Synthesis and Pharmacological Evaluation of 4-Halo Progesterone Derivatives as Antiandrogen

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The pharmacological activity of eight pregnane derivatives 17-α acetoxyprogesterone 9, 17-α acetoxy-4, 5-epoxypregnan-3, 20-dione 10, 17-α acetoxy-4-chloro-4-pregnene-3, 20-dione 11, 17-α acetoxy-4-bromo-4-pregnene-3, 20-dione 12, 17-α hydroxy-4-bromo-4-pregnene-3, 20-dione 13, 4-chloro-17-α hydroxy-4-pregnene-3, 20-dione 14, 17-α benzoyloxy-4-bromo-4-pregnene-3, 20-dione 15 and 17-α benzoyloxy-4-chloro-4-pregnene-3, 20-dione 16 was determined. These compounds were evaluated as antiandrogens on gonadectomized hamster seminal vesicles.

The pharmacological data in this study indicate that compounds 15 and 16 having a C-17 benzoyloxy moiety showed the highest antiandrogenic activity as measured by the reduction of the weight of the seminal vesicles, followed by the steroids 11 and 12 (17-α acetoxy group). The free alcohols 13 and 14 exhibited a lower antiandrogenic activity. Apparently, the ester moiety at C-17 is a necessary requirement for the presence of high antiandrogenic activity. shows the inhibitory effect on the conversion of testosterone (T) to DHT, of the above described steroids as measured by the amount of produced DHT 2 expressed as pmoles of DHT/g of protein/h.

Steroids 11, 12 and 16 showed a much higher inhibitory activity on the conversion of testosterone (T) to dihydrotestosterone (DHT) than presently used finasteride 3.

Key words seminal vesicles; 5α-reductase; testosterone-conversion; halogen substituents; progesterone derivatives

Enzymatic 5-α reduction of testosterone (T) **1** (Fig. 1) which produces the more potent androgen, 5-α dihydrotestosterone (DHT) **2**, occurs in those organs classically categorized as androgen target organs, chiefly the male accessory glands.[1] This tissue enzyme profile results in the accumulation of the potent androgen **2** in the classical androgen target organs.

In several endocrine abnormalities, including benign prostate hyperplasia (BPH), male pattern baldness, acne and hirsutism, 5-α reduction plays a principal role. For this reason it is important to develop 5-α reductase inhibitors for the treatment of these afflictions.

The most extensively studied class of 5-α reductase inhibitors are the 4-azasteroids[2,3] which includes the drug finasteride **3** (Fig. 1). Finasteride is the first 5-α reductase inhibitor approved in the U.S.A. for the treatment of BPH. This drug has approximately a 100-fold greater affinity for type 2 5-α reductase than for the type 1 enzyme. In humans, finasteride decreases prostatic DHT **2** levels by 70—90% and reduces prostate size, while T tissue levels remain constant.[4] The use of finasteride **3** demonstrated a sustained improvement in the treatment of androgen dependent diseases and reduction in prostate specific antigen (PSA) levels.[5] Related analogs **4**, **5** and **6** (Fig. 1) have also demonstrated effectiveness *in vitro* and *in vivo*.[6,7]

Androstane-3-carboxylic acids **7** and **8** (Fig. 1) were recently synthesized and have shown a potent uncompetitive inhibition of type 2 5-α reductase.[8,9] Epristeride **7** has exhibited the ability to lower serum DHT **2** levels by 50%.[10]

In this study we evaluated the following compounds as antiandrogens: 17-α acetoxyprogesterone **9** (Fig. 2), 17-α acetoxy-4, 5-epoxypregnan-3, 20-dione **10**, 17-α acetoxy-4-

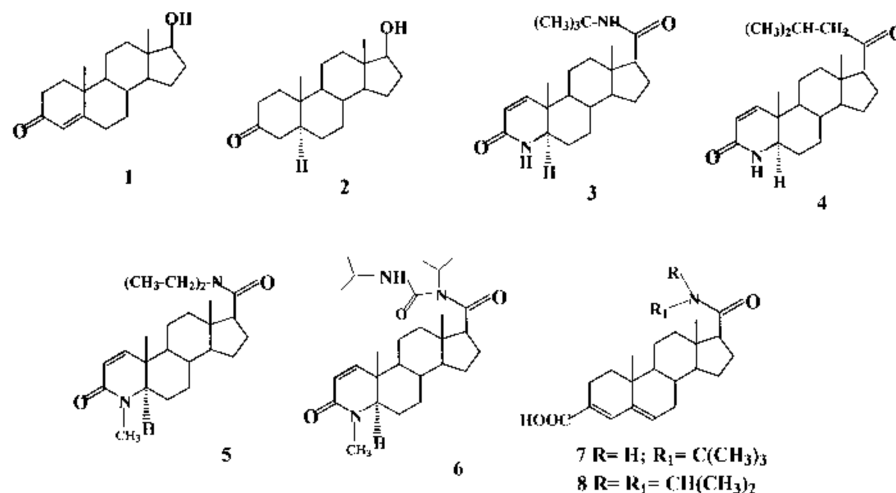

Fig. 1. Steroidal Antiandrogens

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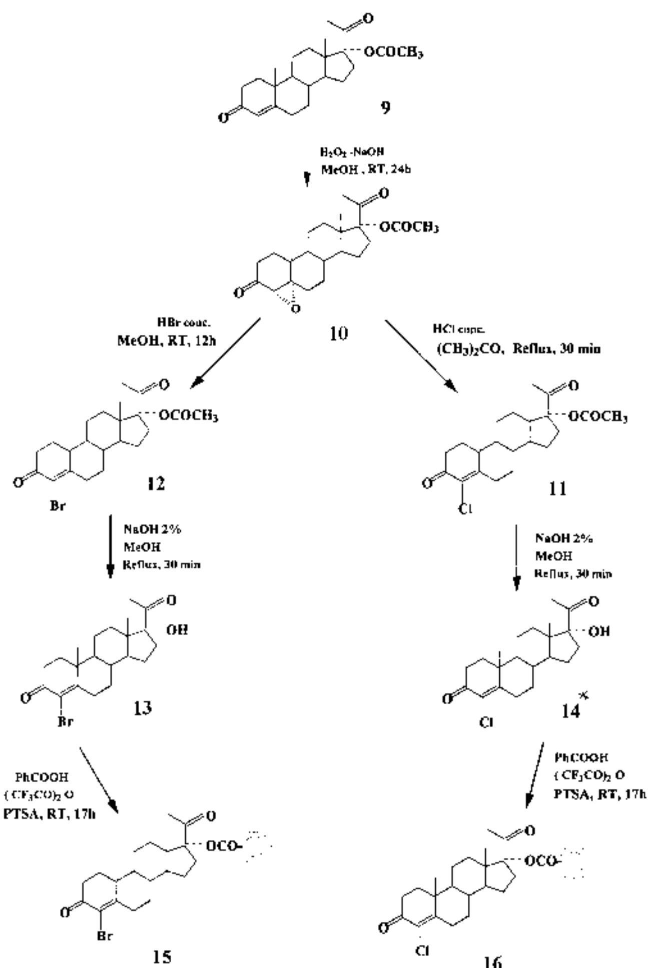

Fig. 2. Synthesis of 4-Haloderivatives

chloro-4-pregnene-3, 20-dione **11**, 17-α acetoxy-4-bromo-4-pregnene-3, 20-dione **12**, 17-α hydroxy-4-bromo-4-pregnene-3, 20-dione **13**, 4-chloro-17-α hydroxy-4-pregnene-3, 20-dione **14**, 17-α benzoyloxy-4-bromo-4-pregnene-3, 20-dione **15** and 17-α benzoyloxy-4-chloro-4-pregnene-3, 20-dione **16**.

The biological activity of these steroids was determined on *in vitro* metabolic change in the T (**1**) molecule produced by 5-α reductase[11,12] and by the *in vivo* steroid action upon the seminal vesicles,[12] where the compound is bound to its cognate receptors.[12,13]

To test the antiandrogenic effect of the synthesized compounds, we used the seminal vesicles of gonadectomized male hamsters.[11,12] Seminal vesicles are male accessory glands that are androgen dependent. These organs are capable of reducing T (**1**) to DHT (**2**) in both intact and gonadectomized animals.[14]

Chemistry As shown in Fig. 2 the active compounds **11**, **12**, **15** and **16** were synthesized from the commercially available 17-α acetoxyprogesterone **9**. Previous reports indicated that compound **9** was synthesized by Mukawa in Japan.[15] In the first step, the C-4 double bond of **9** was epoxydized with hydrogen peroxide in basic medium[16,17]; the desired compound **10** underwent a ring opening reaction with hydrogen chloride or hydrogen bromide to form the corresponding chloroderivative[18] **11** and the bromo compound[18] **12**. Treatment of **11** and **12** with aqueous sodium hydroxide solution[19] afforded the corresponding alcohols **13** and **14**. The esterification of the C-17 hydroxyl group in **13** and **14** with benzoic acid and trifluoroacetic anhydride[19] afforded the desired final compounds **15** (4-bromo derivative) and **16** (4-chloro derivative).

Table 1. Weight and Protein Content of Seminal Vesicles from Animals Receiving Different S.C. Treatments

Treatment (mg)	Weight of seminal vesicles (mg)	Protein content mg/mg of tissue
T	99.0±5.0	0.0064±0.0010
T+**3**	81.0±2.1	0.0096±0.0005
T+**9**	86.1±10	0.0130±0.0005
T+**10**	95.7±8.0	0.0090±0.0002
T+**11**	79.6±3.0	0.0124±0.0006
T+**12**	76.8±2.8	0.0137±0.0005
T+**13**	86.7±8.0	0.0120±0.0020
T+**14**	73.9±11.0	0.0110±0.0020
T+**15**	68.4±3.0	0.0137±0.0080
T+**16**	56.1±3.0	0.0149±0.0010

Significant differences were observed between the weights of castrated testosterone-treated animals (T) and testosterone+finasteride (**3**)-treated animals. Compounds **11**—**16** decreased the weight significantly ($p<0.05$) compared with T-treated animals. Protein content changes in an inverse way relative to the seminal vesicles weight in all cases.

Methods and Results

Seminal Vesicles When **1** (T) and finasteride **3** were injected together, the weight of the seminal vesicles decreased significantly ($p<0.005$) as compared to T (**1**) treated animals (Table 1). The starting material **9** and the intermediate epoxy compound **10** failed to show a substantial decrease of the weight of the seminal vesicles (lack of an antiandrogenic effect). Steroids **11**—**16** decreased the weight of the glands significantly ($p<0.005$) as compared to the T (**1**) treated animals. Table 1 shows that compound **16** (17-α benzoyloxy-4-chloroderivative) has the highest antiandrogenic effect; the corresponding 4-bromoderivative **15** is slightly less active. On the other hand, compound **12** (17-α acetoxy-4-bromoderivative) is more active than the corresponding chloroderivative **11**. Surprisingly the benzoyloxyderivatives **15** and **16** have a much higher antiandrogenic activity than the corresponding acetoxy compounds, **11** and **12**. The starting material **9**, showed a very weak antiandrogenic effect, whereas the alcohols **13** and **14**, the epoxyderivative **10** did not show a comparable pharmacological activity.

Since the weight of the seminal vesicles depends on the 5-α reduced androgens,[1,20] it was important to determine the effect of these compounds on the conversion of T (**1**) to DHT (**2**) in this tissue.

The hexane extract from castrated male hamster seminal vesicles was subjected to TLC analysis. The zone corresponding to the DHT (**2**) standard (*Rf* value 0.33) of each experimental chromatogram was eluted and the radioactivity determined.

The results (Fig. 3) obtained from two separate experiments performed in duplicate demonstrated a difference between the conversion of [^{3}H]T to [^{3}H]DHT in the T plus finasteride **3**-treated animals thus indicating that finasteride **3** is a good inhibitor for the conversion of T to DHT at pH 6. The radioactive purity of the formed DHT is shown in Table

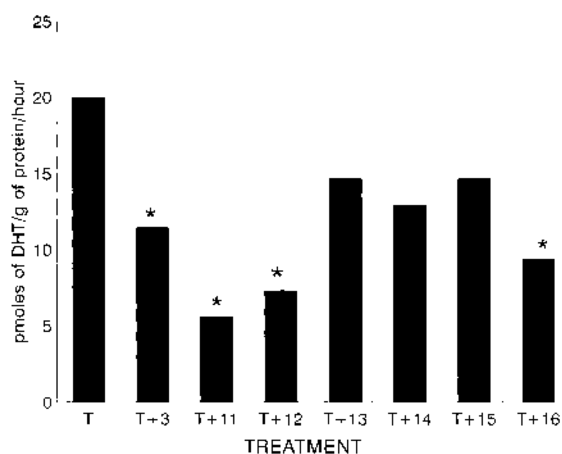

Fig. 3. Effect of Steroidal Structures on T conversion to DHT

T plus finasteride (T+**3**) inhibits significantly, $p<0.005$ (*). T conversion and T+**11** and T+**12** have higher inhibitory effect that the other compounds.

Table 2. Successive Recrystallizations

Treatment	Successive recrystallizations specific activity [^{3}H]DHT (dpm/mg)
T	Cr_1 111
	Cr_2 157
	ML_2 193
T+**3**	Cr_1 160
	Cr_2 200
	ML_2 222
T+**11**	Cr_1 216
	Cr_2 172
	ML_2 216
T+**12**	Cr_1 141
	Cr_2 146
	ML_2 142
T+**15**	Cr_1 181
	Cr_2 196
	ML_2 236
T+**16**	Cr_1 173
	Cr_2 129
	ML 193

The specific activity of the [^{3}H]DHT formed was obtained by reverse isotope dilution technique using pure DHT as standard. The results are given in dpm/mg. Cr, Crystallization number. ML, Mother liquors number.

2.

Figure 3 shows the effect of steroidal structure on the rate of DHT formation expressed as pmoles of DHT/g of protein/h. The most active compounds **11**, **12** and **16** show a lower rate of DHT formation than the control samples, T and T plus finasteride **3** (T+3). The 4-bromo-17-α benzoyloxy compound **15** although showed relatively high antiandrogenic activity (Table 1) in this case, it did not reduce the rate of DHT formation and showed a weak activity as T conversion inhibitor.

Discussion

This paper describes the synthesis of 6 steroidal compounds **11**—**16** (Fig. 2). The starting material **9** and the intermediate **10** are devoid of a halogen atom at C-4 and are biologically inactive. Steroids **12**, **14** and **16** have a chlorine atom at C-4 whereas **11**, **13** and **15** contain a bromine atom at the same position. Compounds **11**, **12** and **14** are known and were previously prepared by Japanese workers.[21,22] All of the bromo and chloro derivatives are biologically active as T conversion inhibitors and also show a significant decrease of the weight of seminal vesicles (Table 1) and therefore could be considered as antiandrogens.

Figure 3 shows the conversion of [^{3}H]T to [^{3}H]DHT expressed as pmoles of DHT/g of protein/h. Finasteride (**3**) reduces the conversion of T to DHT substantially and therefore can be considered as a 5-α reductase inhibitor at pH 6. Other authors who reported the inhibitory effect of similar compounds in agreement with those obtain these results.[23,24] Steroids **11**, **12**, and **16** inhibited significantly ($p<0.005$) the conversion of T to DHT at pH 6 in seminal vesicles homogenates from castrated male hamsters treated with a dose of 200 μg as compared to T (**1**).

This antiandrogenic effect of **11**—**16** is probably due to the presence of a halogen substituent at C-4 on the progesterone molecule (Fig. 2). Similar compounds with a halogen atom at C-6 have shown a very high antiandrogenic activity.[11,12,25] The results in Table 1 clearly indicate that compounds **15** and **16** with the benzoyloxy moiety at C-17 show a much higher antiandrogenic effect as compared to **11** and **12** with an acetoxy group at C-17. In the benzoyloxy series, compound **16** (chlorine at C-4) shows a higher antiandrogenic effect than the analog bromo derivative **15**. In the 17-α-acetoxy series, this behavior is reversed; the chloro derivative **11** shows a lower antiandrogenic activity than the corresponding bromo compound **12**.

The data for protein synthesis (Table 1) indicate that when a significant decrease ($p<0.005$) in the weight of the seminal vesicles has taken place, protein formation is increased. On the other hand, when the T conversion is inhibited, protein synthesis is increased (lower DHT formation) (Table 1). These proteins could be enzymes, membrane proteins, or cytosol receptors. These data are in agreement with those previously published by our group[12] and by Takayasu and Itami[26] who demonstrated that the synthesis of 5-α reductase enzyme is induced by androgen deprivation in hamster sebaceous glands.[20]

Probably in the future, compounds **11**, **12**, and **16** could have a potential application for the treatment of androgen dependent diseases.

Experimental

Chemicals and Radioactive Material 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 a 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; t, triplet; m, multiplet. Mass spectra were obtained with a HP5985-B spectrometer. IR spectra were obtained on a Perkin-Elmer 549B and the UV spectra were recorded on a Perkin-Elmer 200 s spectrometers. The elemental analysis were determined at the Christopher Ingold Laboratories, University College, London.

Ci/mmol, was provided by New England Nuclear Co. (Boston, MA) and radioinert T was kindly provided by Syntex of Cuernavaca (Mexico). D(α)-Glucose, which had a melting point of 146 °C, was purchased from Merck (Mexico). Sigma Chemical Co. (St. Louis, MO) supplied finasteride, 5-α DHT, and NADPH$^+$.

17-α Acetoxy-4, 5-epoxypregnan-3, 20-dione (10) To a solution of **9** (1 g, 2.57 mmol) in methanol (30 ml) was added 0.7 ml, 10% (1.75 mmol) of sodium hydroxide and 1.4 ml, 30%, 14.6 mmol of hydrogen peroxide. The mixture was stirred for 24 h at room temperature. The methanol was evaporated in vacuum. Upon cooling the desired compound **10** precipitated as colorless crystals, 0.99 g, 2.55 mol (99%). mp 192—194 °C. IR (KBr): 1712, 1722, 1730. ^{1}H-NMR ($CDCl_3$) δ: 0.65 (3H, s), 1.16 (3H, s), 2.04 (3H, s),

2.14 (3H, s), 3.01 (1H, s). ^{13}C-NMR, δ: 14.4 (C-18), 16.4 (C-19), 18.9 (C-21), 170.7 (CH_3-C̲OO), 203.9 (C-20), 206.9 (C-3). MS (*m/z*): 388 (M^+).

17-α Acetoxy-4-chloro-4-pregnene-3, 20-dione (11) To a solution of **10** (1 g, 2.6 mmol) in acetone (50 ml) was added concentrated hydrochloric acid (1 ml). The solution was allowed to reflux for 30 min. The organic solvent was evaporated in vacuum and the desired crude product **11** was precipitated upon addition of 200 g of ice. Yield 0.99 g, 2.4 mmol (92%). Recrystallization from methanol afforded the pure product 0.78 g (78%). mp 180—182 °C. UV: 253 nm (log ε, 4.08). IR (KBr) : 1689, 1714, 1732. ^{1}H-NMR ($CDCl_3$) δ: 0.71 (3H, s), 1.31 (3H, s), 2.08 (3H, s), 2.23 (3H, s). ^{13}C-NMR, δ: 14.3 (C-18), 17.7 (C-19), 21.18 (C-21), 170.0 (CH_3-C̲OO), 203.9 (C-20), 127.4 (C=C̲-Cl), 211.5 (C-3). MS (*m/z*): 407 (M^+). Calcd for $C_{23}H_{31}O_4Cl$: C, 67.88; H, 7.68; O, 15.73; Cl, 8.71. Found: C, 67.79; H, 7.75; O, 15.81; Cl, 8.79.

17-α Acetoxy-4-bromo-4-pregnene-3, 20-dione (12) To a solution of **10** (1 g, 2.6 mmol) in methanol (30 ml) was added concentrated hydrobromic acid (15 ml). The mixture was stirred for 12 h at room temperature, water (100 ml) was added and upon cooling the desired crude compounds **12** precipitated 0.98 g. The crude product was recrystallized from methanol to give 0.70 g, 1.55 mmol (60%) of the pure compound **12**. mp 195—196 °C. UV: 260 nm (log ε, 4.12). IR (KBr): 1671, 1710, 1736 and 614. ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s), 1.24 (3H, s), 2.05 (3H, s), 2.11 (3H, s). ^{13}C-NMR, δ: 14.3 (C-18), 17.8 (C-19), 21.20 (C-21), 170.0 (CH_3-C̲OO), 203.0 (C-20), 212.0 (C=C̲-Br), 190.0 (C-3). MS (*m/z*) : 450 (M^+). Calcd for $C_{23}H_{31}O_4Br$: C, 61.20; H, 6.92; O, 14.18; Br, 17.70. Found : C, 61.28; H, 6.87; O, 14.25; Br, 17.67.

17-α Hydroxy-4-bromo-4-pregnene-3,20-dione (13) To a solution of **12** (1 g, 2.6 mmol) in methanol (100 ml) was added a 2% solution of sodium hydroxide (25 ml) and the mixture was allowed reflux for 30 min. The methanol was evaporated in vacuum and to the aqueous phase it was added water (20 ml). The mixture was extracted 3 times with chloroform and the combined organic phases were washed repeatedly with water until a pH of 7. The chloroform solution was dried with sodium sulfate and the organic solvent was -vaporated in vacuum. The crude product was purified by column chromatography (hexane : AcOEt=6 : 4) and recrystallized from methanol to give 0.71 g, 1.74 mmol (66%) of the compound **13**. mp 204—206 °C. UV: 259 nm (log ε, 4.10). IR (KBr) : 1657, 1709, 3400. ^{1}H-NMR ($CDCl_3$) δ: 0.74 (3H, s), 1.17 (3H, s), 2.23 (3H, s), 3.57 (1H, s). ^{13}C-NMR, δ: 15.4 (C-18), 17.5 (C-19), 20.44 (C-21), 89.8 (C-17), 211.6 (C-20), 123.9 (C=C̲-Br), 199.5 (C-3). MS (*m/z*): 408 (M^+).

4-Chloro-17-α hydroxy-4-pregnene-3, 20-dione (14) To a solution of **11** (1 g, 1.2 mmol) in methanol (30 ml) was added a 2% solution of sodium hydroxide (50 ml) and the mixture was allowed to reflux to for 30 min. The methanol was evaporated in vacuum and to the aqueous phase was added water (30 ml). It was extracted 3 times with chloroform and the combined organic phases were washed repeatedly with water to a pH of 7. The chloroform solution was dried with sodium sulfate and the organic solvent was evaporated in vacuum. The crude product was purified by column chromatography (hexane : AcOEt=6 : 4) and recrystallized from methanol to give 0.68 g. 1.9 mmol (79%) of the compound **14**. mp 203—204 °C. UV: 253 nm (log ε, 4.08). IR (KBr) : 1658, 1712, 3420. ^{1}H-NMR ($CDCl_3$) δ: 0.71 (3H, s), 1.14 (3H, s), 2.26 (3H, s), 3.62 (1H, s). ^{13}C-NMR, δ: 15.4 (C-18), 17.5 (C-19), 20.4 (C-21), 89.8 (C-17), 211.6 (C-20), 123.9 (C=C̲-Cl), 194.0 (C-3). MS (*m/z*): 364 (M^+).

17-α Benzoyloxy-4-bromo-4-pregnene-3, 20-dione (15) To a solution of **13** (50 mg, 1.2 mmol) in chloroform (5 ml) was added benzoic acid 0.6 g, 4.9 mmol, PTSA acid (10 mg, 5.7 mmol) and trifluoroacetic anhydride (0.2 ml, 2.7 mmol). The resulting solution was stirred under nitrogen for 17 h at room temperature. Ice water was added and the reaction mixture was neutralized with sodium bicarbonate to a pH of 7. The solution was extracted with chloroform; the combined organic extracts were washed with water, dried over sodium sulfate and the solvent evaporated in vacuum. The crude product was purified by column chromatography (hexane : AcOEt=7 : 3) to give 30 mg, 0.6 mmol (50%) of the pure compound **15**. mp 225—228 °C. UV: 253 nm (log ε, 4.12). IR (KBr): 1669, 1702, 1718. ^{1}H-NMR ($CDCl_3$) δ: 0.77 (3H, s), 1.24 (3H, s), 2.15 (3H, s), 8.06 (2H, m), 7.49 (3H, m). ^{13}C-NMR, δ: 14.5 (C-18), 17.6 (C-19), 20.7 (C-21), 170.7 (PhC̲OO), 203.9 (C-20), 128.5 (C=C̲-Br), 190.5 (C-3). MS (*m/z*): 513 (M^+). Calcd for $C_{28}H_{33}O_4Br$: C, 65.50; H, 6.48; O, 12.46; Br, 15.56. Found: C, 65.62, H, 6.54; O, 12.53; Br, 15.68.

17-α Benzoyloxy-4-chloro-4-pregnene-3, 20-dione (16) To a solution of **14** (50 mg, 1.4 mmol) in chloroform (5 ml) was added benzoic acid (0.6 g, 4.9 mmol), PTSA acid (10 mg, 5.7 mmol) and trifluoroacetic anhydride (0.2 ml, 2.7 mmol). The resulting solution was stirred under nitrogen for 17 h at room temperature. Ice water was added and the reaction mixture was neutralized with sodium bicarbonate to a pH of 7. The solution was extracted with chloroform; the combined organic extracts were washed with water, dried over sodium sulfate and the solvent evaporated in vacuum. The crude product was purified by column chromatography (hexane : AcOEt=7 : 3) to give 26 mg, 0.55 mmol (39%) of the pure compound **16**. mp 223—226 °C. UV: 251 nm (log ε, 4.12). IR (KBr): 1671, 1709, 1714. ^{1}H-NMR ($CDCl_3$) δ: 0.81 (3H, s), 1.39 (3H, s), 2.23 (3H, s), 8.17 (2H, m), 7.53 (3H, m). ^{13}C-NMR, δ: 14.5 (C-18), 17.6 (C-19), 20.7 (C-21), 170.9 (PhC̲OO), 203.9 (C-20), 128.3 (C=C̲-Cl), 190.4 (C-3). MS (*m/z*): 468 (M^+). Calcd for $C_{28}H_{33}O_4Cl$: C, 71.70; H, 7.09; O, 13.64; Cl, 7.55. Found : C, 71.79; H, 7.15; O, 13.73; Cl, 7.68.

Animals and Tissues Adult male Syrian golden hamsters (150—200 g) were obtained from Metropolitan University-Xochimilco of Mexico. The animals were kept in a room with controlled temperature (22 °C) and light–dark periods of 12 h. Food and water were provided *ad libitum*.

Gonadectomies were performed under light ether anesthesia 15 d before treatments. Animals were sacrificed by ether anesthesia. The seminal vesicles were immediately removed, blotted, and weighed prior to their use. Tissues used in the metabolic experiments were homogenized with a tissue homogenizer (model 985-370; variable speed 5000—30000 rpm, Biospec Products, Inc.).

Seminal Vesicles Tests The effect of steroids on seminal vesicles from castrated male hamsters was determined in 10 groups of 5 animals/experiment selected at random and gonadectomized 15 d prior to experimentation. The animals were kept in a room with controlled temperature (22 °C) and light-dark periods of 12 h. Food and water were provided *ad libitum*.

Hormone Treatment of Seminal Vesicles Daily subcutaneous injections of 200 μg of the steroids dissolved in 20 μl of sesame oil were administered for 3 d. After this treatment, the animals were sacrificed by ether anesthesia, and the seminal vesicles were dissected out and weighed on a balance. The following table gives the composition of each treatment:

Group 1	n=4, 200 μg of T[1,2]
Group 2	n=4, 200 μg of T+200 μg **3**
Group 3	n=4, 200 μg of T+200 μg **9**
Group 4	n=4, 200 μg of T+200 μg **10**
Group 5	n=4, 200 μg of T+200 μg **11**
Group 6	n=4, 200 μg of T+200 μg **12**
Group 7	n=4, 200 μg of T+200 μg **13**
Group 8	n=4, 200 μg of T+200 μg **14**
Group 9	n=4, 200 μg of T+200 μg **15**
Group 10	n=4, 200 μg of T+200 μg **16**

Two separate experiments were performed for each group of steroid-treated animals. The results were analyzed using one-way analysis of variance with EPISTAT software.

***In Vitro* Metabolic Studies with Seminal Vesicles** Homogenates from male hamster seminal vesicles (around 3 mg protein) were prepared from intact adult male animals, using Krebs–Ringer–phosphate buffer solution, at pH 6. Tissue preparations were incubated[12] in duplicate with 5.1 mM [^{3}H]T in the presence of 1 mM NADPH$^+$, 0.013 μM of unlabeled T, finasteride **3**, compounds **11**—**16** in a Dubnoff metabolic incubator at 37 °C for 60 min with O_2/CO_2 (95/5) as the gas phase. The final incubation volume was 1 ml. Incubations without tissues were used as controls. Incubation was terminated by addition of dichloromethane, and the [^{3}H]steroid was extracted (4×) using 3 vol of dichloromethane. The solvent was dried under vacuum and the extract washed with hexane in order to remove the remaining lipids. The protein content of the homogenates was determined by Bradford's dye-binding method[4] using bovine serum albumin (BSA) as the standard.

Isolation and purity assessment of radioactive DHT was carried out by the reverse isotope dilution technique. The isolated compound was purified with steroid carriers (T, DHT) in a thin-layer chromatographic system (chloroform–acetone, 9 : 1). The radioactive conversion product was identified on chromatographic plates by autoradiography, while nonradioactive steroid carriers were detected using phosphomolybdic acid reagent and an ultraviolet lamp (254 nm). Radioactivity was determined in a Packard 3255 liquid scintillation spectrometer, using Ultima Gold (Packard, Downers Grove, IL) as the counting solution. The counting efficiency of ^{3}H was 67%. The calculated loss of radioactivity during the procedure was in agreement with the results obtained from a control experiment without tissue. The formation of DHT was calculated and expressed as pmol of DHT/g protein/h.

The specific activity of the DHT formed was determined by double isotope dilution, adding radioinert DHT to the eluting fraction from the TLC. The specific activity was calculated in dpm/mg of crystals.

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