Electronic Supplementary Material (ESI) for Chemical Science.

Tetrazine-Mediated Bioorthogonal Prodrug-Prodrug Activation

Supporting Information

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Abstract: The selective and biocompatible activation of prodrugs within complex biological systems remains a key challenge in medical chemistry and chemical biology. Herein we report, for the first time, a dual prodrug activation strategy that fully satisfies the principle of bioorthogonality by the symbiotic formation of two active drugs without the generation of any by-products. This dual and traceless prodrug activation strategy takes advantage of the $_{INV}$DA chemistry of tetrazines (here a prodrug), generating a pyridazine-based miR21 inhibitor and the anti-cancer drug camptothecin and offers a new concept in prodrug activation.

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Experimental Procedures

General Information

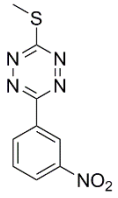
Chemicals were purchased from Sigma Aldrich and used without further purification, unless otherwise indicated. Drugs were purchased from Fluorochem. Anhydrous solvents were purchased from Sigma Aldrich and used under a N_2 atm using Schlenk techniques. For catalysis reactions solvents were degassed by three freeze-thaw cycles.
^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVA500 spectrometer (500 and 125 MHz, respectively) at 298 K in deuterated solvents, unless otherwise indicated. The residual solvent peaks were used as a reference for ^{1}H NMR experiments with $CHCl_3$ (δ_H = 7.26 ppm), CHD_2OD (δ_H = 3.31 ppm), CD_3SOCHD_2 (δ_H = 2.50 ppm), CHD_2CN (δ_H = 1.94 ppm), $(CHD_2)(CD_3)NCOD$ (δ_H = 2.75 ppm or 2.92 ppm) and HDO (δ_H = 1.38 ppm). The deuterated solvent signals were used as a reference for ^{13}C NMR experiments with $CDCl_3$ (δ_C = 77.2 ppm), CD_3OD (δ_C = 49.0 ppm), $(CD_3)_2SO$ (δ_C = 39.5 ppm), CD_3CN (δ_C = 1.3 ppm or 118.3 ppm) and $(CD_3)_2NCOD$ (δ_C = 29.8 ppm, 34.9 ppm or 163.2 ppm).

IR spectra were recorded on a Bruker Tensor 27 Standard System FT-IR spectrophotometer. Low Resolution Mass Spectra were obtained using an Agilent LCMS 110 ChemStation with a G1946B mass detector. Chromatographic purifications were carried out on silica gel 60–120 mesh and analytical TLC on silica gel F254 plates (Merck). Reverse phase analytical HPLC analysis (RP-HPLC) was performed on an Agilent 1100 ChemStation with a Kinetex 5µ XB-C18 (50 × 4 × 60 mm) column, using a flow rate of 1 mL/min with evaporative light scattering detection (ELSD) (Polymer Lab PL-ELS 1000) with simultaneous detection at 220, 254, 260, 282 and 495 nm. Method A: eluting with 95% water for 5 min, then to 95% CH_3CN over 10 min (both with 0.1% HCO_2H). Method B: eluting with 95% H_2O for 5 min, then to 95% CH_3OH over 10 min (both with 0.1% HCO_2H). Reverse phase preparative HPLC (RP-HPLC) was performed on an Agilent 1100 ChemStation with an Eclipse 5µ XB-C18 (50 × 9.5 × 250 mm) column, using a flow rate of 2 mL/min with simultaneous detection at 254, 350 nm and 495 nm. Microwave heating was performed in a Biotage Initiator microwave with an autosampler (Sixty).

Fluorescence and UV/Vis spectra were obtained with a BioTek SynergyHT plate reader in 96-well plates with a typical volume of 100 µL. Absorbance of compounds was translated into concentrations by using a calibration line that typically consists out of 5 data points.

Synthetic Procedures

Compound 1



1

S7[1] (407 mg, 1.6 mmol) was stirred with anhydrous pyridine (100 µL, 1.3 mmol) in anhydrous DMF (2 mL) at room temperature. After 5 min, compound **7** (250 mg, 1.1 mmol) was added to the solution and the reaction mixture was stirred for 2 h at room temperature. The solvent was removed under reduced pressure and the crude dissolved in CH_2Cl_2 (20 mL) and washed with H_2O (2 × 20 mL) and brine (2 × 20 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2 (10 mL) and amyl nitrite (267 µL, 0.87 mmol) was added dropwise. The solution was stirred at room temperature for 1 h. The solvent was removed under reduced pressure and the product purified by flash column chromatography (7:3, hexane/EtOAc) to give a pink powder (25 mg, 9%).

R_f 0.56 (hexane/EtOAc, 7:3).

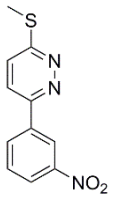
^{1}H NMR (500 MHz, CD_3CN): δ/ppm 9.20 (t, *J* = 1.8 Hz, 1H), 8.84 (ddd, *J* = 7.8, 1.7, 1.0 Hz, 1H), 8.46 (ddd, *J* = 8.2, 2.3, 1.0, 1H), 7.87 (t, *J* = 7.8 Hz, 1H), 2.81 (s, 3H).

^{13}C NMR (125 MHz, CD_3CN): δ/ppm 177.3, 162.0, 150.1, 134.9, 133.9, 131.8, 127.4, 122.7, 13.8.

IR (solid, cm^{-1}): 2926.49, 1519.36, 1396.28, 1346.57, 1160.55.

HRMS (ESI) for $C_9H_8O_2N_5{}^{32}S$ $[M+H]^+$: *calcd*.: 250.03932; *found*: 250.03840.

Compound 2



2

Compound **6** (150 mg, 0.93 mmol) and 3-nitrophenylboronic acid (151 mg, 0.90 mmol) were dissolved in degassed dioxane/H_2O (2:1, 2 mL) under a N_2 atm. $Pd(dppf)Cl_2$ (21 mg, 0.029 mmol) and potassium acetate (6 mg, 0.061 mmol) in degassed dioxane/H_2O (2:1, 1 mL) were added under a N_2 atm to give a suspension. The mixture was heated under microwave irradiation at 80 °C for 2 h. The reaction was cooled down to room temperature, and the mixture was passed through a celite pad and then extracted with CH_2Cl_2 (20 mL). The organic layer was washed with H_2O (5 mL) and brine (5 mL). The organic layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude residue was purified by flash column chromatography (7:3, hexane/EtOAc) to provide **2** as a pale yellow powder (111 mg, 50 %).

R_f 0.42 (hexane/EtOAc, 7:3).

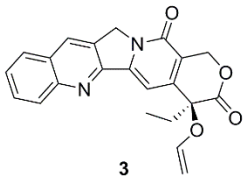
^{1}H NMR (500 MHz, CD_3OD): δ/ppm 8.95 (t, *J* = 2.0 Hz, 1H), 8.45 (ddd, *J* = 7.8, 1.8, 1.0 Hz, 1H), 8.37 (ddd, *J* = 8.2, 2.3, 1.0, 1H), 8.06 (d, *J* = 9.1 Hz, 1H), 7.71 (t, *J* = 8.2 Hz, 1H), 7.50 (d, *J* = 9.1 Hz, 1H), 2.74 (s, 3H).

^{13}C NMR (126 MHz, CD_3OD): δ/ppm 164.9, 155.6, 150.4, 139.1, 133.6, 131.5, 127.9, 125.5, 125.4, 122.5, 13.4.

IR (solid, cm^{-1}): 2926, 1519, 1396, 1347, 1160.

HRMS (EI) for $C_{11}H_9N_3O_2{}^{32}S$ $[M]^{\bullet+}$: *calcd*.: 247.04100; *found*: 247.03995.

Compound 3



3

$[Ir(cod)Cl]_2$ (9.6 mg, 0.014 mmol) was stirred with Na_2CO_3 (91 mg, 0.86 mmol) in dry dioxane (0.5 mL) for 5 min. A solution of camptothecin (4) (50 mg, 0.14 mmol) in dry dioxane (3 mL) was added to the reaction mixture under N_2 atm. Vinyl acetate (26 µL, 0.29 mmol) was added to the solution and it was stirred for 4 h at 100 °C under a N_2 atm. The mixture was allowed to cool to room temperature and the solvent was removed under reduced pressure. The crude was dissolved in CH_3OH resulting in a fine suspension that was filtered prior purification by preparative HPLC (45 min, gradient: 40 % CH_3CN to 60 % in 10 min, 80 % to 95 % in 25 min, 95 % for 5 min, 40 % for 5 min, 350 nm).

R_f 0.27 (CH_2Cl_2/CH_3OH, 98:2).

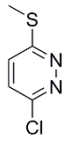
^{1}H NMR (500 MHz, $CDCl_3$): δ/ppm 8.41 (s, 1H), 8.25 (d, *J* = 8.3 Hz, 1H), 7.94 (d, *J* = 8.1 Hz, 1H), 7.85 (ddd, *J* = 8.4, 6.8, 1.4 Hz, 1H), 7.68 (ddd, *J* = 9.5, 7.7, 0.8 Hz, 1H), 7.42 (s, 1H), 6.18 (dd, *J* = 13.8, 6.5 Hz, 1H), 5.71 (d, *J* = 16.9 Hz, 2H), 5.36 – 5.28 (m, 3H), 4.57 (dd, *J* = 13.9, 2.1 Hz, 2H), 4.25 (dd, *J* = 6.5, 2.1 Hz, 2H), 2.19 – 2.07 (m, 2H), 1.05 (t, *J* = 7.5 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$): δ/ppm 168.6, 167.3, 152.5, 149.2, 147.5, 147.4, 146.5, 131.3, 130.9, 130.0, 128.6, 128.3, 128.3, 128.3, 121.1, 98.2, 93.9, 78.8, 66.5, 50.2, 32.5, 7.9.

IR (solid, cm^{-1}): 2921, 2852, 1749. 1661.

HRMS (ESI) for $C_{22}H_{19}N_2O_4$ $[M+H]^+$: *calcd.*: 375.13393; *found*: 375.13290.

Compound 6



6

To a solution of 3,6-dichloropyridazine (250 mg, 1.7 mmol) in dry DMF (5 mL), trimethylamine (705 µL, 5.1 mmol) was added dropwise. Sodium thiomethoxide (142 mg, 2.0 mmol) was slowly added in portions to the reaction mixture over a period of 5 min. The resulting solution was stirred for 18 h at room temperature. After quenching with cold CH_3OH (1 mL), the solution was diluted in CH_2Cl_2 (15 mL) and washed with H_2O (2 × 10 mL). The organic phase was washed with brine (2 × 10 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure and the crude oil was purified by flash column chromatography (7:3, hexane/EtOAc) to give the pure **6** as a white powder (163 mg, 60 %).

R_f 0.39 (hexane/EtOAc, 7:3).

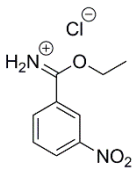
^{1}H NMR (500 MHz, CD_3OD): δ/ppm 7.59 (d, *J* = 9.1 Hz, 1H), 7.54 (d, *J* = 9.1 Hz, 1H), 2.66 (s, 3H).

^{13}C NMR (126 MHz, CD_3OD): δ/ppm 164.7, 154.8, 130.0, 129.1, 13.4.

IR (solid, cm^{-1}): 3063, 1592, 1393, 1161.

HRMS (ESI) for $C_5H_6N_2{}^{35}Cl^{32}S$ $[M+H]^+$: *calcd.*: 160.99347; *found*: 160.99370.

Compound 7



7

To a stirred suspension of 3-nitrobenzonitrile **8** (1.2 g, 8.1 mmol) in dry EtOH (5 mL) at 0 °C, 4N HCl in dioxane (10 mL) was added dropwise and the reaction mixture was stirred for 1 h at 0 °C. The solution was then warmed to room temperature and stirred overnight. The solvent was removed under reduced pressure, THF was added (25 mL) and the product isolated by centrifugation and dried (*in vacuo*) as white powder (1.2 g, 65%).

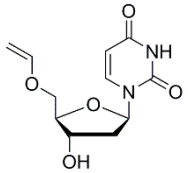
^{1}H NMR (500 MHz, DMF-d_7): δ/ppm 8.74 (ddd, *J* = 2.2, 1.6, 0.4 Hz, 1 H), 8.58 (ddd, *J* = 8.3, 2.4, 1.1 Hz, 1 H), 8.45 (ddd, *J* = 7.8, 1.6, 1.1 Hz, 1 H), 4.46 (q, *J* = 7.1 Hz, 2 H), 1.48 (t, *J* = 7.1 Hz, 3 H).

^{13}C NMR (126 MHz, DMF-d_7): δ/ppm 164.8, 148.4, 135.5, 132.1, 131.1, 128.0, 62.3, 14.0.

IR (solid, cm^{-1}): 3006, 2813, 1635, 1617, 1532, 1348, 1102.

HRMS (ESI) for $C_9H_{11}O_3N_2$ $[M]^+$: *calcd.*: 195.07642; *found*: 195.07570.

Compound 9



9

Compound **12** (30 mg, 0.070 mmol) was dissolved in CH_3OH/H_2O (4:1, 1 mL) at room temperature. Sodium periodate (23 mg, 0.11 mmol) and sodium hydrogen carbonate (7 mg, 0.080 mmol) were added and the solution was stirred for 30 min at room temperature. The solvent was removed under reduced pressure and the solid dissolved in CH_3CN (3 mL). Diisopropylethylamine (64 µL, 0.36 mmol) was added to the reaction mixture and the solution was stirred at 80 °C for 1 h. After cooling to room temperature, the solvent was removed under reduced pressure and the crude was purified by flash column chromatography (95:5, CH_2Cl_2/CH_3OH) to provide the compound **9** (7 mg, 40%) as colourless oil.

R_f 0.40 (CH_2Cl_2/CH_3OH, 95:5).

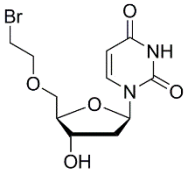
^{1}H NMR (600 MHz, CD_3OD): δ/ppm 7.99 (d, *J* = 8.2 Hz, 1H), 6.85 (t, *J* = 16.2, 9.6, 1H), 6.27 (t, *J* = 6.6 Hz, 1H), 5.89 (d, *J* = 16.2 Hz, 1H), 5.77 (d, *J* = 8.1 Hz, 1H), 5.30 (d, *J* = 9.6 Hz, 1H), 4.38 (dtd, *J* = 6.3 Hz, 3.6, 0.5 Hz, 1H), 3.94 (q, *J* = 3.5 Hz, 1H), 3.79 (dd, *J* = 12.1, 3.3 Hz, 1H), 3.72 (dd, *J* = 12.0, 3.8 Hz, 1H), 2.34 (ddd, *J* = 13.7, 6.2 , 3.7, 1H), 2.21 (ddd, *J* = 13.5, 7.0, 6.3 Hz, 1H).

^{13}C NMR (150 MHz, CD_3OD): δ/ppm 164.2, 151.5, 140.5, 128.1, 112.6, 101.9, 89.1, 87.6, 72.0, 62.7, 41.6.

IR (solid, cm^{-1}): 3253, 3001, 1661, 1462, 1288, 1054.

HRMS (ESI) for $C_{11}H_{15}N_2O_5$ $[M+H]^+$: *calcd*.: 255.09755; *found*: 255.09700.

Compound 10



10

To a solution of 2'-deoxyuridine (250 mg, 1.1 mmol) in DMF (5 mL) at room temperature, sodium hydride (53 mg, 1.4 mmol) was added and the reaction mixture was stirred for 10 min at room temperature before 1,2-dibromoethane (627 µL, 8.9 mmol) was added. The solution was then heated to 65 °C and stirred for 2 h. The reaction mixture was then cooled down to room temperature and concentrated under reduced pressure. The crude residue was purified by flash column chromatography (95:5, CH_2Cl_2/CH_3OH) to provide the product **10** (230 mg, 63%) as pale brown amorphous solid.

R_f 0.41 (CH_2Cl_2/CH_3OH, 95:5).

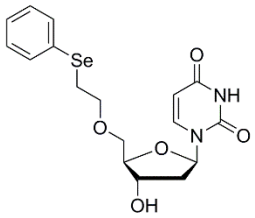
^{1}H NMR (600 MHz, CD_3OD): δ/ppm 8.00 (d, *J* = 8.1 Hz, 1H), 6.27 (t, *J* = 6.6 Hz, 1H), 5.77 (d, *J* = 8.1 Hz, 1H), 5.48 (s, 1H), 4.38 (dt, *J* = 6.7, 3.4 Hz, 1H), 4.29 (td, *J* = 7.1, 2.6 Hz, 2H), 3.94 (q, *J* = 3.4 Hz, 1H), 3.79 (dd, *J* = 12.0, 3.2 Hz, 1H), 3.72 (dd, *J* = 12.0, 3.8 Hz, 1H), 3.56 (t, *J* = 7.2 Hz, 2H), 2.31 (ddd, *J* = 13.6, 6.2, 3.6 Hz, 1H), 2.20 (ddd, *J* = 13.8, 7.7, 6.4 Hz, 1H).

^{13}C NMR (150 MHz, CD_3OD): δ/ppm 164.6, 152.1, 140.9, 101.9, 89.1, 87.6, 72.1, 62.7, 43.0, 41.5, 27.8.

IR (solid, cm^{-1}): 3369, 2929, 1702, 1646, 1458, 1396, 1275, 1216, 1091, 1051, 996.

HRMS (ESI) for $C_{11}H_{16}O_5N_2{}^{79}Br$ $[M+H]^+$: *calcd*.: 335.02371; *found*: 335.02390.

Compound 12



12

Benzeneselenol (13 µL, 0.13 mmol) was dissolved into a solution of caesium hydroxide monohydrate (18 mg, 0.13 mmol) in degassed DMF (1 mL) with molecular sieves and stirred for 1 h at room temperature. **10** (50 mg, 0.15 mmol) was added and the reaction mixture stirred for additional 12 h. The solution was filtered to remove insoluble salts and molecular sieves. The filtrate was extracted with CH_2CL_2 (3 × 20 mL) and the combined organic layers were washed with H_2O (5 mL) and brine (5 mL) and dried over Na_2SO_4. The solvent was removed under reduced pressure. The crude was purified by flash column chromatography (95:5, CH_2Cl_2/CH_3OH) to give compound **12** (16 mg, 26%) as yellow powder.

R_f 0.50 (CH_2Cl_2/CH_3OH, 96:4).

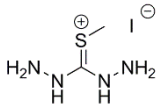
^{1}H NMR (600 MHz, CD_3OD): δ/ppm 7.95 (d, *J* = 8.1 Hz, 1H), 7.55-7.52 (m, 2H), 7.28-7.20 (m, 3H), 6.22 (t, *J* = 6.6 Hz, 1H), 5.71 (d, *J* = 8.1 Hz, 1H), 4.37 (td, *J* = 6.6, 3.5 Hz, 1H), 4.20 (t, *J* = 7.7 Hz, 2H), 3.93 (q, *J* = 3.5 Hz, 1H), 3.78 (dd, *J* = 12.0, 3.3 Hz, 1H), 3.71 (dd, *J* = 12.0, 3.8 Hz, 1H), 3.10 (t, *J* = 7.8 Hz, 2H), 2.31 (ddd, *J* = 13.6, 6.2, 3.6 Hz, 1H), 2.19 (ddd, *J* = 13.5, 7.4, 6.6 Hz, 1H).

^{13}C NMR (150 MHz, CD_3OD): δ/ppm 164.8, 152.1, 140.7, 132.9, 130.9, 130.2, 127.8, 101.9, 89.0, 87.5, 72.1, 62.7, 42.5, 41.5, 23.1.

IR (solid, cm^{-1}): 3090, 2924, 1717, 1635, 1437, 1417, 1223, 734.6.

HRMS (ESI) for $C_{17}H_{21}N_2O_5{}^{80}Se$ $[M+H]^+$: *calcd.*: 413.06102; *found*: 413.05940.

Compound S7



S7

Following a literature procedure[2], a solution of thiocarbohydrazide (**S6**) (2.0 g, 19 mmol) in EtOH (8 mL) was heated to reflux and a solution of methyl iodine (1.3 mL, 21 mmol) in EtOH (8 mL) was added dropwise. The reaction mixture was stirred for 4 h, until a white precipitate had formed. The reaction mixture was cooled to room temperature and the precipitate was collected by filtration and washed with EtOH (3 × 10 mL) to give the product **S7** as a white powder (3.6 g, 77 %).

^{1}H NMR (500 MHz, D_2O): δ/ppm 2.52 (s, 3H).

^{13}C NMR (126 MHz, DMSO-d_6): δ/ppm 170.6, 12.4.

Results and Discussion

Annexin V assay of SK-BR3 cells

Statistical significance and error bars of determination of apoptotic SK-BR3 cells after 14 h incubation of untreated cells, 5'-*O*-vinyl deoxyuridine **9** (20 µM), tetrazine **1** (10 µM), miR21 inhibitor **2** (10 µM), tetrazine **1** (10 µM) with 5'-*O*-vinyl deoxyuridine **9** (20 µM).

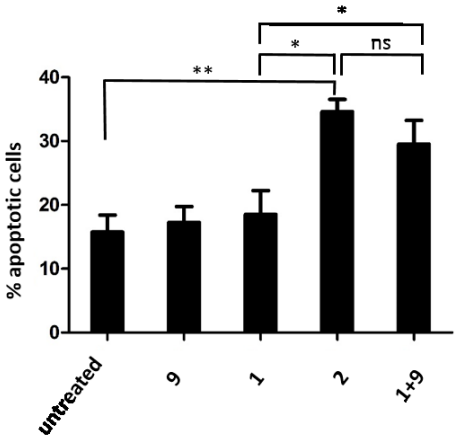


Figure S1. SK-BR3 cells after 14 h incubation of untreated cells = 15.8 ± 2.8, 5'-O-vinyl deoxyuridine 9 (20 µM) = 17.2 ± 2.5 %, tetrazine 1 (10 µM) = 18.5 ± 4.0 %, miR21 inhibitor 2 (10 µM) = 34.6 ± 2.1 %, tetrazine 1 (10 µM) with 5'-*O*-vinyl deoxyuridine 9 (20 µM) = 29.5 ± 4.0 %, (SK-BR3 cells, Annexin V assay, n=3). ** P<0.01 and * P<0.1 by one-way ANOVA with Tukey post-test.

Tetrazine half-lifes

The half-life ($t_{1/2}$) of tetrazine **1** and tetrazine **S5** was determined by measuring the decrease in the absorbance of tetrazine (5 mM) over time in PBS/DMSO (1:1). By plotting the normalised *ln* of concentration c_t against time, the half-lives ($t_{1/2}$) of tetrazine **1** and **S5** k_{obs} were calculated using equation (2) with *m* being the slope of the linear fit.

$$t_{1/2} = \frac{\ln(2)}{m} \quad (2)$$

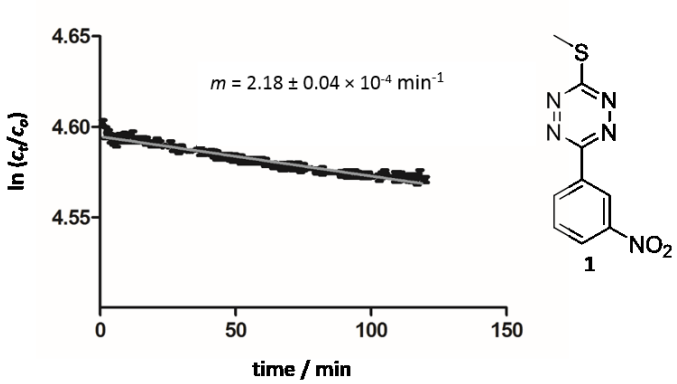


Figure S2. Plot of normalised natural logarithm of tetrazine **1** concentration c_t against time in PBS/DMSO (1:1) with $t_{1/2}$ = 3175 ± 63 min.

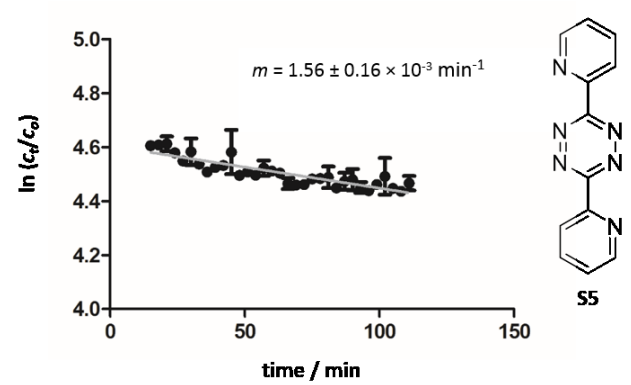


Figure S3. Plot of normalised natural logarithm of tetrazine **S5** concentration c_t against time in PBS/DMSO (1:1) with $t_{1/2}$ = 444 ± 40 min.

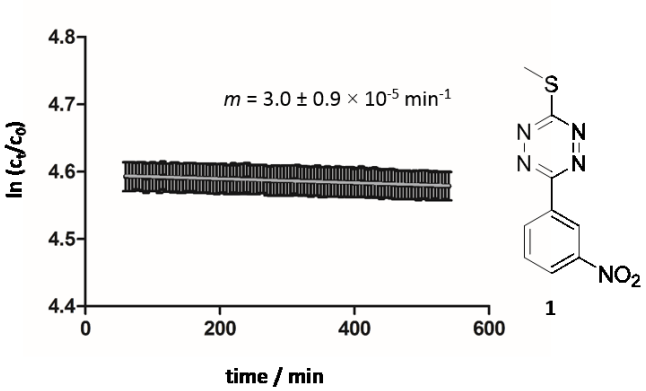


Figure S4. Plot of normalised natural logarithm of tetrazine **1** concentration c_t against time in DMSO/H_2O (1:1) with $t_{1/2}$ = 15.9 ± 4.7 days.

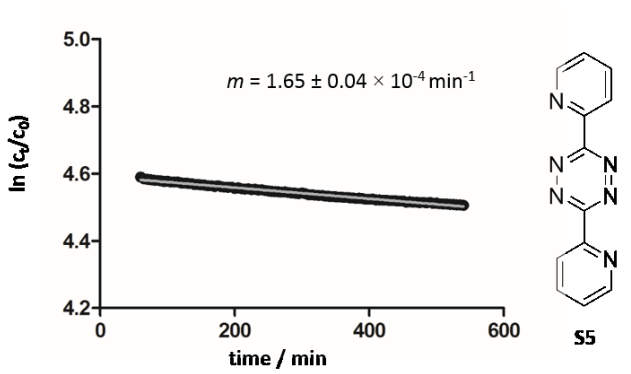


Figure S5. Plot of normalised natural logarithm of tetrazine **S5** concentration c_t against time in DMSO/H_2O (1:1) with $t_{1/2}$ = 2.9 ± 0.1 days.

Stability in the Presence of Glutathione

The stability of tetrazine **1** in the presence of glutathione was determined by HPLC. Tetrazine **1** (2 mM) was incubated together with reduced glutathione (5 mM) in CH_3CN/H_2O (1:1) at 37 °C. In addition, one sample of tetrazine **1** (2 mM) without glutathione was prepared as a reference. After 3 days, a sample (20 µl) was taken and diluted in CH_3CN (100 µL) containing 0.1 % HCO_2H and 1,3,5-trimethoxybenzene as a standard. Samples were analysed by analytical HPLC (10 min, gradient: 5% CH_3CN to 95 % in 6 min, 95 % for 3 min, 5 % for 1 min, 254 nm). After 3, days 77% of tetrazine remained in the presence of GSH.

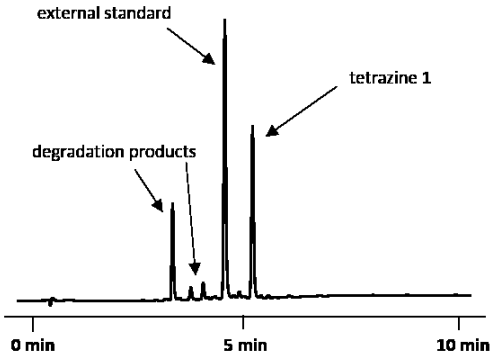
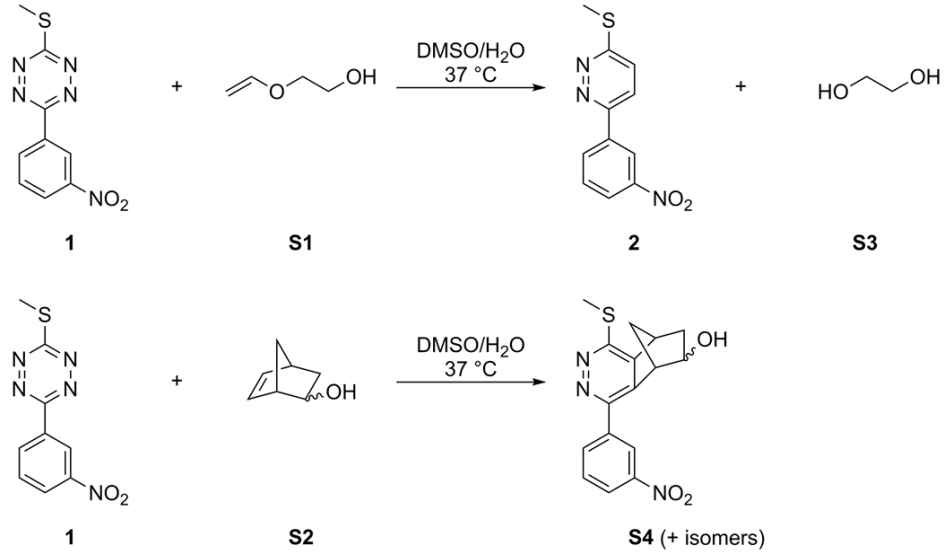


Figure S6. HPLC chromatogram of tetrazine **1** after incubation with glutathione for 3 days.

Water dependency of reaction constants



Scheme S7. A) Reaction between tetrazine **1** and vinyl ether **S1** was monitored over time at 37 °C in DMSO/H_2O; **B)** Reaction between tetrazine **1** and norbornene **S2** was monitored over time at 37 °C in DMSO/H_2O (7:3). Different regio- and stereoisomers of **S4** were produced as expected.

Second order rate constants (k_2) were determined using pseudo-first order conditions that were obtained by monitoring the absorption of tetrazine **1** (5 mM, λ_{abs} 520 nm) during treatment with an excess of vinyl ether **S1** or norbornene **S2** at concentrations between 12.5 mM and 150 mM in DMSO/water with various amount of water at 37 °C for 12 h. **S1** was chosen as a model vinyl ether because it is slightly water soluble allowing the determination of the reaction rate constant with an increasing amount of water. Compared to other vinyl ethers (such as benzyl vinyl ethers), however, is it slightly less reactive most likely due to the minimal hydrophobic interactions. Using calibration lines, the decreasing concentration of tetrazine **1** was plotted over time to give the k_{obs} values (the slope). k_2 was then determined by plotting k_{obs} against the concentrations of vinyl ether **S1** or norbornene **S2**, with k_2 being the slope of the corresponding linear fit (Figure S1 and S2).

In order to confirm the water dependency of the reaction, we carried out kinetic measurements with the more water soluble (but with the slightly less reactive) ethylene glycol vinyl ether (**S1**) as a model dienophile that allowed us to use differing amounts of water (up to 50:50 DMSO/H_2O). Thus for the dienophile ethylene glycol vinyl ether (**S1**) an increase in the second order rate constant by a factor of 10 was observed upon increasing the amount of water from 90:10 to 50:50 (DMSO/H_2O) in accordance with the reviewers belief. This rate constant for the tetrazine (**1**) with ethylene glycol vinyl ether was 2.1 ± 0.3 × 10^{-4} M^{-1} s^{-1} in full agreement with recent literature of tetrazine reactions using non-strained dienophiles.[2,3,4]

Using our data (3 data points; n = 3) allowed plotting and fitting of the rates versus the amount of water and gave an excellent fit to the exponential equation (1) (R^2 of 0.998).

$$k_2 = k_2^0 (1+r)^{\text{water content}} \quad (1)$$

r = 0.0504

k_2^0 = 1.679 × 10^{-5} M^{-1} s^{-1}

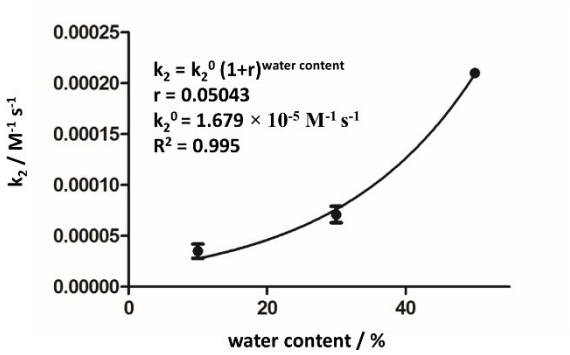


Figure S8. Determined k_2 of the reaction between tetrazine **1** and vinyl ether **S1** plotted versus the water content of the reaction milieu giving an exponential growth.

Extrapolation of this data to 100% water gives a reaction rate of 2.3 × 10^{-3} M^{-1} s^{-1}, similar to those values suggested in the literature for reactions in water.[5,6]

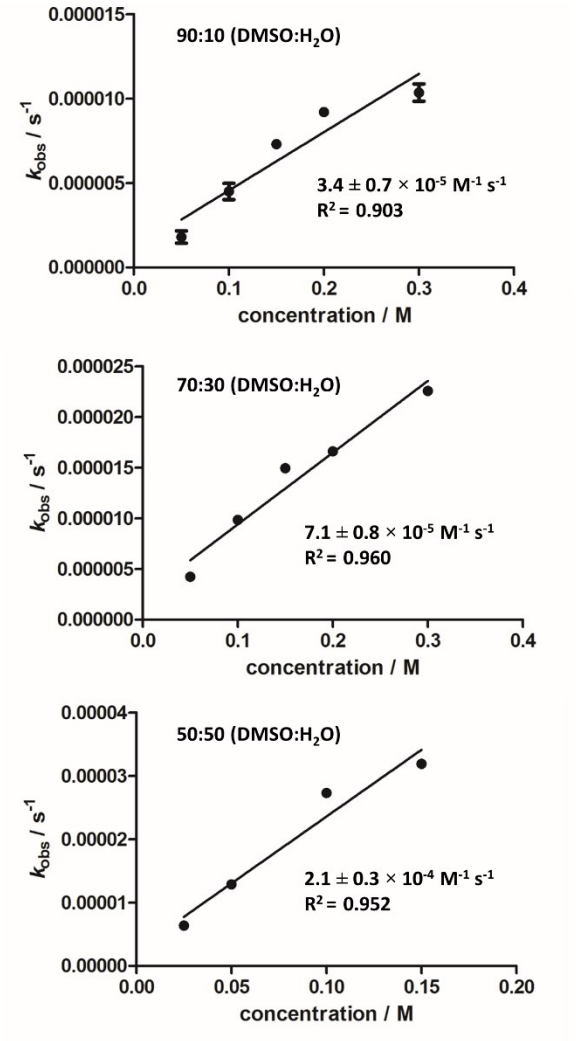


Figure S9. Determination of k_2 for the reaction between tetrazine **1** and vinyl ether **S1** at 37 °C in DMSO/H_2O by plotting k_{obs} *vs* concentration of dienophile.

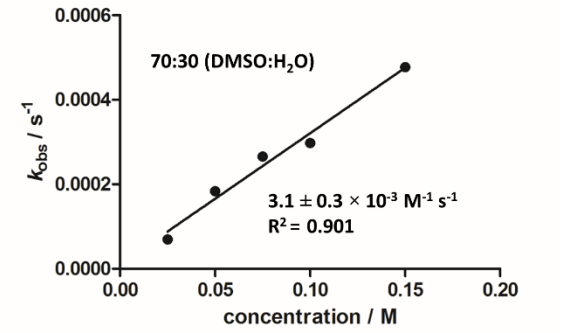


Figure S10. Determination of k_2 for the reaction between tetrazine **1** and norbornene **S2** at 37 °C in DMSO/H_2O (7:3) by plotting k_{obs} *vs* concentration.

Co-treatment of PC3 cells with miR21 inhibitor and campothecin

In order to determine the effect of co-treatment with the two drugs, PC3 cells were co-treated with miR21 inhibitor **2** (10 µM) and campothecin **4** (various concentrations, Figure S9) indicating an additive effect of the cytotoxicity of the two drugs. Cell viability was measured by MTT assay after 72 h of incubation at 37 °C (n = 3).

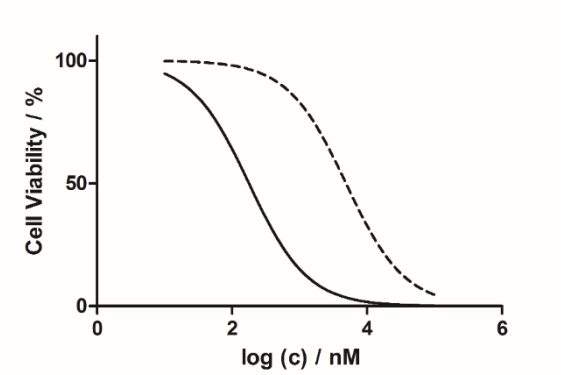


Figure S11. SK-BR3 cell IC_{50} determination with camptothecin **4** (solid line IC_{50} = 0.18 ± 0.07 µM) and vinyl-*O*-camptothecin **3** (dashed line IC_{50} = 4.88 ± 2.09 µM) following incubation for 72 h at 37 °C (MTT assay, n = 3).

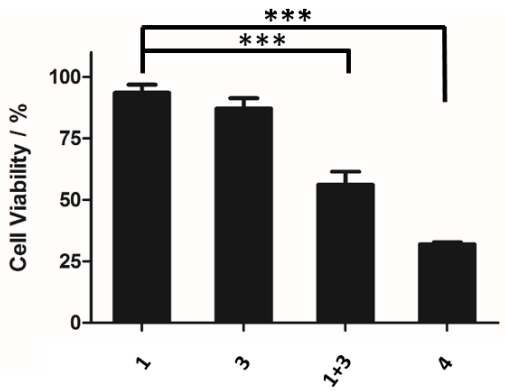
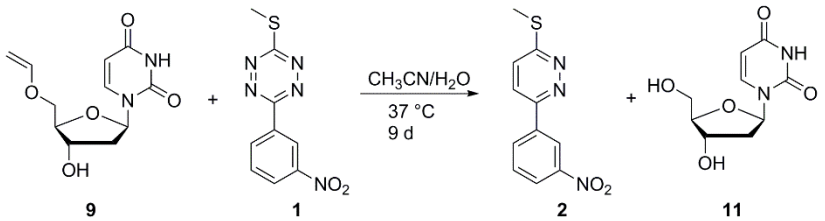


Figure S12. Cell viability of SK-BR3 cells after incubation (72 h) with tetrazine **1** (10 µM) = 94 ± 3 %, vinyl-*O*-camptothecin **3** (0.5 µM) = 88 ± 4 %, co-treatment with tetrazine **1** (10 µM) and vinyl-*O*-camptothecin **3** (0.5 µM) = 60 ± 5 %, camptothecin **4** (0.5 µM) 36 ± 1 %.

HPLC Reaction Monitoring



Scheme S2. Reaction between tetrazine **1** and vinyl-*O*-nucleoside **9** (monitored by HPLC).

The reaction between tetrazine **1** and vinyl-*O*-nucleoside **9** (Scheme S3) was monitored as follows. Stock solutions (100 mM, DMSO) of **1** and **9** were diluted into CH_3CN/H_2O (1:1) to give final concentrations of 32 mM and 16 mM, respectively. The solution was heated to 37 °C and stirred. At certain time points (see Figure S4), a sample (20 µL) was taken and diluted in CH_3CN (100 µL) containing 0.1 % HCO_2H. Samples were analysed by analytical HPLC (10 min, gradient: 5% CH_3CN to 95 % in 6 min, 95 % for 3 min, 5 % for 1 min, 254 nm). It should be noted here that vinyl-*O*-nucleoside **9** gives a very broad signal in HPLC most likely due to dimerization and/or different protonation states; however, the ^1H NMR and ^{13}C NMR spectra showed high purity (see page 22).

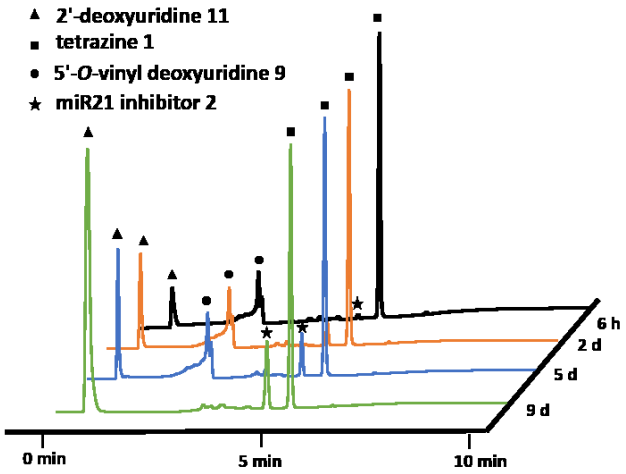
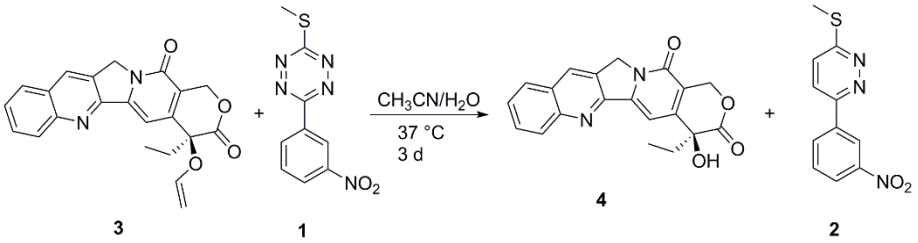


Figure S13. HPLC chromatogram of reaction between tetrazine **1** and vinyl-*O*-nucleoside **9** monitored over 9 days.



Scheme S3. Reaction between tetrazine **1** and vinyl-*O*-camptothecin **3** (monitored by HPLC).

The removal of the vinyl group from vinyl-*O*-camptothecin **3** by tetrazine **1** was monitored by HPLC. Stock solutions (100 mM, DMSO) of **3** and **1** were diluted in $CH_3CN/CH_3OH/H_2O$ (4:5:1) to give final concentrations of 0.5 mM and 10 mM, respectively. At defined time points samples were taken and diluted in CH_3CN containing 0.1 % HCO_2H and analysed by analytical HPLC (10 min, gradient: 5% CH_3CN to 95 % in 6 min, 95 % for 3 min, 5 % for 1 min, 350 nm). After 5 days, 85 % conversion of the vinyl-*O*-camptothecin **3** to camptothecin **4** was observed (Figure S5, signals not labelled belong to the standard (resorufin) and possible to the degraded tetrazine (e.g. tetrazine sulfoxide **S6**)).

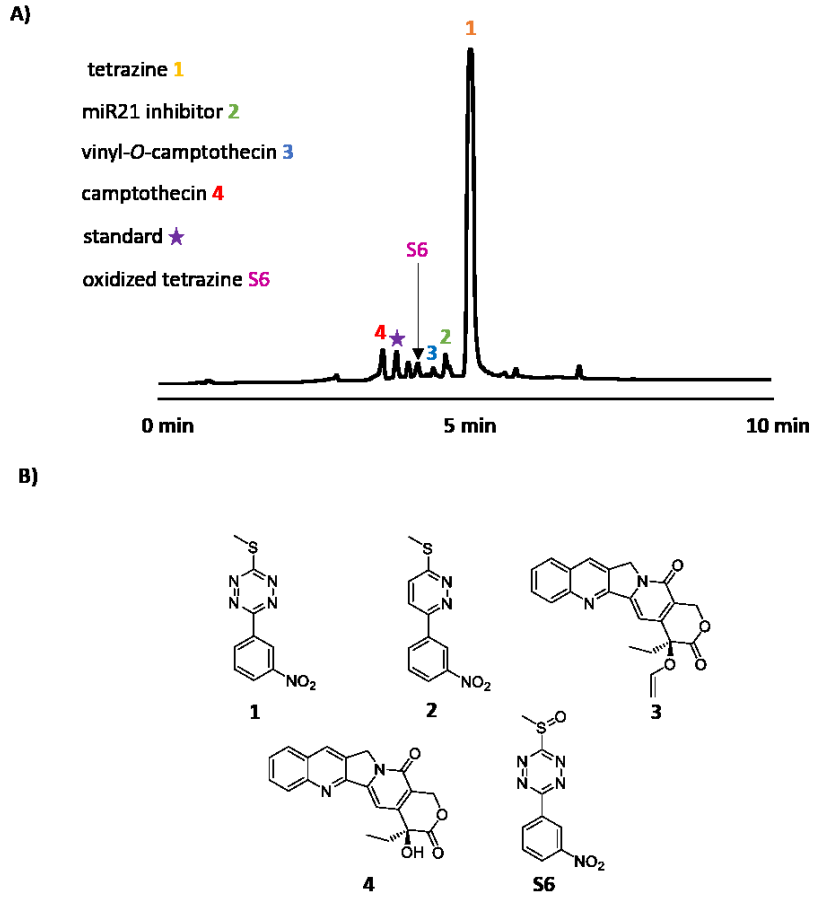


Figure S14. A) HPLC chromatogram of the reaction between tetrazine **1** and vinyl-*O*-camptothecin **3** after 5 days (with detection at 350 nm). **B)** Structures of starting materials (**1** and **3**), products (**2** and **4**) and indicated byproduct **S6**.

Dual Traceless Prodrug Activation with SK-BR3 cells

Activation of vinyl-*O*-camptothecin **3** with tetrazine **1** was investigated in the presence of SK-BR3 cells. Cell viability assays showed a similar cytotoxicity for the free camptothecin **4** (IC_{50} = 0.18 ± 0.07 µM, Figure S12) compared to PC3 cells (IC_{50} = 0.15 ± 0.06 µM, Figure 3) cell viability assays showed a switch-on of cytotoxicity upon co-treatment of vinyl-*O*-camptothecin **3** (0.5 µM) with tetrazine (10 µM) (Figure S8), although not as significant as shown for PC3 cells.

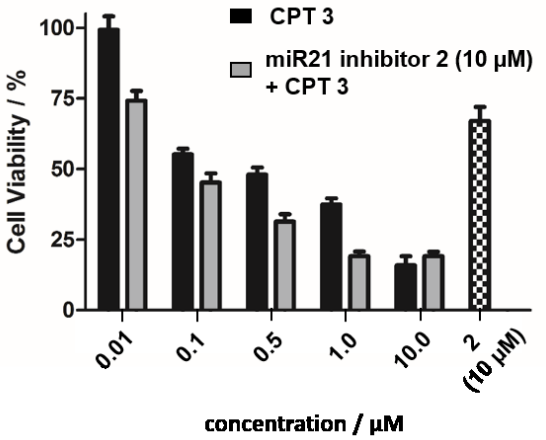


Figure S15. Cell viability of PC3 cells after co-treatment with miR21 inhibitor **2** (10 µM) and campothecin **4** (various concentration) for 72h.

NMR Spectra

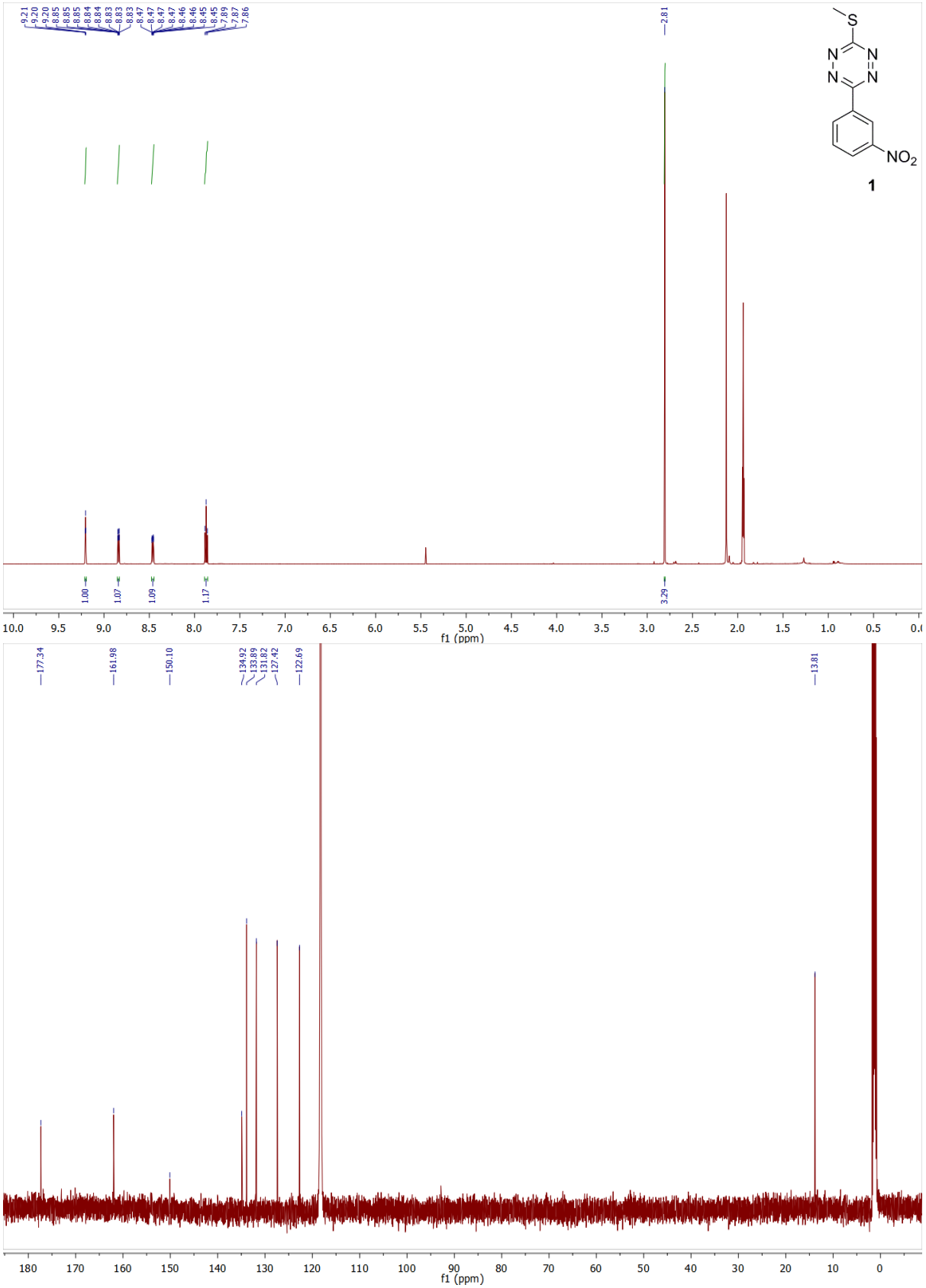


Figure S15. ^{1}H and ^{13}C NMR spectra of compound **1** recorded in CD_3CN at 500 MHz and 126 MHz, respectively.

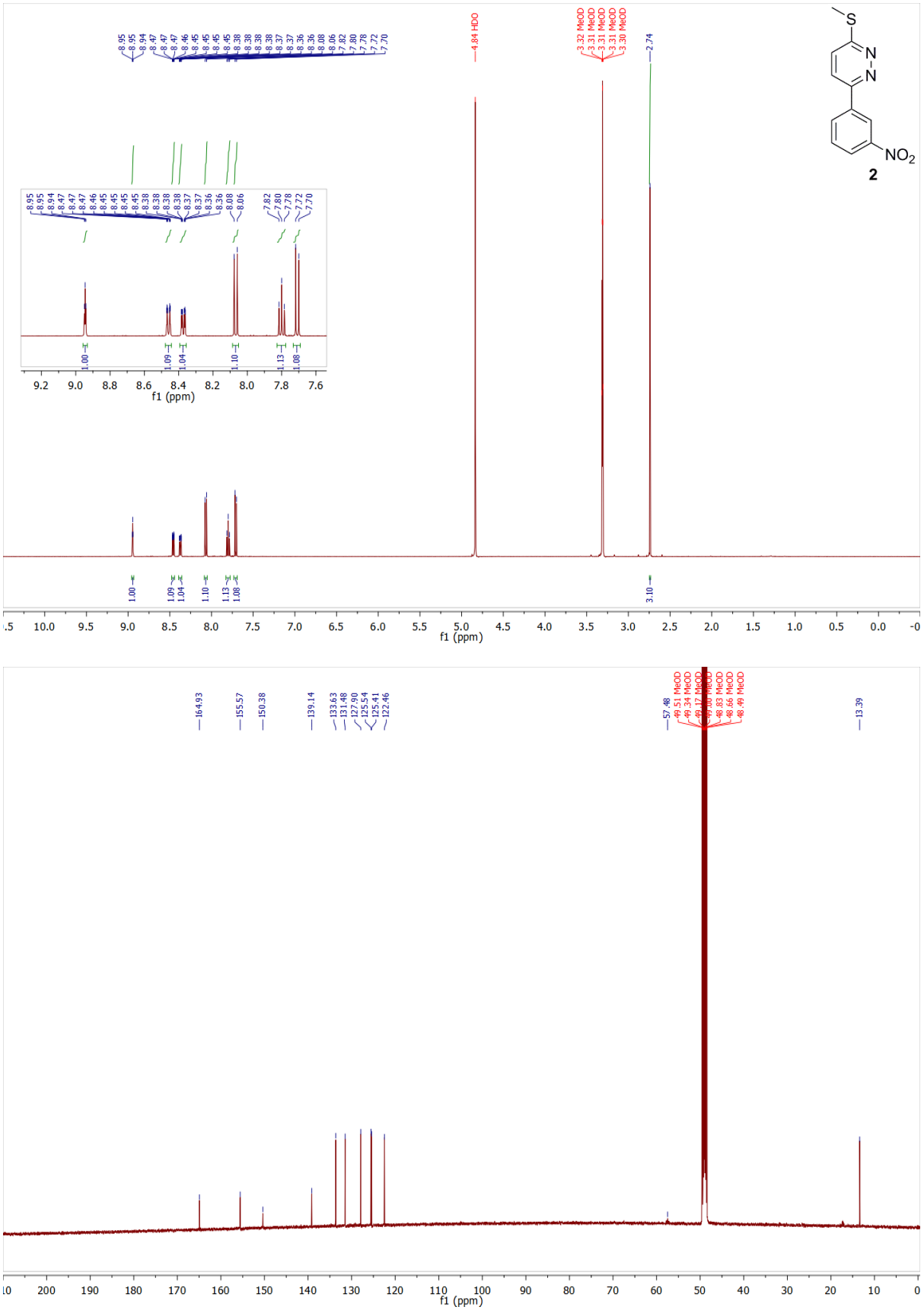


Figure S16. ^{1}H and ^{13}C NMR of compound **2** recorded in CD_3OD at 500 MHz and 126 MHz respectively.

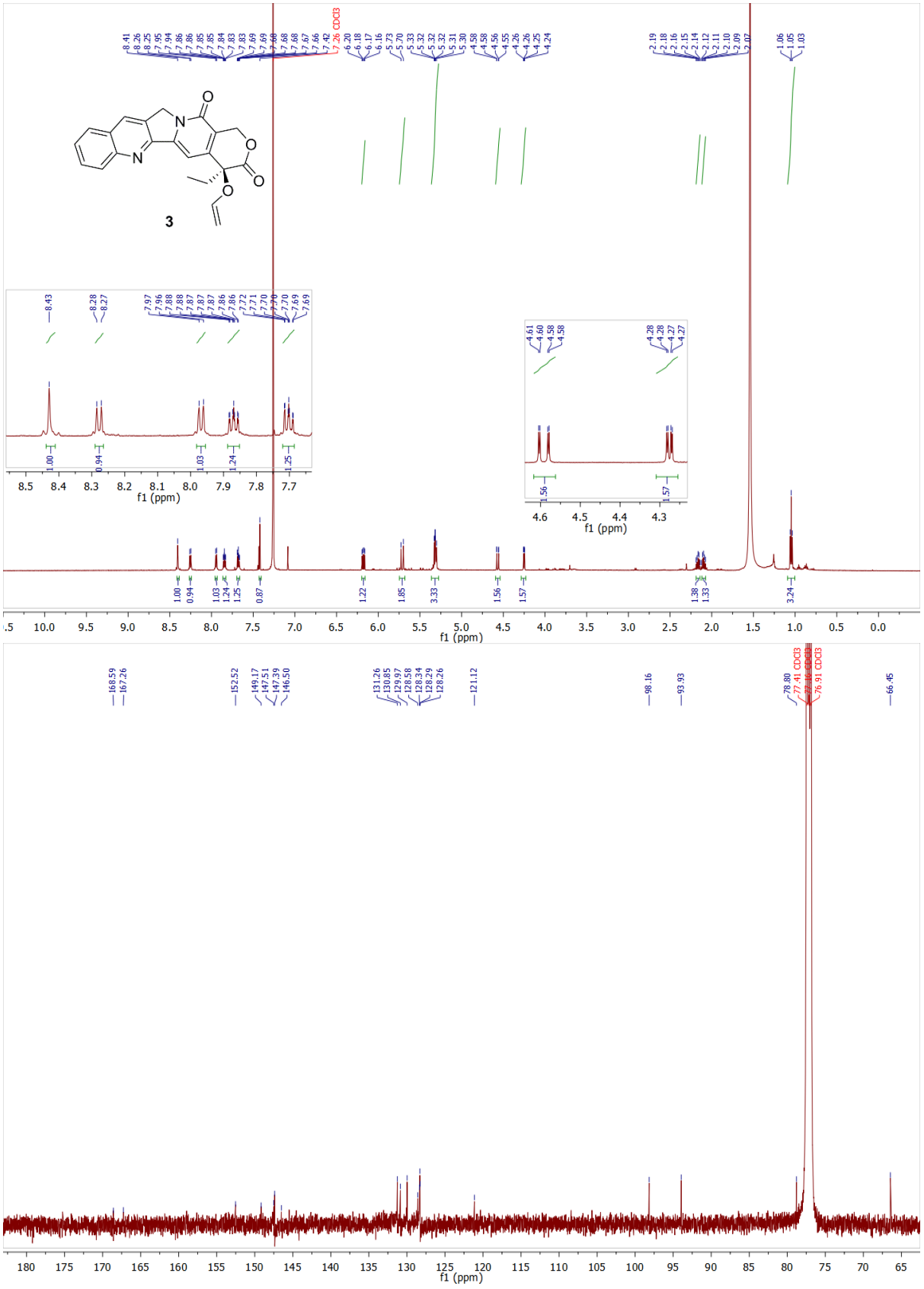


Figure S17. ^{1}H and ^{13}C NMR of compound **3** recorded in $CDCl_3$ at 600 MHz and 150 MHz respectively.

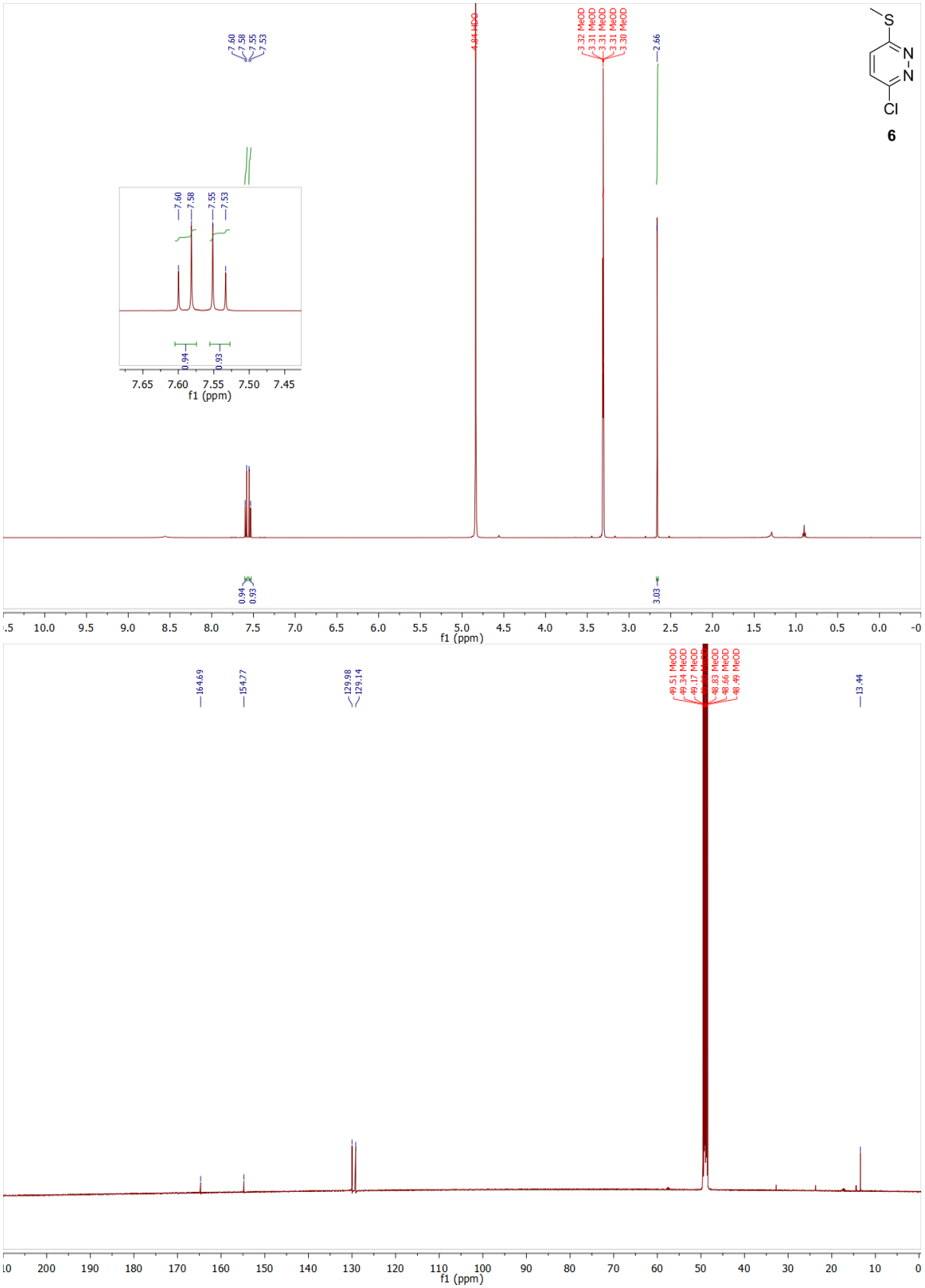


Figure S18. ^{1}H and ^{13}C NMR of compound **6** recorded in CD_3OD at 500 MHz and 126 MHz, respectively.

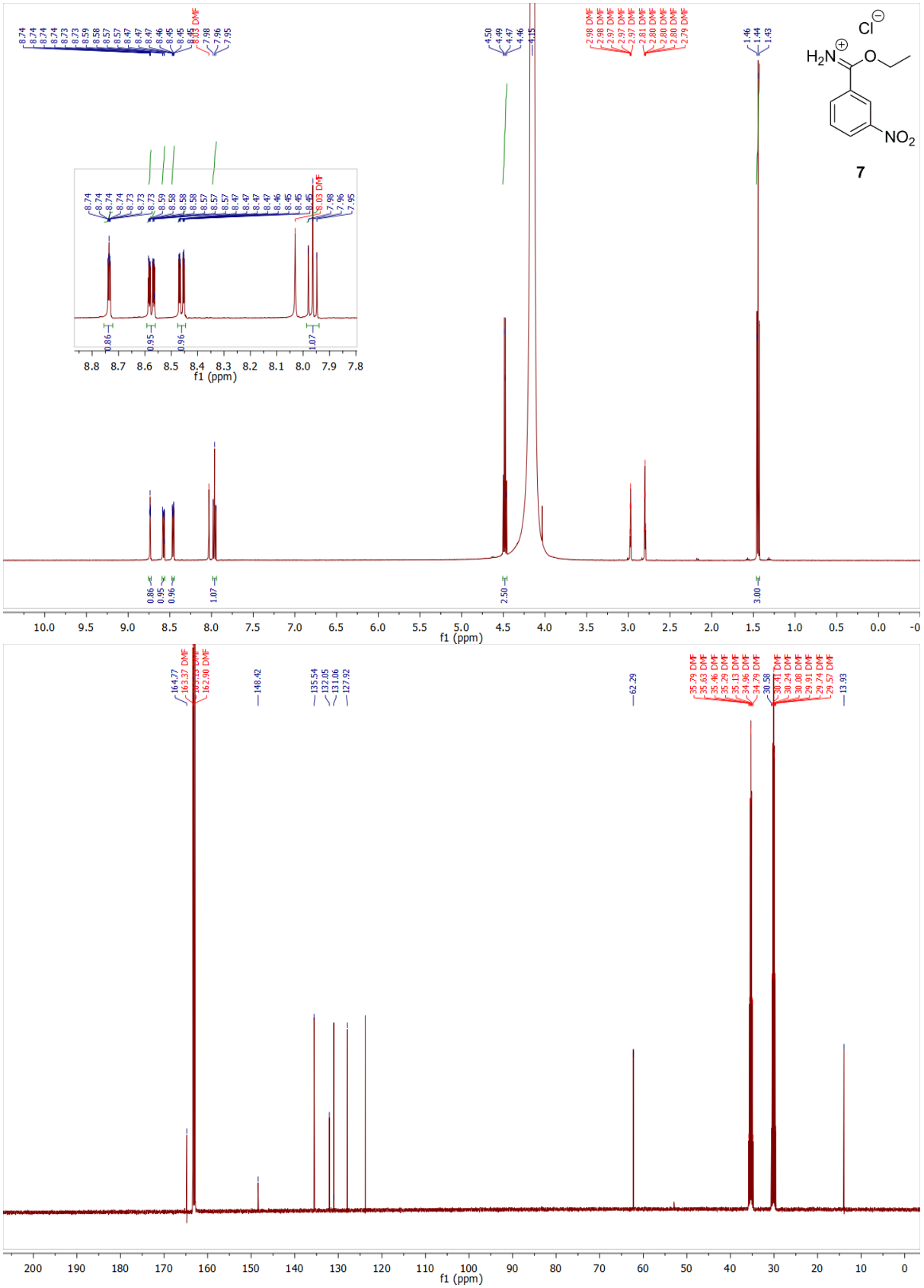


Figure S19. ^{1}H and ^{13}C NMR of compound **7** recorded in DMF-d_7 at 500 MHz and 126 MHz respectively.

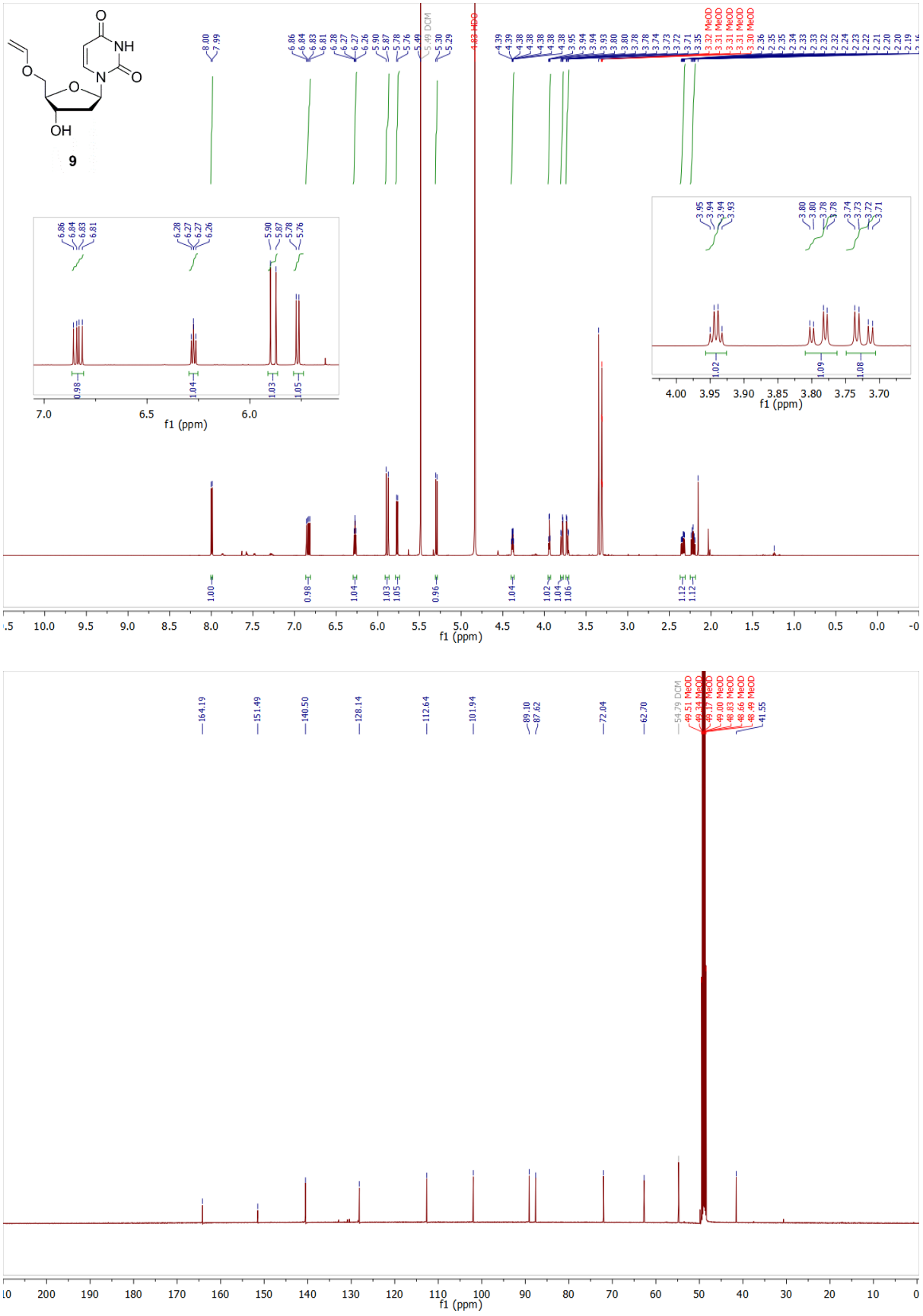


Figure S20. ^{1}H and ^{13}C NMR of compound **9** recorded in CD_3OD at 600 MHz at 150 MHz respectively.

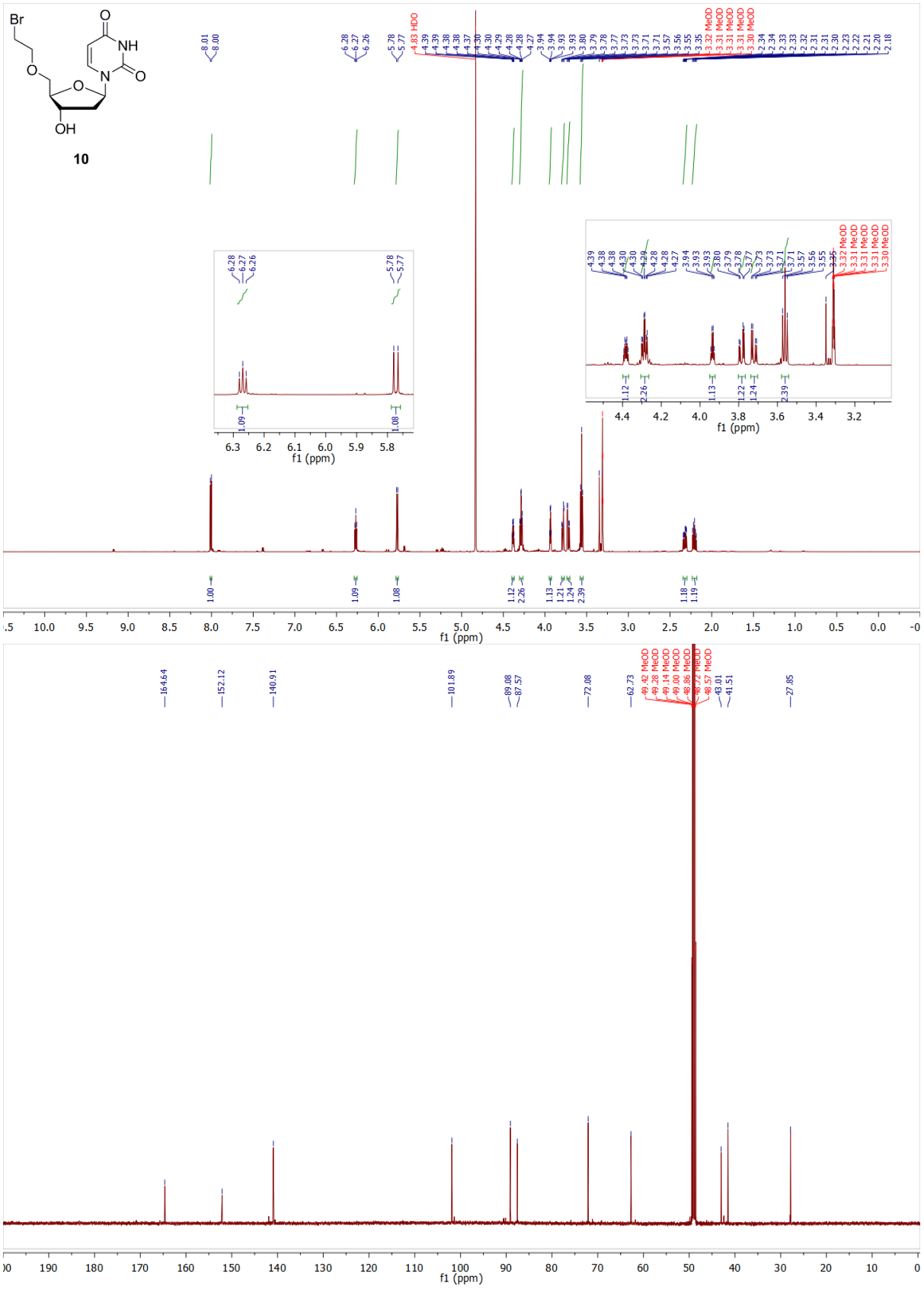


Figure S21. ^{1}H and ^{13}C NMR of compound **10** recorded in CD_3OD at 600 MHz and 150 MHz, respectively.

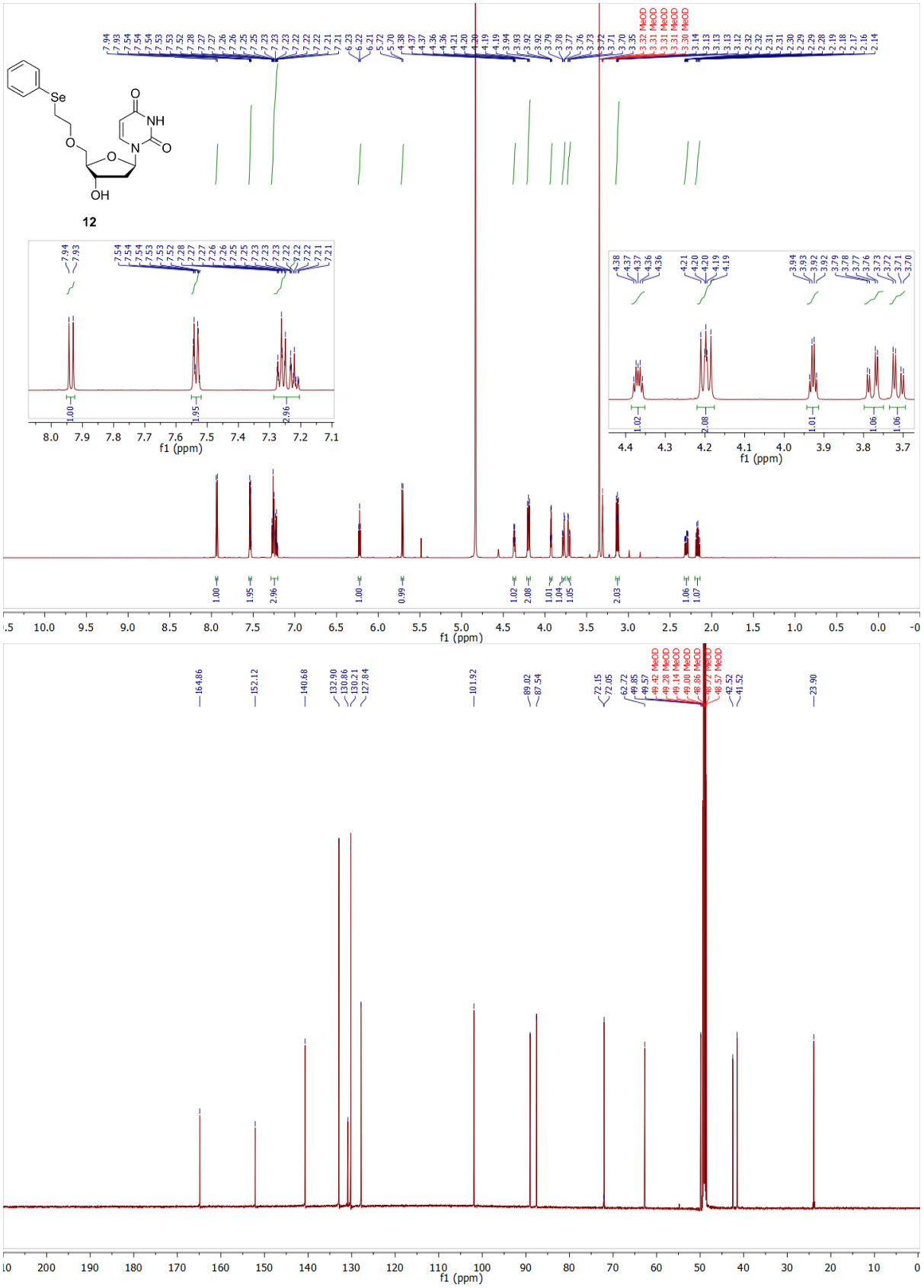


Figure S22. ^{1}H and ^{13}C NMR of compound **12** recorded in CD_3OD at 600 MHz and 150 MHz, respectively.

Biological Assays

General

U87-MG, SK-BR3 and PC3 cells were grown in 25 cm^2 tissue culture flasks (Corning) in DMEM supplemented with 10% FBS (BIOSERA FB-1090/500), L-glutamine (100 units, Gibco) and penicillin/streptomycin (100 units/mL, Sigma P4333). Cells were incubated at 37.4 °C with 5 % CO_2. Flow cytometry was performed on Becton Dickinson FACScan and analysed with FlowJo software.

Flow Cytometry Analysis

For flow cytometry measurements, cells were plated in 24-well plates (80,000 cells per well) and incubated overnight prior to incubation with the corresponding compounds. Stock solutions of **1**, **2**, **3**, **4** and **9** (10 mM, DMSO) were diluted in DMEM to twice the required concentration (e.g. 20 µM for 10 µM incubation of dienophile **9**). Cells were treated with each of these solutions (175 µL) without pre-mixing or any preincubation. Thus, for co-treatment with two compounds (e.g. tetrazine **1** and vinyl-*O*-camptothecin **3**) the final volume added was 350 µL. For single treatments (e.g. only camptothecin **4**) additional DMEM (50 µL) was added to give a final volume of 350 µL.
For miR21 inhibitor induced apoptosis assays, cells were incubated overnight (14 h) and for camptothecin induced apoptosis assays cells were incubated 4 h prior to treatment with the corresponding compounds. After washing with PBS and binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4), cells were stained with 5 µL of FITC labelled phosphatidylserine-binding protein before analysis by flow cytometry (λ_{Ex} 470 nm, λ_{Em} 500–554 nm). In a typical assay, between 5,000 and 10,000 cells were analysed. Forward versus side scatter profiles were used to gate intact cells (Figure S19).

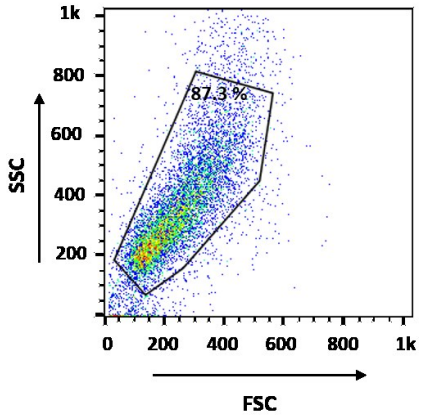


Figure S23. Typical gating of SK-BR3 cells using side scattering versus forward scattering.

MTT Assay

For MTT assays, cells were plated in 96-well plates (10,000 cells per well) and incubated overnight prior to incubation with the corresponding compounds. Stock solutions of **1**, **2**, **3**, **4** and **9** (10 mM, DMSO) were diluted in DMEM to twice the required concentration (e.g. 20 µM for 10 µM incubation of dienophile **9**). Cells were treated with each of these solutions (50 µL) without pre-mixing or any preincubation. Thus, for co-treatment with two compounds (e.g. tetrazine **1** and vinyl-*O*-camptothecin **3**) the final volume added was 100 µL. For single treatments (e.g. only camptothecin **4**) additional DMEM (50 µL) was added to give a final volume of 100 µL. After 72 h, the media was removed and the cells incubated with thiazolyl blue tetrazolium bromide (1.5 mM) in PBS/DMEM (6:2, 100 µL per well) for 2.5 h. MTT solubilising solution (100 µL of a stock solution prepared using 50 mL Triton-X100, 450 ml ^{i}PrOH, 10 drops of 12 M HCl) was added, and the absorbance at 570 nm measured by a plate reader. To determine the IC_{50} values, MTT assays were carried out with concentrations of 10 nM, 100 nM, 500 nM, 1 µM, 10 µM and 100 µM of the corresponding compounds for 72 h at 37.4 °C in a 96-well plate. The cell viability was plotted against concentrations and IC_{50} values were obtained by non-linear fit.

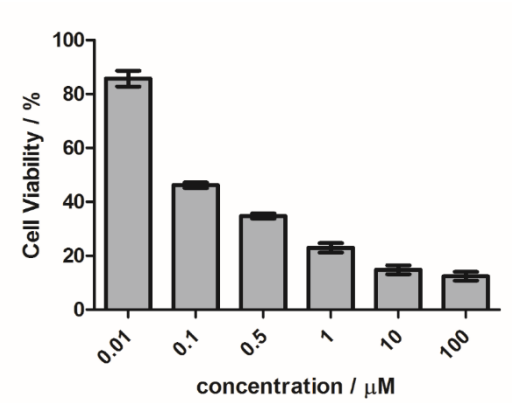


Figure S24. Incubation of PC3 cells with camptothecin **4** (0.01 μM, 0.1 μM, 0.5 μM, 1μM, 10 μM and 100 μM) for 72 h. Cell viability was determined with an MTT assay.

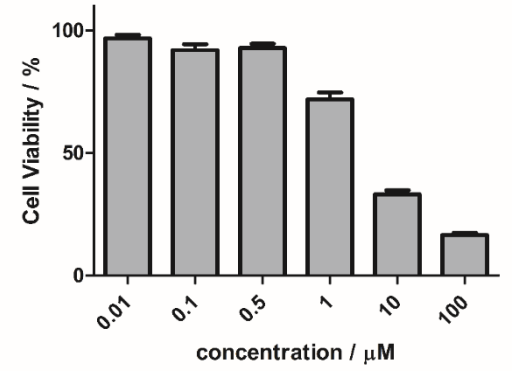


Figure S25. Incubation of PC3 cells with vinyl-*O*-camptothecin **3** (0.01 μM, 0.1 μM, 0.5 μM, 1 μM, 10 μM and 100 μM) for 72 h. Cell viability was determined with an MTT assay.

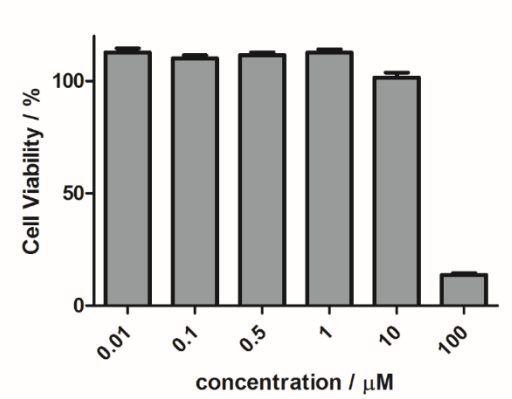


Figure S26. Incubation of PC3 cells with tetrazine **1** (0.01 μM, 0.1 μM, 0.5 μM, 1 μM, 10 μM and 100 μM) for 72 h. Cell viability was determined with an MTT assay.

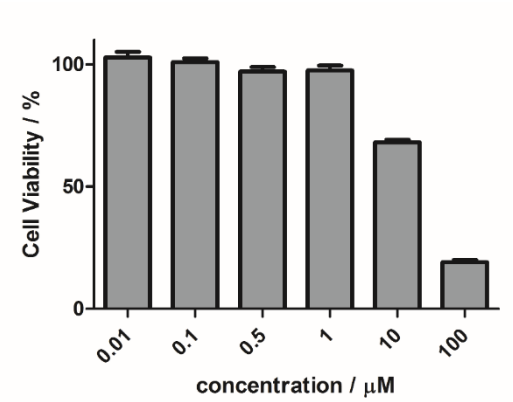


Figure S27. Incubation of PC3 cells with miR21 inhibitor **2** (0.01 μM, 0.1 μM, 0.5 μM, 1 μM, 10 μM and 100 μM) for 72 h. Cell viability was determined with an MTT assay.

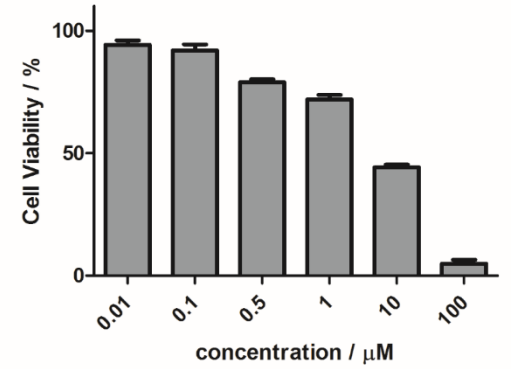


Figure S28. Incubation of SK-BR3 cells with vinyl-O-camptothecin **3** (0.01 μM, 0.1 μM, 0.5 μM, 1 μM, 10 μM and 100 μM) for 72 h. Cell viability was determined with an MTT assay.

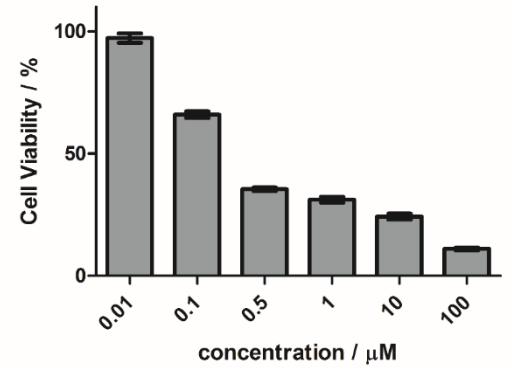


Figure S29. Incubation of SK-BR3 cells with camptothecin **4** (0.01 μM, 0.1 μM, 0.5 μM, 1 μM, 10 μM and 100 μM) for 72 h. Cell viability was determined with an MTT assay.

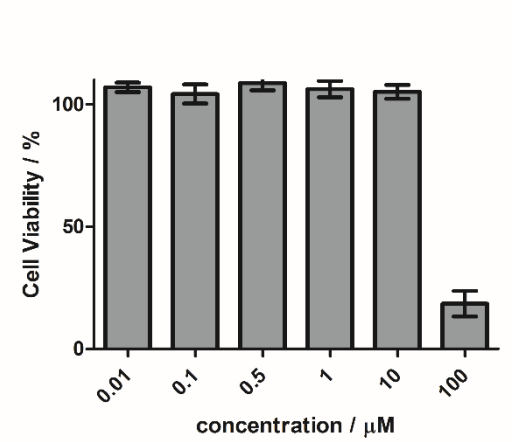


Figure S30. Incubation of SK-BR3 cells with tetrazine **1** (0.01 µM, 0.1 µM, 0.5 µM, 1 µM, 10 µM and 100 µM) for 72 h. Cell viability was determined with an MTT assay.

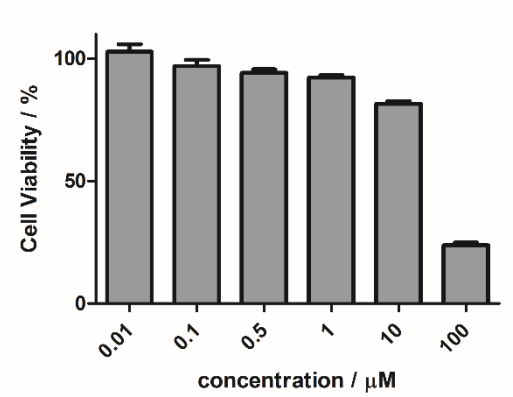


Figure S31. Incubation of SK-BR3 cells with miR21 inhibitor **2** (0.01 µM, 0.1 µM, 0.5 µM, 1 µM, 10 µM and 100 µM) for 72 h. Cell viability was determined with an MTT assay.

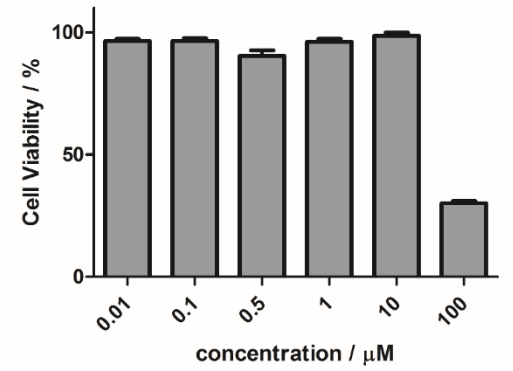


Figure S32. Incubation of U87MG cells with tetrazine **1** (0.01 µM, 0.1 µM, 0.5 µM, 1 µM, 10 µM and 100 µM) for 72 h. Cell viability was determined with an MTT assay.

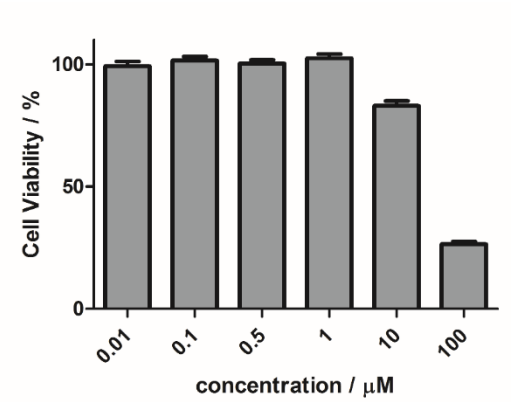


Figure S33. Incubation of U87MG cells with miR21 inhibitor **2** (0.01 μM, 0.1 μM, 0.5 μM, 1 μM, 10 μM and 100 μM) for 72 h. Cell viability was determined with an MTT assay.

References

(1) V. A. Vaillancourt, S. D. Larsen, S. P. Tanis, J. E. Burr, M. C. Connell, M. M. Cudahy, B. R. Evans, P. V. Fisher, P. D. May, M. D. Meglasson, D. D. Robinson, F. C. Stevens, J. A. Tucker, T. J. Vidmar, J. H. Yu, *J. Med. Chem.* 2001, **44** , 1231.

(2) E. Jiménez-Moreno, Z. Guo, B. L. Oliveira, I. S. Albuquerque, A. Kitowski, A. Guerreiro, O. Boutureira, T. Rodrigues, G. Jiménez-Osés, G. J. L. Bernardes, *Angew. Chemie Int. Ed.* 2017, **56**, 243.

(3) H. Wu, S. C. Alexander, S. Jin, and N. K. Devaraj, *JACS* 2016, **138**, 11429.

(4) B. L. Oliveira, Z. Guo, O. Boutureira, A. Guerreiro, G. Jiménez-Osés, G. J. L. Bernardes, *Angew. Chemie Int. Ed.* 2016, **55**, 14683.

(5) A. Meijer, S. Otto, J. B. F. N. Engberts *J. Org. Chem.*, 1998, **63**, 8989.

(6) A. Chanda V. V. Fokin *Chem. Rev.*, 2009, **109**, 725.