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Synthesis and aromatase inhibitory activity of some new 16*E*-arylidenosteroids

Ranju Bansal [a,*], Sridhar Thota [a], Nalin Karkra [a], Maninder Minu [a], Christina Zimmer [b], Rolf W. Hartmann [b]

[a] University Institute of Pharmaceutical Sciences, Sector-14, Panjab University, Chandigarh 160 014, India
[b] Pharmaceutical and Medicinal Chemistry, Saarland University, Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Campus C2.3, D-66123 Saarbrücken, Germany

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ABSTRACT

A new series of 16*E*-arylidene androstene derivatives has been synthesized and evaluated for aromatase inhibitory activity. The impact of various aryl substituents at 16 position of the steroid skeleton on aromatase inhibitory activity has been observed. The 16*E*-arylidenosteroids **6**, **10** and **11** exhibited significant inhibition of the aromatase enzyme. 16-(4-Pyridylmethylene)-4-androstene-3,17-dione (**6**, IC_{50}: 5.2 μM) and 16-(benzo-[1,3]dioxol-5-ylmethylene)androsta-1,4-diene-3,17-dione (**11**, IC_{50}: 6.4 μM) were found to be approximately five times more potent in comparison to aminoglutethimide.

1. Introduction

Aromatase is a cytochrome P-450 dependent enzyme that catalyzes the aromatization of androgens to estrogens and hence plays a key role in endocrine physiology and estrogen-dependent breast cancer. It is comprised of a polypeptide chain of 503 amino-acid residues and a prosthetic heme group at its active site [1,2]. The enzyme complex is bound in the endoplasmic reticulum of the cell. The androgens are converted to estrogens by aromatase *via* three sequential oxidation steps as depicted in Fig. 1. The process utilizes three moles each of oxygen and NADPH for the overall conversion. The first step consists of a typical cytochrome P-450 hydroxylation of the angular C-19 methyl group which stereospecifically forms the 19-hydroxyandrostenedione. The second step is another stereospecific hydroxylation of the C-19 methyl, in which the 19-pro-R hydrogen is displaced to give a C-19,19-gem diol. The gem diol then can readily dehydrate to produce 19-oxoandrostenedione [1,2]. The first two steps entails the hydroxylation of C19-methyl group, which is modulated by three amino acid residues, comprising A306 and T310, and two catalytic water molecules, which activates the ferrous dioxygen to the hydroxylating Fe(IV)=O form. This is followed by a H2β abstraction of the 2,3-enolization process in the aromatization step that essentially involves a nucleophilic attack on 2β hydrogen by A306 and T310 along with concerted electrophilic attack on C3-keto oxygen by D309 to drive H2β abstraction and 2,3-enolization. The final step consists of another oxidation that results in the elimination of the 1β hydrogen as water and incorporation of the C-19 into formic acid [3].

In breast cancer, intratumoral aromatase is the source for local estrogen production and inhibition of this enzyme is an important approach for reducing tumor growth [4]. Over the past two decades, substantial effort has been directed towards developing potent inhibitors of aromatase. There are two classes of AIs, steroidal and nonsteroidal compounds, which cause potent estrogen suppression [5]. The non-steroidal AIs are mostly azole type compounds such as the clinically used anastrozole and letrozole, which compete with the substrate for binding to the enzyme active site [6]. Among steroidal agents, formestane (**1**) was widely used during the early 1990s, but it is not used nowadays because of the need to administer it by intramuscular injection. Therefore, the orally active exemestane (**2**) (Fig. 2) is the main steroidal inhibitor of contemporary importance [7]. These steroidal inhibitors mimic the natural substrate androstenedione and are converted by the enzyme to reactive intermediates, which bind irreversibly to the enzyme active site, resulting in inactivation of aromatase [8].

Despite the success of the third-generation steroidal and nonsteroidal AIs, they still have some major side effects, such as musculoskeletal effects (arthritis, arthralgia, and/or myalgia) and bone toxicity [9]. For this reason, it is important to search for other potent and specific molecules with lower side effects.

Among the various nonsteroidal aromatase inhibitors, a series of pyridyl-substituted indanones, indans, and tetralins have been reported and developed as potent and selective agents. Of these, indanone derivatives 2-(4-pyridylmethylene)-1-indanone (**3**) (Fig. 2) and its corresponding saturated pyridyl–methyl analogue have been reported to possess good selectivity and increased potency toward aromatase as compared to aminoglutethimide [10]. A large number of potent steroidal derivatives with substitution at position 16 have been described in the literature as potent cytotoxic agents [11,12]. Recent work from our laboratory has also demonstrated the effectiveness of 16*E*-arylidenosteroids as potential cytotoxic and aromatase inhibitors [13,14]. These observations prompted us to prepare and study some more new 16*E*-arylidenosteroids as

* Corresponding author. Fax: +91 172 2543101.
E-mail address: ranju29in@yahoo.co.in (R. Bansal).

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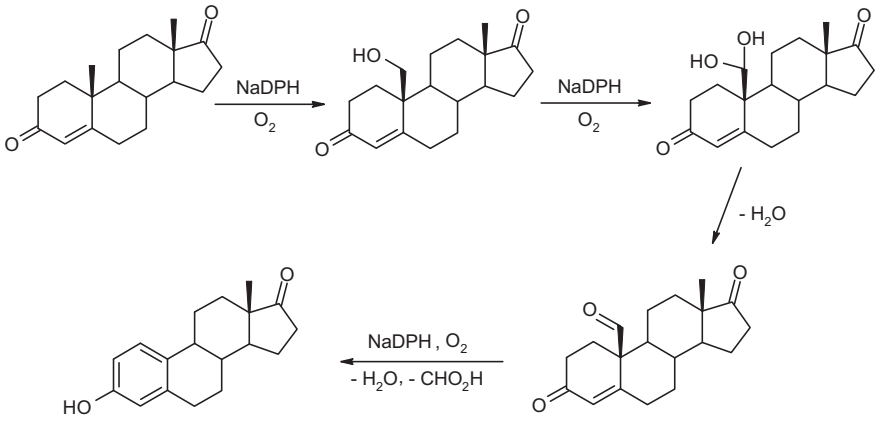


Fig. 1. Aromatization of androgens to estrogens through aromatase enzyme.

aromatase inhibitors, in which structural features of various nonsteroidal aromatase inhibitors have been incorporated.

2. Materials and methods

Melting points were determined on a Veego melting point apparatus and are uncorrected. UV (wavelengths in nm) was recorded on Lambda 15 and IR (wave numbers in cm^{-1}) spectra on Perkin–Elmer spectrum RX 1, FT-IR spectrophotometer models using KBr pellets. ^{1}H NMR spectra were recorded on BrukerAC-300F, 300 MHz using deuterated chloroform ($CDCl_3$) or deuterated dimethylsulfoxide (DMSO-d_6) containing tetramethylsilane as internal standard (chemical shifts in ppm). Mass spectrum was recorded on a Vg-11-250J 70 S model (VG Analytical Ltd., Manchester, England). Elemental analyses were carried out on a Perkin–Elmer-2400 model CHN analyzer. Plates for TLC were prepared according to Stahl (E. Merck) using EtOAc as solvent (activated at 110 °C for 30 min) and were visualized by exposure to iodine vapors. Anhydrous sodium sulfate was used as drying agent. All solvents were distilled prior to use according to standard procedures.

2.1. Chemical synthesis

2.1.1. General method for the preparation of compounds ***1–5***

A mixture of dehydroepiandrosterone (2.60 mmol), the appropriate aldehyde (4-pyridine carboxaldehyde/3,4,5-trimethoxybenzaldehyde/4-methoxy-2-nitrobenzaldehyde/benzo-[1,3]dioxol-5-carboxaldehyde/imidazole-2-carboxaldehyde) and potassium hydroxide (1 g) in methanol (20 ml) was stirred at room temperature for 2 h until the reaction was completed (monitored by TLC). Cold water was added to the reaction mixture and the precipitate obtained was filtered, washed with water, dried and crystallized from methanol to give the corresponding 16-arylideno steroid **1–5**.

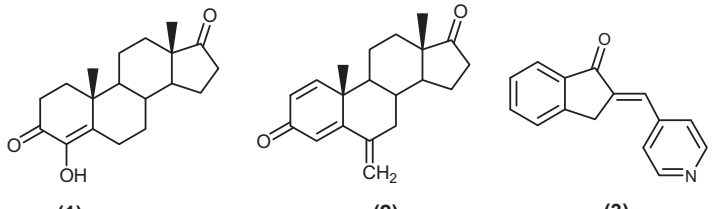
*2.1.1.1. 16-(4-Pyridylmethylene)-17-oxo-5-androsten-3-ol (**1**).* Yield: 61.5%. m.p. 220–224 °C. IRν_{max} (KBr): 3420, 2945, 1720, 1600, 1080. ^{1}H NMR ($CDCl_3$): 0.99 (s, 3H, 18-C*H*$_3$), 1.07 (s, 3H, 19-C*H*$_3$), 3.54 (m, 1H, 3α-H), 5.38 (s, 1H, 6-C*H*), 7.32 (s, 1H, vinylic-*H*), 7.37 (d, 2H, J = 6 Hz, 3-C*H* and 5-C*H*, aromatic), 8.66 (d, 2H, J = 6 Hz, 2-C*H* and 6-C*H*, aromatic). MS: m/z 378 [M^+]. Anal. Calcd for $C_{25}H_{31}NO_2$: C, 79.54; H, 8.27; N, 3.71. Found: C, 79.77; H, 8.11; N, 3.55.

*2.1.1.2. 16-(3,4,5-Trimethoxybenzylidene)-17-oxo-5-androsten-3-ol (**2**).* Yield: 74.1%. m.p. 65–68 °C. IRν_{max} (KBr): 3400, 2971, 1638, 1442, 1224, 1132, 1023. ^{1}H NMR ($CDCl_3$): 0.99 (s, 3H, 18-C*H*$_3$), 1.07 (s, 3H, 19-C*H*$_3$), 3.5 (m, 1H, 3α-*H*), 3.9 (s, 9H, 3× —OC*H*$_3$), 5.39 (s, 1H, J = 4 Hz, 6-C*H*), 6.78 (s, 2H, C*H*, aromatic) and 7.37 (s, 1H, vinylic-*H*). MS: m/z 467 [M^+]. Anal. Calcd for $C_{29}H_{38}O_5$: C, 74.65; H, 8.21. Found: C, 74.97; H, 7.99.

*2.1.1.3. 16-(4-Methoxy-2-nitrobenzylidene)-17-oxo-5-androsten-3-ol (**3**).* Yield: 63.8%. m.p. 158–160 °C. IRν_{max} (KBr): 3400, 2880, 2820, 1720, 1640, 1540, 1280, 1020. ^{1}H NMR ($CDCl_3$): 0.96 (s, 3H, 18-C*H*$_3$), 1.07 (s, 3H, 19-C*H*$_3$), 3.53 (m, 1H, 3α-*H*), 4.01 (s, 3H, —OC*H*$_3$), 5.40 (d, 1H, 6-C*H*), 7.13 (d, 1H, 6-C*H*, J_o = 8.77 Hz, aromatic), 7.35 (s, 1H, vinylic-*H*), 7.67 (dd, 1H, 5-C*H*, J_o = 8.7 Hz and J_m = 1.47 Hz, aromatic), 8.04 (d, 1H, 3-C*H*, J_m = 1.68 Hz, aromatic). MS: m/z 452 [M^+]. Anal. Calcd for $C_{27}H_{33}NO_5$: C, 71.82; H, 7.37; N, 3.10. Found: C, 71.63; H, 7.48; N, 3.01.

*2.1.1.4. 16-(Benzo-[1,3]dioxol-5-ylmethylene)-17-oxo-5-androsten-3-ol (**4**).* Yield: 73.5%. m.p. 238–240 °C. IRν_{max} (KBr): 3400, 2920, 1710, 1460, 1260, 1040. ^{1}H NMR ($CDCl_3$): 0.97 (s, 3H, 18-C*H*$_3$), 1.07 (s, 3H, 19-C*H*$_3$), 3.52 (m, 1H, 3α-*H*), 5.41 (d, 1H, 6-C*H*), 6.02 (s, 2H, —O—C*H*$_2$—O—), 6.86 (dd, 1H, 6-C*H*, J_m = 2.03 Hz, J_o = 6.62 - Hz), 7.06 (d, 2H, 2-C*H* and 5-C*H*, aromatic, J_o = 6.59 Hz), 7.31 (s, 1H, vinylic-*H*). MS: m/z 421 [M^+]. Anal. Calcd for $C_{27}H_{32}O_4$: C, 77.11; H, 7.67. Found: C, 76.93; H, 7.48.

*2.1.1.5. 16-[(1H-imidazol-2-yl) methylene]-17-oxo-5-androsten-3-ol (**5**).* Yield: 73.2%. m.p. 213–215 °C. IRν_{max} (KBr): 3150, 3063, 2930,



(1) (2) (3)

Fig. 2. Steroidal and nonsteroidal aromatase inhibitors.

1712, 1636, 1548, 1441, 1059, 760. ^{1}H NMR ($CDCl_3$): 0.93 (s, 3H, 18-CH_3), 1.05 (s, 3H, 19-CH_3), 3.37 (m, 1H, N*H*), 3.40 (m, 1H, 3α-*H*), 5.35 (s, 1H, 6-C*H*), 7.18 (s, 2H, C*H*, imidazole-*H*), 7.22 (s, 1H, vinylic-*H*). MS: *m*/*z* 367 [M$^+$]. Anal. Calcd for $C_{23}H_{30}N_2O_2$: C, 75.37; H, 8.25; N, 7.64. Found: C, 75.27; H, 8.48; N, 7.40.

*2.1.2. General procedure for the synthesis of compounds **6**–**10***

The aldol products **1**–**5** (2 mmol) were dissolved in a mixture of cyclohexanone (10 ml) and dry toluene (150 ml). Traces of moisture were removed by azeotropic distillation. The distillation was continued at a slow rate while adding a solution of aluminum isopropoxide (1 g) in dry toluene (15 ml) dropwise. The reaction mixture was refluxed for 5 h and allowed to stand at room temperature overnight. The slurry formed was filtered and the residue was washed thoroughly with dry toluene. The combined filtrate and the washings were steam distilled to remove the organic solvent. The solid obtained was filtered, washed with water, dried and treated with diethyl ether and *n*-hexane to furnish the corresponding 4-ene-3-keto steroids **6**–**10**, respectively.

*2.1.2.1. 16-(4-Pyridylmethylene)-4-androstene-3,17-dione (**6**).* Yield: 81.6%. m.p. 112–118 °C. IRν_{max} (KBr): 2925, 1720, 1675, 1550, 1210, 910. ^{1}H NMR ($CDCl_3$): 1.02 (s, 3H, 18-CH_3), 1.25 (s, 3H, 19-CH_3), 5.76 (s, 1H, 4-C*H*), 7.34 (s, 1H, vinylic-*H*), 7.35 (d, 2H, *J* = 6 Hz, 3-C*H* and 5-C*H*, aromatic), 8.67 (br, 2H, 2-C*H* and 6-C*H*, aromatic). MS: *m*/*z* 376 [M$^+$]. Anal. Calcd for $C_{25}H_{29}NO_2$: C, 79.99; H, 7.73; N, 3.75. Found: C, 79.80; H, 7.97; N, 3.57.

*2.1.2.2. 16-(3,4,5-Trimethoxybenzylidene)-4-androstene-3,17-dione (**7**).* Yield: 50.6%. m.p. 218–222 °C. IRν_{max} (KBr): 2910, 1722, 1674, 1631, 1135, 1017, 918. ^{1}H NMR ($CDCl_3$): 1.02 (s, 3H, 18-CH_3), 1.25 (s, 3H, 19-CH_3), 3.90 (s, 9H, 3× —OCH_3), 5.76 (s, 1H, 4-C*H*), 6.78 (s, 2H, C*H*, aromatic), 7.38 (s, 1H, vinylic-*H*). MS: *m*/*z* 465 [M$^+$]. Anal. Calcd for $C_{29}H_{38}O_5$: C, 74.97; H, 7.81. Found: C, 74.68; H, 7.45.

*2.1.2.3. 16-(4-Methoxy-2-nitrobenzylidene)-4-androstene-3,17-dione (**8**).* Yield: 58.6%. m.p. 220–222 °C. IRν_{max} (KBr): 2980, 2920, 1730, 1660, 1550, 1280, 1020. ^{1}H NMR ($CDCl_3$): 1.04 (s, 3H, 18-CH_3), 1.25 (s, 3H, 19-CH_3), 3.99 (s, 3H, —OCH_3), 5.77 (s, 1H, 4-C*H*), 7.13 (d, 1H, 6-C*H*, J_o = 8.76 Hz, aromatic), 7.37 (s, 1H, vinylic-*H*), 7.68 (dd, 1H, 5-C*H*, J_o = 8.76 Hz and J_m = 2.17 Hz, aromatic), 8.05 (d, 1H, 3-C*H*, J_m = 2.15 Hz, aromatic). MS: *m*/*z* 450 [M$^+$]. Anal. Calcd for $C_{27}H_{31}NO_5$: C, 72.13; H, 6.95; N, 3.11. Found: C, 72.23; H, 7.18; N, 3.04.

*2.1.2.4. 16-(Benzo-[1,3]dioxol-5-ylmethylene)-4-androstene-3,17-dione (**9**).* Yield: 58.2%. m.p. 248–250 °C. IRν_{max} (KBr): 2980, 1720, 1660, 1260, 1040, 1020. ^{1}H NMR ($CDCl_3$): 1.01 (s, 3H, 18-CH_3), 1.25 (s, 3H, 19-CH_3), 5.76 (s, 1H, 4-C*H*), 6.03 (s, 2H, —O—CH_2—O—), 6.87 (d, 1H, 6-C*H*, J_o = 8.69 Hz, aromatic), 7.06 (d, 2H, 2-C*H* and 5-C*H*, J_o = 7.37 Hz, aromatic), 7.36 (d, 1H, vinylic-*H*). MS: *m*/*z* 419 [M$^+$]. Anal. Calcd for $C_{27}H_{30}O_{45}$: C, 77.48; H, 7.23. Found: C, 77.29; H, 7.38.

*2.1.2.5. 16-[(1H-imidazol-2-yl)-methylene]-17-oxo-4-androstene-3,17-dione (**10**).* Yield: 56.7%. m.p. 189–190 °C. IRν_{max} (KBr): 3184, 2942, 1712, 1641, 1442, 1094, 1023, 745. ^{1}H NMR ($CDCl_3$): 0.99 (s, 3H, 18-CH_3), 1.24 (s, 3H, 19-CH_3), 3.33 (dd, 1H, 15-C*H*), 5.75 (s, 1H, 4-C*H*), 7.19 (S, 2H, imidazole-*H*), 7.22 (s, 1H, vinylic-*H*). MS: *m*/*z* 365 [M$^+$]. Anal. Calcd for $C_{23}H_{28}N_2O_2$: C, 75.79; H, 7.74; N, 7.69. Found: C, 75.65; H, 8.01; N, 7.53.

*2.1.3. 16-(Benzo-[1,3]dioxol-5-ylmethylene)androsta-1,4-diene-3,17-dione (**11**)*

A solution of 16-(benzo-[1,3]dioxol-5-ylmethlene)-4-androsten-3,17-dione (**9**, 0.66 g, 1.57 mmol) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 1.2 g) was heated under reflux in benzene (100 ml) for 50 h. The progress of the reaction was monitored by TLC. The reaction mixture was cooled to room temperature and filtered. The filtrate was washed several times with an aqueous solution of KOH (1%) and then with water to neutrality. The organic layer was dried over sodium sulfate and removed under vacuum to yield a oily residue, which was crystallized from ether to afford **11**.

Yield: 9.1%. m.p. 232–234 °C. IRν_{max} (KBr): 2920, 2820, 1710, 1660, 1240, 1030. ^{1}H NMR ($CDCl_3$): 1.02 (s, 3H, 18-CH_3), 1.29 (s, 3H, 19-CH_3), 6.03 (s, 2H, —O—CH_2—O), 6.10 (s, 1H, 4-C*H*), 6.25 (dd, 1H, 1-C*H*, J_{cis} = 10.16 Hz and *J* = 1.93 Hz), 6.86 (d, 1H, 6-C*H*, aromatic, J_o = 8.29 Hz), 7.06 (m, 3H, 2-C*H* and 5-C*H*, aromatic and 2-C*H*), 7.37 (s, 1H, vinylic-*H*). MS: *m*/*z* 417 [M$^+$]. Anal. Calcd for $C_{27}H_{28}O_4$: C, 77.86; H, 6.78. Found: C, 77.68; H, 6.53.

*2.1.4. 16-(4-Pyridylmethylene)-17-oximino-5-androsten-3-ol (**12**)*

A solution of sodium acetate trihydrate (0.6 g) and hydroxylamine hydrochloride (0.6 g) in water (20 ml) was added to a refluxing solution of 16-(4-pyridylmethylene)-17-oxo-5-androsten-3β-ol (**1**, 0.3 g) in aldehyde-free ethanol (30 ml). After refluxing for 4 h, the solution was concentrated under reduced pressure, diluted with water and allowed to stand. The precipitate was isolated by filtration, washed with water, dried and crystallized from acetone to afford **12**.

Yield: 35.5%. m.p. 224–230 °C. IRν_{max} (KBr): 3250, 2930, 1600, 1440, 1370, 1040. ^{1}H NMR ($CDCl_3$): 1.05 (s, 3H, 19-CH_3), 1.10 (s, 3H, 18-CH_3), 3.45 (m, 1H, 3α-*H*), 5.33 (d, 1H, 6-C*H*), 7.09 (s), 8.19 (s){1.5:1 ratio,1*H*, vinylic-*H*}, 7.28 (m, 2H, 3-C*H* and 5-C*H*, J_o = 9 Hz, aromatic), 8.55 (m, 2-C*H* and 6-C*H*, J_o = 9 Hz, aromatic), 9.62 ppm (br, 1H, =N—O*H*; disappeared on D_2O exchange). MS: *m*/*z* 393 [M$^+$]. Anal. Calcd for $C_{25}H_{32}N_2O_2$: C, 76.50; H, 8.22; N, 7.14. Found: C, 76.81; H, 8.0; N, 6.96.

*2.1.5. 16-(4-Pyridylmethylene)-3,17-dioximino-4-androstene (**13**)*

To a refluxing solution of 16-(4-pyridylmethylene)-4-androstene-3,17-dione (**6**, 0.5 g) in aldehyde free ethanol (50 ml), a hot solution of sodium acetate trihydrate (1.0 g) and hydroxylamine hydrochloride (1.0 g) in water (20 ml) was added. After refluxing for 4 h, the solution was concentrated, diluted with water (150 ml) and allowed to stand. The precipitate was filtered, washed with water, dried and crystallized from methanol to afford **13**. Yield: 29.9%. m.p. 194–196 °C. IRν_{max} (KBr): 3320, 2960, 1600, 1440, 960. ^{1}H NMR ($CDCl_3$): 1.03 (s, 3H, 19-CH_3), 1.11 (s, 3H, 18-CH_3), 5.77 (s, 1H, 4-C*H*), 7.06 (s), 8.16 (s) {0.5:1 ratio, 1H, vinylic-*H*}, 7.26 (m, 2H, *J* = 9 Hz, 3-C*H* and 5-C*H*, aromatic), 8.54 (m, 2H, J_o = 9 Hz, 2-C*H* and 6-C*H*, aromatic), 8.66 (br, 1H, =N—O*H*; disappeared on D_2O exchange), 8.92 (br, 1H, =N—O*H*; disappeared on D_2O exchange). MS: *m*/*z* 406 [M$^+$]. Anal. Calcd for $C_{25}H_{30}N_3O_2$: C, 74.22; H, 7.47; N, 10.38. Found: C, 74.54; H, 7.75; N, 10.12.

2.2. Aromatase inhibitory activity

2.2.1. Preparation of aromatase

The enzyme was obtained from the microsomal fraction of freshly delivered human term placental tissue according to the procedure of Thompson and Siiteri [15]. The isolated microsomes were suspended in the 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.

2.2.2. Inhibition of aromatase in vitro

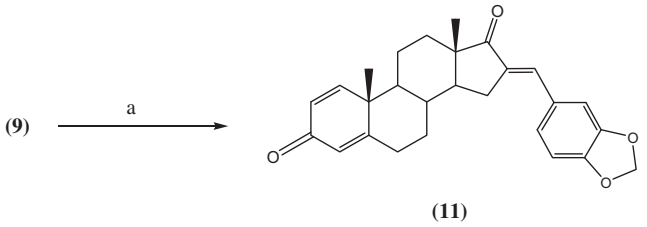
The assay was performed similar to the described methods [16,17] monitoring the enzyme activity by measuring the ^3H_2O formed from [1β-^{3}H]androstenedione during aromatization. Each incubation tube contained 15 nM [1β-^{3}H]androstenedione (0.08 μCi), 485 nM unlabeled androstenedione, 2 mM NADP, 20 mM glucose-6-phosphate, 0.4 U of glucose-6-phosphate-dehydrogenase and inhibitor (in three different concentrations for determining the IC_{50} value) in phosphate buffer (0.05 M, pH 7.4). The test compound had been 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 a shaking water bath. Microsomal protein (0.1 mg) was added to start the reaction. The total volume for each incubation was 0.2 ml. The reaction was terminated by the addition of 200 μl of a cold 1 mM $HgCl_2$ solution. After addition of 200 μl of an aqueous dextran-coated charcoal (DCC) suspension (2%), the vials were shaken for 20 min and centrifuged at 1500*g* for 5 min to separate the charcoal-adsorbed steroids. Aliquots of the supernatant were assayed for ^3H_2O by counting in a scintillation mixture in a 1209 Rackbeta Wallac liquid scintillation spectrometer (Pharmacia LKB, Freiburg, Germany).

3. Results and discussion

3.1. Chemistry

The synthesis of 16*E*-arylidenoandrostene derivatives 1–13 has been carried out as depicted in Schemes 1–3. Aldol condensation of dehydroepiandrosterone with various aromatic aldehydes at room temperature in alkaline medium afforded the corresponding 16*E*-benzylidene steroids 1–5. The methine-bridged proton at C_{16} appeared at ~δ 7.3 ppm in the ^{1}H NMR spectra of all these aldol products. The configuration at C_{16} with respect to the carbonyl at C_{17} has been assigned *E* on the basis of earlier reports from our laboratory [12]. In view of the literature reports that 3,17-diketo steroids with higher degree of unsaturation in A-ring exhibit potent aromatase inhibitory activity [18], Oppenauer oxidation of aldol products 1–5 was carried out to afford the corresponding α,β-unsaturated-3-keto steroids 6–10. A singlet for the 4-C*H* proton at about δ 5.76 ppm was observed in the ^{1}H NMR spectra of all these products.

Insertion of a double bond between C-1 and C-2 allows the steroid to bind irrereversibly to the aromatase enzyme active site, resulting in inactivation of aromatase [18]. Therefore, we attempted to explore such modification in compound **9** by treating

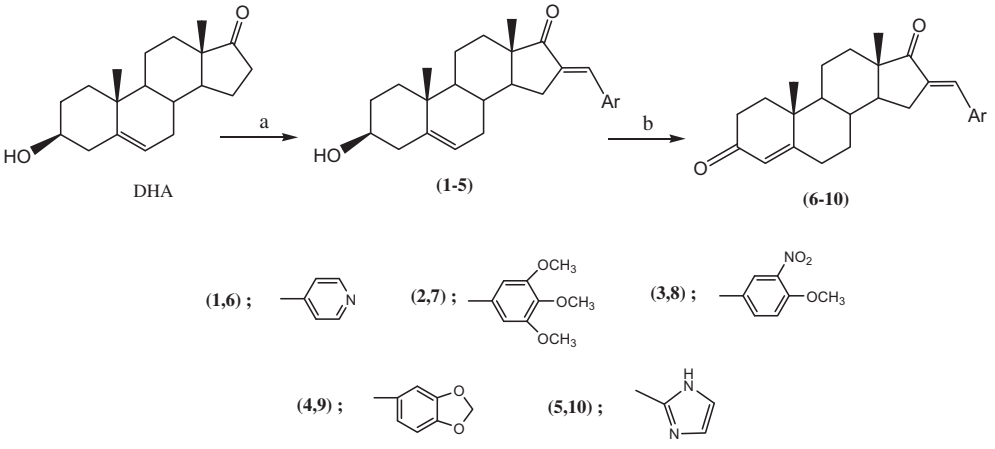


Scheme 2. Synthesis of the compound **11**. Reagents and reaction conditions: (a) 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ).

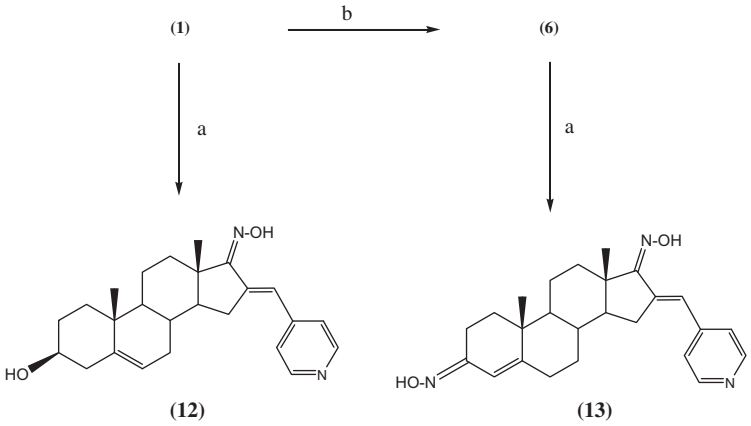
it with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) for 5 h to afford 16-(benzo-[1,3]dioxol-5-ylmethylene)-androsta-1,4-diene-3,17-dione (**11**), which showed ^{1}H NMR singlet at δ 6.1 for 4-C*H* and a doublet at 6.25 ppm for 1-C*H* of A ring of steroid. Oxidation of **1** was affected with hydroxylamine hydrochloride–sodium acetate in aldehyde-free ethanol to get the 17-oximino derivative **12**. Vinyl-*H* of 16-(4-pyridylmethylene) derivative **12** appeared as split singlets at 7.09 and 8.19 in 1.5:1 area ratio. Dioxime derivative **13** was prepared by treating 16*E*-arylidenosteroid **6** with hydroxylamine hydrochloride and sodium acetate trihydrate in refluxing aldehyde free ehanol. Work up of the reaction mixture gave compound **13**, which showed nuclear magnetic resonance signals at 5.77 (s, 1H, 4-C*H*) and split singlets for vinyl-*H* of **13** at 7.06 (s) and 8.16 (s) in 0.5:1 area ratio.

3.2. Aromatase inhibitory activity

Newly synthesized α,β-unsaturated 3-keto-16-arylideno steroids **6**–**13** were screened for aromatase inhibitory activity as described earlier [16]. The data is presented in Table 1. A comparative plot of concentration–response curves of the active compounds **6**, **10**, **11** and standard drug aminoglutethimide for aromatase inhibition has been presented in Fig. 3. Of these compounds, 3-keto-4-ene steroids **6** (IC_{50}: 5.2 μM) and **10** (IC_{50}: 22.7 μM) with a heteroaromatic ring at 16 position exhibited moderate inhibition of the enzyme in comparison to 16-arylidene steroids **7**–**9**, which displayed very weak inhibition of the enzyme even at a higher concentration of 36 μM. It is anticipated that the pyridyl and imidazolyl substituted steroids interfere with steroid hydroxylations by the binding of the sterically available N atom with the heme Fe (III) iron of cytochrome P-450. However 16-pyridyl substituted steroidal oxime **12** with a 3-hydroxy group and 3,17-dioximino derivative **13** displayed reduced binding affinity for aromatase enzyme in comparison to the pyridyl substituted



Scheme 1. Synthesis of the compounds **1**–**10**. Reagents and reaction conditions: (a) Ar—CHO, MeOH, KOH, RT; (b) Al(*t*-BuO)$_3$, cyclohexanone, reflux, 5 h.



Scheme 3. Synthesis of the compounds **12** and **13**. Reagents and reaction conditions: (a) hydroxylamine HCl, sodium acetate trihydrate; (b) Al(*t*-BuO)$_3$, cyclohexanone, reflux, 5 h.

Table 1
Aromatase inhibitory data of various compounds.

S. no.	Compound no.	Inhibition on CYP 19[a] IC_{50} (μM)	RP[b]
1	**6**	5.2 ± 1.5	5.7
2	**7**	24% inhibition ± 0.8 at 36 μM	
3	**8**	36% inhibition ± 0.1 at 36 μM	
4	**9**	45% inhibition ± 3.6 at 36 μM	
5	**10**	22.7 ± 2.9	1.3
6	**11**	6.4 ± 1.8	4.6
7	**12**	7% inhibition ± 3.5 at 36 μM	
8	**13**	32% inhibition ± 1.8 at 36 μM	

[a] Mean value of two independent experiments ±$SD_{absolute}$; substrate [1β-^{3}H]androstenedione, 500 nM.
[b] Relative potency = relative to aminoglutethimide (RP = 1; IC_{50} = 28.5 μM).

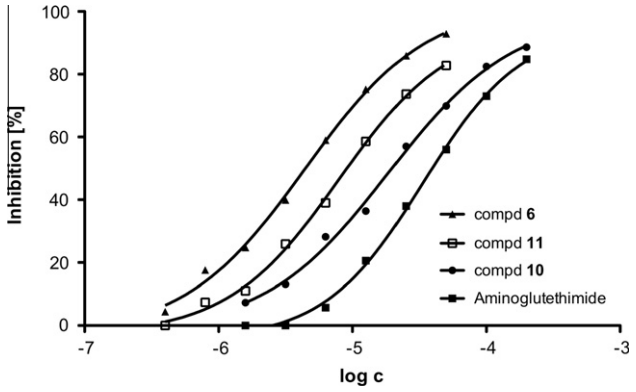


Fig. 3. Concentration–response-curves of compounds **6**, **10**, **11** and aminoglutethimide for aromatase enzyme inhibition. The log (inhibitor) vs. response model (Prism 5.0) was applied for nonlinear regression.

3-keto-4-ene counterpart **6**. This indicates the importance of 3 and 17-carbonyl groups for binding of the molecule with active site of the aromatase in addition to the heteroaromatic ring. On the other side, 16-(benzo-[1,3]dioxol-5-ylmethylene)androstane derivative **11** with increased unsaturation in ring A, although lacks a heteroaromatic ring produced better aromatase inhibitory activity (IC_{50}: 6.4 μM) in contrast to less oxidized analogue **9**, which showed only 45% inhibition at 36 μM. This is also in accordance with earlier literature reports [18]. From the above study it may be concluded that the presence of a heteroaromatic ring so that the nitrogen of the heteroaromatic ring is able to complex with the heme–iron of the enzyme along with 3 and 17 carbonyl groups and enhanced A ring unsaturation in the steroidal nucleus are important structural features for effective binding to the aromatase enzyme complex. Given the significance of azole grouping of many potent aromatase inhibitors [3], introduction of an imidazolyl or pyridyl ring at C-16 position together with C-3 keto and C-1 and 4 double bond of the steroid skeleton seem a rational approach to yield potent and specific enzyme inhibitors. With this design it may be possible to achieve dual inhibition by producing substrate like compounds which not only interact with the steroid binding site of the enzyme, thus introducing high specificity, but also provide a ligand to enzyme heme iron resulting in tight binding.

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