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Research paper

Preparation of novel ring-A fused azole derivatives of betulin and evaluation of their cytotoxicity

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ABSTRACT

An efficient scheme to synthesize novel ring-A fused heterocyclic derivatives of betulin was developed. The starting reaction of this synthesis was one-pot selective bacterial oxidation of betulin to betulone used as the key compound to synthesize the substituted azoles such as C(2)–C(3)-fused 1,2,3-triazoles, oxazoles and 1,2,4-triazine, as well as C(1)–C(2)-fused isoxazoles. The semi-synthetic compounds were screened for their cytotoxic activity against human cancer cell lines A549, HCT 116, HEp-2, MS and RD TE32 with use of the photometric MTT assays. Among the tested compounds, *N*-acetyltriazole of betulin (**10**) displayed impressive cytotoxic activity with IC_{50} 2.3–7.5 μM against HCT 116, HEp-2, MS and RD TE32 cell lines as well as 3-methyl-4-oxido-1,2,4-triazine-derivative of betulonic acid (**12**) that was active against HCT 116 and HEp-2 cell lines with IC_{50} 1.4 and 1.5 μM, respectively. Comparative experiments showed triazole (**10**) to have a lower cytotoxicity to normal epithelial cells, in comparison with compound (**12**). In accord with the *in vivo* acute toxicity test, the LD_{50} of triazole (**10**) exceeded 600 mg/kg. The ability of the most potent active triazole (**10**) to trigger apoptotic cell death was explored in the Annexin V-FITC test and by analyzing of caspase activity and morphological alterations in mitochondria and nuclei of HCT 116 cells.

1. Introduction

The exploratory research for new high-performance and cost-effectiveness anticancer agents with minimal side effects is one of the actively developing areas in modern medicinal chemistry. The pharmacological leaders of anticancer plant-derived natural products and their semi-synthetic derivatives characterized by target activities against malignancies and by lower toxic effects against normal cells are either traditionally used in oncology, or developed as clinical trial candidates [1,2]. The biologically active polycyclic triterpenoids (generally triterpenoids with dammarane, lanostane, lupane, oleanane and ursane skeletons) are one of the most promising groups of naturally occurring compounds being used as starting molecules in the synthetic transformations [3] for novel drug discovery of agents with anticancer [4–8], anti-inflammatory [9,10], antiviral [11–13], antimicrobial [8,14], antidementia [15], antidiabetic and anti-obesity [16,17] effects.

Betulin, a pentacyclic lupane triterpenoid (lup-20(29)-ene-3β,28-diol), is not toxic and featured by a wide spectrum of biological activities [18]. Betulin dominates (up to 30% of dry weight) amongst lipophilic compounds derivable from birch tree bark and can be extracted commercially [19]. That is why betulin is widely usable to develop new pharmacologically promising semi-synthetic derivatives that are often more active as compared with the parent triterpenoids [18,20]. Considering the fact that the structures of many pharmaceuticals currently marketed (incl. anticancer drugs) contain the heterocyclic moiety as a critical building block [21–23], one of the popular directions of triterpene chemistry is related to preparation of novel heterocyclic derivatives [24]. So, different heterocyclic fragments are introduced into the molecules of betulin and of betulinic acid as its derivative. Recently, new series of lupane heterocycles [25–27] including heterocycle-fused triterpenoids with antitumor activity are synthesized [10,28–30]. Interestingly, among the wide range of lupane derivatives prepared by V. Härmä et al. [31] from betulin and betulinic

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acid containing different substituents in the positions 3 and 28 of the lupane core, compounds bearing heterocyclic rings fused to ring A including pyrazine, pyrazole, oxazole, indole, and pyridine moieties are the most promising in suppressing prostate cancer PC-3 cell invasiveness.

Earlier [32], we had reported synthesis of lupane derivatives with a substituted fragment of C(1)–C(2)-fused isoxazole, C(2)–C(3)-fused oxazole and 1,2,3-triazole, starting with allobetulone or methyl ester of betulonic acid. Among the above-mentioned compounds, betulonic acid methyl ester, C(2)–C(3)-fused with *N*-unsubstituted 1,2,3-triazole fragment, display impressive cytotoxicity against rhabdomyosarcoma, lung carcinoma, and melanoma cell lines. In the presented work, we have synthesized new heterocyclic lupane derivatives, starting with betulin. The structure-activity relationship for the betulin derivatives is studied to investigate the effect of C(28) functionalization (mainly, 28-hydroxyl moiety) on cytotoxic activity of lupane azoles.

2. Results and discussion

2.1. Chemistry

To prepare the ring A-fused azole derivatives of lupane type, betulone (**1**) was used as the basic compound. Betulone (**1**) was obtained from betulin by the one-step regioselective biotransformation, as in detail shown previously [33,34]. The heterocyclization method based on the use of triterpene α-hydroximino ketones appeared to be efficient among the approaches to synthesis of cytotoxic A-fused azoles [29,32,35]. Thus, conditions for synthesis of lupane α-hydroximino ketone (**2**) were opted [36,37] (Scheme 1). Similar to the earlier work [32], reaction of oxime (**2**) with an excessive portion of acetyl chloride in pyridine under reflux gave three 28-acetoxy products: enamine (**3**), isoxazole (**4**) and oxazole (**5**) with the 12%, 34% and 24% yields, respectively. 28-Hydroxy derivatives (**6**–**8**) were obtained by means of basic hydrolysis of compounds (**3**–**5**) (Scheme 1).

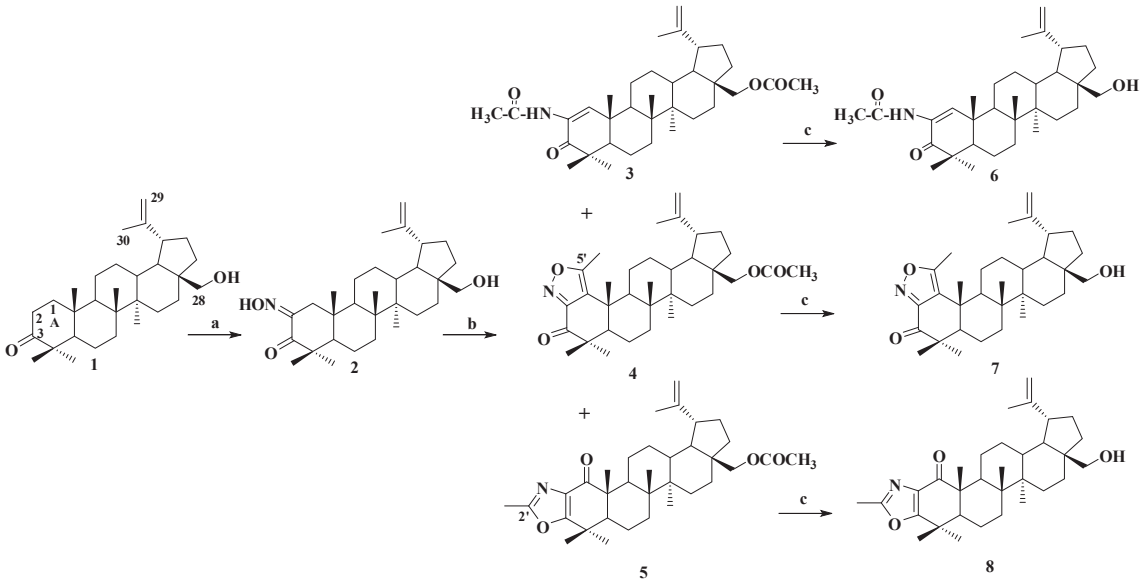
Reaction of (**2**) with hydrazine hydrate in acetic acid afforded 28-acetoxy-2′-*N*-acetyl-1,2,3-triazole (**9**). Basic hydrolysis of the compound (**9**) at room temperature and subsequent extraction of the reaction products with the water/ether mix resulted in formation of 2′-*N*-acetyl-1,2,3-triazole (**10**) with the 28-hydroxy group (Scheme 2). At the same time, pre-treatment of the reaction mixture under acidic conditions resulted in extraction of triazole (**10**) mixed with a less polar (TCL) product, spectral characteristics of which were similar to those of triazole (**10**). The new product was isolated by means of column chromatography and then crystallized from the pet. ether/ethyl acetate mix at the ratio of 5:1. The X-ray analysis identified the product as 4-oxido-1,2,4-triazine (**11**) (Fig. 1) formed as a result of the triazole cycle rearrangement under acidic conditions. Triazole-mediated triazine formation was confirmed by treatment of ethanol solutions of triazole derivatives (**9**) or (**10**) with hydrochloric acid.

According to TLC, HPLC and spectral characteristics, by oxidation of triazole (**10**) or triazine (**11**) with the Jones reagent, formation of the same acid (**12**) with 4-oxido-1,2,4-triazine fragment as a reaction product was recorded (Scheme 2). Selective transformation of 28- hydroxyl group of triazole (**10**) leading to aldehyde (**13**) was attained by using pyridinium chlorochromate (PCC). All the compounds were characterized by spectroscopic methods.

2.2. Biology

The *in vitro* cytotoxic activity was evaluated for all the synthesized compounds against the A549, HEp-2, HCT 116, MS and RD TE32 cell lines. Campthotecine (CPT) as a positive control and the examined compounds (**1**–**13**) were dissolved in DMSO, and then diluted with the culture medium. The control cells were treated with the culture medium containing 0.1% DMSO.

According to the Structure-Activity Relationships (SAR) analysis (Table 1), almost all C(2)–C(3)-fused heterocycles with 28-hydroxyl moiety appeared to be more cytotoxic than their parent 28-acylated azoles. The opposite situation was detected for C(1)-C(2)-fused isoxazole. The acylated precursor (**4**) was more active against all the tested cell lines than its hydrolyzed product (**7**). Interestingly, oxidation of 28-hydroxyl group of triazole (**10**) to 28-aldehyde group leads to a drastic decrease in cytotoxic activity. Whereas, the presence of 28-carboxyl group and triazine fragment in the triterpene structure resulted in a significant increase in cytotoxicity of acid (**12**) against HEp-2 and HCT 116 cells. Judging from the data in Table 1, sensitivity of the tested tumor cell lines to



Scheme 1. Synthesis of lupane derivatives (**2**–**8**). Reagents and conditions: (a) *t*-BuOK, *t*-BuOH, $C_5H_{11}ONO$; (b) CH_3COCl, C_5H_5N; (c) 5% KOH, C_2H_5OH, 25^{0}C.