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Steroidal pyrazolines and pyrazoles as potential 5α-reductase inhibitors: Synthesis and biological evaluation

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ABSTRACT

Taking pregnenolone as the starting material, two series of pyrazolinyl and pyrazolyl pregnenolones were synthesized through different routes. The synthesis of the analogs of both series is multistep and proceeds in good overall yields. While the key step in the synthesis of pyrazolinyl pregnenolones is the heterocyclization of benzylidine derivatives (**3**) in presence of hydrazine hydrate, it is the condensation of 3β-hydroxy-21-hydroxymethylidenepregn-5-en-3β-ol-20-one (**5**) with phenylhydrazine in the synthesis of pyrazolyl derivatives. Compounds of both the series were tested for their 5α-reductase inhibitory activities. Amongst all the compounds screened for their 5α-reductase inhibitory activities, compound **4b**, **4c** and **6b** were found to be the most active.

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1. Introduction

Steroids represent a pharmacologically active class of molecules associated with variety of physiological functions. Steroidal derivatives in which ring D is modified with heterocyclic rings have been of great pharmaceutical interest [1]. Steroids as well as their derivatives have the potential to be developed as drugs for the treatment of a large number of diseases including cardiovascular [2], autoimmune diseases [3], brain tumors, breast cancer, prostate cancer, osteoarthritis, etc. [4]. The promise of using steroids for development of lead molecules lies in the regulation of a variety of biological processes by these molecules and being a fundamental class of signaling molecules [5]. Though steroids and steroid based molecules have been used as active pharmaceutical agents against various diseases, there has recently been a surge in the exploitation of these molecules against cancer. Unfortunately despite the recent advances in the early diagnosis, prevention and therapy, cancer still remains a challenge as it affects millions of people world over and is one of the leading causes of death [6,7]. It thus necessitates the development of new drugs against this dreadful disease which remains the primary focus of various research groups throughout the world. Emerging new molecularly defined targets, such as 5α-reductase (5AR), are being actively explored for treatment of cancer.

The enzyme steroid 5α-reductase (5AR) catalyses the NADPH-dependant reductive conversion of testosterone to dehydrotestosterone. The higher 5AR activity leads to increased levels of dihydrotestosterone in the peripheral tissues, which is implicated in the pathogenesis of prostate cancer, acne and male pattern baldness [8]. However the deficiency of 5AR in males results in an incomplete differentiation of external genitalia at birth [9]. It has been established that there are two genes encoding two distinct isozymes of 5AR that are differentially expressed in human tissues and are referred to as type I 5AR (5AR1) and type II 5AR (5AR2) [10]. While the former is expressed predominantly in the skin and liver, the later is expressed mainly in prostate, seminal vesicles, liver and epididymis [11]. A number of steroidal [12] and non-steroidal [13] inhibitors have been tested against 5AR. The two most important steroid based 5AR inhibitors are Finasteride (PROSCAR, Merck) and dutasteride (Avodart, GlaxoSmithKline). Finasteride, a type II-selective inhibitor, was the first 5AR inhibitor approved in the United States for the treatment of prostate cancer and benign prostatic hyperplasia (BPH). Dutasteride has no selectivity and acts as an inhibitor against type I and type II 5AR. However both the approved drugs suffer from serious side effects such as erectile dysfunction, abnormal ejaculation, impotence, abnormal sexual function, decreased sexual desire, gynecomastia etc. [14]. Recent literature precedents indicate various steroidal

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D-ring heterocyclic derivatives as inhibitors of 5AR and 17α-hydroxylase/C_{17-20}-lyase of which 17-imidazolyl, pyrazolyl, pyrazolinyl, isoxazolyl, oxazolyl and thiazolyl derivatives are very potent [24]. Taking inputs from these literature precedents to obtain the skeleton structure required for 5AR inhibitory activity, we, in continuation of our research program directed towards the development of steroid based lead molecules [15], designed synthesis of two series of novel pyrazolinyl and pyrazolyl analogs from pregnenolone. Further, as it is well established that 17β-heterocycles are more active towards the 5AR inhibition [24], we also designed the heterocycles on the same lines with β-orientation at the 17-position. All the synthesized pregnenolone derivatives were evaluated for their 5AR inhibitory activity. It was observed that compounds **4b**, **4c** and **6b** exhibit excellent activities and are the most potent of all the screened compounds.

2. Experimental

2.1. General methods

Melting points were recorded on Buchi melting point apparatus D-545; IR spectra (KBr discs) were recorded on Bruker Vector 22 instrument. NMR spectra were recorded on Bruker DPX200 instrument in $CDCl_3$ with TMS as internal standard for protons and solvent signals as internal standard for carbon spectra. Chemical shift values are mentioned in δ (ppm) and coupling constants are given in Hz. Mass spectra were recorded on EIMS (Shimadzu) and ESI-esquire 3000 Bruker Daltonics instrument. 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 chromatograms were visualized under UV 254–366 nm and iodine.

2.2. Chemical synthesis

2.2.1. General procedure for the synthesis of pyrazoline derivatives (*4a*–*4j*)

To a solution of pregnenolone 1 (0.316 g, 1 mmol, 1 eq.) in ethanol (10 ml) was added a conc. aq. solution of KOH (2 eq.). Then aldehyde 2 (1.2 eq.) was charged into the reaction mixture to get the corresponding benzylidine derivative 3. After completion, the reaction mixture was precipitated with water. The precipitate was filtered, dried and recrystallized from EtOAc:Hexane to give product as solid white powder. It is to be mentioned that when non-aromatic aldehydes were used, the product was formed in a very minor quantity and that too not stable enough at ambient conditions. Thus the study was restricted to the use of aromatic aldehydes only. The condensation product 3 (1.0 g, 2.4 mmol) was refluxed in ethanol in the presence of hydrazine hydrate (0.24 g, 4.8 mmol) so as to yield the desired pyrazolines. However the products thus obtained were very unstable and they decomposed even at ambient temperature conditions probably because of the inherent instability associated with pyrazolines. The solvent thus used was replaced by acetic acid so as to ensure the formation of N-acetyl pyrazoline **4** (0.99 g, 2.2 mmol, 90%) which was highly stable. The product was precipitated by charging the reaction mass into excessive amounts of ice-cold water. After filtration under suction, the product was obtained in high yields as colorless powder which was later dried in vaccuo. However we observed a diastereomeric mixture of pyrazolines which was separated through the method discussed in the experimental section to yield the desired isomer. The same procedure was followed for the synthesis of all other analogs. Spectral data of various compounds is given as under (Most of the peaks due to steroidal skelton were merged and could not be differentiated. Thus δ values of only those peaks that distinguish the product and could easily be differentiated are reported).

*2.2.1.1. 1-(4,5-Dihydro-3-((10R,13S)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3-hydroxy-10,13-dimethyl-1H-cyclopenta[a]phenanthren-17-yl)-5-phenylpyrazol-1-yl) ethanone (**4a**).* Colourless solid powder. Yield 76%. M.p: 194–197 °C. $[\alpha]_D^{25}$ −85.9 (*c* 1 in $CHCl_3$). IR (KBr, cm^{-1}): 3384, 2926, 1717, 1646, 1404, 1042, 699. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.67 (s, 3H), 1.06 (s, 3H), 1.82–1.90 (m, 6H), 2.17 (s, 3H), 2.65 (t, 1H, *J* = 8.8), 2.79 (m, 2H), 3.26 (m, 1H), 3.49 (m, 1H), 5.33 (s, 1H), 5.44 (m, 1H), 7.15 (d, 2H, *J* = 6.5), 7.22–7.32 (m, 3H). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 14.85, 20.85, 22.40, 23.32, 25.83, 31.13, 33.04, 33.48, 37.99, 38.72, 39.94, 43.68, 45.30, 47.69, 51.53, 53.18, 57.94, 60.56, 73.11, 122.80, 126.78, 128.84, 130.28, 142.29, 160.63, 164.62. ESI-MS: 483 (M^++Na). Anal. Calcd. for $C_{30}H_{40}N_2O_2$: C, 78.22; H, 8.75; N, 6.08; found C, 78.47; H, 8.83; N, 6.21.

*2.2.1.2. 1-(5-(3-Fluorophenyl)-4,5-dihydro-3-((10R,13S)2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3-hydroxy-10,13-dimethyl-1H-cyclopenta[a]phenanthren-17-yl) pyrazol-1-yl)ethanone (**4b**).* Colourless solid. Yield 79%. M.p: 168–171 °C. $[\alpha]_D^{25}$ −40.2 (*c* 1 in $CHCl_3$). IR (KBr, cm^{-1}): 3408, 2936, 1718, 1448, 1021, 756. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.67 (s, 3 H), 1.06 (s, 3H), 1.82–1.90 (m, 6H), 2.15 (s, 3H), 2.67 (t, 1H, *J* = 8.8), 2.77 (m, 2H), 3.26 (m, 1H), 3.35 (m, 1H), 5.30 (s, 1H), 5.39 (m, 1H), 6.80–6.91 (m, 2H), 7.30–7.34 (m, 2H). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 12.24, 18.40, 19.95, 20.82, 23.58, 28.60, 30.59, 31.03, 35.54, 36.27, 37.38, 41.23, 45.07, 50.69, 55.38, 57.66, 70.64, 111.11, 113.16, 120.30, 129.37,139.86, 143.79, 159.69, 164.58, 167.71. ESI-MS: 479 (M^++H). Anal. Calcd. for $C_{30}H_{39}FN_2O_2$: C, 75.28; H, 8.21; N, 5.85; found C, 75.43; H, 8.03; N, 6.04.

*2.2.1.3. 1-(5-(4-Fluorophenyl)-4,5-dihydro-3-(10R,13S)2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3-hydroxy-10,13-dimethyl-1H-cyclopenta[a]phenanthren-17-yl) pyrazol-1-yl)ethanone (**4c**).* Colourless solid powder. Yield 82%. M.p: 222–225 °C. $[\alpha]_D^{25}$ −58.0 (*c* 1 in $CHCl_3$). IR (KBr, cm^{-1}): 3375, 3166, 2928, 1721, 1404, 1042, 756. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.65 (s, 3 H), 1.05 (s, 3H), 1.82–1.90 (m, 6H), 2.17 (s, 3H), 2.76 (m, 2H), 3.26 (m,1H), 3.35 (m, 1H), 5.35 (s, 1H), 5.37 (m, 1H), 7.04 (d, 2H, *J* = 8.6), 7.14 (d, 2H, *J* = 8.4). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 12.34, 19.40, 19.65, 20.82, 23.58, 28.60, 30.59, 31.03, 35.54, 36.27, 38.38, 41.23, 42.91, 45.07, 51.69, 56.38, 57.66, 70.64, 111.11, 113.16, 120.30, 129.37,139.86, 143.79, 159.15, 162.71, 164.56, 167.71. ESI-MS: 479 (M^++H). Anal. Calcd. for $C_{30}H_{39}FN_2O_2$: C, 75.28; H, 8.21; N, 5.85; found C, 75.51; H, 8.05; N, 6.09.

*2.2.1.4. 1-(4,5-Dihydro-3-((10R,13S)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3-hydroxy-10,13-dimethyl-1H-cyclopenta[a]phenanthren-17-yl)-5-p-tolylpyrazol-1-yl)ethanone (**4d**).* Greyish powder. Yield 79%. M.p: 230–233 °C. $[\alpha]_D^{25}$ −33.3 (*c* 1 in $CHCl_3$). IR (KBr, cm^{-1}): 3416, 2936, 1719, 1642, 1455, 1087, 756. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.61 (s, 3 H), 1.08 (s, 3H), 1.82–1.92 (m, 6H), 2.05 (s, 3H), 2.22 (s, 3H), 2.67 (t, 1H, *J* = 8.8), 2.77 (m, 2H), 3.40 (m, 1H), 3.48 (m, 1H), 5.36 (m, 2H), 7.80 (d, 4H, *J* = 6.3). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 12.87, 20.54, 22.46, 23.38, 25.83, 31.13, 33.04, 33.19, 33.48, 37.99, 38.72, 39.94, 43.68, 45.30, 47.38, 47.69, 51.53, 53.18, 57.94, 60.56, 73.11, 122.80, 126.78, 128.84, 130.28, 142.29, 161.65, 162.53. ESI-MS: 497 (M^++Na). Anal. Calcd. for $C_{31}H_{42}N_2O_2$: C, 78.44; H, 8.92; N, 5.90; found C, 78.67; H, 8.73; N, 6.13.

*2.2.1.5. 1-(4,5-Dihydro-3-((10R,13S)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3-hydroxy-10,13-dimethyl-1H-cyclopenta[a]phenanthren-17-yl)-5-o-tolylpyrazol-1-yl) ethanone (**4e**).* Colourless

solid. Yield 83%. M.p: 224–226 °C. $[\alpha]_D^{25}$ −63.4 (*c* 1 in $CHCl_3$). IR (KBr, cm^{-1}): 3406, 2941, 1729, 1642, 1455, 1077, 753. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.61 (s, 3 H), 1.09 (s, 3H), 1.84–1.93 (m, 6H), 2.10 (s, 3H), 2.66 (t, 1H, *J* = 8.8), 2.74 (m, 2H), 3.40 (m, 1H), 3.44 (m, 1H), 5.22 (s, 1H), 5.29 (m, 1H), 6.84 (m, 1H), 7.06 (m, 3H). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 11.76, 14.85, 20.85, 22.40, 23.32, 25.83, 31.13, 33.04, 33.19, 33.48, 37.99, 36.72, 39.84, 43.68, 45.80, 47.35, 47.69, 51.53, 53.18, 57.94, 60.74, 73.11, 123.80, 126.78, 127.84, 130.28, 145.29, 161.63, 166.62, ESI-MS: 497 (M^++Na). Anal. Calcd. for $C_{31}H_{42}N_2O_2$: C, 78.44; H, 8.92; N, 5.90; found C, 78.27; H, 8.85; N, 6.09.

*2.2.1.6. 1-(4,5-Dihydro-3-((10R,13S)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3-hydroxy-10,13-dimethyl-1H-cyclopenta[a]phenanthren-17-yl)-5-m-tolylpyrazol-1-yl) ethanone (**4f**).* Colourless solid powder. Yield 74%. M.p: 220–222 °C. $[\alpha]_D^{25}$ −25.5 (*c* 1 in $CHCl_3$). IR (KBr, cm^{-1}): 3374, 2939, 1719, 1638, 1404, 1041, 756, 702. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.67 (s, 3 H), 1.03 (s, 3H), 1.81–1.92 (m, 6H), 2.19 (s, 3H), 2.20–2.23 (m, 3H), 3.40 (m, 1H), 3.47 (m, 1H), 5.35 (m, 2H), 7.03 (d, 1H, *J* = 7.19), 7.17 (d, 1H, *J* = 7.19). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 12.35, 20.85, 22.40, 23.32, 25.83, 31.13, 33.04, 33.19, 33.48, 37.99, 39.72, 39.94, 43.68, 45.30, 46.38, 47.69, 51.53, 53.18, 57.94, 60.56, 73.11, 122.80, 126.78, 128.84, 131.35, 143.23, 161.63, 167.62. ESI-MS: 475 (M^++H). Anal. Calcd. for $C_{31}H_{42}N_2O_2$: C, 78.44; H, 8.92; N, 5.90; found C, 78.24; H, 8.84; N, 6.11.

*2.2.1.7. 1-(5-(furan-2-yl)-4,5-dihydro-3-((10R,13S)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3-hydroxy-10,13-dimethyl-1H-cyclopenta[a]phenanthren-17-yl)pyrazol-1-yl)ethanone (**4g**).* Colourless Powder. Yield 82%. M.p: 227–229 °C. $[\alpha]_D^{25}$ −55.8 (*c* 1 in $CHCl_3$). IR (KBr, cm^{-1}): 3386, 2936, 1727, 1647, 1451, 1043, 754. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.67 (s, 3H), 1.02 (s, 3H), 1.81–1.90 (m, 6H), 2.17 (s, 3H), 3.10–3.15 (m, 3H), 3.52 (m, 1H), 5.30 (s,1H), 5.37 (s, 1H), 5.51(m,1H), 6.28 (m, 2H), 7.30 (s,1H). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 13.85, 20.85, 22.90, 22.32, 25.84, 31.13, 33.04, 33.19, 33.48, 37.99, 38.82, 39.94, 44.68, 45.30, 47.38, 48.69, 51.78, 53.18, 57.94, 60.56, 73.11, 122.80, 126.78, 128.84, 130.28, 142.29, 160.61, 164.62. ESI-MS: 473 (M^++Na). Anal. Calcd. for $C_{28}H_{38}N_2O_3$: C, 74.63; H, 8.50; N, 6.22; found C, 74.47; H, 8.71; N, 6.47.

*2.2.1.8. 1-(4,5-Dihydro-3-((10R,13S)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3-hydroxy-10,13-dimethyl-1H-cyclopenta[a]phenanthren-17-yl)-5-(4-methoxyphenyl) pyrazol-1-yl)ethanone (**4h**).* Colourless solid. Yield 84%. M.p: 211–215 °C. $[\alpha]_D^{25}$ −48.4 (*c* 1 in $CHCl_3$). IR (KBr, cm^{-1}): 3406, 2930, 2871, 1719, 1642, 1419, 1041, 757. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.67 (s, 3H), 1.01 (s, 3H), 1.82–1.88 (m, 6H), 2.03 (s, 3H), 2.53–2.73 (m, 2H), 3.20 (m, 1H), 3.29 (m, 1H), 3.76 (s, 3H), 5.34 (m, 2H), 6.82 (d, 2H, *J* = 8.1), 7.05 (d, 2H, *J* = 8.1). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 13.38, 20.85, 22.44, 23.32, 25.83, 31.13, 33.04, 33.19, 34.48, 37.99, 38.72, 39.94, 43.68, 45.30, 47.38, 47.69, 51.55, 53.18, 57.94, 60.56, 73.11, 122.80, 126.78, 127.84, 130.28, 142.29, 160.63, 165.62. ESI-MS: 491 (M^++H). Anal. Calcd. for $C_{31}H_{42}N_2O_3$: C, 75.88; H, 8.63; N, 5.71; found C, 75.63; H, 8.81; N, 5.87.

*2.2.1.9. 1-(4,5-Dihydro-3-((10R,13S)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3-hydroxy-10,13-dimethyl-1H-cyclopenta[a]phenanthren-17-yl)-5-(2-methoxyphenyl) pyrazol-1-yl)ethanone (**4i**).* Colourless solid. Yield 80%. M.p: 211–112 °C. $[\alpha]_D^{25}$ −45.1 (*c* 1 in $CHCl_3$). IR (KBr, cm^{-1}): 3399, 2936, 2871, 1719, 1642, 1419, 1039, 757.^1H NMR ($CDCl_3$, 400 MHz): δ 0.66 (s, 3H), 1.01 (s, 3H), 1.81–1.88 (m, 6H), 2.04 (s, 3H), 2.54–2.73 (m, 2H), 3.20 (m, 1H), 3.29 (m, 1H), 3.83 (s, 3H), 5.35 (m, 2H), 6.85–6.93 (m, 2H), 7.24–7.33 (m, 2H). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 13.67, 20.85, 22.40, 23.32, 25.83, 31.13, 31.04, 33.19, 33.48, 37.99, 38.72, 39.94, 43.68, 45.30, 47.38, 43.69, 51.53, 53.18, 57.94, 60.56, 73.11, 122.80, 126.78, 128.84, 130.28, 142.29, 160.73, 164.62. ESI-MS: 491 (M^++H). Anal. Calcd. for $C_{31}H_{42}N_2O_3$: C, 75.88; H, 8.63; N, 5.71; found C, 75.99; H, 8.41; N, 5.65.

*2.2.1.10. 1-(5-(2-Chlorophenyl)-4,5-dihydro-3-((10R,13S)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3-hydroxy-10,13-dimethyl-1H-cyclopenta[a]phenanthren-17-yl) pyrazol-1-yl)ethanone (**4j**).* Colourless white powder. Yield 79%. M.p: 228–230 °C. $[\alpha]_D^{25}$ −45.6 (*c* 1 in $CHCl_3$). IR ($CHCl_3$, cm^{-1}): 3386, 2944, 1720, 1640, 1492, 1407, 1090, 962, 756. ^1H NMR ($CDCl_3$, 400 MHz): δ 0.67 (s, 3 H), 1.08 (s, 3H), 1.81–1.88 (m, 6H), 2.04 (s, 3H), 2.57–2.65 (m, 2H), 3.20 (m, 1H), 3.50 (m, 1H), 5.34–5.42 (m, 2H), 7.09 (d, 2H, *J* = 8.4), 7.37 (d, 2H, *J* = 8.4). ^{13}C NMR ($CDCl_3$, 400 MHz): δ 12.27, 12.46, 18.40, 19.61, 20.80, 23.36, 23.55, 30.53, 30.72, 31.01, 35.53, 36.25, 37.51, 41.17, 45.11, 49.23, 50.68, 55.46, 57.52, 70.66, 120.33, 125.82, 127.98, 132.14, 139.80, 158.60, 166.63, 167.71, 173.88. ESI-MS: 517.4 (M^++Na). Anal. Calcd. for $C_{30}H_{39}ClN_2O_2$: C, 72.78; H, 7.94, Cl, 7.16; N, 5.66; found C,72.96; H, 8.21; Cl, 7.01; N, 5.79.

2.2.2. General procedure for the synthesis of pyrazolyl pregnenolones

All the pyrazolyl pregnenolone derivatives were prepared as per known literature precedents [16]. The method described by Schneider et al. [16] involves the cyclization reaction of 3β-hydroxy-21-hydroxymethylidenepregn-5-en-20-one **5** [17] with phenylhydrazine or its p-substituted derivatives. Compound **5** (2.07 g, 6 mmol) was suspended in CH_2Cl_2 (50 ml) and phenylhydrazine hydrochloride or one of its p-substituted derivatives (1.1 equivalent) was added to the homogenous mixture. This was followed by the dropwise addition of $BF_3{\cdot}OEt_2$ (50%) (2 mmol, 0.25 ml). The reaction mixture was stirred for 5 h. After the disappearance of the starting material as monitored by TLC, saturated $NaHCO_3$ solution (100 ml) was added and the mixture was stirred until bubbling ceased. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was chromatographed on silica gel starting with CH_2Cl_2/hexane (1:1, v/v) as eluant, followed by CH_2Cl_2/hexane (2:1, v/v) and CH_2Cl_2 to afford the desired pyrazolyl pregnenolone derivatives **6a**–**d**. The original work by Schneider et al. [16] may be consulted for further details and the complete spectral data.

3. Determination of 5α-reductase inhibitory activity

3.1. Type I 5α-reductase inhibitory activity

Human prostate was homogenized in 2 volumes of medium A (20 mM sodium phosphate, pH 6.5 containing 0.32 M sucrose, 0.1 mM dithiothreitol Sigma–Aldrich, Inc.) with a tissue homogenizer. Homogenates were centrifuged at 1500 g for 20 min [18,19] in a SW 60 Ti rotor (Beckman Instruments, Palo Alto, CA). The pellets were separated, suspended in medium A and kept at 70 °C. The suspension, 5 mg of protein/mL for human prostates, determined by the Bradford's Method [20] was used as source of 5α-reductase.

The enzyme 5α-reductase was assayed as previously described [18,19]. The reaction mixture for human prostate contained: 1 mM dithiothreitol, sodium phosphate buffer 40 mM, at pH 6.5, 2 mM, NADPH, 2 nM [1,2,6,7-3H] T [20] in a final volume of 1 mL. The reaction in duplicate was started when it was added to the enzymatic fraction (500 lg protein in a volume of 80 lL) incubated at 37 °C for 60 min [19] and stopped by mixing with 1 mL of dichloromethane; this was considered as the end point. Incubation without tissue was used as a control. The mixture (incubation

medium/ dichloromethane) was agitated on a vortex for 1 min and the dichloromethane phase was separated and placed in another tube. This procedure was repeated 4 more times. The dichloromethane extract was evaporated to dryness under a nitrogen stream and suspended in 50 lL of methanol that was spotted on HPTLC Keiselgel 60 F254 plates. T and DHT were used as carriers and were applied in different lanes on both lateral sides of the plates (T, T + DHT and DHT). The plates were developed in chloroform–acetone 9:1 and were air-dried; the chromatography was repeated 2 more times. The DHT steroid carriers were detected using phosphomolibdic acid reagent and T with an UV lamp (254 nm). After the plates were segmented in areas of 1 cm each, they were cut off and the strips soaked in 5 mL of Ultima Gold (Packard). The radioactivity was determined in a scintillation counter (Packard tri-carb 2100 TR). The radioactivity content in the segment corresponding to T and DHT carriers was identified. The radioactivity that has identical chromatographic behavior as the DHT standard was considered as the DHT transformation. Control incubations, chromatography separations and identifications, were carried out in the same manner as described above except that the tubes did not contain tissue. The DHT transformation yields were calculated from the strips, taking into account the entire radioactivity in the plate.

3.2. Type II 5α-reductase inhibitory activity

According to a convenient method already reported in the literature [21], human embryonic kidney cells (HEK293) over-expressing type II 5α-reductase were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Stable HEK293 cells (1×106) were planted in 24-well cell plate and incubated in 5% CO_2 incubator for 24 h at 37 °C. Stable cells were suspended in 50 mM sodium phosphate buffer (pH 5.5) and lysed by sonication for 1 min. The reaction mixture for type II 5α-reductase contained 10 μL (10 μg total protein) of cell extract, 65 μL of reaction buffer [60 mM sodium phosphate, pH 5.5, 50 mM KCl, 1 mM NADPH, and 1 μCi [1,2,6,7-3H]testosterone (65.0 Ci/mmol, Amersham)], and 1 μM and 10 μM of synthesized compounds. The reaction mixtures were incubated at 37 °C for 1 h, followed by steroids extraction with 250 μL of stop solution (70% cyclohexane, 30% ethyl acetate, 40 μg/mL T and 40 μg/mL DHT). Solvent was dried and steroids were dissolved with 20 μL of chloroform, spotted onto thin layer chromatography (TLC) plate (Merck, Darmastadt, Germany) and developed in 80% toluene, 20% acetone. TLC plate was then exposed to Hyperfilm 3H (Amersham, RPN 535B) for three days.

3.3. Determination of IC_{50} values of pregnenolone derivatives **4a–j** and **6a–d** in human prostatic 5α-reductase

In order to calculate the IC_{50} values (the concentration of pregnenolone derivatives **4a–j** and **6a–d** or Finasteride required to inhibit 5α-reductase activity (type I and type II) by 50%), six series of tubes containing increasing concentrations of these steroids (10^{-10}–10^{-4} M) were incubated in triplicate, in the presence of 1 mM of dithiothreitol, 40 mM sodium phosphate buffer pH of 6.5; 2 mM NADPH, 2 nM [1,2,6,7-^{3}H]T and 500 μg of protein from enzymatic fraction in a final volume of 1 mL. The reaction was carried out in triplicate at 37 °C for 60 min; 1 mL of dichloromethane was added to stop the reaction. The extraction and the chromatographic procedures were carried out as described above.

The plates were segmented in areas of one cm each, cut off and the strips were soaked in 10 mL of Ultima Gold (Packard) upon completion of chromatography. The radioactivity was determined in a scintillation counter (Packard tri-carb 2100 TR). The radioactivity content in the segments corresponding to T and DHT carriers was identified. The fraction that has identical chromatographic behavior as the DHT standard was considered as the DHT transformation in the presence of the tested compounds. Control incubations, chromatography separations and identifications, were carried out in the same manner as described above except that these tubes did not contain tissue. The DHT transformation yields were calculated from the strips, taking into account the entire radioactivity in the plate.

4. Results and discussion

The reductive conversion of testosterone (T) to dihydrotestosterone (DHT) by 5α-reductase catalysis is proposed to involve the formation of a binary complex between the enzyme and NADPH, followed by the formation of a ternary complex with the substrate T. This leads to the formation of a delocalized carbocation due to the activation of the enone system by a strong interaction with an electrophilic residue (E^+) present in the active site.

Direct hydride transfer from NADPH to the α-face of the delocalized carbocation generates enolate of DHT which leads to a selective reduction at C-5. Protonation on the β-face at C-4 of the enolate, which is coordinated with $NADP^+$ on the α-face, generates the ternary E-$NADP^+$-DHT complex. However after the departure of DHT, a binary complex of $NADP^+$-enzyme is formed which finally releases $NADP^+$ to leave the enzyme free for further catalytic cycles (Fig. 2). Three different types of inhibitors could be conceived according to the kinetic mechanism of testosterone reduction as shown in Fig. 1: type A inhibitors which are competitive with the cofactor (NADPH) and the substrate (T) and interact with the free enzyme; type B inhibitors which are competitive with the substrate and fit the enzyme-NADPH complex, and type C inhibitors which fits the enzyme-$NADP^+$ complex exhibiting uncompetitive mechanism versus the substrate [22,23]. Recently several steroid based D-ring heterocyclic analogs have been evaluated as efficient 5α-reductase inhibitors acting through one of the mechanisms as discussed above. This is particularly true of nitrogenous heterocyclic analogs at the D-ring of steroids including the very important class of pregnanes. Pyrazolyl and pyrazolinyl analogs are of special significance as these analogs have been found to show interesting biological potential probably because of their better interaction with the target receptors. Taking inspiration from the number of reported biological activities associated with structurally related analogs, we devised a new synthetic design for the pyrazoline derivatives and used an already reported method [16] for the synthesis of the pyazolyl derivatives. Thus, we, in continuation of our efforts towards the synthesis of novel D-ring heterocycles, herein report efficient and simple synthesis of D-ring pyrazolinyl and pyrazolyl derivatives of 20-keto pregnenanes and their evaluation as potential anticancer agents. The preparation of the latter involves the following synthetic approach.

4.1. Synthesis of the pyrazolyl derivatives **4(a–j)**

The stereochemistry of the benzylidine product **3** at the double bond was proved to be trans by the coupling constant value of 16 Hz of the vicinal protons. Compound **3** (1.0 g, 2.4 mmol) was refluxed in ethanol in the presence of hydrazine hydrate (0.24 g, 4.8 mmol) so as to yield the desired pyrazolines. However the products thus obtained were very unstable and they decomposed even at ambient temperature conditions probably because of the inherent instability associated with pyrazolines. The solvent thus used was replaced by acetic acid so as to ensure the formation of N-acetyl pyrazoline **4** (0.99 g, 2.2 mmol, 90%) (Scheme 1) which was highly stable. The product was precipitated by charging the reaction mass into excessive amounts of ice-cold water. After filtration under suction, the product was obtained in high yields as

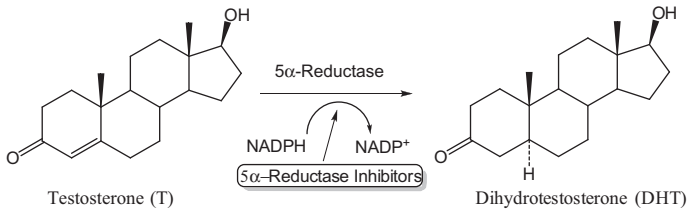


Fig. 1. Site of action of 5α-Reductase inhibitors.

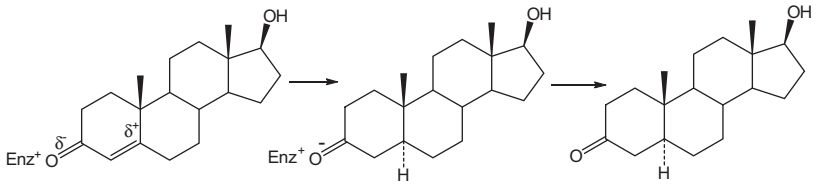
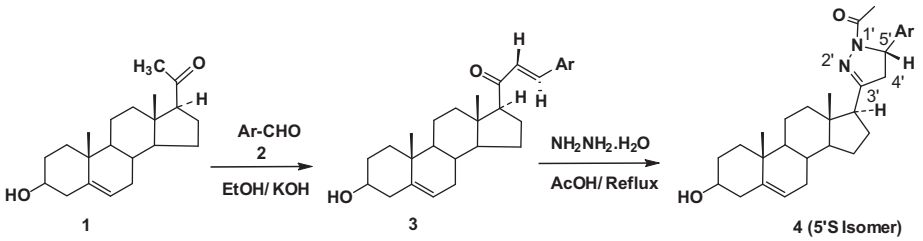


Fig. 2. 5α-reduction of 3-keto-Δ^4 steroids.



Scheme 1. Synthesis of D-ring substituted pyrazolinyl pregnenolones.

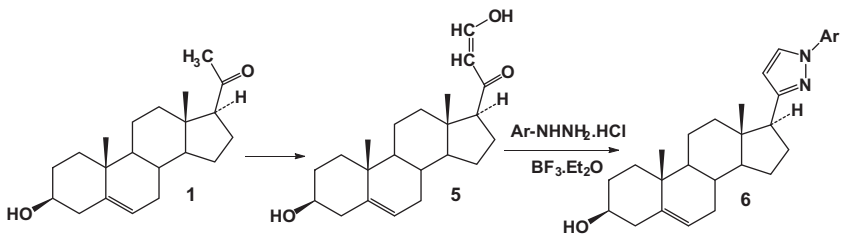
colorless powder which was later dried in vaccuo. In principle, the ring closure would result in two diastereomers at the 5′ position of the pyrazoline ring. However the TLC (Hexane:EtOAc, 7:3) did not show two different spots. The two spots resolved only when the TLC was run in methyl *tert*-butyl ether as eluant. The two diastereomers were isolated in their acetyl forms and the major product was the 5′S isomer. All the pyrazolinyl derivatives were deacetylated to give the required products **4a**–**j** and the same procedure was followed for the synthesis of all analogs.

4.2. Synthesis of the pyrazolyl derivatives **6**(***a***–***d***)

Compound **5** [17] (2.07 g, 6 mmol) was suspended in CH_2Cl_2 (50 ml) and phenylhydrazine hydrochloride or one of its p-substituted derivatives (1.1 equivalent) was added, followed by the drop wise addition of $BF_3{\cdot}OEt_2$ (50%) (2 mmol, 0.25 ml). The reaction mixture was stirred for 6 h. After the disappearance of the starting material (TLC monitoring), saturated $NaHCO_3$ solution (100 ml) was added and the mixture was stirred until bubbling ceased. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was chromatographed on silica gel starting with CH_2Cl_2/hexane (1:1, v/v) as eluent, followed by CH_2Cl_2/hexane (2:1, v/v) and CH_2Cl_2 to afford pyrazolyl pregnenolone derivatives **6a**–**d** (Scheme 2).

4.3. In vitro biological results

The in vitro biological activity of pregnenolone derivatives **4a**–**j** and **6a**–**d** was determined for human 5α-reductase activity. The radioactive zone that had identical chromatographic behavior as the standard T (R_f value of 0.56) corresponds to 70% of the accounted radioactivity in the plate. The radioactivity contained in the zone corresponding to DHT standard (R_f value of 0.67) of the experimental chromatogram was identified as the transformed DHT and corresponds to 27% of the total radioactivity accounted in the plate. This result was considered to be 100% of the activity of 5α-reductase for the development of inhibition plots. Unmodified [^{3}H]T was identified (R_f value of 0.56) from control incubations which did not contain tissue and had identical chromatographic behavior as the non labeled standard. The radioactivity contained



Scheme 2. Synthesis of D-ring substitued pyrazolyl pregnenolones.

Table 1
Nature of aryl group (Ar-) in pregnenolone derivatives **4a–j** and **6a–d**.

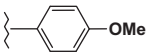
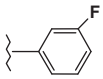
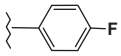
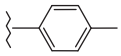
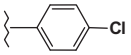
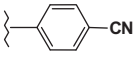
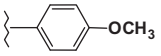
Entry	Nature of Ar	Entry	Nature of Ar
4a		**4h**	
4b		**4i**	
4c		**4j**	
4d		**6a**	
4e		**6b**	
4f		**6c**	
4g		**6d**	

Table 2
IC_{50} values (nM) of pyazolinyl pregnenolones (**4a–j**) and pyrazolyl pregnenolones (**6a–d**) against 5α-reductase inhibition.

Compound	Type I human 5α-reductase, IC 50 ± SD (nM)	Type II human 5α-reductase, IC 50 ± SD (nM)
4a	18.6 ± 0.49	24.7 ± 0.68
4b	14.5 ± 0.48	24.3 ± 0.43
4c	13.9 ± 0.75	27.3 ± 0.65
4d	24.3 ± 0.53	29.7 ± 0.49
4e	17.3 ± 0.48	45.3 ± 0.83
4f	38.3 ± 0.67	23.9 ± 0.53
4g	15.5 ± 0.50	22.3 ± 0.75
4h	27.5 ± 2.43	59.8 ± 0.77
4i	27.3 ± 0.80	27.3 ± 0.67
4j	48.8 ± 0.48	21.3 ± 0.73
6a	27.5 ± 0.63	23.3 ± 0.58
6b	14.2 ± 0.82	35.3 ± 0.85
6c	33.3 ± 0.42	27.5 ± 0.35
6d	45.4 ± 0.53	23.3 ± 0.44
Finasteride	21.6 ± 0.62	15.4 ± 0.58

in the zone corresponding to DHT standard (R_f value of 0.67) of the control chromatogram was 1% of the total radioactivity accounted in the plate and was considered as an error; it was subtracted from the experimental chromatograms. The concentrations of Finasteride and compounds **4a–j**, and **6a–d** (Tables 1 and 2) required for inhibiting 5α-reductase activity by 50% (IC_{50}) were determined from the inhibition plots using Sigma Plot software. The IC_{50} results are shown in the following table with the standard error in each value.

The in vitro 5α-reductase inhibitory studies of various pyrazolinyl pregnenolone (**4a–j**) and pyrazolyl pregnenolone (**6a–d**) derivatives revealed that most of these derivatives inhibited the activity of 5α-reductase. Further it is clear from the IC_{50} values, that the compounds **4b**, **4c** and **6b** exhibit the highest inhibition of 5α-reductase enzyme with lowest IC_{50} values. Interestingly all of the active derivatives are halogenated indicating that halogen substitution increases the activity. Further it evident from the IC_{50} values that the pyrazolines (**4a–j**) are more active and selective towards type-I reductase inhibition whereas the pyrazolines are moderately active with no selectivity.

5. Conclusion

A series of D-ring substituted pyrazolinyl pregnenolone (**4a–j**) and pyrazolyl pregnenolone (**6a–d**) derivatives were synthesized and screened for their 5α-reductase inhibitory activity. From the data it was found that all the compounds exhibit promising inhibitory activity especially against type I human 5α-reductase. However the compounds **4b**, **4c** and **6b** were found to be the most active in this study.

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