Thiol-Containing Androgens as Suicide Substrates of Aromatase

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The thiol-containing androgens 17β-hydroxy-10β-mercaptoestr-4-en-3-one (1) and 19-mercaptoandrost-4-ene-3,17-dione (2) were synthesized and tested in human placental microsomes for their ability to suicide inhibit aromatase. Both compounds showed time-dependent, pseudo-first-order rates of inactivation of aromatase with K_i's of 106 and 34 nM and k_{cat}'s of 3.2×10^{-3} and 1.2×10^{-3} s^{-1} respectively for 1 and 2 at 30 °C. Diffusion dialysis failed to reactivate aromatase previously inactivated by either compound, and both compounds required that NADPH and O_2 be present for the time-dependent inactivation of the enzyme. The presence of the substrate, androst-4-ene-3,17-dione (5.0 μM), protected the enzyme from inactivation while cysteine (1.0 mM) failed to protect aromatase from inactivation by either compound. The above evidence demonstrates that both compounds are potent suicide inhibitors of aromatase.

The observation that approximately 35% of breast cancers are estrogen dependent has stimulated research into methods of limiting estrogen production. Initial efforts were to surgically remove the primary sources of estrogen (ovaries) or the primary sources of their biosynthetic steroid precursors, the androgens (adrenal glands). While these methods are often successful, work has been directed in recent years toward a more effective inhibition of estrogen production in a nonintrusive, less traumatic way.[1] With knowledge of the enzymes involved in the biosynthesis of estrogens from cholesterol, inhibition of the last enzymatic step, the aromatization of the Δ^4, 3-keto androgens to the phenolic estrogens, would seem the most effective and least disruptive process to inhibit. The enzyme responsible for this process is a unique cytochrome P-450 monooxygenase complex known as aromatase.[2]

Work with human placental aromatase has shown the enzyme or enzymes to require 3 equiv of oxygen and 6 reducing equiv in the form of NADPH.[3] Fishman[4] has proposed a mechanism of aromatase that involves three sequential hydroxylations of the androgen precursor followed by a spontaneous chemical aromatization of the A ring to yield the phenolic estrogens (Scheme I). With an understanding of this mechanism, we, as well as others,[5] have developed suicide substrates to aromatase.

On the basis of previous work in which thiol steroids were shown to act as suicide substrates of P-450 steroid hydroxylase activity,[6] we have replaced the 19-methyl group in the androgen nucleus with either a thiol or a methylenethiol group. The normal hydroxylase activity of aromatase at the 19-position might be expected to produce an electrophilic group that could react in a covalent fashion with sites in the active site to irreversibly inhibit the enzyme. There is evidence that aromatase can oxidize a sulfur placed in the 10β-position of an androgen nucleus; oxidation of 17β-hydroxy-10β-(methylthio)estra-1,4-dien-3-one to the sulfenyl ester was proposed as part of a mechanism to explain why this compound is a suicide substrate of aromatase.[5c] However, inhibitor activity appears to be sensitive to minor structural changes in the 10β- and 19-position of the androgen nucleus,[5d,7] (i.e., 10β-aminoandrost-4-ene-3,17-dione is a poor reversible inhibitor of aromatase).

Chemistry. The synthesis of 17β-hydroxy-10β-mercaptoestr-4-en-3-one (1) was a modification of previous work[8] (Scheme II). The known compound **3a**[8a] was converted into the 3-ethylenedioxy 17β-acetate **5**. Reaction with *N*-bromosuccinimide yielded the protected 5β,10α-bromohydrin. Under basic conditions, the bromohydrin grouping in **6** was closed to give the 5α,10α-epoxide **7**. Introduction of the thiol group into position 10 was accomplished with sodium hydrogen sulfide in ethylene glycol at 140 °C to yield the 5α-hydroxy-10β-mercapto steroid **8**. Deprotection of the 3-ketone under acidic con-

Scheme I. Proposed Mechanism of Aromatase-Catalyzed Aromatization of Androgens to Estrogens

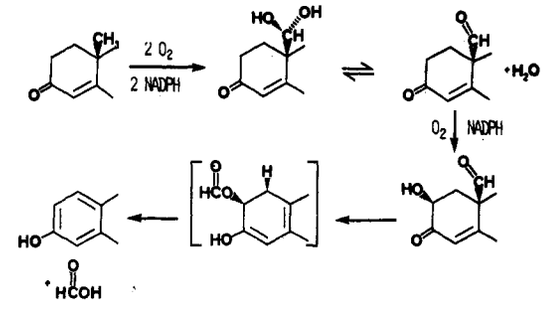

Table I. NADPH and Oxygen Dependency Experiments for 1 and 2 (500 nM)

inhibitor and conditions	% act. remaining[a-c] (mean ± SD)	inhibitor and conditions	% act. remaining[a-c] (mean ± SD)
1	67 ± 2	2-NADPH	94 ± 5
2	77 ± 4	1 + N_2 atmosphere	95 ± 2
1-NADPH	96 ± 3	2 + N_2 atmosphere	95 ± 5

[a] Preincubations with inhibitors were done for 3 min at 30 °C. [b] Values are not corrected for background loss of enzymatic activity since background losses under all three conditions were approximately 5%. Background loss is the amount of enzymatic activity lost with no inhibitor present. [c] Assays were done in triplicate, $N = 9$.

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(1) (a) Abul-Hajj, Y. J. *J. Steroid. Biochem.* **1980**, *13*, 1395. (b) Brodie, A. M. H. *Cancer Res.* **1982**, *42*, 3312s. (c) Brodie, A. M. H.; Brodie, H. J.; Garrett, W. H.; Hendrickson, J. R.; Marsh, D. A.; Tsai-Morris, Chon-Hwa, *Biochem. Pharmacol.* **1982**, *31*, 2017. (d) Powles, T. J. *Semin. Oncology* **1983**, *10*, suppl. 4, 20.
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(4) Fishman, J. *Cancer Res.* **1982**, *42*, 3277s.
(5) (a) Covey, D. F.; Hood, W. F.; Parikh, V. D. *J. Biol. Chem.* **1981**, *256*, 1076. (b) Metcalf, B. W.; Wright, C. L.; Burkhart, J. P.; Johnston, J. O. *J. Am. Chem. Soc.* **1981**, *103*, 3221. (c) Flynn, G. A.; Johnston, J. O.; Wright, C. L.; Metcalf, B. W. *Biochem. Biophys. Res. Commun.* **1981**, *103*, 913. (d) Marcotte, P. A.; Robinson, C. H. *Biochemistry* **1982**, *21*, 2773.
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(8) (a) Baba, S.; Shinohara, Y.; Kasuya, Y. *J. Labelled Compd Radiopharm.* **1978**, *14*, 783. (b) Ponsald, Von K.; Schade, W.; Wanderwold, J. *Prakt. Chem.* **1975**, *317*, 307, 319.

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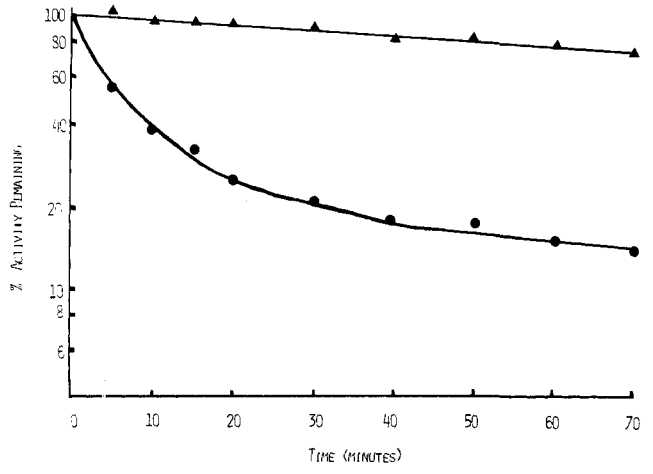


Figure 1. Time-dependent, biphasic inactivation of aromatase by thiol steroids: no inhibitor (▲), 100 nM (●) of 1.

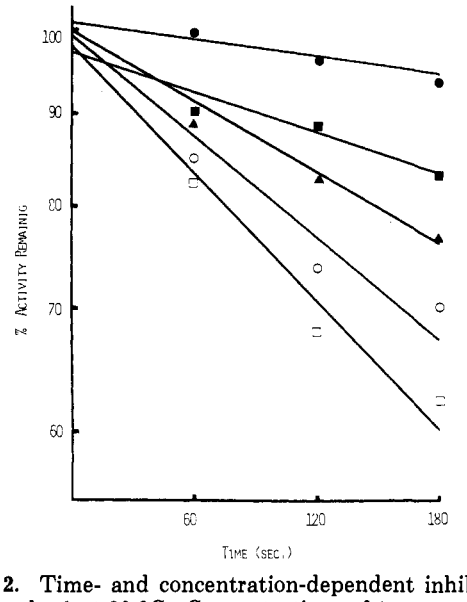


Figure 2. Time- and concentration-dependent inhibition of aromatase by 1 at 30 °C. Concentrations of 1 were as follows: 0 (●), 25 nM (■), 50 nM (▲), 100 nM (○), and 500 nM (□). Each point is the average of two tritium-release incubations. Lines were determined by the least-squares method.

ditions gave 9 and dehydration under basic conditions gave the desired product 1.

The synthesis of the novel 19-mercaptoandrost-4-ene-3,17-dione (2) is described (Scheme III). The hydroxyl group of 19-hydroxyandrosten-4-ene-3,17-dione (10) was activated to the 19-triflate 11 with use of trifluoromethanesulfonic anhydride.[9] Attempts to displace the triflyl group directly using sodium hydrogen sulfide or potassium hydrogen sulfide in the presence of the crown ether 18-crown-6 were unsuccessful. The reaction of 11 with potassium ethylxanthogenate and 18-crown-6 successfully introduced the xanthogenate functionality into the 19-position in good yields. Cleavage of the 19-xanthogenate ester 12 to the 19-thiol 2 was unsuccessful when reacted in pure ethylenediamine;[10] however, when ethylenediamine was diluted in THF, acceptable yields of 2 were obtained.

Biology. Compounds 1 and 2 were tested for their ability to act as suicide substrates of aromatase in human placental microsomes.[5a] Apparent aromatase activity was determined by using a well-established tritium-release assay.[3]

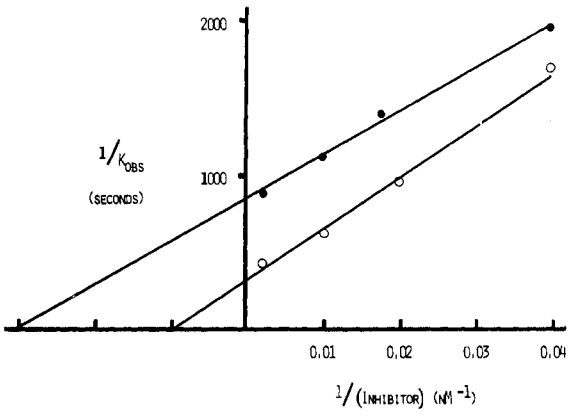


Figure 3. Double-reciprocal plots of the observed pseudo-first-order rate constants of inactivation (k_{obsd}) with respect to inhibitor concentration for 1 (○) and 2 (●). Values of apparent K_i and k_{cat} were 106 nM and 3.2×10^{-3} s^{-1} for 1, respectively, and 34 nM and 1.2×10^{-3} s^{-1} for 2, respectively (k_{obsd} values are corrected for background loss). Lines were determined by the least-squares method.

Table II. Protection Studies of either Androstenedione (5.0 μM) or Cysteine (1.0 mM) for Mercapto Steroids 1 (500 nM) and 2 (167 nM)

inhibitor and conditions	$T_{1/2}$,[a,b] s (mean ± SD)	inhibitor and conditions	$T_{1/2}$,[a,b] s (mean ± SD)
1	281 ± 50	2 and cysteine	412 ± 20
2	462 ± 75	1 and androstenedione	756 ± 50
1 and cysteine	310 ± 58	2 and androstenedione	729 ± 21

[a] $T_{1/2}$ values represent the time it would take for 1 and 2 to bring about a 50% inhibition of aromatase. [b] $T_{1/2}$ values are not correct for background loss of enzymatic activity since background losses were the same under all three conditions.

Table III. Irreversibility Experiments for 1 and 2 (250 nM)

inhibitor	% act. remaining[a,b] (mean ± SD) before dialysis	after dialysis
1	49 ± 6	52 ± 3
2	50 ± 5	58 ± 2

[a] Preincubations with inhibitors were done for 5 min at 30 °C. [b] Assays were done in duplicate; therefore mean and standard deviation values were calculated with $N = 4$.

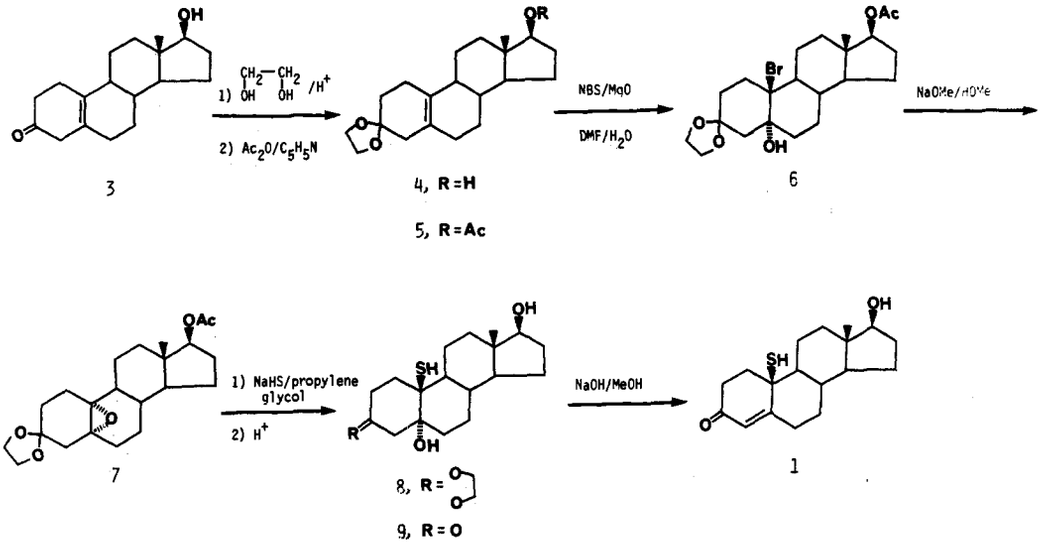
Results

Both compounds gave a time-dependent, biphasic loss of enzymatic activity of 70 min (Figure 1). This loss in activity requires both NADPH and oxygen (Table I). The kinetic parameters of the inhibitors K_i and k_{cat} were determined. With use of increasing concentrations of inhibitors, increasing k_{obsd} values were obtained for both compounds (Figure 2). Double-reciprocal plots of k_{obsd} vs. inhibitor concentration yielded K_i's of 106 and 34 nM and k_{cat}'s of 3.2×10^{-3} and 1.2×10^{-3} s^{-1} for 1 and 2, respectively (Figure 3).

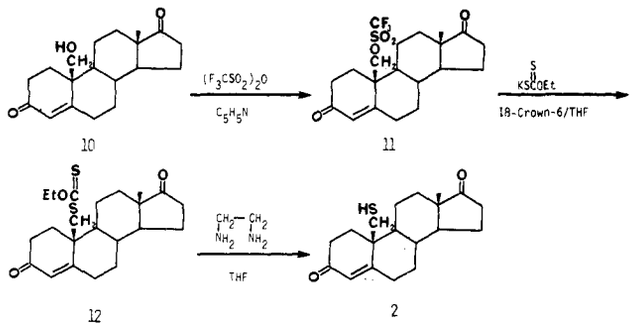
Incubation of inhibitors in the presence of the substrate androstenedione showed an increase in the $t_{1/2}$ of inactivation compared to incubations conducted in the presence of inhibitor alone (Table II). Incubations of the inhibitors in the presence of the nucleophile cysteine had no effect on the $t_{1/2}$ of inactivation compared to incubations conducted in the presence of inhibitor only (Table II). The covalent nature of the inhibition was indicated by using

(9) Beard, C. D.; Baum, K.; Grakanskas, V. *J. Org. Chem.* **1973**, *38*, 3673.
(10) Beretta, E.; Cinquini, M.; Colonna, S.; Formasier, R. *Synthesis* **1974**, 425.

Scheme II. Synthesis of 17β-Hydroxy-10β-mercaptoestr-4-en-3-one (1)



Scheme III. Synthesis of 19-Mercaptoandrost-4-ene-3,17-dione (2)



diffusion dialysis of the inactivated enzyme. The percent activity remaining after dialyzing for 20 h at 0 °C was essentially the same as the activity remaining in the incubations before dialysis (Table III).

Discussion and Conclusions

The cytochrome P-450 monooxygenase aromatase is responsible for the conversion of 3-keto, 4-ene androgens to the phenolic estrogens. Selective and irreversible inhibition of this enzyme can be achieved through the use of suicide substrates.[11]

Five criteria must be met before a compound can be classified as a suicide substrate:[12] (1) exhibits a time-dependent, pseudo-first-order rate of enzymatic inactivation, (2) cofactors that are required for the normal catalytic processes are also required for inactivation, (3) reversible substrates protect the enzyme from time-dependent inactivation, (4) nucleophiles present in the incubate fail to protect from inactivation, and (5) the inhibition is irreversible.

Both compounds exhibit a time-dependent, biphasic loss of enzymatic activity (Figure 1). This biphasic loss is most probably a result of the metabolic dependent elimination of the inhibitors from the incubate since both compounds were stable in phosphate buffer at 30 °C for up to 4 h (data not shown). The observation that the second, linear phase of the biexponential loss is parallel to the normal loss of enzymatic activity adds support to this. Since the slopes of the rates of inactivation are linear only at the initial time points (i.e., 5 min), these earlier times were used to obtain k_{obsd} values of varying concentrations of inhibitor (Figure 2). Double-reciprocal plots of k_{obsd} vs. inhibitor concentration yielded K_i values of 106 and 34 nM and k_{cat}'s of 3.2×10^{-3} and 1.2×10^{-3} s^{-1} for **1** and **2**, respectively (Figure 3).

The conversion of androgens to estrogens by aromatase requires 6 reducing equiv in the form of NADPH. Activation of the thiol androgens would also be expected to require NADPH. Incubations of both inhibitors in the absence of NADPH failed to show time-dependent inactivation of aromatase (Table I). At least 1 equiv of oxygen would be required for the time-dependent inactivation of aromatase by compounds **1** and **2**. Under anaerobic conditions, a complete protection from inactivation by these two inhibitors was achieved (Table I).

Activation of a suicide substrate to its reactive intermediate occurs at the same enzymatic site responsible for catalysis of the normal substrate. Since the suicide substrates **1** and **2** are competing for the same site as the normal substrates of aromatase (i.e., androstenedione), the presence of androstenedione slows the rate of loss of enzymatic activity caused by these two inhibitors (Table II). Suicide inhibition requires that once the suicide substrate is activated, it immediately binds in a covalent fashion at the active site without first diffusing into the incubation medium. If the activated intermediate diffuses out of the active site before it inactivates the enzyme, nucleophiles present in the incubation could react with the electrophilic intermediate and slow the time-dependent loss of activity. For the suicide substrates **1** and **2**, the presence of the nucleophile cysteine in the incubation did not protect aromatase from time-dependent loss of activity (Table II). This indicates that whatever species is formed probably reacts at the enzyme active site before reversible diffusion out of the site. Finally, attempts to reactivate the suicide substrate inactivated enzyme by using diffusion dialysis were unsuccessful, indicating the covalent nature of the inhibitor–enzyme complex (Table III).

Although we do not yet know the mechanism of inhibition by **1** and **2**, requirement of both NADPH and oxygen for inhibition implicate an oxidized species as the reactive intermediate. Oxidation of thiol groups in positions of the androgen nucleus that are normally hydroxylated by aromatase (Scheme I) might be expected to form sulfenic

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acids.[13] These potent electrophiles could bind in a covalent manner with nucleophiles in the enzymatic active site and irreversibly inhibit the enzyme. The specificity of these thiol androgens for aromatase has also not yet been determined. However, Johnston et al.[15] have shown that the suicide substrate 10β-propargylestr-4-ene-3,17-dione is a highly specific inhibitor of aromatase when compared with other steroid metabolizing enzymes.

In conclusion, we have synthesized 17β-hydroxy-10β-mercaptoestr-4-en-3-one (1) and 19-mercaptoandrost-4-ene-3,17-dione (2) and have shown them to be potent suicide substrates to human placental aromatase. Additional experiments are under way to further elucidate the mechanism of suicide inactivation by compounds 1 and 2.

Experimental Section

Estradiol 3-methyl ether and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO). 19-Hydroxyandrostenedione was obtained from the Aldrich Chemical Co. (Milwaukee, WI). NaSH was obtained through Alfa Inorganics (Danvers, MA). Potassium ethylxanthogenate was obtained from Pfaltz and Bauer (Stamford, CT). Androstenedione was obtained through Steraloids (Wilton, NH). 1β,2β-[^{3}H]androstenedione (40–60 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Varian EM 360A spectrometer using Me_4Si as an internal standard. IR were recorded on a Perkin-Elmer 283 instrument and optical rotations were recorded on a Jasco DIP-4 instrument. Electron-impact mass spectra were obtained on a Hewlett Packard 2985 GC/MS instrument using direct insertion. Microanalysis were performed by Galbraith Laboratories (Knoxville, TN). Liquid scintillation counting of radioactive samples was performed on a Beckman LS 7500 instrument. Maxfluor was used as the LSC cocktail and was obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ).

17β-Acetoxyestr-5(10)-en-3-one Ethylene Acetal (5). To a mixture of 150 mL of dry benzene and 16 mL of ethylene glycol were added 17β-hydroxyestr-5(10)-en-3-one (3;[8a] 2.80 g, 10.2 mmol) and *p*-toluenesulfonic acid (0.16 g, 0.93 mmol). The reaction mixture was refluxed for 19 h with continuous removal of the water that was produced by means of a Dean–Stark trap. After cooling, the benzene was washed with 5% $NaHCO_3$ and twice with water, dried over $MgSO_4$, and concentrated under reduced pressure to give 3.31 g of a white solid; ^{1}H NMR ($CDCl_3$) δ 3.70 (m, 1 H), 3.95 (d, 4 H); *IR (Nujol)* 3420 (br), 1670 cm^{-1}.

The crude 17β-hydroxyestr-5(10)-en-3-one ethylene acetal 4 (3.31 g, 10.4 mmol) was dissolved in 10 mL of dry pyridine and acetic anhydride (5.0 mL, 50 mmol) and the solution was allowed to stand 4 h at room temperature. To this were added 50 mL of $CHCl_3$ and 30 mL of water. The organic phase was separated and washed twice with water, twice with ice-cold 3% H_2SO_4, once with 5% $NaHCO_3$, and finally once with water. The organic phase was dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give 3.48 g of a white solid; TLC (CH_2Cl_2) R_f 0.67; ^{1}H NMR ($CDCl_3$) δ 2.05 (s), 3.95 (d, 4 H), 4.68 (to br t, 1 H); IR (Nujol) 1735, 1670, 1240, 1105, 1085, 1055, 1040, 945 cm^{-1}.

17β-Acetoxy-5α,10α-epoxyestran-3-one Ethylene Acetal (7). To a stirred solution of crude 17β-acetoxyestr-5(10)-en-3-one ethylene acetal (5; 3.48 g, 9.7 mmol) in 45 mL of DMF and 12 mL of water were added *N*-bromosuccinimide (3.45 g, 19.4 mmol) and MgO (0.39 g, 9.7 mmol). After the mixture was stirred for 2 h, 100 mL of water was added and the resulting precipitate was collected by suction filtration. The precipitate was washed with water and dissolved in CH_2Cl_2, and the organic phase was washed three times with water, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give 3.59 g of a white, crystalline solid; TLC (CH_2Cl_2) R_f 0.56. Crude 6 (3.59 g, 7.86 mmol) was stirred in 50 mL of MeOH under N_2 at 6 °C and 7 mL of a 21% NaOMe/MeOH solution was added dropwise. The reaction was allowed to stir for 8 h at 6 °C. Following the addition of 120 mL of ice-cold water, the reaction mixture was extracted four times with CH_2Cl_2. The organic phases were combined and washed with water, dried over $MgSO_4$, and concentrated under reduced pressure to afford 2.88 g of a yellow oil. Column chromatography on Florisil using 5% ethyl acetate/$CHCl_3$ as eluant yielded 1.34 g (35% from 3) of a white solid; TLC (5% ethyl acetate/CH_2Cl_2) R_f 0.80; ^{1}H NMR ($CDCl_3$) δ 2.05 (s), 3.90 (s, 4 H), 4.65 (m, 1 H); MS, *m/e 376* (42, M^+), 359 (33), 358 (33), 317 (16), *316* (31), 274 (22), 256 (33), 99 (100).

5α,17β-Dihydroxy-10β-mercaptoestran-3-one Ethylene Acetal (8). Sodium hydrogen sulfide (2.59 g, 46.2 mmol) was added to a suspension of 17β-acetoxy-5α,10α-epoxyestran-3-one ethylene acetal (7; 1.74 g, 4.62 mmol) in 50 mL of ethylene glycol and the reaction was placed under a nitrogen atmosphere. The reaction was warmed to 140 °C for 2 h, cooled, and poured into 200 mL of a 5% Na_2SO_4 solution. This was extracted four times with $CHCl_3$, and the organic phases were combined and washed two times with water, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give 1.49 g of a light tan solid. Column chromatography on Florisil using 50% ethyl ether/cyclohexane as eluant gave 0.60 g (35%) of 8. A recrystallized sample from cyclohexane gave a white, amorphous solid, mp 180–184 °C (lit.[8b] mp 183–186 °C, acetone); ^{1}H NMR ($CDCl_3$) δ 3.70 (m, 1 H), 3.95 (s, 4 H), 4.45 (s, exch); MS, *m/e* 368 (5 M^+), 350 (80), 335 (16), 317 (100), 316 (27), 99 (46); IR (Nujol) 3400 (br), 1115, 1070, 1010, 830 cm^{-1}.

5α,17β-Dihydroxy-10β-mercaptoestran-3-one (9). 5α,17β-Dihydroxy-10β-mercaptoestran-3-one ethylene acetal (8, 0.60 g, 1.6 mmol) and *p*-toluenesulfonic acid (0.20 g, 1.0 mmol) were dissolved in 100 mL of acetone and the solution was stirred overnight under a nitrogen atmosphere. After addition of 75 mL of a 5% $NaHCO_3$ solution, the acetone was removed on the rotary evaporator, and 75 mL of water was added. The aqueous phase was extracted four times with $CHCl_3$, and the organic phases were combined and washed with water, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give 0.65g of a tan solid. Column chromatography on Florisil using 50:50 ethyl ether/chloroform as eluant gave 0.40 g (77%) of 9. A recrystallized sample from benzene gave clear crystals, mp 194–200 °C (lit.[8b] mp 195–205 °C); positive Ellman's test for thiols; ^{1}H NMR ($CDCl_3$) δ 3.70 (m).

17β-Hydroxy-10β-mercaptoestr-4-en-3-one (1). 5α,17β-Dihydroxy-10β-mercaptoestran-3-one (9; 0.38 g, 1.2 mmol) in 25 mL of a 0.1 M NaOH/MeOH solution was refluxed under argon for 1 h. After the mixture was cooled in ice, 5.0 mL of a 1.0 M HCl solution was added and the reaction mixture was concentrated on the rotary evaporator. Water (50 mL) was added and the mixture was extracted three times with $CHCl_3$, and the organic phases were combined, washed with 5% $NaHCO_3$ and water, dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give 0.36 g of an organic solid. Column chromatography on silica gel 60 (0.063–0.200 mm) and 50% ethyl ether/chloroform afforded 0.18 g of 1, which was recrystallized from acetone to give 126 mg (34%) of clear crystals, mp 175–178 °C (lit.[8b] mp 178–182 °C); $[\alpha]_D$ +279° (*c* 0.5 g/100 mL, chloroform) (lit. $[\alpha]_D$ +268°); UV (acetonitrile) λ_{max} 237 nm (ε 14 420 M^{-1} cm^{-1}) [lit.[8b] λ_{max} 241 nm (ε 13 800 M^{-1} cm^{-1})]; *IR (Nujol)* 3380 (br), 2500, 1650, 1605, 1350, 1315, 1275, 1220, 1155, 1130, 1050, 1020, 885, 820, 765 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 3.65 (t, 1 H), 5.65 (s, 1 H); MS, *m/z* 306 (M^+, 13) 273 (100), 272 (62), 255 (20), 213 (40). Anal. ($C_{18}H_{20}O_2S$) C, H, O, S.

19-(Ethylxanthogenyl)androst-4-ene-3,17-dione (12). 19-Hydroxyandrost-4-ene-3,17-dione (10; 0.58 g, 1.92 mmol) in 8 mL of cold pyridine was added dropwise to a stirred solution of trifluoromethanesulfonic anhydride (0.60 mL, 1.0 g, 3.57 mmol) in 5 mL of cold pyridine under nitrogen. The reaction was allowed to warm to room temperature and after 1 h 100 mL of cold CH_2Cl_2 was added. The reaction mixture was washed three times with a cold 1 M H_2SO_4 solution and then washed with cold 5% $NaHCO_3$ and cold water, dried over $MgSO_4$, filtered, and concentrated on the rotary evaporator to give 0.71 g of 11 as a light orange solid;

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(15) Johnston, J. O.; Wright, C. L.; Metcalf, B. W. *Endocrinology* **1984**, *115*, 776.

IR (Nujol) 1735, 1160, 1615, 1405, 1240, 1215, 1195, 1140, 935, 825 cm^{-1}; NMR ($CDCl_3$) δ 4.84 (d of d, 2 H), 6.10 (s, 1 H); MS, m/z 434 (M^+, 79), 283 (100).

A mixture of crude 19-triflylandrost-4-ene-3,17-dione (**11**; 0.71 g, 1.6 mmol), potassium ethylxanthogenate (0.51 g, 3.2 mmol), and 18-crown-6 (42 mg, 0.16 mmol) in 20 mL of dry THF was allowed to stir at room temperature for 20 h under N_2. To the reaction mixture was added 100 mL of CH_2Cl_2 and the mixture was washed three times with a saturated solution of KCl and twice with water, dried over $MgSO_4$, filtered, and concentrated on the rotary evaporator to give 0.71 g of an orange solid. Column chromatography on silica gel 60 (60–200 mesh) using 1:3 ethyl ether/chloroform as eluant gave 0.57 g (76% from **10**) of **12** as a colorless oil; IR (neat) 2935, 1735, 1670, 1615, 1450, 1410, 1370, 1215, 1145, 1110, 1045, 870, 750 cm^{-1}; NMR ($CDCl_3$) δ 1.38 (t, J = 7 Hz), 3.65 (d of d, 2 H), 4.62 (q, 2 H, J = 7 Hz) 5.85 (s, 1 H).

19-Mercaptoandrost-4-ene-3,17-dione (2). Ethylenediamine (0.42 g, 0.42 mL, 7 mmol) was added under nitrogen to a stirred solution of 19-(ethylxanthogenyl)androst-4-ene-3,17-dione (**12**; 0.57 g, 1.40 mmol) in 10 mL of dry THF. After 30 min at room temperature, 10 mL of a cold 1.0 M H_2SO_4 solution was added and the reaction mixture extracted twice with 25 mL of ethyl ether. The organic phases were combined and washed three times with water, dried over $MgSO_4$, filtered, and concentrated on the rotary evaporator. Crude **2** (0.31 g) was recrystallized twice from methylcyclohexane to afford 160 mg (36%) of a white, amorphous solid, mp 130–133 °C; $[\alpha]_D$ +148° (c 0.5 g/100 mL, chloroform); UV (acetonitrile) λ_{max} 235 nm (ϵ 11960 M^{-1} cm^{-1}); IR (neat) 2950, 2845, 2520, 1735, 1660, 1615, 1450, 1370, 1355, 1250, 1225; NMR ($CDCl_3$) δ 5.95 (s, 1 H); MS, m/z 318 (M^+, 62), 285 (15), 272 (100), 271 (89), 253. Anal. ($C_{19}H_{26}O_2S$) C, H, O, S.

Biochemical Methods. Human placenta were obtained from the University Hospital and were used within a few hours of delivery. Placental microsomes were prepared as previously described.[5a] Protein concentration was determined by the method of Lowry.[14]

Time-Dependent Inactivation Experiments. All incubations were carried out in 10 mM phosphate buffer, pH 7.5, 100 mM KCl, 1 mM EDTA. Incubations contained 1.0 mg/mL microsomal protein, 32 mg/mL propylene glycol, 0.36 mM NADPH, 11.5 mM glucose 6-phosphate, 2 units/mL glucose-6-phosphate dehydrogenase, and 10 mM $MgCl_2$ and were allowed to warm to 30 °C for 5 min. Inhibitors **1** or **2** were added as 10 μL/mL from 10 μM stock solutions in ethanol to give final inhibitor concentrations of 100 nM. At various times, from 0 to 70 min, 400-μL aliquots in duplicate were removed and added to 100 μL of a 10 mM phosphate buffer, pH 7.5, 100 mM KCl, 1 mM EDTA containing 40 μM 1β,2β-[^{3}H]androstenedione (20 mCi/mmol). Tritium-release incubations were conducted at 37 °C for 10 min and stopped by addition of 5 mL of 20% acetone/chloroform. Following vortexing at high speeds for 30 s and centrifugation, 100 μL of the water layer was removed and mixed with 5 mL of LSC cocktail and counted. Percent activity remaining is the number of DPM's after a time period divided by the number of DPM's at time zero multiplied by 100.

Concentration-Dependent Inactivation Experiments. Incubations containing 1.0 mg/mL microsomal protein, 32 mg/mL propylene glycol, and 0.36 mM NADPH were warmed to 30 °C for 5 min. Various concentrations of inhibitors were added to the incubations in ethanol (10 μL/mL of incubate). Ethanol (10 μL/mL) was added as a control. After incubating 0, 1, 2, and 3 min at 30 °C, 400-μL aliquots, in duplicate, were removed and added to the tritium release assay, described above.

NADPH Dependency Experiments. Incubations containing 1.0 mg/mL microsomal protein, 32 mg/mL propylene glycol, with or without 0.36 mM NADPH, were warmed to 30 °C for 5 min. Inhibitors were added in ethanol (10 μL/mL of incubate) to give inhibitor concentrations of 500 nM. Controls contained only ethanol and NADPH. After incubating 0 and 3 min at 30 °C, 400-μL aliquots, in duplicate, from the NADPH-containing incubations were added to the tritium release assay. For incubations lacking NADPH, 400-μL aliquots were removed and added to 100 μL of phosphate buffer containing 1.4 mM NADPH and 40 μL of 1β,2β-[^{3}H]androstenedione (20 mCi/mmol). Tritium-release assays were conducted as described above.

Oxygen Dependency Experiments. Incubations containing 1.0 mg/mL microsomal protein, 32 mg/mL propylene glycol, and 500 nM inhibitor or ethanol (10 μL/mL) were prepared. Incubations run under anaerobic conditions were alternatively evacuated and purged with deoxygenated nitrogen 10 times at 0 °C. Both anaerobic and aerobic incubations were allowed to warm to 30 °C for 5 min and 10 μL/mL of a 1.4 mM NADPH solution was added via a syringe. After incubation for 0 and 3 min at 30 °C, triplicate aliquots of 400 μL were removed and added to tritium-release assays.

Androstenedione Protection Experiments. Incubations containing 1.0 mg/mL microsomal protein, 32 mg/mL propylene glycol, and 0.36 mM NADPH were warmed to 30 °C for 5 min. Addition of ethanolic solutions (10 μL/mL) of inhibitors with and without androstenedione gave final concentrations of 500 nM **1**, 167 nM **2**, with and without 5.0 μM androstenedione. After 0, 1, 2, and 3 min at 30 °C, 400-μL aliquots, in duplicate, were removed and added to the tritium-release assay.

Cysteine Protection Experiments. Incubations containing 1.0 mg/mL microsomal protein, 32 mg/mL propylene glycol, and 0.36 mM NADPH, with or without 1.0 mM cysteine, were warmed to 30 °C for 5 min. Addition of inhibitor solution (10 μL/mL) gave final concentrations of 500 nM **1** and 167 nm **2**. After incubation for 0, 1, 2, and 3 min, 400-μL aliquots, in duplicate, were removed and added to tritium-release assays.

Irreversibility Experiments. Incubations containing 1.8 mg/mL microsomal protein, 32 mg/mL propylene glycol, 0.7 mM NADPH, and 250 nM of inhibitor were kept at 4 °C. For zero-time points, 400-μL aliquots, in triplicate, were removed and added to tritium-release assays. Remaining microsomes were incubated at 30 °C for 5 min and cooled in ice to 4 °C and 400-μL aliquots, in triplicate, were added to tritium-release assays to determine remaining activity. Remaining microsomes were added to dialysis tubing (Spectrapore membrane tubing, 3500-dalton cutoff) and dialyzed against a 10 mM phosphate buffer, pH 7.5, 100 mM KCl, 1 mM EDTA, and 5% propylene glycol at 0 °C for 20 h. To serve as controls, incubations containing the same concentrations of inhibitor but no cofactors were carried through the same procedures.

After dialysis, 400-μL aliquots, in duplicate, were added to 100 μL of phosphate buffer containing 2.9 mM NADPH and 40 μM 1β,2β-[^{3}H]androstenedione (20 Ci/mol) and the mixture was incubated at 37 °C for 10 min. The usual workup for the tritium-release assay was followed. Protein content for all incubations fell approximately 25% after dialysis. Enzyme activity fell approximately 60% after dialysis.

Acknowledgment. This work was supported by the National Institutes of Health (Grant GM25418). We thank Drs. Mont Juchau and Moses Namkung for their helpful suggestions during the course of this work. The fine technical assistance of Jeanelle Jackman and preparation of the manuscript by Jay Craver and Arline Jensen are acknowledged.

Registry No. **1**, 51101-80-5; **2**, 90212-02-5; **3**, 1089-78-7; **4**, 15342-09-3; **5**, 18367-54-9; **6**, 28838-86-0; **7**, 24275-29-4; **8**, 51101-78-1; **9**, 51101-79-2; **10**, 510-64-5; **11**, 95936-28-0; **12**, 95936-29-1; ethylene glycol, 107-21-1; trifluoromethanesulfonic anhydride, 358-23-6; potassium ethylxanthogeneate, 140-89-6; aromatase, 9039-48-9.