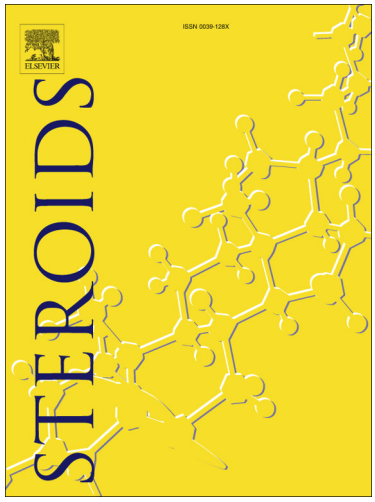

Catalytic cyclometallation in steroid chemistry V: Synthesis of hybrid molecules based on steroid oximes and (5Z,9Z)-tetradeca-5,9-dienedioic acid as potential anticancer agents

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PII: S0039-128X(18)30109-0
DOI: https://doi.org/10.1016/j.steroids.2018.06.002
Reference: STE 8271

To appear in: *Steroids*

Received Date: 13 March 2018
Revised Date: 31 May 2018
Accepted Date: 2 June 2018

Please cite this article as: D'yakonov, V.A., Tuktarova, R.A., Dzhemileva, L.U., Ishmukhametova, S.R., Yunusbaeva, M.M., Dzhemilev, U.M., Catalytic cyclometallation in steroid chemistry V: Synthesis of hybrid molecules based on steroid oximes and (5Z,9Z)-tetradeca-5,9-dienedioic acid as potential anticancer agents, *Steroids* (2018), doi: https://doi.org/10.1016/j.steroids.2018.06.002

Catalytic cyclometallation in steroid chemistry V[1]: Synthesis of hybrid molecules based on steroid oximes and (5Z,9Z)-tetradeca-5,9-dienedioic acid as potential anticancer agents

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ABSTRACT *Synthetic analogues of natural 5Z,9Z-dienoic acids - hybrid molecules based on the oximes of cholesterol, pregnenolone, and androsterone with 1,14-tetradeca-5Z,9Z-dienedicarboxylic acid - were synthesized for the first time and studied for antitumor activity in vitro. The acid was prepared using catalytic cyclomagnesiation of O-containing 1,2-dienes with Grignard reagent in the presence of Cp_2TiCl_2 as the key step. Using flow cytometry, it was shown for the first time that the new molecules are efficient apoptosis inducers in the HeLa, Hek293, U937, Jurkat, and K562.*

Key words: Cross-cyclomagnesiation; Grignard reagents; Steroid oximes; 5Z,9Z-Dienoic acids; Anticancer activity.

1. Introduction

According to published data [1], aliphatic 5Z,9Z-dienolic acids isolated from marine invertebrates possess a broad spectrum of biological activities such as antimalarial, antibacterial, and antifungal activities. In addition, 5Z,9Z-dienoic acids exhibit high inhibitory activity against human topoisomerases I and IIα [2-6], which are considered as intracellular targets of chemotherapeutic agents that prevent the break repair and thus cause accumulation of damaged DNA molecules and promote tumor cell death [7].

In recent years, more and more publications have addressed the use of cytotoxic steroids for the treatment of cancer; most of these steroids are hybrid molecules in which the cytotoxic substituent in linked to one functional group of the steroid [8-10]. In these molecules, the steroid moiety functions as a cytostatic transport, owing to its lipophilic properties and the ability to easily penetrate the lipid bilayer of biological membranes, thus providing access to definite target proteins and increasing the selectivity of drug action. The results indicate that the direct

[1] Steroids, **2016**, 108, 77-84.

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action of steroid molecules on the genetic apparatus of the cell is the key mechanism of their functioning.

Previously [11], we have demonstrated the possibility of synthesis of hybrid molecules based on steroids and 1,14-tetradeca-5Z,9Z-dienedicarboxylic acid, which was prepared using the catalytic cross-cyclomagnesiation of 1,2-dienes with Grignard reagents in the presence of Cp_2TiCl_2 [12,13]. It has been shown that steroid-containing 5Z,9Z-dienoic acids obtained by catalytic esterification of (5Z,9Z)-tetradeca-5,9-dienoic acid with hydroxy steroid derivatives in the presence of DCC/DMAP [14] can efficiently induce apoptosis in tumor cell lines of various etiology and inhibit relaxation of supercoiled DNA by topoisomerase I *in vitro*.

In view of the fact that 5Z,9Z-dienoic acids can selectively inhibit cell cycle enzymes, topoisomerases I and II, and simultaneously exhibit antitumor, anti-inflammatory, antibacterial, and antifungal activities and as continuation of the studies dealing with the synthesis of analogues of natural 5Z,9Z-dienoic acids, we synthesized for the first time hybrid molecules based on cholesterol, pregnenolone, and androsterone oximes and 1,14-tetradeca-5Z,9Z-dienedicarboxylic acid and studied their cytotoxic activity against the Jurkat, K562, U937, Hek293, and HeLa tumor cells.

2. Experimental

2.1. Chemistry

Cholesterol, 5-pregnen-β-ol-20-on, 5-androsten-β-ol-17-on, 4-dimethylaminopyridine (DMAP), *N*-[3-(methylamino)propyl]-*N*'-ethylcarbodiimide hydrochloride (EDC·HCl) were obtained from Sigma-Aldrich and Acros Organics. Dichloromethane was freshly distilled before use. Optical rotations were measured on a Perkin–Elmer 341 polarimeter. IR spectra were recorded on Bruker VERTEX 70V using KBr discs over the range of 400–4000 cm^{-1}. Mass spectra of MALDI TOF/TOF positive ions (matrix of sinapic acid) are recorded on a mass spectrometer Bruker Autoflex^{TM} III Smartbeam. ^{1}H and ^{13}C NMR spectra were obtained using a Bruker Ascend 500 spectrometer in $CDCl_3$ operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Elemental analyses were measured on 1106 Carlo Erba apparatus. Individuality and purity of the synthesized compounds were controlled using TLC on Sorbfil plates; anisic aldehyde in acetic acid was used as a developer. Column chromatography was carried out on Acrus silica gel (0.060–0.200 мм).

*2.1.1. ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectral data and synthesis method for 2,2'-[(5Z,9Z)-tetradeca-5,9-diene-1,14-diylbis(oxy)]bistetrahydro-2H-pyran **3** are described in the literature [15].*

*2.1.2. ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) spectral data for (5Z,9Z)-tetradeca-5,9-dienedioic acid **4** are described in the literature [11].*

*2.1.3. Reaction of steroid oximes with (5Z,9Z)-tetradeca-5,9-dienedioic acid (**4**).*

To a solution of steroid oxime (1.0 mmol) in dichloromethane (50 ml) the (5Z,9Z)-tetradeca-5.9-dienedioic acid **4** (0.51 g, 2.0 mmol) was added followed by *N*-[3-(methylamino)propyl]-*N*'-ethylcarbodiimide hydrochloride (0.48 g, 2.5 mmol) and 4-dimethylaminopyridine (18 mg, 0.15 mmol) under argon. The mixture was stirred at room temperature for 12 h until the reaction was complete (TLC monitoring, hexane/ethyl acetate). The mixture was diluted with H_2O (10 ml) and the CH_2Cl_2 layer was separated, dried over $MgSO_4$, and concentrated. The crude product was purified by column chromatography (silica gel) using hexane/ethyl acetate = 5/1 as the elution solvent to afford 5Z,9Z-dienoic acids of steroid.

*2.1.3.1. (5Z,9Z)-14-({[(3β)-3-(Acetyloxy)cholest-4-en-6E-ylidene]amino}oxy)-14-oxotetradeca-5,9-dienoic acid (**8a**).* White waxy solid, 0.45 g, 65% yield. $[\alpha]\ ^{19}_{D}$ + 4.1 (*c* 0.54, $CHCl_3$); IR (KBr) ν_{max} 2946, 2868, 1737, 1708, 1459, 1375, 1238, 1122, 1042, 1021, 911, 867, 822, 610 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.91 (1H, s, H-4), 5.41 (2H, m, H-6', H-9'), 5.36 (2H, m, H-5', H-10'), 5.29 (1H, t, *J* = 7.5 Hz, H-3), 3.21 (1H, d, *J* = 7.5 Hz, H-7*β*), 2.45 (2H, t, *J* = 7.5 Hz, H-13'), 2.36 (2H, t, *J* = 7.5 Hz, H-2'), 2.11 (4H, m, H-4', H-11'), 2.09 (4H, m, H-7', H-8'), 2.06 (3H, s, $COCH_3$), 2.04–1.00 (25H, m), 1.77 (2H, m, H-12'), 1.71 (2H, m, H-3'), 1.03 (3H, s, H-19), 0.92 (3H, d, *J* = 6.0 Hz, H-21), 0.88 (6H, d, *J* = 6.5 Hz, H-26, H-27), 0.69 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 178.8 (C-1'), 171.4 (C-14'), 171.0 ($COCH_3$), 165.4 (C-6), 141.9 (C-5), 130.3 (C-6', C-9'), 128.9 (C-5', C-10'), 126.3 (C-4), 69.5 (C-3), 56.4 (C-14), 55.9 (C-17), 52.5 (C-9), 42.8 (C-13), 39.5 (C-24), 39.3 (C-12), 38.1 (C-10), 36.1 (C-22), 35.7 (C-20), 34.3 (C-1), 34.2 (C-8), 33.3 (C-2'), 32.4 (C-13'), 32.3 (C-7), 28.0 (C-16, C-25), 27.3 (C-7', C-8'), 26.6, 26.5 (C-4', C-11'), (24.8 (C-12'), 24.6 (C-3'), 24.2 (C-2), 24.1 (C-15), 23.8 (C-23), 22.8 (C-27), 22.6 (C-26), 21.3 ($COCH_3$), 20.8 (C-11), 18.7 (C-19), 18.6 (C-21), 11.9 (C-18); anal. calcd for $C_{43}H_{67}NO_6$: C, 74.42; H, 9.73; found C, 74.36; H, 9.67. MALDI TOF: m/z 716.465 ($[M+Na]^+$, calcd 716.487), 732.427 ($[M+K]^+$, calcd 732.461).

*2.1.3.2. (5Z,9Z)-14-({[(3β)-3-(Acetyloxy)cholest-5-en-7Z-ylidene]amino}oxy)-14-oxotetradeca-5,9-dienoic acid (**8b**).* White waxy solid, 0.44 g, 63% yield. [α] $^{19}_{D}$ – 148.6 (*c* 0.44, $CHCl_3$); IR (KBr) ν_{max} 2950, 2869, 1736, 1709, 1639, 1457, 1439, 1375, 1366, 1241, 1125, 1035, 914, 878, 803, 737, 609 cm^{-1}; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 6.44 (1H, m, H-6), 5.40 (2H, m, H-6', H-9'), 5.36 (2H, m, H-5', H-10'), 4.70 (1H, m, H-3), 2.60–1.00 (26H, m), 2.45 (2H, m, H-13'), 2.36 (2H, t, *J* = 7.5 Hz, H-2'), 2.12 (4H, m, H-4', H-11'), 2.08 (4H, m, H-7', H-8'), 2.06 (3H, s, $COCH_3$), 1.76 (2H, m, H-12'), 1.71 (2H, m, H-3'), 1.16 (3H, s, H-19), 0.94 (3H, d, *J* = 6.5 Hz, H-21), 0.88 (6H, d, *J* = 6.5 Hz, H-26, H-27), 0.72 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 178.8 (C-1'), 171.7 (C-14'), 170.5 ($COCH_3$), 163.0 (C-7), 156.2 (C-5), 130.4, 130.2 (C-6', C-9'), 129.0, 128.8 (C-5', C-10'), 114.2 (C-6), 72.8 (C-3), 54.6 (C-17), 49.9 (C-14), 49.3 (C-9), 42.9 (C-13), 39.5 (C-24), 38.6 (C-8), 38.5 (C-12), 38.4 (C-10), 38.2 (C-4), 36.2 (C-1), 36.1 (C-22), 35.6 (C-20), 33.3 (C-2'), 32.4 (C-13'), 28.3 (C-16), 28.0 (C-25), 27.3 (C-2, C-7', C-8'), 27.1 (C-15), 26.6, 26.5 (C-4', C-11'), 24.8 (C-12'), 24.6 (C-3'), 23.7 (C-23), 22.8 (C-27), 22.6 (C-26), 21.3 ($COCH_3$), 20.8 (C-11), 18.9 (C-21), 17.8 (C-19), 12.1 (C-18); anal. calcd for $C_{43}H_{67}NO_6$: C, 74.42; H, 9.73; found C, 74.37; H, 9.68. MALDI TOF: m/z 716.487 ($[M+Na]^+$, calcd 716.487), 732.448 ($[M+K]^+$, calcd 732.461).

*2.1.3.3. (5Z,9Z)-14-({[(3β)-3-(Acetyloxy)pregn-5-en-20E-ylidene]amino}oxy)-14-oxotetradeca-5,9-dienoic acid (**8c**).* White waxy solid, 0.40 g, 66% yield. [α] $^{19}_{D}$ – 9.7 (*c* 0.85, $CHCl_3$); IR (KBr) ν_{max} 3006, 2962, 2937, 2855, 1732, 1631, 1438, 1368, 1250, 1130, 1037, 802, 756, 611 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.39 (2H, m, H-6', H-9'), 5.36 (3H, m, H-5', H-6, H-10'), 4.62 (1H, m, H-3), 2.44 (2H, t, *J* = 7.5 Hz, H-13'), 2.37 (2H, m, H-2'), 2.38–1.00 (20H, m), 2.14 (4H, m, H-4', H-11'), 2.08 (4H, m, H-7', H-8'), 2.04 (3H, s, $COCH_3$), 1.96 (3H, s, H-23), 1.77 (2H, *J* = 7.5 Hz, H-12'), 1.70 (2H, m, H-3'), 1.03 (3H, s, H-19), 0.69 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 178.9 (C-1'), 171.7 (C-14'), 170.6 ($COCH_3$), 166.7 (C-20), 139.6 (C-5), 130.4, 130.3 (C-6', C-9'), 128.9, 128.8 (C-5', C-10'), 122.4 (C-6), 73.9 (C-3), 56.9 (C-17), 56.2 (C-14), 49.9 (C-9), 44.1 (C-13), 38.6 (C-12), 38.1 (C-4), 36.9 (C-1), 36.6 (C-10), 33.3 (C-2'), 32.5 (C-13'), 31.9 (C-8), 31.7 (C-7), 27.7 (C-2), 27.3 (C-7', C-8'), 26.5, 26.6 (C-4', C-11'), 24.8 (C-12'), 24.6 (C-3'), 24.3 (C-15), 23.1 (C-16), 21.4 ($COCH_3$), 20.9 (C-11), 19.3 (C-19), 17.1 (C-21), 13.3 (C-18); anal. calcd for $C_{37}H_{55}NO_6$: C, 72.87; H, 9.09; found C, 72.69; H, 9.03. MALDI TOF: m/z 632.395 ($[M+Na]^+$, calcd 632.393), 648.370 ($[M+K]^+$, calcd 648.367).

*2.1.3.4. (5Z,9Z)-14-({[(3β)-3-(Acetyloxy)androst-5-en-17E-ylidene]amino}oxy)-14-oxotetradeca-5,9-dienoic acid (**8d**).* White waxy solid, 0.37 g, 63% yield. [α] $^{19}_{D}$ – 36.9 (*c* 0.81,

$CHCl_3$); IR (KBr) ν_{max} 3003, 2963, 2942, 2857, 1732, 1628, 1441, 1363, 1247, 1132, 1033, 805, 754, 612 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 5.40 (2H, m, H-6', H-9'), 5.37 (1H, m, H-6), 5.37 (2H, m, H-5', H-10'), 4.62 (1H, m, H-3), 2.68–1.06 (19H, m), 2.42 (2H, t, *J* = 7.5 Hz, H-13'), 2.36 (2H, m, H-2'), 2.13 (4H, m, H-4', H-11'), 2.08 (4H, m, H-7', H-8'), 2.05 (3H, s, $COCH_3$), 1.74 (2H, m, H-12'), 1.71 (2H, m, H-3'), 1.06 (3H, s, H-19), 1.01 (3H, s, H-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 178.6 (C-17, C-1'), 171.6 (C-14'), 170.6 ($COCH_3$), 139.9 (C-5), 130.4, 130.3 (C-6', C-9'), 128.9 (C-5', C-10'), 121.8 (C-6), 73.8 (C-3), 53.9 (C-14), 49.9 (C-9), 44.9 (C-13), 38.1 (C-4), 36.9 (C-1), 36.7 (C-10), 33.5 (C-12), 33.2 (C-2'), 32.4 (C-13'), 31.3 (C-7, C-8), 27.7 (C-2), 27.3 (C-7', C-8'), 27.2 (C-16), 26.6, 26.5 (C-4', C-11'), 24.8 (C-12'), 24.6 (C-3'), 23.1 (C-15), 21.4 ($COCH_3$), 20.4 (C-11), 19.3 (C-19), 16.8 (C-18); anal. calcd for $C_{35}H_{51}NO_6$: C, 72.26; H, 8.84; found C, 72.14; H, 8.80. MALDI TOF: m/z 620.319 ($[M+K]^+$, calcd 620.335).

2.2. Biological assays

2.2.1. Cell culturing

Cells (Jurkat, K562, U937) were purchased from Russian Cell Culture Collection (Institute of Cytology of the Russian Academy of Sciences) and cultured according to standard mammalian tissue culture protocols and sterile technique. Human cancer cell lines HEK293 and HeLa were obtained from the HPA Culture Collections (UK). All cell lines used in the study were tested and shown to be free of mycoplasma and viral contamination.

HEK293, HeLa cell lines and fibroblasts were cultured as monolayers and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL) supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin solution at 37 °C in a humidified incubator under a 5% CO_2 atmosphere.

Cells were maintained in RPMI 1640 (Jurkat, K562, U937) (Gibco) supplemented with 4 mM glutamine, 10% FBS (Sigma) and 100 units/ml penicillin-streptomycin (Sigma). All types of cells were grown in an atmosphere of 5 % CO_2 at 37 °C. The cells were subcultured at 2-3 days intervals. Adherent cells (HEK293, HeLa, fibroblasts) were suspended using trypsin/EDTA and counted after they have reached 80% confluency. Cells were then seeded in 24 well plates at $5x10^4$ cells per well and incubated overnight. Jurkat, K562, U937 cells were subcultured at 2 day intervals with a seeding density of 1×10^5cells per 24 well plates in RPMI with 10% FBS.

2.2.2. Cytotoxicity assay

Viability (Live/dead) assessment was performed by staining cells with 7-AAD (7-Aminoactinomycin D) (Biolegend). Cells after treatment with compounds **7a-d**, **8a-d** at various concentrations (2, 1, 0.5, 0.25, 0.1 μM) and incubated in an atmosphere of 5 % CO_2 at 37 °C during 24 hours were harvested, washed 1-2 times with phosphate-buffered saline (PBS) and centrifuged at 400g for 5 minutes. Cell pellets were resuspended in 200 uL of flow cytometry staining buffer (PBS without $Ca^{2+}$and Mg^{2+}, 2,5% FBS) and stained with 5 uL of 7-AAD staining solution for 15 minutes at room temperature in the dark. Samples were acquired on NovoCyteTM 2000 FlowCytometry System (ACEA) equipped with 488 nm argon laser. Detection of 7-AAD emission was collected through a 675/30 nm filter in FL4 channel.

2.2.3. Viability and apoptosis

Apoptosis was determined by flow cytometric analysis of Annexin V and 7-aminoactinomycin D staining. K562 Tumor cells after treatment with compound **8d** at concentrations (0.04, 0.03, 0.02, 0.01 μM) during 24 hours were harvested, washed 1-2 times with phosphate-buffered saline (PBS) and centrifuged at 400g for 5 minutes. Cell pellets were resuspended in 200 uL of flow cytometry staining buffer (PBS without $Ca^{2+}$and Mg^{2+}, 2,5% FBS). Then, 200 μl of Guava Nexin reagent (Millipore, Bedford, MA, USA) was added to 5 × 10^5 cells in 200 μl, and the cells were incubated with the reagent for 20 min at room temperature in the dark. At the end of incubation, the cells were analyzed on NovoCyteTM 2000 FlowCytometry System (ACEA).

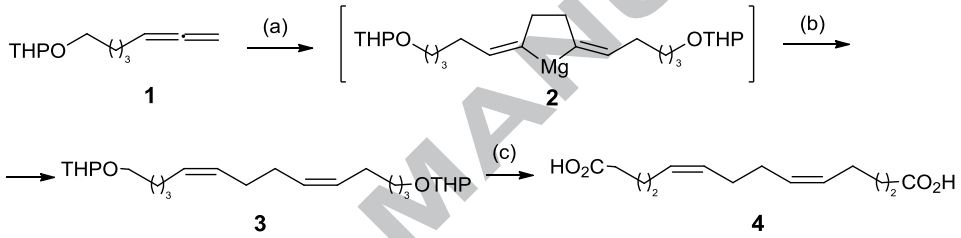
2.2.4. Cell cycle analysis

Cell cycle was analyzed using the method of propidium iodide staining. K562 Tumor cells after treatment with compound **8d** at concentrations (0.04, 0.03, 0.02, 0.01 μM) during 24 hours were harvested, washed 1-2 times with phosphate-buffered saline (PBS) and centrifuged at 400g for 5 minutes. Cell pellets were resuspended in 200 uL of flow cytometry staining buffer (PBS without $Ca^{2+}$and Mg^{2+}, 2,5% FBS). Then, cells were plated in 24-well round bottom plates at a density 10×10^5 cells per well, centrifuged at 450× g for 5 minutes, and fixed with ice-cold 70% ethanol for 24 hour at 0 °C. Cells were then washed with PBS and incubated with 250 μl of

Guava Cell Cycle Reagent (Millipore) for 30 minutes at room temperature in the dark. Samples were analyzed on NovoCyteTM 2000 FlowCytometry System (ACEA).

3. Results and Discussion

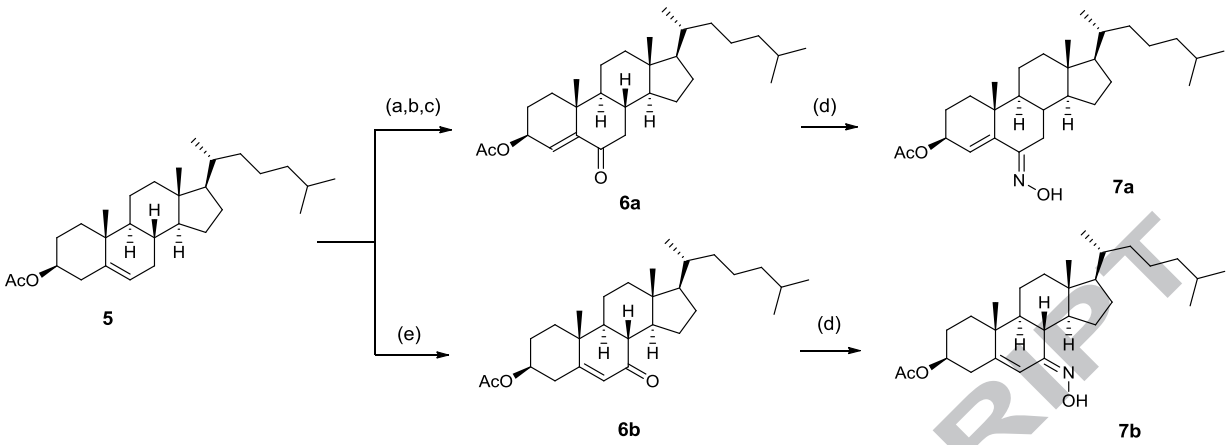
Tetradeca-5Z,9Z-diene-1,14-dicarboxylic acid was synthesized in two steps using the previously developed [15,16] homo-cyclomagnesiation of tetrahydropyran 5,6-heptadien-1-ol ether **1** with EtMgBr in the presence of the Cp_2TiCl_2 catalyst (5 mol. %) to give magnesacyclopentane **2**. The subsequent hydrolysis of organomagnesium compound **2** and oxidation of the resulting 1,14-bis-tetrahydropyranyl-5Z,9Z-diene-1,14-diol **3** with the Jones reagent gives target dicarboxylic acid **4** in an overall yield of ~52 % (Scheme 1).



Scheme 1. Synthesis of (5Z,9Z)-tetradeca-5,9-dienedioic acid **4**. (a): EtMgBr, Mg, Cp_2TiCl_2 (5 mol%), diethyl ether; (b): H_3O^+; (c): H_2CrO_4/H_2SO_4, acetone, CH_2Cl_2.

As the initial stage of our studies dealing with the synthesis of new hybrid molecules based on 1,14-tetradeca-5Z,9Z-dienedicarboxylic acid **4** and steroid oximes, we prepared these compounds from appropriate keto steroids **6a-d**.

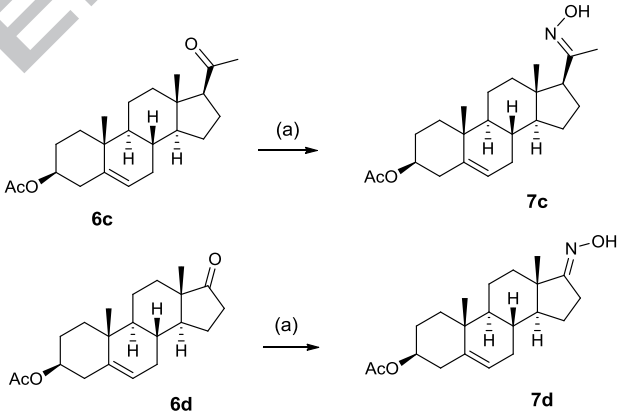
Keto derivative **6a**, needed for the synthesis of oxime **7a**, was prepared by successive reactions including epoxidation of (3*β*)-cholest-5-en-3yl acetate **5**, oxidation, and elimination of the hydroxyl group at C-5 by treatment with thionyl chloride in pyridine [17]. Then keto steroid **6a** was reacted with hydroxylamine hydrochloride in dry pyridine to give (6*E*)-hydroximinocholest-4-en-3*β*-yl acetate **7a** (Scheme 2). The structure of compound **7a** was confirmed by 1D (^{1}H, ^{13}C, Dept 135) and 2D (HSQC, HMBC and HH COSY, NOESY) NMR experiments. The ^{1}H NMR spectrum exhibits a signal at 5.68 ppm for the proton at the double bond and a signal at 3.34 ppm (dd, J = 15.0, 4.5 Hz) for the H-7*β* proton; together with the C-6 carbon signal at 158.2 ppm in the ^{13}C NMR spectrum, are in agreement with those reported in the literature for the *E*-configuration of oxime **7a** [17].



Scheme 2. Synthesis of steroid oximes **7a**,**b**. (a): mCPBA, CH_2Cl_2 (97%); (b): CrO_3, H_2O (90%); (c) $SOCl_2$, Py (92%); (d): $NH_2OH{\cdot}HCl$, Py (~96%); (e): Celite, PDC, *t*-BuOH, benzene (81%).

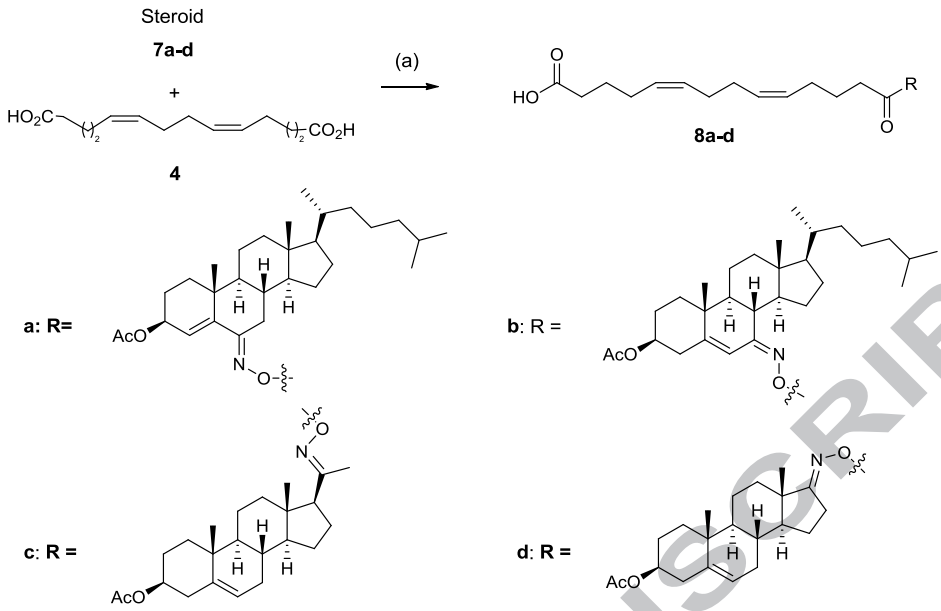
The target (7*Z*)-hydroximinocholest-5-en-3β-yl acetate **7b** was synthesized by oxidation of (3β)-cholest-5-en-3yl acetate **5** with *tert*-butyl hydroperoxide-pyridinium dichromate [18] followed by the reaction of keto derivative **6b** with hydroxylamine hydrochloride (Scheme 2) [19].

The configurations of oximes, (3β)-acetoxy-20-hydroxyimino-5-pregnen-20*E*-one **7c** and (3β)-17*E*-(hydroximino)-5-androsten-3-ol acetate **7d** (Scheme 3), synthesized by the procedure presented above for oximes **7a,b** are consistent with published ^1H and ^{13}C NMR data [20-22].



Scheme 3. Synthesis of steroid oximes **7c**,**d**. (a): $NH_2OH{\cdot}HCl$, Py (~96%).

Having prepared the key steroid oximes **7a-d** and 1,14-tetradeca-5Z,9Z-dienedicarboxylic acid **4**, we synthesized the desired hybrid molecules **8a-d** using *N*-[3-(methylamino)propyl]-*N*'-ethylcarbodiimide hydrochloride (EDC·HCl) and catalytic amounts of 4-dimethylaminopyridine (DMAP) (Scheme 4).



Scheme 4. Synthesis of 5Z,9Z-dienoic acids **8a-d**. (a): DMAP, EDC·HCl, CH_2Cl_2.

Acids **8a-d** and the steroid oxime precursors **7a-d** were tested for the first time for antitumor activity *in vitro* against the Jurkat, K562, Hek293, HeLa, and U937 cell lines with determination of cytotoxicity CC_{50}. Compounds that exhibited the highest activity were additionally studied for the cell viability and effect on the cell cycle by flow cytometry using the Guava Nexin Reagent, Guava Cell Cycle, and Guava ViaCount kits (Millipore).

The *in vitro* cytotoxic activities of compounds **7a-d** and **8a-d** against the Jurkat, K562, U937, and HeLa cultures and the Hek293 human embryonic kidney cells are summarized in Table 1.

Table 1. Inhibition of tumor cell viability by 5Z,9Z-dienoic acid derivatives **8a-d** and steroid oximes **7a-d**, CC_{50} (μM) ± SE.

	Jurkat	**K562**	**U937**	**Hek293**	**HeLa**	**Fibrobl.**
CC_{50} (7a)	1.36±0.026	1.14±0.027	0.81±0.035	0.92±0.033	0.73±0.028	1.52±0.034
CC_{50} (7b)	1.12±0.029	0.61±0.023	0.71±0.029	1.24±0.037	1.04±0.027	1.39±0.045
CC_{50} (7c)	0.82±0.015	1.01±0.022	0.74±0.018	0.62±0.021	0.64±0.024	1.19±0.032
CC_{50} (7d)	0.67±0.012	0.53±0.017	0.45±0.025	0.74±0.021	0.71±0.015	1.12±0.021
CC_{50} (8a)	0.68±0.018	0.27±0.027	0.38±0.035	0.49±0.023	0.30±0.034	1.17±0.047
CC_{50} (8b)	0.51±0.026	0.33±0.021	0.49±0.035	0.76±0.023	0.58±0.017	1.13±0.043
CC_{50} (8c)	0.32±0.035	0.34±0.012	0.41±0.028	0.13±0.011	0.29±0.036	0.79±0.019
CC_{50} (8d)	0.02±0.004	0.02±0.003	0.04±0.003	0.03±0.007	0.17±0.006	0.55±0.023
CC_{50} (camp.)	1.12±0.012	2.10±0.013	1.32±0.011	3.21±0.017	2.18±0.016	4.19±0.067

The highest activities against chronic myeloid leukemia cells (K562) and T-cell leukemia cells (Jurkat) were found for acid **8d** (CC_{50} = 0.025 and CC_{50} = 0.015, respectively), with the

cytotoxic effect being dose-dependent and markedly exceeding that of camptothecin for the same cell lines. The lowest activities against all of the studied cell lines were found for compounds **8a** and **8b** derived from cholesterol (for CC_{50}, see Table 1). It is also noteworthy that acid **8c** has a rather high cytotoxic activity (CC_{50} = 0.13 and 0.031, respectively) against the HEK293 human embryonic kidney cells and HeLa cells.

We found that the cytotoxicity of hybrid molecules **8a-d** substantially exceeds the cytotoxicity of steroid oxime precursors **7a-d** (Table 1). In the CC_{50} study for compounds **8a-d** in normal fibroblast cultures, it has been shown that the cytotoxicity of hybrid molecules is higher than that of tumor cells, indicating a higher sensitivity of tumor lines to synthesized hybrids.

It should be noted that the cytotoxic activity of compound **8d** was 5 times higher than the cytotoxic activity of previously synthesized (5Z,9Z)-dienoic acid on the basis of pregnenolone against the cells of kidney cancer HEK293, and almost 10 times the T cell lines of Jurkat leukemia and myelogenous leukemia K562 [14].

In view of the pronounced cytotoxic effect found for hybrid molecule **8d**, we investigated the apoptosis and cell cycle in the culture cells.

According to published data, steroid hormones have a differentiating effect on various types of cells, namely, prevent apoptosis of some types of cells and induce apoptosis of other cell types. For example, neuroactive steroids, or neurosteroids, can induce apoptosis in neuroglia tissues [23]. Also neurosteroids are actively used to treat multiple sclerosis patients, because the neural tissue of neurodegenerative disease patients was found to be sharply deficient in these steroids [24].

Previously, we found an antiproliferative effect of 3-keto-diosgenin oxime against the cervical carcinoma (HeLa), colon carcinoma (HCT-116), osteosarcoma 1547, hepatocellular carcinoma (HepG2, C3A, and HUH-7), and breast carcinoma (MCF-7) cells [25-29].

In view of our results, we studied in more detail the apoptosis-inducing activities of compounds **8d** on myeloid and T-cell leukemia cells. The highest percentage of apoptosis after the addition of compound **8d** to K562 cell culture was attained for 0.2 μL concentration and amounted to 72.8% (Fig. 1). The percentage of late apoptosis for acid **8d** was 80% for Jurcat cells, 78% for HEK293 cells, 92% for U937 cells, and 65% for HeLa cells.

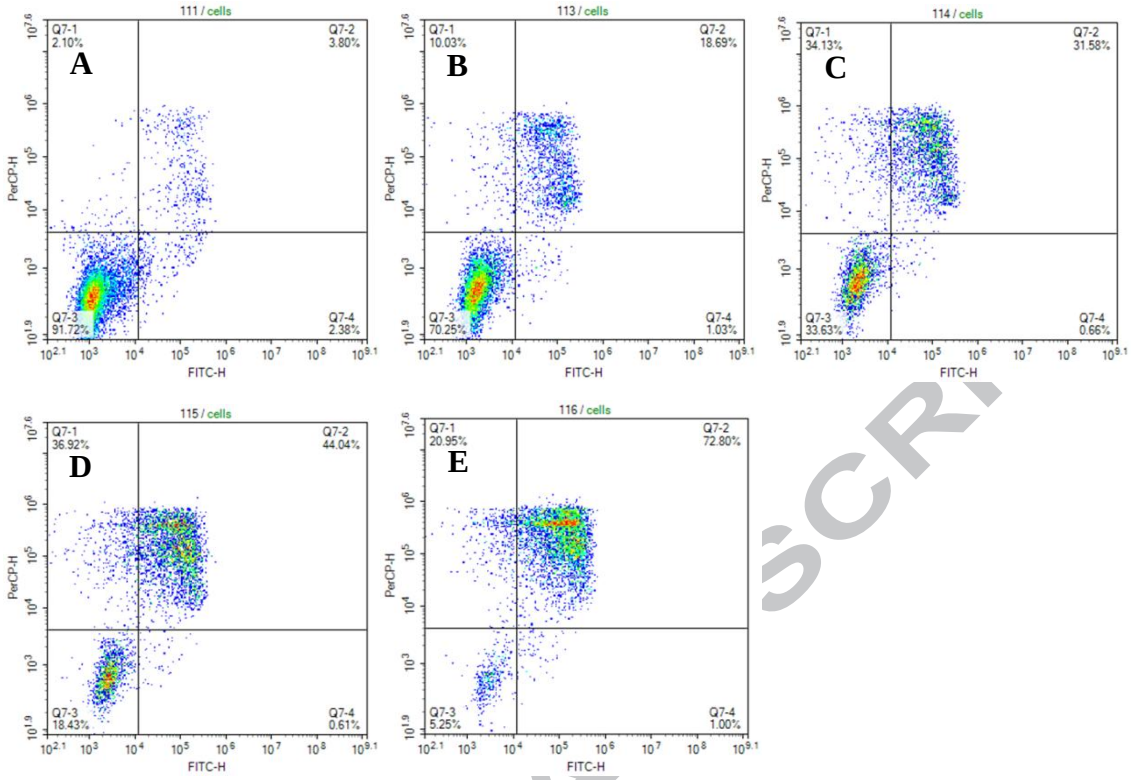


Fig. 1. K562 tumor cells treated with compound **8d** in different concentrations and stained with annexin V/7AAD. Flow cyrometry data: (A) control; (B) **8d** (0.01 μM); (C) **8d** (0.02 μM); (D) **8d** (0.03 μM), (E) **8d** (0.04 μM).

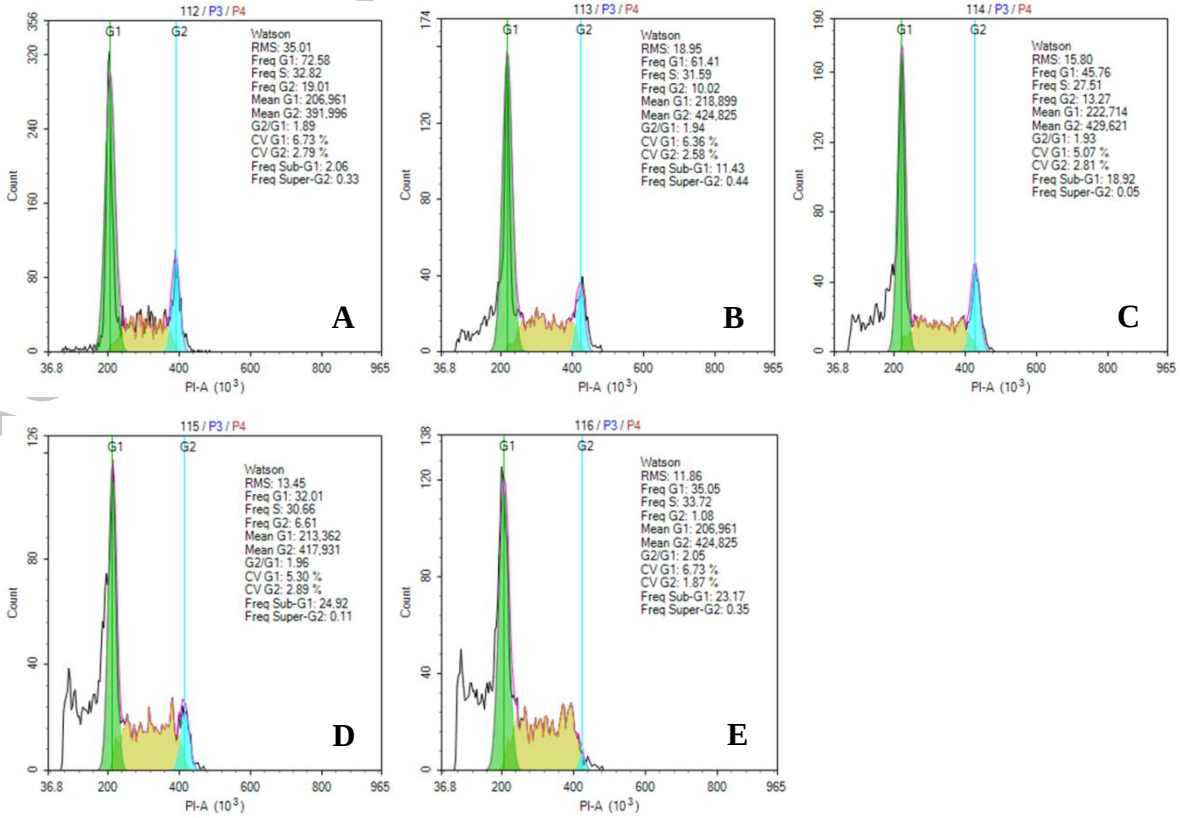


Fig. 2. Cell cycle phases of K562 cells treated with compound **8d**. (A) Control; (B) **8d** (0.01 μM); (C) **8d** (0.02 μM); (D) **8d** (0.03 μM); (E) **8d** (0.04 μM) (The incubation time of **8d** with the cells was 24 hours).

Figure 2 shows the results of a study of the cell cycle phases of cells K562 line, determined by DNA flow cytometry, after treatment with acid **8d** at different concentrations (0.04, 0.03, 0.02, 0.01 μM) after 24 hours.

The cell cycle characteristics of the K562 and Jurkat cells in control samples showed considerable predominance of cells in the G0-G1 phase and a balance between the synthesis (S-phase) and apoptosis (sub-G0-G1 range) processes.

Two days after the exposure to compound **8d**, apoptosis processes predominated (the sub-G0-G1 range increased), while the ability of cells to DNA synthesis (S-phase) was retained or even increased. The percentage of G0-G1 cells decreased, while the proliferation block increased and the proliferation index decreased due to decreasing amount of G2 + M cells.

All the foregoing may be indicative of the cytotoxic activity of acid **8d** against chronic myeloid leukemia cells and T-cell leukemia cells, caused by the ability of this compound to induce apoptosis.

4. Conclusion

Thus, we prepared previously unknown synthetic analogues of natural 5Z,9Z-dienoic acids - hybrid molecules based on cholesterol, pregnenolone, and androsterone oximes and 1,14-tetradeca-5Z,9Z-dienedicarboxylic acid. Using flow cytometry, it was shown for the first time that the new molecules are efficient apoptosis inducers in the HeLa, Hek293, U937, Jurkat, and K562 cell cultures.

Acknowledgments

The work was financially supported by the Russian Science Foundation (Grant No. 16-13-10172), the Russian Foundation for Basic Research (Grant No. 16-03-00543_A) and the Grant of the RF President (Sci. Sh. - 5240.2018.3). The structural studies of the synthesized compounds were performed with the use of Collective Usage Centre "Agidel" at the Institute of Petrochemistry and Catalysis of RAS. The biological studies of Cu complexes were done in the Center for Molecular Design and Drug Bioscreening at the Institute of Petrochemistry and Catalysis of RAS that was created with the financial support of the Russian Science Foundation.

References

[1] N. Carballeira, New advances in fatty acids as antimalarial, antimycobacterial and antifungal agents, Prog. Lipid. Res. 47 (2008) 50–61.

[2] T. Nemoto, G. Yoshino, M. Ojika, Y. Sakagami, Amphimic acids and related long-chain fatty acids as DNA topoisomerase I inhibitors from an Australian sponge, Amphimedon sp.: Isolation, structure, synthesis, and biological evaluation, Tetrahedron 53 (1997) 16699–16710.

[3] N.M. Carballeira, J.E. Betancourt, E.A. Orellano, F.A. Gonzalez, Total synthesis and biological evaluation of (5Z,9Z)-5,9-hexadecadienoic acid, an inhibitor of human topoisomerase I, J. Nat. Prod. 65 (2002) 1715–1718.

[4] V.A. D'yakonov, A.A. Makarov, L.U. Dzhemileva, E.Kh. Makarova, E.K. Khusnutdinova, U.M. Dzhemilev, The facile synthesis of the 5Z,9Z-dienoic acids and their topoisomerase I inhibitory activity, Chem. Commun. 49 (2013) 8401–8403.

[5] V.A. D'yakonov, L.U. Dzhemileva, A.A. Makarov, A.R. Mulyukova, D.S. Baev, E.K. Khusnutdinova, T.G. Tolstikova, U.M. Dzhemilev, nZ,(n+4)Z-Dienoic fatty acids: a new method for the synthesis and inhibitory action on topoisomerase I and IIα, Med. Chem. Res. 25 (2016) 30–39.

[6] V.A.D'yakonov, L.U. Dzhemileva, A.A. Makarov, A.R. Mulyukova, D.S. Baev, E.K. Khusnutdinova, T.G. Tolstikova, U.M. Dzhemilev, 11-Phenylundeca-5Z,9Z-dienoic acid: stereoselective synthesis and dual topoisomerase I/IIα inhibition, Curr. Cancer. Drug. Targets 15 (2015) 504–510.

[7] J.J. Champoux, DNA topoisomerases: structure, function, and mechanism, Annu. Rev. Biochem. 70 (2001) 369–413.

[8] G. Mehta, V. Singh, Hybrid systems through natural product leads: An approach towards new molecular entities, Chem. Soc. Rev. 31 (2002) 324–334.

[9] D.B. Salunke, B.G. Hazra, V.S. Pore, Steroidal conjugates and their pharmacological applications, Curr. Med. Chem. 13 (2006) 813–847.

[10] P.M. Levine, M.J. Garabedian, K. Kirshenbaum, Targeting the androgen receptor with steroid conjugates, J. Med. Chem. 57 (2014) 8224–8237.

[11] V.A. D'yakonov, L.U. Dzhemileva, R.A. Tuktarova, A.A. Makarov, I.I. Islamov, A. R. Mulyukova, U.M. Dzhemilev, Catalytic cyclometallation in steroid chemistry III[1]: Synthesis of steroidal derivatives of 5Z,9Z-dienoic acid and investigation of its human topoisomerase I inhibitory activity, Steroids 102 (2015) 110–117.

[12] V.A. D'yakonov, A.A. Makarov, A.G. Ibragimov, L.M. Khalilov, U.M. Dzhemilev, Novel Mg-organic reagents in organic synthesis Cp_2ZrCl_2 catalized intermolecular cyclomagnesiation of cyclic and acyclic 1,2-dienes using Grignard reagents, Tetrahedron 64 (2008) 10188–10194.

[13] V.A. D'yakonov, L.U. Dzhemileva, U.M. Dzhemilev, Advances in the Chemistry of Natural and Semisynthetic Topoisomerase I/II Inhibitors, Studies Nat. Prod. Chem. 54 (2017) 21–86.

[14] V.A. D'yakonov, L.U. Dzhemileva, R.A. Tuktarova, S.R. Ishmukhametova, M.M. Yunusbaeva, I.R. Ramazanov, U.M. Dzhemilev, Novel hybrid molecules on the basis of steroids and (5Z,9Z)-tetradeca-5,9-dienoic acid: synthesis, anti-cancer studies and human topoisomerase I inhibitory activity, Anti-Cancer Agents in Med. Chem. 17 (2017) 1126–1135.

[15] V.A. D'yakonov, A.A. Makarov, E.Kh. Makarova, U.M. Dzhemilev, Novel organomagnesium reagents in synthesis. Catalytic cyclomagnesiation of allenes in the synthesis of N-, O-, and Si-substituted 1Z,5Z-dienes, Tetrahedron 69 (2013) 8516–8526.

[16] V.A. D'yakonov, A.A. Makarov, E.Kh. Makarova, L.M. Khalilov, U.M. Dzhemilev, Synthesis and transformations of metallacycles 41. Cyclomagnesiation of O-containing 1,2-dienes with Grignard reagents in the presence of Cp_2TiCl_2, Russ. Chem. Bull. 61 (2012) 1943–1949.

[17] N. Deive, J. Rodriguez, C.Jimenez, Synthesis of cytotoxic 6E-hydroximino-4-ene steroids: structure/activity studies, J. Med. Chem. 44 (2001) 2612–2618.

[18] N. Chidambaram, S. Chandrasekaran, Tert-Butyl hydroperoxide-pyridinium dichromate: a convenient reagent system for allylic and benzylic oxidations, J. Org. Chem. 52 (1987) 5048–5051.

[19] F. Ahmad, M. Parveen, M. Alam, S. Azaz, A.M. Malla, M.J. Alam, D-U. Lee, S. Ahmad Synthesis, X-ray crystallography, spectroscopic (FT-IR, 1H &13C NMR and UV), computational (DFT/B3LYP) and enzymes inhibitory studies of 7-hydroximinocholest-5-en-3-ol acetate, Journal of Molecular Structure 1116 (2016) 317–332.

[20] S.A. Amelichev, A.A. Shashkov, I.V. Zavarzin, O.A. Rakitin, Synthesis of 17-(1,2,3-dithiazole) androstene derivatives, Mendeleev Commn. 21 (2011) 186–187.

[21] **S. Kim, E. Ma,** Synthesis of pregnane derivatives, their cytotoxicity on LNCap and PC-3 Cells, and screening on 5α-reductase inhibitory activity, Molecules 14 (2009) 4655–4668.

[22] J. Cui, Q. Lin, C. Gan, J. Zhan, W. Su, D. Zhao, B. Qi, Y. Huang, Synthesis and antiproliferative evaluation of some novel B-nor-D-homosteroids, Steroids 98 (2015) 138–142.

[23] V. Schaeffer, L. Meyer, C. Patte-Mensah, A. Eckert, A.G. Mensah-Nyagan, Sciatic nerve injury induces apoptosis of dorsal root ganglion satellite glial cells and selectively modifies neurosteroidogenesis in sensory neurons, Glia 58 (2010) 169–180.

[24] R. Boghozian, B.A. McKenzie, L.B. Saito, N. Mehta, W.G. Branton, J. Lu, G.B. Baker, F. Noorbakhsh, C. Power, Suppressed oligodendrocyte steroidogenesis in multiple sclerosis: Implications for regulation of neuroinflammation, Glia 65(2017) 1590–1606.

[25] R. Huo, Q. Zhou, B. Wang, S. Tashiro, S. Onodera, T. Ikejima, Diosgenin induces apoptosis in HeLa cells via activation of caspase pathway, Acta Pharmacol. Sin. 25 (2004) 1077–1082.

[26] J. Raju, R.P. Bird, Diosgenin, a naturally occurring furostanol saponin suppresses 3-hydroxy-3-methylglutaryl CoA reductase expression and induces apoptosis in HCT-116 human colon carcinoma cells, Cancer Lett. 255 (2007) 194–204.

[27] S. Moalic, B. Liagre, C. Corbière, A. Bianchi, M. Dauça, K. Bordji, J.L. Beneytout, A plant steroid, diosgenin, induces apoptosis, cell cycle arrest and COX activity in osteosarcoma cells, FEBS Lett. 506 (2001) 225–230.

[28] J. Li, X. Liu, M. Guo, Y. Liu, S. Liu, S. Yao, Electrochemical study of breast cancer cells MCF-7 and its application in evaluating the effect of diosgenin, Anal. Sci. 21 (2005) 561–564.

Highlights

- Synthesis of steroid containing 5Z,9Z-dienoic acids have been developed.
- New molecules are efficient apoptosis inducers in cancer cells.
- The structures of all novel compounds were confirmed by NMR measurements.

Graphical Abstract

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Catalytic cyclometallation in steroid chemistry V: Synthesis of hybrid molecules based on steroid oximes and (5Z,9Z)-tetradeca-5,9-dienedioic acid as potential anticancer agents

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