Novel 17 Substituted Pregnadiene Derivatives as 5α-Reductase Inhibitors and Their Binding Affinity for the Androgen Receptor

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The *in vitro* antiandrogenic activity of four new progesterone derivatives: 4, 5, 6 and 7 (8 is a known compound) was determined. These compounds were evaluated as 5α-reductase inhibitors as well as by their capacity to bind to the androgen receptor in gonadectomized hamster prostate. The IC_{50} value was determined using increasing concentrations of 4, 5, 6, 7 and 8 in the presence of [^{3}H]T and the microsomal fraction of the hamster prostate containing the 5α-reductase enzyme. In this paper we also demonstrated the effect of increasing concentrations of the novel steroids upon [^{3}H]DHT binding to the androgen receptors from hamster prostate which produces competition for the androgen receptor sites. The *in vitro* studies showed that steroids 4, 5, 6, 7 and 8 had an inhibitory activity for the 5α-reductase with IC_{50} of: 4 (0.17 μM), 5 (0.19 μM), 6 (1 μM), 7 (4.2 μM), and 8 (2.7 μM). On the other hand, the IC_{50} value for compounds 4, 5, 6, 7, 8 and DHT showed the following order of affinity for the androgen receptor: 6>7>5>DHT. Surprisingly compounds 4 and 8 did not bind to the androgen receptor. The overall data indicate that all synthesized compounds are inhibitors for the enzyme 5α-reductase present in the hamster prostate. In contrast, compounds 5, 6 and 7, which have a cyclohexyl group in the side chain showed a high affinity for the androgen receptor.

Key words 5α-reductase; prostate; pregnadiene derivative; androgen receptor

Virilization in mammals is mediated by two steroidal hormones, testosterone **1** (T) and dihydrotestosterone **2** (DHT). Both hormones bind to the typical steroidal hormone receptor, the androgen receptor, and this activates the genes containing the androgen-responsive DNA sequence.

The notion that **2** (DHT) is a potent androgen with physiological roles distinct from those of **1** (T) came from two facts. First, androgen target tissues contain an enzyme (steroid 5α-reductase) capable of reducing T to DHT,[1)] and second, the product of this enzyme is accumulated in the nuclei of responsive cells, such as those of rat ventral prostate.[1,2)] The subsequent study of an inborn error of male phenotypical sexual differentiation, now termed steroid 5α-reductase type 2 deficiency, provides genetic proof for the role of DHT in androgen action.[3)]

The deficiency of 5α-reductase in males results in an incomplete differentiation of the external genitalia at birth.[1)] At puberty, these patients have normal to elevated levels of T in plasma and virilization occurs, but the prostate remains small. These individuals never have acne or temporal recession of the hairline. This low level of DHT is typical of male pseudohermaphroditism.[4)] On the other hand, abnormally high 5α-reductase activity in humans results in excessively high DHT levels in peripheral tissues, which is implicated in the pathogenesis of prostate cancer, benign prostatic hyperplasia (BPH), acne, and male patte baldness,[5)] suggesting that both the enzyme and DHT play important physiological and pathological roles in humans. Therefore, the inhibition of androgen action by 5α-reductase inhibitors is a logical treatment for 5α-reductase activity disorders. Furthermore, by the beginning of the past decade two types of 5α-reductase enzyme had been identified as types I and II.[1,5)] 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,[4,5)] which includes the drug finasteride **3**. This compound is the first 5α-reductase inhibitor approved in the USA for the treatment of BPH. This drug has approximately a 100-fold greater affinity for type II-5α-reductase than for the type I enzyme. In humans, finasteride decreases prostatic DHT levels by 70—90% and reduces prostate size, while T tissue levels remain constant.[4)] The use of finasteride demonstrated a sustained improvement in the treatment of androgen dependent diseases and a reduction in prostate specific antigen (PSA) levels.[5)]

Previously it has been stated that β-sitosterol significantly improves the symptoms and urinary flow parameters present in BPH,[6)] and it appears that it can inhibit the growth and migration of one type of prostate cancer cell and to slow the growth of prostate tumors in laboratory mice.[7)] On the other hand, it was reported that β-sitosterol inhibits 5α-reductases type I and II with $IC_{50} > 100$.[8)]

Recently our group synthesized several new steroidal derivatives (17-alfa-acyloyloxypregnadiene and pregnatriene) that decrease the prostate growth produced by T.[9—12)]

In view of the fact that the endocyclic dienes and trienes described above[9—12)] showed a high biological activity, we describe here the synthesis of four new progesterone derivatives having a conjugated exocyclic double bond: 6-methylene-17α-(3-cyclopentylpropionyloxy)-pregn-4-ene-3,20-dione **4**; 6-methylene-17α-(4-cyclohexylbutyryloxy)-pregn-4-ene-3,20-dione **5**; 6-methylene-17α-(3-cyclohexylpropionyloxy)-pregn-4-ene-3,20-dione **6** and 6-methylene-17α-(2-cyclohexylacetoxy)-pregn-4-ene-3,20-dione **7**. These compounds were evaluated as 5α-reductase inhibitors as well as antagonists for the androgen receptor.

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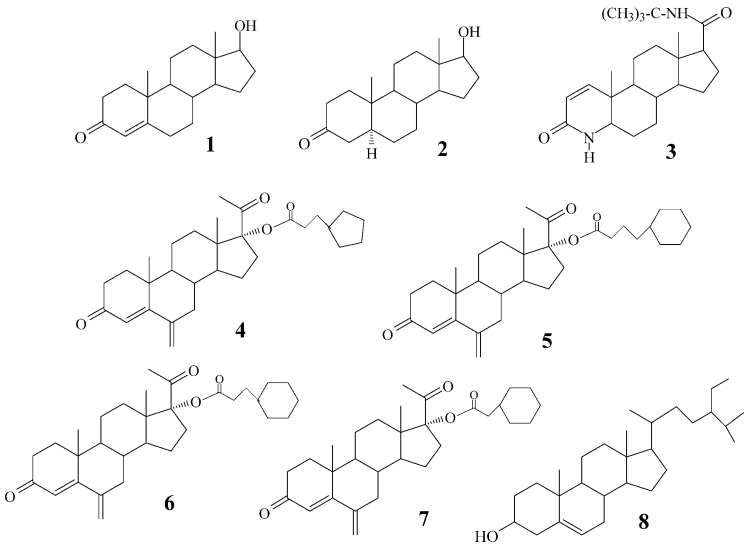

Fig. 1. Steroidal Structures

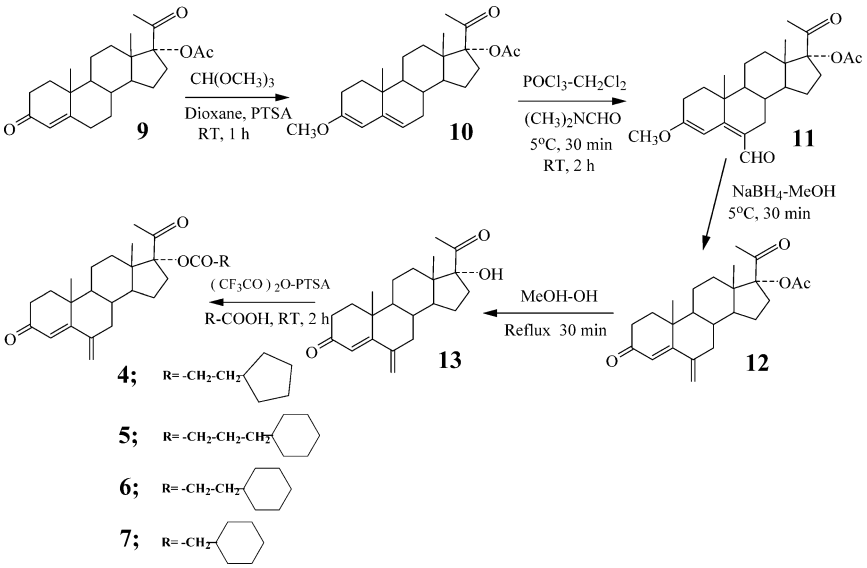

Fig. 2. Synthesis of Compounds **4**, **5**, **6** and **7**

Results

Synthesis of the Steroidal Derivatives 4, 5, 6 and 7 These compounds were prepared from the commercially available 17α-acetoxyprogesterone **9** (Fig. 2). The reaction of steroid **9** in dioxane with trimethyl orthoformate for 1 h at room temperature afforded the enol ether **10**. Treatment of **10** with phosphorous oxychloride in *N*,*N*-dimethylformamide and methylene chloride yielded the 6-formyl enol ether **11** (the Vilsmeyer reaction). Reduction of the formyl group in **11** with sodium borohydride in methanol afforded the 6-hydroxymethylene derivative (not isolated) which dehydrated to the desired exocyclic methylene derivative with a simultaneous hydrolysis of the enol ether to form the desired 17α-acetoxy-6-methylenepregn-4-ene-3,20-dione **12**. Upon treatment with methanol and sodium hydroxide, **12** afforded the free alcohol **13**. This compound was esterified with the corresponding acid in the presence of trifluoroacetic anhydride and *p*-toluenesulfonic acid to form the desired esters **4**, **5**, **6** and **7**.

Biological Activity The biological activity of compounds **4**, **5**, **6**, **7**, **8** was determined in gonadectomized hamster prostate glands homogenized and centrifuged to obtain the prostatic enzyme and cytosolic fractions.

The activity of 5α-reductase was assessed incubating the enzymatic fraction with 2 nM [^{3}H]T. The dichloromethane extract from castrated male hamster prostates was subjected to TLC analysis. The zone corresponding to DHT 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 the Novel Compounds in Hamster Prostate The concentration of compounds **4**, **5**, **6**, **7**, **8** required for inhibiting 5α-reductase activity by 50% (IC_{50}) was determined from the inhibition curves using different concentrations of the steroids and are shown in Table 1.

The IC_{50} value obtained for compounds **4** and **5** showed a higher 5α-reductase inhibitory activity than the derivatives **6,**

7 and **8** (Table 1).

Competition Analysis of Compounds 4, 5, 6, 7 and 8 for the Androgen Receptors The effect of increasing concentrations of non-radioactive steroids upon [^{3}H]DHT binding to androgen receptors from male hamster prostate in two different experiments is shown in Fig. 3. The IC_{50} value for compounds **4**, **5**, **6**, and **7** and DHT shows the following order of affinity for the androgen receptors: **6**>**7**>**5**>DHT. Compounds **5**, **6** and **7** showed high affinity for the androgen receptor, whereas steroids **4** and **8** did not form a complex with the androgen receptor.

Discussion

In this study, we assessed the inhibitory potency of four novel steroids **4**, **5**, **6** and **7**. The 5α-reductase inhibitory activity of these compounds was determined by comparing the individual IC_{50} values. Compound **4** with a cyclopentyl side chain is slightly more active (IC_{50} of 0.17 μM) than steroid **5** (IC_{50} of 0.19 μM) which has a longer side chain and a bulkier cyclohexyl group. Several years ago we determined the 5α-reductase inhibitory activity of similar compounds and the results from this study showed very clearly that when the length and the size of the side chain at C-17 increase, the 5α-reductase inhibitory activity decreases.[13] In view of the fact that steroids **5**, **6** and **7** have a bulkier side chain (cyclohexyl group) than **4** (with a cyclopentyl group), this fact explains very well the lower 5α-reductase inhibitory activity of compounds **5**, **6** and **7**. Previous studies[9] carried out in our laboratory indicated that the inhibition of the enzyme 5α-reductase proceeds *via* a Michael type addition reaction of the nucleophilic portion of the enzyme (amino group) to the conjugated double bond of the steroid. In this study, we also determined that bulky groups at C-17 inhibit this addition and as a consequence of this steric inhibition, compounds **5**, **6** and **7** have a lower activity.

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

Steroid	IC_{50} (μM)
4	0.17
5	0.19
6	1.00
7	4.2
8	2.7

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

Compounds **5**, **6** and **7** exhibited a higher affinity for the androgen receptor than steroid **4** (Fig. 3). Apparently, the higher symmetry of the cyclohexyl substituent in **5**, **6** and **7** enhanced the binding affinity as compared to compound **4** which has a cyclopentyl group in the side chain.

In this study we also reported the 5α-reductase inhibitory activity of β-sitosterol **8** with an IC_{50} value of 2.7 μM. This compound failed to show a binding tendency for the androgen receptor from hamster prostate cytosol. Although Berges *et al.*[6] indicated that compound **8** improved the symptoms of benign prostatic hyperplasia, the low 5α-reductase inhibitory activity as shown by its IC_{50} value makes this steroid a poor candidate for the treatment of androgen dependent diseases.

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.). Radioinert T and 5α-DHT were supplied by Steraloids (Wilton, NH, U.S.A.). Sigma-Aldrich (ST. Louis, MO, U.S.A.) supplied NADPH and pure β-sitosterol (**8**).

Synthesis of the Steroidal Compounds 17α-Acetoxy-3-methoxypregna-3,5-diene-20-one **10**: A solution containing steroid **9** (1 g, 2.68 mmol), PTSA (200 mg), dioxane (10 ml) and trimethyl orthoformate (5 ml, 4.57 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) the crude product **10** precipitated and it 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, CH_3-18), 1.2 (3H, s, CH_3-19), 2.0 (3H, s, CH_3-21), 2.2 (3H, s, CH_3-acetoxy), 3.5 (3H, s, CH_3-enol ether), 5.8 (1H, s, H-4) and 6.0 (1H, d, J=2 Hz, H6). ^{13}C-NMR ($CDCl_3$) δ: 14.3 (C-18), 17.4 (C-19), 26.3 (C-21), 50.8 (enol ether), 170.7 (ester carbonyl), 204 (C-20). MS (m/z) 386 (M^+).

17α-Acetoxy-6-formyl-3-methoxypregna-3,5-diene-20-one **11**: 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

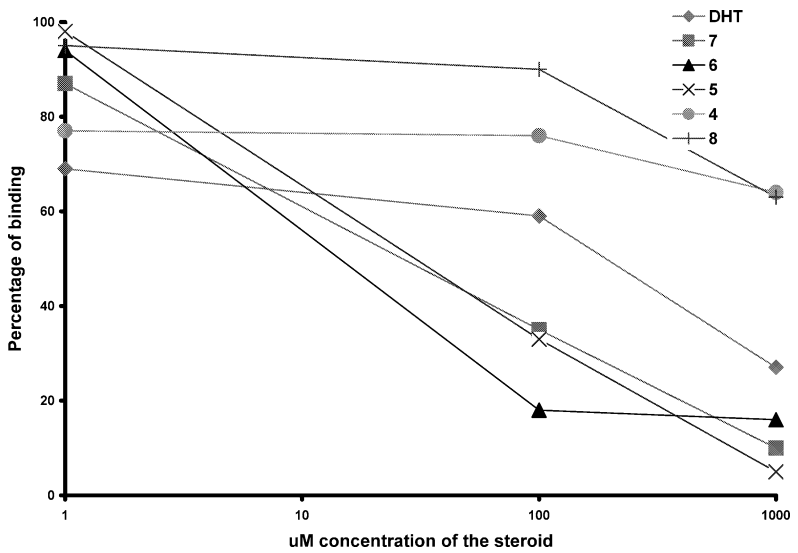

Fig. 3. Binding Specificity

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

5 °C. Steroid **10** (200 mg, 0.52 mmol) dissolved in methylene chloride (2 ml) and pyridine (1.5 ml) was added, keeping the temperature at 0 °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 three 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 (ε=10800) and 315 (ε=15600). IR (KBr) cm^{-1}: 2946, 1731 and 1652. ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s, CH_3-18), 1.2 (3H, s, CH_3-19). 2.0 (3H, s, CH_3-21), 2.2 (3H, s, CH_3-acetoxy), 3.5 (3H, s, CH_3-enol ether), 6.2 (1H, s, H-4) and 10.2 (1H, s, H-formyl). ^{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), 190 (formyl) and 204 (C-20). MS (*m*/*z*) 414 (M^+).

17α-Acetoxy-6-methylenepregn-4-ene-3,20-dione **12**: A suspension containing steroid **11** (100 mg, 0.24 mmol), methanol (2 ml) and sodium borohydride (20 mg, 0.53 mmol) was stirred for 30 min at 5 °C. 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, 1370 and 1262. ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s, CH_3-18), 1.2 (3H, s, CH_3-19), 2.0 (3H, s, CH_3-21), 2.2 (3H, s, CH_3-acetoxy), 5.2 (2H, d, J=2.1 Hz, CH_2 at C-6) and 5.8 (1H, s, H-4). ^{13}C-NMR ($CDCl_3$) δ: 14.2 (C-18), 17.1 (C-19), 26.3 (C-21), 120 (exocyclic methylene at C-6), 150 (C-6), 170.6 (ester carbonyl) and 203.9 (C-20). MS (*m*/*z*) 384 (M^+).

17α-Hydroxy-6-methylene-4-ene-3,20-dione **13**: A solution containing steroid **12** (1 g, 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. The reaction mixture was extracted with chloroform, the organic phase was separated, washed with water and dried over anhydrous sodium sulfate. The solvent was eliminated 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, CH_3-18), 1.22 (3H, s, CH_3-19), 2.0 (3H, s, CH_3-21), 5.2 (2H, d, J=2.1 Hz, CH_2 at C-6) and 6.0 (1H, s, H-4). ^{13}C-NMR ($CDCl_3$) δ: 15.4 (C-18), 17.1 (C-19), 23.8 (C-21), 122 (exocyclic methylene at C-6), 155 (C-6), 175.2 (C-3) and 211.5 (C-20). MS (*m*/*z*) 342 (M^+).

Preparation of Compounds 4, 5, 6 and 7 These esters were prepared according to the following general procedure.

A solution containing steroid **13** (1 g, 2.92 mmol), PTSA (10 mg), trifluoroacetic anhydride (8.29 g, 42.48 mmol) and the corresponding acid (10 mmol) was stirred for 2 h at room temperature (nitrogen atmosphere). The reaction mixture was neutralized with an aqueous solution of sodium hydroxide to a pH of 7 and diluted with chloroform (10 ml). The organic phase was separated, washed with water, dried over anhydrous sodium sulfate, filtered and the solvent was eliminated in vacuum. The crude product was purified by silica gel column chromatography. Hexane–ethyl acetate (8 : 2) eluted the pure ester.

17α-(3-Cyclopentylpropionyloxy)-6-methylenepregn-4-ene-3,20-dione **4**: Yield 40%, mp 140—143 °C. UV (nm): 264 (ε=10800). IR (KBr) cm^{-1}: 2947, 1731, 1715, 1674 and 1264. ^{1}H-NMR ($CDCl_3$) δ: 0.7 (3H, s, CH_3-18), 1.1 (3H, s, CH_3-19), 2.0 (3H, s, CH_3-21), 2.2 (2H, m, CH_2 α to carbonyl of ester), 5.0 (2H, d, J=1.8 Hz, CH_2 at C-6) and 5.9 (1H, s, H-4). ^{13}C-NMR ($CDCl_3$) δ: 14.4 (C-18), 17.1 (C-19), 26.4 (C-21), 118 (exocyclic methylene at C-6), 150 (C-6), 173.4 (ester carbonyl) and 199.8 (C-20). MS (*m*/*z*) 466 (M^+).

6-Methylene-17α-(4-cyclohexylbutyryloxy)-pregn-4-ene-3,20-dione **5**: Yield 47%, mp 114—117 °C. UV (nm): 255 (ε=10600). IR (KBr) cm^{-1}: 2922, 1730, 1710, 1674 and 1226. ^{1}H-NMR ($CDCl_3$) δ: 0.71 (3H, s, CH_3-18), 1.1 (3H, s, CH_3-19), 2.0 (3H, s, CH_3-21), 2.3 (2H, m, CH_2 α to carbonyl of ester), 4.96 (2H, d, J=2 Hz, CH_2 at C-6) and 5.9 (1H, s, H-4). ^{13}C-NMR ($CDCl_3$) δ: 14.4 (C-18), 17.1 (C-19), 26.3 (C-21), 120 (exocyclic methylene at C-6), 152 (C-6), 173.6 (ester carbonyl) and 199.8 (C-20). MS (*m*/*z*) 494 (M^+).

17α-(3-Cyclohexylpropionyloxy)-6-methylenepregn-4-ene-3,20-dione **6**: Yield 46%, mp 146—148 °C. UV (nm): 260 (ε=11000). IR (KBr) cm^{-1}: 2923, 1705, 1673 and 1227. ^{1}H-NMR ($CDCl_3$) δ: 0.7 (3H, s, CH_3-18), 1.1 (3H, s, CH_3-19), 2.0 (3H, s, CH_3-21), 2.2 (2H, m, CH_2 α to ester carbonyl), 5.07 (2H, d, J=1.8 Hz, CH_2 at C-6) and 5.93 (1H, s, H-4). ^{13}C-NMR ($CDCl_3$) δ: 14.4 (C-18), 17.1 (C-19), 26.4 (C-21), 118 (exocyclic methylene at C-6), 155 (C-6), 173.8 (ester carbonyl) and 199.8 (C-20). MS (*m*/*z*) 480 (M^+).

17α-(Cyclohexylacetoxy)-6-methylenepregn-4-ene-3,20-dione **7**: Yield 48%, mp 134—136 °C. UV (nm): 254 (ε=10700). IR (KBr) cm^{-1}: 2924, 1729, 1710, 1673 and 1226. ^{1}H-NMR ($CDCl_3$) δ: 0.72 (3H, s, CH_3-18), 1.09 (3H, s, CH_3-19), 2.0 (3H, s, CH_3-21), 2.3 (2H, m, CH_2 α to ester carbonyl), 5.07 (2H, d, J=2 Hz, CH_2 at C-6), 5.93 (1H, s, H-4). ^{13}C-NMR ($CDCl_3$) δ: 14.3 (C-18), 17.1 (C-19), 26.0 (C-21), 114 (exocyclic methylene at C-6), 145 (C-6), 172.7 (ester carbonyl) and 199.8 (C-20). MS (*m*/*z*) 466 (M^+).

Biological Activity of the Synthesized Compounds The biological activity of steroids **4**, **5**, **6**, **7** and **8**, was determined in *in vitro* experiments using the prostate glands from gonadectomized adult male golden hamsters. The animals (150—200 g) were obtained from the Metropolitan University-Xochimilco of Mexico. Gonadectomies were performed under pentobarbital anesthesia 3 d before the experiments and the animals were sacrificed with CO_2.

The prostate glands were immediately removed, blotted and weighed prior to their use. Unless specified, all procedures were carried out a 4 °C.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 (40 mM Tris–HCl, 3 mM EDTA and 20 mM sodium molybdate, dithiotreitol 0.5 mM, 20% glicerol) at pH 7.5 and at 4 °C with a tissue homogenizer. Homogenates were centrifuged at 140000×***g*** for 60 min[11] 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.[14,15] 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[16]) was used as a source of 5α-reductase.

Determination of 5α-Reductase Activity The 5α-reductase activity was assayed as previously described.[17] 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). This 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 dichloromethane 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 Keiselgel 60 F_{254} plates. T and DHT were used as carriers and the plate was developed in chloroform : acetone=9 : 1. It was air-dried and the chromatography was repeated 2 more times. The T standard was visualized under UV lights (254 nm) and DHT was detected using phosphomolibdic acid reagent with a posterior heating of the plate. DHT developed a classical dark blue color. The DHT containing areas were cut, the strips were soaked in 5 ml of Ultima Gold (Packard) and the radioactivity was determined in a Tri Carb 2100TR liquid scintillation analyzer.

Determination of 50% Inhibitory Concentration Steroids 4, 5, 6, 7 and 8 in Gonadectomized Hamster Prostate In order to calculate the IC_{50} values (the concentration of steroids **4**, **5**, **6**, **7** and **8** required to inhibit 5α-reductase activity by 50%), three series of tubes containing increasing concentrations of **4**, **5**, **6**, **7** and **8** (10^{-11}, 10^{-4} M) were incubated in duplicate as detailed above.

Competitive Studies For competitive studies, tubes containing 3.15 nM of [^{3}H]DHT (specific activity 110 Ci/mmol) plus a range of increasing concentrations (10^{-6}—10^{-3} M) of cold DHT and compounds **4**, **5**, **6**, **7** and **8** were prepared.[14,16]

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[16]) were added and incubated (duplicate) for 18 h at 4 °C in the tubes as previously described. Eight hundred microliters of dextran-coated charcoal in TEDAM buffer (containing dithiotreitol) was then 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 to the mixture. The tubes were vortexed and immediately centrifuged at 800×***g*** for 10 min; aliquots (200 μl) were taken and submitted for radioactive counting. The IC_{50} of each compound was calculated according to the plots.

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References

1) Russell W. D., Wilson J. D., *Annu. Rev. Biochem.*, **63**, 25—61 (1994).
2) Li X., Chen C., Singh S. M., Labrie F., *Steroids*, **60**, 430—441 (1995).
3) Labrie F., Sugimoto Y., Luu-The V., Simard J., Lachance Y., Bachvarov D., Leblanc G., Durocher F., Paquet N., *Endocrinology*, **131**, 1571—1573 (1992).
4) McConnel J., Wilson J. D., George F. G., Geller J., Pappas F., Stoner E., *J. Clin. Endocrin. Metab.*, **74**, 505—509 (1992).
5) Brooks J. R., Harris G. S., Sandler M., Smither H. J. (eds.), "Design of Enzyme Inhibitor as Drug," Vol. 2, Oxford University Press, Oxford, 1984, pp. 495—542.
6) Berges R. R., Windeler J., Trampisch H. J., Senge T. H., *Lancet*, **345**, 1529—1532 (1995).
7) Awad A. B., Fink C. S., Williams H., Kim U., *Eur. J. Cancer Prev.*, **10**, 507—513 (2001).
8) Raynaud J. P., Cousse H., Martin P. M., *J. Steroid Biochem. Mol. Biol.*, **82**, 233—239 (2002).
9) Ramírez E., Cabeza M., Heuze I., Gutiérrez E., Bratoeff E., Membrillo M., Lira A., *Chem. Pharm. Bull.*, **50**, 15—20 (2002).
10) Cabeza M., Heuze I., Bratoeff E., Murillo E., Ramirez E., Lira A., *Chem. Pharm. Bull.*, **49**, 1081—1084 (2001).
11) Cabeza M., Quiroz A., Bratoeff E., Heuze I., Ramirez E., Flores E., *Proc. West Pharmacol. Soc.*, **45**, 166—167 (2002).
12) Cabeza M., Bratoeff E., Flores E., Ramírez E., Calleros J., Montes D., Quiroz A., Heuze I., *Chem. Pharm. Bull.*, **50**, 1447—1452 (2002).
13) Bratoeff E., Herrera H., Ramírez E., Murillo E., Quiroz A., Cabeza M., *Chem. Pharm. Bull.*, **48**, 1249—1255 (2000).
14) Liang T., Cascieri M. A., Cheung A. H., Reynolds G. F., Rasmusson G. H., *Endocrinology*, **117**, 571—579 (1985).
15) Flores E., Bratoeff E., Cabeza M., Ramirez E., Quiroz A., Heuze I., *Med. Chem.*, **3**, 225—237 (2003).
16) Bradford M. M., *Anal. Biochem.*, **72**, 248—254 (1986).
17) Hirosumi J., Nakayama O., Fagan T., Sawada K., Chida N., Inami M., Takahashi S., Kojo H., Notsu Y., Okuhara M., *J. Steroid Biochem. Mol. Biol.*, **52**, 357—363 (1995).