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Steroidal carbonitriles as potential aromatase inhibitors

Mange Ram Yadav [a,*], Prafulla M. Sabale [a], Rajani Giridhar [a], Christina Zimmer [b], Rolf W. Hartmann [b]

[a] Pharmacy Department, Faculty of Technology and Engineering, Kalabhavan, The M.S. University of Baroda, Vadodara 390 001, India
[b] Pharmaceutical and Medicinal Chemistry, Saarland University & Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Campus C 2 3, D-66123 Saarbrucken, Germany

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ABSTRACT

Estrogens, responsible for the growth of hormone-dependant breast cancer are biosynthesized from androgens involving aromatase enzyme in the last rate limiting step. Inhibition of aromatase is an efficient approach for the prevention and treatment of breast cancer. Novel 4-phenylthia derivatives (**2, 3** and **7**) have been synthesized as aromatase inhibitors. The synthesized compounds (**2, 3** and **7**) exhibited noticeable enzyme inhibiting activity. Kinetics study of these compounds (**2**, **3**, and **7**) showed negligible inhibition of the enzyme under conditions conducive for irreversible inhibition of the enzyme. Introduction of unsaturation at C-4, C-1 & 4 or C-4 & 6 (compounds **5, 9** and **11**) was observed to not be an effective strategy for entrancing aromatase inhibiting activity in 17-oxo-16β-carbonitrile derivatives. The D-seco derivatives (**13–15** and **17**) having unsaturation at C-4, C-1 & 4 or C-4 & 6 along with carbonitrile function in ring-D showed complete loss of aromatase inhibiting activity.

1. Introduction

Cancer is the second most important disease leading to death in both developing and developed countries according to WHO [1]. In females, breast cancer is the most frequently diagnosed and leading cause of cancer deaths worldwide, accounting for 23% of the total new cancer cases and 14% of the total cancer deaths. About half of the breast cancer cases and 60% of the deaths are estimated to occur in economically developing countries [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 this disease [3]. Approximately two-thirds of postmenopausal breast cancer patients have estrogen-dependent breast cancer, which contains estrogen receptors (ERs) and requires estrogens for its growth [4,5]. Production of estrogens takes place in many tissues of the body, including the ovaries, adipose tissue, muscle, liver, breast tissue and malignant breast tumors [5]. In pre-menopausal women the ovaries are the main source of circulating estrogens but in postmenopausal women the main source is adipose tissue and muscle [6]. Aromatase is a cytochrome P450 dependent enzyme that catalyzes the aromatization of androgens to estrogens by three sequential oxidation steps, each one requiring one mol each of oxygen and NADPH [7,8]. The third step oxidatively cleaves the C10–C19 bond, resulting in aromatization of the steroid A-ring and release of formic acid [9–11].

Over the past few decades, considerable attempts have been made toward developing potent inhibitors of aromatase [12,13]. Aromatase inhibitors that have been used clinically can be categorized either by generations or by their mechanism of action. They may be described as first-generation [aminoglutethimide (**A**) and testolactone (**B**)], second-generation [formestane (**C**) and fadrozole (**D**)] and third-generation [anastrozole (**E**), letrozole (**F**) and exemestane (**G**)] [14–19] inhibitors according to the order of their clinical development (Fig. 1). They can also be classified as type I or type II according to their mechanism of action. Type I and type II inhibitors are also known as steroidal and nonsteroidal inhibitors respectively [20,21].

Clinical studies initially confirmed that administration of the first generation inhibitor aminoglutethimide, caused regression of hormone-dependent breast cancer in women [22]. This lead served as the thrust to develop second and third generation inhibitors resulting in the development of compounds that are 1000–10,000-fold more effective than aminoglutethimide [13]. Further, the third generation agents are more specific for the aromatase enzyme, associated with less side effects and are adequately long acting to be administered on a once a day basis. The third generation aromatase inhibitors can be divided into two classes: the competitive inhibitors that bind reversibly to the active site of the enzyme and the inactivators that destroy the enzyme by binding covalently to it [13]. Most type I steroidal inhibitors are competitive inhibitors that have structures similar to androgens. Certain steroidal agents inactivate the enzyme irreversibly by blocking the substrate-binding site, and are therefore known as aromatase inactivators. Irreversible steroidal inhibitors such as exemestane form permanent bond with

* Corresponding author.
E-mail address: mryadav11@yahoo.co.in (M.R. Yadav).

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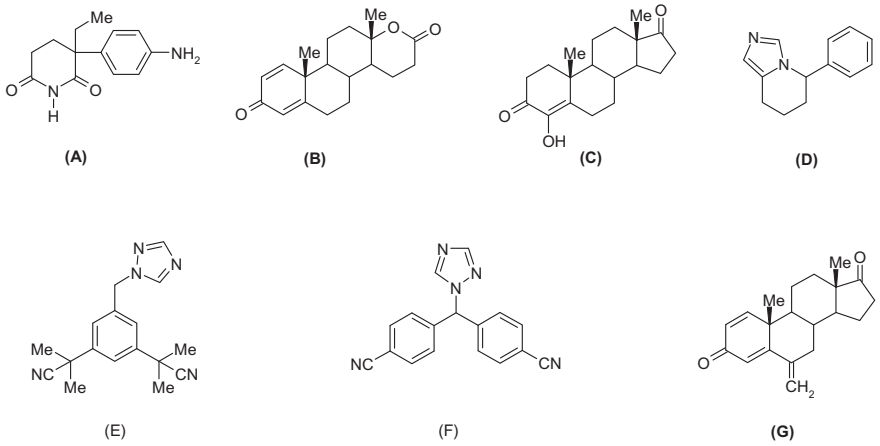


Fig. 1. Steroidal and nonsteroidal aromatase inhibitors.

the aromatase enzyme. Type II inhibitors are usually nonsteroidal and their action is reversible. Two of the most commonly used third generation aromatase inhibitors anastrozole (E) and letrozole (F) are nonsteroidal competitive inhibitors that inhibit the enzyme by reversible binding to it [23].

Inhibition of aromatase enzyme is an efficient approach for the prevention and treatment of breast cancer [24]. Besides attempts to develop novel nonsteroidal compounds, there is a focus on the development of steroidal compounds as potential aromatase inhibitors also [25–27]. Potent steroidal aromatase inhibitors are mainly the C-1, C-2, C-4, C-6, C-16 and C-19 substituted or C-2 and C-19 bridged steroidal compounds, i.e. the A-, B- or D-ring substituted/modified steroids. New analogs of formestane have been synthesized and their biological activity investigated in an attempt to find new aromatase inhibitors and to gain insight into their structure–activity relationships [28].

Extra units of unsaturation in ring A and/or B of several steroidal 4-oxoandrostenes and D-homo-lactones like 3-oxo-4-enes, 3-oxo-1,4-dienes, 3-oxo-4,6-dienes and 3-oxo-1,4,6-trienes showed relatively high anti-aromatase activity [29,30]. In D-seco derivatives the 17-keto group showed higher anti-aromatase activity in comparison to the 17-hydroxy function. The D-secomethyl derivative of 4-androstendione showed the highest activity and competitive type of inhibition of the enzyme [31,32].

In our previous publications [33,34] we have described the synthesis of some novel A-ring fused heterocyclic systems and A- and D-ring substituted/modified androstanes, and their evaluation for aromatase inhibiting activity. It was observed that compound with pyrazole ring fused to 2,3-positions of ring-A offered the highest activity followed by the 2-carbonitrile substituted derivative. Both of these compounds exhibited competitive inhibition in kinetics studies, contrary to our expectations. Another very important outcome of the study was that introduction of basic or neutral nitrogen in ring-A or ring-D of the androstane skeleton was highly deleterious for the molecules for aromatase inhibiting activity [34].

Some nonsteroidal aromatase inhibitors like anastrozole (E), letrozole (F) contain carbonitrile functionality. 2-Carbonitrile derivative, 2-cyano-3,17β-dihydroxy-5α-androstan-2-en-4-one reported earlier from this laboratory [33] showed significant aromatase inhibiting activity. 4-Thioalkyl/aryl derivatives have also been reported [35] to possess good aromatase inhibiting activity. Keeping these observations in mind it was planned to introduce simultaneously cyano group in ring-D and 4-phenylthia function in ring-A. It was of interest to see the impact of both of these functionalities on aromatase inhibiting activity, when combined in a single molecular entity. It was also planned to introduce unsaturation at C-1, C-4, and C-6 positions in some of the targeted compounds with the hope that introduction of unsaturation at these positions could increase binding affinity of the derivatives to the enzymes [29,30] as unsaturation could change the geometry of the molecules for better binding to the active site in the enzyme. It was envisaged to evaluate the synthesized compounds for their aromatase inhibiting activity and determine their kinetics.

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. ^1H NMR spectra were recorded on BRUKER AVANCE II 400 MHz instrument in $CDCl_3$ with TMS as internal standard. Chemical shift values are mentioned in δ, ppm. Chromatographic separations were performed on silica gel columns. The microanalyses for C, H and N were performed on Thermo Scientific FLASH 2000 organic elemental analyzer. Progress of all the reactions was monitored by TLC on 2 cm × 5 cm pre-coated silica gel 60 F254 plates (Merck) of 0.25 mm thickness. The chromatograms were visualized under UV (254 nm) and iodine vapors. 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. 17β-Hydroxy-4-phenylthia-4-androsten-3-one (2)

17β-Hydroxy-4ξ,5-oxido-5ξ-androstan-3-one (**1**) was prepared by the reported method [36]. A solution of 17β-hydroxy-4ξ,5-oxido-5ξ-androstan-3-one (**1**) (0.5 g, 0.0016 mol) in dioxane (10 ml) was stirred with thiophenol (0.26 g, 0.0024 mol) and anhydrous potassium carbonate (0.32 g, 0.0234 mol) for 3 h at room temperature under nitrogen atmosphere. The reaction mixture was poured into water (100 ml) and extracted with dichloromethane (4 × 25 ml). The combined organic extract was washed with water, dried and solvent removed to afford the crude product. The crude product so obtained was further purified by passing it through a column of silica gel. The column was first run with hexane followed by hexane–ethyl acetate (9:1). The solid was crystallized from methanol to afford 17β-hydroxy-4-phenylthia-4-androsten-3-one (**2**) (0.32 g, 55%), m.p. 159–61 °C. UV (MeOH): 250 nm (log ϵ 3.67), IR (KBr):

3499, 1689, 1609, 1532 and 744. ^{1}H NMR: 7.11–7.15 (m, 2H), 7.00–7.04 (m, 3H), 3.55–3.60 (t, 1H), 1.22 (s, 3H) and 0.74 (s, 3H). Calculated for $C_{25}H_{32}O_2S$: C 75.71, H 8.13. Found: C 76.02, H 8.45. MS: m/z 397.9 (M^+).

2.2.2. 4-(4-Aminophenyl)thia-17β-hydroxy-4-androsten-3-one (3)

A solution of 17β-hydroxy-4ξ,5-oxido-5ξ-androstan-3-one [36] (**1**) (0.5 g, 0.0016 mol) in dry tetrahydrofuran (10 ml) was stirred with *p*-aminothiophenol (0.29 g, 0.0021 mol) and anhydrous potassium carbonate (0.32 g, 0.0023 mol) for 3 h at room temperature under nitrogen atmosphere. The reaction mixture was poured into water (100 ml), acidified slightly with hydrochloric acid and extracted with dichloromethane (4 × 25 ml). The combined extract was washed with water, dried and solvent removed to afford a crude product. The crude product so obtained was purified by passing through a column of silica gel using first chloroform and then chloroform–methanol (9:1) as eluents. The solid so obtained was crystallized from methanol to afford 4-(4-aminophenyl)thia-17β-hydroxy-4-androsten-3-one (**3**) (0.33 g, 51%), m.p. 223–25 °C. UV (MeOH): 260 nm (log ϵ 3.18), IR (KBr): 3493, 3360, 1677, 1634, 1492, 1294 and 817. ^{1}H NMR: 6.97–7.01 (m, 2H), 6.48–6.51 (m, 3H), 3.55–3.59 (t, 1H), 1.21 (s, 3H) and 0.72 (s, 3H). Calculated for $C_{25}H_{33}NO_2S$: C 72.95, H 8.08, N 3.40. Found: C 72.76, H 8.25, N 3.24. MS: m/z 412.1 (M^+)

2.2.3. 16β -Cyano-4-androstene-3,17-dione (5)

16β-Cyano-3β-hydroxy-5-androsten-17-one (**4**) was prepared by the reported method [37] using androstenolone as the starting material. A solution of aluminium *i*.propoxide (2.0 g, 0.0097 mol) in dry toluene (20 ml) was added drop by drop during azeotropic distillation to a solution of 16β-cyano-3β-hydroxy-5-androsten-17-one [37] (**4**) (2.0 g, 0.0064 mol) in dry toluene (125 ml) and cyclohexanone (15 ml). Distillate (15–20 ml) was collected further after complete addition of the aluminium *i*.propoxide solution. The reaction mixture was refluxed for 2 h and allowed to stand overnight. Water (1.0 ml) was added to precipitate out aluminium hydroxide. The organic layer was filtered and the precipitate was washed with toluene. The combined organic layer was subjected to steam-distillation until complete removal of organic solvents was effected. The aqueous layer was extracted with chloroform (3 × 50 ml) and the combined chloroform layer was washed with water, dried and the solvent recovered. The residue so obtained was crystallized from hexane–ethyl acetate to afford crystals of 16β-cyano-4-androstene-3,17-dione (**5**) (1.0 g, 50%), m.p. 181–83 °C. UV (MeOH): 240 nm (log ϵ 4.07). (Alk.MeOH): 244 (log ϵ 4.1). IR (KBr): 2237, 1753, 1661, 1615, 1455, 1010 and 734. ^{1}H NMR: 5.76 (s, 1H), 3.06–3.11 (t, 1H), 1.23 (s, 3H) and 1.08 (s, 3H). MS: m/z 312 (M^+).

2.2.4. 16β-Cyano-4ξ,5-oxido-5ξ-androstane-3,17-dione (6)

Cold solutions of sodium hydroxide (20%, 2.0 ml) and hydrogen peroxide (30%, 3.0 ml) were added simultaneously drop by drop to a stirred solution of 16β-cyano-4-androstene-3,17-dione (**5**) (1.0 g, 0.00305 mol) in methanol (25 ml) at −5 °C. The reaction mixture was allowed to stand for 1 h at −5 °C, diluted with water (200 ml) and extracted with dichloromethane (4 × 25 ml). The combined extract was washed with water, dried and the solvent recovered to afford 16β-cyano-4ξ,5-oxido-5ξ-androstane-3,17-dione (**6**) (0.5 g, 57%) which was used as such for the next step without further purification.

2.2.5. 16β-Cyano-17β-hydroxy-4-phenylthia-4-androsten-3-one (7)

Thiophenol (0.5 g, 0.00454 mol) was stirred with sodium hydride (0.1 g, 0.00434 mol) in dry dioxane (5.0 ml) under nitrogen atmosphere at 10 °C. A solution of 16β-cyano-4ξ,5-oxido-5ξ-androstane-3,17-dione (**6**) (0.5 g, 0.00152 mol) in dry dioxane (5.0 ml) was added slowly to the above solution with stirring. The reaction mixture was stirred for 2 h at 10 °C, poured into water (100 ml) and extracted with dichloromethane (4 × 25 ml). The combined extract was washed with water, dried and the solvent removed to afford the crude product. The crude product so obtained was purified by passing through a column of silica gel eluting with hexane first to remove excess thiophenol and then with hexane–ethyl acetate (9:1). The solid so obtained was crystallized from methanol to afford crystals of 16β-cyano-17β-hydroxy-4-phenylthia-4-androsten-3-one (**7**) (0.28 g, 61%), m.p 145–47 °C. UV (MeOH): 250 nm (log ϵ 3.74). IR (KBr): 3336, 2236, 1672, 1563, 1475, 1289, 1108, 1022 and 992. ^{1}H NMR: 7.01–7.44 (m, 5H), 3.56–3.61 (m, 2H), 1.25 (s, 3H) and 0.73 (s, 3H). Calculated for $C_{26}H_{31}NO_2S$: C 74.07, H 7.41, N 3.32. Found: C 74.26, H 7.22, N 3.18.

2.2.6. 16β-Cyano-1,4,6-androstatriene-3,17-dione (8)

16β-Cyano-3β-hydroxy-5-androsten-17-one (**4**) (0.5 g, 0.0016 mol) in dry dioxane (8 ml) was heated with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) (1.5 g, 0.0066 mol) for 20 h at 100 °C on an oil bath. The reaction mixture was cooled and diluted with ethyl acetate (25 ml) and poured into saturated solution of sodium bicarbonate (400 ml). The aqueous layer was extracted with ethyl acetate (3 × 25 ml). The combined organic layer was washed with saturated solution of sodium bicarbonate, dried and the solvent removed under vacuum. The crude residue so obtained was passed through activated neutral alumina, using hexane–ethyl acetate (9:1) as an eluent to afford crystals of 16β-cyano-1,4,6-androstatriene-3,17-dione (**8**) (0.10 g, 20%), m.p. 185–86 °C. UV (MeOH): 296 (log ϵ 4.28), 254 (shoulder peak) (log ϵ 2.11) and 221 (log ϵ 3.58). IR (KBr): 2243, 1754, 1660, 1602, 1290 and 892.

2.2.7. 16β-Cyano-1,4-androstadiene-3,17-dione (9)

A mixture of 16β-cyano-3β-hydroxy-5-androsten-17-one (**4**) (0.5 g, 0.0016 mol) in dry dioxane (8 ml) was refluxed with DDQ (1.1 g, 0.0048 mol) for 8 h on an oil bath. The reaction mixture was cooled and diluted with ethyl acetate (30 ml), mixed well and poured into a saturated solution of sodium bicarbonate (500 ml). The aqueous layer was extracted with ethyl acetate (3 × 20 ml). The combined organic layer was washed with saturated solution of sodium bicarbonate twice, dried and the solvent removed under vacuum. The crude product so obtained was passed through activated neutral alumina, with ethyl acetate as an eluent. The product so obtained was further purified by passing through neutral alumina using hexane–ethyl acetate (9:1) as the eluent. The white solid so obtained was crystallized with hexane–ethyl acetate to get a mixture of 16β-cyano-1,4-androstadiene-3,17-dione (**8**) and compound (**9**) (0.10 g, 20%), m.p. 170–72 °C. UV (MeOH): 246 (log ϵ 3.98) and 296 (shoulder peak) (log ϵ 3.75), (Alk. MeOH): 260 (log ϵ 4.17) and 296 nm (shoulder peak) (log ϵ 3.79). IR (KBr): 2235, 1751, 1647, 1294 and 888. ^{1}H NMR: 7.01–7.06 (m), 6.24–6.38 (m), 6.00–6.11 (m), 3.06–3.15 (m), 1.28 (s), 1.26 (s), 1.16 (s) and 1.11 (s). MS: m/z 307.9 (M^+) and 309.9 (M^+).

2.2.8. 16β-Cyano-3-ethoxy-3,5-androstadien-17-one (10)

A solution of 16β-cyano-4-androstene-3,17-dione (**5**) (0.5 g, 0.0016 mol) in dry dioxane (8.0 ml) was stirred with *p*-toluenesulfonic acid (50 mg) and triethyl orthoformate (1.5 ml, 0.009 mol) for 2 h at room temperature whereby the reaction mixture turned greenish. Freshly prepared mixture of water (10 ml) and pyridine (1.0 ml) was added to the reaction mixture and it was cooled to 0 °C. The yellow sticky solid so formed was extracted with dichloromethane (3 × 20 ml), washed with water containing pyridine (0.5 ml), dried and the solvent removed under vacuum to yield a yellow sticky solid (0.45 g, 83%) which was used as such for the next step without further purification.

2.2.9. 16β-Cyano-4,6-androstadiene-3,17-dione (11)

A solution of 16β-cyano-3-ethoxy-3,5-androstadien-17-one (**10**) (0.5 g, 0.015 mol) in aqueous acetone (95%, 5.0 ml) was stirred with a solution of DDQ (0.42 g, 0.0018 mol) in aqueous acetone (95%, 2.0 ml) for 5–6 min at room temperature. The reaction mixture was diluted with aqueous acetone (95%, 20 ml), poured onto a column of neutral alumina and eluted with acetone until the yellow band moved to the base of the column. The solvent was removed and the crude product so obtained was further purified by passing through activated neutral alumina using hexane–ethyl acetate (9:1) as the eluent. The solid so obtained was crystallized with hexane–ethyl acetate to get crystals of 16β-cyano-4,6-androstadiene-3,17-dione (**11**) (0.130 g, 29%), m.p. 192–94 °C. UV (MeOH): 281 (log ϵ 4.38), (Alk. MeOH): 278 nm (log ϵ 4.47). IR (KBr): 2243, 1751, 1652, 1417, 1206, 1150, 876 and 733. ^{1}H NMR: 6.21–6.24 (d, 1H), 6.08–6.10 (dd, 1H), 5.73 (s, 1H), 3.10–3.15 (m, 1H), 1.15 (s, 3H) and 1.14 (s, 3H). Calculated for $C_{20}H_{23}NO_2$: C 77.64, H 7.48, N 4.50. Found: C 77.31, H 7.53, N 4.59. MS: m/z 309.9 (M^+).

2.2.10. 3β-Hydroxy-16,17-seco-5-androstene-16,17-dinitrile (12)

A solution of 3β-acetoxy-16,17-seco-5-androstene-16,17-dinitrile [38] (2.0 g, 0.0059 mol) and potassium hydroxide (0.4 g, 0.007 mol) in methanol (50 ml) was stirred at room temperature for 30 min. Two-third of the solvent was removed under reduced pressure, the reaction mixture was poured into water (300 ml) and acidified with hydrochloric acid (5%). White precipitate so obtained was filtered off, washed with water, dried and crystallized from methanol to afford crystals of 3β-hydroxy-16,17-seco-5-androstene-16,17-dinitrile (**12**) (1.41 g, 80%), m.p. 168–69 °C. IR (KBr): 3455, 2235, 1053 and 734. ^{1}H NMR: 5.35–5.37 (m, 1H), 3.50–3.60 (m, 1H), 2.66–2.73 (m, 2H), 1.43 (s, 3H) and 1.02 (s, 3H). MS: m/z 316 (M + 18).

2.2.11. 3-Oxo-16,17-seco-4-androstene-16,17-dinitrile (13)

Aluminium *i.*propoxide (2.0 g, 0.0097 mol) in dry toluene (20 ml) was added drop by drop during azeotropic distillation to a solution of 3β-hydroxy-16,17-seco-5-androstene-16,17-dinitrile (**12**) (2 g, 0.0067 mol) in dry toluene (125 ml) and cyclohexanone (15 ml). The reaction mixture was further refluxed for 3 h and allowed to stand overnight. Water (1 ml) was added to precipitate excess of aluminium *i.*propoxide and the organic layer was filtered and the slurry washed with toluene. The combined filtrate was subjected to steam-distillation until complete removal of organic solvents was effected. Aqueous layer was extracted with chloroform (3 × 50 ml) and the combined chloroform layer was washed with water, dried and recovered. The residue so obtained was crystallized from hexane–ethyl acetate to afford crystals of 3-oxo-16,17-seco-4-androstene-16,17-dinitrile (**13**) (1.0 g. 50%), m.p. 140–42 °C. UV (MeOH): 238 nm (log ϵ 4.32). IR (KBr): 2235, 1670, and 1432. ^{1}H NMR: 5.77 (s, 1H), 2.68–2.83 (m, 1H), 1.50 (m, 3H) and 1.16 (s, 3H). Calculated for $C_{19}H_{24}N_2O$: C 76.99, H 8.15, N 9.45. Found: C 77.12, H 8.22, N 9.31. MS: m/z 269.9 (M^+).

2.2.12. 3-Oxo-16,17-seco-1,4,6-androstatriene-16,17-dinitrile (14)

A solution of 3β-hydroxy-16,17-seco-5-androstene-16,17-dinitrile (12) (0.5 g, 0.0017 mol) in dry dioxane (10 ml) and DDQ (1.5 g, 0.0067 mol) was refluxed for 20 h on oil bath. The reaction mixture was diluted with ethyl acetate (80 ml). The organic layer was washed several times with saturated solution of sodium carbonate. The organic extract was dried, solvent recovered and the crude residue so obtained was passed through a column of neutral alumina with hexane–ethyl acetate (9:1) as the eluent. The white solid so obtained was crystallized from hexane–ethyl acetate to afford crystals of 3-oxo-16,17-seco-1,4,6-androstatriene-16,17-dinitrile (14) (0.18 g, 35%), m.p. 214–16 °C. UV (MeOH): 221 (log ϵ 4.01), 255 (log ϵ 3.97) and 295 nm (log ϵ 4.09). IR (KBr): 2235, 1680, 1604, 1229, 893 and 734. ^{1}H NMR: 7.01–7.03 (d, 1H), 6.46–6.47 (dd, 1H), 6.29–6.33 (dd, 1H), 6.07–6.10 (m, 2H), 2.79–3.00 (m, 2H), 1.57(s, 3H) and 1.21(s, 3H). Calculated for $C_{19}H_{20}N_2O$: C 78.05, H 6.88, N 9.58. Found: C 77.94, H 6.43, N 9.39. MS: m/z 292.5 (M^+).

2.2.13. 3-Oxo-16,17-seco-1,4-androstadiene-16,17-dinitrile (15)

A solution of 3β-hydroxy-16,17-seco-5-androstene-16,17-dinitrile (**12**) (0.5 g, 0.0017 mol) in dry dioxane (10 ml) and DDQ (1.0 g, 0.0045 mol) was refluxed for 8 h on oil bath and the reaction mixture was diluted with ethyl acetate (80 ml). The organic layer was washed with saturated solution of sodium carbonate, dried and the solvent removed. The crude residue so obtained was passed through neutral alumina using hexane–ethyl acetate (9:1) as the eluent. The white solid so obtained was crystallized from hexane–ethyl acetate to afford crystals which were characterized to be a mixture of 3-oxo-16,17-seco-1,4-androstadiene-16,17-dinitrile (**15**) and compound (**14**) (0.16 g, 32%), m.p. 174–76 °C. UV (MeOH): 243 (log ϵ 4.15) and 295 nm (shoulder peak) (log ϵ 3.77). IR (KBr): 2232, 1654, 1624, 1604, 890, 734 and 701. ^{1}H NMR: 7.03 (s), 7.00 (s), 6.98 (s), 6.43–6.46 (dd), 6.26–6.33 (m), 6.07–6.12 (m), 1.57 (s), 1.53 (s), 1.27 (s) and 1.22 (s). MS: m/z 292.9 (M^+) and 294.9 (M^+).

2.2.14. 3-Ethoxy-16,17-seco-3,5-androstadiene-16,17-dinitrile (16)

A solution of 3-oxo-16,17-seco-4-androstene-16,17-dinitrile (**13**) (0.5 g, 0.0017 mol), triethyl orthoformate (1.5 ml, 0.009 mol) and *p*-toluenesulfonic acid (0.05 g) in dry dioxane (8 ml) was stirred for 2 h at room temperature. The greenish reaction mixture was treated with a solution of water (10 ml) and pyridine (1 ml) and the resulting yellowish sticky solid was extracted with dichloromethane (3 × 25 ml). The combined organic layer was washed with water containing pyridine (0.2 ml), dried and the solvent removed to get yellowish sticky solid of 3-ethoxy-16,17-seco-3,5-androstadiene-16,17-dinitrile (0.54 g, 93%) (**16**) which was used as such without purification for next step.

2.2.15. 3-Oxo-16,17-seco-4,6-androstadiene-16,17-dinitrile (17)

A solution of 3-ethoxy-16,17-seco-3,5-androstadiene-16,17-dinitrile (**16**) (0.5 g, 0.0016 mol) in aqueous acetone (95%, 5.0 ml) was stirred with a solution of DDQ (0.45 g, 0.002 mol) in aqueous acetone (95%, 2.0 ml) for 6–8 min at room temperature. The reaction mixture was diluted with aqueous acetone (95%, 20 ml), poured onto a column of neutral alumina and eluted with acetone until the yellow band moved to the base of the column. The organic solvent was removed from the eluent to get a crude product which was further purified by passing through a column of neutral alumina using hexane–ethyl acetate (9:1) as an eluent. The solid so obtained was crystallized from hexane–ethyl acetate to obtain 3-oxo-16,17-seco-4,6-androstadiene-16,17-dinitrile (**17**) (0.21 g, 47%), m.p. 185–86 °C. UV (MeOH): 279 nm (log ϵ 4.43). IR (KBr): 2244, 2231, 1666, 1621, 1273, 870, 755 and 735. ^{1}HNMR: 6.28–6.31 (d, 1H), 6.13–6.16 (dd, 1H), 5.76 (s, 1H), 2.79–3.01 (m, 2H), 1.54 (s, 3H) and 1.15 (s, 3H). Calculated for $C_{19}H_{22}N_2O$: C 77.52, H 7.53, N 9.52. Found: C 77.24, H 7.68, N 9.26. MS: m/z 294.9 (M^+).

2.3. Biological

2.3.1. Human placental microsomal aromatase assay

The synthesized compounds were screened for aromatase inhibiting activity in human placental microsomal assay. As human term placenta is a rich source of aromatase enzyme, the assay is a measure of test compounds to bind aromatase enzyme in presence of natural substrates testosterone and androstenedione.

2.3.1.1. 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 [39]. 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.

2.3.1.2. Aromatase inhibition assay. The assay was performed by measuring the ^3H_2O formed from [1β-^{3}H]androstenedione during aromatization [40]. Each incubation tube contained [1β-^{3}H]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 the test compounds (in three different concentrations for determining the IC_{50} value within the linear range of the log-dose response curve, i.e. 20–80% inhibition) 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%. Microsomal protein (0.1 mg) was added to start the reaction. Each tube was incubated for 5 min at 30 °C in water bath. The total volume for 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 200 μl Norit A (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 using a β-Counter. The calculation of the IC_{50} values was performed by plotting the percent inhibition vs. the concentration of inhibitor on a semi-log plot.

2.3.2. Test for irreversible inhibition of aromatase

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 the 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 to adsorb unbound inhibitor. After full-speed centrifugation, 200 μl of the supernatant was supplemented with 50 μl of regenerating system and 50 μl substrate (15nM [1β-^{3}H]androstenedione (0.08 μCi) and 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 samples 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 at 1500*g* for 5 min to separate the charcoal-adsorbed steroids. The supernatant was assayed for ^3H_2O by counting in a scintillation mixture using a β-counter. Exemestane was used as a positive control that irreversibly binds to aromatase. Aminoglutethimide was used as a negative control (not binding irreversibly). The inhibition values after the three different incubation times were related to the DMSO control.

3. Results and discussion

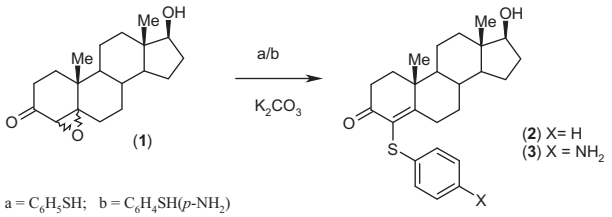
3.1. Chemical

In our earlier studies [33] we have introduced carbonitrile group in ring-A. The carbonitrile containing compound showed enhancement in aromatase inhibiting activity. So, we thought of introducing carbonitrile group in ring-D and to observe its impact on the aromatase inhibiting activity of the molecule. To achieve the synthesis of target compound (7), preliminary setting of the reaction conditions was done using testosterone as the starting material. Testosterone was converted into oxirane derivative (1) using the reported procedure [36]. The oxirane epimers (**1**) were treated with thiophenol in presence of anhydrous potassium carbonate to obtain 17β-hydroxy-4-phenylthia-4-androsten-3-one (**2**) as depicted in Scheme 1. The 4-phenylthia derivative showed sharp peak at 250 nm in methanol in its UV spectrum and characteristic IR bands appeared at 3499 (−OH) and 1689 cm^{-1} (–C=O). It offered characteristic NMR signals at 7.11–7.15 (m, 2H; Ar-*H*), 7.00–7.04 (m, 3H; Ar-*H*) and 3.55–3.60 (t, 1H; 17α-*H*). The mass spectrum showed a peak at m/z 397.9 (M^+) confirming the compound (**2**). The oxirane epimers (**1**) when treated with *p*-aminothiophenol in basic medium in tetrahydrofuran under inert atmosphere afforded 4-(4-aminophenylthia)-17β-hydroxy-4-androsten-3-one (**3**). The thioether (**3**) showed UV_{max} at 260 nm in methanol and prominent peaks appeared at 3493 ($-NH_2$) and 1677 cm^{-1} (–C=O) in its IR spectrum. Characteristic NMR signals appeared at 6.97–7.01 (d, 2H; Ar-*H*), 6.48–6.51 (d, 2H; Ar-*H*) and 3.55–3.59 (t, 1H; 17α-*H*). Its mass spectrum showed peak at m/z 412.1 (M^+).

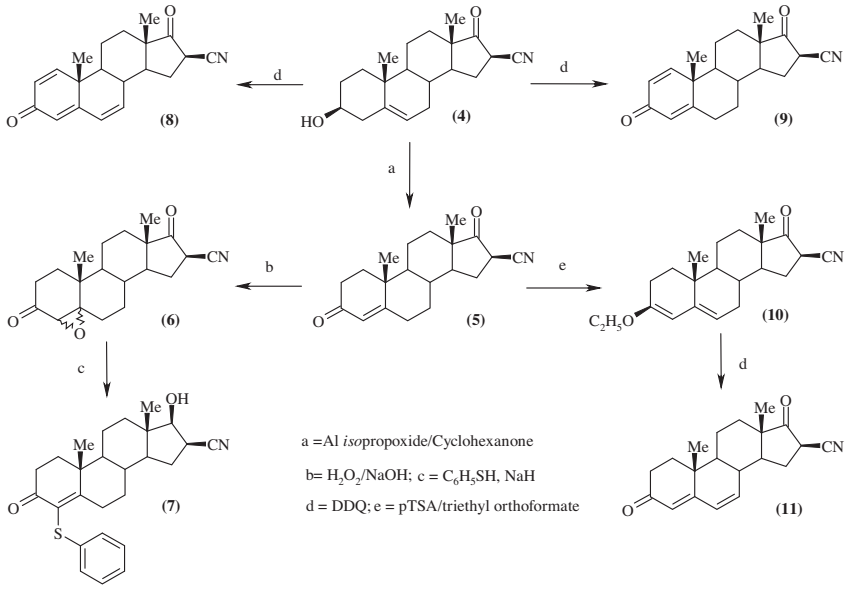
For the preparation of the target compound (**7**), synthesis was planned as given in Scheme 2. Androstenolone was converted into 16β-cyano-3β-hydroxy-5-androsten-17-one (**4**) by the reported procedure [37]. Compound (**4**) showed UV_{max} at 266 nm in methanol and characteristic bands appeared at 3490 (3-OH), 1754 (17-C=O) and 2250 cm^{-1} (-CN) in its IR spectrum. In its NMR spectrum signals appeared at δ 5.36–5.38 (m, 1H; 6-C*H*), 3.50–3,62 (m, 1H; 3α-C*H*) and 3.07–3.09 (dd, 1H; 16α-C*H*). Oppenauer oxidation of the 3β-hydroxy-16β-nitrile (**4**) was carried out using aluminium *i*.propoxide in cyclohexanone-toluene system to afford 16β-cyano-4-androstene-3,17-dione (**5**). The compound (**5**) showed UV_{max} at 240 nm in methanol confirming the formation of α,β-unsaturated ketone. The compound (**5**) showed characteristics IR bands at 1661 (3-C=O), 1753 (17-C = O) and 2237 cm^{-1} (16-CN). It gave NMR signals at δ 5.76 (s, 1H; 4-C*H*) and 3.06–3.11 (t, 1H; 16-C*H*), and its mass spectrum showed peak at m/z 312 (M^+) confirming its structure (**5**).

Treatment of 16β-cyano-3,17-dione (**5**) with alkaline hydrogen peroxide gave the oxirane (**6**), which was found to be a mixture of α,β-epimers. The mixture showed no absorption in its UV spectrum at 240 nm. The oxirane mixture (**6**) on treatment with thiophenol and anhydrous potassium carbonate under various temperature conditions did not yield the desired compound. But, when the oxirane (**6**) was stirred with thiophenol and sodium hydride in dry dioxane under nitrogen atmosphere at −10 °C it gave 16β-cyano-17β-hydroxy-4-phenylthia-4-androsten-3-one **(7)**. We expected the 17-oxo function to be retained but a reduced product (**7**) was obtained. This could be because of the presence of slight excess of sodium hydride present in the reaction mixture.

The compound (**7**) showed UV_{max} at 250 nm in methanol and characteristic IR peaks appeared at 3336 (–OH), 2236 (−CN) and



Scheme 1.



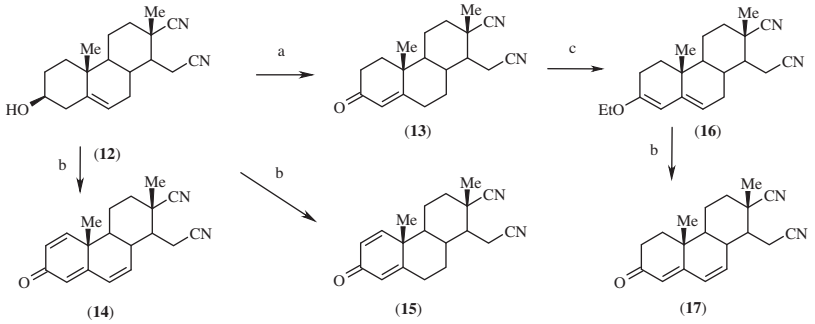
Scheme 2.

1672 cm^{-1} (3-C=O). It offered characteristic NMR signals at δ 7.01–7.44 (m, 5*H*) for aromatic protons and 3.61–3.56 (m. 2*H*) for protons at C_{16} and C_{17} positions. Since *p*-aminothiaphenyl derivative (**3**) did not offer better enzyme inhibiting activity over the thiaphenyl derivative (**2**), the idea of synthesizing *p*-aminothiaphenyl derivative with carbonitrile function in ring-D was dropped.

3β-Hydroxy-16β-nitrile (**4**) was dehydrogenated by refluxing it with 3.3 equivalents of DDQ in anhydrous dioxane for 18 to 20 h to afford 16β-cyano-1,4,6-androstatriene-3,17-dione (**8**) in very poor yield. The compound showed UV_{max} at 221, 254 (shoulder peak) and 296 nm and characteristic IR bands appeared at 2243 (-CN), 1754 (17-C=O) and 1660 cm^{-1} (3-C=O). 3β-Hydroxy-16β-nitrile (**4**) when dehydrogenated by refluxing it with 1.3 equivalents of DDQ in anhydrous dioxane for 8 to 10 h showed a single spot in TLC. The product showed UV_{max} at 246 and 296 nm (shoulder peak). IR peaks appeared at 1647 (3-C=O), 1751 (17-C=O) and 2235 cm^{-1} (16-CN). But, PMR spectrum of this product indicated it to be a 50: 50 mixture of compounds (**8** and **9**).

For the preparation of 4,6-diene derivative (**11**) the 16β-cyano-3,17-dione (**5**) was treated with triethyl orthoformate in presence of *p*-toluenesulfonic acid in dry dioxane for 1 h to afford 16β-cyano-3-ethoxy-3,5-androstadien-17-one (**10**) as an intermediate which showed UV_{max} (MeOH) at 262 nm. The intermediate 3-enolether **(10)** was dehydrogenated with 1.2 equivalents of DDQ in aqueous acetone to afford 16β-cyano-4,6-androstadiene-3,17-dione (**11**). The dienone (**11**) showed UV_{max} at 281 nm in methanol and characteristic IR bands appeared at 1652 (3-C=O), 1751 (17-C=O) and 2243 cm^{-1} (16-CN). In its NMR spectrum signals appeared at δ 6.21–6.24 (dd, 1H; 7-C*H*), 6.08–6.10 (d; 1H; 6-C*H*), 5.73 (s, 1H; 4-C*H*) and 3.10–3.15 (m, 1H; 16-C*H*). Its mass spectrum showed peak at m/z 309.9 (M^+), confirming the compound.

In order to introduce some degree of flexibility to the nitrile group it was planned to break open the ring-D and introduce unsaturation in ring-A and/or ring-B as illustrated in Scheme 3. 3β-Acetoxy-16,17-seco-5-androstene-16,17-dinitrile [38] became handy for this purpose. Alkaline hydrolysis of 3β-acetoxy-16,17-seco-5-androstene-16,17-dinitrile under mild reaction conditions offered 3β-hydroxy-16,17-seco-5-androstene-16,17-dinitrile (**12**). Compound (**12**) showed IR peaks at 3455 (–OH) and, 2235 cm^{-1} (–CN). Characteristic NMR signals appeared at δ 5.35–5.37 (m, 1H; 6-C*H*), 3.50–3.60 (m, 1H; 3α-C*H*) and 2.66–2.73 (m, 2H; 15-CH_2). Its mass spectrum showed peak at m/z 316 (M+18). Oppenauer oxidation of the 3β-hydroxy-16,17-dinitrile (**12**) was carried out using aluminium *i*.propoxide in cyclohexanone-toluene system to afford 3-oxo-16,17-seco-4-androstene-16,17-dinitrile (**13**). The compound showed UV_{max} at 238 nm for the α,β-unsaturated ketone which got confirmed by the presence of IR peak at



Scheme 3.

Table 1
Biological activity (% inhibition of aromatase enzyme) of compounds at 5 μM concentration.

Compd. No	2	3	5	7	9	11	13	14	15	17	Exe
% Inhibition ± S.D.	89.5 ± 7.2	81.3 ± 10.4	0.7 ± 1.2	96.0 ± 3.7	0.0 ± 0.0	0.0 ± 0.0	0.9 ± 1.5	0.0 ± 0.0	1.0 ± 1.3	1.5 ± 2.7	96.1 ± 2.2

The given values are mean values of at least three experiments.

1670 cm^{-1} (C═O). It gave characteristic signals at δ 5.77 (s, 1H; 4-C*H*) and 2.68–2.83 (m, 2H; 15-CH_2) in its PMR spectrum. Its mass spectrum showed peak at m/z 269.9 (M^+), confirming the compound (**13**).

For the preparation of the 1,4,6-triene derivative (**14**) the 3β-hydroxy derivative (**12**) was dehydrogenated by refluxing it with 3.3 equivalents of DDQ in anhydrous dioxane for 18 to 20 h. The compound showed UV_{max} at 221, 255 and 295 nm in methanol and gave characteristic IR bands at 1680 (3-C═O), and 2235 cm^{-1} (–CN). It gave NMR signals at δ 7.01–7.03 (d, 1H; 1-C*H*), 6.46–6.47 (dd, 1H; 7-C*H*), 6.29–6.33 (d, 1H; 2-C*H*), 6.07–6.10 (m, 2H; 4-C*H* and 6-C*H*), and 2.79–3.00 (m, 2H; 15-CH_2). Its mass spectrum showed peak at m/z 292.5 (M^+). Further, it was tried to obtain 1,4 diene derivative (**15**) by dehydrogenating 3β-hydroxy derivative (**12**) with DDQ. The 3β-hydroxy derivative (**12**) was dehydrogenated by refluxing it with 1.3 equivalents of DDQ in anhydrous dioxane for 8 to 10 h to offer a product which showed single spot in TLC. Its UV spectrum showed a peak at 243 nm with a shoulder peak at 295 nm. But, the product was found to be a mixture of compounds (**14** and **15**) as its PMR gave signals for vinylic protons at C-1, 2, 4, 6 and 7 positions.

For the preparation of 3-oxo-4,6-diene derivative (**17**), the enone (**13**) was treated with triethyl orthoformate in presence of *p*-toluenesulfonic acid in dry dioxane for 1 h to yield (**16**) as an intermediate which showed UV_{max} at 262 nm in methanol. The 3-enolether intermediate (**16**) was dehydrogenated using 1.2 equivalents of DDQ in aqueous acetone to afford the desired 3-oxo-16,17-seco-4,6-androstadiene-16,17-dinitrile (**17**). The compound showed UV_{max} at 279 nm in methanol. Characteristic NMR signals appeared at δ 6.28–6.31 (d, 1H; 6-C*H*), 6.13–6.16 (dd, 1H; 7-C*H*), 5.76 (s, 1H; 4-C*H*) and 2.79–3.01 (m, 2H; 15-CH_2) and its mass spectrum showed peak at m/z 294.9 (M^+).

3.2. Biological

All of the synthesized compounds (**2, 3, 5, 7, 11, 13, 14, 15,** and **17**) were evaluated for their aromatase inhibiting activity as shown in Table 1. The assay was performed by monitoring the enzyme activity by measuring the concentration of ^3H_2O formed from [1β-^{3}H] androstenedione as a substrate during its aromatization by the enzyme. In our earlier work [33,34] we have observed that tampering the basic androstane skeleton abolishes aromatase inhibiting activity in the steroidal derivatives. This observation has been further strengthened by this study wherein compounds (**13–15** and **17**) having a fractured androstane skeleton with broken D-ring were totally devoid of aromatase inhibiting activity. The compounds (**2**, **3**, and **7**) bearing phenylthia group at C-4 position exhibited significant enzyme inhibiting activity. Compound (**7**) having phenylthia at C-4 position and nitrile group at 16-position was found to be the most potent compound in the single dose assay. It looks that phenylthia group at C-4 position and suitably placed nitrile group [33] are favorable pharmacophores for aromatase inhibiting activity. But, carbonitrile group all alone could not endow aromatase inhibiting activity. Even introduction of unsaturation at C-4, C-1 & 4 or C-4 & 6 along with carbonitrile function at C-16 failed to incorporate aromatase inhibiting activity as observed for compounds (**5, 9** and **11**).

The three active compounds (**2**, **3**, and **7**) were further evaluated at three concentrations in order to determine their IC_{50} values. Exemestane was used as standard drug for comparison. As is evident from Table 2, the aimed 16β-carbonitrile derivative (**7**) was found to be equipotent to exemestane in inhibiting the enzyme.

The three potent compounds (**2**, **3**, and **7**), have been further investigated for their ability for irreversible inhibition at 2.0 and 20 μM concentration using exemestane as reference compound. The results are given in Table 2. Unlike exemestane the test compounds (**2**, **3**, and **7**) have shown negligible inhibition of the enzyme under conditions which measures the capacity of a compound for irreversible inhibition of the enzyme. Exemestane, an irreversible inhibitor of the enzyme, has shown appreciable activity under these conditions.

Table 2
IC_{50} Values and percentage of irreversible inhibition of aromatase enzyme by test and standard compounds.

Compd. No.	IC_{50} Values (nM) ± S.D.	% Inhibition of aromatase after irreversible binding ± S.D.	
		At 2.0 (μM)	At 20.0 (μM)
2	608.7 ± 117.2	n. i.	n. i.
3	1275.8 ± 253.4	n. i.	7.84 ± 5.78
7	169.3 ± 26.2	n. i.	5.06 ± 7.15
Exemestane	153.9 ± 14.7	55.08 ± 9.74	–

The given IC_{50} values are mean values of at least three experiments.
n. i. = no inhibition (inhibition < 5%).

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