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Full Paper

Synthesis and Identification of Pregnenolone Derivatives as Inhibitors of Isozymes of 5α-Reductase

Alejandra Chávez-Riveros[1], Eugene Bratoeff[1], Yvonne Heuze[2], Juan Soriano[3], Isabel Moreno[2], Araceli Sánchez-Márquez[2], and Marisa Cabeza[2]

[1] Departamento de Farmacia, Facultad de Química, Universidad Nacional Autónoma de México, Ciudad de México, Distrito Federal, México, D. F., Mexico
[2] Departamento de Sistemas Biológicos y de Producción Agrícola y Animal, Universidad Autónoma Metropolitana-Xochimilco, Ciudad de México, México, D. F., Mexico
[3] Departamento de Patología, Hospital General de México, México, D. F., Mexico

Hyperplasia of the prostate gland and prostate cancer have been associated with high levels of serum 5α-dihydrotestosterone. This steroid is formed from testosterone by the activity of the enzyme 5α-reductase (5α-R) present in the prostate. Thus, inhibition of this enzyme could be a goal for therapies to treat these diseases. This study reports the synthesis and effects of five different 21-esters of pregnenolone derivatives as inhibitors of 5α-R types 1 and 2. The activity of these steroidal compounds was determined using *in vivo* and *in vitro* experiments. The results indicate that of the five steroids studied, the 21(*p*-fluoro)benzoyloxypregna-4,16-diene-3,6,20-trione derivative, whose structure has not yet been reported, has the best molecular conformation to inhibit the *in vitro* activity of both types of 5α-R. In addition, this steroid also displayed activity *in vivo*. Apparently, its pharmacological effect was increased by the presence of a keto group at C-6, because this group decreased the possibility that the steroid would be metabolized by hepatic enzymes. In addition, the double bond present at C-4 of this compound also enhanced its inhibitory activity on 5α-R, and the C-21 ester moiety increased its liphophilicity. Therefore, its solubility in the cell membrane and its pharmacological activity were both increased.

Keywords: 5α-Reductase / Hamster prostate gland / Hamster seminal vesicles / (*p*-Fluoro)benzoyloxy 21-esters of pregnenolone derivatives / Steroidal esters

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Introduction

The 5α-reductase enzyme (EC 1.3.99.5) converts Δ^4-3-ketosteroids to 5α-3-ketosteroids in androgen-dependent tissues. The activity of this enzyme in androgen-dependent tissues has long been known; the enzyme converts testosterone (T) to 5α-dihydrotestosterone (DHT) in the prostate gland.

Hyperplasia of the prostate gland and prostate cancer have been associated with high levels of serum 5α-DHT [1–3].

Three types of 5α-reductase (5α-R) isozymes have been described; type 2 5α-R 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 is expressed in the prostate epithelial cells whereas type 2 is mainly located in the stromal compartment [4, 5]. Type 1 5α-R is also located in the liver and skin and acts in a neutral or basic medium, whereas type 2 is active in acidic pH [4]. Recently 5α-R3 has been investigated; this isozyme can

Correspondence: Dr. Marisa Cabeza, Departamento de Sistemas Biológicos, Universidad Autónoma Metropolitana-Xochimilco, Calzada Del Hueso No. 1100, México, D. F., C.P. 04960, México.
E-mail: marisa@correo.xoc.uam.mx
Fax: +52-55-54837260

be found in the brain and pancreas and is related to hormone-refractory prostate cancer (HRPC) [6, 7]. None of these three isozymes have been purified, due to their unstable nature, and as a result, 5α-reductase isozyme inhibitors have been designed by targeting their substrate.

Various 5α-R2 inhibitors have been developed for relieving the symptoms produced in benign prostatic hyperplasia. These 5α-R inhibitors reduce bladder obstruction present in this disease by inhibiting the conversion of T to its more active form DHT. Moreover, these inhibitors eliminate the need for surgery and prevent further progression of the disease. The most extensively studied 5α-R inhibitors are finasteride and dutasteride [8]. Finasteride and dutasteride are 4-azasteroids that have demonstrated low affinity for androgen receptors and thus were not expected to produce undesirable antiandrogen effects such as impotence, muscle growth impairment, or gynecomastia [9]. However, when these drugs are used for long periods, they could induce hepatoxicity [10]; thus, synthesis of new 5α-R inhibitors with low side effects has been a goal for researchers.

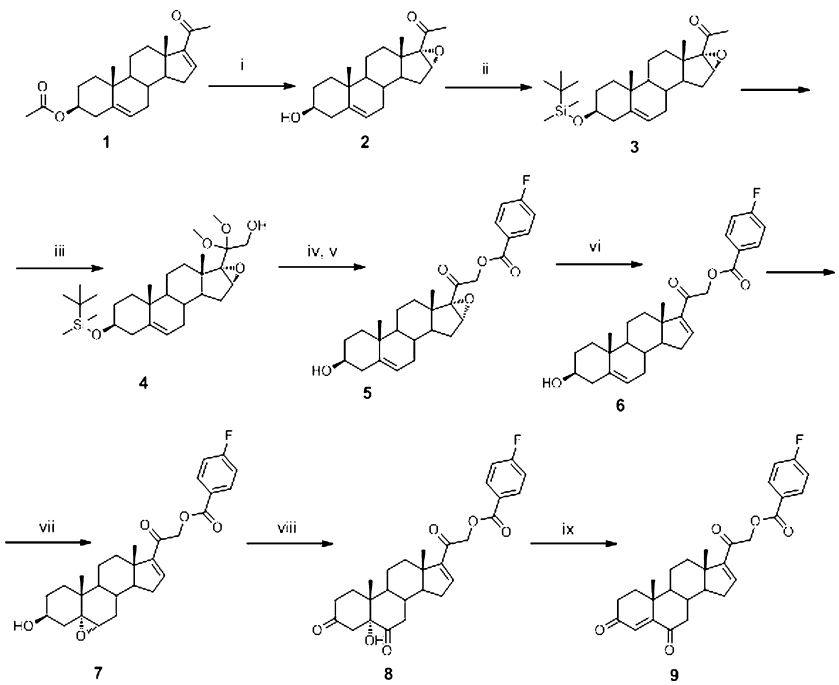
In view of the fact that we have recently synthesized several progesterone derivatives with an ester moiety at C-17, which showed high activity as inhibitors of 5α-R2 and prostate cancer cell growth [11, 12], in this paper we describe the biological activity of five novel steroids based on the progesterone skeleton and with an ester moiety at C-21. They are the following steroidal compounds: 21(*p*-fluoro)benzoyloxy-16α,17α-epoxy-3β-hydroxypregna-5-en-20-one **5**, 21(*p*-fluoro)-benzoyloxy-3β-hydroxypregna-5,16-dien-20-one **6**, 21(*p*-fluoro) benzoyloxy-5α,6α-epoxy-3β-hydroxypregna-16-en-20-one **7**, (*p*-fluoro)benzoyloxy-5α-hydroxypregna-16-en-3,6,20-trione **8**, 21(*p*-fluoro)benzoyloxypregna-4,16-diene-3,6,20-trione **9**.

Results

Chemistry

The target compound 21(*p*-fluoro)benzoyloxypregna-4,16-diene-3,6,20-trione (**9**) was synthesized as shown in Scheme 1. The starting material 16-dehydropregnenolone acetate (**1**) was treated with hydrogen peroxide under basic conditions to form the 16α,17α epoxy compound **2**. Protection of the alcohol at C-3 of compound **2** with *tert*-butyldimethylsilyl chloride followed by oxidation of C-21 using (diacetoxyiodo) benzene afforded compound **4**. The alcohol **4** was esterified using the Steglich method and the C-3 hydroxy was deprotected using acidic conditions to obtain compound **5**. To regenerate the double bond at C-16, the epoxide was reduced using $CrCl_2$ to give compound **6**. The double bond at C-5 of compound **6** was reacted with *m*-chloroperoxybenzoic acid to afford the 5α,6α-epoxy compound **7**. Oxidation of the alcohol **7** with chromic acid yielded the 3,6-diketo-5α-alcohol **8**. Dehydration of the hydroxyl group at C-4 in **8** with thionyl chloride yielded the α,β-unsaturated dicarbonyl analog **9**.

The configuration of the novel pregnenolone derivatives whose geometry resulted from the spatial arrangement of its bonds is shown in Fig. 1. The models of these structures were obtained using the ChemBio3D® program.



Scheme 1. Synthesis of compounds **2**–**9**. Reagents: (i) 30% H_2O_2, 4 N NaOH, MeOH; (ii) TBDMSCL, imidazole, DMF; (iii) C_6H_5I-$(OAc)_2$, NaOH, MeOH, CH_2Cl_2; (iv) *p*-fluoro-benzoic acid, DCC, DMAP, CH_2Cl_2; (v) HCl, acetone; (vi) $CrCl_2$, acetic acid, acetone; (vii) *m*-CPBA, CH_2Cl_2; (viii) CrO_3/H_2O, acetone; (ix) $SOCl_2$, Py, CH_2Cl_2.

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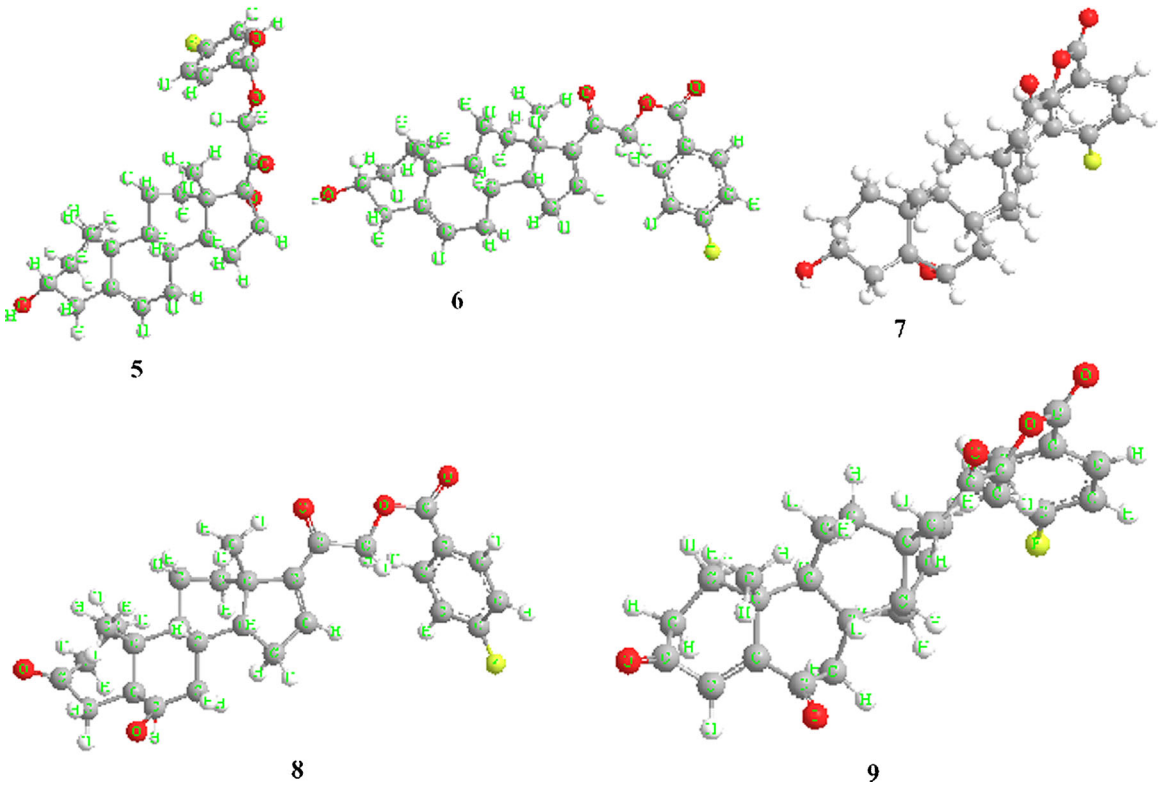

Figure 1. Configuration of the novel 21-esters of pregnenolone derivatives; their geometry resulted from the spatial arrangement of their bonds.

Activity of compounds 5–9 as inhibitors of 5α-R 1 and 2

Figure 2 shows the IC_{50} values of **5–9** required to inhibit 50% of the activity of 5α-reductase isoenzymes 1 and 2. Compared to **5–8**, steroid **9** exhibited the highest inhibitory activity ($IC_{50} = 1.0\,\mu M$) for isoenzyme type 1. For the type 2 5α-reductase isoenzyme, derivative **9** also displayed the highest inhibitory activity ($IC_{50} = 0.179\,nM$). Steroids **5–8** showed lower inhibitory potency than finasteride for 5αR2 (higher IC_{50} value) (Fig. 2).

Weight of the prostate gland and seminal vesicles of hamsters

After sacrificing the animals they were observed statistically significant ($p < 0.05$) reductions in the weights of the prostates and seminal vesicles as compared to glands from intact hamsters (Fig. 3). However, treatment with 1 mg/kg (BW) of T significantly reversed the effect of castration on these glands.

Finasteride (F) and the steroids **9**, when administered together with T, significantly decreased the weight of the prostate and seminal vesicles ($p < 0.05$); however, compared to T, compounds **5–8** did not display any statistically significant effect (Fig. 3).

For 6 days, the castrated hamsters received subcutaneous injections of the various test materials, with the castrated control animals (V) being treated with vehicle only. The experiment was carried out in duplicate. The asterisks show the instances of statistically significant differences ($p < 0.05$) between the group of hamsters treated with testosterone (T) and those treated with T and with either finasteride (F) or with the synthesized steroids (**5–9**; see Scheme 1 for structures).

Discussion

We synthesized and identified the biological activity of five different (*p*-fluoro)benzoyloxy 21-esters of pregnenolone derivatives. On the basis of the results obtained in these experiments, the most accepted spatial conformation of both isozymes of 5α-R was that in steroid **9**. The presence of an ester group at C-21 masks the polar moiety and facilitates its crossing through the cell membranes. Thus, all these steroidal ester derivatives can cross phospholipid membranes and once they are in the bloodstream, they are hydrolyzed at C-21 by esterases in the blood to revert to the corresponding free acid. However, **9** was the only one which displayed a suitable steric

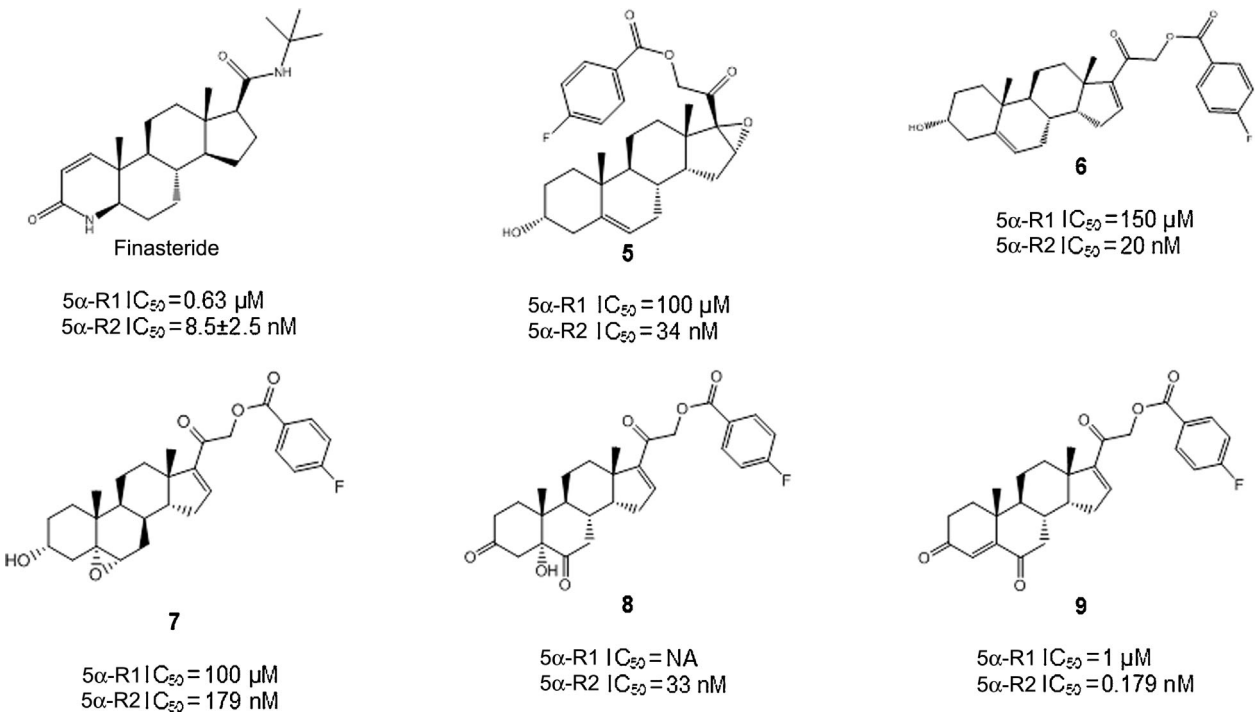

Figure 2. Structures and biological activities of different (*p*-fluoro)benzoyloxy 21-esters of pregnenolone.

conformation to induce pharmacological activity. This observation is consistent with the results previously reported by our group [13]. These previous data indicated that the synthesized steroid pregna-4,6-diene-3,6,20-trione was an inhibitor of 5αR-2, with an IC_{50} value 0.85 nM. This steroid also exhibited slight pharmacological activity, decreasing prostate weight in castrated hamsters stimulated with T [13]. On this basis, we designed the more lipophilic compound **9** to improve the pharmacological activity of this pregna-4,6-diene-3,6,20-trione previously studied, and obtained better results.

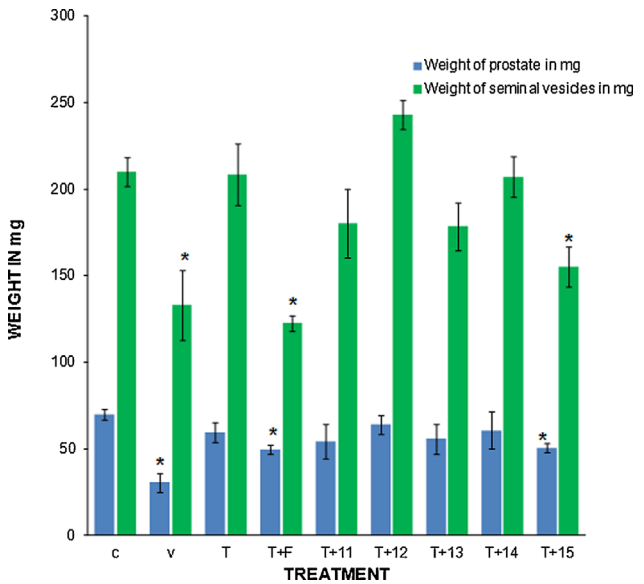

Figure 3. Weights of prostate and seminal vesicles glands (± standard deviation) from castrated (V) and intact animals (C) hamsters. *Statistical significance.

The presence of an OH group in the 5α-position in steroid **8** enables the formation of polar conjugates which can be promptly eliminated in the urine, as has been previously reported [14]. The introduction of a keto group at C-6 as in **9** apparently blocked the hepatic metabolism [14] of this molecule and, as a consequence, an increased effect of this steroid was observed.

The epoxy moiety at C-16 of **5** enhanced the inhibitory effect of 5α-R2 and increased its potency (lower IC_{50} value) as compared with **7**, which has the epoxy moiety in C-5. Thus this spatial arrangement of the molecule was appropriate for inducing the inhibitory effect. However, this increase in activity and potency (**5**) was not observed for the 5α-R1 isoenzyme.

The unsaturated points of these pregnenolone derivatives could also be correlated with the ability of these steroids to inhibit 5α-R activity. The presence of the double bond in Δ^5 as in **6** increased the inhibitory effect on 5α-R2 activity as compared with that produced by **8** (which does not have a double bond in Δ^5). However, the position of this double bond

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did not influence 5α-R1 activity (**8**), whereas the unsaturated point at C-4 in steroid **9** enhanced the inhibitory effect on the activity of both isozymes.

The presence of a keto group at C-3 makes the synthetic steroid a competitive substrate for these both isozymes, because their natural substrates possess this same group. However, the inhibitory activity of compound **8** was not enhanced by the presence of this keto group in C-3, as we would have expected.

In conclusion, **9** proved to have the best molecular conformation for acceptance by both types of 5α-R as a likely substrate. In addition, this steroid also displayed activity *in vivo*; apparently the pharmacological activity was increased by the presence of a keto group at C-6. This could be an advantage for administration of this steroid in the body. As **9** was not metabolized by the liver, this compound could be administered orally. Future studies will enable us to test this hypothesis.

Because the structure of 5α-reductase has not been elucidated, this study contributes to an understanding of the molecular interactions of these two types of 5α-R enzymes.

Experimental

Chemistry

Chemical reagents

Reagents and solvents (laboratory grade) were purchased from commercial sources and were used without further purification. Melting points (uncorrected) were determined on a melting point apparatus (Fisher Johns, Mexico City); ^{1}H NMR and ^{13}C NMR were taken on 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 as follows: s, singlet; d, doublet; t, triplet; m, multiplet). Mass spectra were obtained with an HP5985-B spectrometer (Avantes, Apeldoorn, 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)-Testosterone ([^{3}H]T; specific activity: 95 Ci/mmol) was purchased from Perkin-Elmer Analytical Sciences (Boston, MA). This labeled steroid was purified by thin layer chromatography (TLC) on HPTLC Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany); the solvent system recommended by the manufacturer was used.

Radioinert T and 5α-DHT were purchased from Steraloids (Wilton, NH, USA), Lubrol PX, DL-dithiothreitol (DTT). F 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 an eluant [15].

Syntheses of the steroid derivatives: Overview

As starting material to prepare the steroids, we used commercially available 16-dehydropregnenolone acetate, purity 95%, melting point 172–175°C, Rot: −40° (Sigma–Aldrich) (**1** in Scheme 1). This steroid was hydrolyzed by treatment with 4 N solution of NaOH in methanol. To this mixture, a 30% hydrogen peroxide solution was added drop by drop. The reaction was stirred at room temperature for 4 h, then the solvent was evaporated, and water was added. The precipitate was filtered and washed with water. The crude was purified by recrystallization from methanol to afford 0.87 g of the pure compound.

*16α,17α-Epoxy-3β-hydroxypregna-5-en-20-one **2***

Yield: 95%, mp 180–182°C. IR (cm^{-1}): 3454, 2936, 1642, 1042, 1692. ^{1}H NMR ($CDCl_3$) δ: 1.05 (s, 3H, H-18), 1.02 (s, 3H, H-19), 2.2 (m, 2H, H-21), 2.3 (m, 1H, H-16), 3.5 (m, 1H, H-3), 3.68 (s, 1H, –OH), 5.33 (m, 1H, H-6). ^{13}C NMR ($CDCl_3$) δ: 15.1 (C-18), 19.3 (C-19), 27.51 (C-21), 60.46 (C-16), 71.02 (C-17), 71.06 (C-3), 120.9 (C-6), 141.12 (C-5), 204.9 (C-20). FAB-MS *m/z*: $C_{21}H_{30}O_3$ $[M+H]^+$ calculated 331.2228, found 331.2220.

*16α,17α-Epoxy-3β-tert-butyldimethylsilyloxypregna-5-en-20-one **3***

16α,17α-Epoxy-3β β-hydroxypregna-5-en-20-one **2** (1 g, 3 mmol) in Fig. 1 was dissolved in methanol; *tert*-butyldimethylsilyl chloride (0.76 g, 6.8 mmol) and imidazole (0.450 g, 5 mmol) in 15 mL of *N,N*-dimethylformamide were added to this solution. The mixture was stirred at room temperature for 2 h. After completion of the reaction, the methanol was evaporated and water was added, and the precipitate was filtered and washed with water. The crude was purified by column chromatography using a mixture of 20% ethyl acetate in hexane to give 1.3 g of the pure compound **3**. Yield: 98%, mp 126–127°C. IR (cm^{-1}): 2929, 1659, 1698, 1083. ^{1}H NMR ($CDCl_3$) δ: 0.58 (m, 6H, ($C\underline{H}_3)_2Si$), 0.89 (s, 9H, ($C\underline{H}_3)_3CSi$), 0.99 (s, 3H, H-18), 1.09 (s, 3H, H-19), 2.13 (s, 3H, H-21), 2.2 (m, 1H, H-16), 3.42 (m, 1H, H-3), 5.24 (d, $J = 4.9$ Hz, 1H, H-6). ^{13}C NMR ($CDCl_3$) δ: 19.7 (C-18), 22.8 (C-19), 25.0 (C-21), 30.5 (($\underline{C}H_3)_3CSi$), 32.14 ($\underline{C}$-Si), 65.1 (C-16), 75.5 (C-17), 77.0 (C-3), 125.0 (C-6), 146.5 (C-5), 209.4 (C-20). FAB-MS *m/z*: $C_{27}H_{44}O_3Si$ $[M+H]^+$ calculated 445.3093, found 445.3102.

*16α,17α-Epoxy-21-hydroxy-20-dimethoxy-3β-tert-butyldimethylsilyloxypregna-5-en-20-one **4***

16α,17α-Epoxy-3β-*tert*-butyldimethylsilyloxypregna-5-en-20-one (**3**, 1 g, 2.25 mmol) was dissolved in 10 mL of dichloromethane; 20 mL of a methanolic solution of NaOH (1 g, 25 mmol) and (diacetoxyiodo)benzene (1.2 g, 3.72 mmol) were added. The mixture was stirred for 18 h and then the solvent was evaporated *in vacuo*, water was added, and the precipitate was filtered and washed with water. The crude was purified on silica gel using 10% ethyl acetate in hexane to give 0.74 g of the pure compound **4**. Yield: 65%, mp 205–207°C. IR (cm^{-1}): 3596, 1666, 1070, 1033. ^{1}H NMR ($CDCl_3$) δ: 0.1 (s, 6H, ($C\underline{H}_3)_2Si$), 0.83 (s, 9H, ($C\underline{H}_3)_3CSi$), 0.97 (s, 3H, H-18),

1.2 (s, 3H, H-19), 2.2 (m, 1H, H-16), 3.24 (m, 1H, H-3), 3.25 (m, 6H, C$\underline{H}_3$O–), 3.5 (s, 1H, –OH), 3.25 (d, $J = 16$ Hz, 1H, H-21), 5.24 (m, 1H, H-6). ^{13}C NMR ($CDCl_3$) δ: 17.1 (C-18), 20.7 (C-19), 30.51 (($\underline{C}H_3)_3$CSi), 31.57 ($\underline{C}$-Si), 50.75 (C-16), 50.75 (C$\underline{H}_3$OH–), 63.05 (C-21), 74.22 (C-17), 77.08 (C-3), 125.27 (C-20), 125.27 (C-6), 146.39 (C-5). FAB-MS *m/z*: $C_{29}H_{50}O_5Si$ $[M+H]^+$ calculated 507.3461, found 507.3472.

21(p-Fluoro)benzoyloxy-16α,17α-epoxy-3β-hydroxypregna-5-en-20-one ***5***

16α,17α-Epoxy-21-hydroxy-20-dimethoxy-3β-*tert*-butyldimethylsilyloxypregna-5-en-20-one **4** (1.5 g, 3.05 mmol) was dissolved in methanol; DCC (3.95 g, 19.75 mmol) and DMAP (2.37 g, 19.75 mmol) in dichloromethane (60 mL) was added *p*-fluorobenzoic acid (2.7 g, 19.75 mmol). The resulting solution was stirred at room temperature for 4 h. Ethyl acetate (250 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, the solvent was dried over anhydrous sodium sulfate and evaporated under vacuum. The crude ester was dissolved in acetone (30 mL), and to this solution hydrochloric acid (1 mL) was added drop by drop and the mixture was stirred for 15 min, then water was added and the precipitate was filtered and washed with water. The crude was purified by column chromatography using 10% ethyl acetate in hexane to afford 1.17 g of pure compound **5**. Yield: 82%, mp 210–212°C. IR (cm^{-1}): 3320, 3044, 2936, 1721, 1381, 1055. ^{1}H NMR ($CDCl_3$) δ: 1.02 (s, 3H, H-18), 1.13 (s, 3H, H-19), 3.48 (m, 1H, H-3), 3.87 (s, 1H, –OH), 4.85 (d, $J = 16$ Hz, 1H, H-21), 4.94 (d, $J = 16$ Hz, 1H, H-21), 5.33 (s, 1H, H-6), 7.12 (t, $J = 12$ Hz, 2H, H-Ar), 8.1 (t, $J = 8$ Hz, 2H, H-Ar). ^{13}C NMR ($CDCl_3$) δ: 15.17 (C-18), 19.33 (C-19), 42.18 (C-4), 61.66 (C-21), 66.44 (C-16), 70.66 (C-17), 71.60 (C-3), 115.74 (C-Ar), 120.91 (C-6), 125.54 (C-Ar), 132.43 (C-Ar), 141.13 (C-5), 165.54 (ester carbonyl), 167.26 (C-Ar). FAB-MS *m/z*: $C_{28}H_{33}FO_5$ $[M+H]^+$ calculated 469.2346, found 469.2338.

21(p-Fluoro)benzoyloxy-3β-hydroxypregna-5,16-dien-20-one ***6***

21(*p*-Fluoro)benzoyloxy-16α,17α-epoxy-3β-hydroxypregna-5-en-20-one (**5**) (1 g, 2.1 mmol) dissolved in 100 mL of acetone containing $CrCl_2$ (1.29 g, 10.5 mmol) and 2 mL of acetic acid were stirred at room temperature for 45 min. A mixture of ice in water was then added and the precipitate was filtered and dried under vacuum. The crude was purified on silica gel using 10% ethyl acetate in hexane to afford 0.65 g pure compound **6.** Yield: 69%, mp 280–282°C. IR (cm^{-1}): 3325, 2933, 1723, 1677. ^{1}H NMR ($CDCl_3$) δ: 0.95 (s, 3H, H-18), 1.03 (s, 3H, H-19), 3.51 (m, 1H, H-3), 4.20 (s, 1H, –OH), 5.11 (d, $J = 16$ Hz, 1H, H-21), 5.25 (d, $J = 16$ Hz, 1H, H-21), 5.34 (s, 1H, H-6), 6.82 (s, 1H, H-16), 7.11 (t, $J = 8$ Hz, 2H, H-Ar), 8.11 (t, $J = 8$ Hz, 2H, H-Ar). ^{13}C NMR ($CDCl_3$) δ: 15.88 (C-18), 19.29 (C-19), 66.09 (C-21), 71.68 (C-3), 115.67 (C-Ar), 120.93 (C-6), 125.7 (C-Ar), 132.45 (C-Ar), 141.34 (C-5), 144.26 (C-16), 152.0 (C-17), 165.07 (ester carbonyl), 167.21 (C-Ar), 190.31 (C-20). FAB-MS *m/z*: $C_{28}H_{33}FO_4$ $[M+H]^+$ calculated 453.2396, found 453.2403.

21(p-Fluoro)benzoyloxy-5α,6α-epoxy-3β-hydroxypregna-16-en-20-one ***7***

21(*p*-Fluoro)benzoyloxy-3β-hydroxypregna-5,16-dien-20-one (**6**) (0.45 g, 1 mmol) and *m*-chloroperbenzoic acid (0.52 g, 3 mmol) dissolved in 15 mL of dichloromethane were stirred at room temperature for 45 min. After this, a saturated aqueous solution of sodium bicarbonate (30 mL) containing sodium bisulfite (0.3 g) was added. The reaction mixture was extracted with chloroform; the organic phase was washed with water, dried over sodium sulfate, and the solvent was removed under vacuum. The product was recrystallized from methanol to give 0.36 g of pure compound **7**. Yield: 77%, mp 213–215°C. IR (cm^{-1}): 3517, 2930, 1718, 1678, 1087. ^{1}H NMR ($CDCl_3$) δ: 0.89 (s, 3H, H-18), 1.1 (s, 3H, H-19), 2.93 (d, $J = 4$ Hz, 1H, H-6), 3.84 (s, 1H, –OH), 3.91 (m, 1H, H-3), 4.09 (d, $J = 16$ Hz, 1H, H-21), 5.27 (d, $J = 16$ Hz, 1H, H-21), 6.8 (s, 1H, H-16), 7.12 (t, $J = 8$ Hz, 2H, H-Ar), 8.11 (m, 2H, H-Ar). ^{13}C NMR ($CDCl_3$) δ: 15.14 (C-18), 15.91 (C-19), 58.8 (C-6), 65.87 (C-21), 66.08 (C-5), 68.60 (C-3), 115.47 (C-Ar), 125.71 (C-Ar), 132.56 (C-Ar), 143.97 (C-16), 151.81 (C-17), 165.06 (ester carbonyl), 167.22 (C-Ar), 198.8 (C-20). FAB-MS *m/z*: $C_{28}H_{33}FO_5$ $[M+H]^+$ calculated 469.2346, found 469.2339.

(p-Fluoro)benzoyloxy-5α-hydroxypregna-16-en-3,6,20-trione ***8***

21(*p*-Fluoro)benzoyloxy-5α,6α-epoxy-3β-hydroxypregna-16-en-20-one **7** (0.47 g, 1 mmol) was dissolved in 10 mL of acetone. A solution of chromium trioxide (0.85 g, 8.5 mmol) dissolved in 4.5 mL water at 0°C for 10 min was added dropwise. The resulting mixture was allowed to warm up to room temperature and after 30 min the same amount of chromium trioxide was added in the same manner. The mixture was diluted with cold water (75 mL) and the precipitate was filtered and dried. The product was recrystallized from methanol to afford 0.41 g of the pure compound **8**. Yield: 86%, mp 135–136°C. IR (cm^{-1}): 3332, 2950, 1710, 1685. ^{1}H NMR ($CDCl_3$) δ: 0.95 (s, 3H, H-18), 1.04 (s, 3H, H-19), 2.8 (m, 2H, H-4), 3.89 (s, 1H, –OH), 5.12 (d, $J = 16$ Hz, 1H, H-21), 5.26 (d, $J = 16$ Hz, 1H, H-21), 6.81 (s, 1H, H-16), 7.13 (t, $J = 8$ Hz, 2H, H-Ar), 8.12 (m, 2H, H-Ar). ^{13}C NMR ($CDCl_3$) δ: 13.82 (C-18), 15.98 (C-19), 66.1 (C-21), 82.77 (C-5), 115.52 (C-Ar), 125.68 (C-Ar), 132.59 (C-Ar), 143.62 (C-16), 151.68 (C-17), 165.09 (ester carbonyl), 167.28 (C-Ar), 190.26 (C-20), 210.18 (C-3), 210.87
(C-6). FAB-MS *m/z*: $C_{28}H_{31}FO_6$ $[M+H]^+$ calculated 483.2138, found 483.2144.

21(p-Fluoro)benzoyloxypregna-4,16-diene-3,6,20-trione ***9***

A volume of 4 mL of a cold solution of (*p*-fluoro)benzoyloxy-5α-hydroxypregna-16-en-3,6,20-trione **8** (0.48 g, 1 mmol) was dissolved in dichloromethane. A mixture with 0.4 mL pyridine was added dropwise with thionyl chloride (0.1 mL) under a nitrogen atmosphere. The resulting solution was stirred at room temperature for 2 h. Iced water (100 mL) was added and it was extracted three times with ethyl acetate. The organic phase was washed with 10% aqueous hydrochloric acid, 5%

aqueous sodium bicarbonate, and water. It was dried over sodium sulfate and the solvent was evaporated under vacuum. The product was recrystallized from 20% ethyl acetate in hexane to give 0.38 g of pure compound **9**. Yield: 82%, mp 182–185°C. IR (cm^{-1}): 2942, 1722, 1681. ^{1}H NMR ($CDCl_3$) δ: 1.0 (s, 3H, H-18), 1.21 (s, 3H, H-19), 5.14 (d, $J = 16$ Hz, 1H, H-21), 5.26 (d, $J = 16$ Hz, 1H, H-21), 6.2 (s, 1H, H-4), 6.8 (s, 1H, H-16), 7.13 (s, 2H, H-Ar), 8.11 (s, 2H, H-Ar). ^{13}C NMR ($CDCl_3$) δ: 15.9 (C-18), 17.4 (C-19), 66.0 (C-21), 115.5 (C-Ar), 115.7 (C-Ar), 125.8 (C-4), 127.6 (C-Ar), 132.4 (C-Ar), 143.3 (C-16), 151.4 (C-17), 160.4 (C-5), 165.0 (ester carbonyl), 167.0 (C-Ar), 190.1 (C-20), 199.2 (C-3), 201.3 (C-6). FAB-MS *m/z*: $C_{28}H_{29}FO_5$ $[M+H]^+$ calculated 465.2033, found 465.2026.

Human and animal tissues and procedures

Four hours after a 57-year-old man had died from myocardial infarction, his normal prostate was extirpated in the Pathology Department of the Mexico City General Hospital. 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.

Type 2 5α-R 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 **5–9**, which were designed for inhibition of the activity of this enzyme in humans. In this tissue, type 1 5α-R is not as abundant as type 2.

Human prostate tissue was homogenized in two volumes of buffer A with a tissue homogenizer Ultra-Turrax IKA, T18 basic (Wilmington, NC). The homogenates were centrifuged (1500*g*; 60 min) [16] 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 [17] and was used as a source of type 2 5α-R isozyme.

Type 1 5α-R 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. [18]. 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 (10000*g*; 30 min; 0°C) (Beckman L70K 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 (100000*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 recentrifuged (100000*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 preparation of the solubilized type 1 5α-R steroid.

The solubilization of type 1 5α-R isozyme from rat liver microsomes was carried out according to Levy et al. [18].

Gonadectomized male hamsters

For the experiments *in vivo*, 80 adult male golden hamsters (2.5 months old; 150–200 g) were used. After gonadectomies had been performed on 80 hamsters under isoflurane anesthesia, the castrated hamsters were allowed to recover for 30 days prior to experimentation [19]. 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 ten groups consisting of four animals per group. The hamsters were treated for 6 days as described above and thereafter sacrificed with CO_2. This experiment was carried out twice under the same conditions.

Biological activity of steroidal derivatives: Experiments *in vitro*

The activities of types 1 and 2 5α-reductase were measured by following the conversion of T to DHT, as previously described [16, 18, 20, 21]. 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, the procedure was also carried out without the addition of the enzyme fraction.

In order to extract and purify DHT formed by the activity of types 1 and 2 5α-reductase, after the individual reaction mixtures had been shaken (1 min) using a Type 16700 mixer (Barnstead Thermolyne, Proveedor Científico, México, D. F.), the dichloromethane phase of each was placed into individual tubes. This extraction was repeated for 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 Kieselgel 60 F_{254} plates. T, DHT, and a mixture of both standards were applied to the plate 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 for two additional times. DHT was detected using phosphomolybdic acid reagent (Sigma–Aldrich), and T, by fluorescence (UV lamp; 254 nm). The radioactivity on the plate was scanned by using a Bioscanner AR2000 (Bioscan, Washington, DC). 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 total 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.

Type 1 5α-R activity was calculated from the percentage of 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. Type 2 5α-R activity was calculated taking into consideration the recovery, blank values, and 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 in 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 the 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 that of the non-labeled standard T.

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

Effect of steroid derivatives on activity of types 1 and 2 5α-reductase types

The effect of steroids **5–9** on the activity of types 1 and 2 5α-reductase was determined under the same conditions *in vitro* as described above, but in the presence of six different concentrations ($\{1 \times 10^{-11}$, 1×10^{-10} M,…,1×10^{-3} M$\}$) of each one of derivatives **5–9**. Extraction and purification of the DHT formed from these incubations were carried out as described above.

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. DHT transformations in the presence of **5–9** were calculated from the lanes, taking into account the total radioactivity in the plate, and inhibition curves were plotted using SigmaPlot 12 software.

*Determination of the 50% inhibitory concentration of steroids **5–9** on the activities of types 1 and 2 5α-reductase*

To determine the 50% inhibitory concentration (IC_{50}) of steroids **5–9** and **6** on the activities of types 1 and 2 5α-reductase, the inhibition plots obtained as we indicated above were analyzed with the SigmaPlot 12.5 software (Fig. 4).

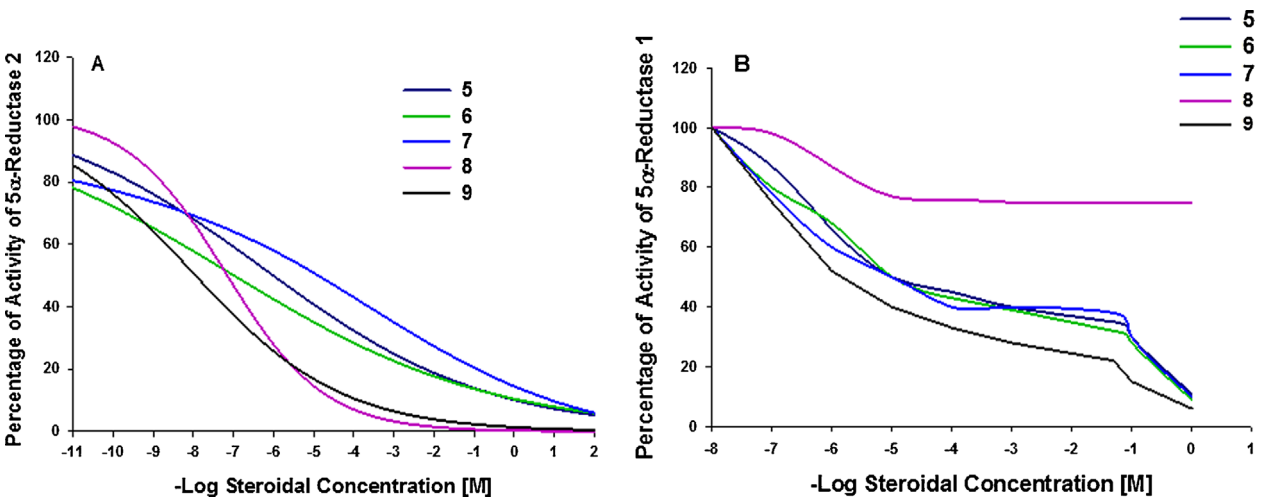

Figure 4. IC_{50} values of steroids **4–9** determined from the curves obtained by plotting the activity of 5α-R types 1 and 2 versus the concentration. The program SigmaPlot was used for curve analysis.

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Biological activity of steroidal derivatives: Experiments *in vivo*

Effect of steroid derivatives on prostate and seminal vesicles of castrated hamsters

For 6 consecutive days, each of the steroid derivatives **5–9** (2 mg/kg body weight (BW)) dissolved in 200 μL sesame oil, together with 1 mg T/kg (BW), was administered by s.c. 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 s.c. 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 an intact control. On the seventh day, the animals were sacrificed with CO_2. The prostate and seminal vesicles of each hamster were dissected and weighed.

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

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