

Article

Betulin-Hippuric Acid Conjugates: Chemistry, Antiproliferative Activity and Mechanism of Action

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Abstract

The structure of betulin enables the formation of conjugates that offer improved activity, selectivity, or pharmacokinetic parameters. It was assumed that combining betulin with hippuric acid could produce a product with favorable biological properties. The bond connecting the conjugate elements was an ester group introduced using a method ensuring mild reaction conditions (Steglich method). In this way, betulin and its acetyl derivatives were converted into conjugates with hippuric acid, with good yields. The obtained compounds were assessed for their in vitro antiproliferative activity against seven different human cancer cell lines (MTT and SRB assays), preceded by in silico prediction (PASS online). Lipophilicity ($\log P_{TLC}$), a significant parameter influencing all stages of the ADME process, was experimentally determined using RP-TLC. $\log P_{TLC}$ values were compared with $\log P$ results obtained from available online computational programs. Antiproliferative activity studies demonstrated the significant sensitivity of MV4-11 cells to the tested compounds. The IC_{50} values ranged from 4.2 to 31.4 μM . The mechanism of anticancer action was investigated for the most active derivatives **4**, **5**, and **7**. For derivative **7**, molecular docking revealed the highest affinity for the FLT3 protein binding site.

Keywords: triterpenoids; betulin; antiproliferative activity; apoptosis

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1. Introduction

Despite the rapid development of new therapeutic strategies, cancer remains the leading cause of death worldwide. Cancer is a pathological condition characterized by uncontrolled cell growth and proliferation resulting from genetic mutations. In 2020, 19.3 million new cancer cases were recorded, with approximately 10 million deaths due to cancer. Commonly used cancer treatment methods include radiotherapy, chemotherapy, and surgery. A major challenge in the search for effective therapeutics is overcoming cancer cell resistance and obtaining agents with low toxicity and high selectivity. An opportunity to obtain compounds that meet these requirements lies in the use of substances of natural origin, which often exhibit anticancer activity. Natural compounds belonging to groups such as alkaloids, taxanes, and flavonoids have been used in medicine for years. Natural products can be used as independent therapeutic agents or as adjuncts to existing chemotherapeutic agents (reducing their toxicity or demonstrating synergistic effects). The chemical structures of natural compounds also provide models for the development of new drugs with similar or improved properties [1]. The use of in silico methods for the initial

assessment of their biological potential is a great support in the process of developing new drugs. The first evaluation criteria used are the rules of drug similarity [2–4]. Unfortunately, rigorous application of these rules, e.g., in relation to molecular weight, can result in premature rejection of some molecules. An opportunity to use such compounds is to conduct broader bioinformatic studies that allow for the prediction of physical and chemical parameters and the interaction of the molecule with the receptor [5].

Among natural compounds, betulin, a pentacyclic triterpene of the lupane type, has attracted considerable research interest. Although this compound has been isolated from over twenty plants belonging to various genera and families, its primary source is birch bark. This raw material contains the highest concentration of betulin (up to 40%) and is readily available in large quantities [6].

The potential biological activity of a new chemical compound results from its structure, i.e., the type of basic skeleton and the presence and method of connecting various functional groups. The introduction of an ester group can improve the solubility, bioavailability, and absorption of the drug substance. The ester functional group is used in the synthesis of prodrugs. The use of such a drug form allows for targeted delivery of the parent drug, the side effects and toxicity of which are reduced, and the active substance is released after absorption or even only after reaching the target site [7]. The human body contains enzymes called esterases that hydrolyze ester bonds, which contributes to the activation of the prodrug and its conversion to the active form at a given place in the body and at the right time [8]. An ester bond is often found in biologically active compounds of natural origin. In the process of developing new drugs, an ester group can be introduced into the molecule using various synthetic methods, as described in the literature [9].

Many chemical modifications of betulin, as well as betulinic acid, are based on the transformation of functional groups present in the structure into ester bonds, enabling further expansion of the molecule and the introduction of new pharmacophores [6]. The new derivatives obtained in this way were subjected to studies aimed at determining their biological activity for specific applications. In this way, compounds with antioxidant, antibacterial, hepatoprotective, and antiviral activity and a large group of molecules active against various types of cancers were synthesized. Examples of ester derivative structures and their types of activity are shown in Figure 1 [10–21].

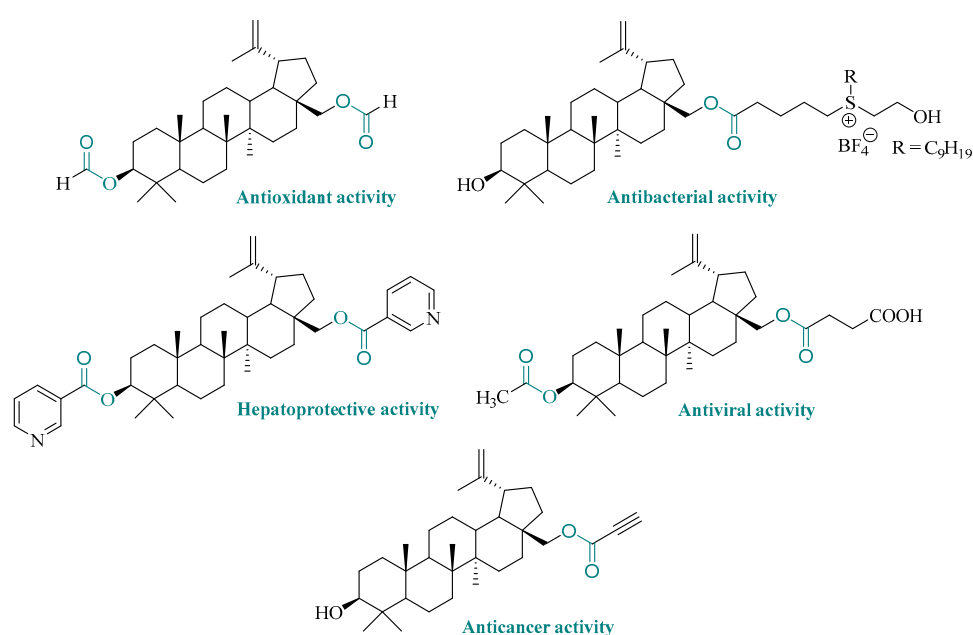


Figure 1. Biological activity of betulin mono- and diester derivatives.

The studies we conducted aimed to obtain new compounds with potential anticancer activity by introducing a hippuric acid fragment into the betulin system through esterification of the hydroxyl groups present in the substrate. In the initial stage of the study, the lipophilicity parameter for the synthesized compounds was determined experimentally and using *in silico* computational methods. *In silico* analysis also allowed us to predict the group of tumors most susceptible to the effects of the designed structures. The next stage of the research involved assessing the anticancer activity on selected cancer cell lines. Mechanistic studies were conducted on the cells most sensitive to the tested compounds. This work was complemented by docking studies to assess the molecular interactions and affinity of the betulin hipuronates as potential tyrosine kinase inhibitors.

2. Materials and Methods

2.1. General Techniques

Silica gel 60 F₂₅₄ plates (Merck, Darmstadt, Germany) were used for thin-layer chromatography (TLC). To purify the compounds, a chromatography column with silica gel 60 (0.063–0.200 mm, Merck, Darmstadt, Germany) was applied. The visibility of compound spots in the chromatograms was achieved by staining with a 10% H₂SO₄ solution in ethanol. Melting points (mp) were determined using an Electrothermal IA 9300 apparatus and were not corrected. Nuclear magnetic resonance spectra (¹H NMR and ¹³C NMR) were obtained by a Bruker Avance III 600 (Bruker Corporation, Billerica, MA, USA) in a CDCl₃ solution. High-resolution mass spectra (HRMS) were recorded on a Bruker Impact II (Bruker Corporation, Billerica, MA, USA). The chemical reagents used were produced by Merck (Darmstadt, Germany), Chempur (Piekary Śląskie, Poland), and Avantor Performance Materials Poland S.A. (Gliwice, Poland). Compounds 1–3 were synthesized by the procedures reported in the literature [22–24].

2.2. Synthesis of Betulin–Hippuric Acid Conjugates 4–6 (General Procedure)

The starting compound (1 mmol betulin or compound 2 or compound 3) and 0.21 g (1.20 mmol) of hippuric acid were dissolved in 6 mL of dichloromethane. The mixture was cooled to −10 °C, and a solution of 0.27 g (1.42 mmol) *N,N'*-dicyclohexylcarbodiimide (DCC) and 0.03 g (0.24 mmol) 4-dimethylaminopyridine (DMAP) in 3 mL of dichloromethane was added dropwise. Then, the reaction mixture was slowly brought to room temperature and stirred for 24 h. The formed dicyclohexylurea was filtered off under reduced pressure and washed with dichloromethane (5 mL). The filtrate was concentrated on a rotary evaporator. Column chromatography (dichloromethane/ethanol: 60:1 *v/v*) was used to isolate the final product. Conjugates 4–6 were obtained in 53–75% yields. Spectroscopic data for compounds 4–6 and their spectra (¹H NMR, ¹³C NMR and HRMS) are provided in the Supplementary Materials (Figures S1–S9).

2.3. Synthesis of Compound 7

In a round-bottomed flask equipped with a magnetic stirrer, 0.30 g (0.45 mmol) of compound 4 was dissolved in 7 mL of dichloromethane. Then 0.15 g (0.69 mmol) of pyridinium chlorochromate (PCC) was added. The mixture was stirred for 2 h at room temperature. After this time, the reaction mixture was filtered under reduced pressure, and the dichloromethane was distilled off using a rotary vacuum evaporator. The crude product was purified by column chromatography (dichloromethane/ethanol: 60:1 *v/v*). Compound 7 was obtained in a 67% yield. Spectroscopic data for compound 7 and its spectra (¹H NMR, ¹³C NMR, and HRMS) are provided in the Supplementary Materials (Figures S10–S12).

2.4. Lipophilicity Study

Reversed-phase thin-layer chromatography (RP-TLC) was used to determine the experimental parameter of lipophilicity ($\log P_{\text{TLC}}$). The method of sample preparation and a detailed description of the procedure were presented in a previous publication [25].

The equation of the calibration curve (dependence of $\log P_{\text{lit}}$ on R_{M0} of reference compounds) and the reference compounds (Table S1) used in the lipophilicity studies are presented in the Supplementary Materials.

2.5. In Silico Study

The first step of the in silico studies was to convert the chemical structures of the compounds into SMILES (Simplified Molecular Linear Input System) codes using Chem Draw (Perkin Elmer Informatics, Waltham, MA, USA). The predictions of the anticancer activity of the studied compounds were calculated using the PASS software [26].

2.6. Biological Evaluation

2.6.1. Biological Materials and Assays

The human cancer cell lines MV4-11 (leukemia; CRL-9591), MiaPaca-2 (pancreatic; CRL-1420), Hs294T (melanoma; HBT-140), and HCT116 (colon; CCL-247) and normal epithelial cells from the mammary gland (MCF-10A; CRL-10317) were obtained from American Type Culture Collection (Manassas, VA, USA), and A549 (lung, 86012804), MCF-7 (breast; 86012803), and PC-3 (prostate; 90112714) were obtained from European Collection of Authenticated Cell Cultures (Salisbury, UK). All the cell lines were maintained at the Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wrocław, Poland).

MV4-11 and PC-3 cells were cultured in RPMI 1640 with 1.0 mM sodium pyruvate (only MV4-11) and 10% fetal bovine serum (FBS). A549 cells were cultured in RPMI 1640 + Opti-MEM (1:1) supplemented with 5% FBS. The MCF-7 cells were cultured in Eagle medium supplemented with 10% FBS, 8 $\mu\text{g/mL}$ of insulin, and 1% of MEM non-essential amino acid. Hs294T cells were cultured in RPMI 1640 + Opti-MEM (1:1) supplemented with 10% FBS and 4.5 g/L glucose. HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% FBS. MiaPaca-2 cells were cultured in Dulbecco's medium supplemented with 10% FBS and 2.5% HS (horse serum). MCF-10A cells were cultured in Ham's F12 medium supplemented with 7.5% HS, 20 ng/mL of EGFh, 10 $\mu\text{g/mL}$ of insulin, 0.5 $\mu\text{g/mL}$ of hydrocortisone, and 0.05 mg/mL of cholera toxin (from *Vibrio cholerae*). All culture media were supplemented with 2 mM L-glutamine, 100 units/mL penicillin, and 100 $\mu\text{g/mL}$ streptomycin. All cell lines were grown at 37 °C in a 5% CO₂ humidified atmosphere.

2.6.2. Determination of Antiproliferative Activity

The tested compounds were dissolved in DMSO (dimethyl sulfoxide) to a concentration of 10 mg/mL and then diluted in culture medium to reach the final concentrations (100–0.03 $\mu\text{g/mL}$). The cells were plated in 384-well plates or in 96-well plates (MV4-11 cells). After 24 h of incubation, the tested compounds were added for 72 h of exposure. The cytotoxic effect was examined using the MTT (MV4-11) or SRB assay, as described previously [27]. The values of IC₅₀ (inhibitory concentration 50%) were calculated by using the Prolab-3 system based on Cheburator 0.4 software [27]. Doxorubicin was applied as a reference drug. The IC₅₀ values in $\mu\text{g/mL}$ were converted to μM .

2.6.3. Apoptosis Studies Using Annexin V

The leukemia MV4-11 cells were seeded on 12-well plates and exposed for 24 or 72 h to the following compounds: **4** at 27 μ M, **5** at 15 μ M, and **7** at 6 μ M (about $1.5 \times \text{IC}_{50}$ value). Camptothecin (0.05 μ M) was used as a positive control. After incubation, the cells were collected, counted, washed with PBS, and then suspended in Hepes binding buffer with APC-Annexin V (BD Pharmingen, Franklin Lakes, NJ, USA). After 15 min of incubation, propidium iodide (PI) solution was added (5 μ g/mL). Cell analysis was performed by flow cytometry using a BD LSRFortessa cytometer (BD Bioscience, San Jose, CA, USA), and double-negative cells were live cells, PI+/AnV+ cells were late apoptotic cells, PI−/AnV+ cells were early apoptotic cells, and PI+/AnV− cells were necrotic cells. Results were analyzed using Flowing software 2.5.1 (Turku, Finland).

2.6.4. Cell Cycle Study

The leukemia MV4-11 cells were seeded on 6-well plates and exposed (24 or 72 h) to the following compounds: **4** at 27 μ M, **5** at 15 μ M, and **7** at 6 μ M (about $1.5 \times \text{IC}_{50}$ value). After incubation, the cells were collected, counted, washed in PBS, and then fixed for 24 h in 70% ethanol at -20°C . Then, the cells were washed with PBS, incubated with RNase (8 μ g/mL, Thermo Scientific, Waltham, MA, USA) at 37°C for 1 h, and then stained for 30 min with PI (50 μ g/mL) at 4°C . The cellular DNA content was analyzed by flow cytometry using a BD LSRFortessa cytometer, and the results were analyzed using Flowing software 2.5.1 (Turku, Finland).

2.6.5. Statistical Analysis

Kruskal–Wallis tests (using GraphPad Prism 7) were performed to indicate significant differences in the comparison of two groups (control vs. study). A statistically significant difference was considered at $p \leq 0.05$.

3. Results and Discussion

3.1. Chemistry

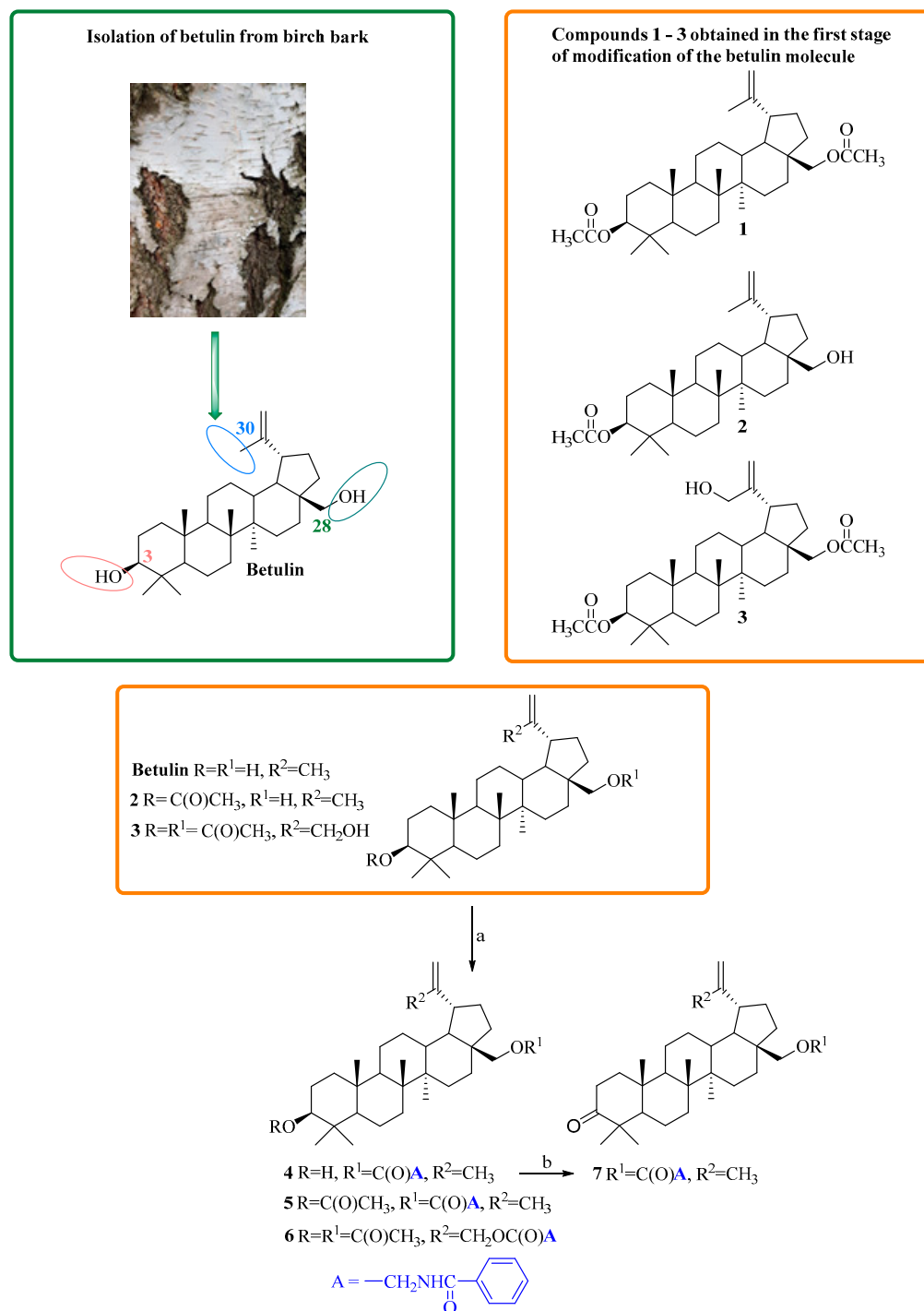
Betulin (Scheme 1) is a lupane-type diol triterpenoid whose presence has been confirmed in various morphological parts of many plant species, such as *Pyrola decorata* H. (leaves), *Pterodon emarginatus* (stem bark), *Matayba elaeagnoides* (bark), and *Xanthium sibiricum* (roots). However, betulin is mostly obtained from birch bark (*Betula pendula*, *Betula pubescens*, *Betula platphylla*, *Betula alba*, *Betula lente*) by extraction using various organic solvents [28–30].

The two hydroxyl groups and the isopropenyl group present in the betulin structure allowed for chemical modifications at the positions C-3, C-28, and C-30, leading to the formation of acetylated derivatives **1–3** (Scheme 1). The chemical structures of the obtained triterpenoids **1–3** were determined based on the analysis of NMR spectra (^1H , ^{13}C) and then compared with the relevant literature data [22–24].

One method often used to introduce an ester bond into natural products is the Steglich reaction, involving the use of a coupling agent. Steglich esterification occurs under mild conditions and allows for the conversion of an alcohol and an acid into an ester in a relatively high yield. The conditions for this method involve the use of *N,N'*-dicyclohexylcarbodiimide (DCC) as a coupling agent and a catalytic amount of 4-dimethylaminopyridine (DMAP) in a suitable organic solvent (dichloromethane, tetrahydrofuran, dimethylformamine, or acetonitrile) [9,31].

Betulin esters **4–6** were obtained from the reaction of betulin and triterpenoids **2–3** with hippuric acid through the Steglich method. The hippurate derivative **7** was formed through

the oxidation of the monoester **4** with pyridinium chlorochromate (PCC) in anhydrous dichloromethane (Scheme 1).



Scheme 1. Synthesis of betulin derivatives bearing a hippuric acid moiety **4–7**. Reagents and conditions: a. hippuric acid, *N,N'*-dicyclohexylcarbodiimide (DCC), 4-dimethylaminopyridine (DMAP), and dichloromethane from $-10\text{ }^{\circ}\text{C}$ to room temperature over 24 h; b. pyridinium chlorochromate (PCC) and dichloromethane at room temperature for 2 h. Photo by E. Bębenek.

3.2. Experimental and Theoretical Studies of Lipophilicity

Lipophilicity is a physicochemical parameter that determines the affinity of a compound to a lipophilic environment, which translates into its transport through biological membranes and the possibility of forming a compound–receptor complex. Lipophilicity is

related to the processes of absorption, distribution, metabolism, and excretion of substances, affecting their biological potential and toxic effects [32].

One of the methods used to determine the lipophilicity of compounds based on their retention coefficients is the reversed-phase chromatography technique, RP-TLC. The advantages of the RP-TLC method include the simplicity of the equipment and low analysis costs. In addition, the RP-TLC method has found a wide range of applications in the study of compounds characterized by higher lipophilicity [33].

In the conducted studies, the RP-TLC method was used to estimate the chromatographic lipophilicity parameters R_{M0} for betulin and compounds 2–7. Based on the calibration curve equation, the experimental $\log P_{TLC}$ values of betulin and its derivatives 2–7 were calculated. The determined experimental lipophilicity values for the tested triterpenoids (R_{M0} , $\log P_{TLC}$) are presented in Table 1.

Table 1. The experimental values of lipophilicity for betulin and its derivatives 2–7.

Compound	R_{M0}	r	b	$\log P_{TLC}$
betulin	5.07	0.993	−0.0573	6.46
2	6.24	0.995	−0.0685	7.82
3	5.75	0.997	−0.0652	7.25
4	5.33	0.988	−0.0623	6.76
5	6.63	0.989	−0.0753	8.27
6	6.04	0.994	−0.0699	7.58
7	5.41	0.996	−0.0627	6.85

The determined lipophilicity values ($\log P_{TLC}$) of the hippurate esters 4–6 are in the range of 6.76–8.27. The lowest value of the lipophilicity parameter in this group of betulin derivatives is characterized by compound 4 ($\log P_{TLC} = 6.76$), with a hydroxyl group at the C-3 position. In the case of compounds containing a hippurate acid moiety (2-(benzoylamino)acetyl group) at the C-28 position, lipophilicity increases in the following order: compound 4 (C-3-hydroxyl group) < compound 7 (C-3-carbonyl group) < compound 5 (C-3-acetyl group). The most lipophilic compound is the diester 5, with acetyl and (2-(benzoylamino)acetyl groups at the C-3 and C-28 position, respectively. It can be seen that the introduction of the 2-(benzoylamino)acetyl group at the C-30 position into the molecule 3 ($\log P_{TLC} = 7.25$) causes a slight increase in the lipophilicity of the hippurate derivative 6 ($\log P_{TLC} = 7.58$).

The theoretical lipophilicity values ($\log P$) of the tested triterpenoids were calculated using computational methods (iLogP, MLOGP, WLOGP, miLogP, CLogP, XLOGP3). The obtained $\log P$ values of the tested compounds ranged from 7.00 to 11.45, as presented in Table 2. The highest $\log P$ values for the hippurate derivatives of betulin were calculated using CLogP, while the lowest $\log p$ values were obtained based on iLogP.

Table 2. The theoretical $\log P$ parameters for betulin and its derivatives 2–7 calculated using the on-line Swiss ADME [34] and Molinspiration [35] databases and Chem Draw [36] software, version 12.

Compound	iLogP ^a	MLOGP ^a	WLOGP ^a	miLogP ^b	CLogP ^c	XLOGP3 ^a
betulin	4.47	6.00	7.00	7.16	8.53	8.28
2	4.74	6.20	7.57	7.87	9.47	8.86
3	4.91	5.60	7.11	7.33	8.63	8.18
4	5.03	6.09	7.98	8.39	10.15	9.86
5	5.47	6.35	8.55	8.78	11.10	10.44
6	6.12	5.83	8.09	8.50	10.26	9.76
7	4.86	6.01	8.19	8.26	9.75	9.54

^a from Swiss ADME; ^b from Molinspiration; ^c from Chem Draw.

3.3. In Silico Prediction of Betulin Derivative Activity

In silico biological activity studies of the hippurate derivatives of betulin **4–7** were conducted using the online tool PASS (Prediction of Activity Spectra for Substances). The PASS program predicts the biological activity of compounds based on two-dimensional chemical structures and molecular fragment descriptors [26]. The Pa values obtained from the program indicate the probability that the compound shows a specific activity, while the Pi values indicate the probability that the compound will be inactive in this direction. Pa values above 0.7 are most often considered the most favorable [37,38].

The prediction of the antiproliferative activity of triterpenoids **4–7** and doxorubicin is presented in Table 3 below.

Table 3. The predicted antiproliferative activity of triterpenoids **4–7** and doxorubicin determined by PASS online.

Activity	Pa (Pi)				
	4	5	6	7	Doxorubicin
Antineoplastic	0.871 (0.002)	0.901 (0.005)	0.900 (0.005)	0.881 (0.005)	0.960 (0.004)
Antineoplastic (lung cancer)	0.738 (0.005)	0.785 (0.004)	0.759 (0.005)	0.709 (0.005)	0.311 (0.038)
Antineoplastic (melanoma)	0.736 (0.004)	0.759 (0.004)	0.747 (0.004)	0.786 (0.003)	0.215 (0.049)
Antineoplastic (colorectal cancer)	0.693 (0.005)	0.783 (0.005)	0.796 (0.005)	0.730 (0.005)	-
Antineoplastic (colon cancer)	0.687 (0.005)	0.780 (0.005)	0.794 (0.005)	0.727 (0.005)	-
Antineoplastic (ovarian cancer)	0.613 (0.005)	0.666 (0.004)	0.584 (0.005)	0.602 (0.005)	-
Antineoplastic (breast cancer)	0.604 (0.010)	0.666 (0.007)	0.569 (0.013)	0.614 (0.009)	0.372 (0.037)
Antineoplastic (cervical cancer)	0.601 (0.004)	0.659 (0.004)	0.719 (0.004)	0.609 (0.004)	0.245 (0.028)
Antineoplastic (thyroid cancer)	0.579 (0.001)	0.621 (0.001)	0.570 (0.001)	0.558 (0.001)	-
Antineoplastic (endocrine cancer)	0.555 (0.002)	0.591 (0.002)	0.546 (0.002)	0.537 (0.002)	-
Prostate cancer treatment	0.432 (0.017)	0.475 (0.013)	0.517 (0.008)	0.448 (0.015)	-
Antileukemic	0.414 (0.023)	0.456 (0.018)	0.349 (0.034)	0.490 (0.015)	0.478 (0.016)
Antineoplastic (pancreatic cancer)	0.355 (0.030)	0.351 (0.031)	0.276 (0.079)	0.398 (0.017)	0.224 (0.143)
Antineoplastic (lymphocytic leukemia)	0.289 (0.019)	0.300 (0.017)	0.392 (0.009)	0.319 (0.015)	-
Antineoplastic (liver cancer)	0.250 (0.042)	-	-	0.187 (0.102)	0.257 (0.038)
Antineoplastic (squamous cell carcinoma)	0.177 (0.027)	0.199 (0.023)	0.141 (0.039)	0.207 (0.021)	0.200 (0.022)
Antineoplastic (glioblastoma multiforme)	-	0.200 (0.063)	0.185 (0.083)	0.201 (0.061)	-
Antineoplastic (renal cancer)	-	0.150 (0.113)	0.147 (0.119)	-	0.231 (0.026)
Antineoplastic (uterine cancer)	-	-	-	-	0.620 (0.003)
Antineoplastic (bladder cancer)	-	-	-	-	0.483 (0.003)
Antineoplastic (brain cancer)	-	-	-	-	0.317 (0.020)
Antineoplastic (sarcoma)	-	-	-	-	0.311 (0.015)

Through in silico analysis, using the PASS program, a preliminary assessment of the anticancer activity of the hippurate esters of betulin **4–7** was performed. The same study was conducted on doxorubicin, an anthracycline antibiotic that effectively inhibits various types of cancer (leukemia, lymphoma, sarcoma, genitourinary system cancers, ovarian cancer, breast cancer). The limitations of doxorubicin use in therapy result from its high toxicity during long-term use (cardiomyopathy, liver damage) [39,40]. In silico studies also indicate the high general anticancer potential of doxorubicin (Pa = 0.960); however, the probability (Pa) of its cytotoxic effect on selected types of cancer is in the range of 0.200–0.620. Analysis of betulin derivatives **4–7** using the PASS program indicates their potential activity against lung cancer, melanoma, colon cancer, ovarian cancer, breast cancer, cervical cancer, thyroid cancer, prostate cancer, leukemia, and pancreatic cancer (Table 3). The obtained results constitute a starting point for further studies on antitumor activity in the in vitro model of synthesized hippuronates.

3.4. Antiproliferative Activity

One possible way to modify natural compound molecules is to introduce an amino acid fragment via an ester linker. Conjugates of natural compounds with amino acids described in the literature have been designed to offer higher pharmacological activity, lower toxicity, improved selectivity, and more favorable pharmacokinetic parameters [41].

A beneficial effect of amino acid conjugation described for betulin is the improved aqueous solubility of the resulting conjugates [42]. Among the betulin amino acid esters, the highest activity against human epidermoid carcinoma cells (A431) was demonstrated by compounds containing a lysine and ornithine fragment (IC_{50} = 7–10 μ M) [43]. For lysine and ornithine derivatives, the lowest IC_{50} values of 2.46 μ M were determined after 72 h of incubation with malignant melanoma cells (Me-45) [42].

The new betulin–hippuric acid conjugates 4–7, as well as betulin and hippuric acid, were tested in vitro on one normal MCF-10A cell line and on seven human cancer cell lines, MV4-11, A549, MCF-7, PC-3, HCT116, MiaPaca-2, and Hs294T. The results of the antiproliferative activity of the tested compounds are presented in Table 4.

Table 4. Antiproliferative activity of hippuric acid, betulin, and its derivatives 4–7.

Compound	IC_{50} [μ M] $\pm$ SD						
	MV4-11	A549	MCF-7	PC-3	HCT116	MiaPaca-2	Hs249T
4	17.6 $\pm$ 4.5	>100	>100	>100	>100	>100	>100
5	9.7 $\pm$ 0.6	98.9 $\pm$ 21.8	>100	>100	92.9 $\pm$ 27.7	>100	>100
6	31.4 $\pm$ 4.3	>100	>100	>100	32.2 $\pm$ 2.1	>100	>100
7	4.2 $\pm$ 1.2	>100	>100	84.9 $\pm$ 31.2	89.5 $\pm$ 14.6	>100	96.5 $\pm$ 33.4
hippuric acid	>100	>100	>100	>100	>100	>100	>100
betulin	32.8 $\pm$ 12.9	14.0 $\pm$ 1.1	43.6 $\pm$ 15.8	51.5 $\pm$ 9.9	37.7 $\pm$ 7.5	>100	58.5 $\pm$ 15.6
doxorubicin	0.021 $\pm$ 0.008	0.040 $\pm$ 0.012	0.150 $\pm$ 0.015	1.27 $\pm$ 0.2	0.118 $\pm$ 0.02	-	-
							0.114 $\pm$ 0.013

In the conducted study, low or no activity was observed for the new betulin–hippuric acid conjugates against most of the cell lines used. These compounds did not exert cytotoxic effects on normal cells, unlike the reference substances betulin and doxorubicin. Hippuric acid was completely inactive.

The cancer cell line most susceptible to the effects of the new triterpenoids 4–7 was biphenotypic B-cell myelomonocytic leukemia (MV4-11). The most active derivative 7 (IC_{50} = 4.2 μ M) contains a carbonyl group at the C-3 position and a hippuric acid fragment at the C-28 position. Its precursor with a hydroxyl group at C-3 (compound 4) exhibits almost four-fold lower activity (IC_{50} = 17.6 μ M). However, the 3-acetyl derivative 5 is more effective than the monoester 4 (IC_{50} = 9.7 μ M). The least favorable structural change is the introduction of a hippuric acid residue at the position C-30 (IC_{50} = 31.4 μ M). In summary, the experimentally determined range of activities of the tested compounds against leukemia cells (7 > 5 > 4 > 6) corresponds to the predicted probability of antileukemic activity determined by PASS online (Table 3).

3.5. Mechanistic Studies

The induction of apoptosis in various types of cancer is one of the possible mechanisms of action of betulin and its derivatives [44–46]. Hata et al. demonstrated that introducing a carbonyl group at the C-28 position of the betulin molecule allows for the preparation of compounds with promising cytotoxic activity against leukemic cells. Analysis of the mechanism of the antileukemic activity of the most active derivative (IC_{50} = 0.48–1.8 μ M) demonstrated the induction of apoptosis in HL60, U937, and K562 cells [47].

Betulin–hippuric acid conjugates tested in vitro demonstrated significant activity against biphenotypic B-cell myelomonocytic leukemia (MV4-11) cells. The lowest IC_{50}

values were determined for compounds 4, 5, and 7, which were selected for analysis of the mechanism of antiproliferative action against MV4-11 cells.

The apoptosis induction assay was performed based on the annexin V (AnV)/propidium iodide (PI) staining method after 24 and 72 h of incubation for compounds 4, 5 and 7 at the following concentrations: 4 at 27 μ M, 5 at 15 μ M, and 7 at 6 μ M, (were approximately $1.5 \times IC_{50}$ value). The results are presented in Figure 2. After 24 h of incubation, we did not observed any influence on cell death. And after 72 h of incubation with compounds 4, 5, and 7, only minimal induction of cells apoptosis was observed (not statistically significant).

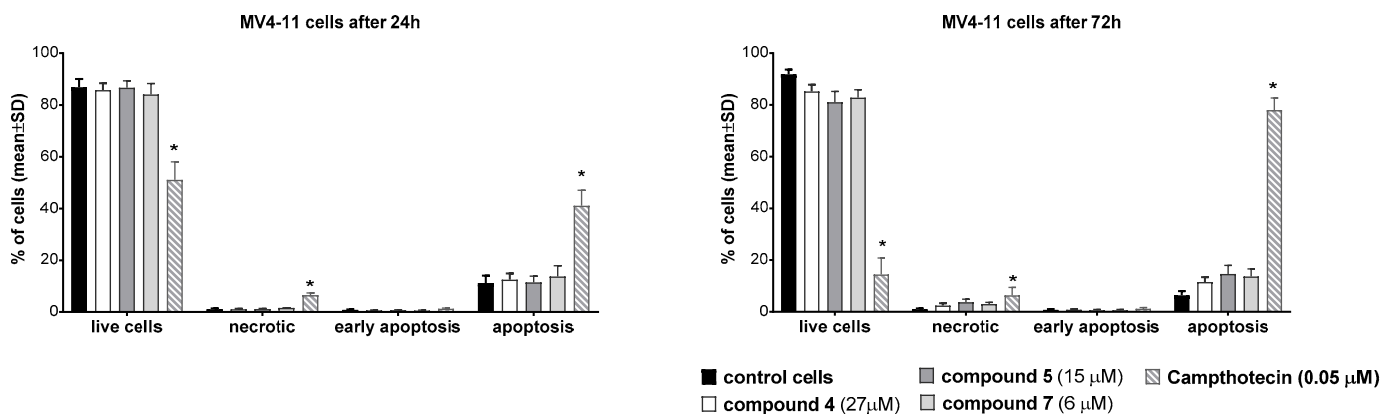


Figure 2. Induction of apoptosis by compounds 4, 5, and 7 after 24 and 72 h. * Statistically significant versus control cells, $p < 0.05$. The Y-axis expresses the number (%) of cells in the stage specified on the X-axis: live or dead cells (necrosis, early apoptosis, or late apoptosis), respectively.

Further studies included analysis of the effect of triterpenoids 4, 5, and 7 on the cell cycle of MV4-11 cells after 24 and 72 h of incubation (Figure 3).

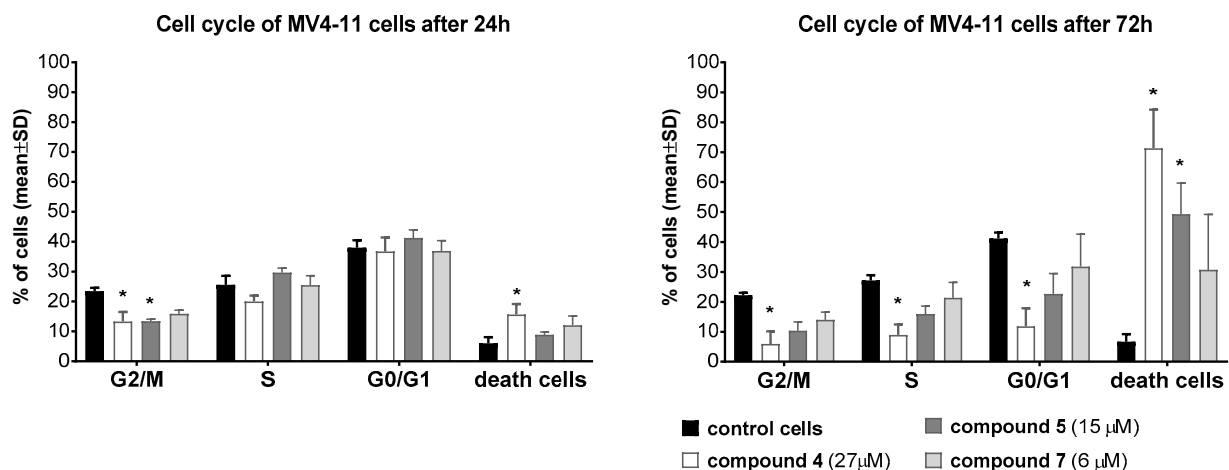


Figure 3. Influence of compounds 4, 5, and 7 on cell cycle after 24 and 72 h incubation. * Statistically significant versus control cells, $p < 0.05$.

The analysis of the cell cycle showed that after 24 h of incubation of compounds 4 and 5, the number of cells in the G2/M phase statistically significantly decreased. After 72 h, the percentage of cells in the G2/M, S and G0/G1 phases decreased for all three compounds. Most cells were dead (necrotic), but this effect was not observed in the study using the AnV/PI staining method. These differences were likely due to the different assay method and the different PI concentration, which was approximately 10 times higher in the cell cycle analysis.

3.6. Molecular Docking Analysis

MV4-11 cells exhibit a high proliferation rate and various genetic abnormalities (FLT3-ITD mutated cell line) [48]. They have been used, among other things, in research on acute myeloid leukemia (AML). AML belongs to a group of primary hematological malignancies and is the most common acute leukemia in adults. Standard treatment involves drugs that are often cytotoxic and do not carry a good prognosis [49].

The course of leukemia depends largely on the genetic background, i.e., the type of genetic changes in the cancer cells. Gene mutations that cause the development of leukemia can activate proliferation, affect the transcription process, or affect the cell cycle and apoptosis of leukemic cells. One of the most common genetic changes in AML cells are mutations in the FLT3 gene, which encodes the tyrosine kinase receptor, causing uncontrolled activation of the receptor. The most common mutations are internal tandem duplication in the fragment encoding the juxtamembrane domain (FLT3-ITD) and duplication of the tyrosine kinase domain (FMS-like tyrosine kinase domain, FLT3-TKD) [50].

Molecular docking was used to assess the potential molecular interactions and affinity of the tested compounds as potential tyrosine kinase inhibitors.

The crystal structure of FLT3 from the RCSB protein data bank (PDB ID: 6JQR) was used in molecular docking studies [51–53]. Interactions between the studied molecules and the protein were verified using AutoDock Vina software 4 [54]. The obtained scoring function values (ΔG) indicate that hippurate derivative 7 and doxorubicin have the highest affinity for the tyrosine kinase (FLT3) receptor (Table 5).

To compare the positioning of ligand 7 (with the best activity and scoring function value) and doxorubicin in the binding pocket of the target protein, 3D visualization of the active complex was performed (Figure 4) [55].

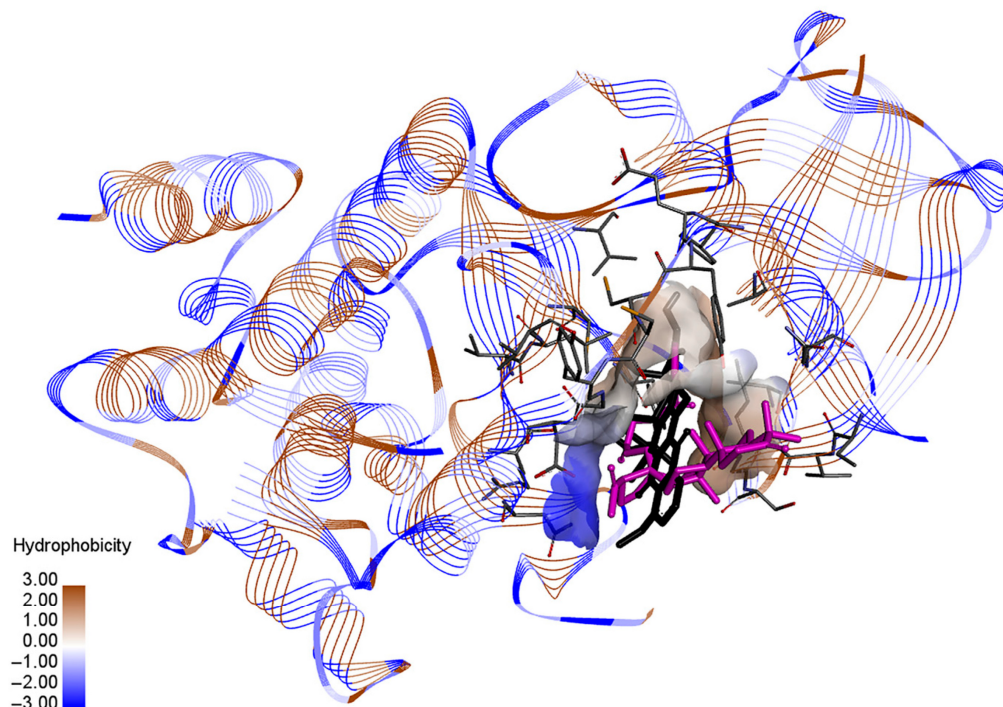
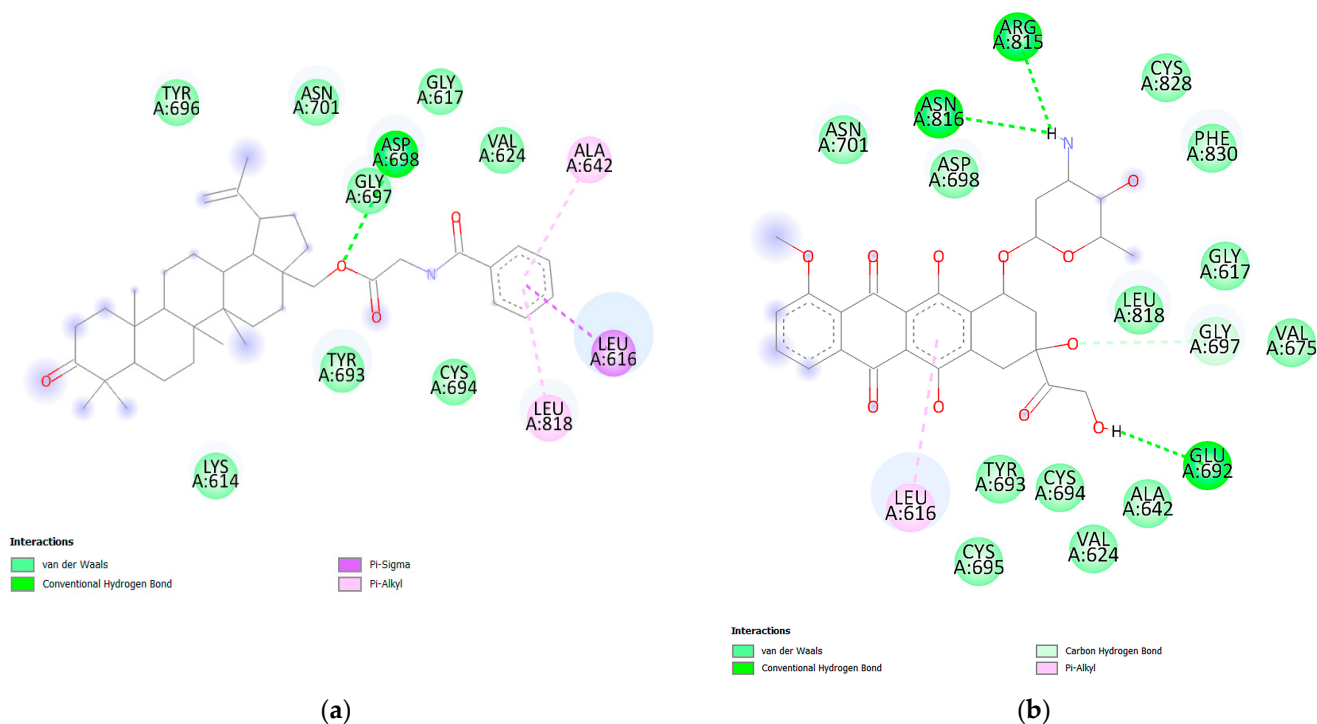


Figure 4. The superposition of docked ligands: compound 7 (magenta) and doxorubicin (black) at the binding site of the protein. The protein is colored according to its hydrophobicity scale.

Table 5. Vina affinity scoring values (ΔG) [kcal/mol] for ligands 4–7.

Compound	(ΔG) [kcal/mol]
4	−6.9
5	−6.3
6	−4.8
7	−7.3
doxorubicin	−7.7

Figure 5 shows the types of interactions between the analyzed ligands (compound 7 and doxorubicin) and the FLT3 protein. The bonds and their distances are summarized in Table S2 (Supplementary Materials). In the case of the ligand 7–protein complex, the phenyl ring of the 2-(benzoylamino)acetyl group undergoes a hydrophobic interaction with leucine LEU616 (pi-sigma), alanine ALA642, and leucine LEU818 (both pi-alkyl). In contrast, the oxygen molecule in the ester group at the C-28 position forms a hydrogen bond with aspartic acid ASP698. Doxorubicin undergoes a hydrophobic interaction with leucine LEU616 (pi-alkyl) and forms hydrogen bonds with arginine ARG815, asparagine ASN816, glutamic acid GLU692, and glycine GLY697.

**Figure 5.** Docking process of protein complex with derivative 4 (a) and doxorubicin (b).

Among the natural compounds used by Egbuna et al. in comparative studies with standard FLT3 drugs in molecular docking simulations for this protein, ursolic acid was selected as a potential inhibitor. The calculated binding energy value for this compound ($\Delta G = -9.9$ kcal/mol) was lower than for the standard drug gilteritinib ($\Delta G = -8.5$ kcal/mol) [56]. Ursolic acid is a lupane-type pentacyclic triterpene with a basic carbon skeleton similar to the compounds we studied. The interactions of this compound with the amino acids in the binding pocket, as determined by the authors, were limited to van der Waals bonds. The presence of a hippuric acid residue in the conjugate molecule 7 increases the number and types of interactions and may increase the stability of the potential inhibitor–protein complex.

4. Conclusions

Steglich esterification, often used in the transformation of natural compounds, allows for the conversion of betulin and its derivatives into conjugates with hippuric acid. Products 4–7 prepared by this method were obtained in good yields (55–88%). Preliminary predictions of the anticancer activity of the tested conjugates indicate a probability ($P_a > 0.7$) of activity against lung cancer, melanoma, and colon cancer. In vitro studies conducted on seven human cancer cell lines confirmed this activity. The tested conjugates demonstrated significant activity against MV4-11 leukemia cells. Compound 7 had the lowest IC_{50} value (4.2 μ M) against this line. Molecular docking showed that among the tested triterpenoids, compound 7 has the highest affinity for the FLT3 protein binding site.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/app15179824/s1>, Spectroscopic data for the new compound 4–7: Figure S1. 1H NMR, compound 4; Figure S2. ^{13}C NMR, compound 4; Figure S3. HRMS, compound 4; Figure S4. 1H NMR, compound 5; Figure S5. ^{13}C NMR, compound 5; Figure S6. HRMS, compound 5; Figure S7. 1H NMR, compound 6; Figure S8. ^{13}C NMR, compound 6; Figure S9. HRMS, compound 6; Figure S10. 1H NMR, compound 7; Figure S11. ^{13}C NMR, compound 7; Figure S12. HRMS, compound 7. Table S1. Lipophilicity parameters of standard compounds, determined experimentally (R_{M0} ; mobile phase acetone/buffer Tris, pH 7.4) and literature values ($\log P_{lit}$). Molecular docking study. Table S2. Interaction of compound 7 and doxorubicin with active site of FLT3 protein.

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Abbreviations

The following abbreviations are used in this manuscript:

mp	Melting points
NMR	Nuclear Magnetic Resonance
HRMS	High-Resolution Mass Spectrometry
TLC	Thin-layer chromatography
DCC	<i>N,N'</i> -Dicyclohexylcarbodiimide
DMAP	4-Dimethylaminopyridine
PCC	Pyridinium chlorochromate
RP-TLC	Reversed-phase thin-layer chromatography

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