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New steroidal lactones as 5α-reductase inhibitors and antagonists for the androgen receptor

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ABSTRACT

This study reports the synthesis of several new steroidal lactones: 5α,6β-dibromo-17a-oxa-D-homoandrostane-3β-yl-3′-oxapentanoate (**11**), 5α,6β-dibromo-17a-oxa-D-homoandrostane-3β-yl-propanoate (**12**), 5α,6β-dibromo-17a-oxa-D-homoandrostane-3β-yl-butanoate (**13**), 5α,6β-dibromo-17a-oxa-D-homoandrostane-3β-yl-pentanoate (**14**), 5α,6β-dibromo-17a-oxa-D-homoandrostane-3β-yl-hexanoate (**15**), 17a-oxa-D-homoandrost-5-en-17-one-3β-yl-3′-oxapentanoate (**16**), 17a-oxa-D-homoandrost-5-en-17-one-3β-yl-propanoate (**17**), 17a-oxa-D-homoandrost-5-en-17-one-3β-yl-butanoate (**18**), 17a-oxa-D-homoandrost-5-en-17-one-3β-yl-pentanoate (**19**) and 17a-oxa-D-homoandrost-5-en-17-one-3β-yl-hexanoate (**20**) with a therapeutic potential as antiandrogens.

The biological effect of these steroids was demonstrated in *in vivo* as well as *in vitro* experiments. In the *in vivo* experiments, we measured the activity of ten new steroidal derivatives on the weight of the prostate and seminal vesicle glands of gonadectomized hamsters treated with testosterone. For the *in vitro* studies, we determined the IC_{50} values by measuring the concentration of the steroidal derivatives that inhibits 50% of the activity of the 5α-reductase enzyme present in human prostate and also its binding capacity to the androgen receptors (AR) obtained from rat's prostate cytosol. The results from these experiments indicated that compounds **11**–**20**, significantly decreased the weight of the prostate and seminal vesicles as compared to testosterone treated animals; this reduction of the weight of these glands was comparable to that produced by Finasteride. On the other hand, compounds **11**–**20** inhibited the enzyme 5α-reductase, with compounds **14**–**19** (IC_{50} values of 4.2 ± 0.95, 0.025 ± 0.003, 1.2 ± 0.45, 1.2 ± 0.1, 0.028 ± 0.003, and 0.069 ± 0.005 nM, respectively) showing the highest inhibitory activity. The results from the *in vitro* experiments indicated that only 1**5**–**17** bind to the AR.

1. Introduction

The 5α-reductase enzyme (EC 1.3.99.5) converts testosterone (T) to 5α-dihydrotestosterone (DHT) in androgen dependent tissues. The activity of this enzyme in androgen dependent tissues has long been known. Two types of 5α-reductase (5α-R) enzymes had been identified: 1 and 2, each encoding for different genes, which have been characterized in several species [1,2]. 5α-R type 2 isozyme plays a major role in prostate cancer and benign prostatic hyperplasia as it is predominantly expressed in this tissue. However, some evidence indicates that type 1 enzyme is expressed in prostate epithelial cells while the type 2 is mainly located in the stromal compartment [3,4]. 5α-Reductase type 1 is also located in the liver and skin and acts in a neutral or basic medium, whereas type 2 is active in acidic pH [1,2].

The primary structures of the rat and human 5α-reductase isozymes were determined from their respective cDNAs [1]. They are hydrophobic proteins composed of 254–260 aminoacids. There are no consensus sequences for N-linked glycosilation (Asn-X-Ser/Thr) or for O-linked glycosilation (Ser/Thr/Pro-rich regions). The hydrophobic aminoacids Cys, Ile, Leu, Met, Phe, Val are distributed throughout both type 1 and 2. 5α-R enzymes which characterized these isozymes as intrinsic membrane proteins deeply embedded into the lipid bilayer [1].

On the other hand, androgen action is mediated by the androgen receptors (AR), a ligand-dependent transcriptional factor. DHT formed from T in target cells interacts more efficiently with the AR than T. The binding of these hydrophobic ligands induces the AR to assume a configuration that leads to transcription activation (or inhibition) and allows transmission of extracellular signals into

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intracellular responses by targeting promote response elements and recruiting cofactors [5].

It had previously been reported that the suppression of androgen action by 5α-reductase inhibitors, as well as with antagonists of AR are the logical treatment for 5α-reductase activity and AR disorders such: prostate cancer, benign prostatic hyperplasia, acne and male pattern baldness [6,7], which are androgen dependent illnesses.

Compounds incorporating a lactone ring are known to interact covalently with biological nucleophiles, and some have been reported to inhibit enzymes that contain in their molecule a cysteine, lysine or serine amino acid or threonine-based enzymes [8].

On the basis of the above mentioned considerations, we synthesized the following dehydroepiandrosterone lactone derivatives: 5α,6β-dibromo-17a-oxa-D-homoandrostane-3β-yl-3′-oxapentanoate (**11**), 5α,6β-dibromo-17a-oxa-D-homoandrostane-3β-yl-propanoate (**12**), 5α,6β-dibromo-17a-oxa-D-homoandrostane-3β-yl-butanoate (**13**), 5α,6β-dibromo-17a-oxa-D-homoandrostane-3β-yl-pentanoate (**14**), 5α,6β-dibromo-17a-oxa-D-homoandrostane-3β-yl-hexanoate (**15**), 17a-oxa-D-homoandrost-5-en-17-one-3β-yl-3′-oxapentanoate (**16**), 17a-oxa-D-homoandrost-5-en-17-one-3β-yl-propanoate (**17**), 17a-oxa-D-homoandrost-5-en-17-one-3β-yl-butanoate (**18**), 17a-oxa-D-homoandrost-5-en-17-one-3β-yl-pentanoate (**19**) and 17a-oxa-D-homoandrost-5-en-17-one-3β-yl-hexanoate (**20**). The effect of these steroidal lactones was evaluated *in vivo* as well as *in vitro*, as potential therapeutic antiandrogens.

2. Materials and methods

2.1. Chemical and radioactive materials

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 a 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 recorded on a Perkin-Elmer 200 spectrometer.

(1,2,6,7-^{3}H) testosterone [^{3}H] T specific activity: 95 Ci/mmol and Mibolerone (17α-methyl-^{3}H) [^{3}H] MIB (Fig. 2) specific activity 70–87 Ci/mmol were provided by a Perkin Elmer Life and Analytical Sciences (Boston, MA). Radioinert T, 5α-dihydrotestosterone and MIB were supplied by Steraloids (Wilton, NH, U.S.A.). Sigma Chemical Co. (St. Louis, MO) provided DCC, DMAP, MMPP and NADPH; Finasteride (Fig. 2) was obtained by extraction from Proscar® (Merck, Sharp & Dohme). The tablets were crushed, extracted with chloroform and the solvent was eliminated in vacuum; the crude product was purified by silica gel column chromatography. The melting point of the isolated Finasteride (252–254 °C) was identical to that reported in the literature.

2.2. Synthesis of the steroidal derivatives (Fig. 1)

2.2.1. General procedure for the preparation of esters

To a solution of steroid (1 g, 3 mmol), DMAP (0.848 g, 6.94 mmol), DCC (2.147 g, 10.41 mmol) in chloroform (15 mL) was added the corresponding acid (14 mmol). The resulting solution was stirred at room temperature for 2 h. Hexane (15 mL) was added and the mixture was filtered to remove the precipitated dicyclohexylurea. The organic phase was washed three times with 10% aqueous hydrochloric acid, 5% aqueous bicarbonate and water. The crude ester was recristallized from methanol.

*2.2.1.1. 17-Oxaandrost-5-ene-3β-yl-3′-oxapentanoate (**1**).* Yield: 96.1% of pure product, mp 143–145 °C. IR (KBr) cm^{-1}: 2954, 1758 and 1740. ^{1}H RMN ($CDCl_3$) δ: 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.3 (3H, t, H-5′), 3.6 (2H, q, H-4′), 4.1 (2H, s, H-2′), 4.7 (1H, m, H-3), 5.5 (1H, dd, $J_1 = 4$ Hz and $J_2 = 2$ Hz, H-6). ^{13}C RMN ($CDCl_3$) δ: 13.7 (C-18), 14.9 (C-5′), 19.5 (C-19), 67.1 (C-4′), 68.2 (C-2′), 74.3 (C-3), 122.1 (C-6), 140.0 (C-5), 168.9 (ester carbonyl), 220.9 (C-17). FAB-MS calc for $C_{23}H_{34}O_4$ 375.3287 (M+H) found 375.5436.

*2.2.1.2. 17-Oxaandrost-5-ene-3β-yl-propanoate (**2**).* Yield: 90.4% of pure product, mp 150–153 °C. IR (KBr) cm^{-1}: 2954, 1739 and 1666. ^{1}H RMN ($CDCl_3$) δ: 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.1 (3H, t, H-3′), 2.3 (2H, q, H-2′), 4.6 (1H, m, H-3), 5.5 (1H, dd, $J_1 = 4$ Hz and $J_2 = 2$ Hz, H-6). ^{13}C RMN ($CDCl_3$) δ: 9.1 (C-3′), 13.7 (C-18), 19.5 (C-19), 31.4 (C-2′), 73.5 (C-3), 121.8 (C-6), 139.9 (C-5), 173.9 (ester carbonyl), 221.0 (C-17). FAB-MS calc for $C_{22}H_{32}O_3$ 345.4378 (M+H) found 345.4583.

*2.2.1.3. 17-Oxaandrost-5-ene-3β-yl-butanoate (**3**).* Yield: 93.0% of pure product, mp 160–162 °C. IR (KBr) cm^{-1}: 2965, 1742 and 1730. ^{1}H RMN ($CDCl_3$) δ: 0.8 (3H, t, H-4′), 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.3 (2H, m, H-3′), 2.3 (2H, t, H-2′), 4.6 (1H, m, H-3), 5.4 (1H, dd, $J_1 = 4$ Hz and $J_2 = 2$ Hz, H-6). ^{13}C RMN ($CDCl_3$) δ: 13.6 (C-18), 13.7 (C-4′), 18.5 (C-3′), 19.5 (C-19), 36.2 (C-2′), 73.4 (C-3), 121.8 (C-6), 139.9 (C-5), 173.1 (ester carbonyl), 221.0 (C-17). FAB-MS calc for $C_{23}H_{34}O_3$ 359.7623 (M+H) found 359.6532.

*2.2.1.4. 17-Oxaandrost-5-ene-3β-yl-pentanoate (**4**).* Yield: 96.7% of pure product, mp 101–103 °C. IR (KBr) cm^{-1}: 2946, 1735 and 1725. ^{1}H RMN ($CDCl_3$) δ: 0.8 (3H, t, H-5′), 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.3 (2H, m, H-4′), 1.5 (2H, m, H-3′), 2.2 (2H, t, H-2′), 4.6 (1H, m, H-3), 5.4 (1H, dd, $J_1 = 4$ Hz and $J_2 = 2$ Hz, H-6). ^{13}C RMN ($CDCl_3$) δ: 13.7 (C-18), 13.8 (C-5′), 19.4 (C-19), 23.8 (C-4′), 31.4 (C-3′), 34.7 (C-2′), 73.2 (C-3), 122.0 (C-6), 139.5 (C-5), 173.3 (ester carbonyl), 221.0 (C-17). FAB-MS calc for $C_{24}H_{36}O_3$ 373.5408 (M+H) found 373.5397.

*2.2.1.5. 17-Oxaandrost-5-ene-3β-yl-hexanoate (**5**).* Yield: 92.7% of pure product, mp 72–74 °C. IR (KBr) cm^{-1}: 2953, 1736 and 1736. ^{1}H RMN ($CDCl_3$) δ: 0.8 (3H, t, H-6′), 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.3 (2H, m, H-5′), 1.5 (2H, m, H-4′), 1.6 (2H, m, H-3′), 2.3 (2H, t, H-2′), 4.8 (1H, m, H-3), 5.5 (1H, dd, $J_1 = 4$ Hz and $J_2 = 2$ Hz, H-6). ^{13}C RMN ($CDCl_3$) δ: 13.7 (C-18), 13.9 (C-6′), 19.4 (C-19), 22.3 (C-5′), 24.7 (C-4′), 31.5 (C-3′), 34.6 (C-2′), 73.3 (C-3), 121.8 (C-6), 138.9 (C-5), 173.1 (ester carbonyl), 220.2 (C-17). FAB-MS calc for $C_{25}H_{38}O_3$ 387.3478 (M+H) found 387.3598.

2.2.2. General procedure for the preparation of 5α,6β-dibromo derivatives

A solution of the corresponding ester (2.9 mmol) in dichloromethane was cooled to 0–5 °C, a solution (2.4 mL) of bromine in carbon tetrachloride (bromine 0.9 mL and carbon tetrachloride 9.1 mL) was slowly added. The mixture was stirred at room temperature for 2 h. Upon termination of the reaction, 10% aqueous sodium thiosulfate was added. The reaction mixture was extracted three times with dichloromethane. It was dried with sodium sulfate and the solvent was removed in vacuum. The crude product was recristallized from methanol.

*2.2.2.1. 5α,6β-Dibromo-17-oxaandrostane-3β-yl-3′-oxapentanoate (**6**).* Yield: 93.1% of pure product, mp 123–125 °C. IR (KBr) cm^{-1}: 2947, 1736 and 1727. ^{1}H RMN ($CDCl_3$) δ: 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.3 (3H, t, H-5′), 3.6 (2H, q, H-4′), 4.1 (2H, s, H-2′), 4.9 (1H, dd, $J_1 = 4$ Hz and $J_2 = 2$ Hz, H-6), 5.6 (1H, m, H-3). ^{13}C RMN ($CDCl_3$) δ: 13.7 (C-18), 13.9 (C-5′), 19.4 (C-19), 55.2 (C-6), 67.2 (C-4′), 68.2

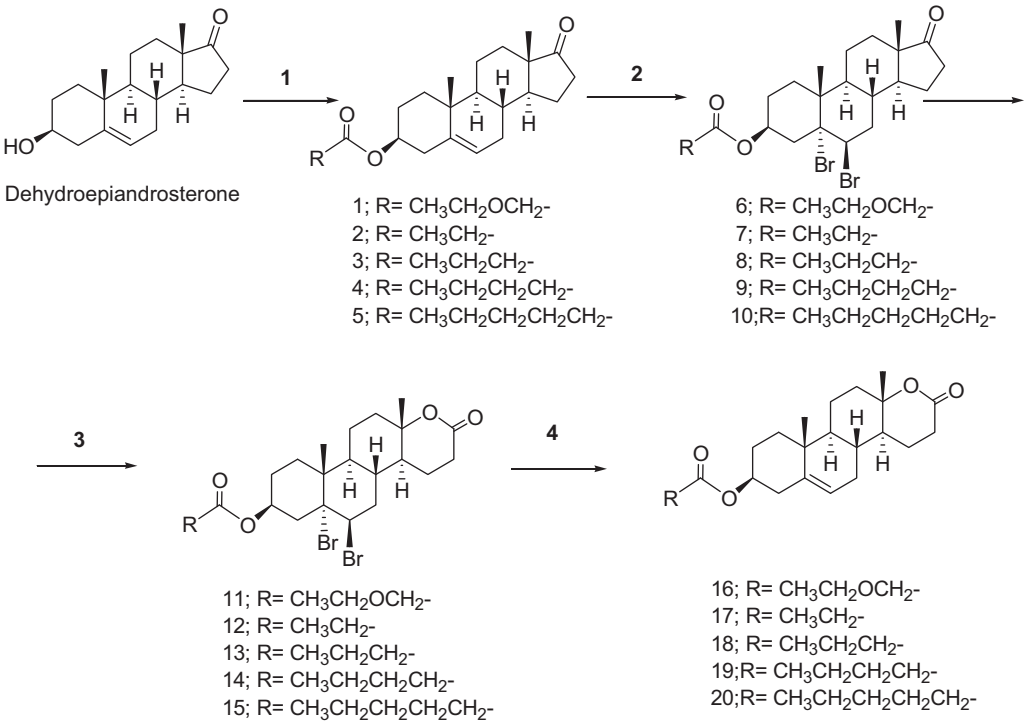

Fig. 1. Synthesis of novel steroidal lactones.

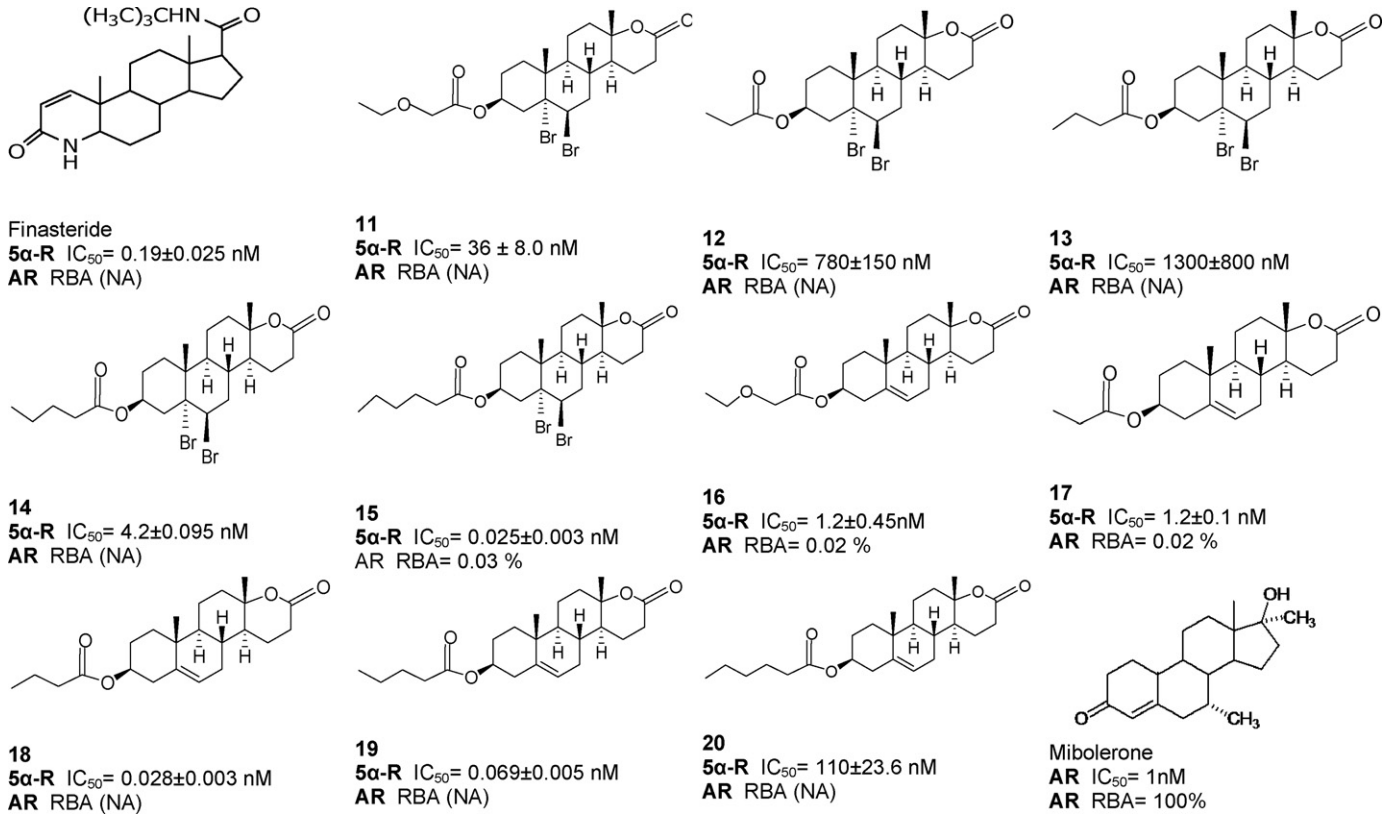

Fig. 2. Steroidal structures and effect of different lactones on the activity of 5α-reductase enzyme (5α-R): **13**–**22**. The IC_{50} values indicate the required concentration of the novel steroids for the inhibition of 50% of the activity of 5α-R. This figure shows also the relative binding affinity (RBA) of these derivatives to the androgen receptor (AR). NA, non active compound.

(C-2′), 72.5 (C-3), 87.1 (C-5), 170.2 (ester carbonyl), 220.1 (C-17). FAB-MS calc for $C_{23}H_{34}Br_2O_4$ 533.8734 (M+H) found 533.6594.

*2.2.2.2. 5α,6β-Dibromo-17-oxaandrostane-3β-yl-propanoate (**7**).* Yield: 94.2% of pure product, mp 96–98 °C. IR (KBr) cm^{-1}: 2947, 1731 and 1766. ^{1}H RMN ($CDCl_3$) δ: 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.1 (3H, t, H-3′), 2.3 (2H, q, H-2′), 4.9 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6), 5.4 (1H, m, H-3). ^{13}C RMN ($CDCl_3$) δ: 9.1 (C-3′), 13.6 (C-18), 19.5 (C-19), 27.7 (C-2′), 55.3 (C-6), 71.6 (C-3), 87.1 (C-5), 173.8 (ester carbonyl), 220.3 (C-17). FAB-MS calc for $C_{22}H_{32}Br_2O_3$ 503.6578 (M+H) 503.4398.

*2.2.2.3. 5α,6β-Dibromo-17-oxaandrostane-3β-yl-butanoate (**8**).* Yield: 88.7% of pure product, mp 128–130 °C. IR (KBr) cm^{-1}: 2946, 1726 and 1665. ^{1}H RMN ($CDCl_3$) δ: 0.8 (3H, t, H-4′), 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.6 (2H, m, H-3′), 2.3 (2H, t, H-2′), 4.9 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6), 5.5 (1H, m, H-3). ^{13}C RMN ($CDCl_3$) δ: 13.7 (C-18), 13.9 (C-4′), 18.5 (C-3′), 19.5 (C-19), 36.4 (C-2′), 55.3 (C-6), 71.5 (C-3), 87.4 (C-5), 172.9 (ester carbonyl), 220.1 (C-17). FAB-MS calc for $C_{23}H_{34}Br_2O_3$ 517.5428 (M+H) 517.5347.

*2.2.2.4. 5α,6β-Dibromo-17-oxaandrostane-3β-yl-pentanoate (**9**).* Yield: 91.6% of pure product, mp 126–128 °C. IR (KBr) cm^{-1}: 2935, 1739 and 1731. ^{1}H RMN ($CDCl_3$) δ: 0.8 (3H, t, H-5′), 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.3 (2H, m, H-4′), 1.5 (2H, m, H-3′), 2.3 (2H, t, H-2′), 4.9 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6), 5.5 (1H, m, H-3). ^{13}C RMN ($CDCl_3$) δ: 13.7 (C-18), 13.8 (C-5′), 19.4 (C-19), 21.5 (C-4′), 31.4 (C-3′), 34.8 (C-2′), 55.3 (C-6), 71.5 (C-3), 87.4 (C-5), 173.1 (ester carbonyl), 220.1 (C-17). FAB-MS calc for $C_{24}H_{36}Br_2O_3$ 533.3488 (M+H) 533.3375.

*2.2.2.5. 5α,6β-Dibromo-17-oxaandrostane-3β-yl-hexanoate (**10**).* Yield: 91.8% of pure product, mp 133–135 °C. IR (KBr) cm^{-1}: 2954, 1736 and 1731. ^{1}H RMN ($CDCl_3$) δ: 0.8 (3H, t, H-6′), 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.3 (2H, m, H-5′), 1.5 (2H, m, H-4′), 1.6 (2H, m, H-3′), 2.3 (2H, t, H-2′), 4.9 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6), 5.5 (1H, m, H-3). ^{13}C RMN ($CDCl_3$) δ: 13.7 (C-18), 13.9 (C-6′), 19.4 (C-19), 22.3 (C-5′), 24.7 (C-4′), 31.5 (C-3′), 34.5 (C-2′), 55.3 (C-6), 71.5 (C-3), 87.4 (C-5), 173.2 (ester carbonyl), 220.2 (C-17). FAB-MS calc for $C_{25}H_{38}Br_2O_3$ 547.3754 (M+H) found 547.3781.

2.2.3. General procedure for the preparation of 3β-acyloxy-5α,6β-dibromo-D-homoandrostane lactones

To a solution of the corresponding 5α,6β-dibromo derivative (0.38 mmol) in dichloromethane-methanol (1:2, 50 mL) was added monoperoxyphthalic acid magnesium salt hexahydrate (MMPP) (6.5 mmol) and 1 mL of water. The mixture was stirred at room temperature for 18 h. Upon termination of the reaction, the reaction mixture was washed three times with 5% aqueous bicarbonate and water. It was dried with sodium sulfate and the solvent was removed in vacuum. The compound was purified by silica gel column chromatography; the corresponding lactone was eluded with hexane/Ethyl acetate (90:10).

*2.2.3.1. 5α,6β-Dibromo-17a-oxa-D-homoandrostane-3β-yl-3′-oxapentanoate (**11**).* Yield: 93.0% of pure product, mp 157–159 °C. IR (KBr) cm^{-1}: 2943 and 1732. ^{1}H RMN ($CDCl_3$) δ: 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.3 (3H, t, H-5′), 3.6 (2H, q, H-4′), 4.1 (2H, s, H-2′), 4.9 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6), 5.6 (1H, m, H-3). ^{13}C RMN ($CDCl_3$) δ: 13.7 (C-18), 14.5 (C-5′), 19.4 (C-19), 54.8 (C-6), 67.2 (C-4′), 68.1 (C-2′), 72.9 (C-3), 82.7 (C-5), 169.9 (carbonyl of the 3β ester function), 171.0 (ester carbonyl of the lactone function). FAB-MS calc for $C_{23}H_{34}Br_2O_5$ 551.3211 (M+H) found 551.3199.

*2.2.3.2. 5α,6β-Dibromo-17a-oxa-D-homoandrostane-3β-yl-propanoate (**12**).* Yield: 92.0% of pure product, mp 147–149 °C. IR (KBr) cm^{-1}: 2943 and 1741. ^{1}H-RMN ($CDCl_3$) δ: 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.1 (3H, t, H-3′), 2.3 (2H, q, H-2′), 4.9 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6), 5.5 (1H, m, H-3). ^{13}C RMN ($CDCl_3$) δ: 9.1 (C-3′), 13.7 (C-18), 19.4 (C-19), 27.8 (C-2′), 54.9 (C-6), 71.5 (C-3), 82.8 (C-5), 171.0 (carbonyl of the 3β ester function), 173.8 (ester carbonyl of the lactone function). FAB-MS calc for $C_{22}H_{32}Br_2O_4$ 521.2951 (M+H) 521.2907

*2.2.3.3. 5α,6β-Dibromo-17a-oxa-D-homoandrostane-3β-yl-butanoate (**13**).* Yield: 89.5% of pure product, mp 129–131 °C. IR (KBr) cm^{-1}: 2952 and 1729. ^{1}H RMN ($CDCl_3$) δ: 0.8 (3H, t, H-4′), 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.7 (2H, m, H-3′), 2.3 (2H, t, H-2′), 4.9 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6), 5.5 (1H, m, H-3). ^{13}C RMN ($CDCl_3$) δ: 13.7 (C-18), 13.9 (C-4′), 18.5 (C-3′), 19.5 (C-19), 38.9 (C-2′), 54.9 (C-6), 71.4 (C-3), 86.4 (C-5), 171.0 (carbonyl of the 3β ester function), 172.9 (ester carbonyl of the lactone function). FAB-MS calc for $C_{23}H_{34}Br_2O_4$ 535.3217 (M+H) 535.3159.

*2.2.3.4. 5α,6β-Dibromo-17a-oxa-D-homoandrostane-3β-yl-pentanoate (**14**).* Yield: 88.6% of pure product, mp 143–145 °C. IR (KBr) cm^{-1}: 2941 and 1730. ^{1}H RMN ($CDCl_3$) δ: 0.8 (3H, t, H-5′), 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.3 (2H, m, H-4′), 1.5 (2H, m, H-3′), 2.3 (2H, t, H-2′), 4.9 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6), 5.5 (1H, m, H-3). ^{13}C RMN ($CDCl_3$) δ: 13.7 (C-18), 13.8 (C-5′), 19.4 (C-19), 22.3 (C-4′), 26.1 (C-3′), 33.5 (C-2′), 54.9 (C-6), 71.4 (C-3), 86.4 (C-5), 171.0 (carbonyl of the 3β ester function), 173.1 (ester carbonyl of the lactone function). FAB-MS calc for $C_{24}H_{36}Br_2O_4$ 549.3482 (M+H) 549.3378.

*2.2.3.5. 5α,6β-Dibromo-17a-oxa-D-homoandrostane-3β-yl-hexanoate (**15**).* Yield: 87.0% of pure product, mp 137–139 °C. IR (KBr) cm^{-1}: 2950 and 1732. ^{1}H RMN ($CDCl_3$) δ: 0.8 (3H, t, H-6′), 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.3 (2H, m, H-5′), 1.5 (2H, m, H-4′), 1.6 (2H, m, H-3′), 2.3 (2H, t, H-2′), 4.9 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6), 5.5 (1H, m, H-3),. ^{13}C RMN ($CDCl_3$) δ: 13.7 (C-18), 13.9 (C-6′), 19.4 (C-19), 22.3 (C-5′), 24.6 (C-4′), 31.6 (C-3′), 34.5 (C-2′), 54.9 (C-6), 71.4 (C-3), 86.4 (C-5), 173.1 (carbonyl of the 3β ester function), 173.2 (ester carbonyl of the lactone function). FAB-MS calc for $C_{25}H_{38}Br_2O_4$ 563.3748 (M+H) found 563.3696.

2.2.4. General procedure for the preparation of the final lactone derivatives

To a solution of the corresponding 5α,6β-dibromo-17a-oxa derivative (0.55 mmol) in 5 mL of tetrahydrofuran was added sodium iodide (3.5 mmol). The mixture was refluxed for 3 h; it was cooled off to room temperature, 20% aqueous sodium thiosulfate was added. The reaction mixture was extracted three times with dichloromethane. It was dried with sodium sulfate and the solvent was removed in vacuum. The crude product was purified by column chromatography (hexane/ethyl acetate, 90:10).

*2.2.4.1. 17a-Oxa-D-homoandrost-5-en-17-one-3β-yl-3′-oxapentanoate (**16**).* Yield: 92.0% of pure product, mp 124–126 °C. IR (KBr) cm^{-1}: 2933 and 1723. ^{1}H RMN ($CDCl_3$) δ: 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.3 (3H, t, H-5′), 3.6 (2H, q, H-4′), 4.1 (2H, s, H-2′), 4.7 (1H, m, H-3), 5.5 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6). ^{13}C RMN ($CDCl_3$) δ: 13.7 (C-18), 14.9 (C-5′), 67.1 (C-4′), 68.2 (C-2′), 74.3 (C-3), 122.0 (C-6), 141.0 (C-5), 170.1 (carbonyl of the 3β ester function), 171.0 (ester carbonyl of the lactone function). FAB-MS calc for $C_{23}H_{34}O_5$ 391.5131 (M+H) found 391.5054.

*2.2.4.2. 17a-Oxa-D-homoandrost-5-en-17-one-3β-yl-propanoate (**17**).* Yield: 93.0% of pure product, mp 170–172 °C. IR (KBr) cm^{-1}: 2950 and 1731. ^{1}H RMN ($CDCl_3$) δ: 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.1 (3H, t, H-3′), 2.3 (2H, q, H-2′), 4.6 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6). ^{13}C RMN ($CDCl_3$) δ: 9.1 (C-3′), 13.7

(C-18), 19.4 (C-19), 27.9 (C-2′), 73.3 (C-3), 121.4 (C-6), 139.6 (C-5), 171.4 (carbonyl of the 3β ester function), 173.9 (ester carbonyl of the lactone function). FAB-MS calc for $C_{22}H_{32}O_4$ 361.4871 (M+H) 361.4807.

*2.2.4.3. 17a-Oxa-D-homoandrost-5-en-17-one-3β-yl-butanoate (**18**).* Yield: 92.3% of pure product, mp 189–191 °C. IR (KBr) cm^{-1}: 2948 and 1731. ^{1}H RMN ($CDCl_3$) δ: 0.8 (3H, t, H-4′), 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.7 (2H, m, H-3′), 2.3 (2H, t, H-2′), 4.6 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6). ^{13}C RMN ($CDCl_3$) δ: 13.6 (C-18), 13.7 (C-4′), 18.5 (C-3′), 19.5 (C-19), 38.9 (C-2′), 73.2 (C-3), 121.4 (C-6), 139.6 (C-5), 171.4 (carbonyl of the 3β ester function), 173.1 (ester carbonyl of the lactone function). FAB-MS calc for $C_{23}H_{34}O_4$ 375.5137 (M+H) 375.5098.

*2.2.4.4. 17a-Oxa-D-homoandrost-5-en-17-one-3β-yl-pentanoate (**19**).* Yield: 91.0% of pure product, mp 106–108 °C. IR (KBr) cm^{-1}: 2949and 1730. ^{1}H RMN ($CDCl_3$) δ: 0.8 (3H, t, H-5′), 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.3 (2H, m, H-4′), 1.6 (2H, m, H-3′), 2.3 (2H, t, H-2′), 4.6 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6). ^{13}C RMN ($CDCl_3$) δ: 13.7 (C-18), 13.9 (C-5′), 19.4 (C-19), 22.3 (C-4′), 31.3 (C-3′), 34.4 (C-2′), 73.2 (C-3), 121.4 (C-6), 139.6 (C-5), 171.2 (carbonyl of the 3β ester function), 173.3 (ester carbonyl of the lactone function). FAB-MS calc for $C_{24}H_{36}O_3$ 389.5402 (M+H) 389.5374.

*2.2.4.5. 17a-Oxa-D-homoandrost-5-en-17-one-3β-yl-hexanoate (**20**).* Yield: 89% of pure product, mp 117–119 °C. IR (KBr) cm^{-1}: 2948 and 1731. ^{1}H RMN ($CDCl_3$) δ: 0.8 (3H, t, H-6′), 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 1.3 (2H, m, H-5′), 1.5 (2H, m, H-4′), 1.6 (2H, m, H-3′), 2.3 (2H, t, H-2′), 4.6 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6), ^{13}C RMN ($CDCl_3$) δ: 13.7 (C-18), 13.9 (C-6′), 19.4 (C-19), 22.3 (C-5′), 24.7 (C-4′), 31.2 (C-3′), 34.4 (C-2′), 73.2 (C-3), 121.4 (C-6), 139.6 (C-5), 171.2 (carbonyl of the 3β ester function), 173.3 (ester carbonyl of the lactone function). FAB-MS calc for $C_{25}H_{38}O_4$ 403.5668 (M+H) found 403.5591.

The biological activity of steroids **1**–**10** is currently been carried out.

2.3. Biological activity of the novel compounds

The human prostate from a man of 53 years old, who died from diabetes and renal insufficiency, was introduced to a solution of NaCl 150 mM and stored at −70 °C. This gland was obtained from the Department of Pathology, The General Hospital (SS) in Mexico City. Frozen human prostate was thawed on ice and minced with scissors. Unless specified, the following procedures were carried out at 4 °C.

2.3.1. Animals and tissues

Adult male golden hamsters 2.5 month old (150–200 g) were obtained from the Metropolitan University in Xochimilco, Mexico. In order to carry out the *in vivo* experiments, several male hamsters were gonadectomized. Gonadectomies were performed under pentobarbital anesthesia and the castrated hamsters were kept in a room with controlled temperature (22 °C) and light-dark periods of 12 h. Food and water were provided *ad libitum*. After 30 days of maintaining these conditions, the hamsters were sacrificed with CO_2 [9]. This protocol was approved by the Institutional Care and Use Committee of the Metropolitan University of Mexico (UAM). This study with gonadectomized animals was carried out on 8 groups of 4 animals/experiment.

In order to determine the binding of steroids **11**–**20** to the androgen receptors, adult rats 8 months old weighing 500 g were used [10,11]. In this study we used rats because they have a bigger prostate gland and there is no difference in the binding activity between rats and hamsters cytosol.

The prostate of the rats was removed, blotted, weighed and soaked in cold TEMD (40 mM Tris–HCl, 3 mM EDTA and 20 mM sodium molybdate, dithiotreitol 0.5 mM, 10% glycerol at pH 8) prior to their use. Unless specified, all procedures were carried out in ice bath. Tissues were homogenized with a tissue homogenizer (Teckmar, Cincinnati, OH).

Tissues were homogenized in one volume of buffer TEMD plus protease inhibitors (2 mM PMSF, 10 μg/mL antipain, 5 mM leupeptin [12]) in an ice bath with a tissue homogenizer. Homogenates were centrifuged at 140,000 × *g* for 60 min [13] in a SW 60 Ti rotor (Beckman Instruments, Palo Alto, CA).

The cytosolic fraction obtained from the supernatant liquid of the rat prostate homogenate described above, was stored at −70 °C. Prostatic cytosol proteins (6 mg of protein in 200 μL) were determined by the Bradford's method [14].

2.3.2. In vitro experiments

2.3.2.1. Competitive studies. For competitive studies, tubes containing 1 nM of [^{3}H] MIB plus a range of increasing concentrations (1×10^{-10}–4×10^{-7} M) of cold MIB (Fig. 2) and **11**–**20** in ethanol or chloroform, or in absence of the competitor were prepared [15]. Incubates contained also 200 nM of triamcinolone, in ethanol (Sigma) to prevent interaction of MIB with glucocorticoid and progesterone receptors. Later, the solvent was evaporated from the tubes.

Aliquots of 200 μL of prostate cytosol were added and incubated in the presence of 300 μL of TEMD buffer containing protease inhibitors (duplicate) for 24 h at 4 °C in the tubes as previously described. After incubation, 0.27 mL saturated ammonium sulfate in TEMD buffer (35%) was added [15,16]. The mixture was further incubated for 1 h with occasional shaking to facilitate the precipitation of the [^{3}H] MIB-complex. The precipitate was collected by centrifugation at 10,000 × *g*, 10 min and the pellet was redissolved in 0.5 mL of TEMD and mixed with 0.5 of 0.1% dextran-coated 1% charcoal in TEMD buffer. 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 agitated on a vortex and immediately centrifuged at 800 × *g* for 10 min to pellet the charcoal; aliquots (600 μL) were taken and submitted for radioactive counting. The IC_{50} of each compound was determined according to the plots of concentration versus percentage of binding using Sigma Plot software.

2.3.2.2. Determination of the activity of 5α-reductase in the nuclear fraction from prostate homogenates. Human prostate was homogenized in 2 volumes of medium A (20 mM sodium phosphate, pH 6.5 containing 0.32 M sucrose, 0.1 mM dithiothreitol Sigma–Aldrich, Inc) with a tissue homogenizer. Homogenates were centrifuged at 1500 × *g* for 20 min [17,18] in a SW 60 Ti rotor (Beckman instruments, Palo Alto, CA). The pellets were separated, suspended in medium A and kept at −70° C. The suspension, 5 mg of protein/mL for human prostates, determined by the Bradford's method [14] was used as source of 5α-reductase.

The enzyme 5α-reductase was assayed as previously described [16,17]. The reaction mixture for human prostate contained: 1 mM dithiothreitol, sodium phosphate buffer 40 mM, at pH 6.5, 2 mM, NADPH, 2 nM [1,2,6,7-^{3}H]T [16] in a final volume of 1 mL. The reaction in duplicate was started when it was added to the enzymatic fraction (500 μg protein in a volume of 80 μL) incubated at 37 °C for 60 min [18] and stopped by mixing with 1 mL of dichloromethane; this was considered as the end point. Incubation without tissue was used as a control. The mixture (incubation medium/dichloromethane) was agitated on a vortex

for one minute and the dichloromethane phase was separated and placed in another tube. This procedure was repeated 4 more times. The dichloromethane extract was evaporated to dryness under a nitrogen stream and suspended in 50 μL of methanol that was spotted on HPTLC Keiselgel 60 F_{254} plates. T and DHT were used as carriers and were applied in different lanes on both lateral sides of the plates (T, T + DHT and DHT). The plates were developed in chloroform–acetone 9:1 and were air-dried; the chromatography was repeated 2 more times. The steroid carriers were detected using phosphomolibdic acid reagent (DHT) and T with an UV lamp (254 nm). After the plates were segmented in areas of 1 cm each, they were cut off and the strips soaked in 5 mL of Ultima Gold (Packard). The radioactivity was determined in a scintillation counter (Packard tri-carb 2100 TR). The radioactivity content in the segment corresponding to T and DHT carriers was identified. The radioactivity that has identical chromatographic behavior as the DHT standard was considered as the DHT transformation. Control incubations, chromatography separations and identifications, were carried out in the same manner as described above except that the tubes did not contain tissue. The DHT transformation yields were calculated from the strips, taken into account the entire radioactivity in the plate.

2.3.2.3. Determination of 50% of the inhibitory concentration of steroids 11–20 in human prostatic 5α-reductase. In order to calculate the IC_{50} values (the concentration of steroids **11**–**20** or finasteride required to inhibit 5α-reductase activity by 50%), six series of tubes containing increasing concentrations of these steroids (10^{-11}–10^{-3} M) were incubated in duplicate, in the presence of: 1 mM of dithiothreitol, 40 mM sodium phosphate buffer pH of 6.5; 2 mM NADPH, 2 nM [1,2,6,7-^{3}H]T and 500 (g of protein from enzymatic fraction in a final volume of 1 mL. The reaction was carried out in duplicate at 37 °C for 60 min; 1 mL of dichloromethane was added to stop the reaction. The extraction and the chromatographic procedures were carried out as described above.

After the chromatography, the plates were segmented in areas of 1 cm each, cut off and the strips were soaked in 10 mL of Ultima Gold (Packard). The radioactivity was determined in a scintillation counter (Packard tri-carb 2100 TR). The radioactivity content in the segments corresponding to T and DHT carriers was identified. The fraction that has identical chromatographic behavior as the DHT standard was considered as the DHT transformation in the presence of the tested compounds. Control incubations, chromatography separations and identifications, were carried out in the same manner as described above except that these tubes did not contain tissue. The DHT transformation yields were calculated from the strips, taken into account the entire radioactivity in the plate.

The IC_{50} of each compound was determined according to the plots of concentration versus percentage of binding using Sigma Plot software.

2.3.2.4. In vivo experiments. For the daily subcutaneous injections, 2 mg/kg of the steroids **11**–**20** were dissolved in 200 μL of sesame oil and administered for 6 days to the gonadectomized animals, together with 1 mg/kg of testosterone. Three groups of gonadectomized animals were kept as control; one was injected with 200 μL of sesame oil, the second one with 1 mg/kg of testosterone and the third one with T plus 1 mg/kg of finasteride for 6 days. After the treatment, the animals were sacrificed by CO_2. The prostate and seminal vesicles of each animal were dissected and weighed. Two separate experiments were performed for each group of steroid treated animals. The results were analyzed using one-way analysis of variance and Dunnett's method for compare means, with JMP IN 5.1 software.

3. Results

3.1. In vitro experiments

3.1.1. Competitive studies

The results showed that non labeled MIB as well as compounds **15**–**17** competed for the androgen receptor with labeled MIB and exhibited an IC_{50} value of 1, 6.2, 9.5 and 9.5 nM respectively. Their relative binding affinities (RBAs) to the AR are shown in Fig. 2. These data showed also that steroids **13**, **14**, **18**– **22** did not inhibit labeled MIB binding to the androgen receptor. The RBAs were calculated according to the following equation:

$$\text{RBA} = \frac{\text{IC}_{50}\text{ of [}^3\text{H] mibolerone}}{\text{IC}_{50}\text{ of inhibitor}} \times 100$$

3.1.2. 5α-Reductase activity

The *in vitro* biological activity of steroids **11**–**20** was determined in human 5α-reductase enzyme. The radioactive zone that had identical chromatographic behavior as the standard T (Rf value of 0.56) corresponds to 70% of the accounted radioactivity in the plate. The radioactivity contained in the zone corresponding to DHT standard (Rf value of 0.67) of the experimental chromatogram was identified as the transformed DHT and corresponds to 27% of the total radioactivity accounted in the plate. This result was considered to be 100% of the activity of 5α-reductase for the development of inhibition plots. Unmodified [^{3}H]T was identified (Rf value of 0.56) from control incubations which did not contain tissue and had identical chromatographic behavior as the non labeled standard. The radioactivity contained in the zone corresponding to DHT standard (Rf value of 0.67) of the control chromatogram is of 1% of the total radioactivity accounted in the plate and was considered as an error; it was subtracted from the experimental chromatograms.

3.1.3. Concentration of the new compounds that inhibits 50% of 5α-reductase activity in human prostate

The concentrations of finasteride and compounds **11**–**20** required for inhibiting 5α-reductase activity by 50% (IC_{50}) were determined from the inhibition plots using Sigma Plot software and are shown in Fig. 2.

These data showed that all steroidal lactones described in this paper inhibited the activity of human 5α-reductase with compounds **14**–**19** exhibiting the highest inhibitory activity with IC_{50} values of 4.2 ± 0.95, 0.025 ± 0.003, 1.2 ± 0.45, 1.2 ± 0.1, 0.028 ± 0.003 and 0.069 ± 0.005 nM respectively (Fig. 2).

3.1.4. In vivo experiments

3.1.4.1. Weight of the prostate and seminal vesicles. After castration, the weight of the hamster prostate and seminal vesicles decreased ($p < 0.05$) compared to the normal glands. Treatment with vehicle alone did not change this condition, whereas s.c. injections of 1 mg/kg of T for 6 days significantly increased ($p < 0.05$) the weight of these glands in castrated male hamsters (Fig. 3). When T (1 mg/kg) and finasteride or compounds **11**–**20** were injected together, the weight of the prostate and seminal vesicles decreased significantly ($p < 0.05$) as compared to that of T-treated animals (Fig. 3). This reduction of the weight of these glands was comparable to that produced by finasteride (2 mg/kg). Compound **20** showed the lowest weight of the prostate and seminal vesicles, thus indicating a higher effect, whereas **13**, **14**, and **19** did not significantly reduce the weight of these glands in the treated hamsters ($p > 0.05$), however the statistical results exhibited a tendency to decrease the weight of these glands.

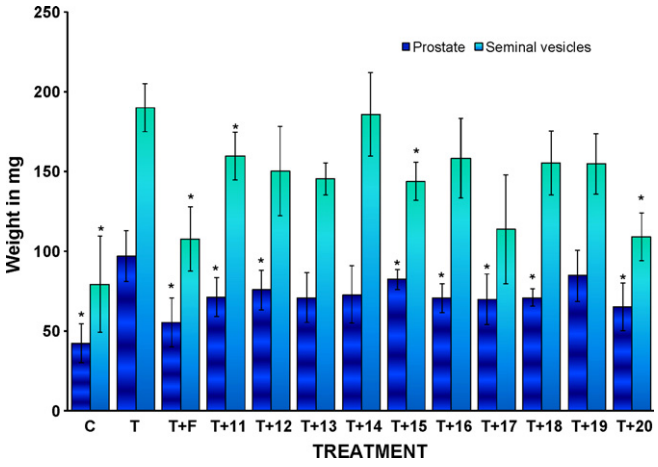

Fig. 3. Weight of prostate and seminal vesicles glands ± standard deviation from castrated hamsters receiving different s.c. treatments for 6 days. The control animals (C) were treated with vehicle only. The pharmacological experiment was carried out in duplicate. The asterisk shows the statistically significant difference between the group of hamsters treated with T and those treated with finasteride or the synthesized steroids.

4. Discussion

In this paper, we report the synthesis and biological activity of several new steroidal lactones **11**–**20**. All compounds described in this paper are good inhibitors for the human 5α-reductase enzyme (*in vitro* evaluation) with compounds **15**, **18** and **19** showing higher inhibitory activity (lower IC_{50} value) as compared to that of finasteride, the drug of choice for the treatment of benign prostatic hyperplasia.

Since all steroidal lactones are lipophilic compounds, they could interact with the enzyme sites, thus forming more readily a steroid-enzyme activated complex and this fact could explain their enhanced activity *in vitro* as well as *in vivo*. On the other hand, the lactone functional group in the D-ring of the steroidal skeleton could react with the nucleophilic residue [8] present in the hydrophobic interior of the enzyme 5α-reductase (cysteine, serine and lysine) to form a thioester, ester or an amide respectively. This reaction could inhibit the enzyme 5α-reductase and as a result of this, the conversion of T to DHT will be blocked.

Previous studies carried out by our group had demonstrated that some dehydroepiandrosterone derivatives inhibited human 5α-reductase enzyme activity. The results from these experiments indicated that a lactone functional group incorporated into the D-ring of the dehydroepiandrosterone skeleton enhanced the inhibitory activity for 5α-reductase enzyme as indicated by the low IC_{50} values. Compounds **11**–**15** have a bromine atom at C-5 and C-6 position showed a lower inhibitory activity for this enzyme as compared to steroids **15**, **18** and **19** lacking the bromine atoms at C-5 and C-6. It is possible that the two halogen atoms decreased the solubility of the steroidal molecule in the reaction medium and thus decreased the efficiency of the nucleophilic reaction of cysteine, lysine and serine with the carbonyl group of the lactone moiety.

Finasteride as well as compounds **15**, **18** and **19** showed a high 5α-reductase inhibitory activity. As a result of this, the decrease of the prostate and seminal vesicles weight produced by these compounds was a consequence of the inhibition of 5α-reductase enzyme present in this tissue [19]. This decrease of the weight of these glands could possibly be explained by considering that steroids **15**–**17** have also a low affinity for the androgen receptor. On the other hand steroids **11**–**14** and **18**–**20** did not inhibit the binding of labeled mibolerone to the androgen receptor.

Furthermore, it has to be considered that steroids **11**, **12**, **15**–**18**, **20** which showed pharmacological activity, have a short ester that could be hydrolyzed *in vivo* during the six day treatment. This reaction could produce another structure in the ring A of the steroidal lactones. As a result of this, they could bind to the AR producing an antagonistic activity. This explanation could elucidate the *in vivo* activity observed with these steroidal derivatives.

In view of the fact that the steroidal lactones **15**, **18** and **19** showed a high 5α-reductase inhibitory activity and also decreased the weight of the prostate and seminal vesicles as compared to the testosterone only treated animals, these steroidal lactones could have a therapeutic potential for the treatment of androgen dependent diseases.

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