Accepted Manuscript

Lupane and 18α-oleanane derivatives substituted in the position 2, their cytotoxicity and influence on cancer cells

Lucie Borkova, Sona Gurska, Petr Dzubak, Renata Burianova, Marian Hajduch, Jan Sarek, Igor Popa, Milan Urban

PII: S0223-5234(16)30425-1

DOI: 10.1016/j.ejmech.2016.05.029

Reference: EJMECH 8621

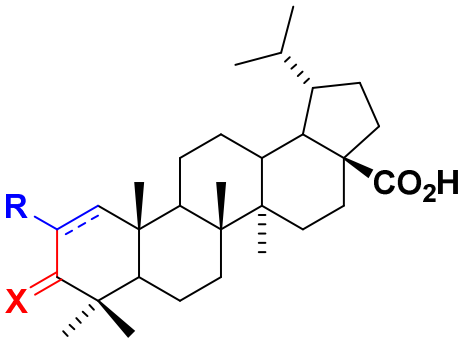
To appear in: *European Journal of Medicinal Chemistry*

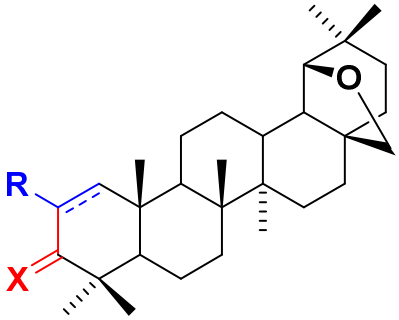
Received Date: 10 March 2016

Revised Date: 11 May 2016

Accepted Date: 13 May 2016

Please cite this article as: L. Borkova, S. Gurska, P. Dzubak, R. Burianova, M. Hajduch, J. Sarek, I. Popa, M. Urban, Lupane and 18α-oleanane derivatives substituted in the position 2, their cytotoxicity and influence on cancer cells, *European Journal of Medicinal Chemistry* (2016), doi: 10.1016/j.ejmech.2016.05.029.





R = N_3, SH, NH_2, NO_2
X = H, OH or O

Lupane and 18α-oleanane derivatives substituted in the position 2, their cytotoxicity and influence on cancer cells

Lucie Borkova,[1] Sona Gurska,[2] Petr Dzubak,[2] Renata Burianova,[2] Marian Hajduch,[2] Jan Sarek,[2] Igor Popa,[1,2] Milan Urban[1,2]*

[1]Department of Organic Chemistry, Faculty of Science, Palacky University Olomouc, 17. listopadu 1192/12, 771 46 Olomouc, Czech Republic.
[2]Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University Olomouc, Hnevotinska 5, 779 00 Olomouc, Czech Republic.

*Milan Urban is the corresponding author. Tel.: +420 585 632 197, Fax: +420 585 632 180; E-mail: milan.urban@gmail.com

Abstract

Lupane derivatives containing an electronegative substituent in the position 2 of the skeleton are often cytotoxic, however, the most active compounds are not selective enough. To further study the influence of a substituent in the position 2 in lupane and 18α-oleanane derivatives on their biological properties, we prepared a set of 38 triterpenoid compounds, 19 of them new, most of them substituted in the position 2. From betulin, we obtained 2-bromo dihydrobetulonic acid and 2-bromo allobetulon and their substitutions yielded derivatives with various substituents in the position 2 such as amines, amides, thiols, and thioethers. Nitration of allobetulon and dihydrobetulonic acid gave 2-nitro and 2,2-dinitro derivatives. Fifteen derivatives had IC_{50} < 50 μM on a chemosensitive CCRF-CEM (acute lymphoblastic leukemia) cell line and were tested on another seven cancer cell lines including resistant and two non-cancer lines. 2-Amino allobetulin had IC_{50} 4.6 μM and caused significant block of the tumor cells in S and slightly in G2/M transition and caused strong inhibition of DNA and RNA synthesis at 5 × IC_{50}. 2-Amino allobetulin is the most active derivative of 18α-oleanane skeletal type prepared in our research group to date.

Keywords

Betulinic acid, allobetulin, cytotoxicity, cell cycle, triterpene, activity

1. Introduction

Triterpenes are natural compounds usually occurring in plants, marine organisms, fungi, and bacteria. There are hundreds of new triterpenes being isolated from natural sources every year.[1] Pentacyclic triterpenes display a wide range of biological activities;[2] they are often cytotoxic,[3] antiviral,[4] antimicrobial,[5] antifungal,[6] antimalarial,[7] anti-inflammatory,[8] antiulcer,[9] hepatoprotective,[10] and cardioprotective.[11] A large number of triterpenes are cytotoxic against various cancer cell lines and anti-tumor activity was also observed in preclinical animal models.[2] Cytotoxicity of the isolated natural terpenes often inspires researchers to prepare quantities of semisynthetic derivatives, such as heterocyclic compounds,[12] derivatives with modified positions C-3,[13-15] C-20,[16-18] or C-28.[19] Recently, a number of patents emerged on betulinic acid derivatives dealing with low solubility in water while retaining selective cytotoxicity against cancer cells.[20] Recent review summarizes synthesis and biological activity of 18α-oleanane (allobetulin) derivatives.[21] Acute toxicity of triterpenes is usually low, which is important for their potential use as therapeutics.

Despite that triterpenes are biologically active *via* various mechanisms of action,[22-26] the parent compounds isolated from natural resources usually have two main drawbacks. Firstly, triterpenes are not active at sufficiently low concentration. The value of IC_{50} reaches low micromolar ranges at best, which usually cannot compete with the already available therapeutics. Therefore, significant improvement of the activity of triterpenoid derivatives is one of the major goals of many research groups. Inappropriate pharmacological properties are the second drawback. Low solubility in water is one of the main reasons why compounds with high *in vitro* activities often fail during *in vivo* screening and why their administration is difficult.

The main interest in our group is to modify the structure of triterpenoid derivatives in order to improve their pharmacological properties and to increase their activity and selectivity against tumors. We had synthesized a number of lupane, oleanane, and other terpenoid derivatives and in some of them, we found significant anticancer effects. In addition to that, we are developing tools to identify molecular targets of active triterpenes.[27] Very promising are

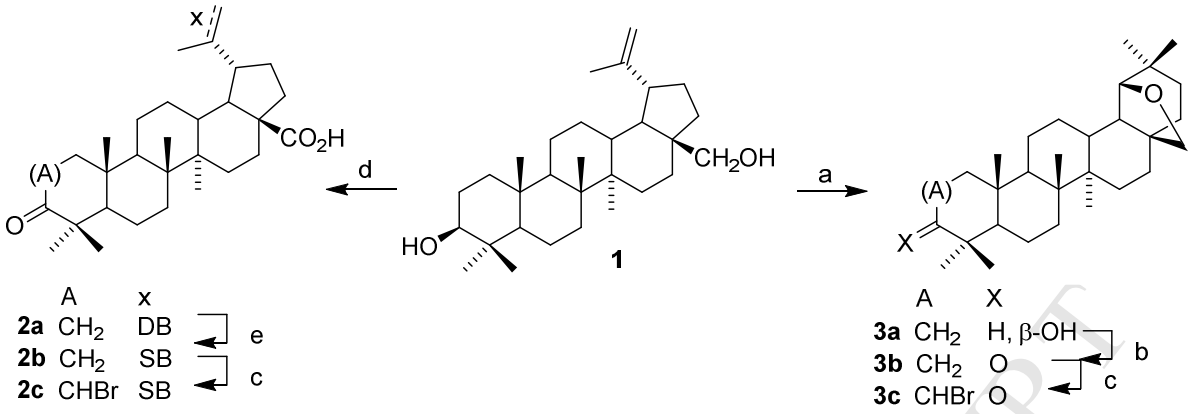
derivatives with modified A-ring[28] and E-ring,[29] heterocyclic structures,[30,31] various esters[32] and fluoroderivatives.[33] Based on this research, we made structure-activity relationship assumptions and discovered trends how the chemical structure may affect the anti-tumor activity. One of the significant trends shows that introducing an electronegative substituent to the position 2 of lupane skeleton increases cytotoxicity significantly and this works especially well for betulinic acid derivatives. The examples are 2,2-difluoroderivatives of dihydrobetulinic acid[28] and diosphenols.[34,35] However, among the most active 2,2-difluoroderivatives, selectivity was compromised.

In this work, we choose to introduce more substituent types to the position 2 of dihydrobetulonic acid (**2b**) and allobetulon (**3b**) *via* nucleophilic substitution of 2-bromo derivatives or by selective nitration of the 2-position in 3-oxocompounds. New derivatives were designed to contain heteroatoms such as nitrogen, sulfur, and oxygen. Resulting compounds were further reduced to get a larger set of more variable derivatives in order to make more accurate assumption about the influence of each substituent at C-2 on cytotoxic activity. Hemisuccinates were prepared from amines because they are soluble in water based γ-cyclodextrine formulation and this would be useful in the intended future *in vivo* experiments.

2. Results and discussion

2.1. Chemistry

From betulin (**1**), we prepared betulonic acid (**2a**), dihydrobetulonic acid (**2b**), and a mixture of diastereoisomers of 2-bromo dihydrobetulonic acid (**2c**).[31,36] From betulin (**1**), we also prepared allobetulin (**3a**), allobetulon (**3b**), and a diastereomeric mixture of 2-bromo allobetulones **3c** by known procedures[37-41] (Scheme 1).

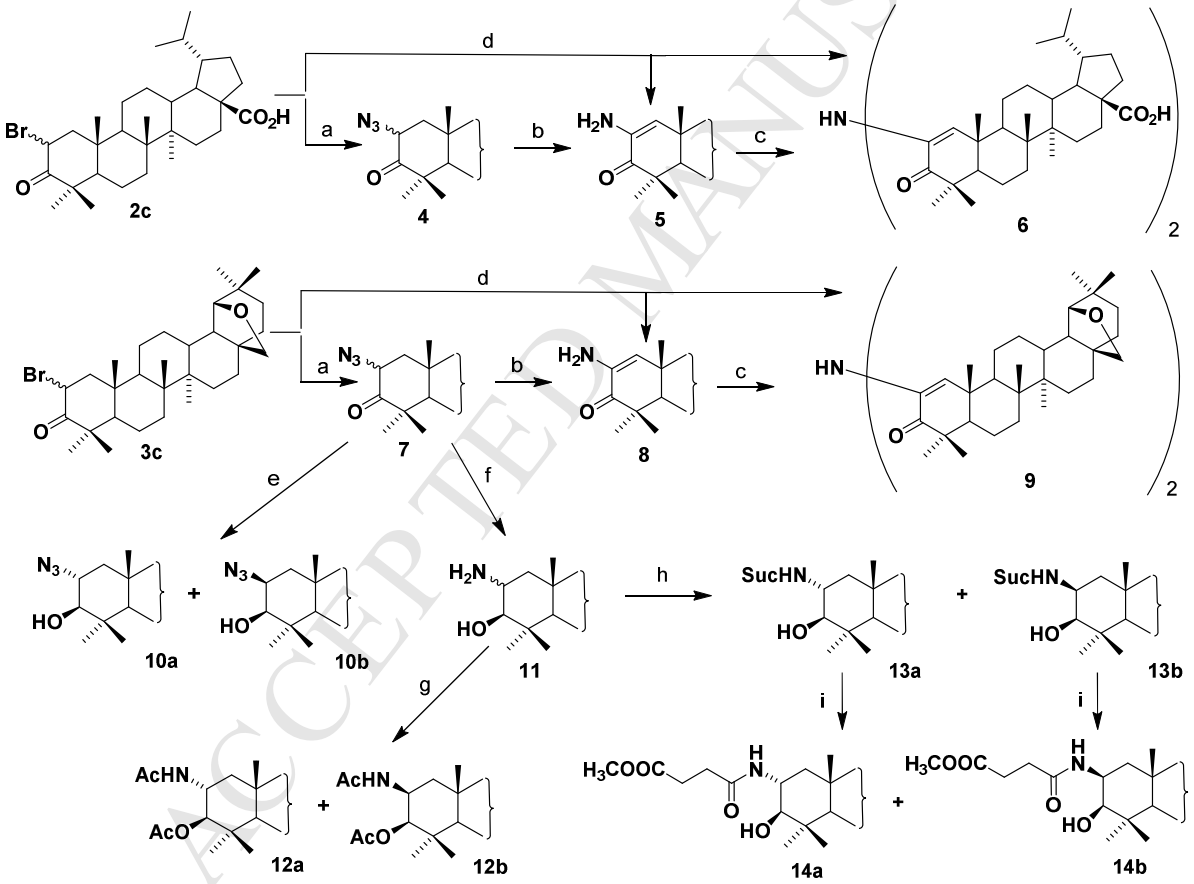


Scheme 1. Preparation of the starting material. DB = double bond, SB = single bond. Reagents and conditions: (a) Montmorillonit-K10, $CHCl_3$, reflux; (b) TEACC, $CHCl_3$, 0 °C; (c) Br_2, $CHCl_3$, r.t.; (d) Jones reagent, aceton, 0 °C; (e) H_2/Raney Nickel, iPrOH, THF, 48 h, 1 MPa, r.t.

Reaction of bromo ketone **2c** with sodium azide was performed in *N*-methylpyrrolidone (NMP) in a presence of a small amount of acetic acid according to a precedent from steroid chemistry.[42] An attempt to purify crude azido acid **4** on a silica gel column failed due to fast decomposition which yielded yellow enaminoketone **5** that spontaneously dimerizes to imine **6** the same way as it was described for analogous betulinic acid derivative.[43] It is worth to mention, that in general, compounds with an electronegative substituent at C-2 often occur in their enolforms.[44] Unlike azide **4**, both enaminoketone **5** and dimer **6** were stable enough that we were able to isolate and characterize them. Moreover compounds **5** and **6** were prepared directly and faster when a reaction of bromo ketone **2c** with sodium azide was stirred in a solution of DMSO containing a drop of H_2SO_4 at 70 °C. All attempts to reduce azide **4** to aminoketone, azido alcohol or aminoalcohol led to compound **5** and **6** (Scheme 2).

Reaction of bromo ketone **3c** with sodium azide in NMP in presence of a small amount of acetic acid yielded azido ketone **7**. Compound **7** was more stable than its analogue **4**. We were able to obtain sufficient amount of pure **7** by HPLC to get the physical and spectral data and also to perform basic cytotoxicity assay on a reference CCRF-CEM cell line. It was observed, that the compound remained stable at -18 ºC for at least 6 months and that slow decomposition was occurring at room temperature which was fastened by heating, especially in oxygen containing solvents such as EtOAc, THF, EtOH (especially during attempts to crystallize the compound) giving enaminoketone **8** in all cases. Attempts to reduce the azido ketone **7** with Ph_3P also gave enaminoketone **8**. Heating **8** in refluxing xylene gave dimer **9** similarly to lit.[43] In order to obtain enaminoketone **8** directly and faster, a reaction of bromo ketone **3c** with sodium azide was performed in DMSO and H_2SO_4 as mentioned earlier.

Higher stability of **7** allowed its reduction with sodium borohydride that gave a mixture of diastereomers of azido alcohols **10a** and **10b** that were separable by HPLC. Reduction of azide **7** with LAH afforded a diastereomeric mixture of aminoalcohols **11**. It was impossible to separate them directly (due to very low solubility) but a reaction of this mixture with acetic anhydride gave less polar diacetates **12a** (2α-acetamidoallobetulin 3β-acetate) and **12b** (2β-acetamidoallobetulin 3β-acetate) which were separable by HPLC and whose structures were unambiguously determined by NMR. Similarly, reaction of aminoalcohols **11** with succinic anhydride gave hemisuccinates **13a** (2α; 3β isomer) and **13b**, (2β; 3β isomer). Because their spectral data was difficult to interpret (very wide signals caused by low solubility), methylesters **14a** and **14b** were prepared and the full characterization was performed on them (Scheme 2).

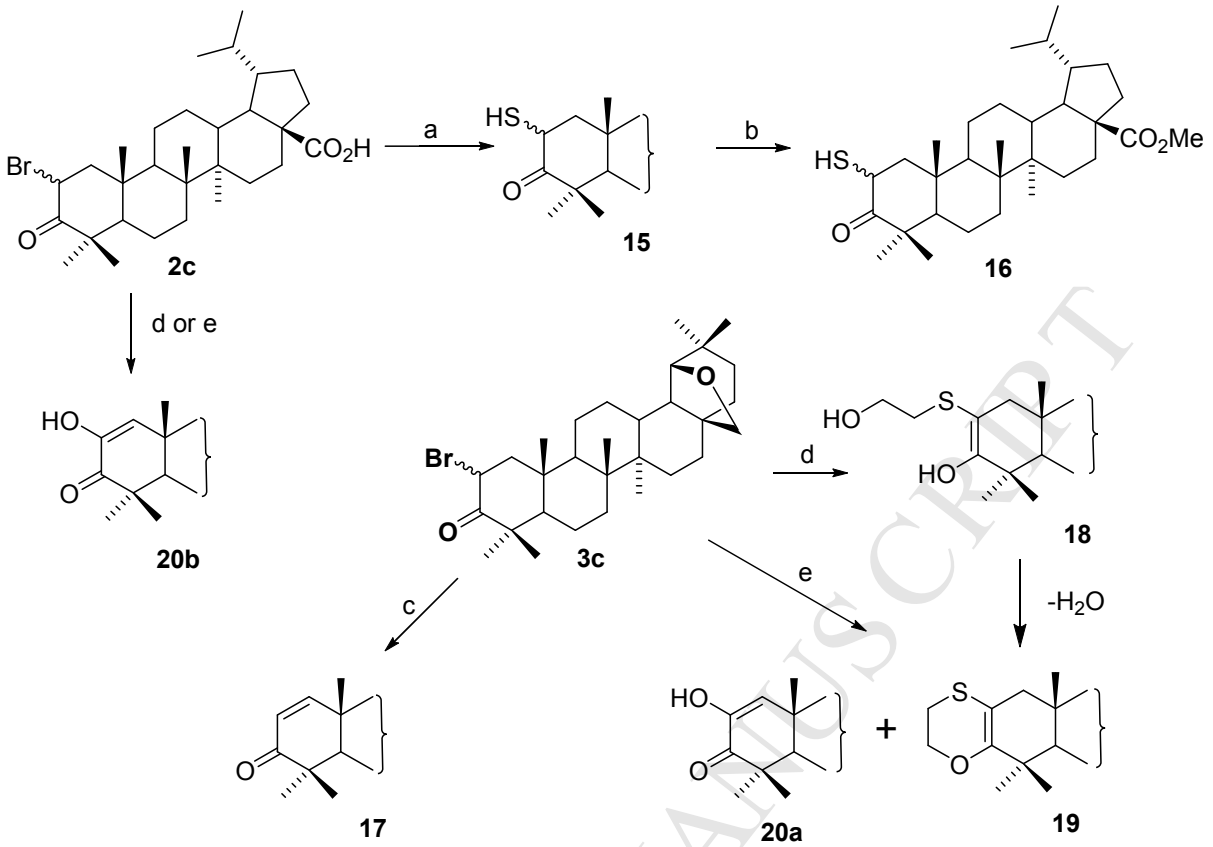


Scheme 2. Reagents and conditions: (a) NaN_3, NMP, AcOH, r.t.; (b, c) spontaneous decomposition at r.t., increased by silica gel, Ph_3P, heating etc.; reflux in xylene affords **9** exclusively; (d) NaN_3, DMSO, H_2SO_4, 70 °C; (e) $NaBH_4$, EtOH, 0 °C to r.t.; (f) LAH, THF, refl.; (g) Ac_2O, py, r.t.; (h) Suc_2O, THF, py, r.t.; (i) CH_2N_2, Et_2O, $CHCl_3$, Et_2O.

A reaction of bromo ketone **2c** with sodium sulfide gave compound **15** which was unstable while chromatographed but when transformed to its methylester before HPLC chromatography, we were able to obtain and characterize it as methyl ester **16**. Both

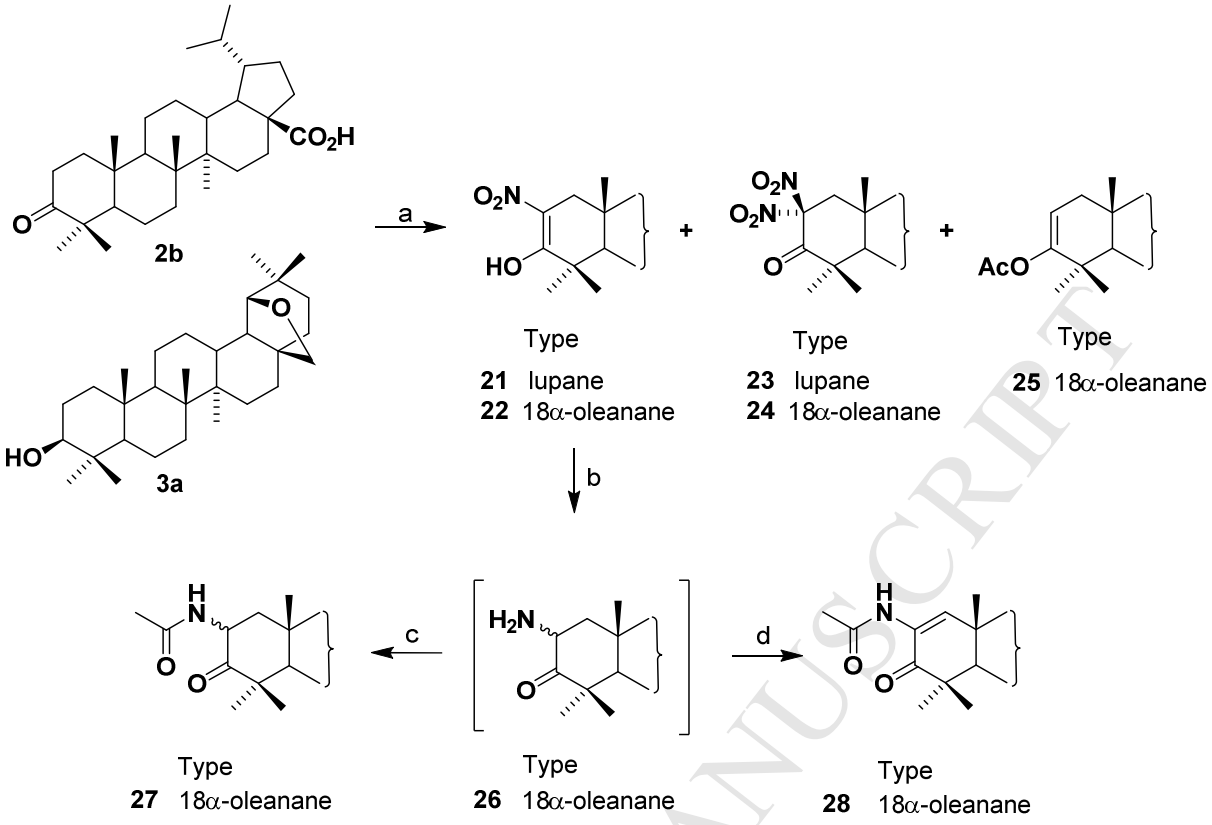
derivatives **15** and **16** succumb to slow decomposition while being purified on silica gel column or using HPLC. In order to obtain 18α-oleanane analogue of compound **15**, a number of reactions of bromo ketone **3c** with variety of reaction conditions were investigated. The most promising reaction gave a mixture of three compounds that was supposed to contain the desired thiol derivative; however, the only isolable product was known compound **17**,[45] the rest was repeatedly lost during the chromatography, probably due to decomposition (Scheme 3).

Nucleophilic substitution of bromo ketone **3c** with mercaptoethanol gave two compounds: 2-hydroxyethylsulfanylderivative **18** and a heterocycle **19**, formed by dehydratation of **18**. Structure of **19** was confirmed by full assignment of NMR signals using multiple 2D techniques; diosphenol **20a** was a byproduct of this reaction. Since 2´,3´-dihydro-1´,4´-oxathiine derivative **19** is a very interesting heterocycle, we attempted to obtain its lupane analogue from bromo ketone **2c**; however, the only isolated product in all experiments was diosphenol **20b**, which is similar result as for many attempts to substitute bromine atom in 2-bromo-3-oxoterpenes described in the lit.[31] Both diosphenols **20a** and **20b** are known compounds.[46,47] It is worth to point out, that it has been observed several times that a reaction that works well at the A-ring of lupane analogues does not work in 18α-oleanane derivatives and *vice versa*. We often see that a chemical modification that occurs on one side of the terpenoid skeleton may interfere with a reactivity on its other side.



Scheme 3. Reagents and conditions: (a) Na_2S, N-methylpyrolidone, r.t.; (b) CH_2N_2, Et_2O, $CHCl_3$, r.t.; (c) Na_2S, DMSO, a drop of water, 75 °C; (d) mercaptoethanol (used as cosolvent), NaOH, EtOH (anh.), 0 °C to r.t.; (e) mercaptoethanol (1 eq.), NaOH, EtOH (anh.), 0 °C to r.t.

Nitroderivatives **21 - 24** were obtained in rather low yields by using nitration conditions analogous to lit.[48,49] to nitrate dihydrobetulonic acid (**2b**) and allobetulin (**3a**). Dihydrobetulonic acid **2b** gave mononitroderivative **21** after 24 h treatment with nitration mixture (Scheme 4) at 25 ºC while dinitroderivative **23** formed in 6 h using the same nitration mixture when temperature was elevated to 35 ºC. Allobetulin (**3a**) gave a mixture of three compounds **22**, **24**, and **25** while the same enolacetate **25** became the only isolated product when the reaction time was extended. Once we had the nitro derivative **21**, it was reduced by zinc in acetic acid to give aminoketone **26** that was directly acetylated to get acetate **27**. It was necessary to acetylate compound **26** because the unprotected product was extremely difficult to isolate during the work-up procedure due to its low solubility in organic solvents and its co-precipitation with inorganic zinc salts, which together diminished its yield. In contrast, extraction of acetates into organic phase proceeded always well. We found that it is also possible to prepare acetylated enaminoketone **28** when heating **26** in toluene and acetic acid under reflux without inert atmosphere **(**Scheme 4**)**.



Scheme 4. Reagents and conditions: (a) HNO_3 (d = 1.35), AcOH, r.t. or 35 °C; (b) Zn, AcOH, reflux; (c) Ac_2O, AcOH, r.t.; (d) toluene, AcOH, reflux.

2.2. Biological assays

2.2.1 Cytotoxicity

Cytotoxic activity of all synthesized compounds and stable intermediates was investigated *in vitro* against human acute T lymphoblastic leukemia cell line CCRF-CEM using the standard MTS test (Table 1). New derivatives with IC_{50} below 50 μM were further examined on seven different cancer cell lines derived from leukemia (K562) and multiresistant counterparts (CEM-DNR, K562-TAX), solid tumors including lung (A549) and colon (HCT116, HCT116p53-/-) carcinomas, osteosarcoma cell line (U2OS), and for comparison, on two human non-cancer fibroblast lines (BJ, MRC-5) (Table 2). From this set of new derivatives, containing a substituent at the position C-2 of triterpenic skeleton, only one compound had activity lower than 10 μM; aminoalcohol **11** with IC_{50} 4.6 μM. Other derivatives with cytotoxicity in low micromolar range prepared within this paper (**2b**, **2c**, **20b**) are already known from[21] and therefore they were not further examined. Aminoalcohol **11** is up to date the most active derivative with 18α-oleanane skeleton found by our research group, but its cytotoxic activity is not limited to the tumor cells.

2.2.2. Cell cycle analysis

As a part of the study into the mechanism of action of compound **11** and to further characterize the anti-tumor properties, we analysed its influence on the cell cycle regulation in highly sensitive CCRF-CEM cells (Table 3). After the 24 hour treatment with 1 × and 5 × IC_{50} concentration, the apoptotic, sub-G1 population was just slightly increased (2.79% / 7.1%). There was visible interference with the cell cycle regulation at 5 × IC_{50}, showing accumulation of the cells in S and slightly in G2/M transition. Phosphorylation of the Histone-3 at Ser10 was not increased in the studied cell population which is indicating that cells were accumulating in the G2 phase and not entering the M phase of the cell cycle. It is in the concordance with the observation that DNA / RNA synthesis was inhibited significantly.

Based on the collected data it seems that some of studied triterpenoid derivatives are showing interesting cytotoxic activity. Unfortunately, selectivity towards cancer cell lines is low and not sufficient to start *in vivo* anticancer tests.

Table 1. Cytotoxic activity of compounds **1** – **28** against the acute T lymphoblastic leukemia CCRF-CEM cell line.

Comp.	IC_{50} (μmol/L[a]) CCRF-CEM	Comp.	IC_{50} (μmol/L[a]) CCRF-CEM	Comp.	IC_{50} (μmol/L[a]) CCRF-CEM	Comp.	IC_{50} (μmol/L[a]) CCRF-CEM
1	>50	**5**[b]	27	**12a**	>50	**20a**	13
2a	14	**6**[b]	27	**12b**	>50	**20b**	6
2b	4	**7**	>50	**13a**	>50	**21**	32
2c	2	**8**	>50	**13b**	>50	**22**	>50
3a	>50	**9**	>50	**14a**	>50	**23**	29
3b	>50	**10a**	>50	**14b**	>50	**24**	>50
3c	>50	**10b**	>50	**16**	45	**27**	32
4	17	**11**	5	**19**	>50	**28**	30

[a]The lowest concentration that kills 50% of cells. The standard deviation in cytotoxicity assays is typically up to 15 % of the average value. Compounds with IC_{50} > 50 μM are considered inactive. [b]Compounds were tested as mixture.

Table 2.

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

Comp.	IC_{50} (μM/L)[a]										
	CCRF-CEM	CEM-DNR	K562	K562-TAX	A549	HCT116	HCT116 $p53^{-/-}$	U2OS	BJ	MRC-5	TI[c]
4	17.5	16.0	14.2	10.6	25.7	>50.0	>50.0	48.9	>50.0	>50.0	>2.9
5[b]	26.6	33.9	>50.0	20.1	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>1.9
6[b]	26.6	33.9	>50.0	20.1	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>1.9
11	4.6	5.9	4.5	3.9	6.2	4.9	4.8	6.2	7.1	6.3	1.4
16	45.3	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>1.1
20a	13.1	>50.0	16.9	45.3	>50.0	41.6	33.4	45.8	>50.0	25.4	>2.9
21	31.8	36.1	10.1	20.6	31.3	>50.0	>50.0	>50.0	>50.0	>50.0	>1.6
23	28.6	46.6	>50.0	29.9	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>1.7
27	32.2	>50.0	32.4	>50.0	>50.0	42.9	38.7	>50.0	>50.0	21.6	>1.1
28	29.9	35.3	37.9	30.5	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>1.7

[a]The lowest concentration that kills 50% of cells. The standard deviation in cytotoxicity assays is typically up to 15 % of the average value. [b]Compounds were tested as mixture. Compounds with IC_{50} > 50 μM are considered inactive. [c]Therapeutic index is calculated for IC_{50} of CCRF-CEM line vs average of both fibroblasts.

Table 3.

Influence of compound **11** on cell cycle, DNA and RNA synthesis at 1^{a} × and 5^{b} × IC_{50}.

	Used conc. (μM)	sub G1 (%)	G0/G1 (%)	S (%)	G2/M (%)	$pH3^{Ser10}$ (%)	DNA synthesis (%)	RNA synthesis (%)
Control	-	1.80	36.41	50.33	13.28	1.66	56.37	43.37
11	4.60[a]	2.79	40.73	47.77	11.50	1.44	53.53	42.98
11	23.00[b]	7.1	28.90	54.92	16.18	0.49	1.88	2.23

3. Conclusion

We prepared a set of 38 triterpenoid compounds (19 of them new) and studied the influence of the substituent in the position C-2 on their biological properties. Higher electronegativity of the substituents resulted in compounds with higher IC_{50} which is in agreement with our previous hypothesis. Aminoallobetulin **11** is the most active compound among 18α-olenanane derivatives. However, in most of the new derivatives, the cytotoxicity was not limited to cancer cell lines exclusively and therefore their use as therapeutics is unlikely. A lot of difficulties had to be overcome during the synthesis. First of all, most of the reactions produced mixtures of epimers. In few cases, it was possible to separate the products or their derivatives by HPLC, however, in some cases it was impossible and therefore the biological testing had to be done with epimeric mixtures. Products with hydrophilic substituents at C-2 (e. g. amines) are strongly amphiphilic and they often do not dissolve well in solvents used for

NMR and therefore their characterization had to be performed after acetylation or methylation of carboxylic groups or amines. Despite all difficulties with synthesis and small chance to find a new therapeutic agent among the presented set of triterpenes, these compounds gave us important data points for our structure-activity relationships evaluations and based on those data, structures of better anti-cancer inhibitors may be proposed in the future research. Aminoallobetulin **11** is most cytotoxic compound of this study and it has influence on cell cycle (cells accumulate in S and slightly in G2/M transition) and causes strong inhibition of DNA and RNA synthesis at 5 × IC_{50}.

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 and ^{13}C NMR spectra were recorded on Varian^{UNITY} Inova 400 (400 MHz for ^1H) or Varian^{UNITY} Inova 300 (300 MHz for ^1H) or Jeol ECX-500SS (500 MHz for ^1H) instruments, using $CDCl_3$, D_6-DMSO or CD_3OD as solvents (25°C). Chemical shifts were eider referenced to the residual signal of the solvent ($CDCl_3$, D_6-DMSO) or to tetramethylsilane added as an internal standard. ^{13}C NMR spectra were eider referenced to $CDCl_3$ (77.00 ppm) or D_6-DMSO (39.51 ppm) or to tetramethylsilane added as an internal standard. 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. HRMS analysis was performed using an Orbitrap Elite high-resolution mass spectrometer (Thermo Fischer Scientific, MA, USA) operating at positive full scan mode (120 000 FWMH) in the range of 200–900 m/z. The settings for electrospray ionization were as follows: oven temperature of 300 °C, sheath gas of 8 arb. units and source voltage of 1.5 kV. The acquired data were internally calibrated with diisooctyl phthalate as a contaminant in methanol (m/z 391.2843). Samples were diluted to a final concentration of 20 μmol/L with 0.1% formic acid in water and methanol (50:50, v/v). The samples were injected by direct infusion into the mass spectrometer. IR spectra were recorded on a Nicolet Avatar 370 FTIR. DRIFT stands for Diffuse Reflectance Infrared Fourier Transform. TLC was carried out on Kieselgel 60 F254

plates (Merck) detected by spraying with 10% aqueous H_2SO_4 and heating to 150 - 200 °C. Starting triterpenes – betulin (**1**), dihydrobetulonic acid (**2b**), and allobetulin (**3a**) were obtained from company Betulinines (www.betulinines.com). All other chemicals and solvents were obtained from Sigma-Aldrich.

4.2. Synthetic procedures

4.2.1. Starting material

2-Bromo dihydrobetulonic acid (**2c**) was obtained from dihydrobetulonic acid (**2b**) using a procedure which we described earlier, all spectral and physical data was in agreement with published data.[31,36]

2-Bromo allobetulone **3c** was obtained from allobetulone (**3b**) using a procedure that we described earlier, all spectral and physical data was in agreement with published data.[37-41]

*4.2.2. Reaction of acid **2c** with sodium azide; 2-azido-4-oxolupan-28-oic acid* **(4)**

Sodium azide (219 mg; 3.37 mmol) was added to a solution of bromo derivative **2c** (300 mg; 0.56 mmol) in N-methylpyrrolidone (NMP, 15 mL). After 15 minutes, bromo derivative **2c** was converted completely to azide **4** according to TLC (toluene/Et_2O 5:1 with a drop of AcOH). Azide **4** was precipitated by pouring the reaction mixture to tenfold volume of water, filtered off, dried under vacuum and lyophilized from *tert*-butanol to give 183 mg (66 %), mp. was not possible to measure due to decomposition of **4** to enaminoketon **5** before melting. All physical and spectral data were obtained from crude mixture of 2α and 2β diastereoisomers (1:1): $[\alpha]_D$ +27.8 (c 0.36). IR (DRIFT): 2500 – 3500, 2100, 1719, 1656 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 0.75, 0.76, 0.77, 0.86, 0.87, 0.92, 0.94, 0.98, 1.00, 1.08, 1.09, 1.13, 1.14, 1.15 (42H, all s, 14 × CH_3 from both diastereoisomers), 2.18 (t, 1H, H-1α from 2β isomer), 2.22 – 2.28 (m, 6H, H-13, H-18, H-19 all from both isomers), 2.32 (dd, 1H, J_1 = 12.6 Hz, J_2 = 6.0 Hz, H-1β from 2α isomer), 4.25 (dd, 1H, J_1 = 13.3 Hz, J_2 = 6.0 Hz, H-2β from 2α isomer), 4.29 (dd, 1H, J_1 = 11.2 Hz, J_2 = 8.7 Hz, H-2α from 2β isomer). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.50, 14.63, 14.66, 15.35, 16.16, 16.31, 17.84, 18.36, 19.07, 19.46, 19.90, 21.14, 21.37, 22.03, 22.72, 22.94, 24.92, 26.60, 26.86, 29.25, 29.57, 29.62, 29.70, 29.73, 30.63, 31.90, 31.98, 32.84, 33.98, 37.02, 37.36, 37.84, 38.12, 38.44, 39.98, 40.56, 40.80, 42.62, 42.74, 44.07, 44.13, 46.56, 46.61, 47.66, 48.59, 48.63, 48.74, 49.48, 49.61, 49.82, 52.03, 56.72, 56.79, 56.95, 59.89, 60.81, 181.80, 181.82, 210.62, 213.43. MS (ESI^+): *m/z* (%)

= 498 (100, $[M+H]^+$), 520 (75, $[M+Na]^+$), 540 (40, $[M+K]^+$). MS (ESI^-): *m/z* (%) = 496 (100, $[M-H]^-$). HRMS (ESI-TOF) *m/z* calcd for $C_{30}H_{47}N_3O_3$ $[M+H]^+$ 498.3696, found 498.3692.

*4.2.3. 2-amino-3-oxolup-1-en-28-oic acid (**5**) and its dimer **6***

Bromo derivative **2c** (500 mg; 0.9 mmol) was dissolved in DMSO (15 mL) and sodium azide (486 mg; 7.5 mmol) and one drop of sulfuric acid were added. The reaction mixture was stirred at 70 °C, monitored on TLC in toluene/Et_2O 5:1. After 4 hours, the reaction mixture was poured to double volume of water and extracted with chloroform. The organic phase was collected, washed with water and the solvents were removed in vacuo. The crude enaminoketone **5** was purified by column chromatography on a silica gel in $CHCl_3$/EtOAc 10:1 and was spontaneously decomposing. Fractions containing mixture of compounds **5** and **6** were collected and evaporated to give 134 mg (30 %); mp. 242 – 243 °C ($CHCl_3$/EtOAc); $[\alpha]_D$ -41° (c 0.20). IR (DRIFT): 3337, 2400 – 3400, 1697, 1675, 1613 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ = 0.78 (d, 9H, *J* = 6.3 Hz, 3 × CH_3), 0.93 (d, 9H, *J* = 6.3 Hz, 3 × CH_3), 0.96 (s, 9H, 3 × CH_3), 1.05 (s, 9H, 3 × CH_3), 1.13 (s, 9H, 3 × CH_3), 1.19 (s, 9H, 3 × CH_3), 1.22 (s, 9H, 3 × CH_3), 2.27 – 2.34 (m, 9H, H-13, H-18, H-19 all from both monomer and dimer), 6.14 (s, 1H, H-1 monomer), 6.55 (s, 3H, H-1, H-1', NH dimer), 11.42 (bs, 2H, NH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.60, 14.72, 16.56, 18.97, 21.12, 21.31, 21.91, 22.77, 23.07, 26.90, 27.72, 29.69, 29.77, 31.96, 34.10, 37.63, 38.17, 38.53, 41.64, 42.97, 44.04, 44.41, 45.74, 48.49, 53.14, 56.96, 132.28, 132.81, 183.45, 200.80. MS (ESI^+): *m/z* (%) = 471 (100, $[M+H]^+$). MS (ESI^-): *m/z* (%) = 469 (100, $[M-H]^-$), 423 (70, $[M-COOH]^-$). HRMS (ESI-TOF) *m/z* calcd for $C_{30}H_{47}NO_3$ $[M+H]^+$ 470.3629, found 470.3628.

*4.2.4. Reaction of 2-bromo allobetulone 3c with sodium azide; 2-azido allobetulon **7***

Bromo derivative **3c** (100 mg; 0.193 mmol) was dissolved in NMP (3 mL) with a drop of acetic acid and then sodium azide (75 mg; 1.154 mmol) was added. The reaction mixture was stirred at r.t. 30 min, while monitored on TLC in hexane/EtOAc 5:1. The reaction mixture was poured to tenfold volume of water and extracted to an organic solvent. The organic phase was collected, washed with water and the solvents were removed in vacuo. A pale yellow honey like product was crystallized from cyclohexane and dried under the flow of N_2 to give white crystals of azide **7** (24 mg; 26 %), mp. 152 – 154 °C (cyklohexane). ^1H NMR was identical to the spectrum in lit.[43]

*4.2.5. 2-amino-18α-olean-1-en-3-one (**8**)*

Sodium azide (5.1 g; 78 mmol) and 20 drops of sulfuric acid were added to the solution of 2-bromallobetulone (**3c**) (5.6 g; 10.8 mmol) in DMSO (150 mL). The reaction mixture was heated 4 hours at 70 °C under continuous stirring. The reaction mixture was poured to double volume of water, extracted to $CHCl_3$, washed with water and solvents were evaporated. The crude product was chromatographed on silica gel (300 g) in toluene/Et_2O 5:1 and crystallized from EtOAc to give enaminoketone **8** (2.5 g; 50 %). 700 mg of enaminoketone **8** was purified by HPLC in hexane/EtOAc 4:1 and crystallized from EtOAc to give pure compound **8** (500 mg; 71 %), mp. 223 – 226 °C (ethylacetate), $[\alpha]_D$ +38° (c 0.35). ^{1}H NMR was identical to the spectrum in lit.[43]

*4.2.6. Dimer **9***

Azido allobetulone **7** (1.1 g; 2.3 mmol) was added to triphenylphosphine (2.4 g; 9.2 mmol) in anhydrous THF (22 mL) under argon atmosphere. The reaction mixture was stirred for 6 hours at r.t. Initially, formation of nitrogen in the mixture was observed as small bubbles and originally colorless solution became yellowish. Then water (1.1 mL) was added to the solution. The reaction mixture was stirred under argon atmosphere for 45 hours while the solution darkened. At this time, azido allobetulone **7** was completely consumed according to TLC (toluene/Et_2O 20:1). The reaction mixture was processed by evaporation of solvents under vacuo; residues of water were removed by 5 × azeotropic distillation with toluene under vacuo. After toluene evaporation the mixture was heated with xylene under reflux for 30 hours. Xylene was evaporated under vacuo, dry residue was dissolved with p-toluenesulfonic acid (40 mg) in EtOH (55 mL) and the solution was vigorously stirred 3 days in presence of air. The resulting precipitate was filtered over on a pad of cellite, washed with EtOH and eluted from the column by chloroform. Chloroform was evaporated under vacuo and crude product was purified by HPLC (hexane/EtOAc 20:3). After the crystallization from the mixture EtOAc/MeOH compound **9** obtained as yellow crystals (200 mg; 17 %), mp. 345 – 348 °C (Lit.[29] > 300 °C); $[\alpha]_D$ +60° (c 0.31) (Lit.[29] +47° (c 0.1, $CHCl_3$)). ^{1}H NMR was identical to the spectrum in lit.[29]

*4.2.7. Reduction of azido allobetulon **7** with sodium borohydride*

Azido ketone **7** (500 mg; 1.0 mmol) was dissolved in EtOH (1 mL) and the solution was cooled to 0 °C. $NaBH_4$ (0.5 g; 13.5 mmol) was added to the mixture and the suspension was stirred for 2 hours while temperature was allowed to reach r.t. The reaction mixture was poured to double volume of diluted hydrochloric acid (1:4) and extracted with an organic

solvent. The organic phase was washed with water and 5% solution of $NaHCO_3$. Solvents were removed on vacuo. The crude product was purified by column chromatography on silica gel (100 g) in $CHCl_3$. Pure azido alcohols **10a** and **10b** were obtained. 2α-azido alcohol **10a** (0.14 g; 28 %), mp. 216 - 219 °C (MeOH); $[\alpha]_D$ +9° (c 0.29). IR (DRIFT): 3605, 2103, 1454 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.80, 0.82, 0.91, 0.93, 0.94, 0.98, 1.03 (21H, all s, 7 × CH_3), 1.67 (1H, dm, *J*(H-1α, H-1β) = 12.5 Hz, H-1α), 2.09 (1H, dd, *J*(H-1β, H-1α) = 12.5 Hz, *J*(H-1β, H-2β) = 4.4 Hz, H-1β), 3.03 (1H, dd, *J*(H-3α, H-2β = 10.7 Hz, *J*(H-3α, H-OH) = 3.1 Hz, H-3α), 3.44 (1H, d, *J* = 7.8 Hz, H-28a), 3.50 (1H, dd, *J*(H-2β, H-3α) = 10.7 Hz, *J*(H-2β, H-1β) = 4.4 Hz, H-2β), 3.53 (1H, s, H-19), 3.77 (1H, d, *J* = 7.8 Hz, H-28b). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.44, 15.67, 16.33, 17.37, 18.12, 21.07, 24.50, 26.19, 26.24, 26.34, 28.33, 28.75, 32.65, 33.66, 34.04, 36.22, 36.68, 38.19, 39.34, 40.49, 40.65, 41.42, 43.91, 46.75, 50.92, 55.23, 61.53, 71.19, 81.30, 87.88. MS-EI: m/z (%) [for $C_{30}H_{49}N_3O_2$: M^+ 483]: 483 (M^+, 14), 455 (31), 441 (100), 424 (5), 412 (86), 382 (31), 370 (12). HRMS (ESI-TOF) *m/z* calcd for $C_{30}H_{49}N_3O_2$ $[M+H]^+$ 484.3903, found 484.3908.

2β-azido alcohol **10b** (0.12 g; 24 %), mp. 260 - 262 °C (MeOH); $[\alpha]_D$ +50° (c 0.31). IR (DRIFT): 3671, 2112, 1454 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.80, 0.85, 0.91, 0.93, 0.94, 1.00, 1.12 (21H, all s, 7 × CH_3), 1.67 (1H, dm, *J*(H-1α, H-1β) = 13.6 Hz, H-1α), 2.23 (1H, dd, *J*(H-1β, H-1α) = 14.7 Hz, *J*(H-1β, H-2α) = 2.9 Hz, H-1β), 3.27 (1H, dd, *J*(H-3α, H-OH) = 9.8 Hz, *J*(H-3α, H-2α) = 4.7 Hz, H-3α), 3.44 (1H, d, *J* = 7.8 Hz, H-28a), 3.53 (1H, s, H-19), 3.78 (1H, dd, J_1 = 7.9 Hz, J_2 = 1.8 Hz, H-28b), 4.04 (1H, ddd, *J*(H-2α, H-1α) = 4.7 Hz, *J*(H-2α, H-3α) = 4.7 Hz, *J*(H-2α, H-1β) = 2.9 Hz, H-2α). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.42, 15.77, 16.35, 16.68, 18.05, 21.19, 24.53, 26.22, 26.27, 26.37, 28.75, 29.47, 32.70, 33.72, 34.04, 36.26, 36.73, 36.94, 38.54, 40.70, 40.87, 41.46, 41.76, 46.79, 50.71, 55.31, 62.65, 71.23, 77.58, 87.89. MS-EI: m/z (%) [for $C_{30}H_{49}N_3O_2$: M^+ 483]: 483 (M^+, 20), 455 (24), 440 (100), 424 (35), 412 (67), 382 (57), 369 (28). HRMS (ESI-TOF) *m/z* calcd for $C_{30}H_{49}N_3O_2$ $[M+H]^+$ 484.3903, found 484.3902.

*4.2.8. Reduction of azido allobetulon **7** with LAH and further modifications of resulting aminoalcohol **11***

LAH (10 g; 0.3 mmol) was added to the solution of azido ketone **7** (10 g; 20.8 mmol) in THF (250 mL) and the reaction mixture was headed under reflux for 2 hours. After cooling to r.t.,

unreacted LAH was slowly decomposed by gradually added EtOAc (10 mL), EtOH (10 mL) and 10% HCl (15 mL). The organic phase was filtered off inorganic salts and THF was removed in vacuo. Crude aminoalcohol **11** was purified in Soxhlet extractor (Et_2O to remove impurities) and by purification over silica gel (50 g) in $CHCl_3$/isopropanol 1:1. Derivative **11** was obtained (5.8 g; 61 %, a mixture of two enantiomers based on NMR analysis 85:15; major one is 2α-amino-18α-oleanane-3α-ol), mp. 280 - 283 °C (non-crystallized); $[\alpha]_D$ +65° (c 0.16 in $CHCl_3$ with 5 % MeOH). IR (nujol): 3648 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.83, 0.92, 0.96, 0.98, 1.04, 1.22, (21H, all s, 7 × CH_3), 3.46 (1H, ddd, *J*(H-2β, H-1α) = 10.5 Hz, *J*(H-2, H-1β) = 7.9 Hz, *J*(H-2, H-3β) = 2.4 Hz, H-2), 3.49 (1H, d, *J* = 7.8 Hz, H-28a), 3.53 (1H, d, *J*(H-3, H-2) = 2.4 Hz, H-3), 3.56 (1H, s, H-19), 3.79 (1H, bd, *J* = 7.6 Hz, H-28b). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.82, 16.19, 19.46, 20.23, 20.68, 23.15, 24.83, 27.09, 27.51, 27.70, 29.24, 29.24, 33.75, 34.32, 35.89, 37.20, 37.57, 38.62, 38.71, 41.27, 42.01, 42.20, 42.69, 49.54, 51.94, 53.00, 72.21, 75.72, 80.00, 89.55. MS-EI: m/z (%) [for $C_{30}H_{51}NO_2$: M^+ 457]: 457 (M^+, 100), 442 (15), 424 (19), 384 (35), 236 (11), 203 (10), 189 (14), 149 (67). HRMS (ESI-TOF) *m/z* calcd for $C_{30}H_{51}NO_2$ $[M+H]^+$ 458.3998, found 458.3991.

Diacetates **12a** and **12b**. Crude aminoalcohol **11** (400 mg; 0.88 mmol) was dissolved in the mixture of pyridine (10 mL) and Ac_2O (3 mL; 30 mmol) and the solution was stirred 4 days at r.t. The reaction mixture was poured to double volume of diluted hydrochloric acid (1:4) and extracted with an organic solvent. The organic phase was washed with water and 5% solution of $NaHCO_3$. Solvents were removed on vacuo. Crude product was chromatographed on HPLC in hexane/EtOAc 1:3. Fractions containing **12a** and **12b** were crystallized from MeOH to give acetates **12a** and **12b**. 2α-acetamido-18α-oleanane-3β-yl acetate **12a** (274 mg; 58 %), mp. 208 – 211 °C (MeOH); $[\alpha]_D$ +10° (c 0.24). IR (DRIFT): 3428, 3375, 1722, 1668, 1520 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.80, 0.88, 0.91, 0.92, 0.93, 0.98, 1.00 (21H, all s, 7 × CH_3), 1.90 (3H, s, 2-NHAc), 2.08 (3H, s, 3-OAc), 3.44 (1H, d, *J* = 7.8 Hz, H-28a), 3.52 (1H, s, H-19), 3.76 (1H, dd, J_1 = 7.8 Hz, J_2 = 1.4 H-28b), 4.25 (1H, m, H-2), 4.45 (1H, d, *J*(H-3, H-2) = 11.0 Hz, H-3), 5.58 (1H, d, *J* = 8.6 Hz, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.44, 15.64, 17.15, 17.20, 18.20, 21.10, 24.51, 26.21, 26.28, 26.37, 28.30, 28.76, 32.67, 33.69, 34.05, 36.23, 36.71, 38.02, 38.86, 40.64, 40.71, 41.43, 46.45, 46.77, 46.77, 50.97, 55.53, 71.20, 82.19, 87.85. MS-EI: m/z (%) [for $C_{34}H_{55}NO_4$: M^+ 541]: 541 (M+, 89), 511 (6), 481 (40), 470 (23), 449 (8), 440 (31), 422 (38), 187 (62), 139 (100). HRMS (ESI-TOF) *m/z* calcd for $C_{34}H_{55}NO_4$ $[M+H]^+$ 542.4209, found 542.4210.

2β-acetamido-18α-oleanane-3β-yl acetate **12b** (85 mg; 18 %), mp. 269 – 271 °C (MeOH); $[\alpha]_D$ +51° (c 0.24). IR (DRIFT): 3446, 1737, 1668, 1510 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.80, 0.86, 0.92, 0.93, 0.98, 1.04, 1.10 (21H, all s, 7 × CH_3), 1.96 (3H, s, 2-NHAc), 2.11 (3H, s, 3-OAc), 3.44 (1H, d, *J* = 7.8 Hz, H-28a), 3.52 (1H, s, H-19), 3.79 (1H, bd, *J* = 7.8 Hz, H-28b), 4.32 (1H, bd, *J* = 6.6 Hz, H-2), 4.85 (1H, d, *J* = 2.6 Hz, H-3), 5.58 (1H, bs, NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.41, 15.70, 18.78, 19.10, 19.10, 21.12, 21.61, 23.49, 24.52, 26.20, 26.32, 26.45, 28.78, 29.96, 32.68, 33.27, 34.32, 36.23, 36.70, 37.49, 37.60, 40.86, 40.96, 41.47, 42.21, 45.39, 46.70, 51.36, 51.53, 71.24, 79.35, 87.93, 169.69, 170.89. MS-EI: m/z (%) [for $C_{34}H_{55}NO_4$: M^+ 541]: 541 (M^+, 85), 481 (21), 470 (9), 440 (20), 422 (17), 203 (19), 187 (60), 139 (100). HRMS (ESI-TOF) *m/z* calcd for $C_{34}H_{55}NO_4$ $[M+H]^+$ 542.4209, found 542.4215.

Hemisuccinates **13a** and **13b** were prepared by reaction of crude aminoalcohol **11** (5 g; 10.9 mmol) with Succinic anhydride (11g; 93 mmol) in the mixture of solvents THF (100 mL) and pyridine (20 mL). The reaction mixture was poured to double volume of diluted hydrochloric acid (1:4) and extracted with an organic solvent. The organic phase was washed with water and 5% solution of $NaHCO_3$. Solvents were removed on vacuo. Crude product was chromatographed by HPLC with reverse phase in the mixture MeCN/H_2O 6:1. Two compounds were obtained: **13a** (1.2 g; 19 %), mp. 290 – 294 °C (MeOH); $[\alpha]_D$ +18° (c 0.27). IR (KBr): 3303, 1718, 1646 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.81, 0.93, 0.97, 1.24, 1.24, 1.43, 1.46 (21H, all bs, 7 × CH_3), 2.5 (3H), 3.45 (1H, m, H-28a), 3.56 (1H, s, H-19), 3.76 (1H, m, H-28b). MS-EI: m/z (%) [for $C_{34}H_{55}NO_5$: M^+ 557]: 557 (M^+, 3), 513 (2), 457 (100), 441 (13). HRMS (ESI-TOF) *m/z* calcd for $C_{34}H_{55}NO_5$ $[M+H]^+$ 558.4158, found 558.4157.

13b (1.3 g; 21 %), mp. 279 – 281 °C (MeOH); $[\alpha]_D$ +58° (c 0.36). IR (KBr): 3284, 1718, 1643 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.80, 0.91, 0.93, 0.97, 1.24, 1.43 (21H, all bs, 7 × CH_3), 2.5 (2H), 3.45 (1H, bd, *J* = 7.4 Hz, H-28a), 3.55 (1H, s, H-19), 3.77 (1H, bd, *J* = 7.4 Hz, H-28b). EI-MS: m/z (%) [all $C_{34}H_{55}NO_5$: M^+ 557]: 557 (M^+, 1), 513 (6), 457 (100), 441 (25). HRMS (ESI-TOF) *m/z* calcd for $C_{34}H_{55}NO_5$ $[M+H]^+$ 558.4158, found 558.4152.

Esters **14a** and **14b**. The solution of the mixture of hemisuccinates **13a** and **13b** (0.5 g; 1.1 mmol) was alkylated by diazomethane in Et_2O according to the common procedure. After

the purification by HPLC in hexane/EtOAc 10:1 and lyophilization from *t*-BuOH methyl-esters **14a** and **14b** were obtained: **14a** (0.25 g; 49 %), mp. 140 – 143 °C (MeOH); $[\alpha]_D$ +23° (c 0.26). IR (DRIFT): 3426, 1741, 1668, 1520 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.80, 0.85, 0.91, 0.93, 0.95, 0.97, 1.01 (21H, all s, 7 × CH_3), 1.95 (1H, dd, *J*(H-1a, H-1b) = 12.4 Hz, *J*(H-1a, H-3α) = 4.1 Hz, H-1a), 2.49 (2H, m, CH_2 - Suc), 2.66 (2H, m, CH_2 - Suc), 2.95 (1H, d, *J* = 10.5 Hz), 3.47 (1H, d, *J* = 8.8 Hz, H-28a), 3.51 (1H, s, H-19), 3.70 (3H, s, O-CH_3), 3.77 (1H, d *J* = 7.3 Hz, H-28b), 4.04 (1H, m, H-2β), 5.66 (1H, d, *J*(H-3α, H-2α) = 7.8 Hz, H-3α). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.47, 15.65, 16.26, 17.32, 18.19, 21.09, 24.53, 26.20, 26.30, 26.35, 26.35, 28.24, 28.78, 31.19, 32.67, 33.71, 34.05, 36.24, 36.70, 38.18, 39.77, 40.63, 40.73, 41.44, 45.75, 46.78, 48.94, 50.95, 51.97, 55.27, 71.21, 83.38, 87.87, 173.11, 173.67. FAB-MS: [for $C_{35}H_{57}NO_5$: M^+ 571]: 572 (M^+ + H), 557, 516. HRMS (ESI-TOF) *m/z* calcd for $C_{35}H_{57}NO_5$ $[M+H]^+$ 572.4315, found 572.4321.

14b (0.21 g; 41 %), mp. 167 – 170 °C (MeOH); $[\alpha]_D$ +70° (c 0.20). IR (DRIFT): 3424, 1734, 1670, 1522 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ: 0.80, 0.91, 0.93, 0.98, 1.10 (21H, all s, 7 × CH_3), 2.38 (1H, bs), 2.47 (2H, t, *J* = 6.9 Hz, CH_2 - Suc), 2.68 (2H, m, CH_2 - Suc), 3.45 (1H, m, H-28a), 3.52 (1H, s, H-19), 3.69 (1H, s, OCH_3), 3.77 (1H, d *J* = 7.7 Hz, H-28b), 4.22 (1H, qd, *J*(H-2α, H-3α) = 7.8 Hz, J_2 = 3.2 Hz, H-2α), 6.02 (1H, d, *J*(H-3α, H-2α) = 7.8 Hz, H-3α). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 13.40, 15.73, 18.63, 18.85, 19.07, 21.49, 24.52, 26.21, 26.30, 26.41, 28.78, 29.39, 30.26, 31.32, 32.38, 33.38, 34.24, 36.23, 36.70, 37.29, 37.74, 40.84, 40.88, 41.45, 42.05, 46.71, 47.37, 51.45, 51.88, 51.93, 71.24, 77.91, 87.89, 171.28, 173.52. FAB-MS: [for $C_{35}H_{57}NO_5$: M^+ 571]: 572 (M^+ + H), 557, 517. HRMS (ESI-TOF) *m/z* calcd for $C_{35}H_{57}NO_5$ $[M+H]^+$ 572.4315, found 572.4318.

*4.2.9. 2-sulfanyl-3-oxolupane-28-oic acid (**15**)*

Bromo derivative **2c** (1.0 g; 1.9 mmol) was dissolved in NMP (100 mL) and sodium sulfide (800 mg; 10.3 mmol) was added. The reaction was stirred 90 minutes at r.t., monitored on TLC in toluene/Et_2O 5:1 with a drop of AcOH. The reaction was quenched when poured to volume of water and extracted to an organic solvent. The organic phase was collected, washed with water and the solvents were removed in vacuo to give 854 mg (81 %) of crude **15**. IR (DRIFT): 2500 – 3300, 1704, 1683 cm^{-1}. MS (ESI^+): *m/z* (%) = 528 (15, $[M+K]^+$). MS (ESI^-): *m/z* (%) = 470 (100, $[M-H_2O]$). Compound **15** decomposed when being purified on column chromatography, probably due to oxidation by atmospheric oxygen.

*4.2.10. Methyl ester **16***

100 mg (0.204 mmol) of crude **15** was transformed to methyl ester by the reaction with diazomethane in a mixture of Et_2O and $CHCl_3$, and purified by HPLC in cyklohexane/EtOAc 13:1 to give methyl ester **16** (8 mg; 8 %), mp. 110 – 112 °C ($CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.76 (d, 3H, *J* = 6.9 Hz), 0.87 (d, 3H, *J* = 6.8 Hz), 0.94 (s, 3H), 0.95 (s, 3H), 0.98 (s, 3H), 1.07 (s, 3H), 1.21 (s, 3H, 7 × CH_3), 2.22 – 2.26 (m, 3H, H-13, H-18, H-19), 2.31 (dd, 1H, J_1 = 13.7 Hz, J_2 = 7.4 Hz, H-2), 3.66 (s, 3H, CH_3), 3.91 (dd, 1H, J_1 = 10.9 Hz, J_2 = 7.5 Hz, SH). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 14.12, 14.50, 14.66, 15.78, 19.65, 20.08, 22.63, 22.74, 22.97, 26.82, 28.24, 29.60, 29.69, 29.73, 30.92, 31.57, 31.91, 31.99, 37.85, 38.07, 40.60, 42.60, 44.11, 47.72, 48.79, 51.21, 55.22, 56.96, 176.85, 201.73. HRMS (ESI-TOF) *m/z* calcd for $C_{31}H_{50}O_3S$ $[M+H]^+$ 503.3559, found 503.3557.

*4.2.11. Reactions of bromo derivative **3c** with sodium sulfide; olefine **17***

Bromo derivative **3c** (200 mg; 0.385 mmol) was dissolved in DMSO (10 mL) with a drop of water and then sodium sulfide (1 g; 12.820 mmol) was added. According to TLC (hexane/EtOAc 5:1), starting bromo derivative **3c** was completely converted to three compounds after 5.5 hours at 75 °C. The reaction mixture was poured to tenfold volume of water and extracted to chloroform. The organic phase was collected, washed with water and the solvents were removed in vacuo to give 165 mg of crude mixture of products which was then chromatographed on silica gel (25 g) in gradient from toluene to toluene/EtOAc 10:1. The only isolated product was olefine **17**. Derivative **17** was crystallized from a mixture of solvents dichloromethan and methanol dried under the flow of N_2 and purified by chromatography on silica gel in hexane/EtOAc 10:1. Pure olefin **17** was obtained (13 mg; 7.7 %), mp. 196 – 198 °C (hexane/EtOAc) (Lit.[45] 249 – 251 °C). ^{1}H NMR was identical to the spectrum in lit.[45] The other two compounds were not obtained probably they decomposed while being chromatographed.

*4.2.12. Reactions of bromo derivative **2c** with mercaptoethanol; diosphenol **20b***

The mixture of sodium hydroxide (19 mg; 0.468 mmol), mercaptoethanol (330 µL; 4.696 mmol) and anhydrous ethanol (15 mL) was stirred at r.t. until the solution was completed. The reaction mixture was cooled to 0 – 5 °C (an ice-bath) and then bromderivative **2c** (250 mg; 0.467 mmol) was slowly added. The ice-bath was removed and the reaction mixture was stirred 3 days at r.t., while monitored on TLC in toluene/Et_2O 5:1 with a drop of

AcOH. The reaction mixture was poured to tenfold volume of water and extracted to an organic solvent. The organic phase was collected, washed with water and the solvents were removed in vacuo. The mixture of products was separated by column chromatography on silica gel in toluene/Et_2O 15:1 with 0.5 % AcOH with gradient of Et_2O to 50 % to give diosphenol **20b** (46 mg; 21 %). The other compounds decomposed during the attempts for theit purification. ^{1}H NMR was identical to the spectrum in lit.[46]

*4.2.13. Thioderivative **18***

Sodium hydroxide (3.9 mg; 0.095 mmol) in mercaptoethanol (7 mL) and anhydrous ethanol (1 mL) was stirred at r.t. until fully dissolved. The reaction mixture was cooled by ice bath and then bromo derivative **3c** (100 mg; 0.19 mmol) was slowly added. The ice-bath was removed and the reaction mixture was stirred 5 days at r.t., while monitored on TLC in hexne/EtOAc 5:1. The reaction mixture was poured to tenfold volume of water and extracted to an organic solvent. Organic phase was collected, washed with water and the solvents were removed in vacuo. Thioderivative **18** was crystallized from EtOAc to give 81 mg (78 %) of pure compound. IR (DRIFT): 1033, 1654, 3341 cm^{-1}. HRMS (FAB) *m/z* calcd for $C_{32}H_{52}O_3S$ $[M+Na]^+$ 537.3457, found 537.3455. Compound was unstable and during NMR measurement and during attempts for its purification, it dehydrated to heterocycle **19**. 40 mg of crystals were purified by chromatography on silica gel in hexane/EtOAc 30:1; however, dehydratation to 2',3'-dihydro-1',4'-oxathiin derivative **19** (17 mg; 36 %) was observed again. Spectral data for **19** are shown in the experiment below that describes intentional synthesis of **19**.

*4.2.14. 2',3'-dihydro-1',4'-oxathiin derivative **19** and diosphenol **20a***

The mixture of sodium hydroxide (79 mg; 1.927 mmol), mercaptoethanol (135 µL; 1.927 mmol) and anhydrous ethanol (10 mL) was stirred at r.t. until the solution was completed. The reaction mixture was cooled to 0 – 5 °C (an ice-bath) and then bromderivative **3c** (850 mg; 1.638 mmol) was slowly added. The ice-bath was removed and the reaction mixture was stirred 3 days at r.t., while monitored on TLC in hexne/EtOAc 5:1. The reaction mixture was poured to tenfold volume of water and extracted to an organic solvent. The organic phase was collected, washed with water and the solvents were removed in vacuo. The mixture of products was separated by column chromatography on silica gel in hexane/EtOAc 10:1 with gradient of EtOAc to 100 % to give white crystals of 2',3'-dihydro-1',4'-oxathiin derivative **19** (89 mg; 11 %), mp. 269 – 271 °C (cyclohexane); $[\alpha]_D$ +88.2° (c 0.34). IR (DRIFT): 1655 cm^{-1}. The full assignment of NMR signals was performed using the 2D NMR

that is part of the supplemental file. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.79 (s, 3H, H-29), 0.90 (s, 3H, H-27), 0.92 (s, 3H, H-30), 0.92 (m, 1H, H-12a), 0.93 (s, 6H, H-24,26), 0.98 (s, 3H, H-25), 1.04 (s, 3H, H-23), 1.27 (m, 1H, H-7a), 1.28 (m, 1H, H-11a), 1.30 (m, 1H, H-a), 1.30 (tt, 1H, J_1 = 12.3 Hz, J_2 = 3.7 Hz, H-21a), 1.38 (m, 1H, H-9), 1.40 (m, 1H, H-6b), 1.42 (m, 1H, H-7b), 1.43 (m, 1H, H-16b), 1.44 (m, 1H, H-12b), 1.45 (m, 1H, H-11b), 1.46 (m, 1H, H-22b), 1.46 (mm, 2H, H-13,18), 1.52 (dd, 1H, *J* = 12.3, 4.7 Hz, H-21b), 1.63 (m, 1H, H-15b), 1.79 (d, 1H, *J* = 15.2 Hz, H-1a), 1.87 (d, 1H, *J* = 15.2 Hz, H-1b), 2.92 (dq, 1H, J_1 = 12.8 Hz, J_2 = 2.5 Hz, H-31a), 3.04 (m, 1H, H-31b), 3.36 (m, 1H, H-6a), 3.43 (d, 1H, *J* = 7.8 Hz, H-28a), 3.52 (m, 1H, H-19), 3.77 (d, 1H, *J* = 7.8 Hz, H-28b), 4.06 (m, 1H, H-32a), 4.27 (m, 1H, H-32b). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 13.56, 15.53, 16.28, 19.79, 19.48, 21.55, 24.65, 26.33, 26.51, 26.52, 27.09, 28.19, 28.89, 32.82, 33.09, 34.37, 36.36, 36.85, 37.12, 38.77, 40.57, 40.78, 41.57, 45.79, 46.87, 49.58, 53.28, 65.27, 71.36, 88.00, 96.28, 149.23. MS (ESI$^+$): *m/z* (%) = 499 (65, $[M+H]^+$), 521 (100, $[M+Na]^+$), 1019 (45, $[2M+Na]^+$). HRMS (ESI-TOF) *m/z* calcd for $C_{32}H_{50}O_2S$ $[M+H]^+$ 499.3604, found 499.3604.

Diosphenol **20a** was obtained as a byproduct (304 mg; 41 %), mp. 204 – 206 °C (cyklohexane), lit.[47] 231.5 – 233 °C ($CHCl_3$/MeOH). ^{1}H NMR was identical to the spectrum in lit.[47]

*4.2.15. 2-nitro-3-oxolupane-28-oic acid (**21**)*

Dihydrobetulonic acid (**2b**) (200 mg; 0.44 mmol) was dissolved in acetic acid (4 mL) by heating. The solution was gradually cooled to r.t. and nitric acid (2 mL, 67 %) was added dropwise. The reaction mixture was vigorously stirred 25 h at r.t., monitored on TLC in hexane/EtOAc 5:1. The reaction mixture was poured to tenfold volume of water. The precipitate was filtered off, washed with $KHCO_3$ and water and crystallized from $CHCl_3$ and cyklohexane. Crude product was chromatographed on silica gel in CH_2Cl_2:MeOH:AcOH 500:10:1 and purified by chromatography on silica gel in cyklohexane/EtOAc 5:1 to give nitrocompound **21** (51 mg; 23 %), mp. 248 – 249 °C ($CHCl_3$/cyklohexane). IR (DRIFT): 2450 – 3200, 1692, 1607, 1570 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.78 (d, 3H, *J* = 6.9 Hz), 0.87 (s, 3H), 0.88 (d, 3H, *J* = 7.4 Hz), 0.98 (s, 6H), 1.19 (s, 3H), 1.27 (s, 3H, 7 × CH_3), 1.98 (d, 1H, *J* = 15.5 Hz, H-1a), 2.23 – 2.29 (m, 3H, H-13, H-18, H-19), 2.84 (d, 1H, *J* = 15.5 Hz, H-1b). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 14.52, 14.64, 15.68, 16.07, 19.45, 20.45, 21.40, 22.71, 22.96, 26.68, 28.62, 29.63, 29.71, 31.91, 33.04, 36.31, 37.34, 38.29, 39.99, 40.12, 40.47, 42.62, 44.10, 48.56, 48.61, 51.77, 56.78, 122.88, 178.38, 181.90. MS (ESI$^+$): *m/z* (%) =

519 (40, $[M+H_2O]^+$). (ESI$^-$): *m/z* (%) = 500 (100, $[M-H]^-$). HRMS (ESI-TOF) *m/z* calcd for $C_{30}H_{47}NO_5$ $[M+H]^+$ 502.3527, found 502.3527.

*4.2.16. 2,2-dinitro-3-oxolupane-28-oic acid (**23**)*

Dihydrobetulonic acid (**2b**) (1.0 g; 2.19 mmol) was dissolved in acetic acid (20 mL) by heating. The solution was gradually cooled to 35 °C and nitric acid (10 mL, 67 %) was added dropwise. The reaction mixture was vigorously stirred 6 h at 35 °C, monitored on TLC in hexane/EtOAc 5:2. The reaction mixture was poured to tenfold volume of water. The precipitate was filtered off, washed with $KHCO_3$ and water and crystallized from $CHCl_3$ and cyklohexane. Crude product was purified by column chromatography on silica gel in CH_2Cl_2:MeOH:AcOH 500:10:1. Fractions containing **23** were collected and evaporated to give shiny white crystals of dinitroderivative **23** (432 mg; 36 %), mp. 226 – 228 °C ($CHCl_3$/hexane); $[\alpha]_D$ +9.6° (c 0.47). IR (DRIFT): 2500 – 3300, 1734, 1692, 1573 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.78 (d, 3H, *J* = 6.3 Hz), 0.88 (d, 3H, *J* = 6.9 Hz), 0.98 (s, 3H), 1.00 (s, 3H), 1.01 (s, 3H), 1.23 (s, 3H), 1.26 (s, 3H, 7 × CH_3), 2.25 – 2.30 (m , 3H, H-13, H-18, H-19), 2.93 (d, 1H, *J* = 16.0 Hz, H-1a), 3.10 (d, 1H, *J* = 16.0 Hz, H-1b), 10.92 (bs, 1H, COOH). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 14.50, 14.63, 15.43, 17.32, 19.59, 21.81, 22.71, 22.94, 23.12, 26.57, 29.53, 29.73, 30.34, 31.82, 32.65, 36.73, 37.34, 38.17, 40.56, 42.83, 44.06, 47.49, 48.48, 48.48, 49.32, 52.26, 56.76, 119.13, 182.19, 197.20. MS (ESI$^+$): *m/z* (%) = 564 (100, $[M+H_2O]^+$). HRMS (ESI-TOF) *m/z* calcd for $C_{30}H_{46}N_2O_7$ $[M+H]^+$ 547.3383, found 547.3385.

*4.2.17. 2-nitroallobetulone **22**, 2,2-dinitroallobetulone **24**, and enolacetate **25***

Allobetulin (**3a**) (500 mg; 1.129 mmol) was dissolved in acetic acid (10 mL) by heating at 80 °C. By gradual cooling to r.t., tiny crystals of starting material precipitated. Nitric acid (5 mL, 58%) was added dropwise to the vigorously stirred reaction mixture. The reaction mixture was stirred for 5 hours at r.t., monitored on TLC in hexane/EtOAc. The reaction mixture was poured to tenfold volume of water and extracted to an organic solvent. The organic phase was collected, washed with water and the solvents were removed in vacuo. The mixture of nitroderivative **22**, dinitroderivative **24** and enolacetate **25** was separated by chromatography on silica gel in dichlormethan. Fractions containing pure nitroderivative **22** or dinitroderivative **24** were collected and evaporated to dryness to give shiny white crystals of 2-nitroallobetulone **22** (111 mg; 20 %), mp. 236 – 238 °C (Lit.[48] 235.8 °C) and white

crystals of 2,2-dinitroallobetulone **24** (109 mg; 18 %), mp. 212 – 213 °C (Lit.[48] 202.4 °C). ^{1}H NMR was in both cases identical to the corresponding spectrum in lit.[48]

By extending the reaction time to 17 hours, allobetulon-1(2)-en-3-acetate (**25**) was the main product of the identical reaction (74 mg; 14 %), mp. 287 °C (evaporated from dichlormethan). Lit.[48] 262 – 264 °C (EtOH/$CHCl_3$). ^{1}H NMR was identical to the spectrum in lit.[48]

*4.2.18. Acetate **27***

Zinc dust (133 mg; 2.046 mmol) was added to the solution of 2-nitroallobetulone **22** (100 mg; 0.206 mmol) in acetic acid (6 mL). The reaction mixture was heated under reflux for 10 min then the heating was stopped and the mixture stirred another 15 min, while being monitored by TLC in hexane/EtOAc 5:1. The reaction was quenched by filtering the zinc dust out of the reaction mixture. Acetic anhydride (3 mL; 31.7 mmol) was added to the reaction mixture. The acylation was completed after 1 hour of stirring at r.t., monitored on TLC in hexane/EtOAc 1:1. The reaction mixture was poured to tenfold volume of water and extracted to an organic solvent. The organic phase was collected, washed with water and the solvents were removed in vacuo. Crude products from the two identical reactions were collected and purified by chromatography on silica gel in hexane/EtOAc 1:1. Fractions containing derivative **27** (mixture of epimers) were collected and evaporated to give white powder of **27** (87 mg; 47 %), mp. 230 – 232 °C (cyklohexane); $[\alpha]_D$ -9.2° (c 0.33). IR (DRIFT): 3426, 1704, 1677 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 0.73, 0.79, 0.81, 0.88, 0.93, 0.95, 1.03, 1.10, 1.11, 1.12, 1.28 (21H, all s, 7 × CH_3), 1.82 – 1.84 (m, 1H, H-1a), 2.01 (s, 3H, Ac), 2.59 – 2.64 (m, 1H, H-1b), 3.45 (d, 1H, *J* = 8 Hz, H-28a), 3.52 (s, 1H, H-19), 3.77 (d, 1H, *J* = 8 Hz, H-28b), 4.86 – 4.97 (m, 1H, H-2α,2β), 6.49 (dd, 1H, *J* = 75, 5.5 Hz, NH). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 13.40, 13.40, 14.17, 15.00, 15.83, 16.67, 19.03, 19.14, 19.30, 19.83, 20.98, 21.35, 22.06, 23.23, 23.33, 24.49, 24.55, 24.58, 26.18, 26.18, 26.24, 26.24, 26.36, 26.36, 28.75, 28.75, 28.94, 32.34, 32.67, 32,67, 33.69, 34.03, 34.41, 36.23, 36.23, 36.68, 36.68, 36.96, 37.98, 40.37, 40.73, 40.75, 40.79, 41.40, 41.43, 46.30, 46.67, 46.75, 48.62, 49.12, 50.00, 50.68, 50.78, 51.53, 51.70, 58.49, 71.19, 71.19, 87.85, 87.91, 169.54, 169.70, 212.98, 215.55. MS (ESI^+): *m/z* (%) = 498 (100, $[M+H]^+$), 996 (18, $[2M+H]^+$). MS (ESI^-): *m/z* (%) = 496 (100, $[M-H]^-$). HRMS (ESI-TOF) *m/z* calcd for $C_{32}H_{51}NO_3$ $[M+H]^+$ 498.3942, found 498.3940.

*4.2.19. Acetate of enaminoketone **28***

Zinc dust (100 mg; 1.538 mmol) was added to the solution of 2-nitroallobetulone **22** (75 mg; 0.155 mmol) in acetic acid (5 mL). The reaction mixture was heated to reflux for 10 min and stirred 15 min more, monitored on TLC in hexane/EtOAc 5:1. The reaction was quenched by filtered zinc off the reaction mixture. Toluene (10 mL) was added to the reaction vial and the mixture was boiled for 3 days, monitored on TLC in hexane/EtOAc 1:1. The reaction mixture was poured to tenfold volume of water and extracted to an organic solvent. The organic phase was collected, washed with water and the solvents were removed in vacuo. Crude product (270 mg) from the two identical reactions was purified by chromatography on silica gel in cyklohexane/EtOAc 5:1. Fractions containing **28** were collected and evaporated to dryness to give yellow-orange powder of acetate **28** (46 mg; 20 %), mp. 135 – 137 °C (acetone); $[\alpha]_D$ +58° (c 0.25). IR (DRIFT): 3383, 1693, 1659, 1632, 1510, 1035 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.81, 0.92, 0.94, 1.05, 1.13, 1.13, 1.18 (21H, all s, 7 × CH_3), 2.09 (s, 3H, Ac), 3.45 (d, 1H, *J* = 7.8 Hz, H-28a), 3.55 (s, 1H, H-19), 3.77 (d, 1H, *J* = 7.5 Hz, H-28b), 7.77 (s, 1H, H-1), 8.16 (s, 1H, NH). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 13.31, 14.10, 16.14, 18.82, 20.52, 21.24, 21.73, 22.61, 24.47, 26.20, 26.29, 27.95, 28.72, 31.55, 32.64, 34.21, 36.21, 36.65, 38.67, 40.99, 41.40, 41.49, 44.03, 45.86, 46.64, 52.67, 71.20, 87.83, 129.13, 140.81, 168.82, 200.09. MS (ESI^+): *m/z* (%) = 497 (100, $[M+H]^+$), 992 (10 [2M]). HRMS (ESI-TOF) *m/z* calcd for $C_{32}H_{49}NO_3$ $[M+H]^+$ 496.3785, found 496.3784.

4.3. Cell lines

All cells (if not indicated otherwise) were purchased from the American Tissue Culture Collection (ATCC). The CCRF-CEM line is derived from T lymphoblastic leukemia, evincing high chemosenzitivity, K562 represent cells from an acute myeloid leukemia patient sample with bcr-abl translocation, U2OS line is derived from osteosarcoma, HCT116 is colorectal tumor cell line and its p53 gene knock-down counterpart (HCT116p53-/-, Horizon Discovery Ltd, UK) is a model of human cancers with p53 mutation frequently associated with poor prognosis, A549 line is lung adenocarcinoma. 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 and P-glycoprotein protein, while K562-TAX cells overexpress P-glycoprotein only. Both proteins belong to the family of ABC transporters and are involved in the primary and/or acquired multidrug resistance phenomenon.[50] MRC-5 and BJ cell lines were used as a non-tumor control and represent human fibroblasts. The cells were maintained in nunc/corning 80 cm^2 plastic tissue culture flasks and cultured in cell culture medium according to ATCC or Horizon

recommendations (DMEM/RPMI 1640 with 5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 10% fetal calf serum, and $NaHCO_3$).

4.4. Cytotoxic MTS assay

MTS assay was performed at Institute of Molecular and Translational Medicine by robotic platform (HighResBiosolutions). Cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (25000 - 35000 cells/mL based on cell growth characteristics). Cells were added by automatic pipetor (30 μL) into 384 well microtiter plates. All tested compounds were dissolved in 100% DMSO and four-fold dilutions of the intended test concentration were added in 0.15 μL aliquots at time zero to the microtiter plate wells by the echoacustic non-contact liquid handler Echo550 (Labcyte). The experiments were performed in technical duplicates and three biological replicates at least. The cells were incubated with the tested compounds for 72 h at 37 °C, in a 5% CO_2 atmosphere at 100% humidity. At the end of the incubation period, the cells were assayed by using the MTS test. Aliquots (5 μL) of the MTS stock solution were pipetted into each well and incubated for additional 1–4 h. After this incubation period, the optical density (OD) was measured at 490 nm with an Envision reader (Perkin Elmer). Tumor cell survival (TCS) was calculated by using the following equation: $TCS = (OD_{\text{drug-exposed well}}/\text{mean } OD_{\text{control wells}}) \times 100\%$. The IC_{50} value, the drug concentration that is lethal to 50% of the tumor cells, was calculated from the appropriate dose-response curves in Dotmatics software.

4.5. Cell Cycle and Apoptosis Analysis

Suspension of CCRF-CEM cells, seeded at a density of 1.10^6 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. Together with the treated cells, control sample containing vehicle was harvested at the same time point after 24 h. After another 24 hours, cells were then washed with cold PBS and fixed in 70% ethanol added dropwise and stored overnight at -20 °C. Afterwards, cells were washed in hypotonic citrate buffer, treated with RNAse (50 μg/mL) and stained with propidium iodide. Flow cytometer using a 488 nm single beam laser (Becton Dickinson) was used for measurement. Cell cycle was analyzed in the program ModFitLT (Verity), and apoptosis was measured in logarithmic model expressing percentage of the particles with propidium content lower than cells in G0/G1 phase (<G1) of the cell cycle. Half of the sample was used for $pH3^{Ser10}$ antibody (Sigma) labeling and subsequent flow cytometry analysis of mitotic cells.[51]

4.6. BrDU Incorporation Analysis (DNA synthesis)

For this analysis, the same procedure of cultivation as previously was used. Before harvesting, 10 μM 5-bromo-2-deoxyuridine (BrDU), was added to the cells for puls-labeling for 30 min. Cells were fixed with ice-cold 70% ethanol and stored overnight. Before the analysis, cellswere 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 $Na_2B_4O_7$ (Borax), cells were washed with PBS containing 0.5% Tween-20 and 1% BSA. Staining with primary anti-BrDU antibody (Exbio) for 30 min at room temperature in the dark followed. Cells were than washed with PBS and stained with secondary antimouse-FITC antibody (Sigma). Cells were then washed with PBS again 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 afterwards analyzed by flow cytometry using a 488 nm single beam laser (FACSCalibur, Becton Dickinson).[51]

4.7. BrU Incorporation Analysis (RNA synthesis)

Cells were cultured and treated as above. Before harvesting, pulse-labeling with 1 mM 5-bromo uridine (BrU) for 30 min followed. The cells were then fixed in 1% buffered paraformaldehyde with 0.05 % of NP-40 in room temperature for 15 min, and then stored in 4°C overnight. Before measurement, they werewashed in PBS with 1% glycin, washed in PBS again, and stained by primary anti-BrDU antibody crossreacting to BrU (Exbio) for 30 min at room temperature in the dark. After another washing step in PBS cells were stained by secondary antimouse-FITC antibody (Sigma). Following the staining, cells were washed with PBS and fixed with 1% PBS buffered paraformaldehyde with 0.05% of NP-40 for 1 hour. Cells were washed by PBS, incubated with propidium iodide (0.1 mg/mL) and RNAse A (0.5 mg/mL) for 1 h at room temperature in the dark, and finally analyzed by flow cytometry using a 488 nm single beam laser (FACS Calibur, Becton Dickinson).[37]

5. Acknowledgment

The chemical part was supported by Czech Science Foundation (15-05620S) and internal grants of Palacky University IGA_PrF_2015_00, IGA-PrF-2016-020, and IGA_LF_2016_19. The biological part was paid by Technology Agency of the Czech Republic (TE01020028). The infrastructural part (Institute of Molecular and Translational Medicine) is supported by the National Sustainability Programme (LO1304). We are grateful to Lukas Najdekr for measurement of HRMS.

6. References

[1] R.A. Hill, J. D. Connolly, Nat. Prod. Rep. 32 (2015) 273.

[2] P. Dzubak, M. Hajduch, D. Vydra, A. Hustova, D. Biedermann, L. Markova, M. Urban, J. Sarek, Nat. Prod. Rep. 23 (2006) 394.

[3] J. Sarek, M. Kvasnica, M. Vlk, D. Biedermann, Semisynthetic Lupane Triterpenes with Cytotoxic Activity, in: J.A.R. Salvador (Ed.), Pentacyclic Triterpenes as Promising Agents in Cancer, Nova Science Publishers, Hauppauge NY USA, 2010, ISBN 978-1-60876-973-5, pp. 159-189. Chapter 7.

[4] Z. Dang, P. Ho, L. Zhu, K. Qian, K.-H. Lee, L. Huang, C.-H. Chem, J. Med. Chem. 56, (2013) 2029.

[5] W.-J. Zuo, H.-F. Dai, J. Chen, H.-Q. Chen, Y.-X. Zhao, W.-L. Mei, J.-H. Wang, Planta Med. 77 (2011) 1835.

[6] B. Innocente, B.B. Casanova, F. Klein, A.D. Lana, D. Pereira, M.N. Muniz, G.G. Sonnet, A.M. Fuentefria, S.C.B. Gnoatto, Chem. Biol. Drug Des. 83 (2014) 344.

[7] G. Chianese, S.R. Yerbanga, L. Lucantoni, A. Habluetzel, N. Basilico, D. Taramelli, E. Fattorusso, O. Tagalialatela-Scafati, J. Nat. Prod. 73 (2010) 1448.

[8] Y. Fu, E. Zhou, Z. Wei, D. Liang, W. Wang, T. Wang, M. Guo, N. Zhang, Z. Yang, FEBS J. 281 (2014) 2543.

[9] S. Yano, M. Harada, K. Watanabe, K. Nakamaru, Y. Hatakeyama, S. Shibata, K. Takahashi, T. Mori, K. Hirabayashi, M. Takeda, N. Nagata, Chem. Pharm. Bull. 37 (1989) 2500.

[10] T. Morikawa, K. Ninomiya, T. Yamaguchi, M. Yoshikawa, T. Hayakawa, O. Muraoka, Phytochemistry 102 (2014) 169.

[11] C. Sanchez-Quesada, A. Lopez-Biedma, F. Warleta, M. Campos, G. Beltran, J.J. Gaforio, J. Agr. Food Chem. 61 (2013) 12182.

[12] M. Kvasnica, M. Urban, N.J. Dickinson, J. Sarek, Nat. Prod. Rep. 32 (2015) 1303.

[13] L. Zhang, X. Jia, J. Dong, D. Chen, J. Liu, L. Zhang, X. Wen, Chem. Biol. Drug. Des. 83 (2014) 297.

[14] M. W. Pertino, C. Lopez, C. Theoduloz, G. Schmeda-Hirschmann, G, Molecules 18 (2013) 7661.

[15] B. Chakraborty, D. Dutta, S. Mukherjee, S. Das, N. C. Maiti, P. Das, C. Chowdhury, Eur. J. Med. Chem. 102 (2015) 93.

[16] I. Khan, S. K. Guru, S. K. Rath, P. K. Chinthakindi, B. Singh, S. Koul, S. Bhushan, P. L. Sangwan, Eur. J. Med. Chem. 63 (2015) 782.

[17] R. Csuk, A. Barthel, R. Sczepek, B. Siewert, S. Schwartz, Arch. Pharm. Chem. Life Sci. 1 (2011) 37.

[18] R. Csuk, A. Barthel, R. Kluge, D. Strohl, Bioorg. Med. Chem. 18 (2010) 7252.

[19] J. Wiemann, L. Heller, V. Perl, R. Kluge, D. Ströhl, R. Csuk, Eur. J. Med. Chem. 106 (2015) 194.

[20] R. Csuk, Expert Opin. Ther. Pat. 24 (2014) 913.

[21] W. Deahaen, A.A. Matshentseva, T.S. Seitembetov, Molecules 16 (2011) 2443.

[22] T. Xu, Q. Pang, D. Zhou, A. Zhang, S. Luo, Y. Wang, X. Yan, PLoS One 9 (2014), e105768, DOI: 10.1371.

[23] T.C. Bernardo, T. Cunha-Oliveira, T.L. Serafim, J. Holy, D. Krasutsky, O. Kolomitsyna, P. Krasutsky, A.M. Moreno, P.J. Oliveira, Bioorg. Med. Chem. 21 (2013) 7239.

[24] L. Li, Y. Du, X. Kong, Z. Li, Z. Jia, J. Cui, J. Gao, G. Wang, K. Xie, Clin. Cancer Res. 19 (2013) 4651.

[25] X. Liu, I. Jutooru, P. Lei, K. Kim, S.O. Lee, L.K. Brents, P.L. Prather, S. Safe, Mol. Cancer Ther. 11 (2012) 1421.

[26] M. Eichenmuller, D. von Schweinitz, D. Kappler, Int. J. Oncol. 35 (2009) 873.

[27] M. Soural, J. Hodon, N.J. Dickinson, V. Sidova, S. Gurska, P. Dzubak, M. Hajduch, J. Sarek, M. Urban, Bioconjugate Chem. 26 (2015) 2563.

[28] M. Urban, J. Sarek, I. Tislerova, P. Dzubak, M. Hajduch, Bioorg. Med. Chem. 13 (2005) 5527.

[29] J. Sarek, M. Kvasnica, M. Urban, J. Klinot, M. Hajduch, Bioorg. Med. Chem. Lett. 15 (2005) 4196.

[30] M. Urban, J. Sarek, M. Kvasnica, I. Tislerova, M. Hajduch, J. Nat. Prod. 70 (2007) 526.

[31] M. Urban, M. Vlk, P. Dzubak, M. Hajduch, J. Sarek, Bioorg. Med. Chem. 20 (2012) 3666.

[32] M. Urban, M. Kvasnica, N.J. Dickinson, J. Sarek, Biologically active triterpenoids usable as prodrugs, in A.R. Bates (Ed.), Terpenoids and Squalene: Biosynthesis, Functions and Health Implications, Nova Science Publishers, Hauppauge NY USA, 2015, ISBN 978-1-63463-656-8, pp. 25-50. Chapter 2.

[33] L. Borkova, L. Jasikova, J. Rehulka, K. Frisonsova, M. Urban, I. Frydrych, I. Popa, M. Hajduch, N.J. Dickinson, M. Vlk, P. Dzubak, J. Sarek, Eur. J. Med. Chem. 96 (2015) 482.

[34] M. Urban, J. Sarek, J. Klinot, G. Korinkova, M. Hajduch, J. Nat. Prod. 67 (2004) 1100.

[35] J. Klinot, A. Vystrcil, Collect. Czech. Chem. Commun. 31 (1966) 1079.

[36] Y.-J. You, Y. Kim, N.-H. Nam, B.-Z. Ahn, Bioorg. Med. Chem. Lett. 13 (2003) 3137.

[37] T.S. Li, J.X. Wang; X.J.J.Zheng, Chem. Soc. Perkin Trans 1 (1998) 3957.

[38] L. Heller, A. Obenauer, R. Csuk, Bioorg. Med. Chem. 23 (2015) 3002.

[39] S. Hunneck, Chem. Ber. 98 (1965) 1837.

[40] T.D. Ngoc, N. Moons, Y. Kim, W. De Borggraeve, A. Mashentseva, G. Andrei, R. Snoeck, J. Balzarini, W. Dehaen, Bioorg. Med. Chem 22 (2014), 3292.

[41] K. Waisser, A. Vystrcil, Collect. Czech. Chem. Commun. 42 (1977) 1220.

[42] A. Wolloch, E. Zbiral, Tetrahedron 32 (1976) 1289.

[43] G.R. Pettit, N. Melody, F. Hempenstall, J.-C. Chapuis, T.L. Groy, L. Williams, J. Nat. Prod. 77 (2014) 863.

[44] H.-O. Kim, G.A. Tolstikov, M.I. Goryaev, Izvestiya Akademii Nauk Kazakhskoi SSR, Seriya Khimicheskaya 20 (1970) 49.

[45] J. Klinot, A. Vystrcil, Collect. Czech. Chem. Commun. 31 (1966) 1079.

[46] Y. Deng, J.K. Snyder, J. Org. Chem. 67 (2002) 2864.

[47] A.V. Korovin, A.V. Tkachev, Russ. Chem. Bull. 50 (2001) 304.

[48] A.V. Shernyukov, I.Y. Mainagashev, D.V. Korchagina, Y.V. Gatilov, N.F. Salakhutdinov, G.A. Tolstikov, Chem. Nat. Compd. 48 (2012) 821.

[49] L. Heller, A. Obenauer, R. Csuk, Bioorg. Med. Chem. 23 (2015) 3002.

[50] V. Noskova, P. Dzubak, G. Kuzmina, A. Ludkova, D. Stehlik, R. Trojanec, A. Janostakova, G. Korinkova, V. Mihal, M. Hajduch, Neoplasma 49 (2002) 418.

[51] A. Bourderioux, P. Nauš, P. Perlíková, R. Pohl, I. Pichová, I. Votruba, P. Džubák, P. Konečný, M. Hajdúch, K.M. Stray, T. Wang, A.S. Ray, J.Y. Feng, G. Birkus, T. Cihlar, M. Hocek, J. Med. Chem. 54 (2011)5498.

Comp.	IC_{50} (μmol/L[a])	Comp.	IC_{50} (μmol/L[a])	Comp.	IC_{50} (μmol/L[a])	Comp.	IC_{50} (μmol/L[a])
	CCRF-CEM		CCRF-CEM		CCRF-CEM		CCRF-CEM
1	>50	**5**[b]	27	**12a**	>50	**20a**	13
2a	14	**6**[b]	27	**12b**	>50	**20b**	6
2b	4	**7**	>50	**13a**	>50	**21**	32
2c	2	**8**	>50	**13b**	>50	**22**	>50
3a	>50	**9**	>50	**14a**	>50	**23**	29
3b	>50	**10a**	>50	**14b**	>50	**24**	>50
3c	>50	**10b**	>50	**16**	40	**27**	32
4	17	**11**	5	**19**	>50	**28**	30

Table 1.

Comp.	CCRF-CEM	CEM-DNR	K562	K562-TAX	A549	HCT116	HCT116 $p53^{-/-}$	U2OS	BJ	MRC-5	TI[a]
4	17.5	16.0	14.2	10.6	25.7	>50.0	>50.0	48.9	>50.0	>50.0	>2.9
5[b]	26.6	33.9	>50.0	20.1	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>1.9
6[b]	26.6	33.9	>50.0	20.1	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>1.9
11	4.6	5.9	4.5	3.9	6.2	4.9	4.8	6.2	7.1	6.3	1.4
16	45.3	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>1.1
20a	13.1	>50.0	16.9	45.3	>50.0	41.6	33.4	45.8	>50.0	25.4	>2.9
21	31.8	36.1	10.1	20.6	31.3	>50.0	>50.0	>50.0	>50.0	>50.0	>1.6
23	28.6	46.6	>50.0	29.9	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>1.7
27	32.2	>50.0	32.4	>50.0	>50.0	42.9	38.7	>50.0	>50.0	21.6	>1.1
28	29.9	35.3	37.9	30.5	>50.0	>50.0	>50.0	>50.0	>50.0	>50.0	>1.7

Table 2.

	Used conc. (μM)	sub G1 (%)	G0/G1 (%)	S (%)	G2/M (%)	$pH3^{Ser10}$ (%)	DNA synthesis (%)	RNA synthesis (%)
Control	-	1.80	36.41	50.33	13.28	1.66	56.37	43.37
11	4.60[a]	2.79	40.73	47.77	11.50	1.44	53.53	42.98
11	23.00[b]	7.1	28.90	54.92	16.18	0.49	1.88	2.23

Table 3.

Highlights:

- 19 new lupane and 18α-oleanane derivatives, substituted at C-2, were prepared
- Cytotoxicity of compounds was measured on 8 cancer and 2 non-cancer cell lines
- Most active compounds interfered with cell cycle and DNA/RNA synthesis
- 2-amino allobetulin was the most active compound of this series
- 2-amino allobetulin almost blocked DNA/RNA synthesis at 5 × IC_{50}