Synthesis and Biological Evaluation of Some Pentacyclic Lupane Triterpenoid Esters

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This paper presents the biological activity of betulin and betulinic acid derivatives with a fatty acid chain tailing, compared with that of native compounds. Their activity as tumor cell inhibiting agents was tested on two tumor cell lines, A431 (skin epidermoid carcinoma) and A375 (human melanoma). The aim of the chemical acylation was to obtain highly hydrophobic compounds with an improved biological activity and lower side effects. Betulin and betulinic acid derivatives were synthesized following the path of chemical esterification in solvent medium, in the presence of triethylamine and fatty acid chloride. Betulin esterification was focused on both hydroxyl groups, C-3 and C-28, while for betulinic acid only the C-3 position was taken into account, due to previous reports that showed a high biological activity conferred by the C-28 carboxylic acid, and C-30 allylic positions.

Keywords: betulin, betulinic acid, esters, cytotoxic activity, A431, A375

Natural compounds are generally derivatized for the purpose of increasing water solubility as well as resistance against oxidation and other metabolic degradation processes within the bloodstream. Recently, fatty acids have captured the interest of scientists as natural compounds and reaction partners which can provide new biocompounds with increased hydrophobicity. The coupling of one bioactive natural molecule with various fatty acids is expected to lead to chemically stable molecules with improved biological activity.

Betulin (Bet), betulinic acid (BA) and their natural derivatives are found in various plant species including the white birch (*Betula pubescens*), pendent birch (*Betula verrucosa Ehrh*. or *Betula pendula Roth*.), downy birch (*B. pubescens Ehrh*.), alder tree (*Aldus subcordata* L.), ber tree (*Ziziphus mauritiana*), and others [1-3]. The two natural products, betulin and betulinic acid, have gained a remarkable attention within the scientific field due to their numerous biological activities: antitumor, anti-inflammatory, antimalarial, antimicrobial and anti-HIV activity [4-8]. Both compounds were chemically modified in order to develop new therapeutic agents with reduced side effects and enhanced activity. Dimethylsuccinoyl derivatives of this pentacyclic triterpenes have proven to be promising inhibitors of *in vitro* HIV replication [9, 10]. A series of recent publications have mentioned a large number of betulin and betulinic acid derivatives with antitumor activity against different types of cell lines. It is well known that cancer is a multistage process and the effort to stop this process at a certain stage has made research in this area to become extremely relevant. A series of betulin and betulinic acid derivatives were synthesized, showing promising effects in suppressing tumor occurrence and development [11, 12]. Short fatty acid derivatives demonstrated excellent chemopreventive activity in both EBV-EA activation and two-stage mouse skin carcinogenesis assays [13]. Kommera *et al.* investigated the antiproliferative activity of thirteen derivatives of betulinic acid and betulin that were tested against five different tumor cell lines [14]. A previous study showed that extracts containing high concentrations of betulin developed a strong *in vitro* antiproliferative effect and an important *in vivo* activity, mainly anti-inflammatory, which adds to its potent antitumor properties [15].

The aim of this study was to obtain highly hydrophobic and stable new compounds starting from betulin and betulinic acid, using fatty acid chloride as esterification partner. Both C-3 and C-28 hydroxyle groups of betulin were acylated while only the C-3 hydroxyl of betulinic acid entered the reaction. The new compounds were then subjected to preliminary *in vitro* tests as antitumor agents, by means of MTT assay, on two tumor cell-lines, A431 (skin epidermoid carcinoma) and A375 (human melanoma).

Experimental part

Materials and methods

Betulin was extracted from white birch bark, betulinic acid was purchased from Sigma Aldrich (Germany), myristoyl chloride and triethylamine were a gift from "Politehnica" University of Timisoara, and all the other chemicals including the solvents (chloroform, isopropanol, *n*-hexane, ethyl acetate, tetrahydrofuran, methanol (HPLC grade), acetonitrile (HPLC grade)), were at least of analytical grade and used as received. The solvent mixture was defined as v/v.

Betulin extraction from birch bark. Dried birch tree bark (*Betula pendula* Roth) was subject to Soxhlet extraction for 8 h, using isopropanol. After the removal of the solvent using a rotary evaporator (300 mmHg, 50°C) the final solid product was dried and re-crystallized from chloroform/ethanol (80/20) mixture. The extraction yield was 16%.

Chemical esterification of betulin and betulinic acid. Betulin (2 mmol) was solubilized in chloroform (20 mL) at reflux temperature under magnetic stirring. The temperature was adjusted at 50°C and triethylamine was added. The esterification started after the myristoyl chloride (4 mmol) was dropped in the solution, betulin:myristoyl chloride molar ratio 1:2. The mixture was then cooled to

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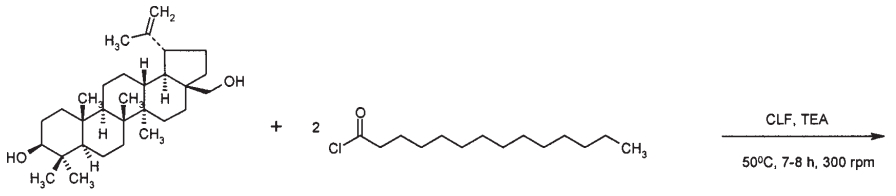
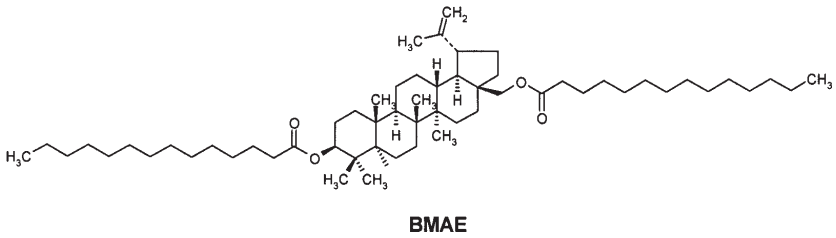
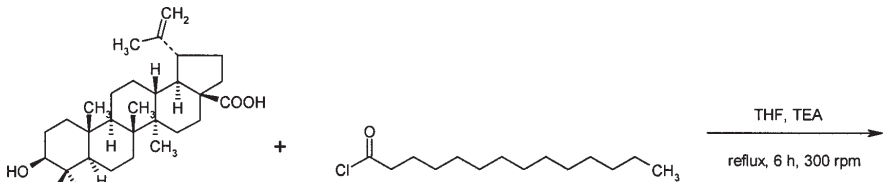


Bet

Fig. 1 Reaction scheme for the formation of betulin ester via myristoyl chloride route (Bet – Betulin and BMAE – Betulin Myristic Acid Ester)

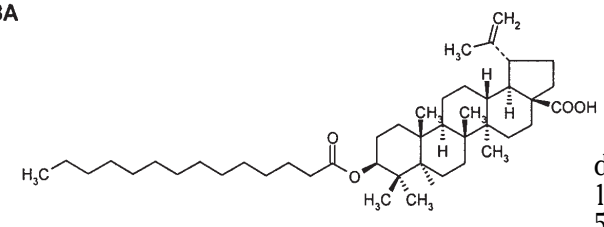


BMAE



BA

Fig. 2 Reaction scheme for the formation of betulinic acid ester via myristoyl chloride route (BA – Betulinic Acid and BAMAE – Betulinic Acid Myristic Acid Ester)



BAMAE

room temperature, washed three times with distilled water until neutral *p*H and dried over anhydrous $MgSO_4$. The solvent was then removed and the crude precipitate was purified by column chromatography to achieve a highly pure betulin ester in 63% yield. (fig. 1). In betulinic acid case, solvent used was tetrahydrofuran, and betulinic acid:myristoyl chloride molar ration 1:1. When the reaction was complete the mixture was filtered and the filtrate was distilled in order to remove the solvent. The mixture was then subject to column chromatography in order to separate the final product (fig. 2)

Three physico-chemical methods were employed to evaluate betulin purity: melting point determination, UV-Vis analysis and HPLC analysis. The techniques used to show and confirm the formation of the final products were TLC, HPLC, FT-IR and MS analyses.

The melting points were determined with a Boetius PHMK (Veb Analytic Dresden). In order to view the purity of betulin a UV-Vis spectrophotometer Spectronic-300, Pye Unicam was used. Working conditions: spectral range 200 – 400 nm, resolution 1.5 nm, data acquisition from 0.5 to 0.5 nm and scan speed 30 nm/min.

TLC analysis. The chemical esterification reactions were monitored qualitatively by thin layer chromatography (silica gel plates 60 F254, Merck) using hexane/ethyl acetate (7/3) as solvent mixtures. Both pentacyclic triterpene and their derivatives were visualized in ultraviolet light (254 nm) after the plates were placed in iodine vapours.

HPLC analysis. Qualitative analysis of the samples was performed using a YL 9100 HPLC System, equipped with vacuum degasser (YL 9101), quaternary pump (YL 9110), column compartment (YL 9131) and spectrophotometric detector (YL 9120) and working conditions were Nucleosil 100 C-18, 250 x 4.6 mm x mm column, particle diameter 5 μm, wavelength 210 nm. Separation of various components from the reaction medium was carried out using as mobile phase acetonitrile/water (86/14) with a flow rate 1 mL/min, at 25°C.

FT-IR spectroscopy. IR spectra were performed using a spectrophotometer FT-IR Jasco 430. Solid samples were prepared and analyzed in potassium bromide tablets while the liquid samples were analyzed as film between the two panes of potassium bromide.

MS spectroscopy. Mass spectra were recorded on a high capacity ion trap, HCT Ultra PTM instrument (Bruker, Daltonics, Bremen), interfaced to a PC running the Compass™ 1.2. integrated software package, which includes the Hystar™ 3.2.37 module for instrument controlling and spectrum acquisition, Esquire Control™ 6.1.512 and Data Analysis™ 3.4.179 modules for storing the ion chromatograms and processing the MS data.

MTT assay. The antiproliferative activity of the new derivatives of betulin and betulinic acid was tested on two tumor cell-lines, A431 (skin epidermoid carcinoma) and A375 (human melanoma), using as standards the native compounds, betulin and betulinic acid. The tumor cells were preserved in minimal essential medium (Sigma Aldrich) in the presence of 10% fetal calf serum (PromoCell, Heidelberg, Germany), with the addition of 1% antibiotic mixture (Pen/Strep, 10,000 IU/mL; PromoCell, Heidelberg, Germany). Briefly, the cells were seeded onto a 96-well microplate; after 24 h, 200 μL new medium (Gibco BRL, Invitrogen, Carlsbad, CA, USA) were added together with the tested substances (BMAE, BAMAE, Bet, BA). The mixture was incubated at 37°C for 72 h in humidified atmosphere with 5% CO_2. The presence of living cell was evaluated by adding 20 μL MTT (5 mg/mL); after a 4 h period the medium was removed and the blue crystals of reduced MTT, formed as a result of the mitochondrial

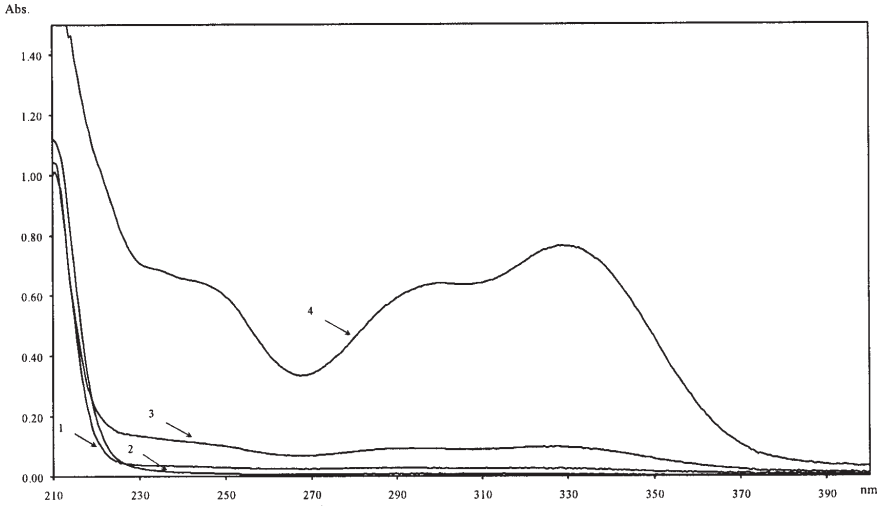


Fig. 3. UV-Vis absorbtion spectra of standard betulin (1, from Sigma Aldrich), recrystallized betulin (2,3) and crude extract of betulin (4)

Compound	Rf values	Retention time (min)
Bet	0.53	14.8
BMAE	0.75	27.5
BA	0.48	7.4
BAMAE	0.61	25.2

Table 1
Rf VALUES AND RETENTION TIMES FOR BETULIN, BETULINIC ACID AND THEIR DERIVATIVES

reductase activity, were dissolved in DMSO (100 μL) and spectrophotometrically analyzed at 545 nm. Untreated cells were used as control. All *in vitro* experiments were performed in triplicate; DMSO was used as solvent for the stock solutions (10 mM) of the active substances. In all cases, the highest used DMSO concentration (0.1%) did not affect in any way cell proliferation.

Results and discussions

The extracted betulin was obtained with a high degree of purity as confirmed by the melting point that was found in the range of 256-258°C. The HPLC chromatogram showed only one peak at 14.8 min retention time. UV-Vis analysis revealed the absorption band of pure betulin at 210 nm while the crude extract, before any treatment, clearly showed the presence of small amounts of impurities, such as polyphenols (fig. 3).

The TLC and HPLC analyses confirmed the formation of the esters and the results are presented in the table 1.

The structure of the new chemical compounds was elucidated using FT-IR and MS spectra. IR spectral data (fig. 4) showed the signals corresponding to the vibrations of carbonyl group of betulin at 1697.28 cm^{-1} this band is missing in betulin derivative, but the band for the carbonyl group of the ester appeared at 1739.71 cm^{-1}. Other signals (cm^{-1}): for betulin – 3446.63 (OH), 2868.01 (CH_2), 1452.33 (OH) and 1375.18 (isopropyl group) and for betulin derivative – 2923.95 and 2852.58 (CH_2), 1463.9 (OH) and 1373.25 (isopropyl group). In the case of betulinic acid derivative the signal corresponding to carbonyl ester group appears at 1732 cm^{-1}. The MS spectra give the values *m/z* 870.31 for betulin diesters and *m/z* 687.13 for betulinic acid ester.

MTT assay is a useful tool for the preliminary evaluation of the antiproliferative activity of active drugs. One can notice that dimethylsulfoxide (DMSO), the generally used solvent for *in vitro* experiments, has only a very small effect on cell viability when used in small concentration (≤ 0.1%). All tested substances exhibited a strong antiproliferative activity on both cell lines, as shown in figure 5; the control was considered as 100%. The results are expressed as the mean ± standard deviation. One-way ANOVA followed by Bonferroni's post-test were used in order to establish the significant differences between experimental and control

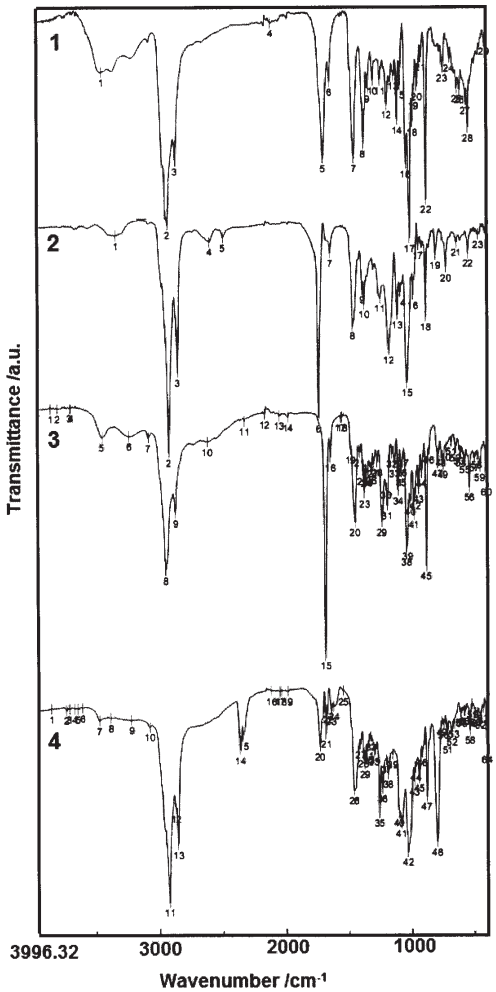


Fig. 4 FT-IR spectra on the entire 400-4000 cm^{-1} spectral range of 1 – Bet, 2 - BMAE, 3 – BA and 4 – BAMAE

lots; *, **and *** indicate $p< 0.05$, $p< 0.01$ and $p< 0.001$, respectively, compared with the control group.

Betulinic acid and betulin revealed a strong antiproliferative activity on both cancer cell lines thus they were used as references for the evaluation of the cytotoxic activity of their myristoyl ester derivatives. Cell viability dropped to 59 and 62% for Bet and 42 and 44% for BA, on A431 cell

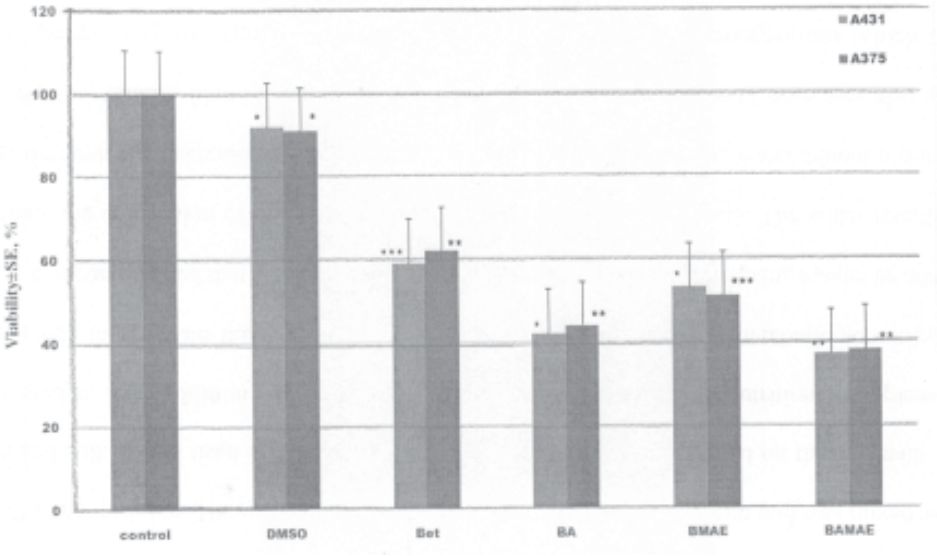


Fig. 5 MTT assay on A431 and A375 tumor cell lines

line and A375 cell line, respectively. The ester derivative of Bet, BMAE, exhibited similar values as its native compound, yet slightly smaller cell viability as follows: 53 and 51%, on A431 and A375, respectively. Betulinic acid derivative, BAMAE, presented a similar activity, cell viability reaching 37 and 38%, on A431 and A375, respectively. Previous studies [16-18,] have also revealed the significant antitumor activity of Bet and BA on several cancer cell lines; therefore their use as reference substances for the evaluation of the new compounds is completely justified.

Conclusions

We have synthesized and evaluated two esters of betulin and betulinic acid, respectively, using myristoyl chloride as reaction partner. BMAE and BAMAE showed a strong antitumor effect on both cancer cell lines, A431 and A375. The increase of the antiproliferative activity following ester derivatization of both betulin and betulinic acid has proven that esterification represents a potentially useful option of generating new compounds; moreover, in biological environment, esters are among the most easily cleaved molecules, due to the large number of esterases which can be found in the human body. Therefore, new esters could serve as pro-drugs for anticancer triterpenes, such as betulin and betulinic acid.

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