

Supplementary materials

Synthesis, pharmacokinetic profile, anticancer activity and toxicity of the new amides of betulonic acid – in silico and in vitro study

Bębenek Ewa¹, Rzepka Zuzanna², Hermanowicz Justyna^{3,4}, Chrobak Elwira¹, Surażynski Arkadiusz⁵, Beberok Artur², Wrześniok Dorota^{2,*}

¹ Department of Organic Chemistry, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia in Katowice, 4 Jagiellońska Str., 41-200 Sosnowiec, Poland

² Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences in Sosnowiec, Medical University of Silesia in Katowice, 4 Jagiellońska Str., 41-200 Sosnowiec, Poland

³ Department of Pharmacodynamics, Medical University of Białystok, Mickiewicza 2c, 15-222 Białystok, Poland

⁴ Department of Clinical Pharmacy, Medical University of Białystok, Mickiewicza 2c, 15-222 Białystok, Poland

⁵ Department of Medicinal Chemistry, Medical University of Białystok, Kilinskiego 1, 15-089 Białystok, Poland

*Corresponding author.

E-mail address: dwrzesniok@sum.edu.pl

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A. Synthesis - Materials and methods

The chemical reagents were purchased from the Sigma-Aldrich. All melting points (mp) were determined using an Electrothermal IA 9300 apparatus, and are uncorrected. ^1H and ^{13}C NMR experiments were performed on a Bruker Avance III 600 spectrometer in CDCl_3 , as solvent. Chemical shifts (δ) were referenced to the signal of the CDCl_3 and are given in parts per million (ppm). The determined coupling constants (J) are reported in Hertz (Hz). Infrared spectra were recorded on an IRAffinity-1 Shimadzu spectrometer. EI MS spectra were recorded on a Finnigan MAT 95 instrument. HRMS spectra were taken on a Bruker Impact II instrument mass spectrometer. The course of the reaction was monitored using Silica gel 60 254F plates (Merck). The spots of the chromatograms were visualized by spraying with an 10% ethanolic solution of H_2SO_4 and heating to 100 $^\circ\text{C}$. Purification was conducted by column chromatography using silica gel 60 (0.063-0.200 mm).

Figure S1. ^1H NMR, compound EB170

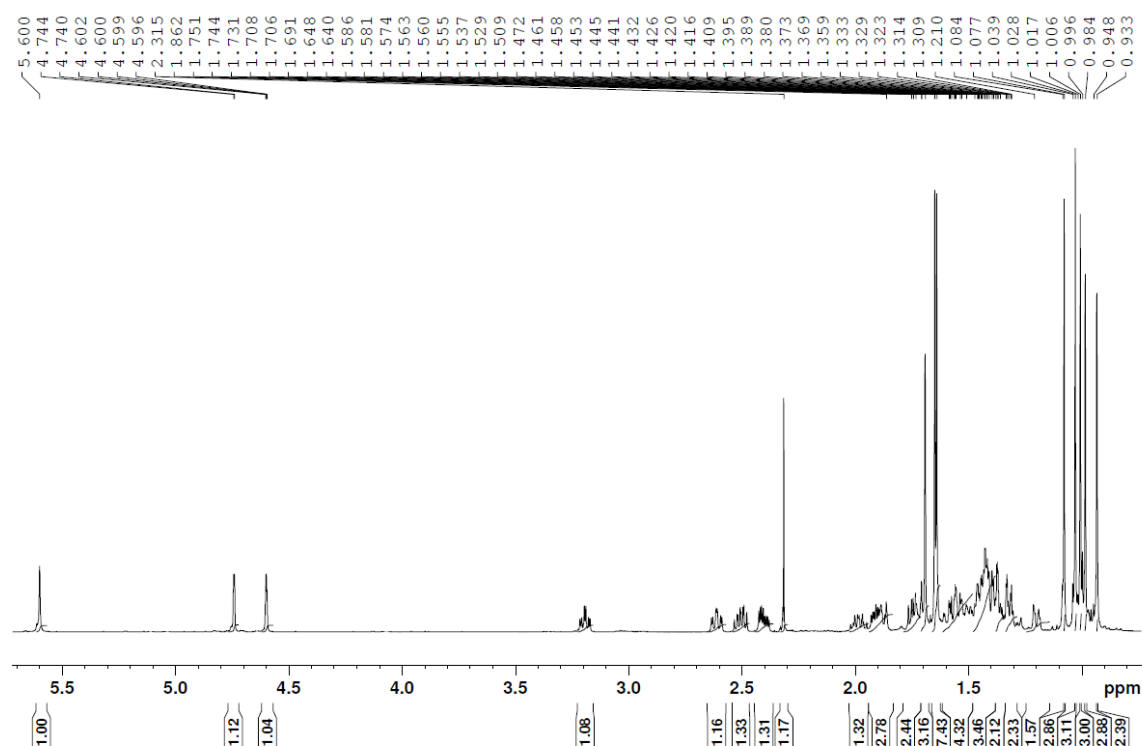


Figure S2. ^{13}C NMR, compound EB170

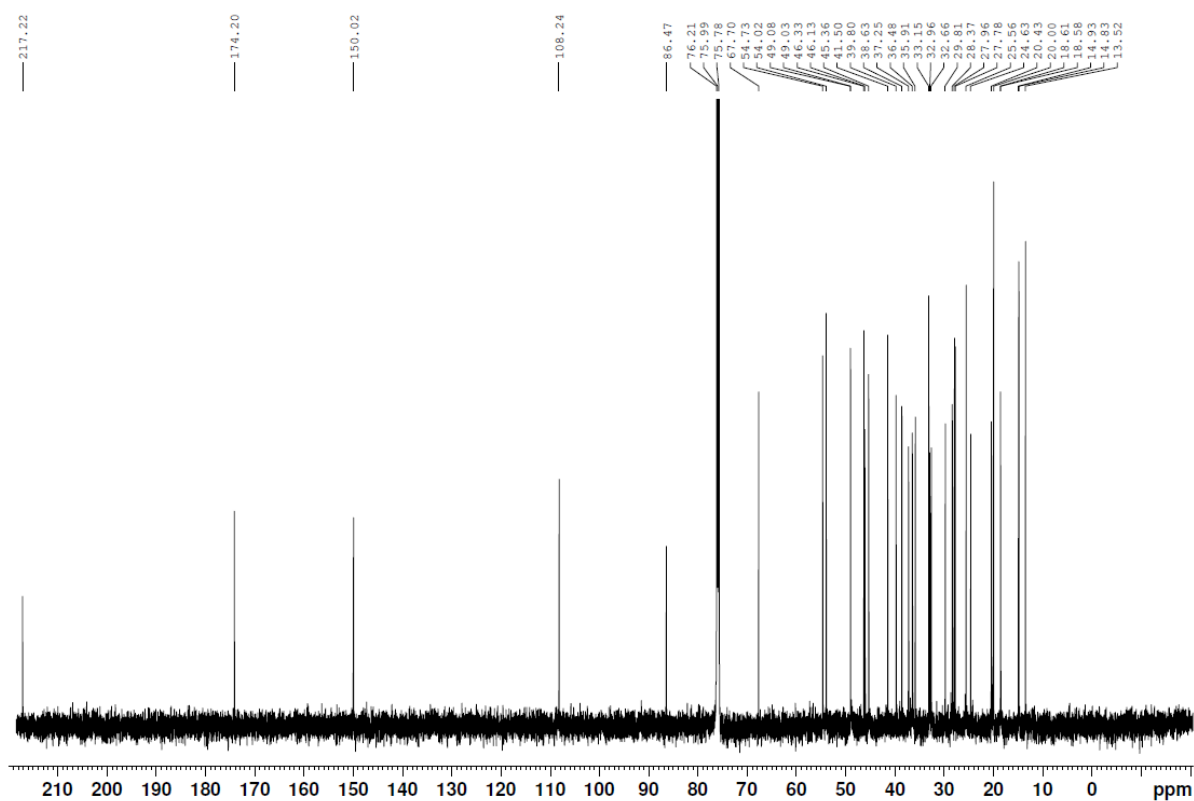


Figure S3. IR, compound EB170

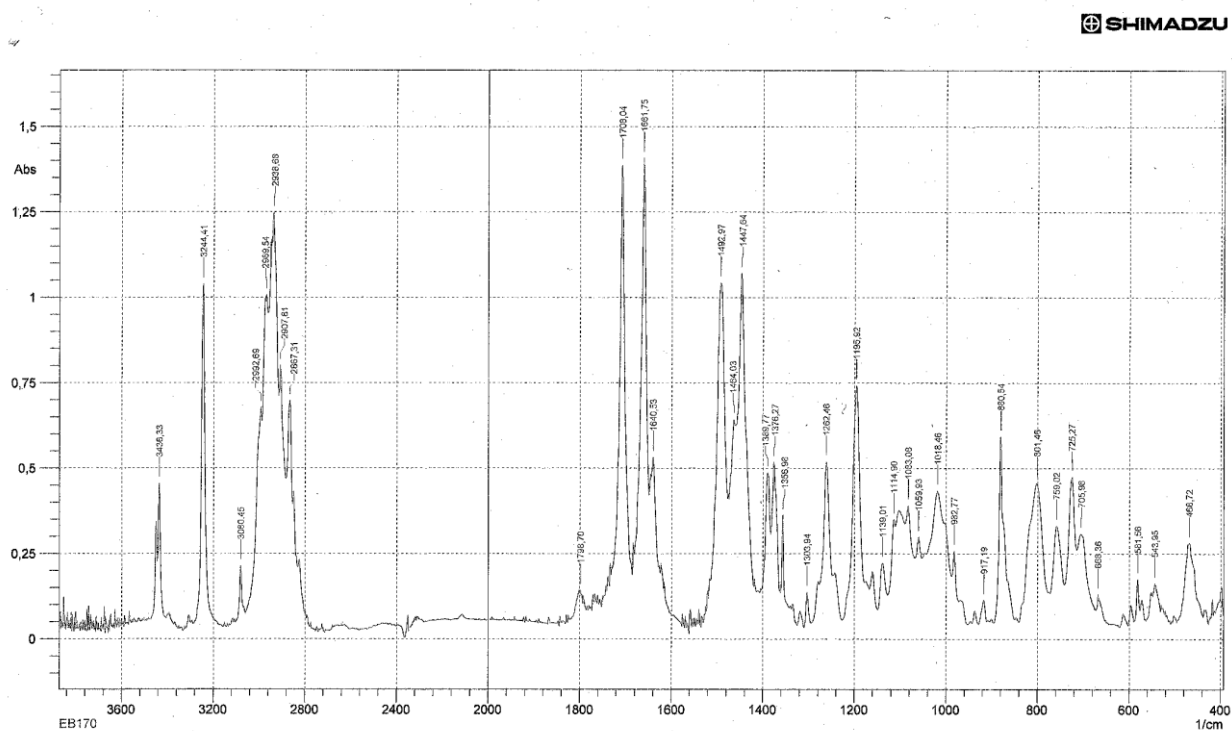


Figure S4. EI MS, compound EB170

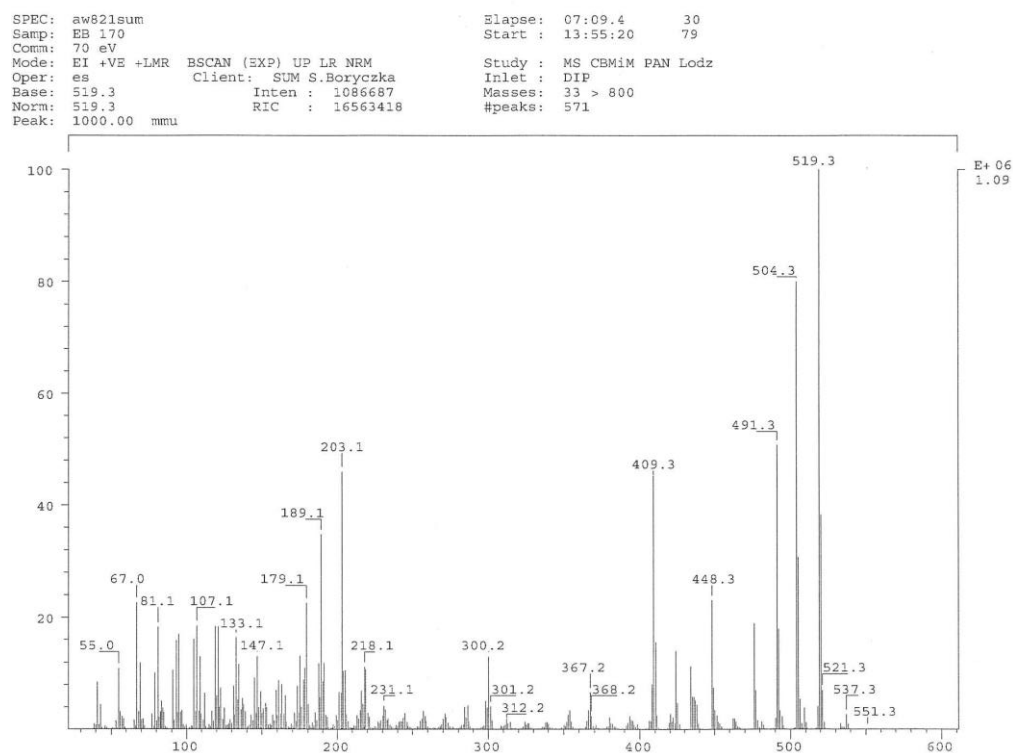


Figure S5. HRMS, compound EB170

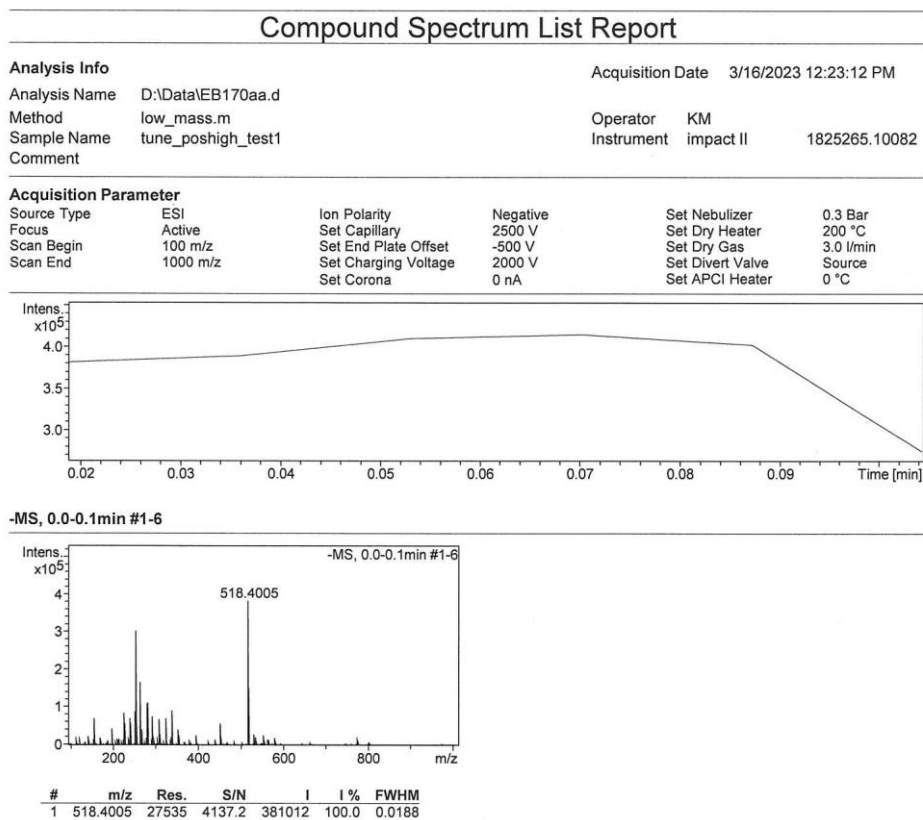


Figure S6. ^1H NMR, compound EB171

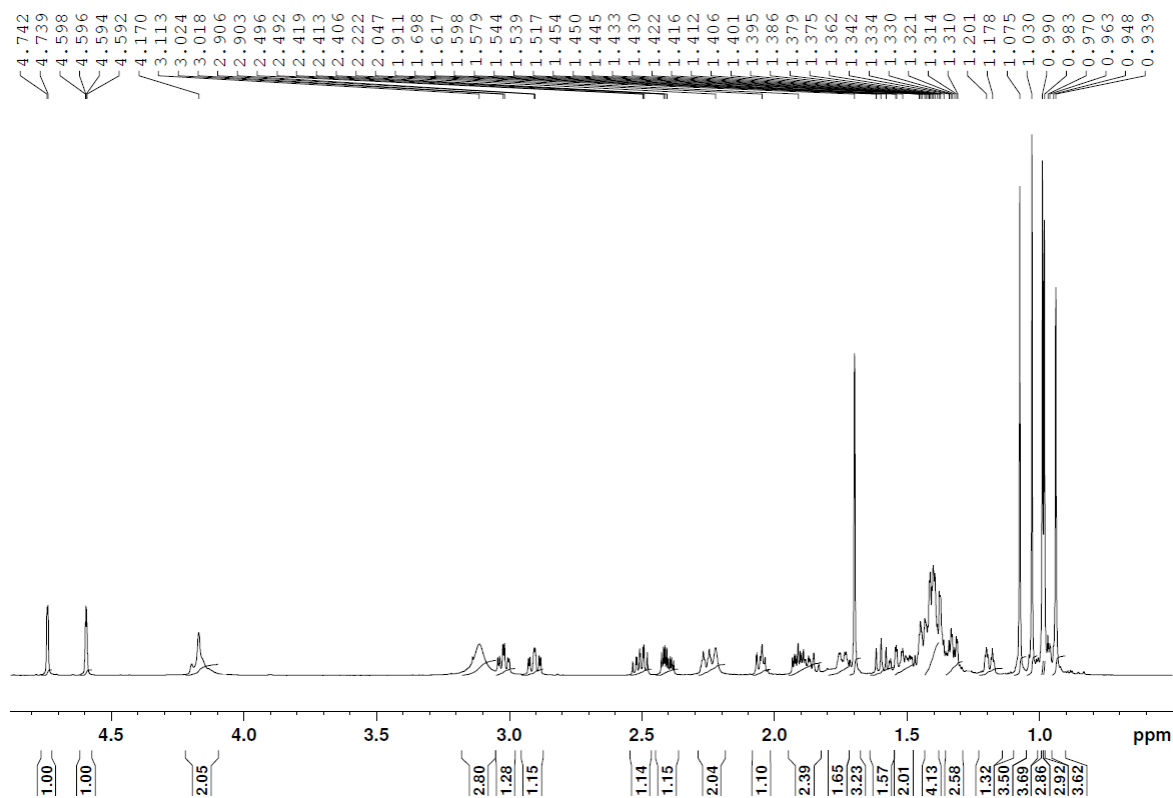


Figure S7. ^{13}C NMR, compound EB171

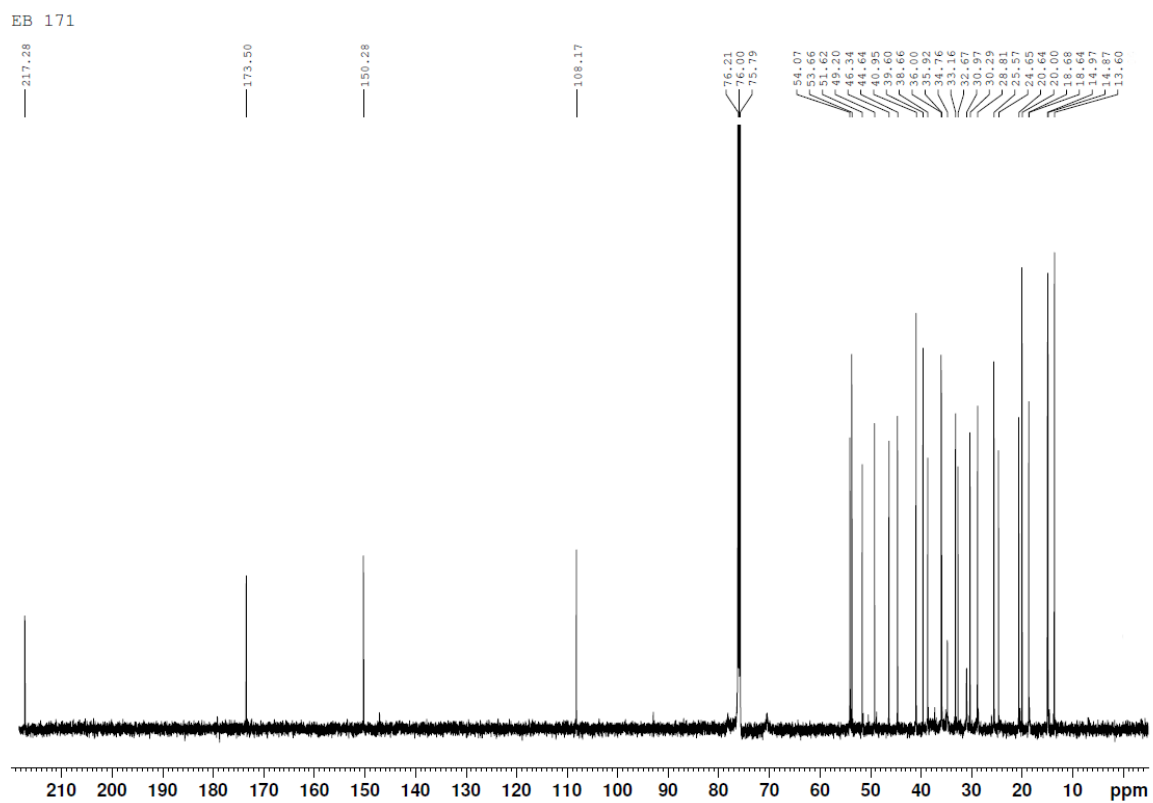


Figure S8. IR, compound EB171

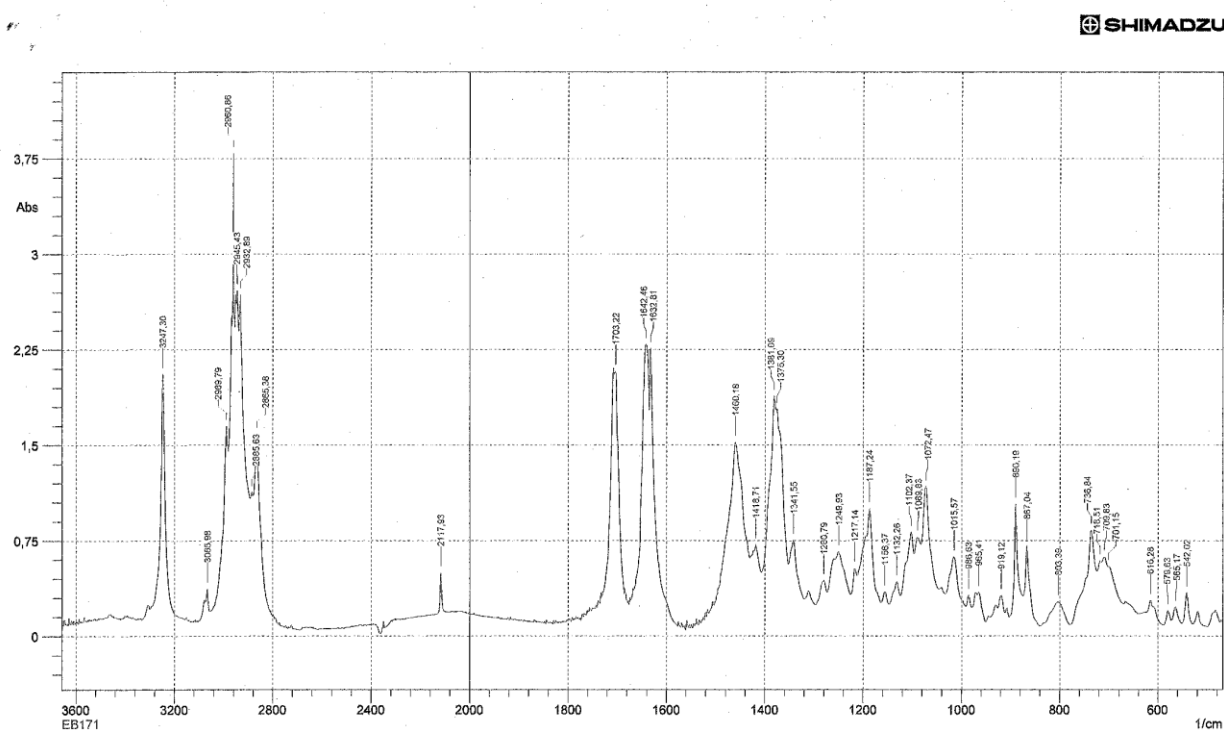


Figure S9. EI MS, compound EB171

SPEC: aw822sum
Samp: EB 171
Comm: 70 eV
Mode: EI +VE +LMR BSCAN (EXP) UP LR NRM
Oper: es
Base: 409.3
Norm: 409.3
Peak: 1000.00 mmu

Elapse: 06:13.1
Start: 14:17:56
Study: MS CBMim PAN Lodz
Inlet: DIP
Masses: 33 > 800
#peaks: 531

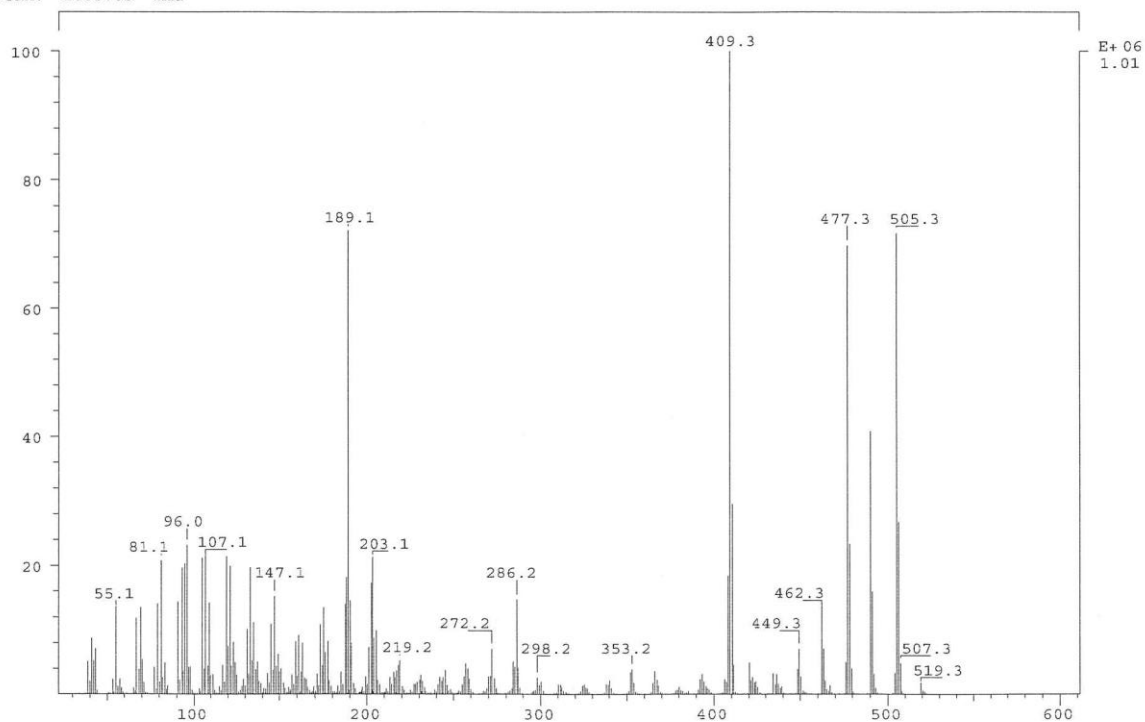


Figure S10. HRMS, compound EB171

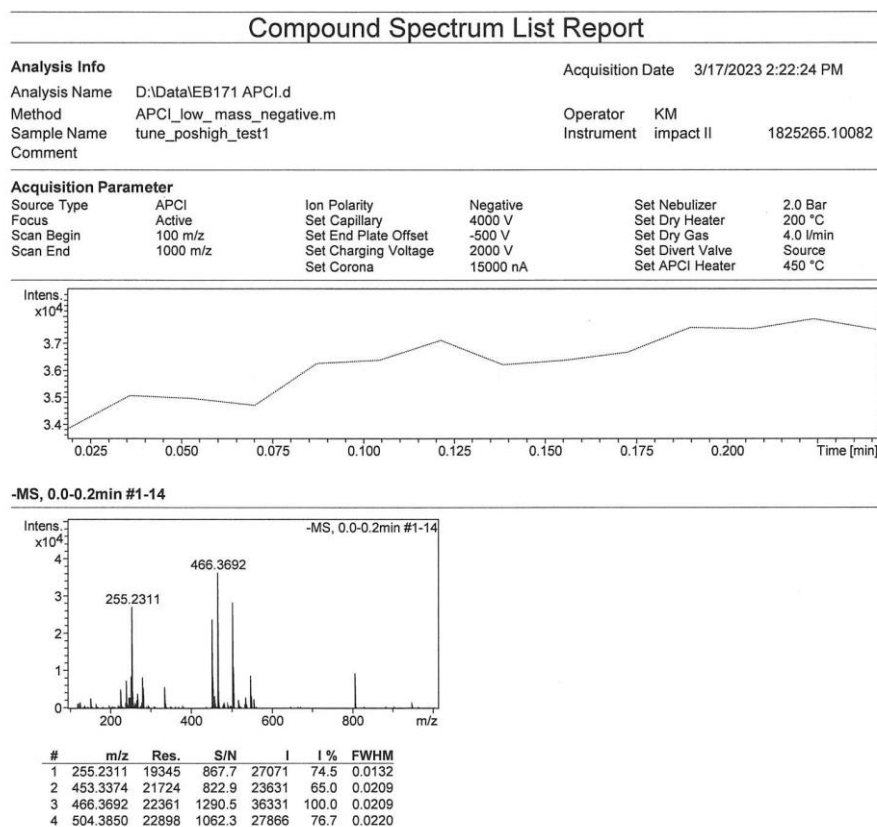


Figure S11. ¹H NMR, compound EB173

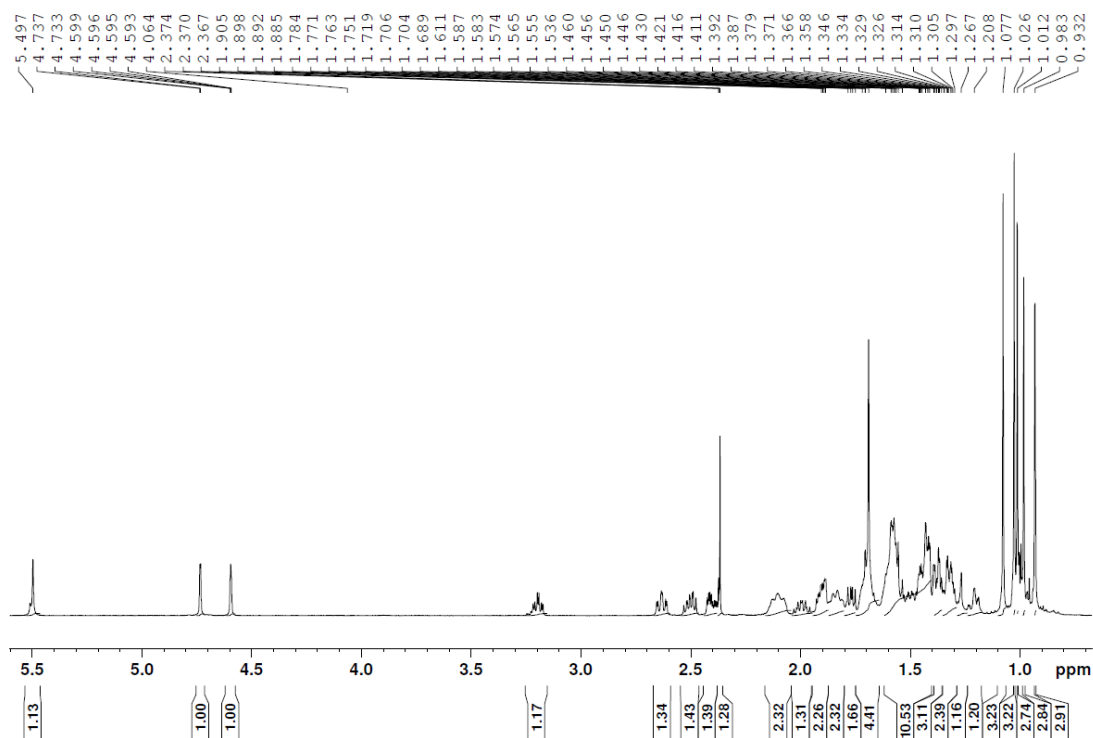


Figure S12. ^{13}C NMR, compound EB173

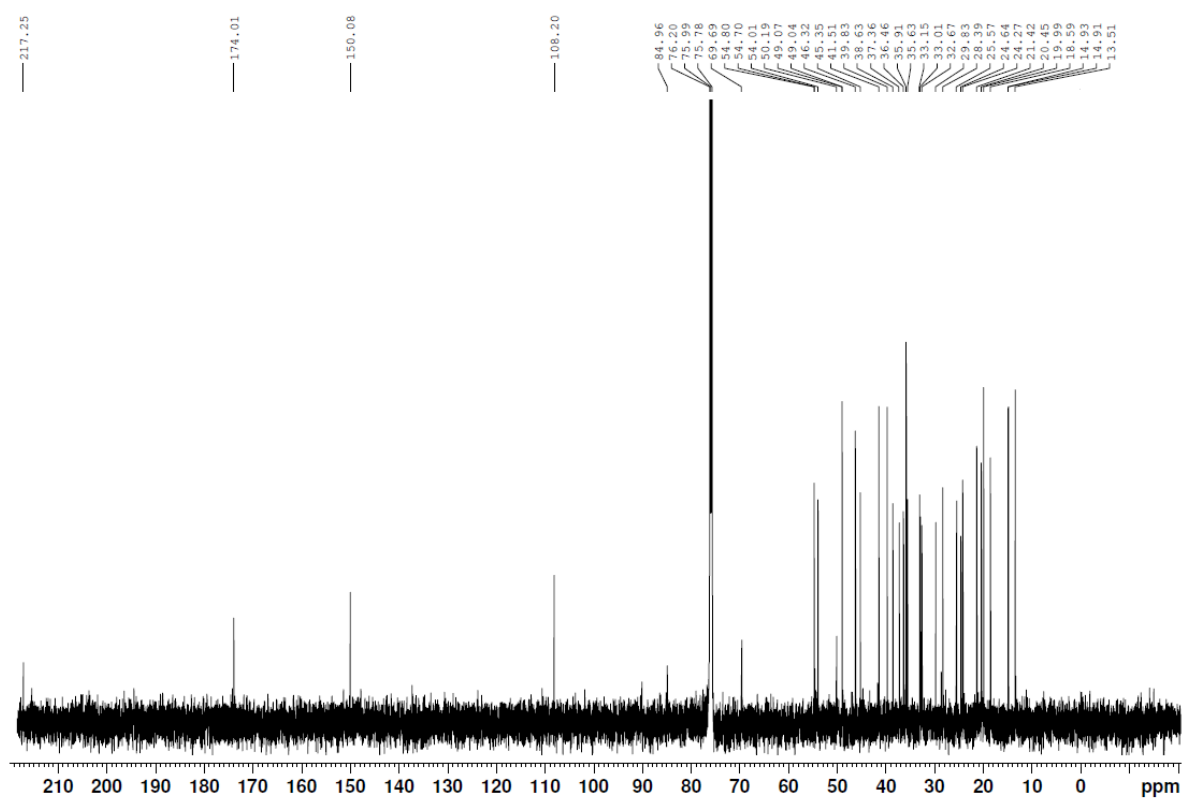


Figure S13. IR, compound EB173

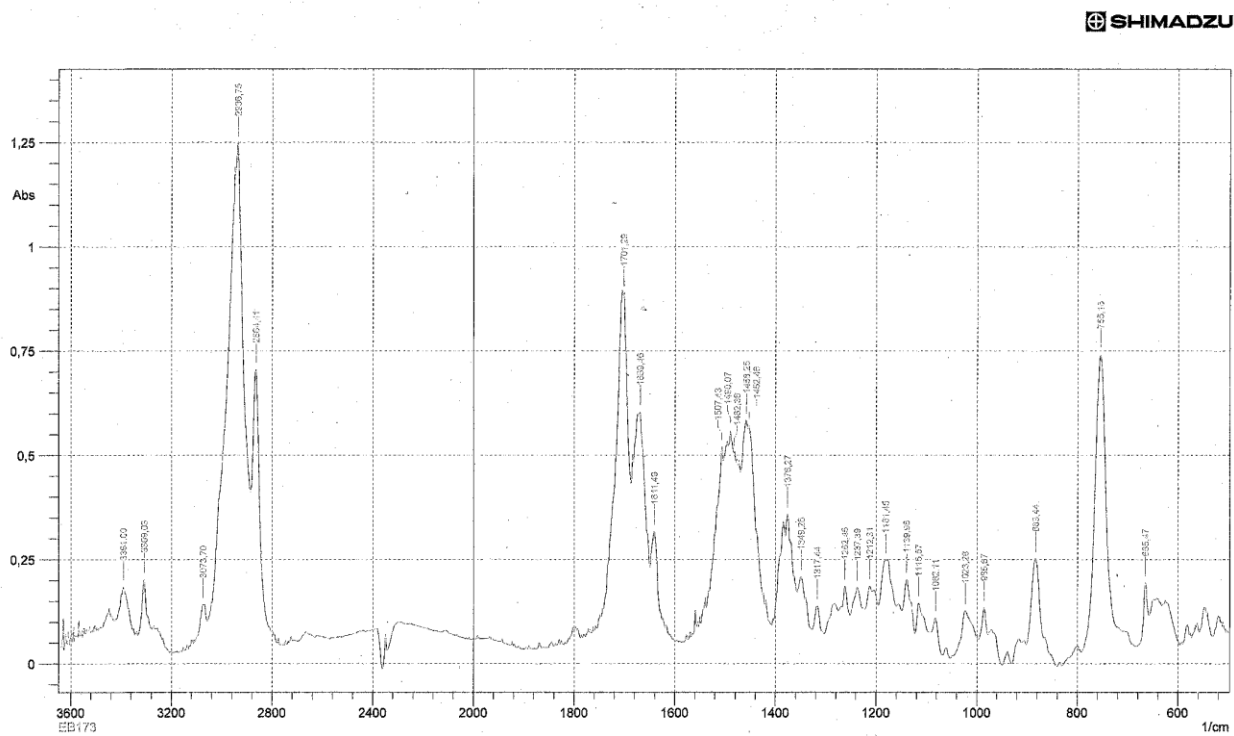


Figure S14. EI MS, compound EB173

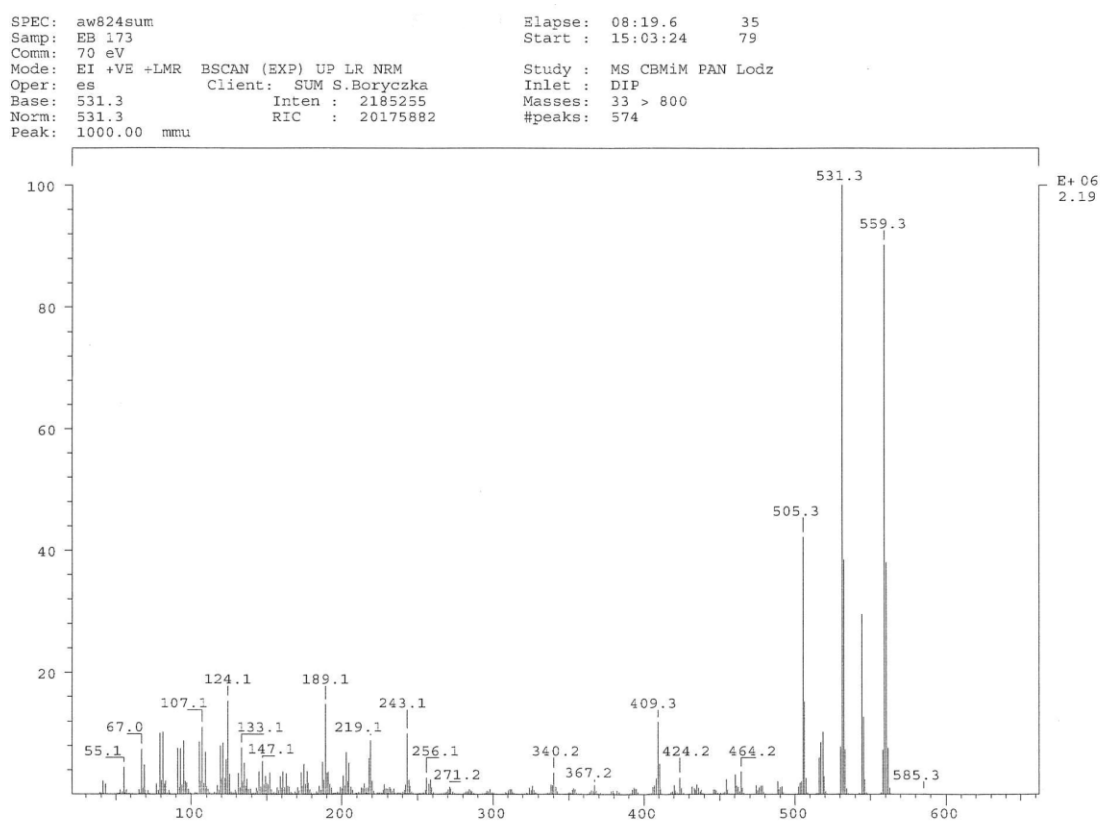
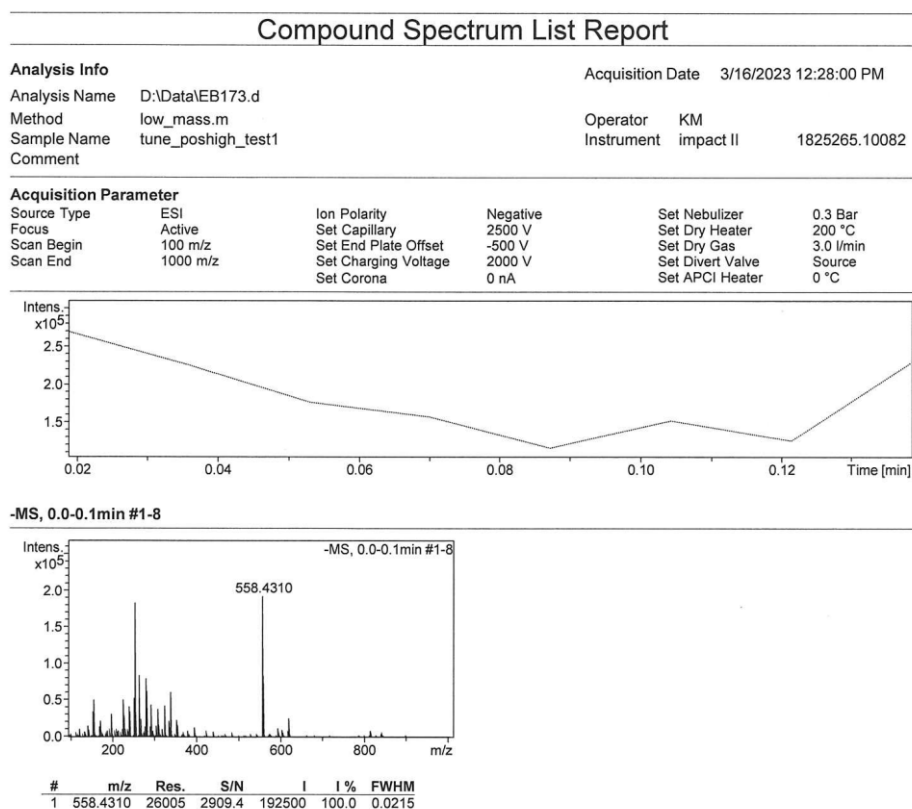


Figure S15. HRMS, compound EB173



B. Lipophilicity

Chromatographic determination of lipophilicity parameters.

The experimental determination of the lipophilicity of the tested substances was carried out using reversed-phase thin-layer chromatography (RP-TLC). For this purpose, aluminum plates covered with modified silica gel RP-18 with F254s fluorescent indicator were used, purchased from Merck. Samples for testing were prepared by dissolving 2 mg of the appropriate substance (standards listed in table S1 or newly synthesized amide derivatives) in 2 ml of chloroform. 2 μ L of the obtained solutions was taken and placed on chromatographic plates, which were then developed in chromatographic chambers saturated with eluent vapors. Mixtures of acetone and Tris buffer (aqueous solution of tris-hydroxymethyl)aminomethane in concentration 0.2 M, pH 7.4) were used as mobile phases in volume proportions taking into account the acetone content from 60 to 90% (every 5%). The chromatographic determination was performed in triplicate for each tested substance. The chromatograms were sprayed with a solution of concentrated sulfuric acid and ethanol (in a volume ratio of 1:10), and then the plates were heated to a temperature of 110°C until spots of the analyzed substances were visible. For all substances, in each eluent system, the distances from the starting line to the center of the spot (a) and from the starting line to the eluent front line were measured, and on this basis the retardation factor (R_f) was calculated (1) and then the value of the R_M coefficient (2):

$$R_f = a/b \quad (1)$$

$$R_M = \log [(1/R_f)-1] \quad (2)$$

The dependence of the R_M parameter on the percentage concentration of acetone in the mobile phase (C) is presented by the Soczewiński-Wachtmeister equation:

$$R_M = R_{M0} + bC \quad (3)$$

where: b – slope of the regression graph.

Extrapolation of the acetone content in the eluent system to zero allows the determination of the chromatographic lipophilicity parameter R_{M0} .

To determine the calibration curve (dependence of logP on R_{M0}), eight reference compounds were used, for which the literature logP_{lit} values are in the range of 1.21–6.38 [1]. R_{M0} values for these compounds were determined in the same way as for the tested amide derivatives and betulonic acid (Table S1).

[1]. Kadela-Tomanek, M.; Jastrzębska, M.; Marciniec, K.; Chrobak, E.; Bębenek, E.; Boryczka, S. Lipophilicity, pharmacokinetic properties, and molecular docking study on SARS-CoV-2 target for betulin triazole derivatives with attached 1,4-quinone. *Pharmaceutics* 2021, 13, 781. <https://doi.org/10.3390/pharmaceutics13060781>.

Table S1. The experimental (R_{M0} and $\log P_{TLC}$) and literature ($\log P_{lit}$) lipophilicity values for reference compounds (mobile phase acetone:buffer Tris)

Reference compound	R_{M0}	$\log P_{lit}$	b	r	$\log P_{TLC}$
acetanilide	0.57	1.21	-0.01	0.982	1.31
prednisone	0.72	1.62	-0.01	0.989	1.48
4-bromoacetophenone	1.82	2.43	-0.02	0.985	2.73
benzophenone	2.23	3.18	-0.03	0.989	3.19
anthracene	3.03	4.45	-0.04	0.993	4.10
dibenzyl	3.51	4.79	-0.04	0.994	4.64
9-phenylanthracene	3.99	6.01	-0.05	0.996	5.19
DDT	5.20	6.38	-0.06	0.997	6.56

b is the slope and r is the correlation coefficient for the linear relationship $R_M = R_{M0} + bC$

The following calibration curve equation was used to determine the experimental lipophilicity values ($\log P_{TLC}$) of the tested amide derivatives and betulonic acid:

$$\log P_{TLC} = 1.1332 R_{M0} + 0.6683 \quad (r = 0.992)$$

Table S2. The theoretical values of lipophilicity for betulonic acid and its amide derivatives

Compound	iLOGP	XLOGP3	WLOGP	MLOGP	SILICOS-IT
betulonic acid	3.62	7.89	7.30	5.73	6.34
EB170	4.76	8.34	7.82	6.16	7.74
EB171	4.77	7.94	7.38	5.98	7.20
EB173	5.12	9.35	8.75	6.69	8.51

Table S3. The correlation matrix for theoretically obtained lipophilicity parameters of tested compounds

	iLOGP	XLOGP3	WLOGP	MLOGP	SILCOS-IT
iLOGP	1.000	0.680	0.703	0.832	0.917
XLOGP3		1.000	0.998	0.972	0.900
WLOGP			1.000	0.979	0.917
MLOGP				1.000	0.972
SILCOS-IT					1.000

Table S4. The correlation between the molecular descriptors and the R_{M0} values for betulonic acid and its amide derivatives

Compound	Molecular descriptors	Equation	<i>r</i>
betulonic acid EB170 EB171 EB173	<i>MW</i>	$R_{M0} = 225.21 MW^2 - 2471.9MW + 7248.3$	0.963
	<i>RefM</i>	$R_{M0} = 76.789 MW^2 - 843.42MW + 2456.2$	0.949
	<i>HA</i>	$HA = -19706 R_{M0}^2 + 21.73 R_{M0} - 57.271$	0.684
	<i>HD</i>	$HD = 1.6764 R_{M0}^2 - 19.04 R_{M0} + 54.489$	0.612
	<i>RB</i>	$RB = 2.1355 R_{M0}^2 - 22.512 R_{M0} + 61.953$	0.783
	<i>TPSA</i>	$TPSA = -1.4233 R_{M0}^2 + 10.82R_{M0} + 30.32$	0.367
	<i>Caco-2 perm</i>	$Caco-2 perm = -0.1189 R_{M0}^2 + 1.2964 R_{M0} - 2.2163$	0.774
	<i>Kp</i>	$Kp = -0.0423 R_{M0}^2 + 0.4754 R_{M0} - 4.0832$	0.345
	<i>BBB</i>	$BBB = -1.1491 R_{M0}^2 + 12.681 R_{M0} - 35.331$	0.746
	<i>PS</i>	$PS = -1.8637 R_{M0}^2 + 20.455 R_{M0} - 57.456$	0.892

Table S5. The correlation between the anticancer activity (IC_{50}) and the R_{M0} values for betulonic acid and its derivatives

Compound	Cell line	Equation	<i>r</i>
betulonic acid EB171 EB173	C32	$IC_{50} = 190.28 R_{M0} + 976.03$	0.962
	Colo829	$IC_{50} = 129.51 R_{M0} + 686.05$	0.858
	A375	$IC_{50} = 188.28 R_{M0} + 1029.1$	0.848
	MCF-7	$IC_{50} = 413.11 R_{M0} + 2242.6$	0.792