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RESEARCH ARTICLE

Synthesis, Aromatase Inhibitory, Antiproliferative and Molecular Modeling Studies of Functionally Diverse D-Ring Pregnenolone Pyrazoles

Abid H. Banday[1,2,*], Bahjat A. Saeed[3] and Najim A. Al-Masoudi[4]

[1]Department of Chemistry and Biochemistry, Auburn University, Auburn, AL, 8572, USA; [2]Department of Chemistry, Islamia College of Science and Commerce, Srinagar, 190002, India; [3]Department of Chemistry, College of Education of Pure Science, University of Basrah 61001, Basrah Iraq; [4]Department of Chemistry, College of Science, University of Basrah, Basrah 61001, Iraq, (Presently Consultant, 78464 Konstanz, Germany)

Abstract: ***Background*:** Aromatase, a cytochrome P450 hemoprotein that is responsible for estrogen biosynthesis by conversion of androgens into estrogens, has been an attractive target in the treatment of hormone-dependent breast cancer. Design of new steroidal aromatase inhibitors becomes imperative.

***Objective*:** Synthesis and biological evaluation of two classes of structurally and functionally diverse D-ring pregnenolone pyrazoles as type I aromatase inhibitors and antiproliferative agents.

***Methods*:** Pregnenolone (**1**) was converted to 3β-hydroxy-21-hydroxymethylidenepregn-5-en-20-one (**2**), which upon cyclization with phenylhydrazine generated regioisomeric pairs of pyrazoles **4** and **5**. Further, Knoevenagel condensation of pregnenolone (**1**) with 3-oxo-3-phenylpropanenitrile (**6**) produced 2-benzoyl-3-(3b-hydroxy-androstan-5-ene-20-ylidene)-but-2-enenitrile (**7**), which upon cyclization with hydrazine or phenylhydrazine generated the pyrazoles **8** and **9**. All new steroidal derivatives were tested for their aromatase inhibition activity using dibenzylfluorescein (DBF) based fluorescence assay developed by Stresser *et al*. Antiproliferative activities were measured using Sulforhodamine B assay. The activities were promising and there was a coherence between aromatase inhibitory and antiproliferative activities.

***Results*:** The study reveals the immense potential of pregnenolone pyrazoles as aromatase inhibitors for the treatment of breast cancer. Molecular docking studies proved efficient binding of the new steroidal analogs on human placental aromatase.

***Conclusion*:** In the overall study, most of the compounds exhibited potential activity for the treatment of hormone dependent breast cancer. Compounds **4c** and **4d** were found to be the most promising pharmacons. Furthermore, compounds **4c** and **4d** were applied for their molecular docking study on human placental aromatase to predict their possible binding modes with the enzyme. These studies revealed that such molecules have high scope and potential for further investigation towards the treatment of estrogen dependent breast cancer.

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1. INTRODUCTION

Steroids represent a promising class of pharmacologically active molecules owing to their versatility in controlling metabolism and signaling pathways [1]. Heterocyclized steroids have especially been shown to hold a great promise and many of such molecules are known for their pharmacological and biological properties [2-6]. Rational attachment of heterocycles to the steroidal framework, based on the defined structure activity relationship (SAR) for various pharmacological objectives, has been an interesting area for synthetic chemists, medicinal chemists and pharmacologists [7, 8]. However, all efforts have not been successful and yet there are limited scaffolds that have been constructed. Steroidal D-ring provides a platform for such chemical modifications altering the functionality, stereochemistry and potency of parent scaffolds [9, 10].

Introduction of azole heterocycles at ring-D of steroids has previously been shown to be of prime importance for inhibition of the hydroxylase/aromatase type enzymes as the lone pair of nitrogen coordinates to the heme iron at the active center of such enzymes [11, 12]. Such coordination inactivates or inhibits the binding of androgen substrates to the active site of the enzyme leading to reduced synthesis of estrogens. Strategies that inhibit the synthesis or block the biosynthetic pathway of estrogens shall be very useful towards therapeutic intervention on breast cancer. This shall be particularly important for post menopausal women where estrogen synthesis takes place in muscle, adipose and breast tissues, rather than in ovaries in premenopausal women. Nearly 30-50% breast cancers are considered estrogen dependant and reduction of estrogen levels in blood and muscle tissues shall lead to regression of such cancers. Two main chemical approaches for estrogen dependent breast cancer regression will be the use of selective estrogen receptor modulators (SERMs) like Tamoxifen or by applying aromatase inhibitors. As previously reported [13-15], the last step in the biosynthesis of estrogens from androgens is catalyzed by the enzyme aromatase encoded by the gene CYP191A, which belongs to the cytochrome P450 superfamily and forms an electron-transfer complex with its partner, NAPDH-cytochrome P450 reductase (CPR). Aromatase mainly catalyzes the conversion of testosterone to estradiol and androstenedione to estrone (Scheme **1**) through an aromatization reaction in which CPR coordinates the transfer of electrons from NADPH to the heme of aromatase and then to the androgen substrate. Since higher blood concentrations of estrogens are linked to increased risks of breast cancer [16],

*Address correspondence to this author at Department of Chemistry and Biochemistry, Auburn University, Auburn, AL, 8572, USA; Fax: +91-194 2429014;
E-mail: abidrrl@gmail.com

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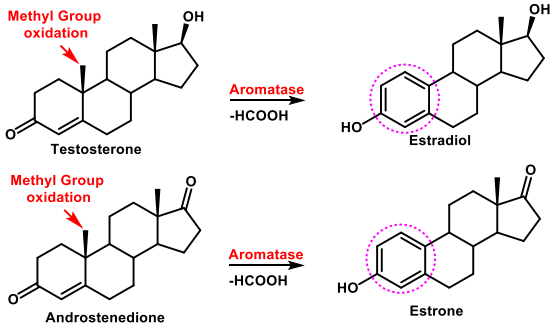
inhibitors of aromatase shall lead to reduced estrogen biosynthesis and thus to less risk of breast cancer of breast cancer. Steroid based type I aromatase inhibitors such as Formestane and Exemestane have already been marketed as "inactivators" for being analogous to androgen substrates binding competitively and irreversibly to the aromatase enzyme [17].



Scheme 1. Aromatase catalyzed conversion of testosterone to estradiol and androstenedione to estrone.

Recently, numerous reports have suggested that 17-pyrazoly, pyrazolinyl, isoxazolyl, imidazolyl, oxazolyl and thiazolyl steroids are very potent inhibitors of hydroxylase and aromatase family of enzymes [18-20]. Inspired by these reports, and our tryst with such heterocycles [21-28], we herein report the facile synthesis and aromatase inhibitory studies of two classes of pregnenolone pyrazoles. Most of the compounds were found to show moderate to good aromatase inhibition and *in-vitro* anticancer activity against breast cancer cell lines. Further, molecular docking study of two active steroids has been performed to understand their binding mode with human placental aromatase enzyme, the results of which were in consonance with the enzyme inhibitory activity data.

2. EXPERIMENTAL

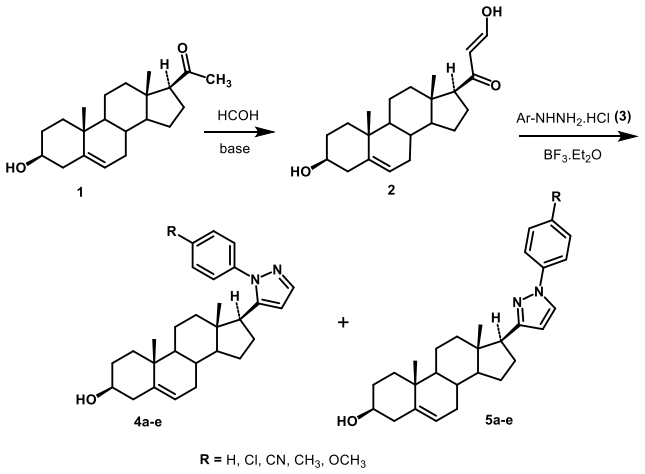
2.1. General Methods

Bruker DPX200 instrument was used for recording NMR spectra in $CDCl_3$ as solvent and TMS as internal standard for protons and solvent signals as an internal standard for ^{13}C spectra. Chemical shift values for both ^{1}H and ^{13}C are mentioned in δ (ppm) and coupling constants in Hz. Bruker Vector 22 instrument was used for recording IR spectra over KBr discs. Melting points were recorded on Buchi Melting point apparatus D-545. ESI-esquire 3000 Bruker Daltonics and EIMS (shimadzu) instruments were used for recording mass spectra. Pre-coated silica gel 60 F254 plates of thickness 0.25mm (Merck) and 2x5 cm dimension TLC plates were used for monitoring all reactions. TLC plates were visualized under UV-254-366 nm and iodine.

2.2. Chemical Synthesis

2.2.1. General Procedure for the Synthesis of Pyrazolyl Pregnenolones (4/5a-e)

Regioisomeric pairs of pyrazolyl pregnenolones **4a-e/5a-e** (Scheme **2**) were prepared as per the procedure based on the method described in our previous paper [26] and as reported previously by Schneider *et al.* [29]. The method involves the cyclization reaction of 5-pregnen-3β-ol-20-one (**2**) with phenylhydrazine (**3**) or its *p*-substituted derivatives. Compound **2** (2.07 g, 6.0 mmol) was treated with phenylhydrazine hydrochloride or its *p*-substituents (**3**, 1.1 equivalent) in CH_2Cl_2 (45 mL) at ambient temperature. $BF_3.OEt_2$ (50%) (0.25 mL, 2.0 mmol) was then added dropwise to the homogenous mixture over a period of 5 min. The reaction mixture was stirred for 5 h. After completion of the reaction as monitored by TLC, a saturated solution of $NaHCO_3$ was added to the reaction mixture till bubbling stopped. Solvent extraction of the organic layer after thorough washing with water was performed and the organic layers were dried ($MgSO_4$), filtered and the combined organic layers were evaporated in *vacuo* to dryness. The residue was purified through chromatography on a SiO_2 column using CH_2Cl_2/hexane (1:1, v/v) as eluent, followed by CH_2Cl_2/hexane (2:1, v/v) and CH_2Cl_2 as eluent to give the desired pyrazolyl pregnenolone derivatives as regioisomeric products **4/5a-e**.



Scheme 2. Synthesis of D-ring substituted pyrazolyl pregnenolones [26, 29].

2.2.1.1. 17β-(1-Phenyl-3-pyrazolyl)androst-5-en-3β-ol (4a)

Yield: 78%; mp: 153-155 °C; $[\alpha]_D^{20}$: - 60 (*c* 1 in $CHCl_3$); ^{1}H NMR (δ, ppm, $CDCl_3$): 0.59 (s, 3H, 18-H3), 1.02 (s, 3H, 19-H_3), 2.80 (t, 1H, J = 8.3 Hz, 17-H), 3.54 (m, 1H, 3-H), 5.38 (d, 1H, *J* = 2.3 Hz, 6-H), 6.27 (d, 1H, *J* = 2.0 Hz, 4'-H), 7.23 (t, 1H, *J* = 6.5 Hz, 4"-H), 7.42 (t, 2H, *J* = 6.5 Hz, 3"- and 5"-H), 7.68 (d, 2H, *J* = 6.5 Hz, 2"- and 6"-H), 7.83 (d, 1H, *J* = 2.0 Hz, 5'-H); ^{13}C NMR (δ, ppm, $CDCl_3$): 13.1, 19.4, 20.9, 24.7, 26.3, 31.7, 32.0, 32.3, 37.3, 37.9, 42.3, 43.7, 50.3, 50.4, 56.2, 71.8, 106.8, 118.8, 121.6, 125.7, 126.6, 129.3, 134.7, 140.9, 153.2, 155.2; MS: *m/z* = 416.2 (M^+, 25%).

2.2.1.2. 17β-(1-Phenyl-5-pyrazolyl)androst-5-en-3β-ol (5a)

Yield:16%; mp: 223-226 °C; $[\alpha]_D^{20}$: -133 (*c* 1 in $CHCl_3$); ^{1}H NMR (δ, ppm, $CDCl_3$): 0.67 (s, 3H, 18-H3), 0.96 (s, 3H, 19-H3), 2.84 (t, 1H, *J* = 8.3 Hz, 17-H), 3.47 (m, 1H, 3-H), 5.31 (d, 1H, *J* = 5.0 Hz, 6-H), 6.27 (d, 1H, *J* = 1.5 Hz, 4'-H), 7.36 (d, 2H, *J* = 6.3 Hz, 2"- and 6"-H), 7.40 (t, 1H, *J* = 6.3 Hz, 4"-H), 7.45 (t, 2H, *J* = 6.3 Hz, 3"- and 5"-H), 7.60 (d, 1H, *J* = 1.5 Hz, 3'-H); ^{13}C NMR (δ, ppm, $CDCl_3$): 13.3, 19.3, 20.7, 24.4, 29.5, 31.6, 31.7, 32.3, 36.5, 37.2, 42.2, 44.3, 46.9, 49.9, 56.0, 71.6, 105.5, 121.3, 126.9 and 128.9, 128.1, 139.3, 140.4, 140.8, 144.4; MS: *m/z* = 416.2 (M^+, 25%).

2.2.1.3. 17β-(1-p-Chlorophenyl-3-pyrazolyl)-androst-5-en-3β-ol (4b)

Yield: 70%; mp: 177-179 °C; $[\alpha]_D^{20}$: -54 (*c* 1 in $CHCl_3$); ^{1}H NMR (δ, ppm, $CDCl_3$): 0.58 (s, 3H, 18-H3), 1.01 (s, 3H, 19-H3), 2.81 (t, 1H, *J* = 9.3 Hz, 17-H), 3.53 (m, 1H, 3-H), 5.38 (s, 1H, 6-H), 6.30 (s, 1H, 4'-H), 7.33 (d, 2H, *J* = 8.3 Hz, 2"- and 6"-H), 7.45 (d, 2H, *J* = 8.3 Hz, 3"- and 5"-H), 7.67 (s, 1H, 3'-H); ^{13}C NMR (δ, ppm, $CDCl_3$): 13.0, 19.4, 20.8, 24.5, 27.4, 31.7, 31.9, 32.3, 36.7, 37.4, 37.9, 42.4, 44.8, 50.1, 50.9, 56.2, 71.8, 106.5, 118.9, 121.6, 125.7, 126.6, 129.4, 134.7, 140.9, 153.2, 155.3; MS: *m/z* = 450.2 (M^+, 35%).

2.2.1.4. 17β-(1-p-Chlorophenyl-3-pyrazolyl)androst-5-en-3β-ol (5b)

Yield: 19%; mp: 133-137 °C; $[\alpha]_D^{20}$: -128 (*c* 1 in $CHCl_3$); ^1H NMR (δ, ppm, $CDCl_3$): 0.66 (s, 3H, 18-H3), 0.96 (s, 3H, 19-H3), 2.78 (t, 1H, *J* = 9.3 Hz, 17-H), 3.47 (m, 1H, 3-H), 5.31 (s, 1H, 6-H), 6.30 (s, 1H, 4'-H), 7.31 (d, 2H, *J* = 8.3 Hz, 2"- and 6"-H), 7.44 (d, 2H, *J* = 8.3 Hz, 3"- and 5"-H), 7.63 (s, 1H, 3'-H); ^{13}C NMR (δ, ppm, $CDCl_3$): 13.3, 19.3, 20.6, 24.4, 29.4, 31.6, 31.7, 32.3, 36.5, 37.2, 37.4, 42.2, 44.6, 46.9, 49.9, 56.1, 71.6, 106.0, 121.2, 128.2, and 129.3, 134.6, 137.8, 138.9, 140.9, 145.4; MS: *m/z* = 450.2 (M^+, 35%).

2.2.1.5. 17β-(1-p-Cyanophenyl-3-pyrazolyl)androst-5-en-3β-ol (4c)

Yield: 71%; mp: 273-275 °C; $[\alpha]_D^{20}$: -47 (*c* 1 in $CHCl_3$); ^1H NMR (δ, ppm, $CDCl_3$): 0.57 (s, 3H, 18-H3), 1.02 (s, 3H, 19-H3), 2.89 (t, 1H, *J* = 9.8 Hz, 17-H), 3.52 (m, 1H, 3-H), 5.37 (s, 1H, 6-H), 6.36 (d, 1H, *J* = 2.0 Hz, 4'-H), 7.55 (d, 2H, *J* = 8.3 Hz, 2"- and 6"-H), 7.69 (d, 1H, *J* = 2.0 Hz, 3'-H), 7.79 (d, 2H, *J* = 8.3 Hz, 3"- and 5"-H); ^{13}C NMR (δ, ppm, $CDCl_3$): 13.1, 19.4, 20.8, 24.5, 29.3, 31.7, 31.9, 32.5, 36.7, 37.2, 37.4, 42.2, 44.8, 47.0, 49.9, 56.1, 71.6, 107.1, 118.8, 121.6, 125.7, 126.6, 129.3, 134.7, 140.9, 153.2, 155.3; MS: *m/z* = 441.2 (M^+, 28%).

2.2.1.6. 17β-(1-p-Cyanophenyl-5-pyrazolyl)androst5-en-3β-ol (5c)

Yield: 9%; mp: 183-186 °C; $[\alpha]_D^{20}$: -129 (*c* 1 in $CHCl_3$); ^1H NMR (δ, ppm, $CDCl_3$): 0.63 (s, 3H, 18-H3), 0.95 (s, 3H, 19-H3), 2.86 (t, 1H, *J* = 9.8 Hz, 17-H), 3.47 (m, 1H, 3-H), 5.31 (s, 1H, 6-H), 6.35 (d, 1H, *J* = 2.0 Hz, 4'-H), 7.53 (d, 2H, *J* = 8.3 Hz, 2"- and 6"-H), 7.67 (d, 1H, *J* = 2.0 Hz, 3'-H), 7.77 (d, 2H, *J* = 8.3 Hz, 3"- and 5"-H); ^{13}C NMR (δ, ppm, $CDCl_3$): 13.3, 19.3, 20.7, 24.3, 29.2, 31.6, 31.7, 32.3, 36.5, 37.2, 37.4, 42.2, 44.8, 47.0, 49.9, 56.1, 71.6, 106.9, 112.2, 118.0, 121.1, 127.3, and 133.1, 139.9, 140.9, 143.2, 145.3; MS: *m/z* = 441.2 (M^+, 28%).

2.2.1.7. 17β-(1-p-Tolylphenyl-3-pyrazolyl)androst-5-en-3β-ol (4d)

Yield: 73%, mp: 167-170 °C; $[\alpha]_D^{20}$: -63 (*c* 1 in $CHCl_3$); ^1H NMR (δ, ppm, $CDCl_3$): 0.59 (s, 3H, 18-H3), 1.01 (s, 3H, 19-H3), 2.36 (s, 3H, 4'-CH_3), 2.84 (t, 1H, *J* = 9.8 Hz, 17-H), 3.53 (m, 1H, 3-H), 5.37 (d, 1H, *J* = 2.5 Hz, 6-H), 6.26 (d, 1H, *J* = 2.3 Hz, 4'-H), 7.22 and 7.54 (d, 4H, *J* = 8.0 Hz, 2"-, 3"-, 5"- and 6"-H), 7.78 (d, 1H, *J* = 2.3 Hz, 5"-H); ^{13}C NMR (δ, ppm, $CDCl_3$): 13.1, 19.4, 20.8, 20.9, 24.7, 26.5, 31.6, 31.9, 32.3, 36.6, 37.3, 37.7, 42.3, 43.8, 49.9, 50.3, 56.2, 71.7, 106.5, 119.2, 121.5, 127.1, 129.8, 135.9,137.7, 140.9, 154.7; MS: *m/z* = 430.3 (M^+, 31%).

2.2.1.8. 17β-(1-p-Toly-5-pyrazolyl)androst-5-en-3β-ol (5d)

Yield: 20%; mp: 166-168 °C; $[\alpha]_D^{20}$: -128 (*c* 1 in $CHCl_3$); ^1H NMR (δ, ppm, $CDCl_3$): 0.68 (s, 3H,18-H3), 0.95 (s, 3H, 19-H3), 2.41 (s, 3H, 4"- CH_3), 2.78 (t, 1H, *J* = 9.8 Hz, 17-H), 3.45 (m, 1H, 3-H), 5.29 (s, 1H, 6-H), 6.29 (d, 1H, *J* = 1.5 Hz, 4'- H), 7.24 (overlapping multiplets, 4H, 2"-, 3"-, 5"-, 6"-H), 7.65 (d, 1H, *J* = 1.5 Hz, 3-H); ^{13}C NMR (δ, ppm, $CDCl_3$): 13.4, 19.3, 20.7, 21.2, 24.4, 29.6, 31.6, 31.7, 32.3, 36.5, 37.2 37.3, 42.2, 44.5, 46.9, 49.9, 56.0, 71.6, 105.6, 121.2, 126.8 and 129.7, 136.2, 137.8 (C-5"), 139.0, 140.9, 145.7; MS: *m/z* = 430.3 (M^+, 31%).

2.2.1.9. 17β-(1-p-Methoxyphenyl-3-pyrazolyl)androst-5-en-3β-ol (4e)

Yield: 63%; mp: 150-152 °C; $[\alpha]_D^{20}$: -60 (*c* 1 in $CHCl_3$); ^1H NMR (δ, ppm, $CDCl_3$): 0.58 (s, 3H, 18-H3), 1.02 (s, 3H, 19-H3), 2.80 (t, 1H, *J* = 8.3 Hz, 17-H), 3.54 (m, 1H, 3-H), 3.83 (s, 3H, OCH_3), 5.38 (d, 1H, *J* = 2.0 Hz, 6-H), 6.24 (d, 1H, *J* = 2.0 Hz, 4'-H), 6.94 (d, 2H, *J* = 7.3 Hz, 3" and 5"-H), 7.56 (d, 2H, *J* = 7.3 Hz, 2" and 6"-H), 7.72 (d, 1H, *J* = 2.0 Hz, 5'-H); ^{13}C NMR (δ, ppm, $CDCl_3$): 13.1, 19.4, 20.8, 24.7, 26.4, 31.7, 32.0, 32.3, 36.6, 37.3, 37.8, 42.3, 43.7, 50.3, 50.4, 55.6, 56.2, 71.8, 106.3, 114.4, 120.6, 121.6, 126.7, 134.3, 140.9, 154.7, 157.8; MS: *m/z* = 446.3 (M^+, 32%).

2.2.1.10. 17β-(1-p-Methoxyphenyl-5-pyrazolyl)androst-5-en-3β-ol (5e)

Yield: 22%; mp: 186-188 °C; $[\alpha]_D^{20}$: -134 (*c* 1 in $CHCl_3$); ^1H NMR (δ, ppm, $CDCl_3$): 0.68 (s, 3H, 18-H3), 0.97 (s, 3H, 19-H3), 2.77 (t, 1H, *J* = 8.3 Hz, 17-H), 3.49 (m,1H, 3-H), 3.86 (s, 3H, OCH_3), 5.32 (d, 1H, *J* = 2.0 Hz, 6-H), 6.24 (d, 1H, *J* = 1.5 Hz, 4'-H), 6.95 (d, 2H, *J* = 7.0 Hz, 3"- and 5"-H), 7.26 (d, 2H, *J* = 7.0 Hz, 2"- and 6"-H), 7.57 (d, 1H, *J* = 1.5 Hz, 3"-H); ^{13}C NMR (δ, ppm, $CDCl_3$): 13.3, 19.4, 20.7, 24.4, 29.6, 31.6, 31.8, 32.3, 36.5, 37.2, 37.3, 42.2, 44.1, 47.0, 49.9, 55.5, 56.0, 71.7, 105.1, 114.0, 121.3, 128.2, 133.4, 139.0, 140.8, 144.6, 159.3; MS: *m/z* = 446.3 (M^+, 32%).

2.2.2. Synthesis of Compound 7a/b and the pyrazolyl derivatives (8a/b, 9a/b)

2.2.2.1. 2-Benzoyl-3-(3β-hydroxy-androstan-5-ene-20-ylidene)-but-2-enenitrile (7)

3-Oxo-3-phenyl propanenitrile (**6**) (680 mg, 4.0 mmol) and ammonium acetate (77 mg, 1.0 mmol) were added to pregnenolone (**1**) (1.264 g, 4.0 mmol) in a round bottom flask fitted with a condenser over a silicone oil bath. The mixture was heated for 1 h at 120 °C and then left to cool. Ethanol was added to the reaction mixture affording a solid product **7a/b** (Scheme **3**), which showed two products, presumably E/Z stereoisomers, by TLC. The two products (*E/Z* stereoisomers) were separated by SiO_2 column, using hexane:EtOAc (9:1) as eluent and characterized through spectral techniques. The assignment of configurations is based on the NMR spectra and coupling constants, which vary between E/Z isomers.

*2.2.2.1.1. E-isomer **7a***. Yellow crystals from EtOAc-hexane. Yield: 60%; mp: 220-222 °C; $[\alpha]_D^{20}$:

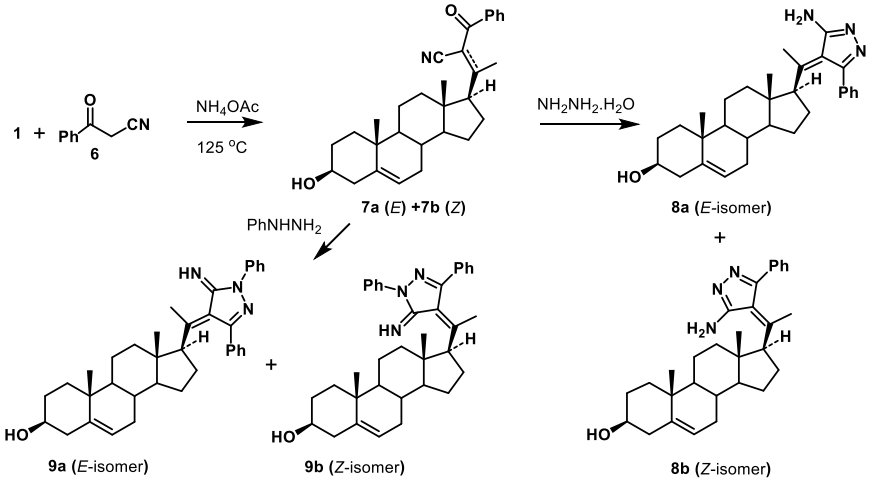
-125 (*c* 1 in $CHCl_3$); ^1H NMR (δ, ppm, $CDCl_3$): 0.63 (s, 3H), 0.93 (s, 3H), 1.81 (s, 3H), 1.61-1.93 (m, 6H), 2.23-2.35 (m, 3H), 2.80 (t, *J* = 9.0, 1H); 3.50 (m, 1H), 5.36 (s, 1H), 6.70 (d, *J* = 14.8, 1H), 7.28-7.35 (m, 5H), 8.25 (s, 1H); ^{13}C NMR (δ, ppm, $CDCl_3$): 12.9, 19.6, 21.9, 22.2, 24.7, 28.6, 31.0, 31.7, 31.9, 37.2, 38.7, 43.6, 45.0, 48.6, 48.8, 48.9, 49.6, 49.3, 50.1, 57.2, 61.7, 71.4, 103.7, 112.9, 115.9, 119.6, 122.4, 123.5, 138.3, 143.2, 148.3, 166.8, 168.2; MS: *m/z* = 443.3 (M^+, 40%).

*2.2.2.1.2. Z-isomer (**7b**)*. Colourless crystals from EtOAc-hexane. Yield: 20%, mp: 228-230 °C; $[\alpha]_D^{20}$:

-80 (*c* 1 in $CHCl_3$); ^1H NMR (δ, ppm, $CDCl_3$): 0.63 (s, 3H), 0.93 (s, 3H), 1.80 (s, 3H), 1.62-1.90 (m, 6H), 2.23-2.37(m, 3H), 2.80 (t, *J* = 7.2, 1H); 3.50 (m,1H), 5.36 (s, 1H), 6.70 (d, *J* = 11.1, 1H), 7.28-7.38 (m, 5H), 8.24(s, 1H); ^{13}C NMR (δ, ppm, $CDCl_3$): 14.1, 19.9, 21.9, 22.3, 24.7, 28.6, 31.0, 31.7,31.9, 37.2, 38.7, 43.6, 45.0, 48.6, 48.8, 48.9, 49.6, 49.3, 50.1, 57.2, 61.6, 71.4, 104.5, 113.9, 113.2, 117.0, 119.6, 122.4, 123.5, 138.3, 143.2, 148.3, 166.8, 168.2; MS: *m/z* = 443.3 (M^+, 25%).

2.2.2.2. (17-E/Z)-3-Amino-4-(3β-hydroxy-androstan-5-ene-20-ylidene)5-phenyl-4H-pyrazole (8)

Hydrazine hydrate (100 mg, 0.098 ml, 2 mmol) was added to a solution of compound **7a/7b** (886 mg, 2.0 mmol) in EtOH (25 mL). The reaction mixture was heated under reflux and monitored regularly by TLC. After the completion of the reaction in 2 h, the reaction mixture was poured onto ice/water, followed by the addition of a few drops of HCl. The product precipitated out as a solid, which was filtered and recrystallized from a suitable solvent.



Scheme 3. Synthesis of new D-ring pregnenolone pyrazole derivatives.

*2.2.2.2.1. E-Isomer (**8a**)*. White crystals from EtOAc-hexane. Yield: 65%; mp: 171-173 °C; $[\alpha]_D^{20}$:

-110 (*c* 1 in $CHCl_3$); ^{1}H NMR (δ, ppm, $CDCl_3$): 0.64 (s, 3H), 0.91 (s, 3H), 1.73 (s, 3H), 1.60-1.93 (m, 6H), 2.27-2.33 (m, 3H), 2.83 (t, *J* = 8.4, 1H); 3.52 (m, 1H), 5.38 (s, 1H), 5.49 (s, 2H, D_2O exchangeable), 6.72 (d, *J* = 14.4, 1H), 7.33-7.40 (m, 5H), 8.26 (s, 1H, D_2O exchangeable); ^{13}C NMR (δ, ppm, $CDCl_3$): 13.3, 19.7, 21.9, 22.3, 24.7, 28.6, 31.2, 31.7, 31.6, 37.0, 38.6, 43.8, 45.3, 48.5, 48.8, 48.9, 49.6, 49.8, 50.0, 57.2, 61.8, 71.4, 112.5, 120.2, 124.4, 129.8, 146.2, 149.3, 155.0, 162.3; MS: *m/z* = 457.1 (M^+, 35%).

*2.2.2.2.2. Z-Isomer (**8b**)*. Shiny white crystals from EtOAc-hexane; Yield: 25%; mp: 177-179 °C; $[\alpha]_D^{20}$:

-80 (*c* 1 in $CHCl_3$);; ^{1}H NMR (δ, ppm, $CDCl_3$): 0.62 (s, 3H), 0.91 (s, 3H), 1.81 (s, 3H), 1.63-1.89 (m, 6H), 2.23-2.36 (m, 3H), 2.82 (t, *J* = 8.6, 1H); 3.52 (m, 1H), 5.34 (s, 1H), 5.45 (s, 2H, D_2O exchangeable), 6.72 (d, *J* = 13.6, 1H), 7.30-7.41 (m, 5H), 8.26 (s, 1H); ^{13}C NMR (δ, ppm, $CDCl_3$): 13.1, 19.8, 21.9, 22.3, 24.7, 28.6, 31.3, 31.7, 31.6, 37.0, 38.6, 43.8, 45.3, 48.5, 48.8, 48.9, 49.6, 49.8, 50.3, 57.2, 61.8, 71.4, 113.6, 120.0, 123.4, 129.8, 146.2, 150.6, 154.9, 164.1; MS: *m/z* = 457.1 (M^+, 18%).

2.2.2.3. (17 E/Z)-3-Imino-4-(3β-hydroxy-androstan-5-ene-20-ylidene)-1,3-diphenyl-4H-pyrazole (9)

Phenylhydrazine (216 mg, 2.0 mmol) was added to a solution of compound **7a/b** (886 mg, 2.0 mmol) in EtOH (25 mL). The same procedure as given under 2.2.2.2 was adopted to afford *E/Z* isomers, which were separated by column chromatography, and further purified by recrystallization. The assignment of configurations is based on the NMR spectra and coupling constants, which vary between E/Z isomers.

*2.2.2.3.1. E-Isomer (**9a**)*. White crystals from EtOAc-hexane; Yield: 70%; mp: 120-132 °C; $[\alpha]_D^{20}$:

-97 (*c* 1 in $CHCl_3$); ^{1}H NMR (δ, ppm, $CDCl_3$): 0.62 (s, 3H), 1.04 (s, 3H), 1.60-1.94 (m, 6H), 2.20-2.38 (m, 3H),2.83 (t, *J* = 7.9, 1H); 3.55 (m, 1H), 5.39 (s, 1H), 6.70 (d, *J* = 13.2, 1H), 7.31-7.39 (m, 10H), 8.24 (s, 1H, D_2O exchangeable), 8.25 (s, 1H); ^{13}C NMR (δ, ppm, $CDCl_3$): 13.3, 19.5, 21.7, 22.4, 23.7, 29.6, 31.2, 32.5, 31.6, 37.2, 39.6, 43.8, 45.3, 47.5, 48.6, 48.9, 49.6, 49.8, 50.0, 57.2, 61.8, 71.4, 115.1, 119.3, 124.4, 126.7, 128.4, 129.6, 138.2, 146.1, 147.2, 153.9, 172.6; MS: *m/z* = 533.2 (M^+, 35%).

*2.2.2.3.2. Z-isomer (**9b**)*. White crystals from EtOAc:hexane. Yield: 20%; mp: 141-142 °C; $[\alpha]_D^{20}$:

-105 (*c* 1 in $CHCl_3$); ^{1}H NMR (δ, ppm, $CDCl_3$): 0.67 (s, 3H), 1.05 (s, 3H), 1.62-1.94 (m, 6H), 2.20-2.36 (m, 3H), 2.83 (t, *J* = 7.8, 1H); 3.53 (m, 1H), 5.38 (s, 1H), 6.71 (d, *J* = 14.9, 1H), 7.30-7.39 (m, 10H), 8.25 (s, 1H, D_2O exchangeable), 8.28 (s, 1H); ^{13}C NMR (δ, ppm, $CDCl_3$): 13.1, 19.6, 21.9, 22.4, 24.7, 28.6, 31.2, 31.7, 31.7, 37.0, 38.2, 43.7, 45.3, 48.5, 48.8, 48.9, 49.6, 49.7, 50.0, 57.2, 61.8, 71.4, 113.9, 120.8, 124.4, 127.3, 128.2, 129.5, 138.3, 146.2, 147.0, 154.6, 177.1; MS: *m/z* = 533.2 (M^+, 27%).

2.3. Ligands and Receptors Preparation

The PDB structure 4GL7 [30] was obtained from the Brookhaven Protein Data Bank (www.rcsb.org). The protein structure was prepared using UCSF Chimera 1.14 [31] software using the Dock Prep tool. The native ligand ((6α,8α)-6-(pent-2-yn-1-yloxy)androsta-1,4-diene-3,17-dione) was stripped out from the PDB structure and the polar hydrogens were added. Avogadro [32] software was used for the preparation of the structures of the studied ligands, which were optimized using the MMFF94 force field [33]. MGLTools software was used for the preparation of the pdpqt files for protein and ligands and visualizing the docking results. MGLTools assigned united atom Kollman charges, fragmental volumes, and solvation parameters to the protein. Docking studies were carried out by employing Autodock4 suite [34]. The grid maps were prepared using Autogrid. The grid size dimensions were 60x60x60 xyz points with a grid spacing of 0.375 angstroms. The grid center dimensions were 85.26, 53.79, and 48.62 for x, y and z, respectively.

2.4. Biological Methods

2.4.1. Aromatase Inhibitory Assay

The aromatase inhibitory activity of the pregnenolone pyrazole derivatives was calculated through the method described by Stresser *et al.* [35] and modified by Prachayasittikul *et al.* [36]. CYP19 and dibenzylfluorescein (DBF) were used as fluorometric substrates for an assay performed using Gentest kit. Aromatase dealkylates DBF and then hydrolyzes it to produce fluorescein product. The references cited above may be consulted for the detailed procedure. Briefly, 100 µL of cofactor, containing 78.4 µL of 50 mM phosphate buffer (pH 7.4); 20 µL of 20×nicotinamide adenine dinucleotide phosphate (NADPH)-generating system (26 mM $NADP^+$, 66 mM glucose-6-phosphate, and 66 mM $MgCl_2$); and 1.6 µL of 100 U/mL glucose-6-phosphate dehydrogenase, were pipetted into a 96-well black plate and preincubated in a water bath (37 °C) for 10 min. The reaction was initiated by addition of 100 µL of enzyme/substrate (E/S) mixture containing 77.3 µL of 50 mM phosphate buffer (pH 7.4); 12.5 µL of 16 pmol/mL CYP19; 0.2 µL of 0.2 mM DBF; and 10 µL of 0.25 mM diluted tested compound (pregnenolone pyrazoles) or 10% DMSO as a negative control and

Formestane or Exemestane as a positive control). To exclude background fluorescence of the sample, E/S was added after the reaction was terminated. To cease the reaction, 50 µL of 2.2 N NaOH was added to the solution after 30 min incubation at 37 °C. The emission wavelength of 530 nm with cut-off of 515 nm and excitation wavelength of 490 was used for measuring the fluorescence signal with reduced background noise. Equation 1, as given below, was used for calculation of percentage inhibition and the samples showing more than 50% inhibition were further diluted and assayed in triplicate to generate IC_{50} values by plotting concentrations against % inhibitions (Table **1**).

% inhibition = 100 - [(sample - blank)/(DMSO - blank) x100] - (1)

Table 1. Inhibition of the human aromatase enzyme by pyrazolyl pregnenolone derivatives[#].

Compd.	IC_{50} (nM)[✚]	Compd.	IC_{50} (nM)[✚]
4a	120.23 ± 0.86	**5e**	87.53 ± 2.83
4b	65.82 ± 1.03	**7a**	Inactive
4c	56.44 ± 1.21	**7b**	Inactive
4d	52.38 ± 0.98	**8a**	ND
4e	165.42 ± 2.54	**8b**	125.02 ± 1.12
5a	ND	**9a**	223.23 ± 1.32
5b	125.23 ± 2.56	**9b**	243.11 ± 3.32
5c	227.45 ± 1.67	**Exemestane**[*]	42.50 ± 2.32
5d	131.23 ± 0.87	**Formestane**[*]	46.30 ± 1.21

[#]Most of the compounds showed more than 50% inhibition and were used for the calculation of IC_{50} values. [✚]All results are presented as mean ± S.D. [*] Exemestane and Formestane were used as reference standards. ND = Not determined

2.4.2. Cell Culture and Anti-breast Cancer Assay

Three human breast cancer cell lines used for the test were MCF-7, BT-20 and T-47D. All these cancer cell lines were obtained from the National cancer institute (NCI), biological testing branch, Federick Research and Development center, USA. Cellular viability in the presence and absence of experimental agents was determined using the standard sulforhodamine B assay [37]. Briefly, cells in their log phase of growth were harvested, counted and seeded (104 cells/well in 100 lL medium) in 96- well microtitre plates. After 24 h of incubation at 37 ^{o}C and 5% CO_2 to allow cell attachment, cultures were treated with varying concentrations (10^{-9}-10^{-4} M) of experimental agents *i.e.*, the steroidal pyrazole analogs kept in six series of tubes. Four replicate wells were set up for each experimental condition. Test samples were left in contact with the cells for 48 h under the same conditions. Thereafter, cells were fixed with 50% chilled trichloroacetic acid (TCA) and kept at 4 ^{o}C for 1 h, washed and air dried. Cells were stained with sulforhodamine B dye. The adsorbed dye was dissolved in Tris-Buffer and plates were gently shaken for 10 min on a mechanical shaker. The optical density (OD) was recorded on ELISA reader at 540 nm. The cell growth was calculated by subtracting the mean OD value of the respective blank from the mean OD value of the experimental set. Percent growth in the presence of test material was calculated considering the growth in the absence of any test material as 100% and in turn percent growth inhibition in the presence of test material was calculated. Finally, the IC_{50} values (Table **2**) were calculated using Sigma Plot software. Exemestane was used as a positive control. The different steroidal derivatives (test material) were dissolved in a mixture of DMSO:H_2O (1:1) and then introduced into the medium containing the cancer cell lines.

Table 2. Anti-breast cancer cell activity (IC_{50}) of the pyrazolyl pregnenolone derivatives.

Entry	MCF-7 (µM)	BT-20 (µM)	T-470 (µM)
4a	47.32 ± 2.23	> 50	> 50
4b	7.32 ± 2.23	ND	9.03 ± 2.12
4c	2.44 ± 0.65	4.62 ± 1.34	2.54 ± 0.83
4d	3.38 ± 0.98	3.64 ± 1.44	2.23 ± 0.54
4e	42.42 ± 0.92	49.90 ± 1.34	40.02 ± 1.34
5a	39.22 ± 1.34	46.32 ± 2.12	ND
5b	43.23 ± 1.89	40.23 ± 1.12	44.34 ± 1.45
5c	35.42 ± 2.54	38.67 ± 1.53	38.87 ± 2.33
5d	45.42 ± 2.03	ND	43.19 ± 2.45
5e	8.54 ± 1.02	7.32 ± 0.72	7.23 ± 1.09
7a	ND	> 50	ND
7b	> 50	49.23 ± 2.43	> 50
8a	44.23 ± 1.54	48.10 ± 1.09	> 50
8b	38.92 ± 2.09	ND	39.52 ± 1.32
9a	> 50	> 50	ND
9b	> 50	ND	48.12 ± 0.82
Exemestane	0.92 ± 0.12	1.33 ± 0.34	1.93 ± 0.43

ND = Not determined. Results presented as mean ± S.D.

3. RESULTS AND DISCUSSION

3.1. Chemistry

Pregnenolone pyrazoles have been of interest and numerous reports about their synthesis and biological evaluation as anticancer agents and hydroxylase inhibitors are available [12, 14]. In the present work, our aim is to evaluate the aromatase inhibitory activity of functionally versatile pregnenolone pyrazoles in which the pyrazole ring is differently substituted. In this direction, we took help of known literature precedents to synthesize steroidal derivatives in which the pyrazoles were substituted at C, N or both C and N atoms [29]. Pregnenolone **1** was converted into 3β-hydroxy-21-hydroxymethyl-idenepregn-5-en-20-one (**2**) through a method described in one of our previous publications [26]. Upon cyclization with arylhydrazine, **2** generated the *N*-aryl pyrazole pregnenolones **4/5a-e** in good overall yield (Scheme **2**). All the pyrazole derivatives thus obtained were screened for their aromatase inhibitory and antiproliferative activities as potential pharmacons for estrogen dependent breast cancers.

Next, the preparation of *E/Z* stereoisomers of **7** was carried out through Knoevenagel reaction by the treatment of pregnenolone **1** with 3-oxo-phenylpropanenitrile (**6**) to furnish good overall yield. The isomers were separated by SiO_2 column chromatography. These analogs were individually treated with hydrazine whereupon the *E*- and *Z*-isomers produced the final pyrazole derivatives **8** (*E*-isomer) and **8** (*Z*-isomer), respectively. Besides, the cyclization of **7***E* and **7***Z* isomers with phenylhydrazine produced.

9*E* and **9***Z* pregnenolone pyrazole derivatives, respectively in quantitative yields (Scheme **3**). The analytical and spectral data of all the compounds are given above.

3.2. Biological Studies

3.2.1. Aromatase Inhibitory Activity

It has earlier been established that aryl substitution at pyrazole skeleton in heterocyclized steroids increases their inhibitory and breast cancer activities [11, 12, 33-38]. In continuation of our efforts towards finding potential type I aromatase inhibitors, we

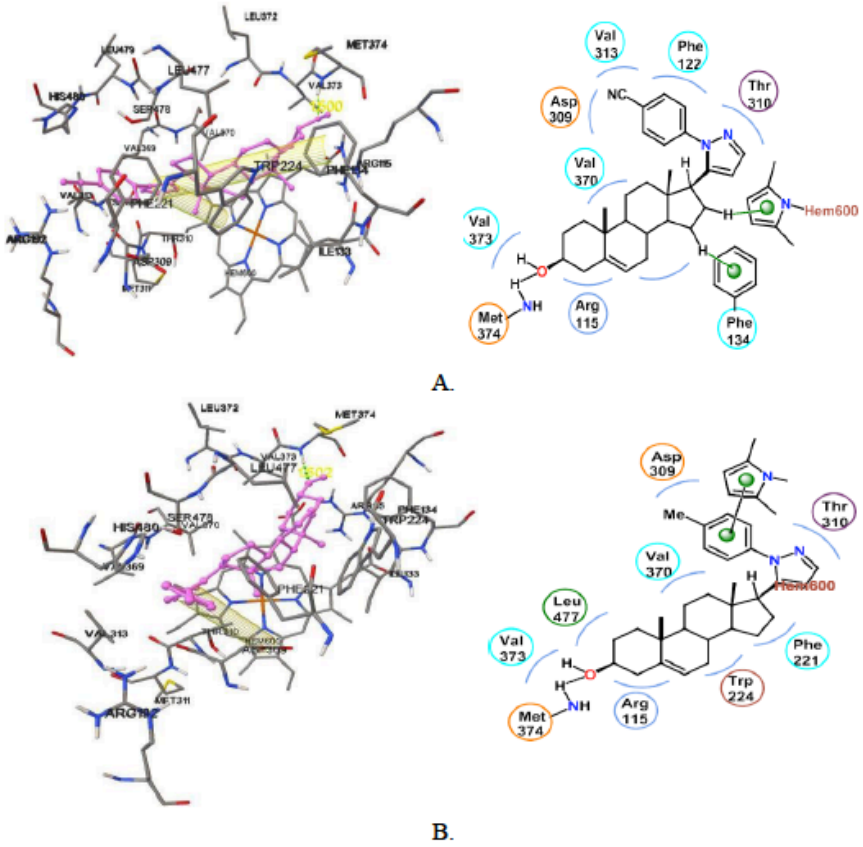


Fig. (1). A. (color online). Docked conformation of **4c** showing H-bonds between Met374 and OH-3 of the steroid scaffold. In addition, two H-p interactions between H-cyclopentane residue ad one of the pyrrole residue of Hem600 as well as the aromatic ring of Phe134 of the aromatase enzyme together with other hydrophobic interactions are observed. B. **4d** shows an H-bond between the oxygen atom OH-3 and NH_2 group of Met374 of the aromatase enzyme. Further, the aromatic ring of the pyrazole residue forms a pi-pi interaction with one of the pyrrole group of Hem600. Several hydrophobic interactions were witnessed.

decided to evaluate the impact of diversifying the functionality of D-ring pyrazolyl pregnenolones. The *in vitro* anti-aromatase activity of compounds **4a- e**; **5a-e**; **7a**,**b**; **8a**,**b** and **9a**,**b;** were valued, following the modified method of Prachayasittikul *et al.* [36] with Exemestane and Formestane as reference drugs. The results are presented in Table **1**, where the IC_{50} values of compounds are in the nanomolar range. Among all the tested analogs, compounds **4b**, **4c**, **4d**, and **5e** exhibited moderate aromatase inhibitory effect in a dose-dependent manner, with IC_{50} of 65.82 ± 1.03, 56.44 ± 1.21, 52.38 ± 0.98, and 87.53 ± 2.83 nM, respectively. These compounds displayed comparable aromatase inhibitory activity for Formestane and Exemestane, which are steroid based type I aromatase inhibitors approved against breast cancer in postmenopausal women. Furthermore, our study revealed that *N*-phenyl pyrazole derivatives **4a-e/5e** were more active than C- phenyl derivatives **8a**,**8b**/**9a**,**9b**.

The preliminary SAR study suggests that the introduction of a moderate hydrophobic substituent, such as *N*-phenyl mono-substituted pyrazoles would buttress the inhibitory activity, whereas introducing more lipophobic substituents through two aryl substitutions at C- and N-centers moderately decreases the activity. Amongst the *N*-phenyl pyrazole regioisomers, derivatives of type 4 (where the steroid backbone is attached at C-17 to C-5 of the pyrazolyl ring) showed moderately better inhibitory activity than the derivatives of type 5 (where the steroid backbone is attached at C-17 to C-3 of the pyrazolyl ring) indicating that the stereochemistry of type 4 regioisomers is more favorable for their androgen agonist property. Further, the acyclic analogs **7a** and **7b** showed very less activity (inhibition < 50%), indicating that heterocyclization at C-17 is essential for the inhibitory activity as expected.

3.2.2. In vitro Anti-breast Cancer Activity

All the synthesized pregnenolone analogs were evaluated for their *in vitro* antiproliferative activities against MCF-7, BT-20 and T-470 breast cancer cell lines by sulforhodamine B assay [37], using Exemestane as a positive control. The 50% inhibitory concentration (IC_{50}, µM) values were determined for these compounds, and the results are summarized in Table **2**. Notably, anticancer evaluation revealed good cytotoxic activities of some derivatives against MCF-7, BT-20 and T-470 cell lines, comparable to the approved drug, Exemestane. Overall, compounds **4b**, **4c**, **4d** and **4e** showed the highest activity in the series. Concerning MCF-7 cell lines, the most active compounds were the 4-cyano- and 4-methylphenyl derivatives **4c** and **4d** with IC_{50} = 2.44 ± 0.65 µM and 3.38 ± 0.98 µM respectively, compared to IC_{50} = 0.92 ± 0.12 µM for the reference drug Exemestane. Furthermore, the most promising activity against BT-20 cell lines was again observed for the compounds **4c** and **4d** with IC_{50}= 4.62 ± 1.34 (µM) and 3.64 ± 1.44 µM, respectively, whereas the IC_{50} of Exemestane was 1.33 ± 0.34 µM. Against T-470 cell lines, **4c** and **4d** exhibited IC_{50} 2.54 ± 0.83 and 2.23 ± 0.54 µM in comparison to those of the reference drug, Exemestane (IC_{50} = 1.93 ± 0.43 µM).

The antiproliferative data against the breast cancer cell lines revealed that there is strong coherence with the results of aromatase inhibition, which supports our understanding that the mechanism of action of the newly synthesized pregnenolone analogs is through the inhibition of aromatase. The compounds which are most active (least IC_{50} values) for aromatase inhibition, exhibit the highest antiproliferative activity against the breast cancer cell lines. This hypothesis is also supported by the molecular docking studies

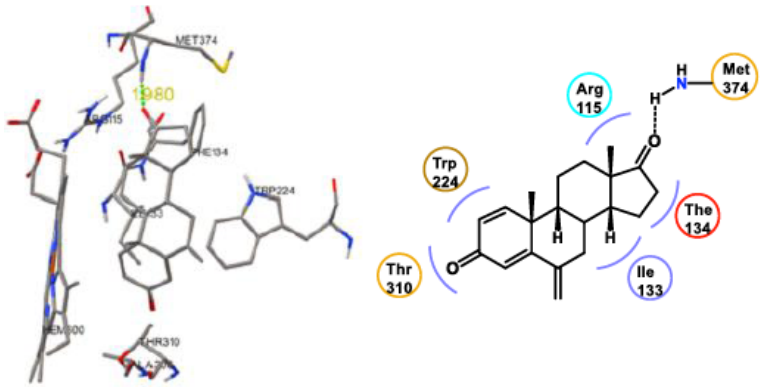


Fig. (2). Computer model of human AR (pdb id 4GL7) with exemestane shows a hydrogen bond between the lone pair of oxygen atom (C=O) of the carbony group at C-17 of pregnene scaffold and the NH2 group of Met374 (1.980 Å) residue. In addition, non-bonded amino acid residues as The134, Arg115, Ile133, Trp224 and Thr310 of the receptor surrounding exemestane molecule were observed.

where the most active analogs *i.e.* **4c** and **4d,** exhibit the highest binding scores against the active site of the aromatase pocket.

4. MOLECULAR DOCKING STUDY

The molecular docking study of the new steroid analogs is based on the modelling studies, which were performed to understand the binding mode of these analogs with the human aromatase enzyme using Autodock4 [34] and the docking results were viewed and analysed by MGLTools. In the docking study, X-ray crystal structure of the human aromatase enzyme (PDB ID: 4GL7) [30] was obtained from the Protein Data Bank server (www.rcsb.org).

Compound **4c** and **4d** have been selected for the docking study as these exhibited significant activity as aromatase enzyme inhibitors of the series. The binding energy scores of these compounds were found -9.22 and -8.89 kcal mol^{-1}, respectively, indicating selectivity and potency profiles of these analogs to bind the active site of aromatase pocket.

Detailed analysis of the binding mode showed that compound **4c** is settled down in the enzyme active site properly (Fig. **1A**). As shown below, cyclopentane ring of **4c** points toward the aromatic ring of the Phe134 residue apparently indicated to H–π stacking interactions with the two residues. In addition, there is an H–π stacking interaction between H-cyclopentane residue and one of the pyrrole residue of Hem600. The pregnene backbone is located in the middle of the binding pocket, anchoring the oxygen atom OH-3 in a favorable position for hydrogen bonding (1.500 Å) with NH_2 group of Met374 of the aromatase enzyme as well as extensive hydrophobic interactions with the surrounding residues, including Val373, Arg115, Thr310, Asp309, Phe221, Val313, and Val370. Overall, the combination of hydrophobic interaction and π stacking appears to govern the binding of **4c** with aromatase enzyme.

The same strategy**,** as described for compound **4c** was employed to identify the core structure of **4d**. Compound **4d** demonstrated the best docking pose based on docking score since it displays compatible settlement with the enzyme active region. As shown in Fig. **1B**, the aromatic ring of the pyrazole residue forms a p-p interaction with one of the pyrrole group of Hem600, meanwhile a H-bond (1.502 Å) between the oxygen atom OH-3 and NH_2 group of Met374 of the aromatase enzyme is observed. The molecule shows hydrophobic interactions with receptors-binding residues of the aromatase enzyme, including Arg115, Trp224 Phe221, Thr310, Asp309, Leu477, and Val373.

The binding mode of the reference drug exemastane in the active site of the human aromatase enzyme at equilibrated state is displayed in Fig. 2 for comparsion purposes with those of the ligands 4c and 4d (Fig. 2). The binding energy score for exemestane is -12.30 kcal mol-1, indicating a good selectivity and potency of this ligand to bind to the active site of the protein receptor pocket (4GL7). The cyclopentanone group in exemestane is pointed to the NH2 group of Met374 group in aromatase active site and coordinate through a hydrogen bonding ((1.980 Å) to the carbonyl group through its oxygen atom at C-17. In addition, it was observed that this ligand occupied hydrophobic pocket with the residues The134, Arg115, Ile133, Trp224 and Thr310

CONCLUSION

In the present work, we have studied the human aromatase inhibitory activity of a library of structurally and functionally versatile pregnenolone pyrazoles so as to have a preliminary idea about the structure- activity relationship (SAR) of various D-ring modified pregnenolone pyrazoles. We modified the position, number and nature of substitution at the pyrazole ring to evaluate its impact on the aromatase inhibition. The aromatase inhibitory activities of these compounds were moderate to good when compared to the reference standards. All the compounds were screened for anticancer activities against three human breast cancer cell lines (MCF-7, BT-20 and T-470) and the IC_{50} values were calculated. IC_{50} values of compounds **4b**, **4c**, **4d**, and **4e** were highest in the series, for both aromatase inhibitory as well as antiproliferative activity. IC_{50} values of compounds **4c** and **4d** were comparable to the control drug exemestane. The coherence between the results of aromatase inhibition and antiproliferative activity supports our analysis that the newly synthesized analogs work as anticancer agents through aromatase inhibition. These studies revealed that such molecules have high scope and potential for further investigation towards the treatment of estrogen dependent breast cancer. Molecular docking studies were in agreement with the enzyme inhibitory activity data. Studies on extensive diversification, mechanistic analysis and application of pharmacognosy principles are currently under process to come up with better leads.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this study are available within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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