Bioorganic Chemistry 87 (2019) 613–628

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

New phosphate derivatives of betulin as anticancer agents: Synthesis, crystal structure, and molecular docking study

Elwira Chrobak[a,*], Monika Kadela-Tomanek[a], Ewa Bębenek[a], Krzysztof Marciniec[a], Joanna Wietrzyk[b], Justyna Trynda[b], Bartosz Pawełczak[c], Joachim Kusz[d], Janusz Kasperczyk[e,f], Ewa Chodurek[e], Piotr Paduszyński[e], Stanisław Boryczka[a]

[a] Medical University of Silesia in Katowice, School of Pharmacy with the Division of Laboratory Medicine in Sosnowiec, Department of Organic Chemistry, 4 Jagiellońska Str., 41-200 Sosnowiec, Poland
[b] Polish Academy of Sciences, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Department of Experimental Oncology, 12 R.Weigla Str., 53-114 Wrocław, Poland
[c] Rainbow Pharmacy, 25 Jana Matejki Str., 43-600 Jaworzno, Poland
[d] University of Silesia, Institute of Physics, 1 75Pułku Piechoty Str., 41 500 Chorzów, Poland
[e] Medical University of Silesia in Katowice, School of Pharmacy with the Division of Laboratory Medicine in Sosnowiec, Department of Biopharmacy, 8 Jedności Str., 41-200 Sosnowiec, Poland
[f] Centre of Polymer and Carbon Materials, Polish Academy of Sciences, 34 Curie-Sklodowska Str., 41-819 Zabrze, Poland

ARTICLE INFO

Keywords:
Betulin
Phosphate
Crystal structure
Molecular docking
Anticancer

ABSTRACT

Betulin derivatives exhibit an antiproliferative activity and have been tested for many cancer cell lines. This paper describes a new series of 3-phosphate derivatives of betulin bearing different substituents at C28 position. The synthesized compounds were tested *in vitro* for their antiproliferative effect against human leukemia (MV-4-11 and CCRF/CEM), lung carcinoma (A549), prostate cancer (DU 145), melanoma (Hs 294T) cell lines, and murine leukemia P388. To explore the possible mechanism of anticancer activity for the most *in vitro* active compounds (**4**, **5**, **7** and **8**) and betulin, molecular docking was performed to the binding sites of potential anticancer targets, described for the various triterpene derivatives, including topoisomerase I and II, epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGFR), transcription factor NF-κB, anti-apoptotic protein Bcl-2 and peroxisome proliferator-activated receptor (PPARγ). According to the results of the docking, the best fit to the binding pocket of PPARγ was shown by compound **4**.

1. Introduction

Cancer is a disease which today belongs to the leading causes of death of people worldwide. The disease is characterized by loss of cellular growth control. Cancer treatment involves the use of surgery, chemotherapy and/or radiotherapy, and still is a significant challenge for modern medicine and pharmacy [1]. For many years, natural products were the main source of anticancer drugs (*e.g.*, adriamycin or taxanes), but in the 1990s, their importance has decreased in favor of targeted therapies based on humanized monoclonal antibodies or small synthetic molecules [2]. These drugs affect all rapidly dividing cells in the body, especially the cancer cells, but also cells of normal tissues such as bone marrow or gastrointestinal tract. For this reason, the majority of the cytotoxic drugs exhibits characteristic side effects [1]. New therapies have been successful in treating certain cancers (leukemia, gastrointestinal, prostate or breast cancers), but in the case of many solid tumors, they are not suitable [2,3]. The problems arising from the use of synthetic drugs resulted in the renewed interest in natural substances, which have a high therapeutic efficacy with low cytotoxicity. More than 60% of the currently used anticancer drugs are of natural origin. They are derived from natural sources such as plants, microbes, marine organisms and animals [4,5]. Nature is not only a source of potential drugs but also the leading structures that can provide a basis and inspiration for creating new semisynthetic biologically active substances [5].

Triterpenes isolated from birch bark have various confirmed biological activities, which may be enhanced by a chemical modification of their structures. For many years, betulin and betulinic acid (Fig. 1) are a subject of interest for many researchers in the field of chemistry and medicinal chemistry.

* Corresponding author.
E-mail address: echrobak@sum.edu.pl (E. Chrobak).

https://doi.org/10.1016/j.bioorg.2019.03.060
Received 19 January 2019; Received in revised form 27 February 2019; Accepted 19 March 2019
Available online 22 March 2019
0045-2068/

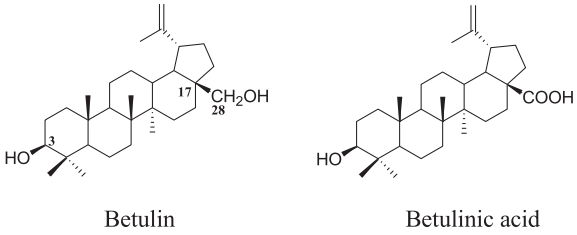


Fig. 1. Structures of betulin and betulinic acid.

These compounds exhibit a broad spectrum of biological activities such as anticancer, antiviral, antibacterial, anti-inflammatory and antimalarial [6]. Their antiproliferative activity was examined in relation to many cancer cell lines, including lung, breast, colorectal, pancreatic, cervical cancers, leukemia and various types of melanoma. In the case of betulin, promising results were obtained with respect to human lung carcinoma cell line A549 [7], human cervical carcinoma HeLa against, skin epidermoid carcinoma A431 and breast adenocarcinoma MCF7 cells [8]. Betulinic acid is selectively cytotoxic against melanoma cell lines, anticancer activity was subsequently also reported against other types of human cancers including neuroblastoma, glioblastoma, medulloblastoma, Ewing tumor, leukemia, as well as several carcinomas, i.e. head and neck, colon, breast, hepatocellular, lung, prostate, renal cell, ovarian or cervix carcinoma [9]. Due to its promising properties, betulinic acid has been extensively studied to understand the mechanism of its anticancer activity [10].

Terpenoids are capable of inhibiting the proliferation of cancer cells and inducing their death by interacting with various molecular targets. They induce apoptosis both on the extrinsic (receptor) and intrinsic (mitochondrial) pathways. The receptor pathway can be independent of the mitochondrial one or can be combined at various levels. It was described that terpene derivatives inhibit the effect of topoisomerase I and II, reduce epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGFR) expression, inhibit of the transcription factor NF-κβ and can suppress anti-apoptotic protein Bcl-2 [11–15].

The poor solubility of betulin and betulinic acid can be a certain limitation in the use of these compounds as therapeutic substances. Introduction of new functional groups to the molecule, creating complexes with cyclodextrins, liposomes, carbon nanotubes or gold nanoparticles, are attempts to overcome these limitations [16–18]. In order to improve various properties like bioavailability of biologically active compounds, their permeation, solubility, or to change the metabolic profile, active substances can be applied in the form of prodrugs [19,20]. Phosphate prodrugs are used as drugs both for parenteral and oral administration. An example of a phosphate prodrug, fosamprenavir is designed to improve the pharmacokinetics of oral antiviral drug – amprenavir [19]. Improved water solubility, compared to the basic active substances, characterizes such phosphate prodrugs as fosphenytoin, fosfluconazole, fludarabine, estramustine (Fig. 2), or propofol [19].

Combretastatin phosphate (anticancer activity) is in the third phase of clinical trials as a prodrug with an anticancer effect [21]. In recent years, such prodrugs as tedizolid phosphate (Fig. 2) (Sivextro - bacterial infections of the skin and subcutaneous tissue) and fostamatinib disodium (Tavalisse - therapy of rheumatic diseases) have been approved by USA Food and Drug Administration - FDA in 2014 and 2018, respectively [22,23]. On the other hand, the introduction of a phosphate group to improve the solubility of the drug may adversely affect the cellular uptake of the drug substance. This is due to the ionic nature of the functional group, which causes a difficulty in penetration of the drug through cell membranes. Hence, a research on derivatives containing esterified phosphate groups (norcantharimide) is carried out. The phosphate group, in addition to improving the bioavailability, may also have a positive impact on the pharmacological activity of compounds [24]. Moreover, introduction of a dialkylphosphate group into the drug molecule can broaden the spectrum of biological activity of the obtained derivatives compared to the starting material. Phosphate derivatives of non-steroidal anti-inflammatory drugs like sulindac, aspirin (Fig. 2), ibuprofen or flurbiprofen have been tested as anticancer agents [25]. So far, only a few betulin derivatives containing the phosphate moiety was described in the chemical literature. In order to overcome the problems associated with the administration and low bioavailability of triterpenoids, they may also be used as phosphate prodrugs, which has been shown in a patent for betulinic acid [26].

Various semisynthetic structural analogs of betulin were evaluated for their antiviral activities towards Epstein-Barr Virus (EBV), and among them, betulin 3,28-diphosphate exhibited significant activity [27]. Russian researchers reported the synthesis of betulin 3,28-diphosphate, which was tested for inhibition of the complement system, however, this compound did not work in this direction [28]. Recently, new scientific papers describing different possibilities of using betulin 3,28-diphosphate have been published. Melnikova *et al.* carried out a study on the physicochemical properties of this compound and its biological activity [29].One can also find patents concerning the examination of antibacterial and antifungal activity of betulin 3,28-diphosphate [30,31]. So far, no betulin derivatives containing a dialkyl phosphate group have been described.

Taking into account the above information, it seemed interesting to obtain novel phosphate derivatives of betulin and betulinic acid and examine them in terms of their antiproliferative activity. In the present paper, synthesis of new phosphate derivatives of betulin and results of an *in vitro* cytotoxic activity study were described. The biological activity was tested in relation to human biphenotypic B myelomonocytic leukemia (MV-4-11), lung carcinoma (A549), prostate cancer (DU 145), melanoma (Hs 294T), human acute lymphoblastic leukemia (CCRF/CEM) and human normal breast epithelial cell lines (MCF-10A), as well murine leukemia (P388) and mouse fibroblasts (Balb3T3).

For betulin and for the most active *in vitro* compounds, molecular docking to various proteins playing an important role in the course of cancer processes was performed. The proposed molecular targets include topoisomerases I and IIα, EGFR, VEGFR-2, catalytic kinase IKKβ, Bcl-2, as well as peroxisome proliferator-activated receptor PPARγ.

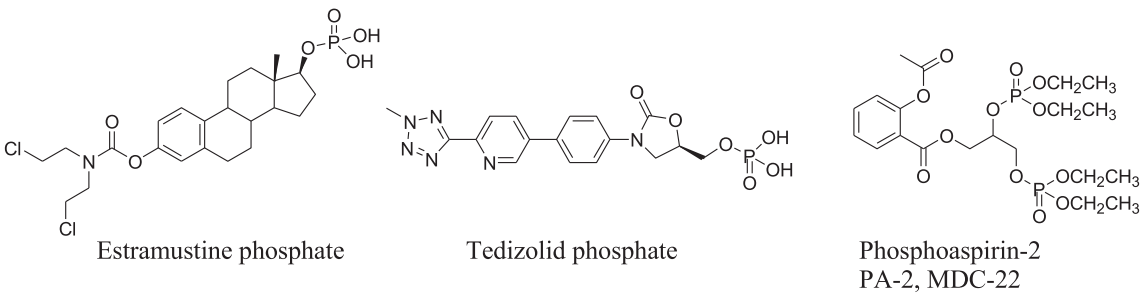
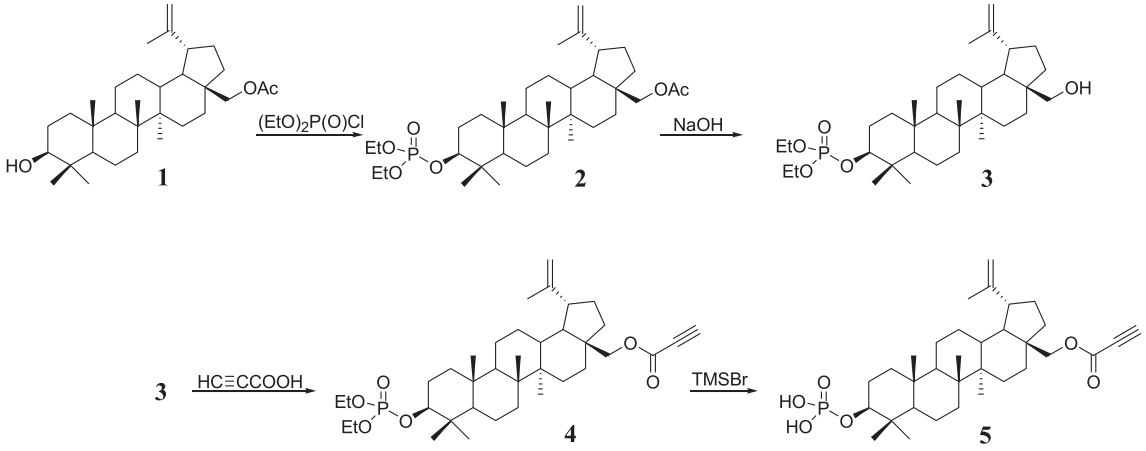


Fig. 2. Chemical structures of phosphate prodrugs.



Scheme 1. Synthesis of 3-diethoxyphosphorylbetulin **3** and derivatives **4**, **5**.

2. Results and discussion

2.1. Chemistry

Hitherto, several procedures for the synthesis of phosphate derivatives have been described. For this purpose, the most common reagents were used, such as phosphorus oxychloride, chlorides of phosphoric acid diesters or trialkyl phosphites with iodine. Herein, we present a convenient and general method using diethyl chlorophosphate [32].

In the first step of the synthesis, we used 28-acetylbetulin **1** as a substrate to obtain the phosphate **2** (69%), which was then subjected to hydrolysis under conventional conditions (NaOH, MeOH/THF). The reaction was carried out at room temperature for 6 h allowing for obtaining the product **3** with a good yield (83%). There was no occurrence of the hydrolysis reaction of the phosphate group leading to the formation of betulin (Scheme 1).

In the next stages of our research we have transformed the 3-diethoxyphosphorylbetulin **3** into acetylenic derivatives. A carbon–carbon triple bond is present in a large number of biologically active compounds, including natural products and some pharmaceuticals, such as, for instance, antifungal (Terbinafine) [33], contraceptive and menopausal hormone therapy (ethinyl estradiol, Norethisterone) [34], antiretroviral (Efavirenz) [35], antidepressant and Parkinson's disease therapy (selegiline) [36], or anticancer therapy (erlotinib) [37].

Syntheses of different betulin and betulinic acid derivatives bearing an alkynyl side chain were described. These compounds were examined for their anticancer [38], antiviral [39], hepatoprotective and anti-inflammatory activity [40].

Likewise, our previous studies have shown that in a reaction of propiolic acid with betulin and its derivatives, compounds having a promising antiproliferative activity are formed [41,42]. In this work, we carried out the synthesis of an acetylenic derivative of betulin phosphate **3** (Scheme 1). The reaction was performed according to the previously described method [41]. The 3-diethoxyphosphoryl-28-propynoylbetulin **4** was obtained with a 50% yield.

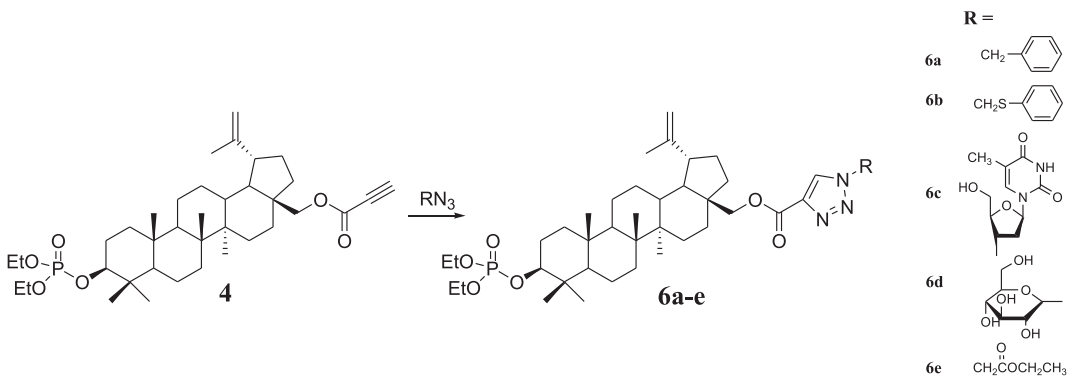
The active substance should be, on one hand, lipophilic enough to overcome the membrane or the metabolic barrier, and on the other hand, it should be sufficiently hydrophilic to have a good solubility and bioavailability [43]. In order to compare the activity of neutral molecule and its charged form, deprotection of the phosphate ester **4** using bromotrimethylsilane was carried out subsequently, to give the monophosphate ester **5** with a good yield (90%).

Compounds which contain an alkyne moiety are used for a variety of further transformations leading to useful organic compounds through the construction of new bonds. Derivatives with a terminal acetylenic group participate in cycloaddition reactions (click chemistry strategy) with substituted azides to form new triazole heterocyclic systems. 1,2,3-Triazole ring is a pharmacophore group, its presence determines a broad spectrum of biological activity, such as anticancer, anti-tubercular, anti-inflammatory, antileishmanial, antitrypanosomal, antimicrobial, antiviral and antimalarial [44].

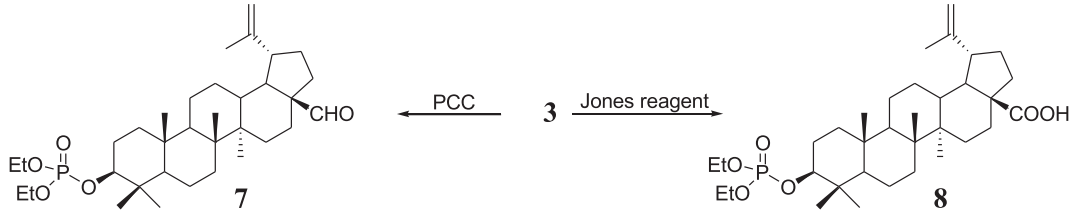
New triazole derivatives of betulin phosphate **6a-e** were synthesized *via* the ring closure of 3-diethoxyphosphoryl-28-propynoylbetulin **4** with various azides (Scheme 2). The derivatives **6a-e** were obtained with yields in the range of 64–73%

Taking into account much information about a high cytotoxic activity of oxidation products at C28 position of betulin, reactions of compound **3** with PCC and Jones reagent were carried out (Scheme 3). Expected 3-diethoxyphosphorylbetulinic aldehyde **7** and 3-diethoxyphosphorylbetulinic acid **8** were obtained with yields 77% and 36%, respectively.

Structure of newly synthesized derivatives were confirmed by ^{1}H,



Scheme 2. Synthesis of triazole derivatives **6a-e**.



Scheme 3. Oxidation of 3-*O*-diethoxyphosphorylbetulin **3**.

^{13}C, ^{31}P NMR, IR and HR MS analysis. Additionally, betulin phosphate **3**, its acetylenic derivative **4** and triazole **6d**, were analyzed by the X-ray diffraction.

2.2. Crystal structures

Molecular structures of 3-diethoxyphosphorylbetulin **3** and 3-diethoxyphosphoryl-28-propynoylbetulin **4** were confirmed by the X-ray diffraction method. The crystals suitable for analysis were grown by slow evaporation of an appropriate solvent, at room temperature. The molecular structures of the compounds **3** and **4** with atom numbering are shown in Figs. 3A and 4A, respectively.

The structure analysis confirms that, in the obtained product, the diethyl phosphate group is connected with the betulin molecule *via* an ester bond and adopts an equatorial orientation. The six-membered rings of the betulin backbone exhibit a chair conformation and are combined in a *trans* system. The five-membered ring assumes the conformation of the twisted envelope, where the C17 carbon atom is located outside the plane formed by the C18-C19-C21-C22 atoms. Compound **3** crystallizes in an orthorhombic $P2_12_12_1$ space group. In the crystal of compound **3**, molecules form (along the *b* axis) chains through hydrogen bonds between the hydroxy group at the C28 position and the oxygen atom of the phosphate group of the second molecule O2—H2···O3, and thus in the "head to tail" system (Fig. 3B).

The phosphate derivative **4** crystallizes in tetragonal $P4_2$ space group. There are two molecules per asymmetric unit, one of them showing rotational disorder of the ethyl groups in phosphate moiety (Fig. 4A).

The molecular structure of **4** is stabilized by weak intermolecular C—H···O bonds (Fig. 4B). The molecules are connected with each other by means of weak (mostly electrostatic) bonds formed between the terminal alkyne group and the oxygen atom of the phosphate group in the next molecule ($\equiv C_{133}—H···O_{24}=P$ and $\equiv C_{233}—H···O_{14}=P$) (Table S3). The relatively high acidity of the $C_{sp}—H$ results in ethynyl group forming some of the shortest C—H···O intermolecular contacts, especially when it is coupled with strong acceptors like O=P [45]. The molecules in the crystal of the compound **4** form springs along the *c* axis (Fig. 4C). The springs are connected to each other by weak hydrogen bonds ($\equiv C_{241}—H_{24H}···O_{26}$ and $\equiv C_{241}—H_{24I}···O_{26}$).

2.3. Antiproliferative activity

All newly synthesized compounds were evaluated for their *in vitro* antiproliferative activity towards normal human mammary gland (MCF-10A) and cancer human cell lines of diverse etiology. Betulin and cisplatin were used as the reference compounds. In the first step of the experiments, the screening of the tested substances on cells of human biphenotypic B myelomonocytic leukemia MV-4-11 was carried out. Betulin concentration causing 50% inhibition of MV-4-11 cells proliferation (IC_{50}) was approx. 8.19 μM. Therefore, only those substances whose IC_{50} level was similar to or greater than betulin were selected for the second stage. The IC_{50} values of the examined compounds towards MV-4-11 cells are shown in Table 1.

Analyzing the obtained results, one may notice that in the case of the compound **3,** the introduction of the diethyl phosphate group into the betulin molecule results in a reduction of activity against leukemia MV-4-11 cells. The values of IC_{50} for betulin and **3** are 8.19 and 25.60 μM, respectively. Among the tested triazole derivatives **6a-e**, the lowest activity is shown by **6a** and **6e** which contain hydrophobic substituents at N3 position. Derivatives with polar substituents in this moiety **6c** and **6d,** are more strongly active. The higher activity of the compound **6b** compared to a similar structure **6a** results from the presence of a sulfur atom (Table 1).

In the case of derivatives, for which the concentration causing 50% inhibition of proliferation was higher than 10 μM, the studies have been terminated at the first stage of testing against of the MV-4-11 cells. From the group of phosphate derivatives of betulin, eight were selected for the second stage of research. The compounds **2**, **4**, **5**, **7**, **8**, **6b**, **6c** and **6d** were evaluated for their *in* vitro antiproliferative activity against three cancer human cell lines A549 (non-small cell lung carcinoma), DU 145 (prostate cancer), Hs 294T (melanoma) and normal human breast epithelial cell lines – MCF-10A. The IC_{50} values for the tested compounds are shown in Table 2. The compounds most active against A549 cells form the following order: **8** > **4** > **7** > betulin > **6c** > **2** > **6b** > **5**. The obtained result is consistent with the studies described in the literature, which confirmed a higher activity of betulinic acid in relation to the A549 line compared to betulin [46]. Other researchers have also reported a high activity of various triterpene derivatives containing a carboxyl group at the C28 position [11]. The phosphate derivative of betulinic acid **8** also showed a good activity in relation to other cell lines with values of IC_{50} 6.10 and 7.42 μM for DU 145 and Hs 294T, respectively. The SI (Selectivity Index) was calculated for the compound **8** using the formula: SI = IC_{50} for normal cell line (MCF-10A)/IC_{50} for respective cancerous cell line. The obtained values equal to 9.34 (A549), 7.00 (DU 145) and 5.76 (Hs 294T) indicate the efficacy of **8** against cancer cells higher than their toxicity against normal cells (SI > 1.0).

The highest activity in the studied group of derivatives in relation to DU 145 and Hs-294T cell lines was demonstrated by the compound **4**. Its activity was significantly higher than that of betulin and higher or comparable to the values for cisplatin (Table 2). This result is consistent with previous reports of a high activity of compounds containing an acetylene group in their structure [41]. A conversion of the acetylene group to a triazole ring with various substituents at the N3 position (compounds **6b-d**) resulted in a decrease in the activity. A similar situation was described by the Csuk et al., for betulin derived compounds bearing an ethynyl side chain at the C28 position [38].

The lowest activity against the tested cell lines is exhibited by the phosphate **5**. The compound **5** showed a poor inhibition of the A549, DU 145 and Hs 294T cells, although its activity was satisfactory in the initial study against leukemia (MV4-11). Additionally, compounds **4** and **5** were evaluated for activity against other human (CCRF/CEM) and murine leukemia (P388) lines (Table 3). With respect to these cell lines, the compound **5** exhibited a better activity (values of IC_{50} 6.87 and 5.46 μM, respectively), however, the derivative **4** showed an inhibition of cell proliferation at lower concentrations (IC_{50} 0.78 and 0.73 μM, respectively).

Comparing the obtained results with the previously described values obtained for 28-*O*-propynoylbetulin in relation to the same cancer cell lines, it can be noticed that the presence of the C3 diethylphosphate

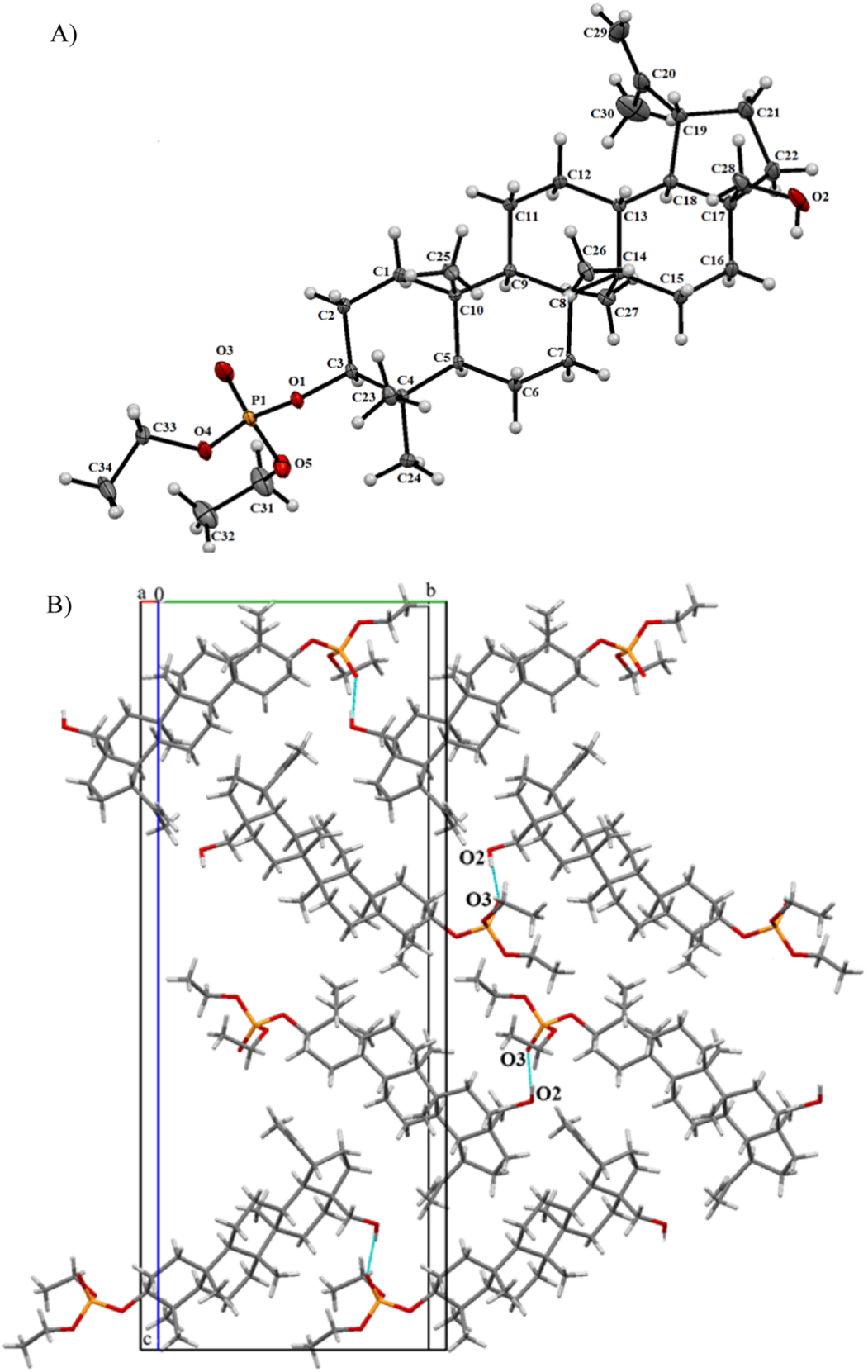


Fig. 3. (A) Molecular structure of the compound **3** showing the atom numbering scheme. Displacement ellipsoids are drawn at the 50% probability level, (B) packing diagram of **3**. Dashed lines indicate hydrogen bonds.

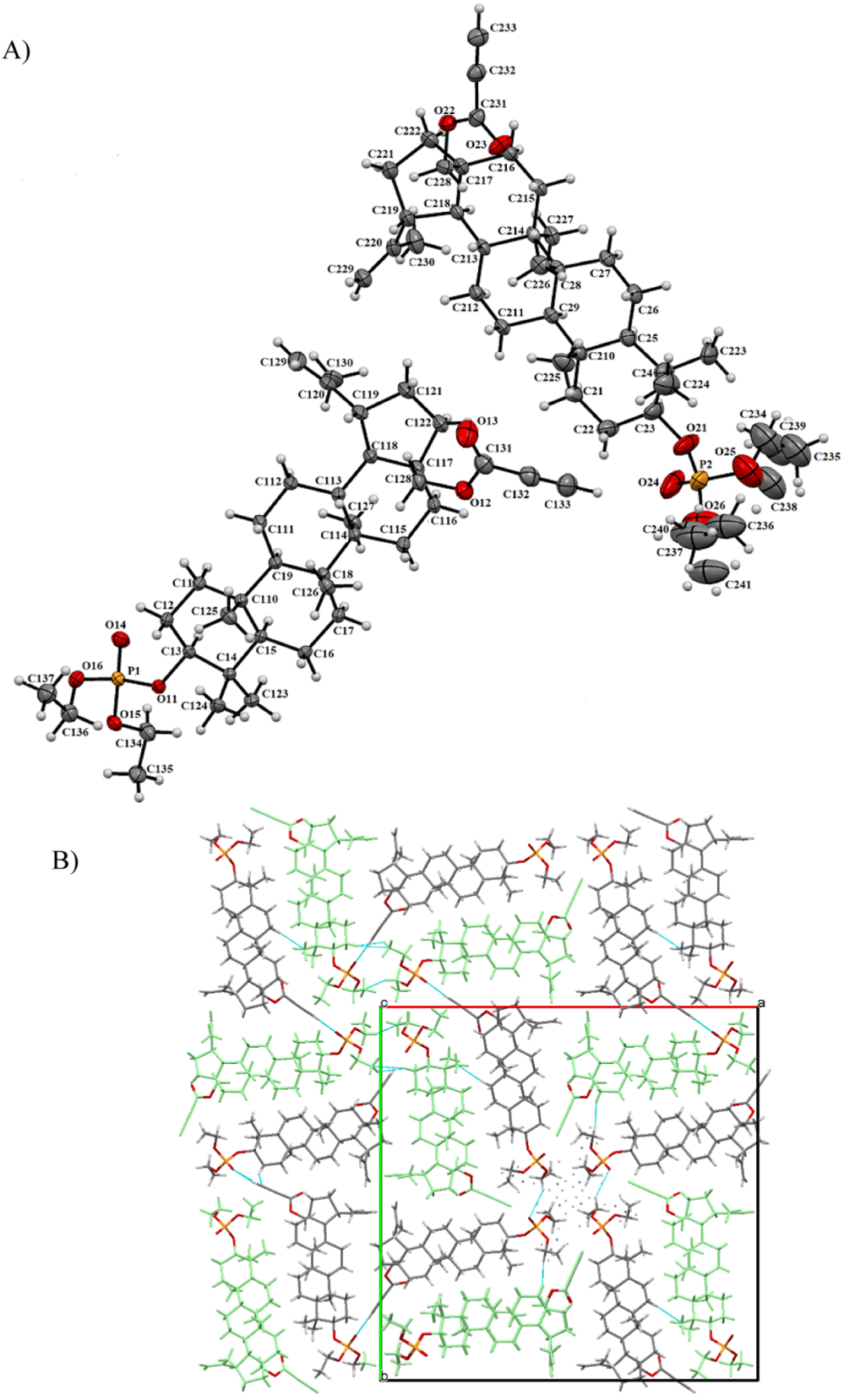


Fig. 4. (A) Molecular structure of the compound **4** showing the atom numbering scheme. Displacement ellipsoids are drawn at the 50% probability level, (B) packing diagram of crystal **4** projected parallel to the *ab* plane, (C) Spring arranged along the *c* axis.

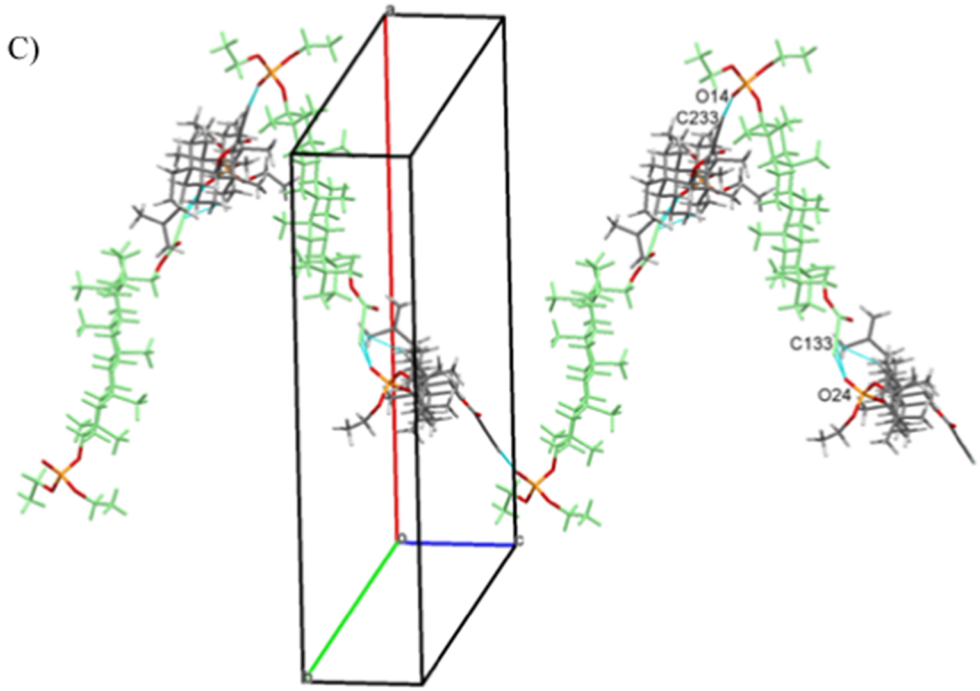


Fig. 4. (*continued*)

Table 1
The *in vitro* antiproliferative activity of phosphate derivatives **2–8** and reference compounds (betulin, cisplatin) against human leukemia MV-4-11 cell line (MTT assay).

MV-4-11 Compound	IC_{50} [μM]	Compound	IC_{50} [μM]
Betulin	8.19 ± 2.84	**6b**	3.08 ± 1.03
Cisplatin	1.63 ± 0.17	**6c**	1.74 ± 0.33
2	3.48 ± 1.67	**6d**	2.93 ± 0.08
3	25.60 ± 6.41	**6e**	76.39 ± 32.57
4	0.43 ± 0.08	**7**	3.85 ± 1.09
5	4.57 ± 0.77	**8**	1.75 ± 0.74
6a	21.86 ± 5.70	–	–

IC_{50} concentration of the tested compounds in μM (mean ± standard deviation).

Table 2
The *in vitro* antiproliferative activity of phosphate derivatives **2**, **4**, **5**, **6b-d**, **7**, **8** and reference compounds (betulin, cisplatin) against human cancer cell lines lung carcinoma A549, prostate cancer DU 145, melanoma Hs 294T and human normal breast epithelial cell lines MCF-10A (SRB assay).

	A549 IC_{50} [μM]	DU 145 IC_{50} [μM]	Hs 294T IC_{50} [μM]	MCF-10A IC_{50} [μM]
Betulin	8.49 ± 0.77	44.13 ± 19.32	44.04 ± 7.20	124.06 ± 41.60
2	9.60 ± 0.55	23.20 ± 6.07	34.72 ± 1.29	41.80 ± 0.66
4	**4.68** ± 2.23	**1.39** ± 0.74	**3.01** ± 1.09	neg
5	71.37 ± 14.80	80.89 ± 8.59	69.15 ± 6.37	neg
6b	12.45 ± 0.16	neg	neg	neg
6c	**6.15** ± 0.42	27.78 ± 2.11	14.89 ± 4.39	41.85 ± 2.29
6d	neg	neg	20.80 ± 6.32	neg
7	**5.82** ± 0.90	9.58 ± 1.65	24.19 ± 2.41	69.17 ± 23.90
8	**4.57** ± 0.96	**6.10** ± 1.37	**7.42** ± 1.35	42.72 ± 15.56
Cisplatin	**4.47** ± 1.20	**1.57** ± 0.37	**6.33** ± 1.33	14.73 ± 1.13

Neg - negative in the concentration used.
Values IC50 < 8 μM for cancer cell line are in bold.

group (compound **4**) causes a significant increase in the cytotoxicity relative to the tested CCRF/CEM and P388 lines. The selectivity of phosphates **4** and **5** is comparable or higher than that for 28-*O*-propynoylbetulin.

2.4. Molecular docking

Molecular modeling carried out in our work was an attempt to predict the possible mode of action of the synthesized compounds and to determine future directions of research in the field of optimizing the structure of the compounds. Four of the most *in vitro* active compounds (**4**, **5**, **7** and **8**) and betulin were docked to binding sites of various reported triterpene derivatives targets. Studies carried out so far, have confirmed the ability of terpenes to inhibit proliferation and induce apoptosis, but the mechanism responsible for causing the above effects is not always known. Numerous scientific papers on the study of the mechanism of action of terpenes describe their ability to inhibit topoisomerase I and IIα [11,47]. The expression of these enzymes (Top I and Top IIα) has been observed in various types of human cancers [48].

According to the results of docking for Top I, all tested compounds showed a higher degree of fit defined in the arbitrary units of the Gold program (Table 4) compared to betulin, however, the phosphate derivatives took a different place in the binding pocket than betulin and showed no differentiation between themselves. Binding interactions of the compounds studied with amino acids residues inside the active side of Top I and nucleotides of single cleavaged DNA are presented in the Table S4.

A high activity of topoisomerase IIα is described in cervical, colon and lung cancer, including non-small cell lung cancer (A549) [48,11]. Among the derivatives tested, the best fit to the binding pocket for Top IIα was obtained for the compound **8** (Table 4).

Docked poses of betulin and the ligand **8,** and their interactions with amino acid residues and nucleotides are presented in Fig. 5.

The top docked pose of betulin in the binding site showed hydrophobic interactions with Arg487, Lys614 (steric) and with Tyr805 (alkyl-π). Hydroxy groups of betulin in positions C3 and C28 have been involved in hydrogen bonds to carbonyl O2 atom of deoxythymidine and to carboxyl oxygen atom of Asp541, respectively (Table S5). The ligand **8** differs from betulin in having a diethoxyphosphoryl group in C3 and a carboxyl group instead the hydroxymethyl moiety in C17 position. The best docked pose of compound **8** was in a place other than

Table 3
The *in vitro* antiproliferative activity of phosphate derivatives **4** and **5** and reference compounds (betulin, cisplatin) against human acute T lymphoblastic leukemia CCRF/CEM, and murine leukemia P388 and mouse fibroblasts Balb3T3, (MTT assay).

	CCRF/CEM IC_{50} [μM]	P388 IC_{50} [μM]	Balb3T3 IC_{50} [μM]	SI* (CCRF/CEM)	SI* (P388)
Betulin	62.73 ± 17.00	12.42 ± 7.45	106.77 ± 17.83	1.7	8.6
4	0.78 ± 0.17	0.73 ± 0.33	3.93 ± 2.01	5.1	5.4
5	6.87 ± 2.85	5.46 ± 0.40	12.57 ± 9.63	1.8	2.3
28-*O*-propynoyl-betulin**	6.46 ± 0.81	5.86 ± 0.40	7.88 ± 5.66	1.2	1.3
Cisplatin	2.17 ± 1.07	1.33 ± 0.97	6.13 ± 2.83	2.8	4.6

* SI, selectivity index calculated as ratio between IC_{50} values for noncancerous cells (Balb3T3) and cancer cells.
** Literature data [40].

Table 4
The top-ranked CHEMPLP fitness score function obtained with GOLD for phosphate derivative **4**, **5**, **7**, **8** and betulin as reference ligand docked to target proteins.

Target protein			CHEMPLP Score (arb. unit)				
Name	PDB entry	PDB chain	**Betulin**	**4**	**5**	**7**	**8**
Top I	1TL8	A	49.67	60.43	59.35	55.94	58.52
Top II α	5GWK	B/A	70.39	73.24	68.77	71.81	61.02
		A/B	62.26	70.81	72.35	56.95	77.64
EGFRK	1M17	A	59.87	66.66	58.08	51.60	51.13
VEGFR2	1Y6A	A	50.82	61.84	58.80	48.46	58.51
IKKβ	3RZF	A	46.40	63.25	61.42	53.05	56.41
Bcl-2	2W3L	A	48.78	57.07	54.87	55.83	55.11
		B	49.51	61.21	52.60	58.88	57.35
PPAR-γ	5LSG	A	46.26	71.49	69.01	53.51	43.06

for betulin, and this is due to the larger number of hydrogen bonds formed by phosphoryl (with Arg487 and deoxyadenine) and carboxyl groups (with Asp541, Gly617 and Leu616). The compound **8**, like betulin, bounds to Tyr805 *via* alkyl-π interactions which are not formed by other ligands. All binding interactions of the phosphate derivatives (**4**, **5**, **7** and **8**) and betulin with residues of Top II α and nucleotides of double cleavaged DNA are presented in Table S6.

Induced by a signal from the outside, so-called “extrinsic”, a pathway leading to cell apoptosis, is associated with membrane death receptors. Receptor tyrosine kinases (RTKs), transmembrane surface receptors regulate numerous cell functions including apoptosis, metabolism and proliferation. These receptors are an important element of the signaling pathway associated with various cancers, *e.g.* prostate, breast, lung and ovarian cancer [14]. As potential receptor molecular targets for the docking of betulin and phosphate ligands (**4**, **5**, **7**, **8**), epidermal growth factor receptor EGFR and vascular endothelial growth factor receptor VEGFR-2, were selected. Analysis of results showed that the docking scores (Table 4) in both cases were the highest for the compound **4**, however, they were comparable with other ligands.

Betulin formed, with the ATP-active site of EGFR, a network of hydrogen bonds with Ala719, Leu764, Thr766 and Arg817 residues in the hinge region area. For the compound **4**, only a single hydrogen bond with the Thr766 residue is observed, but it can get deep into the hydrophobic pocket region forming interactions with Leu694, Val702, Ala719, Leu768, Cys773, Leu775, Arg817, and Leu820 amino acid residues. All docked ligands exhibited alkyl-π contact with Phe699. As can be seen, the top docked poses of betulin and phosphate derivatives do not show hydrophobic interactions with the DFG motif (Asp831, Phe832 and Gly833). According to literature information, interactions with this site play an important role in the regulation of kinase activity [49]. For VEGFR-2, the top docked poses of the tested ligands have adopted a similar binding mode, in which the hydrophobic fragments of the molecule interact with Val846, Leu838, Ala864, Cys917, Leu1033, Cys1043 residues and formed alkyl-π contact with Phe916 and Phe1045. The compound **4** does not form any hydrogen bonds (Table S7).

The nuclear factor kappa B (NF-κB) inhibits apoptosis, stimulates proliferation and angiogenesis, contributes to the formation of metastases, and thus it may be a good therapeutic target in anticancer therapy. NF-κB proteins occur in the inactive cytoplasmic form as a complex with inhibitory proteins from the IκB family. Activation of NF-κB occurs by phosphorylation and degradation of IκB (through IKKβ kinase) [10]. Released from the complex with an NF-κB inhibitor, it is transported to the cell nucleus where it controls the transcription of specific genes. Due to the fact that the main pathway of NF-κB activation depends on IKKβ kinase, inhibition of this subunit with enzymatic properties may be an effective strategy for regulating the NF-κB activity.

Prostate cancer cell lines whose growth is not hormone-sensitive, including DU145, have developed a number of mechanisms that prevent the proper course of apoptosis. In these cells, an increased expression of IKK kinase (IkB kinase) was also observed, affecting the increase in the activity of the transcription factor NF-κB.

As it was described, 16 amino acids residues, namely Leu21, Gly22, Thr23, Val29, Ala42, Lys44, Met96, Glu97, Tyr98, Cys99, Gly102, Asp103, Glu149, Asn150, Ile165s and Asp166, were identified as IKKβ active-site residues [50]. During docking of betulin and the phosphate ligands to IKKβ, the best docked ligand was the compound **4** (Table 4, Fig. 6A). All phosphate compounds (**4**, **5**, **7** and **8**) formed hydrogen bonds with Thr23 and acetylenic derivatives additionally formed hydrogen bonds with Cys99 residue. The betulin molecule and other ligands interact with residues of Leu21, Val29, Cys99, Val152, Ile165 (for betulin, 7 and 8) or Leu21, Val29, Ala42, Ile165, Met96, Lys44 (for compounds **4** and **5**) forming a hydrophobic site in the binding pocket. Compound **7**, **8** and betulin (Fig. 6B) also formed alkyl-π interactions (Tyr98) (Table S8).

Cancer growth may occur not only as a result of an increased proliferation of abnormal cells, but also as a result of a prolonged survival. Many genes involved in the regulation of the process of apoptosis show disturbances of expression in neoplastic processes [13]. The majority of hormone independent prostate cancers show an increased expression of the Bcl-2 proto-oncogene [51]. The Bcl-2 family proteins may be therapeutic targets, and inhibition of those with anti-apoptotic effects may induce apoptosis in cancer cells. Terpenoids are capable of suppressing anti-apoptotic members (Bcl-2, Bcl-XL, Mcl-1) and activating pro-apoptotic members (Bak, Bax, Bid, Bad) of Bcl-2 family [10,52]. The docking results showed that betulin and the phosphate derivatives (**4**, **5**, **7** and **8**) bind to the Bcl-2 protein through steric interactions with Val92, Leu96, Arg105, Ala108, Arg105, Val107 residues and alkyl-π interactions with residues of Tyr (67, 161) and Phe (63, 71, 112). At the A-chain binding site, betulin and derivatives **4**, **5** and **8** formed hydrogen bonds with Asn102, and weak nonconventional hydrogen bonds with Gly104, while the Tyr161 residue was involved in chain B in hydrogen bonds (for compounds **4**, **7** and **8**). The binding interactions of the phosphate derivatives (**4**, **5**, **7** and **8**) and betulin with Bcl-2 residues are presented in Table S9.

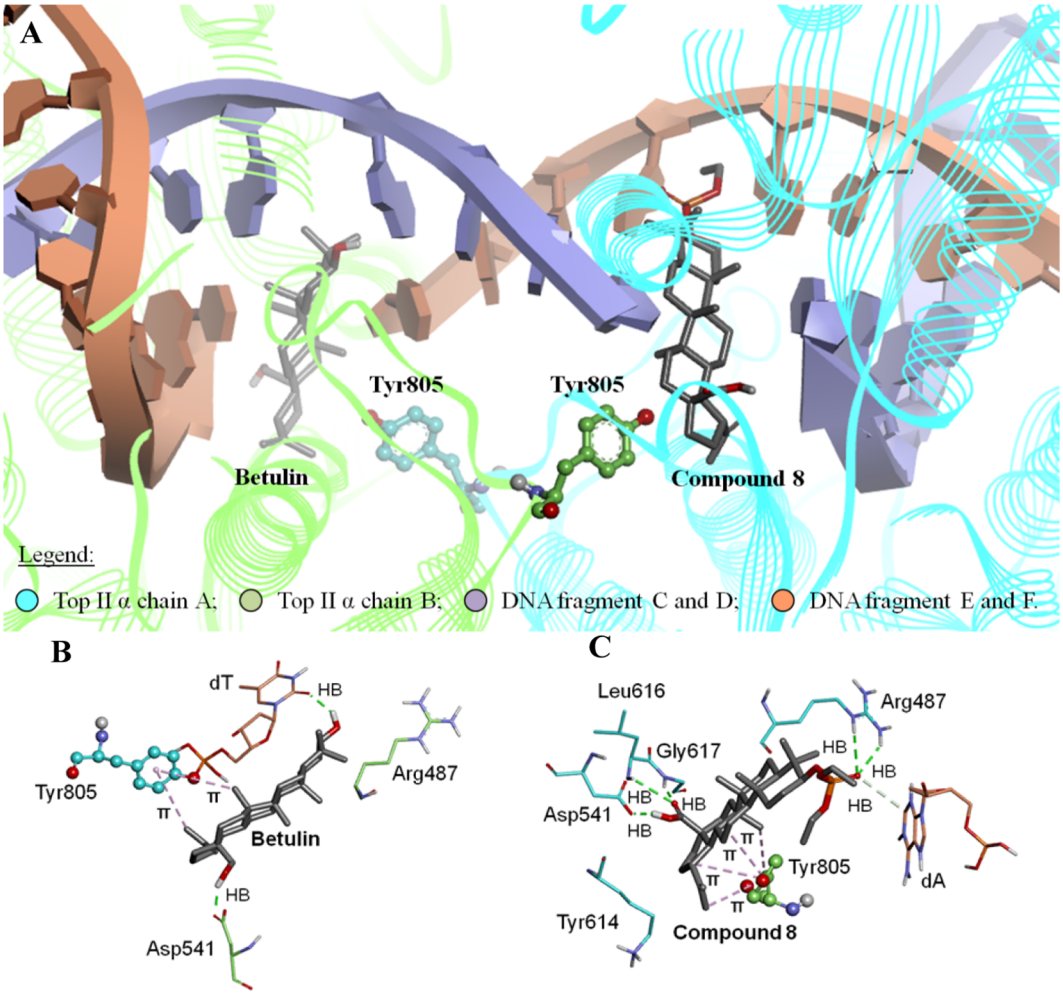


Fig. 5. (A) Topoisomerase IIα and DNA double-stranded cleavage complex (PDB entry 5GWK) stabilizing by betulin and the compound **8** docked structures. (B) Betulin interactions with protein residues and deoxythymidine (dT). (C) The compound **8** interactions with protein residues and deoxyadenosine (dA). Hydrogen bonds (HB) and alkyl-π (π) are indicated. For clarity, non-polar hydrogens and steric interactions are invisible.

Peroxisome proliferator-activated receptors (PPAR*α*, PPAR*β/δ* and PPARγ) belong to the family of nuclear receptors that act as transcription factors. Their main role is to control the metabolism of fatty acids and to maintain glucose homeostasis. In addition, they play an important role in cell proliferation and differentiation and are a key regulator of cellular differentiation [53]. The results of *in vitro* tests on various human cancer cell lines, including breast cancer, lung carcinoma, hematopoietic cancer, glioma, colon, liver cancer, pancreatic cancer, thyroid cancer and prostate cancer indicate that PPAR-γ is often overexpressed in these cancer cells. Compounds which are PPAR-γ ligands have an antiproliferative, pro-apoptotic and differentiation-promoting activities and can be useful in treatment of cancer [54].

The PPARγ receptor is composed of five regions named A-E, arranged from the N- to C-terminus of the protein. Region E contains two functional domains, LBD (ligand binding domain) and AF-2 (ligand-dependent activation fuction). The ligand binding domain is formed of 13 *α*-helices and one *β*-sheet. The LBD secondary structure studies have shown that the ligand-binding pocket is *T*-shaped or Y-shaped with three branches named I, II and III, and is larger than the binding pockets in other nuclear receptors. In addition, it consists mainly of hydrophobic amino acids which makes it able to bind large hydrophobic molecules [53,54].

According to the results from the molecular docking studies for PPARγ, all phosphate derivatives (**4**, **5**, **7** and **8**) showed significantly better scores when compared with betulin (Table 4). The docked poses of betulin and the phosphate derivatives within the active site of PPARγ were in the inside branch III, as its experimentally obtained co-crystallized ligand, betulinic acid. Betulinic acid is a native ligand of crystal structure of this receptor registered in the PDB with ID 5LSG. In the structure described, the carboxyl group present at C17 of the betulin system forms two strong hydrogen bonds with Ser289 (carbonyl oxygen atom) and Tyr327 (oxygen atom of the hydroxyl group) [55]. The PPARγ ligand binding domain with the compounds **4**, **5** and betulin molecule docked inside branch III are shown in Fig. 7.

The major difference in the binging mode of compound **4** compared to betulinic acid is to maintain only one hydrogen bond with Ser289 and carbonyl atom of the ester group present at C17 of the betulin. However, the stability of the complex is increased as a result of additional strong hydrogen bond formed between the oxygen atom of the phosphoryl group and Arg280. The major structural differences between betulin and the compounds **4** and **5** are their hydrophobicity, and the size of the substituent at the C3 and C28 positions. The best docking result was obtained for the compound **4**. The compound **5**, more polar due to the presence of free phosphoric acid groups, assumed a different position and did not get deep into the binding pocket (Fig. 8C). The hydroxy group formed a hydrogen bond with carbonyl oxygen of Glu291. This compound also showed hydrophobic interactions with Cys285, Arg288, Ile341 (steric) and with Phe287 (alkyl-π) (Fig. 8D).

Betulin has adapted to the ligand pocket of PPARγ through hydrophobic interactions as well as a hydrogen bond with Arg280 (Fig. 8E

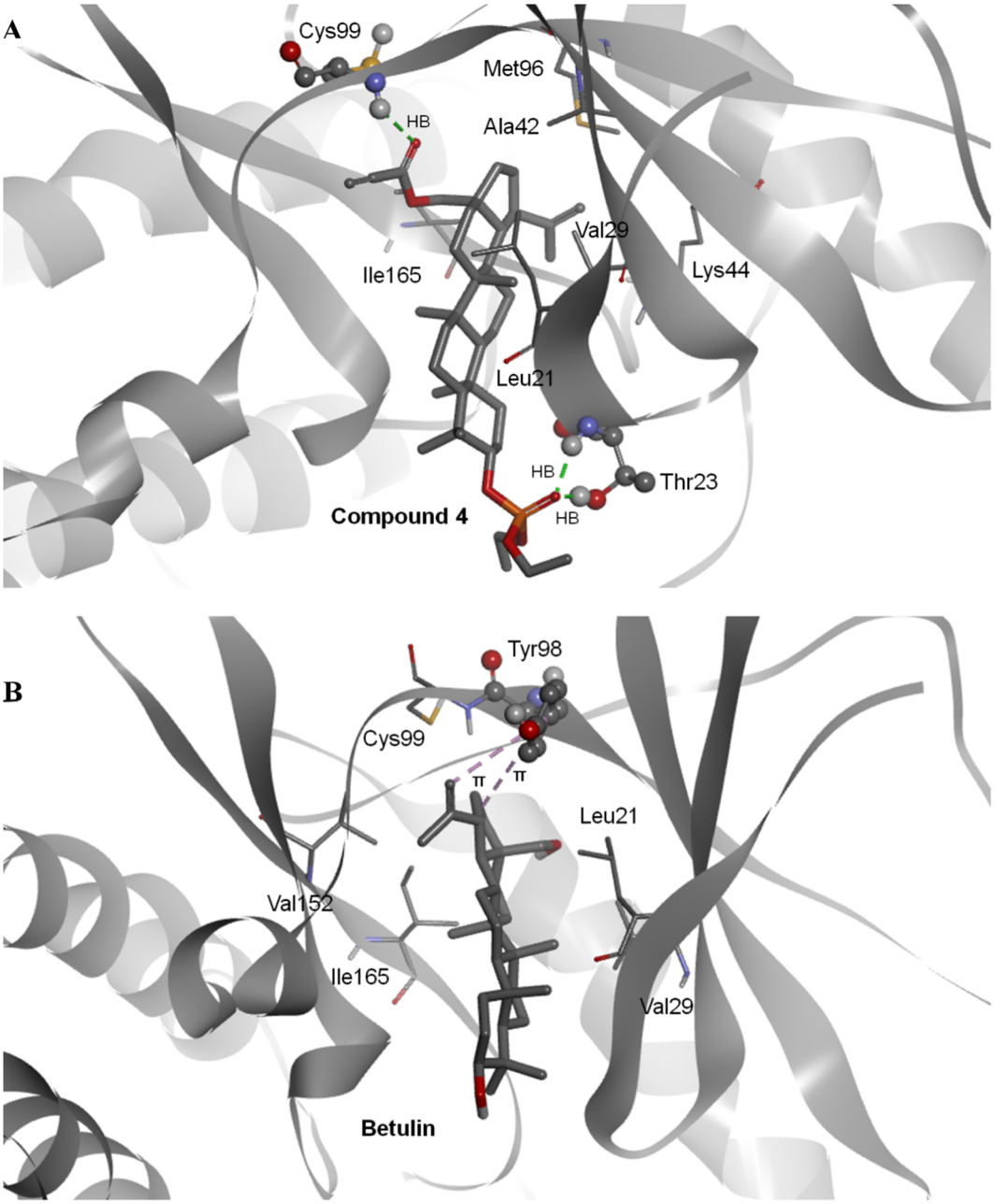


Fig. 6. Docking pose of the compound **4** (A) and betulin (B) inside the IKKβ kinase domain (3RZF). Hydrogen bonds (HB) and alkyl-π interaction are indicated (π). For clarity, non-polar hydrogens and alkyl-hydrophobic interactions are invisible.

and F). The binding interactions of the phosphate derivatives and betulin with PPAR-γ residues are presented in Table S10.

3. Conclusions

In the present study, a new group of betulin 3-phosphate derivatives was synthesized and tested against five human cancer cell lines. The compounds **4** and **8** showed higher activity against four cell lines (MV-4-11, A549, DU 145 and Hs 294T) compared to betulin. In relation to the Hs 294T line, the compound **4** revealed higher activity than cisplatin used as a reference compound (the IC_{50} value was equal to 3.01 μM for **4** and 6.33 μM for cisplatin). Among the studied compounds, the derivative **5** showed the most selectivity, *i.e.* weakly inhibited A549, DU 145 and Hs 294T cells, simultaneously, revealed the activity towards various types of leukemias, human (MV-4–11, CCRF/CEM) and murine (P388). In the studies, carried out against CCRF/CEM cells, the compound **5** was markedly more active than betulin (IC_{50} values were 6.78 μM and 62.73 μM for **5** and betulin, respectively). Against the same leukemia cell line, the compound **4** has a higher activity referred to that previously described for 28-*O*-propynoylbetulin which does not have a diethoxyphosphoryl moiety. Considering the literature reports on various potential mechanisms of anticancer activity of triterpenes as well as cancer cell lines used for *in vitro* research, molecular modeling to the five potential molecular targets has been carried out. The best fit, expressed in arbitrary units of GOLD for all selected molecular targets, showed the compound **4**. The values of the fitness score function were in the range of 57.7–71.5 and did not differ remarkable from the other phosphate ligands and betulin. However, in molecular docking to peroxisome proliferator-activated receptor PPARγ, a significant difference between betulin and the phosphate ligands was observed (fitness score: 71.5 and 69.0 for the compound **4** and **5**, respectively; 46.3 for betulin). This effect suggests a potential

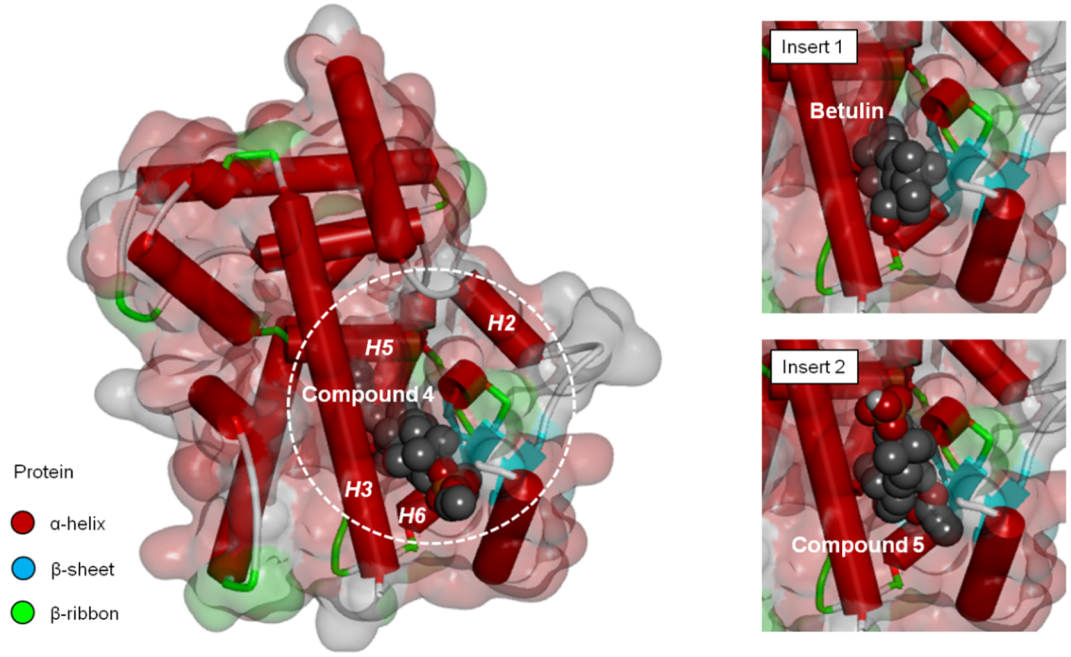


Fig. 7. The PPARγ ligand binding domain (PDB entry 5LSG) with the compound **4** molecule docked inside branch III. In the insert 1 - betulin. In the insert 2 – the compound **5**.

molecular target which determines antiproliferative activity of the studied compounds.

4. Experimental

4.1. Biological activity

4.1.1. Cell culture and medium-is included in the supplementary materials
4.1.1.1. Antiproliferative assay. The *in vitro* cytotoxic effect of the examined substances was determined by means of MTT assay for leukemia cells or SRB assay for adherent cells as it was described by Wietrzyk et al. [56]. Stock solutions of the tested compounds at a concentration of 10 mg/mL were prepared for each experiment *ex tempore* by dissolving 1 mg of substance in 100 μL of DMSO. The solvent for further dilution test was the medium. The compounds were tested in concentration ranges from 0.1 to 100 mg/mL.

The results of experiments were presented in the form of inhibitory concentration 50 (IC_{50}) - the dose of the tested compounds which inhibits proliferation of 50% of the cell population. Calculation of the IC_{50} values were performed separately for each sample, the average values and standard deviation were included in the tables. The compounds in each concentration was tested in triplicate in a single experiment, which was made at least three times.

4.2. Synthesis

4.2.1. Materials and methods–is included in the supplementary materials
28-Acetylbetulin **2** was obtained according to described method. Melting point, ^{1}H and ^{13}C NMR spectra data for compound **2** were consistent with the literature information [57].

*4.2.2. Synthesis of 28-acetoxy-3-diethoxyphosphorylbetulin **2***
Diethyl chlorophosphate (0.28 mL, 1.9 mmol) was added dropwise to a stirred solution of **1** (0.48 g, 1 mmol) and 4-dimethylaminopyridine (DMAP) in pyridine (6 mL), cooled to 0 °C in an ice-water bath. Next, mixture was allowed to warm to room temperature and stirred overnight in an argon atmosphere. After completion the reaction, pyridine was removed in vacuum and the residue was dissolved in 30 mL dichloromethane and washed with 10% HCl, saturated $NaHCO_3$ and water. The organic fraction was dried (anhydrous Na_2SO_4), filtered, and the solvent removed under reduced pressure. The crude product was purified by column chromatography on silica gel (dichloromethane/ethanol, 40:1 v/v) to give the compound **2**.

Yield: 69%, m. p. 67–70 °C.

TLC (dichloromethane/ethanol, 40:1, v/v): $R_f = 0.27$.

^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 4.71 (d, $J = 1.8$ Hz, 1H, H-29), 4.61 (d, $J = 1.8$ Hz, 1H, H-29), 4.26 (d, $J = 10.8$ Hz, 1H, H-28), 4.13 (m, 4H, 2x OCH_2CH_3), 3.99 (m, 1H, H-3), 3.88 (d, $J = 10.8$ Hz, 1H, H-28), 2.46 (m, 1H, H-19), 2.09 (s, 3H, $C(O)CH_3$), 0.85–2.00 (m, 23H, CH, CH_2), 1.70 (s, 3H, H-30), 1.34 (m, 6H, 2x OCH_2CH_3), 1.05 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 0.98(s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.75 (m, 1H, H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 171.7; 150.2; 109.9; 86.3; 63.5; 62.8; 55.3; 48.7; 47.7; 46.3; 42.7; 40.9; 38.8; 38.7; 38.4; 37.6; 36.9; 34.5; 34.1; 29.7; 28.6; 28.0; 25.4; 25.1; 21.1; 20.8; 19.1; 18.3; 16.2; 16.2; 16.1; 15.0; 14.9; 13.7. ^{31}P NMR (243 MHz, $CDCl_3$) δ (ppm): −1.31. IR (KBr, cm^{-1}) ν: 1740 (C═O), 1234 (P═O), 1034 (P−O−C). HR-MS (APCI) *m/z*: $C_{36}H_{60}O_6P$ $[(M-H)^-]$, Calc. 619.4127; Found 619.4123.

*4.2.3. Synthesis of 3-diethoxyphosphorylbetulin **3***
Sodium hydroxide solution (0.25 M) in the water/tetrahydrofuran/methanol system (50 mL in the ratio of 1:2:1 v/v) and 0.62 g (1 mmol) of compound **2** are placed in a round-bottomed flask, the whole mixture was stirred at room temperature for 1 h. Then, dichloromethane (15 mL) was added to the mixture and washed with 10% HCl and water. The organic layer was dried with anhydrous sodium sulfate (VI), then concentrated till dry. The product was purified by column chromatography (SiO_2, dichloromethane/ethanol, 40:1 v/v) yielding compound **3**.

Yield: 83%, m. p. 210–211 °C.

TLC (dichloromethane/ethanol, 40:1, v/v): $R_f = 0.24$.

^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 4.70 (d, $J = 1.8$ Hz, 1H, H-29); 4.60 (d, $J = 1.8$ Hz, 1H, H-29), 4.10 (m, 4H, 2x OCH_2CH_3), 3.98 (m, 1H, H-3), 3.81 (d, $J = 10.8$ Hz, 1H, H-28), 3.35 (d, $J = 10.8$ Hz, 1H, H-28), 2.39 (m, 1H, H-19), 0.90–2.00 (m, 24H, CH, CH_2), 1.70 (s, 3H, CH_3), 1.35 (m, 6H, 2x OCH_2CH_3), 1.07 (s, 3H, CH_3), 1.05 (s, 3H, CH_3),

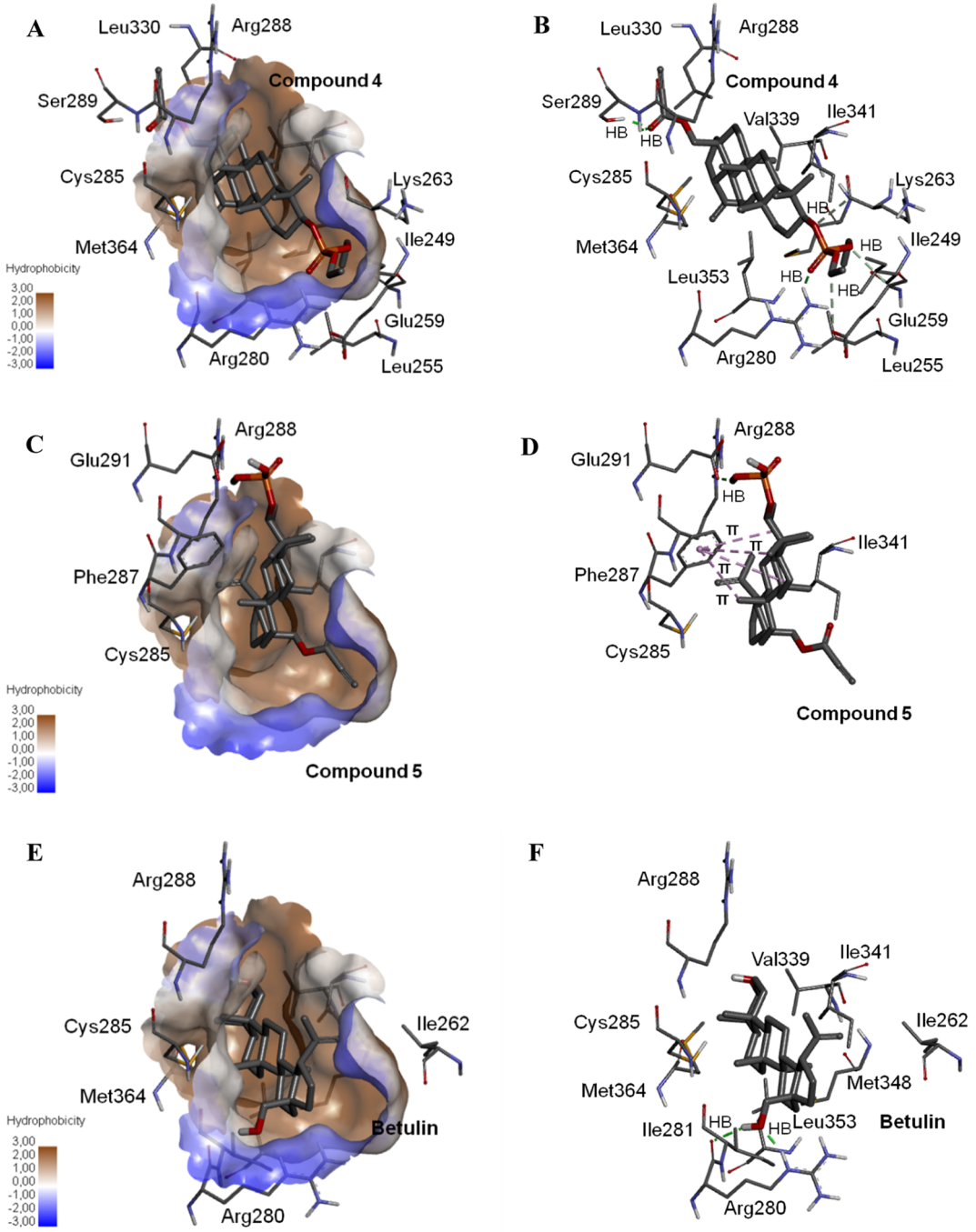


Fig. 8. Binding mode of the compound **4**, **5** and betulin inside branch III of PPARγ ligand binding domain. (A) Hydrophobic view of the compound **4** location; (B) The compound **4** binding interactions; (C) Hydrophobic view of the compound **5** location; (D) The compound **5** binding interactions; (E) Hydrophobic view of betulin location; (F) Betulin binding interactions. Hydrogen bonds (HB) and alkyl-π interactions (π) are indicated. For clarity, non-polar hydrogen atoms and alkyl-hydrophobic interactions are invisible.

1.01 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.75 (m, 1H, H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 150.5; 109.7; 86.3; 63.5; 63.4; 60.5; 55.3; 50.3; 48.7; 47.8; 42.7; 40.9; 38.8; 38.4; 37.3; 36.9; 34.2; 34.0; 29.7; 29.2; 28.0; 27.0; 25.3; 25.1; 20.9; 19.1; 18.3; 16.2; 16.1; 16.0; 14.7. ^{31}P NMR (243 MHz, $CDCl_3$) δ (ppm): −1.35. IR (KBr, cm^{-1}) ν: 3455 (O—H), 1262(P═O), 1036 (P—O—C). HR-MS (APCI) *m/z*: $C_{34}H_{58}O_5P$ [(M − H)$^-$], Calc. 577.4022; Found 577.4011.

4.2.4. Synthesis of 3-diethoxyphosphoryl-28-propynoylbetulin 4

Compound **3** in the amount of 0.29 g (0.5 mmol) was dissolved in 2.5 mL of methylene chloride. The obtained solution was cooled in an ice-water bath to −10 °C, then 0.037 mL (0.59 mmol) of a propiolic acid was added, and next a solution of 0.123 g (0.59 mmol) of DCC (*N,N′*-dicyclohexylcarbodiimide) and 0.005 g (0.04 mmol) of DMAP (4-dimethylaminopyridine) in 0.5 mL of methylene chloride was added dropwise. The reaction was carried out under argon atmosphere for 5 h in a cooling bath, and then at room temperature. After 24 h, the reaction mixture was filtered, the filtrate was concentrated till dry on a vacuum evaporator. The crude product was purified by column chromatography (SiO_2, dichloromethane/ethanol, 40:1, v/v), yielding ester **4**.

Yield: 50%, m. p. 167–168 °C.

TLC (dichloromethane/ethanol, 40:1, v/v): R_f = 0.28.

^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 4.71 (s, 1H, H-29), 4.62 (s, 1H, H-29), 4.40 (d, J = 10.8, 1H, H-28), 4.11 (m, 4H, 2x OCH_2CH_3), 4.00 (d, J = 10.8, 1H, H-28), 3.98 (m, 1H, H-3), 2.91 (s, 1H, C≡CH), 2.44 (m, 1H, H-19), 0.93–2.10 (m, 23H, CH, CH_2), 1.70 (s, 3H, H-30), 1.35 (m, 6H, 2x OCH_2CH_3), 1.03 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 0.85 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 0.74 (m, 1H, H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 153.3; 149.9; 110.0; 86.3; 74.8; 74.6; 64.9; 63.5; 63.4; 55.3; 50.2; 48.8; 47.7; 46.4; 42.7; 40.9; 38.8; 38.4; 37.7; 36.9; 34.4; 34.1; 29.6; 29.5; 28.0; 27.0; 25.3; 25.1; 20.8; 19.1; 18.4; 16.2; 16.1; 16.0; 14.7. ^{31}P NMR (243 MHz, $CDCl_3$) δ (ppm): −1.34. IR (KBr, cm^{-1}) ν: 2112 (C≡CH), 1717 (C═O), 1225 (P═O), 1038 (P−O−C). HR-MS (APCI) m/z: $C_{37}H_{58}O_6P[(M-H)^-]$, Calc. 629.3971; Found 629.3965.

*4.2.5. Synthesis of 3-dihydroxyphosphoryl-28-propynoylbetulin **5***

To a solution of 0.33 g (ca. 0.5 mmol) of compound **4** in 5 mL of dichloromethane, cooled to 0 °C, 0.26 mL (2 mmol) of bromotrimethylsilane (TMSBr) was added and mixed under argon atmosphere for 24 h at room temperature. After evaporation of the solvent, 6 mL of ethanol was added and mixed for another 2 h. The solvent was evaporated again, the residue was triturated with water and the precipitate was filtered, yielding compound **5**.

Yield: 90%, m. p. 146–149 °C.

TLC (ethanol/chloroform, 3:1, v/v) R_f = 0.10.

^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 4.72 (s, 1H, H-29), 4.62 (s, 1H, H-29), 4.42 (d, J = 12 Hz, 1H, H-28), 4.00 (d, J = 12 Hz, 1H, H-28), 3.93 (m, 1H, H-3), 2.91 (s, 1H, C≡CH), 2.45 (m, 1H, H-19), 0.93–2.10 (m, 25H, CH, CH_2), 1.70 (s, 3H, H-30), 1.04 (s, 3H, CH_3), 0.99 (s, 6H, 2x CH_3), 0.85 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.75 (m, 1H, H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 153.3; 149.8; 110.1; 87.3; 74.8; 74.7; 64.8; 55.3; 50.2; 48.8; 47.7; 46.4; 42.7; 40.9; 38.8; 38.5; 37.7; 36.9; 34.5; 34.1; 29.6; 29.5; 27.9; 27.0; 25.1; 25.0; 20.8; 19.2; 18.3; 16.1; 16.0; 15.9; 14.8. ^{31}P NMR (243 MHz, $CDCl_3$) δ (ppm): 1.21. IR (KBr, cm^{-1}) ν: 3303 (C≡C-H), 2122 (C≡C), 1718 (C═O), 1224 (P═O), 972 (P-OR). HR-MS (APCI) m/z: $C_{33}H_{55}O_6P$ [(M − H)$^-$], Calc. 573.3345; Found 573.3329.

*4.2.6. General procedure for the synthesis of the triazoles **6a-e***

The 3-diethoxyphosphoryl-28-propynoylbetulin **4** was dissolved in dry toluene (4 mL). Next, the copper iodide (I) (0.1 eqv, 0.004 g, 0.02 mmol) and a corresponding organic azide (1.05 eqv, 0.213 mmol) were added to the mixture. After 72 h of stirring under reflux, the solvent was removed under reduced pressure. The residue was purified by the column chromatography using various mixtures of organic solvents to give pure triazoles **6a-e**.

*4.2.6.1. 3-Diethoxyphosphoryl-28-(1-benzyl-1H-[1,2,3]-triazol-4-yl)carbonylbetulin **6a**.*

Yield: 81%, m. p. 116–118 °C.

TLC (hexane/ethyl acetate, 3:2, v/v): R_f = 0.23.

^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.97 (s, 1H, CH-triazol), 7.42 (m, 3H, H_{Ar}), 7.32 (m, 2H, H_{Ar}), 5.60 (s, 2H, CH_2), 4.72 (s, 1H, H-29), 4.62 (s, 1H, H-29), 4.56 (d, J = 10.8, 1H, H-28), 4.13 (m, 5H, 2x OCH_2CH_3, H-28), 4.00 (m, 1H, H-3), 2.50 (m, 1H, H-19), 0.83–2.07 (m, 21H, CH, CH_2), 1.71 (s, 3H, H-30), 1.34 (m, 6H, 2x OCH_2CH_3), 1.06 (s, 3H, CH_3), 1.01 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.75 (m, 1H, H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 171.1; 161.2; 150.1; 140.6; 133.8; 129.3; 129.2; 129.0; 128.2; 127.9; 127.1; 110.0; 86.3; 63.6; 63.5; 63.4; 60.4; 55.3; 54.5; 50.3; 48.9; 47.7; 46.7; 42.7; 40.9; 38.8; 38.4; 37.7; 36.9; 34.7; 34.1; 29.8; 29.6; 28.0; 27.1; 25.4; 25.2; 21.1; 20.8; 19.1; 18.3; 16.2; 16.1; 14.7. ^{31}P NMR (243 MHz, $CDCl_3$) δ (ppm): −1.34. IR (KBr, cm^{-1}) ν: 2946 (CH), 1734 (C═O), 1529 (C═N), 1456 (N═N), 1264 (*N*-N═N), 1225 (P═O), 1040 (P−O−C). HR-MS (APCI) m/z: $C_{44}H_{65}N_3O_6P$ [(M − H)$^-$], Calc. 762.4611; Found 762.4602.

*4.2.6.2. 3-Diethoxyphosphoryl-28-(1-phenylthiomethyl-1H-[1,2,3]-triazol-4-yl)carbonylbetulin **6b**.*

Yield: 75%, m. p. 110–111 °C.

TLC (chloroform/ethanol, 15:1, v/v): R_f = 0.60.

^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.95 (s, 1H, CH-triazol), 7.27 (m, 5H, H_{Ar}), 5.59 (s, 2H, CH_2), 4.64 (s, 1H, H-29), 4.54 (s, 1H, H-29), 4.47 (d, J = 10.8, 1H, H-28), 4.04 (m, 5H, 2x OCH_2CH_3, H-28), 3.91 (m, 1H, H-3), 2.43 (m, 1H, H-19), 0.83–2.00 (m, 21H, CH, CH_2), 1.63 (s, 3H, H-30), 1.27 (m, 6H, 2x OCH_2CH_3), 0.98 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.78 (s, 3H, CH_3), 0.74 (s, 3H, CH_3), 0.66 (m, 1H, H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 163.1; 150.0; 138.1; 136.7; 127.4; 111.3; 89.8; 86.8; 85.2; 84.5; 63.5; 62.1; 61.7; 60.0; 59.7; 50.3; 48.9; 47.8; 46.7; 42.7; 40.9; 38.4; 37.7; 37.4; 36.9; 28.0; 25.4; 16.8; 16.1; 14.8; 12.6; 12.5. ^{31}P NMR (243 MHz, $CDCl_3$) δ (ppm): −1.33. IR (KBr, cm^{-1}) ν: 2945 (CH), 1734 (C═O), 1541 (C═N), 1456 (N═N), 1263 (*N*-N═N), 1220 (P═O), 1037 (P−O−C). HR-MS (APCI) m/z: $C_{44}H_{65}N_3O_6PS$ [(M − H)$^-$], Calc. 794.4332; Found 794.4314.

*4.2.6.3. 3-Diethoxyphosphoryl-28-{1-(3′-deoxythymidine-5′-yl)-1H-[1,2,3]-triazol-4-yl}carbonylbetulin **6c**.*

Yield: 83%, m. p. 163–165 °C.

TLC (chloroform/ethanol, 15:1, v/v): R_f = 0.16.

^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.18 (s, 2H, NH-AZT), 8.09 (s, 1H, CH-triazol), 7.36 (s, 1H, AZT), 6.00 (t, 1H, J = 6.6 Hz, AZT), 5.45 (m, 1H, AZT), 4.64 (s, 1H, H-29), 4.54 (s, 1H, H-29), 4.50 (d, J = 10.8, 1H, H-28), 4.34 (m, 1H, AZT), 4.07 (m, 6H, 2x OCH_2CH_3, H-28, 1H-AZT), 3,76 (m, 2H, AZT), 3.02 (m, 1H, AZT), 2.49 (m, 1H, AZT), 2.43 (m, 1H, H-19), 1.96 (m, 1H, AZT), 1.86 (s, 3H, CH_3-AZT), 0.83–1.84 (m, 22H, CH, CH_2), 1.63 (s, 3H, H-30), 1.26 (m, 6H, 2x OCH_2CH_3), 1.01 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.78 (s, 3H, CH_3), 0.77 (s, 3H, CH_3), 0.66 (m, 1H, H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 160.9; 150.0; 140.6; 132.4; 129.7; 126.8; 110.0; 86.3; 63.5; 63.4; 55.3; 54.3;50.3; 48.8; 47.8; 46.7; 42.7; 40.9; 38.8; 38.4; 37.7; 36.9; 34.7; 34.1; 29.8; 29.6; 28.0; 27.1; 20.8; 19.1; 18.3; 16.2; 16.1; 16.0; 14.8; 14.2. ^{31}P NMR (243 MHz, $CDCl_3$) δ (ppm): −1.36. IR (KBr, cm^{-1}) ν: 2945 (CH), 1699 (C═O), 1683 (C═O), 1558 (C═N), 1471 (N═N), 1271 (P═O), 1035 (P−O−C). HR-MS (APCI) m/z: $C_{47}H_{71}N_5O_{10}P$ [(M − H)$^-$], Calc. 896.4939; Found 896.4926.

*4.2.6.4. 3-Diethoxyphosphoryl-28-[1-(1-deoxy-β-D-glucopyranosyl)-1H-1,2,3-triazol-4-yl]carbonylbetulin **6d**.*

Yield: 77%, m. p. 212–213 °C.

TLC (chloroform/ethanol, 5:1, v/v): R_f = 0.10.

^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 9.08 (s, 1H, CH-triazol), 5.61 (d, 1H, J = 5.4 Hz, CH-sugar), 5.45 (d, 1H, J = 5.4 Hz, CH-sugar), 5.34 (d, 1H, J = 5.4 Hz, CH-sugar), 5.20 (d, 1H, J = 5.4 Hz, CH-sugar), 4.73 (s, 1H, H-29), 4.64 (m, 1H, CH-sugar), 4.63 (s, 1H, H-29), 4.59 (d, J = 10.8, 1H, H-28), 4.03 (m, 5H, 2x OCH_2CH_3, H-28), 3.99 (m, 2H, CH-sugar, H-3), 3.83 (m, 1H, OH), 3.47 (m, 1H, OH), 3.40 (m, 1H, OH), 3.28 (m, 1H, H-19), 1.99 (m, 1H, OH), 0.83–1.83 (m, 25H, CH, CH_2), 1.71 (s, 3H, H-30), 1.23 (m, 6H, 2x OCH_2CH_3), 1.04 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 0.76 (s, 3H, CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 160.9; 150.3; 139.2; 129.1; 110.5; 88.3; 85.7; 80.6; 79.7; 77.2; 72.4; 69.9; 63.5; 63.4; 62.7; 61.2; 54.8; 49.9; 48.7; 47.5; 46.8; 42.8; 40.9; 38.8; 38.1; 37.6; 36.9; 34.6; 34.1; 29.6; 29.4; 28.1; 27.1; 25.4; 25.2; 20.8; 19.3; 18.3; 16.5; 16.4; 16.2; 16.1; 15.0. ^{31}P NMR (243 MHz, $CDCl_3$) δ (ppm): −1.43. IR (KBr, cm^{-1}) ν: 2967 (CH), 1732 (C═O), 1543 (C═N), 1456 (N═N), 1238 (P═O), 1031 (P−O−C). HR-MS (APCI) m/z: $C_{43}H_{69}N_3O_{11}P$ [(M − H)$^-$], Calc. 834.4670; Found 834.4657.

*4.2.6.5. 3-diethoxyphosphoryl-28-(1-ethylacetyl-1H-1,2,3-triazol-4-yl) carbonylbetulin **6e**.*

Yield: 64%, m. p. 150–153 °C.

TLC (chloroform/ethanol, 15:1, v/v): R_f = 0.56.

^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.15 (s, 1H, CH-triazol), 5.15

(s, 2H, CH_2), 4.64 (s, 1H, H-29), 4.53 (s, 1H, H-29), 4.49 (d, J = 10.8, 1H, H-28), 4.22 (q, 2H, J = 7.2 Hz, OCH_2), 4.04 (m, 5H, 2x OCH_2CH_3, H-28), 3.90 (m, 1H, H-3), 2.43 (m, 1H, H-19), 0.83–1.98 (m, 26H, CH, CH_2), 1.63 (s, 3H, H-30), 1.27 (m, 6H, 2x OCH_2CH_3), 0.98 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.78 (s, 3H, CH_3), 0.74 (s, 3H, CH_3), 0.66 (m, 1H, H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 165.7; 160.9; 150.1; 140.7; 128.7; 110.0; 86.3; 63.7; 63.5; 63.4; 62.8; 55.3; 51.0; 50.3; 48.9; 47.8; 46.7; 42.7; 40.9; 38.8; 38.4; 37.7; 36.9; 34.7; 34.1; 29.8; 29.6; 28.0; 27.1; 25.4; 25.2; 20.8; 19.1; 18.3; 16.2; 16.1; 16.0; 14.8. ^{31}P NMR (243 MHz, $CDCl_3$) δ (ppm): −1.33. IR (KBr, cm^{-1}) ν: 2945 (CH), 1757 (C═O), 1718 (C═O), 1558 (C═N), 1456 (N═N), 1265 (P═O), 1045 (P—O—C). HR-MS (APCI) m/z: $C_{41}H_{65}N_3O_8P$ [$(M-H)^-$], Calc. 758.4509; Found 758.4500.

4.2.7. Synthesis of 3-diethoxyphosphorylbetulinic aldehyde 7

Compound **3** in the amount of 0.29 g (0.5 mmol) was dissolved in 6 mL of dichloromethane and 0.65 g (3 mmol) of pyridinium chlorochromate (PCC) was added. The reaction was carried out at room temperature for 2 h. After completion of the reaction, 24 mL of diethyl ether was added to the mixture and filtered through silica gel. The filtrate was concentrated till dry on a vacuum evaporator. The crude product was purified by column chromatography (SiO_2, methylene chloride/ethanol, 20:1 v/v), yielding compound **7**.

Yield: 77%, m. p. 109–112 °C.

TLC (dichloromethane/ethanol, 40:1, v/v): R_f = 0.33.

^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 9.61 (d, J = 1.2 Hz 1H, CHO), 4.69 (br. s, 1H, H-29), 4.56 (br. s, 1H, H-29), 4.03 (m, 4H, 2x OCH_2CH_3) 3.91 (m, 1H, H-3), 2.81 (m, 1H, H-19), 0.95–2.10 (m, 23H, CH, CH_2), 1.69 (s, 3H, H-30), 1.27 (m, 6H, 2x OCH_2CH_3), 0.94 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.78 (s, 3H, CH_3), 0.74 (s, 3H, CH_3), 0.67 (m, 1H, H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 206.7; 149.7; 110.2; 86.3; 63.5; 63.4; 59.3; 55.3; 50.4; 48.0; 47.5; 42.6; 40.8; 38.8; 38.4; 36.9; 34.3; 33.2; 29.9; 29.2; 28.8; 28.0; 25.5; 25.3; 20.8; 19.0; 18.3; 16.2; 16.1; 16.0; 15.9; 14.2. ^{31}P NMR (243 MHz, $CDCl_3$) δ (ppm): −1.33. IR (KBr, cm^{-1}) ν: 1728 (C═O), 1262 (P═O), 1038 (P—O—C). HR-MS (APCI) m/z: $C_{34}H_{56}O_5P$ [$(M - H)^-$], Calc. 575.3485; Found 575.3498.

4.2.8. Synthesis of 3-diethoxyphosphorylbetulinic acid 8

A solution of 0.29 g (0.5 mmol) of compound **3** in 16 mL of acetone was cooled for 15 min in an ice-water bath, then 1.4 mL of Jones reagent was added dropwise gradually so as to maintain the temperature not exceeding 0 °C. Stirring was continued for 1.5 h at room temperature. Then, the flask was placed in a water bath with a temperature of 10 °C, 6 mL of ethanol was added gradually and mixed for another 30 min. After this time, the mixture was poured into 15 mL of water with crushed ice, obtaining a pale-green precipitate. The precipitate was filtered, washed with water and dried in air. The crude product was purified by column chromatography (SiO_2, dichloromethane/ethanol, 20:1 v/v), yielding compound **8**.

Yield: 36%, m. p. 244–245 °C.

TLC (dichloromethane/ethanol, 40:1, v/v): R_f = 0.25.

^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 4.67 (br. s, 1H, H-29), 4.54 (br s, 1H, H-29), 4.02 (m, 4H, 2x OCH_2CH_3), 3.91 (m, 1H, H-3), 2.94 (m, 1H, H-19), 0.95–2.10 (m, 24H, CH, CH_2), 1.74(s, 3H, H-30), 1.36 (m, 6H, 2x OCH_2CH_3), 0.92 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.77 (s, 3H, CH_3), 0.73 (s, 3H, CH_3), 0.75 (m, 1H, H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ (ppm): 180.9; 150.4; 140.7; 109.7; 86.4; 63.5; 63.4; 56.3; 55.3; 50.4; 49.3; 46.9; 42.4; 40.7; 38.9; 38.8; 38.4; 37.0; 36.9; 34.3; 32.2; 30.6; 29.7; 28.0; 25.5; 25.4; 20.9; 19.3; 18.3; 16.2; 16.2; 16.1; 16.0; 4.7. ^{31}P NMR (243 MHz, $CDCl_3$) δ (ppm): −1.35. IR (KBr, cm^{-1}) ν: 3068 (O—H), 1715 (C═O), 1243 (P═O), 1019 (P—O—C). HR-MS (APCI) m/z: $C_{34}H_{56}O_6P$ [$(M-H)^-$], Calc. 591.3804; Found 591.3789.

4.3. X-ray diffraction experiment

A single crystal X-ray diffraction study was conducted for compound **3** and **4**. Colourless crystals were obtained from slow evaporation of a solution in acetone/DMF 10:1 and hexane/acetone 3:1, for **3** and **4** respectively.

The crystallographic data, data collection and structure refinement details as well as selected hydrogen-bond parameters are given in Tables S1–S3.

4.4. Molecular docking

All target macromolecules for molecular docking studies were obtained from the Protein Data Bank (https://www.rcsb.org/). We used 3D crystal structure of topoisomerase I (ID: 1TL8), topoisomerase IIα (ID: 5GWK), EGFRK (ID: 1M17), VEGFR2 (ID: 1Y6A), IKKβ (ID: 3RZF), Bcl-2 (ID: 2W3L) and PPARγ (ID: 5LSG).

The three-dimensional (3D) structures of all studied compounds required for virtual screening were generated in their low-energy conformation using Gaussian 16 (revision A.03) computer code [58] at the density functional theory (DFT, B3LYP [59]) and 6-311 + G(d,p) basis sets. In case of compounds **3** and **4**, calculations were performed using the X-ray coordinates as the input structure. Genetic Optimisation for Ligand Docking (GOLD) 5.6.3 [60] was used for the docking. The Hermes visualiser in the GOLD Suite was used to further prepare receptors for docking. The region of interest used for Gold docking was defined as all the protein residues within the 10 Å of the reference ligands that accompanied the downloaded protein complexes. Default values of all other parameters were used and the complexes were submitted to 10 genetic algorithm runs using the GOLDScore fitness function. Molecular docking details were visualized using the BIOVIA Discovery Studio virtual environment [61].

Acknowledgements

This work was supported by the National Science Centre, Poland [grant No. 2015/18/M/ST5/00060].

Calculations have been carried out using resources provided by Wroclaw Centre for Networking and Supercomputing (http://wcss.pl), Grant No. 382.

The authors thank dr Maria Książek (University of Silesia, Institute of Physics, Department of Physics of Crystals and Department of Solid State Physics, Katowice, Poland) for help in elaboration of the X-ray experiment.

Appendix A. Supplementary material

Supplementary files contain the following information: Experimental data such as information on cell culture and medium for biological activity tests as well as materials and methods used in the synthesis. The methodology for X-ray analysis (Table S1), monocrystals characteristics (Table S2) and selected hydrogen-bond parameters (Table S3). The detailed crystallographic data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/structures. The crystal structures have been allocated the deposition numbers CCDC: 1853751 for **3** and 1853752 for **4**. The ^{1}H, ^{13}C, ^{31}P NMR spectra of selected compounds. Interactions of betulin and phosphate derivatives with amino acids residues in binding pocket of topoisomerase I and IIα, EGFRK, VEGFR2, IKKβ, Bcl-2 and PPAR-γ (Tables S4–S10). Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.03.060.

References

[1] S. Nussbaumer, P. Bonnabry, J.L. Veuthey, S. Fleury-Souverain, Analysis of anticancer drugs: a review, Talanta 85 (2011) 2265–2289, https://doi.org/10.1016/j.

talanta.2011.08.034.
[2] C. Basmadjian, Q. Zhao, E. Bentouhami, A. Djehal, C.G. Nebigi, R.A. Johnson, M. Serova, A. Gramont, S. Faivre, E. Raymond, L.G. Désaubry, Cancer wars: natural products strike back, Front. Chem. 20 (2014) 1–18, https://doi.org/10.3389/fchem.2014.00020.
[3] G.M. Cragg, P.G. Grothaus, D.J. Newman, Impact of natural products on developing new anti-cancer agents, Chem. Rev. 109 (2009) 3012–3043, https://doi.org/10.1021/cr900019j.
[4] A.B. da Rocha, R.M. Lopes, G. Schwartsmann, Natural products in anticancer therapy, Curr. Opin. Pharmacol. 1 (4) (2001) 364–369, https://doi.org/10.1016/S1471-4892(01)00063-7.
[5] D.J. Newman, G.M. Cragg, Natural products as sources of new drugs over the 30 years from 1981 to 2010, J. Nat. Prod. 75 (2012) (1981) 311–335, https://doi.org/10.1021/np200906s.
[6] S. Alakurtti, T. Mäkelä, S. Koskimies, J. Yli-Kauhaluoma, Pharmacological properties of the ubiquitous natural product betulin, Eur. J. Pharm. Sci. 29 (2006) 1–13, https://doi.org/10.1016/j.ejps.2006.04.006.
[7] M.H. Cháirez-Ramírez, M.R. Moreno-Jiménez, R.F. González-Laredo, J.A. Gallegos-Infante, N.E. Rocha-Guzmán, Lupane-type triterpenes and their anti-cancer activities against most common malignant tumors: a review, EXCLI J. 15 (2016) 758–771, https://doi.org/10.17179/excli2016-642.
[8] C.A. Dehelean, S. Feflea, J. Molnár, I. Zupko, C. Soica, Betulin as an antitumor agent tested in vitro on A431, HeLa and MCF7, and as an angiogenic inhibitor *in vivo* in the CAM Assay, Nat. Prod. Commun. 7 (2012) 981–985.
[9] S. Fulda, Betulinic acid for cancer treatment and prevention, Int. J. Mol. Sci. 9 (2008) 1096–1107, https://doi.org/10.3390/ijms9061096.
[10] R. Luo, D. Fang, P. Chu, H. Wu, Z. Zhang, Z. Tang, Multiple molecular targets in breast cancer therapy by betulinic acid, Biomed. Pharmacother. 84 (2016) 1321–1330, https://doi.org/10.1016/j.biopha.2016.10.018.
[11] M. Novakovic, J. Nikodinovic-Runic, J. Veselinovic, T. Ilic-Tomic, V. Vidakovic, V. Tesevic, S. Milosavljevic, Bioactive pentacyclic triterpene ester derivatives from *Alnus viridis* ssp. *viridis* Bark, J. Nat. Prod. 80 (2017) 1255–1263, https://doi.org/10.1021/acs.jnatprod.6b00805.
[12] O. Kadioglu, T. Efferth, Pharmacogenomic characterization of compounds from *Salvia officinalis* in cancer cells, J. Nat. Prod. 78 (2015) 762–775, https://doi.org/10.1021/np501007n.
[13] N. Sathishkumar, S. Sathiyamoorthy, M. Ramya, H.D.-U. Yang, Y. Lee, D.-C. Yang, Molecular docking studies of anti-apoptotic BCL-2, BCL-XL and MCL proteins with ginsenosides from Panax ginseng, J. Enzyme Inhib. Med. Chem. 27 (2012) 685–692, https://doi.org/10.3109/14756366.2011.608663.
[14] Y.-A. Yang, W.-J. Tang, X. Zhang, J.-W. Yuan, X.-H. Liu, H.-L. Zhu, Synthesis, molecular docking and biological evaluation of glycyrrhizin analogs as anticancer agents targeting EGFR, Molecules 19 (2014) 6368–6381, https://doi.org/10.3390/molecules19056368.
[15] B.S. Gill, Navgeet, R. Mehra, V. Kumar, S. Kumar, Ganoderic acid, lanostanoid triterpene: a key player in apoptosis, Invest. New Drugs 36 (2018) 136–143, https://doi.org/10.1007/s10637-017-0526-0.
[16] A. Falamas, S. Cînta Pînzaru, V. Chis, C. Dehelean, Spectroscopic investigations of newly formed betulin-cyclodextrin guest–host type complexes as potential anti skin cancer candidates, J. Mol. Struct. 993 (2011) 297–301, https://doi.org/10.1016/j.molstruc.2010.11.044.
[17] R. Csuk, A. Barthel, R. Kluge, D. Ströhl, Synthesis, cytotoxicity and liposome preparation of 28-acetylenic betulin derivatives, Bioorg. Med. Chem. 18 (2010) 7252–7259, https://doi.org/10.1016/j.bmc.2010.08.023.
[18] M. Mioc, I.Z. Pavel, R. Ghiulai, D.E. Coricovac, C. Farca, C.-V. Mihali, C. Oprean, V. Serafim, R.A. Popovici, C.A. Dehelean, M.I. Shtilman, A.M. Tsatsakis, C. Soica, The cytotoxic effects of betulin-conjugated gold nanoparticles as stable formulations in normal and melanoma cells, Article 249 (1-16), Front. Pharmacol. 9 (2018), https://doi.org/10.3389/fphar.2018.00429.
[19] J.B. Zawilska, J. Wojcieszak, A.B. Olejniczak, Prodrugs: a challenge for the drug development, Pharmacol. Rep. 65 (2013) 1–14.
[20] J.B. Rodriguez, C. Gallo-Rodriguez, The role of the phosphorus atom in drug design, ChemMedChem 14 (2019) 190–216, https://doi.org/10.1002/cmdc.201800693.
[21] S.-D. Clas, R.I. Sanchez, R. Nofsinger, Chemistry-enabled drug delivery (prodrugs): recent progress and challenges, Drug Discov. Today 19 (2014) 79–87, https://doi.org/10.1016/j.drudis.2013.08.014.
[22] J. Tatarkiewicz, A. Staniszewska, M. Bujalska-Zadrożny, New agents approved for treatment of acute staphylococcal skin infections, Arch. Med. Sci. 12 (2016) 1327–1336, https://doi.org/10.5114/aoms.2016.59838.
[23] https://www.accessdata.fda.gov/drugsatfda_docs/nda/2018/209299Orig1s000TOC.cfm; May 15, 2018.
[24] M.J. Robertson, C.P. Gordon, J. Gilbert, A. McCluskey, J.A. Sakoff, Norcantharimide analogues possessing terminal phosphate esters and their anti-cancer activity, Bioorg. Med. Chem. 19 (2011) 5734–5741, https://doi.org/10.1016/j.bmc.2011.01.031.
[25] L. Huang, G.G. Mackenzie, Y. Sun, N. Ouyang, G. Xie, K. Vrankova, D. Komninou, B. Rigas, Chemotherapeutic properties of phospho-nonsteroidal anti-inflammatory drugs, a new class of anticancer compounds, Cancer Res. 71 (2011) 7617–7627, https://doi.org/10.1158/0008-5472.CAN-11-2349.
[26] J.M. Pezzuto, J.W. Kosmeder, Z.-Q. Xu, N.E. Zhou, M.E. Goldsmith, Method of preparing and use of prodrugs of betulonic acid derivatives, US Patent 6569842 B2, 2003.
[27] M. Amjad, R.M. Carlson, P. Krasutsky, M.R.-U. Karim, Inhibition of Epstein-Barr Virus by the triterpenoid betulin diphosphate and uvaol, J. Microb. Biotechnol. (2004) 1086–1088.
[28] S. Bureeva, J. Andia-Pravdivy, A. Symon, A. Bichucher, V. Moskaleva, V. Popenko, A. Shpak, V. Shvets, L. Kozlov, A. Kaplun, Selective inhibition of the interaction of C1q with immunoglobulins and the classical pathway of complement activation by steroids and triterpenoids sulfates, Bioorg. Med. Chem. 15 (2007) 3489–3498, https://doi.org/10.1016/j.bmc.2007.03.002.
[29] O. Vorobyova, O. Deryabina, D. Malygina, N. Plotnikova, A. Solovyeva, K. Belyaeva, N. Melnikova, Betulin-3,28-diphosphate as a component of combination cytostatic drugs for the treatment of Ehrlich Ascites Carcinoma in vitro and in vivo experiments, Sci. Pharm. 86 (2018) 1–14, https://doi.org/10.3390/scipharm86020017.
[30] P.A. Krasutsky, R.M. Carlson, R. Karim; Triterpenes having human antifungal and antiyeast activity, US Patent 6642217 B2, 2003.
[31] P.A. Krasutsky, R.M. Carlson, R. Karim; Triterpenes having antibacterial activity. US Patent 0119935 A1, 2002.
[32] C. Grison, N. Barthes, C. Finance, R.E. Duval, Synthesis and antibacterial activity of novel enolphosphate derivatives, Bioorg. Chem. 38 (2010) 218–223, https://doi.org/10.1016/j.bioorg.2010.06.006.
[33] B. Favre, N.S. Ryder, Characterization of squalene epoxidase activity from the dermatophyte Trichophyton rubrum and its inhibition by Terbinafine and other antimycotic agents, Antimicrob. Agents Chemother. 40 (1996) 443–447.
[34] D.F. Archer, F.Z. Stanczyk, A. Rubin, M. Foegh, Ethinyl estradiol and levonorgestrel pharmacokinetics with a low-dose transdermal contraceptive delivery system, AG200-15: a randomized controlled trial, Contraception 85 (2012) 595–601, https://doi.org/10.1016/j.contraception.2011.10.006.
[35] P. Villani, M.B. Regazzi, F. Castelli, P. Viale, C. Torti, E. Seminari, R. Maserati, Pharmacokinetics of efavirenz (EFV) alone and in combination therapy with nelfinavir (NFV) in HIV-1 infected patients, Br. J. Clin. Pharmacol. 48 (1999) 712–715.
[36] K.C. Lee, J.J. Chen, Transdermal selegiline for the treatment of major depressive disorder Neuropsych, Dis. Treat. 3 (2007) 527–537.
[37] L. Landi, F. Cappuzzo, Experience with erlotinib in the treatment of non-small cell lung cancer, Ther. Adv. Respir. Dis. 9 (2015) 146–163, https://doi.org/10.1177/1753465815588053.
[38] R. Csuk, A. Barthel, R. Sczepek, B. Siewert, S. Schwarz, Synthesis, encapsulation and antitumor activity of new betulin derivatives, Arch. Pharm. Chem. Life Sci. 1 (2011) 37–49, https://doi.org/10.1002/ardp.201000232.
[39] O.B. Kazakova, G.V. Giniyatullina, E.Yu. Yamansarov, G.A. Tolstikov, Betulin and ursolic acid synthetic derivatives as inhibitors of *Papilloma virus*, Bioorg. Med. Chem. Lett. 20 (2010) 4088–4090, https://doi.org/10.1016/j.bmcl.2010.05.083.
[40] S.F. Vasilevsky, A.I. Govdi, E.E. Shults, M.M. Shakirov, I.V. Sorokina, T.G. Tolstikova, D.S. Baev, G.A. Tolstikov, I.V. Alabugin, Efficient synthesis of the first betulonic acid–acetylene hybrids and their hepatoprotective and anti-inflammatory activity, Bioorg. Med. Chem. 17 (2009) 5164–5169, https://doi.org/10.1016/j.bmc.2009.05.059.
[41] S. Boryczka, E. Bębenek, J. Wietrzyk, K. Kempińska, M. Jastrzębska, J. Kusz, M. Nowak, Synthesis, structure and cytotoxic activity of new acetylenic derivatives of betulin molecules, Molecules 18 (2013) 4526–4543, https://doi.org/10.3390/molecules18044526.
[42] E. Chrobak, E. Bębenek, M. Kadela-Tomanek, M. Latocha, C. Jelsch, E. Wenger, S. Boryczka, Betulin phosphonates; synthesis, structure, and cytotoxic activity, Molecules 21 (2016) 1123, https://doi.org/10.3390/molecules21091123.
[43] V. Abet, F. Filace, J. Recio, J. Alvarez-Builla, C. Burgos, Prodrug approach: An overview of recent cases, Eur. J. Med. Chem. 127 (2017) 810–827, https://doi.org/10.1016/j.ejmech.2016.10.061.
[44] D. Dheer, V. Singh, R. Shankar, Medicinal attributes of 1,2,3-triazoles: Current developments, Bioorg. Chem. 71 (2017) 30–54, https://doi.org/10.1016/j.bioorg.2017.01.010.
[45] G.R. Desiraju, T. Steiner, The weak hydrogen bond, Structural Chemistry and Biology, Oxford University Press, 2001.
[46] C.-M. Wang, K.-L. Yeh, S.-J. Tsai, Y.-L. Jhan, C.-H. Chou, Anti-proliferative activity of triterpenoids and sterols isolated from *Alstonia scholaris* against non-small-cell lung carcinoma cells, Molecules 22 (2017) 2119, https://doi.org/10.3390/molecules22122119.
[47] A. Mandal, A. Ghosh, S. Ghosh, S. Shil, A.K. Bothra, P. Ghosh, 3-Epihydroxy lup-20(29)-en-19(28)-olide: partial synthesis, antitopoisomerase activity, and 3D molecular docking, Med. Chem. Res. 25 (2016) 1087–1095, https://doi.org/10.1007/s00044-016-1551-9.
[48] H.L. McLeod, F. Douglas, M. Oates, R.P. Symonds, D. Prakash, A.G. van der Zee, S.B. Kaye, R. Brown, W.N. Keith, Topoisomerase I and II activity in human breast, cervix, lung and colon cancer, Int. J. Cancer 59 (1994) 607–611.
[49] S.A. Abdelatef, M.T. El-Saadi, N.H. Amin, A.H. Abdelazeem, H.A. Omar, K.R.A. Abdellatif, Design, synthesis and anticancer evaluation of novel spirobenzo [h]chromene and spirochromane derivatives with dual EGFR and B-RAF inhibitory activities, Eur. J. Med. Chem. 150 (2018) 567–578, https://doi.org/10.1016/j.ejmech.2018.03.001.
[50] F. Tian, P. Zhou, W. Kang, L. Luo, X. Fan, J. Yan, H. Liang, The small-molecule inhibitor selectivity between IKKα and IKKβ kinases in NF-κB signaling pathway, J. Recept. Signal Transduct. Res. 35 (2015) 307–318, https://doi.org/10.3109/10799893.2014.980950.
[51] J. Pinski, T.B. Dorff, Prostate cancer metastases to bone: pathophysiology, pain management and the promise of targeted therap, Eur. J. Cancer 41 (2005) 932–940, https://doi.org/10.1016/j.ejca.2004.12.026.
[52] H. Yang, Q.P. Dou, Targeting apoptosis pathway with natural terpenoids: Implications for treatment of breast and prostate cancer, Curr. Drug Targets 11 (2010) 733–744.
[53] A.J. Kroker, J.B. Bruning, Review of the structural and dynamic mechanisms of PPAR?? Partial agonism, Hindawi Publishing Corporation, 2015 Article ID 816856. https://doi.org/10.1155/2015/816856.

[54] P. Sertznig, M. Seifert, W. Tilgen, J. Reichrath, Present concepts and future outlook: function of Peroxisome Proliferator-Activated Receptors (PPARs) for pathogenesis, progression, and therapy of cancer, J. Cell. Physiol. 212 (2007) 1–12, https://doi.org/10.1002/jcp.20998.

[55] G. Brusotti, R. Montanari, D. Capelli, G. Cattaneo, A. Laghezza, P. Tortorella, F. Loiodice, F. Peiretti, B. Bonardo, A. Paiardini, E. Calleri, G. Pochetti, Betulinic acid is a PPARγ antagonist that improves glucose uptake, promotes osteogenesis and inhibits adipogenesis, Sci. Rep. 7 (2017) 1–14, https://doi.org/10.1038/s41598-017-05666-6.

[56] J. Wietrzyk, M. Chodynski, H. Fitak, E. Wojdat, A. Kutner, A. Opolski, Antitumor properties of diastereomeric and geometric analogs of vitamin D3, Anticancer Drugs 18 (2007) 447–457, https://doi.org/10.1097/CAD.0b013e3280143166.

[57] Y. Deng, J.K. Snyder, Preparation of a 24-nor-1,4-dien-3-one triterpene derivative from betulin: A new route to 24-nortriterpene analogues, J. Org. Chem. 67 (2002) 2864–2873, https://doi.org/10.1021/jo010929h.

[58] M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, G. Scalmani, V. Barone, G.A. Petersson, H. Nakatsuji, X. Li, M. Caricato, A.V. Marenich, J. Bloino, G. Janesko, R. Gomperts, B. Mennucci, H.P. Hratchian, J.V. Ortiz, A.F. Izmaylov, J.L. Sonnenberg, D. Williams-Young, F. Ding, F. Lipparini, F. Egidi, J. Goings, B. Peng, A. Petrone, T. Henderson, D. Ranasinghe, V.G. Zakrzewski, J. Gao, N. Rega, G. Zheng, W. Liang, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, K. Throssell, J.A. Montgomery, J.E. Peralta, F. Ogliaro, M.J. Bearpark, J.J. Heyd, E.N. Brothers, K.N. Kudin, V.N. Staroverov, T.A. Keith, R. Kobayashi, J. Normand, K. Raghavachari, A.P. Rendell, J.C Burant, S.S. Iyengar, J. Tomasi, M. Cossi, J.M. Millam, M. Klene, C. Adamo, R. Cammi, J.W. Ochterski, R.L. Martin, K. Morokuma, O. Farkas, J.B. Foresman, D.J. Fox, Gaussian 16, Revision A. 03. 2016, Gaussian Inc., Wallingford CT, 2016.

[59] P.J. Stephens, F.J. Devlin, C.F. Chablowski, M. Frisch, Ab initio calculation of vibrational absorption and circular dichroism spectra Rusing density functional force fields, J. Phys. Chem. 98 (1994) 11623–11627, https://doi.org/10.1021/j100096a001.

[60] J.C. Cole, J.W.M. Nissink, In: R. Taylor, B. Shoichet, J. Alvarez (Eds.), Protein-Ligand Docking and Virtual Screening with GOLD in Virtual Screening in Drug Discovery, Taylor & Francis CRC Press, Boca Raton, FL, USA, 2005 https://doi.org/10.1201/9781420028775.ch15.

[61] Dessault Systemes BIOVIA. Discovery Studio Modeling Environment; Release 2017; De-ssault Systemes: San Diego, CA, USA, 2016.