

SUPPLEMENTARY MATERIAL

Derivatives of Usnic Acid cause Cytostatic Effect in Caco-2 cells

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Abstract

Usnic acid has anti-cancer activity, however, low solubility and toxicity limit the potential. To investigate biological activity of usnic acid derivatives, enantiopure derivatives were synthesised by reacting usnic acid with ethylenediamine, which yielded one dimer product ((+)-**1**), and two tetra cyclic compounds ((+)-**2** and (-)-**2**). The products were characterised with NMR, and evaluated *in vitro* in human colon cancer cell line Caco-2 by cell count, phase-contrast microscopy, MTT-assay, measurement of DNA content and cell cycle distribution. All compounds tested showed cytostatic effect in Caco-2 cells, but each compound had a distinct effect. Compound (+)-**1** showed anti-proliferative activity by increasing the percentage of cells in S-phase with 25 % compared to the control. Compounds (+)-**2** and (-)-**2** induced paraptosis, but only compound (+)-**2** modulated cell cycle distribution by accumulating cells in G₂/M-phase by 47 % and reduced DNA content by 60 %. All compounds express interesting cellular and potential anti-proliferative activity.

Keywords: Synthesis; enantiomers; anti-cancer effect; cellular response; cell cycle distribution; paraptosis

Experimental section

1. Synthesis

(+)-Usnic acid (CAS: 7562-61-0) and Ethylenediamine (CAS: 107-15-3) used in this study were obtained from Sigma-Aldrich. (-)-Usnic acid (CAS: 7562-61-0) was obtained from ICN Medical inc. Solvents of high quality was used. All NMR spectra were measured on a Bruker 400 MHz Avance – III HD NMR spectrometer along with Topspin software. The solvent was CDCl₃, and TMS was used as reference. All samples were recorded at 25°C.

1.1 General method for preparation of compound 1 and 2

UA (1.5 mmol) was suspended in a mixture of EtOH (28 mL) and THF (7 mL). One addition of ethylenediamine (2.5 mmol) was added to the suspension resulting in a clear brightly yellow solution. The solution was heated to reflux for 4 hours while stirring. The colour of the solution intensified gradually, and a finely dispersed precipitate appeared along the edge of the glassware. The precipitate was filtered off (compound 1). The solution was concentrated under reduced pressure yielding a yellow compound. The compound was dissolved in 20 mL water resulting in a yellow suspension, and compound 2 was extracted with CHCl₃ (3.5 mL) and purified with preparative chromatography.

(S,E)-6-acetyl-2-(1-(((E)-1-((R)-6-acetyl-7,9-dihydroxy-8,9b-dimethyl-1,3-dioxo-3,9b-dihydrodibenzo[b,d]furan-2(1h)-ylidene)ethyl)amino)ethyl)amino)ethylidene)-7,9-dihydroxy-8,9b-dimethyldibenzo[b,d]furan-1,3(2H,9bH)-dione ((+)-1).

Yellow solid (498 mg, yield 47 %); mp. 188.2-189.5°C. ¹H NMR (400 MHz, CDCl₃) δ 1.72 (s, 6H, CH₃-10), 2.09 (s, 6H, CH₃-15), 2.67 (s, 12H, CH₃-12, CH₃-14), 3.87 (m, 4H, CH₂-16), 5.81 (s, 2H, H-4), 13.35 (s, 2H, OH-9), 13.95 (s, 2H, OH-7). ¹³C NMR (MHz, CDCl₃) δ 7.49 (CH₃-15), 18.24 (CH₃-12), 31.30 (CH₃-14), 31.85 (CH₃-10), 42.62 (CH₂-16), 57.60 (C-9b), 101.42 (C-6), 102.16 (C-2), 102.65 (C-4), 104.81 (C-9a), 108.27 (C-8), 155.76 (C-5a), 158.10 (C-9), 163.60 (C-7), 174.50 (C-4a), 175.49 (C-11), 191.60 (C-3), 198.76 (C-1), 200.65 (C-13).

(S)-9-acetyl-10,12-(dihydroxy-5,11,12b-trimethyl-3,4-dihydro-2H-benzo[2,3]benzofuro[4,5-*e*][1,4]diazepin-6(12bH)-one ((+)-**2**).

Yellow solid (283 mg, yield 25.6 %); mp. 177.0-177.8. ¹H NMR (400 MHz, CDCl₃) δ 1.73 (s, 3H, CH₃-10), 2.07 (s, 3H, CH₃-15), 2.60 (s, 3H, CH₃-12), 2.67 (s, 3H, CH₃-14), 3.52-3.58 (m, 1H, CH₂-17), 3.73-3.81 (m, 2H, CH₂-16, CH₂-17), 4.20-4.25 (two d, 1H, CH₂-16), 5.76 (s, 1H, CH-4), 5.85 (bs, 1H, NH), 13.42 (s, 1H, OH-7), 15.18 (s, 1H, OH-9). ¹³C NMR (400 MHz, CDCl₃) δ 7.59 (CH₃-15), 27.66 (CH₃-12), 31.17 (CH₃-14), 31.26 (CH₃-10), 49.20 (CH₂-17), 49.98 (CH₂-16), 55.96 (C-9b), 100.30 (C-6), 100.60 (C-2), 103.58 (C-4), 106.87 (C-9a), 107.92 (C-8), 156.08 (C-5a), 160.78 (C-9), 163.58 (C-7), 165.42 (C-11), 173.76 (C-5), 176.25 (C-1), 187.66 (C-3), 200.43 (C-13).

(R)-9-acetyl-10,12-(dihydroxy-5,11,12b-trimethyl-3,4-dihydro-2H-benzo[2,3]benzofuro[4,5-*e*][1,4]diazepin-6(12bH)-one ((-)-**2**).

Yellow solid (160 mg, yield 29.0 %); mp. 176.8-177.4. ¹H and ¹³C NMR as above.

2 Biological procedures

2.1 Cell culture

Human cancer cell line Caco-2 (epithelial colorectal adenocarcinoma) was used to investigate the *in vitro* effect of compound **1** and **2**. Cells were grown in DMEM AQmedia (Sigma LifeScience, D0819) supplemented with 10 % fetal bovine serum and 5 mL pen/strep (10000 u penicillin, 10 mg/mL streptomycin) in a humidified incubator containing 5 % CO₂ at 37°C.

2.2 Cell count and cell size measurement with Coulter Counter

Cell number and average cell size were determined with a Coulter Counter (Beckmann Coulter Inc.) and Multisizer software (Coulter®AccuComp, version 3.01). Particles below 12.5 μm were considered dead cells and debris, thus excluded. The cells were seeded in 24-well plates at a density of 5 × 10⁴ cells/mL. After overnight incubation, media was replaced with treatments. Compounds were dissolved in DMSO (AMRESCO) and diluted in media to a final concentration of 0.55 % (0.57 % for **1**) DMSO. Cells in the control wells were treated with equivalent amount of DMSO in

media. Cells were treated for 24 or 48 hours. To investigate the effect on Caco-2 monolayer of cells, the cells were grown to confluency in 24 well plates. As the cells reach confluency, they will start to differentiate and stop proliferating resulting in a monolayer of Caco-2 cells. The cells were treated for 48 hours.

2.3 MTT assay

Caco-2 cells were seeded in 96-well plates at a density of 5×10^3 cells/ 100 μ L. Compounds were added after overnight incubation. The cells were treated for 24 or 48 hours, and cell viability was determined by measuring formation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (absorbance 570 nm).

2.4. Hoechst 33342 assay

Caco-2 cells were seeded in 96-wells plates at a density of 5×10^3 cells/ 100 μ L. The cells were treated for 24 or 48 hours. After exposure, 5 μ g/mL Hoechst solution was added, and incubated for 1.5 hours. Intensity as an estimate for DNA content was determined by measuring fluorescence (excitation 360nm and emission 450nm).

2.5. Fluorescence-activated cell sorting

Caco-2 cells were seeded at a density of 1×10^6 cells/mL in T75 flasks and treated after overnight incubation. Cells were collected after 24 hours of treatment, washed with Na-citrate, and fixated in EtOH. Cells were treated with 50 μ L RNase (1 mg/mL), stained with 50 μ L propidium iodide (0.4 mg/mL) and analysed on a FACSCalibur (BD Bioscience), processed in CellQuestPro (BD Bioscience).

2.6. Statistical analysis

All data are normalised according to the control in the experiment. The mean control value is set 100 % and all data relates to this value. To analyse the results, one-way ANOVA (Bonferroni) was used to determine the significance ($P < 0.05$) using GraphPad Prism 6.07 (GraphPad Software Inc.). All data are expressed as means \pm standard deviation (SD) of at least three independent experiments.

Figure S1. ¹H NMR of compound 1.

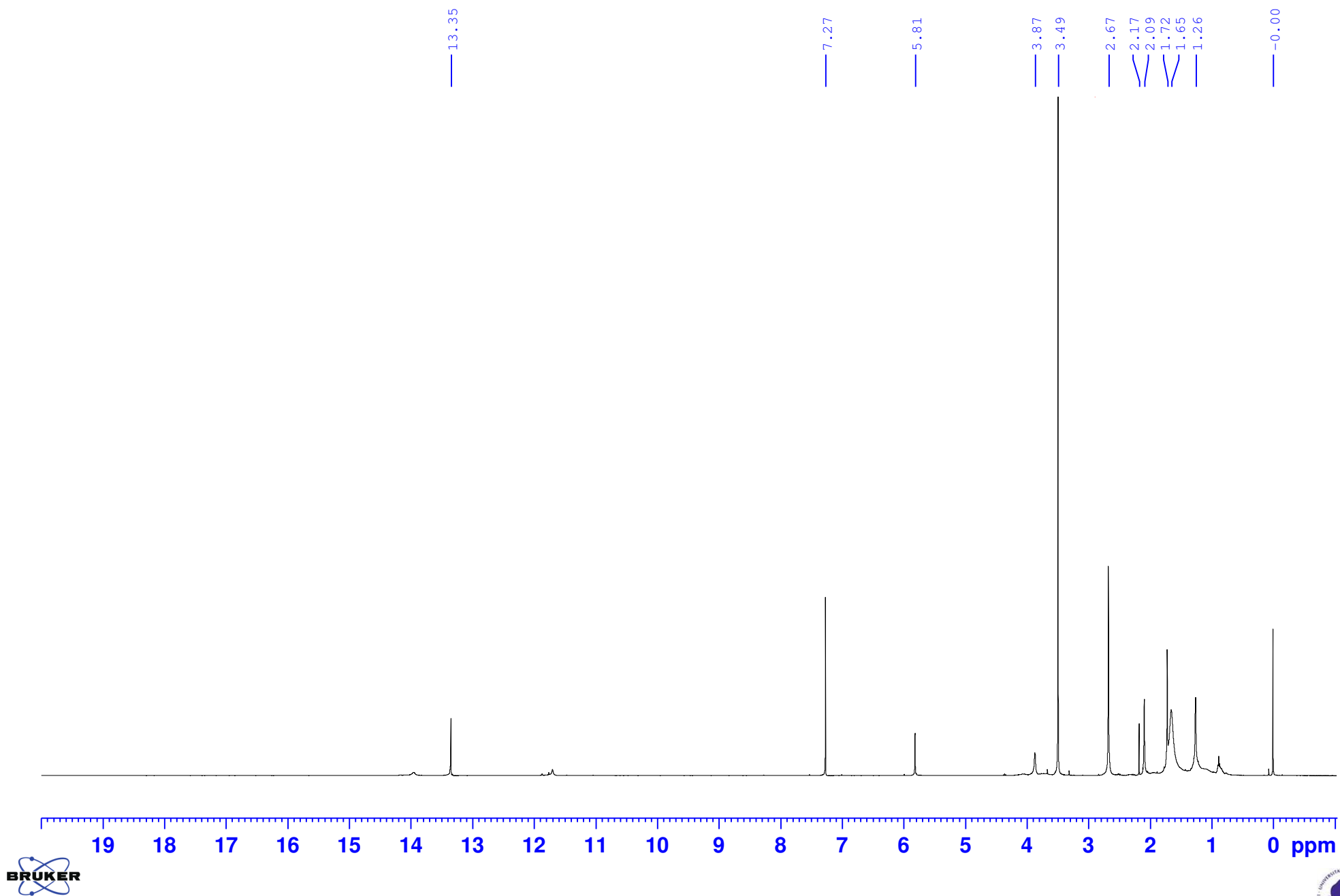


Figure S2. ¹³C NMR of compound 1.

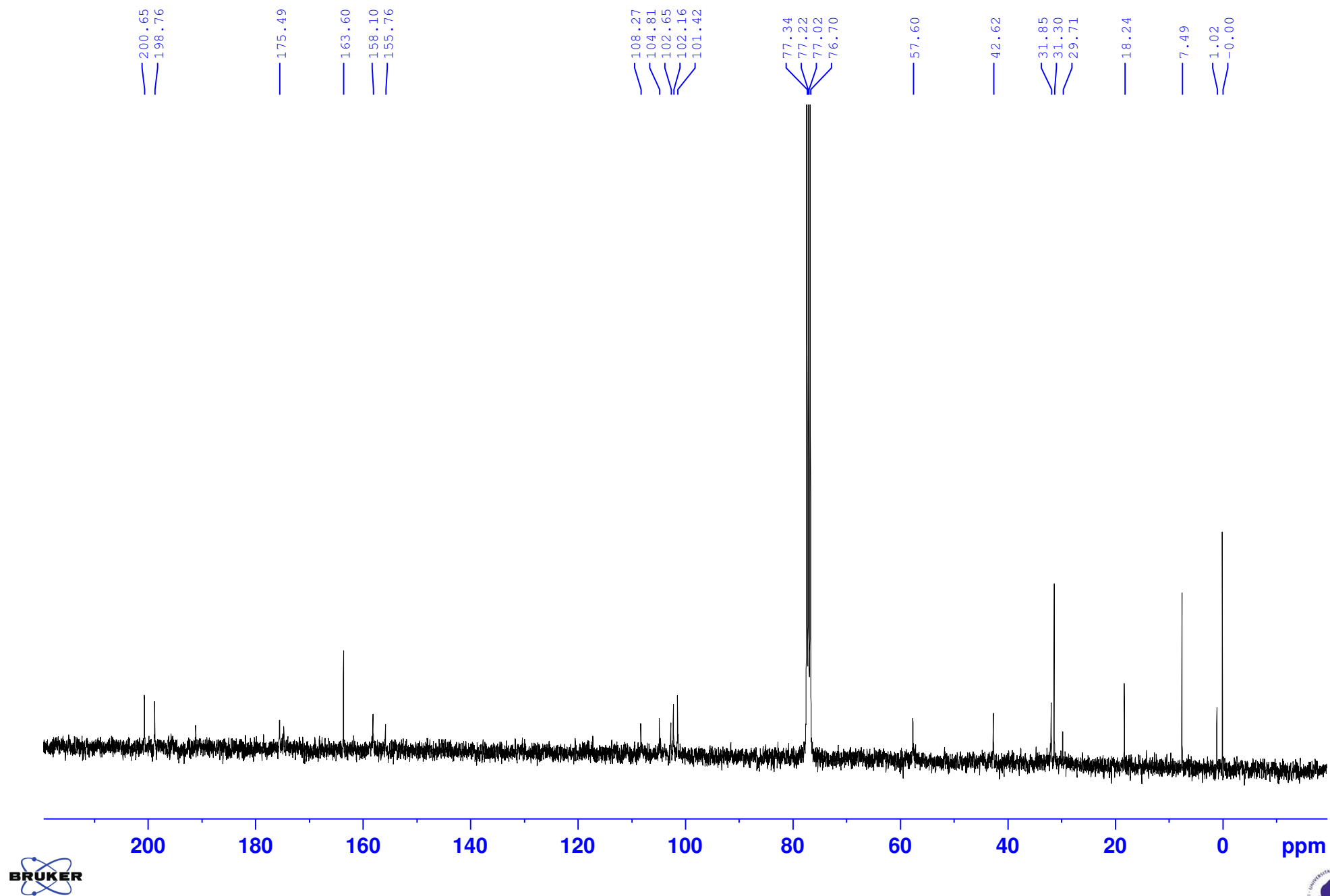


Figure S3. ¹H NMR of compound 2.

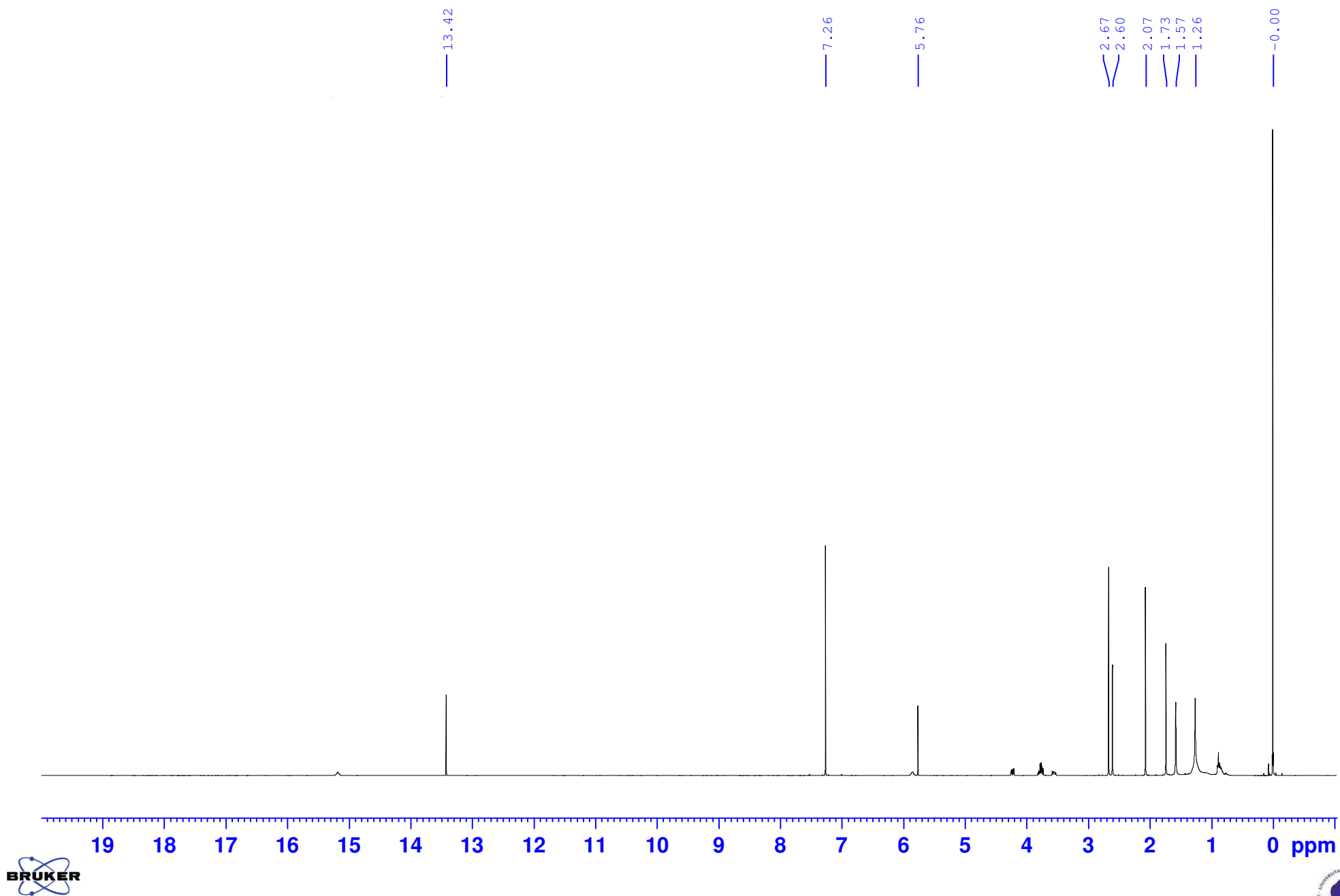


Figure S4. ¹³C NMR of compound 2.

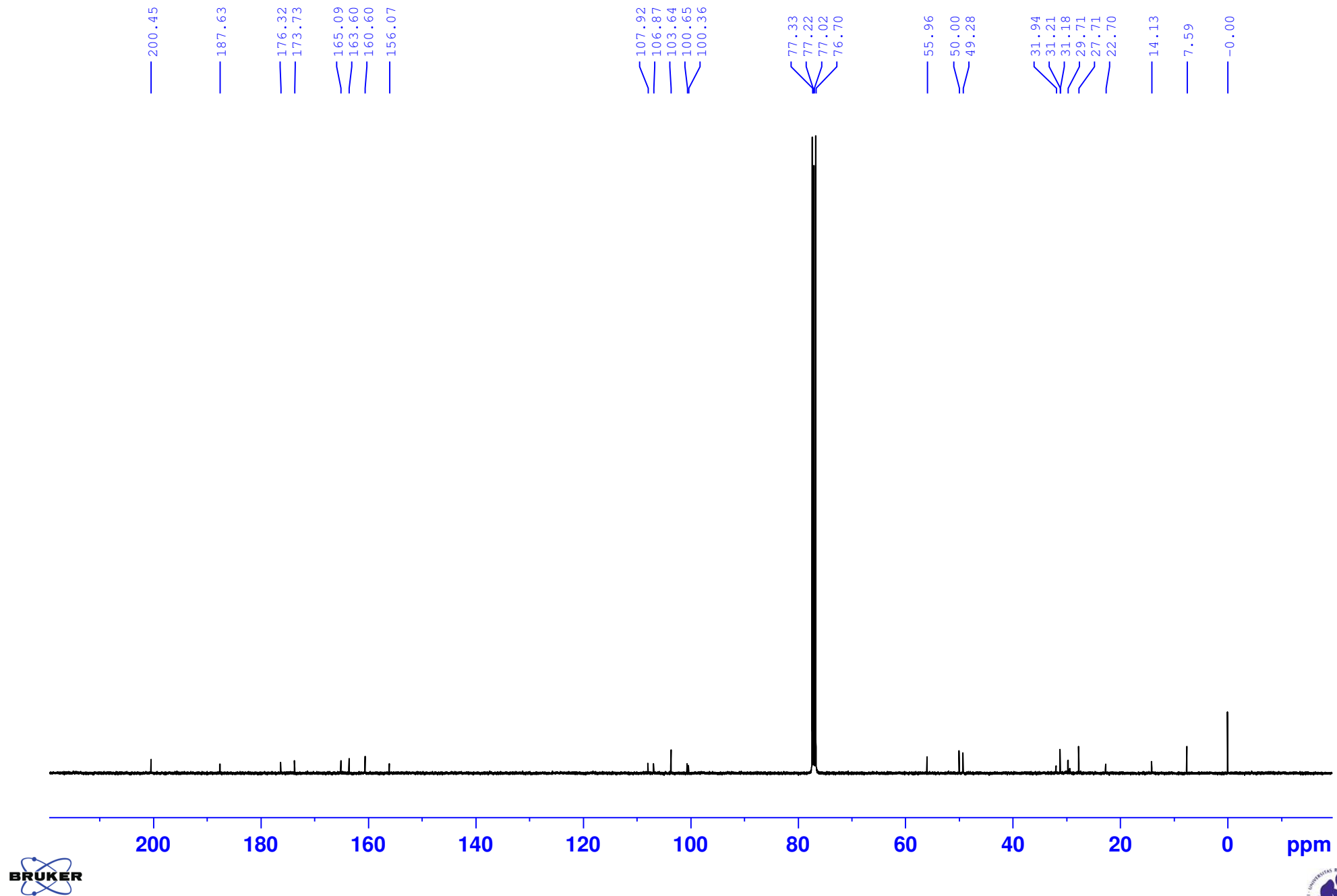
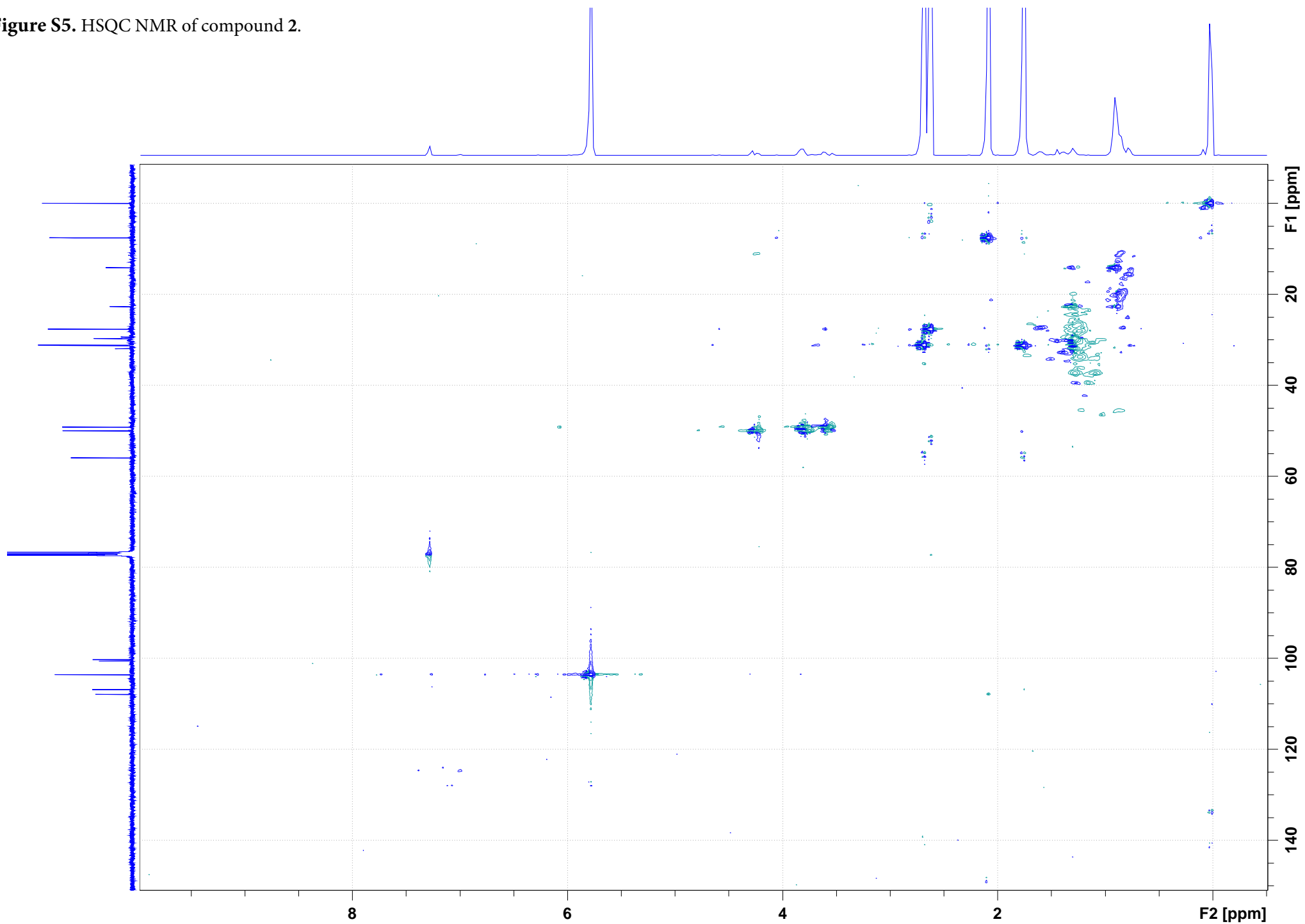


Figure S5. HSQC NMR of compound 2.



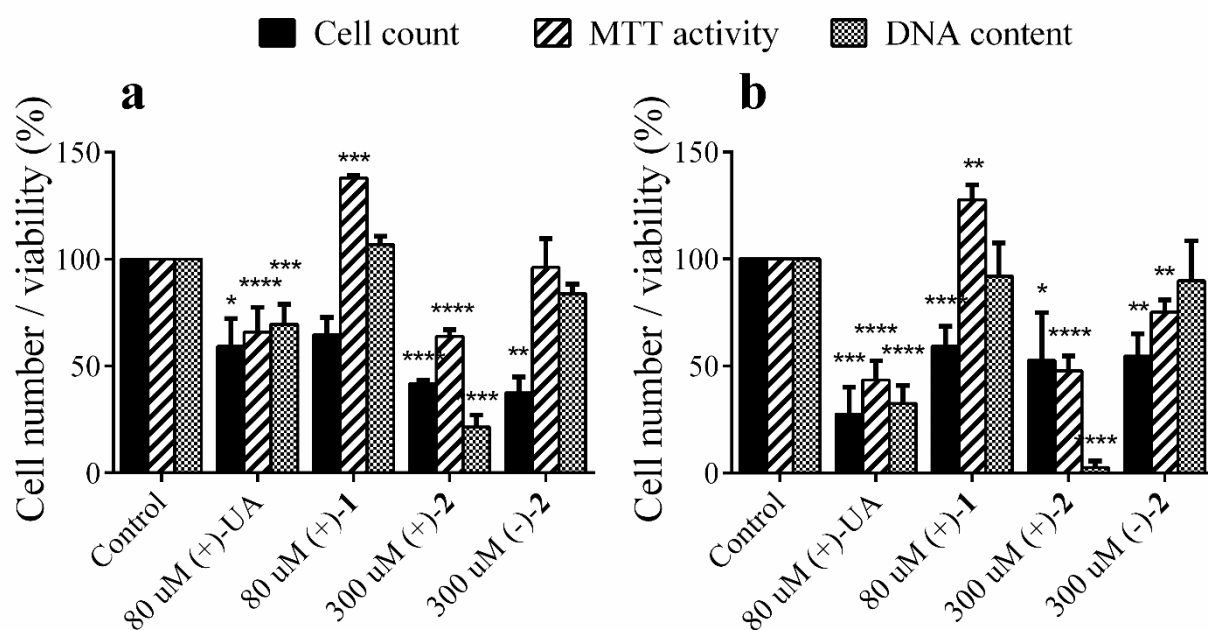


Figure S6. Relative inhibition of undifferentiated Caco-2 cells based on cell count, MTT assay and DNA-content after 24h (a) and 48h (b) of treatment with UA, (+)-1, (+)-2 and (-)-2. The mean \pm SD values were calculated from the results of three independent experiments performed in at least triplicates. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. **** $p < 0.0001$.

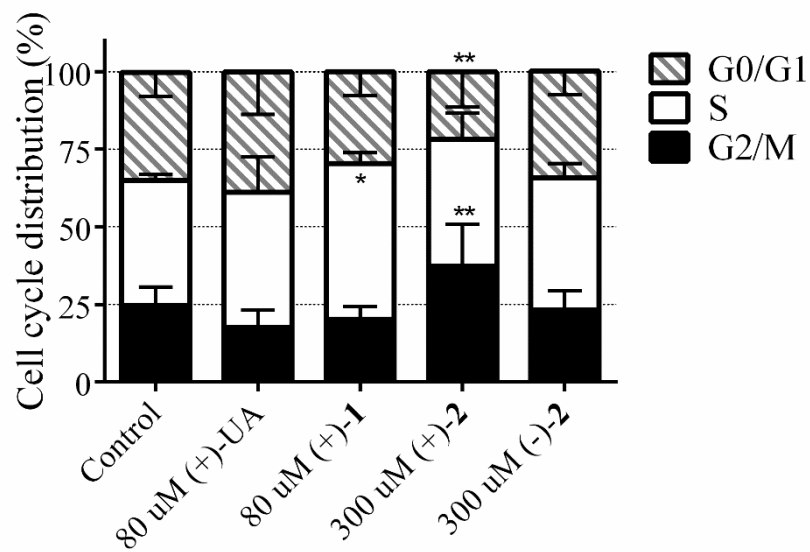


Figure S7. Cell cycle distribution of Caco-2 cells after treatment with UA, (+)-1, (+)-2 and (-)-2 for 24 hours. The mean \pm SD values were calculated based on three independent experiments, performed in triplicates. * $p < 0.05$. ** $p < 0.01$.

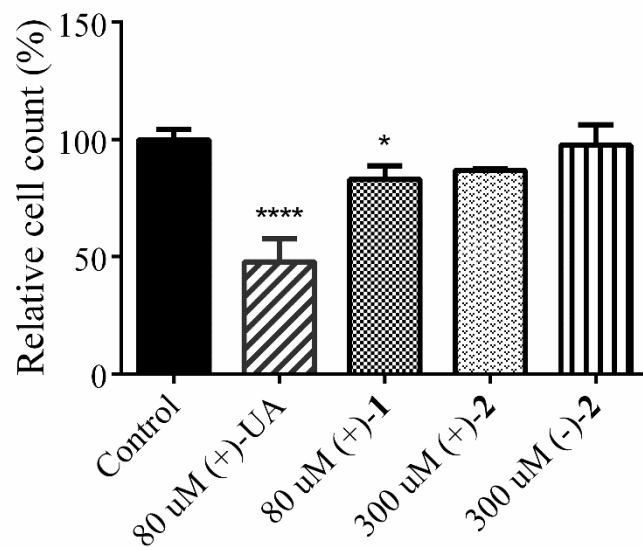


Figure S8. Inhibition of the cell number of Caco-2 monolayer after 48 hours. The mean \pm SD values were calculated from the results of four experiments performed in at least triplicates. * $p < 0.05$. **** $p < 0.0001$.

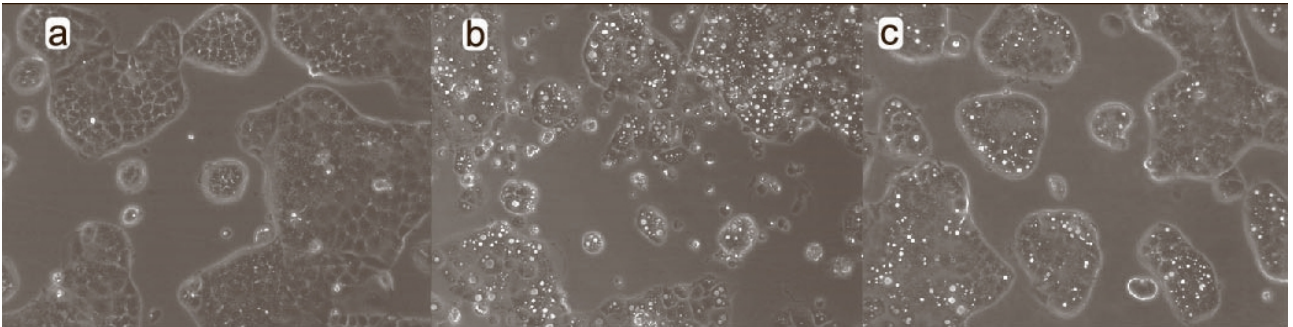


Figure S9. Effect on cell morphology of undifferentiated Caco-2 cells, control (a), exposed to 300 μM (+)-2 (b) or exposed to 300 μM (-)-2 (c) for 24 hours. Pictures were made by phase-contrast microscopy, and representative examples are shown. The white spheres inside the cells (B and C) are cytoplasmic vacoulation. Magnification: 200x.

Table S1. Calculated physicochemical data for UA and derivatives.

	Solubility water (mg/L)	logP ¹
UA	82	1.99
1	16	3.43
2	6.1	3.02

The physicochemical data are calculated with ACD/Labs software.

¹logP = un-ionized octanol-water partition coefficient.

Solubility and the water-octanol distribution coefficient (logP), were estimated by QSAR calculations (ACD/I-Lab. 2017.

<https://ilab.acdlabs.com/iLab2/>)