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Heterocyclizations of pregnenolone: Novel synthesis of thiosemicarbazone, thiophene, thiazole, thieno[2,3-*b*]pyridine derivatives and their cytotoxicity evaluations

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ABSTRACT

Pregnenolone (**1**) was used as a template to develop new anticancer compounds. Ring D modification of **1** through its reaction with 4-phenyl-3-thiosemicarbazide gave the thiosemicarbazone derivative **3**. The latter compound underwent heterocyclization reactions to give the thiazolyl hydrazonoandrostane and pyrazolyl semicarbazidoandrostane derivatives **5a–d**, and **9a–d**, respectively. On the other hand compound **1** reacted with either malononitrile or ethyl cyanoacetate to give the Knoevenagel condensated products **11a** and **11b**, respectively. Compounds **11a,b** afforded the thiophenyl pregnane derivatives **12a** and **12b**, respectively, their reactivity toward some chemical reagents was studied. The cytotoxicity of the newly synthesized heterocyclic steroids against three human tumor cell lines namely breast adenocarcinoma (MCF-7), non-small cell lung cancer (NCI-H460) and CNS cancer (SF-268) were studied. Some of tested compounds were found to exhibit much higher inhibitory effects toward the three tumor cell lines than the reference drug, doxorubicin.

1. Introduction

Steroids have been the prime focus of research throughout the scientific history [1–4]. But the recent past has seen an exhaustive focus of research being diverted toward these biologically important molecules [5–10]. This is pertinently true of the rational semi synthetic modifications of steroidal molecules. Probably, it is because of the various advantages associated with steroid based chemotherapeutics [11–13]. These compounds turn out to be non-toxic, less vulnerable to multi-drug resistance (MDR) and highly bioavailable because of being capable of penetrating the cell wall. Although various modifications of steroids including derivatization, cyclization, heterocyclization, etc. have been tried [8,14–17] but as far the literature precedents are concerned, little efforts have been made toward the efficient synthesis and simultaneous biological analysis of steroid based heterocyclic derivatives. Steroids form an important class of biologically active compounds which exhibit diverse biological activities. Up to now, several steroidal derivatives have been investigated as new curative agents for cancers, and other diseases [18–20]. Several modified steroids, bearing heterocyclic systems as a part of rings-A and -D, have exhibited diverse biological activities, such as anti-microbial, anti-inflammatory, hypotensive, hypocholesterolemic, and diuretic activities. Among these derivatives, 16,17-condensed steroidal pyrazolines have immense pharmacological significance [21–24]. Pregnenolone (**1**), a naturally occurring steroid, is known as a precursor to other hormones, including cortisone, estrogen, testosterone, and progesterone [25,26]. Previously different pregnenolone derivatives have been synthesized, and evaluated for various biological activities. The pregnenolone analogs, hydroxylated at C-20, are known to effect calcium-dependent processes, and also affect the degree of depolarization of smooth muscles [27]. The hemisuccinate of pregnenolone-derivative significantly increases the perfusion pressure, and vascular resistance in isolated rat heart [28]. The nitrochlorambucil ester of pregnenolone exhibited a significant cytotoxic activity toward brain posterior fossa, medullablastoma (Daoy), and lung large cell carcinoma (H460) cell lines [29]. In the present work we investigated some heterocyclization reactions of pregnenolone together with anti-tumor evaluation of the newly synthesized products toward cancer cell lines.

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2. Experimental

2.1. Synthetic methods, analytical and spectral data

The starting steroid, pregnenolone, was purchased from Sigma Company, USA. All solvents were dried by distillation prior to using. Melting points were recorded on Buchi melting point apparatus D-545; IR spectra (KBr disks) were recorded on Bruker Vector 22 instrument. NMR spectra were recorded on Bruker DPX200 instrument in $CDCl_3$ and DMSO-d_6 with TMS as internal standard for protons and solvent signals as internal standard for carbon spectra. Chemical shift values are mentioned in δ (ppm). Mass spectra were recorded on EIMS (Shimadzu) and ESI-esquire 3000 Bruker Daltonics instrument. Elemental analyses were carried out by the Microanalytical Data Unit at the National Research Center, Giza, Egypt and the Microanalytical Data Unit at Cairo University, Giza, Egypt. The progress of all reactions was monitored by TLC on 2×5 cm pre-coated silica gel 60 F254 plates of thickness of 0.25 mm (Merck). The purity of tested compounds was determined by HPLC (Waters Associates, Milford, MA, USA) by using an ultraviolet detector (215 nm) where all compounds showed more than 90% purity. All described compounds showed the characteristic spectral data of cyclopentanoperhydrophenanthrene nuclei of pregnene and androstane series were similar to those reported in the literature [30,31]. For the nomenclature of steroid derivatives, we used the definitive rules for the nomenclature of steroids published by the Joint Commission on the Biochemical Nomenclature (JCBN) of IUPAC [32,33].

2.1.1. 1-(3β-Hydroxy-pregn-5-ene-20E-ylidene)-4-phenylthiosemicarbazone (**3**)

To a solution of pregnenolone (**1**) (0.316 g, 1 mmol) in ethanol (30 mL) 4-phenyl-3-thiosemicarbazide (0.167 g, 1 mmol) was added. The reaction mixture was heated at 60 °C for 30 min then left to cool. The solid product formed upon keeping the reaction mixture overnight was collected by filtration. The precipitate was filtered, dried and monitored through TLC for the purity. Thin layer chromatography revealed just a single spot which proved the presence of a single product. EtOAc:Hexane to give product as solid white powder. HPLC purity = 98% (C-18 NovaPak column; MeCN:MeOH:H_2O/45:20:35), t_r = 19 min; white crystals from EtOAc:hexane (88%), m.p. 120–123 °C; IR (KBr) cm^{-1}: 3425–4322, 3010, 2938, 1804, 1637, 1403, 1041; ^{1}H NMR ($CDCl_3$): δ 0.63 (s, 3H), 1.00 (s, 3H), 1.61–1.90 (m, 6H), 2.20–2.38 (m, 3H), 2.82 (t, J = 8.6, 1H), 3.51 (m, 1H); 5.33 (s, 1H), 6.78 (d, J = 14.7, 1H), 7.29–7.37 (m, 5H), 8.22, 8.30 (2s, 2H); ^{13}C NMR (500 MHz, $CDCl_3$): δ 13.4, 19.5, 21.9, 22.9, 24.6, 31.1, 31.7, 31.9, 37.2, 43.8, 45.1, 48.6, 48.8, 48.9, 49.3, 49.3, 50.1, 57.2, 71.5, 119.0, 120.6, 121.3, 121.7, 126.8, 128.3, 128.4, 130.1, 134.8, 140.6, 141.7, 168.3, 180.3; MS: m/e = 465 (M^+); Anal. Calcd. for $C_{28}H_{39}N_3OS$: C, 72.21; H, 8.44; N, 9.02; S, 6.89. Found C, 72.09; H, 8.73; N, 9.31; S, 7.05.

2.1.2. General procedure for the synthesis of the thiazole derivatives **5a–d**

To a solution of compound **3** (0.465 g, 1 mmol) in ethanol (30 mL) either phanacylbromide (0.20 g, 1 mmol), 4-chlorophenacylbromide (0.23 g, 1 mmol), 4-bromophenacylbromide (0.280 g, 0.01 mol) or 4-methoxyphenacylbromide (0.23 g, 0.01 mol) were added. The reaction mixture was heated under reflux for 2 h then was left to cool. The formed solid product, in each case, upon pouring onto ice/water followed by the addition of few drops of sodium carbonate solution, was collected by filtration and crystallized from the appropriate solvent.

2.1.3. 1-(3β-Hydroxy-pregn-5-ene-20E-ylidene)-2-(3,4-diphenylthiazol-2(3H)-ylidene)azine (**5a**)

HPLC purity = 97.1% (C-18 NovaPak column; MeOH:H_2O/85:15), t_r = 15 min; yellow crystals from ethanol (68%), m.p. 177–180 °C; IR (KBr) cm^{-1}: 3465–4332, 3040, 2936, 1650, 1633, 1405, 1047; ^{1}H NMR (DMSO): δ 0.61 (s, 3H), 1.03 (s, 3H), 1.62–1.92 (m, 6H), 2.24–2.38 (m, 3H), 2.83 (t, J = 8.05 Hz, 1H); 3.50 (m, 1H); 6.78 (s, J = 16.23 Hz, 1H), 5.38 (s, 1H), 6.89 (s, 1H), 7.27–7.38 (m, 10H); ^{13}C NMR (500 MHz, $CDCl_3$): δ 13.5, 19.5, 21.8, 22.9, 24.6, 31.1, 31.7, 31.8, 37.3, 43.8, 45.1, 48.6, 48.8, 48.9, 49.3, 49.3, 50.1, 57.0, 71.6, 119.0, 119.3, 120.6, 121.3, 121.7, 126.8, 127.4, 128.3, 128.4, 130.1, 134.8, 140.6, 141.7, 168.6, 172.4; MS: m/e = 565 (M^+); Anal. Calcd. for $C_{36}H_{43}N_3OS$: C, 76.42; H, 7.66; N, 7.43; S, 5.67. Found C, 76.28; H, 7.92; N, 7.51; S, 5.65.

2.1.4. 1-(3β-Hydroxy-pregn-5-ene-20E-ylidene)-2-(3-phenyl-4-(4-chloro-pheny)lthiazol-2(3H)-ylidene)azine (**5b**)

HPLC purity = 98.2% (C-18 NovaPak column; MeOH:H_2O/90:10), t_r = 24 min; pale yellow crystals from 1,4-dioxan (68%), m.p. 188–191 °C; IR (KBr) cm^{-1}: 3466–4321, 3053, 2932, 1653, 1631, 1408, 1037; ^{1}H NMR (DMSO): δ 0.62 (s, 3H), 1.04 (s, 3H), 1.64–1.90 (m, 6H), 2.23–2.38 (m, 3H), 2.83 (t, J = 7.99 Hz, 1H); 3.52 (m, 1H); 6.74 (d, J = 16.03 Hz, 1H), 5.34 (s, 1H), 6.87 (s, 1H), 7.26–7.39 (m, 9H); ^{13}C NMR (500 MHz, DMSO): δ 13.5, 19.5, 21.8, 22.9, 24.6, 31.1, 31.7, 31.8, 37.3, 43.8, 45.1, 48.6, 48.8, 48.9, 49.3, 49.3, 50.1, 57.0, 71.6, 106.3, 119.3, 120.6, 121.3, 121.7, 126.8, 127.4, 128.3, 128.4, 130.1, 134.8, 140.7, 141.8, 143.2, 168.7, 173.0; MS: m/e = 599 (M^+); Anal. Calcd. for $C_{36}H_{42}ClN_3OS$: C, 72.03; H, 7.05; Cl, 5.91. N, 7.00; S, 5.34. Found C, 72.26; H, 7.12; Cl, 6.22; N, 7.22; S, 5.57.

2.1.5. 1-(3β-Hydroxy-pregn-5-ene-20E-ylidene)-2-(3-phenyl-4-(4-bromopheny)-lthiazol-2(3H)-ylidene)azine (**5c**)

HPLC purity = 96.8% (C-18 NovaPak column; MeOH:H_2O/85:15), t_r = 18 min; pale yellow crystals from 1,4-dioxan (79%), m.p. 203–206 °C; IR (KBr) cm^{-1}: 3471–4320, 3060, 2935, 1650, 1638, 1406, 1039; ^{1}H NMR (DMSO): δ 0.62 (s, 3H), 1.06 (s, 3H), 1.64–1.92 (m, 6H), 2.22–2.39 (m, 3H), 2.83 (t, J = 8.6, 1H), 3.52 (m, 1H); 5.36 (s, 1H), 6.74 (d, J = 14.7, 1H), 6.86 (s, 1H), 7.26–7.39 (m, 9H); ^{13}C NMR (500 MHz, DMSO): δ 13.4, 19.6, 21.8, 22.9, 24.6, 31.1, 31.7, 31.8, 37.3, 43.9, 45.1, 48.6, 48.8, 48.9, 49.3, 49.6, 50.1, 57.1, 71.6, 106.8, 119.3, 120.8, 121.4, 121.9, 126.9, 127.4, 128.6, 128.6, 130.4, 134.8, 140.7, 142.0, 143.2, 168.8, 172.1; MS: m/e = 643 (M^+); Anal. Calcd. for $C_{36}H_{42}BrN_3OS$: C, 67.07; H, 6.57; N, 6.52; S, 4.97. Found C, 66.94; H, 6.42; N, 6.72; S, 4.87.

2.1.6. 1-(3β-Hydroxy-pregn-5-ene-20E-ylidene)-2-(3-phenyl-4-(4-methoxypheny)lthiazol-2(3H)-ylidene)azine (**5d**)

HPLC purity = 98.2% (C-18 NovaPak column; MeOH:H_2O/90:10), t_r = 18 min; pale yellow crystals from 1,4-dioxan (88%), m.p. 166–168 °C; IR (KBr) cm^{-1}: 3468–4322, 3048, 2938, 1652, 1632, 1410, 1044; ^{1}H NMR (DMSO): δ 0.63 (s, 3H), 1.06 (s, 3H), 1.64–1.92 (m, 6H), 2.22–2.38 (m, 3H), 2.83 (t, J = 7.95 Hz, 1H), 3.02 (s, 2H), 3.52 (m, 1H), 5.33 (s, 1H), 6.76 (d, J = 16.08 Hz, 1H), 7.28–7.35 (m, 9H); ^{13}C NMR (500 MHz, DMSO): δ 13.5, 18.6, 19.8, 21.6, 22.8, 24.6, 31.1, 31.7, 31.8, 37.3, 43.8, 45.1, 48.5, 48.7, 49.0, 49.3, 49.6, 50.2, 55.08, 57.1, 71.6, 119.0, 119.5, 120.8, 121.6, 121.9, 126.9, 127.4, 128.6, 128.6, 130.4, 134.8, 139.9, 142.3, 143.2, 168.5, 172.2; MS: m/e = 595 (M^+); Anal. Calcd. for $C_{37}H_{45}N_3O_2S$: C, 74.58; H, 7.61; N, 7.05; S, 5.38. Found C, 74.74; H, 7.09; N, 7.04; S, 5.67.

2.1.7. General procedure for the synthesis the pentan-3-ylidene derivatives **7a,b**

To a solution of compound **3** (0.465 g, 1 mmol) in 1,4-dioxan (20 mL) either acetylacetone (0.01 g, 1 mmol) or ethyl acetoacetate

(0.13 g, 0.01 mol) was added. The whole reaction mixture was heated in a boiling water bath for around 12 h till evolution of H_2S was seized. The solid formed upon pouring onto ice/water was collected by filtration and crystallized from the appropriate solvent.

*2.1.8. 1-(3β-Hydroxy-pregn-5-ene-20E-ylidene)-4-phenyl-3-(2,4-dioxopentan-3-ylidene)semi-hydrazide (**7a**)*

HPLC purity = 99.0% (C-18 NovaPak column; MeCN:MeOH:H_2O/55:20:25), t_r = 23 min; colorless crystals from ethanol (80%), m.p. 127–129 °C; IR (KBr) cm^{-1}: 3480–4336, 3066, 2938, 1688, 1684, 1652, 1633, 1405, 1048; ^{1}H NMR (DMSO): δ 0.61 (s, 3H), 1.03 (s, 3H), 1.62–1.92 (m, 6H), 2.24–2.38 (m, 3H), 2.63, 2.69 (2s, 6H), 2.85 (t, *J* = 8.34 Hz, 1H), 3.50 (m, 1H), 5.34 (s, 1H), 6.74 (d, *J* = 15.99 Hz, 1H), 7.26–7.39 (m, 5H), 8.26, 8.32 (2s, 2H); ^{13}C NMR (500 MHz, DMSO): δ 13.5, 19.5, 21.8, 22.0, 22.3, 22.9, 24.6, 26.0, 26.3, 31.1, 31.7, 31.8, 37.3, 43.8, 45.1, 48.6, 48.8, 48.9, 49.3, 49.3, 50.1, 57.0, 71.6, 119.3, 120.6, 121.3, 121.7, 126.8, 127.4, 128.6, 128.4, 130.1, 132.1, 134.5, 140.9, 141.5, 162.3, 164.6, 168.8, 172.6; MS: *m*/*e* = 531 (M^+); Anal. Calcd. for $C_{33}H_{45}N_3O_3$: C, 74.54; H, 8.53; N, 7.90. Found C, 74.28; H, 8.35; N, 7.83.

*2.1.9. 1-(3β-Hydroxy-pregn-5-ene-20E-ylidene)-4-phenyl-3-(ethyl 3-oxo-butanoato-2-ylidene)semihydrazide (**7b**)*

HPLC purity = 87.1.0% (C-18 NovaPak column; MeCN:MeOH:H_2-O/45:30:25), t_r = 20 min; Colorless crystals from ethanol (69%), m.p. 158–160 °C; IR (KBr) cm^{-1}: 3483–4338, 3050, 2929, 1692, 1686, 1650, 1634, 1402, 1044; ^{1}H NMR (DMSO): δ 0.62 (s, 3H), 1.03 (s, 3H), 1.13 (t, 3H, 7.11 Hz, CH_3), 1.62–1.93 (m, 6H), 2.22–2.37 (m, 3H), 2.64 (s, 3H), 2.86 (t, *J* = 8.23 Hz, 1H), 3.50 (m, 1H), 4.22 (q, 7.11 Hz, 2H), 5.38 (s, 1H), 6.74 (d, *J* = 15.98 Hz, 1H), 7.26–7.39 (m, 5H), 8.26, 8.31 (2s, 2H); ^{13}C NMR (500 MHz, DMSO): δ 13.5, 16.5, 19.3, 19.5, 21.8, 22.0, 22.3, 22.9, 24.6, 31.1, 31.7, 31.8, 37.3, 43.8, 44.0, 45.1, 48.6, 48.8, 48.9, 49.3, 49.3, 50.1, 57.0, 71.6, 119.3, 120.8, 121.4, 121.7, 126.9, 127.6, 128.8, 128.4, 130.4, 132.6, 134.6, 140.9, 141.6, 167.8, 168.4. MS: *m*/*e* = 561 (M^+); Anal. Calcd. for $C_{34}H_{47}N_3O_4$: C, 72.69; H, 8.43; N, 7.48. Found C, 72.73; H, 8.39; N, 7.57.

*2.1.10. General procedure for the synthesis the pyrazole derivatives **9a–d***

To a solution of either **7a** (0.531 g, 1 mmol) or **7b** (0.527 g, 0.01 mol) in ethanol (40 mL) either hydrazine hydrate (0.05 g, 1 mmol) or phenylhydrazine (0.108 g, 1 mmol) was heated under reflux for 1 h then poured onto ice/water containing one drop of hydrochloric acid. The solid product formed, in each case was collected by filtration and crystallized from the appropriate solvent.

*2.1.11. 1-(3β-Hydroxy-pregn-5-ene-20E-ylidene)-4-phenyl-3-(3,5-dimethylpyrazolo-)semicarbazide (**9a**)*

HPLC purity = 97.6% (C-18 NovaPak column; MeCN:MeOH:H_2O/45:30:25), t_r = 21 min; Colorless crystals from ethanol (71%), m.p. 133–135 °C; IR (KBr) cm^{-1}: 3488–4316, 3050, 2935, 1650, 1630, 1411, 1046; ^{1}H NMR (DMSO): δ 0.62 (s, 3H), 1.03 (s, 3H), 1.63–1.90 (m, 6H), 2.25–2.38 (m, 3H), 2.65, 2.72 (2s, 6H), 2.86 (t, *J* = 8.25 Hz, 1H), 3.50 (m, 1H), 5.33 (s, 1H), 6.74 (d, *J* = 16.01 Hz, 1H), 7.24–7.36 (m, 5H), 8.05, 8.31 (2s, 2H); ^{13}C NMR (500 MHz, DMSO): δ 13.5, 19.6, 21.9, 22.0, 22.3, 22.9, 24.6, 31.1, 31.4, 31.9, 37.3, 43.8, 45.3, 48.6, 48.8, 48.9, 49.8, 49.3, 50.1, 57.0, 71.6, 119.3, 120.6, 121.3, 124.2, 125.4, 127.4, 128.6, 128.4, 130.1, 134.5, 140.9, 141.5, 144.2, 148.0, 164.6, 172.6; MS: *m*/*e* = 527 (M^+); Anal. Calcd. for $C_{33}H_{45}N_5O$: C, 75.10; H, 8.59; N, 13.27. Found C, 75.29; H, 8.59; N, 13.42.

*2.1.12. 1-(3β-Hydroxy-pregn-5-ene-20E-ylidene)-4-phenyl-3-(3-methyl-5-hydroxypyrazolo)-semicarbazide (**9b**)*

HPLC purity = 96.8% (C-18 NovaPak column; MeCN:MeOH:H_2O/70:20:10), t_r = 25 min; Colorless crystals from ethanol (84%), m.p. 110–112 °C; IR (KBr) cm^{-1}: 3466–4342, 3055, 2924, 1652, 1636, 1403, 1046; ^{1}H NMR (DMSO): δ 0.62 (s, 3H), 1.04 (s, 3H), 1.62–1.93 (m, 6H), 2.21–2.38 (m, 3H), 2.67 (s, 3H), 2.86 (t, *J* = 8.21 Hz, 1H), 3.50 (m, 1H), 5.32 (s, 1H), 6.74 (d, *J* = 16.04 Hz, 1H), 7.26–7.39 (m, 5H), 8.12, 8.34 (2s, 2H), 10.27 (s, 1H); ^{13}C NMR (500 MHz, DMSO): δ 13.6, 19.5, 21.8, 22.0, 22.8, 22.9, 24.6, 31.1, 31.7, 31.9, 37.3, 43.8, 44.0, 45.6, 48.6, 48.8, 48.9, 49.3, 49.3, 50.1, 57.0, 71.6, 119.8, 120.5, 121.6, 121.7, 126.4, 127.8, 128.0, 128.8, 128.4, 130.4, 134.6, 140.9, 141.6, 154.3, 164.4, 168.2; MS: *m*/*e* = 529 (M^+); Anal. Calcd. for $C_{32}H_{43}N_5O_2$: C, 72.56; H, 8.18; N, 13.22. Found C, 72.60; H, 8.22; N, 13.48.

*2.1.13. 1-(3β-Hydroxy-pregn-5-ene-20E-ylidene)-4-phenyl-3-(3,5-dimethyl-1-phenyl-pyrazolo)semicarbazide (**9c**)*

HPLC purity = 97.5% (C-18 NovaPak column; MeCN:MeOH:H_2O/60:20:20), t_r = 22 min; Colorless crystals from ethanol (55%), m.p. 193–196 °C; IR (KBr) cm^{-1}: 3448–4348, 3048, 2931, 1660, 1656, 1632, 1411, 1046; ^{1}H NMR (DMSO): δ 0.62 (s, 3H), 1.04 (s, 3H), 1.63–1.90 (m, 6H), 2.25–2.38 (m, 3H), 2.68, 2.73 (2s, 6H), 2.87 (t, 8.33 Hz, 1H), 3.50 (m, 1H), 5.38 (s, 1H), 6.72 (d, 15.78 Hz, 1H), 7.26–7.34 (m, 5H), 8.11 (s, 1H); ^{13}C NMR (500 MHz, DMSO): δ 13.6, 19.8, 21.4, 22.6, 22.3, 23.2, 24.6, 31.1, 31.4, 31.9, 37.3, 43.9, 45.3, 48.6, 48.8, 48.9, 49.8, 49.3, 50.2, 57.0, 71.3, 119.6, 120.8, 121.0, 121.4, 124.2, 125.4, 127.4, 128.6, 128.4, 130.1, 134.5, 135.0, 140.9, 141.3, 148.6, 164.2, 168.4; MS: *m*/*e* = 603 (M^+); Anal. Calcd. for $C_{39}H_{49}N_5O$: C, 77.57; H, 8.18; N, 11.60 Found C, 77.49; H, 8.24; N, 11.80.

*2.1.14. 1-(3β-Hydroxy-pregn-5-ene-20E-ylidene)-4-phenyl-3-(3-methyl-5-hydroxy-1-phenyl-pyrazolo)semicarbazide (**9d**)*

HPLC purity = 98.1% (C-18 NovaPak column; MeCN:MeOH:H_2O/55:35:10), t_r = 26 min; yellow crystals from ethanol (89%), m.p. 150–153 °C; IR (KBr) cm^{-1}: 3489–4312, 3050, 2928, 1659, 1633, 1413, 1044; ^{1}H NMR (DMSO): δ 0.62 (s, 3H), 1.05 (s, 3H), 1.61–1.92 (m, 6H), 2.20–2.39 (m, 3H), 2.68 (s, 3H), 2.87 (t, *J* = 7.91 Hz, 1H), 3.50 (m, 1H), 5.32 (s, 1H), 6.78 (d, *J* = 16.09 Hz, 1H), 7.28–7.41 (m, 10H), 8.05 (s, 1H), 10.27 (s, 1H); ^{13}C NMR (500 MHz, DMSO): δ 13.6, 19.5, 21.8, 22.0, 22.8, 22.9, 24.6, 31.1, 31.7, 31.9, 37.3, 43.8, 44.0, 45.6, 48.6, 48.8, 48.9, 49.3, 49.3, 50.1, 57.0, 71.6, 119.8, 120.5, 121.6, 121.7, 123.4, 123.9, 126.4, 127.8, 128.0, 128.4, 128.8, 128.4, 130.4, 134.6, 140.9, 141.7, 154.6, 164.6, 168.0; MS: *m*/*e* = 605 (M^+); Anal. Calcd. for $C_{38}H_{47}N_5O_2$: C, 75.34; H, 7.82; N, 11.56. Found C, 75.77; H, 8.02; N, 11.68.

*2.1.15. General procedure for the synthesis of the ylidene derivatives **11a,b***

To a dry solid of pregnenolone **1** (0.316 g, 1 mmol), either malononitrile (0.066 g, 1 mmol) or ethyl cyanoacetate (0.113 g, 1 mmol) was added followed by ammonium acetate (0.250 g). The whole reaction mixture was heated in a boiling water bath for 8 h then left to cool. The formed solid product was triturated with diethyl ether and the formed solid product, in each case, was collected by filtration.

*2.1.16. 2-(3β-Hydroxy-pregn-5-ene-20-ylidene)malononitrile (**11a**)*

HPLC purity = 98.0% (C-18 NovaPak column; MeOH:H_2O/80:20), t_r = 16 min; pale yellow crystals from ethanol (80%), m.p. 160–163 °C; IR (KBr) cm^{-1}: 3494–4326, 2923, 2223, 2220, 1645, 1630, 1414, 1048; ^{1}H NMR (DMSO): δ 0.61 (s, 3H), 1.02 (s, 3H), 1.63–1.88 (m, 6H), 2.22–2.37 (m, 3H), 2.85 (t, *J* = 8.09 Hz, 1H), 3.52 (m, 1H), 5.36 (s, 1H), 6.74 (d, *J* = 15.69 Hz, 1H); ^{13}C NMR (500 MHz, DMSO): δ 13.5, 19.6, 22.0, 22.3, 22.9, 24.4, 31.1, 31.4, 31.9, 37.3,

43.8, 45.3, 48.6, 48.8, 48.9, 49.8, 49.3, 50.1, 57.0, 71.6, 89.6, 116.8, 117.3, 119.3, 120.6, 122.8, 129.5, 168.2; MS: m/e = 364 (M^+); Anal. Calcd. for $C_{24}H_{32}N_2O$: C, 79.08; H, 8.85; N, 7.68. Found C, 79.19; H, 8.69; N, 7.88.

*2.1.17. 2-Cyano-3-(3β-hydroxy-pregn-5-ene-20E-yl)but-2-enoate (**11b**)*

HPLC purity = 96.4.0% (C-18 NovaPak column; MeOH:H_2O/85:15), t_r = 24 min; pale yellow crystals from ethanol (65%), m.p. 85–87 °C; IR (KBr) cm^{-1}: 3485–4322, 2923, 2221, 1680, 1652, 1630, 1414, 1043; ^{1}H NMR (DMSO): δ 0.63 (s, 3H), 1.02 (s, 3H), 1.12 (t, J = 6.72 Hz, 3H), 1.61–1.82 (m, 6H), 2.21–2.37 (m, 3H), 2.88 (t, J = 8.09 Hz, 1H); 3.53 (m, 1H), 4.22 (q, J = 6.72 Hz, 2H), 5.36 (s, 1H), 6.72 (s, J = 16.21 Hz, 1H), ^{13}C NMR (500 MHz, DMSO): δ 13.5, 16.3, 19.6, 22.0, 22.3, 22.9, 24.4, 31.1, 31.4, 31.9, 37.3, 42.1, 43.8, 45.3, 48.6, 48.8, 48.9, 49.8, 49.2, 50.1, 57.1, 71.3, 88.3, 116.2, 117.3, 118.8, 119.7, 129.6, 168.2, 172.0; MS: m/e = 411 (M^+); Anal. Calcd. for $C_{26}H_{37}NO_3$: C, 75.87; H, 9.06; N, 3.40. Found C, 79.09; H, 8.69; N, 3.65.

*2.1.18. General procedure for the synthesis of the thiophene derivatives **12a,b***

To a solution of either **11a** (0.364 g, 1 mmol) or **11b** (0.431 g, 1 mmol) in 1,4-dioxan (30 mL) containing catalytic amount of triethylamine, sulfur (0.032 g, 1 mmol) was added. The reaction mixture was heated under reflux for 30 min then left at room temperature for one night then poured onto ice/water and the formed solid product was collected by filtration.

*2.1.19. 2-Amino-4-(3β-hydroxy-androst-5-ene-17-yl)thiophene-3-carbonitrile (**12a**)*

HPLC purity = 97.2.0% (C-18 NovaPak column; MeOH:H_2O/80:20), t_r = 18 min; yellow crystals from AcOH (69%), m.p. 197–200 °C; IR (KBr) cm^{-1}: 3444–4316, 3030, 2920, 2222, 1656, 1630, 1414, 1046; ^{1}H NMR (DMSO): δ 0.61 (s, 3H), 1.02 (s, 3H), 1.63–1.88 (m, 6H), 2.22–2.37 (m, 3H), 2.85 (t, J = 8.11 Hz, 1H), 3.52 (m, 1H), 4.77 (s, 2H), 5.35 (s, 1H), 6.78 (d, J = 15.66 Hz, 1H), 6.99 (s, 1H); ^{13}C NMR (500 MHz, DMSO): δ 13.3, 19.2, 22.0, 22.3, 22.9, 24.4, 31.1, 31.4, 31.9, 37.3, 43.8, 45.3, 48.6, 48.8, 48.9, 49.8, 49.3, 50.1, 57.0, 71.6, 116.5, 119.3, 122.8, 129.5, 133.5, 138.9, 140.3, 144.8; MS: m/e = 396 (M^+); Anal. Calcd. for $C_{24}H_{32}N_2OS$: C, 72.68; H, 8.13; N, 7.06; S, 8.09. Found C, 72.83; H, 6.89; N, 7.18; S, 7.84.

*2.1.20. Ethyl 2-Amino-4-(3β-hydroxy-androst-5-ene-17-yl)thiophene-3-carboxylate (**12b**)*

HPLC purity = 97.8% (C-18 NovaPak column; MeOH:H_2O/85:15), t_r = 21 min; pale yellow crystals from AcOH (73%), m.p. 105–107 °C; IR (KBr) cm^{-1}: 3439–4312, 3040, 2922, 1687, 1650, 1632, 1416, 1048; ^{1}H NMR (DMSO): δ 0.63 (s, 3H), 1.02 (s, 3H), 1.11 (t, 3H), 1.14 (t, J = 7.41 Hz, 3H), 1.61–1.82 (m, 6H), 2.21–2.37 (m, 3H), 2.88 (t, 1H), 3.53 (m, 1H); 4.22 (q, J = 7.41 Hz, 2H), 4.72 (s, 2H), 5.35 (s, 1H), 6.71 (s, J = 15.78 Hz, 1H), 7.07 (s, 1H); ^{13}C NMR (500 MHz, DMSO): δ 13.5, 16.3, 19.6, 22.5, 22.3, 22.9, 24.4, 31.1, 31.4, 31.9, 37.3, 42.4, 43.6, 45.2, 48.6, 48.8, 48.9, 49.8, 49.2, 50.1, 57.1, 71.3, 119.4, 122.6, 129.2, 138.4, 140.8, 147.3, 162.8; MS: m/e = 443 (M^+); Anal. Calcd. for $C_{26}H_{37}NO_3S$: C, 70.39; H, 8.41; N, 3.16; S, 7.23. Found C, 70.41; H, 8.52; N, 3.26; S, 7.44.

*2.1.21. General procedure for the synthesis of the arylazothiophene derivatives **14a–f***

To a solution of either compound **12a** (0.396, 1 mmol) or **12b** (0.443 g, 1 mmol) in ethanol (30 mL) containing sodium acetate (2.5 g), either benzenediazonium chloride (0.01 mol), 4-methylbenzenediazonium chloride (1 mmol) or 4-chlorobenzenediazonium chloride (1 mmol) [prepared by adding sodium nitrite solution (0.007 g, 1 mmol) to a cold solution of the appropriate aniline or its derivative (1 mmol) in concentrated hydrochloric acid (3 mL, 18 N) with continuous stirring] was added with stirring. The reaction mixture was kept at room temperature for 1 h and the formed solid product, in each case, was collected by filtration.

*2.1.22. 2-Amino-4-(3β-hydroxy-androst-5-ene-17-yl)-5-(phenylazo)thiophene-3-carbonitrile (**14a**)*

HPLC purity = 98.8% (C-18 NovaPak column; MeOH:H_2O/80:20), t_r = 19 min; orange crystals from ethanol (86%), m.p. 188–191 °C; IR (KBr) cm^{-1}: 3474–4326, 3062, 2920, 2220, 1653, 1633, 1414, 1046; ^{1}H NMR (DMSO): δ 0.64 (s, 3H), 1.04 (s, 3H), 1.63–1.86 (m, 6H), 2.21–2.38 (m, 3H), 2.86 (t, J = 7.94 Hz, 1H), 3.54 (m, 1H), 4.20 (s, 2H), 4.77 (s, 2H), 5.33 (s, 1H), 6.79 (s, J = 15.93 Hz, 1H), 7.28–7.39 (m, 5H); ^{13}C NMR (500 MHz, DMSO): δ 13.4, 19.2, 22.5, 22.3, 22.6, 24.8, 31.1, 31.2, 31.9, 37.3, 43.9, 45.4, 48.6, 49.8, 49.3, 50.1, 57.0, 71.6, 116.5, 119.3, 120.6, 121.0, 122.8, 129.5, 133.5, 144.6; MS: m/e = 500 (M^+); Anal. Calcd. for $C_{30}H_{36}N_4OS$: C, 71.96; H, 7.25; N, 11.19; S, 6.40. Found C, 71.87; H, 7.04; N, 10.98; S, 6.54.

*2.1.23. 2-Amino-4-(3β-hydroxy-androst-5-ene-17-yl)-5-(4-methylphenylazo)thiophene-3-carbonitrile (**14b**)*

HPLC purity = 97.4% (C-18 NovaPak column; MeOH:H_2O/85:15), t_r = 17 min; Orange crystals from ethanol (86%), m.p. 188–191 °C; IR (KBr) cm^{-1}: 3491–4346, 3055, 2926, 2225, 1652, 1630, 1414, 1048; ^{1}H NMR (DMSO): δ 0.62 (s, 3H), 1.06 (s, 3H), 1.60–1.87 (m, 6H), 2.23–2.37 (m, 3H), 2.88 (t, J = 8.47 Hz, 1H), 2.92 (s, 3H), 3.56 (m, 1H); 4.21 (s, 2H), 4.78 (s, 2H), 5.39 (s, 1H), 6.75 (d, J = 16.18 Hz, 1H), 7.03–7.36 (m, 4H); ^{13}C NMR (500 MHz, DMSO): δ 13.1, 18.3, 19.3, 22.7, 22.2, 22.8, 24.6, 31.2, 31.2, 31.9, 37.6, 43.9, 45.7, 48.2, 48.4, 48.6, 49.8, 49.9, 50.2, 57.0, 61.5, 71.3, 116.7, 119.3, 119.2, 120.2, 120.5, 121.4, 122.2, 125.3, 129.1, 133.8, 144.4; MS: m/e = 514 (M^+); Anal. Calcd. for $C_{31}H_{38}N_4OS$: C, 72.34; H, 7.44; N, 10.88; S, 6.23. Found C, 72.48; H, 7.34; N, 10.73; S, 6.44.

*2.1.24. 2-Amino-4-(3β-hydroxy-androst-5-ene-17-yl)-5-(4-chlorophenylazo)thiophene-3-carbonitrile (**14c**)*

HPLC purity = 98.8% (C-18 NovaPak column; MeOH:H_2O/80:20), t_r = 23 min; Orange crystals from ethanol (69%), m.p. 222–225 °C; IR (KBr) cm^{-1}: 3496–4324, 3052, 2924, 2220, 1650, 1631, 1416, 1049; ^{1}H NMR (DMSO): δ 0.60 (s, 3H), 1.03 (s, 3H), 1.62–1.86 (m, 6H), 2.24–2.38 (m, 3H), 2.86 (t, J = 7.93 Hz, 1H); 3.54 (m, 1H); 4.24 (s, 2H), 4.75 (s, 2H), 5.33 (s, 1H), 6.72 (d, J = 16.07 Hz, 1H), 7.28–7.38 (m, 4H); ^{13}C NMR (500 MHz, DMSO): δ 13.0, 18.6, 18.9, 19.4, 22.3, 22.5, 22.8, 24.6, 31.1, 31.2, 31.9, 37.6, 43.9, 45.4, 48.2, 48.4, 48.2, 49.5, 49.9, 50.2, 56.8, 61.6, 71.4, 116.8, 119.3, 119.6, 120.4, 120.6, 121.8, 122.3, 129.5, 133.6, 144.9; MS: m/e = 534 (M^+); Anal. Calcd. for $C_{30}H_{35}ClN_4OS$: C, 67.33; H, 6.59; N, 10.47; S, 5.99. Found C, 67.08; H, 6.84; N, 10.67; S, 6.04.

*2.1.25. Ethyl 2-amino-4-(3β-hydroxy-androst-5-ene-17-yl)-5-(phenylazo)thiophene-3-carboxylate (**14d**)*

HPLC purity = 98.4% (C-18 NovaPak column; MeOH:H_2O/90::10), t_r = 22 min; Orange crystals from ethanol (87%), m.p. 160–163 °C; IR (KBr) cm^{-1}: 3481–4320, 3054, 2922, 1653, 1635, 1412, 1050; ^{1}H NMR (DMSO): δ 0.62 (s, 3H), 1.02 (s, 3H), 1.14 (t, J = 6.94 Hz, 3H), 1.60–1.85 (m, 6H), 2.24–2.39 (m, 3H), 2.82 (t, 1H); 3.53 (m, 1H); 4.21 (s, 2H), 4.24 (q, J = 6.94 Hz, 2H), 4.75 (s, 2H), 5.36 (s, 1H), 6.74 (d, J = 16.42 Hz, 1H), 7.26–7.39 (m, 5H); ^{13}C NMR (500 MHz, DMSO): δ 13.1, 16.2, 18.5, 18.9, 19.4, 22.2, 22.5, 22.6, 24.6, 31.0, 31.2, 32.3, 37.5, 43.6, 45.4, 48.2, 48.4, 48.2, 49.3, 49.8, 50.3, 56.6, 71.6, 119.4, 119.6, 120.6, 120.9, 121.8, 122.6, 129.5, 134.5, 143.2, 162.4; MS: m/e = 547 (M^+); Anal. Calcd. for $C_{32}H_{41}N_3O_3S$: C, 70.17; H, 7.54; N, 7.67; S, 5.85. Found C, 70.33; H, 7.64; N, 7.71; S, 6.13.

*2.1.26. Ethyl 2-amino-4-(3β-hydroxy-androst-5-ene-17-yl)-5-(4-methylphenylazo)thiophene-3-carboxylate (**14e**)*

HPLC purity = 98.8% (C-18 NovaPak column; MeOH:H_2O/85:15), t_r = 23 min; orange crystals from ethanol (69%), m.p. 144–146 °C; IR (KBr) cm^{-1}: 3467–4322, 3053, 2920, 1655, 1635, 1415, 1052; ^1H NMR (DMSO): δ 0.63 (s, 3H), 1.01 (s, 3H), 1.15 (t, *J* = 5.94 Hz, 3H), 1.62–1.88 (m, 6H), 2.25–2.38 (m, 3H), 2.80 (t, *J* = 7.83 Hz, 1H), 2.90 (s, 3H), 3.53 (m, 1H), 4.22 (s, 2H), 4.23 (q, *J* = 5.94 Hz, 2H), 4.73 (s, 2H), 5.36 (s, 1H), 6.72 (d, *J* = 15,72 Hz, 1H), 7.28–7.38 (m, 4H); ^{13}C NMR (500 MHz, DMSO): δ 13.0, 16.6, 18.5, 18.9, 19.4, 22.2, 22.8, 22.9, 24.2, 31.4, 31.8, 32.4, 37.5, 43.8, 45.4, 48.1, 48.8, 49.3, 49.8, 50.3, 56.6, 61.6, 71.6, 119.4, 119.9, 120.6, 120.9, 122.0, 122.6, 129.5, 134.8, 143.8, 154.8, 164.2; MS: *m*/*e* = 561 (M^+); Anal. Calcd. for $C_{33}H_{43}N_3O_3S$: C, 70.55; H, 7.72; N, 7.48; S, 5.71. Found C, 70.82; H, 7.59; N, 7.33; S, 5.90.

*2.1.27. Ethyl 2-amino-4-(3β-hydroxy-androst-5-ene-17-yl)-5-(4-chlorophenylazo)thiophene-3-carboxylate (**14f**)*

HPLC purity = 98.8% (C-18 NovaPak column; MeOH:H_2O/80:20), t_r = 25 min; orange crystals from ethanol (84%), m.p. 203–206 °C; IR (KBr) cm^{-1}: 3477–4352, 3056, 2922, 1655, 1635, 1417, 1052; ^1H NMR (DMSO): δ 0.64 (s, 3H), 1.02 (s, 3H), 1.14 (t, *J* = 7.41 Hz, 3H), 1.64–1.86 (m, 6H), 2.26–2.39 (m, 3H), 2.83 (t, *J* = 8.05 Hz, 1H), 2.92 (s, 3H), 3.56 (m, 1H), 4.20 (s, 2H), 4.24 (q, *J* = 7.41 Hz, 2H), 4.77 (s, 2H), 5.34 (s, 1H), 6.74 (d, *J* = 15.89 Hz, 1H), 7.28–7.39 (m, 4H); ^{13}C NMR (500 MHz, DMSO): δ 13.1, 16.8, 18.5, 18.9, 19.4, 22.5, 22.8, 22.9, 24.2, 31.6, 31.7, 32.6, 37.3, 43.6, 45.6, 48.2, 48.6, 49.0, 49.4, 50.5, 56.6, 61.8, 71.6, 119.6, 119.9, 120.4, 120.9, 122.1, 122.6, 129.6, 133.6, 144.0; MS: *m*/*e* = 581 (M^+); Anal. Calcd. for $C_{32}H_{40}ClN_3O_3S$: C, 66.02; H, 6.93; N, 7.22; S, 5.51. Found C, 65.89; H, 6.79; N, 7.60; S, 5.82.

*2.1.28. General procedure for the synthesis of the thieno[2,3-b]pyridine derivatives **15a–d***

To a solution of either compound **12a** (0.396, 1 mmol) or **12b** (0.443 g, 1 mmol) in 1,4-dioxan (30 mL) containing triethylamine (0.25 mL) either malononitrile (0.066 g, 0.01 mol) or ethyl cyanoacetate (0.11 g, 1 mmol) was added. The reaction mixture was heated under reflux for 2 h then left to cool and evaporated under vacuum. The remaining product was triturated with diethyl ether and the formed solid product in each case, was collected by filtration.

*2.1.29. 4,6-Diamino-3-(3β-hydroxy-androst-5-ene-17-yl)thieno[2,3-b]pyridine-5-carbonitrile (**15a**)*

HPLC purity = 97.2% (C-18 NovaPak column; MeOH:H_2O/80:20), t_r = 19 min; pale yellow crystals from ethanol (90%), m.p. >300 °C; IR (KBr) cm^{-1}: 3488–4336, 2936, 2227, 1655, 1636, 1412, 1046; ^1H NMR (DMSO): δ 0.61 (s, 3H), 1.02 (s, 3H), 1.63–1.89 (m, 6H), 2.23–2.39 (m, 3H), 2.84 (t, *J* = 8.04 Hz, 1H); 3.53 (m, 1H); 4.21 (s, 2H), 4.68, 4.80 (2s, 4H), 5.34 (s, 1H), 6.79 (d, *J* = 15.84 Hz, 1H), 6.93 (s, 1H), ^{13}C NMR (500 MHz, DMSO): δ 13.1, 19.2, 22.5, 22.4, 22.6, 24.4, 30.8, 31.2, 31.9, 37.3, 43.9, 45.4, 48.6, 48.9, 49.8, 50.1, 57.2, 61.7, 71.8, 116.8, 119.3, 120.6, 122.8, 129.5, 130.3, 134.6, 142.1, 144.2, 161.8; MS: *m*/*e* = 462 (M^+); Anal. Calcd. for $C_{27}H_{34}N_4OS$: C, 70.09; H, 7.41; N, 12.11; S, 6.93. Found C, 70.15; H, 7.25; N, 12.32; S, 6.84.

*2.1.30. 6-Amino-3-(3β-hydroxy-androst-5-ene-17-yl)-4-hydroxythieno[2,3-b]pyridine-5-carbonitrile (**15b**)*

HPLC purity = 96.8% (C-18 NovaPak column; MeOH:H_2O/85:15), t_r = 22 min; pale yellow crystals from ethanol (73%), m.p. 273–276 °C; IR (KBr) cm^{-1}: 3441–4316, 2922, 2224, 1652, 1638, 1418, 1042; ^1H NMR (DMSO): δ 0.60 (s, 3H), 1.01 (s, 3H), 1.62–1.86 (m, 6H), 2.24–2.36 (m, 3H), 2.85 (t, *J* = 8.41 Hz, 1H), 3.53 (m, 1H), 4.24 (s, 2H), 4.66 (s, 2H), 5.36 (s, 1H), 6.66 (d, *J* = 15.85 Hz, 1H), 6.90 (s, 1H), 10.33 (s, 1H); ^{13}C NMR (500 MHz, DMSO): δ 13.0, 19.4, 22.5, 22.4, 22.6, 24.4, 30.8, 31.2, 31.4, 37.3, 43.7, 45.4, 48.4, 48.8, 49.6, 50.3, 57.2, 61.7, 71.8, 116.8, 119.3, 120.6, 122.2, 129.3, 130.3, 133.6, 134.8, 142.3, 144.6, 161.5; MS: *m*/*e* = 463 (M^+); Anal. Calcd. for $C_{27}H_{33}N_3O_2S$: C, 69.94; H, 7.17; N, 9.06; S, 6.92.

*2.1.31. Ethyl 4,6-diamino-3-(3β-hydroxy-androst-5-ene-17-yl)thieno[2,3-b]pyridine-5-carboxylate (**15c**)*

HPLC purity = 98.0% (C-18 NovaPak column; MeOH:H_2O/80:20), t_r = 18 min; pale yellow crystals from ethanol (71%), m.p. 164–166 °C; IR (KBr) cm^{-1}: 3492–4316, 3021, 2928, 1655, 1636, 1417, 1040; ^1H NMR (DMSO): δ 0.60 (s, 3H), 1.02 (s, 3H), 1.12 (t, *J* = 6.63 Hz, 3H), 1.63–1.89 (m, 6H), 2.22–2.37 (m, 3H), 2.86 (t, *J* = 7.83 Hz, 1H); 3.53 (m, 1H); 4.21 (s, 2H), 4.25 (q, *J* = 6.63 Hz, 2H), 4.49, 4.69 (2s, 4H), 5.38 (s, 1H), 6.80 (s, *J* = 16.06 Hz, 1H), 6.92 (s, 1H), ^{13}C NMR (500 MHz, DMSO): δ 13.1, 16.6, 19.3, 22.7, 22.4, 22.6, 24.4, 30.8, 31.2, 31.9, 37.3, 42.8, 43.9, 45.4, 48.6, 48.9, 49.6, 50.2, 57.2, 61.7, 71.8, 119.0, 116.8, 119.3, 120.6, 122.8, 129.5, 130.3, 133.8, 136.4, 138.8, 140.3, 147.3, 164.2; MS: *m*/*e* = 509 (M^+); Anal. Calcd. for $C_{29}H_{39}N_3O_3S$: C, 68.34; H, 7.71; N, 8.24; S, 6.29. Found C, 68.41; H, 7.65; N, 8.38; S, 6.44.

*2.1.32. Ethyl 6-amino-3-(3β-hydroxy-androst-5-ene-17-yl)-4-hydroxythieno[2,3-b]pyridine-5-carboxylate (**15d**)*

HPLC purity = 97.0% (C-18 NovaPak column; MeOH:H_2O/90:10), t_r = 24 min; pale yellow crystals from ethanol (66%), m.p. 188–191 °C; IR (KBr) cm^{-1}: 3496–4310, 3010, 2925, 1650, 1636, 1418, 1040; ^1H NMR (DMSO): δ 0.61 (s, 3H), 1.02 (s, 3H), 1.12 (t, 8.52 Hz, 3H), 1.60–1.86 (m, 6H), 2.22–2.36 (m, 3H), 2.86 (t, 1H); 3.56 (m, 1H); 4.21 (s, 2H), 4.23 (q, 2H), 4.69 (s, 2H), 5.35 (s, 1H), 6.81 (d, *J* = 16.07 Hz, 1H), 6.93 (s, 1H), 10.2 (s, 1H); ^{13}C NMR (500 MHz, DMSO): δ 13.1, 16.6, 19.3, 22.7, 22.4, 22.6, 24.4, 30.8, 31.2, 31.9, 37.3, 42.8, 43.9, 45.6, 48.6, 48.9, 49.6, 50.1, 57.2, 61.7, 71.6, 119.3, 120.6, 122.8, 130.1, 133.8, 139.5, 143.7, 148.8, 164.3; MS: *m*/*e* = 510 (M^+); Anal. Calcd. for $C_{29}H_{38}N_2O_4S$: C, 68.20; H, 7.50; N, 5.49; S, 6.28. Found C, 68.46; H, 7.81; N, 5.79; S, 6.51.

*2.1.33. General procedure for synthesis of the 3-oxobutanamido)thiophene derivatives **16a,b***

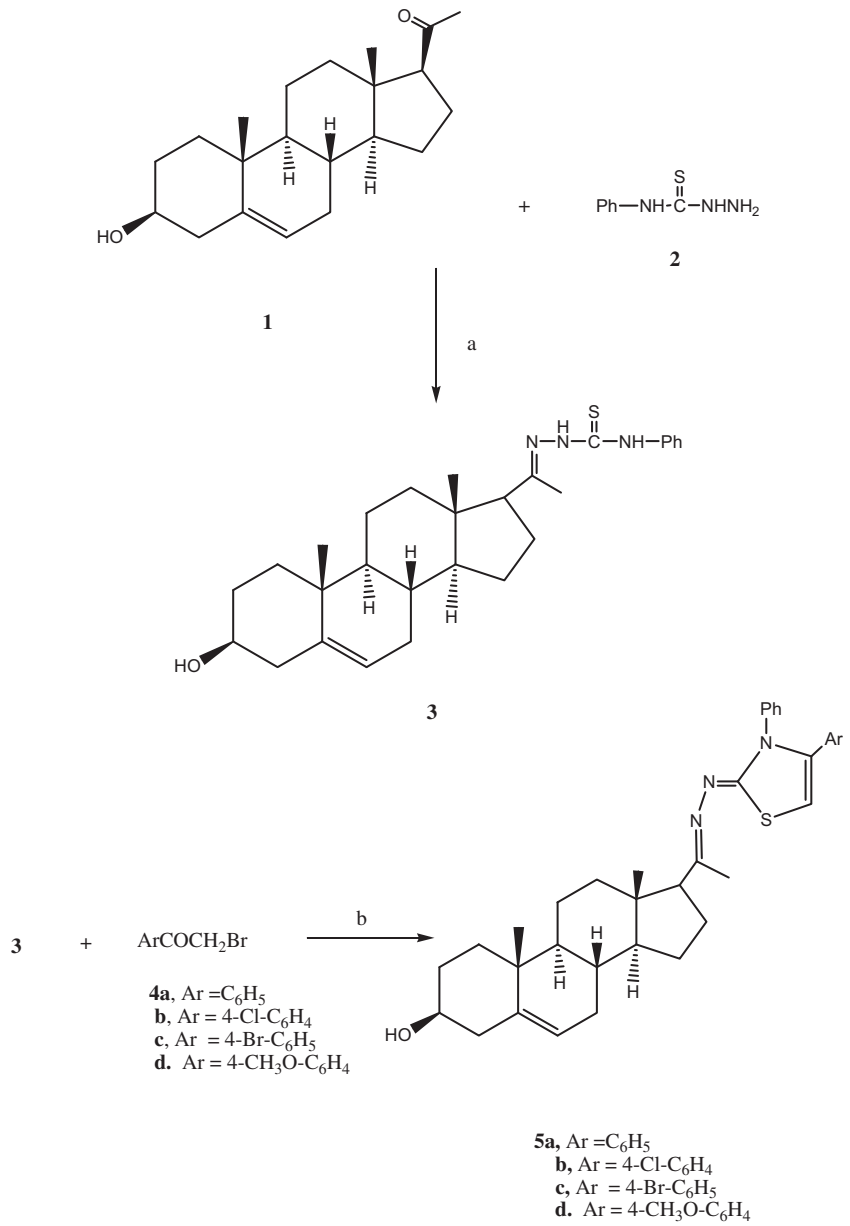
To a dry solid of either compound **12a** (0.396, 1 mmol) or **12b** (0.443 g, 1 mmol) ethyl acetoacetate (0.130 g, 1 mmol) was added. The reaction mixture was heated in an oil bath at 120 °C for 8 h then was left to cool and the solidified product was triturated with diethyl ether and the formed solid product, in each case, was collected by filtration.

*2.1.34. 4-(3β-Hydroxy-androst-5-ene-17-yl)-2-(3-oxobutanamido)thiophene-3-carbonitrile (**16a**)*

HPLC purity = 98.4% (C-18 NovaPak column; MeCN:MeOH:H_2O/65:25:10), t_r = 18 min; pale yellow crystals from ethanol (55%), m.p. >300 °C; IR (KBr) cm^{-1}: 3459–4312, 3039, 2924, 2222, 1688, 1683, 1658, 1633, 1418, 1040; ^1H NMR (DMSO): δ 0.60 (s, 3H), 1.01 (s, 3H), 1.62–1.84 (m, 6H), 2.02 (s. 2H), 2.23–2.36 (m, 3H), 2.88 (t, *J* = 7.83 Hz, 1H), 3.54 (m, 1H), 3.88 (s, 2H), 4.24 (s, 2H), 4.68 (s, 2H), 5.38 (s, 1H), 6.66 (d, *J* = 15.78 Hz, 1H), 6.88 (s, 1H), 8.30 (s, 1H); ^{13}C NMR (500 MHz, DMSO): δ 13.0, 19.7, 22.5, 22.4, 22.6, 24.4, 29.6, 30.8, 31.0, 31.4, 37.3, 43.7, 45.4, 48.4, 48.8, 49.6, 50.3, 50.9, 57.2, 61.7, 71.3, 116.6, 119.3, 121.3, 133.6, 136.2, 142.3, 149.2, 164.7, 169.1; MS: *m*/*e* = 480 (M^+); Anal. Calcd. for $C_{28}H_{36}N_2O_3S$: C, 69.97; H, 7.55; N, 5.83; S, 6.67. Found C, 70.11; H, 7.42; N, 5.63; S, 6.82.

*2.1.35. Ethyl 4-(3β-hydroxy-androst-5-ene-17-yl)-2-(3-oxobutanamido)thiophene-3-carboxylate (**16b**)*

HPLC purity = 99.1% (C-18 NovaPak column; MeCN:MeOH:H_2O/70:20:10), t_r = 21 min; pale yellow crystals from ethanol (55%),



Scheme 1. Synthesis of pregnenolone derivatives. Reagents and conditions: (a) ethanol, 60 °C, (b) ethanol, reflux 2 h.

m.p. 231–233 °C; IR (KBr) cm^{-1}: 3473–4352, 3030, 2921, 1688, 1683–1680, 1658, 1633, 1418, 1042; ^1H NMR (DMSO): δ 0.64 (s, 3H), 1.01 (s, 3H), 1.12 (t, J = 6.88 Hz, 3H), 1.59–1.86 (m, 6H), 2.01 (s. 2H), 2.20–2.38 (m, 3H), 2.92 (t, J = 8.04 Hz, 1H), 3.56 (m, 1H), 3.88 (s, 2H), 4.21 (q, J = 6.88 Hz, 2H), 4.24 (s, 2H), 4.68 (s, 2H), 5.33 (s, 1H), 6.63 (d, J = 16.11 Hz, 1H), 6.86 (s, 1H), 8.33 (s, 1H); ^{13}C NMR (500 MHz, DMSO): δ 13.0, 16.3, 19.7, 22.7, 22.4, 22.8, 24.4, 29.8, 30.6, 31.2, 31.6, 37.3, 42.0, 43.7, 45.4, 48.4, 48.8, 49.6, 50.3, 50.9, 57.2, 61.7, 71.3, 119.3, 121.8, 133.6, 136.2, 143.2, 149.8, 163.8, 164.5, 189.4; MS: m/e = 527 (M^+); Anal. Calcd. for $C_{30}H_{41}NO_5S$: C, 68.28; H, 7.83; N, 2.65; S, 6.08. Found C, 68.50; H, 7.55; N, 2.91; S, 5.83.

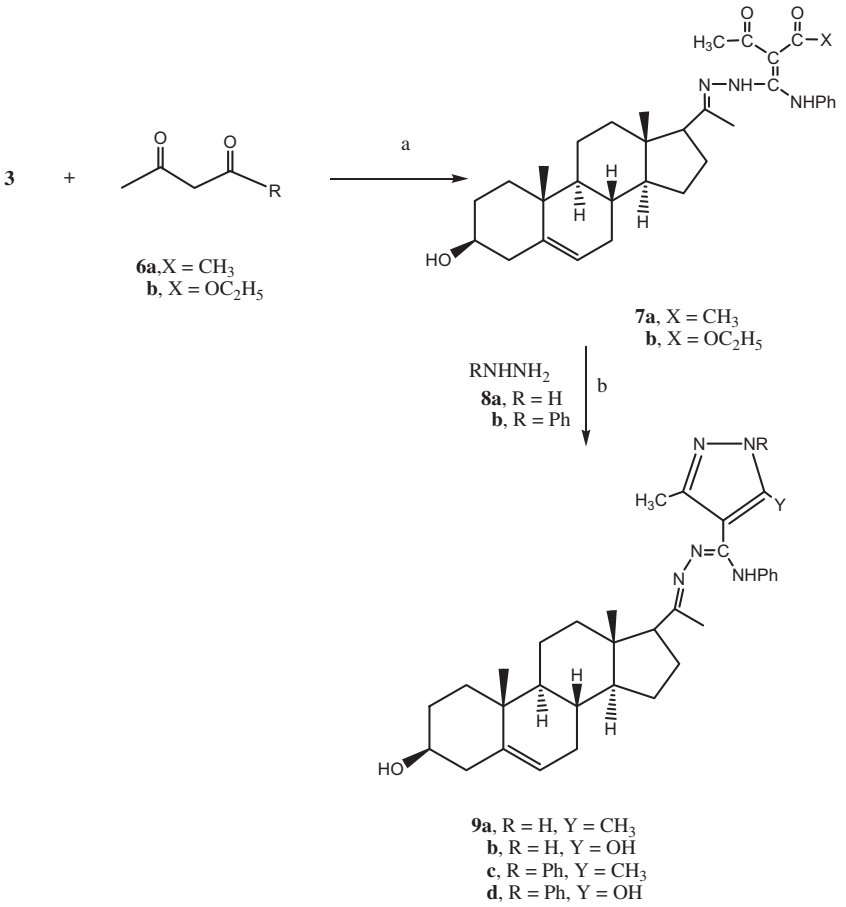
2.2. In vitro cytotoxic assay

2.2.1. Chemicals

Fetal bovine serum (FBS) and L-glutamine, were purchased from Gibco Invitrogen Co. (Scotland, UK). RPMI-1640 medium was purchased from Cambrex (New Jersey, USA). Dimethyl sulfoxide (DMSO), doxorubicin, penicillin, streptomycin and sulforhodamine B (SRB) were purchased from Sigma Chemical Co. (Saint Louis, USA).

2.2.2. Cell cultures

Three human tumor cell lines, MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), and SF-268 (CNS cancer) were used. MCF-7 was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and NCI-H460 and SF-268 were kindly provided by the National Cancer Institute (NCI, Cairo, Egypt). They grow as monolayer and routinely maintained in RPMI-1640 medium supplemented with 5% heat inactivated FBS, 2 mM glutamine and antibiotics (penicillin 100 U/mL, streptomycin 100 μg/mL), at 37 °C in a humidified atmosphere containing 5% CO_2. Exponentially growing cells were obtained by plating 1.5×10^5 cells/mL for MCF-7 and SF-268 and 0.75×10^4 cells/mL for NCI-H460, followed by 24 h of incubation. The effect of the



Scheme 2. (a) 1,4-Dioxan, water bath, 12 h; (b) ethanol, reflux 1 h.

vehicle solvent (DMSO) on the growth of these cell lines was evaluated in all the experiments by exposing untreated control cells to the maximum concentration (0.5%) of DMSO used in each assay.

2.2.3. Tumor cell growth assay

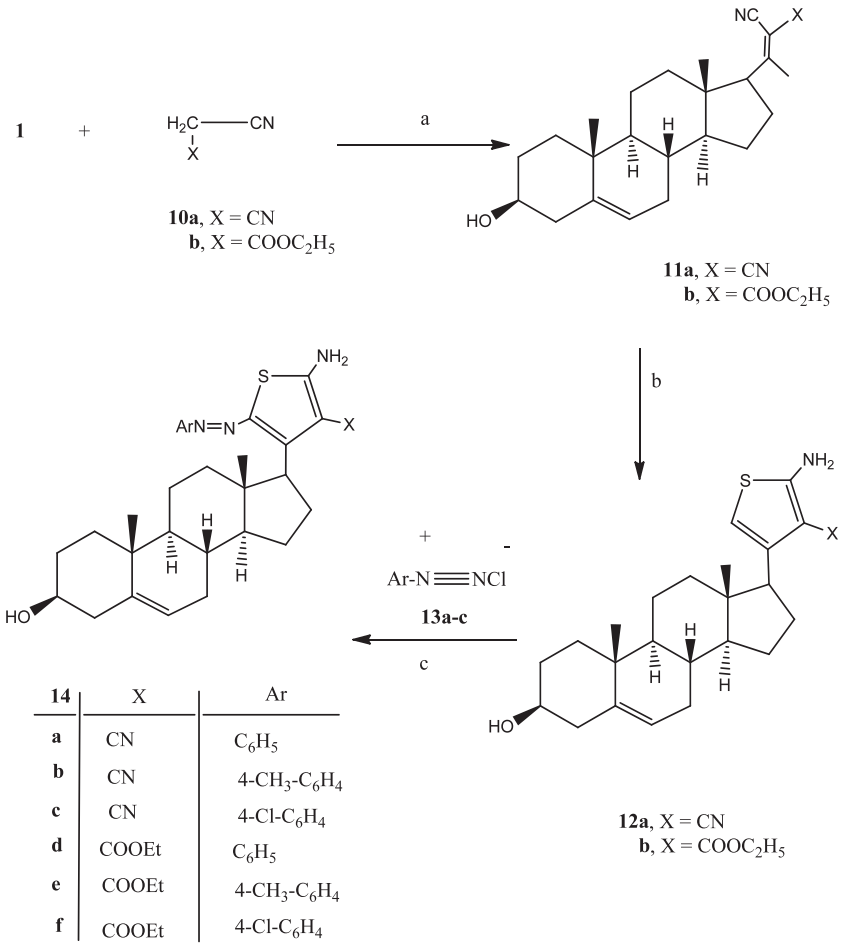
The effects of compounds **3–16a–b** on the *in vitro* growth of human tumor cell lines were evaluated according to the procedure adopted by the National Cancer Institute (NCI, USA) in the '*In vitro* Anticancer Drug Discovery Screen' that uses the protein-binding dye sulforhodamine B to assess cell growth [34]. Briefly, exponentially, cells growing in 96-well plates were then exposed for 48 h to five serial concentrations of each compound, starting from a maximum concentration of 150 μM. Following this exposure period adherent cells were fixed, washed, and stained. The bound stain was solubilized and the absorbance was measured at 492 nm in a plate reader (Bio-Tek Instruments Inc., Powerwave XS, Wincoski, USA). For each test compound and cell line, a dose–response curve was obtained and the growth inhibition of 50% (GI_{50}), corresponding to the concentration of the compounds that inhibited 50% of the net cell growth, was calculated as described elsewhere [35]. Doxorubicin was used as a positive control and tested in the same manner.

3. Results and discussion

3.1. Chemistry

The reaction of pregnenolone (**1**) with 1-phenyl-3-thiosemicarbazide gave the 20-thiosemicarbazonopregnenolone derivative **3**. The ^{1}H NMR and ^{13}C NMR spectra of **3** were the basis of its structure elucidation. The ^{1}H NMR spectrum showed beside the expected signals for pregnenolone moiety a multiplet at δ 7.29–7.37 indicating the phenyl protons and two singlets (D_2O exchangeable) at δ 8.22, 8.30 ppm indicating the NH groups. Moreover, the ^{13}C NMR spectrum showed beside the characteristic signal for the C_6H_5 group, two signals at δ 168.3 and 180.3 indicating the C=N and C=S groups, respectively. Compound **3** reacted with the ω-bromoacetophenone derivatives to give the 20-thiazolyl hydrazonopregnenolone derivatives **5a–d** (Scheme 1), their structures were based on analytical and spectral data (see Section 2). On the other hand, the reaction of compound **3** with either acetylacetone (**6a**) or ethylacetoacetate (**6b**) in ethanol solution under reflux gave the condensated products **7a** and **7b**, respectively. The latter products were good candidates for pyrazolyl steroids formation through their respective reactions with hydrazines. Thus, the reaction of either **7a** or **7b** with either hydrazine hydrate (**8a**) or phenylhydrazine (**8b**) gave the pyrazolyl semicarbazidopregnenolone derivatives **9a–d**, respectively (Scheme 2). The analytical and spectral data of such compounds are in analogy with their respective structures. Thus the ^{1}H NMR spectrum of **9a** showed two singlets at δ 2.65, 2.72 indicating the two CH_3 groups, a multiplet at δ 7.24–7.36 corresponding to the phenyl protons and two singlets (D_2O exchangeable) at δ 8.05, 8.31 ppm indicating the two NH groups. Moreover, the ^{13}C NMR spectrum showed δ at 22.9, 24.6 indicating the two CH_3 groups and two signals at δ 164.6 and 172.6 indicating the two C=N groups.

Next we studied the reaction of the acetyl group present in pregnenolone (**1**) toward active methylene reagents in the aim of formation of condensated products followed by their heterocyclization



14	X	Ar
a	CN	C_6H_5
b	CN	4-CH_3-C_6H_4
c	CN	4-Cl-C_6H_4
d	COOEt	C_6H_5
e	COOEt	4-CH_3-C_6H_4
f	COOEt	4-Cl-C_6H_4

Scheme 3. (a) NH_4Ac, water bath, 8 h; (b) 1,4-dioxan, Et_3N, S_8, reflux 30 min; (c) ethanol, NaOAc, 0–5 °C.

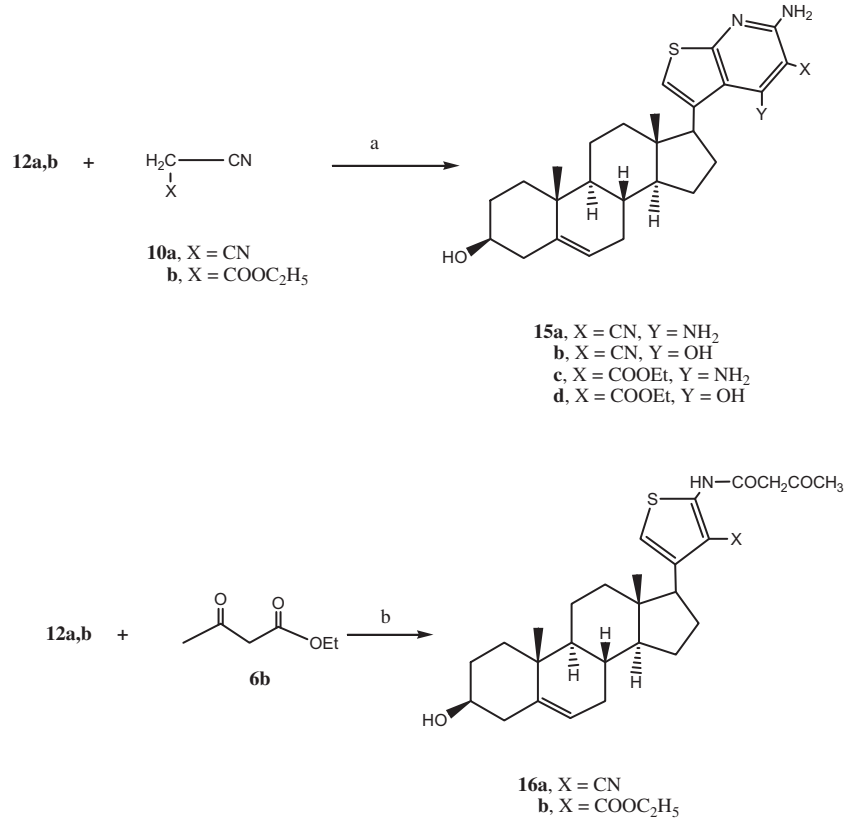
into thiophene derivatives in a similar way to the reported Gewald's thiophene synthesis [36–39]. Thus, the reaction of 1 with either malononitrile (**10a**) or ethyl cyanoacetate (**10b**) in dry ammonium acetate at 120 °C gave the Knoevenagel condensated products **11a** and **11b**, respectively [40,41]. The reaction of either **11a** or **11b** with sulfur in 1,4-dioxan solution containing a catalytic amount of triethylamine gave the 17-thiophenyl androstene derivatives **12a** and **12b**, respectively. The ^{1}H NMR and ^{13}C NMR spectra were the basis of their structures elucidation. Thus, the ^{1}H NMR spectrum of **12a** showed the presence of a singlet at δ 4.77 ppm indicating the presence of NH_2 group and a singlet at δ 6.99 ppm corresponding to the thiophene H-5. Moreover, the ^{13}C NMR spectrum showed the presence of a CN group at δ 116.5 and the characteristic signals of the thiophene C at δ 138.9, 140.3, and 144.8.

Encouraged by the excellent yields for **12a** and **12b** a series of novel arylhydrazono derivatives were obtained with the aim of improving the inhibitory effect against the tested cancer cell lines. Moreover, in order to establish a relationship between the structure and activity for this type of compounds we have studied the affects of substitution on the aryl azo compounds as well as the effect of CN group in case of **12a** and the ester group in case of **12b**. Thus, the reaction of either **12a** or **12b** with either of the aryldiazonium salts namely the benzenediazonium chloride **13a**, the 4-methylphenyldiazonium salt **13b** or the 4-chlorophenyl diazonium salts **13c** in 0–5 °C gave the aryl hydrazono derivatives **14a-f**, respectively (Scheme 3). On the other hand, the reaction of either **12a** or **12b** with either malononitrile (**10a**) or ethyl cyanoacetate (**10b**) gave the 17-thieno[2,3-*b*]pyridinyl androstene derivatives **15a–d**, respectively. Finally, the reaction of either **12a** or **12b** with ethyl acetoacetate (**6b**) gave the 2-oxobutanamidothiophenyl androstene derivatives **16a** and **16b**, respectively (Scheme 4).

Most of compounds synthesized in this work could exist in two stereoisomeric forms E or Z. Trials to obtain pure isomers were carried out by flash chromatography of crude compounds (0.5 mmol) in hexane/acetone mixture (3:1). Thin layer chromatography examination of the reaction mixture revealed minor products probably due to the presence of other isomers. In all experiments our compounds were recovered from the low mobility phases and thus considered to be in the (E)-isomeric forms [42]. The extracts of the low chromatographic mobility for compounds **3**, **5a–d**, **7a,b**, **9a–d** and **11b** which are the (E) isomers showed high purity (higher than 95%) toward HPLC by using an ultraviolet detector (215 nm). It is important to note that the presence of the diene moiety in the azole derivatives **5a–d** and **9a–d**, orient such compounds toward the more stable transforms.

3.2. In vitro evaluation of cytotoxic activity

The effect of compounds **3–16a,b** was evaluated on the *in vitro* growth of three human tumor cell lines representing different tumor types, namely, breast adenocarcinoma (MCF-7), non-small cell lung cancer (NCI-H460) and CNS cancer (SF-268) after a continuous exposure for 48 h. The results are summarized in Table 1. All of the tested compounds were able to inhibit the growth of the tested human tumor cell lines in a dose-dependent manner. The results indicated through Table 1 revealed that compounds **5b**, **5c**, **9b**, **9d**, **11a**, **12a, 14c**, **14f**, **15b**, **15d**, **16a** and **16b** showed the highest inhibitory effect against all the three tumor cell lines.



Scheme 4. (a) 1,4-Dioxan, Et_3N, reflux 2 h; (b) oil bath, 120 °C, 8 h.

Table 1
Effect of compounds **3**–**16a**,**b** on the growth of three human tumor cell lines.

Compound	GI_{50} (μ mol L^{-1})		
	MCF-7	NCI-H460	SF-268
3	4.1 ± 0.6	8.3 ± 1.4	6.3 ± 1.5
5a	6.6 ± 0.4	8.3 ± 0.8	4.1 ± 0.8
5b	0.6 ± 0.4	0.2 ± 0.08	0.8 ± 0 .4
5c	0.2 ± 0.2	0.6 ± 0.02	0.4 ± 0.08
5d	4.6 ± 2.9	6.9 ± 1.8	2.8 ± 1.6
7a	22.7 ± 17.5	12.2 ± 4.8	16.0 ± 4.3
7b	8.1 ± 0.6	4.5 ± 0.8	6.7 ± 1.6
9a	50.1 ± 0.7	23.2 ± 4.8	18.4 ± 1.8
9b	0.01 ± 0.002	0.06 ± 0.004	0.04 ± 0.006
9c	38.0 ± 1.8	44.0 ± 0.8	20.5 ± 1.1
9d	0.02 ± 0.006	0.01 ± 0.004	0.03 ± 0.001
11a	0.01 ± 0.006	0.01 ± 0.006	0.03 ± 0.005
11b	11.9 ± 0.9	12.6 ± 1.8	10.8 ± 0.8
12a	0.02 ± 0.007	0.03 ± 4.8	0.04 ± 0.008
12b	4.1 ± 0.7	2.2 ± 0.8	2.4 ± 0.6
14a	8.1 ± 0.7	6.2 ± 2.8	4.4 ± 2.8
14b	10.1 ± 0.7	18.2 ± 4.8	12.4 ± 1.8
14c	0.01 ± 0.007	0.02 ± 0.008	0.04 ± 0.08
14d	14.1 ± 0.7	12.2 ± 4.8	10.4 ± 1.8
14e	22.1 ± 2.3	20.2 ± 4.8	22.4 ± 2.8
14f	0.03 ± 0.002	0.02 ± 0.004	0.02 ± 0.001
15a	30.1 ± 2.6	33.2 ± 4.6	22.4 ± 4.4
15b	2.1 ± 0.4	2.2 ± 0.9	1.4 ± 0.6
15c	50.1 ± 12.4	23.2 ± 4.8	18.4 ± 1.6
15d	2.1 ± 0.3	1.2 ± 0.2	2.4 ± 0.4
16a	0.01 ± 0.003	0.01 ± 0.002	0.04 ± 0.005
16b	2.1 ± 0.4	4.2 ± 4.8	2.4 ± 0.6
Doxorubicin	0.04 ± 0.008	0.09 ± 0.008	0.09 ± 0.007

Results are given in concentrations that were able to cause 50% of cell growth inhibition (GI_{50}) after a continuous exposure of 48 h and show means ± SEM of three-independent experiments performed in duplicate.

Compounds **9b, 9d, 11a, 12a**, **14c**, **14f** and **16a** showed high inhibitory effects against non-small cell lung cancer (NCI-H460) and breast adenocarcinoma (MCF-7), which are much higher than the reference doxorubicin. Compounds **9a**, **9c**, **15a** and **15c** showed the lowest inhibitory effect. The rest of the compounds showed a moderate growth inhibitory effect.

3.3. Structure–activity relationship

Comparing the cytotoxicity of 20-thiazolyl hydrazonopregnenolone derivatives **5a**, **5b**, **5c** and **5d**, it is obvious that the cytotoxicity of compounds **5b** and **5c** are much higher than those of compounds **5a** and **5b** as the *p*-chloro group in **5b** and the *p*-bromo group in **5c** are responsible for the increasing cytotoxicity toward the three cell lines. On the other hand the cytotoxicity of compound **7b** is higher than that of **7a** the reason is due to the presence of COOEt group in **7b**. Comparing the cytotoxicity of the pyrazolyl semicarbazidopregnenolone derivatives **9a**, **9b**, **9c** and **9d**, it is obvious that compounds **9b** and **9d** showed high inhibitory effects through the three cancer cell lines which are higher than those of compounds **9a** and **9c**. The presence of the electron withdrawing OH group in **9b** and **9d** is responsible for such reactivity. Similarly, the presence of the CN group in compounds **11a, 12a** and **16a** is responsible for the high cytotoxicity activity toward the three cancer cell lines while compounds **11b**, **12b** and **16b** with the COOEt group showed lower cytotoxicity.

Comparing the arylazo derivatives **14a–f** it is clear that the *p*-chloro group present in compounds **14c** and **14f** is responsible for their high cytotoxicity among the six compounds. Considering the cytotoxicity of the thieno[2,3-*b*]pyridinyl androstene derivatives **15a–d**, it is clear that the presence of the OH group in compounds **15b** and **15d** is responsible for such high activity.

4. Conclusion

The objective of the present study was to synthesize a series of heterocyclic derivatives of pregnenolone. The cytotoxicity of the newly synthesized products against three cancer cell lines were tested and the results showed that compounds **9b, 9d, 11a, 12a**, **14c**, **14f** and **16a** showed high inhibitory effects against non-small cell lung cancer (NCI-H460) and breast adenocarcinoma (MCF-7), which are much higher than the reference doxorubicin.

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