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IRREVERSIBLE ACTIVE-SITE-DIRECTED INHIBITION OF Δ^5-3-KETOSTEROID ISOMERASE BY STEROIDAL 17-β-OXIRANES. EVIDENCE FOR TWO MODES OF BINDING IN STEROID-ENZYME COMPLEXES

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SUMMARY

The spiro-17β-oxiranyl derivatives of d-equilenin, epiandrosterone, dehydroepiandrosterone, and Δ^4-androsten-3,17-dione are active-site-directed irreversible inhibitors of Δ^5-3-ketosteroid isomerase of P. testosteroni. The 17β-oxiranyl steroids rapidly inhibit the isomerase in a time-dependent manner which exhibits saturation kinetics. The enzyme is protected against inactivation by the competitive inhibitor 19-nortestosterone. In addition, prolonged dialysis against neutral buffer leads to no regeneration of enzyme activity. Fluorescent spectral changes associated with the incubation of the enzyme with the 17β-oxirane derived from d-equilenin indicate that there are two modes of binding for steroids to the isomerase. These results suggest that 17β-oxiranes may inhibit the enzyme by a mechanism similar to that for the previously studied spiro-3β-oxiranyl steroids.

The enzyme Δ^5-3-ketosteroid isomerase (E.C. 5.3.3.1) from Pseudomonas testosteroni catalyzes the conversion of Δ^5-3-ketosteroids to their Δ^4-isomers (1,2). Although much effort has been expended on both the mechanism of action and the requirements for effective binding of substrates and inhibitors to the active site, neither of these processes is fully understood. In order to probe the active site, several active-site-directed irreversible inhibitors have been synthesized, including 6β-bromotestosterone acetate (3), spiro-3β-oxiranyl-5α-androstan-17β-ol (4), and the mechanism-based acetylenic 5,10-secosteroids (5,6). In addition Δ^4-3-ketosteroids have been shown to photoinactivate the enzyme (7,9). In all of these compounds, the reactive groups have been in positions on the A and B rings of the steroid nucleus which are expected to bind near the enzymatic amino acid residues responsible for catalysis.

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We wish to report here the active-site-directed irreversible inhibition of Δ^5-3-ketosteroid isomerase by spiro-17β-oxiranyl steroids, compounds with a reactive functional group far removed from the A and B rings. This finding, along with fluorescence measurements of inactivation by the 17β-oxirane derived from d-equilenin, suggests that there are at least two distinct binding modes for steroids to Δ^5-3-ketosteroid isomerase.

MATERIALS AND METHODS

The purification of the isomerase and the kinetic methods have been described previously (4).

Spectrofluorometric Studies Fluorescence measurements were made using an Aminco-Bowman spectrofluorometer equipped with a constant temperature cell compartment and an Aminco data aquisition and retrieval system. The raw data were corrected by subtracting the value for a buffer blank containing all components except steroid and enzyme and then taking the ratio relative to a reference solution of steroid in the absence of enzyme.

Analysis by both hplc and uv spectrophotometry showed no detectable changes in the steroid solution in the absence of enzyme over the time course of these experiments.

Steroids All melting points are uncorrected. Nmr and ir spectra were obtained in $CDCl_3$ and $CHCl_3$ unless otherwise specified. Analytical and preparative hplc procedures were carried out on a μBondapak/C_{18} column (7.8 mm x 30 cm) with MeOH-H_2O mixtures. Column chromatography was performed on silica gel (activity III) using CH_2Cl_2-hexane solvent mixtures.

17β-Oxiranes. Reactions of 17-ketosteroids with dimethylsulfonium methylide are highly stereoselective and yield 17β-oxiranes (10a,10b). Using the general procedure of Cook, et al (10a), 5α-androstan-3β-ol-17-one, Δ^5-androsten-3-ol-17-one, and d-equilenin were converted to 2β, 3β, and 5β, respectively. After column chromatography and recrystallization, hplc analysis showed all the 17β oxiranes to be homogeneous (> 99%) and free of the 17α-isomers.

Spiro-17β-oxiranyl-5α-androstan-3β-ol (2β). Recrystallized from ether: mp 171-172° (lit. (10b) 171-173°); NMR: δ 0.82 (19-CH_3), 0.88 (18-CH_3), 2.73 (AB pattern, J = 5 Hz, oxirane CH_2).

Spiro-17β-oxiranyl-Δ^5-androsten-3β-ol (3β). Recrystallized from ether: mp 167-170° (lit. (10b) 166-168°); NMR (10c): δ 0.91 (18-CH_3), 1.04 (19-CH_3), 2.77 (AB pattern, J = 5 Hz, oxirane CH_2), 5.38 (C=CH).

Spiro-17β-oxiranylestra-1,3,5(10), 6,8-pentaene-3-ol (5β). Recrystallized from MeOH: mp 195-200° dec; UV (H_2O, pH 7): λ_m 229 nm (log ε 4.74). Acetylation (pyridine-acetic anhydride) yielded the 3-acetate: mp 131-134°; IR: 1750 cm^{-1} ($COOCH_3$); NMR: δ 0.81 (18-CH_3), 2.33 ($COOCH_3$), 2.84 (AB pattern, J = 5 Hz, oxirane CH_2), 7.15-8.1 (aromatic).

Spiro-17β-oxiranyl-Δ^4-androsten-3-one (4β). Oxidation (CrO_3/pyridine) of 3β and column chromatography yielded pure Δ^5-3-ketosteroid which was treated with 2 mM KOH in MeOH (room temperature for 30 min) to give the conjugated ketone. Recrystallization from hexane/ether gave 17β oxirane 4β: mp 172-174°; IR: 1660 (C=O), 1615 (C=C) cm^{-1} NMR: δ 0.94 (18-CH_3), 1.23 (19-CH_3), 2.76 (AB pattern, J = 5 Hz, oxirane CH_2), 5.77 (CH=C); UV (MeOH): λ_m 240 nm (log ε 4.23).

17α-Oxiranes. Peracid epoxidation of 17(20) unsaturated steroids yields predominantly the 17α-oxiranes (10b,10d). Compounds 2α, 4α, and 5α were synthesized by stirring the 17-methylene precursors with m-chloroperbenzoic acid in a biphasic solution of CH_2Cl_2 and 0.3 M phosphate buffer (pH 7) at room temperature overnight. The crude 17α-oxiranes contained 20-25% of

the 17β-epimers. After column chromatography and recrystallization, the 17α-oxiranes still contained 1-5% of the 17β isomers. Therefore, 2α, 4α, and 5α were all purified further (> 99.8%) by preparative hplc before testing as irreversible inhibitors.

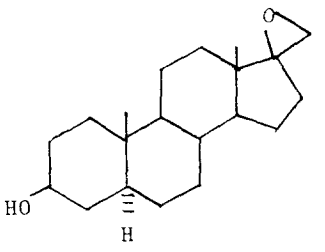
Spiro-17α-oxiranyl-5α-androstan-3β-ol (2α). Prepared from 17-methylene-5α-androstan-3β-ol (10d): mp 143-145°; NMR: δ 0.82 (18-CH_3 and 19-CH_3), 2.68 (AB pattern, J = 4 Hz, oxirane CH_2).

Spiro-17α-oxiranyl-Δ^4-androsten-3-one (4α). Prepared from 17-methylene-Δ^4-androsten-3-one (10c): mp 191-193°; IR: 1660 (C=O), 1615 (C=C) cm^{-1}; NMR: δ 0.87 (18-CH_3), 1.21 (19-CH_3), 2.71 (AB pattern, J = 4 Hz, oxirane CH_2), 5.76 (CH=C).

Spiro-17α-oxiranylestra-1,3,5(10), 6,8-pentaene-3-ol (5α). Reaction of d-equilenin with triphenylphosphonium methylide gave 17-methylene estra-1,3,5(10), 6,8-pentaene-3-ol. IR: 3050, 1650, 880 ($C=CH_2$) cm^{-1}; NMR: δ 0.72 (18-CH_3), 4.85 ($C=CH_2$). Attempts to epoxidize the above compound directly were unsuccessful. Acetylation (pyridine-acetic anhydride) followed by epoxidation and careful hydrolysis of the 3-acetate yielded 5α: mp 176-179° dec; NMR (CD_3OD): δ 0.77 (18-CH_3); UV (H_2O, pH 7): λ_m 229 nm.

RESULTS AND DISCUSSION

Several spiro-17-oxiranyl steroids (2β-5β) were synthesized and examined as possible irreversible inhibitors of the isomerase.



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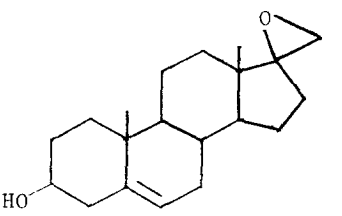

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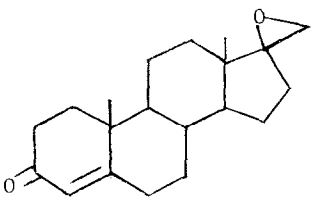

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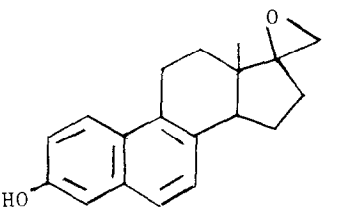

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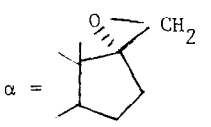
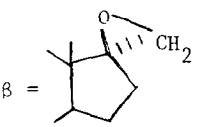




All four 17β-oxiranes show rapid time-dependent irreversible inhibition which obeys pseudo-first-order kinetics over 3-4 half-lives and leads to complete inactivation of the enzyme. The rate constants were analyzed

TABLE I

INHIBITION OF Δ^5-3-KETOSTEROID ISOMERASE

Compound	K_I, μM	k_3, sec^{-1}	k_3/K_I
1β[a,b]	17 ± 3	$1.13 \pm 0.19 \times 10^{-3}$	$6.7 \pm 0.4 \times 10^{-5}$
2β[a]	40 ± 25	$1.2 \pm 0.7 \times 10^{-2}$	$3.0 \pm 0.2 \times 10^{-4}$
3β[a]	47 ± 12	$7.2 \pm 1.9 \times 10^{-3}$	$1.5 \pm 0.2 \times 10^{-4}$
4β[a]	50 ± 7	$4.3 \pm 0.6 \times 10^{-3}$	$8.5 \pm 0.3 \times 10^{-5}$
5β[c]	1.7 ± 0.3	$2.3 \pm 0.2 \times 10^{-4}$	$1.3 \pm 0.2 \times 10^{-4}$

a. 21.0°, 3.8% methanol, [Enz] = 0.3 μM

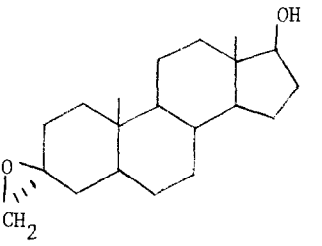
b. ref. 4

c. 27.0°, 1.7% methanol, [Enz] = 0.3 μM

according to the method of Kitz and Wilson (4,11) and are consistent with saturation of the enzyme by inhibitor (eq. 1). Values of the binding constants (K_I) and rate constants (k_3) for 2β-5β are given in Table I along with

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E \cdot I \xrightarrow{k_3} E\text{-}I \quad \text{(inactive enzyme)} \qquad (1)$$

the corresponding values for spiro-3β-oxiranyl-5α-androstan-17β-ol (1β).



1β

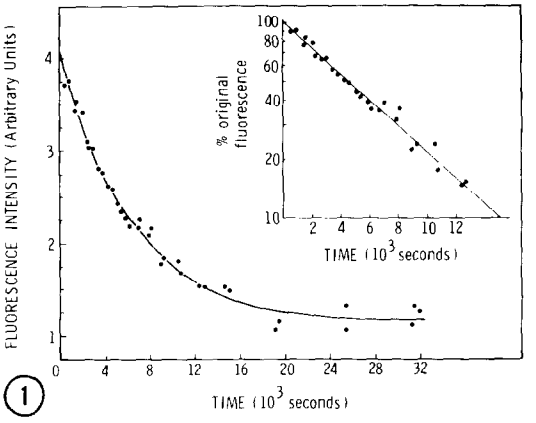
Several lines of evidence, in addition to the existence of saturation kinetics, point to active-site-directed irreversible inhibition by the 17β-oxiranes. 1) The competitive inhibitor 19-nortestosterone (19NT) protects the enzyme from inactivation by the 17β-oxiranes. The extent of protection agrees, in all cases, with that predicted from the binding constant of 19NT. 2) Dialysis of enzyme inhibited by 2β against 0.01 M phosphate (pH 7, 1% meth-

anol) for 24 hours leads to no regeneration of enzyme activity. 3) The corresponding α-oxiranes, 2α, 4α, and 5α show no evidence of irreversible inhibition after 24 hours incubation with the enzyme (12). 4) A variety of other steroidal epoxides (including 4,5α; 4,5β; 5,6α; 5,6β; and 5,10β epoxides) do not act as irreversible inhibitors of the isomerase (13).

Two possible explanations could account for the remarkable ability of the 17β-oxiranes to irreversibly inhibit the isomerase. The first, and simplest, is that the 17β-oxiranes are reacting with an amino acid residue at a portion of the active site (the D-ring binding site) which is far removed from the catalytic machinery of the enzyme. The second hypothesis is that the 17β-oxiranes can bind "backwards", that is with the A-ring in the D-ring binding site and the D-ring in the A-ring binding site. The 17β-oxirane could then react with the same group on the enzyme which is attacked by the corresponding 3β-oxirane (1β). Interestingly, molecular models suggest an overall similarity of molecular shape of 1β and "backwards" 2β (14). Furthermore, these models show that it is 2β rather than 2α which resembles 1β in the positioning of the oxirane in the "backwards" binding mode.

In order to probe the possibility of multiple binding modes of the substrate to the enzyme, we examined the fluorescence spectrum of a solution of 5β and isomerase as a function of time. It has previously been reported that 17β-dihydroequilenin, a competitive inhibitor of the isomerase, shows a marked increase in its fluorescence spectrum at 420 nm upon binding to the enzyme (15). In short term experiments, 5β shows a similar effect. However, prolonged incubation of 5β with the isomerase leads to a decrease in the intensity at 420 nm to a value similar to that for 5β in the absence of enzyme. When this spectral change was monitored as a function of time at 420 nm (excitation at 360 nm), first-order kinetics were observed with rates virtually identical to the rates of enzyme inactivation by 5β under the same conditions (Figures 1,2).

Although there are several possible explanations for these results, we believe the most plausible one to be the following. Initial binding of 5β



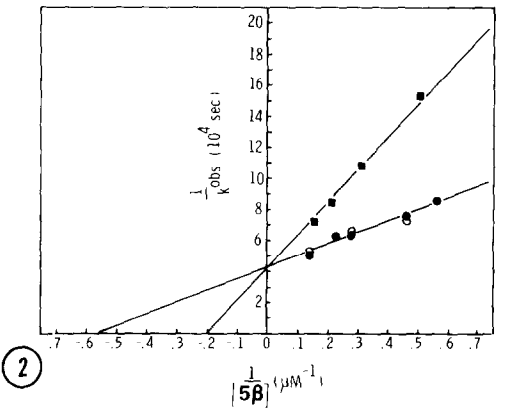


Figure 1. Fluorescent spectral changes of 5β upon incubation with Δ5-3-ketosteroid isomerase at 27°C. The medium contained 7.16 μM 5β, 0.3 μM enzyme, 1.7 percent methanol and 33 mM potassium phosphate (pH 7) in a total volume of 2.1 mls. The excitation wavelength was 360 nm and emission was monitored at 420 nm. The line shown is the computer generated best fit exponential function of the data.

Figure 2. Double reciprocal plot of the pseudo-first-order rate constant of inactivation (k^{obs}) with respect to inhibitor concentration ([5β]) at 27°. The medium contained 0.3 μM enzyme, 33 mM potassium phosphate (pH 7), 1.7 percent methanol, and various concentrations (1.78-7.16 μM) of 5β in a total volume of 520 μl (●). Inactivation was also studied in the presence of 11.8 μM 19-nortestosterone (■). Also presented are rate constants obtained from fluorescence measurements as described in figure 1 (o).

to form a reversible non-covalent complex with the enzyme takes place primarily in the "normal" mode, which corresponds to the catalyticly active mode for substrates (mode A). The enhancement of fluorescence at 420 nm is due to this type of binding. Inactivation, however, occurs in the "backwards" mode (mode D) and inhibitor in mode D does not exhibit enhanced fluorescence. The decrease in fluorescence then is diagnostic of a change in the environment of the inhibitor upon covalent attachment to the enzyme. The relatively low rate constant for inhibition, as well as the relatively high affinity of 5β for the enzyme compared to the other 17β-oxiranes, is a consequence of the preference of 5β for binding in mode A.

Further investigations into the nature of the inactivation of the isomerase by 17β-oxiranes are currently in progress.

ACKNOWLEDGMENT

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