Synthesis and Pharmacological Evaluation of New 16-Methyl Pregnan Derivatives

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The pharmacological activity of several new pregnane derivatives 15—19 was determined on gonadectomized male hamster flank organs, seminal vesicles and in vitro conversion of testosterone (T) to dihydrotestosterone (DHT) as 5α-reductase inhibitors. Steroids 15—19 decreased the diameter of the pigmented spot in the flank organs as compared to the T treated animals; in this model, steroids 16 and 19 showed a higher activity than the commercially available finasteride. Injection of T increased the weight of the seminal vesicles. Compounds 15—19 when injected together with T decreased the weight of the seminal vesicles thus showing an antiandrogenic effect. The trienone 19 exhibited a considerably higher activity than finasteride. Steroids 15—19 inhibited the in vitro metabolism of [3H]T to [3H]DHT in seminal vesicles homogenates of gonadectomized male hamsters. Compounds 18 and 19 showed a much higher antiandrogenic effect than finasteride. This enhancement of the biological activity could probably be attributed to the coplanarity of the steroid skeleton as previously observed by our group. The high antiandrogenic activity of the epoxy compound 16 is probably the result of the ring opening of the oxiran ring with the nucleophilic part of the enzyme 5α-reductase thus leading to a stable adduct with concomitant deactivation of this enzyme.

Key words flank organ; seminal vesicle; 5α-reduction; testosterone conversion; C-16 substituent

The androgen metabolism of human prostate is regulated by a variety of androgen metabolizing enzymes. Among those, the 5α-reductase enzyme possesses the highest potential activity. It converts testosterone (T) (Fig. 1) into the more potent androgen dihydrotestosterone (DHT) (2). Thus 5α-reductase regulates the cellular availability of DHT and consequently the androgen responsiveness of the human prostate. As a result of this, several new clinically useful inhibitors for the treatment of benign prostatic hyperplasia and male pattern baldness were developed shortly after the discovery of the enzyme 5α-reductase.

Recently several new inhibitors of 5α-reductase were described as potential clinical candidates for the treatment of benign prostatic hyperplasia. These compounds (Fig. 1) include finasteride 3,4 episteride 4,5 cyproterone acetate 5,6 BOMT 6* and FCE 282607 7,9 Finasteride 3 inhibits the conversion of T to DHT in epithelium and stroma of human benign prostatic hyperplasia. This steroid has a greater affinity for 5α-reductase type 2 than for the type 1 enzyme, both present in the human prostate. The type 2 isozyme, is the dominant form in genital tissue, however, the exact physiological roles of these enzymes has yet to be fully elucidated.

In this study we evaluated the following new steroidal compounds as 5α-reductase inhibitors: 15—19 (Figs. 2, 3). The pharmacological evaluation was determined in flank organs, seminal vesicles, and the effect of the new steroidal compounds on the in vitro metabolism of [3H]T to [3H]DHT in seminal vesicle homogenates of gonadectomized male hamsters.

Flank organs are pilosebaceous structures androgen dependent. In males, these glands measure 8 mm and shrink upon castration, until they look as those of females: however daily injections of 1 or 2 restores their original size. The presence of 5α-reductase in flank organs as well the inhibition of this enzyme by finasteride has been demonstrated several years ago. Many steroidal and non steroidal compounds have been evaluated as antiandrogens using the flank organs as a model. Seminal vesicles are male accessory glands that are also androgen dependent. These organs are capable of reducing 1 to 2 in both intact and gonadectomized animals and have also been used for evaluation of steroidal and non-steroidal compounds as antiandrogens.

Chemistry

Synthesis of Steroids 15—19 Steroids 15—19 were prepared from the commercially available 16-dehydropregn-9-en-17-one acetate. Epoxydation of the double bond at C-16 in 8 with hydrogen peroxide and sodium hydroxide afforded the epoxy derivative 9 (Fig. 2). Acetylation of 9 in the usual manner yielded the acetoxy compound 10. The protection of the carbonyl group in 10 was effected with ethylene glycol, trimethyl orthoformate and p-toluensulfonic acid (PTS). The resulting dioxolane derivative 11 was allowed to reflux with methylmagnesium chloride in tetrahydrofuran (THF); this reaction afforded the 16-methyl substituted dioxolane derivative 12 (Fig. 2). Hydrolysis of the dioxolane ring in 12 to recover the carbonyl moiety was carried out with PTS in acetone thus forming the carbonyl derivative 13. The oxidation of the hydroxyl group at C-3 in 13 was effected with lithium carbonate, lithium bromide and bromine in N,N-dimethylformamide (DMF) thus affording the 4,6-diene-3,20-dione moiety 14. Esterification of the hydroxyl group in 14 with trifluoroacetic anhydride and valeric acid yielded the desired valeroyloxy derivative 15.

Treatment of 13 with 2,3-dichloro-5,6-dicyanobenzquinone (DDQ) in dioxan (Fig. 3) afforded the triene–dione compound 18, which upon esterification with valeric acid yielded the ester 19.

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The epoxidation of the double bond at C-6 in 15 (Fig. 3) was carried out with \( m \)-chloroperbenzoic acid in chloroform. The resulting epoxy compound 16 was treated with hydrobromic acid in acetic acid to give the desired 6-bromo derivative 17.

**Biological Activity**

The biological activity of steroids 15—19 was determined in gonadectomized male hamsters divided in several groups. The animals in the control group were injected with the vehicle (sesame oil); in the other groups the hamsters were treated with \( 1^{17} \) a combination of T with finasteride 3 or T with the new steroids. After 6d of treatment, the animals
were anesthetized with ether and sacrificed.

**Flank Organs** The diameter of the pigmented spot on the flank organs of the control animals (vehicle treated), T, +3, finasteride and with the new steroids treated animals is shown in Table 1. T significantly increased \((p, 0.05)\) the diameter of the pigmented spot (4.25 mm) whereas finasteride decreased it \((p, 0.005)\) (3 mm). The new steroids — decreased the diameter of the pigmented spot as compared to T, thus suggesting an inhibitory effect on the enzyme 5\(\alpha\)-reductase and also the presence of an antiandrogenic effect. The most effective compound in this model was steroid 19 which reduced the diameter of the pigmented spot on the flank organs to 1.8 mm.

**Seminal Vesicles** After castration, the weight of the seminal vesicles of the male hamsters significantly decreased \((p, 0.05)\) as compared to that of the normal glands. Treatment with vehicle alone (control) did not change this condition whereas subcutaneous injection of 200 \(\mu g\) of 1 for 6 d significantly increased \((p, 0.05)\) the weight of the seminal vesicles in castrated male hamsters (317 mg) (Table 1). When 1 and finasteride 3 were injected together, the weight of the seminal vesicles decreased (216 mg). The injection of steroids 15—19 together with T decreased \((p, 0.005)\) the weight of the seminal vesicles as compared to the T treated hamsters thus suggesting an inhibitory effect on 5\(\alpha\)-reductase enzyme type 2. The most effective compound showing the highest inhibitory effect for 5\(\alpha\)-reductase enzyme was steroid 19 having the lowest value of the weight of the seminal vesicles (202 mg).18,19) In the flank organs, both types of 5\(\alpha\)-reductase enzyme are present, however the type 1 enzyme predominates. On the other hand, in the seminal vesicles the predominant enzyme is type 2 and therefore it is not possible to compare rigorously both experiments.

**Conversion of T to DHT** Since the weight of the seminal vesicles depends on the 5\(\alpha\)-reduced androgens,19) it was important to determine the effect of the new steroids 15—19 on the conversion of \(^{[\text{3H}]\text{T}}\) to \(^{[\text{3H}]\text{DHT}}\) in castrated male hamster flank organs.

![Synthesis of New Steroids](image)

**Table 1. The Diameter of Flank Organs and the Weight of Seminal Vesicles Were Measured from Animals That Received sc Treatments of C-16 Substituted Steroids**

<table>
<thead>
<tr>
<th>Treatment (mg)</th>
<th>Diameter of the pigmented spot (mm)</th>
<th>Weight of seminal vesicles (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.75 ± 0.05</td>
<td>177.45 ± 30.7</td>
</tr>
<tr>
<td>T 0.2</td>
<td>4.25 ± 0.50</td>
<td>317.83 ± 30.1</td>
</tr>
<tr>
<td>T + 3</td>
<td>3.00 ± 0.02</td>
<td>216.00 ± 27.8</td>
</tr>
<tr>
<td>T + 15</td>
<td>3.00 ± 0.00</td>
<td>265.15 ± 30.4</td>
</tr>
<tr>
<td>T + 16</td>
<td>2.00 ± 0.00</td>
<td>208.75 ± 24.5</td>
</tr>
<tr>
<td>T + 17</td>
<td>3.00 ± 0.50</td>
<td>246.00 ± 49.1</td>
</tr>
<tr>
<td>T + 18</td>
<td>3.00 ± 0.50</td>
<td>231.25 ± 35.2</td>
</tr>
<tr>
<td>T + 19</td>
<td>1.80 ± 0.50</td>
<td>202.00 ± 30.1</td>
</tr>
</tbody>
</table>

The results are given mean ± standard deviation.

![Effect of Different Synthetic Steroids on in Vitro Conversion of \(^{[\text{3H}]\text{T}}\) to \(^{[\text{3H}]\text{DHT}}\) in Castrated Male Hamster Flank Organs](image)
good inhibitor for the conversion of T 1 to DHT 2 at pH 6.

The effect of different steroidal structures on the rate of DHT formation is given in Fig. 4. Compounds 15 and 17 showed a higher in vitro conversion of 1 to 2 ($p<0.05$) than the other steroids. On the other hand, compounds 16, 18 and 19 exhibited a lower DHT conversion than the 1 plus finasteride 3 treated animals thus showing a high 5α-reductase inhibitory activity.

**Discussion**

This study reports the 5α-reductase inhibitory effect of 16β-methyl substituted-4,6-pregnadiene-3,20-dione derivative 15, a 6,7-epoxy-16β-methyl substituted 4-ene-3,20-dione derivative 16, a 6-bromo-16β-methyl-17α-valeryl-oxypregnadiene-3,20-dione compound 17 and the triene-dienone derivatives 18 and 19.

As can be seen in Table 1 (diameter of the pigmented spot on the flank organs), finasteride decreases the diameter of the spot (3 mm) and thus shows a high inhibitory effect for the enzyme 5α-reductase type 2, a fact previously reported by other authors.19 Furthermore, this compound has also been shown to inhibit hair loss and improves hair growth.19,20 These results could be related to the presence of 5α-reductase enzyme type 2 in the hair follicles as previously reported.21 Compounds 15—19 decreased also the diameter of the pigmented spot, and this fact indicates that these compounds are also efficient inhibitors for the 5α-reductase enzyme present in the flank organs. The triene–dienone ester 19 exhibited the highest activity probably due to the presence of the 3 double bonds and the C-17 ester group.

Table 1 also shows the 5α-reductase inhibitory effect of steroids 15—19 related to the weight of the seminal vesicles of castrated male hamsters treated with 1 and a combination of 1 with finasteride 3. These data clearly indicate that compounds 15—19 decreased the weight of the seminal vesicles as compared to the T treated hamsters, thus showing an antiandrogenic effect; steroids 16, 18 and 19 exhibited the highest activity. The high antiandrogenic effect of 16, 18 and 19 could also involve inhibition of the synthesis and/or release of pituitary gonadotropins.22

Figure 4 shows the conversion of [3H]T to [3H]DHT expressed as pmoles of protein per hour. In the control animals (C) (vehicle treated), the weight of the seminal vesicles decreased after 15 d of castration thus showing the effect of orchidectomy.

As can be seen from Fig. 4, the trienones 18 and 19 and the epoxy compounds 16 showed a higher antiandrogenic effect than the corresponding dienones 14 and 15.13,23–25 Several years ago, we carried out a theoretical computer assisted study26 with similar compounds. The results from this study indicated that the first step in the inhibition of the enzyme 5α-reductase consists in the formation of an enzyme–antiandrogen activated complex. In a subsequent step, the nucleophilic portion of the enzyme (amino group) attacks the conjugated double bond of the steroid in a Michael type addition reaction to form an irreversible adduct. This explain very well the higher biological activity of the trienones 18 and 19 as compared to the dienones 14 and 15. The trienones have a more coplanar structure react faster with the nucleophilic portion of the enzyme in a Michael type addition reaction than the dienones.

This hypothesis explains also the fact that 17α-acetoxy-6-methylenepregn-4-ene-3,20-dione has much higher antiandrogenic activity than the corresponding 17α-acetoxypregna-4,6-diene-3,20-dione, both compounds previously synthesized by Petrov.27 The 6-methylene compound having an exocyclic double bond can reacts much easier with the nucleophilic part of the enzyme than the corresponding endocyclic 4,6-dieneone.

In the case of the active compound 16, it is also possible that the nucleophilic portion of the enzyme reacts with the electrophilic carbon C-7 of the oxiran ring with a subsequent opening of the epoxy ring with the concomitant formation of a stable steroid–enzyme adduct. As a result of this, the enzyme 5α-reductase is inhibited; this reaction explains the high pharmacological activity of the epoxy compound 16.

At the present time, we are synthesizing several new exocyclic and endocyclic dienones and trienones with the purpose of clarifying this hypothesis. The intermediates 9—14 as well as starting material 8 showed a low pharmacological activity.

**Experimental**

**Chemical and Radioactive Material** Solvent were laboratory grade or better. Melting points were determined on a Fisher John’s melting point apparatus and are uncorrected.1H- and 13C-NMR spectra were taken on Varian Gemini 200 and VRX-300 spectrometers respectively. Chemical shifts are given in ppm relative to that of MeSi (δ=0) in CDCl3. The abbreviations of signal patterns are as follows: s, singlet; d, doublet; t, triplet; m, multiplet. High resolution mass spectra were recorded on a HP5985B spectrometer, IR spectra were recorded on a Perkin Elmer 549-B and the UV spectra on a Perkin Elmer 200s spectrometers. Finasteride was obtained from the commercially available Proscar. The tablets were crushed, extracted with chloroform and the solvent was eliminated in vacuum; the crude product was purified by silica gel column chromatography.

(1,2,6,7-3H) T ([3H]T, specific activity: 95 Ci/mmol) was 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.). Sigma Chemical Co. supplied NADPH.

**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 30 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 experiment were homogenized with a tissue homogenizer (model 985-370; variable speed 5000—30000 rpm, Bisspec Products Inc.).

**Flank Organ Test** The flank organ test was performed as previously reported.45 The effect of the new steroids on the flank organs of male hamsters, which were gonadectomized 30 d before the experiments, was determined on 8 groups of 4 animals/experiment, selected at random.

Daily subcutaneous injections of 200 μg of the steroids 15—19 dissolved in 200 μl of sesame oil were administered for 6 d together with 200 μg of T. Three groups of animals were kept as control, one was injected with 200 μl of sesame oil, the second with 200 μg of T for 6 d and the third with T plus finasteride. After this treatment, 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 results were analyzed using one way of variance with Epistat software.

**Seminal Vesicles Test** The effect of the synthesized steroids on the seminal vesicles from castrated male hamsters was determined. After the sacrifice, the seminal vesicles were dissected out and weighed on a balance. Two separate experiments were performed for each group of steroid-treated animals. The results were analyzed using one way of variance with Epistat software.

**In Vitro Metabolic Studies with Seminal Vesicles** Homogenates from male hamsters seminal vesicles (around 29 mg of protein) were prepared from castrated adult male animals, using Krebs–Ringer buffer solution at pH of 6. Tissue preparations were incubated in duplicate with 2.3 μC [3H]T in
the presence of 1 mm NADPH, 8.7 μM of flaneridene 3 and compounds 15—19 in a Dubnoff metabolic incubator at 37°C for 60 min in the presence of air. The final incubation volume was 3 ml. Incubations without tissues were used as controls. Incubation was terminated by addition of dichloromethane and the [1H]steroid was extracted (4X) using 3 vol. of dichloromethane. The protein content of the homogenates was determined by the Bradford's dye-binding method using bovine serum albumin (BSA) as the standard.

The isolated compound was purified with steroid carriers (T, DHT) in a thin-layer chromatographic system (chloroform-acetone, 9:1). The non-radioactive steroid carriers were located by ultraviolet lamp (254 nm) and by spraying with phosphomolybdic acid reagent 8% in methanol followed by heating. The region containing DHT was scraped off; it was purified by silica gel column chromatography. Hexane–ethyl acetate (8:2) eluted 0.84 g, 1.97 mmol (70%) of pure sodium sulfate and the solvent was eliminated in vacuum. The crude product was purified by silica gel column chromatography. Hexane–ethyl acetate (8:2) eluted 0.81 g, 1.60 mmol (71%) of the pure product.

The reaction mixture was neutralized with an aqueous sodium bicarbonate solution 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 140 mg, 2.03 mmol (69%) of the pure product 19. mp 149—149°C. UV (nm): 221, 255, 300 (ε = 14600, 12400, 17300 respectively). IR (KBr) cm⁻¹: 1720, 1707, 1664, 1604. 1H-NMR (CDCl₃) δ: 0.8 (3H, s), 1.0 (4H, m), 1.3 (3H, s), 1.5 (3H, d, J = 4 Hz), 1.7 (7H, t, J = 4 Hz), 2.0 (3H, s), 2.3 (2H, t, J = 3 Hz), 6.1 (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). 13C-NMR (CDCl₃) δ: 15.7 (CH, at C-16), 17.6 (C-18), 20.6 (C-19), 83.9 (C-17), 163.5 (C-5), 172.6 (ester carbonyl), 182.2 (C-3), 211.6 (C-20). MS (m/z): 424 (M⁺).

16β-Methyl-17α-acetoxyprogesterone-4,6-diene-3,20-dione 18 A solution containing steroid 18 (1 g, 2.94 mmol), PTSE acid (10 mg), trifluoroacetic anhydride (8.92 g, 42.48 mmol) and valeric acid (1.41 g, 14.13 mmol) was neutralized with an aqueous sodium bicarbonate solution 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 (6:4) eluted 840 mg, 2.03 mmol (65%) of the pure product.

Synthesis of the Steroidal Compounds The synthesis of the intermediates 9—14 (Fig. 2) is given in refs. 23 and 24. The preparation of the new compounds 15—19 is briefly described below.

A solution of steroid 14 (1 g, 2.82 mmol), PTSE acid (10 mg), trifluoroacetic anhydride (8.92 g, 42.48 mmol) and valeric acid (1.41 g, 13.79 mmol) was neutralized with an aqueous sodium bicarbonate solution 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 and the crude product was purified by silica gel column chromatography. Hexane–ethyl acetate (7:3) eluted 0.81 g, 1.60 mmol (71%) of the pure product.

The synthesis of the intermediates was briefly described below.

References
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