Synthesis and Evaluation of 19-Aza- and 19-Aminoandrostenedione Analogues as Potential Aromatase Inhibitors[1]

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Derivatives of 19-azaandrostenedione (10β-amino-4-estrene-3,17-dione, **2**) and 19-amino-4-androstene-3,17-dione (**3**) were synthesized as potential inhibitors of aromatase (estrogen synthetase). Compound **2** and its derivatives were synthesized from 3,17-dioxo-4-androsten-19-oic acid (**5**) via a Curtius rearrangement. Derivatives of **3** were synthesized from the intermediate 3,17-bis(ethylenedioxy)-5-androsten-19-al oxime (**14**), which was reduced to the corresponding amine (**16**) with Raney nickel. However, attempts to synthesize the parent compound, **3**, from **16** by several different methods were unsuccessful. The compounds obtained were tested for inhibitory activity in the tritiated water assay for aromatase, with human placental microsomes as the enzyme source and [1-^{3}H]-androst-4-ene-3,17-dione (83% ^{3}H 1β) as the substrate. All of the compounds caused less than 20% inhibition of enzyme activity when tested at one and five times the substrate concentration (0.25 μM, 1.25 μM) and were poorer inhibitors than two known inhibitors, 7α-[(*p*-aminophenyl)thio]androstenedione (7-APTA) and 4-hydroxy-4-androstene-3,17-dione (4-OHA).

Estrogens play a critical role in the regulation of reproduction, as well as having a number of beneficial clinical uses. However, they have also been implicated in the development or progression of several diseases, most notably breast cancer and endometrial carcinoma. This association with certain types of cancers is one reason that many investigators are focusing their attention on inhibitors of estrogen biosynthesis.

The enzyme responsible for the production of estrogens from androgens is aromatase or estrogen synthetase, a microsomal P_{450} enzyme. Although the exact mechanism for aromatization is not fully understood, it is known that the reaction involves two hydroxylations at C-19 of the androgens, with subsequent loss of the 19-carbon as formic acid.[3]

Since the initial report by Schwarzel et al.,[4] several groups have examined a wide variety of modified androgens as potential aromatase inhibitors in vitro, using either human placental microsomes or pregnant mare's serum gonadotrophin (PMSG) primed rat ovarian microsomes[5] as the enzyme source. Of these compounds, the most potent inhibitors are 4-hydroxyandrostenedione (4-OHA), 4-acetoxyandrostenedione (4-OAcA), 1,4,6-androstatrienedione (ATD), and androstene-3,6,17-trione.[4,5] These compounds show classic competitive inhibition kinetics when both substrate and inhibitor are incubated together. However, recent studies indicate that when they are preincubated with the enzyme in the presence of NADPH but in the absence of substrate, these compounds, along with 1,4-androstadienedione (but not 4,6-androstadienedione), inactivate the enzyme in a time-dependent fashion.[6-9] Δ^1-Testololactone, a C_{19} steroid devoid of androgenic activity that has been used in breast cancer therapy for many years, has been shown to inhibit aromatase.[10] It also causes a time-dependent decrease in enzymatic activity.[9] Androgens with modifications at other positions that have been examined as aromatase inhibitors include 6α- and 16α-brominated androgens[11] and 7α-substituted androgens.[12] 7α-[(*p*-Aminophenyl)thio]androstenedione, synthesized in this laboratory, is among the most potent competitive inhibitors of aromatase known.[12]

Several of these aromatase inhibitors have been examined further in various in vitro and in vivo experiments. For example, Abul-Hajj[13] showed that 4-OHA and 7-APTA were effective inhibitors of estrogen biosynthesis in breast cancer cells in vitro. Moreover, Brodie et al.[14-17] have demonstrated that 4-OHA, ATD, and 4-OAcA can inhibit not only ovulation and implantation, but also the growth of estrogen-dependent mammary tumors in vivo.

In order to probe the structural restrictions imposed by the enzyme active site, we decided to design potential competitive inhibitors which contained structural modifications at C-19, the site of the initial enzymatic hydroxylation. The choice of our target compounds was based upon our earlier observations with inhibitors of the cholesterol side-chain cleavage enzyme,[18-20] a cytochrome P_{450} enzyme that converts cholesterol to pregnenolone.

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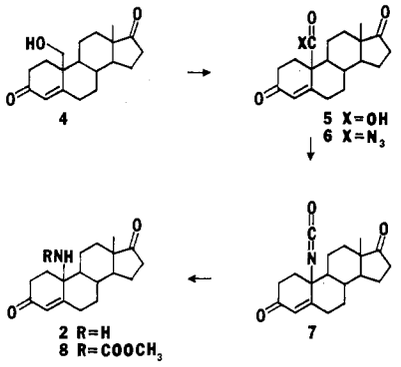
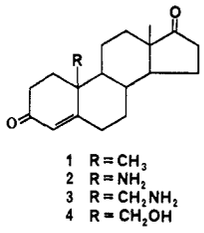
Scheme I



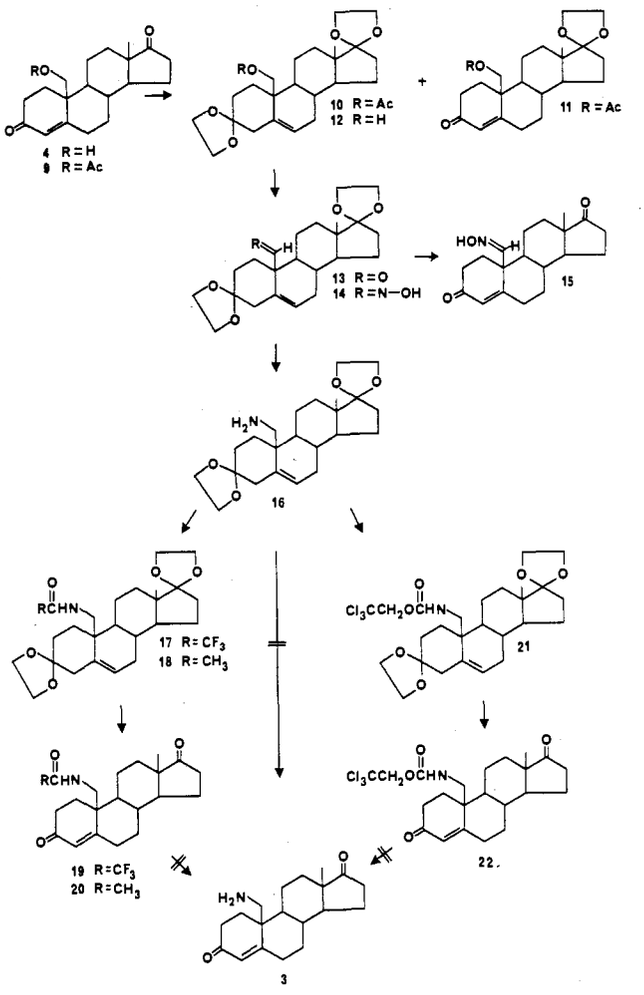
The course of this enzymatic reaction involves hydroxylations at C_{20} and C_{22}, followed by C–C bond cleavage. In that study, 20-aza- and 22-azacholesterol were found to be the most potent inhibitors among a series of cholesterol bioisosteres. Since placement of an heteroatom at the site of enzymatic hydroxylation yielded potent inhibitors in that series, perhaps similar modification of androstendione (1) would afford inhibitors of aromatase. This paper de-



scribes the synthesis and evaluation of 19-aza-androstenedione (10β-amino-4-estrene-3,17-dione, **2**) and derivatives of its homologue, 19-amino-4-androstene-3,17-dione (**3**). These aza analogues can be viewed as bioisosteres of androstenedione (**1**) and 19-hydroxy-androstenedione (**4**), respectively, which are both natural substrates for aromatase. Since initiation of this project, several groups have synthesized and evaluated a number of 19-substituted androstenedione derivatives as aromatase inhibitors, several of which have been found to irreversibly inactivate the enzyme.[21-26]

Chemistry. Synthesis of 10β-Amino-4-estrene-3,17-dione (2) and Its Derivatives. The first target compound, 10β-amino-4-estrene-3,17-dione (**2**), was synthesized from 3,17-dioxo-4-androsten-19-oic acid (**5**) by a modification of de Ruggieri's procedure (Scheme I).[27] The 19-carboxylic acid **5** was prepared by oxidizing 19-hydroxy-4-androstene-3,17-dione (**4**) with Jones reagent,[28,29] with and without the isolation of the intermediate aldehyde.[30] Because of the acid's propensity for thermal de-

Scheme II



carboxylation to 19-norandrogens,[28,29,31] all procedures during its preparation and subsequent utilization were conducted at or below room temperature. The mixed anhydride, rather than the acid chloride, was used to prepare the acid azide **6**, since steroidal conjugated enones appear to be sensitive to reagents such as thionyl chloride[32] or oxalyl chloride,[33] which are generally used in the preparation of acid chlorides. Even under these mild conditions, the yield of the azide **6** was low (34%), but the starting acid could be recovered for recycling. No significant improvement in yield was noted when other mixed anhydrides of **5** (i.e., isobutyl and benzyl) were employed. Apparently, nucleophilic attack at the carbalkoxy carbonyl can occur along with attack at the sterically hindered 19-carbonyl, irrespective of the size of the group forming the mixed anhydride. Once the acid azide **6** was obtained, it was readily rearranged to the isocyanate **7** by heating in toluene. In fact, the rearrangement was so facile that some isocyanate **7** formed during isolation of the acid azide **6**. Acid hydrolysis of **7** afforded the target compound **2**. The use of mild hydrolysis conditions (50% acetic acid or 1 N HCl) required longer reaction times but gave better yields (70–80 vs. 40%) of **2** than did the use of strong acid (20% or 6.5 N HCl). In addition, the methyl carbamate **8** was prepared by refluxing **7** in anhydrous methanol.

Synthesis of Derivatives of 19-Amino-4-androstene-3,17-dione (3). The most direct method for

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preparing the 19-aminoandrostenedione derivatives would be nucleophilic displacement of the 19-mesylate or other readily displaceable group. Although such reactions often lead to the rearranged 5β,10β-cyclo-6α-substituted or 6β,10β-cycloandrogens,[34] there are cases where nucleophilic displacement reactions have successfully yielded 19-substituted androgens.[35-38] However, all attempts to obtain 19-aminoandrostenedione derivatives in this manner were unsuccessful.

At this point, reductive amination of a C-19 aldehyde seemed an attractive alternative. As outlined in Scheme II, 3,17-bis(ethylenedioxy)5-androsten-19-al (13) was prepared in four steps from 19-hydroxyandrostenedione (4) by modification of the procedures described by Amorosa et al.[39] and Hill et al.[40] Since direct ketalization of 19-hydroxyandrostenedione (4) was unsuccessful, the 19-hydroxyl moiety was first protected as the 19-acetate 9.[41] Ketalization of 9 was accomplished with ethylene glycol and p-toluenesulfonic acid in refluxing benzene, with azeotropic distillation of the water formed during the reaction. Even after extended reaction times (30 h) with excess reagents, this procedure afforded a mixture of the desired bisketal 10 and the monoketal, 17-(ethylenedioxy)-19-hydroxy-4-androstene-3-one acetate (11), in isolated yields of 78 and 17%, respectively. Although earlier investigators[39] do not mention the formation of the monoketal as a byproduct of this reaction and used the crude bisketal 12 without purification, we found that in order for hydrolysis of the acetate to proceed cleanly, it was necessary to first remove the monoketal byproduct 11 from the bisketal 12. Because of the acid sensitivity of the ketal groups, the nonacidic reagent pyridinium dichromate[42] was employed to oxidize 12 to the aldehyde 13. However, high yields of 13 (93%) and minimal formation of byproducts was only achieved when acid-free solvents were employed (see Experimental Section). Unfortunately, reductive amination of 13, with either sodium cyanoborohydride and ammonium acetate in methanol/THF[43] or a more recent modification employing a substituted cyanoborohydride and ammonia or ammonium chloride in a nonpolar solvent (e.g., CH_2Cl_2),[44] failed to yield the desired product. A possible explanation for the failure of these procedures may be the low solubility of 13 and the amine salt in the solvents (MeOH and CH_2Cl_2) used in the original and modified procedures, respectively.

The amino group was successfully introduced by converting 13 to the oxime (14) and then reducing the oxime with Raney nickel alloy in an alkaline ethanol solution.[45] Since it has been shown that heating oximes with Raney nickel can result in regeneration of the carbonyl compound,[45] the reaction was conducted without external heating. The reaction proceeded smoothly in high yield to give analytically pure, crystalline 19-amine (16). However, the reduction required much longer reaction times than typically used in the literature (>20 vs. 1 h), presumably as a result of the hindered nature of the 19-oxime. Raney nickel alloy can also reduce α,β-unsaturated ketones to saturated alcohols,[46] so the presence of the ketals was essential for the success of the reaction. This procedure gave a higher yield and a cleaner product than did catalytic hydrogenation using a very active Raney nickel catalyst, T-1.[47] 3,17-Dioxo-4-androsten-19-al oxime (15) was also prepared from 14 by nonselective hydrolysis of the ketal protecting groups with sulfuric acid in dioxane.

The next and final step in the proposed synthesis of 19-aminoandrostenedione (3) was the hydrolysis of the bisketal (16). Standard procedures to remove the ketals, i.e., hydrolysis with acid,[48,49] failed to yield the desired product. Since removal of ketals in the presence of an amino group can be difficult,[50] an alternate route to 3 involved protection of the amino group prior to hydrolysis of the ketal groups. Conversion of 16 to the trifluoroacetamide (17) was accomplished in high yield with trifluoroacetic anhydride and dimethylaminopyridine (DMAP). Once the amino group had been protected, the ketals were easily removed with sulfuric acid in acetone to give pure 19-[(trifluoroacetyl)amino]-4-androstene-3,17-dione (19). 19-(Acetylamino)androstenedione (20) was prepared from 16 in an analogous fashion. However, even under fairly mild conditions (7% K_2CO_3 in aqueous methanol at room temperature),[51] hydrolysis of the trifluoroacetamide (19) failed to yield 19-aminoandrostenedione (3).

Since both acid hydrolysis of the bisketal (16) and basic hydrolysis of the trifluoroacetamide (19) resulted in extensive decomposition of the respective starting materials, we then examined an alternate approach in which the amine group was protected with a group that could be removed under nonaqueous conditions, namely, the 2,2,2-trichloroethyl carbamate.[52] The yields and purity of the 2,2,2-trichloroethyl carbamate derivates, however, were lower than those of the corresponding trifluoroacetamide compounds. Preparation of the 2,2,2-trichloroethyl carbamate (21), using 2,2,2-trichloroethyl chloroformate in either methylene chloride with DMAP or pyridine, afforded material that required chromatographic purification. Removal of the ketals from purified 21 with sulfuric acid in acetone afforded 22, which could not be obtained analytically pure by recrystallization, preparative layer chromatography, or isocratic HPLC. However, since spectral data indicated that the above material was predominantly the desired product (22), the removal of the carbamate protecting group by reduction with zinc in refluxing methanol[52] was attempted. However, this reaction gave a mixture of products and failed to afford the desired amino steroid (3) in quantities sufficient for isolation and characterization.

Biochemistry. The assay used for evaluating the newly synthesized compounds as aromatase inhibitors was the

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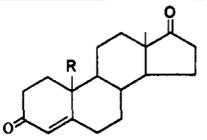
Table I. Results of the Screening Assay



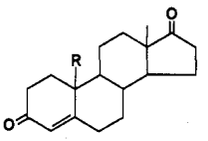
compd	R	% inhibn (±SD) [I] = 0.25 μM	% inhibn (±SD) [I] = 1.25 μM
7-APTA[a]		54.8 ± 5.8	83.1 ± 7.6
4-OHA[b]		50.1 ± 4.5	81.6 ± 2.2
2	NH_2	9.4 ± 4.8	19.2 ± 0.9
		6.9 ± 2.0	10.9 ± 1.7
7	N=C=O	<2	<2
8	$NHCO(=O)CH_3$	<2	<2
15	CH=NOH	8.6 ± 5.6	4.1 ± 3.9
19	$CH_2NHC(=O)CF_3$	2.5 ± 5.1	11.4 ± 1.3[c]
20	$CH_2NHC(=O)CH_3$	<2	13.2 ± 1.0

[a] Average of five experiments. [b] Average of three experiments. [c] $n = 2$.

tritiated water assay, which was developed by Thompson and Siiteri[53] and further refined by Reed and Ohno.[54] This assay depends upon the amount of tritiated water released from a 1- or 1,2-^{3}H-labeled androgen as a measure of the amount of estrogen produced by the enzyme. The assay is based upon the stereospecificity of the enzyme; only tritium at the 1β- and 2β-position of the substrate is released as tritiated water. The substrate chosen for the assay was [1-^{3}H]androstenedione, since this substrate avoids the problems associated with the chemical[55] and metabolic[54] lability of tritium at the 2-position. The source of aromatase for this study was twice-washed human placental microsomes. These were prepared by differential centrifugation according to Ryan's procedure,[56] with precautions taken to minimize cytosolic contamination and the presence of 17β-hydroxysteroid dehydrogenase. Moreover, the enzyme was lyophilized to minimize loss of enzyme activity.[57]

The substrate concentration used for screening potential inhibitors was fixed relative to the K_M of the enzyme for androstenedione at $5 \times K_M$. This concentration is on the plateau of the Michaelis–Menten curve, so minor changes in substrate concentration, either inter- or intraassay, should not significantly alter the reaction's velocity. The K_M for androstenedione, as determined from a Lineweaver–Burk plot and a weighted linear regression,[58] was 42.6 nM, a value consistent with previous reports.[12,59] Therefore, the substrate concentration for the screening assay was set at 0.25 μM. Potential inhibitors were assayed at two concentrations, 1 and 5 × [S], or 0.25 and 1.25 μM, respectively. Two known inhibitors, 7-APTA[12] and 4-OHA[14] were included in the assays for comparison purposes. The results for 7-APTA agree remarkably well with the previously reported values,[12] despite marked difference in assay conditions. As expected, both 7-APTA and 4-OHA showed excellent inhibition (Table I).

In contrast to these known inhibitors, the 10β- and 19-substituted compounds were poor inhibitors (Table I). The maximum inhibition was less than 20% for all of these compounds. Among the 10β-substituted androgens, the parent compound, 10β-amino-4-estrene-3,17-dione (**2**), was a better inhibitor than either the isocyanate (**7**) or the carbamate (**8**). The lack of inhibition by these latter two compounds suggests that the poor inhibition by 2 was not due to the basicity of the amine function. In the 19-substituted androgens there was also very little variation in percent inhibition. Even though the 19-oxime (**15**) has a similar structure to 19-hydroxyandrostenedione (**4**) and 19-oxandrostenedione (**23**), intermediates in the biosynthesis of the estrogens, it still showed poor inhibitory activity.



23 R=CH=O
24 $R=CH_2CH_2X$ (X=F, Cl)
25 $R=CH_2X$ (X=F, Cl)
26 $R=CH_2C\equiv CH$
27 $R=CH=C=CH_2$
28 $R=C\equiv N$

These results are very interesting in light of the recent findings with other 10β- and 19-substituted androgens.[21-26] The 19-(halomethyl)- (**24**) and 19-haloandrostenedione derivatives (**25**) are good inhibitors.[24,25] Moreover, compounds containing π electrons involved in C–C multiple bonds showed good inhibitory activity. Two of these compounds, 19-ethynylandrostenedione (**26**)[21,22,24] and the related 10β-allenyl-4-estrene-3,17-dione (**27**),[22] cause a time-dependent inactivation of the enzyme. In contrast, compounds with π bonds containing a heteroatom, such as 10β-cyano-4-estrene-3,17-dione (**28**; tested by Marcotte and Robinson[24]), the 10β-isocyanate **7** and the 19-oxime **15**, examined in this study, were poor inhibitors.

There is now substantial structure–activity relationship data for the 10β- and 19-substituted androgens as aromatase inhibitors. The enzyme is very specific as to what groups it will tolerate at these positions. It appears clear from this study that insertion of a nitrogen function at this position fails to achieve the desired goal. Although synthesis of aza bioisosteres of cholesterol was found to afford potent inhibitors of cholesterol side-chain cleavage, this strategy failed to carry over to aromatase.

Experimental Section

Infrared spectra of KBr pellets or $CHCl_3$ solutions were recorded on a Perkin-Elmer 281 spectrophotometer. Proton magnetic resonance spectra in $CDCl_3$ with tetramethylsilane as an internal reference were recorded on a Varian EM-360A spectrophotometer. High-field Fourier-transform NMR, both proton and carbon-13, were performed an a Brueker 360-MHz spectrophotometer in $CDCl_3$; residual $CHCl_3$ (δ 77.247) and Me_4Si were used as internal standards for proton and carbon, respectively. ^{13}C NMR were done with broad-band decoupling; tentative assignments were made based upon analogy to the assigments made for androstenedione and dehydroepiandrosterone.[60] Melting points were determined with a Thomas-Hoover capillary melting point apparatus and are corrected. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ, or Midwest Microlab, Ltd., Indianapolis, IN. Analytical thin-layer chromatography was performed on either polyethylene-backed silica gel plates obtained from Eastman Kodak, Rochester, NY, or E. Merck, Rahway, NJ, or on glass-backed reverse-phase plates from Analtech, Newark, DE. Preparative layer plates were from E. Merck. Silica gel for column chromatography, 60–200 mesh, was from Davidson Chemical, Baltimore, MD. Silica gel or reverse-phase Sep-Pak cartridges were purchased from Whatman, Inc., Milford, MA. 19-Hydroxy-4-androstene-3,17-dione was a gift from Searle Laboratories, Skokie, IL. Dioxane and benzene were dried

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over sodium metal and distilled under N_2, THF was distilled from $LiAlH_4$ under N_2, and pyridine was dried and distilled under N_2 from BaO. Acetone was fractionally distilled under N_2 from $KMnO_4$. Methylene chloride was distilled and stored over K_2CO_3 to absorb any traces of acid. Other reagents were purchased from Aldrich Chemical Co., Milwaukee, WI.

10β-Isocyanato-4-estrene-3,17-dione (7). Triethylamine (3.30 g, 32.6 mmol, 1.25 equiv), followed by ethyl chloroformate (3.54 g, 32.6 mmol, 1.25 equiv), were added to a cold (0–5 °C) solution of 3,17-dioxo-4-androsten-19-oic acid[28,29] (**5**; 8.25 g, 26.1 mmol, 1.0 equiv) in freshly distilled THF (200 mL), and the mixture was stirred for 1.5 h. IR indicated the presence of the mixed anhydride (1810 cm^{-1}). Triethylamine hydrochloride was filtered, the filtrate was cooled again to 0 °C, and a solution of sodium azide (2.70 g, 39.7 mmol, 1.5 equiv) in H_2O (60 mL) was added. The reaction mixture was stirred at 0 °C under N_2 for 3.25 h. The THF was then removed in vacuo, H_2O (100 mL) was added, and the aqueous layer was extracted with EtOAc. The aqueous layer was acidified with concentrated HCl, and the starting acid (**5**) that precipitated was collected (4.54 g, 55.0%). The EtOAc extract was washed with saturated NaCl and dried (Na_2SO_4). Removal of the solvent gave a mixture of 3,17-dioxo-4-androsten-19-oic acid azide (**6**) plus 10β-isocyanato-4-estrene-3,17-dione (**7**) as a white glass (2.99 g, 33.6% based on the acid azide): IR ($CHCl_3$) 3005, 2945, 2860, 2255 (isocyanate), 2130 (acid azide), 1740, 1675, 1630 cm^{-1}. The mixture of **6** and **7** was dissolved in toluene (250 mL), and the solution was heated on a steam bath for 3 h. The solvent was removed in vacuo, and the material was applied to a silica gel column and eluted with benzene/EtOAc (10%). Compound **7** (1.76 g, 21.6%) when recrystallized from acetone/hexane gave white needles (1.61 g, 19.7%): mp 140.5–141.5 °C; IR ($CHCl_3$) 3030, 3010, 2945, 2890, 2860, 2250, 1735, 1680, 1620 cm^{-1}; NMR ($CDCl_3$) δ 0.95 (s, 3 H, C_{18}), 5.85 (s, 1 H, vinyl). Anal. ($C_{19}H_{23}O_3N$) C, H, N.

10β-Amino-4-estrene-3,17-dione (2). A solution of **7** (1.00 g, 3.2 mmol) in 50% acetic acid (50 mL) was heated on a steam bath for 4.25 h. The acetic acid was removed in vacuo, and 5% HCl (100 mL) was added. After extraction with EtOAc (3 × 80 mL), the aqueous solution was made basic with 20% NaOH and again extracted with EtOAc (4 × 100 mL). This extract was washed with H_2O and saturated NaCl and then dried (Na_2SO_4), and the solvent was removed. The resulting material was applied to a short silica gel column and eluted with acetone/EtOAc (1:1) to give **2** (642 mg, 70.0%), which was recrystallized from acetone/EtOAc to give off-white needles (473 mg, 51.6%): mp 201–203 °C (lit.[27] 203–205 °C); IR (KBr) 3375, 3315, 3010, 1730, 1655, 1610 cm^{-1}; NMR ($CDCl_3$) δ 0.95 (s, 3 H, C_{18}), 2.60 (s, 2 H, NH_2), 5.80 (s, 1 H, vinyl).

10β-[[(Methyloxy)carbonyl]amino]-4-estrene-3,17-dione (8). Compound **7** (140 mg, 0.45 mmol) was refluxed in anhydrous MeOH (15 mL) under N_2 for 5 h. The MeOH was evaporated in vacuo, and the white solid was recrystallized from acetone/Et_2O to give white plates (126 mg, 81.5%): mp 163–164 °C; IR ($CHCl_3$) 3455, 3010, 2950, 2890, 2860, 1760, 1670, 1630, 1500 cm^{-1}; NMR ($CDCl_3$) δ 0.95 (s, 3 H, C_{18}), 3.65 (s, 3 H, OCH_3), 5.10 (s, 1 H, NH), 5.95 (s, 1 H, vinyl). Anal. ($C_{20}H_{27}O_4N$) C, H, N.

3,17-Bis(ethylenedioxy)-5-androsten-19-yl Acetate (10). 3,17-Dioxo-4-androsten-19-yl acetate[41] (**9**; 5.20 g, 15.10 mmol), ethylene glycol (35 mL), *p*-toluenesulfonic acid (350 mg), and benzene (250 mL) were refluxed for 31 h with azeotropic distillation of H_2O, and, after the reaction mixture was made alkaline (1 mL 5% NaOH), the product was isolated as described by Amorosa et al.[39] The resulting oil was applied to a silica gel column (350 g, 5 × 60 cm) packed in benzene and eluted with a benzene/EtOAc gradient. Two products were obtained from the column. The first product eluted was 3,17-bis(ethylenedioxy)-5-androsten-19-yl acetate (**10**; 5.10 g, 78.1%), which was recrystallized from hexane to give white needles (4.45 g, 68.1%): mp 90.5–95 °C; IR ($CHCl_3$) 3005, 2950, 2880, 1735 cm^{-1}; NMR (360 MHz, $CDCl_3$) δ 0.852 (s, 3 H, C_{18}), 2.026 (s, 3 H, CH_3CO_2), 3.904 (m, 9 H, C_3 and C_{17} OCH_2CH_2O + C_{19} H), 4.440 (d, J = 11.92 Hz, 1 H, C_{19}), 5.575 (br m, 1 H, vinyl). Anal. ($C_{25}H_{36}O_6$) C, H.

The second product was 17-(ethylenedioxy)-19-hydroxy-4-androsten-3-one acetate (**11**; 1.02 g, 17.4%), which was recrystallized from hexane to give white crystals: mp 118–119.5 °C; IR ($CHCl_3$) 3005, 2950, 2875, 1735, 1665, 1620 cm^{-1}; NMR (360 MHz, $CDCl_3$) δ 0.875 (s, 3 H, C_{18}), 1.991 (s, 3 H, CH_3CO_2), 3.820 (m, 2 H, C_{17} OCH_2CH_2O), 3.902 (m, 2 H, C_{17} OCH_2CH_2O), 4.150 (d, J = 11.28 Hz, 1 H, C_{19}), 4.650 (d of d, J_1 = 11.26 Hz, J_2 = 1.09 Hz, 1 H, C_{19}), 5.894 (d, J = 0.99 Hz, 1 H, vinyl). Anal. ($C_{23}H_{32}O_5$) C, H.

3,17-Bis(ethylenedioxy)-5-androsten-19-al (13). Hydrolysis of **10** (2.02 g, 4.67 mmol) in 5% KOH in CH_3OH (100 mL) at room temperature for 3 h and recrystallization of the crude precipitated product (1.75 g, 96.0%) from acetone/hexane afforded pure 3,17-bis(ethylenedioxy)-5-androsten-19-ol (**12**; 1.50 g, 82.1%): mp 202.5–204 °C (lit.[39] 199–200 °C). Oxidation of **12** (560 mg, 1.43 mmol, 1.0 equiv) with pyridinium dichromate[42] (780 mg, 2.24 mmol, 1.50 equiv) in acid-free CH_2Cl_2 (10 mL) at room temperature under N_2 for 24 h afforded, after dilution with Et_2O, filtration through Celite, evaporation, and subsequent filtration of an Et_2O solution through silica gel, crude **13** (520 mg, 93.3%). Recrystallization from acetone/hexane yielded white crystals (393 mg, 70.5%): mp 168.5–172 °C (lit.[40] 169–171 °C); IR ($CHCl_3$) 3000, 2940, 2880, 2825, 2700, 1715 cm^{-1}; NMR (360 MHz, $CDCl_3$) δ 0.785 (s, 3 H, C_{18}), 3.874 (m, 8 H, C_3 and C_{17} OCH_2CH_2O), 5.814 (br d, J = 5.48 Hz, 1 H, vinyl), 9.663 (s, 1 H, CHO).

3,17-Bis(ethylenedioxy)-5-androsten-19-al Oxime (14). Compound **13** (1.95 g, 5.02 mmol, 1.0 equiv) and hydroxylamine hydrochloride (400 mg, 5.75 mmol, 1.15 equiv) in pyridine (25 mL) were stirred under N_2 at room temperature for 22.5 h. The solution was then poured into H_2O (250 mL) and cooled. The resulting solid was collected by filtration, washed well with H_2O, and dried to yield **14** (2.02 g, 100%). Recrystallization from acetone/hexane and EtOAc afforded white needles (1.77 g, 87.7%): mp 210.5–214 °C; IR (KBr) 3440, 2935, 2880 cm^{-1}; NMR (360 MHz, $CDCl_3$) δ 0.789 (s, 3 H, C_{18}), 3.887 (m, 8 H, C_3 and C_{17} OCH_2CH_2O), 5.613 (br m, 1 H, vinyl), 7.255 (s, 1 H, OH), 7.332 (s, 1 H, N=CH); ^{13}C NMR ($CDCl_3$) δ 14.18 (C_{18}), 21.48, 22.75, 30.63, 30.93, 31.92, 32.62, 32.96, 34.27, 42.42 (C_4), 43.81 (C_{10}), 45.78 (C_{13}), 49.63, 50.22, 64.38 + 64.56 + 64.64 + 65.28 (C_3 and C_{17} OCH_2CH_2O), 109.38 (C_3), 119.55 (C_{17}), 125.01 (C_6), 135.57 (C_5), 155.10 (C_{19}). Anal. ($C_{23}H_{33}O_5N$) C, H, N.

3,17-Dioxo-4-androsten-19-al 19-Oxime (15). Sulfuric acid (2.5 N, 2 mL) was added to a solution of **14** (200 mg, 0.50 mmol) in dioxane (18 mL), and the reaction was stirred at room temperature for 22 h. Sodium bicarbonate (1.0 g) in H_2O (40 mL) was added slowly to neutralize the acid. The solution was concentrated in vacuo, and the solid (100 mg) was collected by filtration, washed well with H_2O, and dried. Additional product was obtained by extracting the filtrate with EtOAc, washing the extract with H_2O and saturated NaCl, drying (Na_2SO_4), and removing the solvent in vacuo (total yield 142 mg, 90.8%). Recrystallization from EtOAc/hexane gave **15** as white needles (107 mg, 68.5%): mp 204–207.5 °C; IR (KBr) 3230, 3090, 2970, 2935, 2905, 2850, 2730, 1735, 1655, 1650, 1615 cm^{-1}; NMR (360 MHz, $CDCl_3$) δ 0.880 (s, 3 H, C_{18}), 5.900 (d, J = 0.96 Hz, 1 H, vinyl), 7.570 (s, 1 H, N=CH), 7.942 (s, 1 H, OH); ^{13}C NMR ($CDCl_3$) δ 13.77 (C_{18}), 20.77, 21.77, 30.83, 31.41, 32.18, 33.06, 34.02, 35.76, 36.12, 45.09 (C_{10}), 47.43 (C_{13}), 51.05, 54.09, 126.73 (C_4), 156.76 (C_9), 163.75 (C_5), 198.97 (C_3), 219.94 (C_{17}). Anal. ($C_{19}H_{25}O_3N$) C,H,N.

3,17-Bis(ethylenedioxy)-5-androsten-19-amine (16). The 19-oxime (**14**; 1.12 g, 2.78 mmol) was dissolved in EtOH (55 mL) with heating, and after cooling the solution slightly, 2 N NaOH (45 mL) was added. Raney nickel alloy (6.72 g) was added in one portion to the stirred solution, and stirring was continued, with no external heating, for 21.5 h. Vigorous gas evolution occurred during the first 10–15 min of the reaction. At the end of the reaction, the mixture was filtered through Celite, and the Celite was washed with more EtOH. Most of the EtOH was removed in vacuo, additional H_2O was added slowly, and, after cooling, two crops of the resulting white needles of analytically pure **16** were collected by filtration and then dried (963 mg, 89.2%): mp 165–165.5 °C; IR (KBr) 3360 + 3300 (NH_2 doublet), 2960, 2940, 2925, 2880, 2825, 1580 cm^{-1}; NMR (360 MHz, $CDCl_3$) δ 0.896 (s, 3 H, C_{18}), 1.143 (br s, 2 H, NH_2), 2.677 (d, J = 13.48 Hz, 1 H, C_{19}), 3.004 (d, J = 13.46 Hz, 1 H, C_{19}), 3.899 (m, 8 H, C_3 and C_{17} OCH_2CH_2O), 5.641 (br d, J = 5.27 Hz, 1 H, vinyl); ^{13}C NMR ($CDCl_3$) δ 14.83 (C_{18}), 21.55, 22.73, 30.59, 31.10, 31.64, 34.36, 34.37, 41.44 (C_{10}), 42.17 (C_4), 43.02 (C_{19}), 46.17 (C_{13}), 50.54, 51.76, 64.36 + 64.54 + 64.68 + 65.26 (C_3 and C_{17} OCH_2CH_2O), 109.50 (C_3), 119.62 (C_{17}), 125.88 (C_6), 136.34 (C_5). Anal. ($C_{23}H_{35}O_4$) C, H, N.

19-[(Trifluoroacetyl)amino]-3,17-bis(ethylenedioxy)-5-androstene (17). The 19-amine **16** (150 mg, 0.385 mmol, 1.0 equiv) and 4-(dimethylamino)pyridine (DMAP; 47 mg, 0.385 mmol, 1.0 equiv) were dissolved in CH_2Cl_2 (5 mL) in a flame-dried 25-mL round-bottom flask. Trifluoroacetic anhydride (65 μL, 96.7 mg, 0.46 mmol, 1.19 equiv) was added slowly from a syringe, and the reaction stirred at room temperature under N_2 for 50 min. The reaction mixture was evaporated in vacuo, and the residue was dissolved in CH_2Cl_2 (50 mL). The organic layer was washed with H_2O (2 × 25 mL) and saturated NaCl (25 mL) and then dried (Na_2SO_4), and the solvent was evaporated to give **17** (174 mg, 93.4%), which recrystallized from Et_2O/hexane as white plates (165 mg, 88.3%): mp 161–162.5 °C; IR (KBr) 3420, 2950, 2880, 1730 cm^{-1}; NMR (360 MHz, $CDCl_3$) δ 0.794 (s, 3 H, C_{18}) 3.308 (d, J = 15.23 Hz, 1 H, C_{19}), 3.768 (d of d, J_1 = 14.24 Hz, J_2 = 8.54 Hz, 1 H, C_{19}), 3.907 (m, 8 H, C_3 and C_{17} OCH_2CH_2O), 5.776 (d, J = 5.12 Hz, 1 H, vinyl), 6.074 (br d, J = 7.18 Hz, 1 H, NH); ^{13}C NMR ($CDCl_3$) δ 14.09 (C_{18}), 21.40, 22.51, 30.16, 30.68, 31.35, 33.95, 34.16, 34.20, 39.55 (C_{19}), 39.68 (C_{10}), 41.93 (C_4), 45.97 (C_{13}), 49.88, 51.62, 64.51 + 64.63 + 64.68 + 65.31 (C_3 and C_{17} OCH_2CH_2O), 108.76 (C_3), 116.10 (q, J = 286.9 Hz, CF_3), 119.28 (C_{17}), 127.76 (C_6), 134.78 (C_5), 156.78 (q, J = 36.6 Hz, CON). Anal. ($C_{25}H_{34}O_5NF_3$) C, H, N, F.

19-(Acetylamino)-3,17-bis(ethylenedioxy)-5-androstene (18). The 19-amine **16** (130 mg, 0.33 mmol, 1.0 equiv) in CH_2Cl_2 (5 mL) was treated with acetic anhydride (0.1 mL, 0.108 g, 1.06 mmol, 3.2 equiv) and DMAP (8 mg, 0.065 mmol, 0.20 equiv). After stirring under N_2 overnight at room temperature, the reaction mixture was diluted with additional CH_2Cl_2 (45 mL), washed with saturated $NaHCO_3$ (2 × 40 mL) and saturated NaCl, and then dried (Na_2SO_4), and the solvent was evaporated. The material was applied to a short (1.5 × 3.0 cm, 1.5 g) silica gel column (to remove the DMAP) and eluted with EtOAc to yield **18** (140 mg, 98.3%), which was recyrstalized from EtOAc/hexane to give white crystals (125.5 mg, 88.1%): mp 165–166.5 °C; IR (KBr) 3320, 3080, 2940, 2870, 1660 cm^{-1}; NMR (360 MHz, $CDCl_3$) δ 0.809 (s, 3 H, C_{18}), 1.947 (s, 3 H, CH_3CO_2), 3.177 (d, J = 13.84 Hz, 1 H, C_{19}), 3.711 (d of d, J_1 = 14.04 Hz, J_2 = 8.66 Hz, 1 H, C_{19}), 3.894 (m, 8 H, C_3 and C_{17} OCH_2CH_2O), 5.167 (br d, J = 8.00 Hz, 1 H, NH), 5.697 (br d, J = 5.16 Hz, 1 H, vinyl). Anal. ($C_{25}H_{37}O_5N$) C, H, N.

19-[(Trifluoroacetyl)amino]-4-androstene-3,17-dione (19). Compound **17** (121 mg, 0.25 mmol) in acetone (4 mL) was hydrolyzed for 2.75 h at room temperature with 2.5 N H_2SO_4 (1.0 mL). The reaction mixture was worked up according to the procedure described for compound **15**, using $NaHCO_3$ (0.25 g) in H_2O (20 mL) plus additional H_2O (10 mL) to precipitate the product, **19** (80 mg). Extraction with CH_2Cl_2 (3 × 25 mL), followed by the usual workup, yielded additional **19** (total yield 98.5 mg, 99.5%). Recrystallization from acetone/hexane gave white plates (89.8 mg, 90.7%): mp 207–209 °C; IR (KBr) 3240, 3080, 2950, 2930, 2860, 1740, 1725 + 1715, 1665, 1615 cm^{-1}; NMR (360 MHz, $CDCl_3$), δ 0.911 (s, 3 H, C_{18}), 3.765 (s, 1 H, C_{19}), 3.782, (s, 1 H, C_{19}), 5.971 (s, 1 H, vinyl), 6.564 (br s, 1 H, NH); ^{13}C NMR ($CDCl_3$) δ 13.87 (C_{18}), 20.99, 21.72, 31.64, 31.71, 32.69, 33.04, 34.29, 35.65, 35.76, 42.64 (C_{10}), 42.90 (C_{19}), 47.61 (C_{13}), 51.31, 54.27, 115.92 (q, J = 288.2 Hz, CF_3), 127.87 (C_4), 157.84 (q, J = 37.1 Hz, CON), 164.63 (C_5), 197.63 (C_3), 219.24 (C_{17}). Anal. ($C_{21}H_{26}O_3NF_3$) C, H, N, F.

19-(Acetylamino)-4-androstene-3,17-dione (20). Compound **18** (100 mg, 0.23 mmol), 2.5 N H_2SO_4 (1 mL), and acetone (4 mL) were allowed to react under N_2 for 1.25 h. After the solution was neutralized (1.0 g $NaHCO_3$ in 20 mL of H_2O) and concentrated in vacuo, the product (77 mg, 96.7%) was obtained by extraction with CH_2Cl_2 (4 × 25 mL), followed by the usual workup. Recrystallizion from acetone/Et_2O gave **20** as white crystals (74.5 mg, 93.6%): mp 200.5–201.5 °C; IR (KBr) 3320, 3080, 2930, 2850, 1735, 1660, 1620 cm^{-1}; NMR (360 MHz, $CDCl_3$) δ 0.908 (s, 3 H, C_{18}), 1.930 (s, 3 H, CH_3CO_2) 3.579 (d of d, J_1 = 13.87 Hz, J_2 = 4.82 Hz, 1 H, C_{19}), 3.766 (d of d, J_1 = 13.78, J_2 = 7.48, 1 H, C_{19}), 5.367 (br t, J = 5.79 Hz, 1 H, NH), 5.964 (d, J = 1.31 Hz, 1 H, vinyl). Anal. ($C_{21}H_{29}O_3N$) C, H, N.

19-[[[(2,2,2-Trichloroethyl)oxy]carbonyl]amino]-3,17-bis(ethylenedioxy)-5-androstene (21). The 19-amine **16** (195 mg, 0.50 mmol, 1.0 equiv) was dissolved in pyridine (5 ml). 2,2,2-Trichloroethyl chloroformate (85 μL, 131 mg, 0.62 mmol, 1.23 equiv) was added dropwise from a syringe, and the solution was stirred under N_2 at room temperature overnight. The solution was poured into H_2O (50 mL) with stirring, the resulting oil was extracted with CH_2Cl_2 (3 × 40 mL), and the extract was washed with saturated $NaHCO_3$ (3 × 30 mL), H_2O (2 × 50 mL), and saturated NaCl. The extract was dried (Na_2SO_4) and evaporated, and residual pyridine was removed by azeotropic distillation with toluene (2 × 50 mL). The resulting yellow oil (316 mg) was applied to 2 mm silica gel preparative plates, and the plates were developed with hexane/EtOAc (40%). Compound **21** was eluted with EtOAc, the extract was evaporated, and the crude product (220 mg, 77.9%) was recrystallized from Et_2O/hexane (186 mg, 65.8%): mp 150.5–152 °C; IR (KBr) 3430, 2940, 2870, 1735 cm^{-1}; NMR (360 MHz, $CDCl_3$) δ 0.810 (s, 3 H, C_{18}), 3.343 (d of d, J_1 = 14.03 Hz, J_2 = 2.16, 1 H, C_{19}), 3.541 (d of d, J_1 = 13.73 Hz, J_2 = 8.88 Hz, 1 H, C_{19}), 3.899 (m, 8 H, C_3 and C_{17} OCH_2CH_2O), 4.679 (br s, 1 H, NH), 4.702 (s, 2 H, CCl_3CH_2), 5.694 (br d, J = 4.25 Hz, 1 H, vinyl); ^{13}C NMR ($CDCl_3$) δ 14.41 (C_{18}), 21.33, 22.61, 30.37, 30.84, 31.39, 33.68, 34.16, 34.26, 39.94 (C_{10}), 41.54 + 41.93 (C_4 and C_{19}), 46.04 (C_{13}), 50.00, 51.66, 64.44 + 64.59 + 64.67 + 65.27 (C_3 and C_{17} OCH_2CH_2O), 74.83 (CH_2OCON), 95.78 (CCl_3), 109.01 (C_3), 119.39 (C_{17}), 127.10 (C_6), 135.26 (C_5), 154.23 (CON). Anal. ($C_{26}H_{36}O_6NCl_3$) C, H, N, Cl.

Biochemical Methods. Materials and Instruments. Unlabeled steroids for the biochemical assays were either prepared in this laboratory or were generous gifts from Searle Laboratories, Skokie, IL. The following materials were puchased from Sigma Chemical Co., St. Louis, MO: NADP (monosodium salt, Sigma grade), glucose 6-phosphate (dipotassium salt hydrate), lyophilized glucose-6-phosphate dehydrogenase (type XV from baker's yeast), sodium pyruvate solution (0.02 M sodium pyruvate in 0.1 M potassium phosphate buffer, pH 7.5), NADH (disodium salt, grade III, 0.2- or 2-mg preweighed vials), Sigma Enzyme Control 2N (normal range), bovine serum albumin (fraction V), and activated charcoal (washed with HCl). The sources of other compounds needed for the assays were as follows: dithiothreitol (Cleland's reagent), Calbiochem-Behring Corp., LeJolla, CA, or P-L Biochemicals, Inc., Milwaukee, WI. All reagents and solvents were analytical grade. Linear-K glass-backed silica gel thin-layer plates (LK6F, Whatman, Inc., Clifton, NJ) were purchased through Anspec Co., Ann Arbor, MI.

Centrifugations during enzyme preparation were performed on a Beckman J21B centrifuge (JA-20 rotor) and a Beckman L5-50 ultracentrifuge (50.2 Ti rotor). Routine centrifugations during the ^3H_2O assays were done with an International centrifuge, either Model UV or Model CS. All incubations were performed in air in a Dubnoff PS metabolic shaking incubator. All evaporations were done under N_2 at temperatures between room temperature and 40 °C.

Radiolsotopes and Liquid Scintillation Counting. [1-^{3}H]Androst-4-ene-3,17-dione was prepared from [1,2-^{3}H]-androst-4-ene-3,17-dione, purchased from New England Nuclear, Boston, MA, by refluxing in 2% methanolic KOH.[55,61] The stereochemical distribution of the tritium in [1-^{3}H]androst-4-ene-3,17-dione, as determined by the procedure described by Reed and Ohno[54] and later confirmed by ^{3}H NMR of the starting 1,2-^{3}H androgen,[62] was 83% 1β and 17% 1α. These values were consistent with those reported previously.[54,55,61,63] The purity of the substrate was evaluated prior to use by thin-layer chromatography on silica gel plates (Eastman) in benzene/EtOAc (2:1, v/v), followed by scanning the radioactive chromatographic plates on a Berthold TLC-Scanner LB-2723. When necessary, the substrate was purified prior to use by preparative TLC (LK6F silica gel plates, 5 × 20 cm, Whatman)[64] or purified by filtration through a silica gel Sep-Pak, with benzene/EtOAc (2:1) as the solvent

(61) Brodie, H. J.; Warg, P. A. *Tetrahedron* **1967**, *23*, 535.
(62) The ^{3}H NMR spectrum of [1,2-^{3}H]adrostendione (lots 1163-039 and 1319-080) was kindly furnished by New England Nuclear.
(63) Brodie, H. J.; Raab, K.; Possanza, G.; Seto, N.; Gut, M. *J. Org. Chem.* **1969**, *34*, 2697.
(64) The steroid was first concentrated at the origin of the plate by developing the application zone (3–3.5 cm) with $CHCl_3$/MeOH (2:1, v/v). The purified steroid was eluted from the plate with $CHCl_3$/MeOH (2:1).

system. The purified steroids were generally stored in benzene/EtOH (9:1, v/v).

Radioactive samples were counted in glass LS vials in 10 mL of Formula 963 (New England Nuclear) on a Tracor Analytical Mark III scintillation counter, Model 6881, or a Packard Tri-Carb scintillation counter, Model 3320. Quenched [^{3}H]toluene and [^{3}H]water standards, prepared in Formula 963, were used to determine counting efficiencies; quench correction was performed automatically by the Tracor Analytical scintillation counter. Addition of assay buffer components to the LS samples did not alter the quench curve.

Enzyme Preparation. Human term placental tissue was washed extensively with 0.15 M KCl, minced, and homogenized in homogenization buffer (0.25 M sucrose, 0.04 M nicotinamide, 0.05 M potassium phosphate, pH 7.0; two parts tissue to one part buffer, w/v), and the microsomal fraction was obtained by differential centrifugation as previously described.[12,56] The microsomal pellet, after two washs and recentrifugations in 0.05 M potassium phosphate buffer, pH 7.0, was checked for lactate dehydrogenase activity (Sigma's Technical Bulletin no. 340-UV, based upon the procedure of Wroblewski and LaDue[65]), a marker enzyme for cytosol contamination,[66] and the activity was found to be negligable. The microsomal pellet was then washed with distilled water, resuspended in distilled water, and lyophilized, and the lyophilized enzyme preparation was stored desiccated at −20 °C. Prepared and stored in this manner, the enzyme can be kept for at least 1.5 years without loss of activity.[57] The protein content of the lyophilized enzyme preparation, as determined by Lowry's procedure,[66] was 0.574 ± 0.027 mg of protein/mg of solid.

Screening Assay Procedure. The procedure is essentially that described by Reed and Ohno.[54] [1-^{3}H]Androst-4-ene-3,17-dione in EtOH was added to propylene glycol (3.3 μL per sample), followed by evaporation of the EtOH under N_2. A cofactor solution in a 15 mM potassium phosphate buffer, pH 8.0 at 25 °C, containing KCl, EDTA, and dithiothreitol, was added, and the solution was incubated at room temperature for at least 30 min to ensure reduction of NADP. Inhibitors in propylene glycol (6.67 μL per sample) were prepared as described above and diluted with 15 mM potassium phosphate buffer (53.3 μL per sample). The substrate/cofactor solution (0.44 mL per sample) and inhibitor solutions (60 μL per sample) were combined in large test tubes, and the reaction was started by the addition of a solution of lyophilized enzyme in a 5 mM potassium phosphate buffer, pH 7.5 at 4 °C (0.5 mL per sample), containing KCl, EDTA, and dithiothreitol. The final concentration of the components in the incubations were as follows: [1-^{3}H]androst-4-ene-3,17-dione, 0.25 μM, (0.25 μCi); inhibitor, 0, 0.25, or 1.25 μM; propylene glycol, 10 μL; lyophilized human placental microsomes, 0.1 mg of protein; NADP, 0.5 mM; glucose 6-phosphate, 2.5 mM; glucose-6-phosphate dehydrogenase, 1 unit; KCl, 100 mM; EDTA, 1.0 mM; dithiothreitol, 10 mM; potassium phosphate buffer, 10 mM. The samples were incubated in a shaking water bath at 25 °C for 15 min, and the reaction was stopped by the addition of $CHCl_3$ (10 mL), followed by vortexing the samples for 50 s. Blanks (0 min incubations) were run in duplicate, and all other samples were run in triplicate.

The samples were centrifuged for 10–15 min (ca. 1200g), and the aqueous layers were transferred to disposable polystyrene conical tubes. The aqueous layers were treated with acid-washed charcoal (50 mg per sample) and centrifuged again, and aliquots (2 × 0.2 mL) were counted for radioactivity. Aliquots of the substrate/cofactor solution were also counted for radioactivity to determine the actual substrate concentration. The ^3H_2O released was found to be proportional to the amount of estrogen produced (data not shown), and the amount of estrogen can be calculated as described by Reed and Ohno.[59] Under these conditions, product formation in the control samples (no inhibitor added) was 13.4 ± 3.4% (mean ± SD for five experiments), and the rate of production formation was constant for 15 min (data not shown). Blanks generally showed negligible conversion (<0.1%). Production formation for samples containing an inhibitor was compared to that of the control samples run simultaneously and are reported as percent inhibition of control samples (Table I).

For the K_M determination, where the substrate concentration was varied, the substrate was omitted from the stock cofactor solution and added to individual samples as a more concentrated propylene glycol/buffer (1:8) solution, prepared as described above. The substrate (0.0054–0.157 μM) was incubated with enzyme (0.025 mg of microsomal protein per sample for 5 min), and the remainder of the procedure was completed as described for the screening assay. Under these conditions, the conversion of substrate to product did not exceed 12.5%, and the reaction velocity was constant, even at the lowest substrate concentration (data not shown).

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