European Journal of Medicinal Chemistry 96 (2015) 482–490

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis of cytotoxic 2,2-difluoroderivatives of dihydrobetulinic acid and allobetulin and study of their impact on cancer cells

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ARTICLE INFO

Article history:
Received 13 November 2014
Received in revised form
29 March 2015
Accepted 31 March 2015
Available online 1 April 2015

Keywords:
Betulinic acid
Fluoroderivatives
Synthesis
Cytotoxic
Testing

ABSTRACT

In this article, we describe the preparation and cytotoxic properties of a small focused library of lupane and 18α-oleanane triterpenoids that contain a combination of two structural motifs known to enhance the biological activities. First, we introduced two fluorine atoms to position 2 of the skeleton. Second, we synthesized a set of hemiester prodrugs, which were intended to increase the solubility and activity. Starting from betulin, we obtained two hydroxyketones (derivatives of dihydrobetulinic acid and allobetulin) and their fluorination using DAST provided 2,2-difluoro-3-oxo-compounds as the main products. Then the 3-oxo group in each derivative was reduced by $NaBH_4$ to obtain 3β-hydroxy compounds suitable for modifying by various hemiesters. We prepared 21 compounds, 11 of them new, their cytotoxicity was tested on T lymphoblastic leukemia CCRF-CEM cells first and the most active derivatives were selected for screening on another six tumor and two non-tumor cell lines. All of them showed selectivity against cancer lines with therapeutic index between 2 and 8. All hemiesters had activity in the same range as the free hydroxyl derivatives and they would be suitable prodrugs for future *in vivo* experiments. Interestingly, all hemiesters of 2,2-difluorodihydrobetulonic acid had higher activity against p53 knock-out p53−/− cancer cell line than against the non-mutated analog. In active derivatives, the cell cycle was analyzed by flow cytometry and several compounds slowed down cell cycle progression through G0/G1 or S-phase.

1. Introduction

Triterpenoids belong to a large family of natural compounds with a wide range of biological activities [1,2]. Pentacyclic triterpenoids have anticancer [3], chemopreventive [4], immunosuppressive [5], anti-inflammatory [6], anti-HIV [7,8], antitrypanosomal [9], and antimicrobial properties [6]. The promising anticancer and anti-HIV activities led to an increased research activity in this field. Commonly, however, the pharmacological properties (such as low solubility in water based media and low bioavailability) of those active compounds are not suitable for their use in medicine. Also, the IC_{50} values are often insufficient. Many research groups have tried to improve the properties of the most promising terpenoids, which would make them useful candidates for HIV and cancer treatment. These studies have either explored new plant species or modified the structures of known active compounds. Among hundreds of new compounds, we have synthesized many derivatives of lupane that are highly cytotoxic on a variety of cancer cell lines *in vitro*, including those that are resistant to current cytostatics [10–16]. This allowed us to make simplified structure-activity relationship assumptions and to find some trends of how the chemical structure may affect the anti-tumor activity. We found that in general, increasing hydrophobicity leads to less

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http://dx.doi.org/10.1016/j.ejmech.2015.03.068
0223-5234/

active compounds while introducing an electronegative substituent to the position 2 of lupane derivatives increases the cytotoxicity.

Previously, we studied a general possibility of introducing one or two atoms of fluorine into the terpenic structures using DAST, and as expected, the result was a group of hydrophobic triterpenes where fluorine replaced carboxyl, carbonyl or hydroxy groups [17]. In agreement with the general trend, there was no cytotoxic activity within this group of hydrophobic fluoroderivatives. However, there is a literature precedent of addition of dichloro- and dibromocarbene to 20(29)-double bonds of some terpenic structures. Resulting products had very high inhibitory activity on human melanoma cells Colo 38 and Bro.92 [18]. This motivated us to synthesize triterpenic difluorocyclopropanes, analogous adducts of difluorocarbene to the activated double bonds of several triterpenes that we prepared earlier. The resulting compounds, however, were inactive [19].

Based on all our previous work with cytotoxic triterpenes, knowing that an electronegative substituent (EWG) in the position 2 always improved the cytotoxicity [11,14,17,19], we expected the only way to increase the cytotoxicity by adding fluorine to a triterpene is to add the fluorine atoms to the position 2 of the triterpenoid skeleton (preferably dihydrobetulinic acid). Therefore in this work, we used and further optimized the previously [17] developed method of fluorination of 2-hydroxyallobetulone (**12**) with DAST and introduced two fluorine atoms to C-2. It was expected, that the resulting compounds may be highly hydrophobic which can cause associated problems, such as low solubility and bioavailability. Therefore we decided to prepare hemiester prodrugs from the resulting difluoroderivatives in order to decrease the overall hydrophobicity. Hemiesters are well known from Bevirimat (3β-*O*-3′,3′-dimethylsuccinylbetulinic acid), a compound that inhibits the maturation of the HIV particles by interacting with the GAG protein [20]. Solubility of Bevirimat in water-based media was increased significantly by using cyclodextrines and we expected that it would be possible to use similar method in our new fluoroderivatives.

2. Results and discussion

2.1. Chemistry

A three-step process was used to transform betulin (**1**) to dihydrobetulin-3β-*O*-acetate (**2**), which was further transformed to acetyldihydrobetulinic acid (**3**), dihydrobetulinic acid (**4**), and benzyl dihydrobetulinate (**5**), consecutive oxidation of **5** gave benzyl-dihydrobetulonate (**6**) − **64 % over all 4 steps** [21,22]. Derivative **6** was oxidized by MCPBA to give benzyl-2α-hydroxydihydrobetulonate (**7**) (Scheme 1), which was then treated with DAST in chloroform in presence one equivalent of pyridine [17] to give difluoroderivative **8** at 68% yield (without pyridine, this reaction only gives yield of 40%). Benzyl-esters were deprotected using a catalytic hydrogenolysis on Pd/C catalyst at room temperature under 0.5 MPa of hydrogen. 3-oxoderivatives **8** and **9** were reduced by $NaBH_4$ in THF/MeOH yielding 3β-hydroxyderivatives **10** and **11**. In theory both, α and β-epimers may form by a reduction of 3-oxocompounds to the corresponding alcohols. However, it is well known [23,24] that in triterpenes, the sterical arrangement of the skeleton directs the reaction towards beta alcohols predominantly; and in this case, we did not isolate any α-epimers. We used the original procedure [17] to transform hydroxyketone **12** to 2,2-difluorollobetulone **13** from which 2,2-difluoroallobetulin **14** was accessible by reduction with $NaBH_4$. As a result, we obtained a set of 2,2-difluoroderivatives **8**–**11**, **13**, and **14**.

Knowing that the hemiester type may play significant role in the biological activity (for example see anti HIV activity of bevirimat analogs), we also prepared small sets of various hemiesters from both **11** and **14** and obtained compounds **15**–**21**. While the derivatives of lupane **9**, **11**, **15**–**18** had always interesting IC_{50}, the allobetulin analogs **13**, **14**, **19**–**21** were always inactive, therefore we only synthesized three hemiesters (**19**–**21**) allobetulin analogs (Scheme 2).

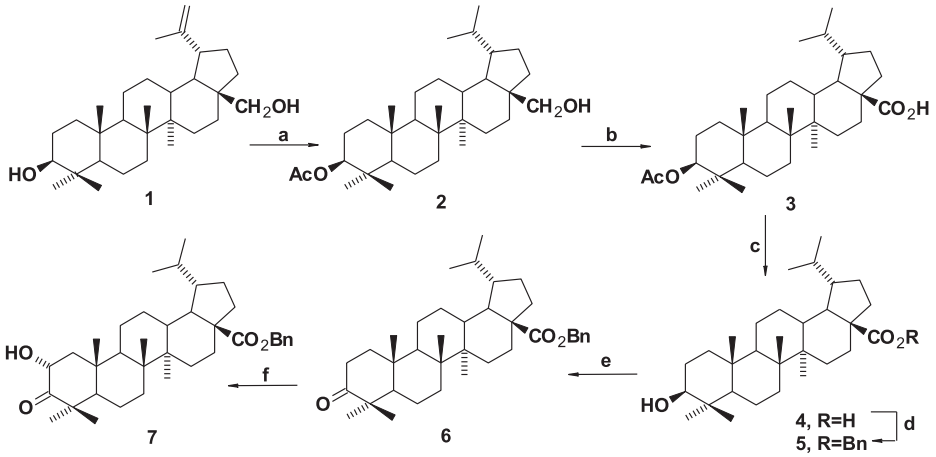
All acylations were performed using the corresponding anhydride in a presence of DMAP in refluxing *sym*-collidine (2,4,6-trimethylpyridine) under argon atmosphere to give desired products **15**–**21**. Yields of hemiesters were generally low due to harsh reaction conditions (about 180 °C), which caused side reactions. Also, during the general work up procedure a portion of the organic material was not extracted from the aqueous phase because of the ambivalent characters of the products. The biological part of this study was extended by a known [16] 2,2-dibromodihydrobetulonic acid (**22**) because we wanted to compare the activities of the compound **22** with our new 2,2-difluoroderivatives.

2.2. Biological assay

2.2.1. Cytotoxicity

Cytotoxic activity of all synthesized compounds and intermediates was investigated. All prepared compounds were tested for their *in vitro* cytotoxic activity against human T-lymphoblastic leukemia cell line CCRF-CEM using the standard MTT test (Table 1).

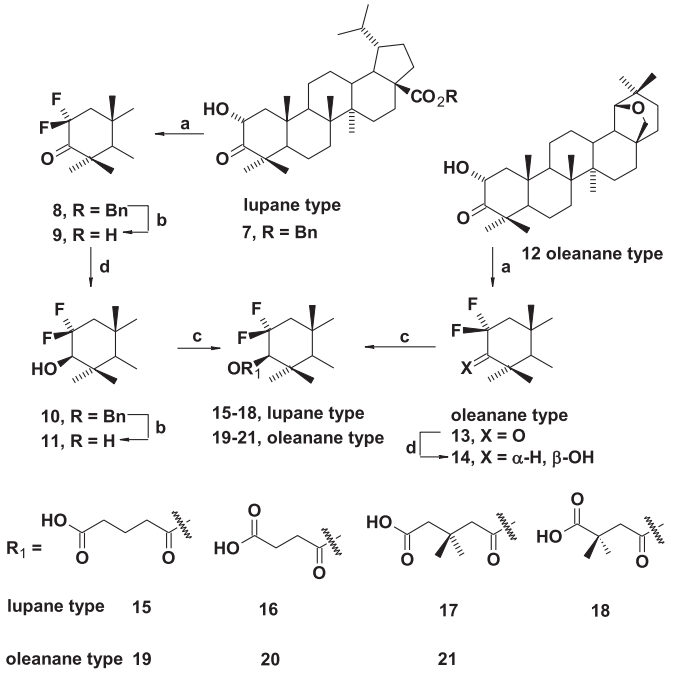
All 18α-oleanane derivatives had cytotoxicity in high micromolar ranges. New 2,2-difluoroderivatives of dihydrobetulinic acid had high cytotoxic activity (IC_{50} < 10 μmol/L) when they contained a free 28-COOH group. The most active derivative of the presented set of compounds was 2,2-difluorodihydrobetulonic acid (**9**) and its analog 2,2-dibromodihydrobetulonic acid (**22**), which is in agreement with our previous results, that lupane 3-oxoderivatives are generally more cytotoxic than 3-hydroxyderivatives. Introduction of halogen atoms to position 2 increased the cytotoxicity from IC_{50} = 4 μM (dihydrobetulonic acid, see Ref. [16]) to 2.4 μM (2,2-difluorodihydrobetulonic acid **9**) or to 1.0 μM (2,2-dibromorodihydrobetulonic acid **22**). A greater improvement was found in 3-hydroxyderivatives, where the activity went from IC_{50} = 9 μM (dihydrobetulinic acid **4**) to 4.0 μM (2,2-difluorodihydrobetulinic acid **11**), although the cytotoxicity was worse than in 3-oxoderivatives. This confirmed our original hypothesis that a strong electron withdrawing group(s) in the position 2 of a triterpenic skeleton increases the cytotoxicity of lupane derivatives. The activity of all lupane hemiesters was in the same range and it was the same or slightly worse than the activity of a free 2,2-difluorodihydrobetulinic acid (**11**). We concluded that the hemiester group is not an important part of the pharmacophore, which is in sharp contrast to the role of the dimethylhemisuccinate group in bevirimat in which the hemiester is essential to improve the strong non covalent interaction between betulinic acid and the viral GAG protein which results in blocking of the HIV protease and maturation of the virus particles as found in lit [20]. Although not important for the activity, the introduction of a hemiester improved the solubility in water-based media and in combination with additives such as cyclodextrines, compounds fully soluble in water may be obtained. These will be a major focus of our future *in vivo* tests. Hemiesters made from 2,2-difluoroallobetulin (**14**), compounds **19**–**21** were much less active than those made from 2,2-difluorobetulinic acid (**11**), compounds **15**–**18**, however, they were more active than 2,2-difluoroallobetulin (**14**). This corresponds with the fact that allobetulin derivatives are almost always less active than analogous lupane derivatives and even the presence of a hemisuccinate is not sufficient to decrease the IC_{50} to low micromolar ranges. Hemiesters though are suitable prodrugs for 2,2-difluoroderivatives of triterpenes, as they possibly improve the



Scheme 1. Reagents and conditions: (a) i: Ac_2O, pyridine, r.t., 14 h; ii: H_2/Ra–Ni, methoxyethanol, THF, 48 h, 1 MPa, r.t.; iii: KOH (1 equiv), toluene, EtOH, r.t.; (b) RuO_2, $NaIO_4$, EtOAc, MeCN, H_2O, 55 °C, 140 min; (c) excess KOH, toluene, EtOH, reflux; (d) BnBr, K_2CO_3, DMF, MeCN; (e) $Na_2Cr_2O_7$, NaOAc, AcOH, Ac_2O, toluene, 22 h, r.t.; (f) MCPBA (added in several portions), H_2SO_4, CH_2Cl_2, MeOH, 17 h, r.t.

pharmacological properties including their bioavailability. Since the activities of the hemiesters prepared in this study are in the same range of IC_{50}, we may conclude that the simplest hemiester – hemisuccinate is fully acceptable.

The most active derivatives (**9**, **11**, **15**–**18**) were further tested on six cancer cell lines (Table 2) of different histogenetic origin and on two types of normal human fibroblasts. All active derivatives showed selective cytotoxicity in low micromolar range on most of the cancer cell lines, compound **9** proved to be the best. Hemiesters **15**–**18** showed interesting selectivity against p53 knock-out HCT116p53−/− cancer cell line. On the other hand, they were less active against daunorubicin resistant CEM cell line than against nonresistant. Although selective, the calculated therapeutic index is between 2 and 8, therefore our future research must be oriented towards the improvement of the selectivity.

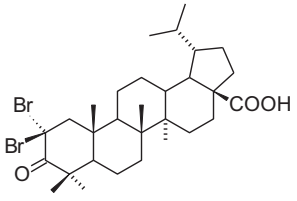


Scheme 2. Reagents and conditions: (a) DAST, pyridine, $CHCl_3$, r.t. 11 h; (b) H_2, Pd/C (10%), THF, MeOH, r.t., 0.5 MPa; (c) anhydride of each diacid, DMAP, *sym*-collidine, reflux under Ar; (d) $NaBH_4$, MeOH, THF, 0 °C.

2.2.2. Cell cycle analysis

As a part of the study into the mechanism of action of these compounds, and to further characterize the anti-tumor properties, we used flow cytometry to analyze the influence of the most active compounds on the cell cycle in most sensitive CCRF-CEM cells (Table 3). After the 24 h treatment with 1×, 2× and 5× IC_{50} concentration, the sub-G1 population was below 10% of total cells (with two exceptions, derivatives **19** and **22**), which indicates the use of reasonable dosing range and timing of the cell death. In derivative **19**, rapid induction of apoptosis demonstrated by high percentage of apoptotic cells within 24 h was observed with dependency on concentration. This can be caused either by the prodrug activity or by a different mechanism of action in this compound. There was no dominant interference with the cell cycle regulation caused by any of the derivatives, however compounds **4**,

Table 1
Cytotoxic activities of all compounds from this work on reference cell line CCRF-CEM. The MTT assays were performed in doublets.



Compound	IC_{50} (μmol/L[a])	Compound	IC_{50} (μmol/L[a])
	CEM		CEM
1	250	**12**	250
2	250	**13**	250
3	14	**14**	250
4	9	**15**	4
5	69	**16**	8
6	68	**17**	5
7	16	**18**	5
8	70	**19**	24
9	2	**20**	102
10	13	**21**	127
11	4	**22**	1[b]

The standard deviation in cytotoxicity assays is typically up to 10% of the average value.
[a] 50% inhibiton concentration.
[b] Published in Ref. [16].

Table 2
Cytotoxic activities of selected compounds on 7 tumor (including resistant) and two normal fibroblast cell lines.

Compound	IC_{50} (μmol/L)									
	CEM	CEM-DNR	A549	K562	K562-TAX	HCT116	HCT116p53$^{-/-}$	BJ	MRC-5	TI[a]
9	02-Apr	06-Feb	05-Aug	02-Jun	02-Mar	04-Apr	04-Jul	15-Apr	09-Jan	05-Jan
11	4.0	10-Sep	06-Jul	05-May	04-Jan	07-Mar	05-Aug	18-Jul	14-Jul	04-Feb
15	04-Jan	14-Jul	10-Jul	06-May	05-Aug	06-Jan	05-Sep	34.7	29.2	07-Aug
16	08-Mar	24-Apr	17-Apr	18-Mar	19-Aug	14-Aug	08-Jun	76.6	27-Sep	06-Mar
17	06-Feb	15-Jul	13-Mar	12-Sep	16.0	14-Feb	07-Jan	39.6	14-Jul	04-Apr
18	04-May	11-Apr	11-Apr	11-Aug	11-May	08-May	03-Jul	26.0	12-Aug	04-Mar

[a] Therapeutic index calculated for CCRF-CEM line vs both fibroblasts.

7, **11**, **15**, **19**, **22** blocked or slowed down the cell cycle progression through G0/G1 or S-phase. Dihydrobetulinic acid (**4**) and 2,2-dibromodihydrobetulonic acid (**22**) were prepared during our previous work [16] and they had high cytotoxic activity. It was interesting to study how the cell cycle and DNA or RNA synthesis would be influenced by replacing hydrogen atoms in the position 2 by isosteric fluorine or bromine atoms. We found that both compounds **4** and **22** had similar influence on the cell cycle and DNA or RNA synthesis, however, 2,2-difluorodihydrobetulinic acid (**11**) was inhibiting RNA synthesis in 5× concentration. This is in concordance with the accumulation of the cells in the S phase of the cell cycle. Compounds **11**, **15**, **19**, and **22** in general decreased the DNA synthesis rate of CCRF-CEM cells. All 2,2-difluoroderivatives of betulinic acid were highly cytotoxic, which supports our hypothesis, that electronegative substituents such as fluorine or bromine in the position 2 of betulinic acid (with free carboxyl) enhance the cytotoxic activity.

2.2.3. Structure-property relationships

The solubility of both, betulinic acid and allobetulin derivatives in water based media is low but it can be significantly enhanced by esterification of the 3-OH group with a hemiester and using cyclodextrine formulas.

When we put our results of this simplified structure-activity relationship study into the context with the literature precedents, we get some general trends. It should be mentioned, however, that those assumption may be ambiguous because researchers worldwide often use different cell lines and their protocols vary in details. The trends we found are:

Table 3
Summary of conventional cell cycle, apoptosis, RNA/DNA – BrU/BrDU analysis of CEM leukemia cell line treated with the most potent compounds **4**, **7**, **11**, **15**, **19** and **22**. Data are expressed as a percentage of positive cells in the total cell population.

Derivatives	Cell cycle phases (%)						
	IC_{50}	Apoptosis	$G_{0/1}$	S	G_2/M	BrDU+	BrU+
control	0	2.20	43.00	45.00	12.00	62.50	47.80
4	1×	5.30	34.40	51.40	14.20	64.50	46.70
	2×	5.50	33.10	49.60	17.30	64.71	37.14
	5×	5.30	28.80	62.30	9.00	55.60	33.00
7	1×	2.00	38.30	50.00	11.70	61.90	53.60
	2×	1.90	36.20	49.60	14.20	63.38	50.89
	5×	2.00	32.40	56.40	11.20	61.30	40.00
11	1×	2.50	39.70	50.20	10.20	61.40	35.60
	2×	2.70	46.80	42.60	10.60	62.96	37.16
	5×	8.50	31.60	57.50	11.00	34.80	1.70
15	1×	3.00	45.60	43.90	10.50	60.00	39.40
	2×	3.60	48.60	40.00	11.40	60.87	42.91
	5×	2.30	44.20	39.40	16.40	41.30	30.13
19	1×	3.10	42.00	45.60	12.40	57.50	48.00
	2×	9.10	48.20	41.50	10.30	52.46	46.29
	5×	16.50	41.70	43.26	15.03	36.73	30.02
22	1×	4.80	37.60	51.00	11.40	64.40	52.50
	2×	18.10	43.60	48.30	8.10	65.88	55.26
	5×	26.20	33.20	52.90	13.90	45.10	39.60

1. The lowest IC_{50} can be found among the derivatives of betulinic acid while the activities of betulin and allobetulin analogs are generally worse [13,16,25]. In addition, in betulinic acid derivatives, the carboxylic function should be free [11,12,26].
2. Electronegative substituent at C-2 lowers the IC_{50} (improves the activity). It has previously been found in 2-carbonitrile derivatives [27], diosphenols [11], 2-halogenoderivatives [16,27], enamines [28], and here we report it in 2-fluoroderivatives.
3. 3-oxoderivatives are often more cytotoxic than 3-hydroxyderivatives, we saw it in our previous work [11,14,29] and we report it here. Dihydrobetulinic derivatives are slightly more active than the same analogs of betulinic acid (containing double bond 20(29)) [16,29].

Our recent book chapter [26] summarizes information from all papers about the influence of various prodrug-like substituents at 28-COOH and 3-OH in triterpenes on their biological activity and properties.

3. Conclusion

We prepared a small library of 21 compounds (11 of them new), derivatives of lupane and 18α-oleanane. The main aim was to investigate cytotoxicity of 2,2-difluoroderivatives because based on the data from analogs previously investigated; an electronegative substituent (EWG) on C-2 can enhance the biological activity. Starting from betulin (**1**), we prepared several derivatives with two fluorine atoms on C-2 and from the hydroxyanalogues, small sets of various hemiesters. The tests of cytotoxicity showed that the introduction of fluorine atoms to position 2 of triterpenic skeleton increased the cytotoxicity significantly (2.25× in lupane derivatives). The activity was selective against six cancer cell lines with rather low therapeutic indexes of 2–8. Hemiesters prepared from the active lupane derivatives had similar cytotoxicity to the parent compounds and there was almost no difference between various types of hemiesters, however, in allobetulin derivatives, the presence of the hemiester enhanced the activity >10×. We expect that they will show better pharmacological properties and bioavailability in *in vivo* experiments to follow than the parent hydroxyderivatives. Selectivity against p53 knock-out cancer line HCT116p53−/− was found in comparison to non-mutated line. From the cell cycle analysis we know that compounds **4**, **7**, **11**, **15**, **19**, **22** blocked or slowed down cell cycle progression through G0/G1 or S-phase which is in concordance with transcription inhibition by an unknown mechanism of action. From the comparison of dihydrobetulinic acid (**4**) to its 2,2-difluoro and 2,2-dibromoderivatives **9**, **11** and **22** it is clear that the introduction of two fluorine or bromine atoms enhanced the cytotoxicity. It seems that the mechanism of action is dependent on the halogen atom, however, along with an enhancement in activity, the selectivity decreases.

4. Experimental part

4.1. General experimental procedures

4.1.1. Materials and instruments

Melting points were determined using a Büchi B-545 apparatus and are uncorrected. Optical rotations were measured on an Autopol III (Rudolph Research, Flanders, USA) polarimeter in MeOH at 25 °C unless otherwise stated and are in [10^{-1} deg cm^2 g^{-1}]. ^1H, ^{19}F, and ^{13}C NMR spectra were recorded on Jeol (500 MHz for ^1H) instrument, using $CDCl_3$ or CD_3OD as a solvent (25 °C). Chemical shifts are expressed in ppm either with tetramethylsilane as an internal standard for ^1H spectra ($CDCl_3$) or were referenced to the residual signal of the solvent (CD_3OD). ^{13}C NMR spectra were referenced to $CDCl_3$ (77.00 ppm) or CD_3OD (49.15 ppm). EI MS spectra were recorded on an INCOS 50 (Finigan MAT) spectrometer at 70 eV and an ion source temperature of 150 °C. The samples were introduced from a direct exposure probe at a heating rate of 10 mA/s. Relative abundances stated are related to the most abundant ion in the region of $m/z > 180$. MS-ESI and MS-APCI spectra of prepared compounds were recorded on a Micromass Platform LC (Waters), samples were introduced as methanolic solutions. The concentrations and time of data collections were chosen to get optimal spectra, only $[M + H]^+$ or $[M + Na]^+$ ions are given. IR spectra were recorded on a Nicolet Avatar 370 FTIR. DRIFT stands for Diffuse Reflectance Infrared Fourier Transform. Elemental analyses were recorded at the Institute of Organic Chemistry Academy of Sciences of the Czech Republic on the PE 2400 Series II CHNS/O Analyzer (Perkin Elmer, USA).

TLC was carried out on Kieselgel 60 F_{254} plates (Merck) detected by spraying with 10% aqueous H_2SO_4 and heating to 150–200 °C. Column chromatography was performed using silica gel 60 (Merck 7734). The HPLC system consisted of a high-pressure pump (Gilson model 361), a Rheodyne injection valve, and a preparative column (25 × 250 mm) filled with silica gel (Biospher 7 μm), the differential refractometer detector (Laboratorni Pristroje, Praha, CZ) was connected with a PC (software Chromulan) and an automatic fraction collector (Gilson model 246). A mixture of EtOAc and hexane was used as the mobile phase, its composition specified in each experiment. Work-up refers to pouring the reaction mixture into water, extracting the product into organic solvent, washing the organic layer successively in this order with water, diluted aqueous HCl, water, saturated aqueous sodium hydrogencarbonate, and again water. Then the organic phase was dryed over magnesium sulfate, filtered, and the solvents evaporated under reduced pressure. Analytical samples were dried over P_2O_5 under diminished pressure. Bulk syntheses of the starting compounds were carried out in 30 L duplicated glass reaction vessel with shaft stirrer. Betulin (**1**) and hydroxyketone **12** were obtained from company Betulinines (www.betulinines.com). DAST and dimethylsuccinic anhydride were purchased from Acros Organics. All other chemicals and solvents were obtained from Sigma–Aldrich.

4.1.2. General procedure for deprotection of benzyl esters

Palladium on charcoal (0.12–3 g; 10%) was added to each benzyl ester (1.7–3.4 mmol) in mixture THF (10–25 mL) and methanol (10–25 mL), the solution was placed into a cylinder and pressurized with H_2 (0.5 MPa) while vigorously stirred at r.t. The reaction progress was monitored using TLC in toluene/Et_2O 6:1. Upon completion, the catalyst was filtered off using cellite column, then solvents were evaporated under vacuo and the crude acid was purified on silica gel (100 g, eluted with EtOAc, unless otherwise stated at the experiment). The solvents were evaporated under vacuo and pure acid was precipitated/crystallized using cyclohexane and dried under vacuo.

4.1.3. General procedure for acylation of hydroxyderivatives

DMAP (0.4 mmol) was added to a solution of each hydroxyderivative (1.0–1.5 mmol) followed by addition of each anhydride (5.0–6.1 mmol) in *sym*-collidine (15–20 mL) under argon. The mixture was heated under reflux and monitored by TLC in $CHCl_3$/EtOAc/AcOH 100:20:1. After the completion (30–50 h), the mixture was cooled to r.t. and then worked up by pouring into HCl (3%, 200 mL). The product was extracted to EtOAc (3×). Organic phase was washed with HCl (8%, twice) and with water, then dried and solvents were removed under vacuo. Crude product was purified by chromatography on silica gel (100 g) eluted with gradient from hexane/EtOAc 10%–100% EtOAc. Mobile phase was evaporated and the oily product was precipitated by heating with 2,2,4-trimethylpentane (or cyclohexane), filtered off and dried under reduced pressure.

4.2. Synthetic procedures

4.2.1. Benzyl dihydrobetulonate (**6**)

Betulin (**1**) was converted to dihydrobetulin-3β-acetate (**2**) by a standard procedure [21,22] with yield of 54% over three steps. All spectral data were identical to those in lit [22].

Benzyl dihydrobetulinate (**5**) was prepared by multistep procedure where some steps were different from the literature [23] however, all intermediates are well known compounds and their spectral data were identical to the authentic samples. Therefore we describe the procedures but we publish the spectral data only for the final product. Solution of sodium periodate (650 g, 2 mol) in water (3.8 L) was slowly added to a solution of **2** (150.0 g; 0.31 mol) in EtOAc (8 L), MeCN (0.5 L) and water (2.1 L) while the mixture was vigorously stirred. Ruthenium (IV) oxide was added and the mixture was heated to 55 °C while still vigorously stirred for 140 min. The reaction was monitored using TLC toluene/Et_2O 6/1. The reaction was quenched by adding isopropyl alcohol (100 mL). After another 15 min, ruthenium (IV) oxide was filtered off and water phase was removed. The organic phase was washed with water solution of sodium sulfite and sodium metabisulfite and solid precipitate was filtered off and discarded. Organic phase was filtered over a pad of silica gel (200 g) and the pad was washed with EtOAc. The solvents were removed under vacuo and the product was crystallized from isopropyl alcohol to give white crystals of 3β-acetyldihydrobetulinic acid (**3**) (125 g; 81%) which was immediately deacetylated by standard procedure (reflux with abundant KOH in mixture toluene and ethanol). Isolated dihydrobetulinic acid (**4**) was arylated with benzyl bromide (1.2 equiv.) and K_2CO_3 in a mixture of DMF (1 L) and MeCN (200 mL) to give crude benzyl-dihydrobetulinate (**5**). Benzyl-dihydrobetulinate (**5**) (120.0 g; 0.22 mol) and sodium acetate (120.0 g; 0.88 mol) was dissolved in a mixture of acetic acid (1.2 L), toluene (1 L) a acetic anhydride (72 mL) and sodium dichromate (60.0 g; 0.20 mol) was added. The reaction mixture was stirred 22 h at r.t. and the progress was monitored on TLC in toluene/Et_2O (6:1). After the completion, the reaction mixture was poured into water (12 L) and crude product was extracted to toluene. Organic phase was washed with solution of sodium bicarbonate (3×), water and the solvents were removed under vacuo. Crude benzylester **6** was chromatographed on silica gel in toluene and crystallized from methanol to give white crystals (102.8 g; 64% over all 4 steps). Benzyl dihydrobetulonate (**6**).

Yield 102.8 g (64% over 4 steps); mp 151–153 °C (Lit.[23] 148–150 °C); $[\alpha]_D$ +13.7 (c 0.35) {Lit [23]. $[\alpha]_D$ +14.3 (c 0.70)}. IR (DRIFT): 1701 (C═O), 1717 (C═O) cm^{-1}. ^1H NMR and ^{13}C NMR are identical to the spectra in lit [23]. MS (ESI^+): m/z (%) = 569 (12, $[M + Na]^+$). Anal. Calcd for $C_{37}H_{54}O_3$: C, 81.27; H, 9.95; O, 8.78. Found: C, 81.30; H, 9.95; O, 8.77.

*4.2.2. Benzyl 2α-hydroxydihydrobetulonate (**7**)*

Benzyl dihydrobetulonate (**6**) (87.4 g; 0.16 mol) was dissolved in dichloromethane (1.4 L) and 0.1% (vol.) solution of H_2SO_4 in MeOH (2.8 L), then 3-chloroperoxybenzoic acid (75%; 108.6 g; 0.44 mol) was slowly added in several portions within 17 h while the mixture was vigorously stirred. The reaction was monitored on TLC in toluene/Et_2O 6:1. The reaction was diluted with water (8 L, twice), the organic phase was separated, washed with a concentrated solution of sodium sulfite (2×) and then with a mixture of solution of potassium iodide (10%) and 25% solution of sodium thiosulfate in water. The last washing was performed using concentrated water solution of $NaHCO_3$. Organic phase was separated, dried over calcium chloride and the solvents were removed under vacuo. Crude benzylester **7** was purified by chromatography on silica gel (2.5 kg) in toluene/Et_2O 10:1. Product was crystallized from MeOH// acetone. Yield 32.4 g (36%); mp 157–159 °C; $[\alpha]_D$ +8.9 (c 0.45). IR (DRIFT): 1708 (C═O), 1753 (C═O), 3481 (O–H) cm^{-1}. ^1H NMR: δ = 0,74 (d, 3H, *J* = 8.8 Hz), 0.78 (s, 3H), 0.85 (d, 3H, *J* = 9.2 Hz), 0.89 (s, 3H), 1.09 (s, 3H), 1.13 (s, 3H), 1.15 (s, 3H), 2.15–2.35 (m, 4H, H-1α, H-13, H-16, H-19), 2.47 (dd, 1H, *J* = 12.6, 6.9 Hz, H-1β), 4.53 (dd, 1H, *J* = 12.3, 6.6 Hz, H-2β), 5.08 (d, 1H, *J* = 12.4 Hz, H-31a), 5.13 (d, 1H, *J* = 12.4 Hz, H-31b), 7.32 (m, 5H, Ph). ^{13}C NMR: δ = 14.5, 14.6, 16.0, 16.6, 19.1, 21.2, 21.4, 22.7, 23.0, 24.5, 26.7, 29.5, 29.7, 32.0, 34.1, 37.2, 37.9, 37.9, 40.76, 42.6, 44.2, 47.7, 48.9, 49.9, 49.9, 56.9, 57.8, 65.6, 69.3, 128.0, 128.2, 128.3, 128.4, 128.4, 136.5, 175.9, 216.7. MS (ESI^+): *m*/*z* (%) = 585 (5, $[M + Na]^+$). Anal. Calcd for $C_{37}H_{54}O_4$: C, 78.96; H, 9.67; O, 11.37. Found: C, 78.98; H, 9.66; O, 11.40.

*4.2.3. Benzyl 2,2-difluorodihydrobetulonate (**8**)*

DAST (20 mL; 0.15 mol) was added to a solution of benzyl ester **7** (13.3 g; 23.7 mmol) v $CHCl_3$ (250 mL) and immediately after was added pyridine (13.5 mL; 0.17 mol) and the mixture was stirred 11 h at r.t. while monitored on TLC toluene/hexane 1:1. After the completion, the reaction was quenched with isopropyl alcohol (20 mL) that was added slowly while the mixture was cooled in ice bath. Then ice was added (30 g) and the mixture was carefully neutralized by a solution of NaOH (10%, 30 mL), then more water (1 L) was added and the product was extracted to $CHCl_3$ (3×). Organic phase was washed twice with diluted HCl (1:4), water (3×), NaOH (1% in water) and water. Crude **8** (11.7 g) was chromatographed on silica gel in mixture of toluene/hexane 1:1 to give difluoroketone **8** (9.4 g; 68%); mp 143–145 °C (isopropyl alcohol); $[\alpha]_D$ +52.5 (c 0.40). IR (DRIFT): 1725 (C═O), 1740 (C═O) cm^{-1}. ^1H NMR; ^{13}C NMR; ^{19}F NMR with the full assignment of all signals are in the Supplementary Information. MS (ESI^+): *m*/*z* (%) = 605 (12, $[M + Na]^+$). Anal. Calcd for $C_{37}H_{52}F_2O_3$: C, 76.25; H, 8.99; F, 6.52; O, 8.24. Found: C, 76.27; H, 9.00; F, 6.52; O, 8.24.

*4.2.4. 2,2-Difluorodihydrobetulonic acid (**9**)*

Benzyl ester **7** (1.0 g; 1.7 mmol) was deprotected according to the general procedure (except $CHCl_3$ was used as a mobile phase for the chromatography) to give acid **7a** (720 mg; 85%); mp 265–267 °C (cyclohexane); $[\alpha]_D$ +65.0 (c 0.40). IR (DRIFT): 1688 (C═O), 1741 (C═O), 2400–3350 (COOH) cm^{-1}. ^1H NMR: δ = 0.78 (d, 3H, *J* = 6.8 Hz), 0.87 (d, 3H, *J* = 6.8 Hz), 0.88 (s, 3H), 0.95 (s, 3H), 1.00 (s, 3H), 1.15 (s, 3H), 1.23 (d, 3H, *J* = 1.2 Hz), 1.71 (dd, 1H, J_1 = 12.6 Hz, J_2 = 3.4 Hz), 1.79 (m, 2H), 1.91 (dd, 1H, J_1 = 13.0 Hz, J_2 = 7.5 Hz), 2.05 (m, 1H, H-1a), 2.15–2.35 (m, 4H, H-1b, H-13, H-16, H-19). ^{13}C NMR: δ = 14.5, 14.6, 15.4, 18.2, 19.6, 21.0, 21.8, 22.7, 23.0 26.7, 28.0, 29.6, 29.7, 31.9, 32.8, 37.0 (q, *J* = 3.3 Hz), 37.3, 38.3, 40.6, 42.7, 44.1, 46.0, 48.6, 49.4, 50.9, 51.5 (m, Σ*J* = 50.3 Hz), 56.8, 181.4. ^{19}F NMR: δ = −99.96 (ddd, *J* = 262.4, 20.0, 6.8 Hz), -88.00 (ddd, *J* = 262.2, 29.3, 20.0 Hz). MS (ESI^+): *m*/*z* (%) = 515 (30, $[M + Na]^+$). Anal. Calcd for $C_{30}H_{46}F_2O_3$: C, 73.13; H, 9.41; F, 7.71; O, 9.74. Found: C, 73.17; H, 9.40; F, 7.70; O, 9.74.

*4.2.5. Benzyl-2,2-difluorodihydrobetulinate (**10**)*

$NaBH_4$ (700 mg; 3.7 mmol) was slowly added to a vigorously stirred solution of benzyl ester **8** (5.0 g; 8.6 mmol) in methanol (60 mL) and THF (70 mL) at 0 °C. The mixture was allowed to heat up to r.t. and stirred another 16 h while monitored on TLC toluene/Et_2O 6:1. The reaction was quenched by adding diluted HCl (1:5, 150 mL) and the product was extracted to EtOAc. Organic phase was collected, washed with water (3×) and the solvents were removed in vacuo. Crude product was crystallized from isopropyl alcohol to give benzyl ester **10** (4.9 g; 98%); mp 175–176 °C; $[\alpha]_D$ +0.0 (c 0.40). IR (DRIFT): 1683 (C═O), 3496 (O–H) cm^{-1}. ^1H NMR (300 MHz; $CDCl_3$): δ = 0.75 (d, 3H, *J* = 6.9 Hz), 0.74 (s, 3H), 0.85 (d, 3H, *J* = 6.9 Hz), 0.88 (s, 3H), 0.93 (s, 3H), 0.97 (s, 3H), 1.08 (s, 3H), 2.20 (m, 1H, H-1a); 2.21–2.47 (m, 4H, H-1b, H-13, H-16, H-19), 3.34 (dd, 1H, *J* = 22.3, 6.9 Hz, H - 3α), 5.09 (d, 1H, *J* = 13.1 Hz, H - 31a), 5.13 (d, 1H, *J* = 11.0 Hz, H − 31b), 7.31 (m, 5H, Σ*J* = 28.5 Hz, Ph). ^{13}C NMR (75.45 MHz; $CDCl_3$): δ = 14.5, 14.6, 15.4 (d), 15.5 (d), 15.8, 18.0, 21.15, 22.7, 23.0, 26.7, 29.1, 29.4, 29.7, 32.0, 34.0, 37.3, 37.9, 38.2 (d), 39.6 (d), 40.8, 42.7, 44.2, 46.1 (t), 48.9, 50.7, 54.9, 56.9, 65.6, 78.5 m, 128.0, 128.3, 128.3, 128.5, 128.5, 136.5, 175.9. ^{19}F NMR: δ = −90.26 (d, 2 F, *J* = 244.7 Hz). MS ($APCI^-$): *m*/*z* (%) = 493 (100, $[M\text{-}Bn]^-$). MS ($APCI^+$): *m*/*z* (%) = 607 (15, $[M + Na]^+$). Anal. Calcd for $C_{37}H_{54}F_2O_3$: C, 76.06; H, 9.28; F, 6.48; O, 8.18. Found: C, 75.99; H, 9.31; F, 6.50; O, 8.21.

*4.2.6. 2,2-Difluorodihydrobetulinic acid (**11**)*

Benzyl ester **10** (2.0 g; 3.4 mmol) was deprotected using the general procedure to give difluorodihydrobetulinic acid (**11**) (1.6 g; 95%); mp 260 - 201 °C (cyclohexane); $[\alpha]_D$ −8.6 (c 0.35). IR (DRIFT): 1685 (C═O), 2300–3610 (COOH) cm^{-1}. ^1H NMR (300 MHz; $CDCl_3$): δ = 0.77 (d, 3H, *J* = 6.9 Hz), 0.87 (d, 3H, *J* = 7.2 Hz), 0.89 (s, 3H), 0.95 (s, 3H), 0.96 (s, 3H), 0.99 (s, 3H), 1.09 (s, 3H), 1.89 (m, 1H, H-1a); 2.28–2.55 (m, 4H, H-1b, H-13, H-16, H-19), 3.35 (dd, 1H, *J* = 12.3 Hz, 6.9 Hz, H − 3α). ^{13}C NMR (75.45 MHz; $CDCl_3$): δ = 14.5, 14.6, 15.3 (d), 15.5 (d), 16.0, 18.0, 21.13, 22.7, 23.0, 26.7, 29.1, 29.5, 29.7, 32.0, 34.0, 37.4, 38.1, 38.3 (d), 39.6 (d), 40.8, 42.7, 44.2, 46.1 (t), 48.7, 50.6, 54.9, 56.8, 78.5 (m), 182.4. ^{19}F NMR δ = −110.31 (dd, *J* = 242.5, 76.14 Hz), -90.88 (dd, *J* = 248.2 Hz, 5.6 Hz). MS ($APCI^-$): *m*/*z* (%) = 493 (6, $[M-H]^-$). MS ($APCI^+$): *m*/*z* (%) = 517 (13, $[M + Na]^+$). Anal. Calcd for $C_{30}H_{48}F_2O_3$: C, 72.90; H, 9.76; F, 7.66; O, 9.68. Found: C, 72.84; H, 9.78; F, 7.68; O, 9.70.

*4.2.7. Difluoroallobetulone **13***

Difluoroketone **13** was prepared using a original procedure (without pyridine) from lit [17] DAST (3.5 mL; 26.50 mmol) was added to a solution of known[17] hydroxyketone **12** (3.50 g; 8.0 mmol) in $CHCl_3$ (20 mL) and the mixture was stirred at r.t. for 4 days. The reaction progress was monitored on TLC in toluene/Et_2O 15:1. Then, water was added followed with solid $NaHCO_3$ until CO_2 was developing. The organic phase was separated, washed with water and the solvents were removed in vacuo. Crude compound was chromatographed on silica gel (100 g) in mixture of toluene/hexane 1:1 to give difluoroketone **13** (1.5 g; 41%), mp. 247–249 °C (MeOH), $[\alpha]_D$ +119 (c 0.52) (lit [17]. mp. 243–245 °C, $[\alpha]_D$ +123. ^1H NMR spectrum was identical to a spectrum in literature [17].

*4.2.8. Difluoroallobetulin **14***

Difluoroalcohol **14** was prepared using a modified procedure from lit.[17] $NaBH_4$ (200 mg, 5.3 mmol) was slowly added to a cooled (0 °C) solution of difluorketone **13** (1.5 g; 3.1 mmol) in a mixture of THF (145 mL) and MeOH (135 mL) and the mixture stirred for 3 h and then poured into diluted HCl (1:5, 500 mL), extracted to $CHCl_3$, collected organic phase was washed with water and the solvents were removed in vacuo. The crude product was chromatographed on silica gel (150 g) in toluene to give compound **14**. Since the

purity wasn't sufficient yet, the sample was chromatographed on preparative HPLC in hexane/EtOAc 9:1 to give difluoroallobetulin **14** (850 mg; 57%); mp. 297–300 °C (MeCN), $[\alpha]_D$ +46 (c 0.39) (lit [17]. mp. 299–301 °C, $[\alpha]_D$ +48).

^{1}H NMR was identical to the spectrum in lit [17].

4.2.9. 3β-O-hemiglutaryl-2,2-difluorodihydrobetulinic acid (*15*)

Difluorodihydrobetulinic acid (**11**) (500 mg; 1.01 mmol) and glutaric anhydride (580 mg; 5.08 mmol) reacted according to the general procedure to give ester **15** (445 mg; 72%); mp 219–220 °C (2,2,4-trimethylpentane). IR (DRIFT): 1694 (C=O), 1729 (C=O), 2500–3300 (COOH) cm^{-1}. ^{1}H NMR (500 MHz; $CDCl_3$): δ = 0.75 (d, 3H, *J* = 6.9 Hz), 0.86 (d, 3H, *J* = 6.9 Hz), 0.90 (s, 3H), 0.92 (s, 3H), 0.93 (s, 3H), 0.95 (s, 3H), 1.02 (s, 3H), 1.68 (bd, 1H, *J* = 13.2 Hz), 1.73–1.83 (m, 1H), 1.87 (dd, 1H, *J* = 12.6 Hz, 7.5 Hz), 1.95–2.06 (m, 2H, HOOCCH$_2$C**H$_2$**CH$_2$COO), 2.13–2.35 (m, 4H, HOOCC**H$_2$**CH$_2$C**H$_2$**COO), 2.40–2.60 (m, 4H, H-1b, H-13, H-16, H-19), 4.86 (dd, 1H, *J* = 22.9 Hz, 5.7 Hz, H - 3α). ^{13}C NMR (125.77 MHz; $CDCl_3$) δ = 14.5, 14.6, 15.6, 15.6, 16.2, 16.5, 18.0, 19.9, 21.1, 22.7, 23.0, 26.6, 28.8, 29.5, 29.7, 30.1, 32.0, 32.9, 33.2, 34.0, 37.3, 38.1, 38.4 (d, *J* = 9.6 Hz), 39.4 (d, *J* = 4.8 Hz), 40.8, 42.7, 44.2, 46.4 (t, *J* = 19.2 Hz), 48.7, 50.5, 54.8, 56.8, 172.3, 179.0, 183.0. ^{19}F NMR δ = −105.59 (dm, *J* = 251.7 Hz), −90.04 (d, *J* = 251.9 Hz). MS (ESI$^+$): *m*/*z* (%) = 631 (100, [M + Na]$^+$). MS (ESI$^-$): *m*/*z* (%) = 607 (100, [M − H]$^-$). Anal. Calcd for $C_{36}H_{54}F_2O_6$: C, 69.05; H, 8.94; F 6.24; O, 15.77. Found: C, 69.11; H, 8.95; F 6.12; O, 15.70.

4.2.10. 3β-O-hemisuccinyl-2,2-difluorodihydrobetulinic acid (*16*)

Difluorodihydrobetulinic acid (**11**) (500 mg; 1.0 mmol) and succinic anhydride (500 mg; 5.0 mmol) reacted according to the general procedure to give ester **16** (344 mg; 57%); mp 256–257 °C (2,2,4-trimethylpentane); $[\alpha]_D$ −10.0 (c 0.40). IR (DRIFT): 1706 (C=O), 1761 (C=O), 2350–3300 (COOH) cm^{-1}. ^{1}H NMR (300 MHz; $CDCl_3$) δ = 0.76 (d, 3H, *J* = 6.0 Hz), 0.86 (d, 3H, *J* = 6.0 Hz), 0.90 (d, 3H, *J* = 6.0 Hz), 0.94 (s, 3H) 0.95 (s, 3H) 0.96 (s, 3H), 1.02 (s, 3H), 1.79 (m, 1H), 1.88 (m, 1H), 2.10–2.45 (m, 4H, H-1b, H-13, H-16, H-19), 2.78 (m, 4H, Σ*J* = 51 Hz, 2× CH_2), 4.89 (dd, 1H, *J* = 12.9 Hz, 5.2 Hz, H − 3α). ^{13}C NMR (75.45 MHz; $CDCl_3$) δ = 14.5, 14.7, 15.6 (d), 16.0, 16.4, 18.0, 21.3, 22.8, 23.0, 26.8, 28.8, 29.75, 29.8, 32.2, 34.1, 37.5, 38.1, 38.4 (d), 39.6 (d), 40.9, 42.8, 44.3, 44.3, 44.3, 46.5 (t), 48.7, 50.0, 50.7, 54.9, 56.7, 77.2, 171.7, 178.4, 183.2. ^{19}F NMR δ = −105.54 (dm, *J* = 251.9 Hz), −90.01 (d, *J* = 251.9 Hz). MS (ESI$^-$): *m*/*z* (%) = 593 (100, [M−H]$^-$). MS (ESI$^+$): *m*/*z* (%) = 617 (75, [M + Na]$^+$). Anal. Calcd for $C_{34}H_{52}F_2O_6$: C, 68.69; H, 8.80; F, 6.37; O, 16.14. Found: C, 68.66; H, 8.81; F, 6.39; O, 16.14.

4.2.11. 3β-O-3′,3′-dimethylhemiglutaryl-2,2-difluorodihydrobetulinic acid (*17*)

Difluorodihydrobetulinic acid (**11**) (500 mg; 1.0 mmol) and 3,3-dimethylglutaric anhydride (700 mg; 4.9 mmol) reacted according to the general procedure to given ester **17** (516 mg; 80%); mp 204–205 °C (2,2,4-trimethylpentan); $[\alpha]_D$ −14.3 (c 0.35). IR (DRIFT): 1702 (C=O), 1736 (C=O), 2500–3300 (COOH) cm^{-1}. ^{1}H NMR (300 MHz; $CDCl_3$): 0.76 (d, 3H, *J* = 6.0 Hz), 0.86 (d, 3H, *J* = 6.0 Hz), 0.89 (s, 3H), 0.92 (d, 3H, *J* = 3.0 Hz), 0.94 (s, 3H), 0.97 (s, 3H), 1.03 (s, 3H), 1.10 (s, 3H), 1.18 (s, 3H), 1.88 (m, 1H), 2.10–2.50 (m, 7H, H-1a, H-1b, H-13, H-16, H-19, 2× H-hemiester), 2.83 (d, 1H, *J* = 16.3 Hz, 1× H-hemiester), 3.80 (d, 1H, *J* = 16.3 Hz, 1× H-hemiester), 4.86 (dd, 1H, *J* = 22.9.0, 6.2 Hz, H - 3α). ^{13}C NMR (75.45 MHz; $CDCl_3$): δ = 14.1, 14.4, 15.7 (d), 16.5 (d), 18.1, 21.1, 22.8, 23.0, 25.4, 26.3, 28.4, 28.5, 29.6, 30.1, 32.2, 33.7, 37.2, 38.1, 38.4 (d), 39.5 (d), 40.6, 42.5, 43.2, 44.3, 44.5, 48.9, 49.9, 53.1, 54.5, 56.9, 171.4, 178.3, 183.1. ^{19}F NMR δ = −102.52 (dm, *J* = 244.7 Hz), −88.54 (d, *J* = 251.9 Hz). MS (ESI$^-$): *m*/*z* (%) = 635 (45, [M − H]$^-$). MS (ESI$^+$): *m*/*z* (%) = 659 (80, [M + Na]$^+$). Anal. Calcd for $C_{37}H_{58}F_2O_6$: C, 69.79; H, 9.17; F, 5.96; O, 15.08. Found: C, 69.78; H, 9.18; F, 5.97; O, 15.07.

4.2.12. 3β-O-3′,3′-dimethylhemisuccinyl-2,2-difluorodihydrobetulinic acid (*18*)

Difluorodihydrobetulinic acid (**11**) (760 mg; 1.5 mmol) and dimethylsuccinic anhydride (650 mg; 5.1 mmol) reacted according to the general procedure to give ester **18** (264 mg; 28%); mp 203–205 °C (2,2,4-trimethylpentane); $[\alpha]_D$ −22.9 (c 0.35). IR (DRIFT): 1682 (C=O), 1706 (C=O), 1742 (C=O), 2400–3400 (COOH) cm^{-1}. ^{1}H NMR (300 MHz; $CDCl_3$): δ = 0.76 (d, 3H, *J* = 9.0 Hz), 0.86 (d, 3H, *J* = 6.0 Hz), 0.89 (s, 3H), 0.92 (s, 3H), 0.94 (d, 3H, *J* = 9.0 Hz), 0.95 (s, 3H), 1.01 (s, 3H), 1.33 (s, 6H, 2× CH_3), 1.88 (m, 1H), 2.10–2.50 (m, 5H, H-1a, H-1b, H-13, H-16, H-19), 2.65 (d, 1H, *J* = 16.0 Hz, 1× H-hemiester), 3.80 (d, 1H, *J* = 16.0 Hz, 1× H-hemiester), 4.86 (bd, 1H, *J* = 22.0, Hz, H − 3α). ^{13}C NMR (75.45 MHz; $CDCl_3$): δ = 14.6, 14.8, 15.8, 16.3, 16.6, 18.2, 21.3, 22.9, 23.1, 25.3, 25.7, 26.8, 28.8, 29.8, 30.2, 32.2, 34.2, 37.6, 38.2, 38.5, 39.6, 40.5, 41.0, 42.9, 44.3, 44.3, 44.3, 48.8, 50.0, 50.7, 55.0, 56.8, 77.2, 171.2, 180.6, 180.6. ^{19}F NMR δ = −103.59 (dm, *J* = 251.9 Hz), −88.70 (d, *J* = 244.7 Hz). MS (ESI$^-$): *m*/*z* (%) = 621 (75, [M − H]$^-$). MS (ESI$^+$): *m*/*z* (%) = 645 (100, [M + Na]$^+$). Anal. Calcd for $C_{36}H_{56}F_2O_6$: C, 69.44; H, 9.06; F, 6.09; O, 15.41. Found: C, 69.42; H, 9.06; F, 6.10; O, 15.41.

4.2.13. 2,2-Difluoro-19β,28-epoxy-18α-oleanane-3β-yl hemiglutarate (*19*)

Hemiglutarate **19** was prepared by the general procedure from **14** (200 mg; 0.40 mmol). Crude product was chromatographed on silica gel (40 g) in gradient from 40% EtOAc in hexane to 100% EtOAc to give **19** (110 mg; 44%); mp. 230.0 °C (lyophylized from *t*-BuOH); $[\alpha]_D$ +41.5 (c 0.24). IR (DRIFT): 1710 (C=O), 1744 (C=O), 2866 (COOH), 2946 (COOH) cm^{-1}. ^{1}H NMR (400 MHz; $CDCl_3$): δ = 0.80 (s, 3H), 0.90 (s, 3H), 0.92 (s, 3H), 0.93 (d, 3H, *J* = 0.9 Hz), 0.93 (s, 3H), 1.00 (s, 3H), 1.04 (s, 3H), 1.97–2.10 (m, 2H, HOOCCH$_2$C**H$_2$**CH$_2$COO), 2.31 (td, 1H, *J* = 13.7, 4.9 Hz, H-1α), 2.40–2.60 (m, 4H, HOOCC**H$_2$**CH$_2$C**H$_2$**COO), 3.46 (d, 1H, *J* = 7.8 Hz, H-28a), 3.54 (s, 1H, H-19α), 3.78 (d, 1H, *J* = 7.6 Hz, H-28b), 4.87 (dd, 1H, *J* = 23.3, 5.8, H-3α). ^{13}C NMR (125 MHz; $CDCl_3$): δ = 13.42, 15.67, 15.94 (d, *J* = 6 Hz), 16.48 (d, *J* = 3 Hz), 17.95, 19.96, 21.28, 24.51, 26.17, 26.22, 26.29, 28.75, 28.75, 32.60, 32.66, 33.16, 33.56, 33.99, 36.23, 36.68, 38.43 (d, *J* = 9 Hz), 39.37 (d, *J* = 6 Hz), 40.77, 40.88, 41.44, 46.66 (t, *J* = 19 Hz), 46.74, 51.42, 55.04, 71.16, 77.20, 87.92, 121.54 (*J* = 248, 240 Hz), 172.41, 177.69. ^{19}F NMR: δ = −90.06 (d, 2 F, *J* = 244.7 Hz). MS (EI): *m*/*z* (%) = 591 (M$^+$−H, 100), 544 (5), 425 (13), 361 (13), 32 (13), 265 (8). Anal. Calcd for $C_{35}H_{54}F_2O_5$: C 70.91; H 9.18; F 6.41. Found: C 70.89; H 9.17; F 6.44.

4.2.14. 2,2-Difluoro-19β,28-epoxy-18α-oleanane-3β-yl hemisuccinate (*20*)

Hemisuccinate **20** was prepared by the general procedure from **14** (200 mg; 0.40 mmol). Crude product was chromatographed on silica gel (40 g) in gradient from 40% EtOAc in hexane to 100% EtOAc to give **20** (100 mg; 41%); mp. 109.3 °C (acetonitrile); $[\alpha]_D$ +48,1 (c 0.26). IR (DRIFT): 1731 (C=O), 1751 (C=O), 2736 (COOH), 2851 (COOH), 2957 (COOH), 3407 (O–H) cm^{-1}. ^{1}H NMR (400 MHz; $CDCl_3$): δ = 0.80 (s, 3H), 0.89 (s, 3H), 0.92 (s, 3H), 0.93 (s, 3H), 0.94 (d, 3H, *J* = 0.9 Hz), 1.00 (s, 3H), 1.04 (s, 3H), 2.32 (td, 1H, *J* = 13.8, 4.8 Hz, H-1α), 2.67–2.81 (m, 4H, HOOCC**H$_2$**C**H$_2$**COO), 3.46 (d, 1H, *J* = 7.8 Hz, H-28a), 3.54 (s, 1H, H-19α), 3.78 (d, 1H, *J* = 7.5 Hz, H-28b), 4.88 (dd, 1H, *J* = 23.1, 5.7 Hz, H-3α). ^{13}C NMR (125 MHz; $CDCl_3$): δ = 13.42, 15.67, 15.93 (d, *J* = 6 Hz), 16.41 (d, *J* = 3 Hz), 17.93, 21.30, 24.51, 26.18, 26.22, 26.29, 28.57, 28.76, 28.80, 28.90, 32.66, 33.56, 34.00, 36.23, 36.68, 38.42 (d, *J* = 9 Hz), 39.44 (d, *J* = 6 Hz), 40.77, 40.88, 41.44, 46.68 (t, *J* = 19 Hz), 46.75, 51.41, 55.09, 71.17, 77.65 (t, *J* = 19 Hz), 87.93, 121.47 (dd, *J* = 242, 250 Hz), 171.64, 177.00. ^{19}F NMR spectrum: −90.60 (dd, *J* = 248, 4.8 Hz), −106.32 (dddd, *J* = 249.2, 34.7, 22.9, 14.1). MS (EI): *m*/*z* (%) = 577 (M$^+$ − H, 90), 478 (56), 458 (61), 407 (93), 342 (22), 323 (19), 297 (7), 269 (10), 236

(25), 205 (73), 191 (100). Anal. Calcd for $C_{34}H_{52}F_2O_5$: C 70.56; H 9.06; F 6.57. Found: C 70.61; H 9.07; F 6.55.

4.2.15. 2,2-Difluoro-19β,28-epoxy-18α-oleanane-3β-yl 3′,3′-dimethylhemiglutarate (**21**)

Dimethylhemiglutarate **21** was prepared by the general procedure from **14** (200 mg; 0.40 mmol). Crude product was chromatographed on silica gel (40 g) in gradient from 40% EtOAc in hexane to 100% EtOAc to give **21** (190 mg; 73%); mp. 111.4 °C (lyophilized from t-BuOH); $[\alpha]_D$ +20.8 (c 0.39). IR (DRIFT): 1689 (C=O), 2700 (COOH), 2964 (COOH) cm^{-1}. ^1H NMR (400 MHz; $CDCl_3$): δ = 0.81 (s, 3H), 0.91 (s, 3H), 0.92 (s, 3H), 0.93 (s, 3H), 0.93 (s, 3H), 1.00 (s, 3H), 1.03 (s, 3H), 1.17 (s, 3H), 1.18 (s, 6H), 2.31 (td, 1H *J* = 13.7, 4.8 Hz, H-1α), 2.52 (s, 2H), 2.53 (d, 1H, *J* = 14.8 Hz), 2.67 (d, 1H, *J* = 14.8 Hz, HOOCC**H**$_2$CC**H**$_2$COO), 3.47 (d, 1H, *J* = 7.7 Hz, H-28a), 3.59 (s, 1H, H-19α), 3.81 (d, 1H, *J* = 7.8 Hz, H-28b), 4.88 (dd, 1H, *J* = 23.4, 5.9 Hz, H-3α). ^{13}C NMR (125 MHz; $CDCl_3$): δ = 13.42, 15.66, 15.89 (d, *J* = 6 Hz), 16.48 (d, *J* = 3 Hz), 17.95, 21.28, 24.50, 26.13, 26.20, 26.29, 27.77, 27.77, 28.72, 28.72, 32.52, 32.64, 33.57, 33.94, 36.18, 36.63, 38.41 (d, *J* = 9 Hz), 39.32 (d, *J* = 6 Hz), 40.77, 40.87, 41.44, 44.67, 45.07, 46.65 (t, *J* = 19 Hz), 46.71, 51.40, 55.06, 71.05, 77.12, 87.99, 121.52 (dd, *J* = 249, 240 Hz), 171.56, 178.03. ^{19}F NMR: δ = −89.29 (d, 2 F, *J* = 251.9 Hz). MS (EI): *m*/*z* (%) = 619 (M^+ − H, 100), 508 (3), 465 (3), 385 (4), 325 (5), 265 (4), 181 (3). Anal. Calcd for $C_{37}H_{58}F_2O_5$: C 71.58; H 9.42; F 6.12. Found: C 71.55; H 9.41; F 12.95.

4.3. MTT assay

Cell culture and cytotoxic MTT assay were performed as described in Refs. [30,31]. All cells were purchased from the American Tissue Culture Collection (ATCC), unless otherwise indicated: the CCRF-CEM line are highly chemosensitive T-lymphoblastic leukemia cells, K562 cells were derived from patient with acute myeloid leukemia with bcr-abl translocation, A549 line is lung adenocarcinoma, HCT116 is colorectal tumor cell line and its p53 gene knock-down counterpart (HCT116p53−/−, Horizon Discovery, UK) is a model of human cancers with p53 mutation frequently associated with poor prognosis. The daunorubicin resistant subline of CCRF-CEM cells (CEM-DNR bulk) and paclitaxel resistant subline K562-Tax were selected in our laboratory by the cultivation of maternal cell lines in increasing concentrations of daunorubicine or paclitaxel, respectively. The CEM-DNR bulk cells overexpress MRP-1 protein, while K562-Tax cells overexpress P-glycoprotein, both proteins belong to family of ABC transporters and are involved in primary and/or acquired multidrug resistance phenomenon [32,33]. MRC-5 and BJ are non-tumor human fibroblasts. The cells were maintained in Nunc/Corning 80 cm^2 plastic tissue culture flasks and cultured in cell culture medium (DMEM/RPMI 1640 with 5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal calf serum, and $NaHCO_3$). Cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (25,000–30,000 cells/well based on cell growth characteristics). Cells were added by pipette (80 μL) into 96-well microtiter plates. Inoculates were allowed a pre-incubation period of 24 h at 37 °C and 5% CO_2 for stabilization. Four-fold dilutions, in 20-μL aliquots, of the intended test concentration were added to the microtiter plate wals at time zero. All test compound concentrations were examined in duplicate. Incubation of the cells with the test compounds lasted for 72 h at 37 °C, in a 5% CO_2 atmosphere at 100% humidity. At the end of the incubation period, the cells were assayed using MTT. Aliquots (10 μL) of the MTT stock solution were pipetted into each well and incubated for further 1–4 h. After this incubation period the formazan produced was dissolved by the addition of 100 μL/well of 10% aq SDS (pH 5.5), followed by a further incubation at 37 °C overnight. The optical density (OD) was measured at 540 nm with a Labsystem iEMS Reader MF. Tumour cell survival (IC_{50}) was calculated using the following equation: IC = (ODdrug-exposed well/mean ODcontrol wells) x 100%. The IC_{50} value, the drug concentration lethal to 50% of the tumour cells, was calculated from appropriate dose–response curves.

4.4. Cell cycle and apoptosis analysis

Apoptosis and cell cycle analysis by FACS, BrdU/BrU incorporation and flow cytometric analysis of DNA/RNA synthesis were performed according our previous publication [33]. Subconfluent CCRF-CEM cells (ATCC), seeded at the density of 5.10^5 cells/ml in 6-well panels, were cultivated with the 1 or 5× IC_{50} of tested compound in a humidified CO_2 incubator at 37 °C in RPMI 1640 cell culture medium containing 10% fetal calf serum, 10 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Control containing vehicle was harvested at the same time point (24 h). Cells were washed with cold PBS and fixed in 70% ethanol overnight at 20 °C. The next day, the cells were washed in hypotonic citrate buffer, treated with RNase (50 μg/mL), stained with propidium iodide, and analyzed by flow cytometry using a 488 nm single beam laser (Becton Dickinson). Cell cycle was analyzed in the program ModFitLT (Verity), and apoptosis was measured in logarithmic model as a percentage of the particles with propidium content lower than cells in G0/G1 phase (<G1) of the cell cycle.

4.5. BrDU incorporation analysis

Cells were cultured and treated as for cell cycle analysis. Before harvesting, they were pulse-labeled with 10 μM 5-bromo-2-deoxyuridine (BrDU) for 30 min. The cells were trypsinized, fixed with ice-cold 70% ethanol, incubated on ice for 30 min, washed with PBS, and resuspended in 2 M HCl for 30 min at room temperature to denature their DNA. Following neutralization with 0.1 M $Na_2B_4O_7$, the cells were washed with PBS containing 0.5% Tween-20 and 1% BSA. They were then stained with primary anti-BrdU antibody (Exbio) for 30 min at room temperature in the dark. Cells were than washed with PBS and stained with secondary antimouse-FITC antibody (Sigma). The cells were then washed with PBS and incubated with propidium iodide (0.1 mg/mL) and RNase A (0.5 mg/mL) for 1 h at room temperature in the dark and finally analyzed by flow cytometry using a 488 nm single beam laser (FACSCalibur, Becton Dickinson).

4.6. BrU incorporation analysis

Cells were cultured and treated as for cell cycle analysis. Before harvesting, they were pulse-labeled with 1 mM 5-bromouridine (BrU) for 30 min. The cells were fixed in 1% buffered paraformaldehyde with 0.05% of NP-40 in room temperature for 15 min, and then in the refridgerator overnight. They were then washed in 1% glycin in PBS, washed in PBS, and stained with primary anti-BrDU antibody crossreacting to BrU (Exbio) for 30 min at room temperature inthe dark. Cells were than washed with PBS and stained with secondary antimouse-FITC antibody (Sigma). Following the staining, the cells were washed with PBS and fixed with 1% PBS buffered paraformaldehyde with 0.05% of NP-40. The cells were then washed with PBS, incubated with propidium iodide (0.1 mg/mL) and RNase A (0.5 mg/mL) for 1 h at room temperature in the dark, and finally analyzed by flow cytometry using a 488 nm single beam laser (FACSCalibur, Becton Dickinson).

Acknowledgment

The biological part of this work (MTT tests, cell cycle analysis etc.) was supported by grant from the Ministry of Education, Youth and Sports of the Czech Republic (No. CZ.1.07/2.3.00/30.0041) and internal grant of Palacky University IGA_LF_2014_010. The infrastructural part (Institute of Molecular and Translational Medicine) was supported by the grant LO1304 from the National Program of Sustainability II by the Ministry of Education, Youth and Sport of the Czech Republic. The chemical part was supported by Technology Agency of the Czech Republic (TE01020028 and LK21310; synthesis of fluoroderivatives) and by the Ministry of Education, Youth and Sport of the Czech Republic and by the European Social Fund (CZ.1.07/2.3.00/30.0060; synthesis of hemisuccinates). Characterization of new compounds was paid from the Czech Science Foundation (project 15-05620S).

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2015.03.068. These data include MOL files and InChiKeys of the most important compounds described in this article.

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