Tritium Release from [19-^{3}H]-19,19-Difluoroandrost-4-ene-3,17-dione during Inactivation of Aromatase†

Paul S. Furth and Cecil H. Robinson*

Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received July 7, 1988; Revised Manuscript Received September 16, 1988

ABSTRACT: Aromatase is a cytochrome P-450 enzyme involved in the conversion of androst-4-ene-3,17-dione to estrogen via sequential oxidations at the 19-methyl group. Previous studies from this laboratory showed that 19,19-difluoroandrost-4-ene-3,17-dione (**5**) is a mechanism-based inactivator of aromatase. The mechanism of inactivation was postulated to involve enzymic oxidation at, and hydrogen loss from, the 19-carbon. The deuteriated analogue **5b** has now been synthesized and shown to inactivate aromatase at the same rate as the nondeuteriated parent (**5**). We conclude that C19–H bond cleavage is not the rate-limiting step in the overall inactivation process caused by **5**. [19-^{3}H]-19,19-Difluoroandrost-4-ene-3,17-dione (**5b**) with specific activity of 31 mCi/mmol was also synthesized to study the release of tritium into solution during the enzyme inactivation process. Incubation of [19-^{3}H]19,19-difluoroandrost-4-ene-3,17-dione with human placental microsomal aromatase at differing protein concentrations resulted in time-dependent, NADPH-dependent, and protein-dependent release of tritium. This tritium release is not observed in the presence of (19*R*)-10β-oxiranylestr-4-ene-3,17-dione, a powerful competitive inhibitor of aromatase. We conclude that aromatase attacks the 19-carbon of 19,19-difluoroandrost-4-ene-3,17-dione, as originally postulated.

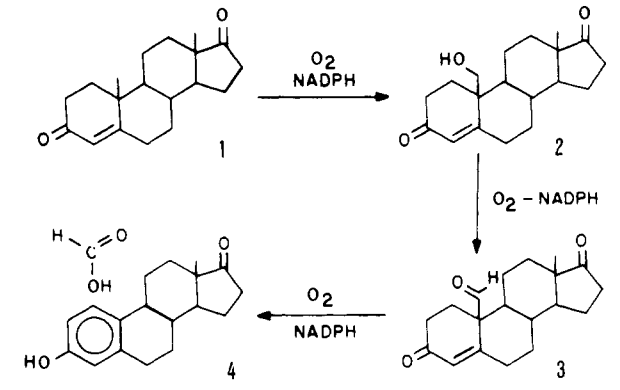
Aromatase cytochrome P-450 is an important biosynthetic system which converts androgens to estrogens. The transformation of androstenedione (**1**) to estrone (**4**) proceeds via the 19-hydroxy and 19-oxo intermediates (**2** and **3**, respectively), with loss of C19 as formic acid (Meyer, 1955a,b; Ryan, 1959; Thompson & Siiteri, 1974a,b) (Scheme I). Although the process is generally accepted to involve successive oxidations at the C19 position [cf. Akhtar and Skinner (1969)], the precise nature of the third step is still not established (Townsley & Brodie, 1968; Hosoda & Fishman, 1974; Akhtar et al., 1976; Caspi et al., 1984). The biological importance of the androgen–estrogen conversion has led to the synthesis of many inhibitors [cf. Brodie et al. (1983) and Johnston and Metcalf (1984)] some of which were designed as mechanism-based (suicide) inactivators (Covey et al., 1981; Metcalf et al., 1981; Marcotte & Robinson, 1982a,b).

This laboratory previously described (Marcotte & Robinson, 1982a) the synthesis and inhibitory properties of the mechanism-based inactivator 19,19-difluoroandrost-4-ene-3,17-dione (**5**). This compound was designed so that the normal enzymatic hydroxylation at C19 might generate an electrophilic acyl fluoride (Scheme II), resulting in covalent modification of the enzyme's active site.

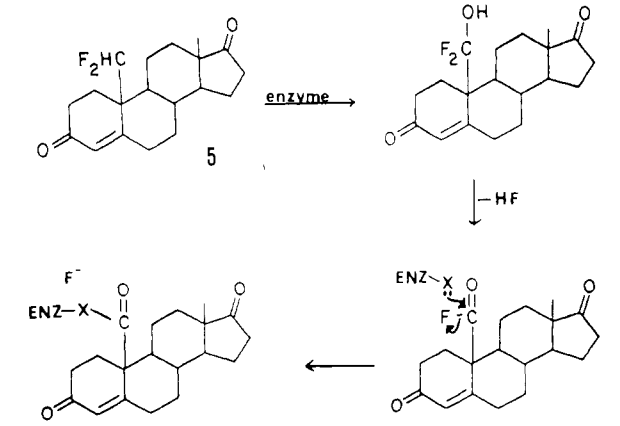
The difluoro compound (**5**) indeed caused NADPH-dependent time-dependent inactivation of human placental microsomal aromatase. Furthermore the *mono*fluoro analogue (**6**) proved to be a noninactivating substrate for the enzyme and was shown to be converted to estrone (**4**) presumably via hydroxylation at the 19-carbon (Marcotte & Robinson, 1982a). These and related data led us to conclude that the difluoro compound (**5**) was indeed hydroxylated at C19 by aromatase.

We now describe experiments which confirm that the difluoro compound (**5**) is indeed attacked at C19 by aromatase.

Scheme I



Scheme II

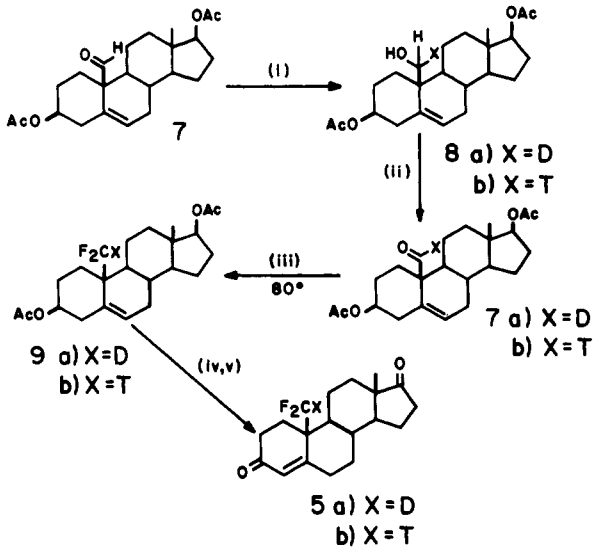


We believe these experiments represent the only such demonstration for an aromatase inactivator. We have synthesized [19-^{3}H]-19,19-difluoroandrost-4-ene-3,17-dione (**5b**) (Scheme III) and have shown that incubation of this compound with human placental aromatase results in time-dependent release of tritium from **5b** into the aqueous medium. This tritium-

†This work was supported in part by National Institutes of Health Grant HD-11840.

0006-2960/89/0428-1254$01.50/0

Scheme III[a]



[a] (i) $NaBD_4$ or $NaBT_4$ in MeOH at 25 °C; (ii) Collins reagent in CH_2Cl_2 at 25 °C; (iii) DAST at 80 °C; (iv) KOH–CH_3OH at 25 °C; (v) Oppenauer oxidation.

release process requires *both* enzyme and NADPH cofactor.

We have also examined the interaction of the 19-deuteriated analogue [19-^{2}H]-19,19-difluoroandrost-4-ene-3,17-dione (**5a**) with aromatase. Comparison of the inactivation of aromatase by **5a** with that induced by the unlabeled inhibitor **5** showed essentially identical kinetics of inactivation. Therefore, loss of the 19-hydrogen is not rate limiting in the inactivation process.

Materials and Methods

General

Human placental microsomes were prepared as described by Ryan (1959). Protein was determined with a Bio-Rad protein assay kit, obtained from Bio-Rad Laboratories. The aromatase activity of the microsomes 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 (1974a). The microsomal preparation had a specific activity of 265 pmol of estrogen min^{-1} (mg of protein)$^{-1}$. All biochemical reagents were purchased from Sigma Chemical Co. [^{3}H]Sodium borohydride (350 mCi/mmol) was purchased from Amersham Corp. [^{2}H]Sodium borohydride was purchased from Merck & Co. 19-Oxoandrost-5-ene-3β,17β-diol diacetate and 19,19-difluoroandrost-4-ene-3,17-dione were prepared as described by Marcotte and Robinson (1982a). (Diethylamino)sulfur trifluoride (DAST) was purchased from Aldrich Chemical Co.

Melting points were obtained with a Kofler hot stage and are uncorrected. Proton NMR spectra were recorded on an IBM FT-80 spectrometer in $CDCl_3$ solutions (Me_4Si as internal standard). Chemical shifts are expressed as δ values (Me_4Si, δ 0) with signal multiplicities shown as s (singlet), d (doublet), t (triplet), and m (multiplet). Fluorine NMR spectra were obtained at 75.4 MHz on an IBM FT-80 instrument with $CDCl_3$ solutions, and chemical shifts are expressed as δ values (Freon 11 as internal standard). Infrared spectra were recorded on a Perkin-Elmer 521 spectrometer in chloroform solutions. Mass spectra were determined on an LKB-9000 spectrometer. Elemental analyses were determined by Galbraith Laboratories, Inc., Knoxville, TN. Chromatographic separations were performed on Woelm dry-column silica gel (EM Reagents), and analytical thin-layer chromatography was carried out on precoated silica gel plates (Macherey-Nagel and Co). Flash chromatography was carried out on silica gel (J. T. Baker Chemical Co.) according to Still's method (Still et al., 1978). HPLC solvents were purchased from Burdick Jackson, and HPLC separations were performed on a Waters Associates Model 6000 instrument.

Synthesis

[*19-^{2}H*]-*19-Hydroxyandrost-5-ene-3β,17β-diol Diacetate* (**8a**). To a stirred solution of 19-oxoandrost-5-ene-3β,17β-diol diacetate (**7**, 2.4 g) in methanol (192 mL) at 25 °C was added [^{2}H]sodium borohydride (405 mg). After this was stirred for 6 min at 25 °C, water (200 mL) was added, and the mixture was extracted twice with methylene chloride (100-mL portions) and once with chloroform (100 mL). The organic phases were combined, dried (Na_2SO_4), and evaporated in vacuo to give **8a**, which was used without purification in the next reaction.

[*19-^{2}H*]-*19-Oxoandrost-5-ene-3β,17β-diol Diacetate* (**7a**). A suspension of Collins reagent (13.5 g) (Collins & Hess, 1972) in methylene chloride (85 mL) was added to a stirred solution of the total crude product (**8a**) described above, in methylene chloride (190 mL). The mixture was stirred for 15 min and then filtered through a sintered glass funnel. The residue on the filter was washed thoroughly with methylene chloride. The filtrate and washings were combined, concentrated in vacuo, and filtered through a Florisil column (30 g). The column was eluted with ethyl acetate, and the combined methylene chloride and ethyl acetate eluates were passed through a silica gel column. Evaporation in vacuo gave crude product (**7a**, 2.40 g). This material was then reduced with [^{2}H]sodium borohydride in methanol exactly as described above and then oxidized back to compound **3a** with Collins reagent, again as described above. The crude product was crystallized from methanol to give pure [19-^{2}H]-19-oxoandrost-5-ene-3β,17β-diol diacetate (**7a**, 1.95 g). Comparison of the ^{1}H NMR and mass spectra with those of the nondeuteriated compound confirmed its structure and indicated the presence of greater than 95% deuterium at the 19-carbon, while its purity was confirmed by TLC and HPLC.

[*19-^{2}H*]-*19,19-Difluoroandrost-4-ene-3,17-dione* (**5a**). A solution of the above 19-^{2}H-labeled 19-oxo compound (**7a**, 1.92 g) in (diethylamino)sulfur trifluoride (DAST, 12 mL) was heated at 80 °C for 2 h. The mixture was cooled to 25 °C, diluted with methylene chloride, and added slowly, dropwise, to crushed ice. The organic phase was washed with saturated aqueous $NaHCO_3$, dried (Na_2SO_4), and evaporated in vacuo to give a brown oil which was dissolved in ethyl acetate and filtered through a short silica gel column. The eluate was evaporated in vacuo. The resulting oil was dissolved in MeOH (150 mL), and sodium borohydride (300 mg) was added. After being stirred for 5 min, the mixture was diluted with water (150 mL) and partitioned between methylene chloride and water. The organic phase was dried (Na_2SO_4) and evaporated in vacuo to give impure [19-^{2}H]-19,19-difluoroandrost-5-ene-3β,17β-diol diacetate. The acetate groups were then hydrolyzed in 2.5% methanolic KOH (180 mL) at 25 °C for 2 h. After glacial acetic acid (3.6 mL) was added, the mixture was diluted with water and extracted successively with chloroform and ethyl acetate. The combined organic extracts were washed with water, dried (Na_2SO_4), and evaporated to give an amber oil which was flash chromatographed on silica gel (ethyl acetate–hexane, 4:1) to give [19-^{2}H]-19,19-difluoroandrost-5-ene-3β,17β-diol (681 mg) after crystallization from hexane. This material was then oxidized by a modified Oppenauer procedure (Raggio & Watt, 1976) as follows: The

foregoing 3β,17β-diol (681 mg) was dissolved in toluene (125 mL) and *N*-methyl-4-piperidone (12.8 mL). After distillation of 10 mL of the solution into a Dean-Stark trap, aluminum isopropoxide (5.4 g) was added, and the reaction mixture was heated to reflux under the Dean-Stark trap for 1 h. The mixture was cooled and diluted with ethyl acetate, then was washed several times with 5% aqueous HCl (300mL, total) and then with water, and dried ($MgSO_4$). Evaporation in vacuo gave an oil whose infrared spectrum indicated incomplete oxidation at the 17-position. The oil was therefore dissolved in methylene chloride (20 mL) and treated with Collins reagent (1.50 g), in methylene chloride (40 mL) for 15 min with stirring at 25 °C to complete oxidation of the 17β-hydroxyl group. The oxidation mixture was filtered through a sintered glass funnel, and the residue on the filter was washed exhaustively with methylene chloride. The combined filtrate and washings were concentrated and filtered through a short column (3 g) of silica gel (ethyl acetate–hexane, 4:1). The resulting product was purified by flash chromatography on silica gel (ethylene dichloride–ethyl acetate, 4:1) followed by reversed-phase HPLC (Whatman M9-ODS column; acetonitrile–water, 4:1). The resulting pure [19-^{2}H]-19,19-difluoroandrost-4-ene-3,17-dione (**5a**, 185 mg) had the following data: mp 108–109.5 °C (from hexane); IR 2940, 1730, 1660, 1620 cm^{-1}; ^{1}H NMR δ 6.02 (s, 1, C4-H), 0.94 s, 3, 18-CH_3); ^{19}F NMR δ −117.17 (t of d, J_{DF} = 8 Hz, J_{FF} = 285 Hz, 19-C^2HF_2); mass spectrum *m/z* 323 (M^+), 305, 279, 237, 161. Comparison of mp, proton and fluorine NMR spectra, and mass spectra with those of the nondeuteriated compound confirmed the structure. The NMR and mass spectra indicated greater than 95% deuterium content at the 19-carbon. The ^{19}F spectra of compounds **5** and **5a** are shown in Figure 1.

[19-^{3}H]-19,19-Difluoroandrost-4-ene-3,17-dione (**5b**). A solution of [^{3}H]sodium borohydride (25 mCi, sp act. 350 mCi/mmol) in methanol (0.45 mL) was added to a stirred solution of 19-oxoandrost-5-ene-3β,17β-diol diacetate (**7**, 13.7 mg) in methanol (0.70 mL) at 25 °C, and stirring was continued for 5 min. Water (2 mL) was added, and the mixture was extracted with chloroform (10 mL). The organic extract was dried (Na_2SO_4) and evaporated under a stream of nitrogen. The resulting [19-^{3}H]-19-hydroxyandrost-5-ene-3β,17β-diol 3,17-diacetate (**8b**) was dissolved in methylene chloride (2 mL), and Collins reagent (100 mg) was added. The mixture was stirred for 15 min at 25 °C and then diluted with ethyl acetate (125 mL) and filtered through a small Florisil column (0.70 g). The eluate was evaporated under a stream of nitrogen to give crude [19-^{3}H]-19-oxoandrost-5-ene-3β,17β-diol diacetate (**7b**, 13 mg). This material was dissolved in (diethylamino)sulfur trifluoride (DAST, 0.20 mL) together with unlabeled 19-oxoandrost-5-ene-3β,17β-diol diacetate (**7**, 7 mg), and the solution was heated at 80 °C for 2 h. The reaction mixture was cooled to 25 °C and diluted with chloroform (10 mL), and the organic extract was washed with saturated aqueous $NaHCO_3$ and dried (Na_2SO_4). Evaporation under a stream of nitrogen gave a mixture containing [19-^{3}H]-19,19-difluoroandrost-5-ene-3β,17β-diol diacetate. Without purification, this material was hydrolyzed in 2.5% methanolic KOH (2.0 mL) at 25 °C for 2 h. Acetic acid (0.5 mL) and water (10 mL) were added, and the mixture was extracted with chloroform (17 mL). The organic extract was dried (Na_2SO_4) and evaporated under a stream of nitrogen to give crude [19-^{3}H-19,19-difluoroandrost-5-ene-3β,17β-diol. This material was then subjected to modified Oppenauer oxidation, using toluene (8 mL), *N*-metyl-4-piperidone (0.5 mL), and aluminum isopropoxide (334 mg) for 1 h, by the procedure described above for the 19-^{2}H series. The crude Oppenauer oxidation product was dissolved in methylene chloride (2.5 mL) and treated with Collins reagent (100 mg) for 15 min at 25 °C, to complete oxidation at the 17-position. The reaction mixture was applied to a small Florisil column (605 mg) which was eluted with ethyl acetate (20 mL). The eluate was evaporated under a stream of nitrogen and then purified by HPLC (twice) using a Whatman Partisil 10/50 column (hexane–ethyl acetate–acetonitrile, 59:37:4) to give [19-^{3}H]-19,19-difluoroandrost-4-ene-3,17-dione (**5b**, 1.91 mg). This purified material had a specific activity of 31 mCi/mmol. A small sample was diluted with unlabeled 19,19-difluoroandrost-4-ene-3,17-dione to give material which, after one recrystallization from methylene chloride–hexane, had specific activities of 6250 cpm/mg (crystals) and 7765 cpm/mg (mother liquors material). Two more crystallizations (first from hexane–acetone then from aqueous methanol) gave specific activities of 6147 cpm/mg (crystals) and 6365 cpm/mg (mother liquor).

Enzyme Studies

Studies of Competitive Inhibition and Time-Dependent Inactivation of Microsomal Placental Aromatase. These studies with 19,19-difluoroandrost-4-ene-3,17-dione (**5**) and [19-^{2}H]-19,19-difluoroandrost-4-ene-3,17-dione (**5a**) were carried out as described previously for **5** by Marcotte and Robinson (1982a) using inhibitor concentrations between 1 and 10 μM.

Incubation of [19-^{3}H]-19,19-Difluoroandrost-4-ene-3,17-dione (**5c**) *with Human Placental Microsomal Aromatase.* The total volume of the standard assay system was 3.0 mL containing 10 mM potassium phosphate (pH 7.4), 100 mM potassium chloride, 1 mM EDTA, 5 mM dithiothreitol, 100 μM NADPH, 981 μg of placental microsomes, and [19-^{3}H]-19,19-difluoroandrost-4-ene-3,17-dione (11 μg; 31 mCi/mmol) at 37 °C. At appropriate time intervals, aliquots (900 μL) were removed and added to chloroform (1.60 mL). The mixture was vortexed for 1 min and then centrifuged in a clinical centrifuge for 10 min. The aqueous layer (700 μL) was back-washed with chloroform (2.3 mL), vortexed (1 min), and centrifuged (10 min). Duplicate portions (200 μL) of the resulting aqueous layer were counted in ACS (10 mL). Control experiments were set up, exactly as above, but without NADPH, or without microsomes, or without NADPH and microsomes.

Additional experiments were carried out in which larger quantities of microsomal protein were used. The composition of the 3-mL incubation mixture differed from the above only in the increased quantity of placental microsomes (1.473 or 1.962 mg) with corresponding increases in dithiothreitol (7.5 or 10 mM, respectively) and NADPH (150 or 200 μM, respectively). All other constituents remained unchanged.

Incubation of [19-^{3}H]-19,19-Difluoroandrost-4-ene-3,17-dione (**5c**) *with Human Placental Microsomal Aromatase in the Presence of (19R)-10β-Oxiranylestr-4-ene-3,17-dione.* A 3-mL preincubation mixture contained 10 mM potassium phosphate (pH 7.4), 100 mM potassium chloride, 1 mM EDTA, 5 mM dithiothreitol, 100 mM NADPH, 981 μg of microsomal placental aromatase, and 10 μM (19*R*)-10β-oxiranylestr-4-ene-3,17-dione (Shih et al., 1987; Kellis et al., 1987). A second mixture was prepared exactly as above, but without (19*R*)-10β-oxiranylestr-4-ene-3,17-dione. A third mixture contained all the components of the first except that microsomal protein was omitted. The mixtures were kept at 37 °C for 15 min, and 11 μg of [19-^{3}H]-19,19-difluoro-

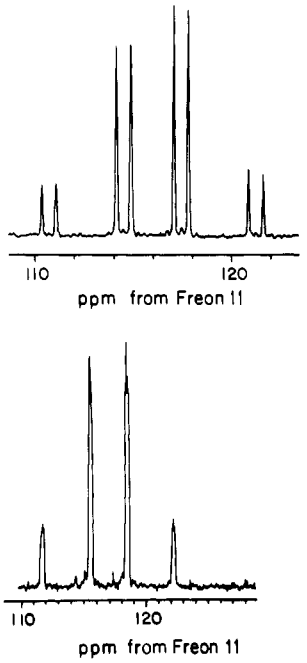


FIGURE 1: (Top) ^{19}F NMR spectrum of 19,19-difluoroandrost-4-ene-3,17-dione (**5**). (Bottom) ^{19}F NMR spectrum of [19-^2H]-19,19-difluoroandrost-4-ene-3,17-dione (**5a**).

androst-4-ene-3,17-dione (**5c**) was added to each. After incubation times of 20, 40, and 60 min, aliquots of each mixture were assayed for tritium release into water by the method described in the previous section.

Results

The isotopically labeled substrate analogues **5a** and **5b** have been synthesized to find out if aromatase attacks the 19-methyl group. Our final target was the tritiated analogue **5b**. The synthesis was first validated with deuterium labeling to give [19-^2H]-19,19-difluoroandrost-4-ene-3,17-dione (**5a**). In addition to validation of the synthetic route, **5a** provided an opportunity to see if replacement of hydrogen by deuterium at C19 in the inactivator (**5**) resulted in a change in the rate of inactivation of aromatase.

The synthesis of the deuterium-labeled inactivator (**5a**) was straightforward (Scheme III). The 19-oxo compound (**7**) was reduced to the 19-^2H-labeled 19-ol (**8a**) by brief treatment with sodium borodeuteride in methanol, essentially following the procedure of Akhtar (Skinner & Akhtar, 1969), who synthesized [19-^2H]- and [19-^3H]-19-oxoandrostenes. The resulting crude product was oxidized to the deuteriated 19-oxo compound (**7a**) with Collins reagent in methylene chloride. In order to get substantially completely deuteriated **7a**, we repeated the cycle (i.e., reduction with sodium borodeuteride, followed by oxidation with Collins reagent). The resulting 19-oxo compound (**7a**) had proton NMR and mass spectra which showed greater than 95% incorporation of deuterium at C19.

This 19-oxo compound (**7a**) was converted to [19-^2H]-19,19-difluoroandrost-5-ene-3β,17β-diol via fluorination with DAST at 80 °C [cf. Marcotte and Robinson (1982a)] followed by hydrolysis of the acetate groupings. Oxidation by the modified Oppenauer procedure then gave the desired [19-^2H]-19,19-difluoroandrost-4-ene-3,17-dione (**5a**). The pure compound showed proton NMR and mass spectra indicating greater than 95% deuterium content at the 19-carbon. The ^{19}F-NMR spectrum of **5a** showed the appropriate fluorine resonances (centered on δ −117.17, J_{FF} = 285 Hz, J_{DF} = 8 Hz) (Figure 1).

Table I: Aromatase-Induced Tritium Release from Compound **5b**[a]

time (min)	cpm of tritium released into water: complete incubation	control I (no enzyme)	control II (no NADPH)
10	510	33	127
15	878	109	171
30	1492	159	142
60	2310	403	439

[a] These data come from a typical 3-mL incubation experiment using 981 μg of placental microsomes and 11 μg of [^3H]-19,19-difluoroandrost-4-ene-3,17-dione at 37 °C as described under Materials and Methods.

We thought it of interest to compare the kinetics of inactivation of placental aromatase by 19-deuteriated inhibitor (**5a**) and the unlabeled compound (**5**). Kitz–Wilson analysis (Kitz & Wilson, 1964) gave K_i values for **5** and **5a** which were indistinguishable (0.83 μM) and very similar k_{inact} values which were respectively 0.048 min^{-1} and 0.055 min^{-1}. Our previously reported values for the nondeuteriated inhibitor (**5**) were K_i = 1 μM and k_{inact} = 0.023 min^{-1} (Marcotte & Robinson, 1982a). The inactivation rates seen with **5** and **5a** under identical conditions were essentially the same, indicating that loss of hydrogen at C19 is not the rate-limiting step in the overall inactivation process. There may nevertheless be a concealed isotope effect associated with the cleavage of the C19–H bond.

We now set about synthesizing the tritiated analogue of **5**, by exactly the same route as for **5a**, but substituting [^3H]sodium borohydride in the first step. Repetition of the reduction–oxidation sequence was not necessary in this case. We confirmed the identity of [19-^3H]-19,19-difluoroandrost-4-ene-3,17-dione (**5b**) by TLC and HPLC, as well as by crystallization to constant specific activity.

Compound **5b**, with specific activity of 31 mCi/mmol, was then incubated with placental aromatase to test for time-dependent release of tritium from C19 into the aqueous medium. All experiments employed high concentrations (11.4 μM) of the tritiated inhibitor (K_i = 0.83 μM) and involved incubation at 37 °C with microsomes and the periodic removal of aliquots to determine tritium release into the aqueous medium. The three control incubations lacked either microsomes, NADPH, or both microsomes and NADPH. Other experiments differed only in the use of greater quantities of microsomes. Table I shows data from one such experiment.

These experiments showed that incubation of **5b** with placental aromatase caused time-dependent release of tritium into the aqueous medium. Furthermore, this tritium release required the presence of both NADPH and microsomes and was proportional to the quantity of aromatase preparation used (Figure 2). The figures for ^3H released are corrected for the controls.

A second experiment differed from the above in that a portion of the incubation was removed after 60 min and monitored for tritium release for a further 30 min. The remaining portion was treated with additional fresh microsomal aromatase and was also monitored for an additional 30 min. The portion treated with additional aromatase showed increase tritium release compared with the untreated portion (Figure 3).

In a third series of experiments, the microsomal aromatase preparation was preincubated with a very potent and specific competitive aromatase inhibitor prior to the addition of **5b**. This inhibitor, (19*R*)-10β-oxiranylestr-4-ene-3,17-dione (Shih et al., 1987; Kellis et al., 1987), has K_i = 7 × 10^{-9} M and was used at a concentration of 10 μM. If the release of tritium

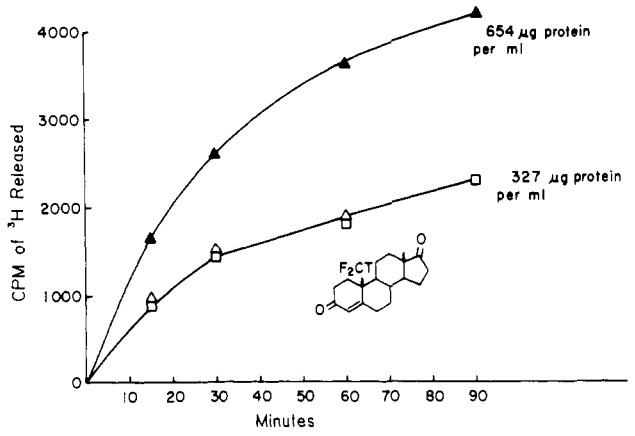


FIGURE 2: Aromatase-induced time-dependent release of tritium into water from [19-^{3}H]-19,19-difluoroandrost-4-ene-3,17-dione (11.4 μM), corrected for controls lacking microsomal aromatase and NADPH: (□, ▲) 0.981 mg of protein/3-mL incubation; (▲) 1.962 mg of protein/3-mL incubation.

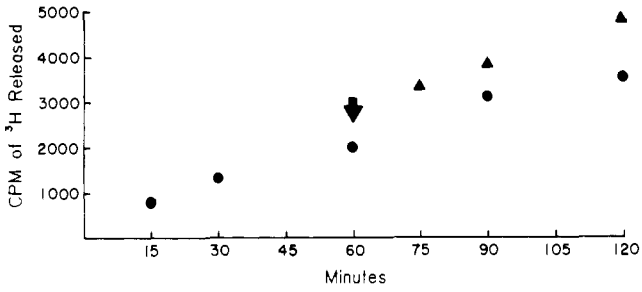


FIGURE 3: (●) Aromatase-induced time-dependent release of tritium into water from [19-^{3}H]-19,19-difluoroandrost-4-ene-3,17-dione (11.4 μM) with 0.981 mg of protein/mL of incubation mixture. Corrected for controls lacking microsomal aromatase and NADPH. The arrow denotes the point at which the incubation mixture was divided into two portions and fresh microsomal aromatase was added to one of the portions (▲).

from **5b** in the previous experiments we really induced by aromatase, the presence of the oxirane at these concentrations should result in diminished tritium loss from **5b**. The control experiments omitted either (19*R*)-10β-oxiranylestr-4-ene-3,17-dione or microsomal aromatase, and incubations were followed for 60 min to assess tritium release from **5b** in to the aqueous medium. The aromatase-induced release of tritium from the difluoro compound (**5b**) was, in fact, prevented by the presence of the (19*R*)-oxiranyl steroid.

Discussion

We have synthesized [19-^{2}H]- and [19-^{3}H]-19,19-difluoroandrost-4-ene-3,17-dione (**5a** and **5b**, respectively) and have established their structures as indicated above.

The 19-deuterio compound (**5a**) was virtually completely deuteriated at C19, as shown by mass spectra and by proton and fluorine NMR spectra. The fluorine NMR spectra of compounds **5** and **5a** illustrated this point well (Figure 1). We decided to compare the kinetics of inactivation of aromatase by **5a** with those of the parent unlabeled **5**. It has been shown (Holland & Taylor, 1981) that a kinetic isotope effect of 3.2 accompanies aromatization of a 19,19,19-$^2H_3$-labeled steroid. A tritium isotope effect of the same magnitude was observed for aromatization of 19,19,19-$^3H_3$-labeled substrate (Miyairi & Fishman, 1983). We recognized that our postulated inactivation process by compound **5** involves not only oxidative attack at C-19 but also a subsequent inactivation sequence, but we thought the comparison of **5** with **5a** would be of interest. The inactivation rates of aromatase caused by concentrations of up to 10 μM **5** and **5a** proved to be indistinguishable, within experimental error. Consequently, we can say only that cleavage of the C19–H bond is not rate determining in the overall inactivation process.

We then undertook a series of experiments to determine whether [19-^{3}H]-19,19-difluoroandrost-4-ene-3,17-dione (**5b**) is indeed attacked at C19 by aromatase, with release of the 19-tritium into water. The first series of experiments demonstrated that incubation of **5b** with microsomal aromatase and NADPH resulted in release of tritium into the aqueous medium. Furthermore, this release was time dependent (Figure 2) and also required the presence of both NADPH and microsomal aromatase. The tritium release was also proportional to protein concentration.

We extended these experiments by replenishing the incubation mixture with fresh microsomal aromatase, in view of the progressive loss of enzyme by inactivation. Indeed, addition of fresh aromatase after 60 min provoked an increased rate of tritium release from **5b** into the aqueous medium (Figure 3).

A third experiment was performed to confirm that the tritium release was specifically induced by aromatase. This involved preincubation of microsomal aromatase with 10 μM (19*R*)-10β-oxiranylestr-4-ene-3,17-dione, a powerful and specific competitive inhibitor of aromatase (Shih et al., 1987; Kellis et al., 1987) with $K_i = 7 \times 10^{-9}$ M. We reasoned that, in the presence of such a high concentration of this very potent inhibitor, the aromatase would be protected against our compound (**5b**) ($K_i = 0.83$ μM). Indeed, this experiment revealed virtually no tritium release by **5b** relative to the inhibitor-free control. We take this to be good evidence that the tritium release observed in our experiments is indeed the result of the action of aromatase.

Detailed analysis of these processes is difficult because the time course of aromatase-induced tritium release from the difluoro compound (**5b**) is associated with progressive inactivation of the enzyme. Furthermore, there may be a kinetic isotope effect associated with cleavage of the C19–H bond in **5**. A tritium isotope effect of 3.2 has been observed on aromatization of a 19-$^3H_3$-labeled substrate (Miyairi & Fishman, 1983). This effect has been associated with the first hydroxylation step only (Miyairi & Fishman, 1985). Although we view compound **5** as an analogue of the substrate, androst-4-ene-3,17-dione, we cannot exclude the alternate possibility that it should be regarded as an analogue of the intermediates, 19-hydroxy- or 19,19-dihydroxyandrostenedione. Consequently, a reliable calculation for the turnover of **5b** by aromatase, based on tritium release, is not possible.

In summary, we have synthesized 19-deuteriated and -tritiated analogues of 19,19-difluoroandrost-4-ene-3,17-dione (**5**) and have studied their interactions with human placental aromatase. The 19-^{3}H-labeled analogue (**5b**) has been incubated with microsomal aromatase, resulting in time-dependent release of tritium into the aqueous medium. The tritium release requires both enzyme and NADPH cofactor and is also proportional to enzyme concentration. Saturating levels of 10β-oxiranylestr-4-ene-3,17-dione, a powerful competitive inhibitor of aromatase, prevented this tritium release from **5b**. Our experiments confirm that the difluoro compound (**5**) is indeed an alternate substrate for aromatase and undergoes attack at the 19-carbon, presumably as part of the inactivation process.

Acknowledgments

We thank Dr. H. Fales and W. Comstock (NHLBI, Laboratory of Chemistry) for mass spectra. We thank P. Ryb-

czynski for technical assistance. Finally, we thank Dr. Paul Talalay (The Johns Hopkins University School of Medicine) and Dr. J. O. Johnston (Merrell Dow) for helpful discussions.

Registry No. **5**, 7764-45-6; **5a**, 118247-43-1; **5b**, 118247-44-2; **7**, 2951-52-2; **7a**, 118247-45-3; **7b**, 118247-46-4; **8a**, 118247-47-5; **8b**, 118247-48-6; **9a**, 118247-49-7; **9b**, 118247-50-0; aromatase, 9039-48-9.

References

Akhtar, M. & Skinner, S. J. M. (1968) *Biochem. J. 109*, 318–321.

Akhtar, M., Corina, D., Pratt, J., & Smith, T. (1976) *J. Chem. Soc., Chem. Commun.*, 854–856.

Brodie, A. M. H., Garrett, W. M., Hendrickson, J. R., Tsai-Morris, C. M., & Williams, J. G. (1983) *J. Steroid Biochem. 19*, 53–58.

Caspi, E., Wicha, J., Arunachalam, T., Nelson, P., & Spiteller, G. (1984) *J. Am. Chem. Soc. 106*, 7282–7283.

Collins, J. C., & Hess, W. W. (1972) *Org. Synth. 54*, 5–10.

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

Holland, H. L., & Taylor, G. J. (1981) *Can. J. Chem. 59*, 2809–2819.

Hosoda, H., & Fishman, J. (1974) *J. Am. Chem. Soc. 96*, 7325–7329.

Johnson, J. O., & Metcalf, B. W. (1984) in *Novel Approaches to Cancer Chemotherapy* (Sunkara, P., Ed.) Chapter 9, pp 307–328, Academic Press, New York.

Kellis, J. T., Childers, W. E., Robinson, C. H., & Vickery, L. E. (1987) *J. Biol. Chem. 262*, 4421–4426.

Kitz, R., & Wilson, I. B. (1964) *J. Biol. Chem. 237*, 3245–3249.

Marcotte, P. A., & Robinson, C. H. (1982a) *Biochemistry 21*, 2773–2778.

Marcotte, P. A., & Robinson, C. H. (1982b) *Steroids 39*, 325–344.

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

Meyer, A. S. (1955a) *Biochim. Biophys. Acta. 17*, 441–442.

Meyer, A. S. (1955b) *Experientia 11*, 99–102.

Miyairi, S., & Fishman, J. (1983) *Biochem. Biophys. Res. Commun. 117*, 392–398.

Miyairi, S., & Fishman, J. (1985) *J. Biol. Chem. 260*, 320–325.

Raggio, M. L., & Watt, D. S. (1976) *J. Org. Chem. 41*, 1873–1875.

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

Shih, M.-J., Carrell, M. H., Carrell, H. L., Wright, C. L., Johnston, J. O., & Robinson, C. H. (1987) *J. Chem. Soc., Chem. Commun.*, 213–214.

Skinner, S. J. M., & Akhtar, M. (1969) *Biochem. J. 114*, 75–81.

Still, W. C., Kahn, M., & Mitra, A. (1978) *J. Org. Chem. 43*, 2923–2925.

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

Thompson, E. A., & Siiteri, P. K. (1974b) *J. Biol. Chem. 249*, 5373–5378.

Townsley, J. D., & Brodie, H. J. (1968) *Biochemistry 7*, 33–40.

Structural Aspects of Pressure Effects on Infrared Spectra of Mixed-Chain Phosphatidylcholine Assemblies in D_2O†

Patrick T. T. Wong*,‡ and Ching-hsien Huang§

Division of Chemistry, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6, and Department of Biochemistry, School of Medicine, University of Virginia, Charlottesville, Virginia 22908

Received March 14, 1988; Revised Manuscript Received September 12, 1988

ABSTRACT: The barotropic behavior of D_2O dispersions of 1-stearoyl-2-caproyl-*sn*-glycero-3-phosphocholine, C(18):C(10)PC, a highly asymmetric phospholipid in which the length of the fully extended acyl chain at the *sn*-1 position of the glycerol backbone is twice as long as that at the *sn*-2 position, has been investigated by high-pressure Fourier transform infrared spectroscopy. This asymmetric phosphatidylcholine bilayer at room temperature displays a pressure-induced phase transition corresponding to the liquid-crystalline → gel phase transition at 1.4 kbar. A conformational ordering of the lipid acyl chains is observed to take place abruptly at the transition pressure of 1.4 kbar. However, the lamellar lipid molecules and their acyl chains remain to be orientationally disordered in the gel phase until the applied pressure reaches 5.5 kbar. In the gel phase of fully hydrated C(18):C(10)PC, the asymmetric lipid molecules assemble into mixed interdigitated bilayers with perpendicular orientation of the zigzag planes among neighboring acyl chains. The role of excess water played in the interchain structure and the behavior of excess water and bound water under high pressure are also discussed.

It is well recognized that in biological membranes all of the component lipids are amphipathic in nature and that phospholipids are a major class of membrane lipids. In general, phospholipids consist of a pair of long acyl chains and a compact polar headgroup composed of a phosphate moiety and the esterified alcohol. These amphipathic phospholipid molecules spontaneously assemble into multiple arrays of two-dimensional lamellar structure called liposomes in excess water. If the chain length difference between the *sn*-1 and *sn*-2 acyl chains of the phospholipid molecule is large, the asymmetric phospholipids in liposomes can be packed at $T < T_m$ into an interdigitated mode [for a review, see Huang and

† Issued as NRCC No. 29380.
‡ National Research Council of Canada.
§ University of Virginia.

0006-2960/89/0428-1259$01.50/0