Journal of the Serbian Chemical Society
JSCS@tmf.bg.ac.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. *87* (9) S299–S306 (2022) *Supplementary material*

SUPPLEMENTARY MATERIAL TO

Apoptosis induction in HeLa cervical cancer cell line by steroidal 16,17-seco-16,17*a*-dinitriles

LIDIJA D. ALEKSIĆ[1], ANDREA R. NIKOLIĆ[2#], VESNA V. KOJIĆ[1], MARIJA. N. SAKAČ[2] and SUZANA S. JOVANOVIĆ-ŠANTA[2*#]

[1]*Oncology Institute of Vojvodina, Faculty of Medicine, University of Novi Sad, Put Doktora Goldmana 4, 21204 Sremska Kamenica, Serbia and* [2]*University of Novi Sad, Faculty of Sciences, Department of Chemistry, Biochemistry and Environmental Protection, Trg Dositeja Obradovica 3, Novi Sad, Serbia*

J. Serb. Chem. Soc. *87* (9) (2022) 969–981

EXPERIMENTAL

Experimental conditions, yields and spectral characterisation of novel compounds ***4*** *and* ***9*** *(3*E*)- and (3Z)-hydroxyimino-16,17-secoandrost-4-ene-16,17*a*-dinitrile (****4a*** *and* ***4b****)*

To a solution of compound **3** (0.1 g, 0.32 mmol) in ethanol (16 mL), sodium acetate (34 mg, 0.41 mmol) and hydroxylamine-hydrochloride (37 mg, 0.53 mmol) were added and the reaction mixture was stirred at 70 °C for 2 h. Then, the mixture was poured into water (10 mL) and extracted with dichloromethane (3×10 mL). The combined organic extracts were dried and evaporated. The crude product was purified by column chromatography (toluene – acetone, 20:1) affording mixture of 3*E*- and 3*Z*- isomers of compound **4** (94.4 mg, 90 %, mp 210-212 °C after recrystallization from *n*-hexane – acetone) in the form of white solid.

IR (KBr, cm^{-1}): 3195, 3019, 2942, 2857, 2244, 1637, 1434, 1390, 962, 754.

^{1}H NMR (250 MHz, DMSO-D6, δ): 0.95 (s, 3H, CH_3); 1.00 (s, 3H, CH_3); 5.73 (s, 1H, H-4, *E*-isomer); 6.36 (s, 1H, H-4, *Z*-isomer); 10.27 (s, 1H, NOH, *Z*-isomer); 10.54 (s, 1H, NOH, *E*-isomer).

^{13}C NMR (62.5 MHz, DMSO-D6, δ): 15.23 (C-15); 17.72 (CH_3-18); 17.91 (CH_3-19); 18.49 and 20.08; 20.28 (CH_2); 29.51 (CH_2); 31.45 and 31.78 (CH_2); 31.97 and 32.60; 34.07; 35.76 and 36.23; 36.30 (CH); 36.43; 45.12 (CH); 52.04 and 52.28 (CH); 110.81 and 117.97 (C-4); 118.76 (CN); 120.34 (CN); 151.01 and 151.61 (C-5); 154.16 and 155.43 (C-3).

* Corresponding author. E-mail: suzana.jovanovic-santa@dh.uns.ac.rs
# Serbian Chemical Society member.
https://doi.org/10.2298/JSC210723035A

*3*β*-Hydroxy-6-oxo-16,17-secoandrost-4-ene-16,17*a*-dinitrile (**9**)*

To the solution of **8** (0.11 g, 0.34 mmol) in methanol (5 mL) cobalt (II)-chloride hexahydrate (0.06 g, 0.25 mmol) was added, then sodium borohydride (0.04 g, 0.10 mmol), portionwise, in couple of minutes. After reaction completion (15 minutes at room temperature) reaction mixture was neutralized with hydrochloric acid (1:1) and methanol was evaporated. Residue was dissolved in ethyl acetate (10 mL), washed with water (10 mL) and saturated sodium chloride solution (10 mL), then dried and evaporated to dryness. Crude product was recrystalized from *n*-hexane – acetone mixture, affording amorphous compound **9** (0.07 g, 64 %).

IR (film, cm^{-1}): 3480, 2949, 2928, 2870, 2855, 2244, 1673, 1612, 1400, 1259.

^{1}H NMR (250 MHz, DMSO-D6, δ): 0.90 and 0.94 (s, 3H, H-18 and H-19); 4.06 (m, 1H, H-3); 5.03 (d, 1H, *J*=5.98, OH); 6.05 (s, 1H, H-4).

^{13}C NMR (62.5 MHz, DMSO-D6, δ): 14.74 (C-15); 17.85 (CH_3-18); 19.32; 19.37 (CH_3-19); 27.76; 29.25; 33.86; 34.21 (CH); 36.16; 37.09; 37.15; 44.61; 45.07 (CH); 48.75 (CH); 65.30 (C-3); 118.50 (CN); 119.97 (CN); 135.04 (C-4); 143.52 (C-5); 200.19 (C-6).

HRMS (TOF) *m*/*z*: $C_{20}H_{26}N_2O_2$: calculated for $[C_{20}H_{26}N_2O_2+H]^+$ 327.20670, observed 327.20618.

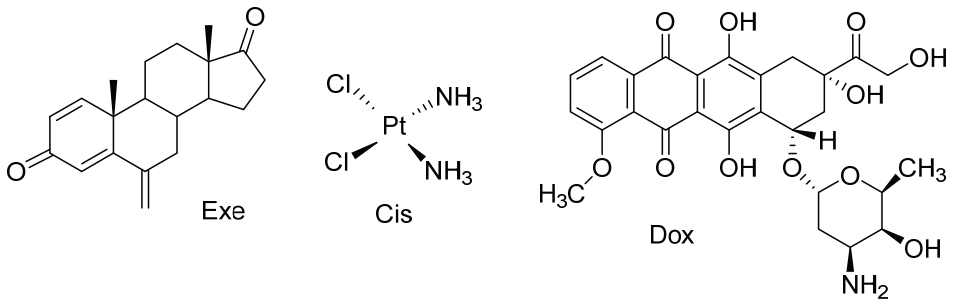


Fig. S-1 Structures of the reference compounds - exemestane (Exe), cisplatin (Cis) and doxorubicin (Dox)

Dot plots, tables and distribution of the cells, referring the results of flowcytometric analysis

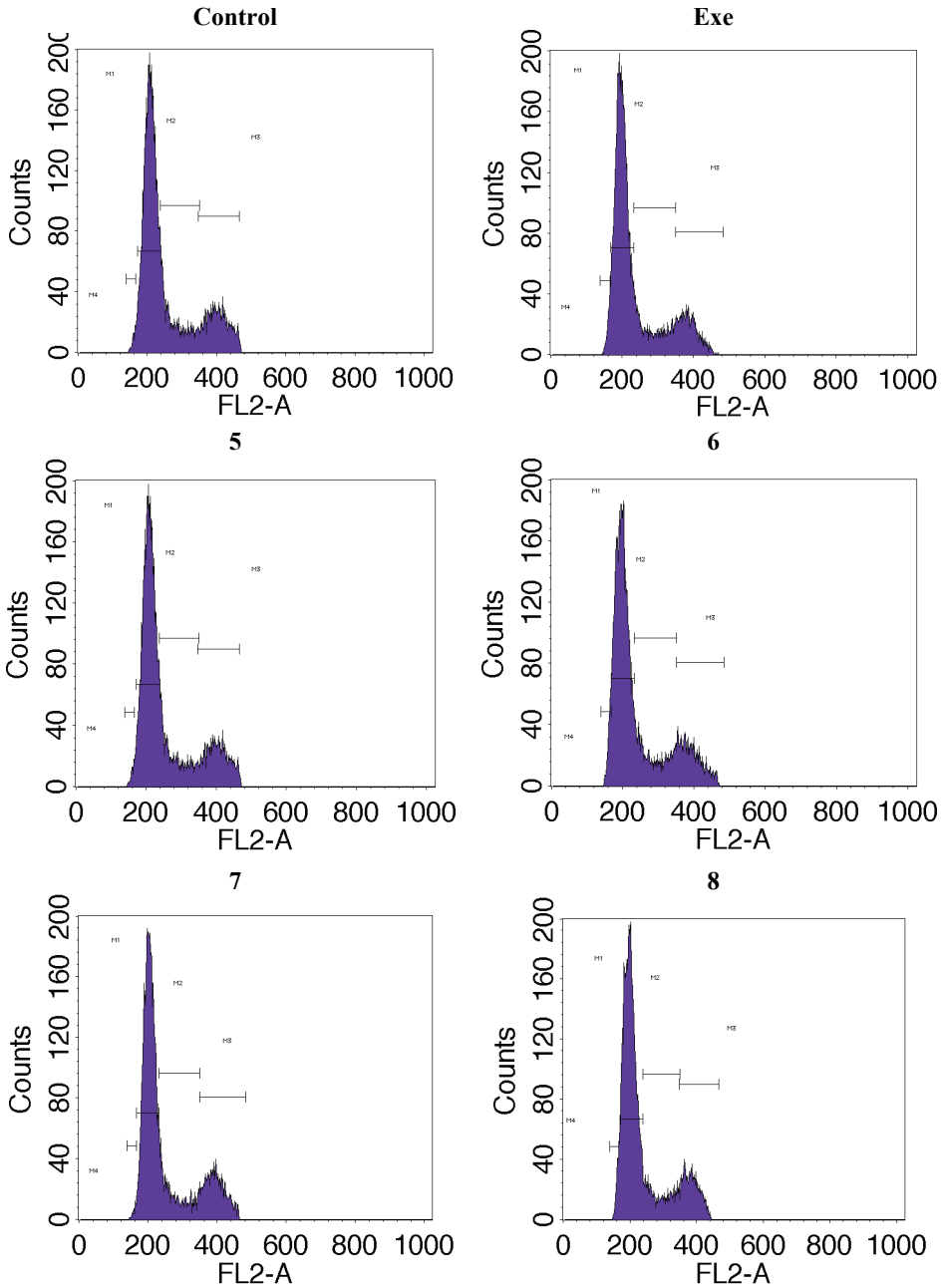


Figure S-2. Flow cytometric analysis of cell cycle distribution of control and HeLa cells treated with compounds **5**–**8** or Exemestane; The one-parameter histogram represents the ratio

of FL2-A peak area of fluorescent cells (*x*-axis) to intensity (counts, *y*-axis), where M_1-M_4 fractions correspond to G0/G1, S, G2/M and subG1 cell cycle phases.

TABLE S-1 Cell cycle distribution of control and HeLa cells treated with compounds **5**–**8** or Exemestane

Compound	Cell cycle distribution, %			
	HeLa, 72 h			
	sub G1	G0/G1	S	G2/M
Control	0.27±0.17	76.71 ± 0.85	11.87 ± 0.37	11.16 ± 1.05
5	3.33± 2.14	63.96 ± 0.45	11.83 ± 5.25	20.9 ± 3.56
6	2.72± 0.96	66.35 ±4.13	12.83 ± 4.97	18.1 ± 1.80
7	1.14±0.34	62.24 ± 2.37	17.23 ± 0.79	19.4 ± 1.25
8	1.78± 1.03	66.3 ± 0.35	11.18 ±3.96	20.7 ± 5.54

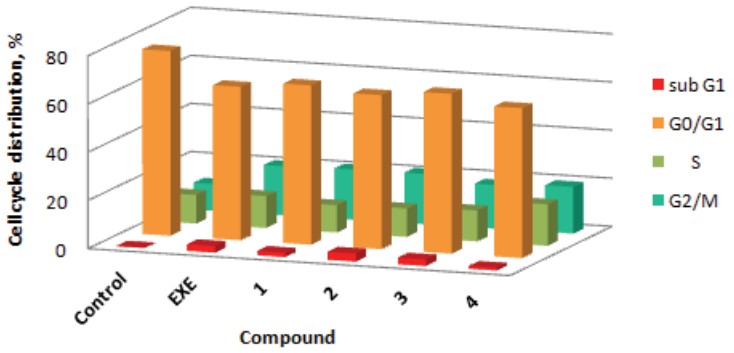


Fig. S-3 Cell cycle distribution of HeLa cells after 72 h treatment with steroidal 16,17-seco-16,17*a*-dinitriles and exemestane

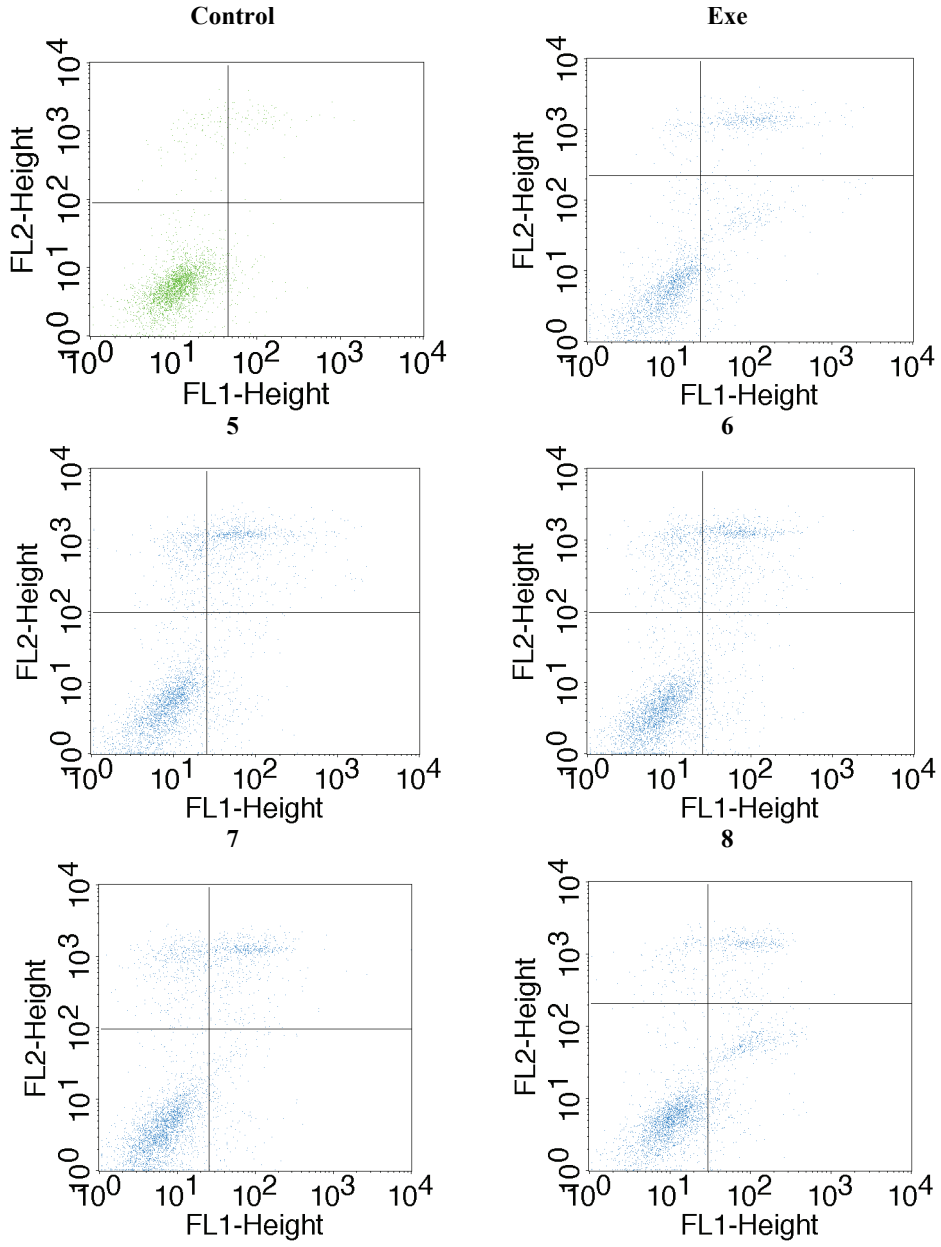
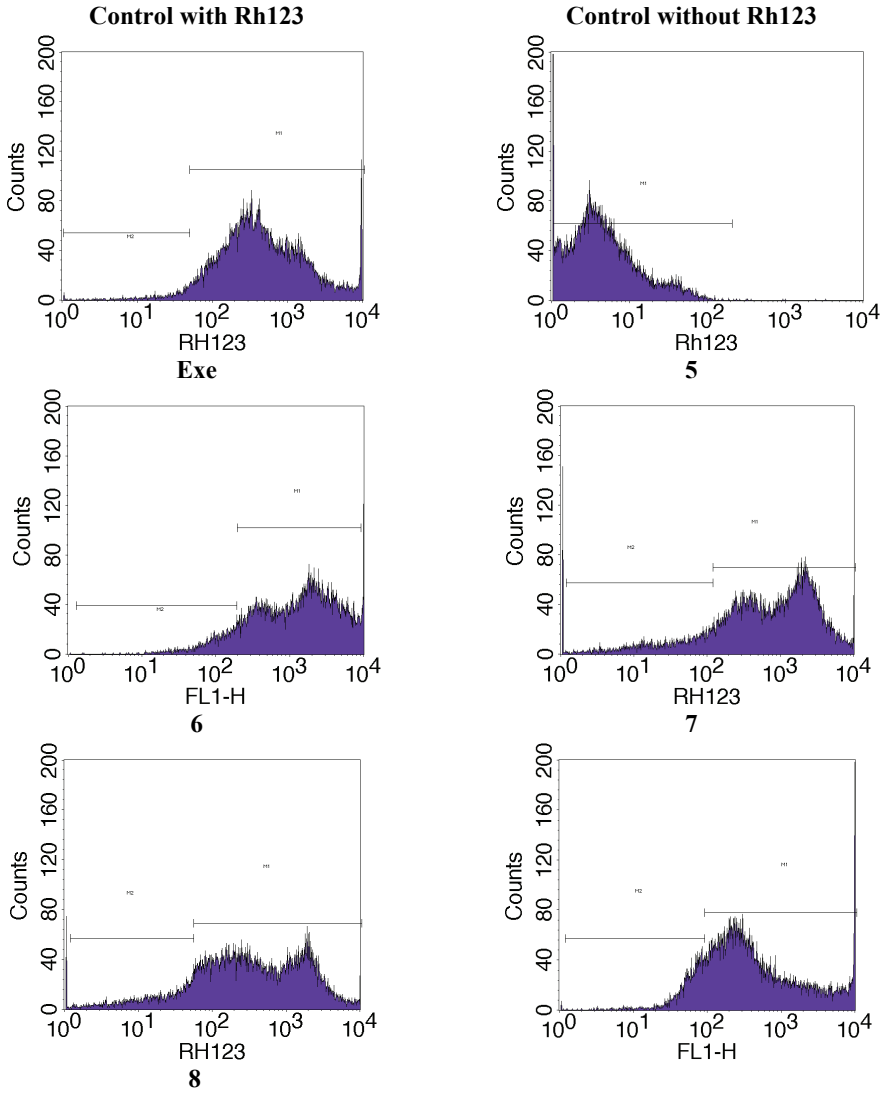


Figure S-4. Flow cytometric quantification of apoptosis and necrosis in control and HeLa cells treated with compounds **5-8** or Exemestane; Dot plots represent portions of apoptotic (lower and upper right quadrant for early and late apoptosis), necrotic (upper left quadrant) or live cells (lower left quadrant) detected with Annexin V flowcytometric test, where FL1 and FL2

parameters correspond to detected Annexin-V-FITC-positive and PI-positive cells, respectivelly.

TABLE S-2 Content of apoptotic and necrotic in control and HeLa cells treated for 72 h with compounds **5**-**8** or Exemestane, estimated by flow cytometry

Compound	Content of cells, %			
	Normal	Early apoptosis	Late apoptosis	Necrosis
5	41.69 ±12.93	11.07 ±0.93	40.21 ±21.63	7.03±3.11
6	46.39 ±29.94	20.99 ± 1.96	22.31 ± 16.62	10.31±1.77
7	53.14 ±15.13	11.16 ± 6.29	25.9 ±10.44	9.8±1.59
8	49.28±22.32	16.13 ± 0.3	29.47 ±18.82	4.52±0.2
Control	92.56 ± 0.4	2.61± 0.84	1.59±0.84	3.24±0.27
Exe	57.53 ± 9.09	12.98± 0.19	25.68±8.77	3.5±0.76


Control with Rh123
Control without Rh123
Counts
RH123
Rh123
FL1-H
Exe
5
6
7
8

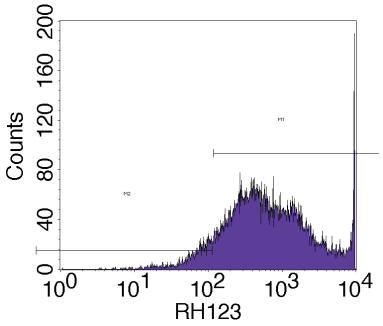


Figure S-5. Flow cytometric analysis of mitochondrial membrane potential of HeLa cells after 72 h treatment with compounds **5**–**8** or exemestane; The one-parameter histogram represents the ratio of the peak area of rhodamine 123-based fluorescent cells (*x*-axis) to intensity (counts, *y*-axis), where M_1 and M_2 fractions correspond to cells with intact mytochondrial membrane integrity, and cells with membrane integrity loss, respectively.

TABLE S-3 Content of cells with membrane integrity loss in control and HeLa cells treated with compounds **5**-**8** or Exemestane, estimated by flow cytometry

Compound	Contribution of cells with membrane integrity loss, %
Control	6.87 ± 3.54
5	15.08 ± 0.19
6	14.49 ± 0.16
7	14.43 ± 1.16
8	10.98 ± 2.19
Exe	14.16 ± 3.92

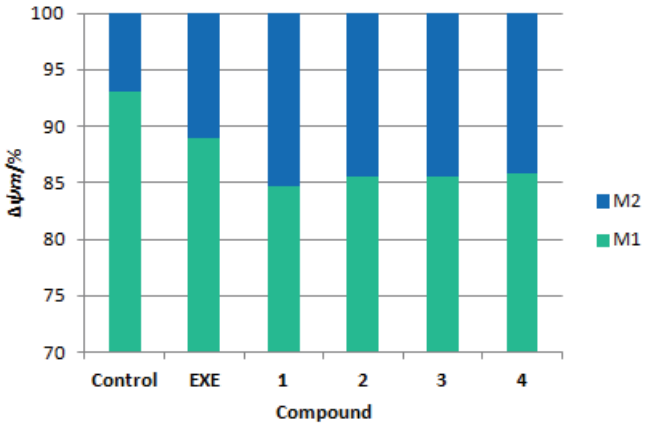


Fig. S-6 Mitochondrial membrane potential of HeLa cells after 72 h treatment with steroidal 16,17-seco-16,17*a*-dinitriles and exemestane; M1 - cells with intact mytochondrial membrane integrity, M2 - cells with membrane integrity loss.