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First synthesis of 7α- and 7β-amino-DHEA, dehydroepiandrosterone (DHEA) analogues and preliminary evaluation of their cytotoxicity on Leydig cells and TM4 Sertoli cells

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Abstract—Efficient syntheses of new DHEA analogues, and their apoptotic and necrotic effects on Leydig cells and TM4 Sertoli cells are described. The key step in the synthetic strategy of 7-amino-DHEA derivatives involves a bromination on C-7 position to give an epimeric mixture of bromides which were substituted by azides and reduced to give 7α- and 7β-amino-3β-hydroxyandrost-5-en-17-ones. No cytotoxic effect induced by apoptosis mechanism was observed on Leydig and TM4 Sertoli cells by treatment with these amino-DHEA analogues. A necrotic effect was induced only in TM4 Sertoli cells. The best activity was obtained with 7α,β-amino-androst-5-en-3β-ol and 7β-amino-3β-hydroxy-androst-5-en-17-one.

1. Introduction

Dehydroepiandrosterone (3β-hydroxy-androst-5-en-17-one or DHEA) is produced in the adrenals and brain.[1] It is the most abundant steroid in human plasma.[2] Plasma concentrations of this steroid reach maximum levels during adolescence and decline with age.[3] Studies have shown that DHEA exerts physiological effects that are supposed to play a beneficial role in diseases/disorders as cancers,[4] diabetes,[5] atherosclerosis,[6] cardiovascular disease,[7] depression,[8] immune function,[9] acquired immune deficiency syndrome (AIDS),[10] aging,[11] obesity,[12] memory,[13] and Alzheimer's disease.[14] Beneficial effect of additional DHEA treatment in the elderly population is currently under study and seems to prevent these disorders.[15]

On the other hand, DHEA is an intermediate in the production of both androgens and estrogens in the peripheral tissues. Effects of DHEA could be either estrogenic or androgenic. The direct effect of DHEA is to act as a weak androgen and that more pronounced androgenicity/estrogenicity are secondary effects due to metabolic conversion into testosterone/estradiol and derivatives. Controlling estrogenic and androgenic steroid concentrations by inhibiting steroidogenic enzymes is a good way to prevent and potentially cure breast and prostate hormone-dependent cancers.[16]

These previous studies showed clearly the essential role of DHEA in various biological mechanisms. However, the broad effect of DHEA itself limits its therapeutic use. To circumvent the side effects of DHEA, one of the promising ways is to develop DHEA derivatives with a greater specificity. With this goal, we are researching on DHEA analogues having targeted properties including cytotoxicity towards testicular cells and we present herein the first promising results obtained with new amino-DHEA derivatives.

It can be noticed that few DHEA analogues have been synthesized to our knowledge. 7-Oxygenated DHEA including DHEA metabolites have been mainly developed and their activities have been widely studied.[17–19] However, DHEA and its androgenous metabolites (7-oxo-DHEA, 7α- and 7β-hydroxyDHEA) have side

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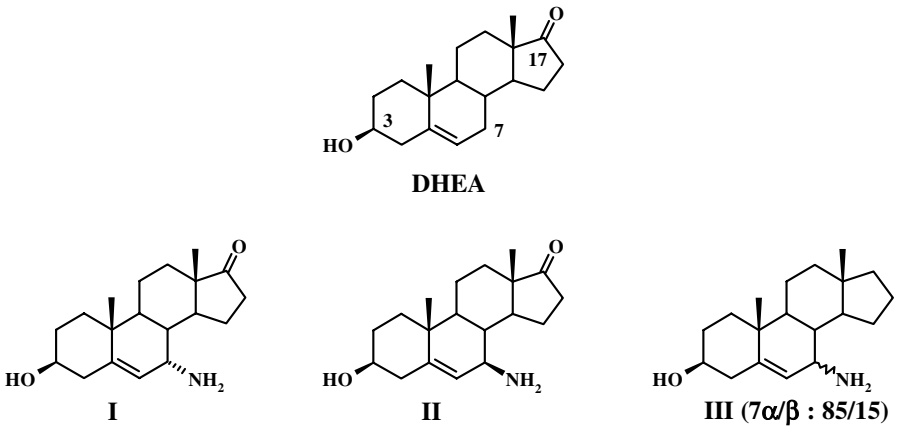


Scheme 1. DHEA and DHEA analogues.

effects that may limit their use in humans.[20–24] Several monohydroxylated derivatives of DHEA (4α, 5α, 7α, 7β, 11β, 16α) and androst-5-ene-3β,17β-diol have been synthesized and evaluated on the induction of the thermogenic enzymes. Only 7-oxygenated derivatives induced thermogenic enzymes.[25] In this pathway, and in our studies related to the synthesis of amino and polyaminosterols,[26–29] we now report the synthesis of 7-aminosteroids as new DHEA analogues (Scheme 1). A first preliminary study consists to evaluate their biological activities on purified Leydig cells and TM4 Sertoli cell lines.

2. Results and discussion

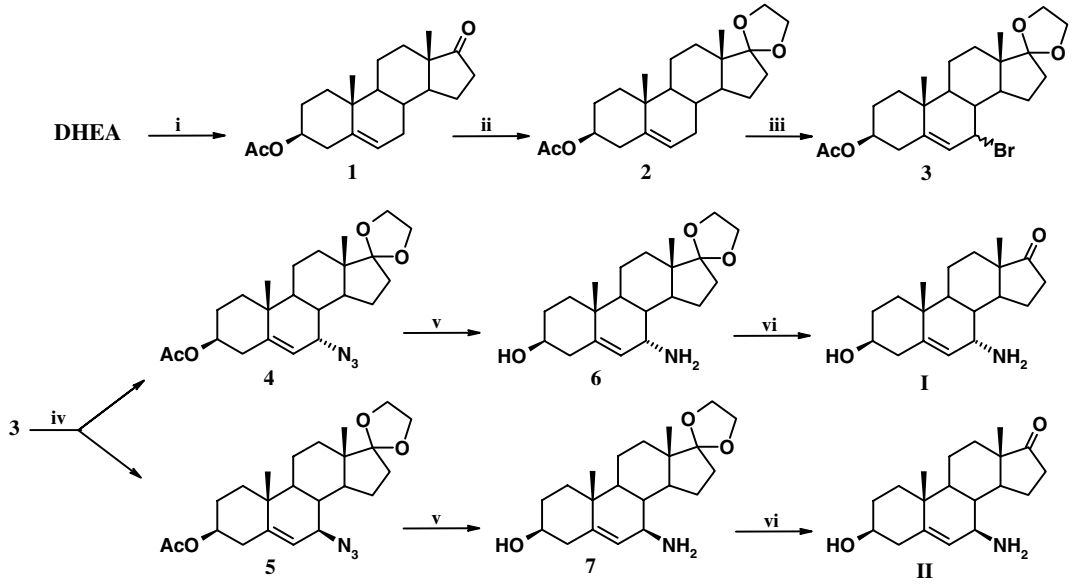
2.1. Chemistry

7α- and 7β-amino-3β-hydroxy-androst-5-en-17-ones (**I** and **II**) were prepared from dehydroepiandrosterone. As depicted in Scheme 2, the hydroxyl group of DHEA was acetylated with acetic anhydride in pyridine to give the acetate **1** (95%) and ketone functionality was protected using ethylene glycol and *p*-toluene sulfonic acid (PTSA) in refluxing toluene to give compound **2** (80%). The exact catalytic quantities (0.5%) of PTSA are necessary to obtain a good yield of ketal. Allylic bromination was accomplished with 1,3-dibromo-5,5-dimethylhydantoin (dibromantin) and sodium bicarbonate in *n*-hexane at 60 °C to afford 7α/β bromides. The literature reported the bromination in situ of cholesteryl 3β-acetate with dibromantin followed by dehydrobromination to give cholesta-4,6-dien-3β-ol.[30] This synthetic way has been applied to a variety of cholesterol derivatives relevant to the synthesis of the human metabolites of vitamin D_3.[31] In our case, we have described the intermediate 7-bromosteroid because its formation in the reaction must be confirmed. In addition, this intermediate could be transformed by dehydrobromination to undesired 3β-acetoxy-17,17-ethylenedioxy-androst-5,7-diene (**3a**) observed in a small amount (10–15%) and confirmed by NMR and MS spectra. Moreover, 3β-acetoxy-17,17-ethylenedioxy-androst-5-en-7α,β-ol (**3b**) could be formed when anhydrous conditions were not respected (Scheme 3). The structure and 7α/7β bromides ratio were established by ^{1}H NMR spectra. Due to the relative instability of the bromide product, the crude product was used in next step without further purification. 7-Bromo derivatives **3** substituted by sodium azide gave 7α- and 7β-azido-androst-5-ene (compounds **4** and **5**) easily separable by column chromatography on silica gel. The stereochemistry of each azido epimer was established by ^{1}H NMR spectra.

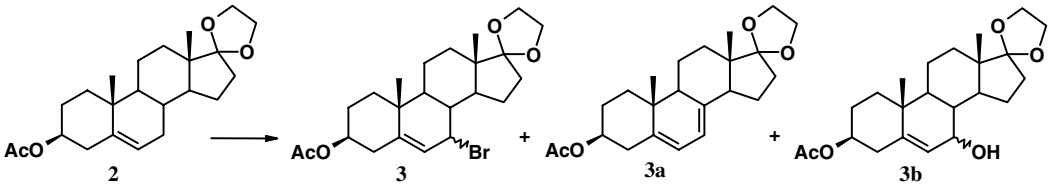
However, direct azidation on allylic position was attempted using trimethylsilyl azide and lead(IV) acetate in Zbiral conditions (Scheme 4).[32] In this case, 7α,β-azidosteroid mixture was not separable in classical chromatography conditions.

The reduction of azides to afford primary amines is an important synthetic operation. A number of reagents described in the literature have been used for this reductive process including Staudinger reaction,[33] borohydrides,[34] samarium diiodide[35] and lithium aluminium hydride.[36] But most of these reagents have some disadvantages in relation to their general applicability. 7-Aminosteroids have been obtained in low yields (0–25%). On the other hand, unfortunately, reduction of azides **4** and **5** using recent methods such as either Sm/$NiCl_2{\cdot}6H_2O$[37] or Fe/$AlCl_3$ in aqueous ethanol[38] failed to yield the desired product. Moreover, catalytic hydrogenation could not be used because it has been limited for being applied to unsaturated compounds. We attempted a reduction of azides **4** and **5** using a high excess of lithium aluminium hydride in presence of nickel(II) chloride hexahydrate. Amines **6** and **7** were, respectively, successfully obtained but with serious purification problems. These problems have been known in the literature for purification of aminosteroids such as aminobrassinosteroids.[39] Finally, the ketal was removed with hydrochloric acid in tetrahydrofuran to give aminosteroids **I** and **II**.

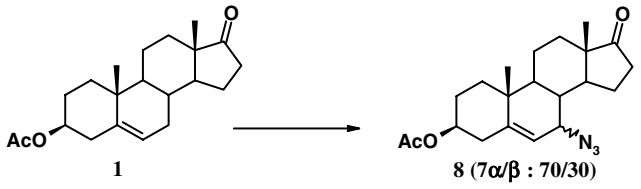
As outlined in Scheme 5, 7α,β-amino-3β-hydroxy-androst-5-enes (**III**) were prepared in the same way.



Scheme 2. Synthesis of 7α- and 7β-amino-3β-hydroxy-androst-5-en-17-one (**I** and **II**). Reagents and conditions: (i) Ac_2O, pyridine, rt, 12 h, 95%; (ii) ethylene glycol, PTSA, toluene, reflux, 8 h, 80%; (iii) dibromantin, $NaHCO_3$, *n*-hexane, 60 °C, 45 min; (iv) NaN_3, DMF, rt, 18 h, 45% (35% 7α and 65% 7β); (v) $LiAlH_4$, $NiCl_2{\cdot}6H_2O$, THF, reflux, 45 min, 39–70%; (vi) 5% aqueous HCl, THF, rt, 18–24 h, 92–95%.

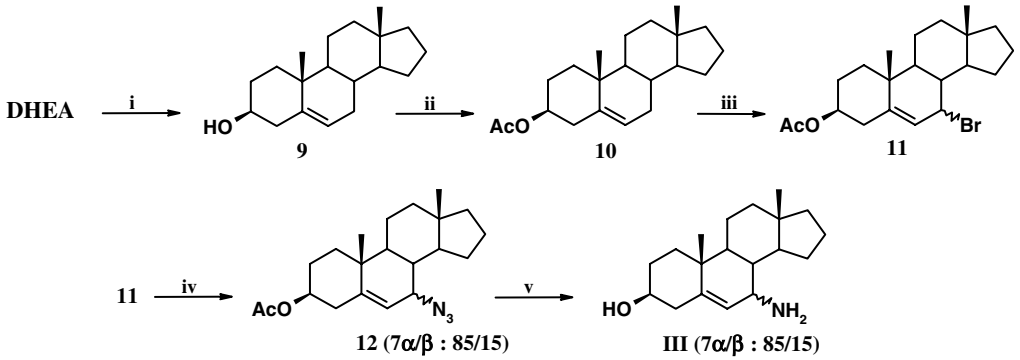


Scheme 3. Allylic bromination of 3β-acetoxy-17,17-ethylenedioxy-androst-5-ene with formation of secondary compounds.



Scheme 4. Azidation in Zbiral conditions. Reagents and conditions: $(AcO)_4Pb$, Me_3SiN_3, CH_2Cl_2, rt, 3 h, 70%.

Dehydroepiandrosterone was deoxygenated in Wolff–Kishner modified conditions following the procedure described by Huang-Minlon[40] in a good yield (**9**). The hydroxyl group was acetylated and allylic bromination was accomplished by dibromantin/sodium bicarbonate. The epimeric allylic bromides **11** were substituted by sodium azide to give 7α,β-azido-androst-5-ene epimers (**12**) inseparable in this case in classical chromatography conditions. The aminosteroids mixture **III** was obtained after reduction of azide and ester.



Scheme 5. Synthesis of 7α,β-amino-3β-hydroxy-androst-5-enes (**III**). Reagents and conditions: (i) hydrazine hydrate, KOH, ethylene glycol, reflux, 24 h, 74%; (ii) Ac_2O, pyridine, rt, 12 h, 88%; (iii) dibromantin, $NaHCO_3$, *n*-hexane, 60 °C, 45 min; (iv) NaN_3, DMF, rt, 18 h, 75% (85% 7α and 15% 7β); (v) $LiAlH_4$, $NiCl_2{\cdot}6H_2O$, THF, reflux, 45 min, 31%.

Table 1. Cell viability percentage after 24 h treatment with either DHEA or DHEA analogues (DAPI test for determination of cell apoptosis percentage)

	Cell viability % (mean ± SD)								
	Control	I		II		III		DHEA	
Dose	Untreated (0 μM)	35 μM	70 μM	35 μM	70 μM	35 μM	70 μM	35 μM	70 μM
Leydig cells	95 ± 5	92 ± 3	93 ± 4	92 ± 3	89 ± 5	92 ± 3	94 ± 4	95 ± 5	95 ± 5
TM4 Sertoli	90 ± 3	87 ± 5	86 ± 4	82 ± 3	79 ± 3*	88 ± 3	77 ± 1*	90 ± 3	90 ± 3

*$p < 0.05$, compared to untreated cells.

Table 2. Cell viability percentage after 24 h treatment with either DHEA or DHEA analogues (Trypan blue test for determination of cell necrosis percentage)

	Cell viability % (mean ± SD)								
	Control	I		II		III		DHEA	
Dose	Untreated	35 μM	70 μM	35 μM	70 μM	35 μM	70 μM	35 μM	70 μM
Leydig cells	95 ± 5	85 ± 5	78 ± 6	93 ± 1	85 ± 4	82 ± 2	**45 ± 6****	86 ± 6	76 ± 4*
TM4 Sertoli	92 ± 6	88 ± 8	**18 ± 3*****	**64 ± 3****	**0**	**43 ± 6****	**0**	92 ± 6	92 ± 6

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, compared to untreated cells.

2.2. Biology

DHEA analogues (**I**, **II** and **III**) were tested on two types of testicular cells (rat Leydig cells and mouse TM4 immortalized Sertoli cell line). Leydig cells were isolated from testis of mature Sprague–Dawley rats and characterized as previously described.[41] TM4 Sertoli cell line derived from testis of immature BALB/c mice provides an ideal system for the in vitro analysis of function and responsiveness to biochemical and hormonal factors.[42]

The cytotoxicity was studied via two separated processes: apoptosis or necrosis.

To assess drug-induced apoptotic cell death, apoptosis was measured by morphological and quantitative evaluation of DNA stained with 4′,6-diamidino-2-phenylindole (DAPI) by fluorescence microscopy.

Necrosis process was determined using Trypan blue exclusion assays.

To evaluate viability of cells treated or not by DHEA or DHEA analogues, the percentage of viable and non-viable cells was determined (Tables 1 and 2). Cells were incubated for 24 h with or without DHEA and DHEA analogues at two different concentrations (35 and 70 μM). Preliminary tests showed that lower doses did not affect cell viability. Viability and total number of cells were determined, and the percentage of cell viability was calculated by the formula (Fig. 1).

$$\text{cell viability} = \frac{\text{number of viable cells}}{\text{total number of control cells}} \times 100$$

Figure 1.

These results indicate that viability of purified Leydig cells was not affected via apoptosis or necrosis process after 24 h treatment with DHEA analogues **I** and **II**. But, 35 and 70 μM of DHEA analogues **II** and **III** significantly increased the percentage of necrotic cells.

No TM4 cells' death induced by apoptosis after treatment with 35 μM of DHEA analogues was observed. With increasing dose of DHEA analogue **I** (70 μM), no significant effect was observed. But with 70 μM of DHEA analogues **II** and **III**, the percentage of apoptotic cells was increased. In contrast, DHEA analogues induced more death by necrosis. When cells were exposed to 35 μM, **II** and **III** appeared to induce necrosis death on TM4 Sertoli cells. Increasing concentration to 70 μM, a high and significant necrosis was obtained by **III** or by **II** exposures, less by **I**.

Finally, these results were then compared to those of DHEA. DHEA did not appear to induce either apoptosis or necrosis of Leydig or Sertoli cells (except for necrosis induced by a dose of 70 μM in Leydig cells).

3. Conclusion

7-Amino-DHEA derivatives constitute new analogues of DHEA. 7α- and 7β-amino-3β-hydroxy-androst-5-en-17-one and 7α,β-amino-3β-hydroxy-androst-5-enes were successfully prepared as new analogues of DHEA. The key step in the synthetic strategy involves an allylic bromination to give an epimeric mixture of 7α,β-bromosteroids which were substituted by an azide function. 3β-Acetoxy-7α,β-azido-17,17-ethylenedioxy-androst-5-enes were easily separated by classical chromatography. In contrast, the intermediate epimer mixture 3β-acetoxy-7α,β-azido-androst-5-ene and 3β-acetoxy-7α,β-azido-androst-5-en-17-one was inseparable in the same conditions. It seems that the presence of ketal was

probably essential for the success of azide epimers' separation. The azides were then reduced to amines.

In this preliminary study, no cytotoxic effect induced by apoptosis or necrosis mechanism was observed on purified Leydig cells by treatment with DHEA analogues (except with the highest dose of **III** that induced necrosis in Leydig cells).

DHEA analogues' exposure to TM4 cells induced necrosis but no apoptosis (except with the highest dose of **II** and **III** that induced apoptosis). A significant effect was obtained by **III** and **II**, less by **I**. These results showed that 7β-epimer (**II**) has a more cytotoxic effect than 7α-epimer (**I**) at a concentration value of 70 μM. It is clear that these results showed structure dependent-cytotoxic effects on purified Leydig and immortalized TM4 Sertoli cells via necrosis process. Further investigation in other cell lines and the assessement of androgenic and other effects will be developed.

4. Experimental

4.1. Chemistry

4.1.1. General remarks. All solvents were distilled and dried prior to use. Reagents and materials were obtained from commercial suppliers and were used without further purification. The reactions were monitored by TLC on Kieselgel-G (Merck Si 254 F) layers (0.25 mm thick). The spots were detected by spraying sulfuric acid/ethanol (2:8) on TLC and heating. Column chromatography was carried out using silica gel 60 (0.063–0.2 mm) (Merck). Melting points were determined on a Kofler block and are uncorrected. IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer. Specific rotation was measured in methanol with a Perkin-Elmer 353 polarimeter. EI mass spectra were recorded on a Jeol-GCmate (GC–MS system) spectrometer with ionisation energy from 30 to 40 eV. ^{1}H NMR and ^{13}C NMR spectra were recorded using $CDCl_3$ or CD_3OD, respectively, at 400 MHz (Jeol Lambda 400 spectrometer) and at 100 MHz. Chemical shifts are reported relative to TMS; *J* values are given in Hertz. ^{13}C NMR spectra are ^{1}H-decoupled. Elemental analyses were performed at the 'Institut de Recherche en Chimie Organique Fine' (Rouen).

4.1.2. 3β-Acetoxy-androst-5-en-17-one (1). Acetic anhydride (26 mL, 277 mmol) was added dropwise to a solution of dehydroepiandrosterone (10 g, 35 mmol) in pyridine (25 mL). The solution was stirred at room temperature and under argon for 12 h. Ice water (20 mL) was poured into the mixture. The white precipitate formed was dissolved in CH_2Cl_2 (200 mL). The organic layer was washed with 1 M HCl (3× 20 mL), 5% $NaHCO_3$ (1× 30 mL), brine (1× 30 mL) and water (1× 30 mL), dried over anhydrous sodium sulfate and evaporated under reduced pressure. The crude product was recrystallized from acetone to give **1** (10.9 g, 95%) as a white powder. Mp 172 °C (lit.[43] 169–171 °C); R_f = 0.28 (cyclohexane/ethyl acetate, 80:20). IR (KBr): ν(cm^{-1}): 1740 (C=O ester), 1725 (C=O ketone). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.89 (s, 3H, 18-Me), 1.05 (s, 3H, 19-Me), 2.04 (s, 3H, 3-CH_3COO), 4.60 (m, 1H, 3-H), 5.41 (d, $^3J_{6\text{-}7\beta}$ = 5.0 Hz, 1H, 6-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 13.5 (C-18), 19.3 (C-19), 20.2, 21.3 (3-OCO*C*H$_3$), 21.8, 27.6, 30.7, 31.3, 31.4, 35.7, 36.6, 36.8, 38.0, 47.4, 50.0, 51.6, 73.6 (C-3), 121.8 (C-6), 139.8 (C-5), 170.4 (3-O*C*OCH$_3$), 220.9 (C-17) ppm. MS (30 eV, EI): *m*/*z* (%) = 270 (100) [M$^+$−AcOH], 255 (46), 121 (75). Anal. Calcd for $C_{21}H_{30}O_3$ (330.47): C, 76.33; H, 9.15. Found: C, 76.22; H, 9.06.

4.1.3. 3β-Acetoxy-17,17-ethylenedioxy-androst-5-ene (2). *p*-Toluene sulfonic acid (0.029 g, 0.15 mmol) and ethylene glycol (25 mL, 454 mmol) were added to a solution of compound **1** (10 g, 30 mmol) in toluene (120 mL) under argon atmosphere. The solution was refluxed for 8 h with azeotropic distillation of water. After cooling, the reaction mixture was made alkaline with saturated aqueous $NaHCO_3$ and extracted with ethyl acetate (4× 30 mL). The organic layer was washed with brine, dried over anhydrous sodium sulfate and evaporated. Recrystallization from a methanol/ethanol mixture (1:1) afforded **2** as a white powder (9.1 g, 80%). Mp 140 °C (lit.[44] 140–141 °C); R_f = 0.38 (cyclohexane/ethyl acetate, 80:20). IR (KBr): ν(cm^{-1}): 1732 (C=O ester). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.86 (s, 3H, 18-Me), 1.03 (s, 3H, 19-Me), 2.04 (s, 3H, 3-CH_3COO), 3.86–3.94 (m, 4H, –O(CH_2)$_2$O–), 4.61 (m, 1H, 3-H), 5.38 (d, $^3J_{6\text{-}7\beta}$ = 4.9 Hz, 1H, 6-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 14.2 (C-18), 19.3 (C-19), 20.4, 21.4 (3-OCO*C*H$_3$), 22.7, 27.7, 30.5, 31.2, 32.0, 35.1, 36.5, 36.9, 38.0, 45.6, 49.8, 50.5, 64.5 and 65.1 (–O(CH_2)$_2$O–), 73.8 (C-3), 119.4 (C-17), 122.4 (C-6), 139.5 (C-5), 170.5 (3-O*C*OCH$_3$) ppm. MS (30 eV, EI): *m*/*z* (%) = 314 (51) [M$^+$−AcOH], 252 (95), 226 (59), 99 (100). Anal. Calcd for $C_{23}H_{35}O_4$ (374.53): C, 73.76; H, 9.15. Found: C, 73.72; H, 9.53.

4.1.4. 3β-Acetoxy-7α,β-bromo-17,17-ethylenedioxy-androst-5-ene (3). Sodium bicarbonate (6.1 g, 72 mmol) was added to a solution of compound **2** (5 g, 13 mmol) and 1,3-dibromo-5,5-dimethylhydantoin (2.30 g, 8 mmol) in *n*-hexane (50 mL). The mixture was refluxed for 40 min under argon atmosphere. The solution was cooled, filtered and evaporated. The crude product was used in next step without purification. IR and ^{1}H NMR spectra have been carried out for characterization. IR (KBr) ν(cm^{-1}): 2971–2832 (C–H alkane), 1729 (C=O ester), 560 (C–Br). ^{1}H NMR ($CDCl_3$, 400 MHz, 25 °C), δ = 0.90 (s, 3H, 18-Me), 1.06 (s, 3H, 19-Me), 2.05 (s, 3H, CH_3COO–), 3.82–3.97 (m, 4H, –O(CH_2)$_2$O–), 4.60 (m, 1H, 3-H), 4.66 (dd, $^3J_{7\alpha\text{-}8}$ = 8.0 Hz, $^3J_{7\alpha\text{-}6}$ = 2.1 Hz, 0.4H, 7α-H of β epimer), 4.69 (dd, $^3J_{7\beta\text{-}8}$ = 5.0 Hz, $^3J_{7\beta\text{-}6}$ = 5.0 Hz, 0.6H, 7β-H of α epimer), 5.41(d, $^3J_{6\text{-}7\alpha}$ = 2.1 Hz, 0.4H, 6-H of β epimer), 5.77 (d, $^3J_{6\text{-}7\beta}$ = 5.2 Hz, 0.6H, 6-H of α epimer).

4.1.5. 3β-Acetoxy-7α-azido-17,17-ethylenedioxy-androst-5-ene (4) and 3β-acetoxy-7β-azido-17,17-ethylenedioxy-androst-5-ene (5). The crude product **3** was dissolved in DMF (15 mL) and sodium azide (8.7 g, 133 mmol) was

added to the mixture that was stirred for 18 h at room temperature under argon atmosphere. The reaction mixture was poured into water (50 mL) and extracted with diethyl ether (4× 50 mL). The combined organic layers were dried over anhydrous sodium sulfate and evaporated. The crude product was purified by column chromatography (silica gel, cyclohexane/ethyl acetate, 98:2) to give two separated epimers as white amorphous solids 7α (16%) and 7β (29%).

4.1.5.1. Compound 4. $R_f = 0.35$ (cyclohexane/ethyl acetate, 80:20). $[\alpha]_D$ −86° (*c* 0.1 in methanol). IR (KBr): $\nu(cm^{-1})$: 2098 (N_3), 1726 (C=O ester). ^1H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.85$ (s, 3H, 18-Me), 1.04 (s, 3H, 19-Me), 2.05 (s, 3H, 3-CH_3COO), 3.60 (m, 1H, 7β-H), 3.85–3.95 (m, 4H, –$O(CH_2)_2O$–), 4.67 (m, 1H, 3-H), 5.37 (d, $^3J_{6\text{-}7\beta} = 4.8$ Hz, 1H, 6-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): $\delta = 14.3$ (C-18), 19.2 (C-19), 20.5, 21.4 (3-OCO*C*H_3), 24.1, 27.6, 29.9, 35.2, 36.4, 36.6, 37.0, 37.6, 46.2, 48.5, 49.8, 63.9, 64.5 and 65.3 (–$O(CH_2)_2O$–), 73.1 (C-3), 118.8 (C-17), 121.2 (C-6), 145.2 (C-5), 170.5 (3-O*C*OCH$_3$) ppm. MS (30 eV, EI): *m*/*z* (%) = 373 (15) [$M^+ - N_3\cdot$], 314 (37), 255 (51), 226 (27), 99 (100). HRMS-EI (20 eV): *m*/*z* [M^+] Calcd for $C_{23}H_{33}N_3O_4$: 415.2471. Found: 415.2455.

4.1.5.2. Compound 5. $R_f = 0.32$ (cyclohexane/ethyl acetate, 80:20). $[\alpha]_D$ +86° (*c* 0.1 in methanol). IR (KBr): $\nu(cm^{-1})$: 2100 (N_3), 1732 (C=O ester). ^1H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.89$ (s, 3H, 18-Me), 1.07 (s, 3H, 19-Me), 2.05 (s, 3H, 3-CH_3COO), 3.40 (m, 1H, 7α-H), 3.83–3.96 (m, 4H, –$O(CH_2)_2O$–), 4.61 (m, 1H, 3-H), 5.35 (s, 1H, 6-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): $\delta = 14.3$ (C-18), 19.2 (C-19), 20.5, 21.3 (3-OCO*C*H_3), 24.1, 27.6, 30.0, 35.2, 36.5, 36.6, 37.0, 37.6, 46.2, 48.4, 49.8, 63.8, 64.5 and 65.2 (–$O(CH_2)_2O$–), 73.1 (C-3), 118.8 (C-17), 121.2 (C-6), 145.2 (C-5), 170.4 (3-O*C*OCH$_3$) ppm. MS (30 eV, EI): *m*/*z* (%) = 373 (15) [$M^+ - N_3\cdot$], 372 (30) [$M^+ - CH_3CO\cdot$], 314 (24), 255 (100), 240 (63), 99 (86). ESI-MS: *m*/*z* = 438.2 $[M+Na]^+$.

4.1.6. 7α-Amino-17,17-ethylenedioxy-androst-5-en-3β-ol (6). Azide **4** (0.40 g, 0.96 mmol) and nickel(II) chloride hexahydrate (0.23 g, 0.96 mmol) were dissolved in anhydrous THF (40 mL) under argon atmosphere. The solution was stirred at room temperature for 30 min. Lithium aluminium hydride (0.37 g, 9.6 mmol) was then added to the solution and the mixture was refluxed for 6 h, cooled and quenched by dropwise addition of water (20 mL). The product was diluted with 2 M aqueous sodium hydroxide solution (100 mL) and extracted with dichloromethane (4× 50 mL). The combined dichloromethane extracts were washed with water (2× 30 mL), dried over calcium chloride and magnesium sulfate and filtered. Removal of the solvent in vacuo and purification by chromatography (silica gel, dichloromethane/methanol, 90:10 then 80:20) afforded a pure product **6** (0.13 g, 39%) as a beige solid. Mp > 260 °C; $R_f = 0.22$ (dichloromethane/methanol, 90:10). $[\alpha]_D$ −193° (*c* 0.1 in methanol). IR (KBr): $\nu(cm^{-1})$: 3386 and 3295 (O–H and N–H). ^1H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.88$ (s, 3H, 18-Me), 1.01 (s, 3H, 19-Me), 3.09 (ddd, $^3J_{7\beta\text{-}8} = 4.8$ Hz, $^3J_{7\beta\text{-}6} = 4.8$ Hz, $^5J_{7\beta\text{-}4} = 2.0$ Hz, 1H, 7β-H), 3.55 (m, 1H, 3-H), 3.84–3.96 (m, 4H, –$O(CH_2)_2O$–), 5.54 (dd, $^3J_{6\text{-}7\beta} = 5.1$ Hz, $^4J_{6\text{-}4} = 1.5$ Hz, 1H, 6-H) ppm. ^{13}C NMR (100 MHz, CD_3OD, 25 °C): $\delta = 14.5$ (C-18), 19.2 (C-19), 21.2, 23.7, 31.3, 32.1, 35.0, 38.3, 38.4, 38.6, 42.9, 43.0, 45.1, 46.8, 47.7, 65.6 and 66.2 (–$O(CH_2)_2O$–), 72.1 (C-3), 120.4 (C-17), 125.6 (C-6), 145.5 (C-5) ppm. MS (30 eV, EI): *m*/*z* (%) = 357 (2) [M^+], 235 (5), 100 (84), 85 (100). HRMS-EI (20 eV): *m*/*z* [M^+] Calcd for $C_{21}H_{33}NO_3$: 347.2460. Found: 347.2464.

4.1.7. 7β-Amino-17,17-ethylenedioxy-androst-5-en-3β-ol (7). Azide **5** (0.8 g, 1.93 mmol) was reduced as described for compound **6** to yield **7** (0.47 g, 70%) as beige crystals. Mp 176 °C; $R_f = 0.20$ (dichloromethane/methanol, 90:10 then 80:20). $[\alpha]_D$ +189° (*c* 0.1 in methanol). IR (KBr): $\nu(cm^{-1})$: 3386 and 3310 (O–H and N–H). ^1H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.84$ (s, 3H, 18-Me), 0.98 (s, 3H, 19-Me), 3.09 (d, $^3J_{7\alpha\text{-}8} = 8.0$ Hz, 1H, 7α-H), 3.47 (m, 1H, 3-H), 3.80–3.92 (m, 4H, –$O(CH_2)_2O$–), 5.11 (s, 1H, 6-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): $\delta = 14.3$ (C-18), 19.3 (C-19), 20.5, 25.2, 30.1, 31.5, 35.2, 36.1, 37.0, 41.8, 42.0, 46.3, 49.0, 50.2, 53.8, 64.4 and 65.1 (–$O(CH_2)_2O$–), 70.9 (C-3), 118.6 (C-17), 126.4 (C-6), 142.1 (C-5) ppm. MS (40 eV, EI): *m*/*z* (%) = 357 (14) [M^+], 315 (11), 235 (24), 99 (100). HRMS-EI (20 eV): *m*/*z* [M^+] Calcd for $C_{21}H_{33}NO_3$: 347.2460. Found: 347.2449.

4.1.8. 7α-Amino-3β-hydroxy-androst-5-en-17-one (I). Five percent aqueous HCl solution (5 mL) was added to a solution of compound **6** (0.05 g, 0.13 mmol) in THF (5 mL). The mixture was stirred for 24 h at room temperature. The solution was cooled to 0 °C and treated with 10% $NaHCO_3$ (10 mL) and extracted with chloroform (4× 10 mL). The organic layer was washed with brine, dried over anhydrous sodium sulfate and evaporated. The crude product was purified by chromatography (silica gel, dichloromethane/methanol/triethylamine, 80:18:2) to afford 0.048 g (95%) of **I** as an amorphous solid. $R_f = 0.21$ (dichloromethane/methanol, 80:20). $[\alpha]_D$ −193° (*c* 0.1 in methanol). IR (KBr): $\nu(cm^{-1})$: 3380 and 3154 (O–H and N–H), 1738 (C=O ketone). ^1H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.89$ (s, 3H, 18-Me), 1.02 (s, 3H, 19-Me), 3.19 (s, 1H, 7β-H), 3.55 (m, 1H, 3-H), 5.54 (d, $^3J_{6\text{-}7\beta} = 5.4$ Hz, 1H, 6-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): $\delta = 13.3$ (C-18), 18.6 (C-19), 20.0, 22.1, 31.1, 31.4, 35.6, 36.6, 37.2, 37.5, 42.0, 42.1, 45.4, 46.0, 47.1, 71.3 (C-3), 125.3 (C-6), 142.8 (C-5), 220.4 (C-17) ppm. MS (30 eV, EI): *m*/*z* (%) = 303 (57) [M^+], 286 (57), 150 (80), 83 (100). HRMS-EI (20 eV): *m*/*z* [M^+] Calcd for $C_{19}H_{29}NO_2$: 303.2198. Found: 303.2185.

4.1.9. 7β-Amino-3β-hydroxy-androst-5-en-17-one (II). Compound **7** (0.35 g, 1 mmol) was deprotected in the same manner as **6** to yield product **II** (0.28 g, 92%) as an amorphous solid. $R_f = 0.20$ (dichloromethane/methanol, 80:20). $[\alpha]_D$ +191° (*c* 0.1 in methanol). IR (KBr): $\nu(cm^{-1})$: 3385 and 3256 (O–H and N–H), 1742 (C=O ketone). ^1H NMR (400 MHz, $CDCl_3$, 25 °C): $\delta = 0.84$

(s, 3H, 18-Me), 1.00 (s, 3H, 19-Me), 3.20 (m, 1H, 7α-H), 3.46 (m, 1H, 3-H), 5.16 (s, 1H, 6-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 13.6 (C-18), 19.1 (C-19), 20.5, 20.9, 24.0, 29.6, 31.2, 31.2, 35.8, 36.3, 36.9, 41.6, 48.0, 51.3, 60.4, 70.8 (C-3), 125.3 (C-5), 142.8 (C-6), 220.4 (C-17) ppm. MS (30 eV, EI): *m/z* (%) = 303 (17) [M^+], 270 (31), 99 (85), 83 (100). HRMS-EI (20 eV): *m/z* [M^+] Calcd for $C_{19}H_{29}NO_2$: 303.2198. Found: 303.2213.

4.1.10. 3β-Acetoxy-7α,β-azido-androst-5-en-17-one (8). Trimethylsilyl azide (8.0 mL, 60.5 mmol) was added dropwise to a solution of **1** (2 g, 6.1 mmol) and lead(IV) acetate (5.37 g, 12.1 mmol) in methylene chloride (20 mL). The mixture was stirred for 2 h at room temperature. The solution was diluted with water and the lead(II) azide precipitate was removed by filtration and decomposed with sodium nitrite/dilute hydrochloric acid. The organic layer was dried over sodium sulfate and evaporated. The crude product was purified by chromatography (cyclohexane/ethyl acetate, 8:2) to give 1.6 g (70% of epimeric mixture: α epimer 70% and β epimer 30%) of **8** as a white amorphous solid. IR: *v*: 2095 (N_3), 1738 (C=O ester), 1725 (C=O ketone). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.86 (s, 3H, 18-Me), 1.02 (s, 3H, 19-Me), 2.16 (s, 3H, 3-CH_3COO), 3.59 (d, $^3J_{7\alpha\text{-}8}$ = 9.4 Hz, 0.3H, 7α-H of azide β epimer), 3.73 (m, 0.7H, 7β-H of azide α epimer), 4.65 (m, 1H, 3-H), 5.35 (s, 0.3H, 6-H of azide β epimer), 5.55 (dd, $^3J_{6\text{-}7\beta}$ = 5.1 Hz and $^3J_{6\text{-}4}$ = 1.4 Hz, 0.7H, 6-H of azide α epimer) ppm.

4.1.11. Androst-5-en-3β-ol (9). Hydrazine hydrate (2.16 mL, 69 mmol) was added to a solution of dehydroepiandrosterone (2 g, 7 mmol) in ethylene glycol (20 mL) and heated to 100 °C for 2 h. Potassium hydroxide (4.2 g, 75 mmol) was added to the stirred solution and stirring was continued with no external heating over 24 h. The solution was cooled to 0 °C. The precipitate was filtered and dissolved in chloroform (30 mL). The organic phase was washed with 1 M HCl (3× 15 mL), 10% $NaHCO_3$ (1× 15 mL), brine and dried over calcium chloride and magnesium sulfate. The solution was evaporated and the crude product was purified by chromatography (silica gel, cyclohexane/ethyl acetate, 80:20) to afford compound **9** as a white solid (1.39 g, 74%). Mp 136 °C (lit.[45] 133–134 °C); R_f = 0.23 (cyclohexane/ethyl acetate, 70:30). IR (KBr): *v*(cm^{-1}): 3246 (O–H). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.72 (s, 3H, 18-Me), 1.02 (s, 3H, 19-Me), 3.53 (m, 1H, 3-H), 5.36 (d, $^3J_{6\text{-}7\beta}$ = 6.6 Hz, 1H, 6-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 17.2 (C-18), 19.4 (C-19), 20.5, 21.1, 25.6, 31.7, 32.1, 32.1, 36.6, 37.3, 38.7, 40.3, 40.6, 42.3, 50.4, 54.8, 71.8 (C-3), 121.7 (C-6), 140.7 (C-5) ppm. MS (30 eV, EI): *m/z* (%) = 274 (100) [M^+], 256 (63), 241 (74), 189 (64), 163 (65). Anal. Calcd for $C_{19}H_{30}O$ (274.45): C, 83.15; H, 11.02. Found: C, 83.37; H, 11.16.

4.1.12. 3β-Acetoxy-androst-5-ene (10). Using the same method described for compound **1**, compound **9** (1.3 g, 4.7 mmol) was converted to **10** (1.14 g, 88%) as a white powder. Mp 98 °C; R_f = 0.64 (cyclohexane/ethyl acetate, 70:30). IR (KBr): *v*(cm^{-1}): 1732 (C=O ester). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.72 (s, 3H, 18-Me), 1.03 (s, 3H, 19-Me), 2.04 (s, 3H, 3-CH_3COO), 4.60 (m, 1H, 3-H), 5.38 (d, $^3J_{6\text{-}7\beta}$ = 4.9 Hz, 1H, 6-H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 17.2 (C-18), 19.3 (C-19), 20.5, 21.1 (3-OCO*C*H_3), 21.4, 25.6, 27.8, 32.1, 32.2, 36.7, 37.1, 38.1, 38.6, 40.2, 40.6, 50.3, 54.8, 73.9 (C-3), 122.6 (C-6), 139.6 (C-5), 170.5 (3-O*C*OCH_3) ppm. MS (30 eV, EI): *m/z* (%) = 316 (1) [M^+], 256 (100) [M^+−AcOH], 241 (16), 148 (23), 135 (21). Anal. Calcd for $C_{21}H_{32}O_2$ (316.49): C, 79.70; H, 10.19. Found: C, 79.98; H, 10.33.

4.1.13. 3β-Acetoxy-7α,β-azido-androst-5-ene (12). Compound **12** was obtained from compound **10** (0.5 g, 1.6 mmol) via **11** as described for the preparation of compounds **4** and **5** to yield (0.42 g, 75%) azide epimers mixture (7α/β: 15/85 ratio, respectively) inseparable by chromatography (silica gel, cyclohexane/ethyl acetate, 95:5) as a colourless oil. R_f = 0.60 (cyclohexane/ethyl acetate, 70:30). IR (KBr): *v*(cm^{-1}): 2094 (N_3), 1735 (C=O ester). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.71 (s, 3H, 18-Me), 1.08 (s, 3H, 19-Me), 2.04 (s, 3H, 3-CH_3COO), 3.37 (d, $^3J_{7\alpha\text{-}8}$ = 9.0 Hz, 0.85H, 7α-H), 3.61 (m, 0.15H, 7β-H), 4.61 (m, 1H, 3-H), 5.36 (s, 0.85H, 6-H of azide β epimer), 5.57 (d, $^3J_{6\text{-}7\beta}$ = 5.1 Hz, 0.15H, 6-H of azide α epimer) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 16.9, 17.3, 18.5, 19.2 (C-18 and C-19), 20.5, 20.8, 21.1 and 21.3 (*C*H_3COO), 25.3, 25.9, 26.8, 27.1, 27.4, 27.6, 30.1, 30.4, 32.8, 33.2, 36.6, 36.7, 37.0, 37.2, 37.5, 37.6, 37.9, 38.3, 40.4, 41.1, 48.6 and 48.8 (C-7), 53.9, 58.5, 64.1, 64.2, 73.1 and 73.6 (C-3), 119.1 and 121.3 (C-6), 145.2 and 147.3 (C-5), 170.3 and 170.4 (3-CH_3*C*OO) ppm. MS (30 eV, EI): *m/z* (%) = 269 (9) [M^+−AcOH, –N_2], 255 (56) [M^+−AcOH, –N_3·], 186 (7), 159 (18), 133 (36), 108 (85), 95 (67), 82 (100). ESI-MS: *m/z* = 480.4 $[M+Na]^+$.

4.1.14. 7α,β-Amino-androst-5-en-3β-ol (III). Epimeric azide mixture **12** (0.38 g, 1.1 mmol) was reduced in the same manner as compound **6** to yield **III** (0.12 g, 31%) after purification by chromatography (silica gel, dichloromethane/methanol, 90:10) as an amorphous solid. R_f = 0.19 (dichloromethane/methanol, 90:10). IR (KBr): *v*: 3362 and 3280 (O–H and N–H). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ = 0.73 (s, 3H, 18-Me), 1.03 (s, 3H, 19-Me), 3.01 (ddd, $^3J_{7\alpha\text{-}8}$ = 7.8 Hz, $^3J_{7\alpha\text{-}6}$ = 2.1 Hz, $^5J_{7\alpha\text{-}4}$ < 1 Hz, 0.55H, 7α-H), 3.10 (ddd, $^3J_{7\beta\text{-}8}$ = 5.1 Hz, $^3J_{7\beta\text{-}6}$ = 5.0 Hz, $^5J_{7\beta\text{-}4}$ = 1.5 Hz, 0.45H, 7β-H), 3.53 (m, 1H, 3-H), 5.18 (dd, $^3J_{6\text{-}7\alpha}$ = 2.1 Hz, $^4J_{6\text{-}4}$ < 1 Hz, 0.55H, 6-H of amine β epimer), 5.52 (dd, $^3J_{6\text{-}7\beta}$ = 5.0 Hz, $^4J_{6\text{-}4}$ < 1 Hz, 0.45H, 6-H of amine α epimer) ppm. ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ = 17.0, 17.4, 18.7, 19.4 (C-18 and C-19), 20.5, 20.8, 25.9, 27.1, 27.6, 27.9, 30.1, 30.6, 31.4, 31.7, 36.3, 37.2, 37.4, 37.5, 38.2, 38.7, 39.7, 40.1, 40.5, 41.4, 42.0, 42.1, 42.2, 42.5, 47.4 and 48.1 (C-7), 54.1, 54.4, 71.3 and 71.4 (C-3), 125.9 and 126.9 (C-6), 141.9 and 143.1 (C-5) ppm. MS (30 eV, EI): *m/z* (%) = 289 (48) [M^+], 272 (27) [M^+−NH_3], 177 (100), 136 (38). HRMS-EI (20 eV): *m/z* [M^+] Calcd for $C_{19}H_{31}NO$: 289.2406. Found: 289.2402.

4.2. Biology

4.2.1. Animals. Seventy-day-old male Sprague–Dawley rats, obtained from our own colony, were maintained on a 12 h light/dark cycle at 20–22 °C. Food and water were provided ad libitum.

4.2.2. Isolation and purification of Leydig cells. Rats were killed and the testes removed, decapsuled and placed in DMEM/Ham F12 medium (1:1; v/v). The crude interstitial cells were isolated following a collagenase/dispase (0.05%) treatment in the presence of soybean trypsin inhibitor (STI) (0.005%) and DNase (0.001%). The interstitial cells were separated from the seminiferous tubular pellet by filtration through a nylon mesh. The Leydig cells were purified on discontinuous (20–80%) Percoll gradients as described previously.[41] Leydig cell fractions were collected, washed with medium and their purity was analyzed by histochemical staining for the specific 3β-hydroxysteroid dehydrogenase (3β-HSD positive cells).[46] Leydig cells' viability was evaluated by Trypan blue test. Purity and cell viability were, respectively, around 90% and 95%.

4.2.3. Treatment of Leydig cells. Cells were incubated for 24 h at a density of 3×10^5 cells/mL of medium under an atmosphere of 95% air-5% CO_2. DHEA and DHEA analogues were dissolved in ethanol before being added to cell culture media. Then, the Leydig cells were treated during 24 h with 0, 35 and 70 μM of DHEA or DHEA analogues (0.007% ethanol in culture media).

4.2.4. Culture and treatment of TM4 cells. Cells were incubated for 24 h at a density of 10^5 cells/mL of DMEM/Ham F12 medium (1:1; v/v) with horse serum (5%), foetal calf serum (2.5%) and glutamine (0.2%) under an atmosphere of 95% air-5% CO_2. Then, TM4 cells were treated during 24 h with 0, 35 and 70 μM of DHEA or DHEA analogues.

4.2.5. In situ analysis of viability

4.2.5.1. Trypan blue test. At the end of the culture, cells were incubated for 5 min in presence of 10 μL of 0.5% Trypan blue. Presence of holes in the cell membrane of necrotic cells allows penetration of the colouring (Apoptotic cells have membrane integrity, so Trypan blue cannot penetrate inside). Observation of blue cells with a microscope revealed necrotic cells and percentage of necrosis.

4.2.5.2. DAPI test. At the end of the incubation, the medium was removed and cells were fixed in absolute ethanol/chloroform/acetic acid (6:3:1; v/v/v) for one day at −20 °C. Each well was washed with phosphate-buffered saline (PBS, pH 7.4) and incubated with 1 μg/mL of DAPI solution. The morphology of the cells' nuclei was observed using a fluorescence microscope at 350 nm excitation wavelength. Labelled DNA of viable cells scattered throughout the nucleus. Apoptotic nuclei can be identified by the condensed chromatin gathering at the periphery of the nuclear membrane or a total fragmented morphology of nuclear bodies. The percentage of cells with apoptotic nuclei was determined.

4.2.6. Statistical analysis. Data are presented as means ± SD of triplicate determinations performed in three different experiments. Student's *t*-test was used to compare the effects of DHEA or DHEA analogues on viability of Leydig cells or TM4 Sertoli cell line. Significance was assumed at $p < 0.05$ (*) $p < 0.01$ (**) or $p < 0.001$ (***).

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