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Synthesis, structural analysis and antiproliferative activity of some novel D-homo lactone androstane derivatives†

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An efficient synthesis of several A,B-modified D-homo lactone androstane derivatives is reported. The synthetic scheme shows the transformation of 17-oxa-D-homoandrost-5-en-16-on-3β-yl acetate **1** into the 5α-hydroxy-17-oxa-D-homoandrostane-6,16-dion-3β-yl acetate (**4**). After the dehydration of **4**, the newly synthesized 6-keto-androst-4-ene-3β-yl acetate derivative **5** was oximinated to give the 6-hydroximino derivative **6**, which was converted to A,B-condensed isoxazole derivatives **7** and **8**. Compound **4** was also converted (*via* 6(*E*)- and 6(*Z*)-hydroximino derivatives **9** and **10**) to the B-seco-cyano derivative **11** under a Beckmann fragmentation, while compound **5** was transformed to the 4β,5β-epoxy derivative **12**. Structures were confirmed by IR, ^{1}H NMR, ^{13}C NMR, and HRMS, and for **7** and **8** by X-ray crystallography. All compounds were tested *in vitro* on six malignant cell lines (MCF-7, MDA-MB-231, PC-3, HeLa, HT-29, K562) and one non-tumor MRC-5 cell line. Significant antiproliferative activity was observed for specific compounds against prostate (PC-3), cervical (HeLa) and colon (HT-29) cancer cells, while no compounds showed antiproliferative activity to non-cancerous control cells (MRC-5). Interestingly, **1–8** displayed selective antiproliferative activity against estrogen-independent (ER−, MDA-MB-231) breast cancer cells over estrogen-dependent (ER+, MCF-7) breast cancer cells.

1. Introduction

Hormone-responsive cancers, including endometrial, ovarian, prostate and breast cancers, comprise over 30% of all newly diagnosed cancers.[1] In addition, steroidal hormones have been proposed to modulate the proliferation of other cancers, such as colorectal[2] and cervical cancer.[3] Synthetic and natural steroidal estrogen, androgen, and progesterone derivatives influence the growth and progression of hormone-sensitive cancers, and modulation of the levels of circulating sex hormones represents a major therapeutic strategy in cancer treatment.[1] We are interested in the synthesis and characterization of heterocyclic steroidal compounds for potential applications in the treatment of steroid-responsive tumors.[4–7] Modification of steroidal compounds by conversion of functional group(s) to a heterocyclic moiety can result in dramatically altered pharmacological properties, and steroidal heterocycles have applications in the treatment of a range of disorders, including breast and prostate cancer.[8–10] Numerous steroidal heterocycles have been synthesized with antitumor activity.[11,12] D-Homo lactone testosterone derivatives, such as Testolactone, represent one of the first steroidal breast cancer treatments in clinical use,[13] while A- and B-ring modified steroids have been reported with high antiproliferative activity against human tumor cells.[6,7,14–17] In particular, B-ring oxygenated steroids displayed strong antiproliferative activity toward several human cancer cells, including selectivity towards leukemia cell lines.[17] Steroidal heterocycles with isoxazole modifications have also been synthesized with antitumor properties,[18] and are convenient intermediates for the synthesis of multifunctional compounds.[19] Clinically, steroidal isoxazoles, such as Danazol are important in the treatment of endometriosis,[20] and inhibit endometrial cancer proliferation *in vitro*.[21,22] Other isoxazole derivatives have been reported with wide-ranging pharmacological properties, including antibacterial and antiviral activities.[23]

Because of their pharmacological potential, we are interested in developing convenient synthetic routes to obtain D-homo lactone steroidal heterocycles with A,B-ring modifications. We

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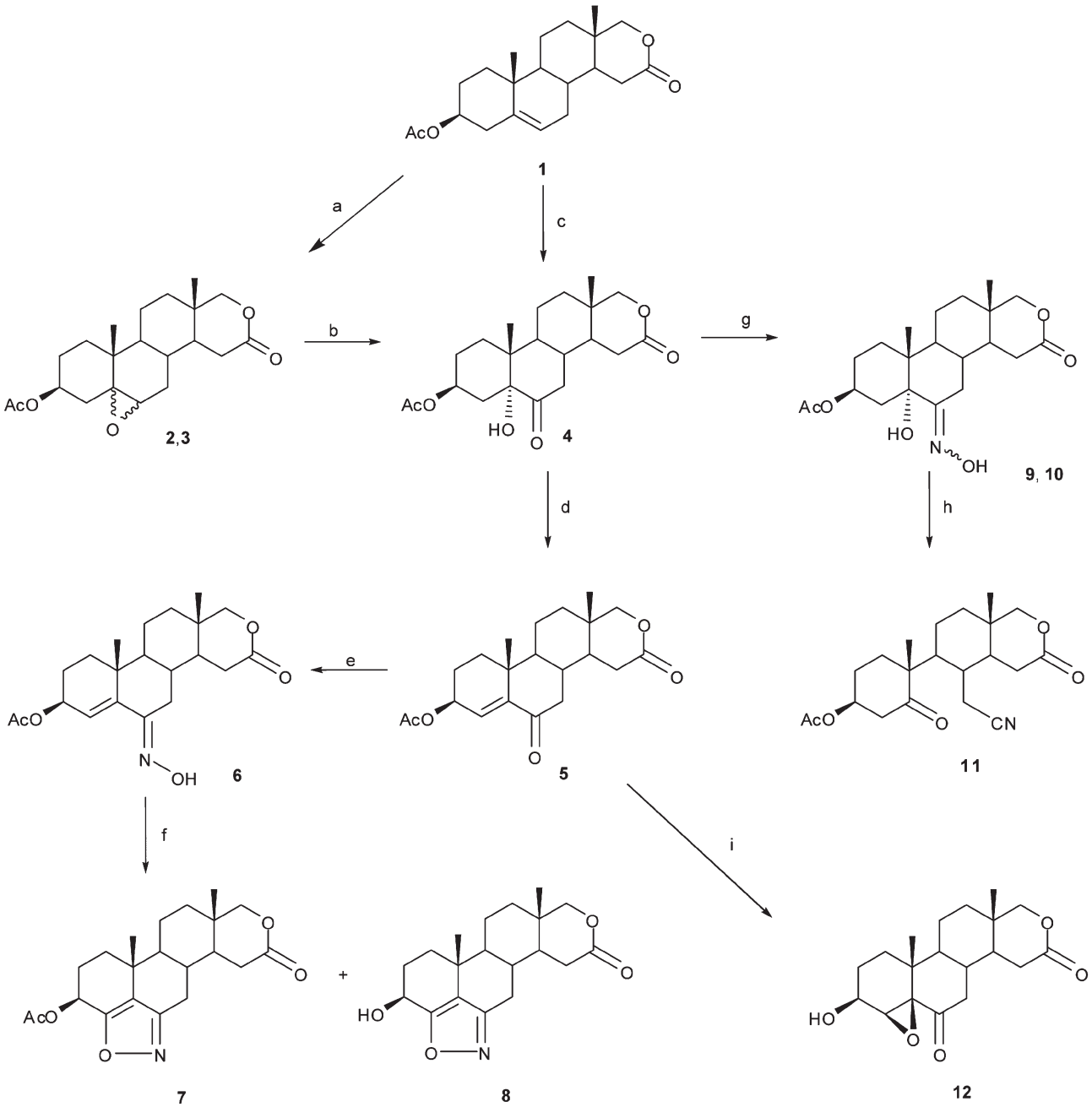

Scheme 1 Reagents and reaction conditions: (a) MCPBA, $NaHCO_3$, CH_2Cl_2, 0 °C, 1 h; (b) CrO_3, acetone, water, 0 °C, 30 min → rt, 40 min; (c) MCPBA, CH_2Cl_2, 0 °C, 90 min → rt, 60 min, then CrO_3, rt 70 min; (d) $SOCl_2$, abs. pyridine, 0 °C, 2 h, HCl (1 : 1); (e) $NH_2OH{\cdot}HCl$, CH_3COONa, C_2H_5OH, rt, 24 h; (f) I_2, KI, $NaHCO_3$, THF–water (4 : 1), reflux, 5.5 h, HCl (1 : 1); (g) $NH_2OH{\cdot}HCl$, CH_3COONa, C_2H_5OH, 60 °C, 1 h; (h) Ac_2O, abs. pyridine, 70 °C, 2h, then 0 °C HCl (1 : 1); (i) H_2O_2, NaOH, CH_3OH, 0 °C, 3 h, HCl (1 : 1).

have previously reported syntheses of steroidal D-homo lactone derivatives with marked antiproliferative activity against human cancer cells.[6,24] Here we present an efficient synthesis of several new A,B-modified D-homo lactone androstanes, including A,B-fused isoxazole derivatives. Synthesized products were characterized by spectroscopic methods, X-ray crystallography and computational methods, and evaluated for antiproliferative activity against six different human cancer cell lines: PC-3 (AR− prostate cancer), MCF-7 (ER+ breast adenocarcinoma), MDA-MB-231 (ER− breast adenocarcinoma), HeLa (cervical cancer), HT-29 (colon cancer) and K562 (chronic myelogenous leukemia), and non-cancerous cells (MCR-5). Results were rationalized using ligand-based inverse docking.

2. Results and discussion

2.1. Synthetic studies

Scheme 1 outlines the synthetic procedures used to obtain compounds **1–12**. Compounds **1–4** were synthesized as

described in our previous work.[5,25] In this paper we report a rapid and simple one-pot procedure[26] which was modified from our previous work[25] for the synthesis of compound **4** from starting compound **1**. Treatment of **4** with thionyl chloride (absolute pyridine, 0 °C for 2 h) gave 17-oxa-D-homoandrost-4-ene-6,16-dion-3β-yl acetate (**5**). Reaction completion was monitored by NMR: the doublet signal at 6.14 ppm in the ^{1}H-NMR spectra of compound **5** is indicative of the presence of an H-4 proton, and a clear C-4 signal was observed at 129.83 ppm by ^{13}C-NMR. The hydroximino derivative **6** was then obtained by the reaction of compound **5** with hydroxylamine hydrochloride in 95% ethanol, in the presence of sodium acetate at room temperature for 24 h. The presence of a 6(*E*)-hydroximino substituent on compound **6** was confirmed by comparing spectroscopic data (of **6**) with reported values for closely related steroidal compounds.[27–29]

One aim of the present study was to fuse an isoxazole moiety with the A,B-ring of a D-homo lactone steroid. 3β-Acetoxy isoxazole derivative **7** and 3β-hydroxy isoxazole derivative **8** were prepared by the reaction of compound **6** with iodine and potassium iodide[30,31] in THF–H_2O under reflux for 5.5 h. Reaction completion was monitored by comparison of the NMR spectra of **6** with those of reaction products **7** and **8**; ^{1}H-NMR spectra of pure **7** and **8** did not contain signals at 10.83 ppm (assigned to 6(*E*)-hydroximino in **6**) or 5.53 ppm (H-4 in **6**), respectively. The structures of A,B-fused isoxazole D-homo androstane derivatives **7** and **8** were then unambiguously confirmed by single crystal X-ray diffraction (Fig. 1, Table 1).

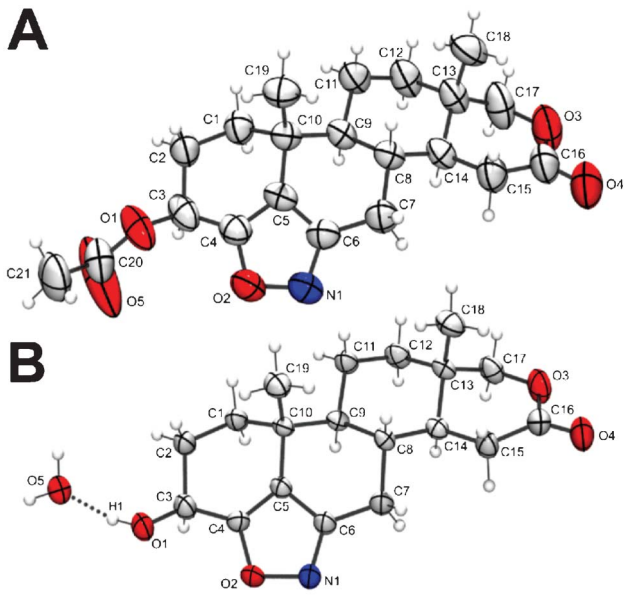

Fig. 1 X-ray crystal structures of A,B-isoxazole D-homo lactone androstane derivatives **7** and **8**. (A) ORTEP drawing of the molecular structure of compound **7** with labeled non-H atoms. Displacement ellipsoids are shown at 50% probability, and H atoms are drawn as spheres of arbitrary radii. (B) ORTEP drawing of the molecular structure of compound **8** with labeled non-H atoms. Displacement ellipsoids are shown at 50% probability level and H atoms are drawn as spheres of arbitrary radii. A hydrogen bond between **8** and an ordered water is shown as a dashed line.

Interestingly, analysis of the crystal lattice packing in the crystal structure of compound **8** revealed intermolecular O–H···O and O–H···N hydrogen bonds with ordered water, mediated by N1 of the A,B-isoxazole moiety and O1 of the C3 hydroxyl group (Fig. 2, Table 2). Consistent with this, molecular electrostatic potential (MEP) maps calculated from the crystal structures of **7** and **8** suggest that isoxazole addition introduces a significant negative partial charge to the A,B-ring (Fig. 3).

An alternative reaction protocol for compound **4** was also explored. The reaction of **4** with hydroxylamine hydrochloride in 95% ethanol in the presence of sodium acetate at 60 °C for 1 h afforded a mixture of 6(*Z*)-(**9**) and 6(*E*)-hydroximino (**10**) derivatives in the ratio $E : Z = 1.47 : 1$, which could not be separated. This mixture was used for the synthesis of a new B-seco-cyano derivative **11**, which was synthesized by a Beckmann fragmentation of **9** and **10** with acetic anhydride in absolute pyridine at 70 °C for 2 h. The structure of compound **11** was confirmed spectroscopically by IR and NMR: IR absorption bands at 2245 cm^{-1} and 1732 cm^{-1} are consistent with the presence of cyano and keto functions, while ^{13}C-NMR signals at 117.26 ppm and 215.66 ppm support the presence of a cyano and C_5=O group, respectively.

Finally, the reaction of a 6-keto derivative **5** with 30% hydrogen peroxide under alkaline conditions in methanol at 0 °C for 3 h gives the 4β,5β-epoxy-3β-hydroxy derivative **12**. The structure of compound **12** was confirmed spectroscopically by IR and NMR. ^{1}H NMR revealed a doublet at 3.32 ppm assigned to the H-4 proton, which in compound **5** is shifted to 6.14 ppm. Moreover, no signal was observed at 170.63 ppm from the 3β-acetoxy function in compound **5** by ^{13}C NMR, while the presence of a broad IR band at 3446 cm^{-1} confirmed the hydroxy group in compound **12**. The β-configuration of the 4,5-epoxy group in compound **12** was assigned by ^{1}H-NMR NOE: irradiation of the H-4 proton at 3.32 ppm resulted in NOE enhancement at H-3α (4.07 ppm), also irradiation of H-1α (2.16 ppm) gave an NOE enhancement at H-4 (3.32 ppm). The same 4β,5β-configuration of the epoxide was confirmed in 4β,5β-epoxy-cholest-4-en-6-one-3β-yl acetate.[32]

2.2. Antiproliferative activity

Synthesized compounds were evaluated for antiproliferative activity against PC-3 (AR− prostate cancer), MCF-7 (ER+ breast adenocarcinoma), MDA-MB-231 (ER− breast adenocarcinoma), HeLa (cervical cancer), HT-29 (colon cancer) and K562 (chronic myelogenous leukemia), as well as control non-cancerous MRC-5 (normal fetal lung fibroblast) cells. Antiproliferative activity was evaluated *in vitro* using the SRB assay,[33] following 48 h treatment with test compounds as previously described.[25] Results were compared with the non-selective antiproliferative drug, Doxorubicin, as well as a clinically approved androstane derivative (Formestane) used in the treatment of ER+ breast cancer, and are presented in Table 3 as IC_{50} values (μM). All of the newly synthesized compounds were non-toxic to normal MRC-5 cells, whereas the Doxorubicin was highly toxic to these cells, consistent with the severe side effects associated with Doxorubicin chemotherapy. As can be seen, strong antiproliferative activity was observed against prostate cancer (PC-3), cervical cancer (HeLa)

Table 1 X-ray data and crystal structure refinement[a]

Compound No.	7	8
Empirical formula	C_{21} H_{27} N O_5	C_{19} H_{27} N O_5
Formula weight	373.44	349.42
Temperature	293(2) K	293(2) K
Wavelength	1.54180 Å	0.71073 Å
Crystal system	Orthorhombic	Orthorhombic
Space group	*P* 21 21 21	*P* 21 21 21
Unit cell dimensions	*a* = 7.6964(6) Å	*a* = 7.6411(3) Å
	b = 9.0385(9) Å	*b* = 8.3284(4) Å
	c = 28.1815(19) Å	*c* = 27.4713(13) Å
Volume	1960.4(3) Å^3	1748.2(2) Å^3
Z	4	4
Density (calculated)	1.265 mg m^{-3}	1.328 mg m^{-3}
Absorption coefficient	0.734 mm^{-1}	0.096 mm^{-1}
F(000)	800	752
Crystal size	0.232 × 0.114 × 0.041 mm^3	0.237 × 0.162 × 0.045 mm^3
θ range for data collection	3.14 to 74.74°	2.97 to 24.99°
Index ranges	$-9 \leqslant h \leqslant 8$	$-9 \leqslant h \leqslant 9$
	$-10 \leqslant k \leqslant 10$	$-6 \leqslant k \leqslant 9$
	$-34 \leqslant l \leqslant 34$	$-32 \leqslant l \leqslant 31$
Reflections collected	4426	5375
Independent reflections	3150 [*R*(int) = 0.0171]	2871 [*R*(int) = 0.0258]
Completeness to θ = 67.50°	99.8%	99.8%
Absorption correction	Semi-empirical from equivalents	Semi-empirical from equivalents
Max. and min. transmission	1.00000 and 0.96820	1.00000 and 0.98354
Refinement method	Full-matrix least-squares on F^2	Full-matrix least-squares on F^2
Data/restraints/parameters	3150/0/264	2871/0/256
Goodness-of-fit on F^2	1.028	1.075
Final *R* indices [$I > 2\sigma(I)$]	R_1 = 0.0448, wR_2 = 0.1108	R_1 = 0.0465, wR_2 = 0.0901
R indices (all data)	R_1 = 0.0711, wR_2 = 0.1413	R_1 = 0.0571, wR_2 = 0.0946
Extinction coefficient	0.0024(4)	
Largest diff. peak and hole	0.251 and −0.256 e Å^{-3}	0.128 and −0.155 e Å^{-3}

[a] Computer programs: CrysAlisPro, Oxford Diffraction Ltd., Version 1.171.34.36 (release 02-08-2010 CrysAlis171.NET) (compiled Aug 2 2010, 13:00:58), *SIR92* (Altomare, 1993), *SHELXL97* (Sheldrick, 1997).

and colon cancer (HT-29) cells. Consistent with these results, steroidal compounds have been shown to modulate the proliferation of colorectal and cervical cancer,[1,3] and steroid-based anti-androgen drugs can interfere with androgen receptor function, leading to targeted apoptosis and inhibition of androgen-dependent prostate cancer growth.[34] Compound **2**, which contains a 5α,6α-epoxy function, displayed the strongest antiproliferative activity against prostate cancer cells (IC_{50} 2.64 μM), while compound **4** (obtained from mixture of **2** and **3**) was only slightly toxic (IC_{50} 66.87 μM), suggesting the importance of the 5α,6α-epoxy group on the A,B-ring. In support of this, other steroidal 5α,6α-epoxides have also been reported with significant antiproliferative activity against PC-3 tumor cells.[14] Furthermore, compound **12**, which contains 4β,5β-epoxy and 6-keto functions, was nontoxic to PC-3 cells, possibly suggesting a specific role for the 5α,6α-epoxy group in PC-3 antiproliferative activity. Along the same lines, compound **6**, which contains a 6(*E*)-hydroximino function, displayed the strongest antiproliferative effect against colon cancer cells (IC_{50} 3.97 μM), while **5** was nontoxic, suggesting the importance of the 6(*E*)-hydroximino in the antiproliferative properties of **6**. Only compound **12** (IC_{50} 4.97 μM) showed strong antiproliferative activity against cervical cancer (HeLa) cells. Because **12** was non-toxic to all other cells, this antiproliferative activity may be selective for cervical cancer cells.

Unexpectedly, compounds **1–8** displayed selective antiproliferative activity against estrogen receptor negative (ER−, MDA-MB-231) over estrogen receptor positive (ER+, MCF-7) breast cancer cell lines (Table 3). Of these, compounds **2**, **6** and **8** showed significant antiproliferative activity (IC_{50} 12.98 μM, 11.88 μM and 11.24 μM, respectively) against MDA-MB-231 breast cancer cells. MDA-MB-231 breast adenocarcinoma cells are triple-negative,[35] they are associated with poorer prognosis, and no targeted therapies have been developed to date for the treatment of triple-negative breast cancers.[36] Identification of compounds which selectively inhibit MDA-MB-231 cell growth may therefore be important for the development of more effective breast cancer chemotherapies. In an attempt to provide a theoretical rationale for these results, we conducted *in silico* ligand-based virtual screening using the program idTarget.[37] Ligand-based inverse screening for protein targets (inverse-docking) has been used to suggest possible molecular mechanisms for the function of new compounds, and to identify possible off-target pathways for known drugs.[38–41] Compound **8** was chosen for initial screening since it showed antiproliferative activity *only* against MDA-MB-231 cells, and we had determined its crystal structure. idTarget predicts protein targets for a given small molecule using a rapid molecular docking-based scoring function which enables molecular docking simulations to be performed against the entire protein databank (PDB).[37] idTarget identi-

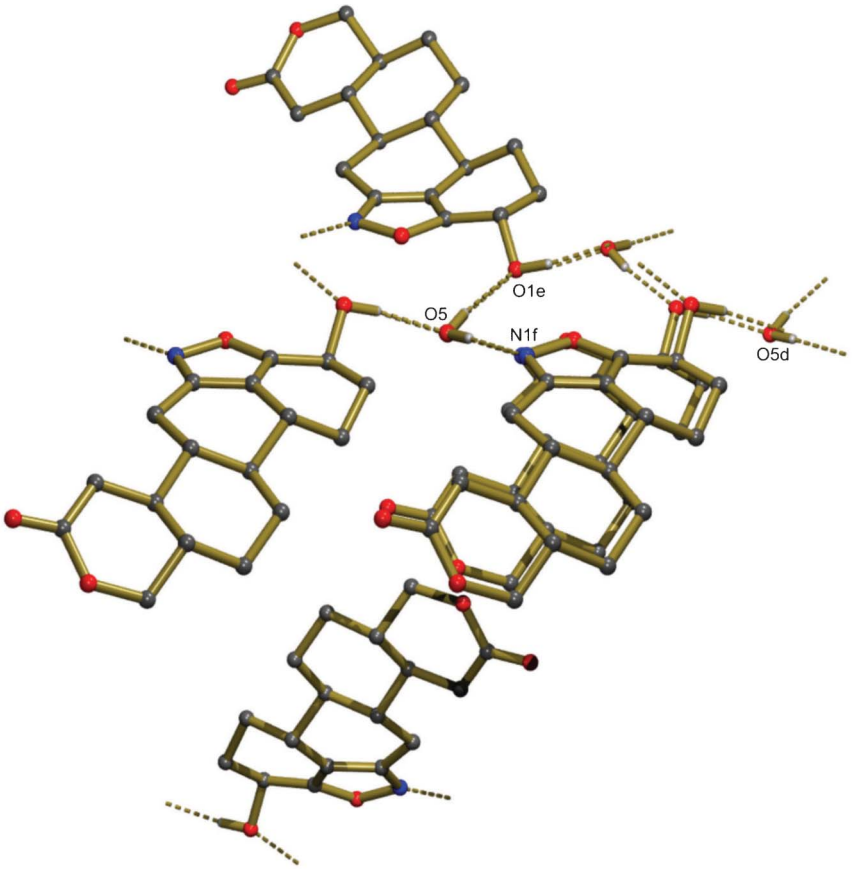

Fig. 2 PLATON drawing showing the crystal packing of compound **8** along the *a* axis. Intermolecular hydrogen bonds are shown as dashed lines. Some atoms in the molecule are not shown for clarity.

fied 400 proteins with an AutoDock4 binding energy of −9.5 kcal mol^{-1} or better and a negative Z-score. These results were then filtered to include only proteins shown in the literature to have an increased expression in estrogen receptor negative breast cancers *versus* ER+ breast cancer cells,[42–44] resulting in 23 potential protein targets (Table 4) for future experimental investigation. Importantly, based on our experimental results, compounds **2**, **6** and **8** could represent possible starting points for the development of triple-negative specific antiproliferative compounds.

Compound **4** exerted the highest antiproliferative activity at lower concentrations, while compound **2** did not show appreciable antiproliferative activity at these concentrations. However, both compounds **2** and **4** had similar activity at higher concentrations (Fig. 4 A). Compounds **5** and **6** showed similar antiproliferative activity, which was slightly higher compared to Formestane (Fig. 4 B). Compound **7** showed activity similar to Formestane, while compound **8** was more toxic at concentrations above 1 μM (Fig. 4 C). Compound **12** had the lowest activity against MDA-MB-231 cells over the whole range of concentrations (Fig. 4 D).

Considering the results of the antiproliferative activity and IC_{50} values of the investigated compounds against the MDA-MB-231 cell line (Fig. 4, Table 3), it could be concluded that compound **8** is the most promising derivative. With the lowest IC_{50} value (11.24 μM), this compound produced most of its antiproliferative activity effects at higher concentrations (>1 μM), while at the same time was less toxic compared to other derivatives at lower concentrations.

Table 2 Intermolecular O–H···O and O–H···N hydrogen-bond parameters (Å, °)[a]

D–H···A	D–H	H···A	D···A	D–H···A
Compound 8				
O(1)–H(1)···O(5)[b]	0.89(3)	1.85(3)	2.725(4)	168(3)
O(5)–H(5A)···O(1)[c]	0.78(3)	2.04(3)	2.818(4)	173(3)
O(5)–H(5B)···N(1)[d]	0.87(3)	2.07(3)	2.936(4)	175(3)

[a] Symmetry transformations used to generate equivalent atoms. [b] −1 + *x*,1 + *y*, *z*. [c] −*x*,−1/2 + *y*,1/2 − *z*. [d] 1 + *x*,*y*,*z*.

3. Conclusions

Here we report a convenient and efficient scheme for the synthesis of A,B-modified D-homo lactone androstane derivatives, including A,B-condensed isoxazoles. Structures of reaction products were confirmed by IR, ^{1}H NMR, ^{13}C NMR, and HRMS, and by X-ray crystallography for the A,B-condensed isoxazoles. The introduction of an isoxazole group increases

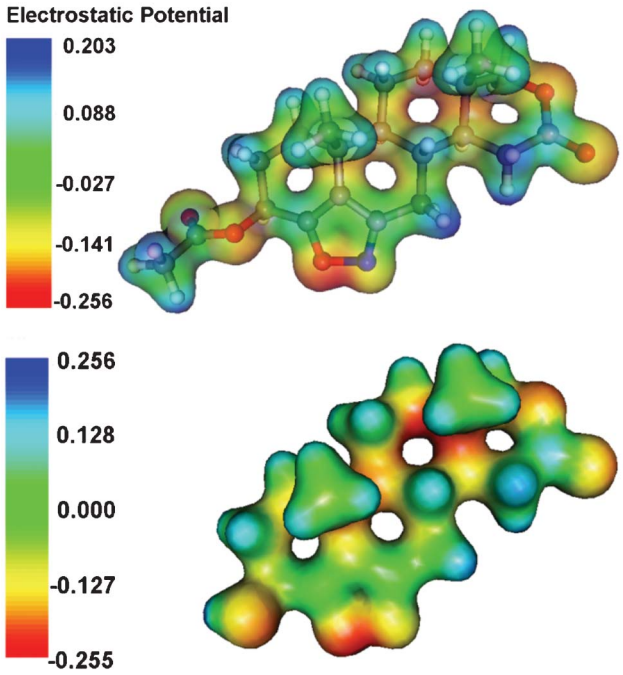

Fig. 3 Calculated molecular electrostatic potential (MEP) map of A,B-isoxazole D-homo lactone androstane derivatives **7** (upper) and **8** (lower). MEP partial charges are colored according to the gradient shown to the left of each structure. Note the significant negative partial charge gained upon addition of the isoxazole group.

the hydrogen bonding potential of the steroidal A,B-ring. Strong antiproliferative activity was observed for three compounds against prostate (PC-3), cervical (HeLa) and colon (HT-29) cancer cells, while no compounds showed antiproliferative activity to K562 cells and non-cancerous control cells (MRC-5). In addition, several compounds displayed selective antiproliferative activity against ER− *vs.* ER+ breast cancer cells. Steroidal isoxazole **8** showed antiproliferative activity only to ER− breast cancer cells and inverse docking suggests that **8** interacts with proteins differentially expressed in ER− *vs.* ER+ breast cancers. Based on the present study, A,B-modified D-homo lactone androstane derivatives could be used to develop new, selective antiproliferative agents, particularly against ER− breast cancers.

Table 3 Antiproliferative activity against a panel of human cancer cell lines

	IC_{50} (μM)						
Compounds	MCF-7	MDA-MB-231	PC-3	HeLa	HT-29	K562	MRC-5[c]
1[25]	>100	23.54	78.31	>100	61.16	>100	>100
2[25]	>100	12.98	2.64	>100	>100	>100	>100
4[25]	>100	15.67	66.87	>100	>100	>100	>100
5	>100	35.47	45.21	97.34	>100	>100	>100
6	81.32	11.88	36.65	35.97	3.97	>100	>100
7	>100	49.76	25.56	>100	>100	>100	>100
8	>100	11.24	>100	>100	>100	>100	>100
12	79.59	>100	>100	4.97	>100	>100	>100
Formestane[a]	>100	55.5	48.36	5.55	>100	>100	>100
Doxorubicin[b]	0.75	0.12	95.61	1.17	0.32	0.36	0.12

[a] Control steroidal compound in clinical use for treatment of breast cancer. [b] Control antiproliferative compound. [c] Normal control cells.

4. Experimental

4.1. General

Melting points were determined using a Electrothermal 9100 apparatus and are uncorrected. Infrared spectra (wave numbers in cm^{-1}) were recorded in KBr pellets (for crystals) on a NEXUS 670 SP-IR spectrometer. NMR spectra were recorded using a Bruker AC 250E spectrometer operating at 250 MHz (proton) and 62.5 MHz (carbon) with tetramethylsilane as the internal standard. Chemical shifts are given in ppm (δ-scale). High resolution mass spectra (HRMS) 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.

4.2. 5α-Hydroxy-17-oxa-D-homoandrostane-6,16-dion-3β-yl acetate (4)

In a solution of compound **1** (0.2205 g, 0.64 mmol) in dichloromethane (3 mL) at 0 °C, MCPBA (0.1580 g, 0.92 mmol) was added and the mixture was stirred for 1 h 30 min at 0 °C, and after that for 1 h at room temperature. Acetone (8 mL) was added and the mixture was cooled to 0 °C in an ice bath before the addition of a solution of CrO_3 (0.3235 g, 3.24 mmol) in water (1 mL). The ice bath was removed and the mixture stirred at room temperature for 20 min, then again cooled to 0 °C in the ice bath prior to the drop wise addition of a solution of CrO_3 (0.1617, 1.62 mmol) in water (0.5 mL). The ice bath was removed and the mixture stirred at room temperature for 50 min. The reaction mixture was poured into water (25 mL) and extracted with dichloromethane (3 × 20 mL). The organic layer was washed with water (1 × 20 mL), 10% $NaHCO_3$ solution (1 × 20 mL) and saturated NaCl solution (1 × 20 mL), dried (anh. Na_2SO_4) and evaporated to afford the crude product, which was purified on a silica gel column (5 g, hexane–ethyl-acetate, 2 : 1 and 1 : 1) to give pure compound **4** (0.1323 g, 54.9%, mp >300 °C after recrystallization from hexane–ethyl-acetate). IR (KBr) ν_{max} cm^{-1}: 3431, 2944, 1716, 1381, 1243, 1031, 754. ^{1}H NMR (250 MHz, $CDCl_3$): δ_H = 0.82 (3 H, s, H-18), 1.01 (3 H, s, H-19), 2.03 (3 H, s, CH_3 from OAc), 2.60 (1 H, d, J = 5.8 Hz, H-7), 2.69 (1 H, dd, J_1 = 5.6 Hz, J_2 = 18.5 Hz, H-15b), 2.80 (1 H, bs, 5α-OH), 3.92 (1 H, d, J = 10.8 Hz, H-17a), 4.00 (1 H, d, J = 10.9 Hz, H-17b), 5.03 (1 H, m, H-3). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ_C = 15.04 (C-18), 19.67 (C-19), 21.33 (CH_3 from OAc), 26.11, 29.25, 29.64, 31.42, 32.10, 32.71, 34.21, 37.56, 39.51, 42.08, 43.11, 44.43, 70.50 (C-3), 79.63 (C-5), 80.83 (C-17), 170.28 (C_{16}=O), 171.17 (C=O from OAc), 211.02 (C_6=O). HRMS (TOF), m/z: for $C_{21}H_{30}O_6$: $[M + H]^+$ calcd. 379.21152, found 379.21164.

4.3. 17-Oxa-D-homoandrost-4-ene-6,16-dion-3β-yl acetate (5)

Thionyl chloride (0.3 mL, cooled to 0 °C) was added to 5α-hydroxy-17-oxa-D-homoandrostane-6,16-dion-3β-yl acetate

Table 4 *In silico* ligand-based screening for possible protein targets of compound **8**

Protein[a]	Binding energy	PDB
ADP-ribosylation factor 1	−10.83	1S9D
Cyclin homolog	−10.73	1XO2
5′-nucleotidase	−10.66	1HO5
Glycogen phosphorylase	−10.24	1KTI
14-3-3-like protein C	−9.99	2O98
Phosphoglycerate kinase 2	−9.97	2PAA
Adenylate kinase	−9.95	1KHT
Cell division protein kinase 2	−9.94	2IW6
6-phosphofructo-2-kinase	−9.89	2DWO
Tyrosine-protein kinase Lyn	−9.88	2ZV9
Dual specificity mitogen-activated protein kinase kinase 1	−9.86	3EQC
Serine/threonine-protein kinase Chk1	−9.85	2WMU
Pyridoxal kinase	−9.85	3IBQ
Eukaryotic initiation factor 4A-III	−9.79	3EX7
Peptidyl-prolyl *cis–trans* isomerase A	−9.79	1VBS
Death-associated protein kinase 1	−9.78	3EH9
Dual specificity mitogen-activated protein kinase kinase 1	−9.72	3EQF
Phosphatidylinositol 3-kinase catalytic subunit type 3	−9.7	3LS8
Ribosomal protein S6 kinase alpha-1	−9.67	2Z7S
Serine/threonine-protein kinase	−9.62	2RIO
Aldose reductase	−9.58	2IKH
Cyclin-dependent protein kinase PHO85	−9.57	2PMI
Fatty acid-binding protein	−9.51	3FR2

[a] Proteins identified by *in silico* screening to bind compound **8** which are reported to have increased expression in ER-negative (MB-MDA-231) *versus* ER-positive (MCF-7) breast cancer cells.

(**4**, 0.173 g, 0.47 mmol) dissolved in absolute pyridine (7 mL, cooled to 0 °C) and the reaction mixture was stirred at 0 °C for 2 h. When the reaction was complete, the mixture was poured into water (10 mL), acidified with HCl (1 : 1) and extracted with dichloromethane (4 × 10 mL). The combined organic extract was dried (anh. Na_2SO_4) and solvent was evaporated to yield a crude product (0.086 g). Pure compound **5** (0.062 g, 37.7%, mp 168–170 °C) was obtained in the form of white crystals after purification by column chromatography (1 g silica gel, hexane–ethyl-acetate 1 : 1). IR (KBr) ν_{max} cm^{-1}: 2947, 1734, 1691, 1637, 1406, 1237, 1134, 1164, 1061. ^{1}H NMR (250 MHz, $CDCl_3$): δ_H = 1.03 (3 H, s, H-18), 1.05 (3 H, s, H-19), 2.07 (3 H, s, CH_3 from OAc), 2.61 (1 H, dd, J_1 = 3.9 Hz, J_2 = 14.0 Hz, H-15), 2.68 (1 H, d, J = 12.6 Hz, H-7), 3.92 (1 H, d, J = 10.8 Hz, H-17a), 4.01 (1 H, d, J = 10.9 Hz, H-17b), 5.34 (1 H, m, H-3), 6.14 (1 H, d, J = 3.6 Hz, H-4). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ_C = 14.95 (C-18), 18.96 (C-19), 19.53, 21.11 (CH_3 from OAc), 24.00, 31.41, 32.29, 33.86, 34.05, 34.52, 37.90, 44.00, 44.90, 49.74, 68.97 (C-3), 80.59 (C-17), 129.83 (C-4), 146.57 (C-5), 169.74 (C_{16}=O), 170.63 (C=O from OAc), 200.24 (C_6=O). HRMS (TOF), m/z: for $C_{21}H_{28}O_5$: $[M + H]^+$ calcd. 361.20095, found 361.20081.

4.4. 6(*E*)-Hydroximino-17-oxa-D-homoandrost-4-en-16-on-3β-yl acetate (6)

Hydroxylamine-hydrochloride (1.10 g, 15.3 mmol) and sodium acetate (0.464 g, 5.66 mmol) were dissolved in 95% ethanol (30 mL) and added to a solution of compound **5** (0.746 g, 2.07 mmol) in 95% ethanol (50 mL). The reaction mixture was stirred at room temperature for 24 h. After reaction completion, water (100 mL) was added and the reaction mixture extracted with dichloromethane (3 × 10 mL). The combined organic extract was dried (anh. Na_2SO_4) and solvent was evaporated to yield a crude product (0.835 g), which was purified by column chromatography (10 g silica gel, hexane–ethyl-acetate 1 : 3), affording pure compound **6** (43.2%, 0.336 g, mp 179–181 °C) after recrystallization from ethyl-acetate–hexane. IR (KBr) ν_{max} cm^{-1}: 3355, 2945, 1732, 1406, 1380, 1241, 1195, 1020, 754. ^{1}H NMR (250 MHz, $CDCl_3$): δ_H = 1.02 (3 H, s, H-18), 1.04 (3 H, s, H-19), 2.07 (3 H, s, CH_3 from OAc), 2.83 (1 H, dd, J_1 = 5.4 Hz, J_2 = 18.6 Hz, H-15), 3.44 (1 H, dd, J_1 = 3.2 Hz, J_2 = 14.5 Hz, H-7), 3.91 (1 H, d, J = 10.8 Hz, H-17a), 3.99 (1 H, d, J = 10.8 Hz, H-17b), 5.30 (1 H, m, H-3), 5.76 (1 H, s, H-4). ^{1}H NMR (250 MHz, DMSO-d_6): δ_H = 0.89 (3 H, s, H-18), 0.90 (3 H, s, H-19), 2.01 (3 H, s, CH_3 from OAc), 5.53 (1 H, s, H-4), 10.83 (1 H, s, =NOH). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ_C = 15.00 (C-18), 18.61 (C-19), 19.09, 21.26 (CH_3 from OAc), 21.45, 25.24, 27.62, 31.67, 32.38, 34.02, 34.21, 37.73, 44.88, 51.15, 69.34, 80.75 (C-17), 123.94 (C-4), 142.66 (C-5), 156.67 (C_6=NOH), 170.10 (C_{16}=O), 170.88 (C=O from OAc). HRMS (TOF), m/z: for $C_{21}H_{29}NO_5$: $[M + H]^+$ calcd. 376.21185, found 376.21186.

4.5. Isoxazolo[5′,4′,3′ : 4,5,6]-17-oxa-D-homoandrost-4-en-16-on-3β-yl acetate (7) and 3β-hydroxy-isoxazolo[5′,4′,3′ : 4,5,6]-17-oxa-D-homoandrost-4-en-16-one (8)

Iodine (0.165 g, 0.65 mmol), potassium iodide (0.280 g, 1.6 mmol), sodium hydrogencarbonate (0.20 g, 2.3 mmol) and water (3 mL) were added to 6(*E*)-hydroximino-17-oxa-D-homoandrost-4-en-16-on-3β-yl acetate (**6**, 0.238 g, 0.63 mmol) dissolved in tetrahydrofuran (10 mL), and the reaction mixture was stirred under reflux for 5.5 h. When the reaction was completed, the reaction mixture was poured into water (20 mL), acidified with HCl (1 : 1), and extracted with dichloromethane (4 × 10 mL). The combined organic extract was washed with a 5% solution of sodium thiosulfate and brine, dried and the solvent was removed to give the crude product (0.178 g). The pure compound **7** (0.066 g, 27.9%, mp 225–227 °C) was obtained in the form of white crystals after purification by column chromatography (5 g silica gel, hexane–ethyl-acetate 1 : 1 and 1 : 2). Pure compound **8** was obtained in a yield of 6.2% (0.013 g, mp 150–152 °C). Compound **7**: IR (KBr) ν_{max} cm^{-1}: 3058, 2943, 1736, 1475, 1427, 1405, 1380, 1355, 1235, 1195, 1107, 1051, 1030, 735. ^{1}H NMR (250 MHz, $CDCl_3$): δ_H = 1.08 (3 H, s, H-18), 1.22 (3 H, s, H-19), 2.14 (3 H, s, CH_3 from OAc), 2.82 (1 H, dd, J_1 = 5.8 Hz, J_2 = 18.5 Hz, H-15), 3.03 (1 H, dd, J_1 = 7.1 Hz, J_2 = 17.8 Hz, H-7), 3.92 (1 H, d, J = 10.9 Hz, H-17a), 4.01 (1 H, d, J = 10.9 Hz, H-17b), 5.86 (1 H, t, H-3, J = 8.7 Hz). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ_C = 14.97 (C-18), 19.04, 19.93 (C-19), 20.96 (CH_3 from OAc), 24.77, 26.52, 31.84, 32.61, 32.65, 32.93, 33.61, 36.48, 45.46, 48.29, 65.09 (C-3), 80.52 (C-17), 125.12 (C-5), 157.87 (C-6), 161.42 (C-4), 169.83 (C_{16}=O), 170.39 (C=O from OAc). HRMS (TOF), m/z: for $C_{21}H_{27}NO_5$: $[M + H]^+$ calcd. 374.19620, found 374.19580. Compound **8**: IR (KBr) ν_{max} cm^{-1}: 3409, 2939, 1727, 1405, 1381, 1339, 1233, 1195, 1051, 1028. ^{1}H-NMR (250 MHz, Py-d_5): δ_H = 0.89 (3 H, s, H-18), 1.05 (3 H, s, H-19), 2.89 (1 H, dd, J_1 = 5.9 Hz, J_2 = 18.5 Hz, H-15), 3.00 (1 H, dd, J_1 = 7.2 Hz, J_2 = 17.7 Hz, H-7), 3.86 (1 H, d, J = 5.5

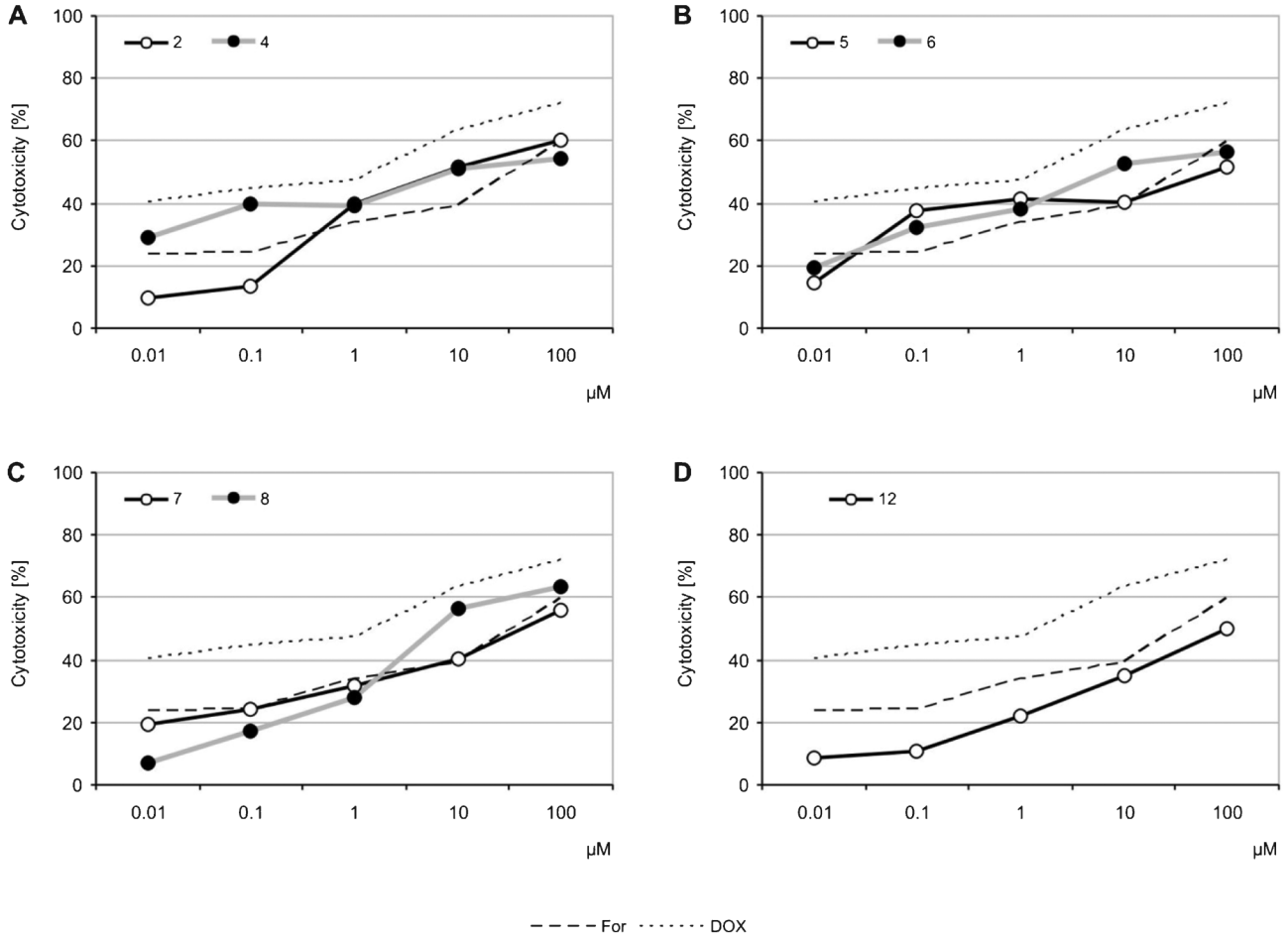

Fig. 4 Antiproliferative activity of selected synthesized compounds against MDA-MB-231 cells. For- Formestane; DOX- Doxorubicin.

Hz, H-17a), 3.90 (1 H, d, J = 10.7 Hz, H-17b), 5.10 (1 H, m, H-3), 8.52 (1 H, s, OH). ^{13}C NMR (62.5 MHz, Py-d_5): 16.54 (C-18), 21.94 (C-19), 26.84, 32.63, 32.95, 34.05, 34.57, 34.75, 35.49, 38.63, 47.25, 50.02, 50.33, 65.67 (C-3), 82.37 (C-17), 131.68 (C-5), 160.35 (C-6), 168.82 (C-4), 171.85 (C_{16}=O). HRMS (TOF), m/z: for $C_{19}H_{25}NO_4$: $[M + H]^+$ calcd. 332.18563, found 332.18589.

4.6. 6(*E*)-Hydroximino-5α-hydroxy-17-oxa-D-homoandrostan-16-on-3β-yl acetate (9) and 6(*Z*)-hydroximino-5α-hydroxy-17-oxa-D-homoandrostan-16-on-3β-yl acetate (10)

Hydroxylamine-hydrochloride (1.75 g, 25.36 mmol) and sodium acetate (0.74 g, 9.02 mmol) dissolved in 95% ethanol (20 mL) were added to compound **4** (1.189 g, 3.15 mmol) dissolved in 95% ethanol (140 mL) and the reaction mixture was stirred at 60 °C for 1 h. After reaction completion, water (50 mL) was added and the mixture was extracted with dichloromethane (5 × 20 mL). The combined organic extract was dried (over anh. Na_2SO_4) and evaporated to give a crude product (1.119 g), which was purified by column chromatography (11 g silica gel, hexane–ethyl-acetate 1 : 1, 1 : 4), affording a mixture of compounds **9** and **10** with a combined yield of 25.2% (0.312 g, mp 284–286 °C). The mixture of compounds **9** and **10**: IR (KBr) ν_{max} cm^{-1}: 3435, 3017, 2945, 1716, 1574, 1474, 1405, 1381, 1367, 1244, 1196, 1163, 1031, 755. ^{1}H NMR (250 MHz, Py-d_5): δ_H = 0.81 and 0.86 (6 H, 2s, 2H-18), 0.84 and 0.93 (6 H, 2s, 2H-19), 1.95 and 2.00 (6 H, 2s, 2CH_3 from 2OAc), 3.86 (2 H, m, 2H-17), 5.62 and 5.79 (2 H, m, 2H-3), 7.77 (1 H, s, 5α-OH), 12.66 (1 H, s, =NOH). ^{13}C NMR (62.5 MHz, Py-d_5): δ_H = 15.28 (2C-18), 16.41 (2C-19), 21.57, 22.74 (CH_3 from OAc), 22.79 (CH_3 from OAc), 24.89, 28.40, 31.36, 33.33, 34.30, 35.82, 37.10, 39.26, 41.47, 42.83, 43.90, 45.04, 72.70 and 73.25 (2C-3), 77.55 and 81.01 (2C-5), 82.26 and 82.41 (2C-17), 161.44 (C_6=NOH), 171.44 and 171.87 (2C=O from 2OAc).

4.7. 6-Nitrile-17-oxa-5,6-seco-D-homoandrostane-5,16-dion-3β-yl acetate (11)

Acetic anhydride (2 mL) was added to a mixture of 6(*E*)-hydroximino-5α-hydroxy-17-oxa-D-homoandrostan-16-on-3β-yl acetate (**9**) and 6(*Z*)-hydroximino-5α-hydroxy-17-oxa-D-homoandrostan-16-on-3β-yl acetate (**10**, 0.17 g, 0.43 mmol) in absolute pyridine (4 mL), and the reaction mixture was stirred at 70 °C for 2 h. After reaction completion, the mixture was poured into cold water (15 mL) and acidified with HCl (1 : 1), then extracted with dichloromethane (5 × 5 mL). The extract was dried (anh. Na_2SO_4) and evaporated to give a crude product (0.160 g), which was further purified by column chromatography (5 g silica gel, hexane–ethyl-acetate, 1 : 1, 1 : 3), affording compound **11** in the form of an oil (0.045 g,

27.8%). IR (film) ν_{max} cm^{-1}: 2947, 2245, 1732, 1638, 1474, 1441, 1406, 1367, 1243, 1197, 1031, 755. ^1H NMR (250 MHz, $CDCl_3$): δ_H = 1.02 (1 H, s, H-18), 1.03 (1 H, s, H-19), 2.04 (3 H, s, CH_3 from OAc), 2.66 (3 H, m, H-4, H-7, H-15a), 3.37 (1 H, dd, J_1 = 4.4 Hz, J_2 = 10.5 Hz, H-15b), 3.95 (1 H, d, J = 10.9 Hz, H-17a), 4.03 (1 H, d, J = 10.9 Hz, H-17b), 5.43 (1 H, bs, H-3). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ_C = 14.84 (C-18), 16.92 (C-19), 17.41, 20.53, 20.72, 21.18, 24.98, 31.59, 32.33, 33.88, 36.67, 40.81, 41.51, 43.52, 52.10, 72.92 (C-3), 80.17 (C-17), 117.26 (C≡N), 168.96 (C_{16}=O), 170.05 (C=O from OAc), 215.66 (C_5=O). HRMS (TOF), m/z: for $C_{21}H_{29}NO_5$: $[M + Na]^+$ calcd. 398.19379, found 398.19353.

4.8. 4β,5β-Epoxy-3β-hydroxy-17-oxa-D-homoandrostane-6,16-dione (12)

Sodium hydroxide (3 mL, 4 mol dm^{-3}) and 30% hydrogen peroxide (4.5 mL) were added to a solution of 17-oxa-D-homoandrost-4-ene-6,16-dion-3β-yl acetate (**5**, 0349 g, 0.96 mmol) in methanol (10 mL), and the reaction mixture was stirred for 3 h at 0 °C. Upon reaction completion, the mixture was acidified with HCl (1 : 1) and extracted with dichloromethane (4 × 10 mL). The extract was dried (anh. Na_2SO_4), and evaporated to give a crude product (0.278 g), which was further purified by column chromatography (6 g silica gel, hexane–ethyl-acetate, 6 : 1 and 1 : 5), affording compound **12** (38.0%, 0.123 g, mp 253–255 °C). IR (KBr) ν_{max} cm^{-1}: 3446, 2948, 1723, 1456, 1406, 1383, 1268, 1245, 1196, 1060, 1036, 926. ^1H NMR (250 MHz, $CDCl_3$): δ_H = 1.05 (3 H, s, H-18), 1.06 (3 H, s, H-19), 2.67 (1 H, dd, J_1 = 3.4 Hz, J_2 = 9.3 Hz, H-15), 2.72 (1 H, d, J = 3.2 Hz, H-7), 3.32 (1 H, d, J = 3.1 Hz, H-4α), 3.92 (1 H, d, J = 10.9 Hz, H-17a), 4.03 (1 H, d, J = 11.0 Hz, H-17b), 4.07 (1 H, s, H-3α). ^{13}C NMR (62.5 MHz, $CDCl_3$): δ_C = 14.87 (C-18), 18.18 (C-19), 19.65, 25.53, 29.22, 31.49, 32.33, 33.94, 35.13, 37.70, 44.33, 44.79, 47.08, 62.85 (C-4), 64.63 (C-3), 69.20 (C-5), 80.51 (C-17), 169.54 (C_{16}=O), 203.78 (C_6=O). HRMS (TOF), m/z: for $C_{19}H_{26}O_5$: $[M + H]^+$ calcd. 335.18530, found 335.18451.

4.9. X-ray crystal structure determination

X-ray diffraction data for compounds **7** and **8** were collected at room temperature on an Oxford Diffraction Gemini S diffractometer. Data reduction for compounds was performed with the program package CrysAlis RED.[45] Space group determinations were based on the analysis of the Laue class and systematically absent reflections. Structures were solved by direct methods using SIR92.[46] Structures were refined using full-matrix least-squares. Non-hydrogen atoms were refined anisotropically for both compounds, H atoms were treated by a mixture of independent and constrained refinement. All calculations were performed using SHELXL97,[47] PARST[48] and PLATON,[49] as implemented in the WINGX[50] system of programs. Crystal data and refinement parameters are shown in Table 1.

4.10. Molecular electrostatic potential map calculations

Molecular electrostatic potential (MEP) maps were calculated from the crystal structures of **7** and **8** using a GAMESS *ab initio* quantum chemistry package[51] (RHF/3-21G level of theory) and were visualized using the program MOLEKEL.[52]

4.11. Determination of antiproliferative activity

Antiproliferative activities were measured against six different human cancer cell lines: PC-3 (AR− prostate cancer), MCF-7 (ER+ breast adenocarcinoma), MDA-MB-231 (ER− breast adenocarcinoma), HeLa (cervical cancer), HT-29 (colon cancer) and K562 (chronic myelogenous leukemia) and one human non-tumor cell line (normal fetal lung fibroblasts MRC-5). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 4.5% glucose. Media were supplemented with 10% fetal calf serum (FCS, NIVNS) and antibiotics: 100 IU mL^{-1} penicillin and 100 lg mL^{-1} streptomycin (ICN Galenika). All cell lines were cultured in flasks (Costar, 25 cm^2) at 37 °C in a 100% humidity atmosphere supplemented with 5% CO_2. Only viable cells were used in the assays, and cell viability was determined by the dye exclusion assay using trypan blue.

Antiproliferative activity was evaluated using a colorimetric sulforhodamine B (SRB) assay.[33] Briefly, single cell suspensions were plated onto 96-well microtiter plates (Costar, flat bottom): 5 × 10^3 cells per 180 mL of medium. Plates were pre-incubated for 24 h at 37 °C, 5% CO_2. Test substances were added to all the wells (except controls) at concentrations ranging from 10^{-8} to 10^{-4} mol L^{-1}. After the incubation period (48 h/37 °C/5% CO_2), the SRB assay was carried out as follows: 50 μL of 80% trichloroacetic acid (TCA) was added to all the wells; after 1 h incubation, the plates were washed with distilled water, and 75 μL of 0.4% SRB was added to all the wells; 30 min later the plates were washed with citric acid (1%) and dried at room temperature. Finally, 200 μL of 10 mmol Tris (pH = 10.5) base was added to all the wells. Absorbance (λ 540/690 nm) was measured using a microplate reader (Multiscan MCC340, Labsystems). Wells containing complete media only were used as blanks.

Antiproliferative activity was calculated according to the formula:

$$\left(\frac{1-A_{test}}{A_{control}}\right)100$$

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

Data Analysis Two independent experiments were set out in quadruplicate for each concentration of the test compounds. Reported IC_{50} values define the dose of compound that inhibits cell growth by 50%. The IC_{50} of the test compounds was determined by median effect analysis.

4.12. Ligand-based reverse molecular docking

Ligand-based virtual screening was conducted *in silico* against the entire protein data bank (PDB) using the program idTarget.[37] idTarget requires the three-dimensional structure of an input ligand molecule and performs rapid molecular docking against protein structures in the PDB using MEDock, followed by rescoring using an Autodock4 scoring function.[37] Proteins were considered to be possible ligand targets if the Autodock4 binding energy was stronger than −9.5 kcal mol^{-1} and the idTarget Z-score was negative. This preliminary list was then further filtered by comparison with published lists of proteins shown to have increased expression in estrogen receptor negative breast cancers *versus* ER+ breast cancer cells.[42–44]

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