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Synthesis, structural analysis and antitumor activity of novel 17α-picolyl and 17(*E*)-picolinylidene A-modified androstane derivatives

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ABSTRACT

The heterocyclic ring at C-17 position of the androstane compounds plays an important role in biological activity. The aim of the present study was to synthesize and evaluate potential antitumor activity of different A-modified 17α-picolyl and 17(*E*)-picolinylidene androstane derivatives. In several synthetic steps, novel derivatives bearing the hydroximino, nitrile or lactame functions in A-ring were synthesized and characterized according to the spectral data, by mass analysis as well as XRD analysis (compounds **6**, **13** and **15**). The structurally most promising compounds **6**, **11**–**17** were investigated as antitumor agents. The in vitro antiproliferative activity was evaluated against six human cancer cell lines: estrogen receptor negative (ER−) breast adenocarcinoma (MDA-MB-231); estrogen receptor positive (ER+) breast adenocarcinoma (MCF-7); prostate cancer (PC-3); human cervical carcinoma (HeLa); lung adenocarcinoma (A549) and colon adenocarcinoma (HT-29) using MTT assay. The results of the 48 h incubation time in vitro tests showed that compound **15** was the most effective against PC-3 (IC_{50} 6.6 μM), compound **17** against MCF-7 (IC_{50} 7.9 μM) cells, while compound **16** exhibited strong antiproliferative effect against both, MCF-7 (IC_{50} 1.7 μM) and PC-3 (IC_{50} 8.7 μM) cancer cells. It was also found that compounds **16** and **17** induced apoptosis in MCF-7 cells (dicyano derivative **17** stronger then dioxime **16** and reference formestane), with no distinct changes in the cell cycle of MCF-7 cells.

1. Introduction

Most of the steroid based pharmaceuticals are semi-synthetic compounds prepared by connecting a special functionality to the core structure of a steroid.[1] Attention has been devoted in the literature to the synthesis of steroidal compounds because of their potent receptor binding properties and valuable pharmacological activities.[2–4]

Steroidal oximes are significant class of compounds and have well validated biological effects. These molecules have always attracted considerable attention because of being a fundamental class of biological signaling molecules and their profound biological, scientific and clinical importance.[5] They can regulate a variety of biological processes and thus have the potential to be developed as drugs for the treatment of cardiovascular,[6] autoimmune,[7] anticancer diseases,[8,9] and they also express anti-inflammatory,[10] antiallergic,[11] antidotal[12] and antiepileptic[13] properties. Some oximino derivatives, such as 6-hydroximinoandrost-4-ene-3,17-dione and 6-hydroximinoandrosta-1,4-diene-3,17-dione, showed a high affinity for human placental aromatase and to function as competitive inhibitors of this enzyme.[14] Also, some oximes have different industrial applications, for example, as anti-skinning agents in paint, blocking agents in the polymer industry, and chelators in the metals industry.[15]

The synthesis and isolation of some steroidal oximes with interesting structures have been reported recently.[8,9,16–20] These compounds showed cytotoxicity against cancer cells which is independent on the location of the hydroximino group on the steroidal nucleus.[21] Despite the enormous number of steroids found to have unusual and intriguing structures,[22] marine steroids with an oxime group were biologically active,[18] including cytotoxic activity.[21]

The advantage of employing hydrophobic steroid units with oxime group enhance their ability to interact with cell membranes and thus pave the way for biological activity of such molecules. This has been proved by different ring modification studies of steroidal molecules and their derivatives, involving the A- and D-ring,

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whereby incorporation of heteroatom (N or O) have been reported to enhance the biological activities of these molecules.

In our previous work,[23–27] we described the synthesis and characterization of a series of 17-substituted androstane derivatives with strong activity against selected tumor cell lines and/or antiaromatase inhibitory activity. In order to evaluate the combined effect of the 17-heterocyclic ring and hydroximino, nitrile or lactame function on antiproliferative activity of the steroidal compound, we report here the synthesis of new A-modified 17α-picolyl and 17(*E*)-picolinylidene androstane derivatives and their antiproliferative activity against several types of human cancer cells including breast, prostate, cervical, colon and lung adenocarcinoma (MDA-MB-231, MCF-7, PC-3, HeLa, HT-29, A549), as well as healthy fetal lung fibroblasts (MRC-5). Correlations between structure and antiproliferative activity (SAR) of investigated steroid derivatives, as well as inducing of apoptosis and cell cycle changes caused by compounds **16** and **17**, were investigated.

2. Results and discussion

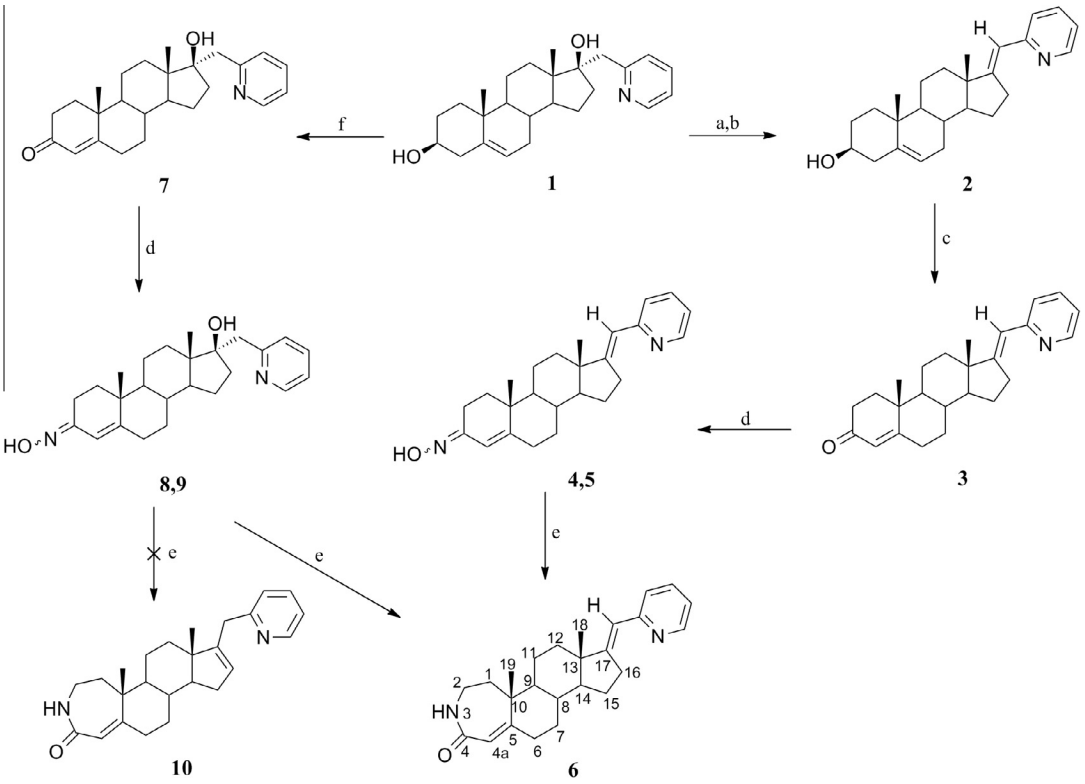
2.1. Synthesis of novel 17α-picolyl and 17(*E*)-picolinylidene A-modified androstane derivatives

To determine the effect of the hydroximino group on the biological role of a steroidal compound, we have synthesized several oximino androstane derivatives, wherein some of them were used as precursors for synthesis of new A-modified derivatives with potential cytotoxic activity. All new compounds were fully characterized by IR, ^{1}H NMR, ^{13}C NMR spectra as well as by mass analysis.

The steroidal 17(*E*)-picolinylidene oximes **4** and **5**, or 17α-picolyl oximes **8** and **9**, with a hydroximino group at C-3 position, were synthesized from **1**[28,29] in 4 or 2 steps, respectively, according to the sequence shown in Scheme 1. The key intermediates in the syntheses of 3-hydroximino derivatives were the corresponding androst-4-en-3-ones **3** or **7**,[23] whose oximation was carried out with hydroxylamine-hydrochloride and sodium-acetate in refluxing ethanol. Resulting mixtures of the stereoisomeric 3(*E*)- and 3(*Z*)-hydroximino compounds **4** and **5**, or **8** and **9**, obtained in good yields, were then subjected to a Beckmann rearrangement, using thionyl-chloride in dioxane, according to known procedure.[30] In this reaction, new enamide-type lactame derivative, 17(*E*)-picolinyliden-3-aza-A-homoandrost-4a-en-4-one (**6**) was obtained from 17(*E*)-picolinylidene oximes **4** and **5**. When the mixture of 17α-picolyl oximes **8** and **9** underwent the same reaction conditions, beside Beckmann rearrangement of A ring, dehydration in D ring also took place, where only thermodynamically more stable 17(*E*)-picolinylidene A-homo lactame **6** was obtained (but not compound **10**).

Formation of the mixture of the *E* and *Z* isomeric oximes **4** and **5**, or **8** and **9** was confirmed by spectroscopic data. Broad IR spectra bands at 3177 (for **4** and **5**), or 3410 cm^{-1} (for **8** and **9**), indicate the presence of a hydroxyl group from =NOH. Two broad singlets at 10.24 and 10.49 ppm in the ^{1}H NMR spectra of compounds **4** and **5**, or at 10.23 and 10.48 ppm in the spectra of compounds **8** and **9**, correspond to the protons from 3(*E*) and 3(*Z*) oximino groups, and are exchangeable in the presence of D_2O. In ^{1}H NMR spectra of the A-homo lactame **6**, the signals for the olefinic H-4a proton, appearing at 5.74 ppm due to deshielding influence by the C_4=O group, and the NH proton at 6.67 ppm were recorded. The structure of A-ring of **6** was established by X-ray diffraction analysis (Fig. 1), and crystallographic data was deposited at the Cambridge Crystallographic Data Centre (CCDC 997657).

Based on the above, we further investigated chemical transformations of starting compounds **3** with 17(*E*)-picolinylidene, and **7** with 17α-picolyl substituent (Scheme 2). Since our first objective was the synthesis of the 2-hydroximino derivatives, for this purpose compounds **3** or **7** were allowed to react with ethyl formate in benzene in the presence of sodium hydride,[31] to give the



Scheme 1. Reagents and reaction conditions: (a) Ac_2O, reflux, 6 h; (b) KOH, MeOH, reflux, 1 h; (c) cyclohexanone, Al(*i*-PrO)$_3$, reflux, 4 h; (d) $NH_2OH{\cdot}HCl$, AcONa, 95% EtOH, reflux, 1.5 h; (e) $SOCl_2$, dioxane, rt, 2 h (for **4** and **5**) or 1 h (for **8** and **9**); (f) cyclohexanone, Al(*t*-BuO)$_3$, reflux, 3 h.

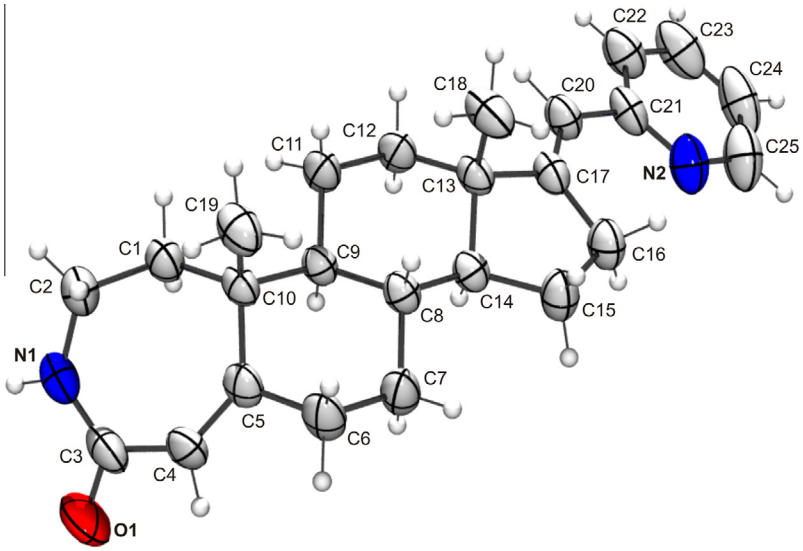
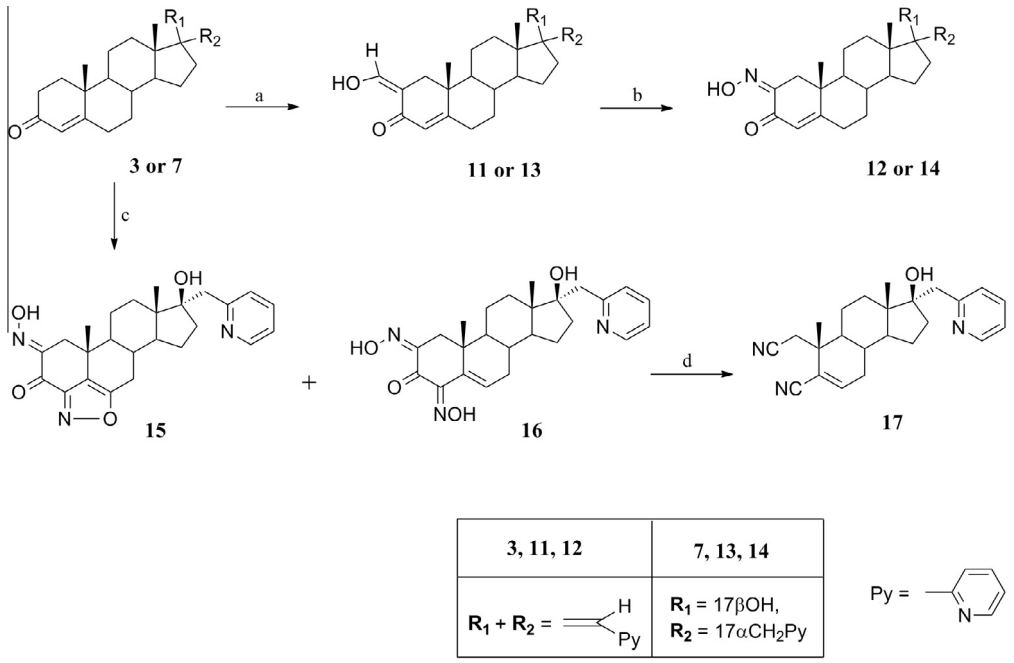


Figure 1. ORTEP drawing of molecular structure of compound **6** with the labelling of non-H atoms. Displacement ellipsoids are shown at the 50% probability level and H atoms are drawn as spheres of arbitrary radii.



Scheme 2. Reagents and reaction conditions: (a) HCOOEt, NaH, abs. benzene, rt, 2.5 h (for **11**) or 2 h (for **13**); (b) 7 M $NaNO_2$, 95% EtOH, 35% HCl, 0 °C, 1 h; (c) *i*-AmONO, 1 M *t*-BuOK, *t*-BuOH, rt, 24 h; (d) Ac_2O, abs. Py, rt, 1.5 h.

corresponding 17(*E*)-picolinylidene or 17α-picolyl 2-hydroxymethylidene derivatives **11** or **13**. Compounds **12** or **14**, containing 2-hydroximino function, were obtained from 2-hydroxymethylidene-3-ketones **11** or **13**, respectively, in reaction with sodium nitrite and hydrochloric acid,[32] at 0 °C for 45 min. The introduction of a 2-hydroxymethylene group in compounds **11** or **13** was confirmed by the presence of an ^{1}H NMR signals at 6.26 and 7.24 ppm corresponding to vinyl protons, and signals for OH protons at 13.74 ppm (for **11**) or 13.76 ppm (for **13**), exchangeable with D_2O. ^{13}C NMR spectral signals were assigned for double bond carbons at 165.29 (=*C*HOH) and 106.42 (C-2) ppm for compound **11**, or at 165.11 (=*C*HOH) and 106.48 (C-2) ppm for compound **13**. The stereochemistry at exocyclic double bond in **13** was established by X-ray diffraction analysis (Fig. 2) (CCDC 997658).

The identity of compounds **12** and **14** was also confirmed by spectroscopic data (IR, ^{1}H NMR, ^{13}C NMR and HRMS), where significant singlet (exchangeable with D_2O) at 12.23 ppm in the ^{1}H NMR spectra of compound **12**, or at 12.20 ppm in the spectra of compound **14**, indicates the presence of 2-hydroximino group. In the ^{13}C NMR spectra signals for C-2 carbons are shifted downfield, at 151.36 ppm for **12** and 151.38 ppm for **14**, compared to starting compound (106.42 ppm for **11** and 106.48 ppm for **13**).

In addition to the above, we investigated the reactivity of 4-en-3-one **7** in the presence of *i*-amyl nitrite and potassium *t*-butoxide,[32] at room temperature for 24 h, where, beside 17β-hydroxy-2(*Z*),4(ξ)-dihydroximino-17α-picolyl-androst-5-en-3-one (**16**), unexpected 2(*E*)-hydroximino-17β-hydroxy-17α-picolyl-isoxazolo[3′,4′,5′:4,5,6]-androst-5-en-3-one (**15**) was also obtained

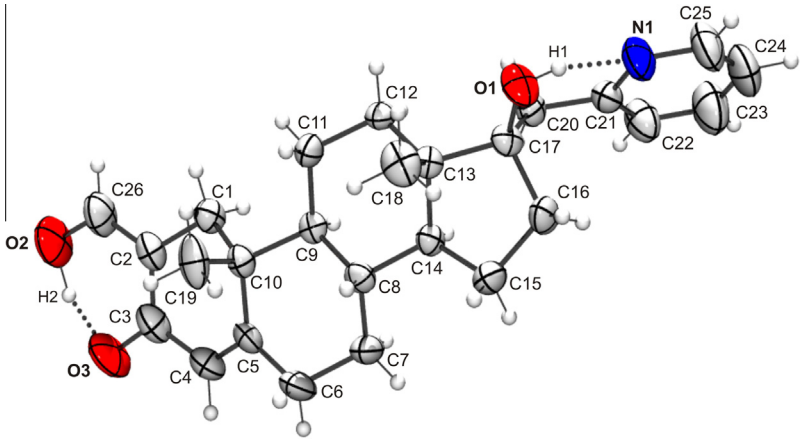


Figure 2. ORTEP drawing of molecular structure of compound **13** with the labelling of non-H atoms. Displacement ellipsoids are shown at the 50% probability level and H atoms are drawn as spheres of arbitrary radii. Intramolecular hydrogen bonds are shown as dashed lines.

(Scheme 2). In the ^{1}H NMR spectra of compound **15**, signal for proton exchangeable with D_2O, from hydroximino group, was recorded at 13.02 ppm, while in ^{13}C NMR spectra new signals at 153.51, 169.38 and 176.79 ppm corresponded quaternary C-4, C-2 and C-6 atoms from hydroximino and isoxazolo groups. The structure of compound **15** was, beside spectroscopic data, unambiguously confirmed by the X-ray structural analysis (Fig. 3) (CCDC 997659). The ^{1}H NMR spectra of the compound **16** indicates two singlets of two newly formed hydroximino groups at 11.85 and 12.69 ppm, while in ^{13}C NMR spectra two quaternary signals for C-2 and C-4 hydroximino groups were identified at 152.00 and 169.34 ppm.

Finally, compound **16** in the treatment with acetic anhydride and pyridine at room temperature was converted into a cleaved dinitrile product **17** by 'abnormal Beckmann rearrangements of the second order'.[33] In the IR spectra of the A-seco nor dinitrile **17**, two bands at 2216 and 2245 cm^{-1} characteristic for nitrile group were found, while the ^{13}C NMR spectra revealed the appearance of the new signals for two nitrile carbons (C-2 and C-4) at 119.56 and 117.20 ppm. In the ^{1}H NMR spectra multiplet at 6.79 ppm, corresponding to H-6 vinyl proton, was identified.

2.2. In vitro antiproliferative activity of A-modified 17-picolyl and 17-picolinylidene androstane derivatives

To determine the effect of different types of transformation in A- and D-ring of the 17-substituted androstane derivatives, we evaluated the antiproliferative activity of selected synthesized compounds against several common human tumor cell lines, originating from 5 different solid tumor types. To evaluate selectivity of cytotoxicity, the selected compounds were also tested against normal fetal lung fibroblasts, MRC-5. Antiproliferative activity was evaluated in vitro after 48 h treatment using the MTT assay.[34] Previously, we reported the cytotoxic activities of several compounds (**1**, **2**, **3** and **7**) with 17α-picolyl or 17(*E*)-picolinylidene substituent groups.[23,24] In the present study, we compare these results with those obtained for newly synthesized compounds **6** and **11**–**17**. Nonselective cytotoxic drug doxorubicin (DOX) and steroidal aromatase inhibitor formestane were used as a positive control for general toxicity. The results of the antiproliferative activity of tested steroidal compounds are given in Table 1.

Interestingly, our results revealed that most of the synthesized compounds demonstrated significant antiproliferative activity against ER+ human breast and AR- prostate cancer cell lines. With respect to breast cancer cells, compounds bearing nitrogen atoms in A-ring: **6** (with A-homo lactame function), **16** (with 2,4-dihydroximino functions), and **17** (with A-seco nor dinitrile system) exhibited strong antiproliferative activity (IC_{50} 12.7, 1.7 and 7.9 μM, respectively) against MCF-7 cells, while 2-hydroxymethylene derivative **13** showed moderate activity to these cells (IC_{50} 19.0 μM), suggesting the importance of the heteroatoms for breast cancer cell cytotoxicity. In contrast, against MDA-MB-231

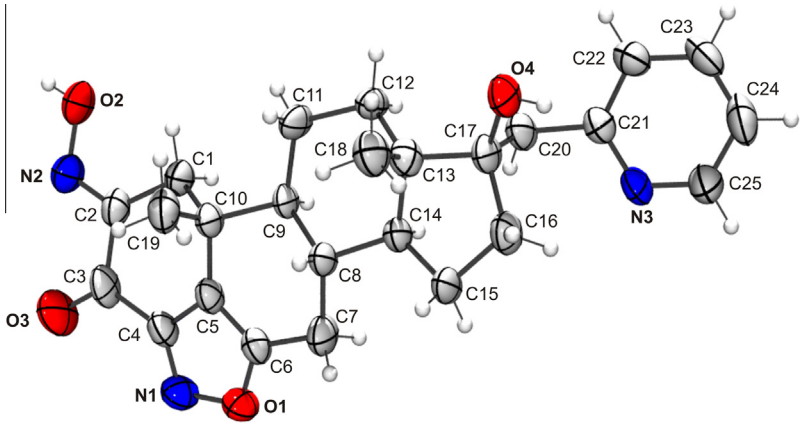


Figure 3. ORTEP drawing of molecular structure of compound **15** with the labelling of non-H atoms. Displacement ellipsoids are shown at the 50% probability level and H atoms are drawn as spheres of arbitrary radii.

Table 1
IC_{50} values (50% inhibitory concentration) of the tested steroidal compounds **1–3**, **6**, **7**, **11** and **13–17** and reference compounds doxorubicin and formestane for different cell lines

Compounds	IC_{50} (μM)						
	MCF-7	MDA-MB-231	PC-3	HeLa	HT-29	A549	MRC-5
1 [23]	>100	96.1	**6.3**	75.2	>100	48.2	>100
2 [23]	>100	>100	>100	>100	>100	>100	>100
3 [23]	>100	>100	**12.9**	>100	>100	>100	>100
6	**12.7**	32.2	60.1	>100	**19.8**	>100	>100
7 [23]	>100	>100	>100	57.8	57.5	>100	>100
11	>100	>100	>100	>100	>100	>100	>100
13	**19.0**	34.4	>100	>100	>100	>100	>100
14	>100	51.3	>100	>100	>100	>100	>100
15	50.4	**25.3**	**6.6**	>100	>100	>100	>100
16	**1.7**	40.1	**8.7**	81.8	**10.3**	56.0	>100
17	**7.9**	**18.2**	31.5	>100	>100	71.8	>100
Doxorubicin	0.75	0.12	95.61	1.17	0.32	7.86	0.12
Formestane	>100	55.5	48.36	5.55	>100	>100	>100

Two experiments in quadruplicate were performed with each test compound, and the standard deviations of the mean results were within ±10%.
Bold values indicate the best antiproliferative effects of the examined compounds.

cells only compound **17** showed moderate antiproliferative activity (IC_{50} 18.2 μM).

With respect to prostate cancer cells, 17α-picolyl androstane derivatives **1**, **15** and **16** displayed markedly strong antiproliferative effect (IC_{50} 6.3, 6.6 and 8.7 μM, respectively), as well as 17-picolinylidene compound **3** with 4-en-3-one function (IC_{50} 12.9 μM).

Against the colon cancer cell line (HT-29), compounds **16** and **6** revealed, according to IC_{50} values, strong or moderate activity (10.3 and 19.8 μM, respectively). Interestingly, none of the tested androstane derivatives were found to inhibit the growth of cervix (HeLa) and lung (A549) cancer cell lines.

It is important to emphasize that all of the tested compounds were confirmed to be nontoxic against healthy MRC-5 cells, whereas doxorubicin was extremely toxic to these cells, consistent with its nonspecific cellular cytotoxicity.[35]

2.3. Structure–activity relationship analysis

The next stage of our research was related to the establishment of correlations between the structure and antiproliferative activity of the tested steroidal derivatives, for three most sensitive cell lines. The effect of structure variations on antitumor activity was determined by comparing activity of pairs of the newly synthesized steroidal derivatives. Antiproliferative activity was quantified as $\Delta \log(IC_{50})$, and represents the difference of the decade logarithms of IC_{50} values of the compared derivatives [log(IC_{50} of chosen compound) – log(IC_{50} of compared compound)]. The negative value of $\Delta \log(IC_{50})$ indicates that the first of compared derivatives has stronger antiproliferative activity, while the positive value corresponds to a higher activity of the second compared derivative with respect to the first. The results of structure–activity relationship analysis are given in Figure 4.

Matching antiproliferative activity of oxime **14** with activities of isoxazol **15** and dioxime **16** it can be seen that **14** was less active in case of all tested cell lines. The biggest difference between the activities of compound **14** and **15** was in case of PC-3 cells, while for pair of compounds **14** and **16** that were MCF-7 and PC-3 cell lines. Comparing derivative **16** with **15** reveals that 2,4-dioxime **16** exhibited stronger impact only to MCF-7 cell line. Comparing the activity of compounds **16** and **17** it can be seen that dioxime **16** showed better antiproliferative effect against MCF-7 and PC-3 cells.

2.4. The influence of the steroidal derivatives 16 and 17 on the cell cycle of MCF-7 cells

Since almost all of the tested compounds were toxic against some cancer cells, we focused our research on identifying preliminary mechanism of action for compounds **16** and **17** against estrogen receptor positive MCF-7 breast cancer cells, as the most active. Hence we performed cell cycle analysis and studied the morphological changes in MCF-7 cells treated with equitoxic doses (IC_{50} concentrations) of the tested compounds over a 48 h time period. Figure 5 shows the effect of the tested compounds on cell cycle of treated cells. The percentages of cells in G1/M, S, G2/M and subG1 phases were calculated and presented on pie chart.

Treatment of MCF-7 cells for 48 h with compounds **16** and **17** resulted in almost the same number of cells in G1/M1 phase, while treatment with formestane resulted in a slight increase, compared to control. Similar situation was in case of G2/M phase. The cell population in the synthetic (S) phase in cultures treated with compounds **16** and **17** was very similar to the control sample, while formestane slightly increased the number of cells in S-phase. The number of hypodiploid (subG1) cells, which are generally considered as an apoptotic population, was very low after 48 h incubation, and very similar as for the control sample.

2.5. Induction of apoptotic cellular morphology by steroidal derivatives

We selected compounds **16** and **17** to study if inducing of apoptosis in MCF-7 cells at the single cell level is underlying the antiproliferative activity expressed by these novel androstane derivatives. As a reference molecule we used formestane. Treated and untreated MCF-7 cells were stained with Giemsa stain. Since morphology represents an important experimental proof of the underlying processes, apoptotic cells were identified by a series of typical morphological changes. Morphology of MCF-7 cells was studied by visual observations using a light microscope. No significant difference was found concerning the quality of specimens between control and treated cells by light microscopy. The quality and clearness of the treated specimens was comparable to those of the control, regardless to the test compound. Figure 6 shows the morphology of MCF-7 cells after treating with equitoxic doses of the tested compounds (**16** and **17** and formestane) for 48 h, as well as untreated cells.

Most cells had normal morphology. Numerous morphological changes indicative of apoptosis were detected: nuclear condensation, vacuolated cytoplasm, degradation of nuclei and cytoplasm, membrane blabbing and apoptotic bodies formation. The cells in different stages of apoptosis in treated cultures were easily distinguishable. For results of cells counting see Supporting information S2, while the percentage of apoptotic cells, as estimated by visual observation of cell morphology, is given in Figure 7. It was found that the number of cells with apoptotic morphology increased

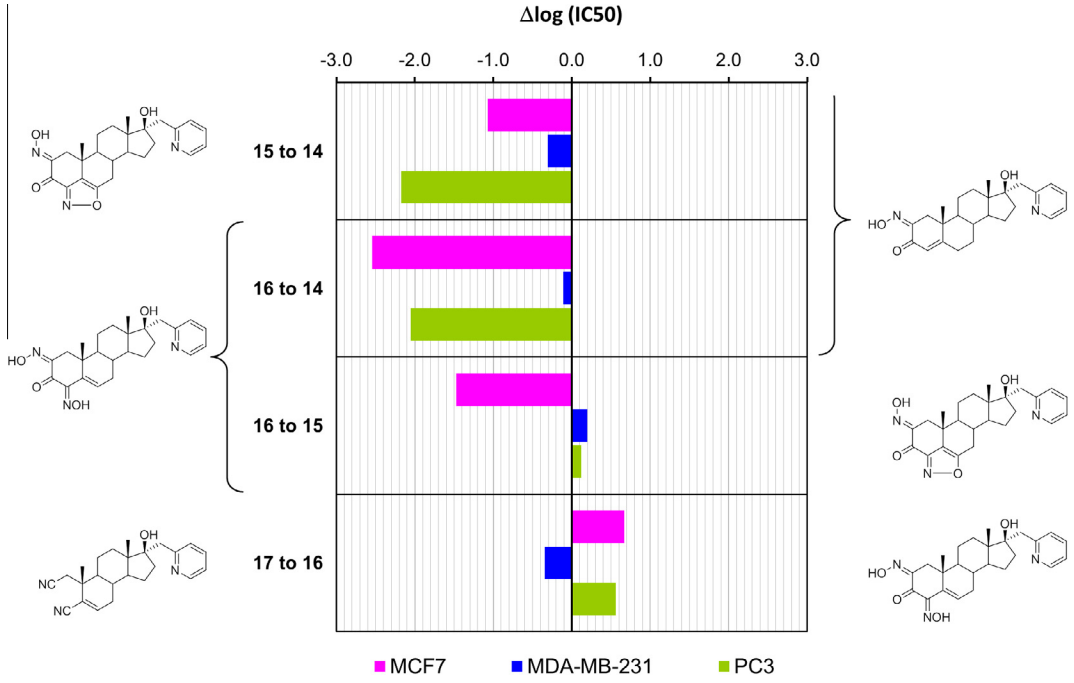


Figure 4. Structure–activity relationship analysis of the tested compounds.

distinctly after treatment with investigated steroidal derivatives. Dioxime **16** and formestane showed similar apoptosis induction effect (12.2% and 11.5%, respectively, compared to 7.3% of control sample), whereas secodinitrile **17** was the most effective in increasing the number of cells featuring apoptotic cellular morphology (16.8%).

Although the number of hypodiploid (subG1) cells in the cell cycle analysis, representing apoptotic cells, indicated low apoptosis rate, test based on quantification of apoptotic cells, identified by a series of typical morphological changes suggested that apoptosis is underlying antiproliferative effect of the tested steroidal compounds.

3. Conclusions

In conclusion, twelve novel 17-substituted A-modified androstane derivatives were synthesized. The in vitro antiproliferative activity for almost all of them was evaluated on six human tumor cell lines. It can be observed that the majority of derivatives showed potent antiproliferative activity against some tumor cell lines, particularly MCF-7, MDA-MB-231 and PC-3 cells. The most consistent cytotoxicity through the whole range of concentrations exerted derivatives **6**, **13**, **16** and **17** on human breast MCF-7 cells, wherein compounds **16** and **17** induced apoptosis in these cells. Based on these results, it can be concluded that incorporation of nitrogen and/or oxygen atoms in steroid A-ring increase the antitumor activity of these molecules. Therefore, these androstane derivatives represent promising candidates for the development of new steroid-based compounds for the treatment of breast and prostate cancer, indicating the need for more detailed future studies.

4. Experimental

4.1. Chemical synthesis

4.1.1. General

Melting points were determined using an electrothermal 9100 apparatus and are reported uncorrected. IR spectra were measured on a NEXUS 670 SP-IR spectrometer. NMR spectra were recorded on a Bruker AC 250E spectrometer, and are reported in ppm (δ-scale) downfield from the tetramethylsilane internal standard. 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 grade. All solutions were dried over anhydrous sodium sulfate.

4.1.2. General procedure for 17(*E*)-picolinylidene and 17α-picolyl 3-hydroximino derivatives (4,5 and 8,9)

Compound **3** (0.262 g, 0.72 mmol) or **7** (0.190 g, 0.50 mmol) was dissolved in 95% ethanol (30 ml), and sodium acetate (0.224 g, 2.73 mmol for **3** or 0.207 g, 2.53 mmol for **7**) and hydroxylamine hydrochloride (0.229 g, 3.33 mmol for **3** or 0.195 g, 2.80 mmol for **7**) were added. The reaction mixture was stirred under reflux for 1.5 h, then poured into water and adjuced to pH 8 with saturated $NaHCO_3$. The white precipitate that formed was filtered off, washed with water and recrystallized from methanol. Mixtures of the isomeric *E* and *Z* oximes **4** and **5** (molar ratio **4**:**5** = 2.6:1), or **8** and **9** (molar ratio **8**:**9** = 2.8:1) were obtained.

4.1.2.1. Mixture of 3(*E*)- and 3(*Z*)-hydroximino-17(*E*)-picolinylidene-androst-4-ene (4 and 5). Yield 90% (0.245 g), white amorphous solid; IR (KBr, ν_{max}/cm^{-1}) 3177, 2938, 2853, 1652, 1590, 1566, 1469, 1435, 1373, 1215, 1153, 1106, 970, 873, 753, 666; ^{1}H NMR (250 MHz; DMSO-d_6; Me_4Si): δ 0.85 (3H, s, H-18), 1.03 (3H, s, H-19), 5.70 (1H, s, H-4), 6.12 (1H, s, H-20), 6.33 (1H, s, H-4), 7.09 (1H, t, $J_{4',5'} = J_{5',6'}$ 4.9 Hz, H-5′ Py), 7.28 (1H, d, $J_{3',4'}$ 7.8 Hz, H-3′ Py), 7.68 (1H, t, $J_{4',3'} = J_{4',5'}$ 7.8 Hz, H-4′ Py), 8.49 (1H, d, $J_{6',5'}$ 4.4 Hz, H-6′ Py), 10.24 (1H, s, =NO*H*), 10.49 (1H, s, =NO*H*); ^{13}C NMR (62.5 MHz; DMSO-d_6; Me_4Si): δ 17.56 (C-18), 18.55, 18.72 (C-19), 21.06, 24.56, 29.90, 31.72, 32.22, 34.43, 35.13, 35.48, 37.56, 40.16 (C-10), 45.34 (C-13), 52.94 (C-9), 53.54 (C-14), 53.82, 110.83 (C-4 from *Z*-oxime), 117.24 (C-20), 117.98 (C-4 from *E*-oxime), 120.43 (C-5′ Py from Z-oxime), 123.03 (C-3′ Py), 136.18

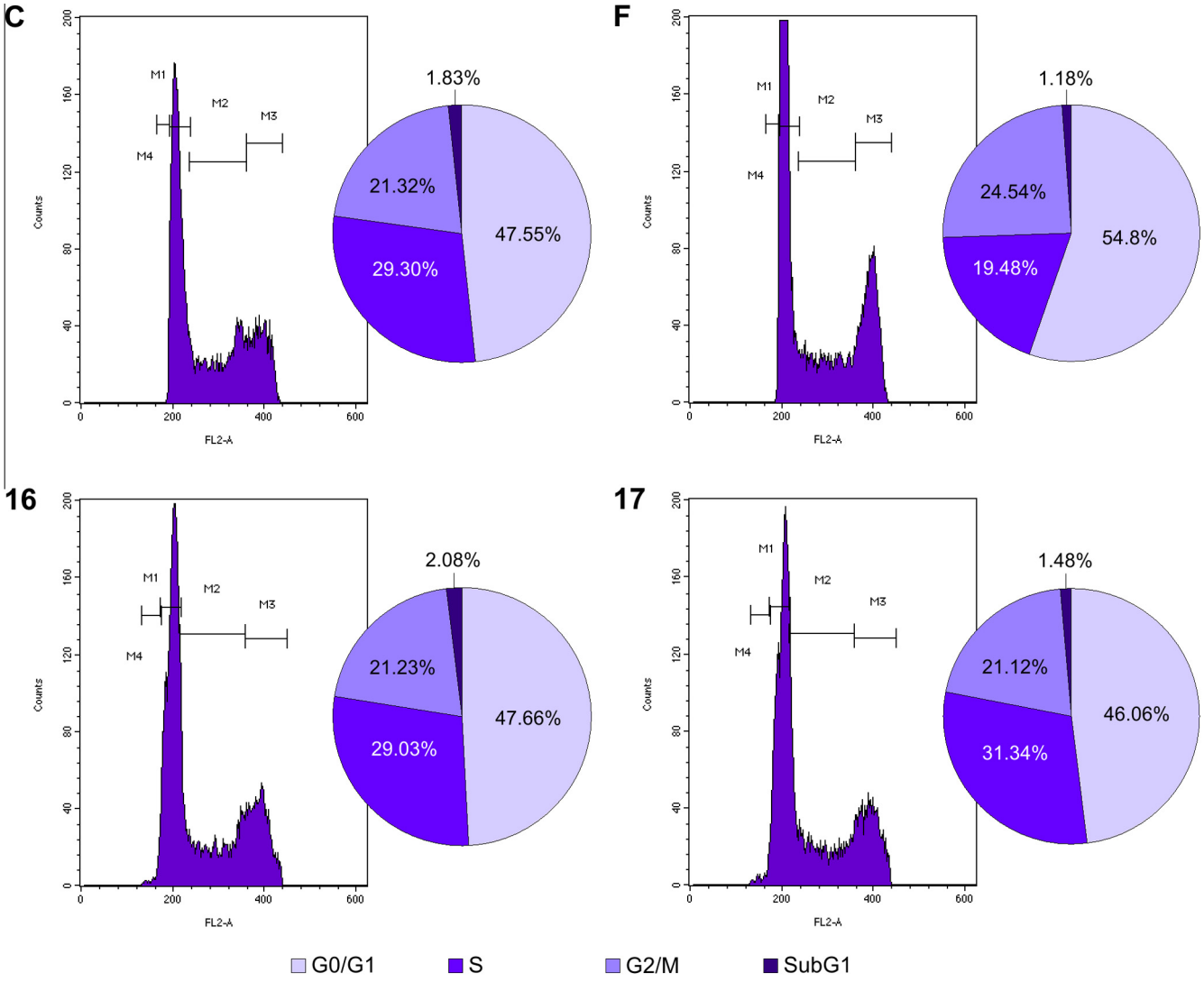


Figure 5. The effects of selected steroidal derivatives (**16** and **17**) and formestane (**F**) on the cell cycle of MCF-7 cells. Cells were collected, stained with propidium iodide and analyzed by flow cytometry, after treatment with equitoxic doses of tested compounds during 48 h. Un-treated cells were used as control (**C**). Marks M1, M2, M3 and M4 on the histograms correspond to G1/M, S, G2/M and subG1 phases of cell cycle.

(C-4′ Py), 149.00 (C-6′ Py), 151.08 (C-3 from *Z*-oxime), 152.59 (C-3 from *E*-oxime), 154.22 (C-5 from *E*-oxime), 156.42 (C-5 from *Z*-oxime), 156.94 (C-17), 159.91 (C-2′, Py).

4.1.2.2. Mixture of 3(*E*)- and 3(*Z*)-hydroximino-17α-picolyl-androst-4-en-17β-ol (8 and 9). Yield 94% (0.186 g), white amorphous solid; IR (KBr, ν_{max} cm^{-1}) 3410, 2932, 2856, 2363, 1638, 1599, 1570, 1438, 1382, 1307, 1198, 1052, 968, 862, 759, 630; ^{1}H NMR (250 MHz; DMSO-d_6; Me_4Si): δ 0.85 (3H, s), 1.02 (3H, s), 1.05 (3H, 1s), 2.84 (1H, m, C*H*$_2$Py), 3.45 (1H, m, C*H*$_2$Py), 5.29 (1H, s, 17β-O*H*), 5.70 (1H, s, H-4), 6.33 (1H, s, H-4), 7.23 (1H, m, H-3′ Py), 7.37 (1H, m, H-5′ Py), 7.69 (1H, t, $J_{4',5'} = J_{4',3'}$ 7.6 Hz, H-4′ Py), 8.45 (1H, d, $J_{6',5'}$ 4.1 Hz, H-6′ Py), 10.23 (1H, s, =NO*H*), 10.48 (1H, s, =NO*H*); ^{13}C NMR (62.5 MHz; DMSO-d_6; Me_4-Si): δ 14.36 (C-18), 17.53 (C-19), 18.52, 20.73, 23.49, 31.26, 31.75, 33.54, 34.43, 36.15, 37.51, 43.35 (C-10), 46.14 (C-13), 49.36 (C-9), 53.28 (C-14), 56.03, 82.50 (C-17), 110.69 (C-4 from *Z*-oxime), 117.86 (C-4 from *E*-oxime), 120.38 (C-5′ Py from *Z*-oxime), 121.32 (C-5′ Py from *E*-oxime), 125.43 (C-3′ Py), 136.30 (C-4′ Py), 147.93 (C-6′ Py), 151.02 (C-3 from *Z*-oxime), 152.72 (C-3 from *E*-oxime), 154.17 (C-5 from *E*-oxime), 156.55 (C-5 from *Z*-oxime), 160.32 (C-2′ Py).

4.1.3. Synthesis of 17(*E*)-picolinyliden-3-aza-A-homoandrostene derivative (6)

To a solution of a mixture of compounds **4** and **5** (0.153 g, 0.41 mmol) or compounds **8** and **9** (0.195 g, 0.49 mmol) in dioxane (8 ml or 10 ml), thionyl chloride (0.83 ml, 11.4 mmol or 1.0 ml, 13.8 mmol) was added. The reaction mixture was stirred at room temperature for 2 h or 1 h, poured into water and adjuced to pH 8 with saturated $NaHCO_3$, and then extracted with dichloromethane (4 × 20 ml). Combined extracts were dried, solvent removed and the resulting crude product was separated and purified by column chromatography (10 g silica gel, chloroform–acetone 3:1), giving pure compound **6**.

4.1.3.1. 17(*E*)-Picolinyliden-3-aza-A-homoandrost-4a-en-4-one (6). Yield 32% (0.048 g) or 29% (0.056 g), light yellow crystals, mp 297–299 °C (from chloroform–hexane); IR (KBr, ν_{max} cm^{-1}) 3271, 2924, 2853, 1658, 1637, 1600, 1468, 1376, 1341, 1149, 864, 747; ^{1}H NMR (250 MHz; $CDCl_3$; Me_4Si): δ 0.92 (3H, s, H-18), 1.18 (3H, s, H-19), 3.18 (2H, m, H-2), 5.74 (1H, s, H-4a), 6.19 (1H, s, H-20), 6.67 (1H, bs, N-*H*), 7.03 (1H, m, H-5′ Py), 7.27 (1H, m, H-3′ Py), 7.58 (1H, td, $J_{4',3'} = J_{4',5'}$ 7.6 Hz, $J_{4',6'}$ 1.9 Hz, H-4′ Py), 8.55

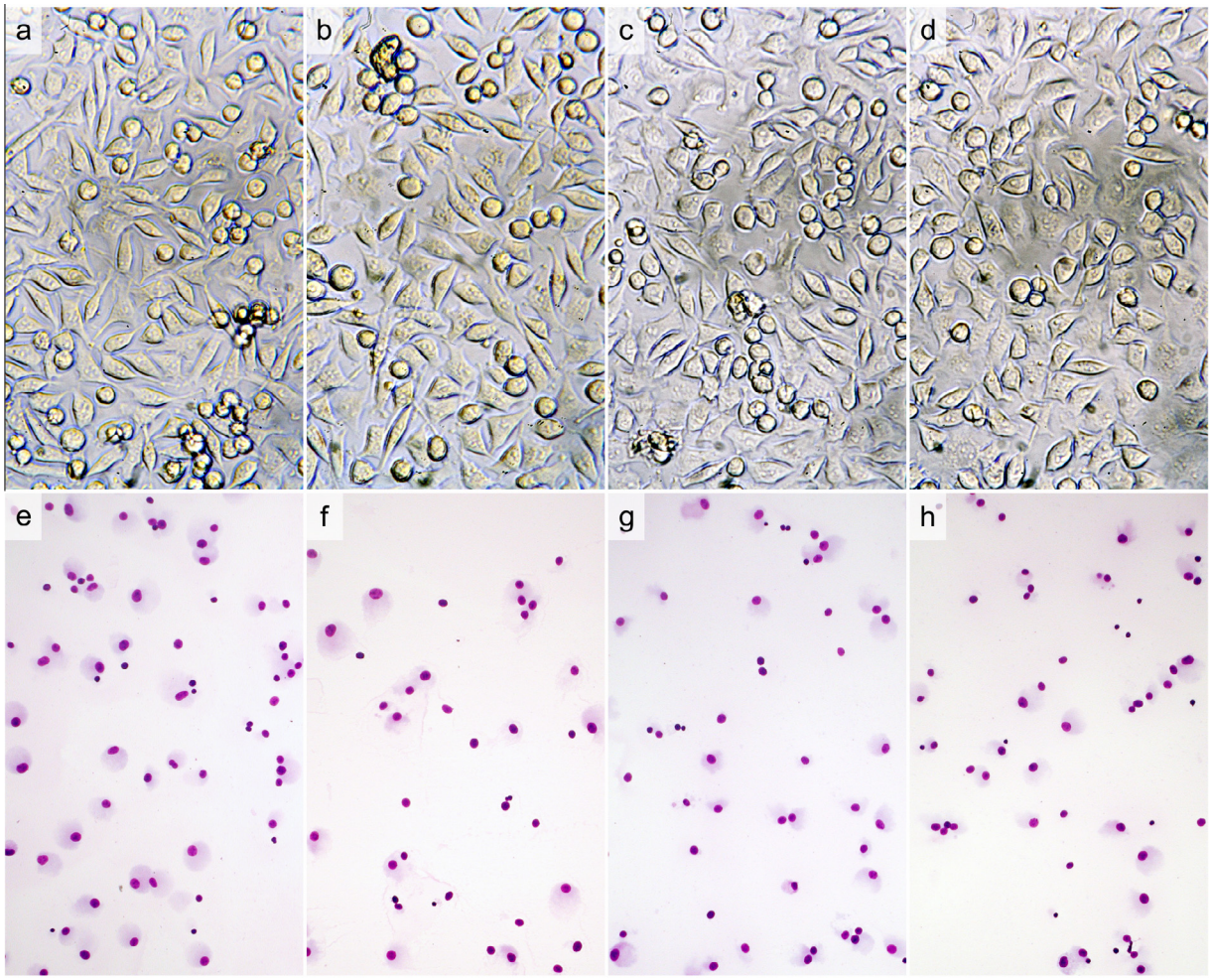


Figure 6. Morphology of MCF-7 cells treated with no substance (a, e; control), with formestane (b, f), compound **16** (c, g) or **17** (d, h) for 48 h. Images in the 1st row were photographed with a Canon 350D digital camera attached to a Reichart BioStar inverted microscope at 20 × 10 magnification; Images in the 2nd row were photographed with an Olympus Camedia 3040 digital camera attached to an Olympus BX51 microscope at 10x15 magnification, after staining with Giemsa.

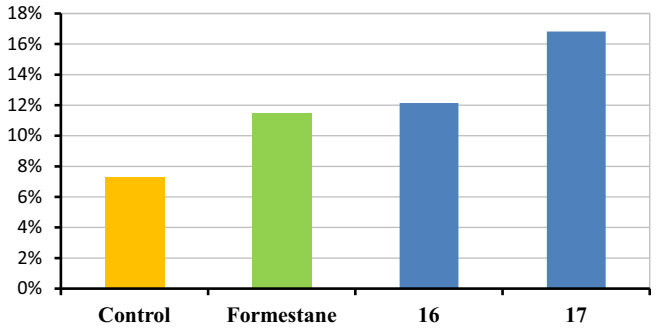


Figure 7. Percentage of apoptotic cells, as estimated by visual observation of the morphology of MCF-7 cells treated for 48 h with equitoxic doses of steroidal derivatives **16** and **17** and formestane. Standard deviations of the results were within ±10%.

(1H, d, $J_{6',5'}$ 4.6 Hz, H-6′ Py); ^{13}C NMR (62.5 MHz; $CDCl_3$; Me_4Si): δ 18.84 (C-18), 21.31 (C-19), 21.84, 24.88, 29.77, 33.51, 35.39, 35.75, 36.31, 36.66, 42.03 (C-2), 44.52, 45.79, 53.05, 53.50, 118.11 (C-20), 118.97 (C-4a), 120.22 (C-5′ Py), 122.73 (C-3′ Py), 135.78 (C-4′ Py), 149.14 (C-6′ Py), 157.44 (C-17), 159.71 (C-2′ Py), 160.78 (C-5), 170.12 (C-4); HRMS (*m*/*z*): for $C_{25}H_{33}N_2O$ $[M+H]^+$ calcd. 377.25874, found 377.25835.

4.1.4. General procedure for 17(*E*)-picolinylidene and 17α-picolyl 2-hydroxymethylidene derivatives (11 and 13)

A solution of compound **3** (0.248 g, 0.69 mmol) or **7** (0.670 g, 1.76 mmol) in absolute benzene (2 ml or 6.5 ml), was stirred with ethyl formate (0.36 ml or 1.0 ml) and sodium hydride (0.142 g, 5.90 mmol for **3** or 0.450 g, 18.75 mmol for **7**) in an argon atmosphere, at room temperature for 2.5 h (for **3**) or 2 h (for **7**). After reaction completion, resulting reaction mixture was diluted with absolute benzene (20 ml) and organic layer was then washed successively with saturated NaH_2PO_4 (2 × 10 ml), saturated $NaHCO_3$ (2 × 10 ml) and water (2 × 10 ml). After drying and solvent removal, crude product was separated by column chromatography (10 g silica gel, petroleum ether–ethyl acetate 4:1), affording pure compound **11**, or pure compound **13**.

4.1.4.1. 2(*Z*)-Hydroxymethylidene-17(*E*)-picolinyliden-androst-4-en-3-one (11). Yield 36% (0.096 g), yellow oil; IR (film, ν_{max} cm^{-1}) 3423, 2940, 2883, 2853, 1643, 1585, 1566, 1470, 1428, 1372, 1235, 1201, 1151, 885, 754; ^{1}H NMR (250 MHz; $CDCl_3$; Me_4Si): δ 0.94 (3H, s, H-18), 1.07 (3H, s, H-19), 5.79 (1H, s, H-4), 6.26 (1H, m, =C*H*(OH)), 6.31 (1H, s, H-20), 7.09 (1H, m, H-5′ Py), 7.35 (1H, m, H-3′ Py), 7.66 (1H, m, H-4′ Py), 8.56 (1H, d, $J_{6',5'}$ 4.7 Hz, H-6′ Py), 13.74 (1H, bs, =CH(O*H*)); ^{13}C NMR (62.5 MHz; $CDCl_3$; Me_4Si): δ 18.03 (C-18), 18.65 (C-19), 21.40, 24.92, 29.79, 31.18, 32.37, 35.51, 35.58, 37.53, 39.94 (C-10), 45.87, 53.01 (C-9),

53.31 (C-14), 106.42 (C-2), 117.32 (C-20), 120.46 (C-5′ Py), 122.96 (C-4 and C-3′ Py), 136.63 (C-4′ Py), 148.18 (C-6′ Py), 156.77 (C-17), 160.93 (C-2′ Py), 165.29 (=*C*H(OH)), 169.83 (C-5), 189.01 (C-3); HRMS (*m*/*z*): for $C_{26}H_{32}NO_2$ $[M+H]^+$ calcd. 390.24276, found 390.24168.

4.1.4.2. 17β-Hydroxy-2(*Z*)-hydroxymethylidene-17α-picolyl-androst-4-en-3-one (13). Yield 38% (0.271 g), yellow needles, mp 184–186 °C (from dichloromethane–hexane); IR (KBr, ν_{max} cm^{-1}) 3312, 2939, 1642, 1597, 1569, 1474, 1440, 1373, 1325, 1236, 1186, 1152, 1064, 1026, 885, 755; ^{1}H NMR (250 MHz; $CDCl_3$; Me_4Si): δ 0.99 (3H, s, H-18), 1.07 (3H, s, H-19), 2.84 (1H, d, J_{gem} 14.3 Hz, C*H*$_2$Py), 3.21 (1H, d, J_{gem} 14.3 Hz, C*H*$_2$Py), 5.79 (1H, s, H-4), 7.24–7.37 (3H, m, =C*H*(OH), H-3′ and H-5′ Py), 7.75 (1H, t, $J_{4',5'} = J_{4',3'}$ 7.6 Hz, H-4′ Py), 8.49 (1H, d, $J_{6',5'}$ 4.4 Hz, H-6′ Py), 13.76 (1H, bs, =CH(O*H*)); ^{13}C NMR (62.5 MHz; $CDCl_3$; Me_4Si): δ 14.17 (C-18), 18.07 (C-19), 21.08, 23.81, 29.66, 31.23, 31.99, 35.44, 36.50, 37.56, 39.97, 42.43 (C-10), 46.54 (C-13), 50.18 (C-9), 53.19 (C-14), 83.26 (C-17), 106.48 (C-2), 121.82 (C-5′ Py), 122.93 (C-3′ Py), 125.43 (C-4), 137.99 (C-4′ Py), 146.69 (C-6′ Py), 159.85 (C-2′ Py), 165.11 (=*C*H(OH)), 170.18 (C-5), 189.17 (C-3); HRMS (*m*/*z*): for $C_{26}H_{34}NO_3$ $[M+H]^+$ calcd. 408.25332, found 408.25283.

4.1.5. General procedure for 17(*E*)-picolinylidene and 17α-picolyl 2-hydroximino derivatives (12 and 14)

To a solution of compound **11** (0.053 g, 0.14 mmol) or compound **13** (0.097 g, 0.24 mmol) in 95% ethanol (1 ml or 2 ml), aqueous solution of 7 M $NaNO_2$ (0.11 or 0.2 ml) was added. Reaction mixture was stirred at room temperature for 10 min., then chilled to 0 °C, and 35% HCl (0.08 ml or 0.15 ml) was added dropwise. After 1 h reaction mixture was poured into water (10 ml) and 5% $NaHCO_3$ (to pH 8) was added. Crude product was precipitated and purified by recrystallization, yielding compound **12** or compound **14**.

4.1.5.1. 2(*Z*)-Hydroximino-17(*E*)-picolinyliden-androst-4-en-3-one (12). Yield 62% (0.033 g), light yellow solid, mp 232–234 °C (from dichloromethane–hexane); IR (KBr, ν_{max} cm^{-1}) 3420, 2944, 1651, 1591, 1436, 1375, 1217, 1096, 993, 753, 666; ^{1}H NMR (250 MHz; DMSO-d_6; Me_4Si): δ 0.88 (3H, s, H-18), 1.09 (3H, s, H-19), 5.89 (1H, s, H-4), 6.15 (1H, s, H-20), 7.11 (1H, m, H-5′ Py), 7.30 (1H, d, $J_{3',4'}$ 7.6 Hz, H-3′ Py), 7.69 (1H, t, $J_{4',5'} = J_{4',3'}$ 7.6 Hz, H-4′ Py), 8.51 (1H, d, $J_{6',5'}$ 2.8 Hz, H-6′ Py), 12.23 (1H, s, =NO*H*); ^{13}C NMR (62.5 MHz; DMSO-d_6; Me_4Si): δ 18.68 (C-18), 20.50 (C-19), 20.93, 24.47, 29.89, 31.22, 31.85, 34.66, 35.28, 36.09, 40.57 (C-13), 45.27 (C-10), 52.39 (C-9), 52.76 (C-14), 117.27 (C-20), 120.44 (C-5′ Py), 123.07 (C-3′ Py), 124.83 (C-4), 136.18 (C-4′ Py), 149.00 (C-6′ Py), 151.36 (C-2), 156.90 (C-17), 159.66 (C-2′ Py), 172.46 (C-5), 183.23 (C-3); HRMS (*m*/*z*): for $C_{25}H_{31}N_2O_2$ $[M+H]^+$ calcd. 391.23800, found 391.23801.

4.1.5.2. 2(*Z*)-Hydroximino-17β-hydroxy-17α-picolyl-androst-4-en-3-one (14). Yield 93% (0.090 g), light yellow solid, mp 224–226 °C (from dichloromethane–hexane); IR (KBr, ν_{max} cm^{-1}) 3416, 2936, 1675, 1605, 1438, 1381, 1300, 1236, 1058, 1027, 986, 890, 761, 616; ^{1}H NMR (250 MHz; DMSO-d_6; Me_4Si): δ 0.86 (3H, s, H-18), 1.08 (3H, s, H-19), 2.83 (1H, d, J_{gem} 15.3 Hz, C*H*$_2$Py), 3.49 (1H, d, J_{gem} 15.3 Hz, C*H*$_2$Py), 5.29 (1H, bs, 17β-O*H*), 5.89 (1H, s, H-4), 7.23 (1H, m, H-3′ Py), 7.38 (1H, m, H-5′ Py), 7.70 (1H, t, $J_{4',5'} = J_{4',3'}$ 7.6 Hz, H-4′ Py), 8.46 (1H, d, $J_{6',5'}$ 4.1 Hz, H-6′ Py), 12.20 (1H, s, =NO*H*); ^{13}C NMR (62.5 MHz; DMSO-d_6; Me_4Si): δ 14.34 (C-18), 20.48 (C-19), 20.65, 23.42, 31.08, 31.21, 31.90, 33.43, 35.78, 36.09, 38.50, 43.37 (C-10), 46.16 (C-13), 49.23 (C-9), 52.21 (C-14), 82.43 (C-17), 121.34 (C-5′ Py), 124.76 (C-3′ Py), 125.47 (C-4), 136.31 (C-4′ Py), 147.97 (C-6′ Py), 151.38 (C-2), 160.24 (C-2′ Py), 172.61 (C-5), 183.24 (C-3); HRMS (*m*/*z*): for $C_{25}H_{33}N_2O_3$ $[M+H]^+$ calcd. 409.24857, found 409.24684.

4.1.6. Synthesis of 2-hydroximino-17α-picolyl isoxazolo derivative (15) and 2,4-dihydroximino-17α-picolyl derivative (16)

Compound 7 (0.194 g, 0.51 mmol) was dissolved in freshly prepared 1 M potassium *tert*-butoxide in *tert*-butanol (5 ml), and *iso*-amyl-nitrite (0.35 ml, 3.26 mmol) was added dropwise. Reaction mixture was stirred at room temperature for 24 h, then poured into water (10 ml) and washed with diethyl ether (2 × 5 ml) to remove impurities. After that, resulting mixture was acidified with HCl (1:4, 1 ml) to adjuced to pH 6, yielding a precipitate, which was filtered and purified by column chromatography (1 g silica gel, hexane–ethyl acetate 1:1, then chloroform–methanol 1:1).

4.1.6.1. 2(*E*)-Hydroximino-17β-hydroxy-17α-picolyl-isoxazolo[3′,4′,5′:4,5,6]-androst-5-en-3-one (15). Yield 10% (0.022 g), white crystals, mp 187–188 °C (from DMSO); IR (KBr, ν_{max} cm^{-1}) 3243, 2957, 2871, 1713, 1598, 1570, 1552, 1455, 1386, 1295, 1216, 1049, 1023, 953, 923, 845, 755, 667; ^{1}H NMR (250 MHz; DMSO-d_6; Me_4Si): δ 0.88 (3H, s, H-18), 1.08 (3H, s, H-19), 2.82 (3H, m, CH_2Py and H-7), 5.35 (1H, bs, 17β-O*H*), 7.24 (1H, m, H-3′ Py), 7.38 (1H, m, H-5′ Py), 7.70 (1H, td, $J_{4',3'} = J_{4',5'}$ 7.6 Hz, $J_{4',6'}$ 1.8 Hz, H-4′ Py), 8.47 (1H, d, $J_{6',5'}$ 4.0 Hz, H-6′ Py), 13.02 (1H, s, =NO*H*); ^{13}C NMR (62.5 MHz; DMSO-d_6; Me_4Si): δ 14.40 (C-18), 20.56, 22.49 (C-19), 23.56, 27.41, 30.63, 32.13 (C-10), 33.18, 34.35, 36.46, 43.44 (C-20), 46.58 (C-13), 47.64 (C-9), 49.73 (C-14), 82.56 (C-17), 121.41 (C-5′ Py), 125.14 (C-5), 125.52 (C-3′ Py), 136.35 (C-4′ Py), 148.01 (C-6′ Py), 153.51 (C-4), 160.17 (C-2′ Py), 169.38 (C-2), 176.79 (C-6), 196.71 (C-3); HRMS (*m*/*z*): for $C_{25}H_{30}N_3O_4$ $[M+H]^+$ calcd. 436.22308, found 436.22110.

4.1.6.2. 17β-Hydroxy-2(*Z*),4(ξ)-dihydroximino-17α-picolyl-androst-5-en-3-one (16). Yield 28% (0.063 g), yellow powder, mp 184–186 °C (from chloroform–methanol); IR (KBr, ν_{max} cm^{-1}) 3411, 2956, 2870, 1702, 1598, 1570, 1439, 1386, 1289, 1231, 1120, 1017, 976, 888, 756; ^{1}H NMR (250 MHz; DMSO-d_6; Me_4Si): δ 0.85 (3H, s, H-18), 0.92 (3H, s, H-19), 2.85 (3H, m, C*H*$_2$Py and H-7), 5.33 (1H, bs, 17β-O*H*), 7.15 (1H, m, H-6), 7.23 (1H, m, H-3′ Py), 7.40 (1H, m, H-5′ Py), 7.71 (1H, t, $J_{4',3'} = J_{4',5'}$ 7.5 Hz, H-4′ Py), 8.46 (1H, d, $J_{6',5'}$ 4.2 Hz, H-6′ Py), 11.85 (1H, bs, C_4=NO*H*), 12.69 (1H, bs, C_2=NO*H*); ^{13}C NMR (62.5 MHz; DMSO-d_6; Me_4Si): δ 14.41 (C-18), 20.82, 22.08 (C-19), 23.47, 31.11, 31.55, 31.69 (C-10), 33.53, 35.21, 35.75, 43.41 (C-20), 46.05 (C-13), 48.07 (C-9), 49.81 (C-14), 82.54 (C-17), 121.37 (C-5′ Py), 125.52 (C-3′ Py), 132.60 (C-5), 134.35 (C-6), 136.33 (C-4′ Py), 147.98 (C-6′ Py), 152.20 (C_4=NOH), 160.28 (C-2′ Py), 169.34 (C_2=NOH), 181.37 (C-3); HRMS (*m*/*z*): for $C_{25}H_{32}N_3O_4$ $[M+H]^+$ calcd. 438.23873, found 438.23715.

4.1.7. Synthesis of 17α-picolyl-A-nor-secoandrostene 2,4-dinitrile derivative (17)

To a solution of compound **16** (0.120 g, 0.28 mmol) in absolute pyridine (1.2 ml), acetic anhydride (0.25 ml) was added in drops. Reaction mixture was stirred at room temperature for 1.5 h, and then poured into water (20 ml), yielding a precipitate which was filtered, and the mother liquor was extracted with ethyl acetate (3 × 15 ml). After work-up, crude product was combined with previously separated precipitate and purified by column chromatography (1 g silica gel, petroleum ether–ethyl acetate 3:1), giving pure compound **17**.

4.1.7.1. 17β-Hydroxy-17α-picolyl-2,3-seco-A-norandrost-5-ene-2,4-dinitrile (17). Yield 46% (0.048 g), fragile white crystals, mp 166–168 °C (from dichloromethane–hexane); IR (KBr, ν_{max} cm^{-1}) 3313, 2958, 2870, 2245, 2216, 1728, 1638, 1596, 1570, 1439, 1389, 1253, 1023, 907, 756; ^{1}H NMR (250 MHz; $CDCl_3$; Me_4Si): δ 0.98 (3H, s, H-18), 1.20 (3H, s, H-19), 2.31 (2H, m, H-7), 2.63 (1H, d, J_{gem} 17.2 Hz, H-1a), 2.77 (1H, d, J_{gem} 17.2 Hz, H-1b), 2.88 (1H, d, J_{gem} 14.2 Hz, C*H*$_2$Py), 3.33 (1H, d, J_{gem} 14.2 Hz, C*H*$_2$Py), 6.79 (1H, m, H-6),

7.34 (2H, m, H-3′ and H-5′ Py), 7.84 (1H, t, $J_{4',3'} = J_{4',5'}$ 7.3 Hz, H-4′ Py), 8.51 (1H, d, $J_{6',5'}$ 4.6 Hz, H-6′ Py); ^{13}C NMR (62.5 MHz; $CDCl_3$; Me_4Si): 14.18 (C-18), 21.23 (C-19), 21.91, 23.70, 27.40, 31.58, 31.62, 32.16 (C-10), 35.31, 39.41, 42.00 (C-20), 43.66, 46.60, 50.35, 83.11 (C-17), 116.68 (C-5), 117.20 (C-4), 119.56 (C-2), 122.21 (C-5′ Py), 125.93 (C-3′ Py), 138.89 (C-4′ Py), 145.96 (C-6), 146.58 (C-6′ Py), 159.11 (C-2′ Py); HRMS (*m*/*z*): for $C_{24}H_{30}N_3O$ $[M+H]^+$ calcd. 376.23834, found 376.23772.

4.2. Antiproliferative activity

4.2.1. Cell lines and cell culture

Six human tumor cell lines: breast adenocarcinoma ER−, MDA-MB-231; breast adenocarcinoma ER+, MCF-7; prostate cancer, PC-3; cervical carcinoma, HeLa; colon adenocarcinoma, HT-29 and lung adenocarcinoma, A549, and one human noncancerous cell line (normal fetal lung fibroblasts MRC-5), were used in the present study. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5% of glucose, supplemented with 10% of fetal calf serum (FCS, Sigma) and antibiotics: 100 IU mL^{-1} of penicillin and 100 μg mL^{-1} of streptomycin (ICN Galenika). Cells were cultured in flasks (Costar, 25 cm^2) at 37 °C in 100% humidity with 5% CO_2. Only viable cells, as determined by trypan blue dye exclusion, were used in subsequent assays.

4.2.2. Antiproliferative assay

Compounds were evaluated for anti-proliferative activity using the tetrazolium colorimetric MTT assay, after exposure to test compounds, in concentrations ranging from 10^{-8} to 10^{-4} M, for 48 h. Doxorubicin (DOX) as a nonselective anti-proliferative agent, and formestane as steroidal aromatase inhibitor, were used as reference compounds. Exponentially growing cells were harvested, counted by trypan blue exclusion test, seeded onto 96-well plates at a density of 5000 cells/well and allowed to stand overnight, after which the medium containing the tested compound was added (10 μM/well). After a 48 h incubation period, viability was determined by the addition of 10 μL of sterile MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) solution (5 mg/mL). The precipitated formazan crystals were solubilized with acidified 2-propanol (100 μL of 0.04 M HCl in 2-propanol) and the absorbance was read (Multiscan MCC340, Labsystems) at 540/690 nm after a few minutes incubation at room temperature. Wells containing cells without tested compounds were used as a control. Wells without cells containing only complete medium and MTT only were used as blank. Two independent experiments were conducted in quadruplicate for each concentration of tested compound. Mean values and standard deviations (SD) were calculated for each concentration. Anti-proliferative activity was expressed as IC_{50} value, which is defined as the dose of compound that inhibits cell growth by 50% of untreated control cells.

4.2.3. Data analysis

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

4.3. Study of change in cell cycle and apoptosis of the treated tumour cells

We selected two the most promising compounds (**16** and **17**) for study the possible mechanism which forms the basis of tumor cells proliferation decreasing. Hence we performed tumor cell cycle analysis and measured apoptosis induction potential of these substances.

4.3.1. Cell culture and cell treatment

Cells were grown in the similar conditions as for the MTT test. The cells, seeded in flasks, were allowed to adhere to the flask surface during 24 h at 37 °C, in an atmosphere comprising 5% of CO_2. Each culture of all control and experimental groups were seeded in duplicate. After 24 h, test steroid compounds (**16**, **17** and the reference formestane) were added to experimental cultures in 1 mL volume, in order to achieve the appropriate final concentrations, equal to their IC_{50} concentrations, which were estimated earlier by cytotoxicity assay (equitoxic doses). Cells were then again incubated in total darkness at 37 °C in a 100% humidity atmosphere with 5% CO_2. Total incubation time with steroid compounds was 48 h, the same as in the control sample.

4.3.2. Cell harvesting

Cell harvesting and slide preparation was conducted under aseptic conditions, following a modified cytogenetic preparation procedure for micronuclei testing.[36,37] Each culture was harvested and processed separately. Cell suspension preparations involved hypotonic treatment, in order to achieve adequate cell spreading and high-quality cell preparation for scoring. Hypotonic treatment, fixation and centrifugation were adapted to preserve the cell cytoplasm. The cell cytoplasm was retained to enable reliable detection and identification of all morphological features related to apoptosis. After 48 h of treatment, cells were separated from culture flask walls by trypsinization (0.5% solution of trypsin). One part was used for slide preparation, and the other for cell cycle analysis.

4.3.3. Flow cytometric cell cycle analysis

Analysis of the cell distribution is based on cellular DNA content measuring of ethanol-fixed MCF-7 cells, stained with propidium iodide (PI), using a Becton Dickinson (BD) Immunocytometry System.[38] After 48 h treatment, trypsinization, cell fixation (70% ethanol 30 min on ice) and centrifugation, solutions of ribonuclease A (RNase A, 100 units ml^{-1}) and propidium iodide (400 mg ml^{-1}) were added to each cell pellet. Cell suspensions were incubated in the dark at room temperature for 30 min. Each sample was filtered then through a 35 μm grid and analyzed by flow cytometry. The cell excitation wavelength was 488–514 nm, and the fluorescence emission of PI was approximately 610 nm. The FL2 parameter of BD FACSCalibur was used for one parametric analysis. In each analysis, 20,000 events were recorded, and the percentages of the cells in the different cell-cycle phases (subG1, G1, S and G2/M) were calculated by using CellQuest software (Becton Dickinson). The subG1 fractions were regarded as the apoptotic cell population.

4.3.4. Slide preparation for morphological analysis

The culture medium was removed from the cells after gentle centrifuging (1200 RPM/5 min). Cells were then hypotonically treated with 7.0 mL of cold (4 °C) 0.075 M KCl and centrifuged immediately at 1200 RPM for 8 min. The supernatant was removed and fixative (5 mL of mixture of methanol and acetic acid (3:1) with 1% formaldehyde) was added while agitating the cells, in order to prevent clump formation. The cells were then centrifuged again (1100 RPM/8 min), washed twice with fixative formaldehyde and resuspended gently. The final suspension was dropped onto clean glass slides and allowed to dry. Specimen staining (after 24 h) was performed using 2% Giemsa stain (Merck) in potassium phosphate buffer (pH 7.3).

4.3.5. Analysis and scoring criteria

Slides with stained cells were analyzed by light microscopy (Olympus BX51). All specimens, including controls, were independently coded before microscopic analysis and analyzed with no prior information regarding the origin of the material. Cells were counted and at least 1000 cells were scored for each specimen.

Table 2
The crystal data and refinement parameters

	6	13	15
Chemical formula	$C_{25}H_{32}N_2O$	$C_{26}H_{33}NO_3$	$C_{25}H_{29}N_3O_4$
M_r	376.53	407.53	435.51
Cell setting, space group	Orthorhombic, $P2_12_12_1$	Monoclinic, $P2_1$	Orthorhombic, $P2_12_12_1$
Temperature (K)	293	293	293
a (Å); b (Å); c (Å)	7.3301 (3), 8.5393 (4), 33.1817 (17)	11.161 (2), 6.2498 (13), 16.253 (3)	12.2836 (5), 13.2366 (6), 16.5473 (10)
β (°)	-	91.564 (19)	—
V (Å^3)	2076.96 (17)	1133.3 (4)	2690.6 (17)
Z	4	2	4
Radiation type	Cu Kα	Mo Kα	Mo Kα
μ (mm^{-1})	0.56	0.08	0.07
Crystal size (mm)	0.42 × 0.28 × 0.17	0.44 × 0.26 × 0.18	0.49 × 0.31 × 0.22
No. of measured, independent and observed reflections	4827, 3222, 2874	4324, 2844, 2296	7846, 4487, 3337
R_{int}	0.019	0.027	0.037
$(\sin\theta/\lambda)_{max}$ (Å^{-1})	0.597	0.595	0.595
$R[F^2 > 2\sigma(F^2)]$, $wR(F^2)$, S	0.039, 0.101, 1.05	0.058, 0.178, 1.05	0.079, 0.212, 1.12
No. of reflections	3222	2844	4487
No. of parameters	279	301	293
$\Delta\rho_{max}$, $\Delta\rho_{min}$ (e Å^{-3})	0.13, −0.20	0.15, −0.19	0.30, −0.25

Computer programs: *CrysAlis PRO*, Agilent Technologies, Version 1.171.36.24 (release 03-12-2012 CrysAlis171 .NET) (compiled Dec 3 2012, 18:21:49), *SIR92*,[41] *SHELXL97*.[43]

Scored features included normal cells and all forms of induced morphological changes that can be attributed to apoptosis.[39]

4.3.6. Image capturing and data processing

Images were captured with a 12 megapixel digital camera (Canon 350D) attached to a computer. Data were processed with Microsoft Excel.

4.4. X-ray crystal structure determination

The diffraction data for compounds **13** and **15** were collected at room temperature on an Oxford Diffraction Gemini S diffractometer with graphite-monochromated MoKα radiation (λ = 0.7107 Å) and for compound **6** also at room temperature using CuKα radiation (λ = 1.5418 Å). The data reduction for all compounds (**6**, **13** and **15**) was performed with program package CrysAlis RED.[40] The space group determinations were based on an analysis of the Laue class and the systematically absent reflections. The structures were solved by direct methods using SIR92.[41] All structures were refined using full-matrix least-squares. For all three compounds non-hydrogen atoms were refined anisotropically. The positions of C–H, N–H and O–H hydrogen atoms in compounds **6** and **13** were found from the inspection of the difference Fourier maps, but other H atoms were included on calculated positions. In compound **15** the hydrogen atoms were included on calculated positions riding on their attached atoms with fixed distances 0.98 Å (CH), 0.97 Å (CH_2) and 0.96 Å (CH_3). At the final stage of the refinement, H atoms from the hydroxyl group were positioned geometrically (O–H = 0.82 Å) and refined using a riding model with fixed isotropic displacement parameters. Compound **15** had contributions from disordered solvent molecules which were removed by the SQUEEZE routine (PLATON)[42] and the output from the SQUEEZE calculations is attached to CIF file. All calculations were performed using SHELXL97,[43] PARST[44] and PLATON,[42] as implemented in the WINGX[45] system of programs. The crystal data and refinement parameters are summarized in Table 2.

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Supplementary data

Supplementary data (S1—^{1}H and ^{13}C NMR spectra of newly synthesized compounds; S2—Results of cells counting; S3—Intra- and intermolecular hydrogen bond parameters and crystal packing of compounds **6** and **15**.) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.02.001.

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