Synthesis and Evaluation of 4-(Substituted thio)-4-androstene-3,17-dione Derivatives as Potential Aromatase Inhibitors

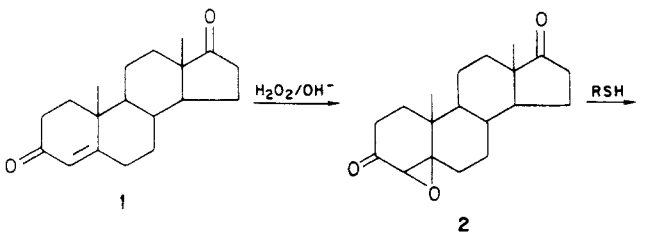
Yusuf J. Abul-Hajj

Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Minnesota, Minneapolis, Minnesota 55455. *Received July 22, 1985*

The synthesis and evaluation of 4-(substituted thio)-4-androstene-3,17-dione derivatives as inhibitors of estrogen synthetase (aromatase) are described. All compounds were prepared by the addition of various thiol reagents to 4β,5β-epoxyandrostanedione. Inhibitory activity of synthesized compounds was determined with use of 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 36 to 73 nM, with the apparent K_m for androstenedione being 53 nM. The results of these studies demonstrate a tightly fitted enzyme pocket that can accommodate bulk up to about 5.5 Å.

Current therapy of breast cancer is by treatment with (a) hormone additive therapy,[1] (b) antiestrogens that block the uptake of estradiol binding by estrogen receptors in tumor cells,[2] and (c) endocrine ablative therapy that results in removal of circulating estrogens from the system.[3] Although the levels of circulating estrogens is greatly reduced following endocrine ablation,[3] recent studies have shown that extraglandular estrogens account for nearly all estrogen produced by postmenopausal women.[4-6] Furthermore, estrogen synthesis has been shown to occur in some breast tumors.[7-10] Thus, peripheral estrogen formation could play a significant role, particularly in women with hormonally dependent metastatic breast cancer. In such patients, effective aromatase inhibitors could be of potential clinical use. Several in vitro studies using aminoglutethimide,[11,13] 4-hydroxy-4-androstenedione (4-OHA),[12,13] 7α-[(4′-aminophenyl)thio]androstenedione (7-APTA),[13,14] and 6α- and 6β-bromoandrostenedione[15,16] were shown to be effective competitive inhibitors of human placental and mammary tumor aromatase. Furthermore, 4-OHA was shown to inhibit ovarian aromatase estrogen production[17] and peripheral aromatization[18] in laboratory animals and causes regression of hormone-dependent carcinogen-induced tumors in rats.[12,18] AG is currently used for treatment of breast cancer,[19] and 4-OHA is undergoing phase II clinical trials. The recent studies by Coomb et al.[20] indicate that 4-OHA shows promise for treatment of metastatic breast cancer.

Scheme I



3, R = $COCH_3$
4, R = H
5, R = CH_3
6, R = CH_2CH_3
7, R = $(CH_2)_2CH_3$
8, R = $(CH_2)_3CH_3$
9, R = $(CH_2)_4CH_3$
10, R = $CH_2C_6H_5$
11, R = C_6H_5
12, R = C_6H_4-p-CH_3
13, R = C_6H_4-p-OCH_3
14, R = C_6H_4-p-NH_2
15, R = C_6H_4-p-Br
16, R = C_6H_4-p-NO_2

In order to gain more insight into the mechanism of aromatase inhibition, the synthesis of a series of 4-(substituted thio)-4-androstene-3,17-dione derivatives has been carried out and their resultant in vitro aromatase inhibitory activity determined.

Chemistry. The synthesis of the 4-(substituted thio)-4-androstene-3,17-dione derivatives was carried out as shown in Scheme I. The 4β,5β-epoxide **2** was prepared by the method of Mann and Pietrzak.[21] Compounds **3** and **4** were prepared according to the procedure of Marsh et al.[22] The synthesis of compounds **5–16** was carried out

(1) McGuire, W. L.; Chamness, G. C.; Costlow, M. E.; Shepherd, R. *Metab. Clin. Exp.* **1974**, *23*, 75.
(2) Sutherland, R. L.; Murphy, L. C. *Mol. Cell. Endocrinol.* **1982**, *25*, 5.
(3) Dao, T. L.; Huggins, C. *Arch. Surgery* **1955**, *71*, 645.
(4) Siiteri, P. P.; MacDonald, P. C. In "Handbook of Physiological Endocrinology"; Greep, R. O., Astwood, E., Eds.; American Physiological Society: Washington, DC, 1973, Vol. 2, Part 1, pp 615–626.
(5) Grodin, J. M.; Siiteri, P. K.; MacDonald, P. C. *J. Clin. Endocrinol. Metab.* **1973**, *36*, 207.
(6) Edman, C. D.; Aiman, E. J.; Porter, J. C.; MacDonald, P. C. *Am. J. Obstet. Gynecol.* **1978**, *130*, 439.
(7) Abul-Hajj, Y. J. *Steroids* **1975**, *26*, 488.
(8) Abul-Hajj, Y. J.; Iverson, R.; Kiang, D. T. *Steroids* **1979**, *33*, 205.
(9) Li, K.; Adams, J. B. *J. Steroid Biochem.* **1981**, *14*, 269.
(10) Miller, W. R.; Forrest, A. P. M. *Lancet* 1974, 866.
(11) Thompson, E. A., Jr.; Siiteri, P. K. *J. Biol. Chem.* **1974**, *249*, 5373.
(12) Brodie, A. M. H.; Schwarzel, W. C.; Shaikh, A. A. *Endocrinology* **1977**, *100*, 1684.
(13) Abul-Hajj, Y. J. *J. Steroid Biochem.* **1980**, *13*, 1395.
(14) Bruggemeier, R. W.; Floyd, E. E.; Counsell, R. E. *J. Med. Chem.* **1978**, *12*, 1007.
(15) Bellino, F. L.; Gilani, S. S. H.; Eng, S. S.; Osawa, Y.; Duaz, W. L. *Biochemistry* **1976**, *15*, 4730.
(16) Dao, T. L. *Cancer Res.* **1982**, *42*, 3338s.
(17) Brodie, A. M. H.; Garrett, W. M.; Hendrickson, J. R.; Tsain Morris, C. H. *Cancer Res.* **1982**, *42*, 3360s.
(18) Brodie, A. M. H.; Longcope, C. *Endocrinology* **1980**, *106*, 19.
(19) Salhanick, H. A. *Cancer Res.* **1982**, *42*, 3315s.
(20) Coombs, R. C.; Goss, P.; Dowse, H. M.; Gazet, J-C.; Brodie, A. M. H. *Lancet* **1984**, 1237.
(21) Mann, J.; Pietrzak, B. *J. Chem. Soc., Perkin Trans.* **1983**, 2681.
(22) Marsh, D. A.; Brodie, H. J.; Garrett, W.; Tsai-Morris, C.; Brodie, A. M. H. *J. Med. Chem.* **1985**, *28*, 788.

0022-2623/86/1829-0582$01.50/0

Table I. Results of Screening for Inhibition of Aromatase by 4-(Substituted thio)-4-androstenediones

no.	R	mp, °C	yield, %	% inhibition of aromatase[a] 0.25 μM	0.75 μM	1.5 μM
3	$COCH_3$	198–200	17	19	42	58
4	H	253–255	24	31	52	63
5	CH_3	116–117	74	39	61	74
6	CH_2CH_3	134–135	85	26	45	52
7	$(CH_2)_2CH_3$	113–114	62	25	42	49
8	$(CH_2)_3CH_3$	120–122	71	8	12	18
9	$(CH_2)_4CH_3$	130–132	48	2	4	8
10	$CH_2C_6H_5$	137–138	56	2	5	8
11	C_6H_5	171–172	83	36	55	68
12	C_6H_4-*p*-CH_3	145–146	61	14	27	31
13	C_6H_4-*p*-OCH_3	173–174	32	0	0	2
14	C_6H_4-*p*-NH_2	186–188	48	0	0	0
15	C_6H_4-*p*-Br	140–142	56	0	4	6
16	C_6H_4-*p*-NO_2	214–216	49	0	0	0
4-OHA[b]				49	74	84
4-OHAc[b]				38	70	78
7-APTA[b]				46	70	82
AG[b]				39	61	79

[a] Values are reported for average of five experiments. [b] Included for comparison. 4-OHA = 4-hydroxyandrostenedione, 4-OHAc = 4-acetoxyandrostenedione, 7-APTA = 7α-[(4′-aminophenyl)thio]androstenedione, and AG = aminoglutethimide = 3-(*p*-aminophenyl)-3-ethyl-2,6-piperidinedione.

by reaction of the sodium salt of thiol with **2**.

Biochemical Results

The enzyme used in this study was obtained from twice-washed human placental microsomes, as described previously by Ryan,[23] and lyophilized to minimize loss of enzyme activity.[24] The method of Thompson and Siiteri,[11] as modified by Reed and Ohno,[25] was used for evaluating the newly synthesized compounds as aromatase inhibitors. The substrate concentration was 0.25 μM, which is approximately 5 times the K_m value for the enzyme preparation. Inhibitors were assayed at concentrations of 0.25, 0.75, and 1.5 μM.

Table I summarizes the results of the initial screening assays for the 4-(substituted thio)-4-androstene-3,17-dione derivatives synthesized in this study. For comparative purposes, we have included four compounds previously reported as inhibitors of aromatase: 4-OHA,[18] 4-acetoxy-4-androstenedione (4-OHAc),[18] 7-APTA,[14] and aminoglutethimide (AG).[11] Analysis of the data in Table I shows inhibitory activity ranging from 0% to 74% (at 1.5 μM inhibitor concentration) for compounds **3–16**. As reported by Marsh et al.[22] we found that the inhibitory activity of compounds **3** and **4** is somewhat lower than the corresponding O isosteres (4-OHA and 4-OHAc). Substitution with short-chain alkyl groups gave compounds **5–7** that were quite active inhibitors of aromatase, while substitution with longer straight-chain alkyl groups gave inactive compounds **8** and **9**. On the basis of these results, it does seem that increasing the alkyl side chain results in a considerable decrease in inhibitory activity as shown for compounds **8** and **9**, indicating that bulky groups may not be tolerated at the enzyme active site, suggesting an optimum steric condition for the binding of the alkyl series of compounds to the active site.

The benzyl derivative **10** was inactive, and this is consistent with our observation that steric bulk may be important in limiting binding to enzyme. However, the thiophenyl derivative **11** exhibited a surprisingly high inhibitory activity, suggesting that the hydrophobic character of the molecule may play an important role in its increased binding to the active site. It is interesting to note that substances with either electron-donating or electron-withdrawing substituents at the para position of the phenyl ring were either weakly active or inactive. This result appears to suggest that electronic factors are not of importance in enhancing the interaction of these analogues with the enzyme active site. These results are of particular interest in light of the recent studies by Darby et al.[26] in which they showed no apparent correlation between para-substituent electronic effects and inhibitory activity in a series of 7α-(phenylthio)androstanedione.

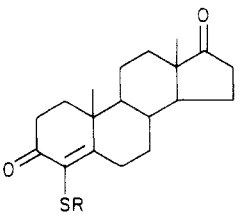
Examination of the results obtained from this study seem to indicate that 4-thio-substituted alkyl analogues of 4-androstenedione require no more than three-atom chain length to exert inhibitory activity. The question as to why the phenylthio analogue (**11**) showed appreciable inhibitory activity may also be explained on the basis of steric factors. Examination of Dreiding models showed values of 4.9–5.0 Å between the S atom and the C-3′ proton in the zig-zag conformation in compound **7**. The distance between the S atom and the C-4′ proton in (phenylthio)-androstenedione (**11**) gave values of 5.2–5.3 Å. The addition of a fourth carbon atom to the propyl side chain increased the S to C-4′ proton in **8** to values of about 6.0–6.1 Å. Similarly, the para substitutions made in compounds **12–16** increases the S to terminal atom distances to more than 6 Å. Thus, the results obtained from this study suggest a tightly fitted enzyme pocket that is capable of accommodating bulk up to about 5.5 Å.

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 II. All compounds demonstrated competitive inhibition and showed K_i values similar to the K_m for androstenedione (0.053 μM).

In conclusion, our studies have shown that several 4-(substituted thio)-4-androstene-3,17-dione derivatives possess considerable inhibitory activity. Even though none of the compounds synthesized are more potent than 4-OHA, 7-APTA, or AG, our studies have delineated the optimum steric conditions for substitution at C-4 of androstenedione and provided information on the geometry

(23) Ryan, K. J. *J. Biol. Chem.* **1959**, *234*, 268.
(24) Kelly, W. G.; Judd, D.; Stolee, A. *Biochemistry* **1977**, *16*, 140.
(25) Reed, K. C.; Ohno, S. *J. Biol. Chem.* **1976**, *251*, 1625.
(26) Darby, M. V.; Lovett, J. A.; Brueggemeier, R. W.; Groziak, M. P.; Counsell, R. E. *J. Med. Chem.* **1985**, *28*, 803.

Table II. K_i Values of Selected Inhibitors[a]



compd	R	app K_i, μM	inhibition
3	$COCH_3$	0.073	competitive
5	CH_3	0.059	competitive
6	CH_2CH_3	0.041	competitive
7	$(CH_2)_2CH_3$	0.065	competitive
11	C_6H_5	0.036	competitive
4-OHA[b]		0.054	competitive
7-APTA[b]		0.018	competitive

[a] Apparent K_m for androstenedione, 0.053 μM. [b] 4-OHA = 4-hydroxyandrostenedione, 7-APTA = 7α-[(4′-aminophenyl)thio]-androstenedione.

of the enzyme active site cavity. The synthesis and evaluation of branched chain alkyl, as well as (*o*- and *m*-substituted phenyl)thio analogues, is being studied, since it may help to further define the structural requirements that have to be controlled in the design of new inhibitors of aromatase.

Experimental Section

A. Synthetic Methods. Melting points (uncorrected) were obtained on a Fisher-Johns apparatus. Infrared spectra were recorded with a Perkin-Elmer 281 spectrophotometer. NMR spectra were obtained with a JEOL-90Q spectrometer. Elemental analyses were performed by Microanalytical Laboratory, Ann Arbor, MI. Dioxane was dried and distilled from CaH_2. 4-Androstenedione was purchased from Steraloids, Wilton, NH. All other thio reagents were obtained from Aldrich Chemical Co., Milwaukee, WI. All compounds were synthesized by one of two methods, depending upon whether the thiol reagent was a solid or a liquid.

4-(Phenylthio)-4-androstene-3,17-dione (11). Sodium metal (40 mg, 1.8 mmol) was added to a solution of **2** (240 mg, 0.8 mmol) in thiophenol (8 mL) with stirring under nitrogen. The reaction was maintained at 60 °C for 8 h, poured over cold water, and extracted with ethyl ether. The ether extract was dried with use of anhydrous Na_2SO_4 and evaporated to dryness under vacuum to yield a semisolid that was crystallized from acetone–hexane: 265 mg, 83%; mp 171–172 °C; IR (KBr) 3060, 2970, 2940, 2920, 2850, 1735, 1685, 1670, 1585, 1560 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.94 (s, 3 H, C-18), 1.22 (s, 3 H, C-19), 7.07–7.22 (m, 4 H, aromatic). Anal. ($C_{25}H_{30}O_2S$) C, H.

4-[(4′-Nitrophenyl)thio]-4-androstene-3,17-dione (16). To a solution of **2** (450 mg, 1.5 mmol) and 4-nitrothiophenol (1 g, 6.4 mmol) in anhydrous dioxane (8 mL) was added sodium metal (25 mg, 1 mmol), and the reaction mixture 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 **16** (190 mg, 29%): mp 214–216 °C; IR (KBr) 3490, 3100, 2940, 2870, 2700, 1745, 1680, 1580, 1560, cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.94 (s, 3 H, C-18), 1.20 (s, 3 H, C-19), 7.11 (m, 2 H, 2′,6′ aromatic), 8.06 (m, 2 H, 3′,5′ aromatic). Anal. ($C_{25}H_{29}NO_2S$) C, H.

B. Biochemical Methods. Enzyme Preparation. Microsomes were obtained from human placentas after normal deliveries and prepared as described previously.[23] 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,[11] as modified by Reed and Ohno,[25] was used in our studies. This assay quantitates the production of $[^3H]H_2O$ released from [1β-^{3}H]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, 0.25, 0.75, or 1.5 μM inhibitor; and 0.25 μM (0.25 μCi) [1β-^{3}H]androstenedione; 1.0 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. 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 0.005–0.25 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 were incubated simultaneously, and blank samples were incubated at 0 min. Each inhibitor was examined at two concentrations (0.1 and 0.3 μM).

Acknowledgment. This investigation was supported by grants from the Graduate School, University of Minnesota, and the Biomedical Research Support Grant. The technical assistance of Mary Harkins and Lucia Liens is greatly appreciated.