Substrate-Induced Inactivation of Aromatase by Allenic and Acetylenic Steroids

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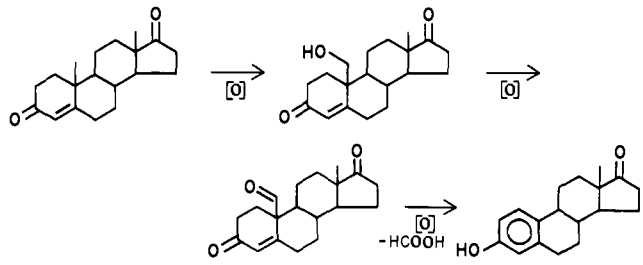
Aromatase catalyzes the conversion of androst-4-ene-3,17-dione and testosterone (androgens) to estrone and estradiol (estrogens). Its selective inactivation may offer a means to control estrogen action. In fact, the competitive inhibitor, 4-acetoxyandrost-4-ene-3,17-dione has been shown to prevent the estrogen surge required for ovulation in rats[1] and cause regression of estrogen-dependent mammary tumors in rats.[2] Aromatization of androst-4-ene-3,17-dione is cytochrome P-450 dependent[3] and involves sequential hydroxylations at C-19 as shown in Scheme I.[4] The nature of the reactive oxygenation species is still incompletely resolved but is considered compatible with activation of oxygen via an NADPH-requiring species to generate an oxene–iron complex which can insert an oxygen atom into the C–H bond.[5] As other cytochrome P 450 mediated reactions involve addition of oxygen across a carbon–carbon double bond to give an epoxide,[5] we proposed that aromatase would be susceptible to irreversible inactivation by an allenic substrate. Thus, if the 10-allenyl steroid (**1**) undergoes oxygen atom insertion by aromatase (Scheme II), a highly reactive allene oxide species would be formed which could alkylate the enzyme via its cyclopropanone rearrangement product or by addition to prosthetic heme. A similar mechanism has been proposed by Ortiz de Montellano and Kunze for the destruction of a hepatic microsomal cytochrome P 450 of broad substrate specificity by a variety of allenic substrates.[6]

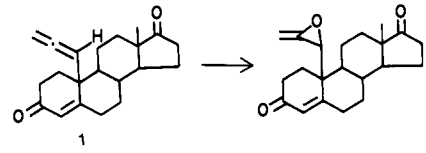
The success of this approach will depend on the ability of an allenic substrate to induce what would be an abnormal oxygenation for aromatase. However, a Michael acceptor could be generated by the enzyme's normal mode of action if the propargylic steroid **2** could undergo two sequential hydroxylations at C-19, in the same manner as the 19-methyl substrate. Dehydration would then lead to the formation of an acetylenic ketone (Scheme III). Covey et al.[7] following this reasoning have very recently reported that **2** as well as the propargylic alcohol **3** and the propargylic ketone **4** do inactivate aromatase. We feel that the reported difference in activity between **2**, **3**, and **4** (the K_I for **2** is approximately 1000 times lower than that for **3**)[7] suggests that the mechanism of inactivation induced by **2** is not via a Michael addition process but more likely may involve oxygen insertion into the carbon–carbon triple bond to generate the highly reactive oxirene species which could covalently bind to the prosthetic heme via its α-ketocarbene tautomer (Scheme IV). This would be an analogous mechanism to that proposed by Ortiz de Montellano and Kunze for the inactivation of hepatic cytochrome P 450 by acetylene itself.[8]

With these considerations in mind, we have synthesized **1**, **2**, and 19,19-dideuterio-**2** and examined their inhibitory activity toward a microsomal preparation containing aromatase from human placenta. Incubation of the aromatase preparation,[9] to which an NADPH-generating system had been added, for varying time periods with different concentrations of **1** and **2** resulted in a time-dependent loss of enzyme activity which followed pseudo-first-order kinetics for more than two half-lives (Figures 1 and 2, respectively). When the enzyme half-life at these different inhibitor concentrations is plotted against the reciprocal of the inhibitor concentration as described by Kitz and Wilson,[10] the K_I's for **1** and **2** are found to be 1.4×10^{-8} and 1.0×10^{-8} M (K_M for testosterone is 4.5×10^{-8} M)[11] and the calculated enzyme half-lives at infinite inhibitor concentration are 24 and 7 min respectively. These observed saturation kinetics indicate that a step subsequent to reversible formation of the Michaelis–Menten complex is rate limiting. The inhibition process is active-site directed as demonstrated by protection against inactivation by the substrate testosterone; at a concentration of **1** of 6.8×10^{-8} M, the enzyme half-life increases from 55 to 90 min when the testosterone concentration during preincubation is increased from 4.5×10^{-8} to 4.5×10^{-7} M. There is no time-dependent loss of enzyme activity in the absence of the NADPH-generating system. This latter result is evidence that both inhibitors require activation by a functioning aromatase before inactivation can occur. The presence of dithiothreitol (10 mM) in the preincubation medium and the absence of lag time before the onset of inhibition rule out the possibility of inhibition via an affinity labeling mode by a diffusible alkylating species. The inhibition observed at zero time presumably represents a competitive component of inhibition. The irreversibility of the process is demonstrated by the inability of

Scheme I



Scheme II



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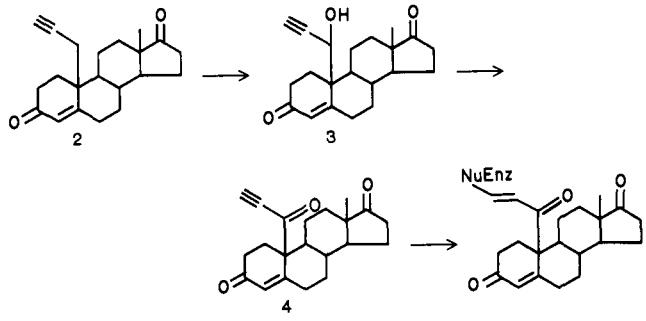
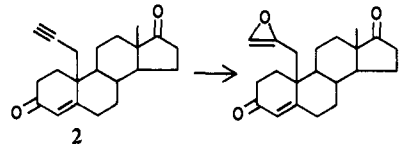
Scheme III



2 3 4

Scheme IV



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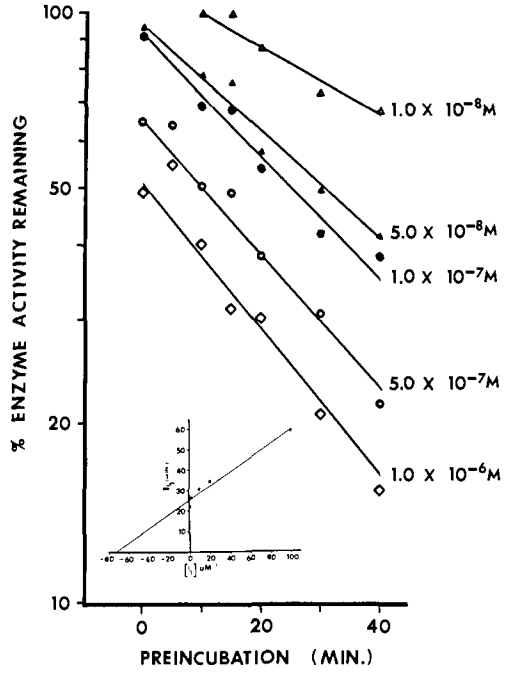

Figure 1. Aromatase inhibition by 10-allenyl steroid (**1**). The incubation media contained 100 mM KCl, 10 mM KH_2PO_4, 10 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid to which is added a NADPH-generating system of 0.5 mM $NADP^+$, 2.5 mM glucose 6-phosphate and 1 unit/mL glucose-6-phosphate dehydrogenase at pH 8.0. For a typical experiment, inhibitor, 10^{-9}–10^{-6} M, in 100 μL of media was added to 600 μL of NADPH-generating system, and then the reaction was started by addition of 700 μL of a crude aromatase preparation containing 450 μg of human placental microsomal protein. After 0, 10, 20, or 40 min of incubation at 25 °C under 1 atm of 95% O_2, 5% CO_2, 100 μL of [1β,2β-^{3}H]testosterone (1.5×10^6 dpm) was added to provide an assay substrate concentration of 4.5×10^{-7} M (K_M for testosterone is 4.5×10^{-8} M). Following 10-min incubation, the reaction was terminated by addition of 10 mL of $CHCl_3$, aqueous/organic phases separated by centrifugation. Then duplicate 500 μL of aqueous-phase samples were treated with 500 μL of 0.25% dextran-coated charcoal, incubated for 15 min at 4 °C, and centrifuged. These supernatants were decanted into vials and scintillation cocktail added. Radioactivity of ^3H_2O from the stereospecific elimination of 1β,2β-^{3}H substrate was utilized to determine product formation.[9]

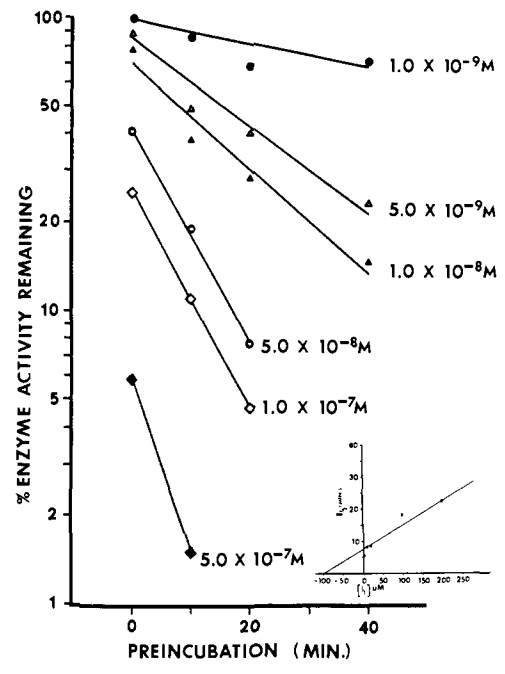

Figure 2. Aromatase inhibition by 10-propargylic steroid **2**. See Figure 1 caption for conditions.

dialysis at 4 °C against the incubation medium to regenerate enzyme activity over a 24-h period (conditions where a control preparation is stable).

The synthesis of **1** relies on the mixed cuprate-mediated reductive elimination of acetate[12] from 10-[1-(acetyloxy)-2-propynyl]estr-5-ene-3,12-dione bis(1,2-ethanediyl acetal) (**6**) to afford the corresponding allene **7**[13] (mp 113–114 °C) in overall 50% yield from the propargylic alcohol **5** which had previously been prepared by Covey et al.[16] The diketal allene **7** was then deprotected (*p*-TosH, acetone) to give **1**[13,14] (mp 104–105 °C) in 70% yield.

Our synthesis of the acetylene **2** involves Claisen rearrangement (collidine, 4 h, 165 °C) of the vinyl ether **9**[13] (mp 82–83 °C), prepared in 78% yield from (6β)-6-hydroxyestr-5(10)-ene-3,17-dione bis(1,2-ethanediyl acetal) (**8**) [ethyl vinyl ether, $Hg(OAc)_2$], to afford the 19β carboxaldehyde **10**[13,17] (mp 128–130 °C) in 66% yield. Wittig reaction of **10** with chloromethylenephosphorane afforded the mixture of *cis*- and *trans*-vinyl chlorides **11** (84% yield). Dehydrochlorination (lithium diisopropylamide, −70 °C, 1 h) of **11** then gave the acetylene **12**[13] (mp 152–153 °C) in 95% yield. Ketone deprotection provided **2**[13,15] (mp 174–175 °C) in 79% yield.

Although compounds bearing allenic or acetylenic functions are inactivators of the broadly specific cytochrome P-450 of rat hepatic microsomes,[6,8] neither **1** nor **2** has any inhibitory activity (10^{-6} M, 1-h incubation) toward the cytochrome P-450 dependent dealkylation of ethyl morphine.[18,19] In addition, no significant inhibition of the steroid 11β-hydroxylase system was observed on incubation of **1** or **2** at 10^{-6} M for 30 min prior to monitoring the conversion of [1,2-^{3}H]deoxycorticosterone to corticosterone.[19,20] Evidently, in the case of **1** and **2** the positioning of the allene or propargylic function at the 10β-position confers specificity toward aromatase. While the inactivation of aromatase by **1** most likely involves the intermediacy of an allene oxide, two distinct possibilities exist for inactivation by **2**. If **2** induced inactivation according to Scheme III, the proposed intermediate **4**[16] could be expected to be an affinity label for aromatase of comparable activity to **2**. In addition a kinetic isotope effect on the rate of inactivation induced by 19,19-dideuterio-**2** may be observable[21] if the mechanism of Scheme III, but not that of Scheme IV, is operative and binding of substrate is not rate determining. We find that **4** does not significantly inactivate aromatase at a concentration of 10^{-6} M (during a 20-min incubation in presence of the NADPH-generating system and absence of dithiothreitol and ethylenediaminetetraacetic acid) nor is the rate of inactivation induced by 19,19-dideuterio-**2**[22] significantly different from that induced by **2**. Thus we favor the mechanism depicted in Scheme IV for the inactivation of aromatase by **2**.

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(14) **1**: IR (KBr) ν_{max} 5.16, 5.77, 6.05 μm; ^{1}H NMR (80 MHz, $CDCl_3$) δ 0.94 (s, 3), 0.96–2.75 (m, 19), 4.85 (d, J = 8 Hz, 2), 5.28 (t, J = 8 Hz, 1), 5.82 (s, 1).
(15) **2**: IR (KBr) ν_{max} 3.10, 4.74, 5.80, 6.00 μm; ^{1}H NMR (80 MHz, $CDCl_3$) δ 0.92 (s, 3), 0.93–2.86 (m, 22), 5.87 (s, 1).
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