Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supplementary Information

Poloxin-2HT+: Changing the hydrophobic tag of Poloxin-2HT increases Plk1 degradation and apoptosis induction in tumor cells

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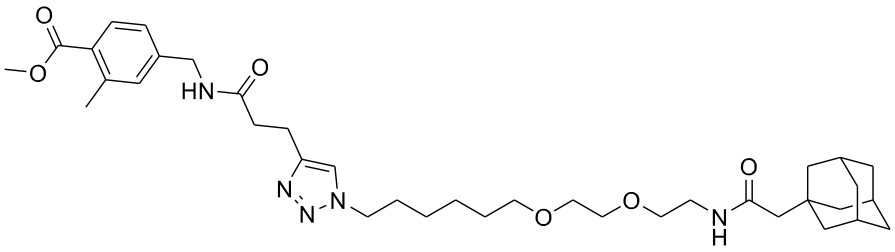
Figure S1



9

Figure S1. Structure of the negative control compound **9**.

Figure S2

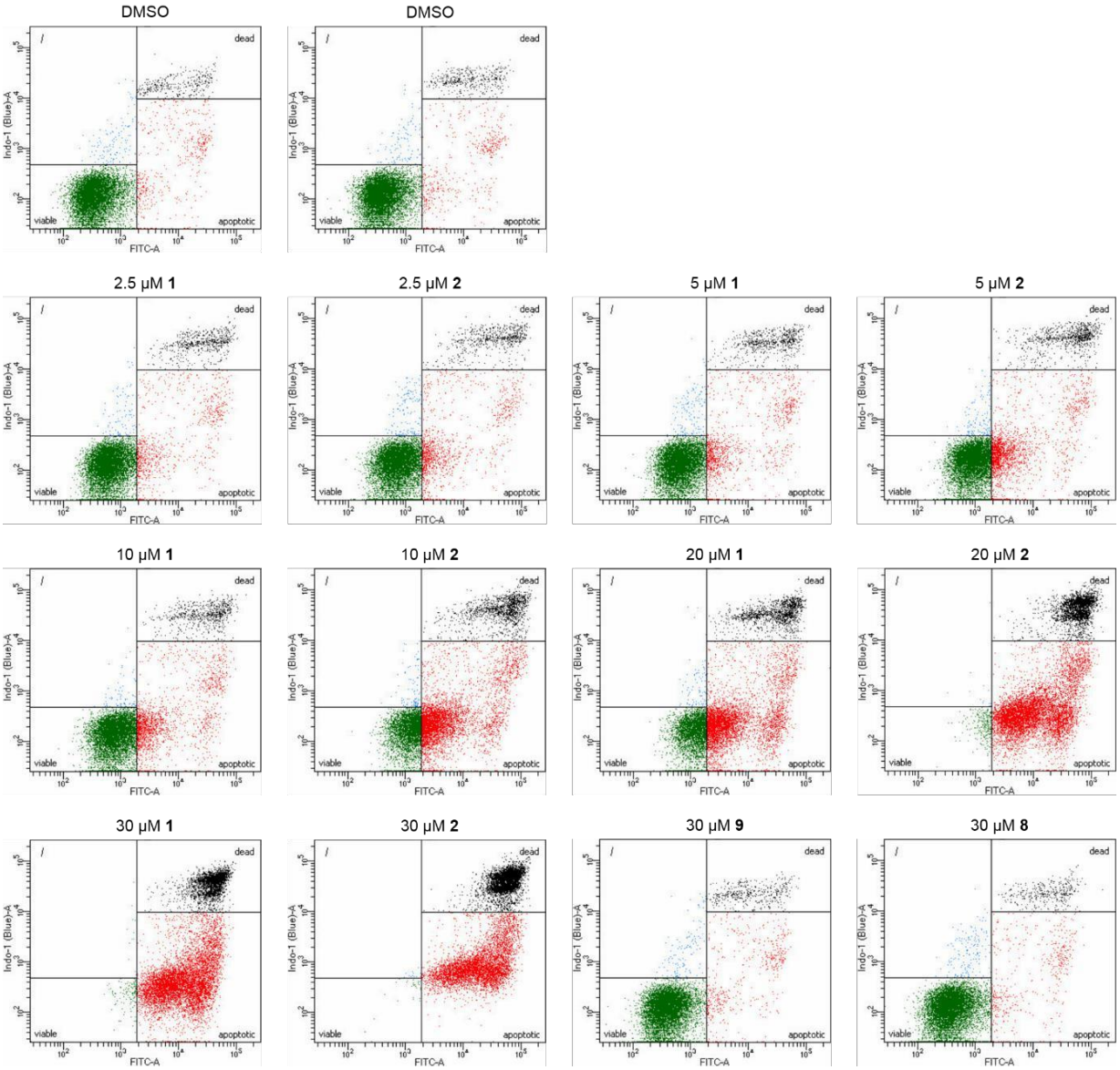


Figure S2. Representative results of flow cytometry analysis using the compounds **1**, **2**, **8**, and **9** at the indicated concentrations. Apoptotic cells, characterized by Annexin V FITC staining and the absence of propidium iodide staining, are depicted in the lower right-hand section of the flow cytometry plot.

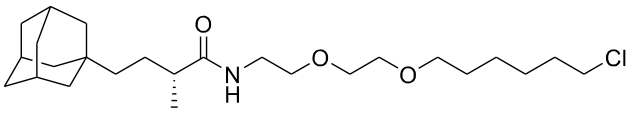
Table S1

Compound	Plk1 PBD app. IC_{50} (µM)	Plk2 PBD app. IC_{50} (µM)	Plk3 PBD inhibition at 100 µM
1	10.5 ± 1.6	51.9 ± 3.5	47 ± 4%
2	48.2 ± 4.9	54.8 ± 11.0	34 ± 4 %

Table 1. Comparison of activity data of compounds **1** and **2** in fluorescence polarization assays. Data for compound **1** are taken from the published reference.[1] See methods section for experimental details.

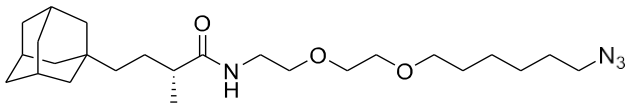
Chemical synthesis and spectroscopic characterization

(*R*)-4-((*3R*,*5R*,*7R*)-adamantan-1-yl)-*N*-(2-(2-((6-chlorohexyl)oxy)ethoxy)ethyl)-2-methylbutanamide (**HyT36**)[2]



Synthesis of **HyT36** was carried out based on the published protocol.[2]

(*R*)-4-((*3R*,*5R*,*7R*)-adamantan-1-yl)-N-(2-(2-((6-azidohexyl)oxy)ethoxy)ethyl)-2-methylbutanamide (**3**)



Under an argon atmosphere, 0.054 g HyT36 (1 eq., 0.12 mmol) were dissolved in 2 mL DMF. 0.014 g sodium azide (0.5 eq., 0.18 mmol) were added. The reaction mixture was stirred for 16 hours. DMF was removed under reduced pressure and the raw product purified with flash chromatography (eluent: 2 % MeOH in DCM) and yielded as an oil.

Yield 0.038 g (0.085 mmol), 71 %.

R_f: 0.42 (eluent: 2% MeOH in DCM).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 5.98 – 5.88 (m, 1H), 3.64 – 3.60 (m, 2H), 3.59 – 3.53 (m, 4H), 3.50 – 3.41 (m, 4H), 3.26 (t, *J* = 6.9 Hz, 2H), 2.10 – 2.00 (m, 1H), 1.95 – 1.90 (m, 3H), 1.73 – 1.53 (m, 12H), 1.48 – 1.42 (m, 6H), 1.41 – 1.36 (m, 4H), 1.13 (d, *J* = 6.8 Hz, 3H), 1.04 – 0.97 (m, 2H).

^{13}C NMR (APT, 101 MHz, $CDCl_3$) δ = 176.82, 71.42, 70.43, 70.18, 70.16, 51.53, 42.53, 42.43, 42.30, 39.10, 37.37, 32.24, 29.65, 28.95, 28.86, 27.43, 26.72, 25.85, 18.07.

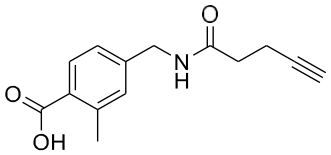
HR-ESI-MS: $C_{25}H_{44}N_4O_3$ calcd. $[M+H]^+$ 449.3486, found 449.3490.

IR: $\tilde{\nu}$ = 3442 (m), 3319 (m), 2926 (s), 2903 (s), 2846 (m), 2817 (w), 2095 (s), 1650 (m), 1548 (m), 1452 (m), 1384 (w), 1371 (w), 1360 (w), 1348 (w), 1286 (w), 1247 (w), 1105 (m), 1038

(w), 754 (m), 701 (w), 665 (w), 638 (w), 620 (w), 604 (w), 504 (w), 491 (w) 472 (w), 462 (w), 451 (w), 451 (w), 435 (w), 405 (w).

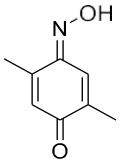
UV-Vis (DCM) $\lambda_{max}(\varepsilon)$ = 268, 228 nm.

2-Methyl-4-(pent-4-ynamidomethyl)benzoic acid (**4**)[3]



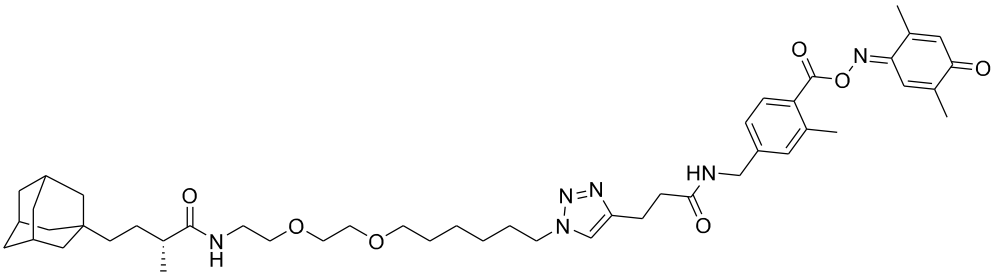
Synthesis of **4** was carried out as described.[3]

(*E*)-4-(Hydroxyimino)-2,5-dimethylcyclohexa-2,5-dien-1-one (**6**)[4]



Synthesis of **6** was carried out as described.[4]

(*R*)-4-((*3R*,*5R*,*7R*)-adamantan-1-yl)-N-(2-(2-((6-(4-(3-((4-(((((*E*)-2,5-dimethyl-4-oxocyclohexa-2,5-dien-1-ylidene)amino)oxy)carbonyl)-3-methylbenzyl)amino)-3-oxopropyl)-1H-1,2,3-triazol-1-yl)hexyl)oxy)ethoxy)ethyl)-2-methylbutanamide (**2**)



0.173 g **3** (1 eq., 0.388 mmol), 0.096 g **4** (1 eq., 0.388 mmol) and 0.0097 g (0.1 eq, 0.039 mmol) $CuSO_4$ x 5 H_2O were dissolved in THF/H_2O (3:1). The reaction mixture was degassed. 0.031 g sodium ascorbate (0.04 eq., 0.18 mmol) were dissolved in water and added to the reaction mixture. It was stirred for 5 h at room temperature. 5 ml $NaHCO_3$ sat. were added. It was extracted with ethyl acetate. The aqueous phase was acidified with 1 M HCl until precipitation started. It was extracted four times with EtOAc. The solvent was evaporated and 0.130 mg raw product **5** were obtained, which was dissolved in 5 ml DCM. 0.028 g **6** (1 eq., 0.187 mmol) and a catalytic amount of DMAP was added. 0.046 g DCC (1.2 eq., 0.224 mmol) were dissolved in 1 ml DCM and added dropwise to the reaction mixture. It was stirred for 16 hours at room temperature. The urea was filtered off and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (DCM/acetone, 1:2), yielding **2** as a semi-solid.

Yield 0.110 g (0.158 mmol), 41 % over two steps.

R_f: 0.47 (DCM/acetone, 1:2)

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.89 (d, *J* = 8.5 Hz, 1H), 7.51 (q, *J* = 1.5 Hz, 1H), 7.35 (s, 1H), 7.19 (s, 1H), 7.18 – 7.16 (m, 1H), 6.63 – 6.56 (m, 1H), 6.42 (q, *J* = 1.5 Hz, 1H), 5.97 – 5.92 (m, 1H), 4.45 (d, *J* = 5.9 Hz, 2H), 4.29 (t, *J* = 7.2 Hz, 2H), 3.62 – 3.58 (m, 2H), 3.57 – 3.53 (m, 4H), 3.46 – 3.41 (m, 3H), 3.09 (t, *J* = 7.0 Hz, 2H), 2.74 (t, *J* = 7.0 Hz, 2H), 2.64 (s, 3H), 2.36 – 2.32 (m, 3H), 2.08 – 2.06 (m, 3H), 2.06 – 2.02 (m, 1H), 1.94 – 1.90 (m, 4H), 1.90 – 1.84 (m, 2H), 1.71 – 1.68 (m, 1H), 1.68 – 1.65 (m, 2H), 1.62 – 1.52 (m, 6H), 1.44 – 1.42 (m, 5H), 1.40 – 1.27 (m, 5H), 1.25 (s, 1H), 1.11 (d, *J* = 6.8 Hz, 3H), 1.03 – 0.97 (m, 2H).

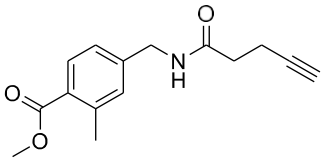
^{13}C NMR (101 MHz, $CDCl_3$) δ = 186.96, 176.87, 172.32, 163.36, 154.50, 146.48, 146.37, 143.98, 142.35, 141.93, 131.18, 131.04, 130.86, 126.42, 125.11, 122.23, 121.61, 71.24, 70.42, 70.23, 70.19, 50.43, 43.19, 42.56, 42.42, 42.32, 39.15, 37.39, 35.62, 32.26, 30.34, 29.53, 28.88, 27.45, 26.44, 25.71, 21.79, 21.50, 18.09, 17.48, 16.39.

HR-ESI-MS: $C_{47}H_{66}N_6O_7$ calcd. $[M+H]^+$ 827.5066, found 827.5062.

IR (KBr): 3388 (m), 3293 (m), 3074 (w), 2926 (s), 2901 (s), 2846 (s), 1764 (m), 1650 (s),1633 (s), 1612 (m), 1547 (m), 1449 (m), 1304 (w), 1263 (m), 1226 (s), 1163 (m), 1139 (m), 1023 (m), 1005 (s), 939 (m), 896 (m), 836 (w), 803 (w), 761 (w), 735 (m), 698 (w), 686 (m), 636 (w), 535 (w), 408 (w).

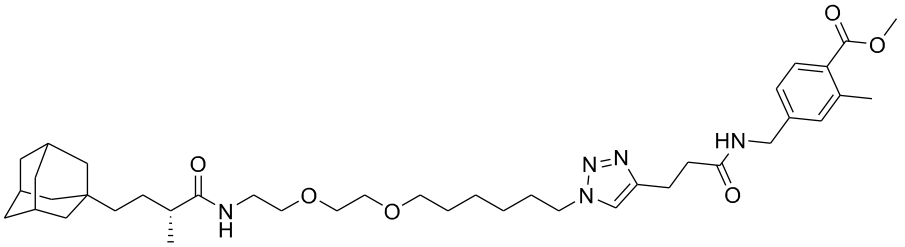
UV-Vis (DCM) $\lambda_{max}(\varepsilon)$ = 290, 238 nm.

Methyl 2-methyl-4-(pent-4-ynamidomethyl)benzoate (**7**)[3]



Synthesis of **7** was carried out as described.[3]

Methyl 4-((3-(1-(6-(2-(2-((*R*)-4-((*3R*,*5R*,*7R*)-adamantan-1-yl)-2 methylbutanamido)ethoxy) ethoxy)hexyl)-1H-1,2,3-triazol-4-yl)propanamido)methyl)-2-methylbenzoate (**8**)



0.1 g **3** (1 eq., 0.223 mmol), 0.06 g **7** (1 eq., 0.223 mmol) and 0.0056 g $CuSO_4 \cdot 5H_2O$ (0.1 eq., 0.023 mmol) were dissolved in degassed THF/water (4:1). 0.18 g sodium ascorbate were dissolved in degassed H_2O and added to the reaction mixture. The mixture was stirred for 16 h

at room temperature. The solvent was removed and the residue purified with flash chromatography (eluent: DCM/acetone, 2:1)

Yield: 0.14 g (0.199 mmol), 89 %.

R_f: 0.21 (eluent: DCM/acetone, 2:1)

m.p.: 59 °C

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.84 (d, *J* = 8.0 Hz, 1H), 7.31 (s, 1H), 7.08 (s, 1H), 7.05 (d, *J*= 8.0 Hz, 1H), 6.32 – 6.24 (m, 1H), 5.98 – 5.90 (m, 1H), 4.40 (d, *J* = 5.9 Hz, 2H), 4.27 (t, *J* =7.2 Hz, 2H), 3.88 (s, 3H), 3.63 – 3.58 (m, 2H), 3.57 – 3.53 (m, 4H), 3.47 – 3.41 (m, 4H), 3.07 (t, *J* = 7.0 Hz, 2H), 2.69 (t, *J* = 7.0 Hz, 2H), 2.57 (s, 3H), 2.09 – 2.00 (m, 1H), 1.95 – 1.89 (m, 3H), 1.89 – 1.82 (m, 2H), 1.72 – 1.60 (m, 6H), 1.56 – 1.51 (m, 2H), 1.46 – 1.41 (m, 6H), 1.41 – 1.24 (m, 6H), 1.12 (d, *J* = 6.9 Hz, 3H), 1.04 – 0.97 (m, 2H).

^{13}C NMR (101 MHz, $CDCl_3$) δ = 176.86, 172.19, 167.85, 142.59, 140.85, 131.17, 130.88, 128.62, 124.82, 71.25, 70.40, 70.18, 51.95, 50.25, 43.15, 42.53, 42.39, 42.30, 39.11, 37.36, 35.76, 32.23, 30.34, 29.51, 28.85, 27.42, 26.43, 25.67, 21.92, 21.60, 18.07.

HR-ESI-MS: $C_{40}H_{61}N_5O_6$ calcd: $[M+H]^+$ 708.4695 found: 708.4694.

IR (KBr): 3432 (m), 3324 (m), 3131 (w), 3072 (w), 2928 (s), 2903 (s), 2844 (m), 2807 (w), 1721 (s), 1651 (s), 1613 (m), 1542 (s), 1450 (m), 1436 (m), 1384 (w), 1361 (m), 1348 (m), 1264 (s), 1217 (m), 1191 (w), 1137 (m), 1123 (m), 1084 (m), 1057 (w), 1027 (m), 838 (w), 813 (w), 772 (m), 742 (w), 702 (w).

UV-Vis (DCM) $\lambda_{max}(\varepsilon)$ = 280, 237 nm.

Fluorescence polarization assays

Fluorescence polarization assays were carried out essentially as described.[1] In brief, the abilities of the test compounds to inhibit binding of the Plk1 PBD (aa 326-603), the Plk2 PBD (aa 355-685), and the Plk3 PBD (aa 335-646) and fluorescent-labeled peptides were analyzed.[5] Test compounds were incubated with the proteins for 1 h at room temperature, followed by addition of fluorescent peptide (for Plk1 PBD: 5-carboxyfluorescein-GPMQSpTPLNG-OH; for Plk2 PBD: 5-carboxyfluorescein-GPMQTSpTPKNG-OH; for Plk3 PBD: 5-carboxyfluorescein-GPLATSpTPKNG-OH, all at a final concentration of 10 nM). A Biomek FXp workstation (Beckman-Coulter) was used for some of the pipetting work. 75 min after addition of the fluorescent peptide, fluorescence polarization (excitation: 485 nm, emission: 535 nm) was measured using a Tecan Infinite F500 384-well plate reader. Final protein concentrations used: Plk1 PBD: 20 nM; Plk2 PBD: 80 nM; Plk3 PBD: 250 nM. Buffer composition: 10 mM Tris (pH 8.0), 50 mM NaCl,1 mM EDTA, 0.1 % Nonidet P-40 substitute, final DMSO concentration: 2 %. Data were analyzed using OriginPro 8G software (OriginLab). Fluorescence polarization values were converted to % inhibition via the binding curve between

fluorescent peptide and protein. Compound concentrations at which 50 % inhibition was observed are given as apparent IC_{50} values.

Cell culture

HeLa cells were cultured in high glucose (4.5 g/L) DMEM (Gibco Life Technologies), supplemented with 10 % (v/v) fetal bovine serum (FBS), 2 mM L-glutamine (Gibco Life Technologies), 1 mM sodium pyruvate (Gibco Life Technologies) and 1 % (v/v) penicillin/streptomycin (Gibco Life Technologies), at 37 °C, 5 % CO_2 and 95 % humidity.

Compound treatment and cell lysates

3.5 x 10^5 HeLa cells per well were seeded into 6-well plates (Corning). After adherence for 16 h, cells were treated with test compound at the indicated concentrations or DMSO (final DMSO concentration: 0.1 %) for 24 h. After compound incubation, cells were washed with cold phosphate buffered saline (PBS) and lysed with lysis buffer (lysis buffer composition: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM $Na_4P_2O_7$, 10 % glycerol, 1 % Triton X-100, 1 mM EDTA, protease inhibitors 1 mM PMSF and 100 ng/ml aprotinin were added freshly prior use). Cell lysates were snap frozen in liquid nitrogen and stored at -80 °C until further use. The protein concentration of the cell lysates was determined using a Bicinchoninic Acid (BCA) assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific) following the manufacturer‘s instructions.

Western Blot

Western Blots were carried out essentially as described.[1] Cell lysates components (total protein amount of 10 - 40 µg per sample) were separated by SDS-PAGE (10 %) under denaturing conditions and then transferred to a nitrocellulose membrane. Plk levels were analyzed using rabbit monoclonal antibodies against Plk1, Plk2 or Plk3 (all 1:1000, all Cell Signaling, at 4 °C for 16 h) and reblotted with β-actin (1:1000, Cell Signaling, at 4 °C for 16 h). Primary antibodies were incubated and detected with a secondary antibody swine anti-rabbit HRP (1:3000, Dako, at room temperature for 1 h) and ECL was performed using Western Lightning Plus chemiluminescence reagent (Perkin-Elmer). For visualization, an ImageQuant digital imaging system (GE Healthcare) was used, and ImageJ software (NIH) was used for quantitative analysis.[6] Experiments were carried out at least in triplicate.

Cell viability assay

Cell viability assays were carried out essentially as described.[1] 4 x 10^3 HeLa cells per well were seeded into 96-well plates (Corning). After adherence for 16 h, cells were treated with test compound at the indicated concentrations or DMSO (final DMSO concentration: 0.1 %) for

24 h. WST-1 solution (Roche, final dilution of 1:10 with cell culture medium) was added to each well, and incubated for 1 h. The absorbance at 450 nm was analyzed, using the absorbance at 650 nm as a reference. Experiments were carried out in triplicate.

Apoptosis Assay

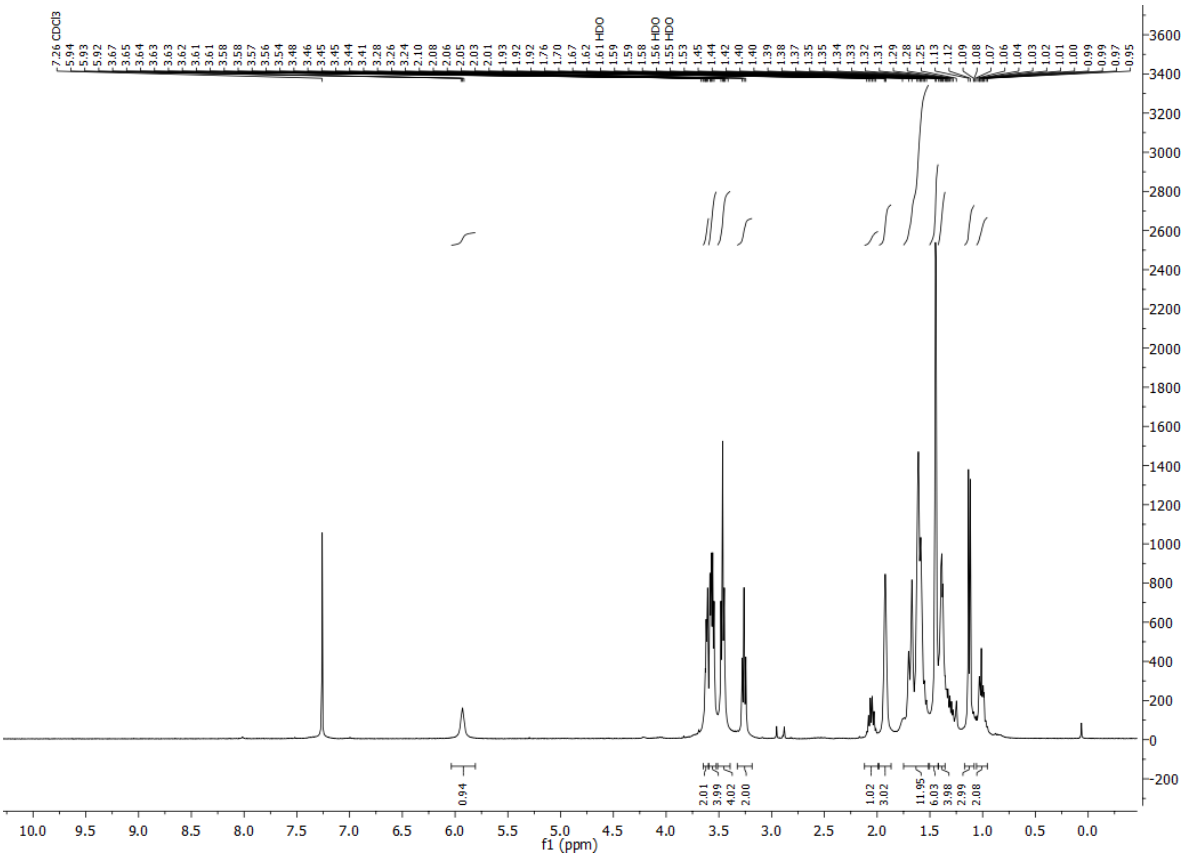
Apoptosis assays were carried out essentially as described.[1] 1 x 10^5 HeLa cells per well were seeded into 24-well plates (Corning). After adherence for 16 h, cells were treated with test compound at the indicated concentrations or DMSO (final DMSO concentration: 0.1 %) for 24 h. After 24 h incubation time, the cell culture supernatant from each well was collected, and the cells were washed twice with warm PBS. Subsequently, cells were detached using Accutase (BD Bioscience) at 37 °C for 10 min, and the supernatants were returned to each well to neutralize the Accutase solution. Cells were centrifuged at 3,000 rpm, at 4 °C for 5 min. The cell pellets were washed twice with cold PBS and then centrifuged again. Cells were stained using the FITC Annexin V Apoptosis Detection Kit I (BD Bioscience). Cells were resuspended in 1 x binding buffer. For staining cells were incubated with FITC annexin V and propidium iodide at 4 °C for 30 min. Apoptosis was measured using a LSR II flow cytometer (BD Bioscience) within 1 h. Experiments were carried out in quadruplicate.

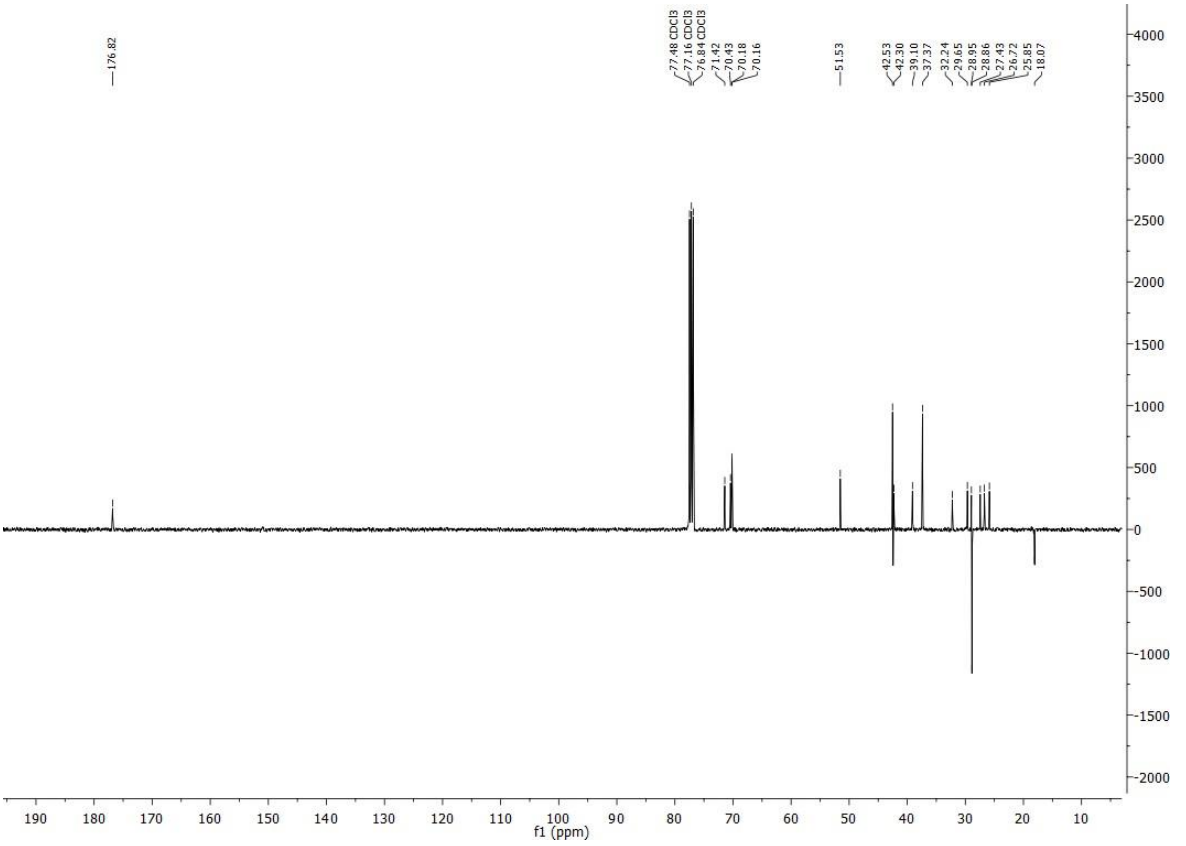
Supplementary references

1. S. Rubner, A. Scharow, S. Schubert and T. Berg, *Angew. Chem. Int. Ed.*, 2018, **57**, 17043-17047.
2. T. K. Neklesa, H. S. Tae, A. R. Schneekloth, M. J. Stulberg, T. W. Corson, T. B. Sundberg, K. Raina, S. A. Holley and C. M. Crews, *Nat. Chem. Biol.*, 2011, **7**, 538-543.
3. A. Scharow, D. Knappe, W. Reindl, R. Hoffmann and T. Berg, *ChemBioChem*, 2016, **17**, 759-767.
4. A. Scharow, M. Raab, K. Saxena, S. Sreeramulu, D. Kudlinzki, S. Gande, C. Dotsch, E. Kurunci-Csacsko, S. Klaeger, B. Kuster, H. Schwalbe, K. Strebhardt and T. Berg, *ACS Chem. Biol.*, 2015, **10**, 2570-2579.
5. W. Reindl, J. Yuan, A. Krämer, K. Strebhardt and T. Berg, *Chem. Biol.*, 2008, **15**, 459-466.
6. C. A. Schneider, W. S. Rasband and K. W. Eliceiri, *Nat. Methods*, 2012, **9**, 671-675.

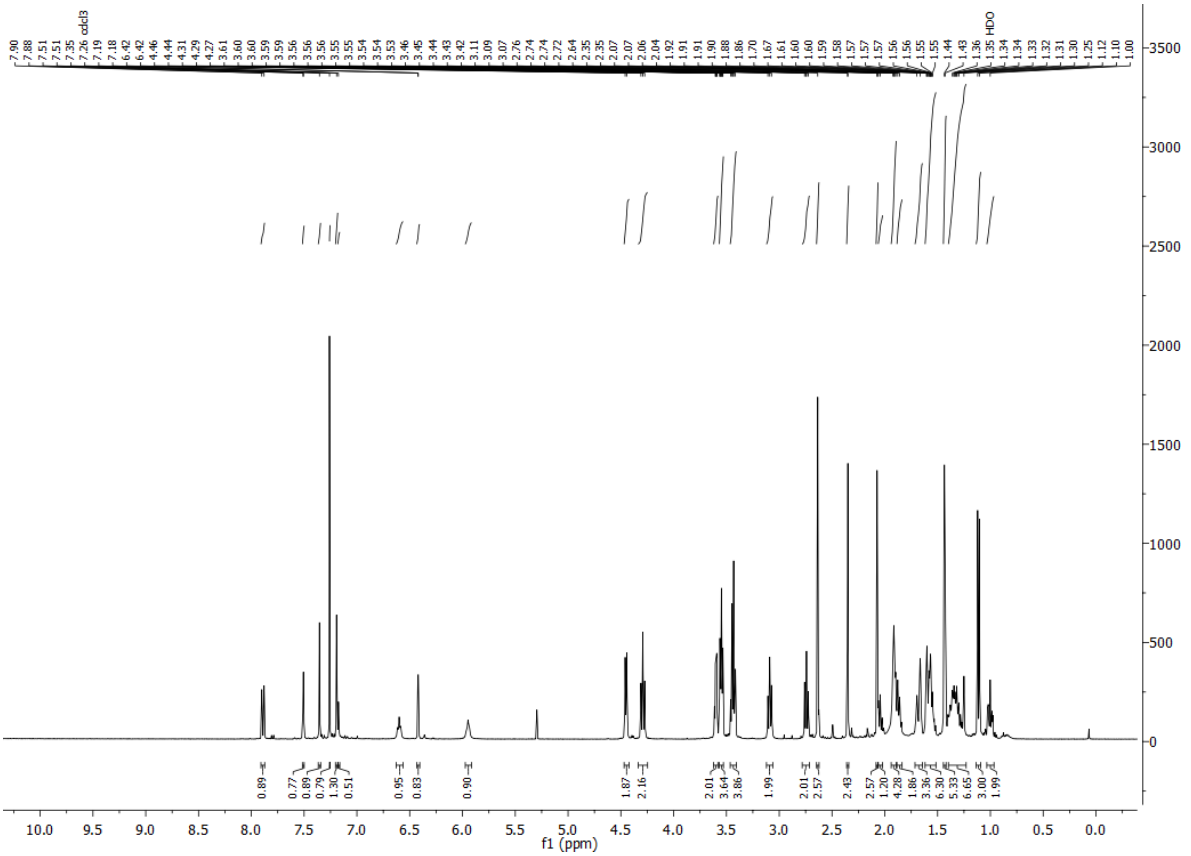
NMR spectra

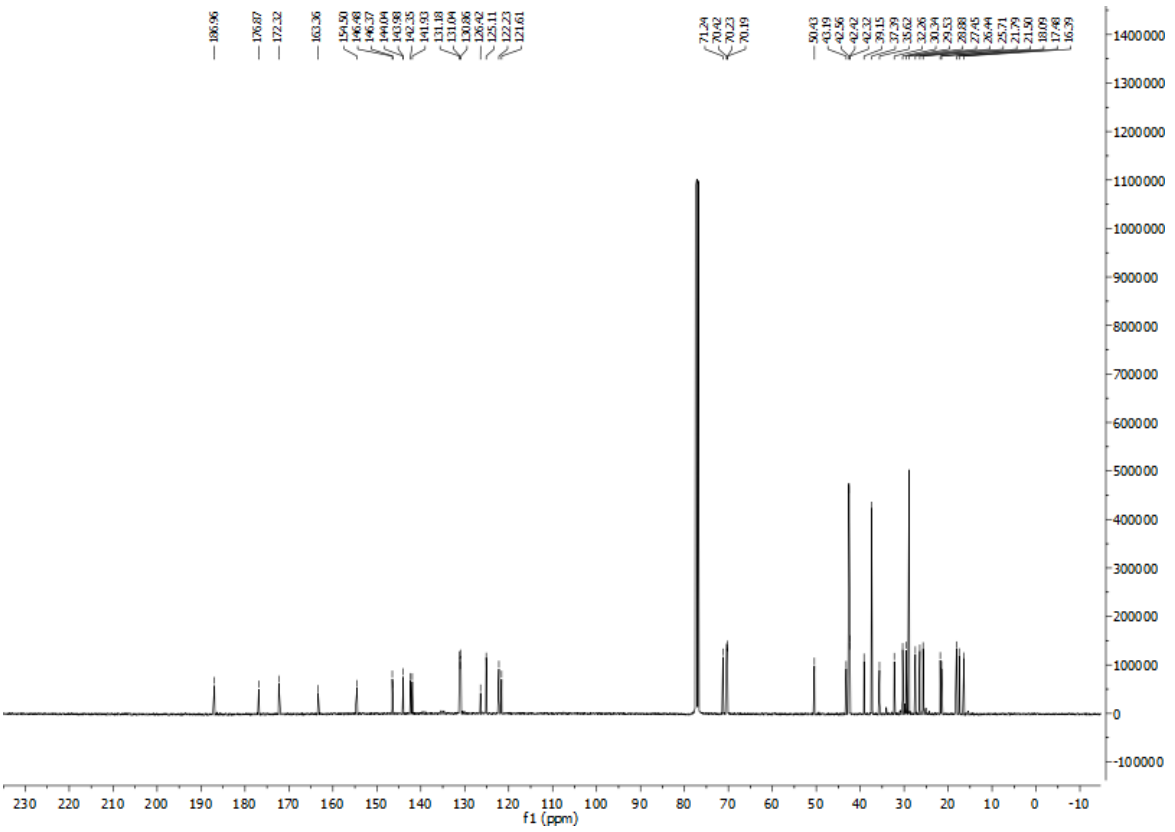
^{1}H and ^{13}C NMR of **3**





^{1}H and ^{13}C NMR of **2**





^{1}H and ^{13}C NMR of **8**