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SYNTHESIS OF 2,2-DIMETHYL-4-HYDROXY-4-ANDROSTENE-3,17-DIONE AS AN INHIBITOR OF AROMATASE

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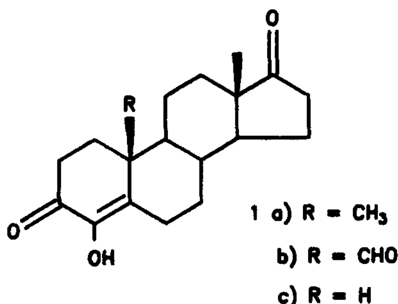
2,2-Dimethyl-4-hydroxy-4-androstene-3,17-dione (**4**) has been synthesized and has been shown to be a powerful competitive inhibitor of aromatase (K_i = 11.4 nM). However, compound **4** does not cause time-dependent loss of enzyme activity, in contrast to the unmethylated parent compound, 4-OHA.

KEY WORDS: Human placental aromatase, 2,2-dimethyl steroids, inhibitors.

INTRODUCTION

The development and study of inhibitors of aromatase (P-450_{arom}) remains an important task, because of the cancer chemotherapeutic potential of such compounds. One aromatase inhibitor which has reached clinical trials[1] for the treatment of cases of breast tumors is the substrate analog 4-hydroxy-4-androstene-3,17-dione (4-OHA, **1a**). This compound was originally described[2,3] as a competitive inhibitor of human placental aromatase in 1977 by the Brodies. It was later shown[4] that 4-OHA causes time-dependent inactivation of the enzyme ($t_{1/2}$ *ca.* 2 min, K_i = *ca* 50 nM). The time-dependent inactivation required NADPH, and was irreversible, as demonstrated by the lack of enzyme activity after prolonged dialysis to remove the inhibitor. The inactivation rate was slowed when substrate (androstenedione) was included in the preincubation step. This protection by substrate supports the notion that the inactivation is an active site process. Covey and Hood showed[4b] that 19-nor-4-hydroxyandrostenedione (**1c**), while capable of binding to the enzyme, did not produce aroma-



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tase inactivation. This led them to speculate that enzyme-produced 19-oxygenated intermediates might be the inactivating species. On the other hand, 19-oxo-4-hydroxy-androstenedione (**1b**) has been found[5] to be a poor inactivator relative to 4-OHA, which argues against the involvement of the enzyme-generated 19-oxo product. However no studies have been reported with the 19-hydroxy analogue of 4-OHA. The 4-acetoxy analog of 4-OHA was also demonstrated[6] to be a time-dependent inactivator of aromatase. However it is likely that this compound is hydrolyzed to 4-OHA before inactivation occurs, as there has been shown to be esterase activity in human placental microsomes that can convert[4b] the 4-*O*-acetyl derivative of **1a** to the 4-OH compound.

The mechanism of inactivation of aromatase by 4-OHA has not yet been established. One may speculate that the mechanism of action of 4-OHA involves formation of a 2,3-olefinic intermediate. Consequently, we embarked on the synthesis and testing of 2,2-dimethyl 4-OHA (**4**) which obviously cannot form such intermediates. We were encouraged in this effort by other studies in this laboratory, which showed that the substrate analog 2,2-dimethyl androst-4-ene-3,17-dione (**2**) is an excellent competitive inhibitor of placental microsomal aromatase (K_i = 2.3 nM, Km for androst-4-ene-3,17-dione = 20 nM).

MATERIALS AND METHODS

General

Melting points were determined on a Kofler hot stage and are uncorrected. IR spectra were recorded on a Perkin-Elmer 521 spectrometer, in $CHCl_3$ unless otherwise noted. UV spectra were obtained on a Perkin-Elmer Lambda 3 instrument. Proton NMR spectra were recorded in $CDCl_3$, with either an IBM FT (80 MHz) spectrometer or a Varian XL-200 MHz spectrometer. Mass spectra were obtained on a DuPont DP-102 instrument. Column chromatography was performed using dry column silica gel (Woelm). [1β-^{3}H]-androst-4-ene-3,17-dione was purchased from New England Nuclear Corporation.

2,2-Dimethyl-4-androstene-3,17-dione (2)

To an ice-cooled solution of 2,2-dimethyl-4-androsten-17β-ol-3-one[7] (1.93 g) in acetone (50 ml) was added Jones reagent (3.7 ml), dropwise with stirring, until a permanent orange coloration remained. After 5 min, excess oxidant was destroyed by the addition of propan-2-ol (6 ml), and the reaction mixture was diluted with water, dried (Na_2SO_4) and evaporated *in vacuo*. The resulting crude product (1.64 g) was chromatographed on silica gel using acetone-$CHCl_3$ (1:49) giving pure **2**, mp 150–151°C (from acetone-hexane), UV EtOH λ_{max} 237.5 nm (ε 15,880); IR ($CHCl_3$) 1730, 1665, 1620 cm^{-1}; NMR δ 5.68 (s, 1, C-4*H*), 1.31 (s, 3), 1.17 (s, 3), and 1.10 (s, 3) [2- and 19-CH_3], 0.91 (s, 3, 18-CH_3); MS, M^+ 314, 299. (Found: C, 80.23; H, 9.73. $C_{21}H_{30}O_2$ requires: C, 80.21; H, 9.62%).

2,2-Dimethyl-4,5-oxidoandrostane-3,17,dione (3)

To a stirred solution of 2,2-dimethyl-4-androstene-3,17-dione (**2**, 1.2 g) in methanol (30 ml) at 0°C was added, dropwise, a mixture of 30% aqueous hydrogen peroxide

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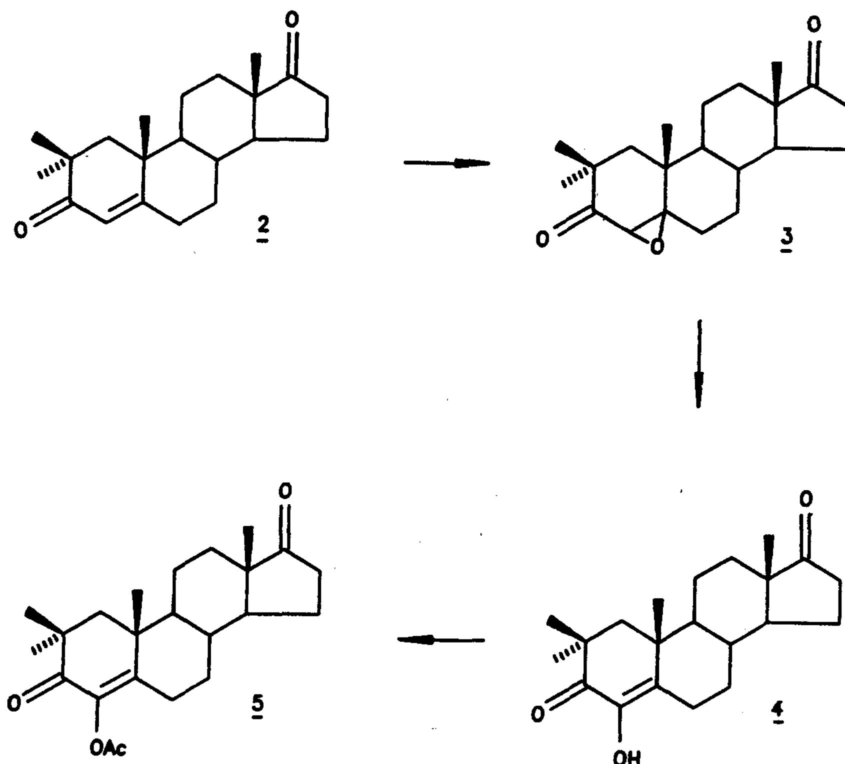
Scheme 1



(3.2 ml) and 20% aqueous NaOH (1.8 ml). The reaction mixture was stirred for 30 min at 0°C, and was then left in the refrigerator for 18 h. Excess hydrogen peroxide was then destroyed by the addition of cold 10% aqueous sodium sulfite (40 ml). After 1 h, the mixture was extracted with $CHCl_3$ and the $CHCl_3$ was washed successively with 10% aqueous sodium sulfite and water, dried (Na_2SO_4) and evaporated *in vacuo*. The residue was chromatographed on silica gel, using acetone-$CHCl_3$ (1:99), giving pure **3** (607 mg), as well as unchanged starting material (**2**, 221 mg) and mixed fractions. The oxidoketone **3** had mp 178–179°C (from CH_2Cl_2-hexane). IR ($CHCl_3$) 1735, 1695 cm^{-1}; NMR δ 3.09 (s, 1, C4-*H*), 1.12 (s, 3), 1.11 (s, 3) and 1.09 (s, 3) [2- and 19-CH_3], 0.89 (s, 3, 18-CH_3); MS, M^+ 330. (Found: C, 76.57; H, 9.09. $C_{21}H_{30}O_3$ requires: C, 76.32; H, 9.15%).

2,2-Dimethyl-4-hydroxy-4-androstene-3,17-dione (4)

A solution of the 4,5-oxido-3-ketone (**3**, 227 mg) in 2.6 ml of a 2% solution of conc. sulfuric acid in acetic acid was left for 4 h at room temperature. Ice was added, and the mixture was kept in a refrigerator for 18 h. The aqueous mixture was then extracted with $CHCl_3$, and the organic layer was washed successively with 5% aqueous $NaHCO_3$ and H_2O, dried (Na_2SO_4) and evaporated *in vacuo*. The residue

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was chromatographed on silica gel, using $CHCl_3$ containing 0.1% acetone. The product was crystallized first from CH_2Cl_2, then from aqueous methanol, to give the desired product (**4**, 37 mg), mp 145–146°C; UV EtOH λ_{max} 275 nm (ε 12,570); IR ($CHCl_3$) 3425, 1730, 1667, 1637, 1570 cm^{-1}; NMR δ 6.17 (s, 1, 4-O*H*) 1.30 (s, 3), 1.22 (s, 3), 1.18 (s, 3) [2- and 19-CH_3], 0.91 (s, 3, C-18 CH_3); MS, M^+ 330. (Found: C, 76.48; H, 9.23. $C_{21}H_{30}O_3$ requires: C, 76.32; H, 9.15%.

*2,2-Dimethyl-4-hydroxy-4-androstene-3,17-dione 4-acetate (**5**)*

A solution of the 2,2-dimethyl-4-hydroxy compound (**4**, 360 mg) in pyridine (1 ml) and acetic anhydride (0.5 ml) was kept at room temperature for 4 h. The mixture was evaporated to dryness, *in vacuo*, using a Rotavapor connected to an oil pump, and the residue was chromatographed on dry column silica gel, using $CHCl_3$-acetone (99:1). The fractions containing the product were combined and crystallized from methylene chloride-hexane to give the pure 4-acetoxy compound (**5**, 124 mg), mp 203–205°C; UV EtOH λ_{max} 243 nm (ε 10,568); IR ($CHCl_3$) 1750, 1730, 1675, 1630, 1220 cm^{-1}. NMR δ 2.32 (s, 3,4-OCOCH_3), 1.35 (s, 3H), 1.25 (s, 3H), and 1.15 (s, 3H) [2- and 19-CH_3], 0.91 (s, 3H, 19-CH_3). MS, M^+ 372, 330. (Found: C, 74.37; H, 8.71. $C_{23}H_{32}O_4$ requires: C, 74.16; H, 8.66%).

ENZYME STUDIES

Enzyme kinetic data were obtained using human placental microsomes prepared as described by Ryan.[8] Competitive inhibition and time-dependent inactivation were assessed using [1β-^{3}H]-androst-4-ene-3,17-dione in a tritium release assay[9] (Thompson and Siiteri) using modifications of the procedures[10] of Marcotte and Robinson, as follows:

Competitive inhibition assays (0.5 ml) contained 10 mM potassium phosphate buffer, 100 mM KCl, 5 mM DTT, 1 mM EDTA, 125 μM NADPH. The stock solution of 1β-^{3}H-androstenedione (2.74 × 10^4 Ci/mol, 3:1 1β-^{3}H: 1α-^{3}H) substrate which was used was 3.71 μM (95% H_2O-EtOH). Seven 1β-^{3}H-androstenedione substrate concentrations were used; 18.6 nM, 24.5 nM, 36.4 nM, 60.8 nM, 120.9 nM, 161 nM, and 282 nM. Stock inhibitor solutions were prepared in *ca* 1% propylene glycol-H_2O solutions. 2,2-Dimethylandrost-4-ene-3,17-dione and 2,2-dimethylandrost-4-en-4-ol-3,17-dione were each run at 40 nM and 100 nM. 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 vortexing with $CHCl_3$ (5 ml) for 20 s. The mixture was centrifuged at low speed for 10 min, and an aliquot from the aqueous phase (0.2 ml) was counted using 10 mL ACS. The K_m, K_i, and V_{max} values were obtained using a double rciprocal analysis and least squares line-fitting, with correlation coefficients greater than 0.95.

Time-dependent inactivation assays were carried out over a period of 20 min at 37°C, using inhibitor concentrations of 0.2 and 0.5 μM.

RESULTS AND DISCUSSION

The synthetic route was straightforward, starting with the known[7] 2,2-dimethyl-4-androsten-17β-ol-3-one (**2**). Reaction of **2** with hydrogen peroxide-NaOH gave 2,2-

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dimethyl-4,5-oxidoandrostane-3,17-dione (**3**). This reaction gave predominantly one 4,5-oxido-3-ketone, which was readily obtained pure by column chromatography. Although a small amount of the isomeric 4,5-oxido-3-ketone was formed in the oxidation reaction, as shown by NMR, the isomer could not be obtained pure, and was not studied further. However the chemical shift for the 4-H signal of the minor isomer appeared at 3.15 ppm, compared with that of the 4-H for the major, pure, isolated product (**3**) which was at 3.09 ppm. On this basis the epoxide group in **3** is assigned the 4β, 5β-configuration, by analogy with the NMR data for other 4,5-oxido-3-keto steroids.[11] Typically, the 4-H signals for 4α, 5α-oxido-3-ketones appear downfield from the corresponding signals in the 4β, 5β-oxido isomers. Compound **3** was then converted to the desired 4-hydroxy compound (**4**) in modest yield by treatment with sulfuric acid-acetic acid. Compound **4** was characterized further as the derived 4-acetate (**5**) by the action of acetic anhydride-pyridine. Compounds **4** and **5** showed the expected[3,4b,6] ultraviolet maxima at 275 nm and 243 nm respectively, and the infrared, nuclear magnetic resonance and mass spectra were also entirely consistent with the postulated structures.

We then assessed the inhibitory properties of 2,2-dimethyl 4-OHA (**4**) using placental microsomal aromatase. Although a powerful competitive inhibitor (K_i = 11.4 nM; Km for androst-4-ene-3,17-dione = 15.5 nM) compound **4** did not induce time-dependent inactivation of the enzyme. This loss of enzyme-inactivating ability without diminution of competitive inhibitory potency is a provocative finding. One possible explanation is that the mechanism of inactivation of aromatase by 4-OHA is related to a process involving the formation of a 2,3-double bond, or some other process involving the C-2 hydrogens. However, this conclusion rests on the assumption that the 2,2-dimethyl analog**4** is bound at the active site of aromatase exactly like substrate or 4-OHA, rather than in some skewed arrangement. It would clearly be of interest to determine if 2,2-dimethyl steroids are processed by aromatase, with oxidation at the 19-carbon, which would support the notion that they bind normally at the active site.

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