sub>4</sub> [M + H]<sup>+</sup> 713.3558, found 713.3556.

### 11(2,2,3)

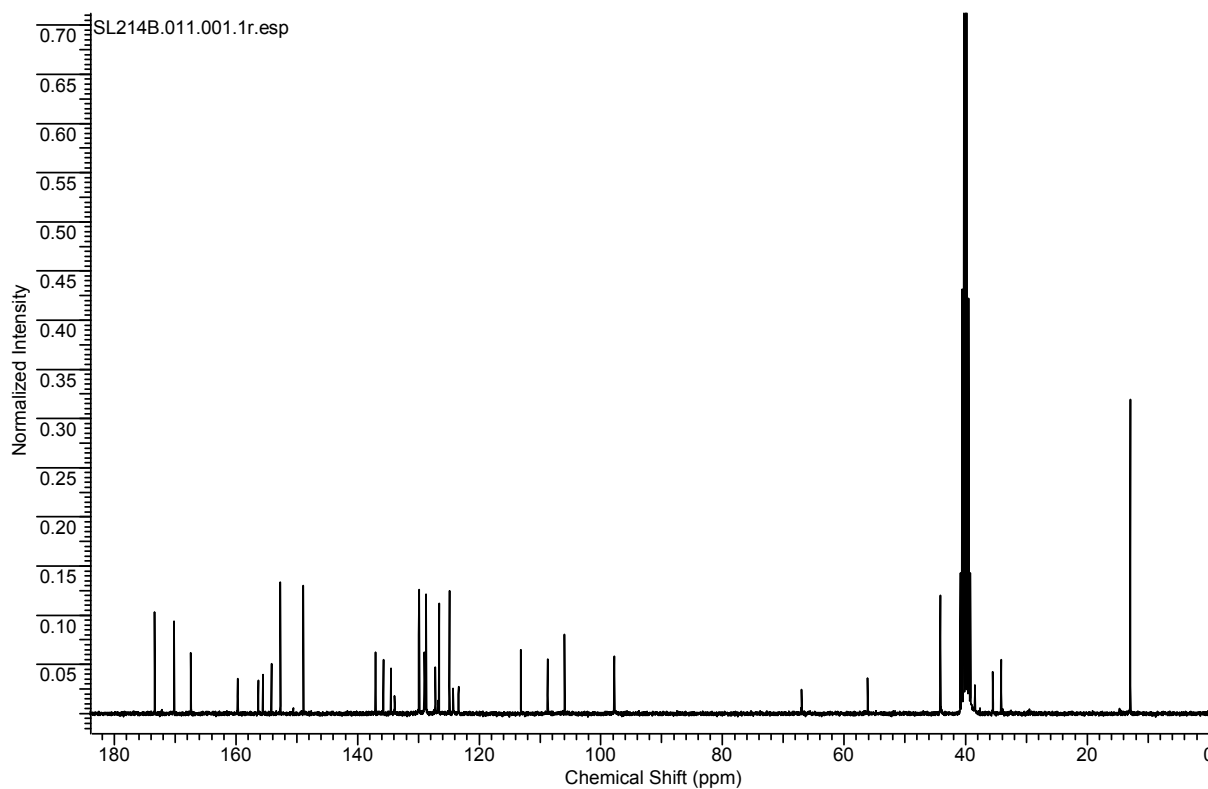
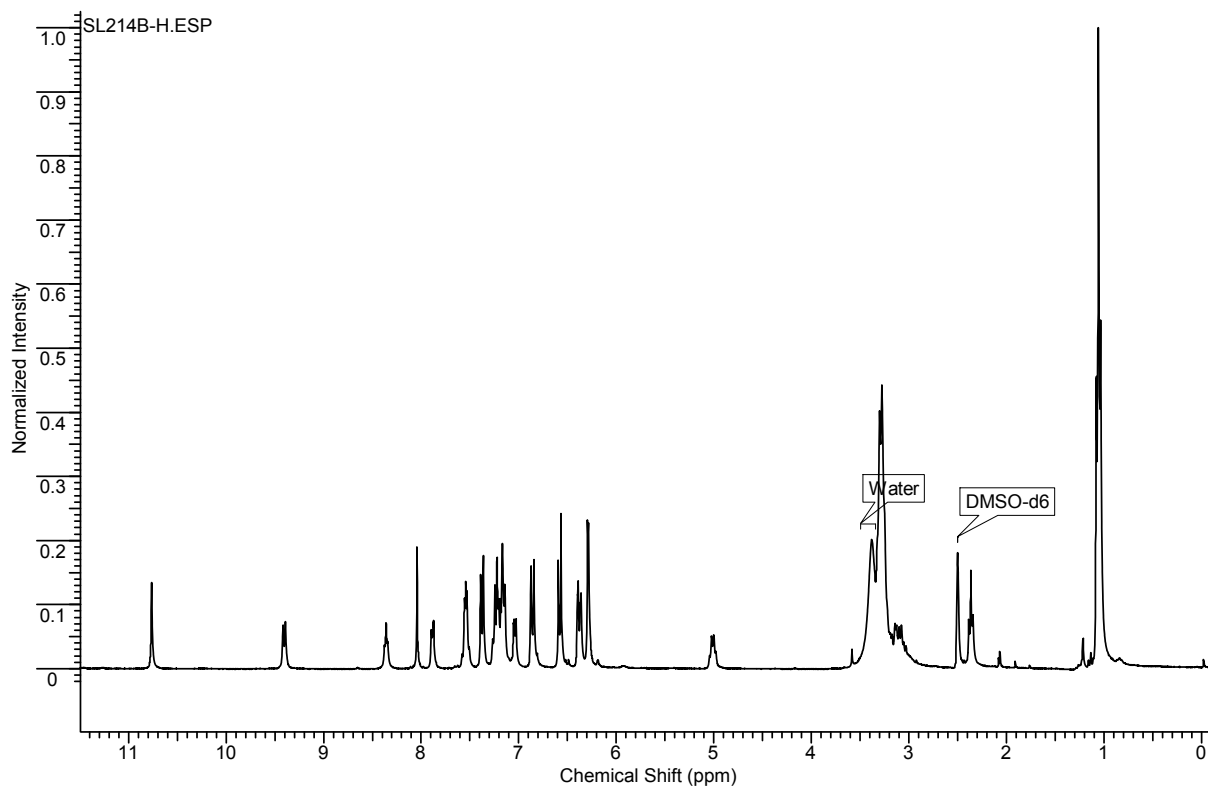
3-((5-amino-2-((2-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)propanoic acid



Yield 11 mg (10 %) of pink amorphous powder.  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  8.04 (d,  $J$  = 6.7 Hz, 1 H), 7.98 – 7.94 (m, 1 H), 7.61 - 7.73 (m, 2 H), 7.27 (br. s., 1 H), 7.20 (d,  $J$  = 5.4 Hz, 1 H), 7.13 – 7.05 (d,  $J$  = 6.5 Hz, 1 H), 6.46 - 6.65 (m, 5 H), 6.27 – 6.17 (m, 3 H), 6.04 (d,  $J$  = 6.5 Hz, 1 H), 3.51 – 3.43 (m, 2 H), 1.12 – 1.10 (m, 12 H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO-}d_6$ )  $\delta$  173.3, 166.6, 154.6, 153.8, 152.7, 152.1, 149.3, 139.2, 133.8, 131.6, 129.3, 128.9, 128.4, 125.3, 124.8, 123.4, 121.4, 121.3, 120.7, 108.9, 106.4, 98.2, 68.3, 44.2, 37.1, 34.2, 12.8. HRMS (ESI)  $m/z$  calcd for  $\text{C}_{41}\text{H}_{45}\text{N}_8\text{O}_4$   $[\text{M} + \text{H}]^+$  713.3558, found 713.3556.

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed at  $70^\circ\text{C}$ .

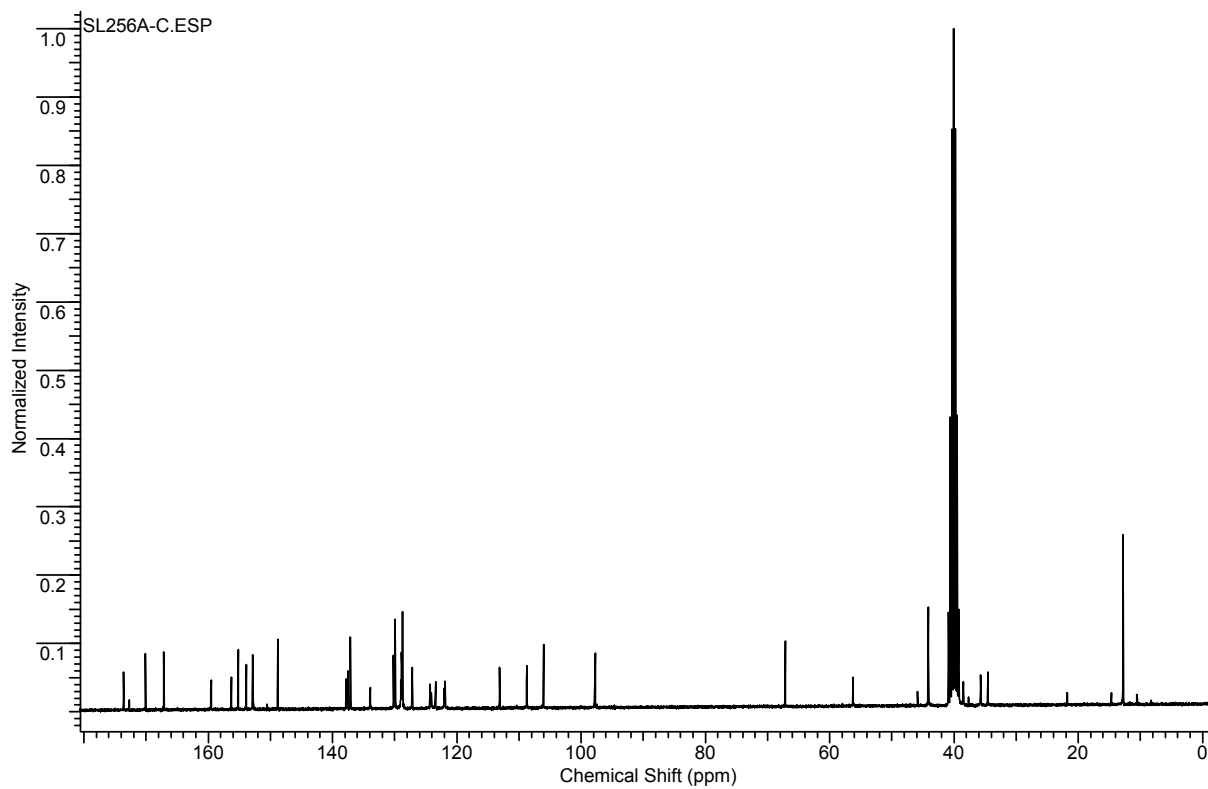
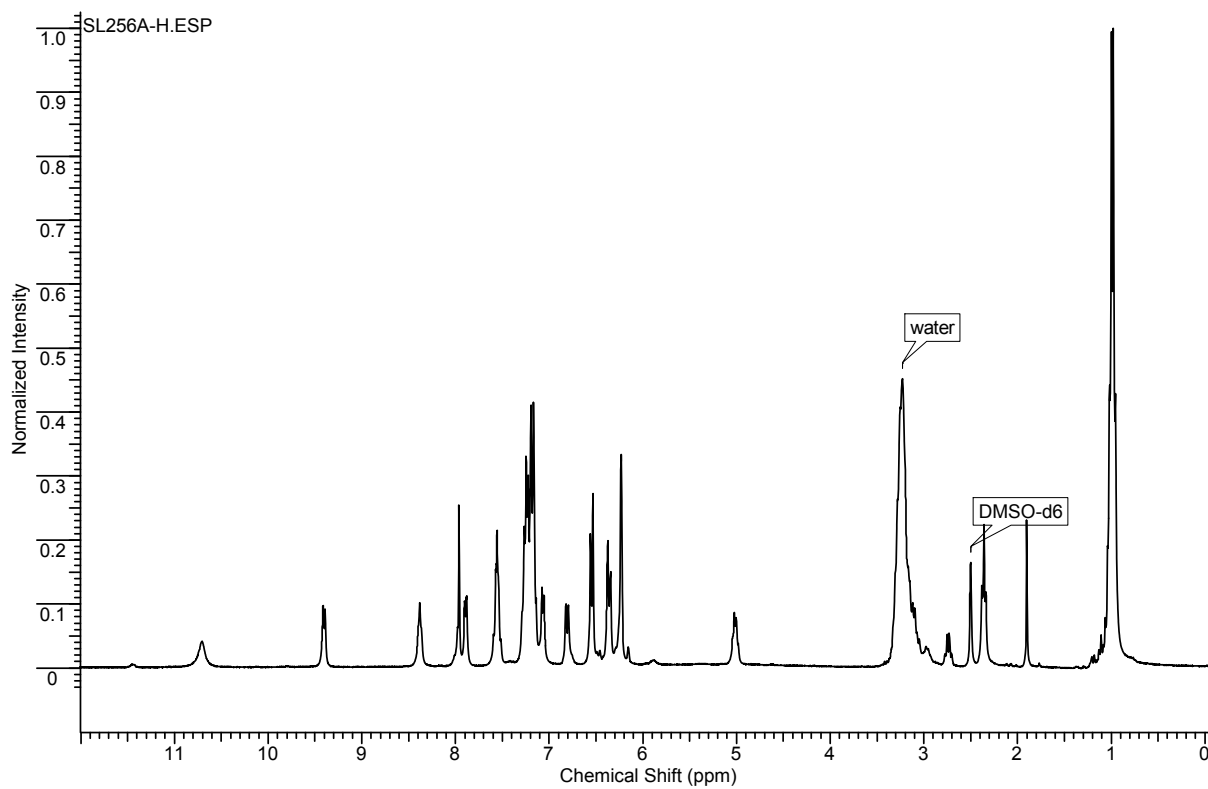
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-(2-((6-((4-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid **6(1,1,1)**



$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of

3-(2-((6-((3-(2-(6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl)benzamido)phenyl)amino)-5-nitropyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid

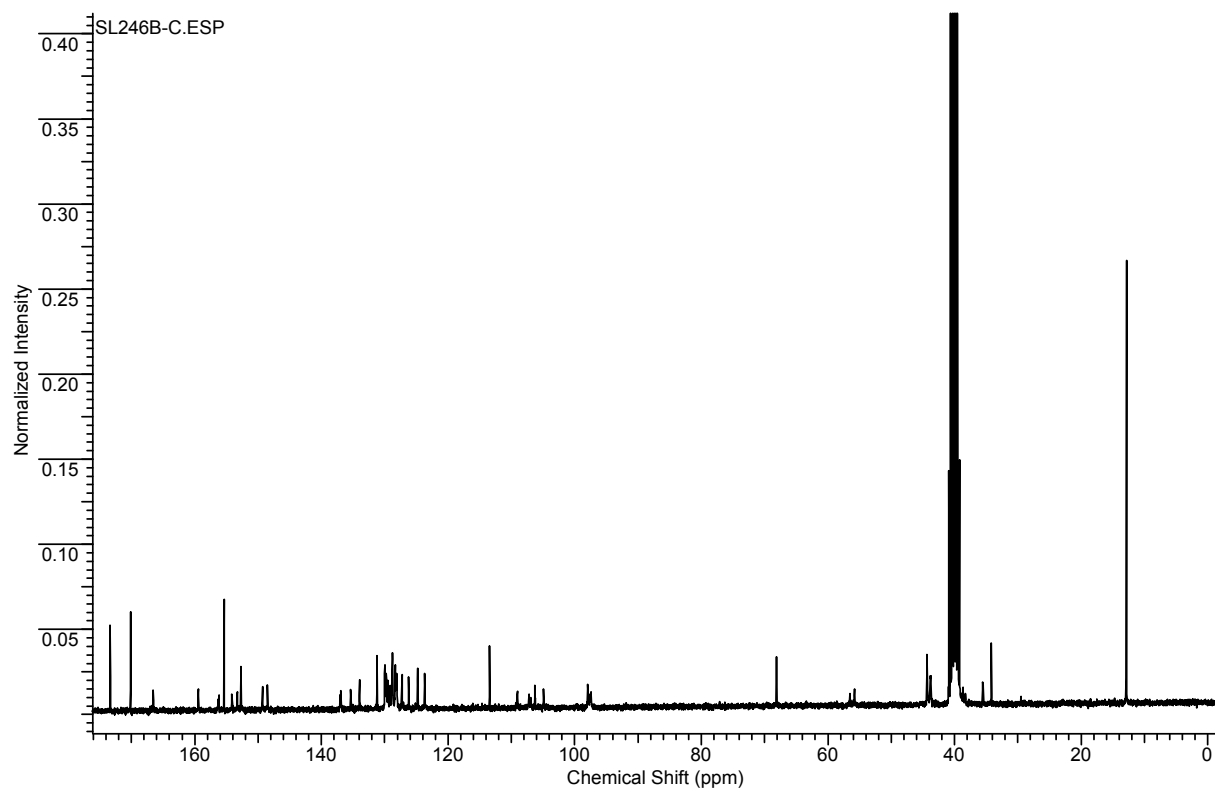
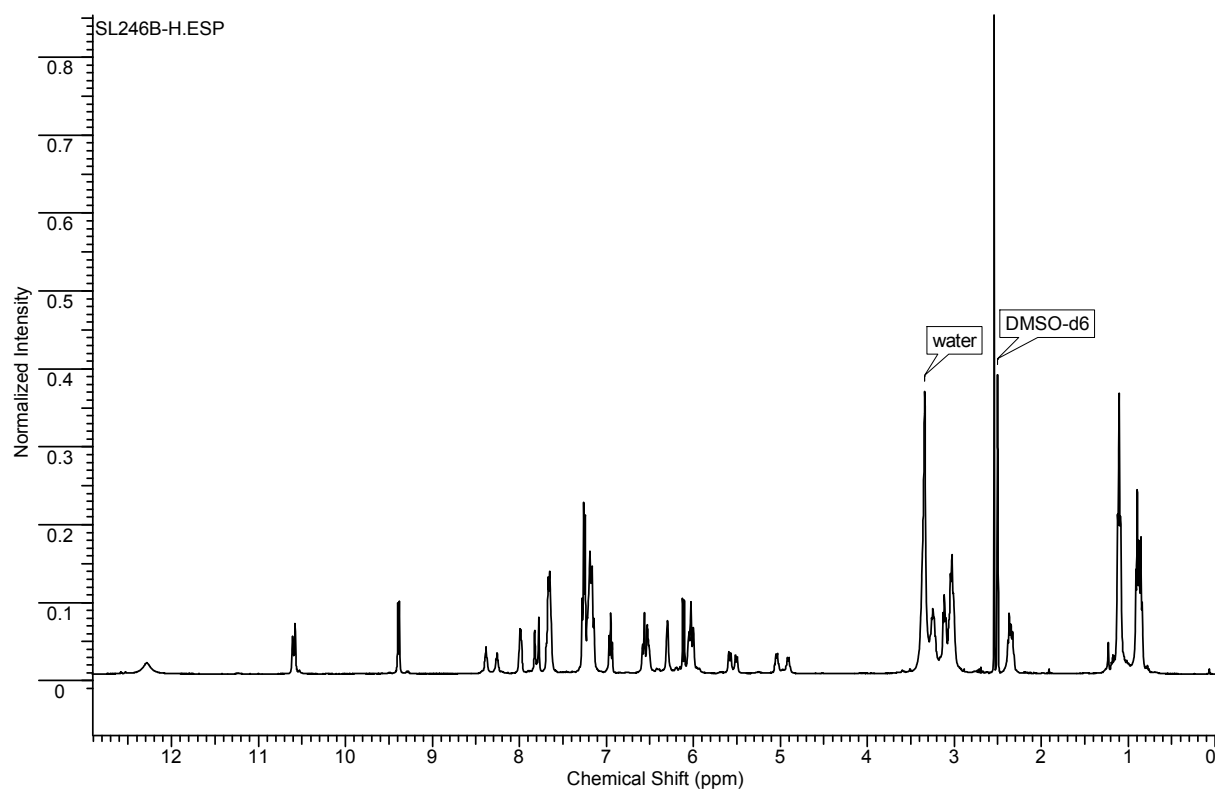
**6(1,1,2)**



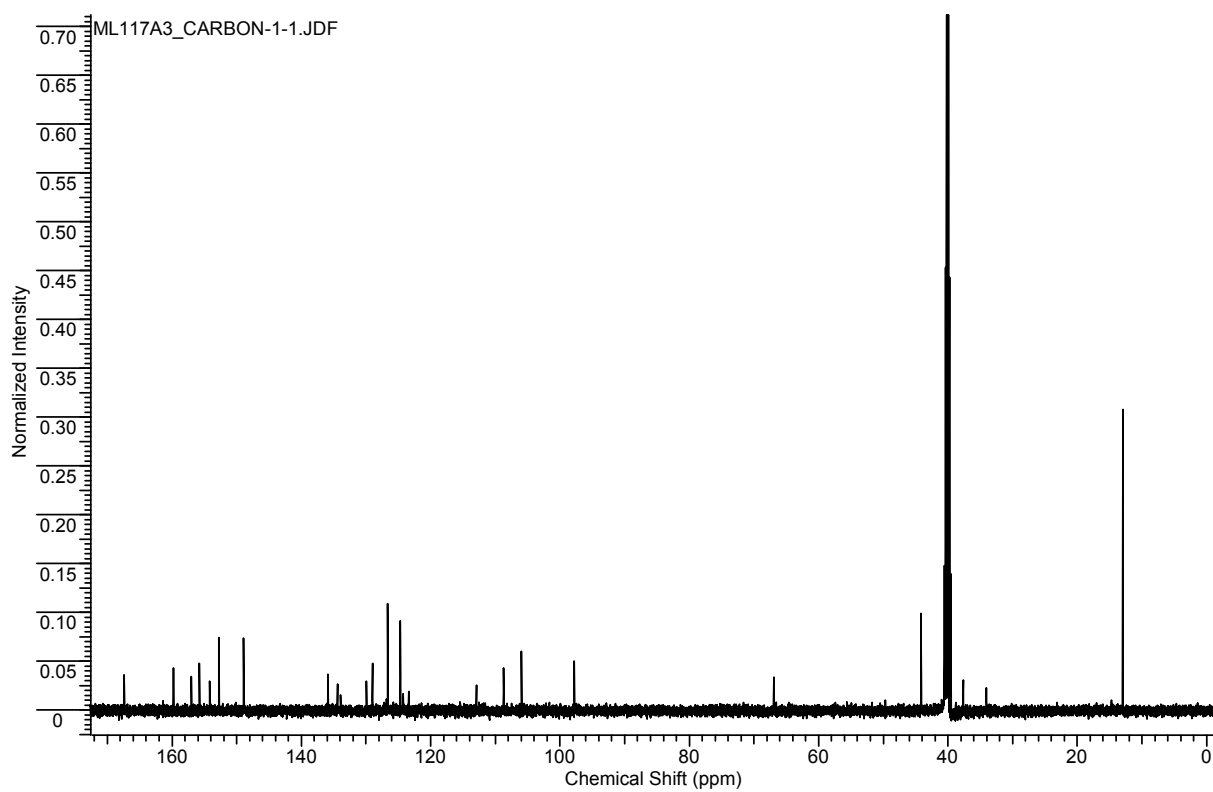
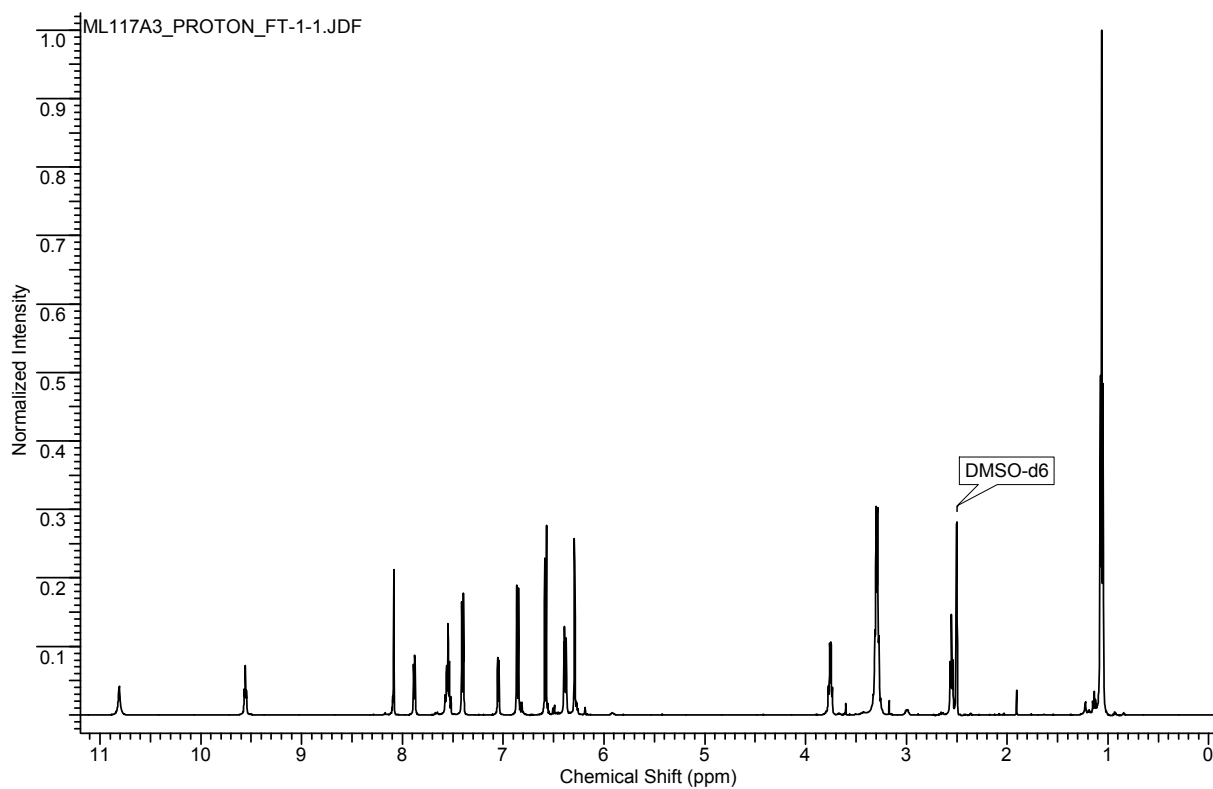
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of

3-(2-((6-((2-(2-(6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl)benzamido)phenyl)amino)-5-nitropyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid

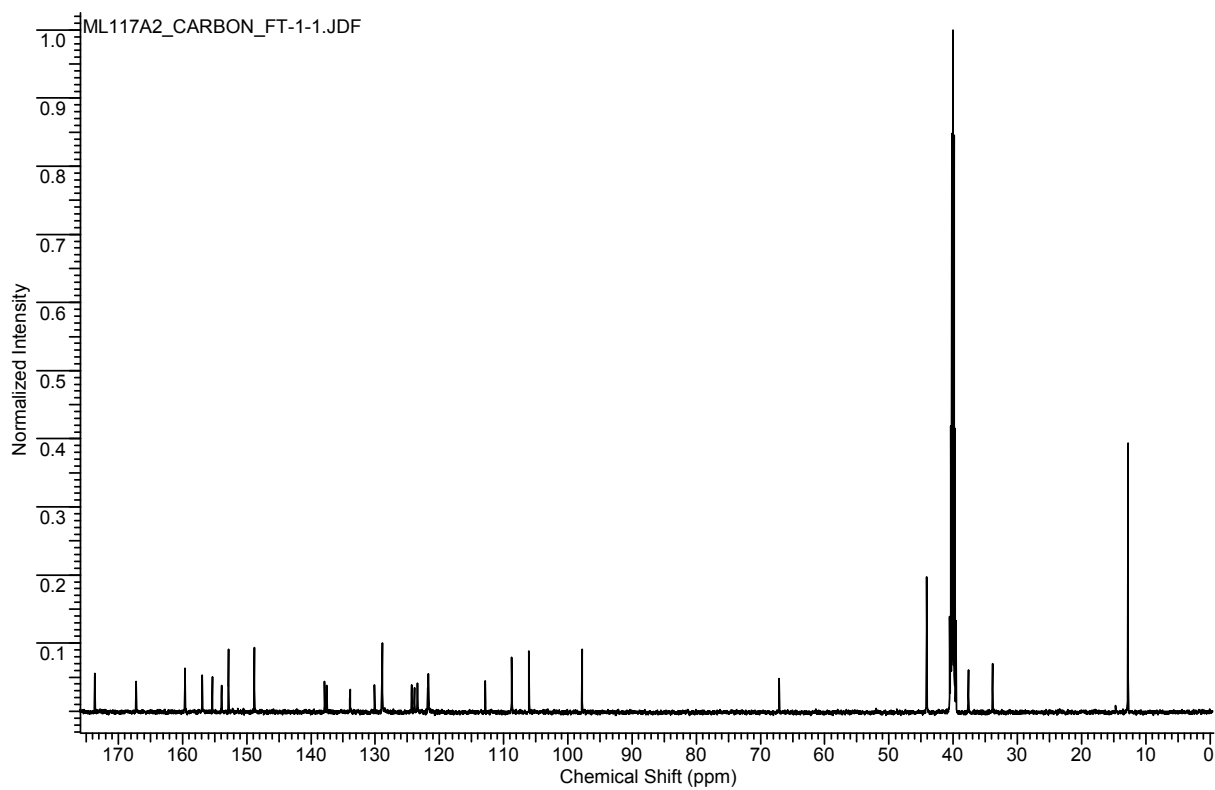
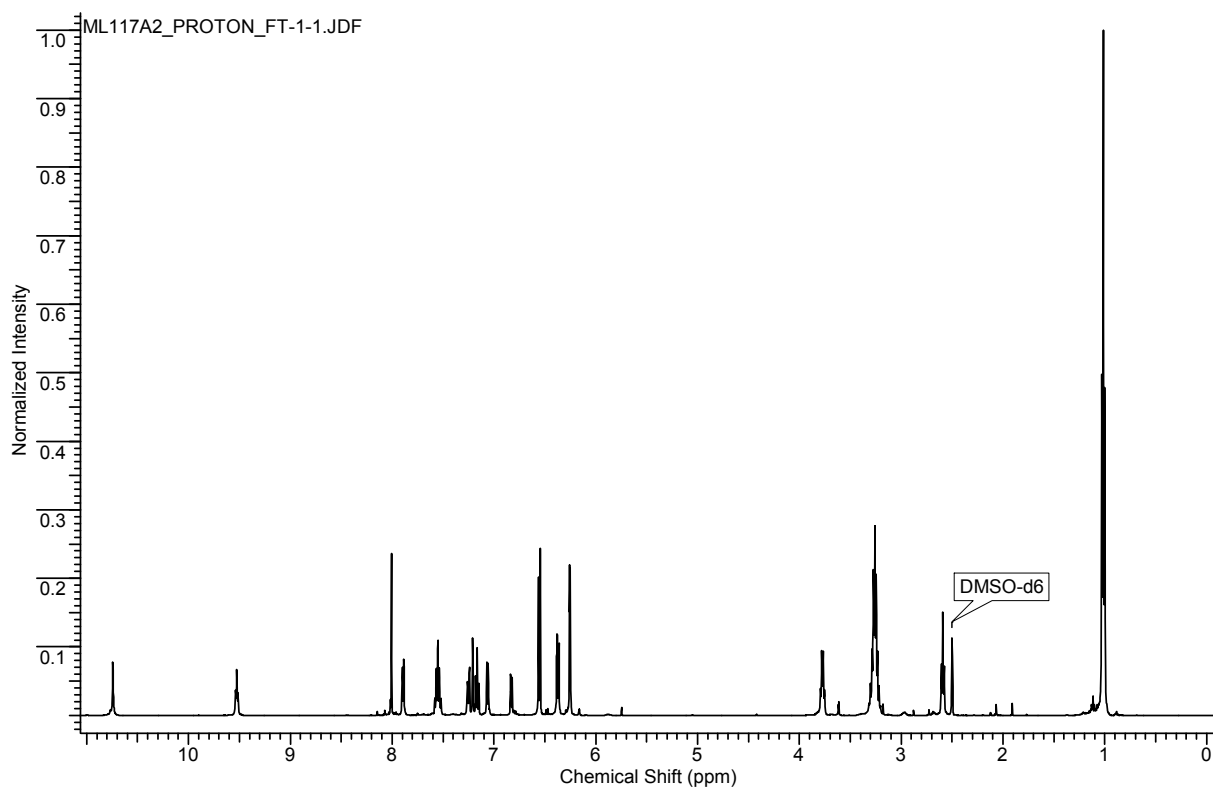
**6(1,1,3)**



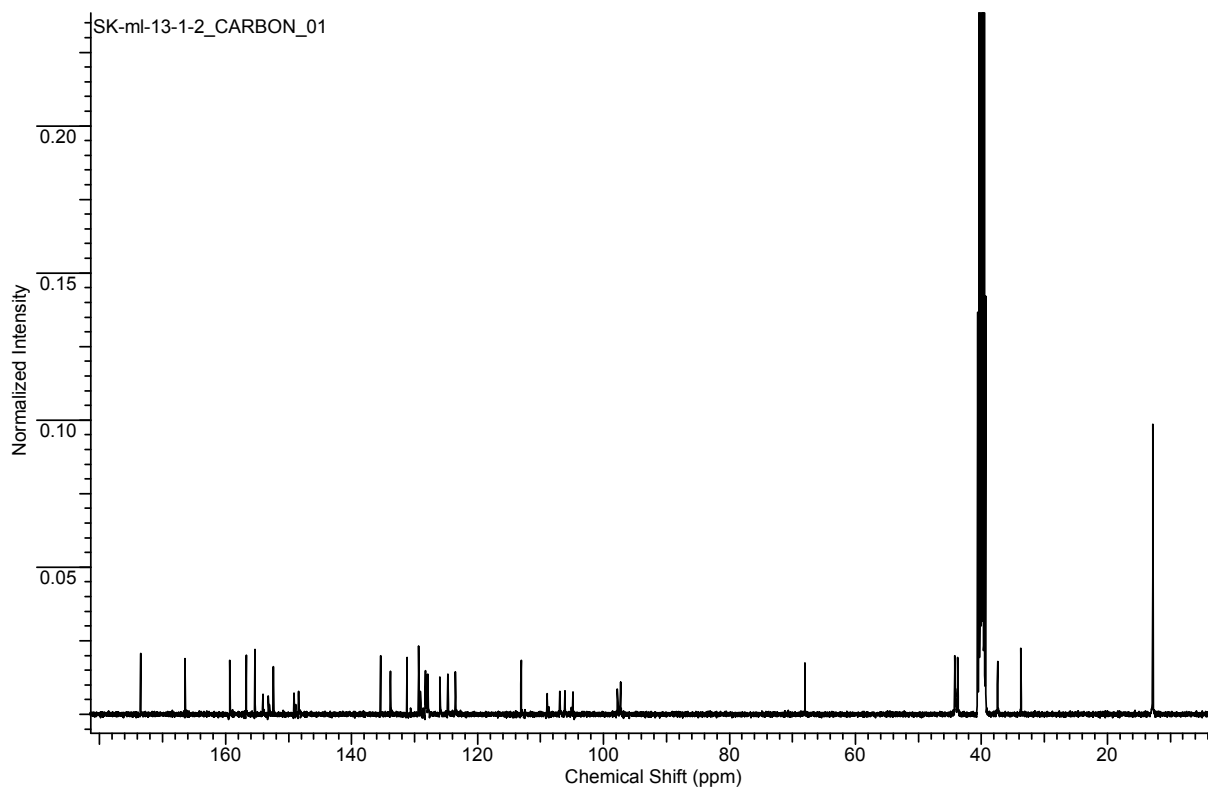
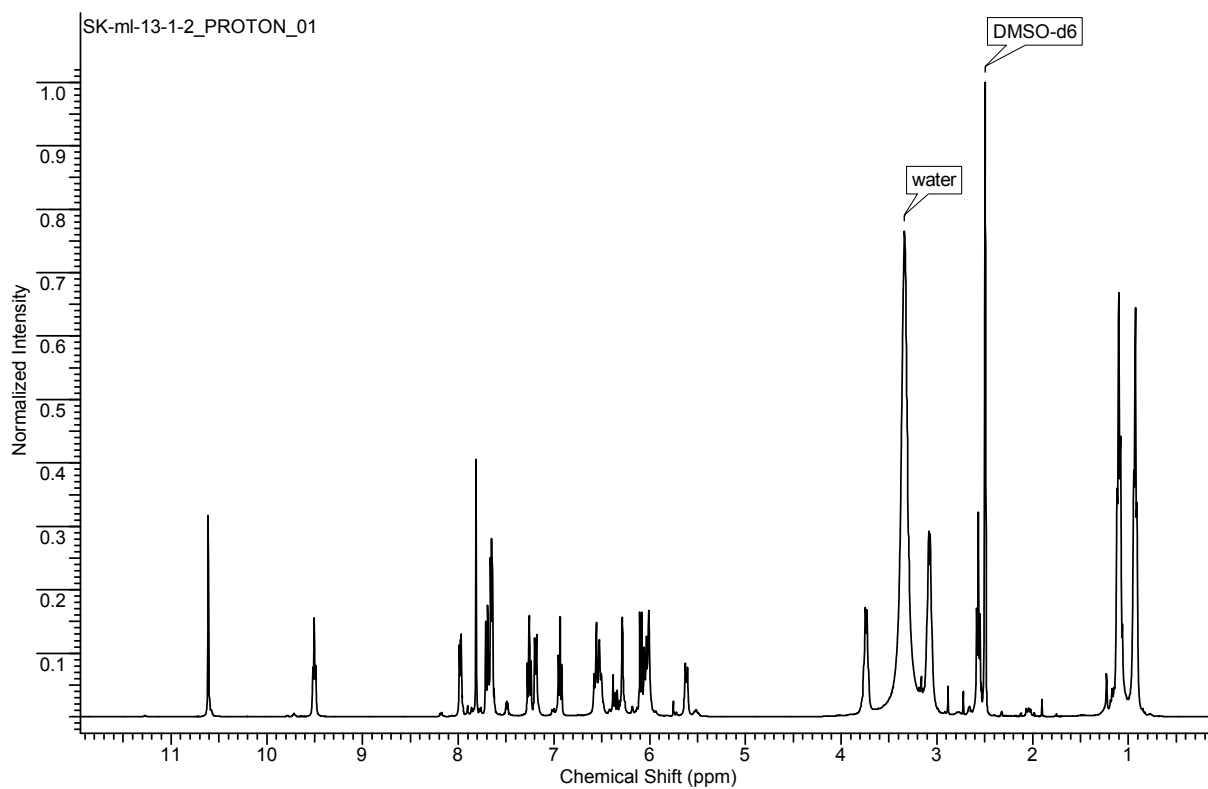
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-((6-((4-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)propanoic acid **6(2,1,1)**



$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-((6-((3-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)propanoic acid **6(2,1,2)**

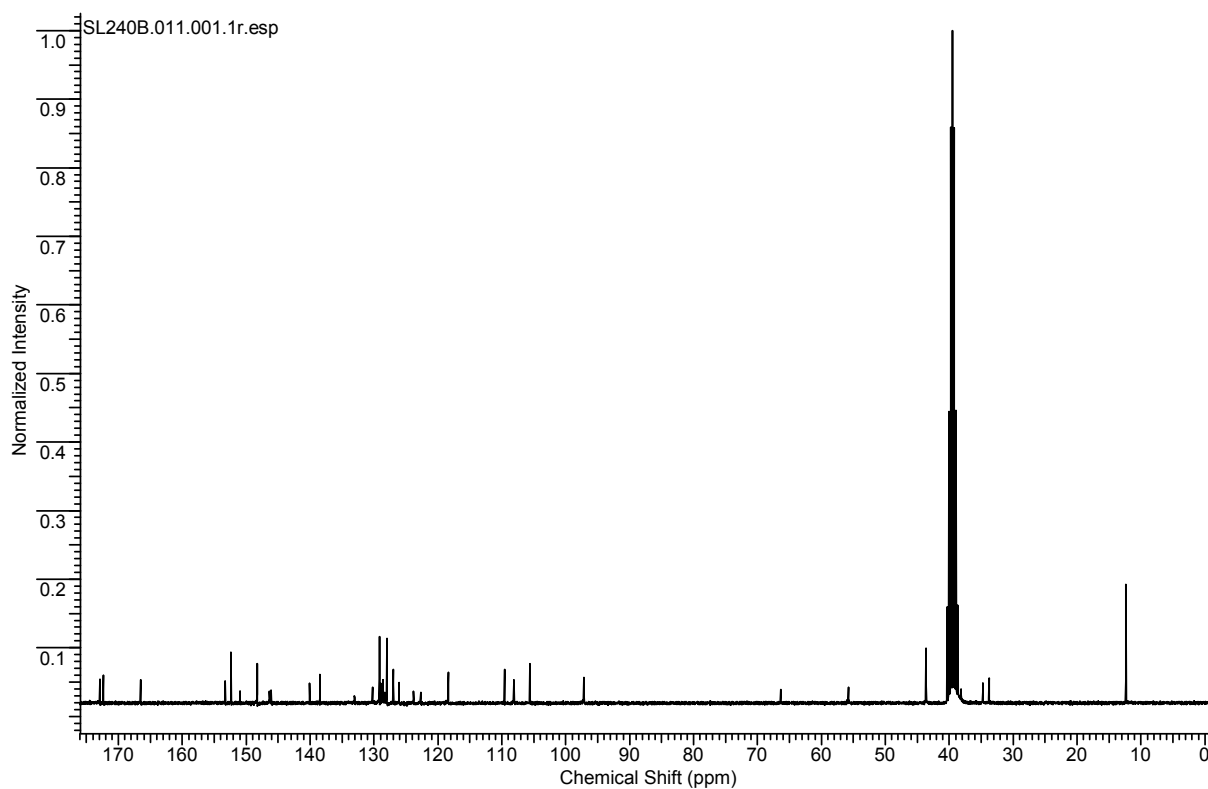
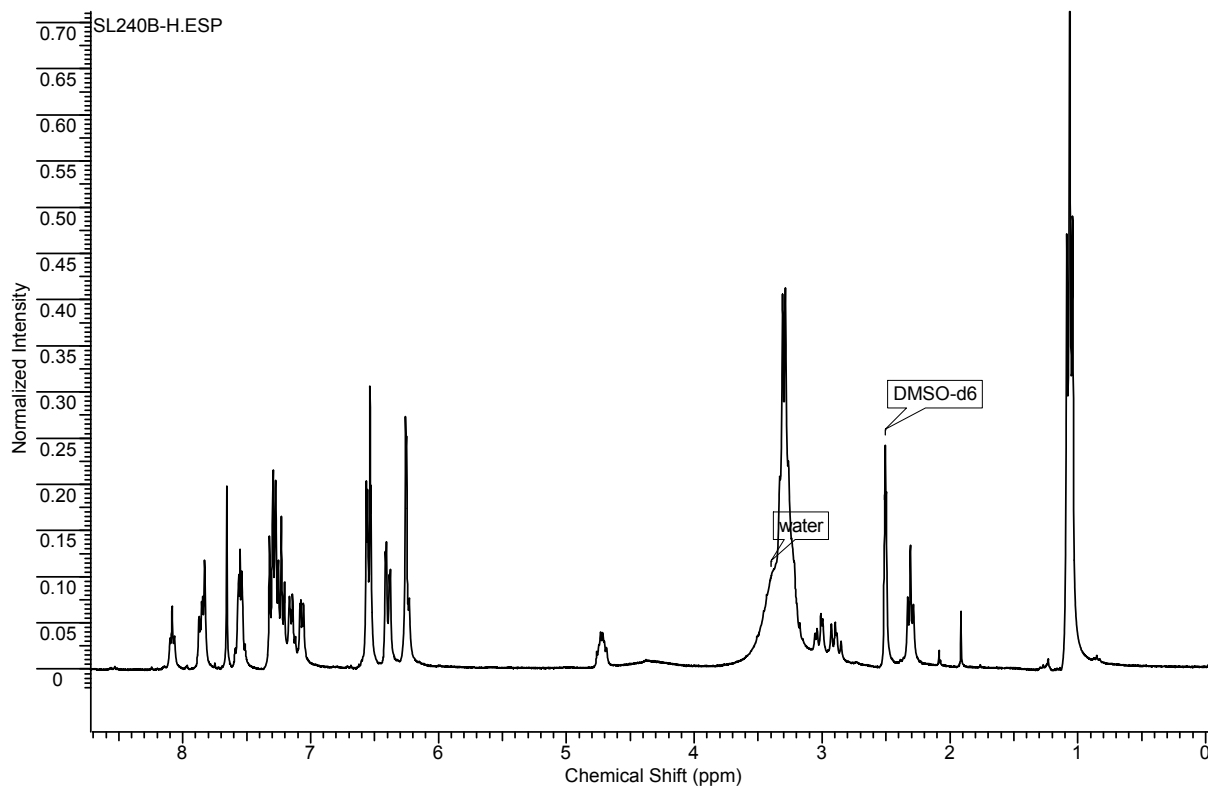


$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-((6-((2-(2-(6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl)benzamido)phenyl)amino)-5-nitropyrimidin-4-yl)amino) propanoic acid **6(2,1,3)**

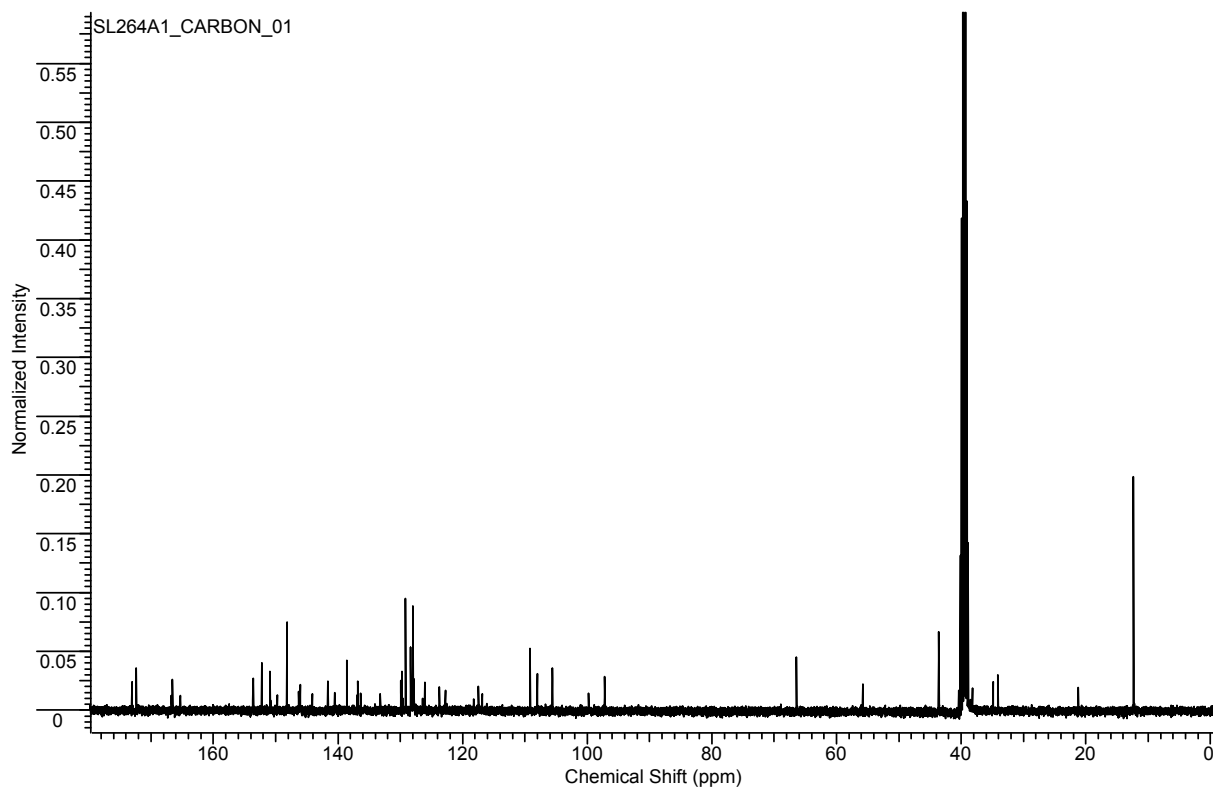
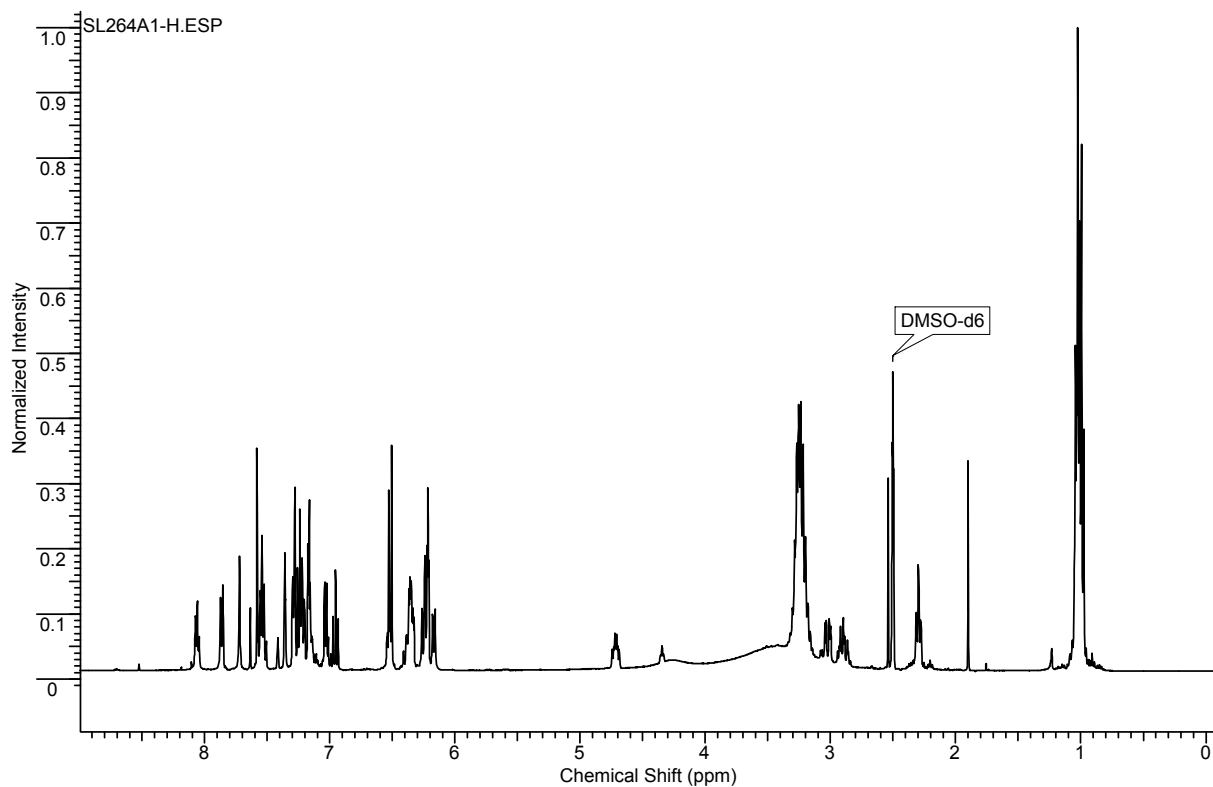




<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3-(2-((5-amino-6-((4-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid **6(1,2,1)**

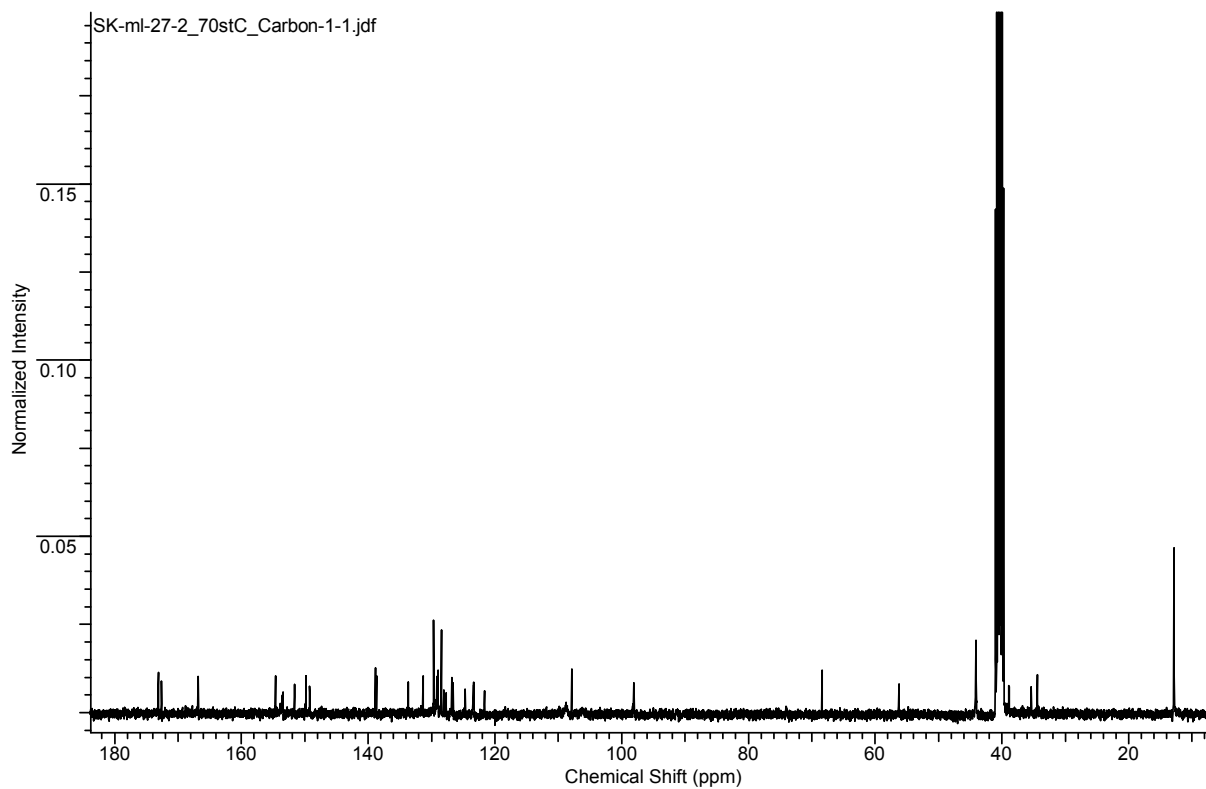
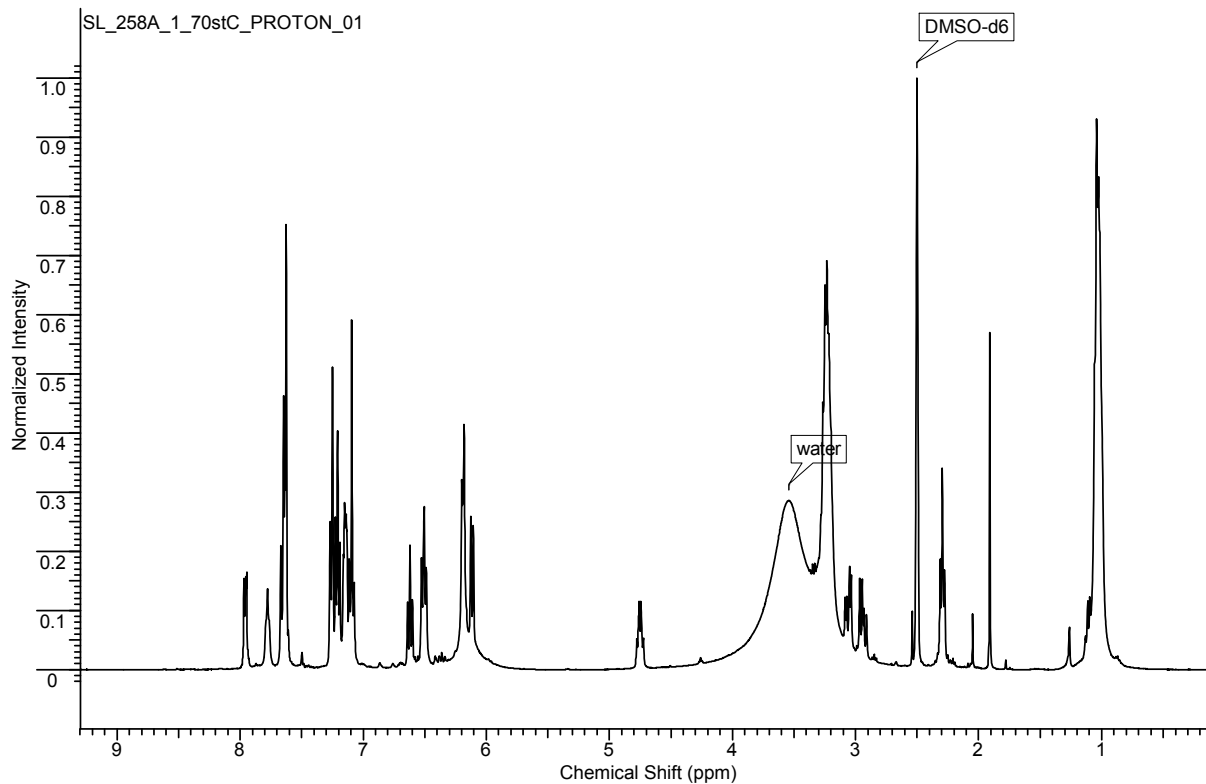


<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3-(2-((5-amino-6-((3-(2-(6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl)benzamido)phenyl)amino)pyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid **6(1,2,2)**

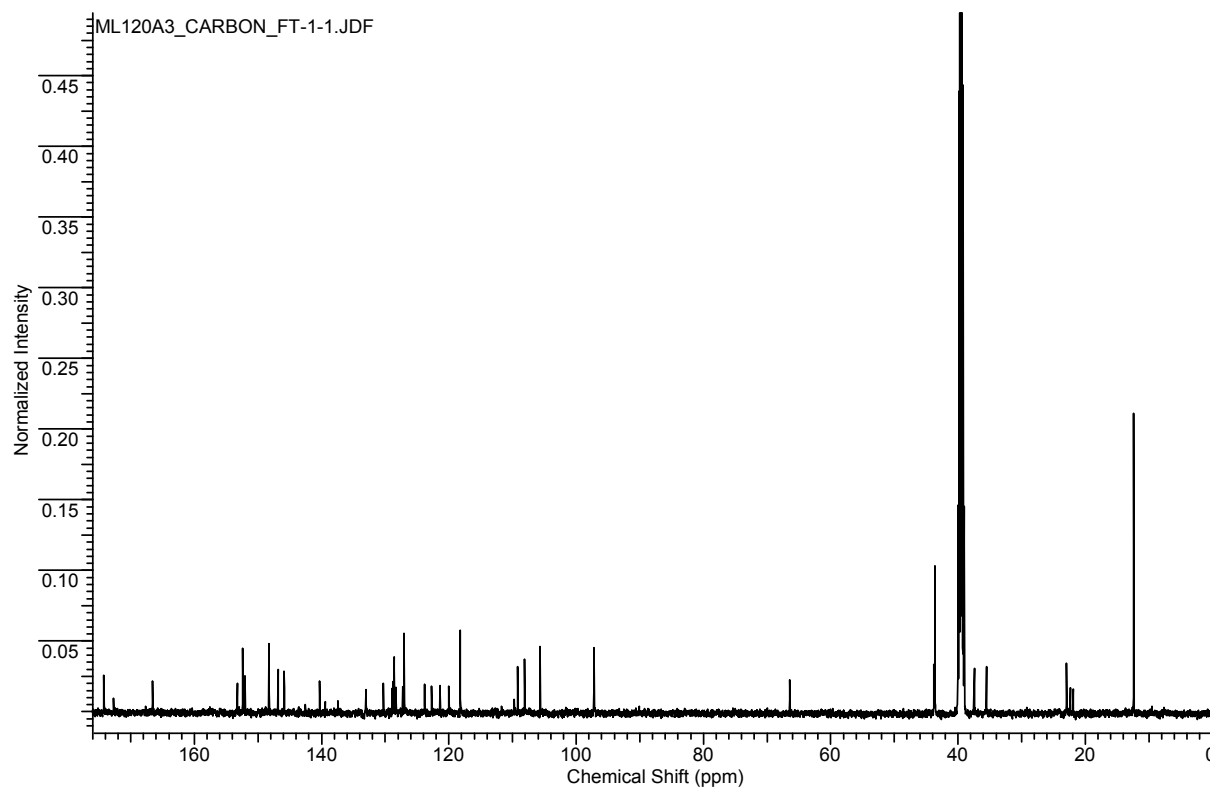
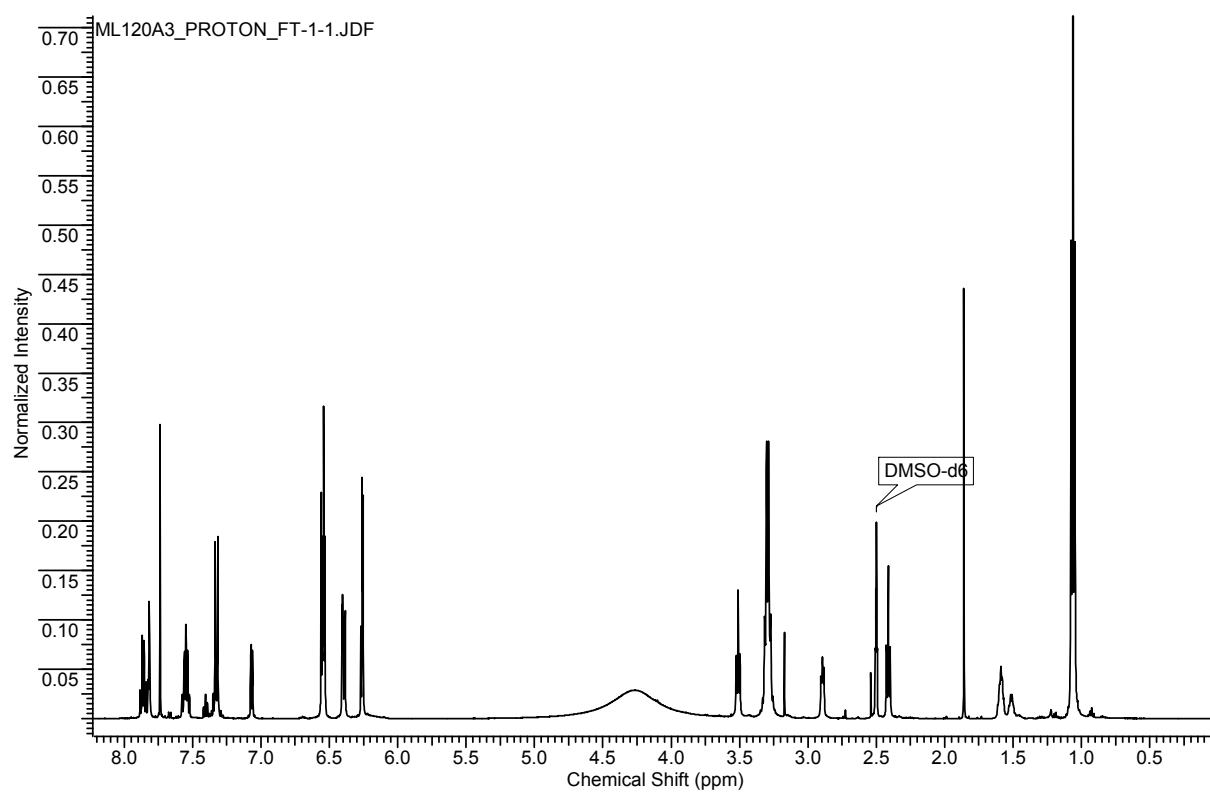


$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-(2-((5-amino-6-((2-(2-(6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl)benzamido)phenyl)amino)pyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid **6(1,2,3)**

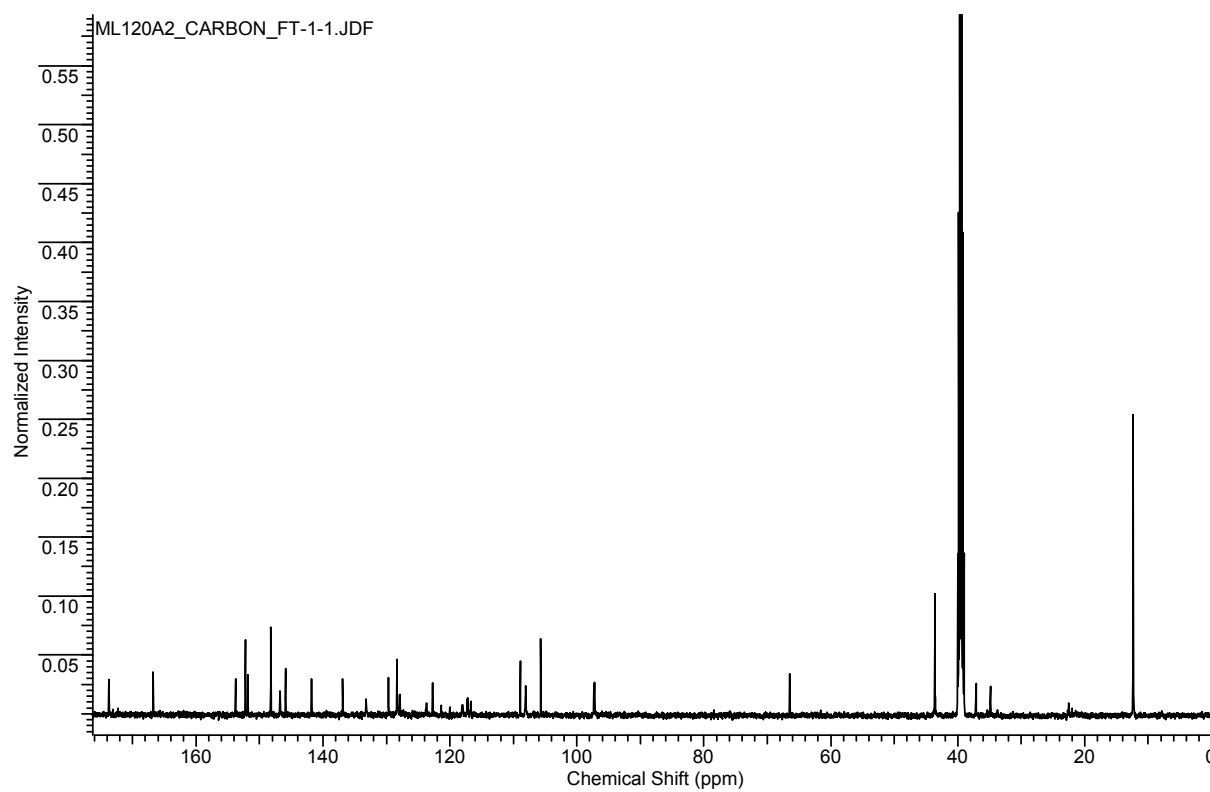
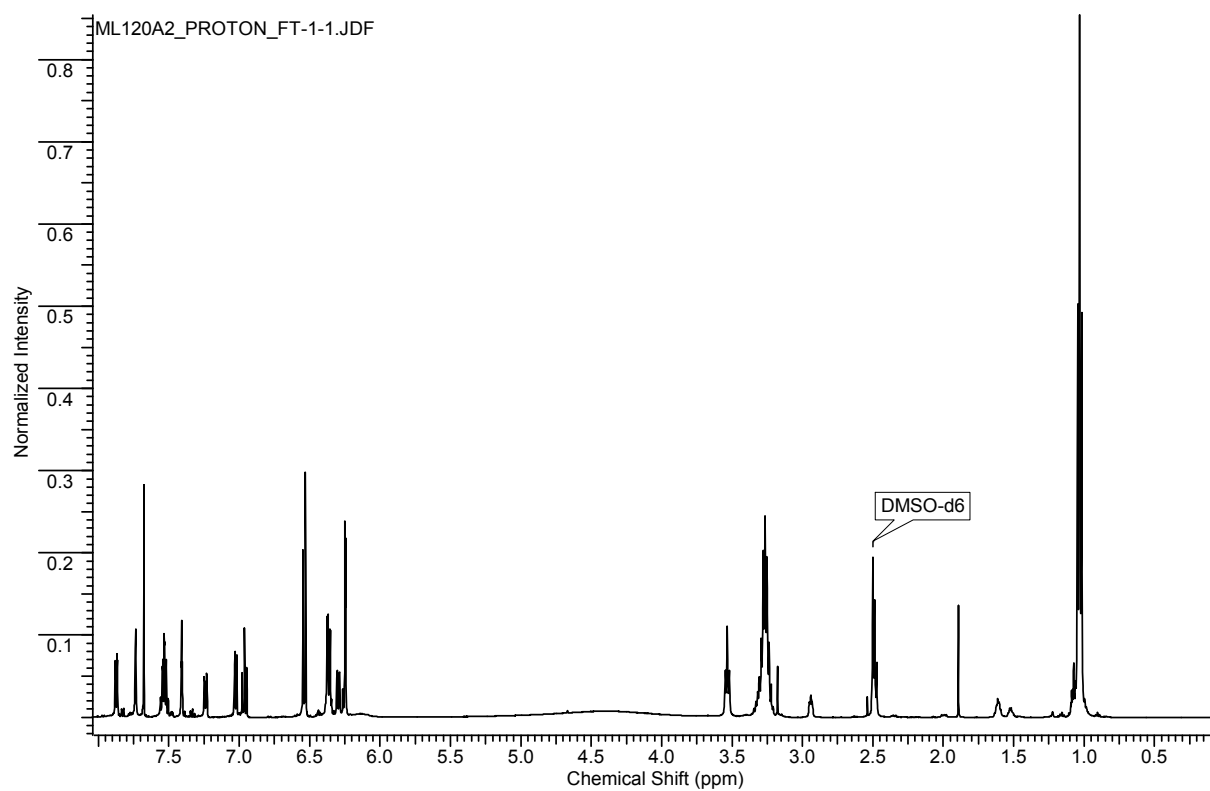
The  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed at 70°C.



<sup>1</sup>H and <sup>13</sup>C NMR spectra of 3-((5-amino-6-((4-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)propanoic acid **6(2,2,1)**

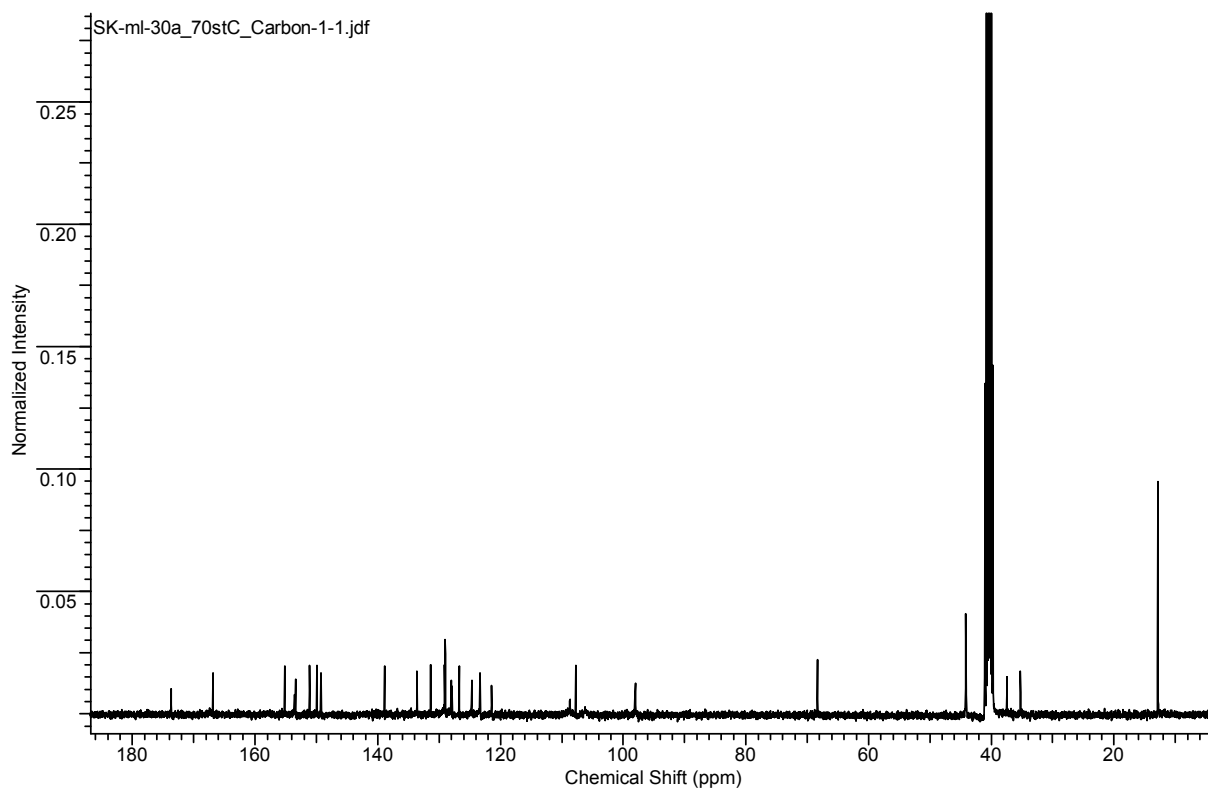
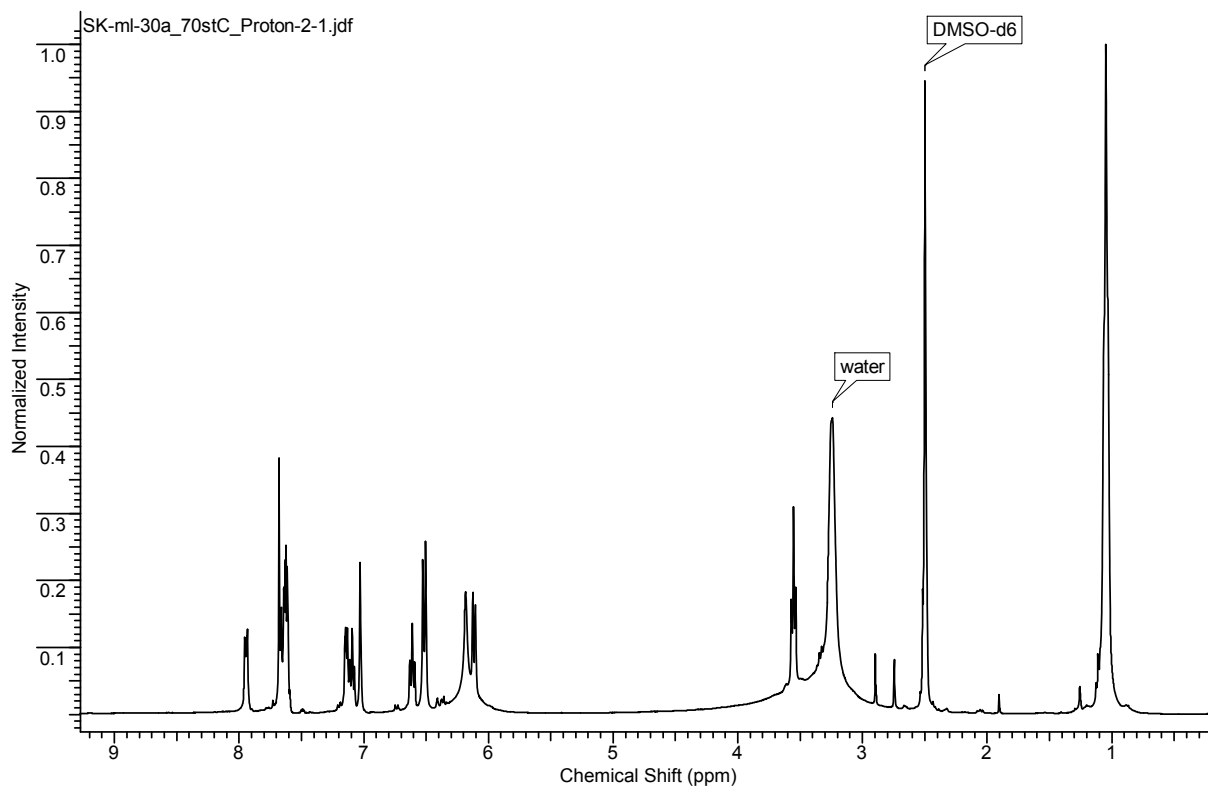


$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-((5-amino-6-((3-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)propanoic acid **6(2,2,2)**



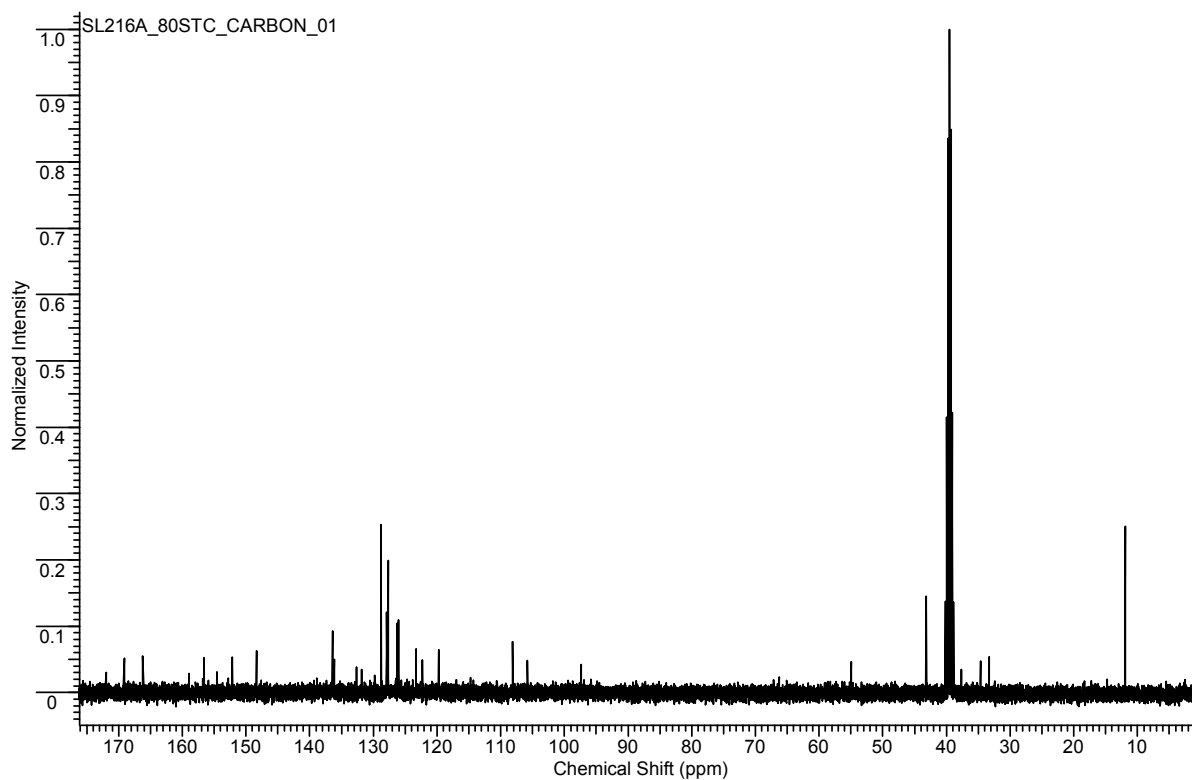
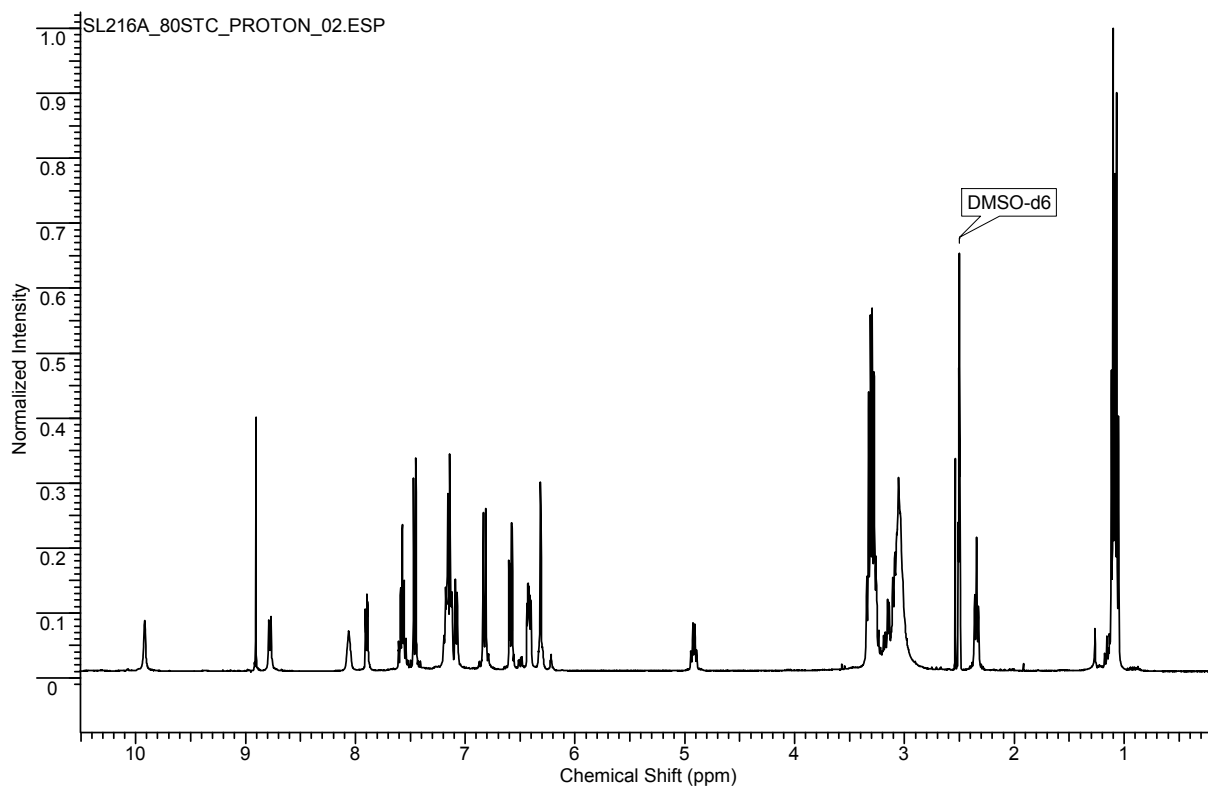
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-((5-amino-6-((2-(2-(6-(diethylamino)-3-(diethyliminio)-3*H*-xanthen-9-yl)benzamido)phenyl)amino)pyrimidin-4-yl)amino) propanoic acid **6(2,2,3)**

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed at 70°C.

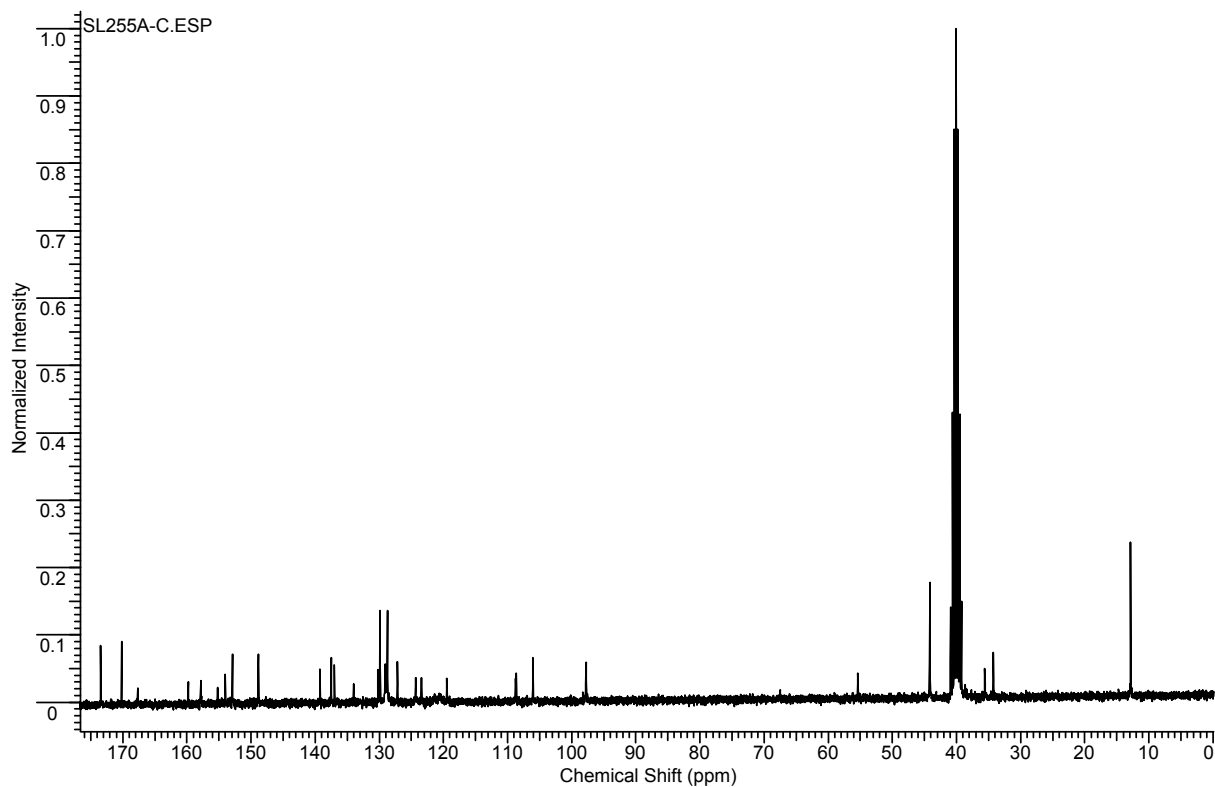
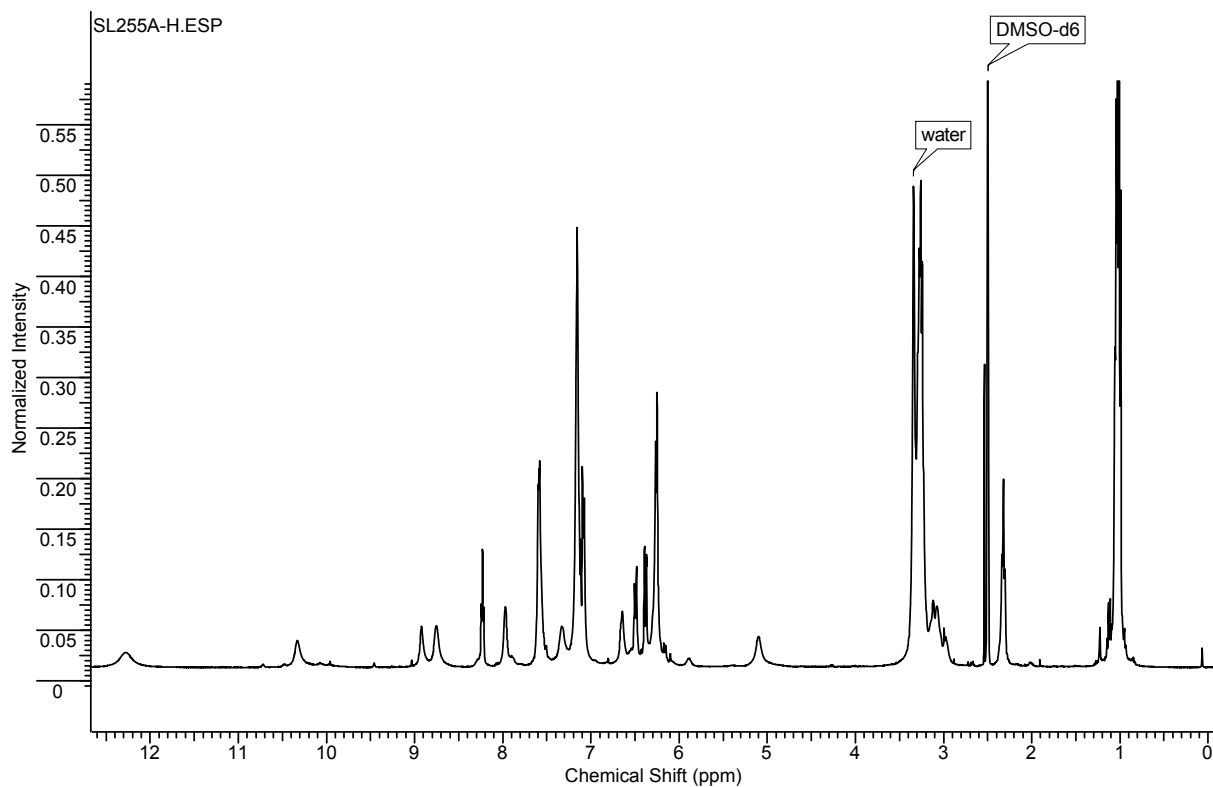


$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-(2-((2-((4-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid **11(1,1,1)**

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed at 80°C.



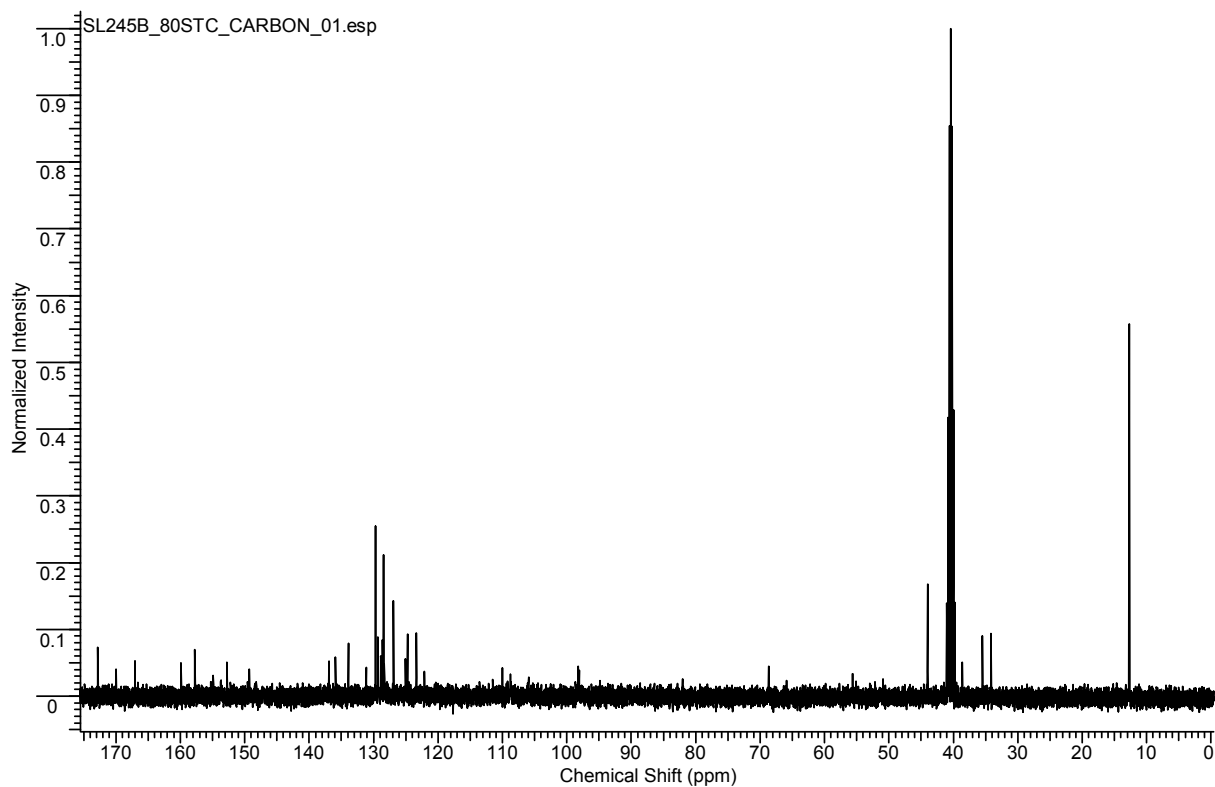
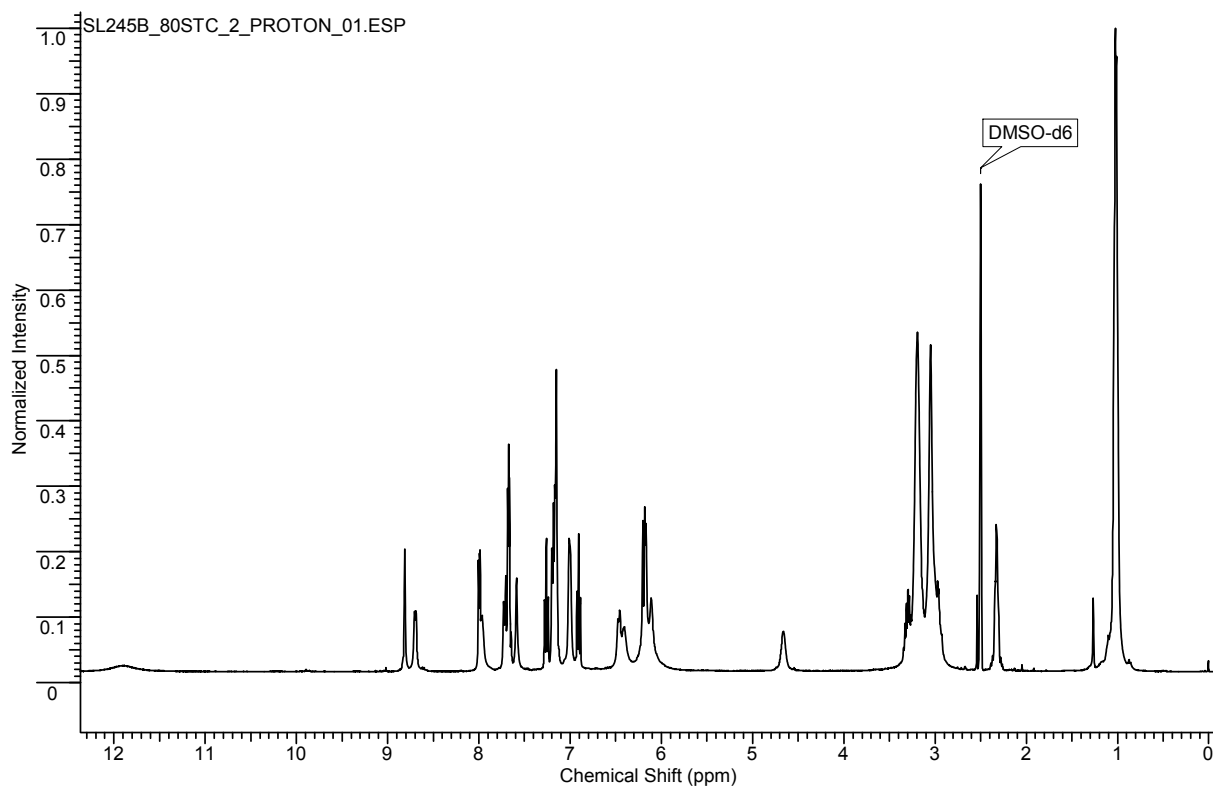
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-(2-((2-((3-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid **11(1,1,2)**



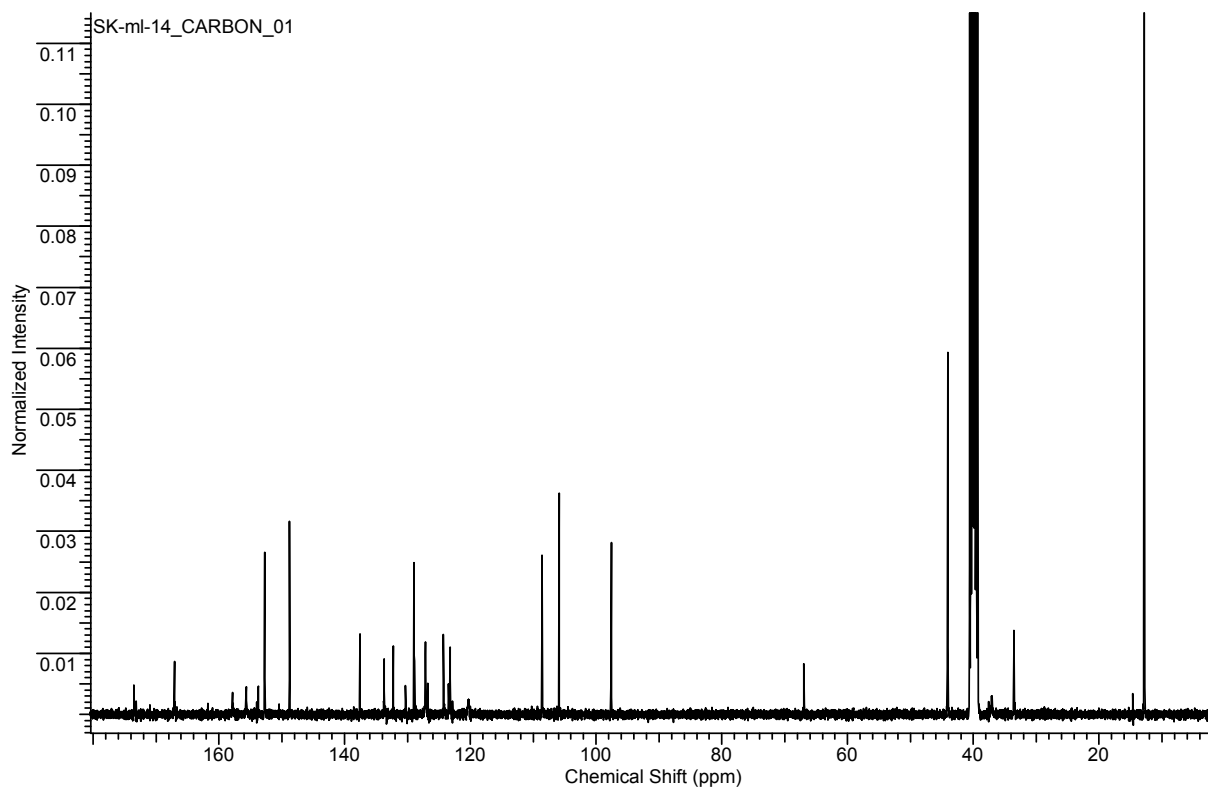
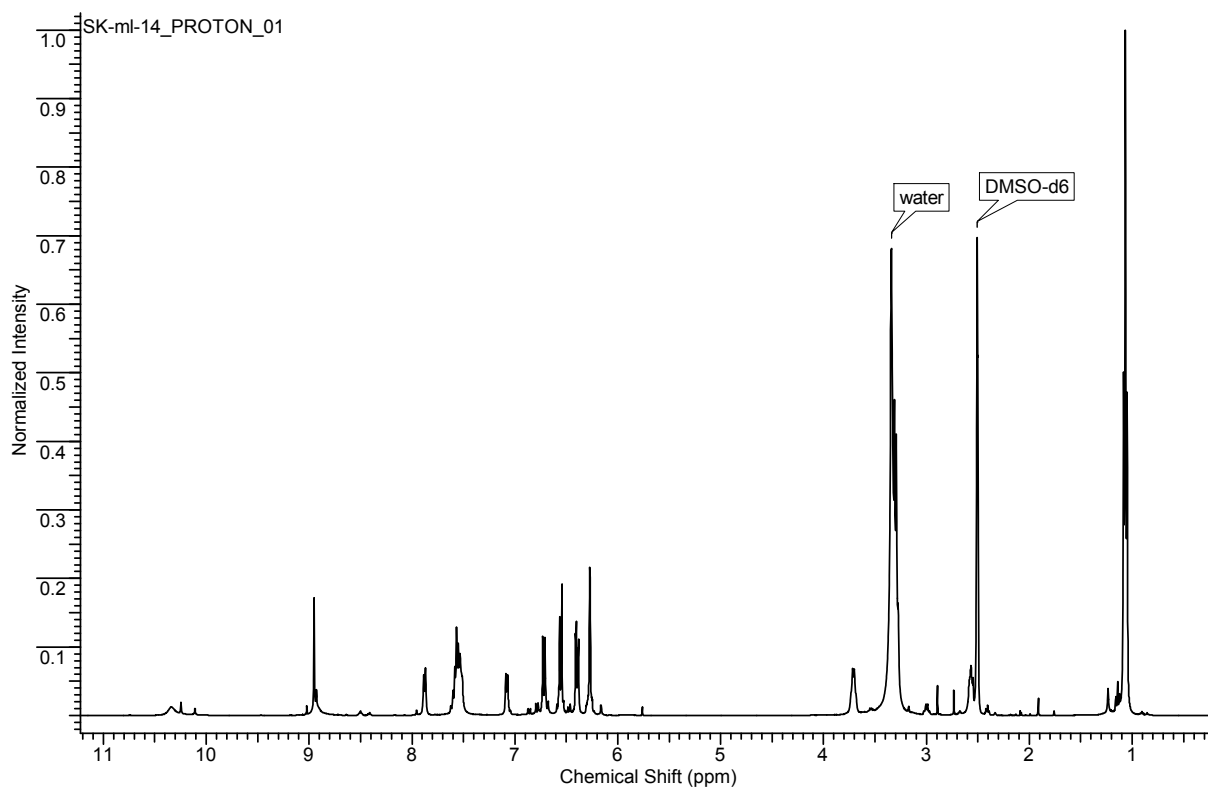


$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-(2-((2-((2-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid **11(1,1,3)**

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed at  $80^\circ\text{C}$ .

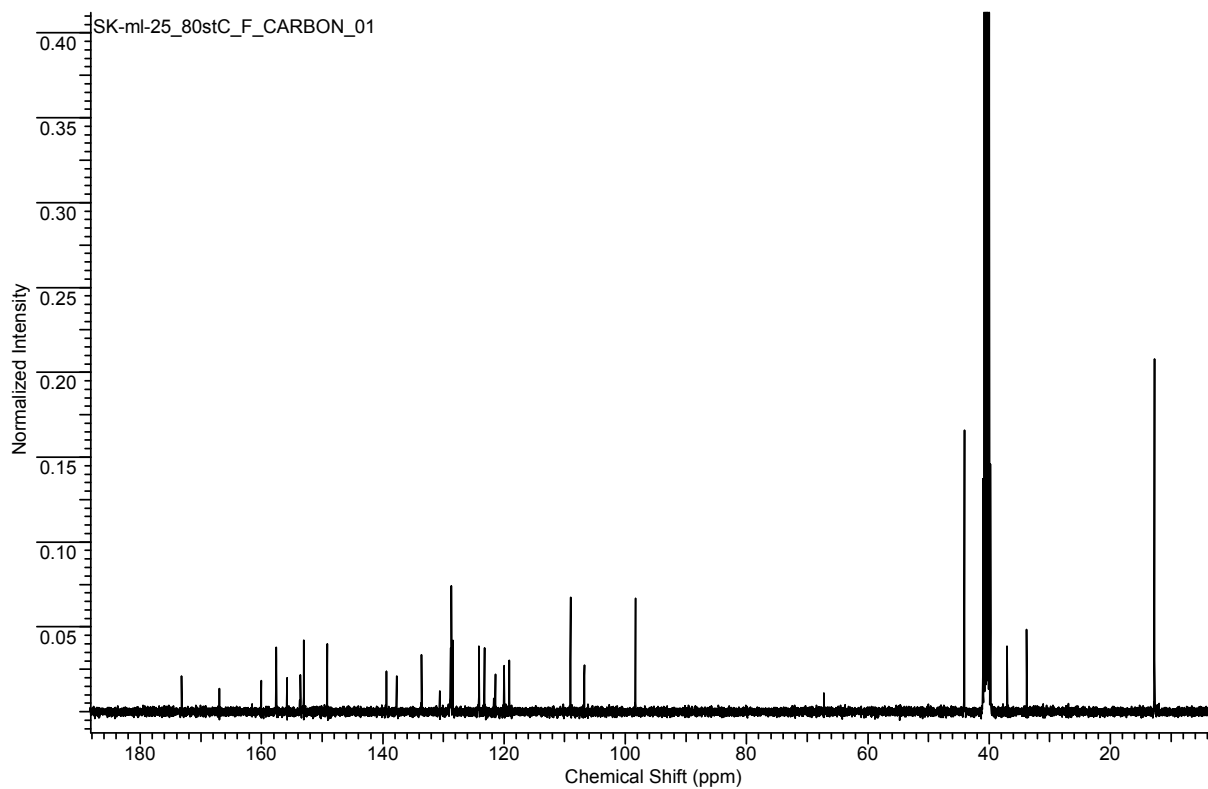
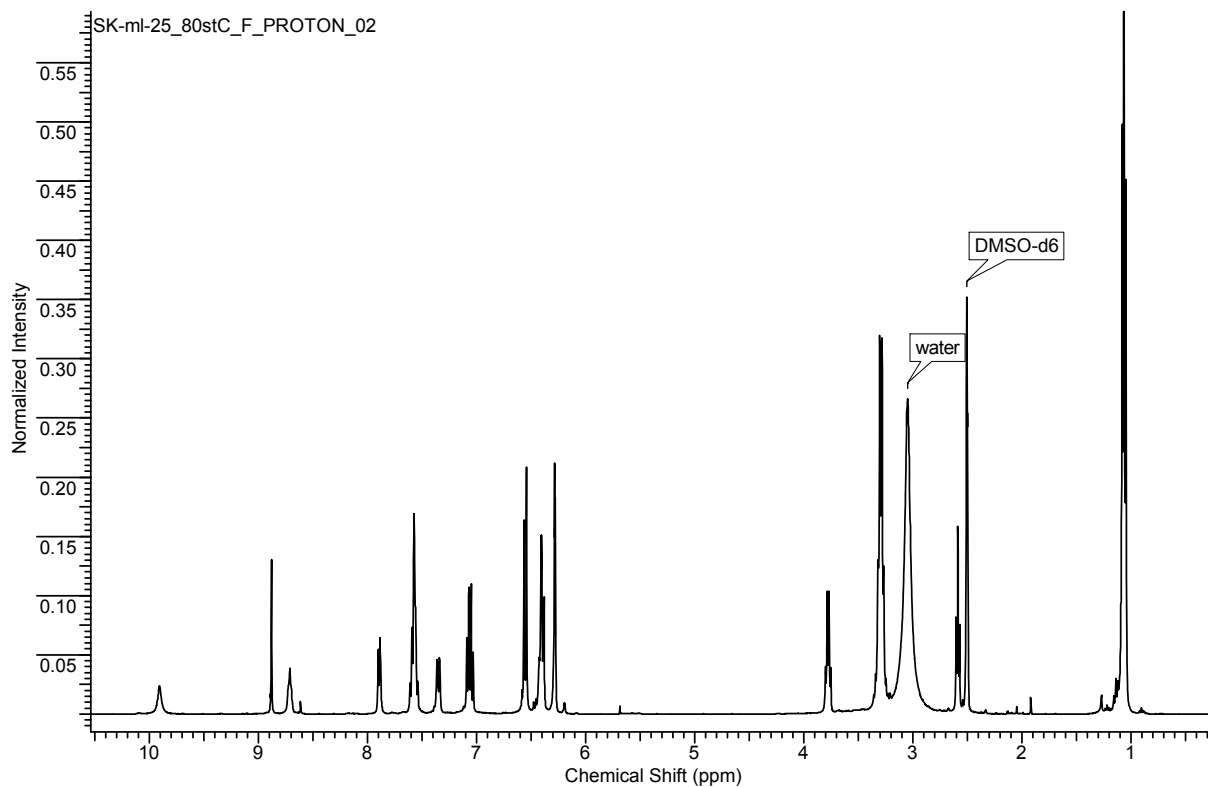


$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-((2-((4-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)propanoic acid **11(2,1,1)**



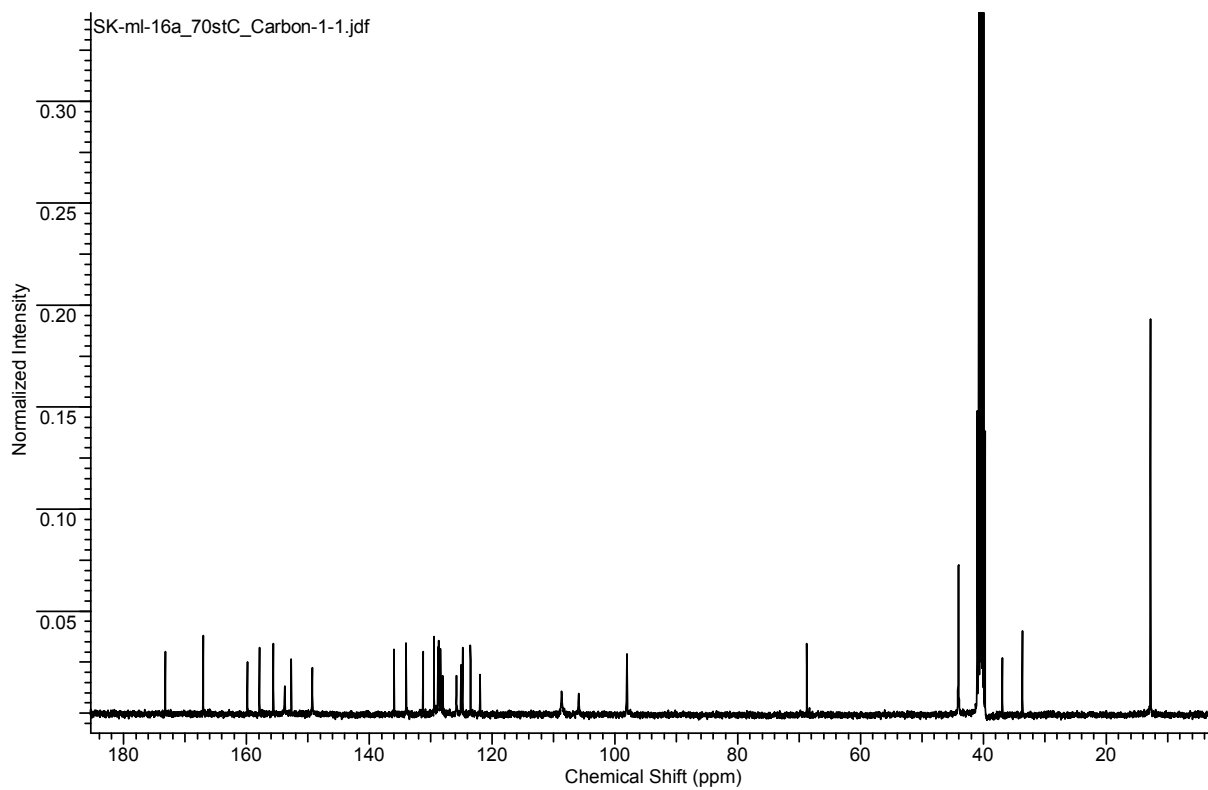
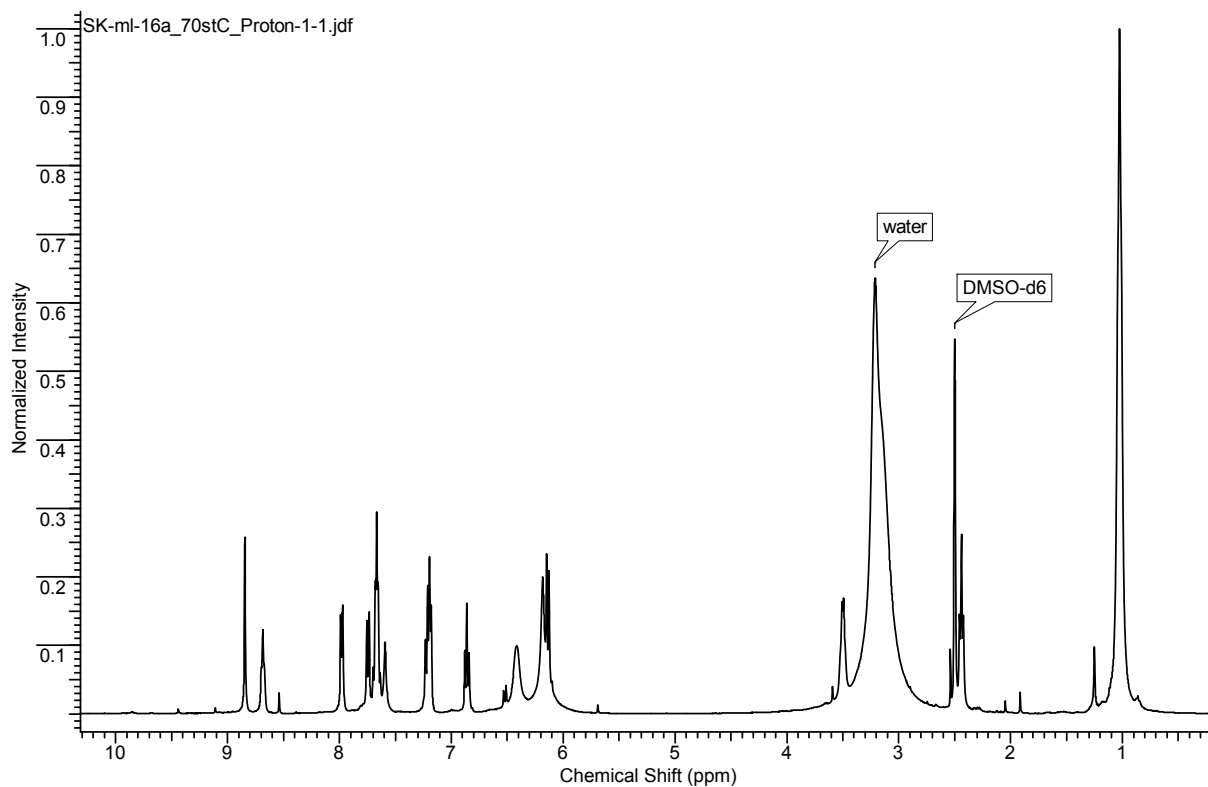
$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-((2-((3-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)propanoic acid **11(2,1,2)**

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed at  $80^\circ\text{C}$ .

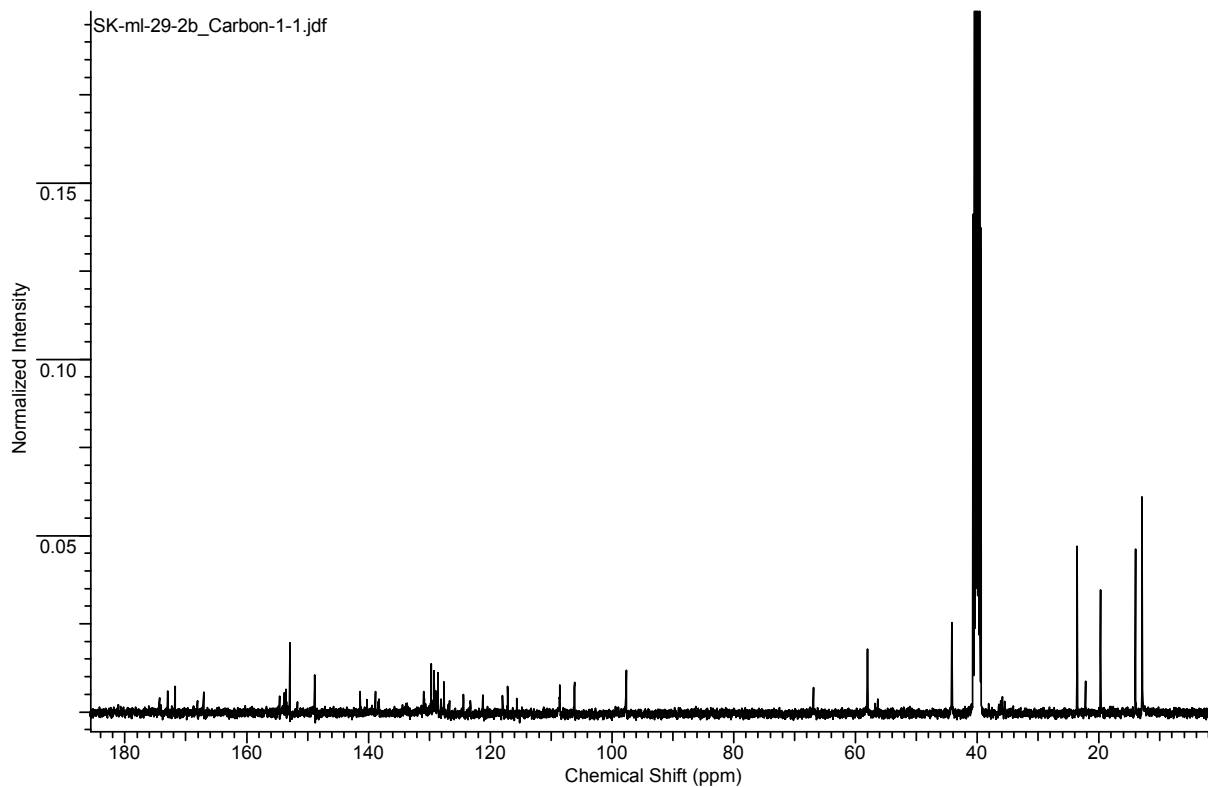
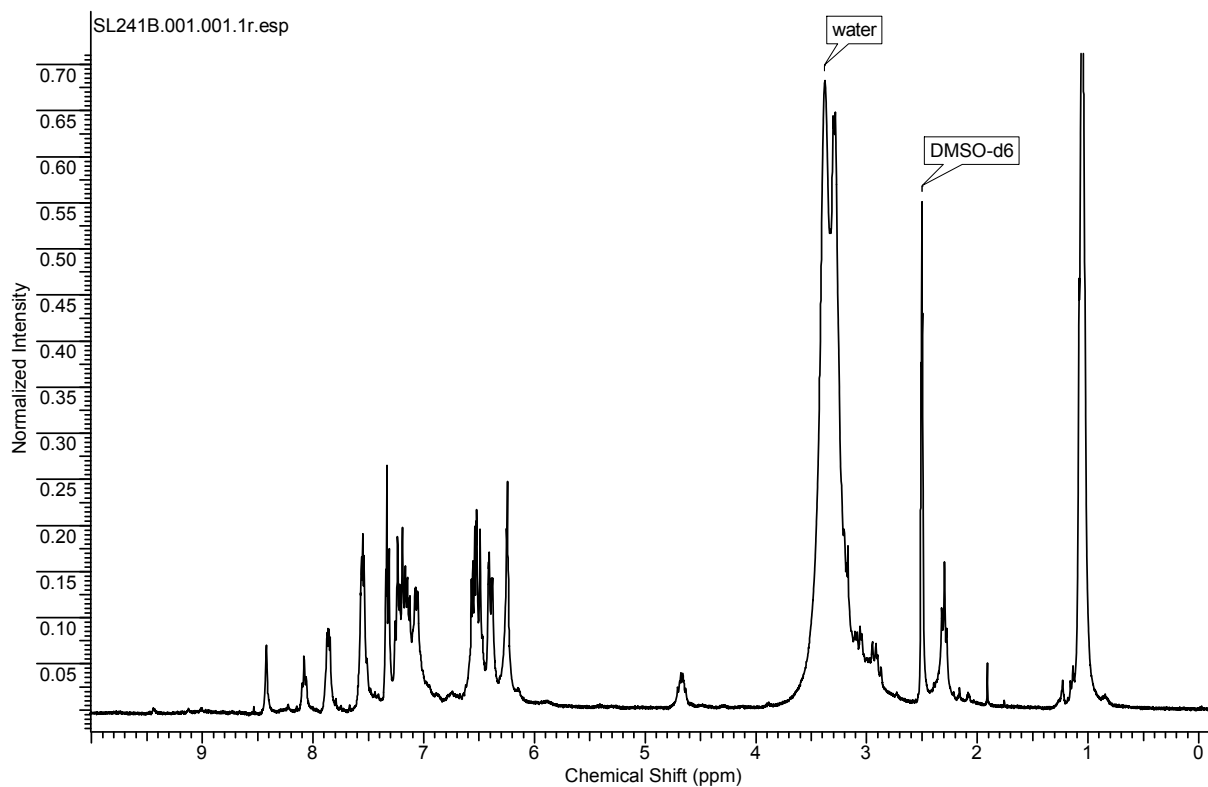


$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-((2-((2-(3',6'-bis(diethylamino)-3-oxospiro[isoindoline-1,9'-xanthen]-2-yl)phenyl)amino)-5-nitropyrimidin-4-yl)amino)propanoic acid **11(2,1,3)**

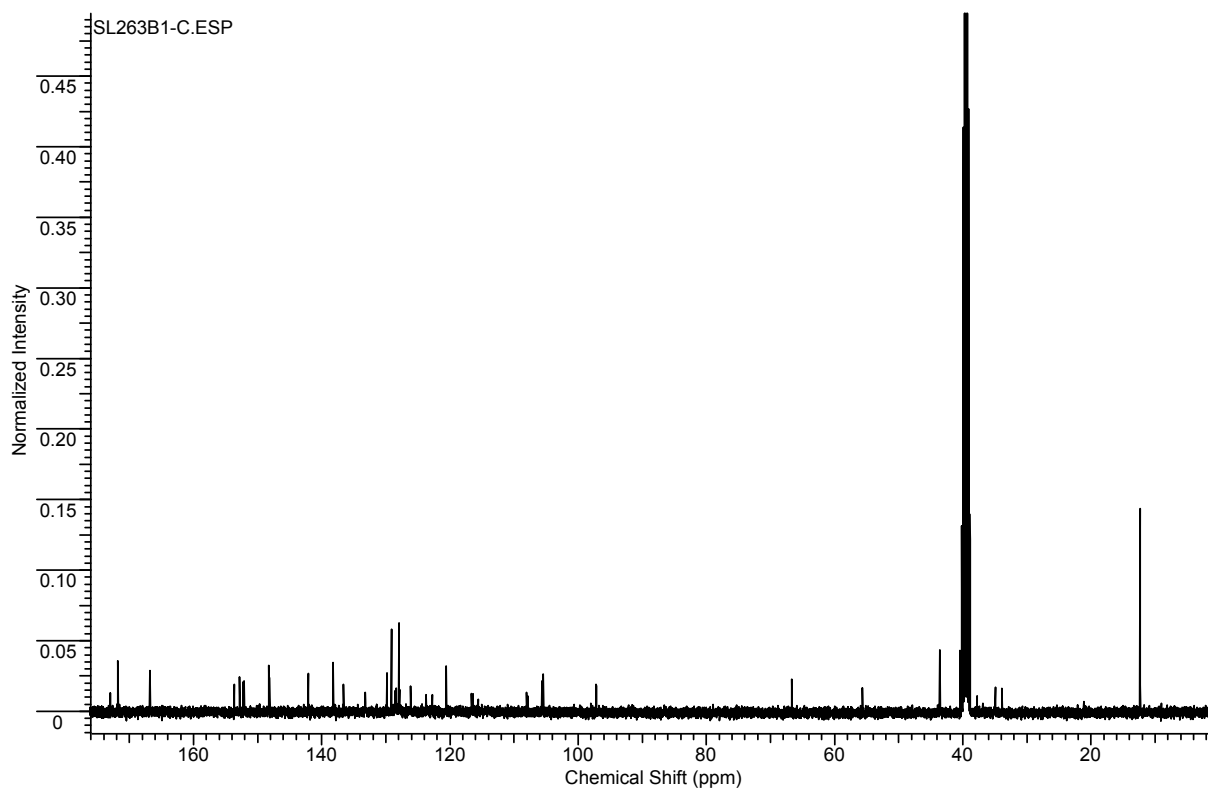
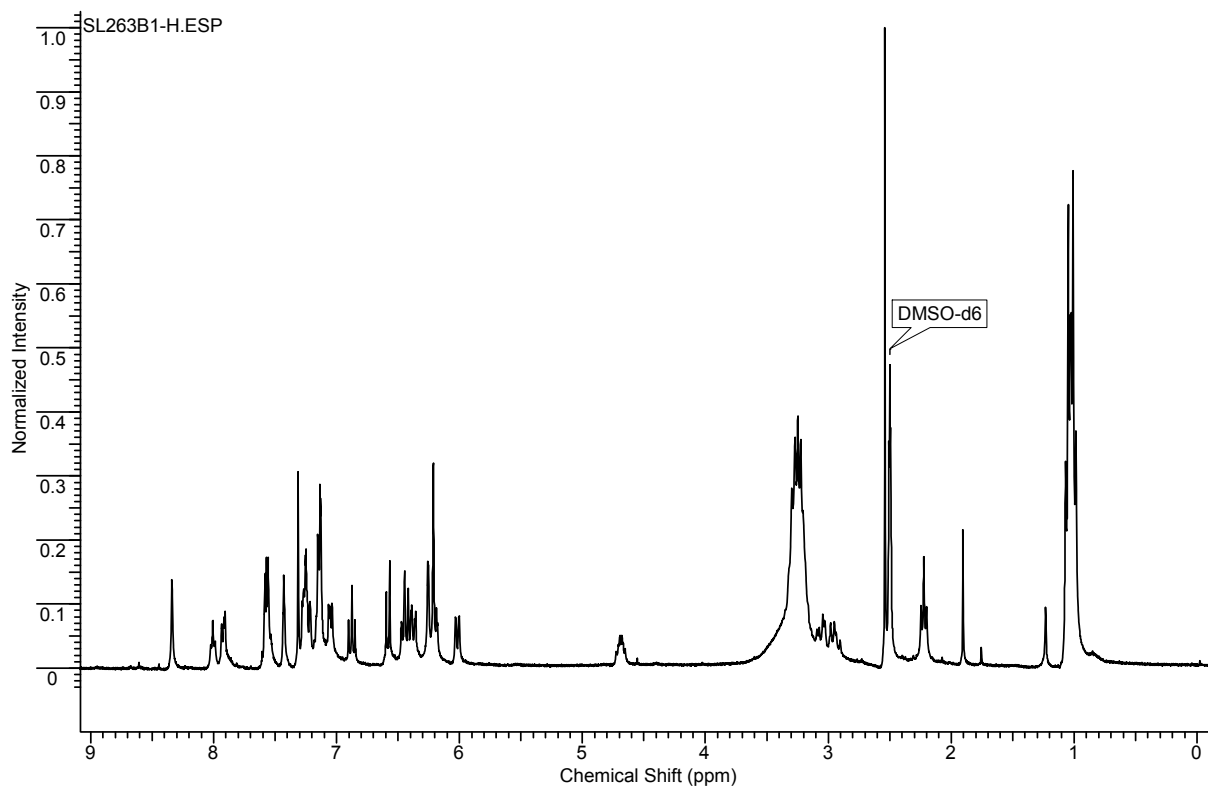
The  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed at 70°C.



<sup>1</sup>H and <sup>13</sup>C NMR spectra of 33-(2-((5-amino-2-((4-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid **11(1,2,1)**

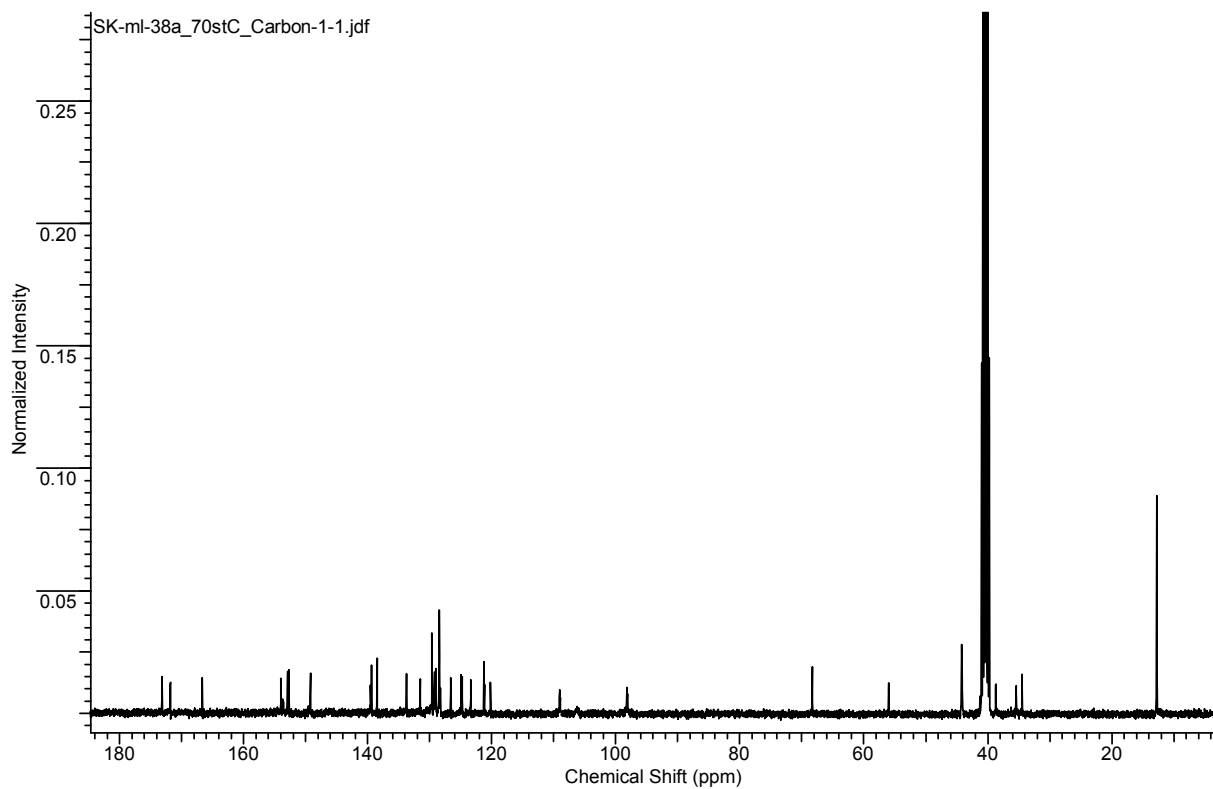
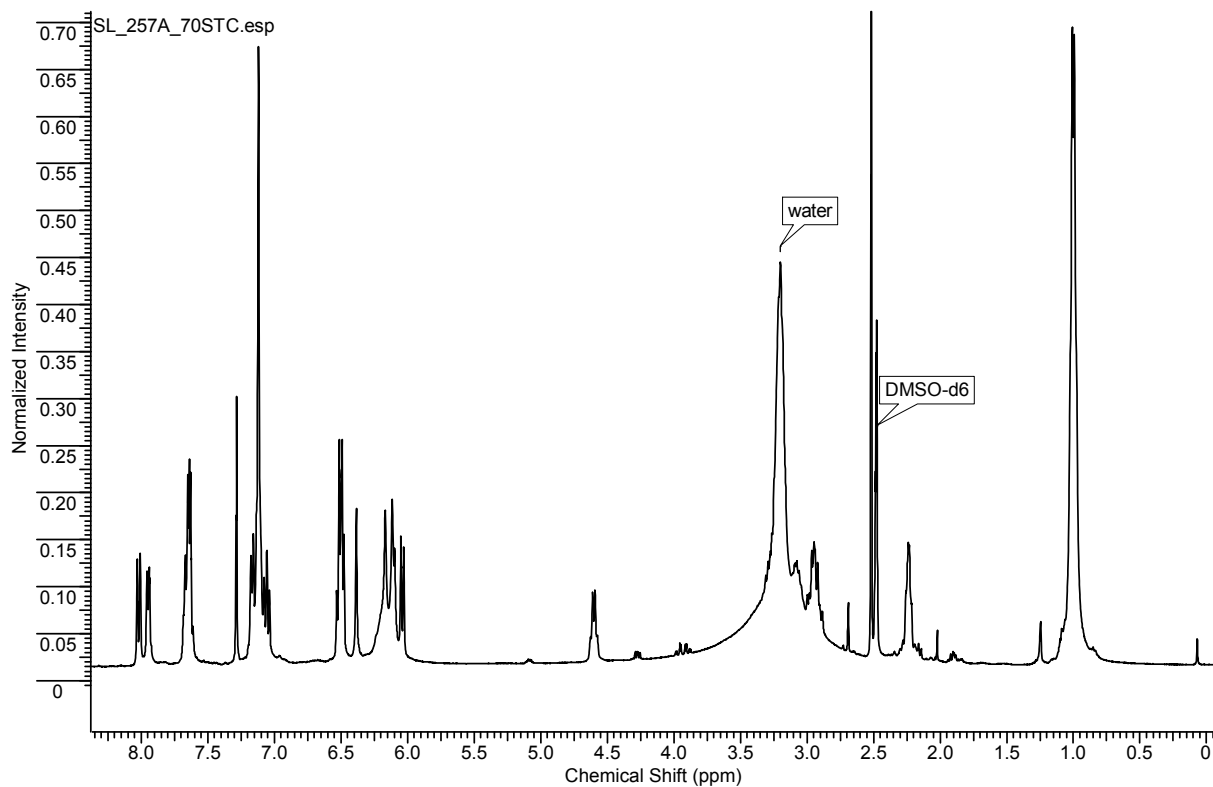


$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-(2-((5-amino-2-((3-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid **11(1,2,2)**

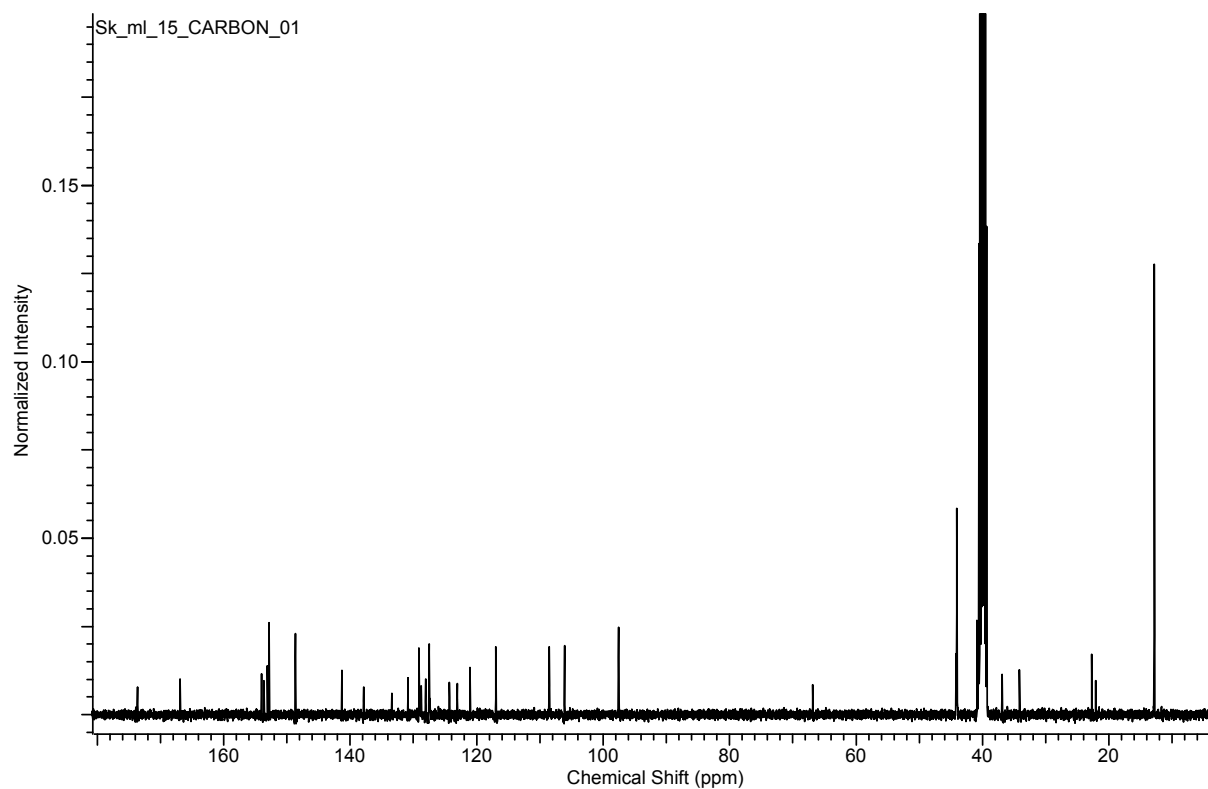
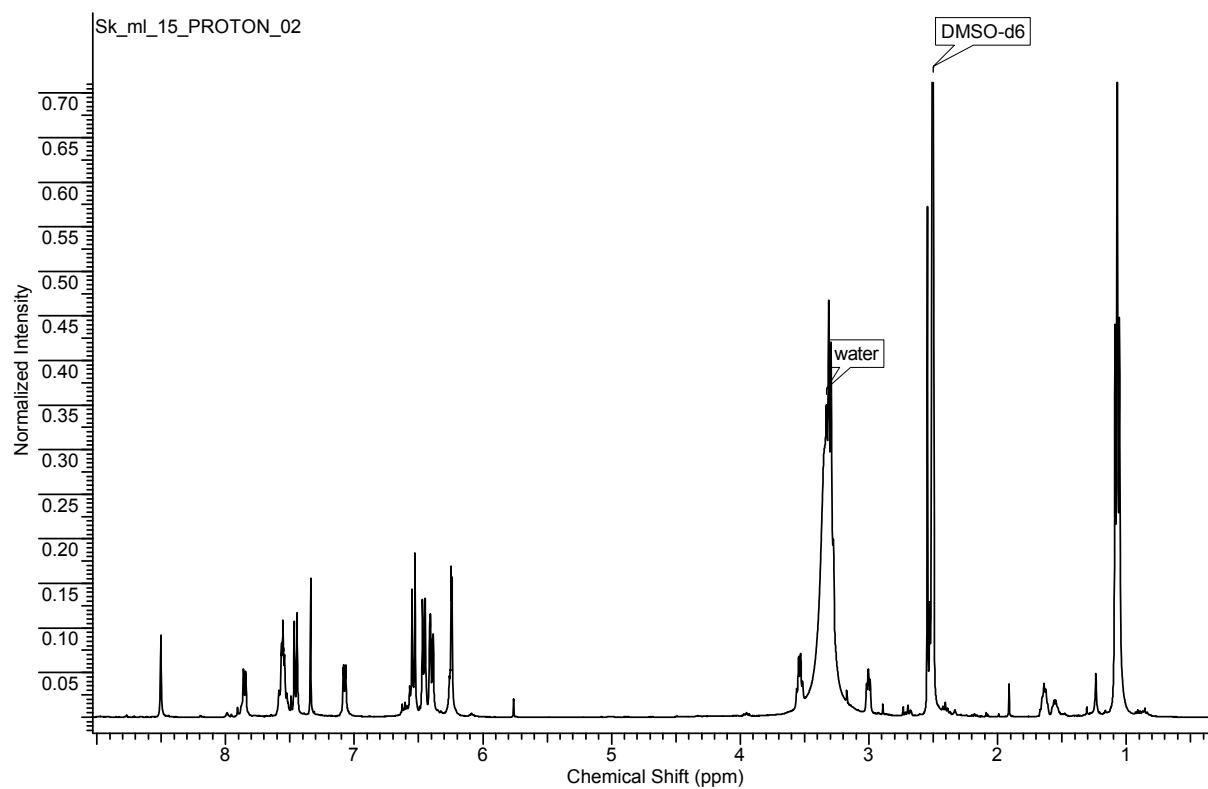


$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-(2-((5-amino-2-((2-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)-3-phenylpropanamido)propanoic acid **11(1,2,3)**

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed at 70°C.

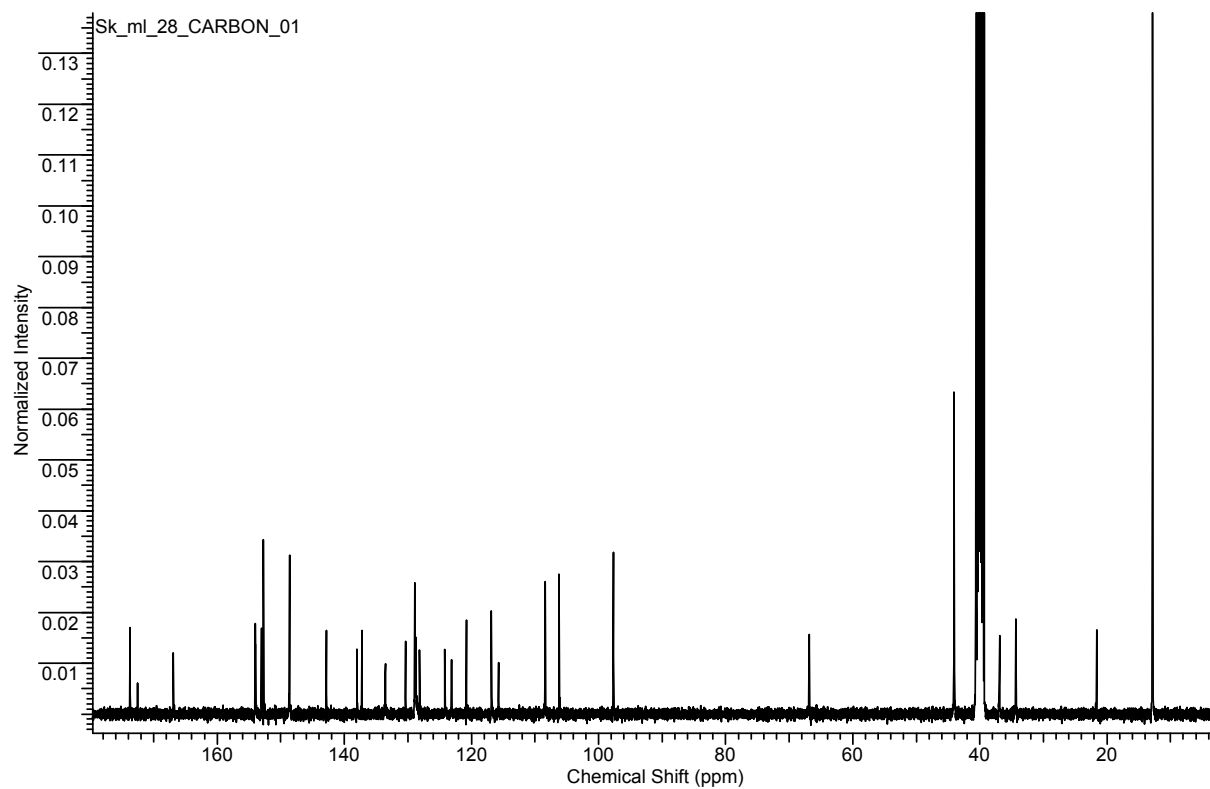
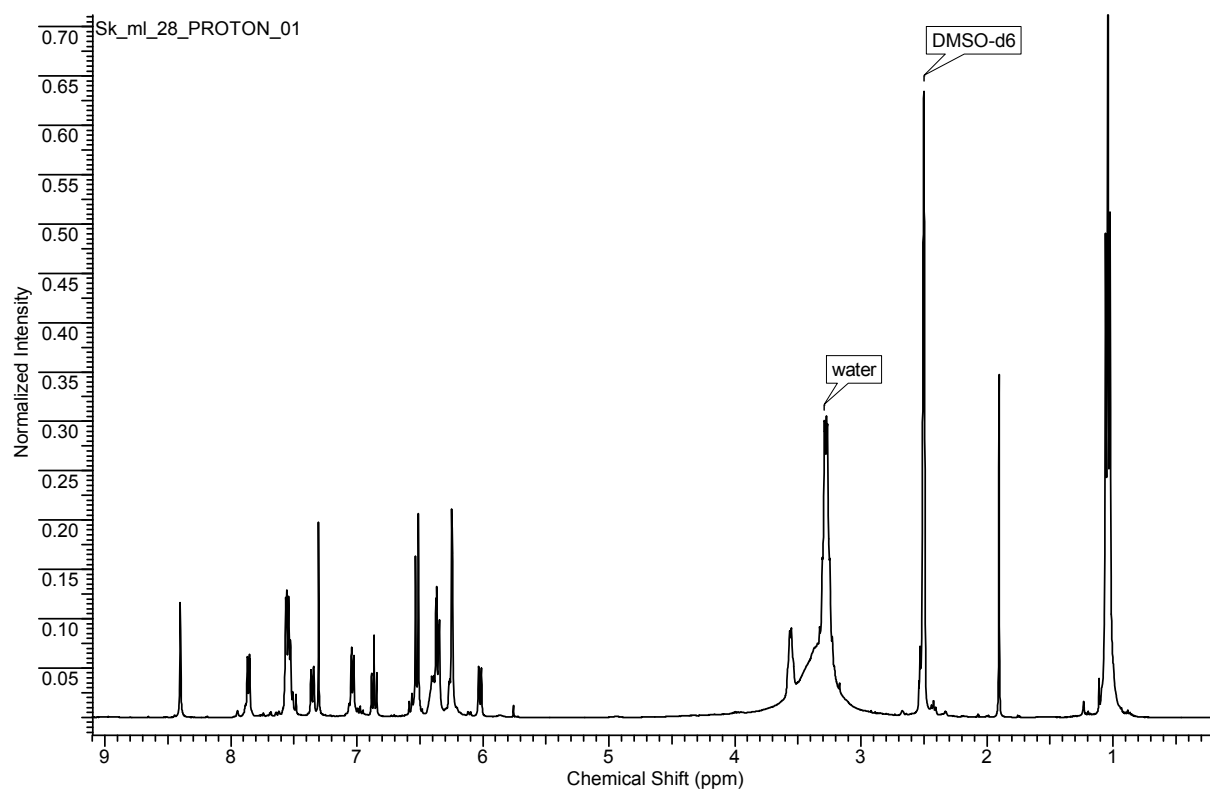


$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-((5-amino-2-((4-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)propanoic acid **11(2,2,1)**



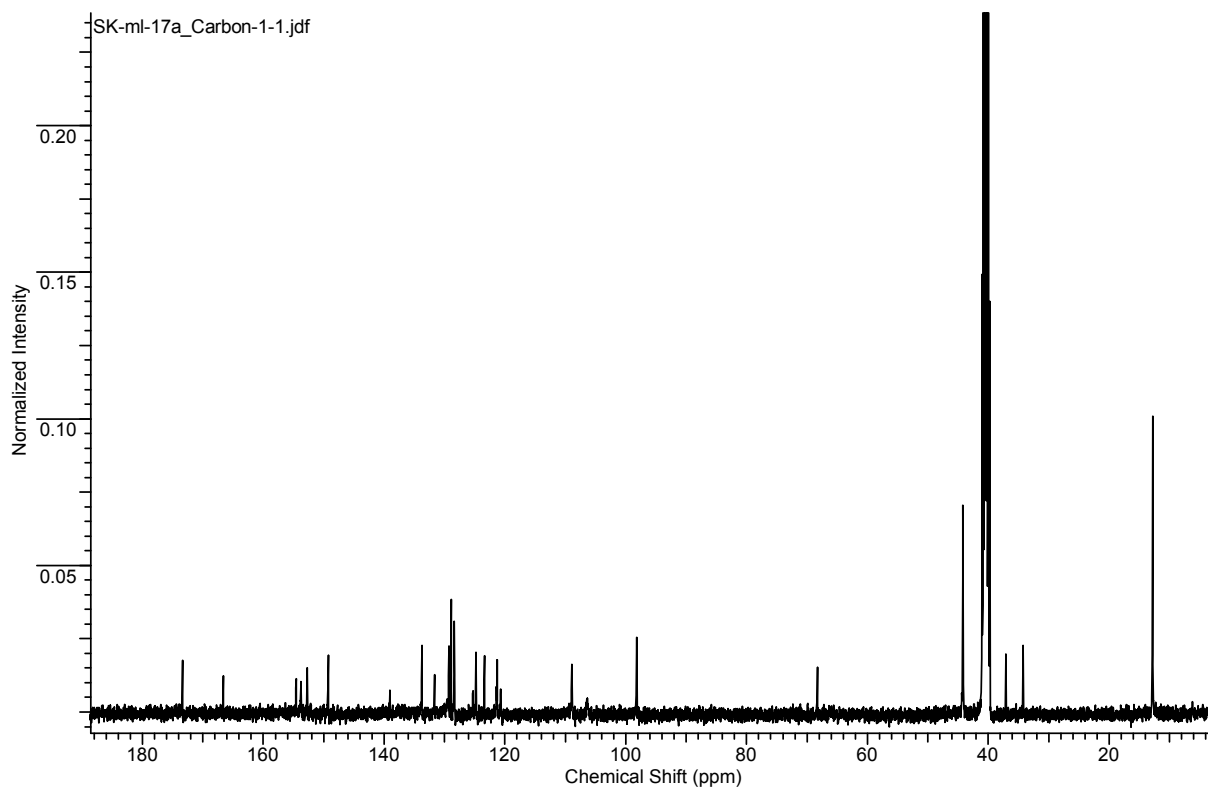
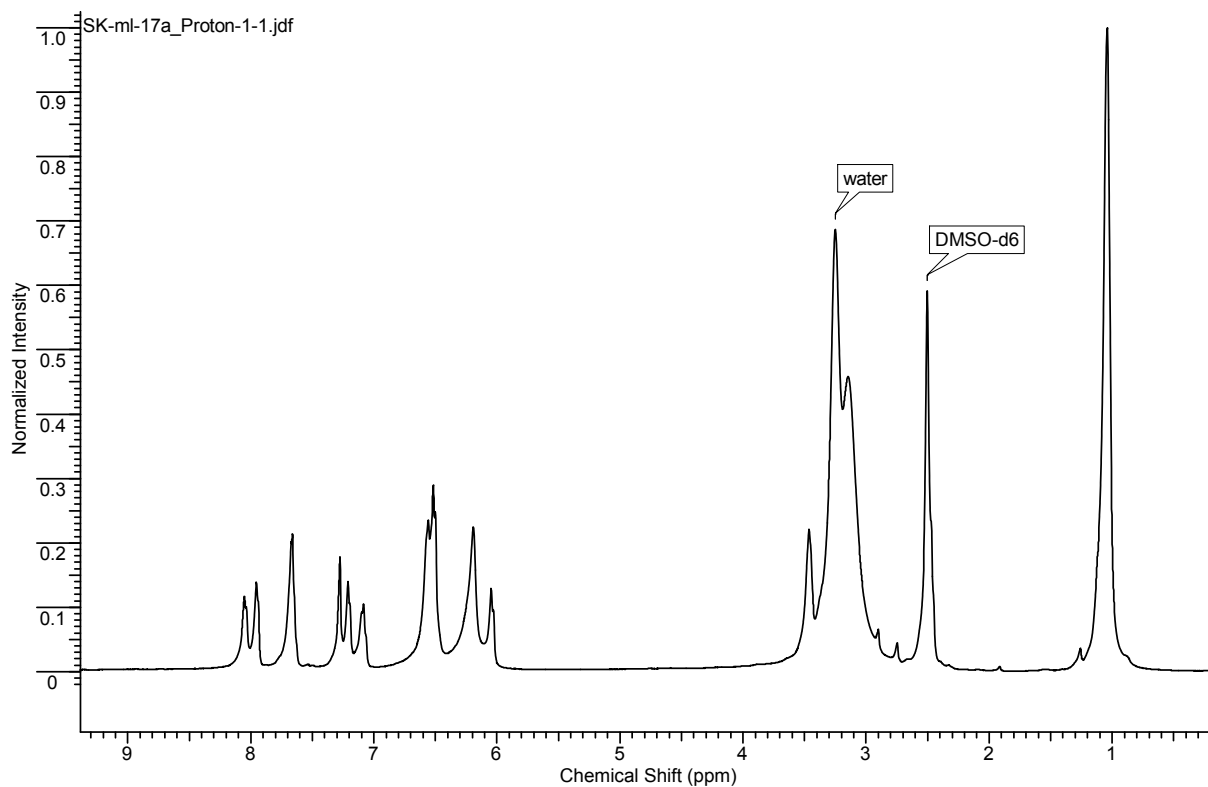


$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-((5-amino-2-((3-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)propanoic acid **11(2,2,2)**

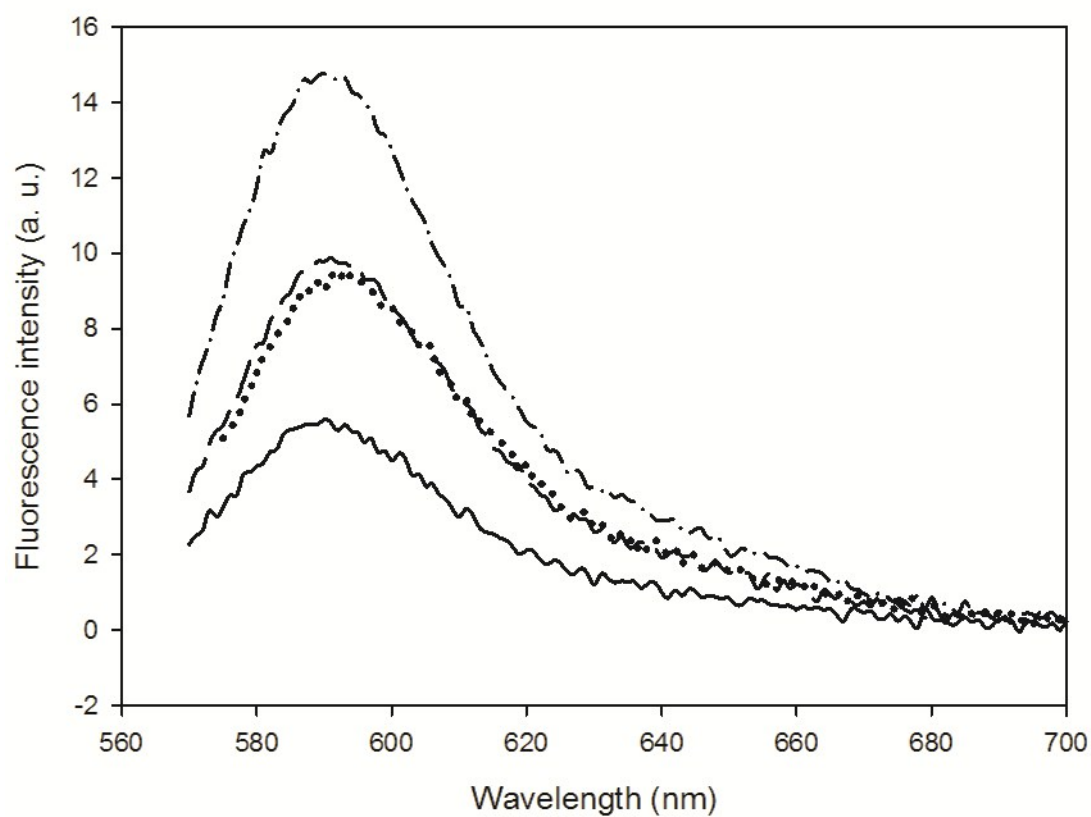


$^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of 3-((5-amino-2-((2-(3',6'-bis(diethylamino)-3-oxospiro[isindoline-1,9'-xanthen]-2-yl)phenyl)amino)pyrimidin-4-yl)amino)propanoic acid **11(2,2,3)**

The  $^1\text{H}$  and  $^{13}\text{C}$  NMR experiments were performed at 70°C.

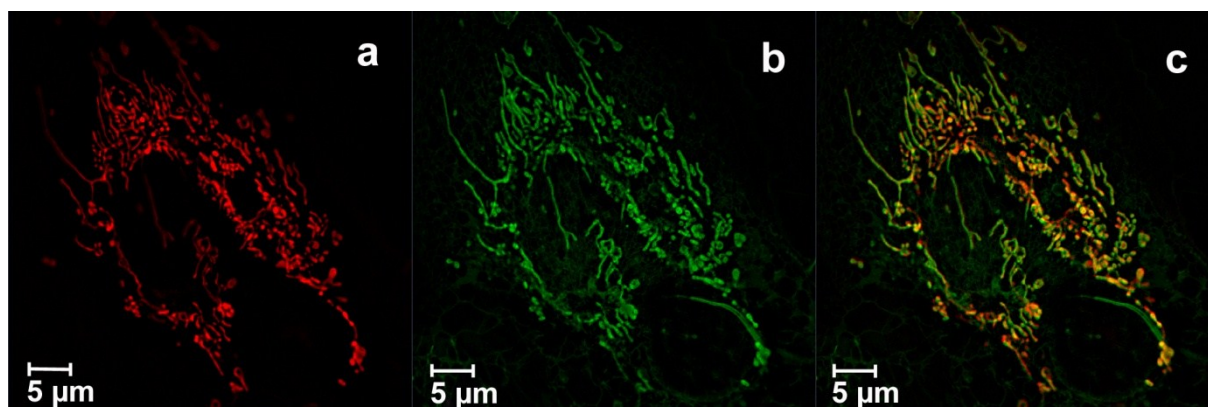


Fluorescence emission spectra of the compounds **6(1,1,2)**, **6(1,1,3)**, **6(2,1,3)** and **11(2,1,3)**.

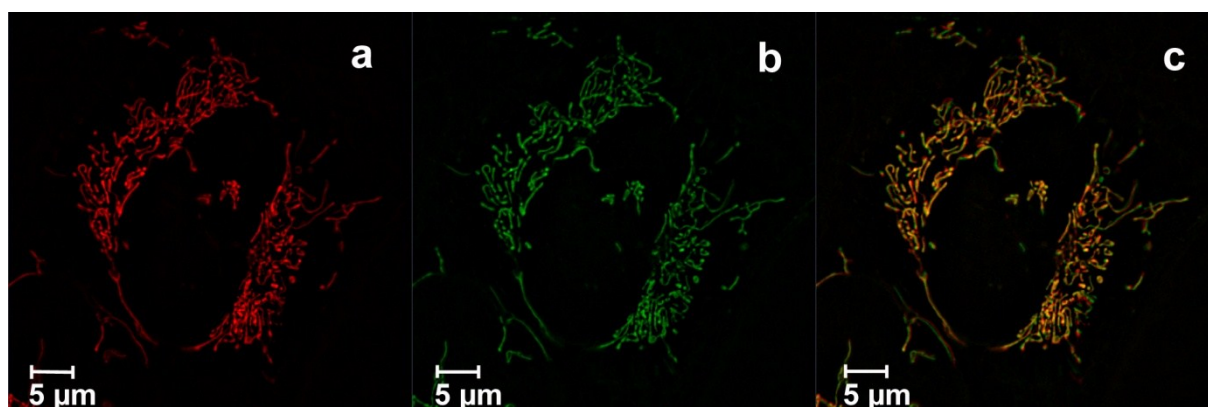


**6(1,1,2)**, solid line; **6(1,1,3)**, dash-dot; **6(2,1,3)**, dotted; **11(2,1,3)**, dash. Conc. 0.1 mg/mL, in DMSO, U(PMT) = 400 V. For excitation wavelength see Table 5.

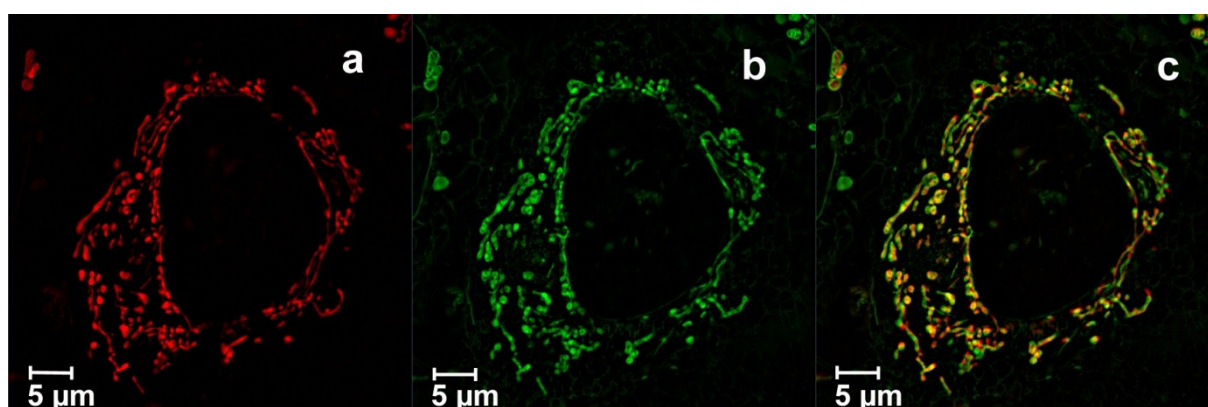
The fluorescence images of the U-2-OS cells treated with the selected compounds.



a) cells after 30 min. of treatment with **6(1,1,2)** excited at 561 nm; b) live mitochondria visualized by MitoTracker® Green FM c) overlay of (a) and (b).

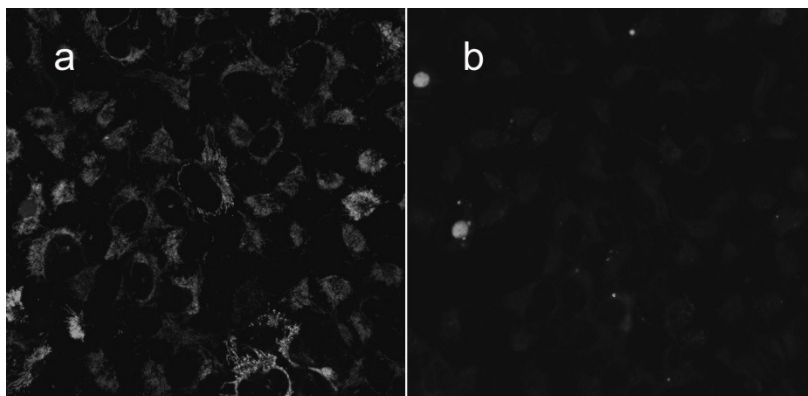


a) cells after 30 min. of treatment with **6(1,1,3)** excited at 561 nm; b) live mitochondria visualized by MitoTracker® Green FM c) overlay of (a) and (b).

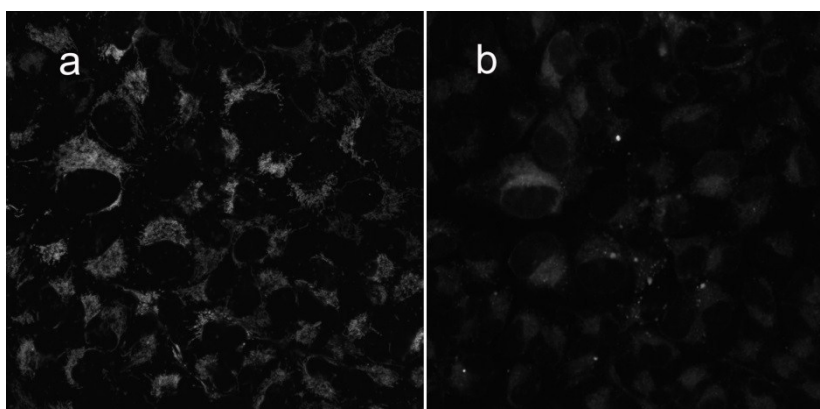


a) cells after 30 min. of treatment with **6(2,1,3)** excited at 561 nm; b) live mitochondria visualized by MitoTracker® Green FM c) overlay of (a) and (b).

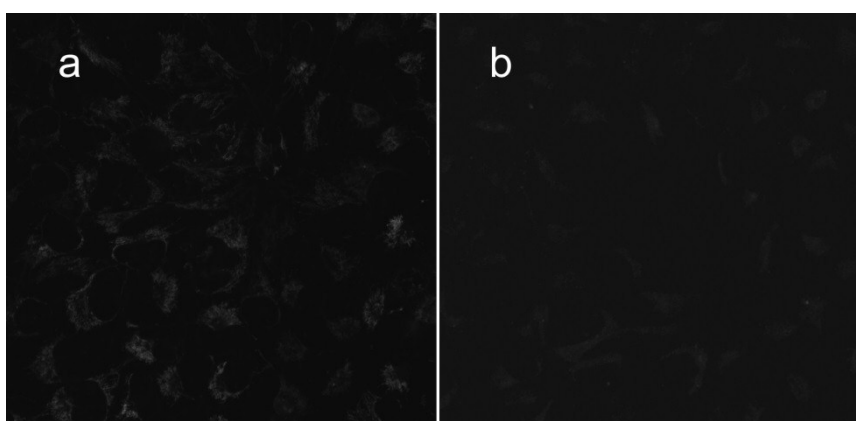
The fluorescence images of the U-2-OS cells treated with the selected compounds before and after cell fixation.



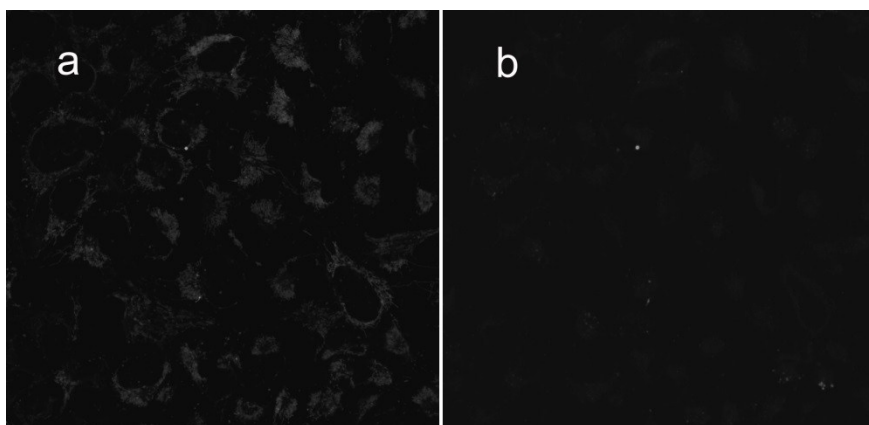
The fluorescence image of the U-2-OS cells treated with the compound **6(1,1,2)** a) before cell fixation; b) after cell fixation.



The fluorescence image of the U-2-OS cells treated with the compound **6(1,1,3)** a) before cell fixation; b) after cell fixation.

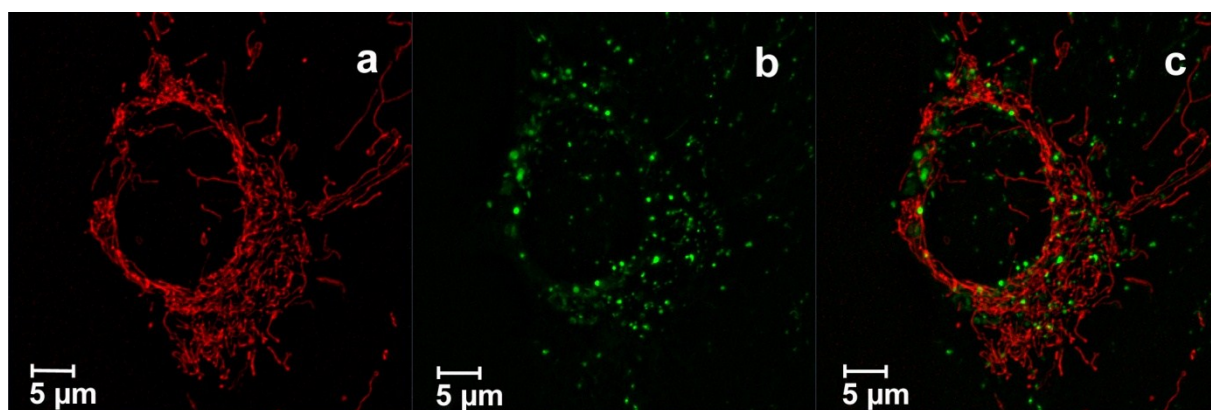


The fluorescence image of the U-2-OS cells treated with the compound **6(2,1,3)** a) before cell fixation; b) after cell fixation.

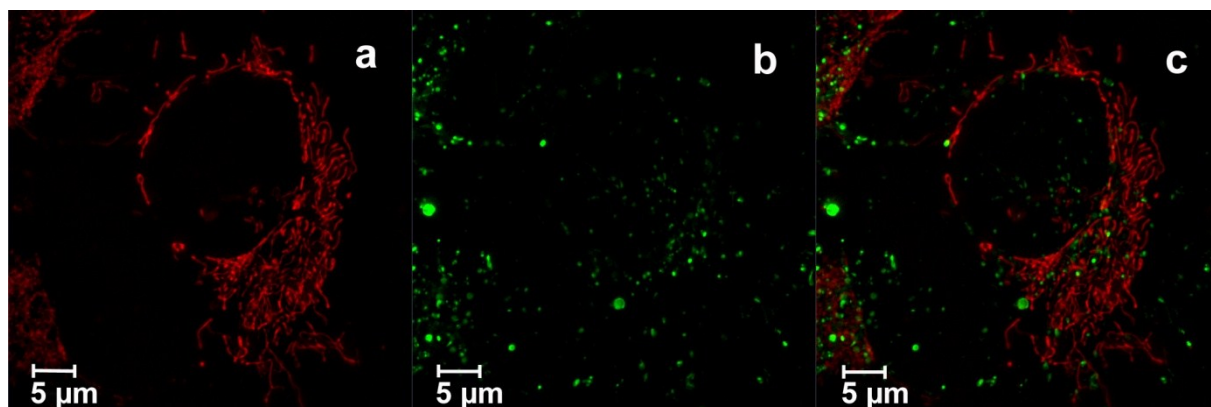


The fluorescence image of the U-2-OS cells treated with the compound **11(2,1,3)** a) before cell fixation; b) after cell fixation.

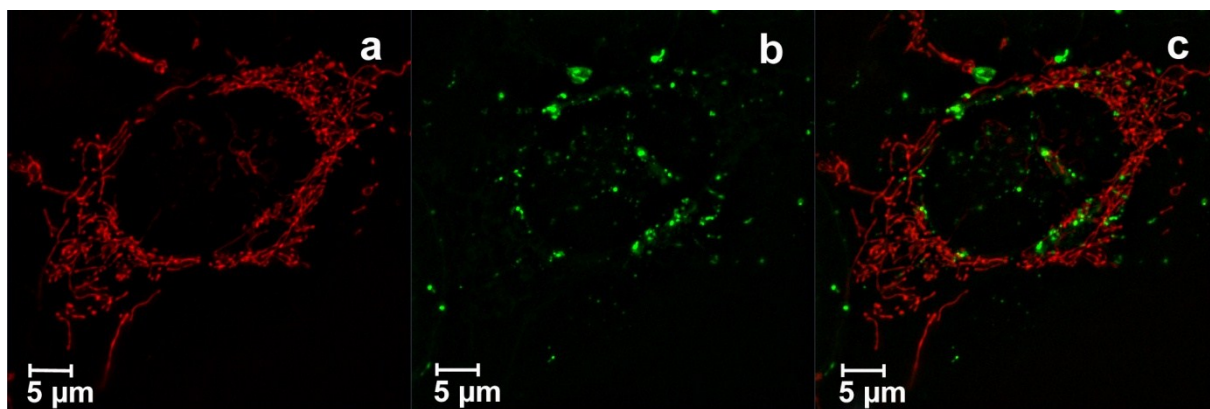
The comparison of the selected compounds and LysoTracker accumulation in live U-2-OS cells.



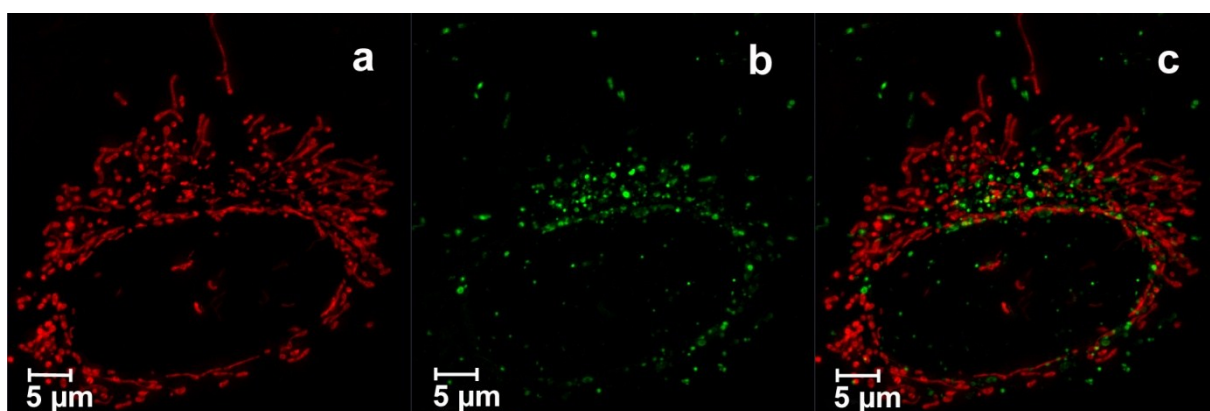
a) cells after 30 min. of treatment with **6(1,1,2)** excited at 561 nm; b) cells after 30 min. of treatment with LysoTracker® Green DND - 26; c) overlay of (a) and (b).



a) cells after 30 min. of treatment with **6(1,1,3)** excited at 561 nm; b) cells after 30 min. of treatment with LysoTracker® Green DND - 26; c) overlay of (a) and (b).

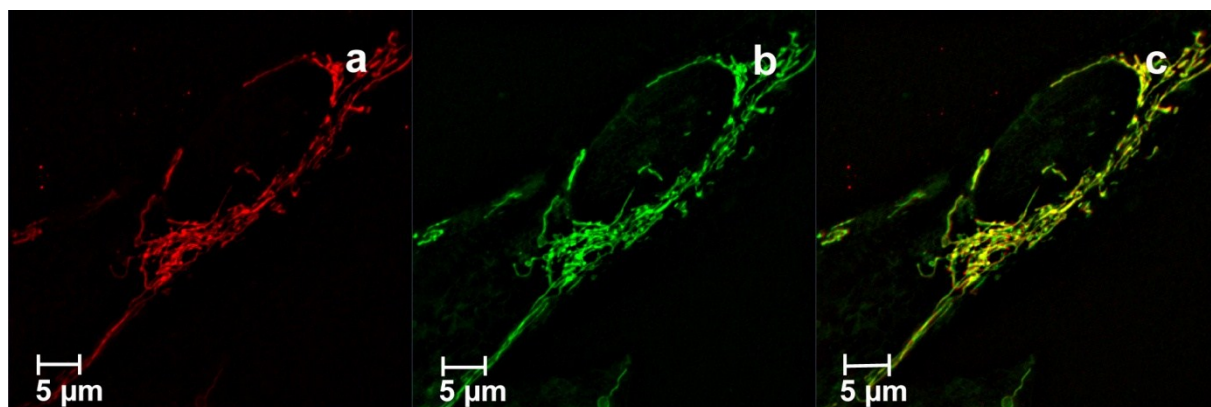


a) cells after 30 min. of treatment with **6(2,1,3)** excited at 561 nm; b) cells after 30 min. of treatment with LysoTracker® Green DND - 26; c) overlay of (a) and (b).

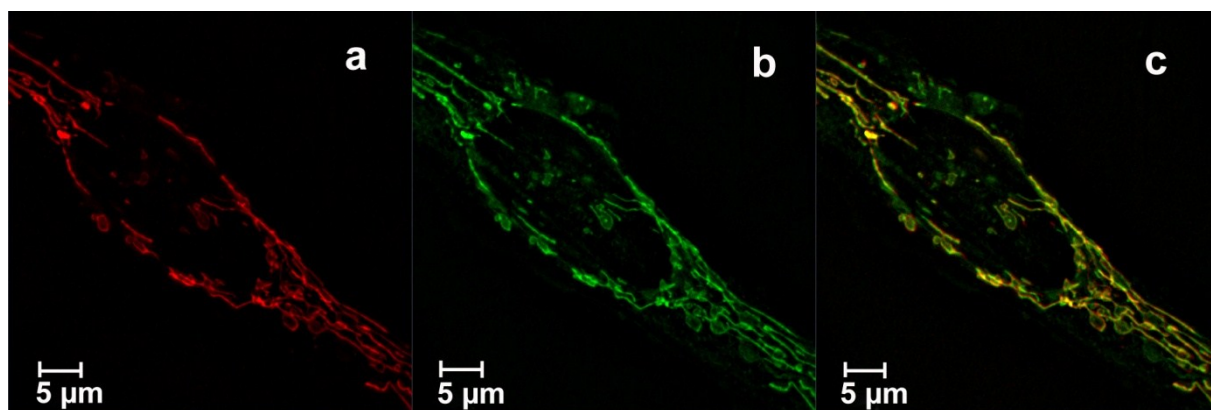


a) cells after 30 min. of treatment with **11(2,1,3)** excited at 561 nm; b) cells after 30 min. of treatment with LysoTracker® Green DND - 26; c) overlay of (a) and (b).

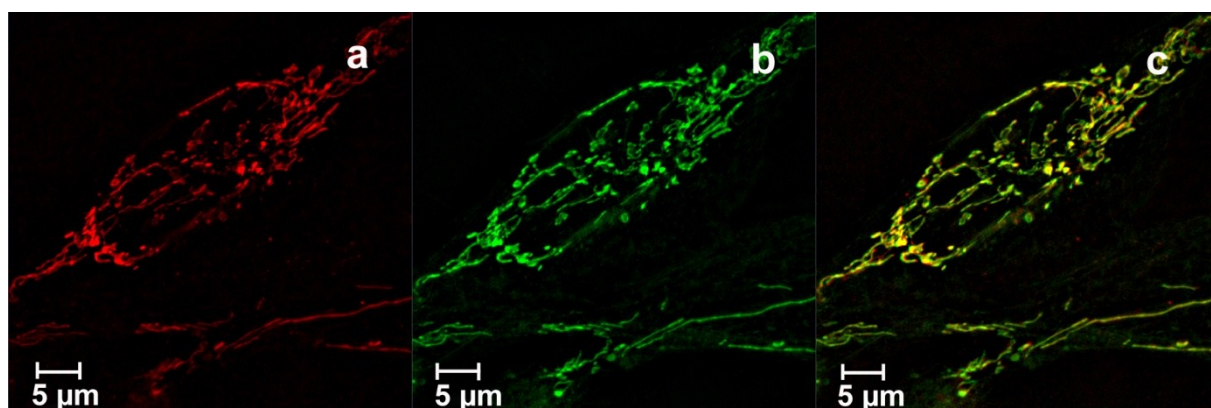
**Fluorescence images of the astrocyte cells treated with the selected compounds.**



a) cells after 30 min. of treatment with **6(1,1,2)** excited at 561 nm; b) cells after 30 min. of treatment with MitoTracker® Green FM; c) overlay of (a) and (b).

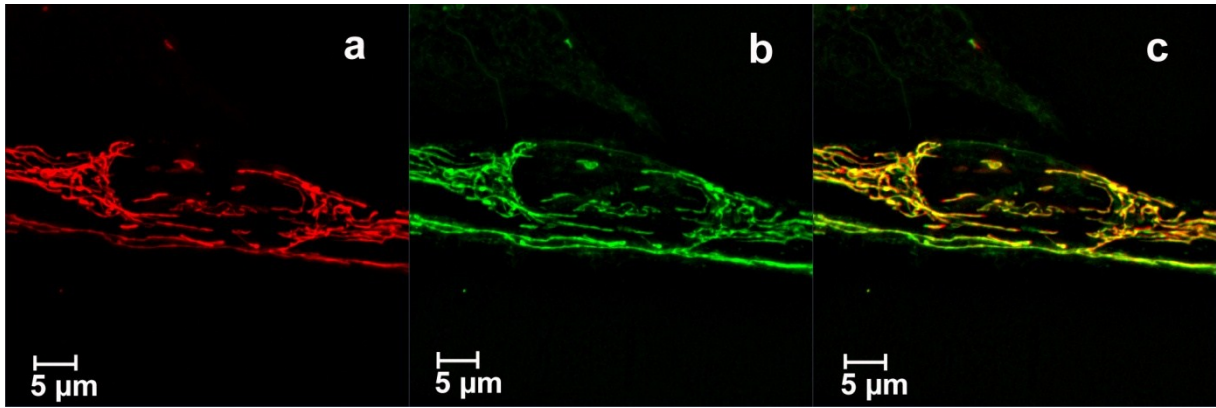


a) cells after 30 min. of treatment with **6(1,1,3)** excited at 561 nm; b) cells after 30 min. of treatment with MitoTracker® Green FM; c) overlay of (a) and (b).



a) cells after 30 min. of treatment with **6(2,1,3)** excited at 561 nm; b) cells after 30 min. of treatment with MitoTracker® Green FM; c) overlay of (a) and (b).





a) cells after 30 min. of treatment with **11(2,1,3)** excited at 561 nm; b) cells after 30 min. of treatment with MitoTracker® Green FM; c) overlay of (a) and (b).