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Synthesis and biological evaluation of a series of A,B-ring modified 16,17-secoandrostane derivatives

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Abstract

The starting compound for the synthesis of 16,17-secoandrostane derivatives with the 4-en-3-on, 1,4-dien-3-on, 4,6-dien-3-on, and 1,4,6-trien-3-on systems was 3β-hydroxy-17-methyl-16,17-secoandrost-5-en-16-nitrile-17-one (**1**), the Oppenauer oxidation of which yielded the corresponding 4-en-3-one derivative **2**. Dehydrogenation of compound **2** with the aid of 2,3,5,6-tetrachloro-1,4-benzoquinone (chloranil) gave the three products: 17-methyl-16,17-secoandrosta-1,4-dien-3,17-dione-16-nitrile (**3**), 17-methyl-16,17-secoandrosta-4,6-dien-3,17-dione-16-nitrile (**4**), and 17-methyl-16,17-secoandrosta-1,4,6-trien-3,17-dione-16-nitrile (**5**). On the other hand, epoxidation of compound **2** resulted in a mixture of α and β isomers of 4,5-epoxy-17-methyl-16,17-secoandrosta-3,17-dione-16-nitrile (**6** and **7**). Opening of the oxirane rings of the mixture of **6** and **7** by the action of formic acid yielded the 4-hydroxy-4-en derivative **8**. Antiaromatase activity and in vitro cytotoxicity against three tumor cell lines (human breast adenocarcinoma ER+, MCF-7, human breast adenocarcinoma ER−, MDA-MB-231, and prostate cancer PC3) of selected compounds were evaluated. Compound **2** exhibited a relatively strong inhibition of aromatase and extremely potent cytotoxicity against PC3 cells. Compound **8** showed satisfactory cytotoxicity against MCF-7 cells.

Keywords: 16,17-Secosteroids; Androstane derivatives; In vitro cytotoxicity; Aromatase inhibitors

1. Introduction

There is compelling evidence that estrogens both increase risk of developing breast cancer and maintain the growth and progression of established tumors [1]. Recently, drugs have been developed which specifically and potently block estrogen biosynthesis—aromatase inhibitors. Namely, there are two mechanisms whereby estrogen may cause breast cancer: via the estrogen receptor (ER) and via metabolism of estrogen. Anti-estrogens block only ER-mediated events, whereas aromatase inhibitors block both ER and metabolic pathways. Aromatase inhibitors occupy now a central role in the treatment of postmenopausal patients with breast cancer, and are being considered as preventative agents in women at high risk of the disease [2]. Furthermore, these inhibitors have also been shown to be clinically relevant in the management of other estrogen-dependent pathological processes such as endometriosis [3], prostatic hyperplasia, and prostate cancer [4]. Aromatase inhibitors can be subdivided into steroidal and non-steroidal agents. Among steroidal agents the formestane was used widely during the early 1990s, but it is not used often nowadays because of the need to administer it by intramuscular injection [5]. So, the orally active exemestane (Fig. 1) is the main steroidal inhibitor of contemporary importance [6].

In our previous papers we described the synthesis of some 16,17-seco-estrane and androstane derivatives

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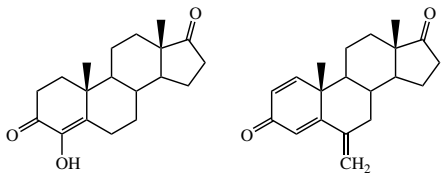


Formestane **Exemestane**

Fig. 1. Potent steroidal aromatase inhibitors.

[7–10]. Some of them exhibited certain anti-estrogen [7] or potent antiaromatase [9] effect. To continue our investigation of D-secoandrostane derivatives as aromatase inhibitors with the objective of studying the effect of the conjugated systems of double bonds in the A and B rings on antiaromatase activity, in this work we synthesized three compounds having the same 17-methyl-16,17-seco-16-nitrile-17-one moiety, with 1,4-dien-3-on, 4,6-dien-3-on and 1,4,6-trien-3-on systems, respectively. These compounds have a cyano function, which is also present in the non-steroidal aromatase inhibitors letrozole [11] and anastrozole [12]. Besides, we also synthesized a D-seco compound with the 4-hydroxy-4-en-3-one system (like formestane), having also in its structure a cyano function. In addition to studying antiaromatase activity, cytotoxicity of selected compounds was tested against three tumor cell lines: human breast adenocarcinoma ER+, MCF-7, human breast adenocarcinoma ER−, MDA-MB-231, and prostate cancer PC3.

2. Materials and methods

Melting points were determined using a Büchi SMP 20 apparatus and are uncorrected. The infrared spectra (wave numbers in cm^{-1}) were taken on a Nexus 670 FT-IR spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AC 250 NMR apparatus operating at 250 MHz (proton) and 62.9 MHz (carbon), using standard Bruker software; the tetramethylsilane peak (δ 0.00) was used as reference for ^{1}H NMR, whereas the central carbon line of chloroform-d was set at 77.0 ppm for ^{13}C NMR. Mass spectral assays were taken on a Finnigan MAT 8230 instrument, using chemical ionization (isobutane) technique. Organic solutions were dried over Na_2SO_4 and evaporated on a rotary evaporator under reduced pressure. Column chromatography was performed on Merck grade 60 silica gel (0.063–0.2 mm). Flash column chromatography was performed on Merck grade 60 silica gel (0.04–0.063 mm). All reagents used were of analytical reagent grade.

2.1. Chemical synthesis

2.1.1. 17-Methyl-16,17-secoandrosta-1,4-dien-3,17-dione-16-nitrile (3), 17-methyl-16,17-secoandrosta-4,6-dien-3,17-dione-16-nitrile (4), and 17-methyl-16,17-secoandrosta-1,4,6-trien-3,17-dione-16-nitrile (5)

The mixture of compound **2** (0.13 g, 0.42 mmol), *p*-toluenesulfonic acid (3.6 mg, 0.02 mmol), and 2,3,5,6-tetrachloro-1,4-benzoquinone (chloranil) (0.15 g, 0.16 mmol) in xylene (16 mL) was refluxed during 2 h. After cooling, xylene was evaporated and residue was separated by column chromatography on silica gel (14 g, toluene–ethyl acetate, 5:1) to give compounds **3** [9] (0.028 g, 22%) and **4** (0.04 g, 31%) as yellow oils, and **5** (0.044 g, 34%, mp 151–153 °C, lit. [9] mp 151–153 °C).

Compound **4**: IR (film): 2260, 1700, 1670, 1620, 1580, 1440, 1360, 1270, 1220, 1100, 880, 750. ^{1}H NMR ($CDCl_3$): 1.16 (s, 3H, H-19); 1.38 (s, 3H, H-18); 2.20 (s, 3H, CH_3, C-20); 5.73 (s, 1H, H-4); 6.21 (d, 1H, H-6, $J_{6,7} = 10.5$ Hz); 6.26 (dd, 1H, H-7, $J_{6,7} = 10.5$ Hz, $J_{7,8} = 2.1$ Hz). ^{13}C NMR ($CDCl_3$): 15.5 (C-18); 16.3 (C-19); 17.9; 19.3; 25.2 (C-20); 33.6; 33.7; 36.0; 36.2; 37.4; 38.9; 49.2; 52.1; 118.6 (CN); 124.3 (C-4); 129.5 (C-6); 136.2 (C-7); 161.5 (C-5); 199.0 (C-3); 212.2 (C-17). CI MS: *m/z* 312 (MH^+).

2.1.2. 4α,5α-Epoxy-17-methyl-16,17-secoandrosta-3,17-dione-16-nitrile (6) and 4β,5β-epoxy-17-methyl-16,17-secoandrosta-3,17-dione-16-nitrile (7)

Compound **2** (0.35 g, 1.1 mmol) was dissolved in methanol (10 mL), cooled to 0 °C, and NaOH (4 M, 0.9 mL) and H_2O_2 (30%, 1.4 mL) were added. Reaction mixture was left at −15 °C for 1 h, then was poured into water (100 mL) and extracted with ethyl acetate. The organic layer was separated, dried and evaporated to dryness. Column chromatography of the crude product on silica gel (35 g; toluene–acetone, 3:1) yielded the mixture of compounds **6** and **7** (0.23 g; 63%; ratio **6**:**7** = 1:1.7). The recrystallization from benzene afforded only compound **7** as colorless crystals, mp 225–227 °C.

Compound **7**: IR: 2975, 2241, 1712, 1693, 1466, 1451, 1420, 1387, 1249, 1144, 1075, 835. ^{1}H NMR ($CDCl_3$): 1.19 (s, 3H, H-19); 1.33 (s, 3H, H-18); 2.18 (s, 3H, CH_3, C-20); 3.03 (s, 1H, H-4). ^{13}C NMR ($CDCl_3$): 15.6 (C-18); 18.1 (C-15); 18.8 (C-19); 20.2; 25.3 (C-20); 26.0; 29.3; 29.7; 32.4; 35.1; 36.4; 37.2; 41.2; 45.3; 51.8; 62.4 (C-4); 69.5 (C-5); 118.8 (CN); 205.8 (C-3); 212.6 (C-17). CI MS: *m/z* 330 (MH^+). Anal. Calcd for $C_{20}H_{27}NO_3$: C, 72.92; H, 8.26; N, 4.25. Found: C, 73.09; H, 8.42; N, 4.33.

2.1.3. 4-Hydroxy-17-methyl-16,17-secoandrost-4-en-3,17-dione-16-nitrile (8)

Compound **2** (0.136 g, 0.4 mmol) was refluxed in formic acid (1.85 mL) for 2.5 h. After cooling the reaction mixture was poured into water (70 mL) and 5% $NaHCO_3$ was added to pH 7. The extractive work up with ethyl acetate gave crude product as a brown oil, whose purification by flash chromatography (toluene–ethyl acetate, 4:1) gave the pure compound **8** (0.027 g; 20%) as a colorless oil.

IR (film): 3430, 2945, 2244, 1712, 1700, 1388, 1358, 734. ^{1}H NMR ($CDCl_3$): 1.22 (s, 3H, H-19); 1.36 (s, 3H, H-18); 2.16 (s, 3H, CH_3-C17); 3.10 (m, 1H, H-15a); 6.10 (s, 1H, OH). ^{13}C NMR ($CDCl_3$): 15.7; 17.3; 18.1; 19.6; 22.7; 25.3; 30.1; 31.6; 34.4; 35.1; 36.5; 37.8; 41.1; 51.7; 52.8; 118.9 (CN); 137.8 (C-5); 141.2 (C-4); 193.2 (C-3); 213.0 (C-17). CI MS: *m/z* 330 (MH^+).

2.2. Biological methods

All experiments were approved by the Local Ethical Committee of the University of Novi Sad and were performed in accordance with the principles and procedures of the NIH Guide for Care and Use of Laboratory Animals.

2.2.1. Antiaromatase activity

2.2.1.1. Chemicals. Anti-estradiol serum no. 244 was kindly supplied by Dr. G.D. Niswender (Colorado State University, CO, USA). Pregnant Mares Serum Gonadotrophin (PMSG) was supplied by the Veterinary Institute Subotica (Serbia). [1,2,6,7-^{3}H(N)] Estradiol was obtained from New England Nuclear (Belgium). NADPH and testosterone were from Sigma (St. Louis, MO). All other chemicals were of analytical reagent grade.

2.2.1.2. Animals (female rats), treatment, and assays. Preparation of denucleated ovarian fraction from PMSG pretreated rats and the determination of aromatase activity in ovarian homogenate were carried out as described previously [9].

For a preliminary assessment of potential antiaromatase activity of the synthesized compounds, compound **8** and formestane were added in concentrations of 1 μM and 50 μM to the incubation mixture containing 50 nM of testosterone as a substrate (subsaturated concentration) and 50 μM of compounds **1**–**5** and 500 nM of testosterone (saturated concentration).

2.2.1.3. Statistics. The statistical significance was evaluated by two-tailed non-parametric Mann–Whitney test.

2.2.2. Antiproliferative activity

2.2.2.1. Cell lines. Three human tumor cell lines and one human non-tumor cell line were used in the study: human breast adenocarcinoma ER+, MCF-7, human breast adenocarcinoma ER−, MDA-MB-231, prostate cancer PC3 and normal fetal lung fibroblasts, MRC-5.

The cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5% of glucose. Media were supplemented with 10% of fetal calf serum (FCS, NIVNS) and antibiotics: 100 IU/mL of penicillin and 100 μg/mL of streptomycin (ICN, Galenika). All cell lines were cultured in flasks (Costar, 25 cm^2) at 37 °C in the 100% humidity atmosphere and 5% of CO_2. Only viable cells were used in the assay. Viability was determined by dye exclusion assay with trypan blue.

2.2.2.2. SRB assay. Cytotoxicity was evaluated by colorimetric sulforhodamine B (SRB) assay after Skehan et al. [13]. Briefly, single cell suspension was plated into 96-well microtitar plates (Costar, flat bottom): 5×10^3 cells (MCF-7; MDA-MB-231; PC3; MRC-5), per 180 mL of medium. Plates were pre-incubated for 24 h at 37 °C, 5% CO_2. Tested substances at concentrations ranging from 10^{-8} to 10^{-4} M were added to all wells except for the controls. After incubation period (48 h/37 °C/5% CO_2) SRB assay was carried out as follows: 50 μL of 80% trichloroacetic acid (TCA) was added to all wells; an hour later the plates were washed with distilled water, and 75 μL of 0.4% SRB was added to all wells; half an hour later the plates were washed with citric acid (1%) and dried at room temperature. Finally, 200 μL of 10 mmol Tris (pH 10.5) was added to all wells. Absorbance (A) was measured on the microplate reader (Multiscan MCC340, Labsystems) at 540/690 nm. The wells without cells, containing complete medium only, served as blank.

Cytotoxicity was calculated according to the formula:

$$(1 - A_{\mathrm{TEST}}/A_{\mathrm{CONTROL}}) \times 100$$

and expressed as a percent of cytotoxicity (CI%).

2.2.2.3. Data analysis. Two independent experiments were set out in quadruplicate for each concentration of the compound. IC_{50} value defines the dose of compound that inhibits cell growth by 50%. The IC_{50} of compounds was determined by Median effect analysis.

3. Results and discussion

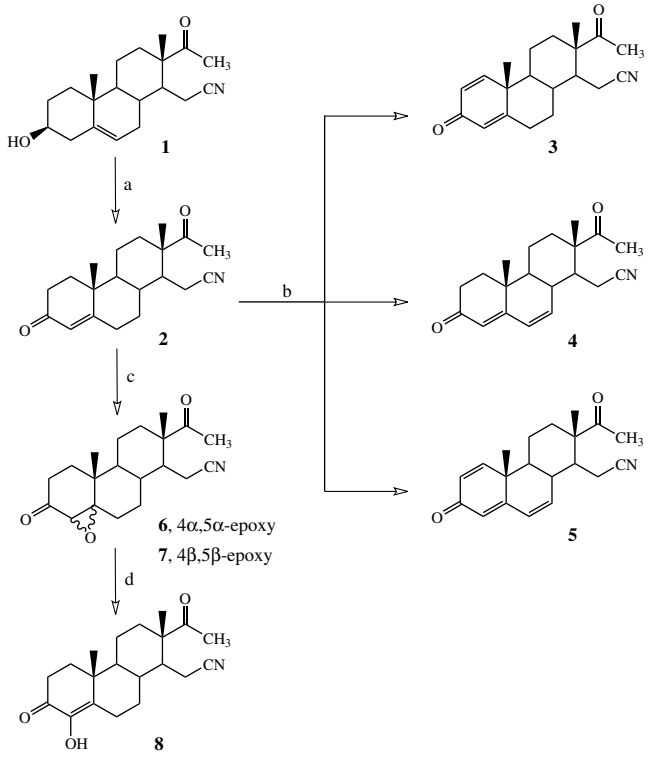
3.1. Chemistry

The starting compound for the synthesis of 16,17-secoandrostane derivatives with the 4-en-3-on, 1,4-dien-3-on, 4,6-dien-3-on, and 1,4,6-trien-3-on systems was 3β-hydroxy-17-methyl-16,17-secoandrost-5-en-16-nitrile-17-one (**1**). The Oppenauer oxidation of this compound yielded the corresponding 4-en-3-one derivative **2** (Scheme 1). Compounds **1** and **2** were obtained according to earlier described procedures [9]. Dehydrogenation of compound **2** with the aid of chloranil, in the presence of *p*-toluenesulfonic acid in boiling xylene, gave the following three products: 17-methyl-16,17-secoandrosta-1,4-dien-3,17-dione-16-nitrile (**3**), 17-methyl-16,17-secoandrosta-4,6-dien-3,17-dione-16-nitrile (**4**), and 17-methyl-16,17-secoandrosta-1,4,6-trien-3,17-dione-16-nitrile (**5**).

The epoxidation of compound **2** with 30% hydrogen peroxide and aqueous solution of sodium hydroxide in methanol at −15 °C resulted in a mixture of α and β isomers of 4,5-epoxy-17-methyl-16,17-secoandrosta-3,17-dion-16-nitrile (**6** and **7**) (Scheme 1). Recrystallization from benzene afforded the β isomer, the other isomer remaining in the mother liquor as an oil. The opening of the oxirane rings of compounds **6** and **7** was performed with the aid of formic acid at the boiling temperature of the reaction mixture. The result was the 4-hydroxy-4-en derivative **8**.

3.2. Biological properties

Results of the biological tests indicate the effect of conjugation in the A and/or B rings of the D-secoandrostane



Scheme 1. Reagents and reaction conditions: (a) Al(*t*-BuO)$_3$, cyclohexanone, reflux, 2 h; (b) Chloranil, *p*-TsOH, xylene, reflux, 2 h; (c) 30% H_2O_2, 4 M NaOH, MeOH, −15 °C, 1 h; (d) HCOOH, reflux, 2.5 h.

derivatives **1-5** on aromatase inhibition. As can be seen from Table 1, the conjugated 4-en-3-on system (compound **2**) contributes to stronger inhibitory activity (100%) compared to its precursor, 3β-hydroxy-5-en derivative **1** (79.2%). Expanding conjugation in the B ring gives the 4,6-dien-3-on derivative **4**, which also shows a stronger inhibitory effect (95.0%) than that of compound **1**. However, the presence of the 1,2-double bond in the ring A, as is the case with the 1,4-dien-3-on analog (**3**) and 1,4,6-trien-3-on analog (**5**), causes a decrease in the inhibitory activity (66.9% for both compounds). This means that the introduction of a double bond in the Δ^1 position of both 4-en-3-on- and 4,6-dien-3-on-16,17-secoandrostane derivatives has an unfavorable effect on the resulting antiaromatase activity.

In Table 2 are given the IC_{50} values for compounds **1**, **2**, **4** and **5**, as well as for the known non-steroidal inhibitor of aromatase, aminoglutetimide (AG). It is evident that the activity of compound **2** ($IC_{50} = 0.42$ μM) is 13 times higher than of its precursor **1** ($IC_{50} = 5.45$ μM), whereas compound **4** ($IC_{50} = 0.22$ μM) is twice as active as compound **2,** and even 76 times more active than compound **5**. The IC_{50} of 4,6-dien-3-on (**4)** is only 1.6 times higher than that of aminoglutetimide ($IC_{50} = 0.14$ μM).

Compound **8** was also tested on antiaromatase activity and the results were compared with those obtained with formestane (Table 3).

Table 1
Inhibitory effects of compounds **1**–**5** on the aromatase activity in the denucleated fraction of ovaries from PMSG pretreated rats

Compound	Aromatase activity (percent of inhibition vs. control)
1 (5-en-3-ol)	$79.2 \pm 3.8^*$
2 (4-en-3-one)	100^*
3 (1,4-dien-3-one)	$66.9 \pm 4.9^*$
4 (4,6-dien-3-one)	$95.0 \pm 2.8^*$
5 (1,4,6-trien-3-one)	$66.9 \pm 2.7^*$

Purified denucleated fraction of ovaries from PMSG pretreated female rats was incubated in the medium with saturated (0.5 μM) solution of substrate testosterone and NADPH (1 mM) and in the absence (control) or presence of different androst-5-ene derivatives (50 μM) for measuring aromatase activity. Estradiol level was determined by RIA. Results are presented as percent of inhibition vs. control. Numbers represent mean ± SEM of six replicates. Significance: $^*p < 0.005$ vs. control (Mann–Whitney non-parametric test).

Table 2
In vitro aromatase inhibition by androstene derivatives and AG

Compound	IC_{50} (μM)
1 (5-en-3-ol)	5.45
2 (4-en-3-one)	0.42
4 (4,6-dien-3-one)	0.22
5 (1,4,6-trien-3-one)	16.84
AG	0.14

Purified denucleated fraction of ovaries from PMSG pretreated female rats was incubated for 15 min. at 37 °C in the presence of saturated (0.5 μM) concentration of testosterone and NADPH (1 mM) in the absence (control) or presence of five different concentrations of compounds **1**, **2**, **4**, **5** or AG. Estradiol level was determined by RIA. IC_{50} was calculated from least-squares lines.

Table 3
Inhibitory effects of compound **8** and formestane on the aromatase activity in the denucleated fraction of ovaries from PMSG pretreated rats

Compound	Aromatase activity (percent of inhibition vs. control)	
	1 μM	50 μM
8	$45.6 \pm 2.3^*$	$111.3 \pm 3.6^*$
Formestane	$101.7 \pm 2.0^*$	$117.9 \pm 1.1^*$

Purified denucleated fraction of ovaries from PMSG pretreated female rats was incubated in the medium with subsaturated (0.05 μM) concentration of substrate testosterone and NADPH (1 mM) and absence (control) or presence of compound **8** or formestane (1 μM or 50 μM). Estradiol level was determined by RIA. Results shown are percent of inhibition vs. control. Numbers represent mean ± SEM of 10 replicates. Significance: $^*p < 0.005$ vs. control (Mann–Whitney non-parametric test).

Table 4
In vitro cytotoxicity of compounds **2**, **7**, **8**, and formestane

Compound	IC_{50} (μM)			
	MCF-7	MDA-MB-231	PC3	MRC-5
2	54.8	27.2	0.62	>100
7	66.7	30.2	25.9	>100
8	27.8	40.0	>100	>100
Formestane	>100	55.5	48.4	>100

Like formestane, in the presence of a subsaturation dose of testosterone, compound **8** showed total inhibition of aromatase at the concentration of 50 μM. However, at the concentration of 1 μM, the inhibitory activity was significantly weaker, about 45.6%. It is clear that the modification of the formestane D-ring to obtain a 16,17-seco system resulted in a decrease of antiaromatase activity. A similar effect was also observed in the case of D-homo derivatives of formestane [14]. These results indicate the advantage of the original steroidal cyclopentane D-ring in the formestane.

The synthesized compounds **2**, **7** and **8** were evaluated for their antiproliferative activity against human breast adenocarcinoma ER+, MCF-7, human breast adenocarcinoma ER−, MDA-MB-231, and prostate cancer PC3, and normal fetal lung fibroblasts, MRC-5. In vitro cytotoxicity was evaluated after 48-h cells treatment by the SRB assay [13]. Formestane served as the reference compound. The results are presented in Table 4.

A moderate cytotoxicity against MCF-7 cells was observed for compound **8** ($IC_{50} = 27.8$ μM), which has the same structure in the A ring as formestane. A weak cytotoxicity against MCF-7 exhibited the precursors of compound **8**, that is the conjugated ketone **2** ($IC_{50} = 54.8$ μM) and epoxide **7** ($IC_{50} = 66.7$ μM). As can be seen from Table 4, compound **8**, i.e. the 4-hydroxy derivative of compound **2**, was twice as active as compound **2**. In the case of MDA-MB-231 cells, the results are different. Compared to the results obtained on the MCF-7 cells, compounds **2** ($IC_{50} = 27.2$ μM) and **7** ($IC_{50} = 30.2$ μM) were two fold more cytotoxic against the MDA-MB-231 cells. Moreover, compound **2** showed a remarkably strong cytotoxicity against prostate carcinoma PC3 cells ($IC_{50} = 0.62$ μM). A significantly weaker activity was observed for the epoxide **7** ($IC_{50} = 25.9$ μM), whereas compound **8** was inactive against PC3 cells.

In conclusion, the 16,17-seco-4-en-3-on derivative **2** represents a relatively good inhibitor of aromatase ($IC_{50} = 0.42$ μM), and a remarkably potent cytotoxic agent against PC3 cells ($IC_{50} = 0.62$ μM). This compound is also active against MDA-MB-231 and MCF-7 cells, but showed certain selectivity with respect to PC3 cells. Compound **8** presented a moderate inhibitory activity against aromatase and was cytotoxic against MCF-7 cells.

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