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Aromatic esters of progesterone as 5α-reductase and prostate growth inhibitors

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Abstract

The aim of this study was to determine the biological activity of 4 steroidal derivatives (**9a**, **9b** and **10a**, **10b**) prepared from the commercially available 17α acetoxyprogesterone, where **9a**, **9b**, have the Δ^4-3-oxo structure and **10a** and **10b** an epoxy group at C-4 and C-5.

These steroids were tested as inhibitors of 5α-reductase enzyme, which is present in androgen-dependent tissues and converts testosterone to its more active reduced metabolite dihydrotestosterone.

The pharmacological effect of these steroids was demonstrated by the significant decrease of the weight of the prostate gland of gonadectomized hamsters treated with testosterone plus finasteride or with steroids **10a** and **10b**. For the studies *in vitro* the IC_{50} values were determined by measuring the steroid concentration that inhibits 50% of the activity of-5α-reductase. In this study we also determined the capacity of these steroids to bind to the androgen receptor present in the rat prostate cytosol.

The results from this work indicated that compounds **9a**, **9b**, **10a**, and **10b** inhibited the 5α reductase activity with IC_{50} values of 360, 370, 13 and 4.9 nM respectively. However these steroids did not bind to the androgen receptors since none competed with labeled mibolerone. Steroid **10b**, an epoxy steroidal derivative containing bromine atom in the ester moiety, was the most active inhibitor of 5α-reductase enzyme, present in human prostate homogenates with an IC_{50} value of 4.9 nM and also showed *in vivo* pharmacological activity since it decreased the weight of the prostate from hamsters treated with testosterone in a similar way as finasteride.

Keywords: *Human prostate, aromatic progesterone esters, androgen receptor, 5α-reductase inhibitors, 5α-reductase, inhibition*

Introduction

Androgen target tissues, contain the NADPH-dependent 5α-reductase enzyme (EC 1.399.5) which is capable of reducing testosterone (T) **1** to dihydrotestosterone (DHT) **2** (Figure 1) [1]. The product of this enzyme is accumulated in the nucleus of responsive cells, such as those of the animal prostate [2]. Males with steroid 5α-reductase 2 deficiency have a biochemical defect for the formation of dihydrotestosterone in the embryo [3]; which in turn leads to a developmental defect in the formation of the external genitalia and the prostate. The fact that DHT mediates the growth of prostate gland and in individuals who lack 5α-reductase enzyme fail to develop a normal prostate, led to the development of therapeutic inhibitors for this enzyme. These drugs are used in the treatment of androgen dependent diseases such as: acne, male pattern baldness, benign prostatic hyperplasia and prostate cancer.

The most extensively studied 5α-reductase inhibitors are: finasteride **3** (F) "Proscar" (Figure 1) and dutasteride **4** "Avodart" [4]. Compound 4-MA **5** (Figure 1) another 4-azasteroid derivative with 5α-reductase inhibition activity, has shown a very low affinity for androgen receptors and thus was not expected to produce undesirable anti-androgen effects such as: impotence, muscle growth impairment

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Figure 1. Structures of the reference compounds **1** testosterone, **2** dihydrotestosterone, **3** finasteride, **4** dutasteride, **5** 4-MA, **6** mibolerone.

and gynecomastia [5]. However, this drug was subsequently shown to be an inhibitor of another steroid-metabolizing enzyme (3β-hydrosteroid dehydrogenase) [6] and causes hepatotoxicity.

Finasteride **3** is a potent inhibitor of both rat and human 5α-reductase enzyme activities in an irreversible way. This compound is a time-dependent inactivator of type 2 5α-reductase enzyme and together with dutasteride **4** are the only steroids approved for the treatment of benign prostatic hyperplasia in the United States [7].

Previously, we demonstrated that the steroidal C-5, C-6 epoxy derivative of 16-pregnen-20-one compound with an ester moiety in C-3, inhibited the 5α-reductase activity and also the growth of the prostate gland in castrated animals, treated with testosterone without any toxicological effect [8]. Therefore on the basis of these results, it was of interest to ascertain if the progesterone epoxydized derivatives: having at C-17 an ester moiety with bromine atom in para position produce this effect as compared with the non-epoxydized compounds.

Materials and methods

Chemical and radioactive materials

Solvents were laboratory grade or better. Melting points were determined on a Fisher Johns melting point apparatus and are uncorrected. ^{1}H-NMR and ^{13}C-NMR were taken on Varian Inova-300 and VRX-400 respectively. The starting material 17α-acetoxyprogesterone (**7**) was purchased from Sigma-Aldrich (ST Louis, MO). Chemical shifts are given in ppm relative to that of Me_4Si ($\delta = 0$) in $CDCl_3$ (the abbreviations of signal patterns are as follows: s, singlet; d, doublet t, triplet, m, multiplet). Mass spectra were obtained with a HP5985-B spectrometer. IR spectra were recorded on a Perkin-Elmer 1605 spectrometer. Purity of the final compounds was measured using Agilent 1100 series high-performance liquid chromatography (HPLC) system with UV detector at 254 nm.

(1, 2, 6, 7-^{3}H) Testosterone [^{3}H] T specific activity: 95 Ci/mmol and Mibolerone (17α-methyl- ^{3}H) ([^{3}H] MIB, **6**, Figure 1) specific activity 70-87 Ci/mmol were provided by Perkin Elmer Life and Analytical Sciences. (Boston, MA). Radioinert T, 5α-dihydrotestosterone and MIB were supplied by Steraloids (Wilton, NH, U.S.A.). Sigma Chemical Co. (ST. Louis, Mo) provided NADPH. Finasteride was obtained by extraction from Proscar® (Merck, Sharp & Dohme). The tablets were crushed, extracted with chloroform and the solvent was eliminated in vacuum; the crude product was purified by silica gel column chromatography.

Synthesis of steroidal derivatives

17α-Hydroxypregn-4-ene-3, 20-dione, ***8***. A solution of steroid **7** (1 g, 2.67 mmol) in methanol (60 mL) was allowed to reflux for 30 min. An aqueous sodium hydroxide (10%) solution (20 mL) was added and allowed to reflux for an additional 60 min. The solvent was eliminated in vacuum and ice (20 g) was added. The precipitated crystalline product was filtered and dried in vacuum. It was recrystallized from methanol, yield 0.64 g (1.93 mmol) 71.7% of pure product **8**, mp 217-218°C. UV (nm) 240 (ε:15,600). IR (KBr) cm^{-1} 3424, 1703, 1664, 1242. ^{1}H-NMR ($CDCl_3$) δ: 0.77 (3 H, s), 1.19 (3 H, s), 2.28 (3 H, s), 3.05 (1H, s), 5,74 (1H,s). ^{13}C-NMR ($CDCl_3$) δ: 13.85 (C-18), 17.39 (C-19), 23.78 (C-21), 123.79 (C-4), 171.02 (C-5), 199.36 (C-3 carbonyl), 199. 45 (C-20 carbonyl). The DEPT spectrum confirmed the

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presence of 3 methyl, 8 methylene and 4 methine carbon atoms. HPLC purity:99%. FAB-MS (m/z) 331 ($M^+ + H$) 100%; high-resolution FAB-MS (m/z) calcd for ($C_{21}H_{30}O_3$) 331.2195 ($M^+ + H$), found 331.2135.

Preparation of compounds ***9a–b***. A solution containing the corresponding acid (3.03 mmol), p-toluenesulfonic acid (100 mg, 0.53 mmol), trifluoroacetic anhydride (0.85 mL, 6 mmol) in methylene chloride (3 mL) was stirred at room temperature until complete dissolution of the acid (about 1 h). The steroidal alcohol **8** (500 mg, 1.51 mmol) was added and the reaction mixture was stirred for additional 3 h. A saturated aqueous solution of sodium bicarbonate (10 mL) was added and the reaction mixture was thrice extracted with chloroform. The organic phase was washed with water, dried over anhydrous sodium sulfate and the solvent was removed in vacuum. The crude product was purified by silica gel (70-30 mesh ASTM) column (size 50 cm long and 0.9 cm wide) using a solvent system of hexane:ethyl acetate (85:15).

17α-Benzoyloxypregn-4-ene-3, 20-dione ***9a***. Yield 220 mg (0.51 mmol) 33.5%, mp 185-187°C. UV (nm) 232 (ε, 12,400). IR (KBr) cm^{-1} 3064,1716, 1665, 1600. ^{1}H-NMR ($CDCl_3$) δ: 0.76 (3H, s), 1.23 (3H, s), 2.09 (3H, s), 5.77 (1H,s), 7.50 (2H, t, J = 7.8 Hz), 7.63 (1H, t, J = 7.4), 8.05 (2H, d, J = 7.2),. ^{13}C-NMR ($CDCl_3$) δ: 14.48 (C-18), 17.39 (C-19), 26.35 (C-21), 124.02 (C-4), 128.60 (C-meta aromatic ring), 129.62 (C-ortho aromatic ring), 133.51 (C-para aromatic ring), 165.91 (ester carbonyl), 170.54 (C-5), 199.27 (C-3 carbonyl), 203.82 (C-20 carbonyl). The DEPT spectrum confirmed the presence of 3 methyl, 8 methylene and 9 methine catbon atoms. HPLC purity: 98%. FAB-MS (m/z) 435 ($M^+ + H$) 100%; high resolution FAB-MS (m/z) calcd for ($C_{28}H_{34}O_4$) 435.2457 ($M^+ + H$), found 435.2489.

*17α-*p*-bromobenzoyloxypregn-4-ene-3, 20-dione* ***9b***. Yield 216 mg (0.42 mmol) 27.8%, mp 195-197°C. UV (nm) 243 (ε, 12,200). IR (KBr) cm^{-1}: 3087, 1709, 1667, 1614. ^{1}H-NMR ($CDCl_3$) δ: 0.76 (3H, s), 1.26 (3H, s), 2.08 (3H, s), 5.77 (1H, s), 7.63 (2H AA′ BB′, t, J = 9.2 Hz), 7.89 (2H,d, J = 9.2 Hz). ^{13}C-NMR ($CDCl_3$) δ: 14.50 (C-18), 17.40 (C-19), 26.43 (C-21), 124.09 (C-4), 128.75 (C′4 aromatic ring), 131.09 (C′3 aromatic ring), 132.01 (C′2 aromatic ring), 165.26 (ester carbonyl), 170.36 (C-5), 199.19 (C- carbonyl), 203.54 (C-20 carbonyl). The DEPT spectrum confirmed the presence of 3 methyl, 8 methylene and 8 methine carbon atoms. HPLC purity: 98%. FAB-MS (m/z) 513 ($M^+ + H$) 98.9%, 515 ($M^+ + H$) 100%; high resolution FAB-MS (m/z) calcd for ($C_{28}H_{33}Br^{79}O_4$) 513.1562 ($M^+ + H$), found 513.1545, calcd for ($C_{28}H_{33}Br\ ^{81}O_4$) 515.1542. ($M^+ + H$), found 515.1587.

Preparation of compounds ***10a–b***. To a solution of steroid **9a** or **9b** (100 mg) in methylene chloride (1.5 mL) and methanol (3 mL) was added a 10% aqueous sodium hydroxide solution (0.06 mL) and 30% aqueous hydrogen peroxide (0.12 mL). The reaction mixture was stirred for 12 h at room temperature. The organic solvents were eliminated in vacuum and ice (2 g) was added; the precipitated product was isolated by filtration. The crude product was recrystallized from methanol.

17α-Benzoyloxy-4α, 5α-epoxypregn-4-ene-3, 20-dione ***10a***. Yield 60 mg (0.14 nM) 57.0%, mp 206-208°C. UV (nm) 231 (ε, 12,800). IR (KBr) cm^{-1}: 2947, 1716, 1600, 1290. ^{1}H-NMR ($CDCl_3$) δ: 0.73 (3H, s), 1.20 (3H, s), 2.09 (3H, s), 3.04 (1H, s), 7.54 (2H, m), 7.64 (2H, m), 8.07 (2H, m). ^{13}C-NMR ($CDCl_3$) δ: 14.56 (C-18), 18.98(C-19), 24.08 (C-21), 62.73 (C-4), 70.11 (C-5), 128.69 (C-meta aromatic ring), 129.65 (C-ortho aromatic ring), 133.58 (C-para aromatic ring), 166.08 (ester carbonyl), 203.77 (C-3 carbonyl), 206.99 (C-20 carbonyl). The DEPT spectrum confirmed the presence of 3 methyl, 8 methylene and 8 methine carbon atoms. HPLC purity: 98%. FAB-MS (m/z) 451 ($M^+ + H$) 100%; high resolution FAB-MS calcd for ($C_{28}H_{34}O_5$) 451.2406 ($M^+ + H$), found 451.2465.

17α-p-Bromobenzoyloxy-4α, 5α-epoxypregn-4-ene-3, 20-dione ***10b***. Yield 51 mg (0.096 mmol) 49.5%, mp 235-238°C. UV (nm) 247 (ε, 12,200). IR (KBr)) cm^{-1}: 2951, 1721, 1589. ^{1}H-NMR ($CDCl_3$) δ: 0.73 (3H, s), 1.20 (3H, s), 2.08 (3H, s), 3.04 (1H, s), 7.68 (2H, d, J = 8.4 Hz), 7.90 (2H AA′ BB′, d, J = 8.4 Hz). ^{13}C-NMR ($CDCl_3$), δ: 14.55 (C-18),18.97 (C-19), 24.05 (C-21), 62.74 (C-4),70.12(C-5), 127.95 (C-para aromatic ring), 131.11 (C-meta aromatic ring), 132.10 (C-ortho aromatic ring), 165.42 (ester carbonyl), 203.54 (C-3 carbonyl), 207.01 (C-20 carbonyl). The DEPT spectrum confirmed the presence of 3 methyl, 8 methylene and 8 methine carbon atoms. HPLC purity: 98%; FAB-MS (m/z) 529($M^+ + H$) 99%, 531($M^+ + H$) 100%; high resolution FAB-MS calcd for ($C_{28}H_{33}Br^{79}O_5$) 529.1511 ($M^+ + H$), found 529.1563, calcd for ($C_{28}H_{33}Br^{81}O_5$) 531.1491 ($M^+ + H$), found 531.1445.

Biological activity of the new compounds

Human prostate from cadavers was kindly provided by Dr Avissai Alcántara from The Department of Pathology, The General Hospital (SS) in Mexico City, and stored at −70°C. Frozen human prostate was thawed on ice and minced with scissors. Unless specified, the following procedures were carried out at 4°C.

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Animals and tissues. Adult male golden hamsters (150-200 g) and Wistar rats (500 g) were obtained from the Metropolitan University in Xochimilco, Mexico. The hamsters were gonadectomized under pentobarbital anesthesia 30 days prior to the experiments and were maintained in a room with controlled temperature (22°C) and light- dark periods of 12 h. Food and water were provided *ad libitum* until the hamsters were sacrificed with CO_2. The Institutional Care and Use Committee of the Metropolitan University of Mexico (UAM) approved this protocol. The experiment with the gonadectomized hamsters was carried out on 8 groups of 4 hamsters/experiment, which were selected at random. Animals were kept at room with controlled temperature (22°C) and light- dark periods of 12 h. Food and water were provided *ad libitum*.

In order to determine the binding of steroids **9a**, **9b**, **10a**, **10b** to the androgen receptors, the rats were gonadectomized 48 h before the experiment. For these studies we used rat's prostate, which were bigger than the hamster's and yielded more cytosol. There is no difference in the binding to mibolerone between hamsters or rats prostate's androgen receptors present in cytosol.

The prostate of the rats was removed, blotted, weighed and soaked in cold TEMD (40 mM tris-HCl, 3 mM EDTA and 20 mM sodium molybdate, dithiothreitol 0.5 mM, 10% glycerol at pH 8) prior to their use. Unless specified, all procedures were carried out in ice bath. Tissues used were homogenized with a tissue homogenizer (Teckmar, Cincinnati, OH) in one volume of buffer TEMD plus protease inhibitors (2 mM PMSF, 10 μg/mL antipain, 5 mM leupeptin [9] in ice bath with a tissue homogenizer. Homogenates were centrifuged at 140,000 × g for 60 min [10] in a SW 60 Ti rotor (Beckman Instruments, Palo Alto, CA).

The cytosolic fraction obtained from the supernatant liquid of the rat prostate homogenate described above, was stored at −70°C. Prostatic cytosol proteins (6 mg of protein in 200 μL) were determined by the Bradford method [11].

In vivo *experiments*

Subcutaneous treatment with steroids. For the daily subcutaneous injections, 2 mg/Kg of the steroids **9a**, **9b**, **10a**, **10b** were dissolved in 200 μL of sesame oil and administered for 6 days to the gonadectomized hamsters, together with 1 mg/Kg of testosterone. Three groups of gonadectomized animals were kept as control: one was injected with 200 μL of sesame oil, the second one with 1 mg/Kg of testosterone and the third one with T plus 1 mg/Kg of finasteride for 6 days. After the treatment, the animals were sacrificed with CO_2. In addition, the prostate gland from the animals of each group was dissected and weighed. The experiments were carried out in two different occasions for each group (4 hamsters each) treated with a steroid and for the control groups (4 hamsters each). The results from this study were analyzed using one-way analysis of variance and the Dunnett's test to compare means, utilizing the JMP IN 7 software.

In vitro *experiments*. Human prostate was homogenized in 2 volumes of medium A (20 mM sodium phosphate, pH 6.5 containing 0.32 M sucrose, 0.1 mM dithiothreitol Sigma-Aldrich, Inc) with a tissue homogenizer. Homogenates were centrifuged at 1,500 × g for 20 min [12] in a SW 27 rotor (Beckman instruments, Palo Alto, CA). The pellets were suspended in medium A and kept at −70°C. The suspension, 5 mg of protein/mL for human prostate, determined by the Bradford method [11] was used as source of 5α-reductase.

Determination of 5α-reductase activity. The enzyme 5α-reductase was assayed as previously described [12,13] The reaction mixture contained a final volume of one mL: 50 μL of DMSO, 1 mM dithiothreitol, sodium phosphate buffer 40 mM, at pH 6.5 for human prostates, 2 mM, NADPH, 2 nM [1,2,6,7-^{3}H]T [24]. The reaction in duplicate was started when it was added to the enzymatic fraction (237 (g protein in a volume of 80 (L) incubated at 37°C for 60 min [12] and stopped by mixing with 1 mL of dichloromethane; this was considered as the end point. Incubation without tissue was used as a control. The fraction of dichloromethane was separated and the extraction was repeated 4 more times. The combined extract was evaporated under a nitrogen stream to dryness and suspended in 50 (L of methanol that was spotted on HPTLC Keiselgel 60 F_{254} plates (Merck, Darmstadt, Germany). T and DHT were used as carriers and were applied in different lanes on both lateral sides of the plates (T, T + DHT and DHT). The plates were developed in chloroform-acetone (9:1) and were air-dried; the chromatography was repeated 2 more times. The steroid carriers were detected using phosphomolibdic acid reagent (DHT) and T with an UV lamp (254 nm). After, the plates were segmented in areas of one cm each, they were cut off and the strips soaked in 5 mL of Ultima Gold (Packard). The radioactivity was determined in a scintillation counter (Packard tri-carb 2100 TR). The radioactivity content in the segment corresponding to T and DHT carriers was identified. The radioactivity that has identical chromatographic behavior as the DHT standard was considered as the DHT transformation. Control incubations, chromatography separations and identifications, were carried out in the same manner as described above except that the tubes did not contain tissue. The DHT transformation yields were calculated from the strips, taken into account the entire radioactivity in the plate.

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Table I. Weight of prostate glands ± standard deviation from animals receiving s.c. treatments for 6 days of differenct compounds.

Treatment	Weight of prostate (mg)
Intact	224.8 ± 35
Control	56.3 ± 12.5*
T	125.8 ± 27
T + F	80.1 ± 10.4*
T + **9a**	89.2 ± 10.5
T + **9b**	85.85 ± 7.6
T + **10a**	80.0 ± 11.1*
T + **10b**	74.0 ± 9.6*

F, finasteride; * Significant differences between controls and treated groups ($P < 0.05$)

*Determination of 50% inhibitory concentration of steroids **9a, 9b, 10a, 10b** in human prostatic 5α-reductase*. In order to calculate the IC_{50} values (the concentration of steroids **9a**, **9b**, **10a**, **10b** or finasteride required to inhibit 5α-reductase activity by 50%), six series of tubes containing increasing concentrations of these steroids (10^{-11}-10^{-3} M) in 50 μL of DMSO, were incubated, extracted and purified in duplicate, as described above. The radioactivity content in the segments corresponding to T and DHT carriers was identified. The fraction that has identical chromatographic behavior as the DHT standard was considered as the DHT transformation in the presence of the tested compounds. Control incubations, chromatography separations and identifications, were carried out in the same manner as described above except that these tubes did not contain tissue. The DHT transformation yields were calculated from the strips, taken into account the entire radioactivity in the plate.

Competitive studies. For competitive studies, tubes containing 1 nM of [^{3}H] MIB plus a range of increasing concentrations (1×10^{-10}-4×10^{-7} M) of cold MIB **6** (Figure 1) and **9a**, **9b**, **10a**, **10b** in ethanol or chloroform, or in absence of the competitor were prepared [10] Incubates also contained 200 nM triamcinolone, in ethanol, (Sigma) to prevent interaction of MIB with glucocorticoid receptors and progesterone receptors, the solvent was evaporated.

Aliquots of 200 μL of prostate cytosol were added and incubated in the presence of 300 μL of TEMD buffer containing protease inhibitors [9] (duplicate) for 18 h at 4°C in the tubes as previously described. After incubation 0.27 mL saturated ammonium sulfate in TEMD buffer (35%) was added [14]. The mixture was further incubated for 1 h with occasional shaking to facilitate the precipitation of the [^{3}H] MIB-complex. The precipitate was collected by centrifugation at 10,000 × g, 10 min and the pellet was redissolved in 0.5 mL of TEMD and mixed with 0.5 of 0.1% dextran-coated 1% charcoal in TEMD buffer. The mixture was incubated for 40 min at 4°C. To prepare the dextran-coated charcoal mixture, the dextran was agitated for 30 min before adding the charcoal to the mixture. The tubes were vortexed and immediately centrifuged at 800 × g for 10 min to pellet the charcoal; aliquots (600 μL) were taken and

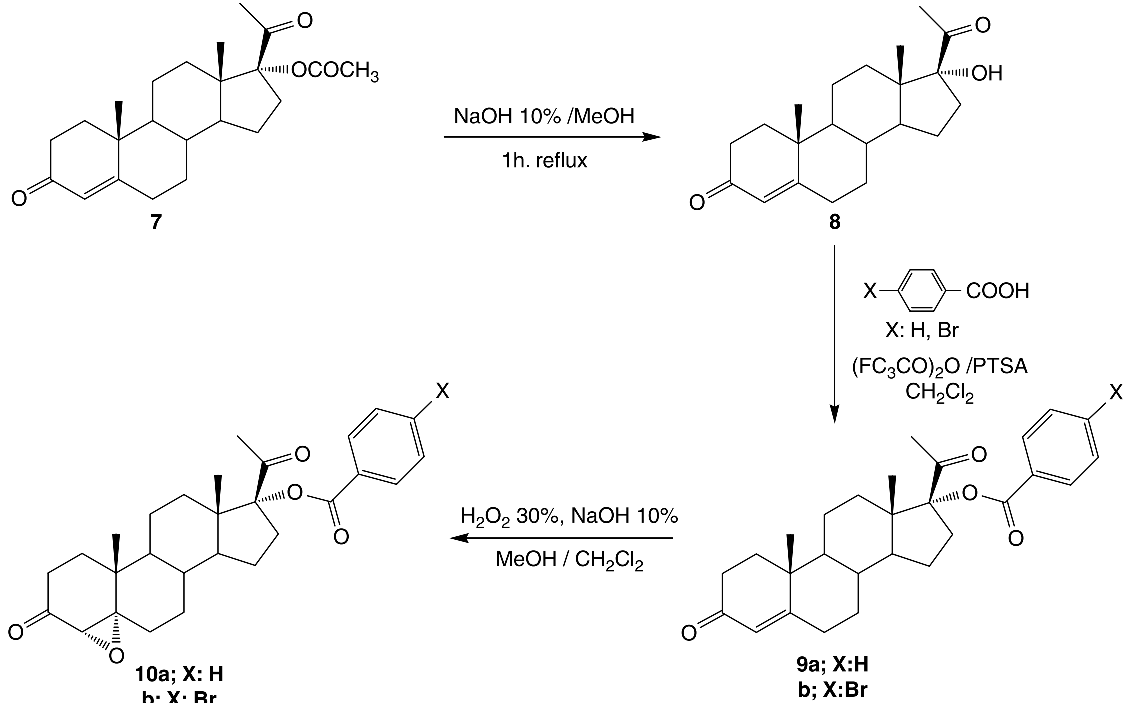

Figure 2. Synthesis of steroidal compounds **9a, 9b, 10a, 10b** and their preparation from the commercially available 17α-acetoxyprogesterone.

submitted for radioactive counting. The IC_{50} of each compound was calculated according to the plots of concentration versus percentage of binding.

Results

In vivo *experiments*

Weight of the prostate. After castration, the weight of the hamster prostate decreased ($p < 0.05$) compared to the normal glands. Treatment with vehicle alone did not change this condition, whereas s.c. injections of 1 mg/Kg of T for 6 days significantly increased ($p < 0.05$) the weight of these glands in castrated male hamsters (Table I). When T (1 mg/Kg) and finasteride or compounds **9a**, **9b**, **10a**, **10b** (2 mg/Kg) were injected together, the weight of the prostate and seminal vesicles decreased as compared to those of T-treated animals (Table I). However non significant difference in the prostate weighs was observed between hamsters treated with **10a** and **10b**.

In vitro *experiments*

The *in vitro* biological activity of steroids **9a**, **9b**, **10a**, **10b** (Figure 2) was determined in human prostate gland homogenized and centrifuged to obtain the prostatic enzyme fraction. The activity of human 5α-reductase was assessed incubating the enzymatic fractions with 2 nM [^{3}H]T. The dichloromethane extracts from human prostates were subjected to TLC analysis. The radioactive zone that had identical chromatographic behavior as the standard T (R_f value of 0.56) corresponds to 70% of the accounted radioactivity in the plate. The radioactivity contained in the zone corresponding to DHT standard (R_f value of 0.67) of the experimental chromatogram was identified as the transformed DHT and corresponds to 27% of the total radioactivity accounted in the plate. This result was considered to be 100% of the activity of 5α-reductase for the development of inhibition plots. Unmodified [^{3}H] T was identified (Rf value of 0.56) from control incubations which did not contain tissue and had identical chromatographic behavior as the non labeled standard (identified by UV lamp, 254 nm). The radioactivity contained in the zone corresponding to DHT standard (R_f value of 0.67) of the control chromatogram was of 1% of the total radioactivity accounted in the plate and was considered as an error; it was subtracted from the experimental chromatograms.

Concentration of the new compounds that inhibits 50% of activity of 5α-reductase in human prostate. The concentration of finasteride and compounds **9a**, **9b**, **10a**, **10b** required for inhibiting 5α-reductase activity by 50% (IC_{50}) were determined from the inhibition plots using different concentrations of the steroids and are shown in Table II. Finasteride exhibited an IC_{50} value of 8.5 nM, whereas that for compound **10b** was lower (4.9 nM)., on the other hand steroid **10a** exhibited a slightly higher IC_{50} than that of finasteride (13 mM).These data indicate that **10b** has a higher inhibitory activity than finasteride, the compound of choice for the treatment of benign prostatic

Table II. The IC_{50} values for finasteride and the synthesized steroids **9a, 9b, 10a, 10b** with human prostate 5α−reductase enzyme. They represent the concentration of the steroid that inhibits 50% of 5α−reductase activity and were determined as described in the Experimental section.

Compound	IC_{50} [nM]
3 (NHC(CH$_3$)$_3$)	8.5
9a	360
9b (Br)	370
10a	13
10b (Br)	4.9

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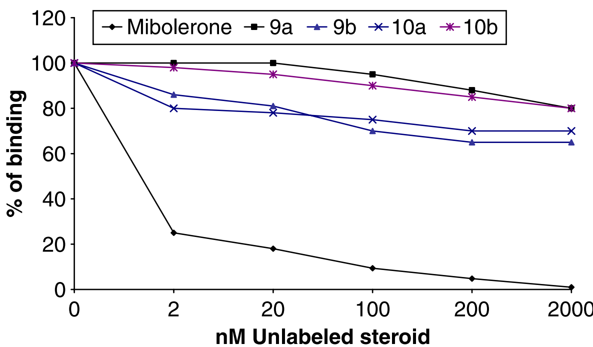

Figure 3. Inhibition of [^{3}H] mibolerone binding to the androgen receptors by unlabeled steroids, as described in Methods section.

hyperplasia. On the other hand, compounds **9a, 9b** showed a lower inhibitory activity for the enzyme 5α-reductase (higher IC_{50} values, Table II).

Competitive studies. The IC_{50} value of each compound was calculated according to the plots of concentration versus percentage of binding (Figure 3). The IC_{50} value of MIB was in the order of 1 nM, whereas steroids **9a**, **9b**, **10a**, **10b** failed to inhibit the radiolabeled MIB binding to the androgen receptor (Figure 3).

Discussion

In this study we report the activity of four novel pregnane derivatives **9a**, **9b**, **10a**, **10b** as 5α-reductase inhibitors (Figure 2). Compounds **9a**, **9b**, **10a**, **10b** exhibited *in vitro*, an inhibitory activity for the enzyme 5α-reductase at pH 6.5; therefore these compounds are inhibitors of 5α-reductase type 2. It is well known that at pH 6.5 and a concentration of 2 nM of T, human prostate 5α-reductase type 2 isozyme, shows higher affinity for T than at pH 5 [12]. On the other hand these steroids did not inhibit MIB binding to the androgen receptor, therefore compounds **9a**, **9b**, **10a**, **10b** did not bind to the androgen receptors.

In this work, we also demonstrated that compounds containing an epoxy group in the molecule (**10a, 10b**) produced a significant decrease of the prostate weight in castrated hamsters treated with T as compared to the non epoxydized steroids. On the other hand steroids **9a** and **9b** (lacking an epoxy group) decreased also the weight of prostate but in a non statistically significant way. This fact could be explained by considering the lower affinity of the nonepoxidated steroids (**9a** and **9b**) for the 5α-reductase; steroids **10a** and **10b** having and epoxy ring (higher polarity) show a higher association to 5α-reductase enzyme due to a dipole-dipole interaction and as a result of this exhibited a lower weight for the prostate

The *in vivo* activity of steroids **10a** and **10b** seems to be the same, since no significant differences in the weight of prostate is observed between gonadectomized hamsters treated with T plus **10a** or T plus**10b**. However both of them decrease the hamster's prostate weight as compared with the gonadectomized hamster treated with T.

On the other hand the presence of a bromine atom (**10b**) in the ester side chain in the epoxydized steroid seems to improve the *in vitro* inhibition of the 5α-reductase activity as determined by the decrease in the IC_{50} values as compared to steroid **10a** (without bromine atom). These results could be explained on the basis that compound **10b**, with a bromine atom in the aromatic ring has a higher lipophilicity than **10a**, which could enhance the association of the steroid with the 5α-reductase amino acid residues lining the enzyme active site and thus shows a higher inhibitory activity in a similar way as observed for finasteride which contains a hydrophobic amido substituent N-t-butyl group, which interacts with amino acids residues lining the active site [7].

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