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SYNTHESIS AND PHARMACOLOGICAL ACTIVITY OF 20-KETO-29-NORLUPANE DERIVATIVES

O. B. Flekhter,[1] G. V. Giniyatullina,[1] F. Z. Galin,[1]
N. Zh. Baschenko,[1] N. S. Makara,[1] F. S. Zarudii,[1]
E. I. Boreko,[2] O. V. Savinova,[2] N. I. Pavlova,[2]
Z. A. Starikova,[3] and G. A. Tolstikov[4]

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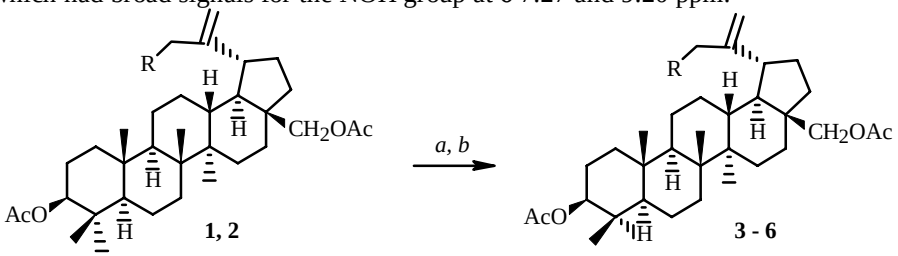
New 20-oxo- and hydroxyimino-derivatives of betulin that exhibit immunotropic and antiviral activities were synthesized.

Key words: triterpenoids, ozonolysis, oximes, antiviral activity, immunotropic activity.

In continuation of our studies on the synthesis of new triterpene derivatives with valuable pharmacological activity [1-5], we synthesized betulin 29-norlup-20-ketones and their hydroxyimino derivatives and studied the antiviral and immunotropic activties of several compounds.

Ozonation of 3β,28-di-O-acetylbetulin (**1**) and its 30-bromo derivative (**2**) produced the corresponding 20-ketones **3** and **4** in yields of 73 and 65%, respectively. The structures of the compounds were established using NMR spectroscopy. Thus, the ^{13}C NMR spectra of **3** and **4** lacked signals for the C-20(29) double bonds typical of starting acetates **1** and **2** but contained signals for a C-20 carbonyl at δ 211 ppm. The PMR of **3** and **4** exhibited a weak-field shift for the signals of H-30 to δ 2.11-2.16 ppm. 3β,28-Di-O-acetyl-29-norlup-20-one (**3**) crystallized from ethanol as colorless needles. This enabled its structure to be confirmed by an x-ray structure analysis (XSA) (Fig. 1). Boiling **3** and **4** with hydroxylamine in pyridine produced in quantitative yield the corresponding oximes **5** and **6**, the ^{13}C NMR spectra of which gave signals for C-20 at δ 162.3 ppm and the PMR spectra of which had broad signals for the NOH group at δ 7.27 and 9.20 ppm.



1, 3, 5: R = H; **2, 4, 6:** R = Br; **3, 4:** X = O; **5, 6:** X = NOH

a. O_3, CH_2Cl_2, -60° C, Zn/AcOH; *b.* $NH_2OH \cdot HCl$, pyridine, 115°C, 2h

A study of the antiviral activities of **2-5** toward flu and Type I herpes simplex viruses showed that only oxime **5** possessed weak ability to suppress reproduction of flu virus. Ketone **4** had a comparable degree of activity toward herpes virus whereas the 30-bromo derivative of 3β,28-di-O-acetylbetulin (**2**) was even less active (Table 1). Despite the low activities of the studied compounds, the results confirm that derivatives of lupane triterpenoids can exhibit antiviral properties toward flu virus, which we first established [6].

1) Institute of Organic Chemistry, Ufa Scientific Center, Russian Academy of Sciences, 450054, Ufa, pr. Oktyabrya, 71, e-mail: obf@anrb.ru; 2) Scientific Research Institute of Epidemiology and Microbiology, Ministry of Health of Belarus, Minsk; 3) N. A. Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences, Moscow; 4) N. N. Vorozhtsov Novosibirsk Institute of Organic Chemistry, Siberian Division, Russian Academy of Sciences.
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TABLE 1. Antiviral Activity of Betulin Derivatives

Compound	Flu virus		Herpes virus	
	EC_{50} (I_{95})/EC_{90}(I_{95}) μM	MTC/EC_{50} MTC/EC_{90}	EC_{50} (I_{95})/EC_{90} (I_{95}) μM	MTC/EC_{50} MTC/EC_{90}
2	>627.26	<1	180.02	3.48
		<1		1.21
3	277.98/5072.32	0.64	55.85/248.66	6.38
		0.03		1.43
4	87.91/202.73	0.89	2.09/16.54	18.6
		0.25		2.36
5	5.94/31.76	7.31	86.84	<1
		1.37		<1

MTC is the maximum tolerated concentration; EC_{50}, the average effective concentration, EC_{90}, the concentration for 90% suppression of virus multiplication; I_{95}, the confidence range at 95% probability.

TABLE 2. Effect of Betulin Derivatives on Functional Activity of the Immune System of White Mice

Compound	Dose, mg/kg	Body mass		Spleen mass		Thymus mass		Antibody titer, lg_2	
		initial, g	on day of sacrifice, g	absolute, mg	relative, %	absolute, mg	relative, %	hemagglutinin	hemolysine
1	50	20.0±0.6	20.5±0.5	201.0±13.8	0.98±0.10	30.9±2.3	0.15±0.08	6.5±0.35	9.70±0.1
5	50	21.0±1.2	20.8±0.7	191.0±27.0	0.91±0.14	33.0±3.1	0.15±0.02	6.0±0.3	10.0±0.1
Betulinic acid	50	20.7±0.9	21.5±0.8	298.0±27.0	1.38±0.16	30.5±3.2	0.1±0.01	7.3±0.5	11.0±0.01
Hydroxymethyluracil	50	21.7±0.9	21.5±0.8	291.6±19.7	1.35±0.18	34.1±3.5	0.15±0.02	7.0±0.3	10.2±0.1
Control		21.0±1.1	22.5±0.8	198.0±11.5	0.88±0.04	38.5±2.0	0.17±0.09	5.4±0.2	9.3±0.2

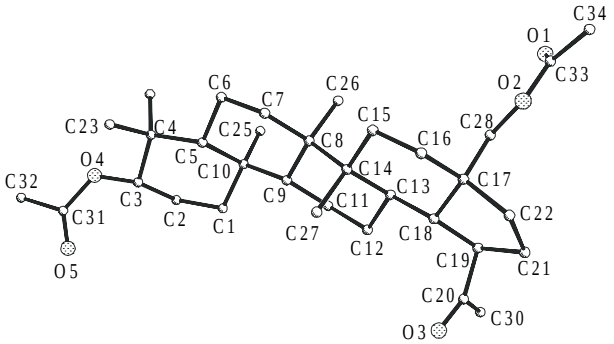

Fig. 1. Structure of 3β,28-di-O-acetyl-29-norlup-20-one (**3**).

EXPERIMENTAL

PMR and ^{13}C NMR spectra were recorded on a Bruker AM-300 spectrometer (300 and 75.5 MHz, respectively) in $CDCl_3$ with TMS internal standard. Melting points were determined on a Boetius microstage; optical density, on a Perkin—Elmer 241 MC polarimeter in a 1-dm tube. TLC was performed on Silufol (Chemapol, Czech Rep.) plates using $CHCl_3$:CH_3OH (25:1). Compounds were developed with phosphotungstic acid solution (5%) in ethanol with subsequent heating at 100-120°C for 2-3 min. **1** and **2** were prepared as before [7, 8].

3β,28-Di-O-acetyl-29-norlup-20-one (3). Ozonated oxygen was passed through a solution of **1** (2 mmol, 0.9 g) in CH_2Cl_2 (50 mL) at -60°C until ozone broke through. The temperature was adjusted to 0°C. Glacial AcOH (10 mL) and zinc dust (1 g) were added. The solution was stirred at 0°C for 1 h. The zinc dust was filtered off. The organic layer was washed with saturated Na_2CO_3 solution (3 × 20 mL) and water (3 × 25 mL), dried over Na_2SO_4, and evaporated in vacuo (water aspirator). The solid was chromatographed over a column of Al_2O_3 with elution by $CHCl_3$. Yield 0.59 g (73%), white needles, R_f 0.55, mp 188-190°C, lit. [9] mp 190-191°C, $C_{33}H_{52}O_5$ (MW 528.769).

PMR spectrum (δ, ppm, J/Hz): 0.81, 0.82, 0.94, 0.96, 1.00 (15H, 5s, $5CH_3$), 1.00-2.00 (24H, m, CH_2, CH), 1.99 and 2.03 (6H, 2s, 2OAc), 2.11 (3H, s, H-30), 2.57-2.64 (1H, m, H-19), 3.77 and 4.23 (2H, both d, J = 11, H-28), 4.43 (1H, dd, J = 5, 9, H-3).

^{13}C NMR spectrum (δ, ppm): 14.4, 15.8. 16.0, 16.3, 18.0, 20.6, 20.8, 21.1 ($\underline{C}H_3CO$), 23.6, 26.8. 27.0, 27.4, 27.8, 29.2, 29.5, 33.0, 34.3, 36.3, 36.9, 37.6, 38.2, 40.7, 42.5, 46.2, 49.2, 51.5, 55.2, 62.4 (C28), 80.6 (C3), 170.7 and 171.2 ($CH_3\underline{C}O$), 211.2 (C20).

XSA of 3. Colorless needlelike crystals $C_{33}H_{52}O_5$ (MW = 528.75) were orthorhombic, at 293 K a = 12.626(3), b = 15.258(3), c = 15.987(3) Å, V = 3080(1) Å^3, space group $P2_12_12_1$, Z = 4, d_{calc} = 1.140 g/cm^3. A data set of reflections was obtained on a Enraf—Nonius CAD4 diffractometer at 293 K (λ Mo Kα-radiation, $2\theta_{max}$ = 49°) from a single crystal of dimensions 0.40 × 0.30 × 0.25 mm. Equivalent reflections were averaged to produce 5366 independent reflections [R(int) = 0.0222] that were used to solve and refine the structure. The structure was solved by direct methods and refined anisotropically (H atoms were placed at positions calculated geometrically and refined isotropically using a rocker model) over F_{hkl}^2. The final agreement factors were R1 = 0.0514 [calculated over F_{hkl} for 2749 reflections with I > 2σ(I)], wR2 = 0.1389 (calculated over F_{hkl}^2 for all 5366 reflections), GOOF = 1.014, 343 refined parameters. All calculations were performed using the SHELXTL PLUS 5 programs. Atomic coordinates and temperature factors were deposited in the Cambridge Crystallographic Database (CCDC 277178).

3β,28-Di-O-acetyl-29-norlup-30-bromo-20-one (4) was prepared analogously to **3** from **2** (1 g). Yield 0.65 g (65%), yellow compound, R_f 0.39, mp 93-94°C, $[\alpha]_D^{20}$ -11.3° (*c* 0.013, $CHCl_3$), $C_{33}H_{51}BrO_5$ (MW 607.665).

PMR spectrum (δ, ppm, J/Hz): 0.95, 0.97, 0.98, 0.99, 1.04 (15H, 5s, $5CH_3$), 1.00-1.90 (24H, m, CH_2, CH), 2.02 and 2.05 (6H, 2s, 2OAc), 2.16 (2H, s, H-30), 2.60 (1H, m, H-19), 3.75 and 4.18 (2H, both d, J = 11, H-28), 4.43 (1H, dd, J = 6, 9.9, H-3).

^{13}C NMR spectrum (δ, ppm): 14.6, 15.9, 16.0, 16.4, 18.1, 20.7, 20.9, 21.2 ($\underline{C}H_3CO$), 23.6, 26.9, 27.1, 27.4, 27.9, 29.3, 29.5, 33.9 (C30), 34.4, 36.4, 37.0, 37.7, 38.3, 40.7, 42.5, 46.3, 49.3, 50.1, 51.6, 55.3, 62.4 (C28), 80.7 (C3), 170.7, 171.3 ($CH_3\underline{C}O$), 211.4 (C20).

3β,28-Di-O-acetyl-29-norlup-20-oxime (5). A solution of **3** (1 mmol, 0.46 g) in anhydrous pyridine (30 mL) was treated with $NH_2OH{\cdot}HCl$ (7 mmol, 0.5 g), refluxed for 2 h, cooled, and poured into HCl solution (150 mL, 5%). The resulting solid was filtered off, washed with water, and dried. Yield 0.39 g (85%), light yellow compound, R_f 0.30, mp 133-135°C, $[\alpha]_D^{20}$ +21.4° (*c* 0.02, $CHCl_3$), $C_{33}H_{53}NO_5$ (MW 543.783).

PMR spectrum (δ, ppm, J/Hz): 0.81, 0.82, 0.94, 0.96, 1.00 (15H, 5s, $5CH_3$), 1.00-1.90 (24H, m, CH_2, CH), 1.81 (3H, s, H-30), 1.97, 2.01 (6H, 2s, 2OAc), 2.58-2.67 (1H, m, H-19), 3.77, 4.23 (2H, both d, J = 11, H-28), 4.43 (1H, dd, J = 5, 9, H-3), 9.20 (1H, br.s, NO$\underline{H}$).

^{13}C NMR spectrum (δ, ppm): 10.8, 14.4, 15.8, 16.0, 16.3, 18.0, 20.5, 20.8, 21.2 ($\underline{C}H_3CO$), 23.5, 25.2, 26.8, 27.2, 27.8, 29.6, 33.9, 34.5, 36.7, 36.9, 37.6, 38.2, 40.7, 42.5, 44.9, 46.0, 49.1, 49.9, 55.2 (C5), 62.4 (C28), 80.7 (C3), 162.3 (C20), 170.9, 171.3 ($CH_3\underline{C}O$).

3β,28-Di-O-acetyl-29-norlup-30-bromo-20-oxime (6) was prepared analogously to **5** from **4** (0.46 g). Yield 0.41 g (89%), R_f 0.51, mp 112-115°C, $[\alpha]_D^{20}$ +17.6° (*c* 0.07, $CHCl_3$), $C_{33}H_{52}BrNO_5$ (MW 622.68).

PMR spectrum (δ, ppm, J/Hz): 0.90, 0.96, 0.98, 1.03 (15H, 4s, $5CH_3$), 1.10-2.00 (24H, m, CH_2, CH), 1.82 (2H, s, H-30), 2.05, 2.07 (6H, 2s, 2OAc), 2.58 (1H, m, H-19), 3.81, 4.27 (2H, both d, J = 11, H-28), 4.45 (1H, dd, J = 6, 9.9, H-3), 7.27 (1H, br.s, NO$\underline{H}$).

^{13}C NMR (δ, ppm): 10.8, 14.6, 16.0, 16.1, 16.5, 18.2, 20.7, 21.0, 21.3, 23.7, 25.4, 26.9, 27.3, 27.9, 29.7, 34.1, 34.6, 36.9, 37.0, 37.8, 38.4, 40.9, 42.6, 45.3, 46.2, 49.1, 50.1, 55.4, 62.6 (C28), 80.9 (C3), 162.5 (C20), 171.0, 171.4 ($CH_3\underline{C}O$).

Antiviral properties were determined in experiments on cell cultures with A/FPV/Rostock/34 (H7N1) flu virus and herpes simplex virus type I (HSV-I). The studies were carried out by reduction of plaques in culture of primary chicken embryo fibroblasts with FPV and estimation of the cytopathic effect on culture of human rhabdomyosarcoma cells with HSV-I by the

literature method [3]. The studied compounds were dissolved beforehand in ethanol (10%) and then in the support medium until the required concentrations were attained. The criterion for antiviral activity was a reduction of the virus titer in the presence of the studied compounds compared with the control. The concentration for 50% (average effective concentration, EC_{50}) and 90% (EC_{90}) suppression of virus multiplication and the ratios of the maximal tolerated concentration (MTC) to EC_{50} and EC_{90} was also calculated. The MTC of the compounds for uninfected cell cultures were determined after 72 h of incubation. Table 1 lists the results.

The immunotropic activity of **1**, **5**, and betulinic acid were estimated from the titers of circulating antibodies (hemolysine and hemagglutinin) [10]. Sheep erythrocytes (SE) were used as a thymus-dependent antigen. Immunization was carried out by i.p. injection to white mice of the optimal (108 cells) dose of SE in 0.2 mL per 20 g of body mass. The reaction was estimated using antibody titers on the 7th day after immunization by recording the masses of the body and lymphatic organs (thymus, spleen). The studied compounds were administered *per os* during 7 d beginning with the first day of immunization. A single dose of the tested compounds did not exceed 50 mg/kg. The controls were animals that received oral hydroxymethyluracil (50 mg/kg) and water during 7 d after immunization with SE. Table 2 lists the results.

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