

Supporting Information

Live-Cell Imaging of Sterculic Acid—a Naturally Occurring 1,2-Cyclopropene Fatty Acid—by Bioorthogonal Reaction with Turn-On Tetrazine-Fluorophore Conjugates

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SUPPORTING INFORMATION

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Supporting figures and tables

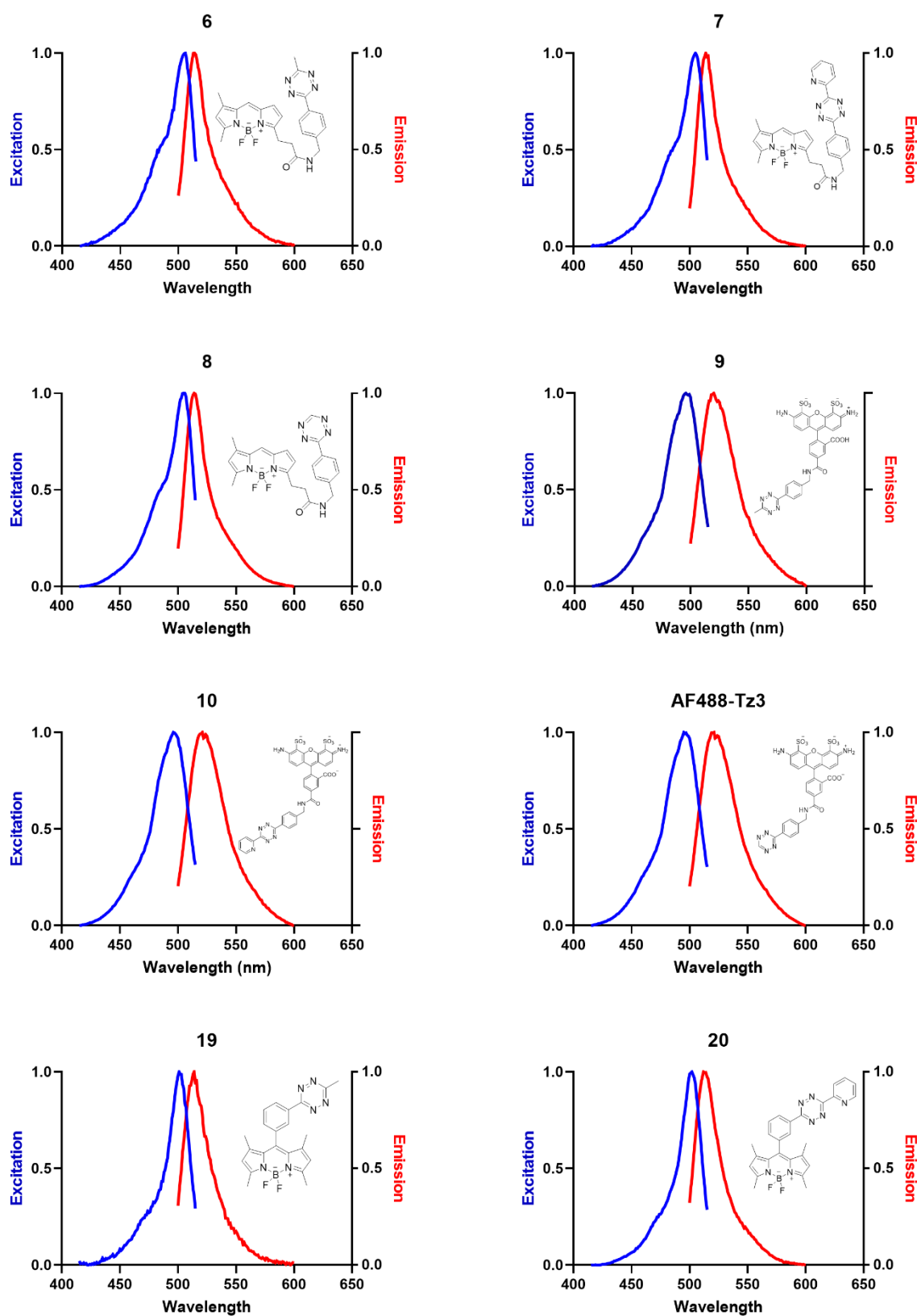


Figure S1: Normalised excitation and emission spectra of fluorophores **6-11** and **19-20** measured at a final concentration of 1 μM in PBS on a CLARIOstar plate reader.

Table S1: Characterisations of the photophysical properties of tetrazine-fluorophore conjugates **6-11** and **19-20**, and their kinetics upon reaction with **1**. Absorption maximum (λ_{abs}) and extinction coefficient (E_{max}) of the tetrazine-fluorophore conjugates (1 μM) in DMSO/H₂O (1:1) and PBS. Second-order rate constants (k_2) for the conjugates (1 μM) upon reaction with **1** (5 μM) in PBS.

Fluorophore	λ_{abs} (nm) DMSO/H ₂ O (1:1)	E_{max} (M ⁻¹ cm ⁻¹) DMSO/H ₂ O (1:1)	λ_{abs} (nm) PBS	E_{max} (M ⁻¹ cm ⁻¹) PBS	k_2 (M ⁻¹ s ⁻¹)
6	506	60000	506	34000	125 ± 41
7	508	28000	506	16000	660 ± 28
8	506	43000	506	24000	ND
9	500	58000	492	48000	ND
10	500	45000	492	36000	ND
11	500	39000	494	39000	ND
19	500	59000	500	26000	658 ± 14
20	502	82000	500	26000	328 ± 36

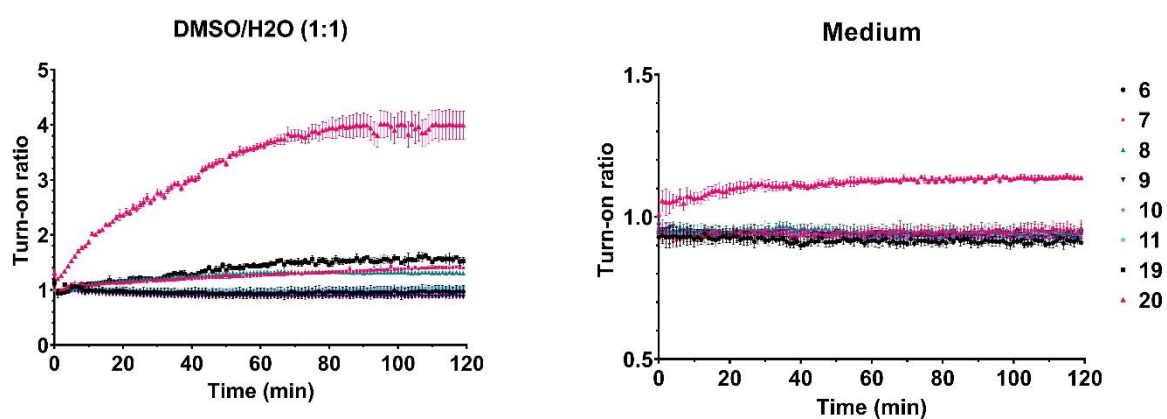


Figure S2: Average turn-on ratio of tetrazine-fluorophore conjugates **6-11** and **19-20** upon reaction with sterculic acid in DMSO/H₂O (1:1, v/v) and complete medium at 25°C. All conditions were measured in triplicate, and standard deviations are indicated.

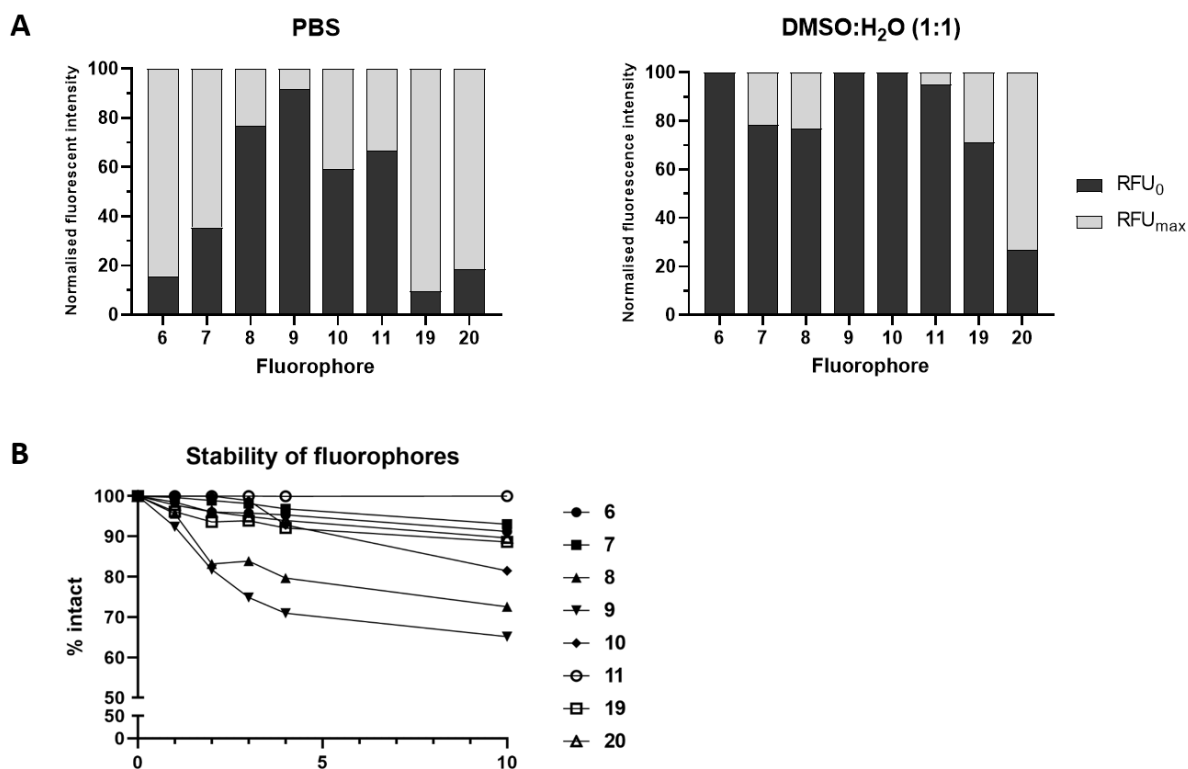


Figure S3: Further insight into the turn-on ability and stability of tetrazine-fluorophore conjugates **6-11** and **19-20**. **(A)** Each fluorophore (1 μM) was reacted with **1** (5 μM) in PBS and DMSO:H₂O (1:1) at RT, and the fluorescent intensity at the start-point of measurement (RFU₀) is compared to the maximum fluorescent intensity (RFU_{max}). RFU_{max} for each fluorophore is set to 100%. **(B)** Stability of the fluorophores (10 μM) in PBS at RT for 10 h as measured by LCMS. The integral of the peak at 0 min was set as 100%.

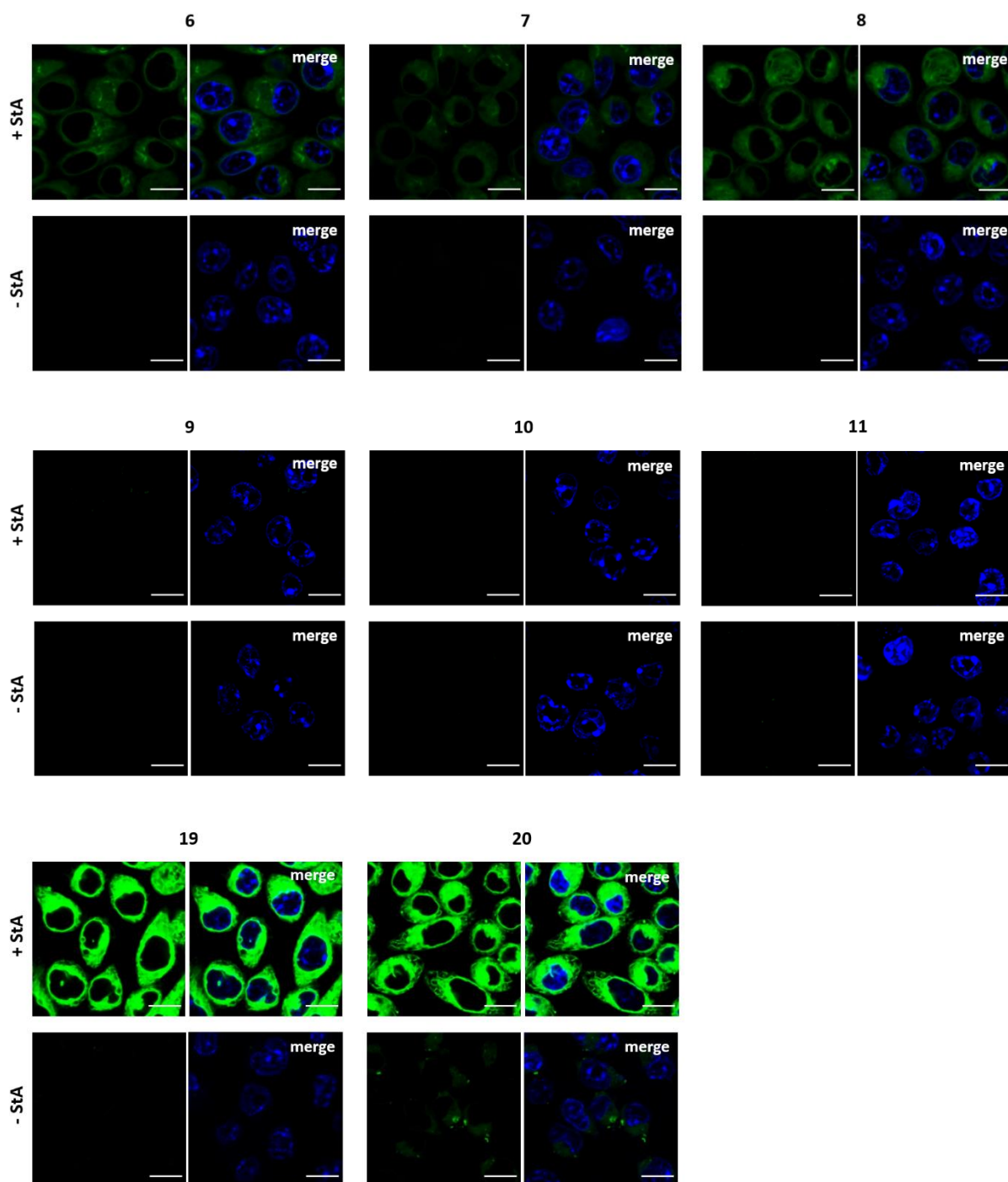


Figure S4: Confocal live-cell imaging of tetrazine-fluorophore conjugate library (6-11 and 19-20). DC2.4 cells were incubated with sterculic acid (+StA, 50 μ M) or no probe (-StA) for 1 h, followed by click reaction with the respective fluorophore (all 5 μ M) for 1 h. Images are presented as single slices. All samples were routinely washed after metabolic incorporation of **1** and after ligation with the fluorophores, and were imaged with minimum 4 technological replicates. DNA was counterstained with Hoechst 33342. All scale bars represent 10 μ m.

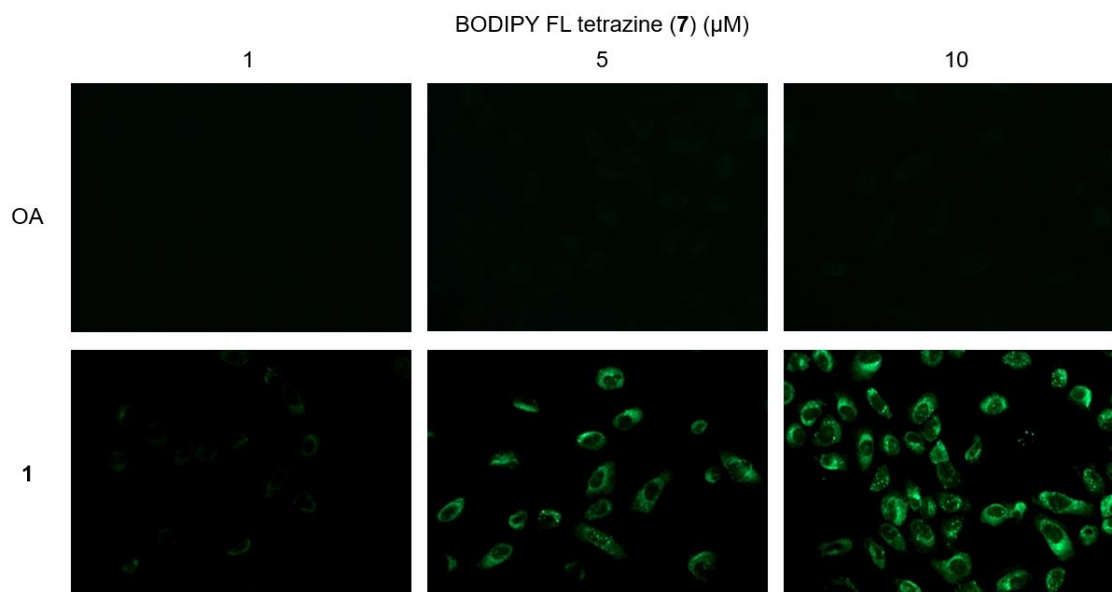


Figure S5: Sterculic acid is dose-dependently labeled by ligation to BODIPY-tetrazine in live cells. Live-cell images of U2OS cells incubated with oleic acid (OA, 50 μM , negative control) or **1** (50 μM) for 1 h, washed and incubated with indicated concentration of BODIPY FL tetrazine **7** for 2 h before washing and imaging.

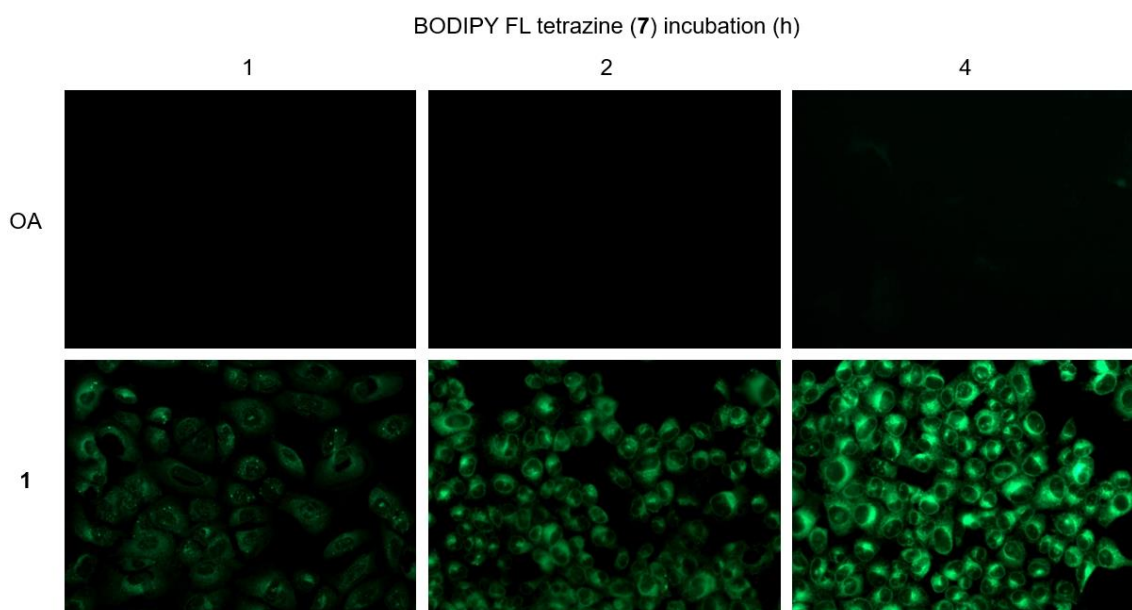


Figure S6: Sterculic acid reacts over time with BODIPY-tetrazine in live cells. Live-cell images of U2OS cells incubated with oleic acid (OA, 50 μM , negative control) or **1** (50 μM) for 1 h, washed and incubated with of BODIPY FL tetrazine **7** (10 μM) for indicated time before washing and imaging.

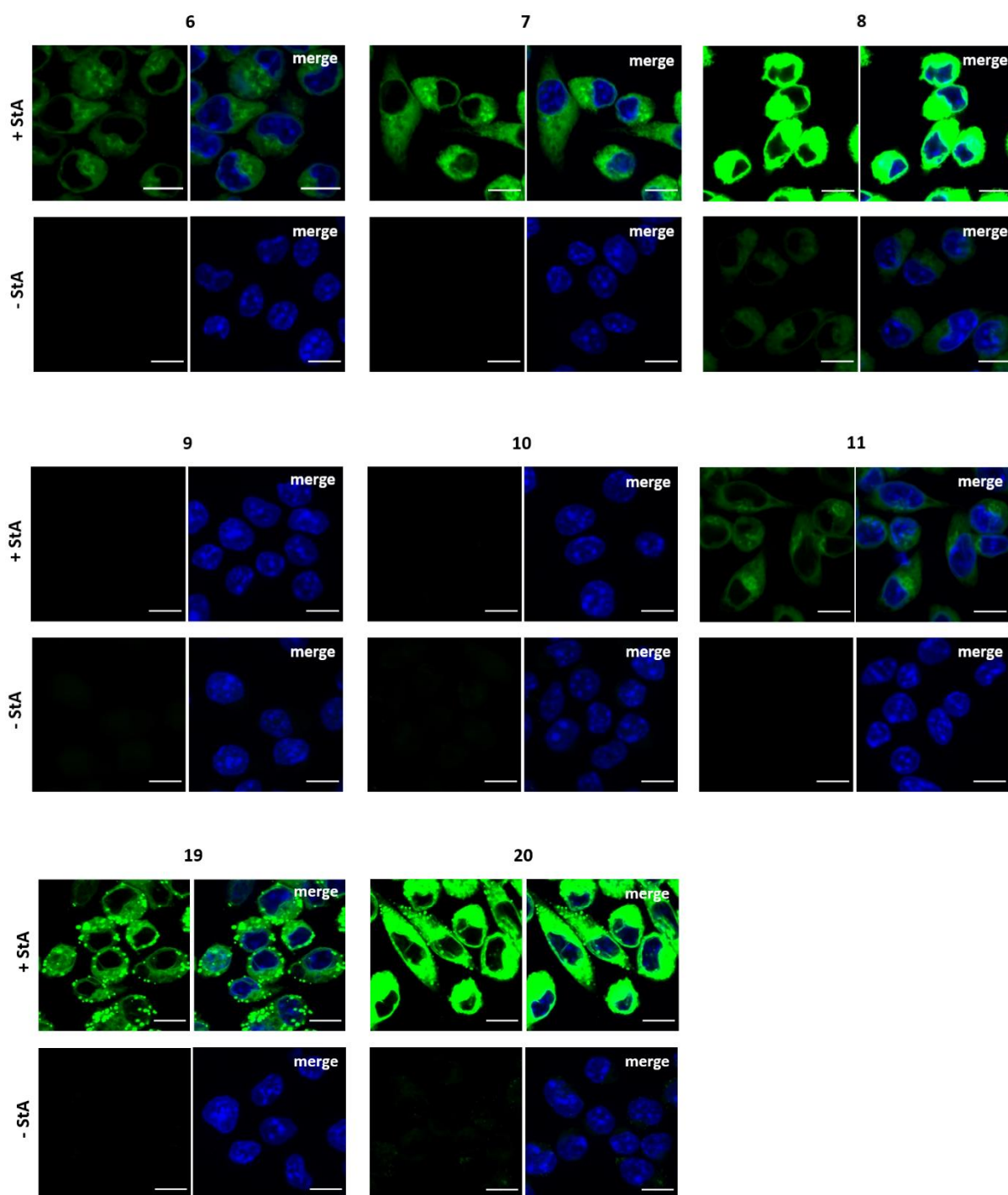


Figure S7: Confocal fixed-cell imaging of tetrazine-fluorophore conjugate library (6-11 and 19-20). DC2.4 cells were incubated with stercularic acid (+StA, 50 μ M) or no probe (-StA) for 1 h, fixed and permeabilised, followed by click reaction with the respective fluorophore (all 5 μ M) for 1 h. Images are presented as maximum intensity projections of z-stacks. All samples were routinely washed after metabolic incorporation of **1** and after ligation with the fluorophores, and were imaged with minimum 4 technological replicates. DNA was counterstained with Hoechst 33342. All scale bars represent 10 μ m.

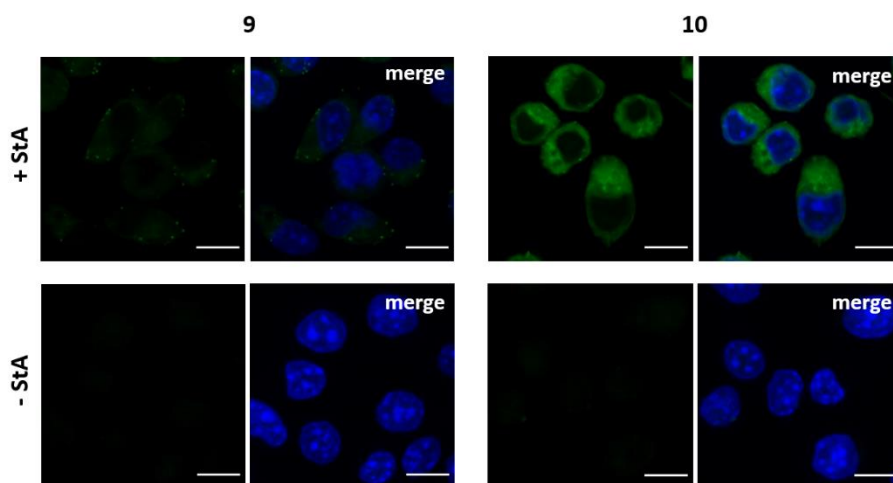


Figure S8: Confocal fixed-cell imaging of fluorophores **9-10** with higher laser power. DC2.4 cells were incubated with stercularic acid (+StA, 50 μ M) or no probe (-StA) for 1 h, fixed and permeabilised, followed by click reaction with the respective fluorophore (5 μ M) for 1 h. Images are presented as maximum intensity projections of z-stacks. All samples were routinely washed after metabolic incorporation of **1** and after ligation with the fluorophores. DNA was counterstained with Hoechst 33342 (blue). All scale bars represent 10 μ m.

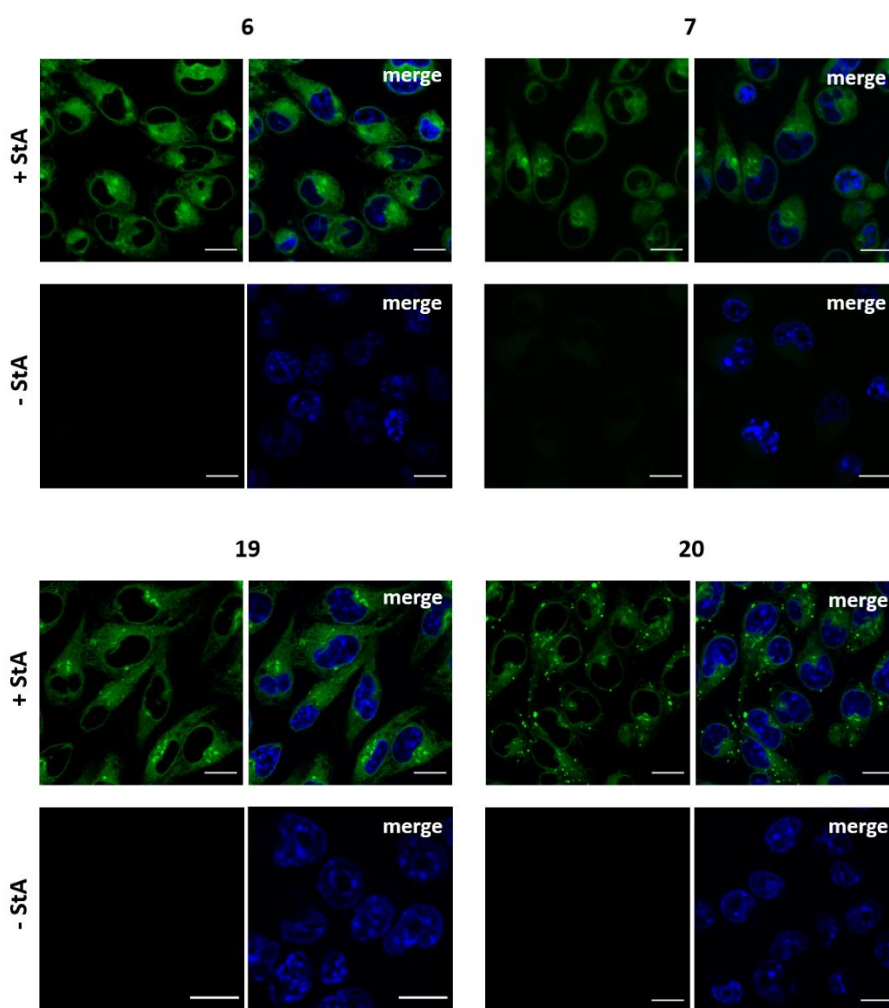


Figure S9: Confocal live-cell imaging without washing steps after ligation between **1** and fluorophores **6-7** and **19-20**. DC2.4 cells were incubated with sterculic acid (+StA, 50 μ M) or no probe (-StA) for 1 h, followed by a wash step to remove excess **1**, and click reaction with the respective fluorophore (all 5 μ M) for 1 h. DNA was counterstained with Hoechst 33342, and samples were imaged directly without further wash steps. Images are presented as single slices, and were imaged with minimum 3 technological replicates. Fluorophores **19-20** were imaged at a lower laser intensity than **6-7**. DNA was counterstained with Hoechst 33342 (blue). All scale bars represent 10 μ m.

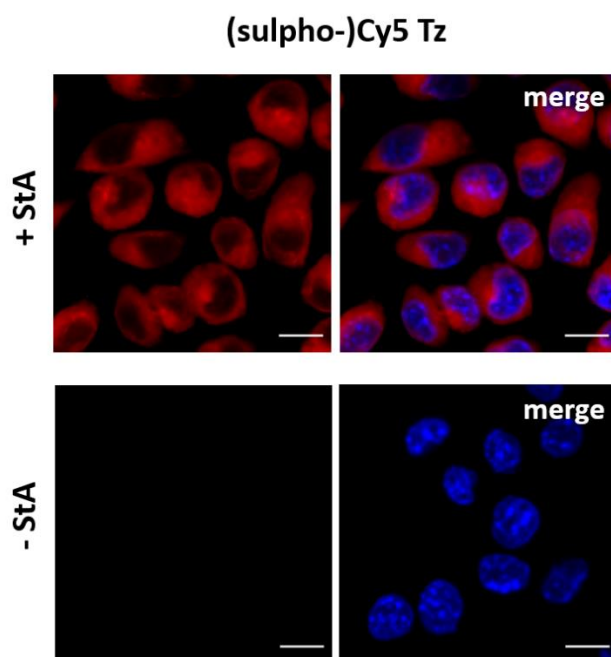
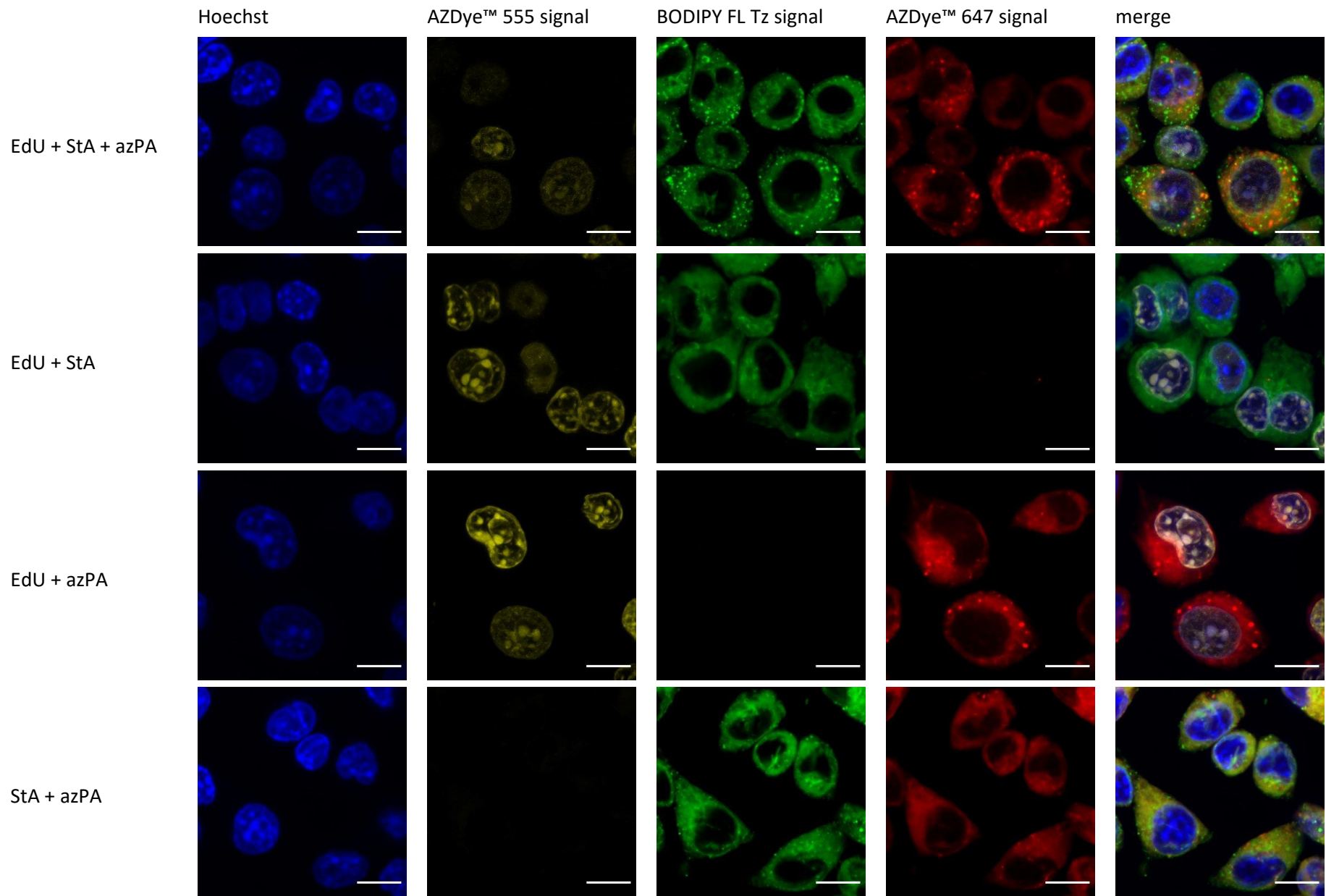


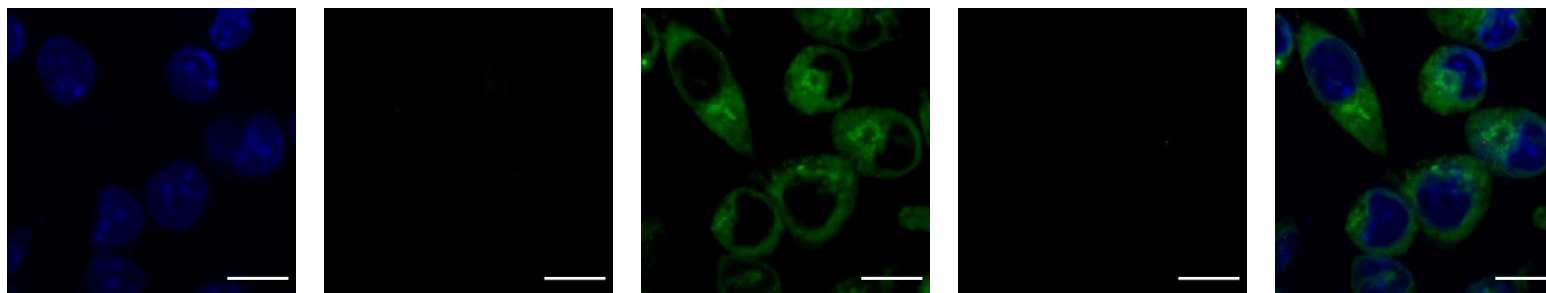
Figure S10: Confocal fixed-cell imaging with (sulpho-)Cy5 tetrazine. DC2.4 cells were incubated with sterculic acid (+StA, 50 μ M) or no probe (-StA) for 1 h, fixed and permeabilised, followed by click reaction with (sulpho-)Cy5 tetrazine (20 μ M) for 1 h. Images are presented as maximum intensity projections of z-stacks. All samples were routinely washed after metabolic incorporation of **1** and after ligation with the fluorophores, and were imaged with 4 technological replicates. DNA was counterstained with Hoechst 33342 (blue). All scale bars represent 10 μ m.



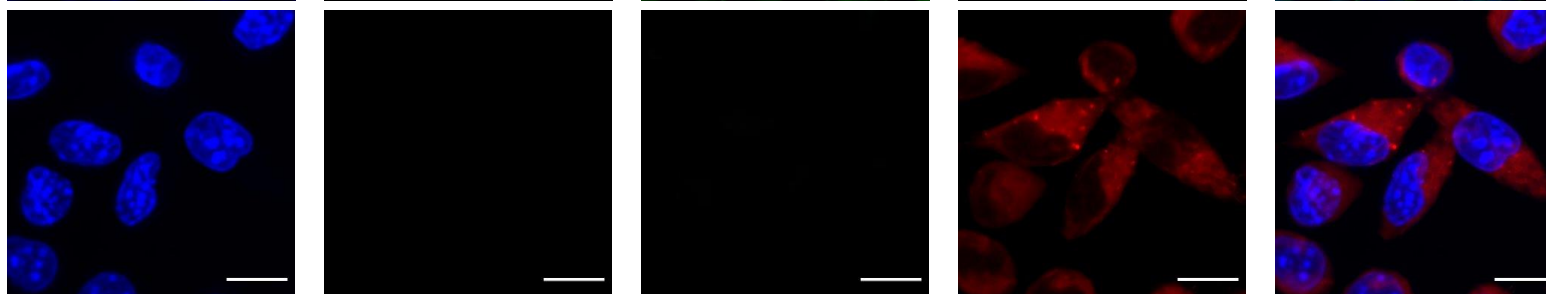
Only EdU



Only StA



Only azPA



No probes



Figure S11: Confocal imaging of triple labelled sample with all respective controls. DC2.4 cells were incubated with EdU (10 μM) for 20 h, followed by sterculic acid (StA, 50 μM) and azido palmitic acid (azPA, 100 μM) for 1 h, or the respective combination of these probes. The cells were then fixed and permeabilised, followed by a triple click strategy. All samples were first clicked with fluorophore **7** (5 μM) for 1 h, followed by two subsequent CuAAC reactions with AZDye™ 555-azide (5 μM) and AZDye™ 647-alkyne (5 μM) for 1 h each. All samples were routinely washed between each metabolic incorporation and between each respective bioorthogonal reaction, and were imaged with 3 technological replicates. Images are presented as maximum intensity projections of z-stacks. DNA was counterstained with Hoechst 33342 (blue) as a reference. All scale bars represent 10 μm .

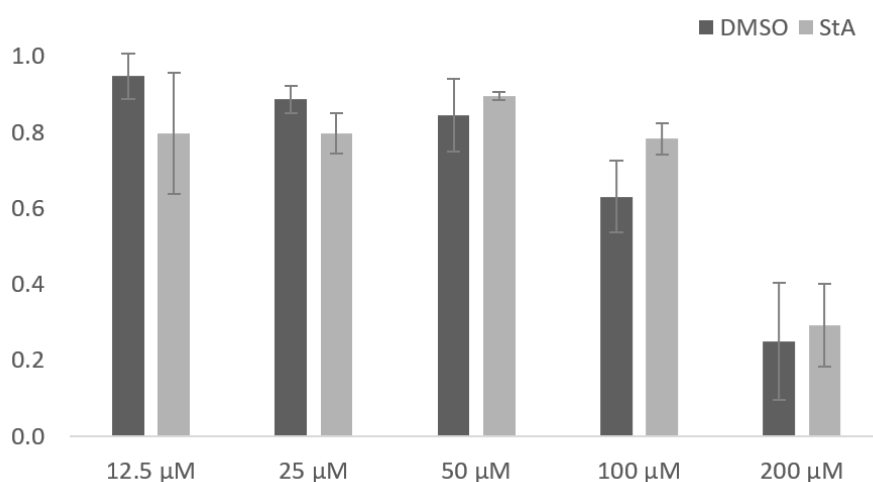


Figure S12: Toxicity of sterculic acid (StA) was assessed by MTT assay. DC2.4 cells were incubated with given concentrations (μM) of StA in DMSO (labelled StA), or the corresponding amount of DMSO (ranging from 0.125-2%) without StA (labelled DMSO) for 24 h. Absorbance was measured at 570 nm. Absorbance values were measured in triplicate and normalised, and standard deviations are indicated.

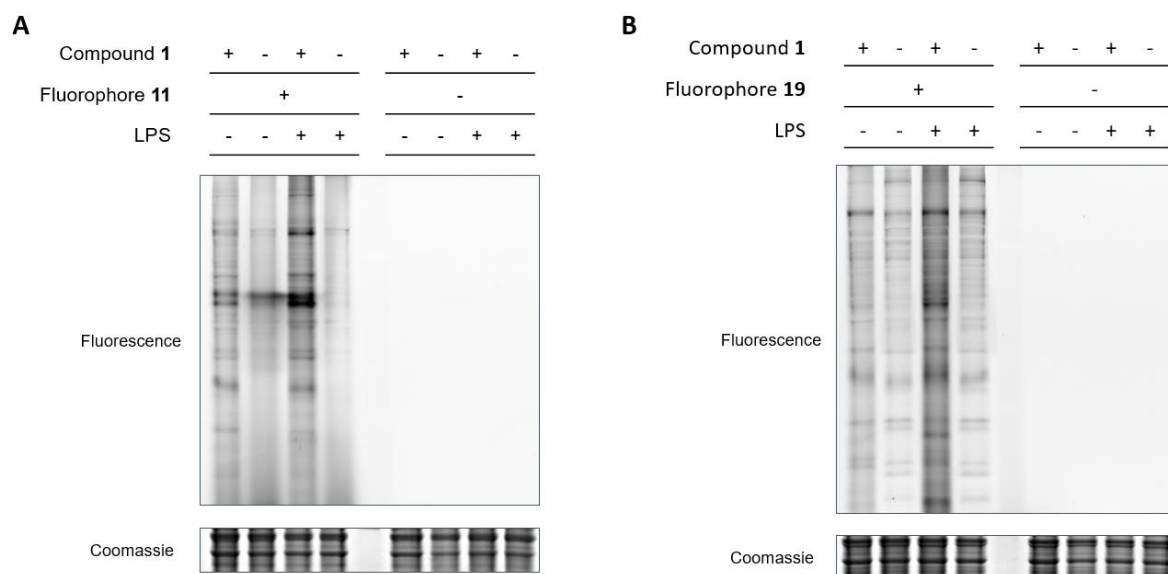


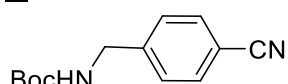
Figure S13: in-gel fluorescence (A-B) of protein oleylation. DC2.4 cells were stimulated with or without LPS (100 ng/mL) for 24 h and treated with **1** (100 μ M) for 20 h. (A) Cells were harvested and lysed before click reaction with fluorophore 11 (10 μ M) or vehicle for 2h at RT. (B) Live cells were subjected to click reaction with fluorophore 19 (10 μ M) or vehicle for 2 h at 37 $^{\circ}$ C, 5% CO₂, before being harvested and lysed. All lysates were analysed by SDS-PAGE and in-gel fluorescence was measured. Coomassie staining served as a protein loading control.

Materials & methods

Chemical synthesis.

All commercially available reagents and solvents were used as received unless stated otherwise. Reaction progress was determined via TLC (Sigma, TLC Silica gel 60 F254) via UV visualisation ($\lambda = 254$ nm) and ninhydrin stain. All column chromatography purifications were performed using solvents as received and silica gel (Macherey-Nagel, Kieselgel 60 M, 0.04 – 0.63 mm). LC-MS analysis was performed on a Finnigan Surveyor HPLC system (detection at 200-600 nm) with an analytical C18 column (Gemini, 50 x 4.6 mm, 3 μ m particle size, Phenomenex) coupled to a Finnigan LCQ Advantage MAX ion-trap mass spectrometer (ESI+). Solvent system: A: water (gradient, 10-90%), B: CH₃CN (gradient, 90-10%), C: (constant, 10%) 1% TFA (aq). High resolution mass spectrometry (HRMS) spectra were recorded by direct injection (2 μ L of a 1 μ M solution in CH₃CN/H₂O/tBuOH) on a Thermo Scientific Q Exactive HF Orbitrap mass spectrometer equipped with an electrospray ion source in positive-ion mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 275 °C) with resolution $R = 240,000$ at m/z 400 (mass range m/z 160 – 2000) correlated to an external calibration (Thermo Finnigan). ¹H and ¹³C NMR spectra were recorded using a Brüker AV-400 (400/101 MHz). Recorded data was interpreted and analysed using MestReNova software.

2.

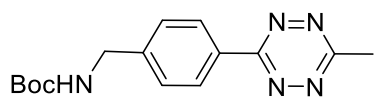


(4-Aminomethyl)benzonitrile (1.19 g, 10 mmol) was dissolved in 20 mL DCM. Boc₂O (2.4 g, 11 mmol) and DMAP (0.244 g, 0.2 mmol) were added and the reaction was stirred overnight. After completion on TLC, the reaction mixture was concentrated under reduced pressure. The crude product was purified by column chromatography (25% EtOAc in PE) and **9** is isolated as white crystals in 76% yield (1.76 g, 7.6 mmol).

¹H NMR (300 MHz, CDCl₃) δ 7.64 – 7.58 (m, 2H), 7.39 (dq, $J = 7.5, 0.7$ Hz, 2H), 5.16 (s, 1H), 4.36 (d, $J = 6.3$ Hz, 2H), 1.46 (s, 9H).

¹³C NMR (75 MHz, CDCl₃) δ 155.93 (s), 144.76, 132.35 (s), 127.76 (s), 118.79 (s), 110.98 (s), 85.20 (s), 79.96 (s), 44.14 (s), 28.33 (s), 27.38 (s).

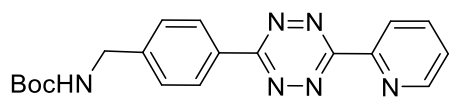
3.



2 (0.464 g, 2 mmol) was dissolved in acetonitrile (0.522 mL, 10 mmol) in a pressure tube. Zn(OTf)₂ (182 mg, 0.50 mmol) and hydrazine monohydrate (4.84 mL, 100 mmol) were added, pressure tube was capped and reaction mixture was stirred overnight at 80 °C. The reaction was cooled to room temperature and the rubber seal was carefully punctured, slowly releasing the generated NH₃ gas. The reaction mixture was slowly added to 40 mL of AcOH/DCM (1/1) and NaNO₂ (2.76 g, 40 mmol) was added slowly to the reaction. When no more gas formation was observed, the reaction mixture was concentrated and redissolved into EtOAc. Reaction mixture was washed with 3x H₂O and 3x saturated NaHCO₃ solution. The organic layer was dried over MgSO₄, filtered and concentrated. The product was purified by column chromatography (2 -> 10% EtOAc in DCM) and isolated as a pink solid in 17% yield (102 mg, 0.34 mmol).

¹H NMR (300 MHz, CDCl₃) δ 8.53 – 8.43 (m, 2H), 7.50 – 7.44 (m, 2H), 5.35 (t, $J = 6.4$ Hz, 1H), 4.42 (d, $J = 6.2$ Hz, 2H), 3.08 (s, 3H), 1.48 (s, 9H).

¹³C NMR (75 MHz, CDCl₃) δ 167.13 (s), 163.79 (s), 156.01 (s), 144.08 (s), 130.62 (s), 128.04 (s), 127.94 (s), 79.66 (s), 44.28 (s), 28.39 (s), 21.10 (s).

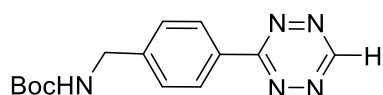
4.

2 (0.232 g, 1.0 mmol) was dissolved in 2-cyanopyridine (0.481 mL, 5.0 mmol) in a pressure tube. Zn(OTf)₂ (91 mg, 0.25 mmol) and hydrazine monohydrate (2.42 mL, 50 mmol) were added,

the pressure tube was capped and reaction mixture was stirred overnight at 80 °C. The reaction was cooled to room temperature and the rubber seal was carefully punctured, slowly releasing the generated NH₃ gas. The reaction mixture was slowly added to 20 mL of AcOH/DCM (1/1) and NaNO₂ (1.38 g, 20 mmol) was added slowly to the reaction. When no more gas formation was observed, the reaction mixture was concentrated and redissolved into EtOAc. Reaction mixture was washed with 3x H₂O and 3x saturated NaHCO₃ solution. The organic layer was dried over MgSO₄, filtered and concentrated. The product was purified by column chromatography (2 → 30% EtOAc in DCM) and isolated as a pink solid in 41% yield (148.0 mg, 0.41 mmol).

¹H NMR (300 MHz, CDCl₃) δ 8.96 (dt, *J* = 4.8, 1.4 Hz, 1H), 8.66 (dt, *J* = 7.9, 1.1 Hz, 1H), 8.64 – 8.56 (m, 2H), 7.99 (td, *J* = 7.8, 1.8 Hz, 1H), 7.56 (ddd, *J* = 7.6, 4.8, 1.2 Hz, 1H), 7.53 – 7.47 (m, 2H), 5.36 (t, *J* = 6.3 Hz, 1H), 4.45 (d, *J* = 6.2 Hz, 2H), 1.48 (s, 9H).

¹³C NMR (75 MHz, CDCl₃) δ 164.09 (s), 163.31 (s), 156.03 (s), 150.87 (s), 150.23 (s), 144.71 (s), 137.42 (s), 130.36 (s), 128.58 (s), 128.04 (s), 126.30 (s), 123.86(s), 79.69(s), 44.32 (s), 28.39 (s).

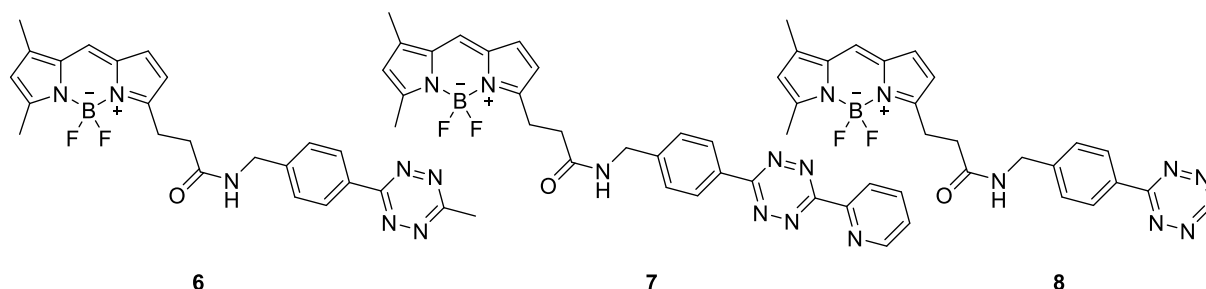
5.

2 (0.232 g, 1 mmol) was added in a pressure tube. Zn(OTf)₂ (91 mg, 0.25 mmol), formamidine acetate (1040 mg, 10 mmol) and hydrazine monohydrate (2.42 mL, 50 mmol) were added, the

pressure tube was capped and reaction mixture was stirred overnight at 60 °C. The reaction was cooled to room temperature and the rubber seal was carefully punctured, slowly releasing the generated NH₃ gas. The reaction mixture was slowly added to 20 mL of AcOH/DCM (1/1) and NaNO₂ (1.38 g, 20 mmol) was added slowly to the reaction. When no more gas formation was observed, the reaction mixture was concentrated and redissolved into EtOAc. Reaction mixture was washed with 3x H₂O and 3x saturated NaHCO₃ solution. The organic layer was dried over MgSO₄, filtered and concentrated. The product was purified by column chromatography (1 → 5% EtOAc in DCM) and isolated as a pink solid in 6% yield (18.2 mg, 6.34 mmol).

¹H NMR (300 MHz, CDCl₃) δ 10.21 (s, 1H), 8.56 (d, *J* = 7.9 Hz, 2H), 7.51 (d, *J* = 7.9 Hz, 2H), 5.20 (s, 1H), 4.44 (d, *J* = 6.2 Hz, 2H), 1.48 (s, 9H).

¹³C NMR (75 MHz, CDCl₃) δ 166.24 (s), 157.76 (s), 155.98 (s), 144.74 (s), 130.49(s), 128.52 (s), 128.09 (s), 79.81 (s), 44.32 (s), 28.39 (s).

General synthesis BODIPY FL-Tz (6-8)

BODIPY-FL-NHS (1.1 mg, 2.83 μmol) was dissolved in 0.2 mL DCM. 1.5 eq. of the respective tetrazine and 2 eq. DIPEA was added and the reaction mixture was stirred for 1 h at room temperature. After complete consumption of the BODIPY-FL-NHS on TLC, the reaction mixture was concentrated and

purified by HPLC. All fractions containing the product were pooled and lyophilised.

6.

6 was isolated as an orange powder in 15% yield (0.20 mg, 0.42 μmol)

^1H NMR (500 MHz, CDCl_3) δ 8.49 – 8.44 (m, 2H), 7.37 – 7.32 (m, 2H), 7.12 (s, 1H), 6.93 – 6.89 (m, 1H), 6.33 (d, $J = 4.0$ Hz, 1H), 6.10 (s, 1H), 4.52 (d, $J = 5.9$ Hz, 2H), 3.34 (t, $J = 7.3$ Hz, 2H), 3.11 (s, 3H), 2.79 (t, $J = 7.3$ Hz, 2H), 2.55 (s, 3H), 2.25 (s, 3H).

HRMS: calculated for $\text{C}_{24}\text{H}_{24}\text{BF}_2\text{N}_7\text{O}$ $[\text{M}+\text{H}]^+$ 476.21035; found 476.21765.

7.

7 was isolated as an orange powder in 19% yield (0.29 mg, 0.54 μmol)

^1H NMR (500 MHz, CDCl_3) δ 9.01 (ddd, $J = 4.8, 1.8, 0.9$ Hz, 1H), 8.73 (dt, $J = 8.0, 1.0$ Hz, 1H), 8.58 – 8.54 (m, 2H), 8.05 (td, $J = 7.8, 1.8$ Hz, 1H), 7.62 (ddd, $J = 7.7, 4.8, 1.2$ Hz, 1H), 7.38 – 7.34 (m, 2H), 7.12 (s, 1H), 6.91 (d, $J = 4.0$ Hz, 1H), 6.34 (d, $J = 4.0$ Hz, 1H), 6.09 (s, 1H), 4.53 (d, $J = 5.9$ Hz, 2H), 3.34 (t, $J = 7.3$ Hz, 2H), 2.82 (t, $J = 7.3$ Hz, 2H), 2.55 (s, 3H), 2.25 (s, 3H).

HRMS: calculated for $\text{C}_{28}\text{H}_{25}\text{BF}_2\text{N}_8\text{O}$ $[\text{M}+\text{H}]^+$ 539.22124; found 539.22859.

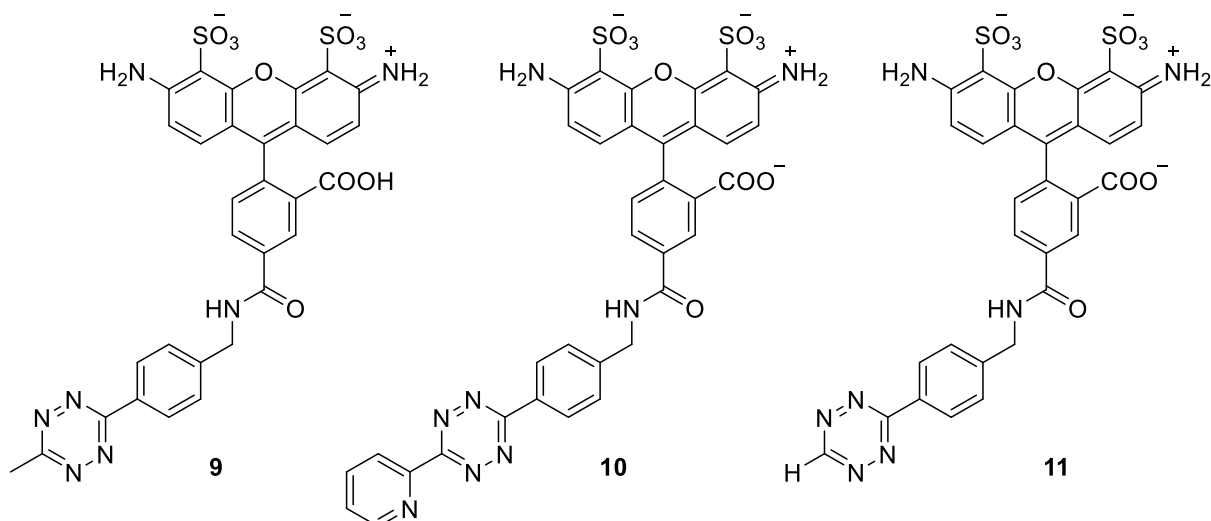
8.

8 was isolated as an orange powder in 17% yield (0.23 mg, 0.51 μmol)

^1H NMR (500 MHz, CDCl_3) δ 10.22 (s, 1H), 8.53 – 8.47 (m, 2H), 7.38 – 7.32 (m, 2H), 7.12 (s, 1H), 6.91 (d, $J = 4.0$ Hz, 1H), 6.33 (d, $J = 4.0$ Hz, 1H), 6.09 (s, 1H), 4.53 (d, $J = 5.9$ Hz, 2H), 3.34 (t, $J = 7.3$ Hz, 2H), 2.80 (t, $J = 7.3$ Hz, 2H), 2.55 (s, 3H), 2.25 (s, 3H).

HRMS: calculated for $\text{C}_{23}\text{H}_{22}\text{BF}_2\text{N}_7\text{O}$ $[\text{M}+\text{H}]^+$ 462.19470; found 462.20199.

General synthesis AF488-Tz (9-11)



AF488-NHS (1 mg, 1.55 μmol) was dissolved in 0.2 mL DCM. 1.5 eq. of the respective tetrazine and 2 eq. DIPEA was added and the reaction mixture was stirred overnight at room temperature. After complete consumption of the AF488 on TLC, the reaction mixture was concentrated and purified by HPLC. All fractions containing the product were pooled and lyophilised.

9.

9 was isolated as an orange powder in 9% yield (0.10 mg, 0.14 μmol)

HRMS: calculated for $\text{C}_{31}\text{H}_{23}\text{N}_7\text{O}_{10}\text{S}_2$ $[\text{M}+\text{H}]^+$ 718.09478; found 718.10161.

10.

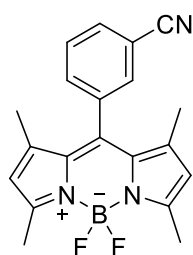
10 was isolated as an orange powder in 26% yield (0.31 mg, 0.4 μmol)

HRMS: calculated for $\text{C}_{35}\text{H}_{24}\text{N}_8\text{O}_{10}\text{S}_2$ $[\text{M}+\text{H}]^+$ 781.10568; found 781.11313.

11.

11 was isolated as an orange powder in 26% yield (0.28 mg, 0.4 μmol)

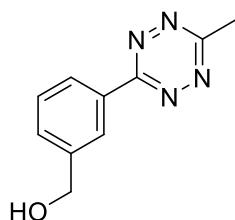
HRMS: calculated for $\text{C}_{30}\text{H}_{21}\text{N}_7\text{O}_{10}\text{S}_2$ $[\text{M}+\text{H}]^+$ 704.07913; found 704.08656

12.

4-Formylbenzonitrile (500 mg, 3.8 mmol) was dissolved in 100 mL DCM. 2,4-dimethylpyrrole (0.85 mL, 8.25 mmol) and TFA (10 μL) were added and reaction mixture was stirred for 30 minutes at room temperature. 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 870 mg, 3.8 mmol) was dissolved in 100 mL DCM was added to the reaction. DIPEA (7.75 mL, 44.5 mmol) and $\text{BF}_3 \cdot \text{OEt}_2$ (7.75 mL) were added and the reaction was left to stir overnight. Afterwards, 100 mL H_2O was added and the aqueous phase was extracted with 3 times 100 mL DCM. The organic layer was dried over MgSO_4 , filtered and concentrated. The product was purified by column chromatography (DCM) was isolated as an orange powder in 38% yield (500 mg, 1.43 mmol).

^1H NMR (400 MHz, CDCl_3) δ 7.81 (dt, $J = 7.8, 1.5$ Hz, 1H), 7.66 (t, $J = 7.8$ Hz, 1H), 7.61 (t, $J = 1.7$ Hz, 1H), 7.54 (dt, $J = 7.8, 1.5$ Hz, 1H), 6.02 (s, 2H), 2.54 (s, 6H), 1.36 (s, 6H).

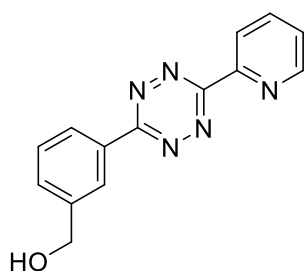
^{13}C NMR (101 MHz, CDCl_3) δ 156.52 (s), 142.60 (s), 136.42 (s), 132.84 (s), 132.82 (s), 131.71 (s), 130.30 (s), 121.91 (s), 117.91 (s), 113.49 (s), 14.70 (s), 14.62 (s).

13.

3-Cyanobenzyl alcohol (133.15 mg, 1 mmol) was dissolved in CH_3CN (0.552 mL, 10 mmol) in a pressure tube. $\text{Zn}(\text{OTf})_2$ (91 mg, 0.25 mmol) and hydrazine monohydrate (2.42 mL, 50 mmol) were added, the pressure tube was capped and reaction mixture was stirred overnight at 60 $^\circ\text{C}$. The reaction was cooled to room temperature and the rubber seal was carefully punctured, slowly releasing the generated NH_3 gas. The reaction mixture was slowly added to 20 mL of AcOH/DCM (1/1) and NaNO_2 (1.38 g, 20 mmol) was added slowly to the reaction. When no more gas formation was observed, the reaction mixture was concentrated and redissolved into EtOAc. Reaction mixture was washed with 3x H_2O and 3x saturated NaHCO_3 solution. The organic layer was dried over MgSO_4 , filtered and concentrated. The product was purified by column chromatography (10 \rightarrow 20% EtOAc in DCM) and isolated as a pink solid in 29% yield (58.4 mg, 0.29 mmol).

^1H NMR (300 MHz, CDCl_3) δ 8.43 (td, $J = 1.8, 0.8$ Hz, 1H), 8.34 (dt, $J = 7.7, 1.6$ Hz, 1H), 7.56 (dt, $J = 7.8, 1.6$ Hz, 1H), 7.47 (t, $J = 7.7$ Hz, 1H), 4.76 (s, 2H), 3.68 (s, 1H), 3.05 (s, 3H).

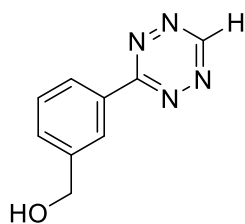
^{13}C NMR (75 MHz, CDCl_3) δ 167.17 (s), 163.82 (s), 142.32 (s), 131.65 (s), 130.95 (s), 129.32 (s), 126.79 (s), 126.05 (s), 64.46 (s), 21.06 (s).

14.

3-Cyanobenzyl alcohol (133.15 mg, 1 mmol) was dissolved in 2-cyanopyridine (0.963 mL, 10 mmol) in a pressure tube. Zn(OTf)₂ (91 mg, 0.25 mmol) and hydrazine monohydrate (2.42 mL, 50 mmol) were added, pressure tube was capped and reaction mixture was stirred overnight at 80 °C. The reaction was cooled to room temperature and the rubber seal was carefully punctured, slowly releasing the generated NH₃ gas. The reaction mixture was slowly added to 20 mL of AcOH/DCM (1/1) and NaNO₂ (1.38 g, 20 mmol) was added slowly to the reaction. When no more gas formation was observed, the reaction mixture was concentrated and redissolved into EtOAc. Reaction mixture was washed with 3x H₂O and 3x saturated NaHCO₃ solution. The organic layer was dried over MgSO₄, filtered and concentrated. The product was purified by column chromatography (20 → 50% EtOAc in DCM) and isolated as a pink solid in 50% yield (132.1 mg, 0.50 mmol).

¹H NMR (300 MHz, CDCl₃) δ 8.97 (ddd, *J* = 4.8, 1.8, 0.9 Hz, 1H), 8.72 – 8.66 (m, 2H), 8.59 (dt, *J* = 7.7, 1.6 Hz, 1H), 8.01 (td, *J* = 7.8, 1.8 Hz, 1H), 7.68 (dt, *J* = 7.7, 1.6 Hz, 1H), 7.63 – 7.54 (m, 2H), 4.89 (s, 2H).

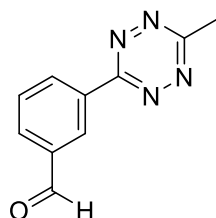
¹³C NMR (75 MHz, CDCl₃) δ 164.31 (s), 163.40 (s), 150.89 (s), 142.48 (s), 137.50 (s), 131.67 (s), 131.51 (s), 129.57 (s), 127.54 (s), 126.69 (s), 126.39 (s), 123.97 (s), 64.80 (s), 29.69 (s).

15.

3-Cyanobenzyl alcohol (133.15 mg, 1 mmol) was added in a pressure tube. Zn(OTf)₂ (91 mg, 0.25 mmol), formamidine acetate (1040 mg, 10 mmol) and hydrazine monohydrate (2.42 mL, 50 mmol) were added, the pressure tube was capped and reaction mixture was stirred overnight at 60 °C. The reaction was cooled to room temperature and the rubber seal was carefully punctured, slowly releasing the generated NH₃ gas. The reaction mixture was slowly added to 20 mL of AcOH/DCM and NaNO₂ (1.38 g, 20 mmol) was added slowly to the reaction. When no more gas formation was observed, the reaction mixture was concentrated and redissolved into EtOAc. Reaction mixture was washed with 3x H₂O and 3x saturated NaHCO₃ solution. The organic layer was dried over MgSO₄, filtered and concentrated. The product was purified by column chromatography (10 → 30% EtOAc in DCM) and isolated as a pink solid in 3% yield (4.8 mg, 0.026 mmol).

¹H NMR (400 MHz, CDCl₃) δ 10.21 (s, 1H), 8.61 – 8.53 (m, 1H), 8.47 (dt, *J* = 7.8, 1.6 Hz, 1H), 7.64 (dt, *J* = 7.9, 1.5 Hz, 1H), 7.55 (t, *J* = 7.7 Hz, 1H), 4.81 (s, 2H).

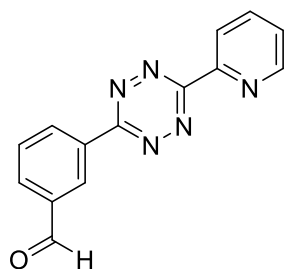
¹³C NMR (101 MHz, CDCl₃) δ 166.39 (s), 157.86 (s), 142.44 (s), 131.71 (s), 131.65 (s), 129.64 (s), 127.43 (s), 126.58 (s), 64.67 (s).

16.

13 (58.4 mg, 0.29 mmol) was dissolved in DCM. Dess-Martin periodinane (0.32 mmol, 136 mg) was added and reaction mixture was stirred for 2 h at room temperature. The reaction mixture was filtered and concentrated. The crude product was purified by column chromatography (30% EtOAc in PE) and **4** was isolated as pink crystals in 99% yield (120.4 mg, 0.46 mmol).

¹H NMR (400 MHz, CDCl₃) δ 10.15 (s, 1H), 9.05 (dq, *J* = 4.2, 1.9 Hz, 1H), 8.86 – 8.78 (m, 1H), 8.18 – 8.10 (m, 1H), 7.78 (td, *J* = 7.9, 2.8 Hz, 1H), 3.14 (s, 3H).

¹³C NMR (101 MHz, CDCl₃) δ 191.41 (s), 167.84 (s), 163.21 (s), 137.23 (s), 133.20 (s), 132.90 (s), 132.49 (s), 130.08 (s), 21.26 (s).

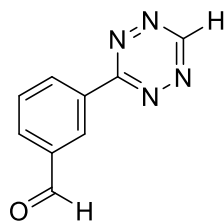
17.

14 (121.1 mg, 0.46 mmol) was dissolved in DCM. Dess-Martin periodinane (0.50 mmol, 213 mg) was added and reaction mixture was stirred for 2 h at room temperature. The reaction mixture was filtered and concentrated. The crude product was purified by column chromatography (50% EtOAc in PE) and **4** was isolated as pink crystals in 99% yield (57.6 mg, 0.29 mmol).

^1H NMR (300 MHz, CDCl_3) δ 10.21 (s, 1H), 9.20 (t, $J = 1.6$ Hz, 1H), 9.04 – 8.93 (m, 2H), 8.73 (dt, $J = 7.9, 1.1$ Hz, 1H), 8.20 (dt, $J = 7.7, 1.4$ Hz, 1H), 8.03 (td, $J = 7.8, 1.8$ Hz, 1H), 7.83 (t, $J = 7.8$ Hz, 1H), 7.60 (ddd, $J = 7.6, 4.8, 1.2$ Hz, 1H).

^{13}C NMR (75 MHz, CDCl_3) δ 191.47 (s), 163.89 (s), 163.75 (s), 151.17 (s), 150.14 (s), 137.6 (s), 133.84 (s), 132.98 (s), 132.82 (s), 130.32 (s), 126.70 (s), 124.30 (s).

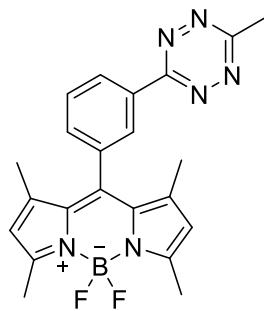
LCMS found: 264.00

18.

15 (4.8 mg, 0.026 mmol) was dissolved in DCM. Dess-Martin periodinane (0.028 mmol, 12 mg) was added and reaction mixture was stirred for 2 h at room temperature. The reaction mixture was filtered and concentrated. The crude product was purified by column chromatography (30% EtOAc in PE) and **4** was isolated as pink crystals in 99% yield (4.8 mg, 0.026 mmol).

^1H NMR (300 MHz, CDCl_3) δ 10.33 (s, 1H), 10.18 (s, 1H), 9.13 (t, $J = 1.8$ Hz, 1H), 8.90 (dt, $J = 7.8, 1.5$ Hz, 1H), 8.20 (dd, $J = 7.7, 1.5$ Hz, 1H), 7.82 (t, $J = 7.7$ Hz, 1H).

^{13}C NMR (75 MHz, CDCl_3) δ 191.25, 165.62, 158.16, 137.32, 133.56, 133.04, 130.21, 130.04.

19.

16 (57.6 mg, 0.29 mmol) was dissolved in 7 mL THF. 2,4-Dimethylpyrrole (37 μL , 0.58 mmol) and TFA (6.5 μL) were added and reaction mixture was stirred overnight at room temperature. The reaction mixture was cooled to 0 $^\circ\text{C}$ and a solution of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 66 mg, 29 mmol) in 4 mL THF was added to the reaction. Reaction mixture was slowly warmed to room temperature and left for 5 h. The reaction mixture was cooled down to 0 $^\circ\text{C}$ and triethylamine (TEA, 404 μL , 2.9 mmol) was added. The reaction mixture was left to stir for 30 minutes at 0 $^\circ\text{C}$ and $\text{BF}_3 \cdot \text{OEt}_2$ (546 μL , 4.35 mmol) was added dropwise at 0 $^\circ\text{C}$. The reaction mixture was slowly warmed to room temperature and left to stir overnight. Afterwards, the reaction was concentrated and dissolved in DCM. The crude mixture was washed with 3x NH_4Cl and 3x brine. The organic layer was dried over MgSO_4 , filtered and concentrated. The product was purified by column chromatography (20% EtOAc in PE) was isolated as an orange powder in 27% yield (32.8 mg, 0.078 mmol).

^1H NMR (400 MHz, CDCl_3) δ 8.75 (dt, $J = 7.9, 1.5$ Hz, 1H), 8.61 (t, $J = 1.7$ Hz, 1H), 7.78 (t, $J = 7.7$ Hz, 1H), 7.60 (dt, $J = 7.6, 1.4$ Hz, 1H), 6.03 (s, 2H), 3.14 (s, 3H), 2.60 (d, $J = 1.2$ Hz, 6H), 1.46 (s, 5H), 1.28 (s, 4H).

HRMS: calculated for $\text{C}_{22}\text{H}_{21}\text{BF}_2\text{N}_6$ [M+H] $^+$ 419.18888; found 419.19602.

20.

17 (120.4 mg, 0.46 mmol) was dissolved in 10 mL THF. 2,4 dimethylpyrrole (58 μ L, 0.92 mmol) and TFA (10 μ L) were added and reaction mixture was stirred overnight at room temperature. The reaction mixture was cooled to 0 °C and a solution of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 104 mg, 46 mmol) in 6 mL THF was added to the reaction. The reaction mixture was slowly warmed to room temperature and left for 5 h. The reaction mixture was cooled down to 0 °C and triethylamine (TEA, 641 μ L, 4.6 mmol) was added. The reaction mixture was left to stir for 30 minutes at 0 °C and $\text{BF}_3 \cdot \text{OEt}_2$ (866 μ L, 6.9 mmol) was added dropwise at 0 °C. The reaction mixture was slowly warmed to room temperature and left to stir overnight. Afterwards, the reaction was concentrated and dissolved in DCM. The crude mixture was washed with 3x sat. aq. NH_4Cl and 3x brine. The organic layer was dried over MgSO_4 , filtered and concentrated. The product was purified by column chromatography (10% EtOAc in DCM) was isolated as an orange powder in 6% yield (12.4 mg, 0.026 mmol).

^1H NMR (500 MHz, CDCl_3) δ 9.00 (ddd, $J = 4.7, 1.8, 0.9$ Hz, 1H), 8.85 (dt, $J = 8.0, 1.4$ Hz, 1H), 8.74 – 8.70 (m, 2H), 8.03 (td, $J = 7.8, 1.8$ Hz, 1H), 7.81 (t, $J = 7.7$ Hz, 1H), 7.65 – 7.58 (m, 2H), 6.04 (s, 2H), 2.60 (s, 6H), 1.48 (s, 6H).

HRMS: calculated for $\text{C}_{26}\text{H}_{22}\text{BF}_2\text{N}_7$ $[\text{M}+\text{H}]^+$ 482.19978; found 482.20704.

Photophysical measurements. Samples for absorption measurements were prepared from 10 mM stock solutions of the fluorophores in DMSO. The fluorophores were diluted to 1 μM in either PBS buffer or DMSO/ H_2O (1:1). Absorption was measured using a Genesys 50 UV spectrophotometer using 1 cm quartz cuvettes. Extinction coefficients were calculated using the Lambert-Beer law. Excitation and Emission spectra were measured at a final concentration of 1 μM in PBS. Spectra were measured in a black 96-well plate on a CLARIOstar plate reader (BMG LABTECH).

Stability of fluorophores in PBS. The samples for stability measurements were prepared from 10 mM stock solutions of the fluorophores in DMSO. Each fluorophore was diluted to 10 μM in PBS. At each timepoint, a sample from the solution was taken and injected into the LCMS. The peak corresponding with the fluorophore was integrated using the Thermo Xcaliber software. The integral of the peak at 0 min was set as 100%.

Biological experiments.

Mammalian cell culture. DC2.4 cells (kindly gifted by Ken Rock) were cultured in RPMI 1640 culture medium (Thermo Fisher Scientific) supplemented with 10% FCS, GlutaMAX (2 mM), sodium pyruvate (1 mM), 1x non-essential amino acids (NEAA, Thermo Fisher Scientific), penicillin (100 I.U./mL), streptomycin (50 $\mu\text{g}/\text{mL}$), and 2-mercaptoethanol (50 μM , Thermo Fisher Scientific), and incubated at 37 °C, 5% CO_2 . The cells were grown to 70-80% confluency and passaged every 2-3 days by trypsinisation.

U2OS cells were cultured at 37 °C under 7% CO_2 in DMEM (Sigma Aldrich, D1145) containing stable glutamine, 10% (v/v) New Born Calf Serum (Thermo Fisher Scientific) and penicillin and streptomycin (200 $\mu\text{g}/\text{mL}$ each, Duchefa). Cells were passaged twice a week at 80-90% confluence by trypsinisation and resuspension in fresh medium. Cell lines were purchased from ATCC and were tested regularly for mycoplasma contamination. Cultures were discarded after 2-3 months of use.

Measuring turn-on of the tetrazine-fluorophore conjugates. Dilutions of the fluorophores **6-11** and **19-20** (2 μM) and sterculic acid (10 μM , Cayman Chemical) were prepared in three solvents; PBS, DMSO/ H_2O (1:1, v/v), and phenol red-free RPMI 1640 culture medium (Thermo Fisher Scientific) supplemented with 10% FCS, GlutaMAX (2 mM), pyruvate (1 mM) and 1x NEAA. The solvents were

analysed one at a time by adding 50 μL of the fluorophore dilutions in triplicate in a black 96-well plate, followed by addition of 50 μL sterculic acid dilution or 50 μL only solvent (negative control). This gives final concentrations of 1 μM fluorophore, and 5 μM sterculic acid. The plate was immediately scanned for fluorescence on a CLARIOstar plate reader (BMG LABTECH) with excitation/emission at 477-14/530-40 and dichroic filter 497. Fluorescence was measured once every 60 sec for 120 min. The results were processed and plotted in GraphPad Prism version 9.3.1 showing the turn-on ratio between the sample and negative control, as an average of the triplicated samples and with indicated standard deviations.

Reaction kinetics between tetrazine-fluorophore conjugates and sterculic acid. To calculate the second-order rate constants (k_2) for the reaction between tetrazines **6-11** and **19-20** and **1**, the data assembled for the turn-on measurements (described earlier) were used and a previously published procedure was followed.^[1] After having subtracted background fluorescence values from the data, each curve was capped at its initial plateau, after the exponential phase, to prevent photobleaching from affecting the calculations. Each replicate ($n=3$) was processed separately for the whole analysis. Since the experiment was performed under pseudo first-order conditions, the pseudo first-order rate constant k_1' was determined by the use of the 'One phase decay' regression in GraphPad Prism version 9.3.1 on the data points. The second-order rate constant was then calculated as $k_2 = k_1'/c_{\text{sterculic acid}}$, and standard deviations were calculated for the replicates.

Preparation of live-cell samples. For microscopy, 6×10^5 DC2.4 cells were seeded per well on an 8-well chamber slide (Ibidi) and allowed to attach for 2 h. The cells were incubated with sterculic acid (50 μM) or no probe (negative control) for 1 h in complete medium (as described above) with 0.1% fatty acid-free BSA (Sigma Aldrich) instead of FCS. Cells were washed with fresh medium x3, followed by incubation with the respective fluorophores from our tetrazine library (5 μM) in complete medium for 1 h. For Figures 3A and S2, the cells were washed with fresh medium x3 after fluorophore ligation to remove unreacted probe, and DNA was counterstained with Hoechst 33342 (5 $\mu\text{g}/\text{mL}$, Sigma Aldrich) in PBS for 5-10 min followed by washing with PBS x3. For Figure S7, labelling medium was aspirated and DNA was immediately counterstained with Hoechst 33342 (5 $\mu\text{g}/\text{mL}$, Sigma Aldrich) in PBS for 5-10 min without washing steps. Each incubation step was done at 37 $^\circ\text{C}$, 5% CO_2 . All samples were directly imaged in phenol red-free DMEM (Sigma Aldrich) supplemented with GlutaMAX (2 mM), sodium pyruvate (1 mM), penicillin (100 I.U./mL), and streptomycin (50 $\mu\text{g}/\text{mL}$), by confocal imaging (see below).

2.0×10^4 U2OS cells were plated in 12-well plates 24 h prior to microscopy experiments. Cells were treated with 600 μL of 50 μM oleic acid (negative control) or sterculic acid in phenol red free DMEM with serum for 1 h at 37 $^\circ\text{C}$ under 7% CO_2 . The medium was aspirated, cells were washed twice with PBS and treated with indicated concentrations of BODIPY FL tetrazine **7** in phenol red-free DMEM with serum for the indicated incubation time at 37 $^\circ\text{C}$ under 7% CO_2 . Medium was aspirated, cells were washed three times with PBS and 600 μL of PBS was added for imaging, which was done using a EVOS M7000 Imaging System (Thermo Fisher Scientific).

Preparation of fixed-cell samples. The same protocol was followed as for live-cell imaging (described above) until the point of incubation with sterculic acid, after which the cells were fixed with 4% paraformaldehyde (PFA) in PBS for 30 min at room temperature. After fixation the cells were washed with PBS followed by glycine (20 mM) in PBS to quench unreacted aldehyde. The cells were permeabilised with 0.01% saponin in PBS for 20 min and washed with PBS x2. The permeabilised sample was incubated with the tetrazine-fluorophore conjugate library (**6-11** and **19-20**, 5 μM) or (sulpho-)Cy5 tetrazine (Click Chemistry Tools, 20 μM) in PBS for 1 h, followed by washing with PBS x2 and blocking with 1% BSA in PBS for 30 min. DNA was counterstained with Hoechst 33342 (2 $\mu\text{g}/\text{mL}$) in PBS for 5-10 min. The incubation steps were done at room temperature and separated by

intermediate washing steps with PBS. The cells were then imaged directly in glycerol/DABCO mounting medium to minimise photo bleaching.

Preparation of triple-labelled sample. For microscopy, 6×10^5 DC2.4 cells were seeded per well on an 8-well chamber slide (Ibidi) and allowed to attach for 3 h. The triple metabolic labelling started with incubating the cells with 5-ethynyl-2'-deoxyuridine (EdU, 10 μ M, Sigma Aldrich) in complete medium for 20 h, followed by washing with PBS x2. Then a metabolic label cocktail containing sterculic acid (50 μ M) and azido palmitic acid (100 μ M, Click Chemistry Tools) in medium with 0.1% fatty acid-free BSA (Sigma Aldrich) instead of FCS, was added to the sample and it was incubated for 1 h. The sample was washed with fresh medium x1 and PBS x1, and fixed in 2% PFA in 0.1M phosphate buffer pH 7.2 at room temperature overnight. Fixation solution was aspirated and sample was washed with PBS and glycine (20 mM) in PBS, and permeabilised with 0.01% saponin in PBS for 20 min. The sample was first reacted with fluorophore **7** (5 μ M) in PBS for 1h, followed by AZDye™ 555-azide (5 μ M, Click Chemistry Tools) in copper-click cocktail (1 mM CuSO_4 , 10 mM sodium ascorbate, 1 mM THPTA ligand, 10 mM amino-guanidine, 0.1 M HEPES pH 7.2) for 1 h and finally AZDye™ 647-alkyne (5 μ M, Click Chemistry Tools) in copper-click cocktail for 1 h. All bioorthogonal reactions were performed with intermediate washing steps with PBS x2, and after the reactions unreacted fluorophores were blocked with 1% BSA in PBS for 30 min. DNA was counterstained with Hoechst 33342 (2 μ g/mL) in PBS for 5-10 min and the sample was imaged directly in glycerol/DABCO mounting medium.

Confocal microscopy. All slides were imaged on an AR1 HD25 confocal microscope (Nikon), equipped with a Ti2-E inverted microscope, LU-NV Series laser unit, and CFI Plan Apo Lambda 100x/1.45 oil objective. Hoechst, the tetrazine-fluorophore conjugates, AZDye™ 555, and AZDye™ 647 and (sulpho-) Cy5 tetrazine were excited using the 405 nm, 488 nm, 561 nm, and 647 nm laser lines, respectively, and images were acquired using the Resonant scanner and DU4 detector (495LP (450/50), 560LP (525/50), 640LP (595/50)). Poisson noise was immediately removed from the images by the built-in Nikon Denoise.ai software. Z-stacks were acquired from the fixed-cell and triple-labelled samples with 0.20 micron steps and are all presented as maximum intensity projections. Brightness and contrast were adjusted for all samples using ImageJ. Brightness and contrast were adjusted identically for samples that are directly compared to each other to make sure the relative intensity between the samples remain the same.

Assessing the cytotoxicity of sterculic acid. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to ensure the concentration of sterculic acid used in the following experiments was not cytotoxic. In a 96-well plate (Sarstedt), 1×10^5 DC2.4 cells were seeded per well and allowed to attach for 2 h. Serial dilutions (1:2) of sterculic acid (stock concentration 10 mM in DMSO) in complete medium, in the range of 12.5-200 μ M, were prepared. These were added in triplicate, together with the appropriate controls and blanks and incubated for 24 h. The cells were spun down (300 g, 5 min) and the culture medium was replaced with complete medium containing MTT (0.5 mg/mL). The plate was incubated for 3 h and spun down at (300 g, 5 min) before the medium was aspirated. The formed formazan crystals were dissolved in DMSO and the plate was incubated for 2 min with 400 rpm shaking. All incubation steps were done at 37 °C, 5% CO_2 . Absorbance was measured at 570 nm on a CLARIOstar plate reader (BMG LABTECH). The data were normalised against cells that had no sterculic acid added, and the normalised standard deviations are given.

SDS-PAGE analysis of protein lipidation with sterculic acid. 3.4×10^5 cells were seeded per well in a 6-well plate (Sarstedt) and allowed to attach for 20 h at 37 °C, 5% CO_2 . Half the samples were stimulated with LPS-EB Ultrapure (100 mg/mL, InvivoGen) in fresh medium, while the rest got refreshed medium. All dishes were incubated 24 h at 37 °C, 5% CO_2 , and washed with medium x2. Sterculic acid (100 μ M) or DMSO vehicle (0.1%) in fresh medium were added to the dishes and incubated for 20 h at 37 °C, 5%

CO₂. Medium was aspirated and all cells were washed with PBS x2, harvested by scraping in ice-cold PBS, and centrifuged at 1000 *g*, 5 min. Supernatant was aspirated and cell pellets were lysed by resuspension in cold lysis buffer (sucrose (250 mM) and MgCl₂ (1 mM) in PBS supplemented with 1x EDTA-free protease inhibitor (Roche)). Samples were incubated 10 min on ice with occasional vortexing followed by sonication in a bath sonicator (2 x 10 s, 0 °C). Protein concentration was measured by Qubit assay (Invitrogen) and all samples were adjusted to 1 mg/mL in 10 μL lysis buffer. 5 μL of 3x concentrated click-mix (30 μM fluorophore **11** in PBS) were added to the respective samples to give a final fluorophore concentration of 10 μM. Samples were incubated in the dark at RT for 2 h, before addition of 5 μL 4x Laemmli buffer (without 2-mercaptoethanol) and incubation at RT for 20 min to ensure all proteins were completely dissolved. The samples were resolved by SDS-PAGE (12.5% acrylamide gel, ±80 min, 175 V) alongside protein marker (PageRuler Plus, Thermo Fisher Scientific) until the bromophenol blue had just come off the gel. In-gel fluorescence was measured in the AlexaFluor 488- Cy3-, and Cy5-channels on a Chemidoc MP imaging system (Bio-Rad), before subsequent staining with Coomassie, followed by destaining and imaging as a loading control.

Chemical proteomics on StA-treated DC2.4 cells. For the preparation of proteomics samples, 7.5x10⁵ DC2.4 cells were seeded in 6 cm dishes in triplicate and allowed to attach for 20 h at 37 °C, 5% CO₂. Cells were then either treated with LPS-EB Ultrapure (100 ng/mL, InvivoGen) or vehicle (PBS) in fresh medium for 24 h. Cells were then washed with medium twice and sterculic acid (10 μM) or vehicle (0.1% DMSO) was added. After 20 h of incubation, medium was aspirated, cells were washed with DBPS twice and harvested by scraping in ice-cold DPBS. Cells were pelleted by centrifugation (1000 *g*, 5 min), supernatant was aspirated and cell pellets were lysed by resuspension in cold lysis buffer (Sucrose (250 mM) and MgCl₂ (1 mM) in PBS with 2x EDTA-free protease inhibitor (Roche)). Cells were lysed by vortexing followed by sonication (Qsonica Q700 Microplate Sonicator, 2 x 10 s pulses, 10% amplitude). Protein concentration was measured by Qubit assay (Invitrogen) and all samples were adjusted to the lowest protein concentration. To assist membrane solubility, 0.1% Triton X-100 was added to all samples followed by click reaction with biotin-PEG4-tetrazine (200 μM, Conju-Probe) at 37 °C for 4 h. Volume was adjusted to 520 μL with PBS and proteins were precipitated by addition of MeOH (666 μL), CHCl₃ (166 μL) and MilliQ (150 μL), vortexing after each addition. After spinning down (1500 *g*, 10 min) the upper and lower layer were aspirated and the protein pellet was resuspended in MeOH (600 μL) by sonication (Qsonica Q700 Microplate Sonicator, 2 x 10 s pulses, 10% amplitude). The proteins were spun down (20 000 *g*, 5 min) and the supernatant was discarded.

The proteins were redissolved in 500 μL PBS containing 0.5% SDS and 5 mM DTT by heating to 65 °C for 15 minutes, allowed to cool to RT and alkylated by addition of IAA (15 μL, 0.5 M) for 30 min. Excess IAA was quenched with DTT (5 μL, 1 M) and samples were transferred to Eppendorf tubes containing 500 μL PBS and 25 μL prewashed high capacity streptavidin agarose slurry. Samples were agitated (1000 rpm) for 2 h to ensure binding to the beads, which were then spun down (3.000 *g*, 2 min). Supernatant was discarded and beads were washed with PBS containing 0.5% SDS (3X) and PBS (3X). Beads were resuspended in MilliQ, transferred to low-binding tubes, spun down (3000 *g*, 2 min) and supernatant was discarded. The beads were resuspended in digestion buffer (200 μL, 100 mM Tris pH 7.8, 100 mM NaCl, 1 mM CaCl₂, 2% (v/v) acetonitrile, sequencing grade trypsin (Promega, 0.25 μg)) and incubated while shaking overnight (16 h, 37 °C, 1000 rpm). Beads were spun down (3000 *g*, 2 min) and supernatant containing tryptic peptides were transferred to new low-bind tubes. The beads were washed with a 10% formic acid solution (100 μL), which was transferred to the previously isolated peptides. Peptides were desalted using C18 StageTips preconditioned with 50 μL MeOH, 50 μL of 0.5% (v/v) FA in 80% (v/v) acetonitrile/MilliQ (solution B) and 50 μL 0.5% (v/v) FA in MilliQ (solution A) by centrifugation (600 *g*, 2 min). The peptides were washed with solution A (100 μL, 800 *g*, 3 min) and

eluted into new low-binding tubes using solution B (100 μ L, 800 g, 3 min). Samples were concentrated using an Eppendorf speedvac (Eppendorf Concentrator Plus 5301).

Nano-LC-MS settings for pulldown samples. Desalted peptide samples were reconstituted in 35 μ L LC-MS solution (97:3:0.1 H₂O, ACN, FA) containing 10 fmol/ μ L yeast enolase digest (cat. 186002325, Waters) as injection control. Injection amount was titrated using a pooled quality control sample to prevent overloading the nanoLC system and the automatic gain control (AGC) of the QExactive mass spectrometer. The desalted peptides were separated on a UltiMate 3000 RSLCnano system set in a trap-elute configuration with a nanoEase M/Z Symmetry C18 100 Å, 5 μ m, 180 μ m x 20 mm (Waters) trap column for peptide loading/retention and nanoEase M/Z HSS C18 T3 100 Å, 1.8 μ m, 75 μ m x 250 mm (Waters) analytical column for peptide separation. The column was kept at 40 °C in a column oven. Samples were injected on the trap column at a flow rate of 15 μ L/min for 2 min with 99% mobile phase A (0.1% FA in ULC-MS grade water (Biosolve)), 1% mobile phase B (0.1% FA in ULC-MS grade acetonitrile (Biosolve)) eluent. The 85 min LC method, using mobile phase A and mobile phase B controlled by a flow sensor at 0.3 μ L/min with average pressure of 400-500 bar (5500-7000 psi), was programmed as gradient with linear increment to 1% B from 0 to 2 min, 5% B at 5 min, 22% B at 55 min, 40% B at 64 min, 90% B at 65 to 74 min and 1% B at 75 to 85 min. The eluent was introduced by electro-spray ionization (ESI) via the nanoESI source (Thermo) using stainless steel Nano-bore emitters (40 mm, OD 1/32", ES542, Thermo Scientific).

The QExactive HF was operated in positive mode with data dependent acquisition without the use of lock mass, default charge of 2+ and external calibration with LTQ Velos ESI positive ion calibration solution (88323, Pierce, Thermo) every 5 days to less than 2 ppm. The tune file for the survey scan was set to scan range of 350 – 1400 m/z, 120,000 resolution (m/z 200), 1 microscan, automatic gain control (AGC) of 3e6, max injection time of 100 ms, no sheath, aux or sweep gas, spray voltage ranging from 1.7 to 3.0 kV, capillary temp of 250 °C and an S-lens value of 80. For the 10 data dependent MS/MS events the loop count was set to 10 and the general settings were resolution to 15,000, AGC target 1e5, max IT time 50 ms, isolation window of 1.6 m/z, fixed first mass of 120 m/z and normalized collision energy (NCE) of 28 eV. For individual peaks the data dependent settings were 1.00e3 for the minimum AGC target yielding an intensity threshold of 2.0e4 that needs to be reached prior of triggering an MS/MS event. No apex trigger was used, unassigned, +1 and charges >+8 were excluded with peptide match mode preferred, isotope exclusion on and dynamic exclusion of 10 sec.

In between experiments, routine wash and control runs were done by injecting 5 μ L LC-MS solution containing 5 μ L of 10 fmol/ μ L BSA or enolase digest and 1 μ L of 10 fmol/ μ L angiotensin III (Fluka, Thermo)/oxytocin (Merck) to check the performance of the platform on each component (nano-LC, the mass spectrometer (mass calibration/quality of ion selection and fragmentation) and the search engine)

Data processing. Raw files were analyzed with MaxQuant (Version 2.0.1.0). The following changes were made to the standard settings of MaxQuant: Label-free quantification was chosen with an LFQ minimal ratio count of 1. Match between runs and iBAQ quantification was enabled. Searches were performed against a Uniprot database of the *Mus Musculus* proteome (UPID: UP000000589, downloaded April 13th, 2022) including the internal standard (yeast enolase P00924). The "proteingroups.txt" file was used for further analysis in Microsoft Excel and GraphPad Prism 8.1.1. for Windows (GraphPad Software Inc., San Diego, CA). Sterculic acid-enriched proteins were selected by filtering the proteins for detection by 2+ unique peptides, not found in the reverse decoy FASTA file, and a 2.5-fold difference between average LFQ values between Sterculic acid- or vehicle-treated samples with a p-value of <0.05 using a Student's t-test. At most 1 missing LFQ value in the positive controls was allowed. The mass spectrometry proteomics data have been deposited to the

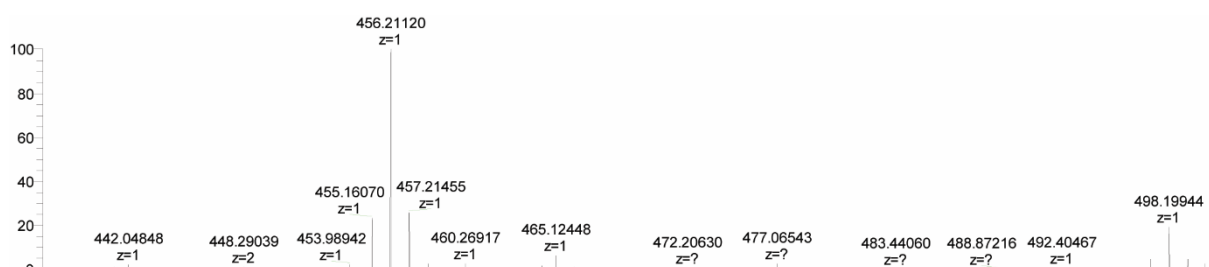
ProteomeXchange Consortium via the PRIDE^[2] partner repository with the dataset identifier PXD033831.

References:

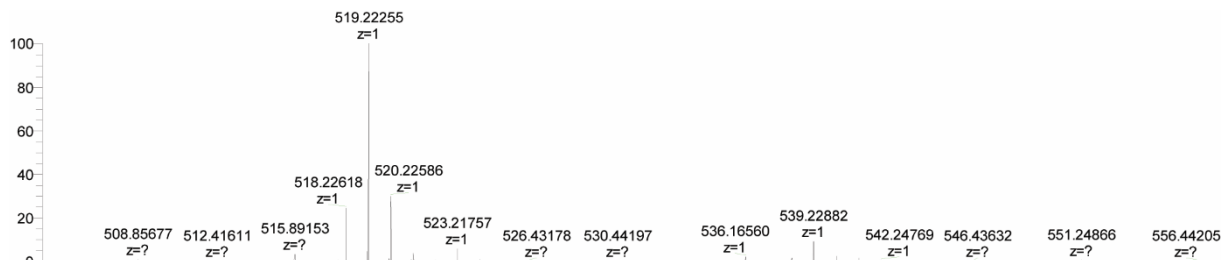
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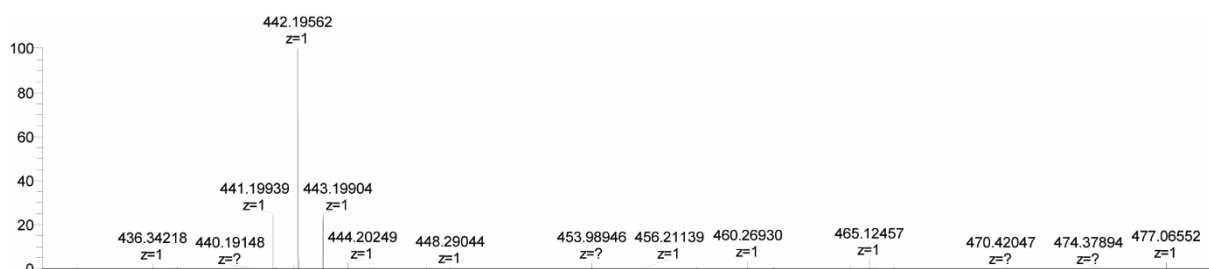
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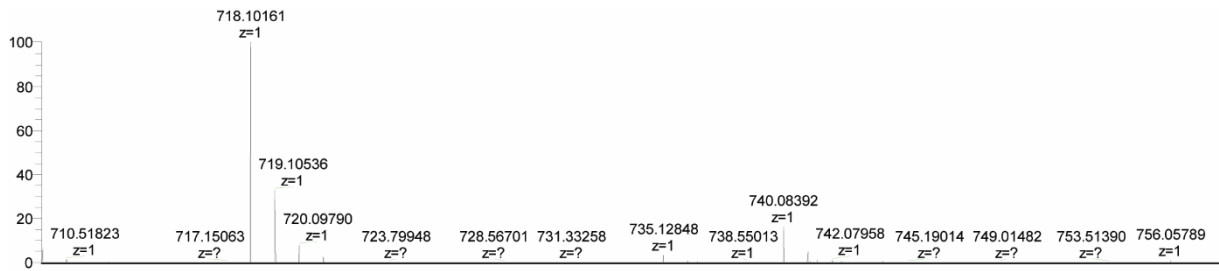
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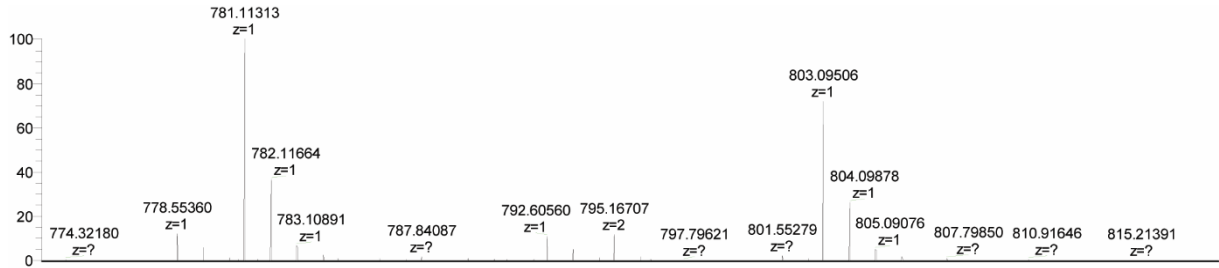
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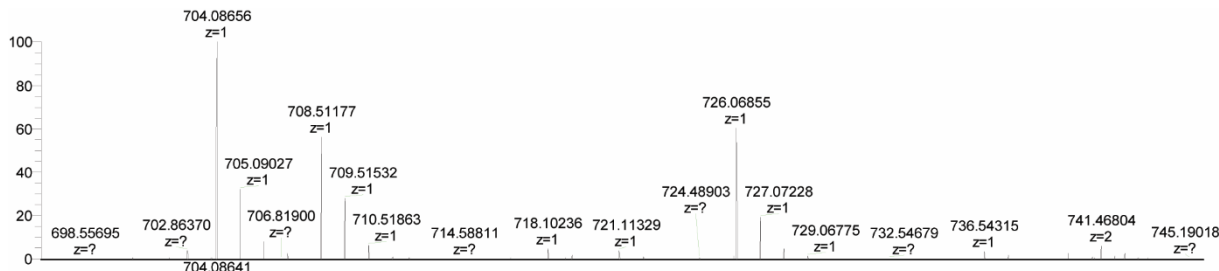
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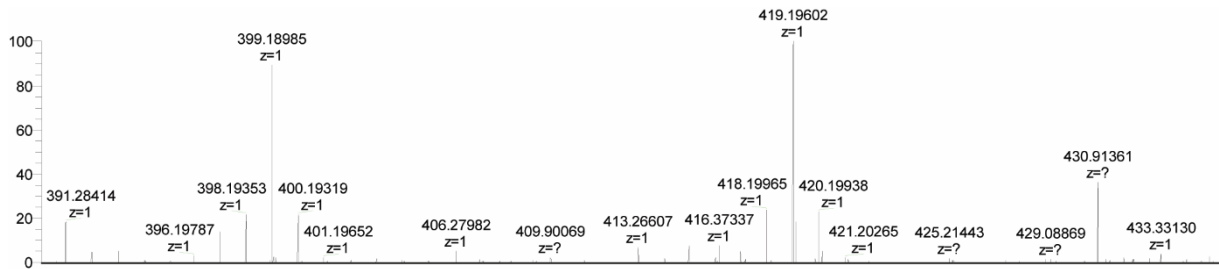
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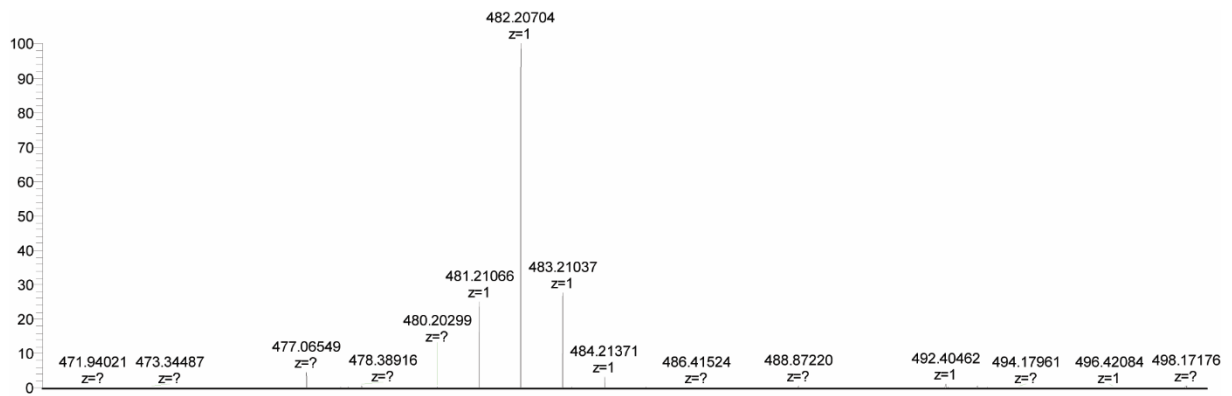
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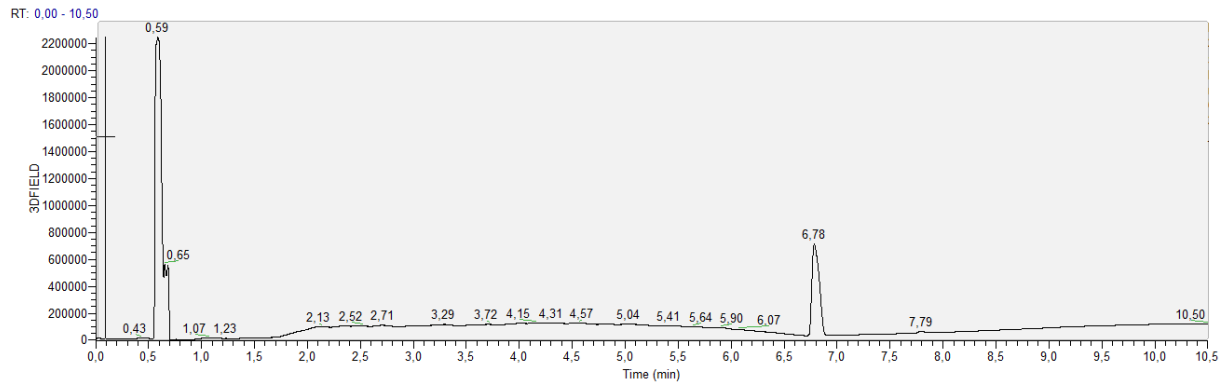


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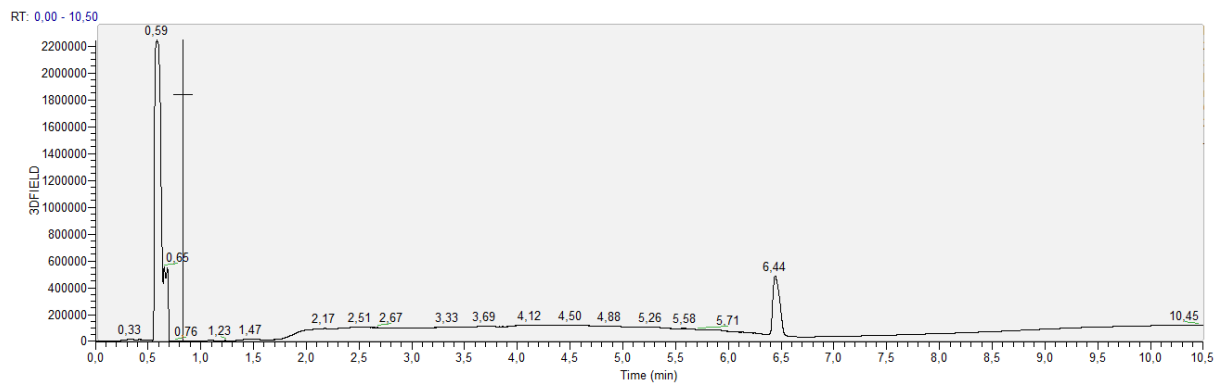


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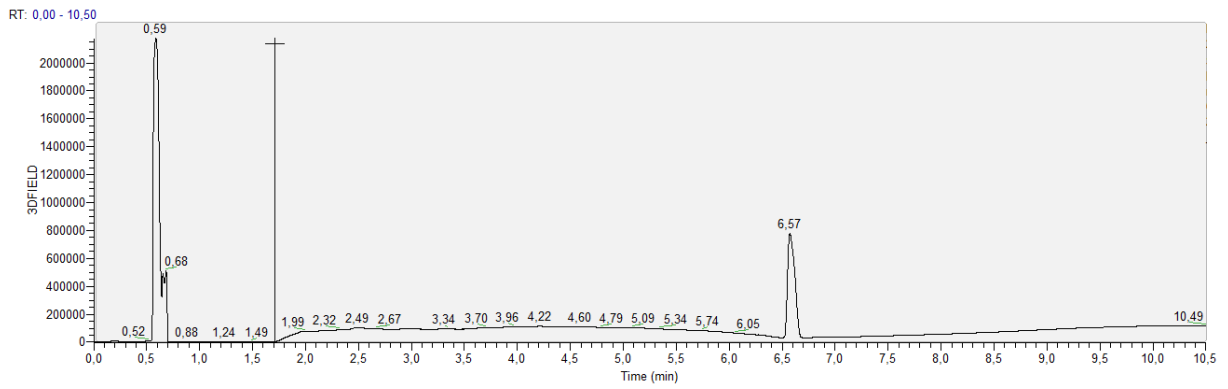
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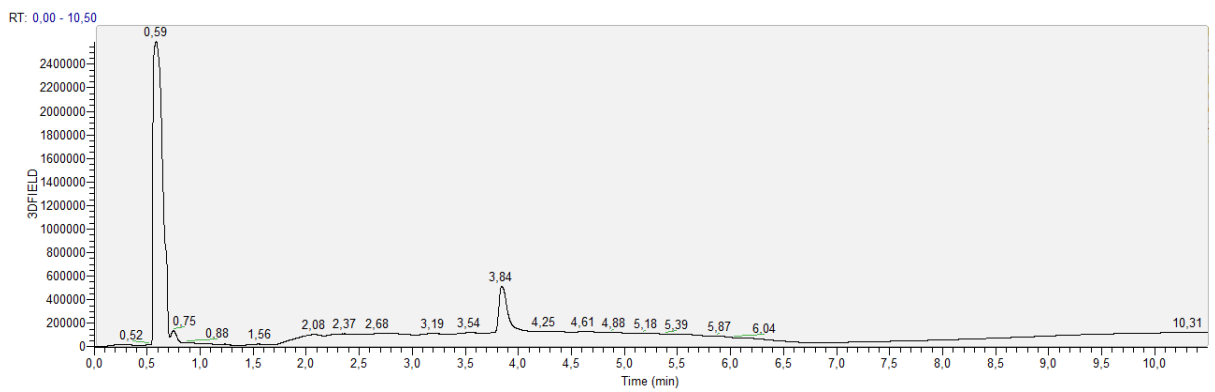
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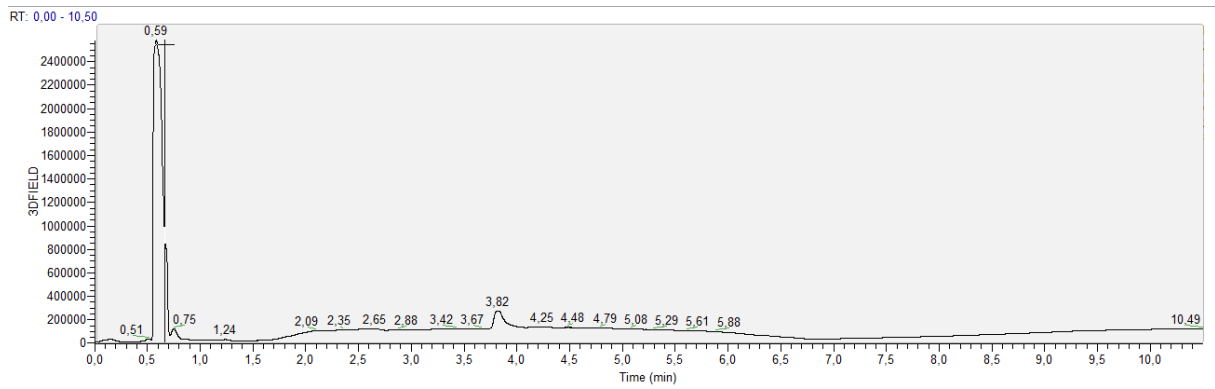
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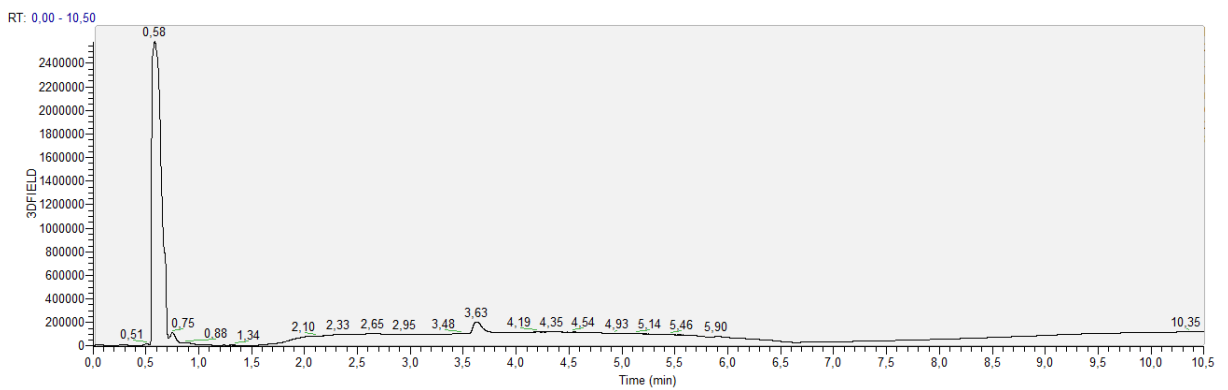
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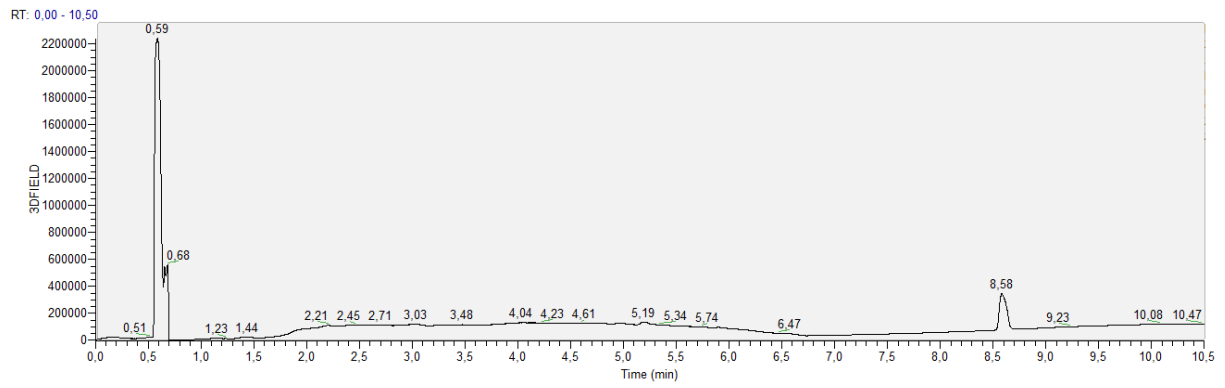
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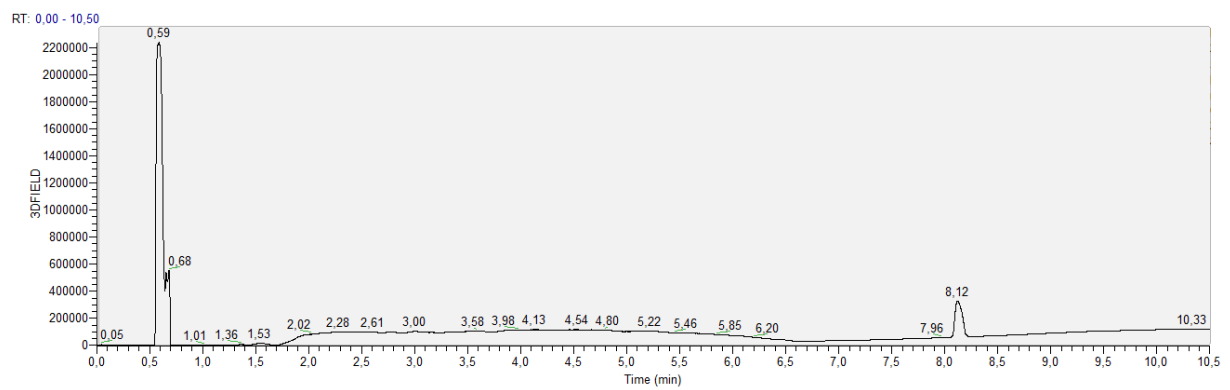
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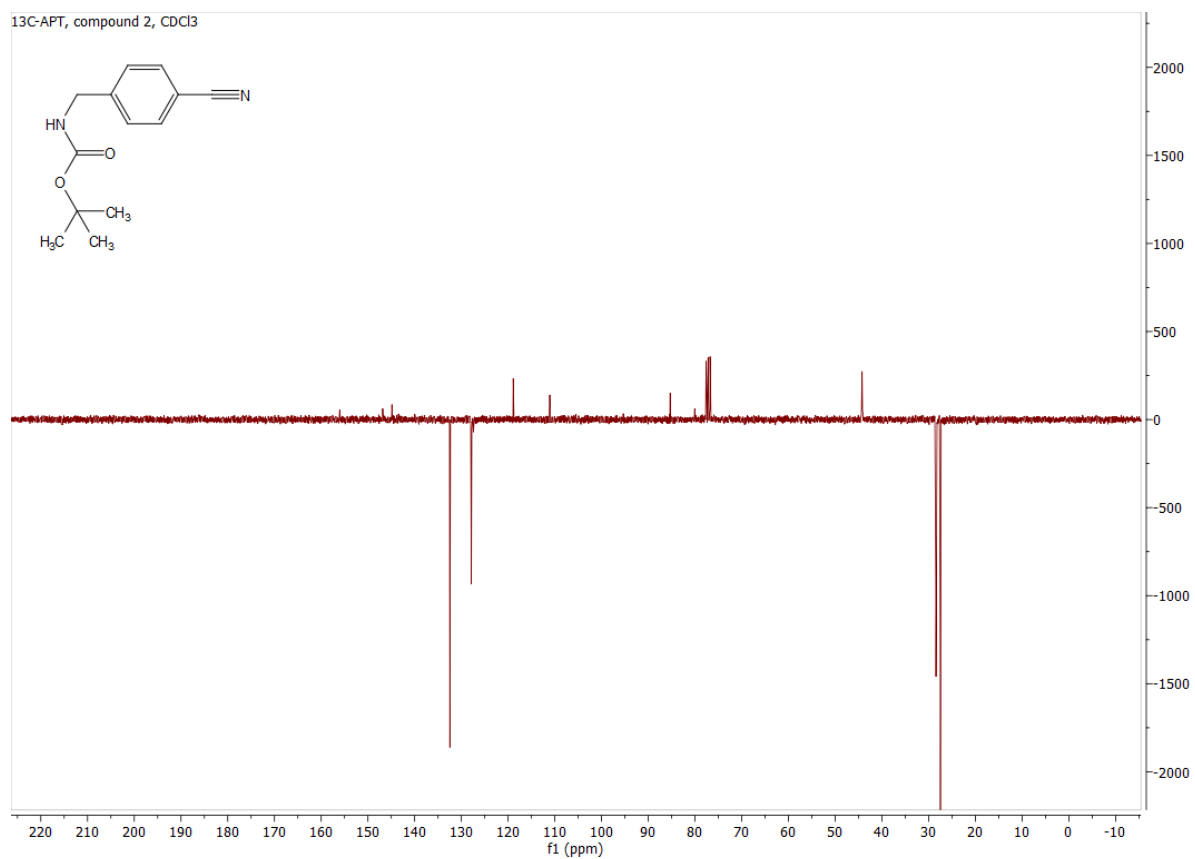
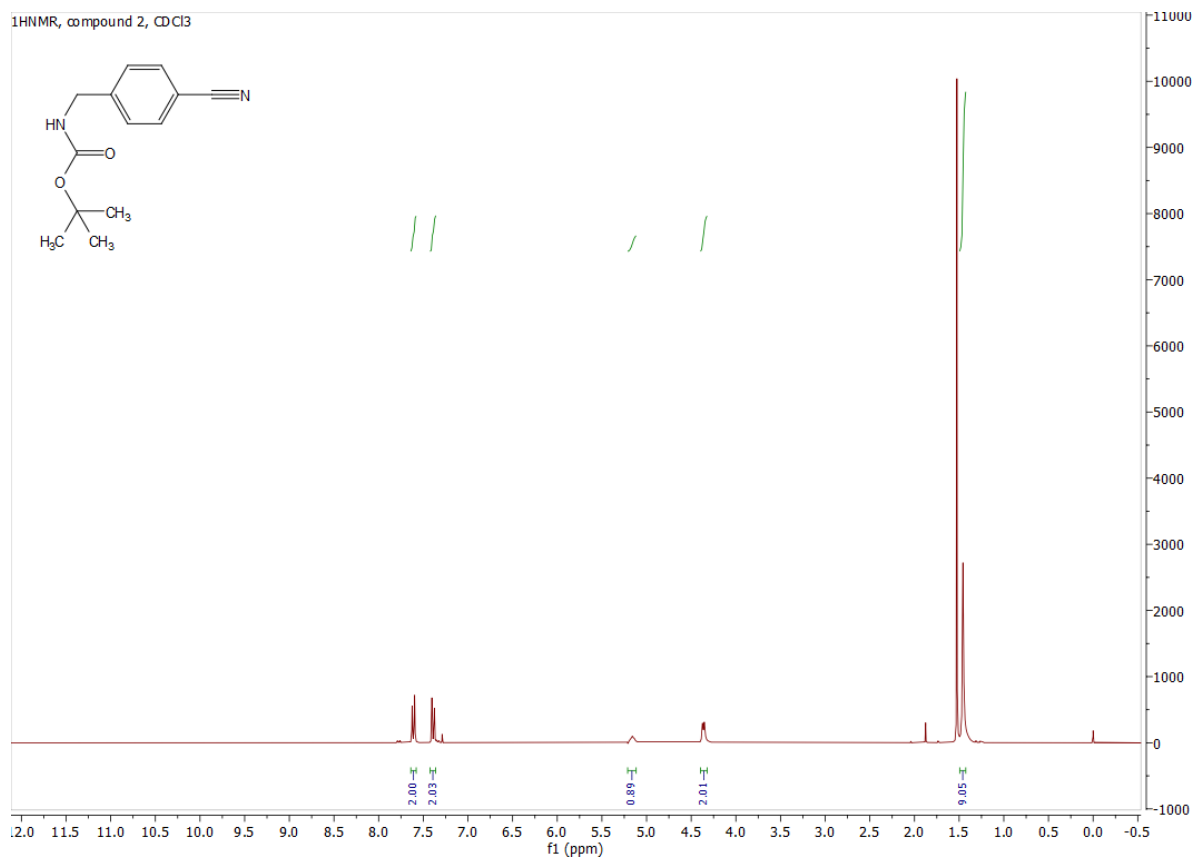


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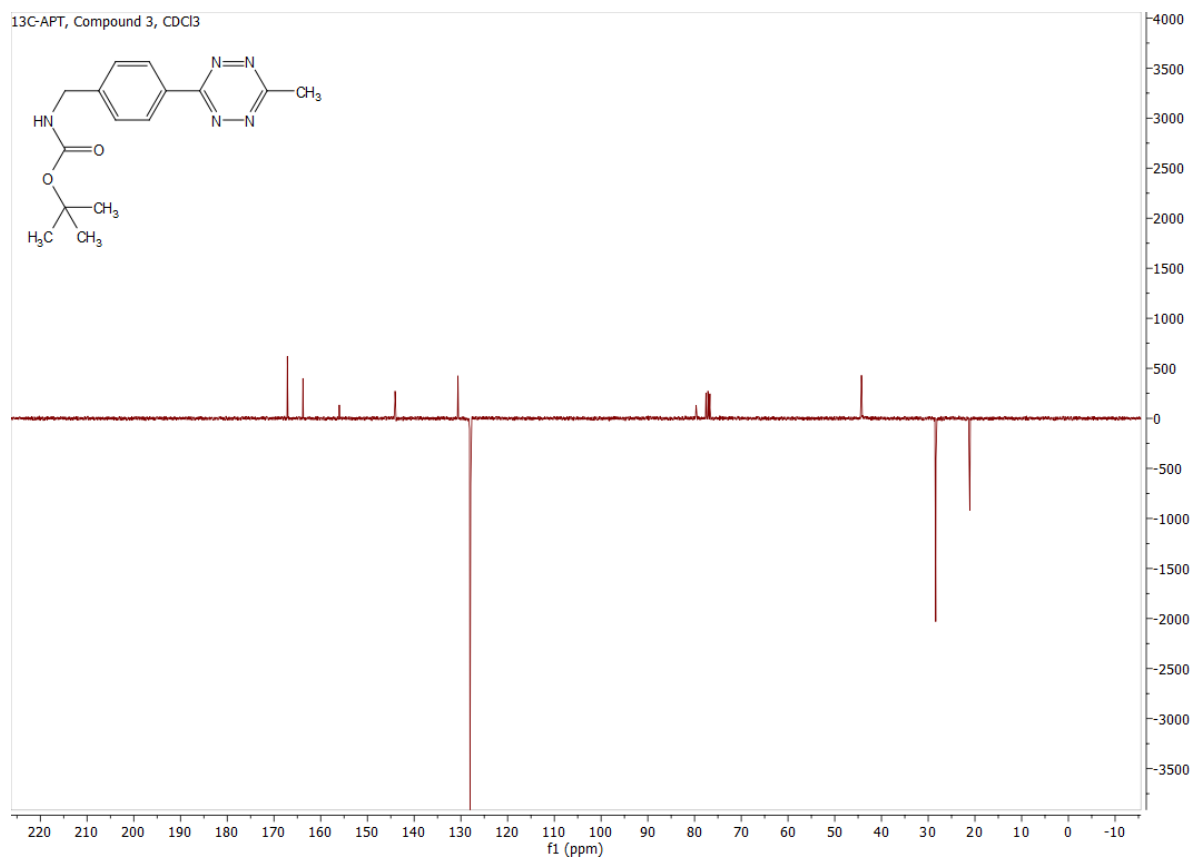
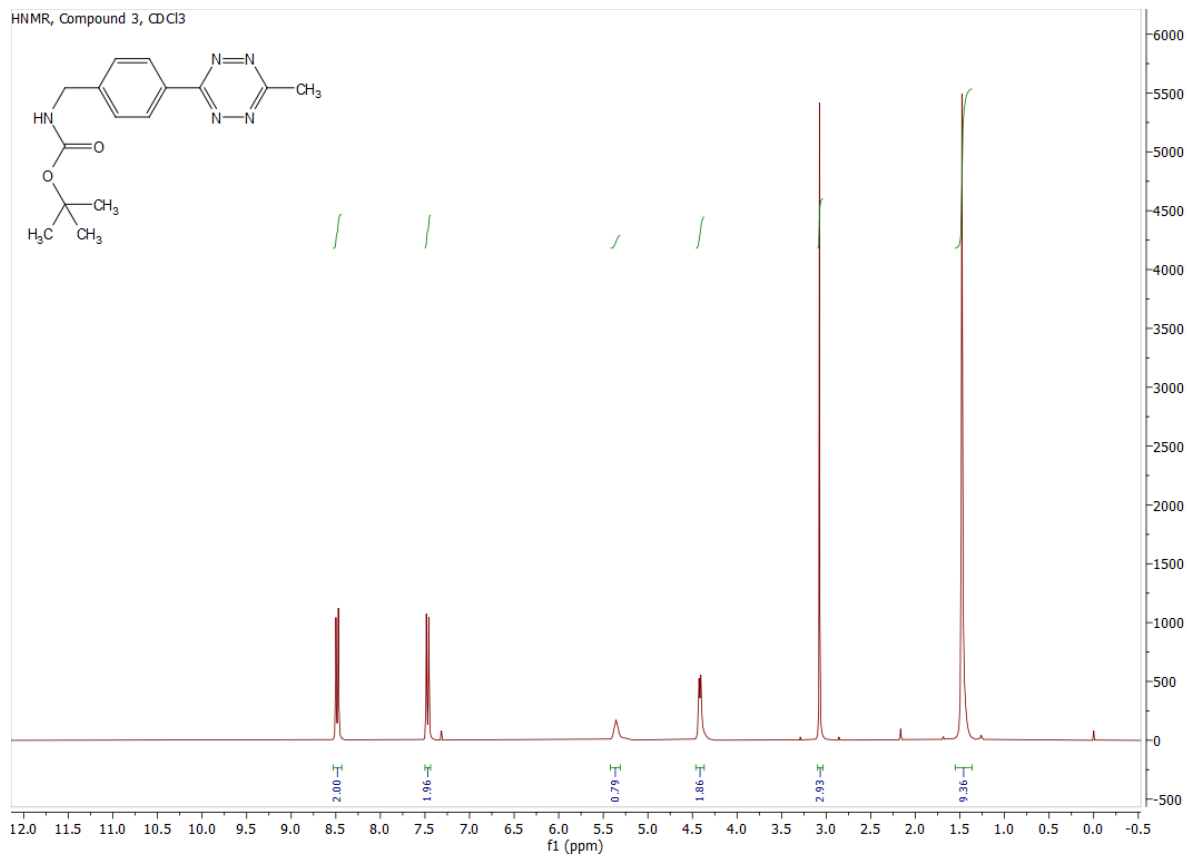


NMR spectra

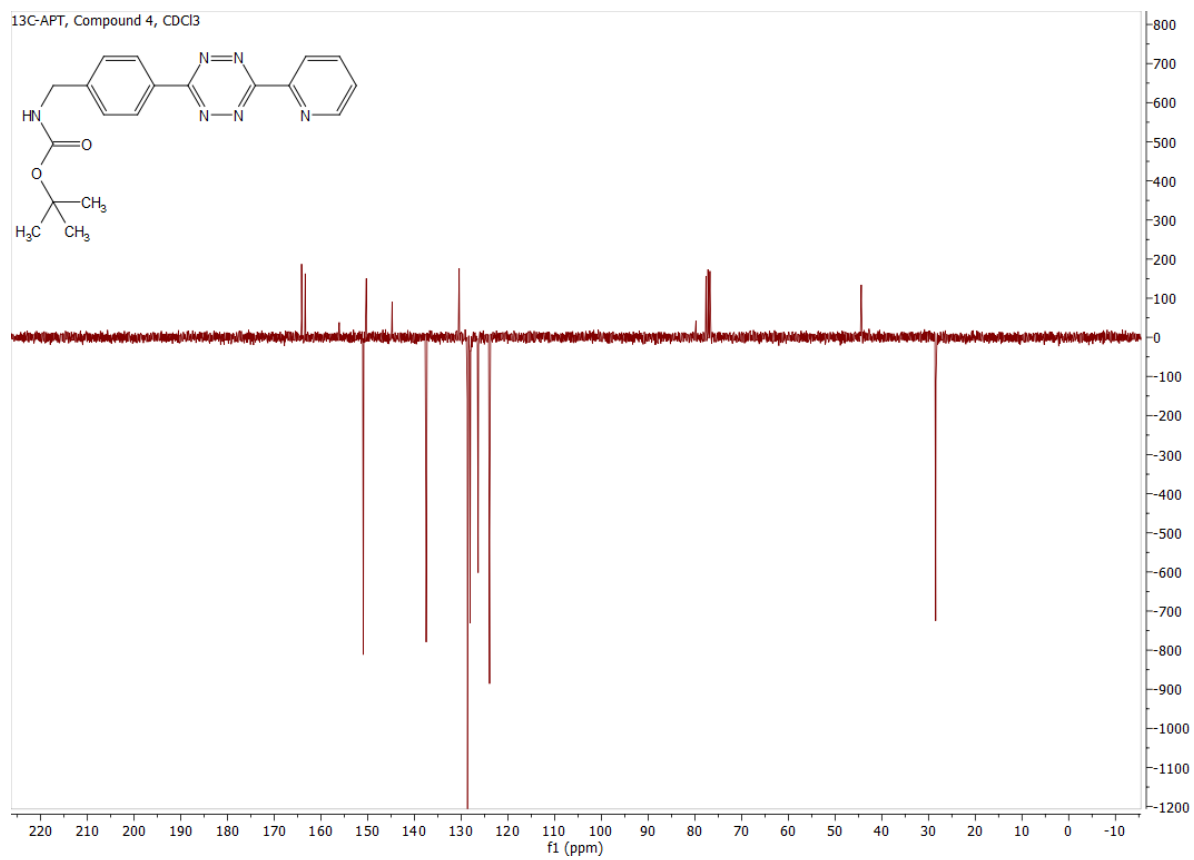
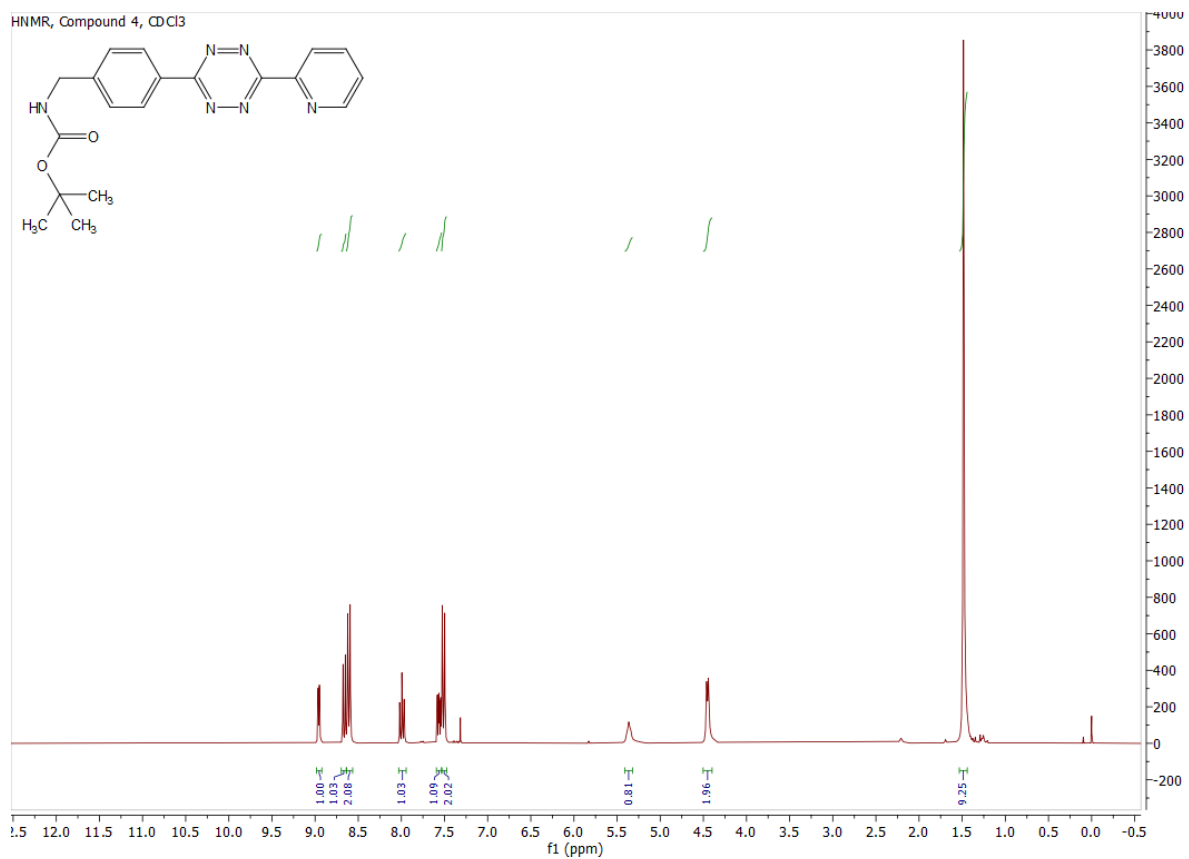
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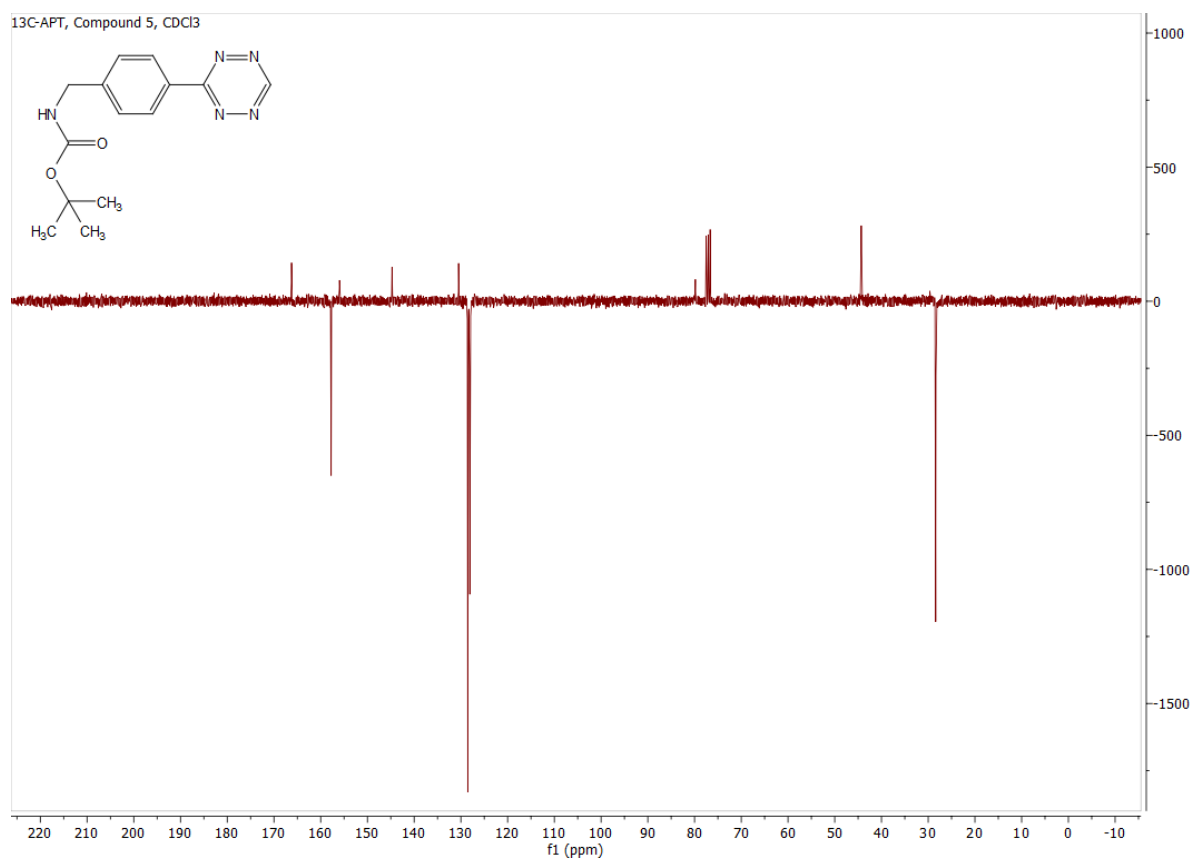
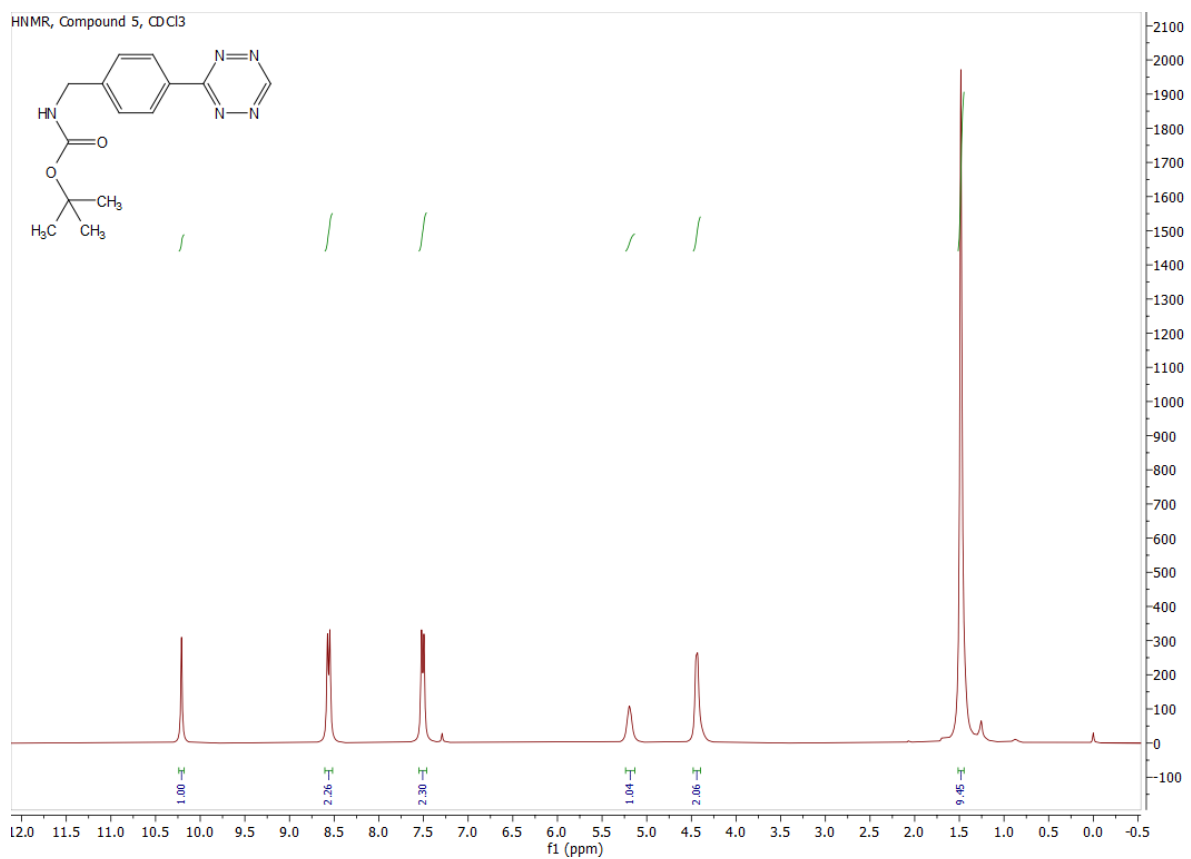
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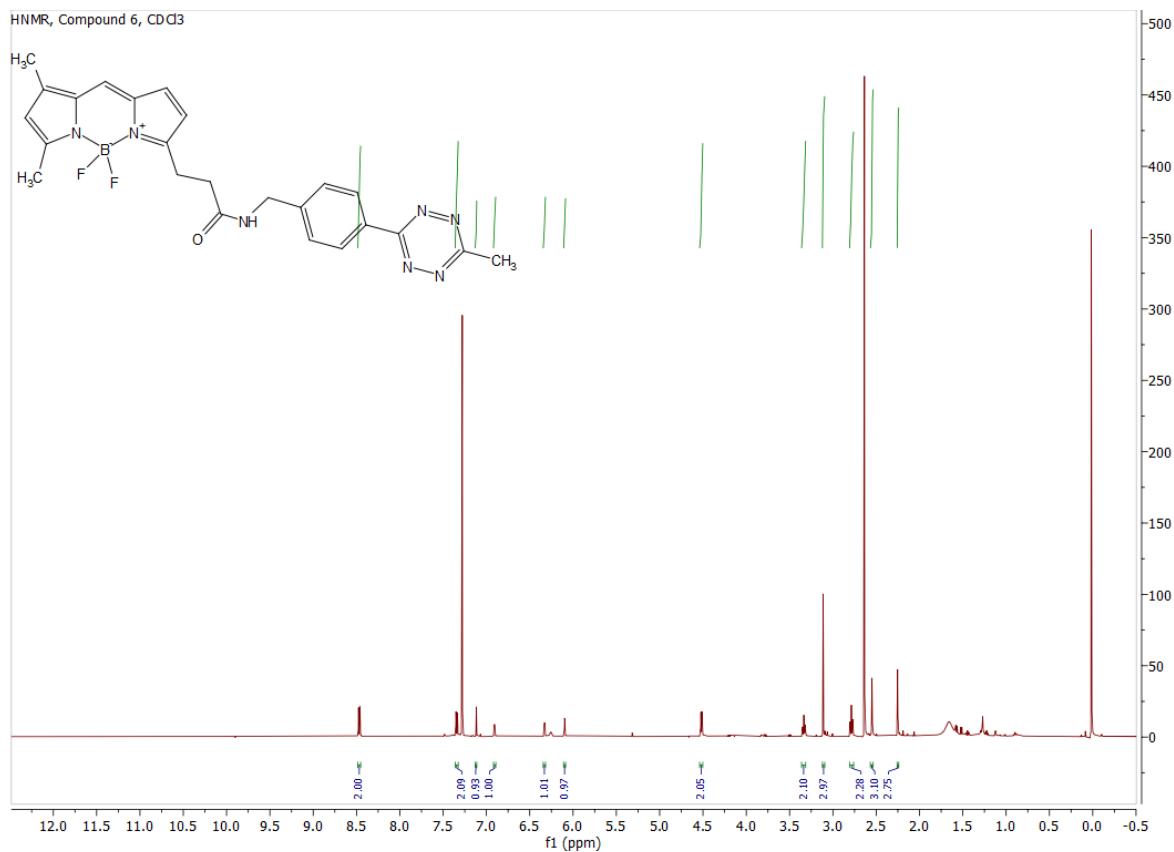
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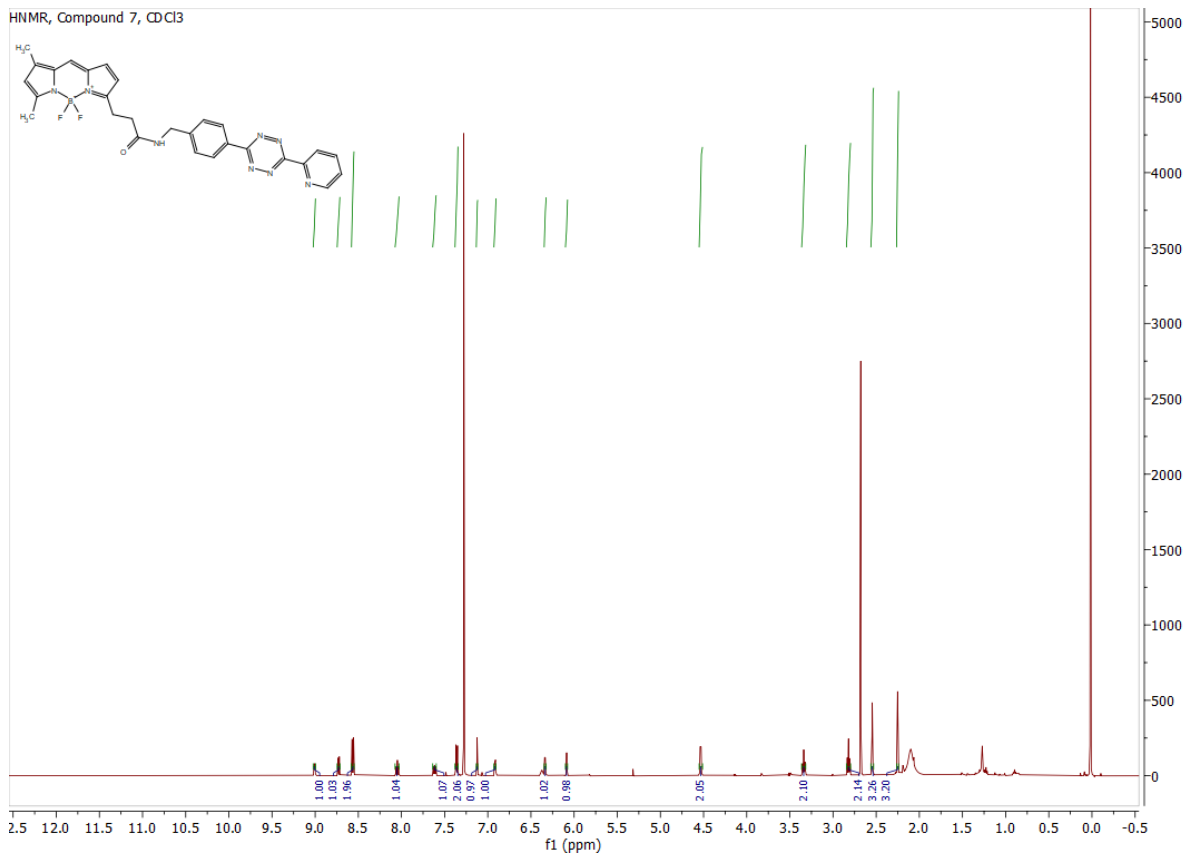
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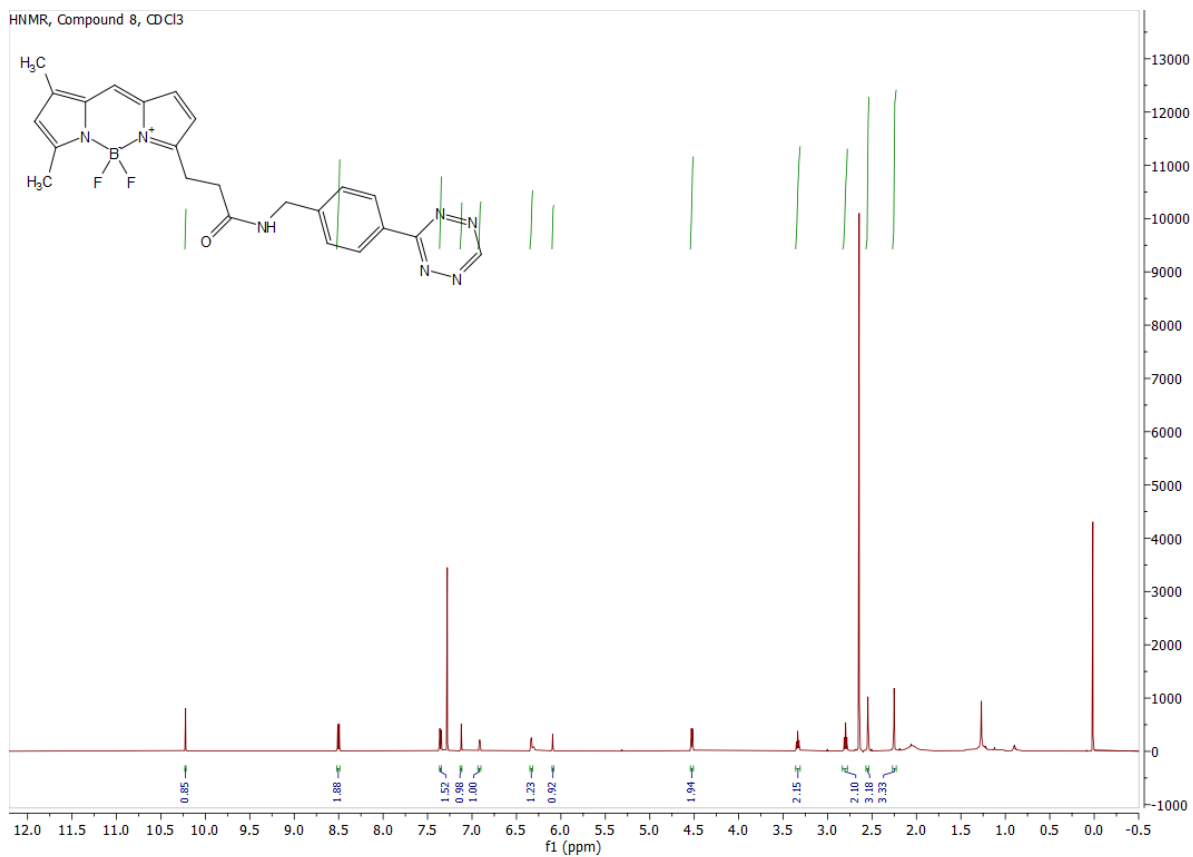
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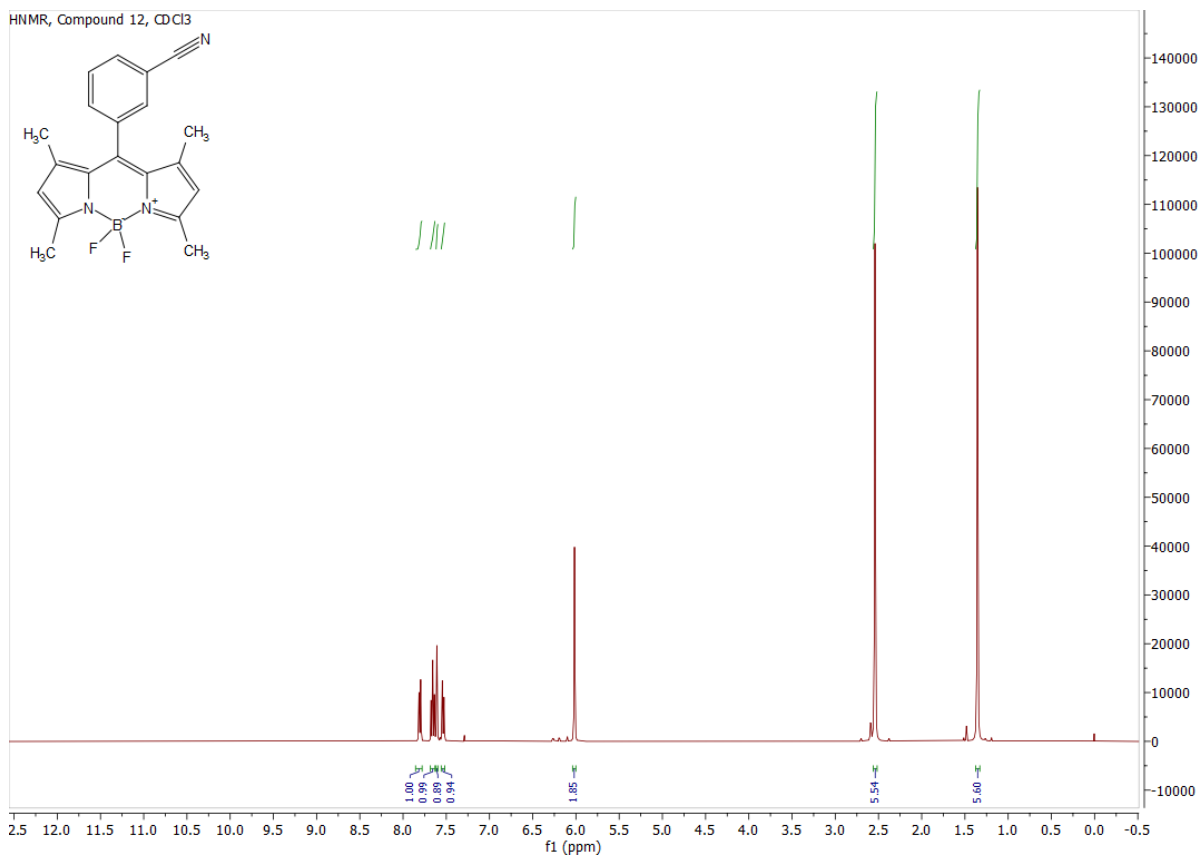
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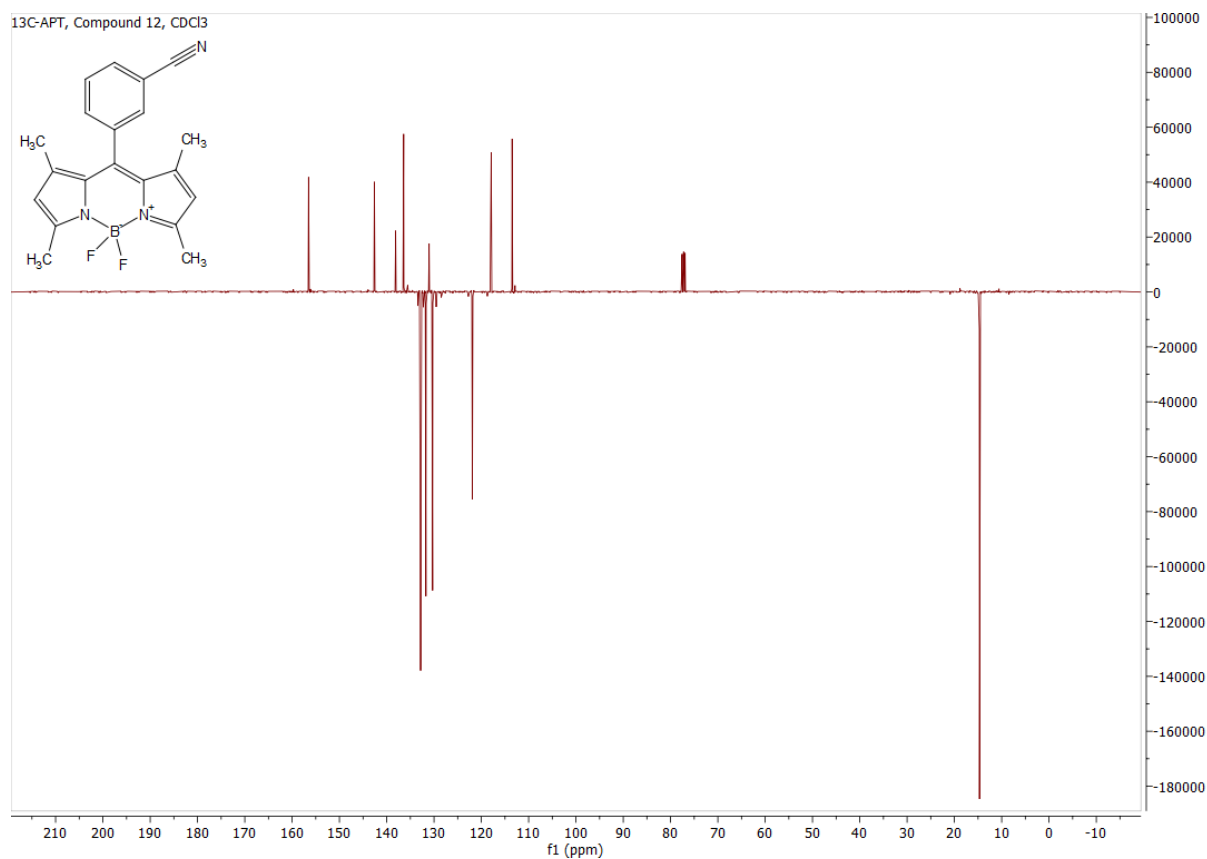


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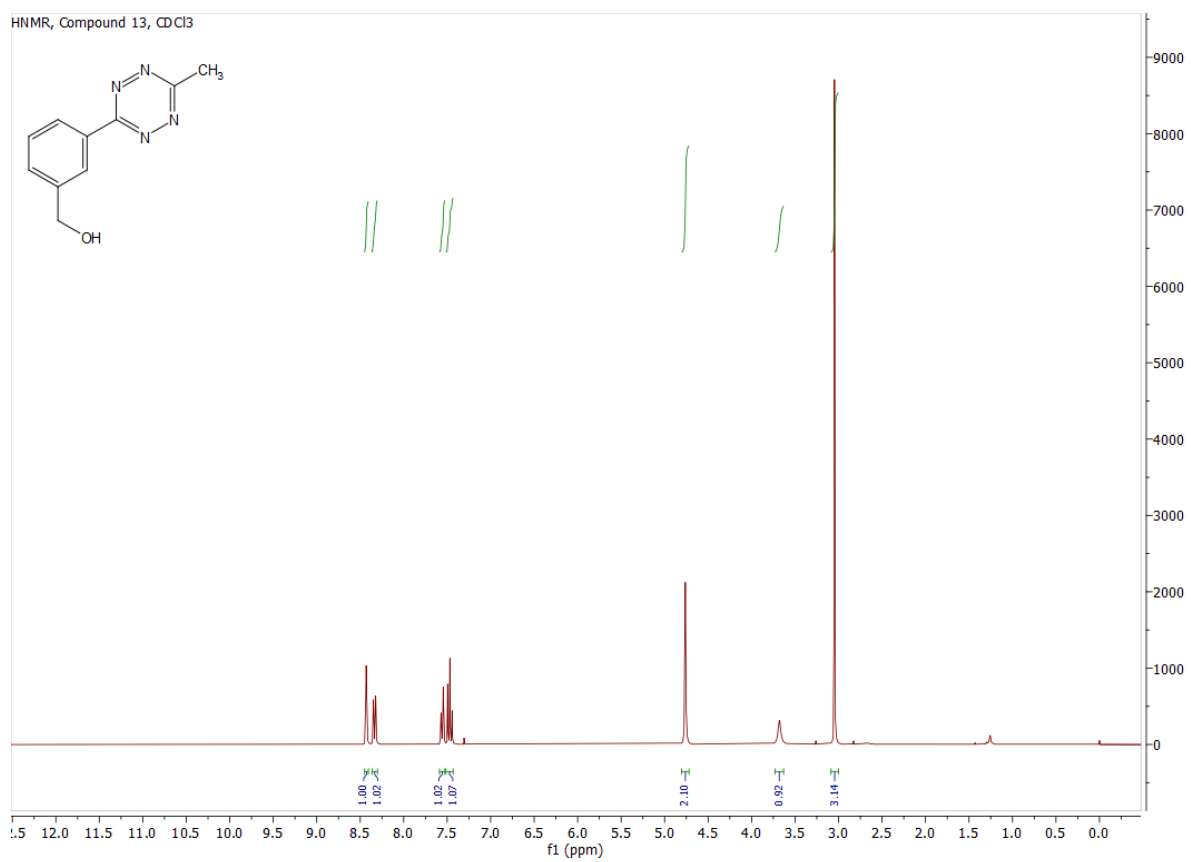


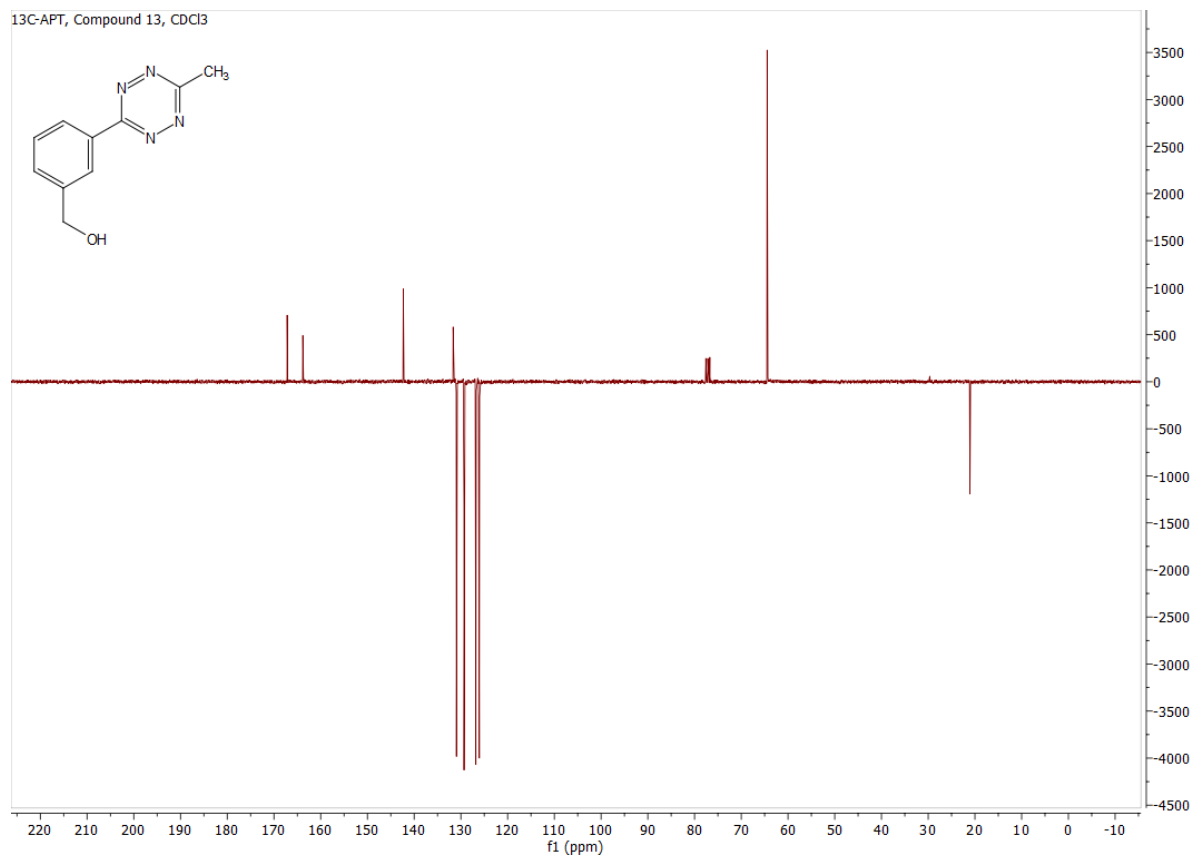
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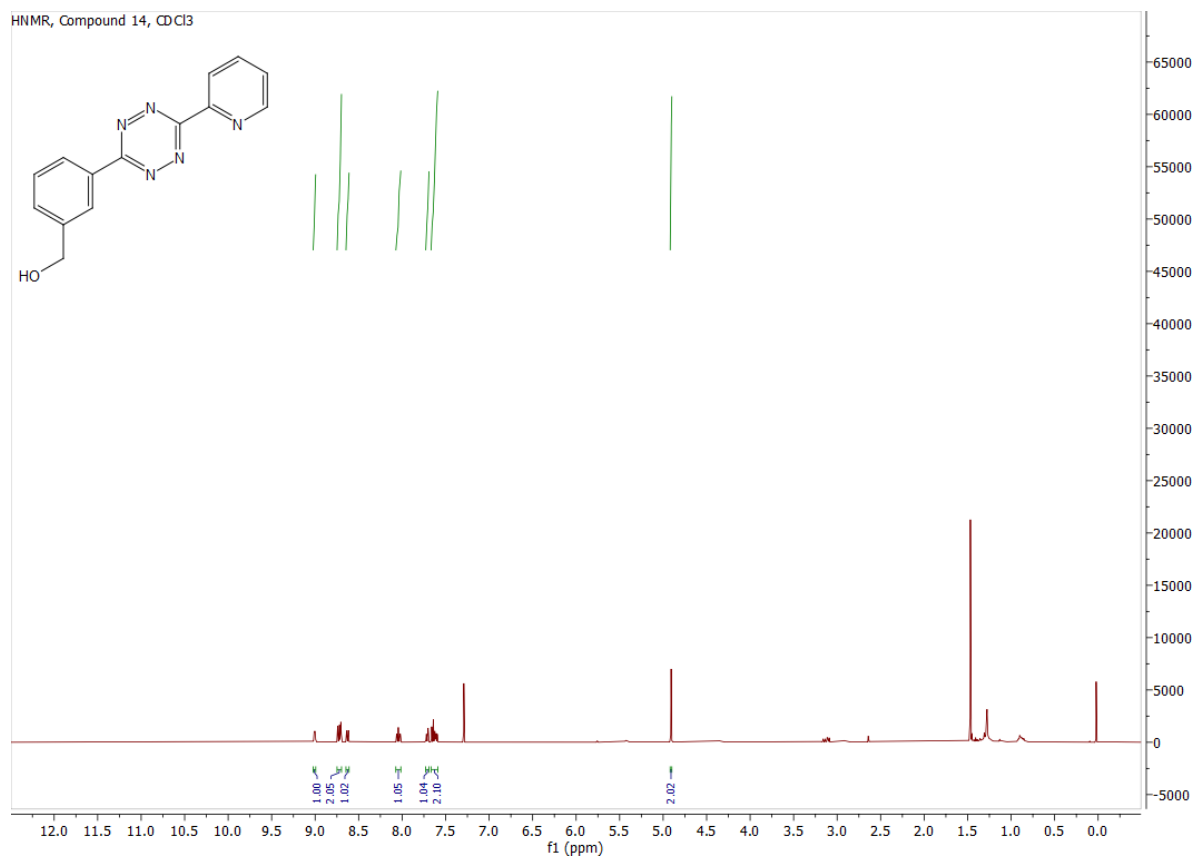


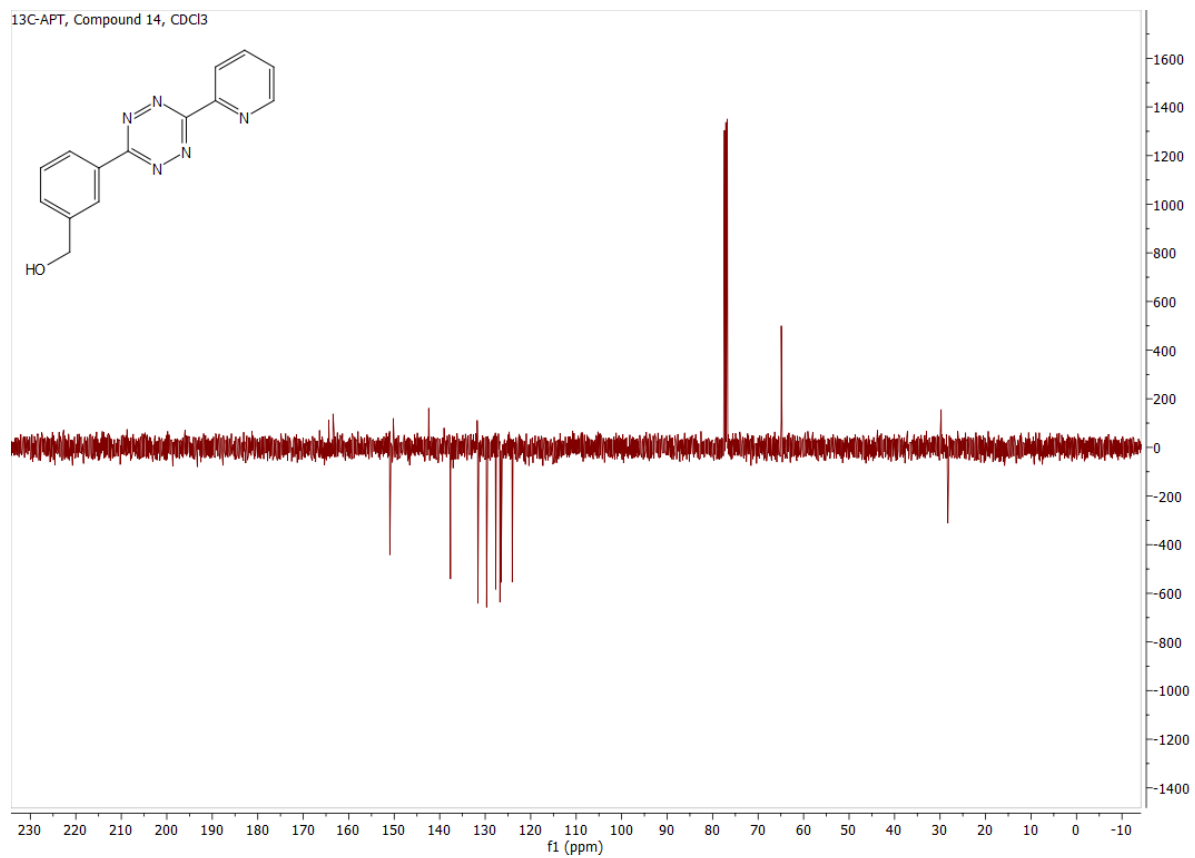
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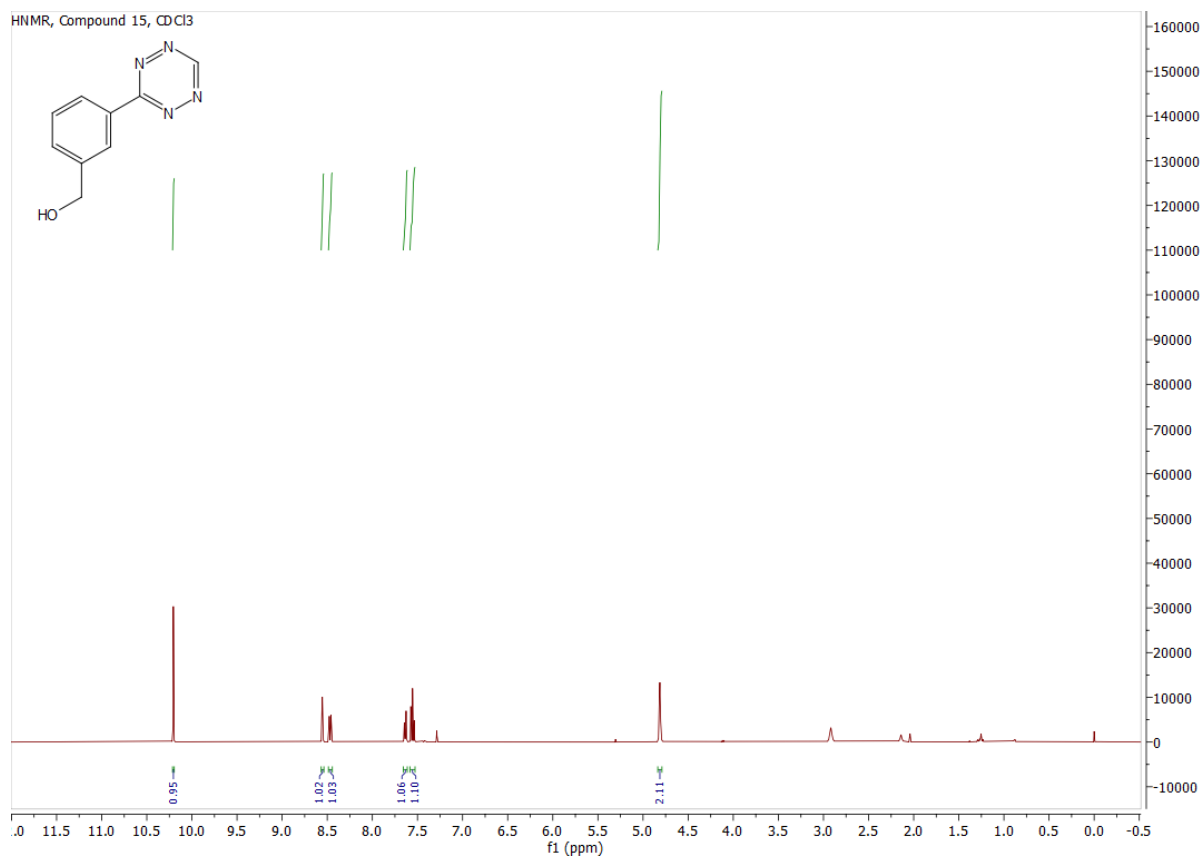


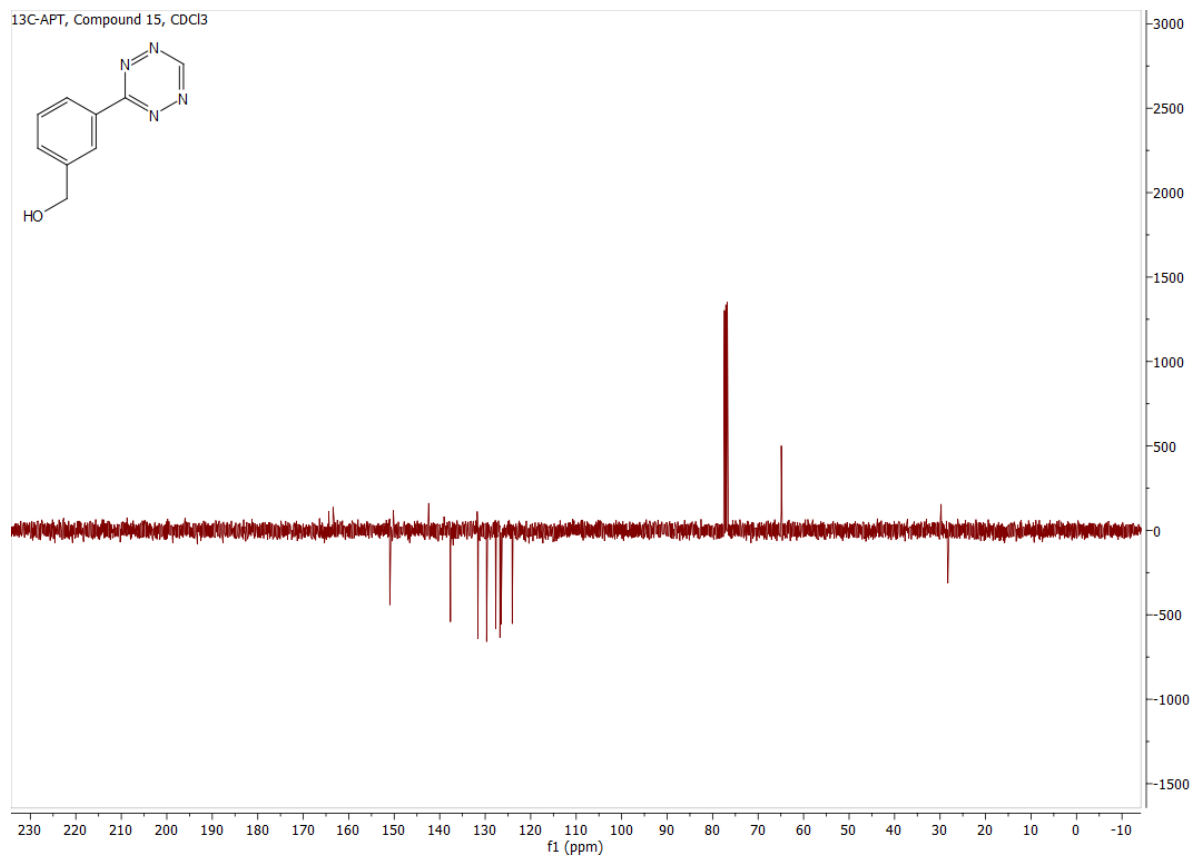
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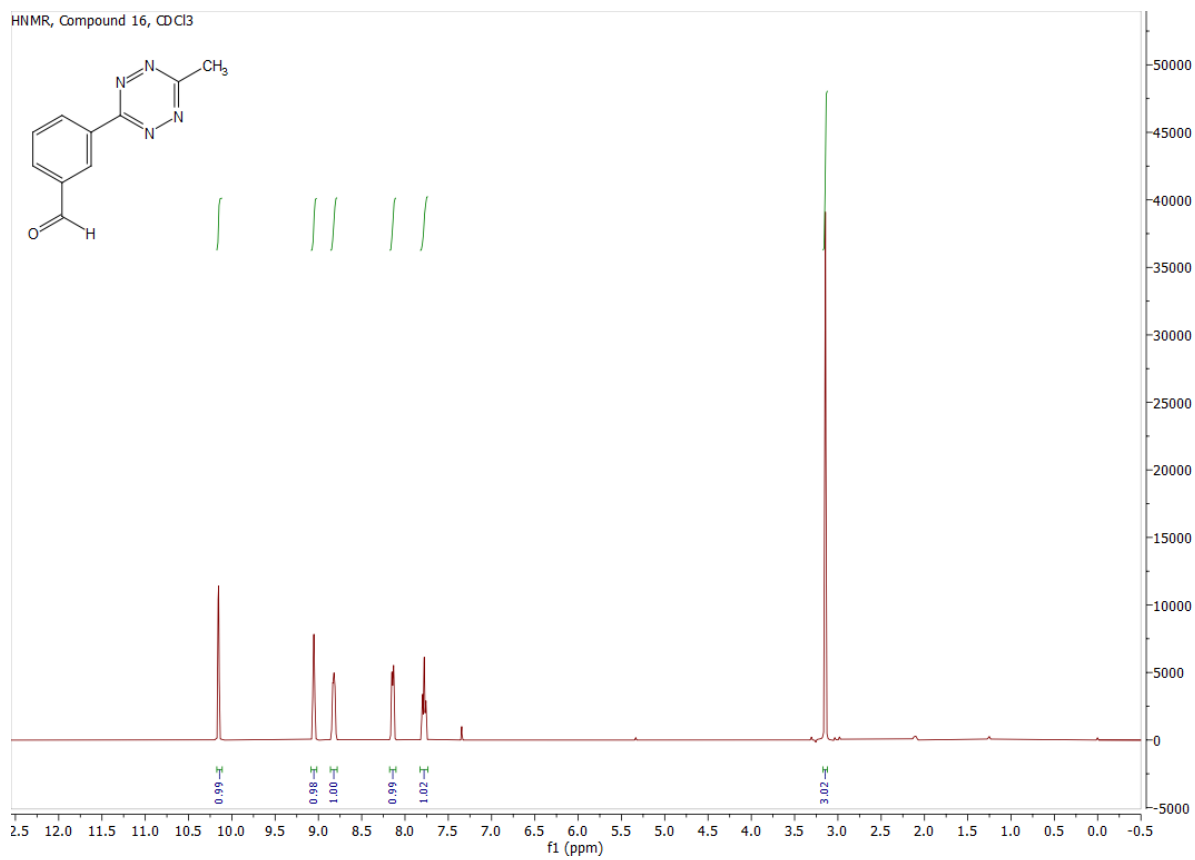


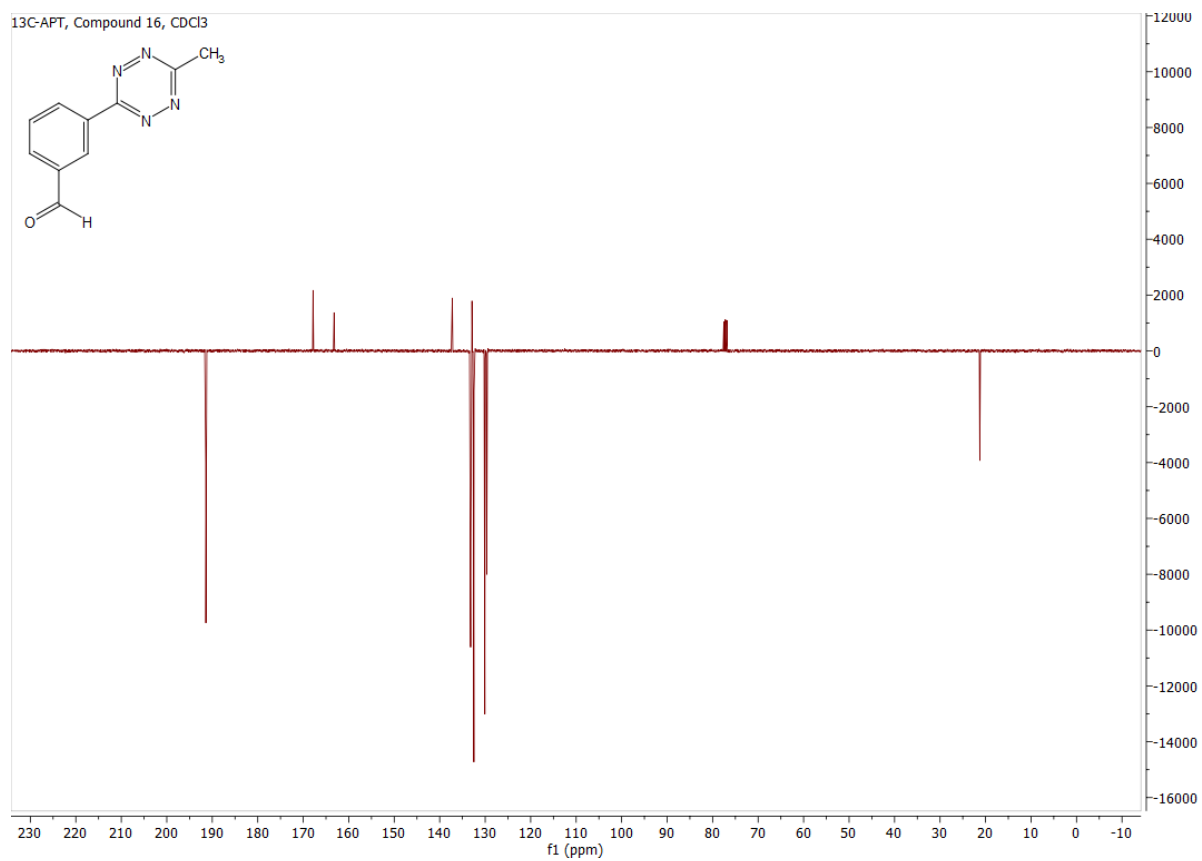
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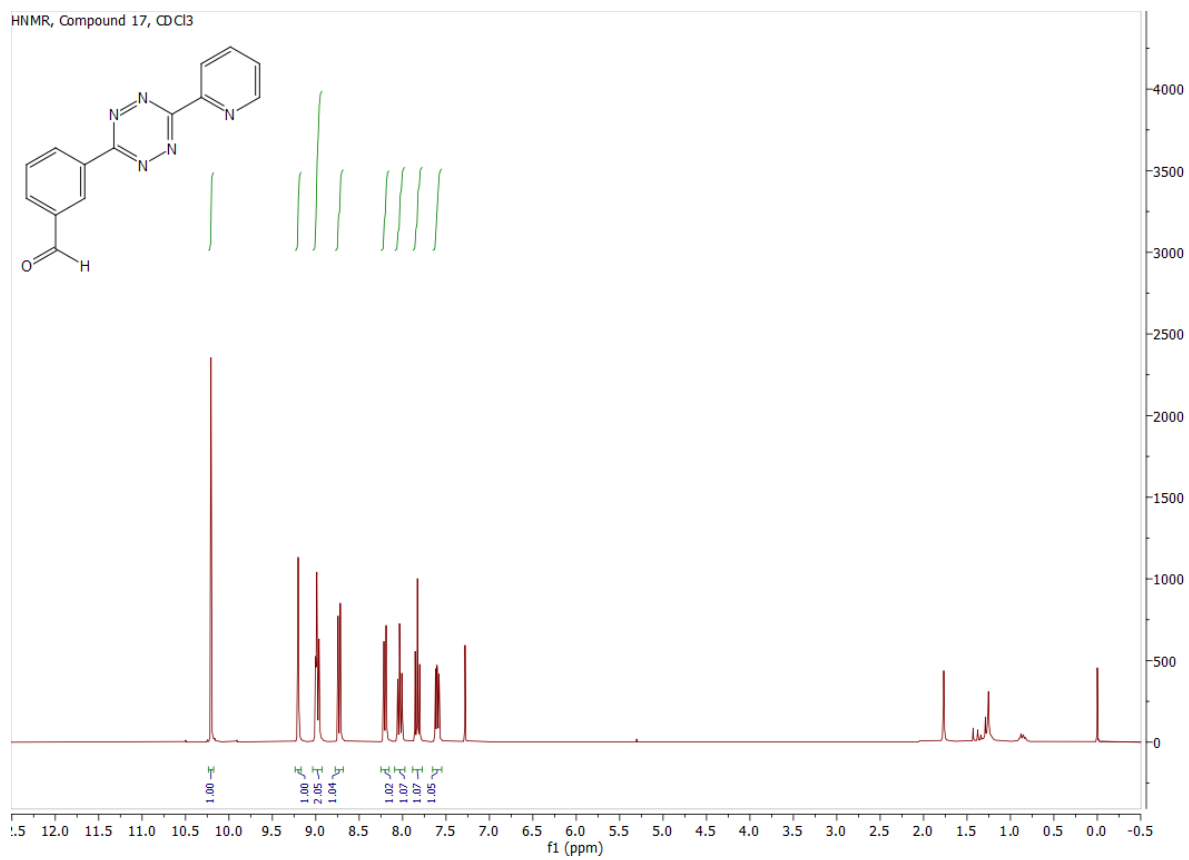


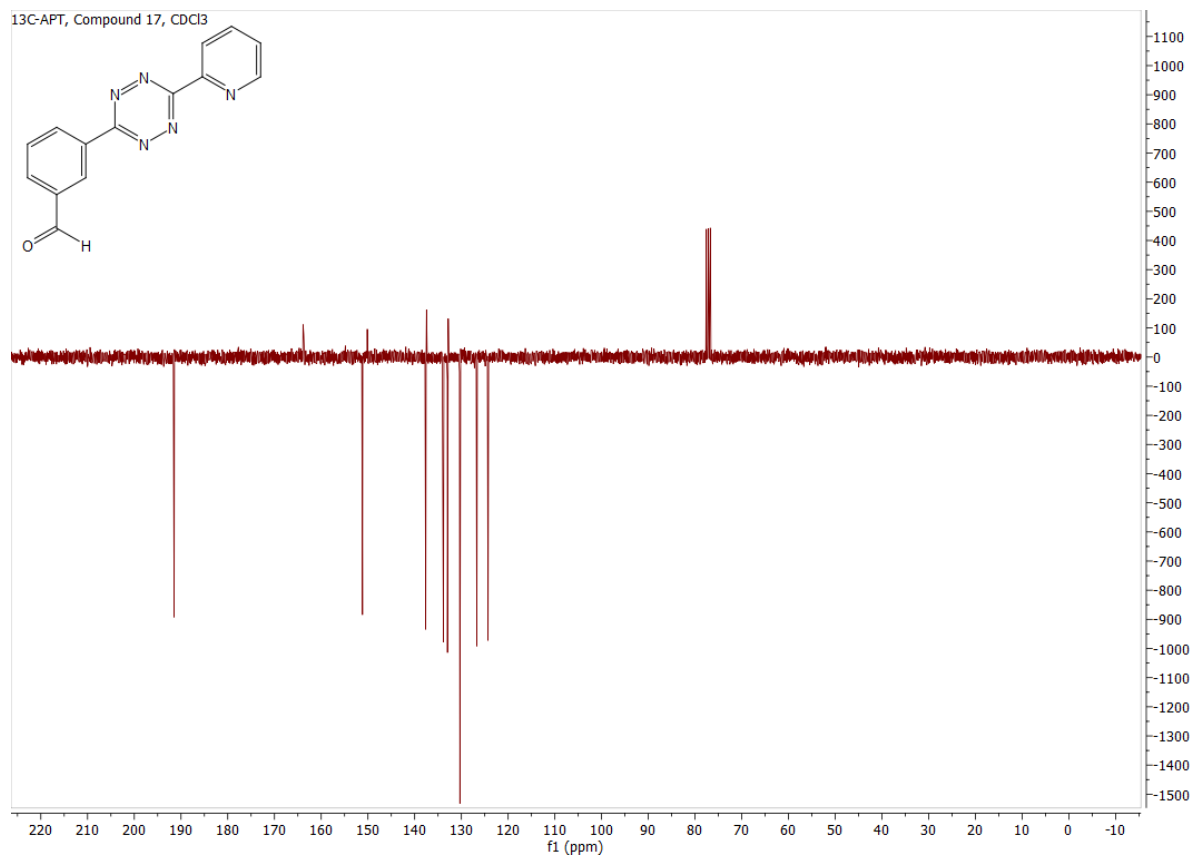
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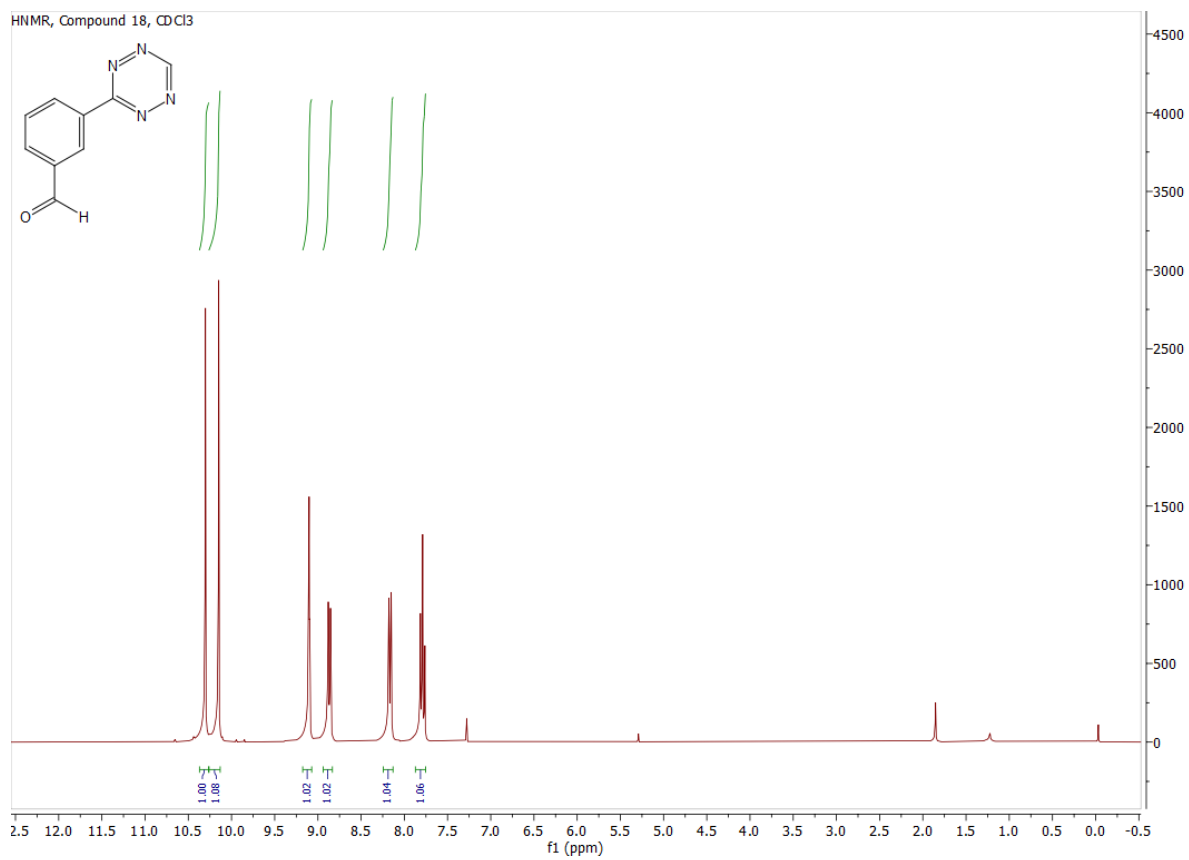


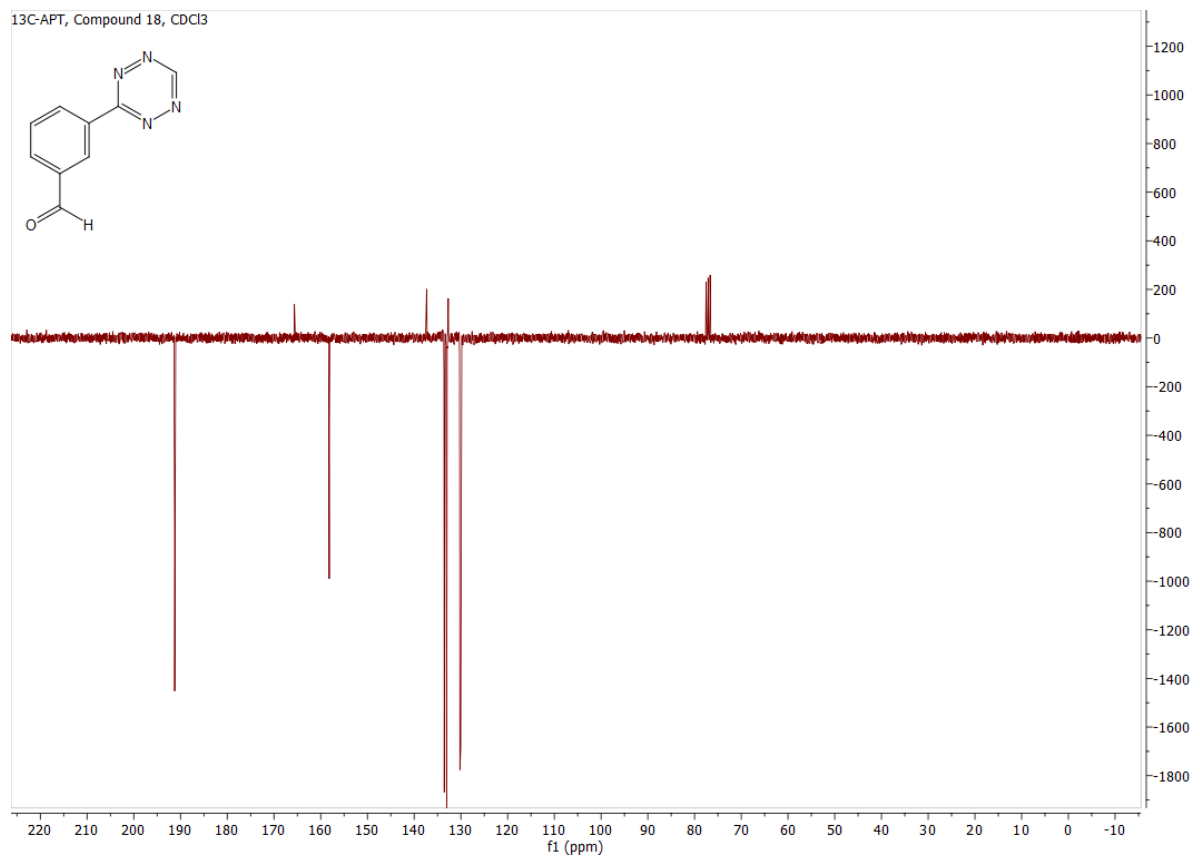
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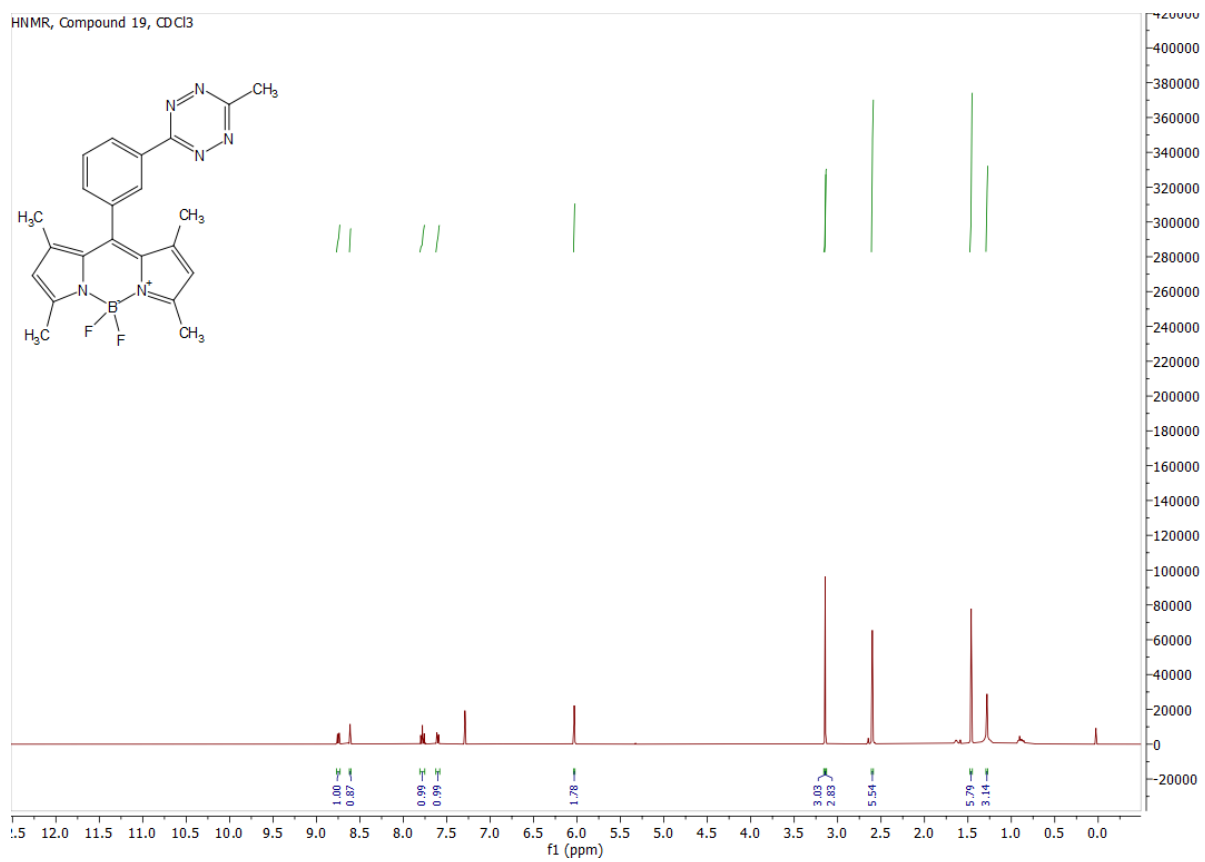


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