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Novel Dehydroepiandrosterone Derivatives with Antiapoptotic, Neuroprotective Activity

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DHEA analogues with modifications at positions C3 or C17 were synthesized and evaluated for neuroprotective activity against the neural-crest-derived PC12 cell model of serum deprivation-induced apoptosis. The most potent compounds were the spiro-epoxy derivatives 17β-spiro[5-androstene-17, 2′-oxiran]-3β-ol (**20**), (20*S*)-3β,21-dihydroxy-17β,20-epoxy-5-pregnene (**23**), and (20*R*)-3β,21-dihydroxy-17α,20-epoxy-5-pregnene (**27**) with IC_{50} values of 0.19 ± 0.01, 99.0 ± 4.6, and 6.4 ± 0.3 nM, respectively. Analogues **20**, **23**, and **27**, up to the micromolar range of concentrations, were unable to activate estrogen receptor α and β (ERα and ERβ) or to interfere with ER-dependent gene expression significantly. In addition, they were unable to stimulate the growth of Ishikawa, MCF-7, and LNCaP cells. Our results suggest that the spiro-epoxyneurosteroid derivatives **20**, **23**, and **27** may prove to be lead molecules for the synthesis of novel neuroprotective agents.

Introduction

Neuronal cell death by apoptosis is the "end point" of many human neurological disorders, including Alzheimer's, Parkinson's, and Huntington's diseases, stroke/trauma, and multiple and amyotrophic lateral sclerosis.[1,2] Apoptotic death of hippocampal and cortical neurons is responsible for the symptoms of Alzheimer's disease, while death of midbrain neurons that use the neurotransmitter dopamine underlies Parkinson's disease. Huntington's disease involves the death of neurons in the striatum, which control body movements, and death of lower motor neurons manifests as amyotrophic lateral sclerosis. Additionally, brain ischemia and trauma induce necrosis of a small brain area, which then propagates neuronal cell loss by apoptosis to a larger brain area, due to the neurotoxic material released by the necrotic cells. Apoptotic neuronal cell loss is also observed in the aging brain, as a physiological process.[1] Currently, there is little or no treatment for most neurodegenerative diseases. At best, available treatments are symptomatic in nature and do not prevent or slow the progression of disease.[3] A fundamental approach for reducing the burden of neurodegenerative diseases is to slow or halt progression and, ultimately, to prevent the onset of the disease process. Thus, strategies for neuroprotection, preventing apoptotic neuronal cell loss may offer new therapeutic interventions.

Neurosteroids are synthesized in the central and peripheral nervous system, particularly in myelinating glial cells but also in astrocytes and neurons. Neuroactive steroids, which act on the nervous system,[4] affect neuronal function and differentiation[5] and provide for neuroprotection against ischemia and stroke.[6] Neuroprotective effects include recovery of motor function after spinal cord injury,[7] regulation of myelination,[8] proliferation of neuronal stem cells,[9,8f] neurogenesis in the hippocampus,[8f,9,10] and induction of analgesia.[11] Dehydroepiandrosterone (DHEA)[a] (Figure 1) and its sulfate ester (DHEAS) are produced in the brain as well as the adrenals,[12,13] with brain DHEA acting locally in a paracrine fashion and adrenal DHEA having a systemic role. DHEA is one of the most potent neuroactive neurosteroids, and the precipitous decline of both brain and circulating DHEA with advancing age has been associated to neuronal dysfunction and degeneration.[14] It is now experimentally supported that DHEA protects neurons from anoxia,[15] that it transiently increases NGF mRNA level,[16] that it exhibits neuroprotective properties in various types of CNS insults,[17] and that it induces a protective effect against spinal cord injury.[18]

Recent studies investigating the physiopathological significance of neurosteroids in Alzheimer's disease (AD) have shown a significant decline of neurosteroid concentrations in individual brain regions of AD patients compared to age-matched nondemented controls.[19,20] Additionally, a significant negative correlation was found between the levels of phosphorylated tau proteins and DHEAS in the hypothalamus.[20,21] These studies strongly suggest a possible neuroprotective role for

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[a] Abbreviations: DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate ester; ERα, estrogen receptor α; ERβ, estrogen receptor β; E2, estradiol; AD, Alzheimer's disease; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CSA, camphor-10-sulfonic acid; PCC, pyridinium chlorochromate; BSA, bovine serum albumin; PBS, phosphate-buffered saline; AlkP, alkaline phosphatase; mDBS, membrane DHEA-binding site(s); NGF, nerve growth factor; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NF-κB, nuclear factor κB; PMSF, phenylmethanesulfonyl fluoride; FBS, fetal bovine serum; CNS, central nervous system.

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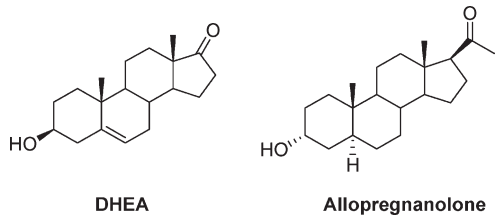


Figure 1. Structures of dehydroepiandrosterone (DHEA) and allopregnanolone.

endogenous neurosteroids in AD. It is also of interest that DHEA is able to potentiate locomotor activity of hemi-Parkinsonian monkeys, alleviating impairment symptoms of the moderately and severely affected MPTP-treated animals.[14b,22] In addition, DHEA affects the catecholaminergic system, upregulating the expression of tyrosine hydroxylase and, thus, increasing catecholamine *de novo* synthesis and release.[23]

The adult central nervous system (CNS) is classically known as a structure with very limited regenerative capacity. However, several pathological conditions, e.g., ischemia, epilepsy and trauma, have been shown to upregulate neural stem cell activity in the subventricular zone and the dentate gyrus. These findings suggest that signals are present throughout the adult brain, which allow limited neuronal regeneration to occur. This fundamental observation changes our view on neurodegeneration and the brain's regenerative capacity,[23] giving us the potential ability to regenerate specific brain areas. The neurosteroids DHEA and allopregnanolone (Figure 1) have recently been shown to induce neurogenesis in various experimental models.[9,24]

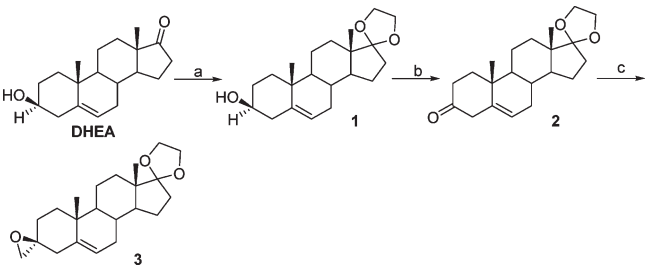
The lack of effective treatment for devastating neurodegenerative diseases has stimulated great interest in the development of neuroprotective means that can prevent or treat progressive loss of neural function. There is a sustained need for the development of new compounds for neural cell protection, repair, and rescue by targeting neural cell apoptosis and survival or neurogenesis. Natural neurosteroids such as DHEA possess important neuroprotective and neurogenic properties *in vitro* and *in vivo*, in experimental animals, as described above. However, naturally occurring neurosteroids are metabolized in humans into estrogens, androgens, or progestins which are known to exert important generalized endocrine side effects, including hormone-dependent neoplasias,[4d] thus limiting their clinical use.

As a continuation of our ongoing studies on neuroactive steroids[25,26] we were interested in obtaining structure–activity relationships for antiapoptotic–neuroprotective steroid derivatives. Thus, in the present report we describe the synthesis and activity of DHEA analogues modified at positions C3 or C17 with the aim of improving their antiapoptotic–neuroprotective activity and inhibiting conversion to estrogens or androgens. The neuroprotective activity was evaluated using the neural-crest-derived PC12 cell model, which has been extensively employed to study neuronal cell apoptosis and survival.

Chemistry

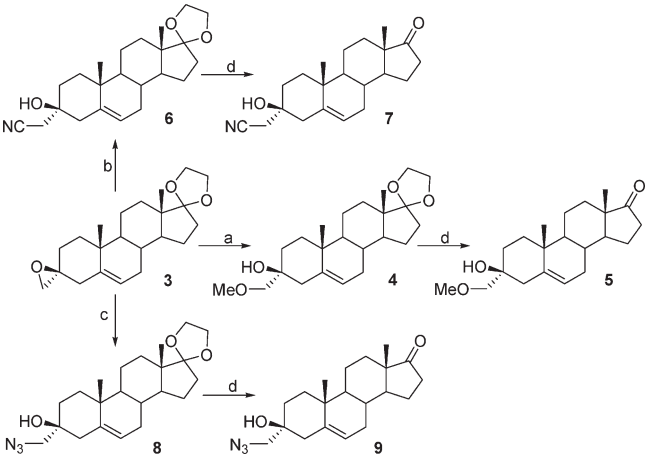
The synthetic strategy followed for the preparation of the neurosteroid analogues **5**, **7**, **9**, **11**, **14**, and **15** is depicted in Schemes 1, 2, and 3. Thus, protection of the 17-keto group of DHEA using ethylene glycol in the presence of CSA to yield the ketal **1**, followed by oxidation of the 3-hydroxy group in **1** using Dess–Martin periodinane in dichlomethane, afforded

Scheme 1[a]



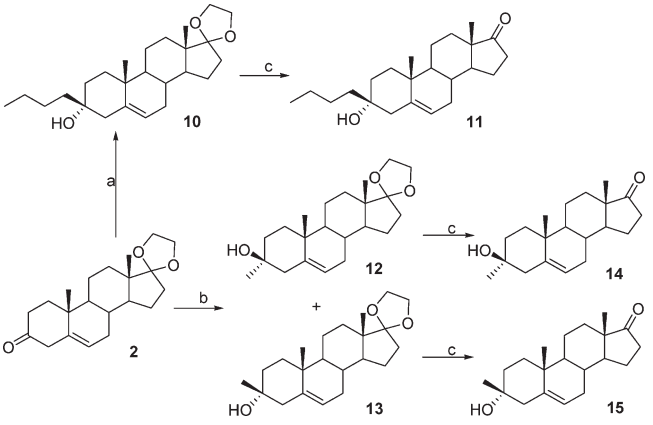
[a] Reagents and conditions: (a) ethylene glycol, CSA, cyclohexane, 80 °C; (b) Dess–Martin periodinane, DCM, room temperature; (c) *n*-BuLi, Me_3SI, THF, −40 °C.

Scheme 2[a]



[a] Reagents and conditions: (a) MeONa, MeOH, reflux; (b) KCN, EtOH, reflux; (c) NaN_3, NH_4Cl, MeOH/H_2O (8:1), reflux; (d) HCl/acetone (1:1), THF, room temperature.

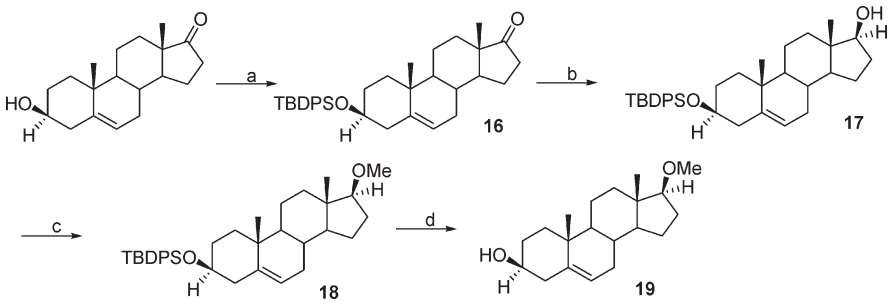
Scheme 3[a]



[a] Reagents and conditions: (a) *n*-BuLi, THF, room temperature; (b) MeLi, THF, room temperature; (c) HCl/acetone (1:1), THF, room temperature.

the unconjugated ketone **2** in 92% overall yield for the two steps. Compound **2** has been previously reported in 50% yield using PCC in the presence of Celite and KOAc.[27] Corey–Chaykovsky epoxidation of **2** using trimethylsulfonium iodide and *n*-BuLi afforded a mixture of the two C3 epimeric spiro-epoxides, which were separated by flash column chromatography to yield the desired epoxide **3**. The stereochemistry of C-3 of compound **3** and the position of the double

Scheme 4[a]



[a] Reagents and conditions: (a) TBDPSiCl, imidazole, DMF, 50 °C; (b) $LiAlH_4$, THF, room temperature; (c) NaH, MeI, THF, room temperature; (d) TBAF, THF, room temperature.

bond were determined by 1D and 2D NMR spectroscopy and more specifically from the correlations observed in the HMBC, COSY, and NOESY spectra (cf.Supporting Information). The peak of the vinylic proton in the ^{1}H NMR spectrum of **3** appears as a doublet (J = 5.4 Hz) at 5.30 ppm and shows correlations in the COSY spectrum with the H-7 protons (H-7α at 2.0 ppm and H-7β at 1.58 ppm) and the H-4α allylic proton at 1.62 ppm. These observations are in agreement with a C-5:C-6 double bond. The other allylic proton at 2.88 ppm also exhibited correlations with carbons C-3, C-10, C-5, C-6, and C-2 and the epoxide methylene which support its position at C-4. The NOE enhancement between the epoxide proton at 2.58 ppm and the allylic proton H-4α (1.62 ppm) and between the epoxide proton at 2.56 ppm and H-2α at 1.19 ppm are in agreement with a 3β-epoxide. Additional proof of the stereochemistry of carbon C-3 of compound **3** was obtained by reaction with $LiAlH_4$ to afford the corresponding 3β-hydroxy-3α-methyl derivative. The 3α-methyl signal in the ^{1}H NMR spectrum resonates at 1.09 ppm as expected from literature reports and is in accordance with the spectra of compound **12**.[28] Nucleophilic opening of epoxide **3** by NaOMe, NaN_3, or KCN afforded compounds **4**, **6**, and **8**, respectively. The vinylic proton H-6 appears as a doublet at 5.29 ppm (J = 5.49 Hz) in **4** and at 5.40 ppm (J = 5.49 Hz) in **6** and as dd at 5.33 ppm (J = 4.89, 2.46 Hz) in **8**, which are in agreement with 5-ene derivatives. Deprotection of the carbonyl group at C-17 of **4**, **6**, and **8** using a mixture of 10% HCl/acetone (1:1) in THF led to the neurosteroid analogues **5**, **7**, and **9**, respectively. Use of 2D NMR spectroscopy in compounds **5**, **7**, and **9** was in agreement with 3β-hydroxy-substituted 5-ene derivatives. In all three compounds we observed NOE enhancement between the H_2-1′ methylene and the proton H-4α (cf. Supporting Information).

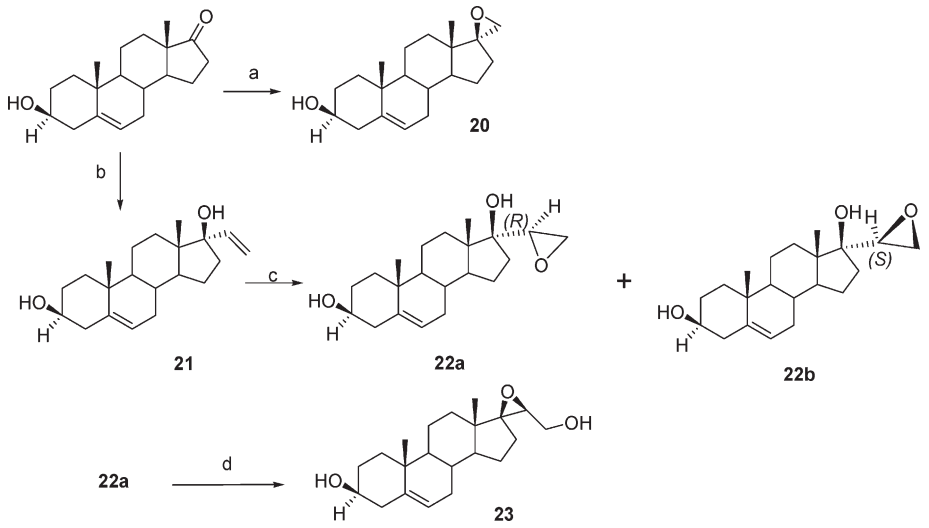
Addition of *n*-BuLi to ketone **2** yielded exclusively the 3α-hydroxy-3β-butyl derivative **10**, since the bulky *n*-BuLi approaches the C-3-carbonyl from the equatorial face of the steroid scaffold. We did not observe double bond isomerization to afford the 4-ene analogue as attested by the peak of the vinylic proton in the ^{1}H NMR spectrum of **10**, which appears as a doublet at 5.39 ppm (J = 5.49 Hz) and shows correlations in the COSY spectrum with the H-7 protons (H-7α at 2.0 ppm and H-7β at 1.60 ppm) and the H-4 allylic protons and NOE enhancement with the H-4α proton. Concerning the stereochemistry of C-3 in compound **10** it was deciphered through the synthesis of both epimers by addition of *n*-BuMgBr to ketone **2** (cf. Supporting Information for experimental procedure). Compound **10** can be differentiated from its C-3 epimer by TLC (silica gel normal phase), the 3β-substituted derivative being less polar (higher R_f value).[28] In addition to their characteristic chromatographic properties the stereoisomers were identified by the ^{13}C NMR signal of the tertiary alcohol at C-3, which is lower in parts per million for the 3β-isomer (axial 3α-OH) than the 3α isomer (equatorial 3β-OH), as has been observed previously.[28] Thus, the C-3 carbon of **10** resonates at 71.9 ppm while its C-3 epimer resonates at 73.4 ppm (cf. Supporting Information). Finally, NOE enhancement between the H_2-1′ and the H_2-2′ protons of the 3β-butyl substituent in **10** and protons H-2α, H-2β, H-4α, and H-4β is in agreement with a 3α-hydroxy-3β-butyl derivative. Conversely, NOE enhancement between the protons H_2-1′ and H-1α, H_2-2′ and H-6, and H_2-3′ and H-6 in the C-3 epimer of **10** supports a 3α-butyl substituent (cf. Supporting Information). Deprotection of the carbonyl group at C-17 in **10** using a mixture of 10% HCl/acetone (1:1) in THF gave analogue **11** (Scheme 3).

Addition of MeLi to the C-3-keto group of **2** afforded the two diasteromeric alcohols **12** and **13** in a 1:2 ratio, which were separated by column chromatography, since the 3α-hydroxy derivative **13** (axial hydroxyl) is less polar than the 3β-congener **12** (equatorial hydroxyl).[28] The position of the double bond in **12** and **13** is at C-5:C-6 as indicated by the resonance of the vinylic proton in the ^{1}H NMR spectra which appears as a doublet at 5.30 ppm (J = 5.49 Hz) for **12** and at 5.39 ppm (J = 5.49) for **13**. Deprotection of the carbonyl group at C-17 in alcohols **12** and **13**, using a mixture of 10% HCl/acetone (1:1) in THF, afforded analogues **14** and **15**, respectively (Scheme 3). Spectroscopic characterization of compounds **14** and **15** using 1D and 2D NMR spectroscopy was in agreement with 5-ene derivatives. In addition, the C-3 carbon of **14** resonates at 72.1 ppm (equatorial hydroxyl) while of its C-3 epimer **15** resonates at 70.1 ppm (axial hydroxyl).[28] The 3α-methyl substitution for **14** was also supported by the observed NOE correlations between the methyl protons H_3-1′ and protons H-2α and H-4α. Conversely, the 3β-methyl substitution for **15** was supported by the observed NOE correlations between the methyl protons H_3-1′ and protons H-2α, H-2β, H-4α, and H-4β (cf. Supporting Information).

The synthesis of the 17β-methoxy derivative **19** is described in Scheme 4. Thus, protection of the C3-hydroxyl group as the *tert*-butyldiphenylsilyl ether[29] followed by reduction of the ketone at C17 using $LiAlH_4$ yielded the 17β-hydroxy derivative **16**, which was subsequently alkylated with MeI using NaH as base, to give the methyl ether **18**. Finally, removal of the silyl protecting group using TBAF afforded analogue **19**.

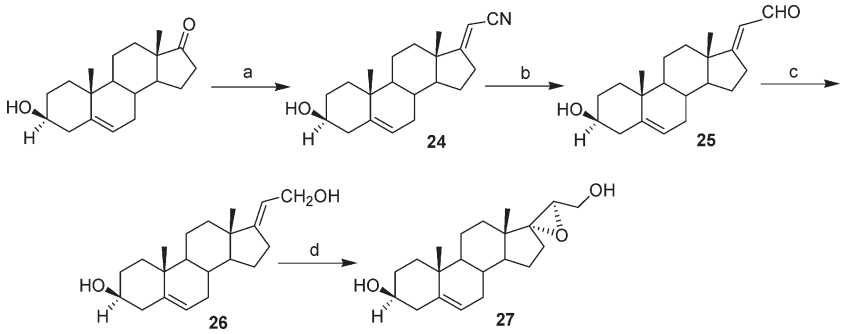
The synthetic route followed for the preparation of derivatives **20** and **23** is depicted in Scheme 5. Epoxidation of the 17-keto group in DHEA using trimethylsulfonium methylide

Scheme 5[a]



[a] Reagents and conditions: (a) *t*-BuOK, $(CH_3)_3SI$, DMF, room temperature; (b) vinyl MgBr, THF, −78 °C to room temperature; (c) $VO(acac)_2$, *t*-BuOOH, CH_2Cl_2, −10 °C; (iv) K_2CO_3, MeOH, room temperature.

Scheme 6[a]



[a] Reagents and conditions: (a) *t*-BuOK, $(EtO)_2P(O)CH_2CN$, THF, 0 °C to room temperature; (b) DIBAL-H, CH_2Cl_2, −78 °C; (c) $NaBH_4$, $CeCl_3 \cdot 7H_2O$, MeOH, room temperature; (d) K_2CO_3, *m*-CPBA, CH_2Cl_2, 0 °C.

afforded the 17,20-spiro-epoxide **20**.[30] The synthesis of epoxide **23** was effected by addition of vinylmagnesium bromide to the C17-keto group of DHEA to afford the quaternary alcohol **21**, followed by epoxidation using *t*-BuOOH in the presence of vanadyl acetonylacetonate as catalyst, to give a mixture of the (20*R*)- and (20*S*)-20,21-epoxides **22a** and **22b** (2:1), respectively. The assignment of the 20*R* configuration was based on previously reported analogous systems.[31] Payne rearrangement of the less hindered epoxide **22a** using K_2CO_3 in methanol afforded the desired epoxide **23**.[31] Derivative **27** was obtained following the synthetic strategy presented in Scheme 6. Horner−Emmons olefination of DHEA with diethyl cyanomethylphosphonate in the presence of *t*-BuOK afforded predominantly the (*E*)-17-cyanomethylene unsaturated nitrile **24** (ratio of **24** and (*Z*)-17-cyanomethylene nitrile (5:1)). Several reaction conditions were tried in order to obtain the *E* isomer selectively (i.e., different ratios of base and phosphonate reagent, temperature, or even performing the reaction on 3-acetyl-DHEA) but resulted either in equimolar formation of the two geometrical isomers or in favor of the undesired *Z* derivative. The previously reported Horner−Emmons olefination of DHEA using NaH as base in DME affords also a mixture of the *E* unsaturated nitrile **24** along with the *Z* isomer.[32] However, compound **24** could be obtained after purification by column chromatography. Treatment of **24** with DIBAL-H in dichloromethane at −78 °C followed by reduction of the resulting aldehyde **25**[33] using the Luche methodology[34] yielded the allylic alcohol **26**. Finally, epoxidation of **26** using *m*-CPBA in the presence of K_2CO_3 afforded the desired analogue **27** in 40% yield. Epoxidation using *t*-BuOOH in the presence of a catalytic amount of vanadyl acetonylacetonate afforded a mixture of the diastereomeric epoxides **27** and **23** in a 2:1 ratio and in 70% yield. However, epoxides **27** and **23** could not be separated by column chromatography.

Results and Discussion

The antiapoptotic activity of the new analogues was evaluated using the neural-crest-derived PC12 cell model, which has been extensively used to study neuronal cell apoptosis and survival. PC12 cells do not express functional $GABA_A$ or NMDA receptors and cannot metabolize DHEA to estrogens and androgens.[8g] Culture of PC12 cells in the absence of serum results in a strong induction of apoptosis, compared to cell cultures supplemented with serum. The antiapoptotic effect of the new compounds at 100 nM was evaluated using the APOPercentage apoptosis assay (Biocolor Ltd., Belfast, N. Ireland), and the results are shown in Figure 2.

More specifically, the new 3β-hydroxy-3α-substituted steroid derivatives **5**, **7**, **9**, and **14** were not capable of protecting

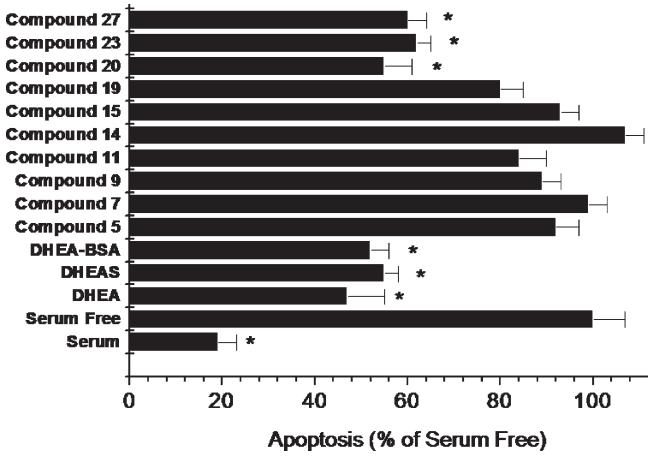


Figure 2. Effect of new neurosteroids against serum deprivation-induced apoptosis of PC12 cells. Cells were cultured for 24 h either in complete media (serum) or in serum-free media supplemented with 1% BSA in the presence of various steroids at 100 nM. Apoptosis was quantified by the APOPercentage assay, depicted as percentage of control (serum-free media in the absence of steroids). Values represent mean ± SEM of at least four independent experiments, each performed in triplicate (*, $p < 0.05$).

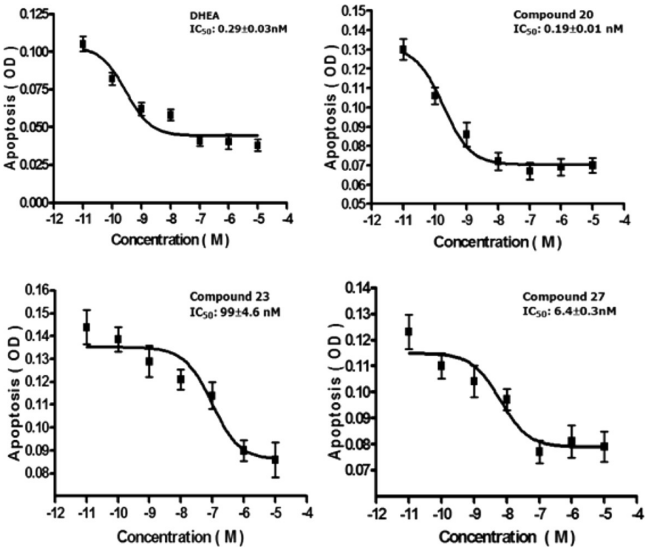


Figure 3. Analogues **20**, **23**, and **27** protected PC12 cells against serum deprivation-induced apoptosis in a dose-dependent manner. Cells were cultured for 24 h either in complete media (serum supplemented) or serum-free media supplemented with 1% BSA in the presence of **20**, **23**, or **27** at concentrations ranging from 10^{-11} to 10^{-5} M. Apoptosis was quantified by the APOPercentage assay, depicted as ratio to control (serum-free media in the absence of steroids). Values represent mean ± SEM of at least four independent experiments, each performed in triplicate.

PC12 cells from apoptosis. This was also observed for the 3α-hydroxy-3β-substituted analogues **11** and **15**. Replacement of the C17-ketone by a 17β-methoxy group in **19** again resulted in loss of antiapoptotic activity. Interestingly, however, 17,20-spiro-epoxide derivatives **20**, **23**, and **27** were capable of protecting neuronal cells against serum deprivation-induced apoptosis, with IC_{50} values of 0.19 ± 0.01, 99.0 ± 4.6, and 6.4 ± 0.3 nM, respectively (Figure 3). This effect at concentrations of 100 nM was also confirmed with flow cytometry (FACS) analysis of annexin V−propidium iodide-stained PC12 cells, as shown in Figure 4.

To further our understanding of the role of substitution of the steroid backbone in the antiapoptotic actions of neuroactive steroids, we also examined the effect of a series of 24 commercially available Δ^5 and Δ^4 androstenes at 100 nM concentration (Figure 5). The Δ^5 androstene series comprised DHEA analogues modified at C3, C7, C10, and C17. More specifically, replacement of the C3-hydroxy by a 3β-chlorine atom, inversion of the hydroxyl group at C3, or oxidation of the 3β-hydroxyl deprived the respective derivatives (3β-chloro-5-androstene-17-one, 3α-hydroxy-5-androstene-17-one, and 5-androstene-3,17-dione) from antiapoptotic activity. Introduction of a 7α-hydroxy or a 7β-hydroxy group in DHEA (3β,7α-dihydroxy-5-androsten-17-one and 3β,7β-dihydroxy-5-androsten-17-one, respectively) abolished the antiapoptotic activity of DHEA. The modifications at C17 included the replacement of the C17-keto group by a *N*-*O*-carboxymethyl oxime, by an ethylene ketal, by an electron-donating 17β-amino group, by a 17β-hydroxyl group, by a 17β-hydroxy-17α-ethynyl group, or by a 17β-hydroxy-17α-methyl group (3β-hydroxy-5-androstene-17-carboxymethyl oxime, 3β-hydroxy-5-androstene-17-ethylene ketal, 17β-amino-5-androsten-3β-ol, 5-androstene-3β-17β-diol, 5-androstene-3β-17α-diol, 17α-ethynyl-5-androstene-3β-17β-diol, and 17α-methyl-5-androstene-3β,17β-diol, respectively) abolished the antiapoptotic activity. Isomerization of the Δ^5 to the Δ^4 derivative 3β-hydroxy-4-androsten-17-one was detrimental to the antiapoptotic effect of DHEA, which was also the case for 5-androstene-3β,11β-diol-17-one, 5-androstene-3β,11α-diol-17-one, and 3β-hydroxy-5-androstene-11,17-dione. DHEA derivatives 5,16-androstadiene-17β-cyano-3β-ol, 5-androstene-3β-ol-16-one, and 5-androstene-16α-bromo-3β-ol-17-one were also inactive, which was also the case for testosterone or testosterone:BSA. Surprisingly, the 17β-carboxylic acid derivative, 5-androsten-3β-ol-17β-carboxylic acid, and the C17-unsubstituted derivative, 5,16-androsten-3β-ol, exhibited moderate antiapoptotic activity (Figure 5).

We further focused our attention on the active synthetic spiro-epoxides **20**, **23**, and **27** in an effort to obtain information on their mechanism of action in comparison to DHEA. It is evident from the previously described SAR that the requirements for neuroprotective activity of DHEA derivatives are very stringent, which may indicate that this effect is mediated through interaction with a specific receptor or enzyme. This is in line with our previous findings that the antiapoptotic effect of DHEA is mediated by specific G-protein-associated membrane binding sites, independent of NMDA or $GABA_A$ receptors.[35] Indeed, DHEA conjugated to bovine serum albumin (BSA), a molecule with no cell penetrating ability, was found to reverse serum deprivation-induced apoptosis by almost 50%, and to protect PC12 cells from apoptosis, with an apparent IC_{50} of 1.5 nM, i.e., in a manner similar to that of nonconjugated DHEA (1.8 nM).[35] Furthermore, DHEA:BSA effectively mimicked DHEA actions on antiapoptotic Bcl-2 proteins by preventing their downregulation by serum deprivation. Competition of [^{3}H]DHEA binding to isolated PC12 cell membranes using increasing concentrations of radioinert DHEA revealed neurosteroid high-affinity binding to membrane DHEA-binding site(s) (mDBS) with an apparent IC_{50} of 1.65 ± 0.2 nM. We were pleased to find that the active synthetic spiro-epoxides **20**, **23**, and **27** displaced [^{3}H]DHEA with apparent IC_{50} values of 0.04 ± 0.002, 0.11 ± 0.01, and 18.5 ± 2.1 nM, respectively, indicating a strong interaction of these compounds with mDBS (Figure 6).

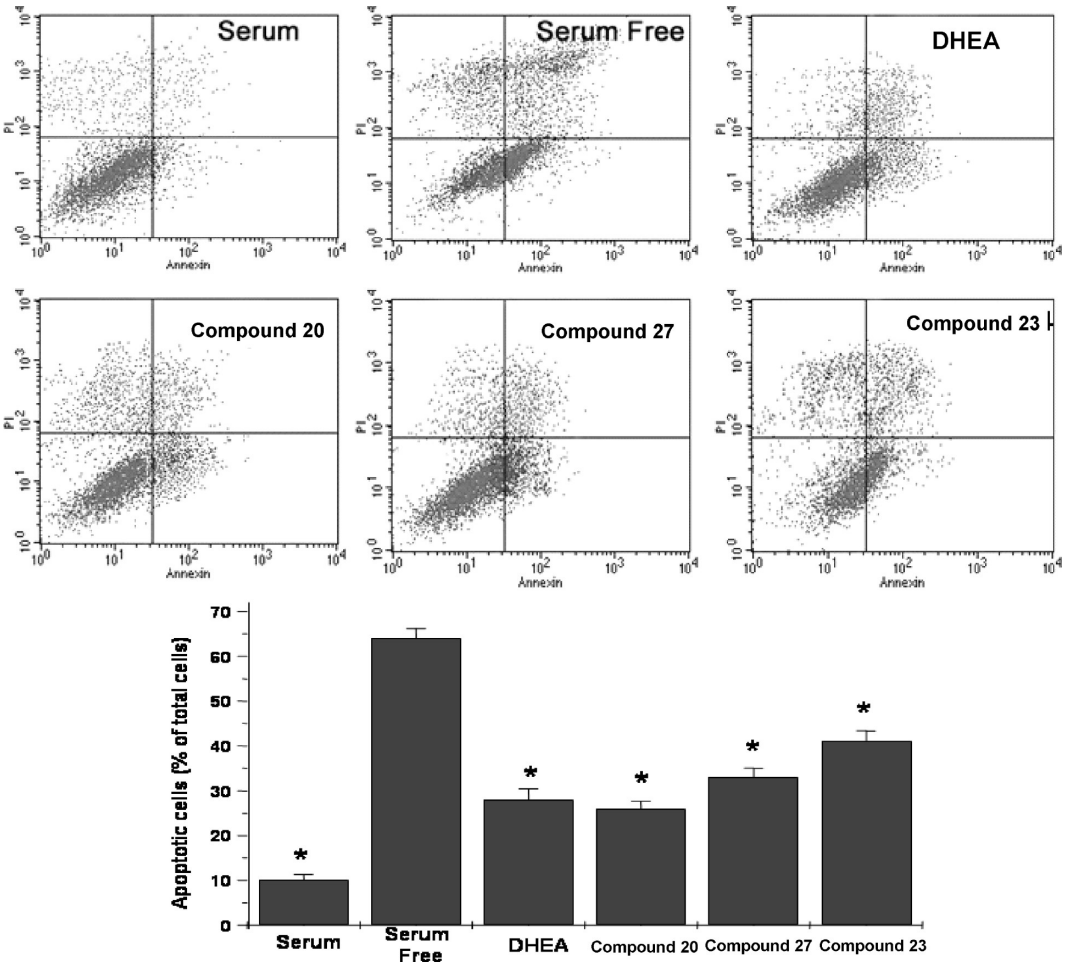


Figure 4. FACS analysis of the antiapoptotic effects of DHEA and compounds **20**, **23**, and **27**. PC12 cells were cultured in 24-well plates (10^6 cells per well) for 24 h either in complete media (serum supplemented) or in serum-free media (supplemented with 1% BSA) in the presence of DHEA, **20**, **23**, or **27** at 100 nM. The *X* axis represents annexin V−FITC while the *Y* axis represents the number of events. A total of 10000 cells were assigned per treatment. Values represent mean ± SEM of at least four independent experiments, each performed in triplicate (*, $p < 0.05$).

We recently reported that DHEA at low nanomolar concentrations (1 nM) protects PC12 cells against apoptosis, activating within minutes the prosurvival transcription factors NF-κB and CREB, two upstream effectors of antiapoptotic Bcl-2 proteins.[36] Serum deprivation of PC12 cells resulted in strong suppression of the levels of antiapoptotic Bcl-2 and Bcl-xL proteins. Analogues **20**, **23**, and **27**, in a similar fashion to DHEA, maintained antiapoptotic protein expression in serum-deprived PC12 cells to levels comparable to those of serum supplementation (Figure 7).

Furthermore, DHEA and DHEAS (at nanomolar concentrations) increase within 10 min the secretion of catecholamines from dopaminergic neural-crest-derived PC12 cells via actin filament disassembly and stimulate the expression of tyrosine hydroxylase, thus stimulating *de novo* synthesis of catecholamines.[23] PC12 cells were exposed to analogues **20** and **27** (10^{-7} M) or to DHEA for short periods of time (from 5 to 30 min), and the concentration of dopamine in the culture media was measured using a radioimmunoassay. We found that the spiro-neurosteroids provoked a fast and statistically significant 2-fold increase of dopamine secretion within 10 min (Figure 8A). We also tested the effect of analogues **20** and **27** for longer periods of time and, more specifically, for 3−48 h. We observed that incubation of PC12 cells with DHEAS or with analogues **20** and **27** resulted in an increase of dopamine levels peaking at 24 h (Figure 8B). These long-term effects of neurosteroids **20** and **27** suggest that these compounds, in addition to their acute effects on dopamine secretion (Figure 8A), may also affect the *de novo* production of dopamine in dopaminergic neurons like DHEAS. Indeed, neurosteroids **20** and **27** provoked a strong induction of the mRNA of the rate-limiting enzyme of dopamine biosynthesis, tyrosine hydroxylase (Figure 8C).

The risk to develop endocrine-related cancer, in particular breast, endometrial, or prostate cancer, is distinctly influenced by endogenous and exogenous hormones, since hormonal induction of cell proliferation has been associated with a higher probability of random mutation errors.[37] The use, for instance, of estrogen to alleviate postmenopausal syndromes is associated with a higher risk of developing breast or endometrial cancer.[38] Endometrial and prostate cancer cells are also highly responsive to estrogen,[39,40] and the risk to develop prostate cancer is reportedly dependent on estrogens as well as androgens.[37,41] It is therefore necessary to scrutinize potentially neuroprotective agents of steroidal structure for possible hormonal effects on the growth of breast, prostate, and uterine adenocarcinoma cells. Therefore, it was imperative to examine the active spiro-neurosteroids **20**, **23**, and **27** for such properties.

Initially, we evaluated the effects of compounds **20**, **23**, and **27** on the expression of ER-regulated genes. Agonism through ERα was assessed by way of induction of ERE-dependent

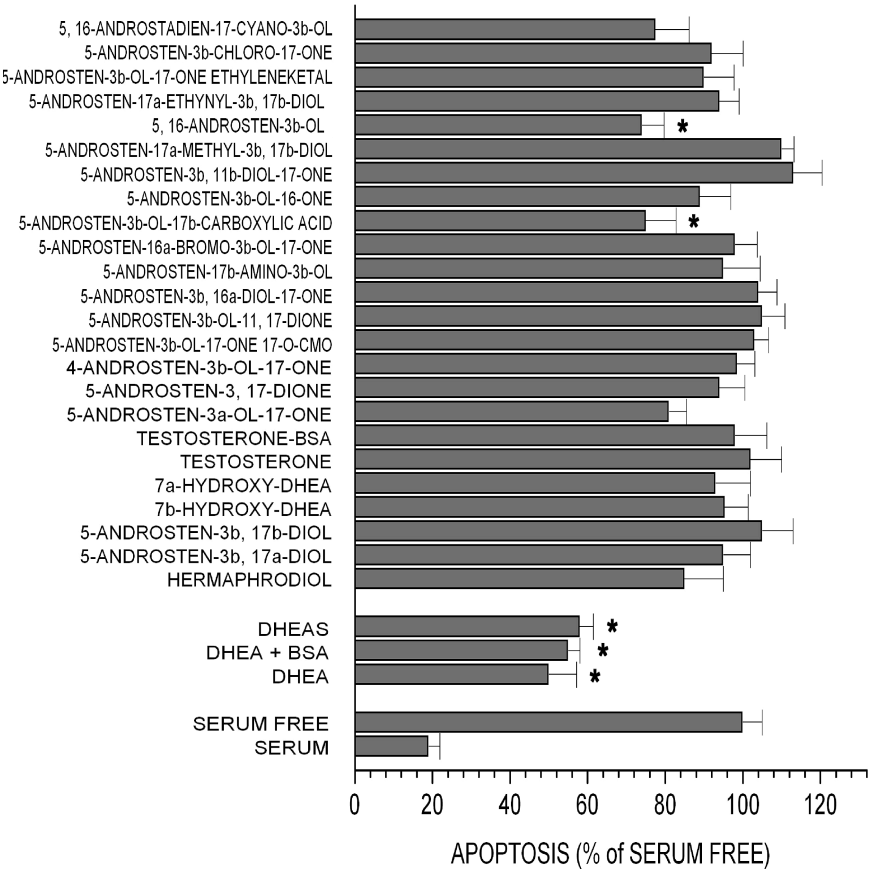


Figure 5. Effects of Δ^5 and Δ^4 androstenes on serum deprivation-induced apoptosis of PC12 cells. Cells were cultured for 24 h either in complete media (serum supplemented) or in serum-free media (supplemented with 1% BSA) in the presence of various steroids at 100 nM. Apoptosis was quantified by the APOPercentage assay, depicted as percentage of control (serum-free media in the absence of steroids). Values represent mean ± SEM of at least four independent experiments, each performed in triplicate (*, $p < 0.05$).

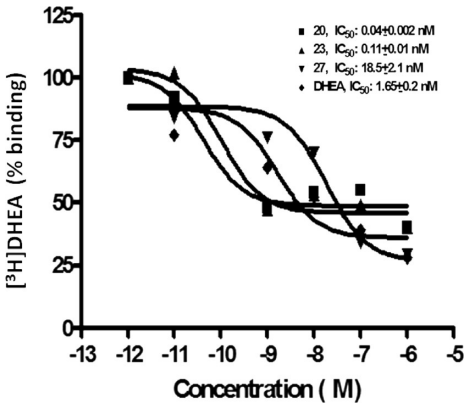


Figure 6. Displacement of [^{3}H]DHEA from membranes isolated from PC12 cells by DHEA and spiro-neurosteroids **20**, **23**, and **27**. Membranes (at a final concentration of 2 mg/mL) from PC12 cells were incubated in the presence of 5 nM [^{3}H]DHEA in the presence or absence of various concentrations of analogues **20**, **23**, and **27**, ranging from 10^{-12} to 10^{-6} M. Following a 30 min incubation in a water bath at 37 °C, membranes were collected on GF/B filters, prewet in 0.5% PEI solution at 4 °C. The filters were washed three times with ice-cold Tris-HCl, dried, and counted in a β-scintillation counter.

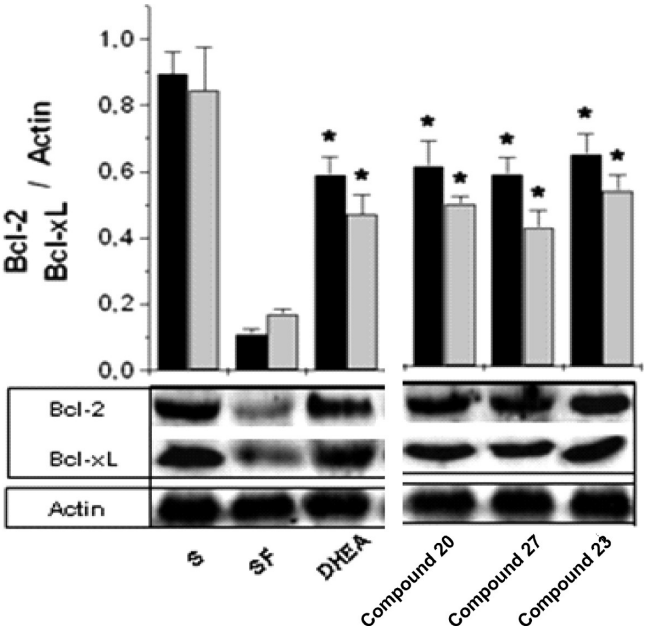


Figure 7. Induction of the expression of the antiapoptotic Bcl-2 proteins in serum-deprived PC12 cells by DHEA and compounds **20**, **23**, and **27**. Cells were cultured for 12 h either in complete or serum-free media containing 10 nM DHEA, **20**, **23**, or **27**. Cellular extracts containing total proteins were collected, and levels of Bcl-2 and Bcl-xL proteins were measured by Western blot, as described in the Experimental Section, and normalized per actin protein content. Values represent mean ± SEM of three independent experiments (*, $p < 0.05$).

luciferase gene expression in MCF-7:D5L cells. In accordance with previous findings[42,43] treatment of estrogen-free MCF-7:D5L cells with 1 nM estradiol results in full (4.2-fold) induction of luciferase, while treatment with **23** or **27** (1 μM) had no effect (Table 1, column 2). Treatment with analogue **20**

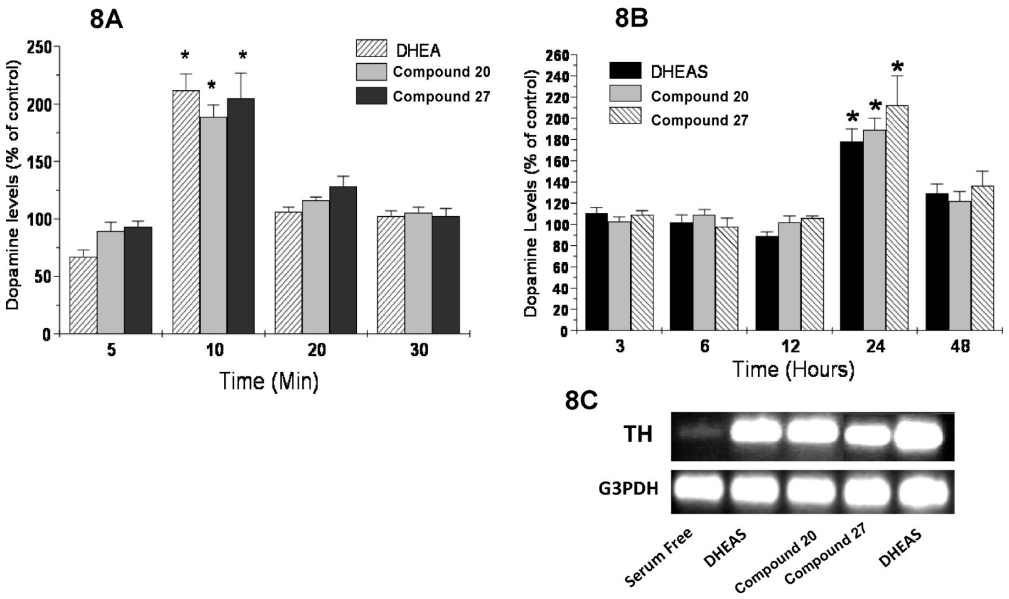


Figure 8. Effect of DHEAS and spiro-neurosteroids **20** and **27** on dopamine secretion and production. PC12 cells were exposed to 10 nM DHEAS, **20**, or **27** for 5−30 min (A) or for 3−48 h (B). At the end of the incubation, dopamine levels were measured in the culture media as described in the Experimental Section. Data are expressed as percentage of control, i.e., cells not exposed to the steroids. Values represent mean ± SEM of four independent experiments. (*, $p < 0.01$ vs control). (C) DHEAS, **20**, and **27** increased the mRNA levels of tyrosine hydroxylase (TH). PC12 cells were incubated for 6 h in the absence (control) or the presence of steroids at 10 nM, and the mRNA levels of TH were measured in cell extracts with RT-PCR analysis. Levels of the TH mRNA were compared to GAPDH mRNA levels.

Table 1. Regulation of the Expression of ER-Dependent Reporter Genes by Spiro-Neurosteroids **20**, **23**, and **27**[a]

	luciferase expression (MCF-7:D5L cells)		luciferase expression (HEK:ERβ cells)		alkaline phosphatase expression (Ishikawa cells)	
compd (1 μM)	ERα agonism (% of 1 nM E2)[b]	ERα antagonism (% of 1 μM ICI)[c]	ERβ agonism (% of 1 nM E2)[b]	ERβ antagonism (% of 1 μM ICI)[c]	ER agonism (% of 0.1 nM E2)[b]	ER antagonism (% of 1 μM ICI)[c]
20	weak (29 ± 1)	ns	ns	ns	weak (22 ± 4)	ns
23	ns	ns	ns	ns	ns	ns
27	ns	ns	ns	weak (31 ± 8)	ns	weak (24 ± 6)

[a] Statistically significant agonism or antagonism (mean ± SEM; $n \geq 3$) was classified as full, partial, or weak depending on whether it was 67−100%, 34−66%, and ≤33%, respectively, of the agonism of E2 or the antagonism of ICI 182,780; ns = nonsignificant. [b] Agonism, expressed as % of that of estradiol (E2), was calculated by (expression in the presence of neurosteroid − $E_{vehicle}$) × 100/(E_{E2} − $E_{vehicle}$). [c] Antagonism of the agonist effect of E2, expressed as % of ICI 182,780 antagonism , was calculated by (E_{E2} − $E_{E2 + neurosteroid}$) × 100/(E_{E2} − $E_{E2 + ICI}$).

at 1 μM resulted in a weak induction which was ERα-dependent, since it was fully inhibited by ICI 182,780, an antiestrogen known to knock out ER expression.[44] Treatment of estradiol (1 nM) supplemented MCF-7:D5L cells with 1 μM ICI 182,780 fully suppressed estradiol induction of luciferase, whereas treatment with any of **20**, **23**, or **27** had no effect up to 1 μM (Table 1, column 3).

Agonism through estrogen receptor β (ERβ) was assessed by way of induction of ERE-dependent luciferase gene expression in HEK:ERβ cells.[45] Treatment of estrogen-free HEK:ERβ cells with 1 nM estradiol induced luciferase by 3.4-fold, while analogues **20**, **23**, and **27** were ineffective up to 1 μM (Table 1, column 4). Treatment of estradiol (1 nM) supplemented HEK:ERβ cells with 1 μM ICI 182,780 fully inhibited estradiol induction of luciferase. Analogue **27** was weakly inhibitory at 1 μM, while **20** and **23** were ineffective (Table 1, column 5).

We also tested the ability of analogues **20**, **23**, and **27** to induce the expression of AlkP in Ishikawa cells, known to express ERβ as well as ERα.[39] Treatment of estrogen-free Ishikawa cells with postmenopausal levels of estadiol (0.1 nM) resulted in full (5-fold) induction of AlkP,[46] treatment with **23** or **27** (1 μM) had no effect, while with compound **20** (1 μM) a weak ER-dependent induction of AlkP was obtained (Table 1, column 6). Treatment of estradiol (0.1 nM) supplemented Ishikawa cells with 1 μM ICI 182,780 fully inhibited estradiol induction of AlkP. In comparison, treatment with **27** (1 μM) resulted in weak suppression of estradiol induction of AlkP, while **20** and **23** had no effect up to this concentration (Table 1, column 7). Taken together, these data suggest that analogues **20** and **27** may impact AlkP expression of Ishikawa cells through ERα and ERβ, respectively, albeit weakly and in opposite directions.

The effects of spiro-neurosteroids **20**, **23**, and **27** on the expression of luciferase suggest that, at concentrations ≤1 μM, **20** is a weak ERα agonist, **27** is a weak ERβ antagonist, and **23** a nonagonist/nonantagonist of either receptor subtype with regard to ER-mediated ERE-dependent regulation of gene expression. In line with these findings we observed that, in Ishikawa cells, **20** induced AlkP expression weakly, presumably through ERα, **27** inhibited estradiol induction of AlkP expression weakly, presumably through ERβ, and **23** was ineffective in either respect, as one would expect from a nonagonist/nonantagonist of ER.

Subsequently, we examined whether **20**, **23**, and **27** could stimulate the growth of MCF-7, LNCaP, and Ishikawa cells. MCF-7 cells are known to express ERα and to proliferate in an estrogen- and ERα-dependent manner.[42,43] Estradiol

at concentrations ≥0.1 nM is known to fully induce the ERα-dependent growth of MCF-7 cells, whereas 1 μM ICI 172,780 is known to fully inhibit the hormonal effect.[42] We found that at 1 μM derivative **20**, but not **23** or **27**, weakly stimulated estrogen-free MCF-7 cells to grow in a manner dependent on ER (Table 2, column 2), since the stimulation was fully inhibited by 1 μM ICI 182,780. These data indicate that **20** is a weak agonist of the ER-dependent growth of MCF-7 cells.

Treatment of estrogen-free Ishikawa cells with 0.1 nM estradiol for 3 days stimulated the growth of these cells marginally (15%), and this effect was inhibited by the presence of 1 μM ICI 182,780. Treatment of Ishikawa cells with any of **20**, **23**, and **27** failed to impact on cell growth significantly (Table 2, column 3).

Table 2. Stimulation of the Growth of Endocrine Cancer Cells by Spiro-Neurosteroids **20**, **23**, and **27**[a]

compd (1 μM)	MCF-7 cell growth induction (% of 0.1 nM E2)	Ishikawa cell growth induction (% of 0.1 nM E2)	LNCaP cell growth induction (% of 10 nM E2)
20	weak (33 ± 11)	ns	ns
23	ns	ns	ns
27	ns	ns	ns

[a] Induction of cell growth, as assessed spectrophotometrically at 550 nm using the MTT assay and expressed as % of that of estradiol (E2), was calculated by (OD at 550 nm in neurosteroid − $OD_{vehicle}$) × 100/(OD_{E2} − $OD_{vehicle}$)]. Statistically significant growth-inducing effects (mean ± SEM; $n \geq 3$) were classified as full, partial, or weak depending on whether they were 67−100%, 34−66%, and ≤33%, respectively, of the effects of E2; ns = nonsignificant.

Finally, we examined the effect of **20**, **23**, and **27** on the growth of LNCaP cells, which are known to express ERα, ERβ, and a mutant form of the androgen receptor (AR).[47,48] The growth of these cells is promoted by estrogens as well as androgens,[40,48] and ERβ is reported to be requisite for stimulation of cell proliferation by either hormone.[48] However, the mechanism of estrogen action and the ER subtype(s) involved in mediating estrogen responsiveness of these cells remain elusive.[40,47−49] The growth of LNCaP cell was significantly stimulated by 10 nM estradiol in a manner fully suppressed by 10 μM ICI 182,780. However, we found that analogues **20**, **23**, and **27** could not stimulate the growth of estrogen-free LNCaP cells (Table 2, column 4). In light of the above it is clear that none of the three compounds had a substantial stimulatory effect on the growth of MCF-7, Ishikawa, or LNCaP cells, even at 1 μM, which is in accordance with the weak ER agonist properties of these compounds, described above.

The effect of the most potent steroids **20**, **23**, and **27** on cell viability was examined in three different cell lines, namely, PC12 (rat adrenal pheochromocytoma), HEK293 (human embryonic kidney), and HT22 (mouse hippocampal neuroblastoma) cells, using the MTT assay. All three compounds were applied to the cell cultures in concentrations 0.1, 1, and 10 μM, and cell survival was assessed after 24, 48, or 72 h. As shown in Figure 9, all three compounds failed to significantly affect cell viability at all concentrations tested.

As an initial step to correlate the *in vitro* results with the active conformations of compounds **20**, **23**, and **27**, a combination of minimization algorithms with stochastic and systematic

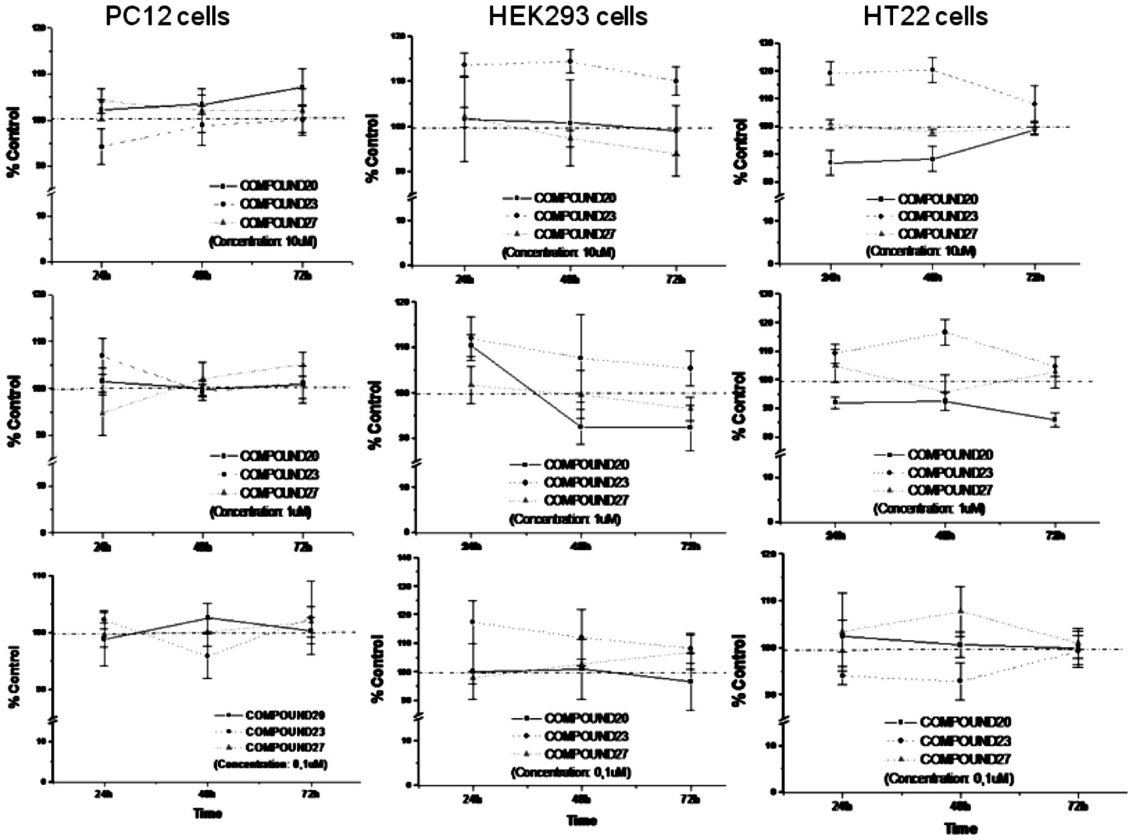


Figure 9. Effects of compounds **20**, **23**, and **27** on cell viability. PC12, HEK293, and HT22 cells (2×10^5 cells/mL) were cultured in serum-supplemented culture media for 24, 48, or 72 h in the absence or the presence of various 0.1, 1, and 10 μM synthetic steroids. At the end of incubation viable cell numbers were determined using the MTT assay. Results (mean ± SEM of three replicate values of three independent experiments) are expressed as percentage of untreated control cultures.

conformational analysis was employed. The initial conformation of the compounds was built using the crystal structure of DHEA,[50] assuming a chair conformation for the A and C rings, a nearly ideal half-chair conformation for ring B, and a 14a envelope conformation for ring D. In a step further, the orientation of the flexible CH_2OH moiety at C21 of **23** and **27** was systematically explored. Thus, grid scan search around torsion angles τ_1 (C17-C20-C21-O) and τ_2 (C20-C21-O-H) resulted in five distinct areas, and the letters A−E and A′−E′ indicate the corresponding conformational local minima for **23** and **27**, respectively. The derived conformers A−E and A′−E′ are depicted in Figure 10, while their energy values and torsion angles are shown in Table 3. The energy map as a function of the dihedral angles τ_1 and τ_2 is plotted in Figure 11, and the isoenergetic curves, plotted with a step of 1.5 kcal mol^{-1}, define the energetic barriers between conformations. The derived minima were further examined for their consistency with experimental NOE data in order to obtain the most probable conformations. The most informative experimentally observed dipole interactions were those between proton H20 of the epoxy ring and protons H12 of the steroid C ring as well as between both methylene protons H21a and H21b and protons H16 of the D ring (Figure 10). Conformers E and E′ adopting an equatorial configuration for the C21-OH group and arranging H21 protons in spatial vicinity with H16 are in full agreement with the NOE data. Analogue **20** was subjected to energy minimization algorithms, and only one lowest energy conformer was found with 35.5 kcal mol^{-1}. Subsequently, the preferred conformations for **23** and **27**, E and E′, respectively, were superimposed with that of **20** and DHEA (energy value calculated at 41.4 kcal mol^{-1}) (Figure 12). The steroid backbone and the 3β-OH group are successfully aligned in all four compounds, which leads to the overlay of the epoxy groups of **20** and **23** in a pseudoequatorial configuration, while **27** arranges the epoxy group below the steroid plane. In DHEA the 17-carbonyl group is positioned in an equatorial orientation with the oxygen atom being equidistant from the epoxide oxygen either in the 17α or 17β configuration.

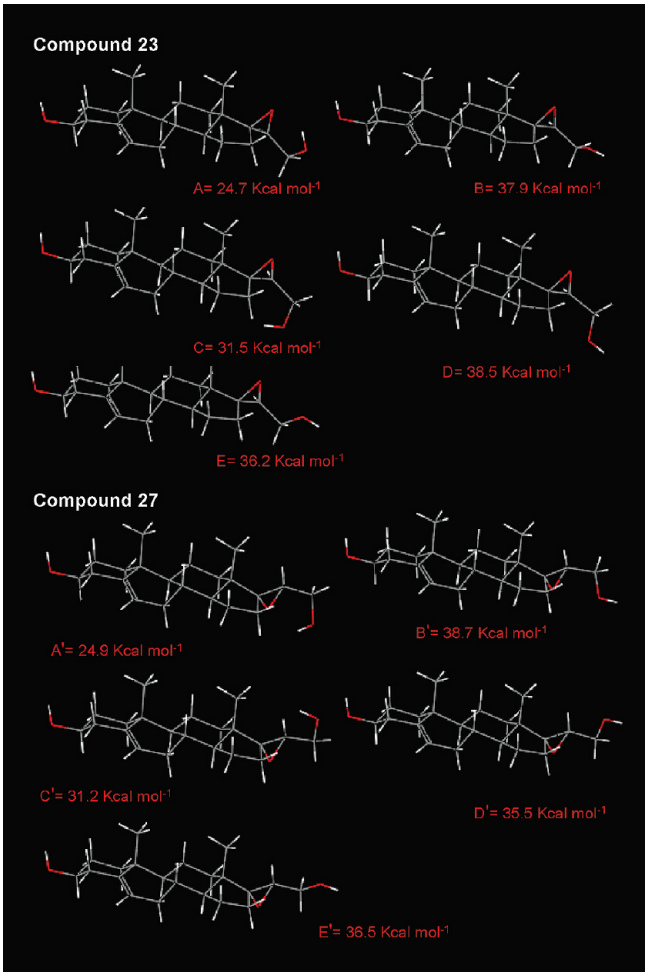


Figure 10. Conformational minima for compound **23** (A−E) and **27** (A′−E′) derived from a grid scan search around dihedral angles τ_1 (C17-C20-C21-O) and τ_2 (C20-C21-O-H).

Concerning the interaction of analogues **20**, **23**, and **27** with the mDBS, and taking into account the apparent K_d values, it is evident that the 17β configuration for the oxirane oxygen is more advantageous (cf. compounds **20** and **23**) than the 17α with both **20** and **23** having higher affinity for the mDBS than DHEA. Furthermore, compounds **20**, **23**, and **27** lack a phenyl hydroxyl, and thus, they cannot effectively mimic 17β-estradiol in forming the key hydrogen bonds with Glu353 and Arg394 that underlie high-affinity binding to ERα or with Glu305/Arg346 for ERβ.[42,51,52] As a result, productive interaction through hydrogen bond formation between the 17 oxygen of the oxirane moiety and His524 of ERα or His475 of ERβ, and the ensuing stabilization of the agonist conformation of the receptor, could be reduced or even abolished. This is more likely in the case of ERβ, due to the inherently lower stability of its agonist conformation and the stricter constraints for optimal fitting into the considerably less spacious binding cavity of this ER subtype.[52] These considerations could account for **20** being a weak agonist through ERα and a nonagonist through ERβ. The structural difference between **20** and **23** is the presence of a C21-hydroxymethylene group which could be prohibitory for binding and, thus, result in activation or inhibition of either form of ER by **23**. Concerning **27**, the opposite configuration of the oxirane with respect to **23** may account for it being a weak antagonist through ERβ and a nonantagonist through ERα.

Our rationale concerning the design of the new DHEA analogues was based on obtaining active compounds that would be devoid of hormonal side effects. Thus, we introduced substituents at position C3 of DHEA in order to inhibit the enzymatic oxidation of the 3β-hydroxyl group to a ketone or the aromatization of the A ring (DHEA → androstenedione → testosterone → estradiol). However, all of the respective compounds (**5**, **7**, **9**, **11**, **14**, and **16**), irrespectively

Table 3. Energy Values and Dihedral Angles of the Conformational Minima Obtained from Grid Scan Search for Compounds **23** and **27**

compd **23** (conformers)	energy value (kcal mol^{-1})	dihedral angle τ_1 (deg)	dihedral angle τ_2 (deg)	compd **27** (conformers)	energy value (kcal mol^{-1})	dihedral angle τ_1 (deg)	dihedral angle τ_2 (deg)
A	24.7	−58	−13	A′	24.9	59	14
B	37.9	−48	−161	B′	38.7	43	166
C	31.5	82	−20	C′	31.2	−75	15
D	38.5	95	180	D′	35.5	−86	−175
E	36.2	−164	165	E′	36.5	173	171

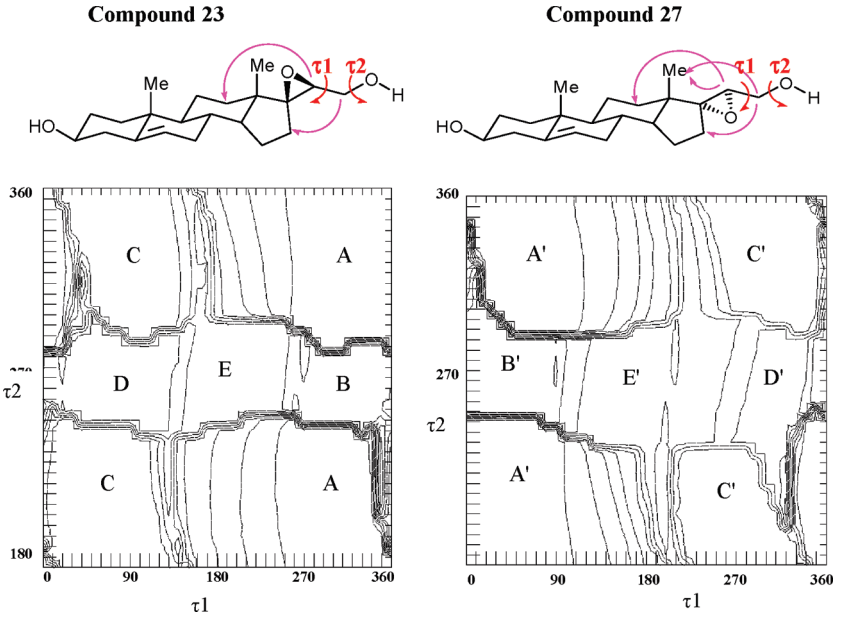


Figure 11. Contour maps for compounds **23** and **27** generated by rotating around dihedral angles τ_1 and τ_2 in increments of 10°. The contour levels which are up to 15 kcal mol^{-1} higher than the lower energy minimum are plotted with a step of 1.5 kcal mol^{-1}. Important NOE interactions are indicated by the pink arrows.

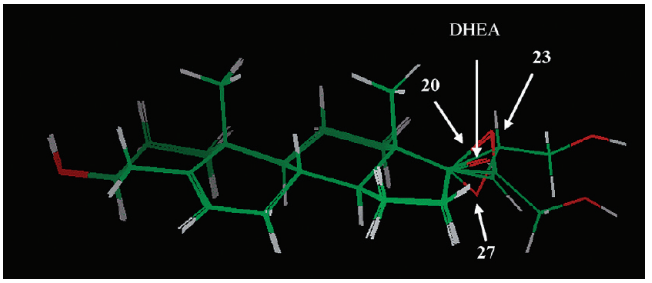


Figure 12. Favorable conformations of **23** (conformer E) and **27** (conformer E′), derived from a grid scan search, are superimposed with energy-optimized structures of **20** and DHEA.

of the nature of the 3α-substituent (aliphatic or polar), were devoid of antiapoptotic activity, at least at 100 nM concentration. Subsequently, we focused our search on modifications at C17, which is also an important position for the metabolic transformation of DHEA to androgens or estrogens. The epoxide group was chosen as a substituent at C17 since it is relatively small, thus disturbing binding to the DHEA membrane receptor sites to a lesser extent, and it has relatively low reactivity, which should reduce the potential for unselective alkylation during passage to the target cells. In addition, the C21-hydroxyl group was introduced since it can participate in hydrogen bond formation and, thus, potentially improve binding. As described above, the spiro-neurosteroid analogues **20**, **23**, and **27** were not only very potent but devoid of hormonal side effects. The order of antiapoptotic potencies (Figure 3: **20** > **27** > **23**) and that of binding affinities for the membrane DHEA binding sites (Figure 6: **20** > **23** > **27**) are similar, which could imply that efficient binding to the DHEA membrane binding sites is the factor predominantly influencing the antiapoptotic activity of these steroid derivatives.

Conclusions

A number of highly effective compounds were developed, which protect neuronal cells against serum deprivation induced apoptosis, with IC_{50} in the range of 0.1−100 nM. These compounds bind with high affinity to membrane DHEA binding sites[35] (K_d at nanomolar concentration) and mimic endogenous neurosteroids in inducing prosurvival antiapoptotic Bcl-2 proteins. The new potent spiro-neurosteroid analogues were found to be practically unable to activate ERα or ERβ up to a concentration of 1 μM and to interfere with estrogen induction of ERE-dependent gene expression, whether in the presence of pre- or postmenopausal levels of estradiol. In addition, they were found to be unable to impact the growth of Ishikawa, MCF-7, and LNCaP cells to any substantial extent. Our results suggest that the spiro-epoxyneurosteroid derivatives **20**, **23**, and **27** might prove to be lead molecules for the synthesis of novel neuroprotective agents.

Experimental Section

NMR spectra were recorded on a Varian 300 spectrometer operating at 300 MHz for ^{1}H and 75.43 MHz for ^{13}C or on a Varian 600 operating at 600 MHz for ^{1}H. ^{1}H NMR spectra are reported in units of δ relative to the internal standard of signals of the remaining protons of deuterated chloroform at 7.24 ppm. ^{13}C NMR shifts are expressed in units of δ relative to $CDCl_3$ at 77.0 ppm. ^{13}C NMR spectra were proton noise decoupled. All NMR spectra were recorded in $CDCl_3$. Silica gel plates (Merck F254) were used for thin-layer chromatography. Chromatographic purification was performed with silica gel (200−400 mesh). Analyses, indicated by the symbols of the elements, were carried out by the microanalytical section of the Institute of Organic and Pharmaceutical Chemistry of the National Hellenic Research Foundation.

17,17-Ethylenodioxy-5-androsten-3β-ol (1). To a solution of DHEA (1 g, 3.46 mmol) in cyclohexane (100 mL) was added ethylene glycol (0.6 mL, 10.38 mmol) and camphor-10-sulfonic acid (CSA) (10 mg, 0.04 mmol), and the resulting mixture was heated at reflux for 4 h using a Dean−Stark apparatus. After completion of the reaction, the mixture was allowed to cool to room temperature and was extracted with ethyl acetate and saturated aqueous $NaHCO_3$. The organic layer was washed with brine and was dried over Na_2SO_4. The solvent was removed *in vacuo* to afford ketal **1** (1 g, 92%) as a white solid which was

used in the next step without purification: mp 162−165 °C; $[\alpha]^{20}_D = -93.42°$ ($c = 0.00152$ g/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.85 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.07−2.27 (m, 19H), 3.47−3.57 (m, 1H, H-3α), 3.83−3.94 (m, 4H, OCH_2CH_2O), 5.34 (d, $J = 5.49$ Hz, 1H, H-6); ^{13}C NMR ($CDCl_3$) δ 14.4, 19.6, 20.6, 22.7, 24.3, 30.5, 31.2, 31.6, 32.2, 34.2, 36.5, 37.2, 42.2, 45.7, 49.9, 50.6, 65.2, 71.6, 119.5, 121.4, 140.7. Anal. ($C_{21}H_{32}O_3$) C, H.

17,17-Ethylenodioxy-5-androsten-3-one (2). To a solution of alcohol **1** (50 mg, 0.15 mmol) in dry dichloromethane (10 mL) was added at 0 °C Dess−Martin periodinane (130 mg, 0.30 mmol), and the mixture was stirred at room temperature for 1 h. Then it was diluted with ether and was quenched with a mixture of saturated aqueous $NaHCO_3/Na_2S_2O_3$ (1:3). The organic layer was washed with brine and was dried over Na_2SO_4, and the solvent was evaporated *in vacuo* to afford unsaturated ketone **2** (49 mg, quantitative), which was pure enough to be used for the next step without purification: mp 141−144 °C; ^{1}H NMR ($CDCl_3$) δ 0.87 (s, 3H, CH_3), 1.17 (s, 3H, CH_3), 1.02−2.77 (m, 17H), 2.80 (dd, $J = 2.44$, 16.48 Hz, 1H), 3.26 (dd, $J = 3.05$, 16.48 Hz, 1H), 3.82−3.92 (m, 4H, OCH_2CH_2O), 5.32 (dd, $J = 5.49$, 2.0 Hz, 1H, H-6); ^{13}C NMR ($CDCl_3$) δ 14.3, 19.2, 20.4, 22.6, 30.4, 31.1, 32.8, 34.1, 35.7, 36.8, 37.6, 45.7, 48.3, 49.0, 49.7, 50.4, 64.5, 65.2, 119.3, 122.6, 138.5, 210.1. Anal. ($C_{21}H_{30}O_3$) C, H.

17,17-Ethylenodioxy-5-androstene-3,2′-oxirane (3). To a solution of trimethylsulfonium iodide (370 mg, 1.82 mmol) in dry THF (5 mL) at 0 °C was added *n*-BuLi (1.6 M solution in hexanes, 1.15 mL, 1.82 mmol), and the resulting mixture was stirred at 0 °C for 30 min. The reaction was cooled at −40 °C, and a solution of ketone **2** (400 mg, 1.21 mmol) in THF (10 mL) was added. The resulting mixture was stirred at −40 °C for 2 h and then at room temperature for 12 h. The solvent was evaporated *in vacuo*, the residue was diluted with ether, the organic layer was extracted with water and brine and was dried over anhydrous Na_2SO_4, and the solvent was removed *in vacuo*. The crude residue was purified by flash column chromatography using petroleum ether, 40−60 °C, and ethyl acetate (92:8) as eluting solvent to afford the desired epoxide **3** (130 mg, 32%) as colorless solid: mp 110−113 °C; $R_f = 0.60$ (petroleum ether, 40−60 °C/ethyl acetate, 8/2); ^{1}H NMR ($CDCl_3$) δ 0.87 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.04−2.18 (m, 18H), 2.56 (d, $J = 4.8$ Hz, 1H, CH_2, epoxide), 2.58 (d, $J = 4.8$ Hz, 1H, CH_2, epoxide), 2.88 (d, $J = 13.7$ Hz, 1H), 3.85−3.91 (m, 4H, OCH_2CH_2O), 5.30 (d, $J = 5.0$ Hz, 1H, H-6). Anal. ($C_{22}H_{32}O_3$) C, H.

17,17-Ethylenodioxy-3α-methoxymethyl-5-androsten-3β-ol (4). To a solution of epoxide **3** (50 mg, 0.14 mmol) in anhydrous methanol (5 mL) was added sodium methoxide (24 mg, 0.44 mmol), and the mixture was refluxed for 5 h. The solvent was evaporated *in vacuo*, and the crude residue was purified by flash column chromatography using petroleum ether, 40−60 °C, and ethyl acetate (8:2) as eluting solvent to afford the desired compound **4** (40 mg, 74%). ^{1}H NMR ($CDCl_3$) δ 0.84 (s, 3H, 19-CH_3), 1.02 (s, 3H, 18-CH_3), 1.02−2.12 (m, 19H), 2.39 (dd, $J = 2.44$, 13.43 Hz, 1H), 3.21 (q, $J = 9.76$ Hz, 2H, 3α-CH_2O), 3.36 (s, 3H, CH_3O), 3.83−3.93 (m, 4H, OCH_2CH_2O), 5.29 (d, $J = 5.49$ Hz, 1H, H-6); ^{13}C NMR ($CDCl_3$) δ 13.3, 19.2, 20.5, 21.2, 30.6, 31.2, 31.5, 31.8, 35.6, 36.5, 36.8, 42.1, 47.5, 50.7, 51.8, 59.6, 65.3, 71.6, 75.1, 119.3, 121.2, 140.5.

3β-Hydroxy-3α-methoxymethyl-5-androsten-17-one (5). To a solution of 17,17-ethylenodioxy-3α-methoxymethyl-5-androsten-3β-ol (**4**) (40 mg, 0.11 mmol) in THF (10 mL) was added a mixture of aqueous 1 M HCl/acetone (1:1) (2 mL), and the reaction mixture was stirred at room temperature for 12 h. Subsequently, the reaction mixture was neutralized by addition of aqueous 1 M NaOH, the organic layer was extracted with ethyl acetate, washed with brine, and dried over anhydrous Na_2SO_4, and the solvent was evaporated *in vacuo*. The crude product was purified by flash column chromatography using petroleum ether, 40−60 °C, and ethyl acetate (7:3) as elution solvent to yield ketone **5** (30 mg, 86%) as a white solid: mp 160−164 °C; $[\alpha]^{20}_D = 25°$ ($c = 0.001$ g/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.88 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.02−2.12 (m, 16H), 2.13 (dd, $J = 1.2$, 13.7 Hz, 1H, H-4α), 2.40 (dd, $J = 2.0$, 13.7 Hz, 1H, H-4β), 2.40−2.44 (m, 1H), 3.17 (d $J = 9.15$ Hz, half of AB system, 1H), 3.24 (d, $J = 9.15$ Hz, half of AB system, 1H), 3.36 (s, 3H, CH_3O−), 5.32 (d, $J = 5.49$ Hz, 1H, H-6). Anal. ($C_{21}H_{32}O_3$) C, H.

(17,17-Ethylenedioxy-3β-hydroxy-5-androsten-3α-yl)acetonitrile (6). To a solution of epoxide **3** (60 mg, 0.17 mmol) in absolute ethanol (12 mL) was added potassium cyanide (14 mg, 0.21 mmol) at room temperatue, and the mixture was stirred for 48 h. The reaction mixture was refluxed for 5 h, followed by stirring at room temperature for 24 h. Subsequently, water and ether were added to the reaction mixture, the organic layer was washed with brine and was dried over anhydrous Na_2SO_4, and the solvent was evaporated *in vacuo*. The crude product was purified by flash column chromatography using petroleum ether, 40−60 °C, and ethyl acetate (8:2) as elution solvent to yield compound **6** (40 mg, 61%); ^{1}H NMR ($CDCl_3$) δ 0.84 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.20−2.30 (m, 19H), 2.47 (br s, 2H, 3α-CH_2CN), 3.85−3.88 (m, 4H, OCH_2CH_2O), 5.40 (d, $J = 5.49$ Hz, 1H, H-6); ^{13}C NMR ($CDCl_3$) δ 14.0, 19.4, 20.2, 21.9, 27.9, 30.6, 31.1, 31.9, 34.2, 35.2, 36.2, 36.5, 44.5, 47.9, 50.6, 51.3, 65.3, 71.7, 117.4, 119.3, 121.2, 140.4.

(3β-Hydroxy-5-androsten-17-oxo-3α-yl)acetonitrile (7). Following the procedure for analogue **5**, ketone **7** was obtained from compound **6** (40 mg, 0.11 mmol) after purification by flash column chromatography (petroleum ether, 40−60 °C/ethyl acetate (8:2)) as a colorless solid, 30 mg (86% yield): mp 190−194 °C; $[\alpha]^{20}_D = 21.74°$ ($c = 0.00115$ g/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.88 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.02−2.22 (m, 16H), 2.20 (dd, $J = 2.7$, 13.7 Hz, 1H, H-4α), 2.43−2.49 (m, 1H), 2.48 (s, 2H, −CH_2CN), 2.50 (dd, $J = 1.7$, 13.7 Hz, 1H, H-4β), 5.45 (d, $J = 5.13$ Hz, 1H, H-6). Anal. ($C_{21}H_{29}NO_2$) C, H, N.

17,17-Ethylenedioxy-3α-azidomethyl-5-androsten-3β-ol (8). To a solution of epoxide **3** (80 mg, 0.23 mmol) in a mixture of methanol and water (8:1) (18 mL) was added sequentially sodium azide (75 mg, 1.15 mmol) and ammonium chloride (28 mg, 0.51 mmol), and the resulting mixture was refluxed overnight. The mixture was filtered from a silica gel pad, and the filtrate was evaporated *in vacuo*. The residue was purified by flash column chromatography (petroleum ether, 40−60 °C/ethyl acetate (8:2)) to obtain product **8** (60 mg, 67% yield): ^{1}H NMR ($CDCl_3$) δ 0.83 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.02−2.13 (m, 18H), 2.40 (dd, $J = 2.44$, 13.43 Hz, 1H), 3.23 (d, $J = 12.21$ Hz, 1H, AB system, 3α-CH_2-N_3), 3.29 (d, $J = 12.21$ Hz, AB system, 1H, 3α-CH_2-N_3), 3.82−3.92 (m, 4H, OCH_2CH_2O), 5.33 (dd, $J = 2.46$, 4.89 Hz, 1H, H-6); ^{13}C NMR ($CDCl_3$) δ 13.3, 19.2, 20.5, 21.3, 30.5, 31.7, 31.5, 31.8, 35.5, 36.1, 36.5, 42.8, 47.7, 50.3, 51.6, 56.7, 65.4, 73.1, 118.2, 121.4, 140.7.

3β-Hydroxyl-3α-azidomethyl-5-androsten-17-one (9). Following the procedure for analogue **5**, using ketal **8** (60 mg, 0.15 mmol), compound **9** was obtained after purification by flash column chromatography using petroleum ether, 40−60 °C/ethyl acetate (8:2) as a colorless solid: yield 45 mg, 85%; mp 168−172 °C; $[\alpha]^{20}_D = 22.22°$ ($c = 0.0018$ g/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.88 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.02−2.17 (m, 17H), 2.45 (m, 2H), 3.26 (d, $J = 12.40$ Hz, 1H, AB system, 3α-CH_2-N_3), 3.30 (d, $J = 12.40$ Hz, AB system, 1H, 3α-CH_2-N_3), 5.38 (d, $J = 5.15$ Hz, 1H, H-6). Anal. ($C_{20}H_{29}N_3O_2$) C, H, N.

3β-*n*-Butyl-17,17-ethylenodioxy-5-androsten-3α-ol (10). To a solution of ketone **2** (100 mg, 0.30 mmol) in dry THF (3 mL) was added at 0 °C *n*-BuLi (1.6 M solution in hexanes, 0.38 mL, 0.60 mmol), and the reaction mixture was stirred at room temperature overnight. The mixture was quenched with saturated aqueous NH_4Cl solution and was extracted with ethyl acetate. The organic layer was washed with brine and dried over Na_2SO_4, and the solvent was removed *in vacuo*. The residue was purified by flash column chromatography (petroleum ether, 40−60 °C/ethyl acetate

(95:5)) to obtain analogue **10** (64 mg, 55%): R_f = 0.60 (petroleum ether, 40−60 °C/ethyl acetate (8/2)); ^{1}H NMR ($CDCl_3$) δ 0.85 (s, 3H, CH_3), 0.90 (t, J = 7.10 Hz, 3H, CH_3), 0.97 (s, 3H, CH_3), 1.02−2.10 (m, 24H), 2.36 (dd, J = 2.30, 14.20 Hz, 1H), 3.91−3.96 (m, 4H, OCH_2CH_2O), 5.39 (d, J = 5.49 Hz, 1H, H-6).

3β-*n*-Butyl-3α-hydroxy-5-androsten-17-one (11). Compound **11** was obtained from ketal **10** (64 mg, 0.16 mmol) following the procedure for analogue **5** after purification by flash column chromatography (petroleum ether, 40−60 °C/ethyl acetate (8:2)) as a colorless solid, 40 mg (71% yield): mp 151−154 °C; ^{1}H NMR ($CDCl_3$) δ 0.87 (m, 3H, CH_3), 0.90 (t, J = 7.10 Hz, 3H, CH_3), 0.99 (s, 3H, CH_3), 1.12−2.14 (m, 24H), 2.36−2.50 (m, 2H), 5.41 (d, J = 5.49 Hz, 1H, 6-CH); ^{13}C NMR ($CDCl_3$) δ 14.5, 15.1, 19.5, 21.1, 22.7, 24.1, 26.2, 31.6, 32.1, 32.2, 33.6, 35.6, 36.5, 37.7, 43.1, 44.5, 47.9, 50.8, 52.1, 72.0, 122.3, 139.4, 218.9. Anal. ($C_{23}H_{36}O_2$) C, H.

17,17-Ethylenodioxy-3α-methyl-5-androsten-3β-ol (12) and 17,17-Ethylenodioxy-3β-methyl-5-androsten-3α-ol (13). To a solution of ketone **2** (100 mg, 0.30 mmol) in dry THF (5 mL) was added MeLi (1.6 M solution in ether, 0.41 mL, 0.60 mmol) at 0 °C, and the resulting mixture was stirred at room temperature for 3 h. The reaction was quenched with saturated aqueous NH_4Cl and was extracted with ethyl acetate. The organic layer was washed with brine and dried over Na_2SO_4, and the solvent was removed *in vacuo*. The residue was purified by flash column chromatography (petroleum ether, 40−60 °C/ethyl acetate (85:15)) to afford products **12** (29 mg) and **13** (58 mg) (75% overall yield).

17,17-Ethylenodioxy-3α-methyl-5-androsten-3β-ol (12). R_f = 0.23 (petroleum ether, 40−60 °C/acetone (9/1)); ^{1}H NMR ($CDCl_3$) δ 0.87 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.09 (s, 3H, 3α-CH_3), 1.02−2.13 (m, 18H), 2.45 (m, 1H), 3.82−3.91 (m, 4H, OCH_2CH_2O), 5.30 (d, J = 5.49 Hz, 1H, H-6).

17,17-Ethylenodioxy-3β-methyl-5-androsten-3α-ol (13). R_f = 0.30 (petroleum ether, 40−60 °C/acetone (9/1)); ^{1}H NMR ($CDCl_3$) δ 0.84 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 1.20 (s, 3H, 3β-CH_3), 1.02−2.37 (m, 18H), 2.40 (dd, J = 2.44, 14.04 Hz, 1H), 3.83−3.92 (m, 4H, OCH_2CH_2O), 5.39 (d, J = 5.49, 1H, H-6).

3β-Hydroxy-3α-methyl-5-androsten-17-one (14). Compound **14** was prepared following the method for **5** using the steroidal ketal **12** (29 mg, 0.08 mmol) after purification by flash column chromatography using (petroleum ether, 40−60 °C/acetone (85:15)) in 87% yield (21 mg) as a white solid: mp 130−133 °C; $[\alpha]^{20}_D$ = 12.55° (c = 0.00255 g/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.87 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.12 (s, 3H, 3α-CH_3), 1.02−2.14 (m, 17H), 2.45 (m, 2H), 5.33 (d, J = 5.13 Hz, 1H, H-6). Anal. ($C_{20}H_{30}O_2$) C, H.

3α-Hydroxy-3β-methyl-5-androsten-17-one (15). Following the procedure for **5** using ketal **13** (58 mg, 0.17 mmol), we obtained ketone **15** (21 mg, 87%) after purification by flash column chromatography (petroleum ether, 40−60 °C/acetone (85:15)): $[\alpha]^{20}_D$ = 24.79° (c = 0.00117 g/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.88 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.22 (s, 3H, 3β-CH_3), 1.02−2.15 (m, 17H), 2.42 (m, 1H, H-4β), 2.45 (m, 1H), 5.42 (d, J = 5.49 Hz, 1H, H-6). Anal. ($C_{20}H_{30}O_2$) C, H.

3β-(*tert*-Butyldiphenylsilyloxy)-5-androsten-17-one (16). To a solution of DHEA (500 mg, 1.73 mmol) in anhydrous DMF (15 mL) was added at 0 °C imidazole (295 mg, 4.32 mmol), and the mixture was stirred for 15 min. Subsequently was added *tert*-butyldiphenylsilyl chloride (1.1 mL, 4.32 mmol), and the resulting mixture was stirred at 50 °C overnight. The reaction mixture was quenched with saturated aqueous NH_4Cl, and the mixture was stirred for an additional 30 min at room temperature. The solvent was evaporated *in vacuo*, the residue was diluted with ethyl acetate, and the organic layer was washed with water and brine and was dried over Na_2SO_4. The solvent was removed *in vacuo* followed by recrystallization from ethanol and petroleum ether, 40−60 °C, to yield analogue **16** (720 mg, 79%) as a white solid: mp 117−120 °C; ^{1}H NMR ($CDCl_3$) δ 0.85 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.05 (s, 9H, (CH_3)$_3$C-Si), 1.10−2.47 (m, 19H), 3.47−3.54 (m, 1H, 3a-H), 5.13 (d, J = 4.89 Hz, 1H, H-6), 7.35−7.38 (m, 6H, ArH), 7.65−7.67 (m, 4H, ArH); ^{13}C NMR ($CDCl_3$) δ 14.3, 15.9, 19.3, 20.1, 20.3, 20.5, 27.3, 29.7, 31.6, 32.2, 34.2, 36.8, 38.8, 39.5, 42.2, 44.0, 58.6, 71.2, 121.2, 128.1, 129.9, 133.4, 135.8, 218.9. Anal. ($C_{35}H_{46}O_2Si$) C, H.

3β-(*tert*-Butyldiphenylsilyloxy)-17β-hydroxy-5-androstene (17). To a suspension of $LiAlH_4$ (36 mg, 0.95 mmol) in anhydrous THF (2 mL) was added dropwise at 0 °C a solution of ketone **16** (250 mg, 0.47 mmol) in anhydrous THF (5 mL), and the resulting mixture was stirred at room temperature for 3 h. Subsequently, the reaction was quenched at 0 °C by a mixture of H_2O/THF (1:1) (3 mL) followed by addition of ethyl acetate (30 mL). The resulting mixture was dried over Na_2SO_4, the solid was filtered, and the filtrate was evaporated *in vacuo*. The residue was purified by flash column chromatography (petroleum ether, 40−60 °C/ethyl acetate (8:2)) to afford alcohol **17** as a white solid in 92% yield (230 mg): mp 161−164 °C; $[\alpha]^{20}_D$ = 37.21° (c = 0.00043 g/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.73 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.06 (s, 9H, (CH_3)$_3$C-Si), 0.90−2.39 (m, 19H), 3.52−3.62 (m, 2H, 3α-H and 17α-H), 5.12 (d, J = 4.89 Hz, 1H, H-6), 7.35−7.39 (m, 6H, ArH), 7.66−7.70 (m, 4H, ArH); ^{13}C NMR ($CDCl_3$) δ 10.9, 19.1, 19.4, 20.6, 23.4, 26.9, 30.5, 31.4, 31.8, 31.9, 36.6, 37.2, 42.4, 42.7, 50.1, 51.3, 73.1, 81.9, 120.8, 127.4, 129.4, 134.8, 135.6, 135.7, 141.4. Anal. ($C_{35}H_{48}O_2Si$) C, H.

3β-(*tert*-Butyldiphenylsilyloxy)-17β-methoxy-5-androstene (18). To NaH (60% dispersion in mineral oil, 46 mg, 1.14 mmol) in anhydrous THF (1 mL) was added at 0 °C a solution of alcohol **17** (300 mg, 0.57 mmol) in anhydrous THF (4 mL), and the resulting mixture was stirred for 30 min at room temperature. Subsequently, MeI (70 μL, 1.14 mmol) was added, and the reaction mixture was stirred at room temperature overnight. The reaction was quenched with saturated aqueous NH_4Cl and was extracted with ethyl acetate. The organic layer was washed with brine and was dried over Na_2SO_4, and the solvent was evaporated *in vacuo*. The residue was purified by flash column chromatography (petroleum ether, 40−60 °C/ether (9:1)) to afford analogue **18** (150 mg, 81%) as a white solid: mp 149−152 °C; $[\alpha]^{20}_D$ = −46.49° (c = 0.00114 g/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.76 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.07 (s, 9H, Si-C(CH_3)$_3$), 0.80−2.39 (m, 19H), 3.20 (t, J = 7.9 Hz, 1H, H- 17α), 3.33 (s, 3H, CH_3O), 3.54−3.58 (m, 1H, H-3a), 5.13 (d, J = 4.89 Hz, 1H, H-6), 7.36−7.39 (m, 6H, ArH), 7.67−7.70 (m, 4H, ArH); ^{13}C NMR ($CDCl_3$) δ 19.1, 20.7, 23.3, 27.0, 27.6, 27.9, 31.4, 31.7, 31.8, 36.5, 37.2, 37.9, 42.5, 42.6, 50.1, 51.6, 57.8, 73.1, 90.7, 120.8, 127.4, 129.4, 134.8, 135.7, 141.2. Anal. ($C_{36}H_{50}O_2Si$) C, H.

17β-Methoxy-5-androsten-3β-ol (19). A solution of analogue **18** (140 mg, 0.26 mmol) in anhydrous THF (2 mL) was treated with a solution of (*n*-Bu)$_4$N$^+$F$^-$ (1 M solution in THF, 6.5 mL, 6.5 mmol), and the resulting mixture was stirred at room temperature overnight. The reaction was quenched with saturated aqueous NH_4Cl and was extracted with ethyl acetate. The organic layer was washed with brine and dried over Na_2SO_4, and the solvent was removed *in vacuo*. The residue was purified by flash column chromatography (dichloromethane/ethyl acetate (9:1)) to yield alcohol **19** (78 mg, 99%) as a white solid: mp 165−168 °C; $[\alpha]^{20}_D$ = −70.92° (c = 0.00141 g/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.75 (s, 3H, 19-CH_3), 0.99 (s, 3H, 18-CH_3), 0.90−2.25 (m, 19H), 3.21 (t, J = 8.55 Hz, 1H, H-17α), 3.33 (s, 3H, CH_3O), 3.47−3.54 (m, 1H, H-3a), 5.31 (d, J = 4.89 Hz, 1H, H-6); ^{13}C NMR ($CDCl_3$) δ 11.4, 19.4, 20.7, 23.3, 27.6, 31.4, 31.6, 31.7, 36.5, 37.2, 37.8, 42.2, 42.6, 50.2, 51.6, 57.8, 71.6, 90.7, 121.2, 140.8. Anal. ($C_{20}H_{32}O_2$) C, H.

17β-Spiro[5-androstene-17,2′-oxiran]-3β-ol (20). To a solution of DHEA (500 mg, 1.73 mmol) in anhydrous DMF (10 mL) were added at 0 °C trimethylsulfonium iodide (530 mg, 2.60 mmol) and *t*-BuOK (292 mg, 2.60 mmol), and the resulting mixture was stirred at room temperature for 2 h. Subsequently, the reaction was quenched by addition of water, and the resulting mixture was

extracted with diethyl ether. The organic layer was washed with brine and dried over anhydrous Na_2SO_4, and the solvent was evaporated *in vacuo*. The residue was purified by flash column chromatography (elution solvent: petroleum ether, 40−60 °C/acetone (8:2)) to obtain the desired epoxide **20** (310 mg, 59%) as a white crystalline solid: mp 170−173 °C; $[\alpha]^{20}_D = -72.80°$ (c = 0.00125 g/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.88 (s, 3H, 19-CH_3), 1.00 (s, 3H, 18-CH_3), 1.02−2.28 (m, 19H), 2.59 (d, J = 4.88 Hz, 1H), 2.89 (d, J = 4.88 Hz, 1H), 3.47−3.54 (m, 1H, H-3a), 5.35 (d, J = 5.49 Hz, 1H, H-6);^{13}C NMR ($CDCl_3$) δ 14.1, 19.4, 20.4, 23.6, 29.0, 31.3, 31.5, 32.0, 33.8, 36.6, 37.2, 39.9, 42.2, 50.1, 53.1, 53.6, 70.5, 71.6, 121.2, 140.9. Anal. ($C_{20}H_{30}O_2$) C, H.

17α-Vinyl-5-androstene-3β,17β-diol (21). A solution of DHEA (1.3 g, 4.5 mmol) in anhydrous THF (13 mL) was added dropwise at 0 °C to a solution of vinylmagnesium bromide (1.0 M in THF, 45 mL, 45 mmol), and the resulting mixture was stirred at room temperature for 12 h. After completion of the reaction saturated aqueous NH_4Cl was added, and the resulting mixture was extracted with ethyl acetate. The organic layer was washed with brine and dried over anhydrous Na_2SO_4, and the solvent was evaporated *in vacuo*. The residue was purified by flash column chromatography (elution solvent: cyclohexane/AcOEt (7:3)) to obtain 17α-vinyl-5-androstene-3β,17β-diol (**21**) (992 mg, 69%) as a white crystalline solid: mp 180−183 °C; ^{1}H NMR ($CDCl_3$) δ 0.93 (s, 3H), 1.02 (s, 3H), 1.10−2.31 (m, 19H), 3.47−3.56 (m, 1H, H-3α), 5.10 (dd, J = 0.90, 10.80 Hz, 1H), 5.15 (dd, J = 0.90, 17.7 Hz, 1H), 5.34 (d, J = 4.92 Hz, 1H), 6.04 (dd, J = 10.80, 17.3 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 14.0, 19.4, 20.6, 23.6, 31.5, 31.6, 32.1, 32.6, 36.0, 36.5, 37.2, 42.2, 46.1, 49.9, 50.3, 71.6, 84.2, 111.9, 121.3, 140.8, 143.02. Anal. ($C_{21}H_{32}O_2$) C, H.

3β,17β-Dihydroxy-20,21-epoxy-5-androstene (22). To a solution of compound **21** (0.992 g, 3.13 mmol) in anhydrous dichloromethane (33 mL) were sequentially added at −10 °C vanadyl acetylacetonate (36 mg, 0.137 mmol) and *tert*-butyl hydroperoxide (∼2.35 M in 1,2-dichloroethane, prepared by extracting *tert*-butyl hydroperoxide (70%, 38.8 mL) with 65.9 mL of 1,2-dichloroethane) (4 mL, 9.39 mmol), and the resulting mixture was stirred at room temperature for 12 h. The reaction mixture was diluted with dichloromethane, the organic layer was extracted with water, saturated Na_2SO_3, and brine and was dried over anhydrous Na_2SO_4, and the solvent was evaporated *in vacuo*. The residue was purified by flash column chromatography (elution solvent: dichloromethane/ethyl acetate (6:1)) to obtain 3β,17β-dihydroxy-20,21-epoxy-5-androstene (**22**) (2:1 mixture of **22a** and **22b**) (0.71 g, 68%) as a white crystalline solid: mp 165−168 °C; $[\alpha]^{20}_D = -53.10°$ (c = 0.00113 g/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.88 (s, 0.99H, −CH_3 compound **22b**), 0.93 (s, 1.99H, −CH_3 compound **22a**), 1.02 (s, 3H, CH_3), 0.96−2.32 (m, 19H), 2.69 (dd, J = 4.2, 5.1 Hz, 0.33H compound **22b**), 2.74−2.77 (m, 1H), 2.87 (dd, J = 2.97, 4.80 Hz, 0.66H compound **22a**), 3.09 (t, J = 4.27 Hz, 0.66H compound **22a**), 3.21 (dd, J = 3.0, 3.90 Hz, 0.33H compound **22b**), 3.45−3.55 (m, 1H), 5.34 (d, J = 5.13 Hz, 1H, H-6); ^{13}C NMR ($CDCl_3$) δ 13.9, 19.4, 20.5, 24.0, 31.6, 32.4, 36.0, 36.6, 37.3, 42.2, 43.2, 45.5, 50.1, 51.4, 51.8, 54.8, 56.2, 71.7, 79.7, 121.2, 140.8. Anal. ($C_{21}H_{32}O_3$) C, H.

(20*S*)-3β,21-Dihydroxy-17β,20-epoxy-5-pregnene (23). To a solution of 3β,17β-dihydroxy-20,21-epoxy-5-androstene (**22**) (0.71 g, 2.13 mmol) in anhydrous MeOH (35 mL) was added K_2CO_3 (0.737 g, 5.33 mmol), and the resulting mixture was stirred at room temperature for 12 h. The reaction mixture was diluted with chloroform, and the organic layer was extracted with water and brine and was dried over anhydrous Na_2SO_4. The solvent was evaporated *in vacuo*, and the residue was purified by flash column chromatography (elution solvent: petroleum ether/acetone (85:15)) to obtain (20*S*)-3β,21-dihydroxy-17β,20-epoxy-5-androstene (**23**) (0.35 g, 48%) as a white crystalline solid: mp 195−197 °C; $[\alpha]^{20}_D = -70.00°$ (c = 0.0009 g/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.87 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.03−2.31 (m, 19H), 3.19 (dd, J = 4.1, 6.2 Hz, 1H, H-20), 3.48−3.54 (m, 1H, H-3α), 3.58 (dd, J = 6.3, 12.0 Hz, 1H, H-21), 3.76 (dd, J = 4.0, 12.0 Hz, 1H, H-21), 5.35 (d, J = 5.10 Hz, 1H, H-6). Anal. ($C_{21}H_{32}O_3$) C, H.

(*E*)-(3β-Hydroxy-5-androsten-17-ylidene)acetonitrile (24). To a solution of *t*-BuOK (0.93 g, 8.28 mmol) in dry THF (10 mL) at 0 °C was added diethyl cyanomethylphosphonate (0.87 mL, 5.52 mmol), and the reaction was stirred for 1 h. To the above mixture was added dropwise a solution of DHEA (0.4 g, 1.38 mmol) in dry THF (10 mL), and the mixture was stirred at room temperature until completion of the reaction. The reaction was quenched by addition of saturated aqueous NH_4Cl and was extracted with ethyl acetate, the organic layer was washed with brine and was dried over anhydrous Na_2SO_4, and the solvent was evaporated *in vacuo*. The residue was purified by flash column chromatography (elution solvent: cyclohexane/ethyl acetate (8:2)) to afford the desired compound **24** (0.28 gr, 65%): mp 164−167 °C; $[\alpha]^{20}_D = -60.10°$ (c = 0.0099 g/mL, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.85 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.03−2.33 (m, 17H), 2.58−2.79 (m, 2H), 3.49−3.56 (m, 1H, 3α-*H*), 5.00 (t, J = 2.46 Hz, 1H, 20-C*H*), 5.36 (d, J = 5.12 Hz, 1H, H-6); ^{13}C NMR ($CDCl_3$) δ 17.8, 19.4, 20.7, 23.9, 26.9, 30.3, 31.5, 31.5, 31.6, 34.6, 36.6, 37.2, 42.2, 45.9, 49.9, 54.1, 71.6, 87.9, 121.1, 140.8, 180.8. Anal. ($C_{21}H_{29}NO$) C, H.

(*E*)-(3β-Hydroxy-5-androsten-17-ylidene)acetaldehyde (25). To a solution of (*E*)-3β-hydroxy-5-androsten-17-ylidene)acetonitrile (**24**) (150 mg, 0.48 mmol) in dry dichloromethane (15 mL) was added at −78 °C a solution of DIBAL-H (1 M in dichloromethane, 1.44 mmol), and the reaction mixture was stirred for 30 min at −78 °C and for an additional 5 h at room temperature. The reaction mixture was diluted with dichloromethane, and a saturated aqueous solution of sodium potassium tartrate was added. The organic layer was extracted with brine and was dried over anhydrous Na_2SO_4, and the solvent was evaporated *in vacuo* to afford (*E*)-(3β-hydroxy-5-androstene-17-ylidene)acetaldehyde (**25**), which was pure enough to be used as such in the next step: yield 151 mg, 93%; mp 169−172 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (s, 3H, CH_3), 1.04 (s, 3H, CH_3), 1.08−2.33 (m, 17H), 2.78−3.01 (m, 2H), 3.47−3.57 (m, 1H, H-3α), 5.37 (d, J = 5.10 Hz, 1H, H-6), 5.75 (d, J = 8.10 Hz, 1H, H-20), 9.87 (d, J = 7.80 Hz, 1H, C*H*O); ^{13}C NMR ($CDCl_3$) δ 17.9, 19.4, 20.8, 24.3, 27.7, 31.58, 31.63, 34.8, 36.6, 37.2, 42.2, 46.3, 50.2, 53.4, 71.6, 119.5, 121.1, 140.8, 180.0, 192.3. Anal. ($C_{21}H_{30}O_2$) C, H.

(*E*)-17-(2-Hydroxyethylidene)-5-androsten-3β-ol (26). To a solution of (*E*)-3β-hydroxy-5-androsten-17-ylidene)acetaldehyde (**25**) (75 mg, 0.24 mmol) in MeOH (2.5 mL) were sequentially added $CeCl_3 \cdot 7H_2O$ (178 mg, 0.48 mmol) and $NaBH_4$ (20 mg, 0.48 mmol). After completion of the reaction saturated aqueous NH_4Cl was added until pH 7. The mixture was diluted with ethyl acetate, the organic layer was extracted with brine and was dried over anhydrous Na_2SO_4, and the solvent was evaporated *in vacuo*. The residue was purified by flash column chromatography (elution solvent: petroleum ether, 40−60 °C/acetone (8:2)) to afford alcohol **26** (70 mg, 92%): mp 128−131 °C; ^{1}H NMR ($CDCl_3$) δ 0.78 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 1.08−2.38 (m, 19H), 3.49−3.53 (m, 1H, H-3α), 4.05−4.19 (m, 2H, CH_2OH), 5.23−5.27 (m, 1H, H-20), 5.36 (d, J = 5.18 Hz, 1H, H-6); ^{13}C NMR ($CDCl_3$) δ 18.8, 19.7, 21.2, 24.6, 26.4, 29.9, 31.8, 31.9, 35.9, 36.9, 37.5, 42.5, 44.1, 50.7, 54.7, 60.6, 71.9, 115.8, 121.7, 141.1, 155.9. Anal. ($C_{21}H_{32}O_2$) C, H.

(20*R*)-3β,21-Dihydroxy-17α,20-epoxy-5-pregnene (27). To a solution of compound **26** (70 mg, 0.22 mmol) in dry dichloromethane (2.2 mL) were added at 0 °C K_2CO_3 (36 mg, 0.26 mmol) and *m*-CPBA 55% (61 mg, 0.22 mmol), and the mixture was stirred at 0 °C for 2 h. The solid was filtered off, and the filtrate was evaporated *in vacuo* and was purified by flash column chromatography (elution solvent: hexane/acetone/25% NH_4OH (5/3/0.1)) to afford epoxide **27** (29 mg, 40%): mp 138−141 °C; $[\alpha]^{20}_D = -77.58°$ (c = 0.0097/mL, MeOH); ^{1}H NMR ($CDCl_3$) δ 0.82 (s, 3H, CH_3), 1.02 (s, 3H, CH_3), 0.99−2.34 (m, 19H), 3.05 (dd, J = 3.6, 6.8 Hz, 1H, 20-C*H*), 3.54−3.60 (m, 1H, 3α-*H*), 3.60 (m, 1H, 21-C*H*), 3.85 (ddd, J = 3.5, 7.0, 11.5 Hz, 1H, 21-C*H*),

5.37 (d, $J = 5.1$ Hz, 1H, 6-C*H*); ^{13}C NMR δ 15.9, 19.4, 20.5, 24.0, 27.5, 30.4, 31.5, 31.9, 36.6, 37.2, 41.9, 42.2, 50.0, 52.5, 57.3, 62.7, 71.7, 73.9, 121.3, 140.8. Anal. ($C_{21}H_{32}O_2$) C, H.

Molecular Modeling and Conformational Analysis. Computer calculations were performed on a Silicon Graphics O2 workstation using QUANTA 97 software purchased from MSI, applying the CHARMm energy function and force field. The initial structures were energy minimized using steepest descents, conjugate gradient and Powell algorithms. The minimized structures were subjected to grid scan search around torsion angles τ_1 and τ_2 from 0° to 360°, applying a torsion step variation of 10°. The derived conformations were optimized by applying the steepest descents algorithm with 2000 steps and an energy gradient tolerance of 0.001 kcal mol^{-1} Å^{-1} as convergence criterion. The dielectric constant was set to $\varepsilon = 1$ to simulate $CDCl_3$ environment. Visualization of the 3D structures has been enabled by the use of Accelrys DS visualizer s/w.

NMR Spectroscopy. NMR spectra were recorded on a Varian 600 MHz spectrometer. The sample concentration was approximately 10 mM. Compounds were dissolved in $CDCl_3$. The DQF-COSY, ^{1}H−^{13}C-edited HSQC, and ^{1}H−^{13}C HMBC, all run with the use of field gradients, facilitated the assignment. The optimum mixing time for the NOESY experiment was found to be 150 ms. Data processing, including sine-bell apodization, Fourier transformation, and phasing, were performed with the use of VNMR routines.

Cell Cultures. MCF-7 (human breast adenocarcinoma) cells and LNCaP (human prostate adenocarcinoma) cells were obtained from ATCC (American Tissue Culture Collection), whereas Ishikawa (human endometrial adenocarcinoma) cells were obtained from ECACC (European Collection of Cell Cultures). All cells were maintained as recommended by the supplier. If not stated otherwise, fine chemicals and culture media were purchased from Sigma-Aldrich, and sera were from Gibco (Invitrogen). ICI 182,780 was purchased from Tocris Bioscience.

PC12 cells were obtained from Dr. M. Greenberg (Children's Hospital, Boston, MA). Cells were maintained in culture at 5% CO_2, at 37 °C, in RPMI 1640 containing 2 mM L-glutamine, 15 mM HEPES, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 10% horse serum, and 5% fetal calf serum (horse serum and fetal calf serum were charcoal-treated for removing endogenous steroids). Serum-free medium was supplemented with 0.1% bovine serum albumin (BSA) and 10 nM sigma1 receptor specific antagonist haloperidol to avoid potential interactions of neurosteroids with sigma1 receptors. Conjugate DHEA−BSA was initially diluted in phosphate-buffered saline (PBS). Prior to experiments, stock solutions of DHEA−BSA were mixed with 3% charcoal−0.3% dextran for 30 min, centrifuged at 3000*g* for 10 min, and finally passed through a 0.22 μm filter in order to remove any free DHEA. In all experiments cells were incubated with neurosteroids diluted in serum-free medium.

Measurement of Apoptosis. Apoptosis in PC12 cells was assayed using two different methods.

(**a**) **The APOPercentage apoptosis assay** (Biocolor Ltd., Belfast, N. Ireland) was used to quantify apoptosis, according to the manufacturer's instructions. The assay uses a dye that stains red the apoptotic cells undergoing the membrane "flip-flop" event when phosphatidylserine is translocated to the outer leaflet. Detection of apoptosis can be readily observed under inverted microscopy. For apoptosis quantitation, the amount of dye within the labeled cells can subsequently be released into solution, and the concentration is measured at a wavelength of 550 nm (reference filter 620 nm) using a color filter microplate colorimeter (Dynatech MicroElisa reader, Chantilly, VA). Apoptosis was measured at 24 h.

(**b**) **Flow cytometry (FACS)** of annexin V−propidium iodide-stained cells was also used. PC12 cells were transferred to a staining tube and washed with 4 mL of PBS, containing 1% BSA, at 4 °C. After medium removal (200 rpm, 10 min, 4 °C), 100 μL of 2 μg/mL annexin V−FITC was added in a staining buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, pH 7.4) and incubated for 10 min in the dark. Then, 1 μg per tube of propidium iodide was added, and cells were analyzed within 20 min by flow cytometry using a Beckton-Dickinson FACSArray apparatus (Beckton-Dickinson, Franklin Lakes, NJ) and analyzed with the CELLQuest (Beckton-Dickinson) and ModFit LT (Verify Software, Topsham, MN) software.

Displacement of Membrane Binding of [^{3}H]DHEA with Synthetic Spiro-Neurosteroids. PC12 cells were cultured in 225 cm flasks, and after being washed twice with PBS, they were detached from the flasks by vigorous shaking. After a centrifugation at 1500*g*, they were homogenized by sonication in a 50 mM Tris-HCl buffer, pH 7.4 at 4 °C, containing freshly added protease inhibitors (1 mM PMSF and 1 μg/mL aprotinin). Unbroken cells were removed by centrifugation at 1500*g* (10 min at 4 °C), and membranes were collected by centrifuging at 102000*g* for 1 h at 4 °C. Membranes were washed once with Tris-HCl, briefly acidified at 4 °C (for 3 min) with 50 mM glycine (pH 5.0), and resuspended in the same buffer. Protein content was assayed by the method of Bradford using reagents from Bio-Rad (Hercules, CA).

Membranes (at a final concentration 2 mg/mL) were incubated with 5 nM [^{3}H]DHEA in the absence or the presence of DHEA or the new spiro-neurosteroids at concentrations varying from 10^{-12} to 10^{-6} M, at a final volume of 100 μL, in Tris-HCl buffer (50 mM, pH 7.4). After a 30 min incubation in a water bath at 37 °C, membranes were collected on GF/B filters, prewet in 0.5% PEI solution at 4 °C. The filters were washed three times with ice-cold Tris-HCl, dried, and counted in a β-scintillation counter (PerkinElmer, Foster City, CA) with 60% efficiency for tritium. All experiments were performed in triplicates.

Western Blot Analysis. PC12 cells were harvested and lysed in lysis buffer supplemented with freshly added proteinase inhibitors phenylmethanesulfonyl fluoride (10 μg/mL) and 1 μg/mL aprotinin for the detection of Bcl-2 proteins (antibodies from Cell Signaling Technologies, Beverly, MA). Membranes were processed according to standard Western blotting procedures. To detect protein levels, membranes were incubated with the appropriate antibodies. Anti-actin monoclonal antibody was supplied by Chemicon (Temecula, CA). Secondary antibodies used were horseradish peroxidase-conjugated anti-mouse IgG (Chemicon) and horseradish peroxidase-conjugated anti-rabbit IgG (Immunotech, France). Then, the membranes were exposed to Kodak X-Omat AR films. A PC-based Image Analysis program was used to quantify the intensity of each band (Image Analysis, Inc., Ontario, Canada).

Measurement of Dopamine. PC12 cells were grown in six-well plates coated with poly(L-lysine) at a concentration of 10^6 cells per well. Cells were incubated with neurosteroids for several time periods; for the short-term experiments the incubation time ranged from 5 to 30 min and for the long-term experiments from 3 to 48 h. One milliliter of supernatants was transferred to tubes containing 200 μL of 0.1 M HCl for measurement of catecholamines. It should be noted that dopamine is stable in acidic environment. Dopamine was measured by radioimmunoassay (TriCatRIA, RE29395; IBL Immuno Biological Lab, Hamburg, Germany) using ^{125}I as tracer. Dopamine was extracted by a *cis*-diol-specific substrate, converted to 3-methoxytyramine using COMT (freshly prepared every time) as enzyme and *S*-adenosyl-L-methionine as coenzyme, and simultaneously acylated to *N*-acyl-3-methoxytyramine. After separation of the bound from the free labeled antigen by precipitation and centrifugation (bound ^{125}I-labeled antigen was precipitated with a second antibody), the amount of bound radioactivity of the precipitates was measured in a gamma counter (1275 minigamma; LKB Wallac). The analytical sensitivity of the method was 30 pg/mL for dopamine, its intraassay CV was 9.5%, and its interassay CV was 16.7%.

RT-PCR. Total RNA was extracted from PC12 cells using Trizol reagent (Invitrogen Life Technologies, CA). One microgram of total RNA was reverse transcribed by the ThermoScript RT-PCR system (Invitrogen) using random hexamers in a total volume of 20 μL. Two microliters of the RT product was used as a template, amplified by PCR using 2 mM $MgCl_2$, one strength PCR buffer, 0.2 mM sense and antisense primers, 0.2 mM dNTPs, and 2.5 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer ABD, Foster City, CA) in a final reaction volume of 50 μL. PCR was performed in a Perkin-Elmer DNA thermal cycler. Primers for TH were 5′-TCGCCACAGCCCAAGGGCTTCAGAA-3′ (sense) and 5-CCTCGAAGCGCACAAAATAC-3 (antisense) and for GAPDH were 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ (sense) and 5′-CATGTGGGCCATGAGGTCCACCAC-3′ (antisense). Oligonucleotides were synthesized by MWG-Biotech AG (Munchen, Germany). After reverse transcription, the cDNA product was amplified by PCR at 33 cycles. The cycle number (33) was chosen such that amplification of the products was in the linear range with respect to the amount of input cDNA. PCR for GAPDH was performed in parallel to ensure good quality of RNA and cDNA preparations. Each cycle consisted of 60 s at 92 °C for denaturation, 120 s at 53 °C for annealing, and 180 s at 72 °C for extension (60 s at 98 °C, 90 s at 55 °C, and 150 s at 72 °C for GAPDH, respectively). Ten microliters of the amplified products (368 bp for TH and 983 bp for GAPDH) was separated on a 2% agarose gel and visualized by ethidium bromide staining.

Effects on the Expression of Estrogen Receptor- (ER-) Regulated Genes. The neurosteroids were assessed for induction of ERE-dependent gene expression through ERα and ERβ using MCF-7:D5L and HEK:ERβ cells, respectively. MCF-7:D5L cells, an ERα-expressing clone of MCF-7 cells that is stably transfected with the estrogen-responsive reporter plasmid pERE-Gl-luciferase, have been described.[42] HEK:ERβ cells, a clone of human embryonic kidney (HEK293) cells that is stably transfected with the estrogen-responsive reporter plasmid pERE-Tk-luciferase and an expression plasmid coding for the full-length human ERβ, have also been described.[45] Assessment of neurosteroid regulation of luciferase expression in MCF-7:D5L and HEK:ERβ cells was carried out as already described[42,45] with minor modifications. Briefly, the cells were plated in 96-well microculture plates at a density of 12000 cells per well in DMEM (Dulbecco's modified minimum essential medium) devoid of phenol red and supplemented with 1 μg/mL insulin and 10% DCC−FBS, i.e., fetal bovine serum (FBS) that was treated with 10% dextran-coated charcoal (DCC) to remove endogenous steroids as already described.[53] After plating (72 h), the cells were exposed to increasing concentrations of test compounds (stock solutions were prepared using tissue culture grade DMSO as vehicle) in the absence (vehicle was added in this case up to a final concentration ≤0.2%) or presence of 1 nM estradiol or 1 μM ICI 182,780 for 16 h, and the induction of luciferase was assessed using the Steady-Glo luciferase assay system (Promega). Controls for full agonism (cells exposed only to 1 nM estradiol), full antagonism (exposed to 1 μM ICI 182,780 as well as to 1 nM estradiol), and nonagonism/antagonism (exposed only to vehicle) served to classify the test compounds that significantly affected luciferase expression as full, partial, or weak ERα or ERβ agonists or antagonists as described in the legend to Table 1.

Test neurosteroids were assessed on the alkaline phosphatase (AlkP) expression of Ishikawa cells using 96-well microculture plates that were plated with 12000 cells per well in phenol red-free MEM supplemented with 1 μg/mL insulin and 5% DCC−FBS. After plating (24 h), cells were exposed to test compounds in the absence or presence of 0.1 nM estradiol or 1 μM ICI 182,780 for 72 h, and compound effects on AlkP activity were assessed as recently described.[46] Cells exposed only to vehicle, ICI 182,780, and/or estradiol served as controls, and those of test neurosteroids that significantly affected AlkP expression were classified as agonists or antagonists as described above.

Effects on the Growth of Endocrine Cancer Cells. The effect of test compounds on the growth of MCF-7 cells was assessed as recently described[54] with minor modifications. Briefly, the cells were plated in 96-flat-bottomed-well microplates at a density of 8000 cells per well in phenol red-free MEM supplemented with 1 μg/mL insulin and 5% DCC−FBS. After plating (72 h), the cells were exposed for 96 h to test compounds, and relative numbers of viable cells were determined by monitoring MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] conversion to formazan at 550 nm. Cells exposed only to vehicle, 1 μM ICI 182,780, and/or 0.1 nM estradiol served as controls. Statistically significant effects of the test compounds on cell growth were classified as full, partial, or weak as described in the legend to Table 2.

The effect of test compounds on the growth of LNCaP cells was assessed as described above, except that cells were plated in polylysine-coated 96-flat-bottomed-well microplates at a density of 5000 cells per well in phenol red-free RPMI supplemented with 10% DCC−FBS. After plating (72 h), the cells were exposed for 96 h to test compounds, and the effects of test compounds on cell growth, as assessed using MTT, were classified as described above. Cells exposed only to vehicle, 10 μM ICI 182,780, and/or 10 nM estradiol served as controls.

The effect of test compounds on the growth of Ishikawa cells was determined as described for MCF-7 cells, except that test compounds were added to the cells 24 h after plating, and the effects of test compounds on cell growth were assessed and classified as above.

MTT Assay for Cell Viability. PC12 (rat adrenal pheochromocytoma), HEK293 (human embryonic kidney), or HT22 (mouse hippocampal neuroblastoma) cells were seeded in 96-well plates at a density of 2×10^4 cells/mL and maintained for 24 h in RPMI 1640 supplemented with 10% horse serum and 5% fetal bovine serum (PC12) or DMEM supplemented with 10% fetal bovine serum (HEK293 and HT22). They were then incubated for various time periods (24−72 h) in the absence or presence of various concentrations of compounds **20**, **23**, and **27** (0.1−10 μM). At the end of incubation cells were treated with 5 mg/mL MTT for 4 h at 37 °C, then the medium was removed, and the cells were dissolved with dimethyl sulfoxide. The formazan reduction product was measured by reading the absorbance at 570 nm in a plate reader. Results (mean ± SEM of three replicate values in three independent experiments) are expressed as percentage of untreated control cultures. This assay is based on the cellular reduction of a tetrazolium salt (MTT) into a colored formazan product by mitochondrial enzymes, and therefore, it is a reliable means to indirectly assess the number of viable cells.[55]

Statistical Analysis. Results (OD in apoptosis measurements or arbitrary densitometric units for Western blot proteins normalized to total protein or actin) were expressed as the percentage of parallel estimates for control cells, treated only with vehicle. Data were subjected to analysis of variance, *post hoc* comparison of means followed by the Fisher's least significance difference test. For percent changes we have used the nonparametric Kruskal−Wallis test for several independent samples. Differences were considered significant for values of $p < 0.05$.

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Supporting Information Available: ^{1}H and ^{13}C data, HMBC correlations, and COSY and NOE interactions for compounds **3**, **5**, **7**, **9**, **10**, **14**, **15**, **23**, and **27** (Tables 1−9); experimental procedure for the synthesis of 3α-*n*-butyl-17,17-ethylenodioxy-5-androstene-3β-ol and spectroscopic data thereof (Table 10); conformational minima of compounds **3**, **5**, **7**, **9**,

10, **14**, and **15** (Figure 1); ^{1}H NMR spectra of compounds **3**, **5**, **7**, **9**, **10**, **14**, **15**, **23**, **27**, and **28**; elemental analysis data for compounds **1**−**3**, **5**, **7**, **9**, **11**, and **14**−**27**. This material is available free of charge via the Internet at http://pubs.acs.org.

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