Aromatase Inhibitors. Synthesis and Biological Activity of Androstenedione Derivatives†

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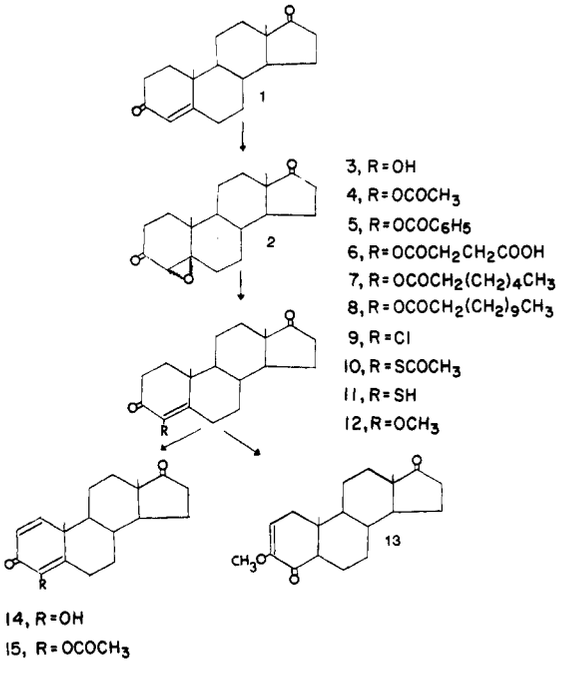
The synthesis and biological evaluation of androstenedione derivatives as inhibitors of estrogen biosynthesis are described. The results show that 4-hydroxy analogues are among the most potent in vitro inhibitors of the series. Esterification of the 4-hydroxy steroids generally reduced activity. Further conjugation of the 3-keto 4-ene system to give 4-hydroxy-4,6-androstadiene-3,17-dione caused more rapid inactivation of aromatase in rat ovarian microsomes than 4-hydroxyandrostenedione. Some compounds exhibited differences in activity when tested for inhibition of human placental microsomes vs. rat ovarian microsomes. The 4-hydroxyandrostenedione derivatives and their nonbulky esters were generally more potent in vitro and in vivo inhibitors than other substituted steroids in the series. Several of the synthesized compounds markedly reduce (50–81%) estrogen levels in rats on proestrus and/or had antifertility action. To date, none of the compounds surpassed the in vivo inhibitory action of 4-hydroxy-4-androstene-3,17-dione or its 4-acetate derivative.

Inhibition of estrogen biosynthesis could be a useful means by which the production of estrogens and their effects might be controlled. Compounds affecting aromatase (estrogen synthetase) inhibition have application as research tools for investigating estrogen-mediated processes[1,2] and are of potential clinical value for controlling estrogen-mediated events, such as ovulation and the growth of estrogen-dependent tumors. In the latter regard, aromatase inhibitors could be more effective than surgical means for reducing production of estrogen as its biosynthesis occurs not only in the ovary but at extragonadal sites including some breast tumors.[3-5]

We sought to evaluate this hypothesis by designing inhibitors of aromatase. The aromatization reaction is specific to estrogen formation. Thus, inhibition of the enzyme would selectively reduce production of estrogens and estrogen metabolites. Since this group of steroids is the last to be produced in the biosynthetic sequence, aromatase inhibition would not interfer with production of other steroids. Aminoglutethimide is an inhibitor of cytochrome P_{450} and therefore of aromatase. Although not specific and requiring coadministration of hydrocortisone, this compound has been shown to lower estrogen levels and be of significant value for treating postmenopausal breast cancer patients.[6-8]

We orginally evaluated over 100 steroid and some nonsteroid[9,10] compounds for in vitro aromatase inhibitory activity using human placental microsomes. The most potent of these steroids have been studied in vivo and several have shown marked inhibitory activity.[11-16] Subsequently, other investigators[17-24] evaluated various steroids in vitro; some of these compounds are also potent inhibitors of aromatase. All of these studies indicate that C_{19} steroids, closely resembling the natural substrates androstenedione and testosterone, are the most effective inhibitors. C_{19} 4-androsten-3-one compounds are usually better inhibitors than the corresponding ring A reduced (5α or 5β) steroids or 4-estren-3-one compounds. We observed no competitive inhibition[9] or aromatase inactivation (unpublished observations) occurring with 19-norethisterone, although this compound was recently reported to cause enzyme inactivation.[25] Further conjugation with

Scheme I



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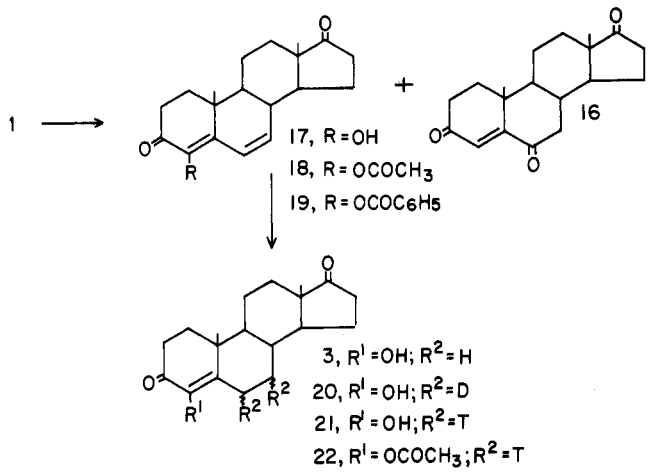
Scheme II

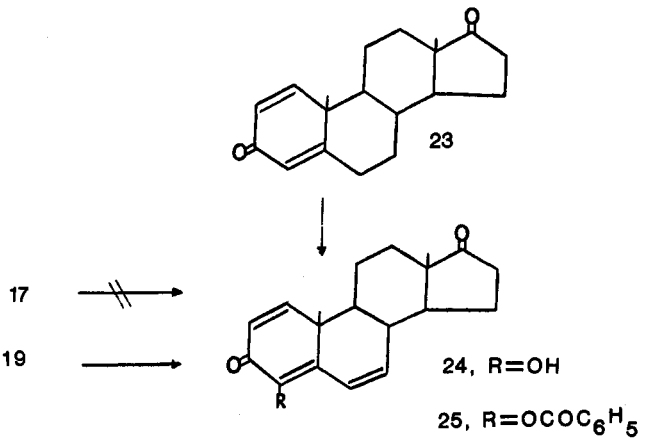


the 4-ene 3-keto system is beneficial (e.g., 1,4,6-trien-3-one, 4,6-en-3-one, and 4-ene-3,6-dione). The effectiveness of C-17 substituents appears to be 17-keto > 17β-ol > 17β-formate > 17β-acetate > 17β-propionate >> other derivatives. The introduction of various substituents on the A or B ring of androstenedione can produce potent inhibitors.

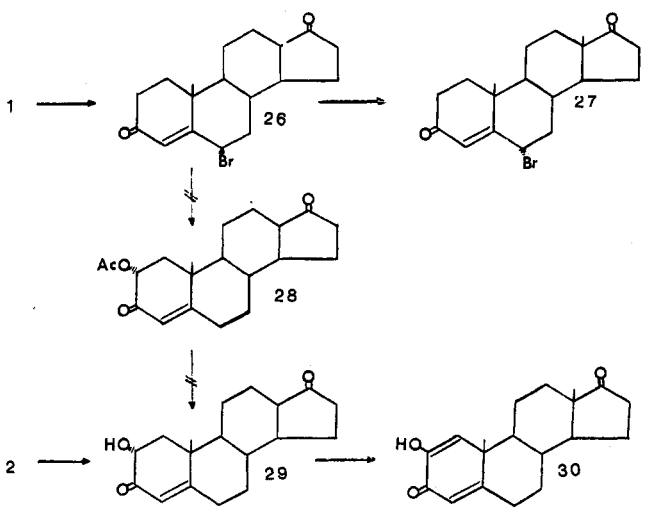
This paper describes the utilization of the steroidal structural features, discussed above, in the synthesis of a series of steroids and the resultant in vitro aromatase inhibitory activity. Also, preliminary in vivo data are presented for the inhibitors with the greatest in vitro activity.

Chemistry. Scheme I shows the synthetic approaches used to prepare a variety of 4-androstene-3,17-dione (1) derivatives. Compound 1 was treated with hydrogen peroxide and sodium hydroxide in methanol to yield a 2:1 mixture of β and α epoxides (2) by a modified literature procedure;[26] improved yields were obtained by reducing

Scheme III



Scheme IV



base strength from 20% to 2%. The two epoxides could be separated on an alumina column. However, separation was not necessary at this step. Treatment of the mixed epoxides with 2% concentrated H_2SO_4 in glacial acetic acid gave 4-hydroxy-4-androstene-3,17-dione[11] (4-OHA; 3). Treatment of 3 with the appropriate acid chloride or acid anhydride in pyridine yielded products 4–8.

Reaction of mixed epoxides 2 with hydrochloric acid in acetone gave 4-chloro-4-androstene-3,17-dione (9).[31] Reaction of epoxide 2 with thioacetic acid yielded 10. Deacetylation of compound 10 with HCl in ether gave pure 4-mercapto-4-androstene-3,17-dione (11). Refluxing steroid 3 with sodium hydroxide in methanol afforded the 4-methoxy derivative 12.[37] Alternatively, treatment of 3 with boron trifluoride etherate in methanol gave 3-methoxy-2-androstene-4,17-dione (13).

By a procedure similar to Burn et al.,[27] steroid 3 was reacted with DDQ and HCl in dioxane to give 4-

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hydroxy-1,4-androstadiene-3,17-dione (14). By an alternative route, compound 14 was prepared in low yield by the treatment of 3 with selenium dioxide in *tert*-butyl alcohol.[28] Acetylation of steroid 14 gave 15. Compound 15 also was prepared by treatment of steroid 4 with DDQ and HCl in dioxane. Hydrolysis of 15 with potassium carbonate in 70% methanol gave 14.

Treatment of steroid 1 (Scheme II) with potassium *tert*-butoxide in *tert*-butyl alcohol gave a mixture of 4-androstene-3,6,17-trione (16) and 4-hydroxy-4,6-androstadiene-3,17-dione (17);[30] the two compounds were separated by column chromatography. Acetylation of 17 gave 18. Reacting 17 with benzoyl chloride in pyridine gave 19. A number of attempts using hydrogen at STP in the presence of tris(triphenylphosphine)rhodium(I) chloride (freshly prepared), failed to reduce steroid 17 to compound 3. However, treatment of 17 in dioxane with 10% Pd/C with hydrogen, deuterium, or tritium gas at atmospheric pressure gave good yields of 3, 20, or 21, respectively, after chromatographic workup. Compound 21 after chromatographic purification cocrystallized with compound 3 without a decrease in specific activity. Acetylation of radiolabeled 21 yielded the corresponding tritiated ester 22 which was radiochemically pure as judged by crystallization data.

Attempts to prepare 4-hydroxy-1,4,6-androstatriene-3,17-dione (24) (Scheme III) from reaction of DDQ with the corresponding 4,6-androstadiene (17) failed to produce significant yields of product. However, compound 24 was prepared in low yields by treatment of 1,4-androstadiene-3,17-dione (23) with potassium *tert*-butoxide in *tert*-butyl alcohol. The triene 24 was treated with benzoyl chloride in pyridine to produce the benzoyl derivative 25. Alternatively, 25 was prepared by refluxing steroid 19 with DDQ in dioxane. Ester 25 could be hydrolyzed to steroid 24 with sodium carbonate in aqueous methanol.

Preparation of 6 β-bromo-4-androstene-3,17-dione (26, Schene IV) was from androstenedione (1) by treatment with *N*-bromosuccinimide in carbon tetrachloride by the method of Bellino et al.[21] The β-bromo was then converted to a mixture of α and β isomers by treatment with acidified carbon tetrachloride. Fractional crystallization, from that solvent, yielded the pure 6α-bromo compound 27. Attempts were made to prepare 2α-hydroxy-4-androstene-3,17-dione (29) through the preparation and subsequent hydrolysis of the 2α-acetoxy derivative 28 from the bromo steroid 26.[30] However, treatment of 26 with potassium acetate produced an inseparable mixture, which (by GC/MS analysis) was thought to be 6α-, 2α-, and 2β-acetoxy-4-androstene-3,17-dione. Alternatively, the pure 2α-hydroxy compound 29 was prepared by rearrangement of epoxide 2 with use of 25% sulfuric acid in acetone.[29] Steroid 29 was oxidized with bismuth trioxide in acetic acid to 2-hydroxy-1,4-androstadiene-3,17-dione (30).[31] The key physicochemical and analytical data for synthesized compounds are tabulated in Table I.

Biochemical Results

Table II reports competitive inhibition of human placental and/or rat ovarian aromatase by the steroids synthesized. In addition to 4-hydroxy- and 4-acetoxyandrostenedione compounds previously reported as inhibitors of aromatase, 1,4,6-androstatriene-3,17-dione,[9] testolactone, and aminoglutethimide[20] are listed for comparison. The latter two compounds have been used in the treatment of human breast cancer.[6-8,32]

The 4-hydroxy derivatives (3, 14, 17, 24) are among the most potent inhibitors of the series. Esterification of the 4-hydroxy steroids (4–8, 15, 18, 19, 25) generally reduced activity, particularly with the more bulky analogs (5, 8, 19, 25). However, the enol esters were expected to hydrolyze in vivo to the corresponding active 4-hydroxy compounds (3, 14, 17, 24); steroid 6 was designed to provide a more water-soluble dosage form of 3, while steroids 5, 7, 8, 19, and 25 were expected to be long-acting drugs by virtue of increased fat depot storage.

In accordance with our previous observations,[9] inhibitory activity is generally maintained when 4-ene 3-keto steroids are further conjugated. This can be seen when steroid 3 is compared to steroids 14, 16, 17, and 24, when compound 4 is compared to 15 and 18, when steroid 5 is compared to 19 and 25, and when the 2-hydroxy derivative 29 is compared to compound 30.

The 4-hydroxyandrostenedione derivatives and their nonbulky esters were generally more potent in vitro inhibitors than other steroidal derivatives. The 4-thioacetate derivative 10 had somewhat lower activity than its isosteric analogue, steroid 4. Removal of the acetate group to produce the 4-mercapto isostere 11 of steroid 3 resulted in a reduction, rather than the expected increase, in activity. The 4-chloro- (9) and the 4-methoxy- (12) substituted androstenedione derivatives had fair to moderate in vitro activity. The 2-hydroxy-substituted steroids 29 and 30 were poor inhibitors while the 2-ene 3-methoxy 4-keto derivative 13 was totally inactive. The 6α- and 6β-bromo steroids 26 and 27 as reported by Bellino and co-worker[21] have moderate in vitro activity in our system. Both the 4α,5α- and the 4β,5β-epoxides were fairly potent in vitro inhibitors of aromatase despite the disruption of the 3-keto 4-ene conjugated system.

It is interesting to note that there are differences between the rat ovarian and the human placental aromatase. Steroid 4 is a significantly weaker competitive inhibitor of rat ovarian aromatase than steroid 3, but the two are equipotent in the human placental aromatase system. Likewise, steroids 16 and 17 are weaker inhibitors in the ovarian than in the placental preparation. There are also significant differences in the potency of these compounds to inactivate human placental and rat ovarian aromatase. 4-Hydroxy steroids 14 and 17 (Figures 1 and 2) were compared to compound 3.[33-36] Inactivation with 4-hydroxy-4,6-androstadiene-3,17-dione was similar to 4-OHA (Figure 1A) in the placental system but occurred more rapidly ($t_{1/2}$ = 6–8 min) than with 4-OHA ($t_{1/2}$ = 20 min) in the ovarian system (Figure 2A). In the presence of 4-hydroxy-1,4-androstadiene-3,17-dione, rapid inactivation of placental aromatase occurred during the first 2.5 min, causing 50% loss of activity (Figure 1B). It appears that this compound may be metabolized by placental enzymes, since when more 4-hydroxy-1,4-androstadienedione was added every 2.5 min, further inactivation occurred (Figure 1B). However, in the ovarian system, aromatase inactivation appeared to follow psuedo-first-order kinetics (Figure 2B) and was similar to the rate of inactivation with 4-OHA. Without the 4-hydroxyl, the 4,6-diene and 1,4-diene have weak activity (Figures 1A and 1B). Other derivatives, the 4-chloro (9), the 4-methoxy (12) preincubated for up to 1 h with as much as 100 μM, and the 4,5-epoxyandrostenedione (2), did not cause significant loss of aromatase activity.

In vivo studies with several of the more potent in vitro inhibitors indicated that a number of the compounds synthesized are also effective in reducing ovarian estrogen secretion in the rat. Figure 3 shows the effect of aromatase inhibitors on endogenous estrogen levels in rat ovarian venous blood during the proestrus surge. Steroids 3, 4, 6,

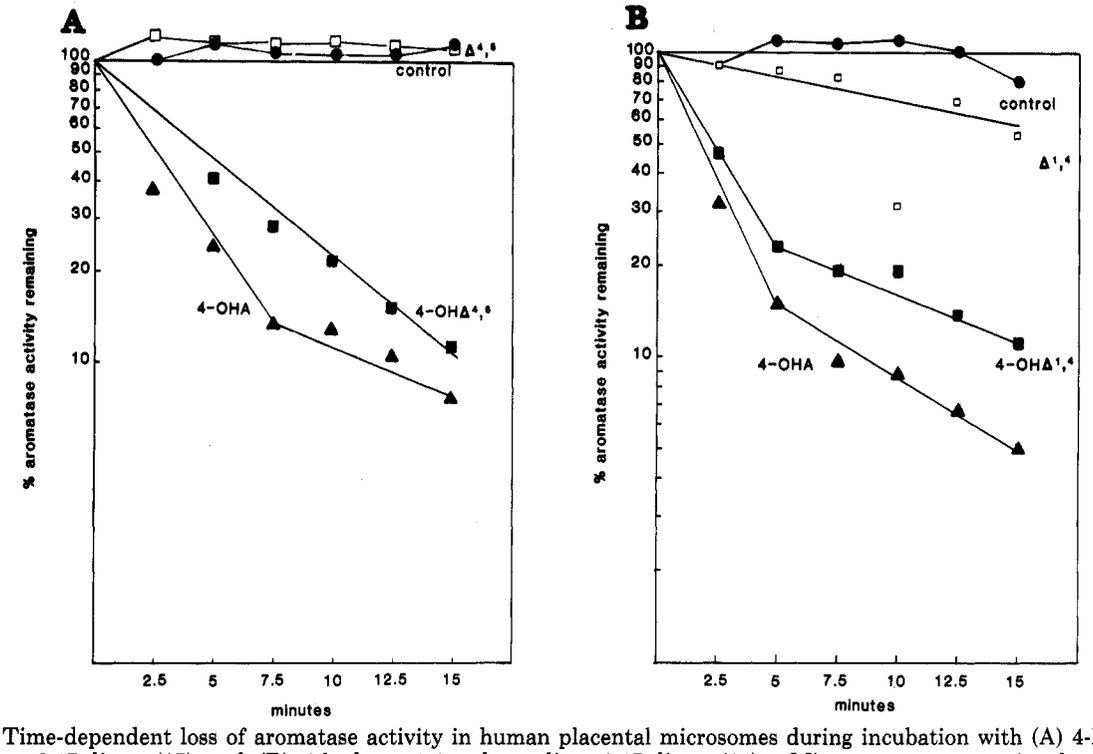

Figure 1. Time-dependent loss of aromatase activity in human placental microsomes during incubation with (A) 4-hydroxy-4,6-androstadiene-3,17-dione (**17**) and (B) 4-hydroxy-1,4-androstadiene-3,17-dione (**14**). Microsomes were preincubated with the NADPH-generating system and with 1 μM 4-OHA (▲), 4-OH $\Delta^{4,6}$ (■) and $\Delta^{4,6}$ (□) (Figure 1A), or 4-OH $\Delta^{1,4}$ (■) and $\Delta^{1,4}$ (□) (Figure 1B) or without added compound (control, ●) for 0, 2.5, 5, 7.5, 10, and 12.5 min. Following preincubation, steroids were removed with charcoal, and aromatase activity was estimated from ^3H_2O released during incubation with [1,2-$^3H_2$]androstenedione (200 000 dpm) for 30 min. The results are representative of four separate experiments.

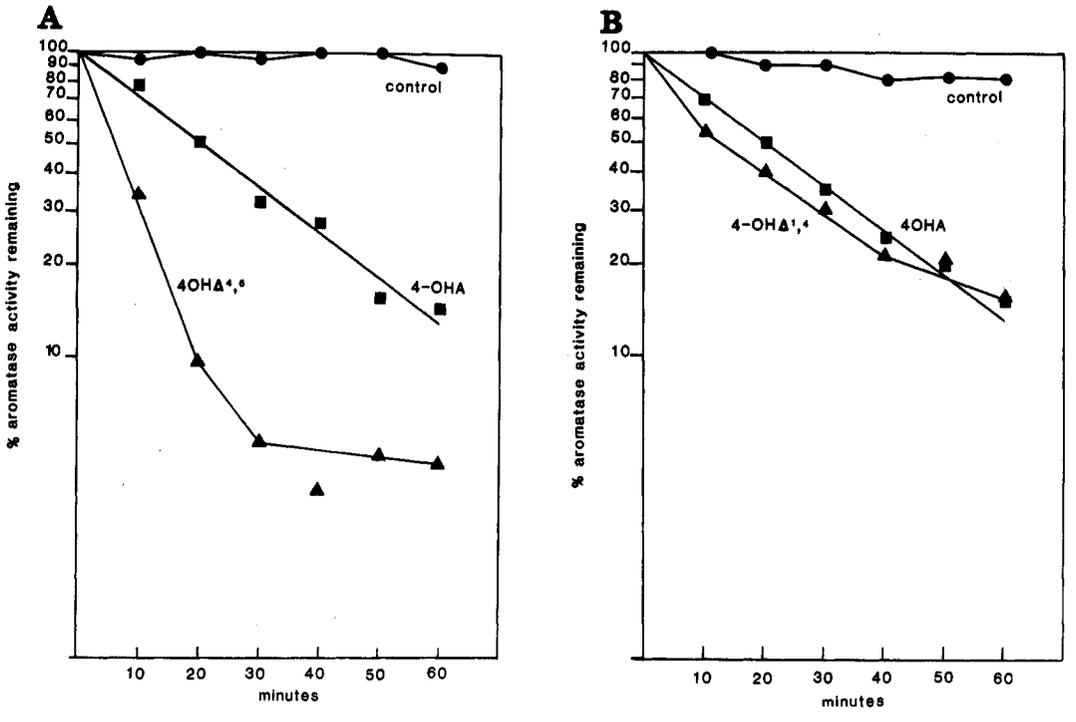

Figure 2. Time-dependent loss of aromatase activity in rat ovarian microsomes during incubation with (A) 4-hydroxy-4,6-androstadiene-3,17-dione (**17**) and (B) 4-hydroxy-1,4-androstadiene-3,17-dione (**14**). Microsomes were preincubated with the NADPH-generating system and 1 μM 4-OHA (■), 4-OH $\Delta^{4,6}$ (▲) (Figure 2A), and 4-OH $\Delta^{1,4}$ (▲) (Figure 2B) or without added compound (control, ●) for 10, 20, 30, 40, 50, and 60 min. Following preincubation, aromatase activity was estimated as described in Figure 1.

14, **16**, and **17** caused between 50% and 81% inhibition of estrogen production as compared to controls. Estrogen levels in animals treated with the 4-chloro (**9**) and 4,5-epoxy (**2**) derivatives were not reduced below control levels. An unexpected finding was increased estrogen levels with compound **9**. None of the compounds were more potent than 4-OHA (**3**) and its acetate derivative (**4**) which showed 84% and 87% reduction in estrogen levels, respectively.

Preliminary results of the effect of a number of inhibitors on fertility are shown in Table III. No adverse toxicity was observed with any of the compounds. However, due to limited amounts of the compounds available,

Table I. Key Physiochemical and Analytical Properties of Synthesized Steroids

no.	mp, °C	cryst solv	yield, %	UV, λ_{max}^{MeOH}, nm (ϵ)	IR[a] (KBr, neat), cm^{-1}	^1H NMR (100 MHz, $CDCl_3$) δ C-18	C-19	other	anal.
4	184–184.5	ethyl acetate	67	347 (14 500)	1754, 1730, 1678	0.93	1.30	2.25 [s, 3, $CH_3C(O)O$]	C, H
5	137.5–138.5	MeOH	48	242 (18 700)	1742, 1692	0.92	1.32	7.56 (m, 3, Ar), 8.16 (m, 2, Ar)	C, H
6	159–164	cyclohexane MeOH	23	245 (12 200)	1730, 1655	0.91	1.28	2.81 [t, 4, $C(O)CH_2CH_2C(O)$], 8.90 (br, 1, COOH)	C, H
7	74.5–75	MeOH	50	246 (13 500)	1752, 1742, 1689	0.89–0.90 (m, 6, 18 CH_3)	1.26 [m, 9, CH_3 and $(CH_2)_3$]		C, H
8	oil		61	247 (13 200)	1764, 1695	0.89 (m, 6, CH_3)	1.26 [m, 23, CH_3 and $(CH_2)_{10}$]		C, H
9	184–186	MeOH then ethyl acetate	24	256 (12 900)	1733, 1715	0.90	1.24	3.28 (m, 1, 6α-H)	C, H, Cl
10	197–199	MeOH	12	247 (10 500)	1818, 1770, 1745	0.92	1.31	2.36 [s, 3, $SC(O)CH_3$], 3.16 (m, 1, 6α-H)	C, H, S
11	256–258	ethyl acetate	31	261 (8277)	1742, 1700	0.92	1.21	4.77 (s, 1, SH)	C, H, S
12	ref 37	ref 37	ref 37	ref 37	ref 37	0.90	1.20	3.14 (m, 1,6α-CH), 3.61 (s, 3, OCH_3)	C, H
13	212–212.5	acetone then ethyl acetate	14	263	1739, 1689	0.85	0.89	3.61 (s, 3, OCH_3), 5.61 (dd, 1, 2-H)	C, H
14	197–199	MeOH	10	222 (8974), 246 (7168), 306 (5190)	3424, 1739, 1661	0.86	1.22	6.21 (s, 1, OH), 6.24 (d, 1, 2-H), 7.05 (d, 1, 1-H)	C, H
15	215–216.5	ethyl acetate	64	245 (13 100)		0.93	1.32	2.21 [s, 3, $CH_3C(O)O$], 6.28 (d, 1, 2-H), 7.08 (d, 1, 1-H)	C, H
17	206–207.5	MeOH	15	319 (20 000)	3400, 1730, 1642	0.97	1.13	610 (dd, 1, 6-H), 6.26 (s, 1, OH), 6.75 (dd, 1, 7-H)	C, H
18	173–175	ethyl acetate	48	290 (23 800)	1767, 1748, 1675	0.96	1.19	2.24 (s, 3, CH_3COO)	C, H
19	260–260.5	MeOH	56	233 (6200)	1760, 1700	0.97	1.26	6.21 (d, 1, 6-H), 6.51 (dd, 1, 7-H)	C, H
				239 (11 200)				7.55 (m, 3, Ar), 8.14 (m, 2, Ar)	
24	204–206.5	MeOH	6	232 (15 500), 343 (6800)	3448, 1757, 1664, 1642	1.00	1.22	5.99 (dd, 1, 6-H), 6.37 (d, 1, 1-H), 6.83 (dd, 1, 7-H), 7.17 (d, 1, 2-H)	C, H
29	ref 32	ref 32	ref 32	ref 32	3436, 1742, 1667	ref 32			C, H
30	ref 33	ref 33	ref 33	ref 33	3333, 1730, 1634	0.97	1.28	6.17 (s, 1, 1-H), 6.30 (s, 1, OH), 7.2 (s, 1, 4-H)	C, H

[a] Key peaks.

Table II. In Vitro Aromatase Inhibitory Activity

		% inhibition of estrogen formation					
		human placental[a]			rat ovarian[b]		
no.	androstenedione deriv	1:1[c]	3:1	6:1	1:1	3:1	6:1
	4-androstene-3,17-diones						
2	4,5α-epoxy					66	58
2	4,5β-epoxy					69	64
3	4-OH	82	91		71	88	
4	4-$OCOCH_3$	81	90		39	68	
5	4-$OCOC_6H_5$					29	
6	4-$OCOH_2CH_2COOH$		70		36	62	
7	4-$OCOCH_2(CH_2)_4CH_3$				44	67	
8	4-$OCOCH_2(CH_2)_9CH_3$					14	
9	4-Cl		9	43	25	49	
10	4-$SCOCH_3$					55	
11	4-SH					46	60
12	4-OCH_3		7	28	27	37	51
16	6-keto		78			33	
26	6α-Br					62	
27	6β-Br					62	74
29	2-OH					10	
	1,4-androstadiene-3,17-diones						
14	4-OH	66	84			78	89
15	4-$OCOCH_3$					62	73
30	2-OH					22	
	4,6-androstadiene-3,17-diones						
17	4-OH	8	30			68	73
18	4-$OCOCH_3$					63	
19	4-$OCOC_6H_5$					38	
	1,4,6-androstatriene-3,17-diones						
24	4-OH				51	75	
25	4-$OCOC_6H_5$						52
	2-androstene-3,17-dione						
13	3-OCH_3, 4-keto						0
	for comparison						
	1,4,6-androstatriene-3,17-dione	69	83		75	87	
	testolactone (teslac)[d]					16	22
	aminoglutethimide[d]					67	80

[a] Microsomes (0.3–0.9 mg of protein) of human placental tissue incubated with test compound, 4-[^{14}C]androstenedione, 0.7 μM androstenedione, and an NADPH-generating system consisting of NADP (5 mg) and glucose 6-phosphate (3 mg) and one unit of glucose-6-phosphate dehydrogenase[9] in 3 mL of 0.1 M phosphate buffer (pH 7.4) for 30 min at 37 °C under oxygen. [b] Microsomes (0.3 mg of protein) from PMSG-primed rat ovarian tissue were incubated with test compound, [1,2-^{3}H]androstenedione, 0.7 μM androstenedione, and an NADPH-generating system in 2.5 mL of phosphate buffer, under the same conditions given in footnote *a*. [c] 1:1 etc. ratio of inhibitor concentration to substrate (0.7 μM androstenedione). [d] Teslac, 17α-oxo-D-homo-1,4-androstadiene-3,17-dione; aminoglutethimide, 3-(4-aminophenyl)-3-ethyl-2,6-piperidinedione.

the number of animals treated was rather small. Both 4-acetoxy-1,4-adrostadiene-3,17-dione (**15**) and 6α-bromoandrostene-3,17-dione (**27**) showed delayed mating and inhibited implantation in a significant number of the rats treated.

Experimental Section

Synthetic Method. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Infrared (IR) spectra of KBr pellets or neat samples were recorded on a Perkin-Elmer 137 spectrophotometer. Ultraviolent (UV) spectra were obtained with a Perkin-Elmer spectrophotometer (Model 202). ^{1}H magnetic resonance (^{1}H NMR) spectra were determined with either a Varian HA-100 (100 MHz) or a Varian EM 360 (60 MHz) spectrometer, and chemical shifts (δ) reported relative to tetramethylsilane. Low-resolution gas chromatographic/mass spectral (GC/MS) data were obtained on a Finnigan modified 3200 mass spectrometer. High-resolution mass spectra were determined by Catherine Costello at the Massachusetts Institute of Technology, Department of Chemistry. Microchemical analyses were performed by Integral Microanalytical Laboratories, Raleigh, NC, or by Schwarzkopf Microanalytical Laboratory, Woodside, NY. 4-Androstene-3,17-dione was purchased from Searle. 4-Androstene-3,6,17-trione was purchased from Steraloids, Inc. and also was isolated as a product during the preparation of steroid **17**. Yields were not optimized.

Preparation of Enol Esters 4, 5, 6, 7, 8, 15, 18, 19, 22, and 25. After initial cooling, a mixture of an enol steroid (**3**, **14**, **17**, **21**, or **24**), an excess of the appropriate acid anhydride or acid chloride, and pyridine was reacted at room temperature and the

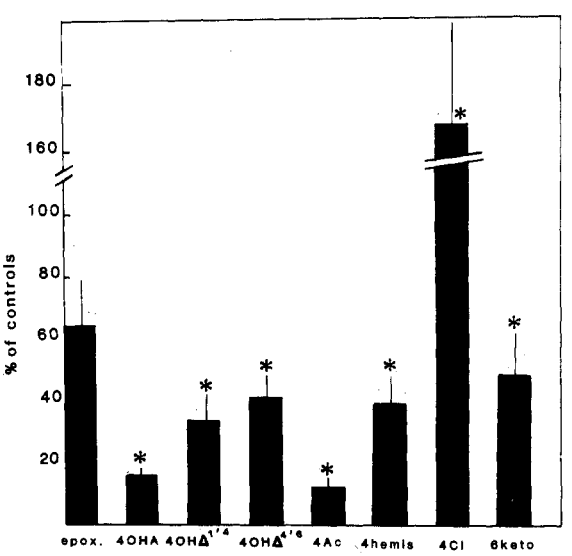

Figure 3. The effect of aromatase inhibitors on estrogen concentrations in rat ovarian venous plasma. At least five rats per group were injected sc with 50 mg/kg of inhibitor in steroid suspending vehicle at 1100 h on proestrus. Each treated group was compared with a group of control rats treated with vehicle at the same time. Ovarian vein blood was collected between 1400 and 1600 h from each animal. Values from the treated groups were compared by the Student's *t* test to their own controls; (*) values significantly different ($p < 0.05$).

Table III. Effect of Aromatase Inhibitors on Fertility in the Rat[a]

no.	androstene-dione deriv	mating day	no. of ova	treatment
	control	1	13, 18, 15, 15	
11	4-mercapto Δ^4	1	14, 14, 15, 12	throughout
	control	1	13, 10, 12, 13	
12	4-methoxy Δ^4	1–2	11, 14, 10, 12, 14, 13	throughout
7 (a)	4-OH Δ^4 hepta-noate	2	10, 10	throughout

no.	androstene-dione deriv	mating day	no. of implants	treatment
7 (b)	4-OH Δ^4 heptanoate	3–10	14, 15, 12, 10	throughout
	control	1	0, 14, 12, 16	
15	4-acetoxy $\Delta^{1,4}$	2–3	0, 0, 0, 10	until day 4
	control	1	17, 16, 13, 7	
17	4-OH $\Delta^{4,6}$	1	11, 15, 12, 14, 15	until day 1
	control	2–3	15, 15, 15, 14	
18	4-acetoxy $\Delta^{4,6}$	1–3	16, 14, 15, 14	until day 5,6
	control	1	15, 15	
16 (a)	6-keto Δ^4	1–2	7, 10, 15, 14, 13, 14	throughout
	control	1	14, 12	
16 (b)	6-keto Δ^4	1	16, 11, 16, 14, 14, 13	throughout
	control	1–2	12, 12, 14, 14, 11	
26	6α-Br Δ^4	2–3	0, 0, 0, 4, 8	until day 4

[a] Except for compound **16** (b), all treatment began on diestrus 1. On the next expected proestrus, each female was housed with two males of proven fertility and checked for evidence of mating (sperm smear or plugs). The next morning (the expected day of mating) was designated Day 1. Rats treated with compound **7** (a) (68 mg/kg per day injected sc twice daily) and compounds **11** and **12** were sacrificed on the day mating was observed and ova flushed from the oviducts. Rats treated with compound **7** (b) (injected as for **7** (a)) and compounds **15–26** were sacrificed 7 days after mating and the uterus examined for implantations. Rats treated with compound **16** (a) were injected with 50 mg/kg per day in dimethyl sulfoxide (Me_2SO) twice daily sc. Rats treated with compound **16** (b) were injected with 50 mg/kg per day in oil as six injections sc per day from day 4 to day 8 of pregnancy. All other compounds were administered from silastic wafers sc containing 100–150 mg of inhibitor and injections of 25 mg/kg per day.

reaction followed to completion by thin-layer chromotography (see Table II). (Compound **6** was prepared in a similar manner but required refluxing in chloroform.) The solvents and reagents were removed in vacuo (<40 °C). The residue was dissolved in chloroform and washed several times with 0.25 N hydrochloric acid, concentrated sodium bicarbonate, and water. (Compound **6** was not washed with bicarbonate.) The organic phase was dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo. The residue was crystallized from a suitable solvent (Table II). [Compound **6** required column chromatography with benzene–ethyl acetate–acetic acid (33:66:1) over silica, followed by vacuum drying at 60 °C.]

4-(Acetylthio)-4-androstene-3,17-dione (10). Under helium atmosphere (dry conditions), 10 g (33.1 mmol) of steroid **2** was dissolved in 100 mL of dry freshly distilled dioxane and cooled in an ice bath. To the stirring solution was added dropwise 3 mL (42 mmol) of thioacetic acid dissolved in 10 mL of dioxane. The reaction was warmed slowly to room temperature and stirring was continued for 4 days. An additional 3-mL volume of thioacetic acid was added and the mixture stirred for 3 more days (TLC showed no starting material remained). The dioxane was evaporated under nitrogen, and then 100 mL of methanol was added and the mixture stirred for 30 min. The solvent was concentrated to about 80 mL, and the solid was filtered and washed with methanol. The steroid was dissolved in 150 mL of methanol, filtered, concentrated (ca 100 mL), and cooled to 8 °C. The pale yellow crystals were filtered and after drying weighed 1.4 g (yield 12%): for analytical data, see Table I.

4-Mercapto-4-androstene-3,17-dione (11). A suspension of 200 mg (0.56 mmol) in 5 mL of methanol of steroid **10** was stirred under a helium atmosphere. Separately, 1 mL of ethyl ether was saturated with HCl gas and then diluted to 5.5 mL with ether. One milliliter of the ether solution was added to the steroid suspension and the mixture was refluxed under helium overnight. After the mixture cooled, the white precipitate was filtered under helium and washed with methanol. The filtrate was evaporated under a stream of helium and the resulting solid was dissolved in boiling ethyl acetate, filtered hot, and allowed to crystallize overnight at 8 °C. The pinkish solid was filtered and, upon drying, weighted 55 mg (yield 31%): for analytical data, see Table I.

3-Methoxy-2-androstene-4,17-dione (13). A solution of 4-hydroxyandrostenedione (**3**; 200 mg, 0.66 mmol), 10 mL of methanol, and 0.5 mL of boron trifluoride etherate was refluxed for 5 h. The reaction mixture was poured into 300 mL of ice water and extracted with 4 × 150 mL of ethyl acetate. The combined organic phases were washed with 2 × 150 mL of saturated sodium bicarbonate solution. The organic phase was dried over anhydrous sodium sulfate, filtered, and evaporated in vacuo to a solid. The solid was crystallized twice from acetone and once from ethyl acetate. The clear crystals were crushed and dried (29 mg, yield 14%): for analytical data, see Table I.

4-Hydroxy-1,4-androstadiene-3,17-dione (14). A solution of steroid **3** (10 g, 3.3 mmol) and 15 g of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (6.6 mmol) in 500 mL of dioxane was treated with 5 mL of concentrated HCl solution. The reaction was followed to completion by TLC (ca. 12 h). The nonsteroidal precipitate was filtered and washed with benzene. The filtrate was concentrated in vacuo (40 °C) and the semisolid residue was dissolved in 300 mL of ethyl acetate, and washed with 4 × 150 mL of 0.5 N NaOH and 5 × 150 mL of water (small quantities of methanol were added to crack emulsions). The aqueous NaOH fractions were combined (600 mL) and washed with 3 × 150 mL of chloroform. Each fraction of chloroform was used to wash the combined water fractions (750 mL). All organic phases (i.e., ethyl acetate + chloroform) were combined, dried over anhydrous sodium sulfate, filtered, and evaporated to a solid (5.16 g). The solid was dissolved and chromatographed on a silica column (1100 g, silica, Grace) with benzene–ethyl acetate (3:1). The product was found, slightly impure, after 2 L of solvent had been eluted. The fractions containing the product were combined and crystallized twice in methanol, giving 1.45 g (yield 10%) of pure product: for analytical Data, see Table I.

4-Hydroxy-4,6-androstadiene-3,17-dione (17). A freshly prepared solution of potassium *tert*-butoxide (34.5 mmol) in 45 mL of *tert*-butyl alcohol) was added to a solution of 3 g (10.5 mmol) of 4-androstene-3,17-dione (**1**) in 120 mL of *tert*-butyl alcohol with stirring. The course of the reaction was followed by TLC on silica gel in benzene–ethyl acetate (1:1) and was stopped when little or no starting material could be detected (ca. 20 h). At this point the UV spectrum of an aliquot showed maxima at 254 and 318 nm. The reaction mixture was acidified with glacial acetic acid (to pH 6) and the *tert*-butyl alcohol was removed under vacuum at 40 °C. The residue was dissolved in 1:1 acetone–methanol and ether was added to precipitate potassium acetate. The solid was filtered and the filtrate was concentrated in vacuo. The solid was dissolved and eluted with benzene–ethyl acetate on a chromatographic column (400 g, ICN 04526, silica). The impure product was found after the first 400 mL (steroid **16** was found in the following 100 mL). The solid was crystallized from methanol, triturated in ethyl ether, and filtered to give 470 mg (yield 15%) of a white solid: for analytical data, see Table I.

4-Hydroxy-1,4,6-androstatriene-3,17-dione (24) was prepared in 6% yield from steroid **23** by a similar procedure to the preparation of steroid **17** from **1**: for analytical data, see Table I.

Biological Methods. Enzyme Preparation. Microsomes were prepared from ovaries obtained from rats that had been pretreated with 100 IU of pregnant mares' serum gonadotropin (PMSG) on alternate days for 12 days.[10] Microsomes from human term placenta were prepared by a similar procedure.[9]

In Vitro Test for Inhibition. Compounds were initially tested by incubating them in various concentrations with microsomes from human placenta[9] or from ovaries of PMSG-primed rats.[10]

Briefly, microsomes (equivalent 50 mg of wet weight ovary) were suspended in 2.5 mL of 0.1 M phosphate buffer, (pH 7.4) containing [1,2-$^3H_2$] (ca. 70% β) 4-androstene-3,17-dione (0.7 μM) and varying concentrations of test compound. The reaction was initiated by the addition of an NADPH-generating system consisting of NADP, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase dissolved in 0.1 M phosphate buffer, and the incubation was carried out for 30 min at 37 °C under oxygen. Chloroform was then added to remove the steroids. The supernatent was further treated with 2.5% charcoal for 10 min. After centrifugation, radioactivity was measured in the aqueous phase. Estrogen formation was calculated from the amount of tritium released as ^3H_2O into the medium during aromatization of [1,2-$^3H_2$]androstenedione.

Microsomes (0.3–0.9 mg) from human placental tissue were incubated with test compounds, [4-^{14}C]androstrenedione, 0.7 μM androstenedione, and the NADPH-generating system in 3 mL of phosphate buffer as above. The incubation was terminated by the addition of recovery markers, [6,7-$^3H_2$]estradiol and estrone, and carrier steroids, and the estrogens were extracted and purified by TLC. Selected samples were further purified as described in detail previously[9] but without change in specific activity, indicating radiochemical purity after TLC.

Enzyme Inactivation. Compounds were preincubated for various lengths of time with microsomes in the absence of substrate but in the presence of the NADPH-generating system. After removal of the compound with charcoal, aromatase activity was assayed as above by quantitation of ^3H_2O released during 30 min of incubation with [1,2-$^3H_1$] androstenedione.[33]

Aromatase Inhibition (in Vivo). All rats (from Charles River Breeding Labs) were fed an ad libitum diet and housed under conditions of controlled temperature and humidity. Rats were maintained on a 12 h light/12 h dark cycle.

Ovarian-Vein Cannulation. Virgin female rats (230–250 g), showing at least two consecutive regular 4-day cycles, were injected with inhibitor (50 mg/kg) sc at 1100 h on the day of proestrus. Blood (1 mL) was collected from the ovarian vein between 1400 and 1600 h.[11] The resulting plasma then was analyzed by radioimmunoassay to determine the concentration of estrogen.

Mating and Blastocyst Implantation. Female rats showing at least two consecutive 4-day cycles were used. On the day of diestrus 1, a silastic wafer was inserted under the dorsal skin of each rat. The wafer, about 25 mm in diameter and 1 mm thick, was prepared by polymerizing 0.5 g of Corning medical grade Silastic 382 with (or without for controls) 100–150 mg of inhibitor with use of stannous octanoate catalyst.[15] Each female was injected sc with 25 mg/kg per day inhibitor or vehicle and housed with two fertile males until mating occurred. The females were checked daily for evidence of mating (i.e., vaginal plugs or sperm). All females were autopsied on day 7 of pregnancy and the number and size of implantation sites were recorded. The ovaries were examined for the presence of corpora lutea (Table III).

Discussion and Conclusion

Substitution at C-4 of the androstenedione molecule provides inhibitory activity, yet of the numerous substitutions tested, none are more potent than the 4-hydroxy as an aromatase inhibitor in vitro or in vivo.[10,11] However, data from Table II suggests that the more water-soluble hemisuccinate ester may have useful properties in vivo.

4-Hydroxy- and 4-acetoxy-4-androstene-3,17-dione[33,34] as well as 1,4,6-androstatriene-3,17-dione[35,36] are competitive inhibitors, but they also cause aromatase inactivation following pseudo-first-order kinetics. Evidence to date suggests that they may be K_{cat} or active-site-directed inhibitors.[33,36] Combining these modifications as in 4-hydroxy 4,6-diene enhanced inactivation of aromatase in rat ovarian microsomes. Differences in activity of several inhibitors in the human placental and rat ovarian microsomes were observed. Whether these represent species or tissue differences is not known at the present time but is in agreement with earlier reports that suggest the existence of more than one type of aromatase.

Only the most potent inhibitors have activity in vivo. Thus, the conjugated compounds and the 4-hydroxy, 4-acetoxy, and the 6α-bromo compounds reduced ovarian estrogen secretion and/or affected estrogen-dependent processes such as ovulation and implantation in the rat. However, it was not determined in this study whether the compounds have other actions that may affect fertility. On the basis of these preliminary results, it may be possible to improve in vivo activities of these compounds relative to 4-OHA, by altering the route of administration, dosage, or dosage schedule.

Surprisingly, neither the 4,5-epoxide nor the 4-chloro or 4-methoxy derivative caused significant inhibition of in vivo processes. These compounds did not cause enzyme inactivation. Although bioavailability and other factors should be considered, studies with 4-OHA indicate that this compound may be irreversibly bound to the enzyme, which may result in sustained activity in vivo.[35] In recent studies, postmenopausal patients with advanced metastatic breast cancer responded to an injection once a week of 4-OHA.[38] It is interestering to speculate that inactivation of aromatase may be required for in vivo activity.[33] The mechanisms by which 4-OHA, 4-(CH_3COO)A, and the conjugated inhibitors cause aromatase inactivation are of considerable interest and are under investigation. Studies with inhibitors that are competitive as well as those that cause inactivation may provide clues in elucidating the biochemical events occurring during the aromatization of androgen to estrogens and the nature of the enzyme.

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Registry No. 1, 63-05-8; 2 (isomer I), 17503-11-6; 2 (isomer II), 7430-11-7; 3, 566-48-3; 4, 61630-32-8; 5, 76942-05-7; 6, 76942-04-6; 7, 76942-02-4; 8, 76942-03-5; 9, 16318-49-3; 10, 95192-05-5; 11, 95192-06-6; 12, 20986-46-3; 13, 95192-07-7; 14, 6561-84-8; 15, 6561-83-7; 16, 2243-06-3; 17, 89617-12-9; 18, 89593-30-6; 19, 95192-08-8; 23, 897-06-3; 24, 95192-09-9; 25, 95192-10-2; 26, 61145-67-3; 27, 38632-00-7; 29, 571-17-5; 30, 2141-15-3; aromatase, 9039-48-9.

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