Evaluation of New Pregnane Derivatives as 5α-Reductase Inhibitor

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The objective of this study was to synthesize several new pregnane derivatives and evaluate them as antiandrogens. From the commercially available 16-dehydropregnenolone acetate (7), two new steroidal compounds were synthesized: 17α-hydroxy-17β-methyl-16β-phenyl-D-homoandrosta-1,4.6-triene-3,20-dione (18) and 17α-acetoxy-17β-methyl-16β-phenyl-D-homoandrosta-1,4.6-triene-3,20-dione (19). The 5α-reductase inhibitory effect of the new compounds 18 and 19 together with the previously synthesized intermediates 7, 8, 13, 16, and 17 was determined in three different models: gonadectomized hamster flank organs diameter size, incorporation of [1,2-^{14}C]sodium acetate into lipids in flank organs and conversion of [^{3}H]testosterone (T) to [^{3}H]dihydrotestosterone (DHT) by *Penicillium crustosum.* The evaluation of these steroids was carried out with three different controls: one group was treated with vehicle, the second with T and the third group with T plus finasteride. The pharmacological results from this work demonstrated that T significantly increases the diameter of the pigmented spot on the flank organs ($p<0.05$) as well as the incorporation of labeled sodium acetate into lipids in gonadectomized hamster flank organs (from 0.125 to 0.255 nmol per gland). In this study we also observed that broth of *Penicillium crustosum* converted [^{3}H]T to [^{3}H]DHT in a manner comparable to that of the flank organs. All experiments indicated that finasteride as well as steroids 7, 8, 13, 16—19 reduced significantly the conversion of T to DHT in *P. crustosum.* These compounds also decrease the size of the pigmented spot in the flank organs as well as reducing the incorporation of radiolabeled sodium acetate into lipids; T and the control sample (treated with vehicle only) were used for comparison. Apparently the presence of the 4,6-diene-3,20-dione moiety and also the C-17 ester group produce a higher inhibitory effect on the parameters used. PPThe data from this study indicated also that the three models used for the pharmacological evaluation exhibited comparable results.

Key words flank organ; lipid synthesis; androgen; lipid metabolism; *Penicillium crustosum*; 5α-reductase

Enzymatic 5α-reduction of testosterone (T) (**1**) (Fig.1) produces a more potent androgen 5α-dihydrotestosterone (5α-DHT) (**2**) which is found in tissues categorized as androgen target organs and considered to be the male accessory glands.[1)] This tissue enzyme profile results in the accumulation of the more potent androgen DHT (**2**) in the classical androgen target organs.

Skin disorders such as acne, seborrhea, hirsutism and androgenic alopecia are secondary side effects associated with a high androgen activity. In view of the fact that the most potent androgen DHT (**2**) is formed from T (**1**) by the action of 5α-reductase enzyme, the inhibition of this enzyme is a logical approach for the reduction of androgen activity. For this reason it is important to develop new 5α-reductase inhibitors which could be used for the treatment of these afflictions.[2)]

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

The purpose of this work was to synthesize new pregnane derivatives as 5α-reductase inhibitors, having a phenyl group at C-16. Previous studies carried out with similar compounds indicated that some of these steroids could strongly inhibit the conversion of T to DHT.[7)]

The influence of sexual steroids on the lipid metabolism of flank organs was previously shown by our group.[8,9)] With this method we determined the effect of several new steroids **7**, **8**, **13**, **16**—**19** upon the incorporation of radiolabeled sodium acetate into lipids.

Methods and Results

Chemistry. Synthesis of the Steroidal Compounds This study reports the 5α-reductase inhibitory effect of a variety of 16-phenyl substituted-D-homoandrostane derivatives **16**—**19** (Fig. 3), the starting material **7**, the intermediate epoxy compound **8** and the 16-methyl substituted 4,5-diene-

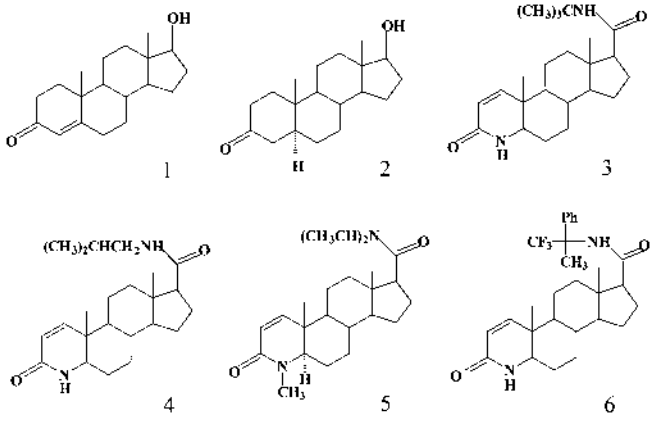

Fig. 1. Steroid Structures

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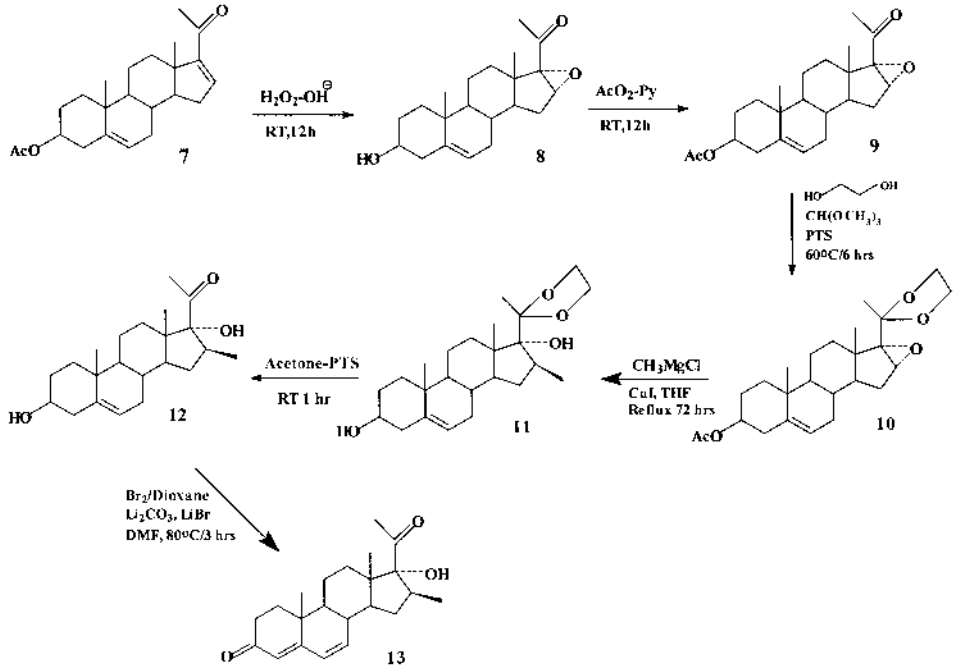

Fig. 2. Synthesis of New Steroids

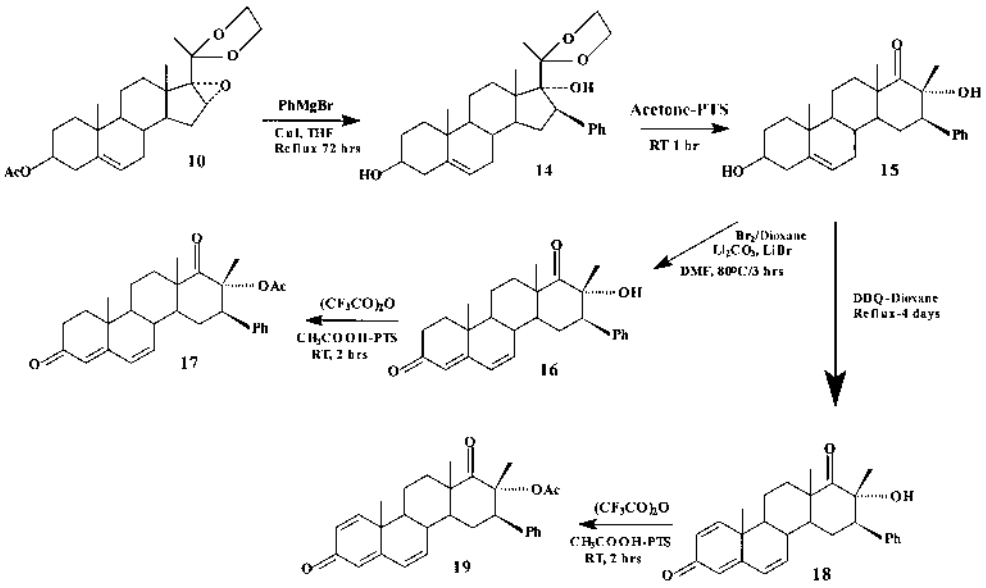

Fig. 3. Synthesis of New Steroids

3,20-dione compound **13** (Fig. 2). All compounds were prepared from the commercially available 16-dehydropregnenolone acetate **7**. Epoxidation of the double bond at C-16 in **7**[10] with hydrogen peroxide and sodium hydroxide afforded the epoxy derivative **8** (Fig. 2). In the strong alkaline medium, the acetoxy group was hydrolyzed. Acetylation of **8** in the usual manner yielded the acetoxy compound **9**.

In view of the fact that in a subsequent step the epoxy moiety at C-16,17 would be opened with phenylmagnesium chloride or methylmagnesium chloride, it was necessary to protect the C-20 carbonyl group and thus avoid a possible reduction. With compound **9**, this protection was carried out using ethylene glycol, trimethyl orthoformate and *p*-toluenesulfonic acid (PTS) as a catalyst.[11] This reaction afforded the dioxolane derivative **10** in good yield. In a subsequent step, the oxiran ring in **10** was opened with methylmagnesium chloride in tetrahydrofuran (THF), thus giving the C-16 methyl derivative **11** (Fig. 2). The hydrolysis of the dioxolane ring in **11** to recover the carbonyl moiety was carried out with perchloric acid in acetone; the resulting C-20 carbonyl derivative **12** was oxidized with bromine, lithium carbonate, lithium bromide in *N*,*N*-dimethylformamide (DMF).[12] This reaction afforded the 4,6-diene-3,20-dione, **13**. The 16-phenyl substituted derivatives were obtained in a similar manner. Compound **10** (Fig. 3) was allowed to reflux with phenylmagnesium bromide in THF to give the C-16 phenyl derivative **14.**[7] Upon hydrolysis (acetone perchloric acid), **14** afforded the D-homosteroid **15**. In this reaction an expansion

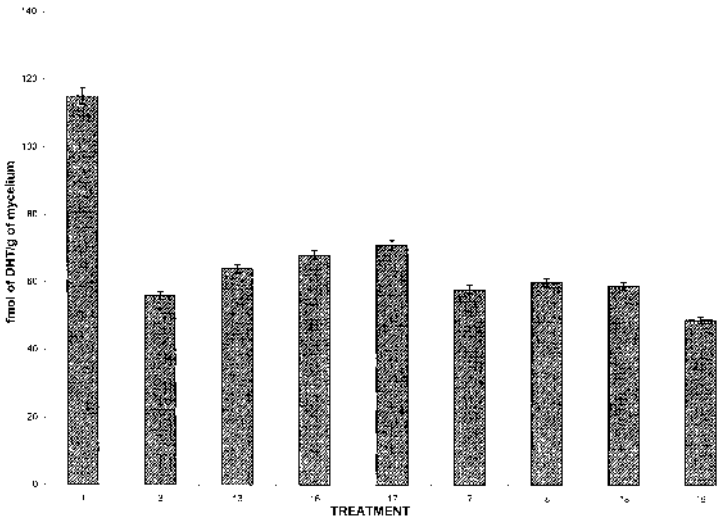

Fig. 4. Effect of Different Synthesized Steroids on the Conversion of [^{3}H]T to [^{3}H]DHT in *Penicillium crustosum* Broth's
Significant differences ($p<0.005$) were observed between T (**1**) and T plus each one of the different steroids added to the growth medium.

of the D-ring took place.[12] Treatment of **15** with lithium carbonate, lithium bromide and bromine in DMF afforded the 4,6-diene-3,20-dione derivative **16** which upon esterification with acetic acid and trifluoroacetic anhydride yielded the acetoxy derivative **17**.[7]

On the other hand, when compound **15** was allowed to reflux in dioxan with dichlorodicyanobenzoquinone (DDQ), this reaction afforded the 1,4,6-triene-3,20-dione moiety **18**. Acetylation of the hydroxyl group in **18** with trifluoroacetic anhydride and acetic acid yielded the desired acetoxy derivative **19**.

The synthesis of the intermediates **7**—**13** (Fig. 2) is described in refs. 5 and 11. The preparation of C-16 phenyl substituted intermediates **14**—**17** (Fig. 3) is described in refs. 5 and 7.

Biological Activity The biological activity of steroids **7**, **8**, **13**, **16**—**19** (Figs. 2, 3) was determined *in vitro* following the transformation of T (**1**) to DHT (**2**) produced by 5α-reductase enzyme in *Penicillium crustosum* broth,[13,14] by the *in vivo* steroid action upon the flank organs where the DHT is bound to its cognate receptor,[15,16] and also by the incorporation of radiolabeled sodium acetate into lipids in these glands.[8,9]

Effect of the Synthesized Steroids on the Conversion of T to DHT in *P. crustosum* Conversion of T (**1**) to DHT (**2**) has been demonstrated in *P. decumbens* and *P. crustosum* broth obtained from fermented pistachios, lemons and corn tortillas.[13,14]

In this experiment, when extracts from *P. crustosum* were spotted onto TLC plates, the *Rf* value (0.33) of the zone corresponding to 5α-DHT standard was determined. After elution, to the fraction corresponding to DHT was added unlabeled 5α-DHT. Constant specific activity of [^{3}]DHT crystals were obtained with successive recrystallizations (data not-shown). The results are in agreement with those we published previously.[14]

The conversion of T to DHT is shown in Fig. 4. Radiolabeled T in the incubated medium increases significantly its conversion to DHT compared to T plus finasteride (**3**). Steroids **7**, **8**, **13**, **16**—**19** ($p<0.005$) present in the cultures decrease the conversion of [^{3}H]T to [^{3}H]DHT effected by the fungi. This data indicated that finasteride as well as the above mentioned steroids are good inhibitors for the conversion of T to DHT in this model. The results from this study correlate well with those obtained from the other two experiments (flank organs and the incorporation of radiolabeled sodium acetate in lipids).

Flank Organ Test To test the effect of the synthesized compounds as 5α-reductase inhibitors, we used the flank organs of gonadectomized male hamsters.[8,16] Hamster flank organs are dorsal spots on the skin that are composed of pilosebaceous tissue. These glands are androgen dependent tissue that decreases upon castration and increase in diameter size with daily injections of T. The flank organs can convert T to DHT and are used for the screening of new antiandrogenic drugs.[15,17]

In this experiment, the diameter of the pigmented spot on the gland 15 d after castration decreased significantly ($p<0.005$) as compared to that of the uncastrated animals. Subcutaneous injections of vehicle alone did not change this condition. However, treatment with T restored the diameter of the pigmented spot (Table 1).

Finasteride (**3**) as well as steroids **7**, **8**, **13**, **16**—**19** decreased significantly ($p<0.005$) the diameter of the flank organs in gonadectomized males treated with T. The most effective compounds in this experiment were steroids **7** and **19** which reduced the diameter of the pigmented spot to 1.5 mm followed by finasteride **3** and steroids **16**—**18**. These data suggest that compounds **7**, **16**—**19** (Table 1) are strong inhibitors of the conversion of T to DHT (finasteride is slightly less active with a diameter of the pigmented spot of 2.5 mm).

***In Vitro* Incorporation of [1,2-^{14}C]Sodium Acetate into Lipids** Flank organs are larger in males than in females and are capable of synthesizing lipids from radioactive precursor; furthermore, they can modify the sebum lipid composition under T or progesterone stimuli.[8,9] The inhibitory effect of finasteride in flank organs was previously demonstrated.[10]

Table 1 shows the percentages of [1,2-^{14}C]sodium acetate incorporation into lipids as well as the rate of this incorporation. Compounds **7**, **18**, and **19** exhibited a much lower per-

Table 1. Effect of Different Compounds on Male Hamster Flank Organ Diameter (mm±S.D.) and *in Vitro* Incorporation of [^{14}C]Acetate in Lipids

Treatment (mg/per gland)	Diameter of the pigmented spot (mm)	Percentage of incorporation	Incorporation 1,2-^{14}C acetate into lipids (nmol/gland)
Control	2.5±0.9	0.46	0.125±0.004
T 0.2	6.0±2.0	0.96	0.255±0.002
T+**3** 0.2	2.5±0.9	0.31	0.080±0.005
T+**7** 0.2	1.5±0.4	0.07	0.055±0.001
T+**8** 0.2	3.0±1.0	0.35	0.090±0.004
T+**13** 0.2	4.3±1.0	0.76	0.115±0.004
T+**16** 0.2	2.0±0.3	0.78	0.115±0.008
T+**17** 0.2	2.0±1.0	0.70	0.100±0.004
T+**18** 0.2	2.0±0.5	0.15	0.080±0.009
T+**19** 0.2	1.5±0.5	0.07	0.056±0.0009

Results are expressed as percentage and nmol/per gland±S.D. Significant difference was observed between the diameter of glands, as well as in the incorporation of [^{14}C]acetate in lipids ($p<0.005$) from castrated control animals (control) and T-treated (T). Finasteride (**3**) decreased both the diameter of the glands as well as the incorporation of [^{14}C]acetate in lipids ($p<0.005$). New steroids (**7**, **8**, **16**—**19**) inhibited significantly ($p<0.005$) the diameter of the flank organs as well as the incorporation of [^{14}C]acetate in lipids.

centage of incorporation of sodium acetate than finasteride (**3**) (Table1). These data which are the result of two different experiments performed in duplicate confirm the importance of sex hormones in lipid metabolism of the flank organs. The vehicle treated control showed a higher percentage of sodium acetate incorporation, although, it is much lower than that of T treated hamsters. The results of these experiments correlate very well with those obtained from the diameter of the pigmented spot in flank organs.

Discussion

In this study, we determined the 5α-reductase inhibitory activity of the above mentioned steroids **7**, **8**, **13**, **16**—**19** in three different models: the conversion of [^{3}H]T to [^{3}H]DHT in *P. crustosum* broth's, the flank organ diameter size, and the incorporation of [1,2-^{14}C]sodium acetate into lipids. From the first experiment it is evident that *P. crustosum* broth's can be used as a model for the evaluation of the 5α-reductase inhibitory effect and thus replace the animal model which involves the sacrifice of dozens of hamsters. In the *P. crustosum* broth, finasteride as well as compounds **7**, **8**, **13**, **16**—**19** inhibited the conversion of T to 5α-DHT, thus confirming the presence of 5α-reductase enzyme in the fungal culture.[14]

The results from the second experiment showed that these steroids as well as finasteride inhibited the 5α-reductase enzyme present in flank organ.[17] A decrease of the diameter of the pigmented spot indicates that these steroids are strong inhibitors and thus confirm the validity of this model for the determination of the antiandrogenic properties of these compounds. When finasteride (**3**) and steroids **7**, **8**, **13**, **16**—**19** were injected daily with T, it caused a reduction in the diameter of the pigmented spots produced by T alone. However, this effect could also be the result of the antagonistic activity of these steroids on the androgen receptors.

In these experiments we showed that T increased the incorporation of the radiolabeled sodium acetate into lipids as compared to the control; this is in agreement with previous results obtained by our group.[8] Table 1 demonstrates very clearly that finasteride as well as steroids **7**, **8**, **13**, **16**—**19** decreased the rate and the percentage of radiolabeled sodium acetate incorporation expressed as nmol/gland. These results show that the above mentioned steroids are strong inhibitors of the incorporation of sodium acetate into lipids in the sebaceous glands and consequently are antiandrogens.

As we expected, the decrease of the diameter of the flank organs produced by the synthesized steroids correlates very well with the reduction of the labeled sodium acetate incorporation into lipids. This corroborates the fact that the antiandrogenic effect is related to labeled sodium acetate incorporation. However, the observed effect could also be the result of the antagonistic activity of the novel steroids on the androgen receptors.

It is important to emphasize that the results from the three experiments correlate very well, thus confirming the fact that steroids **7**, **8**, **13**, **16**—**19** are powerful 5α-reductase inhibitors. The above mentioned steroids inhibit T to DHT conversion in *P. crustosum* broth's, they also decrease the incorporation of radiolabeled sodium acetate into lipids of the flank organs.

As previously observed[16] compounds having the 4,6-diene-3-one moiety exhibited a strong antiandrogenic effect. Therefore, on the basis of this concept compounds **13**, **16**—**19** show a high activity. The antiandrogenic activity in compound **7** is probably the result of the conjugation of the C-16 double bound with the C-20 carbonyl group.

Compounds **18** and **19** having cross conjugated double bounds in A-ring inhibit the incorporation of labeled sodium acetate to a higher degree than the 4,6-dien-3-ones (compounds **13**, **16**, **17**). Apparently when the steroid molecule becomes more coplaner, the inhibition of the incorporation of sodium acetate is increased. Based on this hypothesis, the activity of compound **8** probably could be explained on the grounds that the presence of the oxiran function in ring D flattens the molecule and thus makes it more antiandrogenic.

Experimental

Chemical and Radioactive Material Solvents were laboratory grade or better. Melting points were determined on a Fisher John's melting point apparatus and are uncorrected. ^{1}H- and ^{13}C-NMR spectra were taken on Varian Gemini 200 and VRX-300 spectrometers, 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. High resolution mass spectra were recorded on a HP5985-B spectrometer, IR spectra were recorded on a Perkin Elmer 549-B and the UV spectra on a Perkin Elmer 200s spectrometer. The elemental analysis were determined at the Christopher Ingold Laboratories, University College, London.

(1,2,6,7-^{3}H) T [^{3}H]T, specific activity: 95 Ci/mmole and [1,2-^{14}C] sodium acetate, specific activity, 58.20 mCi/mmole were provided by New England Nuclear Co. (Boston, MA, U.S.A.). Radioinert T and 5α-DHT were supplied by Steraloids (Wilton, NH, U.S.A.). D-α-Glucose, sodium acetate, and albumin were purchased from Merck, Mexico. Sigma Chemical Co. sup-

plied finasteride and $NADPH^+$.

All *P. crustosum* culture media were prepared following Pitt's procedure.[18] The materials were obtained from Baker/or Bioxon (Becton Dickinson), Mexico.

Synthesis of the Steroid Compounds. 17α-Hydroxy-17β-methyl-16β-phenyl-D-homoandrosta-1,4,6-triene-3,17α-dione (18) A solution containing steroid **15** (1.0 g, 2.8 mmol), DDQ (2.2 g, 9.7 mmol) in dioxan (36 ml) was allowed to reflux for 72 h. The reaction mixture was cooled to 0 °C and the precipitated dichlorodicyano hydroquinone was filtered off. The organic phase was washed with an aqueous solution of sodium hydroxide (3%) 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 0.5 g, 1.2 mmol (43%) of pure compound **18**. mp 280—282 °C. UV (nm): 256 (ε=11600), 298 (ε=26500). IR (KBr) cm^{-1}: 3446, 3028, 1694, 1656, 1618, 1126. ^{1}H-NMR ($CDCl_3$) δ: 1.0 (3H, s), 1.1 (3H, s), 1.4 (3H, s), 3.0 (1H, dd, J_1=3, J_2=2 Hz), 6.0 (1H, s), 6.3 (1H, s), 7.1 (2H, d. J=3 Hz), 7.3 (5H, m). ^{13}C-NMR ($CDCl_3$) δ: 12 (C-18), 16 (C-19), 24 (CH_3 at C-17), 54 (C-16), 79 (C-17), 124 (C-4), 138 (C-1), 161 (C-2), 165 (C-5), 121, 124 (phenyl), 185 (C-3 carbonyl), 218 (C-17a-carbonyl). MS (*m*/*z*) 402 (M^+). *Anal.* Calcd for $C_{27}H_{30}O_3$: C, 80.60; H, 7.46; O, 11.94. Found: C, 80.66;H, 7.41; O, 11.93.

17α-Acetoxy-17β-methyl-16β-phenyl-D-homoandrosta-1,4,6-triene-3,17α-dione (19) A solution containing steroid **18** (1.0 g, 2.48 mmol), PTS (50 mg), trifluoroacetic anhydride (0.6 ml) and glacial acetic acid (1.26 ml) was stirred for 1.5 h at room temperature (nitrogen atmosphere). The reaction mixture was diluted with chloroform (10 ml) and was neutralized with an aqueous sodium bicarbonate solution to a pH of 7. 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 0.6 g, 1.3 mmol (52%) of the pure product **19**. mp 240—242 °C. UV (nm): 254 (ε=11800), 300 (ε=26400). IR (KBr) cm^{-1}: 3018, 1698, 1650, 1622. ^{1}H-NMR ($CDCl_3$) δ: 1.2 (3H, s), 1.3 (3H, s), 1.5 (3H, s) 2.2 (3H, s), 2.8 (1H, dd, J_1=3, J_2=2 Hz), 5.9 (1H, s), 6.4 (1H, s), 7.0 (2H, d, J=3 Hz), 7.3 (5H, m). ^{13}C-NMR ($CDCl_3$) δ: 14 (C-18), 17 (C-19), 25 (CH_3 at C-17), 56 (C-16), 80 (C-17), 122 (C-4), 140 (C-1), 160 (C-2), 165 (C-5), 175 (acetoxy), 192 (C-3 carbonyl), 212 (C-17a-carbonyl). MS (*m*/*z*) 444 (M^+). *Anal.* Calcd for $C_{29}H_{32}O_4$: C, 78.38; H, 7.18; O, 14.39. Found: C, 78.43; H, 7.18; O, 14.39.

In this study we observed that when the ketal **14** was hydrolyzed, an expansion of the D-ring took place, thus forming the D-homosteroid **15**. This expansion was due to the presence of the perchloric acid used for the hydrolysis. We actually could never obtain compound **15** with the normal D-ring. In view of the fact that **15** was a new compound, we were very anxious to determine its pharmacological activity as 5α-reductase inhibitor as well as an antiandrogen.

Evaluation of the Synthesized Steroids in *P. crustosum* Cultures The fungus *P. crustosum* was isolated in a solid culture medium consisting of potato dextrose agar (PDA) as described in references 13, 14. Species identification was performed following Pitt's criteria,[18] which uses three different culture media, Czapek yeast autolysate agar (CYA; Pitt, 1973); malt extract agar (MEA; according to Blakeslee, 1915) and 25% glycerol nitrate agar (25Y; Pitt, 1973) as well as three incubation temperatures, 5°, 25° and 37 °C during seven days (Pitt, 1979). One plate of each of CYA, MEA, and G25Y was incubated at 25°, another plate of CYA at 37° and a third at 5 °C.

Conversion of T to DHT by *P. crustosum* The conversion of T to its metabolite DHT was determined by the reverse isotope dilution technique; TLC was used for the isolation and identification of DHT.[14]

Incubation: 16 Erlenmeyer flasks containing 16 ml of sterilized potato dextrose broth[14] were inoculated with *P. crustosum* and stoppered with foam plugs. The inoculated flasks were placed in a water bath at 25 °C and maintained under constant shaking for 24 h. Then, 100 μl of radiolabeled T [^{3}H]T, (5.4 μCi) in 95% ethanol as well as 0.5 mM of each one of the new steroids were added to the medium. The flasks were kept under incubation conditions for on additional 96 h; two Erlenmeyer flasks were prepared as a non-inoculated control.

Extractions: the cultures were extracted with 5 ml of dichloromethane saturated with water (3X). The solvent was evaporated to dryness in a rotary evaporator (Caframo vv 2000).

The extract was spotted on a TLC plate in order to isolate and identify the formed products. The unknown samples were run together with steroid carriers (T, DHT) using; chloroform/acetone, 9 : 1 (v/v) as solvent system. After development, the T standard was identified under 254 nm UV light. DHT was assayed on the same plate after spraying with phosphomolybdic acid reagent (8% in methanol). Recrystallizations to constant specific activity were carried out in order to test the radioactive purity of the isolated DHT.[14] All experiments were performed in duplicate. Radioactivity was determined in a Packard tri-carb 3255 liquid scintillation spectrometer, using the standard solution (Packard, Downers Grove, IL, U.S.A.) as the counting solution. The counting efficiency of ^{3}H was 57%. The formation of 5α-DHT was calculated and expressed as fmol/g of dry mycelium. The results were analyzed with the analysis of variance (Anova) test, using the Epistat software.

Animals and Tissues Adult male Syrian Golden hamsters (150—200 g) were obtained from the 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 treatment. Animals were sacrificed by ether anesthesia. The effect of the synthesized steroids was evaluated in the three different experimental models.

Flank Organ Test The flank organ test was performed as previously reported.[16] The effect of steroids **7**, **8**, **13**, **16**—**19** on the flank organs of male hamsters, which were gonadectomized 15 d before the experiments, was determined on 18 groups of 4 animals/experiment, selected at random.

Daily subcutaneous injections of 200 μg of the steroids **7**, **8**, **13**, **16**—**19** (Figs. 2, 3) dissolved in 20 μl of sesame oil were administered for 4 d together with 200 μg of T. Three groups of animals were kept as control, one was injected with 20 μl of sesame oil, the second with 200 μg of T for 4 d and the third with 200 μg of finasteride.[16]

After these treatments, the animals were sacrificed by ether anesthesia. Both flank organs of the animals were shaven and the diameter of the pigmented spot was measured. The experiments were carried out in quadruplicate on two different occasions. The results were analyzed using one-way Anova with Epistat software.

***In Vitro* Incorporation of [1,2-^{14}C]Sodium Acetate into Lipids** The flank organs of the sacrificed animal were dissected around the pigmented spot.[15,16] To evaluate the effect of the hormone treatments on sodium acetate incorporation into lipids under culture condition, the hamster flank organs were placed in Erlenmeyer flasks (4 whole glands/flask) in a shaking water bath with 3.7 μCi of [1,2-^{14}C]sodium acetate specific activity 58.20 μCi/mmol. To this solution were added 2 mM of nonradioactive glucose,[16] albumin 16 mg/ml, biotin 0.36 mM, ATP 0.66 mM, nonradioactive sodium acetate 1.29 mM, $NADPH^+$ 0.0033 and 10 μg of each steroid. The cultures were incubated in duplicate for 24 h at 37.5 °C in a final volume of 2 ml of Krebs–Ringer buffer (pH 7.4) Incubations without tissue were used as a control.

Determination of Incorporated Radioactivity in Lipids For the lipid extraction, the glands were placed in Folch's solution (4 glands/10ml)[19] for 24 h at room temperature in reviously weighed glass vials. Immediately thereafter, the glands were recovered from the solvent medium. Water, one ml, was added to the organic phase to remove the free [1,2-^{14}C] sodium acetate. The aqueous phase was removed in vacuum and the extract was dried. The extracted lipids were weighed. The incorporated radioactivity was determined with a Packard liquid scintillation spectrometer with Ultima gold (Packard Downers Grove, IL, U.S.A.) as the counting solution. The counting solution efficiency for ^{14}C was 87%. The specific activity of the incorporated radiolabeled sodium acetate in lipids was calculated and expressed as nmol/gland/h[8,9] and as a percentage of incorporation of the total radioactivity used. The results were analyzed using one-way Anova with Epistat software.

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