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Synthesis, structure and *in vitro* biological evaluation of new lupane and dammarane triterpenoids fused with pyrazine heterocycle

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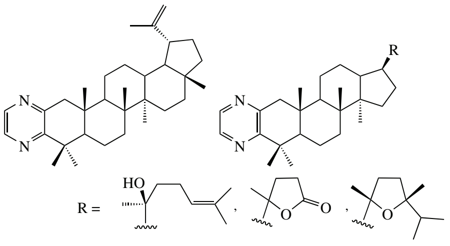
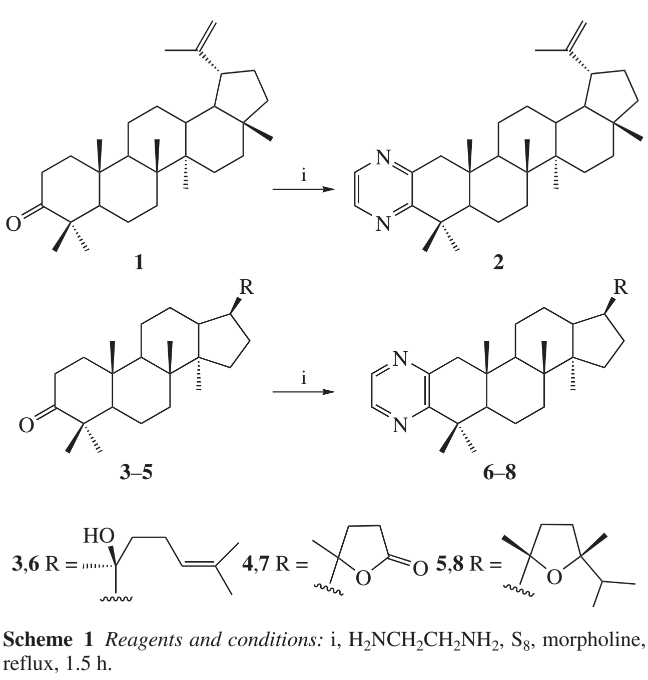
Derivatives of natural lupenone, dipterocarpol, alnincanone and semisynthetic dipterocarpol lactone fused with pyrazine heterocycle along C^2–C^3 positions were obtained by the Willgerodt–Kindler reaction, X-ray analysis having been used for the structure identification. Anti-influenza and anti-proliferative activities of the compounds obtained were estimated.



Lupane and dammarane triterpenoids are widely distributed in plants and some of them exhibit pharmacological activities.[1] Chemically modified triterpenoids equipped with polar N-heterocyclic fragment (*e.g.*, azolyl or azinyl) usually have a better pharmacological profile, possess improved solubility in physiological media and optimal lipophilicity. Dipterocarpol, betulinic acid and some other triterpenoids fused to N-heterocycles along C^2–C^3 atoms were of interest.[2,3] Among them, pyrazines fused to a triterpenoid skeleton turned out to be of particular value. Thus, cephalostatins isolated from marine worm *Cephalodiscus gilchristi* and possessing nanomolar antitumor activity comprise pyrazine heterocycle fused with two steroid molecules.[2] Pyrazine-fused betulinic acid and its derivatives significantly inhibit the growth of various human tumor cells as well as of drug-resistant K562-tax.[4]



Scheme 1 *Reagents and conditions:* i, $H_2NCH_2CH_2NH_2$, S_8, morpholine, reflux, 1.5 h.

Synthesis of pyrazine-fused steroids and terpenoids was previously accomplished by the oxidative annulation of cyclohexanone moiety with ethylenediamine upon the action of elemental sulfur according to the Willgerodt–Kindler protocol. In this way, pyrazino derivatives of natural taraxerone,[5] dipterocarpol lactone,[6] and some other triterpenoids[7] were synthesized and tested for biological activities.

In the present work new analogous derivatives of natural lupenone **1**, dipterocarpol **3**, its lactone **4**, and alnincanone **5** fused with pyrazine heterocycle along C^2–C^3 positions were synthesized. The compounds obtained were fully characterized by spectral methods and X-ray analysis, and their anti-influenza [virus A(H1N1)] and anti-proliferative (cells HeLa, HepG2 and WI-38) activities were studied. The starting compounds **1** and **5** were isolated from the *Alnus incana* bark,[8] while dipterocarpol **3** was isolated from *Dipterocarpus alatus* latex.[9] Lactone **4** was obtained by oxidation of dipterocarpol **3** according to known procedure.[9]

Fused pyrazine derivatives **2**,**6**–**8** were synthesized by boiling 3-keto terpenoids **1**,**3**–**5** in morpholine with sulfur in the presence of ethylenediamine (Scheme 1).[†] Structures of products **2**,**6**–**8** were confirmed by ^{1}D, ^{2}D, ^{1}H and ^{13}C NMR spectra (see Online Supplementary Materials). Their ^{1}H NMR spectra contain signals for

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[†] *General synthesis procedure.* To a solution of triterpenoid **1**,**3**,**4** or **5** (2 mmol) in morpholine (20 ml), elemental sulfur (18 mmol) and ethylenediamine (10 mmol) were added at room temperature, and the mixture was heated under reflux for 1.5 h. After cooling, the mixture was poured into water and extracted with AcOEt (3×50 ml). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. Column chromatography of the residue on silica gel (hexane/AcOEt) and crystallization from AcOEt afforded products **2**,**6**–**8** as colourless crystals in 70–89% yields. For the details and systematic names, see Online Supplementary Materials.

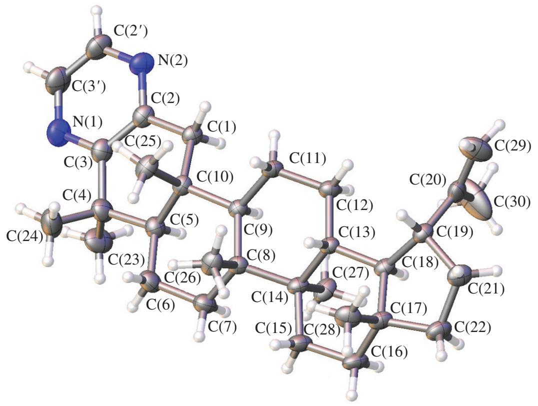

Figure 1 Molecular structure of compound **2** with the key atoms labeled and 50% probability of thermal ellipsoids.

the pyrazine protons at δ 8.28–8.29 ppm (d, 1H, J 2.1–2.5 Hz) and 8.42 ppm (d, 1H, J 2.1–2.5 Hz). Protons of C^1H_2 methylene group resonate separately at 3.05–3.07 ppm (d, 1H, 1H-1β, J 16.7 Hz) and 2.48–2.54 ppm (d, 1H, 1H-1α, J 16.5 Hz). Pyrazine carbon atoms in the ^{13}C NMR spectra are manifested at δ 150.65–150.97 ppm (C^2), 159.59–159.71 ppm (C^3), 142.26–142.42 ppm ($C^{3'}$) and 141.49–141.58 ppm ($C^{2'}$).

Molecular and crystal structures of derivatives **2**,**6**,**7** were elucidated by X-ray diffraction (Figures 1–3, also see Tables S2–S8 in Online Supplementary Materials).[‡] In all cases, pyrazine cycle is practically planar and oriented against the triterpenoid skeleton in the same manner. The endocyclic C–N bonds of the heterocycle were marginally different in length (in limits from 0.01–0.02 Å).

Previously, we have shown that some cyanoethoxy and tetrazole derivatives of dammarane triterpenoids exhibit rather high antiviral activity.[9] Here, antiviral activity of pyrazino triterpenoids **2**,**6**–**8** has been tested with respect to influenza virus A/Puerto Rico/8/34 (H1N1). The antiviral activity, cytotoxicity and selectivity were

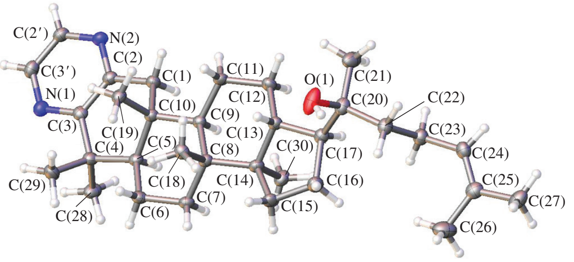

Figure 2 Molecular structure of compound **6** with the key atoms labeled and 50% probability of thermal ellipsoids.

studied *in vitro* in experiments on the MDCK cells according to the reported procedure[15] (Table 1). Compounds **2**,**6**,**7** did not show any noticeable selectivity of the action. More or less appreciable activity has been observed only in the case of alnincanone derivative **8** (SI 10). It should be noted that both compound **8** and natural betulin also reveal a very low cytotoxicity on MDCK cells.

To assess the antitumor potential of compounds **2**,**6**–**8**, their anti-proliferative activity was evaluated *in vitro* against human cervical cancer cells HeLa, liver cancer cells Hep G2, and normal lung fibroblasts cells WI-38. The activity was studied by means of MTT test. All these compounds exhibit only low cytotoxicity (Figure S34). A marked anti-proliferative activity was observed only in the case of pyrazine derivative of lupenone **2** (IC_{50} 42 μM for HeLa, 19 μM for Hep G2 and greater than 60 μM for WI-38).

Incubation of Hep G2 cells with compound **2** at a concentration of IC_{50} induced 32% apoptosis (Table 2). Compounds **6**,**7** and **8** at a concentration of IC_{50} possessed the proapoptic properties and inducing apoptosis at 29.4, 31.8 and 27%, respectively, compared to control.

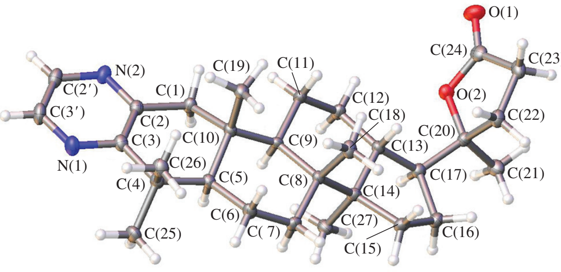

Figure 3 Molecular structure of compound **7** with the key atoms labeled and 50% probability of thermal ellipsoids.

[‡] *Crystallographic data for* **2**. Crystals of $C_{32}H_{48}N_2$ (M = 460.72) are monoclinic, space group $P2_1$, at 100 K: a = 14.42840(18), b = 12.00295(14) and c = 23.1544(2) Å, β = 90.5465(11)°, V = 4009.78(8) A^3, Z = 6, d_{calc} = = 1.145 g cm^{-3}, μ = 0.487 mm^{-1}, F(000) = 1524.0, crystal size 0.34×0.18×0.12 mm, CuKα radiation (λ = 1.54184), 2θ range for data collection 6.126 to 139.99°, index ranges $-17 \leqslant h \leqslant 17$, $-14 \leqslant k \leqslant 14$, $-28 \leqslant l \leqslant 19$, reflections collected 43055, independent reflections 14964 (R_{int} = 0.0434, R_σ = 0.0426). Data/restraints/parameters 14964/2/940, GOOF on F^2 1.043. The final R indexes [$I \geqslant 2\sigma(I)$]: R_1 = 0.0408 and wR_2 = = 0.1037, final R indexes (all data): R_1 = 0.0440 and wR_2 = 0.1064, largest diff. peak/hole: 0.21/–0.21 $eÅ^{-3}$, flack parameter 0.2(2). Single crystals of $C_{32}H_{48}N_2$ were obtained by crystallization from ethyl acetate. A suitable crystal was studied on a SuperNova, Single source at offset/far, HyPix3000 diffractometer. The crystal was kept at 100(2) K during data collection. Using Olex2,[10] the structure was solved with the Superflip[11] structure solution program using Charge Flipping and refined with the ShelXL[12] refinement package using Least Squares minimisation.

Crystallographic data for **6**. Crystals of $C_{32}H_{50}N_2O$ (M = 478.74) are orthorhombic, space group $P2_12_12_1$: a = 6.97320(10), b = 11.8087(2) and c = 33.8433(8) Å, V = 2786.81(9) A^3, Z = 4, d_{calc} = 1.141 g cm^{-3}, μ = = 0.511 mm^{-1}, F(000)=1056.0, crystal size 0.32×0.20×0.12 mm, CuKα radiation (λ = 1.54184), 2θ range for data collection 7.93 to 144.58°, index ranges $-8 \leqslant h \leqslant 8$, $-14 \leqslant k \leqslant 14$, $-41 \leqslant l \leqslant 41$, reflections collected 52955, independent reflections 5485 (R_{int} = 0.0700, R_σ = = 0.0287). Data/restraints/parameters 5485/0/325, GOOF on F^2 1.046. The final R indexes [$I \geqslant 2\sigma(I)$]: R_1 = 0.0411 and wR_2 = 0.1027, final R indexes (all data): R_1 = 0.0440, wR_2 = 0.1043, largest diff. peak/hole: 0.22/–0.21 $eÅ^{-3}$, flack parameter 0.15(14). Single crystals of $C_{32}H_{50}N_2O$ were growth from ethyl acetate. A suitable crystal was studied on a SuperNova, Single source at offset/far, HyPix3000 diffractometer. The crystal was kept at 100(2) K during data collection. Using Olex2,[10] the structure was solved with the ShelXT[13] structure solution program using Intrinsic Phasing and refined with the ShelXL[12] refinement package using Least Squares minimisation.

Crystallographic data for **7**. Crystals of $C_{29}H_{42}N_2O_2$ (M = 450.64) are monoclinic, space group $P2_1$, at 100 K: a = 12.6984(12), b = 15.0386(14) and c = 13.5944(15) Å, β = 108.824(12)°, V = 2457.2(4) A^3, Z = 4, d_{calc} = = 1.218 g cm^{-3}, μ = 0.076 mm^{-1}, F(000) = 984.0, crystal size 0.34×0.22×0.12 mm, MoKα radiation (λ = 0.71073), 2θ range for data collection 5.982 to 52.994°, index ranges $-15 \leqslant h \leqslant 15$, $-18 \leqslant k \leqslant 18$, $-14 \leqslant l \leqslant 17$, reflections collected 11308, independent reflections 8346 (R_{int} = 0.0179, R_σ = 0.0386). Data/restraints/parameters 8346/1/607, GOOF on F^2 1.029, final R indexes [$I \geqslant 2\sigma(I)$]: R_1 = 0.0367 and wR_2 = 0.0850, final R indexes (all data): R_1 = 0.0411, wR_2 = 0.0885, largest diff. peak/hole: 0.22/–0.19 $eÅ^{-3}$, flack parameter 0.8(5). Single crystals of $C_{29}H_{42}N_2O_2$ were obtained by crystallization from ethyl acetate. Suitable crystals of the compound were selected and measured on an Agilent Technologies 'Xcalibur' diffractometer with monochromated MoKα radiation. The crystal was kept at 100(2) K during data collection. Using Olex2,[10] the structure was solved with the ShelXS[14] structure solution program using Direct Methods and refined with the ShelXL[12] refinement package using Least Squares minimisation.

CCDC 1812486, 1813229 and 1812773 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre *via* http://www.ccdc.cam.ac.uk.

Table 1 Cytotoxic (CC$_{50}$) and antiviral (IC$_{50}$) activities and selectivity indices (SI) of dammarane pyrazinyltriterpenoids **2**,**6**,**7**,**8** and betulin against influenza virus A/Puerto Rico/8/34 (H1N1).

Compound	CC$_{50}$/μg ml^{-1}	IC$_{50}$/μg ml^{-1}	SI
6	21.2	$\geqslant$ 11	2
7	34.2	8.2	4
8	$\geqslant$ 300	33	10
2	$\geqslant$ 330	190	1
betulin	> 330	38	9
rimantadine	60	12	5

Table 2 Flow cytofluorometric analysis of Hep G2 cells incubated with compounds **2**,**6**,**7**,**8** in a medium containing 10% FBS for 2 days.

Compound	G1/G0-phase (%)	S-phase (%)	G2/M-phase (%)
6	29.4	12.0	58.6
7	31.8	15.4	52.8
8	27.0	15.9	57.1
2	32.0	13.2	54.8
Control	10.4	6.5	83.1

In summary, a series of novel pyrazines fused with lupenone, dipterocarpol, and alnincanone were synthesized by the Willgerodt–Kindler reaction. The compounds possess only low anti-influenza activity with selectivity index (SI) up to 10 units. A very low cytotoxicity on MDCK cells (CC$_{50}$ $\geqslant$ 300 μg ml^{-1}) was observed. A marked anti-proliferative activity was detected only in the case of pyrazine derivative of lupenone (IC$_{50}$ 42 μM for HeLa and IC$_{50}$ 19 μM for Hep G2), whereas all of investigated compounds demonstrated high pro-apoptotic activity in HepG2 cells.

The authors declare that they have no competing interests. This work was supported by the Russian Science Foundation (grant no. 17-13-01124). Physicochemical measurements were carried out at the X-ray Diffraction Centre, the Centre for Magnetic Resonance, and the Centre for Chemical Analysis and Materials (all belong to St. Petersburg State University).

Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2019.09.007.

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