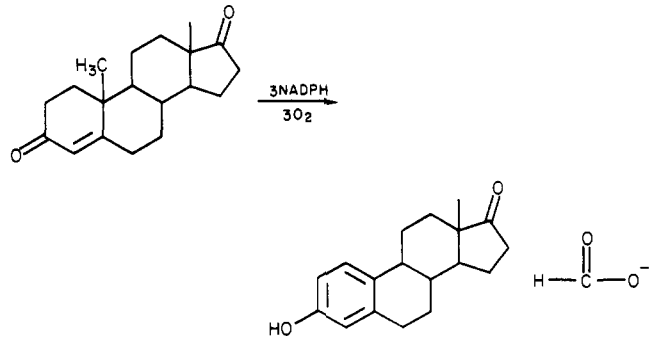
Fraley, R. T., Yen, G. S. L., Lueking, D. R., & Kaplan, S. (1979b) *J. Biol. Chem. 254*, 1987–1991.
Greenstein, J. P., & Winitz, M. (1961) *Chemistry of the Amino Acids*, Vol. 3, Wiley, New York.
Gupta, C. M., Rodhakrishnan, R., & Khorana, H. G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4315–4319.
Ingram, V. M. (1963) *Methods Enzymol. 6*, 831–847.
Kanfer, J., & Kennedy, E. P. (1963) *J. Biol. Chem. 238*, 2919–2922.
Kaplan, S. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp 809–839, Plenum Press, New York.
Kates, M. (1975) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S., & Work, E., Eds.) Vol. 3, American Elsevier, New York.
Katz, J., Lieberman, I., & Barker, H. A. (1953) *J. Biol. Chem. 200*, 417–429.
Kenyon, C. N. (1978) in *The Photosynthetic Bacteria* (Clayton, R. K., & Sistrom, W. R., Eds.) pp 281–313, Plenum Press, New York.
Landon, M. (1977) *Methods Enzymol. 47*, 145–148.
Lascelles, J., & Szilagyi, J. F. (1965) *J. Gen. Microbiol. 38*, 55–64.
Lueking, D. R., & Goldfine, H. (1975) *J. Biol. Chem. 250*, 8530–8535.
Lueking, D. R., Fraley, R. T., & Kaplan, S. (1978) *J. Biol. Chem. 253*, 451–457.
Marinetti, G. V., & Cattieu, K. (1981) *Chem. Phys. Lipids 28*, 241–251.
Matsumoto, M., & Miwa, M. (1973) *Biochim. Biophys. Acta 296*, 350–364.
Nelson, G. J. (1970) *Biochem. Biophys. Res. Commun. 38*, 261–265.
Onishi, J. C., & Niederman, R. A. (1978) Fifth Annual Conference on the Molecular Biology of Photosynthetic Procaryotes, Bloomington, IN.
Poorthius, J. H. M., Yazaki, D. J., & Hostetter, K. (1976) *J. Lipid Res. 17*, 433–437.
Pouchert, C. J., & Campbell, J. R. (1974) *The Aldrich Library of NMR Spectra*, Aldrich Chemical Co., Inc., Milwaukee, WI.
Raetz, C. R. H. (1978) *Microbiol. Rev. 42*, 614–659.
Rothman, J. E., & Kennedy, E. P. (1977) *J. Mol. Biol. 110*, 603–618.
Rouser, G., Kritchevsky, G., Heller, D., & Lieber, E. (1963) *J. Am. Oil Chem. Soc. 40*, 425–454.
Rouser, G., Kritchevsky, G., Yamamoto, A., Simon, G., Galli, C., & Bauman, A. J. (1969) *Methods Enzymol. 14*, 272–316.
Russell, N. J., & Harwood, J. L. (1979) *Biochem. J. 181*, 339–345.
Salem, N., Serpentino, P., Puskin, J. S., & Abood, I. G. (1980) *Chem. Phys. Lipids 27*, 289–304.
Selinger, Z., & Lapidot, Y. (1966) *J. Lipid Res. 7*, 174–175.
Singer, S. J., & Nicholson, G. L. (1972) *Science (Washington, D.C.) 175*, 720–731.
Stadtman, E. R., Katz, J., & Barker, H. A. (1952) *J. Biol. Chem. 195*, 779–785.
Sweeley, C. C. (1969) *Methods Enzymol. 14*, 255–267.
Wraight, C. A., Lueking, D. R., Fraley, R. T., & Kaplan, S. (1978) *J. Biol. Chem. 253*, 465–471.

Inhibition and Inactivation of Estrogen Synthetase (Aromatase) by Fluorinated Substrate Analogues†

Patrick A. Marcotte and Cecil H. Robinson*

ABSTRACT: 19,19-Difluoroandrost-4-ene-3,17-dione (**1**) and 19-fluorcandrost-4-ene-3,17-dione (**2**) have been synthesized, and the interaction of these compounds with the estrogen synthetase (aromatase) activity of human placental microsomes has been studied. **1** has been found to cause time-dependent, irreversible inactivation of this enzyme (K_i = 1 μM, k_{inact} = 0.023 min^{-1}). A possible mechanism of this process is enzymatic generation of an acyl fluoride through oxidation of **1**. Compound **2** does not cause inactivation, and this substrate analogue has been shown to be converted to estrone in high yield by this enzyme system.

Estrogen synthetase (aromatase) is a key enzyme that is involved in the conversion of androgens (male sex hormones) into estrogens (female sex hormones). Aromatase and androgen 5α-reductase are the enzymes that catalyze reactions of an important branch point in biosynthetic steroid transformations. Since the androgen/estrogen balance is considered to be a factor in many biological processes (Motohashi et al., 1979), work in this laboratory and elsewhere has focused on the development of specific inhibitors of the above enzymes (Blohm et al., 1980; Brodie et al., 1977; Covey et al., 1981; Marcotte & Robinson, 1981; Metcalf et al., 1981; Schade & Schubert, 1979; Schwarzel et al., 1973). In particular, the extraordinary importance of aromatase has led to much work with preparations of microsomes from human term placenta

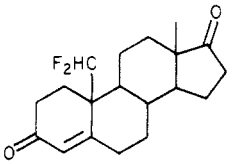


† From the Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received October 19, 1981.* This work was supported in part by Research Grant HD 11840 from the National Institutes of Health.

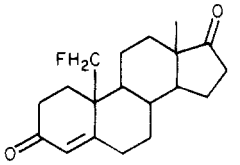
0006-2960/82/0421-2773$01.25/0

despite the limitations introduced by the impurity of this source of enzyme.

We here report the time-dependent, irreversible inactivation of the aromatase activity of human term placental microsomes by 19,19-difluoroandrost-4-ene-3,17-dione (**1**). 19-Fluoro-



1



2

androstene-3,17-dione (**2**)[1] is also oxidized by this enzyme system, but that process does not lead to inactivation: the substrate analogue is converted in high yield to estrone. Evidence as to the probable mechanism for the interaction of these compounds with aromatase is presented.

Materials and Methods

General. Placental microsomes were prepared as described by Ryan (1959). The aromatase activity was measured by the release of tritium into the aqueous medium from [1,2-^{3}H]androst-4-ene-3,17-dione (40–60 Ci/mmol, purchased from New England Nuclear Corp.) as described by Thompson & Siiteri (1974) and Reed & Ohno (1976). Protein was determined by the method of Lowry et al. (1951). Unlabeled androst-4-ene-3,17-dione was purchased from Steraloids, Inc., and all biochemical reagents were from Sigma Chemical Co. Dehydroisoandrosterone was purchased from G. D. Searle and converted to androst-5-ene-3β,17β,19-triol 3,17-diacetate as described by Kalvoda et al. (1963). (Diethylamino)sulfur trifluoride (DAST) was prepared from sulfur tetrafluoride (Matheson) as described by Middleton (1975). Cyanuric fluoride was purchased from Alfa-Ventron Corp.

Melting points were determined on a Kofler hot stage and are uncorrected. NMR[2] spectra were obtained on Perkin-Elmer R-12B (60 MHz) or Bruker FT (300 MHz) spectrometers in $CDCl_3$ solutions with Me_4Si as the internal standard. Chemical shifts are expressed as δ values (Me_4Si = 0), with signal multiplicities shown as s (singlet), d (doublet), t (triplet), and m (multiplet). Infrared spectra were obtained on a Perkin-Elmer 521 spectrometer with chloroform solutions. Mass spectra were determined on a Du Pont DP-102 spectrometer and a Kratos MS-50 instrument. Elemental analyses were determined by Galbraith Laboratories Inc., Knoxville, TN. All chromatographic separations were performed on Woelm dry-column silica gel from EM Reagents, and analytical thin-layer plates were obtained from Macherey Nagel and Co., West Germany. High-pressure liquid chromatographic separations were performed on a Waters Associates Model 6000 instrument.

Syntheses. 19-Oxoandrost-5-ene-3β,17β-diol Diacetate (**3**). A solution of 10 g of androst-5-ene-3β,17β,19-triol 3,17-diacetate was dissolved in 250 mL of acetone and chilled to 0 °C. Jones reagent (24 mL of an aqueous solution containing 2 M CrO_3 and 3 M H_2SO_4) was added over 2 min with vigorous stirring. After 3 min of further reaction at 0 °C, the reaction was quenched with excess 2-propanol, and the product was isolated by partitioning the reaction mixture between chloroform and water. The organic phase was washed with saturated aqueous NaCl, dried with Na_2SO_4, and evaporated to an oil. Crystallization from methanol yielded 7.5 g of **3**: mp 137–140 °C [lit. mp 150–153 °C (Jen & Wolff, 1963)]; NMR δ 9.7 (s, 1, 19-CHO), 5.9 (m, 1, C-6 H), 0.9 (s, 3, 18-CH_3).

19,19-Difluoroandrost-5-ene-3β,17β-diol Diacetate (**4**). A solution of 2.5 g of **3** dissolved in 10 mL of (diethylamino)-sulfur trifluoride (DAST) was heated in an oil bath at 60 °C for 5 h. The mixture was cooled, carefully diluted with methylene chloride, and poured into crushed ice. The organic layer was washed with saturated $NaHCO_3$, dried with Na_2SO_4, and evaporated to a thick oil. After filtration through silica gel (elution with 1:49 acetone–chloroform), the product was crystallized from methanol to yield 750 mg of crude 19,19-difluoroandrost-5-ene-3β,17β-diol diacetate (**4**). An analytical sample was purified by HPLC (Waters μ-Porasil column equilibrated with 67.5% hexane–30% ethylene chloride–2.5% acetonitrile) and recrystallized from hexane: mp 140–143 °C; IR ν_{max} 1730, 1250 cm^{-1}; NMR δ 6.1 (t, 1, J_{HF} = 55 Hz, 19-CHF_2), 5.9 (m, 1, C-6 H), 0.9 (s, 3, 18-CH_3).

19,19-Difluoroandrost-5-ene-3β,17β-diol (**5**). The diacetate (**4**, 750 mg) was treated with 30 mL of 3% methanolic KOH. After 3 h at 25 °C, the reaction was neutralized with acetic acid, the bulk of the methanol evaporated in vacuo, and the product precipitated with water. The solid product was recovered by filtration, washed with cold water, and thoroughly dried, yielding 600 mg of 19,19-difluoroandrost-5-ene-3β,17β-diol (**5**). An analytical sample (from purified diacetate) was crystallized from methanol–water: mp 159–161 °C.

19,19-Difluoroandrost-4-ene-3,17-dione (**1**). The preceding diol (**5**, 600 mg) was dissolved in 100 mL of toluene and 10 mL of cyclohexanone. After distillation of 5 mL (to remove moisture), 1.5 g of aluminum isopropoxide was added and the reaction heated to reflux for 1 h. The product was isolated by extraction with ethyl acetate and purified by column chromatography on silica gel (elution with 1:24 acetone–chloroform). Final purification was effected by reverse-phase HPLC (Whatman M9-ODS column equilibrated with 80% acetonitrile–20% water) and crystallization from ether–hexane. Pure 19,19-difluoroandrost-4-ene-3,17-dione (**1**, 205 mg) showed the following: mp 109–110 °C; IR ν_{max} 1736, 1660, 1610 cm^{-1}; 300-MHz NMR (Figure 1A) δ 6.08 (t, 1, J_{HF} = 55 Hz, 19-CHF_2), 6.04 (s, 1, C-4 H), 0.95 (s, 3, 18-CH_3); mass spectrum, M^+ 322. Anal. Calcd for $C_{19}H_{24}O_2F_2$: C, 70.79; H, 7.50; F, 11.70. Found: C, 70.80; H, 7.63; F, 11.73.

19-Fluoroandrost-4-ene-3,17-dione (**2**). This compound was prepared from androst-5-ene-3β,17β,19-triol 3,17-diacetate (2.5 g) by a procedure identical with the preparation of the 19,19-difluoro compound (**1**) from the 19-oxo compound (**3**). However, the 19-fluoro product was not the major product of the reaction between the 19-alcohol and DAST. The crude reaction mixtures containing a small amount of 19-fluoroandrost-5-ene-3β,17β-diol diacetate and 19-fluoroandrost-5-ene-3β,17β-diol were carried through without characterization and subjected to Oppenauer oxidation. The final product was purified via silica gel chromatography (elution with 1:24 acetone–chloroform), reverse-phase HPLC (Whatman M9-ODS column equilibrated with 80% acetonitrile–20% water), and silica gel HPLC (Waters μ-Porasil column equilibrated with 60% hexane–30% ethylene chloride–10% acetonitrile).

[1] References to these compounds have appeared in the patent literature (U.S. Patents 3 101 356, 3 101 357, and 3 257 430), but we could not find any chemical or biological characterization of the compounds in the literature.

[2] Abbreviations: NMR, nuclear magnetic resonance; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); DAST, (diethylamino)sulfur trifluoride.

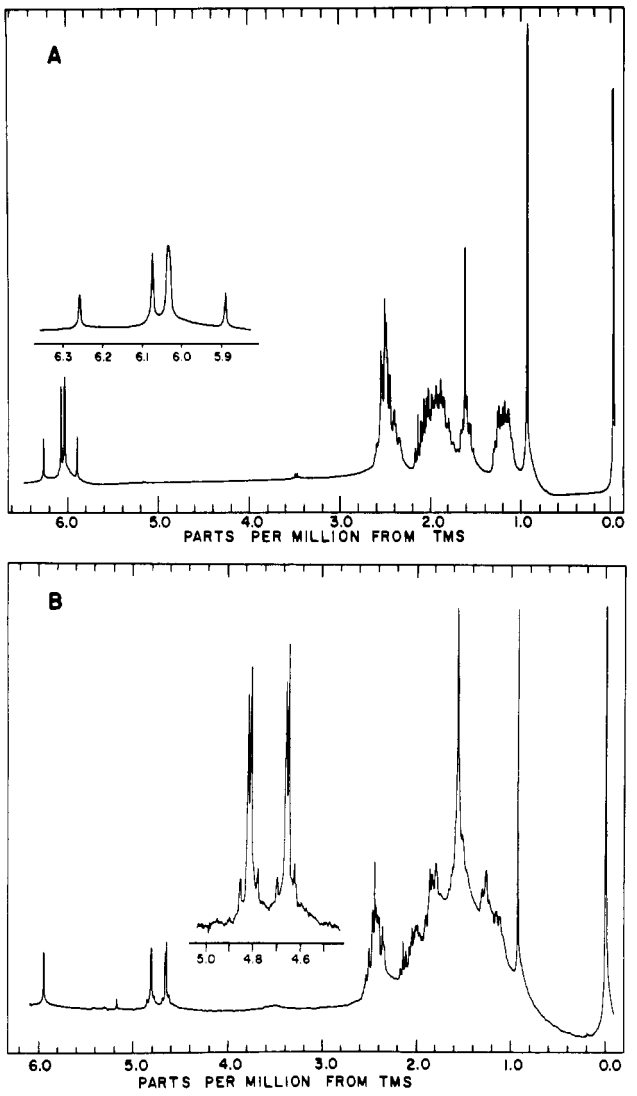


FIGURE 1: (A) 300-MHz NMR spectrum of 19,19-difluoroandrost-4-ene-3,17-dione (**1**). (B) Spectrum of 19-fluoroandrost-4-ene-3,17-dione. Both compounds were dissolved in $CDCl_3$. The peak at δ 1.5 is due to trace water contamination.

This protocol resulted in isolation of 1.8 mg[3] of (chromatographically homogeneous, >95% pure) 19-fluoroandrost-4-ene-3,17-dione (**2**): mp 111–113 °C; in the 300-MHz NMR (Figure 1B) the diastereotopic 19-methylene group (δ 4.6–4.9) exhibits the 50-Hz coupling constant due to fluorine; mass spectrum, M^+ 304. Anal. Calcd for $C_{19}H_{25}O_2F$ (exact mass measurement): 304.1832. Found: 304.1838.

19-Carboxy-3,17-dioxoandrost-4-ene-19-carbonyl Fluoride (**6**). 19-Carboxyandrost-4-ene-3,17-dione (400 mg) was slurried in 10 mL of methanol, treated with 1 equiv of aqueous 1 M NaOH (1.27 mL), evaporated, and thoroughly dried. The residue was slurried in 10 mL of dry acetonitrile, and 1 mL of cyanuric fluoride was added dropwise. The salt dissolved as the reagent was added. After 5 min at 25 °C, the reaction mixture was diluted with ether and washed with water, and the organic phase was dried with Na_2SO_4 and evaporated to dryness. The chloroform-soluble product (the evaporated residue contains a large amount of insoluble byproducts) was purified by column chromatography on silica gel (elution with 1:19 acetone–chloroform), which led to the isolation of material that exhibited absorbance in the ultraviolet and showed the expected R_f on silica gel TLC. HPLC (Waters μ-Porasil column equilibrated with 60% hexane–30% ethylene chloride–10% acetonitrile) resolved the crude product into two components. 19-Carboxy-3,17-dioxoandrost-4-ene-19-carbonyl fluoride (**6**, 21 mg) was crystallized from hexane: mp 155–160 °C; IR ν_{max} 1823, 1733, 1675 cm^{-1}; mass spectrum, M^+ 318. Anal. Calcd for $C_{19}H_{23}O_2F$: C, 71.68; H, 7.28; F, 5.97. Found: C, 71.49; H, 7.34; F, 5.77.

The second product, 19-norandrost-4-ene-3,17-dione (45 mg), was crystallized from hexane: mp 164–166 °C [lit. mp 169–171 °C (Berkoz et al., 1963)]; IR ν_{max} 1735 and 1665 cm^{-1}; mass spectrum, M^+ 272.

Inhibition Experiments. Stock solutions (10 mM) of the 19-fluoro (**2**) and 19,19-difluoro (**1**) substrate analogues in methanol were prepared. A 1:100 dilution of these solutions in water resulted in 1% methanol solutions 100 μM in steroid. A 2.5 mM solution of 19-carboxy-3,17-dioxoandrost-4-ene-19-carbonyl fluoride (**6**) in acetonitrile was prepared, and the steroid was added directly to incubations with a microliter syringe. Up to 1% acetonitrile or methanol in buffer had no significant effect on aromatase activity or stability.

Assays of aromatase activity were carried out in 10 mM potassium phosphate, 100 mM KCl, 1 mM EDTA, which contained 125 μM NADPH, 1 *or* 2 μM [1,2-^{3}H]androst-4-ene-3,17-dione, and varying concentrations of inhibitors in a total volume of 2 mL at 37 °C. Rates of estrogen formation were determined by extraction of aliquots of the incubation with $CHCl_3$ and analysis of the radioactivity released into the aqueous medium. The usual time course was 9 min over which five points were taken.

Inactivation Experiments. Preincubations of substrate analogues were contained in a total volume of 0.5 mL at 37 °C, 0.9 mg of microsomal protein and 1 mM NADPH in the same buffer as above. Glucose 6-phosphate (10 mM), 0.1 mg/mL glucose-6-phosphate dehydrogenase, and 5 mM $MgCl_2$ were added to ensure against depletion of reduced cofactor. The preincubation contained 1–10 μM steroid inhibitors. Aliquots (0.05 mL) were withdrawn at intervals, and the aromatase activity was assayed by dilution into 1 mL of buffer containing 1 μM [1,2-^{3}H]androst-4-ene-3,17-dione and 125 μM NADPH. In some preincubations dithiothreitol (5 mM) or ethanolamine hydrochloride (20 mM) was added. In the absence of any steroid, placental microsomes undergo loss of approximately 35% of their aromatase activity in 1 h at 37 °C.

Irreversibility of Inhibition Caused by 19,19-Difluoroandrost-4-ene-3,17-dione (**1**). In 2 mL of total volume at 37 °C, 1.8 mg of microsomal protein was incubated with 10 μM **1** in the presence of 1 mM NADPH, 10 mM glucose phosphate, 5 mM $MgCl_2$, and 0.2 mg of glucose-6-phosphate dehydrogenase. The buffer was the same as that in kinetic assays. The control incubation lacked NADPH. After 1 h, the incubations were diluted with cold buffer, and the microsomal pellet was precipitated by ultracentrifugation. The pellets were resuspended in buffer, diluted, centrifuged, resuspended a second time, and allowed to stand at 0 °C overnight. The amount of protein and the aromatase activity were assayed after both centrifugations and after standing overnight. The control incubation lost no more than 25% of its initial activity during the experiment.

Aromatization of 19-Fluoroandrost-4-ene-3,17-dione (**2**). In 2 mL of total volume at 37 °C, 10 μM **2** was incubated with 1.8 mg of microsomal protein in buffer containing 1 mM NADPH. A control incubation lacked NADPH. The incu-

[3] This very low yield synthesis was carried out after attempts to reproduce the patent synthesis were unsuccessful. No effort has yet been made to improve the yield, nor have the major products been characterized.

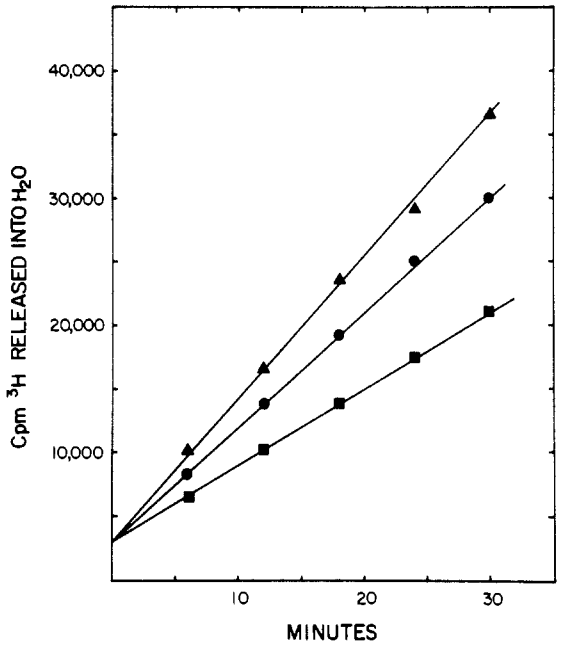


FIGURE 2: Kinetics of competitive inhibition of aromatase by various concentrations of 19-fluoroandrost-4-ene-3,17-dione (**1**): (■) 5 μM; (●) 1 μM; (▲) no inhibitor (vs. 1 μM substrate).

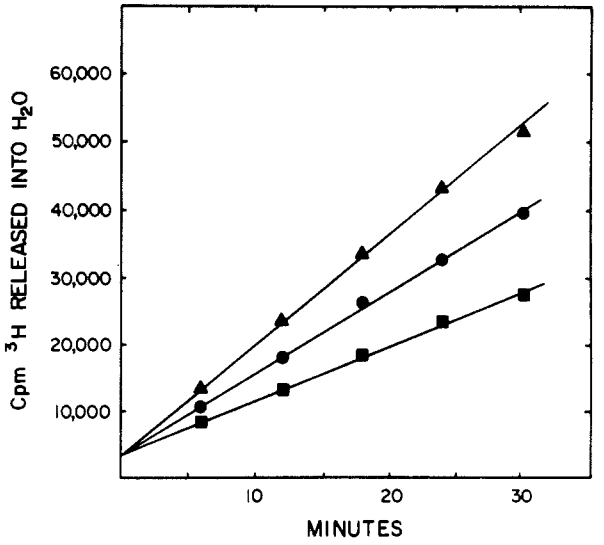


FIGURE 3: Kinetics of competitive inhibition of aromatase by various concentrations of 19,19-difluoroandrost-4-ene-3,17-dione (**2**): (■) 5 μM; (●) 1 μM; (▲) no inhibitor (vs. 1 μM substrate).

bations were quenched by extraction with CH_2Cl_2 (different incubations were quenched at different intervals), the organic extract was dried, and the solvent was evaporated. The composition of the residue was analyzed on a Waters μ-Partisil analytical HPLC column equilibrated with 60% hexane–30% ethylene chloride–10% acetonitrile. Products were detected and quantified by their ultraviolet absorbance at 225 nm. The analysis was calibrated by injection of calculated amounts of standards [**2**, 19-oxoandrost-4-ene-3,17-dione (**8**), estrone, estradiol]. In all extracts, the major components observed were unchanged inhibitor (**2**) and estrone.

Results

Substrate analogues 19-fluoro- (**2**) and 19,19-difluoroandrost-4-ene-3,17-dione (**1**) have been synthesized and found to be inhibitors of the aromatase activity of human term placental microsomes (Figures 2 and 3). The monofluoro compound (**2**) binds more efficiently: with 1 μM substrate 50% inhibition of activity is observed at 2.9 μM. Under the

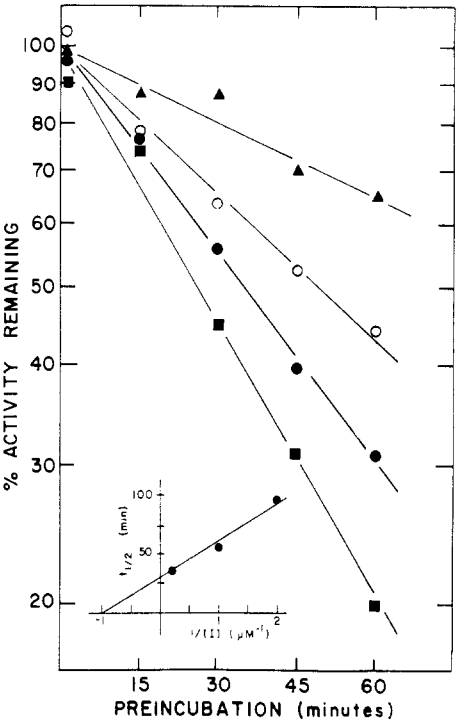
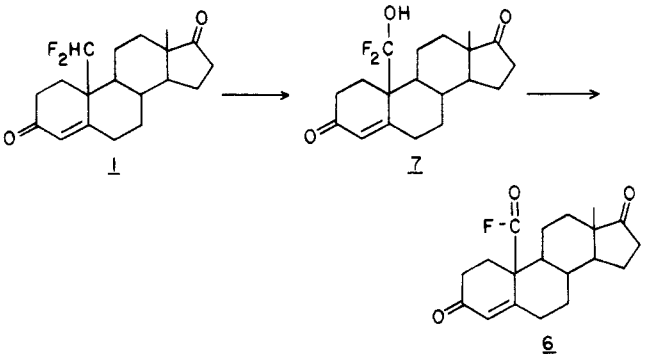


FIGURE 4: Kinetics of NADPH-dependent inactivation of aromatase by various concentrations of 19,19-difluoroandrost-4-ene-3,17-dione (**1**): (■) 5 μM; (●) 1 μM; (○) 0.5 μM; (▲) no inhibitor (background loss of activity). (Inset) Analysis of inactivation rates (corrected for background).

Scheme I



same conditions the difluoro compound (**1**) causes 50% inhibition at 4.8 μM. The inhibition by both substrate analogues has been found to be competitive with substrate (androst-4-ene-3,17-dione).

Both compounds were tested as to their ability to induce inactivation of the aromatase activity of the microsomal preparation. 19,19-Difluoroandrost-4-ene-3,17-dione (**1**) has been found to cause progressive, time-dependent, irreversible loss of activity. Figure 4 demonstrates the kinetics of this process. Analysis[4] by the method of Kitz & Wilson (1962) demonstrates a K_i of 1.0 μM and a k_{inact} of 0.023 min^{-1} ($t_{1/2}$ at saturating inhibitor = 30 min). The inhibition appears irreversible since microsomes preincubated with 19,19-difluoroandrost-4-ene-3,17-dione (**1**) did not regain activity upon exhaustive washing or after standing 20 h following removal of the inhibitor. In the absence of NADPH the rate of inactivation is sharply reduced, implying the inactivation involves oxidative enzymatic activation[5] of the substrate analogue. We

[4] This analysis (inset to Figure 2) subtracts the rate of the background loss of activity from the rate observed in the presence of various concentrations of **1**.

believe it probable that covalent modification of the enzyme takes place; however, we have not yet demonstrated this point with radiolabeled inhibitor.

Oxidation of the 10β-difluoromethyl group of **1** by aromatase would yield a 10β-hydroxydifluoromethyl (**7**) intermediate. Elimination of fluoride would generate acyl fluoride (**6**) (Scheme I). Therefore, authentic acyl fluoride was synthesized from the corresponding carboxylic acid and its interaction with the enzyme studied. These experiments were complicated by the hydrolytic instability of the acyl fluoride in buffered aqueous solution (10 mM potassium phosphate, 100 mM KCl, 1 mM EDTA). The acyl fluoride (**6**) exhibits λ_{max} 242 nm and ϵ 15000 in this system; the carboxylate anion formed by hydrolysis exhibits λ_{max} 253 nm and ϵ 11000. Therefore the hydrolysis could be monitored by changes in the ultraviolet spectrum of the steroid solution (50 μM steroid in 2% acetonitrile–buffer). The half-life of acyl fluoride was found to be 14 min at 37 °C.

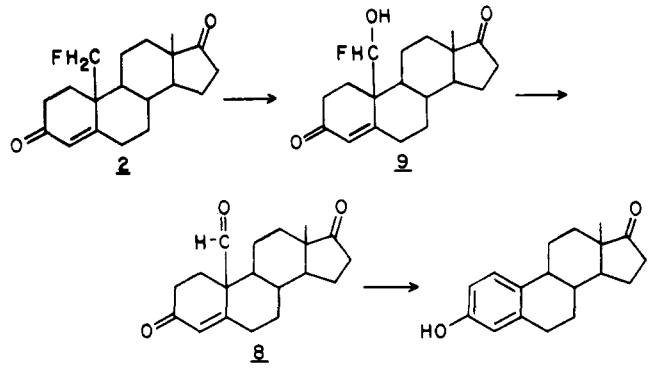
In studies of the initial rate of estrogen synthesis with 1 μM substrate, the acyl fluoride (**6**) was found to be a reversible inhibitor, causing 50% inhibition at 4 μM. In short-term preincubations, **6** appeared to cause some loss of aromatase activity. However, the maximum loss of activity observed in incubation with 10 μM acyl fluoride was approximately 50% (over 30 min); longer incubations did not result in further inactivation possibly because of the disappearance of the acyl fluoride from solution.

We have also been unable to demonstrate protection against inactivation induced by **1** by addition of either thiol (5 mM dithiothreitol) or amine (20 mM ethanolamine hydrochloride) to the incubation, circumstantial evidence that a reactive alkylating agent is not released from the active site. The decomposition of synthetic acyl fluoride (**6**) in buffer containing 20 mM ethanolamine hydrochloride can be monitored by changes in the ultraviolet spectrum (a shift from 242 to 250 nm), and that species has been found to have a half-time of 2 min at 37 °C in this buffer.

Our mechanistic rationale for studying the interaction of 19,19-difluoroandrost-4-ene-3,17-dione (**1**) and placental aromatase was the possibility of enzymatic conversion of the difluoromethyl moiety into an electrophilic acyl fluoride (**6**), with subsequent covalent modification of the enzyme. However, the inconclusive nature of the experiments with synthetic acyl fluoride and aromatase leaves the question of the mechanism of the inactivation caused by **1** as a matter for conjecture. Our results do show that if **6** is the causative agent of enzyme inactivation, it must be produced by fluoride elimination at the active site and not free in solution. Characterization of the enzyme–inhibitor linkage might demonstrate the mechanism of the process, but these experiments await the synthesis of radiolabeled **1** and the isolation of a more purified enzyme preparation.

We turn now to experiments with the related monofluoro compound 19-fluoroandrost-4-ene-3,17-dione (**2**). The same electrophile postulated to be formed from **1** might be produced from **2** if the enzyme were to carry out two rapid successive hydroxylations followed by elimination of one hydroxide anion. This evidently does not happen: the monofluoro substrate analogue (**2**) does not cause time-dependent loss of activity greater than the background. We have shown that **2** is *aromatized* by placental microsomes. Incubation of 1.8 mg of placental microsomal protein, 1 mM NADPH, and **2** (6.06 μg, 20 nmol) resulted in production of 2.8 μg of estrone (52% conversion) in 40 min. The identity of the product isolated by HPLC was confirmed by its mass spectrum. Substantial 19-fluoro starting material (1.8 μg) remained in the incubation, demonstrating that aromatization of **2** (Scheme II) is the predominant pathway in its reaction with aromatase.

Scheme II



No 19-oxoandrost-4-ene-3,17-dione (**8**) could be detected (limits of detection 0.5 μg). Also, 19-fluoroandrost-4-ene-3,17-dione (**2**) was stable to incubation with aromatase in the absence of NADPH, and organic solvent extracts of our placental microsomal preparation did not contain significant quantities of species that interfered with the analysis.

Discussion

This paper reports inactivation of human placental aromatase by 19,19-fluoroandrost-4-ene-3,17-dione (**1**). A probable mechanism of inactivation is enzyme-catalyzed oxidation of the substrate analogue and elimination of hydrogen fluoride, thereby forming an electrophilic acyl fluoride (**6**) (Scheme I). Inactivation would follow from covalent modification of an enzyme nucleophile. The lack of protection observed by the addition of nucleophilic trapping agents and the failure of synthetic acyl fluoride to mimic the inactivation process rule out formation of acyl fluoride (**6**) in solution, followed by rebinding to the enzyme and subsequent inactivation.

Studies of the interaction of aromatase and **6** are complicated by the intrinsic reactivity of the acyl fluoride. There may also be a difference in the conformation of the enzyme and its susceptible nucleophiles when it is carrying out oxidative catalysis on the difluoromethyl substrate analogue (**1**) as opposed to when it binds acyl fluoride (**6**) encountered from solution. Mechanistic arguments must remain speculative until synthesis of a radiolabeled inhibitor and characterization of the enzyme–inhibitor linkage are accomplished.

The related 19-fluoroandrost-4-ene-3,17-dione (**2**) has been shown to be an alternate substrate for aromatase: this substrate analogue is converted to estrone without significant inactivation of the enzyme. The high-yield conversion implies that this transformation takes place (Scheme II) without release of a geminal fluorohydrin (**9**) or an aldehyde intermediate (**8**), but because **8** is also aromatized by the enzyme (Morato et al., 1961) and might not have been detected in small amounts, this point remains uncertain. The enzyme must not oxidize the postulated fluorohydrin intermediate (**9**) to the 19-dihydroxyfluoro moiety as this would presumably result in carboxylic acid (or possibly acyl fluoride) formation and not in aromatization.

Because of the in vivo utility of some other previously discovered aromatase inhibitors (Brodie et al., 1977, 1979, 1981),

[5] The rate at which placental microsomes lose aromatase activity in the absence of any steroidal compound is accelerated severalfold by NADPH (to a maximum of 35% over 1 h at 37 °C). Therefore, in the absence of cofactor, the activity is stabilized with respect to both the inactivation induced by **1** and the background loss of activity.

experiments are in progress to determine whether 19,19-difluoroandrost-4-ene-3,17-dione has in vivo activity. Although the inactivation by this compound occurs more slowly than that caused by other reported inactivators of aromatase (Covey et al., 1981; Metcalf et al., 1981), the difluoromethyl group is chemically stable and resistant to enzymatic degradative processes. Specific action in vivo as a result of hydroxylation at C-19 of the steroid structure by aromatase is therefore a possibility.

Acknowledgments

We thank Joseph Bauer for technical assistance, Joseph Cousins and Dr. L. S. Kan for the 300-MHz NMR spectra obtained at the Johns Hopkins Biomedical Research Center (National Institutes of Health Grant GM 27512), and Sharon Pallante, David Heller, and Dr. C. Fenselau for mass spectra obtained at the Middle Atlantic Mass Spectrometry Laboratory, a National Science Foundation shared instrumentation facility.

References

Berkoz, B., Denot, E., & Bowers, A. (1963) *Steroids 1*, 251–269.

Blohm, T. R., Metcalf, B. W., Laughlin, M. E., Sjoerdsma, A., & Schatzman, G. L. (1980) *Biochem. Biophys. Res. Commun. 95*, 273–280.

Brodie, A. M. H., Schwarzel, W. C., Shaikh, A. A., & Brodie, H. J. (1977) *Endocrinology (Philadelphia) 100*, 1684–1695.

Brodie, A. M. H., Marsh, D. A., & Brodie, H. J. (1979) *J. Steroid Biochem. 10*, 423–429.

Brodie, A. M. H., Garrett, W. M., Hendrickson, J. R., Morris, C.-H. T., Marcotte, P. A., & Robinson, C. H. (1981) *Steroids 38*, 693–702.

Covey, D. F., Hood, W. F., & Parikh, V. D. (1981) *J. Biol. Chem. 256*, 1076–1079.

Jen, T., & Wolff, M. E., (1963) *J. Org. Chem. 28*, 1573–1575.

Kalvoda, J., Heusler, K., Ueberwasser, H., Anner, G., & Wettstein, A. (1963) *Helv. Chim. Acta 46*, 1361–1369.

Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Marcotte, P. A., & Robinson, C. H. (1981) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 40*, 1867.

Metcalf, B. W., Wright, C. L., Burkhart, J. P., & Johnston, J. O. (1981) *J. Am. Chem. Soc. 103*, 3221–3222.

Middleton, W. J. (1975) *J. Org. Chem. 40*, 574–578.

Morato, T., Hayano, M., Dorfman, R. I., & Axelrod, L. R. (1961) *Biochem. Biophys. Res. Commun. 6*, 334–338.

Motohashi, T., Wu, C. H., Abdel-Rahman, H. A., Marymor, N., & Mikhail, G. (1979) *Am. J. Obstet. Gynecol. 135*, 89–95.

Reed, K. C., & Ohno, S. (1976) *J. Biol. Chem. 251*, 1625–1631.

Ryan, K. J. (1959) *J. Biol. Chem. 234*, 268–272.

Schade, K., & Schubert, K. (1979) *Endokrinologie 74*, 90–99.

Schwarzel, W. C., Kruggel, W., & Brodie, H. J. (1973) *Endocrinology (Philadelphia) 92*, 866–880.

Thompson, E. A., & Siiteri, P. K. (1974) *J. Biol. Chem. 249*, 5364–5372.

Inhibition of Chorismate Mutase Activity of Chorismate Mutase-Prephenate Dehydrogenase from *Aerobacter aerogenes*†

Herbert S.-I. Chao and Glenn A. Berchtold*

ABSTRACT: Inhibition data (I_{50} values) have been obtained for inhibitors of the chorismate mutase activity of chorismate mutase-prephenate dehydrogenase from *Aerobacter aerogenes*. Several 1-substituted adamantane derivatives were investigated; the order of decreasing inhibitory activity with the various substituents was $-PO_3^{2-} \gg -P(OCH_3)O_2^- > -CO_2^- > -CH_2CO_2^- > -SO_2^- > -SO_3^-$. 3-Chloroadamantane-1-acetic acid was slightly less effective than adamantane-1-acetic acid. 2-(1-Carboxy-1,4-dihydrobenzyl)acrylic acid (**19**), an analogue of prephenate, was an effective inhibitor. Other substances investigated, including 2,4,10-trioxaadamantane-1-acetic acid (**15**), 5-enolpyruvylshikimic acid (**20**), and 1-(carboxyethyl)-1,4-dihydrobenzoic acid (**18**), failed to inhibit chorismate mutase activity under the conditions investigated.

The classic work of Gibson that established the importance of chorismic acid in the biosynthesis of aromatic amino acids, and numerous other substances, in bacteria, fungi, and higher plants has been reviewed (Gibson & Pittard, 1968; Haslam, 1974; Lingens, 1968; Weiss & Edwards, 1980). Of the metabolic transformations available to chorismate (**1**), the rearrangement to prephenate (**2**), catalyzed by chorismate mutase, has attracted most attention since such enzyme-catalyzed reactions, formally oxy-Cope rearrangements, are rare in biochemical systems. The transformation **1** → **2** (Figure 1) is the first step from the chorismate branch point for the biosynthesis of tyrosine and phenylalanine in the plant world.

Chorismate mutase exists as a monofunctional enzyme in many species of microorganisms and higher plants. *Streptomyces aureofaciens* possesses such a mutase (Görisch & Lingens, 1973, 1974; Görisch, 1978). The occurrence of bifunctional enzymes with chorismate mutase activity is common. The enteric bacteria *Aerobacter aerogenes* and *Escherichia coli*, for example, have a chorismate mutase-prephenate dehydrogenase and a chorismate mutase-prephenate dehydratase. The rearrangement of **1** to **2** occurs in the ab-

†From the Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139. *Received September 22, 1981; revised manuscript received February 10, 1982.* We are grateful to the National Institutes of Health (Grant GM 19103) for financial support.

0006-2960/82/0421-2778$01.25/0