Synthesis and biochemistry of fluorescent aromatase inhibitors

Hsiu-Ho Chen* and Robert W. Brueggemeier

College of Pharmacy, The Ohio State University, Columbus, Ohio, USA

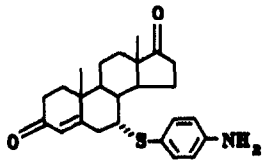
Effective aromatase inhibitors have been developed that contain aryl functionalities at the 7α-position of the steroid nucleus. The exact interactions of 7α-substituted androstenediones with the active site of aromatase is unknown. Fluorescent derivatives may provide a useful spectroscopic method for examining the binding of these inhibitors to the microsomal complex and purified aromatase protein. Dinitrophenyl, dansyl, and naphthyl derivatives of 7α-(4'-amino)phenylthio-4-androstene-3,17-dione and androstenedione were synthesized as potential fluorescent agents. An in vitro assay with human placental microsomes was used to evaluate aromatase inhibitory properties. These fluorescent compounds were effective competitive inhibitors and have apparent K_i values ranging from 24.1 to 86.7 nM. (*Steroids* **55**:123–127, 1990)

Keywords: steroids; aromatase; competitive inhibition; fluorescent agents; enzyme kinetics

Introduction

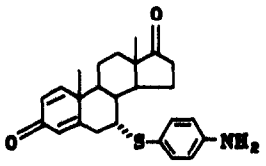
Aromatase is the cytochrome P450 enzyme complex responsible for estrogen biosynthesis in vivo. Inhibitors of this enzyme complex may be useful in controlling reproductive processes and in treating estrogen-dependent disease states such as endometrial cancer and postmenopausal breast cancer.[1–3] The therapeutic efficacies of aromatase inhibitors such as 4-hydroxyandrostenedione and aminoglutethimide are being investigated; these inhibitors produced regression of hormone-dependent breast tumors in both rats[1–3] and humans.[4–6]

Previous work from our laboratory has illustrated that substitution at the 7α position of androstenedione results in inhibitors of enhanced affinity for aromatase.[7–13] This group of inhibitors includes competitive, affinity, photoaffinity, and enzyme-activated irreversible inhibitors. 7α-(4'-Amino)phenylthio-4-androstene-3,17-dione, 7α-APTA (**1**), is one of the most potent competitive inhibitors, with an apparent K_i of 18 nM.[8] Additionally, 7α-(4'-amino)phenylthio-1,4-androstadiene-3,17-dione, 7α-APTADD (**2**), is one of the most effective mechanism-based inhibitors of aromatase reported to date, with an apparent K_i of 9.9 nM and a half-time of inactivation (τ_{50}) of 1.38 minutes.[12]



7α-APTA

1



7α-APTADD

2

The exact interactions of 7α-substituted androstenediones with the active site of aromatase are unknown. Fluorescent derivatives may provide a useful spectroscopic method for examining the binding of these inhibitors to the microsomal complex and purified aromatase protein. Results from these studies could further expand information concerning structure-activity relationships of aromatase inhibitors. Such fluorescent ligands may also be useful in studies of tissue and cellular distribution of the aromatase enzyme complex. We report the synthesis of four fluorescent 7α-substituted steroids and their inhibitory activity in microsomal preparations from human term placenta.

Experimental

All melting points (mp) were taken with a Fisher-Johns (Fisher Scientific, Pittsburgh, PA, USA) melting point apparatus and are uncorrected. Proton nuclear magnetic resonance (NMR) spectra were performed on a multinuclear IBM AF-270 pulse NMR spectrometer, infrared (IR) spectra were recorded on a Beckman IR

* Present address: School of Pharmacy, National Defense Medical Center, P.O. Box 90048, Taipei, Taiwan, R.O.C.
Address reprint requests to Dr. Robert W. Brueggemeier, College of Pharmacy, The Ohio State University, 500 West 12th Avenue, Columbus, OH 43210, USA.
Received August 4, 1989; revised October 16, 1989.

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4230 spectrophotometer, ultraviolet (UV) spectra were obtained on a Beckman DU-40 spectrophotometer, and fluorescent emission spectra were recorded on Farrand spectrofluorometer MK-2 equipped with an x-y recorder. Mass spectra (MS) were taken with a Kratos MS-30 instrument at ionization energy of 70 eV. Elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN).

Steroids were obtained from Searle Laboratories (Skokie, IL) or Research Plus (Bayonne, NJ) and were checked for purity by mp, thin-layer chromatography, or NMR. All other chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). Solvents and chemical reagents were dried and purified prior to use when deemed necessary. Biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). [1β-^{3}H]Androst-4-ene-3,17-dione (28 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Radioactive samples were detected with a Beckman LS 6800 liquid scintillation counter using Formula 963 (New England Nuclear) as the counting solution.

Synthetic Methods

The preparation of 7α-APTA has been described elsewhere.[4-6]

7α-[4'-(2",4"-Dinitrophenyl)amino]phenylthio-4-androstene-3,17-dione (4). 7α-APTA (20 mg, 0.05 mmol) in 0.5 ml ethanol and 40 mg of sodium bicarbonate in 0.5 ml of distilled water were added to a solution of 40 mg (0.028 ml, 0.2 mmol) of 2,4-dinitrofluorobenzene in 1 ml of ethyl alcohol. The mixture was stirred for 15 hours at room temperature. The solution was concentrated to remove ethyl alcohol, and 10 ml of water was then added. It was extracted with ethyl acetate and dried (Na_2SO_4). After rotary evaporation of solvent, the residue was chromatographed over 10 g of silica gel (dichloromethane-ethyl acetate, 10 : 1). Recrystallization in methyl alcohol provided 22 mg (78.3%) of pure **4:** mp 223 to 224 C; IR (KBr) 3,310, 3,080, 2,920, 2,880, 1,735, 1,670, 1,590, 1,490, 1,330, 800 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$) δ 0.93 (s, 3H, C_{18}), 1.23 (s, 3H, C_{19}), 3.64 (m, 1H, C_7H), 5.68 (d, J = 1.2 Hz, 1H, C_4H), 7.19 (d, J = 9.5 Hz, 1H, ArH coupling), 7.25 (d, J = 8 Hz, 2H, ArH), 7.49 (d, J = 8 Hz, 2H, ArH), 8.20 (dd, J = 9.5, 2.6 Hz, 1H, ArH), 9.18 (d, J = 2.6 Hz, 1H, ArH), 9.93 (s, 1H, NH). Analysis calculated for $C_{31}H_{33}N_3O_6S$: C, 64.68; H, 5.78; N, 7.30. Found: C, 64.22; H, 5.78; N, 7.16.

7α-(4'-Dansylamino)phenylthio-4-androstene-3,17-dione (5). To a stirred solution of 20 mg (0.05 mmol) of 7α-APTA in 5 ml of the mixture of acetone and 0.1 M of Na_2CO_3 (1 : 1), pH 9.5 to 10, was added 57 mg (0.2 mmol) of dansyl chloride. The reaction mixture was stirred at room temperature in the dark for 10 hours. The solution was filtered and concentrated in vacuo. The residue was flash-chromatographed over 10 g of silica gel (dichloromethane-ethyl acetate, 4 : 1) to afford a yellow powder **5** (4.5 mg, 14.3%): mp 165 C (dec.); ^{1}H-NMR (270 MHz, $CDCl_3$) δ 0.90 (s, 3H, C_{18}), 1.20 (s, 3H, C_{19}), 2.88 (s, 6H, $N(CH_3)_2$), 3.40-3.41 (m, 1H, C_7H), 5.58 (d, J = 1.2 Hz, 1H, C_4H), 6.87 to 6.91 (m, 3H, ArH & NH), 7.16 to 7.20 (m, 3H, ArH), 7.46 (dd, J = 8.5, 7.5 Hz, 1H, ArH), 7.59 (dd, J = 8.5, 7.5, Hz, 1H, ArH), 8.20 (dd, J = 7.2, 1.2 Hz, 1H, ArH), 8.30 (d, J = 8.5 Hz, 1H, ArH), 8.52 (d, J = 8.5 Hz, 1H, ArH). Exact mass calculated for $C_{31}H_{31}N_3O_6S$ m/e: 643.2664; found m/e: 643.2670.

7α-(1'-Naphthyl)thio-4-androstene-3,17-dione (6). 4,6-Androstadiene-3,17-dione (4,6-ADD, 170 mg, 0.6 mmol) was dissolved in 1-naphthalenethiol (0.25 ml, 1.8 mmol) under argon, and a small piece of sodium metal (approximately 10 mg) was added to the solution. The reaction was heated to 75 to 80 C in oil bath for 18 hours. A crude solid was obtained when cooled, which was then washed with petroleum ether and filtered. The residue was chromatographed over 10 g of silica gel (hexane/ethyl acetate, 2 : 1). The product was recrystallized with ethyl acetate to afford 202 mg (76%) of white crystals of compound **6:** mp 228-230 C (dec.); IR (KBr) 3,010, 2,900 (br), 1,740, 1,650, 1,625 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$) δ 0.93 (s, 3H, C_{18}), 1.23 (s, 3H, C_{19}), 3.64 to 3.68 (m, 1H, C_7H), 5.65 (d, J = 1.6 Hz, 1H, C_4H), 7.43 (dd, J = 8, 7 Hz, 1H, ArH), 7.50 to 7.62 (m, 2H, ArH), 7.73 (dd, J = 7, 1 Hz, 1H, ArH), 7.86 (dd, J = 10, 8 Hz, 2H, ArH), 8.4 (d, J = 8 Hz, 1H, ArH); MS m/e (relative intensity) 444 (M^+, 0.03), 284 (0.35), 160 (0.52), 115 (1.00). Analysis calculated for $C_{29}H_{32}O_2S$: C, 78.34, H, 7.25. Found: C, 78.06; H, 7.13.

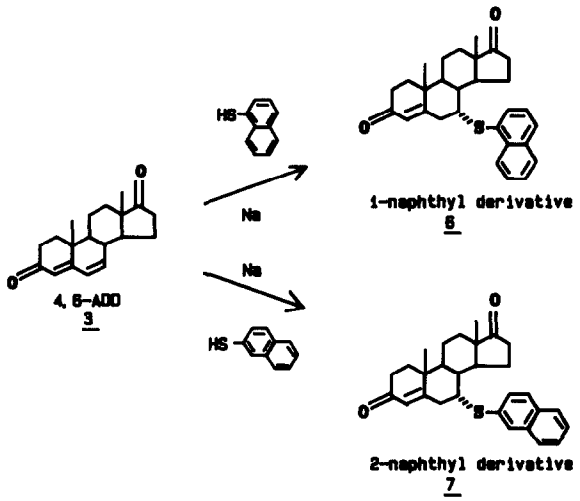
7α-(2'-Naphthyl)thio-4-androstene-3,17-dione (7). 4,6-ADD (170 mg, 0.6 mmol) was added to a melted and stirred solution of 2-naphthalenethiol (200 mg, 1.2 mmol) at 80 C under argon, and a small piece of sodium metal (approximately 10 mg) was then added. The reaction was heated at 80 C for 24 hours. A white powdered solid was obtained when slowly cooled. The solid was then washed with petroleum ether and filtered. The residue was chromatographed over 10 g of silica gel (hexane/ethyl acetate, 4 : 1) and recrystallized with ethyl acetate to get 165 mg (62%) of white crystals of compound **7:** mp 205 to 206 C; IR (KBr) 3,015, 2,900 (br), 1,730, 1,660, 1,640 cm^{-1}; ^{1}H-NMR (270 MHz, $CDCl_3$) δ 0.95 (s, 3H, C_{18}), 1.25 (s, 3H, C_{19}), 3.69 to 3.70 (m, 1H, C_7H), 5.73 (d, J = 1.5 Hz, 1H, C_4H), 7.44 to 7.54 (m, 3H, ArH), 7.64 to 7.89 (m, 4H, ArH); MS m/e (relative intensity) 444 (M^+, 0.12), 284 (0.75), 160 (0.95), 136 (1.00), 115 (0.81). Analysis calculated for $C_{29}H_{32}O_2S$: C, 78.34; H, 7.35. Found: C, 78.61; H, 7.23.

Biochemical Methods

Placental microsomes were prepared as previously described.[13]

Competitive inhibition studies. Various concentrations of 4-androstene-3,17-dione (60-500 nM; 200,000 dpm) and a single concentration of inhibitor were preincu-

bated with propylene glycol (100 μl), nicotinamide adenine dinucleotide phosphate (1.8 mM), glucose-6-phosphate (2.85 mM), and glucose-6-phosphate dehydrogenase (5 U) in 0.5 ml at 37 C for 5 minutes. Placental microsomes (0.07 to 0.12 mg) were resuspended and diluted to 3.0 ml with 0.1 M of sodium phosphate buffer solution, pH 7, and added to the preincubated mixture. The solution was incubated at 37 C for 15 minutes. The reaction was stopped by the addition of 5 ml of $CHCl_3$ to the incubate. After vortexing for 20 seconds, the $CHCl_3$-quenched samples were centrifuged at 1,250 × *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. The results were analyzed by weighted regression programs.[14] Protein concentrations were determined by the Bradford protein assay.[15]

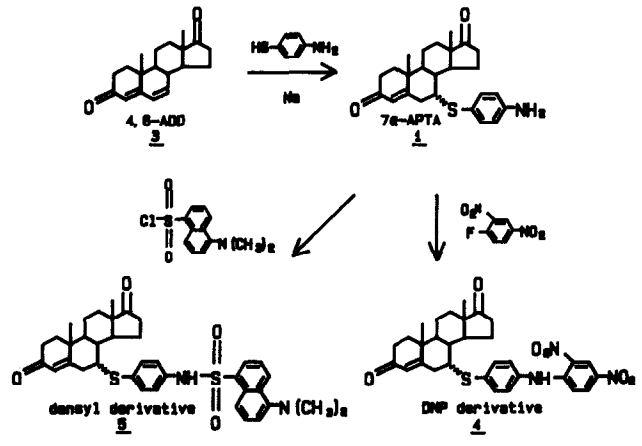


Scheme 2

Results

Two fluorescent analogs were prepared by derivatization of the effective competitive inhibitor, 7α-APTA. The synthesis of derivatives **4** and **5** was accomplished by alkylation of the free amine group of 7α-APTA with 2,4-dinitrofluorobenzene and dansyl chloride, respectively (Scheme 1). In the synthesis of these dansylamine and dinitrophenyl (DNP) derivatives, the pH range of the reactions is critical. 2,4-Dinitrofluorobenzene readily reacts with the amine functionality, with sodium bicarbonate (0.9 to 1.0 nM) used in the reaction to optimize the pH range of 8 to 9. On the other hand, the optimal pH for dansylation is 9.5 to 10.5. However, the dansylamine product **5** decomposed at room temperature and was stored under nitrogen in the dark at 4 C. The yield of DNP derivative **4** was 78.3% and was greater than the yield of 14.3% for derivative **5**.

A second approach to the design of fluorescent aromatase inhibitors was to replace the phenyl substituent. Fluorescent moieties were incorporated directly onto the 7α-position by a Michael addition of the moiety to 4,6-androstadiene-3,17-dione. The naphthylthio derivatives **6** and **7** were prepared from 4,6-androstadiene-3,17-dione and 1- or 2-naphthalenethiol under base catalysis (Scheme 2). The syntheses were straightforward, and the desired compounds were obtained in high yields (62% and 76%, respectively). Ultraviolet absorption and fluorescence emission wavelengths of synthetic aromatase inhibitors **4** through **7** are shown in Table 1.



Scheme 1

Enzyme kinetic studies of aromatase inhibition were performed using preparations of human placental microsomes. Aromatase activity was assayed under initial velocity conditions by the radiometric method which measured the amount of ^3H_2O released as an index of estrogen formation. The dansylamine derivative of 7α-APTA, compound **5**, was not stable in the buffer solution and was not tested for enzyme inhibition. The results of the kinetic analysis using human placental microsomes are shown in Table 2. Compounds **4**, **6**, and **7** exhibited competitive inhibition, as determined from the Lineweaver-Burk plots and the weighted regression analysis.[14] These results are plotted as 1/velocity versus 1/[substrate] (Figures 1 and 2).

Table 1 Ultraviolet absorption and fluorescence emission wavelengths

Compound	UV, λ_{max} (nm)	Fluorescence, λ_{max} (nm)
4	243	365
5	229	515
6	224	394
7	225	362

Table 2 Apparent K_i values for fluorescent aromatase inhibitors

Compound	K_i (nM)	V_{max} (nmol/mg/min)
4	86.7 ± 12.3	147.9 ± 5.6
6	24.1 ± 7.1	230.7 ± 17.8
7	38.9 ± 9.2	203.6 ± 11.4

Apparent K_m for androstenedione in these assays ranged from 58.8 nM (±17.1) to 68.5 nM (±11.1).

Discussion

The introduction of a 7α-substituent on the androstenedione molecule imparts similar or greater affinity of the steroid analog for the aromatase enzyme.[7-12] Furthermore, numerous effective inhibitors in this group of steroid derivatives have phenyl substituents at this 7α-position. Incorporation of other aryl moieties, such as fluorescent aromatic groups, would provide potentially new aromatase inhibitors with unique characteristics.

The dinitrophenyl derivative of 7α-APTA (**4**) and the two 7α-naphthylthio derivatives of androstenedione (**6** and **7**) were prepared in high yields and demonstrated good to excellent inhibition of placental microsomal aromatase. Compounds **6** and **7**, the naphthylthio derivatives, exhibit a high affinity for aromatase, as demonstrated by the excellent apparent K_i values of 24.1 and 38.9 nM. These results indicated that 7α-substituents with bulky, planar functionalities can interact effectively at the active site of aromatase. Additionally, as evidenced by a higher K_i and lower affinity of compound **4**, modifications that extend the 7α-substituents away from the steroid nucleus lead to a decrease in affinity for the enzyme complex. Thus, the studies further extend the structure-activity relationships of this class of important aromatase inhibitors.

The fluorescent derivatives described here, compounds **4**, **6**, and **7**, interact with high affinity to the aromatase enzyme complex. These agents may serve as biochemical probes in spectroscopic studies with the aromatase protein. Additionally, these fluorescent aromatase inhibitors may also be useful as fluorescent ligands in studies of tissue and cellular distribution of the enzyme complex.

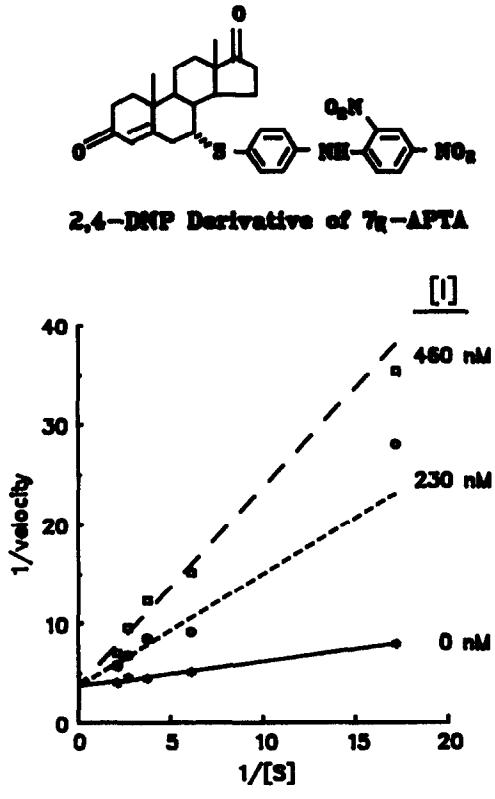


Figure 1 Double reciprocal plot of aromatase inhibition by compound **4**. Varying concentrations of androstenedione were incubated with aromatase at the indicated inhibitor concentrations. Each point represents the average of two determinations with variation of less than 7%.

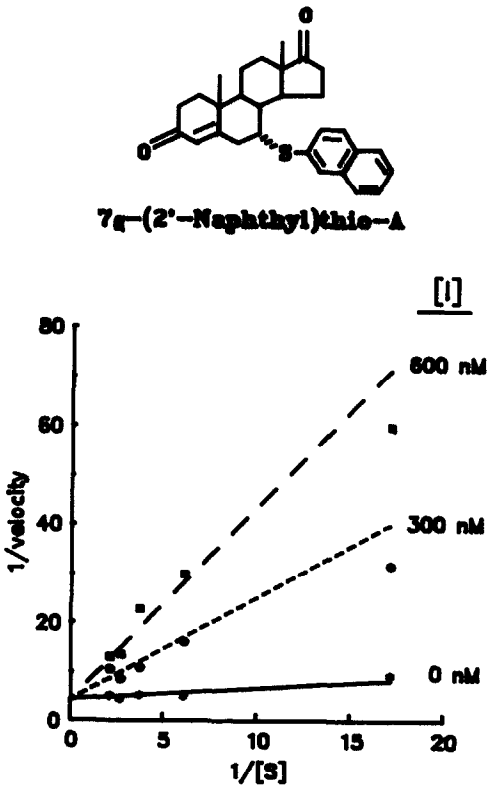


Figure 2 Double reciprocal plot of aromatase inhibition by compound **7**. Varying concentrations of androstenedione were incubated with aromatase at the indicated inhibitor concentrations. Each point represents the average of two determinations with variation of less than 7%.

Acknowledgments

This work was supported in part by NIH Grant R01 DK-40255 and a fellowship (H.-H. Chen) from the Chung Shan Institute of Science and Technology, Taiwan. A portion of this work was presented at the 71st Annual Meeting, the Endocrine Society, June 21–24, 1989, Seattle, WA (abstr no. 703).

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