PROSTATIC CANCER. I. 6-METHYLENE-4-PREGNEN-3-ONES AS IRREVERSIBLE INHIBITORS OF RAT PROSTATIC Δ^4-3 KETOSTEROID 5α-REDUCTASE*

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Received: 12-22-80

ABSTRACT

Some derivatives of 6-methylene-4-pregnen-3-one were studied as inhibitors of Δ^4-3-ketosteroid 5α-reductase. Maximum inhibitory activity was shown by 17-acetoxy-6-methylene-4-pregnene-3,20-dione (AMPD). Irreversible inactivation was observed following preincubation of the enzyme with NADPH and AMPD. This inactivation was found to occur only in the presence of NADPH. As such enzyme inactivation was not due to the formation of a more inhibitory metabolic product, or to the formation of superoxide via a cytochrome P-450/NADPH pathway, it seemed likely that the observed inactivation was derived from an irreversible combination of the enzyme with AMPD. That this was probably the case was established by kinetic studies which revealed a pattern compatible with a k_{cat} type of mechanism.

INTRODUCTION

There is now impressive experimental and clinical evidence that elimination of androgen support from prostatic cancer during its early endocrine-dependent phase represents an important modality in the treatment of the disease (1). Testosterone is generally

*The authors dedicate this paper to Professor David Shemin in honor of his seventieth birthday.

regarded as a proandrogen in this context with 17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone; DHT) as key androgenic support of the neoplasm (2). It follows that inhibition of DHT biosynthesis represents an important requirement in the development of improved forms of treatment.

Testosterone is converted into DHT by the enzyme 5α-reductase. The latter is an NADPH-dependent enzyme that transfers hydride ion from the co-enzyme to C_5 of the testosterone molecule as indicated in Fig. 1A. In seeking to inhibit this transfer we turned our attention to the 6-methylene derivatives of 4-pregnen-3-one (4) in the hope that such structures would inhibit the enzyme irreversibly by a mechanism of the k_{cat} type (5) as proposed in Fig. 1B.

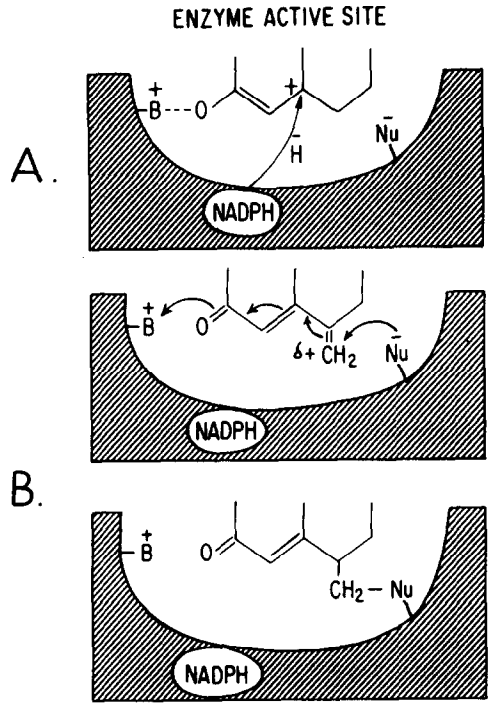

Figure 1. (A) Interaction of testosterone and NADPH at the active site of 5α-reductase (3). Nu is a hypothetical nucleophylic element assumed to reside in this area of the enzyme. (B) Proposed interactions of Δ^4-unsaturated 3-keto steroids bearing a 6-methylene substituent with the enzyme yielding an irreversibly inactivated enzyme.

Preliminary studies described herein show that 6-methyleneprogesterone and certain analogs thereof do, indeed, inhibit 5α-reductase in preparations of rat prostate. Preincubation studies reveal, for the first time, that some of these compounds might serve as irreversible inhibitors provided that the co-enzyme NADPH is present. In addition, kinetic analyses of the rates of inactivation support the concept that this process is of the k_{cat} type.

EXPERIMENTAL

Melting points are uncorrected. Infrared (ir) spectra were in $CHCl_3$, and were obtained on a Perkin-Elmer Grating Infrared-Spectrophotometer Model 337. Ultraviolet absorption spectra were determined using the Beckman DB Spectrophotometer. Optical rotations were determined in $CHCl_3$, using the Perkin-Elmer 141 Polarimeter.

17-Acetoxy-6-dichloromethylene-4-pregnene-3,20-dione 17-Acetoxy-3-methoxy-3,5-pregna-dien-20-one (1.16 g) (7) in dioxane (6 ml) and pyridine (5.5 ml) was treated with bromotrichloromethane (1.2g). After 24 hr at R.T. pyridine halides were removed by filtration and the intermediate trichloromethyl-steroid precipitated with water and triturated with ether. The crude product (1.26 g) was heated with pyridine (17 ml) on the steam bath for 2 hr. Precipitation with water, followed by chromatography [neutral alumina, Woelm, grade 1, 35 g, 2.5 x 4.5 cm column using benzene:ether (4:1) as eluant] and crystallization from benzene-pentane furnished 17-acetoxy-6-dichloromethylene-4-pregnene-3,20-dione as the benzene adduct, needles, mp 91°C (with bubbling) 125-128°C (clear).

Anal. Calcd for $C_{24}H_{30}O_4Cl_2.C_6H_6$: C 67.79; H, 6.83
Found: 67.96; 7.02

ir ($CHCl_3$): ν_{max} 1735 (17-ester C=O), 1720 (20-ketone), 1680 (3-ketone), 1665, 1600 cm^{-1} (C=C).

nmr ($CDCl_3$): δ 0.65 (s; 18-CH_3), 1.04 (s; 19-CH_3), 1.99 (s; 20-$COCH_3$), 2.02 (s; 17-$OCOCH_3$), 5.86 (s; 4-H), 7.26 ppm (s; C_6H_6).

uv (EtOH): λ_{max} 246 (ε=7330) 268-276 (Shoulder ε=5610) nm.

17-Acetoxy-6-dibromomethylene-4-pregnene-3,20-dione was prepared by the method of S. Liisberg et al. (6). Attempts to prepare the 6-difluoromethylene derivative of 17-acetoxyprogesterone using CBr_2F_2 proved unsuccessful.

20β-Acetoxy-6-methylene-4-pregnen -3-one was prepared by the method of Burn et al (7).

20β-Hydroxy-6-methylene-4-pregnen-3-one. The foregoing acetate (0.5 g) and potassium hydroxide (0.3 g) in methanol (18 ml), tetrahydrofuran (10 ml) and water (1.4 ml) was stirred at R.T. for 1 day. The product was precipitated with water and crystallized from acetone, tiny prisms, mp 192-193°C [Lit. (7) mp 189-191°C].

6-Methylene-4-pregnene-3,20-dione. To 20β-hydroxy-6-methylene-4-pregnen-3-one (0.37 g) in acetone (10 ml) was added Jones reagent (0.33 ml) with rapid stirring over 10 min. The product, precipitated with water and crystallized from benzene-ether-hexane, formed prisms, mp 132-134°C [Lit. (7) mp 133-135°C].

3-Oxo-4-pregnene-20α-carboxylic acid. 3-Oxo-4-pregnen-20α-aldehyde (17 g) (ex. Upjohn) was dissolved in 350 ml acetone and the solution treated at room temperature with vigorous stirring with 14.5 ml Jones reagent (26.72 g CrO_3 in 23 ml conc H_2SO_4 and enough ice to make 100 ml) added over a few minutes. After 30 min water (500 ml) was added. The mixture was left at 0°C and the precipitate removed and recrystallized from aq acetic acid, large plates, mp 285-287°C [Lit. (8) mp 268-270°C]. The acid was converted into its methyl ester (2).

Methyl 3-methoxy-3,5-pregnadiene-20α-carboxylate. To the foregoing methyl ester, 13.3 g, in tetrahydrofuran (105 ml) was added trimethyl orthoformate (7 ml), methanol (25 ml) and p-toluene sulfonic acid monohydrate (0.59 g) and the mixture stirred at R. T. for 90 min. Pyridine (4 ml) was added, the solvent removed in vacuo and the residue crystallized from methanol-pentane. The methyl enol ether (13.65 g) formed needles, mp 117-119°C, $[\alpha]_D^{20}$ 469° (c=3), raised to 123-125°C by one crystallization from methanol.

Anal. Calcd for $C_{24}H_{36}O_3$: C, 77.38; H, 9.74
Found: 77.31; 10.23

ir (KBr): ν_{max} 1740 (20-ester CO), 1655, 1630 cm^{-1} (C=C)

uv (EtOH): λ_{max} 240 (ε=19214) nm.

The enol ether was not formed when the methyl ester was treated with trimethyl orthoformate/perchloric acid in dioxane solution.

Methyl 6-formyl-3-methoxy-3,5-pregnadiene-20α-carboxylate. Dimethyl formamide (3.5 ml) in dry methylene chloride (20 ml) was treated dropwise with stirring with phosphorus oxychloride (2.09 g) in methylene chloride (15 ml) over 10 min at 0°C. After a further 10 min methyl 3-methoxy-3,5-pregnadien-20α-carboxylate (4.8 g) in methylene chloride (40 ml) and dimethylformamide (50 ml) was added in one

portion. Stirring was continued for 1 hr at 0°C and 2 hr at 0-20°C. Sodium acetate (6.25 g) in methanol (40 ml of 90%) was added and after a further 10 min the mixture was poured into saturated brine. The product was isolated with chloroform and crystallized from ether-benzene-petroleum ether. The 6-formyl derivative crystallized from methanol, mp 153-156°C, $[\alpha]_D^{20}$ -569° (c=3.9).

Anal. Calcd for $C_{25}H_{36}O_4$: C, 74.96; H, 9.06
Found: 74.65; 9.06

ir ($CHCl_3$): ν_{max} 1730 (20-ester C=O), 1680 (6-formyl C=O) and 1630 cm^{-1} (C=C).

uv (EtOH): λ_{max} 221 (ε= 10411) and 323 (ε=15438) nm.

Methyl 6-methylene-3-oxo-4-pregnene-20α-carboxylate. The foregoing 6-formyl derivative, 1.8 g in tetrahydrofuran (40 ml) and methanol (30 ml) was treated with sodium borohydride (2.7 g). After stirring at R.T. for 35 min water was added and the product isolated with ether. Crude methyl 6-hydroxymethyl-3-methoxy-3,5-pregnadiene-20α-carboxylate formed a glass, mp 100-112°C. It was heated with 80% aq acetic acid (45 ml) for 20 min on the water bath. The product was chromatographed on neutral alumina (Woelm, Grade 1; 20 g; 4 x 3 cm column) with benzene as eluant to give methyl 6-methylene 3-oxo-4-pregnene-20α-carboxylate, prisms, mp 159-159.5°C from ether-benzene-hexane, $[\alpha]_D^{20}$ + 871° (c=3.2).

Anal. Calcd for $C_{24}H_{34}O_3$: C, 77.80; H, 9.25
Found: 77.57; 9.38

ir ($CHCl_3$): λ_{max} 1730 (20-ester C=O), 1675 (3-ketone), 1655, 1600 cm^{-1} (C=C).

nmr ($CDCl_3$): δ 0.73 (s, 18-H), 1.09 (s, 19-H), 1.19 (d, J=6.3 Hz, 21-H), 3.63 (s, 20-$COOCH_3$), 4.90, 5.02 (bs, 6=CH_2), 5.86 ppm (s, 4-H).

uv (EtOH): λ_{max} 261 (ε=12391) nm.

Enzyme Assays. [1,2-^{3}H]Testosterone of specific activity (50-52 Ci/mmol) was from New England Nuclear and was purified by thin layer chromatography prior to use. NADPH, Tween 40, dithiothreitol, EDTA and superoxide dismutase were from Sigma Co. Polygram Sil G pre-coated plastic plates, 0.25 mm without gypsum (Brinkman Instruments, Inc.) were employed for chromatographic analyses. Reference steroids were from Steraloids. All other chemicals and solvents were of the highest purity obtainable.

Prostatic 5α-reductase was prepared and assayed essentially as described by Moore and Wilson (9) employing male Wistar rats as

source of ventral prostate tissue. Twenty-four to thirty rats were processed at a time. Prostatic nuclei, recovered after homogenization as the 800 g pellet, were rehomogenized in 2.0 M sucrose-0.5 mM $CaCl_2$, layered over 2.2 M sucrose-0.5 mM $CaCl_2$, and centrifuged at 56,000 g. The pellet was recovered, resuspended in 0.88 M sucrose-1.5 mM $CaCl_2$, and stored at -70°C. Nuclear extracts were made on the day of the assay as described (10). Nuclei were suspended in 0.05 M potassium phosphate buffer of pH 6.6 containing 10^{-4} M EDTA and 10 mM dithiothreitol (KED buffer) and the suspension triturated through an 18 and 20 gauge needle, centrifuged at 100,000 g for 30 min, resuspended in KED buffer and subjected to sonic disruption. Incubation involved 0.8 ml or 1.0 ml final volumes unless otherwise stated. Final concentrations were 5×10^{-8} [1,2-^{3}H] testosterone and 5×10^{-4} M NADPH in the potassium phosphate-EDTA-dithiothreitol buffer. At the end of the incubation period the enzymatic reaction was terminated by the addition of 5 vol chloroform-methanol (2:1) and processed for analysis by tlc. Reference compounds were added as required. After development of the chromatograms the spots were visualized by heating with the anisaldehyde reagent (9). The lanes were divided and cut as in the original procedure and each fragment transferred to a liquid scintillation vial and assayed for ^{3}H. The fractional reduction of testosterone was assayed by relating the radioactivity found in the 5-androstene-3β,17β-diol and DHT regions of the chromatogram to the total radioactivity found in the lane. Amounts of reduced testosterone were calculated from this value and the amount of testosterone originally present in the incubation. A substrate blank (incubation without enzyme) was always used to correct for slight impurities or non-enzymic degradation. These were found to be within the limits described by Moore and Wilson (9).

Inhibitory potentials of the steroids were determined as follows: The specified amount of the test steroid dissolved in benzene was added to the dry incubation tubes together with 22 µg of Tween 40 (dissolved in 0.2 ml benzene). Tubes were dried under a gentle stream of nitrogen and then placed in a desiccator continuously evacuated by a mechanical vacuum pump for 30 min. Control tubes (no test steroid) as well as the blank tubes containing the same amount of Tween 40 were similarly treated. To each of these dry incubation tubes was added 0.4 ml of the substrate co-enzyme solution in potassium phosphate buffer (9). After vortexing, the tubes were allowed to equilibrate in a 25°C water bath and, at specified times, the reactions were initiated by the addition of 0.4 ml of the enzyme preparation. After 1 hr 4 ml of chloroform-methanol (2:1) were added and the contents of the tube were processed prior to tlc as described in (9). Preliminary experiments showed that Tween 40 at twice the highest concentration employed in these experiments had no effect on enzyme activity.

The preincubation studies involved exposure of the nuclear protein to the test steroid, AMPD, together with the cofactor NADPH.

The ratio of test steroid to the Tween 40 detergent was the same as in the previously described incubations. All control tubes, without test steroid and/or NADPH, had the same amount of detergent. These preincubations were carried out for the stated times as given in the results. Following 1:10 dilution the residual enzymic activity was assayed under the already described conditions.

RESULTS

The results of experiments assessing the potency of selected pregnene derivatives containing methylene or methyl substituents at C_6 as inhibitors of 5α-reductase are given in Fig. 2. Progesterone (A) is included for comparison. Panel B demonstrates that 17-acetoxy-6-methylene-4-pregnene-3,20-dione (AMPD) inhibits the enzyme to the same degree as progesterone. Dihalogenation of the methylene group (C and D) leads to marked loss of activity. Megestrol acetate (E), an isomer of (B), is virtually inactive.

Removal of the 17-acetoxy group from (B) gives 6-methyleneprogesterone (F). This structural change does not significantly alter inhibitory activity. Reduction of the 20-keto group of (F) to give 20β-hydroxy-4-pregnen-3-one (G), in contrast, leads to a marked loss of potency, which, unexpectedly, is largely restored by acetylation (cf. H). Replacement of the 20-oxo group by the 20α-carbomethoxy group (I) is again accompanied by partial loss of activity.

When the 5α-reductase preparation was preincubated with 5×10^{-7}M of 17-acetoxy-6-methylene-4-pregnene-3,20-dione and NADPH, then diluted tenfold and assayed for 5α-reductase activity, 75% of the enzymic activity was lost. This is demonstrated in Table 1, expts. A and B. Preincubation of the enzyme alone, or of enzyme with NADPH but without the 6-methylene-4-pregnen-3-one, had no appreciable

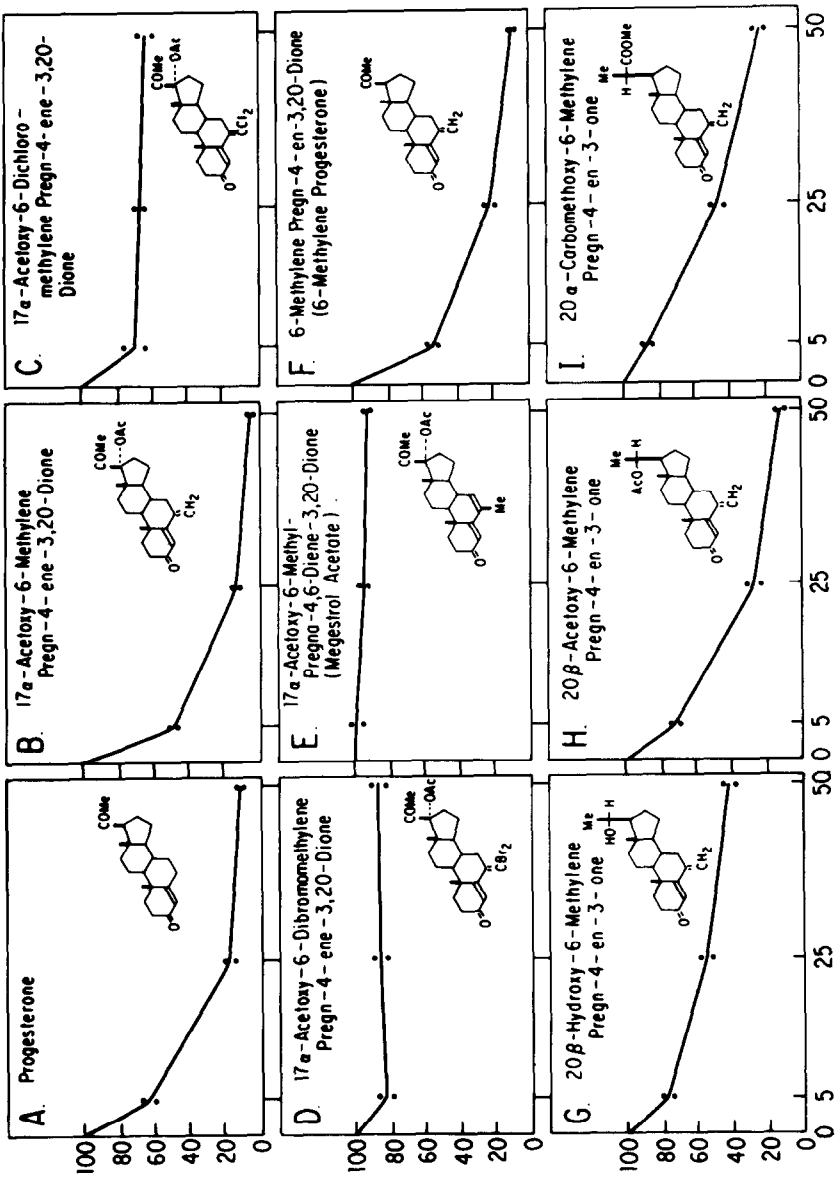

Figure 2. Inhibition of 5α-reductase by various pregnene derivatives.

Table 1. Effect of Preincubation of Enzyme with 17-Acetoxy-6-methylene-4-pregnen-3,20-dione and NADPH on 5α-Reductase Activity

	Preincubation conditions Time: 15 min		Enzymic Assay conditions Time: 45 min			Picomol Testosterone reduced/mg protein in 45 min + SEM
	Inhibitor	NADPH	Inhibitor	Testosterone	NADPH	
	M		M			
A	5×10^{-7}	6×10^{-5}	5×10^{-8}	5×10^{-8}	5×10^{-4}	0.71± 0.018 n = 6
B	0	6×10^{-5}	5×10^{-8}	5×10^{-8}	5×10^{-4}	3.0 ± 0.26 n = 6
C	0	0	5×10^{-8}	5×10^{-8}	5×10^{-4}	2.83± 0.09 n = 6
D	5×10^{-7}	0	5×10^{-8}	5×10^{-8}	5×10^{-4}	2.63± 0.18 n = 6
E	No preincubation		0	5×10^{-8}	5×10^{-4}	4.36± 0.24 n = 4
F	No preincubation		5×10^{-8}	5×10^{-8}	5×10^{-4}	3.14± 0.20 n = 4

n = number of experiments

effect upon residual enzymic activity following dilution and assay (compare expts. B, C and D with F). Preincubation of the enzyme with either progesterone or testosterone (Table 2) under the same experimental conditions did not result in enzyme inactivation. That the saturation conditions for the cofactor during preincubation were optimal is demonstrated in Table 3, when similar inactivation of the enzyme by 17-acetoxy-6-methylene-4-pregnene-3,20-dione took place over an eightfold range of cofactor concentrations. NAD, NADH or NADP could not be substituted for NADPH.

In a further preincubation study, aliquots of a preincubated mixture of enzyme, NADPH, and 5×10^{-7} M of 17-acetoxy-6-methylene-4-pregnene-3,20-dione (Table 4, tube A) were assayed in combination with equal aliquots of untreated enzyme (Table 4, tube B), under equivalent tenfold conditions of dilution. The enzymic activities observed in these combination assays (Table 4, tube D) approximated to the sum of the two separately determined enzymic activities when assayed in the presence of 5×10^{-8} M of inhibitor (Table 4, tubes A and C).

Table 5 demonstrates that inactivation of 5α-reductase following preincubation with NADPH and 17-acetoxy-6-methylene-4-pregnene-3,20-dione is not affected by superoxide dismutase present at a concentration of 10 μg/ml.

Figure 3 shows the progessive inactivation of 5α-reductase activity when the enzyme is preincubated with four concentrations of AMPD together with NADPH for different times. The time course follows pseudo-first order kinetics; the rate constants, also shown,

Table 2. Effect of Preincubation of Enzyme with Progesterone and with Testosterone and NADPH on 5α-Reductase Activity

	Preincubation conditions Time: 15 min		Conditions during enzymic assay 45 min			Picomol Testosterone Reduced/mg protein in 45 min
	Progesterone	NADPH	Progesterone	NADPH	Testosterone	
1	0	6×10^{-5}	5×10^{-8}	5×10^{-4}	5×10^{-8}	3.91
	0	6×10^{-5}	5×10^{-8}	5×10^{-4}	5×10^{-8}	3.41
2	5×10^{-7}	6×10^{-5}	5×10^{-8}	5×10^{-4}	5×10^{-8}	3.91
	5×10^{-7}	6×10^{-5}	5×10^{-8}	5×10^{-4}	5×10^{-8}	3.79
3	No preincubation		0	5×10^{-4}	5×10^{-8}	5.80
	No preincubation		0	5×10^{-4}	5×10^{-8}	5.42
4	No preincubation		5×10^{-8}	5×10^{-4}	5×10^{-8}	3.66
	No preincubation		5×10^{-8}	5×10^{-4}	5×10^{-8}	3.54
	Testosterone	NADPH		NADPH	Testosterone	
1	0	6×10^{-5}		5×10^{-4}	1×10^{-7}	6.9
	0	6×10^{-5}		5×10^{-4}	1×10^{-7}	8.4
2	5×10^{-7}	6×10^{-5}		5×10^{-4}	1×10^{-7}	7.6
	5×10^{-7}	6×10^{-5}		5×10^{-4}	1×10^{-7}	7.7
3	0	0		5×10^{-4}	1×10^{-7}	6.6
	0	0		5×10^{-4}	1×10^{-7}	7.7
4	5×10^{-7}	0		5×10^{-4}	1×10^{-7}	7.4
	5×10^{-7}	0		5×10^{-4}	1×10^{-7}	7.4

Individual results of duplicate incubations are given.

Table 3. Effect of NADPH Concentrations on the Inactivation of 5α-Reductase by 17-Acetoxy-6-methylene-4-pregnene-3,20-dione

	Preincubation conditions Time: 15 min		Conditions during enzymic assay Time: 45 min			Picomol Testosterone reduced/45 min per mg protein
	NADPH	Inhibitor	17-Acetoxy-6-methylene-4-pregnene-3,20-dione	NADPH	Testosterone	
A	6×10^{-5}	5×10^{-7}	5×10^{-8}	5×10^{-4}	5×10^{-8}	0.92
	6×10^{-5}	5×10^{-7}	5×10^{-8}	5×10^{-4}	5×10^{-8}	0.96
B	3×10^{-5}	5×10^{-7}	5×10^{-8}	5×10^{-4}	5×10^{-8}	0.55
	3×10^{-5}	5×10^{-7}	5×10^{-8}	5×10^{-4}	5×10^{-8}	0.57
C	1.5×10^{-5}	5×10^{-7}	5×10^{-8}	5×10^{-4}	5×10^{-8}	0.94
	1.5×10^{-5}	5×10^{-7}	5×10^{-8}	5×10^{-4}	5×10^{-8}	0.77
D	8×10^{-6}	5×10^{-7}	5×10^{-8}	5×10^{-4}	5×10^{-8}	0.51
	8×10^{-6}	5×10^{-7}	5×10^{-8}	5×10^{-4}	5×10^{-8}	0.61
E	0	5×10^{-7}	5×10^{-8}	5×10^{-4}	5×10^{-8}	3.81
	0	5×10^{-7}	5×10^{-8}	5×10^{-4}	5×10^{-8}	4.05

Individual results of duplicate incubations are given.

Table 4. The Effect of Combining Enzyme Exposed to 17-Acetoxy-6-methylene-4-pregnene-3,20-dione and NADPH with Unexposed Enzyme on the Residual Enzymatic Activity

Preincubation conditions Time: 15 min			Conditions during enzymic assay Time: 45 min			Picomol testosterone* reduced/mg protein in 45 min
	NADPH	Inhibitor	Inhibitor	NADPH	Testosterone	
	M		M			
A	6×10^{-5}	5×10^{-7}	5×10^{-8}	5×10^{-4}	5×10^{-8}	0.30
B	0	0	0	5×10^{-4}	5×10^{-8}	2.49
C	0	0	5×10^{-8}	5×10^{-4}	5×10^{-8}	1.19
D	Aliquots of A & B diluted, combined and assayed together		5×10^{-8}	5×10^{-4}	5×10^{-8}	1.37

Table 5. Effect of Preincubation of Enzymes with 17-Acetoxy-6-methylene-4-pregnene-3,20-dione, NADPH and SOD** on 5α-Reductase Activity

Preincubation conditions Time: 15 min			Conditions during enzymic assay Time: 45 min			Picomol testosterone* reduced/mg protein in 45 min
	Inhibitor	NADPH	Inhibitor	Testosterone	NADPH	
A	5×10^{-7}	6×10^{-5}	5×10^{-8}	5×10^{-8}	5×10^{-4}	0.64
B	5×10^{-7}	0	5×10^{-8}	5×10^{-8}	5×10^{-4}	1.97
C	0	6×10^{-5}	5×10^{-8}	5×10^{-8}	5×10^{-4}	2.50
D	0	0	5×10^{-8}	5×10^{-8}	5×10^{-4}	2.33

* Each value is the average of duplicate incubations
** Concentration of superoxide dismutase is 10 γ/ml.

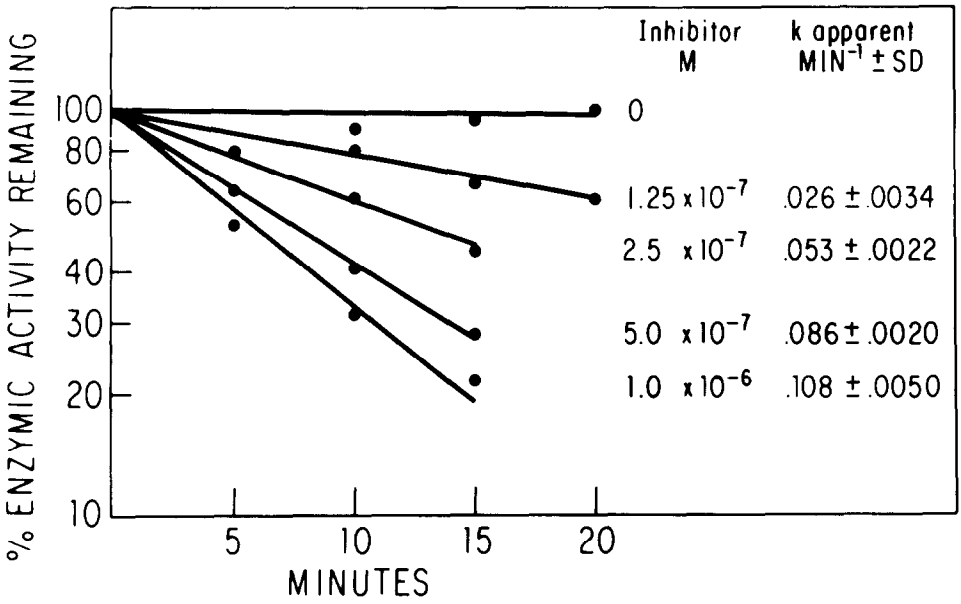

Figure 3. Time course of inactivation of 5α-reductase following incubation of the enzyme with AMPD and NADPH. The assay of residual enzymic activity following the incubation with AMPD utilized 5 x 10^{-6} M testosterone. Each point is the mean of 2 determinations.

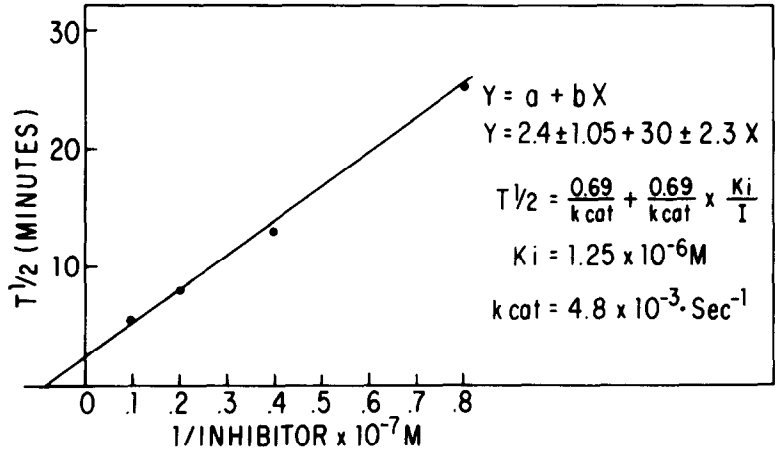

Figure 4. The dependence of the T½ of the inactivation of 5α-reductase upon the reciprocal of the concentration of inhibitor (AMPD).

being obtained from least squares linear regression analyses of the lines produced by plotting the natural logarithm of the concentrations against time. Figure 4 demonstrates the relationship between the reciprocals of these constants represented as their $T\frac{1}{2}$'s against the reciprocals of the concentrations of inhibitor. Also shown is their mathematical expression which represents the modification by Jung and Metcalf (11) of the original formulation by Kitz and Wilson (12) for this type of irreversible inhibition. It should be noted that the intercept on the ordinate is greater than zero by more than two standard deviations (see discussion). Finally, Figure 5

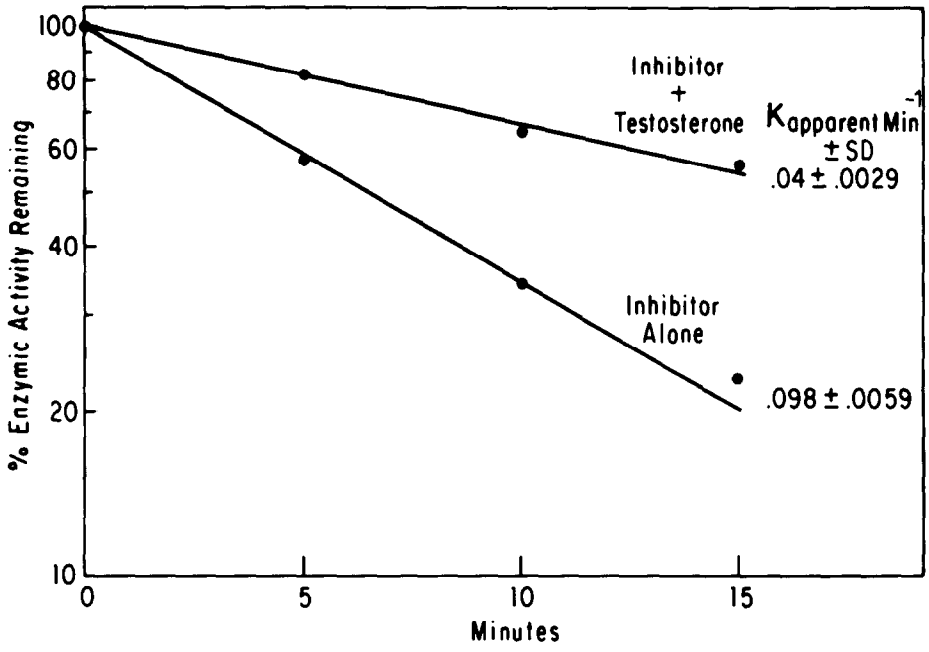

Figure 5. The effect of substrate (testosterone) on the inactivation of 5α-reductase by AMPD in the presence of NADPH. The concentrations of substrate and inhibitor were 5×10^{-7} M. Each point represents two determinations.

demonstrates that the rate constant of inactivation of the enzyme by AMPD (5×10^{-7} M) during preincubation is decreased when an equal amount of testosterone is present.

DISCUSSION

This work demonstrates that certain 4-pregnen-3-one derivatives bearing a 6-methylene substituent can serve as inhibitors of 5α-reductase. Structural requirements for enzyme inhibition appear to be fairly specific in that replacement of the methylene hydrogen atoms by halogen leads to virtual loss of enzyme-inhibitory activity. In addition, transfer of the conjugated methylenic linkage from the exocyclic C_6 position (cf. Figure 2B) to the endocyclic C_6-C_7 position (C) is again accompanied by loss of inhibitory activity. Introduction of a 17-acetoxy group into 6-methylene-progesterone leads to some enhancement of *in vitro* potency. As such 17-acetoxylation of pregnen-20-one derivatives usually leads to structures with oral activity (13), the efficacy of AMPD as a 5α-reductase inhibitor by the oral route can be predicted with considerable certainty. The importance of the 20-keto group is illustrated by comparing the activity of (F) with its 20β--hydroxy analog (G), which is clearly less active. Surprisingly, acetylation of the 20β-hydroxyl group (H) leads to some increase in potency. Methyl 6-methylene-3-oxo-4-pregnene-20α-carboxylate was weakly active.

We believe that irreversible inhibitors of 5α-reductase offer therapeutic advantages over competitive inhibitors in the treatment of androgen-dependent prostatic carcinoma. It was consequently important to establish whether, as predicted in the Introduction,

17-acetoxy-6-methylene-4-pregnene-3,20-dione (AMPD) can function as an enzyme inhibitor of the k_{cat} type. Unfortunately, the use of such standard techniques as prolonged dialysis of the enzyme inhibitor complex to establish irreversibility is not feasible in this instance because of the known instability of the enzyme (9). We have therefore utilized a preincubation technique which provides information on the potential for such compounds to act as irreversible inhibitors.

When an enzyme preparation able to reduce 4.36 picomoles testosterone/45 min/mg protein, or 3.14 picomoles testosterone/45 min/mg protein in the presence of 5×10^{-8} M of AMPD (Table 1, expts E and F) was preincubated with 5×10^{-7} M AMPD and NADPH for 15 min, diluted (x 10) to bring the concentration of the inhibitors to 5×10^{-8}M, and assayed in the usual way, the reducing capacity of the enzyme was found to have dropped to 0.71 picomoles testosterone/45 min/mg protein (Table 1, expt. A). Identical preincubation of the competitive inhibitor progesterone or the substrate testosterone, in contrast, did not show a corresponding loss in potency of the enzyme (Table 2).

The possibility that preincubation of the enzyme with NADPH and AMPD as in Expt A, Table 1, had led to the formation of a metabolic product with enhanced potency as an enzyme inhibitor was excluded by the following experiments employing the enzyme assayed in B, Table 4. A tube containing the enzyme, NADPH and 5×10^{-7}M AMPD was preincubated as before (cf. A, Table 4). An aliquot of the incubated mixture was then mixed with an equal aliquot of the untreated enzyme (cf. C, Table 4) and the combination preincubated, diluted x 10 and assayed.

The enzyme activities determined in the combination experiment (D, Table 4) approximated to the sum of the component enzyme activities (A and B, Table 4) when assayed in the presence of 5×10^{-8} M AMPD. These results are not compatible with formation of a more inhibitory metabolite, which possibility can be excluded from further consideration.

As the enzyme preparation had been obtained from isolated prostatic nuclei, it would probably contain cytochrome P-450 which, in the presence of NADPH, could have inactivated the reductase through an uncoupling phenomenon involving the combination of cytochrome P-450 with NADPH and the methylenic steroid inhibitor with formation of superoxide which could then inactivate the enzyme in a nonspecific manner. The feasibility of such enzyme inactivation has been demonstrated with other substrates (14). The data in Table 5 showing that superoxide dismutase has no effect upon enzyme inactivation eliminates this possibility. The foregoing data suggest that preincubation of the enzyme with NADPH and AMPD leads to irreversible inactivation of the enzyme.

Kinetic studies were next undertaken in order to further delineate the postulated mechanism. When the enzyme was preincubated together with NADPH and the steroid inhibitor, AMPD, and the residual enzymic activity assayed at specified times, the time course of inactivation followed pseudo-first order kinetics (Fig. 3). This is in accord with the postulate that the inhibition evoked by such preincubation exposure is irreversible. When these rate constants were plotted (as the $T_{\frac{1}{2}}$'s) against the reciprocal of the inhibitor

concentrations, a straight line was obtained with a positive intercept on the Y axis, indicating a saturation phenomen. These data are in accord with the idea that the interaction of the inhibitor with the enzyme shows two phases. The first is a reversible combination of the enzyme and inhibitor with a Ki of 1.25×10^{-6} M. The enzyme inhibitor complex then undergoes an irreversible reaction, rendering the enzyme inert. The rate constant for this step (k_{cat}) has been determined to be $4.8 \times 10^{-3} sec^{-1}$. This type of mechanism would require that preincubation of the enzyme with the inhibitor and its substrate (together with its cofactor in this case) demonstrate a decreased rate of enzymic activation. This is shown to be the case (see Figure 5).

ACKNOWLEDGEMENTS

This work was supported by Research Grant No. 1 R26 CA27374 of the National Prostatic Cancer Project (NCI). Technical assistance by Lynette Richelo, Amani Tantawi and Douglas Rockett is gratefully acknowledged. We thank the Upjohn Company, G. D. Searle and Company and Mead Johnson and Company for gifts of steroids.

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