Molecular Interactions of New Pregnenedione Derivatives

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The *in vitro* inhibitory activity of five new progesterone derivatives: 17α-hydroxy-16β-methylpregna-1,4,6-triene-3,20-dione 1; 16β-methyl-17α-toluoyloxypregna-1,4,6-triene-3,20-dione 2; 17α-hydroxy-6-methylenepregn-4-ene-3,20-dione 3; 6-methylene-17α-toluoyloxypregn-4ene-3,20-dione 4 and 17α-(*p*-bromobenzoyloxy)-6-methylenepregn-4-ene-3,20-dione 5 was determined. These compounds were evaluated as 5α-reductase inhibitors as well as antagonists for the androgen receptor. Compounds 1, 2, 3, 4 and 5 showed the following inhibitory activity for the 5α-reductase enzyme with IC_{50} values of: 1 (1.65 μM), 2 (10 μM), 3 (19 nM), 4 (100 nM) and 5 (100 nM). The results of this study also showed the effect of increasing concentrations of the novel steroids upon [^{3}H]dihydrotestosterone binding to androgen receptors from male hamster prostate. The K_i values for compounds 1, 2, 3, 4, 5 and dihydrotestosterone showed the following order of affinity for the androgen receptor: 4>5>dihydrotestosterone>2>3>1. The overall data indicated that all synthesized compounds 1, 2, 3, 4 and 5 are inhibitors of the 5α-reductase enzyme present in the hamster prostate. In addition compounds 1, 2, 3, 4 and 5 also presented an affinity for the androgen receptor.

Key words 5α-reductase inhibition; prostate; pregnenedione derivative; androgen receptor; novel steroid; prostate

The normal activity of the NADPH dependent 5α-reductase enzyme (EC 1.3.99.5) maintains testosterone's **6** (T) biological functions: anabolic actions and spermatogenesis of humans as well as such dihydrotestosterone **7** mediated effects, as increased facial and body hair, acne, scalp hair recession, and prostate enlargement.[1)]

The deficiency of 5α-reductase in males results in incomplete differentiation of the external genitalia at birth.[2)] At puberty, patients have normal to elevated levels of T in plasma, and virilization occurs, but the prostate remains small and there is no acne.[3,4)] Low level of dihydrotestosterone results in male pseudohermaphroditism. On the contrary, abnormally high 5α-reductase activity in humans produces excessively high dihydrotestosterone levels in peripheral tissues,[5,6)] which is implicated in the pathogenesis of prostate cancer, benign prostatic hyperplasia (BPH), acne, and male pate baldness,[1)] thus suggesting that both the enzyme and dihydrotestosterone play important physiological and pathological roles in humans. Therefore, the inhibition of androgen action by 5α-reductase inhibitors is a logical treatment of 5α-reductase activity disorders. Furthermore, by the beginning of the past decade two types of 5α-reductase had been identified (types I, II).[7,8)] The identification of these two isozymes opened the door for specific and selective inhibition of this enzyme.

The most extensively studied class of 5α-reductase inhibitors is the 4-azasteroids[9,10)] which includes the drug finasteride **8** (Fig. 1). This compound is the first 5α-reductase inhibitor approved in the U.S.A. for the treatment of BPH. This drug has approximately 100-fold greater affinity for type II 5α-reductase than for the type 1 enzyme. In humans, finasteride **8** decreases prostatic dihydrotestosterone levels by 70—90% and reduces prostate size, while T tissue levels remain constant.[9)] The use of **8** demonstrated a sustained improvement in the treatment of androgen dependent diseases and reduction in prostate specific antigen (PSA) levels.[10)]

In this paper, we describe the synthesis of four new progesterone derivatives: 17α-hydroxy-16β-methylpregna-1,4,6-triene-3,20-dione **1** (this compound is described in reference 11); 16β-methyl-17α-toluoyloxypregna-1,4,6-triene-3,20-dione **2**; 17α-hydroxy-6-methylenepregn-4-ene-3,20-dione **3**; 6-methylene-17α-toluoyloxypregn-4-ene-3,20-dione **4** and 17α-(*p*-bromobenzoyloxy)-6-methylenepregn-4-ene-3,20-dione **5**. These compounds were evaluated as 5α-reductase inhibitors as well as antagonists for the androgen receptor.

Synthesis of Steroids 1—5 Compounds **1** and **2** (Fig. 1) were prepared from the commercially available 16-dehydropregnenolone acetate **9** (Fig. 2). The three steroids having the exocyclic double bond **3**, **4** and **5** were prepared from 17α-acetoxyprogesterone **18** (Fig. 3).

The synthesis of the intermediates **10**—**14** (Fig. 2) is described in reference 12. When compound **14** (Fig. 2) was allowed to reflux with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in dioxane for 4 d, the trienedione derivative **1** was obtained. Esterification of the free hydroxyl group in **1** with trifluoroacetic anhydride and *p*-toluic acid afforded the desired ester **2**. Compounds **3**, **4** and **5** were prepared from the commercially available 17α-acetoxyprogesterone **18** (Fig. 3). On reflux with trimethyl orthoformate in dioxane, compound **18** afforded the enol ether **19** which on treatment with phosphorous oxychloride in *N*,*N*-dimethylformamide was converted to the 6-formyl derivative **20** (the Vilsmeyer reaction). Reduction of the formyl group in **20** with sodium borohydride in methanol afforded the 6-hydroxymethylene derivative (not isolated) which under the condition of this reaction was dehydrated to the desired exocyclic methylene derivative **21**. In this reaction the enol ether was also hydrolyzed to the original α,β-unsaturated carbonyl moiety. The hydrolysis of the acetoxy group in **21** was effected by refluxing with methanol and an aqueous sodium hydroxide solution. The resulting free alcohol **3** was esterified with *p*-toluic acid and trifluoroacetic anhydride, thus affording the desired ester **4**. When the esterification of **3** was carried out with *p*-bromobenzoic acid under the same conditions, the *p*-

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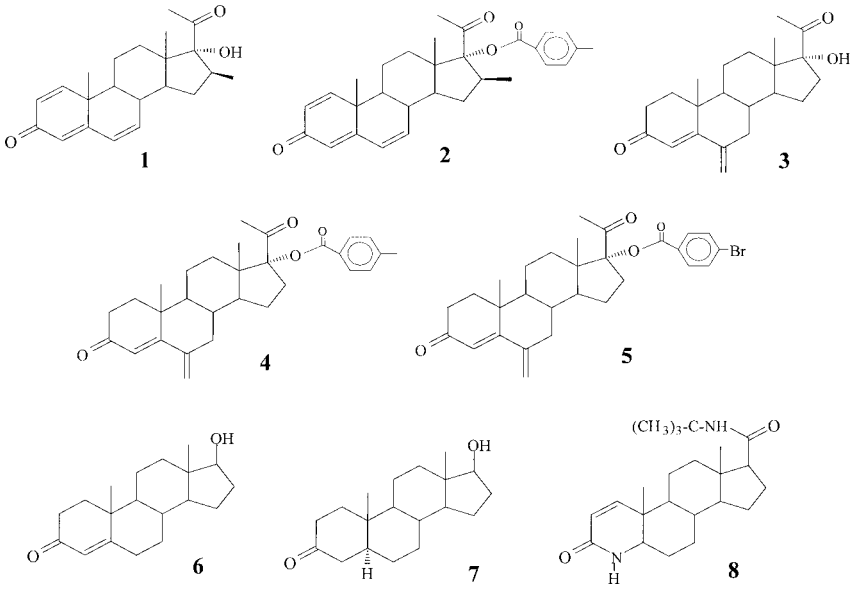

Fig. 1. Steroidal Structures

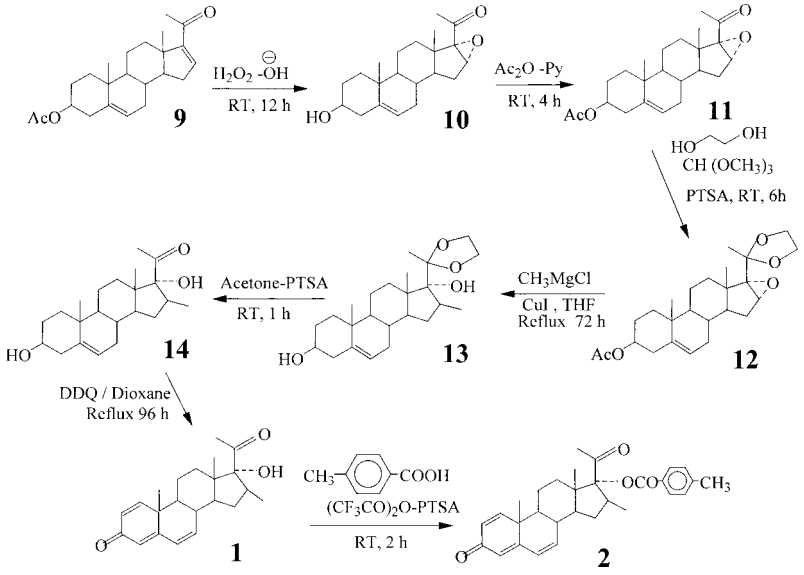

Fig. 2. Steroidal Synthesis

bromobenzoyloxy ester **5** was obtained.

Biological Activity The biological activity of compounds **1**, **2**, **3**, **4** and **5** was determined in gonadectomized hamster prostate glands homogenized and centrifuged to obtain the microsomal and cytosolic fractions.

The activity of 5α-reductase was assessed incubating the enzymatic fraction (microsomal) with 2 nM [^{3}H]T. The dichloromethane extract from castrated male hamster seminal vesicles was subjected to TLC analysis. The zone corresponding to dihydrotestosterone standard (*Rf* value of 0.67) of the experimental chromatogram was eluted and the radioactivity determined. This result was considered to be 100% of the activity of 5α-reductase for development of inhibition plots.

Determination of 50% Inhibitory Concentration of Finasteride and of the Novel Compounds in Hamster Prostate The concentration of finasteride **8** and compounds **1**, **2**, **3**, **4**, and **5** required for inhibiting 5α-reductase activities by 50% (IC_{50}) was determined from the inhibition curves using different concentrations of the steroids and are shown in Table 1. An IC_{50} value of 10 nM at pH of 7 was obtained from the curve using finasteride as inhibitor. This value was lower than those obtained with steroids **1**, **2**, **3**, **4** and **5** thus indicating that finasteride has a higher inhibitory potency that the novel steroids described in this paper. On the other hand, compound **3** with an IC_{50} value of 19 nM had the highest inhibitory potency compared to those of: **4** (100 nM), **5** (100 nM), **2** (10 μM) and **1** (1.65 μM).

Competition Analysis of Compounds 1, 2, 3, 4 and 5 for the Androgen Receptors The effect of increasing concentrations of non radioactive steroids upon [^{3}H]dihydrotestosterone binding to androgen receptors from male hamster prostate in two different experiments is shown in Fig. 4. The K_i values for compounds **1**, **2**, **3**, **4**, **5** and dihydrotestosterone

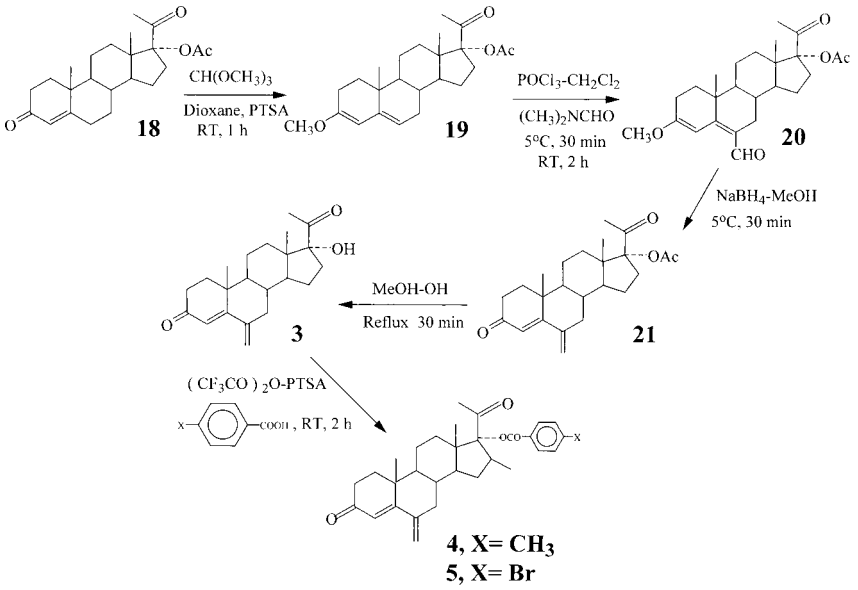

Fig. 3. Steroidal Synthesis

Table 1. IC_{50} Value for the Synthesized Steroids

Steroid	IC_{50} value (μM)
1	1.65
2	10.0
3	0.019
4	0.1
5	0.1
Finasteride **8**	0.01

The IC_{50} value represents the concentration of the steroid that inhibits 50% of 5α-reductase activity and was determined as described in the experimental section.

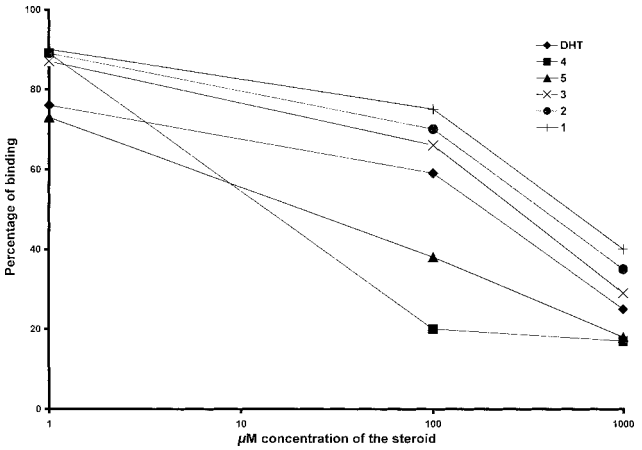

Fig. 4. Binding Specificity

Gonadectomized hamster prostate cytosol incubated for 18—20 h at 4 °C in the presence of 3.15 nM of [^{3}H]dihydrotestosterone and increasing concentrations of radio-inert steroids.

show the following order of affinity for the androgen receptors: **4**>**5**>dihydrotestosterone>**2**>**3**>**1**.

Discussion

In this study, we assessed the inhibitory potency of five new steroids **1**, **2**, **3**, **4** and **5** synthesized by our group by comparing their IC_{50} values. Compounds **4** and **5** which have a very similar ester side chain at C-17, showed the same inhibitory potency as determined by their IC_{50} values of 100 nM. However the presence of the three double bonds in A and B rings of compound **2** decreased its inhibitory potency (IC_{50} 10 μM) as compared to compounds **4** (IC_{50} 100 nM) (Fig. 4) and **5** (IC_{50} 100 nM) having an exocyclic double bond in ring B. On the other hand, compound **3** with a hydroxyl group in C-17 exhibited a higher inhibitory potency (IC_{50} 19 nM) than compound **1** (IC_{50} 1.65 μM) having three endocyclic double bonds and a methyl substituent at C-16. Apparently the presence of the exocyclic double bond in B ring of compound **3** increases its activity.

All five synthesized compounds exhibited affinity for the androgen receptors (Fig. 4). Compounds **4** and **5** showed a higher affinity than its own substrate (dihydrotestosterone) thus indicating that the 6-methylene functional group increases this affinity. On the other hand, compound **2** having three endocyclic double bonds showed a lower affinity as compared to that of **4** and **5** thus indicating an interference of the three endocyclic double bonds with the protein receptor. Compounds **3** and **1** showed a lower affinity than dihydrotestosterone for the androgen receptor. In view of the fact that neither compound has an ester function in C-17, this fact probably explains the low binding affinity of these compounds with the androgen receptor as compared to steroids **4** and **5**, which have a toluoyloxy and *p*-bromobenzoyloxy side chain respectively.

Experimental

Chemical and Radioactive Material Solvents were laboratory grade or better. (1, 2, 6, 7-^{3}H)Testosterone ([^{3}H]T) specific activity: 95 Ci/mmol and (1, 2, 4, 5, 6, 7-^{3}H)dihydrotestosterone ([^{3}H]DHT) specific activity 110 Ci/mmol,, were provided by New England Nuclear Corp. (Boston, MA, U.S.A.). Radioniert T and 5α-dihydrotestosterone were supplied by Steraloids (Wilton, NH, U.S.A.). Sigma Chemical Co. (St. Louis, MO, U.S.A.) supplied 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 Compounds 17-(Hydroxy-16β-methylpregna-1,4,6-triene-3,20-dione **1**: A solution of steroid **14** (1 g, 2.82 mmol) and 2,3-

dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (2.2 g) in dioxane (50 ml) was allowed to reflux for 4 d. Upon cooling, the precipitated 2,3-dichloro-5,6-dicyano-hydroquinone was filtered off. To the filtrate was added 3% aqueous sodium hydroxide solution (100 ml) and chloroform (100 ml); the mixture was stirred for 5 min. The organic phase was separated and washed 3 times with 3% aqueous sodium hydroxide solution and then with water. It was dried over anhydrous sodium sulfate and the solvent was removed in vacuum. The crude product was purified by silica gel column chromatography. Hexane–ethyl acetate (6 : 4) eluted 640 mg, 1.88 mmol (65%) of the pure product **1**, mp 198—2000 °C. UV (nm): 222, 255, 298 (ε=14900, 1270 and 17900) respectively. IR (KBr) cm^{-1}: 3387, 1705, 1656, 1602. ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, s), 1.1 (3H, s), 1.3 (3H, d, J=4 Hz), 2.0 (3H, s), 6.0 (1H, s), 6.2 (1H, d, J=2 Hz), 6.4 (1H, d, J=3 Hz), 6.6 (1H, d, J=3 Hz), 7.1 (1H, d, J=2 Hz). ^{13}C-NMR ($CDCl_3$) δ: 15.0 (C-18), 16.2 (CH_3 at C-16), 20.5 (C-19), 26.5 (C-21), 78.2 (C-17), 186.2 (C-3), 217.0 (C-20). MS m/z: 340 (M^+).

16β-Methyl-17α-toluoyloxypregna-1,4,6-triene-3,20-dione **2**: A solution containing steroid **1** (1 g, 2.94 mmol), PTSA (10 mg) and trifluoroacetic anhydride (8.29 g, 42.48 mmol) was stirred for 2 h at room temperature (nitrogen atmosphere). The reaction mixture was neutralized with an aqueous solution of sodium bicarbonate to a pH of 7 and diluted with chloroform (10 ml) The organic phase was separated and dried over anhydrous sodium sulfate; the solvent was eliminated in vacuum. The crude product was purified by silica gel column chromatography. Hexane–ethyl acetate (8 : 2) eluted 700 mg, 1.74 mmol (59%), mp 186—189 °C. UV (nm): 221, 255 and 300 (ε=14600, 12400 and 17300, respectively). IR (KBr) cm^{-1}: 1720, 1707, 1664 and 1604. ^{1}H-NMR ($CDCl_3$) δ: 0.8 (3H, s), 1.0 (3H, d, J=2 Hz), 1.3 (3H, s), 2.0 (3H, s), 6.0 (1H, s), 6.1 (1H, s), 6.3 (2H, m) and 7.0 (1H, d, J=10 Hz). ^{13}C-NMR ($CDCl_3$) δ: 15.7 (CH_3 at C-16), 17.6 (C-18), 20.6 (C-19), 83.9 (C-17), 165 (C-5), 172 (ester carbonyl), 182 (C-3) and 212 (C-20). MS (m/z) 458 (M^+).

17α-Acetoxy-3-methoxypregna-3,5-diene-20-one **19**: A solution containing steroid **18** (1 g, 2.68 mmol), PTSA (200 mg) and trimethyl orthoformate (5 ml, 45.7 mmol) was stirred for 1 h at room temperature; at this time, pyridine (5 ml) was added and the reaction mixture was stirred for an additional 10 min. It was poured into ice-water (200 ml) and the crude product **19** precipitated; this was filtered and dried. Yield 620 mg, 1.6 mmol (60%); mp 154—156 °C. UV (nm): 238 (ε=12000). IR (KBr) cm^{-1}: 2975, 1736, 1711 and 1651. ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s), 1.2 (3H, s), 2.0 (3H, s), 3.5 (3H, s), 5.8 (1H, s), 6.0 (1H, d, J=2 Hz). ^{13}C-NMR ($CDCl_3$) δ: 14.3 (C-18), 17.4 (C19), 26.3 (C-21), 50.8 (methyl ether), 170.7 (ester carbonyl), 204 (C-20). MS (m/z) 386 (M^+).

17α-Acetoxy-6-formyl-3-methoxypregna-3,5-dien-20-one **20**: A solution containing *N*,*N*-dimethylformamide (0.6 ml, 7.7 mmol), phosphorous oxychloride (0.5 ml, 5.4 mmol) and methylene chloride (5 ml) was cooled to 5 °C. Steroid **19** (200 mg, 0.52 mmol) dissolved in methylene chloride (2 ml) and pyridine (1.5 ml) was added, keeping the temperature at 5 °C. The reaction mixture was stirred for 1 h at 0 °C and for 2 additional hours at room temperature. Sodium acetate (1 g) dissolved in methanol (70 ml) was added and the reaction mixture was stirred for 15 min. It was extracted 3 times with chloroform, the organic phase was separated and washed with an aqueous sodium bicarbonate solution and then with water. It was dried over anhydrous sodium sulfate and the solvent was removed in vacuum. The crude product was recrystallized from ethyl acetate–hexane. Yield 120 mg, 2.9 mmol (60%), mp 205—208 °C. UV (nm): 238 and 315 (ε=10800, 15600 respectively). IR (KBr) cm^{-1}: 2946, 1731 and 1652. ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s), 1.2 (3H, s), 2.0 (3H, s), 2.2 (3H, s), 3.5 (3H, s), 6.2 (1H, s) and 10.2 (1H, s). ^{13}C-NMR ($CDCl_3$) δ: 14.3 (C-18), 18.9 (C-19), 26.5 (C-21), 54.8 (CH_3 enol ether), 177 (ester carbonyl). MS m/z 414 (M^+).

17α-Acetoxy-6-methylenepregn-4-ene-3,20-dione **21**: A suspension containing steroid **20** (100 mg, 0.24 mmol), methanol (2 ml) and sodium borohydride (20 mg, 0.53 mmol) was stirred at 5 °C for 30 min. Water (20 ml) was added and the mixture was transferred to a separatory funnel. It was extracted three times with ethyl acetate, the organic phase was separated, washed with water and dried over anhydrous sodium sulfate. The solvent was removed in vacuum and the resulting crude product was recrystallized from methanol. Yield 64 mg, 0.15 mmol (70%), mp 244—246 °C. UV (nm): 260 (ε=11000). IR (KBr) cm^{-1}: 2955, 1730, 1708, 1673, 1370 and 1262. ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s), 1.20 (3H, s), 2.00 (3H, s), 2.20 (3H, s), 4.95 (1H, d, J=10 Hz), 5.1 (1H, d, J=10 Hz), 6.1 (1H, s). ^{13}C-NMR ($CDCl_3$) δ: 14.2 (C-18), 17.1 (C-19), 26.3 (C-21), 170.6 (ester carbonyl), 203.9 (C-20). MS (m/z) 384 (M^+).

17α-Hydroxy-6-methylenepregn-4-ene-3,20-dione **3**: A solution containing steroid **21** (1 mg, 2.6 mmol), methanol (100 ml) and 2% aqueous sodium hydroxide solution (25 ml) was allowed to reflux for 30 min. Part of the methanol was removed in vacuum and water (20 ml) was added to the resulting solution . It was extracted with chloroform, the organic phase was separated, washed with water and dried over anhydrous sodium sulfate. The solvent was removed in vacuum; the crude product was recrystallized from ethyl acetate–hexane. Yield 660 mg, 1.92 mmol (75%), mp 204—206 °C. UV (nm): 254 (ε=10800). IR (KBr) cm^{-1}: 3436, 2947, 1702 and 1658. ^{1}H-NMR ($CDCl_3$) δ: 0.69 (3H, s), 1.22 (3H, s), 2.0 (3H, s), 2.00 (3H, s), 4.97 (1H, d, J=10 Hz) and 6.0 (1H, s). ^{13}C-NMR ($CDCl_3$) δ: 15.4 (C-18), 17.1 (C-19), 23.8 (C-21), 175.2 (C-3) and 211.5 (C-20). MS (m/z) 342 (M^+).

6-Methylene-17α-toluoyloxypregn-4-ene-3,20-dione **4**: A solution containing steroid **3** (1 g, 2.92 mmol), PTSA (10 mg), trifluoroacetic anhydride (8.29 g, 42.48 mmol) and *p*-toluic acid (1.36 g, 10 mmol) was stirred for two hours at room temperature (nitrogen atmosphere). The reaction mixture was neutralized with an aqueous solution of sodium bicarbonate to a pH of 7 and diluted with chloroform (10 ml). The organic phase was separated and dried over anhydrous sodium sulfate; the solvent was eliminated in vacuum. The crude product was purified by silica gel column chromatography. Hexane–ethyl acetate (8 : 2) eluted 680 mg, 1.48 mmol (50%), mp 214—216 °C. UV (nm): 242 (ε=11000). IR (KBr) cm^{-1}: 2948, 17816, 1672 and 1610. ^{1}H-NMR ($CDCl_3$) δ: 0.74 (3H, s), 1.12 (3H, s), 2.1 (3H, s), 2.45 (3H, s), 4.96 (1H, t, J=1.8 Hz) 5.1 (1H, d, J=1.8 Hz) , 5.94 (1H, s), 7.27 (2H, m) and 8.0 (2H,m). ^{13}C-NMR ($CDCl_3$) δ: 15.2 (C-18), 16.8 (C-19), 24.1 (C-21), 165 (ester carbonyl), 195 (C-3) and 203 (C-20). MS (m/z) 460 (M^+).

17α-(*p*-Bromobenzoyloxy)-6-methylenepregn-4-ene-3,20-dione **5**: A solution containing steroid **3** (1 g, 2.92 mmol), PTSA (10 mg), trifluoroacetic anhydride (8.29 g, 42.48 mmol) and *p*-bromobenzoic acid (2 g, 10 mmol) was stirred for 2 h at room temperature (nitrogen atmosphere). The reaction mixture was neutralized with an aqueous solution of sodium bicarbonate to a pH of 7 and diluted with chloroform (10 ml). The organic phase was separated and dried over anhydrous sodium sulfate; the solvent was eliminated in vacuum. The crude product was purified by silica gel column chromatography. Hexane–ethyl acetate (8 : 2) eluted 710 mg, 1.35 mmol (46%), mp 213—216 °C. UV (nm): 247 (ε=10750). IR (KBr) cm^{-1}: 2948, 1718, 1671, 1589 and 757. ^{1}H-NMR ($CDCl_3$) δ: 0.7 (3H, s), 1.1 (3H, s), 2.1 (3H, s), 5.9 (1H, s), 7.65 (2H, m) and 8.0 (2H, m). ^{13}C-NMR ($CDCl_3$) δ: 15.4 (C-18), 16.3 (C-19), 23.2 (C-21), 170 (ester carbonyl), 197 (C-3) and 205 (C-20). MS (m/z) 524 (M^+).

Biological Activity of the Synthesized Compounds The biological activity of steroids **1**, **2**, **3**, **4** and **5** was determined in *in vitro* experiments using prostate glands from gonadectomized adult male golden hamsters. The animals (150—200 g) were obtained from Metropolitan University-Xochimilco of Mexico. Gonadectomies were performed under light ether anesthesia 30 d before the experiments.

The prostate glands were immediately removed, blotted and weighed prior to their use. Tissues used in the metabolic experiment were homogenized with a tissue homogenizer (model 985—370; variable speed 5000—30000 rpm, Biospec Products, Inc.)

Tissues were homogenized in 3 volumes of buffer TEMD (20 mM Tris–HCl, 1.5 mM EDTA and 10 mM sodium Molybdate) at pH 7.4 and at 4 °C with a tissue homogenizer. Homogenates were centrifuged at 140000×g for 60 min[13] in a SW 60 Ti rotor (Beckman Instruments, Palo Alto, CA, U.S.A.). The pellets were separated, washed with 3 tissue volumes of medium A (20 mM sodium phosphate, pH 6.5 containing 0.32 M sucrose, 0.1 mM dithiothreitol Sigma-Aldrich, Inc.) and centrifuged two additional times at 440×g at 0 °C for 10 min.[14] The washed pellets were suspended in medium A and kept at −70 °C. The suspension (6.8 mg protein/ml determined by the Bradford method)[15] was used as a source of 5α-reductase.

Determination of 5α-Reductase Activity 5α-Reductase was assayed as previously described.[16] The reaction mixture contained a final volume of 1 ml: 1 mM dithiothreitol, sodium phosphate buffer (40 mM), 2 mM, NADPH, 2 nM [1, 2, 6, 7-^{3}H]T (specific activity 95 Ci/mmol). The reaction mixture in duplicate was started when it was added to the enzymatic fraction (250 μg protein), incubated at 37 °C for 60 min, and stopped by mixing with 1 ml of dichloromethane; this was considered as the 100% point. The fraction of dichlorometane was separated and the extraction was repeated 4 more times. The extract was evaporated under a nitrogen stream until dryness and suspended on 50 μl of methanol that was spotted on HPTLC Kieselgel 60 F_{254} plates. T and dihydrotestosterone were used as carriers and the plate was developed in chloroform–acetone (9 : 1). The plates were air-dried and the chromatography was repeated 2 more times. The T standard was visualized under UV light (254 nm) and dihydrotestosterone was detected using molybdatophosphoric acid hydrate reagent with a posterior heating of the plate. Dihydrotestosterone develops a classic dark blue color. The dihy-

drotestosterone containing areas were cut and the strips were soaked in 5 ml of Ultima Gold (Packard) and the radioactivity was counted in a Tri Carb 2100TR liquid scintillation analyzer.

Determination of 50% Inhibitory Concentration of Finasteride and Steroids 1, 2, 3, 4 and 5 in Gonadectomized Hamster Prostate In order to calculate the IC_{50} values (the concentration of finasteride or steroids **1**, **2**, **3**, **4**, and **5** required to inhibit 5α-reductase activity by 50%), three series of tubes containing increasing concentrations of finasteride (10^{-11}, 10^{-8} M) or the steroids (10^{-11}, 10^{-4} M) were incubated in duplicate, in the presence of: 1 mM of dithiothreitol, 20 mM sodium phosphate buffer pHs of 7, 2 mM NADPH, 2 nM [1, 2, 6, 7-^{3}H]T (specific activity 95 Ci/mmol) and 250 μg of protein from enzymatic the fraction in a final volume of 1 ml. The reaction was carried out in duplicate at 37 °C for 60 min. Adding 1 ml of dichloromethane stopped the reaction, and the amount of dihydrotestosterone formed was determined as detailed above.

Competitive Studies, Determination of K_i For competition studies, tubes containing 3.15 nM of [^{3}H]dihydrotestosterone (Specific activity 110 Ci/mmol) plus a range of increasing concentrations (10^{-8}—10^{-4} M) of cold dihydrotestosterone and compounds **1**, **2**, **3**, **4**, and **5** were prepared.[17]

The cytosolic fraction obtained from the supernatant liquid of the prostate homogenate centrifuged at 140000×***g*** as described above, was stored at −70 °C. Aliquots of 200 μl of prostatic cytosol (2.4 mg protein, determined by the Bradford method)[15] were added and incubated (in duplicate) for 18 h at 4 °C in the tubes mentioned above. After this time, 800 μl of dextran-coated charcoal in TEDAM buffer (containing dithiotreitol) was added and 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. The tubes were vortexed and immediately centrifuged at 800×***g*** for 10 min; aliquots (200 μl) were subsequently submitted to radioactive counting. The inhibition constant (K_i) of each compound was calculated according to the procedures described by Cheng and Prusoff.[18]

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References

1) Li X., Chen C., Singh S. M., Labrie F., *Steroids*, **60**, 430—441 (1995).
2) Imperato-McGinley J., Gautier L., Peterson R. E., *Science*, **186**, 1213—1215 (1974).
3) Walsh P. C., Madden J. D., Harrod M. J., Goldtein J. L., McDonald P. C., Wilson J. D., *New Engl. J. Med.*, **291**, 944—949 (1974).
4) Imperato-McGinley J., Peterson R. E., Gautier T., Sturla E., *J. Steroid Biochem.*, **11**, 637—645 (1979).
5) Geller J., Albert J., Lopez D., Geller S., Niwayama G., *J. Clin. Endocrinol. Metab.*, **43**, 486—488 (1976).
6) Sitteri P. K., Wilson J. D., Mayfield J. A., *J. Clin. Invest.*, **49**, 1737—1745 (1970).
7) Anderson S., Berman D. M., Jenkins E. P., Russell D. W., *Nature* (London), **354**, 159—161 (1991).
8) Labrie F., Sugimoto Y., Luu-The V., Simard J., Lachance Y., Bachvarov D., leblanc G., Durocher F., Paquet N., *Endocrinology*, **131**, 1571—1573 (1992).
9) McConnel J., Wilson J. D., George F. G., Geller J., Pappas F., Stoner E., *J. Clin. Endocrin. Meta.*, **74**, 505—509 (1992).
10) Guess H. A., Heyese J. F., Gormely G. J., *Prostate*, **22**, 31—37 (1993).
11) Ramirez E., Cabeza M., Heuze I., Gutierrez E., Bratoeff E., Membrillo M., Lira A., *Chem. Pharm. Bull.*, **50**, 15—20 (2002).
12) Cabeza M., Bratoeff E., Flores E., Ramirez E., Calleros J., Montes D., Quiroz A., Heuze I., *Chem. Pharm. Bull.*, **50**, 1447—1452 (2002).
13) Cabeza M., Heuze I., Bratoeff E., Murillo E., Ramirez E., Lira A., *Chem. Pharm. Bull.*, **49**, 1081—1084 (2001).
14) Liang T., Cascieri M. A., Cheung A. H., Reynolds G. F., Rasmusson G. H., *Endocrinology*, **117**, 571—579 (1985).
15) Bradford M. M., *Anal. Biochem.*, **72**, 248—254 (1986).
16) Hirosumi J., Nakayama O., Fagan T., Sawada K., Chida N., Inami M., Takahashi S., Kojo H., Notsu Y., Okuhara M., *J. Steroid Biochem Molec. Biol.*, **52**, 357—363 (1995).
17) Bratoeff E., Ramirez E., Murillo E., Flores G., Cabeza M., *Curr. Med. Chem.*, **6**, 1107—1123 (1999).
18) Cheng Y. C., Prusoff W. H., *Biochem. Pharmacol.*, **22**, 3099—3108 (1973).