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Effect of dehydroepiandrosterone derivatives on the activity of 5α-reductase isoenzymes and on cancer cell line PC-3

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ABSTRACT

It is well known that testosterone (T) under the influence of 5α-reductase enzyme is converted to dihydrotestosterone (DHT), which causes androgen-dependent diseases. The aim of this study was to synthesize new dehydroepiandrosterone derivatives (**3a–e**, **4a–i**, **6** and **7**) having potential inhibitory activity against the 5α-reductase enzyme. This paper also reports the in vivo pharmacological effect of these steroidal molecules. The results from this study showed that all compounds exhibited low inhibitory activity for 5α-reductase type 1 and 2 enzymes and they failed to bind to the androgen receptor. Furthermore, in the in vivo experiment, steroids **3b**, **4f**, and **4g** showed comparable antiandrogenic activity to that of finasteride; only derivatives **4d** and **7** produced a considerable decrease in the weight of the prostate gland of gonadectomized hamsters treated with (T). On the other hand, compounds **4a**, **f** and **h** showed 100% inhibition of the growth of prostate cancer cell line PC-3, with compound **4g** having a 98.2% antiproliferative effect at 50 μM. The overall data indicated that these steroidal molecules, having an aromatic ester moiety at C-3 (**4f–h**), could have anticancer properties.

1. Introduction

In the prostate, androgens play a crucial role in the development of benign prostatic hyperplasia (BPH) and prostate cancer through androgen receptor regulation. Prostate cancer is still considered a leading cause of male death worldwide. It is estimated that in 2009 around 27,360 men died from prostate cancer.[1,2] BPH is the non-cancerous growth of the prostate; the incidence of BPH afflictions is about 70% at 70 years of age and becomes nearly universal with advancing age.[3] Both diseases are associated with increased levels of the androgen hormone dihydrotestosterone (DHT) and enhanced binding to the androgen receptor (AR). It is well known that DHT binds with higher affinity to the AR than testosterone (T).[4] Testosterone is converted to DHT by the action of 5α-reductase isoenzymes (Fig. 1), which are membrane-bound microsomal proteins that reduce the double bond at C-5 in the steroidal skeleton.

There are three 5α-reductase isoenzymes in the prostate tissue: 5α-R type 1 (5α-R1), 5α-R type 2 (5α-R2) and 5α-R type 3 (5α-R3), which was recently detected. 5α-R1 is expressed in the prostate epithelial cells and is related to the development of prostate cancer. 5α-R2 is mainly located in the stromal compartment and is associated with BPH. 5α-R3 can be found in the brain and pancreas and is related to hormone-refractory prostate cancer (HRPC).[5–7] None of the three isoenzymes have been purified due to their

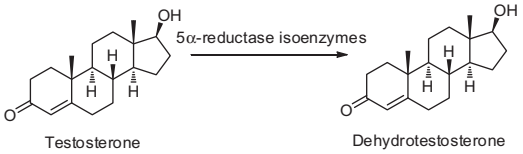

Figure 1. Conversion of testosterone to dehydrotestosterone by the action of 5α-reductase isoenzymes.

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unstable nature, and as a result, 5α-reductase isoenzyme inhibitors have been designed by targeting their substrate. Since the cancer cell lines show different mechanisms of action, it is important to design drugs that have a specific activity for each target. The PC3 cell line shows 17β-hydroxydehydrogenase activity, whereas HeLa cells display increased activity for type 1 5α-reductase.

Finasteride (F) is a 5α-R2 inhibitor with an IC_{50} value of 8.5 nM, but it shows an IC_{50} value of 630 nM for 5α-R1; thus it is more specific for type 2 enzyme.[8] Since 5α-R type 1 and 2 have been associated with prostate cancer development, this inhibition has been an important target for the treatment of the disease. Previously, it has been reported that some dehydroepiandrosterone derivatives with an ester moiety at C-3 exhibited 5α-R inhibitory activity.[8–10] There are several publications which indicate that dehydroepiandosterone derivatives containing an azole group at C-17 showed low 5α-R inhibitory activity but high CYP17 inhibition; it is well known that the inhibition of one or both enzymes could be important for improvement of HRPC-treatments.[11,12] In recent studies, we have demonstrated that the presence of electrophilic centers enhanced 5α-R inhibitory activity.[9]

On the basis of these findings, we decided to synthesize several steroidal derivatives that could be more specific for the inhibition of 5α-R enzymes and could be used for the treatment of androgen-dependent diseases. The structural requirements that we considered include a chlorine atom or an imidazole moiety at C-17 (electrophilic center), a formyl substituent at C-16 (α,β conjugated carbonyl group), and several ester functions at C-3.

In this paper, we report the synthesis of 16-formyl-17-chloroandrosta-5,16-diene-3β-yl derivatives (**3a**–**3e** and **6**) and 16-formyl-17-(1H-imidazole-1-yl)androsta-5,16-diene-3β-yl derivatives (**4a**–**4i** and **7**). These compounds were evaluated (in vitro as well as in vivo) as 5α-R1, 5α-R2, and androgen receptor inhibitors and also in their ability to inhibit the proliferation of prostate cancer cell lines (PC-3).

2. Material and methods

2.1. Chemical and radioactive materials

2.1.1. Reagents

Solvents were laboratory grade. Melting points (uncorrected) were determined on a melting point apparatus (Fisher Johns, Mexico City, Mexico); ^{1}H NMR and ^{13}C NMR were taken on a Varian Gemini 200 and VRX-300 spectrometer, respectively (MR resources NC, USA). Chemical shifts are given in ppm relative to that of Me_4Si ($\delta = 0$) in $CDCl_3$ (the abbreviations of signal patterns are: s, singlet; d, doublet; t, triplet; m, multiplet). Mass spectra were obtained with an HP5985-B spectrometer (Avantes, Apeldoorn, The Netherlands). IR spectra were recorded on a Perkin-Elmer 200s spectrometer (Perkin Elmer Life and Analytical Science, Shelton CT, USA). The UV lamp (254 nm) was from UVP (Upland, CA).

(1,2,6,7-^{3}H) T specific activity: 95 Ci/mmol was purchased from Perking Elmer Life and Analytical Science. (Boston, MA). Labeled T was purified by thin layer chromatography (TLC) on HPTLC Keiselgel 60 F_{254} plates (Merck, Darmstadt, Germany); the solvent system recommended by the manufacturer was used.

Radioinert T, 5α-DHT, and mibolerone (MIB) were purchased from Steraloids (Wilton, NH, USA) and Perkin Elmer Life Sciences, Inc. (Boston, MA, USA). NADPH, Lubrol PX, DL-dithiothreitol (DTT), protease inhibitors, (activated charcoal acid washed with hydrochloric acid), and dextran (Mr: 70,000) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Finasteride was obtained by extraction from Proscar® (Merck, Sharp & Dohm, Mexico City) as follows: 180 Proscar tablets were crushed and extracted with 200 mL chloroform; after the solvent was removed under vacuum, the crude product was purified by silica gel 60 (63–200 μm) column chromatography (Sigma–Aldrich) with ethyl acetate as eluant. The melting point of the isolated Finasteride (252–254 °C) was identical to that reported in the literature. Bio-gel Hydroxyapatite (HAP) was provided by Bio-Rad Laboratories (CA, USA).

2.1.2. Synthesis of the steroidal derivatives: overview

For the synthesis of the new steroidal compounds **3a**–**i**, **4a**–**i**, **6** and **7** (Fig. 2), we used commercially available dehydroepiandrosterone **1** and also its acetate **5**. Compounds **2a**–**i** and **6** were prepared according to the procedure previously reported.[8,13,14] The synthesis of the new compounds is briefly described below.

2.1.2.1. Synthesis of 16-formyl-17-chloroandrosta-5,16-diene-3β-yl derivatives (3 in Fig. 2). Into a round-bottomed flask containing cold phosphorus oxychloride (36 mmol), fitted with a magnetic stirrer, dimethylformamide (43 mmol) was added dropwise followed by a solution of the corresponding ester in chloroform (1 mmol) (5 mL). The mixture was refluxed for 5 h under N_2 atmosphere. The reaction was quenched with a saturated solution of sodium bicarbonate (100 mL). The compound was extracted with chloroform, and the organic phase was washed with water and dried over anhydrous sodium sulfate. The compound was purified on silica gel chromatography.

2.1.2.2. Derivatives 3a–i. *2.1.2.2.1. 16-Formyl-17-chloroandrosta-5,16-diene-3β-yl-cyclopropancarboxylate (**3a**).* Yield: 72%, mp 124–125 °C. UV (nm): 257 ($\varepsilon = 10200$). IR (KBr) cm^{-1}: 2928, 1717, 1674, 1589. ^{1}H NMR ($CDCl_3$) δ: 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 4.6 (1H, m, H-3), 5.3 (1H, dd, $J_1 = 4.0$ Hz and $J_2 = 2.0$ Hz, H-6), 9.9 (1H, s, aldehyde). ^{13}C NMR ($CDCl_3$) δ: 8.4, 13.1, 15.0, 19.3, 20.4, 27.8, 29.9, 30.3, 30.5, 30.8, 31.6, 32.8, 35.9, 38.1, 50.3, 50.6, 53.8, 73.6, 121.8, 136.5, 140.1, 162.3, 174.3, 188.1. HRMS calcd for $C_{24}H_{31}ClO_3$ 402.1932; found 402.1922.

*2.1.2.2.2. 16-Formyl-17-chloroandrosta-5,16-diene-3β-yl-cyclobutancarboxylate (**3b**).* Yield: 69%, mp 152–153 °C. UV (nm): 256 ($\varepsilon = 10100$). IR (KBr) cm^{-1}: 2941, 1719, 1671, 1586. ^{1}H NMR ($CDCl_3$) δ: 0.9 (3H, s, H-18), 1.1 (3H, s, H-19), 4.6 (1H, m, H-3), 5.4 (1H, dd, $J_1 = 4.2$ Hz and $J_2 = 2.1$ Hz, H-6), 9.9 (1H, s, aldehyde). ^{13}C NMR ($CDCl_3$) δ: 14.6, 17.9, 18.9, 19.9, 24.9, 27.3, 28.1, 30.1, 30.4, 32.5, 35.5, 37.7, 37.9, 49.8, 50.5, 53.3, 72.9, 121.4, 136.1, 139.7, 161.9, 174.6, 187.8. HRMS calcd for $C_{25}H_{33}ClO_3$ 416.2188; found 416.2189.

*2.1.2.2.3. 16-Formyl-17-chloroandrosta-5,16-diene-3β-yl-cyclopentancarboxylate (**3c**).* Yield: 70%, mp 180–181 °C. UV (nm): 258 ($\varepsilon = 10300$). IR (KBr) cm^{-1}: 2927, 1728, 1708, 1667, 1590. ^{1}H NMR ($CDCl_3$) δ: 0.9 (3H, s, H-18), 1.1 (3H, s, H-19), 4.6 (1H, m, H-3), 5.4 (1H, dd, $J_1 = 4.4$ Hz and $J_2 = 2.0$ Hz, H-6), 9.9 (1H, s, formyl). ^{13}C NMR ($CDCl_3$) δ: 14.6, 18.9, 19.9, 25.6, 27.3, 28.3, 29.7, 29.9, 30.5, 32.6, 36.4, 36.5, 37.7, 43.7, 49.9, 50.2, 53.3, 72.9, 121.4, 136.1, 139.8, 161.9, 175.8, 187.7. HRMS calcd for $C_{26}H_{35}ClO_3$ 430.2215; found 430.2210.

*2.1.2.2.4. 16-Formyl-17-chloroandrosta-5,16-diene-3β-yl-cyclohexancarboxylate (**3d**).* Yield: 71%, mp 166–167 °C. UV (nm): 257 ($\varepsilon = 10100$). IR (KBr) cm^{-1}: 2929, 1727, 1668, 1592. ^{11}H NMR ($CDCl_3$) δ: 0.9 (3H, s, H-18), 1.1 (3H, s, H-19), 4.6 (1H, m, H-3), 5.4 (1H, dd, $J_1 = 4.5$ Hz and $J_2 = 2.4$ Hz, H-6), 9.9 (1H, s, formyl). ^{13}C NMR ($CDCl_3$) δ: 14.6, 18.9, 19.9, 24.4, 25.1, 25.5, 25.9, 27.3, 28.1, 28.8, 30.1, 30.5, 32.6, 33.3, 36.6, 37.6, 43.0, 49.9, 50.4, 53.4, 72.7, 121.4, 136.1, 139.8, 161.9, 175.2, 187.7. HRMS calcd for $C_{27}H_{37}ClO_3$ 444.2481; found 444.2487.

*2.1.2.2.5. 16-Formyl-17-chloroandrosta-5,16-diene-3β-yl-cycloheptancarboxylate (**3e**).* Yield: 70%, mp 215–216 °C. UV (nm): 257 ($\varepsilon = 10100$). IR (KBr) cm^{-1}: 2946, 1719, 1671, 1585. ^{1}H NMR

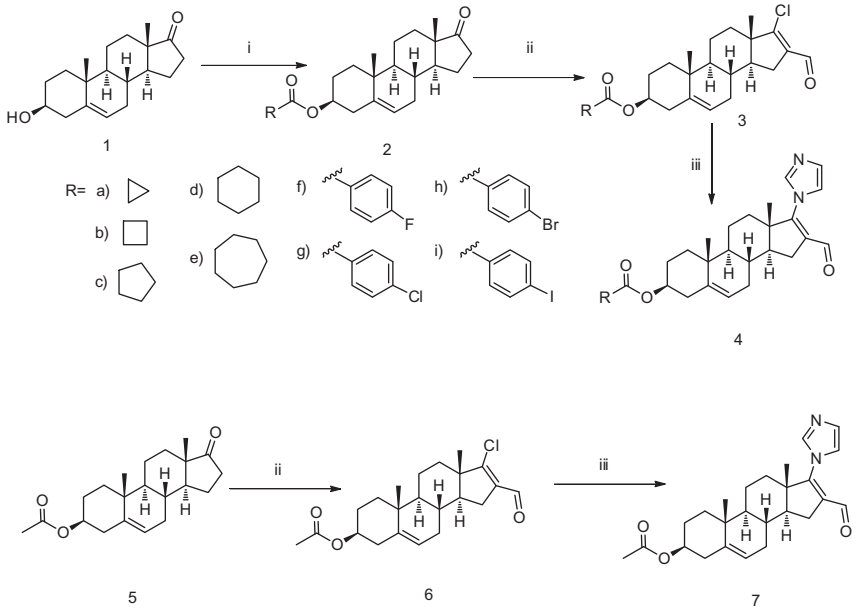


Figure 2. Synthesis of steroidal derivatives. (i) DCC, DMAP, $CHCl_3$, RCOOH; (ii) $POCl_3$, DMF, $CHCl_3$; (iii) imidazole, K_2CO_3, DMF. 4-Dimethylaminopyridine (DMAP), *N,N'*-dicyclohexyl carbodiimide (DCC), dimethyl formamide (DMF), phosphorus oxychloride ($POCl_3$).

($CDCl_3$) δ: 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 4.5 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4.6 Hz and J_2 = 2.1 Hz, H-6), 9.9 (1H, s, formyl). ^{13}C NMR ($CDCl_3$) δ: 15.0, 19.3, 20.4, 26.5, 27.7, 28.3, 28.4, 30.5, 30.8, 30.9, 32.9, 36.9, 38.1, 45.3, 50.3, 50.7, 53.8, 73.1, 121.7, 136.5, 140.1, 162.3, 176.5, 188.1. HRMS calcd for $C_{28}H_{39}ClO_3$ 458.2518; found 458.2514.

*2.1.2.2.6. 16-Formyl-17-chloroandrostan-5,16-diene-3β-yl-4́-flurobenzoate (**3f**).* Yield: 63%, mp 194–195 °C. UV (nm): 231, 256. IR (KBr) cm^{-1}: 2932, 1714, 1663, 1518, 1278. ^{1}H NMR ($CDCl_3$) δ: 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 4.8 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4.5 Hz and J_2 = 2.3 Hz, H-6), 7.1 (2H, ddt, J_1 = 8.8 Hz, J_2 = 8.4 Hz, J_3 = 2.8 Hz, H-aromatic), 8.0 (2H, dd, J_1 = 8.4 Hz, J_2 = 9.0 Hz, H-aromatic), 9.9 (formyl). ^{13}C NMR ($CDCl_3$) δ: 14.9, 19.2, 19.9, 27.6, 28.5, 30.3, 30.9, 32.9, 36.9, 38.1, 50.1, 50.6, 53.4, 74.2, 115.2, 115.5, 122.5, 127.1, 131.4, 136.6, 139.7, 162.2, 164.4, 165.1, 166.4, 187.9. HRMS calcd for $C_{27}H_{30}ClFO_3$ 457.4022; found 457.4029.

*2.1.2.2.7. 16-Formyl-17-chloroandrostan-5,16-diene-3β-yl-4́-chlorobenzoate (**3g**).* Yield: 59%, mp 174–175 °C. UV (nm): 241.66. IR (KBr) cm^{-1}: 2929, 1718, 1669, 1585. ^{1}H NMR ($CDCl_3$) δ: 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 4.8 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4.0 Hz and J_2 = 2.0 Hz, H-6), 7.4 (2H, d, J = 8.4 Hz, H-aromatic), 8.0 (2H, d, J = 8.4 Hz, H-aromatic), 10.0 (formyl). ^{13}C NMR ($CDCl_3$) δ: 14.9, 19.2, 20.4, 27.6, 28.6, 30.4, 30.8, 32.9, 36.8, 38.1, 50.2, 50.6, 53.6, 74.5, 121.9, 128.4, 129.1, 130.9, 135.7, 139.6, 139.9, 162.4, 165.1, 188.1. HRMS calcd for $C_{27}H_{30}Cl_2O_3$ 473.2455; found 473.2451.

*2.1.2.2.8. 16-Formyl-17-chloroandrostan-5,16-diene-3β-yl-4́-bromobenzoate (**3h**).* Yield: 65%, mp 189–190 °C. UV (nm): 255. IR (KBr) cm^{-1}: 2928, 1711, 1668, 1587, 1269. ^{1}H NMR ($CDCl_3$) δ: 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 4.8 (1H, m, H-3), 5.4 (1H, d, J = 4.2 Hz and J_2 = 2.2 Hz, H-6), 7.6 (2H, d, J = 8.4 Hz, H-aromatic), 7.9 (2H, d, J = 8.8 Hz, H-aromatic), 10.0 (formyl). ^{13}C NMR ($CDCl_3$) δ: 14.9, 19.2, 20.3, 27.7, 28.4, 30.4, 30.1, 32.8, 36.7, 36.8, 38.1, 50.1, 50.6, 53.6, 74.6, 122.1, 22.4, 127.7, 127.8, 129.6, 129.8, 131.1, 131.6, 136.4, 139.8, 162.3, 165.2, 188.1. HRMS calcd for $C_{27}H_{30}ClBrO_3$ 517.2186; found 517.2186.

*2.1.2.2.9. 16-Formyl-17-chloroandrostan-5,16-diene-3β-yl-4́-iodobenzoate (**3i**).* Yield: 62%, mp 197–198 °C. UV (nm): 246. IR (KBr) cm^{-1}: 2939, 1707, 1664, 1586, 1271. ^{1}H NMR ($CDCl_3$) δ: 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 4.8 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4.3 Hz and J_2 = 2.4 Hz, H-6), 7.7 (2H, d, J = 8.8 Hz, H-aromatic), 7.9 (2H, d, J = 8.4 Hz, H-aromatic), 10.0 (formyl). ^{13}C NMR ($CDCl_3$) δ: 14.9, 19.2, 20.3, 27.7, 28.4, 30.4, 30.8, 32.9, 36.8, 36.9, 38.1, 50.2, 50.5, 53.7, 74.6, 100.5, 122.1, 130.2, 130.9, 136.4, 137.6, 139.8, 162.2, 165.4, 188.0. HRMS calcd for $C_{27}H_{30}ClIO_3$ 565.4311; found 565.4310.

2.1.2.3. Synthesis of 16-formyl-17-(1H-imidazole-1-yl)androsta-5,16-diene-3β-yl derivatives (4 in Fig. 3). A mixture of imidazole (3 mmol), K_2CO_3 (6 mmol) and the corresponding 16-formyl-17-chloroandrosta-5,16-diene-3β-yl derivative (1 mmol) in dry DMF (5 mL) was heated at 50 °C under N_2 atmosphere for 3 h. Cold water was added and the precipitated was filtered. The compound was purified by column chromatography on florisil.

2.1.2.4. Derivatives 4a–i. *2.1.2.4.1. 16-Formyl-17-(1H-imidazole-1-yl)androsta-5,16-dien-3β-yl-cyclopropancarboxylate (**4a**).* Yield: 83.5%, mp 135–137 °C. UV (nm): 256. IR (KBr) cm^{-1}: 2939, 1720, 1667, 1610. ^{1}H NMR ($CDCl_3$) δ: 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 4.6 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4.6 Hz and J_2 = 2.3 Hz, H-6), 7.1 (1H, s, H-aromatic), 7.2 (1H, s, H-aromatic), 7.6 (1H, s, H-aromatic) 9.7 (formyl). ^{13}C NMR ($CDCl_3$) δ: 8.3, 13.1, 15.9, 19.2, 20.4, 27.7, 28.2, 30.2, 30.8, 33.8, 36.7, 36.8, 49.3, 49.9, 53.8, 73.5, 119.9, 121.7, 133.9, 137.8, 139.9, 160.5, 160.6, 174.2, 187.6. HRMS calcd for $C_{27}H_{34}N_2O_3$ 435.2600; found 435.2601.

*2.1.2.4.2. 16-Formyl-17-(1H-imidazole-1-yl)androsta-5,16-dien-3β-yl-cyclobutancarboxylate (**4b**).* Yield: 79.8%, mp 181–182 °C. UV (nm): 256. IR (KBr) cm^{-1}: 2947, 1727, 1665, 1607. ^{1}H NMR ($CDCl_3$) δ: 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 4.6 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4,2 Hz and J_2 = 2.7 Hz, H-6), 7.1 (1H, s, H-aromatic), 7.2 (1H, s, H-aromatic), 7.6 (1H, s, H-aromatic) 9.7 (formyl). ^{13}C NMR ($CDCl_3$) δ: 8.0, 15.7, 18.1, 18.9, 24.9, 24.9, 27.4, 29.9, 30.5, 35.5, 36.0, 49.1, 49.7, 53.5, 72.9, 119.6, 121.3, 130.4, 133.6, 137.5, 139.8, 160.2, 174.6, 187.4.HRMS calcd for $C_{28}H_{36}N_2O_3$ 449.2726; found 449.2723.

*2.1.2.4.3. 16-Formyl-17-(1H-imidazole-1-yl)androsta-5,16-dien-3β-yl-cyclopentancarboxylate (**4c**).* Yield: 85.7%, mp 188–189 °C. UV (nm): 261. IR (KBr) cm^{-1}: 2948, 1726, 1664, 1606. ^{1}H NMR ($CDCl_3$) δ: 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 4.6 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4.1 Hz and J_2 = 2.0 Hz, H-6), 7.1 (1H, s, H-aromatic),

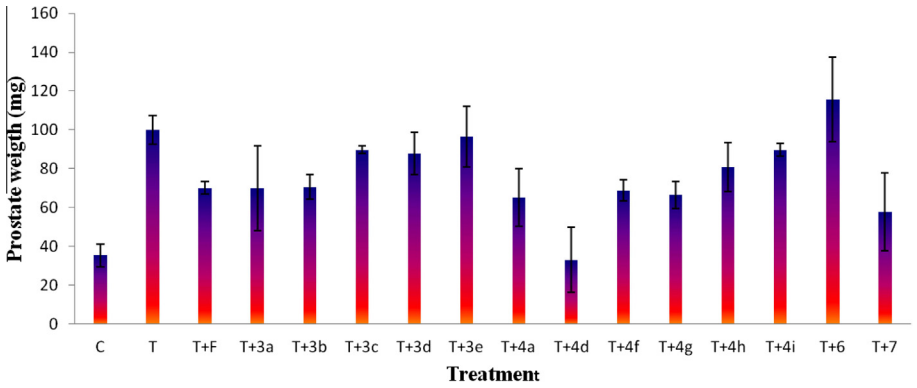


Figure 3. Weight of the prostate gland ± standard deviation from castrated hamsters receiving different treatments for 6 days. The control animals (C) were treated with vehicle only.

7.2 (1H, s, H-aromatic), 7.6 (1H, s, H-aromatic) 9.7 (formyl). ^{13}C NMR ($CDCl_3$) δ: 15.1, 18.4, 19.6, 24.9, 26.8, 27.4, 29.2, 29.4, 29.9, 32.9, 35.9, 36.0, 27.2, 43.2, 48.5, 49.1, 52.9, 72.1, 119.1, 120.8, 129.9, 133.1, 136.9, 139.3, 159.9, 175.4, 186.8. HRMS calcd for $C_{29}H_{38}N_2O_3$ 463.2622; found 463.2623.

*2.1.2.4.4. 16-Formyl-17-(1H-imidazole-1-yl)androsta-5,16-dien-3β-yl-cyclohexancarboxylate (**4d**).* Yield: 80.5%, mp 226–227 °C. UV (nm): 261. IR (KBr) cm^{-1}: 2948, 1726, 1664, 1606. ^{1}H NMR ($CDCl_3$) δ: 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 4.6 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4.05 Hz and J_2 = 2.2 Hz, H-6), 7.1 (1H, s, H-aromatic), 7.2 (1H, s, H-aromatic), 7.6 (1H, s, H-aromatic) 9.7 (formyl). ^{13}C NMR ($CDCl_3$) δ: 15.9, 19.2, 20.4, 25.4, 25.8, 27.6, 28.2, 29.0, 29.1, 30.2, 30.8, 33.8, 36.7, 36.8, 38.0, 43.3, 49.4, 49.9, 53.7, 73.0, 119.9, 121.6, 130.6, 133.9, 135.1, 137.8, 140.1, 160.6, 175.6, 187.6. HRMS calcd for $C_{30}H_{40}N_2O_3$ 477.2990; found 477.2999.

*2.1.2.4.5. 16-Formyl-17-(1H-imidazole-1-yl)androsta-5,16-dien-3β-yl-cycloheptancarboxylate (**4e**).* Yield: 81.3%, mp 216–218 °C. UV (nm): 256. IR (KBr) cm^{-1}: 2926, 1729, 1661, 1610. ^{1}H NMR ($CDCl_3$) δ: 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 4.6 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4.2 Hz and J_2 = 2.3 Hz, H-6), 7.1 (1H, s, H-aromatic), 7.2 (1H, s, H-aromatic), 7.6 (1H, s, H-aromatic) 9.7 (formyl). ^{13}C NMR ($CDCl_3$) δ: 14.9, 18.2, 19.4, 24.4, 24.8, 26.6, 27.2, 28.0, 28.0, 29.2, 29.8, 32.8, 35.7, 35.8, 37.0, 42.3, 48.4, 48.9, 52.8, 72.0, 118.9, 120.6, 129.6, 132.9, 134.1, 136.8, 139.1, 159.6, 174.6, 186.7. HRMS calcd for $C_{31}H_{42}N_2O_3$ 491.2195; found 491.3290.

*2.1.2.4.6. 16-Formyl-17-(1H-imidazole-1-yl)androsta-5,16-dien-3β-yl-4́-fluorobenzoate (**4f**).* Yield: 88%, mp 260–261 °C. UV (nm): 228 IR (KBr) cm^{-1}: 2940, 1713, 1665, 1600, 1273. ^{1}H NMR ($CDCl_3$) δ: 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 4.8 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4.1 Hz and J_2 = 2.1 Hz, H-6), 7.0 (2H, dd, J_1 = 6.4 Hz, J_2 = 5.2 Hz, H-aromatic), 7.1 (1H, s, H-aromatic), 7.2 (1H, s, H-aromatic), 7.6 (1H, s, H-aromatic), 8.0 (2H, dd, J_1 = 9.2 Hz, J_2 = 5.6 Hz, H-aromatic), 9.7 (formyl). ^{13}C NMR ($CDCl_3$) δ: 15.3, 18.6, 19.8, 27.1, 27.7, 29.7, 30.2, 36.1, 37.5, 48.7, 49.5, 53.0, 73.7, 114.6, 114.9, 119.3, 121.3, 126.2, 128.1, 129.9, 130.2, 131.3, 131.4, 133.4, 137.1, 139.2, 159.9, 164.3, 186.9. HRMS calcd for $C_{30}H_{33}FN_2O_3$ 489.2072; found 489.2078.

*2.1.2.4.7. 16-Formyl-17-(1H-imidazole-1-yl)androsta-5,16-dien-3β-yl-4́-chlorobenzoate (**4g**).* Yield: 92%, mp 210–212 °C. UV (nm): 241 IR (KBr) cm^{-1}: 2937, 1710, 1663, 1604, 1267, 1239. ^{1}H NMR ($CDCl_3$) δ: 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 4.8 (1H, m, H-3), 5.5 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6), 7.1 (1H, s, H-aromatic), 7.2 (1H, s, H-aromatic), 7.4 (2H, dd, J_1 = 8.4 Hz, J_2 = 2.4 Hz, H-aromatic), 7.6 (1H, s, H-aromatic), 7.9 (2H, dd, J_1 = 8.8 Hz, J_2 = 2.4 Hz, H-aromatic), 9.7 (formyl). ^{13}C NMR ($CDCl_3$) δ: 15.9, 19.2, 20.4, 27.7, 30.3, 30.9, 36.7, 36.8, 49.3, 49.9, 53.8, 74.5, 119.9, 122.0, 128.6, 129.1, 130.7, 130.9, 133.9, 137.8, 139.2, 139.8, 160.6, 139.8, 160.6, 165.6, 187.6. HRMS calcd for $C_{30}H_{33}ClN_2O_3$ 505.3398; found 505.3394.

*2.1.2.4.8. 16-Formyl-17-(1H-imidazole-1-yl)androsta-5,16-dien-3β-yl-4́-bromobenzoate (**4h**).* Yield: 91%, mp 249–250 °C. UV (nm): 244 IR (KBr) cm^{-1}: 2937, 1710, 1662, 1604, 1267, 1238. ^{1}H NMR ($CDCl_3$) δ: 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 4.9 (1H, m, H-3), 5.5 (1H, dd, J_1 = 4.2 Hz and J_2 = 2.2 Hz, H-6), 7.1 (1H, s, H-aromatic), 7.2 (1H, s, H-aromatic), 7.5 (2H, dd, J_1 = 8.8 Hz, J_2 = 2.0 Hz, H-aromatic), 7.6 (1H, s, H-aromatic), 7.9 (2H, dd, J_1 = 8.8 Hz, J_2 = 2 Hz, H-aromatic), 9.7 (formyl). ^{13}C NMR ($CDCl_3$) δ: 15.7, 18.9, 20.1, 27.4, 29.9, 30.5, 36.4, 36.5, 37.8, 49.1, 49.7, 53.5, 74.1, 119.7, 121.7, 127.6, 129.3, 130.4, 130.7, 131.6, 137.5, 139.5, 160.3, 164.9, 187.3. HRMS calcd for $C_{30}H_{33}BrN_2O_3$ 549.3219; found 549.3212.

*2.1.2.4.9. 16-Formyl-17-(1H-imidazole-1-yl)androsta-5,16-dien-3β-yl-4́-iodobenzoato (**4i**).* Yield: 89%, mp 230–232 °C. UV (nm): 227. IR (KBr) cm^{-1}: 2939, 1709, 1606, 1273. ^{1}H NMR ($CDCl_3$) δ: 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 4.8 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4.1 Hz and J_2 = 2.1 Hz, H-6), 7.1 (1H, s, H-aromatic), 7.2 (1H, s, H-aromatic), 7.5 (2H, d, J = 8.8 Hz, H-aromatic), 7.6 (1H, s, H-aromatic), 7.8 (2H, d, J = 8.8 Hz, H-aromatic), 9.7 (formyl). ^{13}C NMR ($CDCl_3$) δ: 15.9, 19.2, 20.4, 27.7, 30.2, 30.8, 33.8, 36.7, 36.8, 45.5, 49.4, 49.9, 53.8, 68.3, 74.5, 100.5, 119.9, 122.0, 129.1, 130.1, 130.6, 130.9, 133.9, 137.6, 139.9, 159.9, 165.4, 187.6. HRMS calcd for $C_{30}H_{33}IN_2O_3$ 597.3816; found 597.3812.

2.1.2.5. Synthesis of 16-formyl-17-(1H-1,3-imidazole-1-yl)androsta-5,16-dien-3β-yl-acetate (7 in Fig. 3). Yield: 93%, mp 225–226 °C. UV (nm): 257. IR cm^{-1}: 2947, 1731, 1659, 1616, 1234. ^{1}H NMR ($CDCl_3$) δ: 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 2.0 (H-1́), 4.6 (1H, m, H-3), 5.4 (1H, dd, J_1 = 4 .3 Hz and J_2 = 2.1 Hz, H-6), 7.1 (1H, s, aromatic), 7.2 (1H, s, aromatic), 7.6 (1H, s, aromatic) 9.7 (aldehyde). ^{13}C NMR ($CDCl_3$) δ: 15.8, 19.0, 20.2, 21.2, 27.5, 28.1, 30.1, 30.6, 33.6, 35.5, 36.6, 37.9, 49.2, 49.8, 53.6, 73.4, 119.7, 121.5, 130.6, 133.7, 137.6, 139.8, 160.4, 170.2, 187.2. HRMS calcd for $C_{25}H_{32}N_2O_3$ 409.2473; found 409.2476.

2.2. Human and animal tissues and procedures

Four hours after a 53-year-old man had died from diabetes and renal insufficiency, his normal prostate was extirpated in the Pathology Department of the General Hospital in Mexico City. The Ethical Committee of the General Hospital in Mexico City approved this protocol.

The tissue was rinsed and immediately chilled in ice-cold 150 mM NaCl and stored at −20 °C. The frozen human prostate was thawed on ice, rinsed, and minced in buffer A (20 mM sodium

phosphate, pH 6.5, containing 0.32 M sucrose, 0.1 mM DTT (Sigma-Aldrich, Mexico City, Mexico) with an IKA® A11 basic tissue mill (IKA Laboratory Equipment, Mexico City, Mexico). Unless otherwise specified, the following procedures were carried out at 4 °C.

2.2.1. 5a-R type2 isozyme isolated from human prostate

Human prostate was used in this experiment because this tissue is an abundant source of 5α-R2 for the study of the effect(s) of **3a–e**, **6**, **4a–i** and **7**, which were designed for inhibition of the activity of this enzyme in humans. In this tissue, 5α-R type 1 is not as abundant as is type 2; AR is very difficult to study in this model.

The human prostate tissue was homogenized in two volumes of buffer A with a tissue homogenizer Ultra-Turrax IKA, T18 basic (Wilmington, NC). Homogenates were centrifuged (1500×*g*; 60 min)[10] in a SW 60 Ti rotor (Beckman Instruments, Palo Alto, CA). The pellets were resuspended in buffer A, and stored at −70 °C. This suspension had a final concentration of 5 mg protein/mL, as determined by the Bradford method,[15] and was used as source of 5α-R type 2 isozyme.

2.2.2. 5aR type 1 isozyme isolated from rat liver

All procedures involving animals were approved by the Institutional Care and Use Committee of the Metropolitan University of Mexico (UAM; Xochimilco, Mexico). All animals used in this study were obtained from the Animal Care Facility at UAM.

Two adult (8-month old) rats had been fasted overnight to decrease glycogen levels before their livers were extirpated for use as a source of 5α-R1, as recommended by Levy et al.[16] To prepare microsomes, the livers (30 g) were minced in one volume of buffer A using the tissue mill. Unless otherwise specified, the following procedures were carried out at 4 °C. The tissue was homogenized; the suspension was centrifuged (10,000×*g*; 30 min; 0 °C) (Beckman L70 K ultracentrifuge); and the pellet was discarded. The supernatant was filtered through a nylon mesh filter (pore size 11 μm, distributed by OEM-Membrane Solution, Dallas TX) and centrifuged (100,000×*g*; 60 min). The microsomal pellet was resuspended in five volumes of buffer A with a homogenizer, and the protein concentration was determined by the Bradford method. The suspension was re-centrifuged (100,000×*g*; 30 min) and the pellet was resuspended in buffer A to give a final concentration of 20 mg protein/mL. The microsomal suspension was stored at −80 °C prior to the preparation of the solubilized steroid 5α-R type 1.

The solubilization of 5α-R type 1 isozyme from the rat liver microsomes was carried out according to Levy et al.

2.2.3. Cytosol of rat prostate as source of AR

The prostate was extirpated from each of 50 adult rats (8-month old; 500 g). In this study, we used rats because the prostate gland is larger than in the hamster; there is no difference in the binding activity of the AR present in the cytosol of rats and hamsters.[17]

The prostates of the rats were blotted, weighed, and soaked in ice-cold TEMD (40 mM tris-HCl, 3 mM EDTA and 20 mM sodium molybdate, 0.5 mM DTT, 10% glycerol; pH 8) prior to use. Unless otherwise specified, all procedures were carried out at 0 °C on a bed of chopped ice. Tissues were homogenized in one volume of TEMD plus protease inhibitors (2 mM PMSF, 10 μg antipain/mL, 5 mM leupeptin)[18] with a tissue homogenizer immersed in a bed of chopped ice. Homogenates were centrifuged (140,000×*g*; 60 min)[19] in the SW 60 Ti rotor. The final protein concentration of the supernatant containing the cytosolic fraction, as determined by the Bradford method, was 6 mg protein/200 μL. This supernatant was stored at −70 °C until its use.

2.2.4. Gonadectomized male hamsters

For the experiments in vivo, 120 adult male golden hamsters (2.5 months old; 150–200 g) were used. After gonadectomies had been performed on 112 hamsters under isoflurane anesthesia, the castrated hamsters were allowed to recover for 30 days prior to experimentation.[20] The castrated hamsters and the remaining eight intact hamsters were housed in a room with controlled temperature (22 °C) and light-dark periods of 12 h; the hamsters were fed with food and water ad libitum. Thirty days post gonadectomy, the hamsters were separated into nine groups consisting of four animals per group. The hamsters were treated for six days as described in Section 2.3.1.1 and thereafter sacrificed with CO_2. This experiment was carried out twice under the same conditions.

2.3. Biological activity of steroidal derivatives

2.3.1. Experiments in vitro

The activity of the 5α-reductase type 1 and 2 were determined by following the conversion of T to DHT, as previously described.[21,22] The reagent mixture (final volume, 1 mL) contained 1 mM of DTT in 40 mM sodium phosphate buffer (pH 8.0 for type 1; pH 6.5 for type 2), 2 nM [1,2,6,7^{3}H] T, 6.31 μM T (for type 1), and 2 mM NADPH. The reaction, carried out in duplicate, was started when this mixture was added to the enzymatic fraction (for type 1, 90 μg protein/6.7 μL from the solubilized microsomes; for type 2, 50 μg/80 μL from the membrane fraction of human prostate). After incubation (37.5 °C; 60 min), the reaction was stopped by adding dichloromethane (1 mL) and mixing; this was considered the end point. As a control, this procedure was also carried out without the addition of the enzyme fraction.

In order to extract and purify DHT formed by the activities of 5α reductase types 1 and 2, after the individual reaction mixtures had been agitated (1 min) by using a Type 16700 mixer (Barnstead Thermoline, Proveedor Científico, Mexico City), the dichloromethane phase of each was placed into individual tubes. This extraction was repeated four additional times. Each pooled dichloromethane extract was evaporated to dryness under a nitrogen stream and then suspended in methanol (50 μL); each preparation was spotted onto HPTLC Keiselgel 60 F_{254} plates. T, DHT, and a mixture of both standards was applied to the plates in distinct lanes on either side of the spotted preparation samples. The plates were developed in chloroform–acetone (9:1) and were air-dried; the chromatography was repeated two additional times. DHT was detected by using phosphomolybdic acid reagent (Sigma–Aldrich); and T, by fluorescence (UV lamp; 254 nm). The radioactivity on the plate was scanned using a Bioscanner AR2000 (Bioscan, Washington D.C.). The zones that showed chromatographic behavior identical to that of the standards (retention factor, R_f) were quantified as T or DHT. The DHT transformation yields were calculated from the lanes, taking into account the entire radioactivity in the TLC, and were plotted using SigmaPlot 12 software (Systat Software, INC., San Jose, CA). For the control incubations, the chromatographic separations and identifications were carried out in the same manner.

The 5α-R type 1 activity was calculated from the percentage of the labeled DHT that had been formed, taking into consideration the recovery, blank values, specific activity of [^{3}H] T, and the ratio of added [^{3}H] T to unlabeled T. The 5α-R type 2 activity was calculated, taking into consideration the recovery, blank values, and the specific activity of [^{3}H] T.

The R_f of the T standard was 0.56. The radioactive zone that had chromatographic behavior identical to that of the standard T corresponded to 70% of the radioactivity accounted for on the plate. The radioactivity contained in the zone of the experimental chromatogram, which had an R_f identical to that of the DHT standard (R_f: 0.67), was identified as the transformed DHT; it corresponded to 20–27% of total radioactivity accounted for in the TLC. This result

was considered to be 100% of the activity of 5α-R (types 1 or 2) for the development of inhibition plots. Unmodified [^{3}H]T, identified (R_f: 0.56) from control incubations (i.e., those that had not contained tissue), had identical chromatographic behavior to those of non-labeled standard T.

The radioactivity contained in the zone corresponding to DHT standard (R_f: 0.67) of the control chromatogram was 1% of the total radioactivity accounted for on the plate and was considered as an error; it was subtracted from the experimental chromatograms.

2.3.1.1. Effect of steroid derivatives on activity of 5α-reductase types 1 and 2. The effect of steroids **3a–e**, **6**, **4a–i** and **7** on the activity of 5α-reductase type 1 or 2 was determined in the same conditions in vitro as described in Section 2.3.1, but in the presence of six different concentrations (1×10^{-11}, 1×10^{-10} M, ..., 1×10^{-3} M) of each of the **3a–e**, **6**, **4a–i** and **7** derivatives. Extraction and purification of the DHT formed from these incubations were carried out as described in 2.3.1.

Control tubes (without inhibitors) were prepared with the same incubating medium and labeled T plus either type 1 or type 2 5α-reductase, under the same conditions (Section 2.3.1). DHT transformations in presence of **3a–e**, **6**, **4a–i** and **7** were calculated from the lanes, taking into account the entire radioactivity in the plate, and were plotted using SigmaPlot 12 software for inhibition curves.

2.3.1.2. Determination of the 50% inhibitory concentration of steroids 3a–e, 6, 4a–i and 7 on the activities of 5α-reductase types 1 and 2. To determine the 50% inhibitory concentration (IC_{50}) of steroids **3a–e**, **6**, **4a–i** and **7** on the activities of 5α-reductase types 1 and 2, the inhibition plots obtained in Section 2.3.1.1 were analyzed with SigmaPlot 12 software.

2.3.1.3. AR competitive binding assay. For the AR competitive binding studies, a series of tubes containing 1 nM [^{3}H] MIB plus a range of increasing concentrations (1×10^{-10}, 1×10^{-9} M, ..., 1×10^{-7} M) of cold MIB or of one of the steroids **3a–e**, **6**, **4a–i** and **7** (Fig. 1) in ethanol or acetone, or in the absence of competitor were prepared.[20]

To prevent the interaction of MIB with either glucocorticoid or PR receptors that could be present in the cytosol from rat prostates 200 nM triamcinolone (Sigma–Aldrich) in ethanol was added to the incubation mixtures.[23] For each preparation, the solvent was removed under vacuum and the cytosol containing AR (5 mg protein/200μL), together with 300 μL of TEM (pH 8) containing protease inhibitors, were placed into an individual tube. Tubes were incubated (18 h; 4 °C); then the bound MIB-AR was separated from free radiolabeled MIB using hydroxyapatite (Bio-Rad, Mexico, City, Mexico), as previously described.[23] The ethanolic fraction (0.8 mL) obtained by this method was added to Ultima Gold scintillation liquid (10 mL) (Packard Instruments, Mexico City) and counted in a Tri Carb 2100 TR scintillation counter (Packard Instruments).

The IC_{50} value of each compound was calculated according to the plots of concentration versus percentage of binding using SigmaPlot 12. Relative binding affinity (RBA) of **3a–e**, **6**, **4a–i** and **7** values for AR was calculated from the following equation:

$$\mathrm{RBA} = \frac{IC_{50} \text{ of } [^3\mathrm{H}] \text{ labeled reference compound}}{IC_{50} \text{ of inhibitor}} \times 100$$

2.3.2. Experiment in vivo

2.3.2.1. Effect of steroid derivatives on prostate of castrated hamsters. For six consecutive days, each of the steroid derivatives **3a–e**, **6**, **4a–i and 7** (2 mg/kg body weight (BW)) dissolved in 200 μL sesame oil, together with 1 mg T/kg (BW), was administered by SC injection to a group of gonadectomized hamsters (four animals per derivative). Three groups of gonadectomized animals were used as controls; the first group was injected SC with 200 μL sesame oil, the second group with 1 mg T/kg (BW), and the third group with 1 mg T plus 1 mg F/kg (BW) also prepared in sesame oil. Additionally, one group of four intact hamsters was used as the intact control. On the seventh day, the animals were sacrificed with CO_2. The diameters of the flank organs were measured using a vernier caliper; the prostate and seminal vesicles of each hamster were dissected and weighed.[20]

Two separate experiments were performed for each group of steroid-treated hamsters. The results were analyzed by using one-way analysis of variance and Dunnett's method to compare means, using JMP IN 5.1 software (JMP, Statistical Discovery, Cary, NC, USA).

2.3.3. Cytotoxicity assay

2.3.3.1. Prostate cancer cell line (PC-3). PC-3 human prostate cancer cell line was supplied by the National Cancer Institute (USA). The ketoconazol used as a reference in this experiment was purchased from Sigma Life Science.[24] The human tumor cytotoxicity of the synthesized compounds (**3a–e**, **4a–i**, **6** and **7**) was determined using the protein-binding dye sulforhodamine B (SRB) in microculture assay to measure cell growth. The cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B (Gibco) and 1% nonessential amino acids (Gibco). They were maintained at 37 °C in humidified atmosphere with 5% CO_2. The viability of the cells used in the experiments exceeds 95% as determined with trypan blue. The cells were removed from the tissue culture flask and diluted with fresh media. Of this cell suspension, 100 μL containing 5000 or 10000 cells per well (depending on the duplication time), were pipetted into microtiter plates (Costar) and the material was incubated at 37 °C for 24 h in a 5% CO_2 atmosphere. Additionally, a plate was prepared with culture medium only and it was incubated for 1 h. Subsequently, 100 μL of a solution of the test compounds obtained by diluting the stocks were added to each well. The cultures were exposed for 48 h to the synthesized compounds at concentration of 50 μM; after the incubation period, the cells were fixed to the plastic substrate by the addition of 50 μL of cold 50% aqueous trichloroacetic acid. The plates were incubated at 4 °C for 1 h, washed with tap water and air-dried. The trichloroacetic-acid-fixed cells were stained by the addition of 0.4% SRB. Free SRB solution was removed by washing with 1% aqueous acetic acid. The plates were air dried, and the bound dye was solubilized by the addition of 10 mM of unbuffered Tris base (100 μL). The plates were placed on a vortex for 5 min, and absorption at 515 nm was determined using an ELISA plate reader (Bio-Tek Instruments). Percentage cell growth inhibition was calculated according to the following expression:

$$\text{Cell growth inhibition } (\%) = 100 - \frac{(\text{sample absorption}) \times 100}{(\text{vehicle absorption})}$$

2.3.3.2. Isolation and culture of primary peritoneal macrophages. Isolation and culture of primary peritoneal macrophages were conducted as described elsewhere.[25] Swiss female mice, 25–30 g, were treated in accordance with the Animal Care and Use Committee (Mexican Standard NOM-062-Z00-1999). Mice were injected intraperitoneally with 1 mL of 3% (wt vol^{-1}) thioglycollate 3 days before harvesting. Peritoneal exudate cells were harvested, washed and suspended in DMEM. These cells were seeded into 48 well plates (Becton Dickinson, Oxnard, CA, USA) at a

density of 1×10^6 cells ml^{-1}, and then incubated for 2 h at 37 °C in a 5% carbon dioxide incubator. Non-adherent cells were washed off and cultured in DMEM supplemented with 10% FCS. Cell viability was determined by the MTT colorimetric assay. Briefly, 10 mL MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,3-diphenyltetrazolium bromide) was added to the medium after 48 h incubation with the test samples. After 4 h, the medium was removed and DMSO was added to dissolve the formazan solution produced in the cells. The optical density of the formazan solution was measured with a microplated reader at 414 nm.[25]

3. Results

3.1. Chemistry

In this synthesis for the preparation of the final compounds **4a–e** (cycloalkyl aliphatic esters; **4f–i** (aromatic esters) all having a formyl group at C-16, C-16 double bond and imidazole ring at C-17 (compound **7**, 3-acetoxyderivative was used as a reference), we used the commercially available dehydroepiandrosterone **1**. This steroid was treated with the corresponding acid, dicyclohexylcarbodiimide and 4-dimethylaminopyridine (Steglich esterification) to form the desired esters **2a–i**. These compounds were treated with phosphorus oxychloride in dimethylformamide and chloroform (Vilsmeier reaction) to form the 16-formyl-17-chloro-3β-acyloxy compounds **3a–i** with yields ranging from 59% to 82%. The next step in this synthesis involved nucleophilic substitution of the 17-chlorine atom with imidazole. This reaction was carried out with the 17-chloro-l6-formyl-3β ester derivatives **4a–i**, imidazole and potassium carbonate. The desired compounds were obtained with a yield ranging from 80% to 92%. All compounds were isolated in crystalline form and the melting points showed a maximum of one degree difference.

3.2. Activity of compounds 3a–e, 6, 4a–i and 7 as Inhibitors of 5α-R1 and 2

The biological activity of compounds **2a–i** and **3f–i** was previously reported.[8,26]

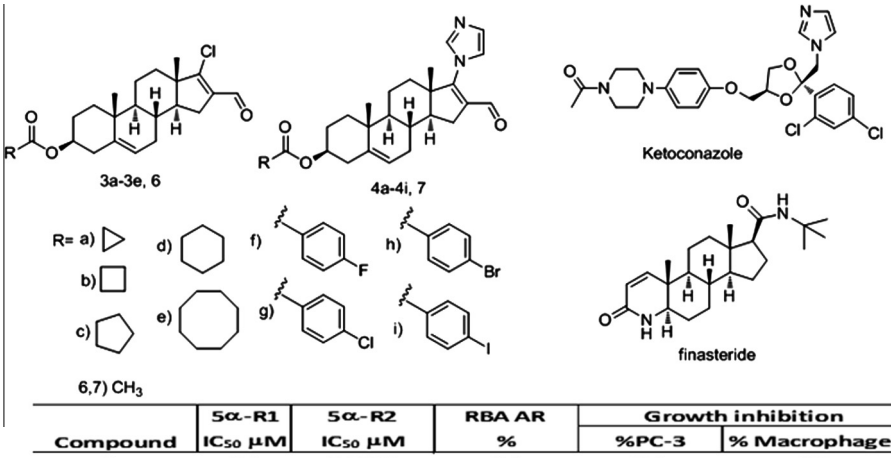
Figure 4 shows the IC_{50} values of **3a–e**, **6**, **4a–i** and **7** required to inhibit 50% of the activity of 5α-reductase isoenzymes 1 and 2. These results indicated that the steroidal derivatives of the 17-chlorine series (**3a–e** and **6)** substantially inhibited the enzyme 5α-reductase type 2. On the other hand, the C-17 imidazole series (**4a–i)** failed to inhibit this enzyme. Surprisingly these compounds inhibited the 5α-reductase type 1 enzyme, albeit to a lower degree.

3.3. Competitive binding of compounds 3a–e, 6, 4a–i and 7 to AR

In the competitive binding assays (Fig. 3), non-labeled MIB competed with the labeled MIB for the androgen receptor and exhibited an IC_{50} value of 1. However, steroids **3a–i**, **4a–i**, **6** and **7** did not inhibit the labeled MIB binding to the androgen receptor.

3.4. Weight of the prostate gland

At sacrifice, there were statistically significant (p <0.05) reductions in the weights of the prostates and seminal vesicles in comparison to glands from intact hamsters (data not shown). However, treatment with 1 mg/kg (BW) of T significantly reversed the effect of castration on these glands (Fig. 4).



Compound	5α-R1 IC_{50} μM	5α-R2 IC_{50} μM	RBA AR %	Growth inhibition %PC-3	Growth inhibition % Macrophages
3a	NA	0.0032	NA	NA	NA
3b	NA	0.013	NA	NA	NA
3c	NA	0.05	NA	NA	NA
3d	NA	0.008	NA	NA	NA
3e	NA	0.015	NA	NA	NA
6	NA	0.05	NA	NA	NA
4a	200	NA	NA	100	41.5
4b	250	NA	NA	NA	NA
4c	265	NA	NA	NA	NA
4d	300	NA	NA	NA	NA
4e	320	NA	NA	NA	NA
4f	320	NA	NA	100	30.1
4g	100	NA	NA	98.2	29.2
4g	94.5	NA	NA	100	40.3
4i	300	NA	NA	NA	NA
7	NA	NA	NA	56.5	36.2
Ketoconazole	ND	ND	ND	85.4	41.5
Finasteride	630	0.0085	NA	NA	NA

Figure 4. Biological activity of several compounds, the IC_{50} value is the concentration of the corresponding compound required to inhibit 50% of the activity of 5α-reductase isoenzymes as well as AR binding assay. Percentage of PC-3 and macrophages growth inhibition at 50 μM. NA: non active compound. ND: non determined.

F and the steroids **3a**–**e**, **4a**–**i** and **7**, when administered together with T, significantly decreased the weight of the prostate (p <0.05); Surprisingly, compound **4d**, having the same chemical structure (the only difference is the cyclohexyl group at C-3), also showed high activity compared to that of finasteride (Fig. 4).

3.5. Cytotoxic activity: effect of the synthesized compounds on PC-3 cell lines and peritoneal mouse macrophages

The effect of the synthesized compounds (**3a**–**e**, **4a**–**i**, **6 and 7**) on the growth of the PC-3 cancer cell line was determined using the SRB assay method. These values for cell growth inhibition and the results of the effect of the steroidal derivatives (**3a**–**e**, **4a**–**i**, **6 and 7**) on the peritoneal mouse macrophages (non-cancerous cell lines) are shown in Figure 4. Ketoconazole and finasteride were used as reference compounds;[26,27] finasteride inhibited neither the growth of PC-3 line cells nor of the macrophages; however, ketoconazole hindered this cell proliferation in PC-3 line cells.

Compounds **4a**, **4f** and **4h** showed 100% inhibition for the growth of prostate cancer cell line PC-3, with compound **4g** having an antiproliferative effect of 98.2% at 50 μM. Otherwise, these compounds did not show the same effect for the PC-3 cancer cell line compared to that of non-cancerous cell lines (peritoneal mouse macrophages) at 50 μM, thus showing higher selectivity (Fig. 4).

4. Discussion

In this paper, we report the synthesis and biological activity of several steroidal derivatives (**3a**–**e**, **4a**–**i**, **6** and **7**). Biological evaluation of compounds **3f**–**i** was not included in this paper; the results are published in Ref. 8. Compounds **3a**–**e** and **6**, having a chlorine atom at C-17 and a conjugated formyl group at C-16, exhibited an inhibitory effect for 5α-R2 enzyme, with compounds **3a** and **d** having higher activity than that of finasteride. When the chlorine atom at C-17 was replaced with an imidazole group, these steroidal derivatives lost their inhibitory effect on the 5α-R2 enzyme and surprisingly acquired an inhibitory effect on the 5α-R1 enzyme. Apparently the rigidity of the imidazole ring does not allow the formation of the complex with the reactive side of the type 2 enzyme and as a consequence, no biological activity was observed.

It is a well known fact that the inhibitory effect of 5α-R2 enzyme consists of a Michael type 1,4 addition of the nucleophilic part of the amino acids such as lysine, cysteine and serine on the double bond conjugated with the carbonyl group,[26–28] thus forming an irreversible adduct, and as a result of this addition, an inhibition of the enzyme 5α-R2 takes place. This explains the much higher activity of the C-17 chlorine series compared to the C-17 imidazole derivatives. The chlorine atom, being very electronegative, polarizes the C-17 carbon atom, thus creating a partial positive charge and makes this carbon atom more electrophilic. As a consequence of this enhanced electrophilicity, the nucleophilic amino acids add in a 1,4 type addition and inhibit the enzyme. In the imidazole series **4a**–**i**, the imidazole group cannot form a partial positive charge on the C-17 atom, a Michael-type addition does not take place, and no 5α-R2 enzyme inhibition is observed. It is important to add that this concept has never been studied with the 5α-R1 enzyme. In view of the fact that **4b** and **c** have a cyclobutyl and cyclopropyl rings in the ester moiety, the conformation of these rings apparently favors the formation of a complex with the esterase enzyme and therefore the hydrolysis of the ester moiety causes a loss their in vivo activity.

On the basis of these results, it could be suggested that the presence of an imidazole substituent at C-17 on the androstane skeleton confers 5α-R1 inhibitory activity. On the other hand, it is evident that the structure of the ester function at C-3 is very important for the inhibition of this enzyme, since steroid **7** did not inhibit the 5α-R type 1. This is supported by the fact that compounds **4a**–**i** showed inhibitory activity only for the 5α-R1 enzyme. The advantage of having different specific inhibitors for each enzyme is that it allows the possibility of better treatments with fewer side effects than other approved molecules.

Apparently the imidazole substituent at C-17 on the androstane skeleton blocks complex formation with the active site of the 5α-R type 2.

The high cytotoxic activity of steroidal derivatives **4a**, **4f**–**h** observed on PC-3 cell lines could be explained by considering the steric effect of the ester moiety at C-3. Compound **4a**, having a planar cyclopropyl group, shows 100% antiproliferative activity. Steroids **4b**–**e**, having bulky non planar ester moieties, lack any effect. This is confirmed by the activity of compounds **4f**–**h** having a planar aromatic ester function, thus showing high cancer cell growth inhibition rates of 100%, 98.2% and 100%, respectively. Compound **4i**, with a bulky iodine atom on the phenyl ring, lacks any antiproliferative activity. It is important to note that these compounds showed selectivity for the inhibition of cancer cell growth compared to non-cancerous cell lines. The mechanism of action of these steroids with this cell line is presently being studied.

The in vivo experiments demonstrated that steroids **3b** (5α-R2 inhibitor), **4d**, **4f**, **4g** (5α-R1 inhibitors) and **7** were also capable of inducing a decrease in the weight of the prostate gland. Compound **7** showed activity in vivo, which indicates that the methyl group in the ester function probably plays a role in in vivo activity on other targets than 5α-R and AR.

5. Conclusion

In conclusion, the results of this study demonstrated that the steroidal derivatives having a chlorine atom at C-17 (**3a**–**e** and **6**) inhibited the enzyme 5α-R2. Surprisingly, when the chlorine atom was replaced with an imidazole group (**4a**–**i**), these compounds lost their inhibitory activity for this isoenzyme but showed an inhibitory effect for the 5α-R1 enzyme. Of the compounds studied in this paper, only **4a**, **4f**–**h** exhibited high antiproliferative activity for PC-3 prostate cancer cell lines as compared to ketoconazole. In spite of the fact that all compounds evaluated in vitro showed a biological effect, the in vivo studies indicated that only compounds **4d** and **7** exhibited a high antiandrogenic effect.

As a consequence of these results, this study could contribute to better understanding of the design and synthesis of more active molecules for the treatment of BPH and prostate cancer.

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