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Biological Activity of Pyrazole and Imidazole- Dehydroepiandrosterone Derivatives on the Activity of 17β -Hydroxysteroid Dehydrogenase

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ABSTRACT: The enzyme type 5 17β -hydroxysteroid dehydrogenase 5 (17β -HSD5) catalyzes the transformation of androstenedione (4-dione) to testosterone (T) in the prostate. This metabolic pathway remains active in cancer patients receiving androgen deprivation therapy. Since physicians seek to develop advantageous and better new treatments to increase the average survival of these patients, we synthesized several different dehydroepiandrosterone derivatives. These compounds have a pyrazole or imidazole function at C-17 and an ester moiety at C-3 and were studied as inhibitors of 17β -HSD5. The kinetic parameters of this enzyme were determined for use in inhibition assays. Their pharmacological effect was also determined on gonadectomized hamsters treated with $\Delta 4$ -androstenedione (4-dione) or testosterone (T) and/or the novel compounds. The results indicated that the incorporation of a heterocyclic at C-17 induced strong 17β -HSD5 inhibition. These derivatives decreased flank organ diameter and prostate weight in castrated hamsters treated with T or 4-dione. Inhibition of 17β -HSD5 by these compounds could have therapeutic potential for the treatment of prostate cancer and benign prostatic hyperplasia.

KEYWORDS: 17β -HSD5 inhibitors, benign prostatic hyperplasia, flank organs, imidazole and pyrazole-dehydroepiandrosterone derivatives, prostate cancer, seminal vesicles

I. INTRODUCTION

Prostatic cancer recurrence has been observed in some castrated patients as well as in patients under androgen ablation therapies. The growth of these tumors could be determined by the adaptive changes of androgen receptors (AR) such as amplifications or mutations, which have been previously, described. However, most prostate cancers are produced in an androgen-dependent manner, and the presence of intraprostatic dihydrotestosterone (DHT) could stimulate AR signaling, since this steroid is only reduced by close to 60% with medical castration using GnRH analogues.

Prostatic tissue is capable of synthesizing DHT either de novo or from weak androgens, dehydroepiandrosterone (DHEA) and androstenedione (4-dione) synthesized the by adrenal glands. These androgens are also present in the plasma of cancer patients under androgen ablation therapies⁵. Therefore, the synthesis of DHT from DHEA and 4-dione is possible, since the enzymes involved in this pathway are increased in the prostates of these patients.

The enzyme 17β -HSD type 5 (17β -HSD5) is well characterized in human prostate tissues, and contributes to local androgen formation. The gene that encodes 17β -HSD5 has been identified in bands 10 p15 and p14 of human chromosome 13 from prostate cells and its mRNA is over-expressed in prostate cancer tissue (77%). Since this enzyme is produced locally in prostatic cancer tumors, it must be considered as a therapeutic target for this illness.

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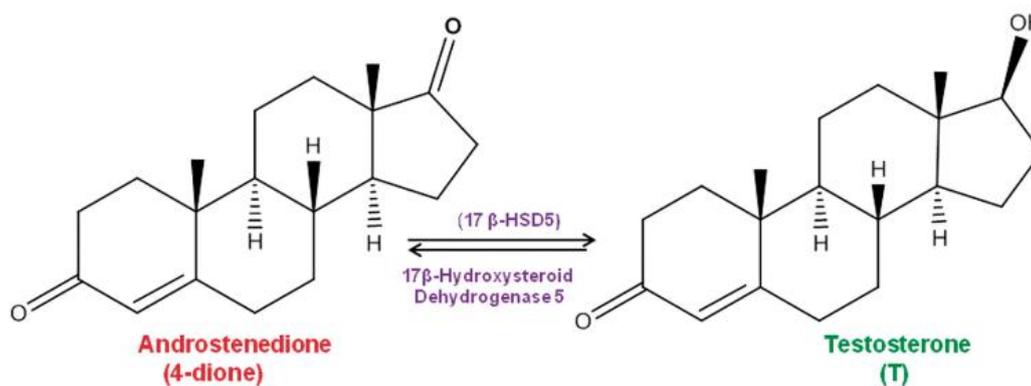


Figure1. Conversion of androstenedione (4-dione) to testosterone (T)

Studies carried out with the prostate cancer cell lines DU-145, PC-3 and LNCaP3–6 showed increased reductive activity for 17β-HSD5 in these cell lines⁹. 17β-HSD5 activity could therefore be relevant in the growth and development of cancerous tumors^{5–10}. Thus the inhibition of this enzyme could offer a new alternative for prostate cancer treatment as compared to currently used endocrine therapies.

Currently, 20 crystalline isoforms of 17β-HSD5 are known; X-ray analysis of these structures has revealed the presence of pockets and sub pockets inside these proteins. On the basis of these findings, a large number of steroidal and non-steroidal 17β-HSD5 inhibitors have been synthesized. Recently, we prepared several new dehydroepiandrosterone derivatives that have a pyrazole group at C-17; some of these compounds entirely inhibited the proliferation of PC-3 cells¹¹. Although the PC-3 prostate cancer cell line exhibits some androgen resistance¹², these cells express 17β-HSD2 mRNA and show oxidative activity. On the basis of these facts, we propose that dehydroepiandrosterone derivatives with a pyrazole group at C-17 could inhibit the activity of 17β-HSD5.

On the basis of these studies, we synthesized several pyrazole–dehydroepiandrosterone derivatives (4a–4f): 16-formyl-17-(1H-pyrazole-1-yl)-androsta-5,16-dien-3β-yl-cyclopropanoate (4a), 16-formyl-17-(1H-pyrazole-1-yl)-androsta-5,16-dien-3β-yl-cyclobutanoate (4b), 16-formyl-17-(1H-pyrazole-1-yl)-androsta-5,16-dien-3β-yl-cyclopentanoate (4c), 16-formyl-17-(1H-pyrazole-1-yl)-androsta-5,16-dien-3β-yl-cyclohexanoate (4d), 16-formyl-17-(1H-pyrazole-1-yl)-androsta-5,16-dien-3β-yl-cycloheptanoate (4e) and the previously synthesized steroid 16-formyl-17-(1H-pyrazole-1-yl)androsta-5,16-diene-3β-yl-p-fluorobenzoate (4f)

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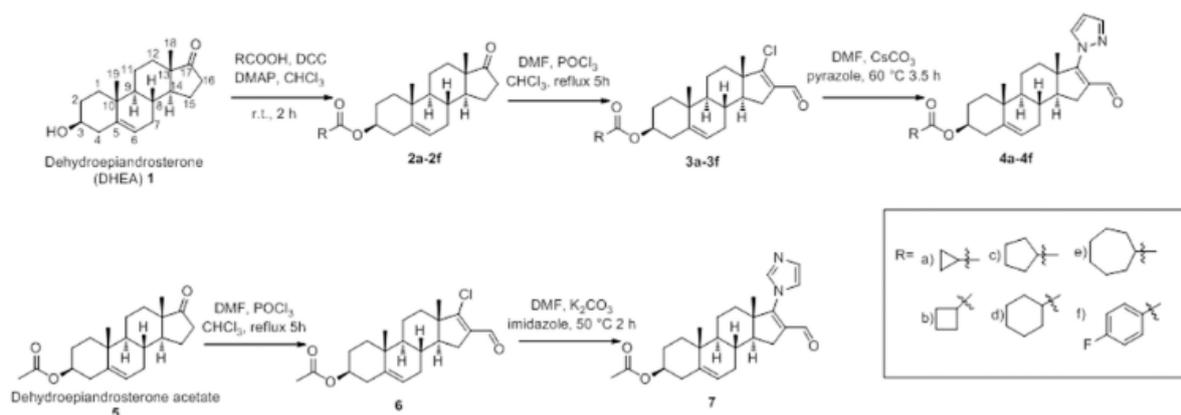


Figure2. Synthesis of dehydroepiandrosterone (1) derivatives.

It has been demonstrated that when the nitrogen atom takes different positions in the pyrazole ring (several isomers are possible), variations in biological activity are observed. Either isomer (pyrazole or imidazole rings) can form a stable complex with the active site of some enzymes, such as active-like kinase 5 (ALK5), thus inhibiting their activity in cell-based luciferase reporter assays, with higher activity of the pyrazole ring. On the basis of this, we synthesized the imidazole derivative 7 (16-formyl-17-(1H-1, 3-imidazole-1-yl)-androsta-5, 16-dien-3 β -yl-acetate) with the aim of comparing its biological activity with the pyrazole derivatives (4a–4f).

II. MATERIALS AND METHODS

2.1 Chemical and radioactive materials

Solvents were of laboratory grade. Melting points (uncorrected) were determined on a melting point apparatus (Fisher Johns, Mexico City, Mexico); ¹H NMR and ¹³C NMR were taken on Varian Gemini 200 and VRX-300 spectrometers, respectively (MR Resources, Fitchburg, MA). Chemical shifts are given in ppm relative to that of Me₄Si (δ = 0) in CDCl₃ (the abbreviations of signal patterns are as follows: s, singlet; d, doublet; t, triplet; m, multiplet). Mass spectra were obtained with an HP5985-B spectrometer (Avantes, Apeldoorn, and The Netherlands). IR spectra were obtained using a Perkin-Elmer 200s spectrometer (Perkin Elmer Life and Analytical Science, Shelton, CT). The UV lamp (254 nm) was from UVP (Upland, CA). UV spectra were determined on a Perkin-Elmer Lambda 2 apparatus using methanol as a solvent. The purity of the synthesized compounds was corroborated by TLC, melting point and Perkin-Elmer High Resolution Mass Spectrometry (HSMS).

(1,2,6,7-3H) T specific activity: 95 Ci/mmol and (1,2,6,7-3H) androstenedione (4-dione) specific activity 98.2 Ci/mmol were supplied by Perkin Elmer Life and Analytical Science (Boston, MA). Radioinert T and 4-dione were supplied by Steraloids (Wilton, NH). Sigma Chemical Co. (St. Louis, MO) provided NADPH and Lubrol PX. Finasteride was obtained by extraction from Proscar (Merck, Sharp and Dohme). The tablets were crushed and 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.

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Vol. 9, Issue 2, February 2020

2.2 Synthesis of the steroidal derivatives: overview

For the synthesis of the new steroidal compounds 4a–4e and 7, we used commercially available dehydroepiandrosterone (1) and also its acetate (5). Compounds 2f, 3f and 4f were prepared according to the procedure reported previously. The synthesis of the new compounds is briefly described below.

2.3 General preparation of esters (2a–2e)

The corresponding acid (14 mmol) was added to a solution of steroid 1 (1 g, 3 mmol), N,N'-dicyclohexylcarbodiimide (2.147 g, 10.41 mmol), 4-dimethylaminopyridine (0.848 g, 6.94 mmol) in chloroform (20 mL). The resulting solution was stirred at room temperature for 2 h. Hexane (15 mL) was added and the precipitated dicyclohexyl urea was filtered. The organic phase was washed three times with 10% aqueous hydrochloric acid, 5% aqueous sodium bicarbonate and water, and was dried over anhydrous sodium sulfate. The crude ester was recrystallized from methanol.

- 17-Oxaandrost-5-ene-3 β -yl-cyclopropanoate (2a): Yield: 85%. m.p. 197–198 °C. IR (KBr) cm^{-1} : 2947, 1736, 1712. ^1H NMR (300 MHz, CDCl_3) δ : 0.9 (3H, s, H-18), 1.1 (3H, s, H-19), 4.6 (1H, m, 3 α -H), 5.4 (1H, dd, $J_1 = 4$ Hz and $J_2 = 2$ Hz, H-6). ^{13}C NMR (100 MHz, CDCl_3) δ : 8.3 (2C-cyclopropyl), 13.2 (C-cyclopropyl), 19.3 (C-18), 20.3 (C-19), 73.6 (C-3), 121.7 (C-6), 139.9 (C-5), 162.2 (C-17), 174.2 (ester carbonyl), 220.9 (C-17). HRMS calcd for $\text{C}_{23}\text{H}_{32}\text{O}_3$ 356.2351, found 356.2299.
- 17-Oxaandrost-5-ene-3 β -yl-cyclobutanoate (2b): Yield: 84%. m.p. 180–181 °C. IR (KBr) cm^{-1} : 2942, 1735, 1720. ^1H NMR (300 MHz, CDCl_3) δ : 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 4.6 (1H, m, 3 α -H), 5.4 (1H, dd, $J_1 = 4$ Hz and $J_2 = 2$ Hz, H-6). ^{13}C NMR (100 MHz, CDCl_3) δ : 14.6 (C-cyclobutyl), 18.2 (C-cyclobutyl), 19.3 (C-18), 20.3 (C-19), 25.0 (2C-cyclobutyl), 73.3 (C-3), 121.6 (C-6), 139.9 (C-5), 174.8 (ester carbonyl), 220.9 (C-17). HRMS calcd for $\text{C}_{24}\text{H}_{34}\text{O}_3$ 370.2508 found 370.2556.
- 17-Oxaandrost-5-ene-3 β -yl-cyclopentanoate (2c): Yield: 88%. m.p. 175–177 °C. IR (KBr) cm^{-1} : 2945, 1735, 1723. ^1H NMR (300 MHz, CDCl_3) δ : 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 4.6 (1H, m, 3 α -H), 5.4 (1H, dd, $J_1 = 4$ Hz and $J_2 = 2$ Hz, H-6). ^{13}C NMR (100 MHz, CDCl_3) δ : 19.3 (C-18), 20.3 (C-19), 25.7 (2C-cyclopentyl), 30.1 (2C-cyclopentyl), 44.1 (C-cyclopentyl), 73.2 (C-3), 121.7 (C-6), 140.3 (C-5), 176.1 (ester carbonyl), 221.0 (C-17). HRMS calcd for $\text{C}_{25}\text{H}_{36}\text{O}_3$ 384.2664 found 384.2695.
- 17-Oxaandrost-5-ene-3 β -yl-cyclohexanoate (2d): Yield: 90%. m.p. 157–158 °C. IR (KBr) cm^{-1} : 2928, 1736, 1725. ^1H NMR (300 MHz, CDCl_3) δ : 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 4.6 (1H, m, 3 α -H), 5.3 (1H, dd, $J_1 = 4$ Hz and $J_2 = 2$ Hz, H-6). ^{13}C NMR (100 MHz, CDCl_3) δ : 19.3 (C-18), 20.3 (C-19), 25.3 (2C-cyclohexyl), 25.8 (C-cyclohexyl), 28.6 (2C-cyclohexyl), 43.1 (C-cyclohexyl), 73.4 (C-3), 121.7 (C-6), 139.91 (C-5), 175.8 (ester carbonyl), 220.8 (C-17). HRMS cal for $\text{C}_{26}\text{H}_{38}\text{O}_3$ 398.2821 found 398.2885.
- 17-Oxaandrost-5-ene-3 β -yl-cycloheptanoate (2e): Yield: 88%. m.p. 160–162 °C. IR (KBr) cm^{-1} : 2923, 1737, 1725. ^1H NMR (300 MHz, CDCl_3) δ : 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 4.6 (1H, m, 3 α -H), 5.4 (1H, dd, $J_1 = 4$ Hz and $J_2 = 2$ Hz, H-6). ^{13}C NMR (100 MHz, CDCl_3) δ : 19.3 (C-18), 20.3 (C-19), 26.2 (2C-cycloheptyl), 28.5 (2C-cycloheptyl), 30.4 (2C-cycloheptyl), 45.1 (C-cycloheptyl), 73.2 (C-3), 121.7 (C-6), 139.9 (C-5), 176.6 (ester carbonyl), 221.4 (C-17). HRMS cal for $\text{C}_{27}\text{H}_{40}\text{O}_3$ 412.2977 found 412.2945.
- 17-Oxaandrost-5-ene-3 β -yl-p-fluorobenzoate (2f): This compound was prepared from dehydroepiandrosterone (1) as described previously¹¹.

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Vol. 9, Issue 2, February 2020

2.4 Synthesis of 17-chloro-16-formyl-androsta-5,16-dien-3 β -yl derivatives (3a–3e and 6)

Dimethylformamide (43 mmol) was added dropwise to a round-bottomed flask fitted with a magnetic stirrer and containing cold phosphorus oxychloride (36 mmol), followed by a solution of the corresponding ester (1 mmol) in chloroform (5 mL). The mixture was refluxed for 5 h under N₂ 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 desired product was purified on silica gel column chromatography, using hexane:ethylacetate (80:20) eluent.

- 17-Chloro-16-formyl-androsta-5, 16-dien-3 β -yl-cyclopropanoate (3a): Yield: 68%, m.p. 114–115 °C. UV (nm): 257 ($\epsilon = 10\,200$). IR (KBr) cm^{-1} : 2928, 1717, 1674, 1589. ¹H NMR (300 MHz, CDCl₃) δ : 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 4.6 (1H, m, H-3), 5.3 (1H, dd, J₁ = 4 Hz and J₂ = 2 Hz, H-6), 9.9 (1H, s, formyl). ¹³C NMR (100 MHz, CDCl₃) δ : 8.3 (2C-cyclopropyl), 13.1 (C-cyclopropyl), 19.2 (C-18), 20.3 (C-19), 73.5 (C-3), 121.7 (C-6), 136.4 (C-16), 140.1 (C-5), 162.2 (C-17), 174.2 (ester carbonyl), 188.0 (formyl). HRMS calcd for C₂₄H₃₁ClO₃ 402.1962 found 402.1922.
- 17-Chloro-16-formyl-androsta-5, 16-dien-3 β -yl-cyclobutanoate (3b): Yield: 69%, m.p. 154–155 °C. UV (nm): 256 ($\epsilon = 10\,100$). IR (KBr) cm^{-1} : 2941, 1719, 1671, 1586. ¹H NMR (300 MHz, CDCl₃) δ : 0.9 (3H, s, H-18), 1.1 (3H, s, H-19), 4.6 (1H, m, 3 α -H), 5.4 (1H, dd, J₁ = 4 Hz and J₂ = 2 Hz, H-6), 9.9 (1H, s, formyl). ¹³C NMR (100 MHz, CDCl₃) δ : 14.6 (C-cyclobutyl), 18.2 (C-cyclobutyl), 19.1 (C-18), 20.2 (C-19), 25.1 (2C-cyclobutyl), 73.2 (C-3), 121.6 (C-6), 136.3 (C-16), 139.9 (C-5), 162.2 (C-17), 174.8 (ester carbonyl), 187.9 (formyl). HRMS cal for C₂₅H₃₃ClO₃ 416.2118 found 416.2189.
- 17-Chloro-16-formyl-androsta-5,16-dien-3 β -yl-cyclopentanoate (3c): Yield: 72%, m.p. 168–170 °C. UV (nm): 258 ($\epsilon = 10\,300$). IR (KBr) cm^{-1} : 2927, 1728, 1708, 1667, 1590. ¹H NMR (300 MHz, CDCl₃) δ : 0.9 (3H, s, H-18), 1.1 (3H, s, H-19), 4.6 (1H, m, 3 α -H), 5.4 (1H, dd, J₁ = 4 Hz and J₂ = 2 Hz, H-6), 9.9 (1H, s, formyl). ¹³C NMR (100 MHz, CDCl₃) δ : 19.2 (C-18), 20.3 (C-19), 25.8 (2C-cyclopentyl), 30.0 (2C-cyclopentyl), 44.0 (C-cyclopentyl), 73.2 (C-3), 121.6 (C-6), 136.4 (C-16), 140.1 (C-5), 162.1 (C-17), 176.2 (ester carbonyl), 186.0 (formyl). HRMS cal for C₂₆H₃₅ClO₃ 430.2275 found 430.2210.
- 17-Chloro-16-formyl-androsta-5,16-dien-3 β -yl-cyclohexanoate (3d): Yield: 71%, m.p. 171–173 °C. UV (nm): 257 ($\epsilon = 10\,100$). IR (KBr) cm^{-1} : 2929, 1727, 1668, 1592. ¹H NMR (300 MHz, CDCl₃) δ : 0.9 (3H, s, H-18), 1.1 (3H, s, H-19), 4.6 (1H, m, 3 α -H), 5.4 (1H, dd, J₁ = 4 Hz and J₂ = 2 Hz, H-6), 9.9 (1H, s, formyl). ¹³C NMR (100 MHz, CDCl₃) δ : 19.2 (C-18), 20.3 (C-19), 25.4 (2C-cyclohexyl), 25.8 (C-cyclohexyl), 28.5 (2C-cyclohexyl), 43.4 (C-cyclohexyl), 72.9 (C-3), 121.6 (C-6), 136.4 (C-16), 140.1 (C-5), 162.1 (C-17), 175.5 (ester carbonyl), 188.0 (formyl). HRMS cal for C₂₇H₃₇ClO₃ 444.2431 found 444.2487.
- 17-Chloro-16-formyl-androsta-5,16-dien-3 β -yl-cycloheptanoate (3e): Yield: 69%, m.p. 168–170 °C. UV (nm): 257 ($\epsilon = 10\,100$). IR (KBr) cm^{-1} : 2946, 1719, 1671, 1585. ¹H NMR (300 MHz, CDCl₃) δ : 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 4.5 (1H, m, 3 α -H), 5.4 (1H, dd, J₁ = 4 Hz and J₂ = 2 Hz, H-6), 9.9 (1H, s, formyl). ¹³C NMR (100 MHz, CDCl₃) δ : 19.2 (C-18), 20.3 (C-19), 26.3 (2C-cycloheptyl), 28.4 (2C-cycloheptyl), 30.4 (2C-cycloheptyl), 45.1 (C-cycloheptyl), 73.1 (C-3), 121.6 (C-6), 136.4 (C-16), 140.1 (C-5), 162.2 (C-17), 176.5 (ester carbonyl), 188.0 (formyl). HRMS cal for C₂₈H₃₉ClO₃ 458.2588 found 458.2514.
- 17-Chloro-16-formyl-androsta-5,16-diene-3 β -yl-p-fluorobenzoate (3f): This compound was prepared from 17-oxaandrost-5-ene-3 β -yl-p-fluorobenzoate (2f) as described previously¹¹.

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Vol. 9, Issue 2, February 2020

- 17-Chloro-16-formyl-androsta-5,16-dien-3 β -yl-acetate (6): Yield: 82%, m.p. 165–167 °C. UV (nm): 257 (ϵ = 10 200). IR (KBr) cm^{-1} : 2928, 1717, 1674, 1589. ^1H NMR (300 MHz, CDCl_3) δ : 0.9 (3H, s, H-18), 1.0 (3H, s, H-19), 2.1 (3H, s, 3 β -OCOCH₃), 4.6 (1H, m, 3 α -H), 5.3 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6), 9.9 (1H, s, formyl). ^{13}C NMR (100 MHz, CDCl_3) δ : 19.2 (C-18), 20.3 (C-19), 21.1 (3 β -OCOCH₃), 73.5 (C-3), 121.7 (C-6), 136.4 (C-16), 140.1 (C-5), 162.2 (C-17), 174.2 (ester carbonyl), 188.0 (formyl). HRMS cal for $\text{C}_{22}\text{H}_{31}\text{ClO}_3$ 379.1962 found 379.1954.

The synthesis of compound 6 as well as its spectroscopy data have been reported previously 17.

2.5 Synthesis of 16-formyl-17-pyrazole-androsta-5,16-diene-3 β -yl derivatives (4a–4e)

Pyrazole (0.6 mmol) was added to a mixture of Cs_2CO_3 (1 mmol) and the corresponding 17-chloro-16-formyl-androsta-5, 16-dien-3 β -yl derivative (0.5 mmol) in dry DMF (2 mL). The mixture was heated at 60 °C under N_2 atmosphere for 3.5 h. Cold water was added and the solid was filtered. The desired pyrazole derivative was purified by column chromatography on Florisil, using hexane: ethyl acetate (70:30) eluent.

- 16-Formyl-17-(1H-pyrazole-1-yl)-androsta-5, 16-dien-3 β -yl-cyclopropanoate (4a): Yield: 57%. m.p.: 212–214 °C. UV (nm): 296 (ϵ = 14 600). IR cm^{-1} : 2915, 1725, 1650, 1597. ^1H NMR (300 MHz, CDCl_3) δ : 1.1 (3H, s, H-18), 1.2 (3H, s, H-19), 4.6 (1H, m, 3 α -H), 5.4 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6), 6.5 (1H, m, aromatic), 7.7 (1H, d, J = 1.6 Hz, aromatic), 7.8 (1H, d, J = 1.6 Hz, aromatic), 10.1 (formyl). ^{13}C NMR (100 MHz, CDCl_3) δ : 8.3 (2C-cyclopropyl), 13.0 (C-cyclopropyl), 19.2 (C-18), 20.5 (C-19), 73.5 (C-3), 107.9 (aromatic), 121.7 (C-6), 129.5 (C-16), 130.5 (aromatic), 139.5 (C-5), 142.4 (aromatic), 160.9 (C-17), 174.2 (ester carbonyl), 190.4 (formyl). HRMS calcd for $\text{C}_{27}\text{H}_{34}\text{N}_2\text{O}_3$ 435.5705 found 435.5699.
- 16-Formyl-17-(1H-pyrazole-1-yl)-androsta-5,16-dien-3 β -yl-cyclobutanoate (4b): Yield: 57%. m.p.: 199–201 °C. UV (nm): 294 (ϵ = 14 500). IR cm^{-1} : 2930, 1722, 1646, 1597. ^1H NMR (300 MHz, CDCl_3) δ : 1.1 (3H, s, H-18), 1.2 (3H, s, H-19), 4.6 (1H, m, 3 α -H), 5.4 (1H, d, J_1 = 5.1 Hz, H-6), 6.5 (1H, m, aromatic), 7.7 (1H, d, J = 2.3 Hz, aromatic), 7.8 (1H, d, J = 1.6 Hz, aromatic), 10.1 (formyl). ^{13}C NMR (100 MHz, CDCl_3) δ : 14.6 (C-cyclobutyl), 18.2 (C-cyclobutyl), 19.2 (C-18), 20.3 (C-19), 25.1 (2C-cyclobutyl), 73.3 (C-3), 107.9 (aromatic), 121.9, 129.5 (C-16), 130.5 (aromatic), 140.0 (C-5), 142.5 (aromatic), 161.0 (C-17), 174.9 (ester carbonyl), 190.4 (formyl). HRMS calcd for $\text{C}_{28}\text{H}_{36}\text{N}_2\text{O}_3$ 449.5970 found 449.5868.
- 16-Formyl-17-(1H-pyrazole-1-yl)-androsta-5, 16-dien-3 β -yl-cyclopentanoate (4c): Yield: 63%. m.p.: 258–260 °C. UV (nm): 297 (ϵ = 14 700). IR cm^{-1} : 2914, 1723, 1653, 1597. ^1H NMR (300 MHz, CDCl_3) δ : 1.1 (3H, s, H-18), 1.2 (3H, s, H-19), 4.6 (1H, m, 3 α -H), 5.4 (1H, d, J = 4.7 Hz), 6.5 (1H, m, aromatic), 7.7 (1H, d, J = 2.3 Hz, aromatic), 7.8 (1H, d, J = 1.6 Hz, aromatic), 10.1 (formyl). ^{13}C NMR (100 MHz, CDCl_3) δ : 19.2 (C-18), 20.3 (C-19), 25.8 (2C-cyclopentyl), 30.1 (2C-cyclopentyl), 44.1 (C-cyclopentyl), 73.3 (C-3), 107.9 (aromatic), 121.9 (C-6), 129.5 (C-16), 130.5 (aromatic), 140.0 (C-5), 142.5 (aromatic), 161.0 (C-17), 174.9 (ester carbonyl), 190.4 (formyl). HRMS calcd for $\text{C}_{29}\text{H}_{38}\text{N}_2\text{O}_3$ 463.6236 found 463.6235.
- 16-Formyl-17-(1H-pyrazole-1-yl)-androsta-5,16-dien-3 β -yl-cyclohexanoate (4d): Yield: 64%. m.p.: 257–259 °C. UV (nm): 297 (ϵ = 14 600). IR cm^{-1} : 2937, 1723, 1650, 1596. ^1H NMR (300 MHz, CDCl_3) δ : 1.1 (3H, s, H-18), 1.2 (3H, s, H-19), 4.6 (1H, m, 3 α -H), 5.4 (1H, dd, J = 5.1 Hz, H-6), 6.5 (1H, m, aromatic), 7.7 (1H, d, J = 2.3 Hz, aromatic), 7.8 (1H, d, J = 1.6 Hz, aromatic), 10.1 (formyl). ^{13}C NMR (100 MHz, CDCl_3) δ : 19.2 (C-18), 20.5 (C-19), 25.3 (2C-cyclohexyl), 25.6 (C-cyclohexyl), 28.5 (2C-cyclohexyl), 43.4 (C-cyclohexyl), 73.1 (C-3), 107.9 (aromatic), 121.8 (C-6), 129.5 (C-16), 130.6 (aromatic), 140.0 (C-5), 142.5 (aromatic), 161.0 (C-17), 175.5 (ester carbonyl), 190.4 (formyl). HRMS calcd for $\text{C}_{30}\text{H}_{40}\text{N}_2\text{O}_3$ 477.6502 found 477.6501.

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: www.ijirset.com

Vol. 9, Issue 2, February 2020

- 16-Formyl-17-(1H-pyrazole-1-yl)-androsta-5,16-dien-3 β -yl-cycloheptanoate (4e): Yield: 59%. m.p.: 225–228 °C. UV (nm): 296 (ϵ = 14500). IR cm^{-1} : 2934, 1726, 1650, 1596. ^1H NMR (300 MHz, CDCl_3) δ : 1.1 (3H, s, H-18), 1.2 (3H, s, H-19), 4.6 (1H, m, 3 α -H), 5.4 (1H, dd, J = 5.1 Hz, H-6), 6.5 (1H, m, aromatic), 7.7 (1H, d, J = 2.3 Hz, aromatic), 7.8 (1H, d, J = 1.6 Hz, aromatic), 10.1 (formyl). ^{13}C NMR (100 MHz, CDCl_3) δ : 19.2 (C-18), 20.5 (C-19), 26.2 (2C-cycloheptyl), 28.3 (2C-cycloheptyl), 30.4 (2C-cycloheptyl), 45.1 (C-cycloheptyl), 73.1 (C-3), 107.9 (aromatic), 121.8 (C-6), 129.5 (C-16), 130.6 (aromatic), 140.0 (C-5), 142.5 (aromatic), 161.0 (C-17), 175.5 (ester carbonyl), 190.4 (formyl). HRMS calcd for $\text{C}_{31}\text{H}_{42}\text{N}_2\text{O}_3$ 491.6359 found 491.6350.
- 16-Formyl-17-(1H-pyrazole-1-yl) androsta-5, 16-diene-3 β -yl-p-fluorobenzoate (4f): This compound was prepared from 17-chloro-16-formyl-androsta-5, 16-diene-3 β -yl-p-fluorobenzoate (3f) as described previously¹¹.

2.6 Synthesis of 16-formyl-17-(1H-1, 3-imidazole-1-yl) androsta-5, 16-dien-3 β -yl-acetate (7)

A mixture of imidazole (3 mmol), K_2CO_3 (6 mmol) and the steroidal derivative 6 (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 precipitate was filtered. The compound was purified by column chromatography on Florisil, using hexane:ethyl acetate (70:30) eluent. Yield: 93%. m.p.: 225–227 °C. UV (nm): 257 (ϵ = 14 400). IR cm^{-1} : 2947, 1731, 1659, 1616, 1234. ^1H NMR (300 MHz, CDCl_3) δ : 1.0 (3H, s, H-18), 1.1 (3H, s, H-19), 2.1 (3H, s, 3 β - OCOCH_3), 4.6 (1H, m, 3 α -H), 5.4 (1H, dd, J_1 = 4 Hz and J_2 = 2 Hz, H-6), 7.1 (1H, s, aromatic), 7.2 (1H, s, aromatic), 7.6 (1H, s, aromatic) 9.7 (formyl). ^{13}C NMR (100 MHz, CDCl_3) δ : 15.8 (C-18), 19.0 (C-19), 21.0 (3 β - OCOCH_3), 73.4 (C-3), 119.7 (aromatic), 121.5 (C-6), 130.6 (C-16), 133.7 (aromatic), 137.6 (aromatic), 139.8 (C-5), 160.4 (C-17), 170.2 (ester carbonyl), 187.2 (formyl). HRMS cal for $\text{C}_{25}\text{H}_{32}\text{N}_2\text{O}_3$ 409.2413 found 409.2476.

2.7 Human, animal and tissue procedures

The prostate of a 53-year-old man who died from renal insufficiency was extirpated 4 h after death in the Pathology Department of the General Hospital of Mexico City. This protocol was approved by the Ethical Committee of the Mexico City General Hospital.

The tissue was chilled in ice-cold 150 mM NaCl and stored at –20 °C. The frozen human prostate was thawed on ice, rinsed and minced with an IKA A11 basic tissue mill (Wilmington, NC). Unless otherwise specified, the following procedures were carried out at 4 °C. The human prostate tissue was homogenized in two volumes of buffer A (20 mM sodium phosphate), pH 6.5 containing 0.32 M sucrose, 0.1 mM dithiothreitol (Sigma-Aldrich, Inc.) with an Ultra-Turrax IKA, T18 basic tissue homogenizer (Wilmington, NC). The homogenates were centrifuged at 1500g for 60 min at 0 °C in a SW 60 Ti rotor (Beckman Instruments, Palo Alto, CA).

The pellets were separated, suspended in buffer A and kept at –70 °C. The suspension, 1.35 mg of protein/mL for human prostates, determined by the Bradford method was used as a source of 17 β -HSD5.

2.8 Determination of 17 β -HSD5 activity

The activity of 17 β -HSD5 using labeled plus unlabeled Δ^4 -androstenedione (4-dione) as the substrate was determined as reported previously¹⁸.

In order to determine the optimum conditions (kinetic parameters) for the measurement of human prostate membrane fraction activity as a source of 17 β -HSD5, different pHs (6.5, 7, 7.5 and 8) and concentrations (43, 87, 174 and

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: www.ijirset.com

Vol. 9, Issue 2, February 2020

313 μM) of unlabeled 4-dione plus 4 nM of labeled 4-dione were used. Different temperatures (36, 36.5, 37, 37.5 $^{\circ}\text{C}$) and human prostate membrane protein (60, 180, 600 and 800 μg), determined by the Bradford Method, were used. Different NADPH concentrations (0.5, 2.5, 5.0 and 10 mM) were also evaluated. Each experiment was repeated twice in duplicate. The reaction in each sample began when human prostate membrane was added to the incubating medium. As a control, this procedure was also carried out without the addition of the enzyme fraction.

The incubating samples of each of these assays and experiments were extracted with ethyl acetate and the solvent from the extractions of each sample were combined in a tube and dried. The extract was dissolved in 50 μL of chloroform:methanol (1:1) and spotted on a thin-layer chromatography plate, HPTLC Keiselgel 60 F254 (Merck, Mexico City). Standards of 4-dione, T, were applied on two lanes, one on each side of the plate. These plates were eluted twice in a mixture of chloroform:acetone (9:1) and air dried; the radioactivity on them was scanned using an AR2000 Bioscanner (Bioscan, Washington DC). Standards were developed by fluorescence (UV lamp; $\lambda = 254 \text{ nm}$ from UVP, Upland, CA) and the data obtained were plotted using SigmaPlot 12 software (Systat Software, Inc., San Jose, CA).

The radioactive zones that showed identical chromatographic behavior to that of the T standard (retention factor, R_f) on the plate were separated in tubes and soaked in 0.2 mL of acetone for 30 min. Each of these fractions was then removed from the tubes and 10 mg of the T standard was added and solubilized in this solvent. A recrystallization of T was carried out; the crystals formed were separated from the acetone (mother liquor). Crystals and mother liquors of each sample were dried under nitrogen steam and the radioactivity contained in 1 mg of each one was determined in a Packard Scintillation Counter (Model 2100 TR) from Perkin Elmer (Boston, MA). This recrystallization procedure was repeated three more times in order to obtain a constant specific activity between the crystals and mother liquors.

2.9 Determination of kinetic parameters of 17 β -HSD5

In order to determine the kinetic parameters, optimum conditions of pH, temperature, protein and NADPH were used. The following parameters were used: pH 7.5, temperature 37.5 $^{\circ}\text{C}$, 600 μg human prostate membrane protein and 2.5 mM of NADPH. The incubating time was 30 min.

The concentrations of non-labeled 4-dione used for this experiment were the following: 187, 303, 417, 512, 735 μM plus labeled 4-dione, 4 nM. The incubation procedure was the same as reported previously. The reaction in each sample began when human prostate membrane was added to the incubating medium. As a control, this procedure was also carried out without the addition of the enzyme fraction.

Extraction, purification, radioactivity determination and recrystallization procedures were the same as described in Section "Determination of 17 β -HSD5 activity". The T transformation yields were calculated from the lanes, taking into account the entire radioactivity in the HPTLC, and were plotted using SigmaPlot 12 software. For the control extraction, chromatographic separations and identifications were carried out in the same manner.

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: www.ijirset.com

Vol. 9, Issue 2, February 2020

III. RESULTS

3.1 Kinetic parameters of 17 β -HSD5

The pH-dependent conversion of labeled androstenedione to testosterone. The conversion of [3H] androstenedione into T in the presence of 2 mM of NADPH by the membrane fractions (600 μ g) of human prostate is shown in panel A, Figure 3. The optimum mean activity of 17 β -HSD5 was obtained at 174 μ M of 4-dione and pH of 7.5. Figure 3, panel B shows the results of the successive recrystallization of T isolated after the chromatographic procedure.

Figure 3. Panel A: Human prostatic 17 β -hydroxysteroid dehydrogenase type 5 (17 β -HSD5) activities in the presence of 2 mM NADPH, determined at different pH values. These data show the effect of labeled plus unlabeled concentrations of 4-Dione (43, 87, 174, 313 μ M) on the formation of labeled T. Panel B: Radiochemical purity of T isolated after HPTLC procedure, and determined by repetitive recrystallizations in the presence of the T standard. Cr, crystallization; ML, mother liquors.

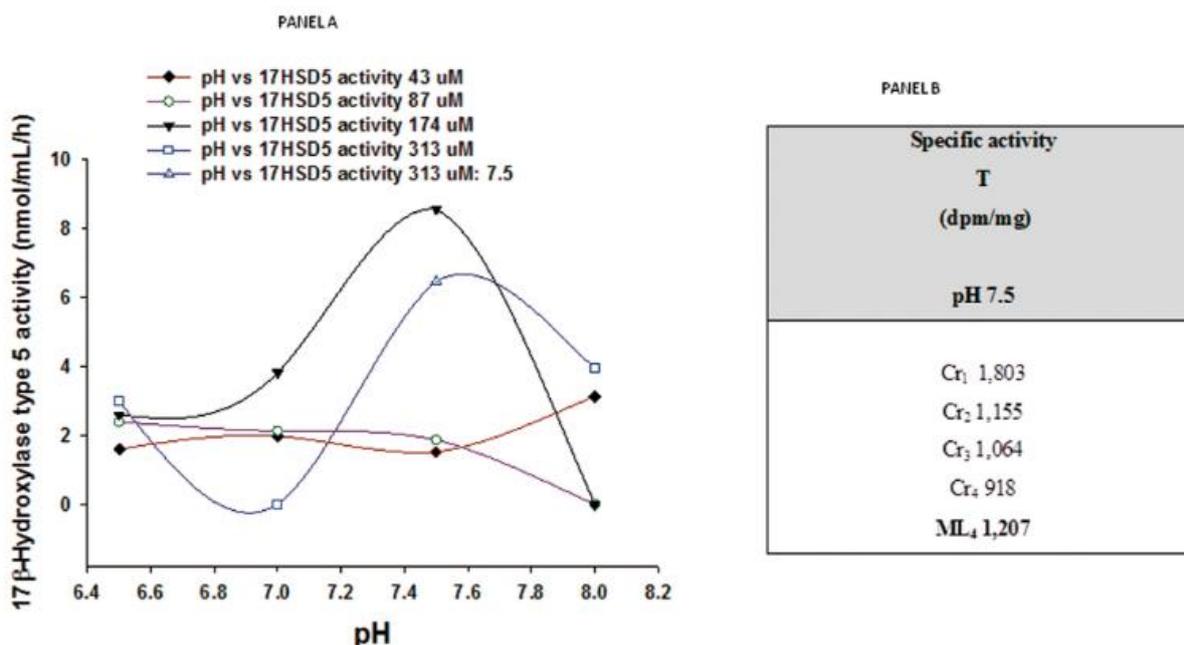


Figure3. Conversion of 4-dione into T in the presence of NADPH at different temperatures

Figure 4, panel A shows that the optimum mean concentration of NADPH for the conversion of labeled 4-dione into T was 2.5 mM. Panel B shows that 37.5 $^{\circ}$ C was the temperature under which the highest conversion of 4-dione to T was obtained. Figure 4. Panel A shows the effect of different concentrations of NADPH on the conversion of labeled plus unlabeled 4-dione (174 μ M) to T. Panel B shows the effect of different temperatures ($^{\circ}$ C) on this conversion in the presence of 2.5 mM of NADPH.

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

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Vol. 9, Issue 2, February 2020

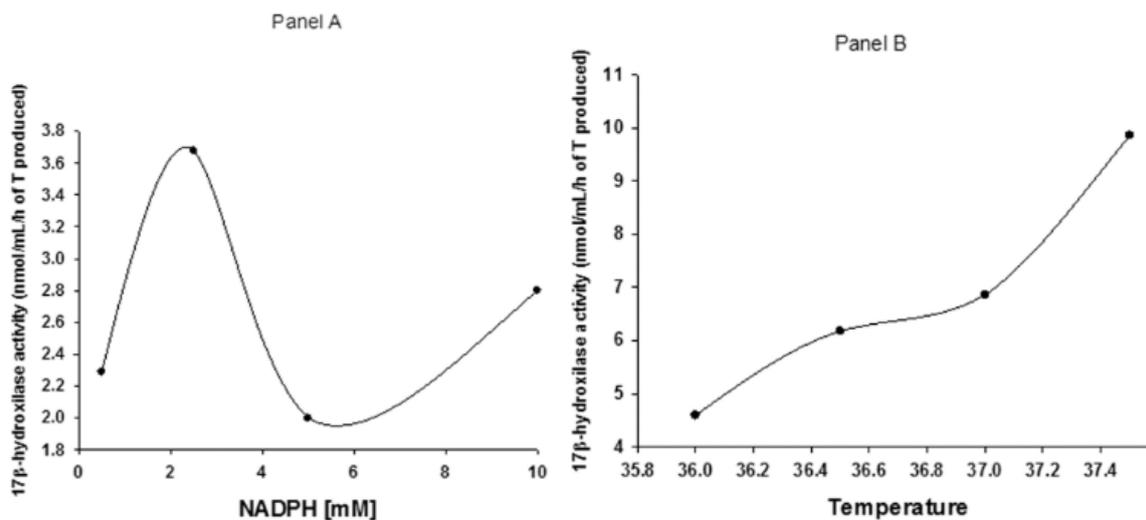


Figure 4. The kinetic parameters were determined from a Lineweaver–Burk plot

Figure 5. Kinetic of 17β-HSD5 enzyme. Lineweaver–Burk plot represents the conversion of labeled plus unlabeled 4-Dione (x axis) to T (y axis). Different concentrations of labeled plus unlabeled 4-dione were incubated with 2.5 mM of NADPH at pH 7.5 and 37.5 °C in the presence of 600 μg protein obtained from the membrane fraction of human prostate.

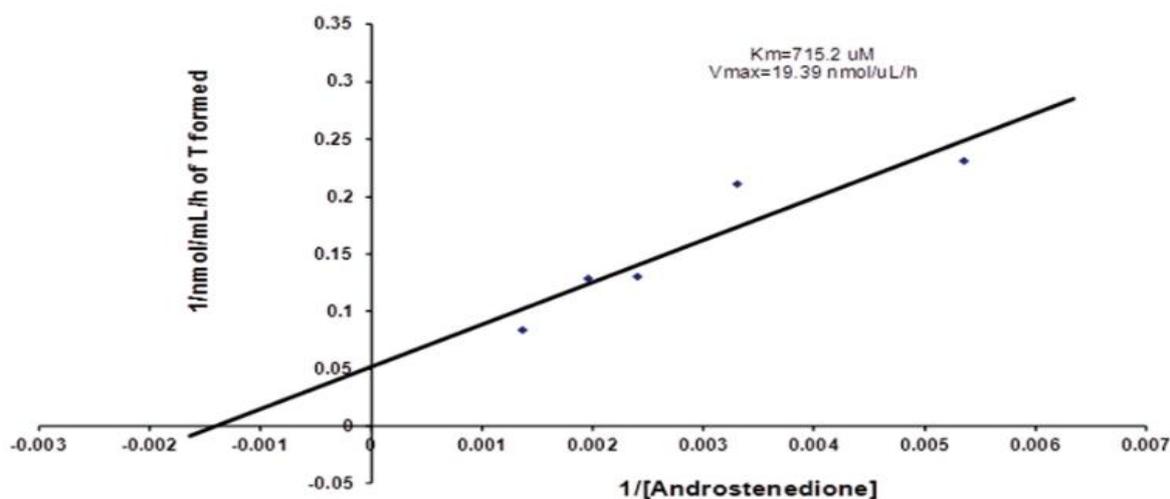


Figure 5. Biological effect of the steroidal derivatives on the inhibition of human 17β-HSD5

The effect of compounds 4a–4f and 7 on the activity of 17-HSD5 is shown in Figure 6. All synthesized compounds inhibited the 17β-HSD5 enzyme. Compound 4a exhibited the highest potency ($IC_{50} = 0.15 \pm 0.02 \text{ nM}$) followed by

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: www.ijirset.com

Vol. 9, Issue 2, February 2020

4c (IC₅₀ = 0.5 ± 0.09 nM), 7 (IC₅₀ = 0.66 ± 0.32 nM),
4f (IC₅₀ = 0.79 ± 0.003 nM),
4b (IC₅₀ = 3.2 ± 0.9 nM),
4d (0.79 ± 0.003 nM),
4d (IC₅₀ = 90 ± 0.02 nM) and
4e (IC₅₀ 17β-HSD5 = 95 ± 3 nM).

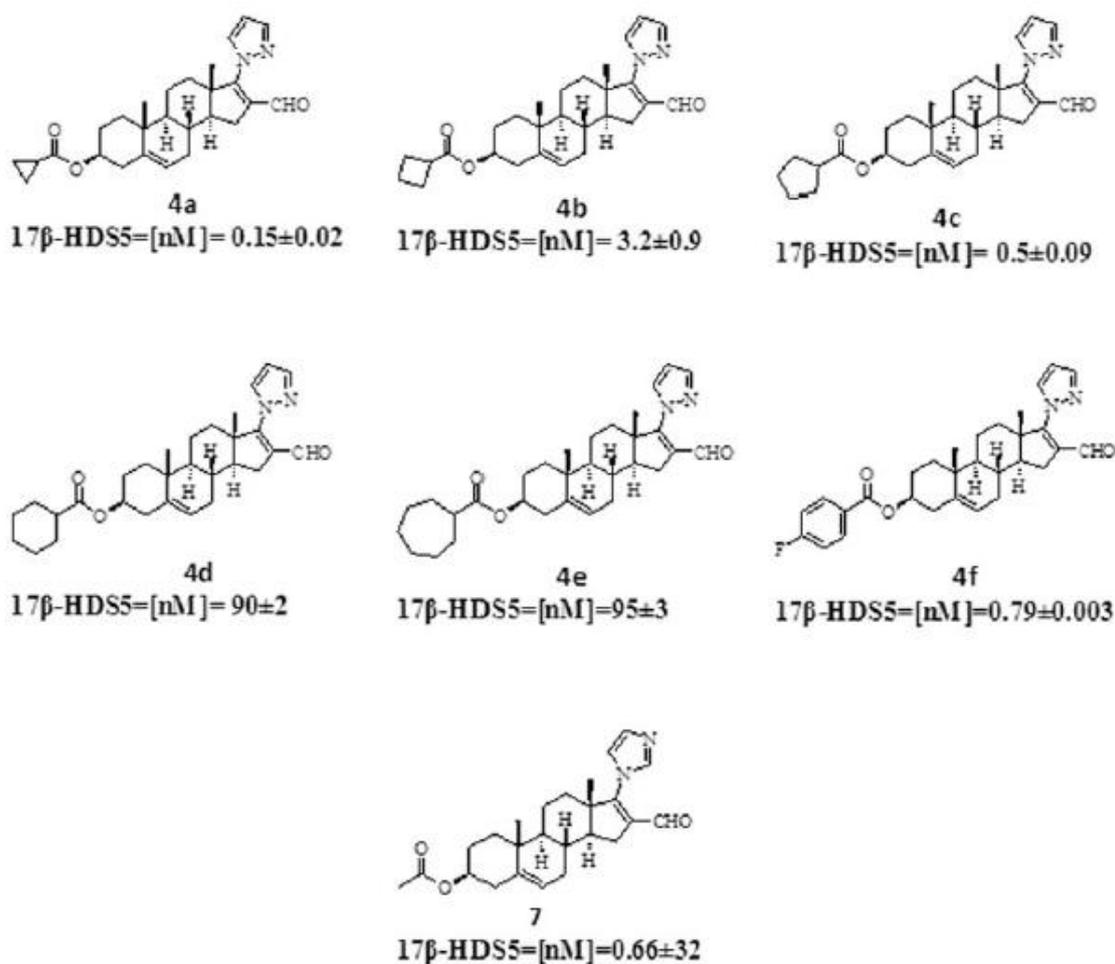


Figure6. Effect of the novel steroids on the activity of 17β-HSD5 enzyme IC₅₀ values show the concentration, for each compound, that inhibited the activity of 17β-HSD5 by 50%.

3.2 Weight of the prostate gland and seminal vesicles

After gonadectomy, the weight of the hamster prostate and seminal vesicle decreased ($p < 0.05$) in contrast to the normal weight. Daily injections with vehicle alone (control C) did not have this effect, but when 1 mg/kg of T or 4-dione was administered for 6 days, the weight of these glands in gonadectomized male hamsters was significantly

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: www.ijirset.com

Vol. 9, Issue 2, February 2020

stimulated ($p < 0.05$) (Figure 7). When T (1 mg/kg) and finasteride (1 mg/kg) or compounds 4a–4f and 7 (2 mg/kg) were injected together, the weight of the prostate and seminal vesicles diminished significantly ($p < 0.05$) in contrast to the T-treated hamsters (Figure 7, Panel A). Surprisingly, compound 4e did not show any significant effect on the weight of prostate and seminal vesicles (Figure 7, Panel A).

The control hamsters were treated with vehicle (sesame oil) only. The pharmacological experiment was carried out in duplicate. The asterisk shows the statistically significant difference between the group of hamsters treated with T (Panel A) or 4-dione (Panel B) and those treated with finasteride (F) or the synthesized steroids (4a–4f and 7). C – Control, T – testosterone, F – finasteride, 4-dione – Δ^4 -androstenedione.

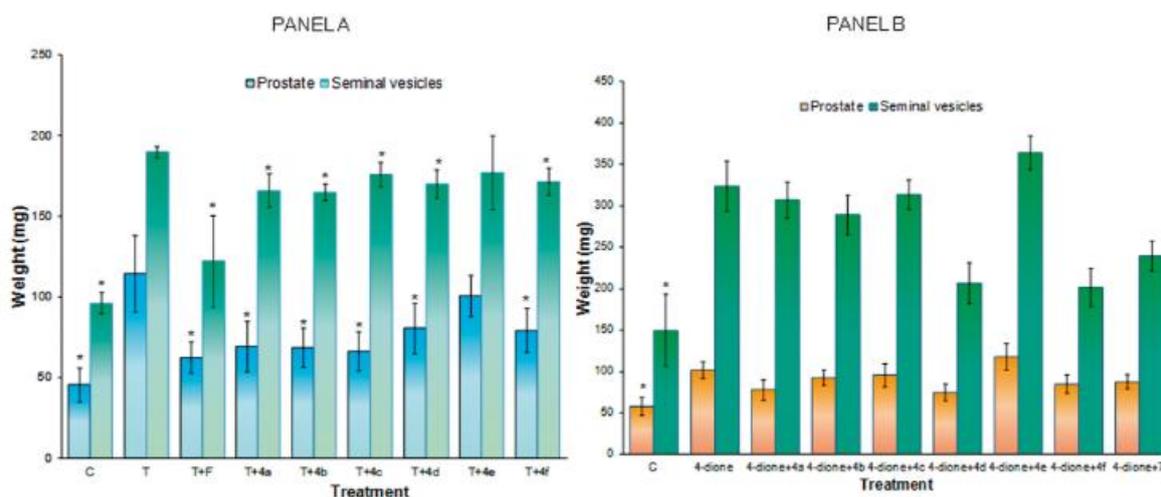


Figure 7. Weight of prostate and seminal vesicles glands \pm standard deviation from castrated hamsters receiving different subcutaneous treatments for 6 days.

The weight of prostate and seminal vesicles from hamsters treated with 4-dione was greater than those of the gonadectomized hamsters treated with sesame oil (C in Figure 7, Panel B). With the exception of steroid 4e, derivatives 4a–4f slightly decreased the weight of prostate and seminal vesicles from hamsters treated with 4-dione (Figure 8, Panel B). However, treatment with 4c did not decrease the weight of seminal vesicles in those hamsters (Figure 7, Panel B). Further studies are required to explain this phenomenon.

Figure 8. Diameter size \pm standard deviations of the pigmented spot from gonadectomized hamster flank organs receiving different treatments with T (Panel A) or 4-dione (Panel B) plus the novel steroids for 6 days. Control gonadectomized hamsters were treated with vehicle (ethanol) only. The asterisk shows the statistically significant difference between the group of hamsters treated with T (Panel A) or 4-dione (Panel B) and those treated with finasteride (F) or the synthesized steroids (4a–4f and 7). C, control, T, testosterone, F, finasteride, 4-dione, Δ^4 -androstenedione.

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

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Vol. 9, Issue 2, February 2020

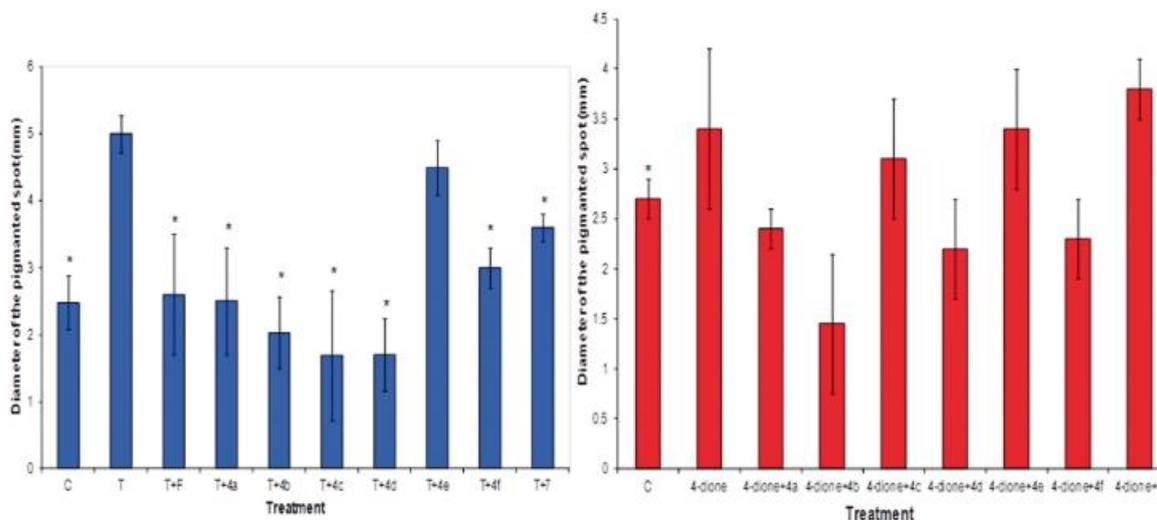


Figure 8. Diameter size \pm standard deviations of the pigmented spot from gonadectomized

3.3 Flank organ test

After castration, the diameter of the male hamster flank organs decreased ($p < 0.005$) compared to that of the intact animals. Treatment with vehicle alone did not change this, but subcutaneous injections of 1 mg/kg of T for 6 days significantly increased ($p < 0.005$) the diameter of the pigmented spot in castrated male hamsters' flank organs (Figure 8, Panel A). With the exception of compound 4e, steroids 4a–4f and 7 (2 mg/kg) injected together with T (1 mg/kg) significantly decreased ($p < 0.005$) the diameter of the pigmented spot in contrast to the hamsters treated with T (Figure 8, Panel A).

The diameter of the pigmented spot of flank organs increased slightly under treatment with 4-dione as compared to the control (Figure 8, Panel B); T-treatment showed more efficacy than 4-dione by producing this effect (Figure 8, Panel A). Steroids 4a, 4b, 4d and 4f decreased the size of the flank organs as compared to 4-dione (Figure 7).

IV. DISCUSSION

The conversion of [3H] 4-dione to T in the presence of NADPH by the membrane fractions of human prostate as well as the radiochemical purity of T demonstrated the presence of 17β -HSD5 enzyme in this fraction. The presence of 17β -HSD5 in prostate has been previously demonstrated. In addition, the expression of 17β -HSD3 activity in normal cells as well as in prostate cancer has also been described²⁴. This type 3 ribozyme is expressed more in seminal vesicles than in prostate; and it is increased in prostate cancer as compared to the normal gland.

Kinetic data indicated that 17β -HSD5 enzyme obtained from the membrane fraction of human prostate clearly followed the Michaelis–Menten kinetic under these experimental conditions (pH 7.5); therefore this fraction can be used as a source of 17β -HSD5 activity to identify novel inhibitors of this enzyme.

Steroids 4a–4f and 7 showed in vitro activity as inhibitors of the 17β -HSD5 enzyme present in the human prostate. With the exception of 4e, these steroids also exhibited pharmacological activity. The overall results suggested

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: www.ijirset.com

Vol. 9, Issue 2, February 2020

compound 4e was not hydrolyzed by the specific esterase present in the membrane fraction of prostate homogenates. This esterase has been previously identified as an oxidized protein hydrolase (OPH), which is overexpressed in the tumorigenic LNCaP cell line. OPH is a serine esterase/protease that has a well-characterized esterase activity towards α -naphthyl butyrate and an exopeptidase activity for removing the N-terminally acetylated amino acid residues from peptides/proteins.

In addition, pharmacological results suggest that steroid 4e could be metabolized by the hepatic or circulatory esterases; as a consequence, an absence of pharmacological effect was observed for this steroid.

Therefore, the incorporation of a pyrazole or imidazole group at C-17 on the dehydroepiandrosterone skeleton having an ester moiety at C-3 (4a–4d and 4f) seems to be the reason for the increase of the ability of these steroids to inhibit the 17β -HSD5 enzyme, since this ester moiety increased the lipophilicity of this molecule and brought on an in vitro inhibitory activity of this enzyme.

The overall results indicated that the incorporation of a pyrazole ring or its isomer imidazole at C-17 of the dehydroepiandrosterone skeleton enhances the inhibition of the 17β -HSD5 enzyme in the in vitro assay using human prostate membrane fraction as a source. Furthermore, compounds 4a–4f and 7 also showed in vivo effect.

The reason for using specific inhibitors of 17β -HSD5 instead of inhibitors of cytochrome P450 in cancer therapy is to decrease side effects. Androgen deprivation therapy using inhibitors of cytochrome P450 as a treatment for prostate cancer could improve existing therapies for this illness. However, these anti-hormonal agents induce a decrease in the production of cortisol by the adrenals and an increase of the synthesis of mineralocorticoids, thus inducing strong metabolic and immunological disturbances.

Currently, arbiterone is the drug of choice for use in combination with prednisone for the treatment of metastatic castration-resistant prostate cancer. This drug was approved by the US Food and Drug Administration (FDA) in April 2011 for this condition. Arbiter one is an inhibitor of 17α -hydroxylase/C17, 20lyase (CYP17A1).

Nevertheless using inhibitors of 17β -HSD5 might offer some advantage for the therapy of prostatic cancer compared with arbiter one, since 17β -HSD5 does not play a part in the synthesis of mineralocorticoids or glucocorticoids. Therefore therapy using 17β -HSD5 inhibitors does not require the additional use of prednisone.

The advantage of using cell cultures expressing pure enzymes instead of homogenates of different tissues for metabolic studies as a methodology to test inhibitors of 5α -Rs and 17β -HSD5s has been an idea on which several research groups have focused. It is true that a clean preparation produces the expected results. However, it has been demonstrated that artificial conditions tend to induce false results; this is true, for example, when different plates of culture cells (plastic or matric ell) are used for the expression of different enzymes by the culture cells³². Other substances such as growth factor or enriched culture medium are not normally added to prostate homogenates³¹. We preferred to use conditions similar to those in the in vivo model. This allowed us to better understand the pharmacokinetic behavior of the novel compounds, which is usually different from that observed in in vitro studies.

4.1 Declaration of interest

This research did not have any real or perceived conflicts of interest or disclosure arising from intellectual, personal or financial circumstances of the researchers. We would like to thank CONACYT project No 165049 and DGAPA project IN 211312 (UNAM) for their generous financial support.

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

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Vol. 9, Issue 2, February 2020

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