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SELECTIVE INHIBITION OF RAT EPIDIDYMAL STEROID Δ^4-5α-REDUCTASE BY CONJUGATED ALLENIC 3-OXO-5,10-SECOSTEROIDS

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SUMMARY

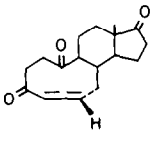
The effects of (4*R*)-5,10-secoestra-4,5-diene-3,10,17-trione (I) and of (4*R*)-5,10-seco-19-norpregna-4,5-diene-3,10,20-trione (II) on epididymal Δ^4-5α-reductase and 3α-hydroxysteroid dehydrogenase were investigated. Assessment by I_{50} values showed that Δ^4-5α-reductase was inhibited approximately 50 and 250 times more effectively than 3α-hydroxysteroid dehydrogenase by compound I and II respectively. The onset of the inhibition of Δ^4-5α-reductase by these compounds occurs in less than five minutes. Both compounds were shown to be non-competitive inhibitors of Δ^4-5α-reductase. For Δ^4-5α-reductase the K_I's for compounds I and II were 5.47×10^{-6} M and 0.98×10^{-6} respectively. The results of equilibrium dialysis experiments suggested that the observed Δ^4-5α-reductase inhibition was irreversible. The relative specificity of these compounds with respect to inhibitor structure and enzyme inhibition is discussed.

INTRODUCTION

The role of androgens in the maintenance of normal epididymal function, maturation and storage of spermatozoa, is well established [1, 2]. A growing body of evidence has demonstrated that 5α-reduced metabolites of testosterone, namely 5α-dihydrotestosterone (DHT) and 5α-androstane-3α,17β-diol (3α-diol) are most active in the maintenance of such functions [3, 4]. The presence in the rat epididymis of testosterone, DHT, and 3α-diol has been demonstrated by a number of investigators [5, 6]. Furthermore, Δ^4-5α-reductase and 3α-hydroxysteroid dehydrogenase, the enzymes which synthesize the latter two steroids have also been found in the epididymis [7–9].

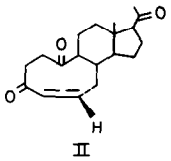
There is to date no report of a non-competitive inhibitor of Δ^4-5α-reductase. If such inhibitors were available, they would be of great value in elucidating the relative roles of testosterone and its 5α-reduced metabolites on target tissues as well as in providing better tools for gaining insight into the molecular properties of Δ^4-5α-reductase.

In contrast, competitive inhibitors of Δ^4-5α-reductase found in skin have been reported by Voigt and Hsia [10]. These molecules are analogs of testosterone; the most potent of these analogs is 4-androsten-3-one-17β-carboxylic acid. We have been unable to find any reports on either competitive or non-competitive inhibitors of epididymal Δ^4-5α-reductase.



I

(4R)-5,10-secoestra-4,5-diene-3,10,17 trione



II

(4R)-5,10-seco-19-norpregna-4,5-diene-3,10,20 trione

Conjugated allenic 3-oxo-5,10-secosteroids (I and II) have been found by Covey and Robinson [11] to be irreversible inhibitors of 5-ene-3-ketosteroid isomerase (EC 5.3.3.1) obtained from *Pseudomonas testosteroni*. X-ray crystallographic studies by Carrell *et al.* [12] have shown that the conjugated allenic 3-oxo-5,10 secosteroid (I) has a closely similar conformation to that of the normal tetracyclic steroid 4-androstene-3,17-dione. Analogous conformational similarity should apply to the allenic secosteroid (II) with respect to progesterone. The conformational similarities between compounds I and II and 4-ene-3-ketosteroids, together with the presence of an alkylating system (the conjugated allenic ketone grouping), led us to expect that compounds I and II might inhibit Δ^4-5α-reductase.

In this communication we report (1) the relative inhibitory potencies of these compounds on both Δ^4-5α-reductase and 3α-hydroxysteroid dehydrogenase found in rat caput-corpus epididymis and (2) the nature of the inhibition observed as determined by kinetic analysis.

METHODS

Preparation of subcellular fractions for assaying Δ^4-5α-reductase and 3α-hydroxysteroid dehydrogenase activities. Caput-corpus epididymides from Sprague–Dawley rats (250–300 g) were removed immediately after decapitation. The epididymal fat pad was carefully trimmed off and the tissues were weighed. All subsequent operations were done at 4°C. The solution used for homogenization and for the washing of pellets was a Krebs–Ringer phosphate buffer (KRP) containing 116 mM NaCl, 4.5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM phosphate buffer and 5% v/v glycerol; the pH was adjusted to 6.9 with 6 N HCl. The tissues were first homogenized (10 ml KRP/g tis-

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sue) in an Omni-Mixer (Ivan Sorvall, Inc.) at maximum speed for two 15 s periods separated by a 15 s interval and then with 10 strokes of a Potter–Elvehjem homengizer (1,000 rev/min). The homegenate was then filtered through a nylon mesh, 93 μ, (Techo Co.), and the filtrate was centrifuged at 1,500 ***g*** for 10 min in an IEC model B-20 centrifuge. The pellet was washed three times with a vol. of KRP buffer equivalent to 30 times the tissue wet weight and the washes were discarded. The washed pellet, nuclear fraction, was resuspended in 10 times the original tissue wet weight and was used for assaying Δ^4-5α-reductase activity. The supernatant was then centrifuged at 100,000 ***g*** for 1 h in a Beckman L ultracentrifuge. The supernatant, cytosol fraction, was used for assaying 3α-hydroxysteroid dehydrogenase activity.

Assay of Δ^4-5α-reductase and 3α-hydroxysteroid dehydrogenase. Δ^4-5α-reductase and 3α-hydroxysteroid dehydrogenase activities were assayed using a modified method of Moore *et al.* [13]. Δ^4-5α-reductase activity was assayed by adding an aliquot of the nuclear fraction to 1 ml of KRP buffer containing 5×10^{-4} M NADPH (Sigma), 5×10^{-7} M testosterone (Sigma, crystallized) and 0.05 μcurie of [1, 2, 6, 7-^{3}H]-testosterone (New England Nuclear). 3α-hydroxy-steroid dehydrogenase activity was assayed in a similar manner using cytosol fraction as the enzyme source and [1,2-^{3}H]DHT and unlabelled DHT (5×10^{-7} M) as substrate. The reaction was allowed to take place for 1 h at 22°C while shaking at 120 cycles/min. It was then stopped and the steroids were extracted by the addition of 10 ml of ethyl acetate containing carrier testosterone, 5α-dihydrotestosterone, 4-androstene-3,17-dione, 5α-androstane-3α, 17β-diol and 5α-androstane-3β, 17β-diol each at a concentration of 10^{-5} M.

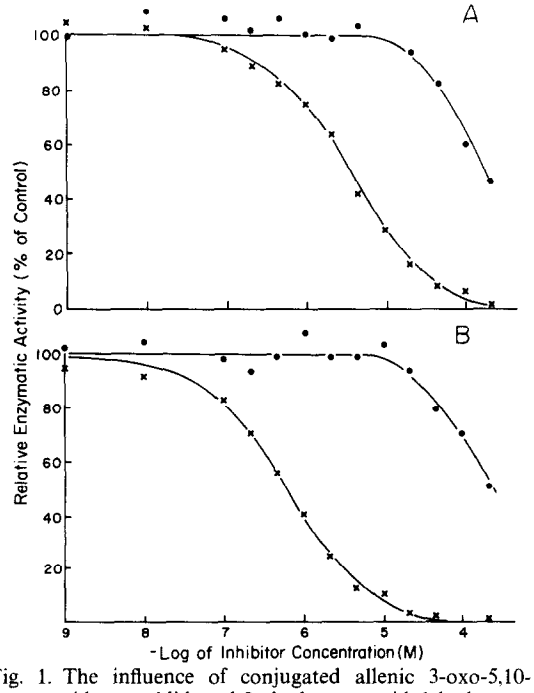

Fig. 1. The influence of conjugated allenic 3-oxo-5,10-secosteroids on epididymal 3α-hydroxysteroid dehydrogenase and Δ^4-5α-reductase. A. The effects of compound I—(4*R*)-5,10-secoestra-4,5-diene-3,10,17-trione on 3α-hydroxysteroid dehydrogenase ●——● and on Δ^4-5α-reductase ×——×. B. The effects of compound II (4*R*)-5,10-seco-19-norpregna-4,5diene-3,10,20-trione on 3α-hydroxysteroid dehydrogenase ●——● and on Δ^4-5α-reductase ×——×.

The solution was immediately vortexed for 15 s, allowed to stand 10 min and vortexed for an additional 15 s. Samples were then centrifuged at 1,000 ***g*** for 10 min in an IEC model V centrifuge. The lower (aqueous) phase was frozen in an acetone-dry ice bath. The upper phase was transferred to a 15 ml conical tube and evaporated to dryness at 45°C under nitrogen. Each tube was washed twice with 2 mls of chloroform–methanol (1 : 1 v/v).

Each sample was applied in chloroform–methanol (1:1 v/v) on silica gel 60 (0.25 mm thickness) plastic plates (E. Merck Co.) and developed in an equilibrated thin layer tank twice using the solvent system benzene–acetone (4 : 1 v/v). The R_F values for 4-androstene-3,17-dione, DHT, testosterone and both 3α- and 3β-androstanediols were 0.52, 0.62, 0.75, 0.85, respectively. In order to visualize the steroids, control lanes were sprayed with 5% w/v phospho-molybdic acid in acetone and heated for 10 min at 80°C. Each lane was then cut into eight sections and the radioactivity contained in each section was determined by scintillation spectrometry (Packard tricord 2000). The scintillation fluid contained 90% v/v toluene, 10% v/v methanol, 0.4% w/v PPO and 0.004 w/v POPOP.

Dihydrotestosterone, the product of the 5α-reductase reaction, and 5α-androstane-3α, 17β-diol, the product of the 3α-hydroxysteroid dehydrogenase reaction, were found to account for more than 90% of the ethyl acetate extractable steroid metabolites from the nuclear and cytosol fractions respectively. The identity of these steroids was demonstrated by recrystallization to constant specific radioactivity [14].

Synthesis of conjugated allenic 3-oxo-5,10 secosteroids. The synthesis of both (4*R*)-5,10, seco-19norpregna-4, 5-diene-3,10,20-trione (I) and (4*R*)-5,10-secoestra-4,5-diene-3,10,17-trione (II) has recently been described by Covey *et al.* [11].

RESULTS

The effects of conjugated allenic 3-oxo-5,10-secosteroids on rat epididymal nuclear Δ^4-5α-reductase and cytoplasmic 3α-hydroxysteroid dehydrogenase are shown in Fig. 1. It is clear from Fig. 1a that the inhibition of 5α-reductase activity by compound I is approximately 100 fold greater than the inhibition of 3α-hydroxysteroid dehydrogenase activity. We did not feel that inhibition of enzymatic activity at drug concentrations exceeding 2×10^{-4} M would be significant because of solubility problems and thus this concentration was not exceeded in our studies. Similarly, compound II was also found to be a much better inhibitor of Δ^4-5α-reductase activity than of 3α-hydroxysteroid dehydrogenase activity (Fig. 1b).

Table 1. I_{50} of (4*R*)-5,10-secoestra-4,5-diene-3,10,17-trione and of (4*R*)-5,10-seco-19-norpregna-4,5-diene-3,10,20 trione for Δ^4-5α-reductase and 3α-hydroxysteroid dehydrogenase

Drug	Δ^4-5α-Reductase (10^{-6} M)	3α-Hydroxysteroid dehydrogenase (10^{-6} M)
(4*R*)-5,10-secoestra-4,5-diene-3,10,17-trione	3.8	180
(4*R*)-5,10-seco-19-norepregna-4,5-diene-3,10,20-trione	0.68	200

Moreover, compound II was a better inhibitor of 5α-reductase activity than compound I. These observations have been quantified by obtaining I_{50} values for each enzyme using both compounds (Table 1). The results show that Δ^4-5α-reductase inhibition obtained with compound II is superior to that obtained with compound I by a factor of 5. Thus, these compounds selectively inhibit Δ^4-5α-reductase over 3α-hydroxysteroid dehydrogenase while the presence of 17β-acetyl side-chain permits better inhibition of Δ^4-5α-reductase than does a 17-keto substituent.

Since the inhibitor is included with both the enzyme and the substrate during the 1 h incubation, more accurate characterization of the time needed to initiate inhibition should be determined. In order to do this, Δ^4-5α-reductase was incubated with 5×10^{-7} M testosterone and the enzyme activity was followed and found to be linear as a function of time. After a 30 min incubation period, 10^{-6} M of compound II (enough to cause 60% inhibition) was added to the reaction mixture and aliquots were then removed and assayed over the next 30 min. The results of this experiment showed that a constant percentage inhibition (60%) was reached in less than five min, thus indicating that the onset of action of the inhibitors is relatively rapid.

We next turned our attention to the nature of the inhibition of Δ^4-5α-reductase by compounds I and II. Since the apparent K_m of the enzyme is needed in order to select proper substrate concentrations for a Dixon plot [15] and since the apparent K_m for testosterone of this enzyme in the epididymis has not been reported, we first obtained this value (Fig. 2). The apparent K_m value obtained by fitting this data to the Wilkinson model (16) is 0.34 μM and the V_{max} is 1.4×10^{-6} mol/h/g wet weight tissue. When these data were analyzed using the Lineweaver–Burk double reciprocal plot [17] (Fig. 2, insert), the values obtained by linear regression analysis were 0.36 μM for the apparent K_m and 1.8×10^{-6} mol/h/g wet weight tissue for the V_{max}.

Based on these observations, Dixon plots were obtained for testosterone concentrations of 200 and 500 nM. The results shown in Fig. 3a and 3b demonstrate that both compounds are non-competitive inhibitors of Δ^4-5α-reductase. The K_i values obtained for compound I by regression analysis of the data shown in Fig. 3a are 5.1×10^{-6} M using 200 nM testosterone and 5.5×10^{-6} M using 500 nM testosterone. The K_i values for compound II were obtained similarly and are 0.84×10^{-6} M using 200 nM testosterone and 0.98×10^{-6} M using 500 nM testosterone. The correlation coefficient for each of the four lines was greater than 0.980. Thus, these results show that compounds I and II are non-competitive inhibitors of epididymal nuclear Δ^4-5α-reductase and that compound II inhibits this enzyme approximately five times better than does compound I.

The question of reversibility of this inhibition remains to be answered. Consequently, equilibrium dialysis experiments were done toward this goal. When Δ^4-5α-reductase was incubated with compound II (10^{-6} M), dialyzed against KRP buffer with changes at least once every 12 h over a three day period and then assayed four times during this three day period, it was found that the drug-treated enzyme

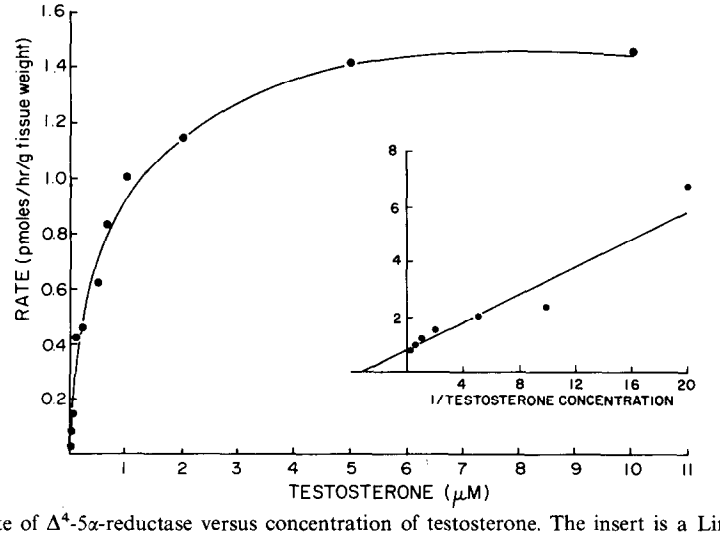

Fig. 2. Rate of Δ^4-5α-reductase versus concentration of testosterone. The insert is a Lineweaver–Burk analysis of these data.

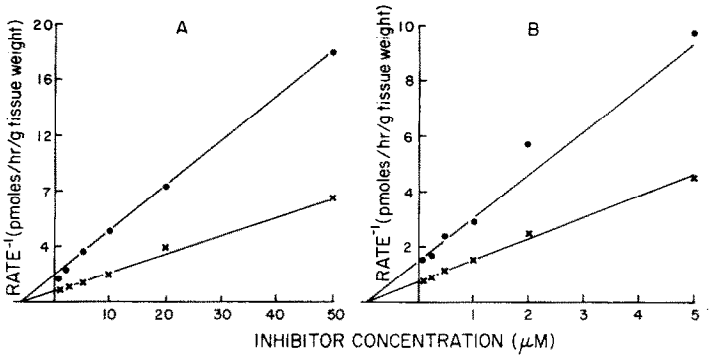

Fig. 3. Dixon plot analysis of the inhibitory effects of conjugated allenic-3-oxo-5,10-secosteroids on rate of reduction of testosterone by epididymal Δ^4-5α-reductase. A. Inhibition by (4*R*)-5,10-secoestra-4,5-diene-3,10,17-trione on Δ^4-5α-reductase at testosterone concentrations of 2×10^{-7} M ●——● and 5×10^{-7} M ×——×. B. Inhibition by (4*R*)-5,10-seco-19-norpregna-4,5 diene-3,10,20-trione on Δ^4-5α-reductase at testosterone concentrations of 2×10^{-7} M ●——● and 5×10^{-7} M ×——×.

had less activity than the untreated dialyzed enzyme. Such experimental results suggest an irreversible type of inhibition by these molecules but until Δ^4-5α-reductase can be further purified, no definite conclusion may be drawn in this regard.

DISCUSSION

The results presented in this manuscript show that compounds I and II are powerful non-competitive inhibitors of epididymal Δ^4-5α-reductase but very poor inhibitors of 3α-hydroxysteroid dehydrogenase. Differences in the degree of inhibition of Δ^4-5α-reductase by compounds I and II suggest that the 17β-acetyl derivative may bind to the active site of the enzyme more tightly than does the 17-keto derivative. This is consistent with the previously known binding preference of prostatic [18] and skin [10] Δ^4-5α-reductase for 4-ene-3-ketosteroids containing a 17β-acetyl substitutent versus those containing a 17-keto grouping. This selectivity might be enhanced by other modifications at the 17 position such as hydroxyl, carboxy, carboxymethyl, etc.

In the studies presented by Voigt and Hsia [10] on the inhibition of skin Δ^4-5α-reductase by a number of testosterone analogs, it was shown that all such analogs, though of varying potency, were pure competitive inhibitors. Thus, these conjugated allenic 3-oxo-5,10-secosteroids appear to be the first molecules to be shown to be non-competitive and possibly irreversible inhibitors of epididymal Δ^4-5α-reductase.

Since at least one other enzyme, bacterial 5-ene-3-ketosteroid isomerase is inhibited by compounds I and II [11], one could speculate that a spectrum of effects on steroid synthesizing and metabolizing enzymes would be obtained by these inhibitors. However, we are encouraged that this may not be the case since one other steroid metabolizing enzyme, epididymal 3α-hydroxysteroid dehydrogenase, is relatively unaffected by these compounds. Since the substrate of 3α-hydroxysteroid dehydrogenase differs from that of Δ^4-5α-reductase by a double bond at the carbon 4–5 positions, a certain degree of specificity by these compounds had been expected in this case. To confirm this hypothesis, the effects of these compounds on other steroid synthesizing and metabolizing enzymes in the epididymis and in other tissues will be elucidated.

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