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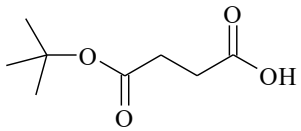
Synthesis and antitumor activity of bis-camptothecin ester of succinic acid

Nuri M. Chobanov, Lilya U. Dzhemileva, Konstantin O. Vakhnin, Usein M. Dzhemilev and Vladimir A. D'yakonov

Experimental

Chemistry

All commercial reagents were purchased from Sigma-Aldrich and Acros Organics. All commercial solvents and reagents used were of analytical grade and did not require further purification. Reactions were monitored by TLC on Sorbfil plates. Column chromatography was performed on Acros silica gel (0.060-0.200 мм). ^{1}H and ^{13}C NMR spectra were obtained using a Bruker AM-300 spectrometer in $CDCl_3$ at 300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C and a Bruker AV 600 spectrometer in CDCl3 at 600.1 MHz for 1H and 150.9 MHz for 13C. Mass spectra of MALDI TOF/TOF positive ions (matrix of sinapic acid) are recorded on a Bruker Au-toflexTM III Smartbeam mass spectrometer.



***tert*-Butyl hydrogen succinate.**[S1] Succinic anhydride (5 g, 50 mmol), *N*-hydroxysuccinimide (1.67 g, 0.15 mmol), and DMAP (0.59 g, 0.5 mmol) were dissolved in toluene (50 ml). *tert*-Butanol (5.85 ml, 62 mmol) and Et_3N (1.52 g, 2.09 ml, 15.0 mmol) were added sequentially. The suspension was heated under reflux for 48 h. The solution was cooled and diluted with EtOAc (50 mL) and was washed with citric acid (10% *w/v*, 100 ml) and brine (100 ml), dried over $MgSO_4$, and concentrated to give a brown solid. The pale yellow solid was recrystallised with ether at -20 °C to give the title monoester (7.9 g, 91%, mp 53 °C).

^{1}H NMR (300 MHz; δ, $CDCl_3$): 11.18(1H, s, CO_2**H**), 2.64 (2H, m, CO_2HC**H**$_2$, CH_2-5), 2.54 (2H, m, HO_2CCH_2C**H**$_2$, CH_2-4), 1.45 (9H, s, 3 × CH_3-1).

^{13}C NMR (75MHz; δ, $CDCl_3$): 178.70 (CO_2H, C-6), 171.46 ($CO_2C(CH_3)_3$, C-3), 81.05 ($C(CH_3)_3$, C-2), 30.14 (*C*H_2), 29.24 (*C*H_2), 28.04 ($C(CH_3)_3$, C-1).

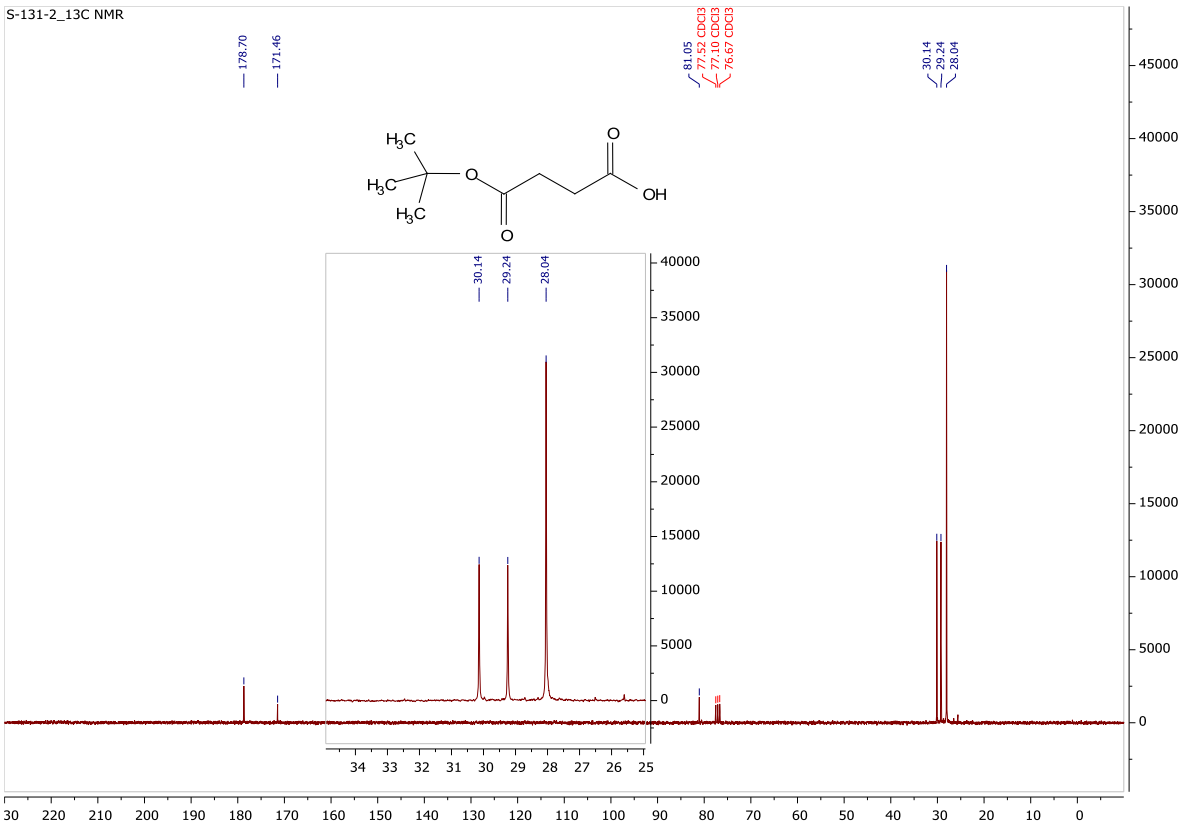


Figure S1. ^{13}C NMR spectrum of *tert*-butyl hydrogen succinate.

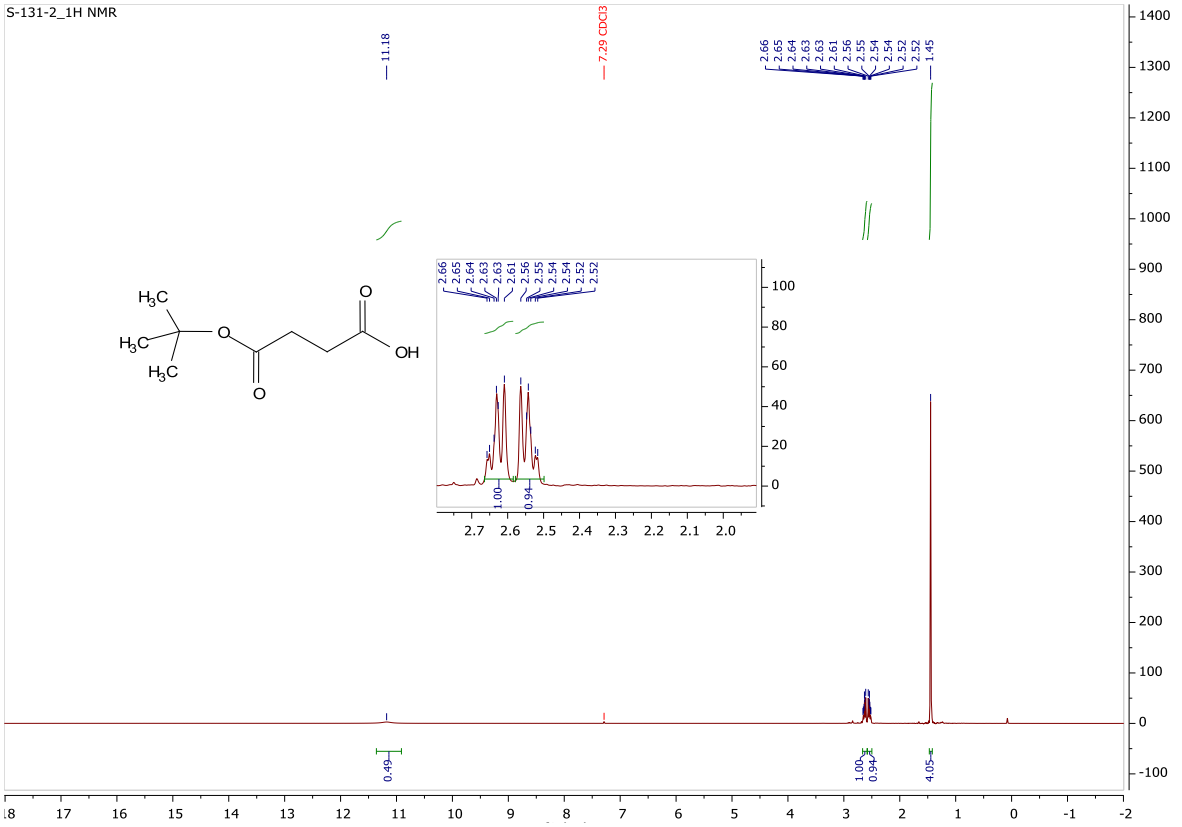
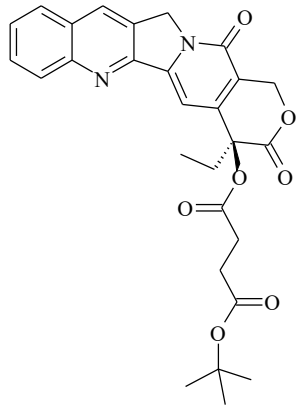


Figure S2. ^{1}H NMR spectrum of *tert*-butyl hydrogen succinate.

Synthesis of CPT-mono-*tert*-Butyl succinate conjugates 2.[S2]



***tert*-Butyl (*S*)-1-ethyl-3,5-dioxo-3,4,5,7-tetrahydro-1*H*-pyrano[4′,3′:6,7]indolizino[1,2-*b*]-quinolin-1-yl succinate** (**2**)**. A** solution of *tert*-butyl hydrogen succinate (348.4 mg, 2.00 mmol), camptothecin **1** (300 mg, 0.86 mmol), DMAP (404 mg, 3.3 mmol) and EDC*HCl (953 mg, 6.14 mmol) in 50 ml of anhydrous CH_2Cl_2 was stirred at room temperature for 12 h. After completion of the reaction the resulting mixture was diluted with 20 ml CH_2Cl_2 and extracted twice with 1M solution HCl.

The organic phase was dried with $MgSO_4$. The solvent was evaporated, the residue recrystallization from methanol or diethyl ether. The resulting precipitate was isolated and dried under vacuum. Compound **2** was obtained as a yellowish powder (399 mg, 92%).

^{1}H NMR (300 MHz, $CDCl_3$): δ_{ppm}: 8.37 (s, 1H), 8.22 (d, *J* = 8.5 Hz, 1H), 7.92 (d, *J* = 8.1 Hz, 1H), 7.87 – 7.75 (m, 1H), 7.64 (t, *J* = 7.5 Hz, 1H), 7.32 (s, 1H), 5.66 (d, *J* = 17.2 Hz, 1H), 5.37 (d, *J* = 17.2 Hz, 1H), 5.24 (s, 2H), 2.84 – 2.72 (m, 2H), 2.60 – 2.49 (m, 2H), 2.35 – 2.04 (m, 2H), 1.38 – 1.32 (m, 9H), 0.98 (t, *J* = 7.5 Hz, 3H).

^{13}C NMR (75MHz, $CDCl_3$): δ_{ppm}: 171.55, 171.05, 167.45, 157.40, 152.38, 148.80, 146.11, 131.20, 130.64, 129.65, 128.22, 128.17, 128.02, 120.13, 96.61, 80.96, 76.20, 67.04, 49.93, 31.80, 30.10, 29.19, 28.00, 7.67.

MS (ESI): 505.1968 [M+H]. Calculated for $C_{28}H_{28}N_2O_7$: 505.1969 [M+H]

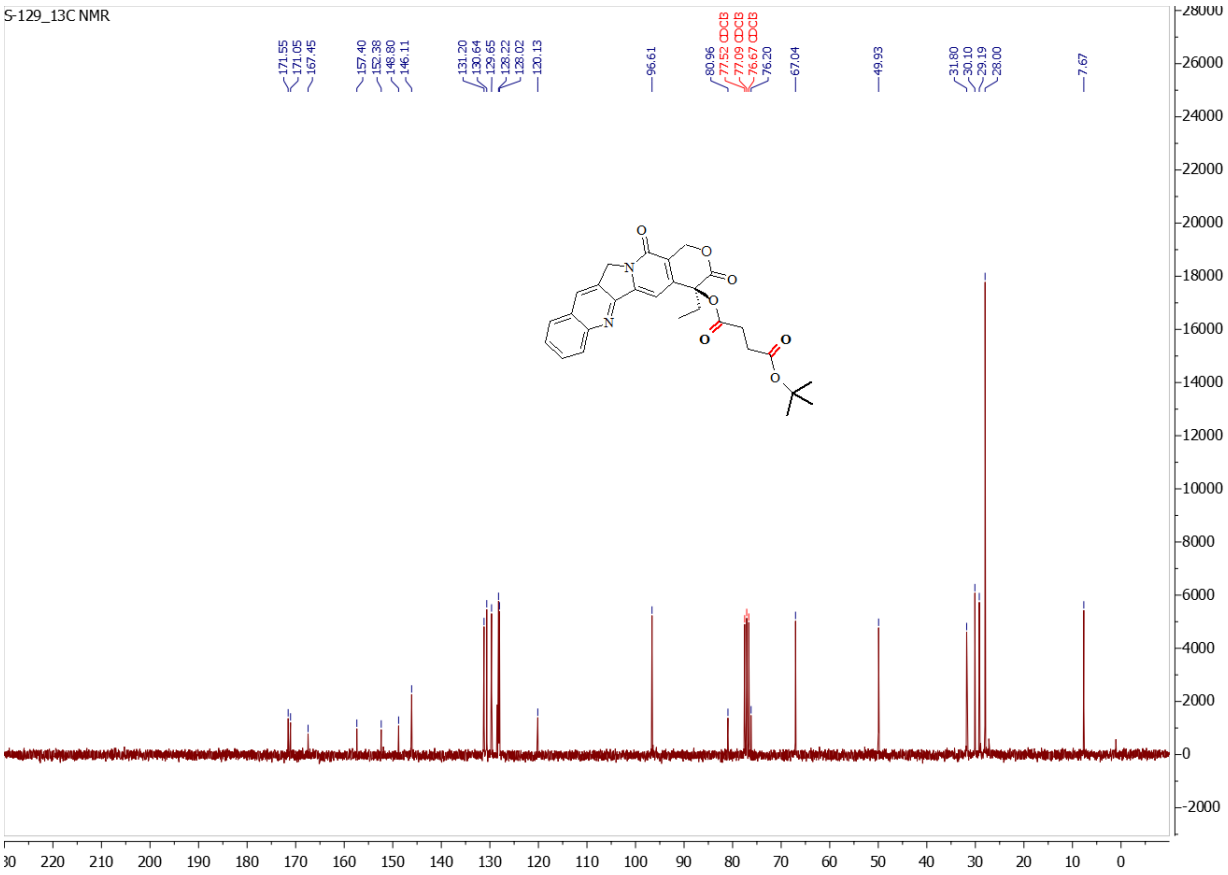


Figure S3. ^{13}C NMR spectrum of compound **2**.

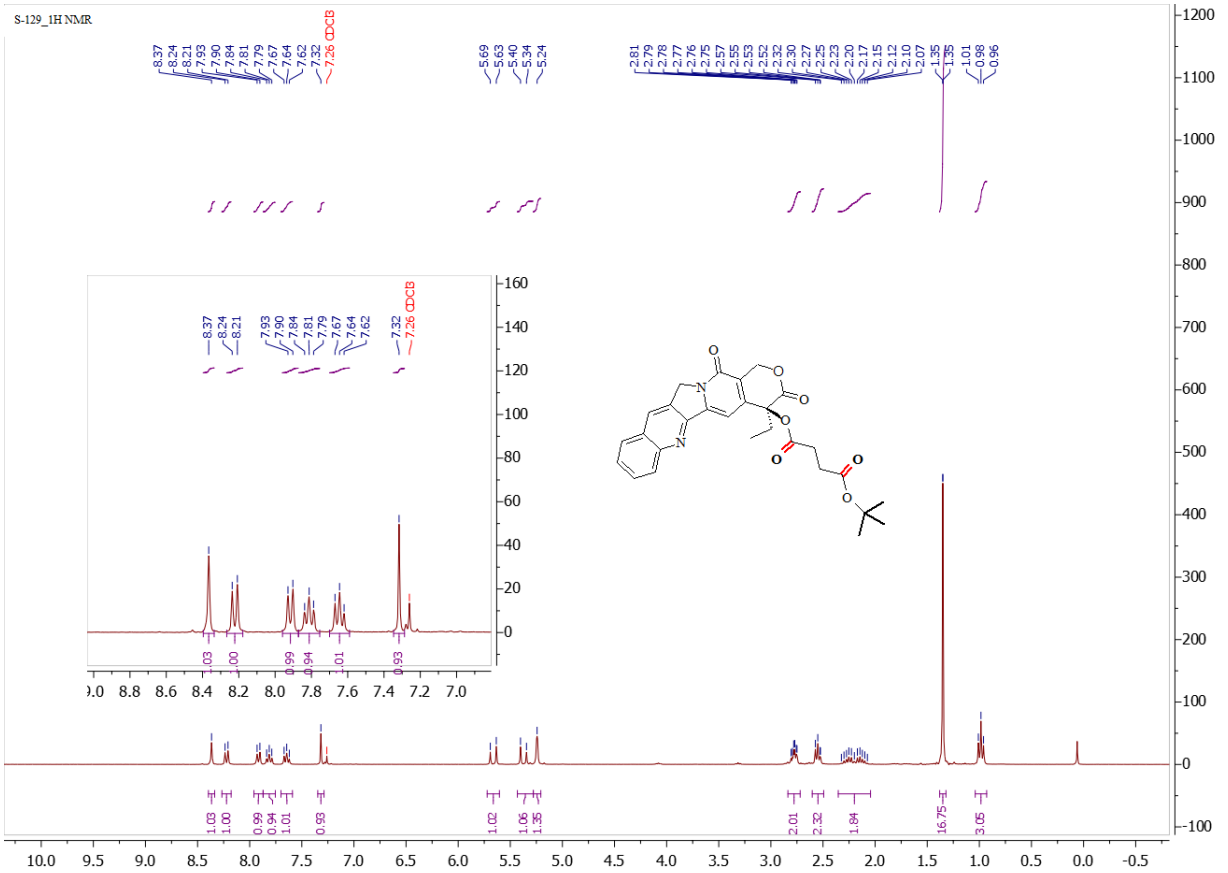
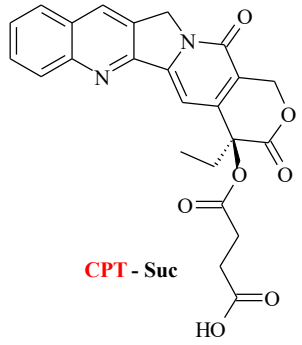


Figure S4. ^{1}H NMR spectrum of compound **2**

Synthesis of CPT-Succinic Acid (CPT-CO_2H) 3.[S2]



(*S*)-4-[(1-Ethyl-3,5-dioxo-3,4,5,7-tetrahydro-1*H*-pyrano[4′,3′:6,7]indolizino[1,2-*b*]quinolin-1-yl)oxy]-4-oxobutanoic acid (3). Conjugate **2** (399 mg, 0.79 mmol) was dissolved in CF_3COOH/CH_2Cl_2 (v/v: 3:2, 16 ml). The solution was stirred for 2 h at room temperature. Trifluoroacetic acid (CF_3COOH) was removed under reduced pressure. The crude product was dissolved in methanol. The precipitate was isolated, washed with methanol (MeOH) and dried under vacuum. A yellowish powder was obtained with a yield of 85% (301 mg).

^{1}HNMR (300 MHz; DMSO-d_6):δ_{ppm}: 8.70 (s, 1H), 8.17 (dd, *J* = 15.9, 8.3 Hz, 2H), 7.88 (t, *J* = 7.7 Hz, 1H), 7.73 (t, *J* = 7.5 Hz, 1H), 7.15 (s, 1H), 5.50 (s, 2H), 5.31 (s, 2H), 2.78 (q, *J* = 7.5 Hz, 2H), 2.47 (d, *J* = 6.9 Hz, 2H), 2.17 (q, *J* = 7.4 Hz, 2H), 0.93 (t, *J* = 7.4 Hz, 3H).

^{13}C NMR (75MHz; DMSO-d_6):δ_{ppm}:172.97, 171.27, 167.16, 156.51, 152.32, 147.86, 145.89, 145.29, 131.50, 130.36, 129.68, 128.96, 128.47, 127.92, 127.66, 118.90, 95.11, 75.89, 66.30, 50.14, 30.41, 28.58, 28.39, 7.53.

MS (ESI): 449.1343 [M+H]. Calculated for $C_{24}H_{20}N_2O_7$: 449.1341 [M+H]

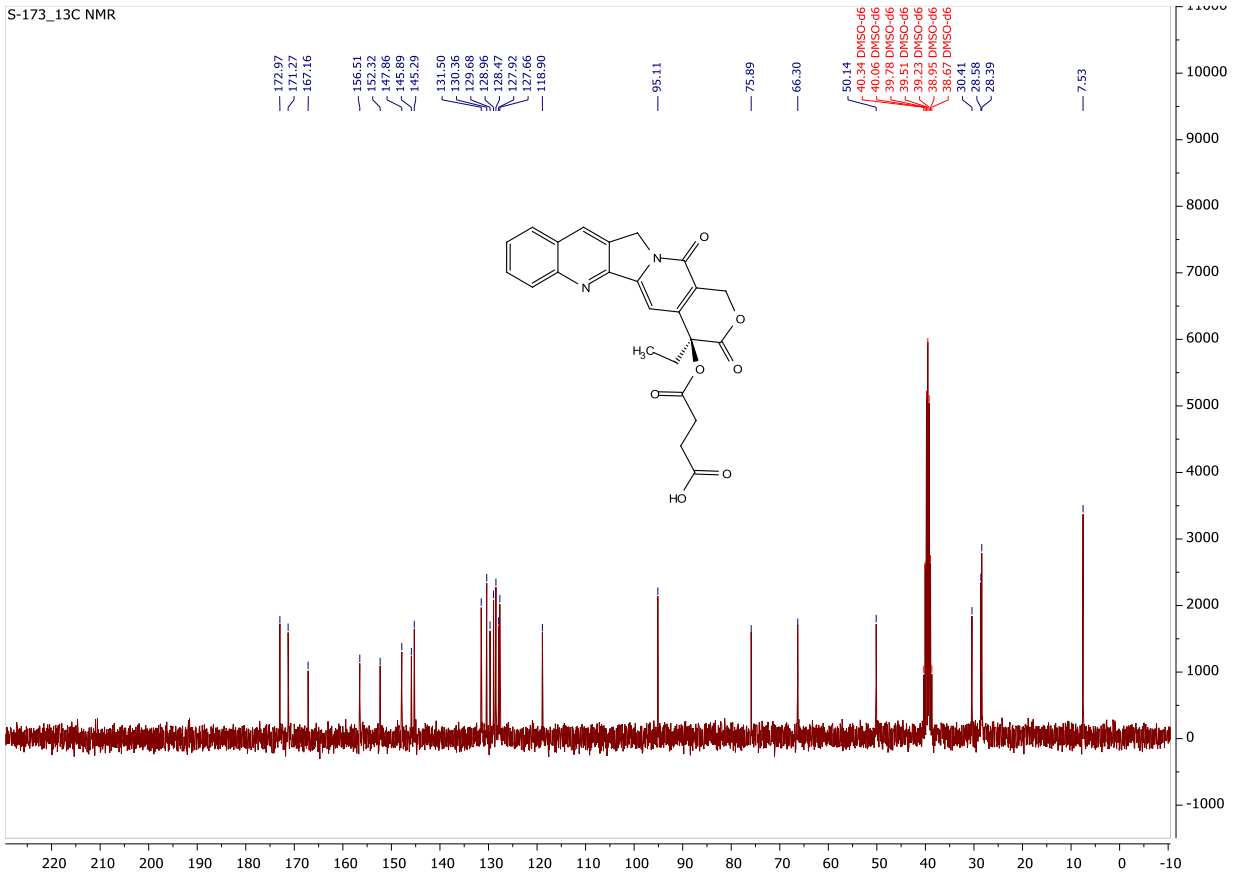


Figure S5. ^{13}C NMR spectrum of compound **3.**

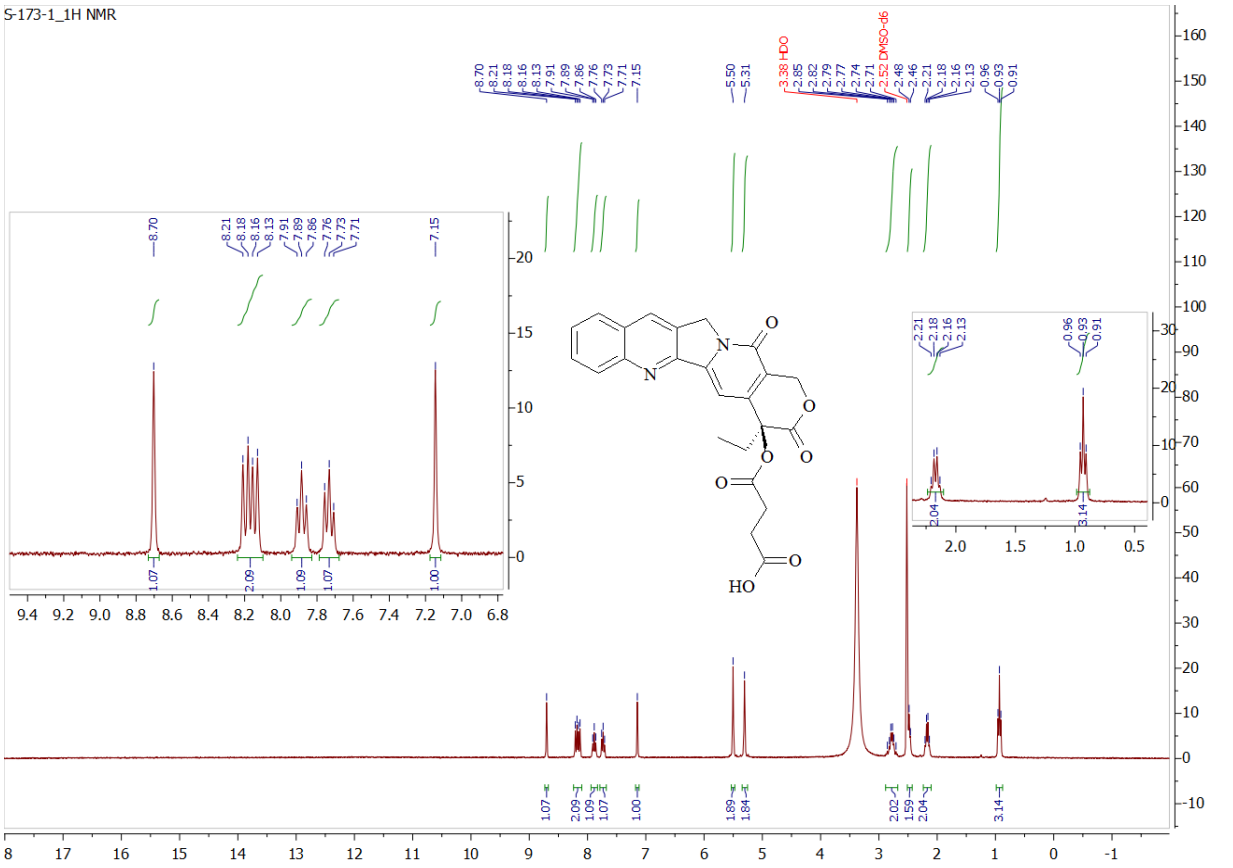


Figure S6. ^{1}H NMR spectrum of compound **3.**

Bis[(*S*)-1-ethyl-3,5-dioxo-3,4,5,7-tetrahydro-1*H*-pyrano[4',3':6,7]indolizino[1,2-*b*]quinolin-1-yl] succinate (4). Conjugate **3** (75 mg, 0.17 mmol) was dissolved in 5 ml of DMF, then EDC*HCl (26.4 mg, 0.17 mmol) and DMAP (21 mg, 0.17 mmol) were successively added to the resulting solution at 0°C. The reaction mixture was brought to room temperature, and CPT **1** (29.61 mg, 0.85 mmol) was added. This was stirred at room temperature (RT) under argon atmosphere for 24 h. Upon completion, the resulting mixture was filtered. The filtrate was evaporated, and the residue was chromatographed on silica gel (SiO_2) (DCM/MeOH, 20:1) to give bis-camptothecin ester of succinic acid **4** (98 mg, 74%).

^{1}H NMR (300 MHz; DMSO-d_6):δ_{ppm}: 8.65 (s, 2H), 8.12 (t, *J* = 8.8 Hz, 4H), 7.86 (t, *J* = 7.8 Hz, 2H), 7.70 (t, *J* = 7.6 Hz, 2H), 7.35 (s, 0H), 7.18 (s, 1H), 5.51 (s, 3H), 5.44 (s, 1H), 5.25 (s, 4H), 2.81 – 2.69 (m, 1H), 2.16 (d, *J* = 7.7 Hz, 3H), 0.96 (t, *J* = 7.2 Hz, 6H).

^{13}C NMR (75MHz; DMSO-d_6):δ_{ppm}:171.11, 170.75, 167.15, 156.50, 152.26, 149.95, 147.84, 145.79, 145.46, 131.41, 130.30, 129.59, 128.84, 128.44, 127.85, 127.59, 119.02, 118.71, 96.66, 95.33, 80.12, 66.17, 50.14, 50.10, 30.20, 29.57, 28.77, 7.76, 7.58.

IR (cm^{-1}): 1748 (O–C=O), 1667 (C=O), 1620 (C=C), 1439 (C=N), 1046 (C–O).

MS (ESI): 779.2348 [M+H]. Calculated for $C_{44}H_{34}N_4O_{10}$: 779.23477 [M+H]

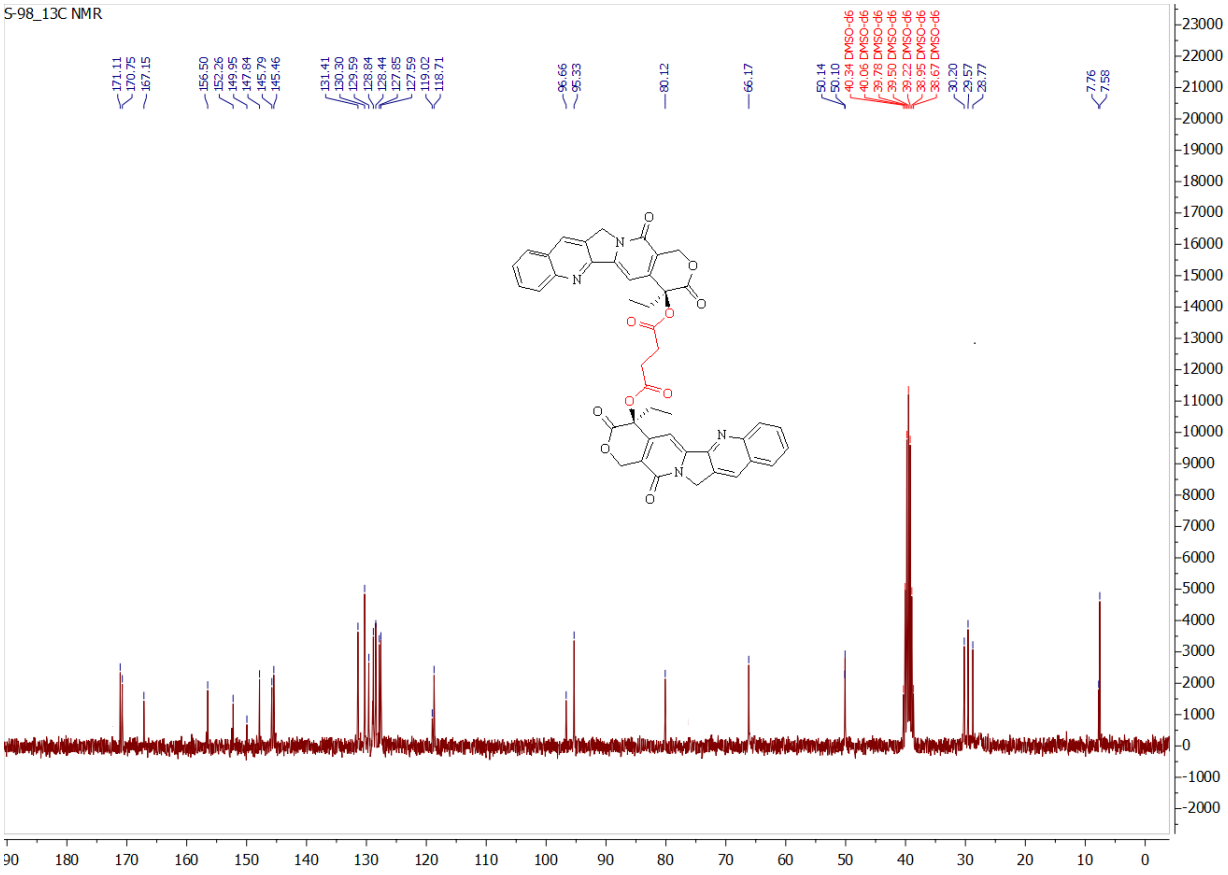


Figure S7. ^{13}C NMR spectrum of compound **4.**

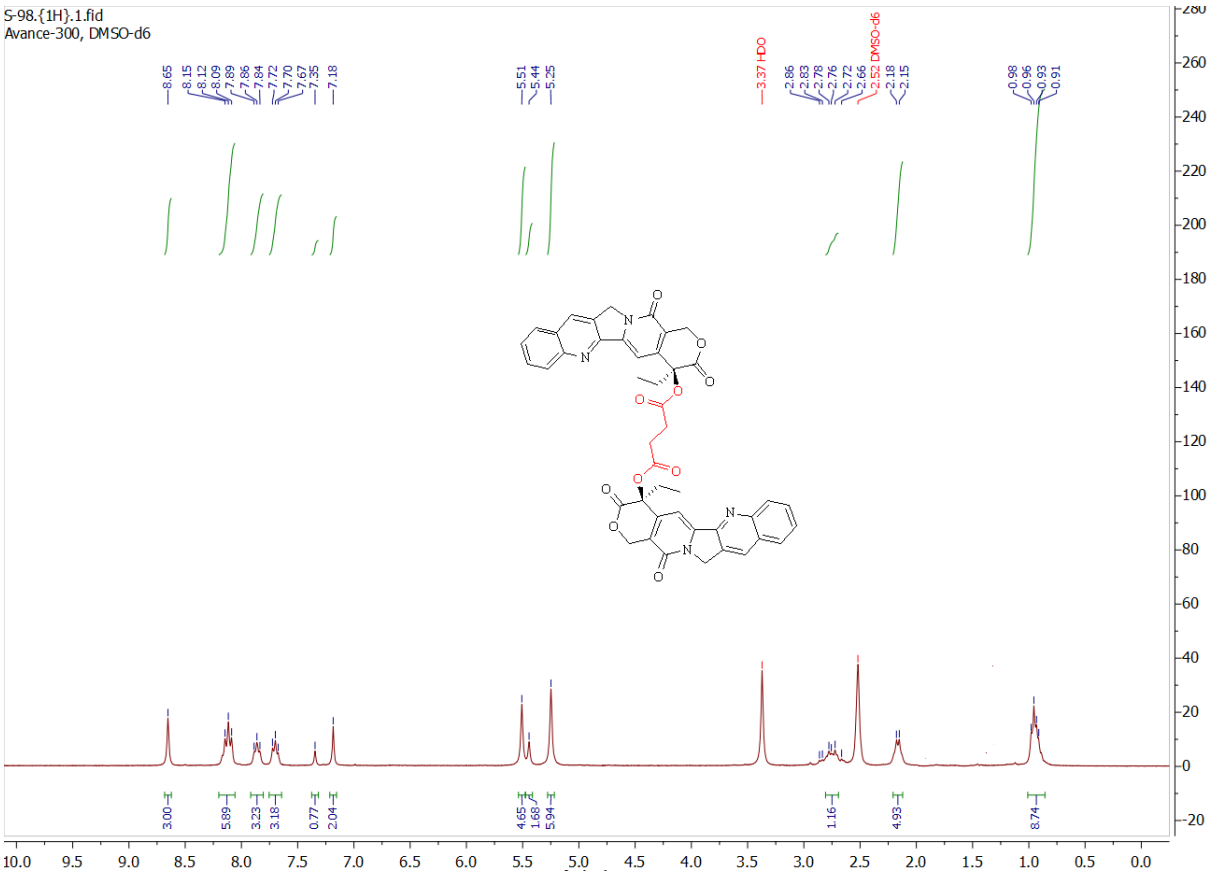


Figure S8. ^{1}H NMR spectrum of compound **4.**

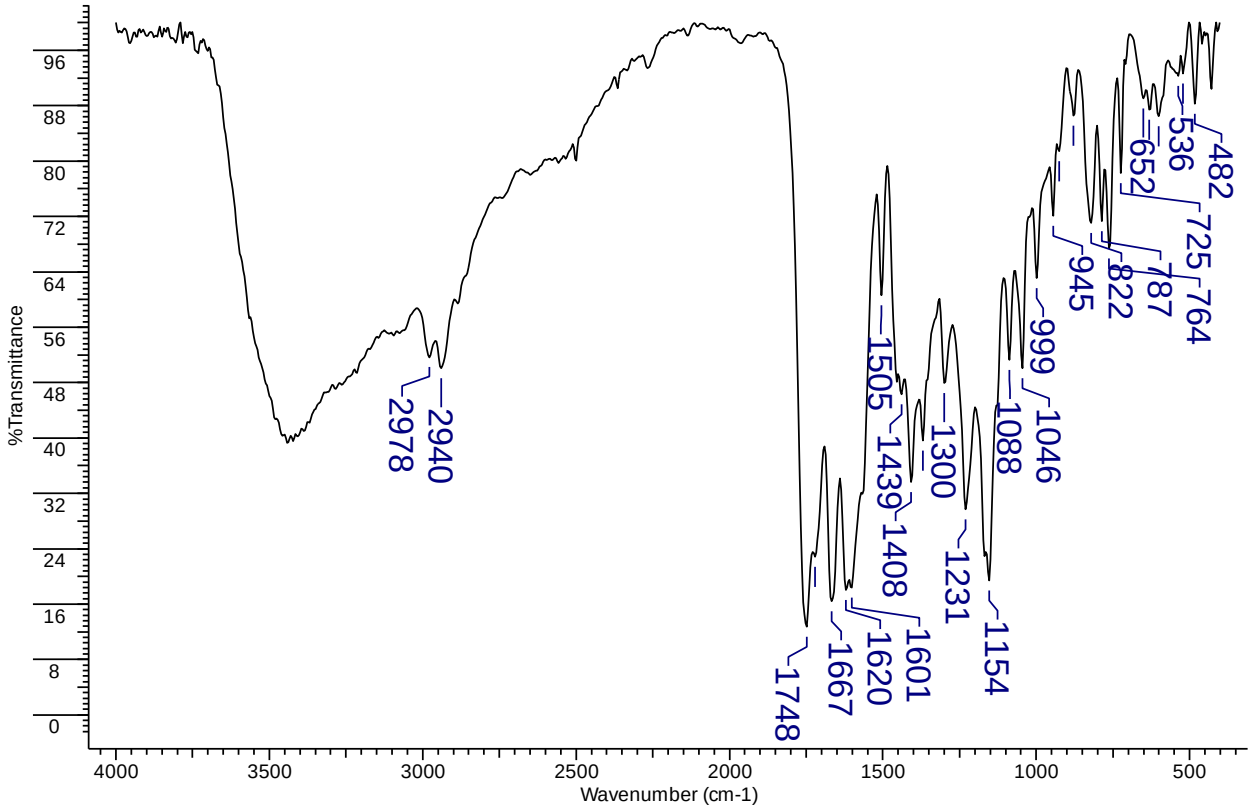


Figure S9. IR-spectrum of compound **4.**

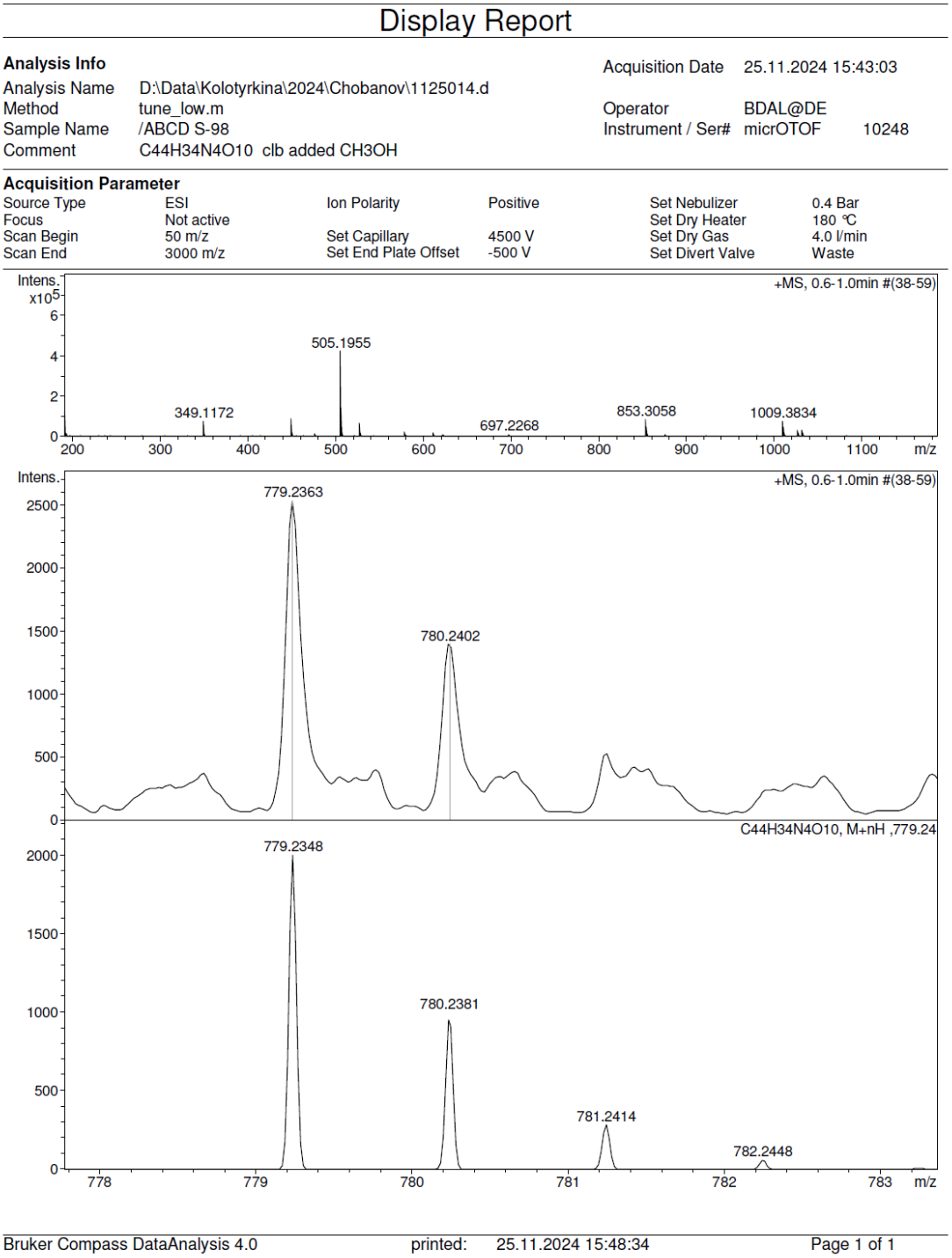


Figure S10. Mass spectrum of compound **4.**

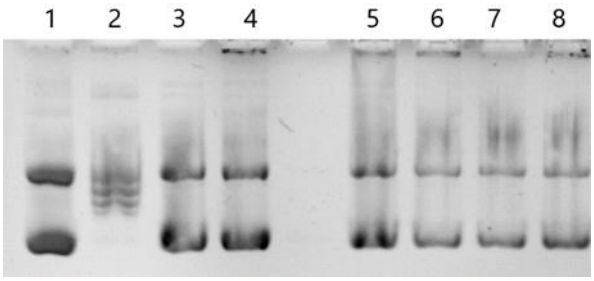


Figure S11. Inhibition of Topoisomerase I by compound **4**. The samples were analyzed on a 1% agarose gel. Lanes 1-4 are samples with addition of topoisomerase I enzyme (2 units), lanes 5-8 are samples without addition of topoisomerase I enzyme. Lane 1 - superhelical HOT1 plasmid; lane 2 - relaxed pHOT1 plasmid (when 2 units of topoisomerase I enzyme were added to 1 μL pHOT1); lane 3 - pHOT1 + TopoI + compound **3**; lane 4 - pHOT1 + TopoI + compound **4**; lanes 5-6 - pHOT1 + compound **3** at concentrations of CC_{50} and $0.5CC_{50}$. Lane 7-8 - pHOT1 + compound **4** at concentrations of CC_{50} and $0.5CC_{50}$, respectively.

Biology

Cell Culturing

The human cancer cell lines Jurkat, K562, A549, and HEK293 were obtained from the European Authenticated Cell Culture Collection and subsequently cultured according to established standard protocols and sterile techniques. Cells were maintained in RPMI 1640 (for Jurkat and K562) and DMEM (for A549 and HEK293) media (Gibco) supplemented with 4 μM glutamine, 10% FBS (Sigma), and 100 units/mL penicillin-streptomycin (Sigma). All cell types were cultured in a humidified atmosphere of 5% CO_2 at 37°C. Subcultures were performed at two- to three-day intervals. The cells were then seeded in 24-well plates at a density of 5×10^4 cells per well and incubated overnight. Cells were subcultured at two-day intervals at a seeding density of 1×10^5 cells per 24-well plate in RPMI medium supplemented with 10% FBS.

Cytotoxicity Assay

Cell viability was assessed using a 7-AAD (7-aminoactinomycin D) dye (eBioscience™). After incubation with the test compounds, the cells were harvested, washed with phosphate-buffered saline (PBS), and centrifuged at 400g for five minutes. The cell precipitates were resuspended in 200 μL flow cytometry staining buffer (PBS without calcium and magnesium, 2.5% FBS) and stained with a 1 mM 7-AAD dye solution for 15 minutes in the dark at room temperature. Cells with damaged membranes that are in the process of dying or are already dead will stain with 7-AAD, while living cells with intact cell membranes will remain unstained. 7-AAD is known to intercalate into double-stranded DNA with a high affinity for GC-rich regions, making it important and useful for studies of different types of chromatin in the cell and allowing this dye, unlike other intercalating dyes that are better at staining euchromatin, to most completely stain DNA regions

in heterochromatin, providing the most realistic picture of cell viability. All experimental and control samples were then analyzed on a NovoCyte Penteon flow cytometer (Agilent, USA).

Apoptosis Assay

Apoptosis was quantified by detecting phosphatidylserine expressed on the outer surface of the membrane. This was done using the Alexa Fluor® 488 Annexin V fluorescence staining kit, designed for the identification of apoptotic and necrotic cells (ThermoFisher, USA). Jurkat cells were incubated with the tested compounds for 24 hours and then washed twice with cold PBS buffer. They were then resuspended in 100 μL annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2; pH 7.4). Annexin V (5 μL) and 7-AAD (1 μL) solutions were then added to the reaction mixture and incubated for 15 minutes at room temperature in the dark. The stained cells were then analyzed using a NovoCyte Pen-teon flow cytometer system (Agilent, USA).

Cell Cycle Analysis

Cell cycle analysis was performed using the MAK344 cell cycle assay kit (Sigma-Aldrich). After 24 hours of incubation with the tested compounds, cells were harvested, washed twice with phosphate-buffered saline (PBS), and centrifuged at 450g for 5 minutes. The cell precipitate was resuspended and fixed with ice-cold 70% ethanol for 24 hours at 0 degrees Celsius. Prior to staining, the cells were washed with PBS and incubated with 0.5 mL of the Cell Cycle Assay Kit working solution for 30 minutes at room temperature in the dark. The samples were then analyzed using the NovoCyte Penteon flow cytometer system (Agilent, USA).

DNA Topoisomerase I Assay

The inhibitory activity and mechanism of inhibition of the synthesized compound were determined using the Topoisomerase I Drug Screening Kit TG-1018-2, (Topogen, USA) (the tested compound was added before topoisomerase I). Relaxation of supercoiled DNA under the action of topoisomerase I was performed as follows: the reaction mixture (20 ml) containing 0. 25 mg of pHOT DNA plasmid (TopoGen, USA), 1 unit of recombinant topoisomerase I (TopoGen, USA) and the test compound was incubated in buffer for 30 min at 37°C using a Biosan thermostat (Latvia). The tested compound was added to the reaction mixture prior to the addition of topoisomerase I enzyme. The inhibitory effect on topoisomerase I was checked by camptothecin alkaloid (TopoGEN, USA). The reaction was terminated by adding sodium dodecyl sulfate to a concentration of 1%. After adding a solution (5 mg/mL) of Proteinase K (Sigma Chemical Co., USA) (1:10), the reaction mixture was incubated at 37°C for 40 minutes. A 0.1% solution of

bromophenol blue (1:10) was added and electrophoresis was performed in the absence of ethidium bromide. The reaction products were separated on a 1% agarose gel (3 V/cm) for 4-6 h. After electrophoresis, the gels were treated with ethidium bromide solution (0.5 mg/mL). The gels were visualized under UV light in an Infinity VX2 1120/Blue X-Press gel documentation system (Vilber Lourmat, France). The possible effect of the test compounds on supercoiled DNA was tested by performing the reaction without Topo I, adding the test compounds at the same concentrations as in the reaction with the enzyme.

Statistics

The normality of the distribution of the results obtained was checked. Chi-square was used for this purpose. Data were expressed as mean ± standard deviation. Student's t-test was used for statistical comparisons of results. A p-value less than 0.01 and less than 0.05 was considered statistically significant. Regression analysis and stepwise analysis of variance (ANOVA) were used for statistical analysis.

References

[S1] R. Srinivasan, M. Uttamchandani and S. Q. Yao, *Org. Lett.*, 2006, **8**, 713–716; https://doi.org/10.1021/ol052895w.

[S2] X. Ma, Q. Sun, Z. Zhou, E. Jin, J. Tang, E. van Kirk, W.J. Murdoch and Y. Shen, *Polym. Chem.*, 2013, **4**, 812–819; https://doi.org/10.1039/C2PY20771K.