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7-SUBSTITUTED STEROIDAL AROMATASE INHIBITORS: STRUCTURE-ACTIVITY RELATIONSHIPS AND MOLECULAR MODELING

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Androstenedione analogs containing 7α-substitutents have proven to be potent inhibitors of aromatase both *in vitro* and *in vivo*. Several of these agents have exhibited higher affinity for the enzyme complex than the substrate does. In order to examine further the interaction(s) of 7-substituted steroids with aromatase, biochemical and molecular modeling studies were performed on 7-substituted 4,6-androstadiene-3,17-diones. 7-Benzyl- and 7-phenethyl-4,6-androstadiene-3,17-diones effectively inhibited microsomal aromatase, with apparent K_i's ranging from 61 to 174 nM. On the other hand, 7-phenyl-4,6-androstadiene-3,17-dione exhibited poor activity, with an apparent K_i of 1.42 μM. Energy minimization calculations and molecular modeling indicated that the 7-substituent is perpendicular to the steroid nucleus in the 7-phenyl analog and can only adopt a pseudo β position. The 7-benzyl- and 7-phenethyl- groups of 4,6-androstadiene-3,17-diones orient themselves in the minimized structure in a way that the phenyl rings can protrude into the 7α pocket. These orientations are similar to those observed in minimized structures for potent 7α-substituted androstenediones.

KEY WORDS: Aromatase inhibition, 7α-APTA, 7α-APTADD, 7-substituted 4,6-androstadiene-3,17-diones, structure-activity relationships, molecular modeling, conformational analysis.

INTRODUCTION

Several 7α-thiosubstituted derivatives of androstenedione have demonstrated very effective inhibition of aromatase activity present in placental microsomes.[1–6] Among the compounds synthesized, 7α-(4′-amino)phenylthio-4-androstene-3,17-dione (7α-APTA) was found to be one of the most potent competitive inhibitors with an apparent K_i of 18 nM.[1] This inhibitor has also demonstrated effectiveness in inhibiting aromatase in cell cultures[6,7] and in treating hormone-dependent rat mammary tumors.[6,8] Affinity labeling derivatives of 7α-APTA produced inactivation of aromatase, with a [^{14}C]-analog demonstrating covalent binding to aromatase.[2,3]

Androstenedione derivaratives with extended linear conjugation in ring A and/or B produced effective inhibition of aromatase.[9] Furthermore, the introduction of an additional double bond in the A ring resulted in inhibitors that inactivated aromatase by an enzyme-catalyzed process.[10–12] Introduction of a 7α-substitutent on 1,4-androstadiene-3,17-dione yielded a potent mechanism-based irreversible inhibitor of aromatase, 7α-(4′-amino)phenylthio-1,4-androstadiene-3,17-dione (7α-APTADD). This compound exhibited an apparent K_i of 9.9 nM and has the most rapid rate of inactivation of aromatase reported to date.[5] The introduction of substituents at C_7 of 4,6-androsta-diene-3,17-dione may lead to enhanced affinity of these analogs for the aromatase complex. In addition, replacement of the carbon-sulfur bond with a

carbon-carbon bond would yield analogs with similar lipophilic character and eliminate potential metabolic oxidation of the thioether linkage. Thus, compounds with both features (extended linear conjugation in ring A and/or B and a carbon-carbon bond at C_7) may result in potent inhibitors. Various 7-substituted 4,6-androstadiene-3,17-dione derivatives have recently been prepared.[13] This paper provides further biochemical data on these agents. In addition, structure-activity relationships (SAR) and molecular modeling of these agents and the 7α-substituted 4-androstene-3,17-diones are described in this report.

MATERIALS AND METHODS

Materials

Steroids were purchased from Searle Laboratories (Skokie, IL) or Steraloids (Wilton, NH) and checked for purity by thick layer chromatography or melting point. The synthesis of the 7-substituted 4,6-androstadiene-3,17-diones has been recently published.[13] Chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). Silica gel was purchased from E. Merck (Darmstadt, Germany) and aluminum oxide (basic) from Fischer Scientific (Fair Lawn, NJ). TLC plates were purchased from Analtech Inc. (Newark, NE). Biochemicals were obtained from Sigma Chemical Co (St. Louis, MO). [1β-^{3}H]-4-Androstene-3,17-dione was purchased from New England Nuclear (Boston, MA). Centrifugation was performed on a Sorvall RC2-B centrifuge and a Beckman L5-50B ultracentrifuge was used for ultracentrifugation. Radioactive samples were detected with a Beckman LS 6800 scintillation counter using Formula 963 (New England Nuclear) as the counting solution.

Biochemical Methods

Human placenta were obtained immediately upon delivery from the Ohio State University Hospital and stored on ice during transportation to the laboratory. The preparation of microsomes was performed according to the method of Ryan.[14] All procedures were carried out at 0–4°C. The placenta was cut free of connective tissue and large blood vessels with scissors. The tissue was then homogenized in a cold Waring blender with two parts of tissues to one part of homogenization buffer consisting of 0.05 M sodium phosphate, 0.25 M sucrose and 0.04 M nicotinamide, pH 7. The homogenate was centrifuged at 10,000 × g for 30 min. The debris was discarded and the supernatant centrifuged at 105,000 × g for 1 h. The microsomal pellet obtained was resuspended in 0.1 M sodium phosphate buffer, pH 7.0, and centrifuged at 105,000 × g for 1 h. The procedure was repeated once again and the resulting pellet was stored at − 70°C until needed.

Competitive Inhibition Studies

Aromatase activity in human placental microsomes was assayed by the radiometric method developed by Siiteri and Thompson[15] in which the tritium from [1β-^{3}H]-4-androstene-3,17-dione was released as ^3H_2O and used as an index of estrogen formation. The procedures for evaluation of inhibition are similar to those previously reported by Brueggemeier *et al.*[6] [1β-^{3}H]-4-Androstene-3,17-dione (300,000 dpm),

various concentration of 4-androstene-3,17-dione (60–500 nM) and a concentration of inhibitor (0–600 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 37°C for 5 min. Placental microsomes (0.07–0.1 mg) were diluted to 3.0 ml with 0.1 M sodium phosphate buffer, pH 7, and warmed to 37°C for 5 min. The enzyme assay began with the addition of the microsomal suspension (3.0 ml) to the mixture of steroids and cofactors. The solution was incubated at 37°C for 15 min a shaking water bath and was stopped by addition of $CHCl_3$ (5 ml), followed by vortexing the samples for 20 s. The samples were then centrifuged for 10 min (1,000 g). Aliquots of water (200 μl) were mixed with scintillation cocktail (5 ml) and counted for radioactivity. Assays were run in duplicate and control samples containing no inhibitor were run simultaneously. Blank samples were obtained by incubating boiled microsomes. Results were analyzed by a weighted regression analysis computer program.[16]

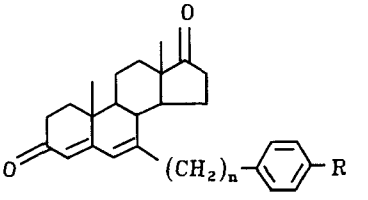
Molecular Modeling

All structure building, energy calculations and conformational analyses were performed on a Silicon Graphics IRIS 4D/70GT system (Mountain View, CA) running Quanta 2.1A and CHARMm 21.1.7b (Polygen Corp., Waltham, MA). Minimizations and volume calculations were performed with SYBYL 5.22 (Tripos Associates, St. Louis, MO). All local minima were displayed and plotted using PLUTO (University Chemical Laboratory, Cambridge, UK).

RESULTS

Several 7-substituted 4,6-androstadiene-3,17-diones were evaluated *in vitro* by enzyme kinetic studies using human placental microsomes. These compounds included 7-phenyl-, 7-benzyl-, and 7-phenethyl-4,6-androstadiene-3,17-diones, compounds **1–7** (Table I).[13] All the inhibitors were evaluated at concentrations ranging from 0 to

TABLE I
Aromatase Inhibition by Various 7-Substituted 4,6-Androstadienediones



cpd.	compound	n	R	K^*_i	inhibition
1	7-phenyl-ADD	0	–H	1424 nM	competitive
2	7-benzyl-ADD	1	–H	61	competitive
3	7-phenethyl-ADD	2	–H	174	competitive
4	7-nitrobenzyl-ADD	1	$-NO_2$	94	competitive
5	7-aminobenzyl-ADD	1	$-NH_2$	88	competitive
6	7-nitrophenethyl-ADD	2	$-NO_2$	95	competitive
7	7-aminophenethyl-ADD	2	$-NH_2$	88	competitive

*K_m for androstenedione = 51 nM (S.E. = 9 nM)

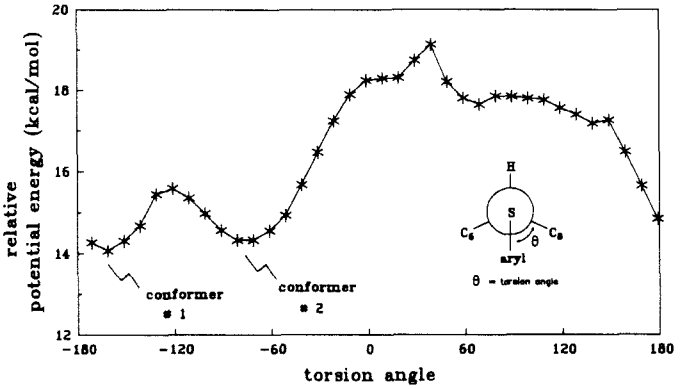


FIGURE 1 Conformational Analysis of 7α-APTA. The torsion angle between the C_7–C_8 bond of the steroid ring, the C_7-sulfur bond of the 7α-substituent, and the sulfur-aryl bond was explored in 10° increments over 360°. The relative potential energy of each resulting conformation was then calculated and plotted vs. torsion angle.

600 nM in initial velocity studies performed under limiting enzyme concentrations. In these microsomal assays, substrate concentrations were varied while the inhibitor concentration remained constant. Each substrate concentration was run in duplicate and the results of the studies were plotted in a typical lineweaver-Burk or double-reciprocal plot as 1/velocity vs 1/[substrate]. The apparent K_i of the inhibitor is an index of the affinity of the enzyme for the inhibitor and is determined by a weighted regression analysis computer program.[16]

The apparent K_i's for 7-substituted 4,6-androstadiene-3,17-diones **1**–**7** ranged from 61 nM to 1.42 μM (Table I). Each inhibitor demonstrated competitive inhibition, as determined from the Lineweaver-Burk plots and V_{max} intercepts. In these studies, the apparent K_m for androstenedione was found to be 0.051 (± 0.009 μM).

To investigate the differences in binding affinity (apparent K_i's) of the aromatase inhibitors, computer-assisted molecular modeling was performed. Analyses were performed on these 7-substituted 4,6-androstadiene-3,17-diones and compared with two of the most potent 7α-substituted 4-androstene-3,17-diones, 7α-APTA and 7α-APTADD. The inhibitors were constructed with Quanta and the energy minimization was performed with CHARMm using the conjugate gradient method. Conformational analyses of each minimized structure was carried out. The torsion angle between the C_7–C_8 bond of the steroid ring, the C_7-carbon (or -sulfur) bond of the 7-sub-

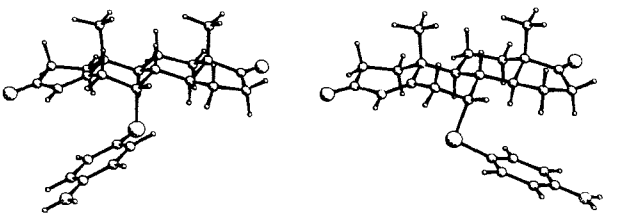


FIGURE 2 Energy-minimized Conformations of 7α-APTA. Conformations of the two local minima for 7α-APTA are viewed along the edge of the steroid nucleus.

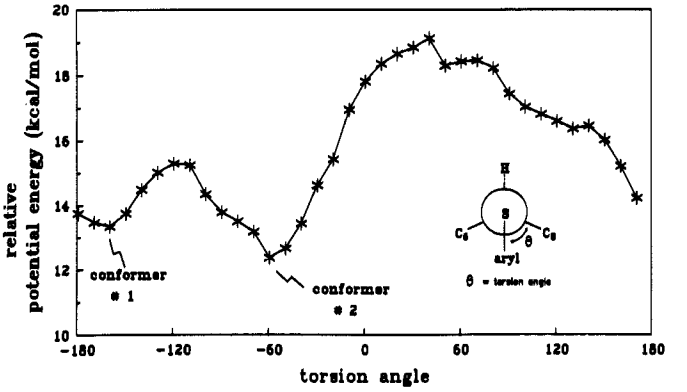


FIGURE 3 Conformational Analysis of 7α-APTADD. The torsion angle between the C_7–C_8 bond of the steroid ring, the C_7-sulfur bond of the 7α-substituent, and the sulfur-aryl bond was explored in 10° increments over 360°. The relative potential energy of each resulting conformation was then calculated and plotted vs. torsion angle.

stituent, and the carbon-aryl (or sulfur-aryl) bond was explored in 10° increments over 360°. The energy of each resulting conformation was then calculated. After the conformational analyses, the local minima of the analyses for each compound were obtained and plotted with PLUTO. Two local minima for 7α-APTA were obtained from this analysis (Figure 1); the conformations of each are shown in Figure 2. Analysis of 7α-APTADD also provided two local minima (Figures 3 and 4). The results for compounds **1**, **2**, and **3** are illustrated in Figures 5 and 6. One local minima was obtained for **1** and **2**, while two minima were obtained for **3**.

DISCUSSION

The inhibitors exhibited a wide range of inhibitory activity, with apparent K_i's of 60.9 nM to 1.424 μM. The 7-benzyl- and 7-phenethyl-4,6-androstadiene-3,17-diones, **2**–**7**, are effective inhibitors of aromatase and exhibited apparent K'_is ranging from 60.9 to 174 nM. 7-Phenyl-4,6-androstadiene-3,17-dione **1**, on the other hand, is a poor inhibitor, with an apparent K_i of 1.424 μM.

Previous studies illustrated that aromatase has considerable tolerance for androstenedione and testosterone derivatives with bulky 7α-substituents and, in fact, has higher affinities for several of these inhibitors than for the substrate.[1–6] The biochemical results of the 7-substituted 4,6-androstadiene-3,17-diones suggest that the orienta-

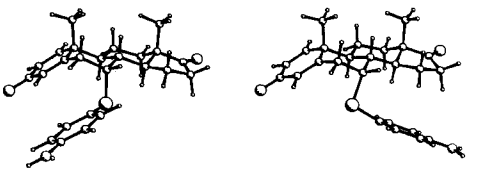


FIGURE 4 Energy-minimized Conformations of 7α-APTADD. Conformations of the two local minima for 7α-APTADD are viewed along the edge of the steroid nucleus.

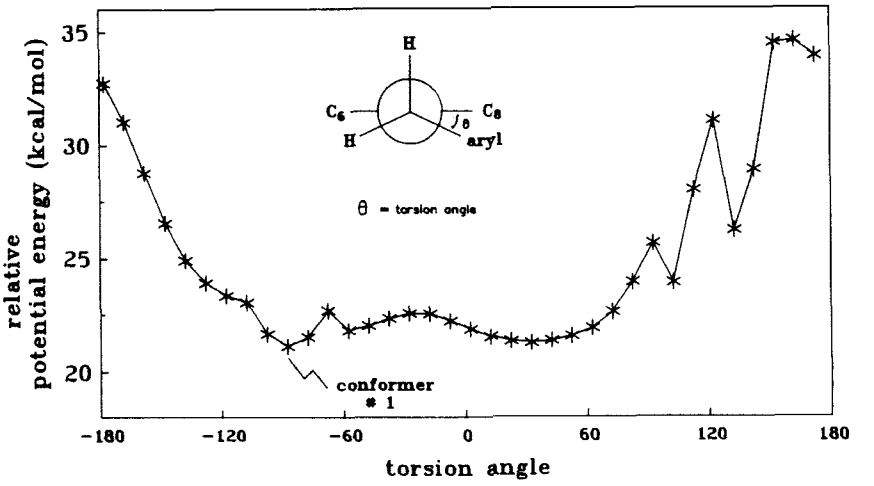


FIGURE 5 Conformational Analysis of 7-Benzyl-4,6-androstadiene-3,17-dione. The torsion angle between the C_7–C_8 bond of the steroid ring, the C_7-carbon bond of the 7-substituent, and the carbon-aryl bond was explored in 10° increments over 360°. The relative potential energy of each resulting conformation was then calculated and plotted vs. torsion angle.

tion of the substituent can affect affinity of the inhibitor to the enzyme complex. The greatest difference in affinities observed in the microsomal incubation was obtained with the poor inhibitor, 7-phenyl analog **1**.

Molecular modeling and energy minimization analyses were performed on these agents to obtain a more quantitative correlation of the influence of conformation of

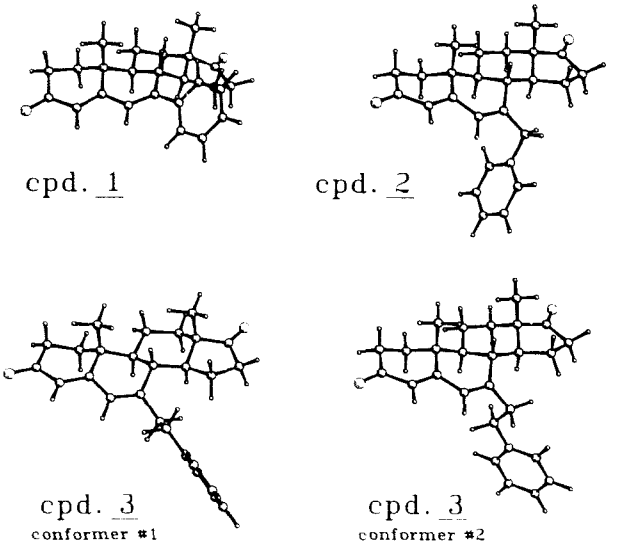


FIGURE Energy-minimized Conformations of 7-Substituted 4,6-androstadiene-3,17-diones. Conformations of the local minima for 7-phenyl-, 7-benzyl-, and 7-phenethyl-4,6-androstadiene-3,17-dione are viewed along the edge of the steroid nucleus.

the 7-substituent and of the 7α-substituent on aromatase inhibition. When the local minima of the inhibitors were examined, a significant difference can be observed in the orientation of the 7-substituents relative to the steroid nucleus. Two local minima are observed for 7α-APTA and 7α-APTADD, with the 7α-aminophenylthioether substituent on both inhibitors (and both conformations for each inhibitor) oriented below the steroid ring (Figures 2 and 4). On the other hand, the 7-substitutents of inhibitors **1**–**7** adopt different orientations, as shown in Figure 6. In the minimized conformation for 7-phenyl-4,6-androstadiene-3,17-diones, **1**, the 7-substitutent is perpendicular to the steroid nucleus and can only adopt a pseudo β position. The 7-benzyl- and 7-phenethyl- groups of 4,6-androstadiene-3,17-diones, **2** and **3**, orient themselves in the minimized structure in a way that the phenyl rings can protrude into the 7α pocket.

Finally, addition of polar substituents ($-NO_2$ or $-NH_2$) on the phenyl ring of 7-benzyl-4,6-androstadiene-3,17-dione resulted in inhibitors **4** and **6** with diminished inhibitory activity. However, the opposite result was observed for 7-phenethyl-4,6-androstadiene-3,17-diones in which addition of polar substituents ($-NO_2$ or $-NH_2$) om the phenyl ring resulted in inhibitors **5** and **7** with increased inhibitory activity. Therefore, additional factors other than geometry may be involved in enhancing the affinity of the inhibitors to the aromatase enzyme complex.

Thus, several 7-substituted 4,6-androstadiene-3,17-dione analogs were prepared and exhibited good competitive inhibiton of aromatase *in vitro* in human placental microsomes. The most effective inhibitors were those with extended linear conjugation (the 4,6-diene-3-one functionality) and flexible 7-substitution, such as the benzyl or phenethyl moiety. However, these inhibitors are not as effective as the previously reported 7α-thiosubstituted androstenediones.[1-6] Nevertheless, the 7-substituted 4,6-androstadiene-3,17-diones may offer an advantage *in vivo* over the 7α-thiosubstituted analogs by providing greater metabolic stability. Further evaluation of these new analogs in cell culture systems and *in vivo* will provide additional information on the efficacy of these new androstadienediones as potential aromatase inhibitors for the treatment of estrogen-dependent cancers.

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