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Original article

Synthesis and steroid sulphatase inhibitory activity of C19- and C21-steroidal derivatives bearing a benzyl-inhibiting group

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Abstract – Two series of compounds, benzyl alkylated at position 17α and 20 of androstane and pregnane, respectively, were synthesised and tested for steroid sulphatase inhibition. We compared the ability of the compounds to inhibit steroid sulphatase obtained from two different sources (homogenates of transfected HEK-293 cells and Jeg-3 cells) and with two types of substrate (DHEAS or E_1S). The inhibitory activity of 17α-benzyl-5α-androstane-3β,17β-diol (**7**), 17α-benzyl-5-androstene-3β,17β-diol (**9**), 17α-benzyl-4,17β-dihydroxy-4-androsten-3-one (**15**) and 20-benzyl-5-pregnene-3β,20α-diol (**16**) has proven to be superior to that of danazol, the first steroid sulphatase inhibitor to be reported, but still lower than that of the potent inhibitor estrone-3-*O*-sulphamate. The inhibitory activity of compound **7** was as potent as that of its previously reported estrane analogue, 17α-benzyl estradiol. Benzyl alkylated compounds with no OH group on the A-ring (with a 4-OCH_3, 4-Cl, or 4-H and their precursor epoxides), as well as a series of basic steroids without a benzyl group (ADT, epi-ADT, 3α-diol, 3β-diol, DHEA, Δ^5-diol, DHT, T, Preg and Prog), did not show steroid sulphatase inhibition. We have thus demonstrated that the steroid sulphatase inhibitory effect of a benzyl group, previously observed for an estrane nucleus, can be extended to certain androstane and pregnane nuclei bearing a 3β-OH or a 4-OH group. Inhibitors **7**, **9**, **15** and **16** did not induce any proliferative effect on androgen-sensitive Shionogi cells. However, when tested on oestrogen-sensitive ZR-75-1 cells, a proliferative effect was observed for **7** and **9**, but not for **15** and **16**. © 2001 Éditions scientifiques et médicales Elsevier SAS

steroid / chemical synthesis / sulphatase / enzyme / inhibitor / cancer

1. Introduction

A strategy expected to bring encouraging improvements when applied to the treatment of hormone-sensitive cancers consists in the inhibition of steroid sulphatase, the enzyme controlling the conversion of steroid sulphates into unconjugated steroids [1, 2]. Dehydroepiandrosterone sulphate (DHEAS), the main secretory product of human adrenals and the second most abundant steroid in circulation after cholesterol, is converted by steroid sulphatase into the steroid DHEA. The transformation of DHEA into active sex steroids requires the presence of a series of enzymes, namely 3β-hydroxysteroid dehydrogenase/Δ^5,Δ^4-isomerases (3β-HSDs), 17β-hydroxysteroid dehydrogenases (17β-HSDs), 5α-reductases, and aromatase. DHEA is thus transformed in peripheral tissues into the active androgen dihydrotestosterone (DHT), or into oestrogens, namely estradiol (E_2), estrone (E_1) and 5-androstene-3β,17β-diol (Δ^5-diol) [3, 4]. Estrone sulphate (E_1S) is the most abundant circulating oestrogen in women [5, 6], and its conversion into E_1 by steroid sulphatase directly provides a substrate to type 1 17β-HSD, which then synthesises estradiol, the most active oestrogen [7].

Recent studies indicated that the enzymes necessary for steroid hormones biosynthesis are expressed in tumoural tissues [8]. In postmenopausal breast cancer, concentrations of E_1S in peripheral blood are relatively high and oestrogen levels in tumours are 10–50-fold higher than in the plasma [9]. The half-life of E_1S

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in the blood is 10–12 h, which is considerably longer than that of the unconjugated oestrogens (30 min) [10]. These facts taken together indirectly support the hypothesis that, in hormone-sensitive tumours, the active steroids are synthesised intracellularly, from circulating steroid sulphates, and through the catalytic activity of the expressed steroidogenic enzymes [11, 12]. Furthermore, direct evidence for in situ oestrogen synthesis involving sulphatase activity has been reported in nitrosomethylurea-induced mammary tumours in rat [13]. Thus, the utilisation of steroid sulphatase inhibitors to reduce hormone synthesis in the endocrine-sensitive cancers might significantly improve therapy.

Some research groups have reported the development of irreversible steroid sulphatase inhibitors derived from E_1, E_1S and E_1-phosphate, and also of non-steroidal inhibitors, the structure of which includes the aromatic ring of E_1 [1, 2, 14–17]. Recently, our group has found that an alkyl, benzyl, or substituted benzyl group introduced at position 17α of estradiol induces a reversible inhibitory effect on steroid sulphatase [18]. We hypothesised that this new family of inhibitors, represented by 17α-benzyl estradiol (**1**) may inhibit steroid sulphatase by a reversible

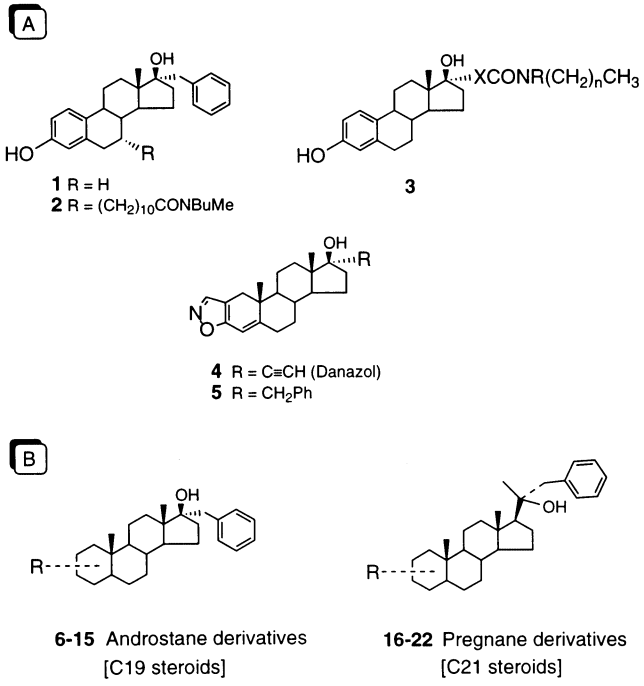

Figure 1. Steroid derivatives developed (A) or proposed (B) to inhibit the steroid sulphatase.

interaction (probably hydrophobic) with a region located within the enzymatic site [19]. Unfortunately, most of these estrane type inhibitors possess intrinsic oestrogenic activity, which is not appropriate for a potential therapeutic agent for oestrogen-sensitive diseases.

In our efforts to develop steroid sulphatase inhibitors free of oestrogenic activity (*figure 1*), we first replaced the Cl8-steroid estradiol nucleus of inhibitor **1** by the pure antioestrogen nucleus of ICI 164384 [20–22]. The long and bulky 7α-alkylamide side chain of compound **2** drastically reduced the inhibitory effect of the 17α-benzyl group [23]. Some promising results were however obtained by introducing at position 17α of estradiol a hydrophobic octyl group within a propanamide side chain (compound **3**), but mixed oestrogenic/antioestrogenic activities were observed that will necessitate further SAR optimisation studies [24]. In another approach, the benzyl group was introduced at position 17α of danazol (**4**), the first inhibitor of steroid sulphatase to be reported [25, 26], but no significant improvement of inhibitory activity was obtained with compound **5** [23]. Finally, we investigated C19 and C21 steroids as nonoestrogenic nuclei, with a number of the C19-steroids maintaining analogy with 4-hydroxy-androstenedione, a strong inhibitor of aromatase [27–29]. Our compounds were designed to inhibit steroid sulphatase via a benzyl group added in position 17α of a C19-steroid (compounds **6**–**15**) or in position 20 of a C21-steroid (compounds **16**–**22**). We here report the chemical synthesis of these compounds, their steroid sulphatase inhibitory activity as well as proliferative activity on androgen-sensitive Shionogi cells and oestrogen-sensitive ZR-75-1 cells.

2. Chemistry

A series of 17 benzyl alkylated C19- and C21-steroids (compounds **6**–**22**) were synthesised following the two pathways reported in *figures 2* and *3*. Compounds **6**–**8** were obtained through the sequence of reactions presented in *figure 2A*. Androsterone (ADT) and epiandrosterone (epi-ADT) were thus alkylated at position 17 using an excess of benzyl magnesium chloride to give diols **6** and **7** in 49 and 90% yield, respectively. For alkylation of epi-ADT, the use of anhydrous cerium(III) chloride as catalyst [30] was responsible for the higher yield of diol **7**. As

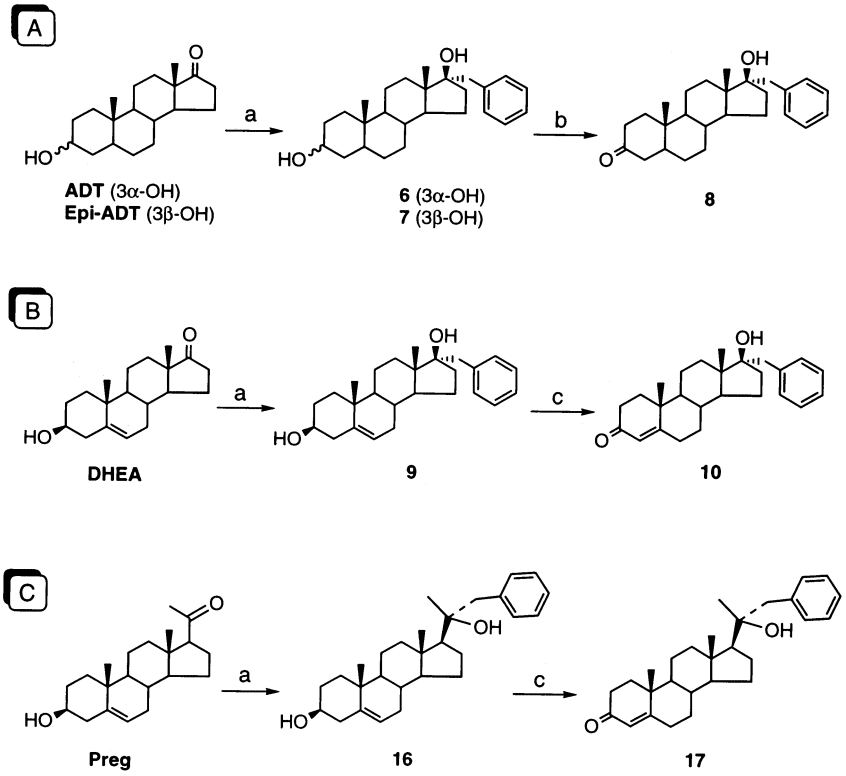

Figure 2. Reagents and conditions: (A) $PhCH_2MgCl$, $CeCl_3$, THF, −78 to −40 °C, 10–12 h; (B) PCC, NaOAc, CH_2Cl_2, 3 h; (C) cyclohexanone, Al(*i*-PrO)$_3$, toluene, reflux, 3 h.

previously reported [19], the addition of a Grignard reagent to a C17-steroidal ketone afforded almost exclusively the 17α-alkylated product. Pyridinium chlorochromate oxidation of the secondary alcohol of **7** in dichloromethane gave the 3-ketosteroid **8** in 81% yield. As reported in *figures 2B* and *2C*, the benzyl Grignard reagent was also added to dehydroepiandrosterone (DHEA) and pregnenolone (Preg) leading to the formation of corresponding tertiary alcohols **9** and **16** in high yields (91 and 91%, respectively) when anhydrous cerium(III) chloride was used as catalyst [30]. In the case of C21-steroid derivative **16**, the stereochemistry at position 20 was determined according to Cram's rule and literature data [31]. A minor 20β-OH isomer (6%) was also observed by NMR of the crude product of alkylation, but only the major 20α-OH isomer (91%) was used for the synthesis of compound **17**. Both compounds **9** and **16** were thereafter submitted to Oppennauer oxidation conditions (cyclohexanone and Al(*i*-PrO)$_3$ in refluxing toluene) to give the α,β-unsaturated ketosteroids **10** (88%) and **17** (73%).

The classical synthesis procedure of 4-hydroxy-androstenedione generally involves formation and opening of a 4,5-epoxy-steroid [32, 33]. As reported in *figure 3*, unsaturated ketones **10** and **17** were transformed into the corresponding epoxides **11**, **12** and **18**, **19** (as a 1:4 mixture of α:β epoxides), which were separated by chromatography or submitted as a mixture to conditions of epoxide opening. In our case, however, the instability under acidic conditions of the tertiary alcohol generated by alkylation at position 17 (androstane series) [34] or position 20 (pregnane series) was not compatible with the strong acid conditions required for epoxide opening. Indeed, acid opening of our 4,5-epoxy-steroids with 2% H_2SO_4 in AcOH or formic acid (with or without reflux) failed, mainly giving products of dehydration or rearrangement. Similarly, no C4-chlorination of both unsaturated ketones **10** and **17** occurred with a sulphuryl chloride treatment, and a mixture of rearrangement products was formed. Instead, chloro derivatives **13** (74%) and **20** (88%) were obtained by a mild acidic treatment (HCl in acetone at room temperature) of

epoxides **11**, **12** and **18**, **19**. No side products resulted from these conditions. In basic conditions (NaOH in refluxing methanol), these epoxides were opened yielding the 4-methoxy derivatives **14** and **21** (57 and 50%, respectively). Since attempts to hydrolyse these methoxy compounds by aqueous HCl in refluxing dioxane and to generate the hydroxy analogues were not successful, another strategy was tried. A direct substitution of vinylic chloride of **13** and **20** using potassium hydroxide (3 equiv. in refluxing *t*-butanol) allowed the formation of the desired 4-hydroxy-steroids **15** and **22** in reasonable yields (42 and 45%, respectively).

3. Biological results

3.1. Inhibition of steroid sulphatase

The level of inhibition of steroid sulphatase by compounds from the androstane and pregnane series, as well as by other compounds of interest, is presented in *tables I and II*, respectively. As a source of steroid sulphatase activity, we used homogenates of transfected human embryonic kidney (HEK)-293 cells or homogenates of human carcinoma Jeg-3 cells. With HEK-293 cells, only DHEAS was used as substrate, whereas for Jeg-3 cells both classical substrates (DHEAS and E_1S) were used for the enzymatic assays. With the Jeg-3 cells, a similar level of inhibition was observed for both substrates. When DHEAS was used as a substrate, the range of inhibition differed from one cell line to the other, but the conclusions regarding the activity of each compound remain valuable. Indeed, for a given concentration of inhibitor, the percentages of inhibition were slightly higher with HEK-293 cells than with Jeg-3 cells. Some reference steroids, such as ADT, epi-ADT, 3α-diol, 3β-diol, DHEA, Δ^5-diol, DHT, T, Preg and Prog, were also tested in order to provide data for substrate comparative studies. However, no inhibition of steroid sulphatase by these steroids was observed. Three known inhibitors, estrone-3-*O*-sulphamate [35], 17α-benzyl estradiol [18, 19] and danazol [25, 26], were also investigated for their inhibitory activity in the same protocol.

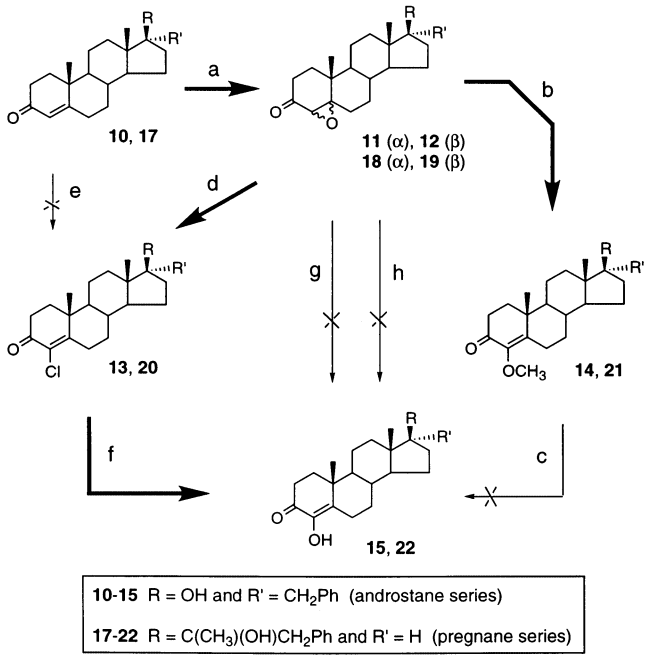

Figure 3. Reagents and conditions: (a) NaOH, H_2O_2, CH_3OH, 0 °C, 12 h; (b) NaOH, MeOH, reflux, 3 h; (c) HCl, H_2O, dioxane, reflux; (d) HCl, acetone, r.t., 1 h; (e) SO_2Cl_2, pyridine, r.t.; (f) KOH (solid), *t*-butanol, reflux, 40 min; (g) HCOOH, reflux; (h) 2% H_2SO_4 in AcOH, 0 °C, 12 h. See *figure 1* for the C20 stereochemistry of compounds **17**–**22**.

In the series of synthesised compounds, ketone **8**, α,β-unsaturated ketones **10**, **13**, **14**, **17**, **20** and **21** and ketoepoxides **11**, **12**, **18** and **19** do not inhibit steroid sulphatase. The absence of a polar hydroxy (or sulphate) group at position 3 of the steroid A-ring might be responsible for a low binding to the active site. Moderate activities were however noted for the keto-enols **15** and **22**, probably due to the presence of the hydroxyl functionality in position 4. Interestingly, no inhibition was obtained with 3α-OH steroid **6**, but a higher inhibitory activity was noted for compounds **7**, **9** and **16**, bearing a 3β-OH. The inhibitory activity of these compounds is higher than that of danazol. In fact, compound **7** is as potent inhibitor than 17α-benzyl estradiol, in both HEK-293 and Jeg-3 cells, using either DHEAS or E_1S as substrate. The inhibitory effect of the benzyl group of **7**, **9** and **16** is made especially clear by comparing it to that of their basic steroidal nuclei, respectively 3β-diol, Δ^5-diol and Preg. Without the benzyl group no inhibition of steroid sulphatase was observed.

3.2. Proliferative effect on AR^+ Shionogi and ER^+ ZR-75-1 cells

In order to be a therapeutic agent for androgen- and oestrogen-sensitive diseases, a steroid sulphatase inhibitor should be devoid of agonist effect on androgen and oestrogen receptors. To assess this, we determined

Table I. Inhibition of steroid sulphatase (%) by compounds **6**–**15** (androstane series) and other compounds of interest. [a,b]

Compounds [c]	HEK-293 cells (DHEAS → DHEA) 0.1 μM/1 μM	Jeg-3 cells (DHEAS → DHEA) 0.1 μM/1 μM	Jeg-3 cells (E_1S → E_1) 0.1 μM/1 μM
6	NA/NA [10/32] [d]	NA/NA	NA/NA
7	46/86 [70/98] [d]	21/71	23/57
8	8/9	0/0	0/9
9	24/69	4/24	13/53
10	4/9	0/0	0/1
11	0/3	0/0	0/2
12	0/0	0/0	0/6
13	27/5	0/0	0/2
14	0/0	0/0	0/2
15	6/62	0/25	3/27
ADT	0/0 [6/12] [d]	0/0	2/1
epi-ADT	0/0 [7/11] [d]	0/0	2/1
3α-Diol	0/0	0/0	0/4
3β-Diol	0/4	0/12	1/4
DHEA	6/24	0/0	4/0
Δ^5-Diol	0/0	0/0	3/2
DHT	0/0	0/0	3/4
T	3/0	6/0	1/2
E_1-3-*O*-sulphamate	89/91 [100/100] [d]	100/100	95/97
17α-Benzyl-E_2	23/72 [79/98] [d]	0/54	18/56
Danazol	18/37	0/29	3/22

[a] Tested at two concentrations of inhibitor (0.1 and 1 μM).
[b] Error ± 5%.
[c] ADT, androsterone; epi-ADT, epiandrosterone; 3α-diol, 5α-androstane-3α,17β-diol; 3β-diol, 5α-androstane-3β,17β-diol; DHEA, dehydroepiandrosterone; Δ^5-diol, 5-androstene-3β,17β-diol; DHT, dihydrotestosterone; T, testosterone.
[d] Tested at two concentrations of 0.3 and 3 μM. N/A: data not available.

Table II. Inhibition of steroid sulphatase (%) by compounds **16**–**22** (pregnane series) and other compounds of interest. [a,b]

Compounds [c]	HEK-293 cells (DHEAS → DHEA) 0.1 μM/1 μM	Jeg-3 cells (DHEAS → DHEA) 0.1 μM/1 μM	Jeg-3 cells (E_1S → E_1) 0.1 μM/1 μM
16	67/100	0/21	2/64
17	0/85	0/0	0/18
18	0/14	0/0	0/2
19	28/0	0/0	0/1
20	12/0	0/0	1/1
21	8/7	0/0	0/1
22	1/34	0/0	7/23
Preg	0/6	24/0	3/8
Prog	0/0	0/0	0/3
E_1-3-*O*-sulphamate	89/91	100/100	95/97
17α-Benzyl-E_2	23/72	0/54	18/56
Danazol	18/37	0/29	3/22

[a] Tested at two concentrations of inhibitor (0.1 and 1 μM).
[b] Error ± 5%.
[c] Preg, pregnenolone; Prog, progesterone.

the proliferative activities of inhibitors **7**, **9**, **15**, **16** and **22** on two AR^+ and ER^+ cell lines. As can be seen in *figure 4*, no proliferative effect on androgen-sensitive (AR^+) Shionogi cells was observed for all tested compounds at either concentration used (0.1 and 1 μM). Conversely, a 2.6-fold proliferative effect was obtained

when Shionogi cells were stimulated by 0.3 nM of the known androgen dihydrotestosterone. In a similar experiment using the oestrogen-sensitive (ER^+) ZR-75-1 cells and reported in *figure 5*, we can see that a 0.1 nM concentration of oestrogen estradiol provided a 3.4-fold stimulation of basal cell proliferation, while no proliferative effect was observed for compounds **15**, **16** and **22** at either concentration used (0.03 and 1 μM). On the other hand, compounds **7** and **9** stimulated the ZR-75-1 cell proliferation, especially at 1 μM. These results were somehow expectable considering their structural similarity to the weak oestrogenic steroids Δ^5-diol [36–38] and 3β-diol [39–41].

4. Conclusions

The inhibitory activity of compounds **7**, **9**, **15** and **16** on steroid sulphatase correspond to, or exceed, the values found for reference inhibitors, danazol and 17α-benzyl estradiol. Their inhibitory activity remains however lower than that of potent inhibitor estrone-3-*O*-sulphamate. When compared to their corresponding steroid nucleus, the results clearly show the inhibitory effect of a benzyl group located at the position 17α or 20 of some C19- and C21-steroids bearing an OH group on the A-ring. They also support the previous observations [19, 42] of a hydrophobic region in the enzyme neighbouring the D-ring of steroid substrates.

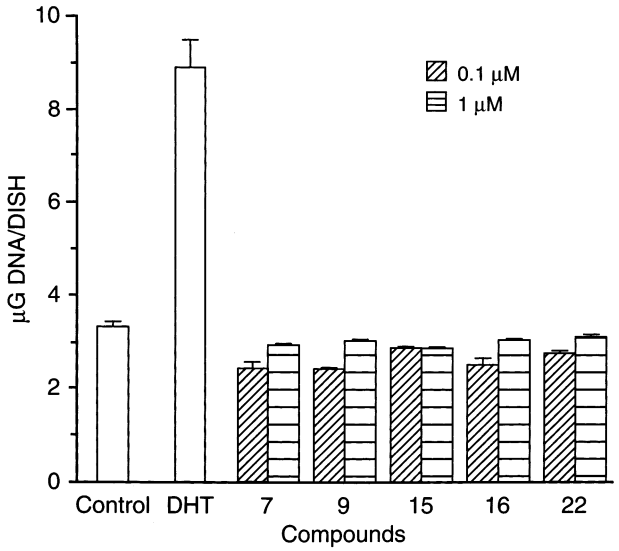

Figure 4. Effect of compounds **7**, **9**, **15**, **16** and **22** on the proliferation of androgen-sensitive (AR^+) Shionogi cells. One day after plating, cells were incubated for ten days with the indicated concentration (0.1 or 1 μM) of tested compounds **7**, **9**, **15**, **16** and **22**. DHT was tested at a concentration of 0.3 nM as a positive control of cell proliferation. Media were changed every 3–4 days. Results are expressed as the mean of DNA content (μg) ±SEM, in triplicate analysis.

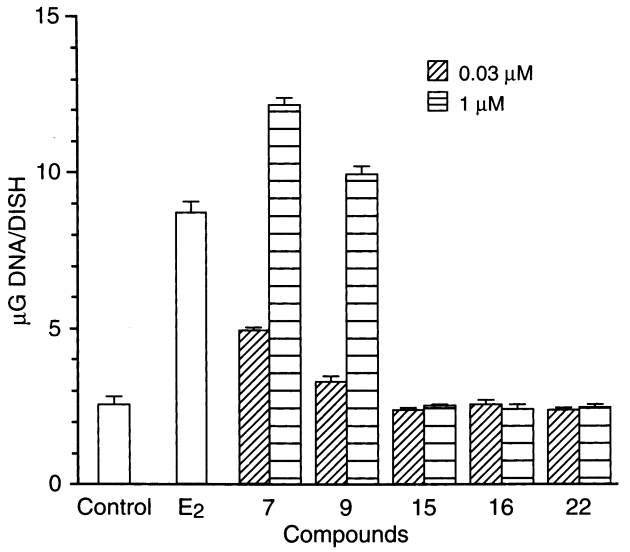

Figure 5. Effect of compounds **7**, **9**, **15**, **16** and **22** on the proliferation of oestrogen-sensitive (ER^+) ZR-75-1 cells. Three days after plating, cells were incubated for nine days with the indicated concentration (0.03 or 1 μM) of test compounds **7**, **9**, **15**, **16** and **22**. E_2 was tested at a concentration of 0.1 nM as a positive control of cell proliferation. Media were changed every second day. Results were expressed as the mean of DNA content (μg) ±SEM, in triplicate analysis.

5. Experimental

*5.1. Chemical synthesis of compounds **6–22***

5.1.1. General methods

Chemical reagents and anhydrous solvents (except THF) were purchased from Aldrich Chemical Company (Milwaukee, WI, USA), while starting steroids and basic steroids tested were from Steraloids (Wilton, NH, USA). Solvents were obtained from BDH Chemicals (Montréal, Canada) or Fisher Chemicals (Montréal, Canada). Dry THF was distilled from sodium benzophenone ketyl. Thin layer chromatography (TLC) was performed on 0.20 mm silica gel 60 F_{254} plates (E. Merck, Darmstadt, Germany), and 230–240 mesh ASTM silica gel 60 (E. Merck) was used for flash

column chromatography. Infrared (IR) spectra are expressed in cm^{-1} and were obtained on a Perkin–Elmer 1600 (series FTIR) spectrophotometer (Norwalk, CT, USA). ^{1}H- and ^{13}C-NMR spectra were recorded with a Bruker AC/F 300 spectrometer (Billerica, MA, USA), respectively, at 300 and 75 MHz. The chemical shifts (δ) are expressed in ppm and referenced to chloroform (7.26 ppm for ^{1}H and 77.00 ppm for ^{13}C). Only specific signals were reported in IR and ^{1}H-NMR whereas all signals were listed in ^{13}C-NMR. Fast atom bombardment-high resolution mass spectra (FAB-HRMS) (nitrobenzylalcohol matrix) were provided by the Centre Régional de Spectrométrie de Masse (Université de Montréal, Montréal, Canada). The purity of synthesised compounds was determined by HPLC (Waters Associates, Milford, MA, USA) by using an ultraviolet detector (205–280 nm). For the nomenclature of pregnane derivatives **16**–**22** having a 20-hydroxyl group, we used the arbitrary convention described by Fieser and Fieser [43].

*5.1.2. Alkylation of 17- and 20-ketosteroids with benzyl magnesium chloride (general procedure for **6**, **7**, **9** and **16**)*

In a 250 mL flask equipped with a magnetic stirrer, under argon atmosphere, was solubilised 1.478 g (6 mmol) of anhydrous cerium(III) chloride in 80 mL of dry THF, at room temperature (r.t.). After stirring 1 h, the mixture was cooled at −78 °C and 3 mL (6 mmol) of 2.0 M solution of benzyl magnesium chloride in THF was added dropwise. Stirring was continued for 1.5 h, and a 0 °C solution of epiandrosterone, dehydroepiandrosterone or pregnenolone (1 mmol) in 25 mL of THF was slowly added to the above mixture. Five minutes after addition, the −78 °C cooling bath was changed for a −40 °C one (dry ice–acetonitrile) and the stirring continued for 10–12 h. The reaction mixture was, thereafter, quenched with an aqueous AcOH solution (2 mL of AcOH in 75 mL of water), and the mixture let warm to r.t. Extraction with EtOAc (3×25 mL) followed by washing the combined organic phase with brine (25 mL), drying over anhydrous $MgSO_4$, and evaporation of the solvent gave the crude product of alkylation, which was further purified by flash column chromatography (hexanes–acetone 75:25 for compounds **7**, **9** and 85:15 for compound **16**). Compound **6** was prepared similarly as above from androsterone, except that 10 equivalents of benzyl magnesium chloride were used without cerium chloride. The purification of **6** by flash chromatography was done with hexanes–EtOAc, 80:20 as eluent.

*5.1.2.1. 17α-Benzyl-5α-androstane-3α,17β-diol (**6**)*

White solid (49% yield or 95% if correction for unreacted ADT). $R_f = 0.19$ (hexanes–acetone, 75:25). IR (KBr) ν 3455 (OH, alcohols). ^{1}H-NMR (acetone-d_6) δ 0.85 (s, CH_3-19), 0.92 (s, CH_3-18), 2.66 and 2.86 (2d, $J = 13.4$ Hz, AB system, CH_2Ph), 3.95 (s, CH-3β), 7.25 (m, 5H, CH_2Ph). ^{13}C-NMR (acetone-d_6) δ 33.01 (C1), 29.33 (C2), 65.91 (C3), 37.36 (C4), 39.75 (C5), 29.34 (C6), 32.66 (C7), 36.80 (C8), 55.37 (C9), 36.80 (C10), 21.18 (C11), 31.97 (C12), 47.57 (C13), 51.32 (C14), 24.07 (C15), 33.28 (C16), 83.49 (C17), 15.05 (C18), 11.57 (C19), 43.21 (CH_2Ph), 140.40 (C1′), 128.16 (C2′ and C6′), 131.92 (C3′ and C5′), 126.29 (C4′). LRMS: Calc. for $C_{26}H_{38}O_2$: 382.4 [M^+] and 400.4 [$M^+ + NH_4$]. HPLC purity = 98.6% (C-18 NovaPak column; MeCN–MeOH–H_2O, 35:35:30).

*5.1.2.2. 17α-Benzyl-5α-androstane-3β,17β-diol (**7**)*

White solid (90% yield). $R_f = 0.26$ (hexanes–acetone, 75:25). IR (KBr) ν 3348 (OH, alcohols). ^{1}H-NMR ($CDCl_3$) δ 0.68 (t_{app}, $J = 10.9$ Hz, CH-14), 0.85 (s, CH_3-19), 0.92 (s, CH_3-18), 2.60 and 2.86 (2d, $J = 13.2$ Hz, AB system, CH_2Ph), 3.59 (m, CH-3α), 7.28 (m, 5H, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 37.05 (C1), 31.40 (C2), 71.23 (C3), 38.14 (C4), 44.97 (C5), 28.62 (C6), 31.85 (C7), 36.42 (C8), 54.41 (C9), 35.59 (C10), 20.92 (C11), 31.48 (C12), 46.58 (C13), 50.54 (C14), 23.61 (C15), 33.67 (C16), 83.01 (C17), 14.55 (C18), 12.34 (C19), 42.36 (CH_2Ph), 138.43 (C1′), 131.00 (C2′ and C6′), 128.03 (C3′ and C5′), 126.20 (C4′). FAB-HRMS: Calc. for $C_{26}H_{37}O_2$: [$M^+ - H$], 381.27936. Found: 381.27870. HPLC purity = 96.1% (C-18 NovaPak column; MeCN–MeOH–H_2O, 35:30:35).

*5.1.2.3. 17α-Benzyl-5-androstene-3β,17β-diol (**9**)*

White solid (91% yield). $R_f = 0.26$ (hexanes–acetone, 75:25). IR (KBr) ν 3330 (OH, alcohols). ^{1}H-NMR ($CDCl_3$) δ 0.95 (s, CH_3-18), 1.06 (s, CH_3-19), 2.61 and 2.87 (2d, $J = 13.2$ Hz, AB system, CH_2Ph), 3.53 (m, CH-3α), 5.37 (d, $J = 4.9$ Hz, CH-6), 7.28 (m, 5H, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 37.31 (C1), 31.74 (C2), 71.67 (C3), 42.24 (C4), 140.88 (C5), 121.32 (C6), 31.28 (C7), 32.87 (C8), 50.91 (C9), 36.61 (C10), 20.77 (C11), 31.61 (C12), 46.32 (C13), 50.17 (C14), 23.71 (C15), 33.74 (C16), 83.04 (C17), 14.39 (C18), 19.39 (C19), 42.36 (CH_2Ph), 138.34 (C1′), 131.00 (C2′ and C6′) 128.06 (C3′ and C5′), 126.24 (C4′). FAB-HRMS: Calc. for $C_{26}H_{35}O_2$: [$M^+ - H$] 379.26370. Found: 379.26210. HPLC purity = 96.9% (C-18 NovaPak column; MeCN–MeOH–H_2O, 35:30:35).

*5.1.2.4. 20-Benzyl-5-pregnene-3β,20α-diol (**16**)*

White solid (91% yield). $R_f = 0.12$ (hexanes–acetone, 85:15); IR (KBr) ν 3400 (OH, alcohols). ^{1}H-NMR ($CDCl_3$) δ 0.89 (s, CH_3-18), 1.00 (s, CH_3-19), 1.19 (s, CH_3-21), 2.61 and 2.82 (2d, $J = 13.1$ Hz, AB system, CH_2Ph), 3.51 (m, CH-3α), 5.35 (d, $J = 5.0$ Hz, CH-6); 7.24 (m, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 37.21 (C1), 31.74 (C2), 71.70 (C3), 42.25 (C4), 140.77 (C5), 121.54 (C6), 31.59 (C7), 31.32 (C8), 49.98 (C9), 36.46 (C10), 20.90 (C11), 40.07 (C12), 42.82 (C13), 56.90 (C14), 23.87 (C15), 22.80 (C16), 58.60 (C17), 13.49 (C18), 19.34 (C19), 74.82 (C20), 26.34 (C21), 49.11 (*C*H_2Ph), 137.59 (C1′), 130.68 (C2′ and C6′), 128.01 (C3′ and C5′), 126.28 (C4′). FAB-HRMS: Calc. for $C_{28}H_{39}O_2$: $[M^+ - H]$ 407.29501. Found: 407.29400. HPLC purity = 96.3% (C-18 NovaPak column; MeCN–MeOH–H_2O, 35:45:20). About 6% of a minor 20β-OH isomer [43] was also observed in the NMR spectra of crude alkylation products, but this later was discarded during the purification by flash column chromatography.

*5.1.3. PCC oxidation of alcohol **7** to ketone **8***

In a 200 mL flask and under argon atmosphere, the alcohol **7** (200 mg, 0.52 mmol) dissolved in dry CH_2Cl_2 (20 mL) was added dropwise to a mixture of pyridinium chlorochromate (172 mg, 0.80 mmol), sodium acetate (131 mg, 1.60 mmol), and molecular sieves (4A) in CH_2Cl_2 (35 mL). After 3 h at r.t., the mixture was filtered on silica gel flash column (hexanes–EtOAc, 85:15) to give 160 mg (81% yield) of ketone **8**.

*5.1.3.1. 17α-Benzyl-17β-hydroxy-5α-androstan-3-one (**8**)*

White solid; $R_f = 0.36$ (hexanes–acetone, 75:25). IR (KBr): ν 3543 (OH, alcohol), 1707 (C=O, ketone). ^{1}H-NMR ($CDCl_3$) δ 0.79 (m, CH-14), 0.95 (s, CH_3-18), 1.05 (s, CH_3-19), 2.61 and 2.87 (2d, $J = 13.3$ Hz, AB system, CH_2Ph), 7.28 (m, 5H, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 38.59 (C1), 38.13 (C2), 211.88 (C3), 44.67 (C4), 46.78 (C5), 28.86 (C6), 31.35 (C7), 36.32 (C8), 53.85 (C9), 35.78 (C10), 21.13 (C11), 31.49 (C12), 46.57 (C13), 50.36 (C14), 23.62 (C15), 33.71 (C16), 82.93 (C17), 14.55 (C18), 11.49 (C19), 42.37 (CH_2Ph), 138.29 (C1′), 130.98 (C2′ and C6′), 128.09 (C3′ and C5′), 126.27 (C4′). FAB-HRMS calculated for $C_{26}H_{37}O_2$: $[M^+ + H]$ 381.27936. Found: 381.28060. HPLC purity = 95.2% (C-18 NovaPak column; MeCN–MeOH–H_2O, 40:30:30).

*5.1.4. Oppennauer oxidation of 3-hydroxy-5-ene-steroids (general procedure for **10** and **17**)*

In a 250 mL three-neck flask equipped with a condenser, a Dean–Stark apparatus, and kept under argon atmosphere, was introduced 1 mmol of 3-hydroxy-5-ene steroids **9** and **16**, 50 mL of toluene (HPLC grade), and 0.80 mL (7.7 mmol) of cyclohexanone. The stirring solution was heated slowly up to distillation temperature and 3×15 mL of toluene was successively removed from the trap. Distillation was continued and a suspension of 246 mg (1.2 mmol) of aluminium isopropoxide in 20 mL of toluene was added with a syringe. A new portion of 15 mL of toluene was extracted from the trap, and the temperature adjusted to keep the reflux without any distillation. After 3 h, the reaction mixture was cooled at r.t. and quenched by adding a 10% HCl solution (30 mL). The crude product was extracted with CH_2Cl_2 (3×40 mL), dried over anhydrous $MgSO_4$, filtered, evaporated, and then purified by flash column chromatography (hexane–EtOAc, 75:25 and 80:20, respectively, for **10** and **17**).

*5.1.4.1. 17α-Benzyl-17β-hydroxy-4-androsten-3-one (**10**)*

White solid (88% yield). $R_f = 0.32$ (hexanes–acetone, 75:25). IR (film) ν 3518 (OH, alcohol), 1670 (C=O, conjugated ketone), 1618 (conjugated C=C). ^{1}H-NMR ($CDCl_3$) δ 0.97 (s, CH_3-18), 1.21 (s, CH_3-19), 2.58 and 2.86 (2d, $J = 13.2$ Hz, AB system, CH_2-Ph), 5.74 (s, CH-4), 7.28 (m, 5H, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 35.69 (C1), 33.90 (C2), 199.47 (C3), 123.81 (C4), 171.14 (C5), 32.79 (C6), 31.10 (C7), 36.47 (C8), 53.77 (C9), 38.62 (C10), 20.69 (C11), 31.70 (C12), 46.35 (C13), 49.95 (C14), 23.52 (C15), 35.56 (C16), 82.75 (C17), 14.43 (C18), 17.36 (C19), 42.30 (*C*H_2Ph), 138.10 (C1′), 130.94 (C2′ and C6′), 128.07 (C3′ and C5′), 126.27 (C4′). FAB-HRMS: Calc. for $C_{26}H_{35}O_2$: $[M^+ + H]$ 379.26370. Found: 379.26240. HPLC purity = 99.4% (C-18 NovaPak column; MeCN–MeOH–H_2O, 35:30:35).

*5.1.4.2. 20-Benzyl-20α-hydroxy-4-pregnen-3-one (**17**)*

White solid (73% yield). $R_f = 0.16$ (hexanes–acetone, 85:15). IR (KBr) ν 3422 (OH, alcohol), 1668 (C=O, conjugated ketone), 1612 (conjugated C=C). ^{1}H-NMR ($CDCl_3$) δ 0.92 (s, CH_3-18), 1.17 and 1.18 (2s, CH_3-19 and CH_3-21), 2.60 and 2.81 (2d, $J = 13.1$ Hz, AB system, CH_2Ph), 5.72 (s, CH-4), 7.22 (m, 5H, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 35.61 (C1), 33.90 (C2), 199.48 (C3), 123.72 (C4), 171.39 (C5), 32.84 (C6), 31.86 (C7), 34.94 (C8), 53.63 (C9), 38.51 (C10), 20.81 (C11), 39.91

(C12), 42.85 (C13), 56.01 (C14), 23.74 (C15), 22.70 (C16), 58.44 (C17), 13.50 (C18), 17.30 (C19), 74.60 (C20), 26.31 (C21), 49.10 (CH_2Ph), 137.42 (C1′), 130.62 (C2′ and C6′), 128.01 (C3′ and C5′), 126.31 (C4′). FAB-HRMS: Calc: for $C_{28}H_{39}O_2$ [M^++H] 407.29501. Found: 407.29601. HPLC purity = 98.7% (C-18 NovaPak column; MeCN–MeOH–H_2O, 35:45:20).

*5.1.5. Epoxidation of enone-steroids **10** and **17** (general procedure for **11**, **12** and **18**, **19**)*

In a 100 mL flask was solubilised 1 mmol of α,β-unsaturated ketone **10** or **17** in 20 mL of CH_3OH–CH_2Cl_2 (9:1). After 30 min at r.t., 0.7 mL of 30% H_2O_2 (w/v) was added dropwise and the mixture stirred for 45 min or 1 h. The mixture was then cooled at 0 °C, and 0.4 mL of 4 M NaOH was slowly added. Stirring was continued for 1 h at 0 °C and for an additional 12 h at 4 °C. The reaction was quenched by 30 mL of water, and the aqueous phase was successively extracted with three portions of CH_2Cl_2 (35 mL). It proved useful to add 2–3 mL of 10% HCl to dissolve the emulsion resulting from the extraction process. The combined organic phase was dried over anhydrous $MgSO_4$, and the solvent evaporated under vacuum. The crude product was then purified by flash column chromatography (hexanes–EtOAc, 85:15 and 87.5:12.5, respectively).

*5.1.5.1. 17α-Benzyl-4α,5α-epoxy-17β-hydroxy-androstan-3-one (**11**)*

White powder (24% yield). R_f = 0.29 (hexanes–acetone, 80:20). IR (KBr) *v* 3548 (OH, alcohol), 1712 (C=O, ketone). ^{1}H-NMR ($CDCl_3$) δ 0.96 (s, CH_3-18), 1.09 (s, CH_3-19), 2.62 and 2.87 (2d, J = 13.2 Hz, AB system, CH_2Ph), 3.05 (s, CH-4β), 7.29 (m, 5H, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 33.08 (C1), 29.16 (C2), 206.85 (C3), 62.89 (C4), 70.08 (C5), 28.72 (C6), 29.65 (C7), 36.34 (C8), 50.76 (C9), 36.80 (C10), 21.11 (C11), 31.20 (C12), 46.48 (C13), 49.79 (C14), 23.59 (C15), 33.63 (C16), 82.86 (C17), 14.46 (C18), 16.50 (C19), 42.35 (CH_2Ph), 138.16 (C1′), 130.97 (C2′ and C6′), 128.13 (C3′ and C5′), 126.33 (C4′). FAB-HRMS: Calc. for $C_{26}H_{35}O_3$: [M^++H] 395.25861. Found: 395.25760. HPLC purity = 97.6% (C-18 Symmetry column; MeCN–MeOH–H_2O, 30:35:35).

*5.1.5.2. 17α-Benzyl-4β,5β-epoxy-17β-hydroxy-androstan-3-one (**12**)*

White powder (71% yield). R_f = 0.22 (hexanes–acetone, 85:15). IR (KBr) *v* 3507 and 3426 (OH, alcohol), 1712 (C=O, ketone). ^{1}H-NMR ($CDCl_3$) δ 0.95 (s, CH_3-18), 1.19 (s, CH_3-19), 2.58 and 2.85 (2d, J = 13.2 Hz, AB system, CH_2Ph), 2.99 (s, CH-4α), 7.27 (m, 5H, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 32.52 (C1), 30.09 (C2), 206.72 (C3), 62.68 (C4), 70.29 (C5), 26.17 (C6), 29.79 (C7), 35.93 (C8), 46.59 (C9), 37.30 (C10), 21.26 (C11), 30.98 (C12), 46.59 (C13), 49.97 (C14), 23.56 (C15), 33.57 (C16), 82.72 (C17), 14.48 (C18), 18.95 (C19), 42.34 (CH_2Ph), 138.07 (C1′), 130.94 (C2′ and C6′), 128.13 (C3′ and C5′), 126.33 (C4′). FAB-HRMS: Calc. for $C_{26}H_{35}O_3$ [M^++H] 395.25861. Found: 395.25800. HPLC purity = 96.0% (C-4 YMCPak column; MeCN–MeOH–H_2O, 35:35:30).

*5.1.5.3. 20-Benzyl-4α,5α-epoxy-20α-hydroxy-pregnan-3-one (**18**)*

White powder (18% yield). R_f = 0.39 (hexanes–acetone, 75:25). IR (KBr) *v* 3588 (OH, alcohol), 1711 (C=O, ketone). ^{1}H-NMR ($CDCl_3$) δ 0.91 (s, CH_3-18), 1.05 (s, CH_3-19), 1.19 (s, CH_3-21), 2.61 and 2.82 (2d, J = 13.0 Hz, AB system, CH_2Ph), 3.03 (s, CH-4β), 7.24 (m, 5H, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 33.09 (C1), 29.70 (C2), 207.00 (C3), 62.84 (C4), 70.14 (C5), 28.81 (C6), 29.70 (C7), 34.80 (C8), 50.55 (C9), 36.65 (C10), 21.23 (C11), 40.03 (C12), 43.00 (C13), 55.82 (C14), 23.80 (C15), 22.72 (C16), 58.61 (C17), 13.54 (C18), 16.47 (C19), 74.67 (C20), 26.34 (C21), 49.11 (CH_2Ph), 137.45 (C1′), 130.67 (C2′ and C6′), 128.06 (C3′ and C5′), 126.36 (C4′). FAB-HRMS: Calc. for $C_{28}H_{39}O_3$ [M^++H] 423.28992. Found: 423.28850. HPLC purity = 97.4% (C-4 YMCPak column; MeOH–H_2O, 80:20 containing 10 mM of NH_4OAc).

*5.1.5.4. 20-Benzyl-4β,5β-epoxy-20α-hydroxy-pregnan-3-one (**19**)*

White powder (53% yield). R_f = 0.25 (hexanes–acetone, 85:15); IR (KBr) *v* 3680 (OH, alcohol), 1705 (C=O, ketone). ^{1}H-NMR ($CDCl_3$) δ 0.90 (s, CH_3-18), 1.14 (s, CH_3-19), 1.18 (s, CH_3-21), 2.60 and 2.80 (2d, J = 13.0 Hz, AB system, CH_2Ph), 2.97 (s, CH-4α), 7.22 (m, 5H, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 32.52 (C1), 30.27 (C2), 206.82 (C3), 62.65 (C4), 70.31 (C5), 26.11 (C6), 29.85 (C7), 34.39 (C8), 46.35 (C9), 37.13 (C10), 21.36 (C11), 39.78 (C12), 43.11 (C13), 56.03 (C14), 23.79 (C15), 22.67 (C16), 58.43 (C17), 13.56 (C18), 18.91 (C19), 74.62 (C20), 26.34 (C21), 49.12 (CH_2Ph), 137.39 (C1′), 130.64 (C2′ and C6′), 128.07 (C3′ and C5′), 126.38 (C4′). FAB-HRMS: Calc. for $C_{28}H_{39}O_3$: [M^++H] 423.28992. Found: 423.28950. HPLC purity = 96.6% (C-4 YMCPak column; MeCN–MeOH–H_2O, 35:35:30).

*5.1.6. Synthesis of 4-chloro-steroids from 4,5-epoxides (general procedure for **13** and **20**)*

In a 100 mL flask, a 1:4 mixture of 4α,5α- and 4β,5β-epoxides (1 mmol) was dissolved in 20 mL of acetone, concentrated HCl (0.3 mL) was slowly added with a syringe, and the reaction mixture was stirred for 1–1.5 h at r.t. Saturated NaCl solution (30 mL) was then added and the reaction mixture extracted with CH_2Cl_2. The organic phase was successively washed with water, 10% Na_2CO_3 solution, and again with water. The dried ($MgSO_4$) organic phase was thereafter filtered and solvent evaporated under vacuum. The crude chloro derivative was further purified by flash column chromatography (hexanes–EtOAc, 80:20).

*5.1.6.1. 17α-Benzyl-4-chloro-17β-hydroxy-4-androsten-3-one (**13**)*

White solid (74% yield). $R_f = 0.24$ (hexanes–acetone, 80:20). IR (KBr) ν 3535 (OH, alcohol), 1694 (C=O, conjugated ketone). ^{1}H-NMR ($CDCl_3$) δ 0.98 (s, CH_3-18), 1.27 (s, CH_3-19), 2.27 (td, $J_1 = 5.2$ Hz and $J_2 = 14.4$ Hz, 1H of CH_2-6), 2.59 (m, 1H of CH_2-2), 2.58 and 2.86 (2d, $J = 13.1$ Hz, AB system, CH_2Ph), 3.27 (td, $J_1 = 3.2$ Hz and $J_2 = 14.8$ Hz, 1H of CH_2-6), 7.28 (m, 5H, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 34.44 (C1), 33.97 (C2), 190.62 (C3), 127.27 (C4), 164.59 (C5), 28.94 (C6), 30.88 (C7), 36.01 (C8), 53.97 (C9), 41.42 (C10), 20.82 (C11), 31.12 (C12), 46.34 (C13), 49.91 (C14), 23.48 (C15), 33.67 (C16), 82.70 (C17), 14.44 (C18), 17.79 (C19), 42.31 (CH_2Ph), 137.99 (C1′), 130.93 (C2′ and C6′), 128.14 (C3′ and C5′), 126.35 (C4′). FAB-HRMS: Calc. for $C_{26}H_{34}^{35}ClO_2$: [M$^+$+H] 413.22473. Found: 413.22580. HPLC purity = 98.4% (C-4 YMCPak column; MeOH–H_2O, 75:25).

*5.1.6.2. 20-Benzyl-4-chloro-20α-hydroxy-4-pregnen-3-one (**20**)*

White solid (88% yield). $R_f = 0.28$ (hexanes–acetone, 80:20); IR (KBr) ν 3480 (OH, alcohol), 1687 (C=O, conjugated ketone). ^{1}H-NMR ($CDCl_3$) δ 0.93 (s, CH_3-18), 1.19 (s, CH_3-19), 1.23 (s, CH_3-21), 2.56 (m, 1H of CH_2-2 and 1H of CH_2-6), 2.60 and 2.82 (2d, $J = 13.1$ Hz, AB system, CH_2Ph), 3.25 (td, $J_1 = 3.2$ Hz and $J_2 = 7.3$ Hz, 1H of CH_2-6), 7.25 (m, 5H, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 34.37 (C1), 33.98 (C2), 190.70 (C3), 127.17 (C4), 164.88 (C5), 29.00 (C6), 31.04 (C7), 34.49 (C8), 53.84 (C9), 41.32 (C10), 20.93 (C11), 39.88 (C12), 42.85 (C13), 55.96 (C14), 23.71 (C15), 22.75 (C16), 58.46 (C17), 13.53 (C18), 17.72 (C19), 74.58 (C20), 26.34 (C21), 49.09 (CH_2Ph), 137.38 (C1′), 130.65 (C2′ and C6′), 128.07 (C3′ and C5′), 126.37 (C4′). FAB-HRMS: Calc. for $C_{28}H_{38}^{35}ClO_2$: [M$^+$+H] 441.25604. Found: 441.25470. HPLC purity = 96.6% (C-4 YMC-Pak column; MeOH–H_2O, 75:25).

*5.1.7. Synthesis of 4-methoxy-steroids from 4,5-epoxides (general procedure for **14** and **21**)*

An aqueous 4 M NaOH solution (3 mL) was added to 3-oxo-4,5-epoxides (1 mmol) dissolved in 12 mL of methanol (HPLC grade) and the reaction mixture was heated to reflux. After completion of the reaction (3 h), the mixture was cooled to r.t. and the solvent evaporated under reduced pressure. The residue was treated with water before extraction with CH_2Cl_2 (3×25 mL). The organic phase was successively washed with water (2×25 mL) and brine (25 mL), followed by drying over anhydrous $MgSO_4$. The filtrate was concentrated in vacuo and the crude product was further purified by flash column chromatography (hexanes–EtOAC, 75:25 and 85:15, respectively).

*5.1.7.1. 17α-Benzyl-17β-hydroxy-4-methoxy-4-androsten-3-one (**14**)*

Slight yellow solid (57% yield). $R_f = 0.25$ (hexanes–acetone, 80:20). IR (film) ν 3463 (OH, alcohol), 1676 (C=O, conjugated ketone). ^{1}H-NMR ($CDCl_3$) δ 0.98 (s, CH_3-18), 1.23 (s, CH_3-19), 2.45 (m, 1H of CH_2-6 and 1H of CH_2-2), 2.59 and 2.86 (2d, $J = 13.1$ Hz, AB system, CH_2Ph), 3.09 (d, $J = 14.5$ Hz, 1H of CH_2-6), 3.60 (s, CH_3O), 7.27 (m, 5H, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 34.88 (C1), 34.26 (C2), 194.06 (C3), 146.64 (C4), 154.85 (C5), 23.40 (C6), 31.32 (C7), 36.23 (C8), 54.26 (C9), 38.78 (C10), 20.74 (C11), 31.23 (C12), 46.39 (C13), 50.09 (C14), 23.59 (C15), 33.74 (C16), 82.81 (C17), 14.48 (C18), 17.54 (C19), 42.36 (CH_2Ph), 138.13 (C1′), 130.98 (C2′ and C6′), 128.16 (C3′ and C5′), 126.36 (C4′), 60.26 (CH_3O). FAB-HRMS: Calc. for $C_{27}H_{37}O_3$: [M$^+$+H] 409.27426. Found: 409.27540. HPLC purity = 99.4% (C-18 Symmetry column; MeCN–MeOH–H_2O, 30:35:35).

*5.1.7.2. 20-Benzyl-20α-hydroxy-4-methoxy-4-pregnen-3-one (**21**)*

Slight yellow solid (50% yield). $R_f = 0.27$ (hexanes–acetone, 80:20); IR (film) ν 3510 (OH, alcohol), 1677 (C=O, conjugated ketone). ^{1}H-NMR ($CDCl_3$) δ 0.92 (s, CH_3-18), 1.18 (s, CH_3-19), 1.19 (s, CH_3-21), 2.43 (m, 1H of CH_2-6 and 1H of CH_2-2), 2.61 and 2.82 (2d, $J = 13.2$ Hz, AB system, CH_2Ph), 3.05 (dm, $J = 14.9$ Hz, 1H of CH_2-6), 3.58 (s, CH_3O), 7.24 (m, 5H, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 34.74 (C1), 34.21 (C2), 194.10 (C3),

146.54 (C4), 155.15 (C5), 23.42 (C6), 31.41 (C7), 34.65 (C8), 54.06 (C9), 38.61 (C10), 20.81 (C11), 39.99 (C12), 42.86 (C13), 56.10 (C14), 23.76 (C15), 22.76 (C16), 58.50 (C17), 13.54 (C18), 17.43 (C19), 74.65 (C20), 26.34 (C21), 49.12 (CH_2Ph), 137.45 (C1′), 130.66 (C2′ and C6′), 128.05 (C3′ and C5′), 126.35 (C4′), 60.23 (CH_3O). FAB-HRMS: Calc. for $C_{29}H_{41}O_3$: $[M^+ + H]$ 437.30557. Found: 437.30710. HPLC purity = 99.1% (C-18 Nova-Pak column; MeCN–MeOH–H_2O, 40:30:30).

*5.1.8. Synthesis of 4-hydroxy-steroids from 5-chloro-steroids **13** and **20** (general procedure for **15** and **22**)*

In a 100 mL flask connected to a reflux condenser and under argon atmosphere chloro-steroids **13** and **20** (1 mmol) was dissolved in 25 mL of *t*-butanol. While slowly stirring started, 168 mg (3 mmol) of solid KOH, as a fine powder, was added in one portion, and the reaction mixture heated slowly to reflux for 35–45 min. Thereafter, the reaction mixture was cooled to r.t. and neutralised (pH 7) with a 5% AcOH solution. Water (30 mL) was then added and the product extracted with EtOAc (3×30 mL). The organic phase was washed with water, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. The resulted crude product was purified by flash column chromatography (hexanes–acetone, 80:20 and 85:15, respectively).

*5.1.8.1. 17α-Benzyl-4,17β-dihydroxy-4-androsten-3-one (**15**)*

White solid (42% yield). $R_f = 0.31$ (hexanes–acetone, 75:25). IR (film) *v* 3440 (OH, alcohols), 1704 and 1667 (C=O, enol), 1635 (C=C, enol). ^{1}H-NMR ($CDCl_3$) δ 0.98 (s, CH_3-18), 1.22 (s, CH_3-19), 2.52 (m, 1H), 2.60 and 2.86 (2d, $J = 13.2$ Hz, AB system, CH_2Ph), 3.04 (dm, $J = 14.5$ Hz, 1H of CH_2-6), 6.09 (s, OH of enol), 7.27 (m, 5H, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 34.73 (C1), 31.78 (C2), 193.58 (C3), 141.20 (C4), 139.91 (C5), 23.00 (C6), 30.82 (C7), 36.16 (C8), 54.28 (C9), 37.89 (C10), 20.70 (C11), 31.24 (C12), 46.36 (C13), 50.14 (C14), 23.64 (C15), 33.75 (C16), 82.88 (C17), 14.49 (C18), 17.20 (C19), 42.35 (CH_2Ph), 138.15 (C1′), 131.01 (C2′ and C6′), 128.17 (C3′ and C5′), 126.37 (C4′). FAB-HRMS: Calc. for $C_{26}H_{35}O_3$: $[M^+ + H]$ 395.25861. Found: 395.25800. HPLC purity = 97.4% (C-4 YMCPak column; MeOH–H_2O, 70:30).

*5.1.8.2. 20-Benzyl-4,20α-dihydroxy-4-pregnen-3-one (**22**)*

White solid (45% yield). $R_f = 0.44$ (hexanes–EtOAc, 75:25); IR (KBr) *v* 3408 (OH, alcohols), 1705 and 1668 (C=O, enol); 1640 (C=C, enol). ^{1}H-NMR ($CDCl_3$) δ 0.92 (s, CH_3-18), 1.17 (s, CH_3-19), 1.19 (s, CH_3-21), 2.50 (m, 1H), 2.61 and 2.82 (2d, $J = 13.1$ Hz, AB system, CH_2Ph), 3.01 (dm, $J = 17.0$ Hz, 1H of CH_2-6), 6.07 (s, OH of enol), 7.23 (m, SH, CH_2Ph). ^{13}C-NMR ($CDCl_3$) δ 34.63 (C1), 31.77 (C2), 193.58 (C3), 141.14 (C4), 140.18 (C5), 23.03 (C6), 30.91 (C7), 34.63 (C8), 54.12 (C9), 37.74 (C10), 20.81 (C11), 40.04 (C12), 42.89 (C13), 56.21 (C14), 23.84 (C15), 22.79 (C16), 58.59 (C17), 13.57 (C18), 17.11 (C19), 74.73 (C20), 26.39 (C21), 49.15 (CH_2Ph), 137.51 (C1′), 130.69 (C2′ and C6′), 128.08 (C3′ and C5′), 126.37(C4′). FAB-HRMS: Calc. for $C_{28}H_{39}O_3$ $[M^+ + H]$ 423.28992. Found: 423.29090. HPLC purity = 96.5% (C-4 YMCPak column; MeCN–MeOH–H_2O, 75:25).

*5.2. Inhibition of steroid sulphatase by compounds **6–22** and basic steroids*

5.2.1. HEK-293 cells as source of steroid sulphatase activity (enzymatic assay)

Human embryonic kidney (HEK)-293 cells (American Type Culture Collection, Rockville, MD), transiently transfected with a sulphatase expression vector (pCMV-sulpha) were used as source of steroid sulphatase activity. The pCMV-sulpha was constructed by inserting a cDNA fragment containing the coding sequence of a human sulphatase gene downstream from the CMV promoter of the pCMV vector (kindly provided by Dr M.B. Mathews, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, USA). The sulphatase cDNA fragment was obtained by screening a human placenta cDNA library (Clontech Laboratories Inc., Palo Alto, CA, USA) using an incomplete cDNA fragment (kindly provided by Dr L.J. Shapiro, Howard Hughes Medical Institute, Los Angeles, CA, USA) as probe. The transfection of the expression vector was performed by the calcium phosphate procedure using 10 μg of recombinant plasmid per 10^6 cells [44]. The cells were initially plated at 10^4 cells cm^{-2} in Falcon culture flasks and grown in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal bovine serum supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU penicillin mL^{-1}, and 100 μg streptomycin sulphate mL^{-1}.

For the assay, the HEK-293 cell homogenate was prepared by repeated freezing (−80 °C) and thawing (5 times), and homogenisation using a Dounce homogeniser. The reaction was carried out at 37 °C in 1.25 mL of 100 mM Tris–acetate buffer (pH 7.4) containing 5 mM of EDTA, 10% glycerol, 100 μM of [^{3}H]DHEAS as substrate, and an ethanolic solution of

compound to test (at appropriate concentrations). After 2 h of incubation, the reaction was stopped by addition of 1.25 mL of xylene. The tubes were then shaken and centrifuged at 2000*g* for 10 min to separate the organic and aqueous phases. Radioactivity in 750 μL of each phase (organic: free steroids; aqueous: sulphated steroids) was determined by liquid scintillating counting with a Beckman LS3801 (Irvine, CA). Data were expressed as percentage (%) of DHEA produced (100% for the control without inhibitor) and the percentage (%) of inhibition then calculated.

5.2.2. Jeg-3 cells as source of steroid sulphatase (enzymatic assay)

The Jeg-3 cells, also used as source of sulphatase activity, were purchased from ATCC, on October 10th 1989, at passage 127. Cells were grown in DMEM medium (Flow) containing $NaHCO_3$ (3.7 g L^{-1}), Hepes (5.96 g L^{-1}) and glucose (4.59 g L^{-1}). The medium, sterilised on Millipore 0.22 μm membrane, was supplemented with foetal bovine serum or bovine calf serum (5%), L-glutamine (1%), penicillin (100 IU mL^{-1}), and streptomycin (50 mg mL^{-1}). Cells were grown in 175 cm^2 flasks seeded at a density of 500,000 cells per flask. The medium was changed every 2–3 days. Cells were subcultured weekly by gentle digestion in Hepes buffered enzyme solution (Pancreatine: EDTA, 0.83%: 3 mM), 15 min at 37 °C. The enzymatic activity was inhibited by diluting the cells in culture medium containing 5% serum. Cells were pelleted, resuspended in culture medium, counted with a haemocytometer and reseeded in culture flasks or harvested for subsequent analysis.

For the assay, the Jeg-3 cell homogenate was prepared by repeated freezing (−80 °C) and thawing (5 times), and homogenisation using a Dounce homogeniser. The reaction was carried out at 37 °C in 1.25 mL of 100 mM Tris–acetate buffer (pH 7.4) containing 5 mM of EDTA, 10% glycerol, 100 μM of [^{3}H]DHEAS or [^{3}H]E_1S as substrate, and an ethanolic solution of compound to test (at appropriate concentrations). After 2 h of incubation, the reaction was stopped by addition of 1.25 mL of xylene. The tubes were then shaken and centrifuged at 2000*g* for 10 min to separate the organic and aqueous phases. Radioactivity in 750 μL of each phase (organic: free steroids; aqueous: sulphated steroids) was determined by liquid scintillating counting with a Beckman LS3801 (Irvine, CA). Data were expressed as percentage (%) of DHEA or E_1 produced (100% for the control without inhibitor) and the percentage (%) of inhibition then calculated.

5.3. Proliferative assays on androgen-sensitive Shionogi cells and oestrogen-sensitive ZR-75-1 cells

5.3.1. Proliferative activity in Shionogi (AR^+) mammary cells

Assays of the proliferation of androgen-sensitive (AR^+) Shionogi mammary carcinoma cells were carried out according to the procedure previously described by Sam et al. [45]. The selected compounds were tested at two concentrations, 0.1 and 1 μM.

5.3.2. Proliferative activity in ZR-75-1 (ER^+) cells

Assays of the proliferation of oestrogen-sensitive (ER^+) ZR-75-1 human breast cancer cells were carried out according to the procedure previously described by Poirier et al. [46]. The selected compounds were tested at two concentrations, 0.03 and 1 μM.

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