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AROMATASE INHIBITION BY 4-THIOSUBSTITUTED-4-ANDROSTENE-3,17-DIONE DERIVATIVES

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Summary—The synthesis and evaluation of 4-thiosubstituted-4-androstenedione analogs as inhibitors of estrogen synthetase (aromatase) is described. All compounds were prepared by the addition of various thiol reagents to 4β,5β-epoxyandrostanedione. Inhibitory activity of synthesized compounds was assessed using a human placental microsomal preparation as the enzyme source and [1β-^{3}H]4-androstene-3,17-dione as substrate. Synthesized compounds exhibiting high inhibitory activity were further evaluated under initial velocity conditions to determine apparent K_i values. Several compounds were effective competitive inhibitors, and have apparent K_i values ranging from 34 to 52 nM, with the apparent K_m for androstenedione being 54 nM. The results of these studies demonstrate a tightly fitted enzyme pocket that can accommodate bulky substituents at the C-4 position of androstenedione not to exceed 4.3 Å in width and 5.5 Å in length.

INTRODUCTION

Peripheral conversion of androgens to estrogens by aromatase accounts for nearly all of the estrone and estradiol produced by post menopausal women [1, 2]. Although much of this conversion occurs in fat and muscle cells [3], certain human breast cancers have the capacity to convert androgens to estrogens locally [4–7]. In view of the fact that estrogens play a significant role in the growth and maintenance of breast cancer, inhibition of estrogen synthesis by aromatase inhibitors may be one significant approach in the treatment of breast cancer.

Numerous aromatase inhibitors have been synthesized and studied *in vitro* using human placental tissue. These include both competitive [8–10] as well as enzyme-activated irreversible inhibitors [11–14]. In a recent study [8] we synthesized a series of 4-thio-substituted *n*-alkyl and *p*-4-thiol phenyl 4-androstenedione analogs in which the optimum steric conditions for substitution at C-4 were determined. To further expand on these results a series of ortho- and meta-4-thiophenyl and 4-thiosubstituted branched chain alkyl analogs were synthesized and their aromatase inhibitory activity determined. The results obtained from these studies are the subject of this paper.

*Abbreviations: EDTA, ethylenediaminetetraacetic acid; IR, infrared spectroscopy; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, tetramethylsilane.

EXPERIMENTAL

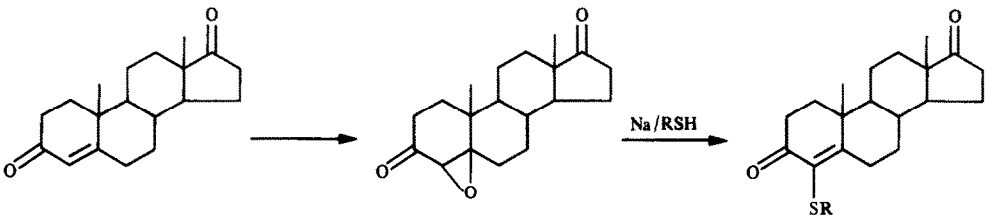
General procedure

Melting points were determined with a Fischer-Jones 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 JEOL FX 90Q spectrometer using TMS* as internal standard. TLC was performed on a precoated silica gel plate (Polygram Sil G/UV 254, Macherey Nagel, Germany). Silica gel (230–400 mesh, E. Merck, Germany) was used for all column chromatography. All chemicals were reagent grade and were purchased from Aldrich Chemical Company (Milwaukee, Wis.) or from Sigma Chemical Company (St. Louis, Mo.). The solvents were distilled according to literature procedure when necessary. Ultracentrifugation was performed on a Beckman L2-65B ultracentrifuge. Radioactivity was determined on a Beckman LS-100 liquid scintillation counter.

Synthesis

The synthesis of the 4-thiosubstituted-4-androstenedione analogs was carried out as shown in Scheme 1. The 4β,5β-epoxide **2** was prepared by the method of Mann and Pietrzak [18]. All compounds were synthesized by the method described previously [8] and are exemplified by the following procedure.

Synthesis of 4-[(2′-Nitrophenyl)thio]-4-androstene-3,17-dione (**3**): To a solution of 4,5-epoxy androstane-3,17-dione (2, 450 mg, 1.5 mmol) and 2-nitrophenol



1 2 3–15

3, R = C_6H_4—*o*—NO_2
4, R = C_6H_4—*o*—CH_3
5, R = C_6H_4—*m*—CH_3
6, R = C_6H_4—*o*—Cl
7, R = C_6H_4—*m*—Cl
8, R = C_6H_4—*p*—Cl
9, R = C_6H_4—*o*—Br
10, R = C_6H_4—*m*—Br
11, R = C_6H_4—*o*—NH_2
12, R = C_6H_5
13, R = $HC(CH_3)_2$
14, R = $HC(CH_3)CH_2CH_3$
15, R = $C(CH_3)_3$

Scheme 1

(1 g, 6.4 mmol) in anhydrous dioxane (8 ml) was added sodium metal (25 mg, 1 mmol), and was stirred and refluxed for 6 h. The reaction mixture was then poured into water to give a solid that was filtered, dried, and purified on a silica gel column. The fractions corresponding to product were pooled and crystallized from acetone-hexane to give pure crystals of **(3)** (220 mg, 39%); m.p. 206–208°C; IR(KBr) 3480, 3050, 2940, 2870, 2700, 1745, 1680, 1580, 1560 cm^{-1}; [^{1}H]NMR ($CDCl_3$) δ 0.94 (s, 3H, C-18), 1.20 (s, 3H, C-19) 6.8–7.30 (m, 2H, 5′,6′-aromatic), 7.52 (m, 1H, 4′-aromatic), 8.11 (dd, 1H, 3′-aromatic).

4-[(2′-Methylphenyl)thio]-4-androstene-3,17-dione **(4)**: m.p. 100–101°C; [^{1}H]NMR ($CDCl_3$) δ 0.93 (s, 3H, C-18), 1.20 (s, 3H, C-19), (s, 3H, 2′-CH_3), 6.52–7.15 (m, 4H, aromatic).

4-[(3′-Methylphenyl)thio]-4-androstene-3,17-dione **(5)**: m.p. 164–165°C; [^{1}H]NMR ($CDCl_3$) δ 0.94 (s, 3H, C-18), 1.22 (s, 3H, C-19), 2.32 (s, 3H, 3′-CH_3), 6.7 (s, 1H, 2′-aromatic), 6.5–7.3 (m, 3H, aromatic).

4-[(2′-Chlorophenyl)thio]-4-androstene-3,17-dione **(6)**: m.p. 179–180°C; [^{1}H]NMR ($CDCl_3$) δ 0.94 (s, 3H, C-18), 1.20 (s, 3H, C-19), 6.6–7.4 (m, 4H, aromatic).

4-[(3′-Chlorophenyl)thio]-4-androstene-3,17-dione **(7)**: m.p. 154–155°C; [^{1}H]NMR ($CDCl_3$) δ 0.94 (s, 3H, C-18), 1.20 (s, 3H, C-19), 6.6–7.3 (m, 4H, aromatic).

4-[(4′-Chlorophenyl)thio]-4-androstene-3,17-dione **(8)**: m.p. 141–142°C; [^{1}H]NMR ($CDCl_3$) δ 0.94 (s, 3H, C-18), 1.20 (s, 3H, C-19), 7.16 (m, 2H, 2′,6′-aromatic), 8.11 (m, 2H, 3′,5′-aromatic).

4-[(2′-Bromophenyl)thio]-4-androstene-3,17-dione **(9)**: m.p. 182–183°C; [^{1}H]NMR ($CDCl_3$) δ 0.94 (s, 3H, C-18), 1.20 (s, 3H, C-19), 6.4–7.6 (m, 4H, aromatic).

4-[(3′-Bromophenyl)thio]-4-androstene-3,17-dione **(10)**: m.p. 168–169°C; [^{1}H]NMR ($CDCl_3$) δ 0.94 (s, 3H, C-18), 1.22 (s, 3H, C-19), 7.05 (s, 1H, 2′-aromatic), 6.6–7.2 (m, 3H, aromatic).

4-[(2′-Aminophenyl)thio]-4-androstene-3,17-dione **(11)**: m.p. 192–193°C; [^{1}H]NMR δ 0.94 (s, 3H, C-18), 1.20 (s, 3H, C-19), 6.5–6.9 (m, 2H, aromatic), 7.50 (m, 1H, 4′-aromatic), 8.11 (dd, 1H, 3′-aromatic).

4-(Phenylthio)-4-androstene-3,17-dione **(12)**: m.p. 171–172°C; [^{1}H]NMR ($CDCl_3$) δ 0.94 (s, 3H, C-18), 1.22 (s, 3H, C-19), 7.07–7.22 (m, 4H, aromatic).

4-(2-Propanethio)-4-androstene-3,17-dione **(13)**: m.p. 149–150°C; [^{1}H]NMR ($CDCl_3$) δ 0.94 (s, 3H, C-18), 1.10–1.23 (1s, 2d, 9H, C-19 & 1′,3′-C-propyl).

4-(1-Methyl-propanethio)-4-androstene-3,17-dione **(14)**: m.p. 129–130°C; [^{1}H]NMR ($CDCl_3$) δ 0.94 (s, 3H, C-18), 0.98–1.22 (m, 8H, isobutyl) 3.7 (m, 1H).

4-(2-Methyl-2-propanethio)-4-androstene-3,17-dione **(15)**: m.p. 172–173°C; [^{1}H]NMR ($CDCl_3$) δ 0.94 (s, 3H, C-18), 1.20 (s, 3H, C-19), 1.3 (s, 9H, t-butyl).

Enzyme preparation. Microsomes were obtained from human placentas after normal deliveries and prepared as described previously [15]. Following isolation of the microsomal pellet (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 [16] as modified by Reed and Ohno [17] was used in our studies. This assay quantitates the production of [^{3}H]H_2O released from [1β-^{3}H]4-androstenedione after 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 2.5 mM glucose-6-phosphate; 0.5 mM NADP; 1 unit of glucose-6-phosphate dehydrogenase; 0.25, 0.75 or 1.5 μM inhibitor; and 0.25 μM (0.25 μCi) [1β-^{3}H]androstenedione; 10 mM EDTA; 10 mM phosphate buffer; and 0.15 mg of protein of lyophilized human placental microsomes. Incubations were carried out for 15 min at 37°C in the air and were terminated by addition of 5 ml of chloroform, followed by vortexing for 40 s. Control samples with no inhibitor present and blank samples containing no microsomes were run simultaneously. After centrifugation at 1500*g* for 5 min, the aqueous layer was treated with acid-washed charcoal and centrifuged again, and aliquots (0.2 ml) were removed and added to scintillation mixture for determination of ^3H_2O production.

K_i assay procedure. This procedure is essentially similar to that employed in the screening assay, except that the substrate concentration was varied at 0.05–0.25 μM and using only 0.025–0.03 mg of microsomal protein that results in a constant initial velocity, even at the lowest substrate concentration. Control samples with no inhibitor and blank samples with no microsomes were incubated simultaneously for 15 min. Each inhibitor was examined at two concentrations (0.1 and 0.3 μM).

RESULTS AND DISCUSSION

Table 1 summarizes the results of the initial screening assays for the 4-thiosubstituted-4-androstenedione derivatives synthesized in this study. For comparative purposes, we have included several compounds previously reported as inhibitors of aromatase: 4-OHA [19], 7-APTA [9], AG [16]. Analysis of the data in Table 1 shows inhibitory activity ranging from 8 to 85% (at 1.5 μM inhibitor concentration).

Previous studies in our laboratory [8] showed that short-chain alkyl substitution (methyl, ethyl, *n*-propyl) gave compounds with good aromatase inhibitory activity, while substitution with longer straight-chain alkyl groups gave compounds with virtually no activity. As shown in Table 1, compounds with branched chain alkyl substituents **13** and **15** showed reasonable inhibitory activity while compound **14** which has a 3-carbon chain and branching showed no inhibitory activity.

On the basis of these results, it does seem that increasing the branching in the alkyl side chain results in a considerable decrease in inhibitory activity as shown for compound **14**, indicating that bulky groups may not be tolerated at the enzyme active site, suggesting an optimal steric environment for the binding of the alkyl series of compounds to the active site.

All phenyl substituted analogs analyzed in this investigation showed reasonable binding affinity. Indeed the 4-thiophenyl substituted analog **12** was found to be more potent than several previously reported aromatase inhibitors, such as 4-OHA and AG. It is interesting to note that the inhibitory activity for the ortho- and meta-substituted analogs were nearly similar and were not affected by the electron-donating or electron-withdrawing character of the substituent indicating that steric factors are probably more important than the electronic characteristics of the substituents. These studies are particularly interesting in light of the recent studies by Darby *et al.*[20] in which no apparent correlation between para-substituent electronic effects and inhibitory activity in a series of 7α-(phenylthio)-androstene-

Table 1. Results of screening of inhibition of aromatase by 4-thiosubstitutede-4-androstene-diones

Compound	R	% Inhibition of aromatase[a] 0.25 μM	0.75 μM	1.5 μM
3	C_6H_4-*o*-NO_2	20	31	49
4	C_6H_4-*o*-CH_3	35	56	70
5	C_6H_4-*m*-CH_3	28	45	63
6	C_6H_4-*o*-Cl	27	38	67
7	C_6H_4-*m*-Cl	25	33	65
8	C_6H_4-*p*-Cl	12	32	41
9	C_6H_4-*o*-Br	29	41	59
10	C_6H_4-*m*-Br	23	46	64
11	C_6H_4-*o*-NH_2	26	41	69
12	C_6H_5	49	72	85
13	$HC(CH_3)_2$	33	55	70
14	$HC(CH_3)CH_2CH_3$	22	45	58
15	$C(CH_3)_3$	17	33	41
16	CH_3[b]	34	58	71
17	$(CH_2)_2CH_3$[b]	25	42	49
18	C_6H_4-*p*-CH_3[b]	14	27	31
19	C_6H_4-*p*-NO_2[b]	0	0	0
4-OHA[c]		46	70	81
7-APTA[c]		46	70	82
AG[c]		39	61	78

[a]Values are reported for average of 5 experiments. [b]Included for comparison. Results obtained from Ref. [8]. [c]Included for comparison. 4-OHA = 4-hydroxyandrostenedione, 7-APTA = 7α[(4'-aminophenyl)thio]-androstenedione, AG = (aminoglutethimide) = 3-(*p*-aminophenyl)-3-ethyl-2,6-piperidinedione.

Table 2. K_i values of selected inhibitors[a]

Compound	R	K_i(app) μM	Inhibition
4	C_6H_4-*o*-CH_3	0.049	Competitive
12	C_6H_5	0.034	Competitive
13	$HC(CH_3)_2$	0.052	Competitive
7-APTA		0.021	Competitive
4-OHA		0.054	Competitive

[a]Apparent K_m for androstenedione, 0.054 μM.

dione was observed. It is interesting to note that 4-(para-substituted phenylthio)-androstenediones were found to have very low or no aromatase inhibitory activity (**18** and **19**, Table 1).

Compounds exhibiting effective inhibition in the initial screening assay were evaluated further to determine the apparent K_i values that were obtained from Lineweaver–Burk plots. The apparent K_i values and the type of inhibition are shown in Table 2. All compounds demonstrated competitive inhibition and showed K_i values similar to the K_m for androstenedione (0.054 μM). It is interesting to note that while 4-OHA, is a competitive irreversible inhibitor [21, 22], preliminary studies on 4-thiosubstituted-androstenediones showed that these compounds act as competitive reversible inhibitors (data not presented).

The results obtained from this study and a previous investigation [8] clearly show that the dominant factor involved in imparting aromatase inhibitory activity to 4-thiosubstituted alkyl and phenyl derivatives of 4-androstenedione is steric factors and not electronic. Examination of Dreiding models showed a distance of 4.9–5.0 Å between the S atom and the C-3′ proton in the zig-zag conformation of 4-(*n*-propylthio)-4-androstenedione (**17**). Also, the distance between the S atom and the C-4′ proton in (phenylthio)-androstenedione (**12**) gave values of 5.2–5.3 Å. Extension of the chain from the *n*-propyl to the *n*-butyl increases the distance from the S to the C-4′ proton to values of about 6.0–6.1 Å. Similarly, para-substitutions with groups such as methyl (**18**) or nitro (**19**) increases the S to the terminal atom distances to more than 6 Å. Although the branched chain alkyl and ortho- and meta-phenyl substituted analogs showed decreased inhibitory activity, the results obtained from this study suggest that bulky substituents at C-4 can be accommodated up to about 4.3 Å in width.

Based on the above studies, the results suggest a tightly fitted enzyme pocket that is capable of accommodating bulky substituents at the C-4 position of androstenedione not to exceed 4.3 Å in width and 5.5 Å in length. A schematic diagram for the interaction of aromatase with the 4-thiosubstituted-4-androstenediones is proposed (Fig. 1). Because the C4-S bond can rotate freely along this axis and since the bond angle for a disubstituted sulfur atom is 100°, the enzyme cavity is significantly larger than one would calculate based on measurements of bond lengths and angles. Indeed, the above assumptions may not be what is actually observed since the enzyme may restrict free rotation around the C4-S bond and limit the size of the enzyme pocket. An additional enzyme pocket that can accommodate

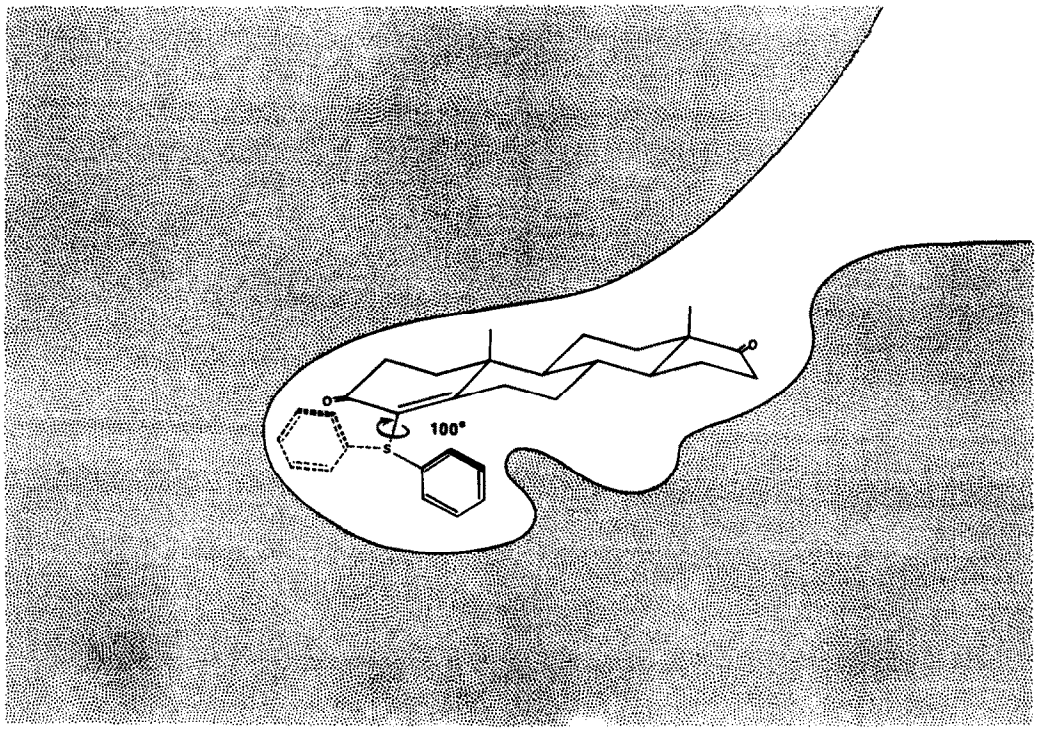


Fig. 1. Proposed model for the interaction of 4-(phenylthio)-4-androstenedione (**12**) with aromatase.

bulky substituents around C-7 is also shown in the diagram based on the results obtained by Brueggemeier's investigations [9, 20]. However, the parameters for this enzyme pocket are not yet defined.

In conclusion, our studies have delineated the optimum steric conditions for substitution at C-4 of androstenedione and provided significant information on the geometry of the enzyme active site cavity. Furthermore, compound **12** was found to be a very potent inhibitor of aromatase *in vitro* as well as capable of inhibiting the growth of hormone dependent rat mammary tumors *in vivo* [23].

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