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Epimerization of Hydroxyl Group in Lupan Series Triterpenoids

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Abstract—Two methods of obtaining 3α-betulinic acid and related compounds from their 3β-epimers were studied: the reaction of bimolecular substitution and the stereoselective reduction of 3-ketoderivatives. The substitution of acyloxy by formyloxy group in 3-*O*-tosyllupeol or of the belulin hydroxyl by benzoyloxy group resulted only in $\Delta^{2,3}$-elimination products, with none of the expected products of bimolecular substitution being found. The catalytic hydrogenation of betulonic acid over Raney nickel resulted only in reduction of the isopropenyl double bond, whereas the use of 5% Ru/C gave a 60 : 40 mixture of epimers of dihydrobetulinic acid. Practically the same mixture of betulinic acid epimers was obtained when reducing betulonic acid with L-Selectride. The cytotoxic activity of 3α-betulinic acid increased toward the Bro melanoma cells and decreased toward the MS melanoma cells.

Key words: *betulinic acid, epimerization, melanoma*

INTRODUCTION

The growth of melanoma morbidity has been 300% during the last 40 years [1]. Cytostatics (dacarbazin and cisplatin) and cytokines (α_2-interferon and interleukin-2) used for the melanoma treatment do not allow complete recovery [2]. In addition, the known anticancer cytostatics are very toxic and, during their use, resistance of the tumors under treatment to the drugs often arise, which reduces their efficiency. Therefore, the search for new compounds displaying anticancer activity remains topical.

It was found in 1995 that betulinic acid (**Xb**) selectively initiates apoptosis of human melanoma cells [3]. It inhibits the growth of tumor cells three times as effectively as dacarbazin and simultaneously displays a low *in vivo* toxicity. Some derivatives of betulinic acid also exhibit a high anti-HIV activity [4–6].

We showed that cytotoxicity of betulonic acid (**VII**) toward the melanoma MS cells is 18 times higher than that of betulinic acid (**Xb**) [7]. Similar results were obtained in Illinois University [8].

Our analysis of the structure–activity relationship in a number of triterpenes demonstrated that their biological activity as a rule increases in the order: 3β-hydroxy–3-keto–3-α-hydroxy compounds. We hypothesized that the antimelanoma activity for betulinic acid derivatives would follow the same regularity and 3α-betulinic acid would display a higher activity. The goal of this work is the test of this hypothesis. This required development of a convenient method of the 3α-betulinic acid (**Xa**) preparation. At present, the only work [9] has been published in which the authors isolated 3-epibetulinic acid by the extraction of the bark of *Picramnia pentandra* sw. (0.8 g from 0.9 kg of the bark) that grows in the United States.

RESULTS AND DISCUSSION

We studied two approaches for the preparation of 3α-betulinic acid (**Xa**): (1) the epimerization of the 3β series compounds and (2) the reduction of betulonic acid (**VII**). Two reactions, which were effective in the case of cholestane-3β-ol, were checked for the epimerization: the substitution of formyloxy for tosyloxy group [10] and the substitution of benzoyloxy for hydroxy group using the Mitsunobu reaction [11]. In our case, both methods gave only $\Delta^{2,3}$-elimination products (Scheme 1 and Table 1): 3-*O*-tosyllupeol (**III**) gave 3-deoxy-2,3-dehydrolupeol (**VI**) and betulin (**I**), 3-deoxy-2,3-dehydrobetulin (**IV**) and 3-deoxy-2,3-dehydrobetulin benzoate (**V**). ^{1}H NMR spectra of these compounds exhibited the resonances of protons at an extra double bond (δ 5.39–5.28 ppm) and the absence of C3 proton resonances. In the spectrum of (**V**), the resonances of the CH_2O methylene protons shifted downfield by 0.65 and 0.75 ppm in comparison with the corresponding resonances in starting betulin (**I**).

Unlike the compounds of cholestane series, the ring A in the lupan compounds contains two heminal methyl group at C4. The sterical and electronic effects resulted from this structural peculiarity favor elimination reac-

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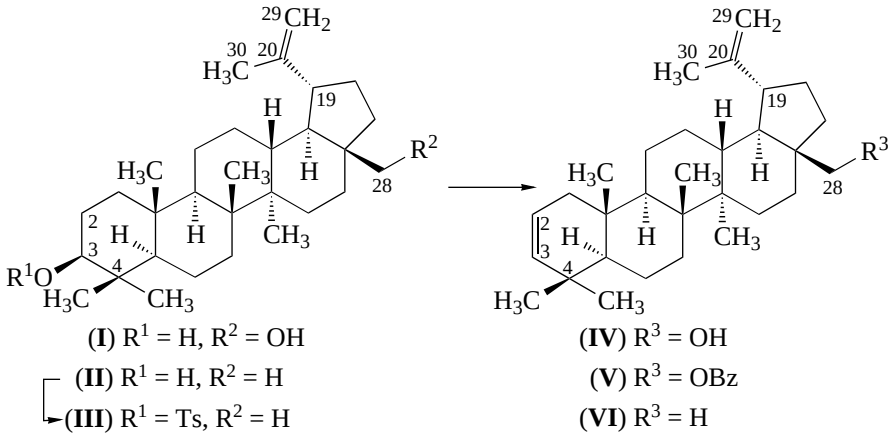


Scheme 1.

tions and hinder the reactions of bimolecular substitution. In this connection, any variants of bimolecular substitution at position 3 of lupans seem to be unpromising.

The catalytic hydrogenation of betulonic acid (**VII**) over Raney nickel up to the temperature of 50°C and the pressure of 50 atm resulted only in the reduction of the double bond of isopropenyl group and the formation of 20,29-dihydrobetulonic acid (**VIII**). Two characteristic doublets of methyl protons at C30 (0.88 ppm) and C29 (0.76 ppm) are observed in the ^{1}H NMR spectrum of this compound. At the same time, the resonance of the C3 proton was not found. The use of a more active catalyst, 5% Ru/C, led to a mixture of α- and β-epimers of 20,29-dihydrobetulinic acid (**IXa** and **IXb**) at a ratio of 60 : 40 according to ^{1}H NMR spectrum (Scheme 2 and Table 1). The presence of a characteristic signal of hydroxy group in its IR spectrum (3430 cm^{-1}) confirmed the reduction of the betulonic acid keto group. In addition, the resonances from the isopropyl group protons and 3-protons corresponding to α- and β-epimers (at 3.29 and 3.15 ppm) appeared in the ^{1}H NMR spectrum of the compound.

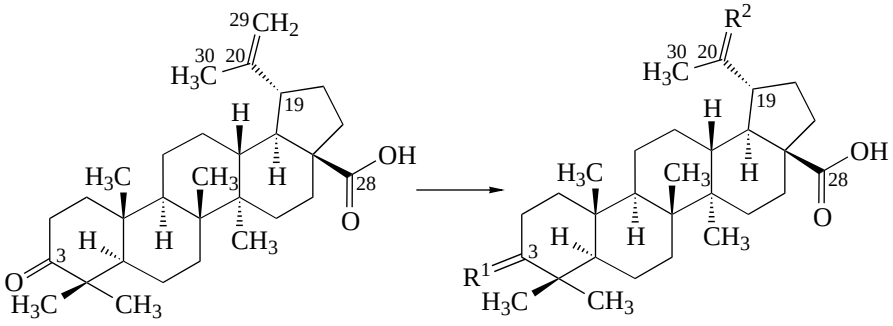
We succeeded to obtain epibetulinic acid (**Xa**) using sterically hindered lithium tri-*sec*-butylborohydride (L-Selectride). According to ^{1}H NMR spectrum, the reduction of betulonic acid with this reagent results in a 62 : 38 mixture of epimers (**Xa**) and (**Xb**) (Scheme 2 and Table 1). Note that the chromatographic mobilities of epibetulinic (**Xa**) and betulonic (**VII**) acids are close, whereas the difference in the mobilities of betulinic acid epimers (**Xa**) and (**Xb**) is rather significant.

Cytotoxic activity of epimers of betulinic acid (**Xa**) and (**Xb**) was studied in human tumor cell cultures of various histogeneses, namely, melanomas Bro and MS and ovary carcinoma CaOv. The cytotoxic effect was determined using the MTT test [12]. The cells used were conventionally sensitive to doxorubicin, a cytostatic agent (IC_{50} 0.1–0.5 μM).

Our results (Table 2) show that betulinic acid (**Xb**) displays cytotoxic properties toward two cell cultures, MS and CaOv (IC_{50} 5 μM) and is not toxic toward melanoma Bro cells (IC_{50} > 10 μM). Epibetulinic acid (**Xa**) did not exhibit an expected rise in cytotoxicity (IC_{50} > 10 μM). However, epimer (**Xa**) demonstrated a low

Table 1. Epimerization of hydroxyl group in triterpenes of lupane series

Starting compounds	Reaction conditions		Reaction products	Yield, %
(**I**)	Ph_3P, $PhCO_2H$, $EtCO_2N{=}NCO_2Et$/THF	20°C/10 h	(**IV**)	44
			(**V**)	16
(**III**)	DMF	78°C/23 h	(**VI**)	17
(**VII**)	H_2/Ni(Raney)/*i*Pr	50°C/50 atm	(**VIII**)	95
	H_2/5% Ru/C/MeOH	80°C/80 atm	(**IXa**)	45
			(**IXb**)	29
	L-Selectride/íHF	–80°C/5 h	(**Xa**)	38
			(**Xb**)	19



(**VII**)

(**VIII**) R^1 = O, R^2 = H, CH_3

(**IXa**) R^1 = H, α-OH, R^2 = H, CH_3

(**IXb**) R^1 = H, β-OH, R^2 = H, CH_3

(**Xa**) R^1 = H, α-OH, R^2 = CH_2

(**Xb**) R^1 = H, β-OH, R^2 = CH_2

Scheme 2.

cytotoxicity toward all the three tumor cell cultures, whose survival was 70–75% (Table 3).

Thus, we did not find the expected increase in cytotoxicity of epibetulinic acid (**Xa**) toward human melanoma MS cells in comparison with the cytotoxic activity of betulinic acid (**Xb**). However, the spectrum of biological activity of epimer (**Xa**) somewhat differs from that of (**Xb**), since it displays cytotoxic properties toward melanoma Bro cells, which are insensitive to betulinic acid.

EXPERIMENTAL

Diisopropyl azodicarboxylate and L-Selectride were from Aldrich (United States); other chemicals were of domestic production of at least the reagent grade.

^{1}H NMR spectra were registered on a Bruker MSL-200 spectrometer (Germany) in $CDCl_3$, CD_3OD, or acetone-d_6 at a working frequency of 200.13 MHz. Chemical shifts are given in a δ scale relative to Me_4Si. IR spectra were recorded on a Hitachi 270-30 data processor (Japan) within the ν range of 800–3600 cm^{-1} in Vaseline oil. Optical absorption was measured on a scanning Multiskan MCC/340 spectrometer (Lab-System, Finland) at 540 nm.

For TLC, Sorbton-Diol plates (Chromdet-Ecologia, Russia) were used in the following systems: (A) chloroform; (B) 100 : 2 : 0.5 chloroform–methanol–formic acid; and (C) 40 : 1 : 0.1 chloroform–methanol–30% ammonia. Triterpenes were detected on the plates using the anisaldehyde-containing reagent [13]; we modified its composition as follows: anisaldehyde (1.85 ml), alcohol (95 ml), H_2SO_4 (7.5 ml), and acetic acid (0.75 ml). An alcoholic solution of 2,4-dinitrophenylhydrazine was used for detecting carbonyl-containing compounds. Column chromatography was performed on Silica gel L 60/100 μ (Chemapol, Czech Republic).

Betulin (**I**) and lupeol (**II**) were isolated from a birch bark [14]. Betulonic acid (**VII**) was obtained by the procedure in [7].

A comparative cytotoxicity of epimers of betulinic acid was studied in the Research Institute of Experimental Diagnostics and Therapy of Tumors, Blokhin Cancer Research Center, Russian Academy of Medical Sciences, using human melanoma (lines Bro and MS) and ovary carcinoma (CaOv) cells.

An attempt of betulin (I) epimerization by Mitsunobu's reaction. Betulin (**I**) (0.20 g, 0.45 mmol), Ph_3P (0.26 g, 0.99 mmol), a solution of benzoic acid (0.12 g, 0.99 mmol) in dry THF (2 ml), and a solution of diisopropyl azodicarboxylate (0.20 g, 0.99 mmol) were added under stirring to dry THF (2 ml); the solution was stirred for 10 h at 20°C and evaporated. The residue was chromatographed on a silica gel column eluted with chloroform to give 3-deoxy-2,3-dehydrobetulin (**IV**) (0.083 g, 44%) as an oil and 3-deoxy-2,3-dehydrobetulin 28-benzoate (**V**) (0.037 g, 16%) as an oil.

(**IV**): R_f 0.50 (A); IR: 3470 (OH), 1660 (C=C); ^{1}H NMR ($CDCl_3$): 5.37 (2 H, m, CH=CH), 4.66 (1 H, d, $=CH_2$), 4.56 (1 H, d, $=CH_2$), 3.81 (1 H, m, H28), 3.26

Table 2. Cytotoxicity of betulinic acid epimers relative to the human tumor cells (IC_{50}, μM)

Cells	Epibetulinic acid (**Xa**)	Betulinic acid (**Xb**)
CaOv	>10	5
MS	>10	5
Bro	>10	>10

Table 3. Viability of the human tumor cells under the action of epibetulinic and betulinic acids (10 μM) (% of control)

Cells	Epibetulinic acid (**Xa**)	Betulinic acid (**Xb**)
CaOv	75	52
MS	70	52
Bro	70	100

(1 H, m, H28), 2.36 (1 H, m, H19), 1.66 (3 H, s, CH_3), 1.23 (3 H, s, CH_3), 0.96 (3 H, s, CH_3), 0.94 (3 H, s, CH_3), 0.80 (3 H, s, CH_3), and 0.74 (3 H, s, CH_3).

(**V**): R_f 0.69 (A); IR: 3030, 3050 (C–H aromatic), 1720 (C=O in PhCOOR), 1650 (C=C), 1590 (C=C aromatic), 1280 (C–O in RCOOR); ^{1}H NMR ($CDCl_3$): 8.03 (2 H, m, aromatic protons), 7.50 (3 H, m, aromatic protons), 5.35 (2 H, m, CH=CH), 4.70 (1 H, d, $=CH_2$), 4.59 (1 H, d, $=CH_2$), 4.50 (1 H, d, H28), 4.12 (1 H, d, H28), 2.38 (1 H, m, H19), 1.66 (3 H, s, CH_3), 1.23 (3 H, s, CH_3), 0.96 (3 H, s, CH_3), 0.94 (3 H, s, CH_3), 0.80 (3 H, s, CH_3), and 0.74 (3 H, s, CH_3).

3-*O*-Tosyllupeol (III). Lupeol (**II**) (0.080 g, 0.19 mmol) and tosyl chloride (0.075 g, 0.39 mmol) were added to pyridine (0.94 ml) at 20°C. The mixture was stirred for 24 h, and water was added until a precipitate was formed. The precipitate was filtered, dissolved in chloroform, washed with 5% HCl (3 × 5 ml), and dried over Na_2SO_4. The solution was evaporated, and the residue was dried over P_2O_5 in a vacuum to give (**III**) as an oil; yield 0.090 g (85%); R_f 0.63 (A); ^{1}H NMR ($CDCl_3$): 7.34 (4 H, m, aromatic protons), 4.66 (1 H, d, $=CH_2$), 4.56 (1 H, d, $=CH_2$), 4.18 (1 H, m, H3), 2.31 (1 H, m, H19), 2.21 (3 H, m, CH_3Ar), 1.66 (3 H, s, CH_3), 1.23 (3 H, s, CH_3), 0.96 (3 H, s, CH_3), 0.94 (3 H, s, CH_3), 0.92 (3 H, s, CH_3), 0.80 (3 H, s, CH_3), and 0.74 (3 H, s, CH_3).

An attempt of 3-*O*-tosyllupeol (III) epimerization in DMF. A solution of (**III**) (0.080 g, 0.14 mmol) in undistilled DMF (3.2 ml) was heated at 78°C for 23 h, evaporated, and chromatographed on a silica gel column eluted with a 1 : 1 hexane–chloroform mixture to give 3-deoxy-2,3-dehydrolupeol (**VI**) as an oil; yield 0.010 g (17%); R_f 0.67 (A); ^{1}H NMR ($CDCl_3$): 5.35 (2 H, m, CH=CH), 4.67 (1 H, m, $=CH_2$), 4.55 (1 H, m, $=CH_2$), 2.38 (1 H, m, H19), 1.66 (3 H, s, CH_3), 1.23 (3 H, s, CH_3), 0.96 (3 H, s, CH_3), 0.94 (3 H, s, CH_3), 0.91 (3 H, s, CH_3),0.80 (3 H, s, CH_3), and 0.74 (3 H, s, CH_3).

Catalytic hydrogenation of betulonic acid (VII). A. Hydrogenation over Raney nickel under pressure. Raney nickel (2 g) was added to a solution of betulonic acid (**VII**) sodium salt (0.500 g, 1.05 mmol) in isopropanol (40 ml), and the reaction mixture was stirred at 20°C under H_2 pressure of 50 atm for 17 h. The temperature was then increased to 50°C, the H_2 pressure remaining constant. After 17 h, the catalyst was filtered off, and the filtrate was evaporated. The residue was diluted with ether (70 ml) and 10% HCl (30 ml) in a separating funnel, shaken up to the complete dissolution of crystals, the ether layer was separated, washed with water, dried with sodium sulfate, and evaporated to give dihydrobetulonic acid (**VIII**) as colorless crystals; yield 0.450 g (95%); mp 250–253°C (lit. mp 251–254°C [15]); R_f 0.42 (C); IR: 1703 (C=O in RCOR), 1687 (C=O in COOH); ^{1}H NMR ($CDCl_3$): 1.07 (3 H, s, CH_3), 1.03 (3 H, s, CH_3), 0.97 (3 H, s, CH_3), 0.93 (3 H, s, CH_3), 0.87 (3 H, d, CH_3), 0.84 (3 H, s, CH_3), 0.75 (3 H, d, CH_3).

B. Hydrogenation in the presence of 5% Ru/C. A solution of betulonic acid (**VII**) (0.20 g, 0.44 mmol) in methanol (15 ml) was hydrogenated over 5% Ru/C (0.10 g) at 40 atm and 20°C. After 22 h, the hydrogen pressure and the temperature were increased to 70 atm and 50°C, respectively. After 7 h, the stirring and heating were stopped, and the mixture was kept in the autoclave for 15 h at 20°C. Then the hydrogen pressure was elevated to 80 atm and the temperature to 80°C. After 4.5 h, the stirring and heating were stopped, and the mixture was kept in the autoclave for 15 h. The reaction mixture was washed off from the catalyst, evaporated, and chromatographed on a silica gel column eluted with chloroform to give 3α-20,29-dihydrobetulinic acid (**IXa**) as colorless crystals; yield 0.090 g (45%); mp 297–300°C (lit. mp 298–301°C [9]); R_f 0.39 (C); IR: 3500 (OH in COOH), 1690 (C=O in COOH); ^{1}H NMR ($CDCl_3$): 3.31 (1 H, m, H3β), 1.04 (3 H, s, CH_3), 0.97 (3 H, s, CH_3), 0.93 (3 H, s, CH_3), 0.85 (3 H, d, CH_3), 0.84 (3 H, s, CH_3), 0.77 (3 H, s, CH_3), and 0.74 (3 H, d, CH_3). The subsequent elution of the column gave 3β-20,29-dihydrobetulinic acid (**IXb**) as colorless crystals; yield 0.060 g (29%); mp > 310–313°C (lit. mp 312–314°C [16]); R_f 0.26 (C); IR: 3500 (OH in COOH), 1690 (CO in COOH); ^{1}H NMR ($CDCl_3$): 3.15 (1 H, m, H3α), 1.06 (3 H, s, CH_3), 0.98 (3 H, s, CH_3), 0.95 (3 H, s, CH_3), 0.85 (3 H, d, CH_3), 0.84 (3 H, s, CH_3), 0.78 (3 H, s, CH_3), and 0.76 (3 H, d, CH_3).

3α-Betulinic acid (Xa). A 1 M solution of L-Selectride in THF (5.6 ml) was added to a solution of betulonic acid (**VII**) (0.33 g, 0.73 mmol) in dry THF (10 ml) at –80°C under argon. The mixture was stirred for 5 h. A 2 N NaOH (18 ml) and 38% H_2O_2 (4 ml) were added to the reaction mixture, stirred for 1 h, THF was evaporated, and 10% HCl (15 ml) and ether (20 ml) were added to the resulting precipitate. The mixture was stirred until the precipitate was completely dissolved, and the ether layer was separated and evaporated to give 254 mg of the residue. It was chromatographed on a silica gel column eluted with chloroform to give 3α-betulinic acid (**Xa**) as colorless crystals; yield 127 mg (38%); mp 278–281°C (lit. mp 279–283°C [9]); R_f 0.38 (C); ^{1}H NMR (1 : 1 $CDCl_3$–acetone-d_6): 4.64 (1 H, m,

=CH_2), 4.51 (1 H, m, =CH_2), 3.29 (1 H, m, H3β), 2.99 (1 H, m, H19), 1.62 (3 H, s, CH_3), 0.94 (3 H, s, CH_3), 0.88 (3 H, s, CH_3), 0.86 (3 H, s, CH_3), 0.78 (3 H, s, CH_3), and 0.75 (3 H, s, CH_3). The subsequent elution gave colorless crystals of 3β-betulunic acid (**Xb**); yield 0.063 g (19%); mp 291–293°C (lit. mp 291–292°C [17]); R_f 0.31 (C); ^{1}H NMR (3 : 1 $CDCl_3$–CD_3OD): 4.71 (1 H, m, =CH_2), 4.59 (1 H, m, =CH_2), 3.15 (1 H, m, H3α), 3.01 (1 H, m, H19), 1.67 (3 H, s, CH_3), 0.96 (3 H, s, CH_3), 0.94 (3 H, s, CH_3), 0.93 (3 H, s, CH_3), 0.81 (3 H, s, CH_3), and 0.74 (3 H, s, CH_3).

Cytotoxicity of betulinic acid epimers. Cell cultures of human melanoma lines MS (replication time *Td* 36 h) and Bro (*Td* 48 h) and human ovary carcinoma line CaOv (*Td* 40 h) were grown as a monolayer on a DMEM medium (Sigma) complemented with 10% FBS (embryonic cow serum from Gibco DRL), 2 mM *L*-glutamine, and gentamycin (40 μg/ml) in the 5% CO_2–95% air atmosphere at 37°C.

The diluted solutions of compounds under study were added to the cell suspensions of the tumor cell cultures in the phase of exponential growth placed into 96-well plates, and was incubated for 72 h. The cell viability was determined using the MTT test [12]. The resulting formazane crystals were dissolved in DMSO, and the optical absorption was measured on a scanning spectrometer at 540 nm. The portion of viable cells was determined according to the ratio of partial absorption in the samples under study and in the control (tumor cells in the growing medium without the compounds under study).

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