# Role of Hydrophilic Interaction in Binding of Hydroxylated 3-Deoxy $C_{19}$ Steroids to the Active Site of Aromatase

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As part of our investigation into the structure−activity relationship of a novel class of aromatase inhibitors, $C_{19}$ steroids having no oxygen function at C-3, we tested aromatase inhibition activity of polar diol compounds 4,19-dihydroxyandrost-5-en-17-ones (**25** and **27**) and 6,19-dihydroxyandrost-4-en-17-ones (**36** and **37**). 4α,19-Diol **25** was synthesized from *tert*-butyldimethylsilyoxyandrost-4-ene steroid (**9**) through its $OsO_4$ oxidation, giving the 4α,5α-dihydroxy derivative **12,** as a key reaction. Acetylation of 5*β*,6α-dihydroxy-19-acetate **30** and its 5α,6*β*-analogue **31** followed by dehydration with $SOCl_2$ and alkaline hydroxysis gave 6α,19-diol **36** and its 6*β*-isomer **37**, respectively. The stereochemistry of a hydroxy group at C-4 of compound **25** and that at C-6 of compounds **36** and **37** were determined on the basis of $^1$H NMR spectroscopy in each case. 4*β*,19-Diol **27**, previously synthesized, was identified as an extremely powerful competitive inhibitor of aromatase ($K_i$ = 3.4 nM). In contrast, its 4α,19-dihydroxy isomer **25** and other series of diol compounds, 6,19-dihydroxy-4-en-17-one steroids, were moderate to poor competitive inhibitors ($K_i$ = 110−800 nM). Through this series of analyses, it was concluded that hydrophilic interaction of a 4*β*,19-diol function with the active site of aromatase plays a critical role in the tight binding of 3-deoxy-5-ene steroids.

## Introduction

Aromatase, a unique cytochrome P-450−enzyme complex, catalyzes the conversion of 4-en-3-one androgens, androst-4-ene-3,17-dione (androstenedione, **1**), and testosterone to the phenolic estrogens, estrone, and estradiol.[1−3] Inhibitors of this enzyme are useful in treating estrogen-dependent diseases such as breast cancer.[4−9] For this reason, various steroids have been tested in a number of laboratories as aromatase inhibitors. Structure−activity studies on aromatase inhibitors[10−19] and a recent structural prediction of aromatase by homology modeling[20−25] predicted the existence of a hydrophobic binding pocket extending roughly in the plane of the substrate androstenedione (**1**) from the position that would be occupied by its $C_4$, $C_6$, and $C_7$ atoms. Thus, introducing a hydrophilic group, such as a hydroxyl, at the C-6[26] and C-19[2] positions of steroid **1** decreases the affinity to the active site of aromatase.

We have previously reported that a 3-deoxy derivative of compound **1**, androst-4-en-17-one (**2**), is an excellent competitive inhibitor of aromatase,[19,27] although this is lacking a carbonyl group at C-3 that is thought to be essential for the proper binding of substrate **1** to the active site of the enzyme.[20−25] On the other hand, androst-5-en-17-one (**6**), an isomer of 3-deoxy steroid **2**, is a fair competitive inhibitor[28] (Figure 1). The structure−activity relationships indicated that a 17-carbonyl function is necessary for an effective binding of 3-deoxy steroids **2** and **6** to the active site and that binding geometries of the two steroids are different in the region of an A−B ring system of the steroid molecule.[28,29] The binding pocket also tolerates a polar hydroxy group at the 6- or 19-position of 4-ene steroid **2**[27,30] and at the 4*β*-position of 5-ene steroid **6**.[31] The 4-ene system plays a critical role in the tight binding of a steroid without a carbonyl function at C-3 to the active site.

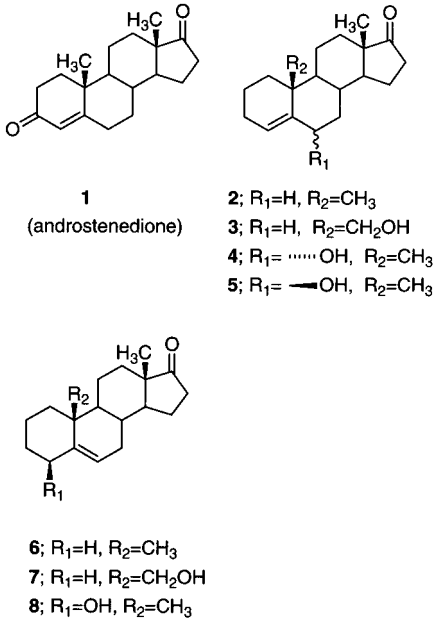


**Figure 1.** Structures of androstenedione and 3-deoxy steroids.

In continuing the study of the 3-deoxy steroids as conformational and catalytic probes for the active site of aromatase, we were interested in steroids that have a hydrophilic structure combining the 19-hydroxy group with a hydroxy function at the allylic position, i.e., C-4 of the 5-ene **6** or C-6 of 4-ene steroid **2.** This study focused on the synthesis and biochemical evaluation of 4,19-dihydroxyandrost-5-en-17-ones and 6,19-dihydroxyandrost-4-en-17-ones. 5-Ene-4*β*,19-diol **27** was found to

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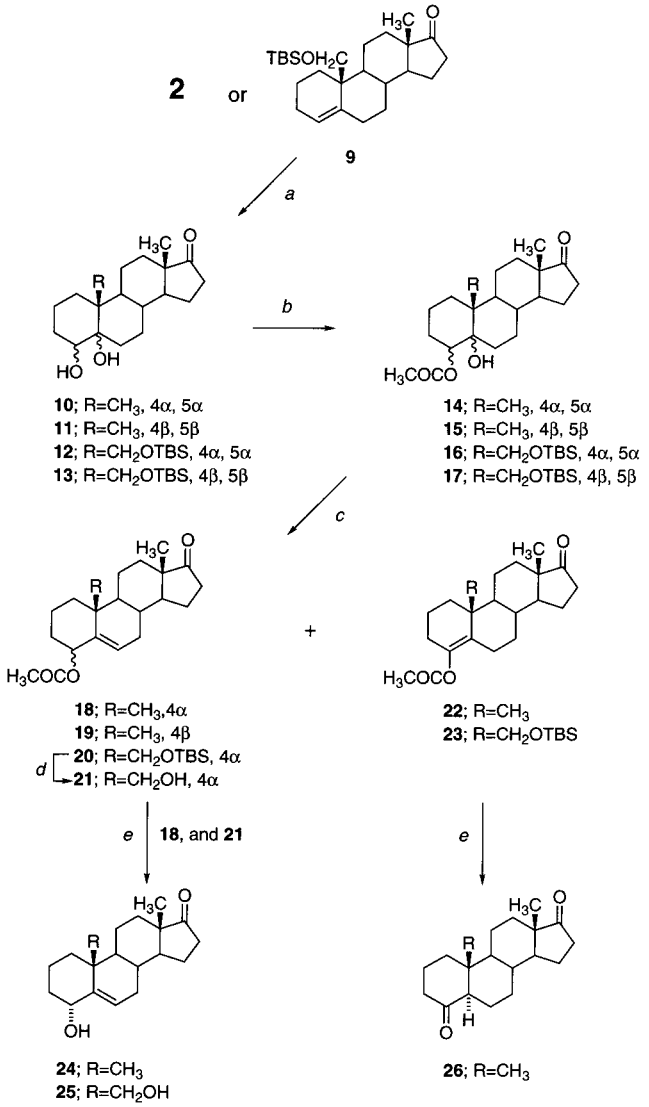
**Scheme 1**[a]



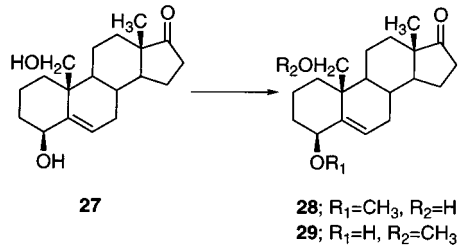

[a] Reagents: (a) (i) $OsO_4$, pyridine, (ii) aqueous $NaHSO_3$; (b) $(CH_3CO)_2O$, pyridine; (c) $SOCl_2$, pyridine; (d) dilute HCl, THF, propan-2-ol; (e) $K_2CO_3$, MeOH, $H_2O$.

be a very powerful competitive inhibitor, indicating that a hydrophilic interaction between the active site and the diol structure plays an important role in the tight binding.

## Results

**Chemistry.** 5-En-4α-ol **24**, having the angular 19-methyl group, was initially prepared to show the effect of the 4α-hydroxy group of 5-ene steroid **6** on the affinity to aromatase (Scheme 1). Treatment of 4-ene compound **2** with $OsO_4$ followed by reductive cleavage of the osmium adduct with $NaHSO_3$ gave an approximate 3:1 mixture of 4α,5α-diol **10** to 4*β*,5*β*-diol **11**. After separation of the mixture using silica gel column chromatography, 5α-compound **10** was acetylated with $Ac_2O$ and pyridine and subsequently dehydrated with $SOCl_2$ to give 5-en-4α-acetate **18** and 4-enol acetate **22** (75% and 18% from 4α-acetate **14**, respectively). The stereochemistry of the 4α-acetoxy group was determined on the basis of $^1$H NMR spectroscopy. Thus, the 41% nuclear Overhauser effect (NOE) enhancement of the 19-methyl protons ($\delta$ = 1.07 ppm) of compound **18** was produced by irradiation of the 4*β*-proton ($\delta$ = 5.43 ppm). Furthermore, the stereochemistry was unambiguously proven by a comparison of this compound with 4*β*-acetate **19** that was previously obtained.[32] The structure of enol acetate **22** was assigned on the basis of NMR spectroscopy [$^1$H, $\delta$ 2.12 ppm (4-OCOCH$_3$); $^{13}$C, 130.2, 140.2, 169.1, and 221.1 ppm (four sp$^2$ carbons)]. Alkaline hydrolysis of compound **22** yielded 5α-4-one product **26**, also supporting the assigned structure. Treatment of acetate **18** with $K_2CO_3$ afforded 4α-ol **24**.

**Scheme 2**[a]



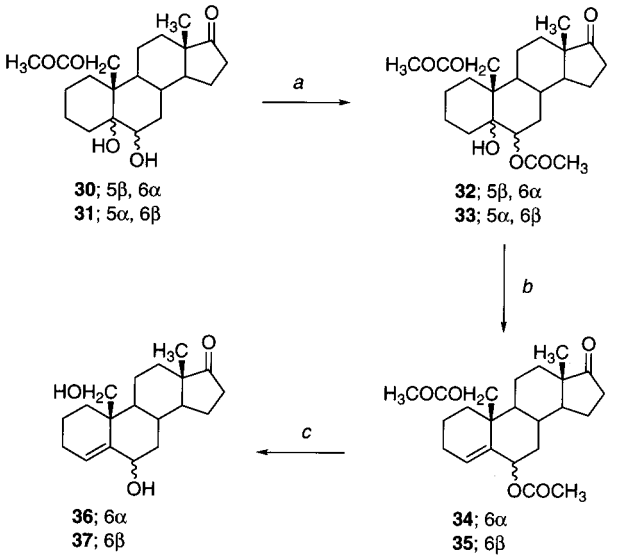

[a] Reagents: $CH_3I$, $Ag_2O$.

4α-Hydroxy derivative of 19-ol **7**, compound **25**, was synthesized in approximate 10% yield, starting from 19-*tert*-butyldimethylsilyloxy-4-ene-compound **9**,[28] in a sequence similar to that described above. This synthesis involves, sequentially, $OsO_4$ oxidation, acetylation of 4α,5α-diol **12** obtained after separation of the oxidation products, dehydration, and deprotection of protecting groups at C-4 with $K_2CO_3$ and at C-19 with dilute HCl (Scheme 1). The stereochemistry of an acetoxy group at C-4 of the intermediate 4α-acetoxy-5-ene steroid **20** was similarly assigned on the basis of $^1$H NMR spectroscopy. Irradiation of the 4*β*-proton ($\delta$ = 5.05 ppm) of 4α-acetate **16** caused 11% NOE enhancement of the 19-methylene proton ($\delta$ = 3.71 ppm). In contrast, there was no NOE enhancement of the 19-methylene proton obtained through irradiation of the 4α-proton ($\delta$ = 5.34 ppm) of 4*β*-acetate **17**. In this sequence, the dehydration of 4*β*-acetoxy-5*β*-ol **17** gave principally 4-acetoxy-4-ene product **23** (56%), and the 5-en-4*β*-acetoxy compound could not be isolated in a pure form. The dehydration of another 4*β*-acetoxy-5*β*-ol **15** also gave 4-enol acetate **22** (47%) as a major product along with 5-en-4*β*-acetate **19** (24%), indicating that not only the conformations at C-4 and C-5 but also the structure at C-19 of a 4-acetoxy-5-ol steroid affect the direction of dehydration of the 5-hydroxy moiety with $SOCl_2$.

Treatment of 4*β*,19-diol **27**, previously synthesized,[33] with $CH_3I$ in the presence of $Ag_2O$ gave 4- and 19-monomethyl ethers **28** and **29**, respectively (Scheme 2).

Previously synthesized 19-acetoxy-5*β*,6α- and 5α,6*β*-*trans*-diols **30** and **31**[34] were separately acetylated to give 6α- and 6*β*-acetates **32** and **33**, respectively, of which treatment with $SOCl_2$ followed by hydrolysis with $K_2CO_3$ yielded 4-ene-6α,19-diol **36** (68% from **32**) and its 6*β*-isomer **37** (47% from **33**), respectively (Scheme 3). The stereochemistry of the C-6 hydroxy moiety of 6,19-diols was determined on the basis of $^1$H NMR spectroscopy [6*β*-H at 4.27 ppm (m) and 4-H at 6.07 ppm (t, $J$ = 4.9 Hz) for the 6α-ol **36** and 6α-H at 4.29 ppm (t, $J$ = 2.9 Hz) and 4-H at 5.86 (t, $J$ = 3.6 Hz) for the 6*β*-ol **37**]. Irradiation of the 6*β*-proton of 6α-ol **36** produced 1.4% and 9.8% NOE enhancement of the 19-methylene protons at 3.60 and 3.84 ppm, respectively, and 2.2%

**Scheme 3**[a]




[a] Reagents: (a) $(CH_3CO)_2O$, pyridine; (b) $SOCl_2$, pyridine; (c) $K_2CO_3$, MeOH, $H_2O$.

NOE enhancement of the C-4 proton at 6.07 ppm. In contrast, no significant NOE enhancement of the 19-methylene protons was detected by irradiation of the 6α-proton of 6β-ol **37** whereas 11.5% NOE enhancement of the 4-proton at 5.86 ppm was observed in the irradiation.

**Biochemical Properties.** Inhibition of aromatase activity in human placental microsomes by 4,19-diols **25** and **27** and their derivatives, 6,19-diols **36** and **37**, and 4α-ol **24** and its acetate **18** was examined in vitro by enzyme kinetics. The results are given in Table 1. In addition to the above compounds, the parent 4- and 5-ene steroids **2** and **6** and their respective 19-ols **3** and **7** are listed for comparison. Aromatase activity in placental microsomes was determined using a radiometric assay in which tritiated water released from [1β-$^3$H]androstenedione (**1**) into the incubation medium during aromatization was measured.[35] To characterize the nature of the inhibitor binding to the active site of aromatase, aromatization was measured at several inhibitor and substrate concentrations. The results of these studies were plotted on a typical Lineweaver−Burk plot. In these studies, the apparent $K_m$ and $V_{max}$ values for androstenedione (**1**) were approximately 33 nM and 128 (pmol/min)/mg of protein, respectively. All the steroids studied exhibited clear competitive-type inhibition. The apparent inhibition constants ($K_i$) were obtained by Dixon plots. The Lineweaver−Burk plot of aromatase inhibition by inhibitor 4β,19-diol **27** is shown in Figure 2.

## Discussion

To clarify the role of hydrophilic interaction in the binding of a series of 3-deoxy steroids to the active site of aromatase, the effect of introducing a 6α- or 6β-hydroxy group into 19-hydroxyandrost-4-en-17-one (**3**), which has a high affinity to aromatase with a $K_i$ of 5.8 nM,[27] and that of a 4α- or 4β-hydroxy group into 19-hydroxyandrost-5-en-17-one (**7**), of which the affinity is very low,[29] on aromatase inhibition were principally tested. Among diol steroids examined in this study,

**Table 1.** Aromatase Inhibition by 4- or 6- Hydroxy and 5α-4-One Steroids[a]

| compound | $IC_{50}$,[b] μM | apparent $K_i$,[c] nM |
|---|---|---|
| 4-Hydroxy-5-ene Series | | |
| 4α-ol **24** | 0.86 | 58 |
| 4α-acetate **18** | 0.70 | 60 |
| 4α,19-diol **25** | 6.8 | 800 |
| 4α-acetoxy-19-ol **21** | 11.0 | |
| 4β-ol **8**[d] | 0.28 | 25 |
| 4β-acetate **19**[d] | 1.0 | 90 |
| 4β,19-diol **27** | 0.031 | 3.4 |
| 4β-methoxy-19-ol **28** | 150 | |
| 4β-hydroxy-19-methoxide **29** | 20% inhibition at 10 μM | |
| 6-Hydroxy-4-ene Series | | |
| 6α-ol **4**[e] | 0.19 | 21 |
| 6α,19-diol **36** | 1.4 | 250 |
| 6β-ol **5**[e] | 0.050 | 6.0 |
| 6β,19-diol **37** | 0.94 | 110 |
| For Comparison | | |
| 4-ene steroid **2**[e] | 0.060 | 6.8 |
| 4-en-19-ol **3**[e] | 0.049 | 5.8 |
| 5-ene steroid **6**[f] | 0.66 | 120 |
| 5-en-19-ol **7**[f] | 6.9 | 1000 |

[a] Inhibition type was determined by Lineweaver−Burk plot. In these experiments, the apparent $K_m$ for the substrate androstenedione was found to be about 33 nM. All of the compounds listed showed a competitive type of inhibition. [b] A concentration of 300 nM of [1β-$^3$H] androstenedione and 20 μg of protein from human placental microsomes were used. [c] Inhibition constant was obtained by Dixon plot. [d] Reference 31. [e] Reference 30. [f] Reference 29.

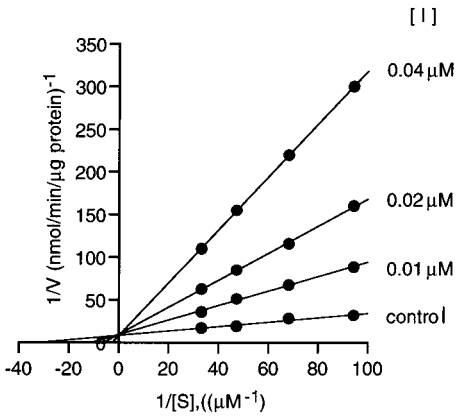



**Figure 2.** Lineweaver−Burk plot of inhibition of human placental aromatase by 4β,19-diol **27** with androstenedione (**1**) as the substrate. Each point represents the mean of two determinations that varied by less than 5% of the mean. The inhibition experiments with the other inhibitors examined in this study gave essentially results similar to the results for **27** (data not shown).

5-ene-4β,19-diol **27** was an extremely potent competitive inhibitor of aromatase in human placental microsomes, and with an apparent $K_i$ value of 3.4 nM, it is one of the most powerful steroidal aromatase inhibitors reported to date. In contrast, the 4α-isomer of inhibitor **27**, compound **25**, and 6,19-diols **36** and **37** with a 4-ene structure were moderate to poor inhibitors of aromatase, with $K_i$ values ranging between 110 and 800 nM.

The introduction of a 4β-hydroxy group into 5-en-19-ol **7** enhanced about 300-fold the affinity to aromatase. This modification is equivalent to the introduction of a 19-hydroxy group into 4β-ol **8** ($K_i$ = 25 nM) where the affinity was increased by 85-fold. The 4β-hydroxylated 19-ol **27** is the first powerful aromatase inhibitor having

such polar functions at the A,B-ring system. In contrast, its 4α-isomer **25** was a poor inhibitor of aromatase with almost the same affinity as the parent 19-ol **7** ($K_i = 800$ vs 1000 nM for **25** vs **7**) (Table 1); however, introducing a 4α-hydroxy function into 19-methyl-5-ene compound **6** slightly increased the affinity to aromatase ($K_i = 58$ vs 120 nM, 4α-ol **24** vs **6**), as seen in the case of the 4*β*-hydroxylation.[31] 4α-Acetylation of 4α,19-diol **25** decreased the affinity ($IC_{50} = 6.8$ and 11.0 $\mu$M for **25** and 4α-acetoxy-19-ol **21**, respectively), although acetylation of 4α-ol **24** does not change the affinity ($K_i$ for 4α-acetate **18** is 60 nM). Methylation of either a 4*β*-hydroxy or a 19-hydroxy group of the powerful inhibitor **27** dramatically decreased the affinity. Taken together, it is shown that a combination of 4*β*- and 19-hydroxy groups of 4*β*,19-diol **27** is important in the formation of a thermodynamically stable inhibitor−aromatase complex.

On the other hand, the introduction of a hydroxyl group at the C-6α or C-6*β* position of 4-en-19-ol **3**, which is equivalent to the introduction of a 19-hydroxy group into either 6α-ol **4** or 6*β*-ol **5**, lowered to a great extent the affinity of each parent inhibitor with a high affinity to aromatase. The results indicate that a 6α,19- or 6*β*,19-diol structure does not tolerate the access of 4-ene-diols **36** and **37** to aromatase; either a 6*β*-hydroxy or a 19-hydroxy moiety existing in the *β*-side region of a steroid nucleus is enough for production of the thermodynamically stable complex with aromatase in the 3-deoxy-4-ene series, and the additional hydroxy group interferes with the production of the stable complex.

Structure−activity relationships[27−30] and the aromatase-catalyzed 19-oxygenation[37] of a series of 3-deoxy $C_{19}$ steroids previously revealed that there is a marked difference in the binding manner between the two parent 3-deoxy-4-ene and 5-ene steroids, **2** and **6**, in the active site and that the binding aspect of the latter is comparable to that of substrate **1**. The mechanistic proposals by Laughton's group as well as Robinson's and Simpson's groups incorporate critical information from site-directed mutagenesis and molecular modeling studies that place glutamic acid ($^{302}$Glu) as an essential active-site amino acid.[20,23,24,37] Substrate androstenedione (**1**) is anchored by a hydrogen bond between an active-site amino acid ($^{128}$His) and the C-3 carbonyl function.[37] On the other hand, it is reported that $^{309}$Asp and $^{310}$Thr are close to the catalytic site in the active site,[20,21,24] suggesting that a carboxyl group of $^{309}$Asp and a hydroxy group of $^{310}$Thr may also play a role in the catalytic mechanism.

On the basis of these previous reports, it is likely that 4*β*,19-diol **27** would be anchored by hydrogen bonds between two hydroxy groups of the inhibitor and two polar amino acid residues in the active site such as $^{128}$His, $^{302}$Glu, $^{309}$Asp, and/or $^{310}$Thr. The remaining carbonyl group at C-17 of compound **27** also becomes very important in anchoring this inhibitor in the active site, similar to the other 3-deoxy steroids.[19,28−30] The present findings regarding a hydrophilic interaction of a series of hydroxylated 3-deoxy $C_{19}$ steroids with aromatase would be useful for understanding spatial and electronic aspects of the binding (active) site of the enzyme as well as for developing an effective aromatase inhibitor.

## Experimental Section

**Materials and General Methods.** Androst-4-en-17-one (**2**)[27] and its 19-*tert*-butyldimethylsiloxy derivative **9**,[28] 4*β*-hydroxyandrost-5-en-17-one (**8**),[30] 5*β*,6α-dihydroxy-19-acetoxyandrostan-17-one (**30**) and its 5α,6*β*-dihydroxy derivative **31**,[33] and 4*β*,19-diol **27**[33] were prepared according to known methods. [1*β*-$^3$H]Androstenedione (27.5 Ci/mmol ($^3$H distribution of 74−79% at 1*β*) was purchased from New England Nuclear Corp. (Boston, MA) and NADPH from Kohjin Co. Ltd. (Tokyo, Japan).

Melting points were measured on a Yanagimoto melting point apparatus and were uncorrected. IR spectra were recorded in KBr pellets, except for oily compounds of which spectra were obtained in neat forms, on a Perkin-Elmer FT-IR 1725X spectrophotometer. $^1$H and $^{13}$C NMR spectra were obtained in $CDCl_3$ solution with JEOL EX 270 (270 MHz for $^1$H and 67.5 MHz for $^{13}$C) and GSX 400 (400 MHz for $^1$H and 100 MHz for $^{13}$C) spectrometers, respectively (Tokyo, Japan), using tetramethylsilane ($\delta = 0.00$) or C*H*$Cl_3$ ($\delta = 7.26$, for *tert*-butyldimethylsilyl derivatives) as an internal standard, and mass spectra (MS; electron impact) were obtained with a JEOL JMS-DX 303 spectrometer. Thin-layer chromatography (TLC) was performed on E. Merck precoated silica gel plates (Darmstadt, Germany). Column chromatography was conducted with silica gel (E. Merck, 70−230 mesh).

**Treatment of the 4-Ene Steroid 2 or 9 with $OsO_4$.** $OsO_4$ (360 mg, 1.42 mmol) was added to a solution of 19-methyl steroid **2** (354 mg, 1.3 mmol) in pyridine (14 mL), and the mixture was stirred at room temperature for 3 h. After this time, $NaHSO_3$ (680 mg, 6.5 mmol) in $H_2O$ (10 mL) was added. The mixture was stirred until the color changed to orange, and then it was diluted with EtOAc, washed with NaCl solution, and dried with $Na_2SO_4$. Evaporation of the solvent gave a brown oil (380 mg) that was purified by column chromatography (hexanes−EtOAc) followed by recrystallization from hexanes−EtOAc to afford two products: 4α,5α-dihydroxyandrostan-17-one (**10**) and 4*β*,5*β*-dihydrosyandrostan-17-one (**11**).

Compound **10** (224 mg, 56%): mp 116−117 °C; $^1$H NMR (270 MHz) $\delta$ 0.86 (3H, s, 18-Me), 0.99 (3H, S, 19-Me), 4.02 (1H, dd, $J = 5.3$ and 11.2 Hz, 4*β*-H); MS *m*/*z* (rel intens) 306 ($M^+$, 85), 288 (48), 270 (18), 234 (100), 219 (42); IR $\nu_{max}$ 3471 (OH), 1740 (C═O) cm$^{-1}$. Anal. ($C_{19}H_{30}O_3$) C, H.

Compound **11** (75 mg, 17%): mp 172−173 °C; $^1$H NMR (270 MHz) $\delta$ 0.86 (3H, s, 18-Me), 0.96 (3H, s, 19-Me), 3.70 (1H, m, 4α-H); MS *m*/*z* (rel intens) 306 ($M^+$, 68), 288 (35), 270 (20), 234 (90), 219 (38), 97 (100); IR $\nu_{max}$ 3478 (OH), 1732 (C═O) cm$^{-1}$. Anal. ($C_{19}H_{30}O_3$) C, H.

19-*tert*-butyldimethylsiloxy steroid **9** (1.91 g, 5 mmol) was treated with $OsO_4$ (1.33 g, 5.2 mmol) in a similar fashion (pyridine, 52 mL; reaction period, 2.2 h; $NaHSO_3$, 3.3 g, 31.7 mmol; $H_2O$, 54 mL). The brown oily product was purified by column chromatography (hexanes−EtOAc) to afford two products: 19-(*tert*-butyldimethylsilyloxy)-4α,5α-dihydroxyandrostan-17-one (**12**) and 19-(*tert*-butyl-dimethylsiloxy)-4*β*,5*β*-dihydroxyandrostan-17-one (**13**).

Compound **12** (480 mg, 23%): mp 174−175 °C (from acetone−hexane); $^1$H NMR $\delta$ 0.06 and 0.07 (3H each, s, 19-OSi*Me*$_2$), 0.87 (3H, s, 18-Me), 0.89 (9H, s, 19-OSiMe$_2$C*Me*$_3$), 3.63 and 3.91 (1H each, d, $J = 10.6$ Hz, 19-$CH_2$), 3.71 (1H, m, 4*β*-H); MS *m*/*z* (rel intens) 436 ($M^+$, 7), 379 (31), 361 (43), 303 (26), 287 (100); IR $\nu_{max}$ 3455 (OH), 1729 (C═O) cm$^{-1}$. Anal. ($C_{25}H_{44}O_4Si$) C, H.

Compound **13** (oil, 572 mg, 25%): $^1$H NMR $\delta$ 0.11 and 0.12 (3H each, s, 19-OSi*Me*$_2$), 0.84 (3H, s, 18-Me), 0.93 (9H, s, 19-OSiMe$_2$C*Me*$_3$), 3.59 and 4.34 (1H each, d, $J = 10.6$ Hz, 19-$CH_2$), 3.89 (1H, m, 4α-H); MS *m*/*z* (rel intens) 436 ($M^+$, 3), 379 (28), 361 (94), 287 (100), 269 (89); IR $\nu_{max}$ 3448 (OH), 1740 (C═O) cm$^{-1}$; HRMS ($C_{25}H_{44}O_4Si$) found 436.3009, calcd 436.3030.

**Acetylation of 4,5-Diols 10−13.** 19-Methyl compounds **10** and **11** (200 mg, 0.68 mmol) and 19-siloxy compounds **12** and **13** (450 mg, 3 mmol) were separately acetylated with acetic anhydride and pyridine (1 and 2 mL for **10** and **11**, respectively, and 2.2 and 4.5 mL for **12** and **13**, respectively) (room

temperature, overnight). After the usual treatment, the crude product obtained was recrystallized from an appropriate solvent.

4α-Acetoxy-5α-hydroxyandrostan-17-one (**14**) (198 mg, 87%): mp 178−179 °C (from EtOAc); $^1$H NMR (270 MHz) $\delta$ 0.85 (3H, s, 18-Me), 1.00 (3H, s, 19-Me), 2.09 (3H, s, 4-OCOMe), 5.38 (1H, m, 4$\beta$-H); MS *m*/*z* (rel intens) 348 ($M^+$, 40), 288 (95), 270 (32), 234 (100), 219 (33); IR $\nu_{max}$ 3538 (OH), 1729 (C=O) cm$^{-1}$. Anal. ($C_{21}H_{32}O_4$) C, H.

4$\beta$-Acetoxy-5$\beta$-hydroxyandrostan-17-one (**15**) (193 mg, 85%): mp 198−199 °C (from acetone); $^1$H NMR (270 MHz) $\delta$ 0.86 (3H, s, 18-Me), 1.01 (3H, s, 19-Me), 2.07 (3H, s, 4-OCOMe), 4.99 (1H, t, $J$ = 8.2 Hz,4α-H); MS *m*/*z* (rel intens) 348 ($M^+$, 60), 330 (10), 288 (100), 270 (36), 234 (91); IR $\nu_{max}$ 3596 (OH), 1737 (C=O) cm$^{-1}$. Anal. ($C_{21}H_{32}O_4$) C, H.

4α-Acetoxy-19-(*tert*-butyldimethylsiloxy)-5α-hydroxyandrostan-17-one (**16**) (330 mg, 67%): mp 220−221 °C (from acetone); $^1$H NMR (400 MHz) $\delta$ 0.082 and 0.085 (3H each, s, 19-OSi*Me*$_2$), 0.86 (3H, s, 18-Me), 0.89 (9H, s, 19-OSiMe$_2$C*Me*$_3$), 2.07 (3H, s, 4-OCOMe), 3.71 and 3.92 (1H each, d, $J$ = 10.7 Hz, 19-CH$_2$), 5.05 (1H, m, 4$\beta$-H); MS *m*/*z* (rel intens) 478 ($M^+$, 1), 421 (25), 362 (100), 269 (22); IR $\nu_{max}$ 3533 (OH), 1736 (C=O) cm$^{-1}$. Anal. ($C_{27}H_{46}O_5Si$) C, H.

4$\beta$-Acetoxy-19-(*tert*-butyldimethylsiloxy)-5$\beta$-hydroxyandrostan-17-one (**17**) (419 mg, 85%): mp 184−185 °C (from acetone−hexane); $^1$H NMR (400 MHz) $\delta$ 0.10 and 0.11 (3H each, s, 19-OSi*Me*$_2$), 0.83 (3H, s, 18-Me), 0.92 (9H, s, 19-OSiMe$_2$C*Me*$_3$), 2.10 (3H, s. 4-OCOMe), 3.62 and 4.30 (1H each, d, $J$ = 10.6 Hz, 19-CH$_2$), 5.34 (1H, m, 4α-H); MS *m*/*z* (rel intens) 421 ($M^+$ − 57, 3), 361 (100), 287 (46), 269 (77); IR $\nu_{max}$ 3480 (OH), 1736 (C=O) cm$^{-1}$. Anal. ($C_{27}H_{46}O_5Si$) C, H.

**Treatment of the 4-Acetoxy-5-ols 14−17 with $SOCl_2$.** The 5-ols **14**-**17** (1 mmol) were separately dissolved in pyridine (11 mL). $SOCl_2$ (8.5 mmol) was added dropwise to this solution at 0 °C, and the mixture was stirred at 0 °C for about 1 h. Water was carefully added to the reaction mixture, and the product was extracted with EtOAc, washed sequentially with 1% HCl, 5% $NaHCO_3$ solution, and water, and dried with $Na_2SO_4$. Evaporation of the solvent gave a solid that was purified by column chromatography (hexanes−EtOAc) and crystallization from an appropriate solvent to give the 4-acetoxy product along with 4-enol acetate in each case.

4α-Acetoxyandrost-5-en-17-one (**18**) (75%): mp 149−151 °C (from EtOAc); $^1$H NMR (270 MHz) $\delta$ 0.88 (3H, s, 18-Me), 1.07 (3H s 19-Me), 2.12 (3H, s, 4α-OCOMe), 5.43 (1H, m, 4$\beta$-H), 5.48 (1H, m, 6-H); $^{13}$C NMR (100 MHz) $\delta$ 13.5, 20.0, 20.1, 20.2, 21.2, 21.9, 30.4, 31.0, 31.5, 33.6, 35.8, 38.9, 39.0, 47.4, 50.8, 51.8, 71.6, 115.4, 141.0, 170.2, 221.0; MS *m*/*z* (rel intens) 330 ($M^+$, 15), 288 (100), 273 (72), 255 (14); IR $\nu_{max}$ 1742 (C=O) cm$^{-1}$. Anal. ($C_{21}H_{30}O_3$) C, H.

4$\beta$-Acetoxyandrost-5-en-17-one (**19**) (24%): mp 129−130 °C (from MeOH) (lit.[32] 120−122 °C); $^1$H NMR (270 MHz) $\delta$ 0.89 (3H, s, 18-Me), 1.15 (3H s 19-Me), 2.02 (3H, s, 4$\beta$-OCOMe), 5.33 (1H, s, 4α-H), 5.77 (1H, t, $J$ = 2.7 Hz, 6-H).

4-Acetoxyandrost-4-en-17-one (**22**) (18% from **14** or 47% from **15**): mp 108−109 °C (from acetone−hexane); $^1$H NMR (270 MHz) $\delta$ 0.88 (3H, s, 18-Me), 1.08 (3H s 19-Me), 2.12 (3H, s, 4-OCOMe); $^{13}$C NMR (100 MHz) $\delta$ 13.7, 18.8, 19.4, 20.9, 21.1, 21.8, 22.3, 27.7, 30.6, 31.5, 35.2, 35.8, 37.1, 37.5, 47.6, 51.0, 54.3, 130.2, 140.2, 169.1, 221.1; MS *m*/*z* (rel intens) 330 ($M^+$, 21), 288 (100), 273 (62); IR $\nu_{max}$ 1746 (C=O) cm$^{-1}$. Anal. ($C_{21}H_{30}O_3$) C, H.

4α-Acetoxy-19-(*tert*-butyldimethylsiloxy)androst-5-en-17-one (**20**) (92%): mp 93−95 °C (from EtOAc); $^1$H NMR (270 MHz) $\delta$ 0.039 and 0.054 (3H each, s, 19-OSi*Me*$_2$), 0.86 (9H, s, 19-OSiMe$_2$C*Me*$_3$), 0.91 (3H, s, 18-Me), 2.12 (3H, s. 4-OCOMe), 3.61 and 3.76 (1H each, d, $J$ = 10.7 Hz, 19-CH$_2$), 5.38 (1H, m, 4-H), 5.70 (1H, m, 6-H); MS *m*/*z* (rel intens) 403 ($M^+$ − 57, 100), 343 (20), 269 (85), 255 (34); IR $\nu_{max}$ 1742 (C=O) cm$^{-1}$. Anal. ($C_{27}H_{44}O_4Si$) C, H.

4-Acetoxy-19-(*tert*-butyldimethylsiloxy)androst-4-en-17-one (**23**) (3% from **16** or 56% from **17**): mp 127−130 °C (from acetone); $^1$H NMR (270 MHz) $\delta$ 0.037 and 0.043 (3H each, s, 19-OSi*Me*$_2$), 0.88 (9H, s, 19-OSiMe$_2$C*Me*$_3$), 0.89 (3H, s, 18-Me), 2.12 (3H, s, 4-OCOMe), 3.71 and 3.83 (1H each, d, $J$ = 10.1 Hz, 19-CH$_2$); MS *m*/*z* (rel intens) 403 ($M^+$ − 57, 33), 273 (100); IR $\nu_{max}$ 1741 (C=O) cm$^{-1}$. Anal. ($C_{27}H_{44}O_4Si$) C, H.

**4α-Acetoxy-19-hydroxyandrost-5-en-17-one (21).** 19-Silyl ether **20** (120 mg, 0.26 mmol) was dissolved in propan-2-ol (3.5 mL) and THF (1.7 mL), and 1 M hydrochloric acid (1.8 mL, 1.8 mmol) was added to this solution. The mixture was stirred at room temperature for 2 days, then diluted with EtOAc (100 mL), washed with 5% $NaHCO_3$ solution and water, and dried with $Na_2SO_4$. Evaporation of the solvent gave an oil that was purified by column chromatography (hexanes−EtOAc) to yield 19-ol **21** (28 mg, 31%) along with the recovered silyl ether **20** (73 mg, 61%).

Compound **21**: mp 77−80 °C (from EtOAc−hexane); $^1$H NMR (270 MHz) $\delta$ 0.94 (3H, s, 18-Me), 2.14 (3H, s. 4-OCOMe), 3.63 and 3.86 (1H each, d, $J$ = 11.9 Hz, 19-CH$_2$), 5.33 (1H, m, 4-H), 5.79 (1H, m, 6-H); MS *m*/*z* (rel intens) 346 ($M^+$, 3), 273 (61), 256 (100), 238 (25); IR $\nu_{max}$ 3547 (OH), 1730 (C=O) cm$^{-1}$. Anal. ($C_{21}H_{30}O_4$) C, H.

**Alkaline Hydrolysis of 4α-Acetates 18 and 21.** Compounds **18** and **21** (0.75 mmol) were separately dissolved in MeOH (70 mL). $K_2CO_3$ (120 mg, 0.87 mmol) in water (24 mL) was added to this solution. The mixture was stirred at room temperature for 15−19 h, diluted with EtOAc, washed with water, and dried with $Na_2SO_4$. Evaporation of the solvent gave a crude product, which was purified by column chromatography and recrystallized from an appropriate solvent to afford 4α-ols **24** and **25**, respectively.

4α-Hydroxyandrost-5-en-17-one (**24**) (75%): mp 152−153 °C (from acetone−hexane); $^1$H NMR (270 MHz) $\delta$ 0.89 (3H, s, 18-Me), 1.02 (3H s 19-Me), 4.25 (1H, d, $J$ = 10.1 Hz, 4$\beta$-H), 5.73 (1H, m, 6-H); $^{13}$C NMR (100 MHz) 13.6, 20.205, 20.218, 20.4, 21.9, 30.5, 31.1, 31.5, 35.9, 37.4, 38.6, 39.3, 47.4, 50.9, 51.9, 69.5, 114.8, 145.7, 221.1; MS *m*/*z* (rel intens) 288 ($M^+$, 100), 273 (99), 255 (41), 199 (22); IR $\nu_{max}$ 3452 (OH), 1727 (C=O) cm$^{-1}$. Anal. ($C_{19}H_{30}O_2$) C, H.

4α,19-Dihydroxyandrost-5-en-17-one (**25**) (88%): mp 167−170 °C (from acetone); $^1$H NMR (270 MHz) $\delta$ 0.95 (3H, s, 18-Me), 3.61 (1H, m, 19-CH$_a$), 3.76 (1H, d, $J$ = 11.5 Hz, 19-CH$_b$), 4.23 (1H, m, 4-OH), 6.13 (1H, m, 6-H); $^{13}$C NMR (100 MHz) 13.9, 20.7, 20.8, 21.7, 29.7, 31.7, 32.5, 35.7, 35.8, 37.2, 43.7, 47.7, 51.1, 52.6, 63.5, 69.2, 120.5, 140.4, 221.1; MS *m*/*z* (rel intens) 304 ($M^+$, 3), 273 (100), 255 (43), 237 (14); IR $\nu_{max}$ 3480 and 3521 (OH), 1708 (C=O) cm$^{-1}$. Anal. ($C_{19}H_{28}O_3$) C, H.

**Treatment of Enol Acetate 22 with $K_2CO_3$.** Compound **22** (100 mg, 0.30 mmol) was treated with 1.2 mol equiv $K_2CO_3$ similarly as described for the hydrolysis of compounds **18** and **21**. The crude product obtained was purified by crystallization to yield androstane-4,17-dione (**26**) (75%): mp 164−165 °C (from acetone) (lit.[38] 162−164 °C); $^1$H NMR (270 MHz) $\delta$ 0.77 (3H, s, 18-Me), 0.87 (3H, s, 19-Me); MS *m*/*z* (rel intens) 288 ($M^+$, 100), 273 (14), 255 (20), 229 (10); IR $\nu_{max}$ 1706 and 1737 (C=O) cm$^{-1}$.

**Methylation of 4α,19-Diol 27 with $CH_3I$.** A mixture of the 4-ol (0.3 mmol), $CH_3I$ (3 mL), and $Ag_2O$ (100 mg, 0.43 mmol) was refluxed with stirring for 24−36 h. The solid material was removed by filtration, and the filtrate was evaporated to give an oil that was purified by preparative TLC (hexanes−EtOAc) and recrystallization from an appropriate solvent.

4$\beta$-Methoxy-19-hydroxyandrost-5-en-17-one (**28**) (15%): mp 159−163 °C (from acetone); $^1$H NMR (270 MHz) $\delta$ 0.93 (3H, s, 18-Me), 3.28 (3H, s, 4-OMe), 3.65 (2H, m, 19-CH$_2$), 3.88 (1H, dd, $J$ = 6.1 and 11.2 Hz, 4-H), 5.83 (1H, dd, $J$ = 2.3 and 4.8 Hz, 6-H); MS *m*/*z* (rel intens) 318 ($M^+$, 3), 286 (60), 257 (100), 238 (43), 228 (23); IR $\nu_{max}$ 3589 (OH), 1735 (C=O) cm$^{-1}$. Anal. ($C_{20}H_{30}O_3$) C, H.

4$\beta$-Hydroxy-19-methoxyandrost-5-en-17-one (**29**) (16%) (oil): $^1$H NMR (270 MHz) $\delta$ 0.91 (3H, s, 18-Me), 3.34 (3H, s, 19-CH$_2$-OMe), 3.46 and 3.82 (1H each, d, $J$ = 9.1 Hz, 19-CH$_2$), 4.22 (1H, s, 4-H), 5.83 (1H, dd, $J$ = 2.1 and 4.7 Hz, 6-H); MS *m*/*z* (rel intens) 318 ($M^+$, 4), 300 (12), 255 (100), 238 (30), 213 (15); IR $\nu_{max}$ 3449 (OH), 1737 (C=O) cm$^{-1}$; HRMS ($C_{20}H_{30}O_3$) found 318.2222, calcd 318.2195.

**Acetylation of 5,6-Diols 30 and 31.** Compounds **30** and **31** (280 mg, 0.77 mmol) were separately dissolved in pyridine (2.8 mL) and acetic anhydride (1.4 mL), and the mixture was allowed to stand at room temperature overnight. After the usual procedure, the crude product was purified by column chromatography (hexanes−EtOAc) to afford 5$\beta$-hydroxy-6,19-diacetoxyandrostan-17-one (**32**) and 5α-hydroxy-6,19-diacetoxy-androstan-17-one (**33**), respectively.

Compound **32** (250 mg, 80%) (oil): $^1$H NMR (270 MHz) $\delta$ 0.86 (3H, s, 18-Me), 2.08 and 2.11 (3H each, s, 6α- and 19-OCOMe), 4.22 and 4.37 (1H each, d, $J$ = 12.0 Hz, 19-$CH_2$), 5.22 (1H, dd, $J$ = 4.8 and 12.0 Hz, 6-H); MS $m/z$ (rel intens) 406 ($M^+$, 24), 364 (51), 346 (23), 303 (37), 286 (100), 268(45); IR $\nu_{max}$ 1738 (C=O) cm$^{-1}$; HRMS found 406.2325, calcd for $C_{23}H_{34}O_6$ 406.2355.

Compound **33** (255 mg, 82%): mp 171−175 °C (from acetone−hexane); $^1$H NMR (270 MHz) $\delta$ 0.88 (3H, s, 18-Me), 2.07 and 2.14 (3H each, s, 6$\beta$- and 19-OCOMe), 4.42 and 4.60 (1H each, d, $J$ = 12.7 Hz, 19-$CH_2$), 4.67 (1H, s, 6-H); MS $m/z$ (rel intens) 406 ($M^+$, 57), 346 (12), 328 (10), 286 (40), 268 (100); IR $\nu_{max}$ 3456 (OH), 1738 (C=O) cm$^{-1}$. Anal. ($C_{23}H_{34}O_6$) C, H.

**Treatment of 6-Acetoxy-5-ols 32 and 33 with $SOCl_2$.** Compounds **32** and **33** (100 mg, 0.25 mmol) were separately treated with $SOCl_2$ (200 mg, 1.68 $\mu$mol) in pyridine (5.5 mL) at 0 °C for 30 min in a similar fashion as described for the synthesis of compounds **18**−**20**. The crude product obtained was purified by column chromatography to give 6α,19-diacetoxyandrost-4-en-17-one (**34**) and 6$\beta$,19-diacetoxyandrost-4-en-17-one (**35**), respectively.

Compound **34** (86 mg, 89%) (oil): $^1$H NMR (270 MHz) $\delta$ 0.89 (3H, s, 18-Me), 2.08 and 2.12 (3H each, s, 6α- and 19-OCOMe), 4.10 and 4.36 (1H each, d, $J$ = 11.4 Hz, 19-$CH_2$), 5.37 (1H, m, 6$\beta$-H), 5.71 (1H, t, $J$ = 1.9 Hz, 4-H); MS $m/z$ (rel intens) 388 ($M^+$, 3), 328(12), 273 (100), 255 (48); IR $\nu_{max}$ 1740 (C=O) cm$^{-1}$; HRMS found 388.2239, calcd for $C_{23}H_{32}O_5$ 388.2250.

Compound **35** (74 mg, 77%): mp 157−161 °C (from acetone); $^1$H NMR (270 MHz) $\delta$ 0.94 (3H, s, 18-Me), 2.00 and 2.03 (3H each, s, 6$\beta$- and 19-OCOMe), 4.27 and 4.33 (1H each, d, $J$ = 11.3 Hz, 19-$CH_2$), 5.35 (1H, m, 6-H), 5.97 (1H, dd, $J$ = 1.8 and 4.5 Hz, 4-H); MS $m/z$ (rel intens) 388 ($M^+$, 11), 346 (24), 328 (20), 273 (100); IR $\nu_{max}$ 1734 (C=O) cm$^{-1}$. Anal. ($C_{23}H_{32}O_5$) C, H.

**Alkaline Hydrolysis of 6,19-Diacetates 34 and 35.** Compounds **34** and **35** (86 mg, 0.22 mmol) were separately treated with $K_2CO_3$ (86 mg, 0.62 mmol) in MeOH (7 mL) and $H_2O$ (1 mL) at room temperature for 24 h as described above. The crude product obtained was purified by column chromatography and recrystallization to give 6α,19-dihydroxyandrost-4-en-17-one (**36**) and 6$\beta$,19-dihydroxyandrost-4-en-17-one (**37**), respectively.

Compound **36** (52 mg, 77%): mp 191−194 °C (from EtOAc); $^1$H NMR (270 MHz) $\delta$ 0.89 (3H, s, 18-Me), 3.59 (1H, dd, $J$ = 7.9 and 10.6 Hz, 19-$CH_a$), 3.84 (1H, d, $J$ = 8.9 Hz, 19-$CH_b$), 4.27 (1H, m, 6$\beta$-H), 6.07 (1H, t, $J$ = 4.9 Hz, 4-H); $^{13}$C NMR (100 MHz) $\delta$ 13.9, 19.5, 20.9, 21.7, 25.0, 31.7, 34.5, 34.5, 35.7, 41.0, 43.1, 47.9, 51.2, 53.4, 66.5, 69.0, 121.3, 141.4, 220.5; MS $m/z$ (rel intens) 286 ($M^+$ − 18, 6), 273 (100), 255 (37), 237 (15); IR $\nu_{max}$ 3321 (OH), 1737 (C=O) cm$^{-1}$. Anal. ($C_{19}H_{28}O_3$) C, H.

Compound **37** (41 mg, 61%): mp 176−178 °C (from acetone); $^1$H NMR (270 MHz) $\delta$ 0.95 (3H, s, 18-Me), 3.65 and 3.86 (1H each, d, $J$ = 10.7 Hz, 19-$CH_2$), 4.29 (1H, t, $J$ = 2.9 Hz, 6-H), 5.86 (1H, t, $J$ = 3.6 Hz, 4-H); $^{13}$C NMR (100 MHz) $\delta$ 14.1, 19.2, 20.6, 21.7, 25.4, 30.9, 31.8, 35.8, 36.0, 38.1, 41.2, 48.1, 51.8, 53.9, 69.3, 74.0, 129.4, 140.9, 221.1; MS $m/z$ (rel intens) 286 ($M^+$ − 18, 6), 256 (100), 238 (15); IR $\nu_{max}$ 3240 (OH), 1730 (C=O) cm$^{-1}$. Anal. ($C_{19}H_{28}O_3$) C, H.

**Preparation of Placental Microsomes.** Human term placental microsomes (particles sedimenting at 105000$g$ for 60 min) were obtained as described by Ryan.[39] They were washed once with 0.5 mM dithiothreitol solution, lyophilized, and stored at −20 °C. No significant loss of aromatase activity occurred over the period of this study (2 months).

**Aromatase Assay Procedure.** Aromatase activity was measured according to the procedure of Siiteri and Thompson,[35] in which tritiated water released from [1$\beta$-$^3$H]AD into the incubation medium during aromatization was used as an index of the enzyme activity. All assays were carried out in 67 mM phosphate buffer, pH 7.5, in the presence of NADPH (180 $\mu$M) at a final incubation volume of 0.5 mL under the conditions similar to those used previously.[16] Briefly, for the screening assay, 20 $\mu$g of protein of the lyophilized microsomes, a 20 min incubation time, 300 nM concentration of the $^3$H-labeled substrate, and various concentrations of each of the inhibitors were used, and for the kinetic study, 20 $\mu$g of protein, various concentrations of each of the inhibitors, 12.4, 15.3, 21.3, and 28.3 nM concentrations of the $^3$H-labeled substrate, and a 5 min incubation time were used.

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