Synthesis and biochemical studies of 7α-substituted androsta-1,4-diene-3,17-diones as enzyme-activated irreversible inhibitors of aromatase

Soheila Ebrahimian, Hsiu-Ho Chen[a], and Robert W. Brueggemeier

The College of Pharmacy and OSU Comprehensive Cancer Center, The Ohio State University, Columbus, OH, USA

Several 7α-thiosubstituted derivatives of androstenedione have demonstrated effective inhibition of aromatase, the cytochrome P450 enzyme complex responsible for the biosynthesis of estrogens. Introduction of an additional double bond in the A ring resulted in 7α-(4'-amino)phenylthioandrosta-1,4-diene-3,17-dione (7α-APTADD), a potent inhibitor that inactivated aromatase by an enzyme-catalyzed process. Additional 7α-thiosubstituted androsta-1,4-diene-3,17-dione derivatives were designed to further examine enzyme-catalyzed inactivation. Two halogenated and one unsubstituted 7α-phenylthioandrosta-1,4-diene-3,17-diones were synthesized via an acid-catalyzed conjugate Michael addition of substituted thiophenols with androsta-1,4,6-triene-3,17-dione. Two 7α-naphthylthioandrosta-1,4-diene-3,17-diones were synthesized via either acid-catalyzed or based-catalyzed conjugate Michael addition of substituted thionaphthols with androsta-1,4,6-triene-3,17-dione. These agents were evaluated for aromatase inhibitory activity in the human placental microsomal preparation. Under initial velocity assay conditions of low product formation, the inhibitors demonstrated potent inhibition of aromatase, with apparent K_i's ranging from 12 to 27 nM. Furthermore, these compounds produced time-dependent, first-order inactivation of aromatase in the presence of NADPH, whereas no aromatase inactivation was observed in the absence of NADPH. This enzyme-activated irreversible inhibition, also referred to as mechanism-based inhibition, can be prevented by the substrate androstenedione. Thus, the apparent K_i values for these inhibitors are consistent with earlier studies on 7α-substituted competitive inhibitors that indicate bulky substituents can be accommodated at the 7α-position. On the other hand, differences in the inactivation half-times at infinite inhibitor concentration, in the k_{app} rates of inactivation, and in the apparent K_{inact}'s were observed, suggesting that binding of 7α-substituted androsta-1,4-diene-3,17-diones can affect enzyme catalysis and enzyme-mediated inactivation. (Steroids **58:**414–422, 1993)

Keywords: aromatase; inhibitors; enzyme-mediated inactivation; 7α-substituted androsta-1,4-diene-3,17-diones

Introduction

Aromatase is the cytochrome P450 enzyme complex located in the endoplasmic reticulum of various endocrine cells that is responsible for the biosynthesis of estrogens. Inhibitors of aromatase may be useful in controlling reproductive processes and in the treatments of endometriosis, iodopathic oligospermia, endometrial and breast cancers, and gynecomastia.[1–8] The therapeutic efficacies of steroidal aromatase inhibitors such as 4-hydroxyandrostenedione and several nonsteroidal compounds are being investigated, and these agents have been shown to be effective in the treatment of hormone-dependent breast cancer in humans and in animal models.[7–13]

Several 7α-thiosubstituted derivatives of androstenedione have demonstrated enhanced affinity for aromatase and produced very effective inhibition of aromatase activity present in human placental microsomes.[14–21] These inhibitors include competitive, affinity, photoaffinity, and enzyme-activated irreversible in-

[a] Present address: School of Pharmacy, National Defense Medical Center, P.O. Box 90048, Taipei, Taiwan, ROC.
Address reprint requests to Dr. Robert W. Brueggemeier, College of Pharmacy, The Ohio State University, 500 W. 12th Avenue, Columbus, OH 43210, USA.
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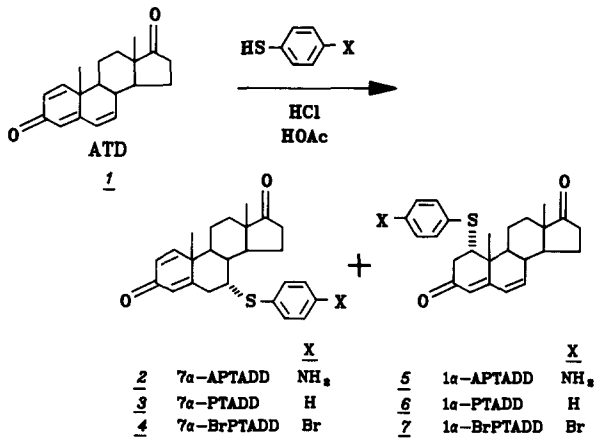


Scheme 1 Synthesis of 7α-APTADD **2**, 7α-PTADD **3**, and 7α-BrPTADD **4**.

hibitors. One of the most potent competitive inhibitors is 7α - (4′ - amino)phenylthioandrost - 4 - ene - 3,17 - dione (7α-APTA), with an apparent K_i of 18 nM.[14] In addition, this compound demonstrated effectiveness in inhibiting aromatase in cell cultures[21,22] and in treating hormone-dependent rat mammary tumors.[21,23]

Androstenedione derivatives with extended linear conjugation in ring A and/or B produced effective inhibition of aromatase.[24] Furthermore, the introduction of an additional double bond in the A ring resulted in inhibitors that inactivated aromatase by an enzyme-catalyzed process.[25] Introduction of a 7α-substituent onto androsta-1,4-diene-3,17-dione (1,4-ADD) yielded a potent enzyme-activated irreversible inhibitor of aromatase, 7α - (4′ - amino)phenylthioandrosta - 1,4 - di-ene-3,17-dione (7α-APTADD, **2**, Scheme 1).[20] An apparent K_i of 9.9 nM for aromatase inhibition was determined under initial velocity conditions. Furthermore, 7α-APTADD inactivated aromatase only in the presence of NADPH, exhibiting a half-time of inactivation of 1.4 minutes and a k_{app} of 8.4×10^{-3} sec^{-1}.[20]

The mechanism of aromatase inactivation following enzymatic catalysis by various steroids containing the 1,4-ADD structural moiety remains unknown. Additionally, the interactions of 7α-thiosubstituents on the androstadienedione molecule as inhibitors with the active site of aromatase are not clearly defined. Therefore, it is attractive to design potential enzyme-activated irreversible inhibitors based on the structure of 7α-APTADD. The synthesis and biochemical evaluation of halogenated and fluorescent 7α-substituted derivatives of androstadienedione are described here. Such inhibitors will provide useful pharmacological tools for probing the active site of the aromatase enzyme complex and further extend the structure-activity relationships of 7α-substituted steroidal aromatase inhibitors.

Experimental

Commercially available steroids were obtained from Steraloids (Wilton, NH, USA). 7α-APTA and 7α-APTADD were prepared as previously described[3,4]. All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). All melting points were taken with a Fischer-Johns melting point apparatus and are uncorrected. Proton nuclear magnetic resonance spectra (NMR) were performed on Bruker HX-90E (90 MHz), IBM AF-250 FT NMR and multinuclear IBM AF-270 pulse NMR spectrometers. Infrared spectra (IR) were recorded on a Beckman IR 4230 spectrophotometer. Ultraviolet spectra (UV) were obtained on a Beckman DU-40 spectrophotometer. Mass spectra (MS) were taken with Kratos MS25RFA double-focusing or Kratos MS-30 instruments at ionization energy of 70 eV. Samples on which exact masses were measured exhibited no significant peaks of m/z greater than that of the parent. Elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN, USA). Analytical thin-layer chromatography was performed with EM laboratories or Analtech, Inc. (Newark, DE, USA) 0.20 mm thick precoated silica gel 60 F-254 plates. Column chromatography materials were purchased from E. Merck (Darmstadt, Germany) and basic aluminum oxide from Fischer Scientific (Fair Lawn, NJ, USA).

Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [1β-^{3}H]Androst-4-ene-3,17-dione was purchased from New England Nuclear (Boston, MA, USA). Sorvall RC 2-B centrifuge was used for centrifuge samples at 4 C and ultracentrifugation was performed on a Beckman L5-50B Ultracentrifuge. Radioactive samples were detected with a Beckman LS 6800 scintillation counter using Formula 963 (New England Nuclear) as the counting solution at counting efficiencies of 35%.

Synthetic methods

7α - (4′ - Amino)phenylthioandrosta - 1,4 - diene - 3,17 - dione (2). To a stirred solution of androsta-1,4,6-triene-3,17-dione (ATD, 75 mg, 0.266 mmol) and a mixture of 8 drops of 12N HCl and 160 drops of glacial acetic acid was added aminothiophenol (30 μL, 32.23 mg, 0.293 mmol). The reaction was stirred for 24 hours at room temperature, then neutralized with saturated NaOH and extracted with ethyl acetate. The organic layer was concentrated in vacuo. Isolation of 7α-APTADD **2** and 1α-APTADD **5** were accomplished as previously described.[19] The yield of 7α-APTADD **2** was 15% and the yield of 1α-APTADD **5** was 30%.

7α-Phenylthioandrosta-1,4-diene-3,17-dione (3). To a stirred solution of androsta-1,4,6-triene-3,17-dione (ATD, 75 mg, 0.266 mmol) and a mixture of 8 drops of 12N HCl and 160 drops of glacial acetic acid was added thiophenol (30 μL, 32.23 mg, 0.293 mmol). The reaction was stirred for 24 hours at room temperature, then neutralized with saturated NaOH and extracted with ethyl acetate. The organic layer was concentrated in vacuo. The residue was chromatographed over 5 g of silica gel (CH_2Cl_2). Recrystallization with ethyl acetate gave 28 mg (27%) of **3**: mp 203–205C. IR (KBr) 3015, 2900 (br), 1730, 1660, 795, 750 cm^{-1}; ^{1}H NMR (270 MHz, $CDCl_3$) δ 0.94 (s, 3H, C_{18}), 1.25 (s, 3H, C_{19}), 3.66–3.67 (m, 1H, C_7H), 6.03 (m, 1H, C_4H), 6.27 (dd, J = 10, 1.8 Hz, 1H, C_2H), 7.04 (d, J = 10 Hz, 1H, C_1H), 7.26–7.42 (m, 5H, ArH); exact mass for $C_{25}H_{28}O_2S$: calcd m/z: 392.1811, found m/z: 392.1813. Anal. ($C_{25}H_{28}O_2S$-0.5H_2O) C,H,S.

7α - (4′ - Bromo)phenylthioandrosta - 1,4 - diene - 3,17 - dione (4). Bromothiophenol (110 mg, 0.58 mmol) was added to a stirred solution of ATD (200 mg, 0.709 mmol) containing a mixture of 3 drops of 12N HCl and 60 drops of glacial acetic acid. The reaction mixture was stirred at room temperature for 15 hours. The solution was concentrated in vacuo and the residue was flash chromatographed twice over 8 g portions of silica gel (ethyl acetate-petroleum ether, 1 : 4), followed by recrystallization with

ethyl acetate to yield white crystals of **4** (50 mg, 15%): mp 215–217C (dec); IR (KBr) 3020, 2915 (br), 1730, 1655, 825 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 1.06 (s, 3H, C_{18}), 1.37 (s, 3H, C_{19}), 3.74–3.78 (m, 1H, C_7H), 6.12 (m, 1H, C_4H), 6.39 (dd, J = 10, 2 Hz, 1H, C_2H), 7.15 (d, J = 10 Hz, 1H, C_1H), 7.37 (d, J = 8.5 Hz, 2H, ArH), 7.55 (d, J = 8.5 Hz, 2H, ArH); MS m/z (relative intensity): 470 (M^+, 52.87), 392 (2.45), 283 (51.36); exact mass for $C_{25}H_{27}O_2SBr$: calcd m/z: 470.0916, found m/z: 470.0938. Anal. ($C_{25}H_{27}O_2SBr$) C,H,S,Br.

7α - [4′ - (3″,3″ - Dimethylazido)phenyl]thioandrosta - 1,4 - diene-3,17-dione (8). A solution of 90 mg (0.22 mmol) of 7α-APTADD in 5 mL of acetone and 1.5 mL of 1.0N HCl was cooled to 0C, and 18 mg (0.26 mmol) of $NaNO_2$ in 0.2 mL of water was added and stirred at 0C for 1 hour. The reaction mixture was treated with 1 mL of 40% aqueous dimethylamine solution, dropwise over a 10-minute period, then poured into a mixture of 20 mL of methylene chloride and 20 mL of water. The organic layer was separated and washed with two 20-mL portions of water, and two 20-mL portions of saturated aqueous sodium chloride. The organic layer was dried (Na_2SO_4) and concentrated in vacuo. The residue (122 mg) was chromatographed over 10 g of silica gel (methylene chloride/ethyl acetate, 25 : 1) to give 87 mg (85.2%) of white crystals **8**, which were recrystallized from acetone/hexane: mp 175–176C. ^1H NMR (300 MHz, $CDCl_3$) δ 0.95 (s, 3H, C_{18}), 1.26 (s, 3H, C_{19}), 3.4 (s, 6H, $N(CH_3)_2$), 3.61–3.62 (m, 1H, C_7H), 6.07 (m, 1H, C_4H), 6.29 (dd, J = 10, 1.8 Hz, 1H, C_2H), 7.05 (d, J = 10 Hz, 1H, C^1H), 7.38 (m, 4H, ArH); exact mass for $C_{27}H_{33}N_3O_2S$: calcd m/z: 463.227, found m/z: 463.228. Anal. ($C_{27}H_{33}N_3O_2S$-0.5H_2O) C,H,N.

7α - (4′ - Iodophenyl)thioandrosta - 1,4 - diene - 3,17 - dione (9). A solution of NaI (15 mg, 0.1 mmol) and trifluoroacetic acid (10 μL, 0.131 mmol) in 0.5 mL of distilled formic acid was added to a stirred solution of **8** (25 mg, 0.0539 mmol) in 3 mL of benzene was added. The solution was stirred vigorously at room temperature for 1 hour, and then diluted with 5 mL of benzene. The benzene layer was removed, and the formic acid was washed with 5 mL of fresh benzene three times. The combined benzene solutions were dried (K_2CO_3) for 30 minutes, then evaporated to yield 27 mg of crude product as a brown oil. It was chromatographed over 5 g of silica gel (methylene chloride-ethyl acetate, 40 : 1) to afford a solid, which was crystallized with ethyl acetate/hexane to yield 22.5 mg (80.6%) pure product **9** as an off-white solid: mp 206–208C. IR (KBr) 3040, 2920 (br), 1740, 1665 cm^{-1}; ^1H NMR (250 MHz, $CDCl_3$) δ 0.99 (s, 3H, C_{18}), 1.23 (s, 3H, C_{19}), 3.64–3.71 (m, 1H, C_7H), 6.03 (m, 1H, C_4H), 6.27 (dd, J = 10.2, 1.8 Hz, 1H, C_2H), 7.03 (d, J = 10.2 Hz, 1H, C^1H), 7.10 (d, J = 8.3 Hz, 2H, ArH), 7.62 (d, J = 8.3 Hz, 2 H, ArH); exact mass for $C_{25}H_{27}O_2SI$: calcd m/z: 518.0778, found m/z: 518.0815. Anal. ($C_{25}H_{27}O_2SI$) C,H.

7α - (1′ - Naphthyl)thioandrosta - 1,4 - diene - 3,17 - dione (10). 1-Thionaphthalene (750 μL, 5.49 mmol) was added to a stirred solution of ATD (500 mg, 1.77 mmol) containing a mixture of 2 mL of 12N HCl and 20 mL of glacial acetic acid. The reaction mixture was stirred under nitrogen at room temperature for 20 hours. The solution was neutralized with saturated $NaHCO_3$ solution, extracted with ethyl acetate, and concentrated in vacuo. The residue was flash chromatographed over 15 g of silica gel using a step gradient of methylene chloride, methylene chloride-ethyl acetate (50 : 1), and then methylene chloride-ethyl acetate (10 : 1). Recrystallization with ethyl acetate-hexane yielded white crystals of **10** (156 mg, 20%): mp 206–208C. IR (KBr) 3010, 2900 (br), 1740, 1665, 1625 cm^{-1}; ^1H NMR (250 MHz, d_6-acetone) δ 0.92 (s, 3H, C_{18}), 1.34 (s, 3H, C_{19}), 3.92–3.94 (m, 1H, C_7H), 5.91 (dd, J = 1.9, 1.9 Hz, 1H, C_4H), 6.18 (m, 1H, C_2H), 7.24 (d, J = 10.2 Hz, 1H, C_1H), 7.48–7.64 (m, 3H, ArH), 7.83–7.98 (m, 3H, ArH), 8.45 (dd, J = 7.3, 2.3 Hz, 1H, ArH); ms m/z (relative intensity): 442 (M^+, 32.2), 283 (36.7), 173 (17.6), 160 (83.4); Anal. ($C_{29}H_{30}O_2S$) C,H.

7α - (2′ - Naphthyl)thioandrosta - 1,4 - diene - 3,17 - dione (11). 2-Thionaphthalene (500 μL, 3.12 mmol) was dissolved in dioxane (30 mL) and a small piece of sodium (approximately 15 mg) was added. ATD (400 mg, 1.42 mmol) was then added and the solution stirred under nitrogen at 80C for 18 hours. The remaining sodium was removed, the reaction poured into ice water, and the suspension extracted with ethyl acetate. The organic phase was washed with aqueous NaCl, dried (Na_2SO_4), and solvent removed in vacuo. Purification was accomplished with flash chromatography over silica gel (15 g) with methylene chloride-ethyl acetate (10 : 1) and recrystallized with ethyl acetate/hexane yielding white crystals of **11** (157 mg, 25%): mp 218–219C. IR (KBr) 3015, 2900 (br), 1730, 1660, 1640 cm^{-1}; ^1H NMR (250 MHz, d_6-acetone) δ 0.95 (s, 3H, C_{18}), 1.35 (s, 3H, C_{19}), 4.01–4.02 (m, 1H, C_7H), 5.95 (m, 1H, C_4H), 6.18 (dd, J = 10.2, 1.9 Hz, 1H, C_2H), 7.22 (d, J = 10.2 Hz, 1H, C_1H), 7.47–7.59 (m, 3H, ArH), 7.88–7.92 (m, 3H, ArH), 8.02 (br s, 1H, ArH); ms m/z (relative intensity): 442 (M^+, 57.3), 283 (23.3), 173 (9.7), 160 (88.6); Anal. ($C_{29}H_{30}O_2S$) C,H.

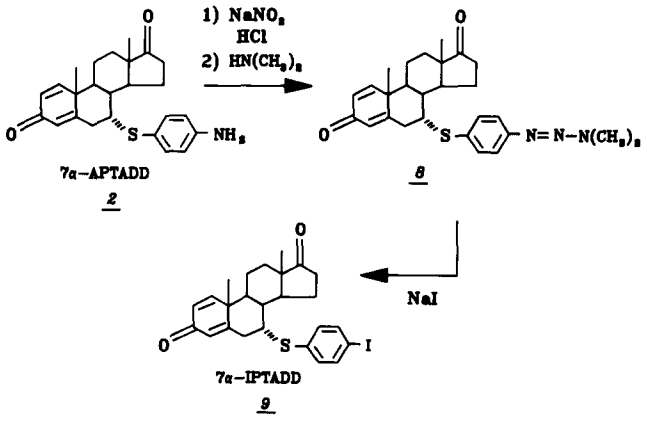
Biochemical methods

Human term–placentas were obtained from non-smoking women on delivery at OSU hospital, were transported on ice to the lab and processed immediately at 4C. All procedures were carried out as described by Brueggemeier et al.[14] to obtain the microsomal preparations.

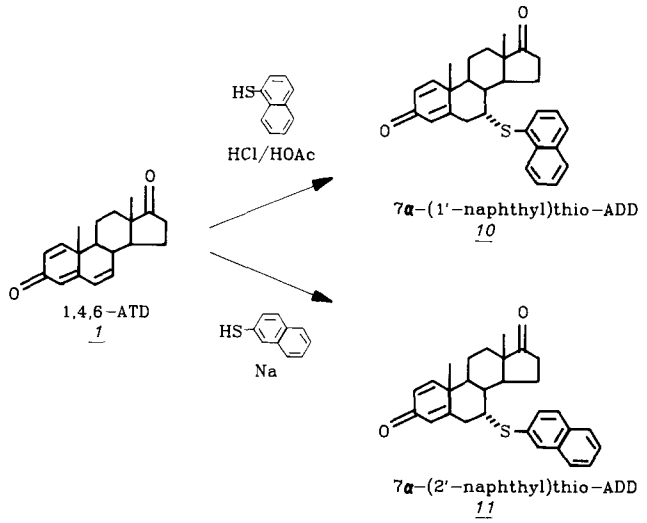
Competitive inhibiton studies. Kinetic studies were carried out under initial velocity conditions and the aromatase activity was measured by the ^3H_2O assay originally developed by Siiteri and Thompson.[26] The procedures for the evaluation of inhibition using this ^3H_2O assay method were similar to those previously reported by Brueggemeier et al.[17,20,21] Various concentrations of androst-4-ene-3,17-dione (60–500 nM; 200,000–300,000 dpm) and a single concentration of inhibitor **3, 4,** or **9–11** (20–500 nM) were preincubated with propylene glycol (100 μL), NADP (1.8 mM), glucose-6-phosphate (2.85 mM) and glucose-6-phosphate dehydrogenase (5 Units) at 37C for 5 minutes. Placental microsomes (0.07–0.12 mg) were homogenized and diluted to a total volume of 3.5 mL with 0.1 M of sodium phosphate buffer solution, pH 7, and then were added to the preincubated mixture. The solution was incubated at 37C for 15 minutes. The reaction was stopped by the addition of 5 mL of $CHCl_3$ into the incubate. After vortexing for 20 seconds, the $CHCl_3$-quenched samples were centrifuged at 1250 × g for 10 minutes. Aliquots (200 μL) of the aqueous layer were mixed with 5 mL of scintillation cocktail, and counted for ^3H radioactivity by liquid scintillation spectrometry. Assays were run in duplicate and control samples containing no inhibitors were run simultaneously. Blank samples were incubated with boiled microsomes. Protein concentrations were determined by the Lowry protein assay.[27] The enzyme kinetic data were analyzed by regression analysis programs.[28]

Time-dependent aromatase inactivation studies. NADPH (0.2 mM, 0.5 mL) was added into an incubation mixture which contained various concentrations of inhibitor **3, 4,** or **9–11** (20–900 nM), placental microsomal protein (0.2–0.3 mg/mL) and propylene glycol (100 μL) in 0.1 M of sodium phosphate buffer solution, pH 7, to give a total volume of 10 mL. Aliquots (1.0 mL) were removed at various time periods (0, 2, 5, 7, 10, and 15 minutes)

and immediately diluted 1 : 10 with ice-cold buffer solution. The remaining aromatase activity was assayed by addition of the diluted solution (3 mL) to a mixture of [1β-^{3}H]androst-4-ene-3,17-dione (200,000–300,000 dpm; 500 nM), propylene glycol (100 μL), NADP (1.8 mM), glucose-6-phosphate (2.8 mM) and glucose-6-phosphate dehydrogenase (5 Units) in 0.1 M of sodium phosphate buffer, pH 7, to give a final volume of 3.6 mL, and incubated at 37C for 30 minutes. The reaction was terminated by addition of 5 mL of $CHCl_3$. The sample was then vortexed for 20 seconds, and centrifuged at 1250 × *g* for 10 minutes Aliquots (1 mL) from the water layer were mixed with 4 mL of scintillation cocktail to form gels, and counted for radioactivity. Controls were run simultaneously without the inhibitor. The inactivation studies in the absence of NADPH were performed in the same manner as those with NADPH in the initial incubation. Protection studies were carried out analogously to the inactivation studies with unlabeled androst-4-ene-3,17-dione (100–1500 nM) and inhibitor (100–700 nM) included in the initial incubation.



Scheme 2 Synthesis of 7α-IPTADD **9**.



Scheme 3 Synthesis of 7α-thionaphthyl analogs **10** and **11**.

Results

Chemistry

The original synthesis of 7α-APTADD (**2**) was accomplished by a base-catalyzed conjugate Michael addition of aminothiophenol at the C_7 position of ATD (**1**).[20] These reaction conditions provided the desired product in only a 14% yield, with the undesired 1α-substituted analog being isolated in a 38% yield. The ratio of 7α-compound to 1α-compound was 1 : 2.7 under these base-catalyzed reaction conditions. Attempts to introduce a C_1–C_2 double bond onto 7α-APTA by dichloro dicyano quinone (DDQ) dehydrogenation or by selenium dioxide were not successful and gave only loss of the 7α-substituent and androsta-4,6-diene-3,17-dione.

An alternate synthetic route to various 7α-substituted androsta-4,6-diene-3,17-diones was employed using acid-catalyzed conjugate Michael addition of various thiophenols at the C_7 position. This approach was attempted in order to improve the yields of the desired product and to avoid potential reaction of substituted thiophenols under basic conditions. Under these acid-catalyzed conditions of HCl and acetic acid, both the 7α- and 1α-substituted androstadienediones were formed. The ratio of 7α-compound **2** to 1α-compound **5** improved slightly to 1 : 2 under the acid-catalyzed conditions, although the overall yield of 7α-APTADD remained the same at 15%.

Other 7α-substituted analogs were synthesized using different thiophenol starting materials. The highest synthetic yields of 27% isolated product were obtained for the synthesis of 7α-phenylthioandrosta-1,4-diene-3,17-dione (7α-PTADD, **3**, Scheme 1). 7α-(4'-Bromo)-phenylthioandrosta - 1,4 - diene - 3,17 - dione (7α-BrPTADD, **4**, Scheme 1) was prepared in a 15% isolated yield.

Another halogenated target compound was 7α-(4'-iodo)phenylthioandrosta - 1,4 - diene - 3,17 - dione (7α-IPTADD, **9**). Since the iodothiophenol starting material is not commercially available, the synthesis of this desired compound was envisioned to occur by the displacement of the amino functionality on 7α-APTADD, as outlined in Scheme 2. This synthesis involving an aryl triazine intermediate is analogous to the preparation of the androstenedione analog.[18] Furthermore, development of this synthetic route would permit introduction of a radioiodine nucline onto the inhibitor at a later date. Synthesis of 7α-IPTADD was accomplished in an 80% yield from 7α-APTADD.

Two 7α-thionaphthylandrosta-1,4-diene-3,17-diones were synthesized via either acid-catalyzed or based-catalyzed conjugate Michael addition of substituted thionaphthols with androsta-1,4,6-triene-3,17-dione (Scheme 3). The synthesis of 7α-(1'-naphthyl)-thioandrosta - 1,4 - diene - 3,17 - dione (**10**) under acid-catalyzed conditions provided the greatest yield of desired product, 20% isolated material. Interestingly, the only other product isolated from reaction of this more hindered thiol with ATD was the 1α,7α-disubstituted analog at a 45% yield. 7α-(2'-Naphthyl)thioandrosta-1,4-diene-3,17-dione (**11**) was prepared under base-catalyzed conditions in a 25% isolated yield, with side products of both 1α-substituted and 1α,7α-disubstituted analogs formed in 35% and 10% yields, respectively.

Table 1 Enzyme kinetic values for aromatase inhibitors

cpd. no.	cpd. abbrev.	Initial velocity assays[a] App. K_i	S.E.	Enzyme-activated inactivation assays[b] $T_{1/2}$	k_{app}	App. K_{inact}	slope	corr. coeff.
2	7α-APTADD[c]	9.9 nM	1.0 nM	1.38 min.	8.40×10^{-3} sec^{-1}	159 nM	0.22×10^3 min-nM	
3	7α-PTADD	20.0	3.5	6.53	1.77×10^{-3}	323	2.11×10^3	0.999
4	7α-BrPTADD	21.9	5.1	7.83	1.47×10^{-3}	151	1.19×10^3	0.980
9	7α-IPTADD	12.4	4.7	5.65	2.04×10^{-3}	483	2.73×10^3	0.981
10	7α-(1′-Naphthyl)ADD	27.7	9.5	3.72	3.11×10^{-3}	1060	3.46×10^3	0.977
11	7α-(2′-Naphthyl)ADD	14.2	3.3	4.28	2.70×10^{-3}	821	3.91×10^3	0.886

[a] Apparent K_i and S.E. values were calculated by a nonlinear regression analysis.[28] The apparent K_m for the substrate androstenedione was 52 nM.
[b] Inactivation kinetic values were determined from plots of the half-time of inactivation versus the reciprocal of the inhibitor concentration (Figure 5). The y-intercept of the resulting line is the half-time of inactivation at infinite inhibitor concentration ($T_{1/2}$) and the k_{app} is equal to $0.693/T_{1/2}$. The slope is equal to the product of the y intercept and apparent K_{inact}. Correlation coefficients (corr. coeff.) from the linear regression analysis are given for each inhibitor.
[c] Data for 7α-APTADD from Reference 20.

Biochemistry

The 7α-substituted androsta-1,4-diene-3,17-diones were first evaluated in human placental microsomes under initial velocity conditions. Aromatase activity in placental microsomes was assayed by the radiometric method developed by Siiteri and Thompson[26] using [1β-^{3}H]androst-4-ene-3,17-dione, with the amount of ^3H_2O released used as an index of estrogen formation. Under initial velocity assay conditions of low product formation, inhibitors were incubated at concentrations ranging from 0–600 nM with placental microsomal suspension and substrate androstenedione at concentrations ranging from 60–500 nM. 7α-Thiophenyl inhibitors **3**, **4**, and **9** demonstrated potent inhibition of aromatase with apparent K_i's of 20.0, 21.9, and 12.4 nM, respectively (Table 1, Figure 1). For comparison, 7α-APTADD exhibited an apparent K_i of 10 nM. The two naphthyl analogs **10** and **11** also demonstrated potent inhibition of aromatase with apparent K_i's of 14.2 and 27.7 nM, respectively.

The 7α-substituted androsta-1,4-diene-3,17-diones were examined in time-dependent inactivation studies to determine the abilities of the inhibitors to inactivate microsomal aromatase. Rapid, first-order inactivations of aromatase activities were produced when incubated in the presence of NADPH with various inhibitor concentrations of 20–900 nM (Figures 2–4). When the enzyme preparations were incubated with inhibitors in the absence of NADPH, no inactivations of aromatase activity were observed. These results suggest that enzyme catalysis is necessary for enzyme inactivations by all five inhibitors tested. Protection studies were performed by including the substrate androstenedione in the inactivation incubation mixture containing enzyme, inhibitor, and NADPH. As androstenedione

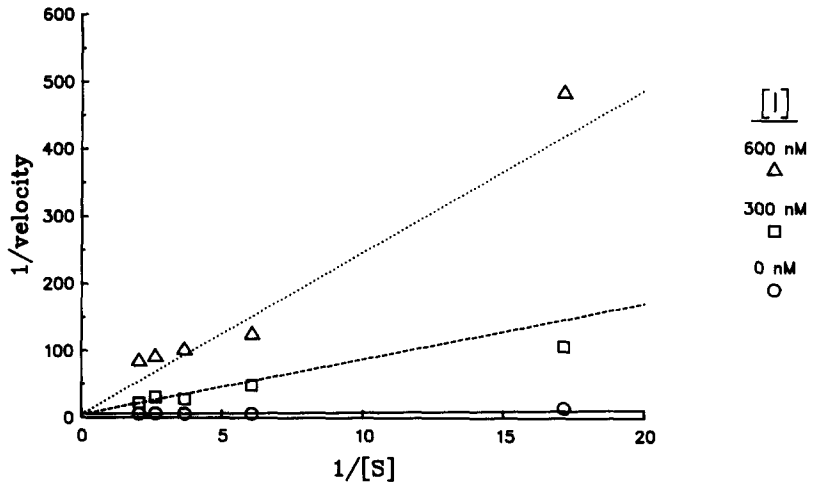


Figure 1 Double-reciprocal plot for aromatase inhibition by 7α-IPTADD **9**. Varying concentrations of androstenedione were incubated with microsomal enzyme preparations at inhibitor concentrations of 0, 300, or 600 nM. Velocity is expressed as nmol product per mg protein per minute and 1/[S] values have units of μM^{-1}. Each point represents the average of two determinations with less than 5% variation from the mean.

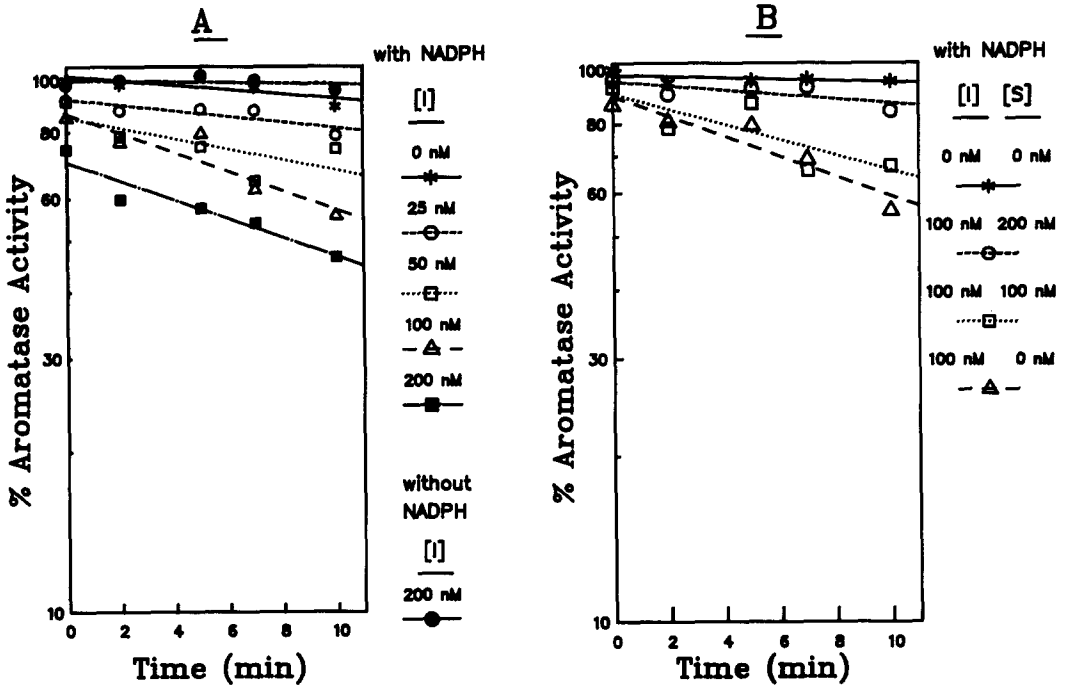


Figure 2 Inactivation of aromatase activity by 7α-BrPTADD **4**. ***(A)*** Time-dependent, first-order inactivations of aromatase activity were produced in the presence of NADPH at inhibitor concentrations of 0 nM, 25 nM, 50 nM, 100 nM, and 200 nM. No inactivation was observed in the absence of NADPH at inhibitor concentration of 200 nM. Each point represents the average of three determinations with less than 5% variation from the mean. ***(B)*** The rate of inactivation by inhibitor (200 nM) was decreased by increasing androstenedione ([S]) concentrations of 0 nM, 100 nM, and 200 nM. Control incubations received no inhibitor and no androstenedione. Each point represents the average of three determinations with less than 5% variation from the mean.

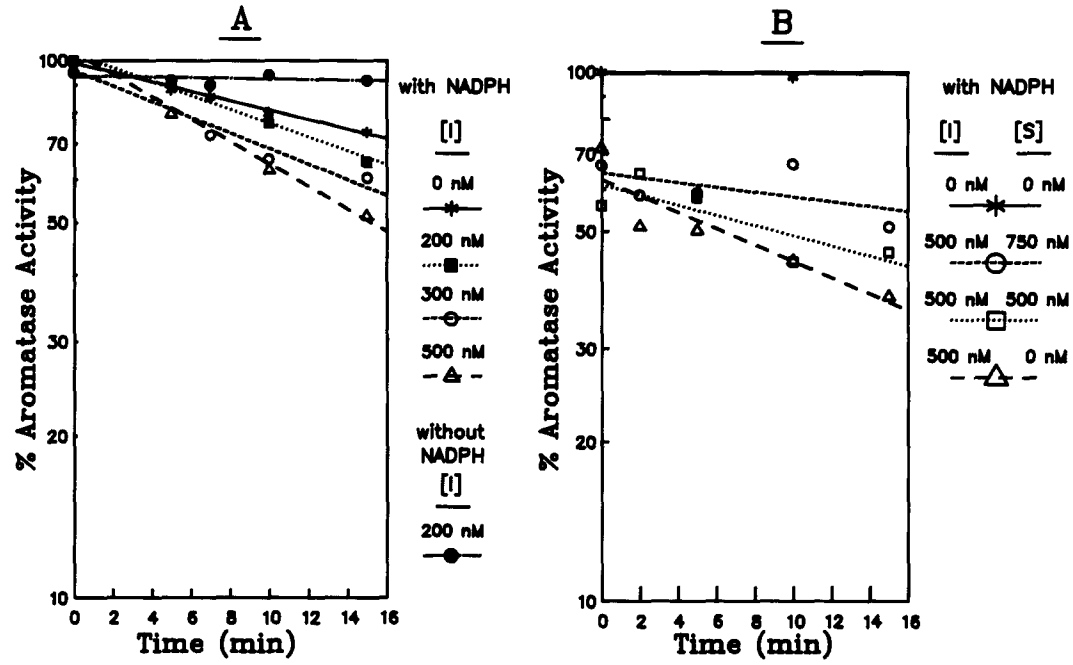


Figure 3 Inactivation of aromatase activity by 7α-IPTADD **9**. ***(A)*** Time-dependent, first-order inactivations of aromatase activity were produced in the presence of NADPH at inhibitor concentrations of 0 nM, 200 nM, 300 nM, and 500 nM. No inactivation was observed in the absence of NADPH at inhibitor concentration of 200 nM. Each point represents the average of three determinations with less than 5% variation from the mean. ***(B)*** The rate of inactivation by inhibitor (500 nM) was decreased by increasing androstenedione ([S]) concentrations of 0 nM, 500 nM, and 750 nM. Control incubations received no inhibitor and no androstenedione. Each point represents the average of three determinations with less than 5% variation from the mean.

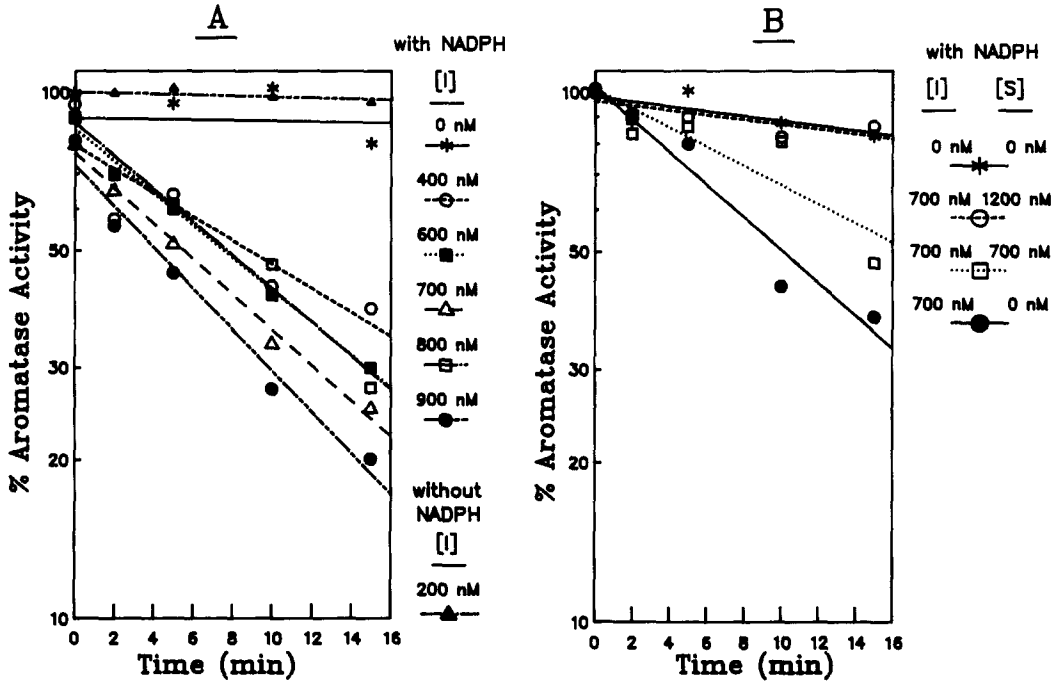


Figure 4 Inactivation of aromatase activity by 7α-(2'-Naphthyl)thio-ADD **11**. ***(A)*** Time-dependent, first-order inactivations of aromatase activity were produced in the presence of NADPH at inhibitor concentrations of 0 nM, 400 nM, 600 nM, 700 nM, 800 nM, and 900 nM. No inactivation was observed in the absence of NADPH at inhibitor concentration of 200 nM. Each point represents the average of three determinations with less than 5% variation from the mean. ***(B)*** The rate of inactivation by inhibitor (700 nM) was decreased by increasing androstenedione ([S]) concentrations of 0 nM, 700 nM, and 1200 nM. Control incubations received no inhibitor and no androstenedione. Each point represents the average of three determinations with less than 5% variation from the mean.

concentrations increased from 0–1500 nM, the half-life for aromatase activity was lengthened, suggesting that the inhibitors were interacting at the active site of microsomal aromatase.

Inactivation kinetic values were determined from plots of the half-time of inactivation, i.e., the time required to decrease the enzymatic activity by 50%, versus the reciprocal of the inhibitor concentration (Figure 5).[29–32] The y-intercept of the resulting line is the half-time of inactivation at infinite inhibitor concentration ($T_{1/2}$) and the rate of inactivation, k_{app}, is equal to 0.693/$T_{1/2}$. The slope is equal to the product of the y-intercept and apparent K_{inact}, which is the inactivation rate constant and is analogous to the Michaelis-Menten K_m. If the k_{app} is slow relative to the reversible binding of inhibitor with the enzyme (K_i), then the K_{inact} value for an inhibitor is greater than the K_i value.

The inactivation kinetics for these newly synthesized compounds are shown in Table 1 and compared with those values for 7α-APTADD. For the 7α-thiophenyl analogs **3, 4,** and **9,** the half-times of inactivation at infinite inhibitor concentration range from 5.65 minutes to 7.83 minutes; the k_{app} values for these inhibitors are approximately 1.5–2.0 × 10^{-3} $second^{-1}$. The apparent K_{inact} values for the three 7α-thiophenyl compounds are 151.6–483.4 nM. For the 7α-thionaphthyl analogs **10** and **11,** the half-times of inactivation are 3.72 minutes and 4.28 minutes, respectively; the k_{app} values for these inhibitors are approximately 2.7 to 3.1 × 10^{-3} $seconds^{-1}$. The apparent K_{inact} values for the two 7α-thionaphthyl compounds are 1060 and 821 nM, respectively.

Discussion

Effective enzyme-activated irreversible inhibitors have been developed by the introduction of aryl substituents at the 7α-position of androsta-1,4-diene-3,17-dione. The most effective agent of this series to date is the inhibitor 7α-(4'-amino)phenylthioandrosta-1,4-diene-3,17-dione (7α-APTADD), first reported by Snider and Brueggemeier in 1987.[20]

Two halogenated and one unsubstituted 7α-thiophenylandrosta-1,4-diene-3,17-diones were synthesized and evaluated for aromatase inhibitory activity in the human placental microsomal preparation. Under initial velocity assay conditions of low product formation, the inhibitors demonstrated potent inhibition of aromatase, with apparent K_i's ranging from 12–22 nM. These apparent K_i values are similar to those for 7α-APTADD, with an apparent K_i of 10 nM. Furthermore, these compounds produced time-dependent, first-order inactivation of aromatase in the presence of NADPH, whereas no aromatase inactivation was observed in the absence of NADPH. The half-time of inactivation at infinite inhibitor concentration ranges from 5.65–7.83 minutes; the k_{app} values for these inhibitors are approximately 1.5–2.0 × 10^{-3} $seconds^{-1}$. However, these values indicate slower rates and longer half-times of inactivation when compared to 7α-APTADD. These results

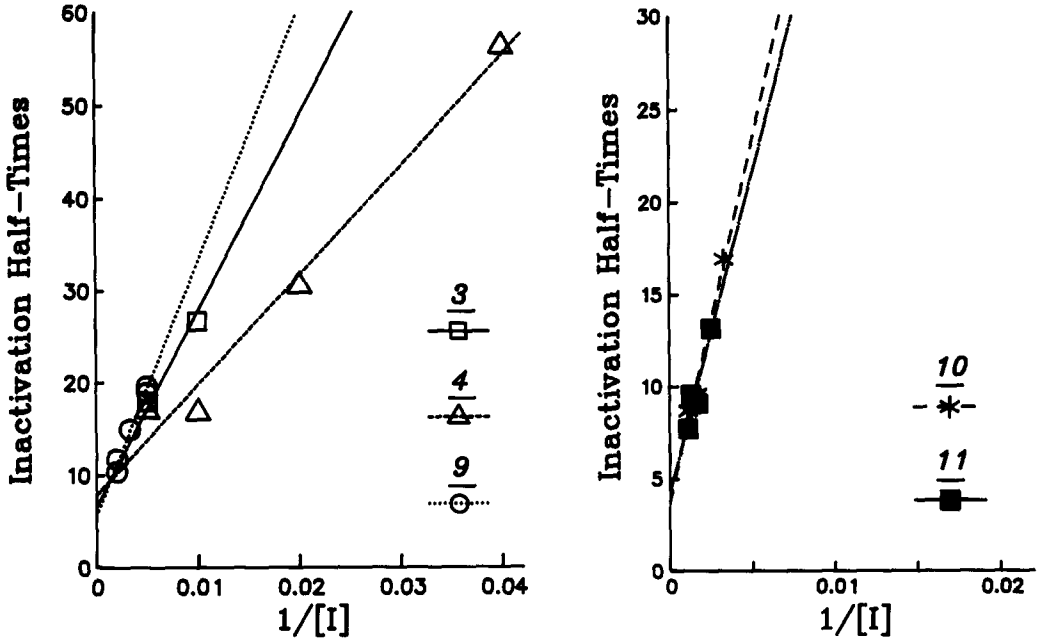


Figure 5 Inactivation Half-Times vs 1/[I] for 7α-Substituted Androstadienediones. Inactivation kinetics are calculated from the slope and intercept of the lines for 7α-PTADD **3**, 7α-BrPTADD **4**, 7α-IPTADD **9**, 7α-(2'-Naphthyl)thio-ADD **10**, and 7α-(2'-Naphthyl)thio-ADD **11**.

on varying aromatase inactivation kinetics by 7α-thiophenyl derivatives having similar affinities suggest that the binding of these compounds leads to microsomal enzyme complexes with varying degrees of efficiency for enzyme catalysis and enzyme-mediated inactivation.

The differences in enzyme catalysis and enzyme-mediated inactivation for 7α-substituted androstadienediones are further illustrated by examining the biochemical results of the two 7α-thionaphthyl analogs. Again, the apparent K_i values for **10** and **11** are similar to the 7α-thiophenyl derivatives **3, 4,** and **9**. However, the slopes of the lines in inactivation half-time vs. 1/[I] plots (Figure 5) are greater, and the resulting inactivation rate constants, apparent K_{inact}'s, are significantly larger. Thus, the efficiencies for enzyme catalysis and enzyme-mediated inactivation upon binding of the 7α-thionaphthyl analogs are significantly diminished when compared to the 7α-thiophenyl analogs and 7α-APTADD.

The 7α-thioarylandrosta-1,4-diene-3,17-diones examined to date all have high affinity for the active site of the enzyme, with approximately 3–5 times greater affinity than shown for the substrate androstenedione. Substitution of the larger halogen atoms, such as bromine or iodine, in place of the 4'-hydrogen did not affect the affinity of the enzyme for the inhibitors. The apparent K_i results of these inhibitors from the biochemical assays are consistent with previous studies on 7α-substituted competitive inhibitors[14,18,19,33] that indicate bulky substituents can be accommodated at the 7α-position.

However, inactivation half-times at infinite inhibitor concentration, the k_{app} rates of inactivation, and the apparent K_{inact}'s suggest that differences can be observed in enzyme catalysis and enzyme-mediated inactivation with 7α-substituted androstadienediones. These differences in the efficiencies of enzyme-mediated inactivations suggest that minor conformational changes induced upon binding of 7α-substituted androstadienediones to the microsomal enzyme complex produce significant effects on catalytic activity. Thus, these 7α-substituted C_{19} inhibitors provide useful pharmacological tools for probing the active site of the aromatase enzyme complex.

Acknowledgments

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