Journal of Enzyme Inhibition and Medicinal Chemistry, August 2006; 21(4): 371–378

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healthcare

New progesterone derivatives as inhibitors of 5α-reductase enzyme and prostate cancer cell growth

MARISA CABEZA[1], EUGENE BRATOEFF[2], IVONNE HEUZE[1], ARELY ROJAS[2], NAYELY TERÁN[2], MARTHA' OCHOA[2], MA TERESA RAMÍREZ-APAN[3], ELENA RAMÍREZ[2], VICTOR PÉREZ[2], & ISABEL GRACIA[2]

[1]*Department of Biological Systems and Animal Production Metropolitan University-Xochimilco, Mexico D. F., Mexico,*
[2]*Department of Pharmacy, Faculty of Chemistry, National University of Mexico City, Mexico D. F., Mexico, and*
[3]*Institute of Chemistry, National University of Mexico City, Mexico D. F., Mexico*

(Received 30 November 2005; in final form 15 March 2006)

Abstract

In this study we report the synthesis and pharmacological evaluation, *in vivo* as well as *in vitro*, of four new progesterone derivatives **4–7**. The evaluation *in vivo* was carried out on gonadectomized male hamsters that were injected subcutaneously daily with 1 mg/Kg of testosterone (T) and/or 1 mg/Kg of finasteride, or with 2 mg/Kg of the novel compounds. It was observed that when testosterone (T) and finasteride or compound **4** were injected together, the weight of the prostate decreased significantly as compared to that of testosterone-treated animals. Compounds **5–7** did not show any *in vivo* activity.

The 5α-reductase inhibitory activity of the novel compounds was determined *in vitro* using human prostate homogenates; the steroids **4–7** inhibited the 5α-reductase activity with IC_{50} values lower than that for the reference compound finasteride. **3**.

The effect of compounds **4–7** on the growth of lymphocytes and prostate cancer culture cells line was that steroid **4** inhibited the growth of both cells lines at a concentration of 50 μM and showed a cytotoxic effect whereas compounds **5–7** showed a much lower inhibition**.** Nevertheless steroids **4–7** didn't exhibit any toxic effects *in vivo* since the animals remained alive during the six days of treatment.

Introduction

Steroid 5α-reductase is a NADPH dependent enzyme responsible for the reduction of testosterone (T) **1** into the more potent androgen dihydrotestosterone (DHT) **2** (Figure 1) [1]. This enzyme is located in androgen-dependent tissue such as prostate, seminal vesicles, epididymis and other reproductive tissues [1]. It has been determined that DHT **2** interacts more efficiently with the androgen receptors than its precursor T **1** [2] and has been implicated in the pathogenesis of prostate cancer, benign prostatic hyperplasia (BPH), acne and male pattern baldness [2–4]. This fact indicates that both the 5α-reductase enzyme and DHT **2** play important physiological and pathological roles in human males. Therefore, the inhibition of DHT formation by inhibition of 5α-reductase enzyme is a logical treatment for androgen-dependent afflictions [5].

The most extensively studied class of 5α-reductase inhibitors are the 4-azasteroids [6–8], which include the drug finasteride **3**. This compound is the first 5α-reductase inhibitor approved in the USA for the treatment of BPH. In humans, finasteride decreases prostatic DHT levels by 70–90% and reduces prostate size, while T tissue levels remain constant [5]. The use of finasteride demonstrated a sustained improvement in the treatment of androgen-dependent

Correspondence: Marisa Cabeza Ph.D., Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana-Xochimilco, Calzada del Hueso No. 1100, México, D.F C.P. 04960, México. Tel: [011-52-55] 5483-72-60. Fax: [011-52-55] 5483-72-60. E-mail: marisa@correo.xoc.uam.mx

ISSN 1475-6366 print/ISSN 1475-6374 online
DOI: 10.1080/14756360600748474

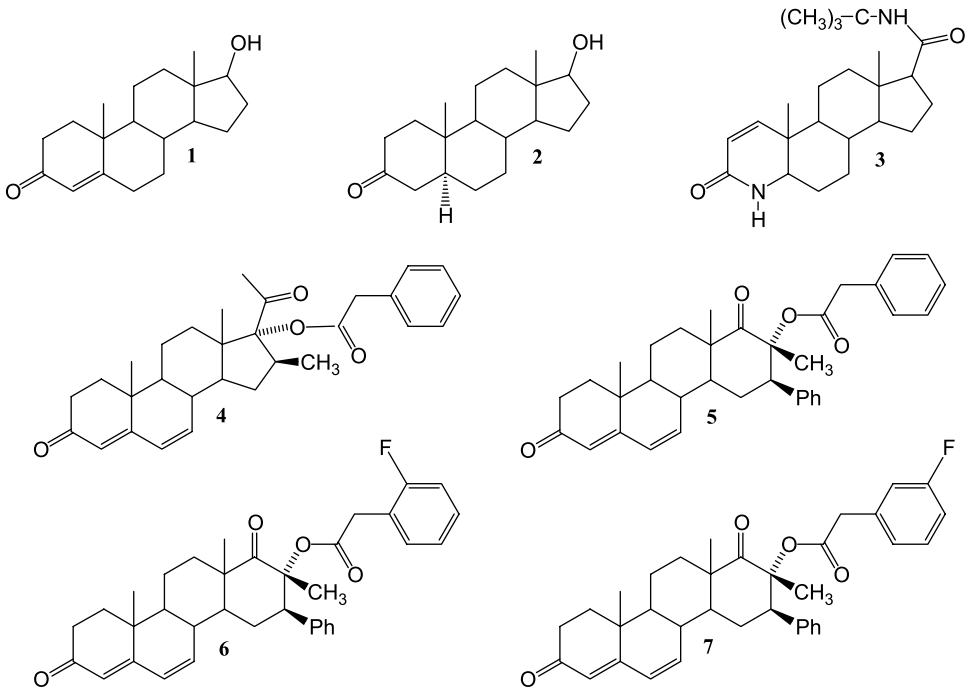

Figure 1. Structures of (**1**–**7**).

diseases and it also reduces the prostate specific antigen (PSA) levels [9].

Recently our group synthesized several new progesterone derivatives that considerably decreased the prostate growth produced by T [10,11]. Since these compounds showed a high biological activity in two different systems [10–12], in this paper we describe the synthesis and pharmacological evaluation of four similar compounds **4–7** based on the progesterone skeleton with antiandrogenic activity [13] and as inhibitors of human 5α-reductase enzyme. In this study, we also determined the effect of the novel steroids on the prostate cancer cell lines.

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 gemini 200 and VRX-300 respectively. 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; triplet, t; m, multiplet). Mass spectra were obtained with a HP5985-B spectrometer. IR spectra were recorded on a Perkin-Elmer 200 s spectrometer. (1, 2, 6, 7-^{3}H) Testosterone [^{3}H] T (specific activity: 95 Ci/mmol) was provided by Perkin Elmer Life and Analytical Sciences. (Boston, MA). Radioinert T and 5α-dihydrotestosterone 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 the steroidal derivatives

The synthesis of the new steroids **4–7** is briefly described below (Figures 2 and 3). The preparation of all intermediates **9–17** is given in references [12] and [14].

17α-Phenylacetoxy-16β-methylpregna-4, 6-diene-3, 20-dione ***4***. Phenylacetic acid (0.06 g, 0.44 mmol), p-toluenesulfonic acid (0.005 g, 0.026 mmol) and trifluoroacetic anhydride (0.092 g, 0.438 mmol) was stirred for 2 h at room temperature. Steroid **14** (0.05 g, 0.15 mmol) was added; the reaction mixture was stirred for an additional 2 h at room temperature (nitrogen atmosphere). It was neutralized with an aqueous sodium bicarbonate solution to pH 7 and diluted with chloroform (10 mL). The organic phase was separated and dried over anhydrous sodium sulfate; the solvent was removed in vacuum and the crude product was purified by silica gel column chromatography. Hexane-ethyl acetate (8:2) eluted 0.033 g; 0.072 mmol (49%) of pure product **4**, mp 166–169°C. UV (nm): 283 ($\varepsilon = 23{,}200$). IR (KBr) cm^{-1}: 1726, 1710, 1665,

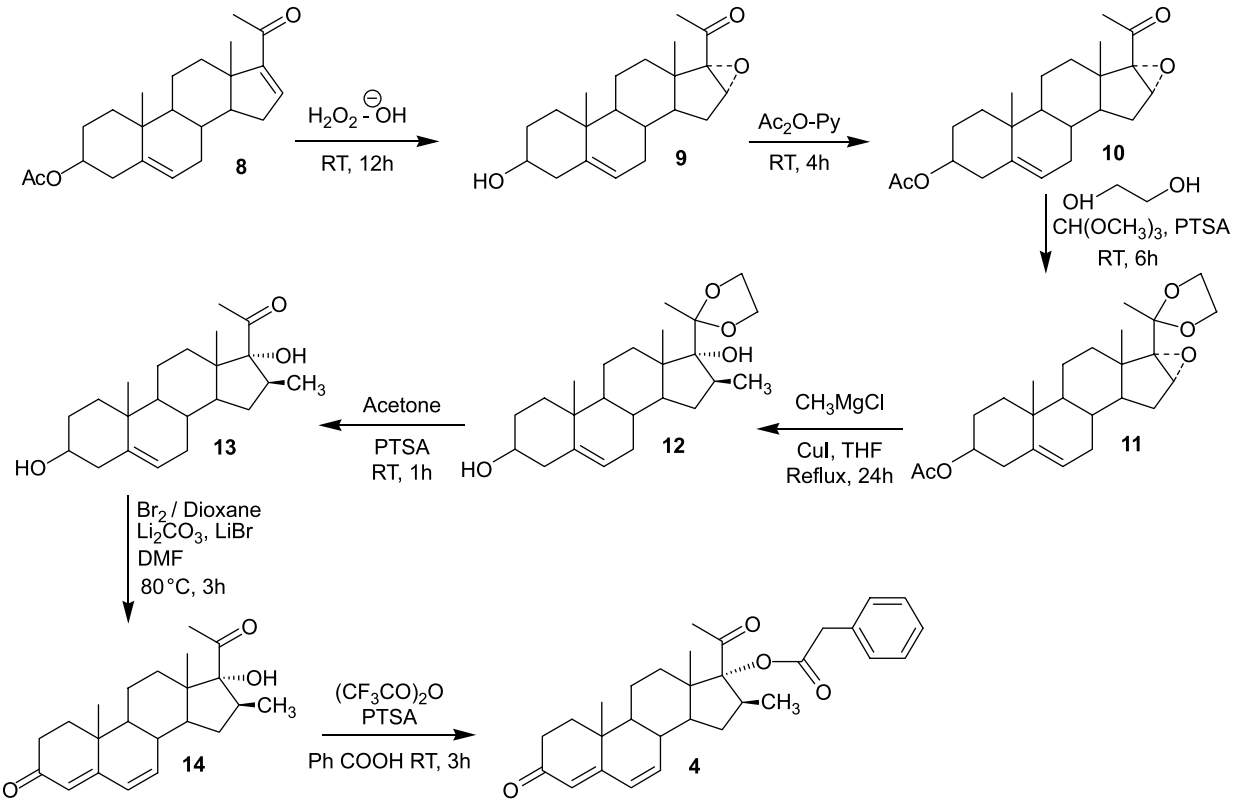

Figure 2. Synthesis of (**4**).

1618, 719. ^{1}H-NMR ($CDCl_3$) δ: 0.74 (3H,s, H-18), 1.11 (3H, s, H-19), 1.34 (3H, d, J = 7 Hz, CH_3 at C-16), 1.84 (3H, s, H-21), 3.67 (2H, s, COO-CH_2-Ph), 5.72 (1H, s, H-4), 6.09 (1H,d, J = 6 Hz, H-6), 7.29 (5H m, aromatic protons). ^{13}C-NMR ($CDCl_3$)δ: 15.0 (CH_3 at C-16), 16.3 (C-18), 19.9 (C-19), 22.2 (C-21), 124.1 (C-4), 127.4 (C-6), 128.1 (C-para), 128.7 (C-meta-2C), 129.4 (C-ortho-2C), 141.2 (C-7), 163.3 (C-5), 173.3 (ester carbonyl), 199.5 (C-3), 204.6 (C-20). MS (m/z): 460 (M^+).

17α-Phenylacetoxy-17β-methyl-16β-phenyl-D-homo-pregna-4, 6-diene-3, 17a-dione ***5***. A mixture of phenylacetic acid (1.22 g, 8.96 mmol), p-toluene-sulfonic acid (0.005 g, 0.026 mmol) and trifluoro-acetic anhydride (0.94 g, 4.03 mmol) was stirred for 2 h at room temperature. Steroid **17** (1 g, 2.47 mmol) was added; the reaction mixture was stirred for an additional 2 h at room temperature (nitrogen atmosphere). It was neutralized with an aqueous sodium bicarbonate solution to pH 7 and diluted with chloroform (10 mL). The organic phase was separated and dried over anhydrous sodium sulfate; the solvent was eliminated in vacuum and the crude product was purified by silica gel column chromatography. Hexane-ethyl acetate (8:2) eluted 0.59 g, 0.97 mmol (39%) of pure product **5,** mp 186–189°C. UV (nm): 283 (e = 22,900). IR (KBr) cm^{-1}: 1735, 1720, 1704, 1667, 1620, 870, 761, 700. ^{1}H-NMR ($CDCl_3$)δ:1.17 (3H,s, H–18), 1.22 (3H, s, H–19), 1.26 (3H, s, H–21), 3.60 (2H,s, COOCH_2-Ph), 5.68 (1H, s, H-4), 6.13 (1H, d, J = 3 Hz, H-7), 6.26 (1 h, d, J = 4 Hz, H-7), 7.23 (5H, phenyl at C-16), 7.35 (5H, m, phenyl of ester). ^{13}C-NMR ($CDCl_3$)δ: 16.2 (C-18), 17.6 (C-19), 22.9 (C-21), 123.8 (C-4), 128.2 (C-6), 139.2 (C-7), 162.6 (C-5), 170.7 (ester carbonyl), 199.4 (C-3), 211.6 (C-17a). MS (m/z) 522 (M^+).

17α-(2-Fluorophenyl) acetoxy-17β-methyl-16β-phenyl-D-homopregna-4, 6-diene-3, 17a-dione ***6***. A mixture of 2-fluorophenylacetic acid (1.38 g, 8.96 mmol), p-toluenesulfonic acid (0.005 g, 0.026 mmol) and trifluoroacetic anhydride (0.94 g, 4.03 mmol) was stirred for 2 h at room temperature. Steroid **17** (1 g, 2.47 mmol) was added; the reaction mixture was stirred at room temperature for an additional 3 h (nitrogen atmosphere). It was neutralized with an aqueous sodium bicarbonate solution to pH 7 and diluted with chloroform (10 mL). The organic phase was separated and dried over anhydrous sodium sulfate; the solvent was eliminated in vacuum and the crude product was purified by silica gel column chromatography. Hexane-ethyl acetate (8:2) eluted 0.5 g, 0.93 mmol (37.4%) of pure product **6**, mp 192-194°C. UV (nm) 284 (ε = 22,700). IR (KBr)cm^{-1}: 1730, 1712, 1662, 1618, 877. ^{1}H-NMR ($CDCl_3$) δ:1.11(3H, s, H-18),

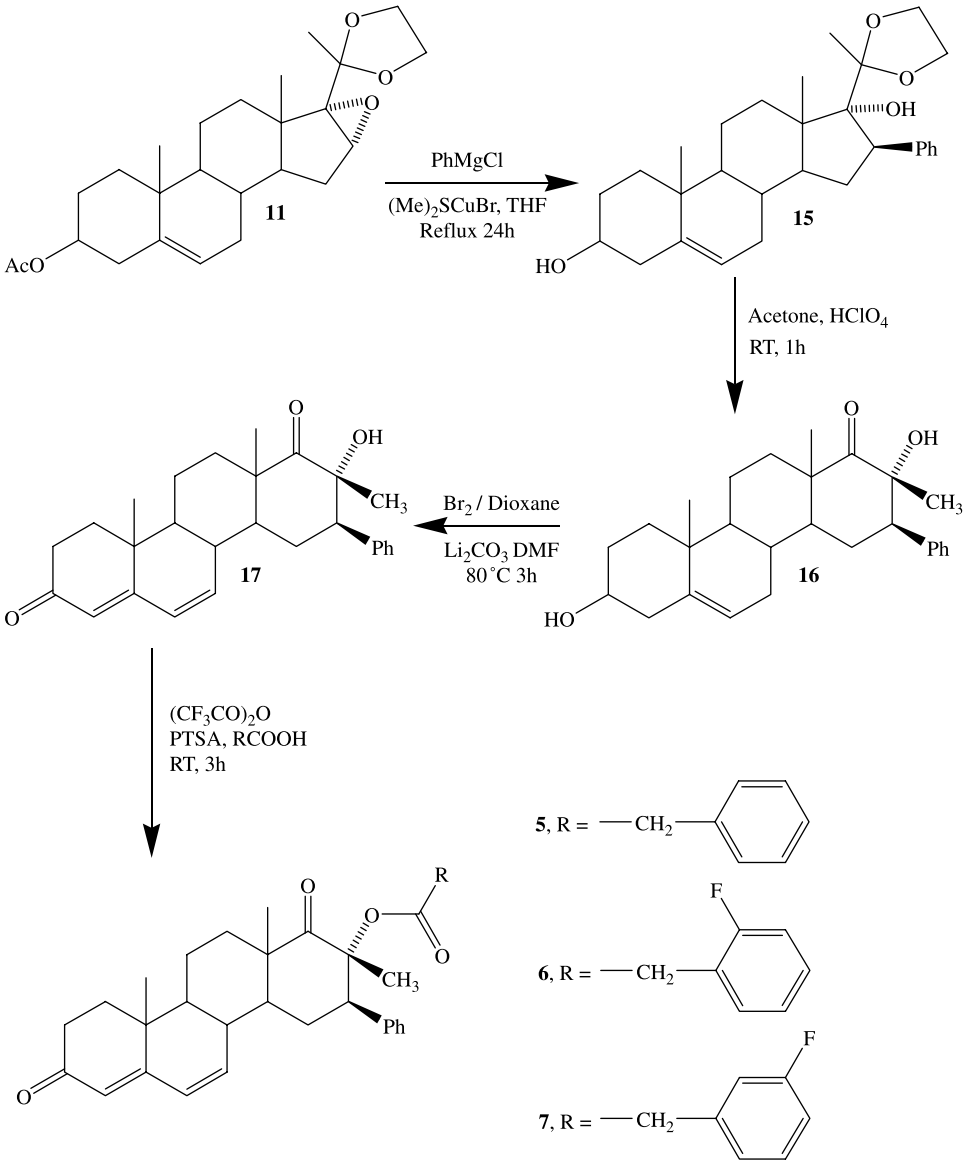

Figure 3. Synthesis of (**5**–**7**).

1.15 (3H, s, H-19), 1.22 (3H, s, H-21), 3.75 (2H, s, COO*CH*$_2$-Ph), 5.67 (1H, s, H-4), 6.13 (1H, d, J = 3 Hz, H-6), 6.27 (1H, d, J = 4 Hz, H-7), 7.04 (5H,m, phenyl at C-16), 7.26 (4H, m, phenyl of ester). ^{13}C-NMR ($CDCl_3$) δ: 16.4 (C-18), 18.0 (C-19), 23.1 (C-21), 127.4(C-4), 128.1 (C-6), 128.4 (C-para, aromatic,1C), 129.1 (C-ortho, aromatic, 1C), 129.5 (C-meta, aromatic, 2C), 130.2 (C-F aromatic, 1C), 138.5 (C-7), 162.9 (C-5), 170.2 (ester carbonyl), 199.9 (C-3), 211.8 (C-17a). MS (m/z): 540 (M^+).

17α-(3-Fluorophenyl) acetoxy-17β-methyl-16β-phenyl-D-homopregna-4, 6-diene-3, 17a-dione **7**. A mixture of 3-fluorophenylacetic acid (1.38 g, 8.96 mmol), p-toluenesulfonic acid (0.005 g, 0.026 mmol) and trifluoroacetic anhydride (0.94 g, 4.03 mmol) was stirred for 2 h at room temperature. Steroid **17** (1 g, 2.47 mmol) was added; the reaction mixture was stirred for an additional 3 h (nitrogen atmosphere). It was neutralized with an aqueous sodium bicarbonate solution to pH 7 and diluted with chloroform (10 mL). The organic phase was separated and dried over anhydrous sodium sulfate; the solvent was eliminated in vacuum and the crude product was purified by silica gel column chromatography. Hexane-ethyl acetate (8:2) eluted 0.5 g, 0.93 mmol (37.4%) of pure product **7**, mp 178–180°C. UV (nm): 285 (ε = 22,500). IR(KBr): 1725, 1711, 1665, 1620. ^{1}H-NMR ($CDCl_3$)δ: 1.11 (3H, s, H-18), 1.14 (3H, s, H-19), 1.23 (3H, s, H-21), 3.66 (2H, s, COO*CH*$_2$-Ph), 5.70 (1H, s, H-4), 6.15 (1H, d, J = 3 Hz, H-6), 6.25 (1H, d, J = 4 Hz, H-7), 6.91 (5H, m, phenyl at C-16), 7.37 (4H, m, phenyl of ester), ^{13}C-NMR ($CDCl_3$)δ:16.22 (C-18), 17.73 (C-19), 22.85 (C-21), 123.85 (C-4), 125.26 (C-6), 127.18 (C-para,

aromatic, 1C), 128.12(C-ortho, aromatic, 2C), 128.75 (C-meta, aromatic, 1C), 130.11 (C-F, aromatic-1C), 138.10 (C-7), 163.90 (C-5), 172.21(ester carbonyl), 199.41 (C-3), 211.45 (C-17a). MS (m/z): 540 (M^+).

Biology

Animals and tissues. Adult male golden hamsters (150–200 g) were obtained from the Metropolitan University in Xochimilco, Mexico. Gonadectomies were performed under pentobarbital anesthesia 30 days prior to the experiments and the animals were sacrificed with CO_2. This protocol was approved by the Institutional Care and Use Committee of the Metropolitan University of Mexico (UAM). The experiment was carried out on 9 groups of 4 randomly selected animals/experiment, which were kept in a room at controlled temperature (22°C) and light- dark periods of 12 h. Food and water were provided *ad libitum*.

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. Tissue was homogenized with a tissue homogenizer (model 985–370; variable speed 5000–30,000 rpm, Biospec Products, Inc.).

In vivo experiments. For the daily subcutaneous injections, 2 mg/Kg of the steroids **4–7** was dissolved in 200 μL of sesame oil and administered for 6 days together with 1 mg/Kg of testosterone **1**. Three groups of animals were kept as control; one was injected with 200 μL of sesame oil, the second with 1 mg/Kg of testosterone and the third with T(**1**) plus 1 mg/Kg of finasteride (**3**) for 6 days. After the treatment, the animals were sacrificed by CO_2. The prostate gland from the animals (4) of each group was dissected and weighed. Two separate experiments were performed for each group of steroid-treated animals. The results (Table I) were analyzed using one-way analysis of variance with EPISTAT software.

Table I. Weight of prostate glands ± standard deviation from rats receiving different s.c. treatments for 6 days.

Treatment	Prostate weight (mg)
Control	39 ± 11
T (*1 mg/Kg*)	86 ± 8
T + **3** (*1 mg/Kg*)	52 ± 12
T + **4** (*2 mg/Kg*)	66 ± 13
T + **5** (*2 mg/Kg*)	90 ± 14
T + **6** (*2 mg/Kg*)	82 ± 9
T + **7** (*2 mg/Kg*)	72 ± 9

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 [10,14,15] in a SW 60 Ti rotor (Beckman instruments, Palo Alto, CA). The pellets were separated, washed with 3 volumes of medium A and centrifuged for an additional 40 min at 440 × g at 0°C for 10 min [10,15]. The washed pellets were suspended in medium A and kept at −70°C. The suspension, 5 mg of protein/ml for human prostates, determined by the Bradford method [16] was used as source of 5α-reductase.

Determination of 5α-reductase activity. The enzyme 5α-reductase was assayed as previously describes [10,14,15]. The reaction mixture in a final volume of 1 mL contained: 1 mM dithiothreitol, sodium phosphate buffer 40 mM, at pH 6.5 for human prostates [14], 2 mM, NADPH, 2 nM [1,2,6,7-^{3}H]T. The reaction, in duplicate, was started by addition of to the enzymatic fraction (134 μg protein in a volume of 80 μL), incubated at 37°C for 60 min [15] 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 dichloromethane fraction was separated and the extraction was repeated 4 more times. The pooled extract was evaporated under a nitrogen stream to dryness and suspended in 50 μL of methanol that was then spotted on HPTLC Keiselgel 60 F_{254} plates. T and DHT were used as carriers and the plates were developed in chloroform-acetone (9:1). The plates were air-dried and the chromatography was repeated on 2 occassions. The steroid carriers were detected using phosphomolybdic acid reagent and a UV lamp (254 nm). The DHT-containing areas were cut off and the strips were soaked in 5 mL of Ultima Gold (Packard) and the radioactivity was determined in a scintillation counter (Packard tri-carb 2100 TR). Control incubations, chromatographic separation and identification were carried out in the same way as described above except that these tubes did not contain tissue.

Determination of 50% inhibitory concentration of steroids 4–7 on human prostatic 5α-reductase. In order to calculate the IC_{50} values (the concentration of steroids **4–7** required to inhibit 5α-reductase activity by 50%), six series of tubes containing increasing concentrations of these steroids (10^{-11}–10^{-3} M) were incubated in duplicate, in the presence of: 1 mM of dithiothreitol, 40 mM sodium phosphate buffer pH 6.5 for human; 2 mM NADPH, 2 nM [1,2,6,7-^{3}H]T and 134 μg of protein from the enzymatic fraction in a final volume of 1 mL. The reaction was carried out in duplicate at 37°C for 60 min followed by addition of 1 mL dichloromethane

to stop the reaction. The amount of DHT formed was determined as detailed above.

Percentage of inhibition of prostate cancer cell growth. Assays were performed by the method with sulforhodamine B (SRB) as previously described [17] with PC3 (prostate) and MT-2 (lymphocyte T) lines. Briefly, this method is based on the bonding of anionic dry SRB to the proteins of cells fixed with 50% trichloroacetic acid. The complex protein-SRB is solubilized with a Tris buffer and the solution which is read at 515 nm in a microtiter plate reader. Preliminary screening was carried out at 50 μM; all compounds were dissolved in ethanol with a maximum concentration of 0.5% ethanol in the test; at this concentration these solutions were completely innoculous.

Cell culture and assay for cytotoxic activity. The prostate cancer (PC-3) cell line was supplied by the National Cancer Institute (NCI), USA. MT2 was provided by Unidad de Servicios en VIH/SIDA in Mexico City.

The cytotoxic of the tumor cells with the test compounds was determined using the protein-binding dye sulforhodamine B (SRB) in microculture assay to measure cell growth (14). The cell lines were cultured in RPMI-1640 (Sigma Chemical Co., Ltd., St. Louis, MO, USA), supplemented with 10% fetal bovine serum, and 2 mM L-glutamine (Gibco). They were maintained at 37 °C in a 5% CO_2 atmosphere with 95% humidity. For the assay 7.5×10^4 cel/well (PC-3) and 10×10^4 cell/well (MT-2), and 100 μl/well of these cell suspensions were seeded in 96-well microtiter plates and incubated to allow for cell attachment. After 24 h, 100 μL of each test compounds and positive substances were added to each well. 48 h later, adherent cell cultures were fixed *in situ* by adding 50 μL of cold 50% (wt/vol) trichloacetic acid (TCA) and incubated for 60 min at 4°C. The supernatant liquid was discarded and the plates were washed three times with water and air-dried. Cultures fixed with TCA were stained for 30 min with 100 μL of 0.4% SRB solution. Protein-bound dye was extracted with 10 mM unbuffered tris base and the absorbances were read on an Ultra Microplate Reader (El_x 808, BIO-TEK Instruments, Inc.), with a test wavelength of 515 nm. Preliminary screening was carried out at 50 μM; all compounds were dissolved in ethanol with a maximum concentration of 0.5% ethanol; at this concentration the solution was completely innocuous. The results were expressed as percentage of growth inhibition at 50 μM.

Results

Synthesis of the steroidal compounds 4–7

Compounds **4–7** (Figures 2 and 3) were prepared from the commercially available 16- dehydropregnenolone acetate **8.** Compound **14** was esterified with the corresponding acid in the presence of trifluoroacetic acid and p-toluenesulfonic acid to form the desired esters **4–7**. The preparation of all intermediates is given in references [12] and [14].

Biological activity

In vivo experiments. After castration, the weight of the male hamster prostate decreased ($p < 0.005$) compared to that of 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 ($p < 0.005$) the weight of this gland in castrated male hamsters (Table I). When testosterone (T) and finasteride (1 mg/Kg) or compound **4 (**2 mg/Kg) were injected together, the weight of the prostate decreased ($p < 0.005$) as compared to that of testosterone-treated animals (Table I). Steroids **5–7** (2 mg/Kg) did not significantly decrease the weight of the prostate gland.

In vitro experiments. The *in vitro* biological activity of compounds **4–7** was determined in human prostate gland, homogenized and centrifuged to obtain the prostatic enzyme fraction. The activity of human 5α-reductase was assessed by incubating the enzymatic fractions with 2 nM [^{3}H]T. The dichloromethane extracts from human prostates were subjected to TLC analysis. The zone corresponding to the DHT standard (R_f value = 0.67) of the experimental chromatogram was cut off, soaked in Ultima Gold and the radioactivity determined. 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 from control incubations which did not contain tissue.

Determination of the 50% inhibitory concentration of the new compounds in human prostate. The concentrations of finasteride **3** and compounds **4–7** required for inhibiting 5α-reductase activity by 50% (IC_{50}) were determined from the inhibition curves using different concentrations of the steroids (Table II). Compounds **6** and **7** had a lower IC_{50} value as compared to that of finasteride. These data indicate that both compounds are more active *in vitro* than

Table II. The IC_{50} values determined for finasteride and the synthesized steroids **4**–7 with human prostate 5α–reductase enzyme.

Compounds	IC_{50} value (nM)
Finasteride 3	8.5
4	1.2
5	310
6	0.5
7	0.7

Table III. Percentage of inhibition of prostate cancer cell growth, and normal lymphocytes produced by 50 μM of the synthesized compounds.

Compounds	Percentage inhibition of prostatic cellular growth	Percentage inhibition of normal lymphocytes growth
8	50	54
4	69	92
5	46	20
6	51	NA
7	31	NA

NA. Non-active compound.

finasteride, the compound of choice for the treatment of benign prostatic hyperplasia.

Percentage of inhibition of prostate cancer cell growth. The percent inhibition of normal lymphocytes and prostate cancer cell growth was determined by the sulforhodamine method. Table III gives the percent inhibition of prostate cancer cell growth and normal lymphocytes cell growth produced by 50 μM of the synthesized compounds (**4–7**). Steroid **4** inhibited the lymphocytes and prostate growth by 92% and 69% respectively. From all evaluated compounds (**4–8**), compound **4** exhibited the highest cytotoxicity.

Discussion

In this study we determined the effect of four novel compounds **4–7** on the weight of hamster prostate gland. (Table I) Steroid **4** was the only one of the series that had *in vivo* an antiandrogenic effect similar to that of finasteride **3**. Steroids **4–7** inhibited *in vitro* the 5α-reductase activity present in human prostate homogenates. The IC_{50} values (Table II) showed that the novel compounds **4, 6** and **7,** had a lower value than that for the commercially available finasteride (Proscar). These data indicated that compound **4** which showed *in vivo* an antiandrogenic effect similar to that of finasteride **3** could be an alternative drug for the inhibition of 5α- reductase activity. The data from our studies indicated that the *in vivo* efficiency of the 5α-inhibitors may depend not only on its affinity for the enzyme but also on the rate of absorption, the half life of the parent compound and the active metabolites in the prostate gland.

The results from lymphocytes and prostate cancer cells growth experiments indicated that in high doses compound **4** had the highest cytotoxic effect in these cells as compared with compounds **5–8**. Nevertheless subcutaneous treatment of the animals for 6 days with 2 mg/Kg of compound **4** did not show any toxic effects. In the future we are planning to synthesize larger amount of compound **4** in order to determine the *in vivo* toxicological doses.

Steroids **6** and **7** showed the most potent *in vitro* 5α-reductase inhibitory activity, as indicated by their IC_{50} values (Table II). This enhanced biological activity is probably due to the presence of the fluorine atom in the phenyl ester group that is not present in compounds **4** or **5.** A similar compound PM-9 (having a fluorine atom) also showed high inhibitory activity on 5α-reductase present in *P. crustosum* broths [12].

Acknowledgement

We gratefully acknowledge the financial support of DGAPA for project 208505.

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