17-Picolinylidene-substituted steroid derivatives and their antiaromatase and cytotoxic activity

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Abstract

Starting from 17(*Z*)-picolinylidene-androst-5-en-3β-yl acetate **1**, the new derivatives **4-8** were synthesized. By oxidation of 3β-hydroxy derivative **2** with *Jones* reagent the corresponding 17(*Z*)-picolinylidene-androst-4-ene-3,6-dione **4** was obtained. The *Oppenauer* oxidation of **2** yielded 4-en-3-one derivative **3**, which reacted with potassium-*t*-butoxide in *t*-butanol to give 4-ene-3,6-dione **4** and 4-hydroxy-4,6-dien-3-one **5** derivatives. Nitration of compound **1** afforded 6-nitro-5-ene derivative **6**. The reaction of compound **3** with $NaBH_4$ in ethanol afforded stereoselectively the 3β-hydroxy-4-ene derivative **7**, the acetylation of which gave 3β-acetoxy derivative **8** whereas 17-picolinylidene derivatives **9-14** have been synthesized earlier. Compounds **4-8** were tested on potential inhibitory activity against the enzyme aromatase. Satisfactory inhibitory activity showed compounds **4**, **5**, **7** and **8**.

Cytotoxicity *in vitro* against three tumor cell lines (human breast adenocarcinoma ER+, MCF-7 as well as human breast adenocarcinoma ER-, MDA-MB-231 and prostate cancer AR-, PC-3) and normal fetal lung fibroblasts, MRC-5, of compounds **1-14** was also evaluated. Strong cytotoxic activity showed compound **5** against MDA-MB-231 (IC_{50} 9.3 μM) and compound **7** against PC-3 (IC_{50} 10.1 μM). All compounds were not toxic to healthy MRC-5 cells.

Keywords: Androstane derivatives, picolinylidene derivatives, aromatase inhibition, cytotoxic activity

Introduction

Breast cancer is the most commonly diagnosed cancer among women and continues to be a major cause of cancer deaths.[1] There are several approaches for the therapy of breast cancer but the most effective way to treat hormone-dependent breast cancer is to deprive the cancer cells of

estrogens by inhibiting their biosynthesis.[2] The two most important identified risk factors for breast cancer are gender and age,[3] and more than 70% of women over the age of 50 with breast cancer do not have any other remarkable risk factor.

Aromatase, a P-450-dependent enzyme, catalyzes the ultimate step in estrogen biosynthesis that converts androgens to estrogens both in pre- and postmenopausal women.[4] While the main source of estrogen is the ovary in premenopausal women, the principal source of circulating estrogens in postmenopausal women is from the aromatization of adrenal and ovarian androgens to estrogens by the enzyme aromatase in peripheral tissues (muscle, body fat). Inhibition of aromatase is an important approach for reducing growth-stimulatory effects of estrogens. Effective aromatase inhibitors have been developed as therapeutic agents for controlling estrogen-dependent breast cancer.[5] Aromatase inhibitors, which were first reported in the 1970s, have been used in the clinic as second line drugs.[6,7] The third generation nonsteroidal aromatase inhibitors (letrozole, anastrozole) have shown considerable advances in the treatment of hormone-dependent breast cancer.[2,8] Among steroidal aromatase inhibitors, formestane (4-hydroxyandrost-4-ene-3,17-dione, 4-OHA - the second generation) and exemestane (third generation), have been approved for clinical use in the treatment of breast cancer in several countries.[8,9]

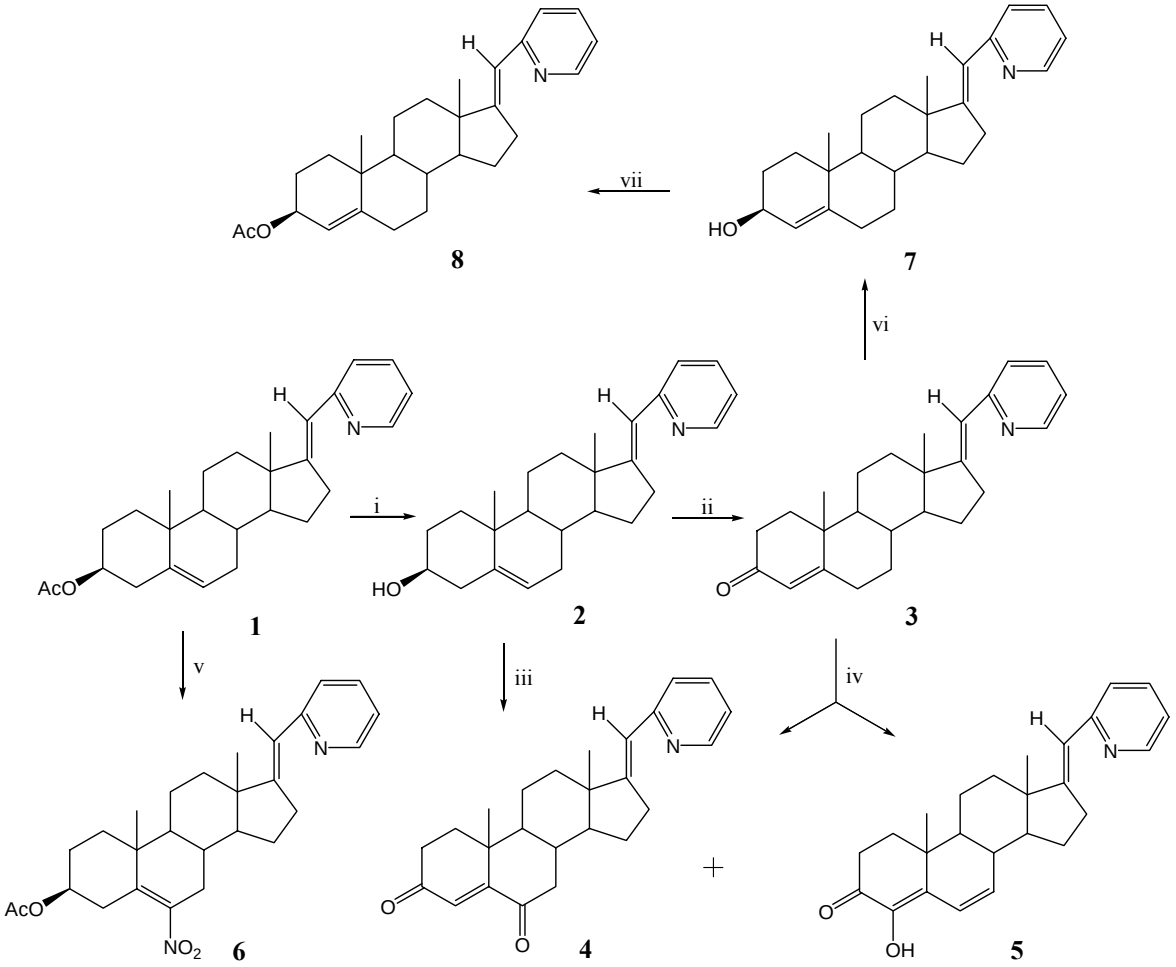
In our previous papers[10,11] we described the synthesis of some 17α-picolyl and 17-picolinylidene-androst-5-ene derivatives and their antiaromatase and antitumor activity against some tumor cell lines. Some tested androstane derivatives showed inhibitory activity against the enzyme aromatase, as well as strong activity against three tumor cell lines (human cervix carcinoma, HeLa, human melanoma, FemX, and human myelogenous leukemia, K562), the IC_{50} values being in the range of 4-10 μM.[10] These compounds were chosen because of presence of a nitrogen in a steroidal structure. It is well known[12] that heteroatom (usually a nitrogen) interferes with steroidal hydroxylation by binding with the iron atom of the hemoprotein of $P\text{-}450_{arom}$. This has been investigated in nonsteroidal inhibitors, such as anastrozole[13] and letrozole[14], two highly potent, competitive and reversibile aromatase inhibitors. Also, antitumor activity against three different tumor cell lines (human breast adenocarcinoma ER+, MCF-7, human breast adenocarcinoma ER-, MDA-MB-231, and prostate cancer AR-, PC-3) was evaluated. Several tested compounds showed strong activity against PC-3, the IC_{50} values being in the range of 0.55-10 μM, whereas 4β,5β-epoxy-17β-hydroxy-17α-picolyl-androstan-3-one showed strong activity against MDA-MB-231 (IC_{50} 10.4 μM).[11] For those reasons, as a continuation of our ongoing efforts concerning 17-picolinylidene-androst-5-ene derivatives, we report in this paper the synthesis of some new A and/or B modified 17(Z)-picolinylidene-androstane derivatives and their antiaromatase activity and cytotoxicity against three tumor cell lines (human breast adenocarcinoma ER+, MCF-7, as well as human breast adenocarcinoma ER-, MDA-MB-231 and prostate cancer AR-, PC-3), and normal fetal lung fibroblasts, MRC-5.

Results and Discussion

Chemistry

According to the known procedures,[15,16] starting compound **1** was prepared by addition of α-picolyllithium to the 17-oxo group of dehydroepiandrosterone, resulting in the 17α-picolyl derivative, which, by boiling in acetic anhydride was transformed into 17(Z)-picolinylidene-androst-5-en-3β-yl acetate **1**. Deprotection with alcoholic KOH gave 3β-hydroxy derivative **2**.[10] An efficient one-pot oxidation of compound **2** with *Jones* reagent at 0 ºC for 1.5 hour (Method A), afforded a new 17(Z)-picolinylidene-androst-4-ene-3,6-dione **4** in a yield of 26% (Scheme 1). The same reaction was carried out for a number of steroidal 5-en-3β-ols using a modified *Jones* oxidation methodology.[17] ^{1}H NMR data for compound **4** showed evidence that a singlet of the H-4 at 6.26 ppm appeared instead of the multiplet at 5.36 ppm (H-6) of compound **2**. In the ^{13}C NMR spectrum, two signals at 199.39 and 201.94 ppm corresponded to the C-3 and C-6 carbonyl atoms. The *Oppenauer* oxidation of compound **2** with cyclohexanone in the presence of aluminum-*i*-propoxide afforded 17(Z)-picolinylidene-androst-4-en-3-one **3**, which was described in our previous paper.[10] According to the method of Marsh et al.,[18] treatment of compound **3** with potassium-*t*-butoxide in *t*-butanol at room temperature for 45 hours afforded compound **4** in a yield of 19% and 4-hydroxy-17(Z)-picolinylidene-androsta-4,6-dien-3-one **5** in a yield of 22% (Method B) (Scheme 1). ^{1}H NMR spectrum of compound **5** showed signals at 6.13 ppm (H-6) and 6.68 ppm (H-7) as double bond hydrogens. ^{13}C NMR spectrum was assigned four double bond carbons at 121.47 (C-6), 134.48 (C-5), 137.47 (C-7), and 140.21 (C-4) ppm.

Compound **1** was converted to compound **6** with the C-6 vinyl nitro group in a yield of 46%, using nitric acid and sodium nitrite in diethyl ether at 5 °C for 1.5 h (Scheme 1), whose structure was confirmed by spectroscopic data. Similar reaction with nitric acid–sodium nitrite reagent was reported by Li et al. for the other steroidal substrate.[19]



Scheme 1. Reagents and reaction conditions: i) KOH, MeOH, reflux, 1 h; ii) cyclohexanone, Al(*i*-PrO)$_3$, reflux, 4 h; iii) *Jones* reagent, acetone, 0 ºC, 1.5 h; iv) *t*-BuOK, *t*-BuOH, r.t., 45 h; v) c.c.HNO_3, $NaNO_2$, diethyl ether, 5 ºC, 1.5 h; vi) $NaBH_4$, EtOH, r.t., 2 h; vii) Ac_2O, Py, r.t., 24 h.

On the other hand, compound **3** was reduced with $NaBH_4$ in absolute ethanol, at room temperature for 2 hours, affording only 17(Z)-picolinylidene-androst-4-en-3β-ol **7** (Scheme 1). The attack of hydride anion to the axial direction of the carbonyl group produced the 3β-hydroxy configuration. The stereochemistry of compound **7** was investigated by selective NOE difference NMR spectroscopy. Selective irradiation of C-10 methyl hydrogens yielded no enhancement of the H-3 signal, so we assumed a half chair conformation for A ring (CH_2-2 up). Since NMR signal for H-4 is a sharp singlet at 5.29 ppm (not coupled with H-3) the dihedral angle between H-3 and H-4 must be close to 90 degree (*Karplus* equation). This dihedral angle is present when hydroxyl group on C-3 has β configuration, only.

In the next step, compound **7** reacted with acetic anhydride in absolute pyridine at room temperature for 24 hours, to give 17(Z)-picolinylidene-androst-4-en-3β-yl acetate **8**, in a yield of 46% (Scheme 1).

Syntheses of compounds **9-14** (Figure 1) were described in our previous papers,[15,16,20,21] and now their antiproliferative activities were investigated in order to see the effect of the saturated AB rings for **11-14** and of the substituent introduced in the ring D on biological activity and compare it with that of compunds **1-8**.

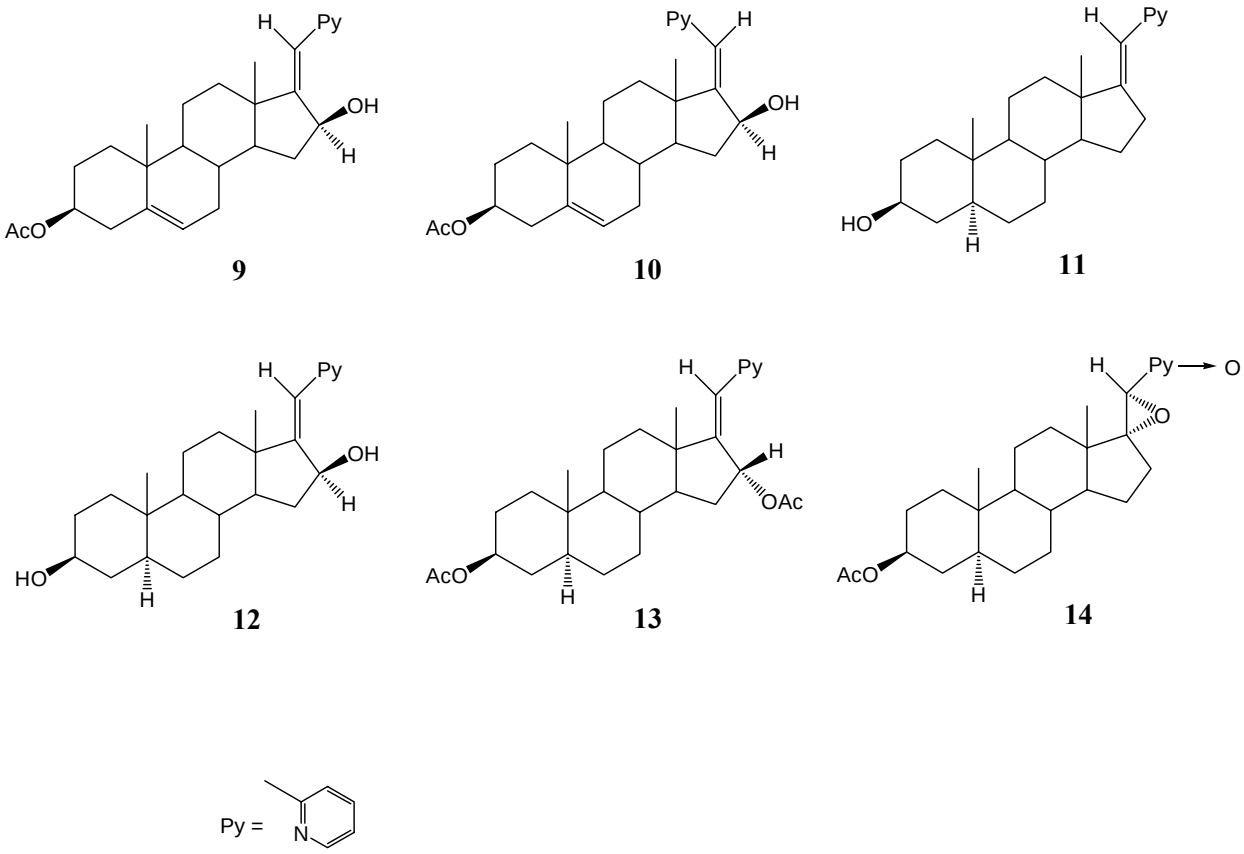


Figure 1. 9, 17(Z)-picolinylidene-androst-5-ene-3β,16β-diol 3 monoacetate;
10, 17(E)-picolinylidene-androst-5-ene-3β,16β-diol 3 monoacetate;
11, 17(Z)-picolinylidene-5α-androstan-3β-ol;
12, 17(Z)-picolinylidene-5α-androstane-3β,16β-diol;
13, 17(Z)-picolinylidene-5α-androstane-3β,16α-diyl diacetate;
14, 17α,20α-epoxy-17β-picolyl-N-oxide-5α-androstan-3β-yl acetate

Biological properties

Screening assay procedures were used to assess the potential inhibitory effects of the synthesized compounds on aromatase.

It is evident from Table 1 that 17-picolinylidene derivatives exhibited a higher inhibitory activity against aromatase if the 3β-hydroxy-5-ene system (**2**, -24.5%) was replaced with the 3-oxo-4-ene system (**3**, 67.9%),[10] and also with the 3,6-dioxo-4-ene system (**4**, 108.54%), or with 4-hydroxy-4,6-dien-3-one system (**5**, 92.03%).

Table 1. Inhibitory effects of the tested compounds on the aromatase activity in the denucleated fraction of ovaries from PMSG pretreated rats

	Percent (%) of inhibition of aromatase activity vs control	
Compounds	(1 μM)	(50 μM)
1	-	-18.2 ± 16.3
2	-	-24.5 ± 12.9
3	-	67.9 ± 0.9**
4	64.92±6.54**	108.54±8.15**
5	64.12±5.68**	92.03±7.57**
6	-	-21.8 ± 10.2
7	65.30±7.14**	86.84±5.88**
8	54.33±5.15**	79.06±6.07**
Formestane	104.95±1.32**	112.02±2.71**

To measure the aromatase activity, the purified denucleated fraction of ovaries from PMSG pretreated female rats was incubated in the environment with subsaturated (50 nM) concentration of substrate testosterone and NADPH (1 mM) and absence (control) or presence of different tested compounds (1 μM or 50 μM). Estradiol level was determined by RIA. Results shown are percents of inhibition of aromatase activity vs control. Numbers represent mean ± SEM of 10-20 replicates. Significance: **p<0.005 vs control (Mann-Whitney non-parametric test)

On the other hand, 3β-hydroxy and 3β-acetoxy-4-ene systems (compounds **7** and **8**, respectively) exhibited a higher inhibitory activity (86.84% for **7** and 79.06% for **8**), compared to the 3β-hydroxy and 3β-acetoxy-5-ene systems (-18.2% for **1** and -24.5% for **2**) and compound **6** (-21.8%) with nitro group in C-6 position.

If compared with formestane, the newly synthesized compounds **4**, **5**, **7** and **8** showed a satisfactory inhibitory activity against aromatase in a concentration of 50 μM.

The synthesized compounds were evaluated for their antiproliferative activity against human breast adenocarcinoma ER+, MCF-7, human breast adenocarcinoma ER-, MDA-MB-231, prostate cancer AR-, PC-3 and normal fetal lung fibroblasts, MRC-5. Their cytotoxicity *in vitro* was evaluated after 48h treatment of the cells by SRB assay.[22] The results are presented in Table 2

Table 2. *In vitro* antiproliferative activity of the tested compounds – IC_{50} values.

Compounds	*IC_{50} (μM)*			
	MCF-7	MDA-MB-231	PC-3	MRC-5
1	>100	>100	>100	>100
2	>100	>100	>100	>100
3	>100	>100	12.9	>100
4	>100	20.2	88.9	>100
5	>100	9.3	>100	>100
6	>100	32.8	24.3	>100
7	>100	>100	10.1	>100
8	37.5	39.3	66.2	>100
9	>100	>100	19.1	>100
10	>100	>100	45.1	>100
11	28.1	64.5	>100	>100
12	22.1	>100	39.9	>100
13	40.9	>100	>100	>100
14	>100	>100	>100	>100
Doxorubicin	0.75	0.12	95.6	0.12
Formestane	>100	55.5	48.4	>100

As can be seen from Table 2, of all the synthesized compounds, compound **5,** with 4-hydroxy-4,6-dien-3-one system, showed a markedly strong cytotoxicity (IC_{50} 9.3 μM) against MDA-MB-231 cancer cell line, exhibiting also a pronounced inhibition of aromatase (92.03%). Compound **4,** with 3,6-dioxo-4-ene system exhibited moderate cytotoxicity against MDA-MB-231 (IC_{50} 20.2 μM), along with a very strong inhibition of aromatase (108.54%). Low cytotoxicity showed compound **6** (IC_{50} 32.8 μM) and compound **8** (IC_{50} 39.3 μM), having 3β-acetoxy-6-nitro-5-en and 3β-acetoxy-4-ene systems, respectively. Compound **8** exhibited a high percent of inhibition of aromatase (79.06%), in contrast to compound **6**. Compound **11**, with A/B *trans* system, showed low cytotoxicity against MDA-MB-231 (IC_{50} 64.5 μM) and against MCF-7 cells (IC_{50} 28.1 μM). Moderate cytotoxicity against MCF-7 cells exhibited only compound **12** (IC_{50} 22.1 μM), whereas **8** (IC_{50} 37.5 μM) and **13** (IC_{50} 40.9 μM) showed low cytotoxicity. Strong cytotoxicity against PC-3 cells exhibited compounds **7** (IC_{50} 10.1 μM) and **3** (IC_{50} 12.9 μM), and

a moderate one compounds **6** (IC_{50} 24.3 μM) and **9** (IC_{50} 19.1 μM), with 3β-acetoxy-5-ene system. On the other hand, compound **10**, having a 16β-hydroxy function, like compound **9** but with opposite configuration of the C_{17}-C_{20} double bond (compared to **9**), showed a low cytotoxicity (IC_{50} 45.1 μM) against PC-3 cells. Compounds **11-14**, which have saturated A and B rings exhibit a moderate cytotoxicity (**11**, IC_{50} 28.1 μM and **12**, IC_{50} 22.1 μM), that is low cytotoxicity (**13**, IC_{50} 40.9 μM) against MCF-7 cells, whereas against MDA-MB-231 cells weak cytotoxicity exhibited only compound **11** (IC_{50} 64.5 μM), compounds **12** and **13** being inactive in this respect. If we compare the cytotoxic activity of the newly synthesized compound **7**, which contains 3β-hydroxy-4-ene system, with that of compound **11**, having also the 3β-hydroxy function but with no 4-ene system, against all cell lines tested, it appears that compound **7** showed strong cytotoxicity against PC-3 cells (IC_{50} 10.1 μM), whereas compound **11** showed a moderate cytotoxicity against MCF-7 and low cytotoxicity against MDA-MB-231. If compared with Doxorubicin, compounds **7** and **3** were 9 and 7 times, respectively, more active against PC-3. Doxorubicin showed low cytotoxicity towards PC-3 cells (IC_{50} 95.6 μM), which was expected according to David-Beabes et al.[23] Formestane also showed low cytotoxicity against MDA-MB-231 (IC_{50} 55.5 μM) and PC-3 (IC_{50} 48.4 μM), but it was nontoxic to MCF-7 cells and to healthy MRC-5 cells compared with Doxorubicin.

All newly synthesized compounds were nontoxic to healthy MRC-5 cells, whereas Doxorubicin was very toxic to these cells.

Experimental Section

General. Melting points were determined using a Büchi SMP 20 apparatus and are uncorrected. IR spectra were recorded on a NEXUS 670 SP-IR spectrometer (wavenumbers in cm^{-1}). NMR spectra were taken on a Bruker AC 250E spectrometer operating at 250 MHz (^1H) and 62.5 MHz (^{13}C), and are reported in ppm (δ-scale) downfield from the tetramethylsilane internal standard; coupling constants (*J*) are given in Hz. High resolution mass spectra (TOF) were recorded on a 6210 Time-of-Flight LC/MS Agilent Technologies (ESI+) instrument. Chromatographic separations were performed on silica gel columns (Kieselgel 60, 0.063-0.20 mm, Merck). All reagents used were of analytical reagent grade. All solutions were dried over anhydrous sodium sulfate.

17(*Z*)-Picolinylidene-androst-4-ene-3,6-dione 4 and 4-hydroxy-17(Z)-picolinylidene-androsta-4,6-dien-3-one 5. Method A for 4. To the solution of 17(Z)-picolinylidene-androst-5-en-3β-ol (**2**, 0.30 g; 0.83 mmol) in acetone (35 mL) at 0 ºC, *Jones* reagent[17] (2 mL) was added dropwise (~1 drop/10 s) with vigorous stirring. Upon complete addition of the reagent, the reaction mixture was stirred for another 1.5 h. The reaction mixture was then quenched with methanol (15 mL) (to destroy the excess of the reagent) and solvent was then removed in vacuo. After that, water (20 mL) and $NaHCO_3$ (to pH 8) were added and crude product was extracted

with dichloromethane (5 × 10 mL). After purification by column chromatography (15 g silica gel, toluene–ethyl acetate 7:1) the pure compound **4** (0.08 g, 26%, m.p. 231-233 ºC after recrystallization from methanol–dichloromethane), was obtained.

Method B for 4 and 5. To the solution of compound **3**[10] (0.20 g, 0.55 mmol) in *t*-BuOH (6 mL), a freshly prepared solution of potassium-t-butoxide in t-BuOH (5 mL, 3.83 mmol) was added in drops. The reaction mixture was stirred at room temperature for 45 h in the dark. Then, the reaction mixture was poured into water (10 mL) and extracted with ethyl acetate (5 × 10 mL) and then with dichloromethane (5 × 10 mL). After drying and removal of solvents, the crude product was separated by column chromatography (15 g silica gel). Elution with *n*-hexane–ethyl acetate 5:1 afforded a pure compound **5** (0.044 g, 22%, m.p. 182-184 ºC after recrystallization from methanol). Further elution with *n*-hexane–ethyl acetate 2:1 afforded a pure compound **4** (0.038 g, 19%, m.p. 231-233 ºC after recrystallization from methanol).

Compound 4. IR (film): 2945, 1686, 1584, 1564, 1469, 1428, 1259, 1220, 1094, 874, 777, 735. ^{1}H NMR ($CDCl_3$): 0.96 and 1.22 (2s, 6H, H-18 and H-19); 2.76-2.93 (m, 2H, H-16); 6.21 (s, 1H, H-20); 6.26 (s, 1H, H-4); 7.07 (m, 1H, H-5', Py); 7.29 (m, 1H, H-3', Py); 7.63 (td, 1H, $J_{4',3'}$ = $J_{4',5'}$ = 7.8 Hz, $J_{4',6'}$ = 1.8 Hz, H-4', Py); 8.58 (d, 1H, $J_{6',5'}$ = 4.2 Hz, H-6', Py). ^{13}C NMR ($CDCl_3$): 17.58 and 18.71 (C-18 and C-19); 35.52 (qC); 39.79; 45.77 (qC); 46.56; 51.14 (CH); 53.78 (CH); 118.58 (C-20); 120.48 (C-5', Py); 122.88 (C-3', Py); 125.67 (C-4); 135.94 (C-4', Py); 149.23 (C-6', Py); 157.17 (C-17); 158.59 (C-2', Py); 160.69 (C-5); 199.39 (C-3); 201.94 (C-6). HRMS (TOF) *m/z*: $C_{25}H_{30}NO_2$ $[M+H]^+$ calcd. 376.22711, found 376.22523.

Compound 5. IR (film): 3033, 2943, 1656, 1610, 1589, 1566, 1467, 1369, 1219, 1174, 1095, 870, 773, 740. ^{1}H NMR ($CDCl_3$): 0.99 and 1.15 (2s, 6H, H-18 and H-19); 2.79-2.98 (m, 2H, H-16); 6.13 (dd, 1H, $J_{6,7}$ = 9.9 Hz, $J_{6,8}$ = 1.9 Hz, H-6); 6.24 (s, 1H, H-20); 6.68 (dd, 1H, $J_{7,6}$ = 9.9 Hz, $J_{7,8}$ = 2.7 Hz, H-7); 7.05 (m, 1H, H-5', Py); 7.29 (m, 1H, H-3', Py); 7.62 (td, 1H, $J_{4',3'}$ = $J_{4',5'}$ = 7.8 Hz, $J_{4',6'}$ = 1.8 Hz, H-4', Py); 8.58 (d, 1H, $J_{6',5'}$ = 3.9 Hz, H-6', Py). ^{13}C NMR ($CDCl_3$): 16.44 and 18.75 (C-18 and C-19); 35.62 (qC); 37.12; 46.59 (qC); 51.01; 51.36; 118.14 (C-20); 120.34 (C-5', Py); 121.47 (C-6); 122.84 (C-3', Py); 134.48 (C-5); 135.87 (C-4', Py); 137.47 (C-7); 140.21 (C-4); 149.18 (C-6', Py); 157.34 (C-17); 159.22 (C-2', Py); 193.65 (C-3). HRMS (TOF) *m/z*: $C_{25}H_{30}NO_2$ $[M+H]^+$ calcd. 376.22711, found 376.22619.

6-Nitro-17(*Z*)-picolinylidene-androst-5-en-3β-yl acetate 6. Compound **1** (0.20 g, 0.50 mmol) was suspended in absolute diethyl ether (6 mL) and cooled to 5 °C, then concentrated nitric acid (5 mL) was added in drops. After that, solid $NaNO_2$ (0.05 g, 0.71 mmol) was added, and the reaction mixture was stirred at 5 °C for 1.5 hour. The reaction mixture was diluted with cold water (20 mL), and solution of 4 M NaOH was added (to pH 10) and the mixture extracted with dichloromethane (5 × 10 mL). The combined organic phases were dried, filtered, and evaporated in vacuo to a solid. The crude product was purified by silica gel column chromatography (20 g, toluene–ethyl acetate 9:1 and 1:1), affording a pure compound **6** (0.103 g, 46%) in the form of colorless oil. IR (film): 2948, 1734, 1653, 1585, 1520, 1470, 1428, 1366, 1240, 1151, 1036, 777, 754. ^{1}H NMR ($CDCl_3$): 0.92 and 1.19 (2s, 6H, H-18 and H-19); 2.04 (s, 3H, from Ac); 2.77-2.85 (m, 2H, H-16); 4.66 (m, 1H, H-3); 6.25 (s, 1H, H-20); 7.06 (m, 1H, H-5', Py); 7.30 (m, 1H, H-3',

Py); 7.63 (td, 1H, $J_{4',3'} = J_{4',5'} = 7.8$ Hz, $J_{4',6'} = 1.8$ Hz, H-4', Py); 8.57 (d, 1H, $J_{6',5'} = 4.8$ Hz, H-6', Py). ^{13}C NMR ($CDCl_3$): 18.58 and 19.78 (C-18 and C-19); 21.21 (CH_3 from Ac); 37.95 (qC); 45.60 (qC); 53.34; 71.83 (C-3); 118.36 (C-20); 120.40 (C-5', Py); 122.86 (C-3', Py); 135.97 (C-4', Py); 137.67 (C-6); 146.37 (C-5); 149.09 (C-6', Py); 157.22 (C-17); 159.13 (C-2', Py); 170.07 (qC from Ac). HRMS (TOF) *m/z*: $C_{27}H_{35}N_2O_4$ $[M+H]^+$ calcd. 451.25913, found 451.25762.

17(*Z*)-Picolinylidene-androst-4-en-3β-ol 7. Compound **3** (0.05 g, 0.14 mmol) was dissolved in absolute ethanol (3 mL) at room temperature, then $NaBH_4$ (0.04 g, 1 mmol) was added and the reaction mixture was stirred for the next 2 h. Then, the reaction mixture was poured into water (20 mL), the crude product was filtered and recrystallized from dichloromethane–methanol, to give a pure compound **7** (0.050 g, 97%, m.p. 147-149 °C). IR (film): 3422, 2934, 2851, 1653, 1588, 1562, 1468, 1433, 1374, 1155, 1110, 1034, 998, 864, 776, 744. ^{1}H NMR ($CDCl_3$): 0.91 and 1.09 (2s, 6H, H-18 and H-19); 2.77-2.84 (m, 2H, H-16); 4.17 (m, 1H, H-3); 5.29 (s, 1H, H-4); 6.21 (s, 1H, H-20); 7.02 (m, 1H, H-5', Py); 7.26 (m, 1H, H-3', Py); 7.60 (td, 1H, $J_{4',3'} = J_{4',5'} = 7.7$ Hz, $J_{4',6'} = 1.8$ Hz, H-4', Py); 8.55 (d, 1H, $J_{6',5'} = 4.7$ Hz, H-6', Py). ^{13}C NMR ($CDCl_3$): 18.77 and 21.03 (C-18 and C-19); 35.80; 37.41 (C-10); 45.82 (C-13); 53.30; 53.70 (C-9); 54.69; 67.81 (C-3); 117.93 (C-20); 120.18 (C-5', Py); 122.73 (C-3', Py); 123.64 (C-4); 135.81 (C-4', Py); 147.23 (C-5); 149.09 (C-6', Py); 157.53 (C-17); 160.33 (C-2', Py). HRMS (TOF) *m/z*: $C_{25}H_{34}NO$ $[M+H]^+$ calcd. 364.26349, found 364.26260.

17(*Z*)-Picolinylidene-androst-4-en-3β-yl acetate 8. Compound **7** (0.03 g, 0.08 mmol) was dissolved in absolute pyridine (1 mL), then Ac_2O (1 mL) was added and the reaction mixture was stirred at room temperature for the next 24 h. After that, the reaction mixture was slowly poured into cold water (20 mL), yielding a precipitate which was filtered and recrystallized from dichloromethane–*n*-hexane affording a pure compound **8** (0.014 g, 46%, m.p. 126-128 °C). IR (film): 2939, 2851, 1731, 1655, 1584, 1471, 1428, 1371, 1242, 1149, 1025, 861, 776, 740. ^{1}H NMR ($CDCl_3$): 0.91 and 1.11 (2s, 6H, H-18 and H-19); 2.06 (s, 3H, from Ac); 2.70-2.85 (m, 2H, H-16); 5.24 (m, 2H, H-3 and H-4); 6.22 (s, 1H, H-20); 7.04 (m, 1H, H-5', Py); 7.27 (m, 1H, H-3', Py); 7.61 (td, 1H, $J_{4',3'} = J_{4',5'} = 7.7$ Hz, $J_{4',6'} = 1.8$ Hz, H-4', Py); 8.56 (d, 1H, $J_{6',5'} = 4.7$ Hz, H-6', Py). ^{13}C NMR ($CDCl_3$): 18.79 and 18.82 (C-18 and C-19); 21.45 (CH_3 from Ac); 37.42 (C-10); 45.84 (C-13); 53.25 (C-9); 54.44 (C-14); 70.86 (C-3); 117.91 (C-20); 119.20 (C-4); 120.21 (C-5`, Py); 122.75 (C-3', Py); 135.87 (C-4', Py); 149.05 (C-6', Py); 149.28 (C-5); 157.50 (C-17); 160.34 (C-2', Py); 170.99 (qC from Ac). HRMS (TOF) *m/z*: $C_{27}H_{36}NO_2$ $[M+H]^+$ calcd. 406.27406, found 406.27288.

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.

Antiaromatase activity

Chemicals. Antiestradiol 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 reagents were of analytical reagent grade.

Animals (female rats), treatment, and assays. Preparation of denucleated ovarian fraction from PMSG pretreated rats and determination of aromatase activity in ovarian homogenate was carried out as described previously.[24]

For a preliminary assessment of potential antiaromatase activity of the synthesized compounds, the given compound was added in a concentrations of 1 μM and 50 μM to the incubation mixture containing 50 nM of testosterone as a substrate (subsaturated concentration; the estimated K_m for testosterone was 49.17 nM and V_{max} 5.76 pM/(min mg) protein).

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

Antiproliferative activity

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 AR-, PC-3, 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^3) 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.

Cytotoxicity assay. Antiproliferative activity was evaluated by colorimetric sulforhodamine B (SRB) assay after Skehan et al.[22] Briefly, single cell suspension was plated into 96-well microtitar plates (Costar, flat bottom): 5 x 10^3 cells (MCF-7; MDA-MB-231; PC-3; MRC-5), per 180 μL of medium. The plates were pre-incubated 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 control ones. 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, acted as blank. Antiproliferative activity was calculated according to the formula:

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

and expressed as a percent of antiproliferative activity (CI %).

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.[25]

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