Interactions of Thiol-Containing Androgens with Human Placental Aromatase

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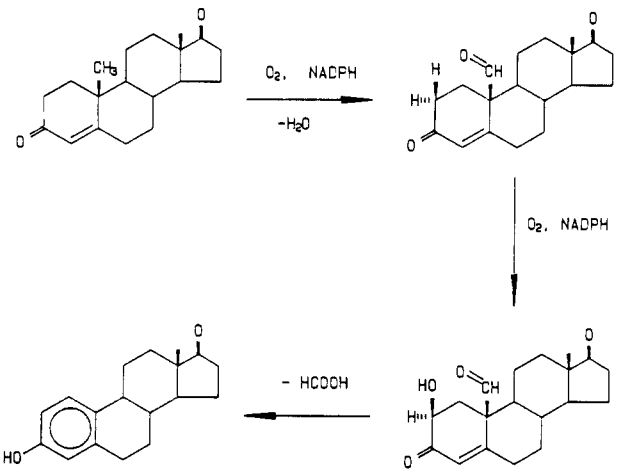
A series of thiol androgens were synthesized and investigated to characterize structural features important for the inhibition of aromatase. Analogues of androstenedione with thiol groups in either the 2α-, 10β-, or 19-positions caused time-dependent inhibition of human placental aromatase. When their K_I and k_{cat} values were compared with those of 4-hydroxyandrost-4-ene-3,17-dione (4-OHa) and 10β-propargylestr-4-ene-3,17-dione (PED), the thiol androgen 10β-mercaptoestr-4-ene-3,17-dione (10β-SHnorA) proved to be the most potent suicide substrate. However, 19-mercaptoandrost-4-ene-3,17-dione (19-SHA) was the best all-around inhibitor. All compounds except 19-SHA exhibited normal type I P-450 difference spectra with partially purified/solubilized, human placental aromatase. The K_s values for the series of compounds compared qualitatively to the K_I values determined from the time and concentration-dependent inhibition experiments. 19-SHA induced split Soret peaks at 380 and 474 nm, which suggested binding of the 19-thiolate directly to the ferric iron of aromatase. This binding could be displaced by aminoglutethimide but not by androstenedione. The inhibitory activity of 19-SHA may be explained by two independent mechanisms: (1) suicide inactivation of aromatase in the ferrous state; and (2) a direct "hyper-type II" binding to the remaining portion of the cytochrome in the ferric state. A free thiol group was necessary for the suicide inhibitory activity of 19-SHA; time-dependent inactivation of aromatase by 19-(acetylthio)androst-4-ene-3,17-dione (19-SAcA) and 19-xanthogenylandrost-4-ene-3,17-dione (19-XanA) could be prevented if the microsomes were preincubated with a carboxyesterase inhibitor. Aromatase previously inactivated by either thiol androgens, 4-OHA, or PED could not be reactivated after incubation with the disulfide reducing agent dithiothreitol, which suggests that a disulfide bond may not be involved in aromatase inactivation by these inhibitors.

The importance of estrogens in both healthy and diseased physiology has prompted an interest in the mechanism of estrogen biosynthesis as well as in ways to inhibit it. The conversion of 4-ene-3-one androgens to the phenolic estrogens represents the last step in the multienzyme transformation of cholesterol to the female sex steroids. A unique microsomal cytochrome P-450-type monooxygenase known as aromatase is responsible for this conversion.[1] Although the details of the mechanism are still a subject of debate, it is generally believed that human placental aromatase carries out three sequential oxidations of the androgen (Scheme I).[2] The 19-methyl group is believed to be the site of the first two oxidations, resulting in an intermediate at the oxidation state of an aldehyde.[1] Evidence has been presented to support 2β-position as the site of the third oxidation.[3] Although there is some dispute about details of the reaction sequence, the last step of aromatization yields the estrogen and formate.[4]

Present knowledge of the mechanism of aromatization has led to the successful design and development of a wide variety of suicide substrates of aromatase.[5] Known suicide substrates primarily have made use of the oxidation of the 19-methyl group in the autoinactivation process. To date, no suicide inhibitors have been designed to take advantage of oxidation at C-2 of the A ring.[6] An aromatase suicide substrate that was specifically oxidized at this position would lend additional support to the mechanism proposed for aromatase.

We have previously reported that substitution of a thiol group at either the 10β- or 19-positions of a 4-ene-3-one androgen molecule (compounds **1** and **2**, respectively)

Scheme I. Proposed Catalytic Mechanism of Aromatase



yields steroids possessing potent suicide inhibitory activity.[5e] Compound **1** replaces the 10β-methyl group of testosterone with the isosteric thiol group, and compound **2** replaces the 19-hydroxyl group of the anticipated first oxidation product of androstenedione with the isoelectronic thiol group.

As a continuing study of this class of aromatase inhibitors, we have investigated further structural requirements necessary for aromatase inhibition by thiol androgens. We prepared the 10β-thiol analogue of androstenedione (10βSHnorA, **3**) and the 2α-thiol derivative of androstenedione (2α-SHA, **4**). We have compared these compounds with two other suicide inhibitors: 10β-propargylestr-4-ene-3,17-dione (PED, **5**)[5b] and 4-hydroxyandrost-4-ene-3,17-dione (4-OHA, **6**).[7] We also report investigations undertaken to understand the mechanism(s) through which the thioandrogens are able to inactivate human placental aromatase. A free thiol group was found to be necessary for aromatase inhibition as was determined by studies with two ester derivatives of **2**, 19-(acetylthio)-androst-4-ene-3,17-dione (19-5AcA, **7**) and 19-

(1) Brodie, A. M. H. *J. Endocrinol. Invest.* **1979**, *2*, 445.
(2) Thomas, E. A.; Siiteri, P. K. *J. Biol. Chem.* **1974**, *249*, 5364.
(3) Fishman, J. *Cancer Res.* **1982**, *42*, 3277s.
(4) Caspi, E.; Wicha, J.; Arunachalam, T.; Nelson, P.; Spiteller, G. *J. Am. Chem. Soc.* **1984**, *106*, 7282.
(5) (a) Covey, D. F.; Hood, W. F.; Parkikh, V. D. *J. Biol. Chem.* **1981**, *256*, 1076. (b) Metcalf, B. W.; Wright, C. L.; Burkart, J. P.; Johnston, J. O. *J. Am. Chem. Soc.* **1981**, *103*, 3221. (c) Marcotte, P. A.; Robinson, C. H. *Steroids* **1982**, *39*, 325. (d) Flynn, G. A.; Johnston, J. O.; Wright, C. L.; Metcalf, B. W. *Biochem. Biophys. Res. Commun.* **1981**, *103*, 913. (e) Bednarski, P. J.; Porubek, D. J.; Nelson, S. D. *J. Med. Chem.* **1985**, *28*, 775. (f) Burkart, J. P.; Weintraub, P. M.; Wright, C. L.; Johnston, J. O. *Steroids* **1985**, 357.
(6) One unsuccessful attempt is reported in ref 5f.
(7) (a) Brodie, A. M. H.; Garrett, W. M.; Hendrickson, J. R.; Tsui-Morris, C.; Marcotte, P. A.; Robinson, C. H. *Steroids* **1981**, *38*, 693. (b) Covey, D. F.; Hood, W. F. *Mol. Pharmacol.* **1981**, *21*, 173.

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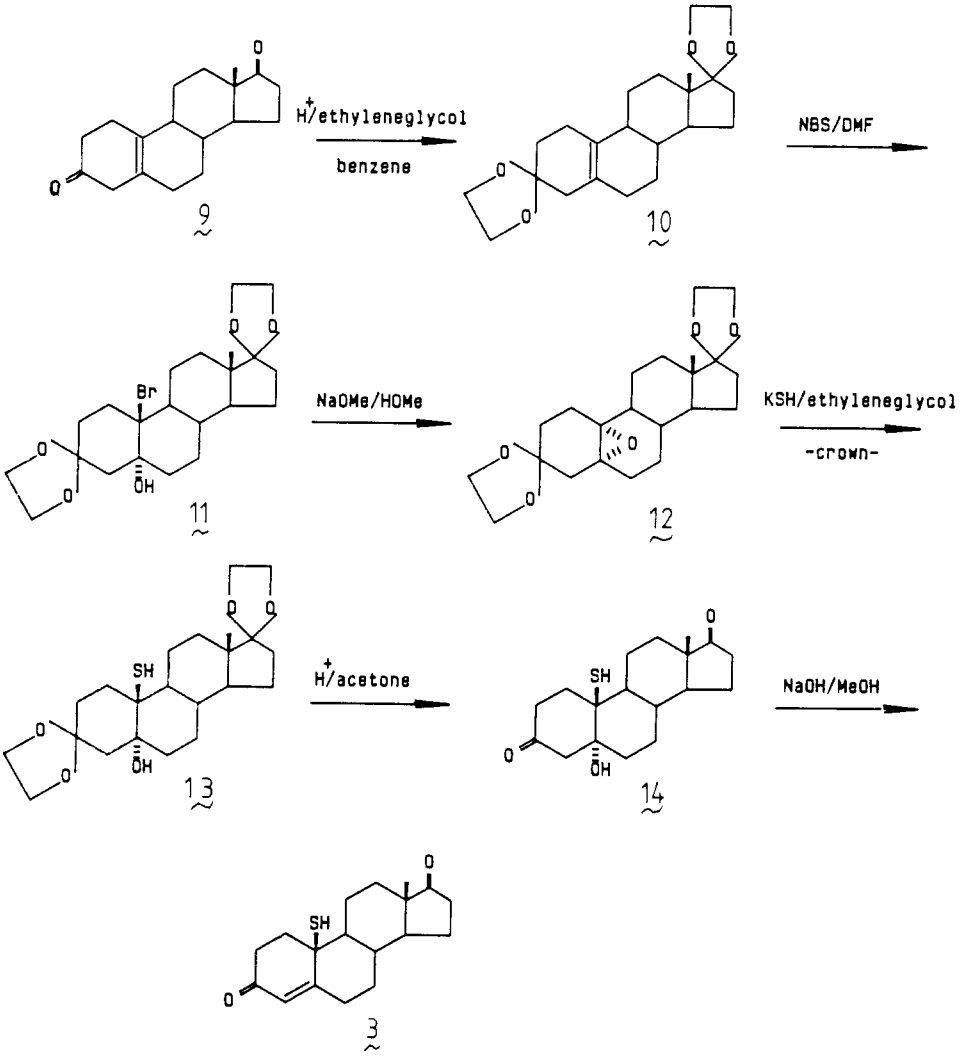
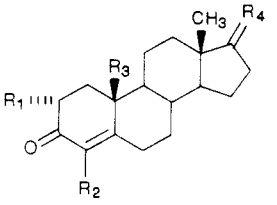
Scheme II. Synthesis of 10β-Mercaptoestr-4-ene-3,17-dione



xanthogenylandrost-4-ene-3,17-dione (19-xanA, 8). The structures of compounds tested (1–8) are shown below.



	R_1	R_2	R_3	R_4
1	H	H	SH	
2	H	H	CH_2SH	O
3	H	H	SH	O
4	SH	H	CH_3	O
5	H	H	$CH_2C{\equiv}CH$	O
6	H	OH	CH_3	O
7	H	H	CH_2SAc	O
8	H	H	$CH_2SC(S)OEt$	O

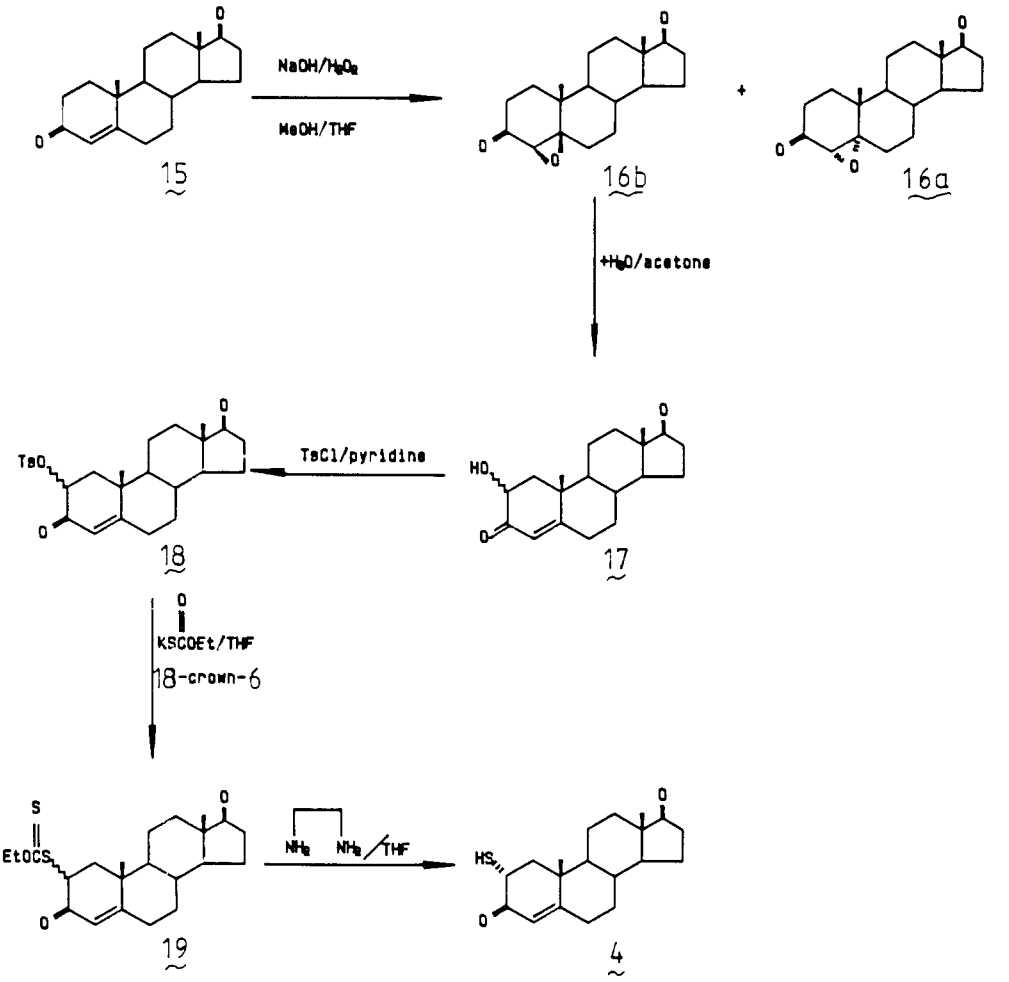
Chemistry

The synthesis of 10β-mercaptoestr-4-ene-3,17-dione (**3**) was carried out by a modification of the synthesis of the 17β-hydroxy analogue **10**[5e] and is described in Scheme II. The commercially available 5(10)-olefin (**9**) was converted to the protected bisketal **10** and reaction with *N*-bromosuccinimide yielded the protected 5α,10β-bromohydrin (**11**). Under basic conditions, the bromohydrin in **11** was closed to give the 5α,10α-epoxiode **12**. Introduction of the thiol group into the 10β-position was accomplished with potassium hydrogen sulfide in ethylene glycol in the presence of the crown ether 18-crown-6 at 140 °C to yield the 5α-hydroxy-10β-mercapto steroid **13**. Deprotection of the ketone functionalities gave **14**, as well as a side product later identified as estr-4-ene-3,17-dione (norandrostenedione). Dehydration of **14** under basic conditions gave the desired product **3** in a 10% overall yield from **9**.

The synthesis of the novel 2α-mercaptoandrost-4-ene-3,17-dione (**4**) is described in Scheme III. Oxidation of androstenedione (**15**) to the isomeric 4,5-epoxides (**16a** and **16b**) was performed as described previously.[8] A rapid separation of the α- and β-isomers was achieved with flash chromatography on silica gel. Acid rearrangement of the β-isomer was carried out as described previously[9] except that an approximate 5:1 ratio of 2α- to 2β-hydroxy products (**17**) was found upon NMR analysis of the product. No attempt was made to separate these isomeric alcohols. The alcohols were converted to their tosylate ester (**18**) and sulfur was introduced into the 2-position through displacement of the tosyl group by potassium ethyl xanthogenate in the presence of 18-crown-6.[10] The xanthogenates (**19**) were cleaved in a dilute solution of ethylenedi-

(8) Henbest, H. B.; Jackson, W. R. *J. Chem. Soc. C* **1967**, 2459.
(9) Burnett, R. D.; Kirk, D. N. *J. Chem. Soc., Perkin Trans. 1* **1973**, 1830.
(10) Beretta, E.; Cinguini, M.; Colonna, S.; Fornasier, R. *Synthesis* **1974**, 425.

Scheme III. Synthesis of 2α-Mercaptoandrost-4-ene-3,17-dione



amine/THF to yield the 2α-thiol **4** in a 5.3% overall yield from **15**. The stereochemistry at the 2-position was confirmed by circular dichroism and NMR studies.

The circular dichroism of 2α- versus 2β-hydroxyandrostenediones have been investigated; 2α-OHA (**17a**) gives a positive Cotton effect while 2β-OHA (**17b**) shows a strong negative effect for the 240–245 nm ($\pi \rightarrow \pi^*$) transition.[9] A positive Cotton effect, although much weaker than that seen with 2α-OHA, was also observed for thiol **4** (Figure 1), indicating an α orientation. Additionally, it was reasoned that if the 2β isomer was formed and did not epimerize under the previous mild basic conditions of the synthesis, then epimerization could be forced under strongly acidic conditions. The 500-MHz NMR spectra before and after refluxing compound **4** in H_2SO_4/ethanol for 24 h were identical (see the Experimental Section).

The synthesis of 10β-propargylestr-4-ene-3,17-dione (**5**) has been reported.[5a-c] However, we used a simpler, alternative route (Scheme IV). (Dialkylcopper)lithium reagents have been found to be superior to alkyllithium and Grignard reagents for nucleophilic addition to epoxides.[11] Therefore, the protected 5α,10α-epoxide (**12**) was opened with a trimethylsilyl-protected (dipropynylcopper)lithium reagent (**20**) prepared by the reaction of 1-(trimethylsilyl)propyne[12] with *n*-BuLi and CuI at −15 °C. Yields of the adduct (**21**) were approximately 60% for this method. Acid hydrolysis of the 3- and 17-ketals gave **22**. Dehydration of the 5α-hydroxyl group and deprotection

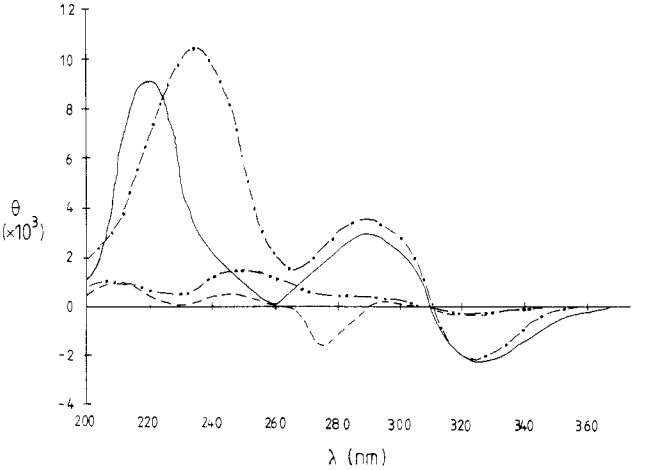


Figure 1. CD: -●●-, **4**; -●-, **17**; -, **18**; --, **19**.

of the ethynyl group were both accomplished in the same reaction under basic conditions to give the desired product **5** in a 23% overall yield from **12**.

The synthesis of 4-hydroxyandrost-4-ene-3,17-dione (**6**) was adapted from the reported synthesis of 4-hydroxytestosterone.[13] A mixture of α- and β-4,5-epoxyandrost-4-ene-3,17-diones (**16a** and **16b**) were rearranged with boron trifluoride etherate in benzene to yield **6** in a 48% yield.

(11) Herr, R. W.; Johnson, C. R. *J. Am. Chem. Soc.* **1970**, *92*, 4979.
(12) Corey, E. J.; Kirst, H. A. *Tetrahedron Lett.* **1968**, *48*, 5041.
(13) Camerino, B.; Patelli, B.; Vercellone, A. *J. Am. Chem. Soc.* **1956**, *78*, 3540.

Table I. Difference Spectra Maxima and Minima, K_s Binding Constants, and Absorbance Maxima of Various Androgens in Partially Purified, Solubilized Human Placental Aromatase, and the K_I and k_{cat} Values for Various Suicide Inhibitors of Aromatase in Human Placental Microsomes

compd[d]	max, nm	min, nm	K_S,[a] nM (PL1)	A_{max}/nmol P-450 ($\times 10^{-3}$) (PL1)	K_I,[b] nM (PL2)	k_{cat} ($\times 10^{-3}$) (PL2)	k_{cat}/K_I ($\times 10^{-6}$) (PL2)
A	392	426	105	177			
T	392	426	549	166			
19-OHA	392	426	368	125			
10β-SHnorT (1)	392	426	785	167	100	4.0	40
19-SHA (2)	380, 474	425	c	179	47	2.5	53
10β-SHnorA (3)	392	426	144	187	28	4.3	154
2α-SHA (4)	392	424	602	169	3100	2.0	1.6
PED (5)	392	424	213	191	6.2	0.7	113
4-OHA (6)	392	424	324	162	542	8.0	15

[a] Determinations done at 23 ± 1 °C. [b] Determinations done at 30 ± 1 °C. [c] See text. [d] Abbreviations include: PL, placenta; A, androstenedione; T, testosterone; 19-OHA, 19-hydroxyandrostenedione.

The acetylation of 19-mercaptoandrost-4-ene-3,17-dione (2) was carried out with acetic anhydride/pyridine in the presence of a catalytic amount of 4-(dimethylamino)-pyridine to give 19-(acetylthio)androst-4-ene-3,17-dione (7).

The syntheses of 17β-hydroxy-10β-mercaptoestr-4-ene-3-one (1), 19-mercaptoandrost-4-ene-3,17-dione (2), and 19-(ethyl xanthogenyl)androst-4-ene-3,17-dione (8) have been described.[5e]

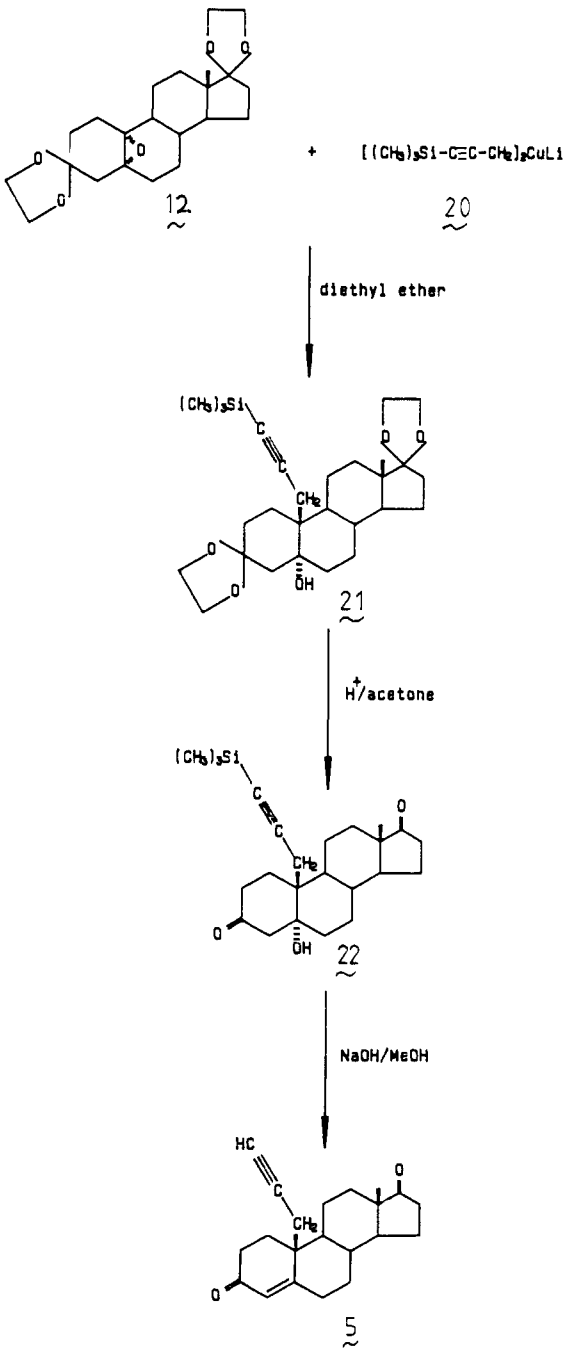
Biochemistry

The ability of substrates and inhibitors to form reversible enzyme–ligand complexes with aromatase was investigated. Partially purified aromatase from human placental microsomes was prepared as described previously,[14] and ligand binding was observed as the P-450 optical differences between the high and low spin states of the ferric heme with and without ligand added. Strong type I binding was seen for all substrates except 19-mercaptoandrostenedione. This compound displayed an unusual difference spectrum with Soret peaks at 380 and 474 nm and a trough at 425 nm. For type I substrates, K_s values were determined by measuring the absorbance differences between the peak (i.e. 392 nm) and trough (i.e. 424 nm) versus increasing ligand concentrations. Scatchard analysis of the data afforded the K_s values (Table I).

For comparative purposes, aromatase from a single placenta was used to determine K_s values for all the steroids. Results from this placenta (Table I) showed that androstenedione bound in a monophasic manner with a K_s value of approximately 100 nM. Reduction of the 17-keto group of androstenedione (A) gives testosterone (T) which had a K_s about 5 times higher than A. A similar decrease in binding affinity was observed for 10β-SHnorT (1) compared to 10β-SHnorA (3). Substitution of the 19-methyl group of either A or T for a thiol group (3 and 1, respectively) had little effect on K_s, whereas introduction of a 2α-thiol group (4) into androstenedione significantly increased the K_s value. PED (5) and 4-OHA (6) showed binding constants approximately 2 times and 3 times that of androstenedione, respectively. All of these compounds exhibited monophasic binding.

Substitution of a thiol group for a hydroxy group at C-19 (19-OHA versus 19-SHA, 2) caused dramatic changes in binding to aromatase. Whereas 19-OHA bound reversibly with a calculated K_s approximately 3–4 times that of A (Table I), 19-SHA bound so tightly that no K_s could be determined. Double-reciprocal plots of the absorbance differences between the trough at 425 nm and the peak at 474 nm versus the concentration of 19-SHA (2) produced a straight line with a slope of 1.0 at concentrations <0.2 μM (Figure 2). The 1:1 increase in absorbance units

Scheme IV. Synthesis of 10β-Propynylandrost-4-ene-3,17-dione



(14) Zachariah, P. K.; Lee, Q. P.; Symms, K. G.; Juchau, M. R. *Biochem. Pharmacol.* **1976**, *25*, 793.

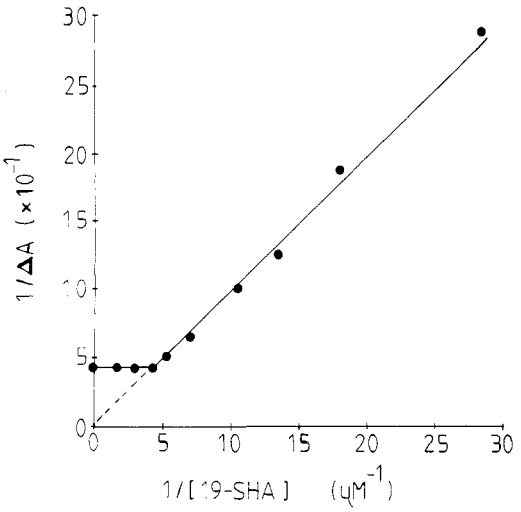


Figure 2. Double-reciprocal plot of absorbance difference versus 19-mercaptoandrost-4-ene-3,17-dione concentration.

versus added **2** ceased after the same concentration was achieved as there was P-450 present in the cuvette. Addition of saturating concentrations of reducing equivalents in the form of sodium dithionite rapidly decreased the intensity of the Soret peak at 474 nm by approximately 30%. However, there was little additional effect on the spectrum during the next 60 min. Additions of androstenedione 100–30 000 times the concentration of 19-SHA (0.250 μM) decreased the intensity of the 19-SHA difference spectra, but did not produce the characteristic type I binding pattern. The addition of aminoglutethimide (0.04–3.20 mM), on the other hand, gradually caused a change in the difference spectra to yield a characteristic type II binding pattern.

For enzymatic activity experiments, the apparent aromatase activity was determined with a well-established tritium-release assay.[2] A V_{max} of 10.7 pmol/mg per min and an apparent K_m of 4.4 μM were found for microsomes from the placenta (PL2) used for determinations of the K_I and k_{cat} values of the inhibitors. These values of V_{max} and apparent K_m for androstenedione are within the rather wide range of values reported by several other groups.[2,15] The reasons for discrepancies in these values is unknown. In fact, we have found considerable variation in these kinetic parameters from one placenta to the next. Therefore, all determinations of K_I and k_{cat} were carried out with microsomes from the same placenta so that relative values could be used for comparison.

Compounds **1–8** all showed time-dependent loss of aromatase activity, which was pseudo-first-order at early time points (i.e., 3 min), and rates of inactivation (k_{obs}) increased with increasing concentrations of each inhibitor. Data for the thiol androgens (**2** and **3**) is shown in Figure 3. From such data, double-reciprocal plots were generated for compounds **1–6** and the apparent kinetic parameters, K_I and k_{cat}, were determined (Table I).[16] PED (**5**) possessed the smallest K_I but also had the smallest k_{cat} value. 4-OHA (**6**) possessed the largest k_{cat} value but also had a large K_I as did 2α-SHA (**4**). Reduction of the 17-ketone

(15) (a) Reed, K. C.; Ohno, S. *J. Biol. Chem.* **1976**, *251*, 1625. (b) Schwarzel, W. C.; Kruggel, W. G.; Brodie, H. J. *Endocrinology* **1973**, *92*, 866. (c) Thompson, E.; Siiteri, P. K. *Ann. N.Y. Acad. Sci.* **1972**, 378. (d) Gibb, W.; Lavoie, J. *Steroids* **1980**, *36*, 507. (e) Canick, J. A.; Ryan, K. J. *Mol. Cell. Endocrinol.* **1976**, *6*, 105. (f) Muto, N.; Tan, L. *Biochem. Biophys. Res. Commun.* **1986**, *136*, 454.

(16) Kitz, R.; Wilson, I. *J. Biol. Chem.* **1982**, *273*, 3245.

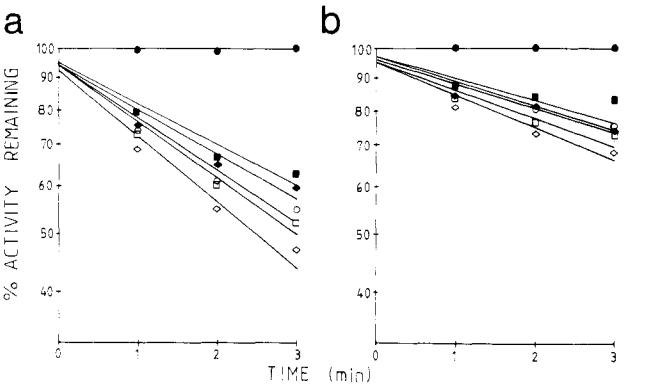


Figure 3. Time- and concentration-dependent loss of aromatase activity by thiol androgens: (a) ●, 0.0; ■, 40; ◆, 60; ○, 100; □, 167; ◇, 500 nM 10β-mercaptoandrost-4-ene-3,17-dione (**3**). (b) ●, 0.0; ■, 35; ◆, 50; ○, 75; □, 100; ◇, 500 nM 19-mercaptoandrost-4-ene-3,17-dione (**2**).

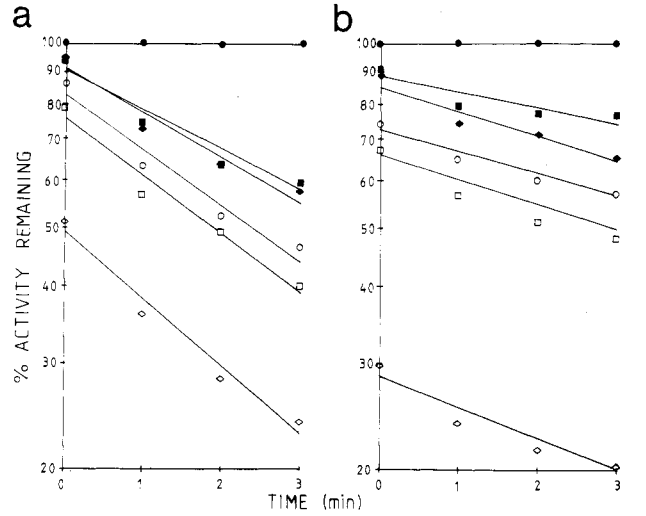


Figure 4. Alternate method for plotting time- and concentration-dependent inhibition by thiol androgens. a: **3**; b: **2**. Concentrations are the same as in Figure 3.

to the 17β-alcohol as in **3** to **1**, increased the K_I values by approximately 4 times while having little effect on the k_{cat} value. Insertion of a methylene group into the 10β-position, as in **3** to **2**, increased slightly the K_I while decreasing slightly the k_{cat}.

Two methods for plotting the ln of the percent activity remaining versus time were compared. The first method, which was chosen to calculate K_I and k_{cat} values, reports activity remaining (DPMs) after a preincubation time period (t_1) with inhibitor present as a fraction of activity (DPMs) at zero time (t_0) for the same incubation (Figure 3):

$$\% \text{ activity remaining} = \frac{\text{DPMs at } t_1 \text{ with inhibitor}}{\text{DPMs at } t_0 \text{ with inhibitor}} \times 100$$

The second method was used to determine to what degree inhibition had occurred after the inhibitor had been added, but before time-dependent inhibition had progressed. In this method the activity remaining (DPMs) at every time point for all inhibitor concentrations is a fraction of the activity (DPMs) present at t_0 with no inhibitor added:

$$\% \text{ activity remaining} = \frac{\text{DPMs at } t_1 \text{ with inhibitor}}{\text{DPMs at } t_0 \text{ without inhibitor}} \times 100$$

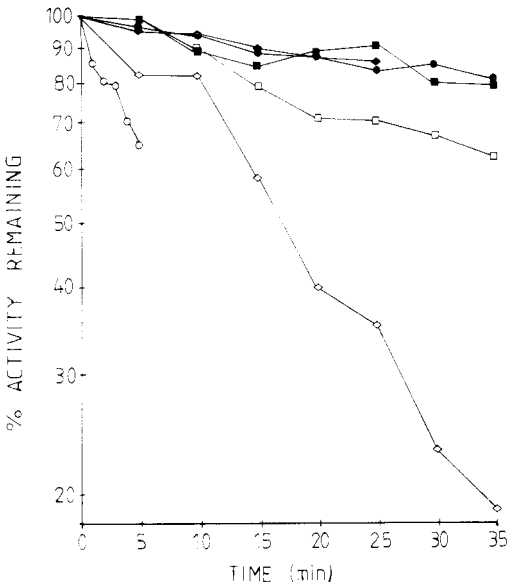


Figure 5. Paraoxon protection experiments: ●, no inhibition; ○, 0.5 μM **2**; ◆, 2.0 μM **7** + paraoxon; ◇, 2.0 μM **7**; ■, 0.5 μM **8** + paraoxon; □, 0.5 μM **8**.

This plotting method revealed that 19-SHA (**2**) caused a large degree of inhibition at t_0 (Figure 4). This inhibition was not due to either competitive inhibition or to suicide inactivation inasmuch as 10β-SHnorA, which possesses a K_I approximately half and a k_{cat} value approximately twice as great as 19-SHA, did not show similar inhibition at t_0 at the same inhibitor concentration.

When placental microsomes were preincubated with 25 mM of the carboxyesterase inhibitor paraoxon, the time-dependent inactivation of aromatase by the 19-thioacetate (**7**) and the 19-xanthogenate ester (**8**) was prevented (Figure 5).

We attempted to reactivate aromatase activity after inhibition with compounds **1–6**. Incubation of the inactivated enzyme in the presence of 10 mM dithiothreitol for 10 min at 30 °C failed to show increased activity compared to inactivated enzyme incubated under identical conditions but without dithiothreitol (Table II).

Discussion and Conclusions

Thiols are known to be oxidized metabolically by cytochrome P-450 monooxygenases to reactive intermediates which can then bind to protein nucleophiles. For example, some thiosteroids have been shown to inhibit P-450 steroid hydroxylase activity by a suicide mechanism.[17] At low concentrations, 7α-mercaptotestosterone caused the selective O_2- and NADPH-dependent destruction of microsomal hepatic testosterone 7α-hydroxyase activity. 6-Mercaptopurine, an antineoplastic agent, also is known to be activated by hepatic microsomal cytochrome P-450 to a reactive intermediate that covalently binds to tissue proteins.[18] Our previous work has shown that the substitution of the 19-methyl group of the 3-keto-4-ene androgens by either a 10β-thiol or a 19-methylene thiol group gives compounds that are suicide substrates for aromatase.[5e] We chose to extend this class of thiol androgens to include a compound with a thiol group in the 2-position,

Table II. Dithiothreitol Reactivation Studies: Attempt Using 10 mM Dithiothreitol To Reactivate Human Placental Aromatase Previously Inactivated by Preincubation with 1.0 μM of **2–6** for either 5 or 10 min

conditions	% activity remaining[a] (±SD); preincubation time: 5 min	10 min
no inhibitor	95 ± 5	88 ± 5
no inhibitor + DDT	97 ± 2	84 ± 3
19-SHA (**2**)	60 ± 3	–
2 + DTT	59 ± 4	–
10β-SHnorA (**3**)	50 ± 6	–
3 + DTT	48 ± 7	–
2α-SHA (**4**)	–	75 ± 2
4 + DTT	–	74 ± 4
PED (**5**)	–	65 ± 1
5 + DTT	–	63 ± 2
4-OHA (**6**)	43 ± 2	–
6 + DTT	41 ± 3	–

[a] $N = 3$.

as well as to investigate some of the other structural requirements for suicide inactivation by this class of compounds.

The binding of thiol androgens **1–4**, in addition to the known aromatase substrates androstenedione, testosterone, and 19-hydroxyandrostenedione, and the two suicide substrates PED (**5**) and 4-OHA (**6**) to solubilized/partially purified placental P-450, was compared (Table I). Thiol androgens **1**, **3**, and **4** formed dissociable enyzme–inhibitor complexes as evidenced by their type I difference spectra. The K_s value for 10β-SHnorA (**3**, 144 nM) was similar to that of androstenedione (A, 105 nM), and the K_s for 10βSHnorT (**1**, 785 nM) was similar to that of testosterone (T, 549 nM). This would appear to validate our original hypothesis that similarities in size and lipophilicity between the thiol and the methyl group allow for the normal binding of 10β-mercapto analogues to the enzyme active site. However, incorporation of a thiol group into the 2α-position of the androstenedione molecule decreased the binding constant significantly. This was not unexpected since substitution of a thiol group for a proton would be expected to cause significant structural pertubation. A 2β-thiol analogue was not synthesized because substitution in the 2β-position is known to markedly distort the conformation of the A ring of androstenedione.[9]

The isoelectronic thiol analogue (19-SHA, **2**) of 19-OHA produced an unusual split Soret difference spectrum. Similar spectra have been reported for the binding mercaptoalkanes[19] and other thiosteroids[17] to rat hepatic cytochrome P-450. More recently the same kind of spectra were observed for the binding of alkane- and arenethiols to cytochrome $P\text{-}450_{CAM}$.[20] Apparent dissociation rate constants have been calculated for such thiol compounds by taking the difference in absorbance between the peak at 380 nm and the trough at 420 nm or the peak at 466 nm and the trough at 420 nm and by plotting the double reciprocals of these absorbance differences versus ligand concentration.[17,19] A similar calculation of a K_s value for 19-SHA revealed a 1:1 binding ratio of steroid to P-450 with no apparent dissociation of the steroid–enzyme complex (Figure 2). This tight association of ligand to enzyme was demonstrated further by the ineffectiveness of androstenedione to alter the 19-SHA-induced difference

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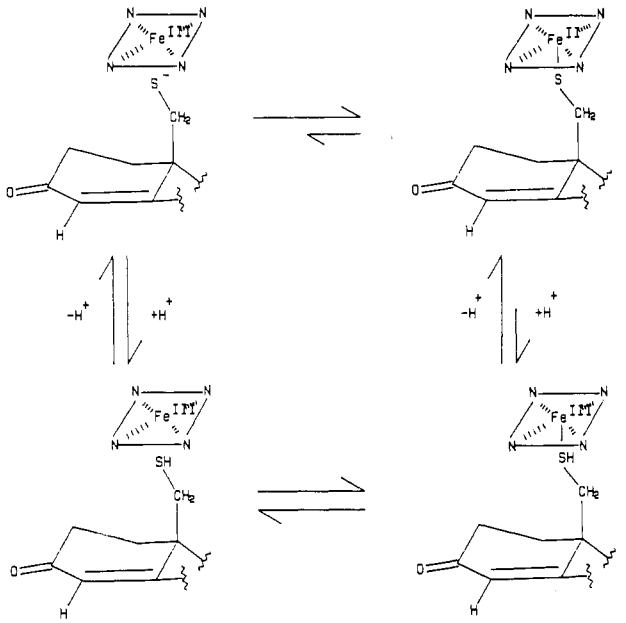
Scheme V. Proposed Binding of 19-Mercaptoandrost-4-ene-3,17-dione to the Ferric Porphyrin of Aromatase



spectra. Displacement of 19-SHA from the enzyme was achieved by addition of the type II substrate aminoglutethimide, as well as with the addition of sodium dithionite.

These results would appear to indicate a direct interaction between sulfur and the iron of the ferric porphyrin. The nature of this interaction most likely involves a thiolate anion equivalent of 19-SHA, such that the heme appears to be spectrally reduced to the ferrous state (Scheme V). Evidence for the involvement of a thiolate anion equivalent comes from two previously published observations: (1) The transformation from a normal type II binding spectra ot a "split Soret" absorption spectrum for 1-propanethiol and *p*-chlorothiophenol was found to be pH dependent; increases in pH and thereby thiolate anion concentration lead to greater "split Soret" and lesser type II binding in the absorption spectrum.[20] (2) The substitution of a methyl group for the proton of the 19-thiol group produces a methyl sulfide incapable of forming a thiolate. 19-(Methylthio)androstenedione has been found to give a classical type II binding spectrum with aromatase, indicating direct coordination of the sulfur atom to the ferric heme but without heme reduction.[21] Type II binding to aromatase also has been observed for 19-episulfide analogues of androstenedione.[22] In particular, the 19*R* isomer of 10-thiiranyl-4-estrene-3,17-dione binds tightly to aromatase and is a potent competitive inhibitor of the enzyme.

Another conclusion of our studies on the binding of the thiol androgens to aromatase is that the homologous structural change from a thiol group at the 10β-position of the A ring to a methylene thiol group markedly affects the way that the steroid interacts with the aromatase enzyme active site. Compounds **1** and **3**, with thiol groups at the 10β-position, appear to bind only at the lipophilic steroid binding site of aromatase without interacting directly with the ferric heme. However, addition of a methylene spacer at the 10β-position moves the sulfur atom to a position which then allows for its direct interaction with the ferric iron. This indicates that the steroid binding site of aromatase is sufficiently inflexible to allow the 10β-thiol group to directly interact with the ferric iron.

All of the compounds that we tested inhibited the aromatization of androstenedione in a time-dependent, pseudo-first-order manner. The rate of inhibition increased linearly with increasing inhibitor concentration, which indicated formation of a dissociable enzyme–inhibitor complex followed by unimolecular inactivation. Literature K_I and k_{cat} values for PED (**5**, 23 nM, 1.11×10^{-3} s^{-1})[5a] and 4-OHA (**6**, 170 nM, 9.21×10^{-3} s^{-1})[7b] compared closely with those obtained in our studies (Table I). The ratio of k_{cat} to K_I provides a value which has been used to make relative comparisons of the in vitro effectiveness of inhibitors. By this criteria, where K_I and k_{cat} values are weighted equally, 10β-SHnorA (**3**) and PED (**6**) would appear to be the most effective suicide inhibitors (Table I). Although 4-OHA (**5**) has the largest k_{cat}, it also has a very large K_I value and ranks 1 order of magnitude below **3** in overall effectiveness. The least effective compound was 2α-SHA (**4**), which had a k_{cat}/K_I ratio more than 2 orders of magnitude less than the best inhibitors in this series.

In addition to its ability to form a tightly bound enzyme–ligand complex with the ferric heme, 19-SHA also showed time-dependent inhibition (Figure 3). That this inhibition was suicidal in nature, rather than due to the continued binding of inhibitor to ferric heme with time, was demonstrated by the requirement for both NADPH and oxygen during the inactivation process.[5e] Interactions of thiols with ferrous porphyrins are weak,[23] and under the NADPH-reducing conditions of the incubations a portion of aromatase is in the reduced ferrous state.[14] Thus, the ferrous cytochrome could bind 19-SHA in a reversible fashion. After oxygen activation and reactive intermediate formation, aromatase would be inactivated. The presence of a large pool of aromatase that is inhibited at the beginning of incubations containing 19-SHA (Figure 4) suggests that rapid inhibition is caused by a direct binding of the thiol to the porpyrin, as previously described (Scheme V). However, this inhibition is diffusion controlled. The involvement of a dissociable enzyme–inhibitor, active-site complex before suicide inactivation by 19-SHA was demonstrated by the protection from inactivation by concurrent incubation of androstenedione with inhibitor.[5e] Thus, while 19-SHA fails to form a freely dissociable enzyme–inhibitor complex with the oxidized ferric heme state, such a complex would appear to form with the reduced ferrous state.

Derivatization of the 19-thiol group of 19-SHA as either an acetate (**7**) or a xanthogenate ester (**8**) produced compounds that still exhibited time-dependent inhibition of aromatase (Figure 5). However, if microsomal carboxyesterase activity was first inhibited by preincubation with paraoxon, neither **7** nor **8** caused time-dependent inhibition. Thus, a free thiol is required for the inactivation. Compounds **7** and **8** apparently are prodrugs of 19-SHA. Such prodrugs may enhance the bioavailability of the thiosteroids.

Dialysis experiments showed the irreversible nature of the inhibition caused by the thiol compounds **1** and **2**.[5e] Since NADPH and oxygen are both required for time-dependent inactivation, an oxidized reactive intermediate is implicated in a covalent interaction with aromatase. If this intermediate were a sulfenic acid, then disulfide bond

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formation between a free active-site cysteine and the thiosteroid would seem likely. However, incubation of aromatase previously inactivated by thiosteroids **1**, **3**, **4**, as well as PED (**5**) and 4-OHA (**6**), with the disulfide reducing reagent dithiothreitol failed to reactivate the aromatase. Dithiothreitol has been found effective at reactivating aromatase previously inactivated by 10β-hydroperoxyestrenedione, and this inactivation is postulated to occur through disulfide formation.[24] Our results indicate that either the covalent attachment between aromatase and thiosteroids is not a disulfide bond, or that dithiothreitol is unable to gain access to the disulfide bond within the inactivated enzymatic site. The nature of this interaction will be addressed by probing purified/reconstituted aromatase with a radiolabeled thioandrogen and determining the site(s) and nature of the covalent attachment.

In conclusion, we have further demonstrated the utility of the thiol group in developing suicide substrates to aromatase. We report here the first synthesis of a 2α-thioandrogen designed to inactivate the aromatase during the proposed 2β-oxidation. However, additional experiments will be required to determine the selectivity of compound **4** for the 2-carbon oxidative versus the 19-carbon oxidative step. A unique bidentate mechanism through which 19-mercaptoandrostenedione (**2**) inactivates both the ferric and ferrous forms of human placental aromatase has been identified. We believe that these special features of the 19-thiol group could give compound **2** an advantage over other aromatase inhibitors in the treatment of estrogen-dependent breast cancer. The antitumor activities of the thiosteroids are presently being investigated.

Experimental Section

Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^{1}H nuclear magnetic resonance spectra (NMR) were recorded with either a Varian EM-360 or a Bruker WM-500 spectrometer. Chemical shifts are reported in parts per million (ppm) with tetramethylsilane (TMS) as the internal standard. Coupling constants (J) are given in hertz; the abbreviations s, d, t, q, and m refer to singlet, doublet, triplet, quartet, and multiplet, respectively. Electron-impact (EI) mass spectra (MS) were recorded routinely on a Hewlett-Packard 5985 GC/MS instrument using direct insertion. High-resolution mass spectra were recorded on a VG-7070H mass spectrometer operated in the chemical-ionization (CI) mode with methane as the reagent gas and a source temperature of 200 °C. Infrared spectra (IR) were recorded on a Perkin-Elmer 283 instrument, optical rotations $[\alpha]_D$ were determined on a JASCO DIP-4 polarimeter, circular dichroism measurements were made on a CNRS-Roussel-Jouan Dichromographe III instrument, and ultraviolet spectra (UV) were recorded with a Hewlett-Packard 8451A diode array spectrophotometer. Optical difference spectra were performed on an American Instrument Co. DW-2 UV-VIS spectrophotometer (Silver Spring, MD). Scintillation counting was carried out on a Beckman LS 7500 instrument (Fullerton, CA); Maxifluor was used as the LSC cocktail and was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ). Microanalyses were performed by Galbraith Laboratories (Knoxville, TN).

Thin-layer chromatography (TLC) was performed with DC-Plastikfolien Kieselgel 60 F_{254} (E. Merck, Darmstadt, West Germany). UV, iodine vapor, or phosphoric acid was used to visualize the developed plates. For phosphoric acid visualization, the developed plates were dipped in a 50% phosphoric acid solution, wiped dry, and heated at 120 °C for approximately 30 min. Column chromatography was performed with either silica gel, Merck (grade 62, 60–200 mesh; Aldrich), silica gel, Merck (grade 60, 230–400 mesh; Aldrich), or Florisil (reagent grade, 100–200 mesh; Aldrich). High-performance liquid chromatography (HPLC) was accomplished on a DuPont 850 liquid chromatograph equipped with a DuPont 850 absorbance detector (DuPont Co., Wilmington, DE) and either a 4.6 mm i.d./25 cm L. Rainin analytical or 10 mm i.d./25 cm L. Rainin semipreparative silica column. Burdick and Jackson (Muskegon, MI) UV solvents were used for all HPLC procedures.

Chemicals were obtained from the following sources and were used as received: androstenedione and estr-5(10)-ene-3,17-dione from Steraloids, Inc., (Wilton, NH), testosterone from Nutritional Biochemical Corp. (Cleveland, OH), 19-hydroxyandrostenedione, NADPH tetrasodium salt, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, diethyl p-nitrophenyl phosphate (paraoxone, and dithiothreitol, from Sigma Chemical Co. (St. Louis, MO), EDTA from the Mallinckrodt Chemical Works (St. louis, MO), propynyllithium, and potassium hydrogen sulfide from Alfa Research Chemicals (Danvers, MA), potassium ethyl xanthogenate from Pfaltz and Bauer, and 18-crown-6 from Aldrich Chemical Co. (Milwaukee, WI).

Estr-5(10)-ene-3,17-dione Bis(ethylene ketal) (10). Estr-5(10-ene-3,17-dione (**9**, 7.0 g, 26 nmol) was dissolved in 300 mL of dry benzene containing 40 mL of ethylene glycol and p-toluenesulfonic acid (0.5 g, 2.6 mmol). A Dean–Stark trap and condenser were attached, and the mixture was refluxed overnight. After cooling, the reaction mixture was washed once with aqueous 5% $NaHCO_3$ solution, the $NaHCO_3$ layer was back-extracted with benzene, and the benzene layers were combined, dried over $MgSO_4$, filtered, and evaporated under reduced pressure to yield 9.65 g (100% crude yield) of a yellow oil. TLC (1:1 ethyl ether/petroleum ether): **9**, R_f = 0.23; **10**, R_f = 0.43. IR (neat): no carbonyl absorbance. NMR ($CDCl_3$): δ 3.90 (8 H, d, ketal methylenes), 0.85 (3 H, s, 18-CH_3).

10β-Bromo-5α-hydroxyestra-3,17-dione Bis(ethylene ketal) (11). To a stirred solution of **10** (9.67 g, 26.9 mmol) in 200 mL of DMF and 50 mL of H_2O were added N-bromosuccinimide (7.48 g, 42 mmol) and MgO (0.85 g, 21 mmol). The reaction was stoppered and allowed to stir at room temperature for 2 h, after which time 500 mL of ice-cold water was added. The white precipitate was collected by suction filtration and washed with generous amounts of cold water. The precipitate was dissolved in 200 mL of $CHCl_3$, washed with 100 mL of saturated NaCl solution and three times with water, dried over $MgSO_4$, filtered, and concentrated on the rotary evaporator to give 9.97 g (80% crude yield) of **11** as a white, crystalline solid. TLC (1:1 ethyl ether/petroleum ether): R_f = 0.22. NMR ($CDCl_3$): δ 4.58 (1 H, s, 5α-OH), 3.92 (8 H, asymmetric doublet, ketal methylenes).

5α,10α-Epoxyestra-3,17-dione Bis(ethylene ketal) (12). To a solution of 5α-bromo-10β-hydroxyestra-3,17-dione bis(ethylene acetal) ketal (**11**, 9.67 g, 26.9 mmol) in 200 mL of methanol and 100 mL of THF stirred at 0 °C under argon was added a suspension of NaOMe (12 g, 0.23 mol) in 100 mL of MeOH. The reaction was stirred at 4 °C for 8 h after which time the contents were poured into 400 mL of cold CH_2Cl_2 and washed with 400 mL of ice-cold water. The aqueous wash was extracted with 400 mL of cold CH_2Cl_2, and organic phases were combined and washed twice more with 400 mL of ice-cold water, dried over $MgSO_4$, filtered, and evaporated under reduced pressure to yield 7.56 g of a white solid. Florisil chromatography (100–200 mesh, 40:1; 1:1 ethyl ether/petroleum ether) gave 5.71 g (71% yield) of a white crystalline solid. TLC (9:1 $CHCl_3$/ethyl ether): **11**, R_f = 0.58; **12**, R_f = 0.34. IR (Nujol): no alcoholic or carbonyl absorption. NMR ($CDCl_3$): δ 3.88 (8 H, d, ketal methylenes), 0.84 (3 H, s, 18-CH_3). MS(EI): m/z 376 (M^+, 100), 258 (10), 348 (14), 333 (18), 331 (15), 315 (68), 290 (29), 288 (30), 272 (16).

5α-Hydroxy-10β-mercaptoestra-3,17-dione Bis(ethylene ketal) (13). In a glove bag filled with N_2, a 200-mL round-bottom flask was charged with 5α,10α-epoxyestra-3,17-dione bis(ethylene ketal) (**12**, 4.0 g, 10.6 mmol), potassium hydrogen sulfide (3.7 g, 51 mmol), and 18-crown-6 (100 mg). After addition of 100 mL of ethylene glycol, the reaction mixture was stoppered and stirred under argon at 140 °C for 2 h. The reaction was then poured into 300 mL of an ice-cold 5% H_2SO_4/5% Na_2SO_4 solution and extracted three times with 200 mL of CH_2Cl_2. The organic phases were combined and washed three times with 200 mL of water, dried over $MgSO_4$, filtered, and evaporated under reduced pressure to give 4.34 g of a light brown solid. Florisil chromatography (100–200 mesh, 70:1; 1:1 ethyl ether/petroleum ether) gave 3.07

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g (71%) of a white solid. TLC (1:1 ethyl ether/petroleum ether): **12**, R_f = 0.23; **13**, R_f = 0.21. IR (Nujol): 3340 cm^{-1} (5α-OH). NMR ($CDCl_3$): δ 4.40 (1 H, s, 10β-SH), 3.95 (4 H, s, ketal methylene), 3.85 (4 H, s, ketal methylene), 0.85 (3 H, s, 18-CH_3). MS(EI): *m/z* 410 (M^+, 3), 392 (20), 377 (17), 357 (98), 315 (20), 297 (100).

5α-Hydroxy-10β-mercaptoestra-3,17-dione (14). 5α-Hydroxy-10β-mercaptoestra-3,17-dione bis(ethylene ketal) (**13**, 3.07 g, 7.5 mmol) and *p*-toluenesulfonic acid (0.5 g) in 250 mL of acetone were stirred overnight at room temperature under argon. The acetone was removed under reduced pressure and chloroform was added. The organic phase was then washed with 5% $NaHCO_3$ and twice with water, dried over $MgSO_4$, filtered, and evaporated under reduced pressure to yield 2.60 g of a green solid. Two products were separated by silica gel chromatography (60–200 mesh, 100:1; 90:8:2 chloroform/ethyl ether/methanol); 0.56 g (27%) of norandrostenedione was collected first followed by the elution of 1.88 g (78% yield) of the desired product **14**. **14**: TLC (90:8:2 chloroform/ethyl ether/methanol): R_f = 0.07. IR (Nujol): 3550 (5α-OH), 1735 (C-17 carbonyl), 1700 cm^{-1} (C-3 carbonyl). NMR ($CDCl_3$): δ 0.95 (3 H, s, 18-CH_3). MS(EI): *m/z* 322 (M^+, 9), 304 (15), 288 (10), 271 (100), 270 (23), 253 (11), 236 (15).

10β-Mercaptoestr-4-ene-3,17-dione (3). 5α-Hydroxy-10β-mercaptoestra-3,17-dione (**14**, 1.88 g, 5.8 mmol) in 230 mL of a 0.1 M NaOH/MeOH solution was refluxed under argon for 2 h. The reaction mixture was then cooled in an ice bath and 230 mL of a 5% H_2SO_4 solution added. The aqueous phase was extracted three times with 230 mL of $CHCl_3$, and organic phases were combined and washed with water, dried over $MgSO_4$, filtered, and concentrated to dryness on the rotary evaporator to give 1.35 g of a light brown solid. Flash silica chromatography (200–400 mesh, 25:1; 10% ethyl ether/$CHCl_3$) gave 0.90 g of **3**. Recrystallization from ethyl ether gave 0.57 g (32%). Mp = 161–162 °C. TLC (90:8:2 chloroform/ethyl ether/methanol): R_f = 0.47. UV (acetonitrile): λ_{max} = 236 nm (ε = 15 300). IR (Nujol): 2500 (10β-SH), 1740 (C-17 carbonyl), 1675 (C-3 conj carbonyl), 1615 cm^{-1} (4,5 conj olefin). NMR (CDCl3): δ 5.70 (1 H, s, 4-H), 0.95 (3 H, s, 18-CH3). MS(EI): *m/z* 304 (M^+, 32), 272 (100), 253 (40). Anal. ($C_{18}H_{24}O_2S$), C, H, S.

4β,5β-Epoxyandrostane-3,17-dione (16b).[8] To a stirred solution of androstenedione (1.0 g, 3.6 mmol) in 100 mL of methanol at 0 °C were added 4.3 mL of 4 N NaOH solution and 12.8 mL of a 30% H_2O_2 solution. After stirring for 2 h at 0 °C, the reaction mixture was extracted three times with CH_2Cl_2. The CH_2Cl_2 layers were combined, washed twice with water, dried over $MgSO_4$, filtered, and evaporated under reduced pressure to yield 1.0 g of a white solid. Flash silica gel chromatography (200–400 mesh, 100:1; 3:2 petroleum ether/ethyl ether) gave 108 mg (20%) of the α-isomer (**16a**), 540 mg (49%) of the β-isomer (**16b**), and 195 mg (18%) of a mixture of the two. **16a**: TLC (1:1 petroleum ether/ethyl ether): R_f = 0.15. $[\alpha]_D$ (*c* = 1.0, chloroform): +6.7° (lit.[8] $[\alpha]_D$ −3°, chloroform). IR (Nujol): 1735 (C-17 carbonyl), 1715 cm^{-1} (C-3 carbonyl). NMR ($CDCl_3$): δ 2.98 (1 H, s, 4-H), 1.02 (3 H, s, 19-Me), 0.85 (3 H, s, 18-Me). **16b**: TLC (1:1 petroleum ether/ethyl ether): R_f = 0.13. Mp (from acetone/ethyl ether): 200–201 °C (lit.[8] mp 202–203 °C. $[\alpha]_D$ (*c* = 0.52, chloroform): +208° (lit.[8] $[\alpha]_D$ +221°, chloroform). IR (Nujol): 1730 (C-17 carbonyl), 1705 cm^{-1} (C-3 carbonyl). NMR ($CDCl_3$): δ 2.97 (1 H, s, 4-H), 1.17 (3 H, s, 19-Me), 0.87 (3 H, s, 18-Me).

2α/β-Hydroxyandrost-4-ene-3,17-dione (17).[9] To a solution of 4β,5β-epoxyandrost-4-ene-3,17-dione (**16b**, 0.63 g, 2.1 mmol) stirred in 30 mL of acetone was added 0.60 mL of 98% H_2SO_4 in 1.8 mL of water, and the solution was allowed to continue stirring at room temperature for 1 week. The reaction was diluted in 80 mL of ethyl acetate, and the organic phase was washed with a 5% sodium bicarbonate solution and twice with water, dried over $MgSO_4$, filtered, and concentrated to dryness on the rotary evaporator to yield 0.50 g of a yellow solid. Flash silica gel chromatography (200–400 mesh, 100:1; 1:1 ethyl ether/dichloromethane) yielded 0.38 g (60%) of **17** as a colorless solid. TLC (1:1 ethyl ether/CCl_4): R_f = 0.06. Mp = 151–154 °C (lit.[9] mp 158–160 °C). IR (Nujol): 3370 (br, 2α-hydroxyl), 1740 (17-carbonyl), 1670 (3 conj carbonyl), 1600 cm^{-1} (3-conj olefin). MS(EI): *m/z* 302 (M^+, 12), 258 (100), 243 (22).

17a: 500-MHz NMR ($CDCl_3$): δ 5.81 (1 H, s, 4-H), 4.26 (1 H, q, *J* = 5.9, 13.8 Hz, 2β-H), 3.51 (1 H, s, 2α-OH), 1.33 (3 H, s, 19-Me), 0.94 (3 H, s, 18-Me) (lit.[9] δ 5.78 (1 H, s, 4-H), 4.24 (1 H, q, *J* = 5.6 Hz, 13.6 Hz 2-H), 3.51 (1 H, s, 2α-OH), 1.32 (3 H, s, 19-Me), 0.92 (3 H, s, 19-Me)).

17b: 500-MHz NMR ($CDCl_3$): δ 5.83 (1 H, s, 4-H), 4.17 (1 H, q, *J* = 5.9, 13.8 Hz, 2α-H), 3.43 (1 H, s, 2β-OH), 1.22 (3 H, s, 19-Me), (lit.[9] δ 5.84 (1 H, s, 4-H), 4.23 (1 H, q, *J* = 5.4 Hz, 14.4 Hz, 2-H), 3.46 (H, s, 2-OH), 1.21 (3 H, s, 19-Me), 0.93 (3 H, s, 18-Me).

2α/β-(Tosyloxy)androst-4-ene-3,17-dione (18). As a solution of 2α- and 2β-hydroxyandrost-4-ene-3,17-dione (**17a and 17b**, 100 mg, 0.33 mmol) in 3.0 mL of dry pyridine stirred in an ice bath under nitrogen was added dropwise tosyl chloride (250 mg, 1.3 mmol) in 2.0 mL of ice-cold, dry pyridine. The reaction was then stirred at 0 °C for 24 h and 25 mL of ice-cold CH_2Cl_2 was added. The organic phase was washed three times with 12 mL of ice-cold water, dried over $MgSO_4$, filtered, and evaporated under reduced pressure to give 0.27 g of a white solid. Flash silica gel chromatography (230–240 mesh, 100:1, 1:1 ethyl ether/CCl_4) yielded 100 mg (66%) of **18**. TLC (1:1 ethyl ether/CCl_4): R_f = 0.14. IR (Nujol): 1735 (C-17 carbonyl), 1690 (C-3 conj carbonyl), 1615 (4,5 conj olefin), 1185 cm^{-1} (S(=O)$_2$). NMR ($CDCl_3$): δ 7.20–8.00 (4 H, m, aromatics), 5.68 (1 H, s, 4-H), 5.10 (1 H, q, *J* = 5 Hz, 14 Hz, 2-H), 2.40 (3 H, s, 4′-Me), 1.30 (3 H, s, 19-Me), 0.90 (3 H, s, 18-Me). MS(EI): *m/z* 456 (M^+, 5), 284 (5), 258 (100).

2α/β-(Ethyl xanthogenyl)androst-4-ene-3,17-dione (19). To a 50-mL round-bottom flask containing 2α-(toxyloxy)androst-4-ene-3,17-dione (**18**, 0.43 g, 0.94 mmol), potassium ethyl xanthogenate (0.45 g, 2.8 mmol), and 18-crown-6 (40 mg) was added 10 mL of dry THF. The reaction was stoppered and stirred for 3 days at room temperature. To the reaction was added CH_2Cl_2, and the organic phase was washed twice with a saturated KCl solution and once with water, dried over $MgSO_4$, filtered, and concentrated on the rotary evaporator to 0.33 g of a yellow glass. Florisil chromatography (100–200 mesh; 100:1; 1:1 CCl_4/ethyl ether) gave 0.25 g (66%) of **19**. TLC (1:1 CCl_4/ethyl ether): R_f = 0.31. IR (Nujol): 1745 (C-17 carbonyl), 1675 (C-3 conj carbonyl), 1620 (4,5-conj olefin), 1220 (br, thionyl), 1050 (=CO), 780 cm^{-1} (br, =CS). UV (acetonitrile): λ_{max} = 237 (ε = 14 000), 282 nm (ε = 9028). NMR ($CDCl_3$): δ 5.87 (1 H, *J* = 1.5 Hz, 4-H), 4.77 (1 H, d of d, *J* = 4.8 and 14.3 Hz, 2-H), 4.62–4.72 (2 H, m, 1′-CH_2), 1.52 (3 H, s, 19-CH_3), 0.93 (3 H, s, 18-Me). MS(EI): *m/z* 406 (M^+, 66), 373 (14), 346 (70), 316 (60), 317 (100), 289 (18), 285 (20).

2α-Mercaptoandrost-4-ene-3,17-dione (4). To a solution of 2α/β-(ethyl xanthogenyl)androst-4-ene-3,17-dione (**19**, 0.25 g, 0.62 mmol) in dry THF stirred in an ice bath under N_2 was added ethylenediamine (45 mg, 50 μL, 0.75 mmol), and the reaction mixture was stirred at 0 °C for 1 h. To the reaction was added ice-cold $CHCl_3$, and the organic layer was washed once with 30 mL of an ice-cold solution of 5% H_2SO_4 and twice with 30 mL of water, dried over $MgSO_4$, filtered, and evaporated under reduced pressure to yield 120 mg of a light yellow solid. Florisil chromatography (100–200 mesh, 125:1, 50:45:5 petroleum ether/ethyl ether/methanol) gave 80 mg (41%) of a white solid. Further purification by HPLC (Microsorb Si-semiprep column, 3:2 hexane/chloroform, 6 mL/min) and recrystallization (ethyl ether/hexane) gave 28 mg of a white powder. Mp = 185–189 °C. TLC (50:45:5 petroleum ether/ethyl ether/methanol): R_f = 0.20. UV(acetonitrile): λ_{max} = 237 nm (ε = 14 400). IR (Nujol): 2540 (2α-SH), 1730 (C-17 carbonyl), 1655 (C-3 conj carbonyl), 1610 cm^{-1} (4,5 conj olefin). NMR ($CDCl_3$): δ 5.85 (1 H, s, 4-H), 3.85 (1 H, d of triplets, *J* = 4 Hz), 1.30 (3 H, s, 19-Me), 0.93 (3 H, s, 18-Me). MS(EI): *m/z* 318 (M^+, 38, 303 (20), 284 (95), 269 (100). Anal. ($C_{19}H_{26}O_2S$), C, H, S.

Attempt To Epimerize the 2-Thiol Group of 4. 2-Mercaptoandrost-4-ene-3,17-dione (**4**, 10 mg, 0.03 mmol) was refluxed in 5 mL of ethanol/2 mL of 5% H_2SO_4 for 24 h under nitrogen. After concentration on the rotary evaporator, 10 mL of CH_2Cl_2 was added, washed with 10 mL of water, the water layer was back-extracted with 10 mL of CH_2Cl_2, and the combined organic extracts were washed twice with 15 mL of water. The organic extract was then dried over $MgSO_4$, filtered, and evaporated under reduced pressure to yield 10 mg of a white solid. Purification by preparative HPLC (Microsorb Si-semiprep column, 3:2 hexane/chloroform, 6.0 mL/min) gave 4 mg of a white

powder. TLC (50:45:5 petroleum ether/ethyl ether/methanol): R_f = 0.22. NMR ($CDCl_3$): δ 5.85 (1 H, s, 4-H), 3.85 (1 H, d of triplets, J = 4.75 Hz, and J = 14.81 Hz, 2β-H), 1.30 (3 H, s, 19-Me), 0.93 (3 H, s, 18-Me).

19-(Acetylthio)androst-4-ene-3,17-dione (7). To 19-mercaptoandrost-4-ene-3,17-dione (**2**, 45 mg, 0.15 mmol) and a catalytic amount of 4-(dimethylamino)pyridine stirred in 1.0 mL of freshly distilled pyridine was added acetic anhydride (100 μM, 102 mg, 1.0 mmol) via a syringe. After the mixture was stirred overnight at room temperature, 20 mL of CH_2Cl_2 was added and the organic layer was washed three times with 20 mL of an ice-cold 5% H_2SO_4 solution, once with a 5% $NaHCO_3$ solution, and once with water, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to yield 60 mg of a yellow oil. Preparative TLC (25% ethyl ether/chloroform) gave 3 mg (6% yield) of a colorless semisolid. NMR ($CDCl_3$): δ 5.82 (1 H, s, 4-H), 3.48 (1 H, d, J = 15 Hz, 19-CH), 3.10 (1 H, d, J = 15 Hz, 19-CH), 2.22 (3 H, s, acetyl CH_3), 0.90 (3 H, s, 18-CH_3). MS(EI): m/z 360 (M^+, 14), 318 (25), 317 (53), 284 (13), 272 (100), 271 (39).

1-(Trimethylsilyl)propyne. To propynyllithium (10 g, 0.21 mol) stirred under nitrogen in 80 mL of freshly opened anhydrous ethyl ether was added dropwise via a syringe trimethylsilyl chloride (20 mL, 17.12 g, 0.16 mol). The reaction was brought to reflux temperature for 30 h and cooled to room temperature and 50 mL of saturated NaCl solution was added. The aqueous phase was acidified with 5% H_2SO_4, and the organic phase was separated. The organic phase was washed twice with saturated NaCl. The NaCl washes were combined and back-extracted with ethyl ether. The organic extracts were combined, dried over $MgSO_4$, filtered, and concentrated on the rotary evaporator. Distillation gave 8.0 mL of a clear liquid (bp 96–99° C/760 mm, lit.[12] bp 98–99 °C). NMR ($CDCl_3$): δ 1.82 (3 H, s, 3-Me), 0.10 (9 H, s, SiMe).

5α-Hydroxy-10β-[3-(trimethylsilyl)propargyl]estra-3,17-dione Bis(ethylene ketal) (21). To 1-(trimethylsilyl)propyne (0.60 g, 5.9 mmol) and *N,N,N',N'*-tetramethylethylenediamine (90 μM, 60 μmol) stirred in 25 mL of anhydrous ethyl ether under argon in a dry ice/acetone bath was added *n*-butyllithium (2.8 mL of a 2.0 M solution, 5.61 mmol) via a syringe. The reaction was allowed to warm to −15 °C for 45 min to generate the alkyllithium. The alkyllithium solution was transferred under argon via a cannula to a stirred suspension of CuI (0.48 g, 2.55 mmol) in 25 mL of anhydrous ether kept in a dry ice/acetone bath. The reaction was warmed to −15 °C and stirred for 30 min to generate the lithium dialkylcuprate **20**. 5α,10α-Epoxyestra-3,17-dione bis(ethylene ketal) (**12**, 0.48 g, 1.3 mmol) in 25 mL of anhydrous ether under argon at −78 °C was then added dropwise to the stirred solution of **20** and allowed to stir for 24 h at −20 °C. The reaction was washed with ice-cold 5% H_2SO_4 and three times with water, and the ether was removed on rotary evaporator to give 0.63 g of a colorless oil. Florisil chromatography (100–200 mesh, 70:1; 1:1 petroleum ether/ethyl ether) gave 0.36 g (57%) of a white solid. TLC (1:1 petroleum ether/ethyl ether): R_f = 0.21. IR (Nujol): 3480 (5α-OH), 2170 (C–C). NMR ($CDCl_3$): δ 3.84 (6 H, d, C-3 and C-17 ethylene ketals), 0.88 (3 H, s, 18-Me). MS(EI): m/z 488 (M^+, 7), 470 (9), 415 (35), 359 (33), 297 (100).

5α-Hydroxy-10β-[3-(trimethylsilyl)propargyl]estra-3,17-dione (22). Ketal **21** (0.36 g, 0.75 mmol) and *p*-toluenesulfonic acid (100 mg) were stirred under N_2 in 50 mL of acetone at room temperature for 18 h. To the reaction was added 25 mL of 5% $NaHCO_3$, and the acetone was removed on a rotary evaporator. To the concentrate was added 25 mL of H_2O and the solution was extracted twice with 60 mL of CH_2Cl_2. The organic phases were combined and washed twice with water, dried over $MgSO_4$, filtered, and evaporated to dryness under reduced pressure to yield 0.25 g of a white solid. TLC (1:1 petroleum ether/ethyl ether): R_f = 0.06. IR (Nujol): 3450 (5α-OH), 2175 (19-C), 1730 (C-17 carbonyl), 1705 cm^{-1} (C-3 carbonyl). NMR ($CDCl_3$): δ 0.95 (18-CH_3), 0.14 (1'-Si(CH_3)$_3$).

10β-Propargylestr-4-ene-3,17-dione (5). Compound **22** (0.25 g, 0.61 mmol) was refluxed under argon in a 1.0 M NaOH/MeOH solution for 2 h, after which the reaction was cooled in ice and 25 mL of a 5% H_2SO_4 solution was added. The reaction was concentrated on the rotary evaporator and 25 mL of H_2O added. The aqueous mixture was extracted twice with 50 mL of CH_2Cl_2, the organic phases were combined and washed twice with H_2O, dried over $MgSO_4$, filtered, and evaporated to dryness under reduced pressure to give 0.18 g of a yellow solid. Flash silica gel chromatography (200–400 mesh, 100:1; 25% ethyl ether/chloroform) gave 0.18 g of a white solid. Recrystallization from ethyl ether/hexane gave 93 mg (40% from **21**) of white needles. Mp = 174–181 °C (lit.[5a,5b] mp 174–175 °C and lit.[5c] mp 183–185 °C). TLC (25% ethyl ether/$CHCl_3$): R_f = 0.34. HPLC (25% hexane/chloroform; 2.0 mL/min; Rainin analytical Si-column): t_R = 11.0 min; UV (acetonitrile): λ_{max} = 237 nm (ε = 14700). IR (Nujol): 3210 (HC≡C), 2125 (C≡C), 1725 (C-17 carbonyl), 1680 (C-3 conj carbonyl), 1625 cm^{-1} (conj olefin). NMR ($CDCl_3$): δ 5.90 (1 H, s, 4-H), 0.92 (3 H, s, 18-CH_3). MS(EI): m/z 310 (M^+, 38), 282 (48), 271 (42), 253 (40), 216 (28), 197 (33), 105 (100). Anal. ($C_{21}H_{26}O_2$) C, H.

4-Hydroxyandrost-4-ene-3,17-dione (6). To 4,5-epoxyandrost-4-ene-3,17-dione (**16a** and **16b**, 0.62 g, 2.1 mmol) stirred under N_2 in benzene was added BF_3·Et_2O (20 μL, 23 mg, 0.165 mmol) via syringe. After the mixture was stirred at room temperature for 19 h, 70 mL of ethyl ether was added and the mixture washed with 50 mL of a 5% $NaHCO_3$ solution and twice with 50 mL of water, dried over $MgSO_4$, filtered, and evaporated to dryness under reduced pressure to give 0.64 g of a yellow solid. Flash silica chromatography (200–400 mesh, 50:1; 1:1 ethyl ether/petroleum ether) gave 0.30 g (47%) of **6**. Three recrystallizations from methanol/water gave 30 mg of white needles. Mp = 199–201 °C (lit.[9] mp 199–202 °C). TLC (1:1 ethyl ether/CCl_4): R_f 0.29. UV (acetonitrile): λ_{max} = 274 nm (ε = 11940). IR (Nujol): 3350 (4-OH), 1730 (C-17 carbonyl), 1655 (C-13 conj carbonyl), 1620 cm^{-1} (4,5 conj olefin). NMR ($CDCl_3$): δ 6.12 (1 H, s, 4-OH), 1.2 (3 H, s, 19-Me), 0.90 (3 H, s, 18-Me). MS(EI): m/z 302 (M^+, 100), 287 (18), 274 (14), 260 (30). Anal. ($C_{19}H_{26}O_3$) C, H.

Biochemical Methods. Protein concentrations were determined according to the method of Lowry et al.[25] Concentrations of cytochrome P-450 were determined by utilizing the absorbance difference between 450 and 474 nM (obtained on a Hewlett-Packard 8451A diode array spectrophotometer) and calculating with an extinction coefficient of 100 nm^{-1} cm^{-1}. Incubations containing the NADPH-regenerating system were readjusted to pH 7.5 after the addition of glucose 6-phosphate with a dilute solution of KOH. Unless otherwise indicated, all microsomal incubations were performed in 10 mM (pH 7.5) potassium phosphate buffer containing 100 mM KCl, 1 mM EDTA, and 5% propylene glycol.

Preparation of Placental Microsomes.[5a] The preparation of microsomes was performed on ice and in refrigerated centrifuges (2 °C). Human placentae were obtained from the University Hospital delivery room within 2 h of delivery at term. Tissue was cut away from fetal membrane and large blood vessels and then washed with generous amounts of ice-cold 0.15 M KCl to remove blood. The pieces of washed tissue were weighed and homogenized in 0.05 M (pH 7.0) potassium phosphate buffer/0.25 M sucrose (1.0 mL/3 g of tissue) with three 30-s bursts in a Waring seven-speed blender set at speed 7. The homogenate was centrifuged for 30 min at 10000*g*. The pellet was discarded and the supernatant was centrifuged for 60 min at 100000*g*. The resulting microsomal pellet was resuspended in 0.25 M sucrose/0.05 M phosphate buffer and centrifuged at 100000*g* for 60 min. The second microsomal pellet was washed with 100 mM KCl/5 mM (pH 7.5) potassium phosphate buffer containing 1 mM EDTA, recentrifuged, and then stored under argon at −70 °C.

Partial Purification of Placental P-450.[14] Placenta microsomes prepared on the same day were resuspended at approximately 15 mg of protein/mL in a 0.16 M sucrose/0.12 M (pH 7.6) potassium phosphate buffer containing 25% glycerol, 2 mM EDTA, and 1 mM dithiothreitol. For each 1.0 mL of suspension was added 0.16 mL of a 10% sodium cholate solution containing 1 mM ascorbate. This suspension, on ice and under argon, was stirred by means of a magnetic stirrer for approximately 30 min and then centrifuged at 22000*g* for 30 min at 2 °C. The supernatant was carefully poured into a prechilled graduated cylinder to measure the volume of the solubilized preparation. With stirring on ice, 240 mg/mL NH_4SO_4 was slowly added to produce a 42% saturation. The pH was maintained at 7.7 by

(25) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.

adding a few drops of 2 N NH_4OH. After stirring on ice and under argon for 20 minutes, the suspension was centrifuged at 20000*g* at 2 °C for 20 min. To the supernatant, 70 mg/mL NH_4SO_4 was added to bring the solution to a 50% saturation while the pH was maintained at 7.7. After stirring on ice and under argon for 20 min, the suspension was centrifuged at 20000*g* for 20 min at 2 °C. The supernatant fraction was carefully removed as completely as possible and the precipitate was redissolved in 10 mM (pH 7.7) potassium phosphate buffer containing 20% glycerol, 0.5 mM EDTA, 0.1 mM dithiothreitol, and 0.05% sodium cholate. Aliquots of 2.0 mL (approximately 5 mg of protein/mL) were stored frozen at −70 °C. An average 2.5-fold purification was obtained. The P-450 content after purification ranged between 0.058 and 0.087 nmol of P-450/mg of protein. This degree of purification was sufficient to allow determinations of binding constants which were unobtainable with the crude microsomal fraction.

Spectral Analyses. Difference spectra were performed in the split-beam mode with a Model DW-2 recording spectrophotometer (Aminco) with a 3.0-nm spectral band width and 1.0 cm path length cuvettes. Partially purified human placental P-450 was diluted to a P-450 concentration of 0.13 nmol/mL in 10 mM (pH 7.7) potassium phosphate buffer containing 20% glycerol, 0.5 mM EDTA, 0.1 mM freshly added dithiothreitol, and 0.05% sodium cholate. Both sample and reference cuvettes contained 1.0 mL of the above P-450 solution previously equilibrated to ambient temperature (23 °C). Steroids were dissolved in propylene glycol to a concentration of 5.0 mM and then serially diluted in propylene glycol to concentrations 1000 times their desired cuvette concentrations. Additions of the steroids to the sample cuvette were made 1.0 μL at a time, with each subsequent addition being added to the total concentration of steroid already in the cuvette. Parallel 1.0-μL additions of propylene glycol were made to the reference cuvette. Each addition was followed by mixing of the cuvette contents by means of a Pasteur pipet. Up to ten 1.0-μL additions were made for each steroid, with steroid concentrations typically ranging between 20 nM and 6.0 μM. Maximal spectral changes (A_{max}) and spectral dissociation constants (K_s) were determined by utilizing ΔA/concentration versus ΔA (Scatchard) plots.

K_m Determination. Microsomal pellets were resuspended in phosphate buffer to 2 mg of protein/mL with and without 0.36 mM NADPH. The microsomes were warmed to 30 °C for 5 min and 400-μL aliquots, in duplicate, were added to 100 μL of phosphate buffer containing 20, 10, 5, 0.5, 0.25, and 0.05 nmol of [1β,2β-^{3}H]androstenedione. The tritium release assay was conducted for 10 min at 30 °C, after which time the incubations were stopped by the addition of 20% acetone/chloroform, vortexed at high speeds for 30 s, and centrifuged, and an aliquot (100 μL) of the aqueous layer was removed and mixed with 5 mL of LSC cocktail prior to LS counting.

The K_m and V_{max} values were determined by subtracting the DPMs from NADPH minus incubations from their respective NADPH positive incubations followed by the least-squares analysis of the double-reciprocals of background corrected DPMs versus androstenedione concentrations.

Time-Dependent/Concentration-Dependent Inhibition Experiments. Incubations contained either 0.5 or 1 mg/mL of resuspended microsomal protein and 0.5 mM NADPH in phosphate buffer. Aliquots of microsomal suspensions were warmed to 30 °C for 5 min, and inhibitors were added at 10 μL from ethanolic stock solutions, which were prepared at 100 times their desired incubation concentrations. Typically, between four and six serially diluted concentrations of inhibitor were used. Ethanol (10 μL/mL) was added as a control. Incubation contents were quickly mixed and after incubation for periods of either 0, 1, 2, or 3 min with compounds **1–3** and **6** or 0, 2.5, 5.0, 7.5, and 10.0 min with compounds **4** and **5**, at 30 °C, 400-μL aliquots, in duplicate, were removed and added to 100 μL of a 10 mM (pH 7.5) potassium phosphate buffer containing 100 mM KCl, 1 mM EDTA, and 20 nmol [1β,2β-^{3}H]androstenedione (20 nCi/nmol). Tritium-release incubations were conducted at 37 °C for 10 min and stopped by the addition of 5 mL of 20% acetone/chloroform. The incubations were then vortexed at high speeds for 30 s and centrifuged. An aliquot (100 μL) of the water layer was removed and mixed with 5 mL of LSC cocktail and counted.

Background radioactivity was determined in incubations lacking NADPH. Background radioactivity was typically less than 15% of that seen in incubations which had been carried out with 0.5 mg of microsomal protein/mL at 30 °C for 10 min that contained NADPH but lacked inhibitors.

The percent of aromatase activity remaining was determined by dividing the activity (DPM) in the presence of inhibitor after an incubation time interval by the activity (DPM) in the presence of inhibitor at the beginning of the preincubation, and multiplying by 100. Thus, a zero minute incubation at an inhibitor concentration [I] was arbitrarily made equal to 100%.

Values of k_{obs} were obtained by plotting the ln of the percent activity remaining versus time for each concentration of inhibitor. The least-squares slope of the line obtained in the absence of inhibitor was then subtracted from each of the least-squares slopes of lines obtained in the presence of inhibitors to give the background corrected k_{obs} values. Least-squares analysis of the double-reciprocal plots of corrected k_{obs} versus inhibitor concentrations allowed the K_I and k_{cat} values to be determined.[16]

Dithiothreitol Reactivation Experiments. Incubations containing resuspended 1.0 mg/mL microsomal protein, 0.35 mM NADPH, 11.5 mM glucose 6-phosphate, and 2 units/mL glucose-6-phosphate dehydrogenase in phosphate buffer were warmed at 30 °C for 4 min. At this time 10 μL of 0.10 mM ethanolic solutions **2–6** were added to each milliliter of incubation to bring the inhibitor concentrations to 1.0 μM. Compounds **2**, **3**, and **6** were incubated for 1 min and compounds **4** and **5** for 1 and 11 min, respectively, at 30 °C. Then 400-μL aliquots, in triplicate, were removed and added either to 20 nmol of [1β,2β-^{3}H]-androstenedione (32 nCi/nmol) in 100 μL of phosphate buffer or to 20 nmol of [1β,2β-^{3}H]androstenedione in 100 μL of a 50 mM dithiothreitol/phosphate buffer solution. Tritium-release assays were performed as previously described.

Paraoxon Protection Experiments. Incubations containing 1.0 mg/mL resuspended microsomal protein, 0.35 mM NADPH, 11.5 mM glucose 6-phosphate, and 2 units/mL glucose-6-phosphate dehydrogenase in phosphate buffer were warmed at 30 °C for 4 min, with or without 25 mM paraoxon present. After addition of either 10 μL of ethanol, 10 μL of a 50 μM ethanolic solution of **7**, or 10 μL of a 200 μM ethanolic solution of **8** to each milliliter of incubation, microsomes were incubated at 30 °C for an additional 0, 5, 10, 15, 20, 25, 30, and 35 min. Controls in which 500 nM 19-SHA (**2**) and paraoxon were incubated together for 0, 1, 2, 3, 4, and 5 min were also carried out. After these time periods, 400 μL, in duplicate, was removed and added to 100 μL of phosphate buffer containing 20 nmol of [1β,2β-^{3}H]-androstenedione (20 nCi/nmol). Tritium-release assays were carried out as previously described.

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Registry No. **2**, 90212-02-5; **3**, 116168-66-2; **4**, 117626-50-3; **5**, 77016-85-4; **6**, 566-48-3; **7**, 90212-29-6; **9**, 3962-66-1; **10**, 2220-74-8; **11**, 116168-68-4; **12**, 102490-33-5; **13**, 116168-69-5; **14**, 116168-70-8; **15**, 63-05-8; **16a**, 17503-11-6; **16b**, 7430-11-7; **17a**, 571-17-5; **17b**, 571-16-4; **18** (isomer 1), 117626-51-4; **18** (isomer 2), 117626-52-5; **19** (isomer 1), 117626-53-6; **19** (isomer 2), 117626-54-7; **20**, 117678-49-6; **21**, 117626-55-8; **22**, 117626-56-9; propynyllithium, 17689-04-2; 1-(trimethylsilyl)propyne, 13361-64-3; aromatase, 9039-48-9.

Supplementary Material Available: Absorbance difference spectra of the thiosteroids with human placental aromatase (3 pages). Ordering information is given on any current masthead page.