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SYNTHESIS AND ANTITUMOR ACTIVITIES OF STEROIDAL 5α,8α-ENDOPEROXIDE DERIVATIVES WITH SIDE CHAIN OF 17-HYDRAZONE AROMATIC HETEROCYCLE

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Abstract – Here a series of novel 5α,8α-epidioxyandrostan-3β-ol-17-hydrazone derivatives possessing various aromatic heterocycle structures in 17-side chain of their steroidal nucleus were synthesized and characterized. The antiproliferative activity of synthesized compounds against some cancer cells was investigated. The results have demonstrated that compound **8b** with quinoline and **8c** with indole structure in side chain display excellent anti-proliferative activity *in vitro* against tested cancer cell lines.

INTRODUCTION

It is well known that N, O, S-heterocycles are important structural units present in many drugs, natural and synthetic products with potent bioactivity profile.[1] They possess hydrogen bond donors and acceptors in a rigid framework, and they can therefore effectively interact with target enzymes and receptors via hydrogen bond interactions.[2] They can enhance binding affinity and improve in vitro potency, thus providing desired pharmacokinetic properties and pharmaceutical properties.[3] Studies have found that introducing some heterocycles into the steroids, changing the steroidal side chain or substitution of the steroidal skeleton, introducing heteroatom or replacing one or more carbon atoms in steroidal molecule with heteroatom may result in change of its biological activities.[4] So far, the steroids containing heterocycles had been widely researched and reported.[5]

Sterol endoperoxides are the important active lead compounds in drug discovery, which are well known for their 5α,8α-peroxy moiety. Among sterol endoperoxides, ergosterol peroxide (5α,8α-epidioxyergosta-6,22-dien-3β-ol, EP) (Figure 1), is a member of a class of fungal secondary metabolites of sterol 5α,8α-endoperoxide derivatives.[6] In our previous study, we found that EP purified from *Ganoderma lucidum*, induced cell death and inhibited cell migration, cell cycle progression, and colony growth of human hepatocellular carcinoma cells. We further examined the mechanism associated with this effect and found that treatment with EP increased expression of Foxo3a mRNA and protein in HepG2 cells. The levels of Puma and Bax, pro-apoptotic proteins, were effectively enhanced. Our results suggest that ergosterol peroxide stimulated Foxo3 activity by inhibiting pAKT and c-Myc and activating pro-apoptotic protein Puma and Bax to induce cancer cell death.[7]

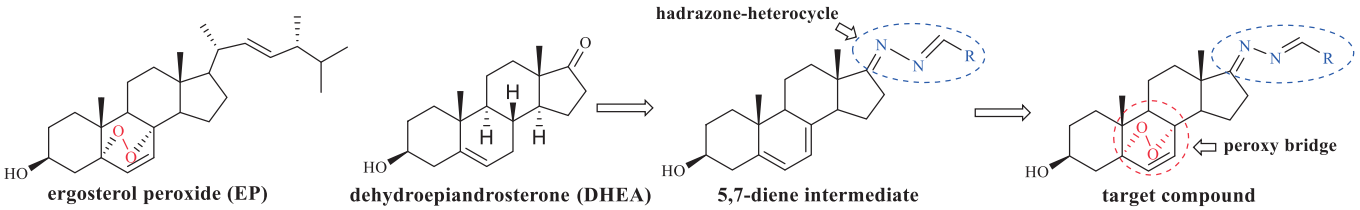
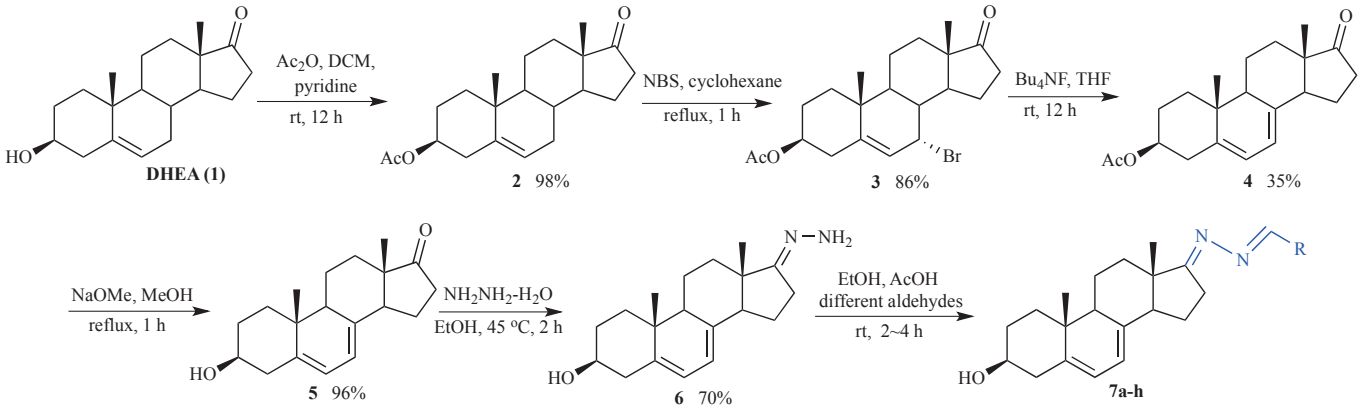


Figure 1. Molecular structures of ergosterol peroxide, DHEA, and designed compound

In order to obtain biologically potent compounds with diverse structures, therefore in this paper, a series of steroidal compounds containing heterocycle attached to the steroidal 17-side chain by a hydrazone structure were designed and synthesized using dehydroepiandrosterone as staring materials (Figure 1). Furthermore, the antiproliferative activity of the compounds against some cancer cells was evaluated *in vitro*.

RESULTS AND DISCUSSION

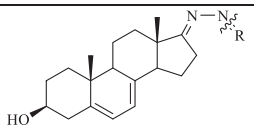
Chemistry. The synthetic route of new steroidal derivatives with side chain of hydrazone aromatic heterocycle **7a-h** is shown in Scheme 1. First, acetylize of **1** to get compound **2**, then bromination of **2** and

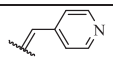
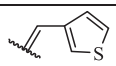
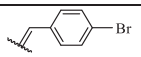
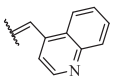
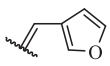
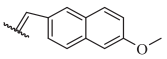
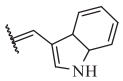


Scheme 1. Synthetic route of the target compounds **7a-h**

dehydrobromination of **3** to afforded 5,7-diene acetate **4**,[8] which was then converted to **5** via solvolysis in 30% overall yield from **1**. Then, **5** was converted to the corresponding steroidal hydrazone **6** via reaction with hydrazine hydrate in anhydrous ethanol.[9] After crystallized, the reaction of pure steroidal hydrazone with appropriate aldehydes gave steroidal hydrazone derivatives **7a-h** (Table 1).

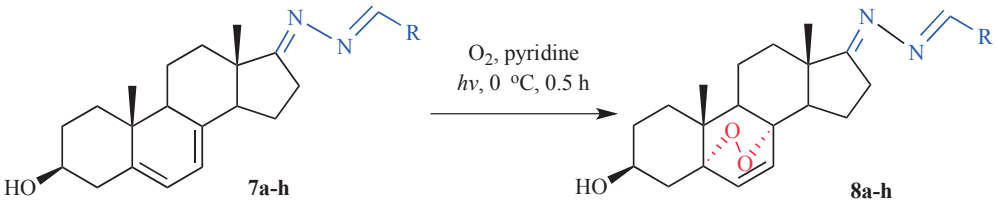
Table 1. Synthesis of the intermediates **7a-h**



Entry	R	Yield[a]	Entry	R	Yield[a]	Entry	R	Yield[a]
7a		78%	**7d**		82%	**7g**		80%
7b		86%	**7e**		77%	**7h**		91%
7c		73%	**7f**		80%			

[a] Isolated yields.

Then treatment of **7a-h** with eosin Y (EY) as photosensitizer in pyridine, irradiated by iodine tungsten lamp and kept bubbling oxygen for 0.5 h to get target compounds **8a-h** (Scheme 2).[10] The synthesis of compounds **8a-h** were achieved by the α-π-facial stereoselectivity of the addition reaction of singlet oxygen with $\Delta^{5,7}$-sterol **7a-h**. Reactions were monitored by thin layer chromatography (TLC) plates and/or by staining with H_2SO_4-EtOH solution (5 mL concentrated H_2SO_4 + 95 mL EtOH). The chemical structures of the compounds synthesized were elucidated on the basis of ^{1}H NMR, ^{13}C NMR, and LC-MS. The detailed physical and analytical data are given in experimental part. In the ^{1}H NMR spectrum, the downfield chemical shifts of C6-H at 6.50 ppm (d) and C7-H at 6.35 ppm (d) demonstrate a formation of 5,8-peroxy bond and 6-CH=CH-7 bond in compound **8a (**Figure 2**)**. In addition, the resonances showing of C22-H at 8.25 ppm (s) and 22-C at 155.3 ppm demonstrate a formation of 17=N−N=C−Ar bond in **8a**.



Scheme 2. Synthetic route of the target compounds **8a-h**

Biology. The newly synthesized compounds **8a-h** and EP were evaluated for their antiproliferative activities against human cancer cell lines derived from various human cancer types, including human hepatocellular carcinoma cell lines (HepG2, SK-Hep1) and human breast cervical cancer cell lines (MDA-MB-231, MCF-7). *In vitro* cell-based evaluation of the antiproliferative activities of the

synthesized compounds was carried out using MTT assay. Cisplatin was employed as the positive control. The anticancer potency of these compounds were indicated by IC_{50} values. The results were summarized in Table 2. As compared to the parent EP molecule, most of compounds **8a-h** possess distinct antiproliferative activity against all the four tested adherent cancer cell lines. The screening results suggested a rough structure-activity relationships: among the steroidal 5α,8α-endoperoxide derivatives with side chain of 17-hydrazone aromatic heterocycle, compound **8b** and **8c** with quinoline and indolyl-group in 17-side chain displayed better antiproliferative activity.

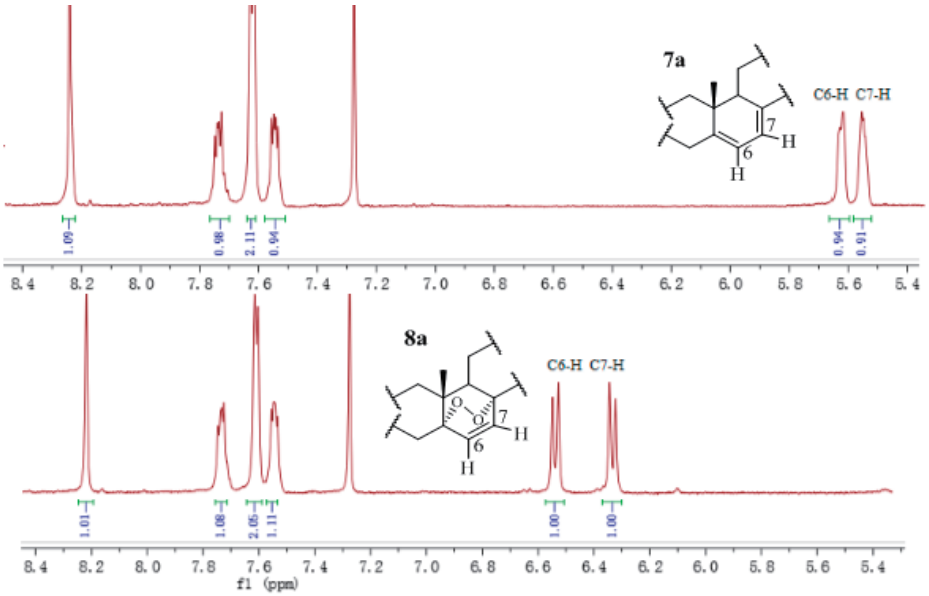


Figure 2. The partial ^{1}H NMR spectrums of compound **7a** and **8a**

Compounds **8a-h** and EP were selected for further cytotoxic activity evaluation *in vitro* using a non-cancer cell line 293T as a control. As shown in Table 2, only compound **8b** showed little cytotoxicity (CC_{50} <50 μM). It is apparent from the results depicted in Table 2 that most of compounds exhibit obvious selectivity between cancer cell line and non-cancer cell line.

In summary, we have prepared a series of steroidal 5α,8α-epidioxyandrost-6-en-3β-ol-17-hydrazone derivatives possessing various aromatic heterocycle structures. The antiproliferative activity of the synthesized compounds against human hepatocellular carcinoma cell lines HepG2, SK-Hep1 and human breast cervical cancer cell lines MDA-MB-231, MCF-7 were investigated. The results had demonstrated that some steroidal endoperoxide-17-hydrazone heterocycle derivatives showed distinct antiproliferative activity. Preliminary SARs were put forward based on the biological results. Compound **8b** and **8c** with quinoline and indole structure in 17-side chain displayed better antiproliferative activity *in vitro* against tested cells. In an overall view, the steroidal 5α,8α-endoperoxide-17-hydrazone derivatives will provide better insight into the effect of the C-17 site on anticancer activity for designing potential steroidal

endoperoxide anticancer agents. Future work will also focus on the synthesis of additional candidate structures with different side-chain to address specific cancer cell lines.

Table 2. *In vitro* antiproliferative activity and cytotoxic activity data of compounds

	IC_{50} (μM)[a]				CC_{50} (μM)[a]
Entry	**HepG2**	**SK-Hep1**	**MCF-7**	**MDA-MB-231**	**293T**
8a	35.40	27.76	31.60	26.53	>80
8b	8.74	13.99	14.30	17.54	44.10
8c	10.63	9.38	15.78	10.66	54.52
8d	19.54	16.34	24.25	35.07	62.60
8e	40.46	34.73	47.62	38.22	>80
8f	>60	43.54	49.34	>60	>60
8g	23.22	20.14	17.76	15.63	54.40
8h	48.62	>60	>60	>60	>80
EP	16.80	17.25	23.31	19.17	56.82
Cisplatin	0.65	2.39	6.63	5.50	24.22

[a] Data represent the mean values of three independent determinations.

EXPERIMENTAL

General methods. All commercially available reagents were used without further purification. Melting points (uncorrected) were determined on a MP120 auto point apparatus (Jinan Hanon Instruments Co. Ltd). ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker Avance DRX400 spectrometer with TMS and solvent signals allotted as internal standards. The chemical shifts of the ^{1}H NMR and ^{13}C NMR were expressed in ppm (δ). MS-ESI mass spectra were obtained on an Esquire 6000 mass spectrometer.

LC-MS data were measured using an Agilent 6120 mass spectrometer. Silica gel (300-400 mesh) was used for analytical and flash chromatography. HRMS data were measured using a Bruker APEX IV Fourier transform ion cyclotron resonance mass spectrometer.

Synthesis of 3β-acetoxyandrost-5-en-17-one (2). To a suspension of DHEA (**1**) (28.8 g, 0.10 mol) in CH_2Cl_2 (40 mL) and pyridine (10 mL) was added Ac_2O (12 mL, 0.13 mol) over 20 min. After the mixture was stirred in room temperature for 6 h, water (30 mL) was added, and then extracted with EtOAc. The combined organic layers were washed with saturated aqueous $NaHCO_3$ and brine. The organic extracts were dried with anhydrous $MgSO_4$ and concentrated to get crude product **2** as a white solid (32 g). Yield: 98%, mp 167-170 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.41 (d, J = 5.1 Hz, 1H), 4.65-4.54 (m, 1H), 2.46 (dd, J = 19.3, 8.6 Hz, 1H), 2.33 (t, J = 7.9 Hz, 2H), 2.10 (dd, J = 18.8, 9.4 Hz, 2H), 2.04 (s, 3H), 1.94 (d, J = 5.7 Hz, 1H), 1.89 (d, J = 2.9 Hz, 1H), 1.87-1.81 (m, 2H), 1.71-1.61 (m, 4H), 1.56 (dd, J = 10.4, 6.4 Hz, 1H), 1.48 (dd, J = 13.3, 4.4 Hz, 1H), 1.33-1.26 (m, 2H), 1.19-1.11 (m, 1H), 1.04 (s, 3H), 0.99 (d, J = 4.6 Hz, 1H), 0.88 (s, 3H). MS (ESI) *m/z*: 353.9 $[M+Na]^+$, 369.7 $[M+K]^+$.

Synthesis of 7α-bromo-3β-(acetyloxy)androst-5-en-17-one (3). A mixture of **2** (32 g, 0.1 mol) and cyclohexane (80 mL) was warmed to 65 °C to obtain a clear solution, then NBS (23.3 g, 0.15 mol) was added and the mixture was heated to reflux for 1 h. The mixture was cooled to room temperature and diluted with 200 mL water. After being stirred for 1 h, the precipitate was collected by filtration and washed with water. Then the solid was dissolved in CH_2Cl_2, the solution was washed with brine, dried with anhydrous $MgSO_4$, and concentrated to get crude 7α-bromo-3β-(acetyloxy)androst-5-en-17-one **3** as a light brown solid (35 g, 86%). The crude product was used for the next step.

Synthesis of 3β-acetoxyandrosta-5,7-dien-17-one (4). A mixture of the crude bromide **3** (35 g, 0.085 mol) prepared above and 1.0 M Bu_4NF in THF (88 mL, 0.127 mol, 1.5 equiv) was stirred overnight. Then, the mixture was diluted with cyclohexane, washed with water, dried with anhydrous $MgSO_4$, and concentrated to get crude product **4** as a brown solid. The product was purified by chromatographic column to give a pale yellow solid as compound **4** (11.5 g). Yield: 35%, mp 112-115 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.6 (1H, s), 5.57 (1H, d, J = 2.6 Hz), 4.7 (1H, m), 2.56-2.49 (2H, m), 2.38 (1H, d, J = 12.5 Hz), 2.24-2.17 (2H, m), 2.06 (3H, s), 1.97-1.92 (2H, m), 1.91 (1H, d, J = 3.7 Hz), 1.74 (2H, d, J = 4.8 Hz), 1.70 (1H, d, J = 5.2 Hz), 1.58 (2H, s), 1.40-1.34 (2H, m), 1.27 (1H, s), 0.98 (3H, s), 0.82 (3H, s). MS (ESI) *m/z*: 351.7 $[M+Na]^+$.

Synthesis of 3β-hydroxyandrosta-5,7-dien-17-one (5). To a suspension of intermediate **4** (11 g, 0.034 mol) in 100 mL of MeOH was added 25% (wt) NaOMe in MeOH (10 mL). After the mixture was reflux for 1 h, 100 mL of water was added dropwise over 1 h. Then, the mixture was stirred for 2 h, the precipitate was collected by filtration, washed with MeOH-H_2O (1:2), and dried at 40 °C under high vacuum to get crude product as a brown solid. The product was purified by chromatographic column to

give a pale yellow solid as intermediate **5** (9.3 g). Yield: 96%, mp 157-159 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.00 (1H, d, J = 9.8 Hz, C6-H), 5.69 (1H, d, J = 9.8 Hz, C7-H), 4.29 (1H, t, J = 7.9 Hz, C3-αH), 3.75-3.58 (1H, m, C3-OH), 2.66-2.40 (2H, m, C16-H), 1.03 (3H, s, C19-CH_3), 0.95 (3H, s, C18-CH_3). MS (ESI) *m/z*: 309.8 $[M+Na]^+$, 325.8 $[M+K]^+$.

Synthesis of 17-hydrazonoandrosta-5,7-dien-3β-ol (6). Intermediate **5** (3.0 g, 10 mmol) was dissolved in 40 mL of EtOH. The mixture was heated to 45 °C, and 4 mL of 85% hydrazine hydrate was added. The solution was stirred for 2 h until no starting material was observed. The reaction was terminated and some white solid was separated out. The solid was filtrated and recrystallized using MeOH as solvent. Compound **6** was obtained as white solid (2.1 g) after drying. Yield: 70%, mp 190-192 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.51 (1H, d, J = 3.7 Hz, C6-H), 5.45 (1H, d, J = 2.7 Hz, C7-H), 5.36 (2H, d, J = 10.9 Hz, NH2), 4.69 (1H, m, C3-αH), 3.17 (1H, m, C3-OH), 2.29 (2H, m, C16-H), 0.88 (3H, s, C19-CH_3), 0.69 (3H, s, C18-CH_3). LC-MS (ESI) *m/z*: 301.2 $[M+H]^+$, 323.2 $[M+Na]^+$.

General procedure for the synthesis of compounds 7a-h. After 0.2 mmol of compound **6** was dissolved in 25 mL of anhydrous EtOH, 0.2 mmol of aldehyde was added. Then the mixture was heated to 50 °C and stirred continually at the temperature until no starting material. Then the reaction was terminated and solvent was evaporated under reduced pressure. The residue was purified by recrystallization or flash chromatography on silica gel to afford the corresponding compounds **7a-h**.

General procedure for the synthesis of compounds 8a-h. For the synthesis of the title compound **8a-h**, a mixture of **7a-h** (100 mg, 0.23 mmol) and eosin Y (1 mg) in pyridine (10 mL) in a quartz tube kept in a water-cooled bath. Then the tube was irradiated with an iodine tungsten lamp. The oxygen was kept bubbling during the irradiation for 0.5 h. The mixture was then poured into cold water and extracted with EtOAc. The combined organic fractions were dried over anhydrous $MgSO_4$ and the solvent was removed under reduced pressure. The product was purified by chromatographic column to give the corresponding compounds **8a-h**.

5α,8α-Epidioxy-17-(pyridin-4-ylmethylene)hydrazonoandrostan-3β-ol (8a). Faint yellow crystals, yield: 62%, mp 197.8-200.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 8.21 (1H, s, C22-H), 7.71 (1H, dd, J = 21.4, 18.0 Hz, 3′-Ar-H), 7.60 (2H, d, J = 4.8 Hz, 2′,5′-Ar-H), 7.55-7.46 (1H, m, 4′-Ar-H), 6.53 (1H, d, J = 8.5 Hz, C6-H), 6.32(1H, d, J = 8.5 Hz, C7-H), 4.03-3.92 (1H, m, C3-αH), 2.82-2.56 (2H, m, C16-H), 1.12 (3H, s, C19-CH_3), 0.92 (3H, s, C18-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.9 (17-C), 155.3 (22-C), 150.1 (4′-Ar-C), 141.5 (3′-Ar-C), 136.3(7-C), 130.9 (2′-Ar-C), 129.9 (6-C), 128.8 (6′-Ar-C), 121.1 (5′-Ar-C), 82.4 (8-C), 79.0 (5-C), 71.8 (3-C), 66.1 (14-C), 51.5 (9-C), 49.1 (4-C), 46.1 (13-C), 37.2 (12-C), 34.7 (10-C), 33.6 (1-C), 30.1 (2-C), 27.9 (16-C), 23.0 (15-C), 20.3 (11-C), 19.1 (18-C), 18.1 (19-C). LC-MS (ESI) *m/z*: 422.2 $[M+H]^+$. HRMS (ESI) *m/z* calcd for $C_{25}H_{31}N_3O_3$ $[M+H]^+$ 422.2444, found 422.2439.

5α,8α-Epidioxy-17-(quinolin-4-ylmethylene)hydrazonoandrostan-3β-ol (8b). White crystals, yield: 64%, mp 207.5-209.6 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 8.98 (1H, d, J = 4.3 Hz, 2′-Ar-H), 8.89 (1H, s, C22-H), 8.73 (1H, d, J = 8.5 Hz, 5′-Ar-H), 8.18 (1H, d, J = 8.4 Hz, 8′-Ar-H), 7.78 (1H d, J = 4.8, 3′-Ar-H), 7.76 (1H, m, 6′-Ar-H),7.64 (1H, t, J = 7.6 Hz, 7′-Ar-H), 6.55 (1H, d, J = 8.4 Hz, C6-H), 6.33 (1H, d, J = 8.4 Hz, C7-H), 4.04-3.92 (1H, m, C3-αH), 2.90-2.63 (2H, m, C16-H), 1.16 (3H, s, C19-CH_3), 0.94 (3H, s, C18-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 182.5 (17-C), 155.6 (22-C), 150.1 (2′-Ar-C), 148.9(4′-Ar-C), 137.6 (10′-Ar-C), 136.3 (7-C), 130.1 (8′-Ar-C), 129.6 (6-C), 128.8 (7′-Ar-C), 127.6 (6′-Ar-C), 125.6 (5′-Ar-C), 124.5 (9′-Ar-C), 121.1 (3′-Ar-C), 82.4 (8-C), 79.0 (5-C), 71.7 (3-C), 66.14 (14-C), 51.5 (9-C), 49.1 (4-C), 46.1 (13-C), 37.0 (12-C), 34.7 (10-C), 34.3(1-C), 30.1 (2-C), 28.3 (16-C), 23.0 (15-C), 20.3 (11-C), 19.1 (18-C), 18.1 (19-C). LC-MS (ESI) *m/z*: 472.2 $[M+H]^+$. HRMS (ESI) *m/z* calcd for $C_{29}H_{33}N_3O_3$ $[M+H]^+$ 472.2600, found 472.2589.

5α,8α-Epidioxy-17-(indol-3-ylmethylene)hydrazonoandrostan-3β-ol (8c). White crystals, yield: 57%, mp 187.4-189.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.65 (1H, s, NH), 8.62 (1H, s, 1′-Ar-H), 8.34 (1H, d, J = 7.3 Hz, C22-H), 7.72 (1H d, J = 3.6 Hz, 4′-Ar-H), 7.53 (1H, d, J = 4.8 Hz, 7′-Ar-H), 7.50 (1H, s, 5′-Ar-H), 7.39 (1H, d, J = 7.4 Hz, 6′-Ar-H), 6.56 (1H, d, J = 8.5 Hz, C6-H), 6.31 (1H, d, J = 8.4 Hz, C7-H), 4.03-3.94 (1H, m, C3-αH), 2.99-2.75 (2H, m, C16-H), 0.99 (3H, s, C19-CH_3), 0.93(3H, s, C18-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 180.3 (17-C), 154.4 (22-C), 136.9 (9′-Ar-C), 136.0 (7-C), 130.9 (2′-Ar-C), 129.6 (6-C), 125.1 (8′-Ar-C), 123.5 (4′-Ar-C), 122.7 (6′-Ar-C), 121.6 (5′-Ar-C), 113.8 (3′-Ar-C), 111.3 (7′-Ar-C), 82.4 (8-C), 79.2 (5-C), 71.8 (3-C), 66.3 (14-C), 51.5 (9-C), 49.2 (4-C), 45.8 (13-C), 37.2 (12-C), 34.7 (10-C), 34.1 (1-C), 30.1 (2-C), 28.2 (16-C), 27.8 (15-C), 20.3 (11-C), 19.2 (18-C), 18.1 (19-C). LC-MS (ESI) *m/z*: 460.3 $[M+H]^+$. HRMS (ESI) *m/z* calcd for $C_{28}H_{33}N_3O_3$ $[M+H]^+$: 460.2600, found 460.2596.

5α,8α-Epidioxy-17-(thiophene-3-ylmethylene)hydrazonoandrostan-3β-ol (8d). Faint yellow crystals, yield: 59%, mp 173.8-175.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.30 (1H, s, C22-H), 7.61 (1H, d, J = 1.6 Hz, 2′-Ar-H), 7.54 (1H, d, J = 4.9 Hz, 5′-Ar-H), 7.34 (1H, d, J = 2.8 Hz, 4′-Ar-H), 6.53 (1H, d, J = 8.4 Hz, C6-H), 6.31 (1H, d, J = 8.4 Hz, C7-H), 4.04-3.89 (1H, m, C3-αH), 2.90-2.51 (2H, m, C16-H), 1.09 (3H, s, C19-CH_3), 0.92 (1H, s, C18-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 180.8 (17-C), 152.6 (22-C), 136.1 (7-C), 131.0 (3′-Ar-C), 130.1 (6-C), 128.8 (2′-Ar-C), 126.6 (5′-Ar-C), 125.5 (4′-Ar-C), 82.4 (8-C), 79.1 (5-C), 71.8 (3-C), 66.2 (14-C), 51.5 (9-C), 49.1 (4-C), 45.9 (13-C), 37.1 (12-C), 34.7 (10-C), 34.0 (1-C), 30.1 (2-C), 27.8 (16-C), 23.0 (15-C), 20.3 (11-C), 19.2 (18-C), 18.0 (19-C). LC-MS (ESI) *m/z*: 427.2 $[M+H]^+$. HRMS (ESI) *m/z* calcd for $C_{24}H_{30}N_2O_3S$ $[M+H]^+$ 427.2055, found 427.2045.

5α,8α-Epidioxy-17-(furan-3-ylmethylene)hydrazonoandrostan-3β-ol (8e). Faint yellow crystals, yield: 55%, mp 165.7-168.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (1H, s, C22-H), 7.81-7.63 (1H, m, 2′-Ar-H), 6.81 (1H, d, J = 3.3 Hz, 4′-Ar-H), 6.52 (1H, s, 3′-Ar-H), 6.51 (1H, d, J = 3.1 Hz, C6-H), 6.32 (1H, d, J =

8.4 Hz, C7-H), 4.03-3.90 (1H, m, C3-αH), 2.72 (2H, m, C16-H), 1.09 (3H, s, C19-CH_3), 0.93 (3H, s, C18-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.2 (17-C), 149.9 (22-C), 147.3 (2′-Ar-C), 145.2 (4′-Ar-C), 136.1 (7-C), 130.1 (6-C), 114.9 (3′-Ar-C), 111.9 (5′-Ar-C), 82.3 (8-C), 79.1 (5-C), 71.8 (3-C), 66.3 (14-C), 51.5 (9-C), 49.1 (4-C), 46.0 (13-C), 37.1 (12-C), 34.7 (10-C), 34.0 (1-C), 30.1 (2-C), 27.8 (16-C), 23.0 (15-C), 20.3 (11-C), 19.2 (18-C), 18.0 (19-C). LC-MS (ESI) *m/z*: 411.2 $[M+H]^+$. HRMS (ESI) *m/z* calcd for $C_{24}H_{30}N_2O_4$ $[M+H]^+$ 411.2284, found 411.2276.

5α,8α-Epidioxy-17-(benzylidene)hydrazonoandrostan-3β-ol (8f). White solid, yield: 61%, mp 72.5-74.4 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.28 (1H, s, C22-H), 7.74 (1H, m, Ar-H), 7.54 (2H, d, J = 5.7 Hz, Ar-H), 7.42 (2H, m, Ar-H), 6.54 (1H, d, J = 8.4 Hz, C6-H), 6.32 (1H, d, J = 8.4 Hz, C7-H), 3.98 (1H, dd, J = 11.2, 5.5 Hz, C3-αH), 2.83-2.60 (2H, m, C16-H), 1.11 (3H, m, C18-CH_3), 0.93-0.88 (3H, m, C18-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.1 (17-C), 162.2 (22-C), 141.1 (5-C), 134.6 (1′-Ar-C), 130.6 (4′-Ar-C), 128.8 (3′-Ar-C), 128.7 (5′-Ar-C), 128.5 (2′-Ar-C), 128.1 (6′-Ar-C), 121.0 (6-C), 71.6 (3-C), 53.6 (14-C), 50.4 (9-C), 44.3 (4-C), 42.2 (13-C), 37.2 (10-C), 36.7 (1-C), 33.9 (12-C), 31.6 (7-C), 31.4 (2-C), 31.3 (8-C), 28.0 (16-C), 23.3 (15-C), 20.7 (11-C), 19.5 (18-C), 16.5 (19-C). LC-MS (ESI) *m/z*: 421.2 $[M+H]^+$. HRMS (ESI) *m/z* calcd for $C_{26}H_{32}N_2O_3$ $[M+H]^+$ 421.2491, found 421.2479.

5α,8α-Epidioxy-17-(4-bromo-benzylidene)hydrazonoandrostan-3β-ol (8g). White crystals, yield: 60%, mp 76.4-78.7 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.23 (1H, s, C22-H), 7.62 (2H, d, J = 8.1 Hz, Ar-H), 7.54 (2H, d, J = 7.8 Hz, Ar-H), 6.53 (1H, d, J = 8.4 Hz, C6-H), 6.31 (1H, d, J = 8.4 Hz, C7-H), 4.03-3.89 (1H, m, C3-αH), 2.82-2.56 (2H, m, C16-H), 1.10 (3H, s, C19-CH_3), 0.93 (3H, s, C18-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.3, 156.7, 136.1, 131.9, 130.9, 130.0, 129.9, 129.5, 128.8, 82.4, 79.0, 71.8, 66.3, 51.5, 49.1, 46.0, 37.2, 36.8, 34.6, 33.9, 30.1, 27.8, 23.0, 20.3, 19.2, 18.1. LC-MS (ESI) *m/z*: 500.2, 502.2 $[M+H]^+$. HRMS (ESI) *m/z* calcd for $C_{26}H_{31}BrN_2O_3$ $[M+H]^+$ 501.1596, found 501.1570.

5α,8α-Epidioxy-17-(6-methoxynaphthalen-2-ylmethylene)hydrazonoandrostan-3β-ol (8h). White crystals, yield: 55%, mp 112.1-114.3 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.41 (1H, s, C22-H), 8.05-7.91 (2H, m, 4′,5′-Ar-H), 7.76 (2H, dd, J = 14.0, 8.7 Hz, 7′-Ar-H), 7.16 (2H, d, J = 9.3 Hz, Ar-H), 6.55 (1H, d, J = 8.4 Hz, C6-H), 6.32 (1H, d, J = 8.5 Hz, C7-H), 3.96 (3H m, OCH_3), 3.72 (1H, m, C3-αH), 2.83-2.71 (2H, m, C16-H), 1.12 (3H, s, C19-CH_3), 0.94 (3H, s, C18-CH_3). LC-MS (ESI) *m/z*: 501.3 $[M+H]^+$. HRMS (ESI) *m/z* calcd for $C_{31}H_{36}N_2O_4$ $[M+H]^+$ 501.2753, found 501.2742.

Biological assays. The effect of derivatives on cell proliferation was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Human hepatic carcinoma cells (HepG2, SK-Hep1) and human breast cancer cells (MCF-7, MDA-MB-231) were used in the study. The cells were cultured in DMEM/RPMI1640 supplemented with 10% FBS, 100 U/mL penicillin/ streptomycin at 37 °C in an incubator containing 5% CO_2. Cells (1×10^4 cells/mL) were seeded into 96-well plates and were incubated at 37 °C overnight in a humidified incubator containing 5% CO_2. Cells

were dosed with compounds at final concentrations ranging from 5 μM to 60 μM in each well of the plates. The cells were incubated for various periods and analyzed by MTT assay to analyze rates of cell proliferation as described. Cell survival was determined by measuring the absorbance at 490 nm using a microplate reader. A calibration curve was prepared using the SPSS to determine the IC_{50} of the target compounds. Cytotoxicity as IC_{50} for each cell line is the concentration of compound which reduced by 50% the optical density of treated cells (48 h) with respect to untreated cells using the MTT assay.

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