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Biochemistry

Conversion of a 3-desoxysteroid to 3-desoxyestrogen by human placental aromatase

(mechanism/inhibition/peroxide/enolization/cytochrome P-450)

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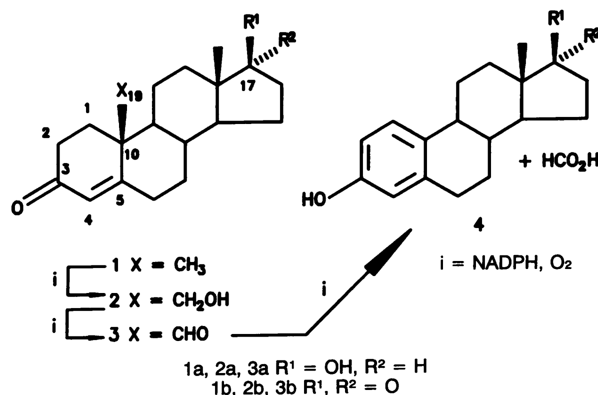
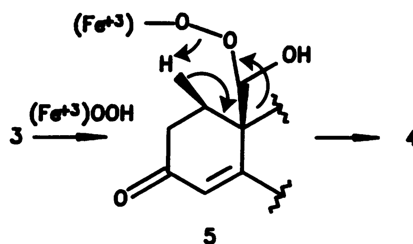
ABSTRACT **Human placental aromatase is a cytochrome P-450 enzyme system which converts androgens to estrogens by three successive oxidative reactions. The first two steps have been shown to be hydroxylations at the androgen 19-carbon, but the third step remains unknown. A leading theory for the third step involves ferric peroxide attack on the 19-oxo group to produce a 19,19-hydroxyferric peroxide intermediate and subsequent collapse to estrogen. We had previously developed a nonenzymatic peroxide model reaction which was based on the above-mentioned theory, and we demonstrated the importance of 3-ketone enolization in facilitating aromatization. This study discusses the synthesis and nonenzymatic and enzymatic study of a 3-desoxy-2,4-diene-19-oxo androgen analogue. This compound was found to be a potent nonenzymatic model substrate and competitive inhibitor of aromatase (K_i = 73 nM). Furthermore, in an unprecedented event, this compound served as a substrate for aromatase, with conversion to the corresponding 3-desoxyestrogen.**

Aromatase is a cytochrome P-450 complex which converts steroidal androgens [testosterone (**1a**), androstenedione (**1b**)] into steroidal estrogens [estradiol (**4a**), estrone (**4b**)]. Since the discovery of human placental aromatase in 1955 by Meyer (1, 2), there has been a sustained interest in elucidating its mechanism of action. The role of estrogens in promoting some forms of neoplasm, particularly breast cancer, is well documented (3–5). In fact, the estrogen receptor antagonist tamoxifen is used widely in the treatment of breast cancer. An alternative approach to blockade of estrogenic activities, which continues to receive attention, involves the lowering of estrogen levels by aromatase inhibition. Presumably the design of specific inhibitors could be improved by a greater understanding of aromatase mechanism. At a more fundamental level, the chemical novelty of the aromatase reaction has also attracted mechanistic enzymologists.

It has been confirmed recently that one cytochrome P-450 polypeptide, working in conjunction with a nonspecific NADPH-dependent reductase, carries out the complete aromatase transformation (6–7). Three separate steps are involved; the first two are sequential hydroxylations at carbon-19 of the substrate androgen to produce 19-hydroxy (**2**) and 19-oxo (**3**) intermediates, respectively. Each step apparently requires one equivalent each of O_2 and NADPH (8). The first two steps involve stereospecific hydroxylations (9–11); the resultant *gem*-diol is then thought to undergo a stereospecific dehydration event (12). In the third step, carbon-19 is eliminated as formic acid which contains oxygen atoms derived from the first and third equivalents of O_2 consumed as well as one of the original methyl hydrogens (13). The aromatization process involves stereospecific removal of the 1β-hydrogen (14–16), but the stereoselectivity of carbon-2 hydrogen loss is substrate dependent [Fronckowiak, M. D. & Osawa, Y., 70th Endocrine Society Meeting, June 8–11, 1988, New Orleans, p. 128 (abstr. 430); ref. 46]. Thus 19-oxoandrostenedione (**3b**) undergoes predominantly 2β-hydrogen loss, whereas there is approximately a 1:1 ratio of 2α-hydrogen to 2β-hydrogen loss from 19-oxotestosterone (**3a**). This has been interpreted as evidence for enzymic assistance in removal of the 2-hydrogen from each substrate.



Despite these and other findings, the precise nature of the third aromatase step has remained unsolved. Some of the theories demonstrated to be unlikely include 2β-hydroxylation (17), Baeyer–Villiger oxygen insertion (13), 4,5-epoxidation (18, 19), and 10β-hydroxyestr-4-ene-3,17-dione formation (20). One of the remaining viable hypotheses, proposed by Akhtar *et al.* (21), involves nucleophilic attack by ferric peroxide on the 19-aldehyde group to afford a 19,19-hydroxyferric peroxide intermediate (**5**).



This intermediate could then decompose by hydride shift (13), proton transfer (as shown) (22), or radical pathways (23) to yield estrogen and formic acid.

While it is currently not possible to test the theory of Akhtar *et al.* rigorously, we recently synthesized several 19-peroxide androstenedione analogues of the proposed intermediate and examined their nonenzymatic reactivity (24).

Abbreviations: THP, tetrahydropyranyl; SEM, 2-(trimethylsilyl)ethoxymethyl; TBDMS, *t*-butyldimethylsilyl; TMEDA, *N,N,N′,N′*-tetramethylethylenediamine; PPTS, pyridinium *p*-toluenesulfonate.

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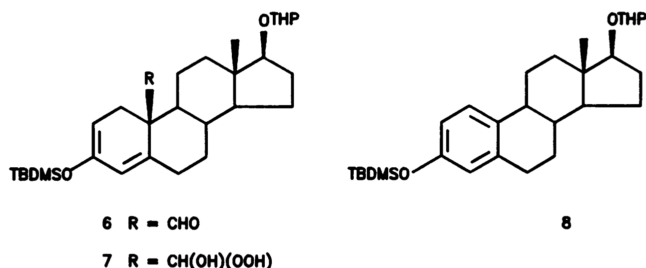
These analogues were not aromatized to estrogen derivatives. We then theorized that prior or concomitant enolization of the 3-ketone group might facilitate aromatization by lowering the activation energy for 1-hydrogen removal. Consequently, we investigated the reaction of 3-silyloxy-2,4-diene-19-oxo compound **6** with hydrogen peroxide (and *t*-butylhydroperoxide).

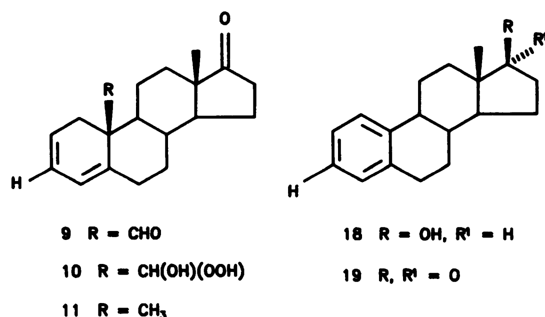


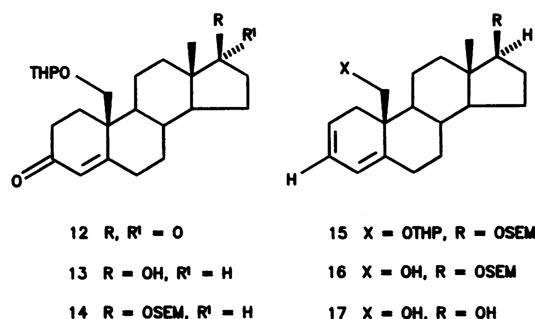
(THP, tetrahydropyranyl; TBDMS, *t*-butyldimethylsilyl.) It was hoped that a 19,19-hydroxyhydroperoxide (**7**) would be formed transiently and that it might fragment to give the corresponding aromatic product. Indeed aromatization occurred in high yield to give the corresponding estrogen derivative **8** along with formic acid (25). Furthermore, the stereoselectivity of 1β-H removal was shown to be faithful to the enzymatic process (unpublished data). Other evidence also supports the intermediacy of the 19,19-hydroxyhydroperoxide **7** in this aromatase model reaction (ref. 25 and unpublished data).

It seemed worthwhile to continue exploring the relevance of this model reaction to the actual aromatase reaction. While we considered the possibility of testing the 3-silyloxy-2,4-diene-19-oxo compound **6** as an actual aromatase substrate, we were discouraged by the possibility for reketonization of **6** under the assay conditions as well as by solubility problems and binding problems caused by the bulky hydrophobic appendages of **6** at its 3 and 17 positions. On the other hand, a potentially more stable analogue which might also be expected to fit better into the aromatase active site was the 3-desoxy-2,4-diene-19-oxo compound **9**. There was also a chance that this compound could serve as a "transition-state" analogue. Furthermore, although we know of no reports of 3-desoxy compounds serving as aromatase substrates, it was felt that **9** might be an alternative substrate for the enzyme.

MATERIALS AND METHODS

General. Proton NMR spectra were run in C^2HCl_3 and referenced to C^1HCl_3 (7.26 ppm). Flash chromatography was performed according to the method of Still *et al.* (26). HPLC was performed with a normal-phase analytical column (Whatman, Partisil 10, 25 cm; flow rate = 1 ml/min). 3-Desoxyestrone **19** was obtained as a gift from Searle (Chicago) and was purified by flash chromatography (15% EtOAc/hexanes). 19-Hydroxyandrost-4-ene-3,17-dione (**2b**) was obtained as a gift from Merrell Dow (Cincinnati). Androsta-2,4-diene-17-one (**11**) was synthesized according to a published procedure (27). Twice-washed placental microsomes were obtained by using minor modifications of previously described methods (28). The BCA (bicinchoninic acid) assay (29) was used to quantify protein concentration. When used in the experimental section, "the usual workup was performed" implies that the reaction mixture was partitioned between CH_2Cl_2 and H_2O, and the combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. Compound **2b** was converted to **12** by published methods (30).





[SEM, 2-(trimethylsilyl)ethoxymethyl.] Compounds **13** and **14** were prepared by standard methods (31, 32) and were fully characterized.

17-*O*-[2-(Trimethylsilyl)ethoxymethyl]-19-*O*-tetrahydropyranylandrosta-2,4-diene-17β,19-diol (15). To a stirred solution of the enone (**14**, 650 mg, 1.25 mmol) in MeOH (7 ml) was added 2,4,6-triisopropylbenzenesulfonylhydrazide (obtained as described in ref. 33, 77% pure based on the ^1H NMR spectrum, 550 mg, 1.42 mmol) under argon at room temperature. To this homogeneous solution was added, dropwise, a 4% solution of HCl in MeOH (0.125 ml). After 15 min, the reaction was cooled to −78°C and H_2O (2 ml) was added to precipitate a white solid. The mixture was concentrated under reduced pressure below 0°C for 1 hr and then kept under high vacuum (100 μm Hg) at room temperature overnight to afford the unstable hydrazone (1.2 g), which was used without further characterization. To a stirred solution of the hydrazone (1.2 g) in 3:1 (vol/vol) *N,N,N',N'*-tetramethylethylenediamine (TMEDA)/hexanes (16 ml) was added 1.6 M *n*-butyllithium (hexane solution, 7 ml, 10.4 mmol) under argon at −78°C, inducing a bright orange color. After 20 min, the dry-ice bath was removed and the mixture was warmed to 0°C. After a further 35 min, the mixture was quenched with H_2O (4 ml), and the usual workup was performed. The residue was flash chromatographed (45 g of SiO_2, gradient from 100% hexanes to 6% EtOAc/hexanes) to afford the homoannular diene (**15**; 380 mg, 0.757 mmol, 61% yield) as a colorless oil. ^1H NMR (400 MHz; multiple resonances for **15** result from the additional diastereomeric center in the THP group) δ 5.74 (m, 1 H), 5.64 (m, 1 H), 5.59 (m, 1 H), 4.67 (s, 2 H, $ROCH_2OCH_2CH_2SiMe_3$), 4.55 (m, 1 H, ROC*H*OR'), 3.9–3.5 (m, 5 H), 3.62 (ABq, J_{AB} = 9.7 Hz, $\Delta\nu_{AB}$ = 95.5 Hz, 1 H, 19-CH_2), 3.60 (ABq, J_{AB} = 9.6 Hz, $\Delta\nu_{AB}$ = 305 Hz, 1 H, 19-CH_2), 0.83 (s, 1.5 H, 18-CH_3), 0.79 (s, 1.5 H, 18-CH_3), 0.02 (s, 9 H, $(CH_3)_3Si$); IR ($CHCl_3$) 2900, 1660 cm^{-1}; UV (MeOH) 266 nm (ε 5400); calculated for $C_{30}H_{50}O_4Si$ *m/z* 502.3478, found 502.3484.

Androsta-2,4-diene-17β,19-diol (17). To a stirred solution of the THP ether (**15**, 358 mg, 0.713 mmol) in 4:1 CH_2Cl_2/MeOH (16 ml) was added pyridinium *p*-toluenesulfonate (PPTS) (560 mg, 2.2 mmol) under N_2 at room temperature. After 18 hr, the reaction appeared nearly complete (TLC), and the usual workup was performed. The residue was flash chromatographed (20 g of SiO_2, gradient from 8% EtOAc/

hexanes to 40% EtOAc/hexanes) to afford the 19-alcohol (**16**, 186 mg, 0.445 mmol, 62% yield) as a colorless oil (fully characterized). The diol (**17**, 20 mg, 0.07 mmol, 10% yield) was also obtained as a white solid. To a stirred solution of the SEM ether (**16**, 183 mg, 0.438 mmol) in CH_3CN (35 ml) was added aqueous 48% HF (6 ml) in a Nalgene bottle at room temperature. After 80 min, the reaction mixture was partitioned between aqueous saturated $NaHCO_3$ and CH_2Cl_2, and the organic phase was dried (Na_2SO_4) and concentrated under reduced pressure. The residue was flash chromatographed (16 g of SiO_2, 31% EtOAc/hexanes) to furnish **17** (64 mg, 0.222 mmol, 51% yield) as a white solid as well as 11 mg of starting material **16** (6% yield). An analytical sample of **17** was obtained after recrystallization from EtOAc/hexanes. HPLC retention time (20% EtOAc/hexanes) = 59 min; mp 140–141°C; ^1H NMR (400 MHz) δ 5.80 (m, 1 H), 5.7 (m, 2 H), 3.83 (dd, J = 10.6, 3.9 Hz, 1 H, 19-H), 3.61 (m, 1 H, 17-H), 3.60 (d, J = 10.6 Hz, 1 H, 19-H), 2.58 (dd, J = 17.4, 4.6 Hz, 1 H, 1β-H), 2.33 (d, J = 17.4 Hz, 1 H, 1α-H), 2.3 (m, 2 H, 6-CH_2), 0.76 (s, 3 H, 18-CH_3); IR ($CHCl_3$) 3570, 2900, 1660 cm^{-1}; UV (MeOH) 266 nm (ε 5600); calculated for $C_{19}H_{28}O_2$ m/z 288.2089, found 288.2090; analysis calculated C 79.12, H 9.79, found C 79.01 H 9.46.

19-Oxoandrosta-2,4-diene-17-one (9). To a stirred solution of diol **17** (11.0 mg, 0.0382 mmol) in CH_2Cl_2 (1.5 ml) containing 4-Å molecular sieves (three pellets) was added 4-methylmorpholine *N*-oxide (18 mg, 1.5 mmol) under N_2 at room temperature. After 15 min, tetrapropylammonium perruthenate (15 mg, 0.043 mmol) was added. After 6 min more, the mixture was quenched with aqueous saturated Na_2SO_3 (2 ml) and partitioned between CH_2Cl_2 (60 ml) and aqueous saturated Na_2SO_3 (40 ml). The aqueous phase was extracted with CH_2Cl_2 (30 ml), and the combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was flash chromatographed (2 g of SiO_2, 11% EtOAc/hexanes) to afford pure (HPLC, TLC) **9** (6.0 mg, 0.0211 mmol, 55% yield) as a colorless oil. HPLC retention time (12.5% EtOAc/hexanes) = 13 min; ^1H NMR (400 MHz; some of the resonances for **9** were assigned based on standard decoupling experiments) δ 9.71 (s, 1 H, 19-H), 5.89 (m, 1 H, 4-H), 5.74 (m, 1 H, 3-H), 5.69 (m, 1 H, 2-H), 3.04 (dd, J = 18, 5.7 Hz, 1 H, 1β-H), 2.46 (d, J = 18 Hz, 1 H, 1α-H), 0.88 (s, 3 H, 18-CH_3); IR ($CHCl_3$) 2900, 1730, 1705 cm^{-1}; UV (MeOH) 269 nm (ε 5500); calculated for $C_{19}H_{24}O_2$ m/z 284.1776, found 284.1777.

3-Desoxyestrone (19). To a solution of the aldehyde **9** (5.5 mg, 0.019 mmol) in 9:1 MeOH/CH_2Cl_2 (1 ml) containing anhydrous $NaHCO_3$ (3 mg) was added aqueous 30% (wt/vol) HOOH (0.1 ml, 30 mg, 0.88 mmol) at 4°C. The mixture was stirred vigorously and allowed to stand at 4°C. After 3 days, reaction appeared nearly complete (TLC), and the mixture was partitioned between aqueous half-saturated sodium thiosulfate (50 ml) and CH_2Cl_2 (50 ml). The aqueous phase was extracted with CH_2Cl_2 (50 ml), and the combined organic phases were dried (Na_2SO_4) and concentrated under reduced pressure. The residue was flash chromatographed (2 g of SiO_2, 11% EtOAc/hexanes) to afford pure 3-desoxyestrone (**19**, 2.8 mg, 0.011 mmol, 58% yield) as a white solid. Compound **19** had identical TLC, ^1H NMR, IR, and UV properties compared to the authentic compound **19** (34).

3-Desoxyestradiol (18). Compound **19** was reduced with $NaBH_4$ to provide the known compound **18** (34).

Aromatase Incubations with 2,4-Diene-19-oxo Compound 9, 3-Desoxyestrone (19), 3-Desoxyestradiol (18), and Testosterone (2a). Reactions were performed according to procedures described elsewhere (46). Solvent systems used for HPLC were 12.5% EtOAc/hexane to elute 2,4-diene-19-oxo compound **9** (retention time = 13 min), 3-desoxyestradiol (**18**) (retention time = 22 min), and 3-desoxyestrone (**19**) (retention time = 7.5 min); 25% EtOAc/hexane to elute estrone **4b** (retention time = 9 min) and estradiol **4a** (retention time = 17 min); and 50% EtOAc/hexane to elute testosterone (**1a**) (retention time = 10 min). Average yields are reported in *Results* with errors representing the span of yields from two separate runs.

Competitive Inhibition. Enzyme activity was measured by 1β-tritium release (36). Assay reaction mixtures (0.5 ml) contained potassium phosphate buffer (10 mM, pH 7.4), KCl (100 mM), dithiothreitol (5 mM), EDTA (1 mM), NADPH (125 μM). Stock [1β-^{3}H]androstenedione substrate [2.74 × 10^4 Ci/mol (1 Ci = 37 GBq); 3:1 1β-^{3}H:1α-^{3}H] used was 3.71 μM (95% H_2O/EtOH solution). Seven [1β-^{3}H]androstenedione substrate concentrations from 18.6 to 282 nM were used. Stock inhibitor solutions were prepared in ≈1% propylene glycol/H_2O solutions. 2,4-Diene-19-methyl compound **11** was present at 500 and 200 nM, 2,4-diene-19-oxo compound **9** was present at 2 μM and 500 nM, and 3-desoxyestrone **19** was present at 7 and 3.5 μM. Reactions were initiated with human placental microsomes (2–3 μg of microsomal protein) and allowed to proceed for 5 min at 37°C in a Dubnoff shaker bath. An aliquot (400 μl) was removed and quenched by vigorous mixing with $CHCl_3$ (5 ml) for 20 s. The mixture was centrifuged at low speed for 10 min and the radioactivity of an aliquot from the aqueous phase (0.2 ml) was measured with 10 ml of ACS scintillation fluid (Amersham). K_m, K_i, and V_{max} values were obtained by using a double reciprocal analysis. K_m values for androstenedione were 15 ± 2 nM. V_{max} values were about 200 pmol/min·mg. Average K_i values are listed in *Results* with the errors representing the span of K_i values obtained from two assays.

Time-Dependent Inactivation Assays. The inactivation assay of Marcotte and Robinson (37) was used with only slight modifications.

RESULTS

It was not possible to synthesize the heretofore undescribed 2,4-diene-19-oxo compound **9** by using regioselective eliminations with appropriately derivatized steroidal 3-hydroxy-4-enes. Consequently, it was decided to use a Bamford–Stevens–Shapiro reaction (38, 39). Reduction of 19-THP-19-hydroxyandrostenedione (**12**) (30) with LiAl (*O*-*t*-butyl)$_3$H (31) gave the known (30) 19-THP-19-hydroxytestosterone (**13**). This compound was then protected as the 17-SEM ether **14** [87% yield from 19-THP-19-hydroxyandrostenedione with SEM chloride and Hunig's base (32)] and converted to the unstable hydrazone derivative, using 2,4,6-triisopropylbenzenesulfonylhydrazide and HCl. The unpurified hydrazone was treated with *n*-butyllithium and TMEDA in hexane to afford the bis-protected homoannular diene **15** in 61% yield. The 19-THP ether group was removed with PPTS in MeOH, giving **16**, and the 17-SEM ether group was then removed with aqueous HF in CH_3CN to afford the desired 2,4-diene-17β,19-diol **17**, in 41% yield from the bis-protected precursor. That this diene **17** contained very little of the 3,5-diene isomer was apparent both from the UV spectrum [which lacked an absorption maximum at 235 nm (40)] and from the 400-MHz ^1H NMR spectrum, which indicated greater than 95% purity. Although attempted oxidations of the 2,4-diene-17β,19-diol **17** using chromium reagents led to significant decomposition, oxidation of the two alcohol functions with tetrapropylammonium perruthenate (41) furnished the 2,4-diene-17-ketone-19-aldehyde **9** (55% isolated yield) as an unstable colorless oil which resisted crystallization. This compound was used within 24 hr after purification because of its lability.

We investigated first the reactivity of the 2,4-diene-19-oxo compound **9** with HOOH. If the structural similarity between the 3-silyloxy-2,4-diene-19-oxo compound **6** and the 2,4-diene-19-oxo compound **9** extended to a similarity in chem-

ical reactivity, then 2,4-diene-19-oxo compound **9** should be converted to 3-desoxyestrone **19**, *via* the 19-hydroxyhydroperoxide **10**. As a control experiment, the known (27) 2,4-diene-19-methyl compound **11** was shown not to react with HOOH after 3 days. In contrast, the 2,4-diene-19-oxo compound **9** did aromatize to form 3-desoxyestrone **19** (58%) yield after 3 days, under similar conditions to the 3-silyloxy-2,4-diene-19-oxo **6** reaction, and at approximately the same rate. The product was shown to be 3-desoxyestrone **19** by chromatographic and spectroscopic comparison with the authentic compound. That HOOH was a necessary component in the reaction was demonstrated by the observation that less than 2% 3-desoxyestrone **19** formation occurred in the absence of HOOH.

In view of the above findings, the 2,4-diene-19-oxo compound **9** was examined as a possible inhibitor/substrate for placental aromatase. When the [1β-^{3}H]androstenedione tritium release assay and double-reciprocal plot analysis were used, the 2,4-diene-19-oxo compound **9** was found to be a potent competitive inhibitor of aromatase (see Fig. 1) with K_i = 73 ± 0 nM, and it showed no detectable time-dependent inactivation. The corresponding 4-ene-3-one-19-oxo compound **3b** is a substrate with K_m = 182 nM (6). In contrast, the known 2,4-diene-19-methyl compound **11** (also exhibiting no detectable time-dependent aromatase inactivation) had K_i = 28 ± 2 nM. Although **11** was a potent competitive inhibitor, it was found to be a somewhat poorer binder than the corresponding 4-ene-3-one-19-methyl compound **1b** [literature (6) value K_m = 14 nM, our experimental K_m value = 15 ± 2 nM].

These results suggest that the steroids with 3-desoxy-2,4-diene A-rings are well accepted by the aromatase active site, and further, that the simultaneous presence of the 19-aldehyde group and the 2,4-diene function are complementary features for binding relative to the case for the 4-ene-3-one compounds. While this complementarity could be a fortuitous result, it is consistent with the interpretation that a 2,4-diene-3-ol function is particularly well stabilized when needed by the enzyme—i.e., in the third oxidative step. This added stabilization is calculated to be about 1 kcal/mol for 2,4-diene-19-oxo binding *versus* 2,4-diene-19-methyl binding in comparison to the corresponding 4-ene-3-one systems.*

It is also worth noting that both 3-desoxyestrone (**19**) and 3-desoxyestradiol (**18**) were analyzed for aromatase inhibition. 3-Desoxyestrone (**19**) had K_i = 1.5 ± 0.4 μM and the K_i for 3-desoxyestradiol (**18**) was too high to be measured under the assay conditions (K_i > 2 μM). Thus the desoxyestrogens are relatively poor aromatase inhibitors, which is consistent with their structural similarity to estrogens, also known to be weak aromatase inhibitors (42).

The K_i data suggest that the 2,4-diene-19-oxo compound **9** binds effectively to the aromatase active site, but they do not address the possibility that this binding is suitable for aromatase processing. For example, while the 19-methyl, 19-hydroxy, and 19-oxo-3-exomethylene androst-4-ene derivatives of Miyairi and Fishman (43) were shown to be effective competitive inhibitors of aromatase, they were not processed by the enzyme to a significant degree.

Therefore, the 2,4-diene-19-oxo compound **9** was incubated with placental microsomal aromatase for 60 min at 37°C in the presence of an NADPH-regenerating system. In the event, there was significant conversion to 3-desoxyestradiol (**18**) (22 ± 1% yield) [and a small amount of desoxyestrone (**19**) (*ca.* 2.5 ± 1% yield)]. The structure of the product **18** was confirmed by TLC, HPLC, ^{1}H NMR, and MS comparison with the authentic material (34). The NADPH-regenerating system was shown to be required, as its absence resulted in formation of less than 1% 3-desoxyestrone (**19**) or 3-desoxyestradiol (**18**).

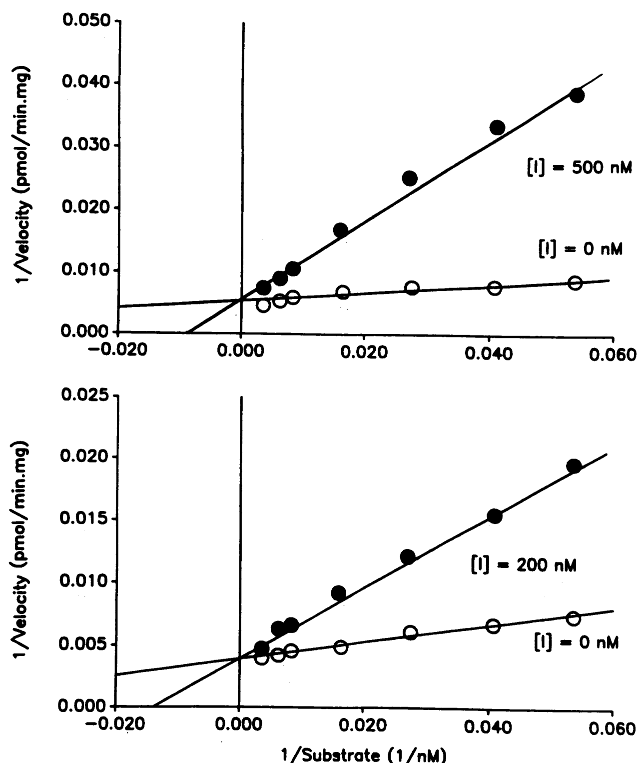


FIG. 1. Lineweaver–Burk aromatase inhibition assays using 2,4-diene-19-oxo compound **9** as the inhibitor and androstenedione as the substrate.

Furthermore, a powerful reversible aromatase inhibitor [the (*19R*)-thiirane (ref. 35, K_i = 1 nM)] was found to block desoxyestrogen formation significantly. At 20 μM inhibitor and 140 μM substrate only *ca.* 1% 3-desoxyestradiol (**18**) was formed (and less than 1% 3-desoxyestrone).† This is in rather good agreement with the expected value (2%) for 3-desoxyestrogen formation, assuming that the K_m of the 2,4-diene-19-oxo compound **9** is equal to its K_i (i.e., 73 nM) and that classical reversible kinetics are followed.

In these cases, nearly all of the isolated 3-desoxyestrogen formed from the 2,4-diene-17,19-dione compound **9** was 3-desoxyestradiol (**18**). It was well known that there can be significant 17β-hydroxysteroid dehydrogenase activity in washed placental microsomes, with the possibility for interconversion of 17-hydroxy and 17-keto androgens and estrogens (44, 45). In our case the formation of 3-desoxyestradiol (**18**) could, in principle, arise from 17β-hydroxysteroid dehydrogenase-catalyzed reduction of initially formed 3-desoxyestrone (**19**). In a separate experiment, it was confirmed that 3-desoxyestrone (**19**) is indeed converted to 3-desoxyestradiol (**18**) (50 ± 4% yield, 7 ± 1% recovered 3-desoxyestrone) under the experimental conditions. However, we cannot exclude some contribution from 17β-hydroxysteroid dehydrogenase-catalyzed reduction of 2,4-diene-17-ketone **9** followed by aromatization to 3-desoxy-

*$\Delta\Delta G = -RT\ln(182 \text{ nM}/14 \text{ nM}) + RT\ln(73 \text{ nM}/28 \text{ nM}) = -1$ kcal/mol; 1 kcal = 4.18 kJ.

†Levels of recovered starting material **9** from aromatase incubations were found to be quite variable, probably because of the instability of **9**, and could not be reliably quantitated. Significant time-dependent decreases in substrate amounts occurred even after the reaction mixture was extracted into CH_2Cl_2. The highest yield of substrate **9** recovered from any reaction was 31% [from the reaction performed in the presence of (*19R*)-thiirane].

estradiol (**18**). This is probably not a major issue, since aromatase processes normal 17-ketone and 17β-hydroxy substrates at comparable rates (6).

The recovery of 3-desoxyestradiol (**18**) when incubated with placental microsomes under the above conditions was 45 ± 5%. Testosterone (**1a**) afforded 23 ± 1% estradiol (**4a**) and 60 ± 3% recovered starting material **1a** under similar conditions. In contrast, 19-oxoandrostenedione (**3b**) and 19-oxotestosterone (**3a**) have been shown previously to be nearly completely converted by aromatase to estrogens (*ca.* 80% with less than 1% recovered starting material) under these conditions (46). On the basis of the relatively low recovery of the desoxyestrogens and the instability of the 2,4-diene-19-oxo compound **9**,[†] it can be surmised that the aromatase k_{cat} value for 2,4-diene-19-oxo compound is probably somewhere between the k_{cat} values for testosterone (**1a**) and 19-oxoandrostenedione (**3b**), which are 5 and 21 min^{-1}, respectively (6).

DISCUSSION

To assess further the validity of a recent model reaction as a probe for aromatase mechanism, a 3-desoxy analogue, **9**, of the original model substrate **6** has been synthesized. Under the conditions developed for the original model reaction, this new substrate, **9**, is smoothly aromatized to 3-desoxyestrone, **19**. Control experiments with 2,4-diene-19-methyl compound **11** and with 2,4-diene-19-oxo compound **9** in the absence of hydrogen peroxide suggest that the mechanism for this model reaction involves 19,19-hydroxyhydroperoxide **10** formation followed by collapse to aromatic product.

The 2,4-diene-19-oxo compound **9** was also found to be a potent aromatase competitive inhibitor (K_i = 73 nM). When data for the corresponding 4-ene-3-one substrates are compared, there appears to be a complementary interaction between the 19-oxo group and the diene function for aromatase binding. This complementarity, worth about 1 kcal/mol binding energy, may be related to increased stabilization of the 2,4-diene-3-ol function in the aromatase third step.

The 2,4-diene-19-oxo compound **9** also was converted in good yield to 3-desoxyestrogen by placental microsomes at an enzymatically significant rate. Such conversion was dependent upon NADPH and could be blocked by *(19R)*-10β-thiiranylestr-4-ene-3,17-dione, a specific and potent aromatase inhibitor. It may be inferred from these results that aromatase is, in fact, responsible for this conversion, although ultimate proof awaits experiments with pure enzyme. This study suggests that aromatase can accommodate and turn over 2,4-diene-3-ol-19-oxo type substrates. These results lend plausibility to the assertion that 3-ketone enolization may play a key role in the aromatase third step. Furthermore, this work provides an arena for direct mechanistic comparisons between a nonenzymatic aromatase model reaction and the corresponding enzymatic conversion.

To our knowledge, the 2,4-diene-19-oxo compound **9** is the first compound lacking 3-oxygen substitution that has been shown to be an aromatase substrate.[‡] Furthermore, the 19-methyl (**11**) and 19-oxo (**9**) compounds may be considered as prototypes for a family of aromatase inhibitors which, through the lack of a 3-oxygen, will be less susceptible to a common route of *in vivo* clearance of steroidal 3-ketone aromatase inhibitors.

[‡]Compound **11** was not aromatized (<1%) to 3-desoxyestrogens by placental microsomes under conditions similar to those used for **9**. In contrast, **17** was aromatized to 3-desoxyestradiol (**18**) in yield comparable to the aromatization of **9**. Furthermore, the aromatization of **17** was NADPH dependent and was inhibited by *(19R)*-10β-thiiranylestr-4-ene-3,17-dione, just like aromatization of **9**.

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