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Substituted dienes prepared from betulinic acid – Synthesis, cytotoxicity, mechanism of action, and pharmacological parameters

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ABSTRACT

A set of new substituted dienes were synthesized from betulinic acid by its oxidation to 30-oxobetulinic acid followed by the Wittig reaction. Cytotoxicity of all compounds was tested *in vitro* in eight cancer cell lines and two noncancer fibroblasts. Almost all dienes were more cytotoxic than betulinic acid. Compounds **4.22**, **4.30**, **4.33**, **4.39** had IC_{50} below 5 μmol/L; **4.22** and **4.39** were selected for studies of the mechanism of action. Cell cycle analysis revealed an increase in the number of apoptotic cells at 5 × IC_{50} concentration, where activation of irreversible changes leading to cell death can be expected. Both **4.22** and **4.39** led to the accumulation of cells in the G0/G1 phase with partial inhibition of DNA/RNA synthesis at 1 × IC_{50} and almost complete inhibition at 5 × IC_{50}. Interestingly, compound **4.39** at 5 × IC_{50} caused the accumulation of cells in the S phase. Higher concentrations of tested drugs probably inhibit more off-targets than lower concentrations. Mechanisms disrupting cellular metabolism can induce the accumulation of cells in the S phase. Both compounds **4.22** and **4.39** trigger selective apoptosis in cancer cells *via* intrinsic pathway, which we have demonstrated by changes in the expression of the crucial apoptosis-related protein. Pharmacological parameters of derivative **4.22** were superior to **4.39**, therefore **4.22** was the finally selected candidate for the development of anticancer drug.

1. Introduction

Pentacyclic triterpenes are natural compounds with a broad spectrum of biological activities [1–7]. Among them, betulinic acid (**1**) is one of the molecules disposing of cytotoxic, antiviral (especially anti-HIV), or antiprotozoal activity [8–17], although its IC_{50} is not sufficiently low for the treatment. Many scientists have been focused on the research of derivatives of acid **1** with much better cytotoxic activity that are promising candidates for the development of anticancer drugs [18–25]. Similarly, derivatives of betulinic acid (**1**) are often being used to investigate its mechanism of action [26–30] or to improve its solubility and bioavailability [31–34]. One part of our research on cytotoxic derivatives of acid **1** has been focused on the compounds modified in their position C-30 (Fig. 1). Oxidation of acid **1** with SeO_2 gives 30-oxobetulinic acid **2,** which has significant cytotoxic activity in multiple cancer cell lines [35]. The main drawback of compound **2** is the low selectivity, which is certainly caused by the presence of an electron-deficient double bond 20(29) (Michaels acceptor), which binds non-specifically to proteins by forming covalent bonds. One of the proposed solutions to diminish this drawback was to replace the oxo-group with an isosteric moiety to weaken the Michaels acceptor potency. In 2018, we published a study about a new set of triterpenoid azines in which the oxygen was replaced with a nitrogen-containing azine group (Fig. 1, general formula 3), and the set showed a significant improvement in selectivity [18]. Azines **3** were cytotoxic against the CCRF-CEM lymphoblastic leukemia cell line (IC_{50} around 3 μmol/L), while their cytotoxicity in other cancer cell lines and noncancer fibroblasts was in the high micromolar range or below the detection limit. Despite this significant improvement of the therapeutic index, the azines **3** were less appropriate for potential drug development because of insufficient stability in an aqueous environment [18]. Besides, some of the synthetic procedures gave low yields when side-products formed instead of the quantitative amounts of the desired products. Here we decided on another

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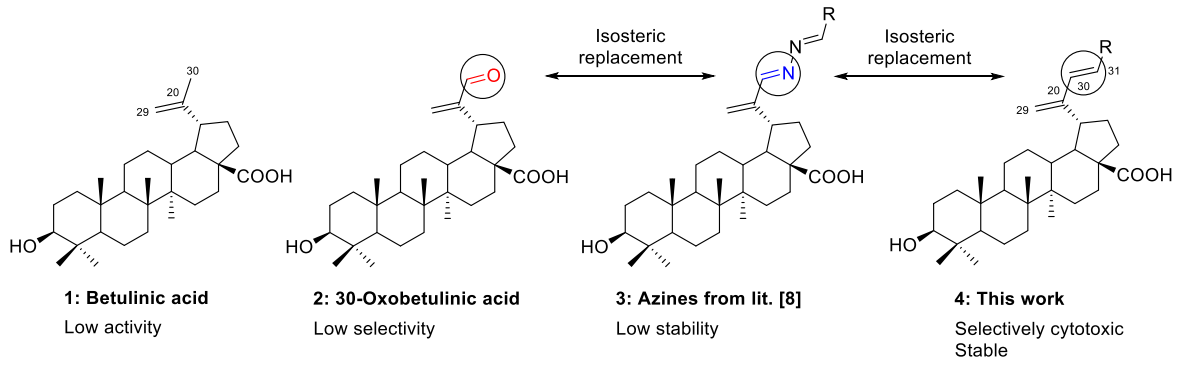


Fig. 1. Isosteric replacements and the context of this work with previous research [18,35].

isosteric replacement of the active moiety: C═O or C═N group for C═C moiety (Fig. 1). This should yield stable compounds that interact with similar molecular targets as active compounds from Refs. [18,35] but they should not bind covalently. We used the same starting compound, 30-oxobetulinic acid (**2**), and modified it in the position 30 by the Wittig reaction. We have prepared 49 triphenylphosphonium salts and optimized their use in the Wittig reaction with the aldehyde **2**. All new compounds were subsequently tested for cytotoxic activity, and the best of them underwent the search for the mechanism of action.

The selection of the substituents was partly motivated by the most cytotoxic azines from our earlier work [18] but the main intention here was to explore their variability. Our main focus was oriented towards variously substituted benzenes (in *o*, *m*, and *p* positions or more substituted), heterocycles are represented by pyridines, alkanes by methyl- and cyclohexyl-. This was expected to show us the differences in both the chemical reactivity of their phosphonium salts and the SAR in biological activity.

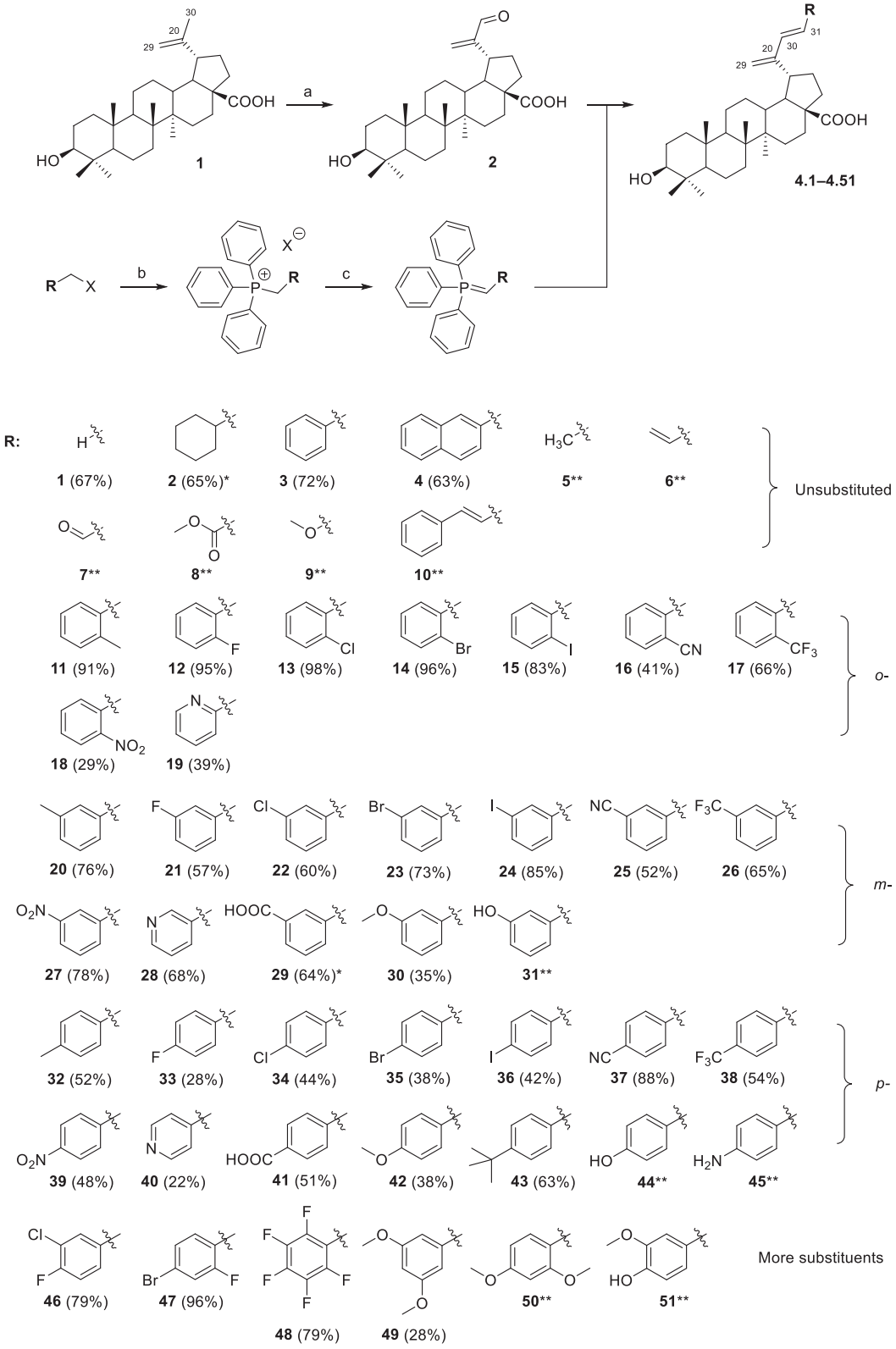
2. Results and discussion

2.1. Chemistry

The starting material of the synthesis was betulinic acid (**1**), which was oxidized in the position C-30 by selenium dioxide in 2-methoxyethanol [35] to form the aldehyde (**2**, Scheme 1). In the next step, a wide range of triphenylphosphonium salts was prepared (or purchased for R = **1** and **7**, see Scheme 1) by a reaction of the corresponding bromide or iodide with triphenylphosphine in refluxing toluene or acetonitrile. This reaction was usually quantitative and did not require special conditions. The last step in the reaction route, the Wittig reaction, was performed under an argon atmosphere using dry tetrahydrofuran (THF), potassium *t*-butoxide (*t*-BuOK), vacuum line, and Schlenk flask to avoid degradation of the ylide. Formation of the ylide in the presence of the base was always characterized by the change of color (a palette from yellow to deep violet), while the completion of the reaction with the aldehyde **2** was usually accompanied by decolorization of the reaction mixture in 2–18 h (overnight). The optimum amount of the triphenylphosphonium salt was 3.0 equivalents accompanied by 4.5 equivalents of *t*-BuOK, which yielded almost 100% conversion of the aldehyde **2** to the product (according to the TLC). There were only two exceptions to this procedure; the –COOH substituted derivatives (**4.29**, **4.41**) required a double amount of the base to complete the reaction.

According to the literature [36], we started an optimization at higher temperatures using toluene as the solvent. After several modifications of the procedure, including attempts with other solvents, we have found THF to be the best choice for the reaction improving the solubility of the starting aldehyde **2** and the product. During the optimization, we found out that the reaction temperature plays an essential role in the reaction stereochemistry outcome. When the Wittig reaction of **2** was performed at 25 °C, the typical outcome was a formation of both *E* and *Z* isomers in various ratios, depending on the nature of the ylide used. For our selection of substituted benzyl-type ylides, we proposed a starting reaction temperature at 0 °C when the formation of the *E* isomers was predominant, and the procedures gave high yields of this isomer. For most of the derivatives, the reaction temperature of 0 °C during the addition of aldehyde into the ylide was an essential requirement for the effective reduction of the amount of the *Z* isomer originating in the reaction mixture. There were some exceptions to this. The temperature of 0 °C was insufficient to complete the reaction of *o*-nitro and *p*-nitro derivatives (**4.18**, **4.39**); the starting material remained mostly unreacted. As a result, increasing the reaction temperature to 70 °C helped to finish the reaction for the *p*-substituted derivative **4.39**. However, it was still insufficient for the *o*-derivative in which the conversion reached only about 40% (visualized by the TLC). Surprisingly, the reaction of both *o*- and *p*-nitro derivatives at 70 °C yielded almost pure *E* isomer, unlike the majority of the other ylides. In contrast to the *o*- and *p*-nitro products, the *m*-nitro isomer (**4.27**) reacted smoothly at 0 °C yielding the pure *E* isomer. Another exception was observed in the case of *m*-COOH derivative (**4.29**), in which we were not successful to prepare only one isomer selectively. Both isomers always formed in a ratio of approximately 2 : 1 (*E*/*Z*), and the mixture was inseparable using the standard column chromatography. The same behavior was observed for the derivative **4.2** bearing cyclohexyl group. Both isomers were always found in a ratio of approximately 2.5 : 1 (*E*/*Z*) as an inseparable mixture.

The stability of the final products is essential for biological evaluation. Since double bonds may undergo isomerization, and since we had a negative experience with previously synthesized azines [18], we paid special attention to this potential problem. We observed that most of the products were stable under standard conditions, in solutions in organic and aqueous solvents, and at room temperature for several weeks; however, there were a few exceptions. Dimethoxy derivative **4.50** (Scheme 1) has always decomposed during column chromatography or in solution after several days at r. t., while the derivative **4.49** seems to be reasonably stable. The next unstable derivative **4.10** was prepared with a longer conjugated chain in a mixture of several isomers. We have also tried to prepare the amino derivative **4.45**. We proved the final product mass by HRMS in a mixture, but we were not able to purify it enough for characterization by NMR, probably due to its instability in silica gel. Similar behavior was partly observed in the case of the derivative **4.29** during longer column chromatography. Triphenylphosphonium salts bearing OH- groups on the benzene ring



Scheme 1. The general reaction scheme for the preparation of the conjugates **4.1**–**4.51**. Reagents and conditions: a) SeO_2, 2-methoxyethanol, reflux, 3 h; b) PPh_3, toluene or acetonitrile, reflux, overnight; X = Br, I (for R = **5**, **6**) or Cl (for R = **7**); c) *t*-BuOK, THF, 30 min; subsequently 0 °C, terpene **2**, then r. t., 2–18 h *Yields for compounds **4.2** and **4.29** are given for a mixture of both *E*/*Z* isomers. **Compounds were either not prepared or they were unstable, see the chemistry section (last paragraph).

(final structures **4.31**, **4.44**, **4.51**) were unfortunately unreactive; this class of salts cannot form reactive ylide form probably due to a resonance deactivation after deprotection of the OH- group in the presence of the base. We have tried some stabilized (**4.7**, **4.8**) and nonstabilized ylides (**4.5**, **4.6**, **4.9**). In these few cases, we were not successful in preparing the desired product, or we obtained a

mixture of inseparable isomers in very low yields. The total number of fully characterized new dienes (pure *E*-isomers) was 40.

2.2. Biology

2.2.1. Cytotoxicity

All prepared compounds were tested *in vitro* for their cytotoxic activity in eight cancer cell lines and two noncancer fibroblasts in the same manner as in Ref. [21]. All compounds were stable in a water-containing environment. The used cancer cell lines were derived from T-lymphoblastic leukemia (CCRF-CEM), chronic myelogenous leukemia (K562), and their MDR counterparts (CEM-DNR, K562-TAX) resistant against daunorubicin or paclitaxel, solid tumors including lung (A549) and colon (HCT116, HCT116 p53$^{-/-}$) carcinomas, osteosarcoma (U2OS), and for comparison, the synthesized derivatives were tested on two human non-cancer fibroblast cell lines (BJ, MRC-5). CCRF-CEM cell line is the most chemosensitive cell line on the panel, and therefore it was used for the SARs assumptions similarly to Ref. [21]. Compounds **4.22**, **4.30**, **4.33**, and **4.39** had high and selective cytotoxic activity (IC_{50} in the range of 3.2–4.9 μmol/L) against the CCRF-CEM cell line. In contrast, the other compounds had moderate to low activity in most cancer cell lines and low activity in noncancer fibroblasts (Table 1). All compounds were less active against the CEM-DNR multiresistant cell line, which can indicate the involvement of general mechanisms of resistance like the presence of MDR transporters. Compounds **4.22** and **4.39** had the highest cytotoxic activity and favorable selectivity index, and therefore they were selected to evaluate their mechanism of action in more detailed biological tests – analysis of cell cycle, synthesis of DNA and RNA, and protein expression analysis in cells treated with these compounds.

Importantly, dienes **4.22**, **4.30**, **4.33**, and **4.39** are far more selective than aldehyde **2**, which makes them much more promising candidates for drug development even though they are less active. In comparison to azines **3** from the lit. [18], dienes **4.22**, **4.30**, **4.33**, and **4.39** have similar cytotoxicity and selectivity, but they are more stable. In conclusion, the Wittig reaction afforded four candidates (**4.22**, **4.30**, **4.33**, and **4.39**) for the anticancer drug development that are superior to our previously published derivatives.

2.2.2. Influence of compounds *4.22* and *4.39* on cell cycle and DNA/RNA synthesis in CCRF-CEM cancer cells

To further understand the mechanism of action of the most active compounds **4.22** and **4.39**, their effect on the cell cycle and DNA and RNA synthesis in cancer cells (CCRF-CEM) was studied (Table 2, Fig. 2). It was found that after incubation with 1 × IC_{50} concentration, the cells were still viable, with only a slight increase in the percentage of apoptotic cells with fragmented DNA. An increase in the numbers of apoptotic cells was observed at a higher 5 × IC_{50} concentration, where we can already expect activation of irreversible changes leading to cell death. Compound **4.22** led to the accumulation of cells at the G0/G1 cell cycle phase with partial inhibition of DNA and RNA synthesis at 1 × IC_{50} and almost complete inhibition at 5 × IC_{50}. Similarly, the cells' accumulation in the G0/G1 phase was observed after treatment with a 1 × IC_{50} concentration of compound **4.39**. Interestingly, at 5 × IC_{50}, the accumulation of cells was observed in the S phase of the cell cycle. To explain such a phenomenon, we can hypothesize that higher concentrations could inhibit more targets (off-targets) than lower and, therefore, more specific cytotoxic concentrations of the tested drugs. Thus, mechanisms disrupting cellular metabolism can lead to the accumulation of cells in the synthetic phase of the cell cycle.

2.2.3. Evaluation of cell death by annexin V/propidium iodide double labeling

For further investigation of the predominant mode of cell death induced by the most active compounds **4.22** and **4.39**, we have used dual Annexin V/propidium iodide labeling with subsequent flow cytometry analysis. Based on the results, both compounds induced a significant, concentration-dependent increase in the number of apoptotic cells compared to the untreated control (Fig. 3). Following individual quadrant analyses, the early (right lower quadrant) together with late (right upper quadrant) apoptotic effects of compounds **4.22** and **4.39** for 1 × IC_{50} doses on the CCRF-CEM cell line were determined as 6.7% and 7.3%, respectively. For 5 × IC_{50} doses were evaluated apoptotic population 10.0% and 27.2%. Compound **4.39** at 5 × IC_{50} concentration proved the most considerable cytotoxic effect leading to a more than nine-fold increase of apoptotic cells number compared to the control. The total number of apoptotic cells from the Annexin V/propidium iodide assay nicely correlates with the sub-G1 apoptotic cell portion resulting from cell cycle analysis (Table 2).

2.2.4. Western blot

Based on the results from cell cycle analysis and Annexin V/propidium iodide assay, we further concentrated on a more detailed analysis of apoptosis-as well as cell cycle-associated proteins expression. For that purpose, we used standard western blot analysis of cell extracts prepared from CCRF-CEM cells treated with **4.22** and **4.39** at 1 × IC_{50} and 5 × IC_{50} concentrations for 24 h. It is well-known that caspases activation represents the critical hallmark of apoptotic cell death. Thus, the expression of the main apoptotic caspases 3, 8, and 9 was analyzed. We have found that both compounds at 5 × IC_{50} concentration induced activation of initiation caspase-9 and execution caspase-3, demonstrated by the presence of caspase's active form (Fig. 4). On the other hand, no activation of caspase-8 was observed. Such results strongly suggest the involvement of the mitochondrial apoptotic pathway. Both compounds at 5 × IC_{50} concentration also induced PARP cleavage, which represents one of the downstream substrates of activated caspase-3. We further examined the possible modulation effect of active compounds on the expression of selected proteins from the Bcl-2 family since they are essential regulators of mitochondria-mediated apoptosis. As presented in Fig. 4, compound **4.39** at 5 × IC_{50} concentration induces downregulation of Bcl-2 protein while BAX expression is slightly (20%) upregulated. Finally, we observed upregulation of p21 and downregulation of Cyclin D1 following treatment with **4.22** and **4.39** at 5 × IC_{50} concentration as additional evidence for their impact on cell cycle progression.

2.2.5. Pharmacological parameters

Two compounds with the highest cytotoxicity – **4.22** and **4.39** – were selected to evaluate the fundamental pharmacokinetic parameters such as absorption, distribution, metabolism, and excretion (ADME). Knowledge of the ADME properties of a new chemical entity is critical during its evaluation to become a leading candidate in a drug discovery program and/or its use as a functional research tool.

Both candidates **4.22** and **4.39** were incubated with human plasma *in vitro* for fast determination if they are susceptible to plasma degradation and to what extent. Compounds **4.22** and **4.39** slowly hydrolyzed in plasma (after incubation for 2 h, 70%–80% of the original amount of each substance was remaining).

For the microsomal stability assay, human liver microsomes and NADPH cofactor were used to assess phase I oxidation by cytochrome P450 and flavin monooxygenases. The intrinsic clearance calculated from the microsomal stability assay indicated a low or medium category. This means that the studied compounds **4.22** and **4.39** were not subject to rapid metabolism by liver microsome enzymes.

Table 1
Cytotoxic activities of the final compounds on eight tumor and two normal fibroblast cell lines.

Comp.	IC_{50} (μmol/L)[a] CCRF-CEM	CEM-DNR	HCT 116	HCT116 $p53^{-/-}$	K562	K562-TAX	A549	U2OS	BJ	MRC-5	SI[b]
2[c]	**0.8**	**3.8**	**1.9**	**1.9**	**3.4**	**1.3**	>50	**2.2**	6.7	**2.6**	5.7
4.1	11.1	25.1	24.1	24.3	15.7	26.2	23.9	24.1	44.6	24.3	3.1
4.2[d]	8.0	45.7	15.2	>50	>50	43.6	>50	>50	>50	>50	>6.3
4.3	6.5	30.2	35.5	39.5	40.0	25.3	41.8	24.2	>50	>50	>7.7
4.4	11.8	47.0	30.2	26.3	>50	45.8	32.9	27.2	>50	43.1	>3.9
4.11	19.1	22.8	29.7	25.9	28.1	25.9	26.8	28.0	33.0	31.5	1.7
4.12	15.4	23.2	24.5	22.6	21.8	25.1	29.7	27.7	42.1	31.7	2.4
4.13	18.7	42.1	26.6	24.8	23.9	39.3	29.4	28.1	42.3	32.6	2.0
4.14	16.8	49.1	27.2	24.5	21.6	47.9	28.6	27.8	46.7	35.3	2.4
4.15	20.6	>50	29.6	29.5	25.1	>50	29.7	28.5	>50	29.5	>1.9
4.16	23.2	26.8	36.6	47.0	>50	24.8	>50	40.4	>50	>50	>2.2
4.17	21.8	>50	33.0	36.1	48.7	>50	37.0	29.6	>50	>50	>2.3
4.18	23.7	30.6	34.8	39.3	>50	29.8	45.2	33.1	>50	>50	>2.1
4.19	19.7	27.7	>50	>50	>50	29.2	>50	>50	>50	>50	>2.5
4.20	9.5	21.4	24.7	22.3	24.9	23.6	36.5	18.2	>50	42.2	>4.9
4.21	11.7	23.1	23.0	21.0	21.6	21.9	28.7	27.7	41.5	32.1	3.1
4.22	**3.2**	25.5	16.4	15.2	27.7	25.6	29.2	17.3	>50	32.6	>13.0
4.23	8.8	35.9	25.3	26.8	23.6	32.8	33.1	27.6	>50	>50	>5.7
4.24	11.6	>50	27.3	29.5	25.2	48.4	38.7	28.4	>50	>50	>4.3
4.25	12.1	21.6	35.1	29.4	>50	22.7	>50	>50	>50	>50	>4.1
4.26	8.0	28.1	23.1	24.8	26.5	25.1	35.1	24.6	>50	42.9	>5.8
4.27	12.4	25.3	37.5	35.5	>50	24.3	48.8	29.3	>50	45.1	>3.8
4.28	11.3	17.4	30.9	32.8	>50	14.2	26.2	30.9	>50	>50	>4.4
4.29[d]	20.1	33.9	43.4	43.1	>50	36.7	>50	>50	>50	>50	>2.5
4.30	**4.6**	30.6	34.6	39.6	41.4	25.6	40.1	25.2	>50	44.1	>10.3
4.32	5.9	26.1	21.4	18.2	>50	26.3	>50	>50	>50	>50	>8.5
4.33	**4.9**	31.4	35.4	41.0	36.2	29.8	40.6	23.9	44.6	38.3	8.5
4.34	5.4	33.1	39.5	38.5	46.3	29.2	45.4	25.8	>50	>50	>9.2
4.35	6.5	28.9	33.2	31.8	>50	29.3	>50	27.0	>50	35.2	>6.6
4.36	16.0	>50	30.7	33.3	>50	>50	>50	32.2	>50	41.2	>2.9
4.37	23.8	27.0	39.1	43.3	>50	27.2	>50	44.1	>50	49.7	>2.1
4.38	21.5	40.9	40.9	31.9	40.5	45.6	41.4	28.2	49.5	42.4	2.1
4.39	**3.6**	22.9	18.6	15.7	11.5	23.4	14.3	19.5	24.3	19.2	6.1
4.40	5.9	20.5	35.0	30.6	43.4	20.6	46.0	29.7	>50	>50	>8.4
4.41	17.5	32.0	39.0	42.5	>50	39.2	>50	49.0	>50	>50	>2.9
4.42	20.9	>50	42.4	48.9	>50	48.6	>50	48.6	>50	>50	>2.4
4.43	45.6	>50	>50	>50	>50	>50	>50	45.1	>50	>50	>1.1
4.46	5.5	27.8	21.8	20.8	40.6	25.2	45.5	26.5	>50	32.4	>7.5
4.47	22.4	>50	33.5	33.6	26.0	>50	32.5	29.7	43.0	33.7	1.7
4.48	22.1	>50	32.2	36.3	29.8	>50	32.2	30.2	>50	43.2	>2.1
4.49	10.0	13.7	17.3	20.8	22.2	16.0	19.0	22.3	47.6	29.2	3.8

[a] The lowest concentration that kills 50% of cells. The maximum standard deviation in cytotoxicity assays is typically 15% of the average value. Compounds with IC_{50} > 50 μmol/L were considered inactive.
[b] Selectivity index is calculated for IC_{50} of CCRF-CEM line vs. the average IC_{50} values of both fibroblasts (BJ and MRC-5).
[c] Parent compound is used as a standard.
[d] IC_{50} value was measured for a mixture of both *E*/*Z* isomers.

Table 2
Cell cycle analysis and influence on DNA and RNA synthesis in CCRF-CEM cancer cells treated with 1 × IC_{50} and 5 × IC_{50} of compounds **4.22** and **4.39**.

		Used conc. (μmol/L)	Sub G1 (%)	G0/G1 (%)	S (%)	G2/M (%)	$pH3^{Ser10}$ (%)	DNA synthesis (%)	RNA synthesis (%)
Control		0	2.77	40.20	38.17	21.62	1.72	41.56	36.54
4.22	1 × IC_{50}	3.19	5.66	42.87	42.75	14.39	1.50	35.56	18.97
	5 × IC_{50}	15.95	9.13	49.79	37.93	12.28	1.52	0.40	11.37
4.39	1 × IC_{50}	3.57	6.54	48.40	28.44	23.16	2.90	43.17	34.98
	5 × IC_{50}	17.85	25.04	29.57	57.61	12.81	0.93	3.06	4.07

The measurement of plasma protein binding was performed using equilibrium dialysis, compounds **4.22** and **4.39** were bound by 79% and 68%, respectively.

Both derivatives showed low ability (−log *P*app > 6 cm/s) to diffuse through an artificial cellular membrane in the Parallel Artificial Membrane Permeability Assay (PAMPA).

Compound **4.22** shows a small improvement in cellular permeability (without category change) compared to compound **4.39** in our PAMPA model and permeability data from cell permeability assay: Caco-2 and MDCK-MDR1 cell lines. The Caco-2 and MDCK-MDR1 permeability assays are established models of intestinal [37] and blood-brain barriers, respectively [38]. Molecule **4.22** showed a moderate ability of intestinal absorption ($P_{app\ (AB)}$ in the range of $5 \cdot 10^{-6}$ cm/s − $20 \cdot 10^{-6}$ cm/s) and ability to cross blood-brain barriers ($P_{app\ (AB)} > 10 \cdot 10^{-6}$ cm/s; CNS positive), while compound **4.39** manifested a low category of permeability in both cell assays. We assessed rates of transport across MDCK-MDR1 and Caco-2 monolayers in both directions (apical to basolateral [A−B], and basolateral to apical [B−A]) across the cell monolayer, which enables us to determine the efflux ratio and shows if the compound undergoes active efflux. Studied derivatives **4.22** and **4.39** were actively exported from the cells in both barrier models as indicated

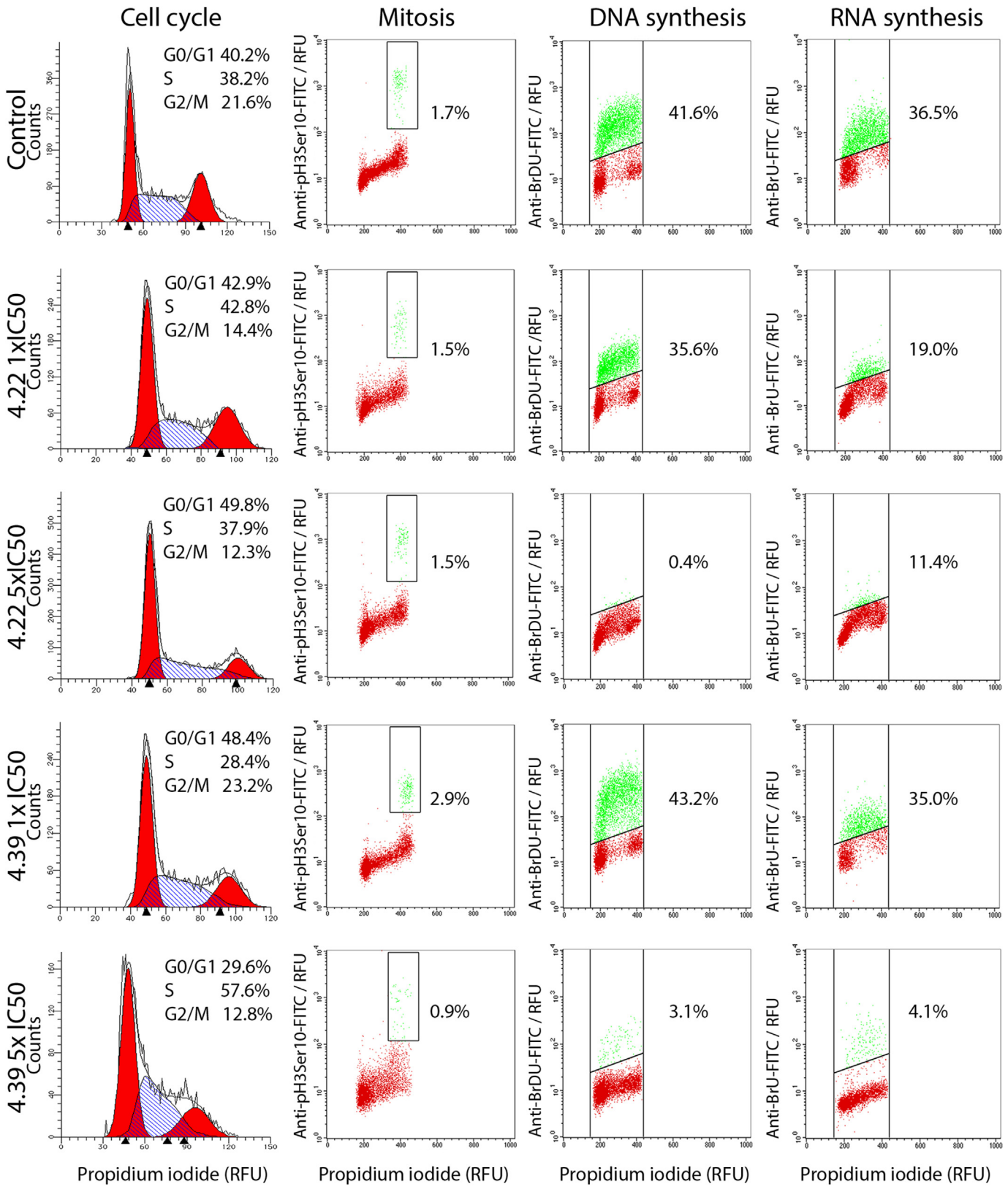


Fig. 2. Graphs and dot plots from flow cytometry analyses show the influence of the best compounds **4.22** and **4.39** on cell cycle and DNA/RNA synthesis in cancer cells monitored by incorporation of BrdU/BrU into DNA/RNA.

by efflux ratios $ER > 2$. Still, the values of efflux ratio were very close to the limit of active and passive efflux that the compounds cannot be clearly identified as substrates of MDR1 efflux pump present in both cell types. Results from all *in vitro* pharmacology testing are summarized in Table 3.

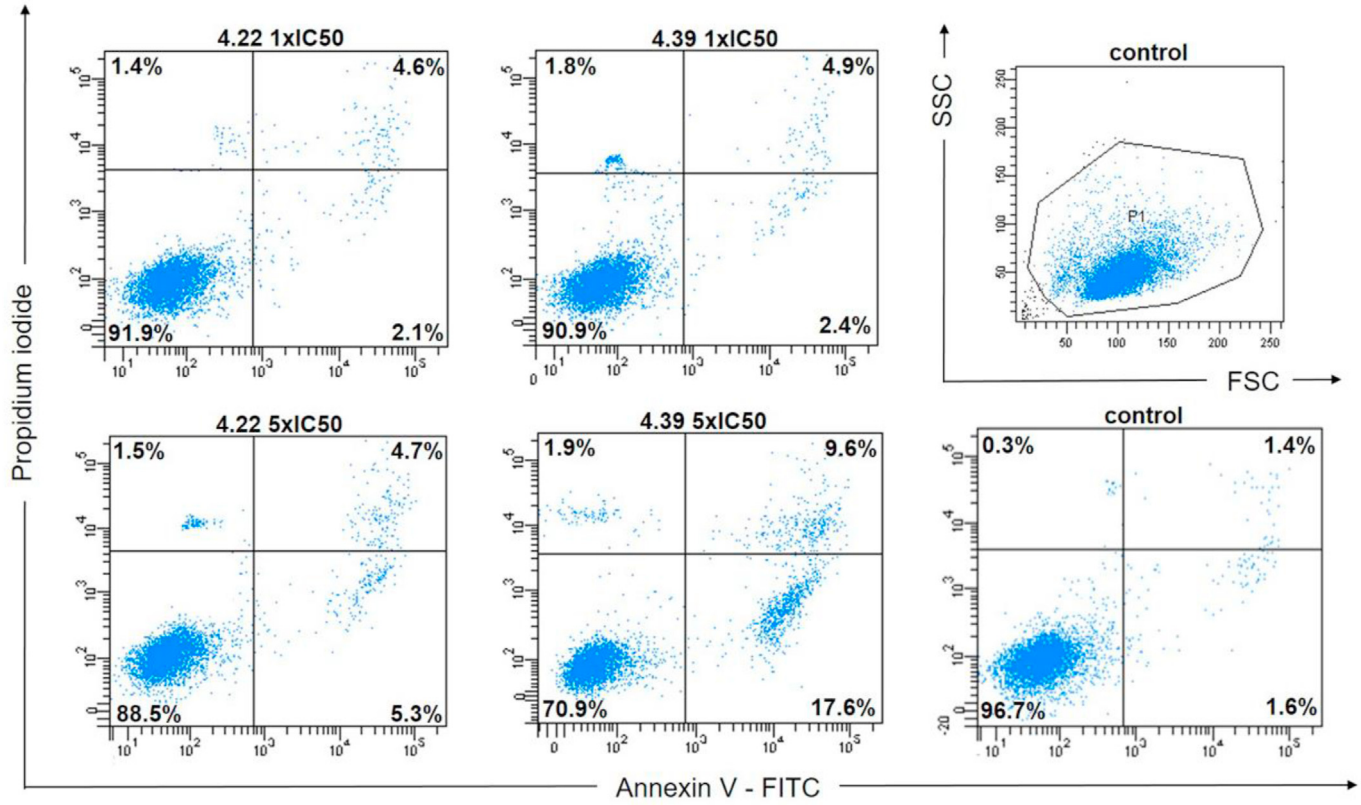


Fig. 3. Representative dot plot diagrams of dual Annexin V/propidium iodide staining of CCRF-CEM cells treated with **4.22** and **4.39** at 1 × IC_{50} and 5 × IC_{50} concentrations for 24 h. The fluorescence signal was measured at green (Annexin V) and red (propidium iodide) channels using FACSAria II flow cytometer. Debris identified by FSC/SSC dot plot was excluded from the PI/FITC channels of the dot plot diagram. At least 10,000 cells were analyzed in each sample and analysis of the individual quadrant was performed. The portion (%) of cell numbers in each quadrant is shown.

3. Conclusions

The main goal of this work was to modify betulinic acid (**1**) at the position C-30 in order to find compounds with selective cytotoxic activity in cancer cell lines and reveal their mechanism of action. From the past work, we knew that the oxidation of **1** gives 30-oxobetulinic acid **2** with high cytotoxicity, which unfortunately is not selective and kills both cancer and healthy cells and therefore is not useable in drug development [18,41]. The first attempt to reach this goal was to mask the reactive Michaels acceptor of compound **2** as azines and as expected, the selectivity improved significantly, the activity was still reasonable, but compounds suffered from low stability in the water environment [18]. In this work, we replaced the reactive part of azines = N– with isosteric = CH– and obtained a set of 40 compounds of which **4.22**, **4.30**, **4.33**, and **4.39** had cytotoxicity below 5 μmol/L and all of them had high selectivity. The goal was achieved. The other compounds had moderate activities but they provide important data points to our long-term SAR studies based on which we may predict the cytotoxicity of proposed new structures in the future and decide whether to synthesize new compounds or not. To better understand the mechanism of action, we studied the cell cycle and DNA/RNA synthesis in CCRF-CEM cells treated with **4.22** and **4.39** and found that both compounds interfere with the cell cycle and induce G1 cell cycle phase block, which may be associated with p21 overexpression and cyclin D1 downregulation [42]. Inhibition of DNA and RNA synthesis was also observed. Similarly, apoptosis was confirmed by analysis of caspase activation and cleavage of their substrates. Interestingly, Bcl-2 expression decreased strongly in contrast to the BAX level, which increased after **4.39** treatment at

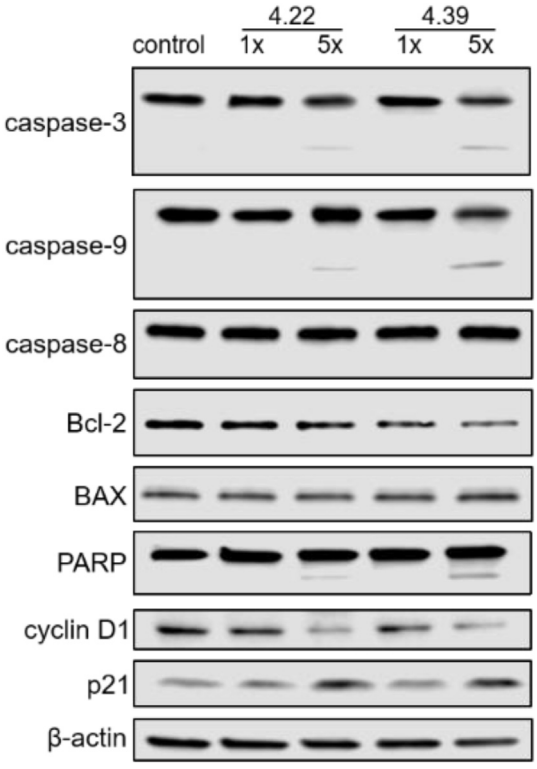


Fig. 4. Western blot analysis of selected proteins involved in apoptosis and cell cycle regulation. Cell extracts were prepared from CCRF-CEM cells treated with **4.22** and **4.39** at 1 × IC_{50} and 5 × IC_{50} for 24 h. As a protein loading control, β-actin was used.

Table 3
Pharmacological parameters of selected compounds **4.22** and **4.39**.

	Plasma stability			Microsomal stability			Category of intrinsic clearance[a]	Plasma protein binding	
	Compound remaining (%)			Compound remaining (%)				Fraction bound (%)	
Comp.	15 min	30 min	60 min	15 min	30 min	60 min			
4.22	92.31	87.89	89.51	90.10	92.37	81.50	low	78.97	
4.39	101.65	79.07	69.29	93.23	78.52	53.54	medium	68.12	

	PAMPA		MDCK-MDR1 Permeability Assay				Caco-2 Permeability Assay				
	log P_{app} (cm/s)	**Category**[b]	**P_{app} × 10^{-6}** (cm/s)	**Category**	**Efflux ratio**	**Active efflux**	**Recovery** (%)	**P_{app} × 10^{-6}** (cm/s)	**Category**	**Efflux ratio**	**Active efflux**	**Recovery** (%)
Comp.												
4.22	−6.63	low	22.36	positive	2.90	yes	103.90	17.50	moderate	2.64	yes	42.13
4.39	−7.84	low	0.43	negative	3.06	yes	56.74	0.96	low	2.50	yes	58.51

[a,b] Reference [39,40]; error deviations are ranges of values lower than 10% (all experiments were done in triplicates except cell-based permeability assays which were done in duplicates).

5 × IC_{50} concentration. Such changes could correspond to a disruption of mitochondrial permeability and lead to the release of cytochrome *c* prior to activation of apoptosis [43]. As part of preclinical development, we tested *in vitro* pharmacological parameters. Good stability and favorable binding to plasma proteins were observed for both compounds. *In vitro* models predicting the permeability of potential drugs across the intestinal and blood-brain barrier showed a favorable profile of compound **4.22**. Derivative **4.39** has worse pharmacological parameters, it does not cross the PAMPA artificial membrane, and the Caco-2 permeability assay indicates poor intestinal absorption. Due to the high cytotoxic activity 3.2 μmol/L against chemosensitive CCRF-CEM cancer cell line, favorable selectivity >12 and advantageous pharmacological parameters, derivative **4.22** is a promising candidate for further preclinical development.

4. Materials and methods

4.1. Chemistry

HRMS analysis was performed using LC chromatograph Dionex UltiMate 3000 (Thermo Fischer Scientific) and mass spectrometer Exactive Plus Orbitrap high-resolution (Thermo Fischer Scientific). Source of ionization: electrospray or APCI. Spectra: positive and negative mode in the range of 400−700 *m/z*. Chromatographic separation: column Phenomenex Gemini (C18, 50 × 2 mm, 3 μm particle), isocratic elution, mobile phases: 80% ACN and 20% buffer (0.01 mol/L ammonium acetate) or 95% MeOH + 5% water + 0.1% HCOOH. Sample preparation: dissolution in MeOH. Spectra were processed in the Thermo Scientific Xcalibur 4.1.31.9 software. All ^{1}H and ^{13}C NMR experiments were recorded at 500 MHz (Jeol JNM-ECX-500) or 400 MHz (Jeol JNM-ECA400II) for ^{1}H NMR, and 126 MHz or 100 MHz for ^{13}C NMR, respectively, at 20 °C in $CDCl_3$, CD_3OD, or DMSO-d_6. Chemical shifts (δ) are reported relative to the residual solvent peak (for $CDCl_3$ δ_H = 7.26 ppm, δ_C = 77.16 ppm; for CD_3OD δ_H = 3.31 ppm, δ_C = 49.00 ppm; for DMSO-d_6 δ_H = 2.50 ppm, δ_C = 39.52 ppm). NMR spectra were processed using MestReNova 6.0.2−5475 and JEOL Delta 5.0.5.1 software. Melting points were determined using a Stuart Melting Point Apparatus SMP30 and are uncorrected. Specific rotations were measured in THF solutions at 22 °C using an Atago POL-1/2 instrument, sample concentrations (*c*) are given in grams per 100 mL. IR spectra were recorded on a Nicolet Avatar 370 FTIR and processed in the OMNIC 9.8.372 software. DRIFT stands for Diffuse Reflectance Infrared Fourier Transform. TLC was carried out on Kieselgel 60 F_{254} plates (Merck) detected first by UV light (254 nm) and then by spraying with 10% aqueous H_2SO_4 and heating to 150−200 °C. Purification was performed using column chromatography on Silica gel 60 (Merck 7734). Betulinic acid was obtained from the company Betulinines (www.betulinines.com). All other chemicals and solvents were obtained from Sigma-Aldrich, Fluorochem, or Across Chemicals in analytical quality, and were used as purchased.

4.1.1. Synthesis of 30-oxobetulinic acid (**2**)

10 g (21.9 mmol) of betulinic acid (**1**) was dissolved in 150 mL of 2-methoxyethanol, and 6.1 g (54.8 mmol) of SeO_2 was added. The reaction was stirred under reflux for 3 h and monitored by TLC. After completion, the hot reaction was immediately filtered over a paper filter into 1 L icy water, where the product precipitated as a yellow solid. The precipitate was then filtered using a Büchner funnel, and the filter cake was dried at r. t. for 3 days. The product was purified by flash column chromatography (toluene/diethyl ether 1 : 1), affording 6.3 g (61%) of white solid. All spectral and physical data agreed with the literature [35].

4.1.2. General procedure for the preparation of bromomethylpyridines

2.8 mmol of the corresponding 2-, 3- or 4-(bromomethyl)pyridine hydrobromide was dissolved in 10 mL of distilled water, then 15 mL of DCM was added, and the solution was placed to stir at 0 °C in an ice bath. While continuous stirring, a solution of 3.0 mmol K_2CO_3 dissolved in 10 mL H_2O was added dropwise. After 30 min, the reaction mixture was extracted with DCM, the organic phases were combined, dried over anhydrous $MgSO_4$, filtered, and evaporated at r. t. The residue was immediately used in the following procedure to prepare the corresponding triphenylphosphonium salt.

4.1.3. General procedure for the preparation of triphenylphosphonium salts

2.1 mmol of triphenylphosphine was dissolved in 5 mL of toluene or acetonitrile (for **4.2**), and 2.0 mmol of the corresponding bromide was added. The mixture was stirred overnight under reflux until white crystals of the salt precipitated. The precipitate was then filtered, washed with diethyl ether, and dried under a vacuum to provide white crystalline salt in quantitative yields. For **4.2**: The mixture was cooled down after reflux, the solvent was almost evaporated, and the residue was precipitated by adding diethyl ether to obtain white crystals, which were filtered and dried under vacuum (56% yield). The salts were used without further purification.

4.1.4. General procedure for the preparation of triphenylphosphonium salts bearing −OH group

4.0 mmol of the corresponding hydroxybenzyl alcohol was dissolved in 10 mL of acetonitrile, and 4.5 mmol of triphenylphosphine hydrobromide was added. The mixture was stirred overnight under reflux until white crystals of the salt precipitated. The precipitate was then filtered, washed with diethyl ether, and dried under a vacuum to provide white crystalline salt in quantitative yield.

*4.1.5. General procedure for the preparation of conjugates **4.1**–**4.51***

0.96 mmol of the triphenylphosphonium salt was introduced in a dry Schlenk flask on the vacuum line, followed by 165 mg (1.43 mmol) of *t*-BuOK. After 30 min of drying under vacuum, 2 mL of dry THF was added, and the reaction was stirred for 1 h to form the ylide. Then, the flask was cooled down in an ice bath, and after 10 min, 150 mg (0.32 mmol) of the aldehyde (**2**) was added under an argon atmosphere. The reaction was stirred at 0 °C until the ice bath melted and then at r. t. 2–18 h. Completion of the reaction was monitored by TLC (toluene/diethyl ether 1 : 1) followed by extraction (1% HCl/DCM). Organic phases were combined, dried over $MgSO_4$, and evaporated under reduced pressure. The residue was purified by flash column chromatography (toluene/diethyl ether 2 : 1), affording the final product in moderate to high yields. Modification of the conditions is described in the respective experiment if necessary.

*4.1.5.1. 30-Methylenebetulinic acid **4.1**.* The product **4.1** was obtained as white solid, 100 mg (67%) by the general procedure (yellow ylide color) while the reaction time was 18 h: m. p. 228–231 °C; $[\alpha]_D^{22}$ −5° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, DMSO-d_6) δ 12.06 (s, 1H, −COOH), 6.35 (dd, 1H, $J_1 = 17.5$ Hz, $J_2 = 11.0$ Hz, H-30), 5.25 (d, 1H, $J = 17.5$ Hz, H-31 *pro-E*), 5.06 (d, 1H, $J = 10.9$ Hz, H-31 *pro-Z*), 5.00, 4.98 (2H, all s, 2 × H-29), 4.24 (d, 1H, $J = 5.2$ Hz, −OH), 3.07 (td, 1H, $J_1 = 11.1$ Hz, $J_2 = 4.7$ Hz, H-19β), 3.01–2.92 (m, 1H, H-3α), 2.02–1.95 (m, 1H), 1.80 (dd, 1H, $J_1 = 12.3$ Hz, $J_2 = 7.9$ Hz), 1.74 (t, 1H, $J = 11.3$ Hz), 0.94, 0.87, 0.86, 0.75, 0.65 (15H, all s, 5 × $-CH_3$) ppm; **^{13}C NMR** (126 MHz, DMSO-d_6) δ 177.16, 152.43, 139.20, 113.27, 112.45, 76.74, 55.40, 54.85, 49.90, 49.75, 41.92, 40.23, 40.11, 38.46, 38.23, 37.62, 36.69, 36.09, 33.93, 32.41, 31.58, 29.28, 28.06, 27.12, 26.52, 20.56, 17.92, 15.91, 15.76, 15.74, 14.40 ppm; **HRMS (**ESI$^+$) *m/z* calcd for $C_{31}H_{49}O_3$ $[M+H]^+$ 469.3676, found 469.3680; **IR** (DRIFT) ν_{max} 2390–3670, 2939, 2868, 1685, 1628, 1593, 1448 cm^{-1}.

*4.1.5.2. 30-(Cyclohexylmethylene)betulinic acid **4.2**.* The product **4.2** was obtained as a mixture of inseparable *E* and *Z* isomers in a ratio approximately 2.5 : 1, white solid, 114 mg (65%) by the general procedure (orange ylide color) while the reaction time was 2 h: m. p. 174–177 °C; $[\alpha]_D^{22}$ +36° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 5.96 (d, $J = 15.8$ Hz, 0.4H, H-30 of *Z*-isomer), 5.76–5.69 (m, 1.4H, H-30 of *E*-isomer and H-31 of *Z*-isomer), 5.43–5.36 (m, 1H, H-31 of *E*-isomer), 4.97 (d, $J = 2.2$ Hz, 1H, H-29a of *E*-isomer), 4.84, 4.82 (2 × 0.4H, both s, 2 × H-29 of *Z*-isomer), 4.72 (s, 1H, H-29b of *E*-isomer), 3.23–3.16 (m, 1.4H, H-3α of both isomers), 3.12–3.00 (m, 1.4H, H-19β of both isomers), 2.53–2.44 (m, 1H), 0.98 (s, 1.2H, CH_3- group of *Z*-isomer), 0.96 (s, 4.2H, CH_3- group of both isomers), 0.93 (s, 3H, CH_3- group of *E*-isomer), 0.93 (s, 4.2H, CH_3- group of both isomers), 0.82 (s, 3H, CH_3- group of *E*-isomer), 0.82 (s, 1.2H, CH_3- group of *Z*-isomer), 0.75 (s, 4.2H, CH_3- group of both isomers); **HRMS** (ESI$^-$) *m/z* calcd for $C_{37}H_{57}O_3$ $[M-H]^-$ 549.4302, found 549.4308; **IR** (DRIFT) ν_{max} 3680, 3340, 2925, 2870, 2851, 2654, 1685, 1449 cm^{-1}.

*4.1.5.3. 30-Benzylidenebetulinic acid **4.3**.* The product **4.3** was obtained as white solid, 125 mg (72%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 170–173 °C; $[\alpha]_D^{22}$ +1° (*c* 0.29, THF); **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.47–7.42 (m, 2H, arom. ring), 7.35–7.29 (m, 2H, arom. ring), 7.25–7.19 (m, 1H, arom. ring), 6.81 (d, $J = 16.2$ Hz, 1H, H-31), 6.70 (d, $J = 16.2$ Hz, 1H, H-30), 5.12. 5.07 (2H, all s, 2 × H-29), 3.29 (td, $J_1 = 11.1$, $J_2 = 4.7$ Hz, 1H, H-19β), 3.20 (dd, $J_1 = 11.1$, $J_2 = 4.9$ Hz, 1H, H-3α), 2.03 (dd, $J_1 = 12.6$, $J_2 = 7.8$ Hz, 1H), 1.92 (t, $J = 11.3$ Hz, 1H), 1.02 (s, 3H), 0.97 (s, 3H), 0.96 (s, 3H), 0.81 (s, 3H), 0.75 (s, 3H) ppm; **^{13}C NMR** (101 MHz, $CDCl_3$) δ 182.32, 152.70, 137.69, 131.72, 128.70, 128.15, 127.48, 126.63, 112.72, 79.21, 56.59, 55.47, 50.79, 50.63, 42.53, 40.85, 40.18, 38.97, 38.83, 38.67, 37.32, 37.07, 34.50, 33.26, 32.37, 29.98, 28.13, 27.40, 27.15, 21.17, 18.40, 16.25 (2C), 15.49, 14.91 ppm; **HRMS** (ESI$^+$) *m/z* calcd for $C_{37}H_{53}O_3$ $[M+H]^+$ 545.3989, found 545.3988; **IR** (DRIFT) ν_{max} 2450–3610, 2939, 2869, 1692, 1601, 1450 cm^{-1}.

*4.1.5.4. 30-(Naphth-3-ylmethylene)betulinic acid **4.4**.* The product **4.4** was obtained as white solid, 119 mg (63%) by the general procedure (red ylide color), while the reaction time was 2 h: m. p. 187–190 °C; $[\alpha]_D^{22}$ +21° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.82–7.76 (m, 4H, arom. ring), 7.69–7.64 (m, 1H, arom. ring), 7.46–7.40 (m, 2H, arom. ring), 6.93 (d, $J = 16.2$ Hz, 1H, H-31), 6.87 (d, $J = 16.2$ Hz, 1H, H-30), 5.17, 5.11 (2H, all s, 2 × H-29), 3.35 (td, $J = 11.1$, 4.7 Hz, 1H, H-19β), 3.18 (dd, $J = 11.5$, 4.6 Hz, 1H, H-3α), 2.22 (td, $J = 12.3$, 3.3 Hz, 1H), 2.05 (dd, $J = 12.8$, 7.7 Hz, 1H), 1.95 (t, $J = 11.4$ Hz, 1H), 1.02 (s, 3H), 0.97 (s, 3H), 0.95 (s, 3H), 0.79 (s, 3H), 0.72 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.70, 152.82, 135.20, 133.88, 133.08, 132.13, 128.32, 128.27, 128.07, 127.79, 126.70, 126.38, 125.89, 123.75, 112.95, 79.17, 56.60, 55.49, 50.98, 50.65, 42.57, 40.89, 40.17, 38.99, 38.84, 38.76, 37.35, 37.10, 34.52, 33.39, 32.41, 30.00, 28.13, 27.50, 27.21, 21.21, 18.40, 16.29, 16.25, 15.45, 14.95 ppm; **HRMS** (ESI$^-$) *m/z* calcd for $C_{41}H_{53}O_3$ $[M-H]^-$ 593.3989, found 593.3994; **IR** (DRIFT) ν_{max} 3629, 3450, 3186, 2931, 2867, 2612, 1948, 1686, 1598, 1450 cm^{-1}.

4.1.5.5. 30-(2-Methylbenzylidene)betulinic acid ***4.11***. The product **4.11** was obtained as a white solid, 162 mg (91%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 167–170 °C; $[\alpha]_D^{22}$ −9° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.51–7.46 (m, 1H, arom. ring), 7.21–7.13 (m, 3H, arom. ring), 6.91 (d, J = 16.0 Hz, 1H, H-31), 6.64 (d, J = 16.0 Hz, 1H, H-30), 5.11, 5.06 (2H, all s, 2 × H-29), 3.30 (td, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-19β), 3.19 (dd, J_1 = 11.5, J_2 = 4.5 Hz, 1H, H-3α), 2.38 (s, 3H, $-CH_3$), 2.03 (dd, J_1 = 12.6, J_2 = 8.0 Hz, 1H), 1.92 (t, J = 11.3 Hz, 1H), 1.02 (s, 3H), 0.97 (s, 3H), 0.96 (s, 3H), 0.82 (s, 3H), 0.75 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 182.32, 152.75, 136.88, 135.78, 132.79, 130.42, 127.43, 126.46, 126.26, 125.54, 112.69, 79.18, 56.63, 55.49, 50.65, 50.51, 42.57, 41.17, 40.87, 39.00, 38.86, 38.70, 37.34, 37.09, 34.52, 32.91, 32.35, 30.01, 28.14, 27.44, 27.06, 21.19, 20.04, 18.42, 16.26, 16.23, 15.47, 14.93 ppm; **HRMS** (ESI^+) m/z calcd for $C_{38}H_{55}O_3$ $[M+H]^+$ 559.4146, found 559.4147; **IR** (DRIFT) ν_{max} 3660, 3447, 2938, 2869, 2615, 1911, 1688, 1602, 1449 cm^{-1}.

4.1.5.6. 30-(2-Fluorobenzylidene)betulinic acid ***4.12***. The product **4.12** was obtained as white solid, 170 mg (95%) by the general procedure (red ylide color) while the reaction time was 4 h: m. p. 161–164 °C (methanol); $[\alpha]_D^{22}$ −8° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.54–7.48 (m, 1H, arom. ring), 7.21–7.15 (m, 1H, arom. ring), 7.11–7.06 (m, 1H, arom. ring), 7.05–6.99 (m, 1H, arom. ring), 6.84 (s, 2H, H-31 and H-30), 5.14, 5.10 (2H, all s, 2 × H-29), 3.28 (td, J_1 = 11.2, J_2 = 4.7 Hz, 1H, H-19β), 3.18 (dd, J_1 = 11.4, J_2 = 4.8 Hz, 1H, H-3α), 2.34 (dt, J_1 = 12.5, J_2 = 2.8 Hz, 1H), 2.02 (dd, J_1 = 12.7, J_2 = 7.9 Hz, 1H), 1.91 (t, J = 11.4 Hz, 1H), 1.00 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.81 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.18, 160.53 (d, J_{CF} = 249.5 Hz), 152.75, 133.85, 128.62 (d, J_{CF} = 8.3 Hz), 127.28 (d, J_{CF} = 3.4 Hz), 125.62 (d, J_{CF} = 12.0 Hz), 124.21 (d, J_{CF} = 3.1 Hz), 120.68, 115.87 (d, J_{CF} = 22.2 Hz), 113.51, 79.20, 56.52, 55.51, 50.74, 50.67, 42.57, 40.88, 40.52, 39.01, 38.86, 38.64, 37.36, 37.07, 34.53, 33.06, 32.37, 29.96, 28.14, 27.52, 27.15, 21.17, 18.43, 16.25, 15.48, 14.90 ppm; **HRMS** (ESI^-) m/z calcd for $C_{37}H_{50}O_3F$ $[M-H]^-$ 561.3739, found 561.3748; **IR** (DRIFT) ν_{max} 3639, 3430, 3176, 2931, 2867, 2607, 1946, 1684, 1485, 1452 cm^{-1}.

4.1.5.7. 30-(2-Chlorobenzylidene)betulinic acid ***4.13***. The product **4.13** was obtained as white solid, 181 mg (98%) by the general procedure (orange ylide color), while the reaction time was 6 h: m. p. 153–156 °C (methanol); $[\alpha]_D^{22}$ −5° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.59–7.55 (m, 1H, arom. ring), 7.36–7.32 (m, 1H, arom. ring), 7.24–7.20 (m, 1H, arom. ring), 7.17–7.13 (m, 1H, arom. ring), 7.08 (d, J = 16.2 Hz, 1H, H-31), 6.72 (d, J = 16.2 Hz, 1H, H-30), 5.15, 5.12 (2H, all s, 2 × H-29), 3.31 (td, J_1 = 11.2, J_2 = 4.8 Hz, 1H, H-19β), 3.19 (dd, J_1 = 11.4, J_2 = 4.8 Hz, 1H, H-3α), 2.38–2.32 (m, 1H), 2.03 (dd, J_1 = 12.7, J_2 = 7.9 Hz, 1H), 1.94 (t, J = 11.4 Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.81 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 180.92, 152.49, 135.91, 133.94, 133.58, 129.87, 128.39, 126.94, 126.60, 124.84, 114.18, 79.18, 56.54, 55.51, 50.67, 50.48, 42.60, 41.13, 40.89, 39.02, 38.87, 38.68, 37.37, 37.06, 34.53, 32.77, 32.36, 29.94, 28.15, 27.54, 27.09, 21.16, 18.44, 16.26 (2C), 15.49, 14.92 ppm; **HRMS** (ESI^-) m/z calcd for $C_{37}H_{50}O_3Cl$ $[M-H]^-$ 577.3443, found 577.3457; **IR** (DRIFT) ν_{max} 3440, 3255, 2927, 2866, 2614, 1912, 1680, 1603, 1465, 1447 cm^{-1}.

4.1.5.8. 30-(2-Bromobenzylidene)betulinic acid ***4.14***. The product **4.14** was obtained as white solid, 191 mg (96%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 155–158 °C; $[\alpha]_D^{22}$ −2° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.57–7.52 (m, 2H, arom. ring), 7.30–7.23 (m, 1H, arom. ring), 7.10–7.07 (m, 1H, arom. ring), 7.04 (d, J = 15.9 Hz, 1H, H-31), 6.67 (d, J = 16.2 Hz, 1H, H-30), 5.14, 5.12 (2H, all s, 2 × H-29), 3.32 (td, J_1 = 11.2, J_2 = 4.8 Hz, 1H, H-19β), 3.18 (dd, J_1 = 11.4, J_2 = 4.8 Hz, 1H, H-3α), 2.37–2.32 (m, 1H), 2.22 (td, J_1 = 12.4, J_2 = 3.4 Hz, 1H), 2.03 (dd, J_1 = 12.7, J_2 = 7.9 Hz, 1H), 1.95 (t, J = 11.4 Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.81 (s, 3H), 0.75 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.51, 152.39, 137.62, 134.05, 133.11, 128.66, 127.63, 127.58, 126.80, 124.34, 114.34, 79.19, 56.58, 55.50, 50.65, 50.41, 42.61, 41.38, 40.89, 39.01, 38.86, 38.71, 37.36, 37.06, 34.52, 32.71, 32.35, 29.94, 28.15, 27.53, 27.06, 21.16, 18.43, 16.27 (2C), 15.49, 14.95 ppm; **HRMS** (ESI^-) m/z calcd for $C_{37}H_{50}O_3Br$ $[M-H]^-$ 621.2938, found 621.2947; **IR** (DRIFT) ν_{max} 3610, 3450, 3219, 2939, 2868, 2371, 1913, 1685, 1602, 1463 cm^{-1}.

4.1.5.9. 30-(2-Iodobenzylidene)betulinic acid ***4.15***. The product **4.15** was obtained as white solid, 177 mg (83%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 156–159 °C; $[\alpha]_D^{22}$ +6° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.85–7.81 (m, 1H, arom. ring), 7.54–7.48 (m, 1H, arom. ring), 7.33–7.28 (m, 1H, arom. ring), 6.95–6.87 (m, 2H, 1 × arom. ring and H-31), 6.60 (d, J = 16.0 Hz, 1H, H-30), 5.14, 5.12 (2H, all s, 2 × H-29), 3.34 (td, J_1 = 11.2, J_2 = 4.9 Hz, 1H, H-19β), 3.18 (dd, J_1 = 11.4, J_2 = 4.7 Hz, 1H, H-3α), 2.22 (td, J_1 = 12.4, J_2 = 3.3 Hz, 1H), 2.04 (dd, J_1 = 12.7, J_2 = 8.0 Hz, 1H), 1.94 (t, J = 11.4 Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.80 (s, 3H), 0.75 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.24, 152.29, 140.79, 139.66, 134.19, 132.68, 128.87, 128.46, 126.32, 114.38, 100.80, 79.18, 56.57, 55.50, 50.66, 50.44, 42.62, 41.45, 40.89, 39.01, 38.87, 38.75, 37.36, 37.07, 34.52, 32.75, 32.31, 29.95, 28.15, 27.54, 27.07, 21.19, 18.44, 16.29, 16.28, 15.50, 15.02 ppm; **HRMS** (ESI^+) m/z calcd for $C_{37}H_{52}O_3I$ $[M+H]^+$ 671.2956, found 671.2954; **IR** (DRIFT) ν_{max} 3439, 2939, 2867, 2363, 1917, 1687, 1624, 1458 cm^{-1}.

4.1.5.10. 30-(2-Cyanobenzylidene)betulinic acid ***4.16***. The product **4.16** was obtained as white solid, 74 mg (41%) by the general procedure (yellow ylide color) while the reaction time was 18 h: m. p. 146–149 °C; $[\alpha]_D^{22}$ −8° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.68–7.64 (m, 1H, arom. ring), 7.63–7.59 (m, 1H, arom. ring), 7.55–7.50 (m, 1H, arom. ring), 7.31–7.27 (m, 1H, arom. ring), 7.03 (d, J = 16.2 Hz, 1H, H-31), 6.91 (d, J = 16.1 Hz, 1H, H-30), 5.22, 5.19 (2H, all s, 2 × H-29), 3.31 (td, J_1 = 11.2, J_2 = 4.8 Hz, 1H, H-19β), 3.19 (dd, J_1 = 11.4, J_2 = 4.7 Hz, 1H, H-3α), 2.22 (td, J_1 = 12.6, J_2 = 3.2 Hz, 1H), 2.04 (dd, J_1 = 12.7, J_2 = 8.1 Hz, 1H), 1.92 (t, J = 11.4 Hz, 1H), 1.00 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H), 0.81 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.31, 151.98, 141.12, 136.09, 133.22, 132.76, 127.41, 125.71, 124.39, 118.10, 115.66, 111.28, 79.20, 56.51, 55.48, 50.60, 50.53, 42.58, 41.49, 40.86, 38.99, 38.85, 38.67, 37.34, 37.00, 34.49, 32.63, 32.28, 29.89, 28.13, 27.47, 27.15, 21.11, 18.41, 16.26, 16.24, 15.48, 14.87 ppm; **HRMS** (ESI^-) m/z calcd for $C_{38}H_{50}O_3N$ $[M-H]^-$ 568.3785, found 568.3800; **IR** (DRIFT) ν_{max} 3447, 2939, 2868, 2224 (C≡N), 1685, 1595, 1448 cm^{-1}.

4.1.5.11. 30-(2-Trifluoromethylbenzylidene)betulinic acid ***4.17***. The product **4.17** was obtained as white solid, 129 mg (66%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 159–162 °C; $[\alpha]_D^{22}$ −19° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.69–7.60 (m, 2H, arom. ring), 7.52–7.46 (m, 1H, arom. ring), 7.34–7.29 (m, 1H, arom. ring), 7.11–7.02 (m, 1H, H-31), 6.69 (d, J = 16.0 Hz, 1H, H-30), 5.15, 5.13 (2H, all s, 2 × H-29), 3.29 (td, J_1 = 11.2, J_2 = 4.8 Hz, 1H), 3.19 (dd, J_1 = 11.4, J_2 = 4.8 Hz, 1H), 2.38–2.31 (m, 1H), 2.03 (dd, J_1 = 12.6, J_2 = 8.0 Hz, 1H), 1.91 (t, J = 11.4 Hz, 1H), 0.99 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.81 (s, 3H), 0.75 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 182.04, 152.36, 136.97, 135.25, 131.90, 127.67 (q, J_{CF} = 29.7 Hz), 127.14, 127.06, 125.97 (q, J_{CF} = 5.6 Hz), 124.60, 124.58 (q, J_{CF} = 274.1 Hz, CF_3 group), 114.66, 79.23, 56.60, 55.50, 50.65, 50.46, 42.57, 41.51, 40.87, 39.01,

38.85, 38.71, 37.35, 37.02, 34.51, 32.49, 32.30, 29.92, 28.14, 27.49, 27.03, 21.11, 18.43, 16.21 (2C), 15.49, 14.84 ppm; **HRMS** (ESI^-) *m/z* calcd for $C_{38}H_{50}O_3F_3$ $[M-H]^-$ 611.3707, found 611.3709; **IR** (DRIFT) ν_{max} 3409, 3188, 2938, 2868, 2556, 1940, 1687, 1603, 1451 cm^{-1}.

4.1.5.12. 30-(2-Nitrobenzylidene)betulinic acid ***4.18***. The product **4.18** was obtained as a yellow solid, 55 mg (29%) by the modified general procedure (violet ylide color), while the reaction time was 18 h and the reaction temperature was 70 °C; conversion of the starting material was not complete: m. p. 167–170 °C; $[\alpha]_D^{22}$ +22° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.97–7.91 (m, 1H, arom. ring), 7.66–7.61 (m, 1H, arom. ring), 7.59–7.53 (m, 1H, arom. ring), 7.40–7.33 (m, 1H, arom. ring), 7.16 (d, J = 16.0 Hz, 1H, H-31), 6.69 (d, J = 16.0 Hz, 1H, H-30), 5.18 (s, 2H, 2 × H-29), 3.26 (td, J_1 = 11.2, J_2 = 4.6 Hz, 1H, H-19β), 3.19 (dd, J_1 = 11.4, J_2 = 4.7 Hz, 1H, H-3α), 2.04 (dd, J_1 = 12.6, J_2 = 8.1 Hz, 1H), 1.91 (t, J = 11.3 Hz, 1H), 1.00 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H), 0.81 (s, 3H), 0.75 (s, 3H) ppm; **^{13}C NMR** (101 MHz, $CDCl_3$) δ 181.48, 152.33, 148.06, 136.47, 133.76, 133.12, 128.52, 127.83, 124.84, 123.93, 115.39, 79.20, 56.55, 55.48, 50.63, 50.59, 42.58, 41.28, 40.86, 39.00, 38.85, 38.66, 37.34, 37.03, 34.50, 32.67, 32.31, 29.92, 28.13, 27.49, 27.12, 21.12, 18.42, 16.24 (2C), 15.48, 14.86 ppm; **HRMS** (ESI^-) *m/z* calcd for $C_{37}H_{50}O_5N$ $[M-H]^-$ 588.3684, found 588.3696; **IR** (DRIFT) ν_{max} 3620, 3482, 3341, 2939, 2868, 1944, 1694, 1605, 1519 (N–O), 1448 cm^{-1}.

4.1.5.13. 30-(Pyridin-2-ylmethylene)betulinic acid ***4.19***. The product **4.19** was obtained as beige solid, 68 mg (39%) by the modified general procedure (brown ylide color) without extraction (the reaction mixture was evaporated after completion followed by purification) while the reaction time was 18 h. Purification was performed using mobile phase $CHCl_3$/MeOH 50 : 1: m. p. 166–170 °C; $[\alpha]_D^{22}$ +13° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 8.60–8.55 (m, 1H, H-pyridine), 7.68–7.61 (m, 1H, H-pyridine), 7.38 (d, 1H, J = 7.9 Hz, H-pyridine), 7.28 (d, 1H, J = 16.3 Hz, H-31), 7.17–7.10 (m, 1H, H-pyridine), 6.79 (d, 1H, J = 16.1 Hz, H-30), 5.25, 5.20 (2H, all s, 2 × H-29), 3.31 (td, 1H, J_1 = 11.1 Hz, J_2 = 4.6 Hz, H-19β), 3.18 (dd, 1H, J_1 = 11.4 Hz, J_2 = 4.6 Hz, H-3α), 2.21 (td, 1H, J_1 = 12.3 Hz, J_2 = 3.1 Hz), 2.01 (dd, 1H J_1 = 12.6 Hz, J_2 = 7.9 Hz), 1.91 (t, 1H, J = 11.3 Hz), 1.00, 0.96, 0.94, 0.79, 0.74 (15H, all s, 5 × $-CH_3$) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.15, 155.95, 152.50, 149.18, 137.00, 136.61, 127.13, 122.06, 115.47, 79.16, 56.53, 55.49, 50.94, 50.66, 42.55, 40.87, 39.96, 39.00, 38.85, 38.64, 37.35, 37.08, 34.52, 33.33, 32.41, 29.99, 28.14, 27.50, 27.31, 21.19, 18.43, 16.26 (2C), 15.49, 14.92 ppm; **HRMS** (ESI^+) *m/z* calcd for $C_{36}H_{52}O_3N$ $[M+H]^+$ 546.3942, found 546.3947; **IR** (DRIFT) ν_{max} 2380–3595, 2929, 2867, 1689, 1587, 1465 cm^{-1}.

4.1.5.14. 30-(3-Methylbenzylidene)betulinic acid ***4.20***. The product **4.20** was obtained as white solid, 135 mg (76%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 158–161 °C; $[\alpha]_D^{22}$ −1° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.28–7.24 (m, 2H, arom. ring), 7.23–7.18 (m, 1H, arom. ring), 7.06–7.01 (m, 1H, arom. ring), 6.79 (d, J = 16.2 Hz, 1H, H-31), 6.67 (d, J = 16.2 Hz, 1H, H-30), 5.10, 5.06 (2H, all s, 2 × H-29), 3.28 (td, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H19β), 3.19 (dd, J_1 = 11.4, J_2 = 4.6 Hz, 1H, H-3α), 2.35 (s, 3H), 2.23–2.15 (m, 1H), 2.02 (dd, J_1 = 12.7, J_2 = 7.9 Hz, 1H), 1.91 (t, J = 11.4 Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 182.14, 152.80, 138.23, 137.65, 131.53, 128.62, 128.31, 128.26, 127.41, 123.79, 112.58, 79.21, 56.60, 55.49, 50.87, 50.65, 42.55, 40.87, 40.21, 39.00, 38.85, 38.70, 37.35, 37.08, 34.51, 33.28, 32.39, 30.00, 28.14, 27.47, 27.13, 21.54, 21.19, 18.41, 16.26 (2C), 15.48, 14.93 ppm; **HRMS** (ESI^+) *m/z* calcd for $C_{38}H_{55}O_3$ $[M+H]^+$ 559.4146, found 559.4147; **IR** (DRIFT) ν_{max} 3660, 3433, 3276, 2935, 2867, 2619, 2362, 1684, 1603, 1449 cm^{-1}.

4.1.5.15. 30-(3-Fluorobenzylidene)betulinic acid ***4.21***. The product **4.21** was obtained as white solid, 102 mg (57%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 159–162 °C; $[\alpha]_D^{22}$ −2° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.29–7.24 (m, 1H, arom. ring), 7.22–7.18 (m, 1H, arom. ring), 7.17–7.13 (m, 1H, arom. ring), 6.94–6.88 (m, 1H, arom. ring), 6.79 (d, J = 16.2 Hz, 1H, H-31), 6.65 (d, J = 16.2 Hz, 1H, H-30), 5.14, 5.11 (2H, all s, 2 × H-29), 3.27 (td, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-19β), 3.20 (dd, J_1 = 11.3, J_2 = 4.7 Hz, 1H, H-3α), 2.30–2.23 (m, 1H), 2.23–2.16 (m, 1H), 2.03 (dd, J_1 = 12.7, J_2 = 7.9 Hz, 1H), 1.90 (t, J = 11.3 Hz, 1H), 1.01 (s, 3H), 0.97 (s, 3H), 0.95 (s, 3H), 0.81 (s, 3H), 0.75 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 182.19, 163.31 (d, J_{CF} = 245.0 Hz), 152.41, 140.13 (d, J_{CF} = 7.6 Hz), 133.08, 130.09 (d, J_{CF} = 8.4 Hz), 129.17, 128.36, 127.00, 125.44, 124.81, 122.60 (d, J_{CF} = 1.5 Hz), 114.62, 114.23 (d, J_{CF} = 21.4 Hz), 113.88, 113.66, 112.89 (d, J_{CF} = 21.6 Hz), 79.21, 56.59, 55.48, 50.89, 50.63, 42.54, 40.86, 40.10, 38.99, 38.84, 38.69, 37.34, 37.04, 34.50, 33.26, 32.36, 29.97, 28.14, 27.44, 27.21, 21.18, 18.40, 16.25 (2C), 15.48, 14.91 ppm; **HRMS** (ESI^+) *m/z* calcd for $C_{37}H_{52}O_3F$ $[M+H]^+$ 563.3895, found 563.3896; **IR** (DRIFT) ν_{max} 3671, 3430, 3279, 2934, 2867, 2615, 1688, 1609, 1578, 1485, 1446 cm^{-1}.

4.1.5.16. 30-(3-Chlorobenzylidene)betulinic acid ***4.22***. The product **4.22** was obtained as white solid, 111 mg (60%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 181–184 °C; $[\alpha]_D^{22}$ +11° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.44–7.42 (m, 1H, arom. ring), 7.32–7.28 (m, 1H, arom. ring), 7.26–7.21 (m, 1H, arom. ring), 7.20–7.17 (m, 1H, arom. ring), 6.79 (d, 1H, J = 16.2 Hz, H-31), 6.62 (d, 1H, J = 16.2 Hz, H-30), 5.14, 5.11 (2H, all s, 2 × H-29), 3.25 (td, 1H, J_1 = 11.1 Hz, J_2 = 4.8 Hz, H-19β), 3.19 (dd, 1H, J_1 = 11.4 Hz, J_2 = 4.7 Hz, H-3α), 2.29–2.22 (m, 1H), 2.22–2.15 (m, 1H), 2.02 (dd, 1H, J_1 = 12.8 Hz, J_2 = 7.7 Hz), 1.90 (t, 1H, J = 11.3 Hz), 1.01, 0.96, 0.95, 0.80, 0.74 (15H, all s, 5 × $-CH_3$) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.80, 152.42, 139.64, 134.70, 133.19, 129.90, 127.37, 126.75, 126.50, 124.85, 113.80, 79.20, 56.57, 55.49, 50.94, 50.64, 42.54, 40.87, 40.06, 39.01, 38.85, 38.67, 37.36, 37.04, 34.51, 33.25, 32.33, 29.97, 28.14, 27.48, 27.20, 21.17, 18.42, 16.26 (2C), 15.49, 14.92 ppm; **HRMS** (ESI^+) *m/z* calcd for $C_{37}H_{52}O_3Cl$ $[M+H]^+$ 579.3599, found 579.3605; **IR** (DRIFT) ν_{max} 2380–3610, 2939, 2869, 1689, 1594, 1574, 1451 cm^{-1}.

4.1.5.17. 30-(3-Bromobenzylidene)betulinic acid ***4.23***. The product **4.23** was obtained as white solid, 145 mg (73%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 165–168 °C; $[\alpha]_D^{22}$ +3° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.61–7.56 (m, 1H, arom. ring), 7.36–7.31 (m, 2H, arom. ring), 7.19–7.15 (m, 1H, arom. ring), 6.78 (d, J = 16.2 Hz, 1H, H-31), 6.60 (d, J = 16.2 Hz, 1H, H-30), 5.14, 5.11 (2H, all s, 2 × H-29), 3.25 (td, J_1 = 11.2, J_2 = 4.7 Hz, 1H, H-19β), 3.20 (dd, J_1 = 11.3, J_2 = 4.7 Hz, 1H, H-3α), 2.22–2.15 (m, 1H), 2.02 (dd, J_1 = 12.7, J_2 = 8.0 Hz, 1H), 1.89 (t, J = 11.3 Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 182.07, 152.42, 139.94, 133.28, 130.25, 130.17, 129.45, 126.59, 125.25, 122.96, 113.83, 79.21, 56.57, 55.47, 50.93, 50.62, 42.52, 40.85, 39.99, 38.98, 38.84, 38.68, 37.33, 37.03, 34.49, 33.29, 32.35, 29.96, 28.14, 27.42, 27.21, 21.18, 18.39, 16.25 (2C), 15.48, 14.91 ppm; **HRMS** (ESI^+) *m/z* calcd for $C_{37}H_{52}O_3Br$ $[M+H]^+$ 623.3094, found 623.3093; **IR** (DRIFT) ν_{max} 3426, 2938, 2867, 1686, 1589, 1559, 1450 cm^{-1}.

4.1.5.18. 30-(3-Iodobenzylidene)betulinic acid ***4.24***. The product **4.24** was obtained as white solid, 182 mg (85%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 177–180 °C; $[\alpha]_D^{22}$ +2° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.82–7.76 (m, 1H, arom. ring), 7.56–7.50 (m, 1H, arom. ring), 7.41–7.36 (m, 1H, arom. ring), 7.07–7.00 (m, 1H, arom. ring), 6.76 (d, J = 16.2 Hz, 1H, H-31), 6.56 (d, J = 16.2 Hz, 1H, H-30), 5.13, 5.11 (2H, all s, 2 × H-29), 3.25 (td, J_1 = 11.3, J_2 = 4.8 Hz, 1H, H-19β), 3.20 (dd, J_1 = 11.3, J_2=4.6 Hz, 1H, H-3α), 2.21–2.15 (m, 1H), 2.02 (dd, J_1 = 12.6, J_2 = 8.0 Hz, 1H), 1.89 (t, J = 11.3 Hz, 1H), 1.01 (s, 3H), 0.97 (s, 3H), 0.94 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 182.11, 152.44, 140.01, 136.23, 135.51, 133.19, 130.33, 126.47, 125.81, 113.81, 94.88, 79.20, 56.57, 55.47, 50.94, 50.62, 42.53, 40.85, 39.96, 38.98, 38.84, 38.69, 37.34, 37.03, 34.49, 33.31, 32.35, 29.96, 28.15, 27.44, 27.21, 21.18, 18.40, 16.26 (2C), 15.49, 14.92 ppm; **HRMS** (ESI^+) *m*/*z* calcd for $C_{37}H_{52}O_3I$ $[M+H]^+$ 671.2956, found 671.2955; **IR** (DRIFT) ν_{max} 3433, 2939, 2868, 1685, 1601, 1584, 1556, 1465, 1451 cm^{-1}.

4.1.5.19. 30-(3-Cyanobenzylidene)betulinic acid ***4.25***. The product **4.25** was obtained as white solid, 94 mg (52%) by the general procedure (yellow ylide color) while the reaction time was 18 h: m. p. 163–166 °C; $[\alpha]_D^{22}$ −3° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.73–7.69 (m, 1H, arom. ring), 7.66–7.62 (m, 1H, arom. ring), 7.50–7.47 (m, 1H, arom. ring), 7.43–7.39 (m, 1H, arom. ring), 6.83 (d, J = 16.2 Hz, 1H, H-31), 6.64 (d, J = 16.2 Hz, 1H, H-30), 5.18, 5.16 (2H, all s, 2 × H-29), 3.26 (td, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-19β), 3.19 (dd, J_1 = 11.3, J_2 = 4.6 Hz, 1H, H-3α), 2.22–2.15 (m, 1H), 2.03 (dd, J_1 = 12.7, J_2 = 7.9 Hz, 1H), 1.90 (t, J = 11.3 Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.76, 152.17, 139.01, 134.43, 130.71, 130.60, 130.04, 129.49, 125.77, 118.93, 114.73, 112.98, 79.16, 56.57, 55.48, 51.00, 50.62, 42.54, 40.86, 39.95, 39.00, 38.84, 38.69, 37.36, 36.99, 34.50, 33.26, 32.31, 29.96, 28.14, 27.48, 27.28, 21.17, 18.40, 16.26 (2C), 15.47, 14.92 ppm; **HRMS** (ESI^-) *m*/*z* calcd for $C_{38}H_{50}O_3N$ $[M-H]^-$ 568.3785, found 568.3793; **IR** (DRIFT) ν_{max} 3506, 3436, 2934, 2867, 2550, 2236 (C≡N), 1992, 1686, 1601, 1450 cm^{-1}.

4.1.5.20. 30-(3-Trifluoromethylbenzylidene)betulinic acid ***4.26***. The product **4.26** was obtained as white solid, 127 mg (65%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 168–171 °C; $[\alpha]_D^{22}$ −1° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.69–7.66 (m, 1H, arom. ring), 7.62–7.58 (m, 1H, arom. ring), 7.48–7.40 (m, 2H, arom. ring), 6.85 (d, J = 16.2 Hz, 1H, H-31), 6.70 (d, J = 16.2 Hz, 1H, H-30), 5.17, 5.14 (2H, all s, 2 × H-29), 3.27 (td, J_1 = 11.1 Hz, J_2 = 4.7 Hz, 1H, H-19β), 3.19 (dd, J_1 = 11.3 Hz, J_2 = 4.7 Hz, 1H, H-3α), 2.39–2.33 (m, 1H), 2.19 (td, J_1 = 12.3 Hz, J_2 = 3.2 Hz, 1H), 2.03 (dd, J_1 = 12.8 Hz, J_2 = 7.9 Hz, 1H), 1.91 (t, J = 11.3 Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 182.07, 152.38, 138.52, 133.71, 131.16 (q, J_{CF} = 32.0 Hz), 129.66, 129.12, 126.61, 124.31 (q, J_{CF} = 272.4 Hz, CF_3 group), 123.93 (d, J_{CF} = 3.2 Hz), 123.30 (d, J_{CF} = 3.4 Hz), 114.13, 79.20, 56.59, 55.48, 50.96, 50.63, 42.54, 40.87, 39.90, 38.99, 38.84, 38.70, 37.35, 37.03, 34.50, 33.34, 32.36, 29.97, 28.14, 27.46, 27.27, 21.18, 18.40, 16.27, 16.24 (2C), 15.47, 14.91 ppm; **HRMS** (ESI^+) *m*/*z* calcd for $C_{38}H_{52}O_3F_3$ $[M+H]^+$ 613.3863, found 613.3862; **IR** (DRIFT) ν_{max} 3445, 2939, 2869, 1689, 1629, 1606, 1449 cm^{-1}.

4.1.5.21. 30-(3-Nitrobenzylidene)betulinic acid ***4.27***. The product **4.27** was obtained as yellow solid, 147 mg (78%) by the general procedure (red-brown ylide color) while the reaction time was 18 h: m. p. 168–171 °C; $[\alpha]_D^{22}$ +3° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 8.31–8.26 (m, 1H, arom. ring), 8.08–8.03 (m, 1H, arom. ring), 7.75–7.71 (m, 1H, arom. ring), 7.50–7.45 (m, 1H, arom. ring), 6.91 (d, J = 16.2 Hz, 1H, H-31), 6.71 (d, J = 16.2 Hz, 1H, H-30), 5.21, 5.18 (2H, all s, 2 × H-29), 3.27 (td, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-19β), 3.18 (dd, J_1 = 11.4, J_2 = 4.6 Hz, 1H, H-3α), 2.23–2.16 (m, 1H), 2.03 (dd, J_1 = 12.8, J_2 = 8.0 Hz, 1H), 1.91 (t, J = 11.3 Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 182.00, 152.15, 148.83, 139.59, 134.96, 132.35, 129.55, 125.66, 121.92, 121.12, 115.00, 79.16, 56.58, 55.46, 51.02, 50.60, 42.52, 40.85, 39.85, 38.98, 38.83, 38.69, 37.34, 36.99, 34.49, 33.33, 32.32, 29.96, 28.13, 27.45, 27.31, 21.17, 18.39, 16.27, 16.24, 15.46, 14.90 ppm; **HRMS** (ESI^-) *m*/*z* calcd for $C_{37}H_{52}O_5N$ $[M-H]^-$ 590.3840, found 590.3837; **IR** (DRIFT) ν_{max} 3580, 3433, 3299, 2932, 2867, 2724, 2556, 1964, 1687, 1629, 1604, 1524 (N–O), 1448, 1346 (N–O) cm^{-1}.

4.1.5.22. 30-(Pyridin-3-ylbenzylidene)betulinic acid ***4.28***. The product **4.28** was obtained as a white solid, 118 mg (68%) by the modified general procedure (orange-brown ylide color) without extraction (the reaction mixture was evaporated after completion followed by purification) while the reaction time was 18 h. Purification was performed using mobile phase $CHCl_3$/MeOH 50 : 1: m. p. 187–190 °C; $[\alpha]_D^{22}$ −10° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 8.96–8.89 (m, 1H, arom. ring), 8.41–8.35 (m, 1H, arom. ring), 8.05–7.97 (m, 1H, arom. ring), 7.37–7.30 (m, 1H, arom. ring), 6.93 (d, J = 16.3 Hz, 1H, H-31), 6.89 (d, J = 16.4 Hz, 1H, H-30), 5.21, 5.16 (2H, all s, 2 × H-29), 3.59 (td, J_1 = 11.1, J_2 = 5.0 Hz, 1H, H-19β), 3.17 (dd, J_1 = 11.3, J_2 = 4.8 Hz, 1H, H-3α), 2.01 (dd, J_1 = 12.5, J_2 = 7.9 Hz, 1H), 1.88 (t, J = 11.3 Hz, 1H), 1.03 (s, 3H), 0.96 (s, 3H), 0.96 (s, 3H), 0.80 (s, 3H), 0.73 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 179.01, 153.13, 148.97, 145.97, 134.91, 134.30, 132.51, 123.96, 123.45, 114.65, 79.20, 56.40, 55.54, 51.42, 50.78, 42.51, 40.93, 39.94, 39.01, 38.87, 38.57, 37.38, 37.04, 34.58, 34.07, 32.43, 30.19, 28.15, 27.60, 27.56, 21.20, 18.49, 16.28 (2C), 15.49, 14.87 ppm; **HRMS** (ESI^+) *m*/*z* calcd for $C_{36}H_{52}O_3N$ $[M+H]^+$ 546.3942, found 546.3946; **IR** (DRIFT) ν_{max} 3431, 2933, 2867, 2539, 2065, 1673, 1586, 1569, 1449 cm^{-1}.

4.1.5.23. 30-(3-Carboxybenzylidene)betulinic acid ***4.29***. The product **4.29** was obtained as a mixture of inseparable *E* and *Z* isomers in a ratio approximately 2 : 1, white solid, 120 mg (64%) by the modified general procedure using double amount of *t*-BuOK (orange ylide color) while the reaction time was 18 h. The compound is not very stable on silica gel and exhibits bright blue fluorescence under UV light. Purification was performed in $CHCl_3$/MeOH 50 : 1: m. p. 183–186 °C; $[\alpha]_D^{22}$ −3° (*c* 0.29, THF); **^{1}H NMR** (400 MHz, CD_3OD) δ 8.22 (s, 0.5H, H-arom. ring of *Z*-isomer), 8.09 (s, 1H, H-arom. ring of *E*-isomer), 7.89–7.82 (m, 1.5H, H-arom. ring of both isomers), 7.65 (m, 1.5H, H-arom. ring of both isomers), 7.41 (t, J = 7.7 Hz, 1H, H-arom. ring of *E*-isomer), 7.35 (t, J = 7.7 Hz, 0.5H, H-arom. ring of *Z*-isomer), 6.91 (d, J = 16.2 Hz, 1H, H-31 of *E*-isomer), 6.73 (d, J = 16.2 Hz, 1H, H-30 of *E*-isomer), 6.52 (d, J = 12.7 Hz, 0.5H, H-31 of *Z*-isomer), 6.12 (d, J = 12.5 Hz, 0.5H, H-30 of *Z*-isomer), 5.14 (d, J = 17.8 Hz, 2H, 2 × H-29 of *E*-isomer), 5.00 (d, J = 22.7 Hz, 2H, 2 × H-29 of *Z*-isomer), 3.15–3.03 (m, 2H), 1.00 (s, 3H, CH_3- group of *E*-isomer), 0.94 (s, 3H, CH_3- group of *E*-isomer), 0.93 (s, 4.5H, CH_3- groups of both isomers), 0.92 (s, 1.5H, CH_3- group of *Z*-isomer), 0.91 (s, 1.5H, CH_3- group of *Z*-isomer), 0.82 (s, 1.5H, CH_3- group of *Z*-isomer), 0.80 (s, 3H, CH_3- group of *E*-isomer), 0.73 (s, 1.5H, CH_3- group of *Z*-isomer), 0.72 (s, 3H, CH_3- group of *E*-isomer) ppm; **HRMS** (ESI^-) *m*/*z* calcd for $C_{38}H_{51}O_5$ $[M-H]^-$ 587.3731, found 587.3744; **IR** (DRIFT) ν_{max} 2939, 2870, 2616, 2362, 2006, 1691, 1602, 1582, 1450 cm^{-1}.

4.1.5.24. 30-(3-Methoxybenzylidene)betulinic acid ***4.30***. The product **4.30** was obtained as white solid, 70 mg (38%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 147–150 °C; $[\alpha]_D^{22}$ +2° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.25–7.20 (m, 1H, arom. ring), 7.06–7.02 (m, 1H, arom. ring),

7.00–6.97 (m, 1H, arom. ring), 6.81–6.75 (m, 2H, arom. ring and H-31), 6.66 (d, 1H, J = 16.2 Hz, H-30), 5.12, 5.07 (2H, all s, 2 × H-29), 3.83 (s, 3H, methoxy $-CH_3$), 3.27 (td, 1H, J_1 = 11.1 Hz, J_2 = 4.7 Hz, H-19β), 3.19 (dd, 1H, J_1 = 11.4 Hz, J_2 = 4.7 Hz, H-3α), 2.38–2.32 (m, 1H), 2.30–2.23 (m, 1H), 2.23–2.16 (m, 1H), 2.02 (dd, 1H, J_1 = 12.8 Hz, J_2 = 7.7 Hz), 1.91 (t, 1H, J = 11.4 Hz), 1.01, 0.96, 0.95, 0.80, 0.74 (15 H, all s, 5 × $-CH_3$) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 182.01, 159.99, 152.66, 139.19, 132.02, 129.66, 128.09, 119.41, 113.32, 112.91, 111.82, 79.20, 56.59, 55.49, 55.39, 50.87, 50.65, 42.55, 40.87, 40.21, 39.00, 38.84, 38.69, 37.35, 37.07, 34.51, 33.25, 32.37, 29.99, 28.14, 27.48, 27.14, 21.18, 18.41, 16.26 (2C), 15.48, 14.92 ppm; **HRMS** (ESI^+) m/z calcd for $C_{38}H_{55}O_4$ $[M+H]^+$ 575.4095, found 575,4090; **IR** (DRIFT) ν_{max} 3450, 2939, 2868, 1686, 1599, 1578, 1485, 1452 cm^{-1}.

4.1.5.25. 30-(4-Methylbenzylidene)betulinic acid ***4.32***. The product **4.32** was obtained as white solid, 93 mg (52%) by the general procedure (red ylide color) while the reaction time was 18 h: m. p. 182–185 °C; $[\alpha]_D^{22}$ +5° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.36–7.32 (m, 2H, arom. ring), 7.14–7.10 (m, 2H, arom. ring), 6.75 (d, 1H, J = 16.2 Hz, H-31), 6.66 (d, 1H, J = 16.2 Hz, H-30), 5.08, 5.03 (2H, all s, 2 × H-29), 3.27 (td, 1H, J_1 = 11.2 Hz, J_2 = 4.7 Hz, H-19β), 3.19 (dd, 1H, J_1 = 11.4 Hz, J_2 = 4.7 Hz, H-3α), 2.34 (s, 3H, $-CH_3$ on arom. ring), 2.29–2.23 (m, 1H), 2.22–2.16 (m, 1H), 2.01 (dd, 1H, J_1 = 12.7 Hz, J_2 = 7.8 Hz), 1.90 (t, 1H, J = 11.4 Hz), 1.01, 0.96, 0.95, 0.80, 0.74 (15H, all s, 5 × $-CH_3$) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 182.02, 152.82, 137.33, 134.92, 130.72, 129.45 (2C), 128.11, 126.56 (2C), 112.20, 79.20, 56.60, 55.50, 50.85, 50.66, 42.55, 40.88, 40.27, 39.01, 38.85, 38.70, 37.36, 37.09, 34.52, 33.27, 32.38, 29.99, 28.15, 27.50, 27.11, 21.35, 21.19, 18.43, 16.27 (2C), 15.49, 14.93 ppm; **HRMS** (ESI^+) m/z calcd for $C_{38}H_{55}O_3$ $[M+H]^+$ 559.4146, found 559.4146; **IR** (DRIFT) ν_{max} 2490–3620, 2939, 2868, 1691, 1605, 1511, 1450 cm^{-1}.

4.1.5.26. 30-(4-Fluorobenzylidene)betulinic acid ***4.33***. The product **4.33** was obtained as a white solid, 68 mg (38%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 149–151 °C; $[\alpha]_D^{22}$ +3° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.43–7.37 (m, 2H, arom. ring), 7.03–6.97 (m, 2H, arom. ring), 6.71 (d, 1H, J = 16.2 Hz, H-31), 6.64 (d, 1H, J = 16.2 Hz, H-30), 5.10, 5.06 (2H, all s, 2 × H-29), 3.26 (td, 1H, J_1 = 11.1 Hz, J_2 = 4.7 Hz, H-19β), 3.19 (dd, 1H, J_1 = 11.4 Hz, J_2 = 4.7 Hz, H-3α), 2.39–2.32 (m, 1H), 2.29–2.23 (m, 1H), 2.19 (td, 1H, J_1 = 12.4 Hz, J_2 = 3.3 Hz), 2.02 (dd, 1H, J_1 = 12.8 Hz, J_2 = 7.8 Hz), 1.90 (t, 1H, J = 11.4 Hz), 1.01, 0.96, 0.95, 0.81, 0.74 (15 H, all s, 5 × $-CH_3$) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.78, 162.35 (d, J_{CF} = 247.0 Hz), 152.56, 133.88 (d, J_{CF} = 2.9 Hz), 131.57, 128.09 (d, J_{CF} = 7.9 Hz), 126.95, 115.64 (d, J_{CF} = 21.5 Hz), 112.78, 79.19, 56.58, 55.50, 50.85, 50.65, 42.55, 40.88, 40.13, 39.01, 38.85, 38.69, 37.37, 37.05, 34.52, 33.27, 32.36, 29.99, 28.15, 27.50, 27.19, 21.19, 18.42, 16.27 (2C), 15.49, 14.93 ppm; **HRMS** (ESI^+) m/z calcd for $C_{37}H_{52}O_3F$ $[M+H]^+$ 563.3895, found 563.3899; **IR** (DRIFT) ν_{max} 2450–3650, 2939, 2869, 1693, 1600, 1508, 1452 cm^{-1}.

4.1.5.27. 30-(4-Chlorobenzylidene)betulinic acid ***4.34***. The product **4.34** was obtained as a white solid, 81 mg (44%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 173–176 °C; $[\alpha]_D^{22}$ +11° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.39–7.34 (m, 2H, arom. ring), 7.30–7.26 (m, 2H, arom. ring), 6.76 (d, 1H, J = 16.2 Hz, H-31), 6.63 (d, 1H, J = 16.2 Hz, H-30), 5.12, 5.09 (2H, all s, 2 × H-29), 3.25 (td, 1H, J_1 = 11.1 Hz, J_2 = 4.7 Hz, H-19β), 3.19 (dd, 1H, J_1 = 11.4 Hz, J_2 = 4.7 Hz, H-3α), 2.29–2.22 (m, 1H), 2.18 (td, 1H, J_1 = 12.5 Hz, J_2 = 3.6 Hz), 2.02 (dd, 1H, J_1 = 12.8 Hz, J_2 = 7.8 Hz), 1.90 (t, 1H, J = 11.3 Hz), 1.01, 0.96, 0.94, 0.80, 0.74 (15H, all s, 5 × $-CH_3$) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.85, 152.42, 136.15, 132.98, 132.34, 128.80 (2C), 127.73 (2C), 126.79, 113.24, 79.10, 56.50, 55.41, 50.81, 50.56, 42.47, 40.80, 39.98, 38.93, 38.77, 38.62, 37.28, 36.96, 34.44, 33.22, 32.28, 29.90, 28.07, 27.42, 27.14, 21.11, 18.34, 16.19 (2C), 15.40, 14.84 ppm; **HRMS** (ESI^+) m/z calcd for $C_{37}H_{52}O_3Cl$ $[M+H]^+$ 579.3599, found 579.3602; **IR** (DRIFT) ν_{max} 2380–3650, 2939, 2868, 1691, 1593, 1490, 1451 cm^{-1}.

4.1.5.28. 30-(4-Bromobenzylidene)betulinic acid ***4.35***. The product **4.35** was obtained as a white solid, 76 mg (38%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 188–191 °C; $[\alpha]_D^{22}$ +7° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.45–7.41 (m, 2H, arom. ring), 7.32–7.28 (m, 2H, arom. ring), 6.77 (d, 1H, J = 16.2 Hz, H-31), 6.61 (d, 1H, J = 16.2 Hz, H-30), 5.12, 5.09 (2H, all s, 2 × H-29), 3.25 (td, 1H, J_1 = 11.1 Hz, J_2 = 4.7 Hz, H-19β), 3.19 (dd, 1H, J_1 = 11.4 Hz, J_2 = 4.7 Hz, H-3α), 2.29–2.22 (m, 1H), 2.18 (td, 1H, J_1 = 12.5 Hz, J_2 = 3.6 Hz), 2.01 (dd, 1H, J_1 = 12.7 Hz, J_2 = 7.8 Hz), 1.90 (t, 1H, J = 11.3 Hz), 1.01, 0.96, 0.94, 0.80, 0.74 (15H, all s, 5 × $-CH_3$) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.82, 152.49, 136.67, 132.53, 131.82 (2C), 128.13 (2C), 126.91, 121.19, 113.42, 79.17, 56.57, 55.49, 50.90, 50.64, 42.55, 40.88, 40.03, 39.01, 38.85, 38.70, 37.36, 37.03, 34.51, 33.30, 32.35, 29.97, 28.15, 27.50, 27.23, 21.18, 18.42, 16.27 (2C), 15.48, 14.92 ppm; **HRMS** (ESI^+) m/z calcd for $C_{37}H_{52}O_3Br$ $[M+H]^+$ 623.3094, found 623.3098; **IR** (DRIFT) ν_{max} 3459, 2938, 2868, 1686, 1589, 1486, 1449 cm^{-1}.

4.1.5.29. 30-(4-Iodobenzylidene)betulinic acid ***4.36***. The product **4.36** was obtained as a white solid, 90 mg (42%) by the general procedure (orange ylide color), while the reaction time was 18 h: m. p. 175–178 °C; $[\alpha]_D^{22}$ −1° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.66–7.59 (m, 2H, arom. ring), 7.21–7.14 (m, 2H, arom. ring), 6.78 (d, J = 16.2 Hz, 1H, H-31), 6.59 (d, J = 16.2 Hz, 1H, H-30), 5.12, 5.09 (2H, all s, 2 × H-29), 3.25 (td, J_1 = 11.1, J_2 = 4.6 Hz, 1H, H-19β), 3.19 (dd, J_1 = 11.3, J_2 = 4.6 Hz, 1H, H-3α), 2.38–2.31 (m, 1H), 2.22–2.14 (m, 1H), 2.01 (dd, J_1 = 12.6, J_2 = 8.0 Hz, 1H), 1.89 (t, J = 11.3 Hz, 1H), 1.00 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H), 0.79 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.83, 152.47, 137.77, 137.24, 132.62, 128.37, 126.98, 113.50, 92.58, 79.17, 56.55, 55.47, 50.89, 50.62, 42.53, 40.86, 39.98, 38.99, 38.83, 38.68, 37.34, 37.03, 34.50, 33.31, 32.35, 29.95, 28.14, 27.47, 27.22, 21.17, 18.40, 16.25 (2C), 15.47, 14.91 ppm; **HRMS** (ESI^-) m/z calcd for $C_{37}H_{50}O_3I$ $[M-H]^-$ 669.2799, found 669.2811; **IR** (DRIFT) ν_{max} 3612, 3430, 3197, 2936, 2867, 2610, 1960, 1681, 1602, 1483, 1449 cm^{-1}.

4.1.5.30. 30-(4-Cyanobenzylidene)betulinic acid ***4.37***. The product **4.37** was obtained as a white solid, 160 mg (88%) by the general procedure (yellow-orange ylide color), while the reaction time was 18 h: m. p. 171–174 °C; $[\alpha]_D^{22}$ −1° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.60–7.57 (m, 2H, arom. ring), 7.52–7.48 (m, 2H, arom. ring), 6.88 (d, J = 16.1 Hz, 1H, H-31), 6.66 (d, J = 16.2 Hz, 1H, H-30), 5.20, 5.19 (2H, all s, 2 × H-29), 3.26 (td, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-19β), 3.19 (dd, J_1 = 11.3, J_2 = 4.7 Hz, 1H, H-3α), 2.22–2.15 (m, 1H), 2.02 (dd, J_1 = 12.7, J_2 = 8.0 Hz, 1H), 1.89 (t, J = 11.3 Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.93 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.97, 152.20, 142.26, 135.56, 132.50, 127.01, 126.30, 119.16, 115.30, 110.47, 79.14, 56.56, 55.45, 50.95, 50.58, 42.51, 40.84, 39.83, 38.97, 38.82, 38.65, 37.33, 36.97, 34.48, 33.27, 32.30, 29.94, 28.13, 27.42, 27.31, 21.15, 18.38, 16.24 (2C), 15.47, 14.89 ppm; **HRMS** (ESI^-) m/z calcd for $C_{38}H_{50}O_3N$ $[M-H]^-$ 568.3785, found 568.3788; **IR** (DRIFT) ν_{max} 3421, 3261, 2931, 2867, 2613, 2223 (C≡N), 1724, 1673, 1601, 1503, 1450 cm^{-1}.

4.1.5.31. 30-(4-Trifluoromethylbenzylidene)betulinic acid ***4.38***. The product **4.38** was obtained as a white solid, 105 mg (54%) by the general procedure (orange ylide color), while the reaction time was 2 h: m. p. 155–158 °C; $[\alpha]_D^{22}$ −3° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.57–7.51 (m, 4H, arom. ring), 6.87 (d, J = 16.2 Hz, 1H, H-

31), 6.69 (d, J = 16.2 Hz, 1H, H-30), 5.18, 5.15 (2H, all s, 2 × H-29), 3.27 (td, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-19β), 3.19 (dd, J_1 = 11.4, J_2 = 4.7 Hz, 1H, H-3α), 2.39–2.33 (m, 1H), 2.22–2.16 (m, 1H), 2.03 (dd, J_1 = 12.7, J_2 = 7.9 Hz, 1H), 1.91 (t, J = 11.3 Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.80 (s, 3H), 0.73 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.88, 152.37, 141.22, 134.32, 129.19 (q, J_{CF} = 32.4 Hz), 126.71 (2C), 125.66 (d, J_{CF} = 3.6 Hz), 124.39 (q, J_{CF} = 271.8 Hz, CF_3 group), 114.39, 79.19, 56.58, 55.48, 50.94, 50.63, 42.55, 40.87, 39.96, 38.99, 38.84, 38.70, 37.35, 37.03, 34.51, 33.31, 32.35, 29.96, 28.13, 27.46, 27.28, 21.19, 18.41, 16.26, 16.25, 15.46, 14.91 ppm; **HRMS** (ESI^-) m/z calcd for $C_{38}H_{50}O_3F_3$ $[M-H]^-$ 611.3707, found 611.3718; **IR** (DRIFT) ν_{max} 3671, 3432, 3216, 2934, 2868, 2615, 9112, 1681, 1613, 1450 cm^{-1}.

4.1.5.32. 30-(4-Nitrobenzylidene)betulinic acid ***4.39***. The product **4.39** was obtained as a yellow solid, 90 mg (48%) by the general procedure (dark violet ylide color), while the reaction time was 18 h and the reaction temperature was 70 °C: m. p. 176–178 °C; $[\alpha]_D^{22}$ +1° (*c* 0.29, THF); **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.21–8.14 (m, 2H, arom. ring), 7.59–7.53 (m, 2H, arom. ring), 6.94 (d, J = 16.2 Hz, 1H, H-31), 6.72 (d, J = 16.2 Hz, 1H, H-30), 5.24, 5.23 (2H, all s, 2 × H-29), 3.28 (td, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-19β), 3.19 (dd, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-3α), 2.03 (dd, J_1 = 12.8, J_2 = 7.8 Hz, 1H), 1.91 (t, J = 11.3 Hz, 1H), 1.02 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (101 MHz, $CDCl_3$) δ 181.78, 152.22, 146.79, 144.29, 136.51, 127.01, 125.92, 124.18, 115.87, 79.14, 56.57, 55.47, 51.00, 50.60, 42.53, 40.86, 39.75, 38.99, 38.84, 38.67, 37.35, 36.98, 34.50, 33.31, 32.31, 29.95, 28.14, 27.46, 27.38, 21.17, 18.40, 16.27 (2C), 15.47, 14.90 ppm; **HRMS** (ESI^-) m/z calcd for $C_{37}H_{50}O_5N$ $[M-H]^-$ 588.3684, found 588.3688; **IR** (DRIFT) ν_{max} 3430, 2934, 2867, 1679, 1592, 1512 (N–O), 1450 cm^{-1}.

4.1.5.33. 30-(Pyridin-4-ylbenzylidene)betulinic acid ***4.40***. The product **4.40** was obtained as a yellowish solid, 38 mg (22%) by the general procedure (brown ylide color) without extraction (the reaction mixture was evaporated after completion followed by purification) while the reaction time was 18 h. Purification was performed using mobile phase $CHCl_3$/MeOH 50 : 1: m. p. 166–169 °C; $[\alpha]_D^{22}$ −2° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 8.57–8.53 (m, 2H, arom. ring), 7.38–7.34 (m, 2H, arom. ring), 7.00 (d, J = 16.2 Hz, 1H, H-31), 6.63 (d, J = 16.2 Hz, 1H, H-30), 5.24, 5.23 (2H, all s, 2 × H-29), 3.32 (td, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-19β), 3.18 (dd, J_1 = 11.3, J_2 = 4.7 Hz, 1H, H-3α), 2.40–2.34 (m, 1H), 2.03 (dd, J_1 = 12.5, J_2 = 8.0 Hz, 1H), 1.87 (t, J = 11.3 Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 179.98, 152.40, 148.89 (2C), 146.34, 137.35, 125.15, 121.39 (2C), 116.14, 79.12, 56.48, 55.50, 51.03, 50.69, 42.55, 40.97, 39.63, 39.00, 38.86, 38.54, 37.35, 37.04, 34.54, 33.43, 32.45, 30.01, 28.14, 27.50, 21.21, 18.44, 16.27, 16.26, 15.50, 14.89 ppm; **HRMS** (ESI^+) m/z calcd for $C_{36}H_{52}O_3N$ $[M+H]^+$ 546.3942, found 546.3946; **IR** (DRIFT) ν_{max} 2400–3670, 2938, 2867, 1932, 1700, 1600, 1549, 1450 cm^{-1}.

4.1.5.34. 30-(4-Carboxybenzylidene)betulinic acid ***4.41***. The product **4.41** was obtained as white solid, 96 mg (51%) by the general procedure using double amount of *t*-BuOK (red ylide color) while the reaction time was 18 h. Purification was performed using mobile phase toluene/MeOH 5 : 1: m. p. 217–220 °C; $[\alpha]_D^{22}$ −3° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, CD_3OD) δ 7.99–7.94 (m, 2H, arom. ring), 7.57–7.51 (m, 2H, arom. ring), 6.99 (d, J = 16.2 Hz, 1H, H-31), 6.76 (d, J = 16.2 Hz, 1H, H-30), 5.21, 5.18 (2H, all s, 2 × H-29), 3.11 (dd, J_1 = 11.4, J_2 = 4.8 Hz, 1H, H-3α), 1.03 (s, 3H), 0.97 (s, 3H), 0.94 (s, 3H), 0.84 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 180.08, 170.20, 154 0.35, 143.47, 135.32, 131.16 (2C), 129.92, 128.09, 127.32 (2C), 114.80, 79.66, 57.54, 56.86, 52.08, 52.01, 43.53, 41.96, 41.27, 40.06, 39.94, 39.75, 38.33, 37.95, 35.65, 34.32, 33.27, 30.97, 28.60, 28.51, 28.03, 22.27, 19.44, 16.71, 16.69, 16.09, 15.14 ppm; **HRMS** (ESI^+) m/z calcd for $C_{38}H_{53}O_5$ $[M+H]^+$ 589.3888, found 589.3889; **IR** (DRIFT) ν_{max} 3664, 3599, 3394, 2932, 2868, 2000, 1702, 1674, 1604, 1465, 1449 cm^{-1}.

4.1.5.35. 30-(4-Methoxybenzylidene)betulinic acid ***4.42***. The product **4.42** was obtained as a white solid, 70 mg (38%) by the general procedure (red ylide color), while the reaction time was 2 h: m. p. 156–159 °C; $[\alpha]_D^{22}$ −1° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.41–7.34 (m, 2H, arom. ring), 6.87–6.84 (m, 2H, arom. ring), 6.67 (d, J = 16.3 Hz, 1H, H-31), 6.63 (d, J = 16.3 Hz, 1H, H-30), 5.06, 5.01 (2H, all s, 2 × H-29), 3.81 (s, 3H, methoxy $-CH_3$), 3.26 (td, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-19β), 3.19 (dd, J_1 = 11.4, J_2 = 4.7 Hz, 1H, H-3α), 2.37–2.32 (m, 1H), 2.01 (dd, J_1 = 12.7, J_2 = 7.9 Hz, 1H), 1.90 (t, J = 11.3 Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.97, 159.20, 152.84, 130.51, 129.73, 127.78 (2C), 127.59, 114.18 (2C), 111.68, 79.19, 56.55, 55.46, 55.41, 50.75, 50.63, 42.52, 40.84, 40.16, 38.96, 38.82, 38.65, 37.31, 37.07, 34.49, 33.30, 32.37, 29.97, 28.12, 27.39, 27.11, 21.17, 18.40, 16.24 (2C), 15.48, 14.90 ppm; **HRMS** (ESI^-) m/z calcd for $C_{38}H_{53}O_4$ $[M-H]^-$ 573.3938, found 573.3951; **IR** (DRIFT) ν_{max} 3447, 2935, 2867, 1680, 1603, 1510, 1450 cm^{-1}.

4.1.5.36. 30-(4-{t-Butyl}benzylidene)betulinic acid ***4.43***. The product **4.43** was obtained as a white solid, 121 mg (63%) by the general procedure (orange ylide color) while the reaction time was 2 h: m. p. 165–168 °C; $[\alpha]_D^{22}$ +4° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.42–7.37 (m, 2H, arom. ring), 7.36–7.33 (m, 2H, arom. ring), 6.77 (d, J = 16.2 Hz, 1H, H-31), 6.68 (d, J = 16.2 Hz, 1H, H-30), 5.10, 5.05 (2H, all s, 2 × H-29), 3.29 (td, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-19β), 3.20 (dd, J_1 = 11.4, J_2 = 4.7 Hz, 1H, H-3α), 2.40–2.33 (m, 1H), 2.03 (dd, J_1 = 12.6, J_2 = 8.0 Hz, 1H), 1.91 (t, J = 11.3 Hz, 1H), 1.32 (s, 9H, *t*-butyl group), 1.02 (s, 3H), 0.97 (s, 3H), 0.96 (s, 3H), 0.81 (s, 3H), 0.75 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 182.26, 152.84, 150.62, 134.92, 130.90, 127.97, 126.36 (2C), 125.64 (2C), 112.24, 79.21, 56.60, 55.49, 50.82, 50.65, 42.55, 40.87, 40.30, 38.99, 38.84, 38.68, 37.34, 37.10, 34.71, 34.51, 33.23, 32.39, 31.44 (3C), 29.98, 28.14, 27.45, 27.07, 21.18, 18.42, 16.26 (2C), 15.49, 14.93 ppm; **HRMS** (ESI^-) m/z calcd for $C_{41}H_{59}O_3$ $[M-H]^-$ 599.4459, found 599.4465; **IR** (DRIFT) ν_{max} 3672, 3448, 3229, 2936, 2867, 2558, 1898, 1734, 1682, 1601, 1518, 1462 cm^{-1}.

4.1.5.37. 30-(3-Chloro-4-fluorobenzylidene)betulinic acid ***4.46***. The product **4.46** was obtained as yellowish solid, 150 mg (79%) by the general procedure (orange ylide color) while the reaction time was 18 h: m. p. 165–168 °C; $[\alpha]_D^{22}$ +4° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.51–7.42 (m, 1H, arom. ring), 7.30–7.26 (m, 1H, arom. ring), 7.10–7.04 (m, 1H, arom. ring), 6.70 (d, J = 16.2 Hz, 1H, H-31), 6.57 (d, J = 16.2 Hz, 1H, H-30), 5.12, 5.10 (2H, all s, 2 × H-29), 3.27–3.16 (m, 2H, H-19β and H-3α), 2.21–2.14 (m, 1H), 2.01 (dd, J_1 = 12.8, J_2 = 7.8 Hz, 1H), 1.89 (t, J = 11.3 Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.90, 157.42 (d, J_{CF} = 249.5 Hz), 152.30, 135.10 (d, J_{CF} = 3.8 Hz), 132.97, 128.41 (d, J_{CF} = 3.6 Hz), 126.19 (d, J_{CF} = 6.2 Hz), 125.69, 121.32 (d, J_{CF} = 17.9 Hz), 116.76 (d, J_{CF} = 21.5 Hz), 113.74, 79.18, 56.56, 55.47, 50.93, 50.62, 42.53, 40.86, 39.95, 38.99, 38.84, 38.68, 37.34, 37.01, 34.50, 33.29, 32.33, 29.96, 28.14, 27.46, 27.23, 21.17, 18.39, 16.26 (2C), 15.47, 14.91 ppm; **HRMS** (ESI^-) m/z calcd for $C_{37}H_{49}O_3ClF$ $[M-H]^-$ 595.3349, found 595.3354; **IR** (DRIFT) ν_{max} 3438, 2939, 2867, 1688, 1597, 1498, 1451 cm^{-1}.

4.1.5.38. 30-(4-Bromo-2-fluorobenzylidene)betulinic acid ***4.47***. The product **4.47** was obtained as a white solid, 196 mg (96%) by the general procedure (orange ylide color), while the reaction time was

6 h: m. p. 177–180 °C; $[\alpha]_D^{22}$ −8° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.40–7.35 (m, 1H, arom. ring), 7.25–7.19 (m, 2H, arom. ring), 6.83 (d, J = 16.4 Hz, 1H, H-31), 6.74 (d, J = 16.4 Hz, 1H, H-30), 5.15, 5.13 (2H, all s, 2 × H-29), 3.25 (td, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-19β), 3.18 (dd, J_1 = 11.4, J_2 = 4.7 Hz, 1H, H-3α), 2.38–2.31 (m, 1H), 2.23–2.17 (m, 1H), 2.02 (dd, J_1 = 12.7, J_2 = 7.9 Hz, 1H), 1.90 (t, J = 11.4 Hz, 1H), 1.00 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.52, 160.11 (d, J_{CF} = 254.0 Hz), 152.55, 134.57, 128.23 (d, J_{CF} = 4.2 Hz), 127.60 (d, J_{CF} = 3.2 Hz), 124.84 (d, J_{CF} = 12.1 Hz), 120.77 (d, J_{CF} = 9.7 Hz), 119.61 (d, J_{CF} = 4.4 Hz), 119.39, 114.17, 79.18, 56.53, 55.49, 50.78, 50.64, 42.55, 40.87, 40.29, 39.01, 38.85, 38.65, 37.36, 37.03, 34.51, 33.11, 32.35, 29.94, 28.14, 27.51, 27.22, 21.16, 18.42, 16.24 (2C), 15.48, 14.89 ppm; **HRMS** (ESI^-) *m/z* calcd for $C_{37}H_{49}O_3BrF$ $[M-H]^-$ 639.2844, found 639.2852; **IR** (DRIFT) ν_{max} 3655, 3450, 3255, 2930, 2867, 2615, 1963, 1727, 1687, 1596, 1562, 1481, 1462, 1450 cm^{-1}.

4.1.5.39. 30-(Pentafluorobenzylidene)betulinic acid ***4.48***. The product **4.48** was obtained as white solid, 160 mg (79%) by the general procedure (yellow ylide color) while the reaction time was 18 h: m. p. 167–170 °C; $[\alpha]_D^{22}$ −12° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.05 (d, J = 16.7 Hz, 1H, H-31), 6.55 (d, J = 16.7 Hz, 1H, H-30), 5.21, 5.20 (2H, all s, 2 × H-29), 3.27–3.16 (m, 2H, H-19β and H-3α), 2.34 (dd, J_1 = 9.7, J_2 = 3.0 Hz, 1H), 2.03 (dd, J_1 = 12.8, J_2 = 7.8 Hz, 1H), 1.87 (t, J = 11.3 Hz, 1H), 1.00 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H), 0.81 (s, 3H), 0.75 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.50, 152.32, 146.00–145.68 (m, 1C), 144.01–143.71 (m, 1C), 140.71, 140.16–139.72 (m, 1C), 139.11–138.45 (m, 1C), 137.06–136.68 (m, 1C), 115.77, 112.99–112.68 (m, 1C), 112.64, 79.18, 56.51, 55.50, 50.79, 50.63, 42.56, 40.87, 40.39, 39.01, 38.86, 38.64, 37.36, 37.01, 34.51, 32.82, 32.30, 29.91, 28.14, 27.50, 27.19, 21.14, 18.41, 16.22 (2C), 15.46, 14.85 ppm; **HRMS** (ESI^-) *m/z* calcd for $C_{37}H_{46}O_3F_5$ $[M-H]^-$ 633.3362, found 633.3372; **IR** (DRIFT) ν_{max} 3682, 3461, 3271, 2934, 2867, 2617, 1731, 1639, 1520, 1494, 1465 cm^1.

4.1.5.40. 30-(3,5-Dimethoxybenzylidene)betulinic acid ***4.49***. The product **4.49** was obtained as white solid, 54 mg (28%) by the general procedure (red ylide color) while the reaction time was 18 h: m. p. 158–161 °C; $[\alpha]_D^{22}$ −1° (*c* 0.29, THF); **^{1}H NMR** (500 MHz, $CDCl_3$) δ 6.76 (d, J = 16.2 Hz, 1H, H-31), 6.65–6.58 (m, 3H, H-30 and 2 × H − arom. ring), 6.38–6.35 (m, 1H, arom. ring), 5.11, 5.07 (2H, all s, 2 × H-29), 3.81 (s, 6H, 2 × methoxy $-CH_3$), 3.26 (td, J_1 = 11.1, J_2 = 4.7 Hz, 1H, H-19β), 3.18 (dd, J_1 = 11.4, J_2 = 4.7 Hz, 1H, H-3α), 2.38–2.32 (m, 1H), 2.22–2.15 (m, 1H), 2.01 (dd, J_1 = 12.8, J_2 = 7.8 Hz, 1H), 1.90 (t, J = 11.3 Hz, 1H), 1.01 (s, 3H), 0.96 (s, 3H), 0.94 (s, 3H), 0.80 (s, 3H), 0.74 (s, 3H) ppm; **^{13}C NMR** (126 MHz, $CDCl_3$) δ 181.43, 161.08, 152.60, 139.76, 132.24, 128.16, 113.08, 104.72, 100.06, 79.18, 56.56, 55.52, 50.90, 50.65, 42.55, 40.87, 40.17, 39.01, 38.85, 38.70, 37.36, 37.05, 34.52, 33.27, 32.37, 29.99, 28.14, 27.51, 27.16, 21.19, 18.41, 16.28, 16.25, 15.47, 14.93 ppm; **HRMS** (ESI^+) *m/z* calcd for $C_{39}H_{57}O_5$ $[M+H]^+$ 605.4201, found 605.4205; **IR** (DRIFT) ν_{max} 3449, 2938, 2867, 1687, 1589, 1455 cm^{-1}.

4.2. Biology

4.2.1. Cytotoxic MTS assay

MTS assay was performed at the Institute of Molecular and Translational Medicine by a robotic platform (HighResBiosolutions) in the same manner as in our previous work [21]. HCT116 and HCT116 $p53^{-/-}$ cell lines were obtained from Horizon Discovery Ltd., CCRF-CEM, K562, A549, U2OS, BJ, MRC-5 cell lines were obtained from ATCC (American Type Culture Collection), and resistant cancer cell lines CEM-DNR and K562-TAX were developed in our laboratory according to our procedure described in Ref. [44]. Cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (25,000–35,000 cells/mL based on cell growth characteristics). Cells were added by automatic pipettor (30 μL) into 384 well microtiter plates. All tested compounds were dissolved in 100% DMSO and four-fold dilutions of the intended test concentration were added in 0.15 μL aliquots at time zero to the microtiter plate wells by the echoacustic noncontact liquid handler Echo550 (Labcyte). The experiments were performed in technical duplicates and three biological replicates at least. The cells were incubated with the tested compounds for 72 h at 37 °C, in a 5% CO_2 atmosphere at 100% humidity. At the end of the incubation period, the cells were assayed by using the MTS test. Aliquots (5 μL) of the MTS stock solution were pipetted into each well and incubated for an additional 1–4 h. After this incubation period, the optical density (OD) was measured at 490 nm with an Envision reader (PerkinElmer). Tumor cell survival (TCS) was calculated by using the following equation:

$$TCS = \frac{OD_{drug-exposed\ well}}{mean\ OD_{control\ wells}} \cdot 100\ \% \quad (1)$$

The IC_{50} value, the drug concentration that is lethal to 50% of the tumor cells, was calculated from the appropriate dose-response curves in Dotmatics software.

4.2.2. Cell cycle and apoptosis analysis

This part was performed in the same manner as in our previous work [21]. Suspension of CCRF-CEM cells, seeded at a density of 10^6 cells/mL in 6-well panels, were cultivated with 1 × or 5 × IC_{50} of the tested compound in a humidified CO_2 incubator at 37 °C in RPMI 1640 cell culture medium containing 10% fetal calf serum, 10 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Together with the treated cells, a control sample containing the vehicle was harvested at the same time point after 24 h. After another 24 h, cells were then washed with cold PBS and fixed in 70% ethanol added dropwise, and stored overnight at −20 °C. Afterward, cells were washed in hypotonic citrate buffer, treated with RNAse (50 μg/mL), and stained with propidium iodide. The flow cytometer using a 488 nm single beam laser (FACSCalibur, Becton Dickinson) was used for measurement. Cell cycle was analyzed in the program ModFitLT (Verity), and apoptosis was measured in a logarithmic model expressing a percentage of the particles with propidium content lower than cells in the G0/G1 phase (<G1) of the cell cycle. Half of the sample was used for $pH3^{Ser10}$ antibody (Sigma) labeling and subsequent flow cytometry analysis of mitotic cells [45].

4.2.3. BrdU incorporation analysis (DNA synthesis)

This part was performed in the same manner as in our previous work [21]. For this analysis, the same procedure of cultivation as previously was used. Before harvesting, 10 μmol/L 5-bromo-2-deoxyuridine (BrdU) was added to the cells for puls-labeling for 30 min. Cells were fixed with ice-cold 70% ethanol and stored overnight. Before the analysis, cells were washed with PBS and resuspended in 2 mol/L HCl for 30 min at room temperature to denature their DNA. Following neutralization with 0.1 mol/L $Na_2B_4O_7$ (Borax), cells were washed with PBS containing 0.5% Tween-20 and 1% BSA. Staining with primary anti-BrdU antibody (Exbio) for 30 min at room temperature in the dark followed. Cells were then washed with PBS and stained with secondary anti-mouse-FITC antibody (Sigma). Cells were then rewashed with PBS and incubated with propidium iodide (0.1 mg/mL) and RNAse A (0.5 mg/mL) for 1 h at room temperature in the dark and afterward analyzed by flow cytometry using a 488 nm single beam laser (FACSCalibur, Becton Dickinson) [45].

4.2.4. BrU incorporation analysis (RNA synthesis)

This part was performed in the same manner as in our previous work [21]. Cells were cultured and treated as above. Before harvesting, pulse-labeling with 1 mmol/L 5-bromouridine (BrU) for 30 min followed. The cells were then fixed in 1% buffered paraformaldehyde with 0.05% of NP-40 at room temperature for 15 min, and then stored in 4 °C overnight. Before measurement, they were washed with PBS with 1% glycine, washed in PBS again, and stained by primary anti-BrdU antibody cross-reacting to BrU (Exbio) for 30 min at room temperature in the dark. After another washing step in PBS, cells were stained by a secondary anti-mouse-FITC antibody (Sigma). Following the staining, cells were washed with PBS and fixed with 1% PBS buffered paraformaldehyde with 0.05% of NP-40 for 1 h. Cells were washed by PBS, incubated with propidium iodide (0.1 mg/mL) and RNAse A (0.5 mg/mL) for 1 h at room temperature in the dark, and finally analyzed by flow cytometry using a 488 nm single beam laser (FACSCalibur, Becton Dickinson) [45].

4.2.5. Annexin V/propidium iodide staining

Apoptosis in CCRF-CEM cells was assessed using Annexin V/propidium iodide double staining as described in detail elsewhere [21]. Briefly, cells were treated with **4.22** or **4.39** derivatives at 1 × IC_{50} and 5 × IC_{50} concentrations for 24 h. Following washing by ice-cold PBS, cells were incubated with Annexin V-FITC (5 μL) and PI (5 μL) at room temperature for 20 min in the dark. After the incubation period, cells were centrifuged, resuspended in 100 μl of annexin binding buffer (10 mmol/L HEPES/NaOH of pH = 7.4, 140 mmol/L NaCl, 2.5 mmol/L $CaCl_2$) and analyzed by FACSAria II (Becton Dickinson) flow cytometer. At least 10,000 cells per sample were acquired.

4.2.6. Western blot

Western blot analysis was performed in the same manner as we described earlier [21]. CCRF-CEM cells were treated with the most active derivatives **4.22** and **4.39** at 1 × IC_{50} and 5 × IC_{50} concentrations for 24 h. Total proteins were extracted from the cells with RIPA lysis buffer (50 mmol/L Tris-HCl of pH = 8.0, 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) supplemented with protease inhibitor cocktail (Roche) and incubated for 30 min on ice with occasional agitation. The lysates were clarified by centrifugation (14,000*g*, 20 min, 4 °C) and protein samples were quantified with a BCA protein assay. Aliquots of lysates were mixed with 4 × concentrated Laemmli buffer and boiled at 95 °C for 5 min. An equal amount (15 μg/well) of protein samples were separated by 10% SDS-PAGE and transferred onto a 0.2-μm pore size nitrocellulose membrane. The membrane was then blocked with 5% skimmed milk in Tris buffer saline Tween-20 (TBST) buffer for 1 h at room temperature and probed with specific primary antibodies (Cell Signaling Technology) at 4 °C overnight. Primary antibodies were diluted in TBST at the ratio of 1 : 1000. After four cycles (5 min each) of washing with TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (mouse or rabbit; Sigma-Aldrich) for 1 h at room temperature. The chemiluminescence signal was visualized with the ECL Prime kit (Amersham) and detected by Li-cor Odyssey (LI-COR Biotechnology) imaging system. As an internal control, an anti-β-actin (Sigma-Aldrich) primary antibody at the 1 : 5000 dilution was used.

4.2.7. Pharmacological parameters

Same instruments and methods for the measurements of pharmacological parameters were used as described in our earlier work [21]. Stability in human plasma, microsomal stability assay, parallel artificial membrane permeability assay, and protein plasma binding assay were performed in the same manner as we described earlier [21].

4.2.7.1. Cell cultures. Caco-2 and MDCK-MDR1 cells were maintained in DMEM at pH = 7.4, supplemented with 10% fetal bovine serum and 0.1% penicillin-streptomycin solution in a humidified atmosphere (5% CO_2, 95% air, 37 °C). The cells were seeded on tissue culture polyester membrane filters (pore size 0.4 μm for Caco-2 and 1 μm for MDCK-MDR1) in 96-well Transwell® plates (Corning). The culture medium was added to both the donor and the acceptor compartment. The medium was changed every second day.

4.2.7.2. Transport studies. Caco-2 and MDCK-MDR1 monolayers grown in Transwell® (96-well) plates [37,38] and were used for transport studies when they had differentiated and the monolayer was intact, as checked by Lucifer Yellow Rejection Assay. Prior to the experiment, the cells were washed twice with HBSS (Gibco) and pre-equilibrated for 1 h with HBSS buffered at pH = 7.4. After removing the medium, the cells were treated with the studied compound (10 μmol/L in HBSS, pH = 7.4) for 1 h for MDCK, and 2 h for Caco-2, respectively. After incubation, followed by lyophilization, the samples were dissolved in a mobile phase containing internal standard and analyzed using an Agilent RapidFire 300 High-Throughput Mass Spectrometry System (Agilent) with subsequent detection on the mass spectrometer Qtrap 5500 (AB Sciex). All experiments were done in duplicates. The apparent permeability coefficient P_{app} (cm/s) was calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{C_0 A} \quad (2)$$

where dQ/dt is the rate of permeation of the drug across the cells (μmol/s), C_0 is the donor compartment concentration at time zero (μmol/L), and A is the area of the cell monolayer (cm^2). C_0 is obtained from the analysis of the dosing solution at the start of the experiment.

The efflux ratio *ER* was calculated using the following equation:

$$ER = \frac{P_{app\ (BA)}}{P_{app\ (AB)}} \quad (3)$$

where $P_{app\ (BA)}$ and $P_{app\ (AB)}$ represent the apparent permeability of the test compound from the basal to apical (B–A) and apical to the basal (A–B) side of the cell monolayer. The compounds having an efflux ratio $ER > 2$ are considered as having potential for P-gp substrate.

The compound recovery can be helpful to interpret permeability data (Equation (4)). If the recovery is very low, this may indicate poor solubility, binding of the compound to the hydrophobic plate, metabolic degradation by cells, or accumulation of the compound in the cell monolayer.

$$\text{Recovery} = \frac{\text{Total compound in donor and receiver at the end of the experiment (nmol)}}{\text{Initial compound present (nmol)}} \cdot 100\,\% \quad (4)$$

For cell permeability assays, reference drugs with established P_{app} and efflux values are used. In Caco-2 cell experiments, three categories are differentiated according to P_{app} values: low ($P_{app} < 5 \cdot 10^{-6}$ cm/s), moderate ($5 \cdot 10^{-6} < P_{app} < 20 \cdot 10^{-6}$ cm/s) and high ($P_{app} > 20 \cdot 10^{-6}$ cm/s). The low P_{app} standard is rhodamine 123, while the high P_{app} reference is verapamil [46]. In MDCK-MDR1 cell assays atenolol was used as a standard for low P_{app} ($P_{app} < 10 \cdot 10^{-6}$ cm/s) and propranolol for high P_{app} reference ($P_{app} > 10 \cdot 10^{-6}$ cm/s) [47].

Author contributions

Conceptualization, J.P., M.U., P.D., and M.H.; methodology, J.P., S.B., J.Š., I.F., J.K., B.L. and S.G.; validation, P.D., and M.U.; formal analysis, M.U.; investigation, J.P., D.O., S.G., and S.B.; resources, P.D., M.H., J.Š., and M.U.; writing – original draft preparation, J.P., P.D. and M.U.; writing – review and editing, P.D., S.B. and M.U.; funding acquisition, P.D., M.H., and M.U.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2021.113706.

Abbreviations

A549	Lung carcinoma cell line
ACN	Acetonitrile
ADME	Absorption, distribution, metabolism, and excretion
APCI	Atmospheric-pressure chemical ionization
BAX	Bcl-2 associated X protein
BCA	Bicinchoninic acid assay
Bcl-2	B-cell lymphoma family
BJ	Human noncancer fibroblast cell line
BrdU	5-Bromo-2-deoxyuridine
BrU	5-Bromouridine
BSA	Bovine serum albumin
Caco-2	Colorectal adenocarcinoma cell line
CCRF-CEM	T-lymphoblastic leukemia cell line
CEM-DNR	T-lymphoblastic leukemia cell line resistant to daunorubicin
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRIFT	Diffuse reflectance infrared Fourier transform
ESI	Electrospray ionization
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
G0	Gap 0 phase of cell cycle
G1	Gap 1 phase of cell cycle
G2	Gap 2 phase of cell cycle
HBSS	Hank's balanced salt solution
HCT116 $p53^{-/-}$	Colon carcinoma cell line
HCT116	Colon carcinoma cell line
HIV	Human immunodeficiency virus
HRMS	High-resolution mass spectrometry
IC_{50}	Half maximal inhibitory concentration
IR	Infrared
K562	Chronic myelogenous leukemia cell line
K562-TAX	Chronic myelogenous leukemia cell line resistant to paclitaxel
LC	Liquid chromatography
M	Mitotic phase of cell cycle
m. p.	Melting point
MDCK	Madin-Darby canine kidney cell line
MDR	Multiple drug resistance
MRC-5	Human noncancer fibroblast cell lines
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, tetrazolium salt
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
NP-40	Nonyl phenoxypolyethoxylethanol, commercially available detergent
OD	Optical density
PAMPA	Parallel artificial membrane permeability assay
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline buffer
P-gp	P-glycoprotein
pH	Potential of Hydrogen
$pH3^{Ser10}$	Phospho-histone H3 Serin10
PI	Propidium iodide
r. t.	Room temperature
RFU	Relative fluorescence unit
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RPMI 1640	Growth medium used in cell culture
S	Synthesis phase of cell cycle
SAR	Structure-activity relationship
SDS-PAGE	Sodium dodecylsulphate – polyacrylamide gel electrophoresis
SSC	Side scatter
TBST	Tris buffer saline Tween-20
t-BuOK	Potassium *tert*-butoxide
TCS	Tumor cell survival
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
U	Unit
U2OS	Osteosarcoma cell line
UV	Ultraviolet

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