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SYNTHESIS AND BIOCHEMICAL EVALUATION OF THE NOVEL STEROID ANDROSTA-4,6,8(9)-TRIENE-3,17-DIONE*

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According to a proposed aromatisation mechanism by which estrogens are biosynthesized from androgens, the novel steroid androsta-4,6,8(9)-triene-3,17-dione (**FCE 24918**) should behave as a suicide substrate for aromatase. The synthesis of this triene steroid has been accomplished starting from androsta-4,7-diene-3,17-dione (**4**) by the acid-catalysed cleavage of the corresponding 7,8α-epoxide, **5**, and it was obtained together with androsta-4,6,8(14)-triene-3,17-dione (**FCE 24917**) as a side product. The time-dependent inactivation of placental aromatase by the two isomers was studied comparatively and showed that the 4,6,8(9)-triene moiety acts as a latent alkylating group.

KEY WORDS: Androsta-4,6,8(9)-triene-3,17-dione synthesis, *O*-ethylperoxycarbonic acid epoxidation, time-dependent aromatase inactivation, latent alkylating group.

INTRODUCTION

The mechanism by which estrogens are biosynthesized from androgens is still a matter of debate. While it is known that this conversion by mean of the cytochrome P-450 aromatase complex involves three subsequent oxidative steps, the first two occurring at C-19, no unanimous view has been reached about the site of the third oxidation, whether at C-2[1–4] or at C-19[5–8].

When we started our research programme aimed at the synthesis and the biochemical and pharmacological evaluation of novel aromatase inhibitors, which eventually led to the selection of 6-methylenandrosta-1,4-diene-3,17-dione (**FCE 24304**)[9–11] for clinical studies and of the follow-up 4-aminoandrosta-1,4,6-triene-3,17-dione (**FCE 24928**)[12–14], we envisaged Covey's mechanistic proposal[8] as a reasonable model for the design of potential mechanism-based (irreversible) steroidal inhibitors.

According to Covey, the third oxidation is carried out by aromatase at C-19, followed by the attack at C-4 by an enzyme nucleophilic group. Endo shift of the 4,5-double bond with cleavage of the peroxy link to the heme of the cytochrome and departure of the C-19 methyl group as formic acid, result in the formation of an enzyme-bound intermediate **1** which forms the estrogen product **2** and the free enzyme upon loss of the 2β-H and enolisation of the keto-diene moiety (Scheme I).

Various structural modifications, therefore, can be introduced in the potential

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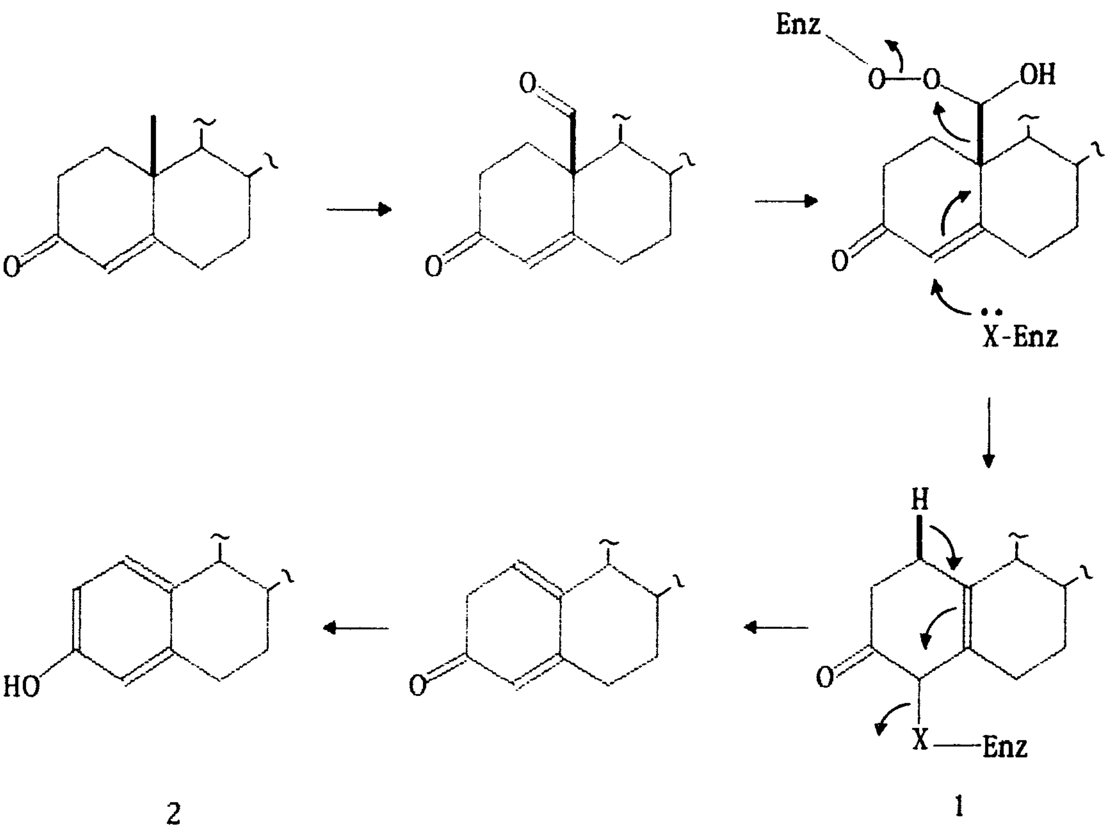
SCHEME I



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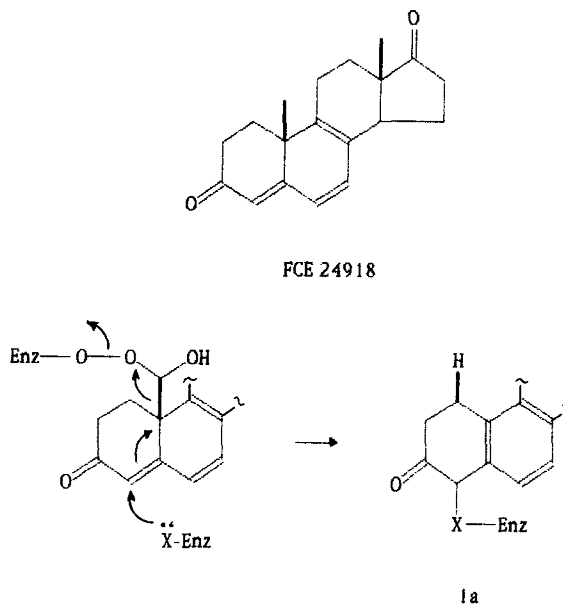
SCHEME II



inhibitor in order to redirect any of the enzymic steps towards an energetically more favourable path, so that the resulting enzyme-bound intermediate remains covalently (irreversibly) attached to the enzyme, instead of being released as a transformed product.

With this mechanism as a hypothesis, it is possible to rationalize why, for example, 4-hydroxyandrost-4-ene-3,17-dione, androsta-1,4-diene-3,17-dione, as well as **FCE 24304** and **FCE 24928**, are aromatase suicide substrates.

We envisioned that, accordingly, the novel steroid androsta-4,6,8(9)-triene-3,17-dione (**FCE 24918**) should behave as a suicide substrate for aromatase. We considered that after the third oxidation at C-19 and the nucleophilic attack at C-4 have occurred, the resulting aromatisation of the B-ring would render the loss of the 2β-H energetically unfavourable and, by precluding the elimination reaction from occurring, would hold the steroid bound to the enzyme (Scheme II, **1a**). Thus, the 4,6,8(9)-triene system should act as a latent alkylating group by stabilising the covalent bond formed between the inhibitor and the enzyme.

RESULTS AND DISCUSSION

A literature survey indicated that this trienone grouping is quite uncommon in the steroid field, and the only two examples reported are in the 22-isospirosten-3-one[15] and 17,21-dihydroxypregnane-3,20-dione[16,17] series. In both cases, the compounds were prepared by manipulations performed on the 9,11-double bond of 3-keto-Δ^4-

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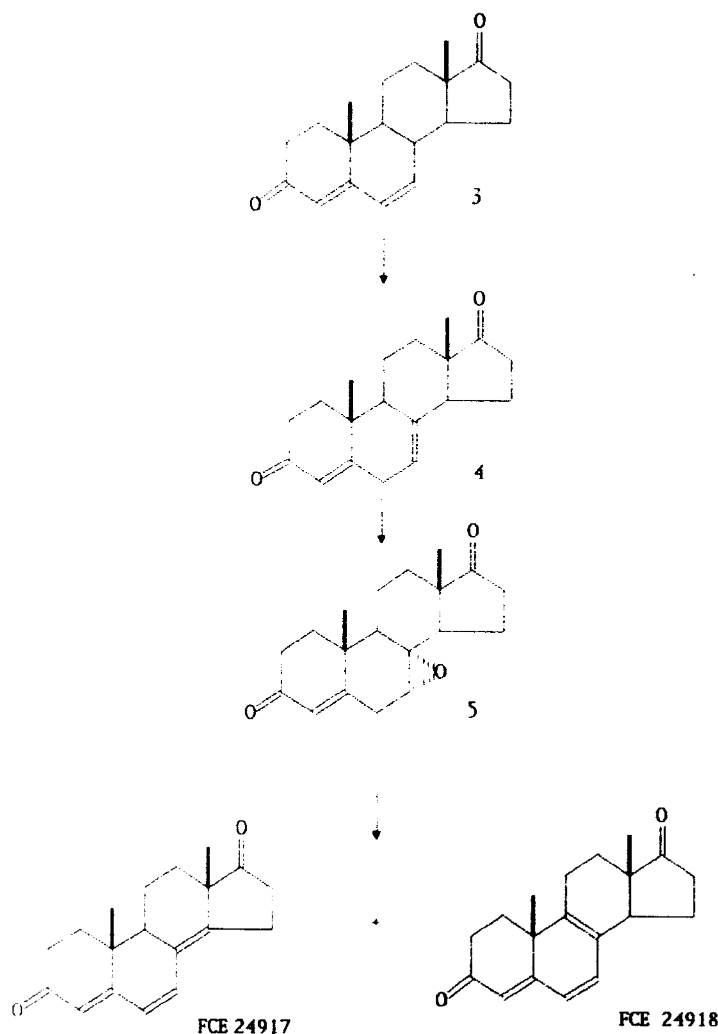
steroid derivatives and, however, their structures were assigned on the sole basis of UV absorptions (λ_{max} at 244, 290 and 385 nm).

By applying the same chemistry to analogous androstenedione derivatives, we obtained a crude product which, albeit displaying a simple UV absorption pattern similar to the published data, was revealed to be a mixture of differently unsaturated isomers form which the desired compound could scarcely, if not, be detected by more accurate spectral analysis.

A different synthetic approach to the 4,6,8(9)-trien-3-one system was then sought (Scheme III). By assuming that the acid-catalysed cleavage of a suitable 7,8-epoxide would, hopefully, generate the 6,8(9)-diene moiety, we prepared androsta-4,7-diene-

SCHEME III



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3,17-dione (**4**) by deconjugation of androsta-4,6-diene-3,17-dione (**3**) under equilibrating conditions with NaOMe in DMSO[18]. Epoxidation of Δ^7-steroids is reported[19] to proceed, if at all, with poor yield due to, (*a*) extensive Baeyer-Villiger oxidation at the A-ring, when present a 4-en-3-one moiety, and/or, (*b*) instability under general epoxidation conditions of the resulting epoxides which easily give allylic alcohols, making their isolation quite troublesome.

However, we could isolate 7,8α-epoxyandrost-4-ene-3,17-dione (**5**) in good yield, avoiding all the aforementioned problems, when *O*-ethylperoxycarbonic acid[20], generated *in situ* in an alkaline biphasic solvent system from H_2O_2 and ethylchloroformate, was used as epoxidizing agent. By treating the epoxysteroid **5** with a catalytic amount of *p*-toluenesulphonic acid in $CHCl_3$ at ambient temperature, two isomeric products, **FCE 24917** and **FCE 24918**, were nicely obtained in the 1:1 ratio. **FCE 24917** had a λ_{max} at 342 nm and showed two doublets in the ^1H n.m.r. spectrum centred at δ 6.15 and δ 6.74 for 7-H and 6-H, respectively, whereas **FCE 24918** had a λ_{max} at 245 and 385 nm and showed a singlet at δ 6.24 (2H) for both 6-H and 7-H. Definite structure assignments were, however, based on the respective ^{13}C n.m.r. spectra and the two isomers were distinguished by the following considerations. The spectrum for **FCE 24918** showed the signal for the C-18 methyl at 13.0 ppm, which is about that for the same methyl in androsta-4,6-diene-3,17-dione, whereas in the spectrum of **FCE 24917** it appeared shifted to 16.7 ppm, indicating the presence of the additional olefinic bond in the 8(14)-position. Likewise, the spectrum for **FCE 24917** showed the signal for the C-19 methyl at 23.4 ppm, which is about that for the same methyl in androsta-4,6-diene-3,17-dione, whereas in the spectrum of **FCE 24918** it appeared shifted to 26.0 ppm, indicating the presence of the additional olefinic bond in the 8(9)-position.

The obtaining of both isomers was considered, as a matter of fact, a positive asset. In initial co-incubation studies with the substrate, both **FCE 24917** and **FCE 24918** were found to inhibit human placental aromatase, showing an IC_{50} of 102 nM and 375 nM respectively. These values, however, could be the result of both competitive and time-dependent enzyme inhibition, and give only a preliminary indication of the compounds' inhibitory potency. According to our former assumptions, **FCE 24917** *should not* behave as a suicide substrate for aromatase. Therefore, the time-dependent inactivation of placental aromatase by these new steroidal compounds was studied comparatively and the results are reported in Figures 1 and 2. **FCE 24918** induced time-dependent enzyme inactivation, causing irreversible inhibition of aromatase, thus indicating that the 4,6,8(9)-triene system acts as a latent alkylating group. Conversely, no time-related decrease in aromatase activity was observed when the isomer **FCE 24917** was pre-incubated with the enzyme for up to 32 min.

EXPERIMENTAL

Chemistry

Melting points were determined with a Buchi SMP 20 apparatus and are uncorrected. UV spectra were recorded on a Varian DMS 300 spectrophotometer and are in 95% EtOH solutions. IR spectra were recorded on a Perkin Elmer 683 spectrophotometer and are in $CHCl_3$ solutions. ^1H n.m.r. spectra and ^{13}C n.m.r. spectra (operating at 50.31 MHz) were obtained with a Brucker 80 MHz for $CDCl_3$ solutions with TMS as

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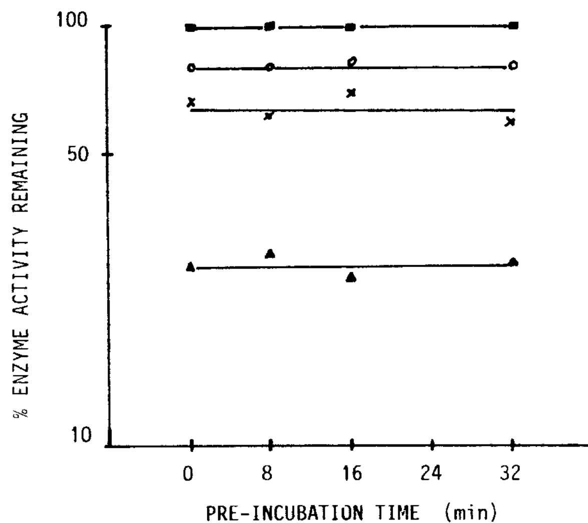

FIGURE 1 Time course for aromatase inhibition by **FCE 24917**. the compound was pre-incubated for 0–32 min with human placental aromatase in the presence of NADPH. No time-related decrease in enzyme activity was observed. (■) control; (○) 100 nM; (×) 200 nM; (▲) 1000 nM.

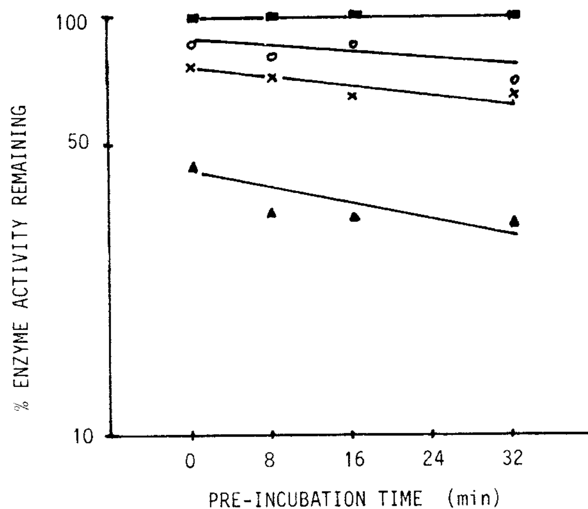

FIGURE 2 Time course for aromatase inhibition by **FCE 24918**. The compound was pre-incubated for 0–32 min with human placental aromatase in the presence of NADPH. Kinetic parameters ($t_{1/2}$ = 69 min, K_i = 72 nM and K_{cat} = 0.17×10^{-3} sec^{-1}) were determined as described by Kitz and Wilson[23]. (■) control; (○) 200 nM; (×) 400 nM; (▲) 2000 nM.

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internal standard. Optical rotational values were obtained with a Perkin Elmer 241 polarimeter for $CHCl_3$ solutions at 23°C. MS were recorded on a Finnigan MAT CH7 spectrometer at 70 eV and fragments are quoted as percentage of the base peak (100%). TCL were carried out on precoated TLC plates with silica gel 60F-254 (Merck). For flash column chromatography, silica gel 60 RS (Farmitalia Carlo Erba Reagenti) was used.

Androsta-4,7-diene-3,17-dione (4). To a stirred solution of 4.39 g (15.5 mmole) of androsta-4,6-diene-3,17-dione (**3**) in 45 ml of dry DMSO was added 10.74 g of NaOMe at room temperature under nitrogen. After 25 min. of additional stirring, the reaction mixture was poured into 600 ml of an iced 2N HCl aqueous solution; 1.5 ml of pyridine were added and the mixture was extracted three times with AcOEt. The combined extracts were washed with a saturated $NaHCO_3$ aqueous solution, a saturated NaCl aqueous solution, water, and then dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The resulting residue was purified by flash column chromatography eluting with hexane: AcOEt (60:40). There was obtained 1.89 g (43%) of **4** after crystallisation from acetone-hexane: mp 130–2°C; UV nm 239 (ε = 13260); IR 1730, 1610 cm^{-1}; ^1H n.m.r. δ 0.79 (3H, s), 1.22 (3H, s), 2.73 (1H, m), 3.33 (1H, m), 5.38 (1H, m), 5.82 (1H, d).

7,8α-epoxyandrost-4-ene-3,17-dione (5). To a stirred solution of 6.08 g of $Na_2HPO_4 \cdot 12H_2O$ in 7 ml of 36% H_2O_2 was added a solution of 1.51 g (5.3 mmole) of **4** and 1.35 ml of ethyl chloroformate in 12.7 ml of CH_2Cl_2 at room temperature. After 24 h of additional stirring, 6.06 g of $Na_2HPO_4 \cdot 12H_2O$ dissolved in 7 ml of 36% H_2O_2 and 1.35 ml of ethyl chloroformate were added to the reaction mixture which was stirred for a further 48 h and then diluted with 120 ml of toluene. The organic phase was separated and the aqueous phase was extracted with CH_2Cl_2. The combined organic phase and extracts were washed with a 10% sodium metabisulphite aqueous solution, a saturated NaCl aqueous solution, water, and then dried over Na_2SO_4, filtered and evaporated *in vacuo*. The resulting residue was purified by flash column chromatography eluting with AcOEt. There was obtained 0.107 g of the starting material **4** followed by 0.92 g (61%) of **5** as white crystals: mp 136–8°C; $[\alpha]_D$ = +170° (c = 1.2); IR 1742, 1660 cm^{-1}; ^1H n.m.r. δ 0.96 (3H, s), 1.21 (3H, s), 2.82 (2H, m), 3.42 (1H, d), 5.77 (1H, br s).

*Androsta-4,6,8(14)-triene-3,17-dione (**FCE 24917**) and androsta-4,6,8(9)-triene-3,17-dione (**FCE 24918**).* A mixture of 0.72 g (2.4 mmole) of **5** and 0.046 g of *p*-toluenesulphonic acid in 50 ml of anhydrous $CHCl_3$ was stirred at room temperature during 3 h. The reaction mixture was then washed with a saturated $NaHCO_3$ aqueous solution, water, and then dried over $CaCl_2$, filtered and evaporated *in vacuo*. The resulting residue was purified by flash column chromatography. Elution with AcOEt: CH_2Cl_2 (1:4) yielded 0.170 g (25%) of **FCE 24917**: mp 146–150°C; $[\alpha]_D$ = +1044° (c = 1); UV nm 342 (ε = 26176); IR 1732, 1638, 1585 cm^{-1}; ^1H n.m.r. δ 1.04 (3H, s), 1.21 (3H, s), 5.79 (1H, s), 6.15 (1H, d), 6.74 (1H, d); ^{13}C n.m.r. ppm 16.7 (C-18), 23.4 (C-19); TLC (AcOEt: CH_2Cl_2 1:5) R_f 0.50; MS 282 (100%, M^+), 267 (13%, M^+-CH_3); followed by 0.160 g (23.6%) of **FCE 24918**: mp 220–5°C; $[\alpha]_D$ = +1158° (c = 1); UV nm 245 (ε = 15360), 385 (ε = 8500); IR 1732, 1640, 1625 cm^{-1}; ^1H n.m.r. δ 0.84 (3H, s), 1.36 (3H, s), 5.81 (1H, s), 6.24 (2H, s); ^{13}C n.m.r. ppm 13.0

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(C-18), 26.0 (C-19); TLC (AcOEt: CH_2Cl_2 1:5) R_f 0.41; MS 282 (100%, M^+) 267 (97%, M^+-CH_3).

Biochemistry

Substrate. [1β, 2β-^{3}H] androst-4-ene-3,17-dione was purchased from New England Nuclear.

Buffers. Aromatase phosphate buffer, pH 7.5, contained 10 mM potassium phosphate buffer, 100 mM KCl, 1 mM EDTA and 1 mM dithiothreitol.

In vitro *aromatase inhibition.* Microsomes were prepared from fresh human placenta according to Ryan[21], resuspended in aromatase phosphate buffer and stored in aliquots at −80°C. Aromatase activity was tested in the assay of Thompson and Siiteri[22], which determines the rate of aromatisation by measuring the release of ^3H_2O from [1β, 2β-^{3}H] androstenedione. All incubations were carried out in a Dubnoff shaking incubator at 37°C in air in aromatase phosphate buffer. Both co-incubation and pre-incubation studies were performed. (a) *Co-incubation assay.* The experiments were carried out in 1 ml final incubation volume containing 50 nM [^{3}H]androstenedione, various concentrations of the inhibitors, 100 μM NADPH and the aromatase preparation (≃ 15 μg of proteins). After 15 min of incubation the enzymatic reaction was terminated by the addition of 4 ml $CHCl_3$. The radioactivity in the water phase was determined in a liquid scintillation counter. (b) *Pre-incubation assay.* Aromatase inactivation was determined by pre-incubating the enzyme preparation (100 μg of proteins) for various periods of time with different concentrations of the inhibitor in the presence of 100 μM NADPH in 1 ml final volume. Each sample was assayed in duplicate. After 0, 8, 16 and 32 min of pre-incubation, [^{3}H]androstenedione was added to each tube to provide a 300 nM final concentration. After 10 min of incubation the assay was terminated as described above. ^3H_2O was determined as an index of residual aromatase activity. Inactivation curves, corrected for the loss of activity in the pre-incubated controls, were analyzed according to Kitz and Wilson[23] and K_i and $t_{1/2}$ values were estimated.

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