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Synthesis of androstanopyridine and pyrimidine compounds as novel activators of the tumor suppressor protein p53

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Abstract: A series of androstane derivatives **2–16** were synthesized from 3β-hydroxyandrostan-17-one derivatives (**1a–e**). Compounds (**1a,b**) were treated with ethyl cyanoacetate, cyanoacetamide, or malononitrile and gave the corresponding derivatives **2–7**, respectively. Additionally, compounds (**1a–e**) were condensed with cyanothioacetamide, urea, or guanidine hydrochloride afforded the corresponding derivatives **8–12**, which then by Moffat oxidation gave the oxidized derivatives **9, 11** and **13**, respectively. Finally, compound (**1**) condensed with acetyl acetone or ethyl acetoacetate gave cyclohexene derivatives (**14a–c**) and (**15a,b**), respectively. Compound **15** was oxidized with a Moffat oxidizing agent and afforded the corresponding oxidized compound **16**. The newly synthesized compounds activated the tumor suppressor p53 in cancer cells through inhibition of the p53-specific ubiquitin E3 ligase HDM2.

Keywords: androstanes; inhibitors of p53 ubiquitination; pyridines; pyrimidines.

[a]Profs. Safwat and Elgamal passed away during the course of this work.

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1 Introduction

In previous work, we found that certain substituted steroidal derivatives exhibited androgenic, anabolic, and anti-inflammatory activities [1, 2]. The tumor suppressor p53 (phosphoprotein p53) is an attractive target for the design and synthesis of chemical agents that could activate the p53 pathway and thus combat cancer. Many of such agents have reached advanced stages in clinical trials. A p53 activator may affect the level of p53 through the inhibition of the E3 ubiquitin-protein ligase MDM2- (Mouse double minute 2 homolog) and MDMX- (essential for MDM2) mediated degradation of p53. The MDM2 (also known as HDM2) and MDMX (also known as HDMX or MDM4) proteins are deregulated in many human cancers and exert their oncogenic activity predominantly by initiating the degradation of the p53 tumor suppressor. However, the MDM proteins modulate and respond to many other signalling networks in which they function as well. More general p53 activators can be discovered by high-throughput screening; for example, the small molecule tenovin-6 was found to maintain p53 in its activated acetylated state by inhibition of the sirtuin 1 (SIRT1) deacetylase.

5-Deazaflavin derivatives have been shown to activate p53 through inhibition of the p53-specific ubiquitin E3 ligase HDM2 [3], which catalyzes the initial step in p53 degradation (Figure 1). Aiming to expand the group of p53 activators interrupting the p53-HDM2 signaling pathway, we are reporting herein a novel steroidal scaffold that incorporates a heterocyclic ring system fused onto ring D. For the most potent p53 activators previously reported see Yang et al. [4], Kitagaki et al. [5], and Wilson et al. [3].

2 Results and discussion

2.1 Chemistry

Arylmethylene derivatives of 3β-hydroxyandrostan-17-one derivatives (**1a–e**) were synthesized according to our

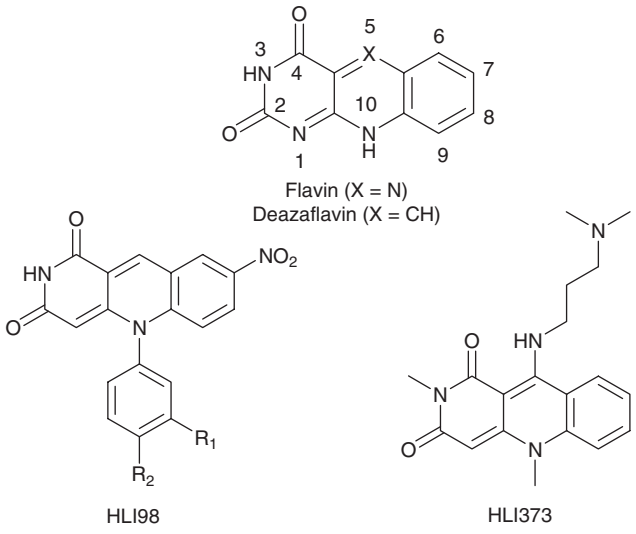
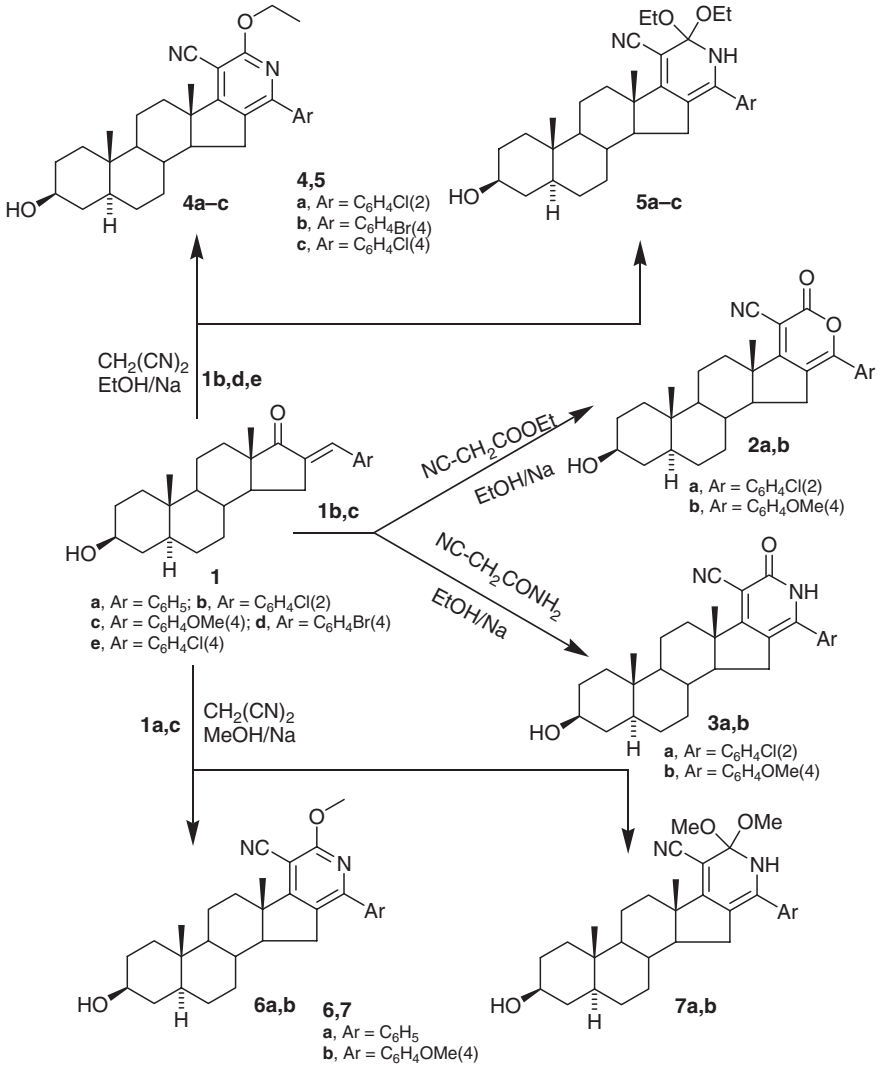


Figure 1: The chemical structures of the flavin and 5-deazaflavin derivatives HLI98 and HLI373 known as HDM2 ligase inhibitors (HLI) [3].

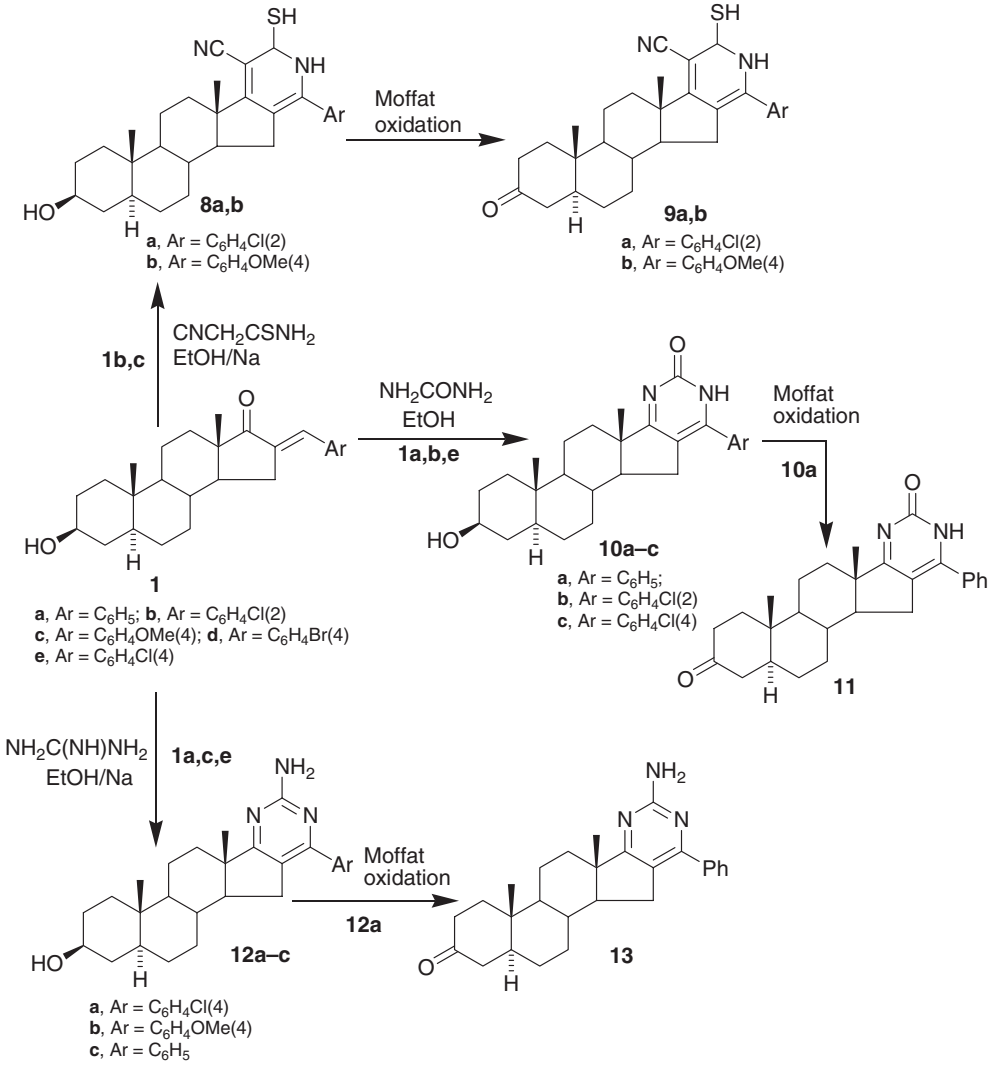
previously reported procedures [6–8]. Compounds (**1b,c**) were treated with ethylcyanoacetate or cyanoacetamide in the presence of sodium ethoxide to give the corresponding pyrano-derivatives (**2a,b**) and (**3a,b**), respectively. Also, appropriate arylmethylenes (**1a–e**) were reacted with malononitrile in the presence of sodium ethoxide or sodium methoxide to afford the corresponding pyridine derivatives **4a–c**, **5a–c**, **6a,b** and **7a,b**, respectively (Scheme 1).

Additionally, compounds **1a-c, e** were condensed with cyanothioacetamide, urea, or guanidine hydrochloride in the presence of sodium ethoxide to give the corresponding pyrimidine thiol derivatives **8a,b, 10a–c** and **12a,b**, respectively. Moffat oxidation of compounds **8a,b, 10a** or **12c** gave the corresponding oxidized derivatives **9a,b, 11** and **13**, respectively (Scheme 2).

Finally, compound (**1**) was condensed with acetyl acetone or ethyl acetoacetate in the presence of sodium



Scheme 1: Synthetic route for compounds **2–7**.



Scheme 2: Synthetic route for compounds **8–13**.

ethoxide to give the corresponding cyclohexene derivatives **14a–c** and **15a,b**, respectively. Compound **15** was converted by Moffat oxidation to the corresponding oxidized compound **16** (Scheme 3).

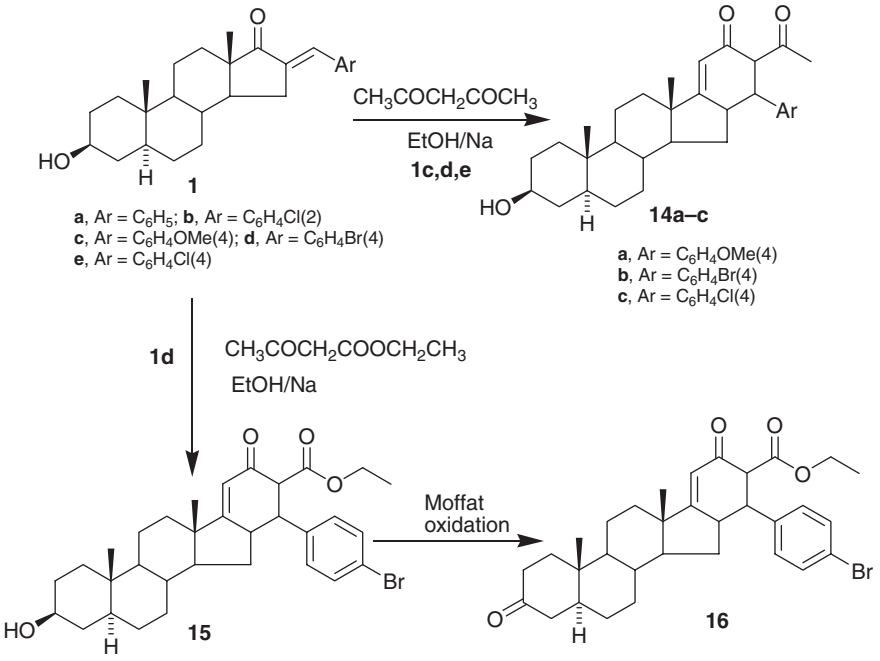
2.2 Biological screening

The test compounds were assayed for inhibition of p53 ubiquitination *in vitro* by incubation of GST-tagged HDM2 immobilized on glutathione-sepharose (glutathione-*S*-transferase-MDM2) with p53, ubiquitin, as well as E1 and E2 (UbcH5B) ligases, in an assay buffer containing ATP [9–11]. The reaction products were then resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and p53 ubiquitination was quantitated by Western blotting using an anti-p53 antibody [12].

Of those test compounds that were inhibitory at concentrations below 100 μM, half-maximal inhibition concentrations (IC_{50}) of p53 ubiquitination were determined by using essentially the same procedure, except that a fluorescently labelled ubiquitin was used. After completion of the ubiquitination reaction, excess fluorescent ubiquitin was separated from the immobilized p53-HDM2 complex by centrifugation, and incorporation of ubiquitin was measured by fluorescence spectroscopy. Compounds active as inhibitors *in vitro* were tested for inhibition of *in vivo* ubiquitination of p53 and all found to be active in this assay as well (Table 1).

The tested compounds exhibited potent *in vivo* and *in vitro* inhibition of p53 ubiquitination Compounds **12a,b,c** and **13** were more potent than the positive control 4,5-diphenylimidazole.

The examination of the relation between the structure of the tested compounds and their inhibitory activity



Scheme 3: Synthetic route for compounds **14–16**.

revealed a descending order of activity as a function of the heterocyclic ring system: aminopyrimidine > pyrimidinone > methoxypyrimidine > ethoxypyrimidine > gem-dimethoxypyrimidine > gem-diethoxypyrimidine > pyridine > pyrane mercaptopiperdine > ethyl cyclohexenone carboxylate > acetyl cyclohexenone, so we can conclude that, as the number of heteroatoms in the ring fused onto ring D increases, the activity increases.

3 Experimental

3.1 Chemistry

All melting points are uncorrected and were measured using an electrothermal capillary melting point apparatus. The IR spectra were recorded on a Shimadzu FT-IR 8101 PC infrared spectrophotometer. The ^{1}H-NMR spectra were determined with a Bruker AM-200 MHz spectrometer. The chemical shifts are expressed on the δ (ppm) scale using TMS as the standard reference. Mass spectra were recorded on a Finnigan SSQ spectrometer operating at 70 eV. Elemental analysis was performed on a Perkin Elmer 240 (microanalysis) at the Microanalysis Center, Cairo University, Cairo, Egypt.

3.2 Synthesis of 2′-oxo-3′-cyano-6′-aryl-androstano[17,16-d]pyran-3β-ols (2a,b).

A solution of **1b,c** (10 mmol) and ethyl cyanoacetate (1.27 ml, 12 mmol) in absolute ethanol (25 ml in the presence of sodium metal (920 mg, 40 mmol) was refluxed for 7 h. The reaction mixture was evaporated under reduced pressure, the obtained residue was washed with 10% HCl, and finally with water. The formed solid was dried and crystallized from ethanol to give androstanopyran-3β-ols (**2a,b**), respectively.

3.3 2′-Oxo-3′-cyano-6′-(2-chlorophenyl)-androstano[17,16-d]pyran-3β-ol (2a)

Yield 76%, mp. 254°C, $[\alpha]_D^{25}$ = +78 (c 1, $CHCl_3$); IR (KBr): 3441 (OH), 3091 (CH-Ar), 2900 (CH-aliph), 2238 (CN), 1728 (C=O), 1618 (C=C) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.81, 0.92 (2s, 6H, $2CH_3$), 0.98–1.12 (m, 1H, CH), 1.18–1.32 (m, 4H, $2CH_2$), 1.40–1.56 (m, 6H, $3CH_2$), 1.65–1.87 (m, 4H, $2CH_2$), 1.97 (m, 1H, CH), 2.25–2.35 (m, 2H, CH_2), 2.50 (m, 1H, CH), 2.60 (m, 1H, 3α-CH), 3.15 (m, 1H, 5α-CH), 7.26–7.68 (m, 4H, Ar-H), 9.96 (s, 1H, OH, exchangeable with D_2O). ^{13}C NMR ($CDCl_3$): δ 19.98, 22.05, 22.65, 26.66, 27.86, 31.95, 32.14, 35.45, 36.10, 36.76, 37.02, 37.45, 44.65, 52.50, 55.86, 63.78, 70.87, 95.01, 109.16, 115.67, 126.75, 127.65, 128.35, 129.08, 130.85, 131.55, 134.56, 149.32, 178.01 (29C). MS (EI): m/z 478 (25%) [M$^+$]. Anal. for $C_{29}H_{32}ClNO_3$ (478.02): Calcd. C, 72.86; H, 6.75; Cl, 7.42; N, 2.93; found C, 72.78; H, 6.70; Cl, 7.32; N, 2.85.

3.4 2′-Oxo-3′-cyano-6′-(4-methoxyphenyl)-androstano[17,16-d]pyran-3β-ol (2b)

Yield 82%, mp. 276°C, $[\alpha]_D^{25}$ = +110 (c 1, $CHCl_3$); IR (KBr): 3434 (OH), 3093 (CH-Ar), 2921 (CH-aliph), 2256 (CN), 1728 (C=O), 1623 (C=C) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 0.86, 0.95 (2s, 6H, $2CH_3$), 0.99–1.05 (m, 1H, CH), 1.15–1.30 (m, 4H, $2CH_2$), 1.39–1.50 (m, 6H, $3CH_2$), 1.64–1.87 (m, 4H, $2CH_2$), 1.94 (m, 1H, CH), 2.21–2.30 (m, 2H, CH_2), 2.51 (m, 1H, CH), 2.61

Table 1: IC_{50} of p53 ubiquitination of the newly synthesized compounds **2-16**.

Comp. No.	IC_{50} (μM) of p53 ubiquitination *in vitro*	IC_{50} (μM) of p53 ubiquitination *in vivo*
2a	6.12 ± 0.08	30.67 ± 0.08
2b	5.60 ± 0.07	25.16 ± 0.07
3a	5.30 ± 0.06	23.78 ± 0.09
3b	4.90 ± 0.08	22.30 ± 0.08
4a	3.54 ± 0.07	15.55 ± 0.09
4b	3.94 ± 0.06	16.11 ± 0.06
4c	3.73 ± 0.05	15.77 ± 0.07
5a	4.65 ± 0.04	20.90 ± 0.08
5b	4.89 ± 0.05	21.23 ± 0.09
5c	4.77 ± 0.09	20.21 ± 0.08
6a	3.45 ± 0.08	15.09 ± 0.09
6b	3.16 ± 0.06	14.98 ± 0.009
7a	4.54 ± 0.07	19.44 ± 0.07
7b	4.12 ± 0.05	18.90 ± 0.06
8a	6.58 ± 0.03	33.89 ± 0.05
8b	6.28 ± 0.04	32.24 ± 0.04
9a	7.89 ± 0.04	37.12 ± 0.06
9b	7.56 ± 0.07	34.98 ± 0.07
10a	2.74 ± 0.04	11.00 ± 0.08
10b	2.23 ± 0.03	9.56 ± 0.09
10c	2.53 ± 0.02	10.87 ± 0.09
11	2.85 ± 0.02	12.09 ± 0.08
12a	0.18 ± 0.01	1.44 ± 0.07
12b	0.12 ± 0.01	1.21 ± 0.06
12c	0.17 ± 0.02	1.33 ± 0.05
13	0.19 ± 0.01	1.71 ± 0.04
14a	9.20 ± 0.08	45.87 ± 0.07
14b	9.34 ± 0.09	47.88 ± 0.07
14c	9.23 ± 0.09	46.87 ± 0.08
15	8.67 ± 0.08	40.76 ± 0.09
16	8.80 ± 0.07	42.76 ± 0.09
4,5-Diphenylimidazole	0.26 ± 0.01	1.88 ± 0.08

Values were calculated from the mean values of data from three separate experiments.

All results are significantly different from control values at $p \leq 0.005$.

All results are significantly different from reference standard values at $p \leq 0.005$.

(m, 1H, 3α-CH), 3.17 (m, 1H, 5α-CH), 3.56 (s, 3H, OCH_3), 7.25–7.65 (m, 4H, Ar-H), 9.94 (s, 1H, OH, exchangeable with D_2O). ^{13}C NMR ($CDCl_3$): δ 19.75, 22.08, 22.64, 26.63, 27.84, 31.90, 32.16, 35.48, 36.08, 36.76, 34.02, 37.42, 44.64, 52.48, 55.02, 55.85, 63.78, 70.85, 95.07, 109.14, 114.02, 115.65, 122.45, 127.12, 134.52, 149.30, 159.65, 178.00 (30 C). MS (EI): m/z 473 (78%) [M^+]. Anal. $C_{30}H_{35}NO_4$ (473.60): Calcd. C, 76.08; H, 7.45; N, 2.96; found C, 76.00; H, 7.37; N, 2.88.

3.5 Synthesis of (1H′)-2′-oxo-3′-cyano-6′-aryl-androstano[17,16-d]pyrido-3β-ols (3a,b)

A mixture of **1b,c** (10 mmol) and cyanoacetamide (1 g, 12 mmol) in absolute ethanol (25 ml in the presence of sodium metal (920 mg, 40 mmol) was refluxed for 4 h. The reaction mixture was evaporated under reduced pressure, the obtained residue was washed with 10% HCl, and finally with water. The formed solid was filtered off, dried and crystallized from acetone/ethylacetate to give androstano [17,16-d]pyridine-3β-ols (**3a,b**), respectively.

3.6 (1H′)-2′-Oxo-3′-cyano-6′-(2-chlorophenyl)-androstano[17,16-d]pyrido-3β-ol (3a)

Yield 73%, mp. 305°C, $[\alpha]_D^{25}$ = +111 (c 1, $CHCl_3$); IR (KBr): 3445–3395 (OH, NH), 3088 (CH-Ar), 2934 (CH-aliph), 2235 (CN), 1723 (C=O), 1617 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.81, 0.91 (2s, 6H, $2CH_3$), 0.97–1.11 (m, 1H, CH), 1.17–1.29 (m, 4H, $2CH_2$), 1.37–1.59 (m, 6H, $3CH_2$), 1.63–1.88 (m, 4H, $2CH_2$), 1.99 (m, 1H, CH), 2.25–2.39 (m, 2H, CH_2), 2.52 (m, 1H, CH), 2.59 (m, 1H, 3α-CH), 3.18 (m, 1H, 5α-CH), 7.18–7.59 (m, 4H, Ar-H), 9.98 (s, 1H, OH, exchangeable with D_2O), 11.64 (s, 1H, NH exchangeable with D_2O). ^{13}C NMR ($CDCl_3$): δ 20.05, 21.98, 22.68, 26.56, 27.84, 31.90, 32.12, 35.36, 36.44, 36.85, 37.00, 37.44, 44.66, 52.51, 55.68, 63.93, 70.80, 114.02, 115.65, 116.34, 126.05, 126.78, 127.65, 128.36, 129.08, 130.87, 135.00, 160.12, 173.76 (29 C). MS (EI): m/z 477 (8%) [M^+]. Anal. $C_{29}H_{33}ClN_2O_2$ (477.04): Calcd. C, 73.02; H, 6.97; N, 5.87; found, C, 72.86; H, 6.90; N, 5.80.

3.7 (1H′)-2′-Oxo-3′-cyano-6′-(4-methoxyphenyl) androstano[17,16-d]pyrido-3β-ol (3b)

Yield 76%, mp. 295°C, $[\alpha]_D^{25}$ = +104 (c 1, $CHCl_3$); IR (KBr): 3420–3388 (OH, NH), 3092 (CH-Ar), 2912 (CH-aliph), 2222 (CN), 1729 (C=O), 1628 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.82, 0.90 (2s, 6H, $2CH_3$), 0.96–1.05 (m, 1H, CH), 1.19–1.34 (m, 4H, $2CH_2$), 1.38–1.58 (m, 6H, $3CH_2$), 1.63–1.84 (m, 4H, $2CH_2$), 1.97 (m, 1H, CH), 2.21–2.31 (m, 2H, CH_2), 2.52 (m, 1H, CH), 2.63 (m, 1H, 3α-CH), 3.17 (m, 1H, 5α-CH), 3.57 (s, 3H, OCH_3), 7.27–7.60 (m, 4H, Ar-H), 9.80 (s, 1H, OH, exchangeable with D_2O), 11.51 (s, 1H, NH exchangeable with D_2O). ^{13}C NMR ($CDCl_3$): δ 20.14, 21.76, 22.65, 26.52, 27.86, 31.84, 32.10, 35.36, 36.52, 36.80, 37.05, 37.46, 44.65, 52.50, 55.70, 55.86, 63.90, 70.82, 113.96, 114.05, 115.68, 116.35, 122.04, 126.80, 127.01, 159.32, 160.15, 173.72 (30 C). MS (EI): m/z 472 (45%) [M^+]. Anal. $C_{30}H_{36}N_2O_3$ (472.62): Calcd. C, 76.24; H, 7.68; N, 5.93; found C, 76.12; H, 7.60; N, 5.86.

3.8 Synthesis of 2′-ethoxy-3′-cyano-6′-aryl-androstano[17,16-d]pyridino-3β-ols (4a-c) and (1H)-2′,2″-gemdiethoxy-3′-cyano-6′-aryl-androstano[17,16-d]pyridino-3β-ols (5a-c)

A mixture of **1b,d,e** (10 mmol) and malononitrile (0.79 g, 12 mmol) in absolute ethanol (25 ml) in the presence of sodium metal (920 mg, 40 mmol) was refluxed for 6 h. The reaction mixture was evaporated under reduced pressure, the obtained product was washed with 10% HCl, the precipitated solid was separated by filtration to give **4a–c**, the filtrate was neutralized with sodium carbonate at pH ~7 to obtain compounds **5a–c**. The obtained products **4a–c** and **5a–c** were crystallized from ethyl acetate.

3.9 2′-Ethoxy-3′-cyano-6′-(2-chlorophenyl)-androstano[17,16-d]pyridino-3β-ol (4a)

Yield. 29%, mp. 259°C, $[\alpha]_D^{25}$ = +156 (c 1, $CHCl_3$); IR (KBr): 3443 (OH), 3077 (CH-Ar), 2928 (CH-aliph), 2231 (CN), 1718 (C=O), 1629 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.84, 0.93 (2s, 6H, $2CH_3$), 0.98–1.14 (m, 1H, CH), 1.17–1.31 (m, 4H, $2CH_2$), 1.37 (t, 3H, CH_3), 1.37–1.57 (m, 6H, $3CH_2$), 1.64–1.84 (m, 4H, $2CH_2$), 1.95 (m, 1H, CH), 2.21–2.37 (m, 2H, CH_2), 2.48 (m, 1H, CH), 2.61 (m, 1H, 3α-CH), 3.19 (m, 1H, 5α-CH), 4.35 (q, 2H, CH_2), 7.18–7.77 (m, 4H, Ar-H), 9.81 (s, 1H, OH, exchangeable with D_2O). ^{13}C NMR ($CDCl_3$): δ 14.62, 20.03, 21.56, 23.54, 26.80, 27.08, 28.00, 31.88, 32.02, 35.42, 36.76, 36.84, 37.04, 44.78, 51.95, 52.01, 54.88, 63.79, 70.90, 91.85, 116.68, 120.66, 126.97, 128.00, 128.86, 129.00, 132.02, 132.78, 159.04, 162.98, 167.45 (31 C). MS (EI): m/z 505 (35%) [M^+]. Anal. $C_{31}H_{37}ClN_2O_2$ (505.09): Calcd. C, 73.72; H, 7.38; Cl, 7.02; N, 5.55; found C, 73.64; H, 7.30; Cl, 6.89; N, 5.48.

3.10 2′-Ethoxy-3′-cyano-6′-(4-bromophenyl) androstano[17,16-d]pyridino-3β-ol (4b)

Yield 36%, mp. 278°C, $[\alpha]_D^{25}$ = +123 (c 1, $CHCl_3$); IR (KBr): 3444 (OH), 3077 (CH-Ar), 2928 (CH-aliph), 2231 (CN), 1718 (C=O), 1629 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.84, 0.93 (2s, 6H, $2CH_3$), 0.98–1.14 (m, 1H, CH), 1.17–1.31 (m, 4H, $2CH_2$), 1.37 (t, 3H, CH_3), 1.38–1.58 (m, 6H, $3CH_2$), 1.62–1.80 (m, 4H, $2CH_2$), 1.90 (m, 1H, CH), 2.20–2.32 (m, 2H, CH_2), 2.45 (m, 1H, CH), 2.60 (m, 1H, 3α-CH), 3.18 (m, 1H, 5α-CH), 4.32 (q, 2H, CH_2), 7.20–7.64 (m, 4H, Ar-H), 9.75 (s, 1H, OH, exchangeable with D_2O). ^{13}C NMR ($CDCl_3$): δ 14.56, 20.00, 21.58, 23.48, 26.77, 27.12, 27.98, 31.86, 32.12, 35.40, 36.75, 36.84, 37.04, 44.79, 51.92, 52.01, 54.85, 63.77, 70.91, 91.85, 116.65, 120.64, 121.56, 129.34, 132.01, 134.89, 159.00, 162.93, 167.40 (31 C). MS (EI): m/z 549 (45%) [M^+]. Anal. $C_{31}H_{37}BrN_2O_2$ (549.54): Calcd. C, 67.75; H, 6.79; N, 5.10; found C, 67.65; H, 6.70; N, 5.02.

3.11 2′-Ethoxy-3′-cyano-6′-(4-chlorophenyl)-androstano[17,16-d]pyridino-3β-ol (4c)

Yield 32%, mp. 236°C, $[\alpha]_D^{25}$ = +106 (c 1, $CHCl_3$); IR (KBr): 3461 (OH), 3087 (CH-Ar), 2947 (CH-aliph), 2217 (CN), 1727 (C=O), 1619 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.87, 0.95 (2s, 6H, $2CH_3$), 0.98–1.06 (m, 1H, CH), 1.18–1.31 (m, 4H, $2CH_2$), 1.37 (t, 3H, CH_3), 1.38–1.54 (m, 6H, $3CH_2$), 1.65–1.86 (m, 4H, $2CH_2$), 1.97 (m, 1H, CH), 2.19–2.31 (m, 2H, CH_2), 2.48 (m, 1H, CH), 2.62 (m, 1H, 3α-CH), 3.12 (m, 1H, 5α-CH), 4.23 (q, 2H, CH_2), 7.31–7.58 (m, 4H, Ar-H), 9.77 (s, 1H, OH, exchangeable with D_2O). ^{13}C NMR ($CDCl_3$): δ 14.55, 19.96, 21.58, 23.45, 26.74, 27.15, 28.00, 31.88, 32.02, 35.45, 36.75, 36.86, 37.04, 44.75, 51.92, 52.04, 54.85, 63.77, 70.92, 91.84, 116.60, 120.68, 129.00, 129.30, 132.82, 134.08, 159.01, 162.90, 167.56 (31 C). MS (EI): m/z 505 (37%) [M^+]. Anal. $C_{31}H_{37}ClN_2O_2$ (505.09): Calcd. C, 73.73; H, 7.33: Cl, 7.03; N, 5.55; found C, 73.64; H, 7.30; Cl, 6.86; N, 5.48.

3.12 (1H)-2′,2″-Gemdiethoxy-3′-cyano-6′-(2-chlorophenyl) androstano[17,16-d]pyridino-3β-ol (5a)

Yield 52%, mp. 270°C, $[\alpha]_D^{25}$ = +170 (c 1, $CHCl_3$); IR (KBr): 3471 (OH), 3391 (NH), 3061 (CH-Ar), 2945 (CH-aliph), 2232 (CN), 1631 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.84, 0.90 (2s, 6H, $2CH_3$), 0.95–1.09 (m, 1H, CH), 1.19–1.31 (m, 4H, $2CH_2$), 1.38 (2t, 6H, $2CH_3$), 1.42–1.59 (m, 6H, $3CH_2$), 1.61–1.89 (m, 4H, $2CH_2$), 1.94 (m, 1H, CH), 2.21–2.38 (m, 2H, CH_2), 2.41 (m, 1H, CH), 2.62 (m, 1H, 3α-CH), 3.13 (m, 1H, 5α-CH), 4.34 (2q, 4H, $2CH_2$), 7.29–7.60 (m, 4H, Ar-H), 9.78 (s, 1H, OH, exchangeable with D_2O), 10.14 (s, 1H, NH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 15.32, 20.15, 22.60, 22.62, 26.98, 27.87, 31.95, 32.01, 35.68, 36.98, 37.05, 37.14, 37.45, 44.88, 52.08, 56.18, 57.04, 64.10, 70.90, 102.02, 103.43, 114.87, 115.10, 117.00, 125.99, 127.16, 128.67, 129.22, 130.98, 135.10, 159.45 (33 C). MS (EI): m/z 551 (66%) [M^+]. Anal. $C_{33}H_{43}ClN_2O_3$ (551.16): Calcd. C, 71.91; H, 7.86; Cl, 6.43; N, 5.08; found C, 71.83; H, 7.78; Cl, 6.35; N, 5.00.

3.13 (1H)-2′,2″-Gemdiethoxy-3′-cyano-6′-(4-bromophenyl) androstano[17,16-d]pyridino-3β-ol (5b)

Yield 40%, mp. 250°C, $[\alpha]_D^{25}$ = +156 (c 1, $CHCl_3$); IR (KBr): 3468 (OH), 3398 (NH), 3061 (CH-Ar), 2933 (CH-aliph), 2218 (CN), 1622 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.85, 0.92 (2s, 6H, $2CH_3$), 0.98–1.11 (m, 1H, CH), 1.18–1.32 (m, 4H, $2CH_2$), 1.35 (2t, 6H, $2CH_3$), 1.41–1.60 (m, 6H, $3CH_2$), 1.64–1.85 (m, 4H, $2CH_2$), 1.95 (m, 1H, CH), 2.25–2.36 (m, 2H, CH_2), 2.47 (m, 1H, CH), 2.61 (m, 1H, 3α-CH), 3.16 (m, 1H, 5α-CH), 4.34 (2q, 4H, $2CH_2$), 7.27–7.58 (m, 4H, Ar-H), 9.79 (s, 1H, OH, exchangeable with D_2O), 10.16 (s, 1H, NH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 15.22, 20.18, 22.56, 22.64, 26.95, 27.89, 31.96, 32.04, 35.68, 36.94, 37.10, 37.18, 37.48, 44.88, 52.08, 56.18, 57.04, 64.12, 70.90, 102.02, 103.43, 114.87, 115.10, 116.86, 122.04, 127.95, 131.32, 133.04, 159.48 (33 C). MS (EI): m/z 595 (45%) [M^+]. Anal. $C_{33}H_{43}BrN_2O_3$ (595.61): Calcd. C, 66.55; H, 7.28; N, 4.70; found C, 66.42; H, 7.20; N, 4.60.

3.14 (1H)-2′,2″-Gemdiethoxy-3′-cyano-6′-(4-chlorophenyl) androstano[17,16-d]pyridino-3β-ol (5c)

Yield 43%, mp. 253°C, $[\alpha]_D^{25}$ = +105 (c 1, $CHCl_3$); IR (KBr): 3485 (OH), 3340 (NH), 3056 (CH-Ar), 2945 (CH-aliph), 2208 (CN), 1615 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.82, 0.95 (2s, 6H, $2CH_3$), 1.02–1.10 (m, 1H, CH), 1.18–1.28 (m, 4H, $2CH_2$), 1.34 (2t, 6H, $2CH_3$), 1.41–1.55 (m, 6H, $3CH_2$), 1.64–1.84 (m, 4H, $2CH_2$), 1.95 (m, 1H, CH), 2.20–2.30 (m, 2H, CH_2), 2.48 (m, 1H, CH), 2.62 (m, 1H, 3α-CH), 3.15 (m, 1H, 5α-CH), 4.35 (2q, 4H, $2CH_2$), 7.26–7.60 (m, 4H, Ar-H), 9.74 (s, 1H, OH, exchangeable with D_2O), 10.08 (s, 1H, NH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 15.30, 20.16, 22.60, 22.68, 26.98, 27.85, 31.90, 31.96, 35.74, 36.95, 37.09, 37.18, 37.42, 44.83, 52.12, 56.19, 57.04, 64.12, 70.95, 102.00, 103.40, 114.84, 115.15, 117.03, 127.87, 128.67, 132.12, 133.14, 159.36 (33 C). MS (EI): m/z 551 (71%) [M^+]. Anal. $C_{33}H_{43}ClN_2O_3$ (551.16): Calcd. C, 71.91; H, 7.86; Cl, 6.43; N, 5.08; found C, 71.80; H, 7.77; Cl, 6.35; N, 5.00.

3.15 Synthesis of 2′-methoxy-3′-cyano-6′-aryl-androstano[17,16-d]pyridino-3β-ols (6a,b) and (1H)-2′,2″-gemdimethoxy-3′-cyano-6′-aryl-androstano[17,16-d]pyridino-3β-ols (7a,b)

A mixture of **1a,c** (10 mmol) and malononitrile (0.79 g, 12 mmol) in absolute ethanol (25 ml) in the presence of sodium metal (920 mg, 40 mmol) was refluxed for 6 h. The reaction mixture was evaporated

under reduced pressure, the obtained residue was washed with 10% HCl. The precipitated product was filtered off, washed with water and dried to give compounds **6a,b**. The obtained filtrate was neutralized with sodium carbonate at pH ~7 to precipitate the compounds **7a,b**. The obtained products **6a,b** and **7a,b** were crystallized from ethyl acetate.

3.16 2′-Methoxy-3′-cyano-6′-phenyl-androstano[17,16-d] pyridino-3β-ol (6a)

Yield 34%, mp. 228°C, $[\alpha]_D^{25}$ = +103.8 (c 1, $CHCl_3$); IR (KBr): 3445 (OH), 3083 (CH-Ar), 2925 (CH-aliph), 2222 (CN), 1718 (C=O), 1622 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.82, 0.93 (2s, 6H, $2CH_3$), 0.98–1.13 (m, 1H, CH), 1.16–1.30 (m, 4H, $2CH_2$), 1.37–1.56 (m, 6H, $3CH_2$), 1.62–1.85 (m, 4H, $2CH_2$), 1.90 (m, 1H, CH), 2.20–2.32 (m, 2H, CH_2), 2.45 (m, 1H, CH), 2.60 (m, 1H, 3α-CH), 3.18 (m, 1H, 5α-CH), 4.32 (s, 3H, CH_3), 7.20–7.64 (m, 5H, Ar-H), 9.75 (s, 1H, OH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 20.10, 21.60, 23.76, 26.95, 27.32, 28.04, 31.92, 32.33, 35.85, 37.12, 37.24, 37.56, 44.92 52.02, 52.16, 55.12, 55.68, 71.01, 92.04, 121.14, 117.06, 127.22, 127.34, 128.96, 136.12, 159.44, 163.34, 167.66 (30 C). MS (EI): m/z 456 (18%) [M^+]. Anal. $C_{30}H_{36}N_2O_2$ (456.62): Calcd. C, 78.91; H, 7.95; N, 6.13; found C, 78.82; H, 7.87; N, 6.02.

3.17 2′-Methoxy-3′-cyano-6′-(4-methoxyphenyl)-androstano[17,16-d]pyridino-3β-ol (6b)

Yield 28%, mp. 262°C, $[\alpha]_D^{25}$ = +156 (c 1, $CHCl_3$); IR (KBr): 3454 (OH), 3086 (CH-Ar), 2942 (CH-aliph), 2218 (CN), 1721 (C=O), 1619 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.85, 0.94 (2s, 6H, $2CH_3$), 0.97–1.08 (m, 1H, CH), 1.18–1.28 (m, 4H, $2CH_2$), 1.34–1.55 (m, 6H, $3CH_2$), 1.60–1.86 (m, 4H, $2CH_2$), 1.95 (m, 1H, CH), 2.18–2.30 (m, 2H, CH_2), 2.50 (m, 1H, CH), 2.60 (m, 1H, 3α-CH), 3.15 (m, 1H, 5α-CH), 3.57 (s, 3H, OCH_3), 4.24 (s, 3H, OCH_3), 7.28–7.66 (m, 4H, Ar-H), 9.80 (s, 1H, OH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 20.15, 21.71, 23.88, 26.99, 27.44, 28.23, 31.98, 32.28, 35.80, 37.42, 37.56, 37.66, 44.90 52.11, 52.22, 55.18, 55.68, 55.85, 69.88, 92.13, 121.18, 116.98, 114.72, 127.9, 128.16, 159.10, 159.65, 163.40, 167.65 (31 C). MS (EI): m/z 486 (27%) [M^+]. Anal. $C_{31}H_{38}N_2O_3$ (486.65): Calcd. C, 76.51; H, 7.87; N, 5.76; found C, 76.43; H, 7.80; N, 5.70.

3.18 (1H)-2′,2″-Gemdimethoxy-3′-cyano-6′-phenyl-androstano[17,16-d]pyridino-3β-ol (7a)

Yield 53%, mp. 258–260°C, $[\alpha]_D^{25}$ = +123 (c 1, $CHCl_3$); IR (KBr): 3465 (OH), 3396 (NH), 3060 (CH-Ar), 2932 (CH-aliph), 2212 (CN), 1620 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.84, 0.91 (2s, 6H, $2CH_3$), 0.99–1.12 (m, 1H, CH), 1.19–1.31 (m, 4H, $2CH_2$), 1.40–1.59 (m, 6H, $3CH_2$), 1.63–1.83 (m, 4H, $2CH_2$), 1.93 (m, 1H, CH), 2.23–2.34 (m, 2H, CH_2), 2.45 (m, 1H, CH), 2.60 (m, 1H, 3α-CH), 3.16 (m, 1H, 5α-CH), 4.35 (s, 6H, $2CH_3$), 7.23–7.62 (m, 5H, Ar-H), 9.82 (s, 1H, OH, exchangeable with D_2O), 10.06 (s, 1H, NH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 20.10, 22.55, 22.65, 27.16, 27.94, 32.08, 32.18, 35.88, 36.82, 37.40, 37.63, 37.78, 45.02, 48.15, 52.32, 56.99, 64.24, 69.86, 104.32, 108.78, 114.89, 115.00, 117.10, 126.22, 127.98, 128.44, 134.12, 160.05 (31 C). MS (EI): m/z 488 (27%) [M^+]. Anal. $C_{31}H_{40}N_2O_3$ (488.66): Calcd. C, 76.19; H, 8.25; N, 5.73; found C, 76.10; H, 8.20; N, 5.62.

3.19 (1H)-2′,2″-Gemdimethoxy-3′-cyano-6′-(4-methoxyphenyl)-androstano[17,16-d]pyridino-3β-ol (7b)

Yield 48%, mp. 178–180°C, $[\alpha]_D^{25}$ = +98 (c 1, $CHCl_3$); IR (KBr): 3485 (OH), 3340 (NH), 3056 (CH-Ar), 2945 (CH-aliph), 2208 (CN), 1615 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.82, 0.95 (2s, 6H, $2CH_3$), 1.02–1.10 (m, 1H, CH), 1.18–1.28 (m, 4H, $2CH_2$), 1.41–1.55 (m, 6H, $3CH_2$), 1.64–1.84 (m, 4H, $2CH_2$), 1.95 (m, 1H, CH), 2.20–2.30 (m, 2H, CH_2), 2.48 (m, 1H, CH), 2.62 (m, 1H, 3α-CH), 3.15 (m, 1H, 5α-CH), 3.57 (s, 3H, OCH_3),4.35 (s, 6H, $2CH_3$), 7.26–7.60 (m, 4H, Ar-H), 9.74 (s, 1H, OH, exchangeable with D_2O), 10.12 (s, 1H, NH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 19.97, 22.50, 22.60, 27.10, 27.90, 32.03, 32.18, 35.86, 36.80, 37.45, 37.60, 37.72, 45.02, 48.10, 52.30, 55.17, 56.94, 64.24, 69.82, 104.32, 108.70, 114.83, 114.05, 115.01, 117.15, 126.52, 127.30, 159.80, 160.12 (32 C). MS (EI): m/z 519 (7%) [M^+]. Anal. $C_{32}H_{42}N_2O_4$ (518.69): Calcd. C, 74.10; H, 8.16; N, 5.40; found C, 74.01; H, 8.10; N, 5.31.

3.20 Synthesis of 2′-mercapto-3′-cyano-6′-aryl-androstano[17,16-c]piperidino-3β-ols (8a,b)

A mixture of **1b,c** (10 mmol) and cyanothioacetamide (1.2 g, 12 mmol) in absolute ethanol (25 ml in the presence of sodium metal (920 mg, 40 mmol) was refluxed for 4 h. The reaction mixture was evaporated under reduced pressure, the obtained residue was washed with 10% HCl, and finally with water. The formed solid was dried and crystallized from ethanol to give 2′-oxo-3′-cyano-6′-aryl-androstano[17,16-d] pyridino-3β-ols (**8a,b**), respectively.

3.21 2′-Mercapto-3′-cyano-6′-(2-chlorophenyl)-androstano[17,16-dc]piperidino-3β-ol (8a)

Yield 70%, mp. 168°C, $[\alpha]_D^{25}$ = +161 (c 1, $CHCl_3$); IR (KBr): 3443 (OH), 3381(NH), 3212 (SH), 3076 (CH-Ar), 2918 (CH-aliph), 2221 (CN), 1616 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.86, 0.94 (2s, 6H, $2CH_3$), 0.98–1.08 (m, 1H, CH), 1.16–1.30 (m, 4H, $2CH_2$), 1.39–1.56 (m, 6H, $3CH_2$), 1.64–1.84 (m, 4H, $2CH_2$), 1.94 (m, 1H, CH), 2.23–2.30 (m, 2H, CH_2), 2.50 (m, 1H, CH), 2.62 (m, 1H, 3α-CH), 2.81 (m, 1H, CH), 3.16 (m, 1H, 5α-CH), 4.34 (s, 1H, NH exchangeable with D_2O), 4.45 (s, 1H, SH exchangeable with D_2O), 7.30–7.65 (m, 4H, Ar-H), 9.85 (s, 1H, OH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 20.16, 22.56, 22.66, 26.99, 27.95, 31.92, 31.98, 35.75, 36.98, 37.02, 37.12, 37.28, 44.96, 48.98, 52.56, 56.12, 64.10, 70.99, 101.03, 114.94, 115.22, 117.00, 126.65, 127.45, 128.42, 129.10, 131.00, 134.14, 159.46 (29 C). MS (EI): m/z 495 (6%) [M^+]. Anal. $C_{29}H_{35}ClN_2OS$ (495.12): Calcd. C, 70.35; H, 7.13; Cl, 7.16; N, 5.66; S, 6.48; found C, 70.28; H, 7.04; Cl, 7.16; N, 5.60; S, 6.40.

3.22 2′-Mercapto-3′-cyano-6′-(4-methoxyphenyl)-androstano[17,16-dc]piperidino-3β-ol (8b)

Yield 61%, mp. 231°C, $[\alpha]_D^{25}$ = +116 (c 1, $CHCl_3$); IR (KBr): 3441 (OH), 3385 (NH), 3202 (SH), 3076 (CH-Ar), 2920 (CH-aliph), 2212 (CN), 1620 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.84, 0.90 (2s, 6H, $2CH_3$), 0.99–1.12 (m, 1H, CH), 1.19–1.31 (m, 4H, $2CH_2$), 1.37–1.59 (m, 6H, $3CH_2$), 1.64–1.84

(m, 4H, $2CH_2$), 1.94 (m, 1H, CH), 2.24–2.34 (m, 2H, CH_2), 2.47 (m, 1H, CH), 2.61 (m, 1H, 3α-CH), 2.79 (m, 1H, CH), 3.16 (m, 1H, 5α-CH), 3.58 (s, 3H, OCH_3), 4.29 (s, 1H, NH exchangeable with D_2O), 4.42 (s, 1H, SH exchangeable with D_2O), 7.23–7.62 (m, 4H, Ar-H), 9.88 (s, 1H, OH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 20.22, 22.55, 22.68, 26.92, 27.90, 31.88, 31.96, 35.70, 36.95, 37.02, 37.18, 37.30, 44.95, 48.95, 52.58, 55.40, 56.15, 64.12, 70.98, 101.05, 114.06, 114.97, 115.25, 117.02, 126.52, 127.35, 159.76, 159.48 (30 C). MS (EI): m/z 491 (14%) [M^+]. Anal. $C_{30}H_{38}N_2O_2S$ (490.70): Calcd. C, 73.43; H, 7.81; N, 5.71; S, 6.53; found C, 73.32; H, 7.72; N, 5.64; S, 6.46.

3.23 Synthesis of 2′-mercapto-3′-cyano-6′-aryl-androstano[17,16-c]pyridino-3-onea (9a,b)

To a solution of **8a,b** (2 mmol) in benzene (3 ml), dimethylsulfoxide (3 ml), pyridine (0.16 ml, 2 mmol) and trifluoroacetic acid (0.08 ml, 1 mmol), dicyclohexylcarbodiimide (1.24 g, 6 mmol) was added in sealed reaction flask. The reaction mixture was kept overnight at room temperature. Ether (50 ml) was added followed by the addition of a solution of oxalic acid (0.54 g, 6 mmol) in methanol (5 ml). After gas evolution had ceased (30 min), water (50 ml) was added and the insoluble dicyclohexylurea was removed by filtration. The organic phase was separated and washed with 5% sodium bicarbonate and once with water, dried over sodium sulfate and evaporated under reduced pressure. The obtained residue was crystallized from ethanol to give compounds (**9a,b**), respectively.

3.24 2′-Mercapto-3′-cyano-6′-(2-chlorophenyl)-androstano[17,16-dc]pyridino-3-one (9a)

Yield 78%, mp. 177°C, $[\alpha]_D^{25}$ = +105 (c 1, $CHCl_3$); IR (KBr): 3385(NH), 3218 (SH), 3079 (CH-Ar), 2919 (CH-aliph), 2228 (CN), 1741 (C=O), 1618(C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.87, 0.94 (2s, 6H, $2CH_3$), 0.97–1.08 (m, 1H, CH), 1.17–1.30 (m, 4H, $2CH_2$), 1.37–1.57 (m, 6H, $3CH_2$), 1.63–1.85 (m, 4H, $2CH_2$), 1.93 (m, 1H, CH), 2.24–2.32 (m, 2H, CH_2), 2.51 (m, 1H, CH), 2.80 (m, 1H, CH), 3.17 (m, 1H, 5α-CH), 4.35 (s, 1H, NH exchangeable with D_2O), 4.45 (s, 1H, SH exchangeable with D_2O), 7.30–7.65 (m, 4H, Ar-H); ^{13}C NMR ($CDCl_3$): δ 20.26, 22.50, 22.62, 26.25, 27.98, 35.75, 36.86, 36.98, 37.28, 38.45, 44.02, 44.12, 44.22, 48.95, 52.57, 56.15, 64.11, 101.07, 114.96, 115.28, 117.02, 126.62, 127.48, 128.40, 129.16, 131.02, 134.18, 159.56, 210.32 (29 C). MS (EI): m/z 493 (78%) [M^+]. Anal. $C_{29}H_{33}ClN_2OS$ (493.10): Calcd. C, 70.64; H, 6.75; Cl, 7.19; N, 5.68; S, 6.50; found C, 70.56; H, 6.66; Cl, 7.10; N, 5.60; S, 6.40.

3.25 2′-Mercapto-3′-cyano-6′-(4-methoxyphenyl)-androstano[17,16-c] pyridino -3-one (9b)

Yield 71%, mp. 128°C, $[\alpha]_D^{25}$ = +113 (c 1, $CHCl_3$); IR (KBr): 3390 (NH), 3210 (SH), 3088 (CH-Ar), 2922 (CH-aliph), 2228 (CN), 1738 (C=O), 1622 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.85, 0.91 (2s, 6H, $2CH_3$), 0.97–1.10 (m, 1H, CH), 1.17–1.30 (m, 4H, $2CH_2$), 1.35–1.55 (m, 6H, $3CH_2$), 1.65–1.85 (m, 4H, $2CH_2$), 1.95 (m, 1H, CH), 2.20–2.31 (m, 2H, CH_2), 2.48 (m, 1H, CH), 2.77 (m, 1H, CH), 3.17 (m, 1H, 5α-CH), 3.65 (s, 3H, OCH_3), 4.29 (s, 1H, NH exchangeable with D_2O), 4.42 (s, 1H, SH exchangeable with D_2O), 7.23–7.62 (m, 4H, Ar-H); ^{13}C NMR ($CDCl_3$): δ 20.20, 22.56, 22.68, 26.22, 27.95, 35.78, 36.86, 36.94, 37.32, 38.46, 44.10, 44.18, 44.34, 48.96, 52.58, 55.38, 56.18, 64.18, 101.08, 114.15, 114.98, 115.34, 116.96, 126.50, 127.38, 159.82, 159.58, 210.16 (30 C). MS (EI): m/z 488 (67%) [M^+]. Anal. $C_{30}H_{36}N_2OS$ (488.68): Calcd. C, 73.73; H, 7.43; N, 5.73; S, 6.56; found C, 73.65; H, 7.32; N, 5.66; S, 6.50.

3.26 Synthesis of (1′H)-2′-oxo-6′-aryl-androstano[17,16-d] pyridino-3β-ols (10a-c)

A mixture of **1a,b,e** (10 mmol) and urea (0.72 g, 12 mmol) in absolute ethanol (25 ml) in the presence of sodium metal (920 mg, 40 mmol) was refluxed for 7 h. The reaction mixture was evaporated under reduced pressure, the obtained residue was washed with 10% HCl, then with water. The formed solid was filtered off, dried and crystallized from benzene/ethanol to give the corresponding products (**10a-c**), respectively.

3.27 (1′H)-2′-Oxo-6′-phenyl-androstano[17,16-d] pyrimidino-3β-ol (10a)

Yield 56%, mp. 278°C, $[\alpha]_D^{25}$ = +109 (c 1, $CHCl_3$); IR (KBr): 3457 (OH), 3368 (NH), 3088 (CH-Ar), 2961 (CH-aliph), 1721 (C=O), 1622 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.83, 0.89 (2s, 6H, $2CH_3$), 0.98–1.10 (m, 1H, CH), 1.17–1.29 (m, 4H, $2CH_2$), 1.35–1.56 (m, 6H, $3CH_2$), 1.63–1.85 (m, 4H, $2CH_2$), 1.94 (m, 1H, CH), 2.24–2.34 (m, 2H, CH_2), 2.47 (m, 1H, CH), 2.61 (m, 1H, 3α-CH), 3.16 (m, 1H, 5α-CH), 7.23–7.62 (m, 5H, Ar-H), 8.54 (s, 1H, NH exchangeable with D_2O), 9.83 (s, 1H, OH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 19.14, 20.42, 22.32, 27.00, 27.42, 32.08, 32.22, 33.54, 33.86, 35.62, 37.35, 37.52, 41.78, 44.86, 52.21, 60.48, 70.94, 103.87, 126.12, 127.38, 128.42, 133.04, 134.10, 155.82, 164.32 (27 C). MS (EI): m/z 418 (56%) [M^+]. Anal. $C_{27}H_{34}N_2O_2$ (418.57): Calcd. C, 77.48; H, 8.19; N, 6.69; found C, 77.40; H, 8.10; N, 6.60.

3.28 (1′H)-2′-Oxo-6′-(2-chlorophenyl)-androstano[17,16-d]pyrimidino-3β-ol (10b)

Yield 46%, mp. 236°C, $[\alpha]_D^{25}$ = +100 (c 1, $CHCl_3$); IR (KBr): 3455 (OH), 3360 (NH), 3080 (CH-Ar), 2954 (CH-aliph), 1718 (C=O), 1620 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.84, 0.90 (2s, 6H, $2CH_3$), 0.99–1.12 (m, 1H, CH), 1.19–1.31 (m, 4H, $2CH_2$), 1.37–1.59 (m, 6H, $3CH_2$), 1.64–1.84 (m, 4H, $2CH_2$), 1.94 (m, 1H, CH), 2.24–2.34 (m, 2H, CH_2), 2.47 (m, 1H, CH), 2.61 (m, 1H, 3α-CH), 3.16 (m, 1H, 5α-CH), 7.23–7.62 (m, 4H, Ar-H), 8.54 (s, 1H, NH exchangeable with D_2O), 9.83 (s, 1H, OH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 18.94, 20.34, 22.24, 27.02, 27.45, 32.12, 32.23, 33.57 33.87, 35.65, 37.35, 37.54, 41.80, 44.85, 52.21, 60.49, 70.94, 103.89, 126.34, 128.38, 128.44, 129.10, 131.02, 133.09, 135.14, 155.84, 164.39 (27 C). MS (EI): m/z 453 (67%) [M^+]. Anal. $C_{27}H_{33}ClN_2O_2$ (453.02): Calcd. C, 71.58; H, 7.34; Cl, 7.83; N, 6.18; found C, 71.50; H, 7.23; Cl, 7.75; N, 6.10.

3.28.1 (1′H)-2′-Oxo-6′-(4-chlorophenyl)-androstano[17,16-d] pyrimidino-3β-ol (10c): Yield 55%, mp. 245°C, $[\alpha]_D^{25}$ = +102 (c 1, $CHCl_3$); IR (KBr): 3490 (OH), 3080 (CH-Ar), 2921 (CH-aliph), 1720 (C=O), 1616 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.87, 0.93 (2s, 6H, $2CH_3$),

0.97–1.06 (m, 1H, CH), 1.15–1.31 (m, 4H, $2CH_2$), 1.38–1.55 (m, 6H, $3CH_2$), 1.64–1.83 (m, 4H, $2CH_2$), 1.92 (m, 1H, CH), 2.21–2.32 (m, 2H, CH_2), 2.51 (m, 1H, CH), 2.61 (m, 1H, 3α-CH), 3.18 (m, 1H, 5α-CH), 7.56–8.12 (m, 4H, Ar-H), 8.54 (s, 1H, NH exchangeable with D_2O), 9.79 (s, 1H, OH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 19.00, 20.16, 22.60, 26.98, 27.85, 31.95, 31.96, 33.74, 34.05, 35.65, 37.26, 37.52, 41.83, 44.76, 52.19, 60.44, 70.95, 103.66, 127.87, 128.67, 132.12, 133.14, 133.45, 155.76, 164.42 (27 C). MS (EI): m/z 453 (67%) [M^+]. Anal. $C_{27}H_{33}ClN_2O_2$ (453.02): Calcd. C, 71.58; H, 7.34; Cl, 7.83; N, 6.18; found C, 71.49; H, 7.24; Cl, 7.74; N, 6.09.

3.29 Synthesis of (1′H)-2′-oxo-6′-phenyl-androstano[17,16-d]pyrimidino-3-one (11)

To a mixture of **10a** (2 mmol) in benzene (3 ml), dimethylsulfoxide (3 ml), pyridine (0.16 ml, 2 mmol) and trifluoroacetic acid (0.08 ml, 1 mmol), dicyclohexylcarbodiimide (1.24 g, 6 mmol) was added in sealed reaction flask, then kept overnight at room temperature. Ether (50 ml) was added followed by the addition of a solution of oxalic acid (0.54 g, 6 mmol) in methanol (5 ml). After gas evolution had ceased (30 min), water (50 ml) was added and the insoluble dicyclohexylurea was removed by filtration. The organic phase was then extracted twice with 5% sodium bicarbonate and once with water, dried over sodium sulfate and evaporated under reduced pressure to dryness, the obtained residue was crystallized from ethanol to give compound (**11**). Yield 58%, mp. 258–259°C, $[\alpha]_D^{25}$ = +87 (c 1, $CHCl_3$); IR (KBr): 3368 (NH), 3076 (CH-Ar), 2955 (CH-aliph), 1738 (C=O), 1632 (C=C) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.84, 0.91 (2s, 6H, $2CH_3$), 0.99–1.13 (m, 1H, CH), 1.191.2930 (m, 4H, $2CH_2$), 1.37–1.59 (m, 6H, $3CH_2$), 1.64–1.88 (m, 4H, $2CH_2$), 1.97 (m, 1H, CH), 2.21–2.31 (m, 2H, CH_2), 2.45 (m, 1H, CH), 3.16 (m, 1H, 5α-CH), 7.31–7.63 (m, 5H, Ar-H), 8.54 (s, 1H, NH exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 19.43, 20.48, 22.39, 26.32, 27.46, 37.08, 39.22, 33.58, 33.85, 35.64, 44.35, 44.52, 41.80, 44.66, 52.25, 60.46, 210.08, 103.89, 126.19, 127.35, 128.45, 133.16, 134.16, 155.87, 164.36 (27 C). MS (EI): m/z 416 (28%) [M^+]. Anal. $C_{27}H_{32}N_2O_2$ (416.56): Calcd. C, 77.85; H, 7.74; N, 6.73; found C, 77.78; H, 7.64; N, 6.65.

3.30 Synthesis of 2′-amino-6′-aryl-androstano[17,16-d] pyrimidine-3β-ols (12a-c)

A solution of **1a,c,e** (10 mmol) and guanidine hydrochloride (1.146 g, 12 mmol) in absolute ethanol (25 ml) in the presence of sodium metal (920 mg, 40 mmol) was refluxed for 5 h. The reaction mixture was evaporated under reduced pressure, the obtained residue was washed with 10% HCl, and finally with water. The formed solid was filtered off, dried and crystallized from methanol/ethyl acetate to give compounds (**12a–c**), respectively.

3.31 2′-Amino-6′-phenyl-androstano[17,16-d]pyrimidine-3β-ol (12a)

Yield 58%, mp. 253°C, $[\alpha]_D^{25}$ = +109 (c 1, $CHCl_3$); IR (KBr): 3470–3415 (OH, NH_2), 3080 (CH-Ar), 2936 (CH-aliph), 1620 (C=C), 1600 (C=N) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.85, 0.95 (2s, 6H, $2CH_3$), 1.00–1.14 (m, 1H, CH), 1.18–1.35 (m, 4H, $2CH_2$), 1.40–1.56 (m, 6H, $3CH_2$), 1.60–1.82 (m, 4H, $2CH_2$), 1.93 (m, 1H, CH), 2.23–2.31 (m, 2H, CH_2), 2.51 (m, 1H, CH), 2.61 (m, 1H, 3α-CH), 3.18 (m, 1H, 5α-CH), 4.68 (s, 2H, NH_2 exchangeable with D_2O), 7.52–8.36 (m, 5H, Ar-H), 9.80 (s, 1H, OH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 20.43, 21.76, 24.20, 27.10, 27.42, 28.55, 32.08, 32.22, 36.04, 37.35, 37.52, 37.76, 44.55, 45.32, 52.00, 55.78, 70.94, 123.07, 127.30, 128.32, 129.01, 132.85, 161.04, 168.32, 169.03 (27 C). MS (EI): m/z 417 (7%) [M^+]. Anal. $C_{27}H_{35}N_3O$ (417.59): Calcd. C, 77.66; H, 8.45; N, 10.06; found C, 77.55; H, 8.34; N, 10.00.

3.32 2′-Amino-6′-(4-methoxyphenyl)-androstano[17,16-d]pyrimidine-3β-ol (12b)

Yield 65%, mp. 245°C, $[\alpha]_D^{25}$ = +115 (c 1, $CHCl_3$); IR (KBr): 3467–3431 (OH, NH_2), 3088 (CH-Ar), 2948 (CH-aliph.), 1616 (C=C), 1615 (C=N) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.88, 0.96 (2s, 6H, $2CH_3$), 0.99–1.12 (m, 1H, CH), 1.17–1.38 (m, 4H, $2CH_2$), 1.42–1.53 (m, 6H, $3CH_2$), 1.61–1.81 (m, 4H, $2CH_2$), 1.93 (m, 1H, CH), 2.20–2.30 (m, 2H, CH_2), 2.55 (m, 1H, CH), 2.68 (m, 1H, 3α-CH), 3.18 (m, 1H, 5α-CH), 3.61 (s, 3H, OCH_3), 4.58 (s, 2H, NH_2 exchangeable with D_2O), 7.28–7.58 (m, 4H, Ar-H), 9.87 (s, 1H, OH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 20.42, 21.78, 24.20, 27.10, 27.45, 28.58, 32.03, 32.28, 36.22, 37.37, 37.57, 37.75, 44.57, 45.36, 52.03, 55.45, 55.74, 70.96, 123.00, 114.32, 125.30, 128.02, 160.54, 161.10, 168.35, 169.14 (28 C). MS (EI): m/z 447 (50%) [M^+]. Anal. $C_{28}H_{37}N_3O_2$ (447.61): Calcd. C, 75.13; H, 8.33; N, 9.39; found C, 75.02; H, 8.22; N, 9.30.

3.33 2′-Amino-6′-(4-chlorophenyl)-androstano[17,16-d] pyrimidine-3β-ol (12c)

Yield 90%, mp. 261°C, $[\alpha]_D^{25}$ = +136 (c 1, $CHCl_3$); IR (KBr): 3478–3444 (OH, NH_2), 3110 (CH-Ar), 2956 (CH-aliph.), 1626 (C=C), 1617 (C=N) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.85, 0.93 (2s, 6H, $2CH_3$), 0.99–1.11 (m, 1H, CH), 1.16–1.32 (m, 4H, $2CH_2$), 1.41–1.57 (m, 6H, $3CH_2$), 1.61–1.81 (m, 4H, $2CH_2$), 1.93 (m, 1H, CH), 2.20–2.30 (m, 2H, CH_2), 2.55 (m, 1H, CH), 2.68 (m, 1H, 3α-CH), 3.18 (m, 1H, 5α-CH), 4.58 (s, 2H, NH_2 exchangeable with D_2O), 7.28–7.58 (m, 4H, Ar-H), 9.87 (s, 1H, OH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 20.36, 21.82, 24.24, 27.16, 27.43, 28.56, 32.10, 32.28, 36.12, 37.32, 37.54, 37.75, 44.57, 45.37, 52.02, 55.78, 70.94, 123.07, 128.33, 129.12, 130.95, 134.35, 161.12, 168.30, 169.16 (27 C). MS (EI): m/z 452 (25%) [M^+]. Anal. $C_{27}H_{34}ClN_3O$ (452.03): Calcd. C, 71.74; H, 7.58; Cl, 7.84; N, 9.30; found C, 71.67; H, 7.50; Cl, 7.75; N, 9.21.

3.34 Synthesis of 2′-Amino-6′-phenyl-androstano[17,16-d]pyrimidine-3-one (13)

To a solution of **12a** (2 mmol) in benzene (3 ml), dimethylsulfoxide (3 ml), pyridine (0.16 ml, 2 mmol) and trifluoroacetic acid (0.08 ml, 1mmol), dicyclohexylcarbodiimide (1.24 g, 6 mmol) was added in sealed reaction flask. The reaction mixture was kept overnight at room temperature, then ether (50 ml) was added followed by the addition of a solution of oxalic acid (0.54 g, 6 mmol) in methanol (5 ml). After gas evolution had ceased (30 min), water (50 ml) was added and the insoluble dicyclohexylurea was removed by filtration. The organic phase was then extracted twice with 5% sodium bicarbonate and once with water, dried over sodium sulfate and evaporated under reduced pressure to dryness, the obtained residue

was crystallized from ethanol to give compound (**113**). Yield 89%, mp. 251°C, $[\alpha]_D^{25}$ = +88.5 (c 1, $CHCl_3$); IR (KBr): 3338 (NH_2), 3077 (CH-Ar), 2921 (CH-aliph), 1616 (C=C), 1580 (C=N) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.81, 0.92 (2s, 6H, $2CH_3$), 0.98–1.09 (m, 1H, CH), 1.11–1.29 (m, 4H, $2CH_2$), 1.39–1.50 (m, 6H, $3CH_2$), 1.62–1.81 (m, 4H, $2CH_2$), 1.90 (m, 1H, CH), 2.21–2.30 (m, 2H, CH_2), 2.48 (m, 1H, CH), 3.17 (m, 1H, 5α-CH), 4.58 (s, 2H, NH_2 exchangeable with D_2O), 7.52–8.36 (m, 5H, Ar-H); ^{13}C NMR ($CDCl_3$): δ 20.40, 21.74, 24.18, 26.10, 27.46, 28.50, 36.12, 37.08, 39.22, 37.37, 44.52, 44.16, 44.56, 44.32, 52.08, 55.78, 210.08, 123.12, 127.30, 128.32, 129.01, 132.85, 161.32, 168.16, 169.18 (27 C). MS (EI): m/z 415 (89%) [M^+]. Anal. $C_{27}H_{33}N_3O$ (415.57): Calcd. C, 78.03; H, 8.00; N, 10.11; found C, 77.90; H, 7.91; N, 10.00.

3.35 Synthesis of androstano[17,16-c]cyclohex-2′-en-3β-ol derivatives (14a-c) and (15)

A solution of the corresponding derivative **1c,d,e** (10 mmol) and acetyl acetone or ethyl acetoacetate (12 mmol) in absolute ethanol (25 ml in the presence of sodium metal (920 mg, 40 mmol) was refluxed for 7 h. The reaction mixture was evaporated under reduced pressure, the obtained residue was washed with 10% HCl, and finally with water. The formed solid was dried and crystallized from ethanol to give compounds (**14a–c**) and (**15**), respectively.

3.36 1′-Oxo-5′-(4-methoxyphenyl)-6′-acetyl-androstano[17,16-c]cyclohex-2′-en-3β-ol (14a)

Yield 73%, mp. 295°C, $[\alpha]_D^{25}$ = +156 (c 1, $CHCl_3$); IR (KBr): 3454 (OH), 3032 (CH, Ar), 2945 (CH, Aliph), 1718, 1678 (2C=O) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.84, 0.95 (2s, 6H, $2CH_3$), 0.99–1.15 (m, 1H, CH), 1.20–1.87 (m, 14H, $7CH_2$), 2.01 (m, 1H, CH), 2.12 (s, 3H, $COCH_3$), 2.21–2.32 (m, 2H, CH_2), 2.38 (m, 1H, CH), 2.44 (m, 1H, C-H), 2.56 (m, 1H, 3α-CH), 3.08 (m, 1H, 5 α-CH), 3.28 (d, 1H, CH), 3.56 (s, 3H, OCH_3), 3.62 (d, 1H, CH), 5.78 (s, 1H, CH=C), 7.15–7.58 (m, 4H, Ar-H), 9.78 (s, 1H, OH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 20.43, 21.78, 22.20, 26.80, 27.01, 27.75, 28.16, 32.10, 32.36, 35.82, 37.38, 37.56, 37.68, 40.15, 41.06, 44.92, 52.25, 52.56, 55.32, 64.67, 69.86, 70.66, 114.30, 124.22, 129.00, 131.02, 157.88, 177.88, 197.07, 203.18 (32 C). MS (EI): m/z% = 490 [M^+, 22]. Anal. $C_{32}H_{42}O_4$ (490.67): Calcd. C, 78.33; H, 8.63; found C, 78.20; H, 8.55.

3.37 1′-Oxo-5′-(4-bromophenyl)-6′-acetyl-androstano[17,16-c]cyclohex-2′-en-3β-ol (14b)

Yield 79%, mp. 308°C, $[\alpha]_D^{25}$ = +98 (c 1, $CHCl_3$); IR (KBr): 3445 (OH), 3012 (CH, Ar), 2941 (CH, Aliph), 1718, 1684 (2C=O) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.85, 0.95 (2s, 6H, $2CH_3$), 0.98–1.12 (m, 1H, CH), 1.17–1.86 (m, 14H, $7CH_2$), 2.00 (m, 1H, 1CH), 2.09 (s, 3H, $COCH_3$), 2.13–2.32 (m, 2H, CH_2), 2.31 (m, 1H, CH), 2.45 (m, 1H, C-H), 2.58 (m, 1H, 3α-CH), 3.12 (m, 1H, 5 α-CH), 3.24 (d, 1H, CH), 3.56 (d, 1H, CH), 5.82 (s, 1H, CH=C), 7.20–7.62 (m, 4H, Ar-H), 9.89 (s, 1H, OH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 20.52, 21.66, 22.18, 26.78, 27.12, 27.76, 28.18, 32.15, 32.44, 35.80, 37.42, 37.64, 37.78, 40.18, 41.02, 44.90, 52.28, 52.65, 64.60, 69.85, 70.68, 120.04, 124.42, 129.95, 131.08, 138.10, 177.75, 197.14, 203.24 (31 C). MS (EI): m/z% = 539 [M^+, 34]. Anal. $C_{31}H_{39}BrO_3$ (539.54): Calcd. C, 69.01; H, 7.29; found C, 68.88; H, 7.20.

3.38 1′-Oxo-5′-(4-chlorophenyl)-6′-acetyl-androstano[17,16-c]cyclohex-2′-en-3β-ol (14c)

Yield 81%, mp. 358°C, $[\alpha]_D^{25}$ = +77 (c 1, $CHCl_3$); IR (KBr): 3467 (OH), 3044 (CH, Ar), 2933 (CH, Aliph), 1720, 1655 (2C=O) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.83, 0.94 (2s, 6H, $2CH_3$), 0.98–1.14 (m, 1H, CH), 1.19–1.86 (m, 14H, $7CH_2$), 2.00 (m, 1H, CH), 2.11 (s, 3H, $COCH_3$), 2.20–2.31 (m, 2H, CH_2), 2.37 (m, 1H, CH), 2.43 (m, 1H, C-H), 2.55 (m, 1H, 3α-CH), 3.07 (m, 1H, 5 α-CH), 3.27 (d, 1H, CH), 3.61 (d, 1H, CH), 5.77 (s, 1H, CH=C), 7.14–7.57 (m, 4H, Ar-H), 9.77 (s, 1H, OH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 20.34, 21.76, 22.28, 26.82, 27.05, 27.75, 28.14, 32.11, 32.31, 35.81, 37.34, 37.55, 37.68, 40.18, 41.06, 44.90, 52.26, 52.58, 64.67, 69.85, 70.65, 124.25, 128.65, 129.34, 131.16, 137.88, 177.86, 197.07, 203.34 (31 C). MS (EI): m/z% = 495 [M^+, 19]. Anal. $C_{31}H_{39}ClO_3$ (495.09): Calcd. C, 75.20; H, 7.94; Cl, 7.16; found C, 75.08; H, 7.85; Cl, 7.10.

3.39 6′-Ethyl-3β-hydroxy-1′-oxo-6′-(4-bromophenyl)-androstan[17,16-c]cyclohex-2′-en-carboxylate (15)

Yield 82%, mp. 305°C, $[\alpha]_D^{25}$ = +141 (c 1, $CHCl_3$); IR (KBr): 3428 (OH), 3032 (CH, Ar), 2950 (CH, Aliph), 1735, 1722 (2C=O) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.87, 0.95 (s, 6H, 2 CH_3), 1.05–1.15 (m, 1H, CH), 1.22 (t, 3H, CH_3), 1.25–1.89 (m, 14H, $7CH_2$), 2.05 (m, 1H, CH), 2.16–2.26 (m, 2H, CH_2), 2.35 (m, 1H, CH), 2.45 (m, 1H, C-H), 2.55 (m, 1H, 3α-CH), 3.12 (m, 1H, 5 α-CH), 3.29 (d, 1H, CH), 3.65 (d, 1H, CH), 4.16 (q, 2H, CH_2), 5.78 (s, 1H, CH=C), 7.12–7.60 (m, 4H, Ar-H), 9.92 (s, 1H, OH, exchangeable with D_2O); ^{13}C NMR ($CDCl_3$): δ 14.65, 20.45, 21.60, 22.19, 26.79, 27.17, 27.77, 61.18, 32.17, 32.44, 35.80, 37.47, 37.64, 37.75, 40.18, 41.07, 44.90, 52.26, 52.63, 64.61, 61.85, 70.61, 120.04, 124.41, 129.95, 131.08, 138.10, 169.96, 177.73, 197.14 (32 C). MS (EI): m/z% = 569 [M^+, 32]. Anal. $C_{32}H_{41}BrO_4$ (569.57): Calcd. C, 67.48; H, 7.26; found C, 67.40; H, 7.18.

3.40 Synthesis of 6′-ethyl-3,1′-dioxo-6′-(4-bromophenyl)-androstano[17,16-c]cyclohex-2′-en-carboxylate (16)

To a solution of **15** (2 mmol) in a mixture of benzene (3 ml), dimethylsulfoxide (3 ml), pyridine (0.16 ml, 2 mmol) and trifluoroacetic acid (0.081 ml, 1 mmol), dicyclocarbodiimide (1.24 g, 6 mmol) was added in a sealed reaction flask. The reaction mixture was kept overnight at room temperature. Ether (50 ml) was added, followed by a solution of oxalic acid (0.54 g, 6 mmol) in methanol (5 ml). After gas evolution had ceased (30 min), water (50 ml) was added and the insoluble dicyclohexylurea was removed by filtration. The organic phase was extracted twice with 5% sodium bicarbonate and once with water, dried over sodium sulphate and evaporated to dryness, the obtained residue was crystallized from ethanol to give compound **16**. Yield 68%, mp. 262°C, $[\alpha]_D^{25}$ = +138 (c 1, $CHCl_3$); IR (KBr): 3032 (CH, Ar), 2950 (CH, Aliph), 1742, 1738, 1722 (3C=O) cm^{-1}. ^1H NMR ($CDCl_3$): δ 0.86, 0.96 (s, 6H, $2CH_3$), 1.06–1.17 (m, 1H, CH), 1.24 (t, 3H, CH_3), 1.28–1.87 (m, 14H, $7CH_2$), 2.04 (m, 1H, CH), 2.13–2.24 (m, 2H, CH_2), 2.33 (m, 1H, CH), 2.43 (m, 1H, C-H), 3.12 (m, 1H, 5 α-CH), 3.31 (d, 1H, CH), 3.67 (d, 1H, CH), 4.17 (q, 2H, CH_2), 5.79 (s, 1H, CH=C), 7.12–7.61 (m, 4H, Ar-H); ^{13}C NMR ($CDCl_3$): δ 14.75, 20.52, 21.61, 21.99, 26.75, 27.14, 27.71, 61.15, 37.04, 39.12, 35.81, 37.78, 40.28, 41.14, 43.67, 44.62, 45.64, 52.30, 52.65, 64.62, 61.84, 209.96, 120.10, 124.43, 129.92, 131.08, 138.15, 169.92,

177.74, 197.18 (32 C). MS (EI): m/z% = 567 [M^+, 31]. Anal. $C_{32}H_{39}BrO_4$ (567.55): Calcd. C, 67.72; H, 6.93; found C, 67.61; H, 6.84.

3.41 Biology

3.41.1 In vitro ubiquitination assay: The expression of recombinant glutathione-*S*-transferase-tagged MDM2 (GST-MDM2) (Sigma-Aldrich, St. Louis, MO, USA) was induced in a 25 ml culture of exponentially growing *Escherichia coli* BL21 cells (OD_{600} 0.6) by 1 mM isopropyl-thio-β-D-galactoside for 3 h. Glutathione-*S*-transferase-MDM2 was purified on glutathione-Sepharose beads (Amersham Biosciences, Amersham, UK). The beads were washed with 50 mM Tris (pH 7.5). Fluorescent ubiquitin (5 μg; Invitrogen, Malvern, PA, USA), 50 ng mammalian E1 (Enzo, New York, NY, USA), 200 ng human recombinant UbcH5B E2 (Enzo) and 200 ng His-p53 (Enzo) were mixed with reaction buffer [50 mM Tris (pH 8.2), mM dithiothreitol, 5 mM $MgCl_2$, 2 mM ATP]. A dose of each newly synthesised tested compound (50 ng) compound or dimethyl sulfoxide (DMSO) was added to the mixture which was then pipetted onto the GS4b-MDM2 beads. The suspension was incubated at 37°C, with shaking at 1200 rpm, for 1 h and then stopped by the addition of 3× sodium dodecyl sulfate sample buffer. Free fluorescent ubiquitin was washed off, and the total fluorescent ubiquitin signal was measured in a monochromator plate reader (Safire, Männedorf, Switzerland).

3.42 In vivo ubiquitination of p53

3.42.1 Cells and transfections: H1299 cells (p53-null human non-small-cell lung adenocarcinoma cells) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum, 1% L-glutamine, 50 U/ml penicillin G and 50 μg/ml streptomycin sulfate at 37°C and 5% CO_2.

Retinal pigment epithelial cells were obtained from ATCC and cultured in DMEM/F12 HAM (Invitrogen), supplemented with 10% fetal calf serum, 1.6% sodium bicarbonate, 1% L-glutamine, 50 U/ml penicillin G and 50 μg/ml streptomycin sulfate at 37°C and 5% CO_2. U2OS-GFP-MDM2 TetOn cells were made by transfecting a U2OS TetOn cell line with a pTRE2 GFP-MDM2 plasmid and pBabe Eco Puro plasmid. Cells were then selected for 2 weeks using puromycin. Colonies resistant to puromycin were picked and grown up in DMEM with supplements and under conditions as above.

Several clones were then tested by fluorescence microscopy to ensure that GFP (green fluorescent protein) positive nuclei were visible after doxocycline induction and were tested for inducible GFP-MDM2 expression by Western blotting. Clone 2 was selected for further work. RKO cells (wild-type and null for p53; obtained from ATCC and authenticated by short-tandem repeat (STR) profiling/karyotyping/isoenzyme analysis) were a gift from Dr. Bert Vogelstein (Sidney Kimmel Comprehensive Cancer Center, Baltimore, MD, USA) and were cultured in DMEM with supplements and under conditions as above.

Cells were transfected with GeneJuice (Merck, Darmstadt, Germany) according to the manufacturer's instructions. Cells were treated with 10 μM Nutlin-3a (Roche) and newly synthesised tested compound at the indicated doses.

The plasmid expressing human wild-type p53 has been described previously [13]. Wild-type MDM2 has been described previously [14]. HA-tagged ubiquitin (Boston Biochem, Cambridge, MA, USA) was kindly provided by Dr. R. Hay (Centre for Gene Regulation and Expression, College of Life Sciences, University of Dundee, UK). The GFP-MDM2 plasmid used to make the U2OS GFP-MDM2 TetOn cell line described above was cloned from peGFPC1 MDM2 (wild-type MDM2 cloned into Clontec backbone) into the pTRE2 plasmid (Clontech, Mountain View, CA, USA).

Cells were grown in DMEM with 10% (v/v) fetal bovine serum and seeded in 10 cm plates to reach 50% confluence 24 h prior to transfection with 1 μg p53, 4 μg MDM2 and 1 μg HIS-ubiquitin with 1.5 μl GeneJuice reagent (Merck). After 20 h, cells were treated with the newly synthesised test compounds for 6 h and MG132 (Sigma-Aldrich) for 4 h, washed and collected in phosphate-buffered saline, the remaining cells were centrifuged for 5 min at 5000 *g* and the cell pellet lysed in 700 μl of ubiquitin buffer A (6 M guanidinium HCl, 300 mM NaCl, 50 mM phosphate [pH 8.0], 100 μg/ml N-ethylmaleimide) and sonicated for 5 min at 20% amplitude (Fisher sonicator model 500). Lysates were incubated overnight with Invitrogen Dynabeads His-Tag matrix, and once washed with each of the ubiquitin buffers A, B and C, and finally phosphate buffered saline (ubiquitin buffer B: Mix ubiquitin buffers A and C 1:1 (v/v); ubiquitin buffer C: 300 mM NaCl, 50 mM phosphate (pH 8.0), 100 μg/ml N-ethylmaleimide). Adsorbed proteins were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting with a p53 (DO-1) antibody (Sigma-Aldrich).

3.43 Non-fluorescent in vitro ubiquitination assay

The procedure was as above except that 5 μg unlabeled ubiquitin (Enzo) was used. Ten microliters of each tested compound or DMSO were added to the mixture and then pipetted onto GS4b-MDM2 beads. After incubation, as described above, reaction products were resolved by SDS-PAGE and analyzed by Western blotting with anti-p53 DO-1. For the MDM2 autoubiquitination assay, GS4b-MDM2 RING beads were prepared as above and used in place of the full length MDM2.

4 Statistical analysis

Results are expressed as mean ± S.E.M. Differences between vehicle control and treatment groups were tested using one-way ANOVA, followed by multiple comparisons by the Dunnett's test. A value of $p \leq 0.005$ was considered statistically significant. Dose-response curves for percent inhibition were fitted by a four-parameter logistic function using a nonlinear least-squares regression. IC_{50} was derived by interpolation from the fitted four-parameter.

The IC_{50} value for each test chemical was determined by the standard curve method using three replicates of each experiment.

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