STEREOSELECTIVE INHIBITION OF HUMAN PLACENTAL AROMATASE

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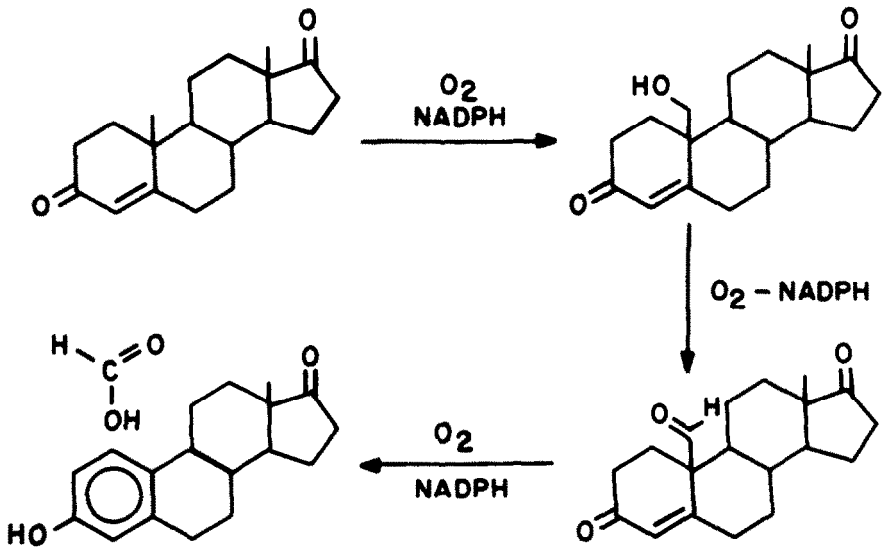
ABSTRACT

We have synthesized the (19R)- and (19S)-isomers (2 and 3 respectively) of 10β-oxiranylestr-4-ene-3,17-dione. The configurations and conformations of these compounds were established by X-ray crystallographic analysis. Each of these compounds is a powerful competitive inhibitor of human placental microsomal aromatase, and stereoselectivity of inhibition was observed (Ki values for 2 and 3 were 7 and 75 nanomolar, respectively). Spectroscopic studies with purified aromatase indicate that the inhibition process involves reversible binding of oxirane oxygen to the heme iron of the enzyme.

The (19R)- and (19S)-10β-thiiranes (6 and 7) corresponding to 2 and 3 have been synthesized from the oxiranes by a stereospecific process. The thiiranes are very effective competitive inhibitors of placental aromatase, and show even greater stereoselectivity in binding than the oxiranes (Ki values for 6 and 7 were 1 and 75 nanomolar, respectively). Spectroscopic studies with purified aromatase indicate that the inhibition process involves reversible binding of thiirane sulfur to heme iron.

Human placental aromatase is a cytochrome P-450 enzyme complex which catalyzes the important biosynthetic transformation of androgens to estrogens. The process is exemplified by the conversion of androst-4-ene-3,17-dione to estrone *via* three steps, each of which requires 1 mol of O_2 and 1 mol of NADPH (Scheme 1) (1,2). There has been considerable recent interest in the development of inhibitors of aromatase, in part because of their potential value in treatment of estrogen-sensitive breast tumors (3).

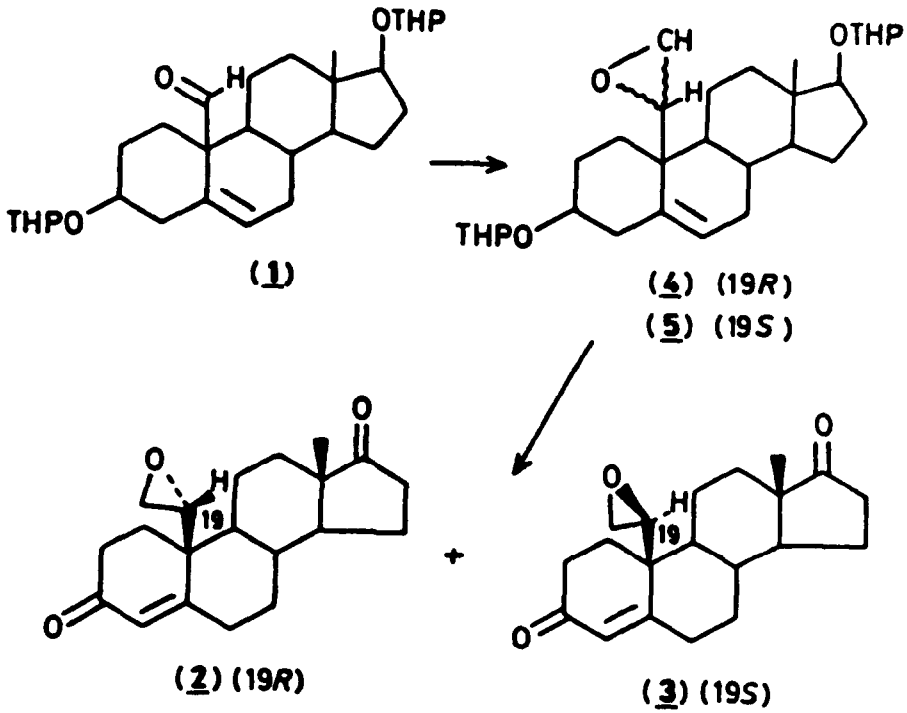
Based on the reported (4) X-ray crystal structure of 19-hydroxyandrost-4-ene-3,17-dione, the first intermediate



SCHEME 1

in the enzyme reaction sequence, and on consideration of molecular models, we undertook the synthesis and evaluation of analogs of androst-4-ene-3,17-dione carrying an epoxide oxygen at the 19-carbon. It was hoped that these would show stereoselectivity in binding to the enzyme, with the (19R)-isomer 2 likely to be a better inhibitor than the (19S)-diastereomer 3. Consequently we prepared the diastereoisomeric 10β-oxiranyl compounds 2 and 3 in an attempt to correlate enzyme inhibition with the configuration at C-19. The results of these studies then led us to synthesize and evaluate the corresponding sulfur analogs, namely (19R)- and (19S)-10β-thiiranyl steroids.

The synthesis of compounds 2 and 3 (Scheme 2) involved reaction of 19-oxo-androst-5-ene-3β,17β-diol 3,17-bistetrahydropyranyl (THP) ether (1) with either dimethylsulfonium methylide or dimethyloxosulfonium methylide (5). Thus a solution of 1 (5 mmol) in dry tetrahydrofuran (THF) was added to dimethyloxosulfonium methylide (50 mmol) in THF under nitrogen at 60°C and the mixture kept for 24 h at 60°C, followed by 48 h at 25°C. The THP ether groups in the crude product were cleaved (pyridinium toluene-p-sulfonate-methanol, 25°C, 4 h) to give the (19R)- and (19S)-isomers of

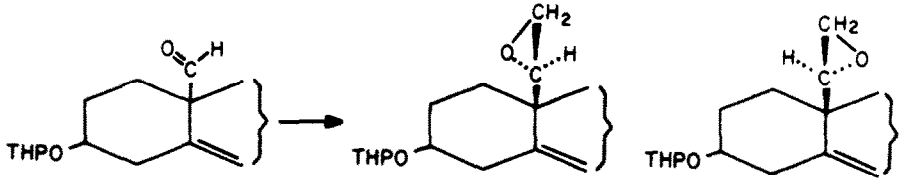


SCHEME 2

10β-oxiranylestr-5-ene-3β,17β-diol. Alternatively, reaction of 1 (4.47 mmol) with dimethylsulfonium methylide(20 mmol) in dimethyl sulfoxide (DMSO)-THF under N_2 at ice-bath temperature for 1 h, followed by 19 h at 25°C, also gave a mixture of the same (19R)- and (19S)-isomers of 10β-oxiranylestr-5-ene-3β,17β-diol after cleavage of the THP groupings. The ratios of (19R)- and (19S)-isomers obtained from the above reactions (determined by HPLC analysis after THP-cleavage) are shown in Table 1. The epoxides were, in all cases, separated by silica gel chromatography and HPLC, and each was oxidized by the Oppenauer procedure to give the desired 10β-oxiranyl-estr-4-ene-3,17-diones 2 and 3 (6).

TABLE 1.

Ratios of (19R)- and (19S)-oxiranes from ylid reactions.



	(19R)	(19S)
$(CH_3)_2\overset{+}{S}(=O)-\bar{C}H_2$	26 %	74 %
$(CH_3)_2\overset{+}{S}-\bar{C}H_2$	89 %	11 %

X-ray crystallographic analysis of 2 and 3 by Carrell and Carrell at the Fox Chase Cancer Institute established (6) the configuration and conformation of the epoxide groupings. The crystal structures are shown in Figure 1, together with the previously reported (4) structure for the

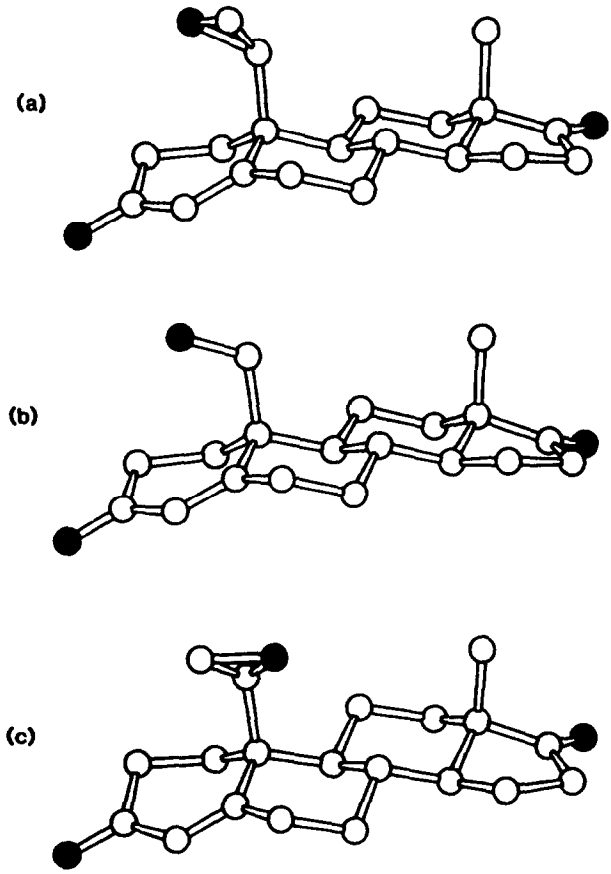


FIGURE 1. MOLECULAR STRUCTURES OF (a) (19R)-OXIRANE, (b) 19-HYDROXYANDROST-4-ENE-3,17-DIONE, AND (c) (19S)-OXIRANE.

aromatase reaction intermediate 19-hydroxyandrost-4-ene-3,17-dione. It can be seen that the oxirane system in the (19R)-isomer 2 is positioned over the steroid A-ring in a manner very similar to the arrangement of the 19-hydroxymethyl group in 19-hydroxyandrost-4-ene-3,17-dione. In the (19S)-isomer 3 on the other hand, the oxygen of the oxirane group is positioned over the B-ring in an arrangement unlike that of 19-hydroxy-androst-4-ene-3,17-dione. If these solid-state conformations can be extrapolated to the arrangements at the enzyme active site, the (19R)-epoxide 2 might be expected to bind to the enzyme more effectively than the (19S)-isomer 3. Indeed, the (19R)-compound 2 proved to be a very powerful competitive inhibitor at 37°C of human placental microsomal aromatase (Ki=7 nM) whereas the (19S)-isomer 3 was less effective (Ki=75 nM). Time-dependent assays at 37°C and 25°C revealed a 25°C time-dependent loss of enzyme activity in the absence of NADPH during the initial 8 min. The limiting t1/2 values for the (R)- and (S)-isomers 2 and 3 were 1.6 and 20 min, respectively. The exact nature of these brief temperature-dependent processes is not known but they may represent conformation changes and new interactions at the active site.

In order to study further the nature of the inhibition of aromatase by these epoxides we synthesized the (19R)-epoxide 2 in radiolabeled form. Starting with the aldehyde

1 carrying ^{3}H at the C-19 position, the 19-^{3}H-(19R)-epoxide 2 was obtained and then shown (in collaborative studies with Dr. O'Neal Johnston at Merrell Dow) not to liberate ^{3}H from C-19 on incubation with placental aromatase. This establishes that compound 2 is not undergoing hydroxylation at C-19. The epoxide compounds reported above are of considerable interest both as active site probes of aromatase and as inhibitors of estrogen synthesis. They are also very unusual in that they inhibit a cytochrome P-450 system. Epoxides are often biosynthesized by such systems, but are not commonly viewed as potential cytochrome P-450 enzyme inhibitors.

Further studies with the epoxides 2 and 3 involving purified aromatase, carried out in collaboration with Dr. L. E. Vickery and J. T. Kellis (Irvine, CA), provided new insight into the inhibition process (7). Spectroscopic titrations of purified aromatase-androstenedione complex with the (19R)-oxirane 2 revealed (Figure 2) that inhibitor binding shifted the Soret maximum of the enzyme to 411 nm. This spectroscopic shift could be reversed by addition of excess androstenedione to give the high-spin enzyme form, with a Soret maximum at 393 nm. The data are consistent with a process involving reversible binding of the epoxide oxygen to heme iron. Although data are available for the binding of oxygen ligands to cytochrome P-450 systems, resulting in complexes with a Soret maximum at 416-418 nm

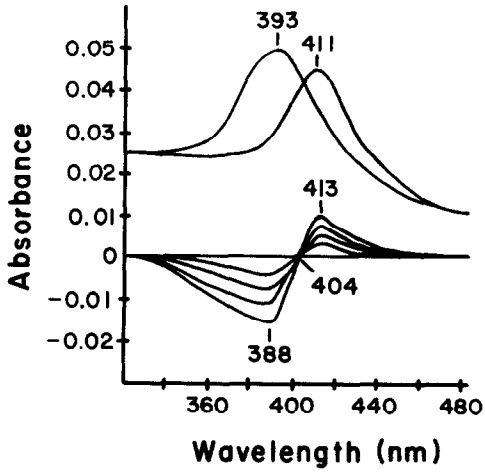


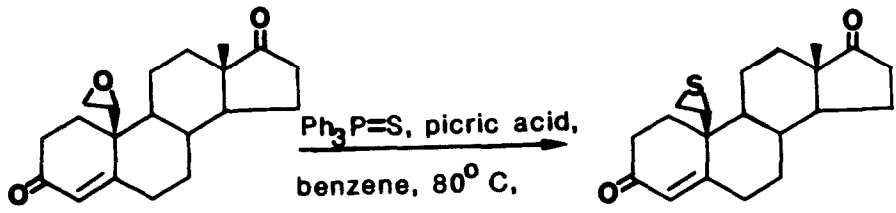
FIGURE 2. SPECTROSCOPIC STUDIES WITH PURIFIED ENZYME AND (19R)-OXIRANE 2. TOP: ABSOLUTE SPECTRA OF 0.3 μM ENZYME PLUS 10 μM ANDROSTENEDIONE BEFORE (393 nm) AND AFTER (411 nm) ADDITION OF (19R)-OXIRANE 2 (10 μM). BOTTOM: DIFFERENCE SPECTRA SHOWING EFFECTS OF TITRATION OF ENZYME-ANDROSTENEDIONE WITH (19R)-OXIRANE 2.

(8, 9), we are not aware of data for such complexes with epoxides. The (19S)-isomer 3, which has a larger Ki, showed difference spectra at partial saturation consistent with coordination of epoxide oxygen to heme, but complete saturation was not possible in this case. In summary, the epoxides 2 and 3 behave as powerful competitive inhibitors and show spectro-scopic behavior consistent with reversible coordination of epoxide oxygen to heme iron.

These results encouraged us to synthesize the corresponding 10β-thiiranyl steroids. On the one hand, sulfur should be a better ligand to iron than is oxygen. On the other hand, it was thought that nucleophilic attack on the thiirane grouping might result in covalent attachment to

the enzyme. Alternatively, enzymatic oxidation of the thiirane might yield a reactive species leading to covalent inactivation of enzyme.

We synthesized the desired thiiranes by a modification of the triphenylphosphine sulfide-trifluoroacetic acid procedure which has been reported to generate thiiranes from oxiranes (10). Thus we were able (11) to generate the (19S)-10β-thiiranyl steroid stereospecifically in good yield (75%) by treatment of 2 with triphenylphosphine sulfide (10 equiv) and picric acid (10 equiv) in benzene at 80°C under nitrogen for 16 h (Scheme 3). The stereochemical assignment at C-19 was made on the basis of the proposed (10) mechanism of the triphenylphosphine sulfide reaction, which would require inversion at C-19, and by comparison with the spectroscopic data and chromatographic behavior of 2 and 3, whose structures are securely established by X-ray crystal structure determinations as noted above.



SCHEME 3

Likewise, exposure of (S)-epoxydione 3 to picric acid-triphenylphosphine sulfide under the conditions described above gave (19R)-10β-thiiranyl estr-4-ene-3,17-dione in 40% yield. Approximately 30% starting material was recovered in this case, and these results suggest greater chemical reactivity for the (R)-epoxydione 2 vs. the (S)-epoxydione 3. To confirm the absence of skeletal rearrangements, the thiiranes were each smoothly desulfurized to the known (12) 10β-vinyl estr-4-ene-3,17-dione by treatment with triphenylphosphine in refluxing toluene.

Both the (R)- and (S)-thiiranes 6 and 7 proved to be excellent competitive inhibitors of placental microsomal aromatase, with Ki values of 1 and 75 nM, respectively (Figure 3). The difference in inhibitory potency due to C-19 stereochemistry which was seen for the epoxides is observed in the thiiranes to an even greater degree. Two recent reports describe effective inhibition of aromatase by 19-thiosteroids (13, 14) and in one case (14) spectroscopic studies implicated coordination of sulfur to heme iron.

Again, collaborative studies with Vickery and Kellis (7) who used using purified human placental aromatase gave important information on the inhibition process. Spectroscopic titration of the purified aromatase-androstenedione complex with the (19R)-10β-thiiranyl inhibitor 6 produced a shift in the Soret maximum to 425 nm (Figure 4). This shift was reversible, as in the case of the corresponding oxirane,

on addition of excess androstenedione. This shift closely parallels that seen (424 nm) when thioether ligands complex

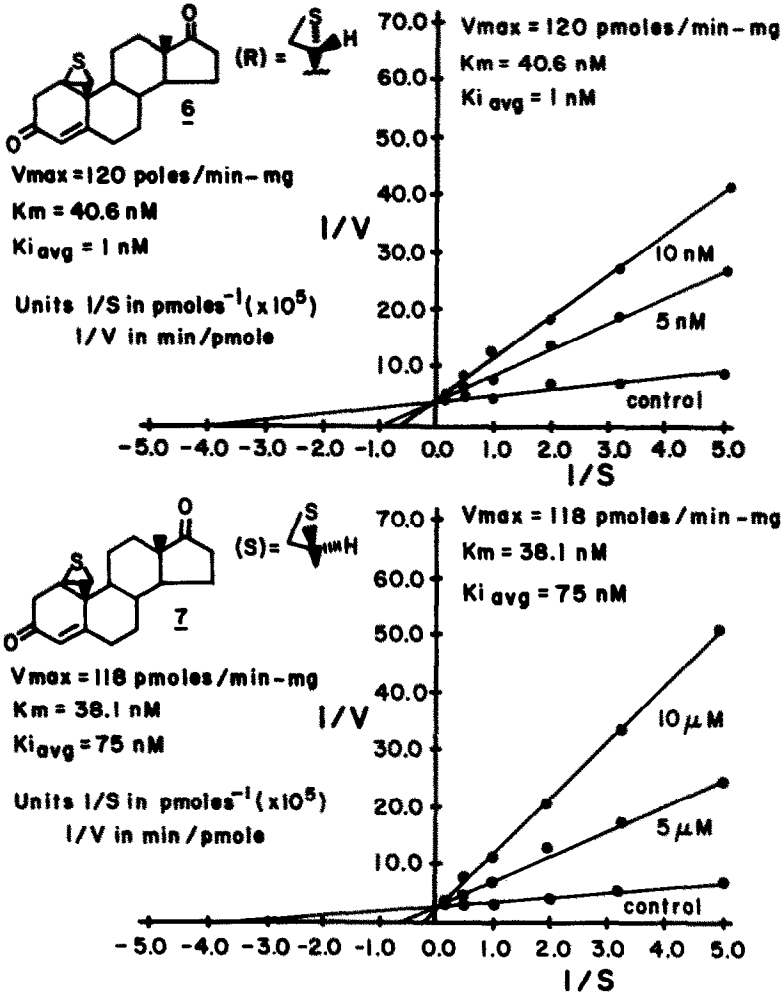


FIGURE 3. LINEWEAVER-BURKE PLOTS FOR THE INHIBITION OF MICROSOMAL PLACENTAL AROMATASE BY THE (19R)- AND (19S)-THIIRANES 6 AND 7.

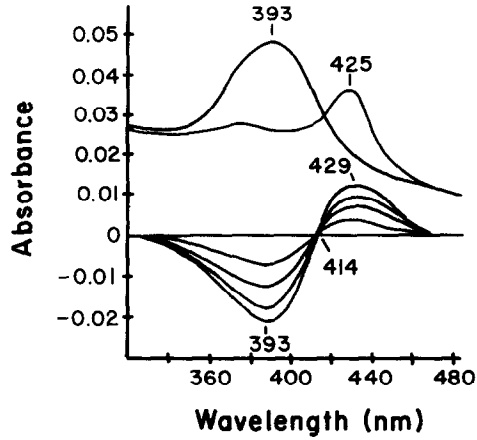


FIGURE 4. SPECTROSCOPIC STUDIES WITH PURIFIED ENZYME AND (19R)-THIIRANE 6. TOP: ABSOLUTE SPECTRA OF 0.3 μM ENZYME PLUS 10 μM ANDROSTENEDIONE BEFORE (393 nm) AND AFTER (425 nm) ADDITION OF (19R)-THIIRANE 6 (10 μM). BOTTOM: DIFFERENCE SPECTRA SHOWING EFFECTS OF TITRATION OF ENZYME-ANDROSTENEDIONE WITH (19R)-THIIRANE 6.

with the cytochrome P-450$_{cam}$ system (15).

In summary, the 10-oxiranyl and 10-thiiranyl heteroatoms appear to coordinate with the heme iron of the aromatase system. We assume that these substrate analogs are binding to the active site just like the natural substrates. If the solid state conformations of the oxiranes, as determined by X-ray crystallography, can be extrapolated to the active site situation, one may infer that the heme grouping is sited adjacent to C-1 and C-2 as well as to C-19. These novel and powerful inhibitors are of interest as active site probes of aromatase *in vitro*. Studies of their *in vivo* activity are in progress.

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NOTE

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