Probing the Hydrophobic Pocket of the Active Site of Aromatase with 4-Phenoxy-7α-(phenylthio)-4-androstene-3,17-dione

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Received June 9, 1995®

In order to examine the nature of the hydrophobic pocket at the active site of aromatase, we carried out the synthesis, biochemical evaluation, and molecular modeling studies on 4-phenoxy-7α-(phenylthio)-4-androstenedione **2**. Aromatase inhibitory activity of **2** was found to be significantly weaker than that of the 4- and 7α-mono(phenylthio)-substituted derivatives of androstenedione. These results along with those obtained from the modeling studies suggest the existence of a single hydrophobic pocket corresponding to the α-face in the C4, C6, C7 region of androstenedione.

Introduction

Aromatase is a cytochrome P-450 dependent enzyme that catalyzes the aromatization of androgens to estrogen[1] and hence plays a key role in endocrine physiology and estrogen-dependent disease. Inhibitors of this enzyme complex may be useful in controlling pathologic conditions associated with estrogens, including breast and endometrial cancer.[2,3] Numerous aromatase inhibitors have been synthesized and studied over the past two decades and have resulted in the identification of structurally diverse groups of compounds including the analogs of 4-androstenedione, aminoglutethimide, and heterocyclic azoles.

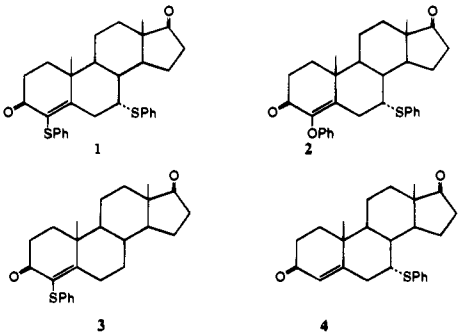
Previous studies in our laboratory[4–6] and those of Brueggemeier[7–9] illustrated that aromatase has considerable tolerance for androstenedione and testosterone derivatives with bulky 4- and 7α-substituents. These results led us to propose a hypothetical model for aromatase in which two hydrophobic pockets exist in the active site near the 4- and 7α-positions of the substrate.[5] Recent modeling studies by Furet *et al.*[10] and Laughton *et al.*[11] showed that several 4- and 7-substituted androstenediones and fadrazole (a nonsteroidal aromatase inhibitor) occupy the same volume, suggesting the presence of a hydrophobic pocket in the region located below the α-face of the steroid corresponding to the 4–7-positions of the steroid skeleton.

As structure–activity relationship studies of 4-androstenedione analogs have revealed that bulky substituents at the 4- and 7α-positions are tolerated by aromatase, the present study was undertaken in an effort to explore the nature of the hydrophobic pocket around the C4, C6, C7 region of androstenedione. In this connection 4,7α-disubstituted analogs were investigated to determine whether a combination of these substituents might afford more potent aromatase inhibitors. We report herein the synthesis and evaluation of 4-phenoxy-7α-(phenylthio)-4-androstenedione **2** as an aromatase inhibitor.

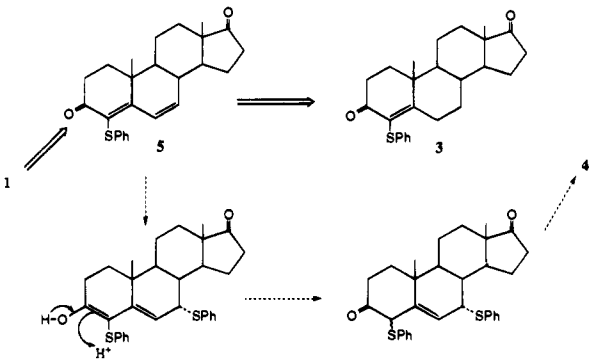
Results and Discussion

Preparation of 4-(phenylthio)-4-androstenedione **3** from 4-androstenedione has been described previously in our laboratory.[4] This procedure relies on base-catalyzed epoxidation of the 4,5-double bond followed by opening with thiophenol. However, this methodology could not be extended to 7α-(phenylthio)-4-androstenedione **4** since treatment of this compound with H_2O_2/OH^- did not result in the formation of the 4,5-epoxide. An alternative method of introducing the 4,7α-bis(phenylthio) groups was thus required.

Chart 1



Scheme 1



Retrosynthesis (Scheme 1) suggested that the desired 4,7α-bis(phenylthio) derivative **1** could potentially be obtained by base-catalyzed 1,6-Michael addition of thiophenol to **5**. This type of reaction has previously been successfully employed for the synthesis of 7α-thio-substituted androstenediones.[7] Although the 7α-phenylthio group did add to **5** by Michael fashion, this procedure resulted in loss of the 4-phenylthio group and gave instead compound **4** as proposed in Scheme 1. Several other attempts at synthesizing **1** resulted in loss of the 4-phenylthio group to give only **4**. Therefore we decided to synthesize the 4-phenoxy-7α-phenylthio derivative **2** instead of **1**.

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® Abstract published in *Advance ACS Abstracts,* September 1, 1995.

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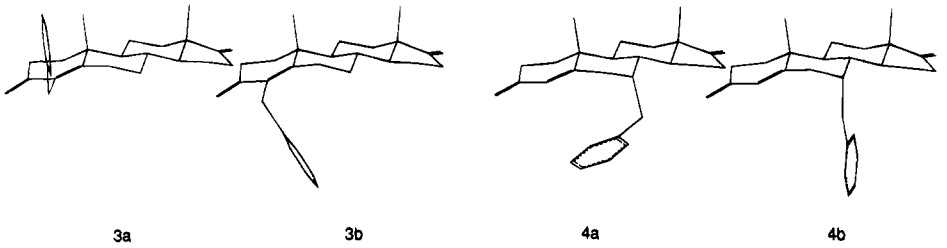
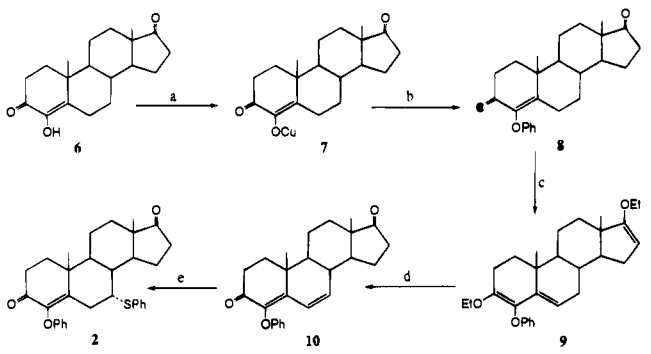


Figure 1. Energy-minimized conformations of **3** and **4**. Conformations of the two global minima are viewed along the edge of the steroid nucleus.

Scheme 2[a]



[a] Reagents: (a) CH_3Cu; (b) PhI; (c) $CH(C_2H_5O)_3$, TsOH; (d) DDQ, acetone; (e) PhSH, Na, dioxane.

Table 1. Inhibition of Aromatase by 4-Substituted and 4,7-Disubstituted Analogs of Androstenedione

compd	IC_{50}, μM[a]	app K_i[b]	inhibition[c]
3	0.19	35	competitive
4	0.65	212	competitive
8	0.53	412	competitive
2	1.65	472	competitive

[a] Incubations were carried out with 0.25 μM substrate and inhibitor concentrations ranging from 10^{-5} to 10^{-9} M. Values are reported for the average of three experiments. [b] Apparent K_i values were obtained from Lineweaver–Burke plots. Apparent K_m for androstenedione, 42 nM. [c] Inhibition type was determined by Lineweaver–Burke plots.

The synthesis of **2** was carried out as shown in Scheme 2. The 4-aryloxy analog **2** was obtained by preparing the 4-copper alkoxide **7** from 4-hydroxy androstenedione **6** (synthesized as described previously by Mann and Pietrzak[12]) followed by treatment with iodobenzene to yield 4-phenyl ether **8** as described by Whiteside *et al.*[13] Introduction of the 6,7-double bond was carried out by treatment of the intermediate ethyl enol ether **9** with 1 equiv of DDQ in aqueous acetone in 50% overall yield. Treatment of **10** with thiophenol gave the Michael addition product **2** in 40% yield. The ^{1}H-NMR and mass spectral data were consistent with the structure of compound **2**.

The biological results summarized in Table 1 indicate that both **3** and **4** are effective agents having similar aromatase inhibitory activity. Energy-minimization studies on **3** and **4** gave two global minima (Figure 1) showing significant differences in the orientation of the phenylthio substituents relative to the steroid nucleus. Our modeling studies on **4** were found to be essentially similar to those reported previously by Li and Brueggemeier.[14] It is noteworthy that energy minimizations of **8** gave two global minima that were quite similar to those observed for conformers **3a,b**. Superposition of conformers **3b** and **4a** (Figure 2) showed that the phenylthio substituents in these analogs occupy the same volume and orient below the plane of the steroid nucleus, suggesting a single hydrophobic pocket in the active site of aromatase.

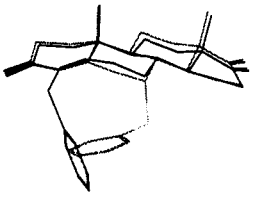


Figure 2. Superposition of conformers **3b** and **4a** (Figure 1).

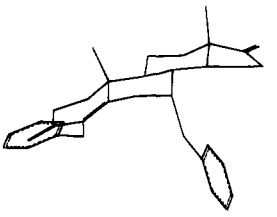


Figure 3. Energy-minimized conformation of **2**. Conformation of the global minima of **2** is viewed along the edge of the steroid nucleus.

Compound **2** was prepared with the aim of furthering our knowledge on the nature of the hydrophobic pocket in the active site of aromatase around the C4, C5, C6, and C7 region of 4-androstenedione. Examination of the results in Table 1 shows a significant decrease in aromatase inhibitory activity by **2** compared to **3**, **4**, and **8**. While we do not know the exact reasons for this observation, it is quite possible that the disubstituted analog may interfere with effective interaction due to increased steric hinderance or to lack of clustering of the hydrophobic groups in the 4- and 7α-positions to occupy the same volume in the active site of aromatase. Support for these results may be obtained from molecular modeling studies which showed that the 4- and 7α-substituents in **2** (Figure 3) adopt orientations similar to those of conformers **3a** and **4b**, respectively, and do not fit into a common hydrophobic pocket as depicted in Figure 2.

In conclusion our studies showed that the similarities in inhibition of aromatase by **3**, **4**, and **8** may be partially explained by a common hydrophobic pocket exemplified by conformers **3b** and **4a** as shown in Figure 2. Furthermore the poor inhibitory activity by **2** argues against the existence of two hydrophobic pockets in the active site of aromatase in the region corresponding to the α-face of the steroid ligand. Although the low-energy conformations we obtained are not necessarily the bioactive conformations, the approach taken here can provide possible insights into the bioactive conformations of the ligands and their possible interactions with the enzyme active site. Analogs with bridged 4,7-disubstituted 4-androstenediones are currently being

synthesized to further probe the nature of the hydrophobic pocket at the active site of aromatase.

Experimental Section

Melting points were determined on a Fisher Johns melting point apparatus and are uncorrected. Infrared spectra were obtained on a Nicolet 5DXC FT-IR spectrometer. Nuclear magnetic resonance spectra were obtained with a GE 300 MHz spectrometer using TMS as internal standard. TLC was performed on a precoated silica gel plate (silica gel GF; Analtech, Inc., Newark, NJ). Silica gel (200–400 mesh; Aldrich Chemical Co., Milwaukee, WI) was used for all column chromatography. Ultracentrifugation was performed on a Beckman L2-65B ultracentrifuge. Radioactivity was determined on a Beckman LS-100 liquid scintillation counter. Androstenedione was purchased from Steraloids (Wilton, NH). [1β-^{3}H]Androstenedione (24 Ci/mmol) was purchased from New England Nuclear (Boston, MA). All other chemicals were reagent grade and obtained from Aldrich Chemical Co. or Sigma Chemical Co. (St. Louis, MO).

Synthetic Methods. The synthesis of 4-(phenylthio)-4-androstene-3,17-dione (4-TPAD) (1) was carried out by reaction of thiophenol with 4,5-epoxyandrostane-3,17-dione as described previously.[4]

7α-(Phenylthio)-4-androstene-3,17-dione (4). The synthesis of this compound was carried out as described previously.[7] The overall yield from 4,6-androstadiene-3,17-dione was 77%: mp 223–225 °C; ^{1}H-NMR ($CDCl_3$) δ 0.94 (s, 3H, 18-CH_3), 1.23 (s, 3H, 19-CH_3), 3.56 (q, 1H, 7-H), 5.71 (d, 1H, 4-H), 7.31–7.44 (m, 5H, ArH). Anal. ($C_{25}H_{30}O_2S$) C,H.

4-Phenoxy-4-androstene-3,17-dione (8). The synthesis of this compound was carried out as described recently in our laboratory.[6] Commercial copper(I) iodide (1.71 g, 9 mmol) was stirred with di-*n*-butyl sulfide (2.63 g, 3.13 mL, 18 mmol) for 30 min at 80 °C, and the resulting amber liquid was filtered and stored at room temperature. To a cooled solution (−78 °C) of copper iodobis(di-*n*-butyl sulfide) (9 mmol) in ether (80 mL) was added methyllithium (6.42 mL, 1.4 M, 9 mmol) resulting in the immediate formation of a bright yellow methyl copper precipitate. The solid was compacted down by centrifugation at room temperature and the supernatant removed by cannula. Fresh ether (80 mL) was added to the methyl copper and the solution kept at −78 °C. A solution of **6** (2.02 g, 9 mmol) in THF (20 mL) and pyridine (80 mL) was added to the methyl copper solution (9 mmol), the mixture was stirred for 2 h at room temperature to give **7** (this compound was not isolated and characterized but used directly for the next reaction) after which iodobenzene (2.24 g, 11 mmol) was added, and the reaction mixture was refluxed with stirring overnight. The reaction mixture was poured over 250 g of ice containing 70 mL of concentrated HCl and extracted with CH_2Cl_2, and the organic extracts were combined, washed with water, dried (Na_2SO_4), and concentrated to give 4.24 g of an oily product of **8**. Chromatography over silica gel gave 1.45 g of **8**. Recrystallization from acetone–hexane gave 902 mg of pure **8** (55% yield): mp 148–149 °C; IR (KBr) 2945, 1739, 1683, 1165 cm^{-1}; ^{1}H-NMR δ 0.93 (s, 3H, 18-CH_3), 1.33 (s, 3H, 19-CH_3), 6.79–7.29 (m, 5H, ArH). Anal. ($C_{25}H_{30}O_3$) C,H.

4-Phenoxy-4,6-androstadiene-3,17-dione (10). To a solution of **8** (1.45 g) in ethanol (40 mL) was added 1 mL of triethyl orthoformate, 5 mg of *p*-toluenesulfonic acid, and 1 mL of triethylamine. The reaction mixture was stirred for 24 h at room temperature and then neutralized with a saturated solution of $NaHCO_3$ and extracted with methylene chloride. The organic layer was washed with water, dried over anhydrous Na_2SO_4, and concentrated in vacuo to give quantitative yield (checked by TLC) of the ethyl enol ether **9** as an oily product: ^{1}H-NMR ($CDCl_3$) δ 0.88 (s, 3H, 18-CH_3), 1.08 (t, 3H, 17-OCH_2CH_3), 1.13 (s, 3H, 19-CH_3), 1.16 (t, 3H, 3-OCH_2CH_3), 3.52 (q, 2H, 17-O$CH_2$$CH_3$), 3.84 (q, 2H, 3-O$CH_2$$CH_3$), 6.79–7.29 (m, 5H, ArH).

To a solution of **9** in aqueous acetone (30 mL, 5% H_2O) was added DDQ (1.0 g, 0.66 mmol) in 5 mL of 5% aqueous acetone. The reaction mixture was stirred for 3 min at room temperature, diluted with 30 mL of CH_2Cl_2, and washed with 1% NaOH solution, water, and brine, and dried over Na_2SO_4. Evaporation of the organic solvent in vacuo gave 920 mg of crude product which was chromatographed over a silica gel column to give **10**. Recrystallization from ethyl acetate–hexane gave 502 mg of pure **10**: mp 179–181 °C; IR (KBr) 2978, 2943, 1736, 1615, 1589, 1490 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 0.98 (s, 3H, 18-CH_3), 1.26 (s, 3H, 19-CH_3), 6.16–6.20 (dd, 1H, C-7H), 6.58–6.62 (dd, 1H, C-6H), 6.82–7.28 (m, 5H, ArH). Anal. ($C_{25}H_{28}O_3$) C,H.

4-Phenoxy-7α-(phenylthio)-4-androstene-3,17-dione (2). To a solution of **10** (500 mg) in dioxane (30 mL) were added 5 mg of sodium metal and thiophenol (1.5 mL), and the reaction mixture was refluxed at 60 °C for 8 h. Dilution with 70 mL of water followed by extraction with CH_2Cl_2 and washing with water gave crude thiophenol product. Purification on column chromatography gave 210 mg of pure **2**. Recrystallization from acetone–hexane gave an analytical sample: mp 210–211 °C; IR (KBr) 2951, 2935, 2845, 1735, 1697, 1591, 1488 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 0.95 (s, 3H, 18-CH_3), 1.36 (s, 3H, 19-CH_3), 3.45 (q, 1H, 7-H), 6.76–7.32 (m, 10H, ArH); MS (EI) (m/z) 487.2 ($M^+ + 1$, 18.5), 486 (M^+, 37.5), 376.2 (M^+ − HSPh). Anal. ($C_{31}H_{34}O_3S$) C,H.

Biochemical Methods. Enzyme Preparation. Microsomes were obtained from human placentas after normal deliveries and prepared as described previously.[4] Following isolation of the microsomal pellets (washed twice), they were lyophilized and stored at −20 °C. These preparations can be kept for 6 months without loss of activity.

Screening Assay Procedure. The method of Thompson and Siiteri,[1] as modified by Reed and Ohno,[15] was used in our studies. This assay quantitates the production of ^3H_2O released from [1β-^{3}H]androstenedione during aromatization. All enzymatic studies were performed in 0.1 M phosphate buffer, pH 7.4, at a final incubation volume of 3.0 mL. The incubation mixture contained 5.3 mM glucose 6-phosphate, 0.6 mM NADP, 2 units of glucose 6-phosphate dehydrogenase, various concentrations of inhibitors ranging from 10^{-5} to 10^{-8} M dissolved in ethanol (10 μL/0.5 mL incubation volume), 0.25 μM (0.25 μCi) [1β-^{3}H]androstenedione, 100 μL of propylene glycol, 1.0 mM EDTA, 10 mM phosphate buffer, and 0.15 mg of lyophilized human placental microsomes (54% protein). Incubations were carried out for 30 min at 37 °C and terminated by addition of 5 mL of $CHCl_3$ followed by vortexing for 40 s. After centrifugation at 1500g for 5 min, a 0.5 mL aliquot of the aqueous layer was removed and added to 5 mL of scintillation mixture for determination of ^3H_2O production.

***K*$_i$ Assay Procedure.** Kinetic analysis conditions employed [1β-^{3}H]androstenedione progressively diluted with inert androstenedione (0.005–0.25 μM). Incubations employed 0.025–0.03 mg of microsomal protein in 1.0 mL of 0.1 M phosphate buffer (pH 7.4) at 37 °C in air for 10 min and were processed as described above. Control samples with no inhibitor were incubated simultaneously, and blank samples were incubated for 0 min. Each inhibitor was examined at two concentrations (100 and 300 nM).

Molecular Modeling. Molecular modeling studies were conducted using Biosym software (Insight II 2.3.0, Discover 2.9)[16] operating in an IRIX 4.0.5 environment on a 35/TG personal Iris workstation. All the atomic charges were assigned by running Mopac (version 6.0).[17] Discover calculations used the consistent valence forcefield supplied with the Biosym package. Molecules were minimized using steepest descent (200 steps) followed by a quasi-Newton–Raphson algorithm[18] (VA09A, until the maximum derivative is less than 0.001 kcal/Å). A systematic search for conformers was carried out by allowing free rotation of the phenyl moieties (360°, by intervals of 10°) around the following dihedral angles: C5–C4–S–Cipso for **3** and C8–C7–S–Cipso for **4**, generating 36 conformers for each compound. In the case of the disubstituted **2**, the two phenyl moieties were allowed to rotate (360°, intervals of 10°) around the C5–C4–O–Cipso and C8–C7–S–Cipso dihedral angles, generating 1296 conformers. The resulting conformers for each compound were again energy-minimized as described above. The local minima for the conformers obtained in this investigation gave the following total energies: **3a**, 100.13; **3b**, 99.77; **4a**, 120.60; **4b**, 124.29; **2**, 170.26 kcal.

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JM950427U