SYNTHESIS AND EVALUATION OF ANALOGUES OF 4-ANDROSTENE-3,17-DIONE AS AROMATASE INHIBITORS

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ABSTRACT

Twenty-three synthetic analogues of 4-androstene-3,17-dione (androstenedione) have been evaluated as inhibitors of human placental microsomal aromatase enzyme. Among the most potent of these compounds were the 4-hydroxy, 6α-fluoro, 6β-fluoro, and 4-fluoroandrostenediones and 4-fluoro-19-nor-4-androstene-3,17-dione. 4-Hydroxy-4-androstene-3,17-dione (4HAD) is an irreversible inhibitor of aromatase in vitro, whereas the four fluoro analogues are reversible inhibitors. 4HAD and 4-fluoro-4-androstene-3,17-dione caused significant regression of the nitrosomethylurea-induced mammary tumor in rats, but the other fluoro derivatives were inactive.

INTRODUCTION

The aromatase (estrogen synthetase) enzyme complex mediates the final stage in the pathway of estrogen biosynthesis and is responsible for the conversion of the androgenic steroids,

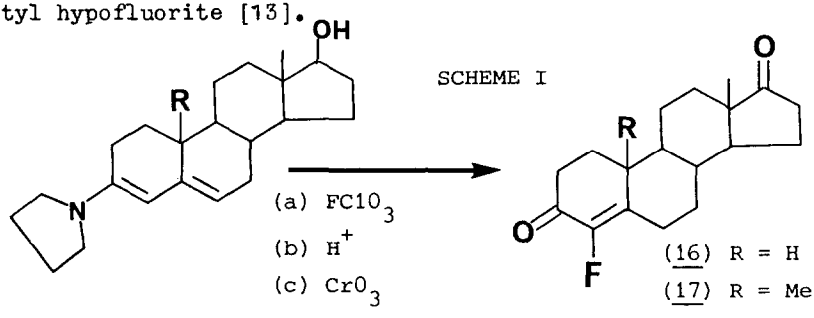
androstenedione and testosterone, into the corresponding estrogens, estrone and estradiol [1]. A non-toxic potent inhibitor of aromatase activity could have potential for the treatment of estrogen-dependent mammary carcinomas.

Many studies have indicated that C_{19} steroids resembling the substrate, 4-androstene-3,17-dione, are effective inhibitors of aromatase activity [2-6] and one of the most potent <u>in vitro</u> inhibitors is 4-hydroxy-4-androstene-3,17-dione (4-HAD) [7]. 4-HAD reduces estrogen secretion in rats and causes regression of the dimethylbenzanthracene - induced hormone-dependent mammary tumor in the rat [8,9]. The early results of Phase I clinical trials with 4-HAD have been encouraging [10]. We now report some biological data on other analogues of androstenedione, including several fluorinated derivatives.

EXPERIMENTAL

<u>Chemical Syntheses</u>. The syntheses of most of the compounds included in this study have already been described [11,12]. The syntheses of the two 4-fluoro steroids are described below and illustrated in Scheme I. An alternative route to the 4-fluoro-4-androstene-3,17-dione is by electrophilic fluorination using acetyl hypofluorite [13].

SCHEME I



(<u>16</u>) R = H
(<u>17</u>) R = Me

3-(N-Pyrrolidinyl)androsta-3,5-dien-17β-ol. Pyrrolidine (2.0mL, 2.89×10^{-2} mol) was added to a refluxing solution, under nitrogen, of testosterone (5.0g, 1.74×10^{-2} mol) in AR methanol (20mL). The resulting bright yellow solution was cooled to room temperature and the crystalline product filtered off and dried *in vacuo*. A quantitative yield of product was obtained and this was used without purification for the next stage.

4-Fluorotestosterone. (The synthesis is a modification of the one used by Njar and co-workers for the synthesis of 4-fluoro-19-nortestosterone [14].) A slow stream of perchloryl fluoride was bubbled through a suspension, at -20°, of the enamine (5.0g, 1.47×10^{-2} mol), in 94% aqueous methanol (135mL) until a clear solution was obtained, and addition of the reagent continued for a further 15 minutes. The solution was poured into ice-water, (*ca.* 1400g) and extracted with ethyl acetate (5 x 100mL). The combined extracts were washed with water (2 x 100mL), dried (Na_2SO_4), the solvent removed *in vacuo*, and the residue redissolved in dimethylformamide (40mL). Hydrochloric acid (12N, 5mL) was added and the solution stirred overnight, poured into water (1400mL), and extracted with ether (5 x 100mL). The combined organic extracts were washed with water (3 x 150mL), dried, the solvent removed *in vacuo*, and residue "flash chromatographed" on silica gel, eluting with dichloromethane/diethyl ether (2:1). The product (2.22g, 48%) was obtained as colorless needles which had m.p. 157-158° (lit. 159-160°); νmax ($CHCl_3$) 3613 (sharp, OH), 3030 (olefin) and 1687 (C-3 ketone) cm-1; δ (1H, 220 MHz, $CDCl_3$) 0.80 (s, 3H, CH_3-18), 1.21 (s, 3H, CH_3-19), 3.66 (t, J 4.5 Hz, H-17) ppm.

4-Fluoro-4-androstene-3,17-dione (17). 4-Fluoro-testosterone (5.6g, 1.83×10^{-2} mol) in AR acetone (100mL) at 0° was treated with Jones' reagent. The reaction mixture was poured into ice-water (*ca.* 300g) and extracted with dichloromethane (5 x 50mL). The combined extracts were washed with water (2 x 50mL), dried (Na_2SO_4), and the solvent removed *in vacuo*. The residue was "flash chromatographed" on silica eluting with dichloromethane/diethyl ether (2:1). Recrystallization from diethyl ether afforded the product (3.73g, 67%) as colorless crystals which had m.p. 174-175°; δ (^{13}C, 22.5 MHz, $CDCl_3$) 220.13 (C-17), 189.80 (d, J_{CF} 20.5 Hz, C-3), 148.47 (d, J_{CF} 254.6 Hz, C-4), 145.57 (d, J_{CF} 6.6 Hz, C-5); (^1H, 220 MHz, $CDCl_3$) 0.9 (s, 3H, CH_3-18), 1.24 (s, 3H, CH_3-19), 3.02 (m, H-6) ppm; found C 74.88%, H 8.24%, calculated for $C_{19}H_{25}O_2$ C 74,9%, H 8.27%.

The route to 4-fluoro-19-nortestosterone was as described in reference [14]; and Jones oxidation provided 4-fluoro-19-nor-4-

androstene-3,17-dione (16), m.p. 167-169°.

In Vitro Experiments

Aromatase Assay. Microsomes were prepared [15] from human placentae, and the aromatase assays were performed [16] with [1,2-^{3}H] 4-androstene-3,17-dione replacing [1,2,-^{3}H] testosterone as the substrate as appropriate. Testosterone, which was used in the initial screening assays, has an apparent Km of 0.13 μM hence a substrate concentration of 1.5 μM was employed. Under these conditions, the I_{50} value is the concentration of inhibitor required to reduce the activity of the enzyme to 50% of the control value. Kinetic analysis of the inhibitors was carried out using 4-androstene-3,17-dione as substrate (Km of 0.038 μM) for the enzyme, and the apparent Ki values were determined [16].

Wash and Centrifugation Experiments. In order to ascertain whether the inhibition of aromatase was reversible or irreversible, placental microsomes were suspended at a protein concentration of 1.25 mg/mL in 50 mM phosphate buffer, pH7.4 and incubated at 37°C in the presence of inhibitor at 20 μM and NADPH for 15 minutes. Before the incubation was started the aromatase activity was determined on a sample as described above. The incubation was terminated by dilution with 4 volumes of ice-cold 50mM Phosphate buffer, pH7.4 followed by centrifugation at 120,000g for 1h. The sedimented microsomes were resuspended in fresh buffer (same volume and conditions) and the enzyme activity was reassayed. Microsomes with no added inhibitor were used as a control.

Inactivation Experiments. Each steroid was preincubated at two concentrations in a total volume of 0.5 mL at 37°C using 1.0 mg of placental microsomal protein and 2 mM NADPH in 50 mM phosphate buffer pH7.4. To ensure against depletion of cofactors, 2mM glucose-6-phosphate, 5mM $MgCl_2$, and 0.2 mg/mL glucose-6-phosphate dehydrogenase were added. Aliquots (0.025 mL) were withdrawn at intervals over 20 minutes and the aromatase activity assayed by dilution into 0.5 mL of 50 mM phosphate buffer pH7.4 containing 0.38 μM [1,2-^{3}H] 4-androstene-3,17-dione and 0.5 mM NADPH. Each sample was incubated for 10 minutes and the assay completed as described [16]. In the absence of any inhibitor, the placental microsomes undergo loss of approximately 10% of aromatase activity in 20 minutes at 37°C as shown by the control sample in Figure 1. Each experimental point in Figure 1 is the average of three determinations which were all within 10% of each other.

In Vivo Tumor Assay

Hormone-dependent mammary tumors were induced in rats with nitrosomethylurea (NMU) [17]. Administration of 4-HAD causes significant reduction of these mammary tumors [18]. The fluoro analogues (7, 8, 16, and 17) of 4-androstene-3,17-dione were tested using the same dose and conditions as 4-HAD.

RESULTS AND DISCUSSION

Each derivative of 4-androstene-3,17-dione was screened for inhibition of placental aromatase *in vitro* using testosterone as substrate, and the results are shown in Table 1. Two groups of

Table 1. Inhibition of Aromatase Activity by Analogues of Androstenedione

Steroid		I_{50} (μM)
4-hydroxy-4-androstene-3,17-dione	1	0.20
4-methoxy-4-androstene-3,17-dione	2	7.2
4-acetoxy-4-androstene-3,17-dione	3	1.5
4-hydroxy-androst-4,6-dien-3,17-dione	4	1.0
4-methoxy-androst-4,6-dien-3,17-dione	5	11.0
4-acetoxy-androst-4,6-dien-3,17-dione	6	8.0
6β-fluoro-4-androstene-3,17-dione	7	0.8
6α-fluoro-4-androstene-3,17-dione	8	0.4
2α-fluoro-4-androstene-3,17-dione	9	1.0
2α6β-difluoro-4-androstene-3,17-dione	10	2.9
4α-hydroxy-5α-fluoroandrostane-3,17-dione	11	4.6
6α-hydroxy-4-androstene-3,17-dione	12	0.7
6β-hydroxy-4-androstene-3,17-dione	13	0.8
6β-bromo-4-androstene-3,17-dione	14	0.4
2α-hydroxy-4-androstene-3,17-dione	15	inactive
4-fluoro-19-nor-4-androstene-3,17-dione	16	0.48
4-fluoro-4-androstene-3,17-dione	17	0.42
19,19-difluorotestosterone-17β-ol,17-benzoate	18	inactive
19,19-difluorotestosterone	19	17.0
19,19-difluoro-4-androstene-3,17-dione	20	1.2
19,19-difluoro-2α-hydroxy-4-androstene-3,17-dione	21	3.8
19,19-difluoro-4-hydroxy-4-androstene-3,17-dione	22	2.5

compounds are discernible, namely, analogues of 4-androstene-3,17-dione (1-17) and compounds which possess a geminal difluoromethyl in place of the C-19 methyl group (18-22).

In the first group, the most active compounds are the 6-hydroxy-4-androstene-3,17-dione derivatives (12 and 13), 6 β-bromo-4-androstene-3,17-dione (14), the 6-fluoro-4-androstene-3,17-dione derivatives (7 and 8), and the 4-fluoro analogues (16 and 17). The aromatase inhibitory properties of 1 and 14 have been reported [8 and 2 respectively]. The inhibitory activities of 7, 8, 12, 13, 16, and 17 approach that of 4-HAD (1). None of the above compounds were more potent than 4-HAD in inhibiting the aromatization of testosterone by the placental microsomes. Compounds 7, 8, 12, and 13 have been synthesized by Bowers and Ringold [19], but the routes reported here are more efficient.

The 19,19-difluoro analogues (18-22) of 4-androstene-3,17-dione were synthesized as potential inhibitors of the hydroxylation of the angular methyl group (C-19), which is the first step in the sequence of reactions that results in the aromatization of ring A [20-22]. The results (I_{50} 1.2 μM versus I_{50} 4.8 μM) for 20 are similar to those reported by Marcotte and Robinson [4], but this was the only compound of this group to possess reasonable inhibitory activity.

Kinetic analysis using 4-androstene-3,17-dione as the substrate (Table 2) demonstrated from the apparent Ki values that

7 and 8 were equipotent with 4-HAD (1) and 17 was about three times as potent (respective Ki values of 1 and 17 are 0.043 and 0.015 μM). 4-HAD has been demonstrated to be an irreversible inhibitor of the aromatase enzyme, because it produces a time-dependent decrease in enzyme activity and irreversibly binds to placental microsomes [23,24]. We have evaluated the fluorinated derivatives 7, 8, 16, and 17 in a similar fashion and compared the results with those for 4-HAD. The placental microsomes incubated with 4-HAD did not regain any aromatase activity after the wash and centrifugation experiments. On the other hand, the inhibition exerted by the fluorinated compounds could be removed and 50-60% of the aromatase activity recovered, compared with control values after such experiments. Figure 1 demonstrates that 4-HAD causes a time-dependent loss of aromatase activity. Under identical conditions and the same concentrations, the fluorinated compounds did not cause any time-dependent loss of activity greater than the control value. These *in vitro* experiments have confirmed the irreversible nature of the inhibition exerted by 4-HAD and demonstrated that the analogues 7, 8, 16, and 17 are reversible inhibitors (Table 2). The activities of these fluorinated derivatives against the hormone-dependent NMU-induced rat mammary tumor are shown in Table 3. Although the results are preliminary, certain trends are evident. Thus, the 6-fluoro compounds 7 and 8 caused regression of the

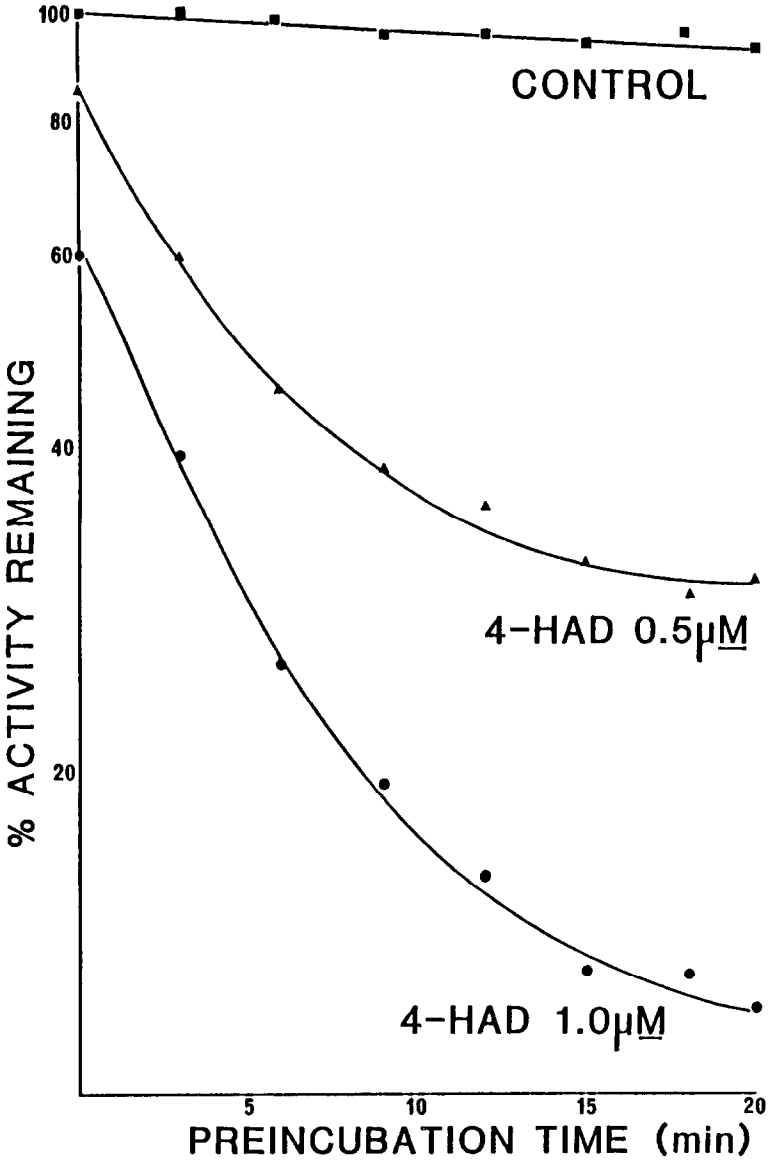


Figure 1. Time-dependent loss of aromatase activity of placental microsomes by 4-HAD. Details are in Experimental section.

Table 2. Inhibition Constants (Ki) and Type of Inhibition Exerted by Potent Inhibitors of Aromatase Activity

Steroid derivative	Ki[a] μM	Type of inhibition
4-HAD	0.043	Irreversible
7	0.055	Reversible
8	0.053	Reversible
16	0.090	Reversible
17	0.015	Reversible

[a]Androstenedione as substrate for aromatase assay.

Table 3. In Vivo Tumor Assay

Steroid[a] derivative	no. of rats	no. of tumours	Tumors with regression (50-100%)	Tumors with progression (>100%)	Response rate
4-HAD (1)	12	27	18	0	67%
7	11	33	4	18	12%
8	11	28	3	10	9%
16	10	12	1	7	8%
17	8	9	4	4	44%

[a] The dosage level for all compounds was 50 mg/kg/day.

tumors in some animals, but the main result was tumor progression associated with an increase in uterine weight. These findings suggest that in vivo (at least in rats) the compounds

are estrogenic, perhaps via conversion into the 6α- and 6β-fluoro estrones. The 4-fluoro-19-nor-4-androstene-3,17-dione (16) had no significant anti-tumor activity, whereas 4-fluoro-4-androstene-3,17-dione (17) elicited a response rate of 44% as compared with a value of 67% for 4-HAD. Due to the small number of animals involved, these tests should be regarded with some caution.

It is not yet clear whether, in the treatment of breast cancer in humans, it is more advantageous to use an irreversible (such as 4-HAD) or a reversible inhibitor (such as aminoglutethimide) of aromatase. The availability of a potent steroid-type reversible inhibitor of aromatase, namely, 4-fluoro-4-androstene-3,17-dione (17), makes further comparisons both possible and desirable.

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