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Original article

Synthesis and cytotoxic activity of some 17-picolyl and 17-picolinylidene androstane derivatives

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ABSTRACT

New 17-picolyl and 17-picolinylidene androstane derivatives, **3**–**10**, **15**, **18**, **19**, **22** and **23**, were synthesized starting from 17α-picolyl-androst-5-en-3β,17β-diol (**1**) and 17(Z)-picolinylidene-androst-5-en-3β-ol (**2**). Reaction of **1** with *m*-chloroperoxybenzoic acid gives 5α,6α-epoxy N-oxide derivative **3**, or, with Jones reagent, 3,6-dione derivative **4**; while 17α-picolyl-androst-5-en-3β,4α,17β-triol (**5**) or 3β,4β,17β-triol (**6**) derivatives are obtainable from **1** using SeO_2 in dioxane. Base-catalyzed tosyl group elimination from 7 or **9** affords AB conjugated derivatives **8** and **10**. Oppenauer oxidation of **1** and **2** yields 4-en-3-one derivatives **11** and **12**, which, with H_2O_2 in 4 M NaOH, affords 4α,5α and 4β,5β-epoxides **13**, **14**, **16** and **17**. New 4-methoxy-3-keto derivatives **15** and **18** were obtained from **13** and **14**, or, with methanol in 4 M NaOH, from **16** and **17**. Reduction of **11** with $NaBH_4$ gives **22,** which was then acetylated to obtain **23**. All new derivatives were screened for antitumor activity against human breast adenocarcinoma ER+, MCF-7; human breast adenocarcinoma ER-, MDA-MB-231; prostate cancer AR-, PC-3; human cervix carcinoma, HeLa; and colon cancer, HT-29 cells; as well as one human non-tumor cell line, MRC-5. Compounds **3**, **5**, **6**, **8**, **10**, **18**, **19** and **22** exhibited significant antitumor activity against MDA-MB-231 breast cancer cells; while **5**, **6** and **10** also showed strong cytotoxicity against HT-29. Only compound **19** exhibited significant activity against MCF-7 breast cancer cells. No compounds displayed cytotoxicity against non-tumor MRC-5 cells.

1. Introduction

Steroids are a fundamental class of natural biological signaling molecules, which have demonstrated potential for development into potent drugs for the treatment of a large number of diseases, including: brain tumors, breast cancer, prostate cancer, cardiovascular disease or autoimmune disorders [1,2].

Androgens are male steroid hormones which have a wide-range of important physiological functions, including central involvement in the development of male sex characteristics and other phenotypes [3–5]. Anti-androgens are a diverse class of drugs which can compete with circulating androgens for binding sites on androgen receptors within prostate cells, leading to targeted apoptosis and inhibition of androgen-dependent prostate cancer growth [5].

Several steroidal derivatives with substitutions at the C-17 position have been previously described in the literature as potent biologically active compounds [6,7]. Similarly, in our own work [8–10], we described the synthesis and characterization of several 17α-picolyl and 17-picolinylidene-androst-5-ene derivatives, with anti-aromatase (CYP19A1) and/or anti-tumor activity against a range of tumor cell lines. Several of these tested androstane derivatives displayed significant aromatase inhibitory activity, and/or strong activity against 3 common tumor cell lines (human cervix carcinoma, HeLa; human melanoma, FemX; and human myelogenous leukemia, K562), with IC_{50} values between 4 and 10 μM [8]. Because it is well known [11] that the presence of nitrogen heteroatoms can interfere with steroidal hydroxylation by directly interacting with the cytochrome P450 heme iron in aromatase; these androstane compounds were specifically designed to incorporate nitrogen in the steroidal structure. In the present study, we continue our investigation of C-17 substitutions, and report a simple synthetic route for the preparation of 17-picolyl and 17-picolinylidene androst-5-ene derivatives. The effect of these new androstane derivatives on cell proliferation was tested against a wide-range of common tumor cell lines, including: human breast

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adenocarcinoma ER+, MCF-7; human breast adenocarcinoma ER-, MDA-MB-231; prostate cancer AR-, PC-3; human cervix carcinoma, HeLa; and colon cancer, HT-29.

2. Results and discussion

2.1. Chemistry

The initial steps in the preparation of 17-picolyl and 17-picolinylidene androstane derivatives consist of a series of different reactions of 17α-picolyl-androst-5-en-3β-ol (**1**) [12,13] and 17-picolinylidene-androst-5-en-3β-ol (**2**) [12,13] (see Fig. 1). Specifically, compound **3** (5α,6α-epoxy N-oxide) was obtained from compound **1** by reaction with *m*-chloroperoxybenzoic acid (MCPBA) at 0 °C, 1 h → 10 °C, 3 h → and room temperature, 24 h, at a molar ratio of **1** : MCPBA = 1:3 (Scheme 1). The presence of 5α,6α-epoxy function in compound **3** was confirmed by ^{1}H and ^{13}C NMR spectroscopy: the doublet at 2.93 ppm was attributed to H-6β and ^{13}C NMR signals for C-5 and C-6 atoms were identified at 65.98 and 59.15 ppm, respectively. In addition, a signal for the 17β-OH proton was visible at 6.65 ppm for compound **3** (but not compound **1**), suggesting a lack of rapid proton exchange; while significant chemical shift changes were also observed for pyridine C-atoms in compound **3** (but not compound **1**). Together, these results strongly suggest the presence of an intramolecular hydrogen bond between 17β-OH and the N-oxide function on the pyridine core in compound **3**, which is absent in compound **1**.

In contrast with the above described synthesis, reaction of compound **1** with Jones reagent [14] at 0 °C for 1.5 h affords 17β-hydroxy-17α-picolyl-androst-4-en-3,6-dione (**4**). Consistent with this product, ^{1}H NMR data for compound **4** shows the expected singlet for the H-4 proton at 6.19 ppm, in place of the H-6 doublet at 5.36 ppm visible in ^{1}H NMR spectra of compound **1**. In addition, signals for C-3 and C-6 carbonyl carbons are visible in ^{13}C NMR spectra of compound **4** at 199.46 and 201.95 ppm, respectively.

Interestingly, reaction of compound **1** with selenium-dioxide in dioxane was found to give a mixture of 4α and 4β hydroxylated derivatives (**5** and **6**). Although a 4β-hydroxylation mechanism was previously reported for steroidal 5-en-3-ols via selenium-dioxide oxidation by Ma and Choi [15], these authors obtained only a 4β-hydroxy group. After separation by column chromatography, the 17α-picolyl-androst-5-ene-3β,4α-diol (**5**) product was identified by the appearance of a new ^{1}H NMR triplet signal at 3.89 ppm (J = 3.8 Hz) corresponding to the H-4 proton. In addition, a C-4 carbon signal was also visible for compound **5** at 65.06 ppm by ^{13}C NMR. Analogously, for compound **6**, a new ^{1}H NMR doublet appeared at 4.16 ppm (J = 3.2 Hz), corresponding to the H-4 proton, and a C-4 carbon signal was observed at 77.24 ppm by ^{13}C NMR. The β-configuration of the 4-hydroxy group in compound **6** was assigned by ^{1}H-NOE NMR: irradiation of the H-4 proton at 4.16 ppm resulted in NOE enhancements at H-3α (3.57 ppm) and H-6 (5.71 ppm). In contrast, for compound **5**, selective irradiation of the H-4 proton signal at 3.89 ppm yielded no such NOE enhancement of the H-3 signal, indicating that the C4-OH proton is directed toward the α-position, consistent with our assignment of an α-configuration for the 4-hydroxy group in compound **5**.

Base-catalyzed tosyl group elimination from **7** or **9** was used to obtain AB conjugated derivatives **8** and **10**. Specifically, compound **8**, which contains a 3,5-diene system, was obtained from compound **1** via **7** (in which the 3β-hydroxy group is tosylated). The tosyl group was then eliminated by reaction of **7** with bis(2-aminoethyl)amine under reflux for 1.5 h, yielding only compound **8**. A similar scheme for formation of a Δ^3 double bond was previously reported by King and Bigelow [16], by reaction of epicholesteryl p-toluenesulfonate with pyridine; and by Numazawa at al. [17], via elimination of the tosyl group at C-3 in basic media with N-methylpirrolidone. The structure of compound **8** was identified by the presence of an ^{1}H NMR multiplet at 5.63 ppm, and a doublet at 5.94 ppm (J = 9.9 Hz), corresponding to H-3 and H-4 protons, respectively. Consistent with this, ^{13}C NMR signals were also recorded for C-3 (128.90 ppm) and C-4 (125.14 ppm).

Similar reactions were performed on the 17-picolinylidene-substituted derivative **2** (Scheme 1), to obtained compound **10** via 3β tosyl derivative **9**. In this case, the tosyloxy group was also eliminated with bis(2-aminoethyl)amine at reflux for 1 h, and compound **10** was isolated from the resulting complex reaction mixture. The identity of compound **10** was also confirmed by NMR. ^{1}H NMR spectra of **10** shows a multiplet at 5.42 ppm for the H-3 proton, and a doublet at 5.95 ppm (J = 9.7 Hz) for the H-4 proton; while another multiplet signal at 5.61 ppm was assigned to the H-6 proton. Similarly, ^{13}C NMR spectra contain characteristic signals for C-3, C-4, C-5 and C-6 atoms, at 128.89, 125.16, 141.57 and 124.72 ppm, respectively.

In addition to the above, we also investigated the effects of applying Oppenauer oxidation [8] to our starting compounds **1** and **2**. Oppenauer oxidation of **1** and **2** yields 4-en-3-one derivatives **11** and **12**, which, with H_2O_2 in 4 M NaOH, affords 4α,5α and 4β,5β-epoxides **13**, **14**, **16** and **17** (Scheme 2), respectively. Compounds **15** and **18**, which contain a 4-methoxy group, were then obtained from 17-picolyl epoxy derivatives **13** and **14** [9] and 17-picolinylidene epoxy derivatives **16** and **17** [9], in reaction with methanol and sodium-hydroxide at reflux for 3 h. Analysis of compounds **15** and **18** by ^{1}H NMR confirmed the expected 4-methoxy group signal at 3.60 and 3.59 ppm, respectively. Similarly, ^{13}C NMR signals were observed for C-4 atoms at 147.23 ppm (**15**) and at 154.96 ppm (**18**), respectively.

We next attempted a series of synthetic schemes involving compounds **11** and **12**. Reaction of compounds **11** and **12** with potassium-*tert*-butoxide in *tert*-butanol at 45 °C for 3 h was found to afford 4,17β-dihydroxy-17α-picolyl-androsta-4,6-dien-

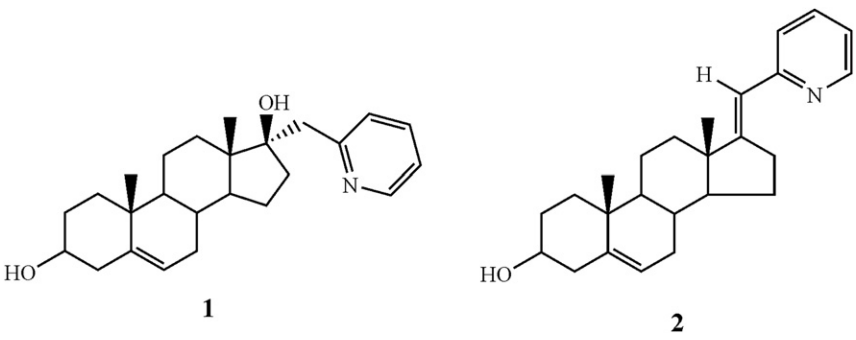
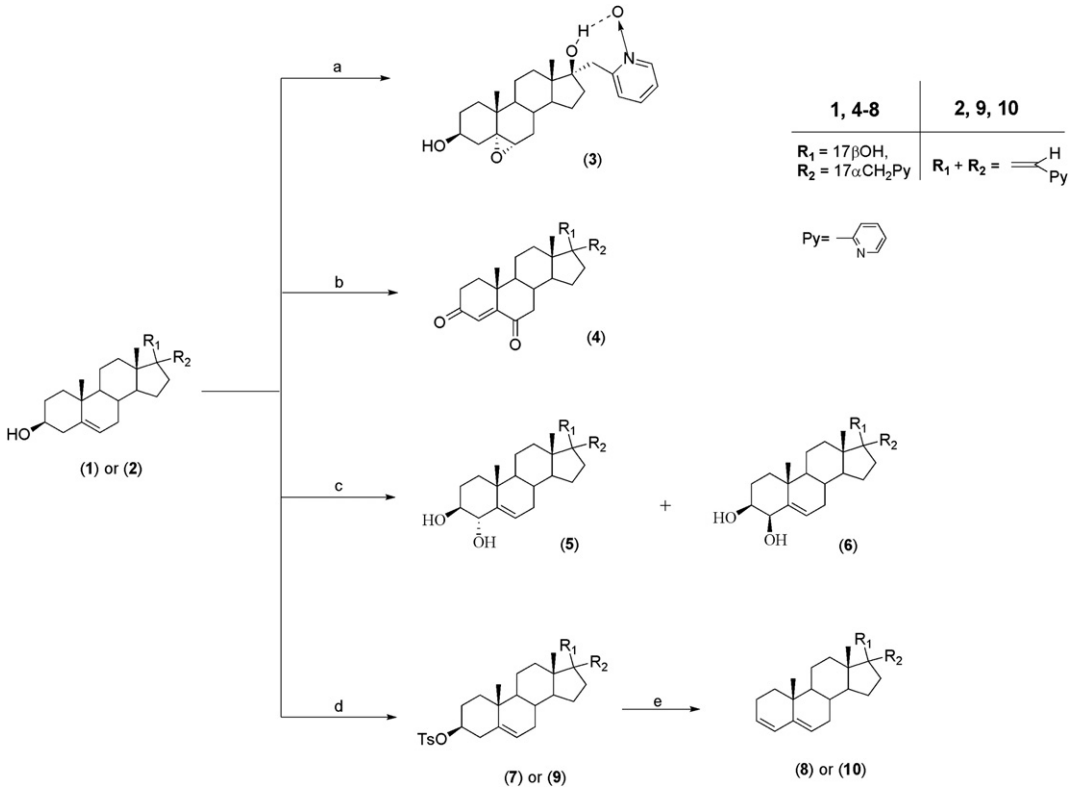


Fig. 1. 17α-Picolyl-androst-5-en-3β,17β-diol (**1**) and 17(Z)-picolinylidene-androst-5-en-3β-ol (**2**).



Scheme 1. Reagents and reaction conditions: a) MCPBA, CH_2Cl_2, 5% $NaHCO_3$, 0 °C, 1 h → 10 °C, 3 h → r.t., 24 h; b) Jones reagent, acetone, 0 °C, 1.5 h; c) SeO_2, dioxane, reflux, 25.5 h; d) *p*-TsCl, Py, r.t., 45 h (for **1**) or 19 h (for **2**); e) bis(2-aminoethyl)amine, reflux, 1 h.

3-one (**19**), accompanied by compound **4**, and 4-hydroxy-17-picolinylidene-androsta-4,6-dien-3-one (**21**) [10] accompanied by compound **20** (Scheme 2). ^{1}H NMR analysis of compound **19** showed a double doublet at 6.06 ppm ($J_{6,7}$ = 9.4 Hz, $J_{7,8}$ = 2.0 Hz) and 6.65 ppm ($J_{6,7}$ = 9.4 Hz, $J_{6,8}$ = 2.8 Hz) for the H-7 and H-6 protons, as expected. Similarly, ^{13}C NMR spectral signals were assigned for double bond carbons at 124.71 (C-6), 134.70 (C-5), 137.10 (C-7) and 140.21 (C-4) ppm. The molecular formula of compound **19** was confirmed by high resolution mass spectra (HRMS) (TOF); and by comparison with spectroscopic data previously reported by our group for compound **21** [10].

Based on the above, we further investigated possible synthetic routes based on compounds **11** and **12**. Interestingly, reduction of compound **11** with $NaBH_4$ in ethanol at room temperature for 2.5 h was found to yield a 3β-hydroxy derivative, compound **22**. Mechanistically, attack of the hydride anion in the axial direction of the carbonyl group could result in generation of the 3β-hydroxy configuration observed for compound **22**. Analogously, reduction of compound **12** with $NaBH_4$ in absolute ethanol at room temperature for 2 h, affords compound **24** [10]. The ^{1}H NMR spectrum of compound **22** was characterized by a multiplet at 4.15 ppm, attributed to the H-3α proton; while ^{13}C NMR spectroscopy revealed the corresponding C-3 atom signal at 67.93 ppm. The stereochemistry of compound **22** was confirmed using selective NOE difference experiments. Because selective irradiation of the C-10 methyl protons did not result in NOE enhancement of the H-3 proton signal, the H-3 and C-10 angular methyl group are assumed to be in *trans*; and the OH group at C-3 is thus directed toward the β position.

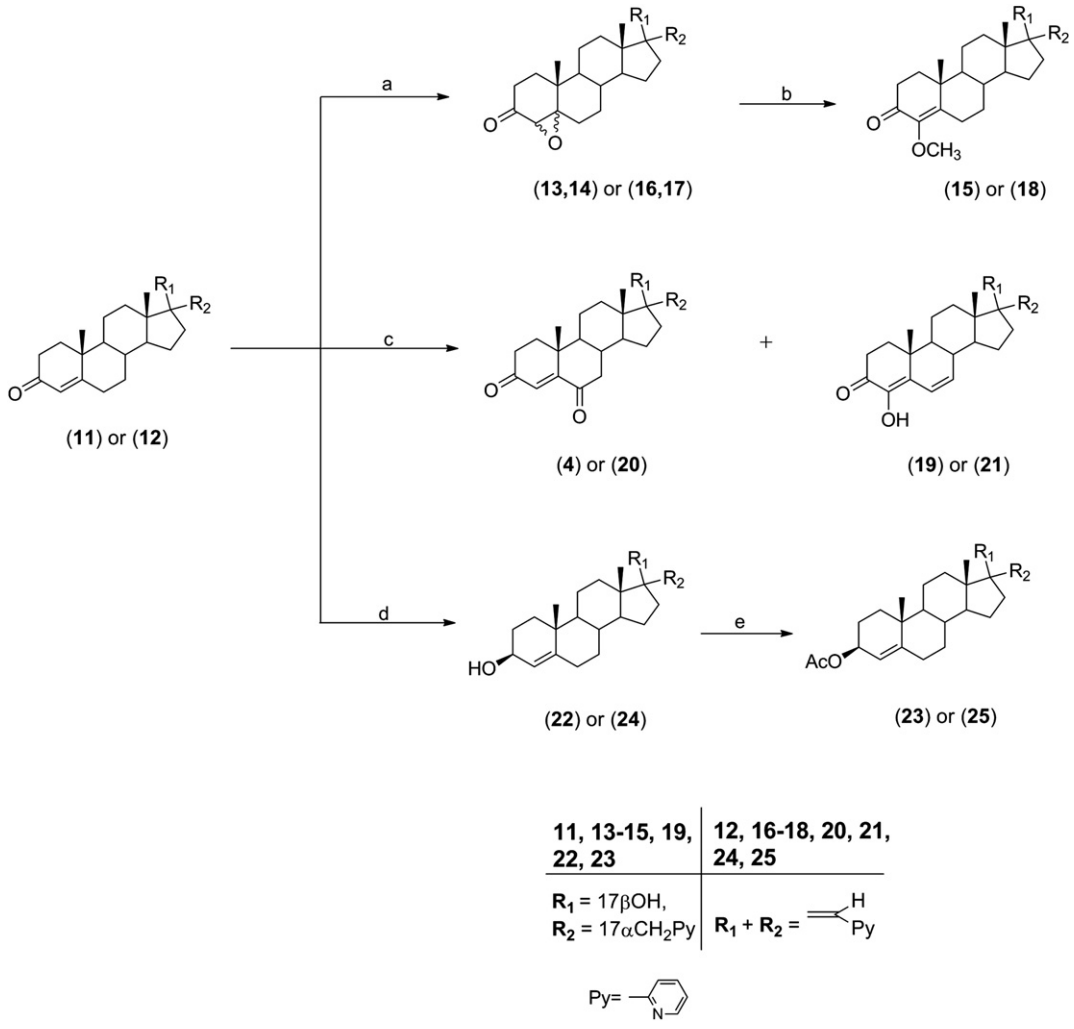
Finally, $NaBH_4$ reduction of compound **11** to **22**, followed by acetylation with acetic anhydride in absolute pyridine at room temperature for 24 h was found to give compound **23**.

2.2. Cytotoxicity

As a first step toward testing the potential of the above synthesized compounds for the development of novel steroidal therapeutics, we performed a series of cytotoxicity assays. The cytotoxic activity of selected synthesized compounds was evaluated against several common human tumor cell lines, originating from 4 different solid tumor types. As a control, the selected compounds were also tested against normal fetal lung fibroblasts, MRC-5. Cytotoxic activity was evaluated *in vitro* after 48 h treatment using the SRB assay, as previously described [18].

Interestingly, all of the synthesized compounds with a 17α-picolyl substituent (**3**, **5**, **6**, **8**, **19** and **22**) appear to have stronger cytotoxicity against ER- human breast adenocarcinoma cells (MDA-MB-231) (see Table 1). Of these, compound **19**, which contains a 4-hydroxy-4,6-dien-3-on system, displayed the strongest cytotoxicity (IC_{50} 1.2 μM); while compound **8**, which contains a 3,5-dien system, also displayed significant cytotoxicity (IC_{50} 2.3 μM). In comparison, the cytotoxicity for the 17-picolynilidene compound **21** (IC_{50} 9.3 μM) [10] was approximately 8-fold less than that of the 17α-picolyl derivative **19** (IC_{50} 1.2 μM); while compound **10** (17-picolynilidene derivate) was 2-fold less cytotoxic (IC_{50} 4.6 μM) than compound **8** (IC_{50} 2.3 μM). One possibly interesting exception to this rule was compound **18**, a 17-picolynilidene derivate with a 4-methoxy group: compound **18** was measured to have 10-fold stronger cytotoxicity (IC_{50} 4.1 μM) than compound **15** (IC_{50} 42.6 μM), which also has a 4-methoxy group, but contains a 17α-picolyl substituent.

Compounds **5** and **6**, which contain an additional 4-hydroxy group, both displayed significantly stronger cytotoxicity against ER- human breast adenocarcinoma cells (MDA-MB-231) (17-fold



Scheme 2. Reagents and reaction conditions: a) 30% H_2O_2, 4M NaOH, MeOH, 0 °C, 1 h → 10 °C, 24 h; b) 4M NaOH, MeOH, reflux, 3.5 h (or 3 h); c) 1M t-BuOK, t-BuOH, 45 °C, 3 h (or r.t., 45 h); d) $NaBH_4$, EtOH, r.t., 24 h (or 2 h); e) Ac_2O, Py, r.t., 24 h.

and 10-fold, respectively) vs. compound **1** [9] (IC_{50} 96.1 μM). Similarly, compound **3**, which contains a 5α,6α-epoxy and N-oxide function, also had significantly stronger cytotoxicity (IC_{50} 7.5 μM) than compound **1** against MDA-MB-231 cells.

Intriguingly, replacement of the 3β-hydroxy group in compound **22** with a 3β-acetoxy group (compound **23**), resulted in a reduction in cytotoxicity (IC_{50} 10.2 μM vs. IC_{50} 3.8 μM for **23** vs. **22**, respectively). Similarly, compound **25**, which also contains a 3β-acetoxy group [10], showed low cytotoxicity (IC_{50} 39.3 μM) against the same cell line. However, compound **24** [10] (17-picolinylidene), with a 3β-hydroxy group, appears to be inactive (IC_{50} > 100 μM) against MDA-MB-231 cells.

Table 1
In vitro cytotoxic activity of the tested compounds – IC_{50} values.

Compounds	IC_{50} (μM)					
	MCF-7	MDA-MB-231	PC-3	HeLa	HT-29	MRC-5
1 [9]	>100	96.1	6.3	–	–	>100
3	>100	7.5	33.6	33.5	25.8	>100
4	>100	>100	4.3	>100	>100	>100
5	>100	5.6	21.3	21.3	2.6	>100
6	>100	9.0	52.1	26.6	8.0	>100
8	17.4	2.3	24.7	27.5	11.7	>100
10	55.8	4.6	19.9	23.3	1.8	>100
15	>100	42.6	54.8	19.1	>100	>100
18	>100	4.1	34.1	15.3	56.0	>100
19	3.6	1.2	25.6	36.6	>100	>100
21 [10]	>100	9.3	>100	–	–	>100
22	>100	3.8	22.3	26.9	>100	>100
23	>100	10.2	31.3	>100	10.6	>100
24 [10]	>100	>100	10.1	–	–	>100
Doxorubicin	0.75	0.12	95.61	1.17	0.32	0.12
Formestane	>100	55.5	48.36	5.55	>100	>100

In contrast with our results from ER- breast adenocarcinoma cell lines, only compound **19** strong cytotoxicity against ER + human breast adenocarcinoma cells (MCF-7 cells) (IC_{50} 3.6 μM); although compound **8** did display moderate MCF-7 cytotoxicity (IC_{50} 17.4 μM). Interstingly, compound **19** also displayed the strongest cytotoxicity against ER- breast cancer cells, followed by compound **8**.

With respect to prostate cancer, PC-3 cells were the most sensitive to compound **1** [9] (IC_{50} 6.3 μM), although the majority of the tested compounds (**3**, **5**, **8**, **10**, **12** [9], **17** [9], **18**, **19**, **22** and **24**) showed moderate PC-3 cytotoxicity: ranging from a maximum IC_{50} of 10.1 μM (**24**) to a minimum IC_{50} of 34.1 μM (**18**). Of these, **5**, **8**, **10**, **18** and **22** also displayed moderate cytotoxicity against HeLa cancer cells (along with **6**). Unlike our results from ER- breast cancer cells, compound **24** [10] had 2-fold *higher* cytotoxicity (IC_{50} 10.1 μM) than compound **22** (IC_{50} 22.3 μM) against PC-3 cells.

Cytotoxicity results against the colon cancer-derived cell line (HT-29) revealed a different set a compounds with significantly

strong cytotoxic activity: according to IC_{50} values, **5** and **10** showed strong cytotoxicity (IC_{50} 2.6 μM and 1.8 μM, respectively) against HT-29 cells; followed by compound **6** (IC_{50} 8.0 μM).

Overall, the ER- breast cancer cell line (MDA-MB-231) appears tobe the most sensitive to the majority of tested compounds (Fig. 2).

In fact, all of the tested compounds induced a dose-dependent cytotoxic response against MDA-MB-231 cancer cells: with some compounds displaying more toxicity than others. For example, compounds **5**, **18** and **19** showed stronger cytotoxic activity for the whole range of concentrations vs. Formestan (a CYP19A1 aromatase inhibitor in clinical use); whereas compounds **8** and **22** only induced higher cytotoxicity at concentrations > 0.1 μM; and compounds **3**, **4**, **6**, **15**, and **23** showed either similar or less cytotoxicity than Formestan.

As an indication of their potential potency, the cytotoxic activities of compounds **19**, **8** and **5** were found to be similar to that of Doxorubicin at concentrations ≥ 0.1 μM. However, only compound **18** displayed higher cytotoxicity than Doxorubicin (up to 10% at concentrations <1 μM against MDA-MB-231 cells).

As a control, all of the newly synthesized compounds were confirmed to be non-toxic to normal MRC-5 cells, whereas Doxorubicin was very toxic to these cells.

3. Conclusion

Novel androstane derivatives **3**–**10**, **15**, **18**, **19**, **22** and **23** were synthesized from 17α-picolyl-androst-5-en-3β,17β-diol (**1**) and 17(Z)-picolinylidene-androst-5-en-3β-ol (**2**), respectively. These compounds were evaluated for cytotoxic activity against five common tumor cell lines, and one normal cell line. Synthesized compounds with a 17-picolyl substituent (**3**, **5**, **6**, **8**, **10**, **18**, **19** and **22**) showed markedly strong cytotoxic activity against ER- breast cancer cells (MDA-MB-231 cells); while compound **19** (followed by **8**) also displayed the strongest cytotoxicity against ER + breast cancer cells (MCF-7); and compounds **5**, **6** and **10** were the most cytotoxic to HT-29 colon cancer cells.

4. Experimental

4.1. Chemistry

All melting points were determined using a Büchi SMP 20 apparatus and are reported uncorrected. IR spectra were recorded on a NEXUS 670 SP-IR spectrometer (wave numbers in cm^{-1}). NMR spectra were recorded on a Bruker AC 250E spectrometer

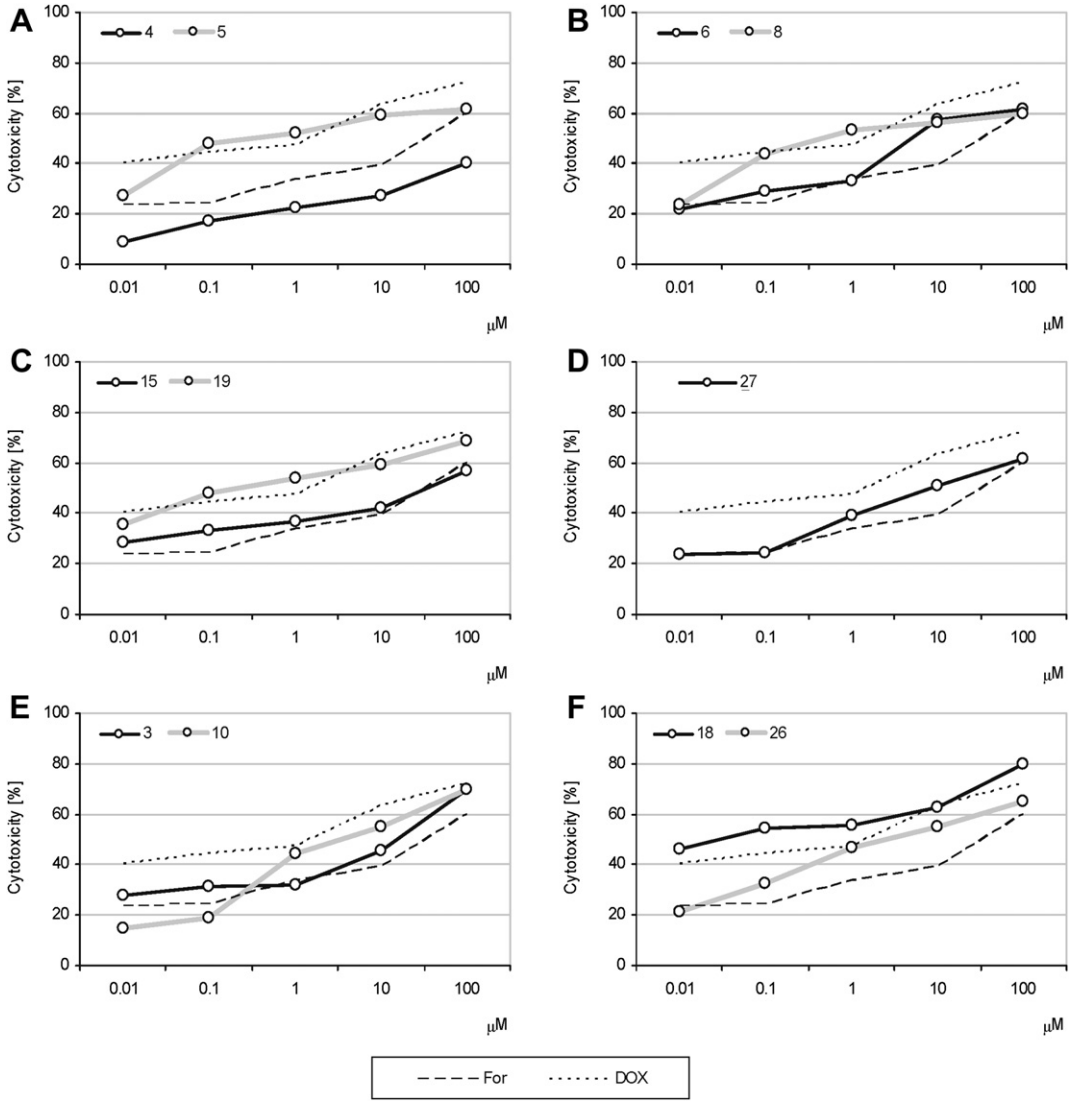


Fig. 2. Cytotoxic activity of selected synthesized compounds against MD-MB-231 cells. For- formestan; DOX- doxorubicin.

operating at 250 MHz (^{1}H) and 62.5 MHz (^{13}C), and are reported in ppm (symbol 100 \f "Symbol" \s 11d-scale) downfield from a tetramethylsilane internal standard; coupling constants (*J*) are given in Hz. GC/MS analyses were performed on an Agilent Technologies GC 6890N instrument with Mass Selective Detector 5973; the first number denotes the *m*/*z* value, while ion abundances are given in parentheses. 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.

4.1.1. 5α,6α-Epoxy-17α-picolyl-androstane-N-oxide-3β,17β-diol (**3**)

A 5% solution of $NaHCO_3$ (2.5 ml) was added to a solution of compound **1** (0.305 g, 0.799 mmol) in dichloromethane (10 ml) at 0 °C, to pH 8. Then, MCPBA (0.414 g, 2.38 mmol) was added and the reaction mixture was stirred for 1 h at 0 °C, 3 h at 10 °C and 24 h at room temperature in the dark. The resulting organic layer was then washed successively with a solution of 10% $Na_2S_2O_3$ (2 × 10 ml), 5% $NaHCO_3$ (2 × 10 ml) and water (2 × 10 ml). After drying and removal of dichloromethane, the mixture of reaction products was separated by column chromatography (15 g silica gel, chloroform-acetone 1:1), yielding pure compound **3** (0.138 g, 42%, m.p. 248–250 °C after recrystallization from dichloromethane-hexane).

IR (KBr, cm^{-1}): 3416, 2940, 2868, 1637, 1560, 1489, 1438, 1377, 1215, 1060, 1034, 771; ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.87 (s, 3H, H-18), 1.09 (s, 3H, H-19), 2.56 (d, 1H, J_{gem} = 13.4 Hz, C<u>H</u>$_2$Py), 2.93 (d, 1H, $J_{6,7}$ = 4.3 Hz, H-6β), 3.79 (d, 1H, J_{gem} = 13.4 Hz, C<u>H</u>$_2$Py), 3.89 (m, 1H, H-3), 6.65 (s, 1H, 17β-O<u>H</u>···O←N-Py), 7.22–7.39 (m, 3H, H-3′, H-4′, H-5′, Py), 8.28 (d, 1H, $J_{5',6'}$ = 6.1 Hz, H-6′, Py); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 14.12 (C-18), 15.96 (C-19), 20.42, 23.04, 28.47, 30.82 (CH), 31.05, 32.52, 33.74, 34.93, 38.44, 39.74, 42.87 (CH), 47.02, 51.45 (CH), 59.15 (CH), 65.98 (C-5), 68.42 (C-6), 69.16 (C-3), 85.18 (C-17), 124.28 (CH), 127.74 (CH), 128.97 (CH), 139.95 (CH), 150.76 (C-2′, Py); GC/MS (m/e, %): 304 ([M-CH$_2$Py-O]$^+$, 100), 286 ([M-CH$_2$Py-O-H$_2$O]$^+$, 29), 271 (24), 253 (28), 227 (16); HRMS (TOF), *m*/*z*: for $C_{25}H_{35}NO_4$ [M + H]$^+$ calcd. 414.26389, found 414.26441.

4.1.2. 17β-Hydroxy-17α-picolyl-androst-4-ene-3,6-dione (**4**)

Jones reagent [14] (2 ml) was added dropwise (~ 1 drop/10 s) to a solution of compound **1** (0.352 g, 0.923 mmol) in acetone (35 ml) at 0 °C, with vigorous stirring, and the reaction mixture was stirred for 1.5 h. The resulting reaction mixture was then quenched with methanol (15 ml, to eliminate any excess reagent) and solvent was removed *in vacuo*. Water (20 ml) and $NaHCO_3$ (to pH 8) were then added, and crude product was extracted with dichloromethane (5 × 10 ml). Finally, purification by column chromatography (15 g silica gel, toluene-ethyl acetate 7:1) afforded pure compound **4** (0.044 g, 12%, m.p. 243–245 °C after recrystallization from dichloromethane-hexane).

IR (KBr, cm^{-1}): 3299, 3063, 2923, 2857, 1726, 1693, 1596, 1569, 1440, 1378, 1264, 1119, 1055, 1022, 869; ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.01 (s, 3H, H-18), 1.20 (s, 3H, H-19), 2.80 (d, 1H, J_{gem} = 14.5 Hz, C<u>H</u>$_2$Py), 3.09 (d, 1H, J_{gem} = 14.5 Hz, C<u>H</u>$_2$Py), 6.19 (s, 1H, H-4), 7.18 (m, 2H, H-3′ and H-5′, Py), 7.65 (td, 1H, $J_{3',4'}$ = $J_{4',5'}$ = 7.7 Hz, $J_{4',6'}$ = 1.7 Hz, H-4′, Py), 8.47 (d, 1H, $J_{6',5'}$ = 4.4 Hz, H-6′, Py); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 14.08 (C-18), 17.50 (C-19), 18.72, 20.56, 23.58, 31.64, 33.95, 34.84, 35.55, 35.73, 39.75, 42.94, 46.55, 50.94, 50.99, 83.06 (C-17), 121.53 (C-5′, Py), 124.68 (C-3′, Py), 125.51 (C-4), 136.93 (C-4′, Py), 148.02 (C-6′, Py), 160.27 (C-2′, Py), 160.82 (C-5), 199.46 (C-3), 201.95 (C-6); HRMS (TOF), *m*/*z*: for $C_{25}H_{32}NO_3$ [M + H]$^+$ calcd. 394.23767, found 394.23581.

4.1.3. 17α-Picolyl-androst-5-en-3β,4α,17β-triol (**5**) and 17α-picolyl-androst-5-en-3β,4β,17β-triol (**6**)

Selene-dioxide (0.089 g, 0.81 mmol) was added to a solution of compound **1** (0.200 g, 0.52 mmol) dissolved in dioxane (4 ml), and the reaction mixture was stirred under reflux for 25.5 h. The resulting mixture was then filtered, and the precipitate washed with dichloromethane (10 ml) and ethyl acetate (10 ml). Water (10 ml) was then added, and the mother liquor was extracted with dichloromethane (5 × 10 ml). After drying and solvent removal, crude products were separated by column chromatography (10 g silica gel, hexane-ethyl acetate 2:1 and 1:2), affording pure compound **6** (0.045 g, 22%) and compound **5** (0.058 g, 28%), both in the form of a colorless oil.

Compound **5**: IR (film, cm^{-1}): 3353, 2926, 2854, 1665, 1596, 1570, 1438, 1377, 1260, 1216, 1053, 1080, 955, 757; ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.98 (s, 3H, H-18), 1.04 (s, 3H, H-19), 2.88 (d, 1H, J_{gem} = 14.5 Hz, C<u>H</u>$_2$Py), 3.10 (d, 1H, J_{gem} = 14.5 Hz, C<u>H</u>$_2$Py), 3.60 (m, 1H, H-3), 3.89 (t, 1H, *J* = 3.8 Hz, H-4), 5.64 (m, 1H, H-6), 7.18 (m, 2H, H-3′ and H-5′, Py), 7.63 (td, 1H, $J_{3',4'}$ = $J_{4',5'}$ = 7.7 Hz, $J_{4',6'}$ = 1.8 Hz, H-4′, Py), 8.46 (d, 1H, $J_{5',6'}$ = 4.1 Hz, H-6′, Py); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 13.90 (C-18), 18.27 (C-19), 20.47, 21.47, 23.99, 29.69, 31.38, 31.78, 36.07, 37.09, 38.45, 42.00, 42.47, 43.01, 46.09, 65.06 (C-4), 71.35 (C-3), 83.52 (C-17), 121.35 (C-5′, Py), 123.81 (C-3′, Py), 124.86 (C-6), 136.79 (C-4′, Py), 146.44 (C-5), 147.90 (C-6′, Py), 160.83 (C-2′, Py); GC/MS (m/e, %): 271 ([M-CH$_2$Py-2OH]$^+$, 41), 260 (100), 253 (58); HRMS (TOF), *m*/*z*: for $C_{25}H_{36}NO_3$ [M + H]$^+$ calcd. 398.26897, found 398.26828.

Compound **6**: IR (film, cm^{-1}): 3362, 2926, 2855, 1661, 1597, 1438, 1379, 1250, 1052, 1027, 967, 757, 666; ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.98 (s, 3H, H-18), 1.22 (s, 3H, H-19), 2.80 (d, 1H, J_{gem} = 14.5 Hz, C<u>H</u>$_2$Py), 3.08 (d, 1H, J_{gem} = 14.5 Hz, C<u>H</u>$_2$Py), 3.57 (m, 1H, H-3), 4.16 (d, 1H, *J* = 3.2 Hz, H-4), 5.71 (m, 1H, H-6), 7.16 (m, 2H, H-3′ and H-5′, Py), 7.64 (td, 1H, $J_{3',4'}$ = $J_{4',5'}$ = 7.7 Hz, $J_{4',6'}$ = 1.8 Hz, H-4′, Py), 8.46 (m, 1H, H-6′, Py); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 14.11 (C-18), 20.27, 21.05 (C-19), 23.96, 25.41, 29.69, 32.13, 32.64, 36.13, 37.01, 38.14, 43.19, 46.34, 50.33, 51.31, 72.48 (C-3), 77.24 (C-4), 83.48 (C-17), 121.35 (C-5′, Py), 124.74 (C-3′, Py), 128.46 (C-6), 136.75 (C-4′, Py), 142.94 (C-5), 148.01 (C-6′, Py), 160.80 (C-2′, Py); GC/MS (m/e, %): 304 ([M-CH$_3$Py]$^+$, 23), 286 ([M-CH$_3$Py-H$_2$O]$^+$, 45), 260 (100), 247 (75); HRMS (TOF), *m*/*z*: for $C_{25}H_{36}NO_3$ [M + H]$^+$ calcd. 398.26897, found 398.26769.

4.1.4. General procedure for 17β-hydroxy-17α-picolyl-androst-5-en-3β-yl p-toluenesulfonylate (**7**) and 17-picolinyliden-androst-5-en-3β-yl p-toluenesulfonylate (**9**)

A solution of p-toluenesulfonylchloride (1.028 g, 5.41 mmol for compound **1** or 0.511 g, 2.69 mmol for compound **2**) was added to **1** (0.506 g, 1.33 mmol) or **2** (0.200 g, 0.55 mmol) dissolved in absolute pyridine (8 ml or 4 ml) at 0 °C; and the resulting mixture was stirred 45 h (for **1**) or 19 h (for **2**) at room temperature. Reactions were stopped by addition of ice, and poured into water (20 or 40 ml). Crude product (**7**) was precipitated and purified by column chromatography (30 g silica gel, toluene-ethyl acetate 20:1 and 15:1), giving pure compound **7** (0.140 g, 20%, m.p. 163–165 °C after recrystallization from dichloromethane-hexane). The reaction mixture for **9** was extracted with dichloromethane (8 × 10 ml), and, after drying and solvent removal, crude product (**9**) was recrystallized from dichloromethane-hexane, giving pure compound **9** (0.145 g, 51%, m.p. 176–178 °C).

Compound **7**: IR (KBr, cm^{-1}): 3323, 2951, 2870, 1597, 1569, 1473, 1438, 1361, 1188, 1175, 1098, 938, 866, 755, 667; ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.94 (s, 3H, H-18), 1.00 (s, 3H, H-19), 2.45 (s, 3H, CH_3 from OTs group), 2.79 (d, 1H, J_{gem} = 14.5 Hz, C<u>H</u>$_2$Py), 3.06 (d, 1H, J_{gem} = 14.5 Hz, C<u>H</u>$_2$Py), 4.36 (m, 1H, H-3), 5.32 (d, 1H, *J* = 4.9 Hz, H-6), 7.16 (m, 2H, H-3′ and H-5′, Py), 7.34 (d, 2H from OTs group), 7.63 (td,

1H, $J_{3',4'} = J_{4',5'} = 7.8$ Hz, $J_{4',6'} = 1.7$ Hz, H-4′, Py), 7.80 (d, 2H from OTs group), 8.46 (d, 1H, $J_{5',6'} = 5.6$ Hz, H-6′, Py); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 14.05 (C-18), 19.13 (C-19), 20.67, 21.62 (CH_3, OTs), 23.93, 28.59, 31.75, 32.05, 32.55, 35.94, 36.45, 36.93, 38.84, 43.11, 46.26, 49.99, 50.94, 82.27 (C-3), 83.42 (C-17), 121.34 (C-5′, Py), 123.23 (C-6), 124.73 (C-3′, Py), 127.57 (C-2′ and C-6′, OTs), 129.72 (C-3′ and C-5′, OTs), 134.71 (C-4′, OTs), 136.76 (C-4′, Py), 138.95 (C-5), 144.38 (C-1′, OTs), 147.95 (C-6′, Py), 160.72 (C-2′, Py); HRMS (TOF), *m/z*: for $C_{32}H_{42}NSO_4$ $[M + H]^+$ calcd. 536.28291, found 536.28358.

Compound **9**: IR (film, cm^{-1}): 2945, 1653, 1586, 1562, 1467, 1432, 1362, 1175, 1098, 938, 865, 753. ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.88 (s, 3H, H-18), 1.01 (s, 3H, H-19), 2.44 (s, 3H, CH_3 from OTs), 4.32 (m, 1H, H-3), 5.32 (d, 1H, $J = 5.0$ Hz, H-6), 6.26 (s, 1H, H-20), 7.07 (m, 2H, H-3′ and H-5′, Py), 7.33 (d, 2H from OTs group), 7.65 (t, 1H, $J_{4',3'} = J_{4',5'} = 9.2$ Hz, H-4′, Py), 7.79 (d, 2H from OTs group), 8.58 (m, 1H, H-6′, Py); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 18.50 (C-18), 19.14 (C-19), 20.94, 21.58 (CH_3, OTs), 24.94, 28.51, 29.81, 31.48, 31.61, 35.54, 36.40, 36.81 (C-10), 38.81, 45.79 (C-13), 50.09, 53.74 (C-9), 82.17 (C-3), 117.33 (C-20), 120.39 (C-5′, Py), 123.16 (C-6), 126.01 (C-3′, Py), 127.57 (C-2′ and C-6′, OTs), 129.70 (C-3′ and C-5′, OTs), 134.53 (C-4′, OTs), 136.55 (C-4′, Py), 138.93 (C-5), 144.38 (C-1′, OTs), 148.39 (C-6′, Py), 156.93 (C-17), 161.24 (C-2′, Py).

4.1.5. General procedure for 17α-picolyl-androsta-3,5-dien-17β-ol (**8**) and 17-picolinyliden-androsta-3,5-diene (**10**)

Compound **7** (0.080 g, 0.149 mmol) or compound **9** (0.379 g, 0.733 mmol) were stirred with bis(2-aminoethyl)amine (4.5 ml, or 20 ml) under reflux for 1 h, cooled to room temperature, poured into water, and extracted with dichloromethane (5 × 10 ml). The organic phase was dried, filtered and evaporated. Crude product was then purified by column chromatography (1 g silica gel, toluene-ethyl acetate 30:1 for **8**, or 6 g silica gel, toluene-ethyl acetate 30:1 for **10**), affording pure compound **8** (0.008 g, 15%) and pure compound **10** (0.018 g, 7%), both in the form of a colorless oil.

Compound **8**: IR (film, cm^{-1}): 3328, 3017, 2926, 2855, 1652, 1596, 1570, 1438, 1377, 1024, 846, 753; ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.99 (s, 3H, H-18), 1.26 (s, 3H, H-19), 2.82 (d, 1H, $J_{gem} = 14.6$ Hz, CH_2Py), 3.09 (d, 1H, $J_{gem} = 14.6$ Hz, CH_2Py), 5.41 (m, 1H, H-3), 5.63 (m, 1H, H-6), 5.94 (d, 1H, $J = 9.9$ Hz, H-4), 7.16 (m, 2H, H-3′ and H-5′, Py), 7.63 (td, 1H, $J_{4',5'} = J_{4',3'} = 7.7$ Hz, $J_{4',6'} = 1.8$ Hz, H-4′, Py), 8.47 (m, 1H, H-6′, Py); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 14.21 (C-18), 18.77 (C-19), 20.66, 23.03, 23.89, 29.68, 31.68, 32.20, 32.56, 33.83, 36.04, 43.22, 46.46, 48.53, 51.33, 83.48 (C-17), 121.31 (C-5′, Py), 122.80 (C-6), 124.74 (C-3′, Py), 125.14 (C-4), 128.90 (C-3), 136.72 (C-4′, Py), 141.56 (C-5), 147.99 (C-6′, Py), 160.88 (C-2′, Py); HRMS (TOF), *m/z*: for $C_{25}H_{34}NO$ $[M + H]^+$ calcd. 364.26349, found 364.26255.

Compound **10**: IR (film, cm^{-1}): 3016, 2963, 2851, 1652, 1586, 1560, 1470, 1428, 1372, 1149, 857, 756; ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.94 (s, 3H, H-18), 1.01 (s, 3H, H-19), 5.42 (m, 1H, H-3), 5.61 (m, 1H, H-6), 5.95 (d, 1H, $J_{4,3} = 9.7$ Hz, H-4), 6.25 (t, 1H, $J = 2.4$ Hz, H-20), 7.03 (m, 1H, H-5′, Py), 7.29 (m, 1H, H-3′, Py), 7.61 (td, 1H, $J_{4',5'} = J_{4',3'} = 7.8$ 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 (62.5 MHz, $CDCl_3$): δ 18.79 (C-18), 18.83 (C-19), 21.01, 23.03, 24.98, 29.87, 31.62, 31.66, 33.77, 35.32, 35.82, 45.90, 48.67, 54.17, 118.02 (C-20), 120.17 (C-5′, Py), 122.77 (C-3′, Py), 124.72 (C-6), 125.16 (C-4), 128.89 (C-3), 135.80 (C-4′, Py), 141.57 (C-5), 149.16 (C-6′, Py), 157.65 (C-17), 160.44 (C-2′, Py); HRMS (TOF), *m/z*: for $C_{25}H_{32}N$ $[M + H]^+$ calcd. 346.25293, found 346.25184.

4.1.6. General procedure for 17β-hydroxy-4-methoxy-17α-picolyl-androst-4-en-3-one (**15**) and 4-methoxy-17-picolinyliden-androst-4-en-3-one (**18**)

A solution of 4 M NaOH (2 ml or 0.6 ml) was added to a solution of a mixture of compounds **13** and **14** [9] (0.175 g, 0.44 mmol) or compounds **16** and **17** [9] (0.056, 0.14 mmol) in methanol (22 ml or 7 ml), and the reaction mixture was stirred under reflux for 3.5 h (or 3 h). After reaction completion, the resulting reaction mixture was extracted with dichloromethane (5 × 10 ml or 5 × 5 ml), solvent was dried and removed, and crude product was separated by column chromatography (5 g or 1 g silica gel). Elution with hexane-ethyl acetate 3:1 (for **15**) or toluene-ethyl acetate 9:1 (for **18**) afforded pure compound **15** (0.007 g, 4%), or pure compound **18** (0.006 g, 10%), both in the form of a colorless oil.

Compound **15**: IR (film, cm^{-1}): 3391, 2929, 1681, 1597, 1570, 1439, 1379, 1317, 1260, 1214, 1086, 1022, 927, 891, 800, 756; ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.00 (s, 3H, H-18), 1.22 (s, 3H, H-19), 2.80 (d, 1H, $J_{gem} = 14.3$ Hz, CH_2Py), 3.07 (d, 1H, $J_{gem} = 14.3$ Hz, CH_2Py), 3.60 (s, 3H, from 4-OCH_3), 4.72 (bs, 1H, 17β-OH), 7.18 (m, 2H, H-3′ and H-5′, Py), 7.63 (td, 1H, $J_{4',5'} = J_{4',3'} = 7.6$ Hz, $J_{4',6'} = 1.7$ Hz, H-4′, Py), 8.46 (d, 1H, $J_{6',5'} = 5.1$ Hz, H-6′, Py); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 14.15 (C-18), 17.50 (C-19), 20.69, 23.41, 23.79, 29.66, 31.90, 34.25, 34.84, 35.91 (C-10), 36.01, 38.78, 43.02, 44.23 (C-13), 50.20 (C-14), 54.33 (C-9), 60.23 (O—CH_3), 83.34 (C-17), 121.43 (C-5′, Py), 124.75 (C-3′, Py), 136.88 (C-4′, Py), 146.21 (C-5), 147.23 (C-4), 147.91 (C-6′, Py), 160.57 (C-2′, Py), 191.39 (C-3); GC/MS (m/e, %): 316 ([M-CH_3Py] $^+$, 59), 284 ([M-CH_3Py-CH_3OH] $^+$, 26), 273 (100); HRMS (TOF), *m/z*: for $C_{26}H_{36}NO_3$ $[M + H]^+$ calcd. 410.26897, found 410.26718.

Compound **18**: IR (film, cm^{-1}): 2924, 1682, 1607, 1586, 1561, 1470, 1435, 1359, 1334, 1314, 1207, 1150, 1095, 1066, 1035, 776, 732. ^{1}H NMR (250 MHz, $CDCl_3$): δ 0.93 (s, 3H, H-18), 1.25 (s, 3H, H-19), 3.59 (s, 3H, from 4-OCH_3), 6.23 (s, 1H, H-20), 7.04 (m, 1H, H-5′, Py), 7.27 (m, 1H, H-3′, Py), 7.62 (td, 1H, $J_{4',5'} = J_{4',3'} = 7.8$ Hz, $J_{4',6'} = 1.8$ Hz, H-4′, Py), 8.57 (d, 1H, $J_{6',5'} = 4.7$ Hz, H-6′, Py); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 17.52 (C-18), 18.80 (C-19), 21.06, 23.40, 24.92, 29.74, 31.40, 34.24, 34.81, 35.14, 35.70 (C-10), 38.80, 45.72 (C-13), 53.15 (C-14), 54.43 (C-9), 60.32 (O—CH_3), 118.14 (C-20), 120.30 (C-5′, Py), 122.82 (C-3′, Py), 135.89 (C-4′, Py), 146.66 (C-5), 149.16 (C-6′, Py), 154.96 (C-4), 157.46 (C-17), 159.82 (C-2′, Py), 194.14 (C-3); GC/MS (m/e, %): 391 (M^+, 100), 377 (59), 348 (6), 170 (7), 130 (20), 93 (15); HRMS (TOF), *m/z*: for $C_{26}H_{34}NO_2$ $[M + H]^+$ calcd. 392.25841, found 392.25724.

4.1.7. 4,17β-Dihydroxy-17α-picolyl-androsta-4,6-dien-3-one (**19**) and 17β-hydroxy-17α-picolyl-androst-4-en-3,6-dione (**4**)

1 M potassium *tert*-butoxide (2.5 ml) was added dropwise to a solution of compound **11** (0.202 g, 0.54 mmol) dissolved in *tert*-butyl alcohol (6 ml) at room temperature, and the reaction mixture was stirred at 45 °C for 3 h (protected from light). When the reaction was complete, the resulting mixture was poured into water (10 ml) and extracted with ethyl acetate (5 × 10 ml), followed by dichloromethane (5 × 10 ml). Combined extracts were then dried and solvent removed. The resulting crude product was separated and purified by column chromatography (20 g silica gel, toluene-ethyl acetate 8:1 and 5:1), giving compound **19** (0.052 g, 25%, m.p. 147–149 °C after recrystallization from methanol) and compound **4** (0.028 g, 13%, m.p. 243–245 °C after recrystallization from methanol).

Compound **19**: IR (KBr, cm^{-1}): 3423, 3054, 2941, 2872, 1655, 1616, 1597, 1570, 1380, 1265, 894; ^{1}H NMR (250 MHz, $CDCl_3$): δ 1.04 (s, 3H, H-18), 1.12 (s, 3H, H-19), 2.80 (d, 1H, $J_{gem} = 14.5$ Hz, CH_2Py), 3.10 (d, 1H, $J_{gem} = 14.5$ Hz, CH_2Py), 6.06 (dd, 1H, $J_{6,7} = 9.4$ Hz, $J_{7,8} = 2.0$ Hz, H-7), 6.65 (dd, 1H, $J_{6,7} = 9.4$ Hz, $J_{6,8} = 2.8$ Hz, H-6), 7.18 (m, 2H, H-3′ and H-5′, Py), 7.66 (td, 1H, $J_{3',4'} = J_{4',5'} = 7.6$ Hz, $J_{4',6'} = 1.8$ Hz, H-4′, Py), 8.47 (d, 1H, $J_{5',6'} = 4.0$ Hz, H-6′, Py); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 14.08 (C-18), 16.44 (C-19), 20.27, 23.43, 29.70, 31.93, 33.29, 35.75, 36.48, 37.93, 42.98, 46.87, 48.04, 51.44, 83.17 (C-17), 121.55 (C-5′, Py), 123.94 (C-3′, Py), 124.71 (C-6), 134.70 (C-5),

136.76 (C-4′, Py), 137.10 (C-7), 140.21 (C-4), 147.83 (C-6′, Py), 160.39 (C-2′, Py), 193.77 (C-3); HRMS (TOF), *m*/*z*: for $C_{25}H_{32}NO_3$ $[M + H]^+$ calcd. 394.23767, found 394.24972.

4.1.8. 17α-Picolyl-androst-4-en-3β,17β-diol (**22**)

$NaBH_4$ (0.142 g, 3.74 mmol) was added to a solution of compound **11** (0.203 g, 0.535 mmol) dissolved in absolute ethanol (15 ml), and the reaction mixture was stirred at room temperature for 2.5 h. When the reaction was complete, the resulting mixture was poured into water (20 ml), and crude product was filtered and recrystallized from dichloromethane-methanol, to give pure compound **22** (0.191 g, 94%, m.p. 96–98 °C) as white crystals.

Compound **22**: IR (KBr, cm^{-1}): 3417, 2934, 2853, 1634, 1597, 1570, 1440, 1380, 1327, 1230, 1193, 1101, 1056, 1031, 860, 760, 657, 588; ^1H NMR (250 MHz, $CDCl_3$): δ 0.97 (s, 3H, H-18), 1.08 (s, 3H, H-19), 2.78 (d, 1H, $J_{gem} = 14.5$ Hz, C$\underline{H}_2$Py), 3.07 (d, 1H, $J_{gem} = 14.5$ Hz, C$\underline{H}_2$Py), 4.15 (m, 1H, H-3), 5.30 (s, 1H, H-4), 7.17 (m, 2H, H-3′ and H-5′, Py), 7.64 (td, 1H, $J_{\underline{4',3'}} = J_{\underline{4',5'}} = 7.7$ Hz, $J_{4',6'} = 1.8$ Hz, H-4′, Py), 8.45 (d, 1H, $J_{6',5'} = 4.4$ Hz, H-6′, Py); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 14.16 (C-18), 18.92 (C-19), 20.68 (C-11), 23.84, 29.50 (C-2), 32.14 (C-7), 32.17, 32.92 (C-1), 35.43, 35.88, 36.65 (C-8), 37.42 (C-10), 43.11 (C-20), 46.28 (C-13), 50.37 (C-14), 54.60 (C-9), 67.93 (C-3), 83.41 (C-17), 121.32 (C-5′, Py), 123.41 (C-4), 124.71 (C-3′, Py), 136.74 (C-4′, Py), 147.90 (C-5), 147.96 (C-6′, Py), 160.77 (C-2′, Py); HRMS (TOF), *m*/*z*: for $C_{25}H_{36}NO_2$ $[M + H]^+$ calcd. 382.27406, found 382.27769.

4.1.9. 17β-Hydroxy-17α-picolyl-androst-4-en-3β-yl acetate (**23**)

Acetic anhydride (1.5 ml) was added to a solution of compound **22** (0.049 g, 0.13 mmol) in absolute pyridine (1.5 ml) and the reaction mixture was stirred at room temperature for 24 h. The resulting reaction mixture was slowly poured into cold water (10 ml), yielding a precipitate, which was filtered and recrystallized from dichloromethane-hexane, affording pure compound **23** (0.031 g, 56%, m.p. 179–181 °C) as white crystals.

Compound **23**: IR (KBr, cm^{-1}): 3437, 2942, 2852, 1730, 1598, 1570, 1441, 1371, 1240, 1097, 1023, 970, 927, 901, 760, 675, 612; ^1H NMR (250 MHz, $CDCl_3$): δ 0.97 (s, 3H, H-18), 1.09 (s, 3H, H-19), 2.06 (s, 3H, CH_3 from OAc), 2.78 (d, 1H, $J_{gem} = 14.6$ Hz, C$\underline{H}_2$Py), 3.05 (d, 1H, $J_{gem} = 14.6$ Hz, C$\underline{H}_2$Py), 5.24 (m, 1H, H-3), 5.30 (m, 1H, H-4), 6.65 (bs, 1H, 17β-O$\underline{H}\cdots$N-Py), 7.15 (m, 2H, H-3′ and H-5′, Py), 7.63 (td, 1H, $J_{4',3'} = J_{4',5'} = 7.8$ Hz, $J_{4',6'} = 1.6$ Hz, H-4′, Py), 8.45 (d, 1H, $J_{6',5'} = 5.2$ Hz, H-6′, Py); ^{13}C NMR (62.5 MHz, $CDCl_3$): δ 14.15 (C-18), 18.84 (C-19), 20.65, 21.45 (CH_3 from OAc), 23.86, 25.08, 32.18, 32.77, 35.04, 35.94, 36.62, 37.42, 43.13, 46.43, 50.32, 54.38, 70.88 (C-3), 83.37 (C-17), 119.04 (C-4), 121.32 (C-5′, Py), 124.73 (C-3′, Py), 136.75 (C-4′, Py), 147.98 (C-5), 149.48 (C-6′, Py), 160.82 (C-2′, Py), 171.04 (C═O from OAc); HRMS (TOF), *m*/*z*: for $C_{27}H_{38}NO_3$ $[M + H]^+$ calcd. 424.28462, found 424.28428.

4.2. Cytotoxicity

Cell lines. Five human tumor cell lines and one human non-tumor cell line were used in the present study: human breast adenocarcinoma ER+, MCF-7; human breast adenocarcinoma ER-, MDA-MB-231; prostate cancer AR-, PC-3; human cervix carcinoma, HeLa; colon cancer, HT-29; and normal fetal lung fibroblasts, MRC-5.

Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with addition of 4.5% glucose. Media were supplemented with 10% fetal calf serum (FCS, NIVNS) and the following 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 a 100% humidity atmosphere with 5% CO_2. Only viable cells were used in the assay. Viability was determined by the dye exclusion assay with trypan blue.

Cytotoxicity assay. Cytotoxic activity was evaluated using the colorimetric sulforhodamine B (SRB) assay, following the method of Skehan et al. [18]. Briefly, a single cell suspension was plated onto 96-well microtitar plates (Costar, flat bottom): 5×10^3 cells/well in 180 μl of medium. Plates were pre-incubated 24 h at 37 °C, 5% CO_2. Tested substances were then added to all wells (except controls), at concentrations ranging from 10^{-8} to 10^{-4} M. After incubation (48 h/37 °C/5% CO_2), the SRB assay was carried out as follows: 50 μl of 50% trichloroacetic acid (TCA) was added to all wells and plates were incubated for 1 h; plates were washed with distilled water, 75 μl of 0.4% SRB was then added to all wells and plates were incubated for 30 min; plates were then 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, and absorbance (A) was measured at 540/690 nm on a microplate reader (Multiscan MCC340, Labsystems). Wells without cells, but containing complete medium only, acted as blanks. Cytotoxic activity was calculated according to the formula:

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

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

Data analysis. Two independent experiments were conducted in quadruplicate for each concentration of tested compound. Mean values and standard deviations (SD) was calculated for each concentration. IC_{50} value defines the dose of compound that inhibits cell growth by 50%. The IC_{50} of each tested compound was determined by median effect analysis [19].

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejmech.2012.06.030.

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