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Synthesis of some novel androstanes as potential aromatase inhibitors

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ABSTRACT

Novel pyrazole and isoxazole derivatives (**6**–**9**) were synthesized as a aromatase inhibitors. Pyrazole was synthesized from hydrazine hydrate and isoxazoles from hydroxylamine hydrochloride under different conditions. Molecular docking studies were carried out for the synthesized compounds. The best score was obtained for the compound (**9**) followed by compound (**6**) while compound (**8**) afforded poorest of the score. Aromatase inhibitory activity for compound (**6**) having pyrazole ring at 2,3 position showed highest activity followed by nitrile derivative (**9**). Isomeric forms of isoxazole (**7** and **8**) showed very poor activity compared to fadrozole and aminoglutethimide. Preliminary kinetic studies have shown that both of the active compounds (**6** and **9**) are reversible inhibitors of the enzyme.

1. Introduction

Estrogens are essential regulators of many physiological processes including maintenance of the female sexual organs, the reproductive cycle and numerous neuroendocrine functions. Along with these normal physiological functions, these hormones also play crucial roles in some disease states, particularly in breast cancer, where through binding to their target receptor, they promote proliferation of breast cancer cells [1]. Breast cancer is the most common cancer diagnosed in women, and despite advances in treatment, remains the second most common cause of death in women in the Western world. Generally, it is thought of as a disease of older women; however 22% of the cases occur in those below the age of 50 [2]. Worldwide, more than one million women develop breast cancer each year with nearly half of these diagnoses occurring in the Unites States and Europe. Moreover, nearly 40% of these women die of their diseases [3]. Approximately two-thirds of postmenopausal breast cancer patients have estrogen-dependent breast cancer, which contains estrogen receptors (ERs) and requires estrogen for tumor growth [4].

Production of estrogens takes place in many tissues throughout the body including the ovaries, adipose tissue, muscle, liver, breast tissue and malignant breast tumors. In premenopausal women the ovaries are the main source of circulating estrogens while in the postmenopausal women the main source is adipose tissue and muscle [5]. Aromatase (CYP19) is the cytochrome P450 enzyme responsible for the conversion of androgens including androstenedione and testosterone, to the estrogen products, estrone and estradiol [6]. Expression of aromatase is highest in or near the breast tumor cells [7,8]. Endocrine-deprivation therapy for breast cancer is based on the knowledge that certain tumors require estrogen for their continued growth [9]. Antagonizing estrogen action is a popular treatment strategy because ER overexpression is observed in about 70% breast cancers [10–12]. Nonsteroidal antiestrogens like tamoxifen are the most commonly used drugs in this category. Apart from its side effects, the use of tamoxifen beyond five years is not indicated leaving a large population of breast cancer survivors at risk for relapse [13]. An alternative strategy of hormone deprivation is to block specifically estrogen biosynthesis, irrespective of site of production [14]. Aromatase has been a particularly attractive target for inhibition in the treatment of hormone-dependant breast cancer since the aromatization of androgen substrates is the terminal and rate limiting step in estrogen biosynthesis [15].

Over the past two decades substantial efforts have been made towards developing potent inhibitors of aromatase [16]. Aromatase inhibitors have been subdivided into two main groups according to their mechanism of action and structure. Type-I inhibitors are steroidal in nature. These steroidal inhibitors associate to the substrate-binding site of the enzyme and might inactivate it irreversibly. Such mechanism-based inactivators of aromatase are very specific and show prolonged effects as neosynthesis of the enzyme is necessary for estrogen formation. The type-II inhibitors are azoles chemically. They are competitive reversible inhibitors and may lack

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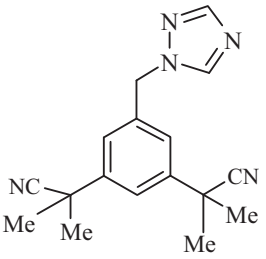
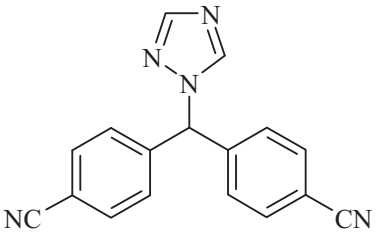
specificity [14]. However, until today several classes of potent and selective type-II inhibitors have been described [16–20]. Among those, three third generation aromatase inhibitors, anastrozole (**1**) [21], letrozole (**2**) [22] and the steroidal compound exemestane (**3**) [23] have been approved by US-FDA for use in breast cancer, with various other clinical applications being currently tested after these products had became commercially available [24–26]. Aromatase inhibitors can be utilized in several adjuvant settings [27]. Sufficient data are available to recommend the use of aromatase inhibitors as better alternatives to tamoxifen for adjuvant therapy [28]. Furthermore, these inhibitors have also been shown to be clinically effective in the management of other estrogen dependant pathological processes such as endometriosis [29], prostate hyperplasia and prostate cancer [30]. In addition, unlike tamoxifen that has mixed estrogen agonist and antagonist properties, the aromatase inhibitors have no estrogenic agonist activity [31].



(1)



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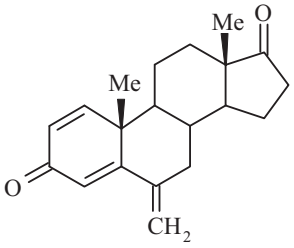
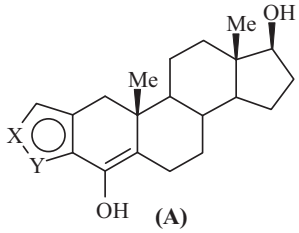


(3)

Treatment with aromatase inhibitors is generally well tolerated with low incidence of serious side effects. However short-term events like hot flushes, vaginal dryness, musculoskeletal pain and headache have been observed [2,32]. Accordingly there is need for new, potent, more selective and less toxic CYP19 inhibitors [33]. Eventually new aromatase inhibitors could also be superior to the current compounds regarding the acquirement of resistance [1].

Mechanism-based inhibitors have distinct advantages in drug design strategy because these inhibitors are highly enzyme specific, produce prolonged inhibition and often exhibit minimum toxicitiy [4]. Exemestane (**3**), a third generation steroidal mechanism-based aromatase inhibitor has not been found to affect cortisol or aldosterone secretion at baseline or in response to adrenocorticotropic hormone at any dose [34]. The apparent lack of cross-resistance between exemestane (**3**) and other aromatase inhibitors is a major benefit of the drug [35]. Of the three agents, exemestane (**3**) is the most recently FDA approved drug. It may differ from the others as the steroidal structure potentially protects bone and lipid metabolism from estrogen ablation [11].

In the steroidal moieties, effective aromatase enzyme inhibition is observed with an androstane skeleton possessing an A/B trans ring junction, a ketone functionality at the C-3 position, unsaturation in the steroid nucleus (4-ene, 4,6-diene or 1,4,6-triene functions) and either a 17-ketone or 17β-hydroxyl group [4]. In light of the above discussion it was planned to synthesize some novel steroidal derivatives with the given structure (**A**) wherein the 2,3 position of the ring-A is fused to five-membered heterocyclic rings like isoxazole (X = O/N and Y = N/O) and pyrazole (X = Y = N).



(A)

2. Experimental

2.1. General

Melting points were determined using a VEEGO make microprocessor based melting point apparatus having silicone oil bath and are uncorrected. IR spectra (wave numbers in cm^{-1}) were recorded on a BRUKER ALPHA T FT-IR spectrophotometer using KBr discs. NMR spectra were recorded on BRUKER AVANCE II 400 MHz instrument in $CDCl_3$ with TMS as internal standard for ^1H NMR. Chemical shift values are mentioned in δ, ppm. Chromatographic separations were performed on silica gel columns. The progress of all reactions was monitored by TLC on 2 cm × 5 cm pre-coated silica gel 60 F254 plates of thickness of 0.25 mm (Merck). The chromatograms were visualized under UV (254 nm) and iodine vapours. The term "dried" refers to the use of anhydrous sodium sulfate. All reagents used were of analytical reagent grade.

2.2. Chemical

2.2.1. 2-Formyl 4,17β-dihydroxy-4-androsten-3-one (*5*)

4-Hydroxytestosterone (**4**) [36] (0.5 g, 0.016 mol) and sodium methoxide (1.0 g) in dry pyridine (15 ml) were stirred under nitrogen at −5 °C for 20 min. Freshly distilled ethyl formate (1.0 ml) was added to the reaction mixture maintaining the temperature at 0 °C. The reaction mixture was left in refrigerator for 72 h with occasional shaking. The reaction mixture was acidified with conc. HCl, diluted with cold water and extracted with solvent ether. The solvent ether extract was washed with water and extracted with 10% NaOH solution. The alkaline solution was washed with solvent ether, acidified with conc. HCl and extracted with solvent ether. The ether layer so obtained was dried and evaporated slowly, to afford compound (**5**) (0.2 g, 40%, mp 208–212 °C) after crystallization.

UV (MeOH): 374 nm (log Є4.22), UV (Alk. MeOH): 379 nm (log Є4.38). IR (KBr): 2942, 2844, 1695, 1640, 1417, 1173, 1070, 939. ^1H NMR: δ 10.29 (s, 1H); 3.64–3.68 (t, 1H); 2.77–2.81 (d, 1H); 2.29–2.33 (dd, 1H), 2.12–2.16 (d, 1H); 1.21–1.35 (m, 4H); 0.89–1.11 (m, 6H); 0.86 (s, 3H); 0.75 (s, 3H).

2.2.2. 17β-Hydroxy-4-oxo-5α-androstano[2,3-d]pyrazole (*6*)

Compound (**5**) (0.5 g, 0.0015 mol) and hydrazine hydrate (0.09 ml, 0.0018 mol) were refluxed in aldehyde-free ethanol (25 ml) for 2 h. Excess ethanol was removed and the reaction mixture was poured into cold water, filtered and dried. The dried residue afforded compound (**6**) after recrystallization from methanol (0.2 g, 50%, mp 245–249 °C).

UV (MeOH): 270 nm (log Є4.31). IR (KBr): 3388, 3258, 3319, 2944, 2844, 1634, 1545, 1447, 1259, 1074, 959. ^1H NMR: δ 12.5 (s, 1H); 7.24 (s, 1H); 3.51–3.55 (t, 1H); 2.70–2.74 (d, 1H); 2.51–2.53 (dd, 1H); 2.20–2.24 (d, 1H); 1.19–1.38 (m, 6H); 0.92–1.18 (m, 4H); 0.68 (s, 3H); 0.64 (s, 3H).

*2.2.3. 17β-Hydroxy-4-oxo-5α-androstano[3,2-c]isoxazole (**7**)*

Compound (**5**) (0.5 g, 0.0015 mol) and hydroxylamine hydrochloride (0.25 g, 0.036 mol) were stirred in aldehyde-free ethanol (25 ml) containing a few drops of pyridine for 2 h at room temperature and left overnight. Excess of ethanol was recovered under reduced pressure and the reaction mixture was poured into water, filtered and dried. The dried residue afforded compound (**7**) after recrystallization from ether (0.3 g, 60%, mp 210–212 °C).

UV (MeOH): 310 nm (log Є3.80), UV (Alk. MeOH): 362 nm (log Є3.89). IR (KBr): 3300, 2941, 2844, 1676, 1451, 1397, 1250, 1001, 944. ^{1}H NMR: δ 8.29 (s, 1H); 3.55–3.58 (t, 1H); 2.82–2.86 (d, 1H); 2.27 (s, 3H); 2.20–2.25 (dd, 1H); 1.83–1.87 (d, 1H); 1.06–1.23 (m, 6H); 0.86–1.03 (m, 4H); 0.85 (s, 3H); 0.71 (s, 3H).

*2.2.4. 17β-Acetoxy-4-oxo-5α-androstano[2,3-d]isoxazole (**8**)*

Compound (**5**) (0.5 g, 0.0015 mol) was heated with hydroxylamine hydrochloride (0.25 g, 0.004 mol) in glacial acetic acid (10 ml) for 8 h at 80 °C and left overnight. Glacial acetic acid was removed under reduced pressure and the reaction mixture was poured into water, filtered and dried. The dried residue afforded compound (**8**) after recrystallization from methanol (0.25 g, 60%, mp 221–225 °C).

UV (MeOH): 256 nm (log Є4.29). UV (Alk. MeOH): 336 nm (log Є4.11). IR (KBr): 2947, 2842, 1732, 1694, 1452, 1364, 1240, 1171, 1042, 937, 906. (^{1}H NMR): δ 8.26 (s, 1H); 4.61–4.65 (t, 1H); 2.81–2.85 (d, 1H); 2.56–2.60 (d, 1H); 2.45–2.49 (dd, 1H); 2.05 (s, 3H); 0.89 (s, 3H); 0.81 (s, 3H).

*2.2.5. 2-Cyano-3,17β-dihydroxy-5α-androst-2-en-4-one (**9**)*

Compound (**8**) (0.2 g, 0.0006 mol) was stirred with sodium methoxide (0.5 g) in dry THF (10 ml) under nitrogen atmosphere at room temperature for 30 min. The reaction mixture was poured into water, acidified with conc. HCl and extracted with dichloromethane (3× 25 ml). The combined organic extracts were washed, dried and the solvent evaporated to yield the crude product. Purification by column chromatography (5 g silica gel, ethyl acetate–n-hexane, 8:2) afforded compound (**9**) 0.12 g, 55%; mp 235–237 °C after recrystallization from acetone-n-hexane.

UV (MeOH): 286 nm (log Є4.29). UV (Alk. MeOH): 336 nm (log Є4.12). IR (KBr): 3127, 2966, 2847, 2210, 1691, 1450, 1380, 1172, 1067, 947. ^{1}H NMR: δ 3.58–3.62 (t, 1H); 2.85 (t, 1H); 2.46–2.50 (d, 1H); 2.39–2.44(d, 1H); 2.29–2.33 (dd, 1H); 0.90 (s, 3H); 0.73 (s, 3H).

2.3. Molecular modeling studies

Molecular modeling studies were performed on a Silicon Graphics Fuel Workstation running on the IRIX 6.5 operating system using SYBYL 7.0 molecular modeling software from Tripos, Inc. [37] and GLIDE from Schrodinger Inc., USA [38] installed on Microsoft Windows XP Professonal (version 2002) based Intel core2 Duo 2.53 GHz PC (with 3.0 GB memory).

2.3.1. Molecular structure generation

All compounds were built from the fragments in the SYBYL database. A set of low energy conformations for each molecule under study were generated by dynamics using simulated annealing technique with Tripos force fields in SYBYL. The molecules were heated to 700 K followed by cooling to 300 K. Time spent for annealing was 1000 fs. Time increament for dynamics computations was 0.5 fs and coupling time for temperature regulation was 2.0 fs. Ten consecutive cycles were calculated. Repeating the cycle many times and collecting the low energy structure results in a set of low energy conformations. The lowest energy conformers thus, obtained were further minimized using the conjugate gradient method in SYBYL 7.0 using Tripos force field, atomic charges assigned by the Gasteiger–Huckel method with a distance dependent dielectric function until a root mean square (rms) deviation of 0.001 kcal/mol Å was achieved. The lowest energy conformer thus obtained was subsequently used for docking studies.

2.3.2. Docking studies

The crystal structure of human placental aromatase (pdb code: 3EQM) [39] obtained from the Protein Data Bank (USA) was refined to remove water molecules. The bond orders and formal charges were adjusted prior to docking. Docking was performed using GLIDE software according to the previously reported protocol except for scaling of van der Waals radii which was modified (scaling factor 0.60).

2.4. Biological

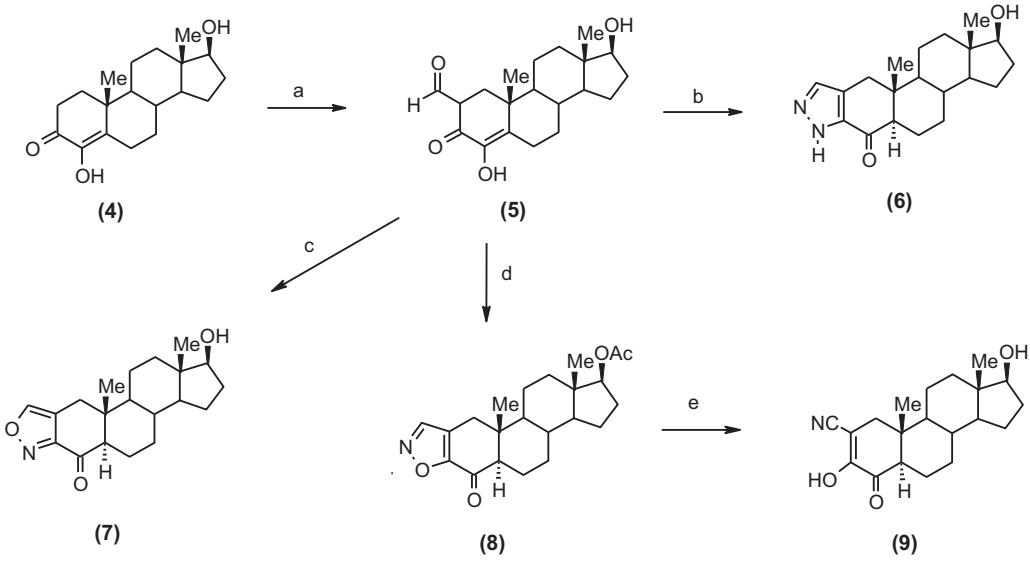
2.4.1. Aromatase inhibiting activity

2.4.1.1. Enzyme preparation. The enzyme was obtained from the microsomal fraction of freshly delivered human term placental tissue as per the procedure described by Thompson and Siiteri [40]. The isolated microsomes were suspended in minimum volume of phosphate buffer (0.05 M, pH 7.4, 20% glycerol). Additionally DTT (dithiothreitol, 10 mM) and EDTA (1 mM) were added to protect the enzyme from degradation. The enzyme preparation was stored at −70 °C.

2.4.1.2. Assay.

2.4.1.2.1. Normal test procedure. The assay was performed according to our procedure [41]. Each incubation tube contained [1β-3H] androstenedione (0.08 μCi, 15 nM), unlabeled androstenedione, (485 nM) NADP, (2 mM), glucose-6-phosphate, (20 mM) glucose-6-phosphate-dehydrogenase (0.4 units) and inhibitor in phosphate buffer (0.05 m, pH 7.4). The test compounds were dissolved in DMSO and diluted with buffer. The final DMSO concentration in the control and inhibitor incubation was 2%. Each tube was preincubated for 5 min at 30 °C in water bath. Microsomal protein was added to start the reaction (0.1 mg). The total volume of each incubation was 0.2 ml. The reaction was terminated by the addition of cold solution of mercuric chloride (1 mM, 200 μl). After addition of Norit A (Serva, Heidelberg, Germany) (2%, 200 μl), the vials were shaken for 20 min and centrifuged at 1500 × *g* for 5 min to separate the charcoal-absorbed steroids. The supernatant was assayed for ^3H_2O by counting in a scintillation mixture using PerkinElmer-Wallac β-Counter. The calculation of the IC_{50} value was performed by plotting the percent inhibition vs. the concentration of inhibitor on a semi-log plot. From this the molar concentration causing 50% inhibition was calculated.

2.4.1.2.2. Inhibition of aromatase by irreversibly binding compounds. The assay was performed similar to that of the normal test procedure. A preincubation of the aromatase containing microsomes was performed along with a regenerating system (2 mM NADP, 20 mM glucose-6-phosphate, 0.4 units of glucose-6-phosphate-dehydrogenase) and inhibitor in phosphate buffer (0.05 M, pH 7.4) for 30 min at 30 °C. The test compounds were dissolved in DMSO and diluted with buffer. The final DMSO concentration in the control and inhibitor incubation was 2%. After preincubation an aqueous dextran coated charcoal (DCC) suspension (2%) (Sigma, St. Louis, MO) was added followed by a shaking step for 20 min at 4 °C. After full-speed centrifugation 200 μl of the supernatant were supplemented with 50 μl of regenerating system and 50 μl substrate (15 nM [1β-^{3}H]androstenedione (0.08 μCi), 485 nM unlabeled androstenedione) to start the enzymatic reaction at 30 °C. After several time points (8, 16, and 24 min) 50 μl of the sample were stopped by the addition of 100 μl of a cold 1 mM $HgCl_2$ solution. After addition of 100 μl of Norit A (2%) (Serva, Heidelberg, Germany), the vials were shaken for 20 min and centrifuged



Scheme 1.

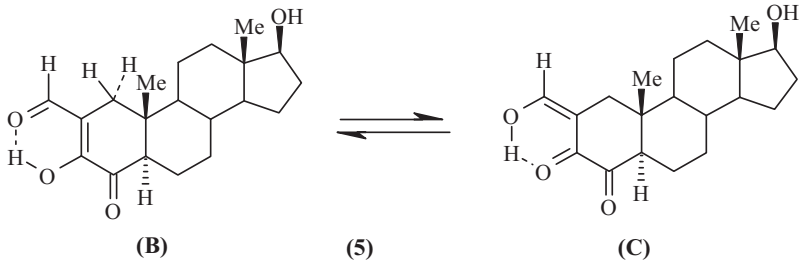
at 1500 × *g* for 5 min to separate the charcoal-absorbed steroids. The supernatant was assayed for ^3H_2O by counting in a scintillation mixture using a LKB-Wallac β-counter. Exemestane was used as a positive control that irreversibly binds to aromatase, as a negative control (not binding irreversibly) aminoglutethimide was used. The inhibition values after the three different incubation times were related to the DMSO control, averaged and compared with the inhibition values after performance of the normal test procedure using the same inhibitor concentration.

3. Results and discussion

3.1. Chemical

4,17β-Dihydroxy-4-androsten-3-one [36] (**4**) was used as the starting material for the synthesis of the desired products as per Scheme 1. Formylation of compound (**4**) with ethyl formate under basic conditions offered the desired product which has been depicted as structure (**5**). Compound (**5**) can exist in various tautomeric forms (like 'B' and 'C'). Form 'B' seems to be the dominant one as inferred from the spectral data [UV (MeOH): 374 nm (log Є4.22). UV (Alk. MeOH): 379 nm (log Є4.38). IR: 1695 cm^{-1} (4-C=O) and 1640 cm^{-1} (2-CHO). PMR: δ 10.29 (s, 1H 2-C*H*O); 2.77–2.81(d, 1H, 1α/β-*H*); 2.29–2.33 (dd, 1H, 5α-*H*); 2.12–2.16 (d, 1H, 1α/β-*H*)]. Molecular modeling studies also indicated higher stability of form (B) over form (C). Both of the structures (**5B** and **5C**) were minimized as per the protocol mentioned in Section 2.

The energy calculated for structures (**B**) (23.771 kcal/mol) and (**C**) (25.771 kcal/mol) clearly favored the dominance of form (**B**) over form (**C**).



According to the objective of this work, the 2-formyl derivative (**5**) was reacted with hydrazine hydrate in ethanol under refluxing conditions to obtain the desired pyrazole (**6**). Structure of compound (**6**) is in conformity with the spectral data obtained for the compound.

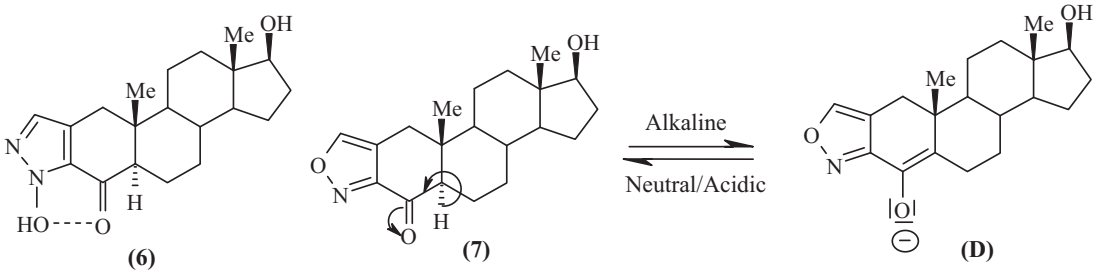


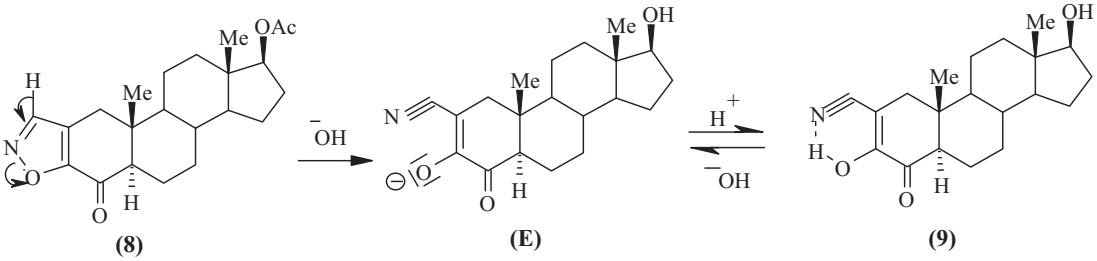
Table 1
G-Score and enzyme inhibition data of the compounds.

Compd.	*G*-score	% Inhibition[a] of aromatase in normal test procedure at		IC_{50} (nM)	% Inhibition[a] of aromatase after irreversible binding at
		0.5 μM conc.	2.0 μM conc.		2.0 μM Conc.
6	−7.77	46.1 ± 11.8	78.7 ± 2.6	512.0 ± 93.3	12.3 ± 2.1
7	−6.90	1.3 ± 2.2	–	–	–
8	−4.44	0.0 ± 0.0	–	–	–
9	−7.90	37.0 ± 10.3	53.6 ± 5.0	1019.8 ± 157.5	2.0 ± 3.5
3	−6.99		88.9 ± 0.8	153.9 ± 14.7	66.3 ± 0.6
Exemestane					
Aminogluthemide	–	–	3.6 ± 1.7	30 μM	2.0 ± 2.6
Androstenedione	−7.13	–	–	–	–
Formestane	−6.96	–	–	–	–
Control	–	–	–	–	–

[a] The given values are mean values of at least three experiments.

[UV (MeOH): 270 nm (log Є4.31). IR: 1634 cm^{-1} (4-C=O). PMR; 2.70–2.74 (d, 1H, 1α/β-*H*); 2.51–2.53 (dd, 1H, 5α-*H*); 2.20–2.24 (d, 1H, 1α/β-*H*)]. It is worth noting that the UV_{max} of compound (**6**) does not show a bathochromic shift in alkaline medium and the vibrational band of 4-keto moved towards lower frequency (1634 cm^{-1}). Both of these effects could be explained by the thermodynamic stabilization of the system due to intramolecular hydrogen bonding. Treatment of compound (**5**) with hydroxylamine hydrochloride in presence of few drops of pyridine in ethanol afforded the isoxazole (**7**). [UV (MeOH): 310 nm (log Є3.80). UV (Alk. MeOH): 362 nm (log Є3.89). IR: 1676 cm^{-1} (4-C=O). PMR: *δ*; 2.82–2.86 (d, 1H, 1α/β-*H*); 2.20–2.25 (dd, 1H, 5α-*H*); 1.83–1.87 (d, 1H, 1α/β-*H*)]. A big bathochromic shift in its UV spectrum in alkaline medium is anticipated due to its conversion into the anion (**D**).

Treatment of compound (**5**) with hydroxylamine hydrochloride under acidic conditions offered the isomeric isoxazole (**8**). [UV (MeOH): 256 nm (log Є4.29). UV (Alk. MeOH): 336 nm (log Є4.11). IR: 1694 cm^{-1} (4-C=O). PMR: *δ* 2.81–2.85(d, 1H, 1α/β-*H*); 2.56–2.60 (d, 1H, 1α/β-*H*); 2.45–2.49 (dd, 1H, 5α-*H*)]. Alkaline hydrolysis of isoxazole (**8**) yielded the desired nitrile derivative (**9**). [UV (MeOH): 286 nm (log Є4.29), UV (Alk. MeOH): 336 nm (log Є4.12); IR: 2210 (CN stretching), 1691 cm^{-1} (4-Oxo); PMR: *δ* 2.46–2.50 (d, 1H; 1α/β-*H*), 2.39–2.44(d, 1H; 1α/β-*H*), 2.29–2.33 (dd, 1H; 5α-*H*)]. Both of the compounds (**8** and **9**) convert to the same chrom-ophoric system (as shown in structure **E**) in alkaline medium. Intramolecular hydrogen bonding explains a slight shift in vibrational group frequency of nitrile in compound (**9**).



3.2. Molecular modeling

It was envisaged to perform molecular modeling studies of the synthesized compounds (**6**–**9**). Structures of these compounds were minimized as per the protocol given in Section 2. The energy minimized structures were docked independently into the active site of the aromatase enzyme. Similarly, the two known inhibitors exemestane (**3**) and formestane and the substrate androstenedione were also docked. Glide scores for the enzyme–drug complexes for all of them were calculated (Table 1). It is evident from the data that the best score was obtained for compound (**9**) followed by compound (**6**) while compound (**8**) afforded poorest of the score (−4.44).

The crystal structure of human placental aromatase [40] shows that the bound ligand- androgen makes a hydrogen bond with the backbone amide of Met 374. Similar hydrogen bonding was observed with oxygen of 17-hydroxyl group for compounds (**6**) and (**9**) (Fig. 1), which indicates that both the compounds bind with the active site in a similar way as the natural substrate (**3**). Moreover, compounds (**6**) and (**9**) showed additional hydrogen bonding of C-4 carbonyl group with amino acid residue Thr 310 (Fig. 1). Compound (**8**) did not show the hydrogen bonding with Met 374 or Thr 310. This could be one of the probable reasons for poor docking score for compound (**8**). Compound (**7**) showed hydrogen bonding with Met 374 with oxygen of C-4 hydroxyl group (instead of C-17) which is contrary to the hydrogen bonding of the natural substrate, flipping the conformer in opposite direction. Thus, poor binding score of the compound (**7**) could be because of its unfavorable positioning in contrast to the natural substrate in the active site. Compound (**6**) and (**9**) showed good contacts with Ala 306, The 310, Trp 224, Ile 133, Phe 134, Val 370, Leu 372, Val 373, Met 374 and Ser 478, active site residues of aromatase.

Takahashi et al. have reported that hydrophobic amino acids such as Ile 132, Ile 133, Ile 305, Phe 148, Met 303, and Ala 306 may play a critical role in the binding to the active site [42]. Similar type of observation was made with the compound (**6**) and compound (**9**) as shown in Fig. 2a and b, respectively. Both of the compounds are having good contacts with β-carbon of Ala 306 through the hydrogen attached to position-3 of the A-ring while, this feature is missing in compounds (**7**) and (**8**) (Fig. 2c). This could be one of the reasons for the good binding score of both the compounds (**6** and **9**).

3.3. Biological

All of the four synthesized compounds (**6**–**9**) were evaluated of their aromatase inhibiting activity. The assay was performed by monitoring the enzyme activity by measur-

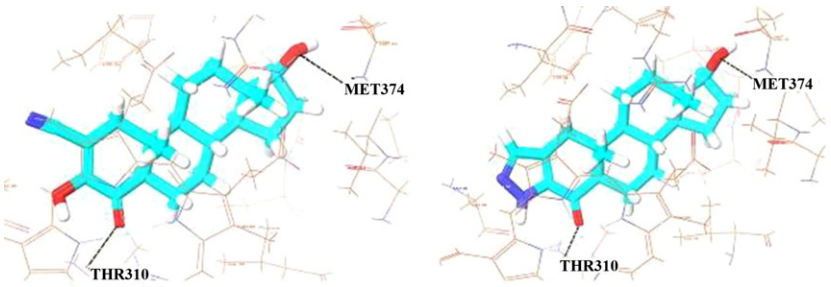


Fig. 1. Docked conformations of (A) compound **6** and (B) compound **9** along with the important amino acid residues of human placental aromatase. Both the compounds are forming two hydrogen bonds.

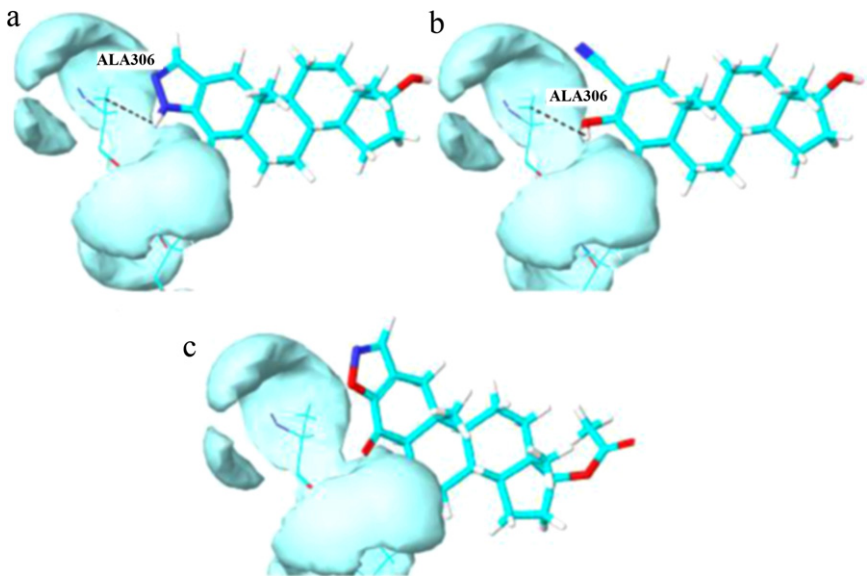


Fig. 2. Interaction of (a) compound (**6**) and (b) compound (**9**) with β-carbon of Ala 306. NH of compound (**6**) and OH of compound (**9**) orients to the hydrophilic pocket where as (c) compound (**8**) dislocates from the hydrophilic pocket.

ing the ^3H_2O formed from [1β-^{3}H] androstenedione during aromatization. The activity profile of the compounds is indicated in Table 1. IC_{50} values of aminoglutethimide and fadrozole were determined to be 30 μM and 30 nM in the test system utilized for the evaluation of the test compounds. It is ample clear from Table 1 that the pyrazole derivative (**6**) showed the highest activity followed by nitrile derivative (**9**). Both of the isomeric isoxazoles were found to be inactive. It is worth noting that both of the active compounds (**6** and **9**) have hydrogen bond donating groups attached to position 3 of the A-ring while, this feature is missing in the two inactive derivatives (**7** and **8**). There is a high probability that these compounds are binding to a site in the aromatase enzyme which acts as a hydrogen bond acceptor thereby increasing the stability of the enzyme–inhibitor complex. Exemestane (**3**) does not have hydrogen bond donor group in ring A or B. As discussed earlier, exemestane (**3**) is a mechanism-based inhibitor as it has electrophilic sites in ring A and B which can lead to covalent bond formation between the enzyme and the drug. Compounds (**6** and **9**) do not have such active sites indicating that these compounds should be acting as reversible competitive inhibitors.

The normal test procedure used for the determination of IC_{50} values would assess both, reversible as well as irreversible binding of the test compounds to the aromatase enzyme. Hence, it was decided to perform a separate test which would assess whether the test compounds were binding to the enzyme irreversibly. The test compounds were allowed to interact with aromatase enzyme in a physiological environment followed by treatment with dextran-coated charcoal (DCC) to remove unbound test compounds. The enzyme preparation so obtained was incubated with the substrate [1β-^{3}H]androstenedione and the enzyme activity evaluated by measuring the concentration of ^3H_2O using a scintillation counter. The experiment clearly indicated (Table 1) that both the compounds (**6** and **9**) bind to the enzyme in a reversible manner. This is contrary to the binding of exemestane (**3**) with the enzyme.

4. Conclusion

The novel pyrazole (**6**), isoxazole (**7** and **8**) and nitrile (**9**) derivatives were synthesized. Molecular docking studies were carried out for the synthesized compounds showed good docking score for compound (**9**) followed by compound (**6**) while compound (**8**) afforded poorest of the score. Aromatase inhibitory activity for compound (**6**) having pyrazole ring at 2,3 position showed the highest activity followed by nitrile derivative (**9**). Isomeric forms (**7** and **8**) of isoxazole showed very poor activity compared to fadrozole and aminoglutethimide.

It is worth noting that both of these compounds (**6** and **9**) bear a hydrogen bond donating group at position 3 of the A-ring. This feature is lacking in compounds (**7** and **8**) which show poor binding affinity for the enzyme. Preliminary enzyme binding studies indicate reversible binding of test compounds (**6** and **9**) to the enzyme contrary to exemestane (**3**) which is a known irreversible inhibitor of aromatase enzyme.

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