Chemical Synthesis, 17β-Hydroxysteroid Dehydrogenase Type 1 Inhibitory Activity and Assessment of *In Vitro* and *In Vivo* Estrogenic Activities of Estradiol Derivatives

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Abstract: 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1) was chosen as a key steroidogenic target enzyme to reduce the formation of estradiol (E2), which is the most potent estrogen. This article completes a previous study by synthesizing and testing 16β-methylene derivatives (Br, Cl and OH) of E2 in order to generate new structure-activity relationships. This study also investigates a series of 2-methoxy (MeO) derivatives synthesized as non-estrogenic inhibitors of 17β-HSD1. The 2-MeO-E_2 derivatives (16β-CH_2Br, 16β-CH_2OH and 16α-$(CH_2)_3$Br) are all less potent inhibitors (IC_{50} = 5.91, 3.80 and 5.80 μM) than analogues without the 2-MeO group (IC_{50} = 1.20, 1.27 and 0.99 μM, respectively) for the reduction of estrone into E2 by 17β-HSD1 overexpressed in HEK-293 cells. Except for one compound, these E2 derivatives have shown an estrogenic-like effect on estrogen-sensitive T-47D cells at 1 μM. A cytotoxic effect was also obtained at higher concentrations for two compounds tested on T-47D cells. However, no estrogenic-like effect was observed in the estrogen-sensitive tissues (uterus and vagina) of the ovariectomized mouse model for 2-MeO-16α-bromopropyl-E_2.

Key Words: Steroid, Estradiol, Estrogen, Inhibitor, Hydroxysteroid dehydrogenase.

INTRODUCTION

17β-hydroxysteroid dehydrogenases (17β-HSDs) form an interesting enzyme family involved in the biosynthesis of sex steroids from cholesterol and targeted for controlling the concentration of estrogens and androgens [1-6]. From a therapeutic standpoint, inhibitors of 17β-HSDs will no doubt be useful tools in the treatment of estrogen-sensitive pathologies (breast, ovarian and endometrium cancers) and androgeno-sensitive pathologies (prostate cancer, benign prostatic hyperplasia, hirsutism, etc.) [7-12]. Since 17β-HSDs exert their action at the end of the steroidogenesis of active steroidal hormones, selective inhibitors of these enzymes should not display undesired effects on the biosynthesis of other steroidal hormones such as aldosterone and cortisol. We are also aware that the activity of the 17β-HSDs in intact cells is mainly unidirectional [4, 13]. Each member of the 17β-HSDs family can therefore be classified either as reductive or oxidative. 17β-HSD1 was the first known of this enzyme family and 3D structures of enzyme, complexed or not with substrate or/and cofactor have been elucidated [14-17]. 17β-HSD1 catalyzes the reductive transformation of the less potent estrogen estrone (E1) into the most potent one, estradiol (E2). It also catalyzes the transformation of dehydroepiandrosterone (DHEA) into 5-androstene-3β,17β-diol (Δ^5-diol), although at a lower rate (Fig. **1**). Inhibition of this enzyme in a way that would decrease the level of E2 in the bloodstream as well as in peripheral tissues could be a good strategy in the fight against estrogen-sensitive diseases.

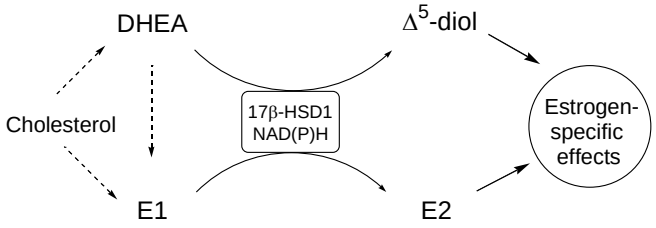

Fig. (1). Formation of weak estrogen Δ^5-diol and potent estrogen E2 by 17β-HSD1 and cofactor NAD(P)H.

In the late nineties, our research team performed a structure-activity relationship study for a series of 16α and 16β-(bromoalkyl)-E2 inhibitors of 17β-HSD1 [11]. The main conclusion was that 16α-(bromopropyl)-E2 (**5a**) and 16β-(bromopropyl)-E2 (**5b**) are the two compounds that offered optimal inhibition of 17β-HSD1 (Table **1**). However, **5b** is not fully stable and undergoes cyclization with loss of inhibitory activity. Although the inhibitory potency is slightly decreased for a 4-carbon side chain, (compounds **4a** and **4b**) there is a drastic decrease of the inhibitory potency for 16α-(bromoethyl)-E2 (**6a**), a 2-carbons side chain, but the result for the 16β analogue was not obtained. In fact the synthesis of 16β-(bromoethyl)-E2 (**6b**) is not possible because of the formation of a highly stable 5-member ring ether. The synthesis of 16β-(bromomethyl)-E2 (**7**) is however possible and

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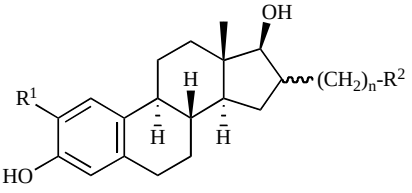
will allow verification by extrapolation, if the inhibitory activity of the various 16β-(bromoalkyl)-E2 compounds follows the same tendency as observed for the 16α analogues. In the meantime, the synthesis of 16β-chloromethyl-E2 (**8**) and 16β-hydroxymethyl-E2 (**9**) will make it possible to evaluate the influence of these two different atoms that, similarly to the 16β-bromomethyl group, closely proximate the tyrosine-155 of the catalytic triad and the nicotinamide nucleus of the cofactor NAD(P)H, both components involved in the catalytic process.

Table 1. E2 Derivatives at Position 16 Previously Reported (1-6**) or Newly Synthesized (**7-12**) as Inhibitors of 17β-HSD1**



#	R^1	n	C16 (α or β)	R^2
1a	H	7	α	Br
1b	H	7	β	Br
2a	H	6	α	Br
2b	H	6	β	Br
3a	H	5	α	Br
3b	H	5	β	Br
4a	H	4	α	Br
4b	H	4	β	Br
5a	H	3	α	Br
5b	H	3	β	Br
6a	H	2	α	Br
6b	H	2	β	Br
7	H	1	β	Br
8	H	1	β	Cl
9	H	1	β	OH
10	CH_3O	1	β	Br
11	CH_3O	1	β	OH
12	CH_3O	3	α	Br

The first part of our work consists in completing the previous study performed by Tremblay *et al.* [18, 11] on 16α/β-(bromoalkyl)-E2 as inhibitors of 17β-HSD1 by synthesizing compounds **7-9** (Table **1**). Based on the fact that 2-methoxy (MeO)-E2 interacts negligibly with the estrogen receptor [19], we also performed the synthesis of 2-MeO-E2 derivatives **10-12**, including the 2-MeO version of the inhibitor **5a** already synthesized by Tremblay and Poirier [18] but having residual estrogenic activity when tested on the estrogen-sensitive cell line ZR-75-1 [11]. This manuscript describes the chemical synthesis and biological evaluation of targeted compounds **7-12** as inhibitors of 17β-HSD1 with a special focus in addressing their *in vitro* and *in vivo* estrogenic effects.

MATERIALS AND METHODOLOGY

Chemical Synthesis

The starting steroid estrone was purchased from Steraloids Inc. (Newport, RI, USA). 2-Methoxy-estrone was synthesized from estrone similarly as reported in literature [20]. Chemical reagents of highest purity and anhydrous solvents were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) and Fisher Scientific (Montréal, QC, Canada). Tetrahydrofuran used in the anhydrous reactions was distilled from benzophenone ketyl. Reactions were run under inert (argon) atmosphere in oven-dried glassware. Analytical thin-layer chromatography (TLC) was performed on 0.20 mm silica gel 60 F254 plates (Fisher Scientific), and compounds were visualized using UV light or ammonium molybdate/sulfuric acid/water (with heating). Flash column chromatographies were performed upon Silicycle R10030B 230-400 mesh silica gel (Québec, QC, Canada). Infrared spectra (IR) were obtained from a thin film of compound usually solubilized in $CDCl_3$ and deposited upon an NaCl pellet. They were recorded with a Perkin-Elmer 1600 FT-IR spectrometer (Norwalk, CT, USA) and only the significant bands were reported in cm^{-1}. Nuclear magnetic resonance (NMR) spectra were recorded at 300 MHz (^1H) and 75.5 MHz (^{13}C) on a Bruker AC/F300 spectrometer (Billerica, MA, USA) or a 400 MHz (^1H) and 100.6 MHz (^{13}C) on a Bruker Avance 400 digital spectrometer and reported in ppm. Low-resolution mass spectra (LRMS) were recorded on a PE Sciex API-150 ex apparatus (Foster City, CA, USA).

Synthesis of 3-tert-butyldimethylsilyloxy-estra-1,3,5(10)-trien-17-one (13):

Estrone was protected as a *t*-butyldimethylsilyl ether as previously reported in literature [21].

Synthesis of 2-methoxy-3-tert-butyldimethylsilyloxy-estra-1,3,5(10)-trien-17-one (14):

To a solution of 2-methoxy-estrone (1.12 g, 3.73 mmol) in dry CH_2Cl_2 (10 mL) cooled at 0 °C were added dry triethylamine (1.56 mL, 11.18 mmol) and *t*-butyldimethylsilyl chloride (TBDMS-Cl) (1.69 g, 11.18 mmol). The mixture was stirred under argon atmosphere from 0 °C to room temperature overnight. The reaction was then quenched with MeOH, water added in excess, and the mixture extracted with CH_2Cl_2. The organic phase was washed several times with water, dried over Na_2SO_4 and evaporated under reduced pressure. The crude product was purified by flash chromatography, using a mixture of hexanes and EtOAc (9/1) as eluent to give **14** in a 76 % yield. White solid; ^1H NMR 300 MHz δ ($CDCl_3$): 0.15 (s, 6H, $Si(CH_3)_2$), 0.91 (s, 3H, 18-CH_3), 0.99 (s, 9H, *t*-Bu of TBDMS), 1.20-2.60 (residual protons from the steroid), 2.77 (m, 2H, 6-CH_2), 3.75 (s, 3H, CH_3O), 6.55 (s, 1H, 4-CH), 6.75 (s, 1H, 1-CH); ^{13}C NMR 75.5 MHz δ ($CDCl_3$): -4.74 (2X), 13.72, 18.25, 21.39, 25.60 (3X), 25.89, 26.48, 28.54, 31.43, 35.69, 38.05, 44.12, 47.82,

50.19, 55.52, 109.43, 120.83, 128.48, 132.34, 142.79, 148.57, 220.78; LRMS calculated for $C_{25}H_{38}O_3Si$: 414.2, found 415.0 $[M+H]^+$.

Synthesis of methyl-1-[3'-tert-butyldimethylsilyloxy-17'-oxo-estra-1',3',5'(10')-trien-16'β-yl]-formate (15):

A solution of diisopropylamine (1.00 mL, 7.14 mmol) in dry THF (15 mL) was stirred under argon at -25 °C and a solution of *n*-butyllithium in hexanes (2.88 mL, 7.20 mmol) was added dropwise. After 45 minutes, the resulting lithium diisopropylamide (LDA) solution was cooled at –78 °C and TBDMS-E1 (**13**) (2.2 g, 5.71 mmol) dissolved in dry THF (15 mL) was added dropwise. The mixture was allowed to stir for 1 hour at 0 °C, then cooled again to –78 °C and hexamethylphosphoramide (HMPA) (1.00 mL, 5.71 mmol) was slowly added followed by methylcyanoformate (0.46 mL, 5.80 mmol). The reaction mixture was allowed to stir at –78 °C for 30 minutes before the reaction was fully completed. Water was added to quench the reaction and the crude product was extracted with EtOAc. The organic phase was washed with water and dried over Na_2SO_4. After evaporation under reduced pressure, the crude product was purified by flash chromatography, using a mixture of hexanes and EtOAc (9/1) as eluent to give **15**. Although a mixture of two C16 isomers (β-major and α-minor) was obtained in a 78 % yield, only the major compound was recovered after chromatography. White solid; IR ν (film): 1752 and 1725 (C=O of ketone and ester); ^{1}H NMR 400 MHz δ ($CDCl_3$): 0.21 (s, 6H, $Si(CH_3)_2$), 1.00 (s, 9H, *t*-Bu of TBDMS), 1.01 (s, 3H, 18'-CH_3), 1.40-2.50 (residual protons from the steroid), 2.88 (m, 2H, 6'-CH_2), 3.23 (dd, J_1 = 8.5 Hz and J_2 = 9.8 Hz, 16'-CH), 3.79 (s, 3H, $COOCH_3$), 6.60 (d, J = 2.5 Hz, 1H, 4'-CH), 6.65 (dd, J_1 = 8.4 Hz and J_2 = 2.6 Hz, 1H, 2'-CH), 7.13 (d, J = 8.4 Hz, 1H, 1'-CH); ^{13}C NMR 75.5 MHz δ ($CDCl_3$): -4.40 (2X), 13.27, 18.15, 25.67 (3X), 26.33, 26.53, 29.36, 31.90, 37.84, 43.97, 47.89, 48.92, 52.58, 54.04, 117.34, 119.99, 126.09, 132.09, 137.47, 153.52, 169.85, 212.12; LRMS calculated for $C_{26}H_{38}O_4Si$: 442.3, found 443.1 $[M+H]^+$.

Synthesis of methyl-1-[2'-methoxy-3'-tert-butyldimethyl silyloxy-17'-oxo-estra-1',3',5'(10')-trien-16'β-yl]-formate (16):

Using the same protocol as for the synthesis of **15**, the ester moiety was introduced upon **14** to give **16** in a 71 % yield. Only the major 16'β-isomer was described. White solid; IR ν (film): 1752 and 1728 (C=O of ketone and ester); ^{1}H NMR 400 MHz δ ($CDCl_3$): 0.17 (s, 6H, $Si(CH_3)_2$), 1.01 (s, 9H, *t*-Bu of TBDMS), 1.02 (s, 3H, 18'-CH_3), 1.40-2.30 (residual protons from the steroid), 2.80 (m, 2H, 6'-CH_2), 3.24 (dd, 1H, J_1 = 8.5 Hz and J_2 = 9.3 Hz, 16'α-CH), 3.78 (s, 3H, $COOCH_3$), 3.79 (s, 3H, 2'-CH_3O), 6.59 (s, 1H, 1'-CH), 6.77 (s, 1H, 4'-CH); ^{13}C NMR 75.5 MHz δ ($CDCl_3$): -4.59 (2X), 13.33, 18.43, 25.74 (3X), 25.93, 26.37, 26.67, 28.63, 31.96, 37.84, 38.30, 44.31, 47.92, 52.61, 54.08, 55.82, 109.72, 121.05, 128.63, 132.21, 143.15, 148.83, 169.88, 212.18; LRMS calculated for $C_{27}H_{40}O_5Si$: 472.5, found 471.6 $[M-H]^-$ and 495.3 $[M+Na]^+$.

Synthesis of 3-tert-butyldimethylsilyloxy-16β-hydroxymethyl-estra-1,3,5(10)-trien-17β-ol (17):

To a stirred solution of **15** (0.830 g, 1.88 mmol) in dry THF (15 mL) at –78 °C under argon atmosphere a solution of $LiAlH_4$ in THF (3.4 mL, 3.40 mmol) was added dropwise. After 1 hour, the reaction mixture was warmed up to 0 °C and allowed to react for 30 minutes before it was fully completed. Water was added to quench the reaction and the crude product was extracted using EtOAc. The organic phase was washed with a saturated NaCl aqueous solution and dried over Na_2SO_4. After evaporation under reduced pressure, the crude product was purified by flash chromatography, using a mixture of hexanes and EtOAc (8/2) as eluent to give **17** in an 83 % yield. White solid; IR ν (film): 3312 (OH); ^{1}H NMR 400 MHz δ ($CDCl_3$): 0.21 (s, 6H, $Si(CH_3)_2$), 0.87 (s, 3H, 18-CH_3), 1.00 (s, 9H, *t*-Bu of TBDMS), 1.05-2.40 (residual protons for the steroid), 2.50 (m, 1H, 16α-CH), 2.82 (m, 2H, 6-CH_2), 3.64 (m, 1H of CH_2OH), 3.87 (m, 1H of CH_2OH), 3.98 (d, J = 9.8 Hz, 1H, 17α-CH), 6.58 (d, J = 2.5 Hz, 1H, 4-CH), 6.64 (dd, J_1 = 8 Hz and J_2 = 2.5 Hz, 1H, 2-CH), 7.14 (d, J = 8 Hz, 1-CH); ^{13}C NMR 75.5 MHz δ ($CDCl_3$): -4.40 (2X), 12.27, 18.15, 25.68 (3X), 26.17, 27.47, 27.78, 29.56, 37.62, 38.05, 41.81, 43.84, 44.24, 49.06, 64.77, 83.03, 117.15, 119.92, 126.06, 132.90, 137.72, 153.27; LRMS calculated for $C_{25}H_{40}O_3Si$: 416.3, found 417.1 $[M+H]^+$.

Synthesis of 2-methoxy-3-tert-butyldimethylsilyloxy-16β-hydroxymethyl-estra-1,3,5(10)-trien-17β-ol (18):

Using the same protocol as for the synthesis of **17**, the keto ester **16** was reduced to give compound **18** in a 76 % yield. White solid; ^{1}H NMR 400 MHz δ ($CDCl_3$): 0.17 (s, 6H, $Si(CH_3)_2$), 0.88 (s, 3H, 18-CH_3), 1.01 (s, 9H, *t*-Bu of TBDMS), 1.05-2.40 (residual protons from the steroid), 2.50 (m, 1H, 16α-CH), 2.74 (m, 2H, 6-CH_2), 3.69 (m, 1H of CH_2OH), 3.79 (s, 3H, CH_3O), 3.87 (m, 1H of CH_2OH), 3.98 (d, J = 9.8 Hz, 1H, 17α-CH), 6.56 (s, 1H, 4-CH), 6.78 (s, 1H, 1-CH); ^{13}C NMR 75.5 MHz δ ($CDCl_3$): -4.61 (2X), 12.27, 18.40, 25.73 (3X), 26.37, 27.57, 27.76, 28.78, 37.68, 37.99, 41.91, 44.15, 44.26, 49.07, 55.82, 64.75, 83.04, 109.80, 120.88, 128.88, 132.99, 142.89, 148.64.

Synthesis of 3-tert-butyldimethylsilyloxy-16β-chloromethyl-estra-1,3,5(10)-trien-17β-ol (19):

A solution of diol **17** (0.494 g, 1.196 mmol), PPh_3 (0.627 g, 2.392 mmol) and CCl_4 (0.793 g, 2.392 mmol) in dry CH_2Cl_2 (20 mL) was stirred at 0 °C under argon. The reaction was monitored by TLC and was completed after 3 hours. The crude mixture was pre-adsorbed on silica gel and purified by flash chromatography using a mixture of hexanes and EtOAc (95/5) as eluent to give **19** in a 60 % yield. White solid; IR ν (film): 3575 (OH); ^{1}H NMR 400 MHz δ ($CDCl_3$): 0.20 (s, 6H, $Si(CH_3)_2$), 0.84 (s, 3H, 18-CH_3), 1.00 (s, 9H, *t*-Bu of TBDMS), 1.10-2.40 (residual protons from the steroid), 2.59 (m, 1H, 16α-CH), 2.83 (m, 2H, 6-CH_2), 3.54 (dd, J_1 = 8.4 Hz and J_2 = 10.7 Hz, 1H of CH_2Cl), 3.88 (dd, J_1 = 6.8 Hz and J_2 = 10.8 Hz, 1H of CH_2Cl), 3.91 (d, J = 9.8 Hz, 1H, 17α-CH), 6.58 (d, J = 2.5 Hz, 1H, 4-CH), 6.64 (dd, J_1 = 8 Hz and J_2 = 2.5 Hz, 1H, 2-CH), 7.14 (d, J = 8 Hz, 1H, 1-CH); ^{13}C NMR 75.5 MHz δ ($CDCl_3$): -4.40 (2X), 12.27, 18.15, 25.68 (3X), 26.17, 27.47, 27.78, 29.56, 37.62, 38.05, 41.81, 43.84, 44.24, 49.06, 64.77, 83.03, 117.15, 119.92, 126.06, 132.90, 137.72, 153.27; LRMS calculated for $C_{25}H_{39}ClO_2Si$: 434.5, found 479.5 for $C_{26}H_{40}ClO_4Si$ $[M + COOH]^-$.

Synthesis of 3-tert-butyldimethylsilyloxy-16β-bromomethyl-estra-1,3,5(10)-trien-17β-ol (20):

A solution of diol **17** (0.519 g, 1.246 mmol), PPh_3 (0.653 g, 2.491 mmol) and CBr_4 (0.826 g, 2.491 mmol) in dry CH_2Cl_2 (20 mL) was stirred at 0 °C under argon. The reaction was monitored by TLC and was completed after 3 hours. The crude mixture was pre-adsorbed on silica gel and purified by flash chromatography using a mixture of hexanes and EtOAc (95/5) as eluent to give **20** in a 64 % yield. White solid; IR ν (film): 3560 (OH); ^{1}H NMR 400 MHz δ ($CDCl_3$): 0.21 (s, 6H, $Si(CH_3)_2$), 0.84 (s, 3H, 18-CH_3), 1.00 (s, 9H, *t*-Bu of TBDMS), 1.10-2.35 (residual protons from the steroid), 2.65 (m, 1H, 16α-CH), 2.83 (m, 2H, 6-CH_2), 3.38 (t, J = 9.5 Hz, 1H of 17α-CH), 3.77 (dd, J_1 = 9.8 Hz and J_2 = 6.5 Hz, 1H of CH_2Br), 3.88 (dd, J_1 = 9.8 Hz and J_2 = 6.5 Hz, 1H of CH_2Br), 6.58 (d, J_2 = 2.4 Hz, 1H, 4-CH), 6.64 (dd, J_1 = 8.4 Hz and J_2 = 2.4 Hz, 1H, 2-CH), 7.14 (d, J_1 = 8.4 Hz, 1H, 1-CH); ^{13}C NMR 75.5 MHz δ ($CDCl_3$): -4.41 (2X), 12.33, 18.15, 25.68 (3X), 26.11, 27.41, 29.53, 31.21, 32.62, 36.53, 37.94, 43.21, 43.90, 44.43, 48.15, 81.92, 117.16, 119.93, 126.05, 132.79, 137.70, 153.33; LRMS calculated for $C_{25}H_{39}{}^{81}BrO_2Si$: 480.2, found 481.1 $[M+H]^+$.

Synthesis of 21:

Using the same protocol as for the synthesis of **20**, the bromination was performed upon diol **18**. After extraction, the crude compound **21** was used without purification for the next step (TBDMS hydrolysis).

Synthesis of final compounds 7-11 (hydrolysis of silylated ethers 17-21):

Each of the silylated ethers **17**-**21** was dissolved in a methanolic solution of HCl (2 % v/v) and the resulting mixture was stirred at room temperature for 3 hours. Water was added, the MeOH evaporated under reduced pressure and the residue extracted with EtOAc. The organic phase was washed with a saturated NaCl aqueous solution and dried over Na_2SO_4. After evaporation under reduced pressure, the crude product was purified by flash chromatography, using a mixture of hexanes and EtOAc (8/2) as eluent to give the following final compounds in 85 % to 95 % yields

16β-Bromomethyl-estra-1,3,5(10)-triene-3,17β-diol (7):

White solid; IR ν (film): 3357 (OH); ^{1}H NMR 300 MHz δ ($CDCl_3$): 0.81 (s, 3H, 18-CH_3), 1.10-2.35 (residual protons from the steroid), 2.65 (m, 1H, 16α-CH), 2.79 (m, 2H, 6-CH_2), 3.36 (t, J = 9.5 Hz, 1H of CH_2Br), 3.74 (dd, J_1 = 6.5 Hz and J_2 = 9.6 Hz, 1H of CH_2Br), 3.87 (d, J = 9.8 Hz, 1H, 17α-CH), 4.80 (broad, OH), 6.57 (d, J = 2.7 Hz, 1H, 4-CH), 6.63 (dd, J_1 = 2.7 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 7.15 (d, J = 8.4 Hz, 1H, 1-CH); ^{13}C NMR 75.5 MHz δ ($CDCl_3$): 12.32, 26.17, 27.31, 29.51, 32.60, 36.47, 37.50, 37.96, 43.19, 43.81, 47.14, 48.10, 81.92, 112.65, 115.20, 126.49, 132.49, 138.15, 153.30; LRMS calculated for $C_{19}H_{25}{}^{79}BrO_2$: 364.5, found 365.0 $[M+H]^+$.

16β-Chloromethyl-estra-1,3,5(10)-triene-3,17β-diol (8):

White solid; IR ν (film): 3380 (OH); ^{1}H NMR 400 MHz δ ($CDCl_3$): 0.84 (s, 3H, 18-CH_3), 1.15-2.40 (residual protons from the steroid), 2.60 (m, 1H, 16α-CH), 2.85 (m, 2H, 6-CH_2), 3.54 (dd, J_1 = 8.3 Hz and J_2 = 10.7 Hz, 1H of CH_2Cl), 3.88 (dd, J_1 = 6.8 Hz and J_2 = 10.8 Hz, 1H of CH_2Cl), 3.91 (d, J = 10.8 Hz, 1H, 17α-CH), 6.59 (d, J = 2.4 Hz, 1H, 4-CH), 6.65 (dd, J_1 = 8.4 Hz, J_2 = 2.4 Hz, 1H, 2-CH), 7.17 (d, J = 8.4 Hz, 1H, 1-CH); ^{13}C NMR 75.5 MHz δ ($CDCl_3$): 12.24, 26.20, 27.33, 29.52, 31.18, 37.49, 37.97, 43.03, 44.28, 47.14, 48.36, 62.51, 81.73, 112.65, 115.20, 126.49, 135.58, 138.15, 153.30; LRMS calculated for $C_{19}H_{25}ClO_2$: 320.5, found 365.1 for $C_{20}H_{26}ClO_4$ $[M+COOH^-]$.

16β-Hydroxymethyl-estra-1,3,5(10)-triene-3,17β-diol (9):

White solid; ^{1}H NMR 400 MHz δ (CD_3OD): 0.82 (s, 3H, 18-CH_3), 1.10-2.50 (residual protons from the steroid), 2.78 (m, 2H, 6-CH_2), 3.54 (dd, J_1 = 10.8 Hz and J_2 = 7.6 Hz, 1H of CH_2OH), 3.84 (m, 1H of CH_2OH), 3.85 (d, J = 10.0 Hz, 17α-CH), 6.49 (d, J = 2.5 Hz, 1H, 4-CH), 6.55 (dd, J_1 = 8.4 Hz and J_2 = 2.5Hz, 1H, 2-CH), 7.09 (d, J_1 = 8.4 Hz, 1H, 1-CH); ^{13}C NMR 75.5 MHz δ (CD_3OD): 12.92, 27.62, 28.76, 30.26, 30.73, 38.85, 39.94, 43.36, 45.30, 45.36, 50.21, 65.55, 83.39, 113.70, 116.00, 127.20, 132.55, 138.76, 155.91; LRMS calculated for $C_{19}H_{26}O_3$: 302.3, found 301.3 $[M-H]^-$.

2-Methoxy-16β-bromomethyl-estra-1,3,5(10)-triene-3,17β-diol (10):

White solid; IR ν (film): 3505 (OH); ^{1}H NMR 400 MHz δ ($CDCl_3$): 0.84 (s, 3H, 18-CH_3), 1.10-2.40 (residual protons from the steroid), 2.70 (m, 1H, 16α-CH), 2.79 (m, 2H, 6-CH_2), 3.38 (t, J = 9.5 Hz, 1H of CH_2Br), 3.77 (dd, J_1 = 6.6 Hz and J_2 = 9.8 Hz, 1H of CH_2Br), 3.88 (s, 3H, OCH_3), 3.89 (d, J = 7.8 Hz, 1H, 17α-CH), 6.67 (s, 1H, 4-CH), 6.81 (s, 1H, 1-CH); ^{13}C NMR 75.5 MHz δ ($CDCl_3$): 12.33, 26.47, 27.45, 28.88, 32.58, 36.44, 37.56, 37.96, 43.22, 44.15, 44.43, 48.10, 56.00, 81.88, 107.94, 114.58, 129.38, 131.50, 143.44, 144.53; LRMS calculated for $C_{20}H_{26}{}^{79}BrO_3$: 394.4, found 395.0 $[M+H]^+$.

2-Methoxy-16β-hydroxymethyl-estra-1,3,5(10)-triene-3,17β-diol (11):

White solid; IR ν (film) cm^{-1}: 3380 (OH); ^{1}H NMR 400 MHz δ ($CDCl_3$) : 0.87 (s, 3H, 18-CH_3), 1.00-2.30 (residual protons from the steroid), 2.50 (m, 1H, 16α-CH), 2.79 (m, 2H, 6-CH_2), 3.69 (dd, J_1 = 4.3 Hz and J_2 = 10.0 Hz, 1H of CH_2OH), 3.87 (m, 1H of CH_2OH), 3.88 (s, 3H, OCH_3), 3.99 (d, J = 9.8 Hz, 1H, 17α-CH), 6.67 (s, 1H, 4-CH), 6.81 (s, 1H, 1-CH); ^{13}C NMR 75.5 MHz δ ($CDCl_3$): 12.27, 26.56, 27.54, 27.79, 28.94, 37.68, 38.09, 41.97, 44.12, 44.27, 49.03, 56.03, 64.74, 83.06, 107.98, 114.58, 129.43, 131.61, 143.46, 144.56; LRMS calculated for $C_{20}H_{28}O_4$: 332.6, found 331.2 $[M-H]^-$.

Synthesis of 2-methoxy-3-tert-butyldimethylsilyloxy-16α-allyl-estra-1,3,5(10)-trien-17β-ol (23):

A solution of diisopropylamine (0.199 mL, 1.42 mmol) in dry THF (20 mL) was stirred under argon at 0 °C and a solution of *n*-butyllithium in hexanes (0.57 mL, 1.42 mmol) was added dropwise. After 45 minutes, the resulting lithium diisopropylamide (LDA) solution was cooled at –78 °C and **14** (0.535 g, 1.29 mmol) dissolved in dry THF (10 mL) was added dropwise. The mixture was allowed to stir for 1 hour at 0 °C, then cooled again to –78 °C and allyl bromide (0.123 mL, 1.42 mmol) was added dropwise. The reaction mixture was stirred overnight from –78 °C to room temperature. Water was added to quench the reaction and the crude product was extracted with EtOAc. The organic phase was washed

with a saturated NaCl aqueous solution, dried over Na_2SO_4 and evaporated under reduced pressure. A solution of $LiAlH_4$ in THF (1.65 mL, 1.65 mmol) was added dropwise to a solution of crude α/β-allyl derivative **22** (0.535 g, 1.18 mmol) dissolved in dry THF (15 mL) and cooled at 0 °C. The reaction was completed after 30 minutes. Water was slowly added at 0 °C to quench the reaction and the crude product was extracted using EtOAc. The organic phase was washed with a saturated NaCl aqueous solution, dried over Na_2SO_4, then filtered and evaporated under reduced pressure. The crude product was purified by flash chromatography, using a mixture of hexanes and EtOAc (9/1) as eluent to give **23** in 66 % yield for two steps (**14** to **23**). Only the 16α-isomer was recovered. White solid; IR ν (film): 3380 (OH); ^{1}H NMR 300 MHz δ ($CDCl_3$): 0.14 (s, 6H, $Si(CH_3)_2$), 0.82 (s, 3H, 18-CH_3), 0.98 (s, 9H, *t*-Bu of TBDMS), 1.20-2.40 (residual protons from the steroid), 2.72 (m, 2H, 6-CH_2), 3.32 (d, J = 7.5 Hz, 1H, 17α-CH), 3.76 (s, 3H, 2-CH_3O), 5.03 (d, J = 10.5 Hz, 1H of $CH=CH_2$), 5.09 (d, J = 17.4 Hz, 1H of $CH=CH_2$), 5.88 (m, 1H, $CH=CH_2$), 6.54 (s, 1H, 1-CH), 6.76 (s, 1H, 4-CH); ^{13}C NMR 75.5 MHz δ ($CDCl_3$): -4.60 (2X), 11.92, 18.41, 25.74 (3X), 26.30, 27.34, 28.79, 29.69, 36.81, 38.40, 39.62, 43.31, 44.07, 44.30, 48.32, 55.82, 87.39, 109.79, 115.72, 120.95, 128.93, 133.11, 137.84, 142.82, 148.61.

Synthesis of 2-methoxy-3-tert-butyldimethylsilyloxy-16α-(3'-hydroxypropyl)-estra-1,3,5(10)-trien-17β-ol (24):

To a stirred solution of **23** (0.287 g, 0.629 mmol) in dry THF (20 mL) at 0 °C a solution of borane in THF (2.83 mL, 2.83 mmol) was added dropwise. The mixture was allowed to react under argon for 3 hours, then a solution of 30 % (w/v) H_2O_2 (286 µL) and NaOAc (257 mg) in water (780 µL) was added. The resulting mixture was stirred at room temperature for 1 hour. The reaction was quenched by addition of water and extracted with EtOAc. The organic phase was washed with a saturated NaCl aqueous solution, dried over Na_2SO_4, then filtered and evaporated under reduced pressure. The crude product was purified by flash chromatography using a mixture of hexanes and EtOAc (7/3) as eluent to give **24** in 66 % yield. ^{1}H NMR 400 MHz δ ($CDCl_3$): 0.17 (s, 6H, $Si(CH_3)_2$), 0.84 (s, 3H, 18-CH_3), 1.01 (s, 9H, *t*-Bu of TBDMS), 1.20-2.40 (residual protons from the steroid), 2.74 (m, 2H, 6-CH_2), 3.33 (d, J = 7.4 Hz, 1H, 17α-CH), 3.72 (m, 2H, 3'-CH_2OH), 3.79 (s, 3H, 2-CH_3O), 6.56 (s, 1H, 4-CH), 6.79 (s, 1H, 1-CH); ^{13}C NMR 75.5 MHz δ ($CDCl_3$): -4.58 (2X), 12.01, 18.43, 25.77 (3X), 26.33, 27.36, 28.82, 30.37, 31.24, 32.20, 36.85, 38.45, 43.37, 44.22, 44.31, 48.35, 55.85, 63.28, 88.01, 109.82, 120.99, 128.95, 133.11, 142.70, 148.65.

Synthesis of 2-methoxy-3-tert-butyldimethylsilyloxy-16α-(3'-bromopropyl)-estra-1,3,5(10)-trien-17β-ol (25):

A solution of **24** (0.095 g, 0.200 mmol), PPh_3 (0.105 g, 0.400 mmol) and CBr_4 (0.133 g, 0.400 mmol) in dry CH_2Cl_2 (10 mL) was stirred at 0 °C under argon. The reaction was monitored by TLC and was completed after 3 hours. The crude mixture was pre-adsorbed on silica gel and purified by flash chromatography using hexanes and EtOAc (95/5) as eluent to give **25** in 64 % yield. White solid; ^{1}H NMR 400 MHz δ ($CDCl_3$): 0.17 (s, 6H, $Si(CH_3)_2$), 0.84 (s, 3H, 18-CH_3), 1.01 (s, 9H, *t*-Bu of TBDMS), 1.20-2.35 (residual protons from the steroid), 2.74 (m, 2H, 6-CH_2), 3.31 (d, J = 7.2 Hz, 1H, 17α-CH), 3.47 (t, J = 6.3 Hz, 1.33 H, 3'-CH_2Br), 3.60 (t, J = 6.3 Hz, 0.67 H, 3'-CH_2Br), 3.79 (s, 3H, 2-CH_3O), 6.57 (s, 1H, 4-CH), 6.78 (s, 1H, 1-CH).

Synthesis of 2-methoxy-16α-(3'-bromopropyl)-estra-1,3,5(10)-triene-3,17β-diol (12):

The silylether **25** was dissolved in a methanolic solution of HCl (2 %, v/v) and the resulting mixture was stirred at room temperature for 3 hours. Water was added, the MeOH evaporated under reduced pressure and the residue extracted with EtOAc. The organic phase was washed with a saturated NaCl aqueous solution and dried over Na_2SO_4. After evaporation under reduced pressure, the crude product was purified by flash chromatography, using a mixture of hexanes and EtOAc (8/2) as eluent to give **12** in 75 % yield. White solid; ^{1}H NMR 400 MHz δ ($CDCl_3$): 0.83 (s, 3H, 18-CH_3), 1.25-2.30 (residual protons from the steroid), 2.78 (m, 2H, 6-CH_2), 3.32 (d, J = 7.3 Hz, 1H, 17α-CH), 3.47 (t, J = 6.7 Hz, 2H, 3'-CH_2Br), 3.88 (s, 3H, 2-CH_3O), 5.50 (broad, 1H, OH), 6.67 (s, 1H, 4-CH), 6.81 (s, 1H, 1-CH); ^{13}C NMR 75.5 MHz δ ($CDCl_3$): 11.87, 26.43, 27.23, 28.91, 30.06, 31.62, 34.21, 36.71, 38.49, 43.10, 44.12, 44.21, 45.34, 48.29, 56.00, 87.98, 107.95, 114.56, 129.44, 131.62, 143.40, 144.52; LRMS calculated for $C_{22}H_{31}{}^{79}BrO_3$: 422.5, found 423.1 $[M+H]^+$ and 405.2 $[M-H_2O]^+$.

***In Vitro* Assay for the Inhibition of 17β-HSD1**

The enzymatic assay was performed as previously described [22]. Briefly, Human Embryonic Kidney (HEK)-293 cells transfected with cDNA encoding for 17β-HSD1 were sonicated to liberate the crude enzyme that was used as the enzymatic pool without further purification. The enzymatic assay was performed as follows: a stock solution was first prepared containing the radiolabeled substrate [^{14}C]-E1 (0.1 µM), NADH (1 mM) in a phosphate buffer (pH 7.4, 50 mM KH_2PO_4, EDTA 1 mM, 20% glycerol). For the assay, 890 µL of the stock solution and 10 µL of a solution of inhibitor dissolved in ethanol were added in a tube. The reaction was started by adding 100 µL of a solution of crude enzyme prepared as described above. The mixture was incubated for 1 hour at 37 °C, and the reaction was stopped by adding an excess of unlabeled E1 and E2. Steroids were extracted with diethyl ether and solvent was removed under reduced pressure. The residue was dissolved in CH_2Cl_2, spotted on a silica gel plate (TLC, 20 cm x 20 cm x 0.2 mm, Kieselgel 60 F254) and eluted with CH_2Cl_2/EtOAc (9:1). Less polar E_1 and more polar E_2 were identified on TLC as two rows of visible spots under UV light. Radioactivity signals associated to [^{14}C]-E1 and [^{14}C]-E2 were detected and quantified using a Storm 860 Imager (Molecular Dynamics, Sunny Vale, CA, USA). The percentage of transformation of [^{14}C]-E1 into [^{14}C]-E2 was calculated as follows: % trans. = 100 X [^{14}C]-E2 (unit) / ([^{14}C]-E1 (unit) + [^{14}C]-E2 (unit)). From the curve of the % trans. versus the concentration of inhibitor, the IC_{50} value was calculated using an unweighted iterative least-sqares method for four-parameter logistic curve fitting (DE_{50} program, CHUL Research Center, Québec QC, Canada).

In Vitro Assay for the Determination of Estrogenic Activity

The ER-positive breast cancer cell line T-47D was obtained from the American Type Culture Collection (ATCC) and maintained in 75 cm^2 culture flasks at 37 ºC under 5 % CO_2 humidified atmosphere in RPMI medium (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10 % foetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 µg/mL) and estradiol (1 nM). For the cell growth assay, T-47D cells were re-suspended in RPMI medium (without E2) supplemented with insulin (50 ng/mL) and 5 % dextran-coated charcoal treated FBS to remove the remaining estrogen present in the serum. Aliquots (100 µL) of the cell suspension were seeded in 96-well plates (3000 cells/well). After 48 hours, the medium was changed with appropriate dilution of the different inhibitors and reference compounds in medium and was replaced every 2 days until 8 days of treatment. Quantification of cell growth was determined using CellTitter 96® AQ_{ueous} Solution Cell Proliferation Assay (Promega, Nepean, ON, Canada) following the manufacturer's instructions. To determine the proliferative (estrogenic) activity, the cells were grown in absence (control fixed as 100 %) or presence of the tested compound at indicated concentrations.

In Vivo Assay for the Determination of Estrogenic Activity

The *in vivo* estrogenic activity of two compounds was measured by stimulation of uterine and vagina weight in adult ovariectomized (OVX) Balb/c mice (body wt – 18-20 g) sacrificed 10 days after ovariectomy (day 0). Compounds **5a** and **12** (at doses of 10 and 50 µg / mouse), estrone (0.06 µg / mouse) and estradiol (0.02 µg) dissolved in ethanol were injected s.c. in each appropriate group of mice in a solution

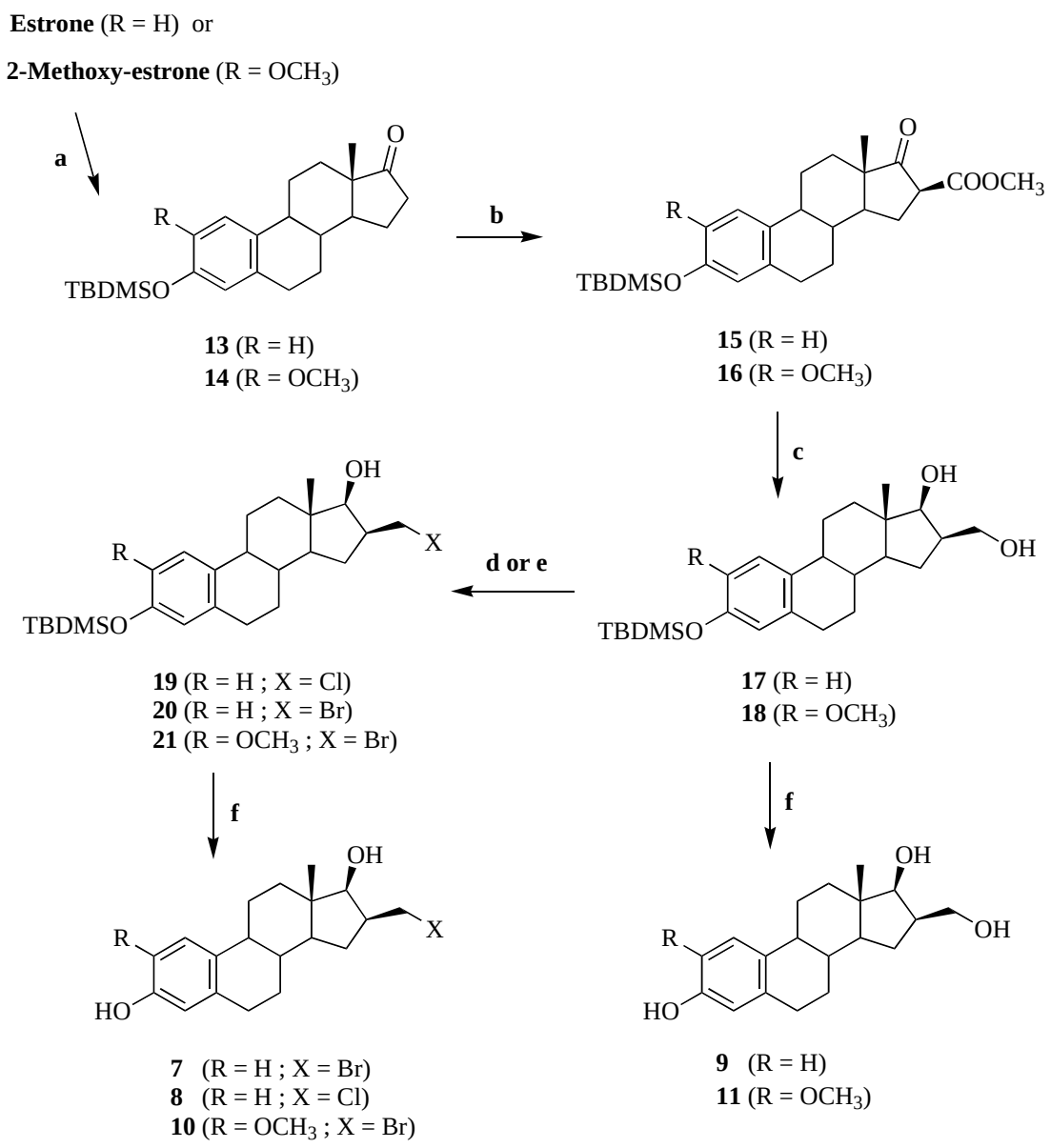


Fig. (2). Synthesis of **7-11**. The reagents are: (**a**) TBDMS-Cl, imidazole; (**b**) LDA, methylcyanoformate, HMPA; (**c**) $LiAlH_4$; (**d**) Ph_3P, CCl_4; (**e**) Ph_3P, CBr_4; (**f**) 2 % HCl in MeOH.

of 0.4 % methylcellulose (8 % ethanol final concentration) for a total volume of 0.2 mL, twice daily for 9 days, starting on day 2 of the protocol for a total of 18 injections. Vehicle only was injected to intact and CTR-OVX groups. After sacrifice, the uteri and vagina were rapidly removed, freed from fat and connective tissue and weighed. Results are the means ± SEM of 8 mice per group.

RESULTS AND DISCUSSION

Chemical Synthesis

The sequences of reactions needed to obtain compounds **7-12** are represented in Fig. (**2**) and (**3**). Following a similar route already employed by us [18], **17** and **18** were synthesized without difficulty starting from E1 and 2-MeO-E1. First, this involves the protection of the phenol group with a *tert*-butyldimethylsilyl (TBDMS) ether followed by the alkylation using methylcyanoformate to afford after a silica gel chromatography, mostly the more stable β-epimer (compounds **15** and **16**). Then, a classical reduction with lithium aluminium hydride of both ketone and ester groups gave us, after purification with silica gel, the desired 16β-hydroxymethyl compounds **17** and **18**. We then used either CBr_4 or CCl_4 with triphenylphosphine to obtain according to the case, the 16β-bromomethyl and 16β-chloromethyl derivatives **19-21**. Another point should be mentioned about the chlorination or bromination of the primary alcohols **17** and **18** (conditions (d) and (e) of Fig. **2**). If the reaction goes on for more than 4 hours, TBDMS hydrolysis occurs with a total removing of the TBDMS protective group after 12 hours. Despite this, the final compounds were easily obtained in expected good yields for these kinds of reactions. The TBDMS hydrolysis of compounds **17-21** was performed using a mild acidic condition (2 % HCl in MeOH) to afford compounds **7-11** in good yields.

The synthesis of 16α-(3'-bromopropyl)-2-MeO-E2 (**12**) follows the route previously developed for inhibitor **5a** [18], and the achievement of the synthesis did not encounter big issues (Fig. **3**). However, after introduction of the allyl moiety upon the starting material **14**, the isolation of the pure 16α isomer is not possible at this stage. The best way to do it is to perform the carbonyl reduction upon the crude mixture of 16α and 16β-allyl isomers **22** into corresponding alcohols. The isolation of the 16α isomer **23** becomes much more feasible by classical flash chromatography of isomeric alcohols instead of isomeric ketones **22**.

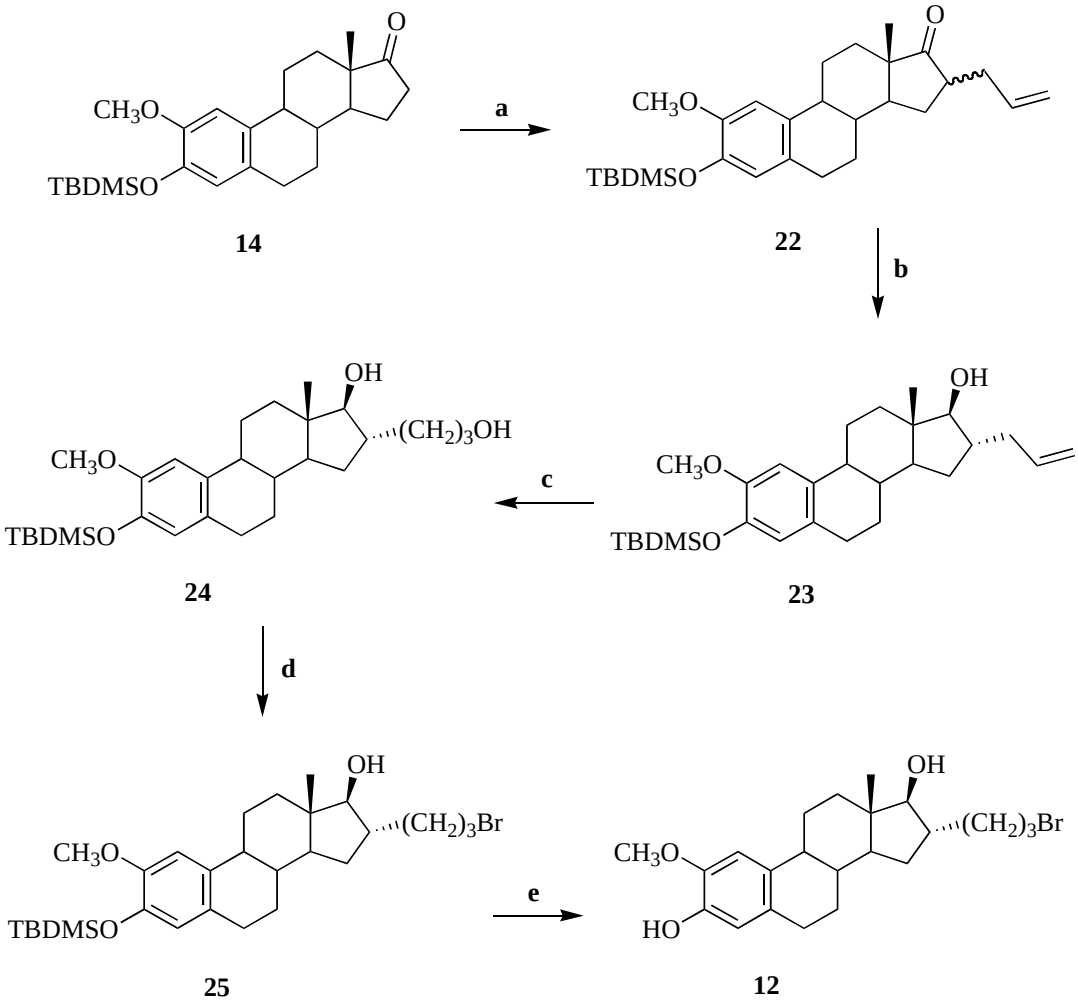


Fig. (3). Synthesis of **12**. The reagents are: (**a**) LDA, allyl bromide; (**b**) $LiAlH_4$; (**c**) *1*. CH_3, *2*. H_2O_2, NaOAc; (**d**) Ph_3P, CBr_4; (**e**) 2 % HCl in MeOH.

The 16β-stereochemistry of final compounds **7-11** and the 16α-stereochemistry of compound **12** were unambiguously confirmed by ^{1}H and ^{13}C NMR analysis. Indeed, we previously reported that proton and carbon signals at position 17 are useful probes for C-16 and C-17 stereochemistry determination [23]. All expected NMR probes were found in NMR spectra of final compounds (Table **2**).

In Vitro Study (17β-HSD1 Inhibitory Activity)

The IC_{50} values of synthesized compounds **7-12** and reference compounds **5a** and E2 were determined in homogenate of HEK-293 cells overexpressing 17β-HSD1 (Table **3**). The enzyme preparation was incubated with 100 nM of [^{14}C]-E1 in presence of the inhibitor at five different concentrations (0.01, 0.1, 1.0, 10 and 30 μM) to obtain a good approximation of the IC_{50} value.

In the first protocol (assay A) we evaluated the inhibitory character of the E2 derivatives at all concentrations mentioned above. We can see that the inhibitor **5a**, already synthesized by our research team [18], has good inhibitory effect upon 17β-HSD1 with IC_{50} values of 1.30 μM. In comparison, the non radioactive natural substrate E2, without a substituent at position 16, weakly inhibited the enzyme (IC_{50} = 7.34 μM). In the series of 16β-methylene derivatives of E2 (compounds **7-9**), we can conclude that **7** (16β-bromomethyl-E2) and **9** (16β-hydroxymethyl-E2), with IC_{50} values of 1.20 and 1.49 μM, have comparable inhibitory effects than reference inhibitor **5a** upon 17β-HSD1. Compound **8** is not as good an inhibitor of 17β-HSD1 as the previous two with IC_{50} values over 3 μM. The 2-MeO-E_2 derivatives **10-12** were next tested in a second protocol (assay B). First, we can see that the IC_{50} values of **5a** and **9** are consistent in both assays indicating that the results of the enzyme assays are reproducible (Table **3**). The IC_{50} values of compounds **10-12** (5.91, 3.80 and 5.80 μM, respectively) are not as good as previously expected and the presence of a 2-MeO group clearly decreases the inhibitory potency on 17β-HSD1. This negative effect of the 2-MeO group was recently observed in other families of 17β-HSD1 inhibitors [24-26].

In Vitro Study (Estrogenic Activity in ER^{+} Cell Line)

The breast cancer T-47D cell line was used to determine the estrogenic profile of inhibitors **7-12** (Fig. **4**). These cells express the estrogen receptor (ER) and proliferate in pres-

Table 2. NMR Probes for 16β- and 16α-Derivatives of 17β-E2

#	Solvent	H-17α (16β-R) ppm	H-17α (16α-R) ppm	C-17 (16β-R) ppm	C-17 (16α-R) ppm
7	$CDCl_3$	3.87	---	81.92	---
8	CD_3OD	3.91	---	81.73	---
9	$CDCl_3$	3.85	---	83.39	---
10	$CDCl_3$	3.89	---	81.88	---
11	$CDCl_3$	3.99	---	83.06	---
12	$CDCl_3$	---	3.32	---	87.98
A*	Acetone-d_6	3.76	---	81.91	---
B**	Acetone-d_6	---	3.27	---	87.11

(*) 16α-Allyl-E2. Data from reference [23]. **(**)** 16β-Allyl-E2. Data from reference [23].

Table 3. Inhibition of 17β-HSD1 Transformation of [^{14}C]-E1 into [^{14}C]-E2 by E2, 5a and 7-12

#	Steroid Nucleus	C-16 Group	α /β	IC_{50} (μM) Assay A	IC_{50} (μM) Assay B
E2	E2	H	---	7.34	---
5a	E2	$(CH_2)_3Br$	α	1.30	0.99
7	E2	CH_2Br	β	1.20	---
8	E2	CH_2Cl	β	3.02	---
9	E2	CH_2OH	β	1.49	1.27
10	2-MeO-E2	CH_2Br	β	---	5.91
11	2-MeO-E2	CH_2OH	β	---	3.80
12	2-MeO-E2	$(CH_2)_3Br$	α	---	5.80

ence of estrogens. Similarly as observed for E2, the E2 derivatives **5a**, **7**, **8** and **9** clearly stimulated the cell growth. The 2-MeO-E2 derivatives **10**-**12** differently affected the cell growth. Thus, compound **10** is not estrogenic at 0.1 μM but estrogenic at 1 μM, compound **11** does not induce proliferation at both concentrations, whereas **12** appears to be estrogenic. This last result is very deceptive considering the presence of a 2-MeO-E2 nucleus. To better understand the role of this nucleus on estrogenicity, the effect of 2-MeO-E2 derivative **12** and the E2 derivative **5a** on T-47D cell proliferation was measured at several concentrations and compared with the 2-MeO-E2 nucleus only (without the 16α-side chain) Fig. (**5**). All three compounds induced the cell proliferation in the following order: **5a** > **12** > 2-MeO-E2. In fact, they produce an estrogenic effect over 0.01 μM and have the same pattern of activity until 0.5 μM. Over 0.5 μM, cell growth decreased markedly in the presence of 2-MeO-E2 but not with **12** and **5a**. For **12** and **5a**, cell growth increased until 3 μM and 5 μM, respectively, and then started to decrease. These results illustrate that the 2-MeO-E2 derivatives are not estrogenic at a low concentration (< 0.01 μM) but are estrogenic in a range of concentrations, which vary according to the substituent added on the nucleus. At high concentrations, however, the effect on cell growth is reversed suggesting a non ER-dependent mechanism. In fact, we

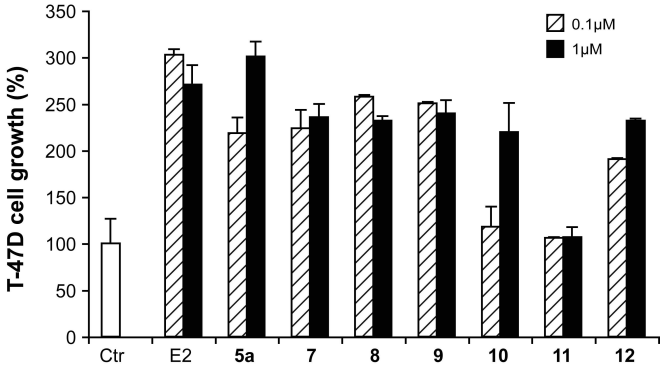


Fig. (4). Effect of E2, **5a** and **7**-**12** on the proliferation of estrogen-sensitive T-47D cells.

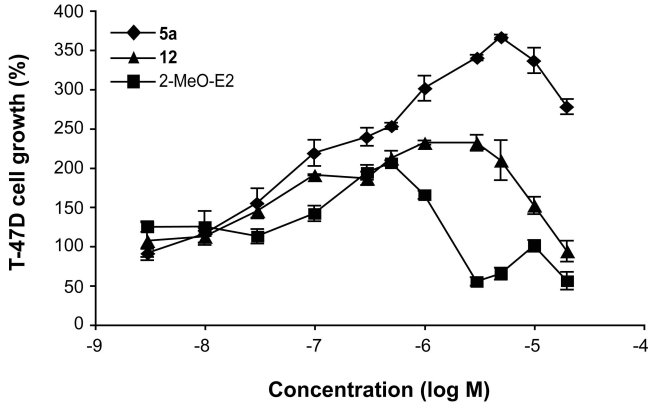


Fig. (5). Effect of **5a**, **12** and 2-MeO-E2 on the proliferation of estrogen-sensitive T-47D cells.

observed cell death over 10 μM. Thus, the 2-MeO-E2 nucleus is less estrogenic than the E2 nucleus, however it kept a residual estrogenic character in T-47D cells before producing a cytotoxic activity at higher concentrations. In estrogen-sensitive MCF-7 cells, La Vallee *et al*. [27] obtained a similar curve of proliferation with the 2-MeO-E2. Such results are in agreement with the conclusions of Sutherland *et al*. [28], which reported that 2-MeO-E2 produces both ER-dependent and ER-independent effects. Moreover, it has been also reported that 2-MeO-E2 enhances inhibitory effects on tubulin polymerization and cancer cell growth, and inhibits the angiogenesis required for the growth of solid tumors [29-33].

In Vivo Study (Estrogenic Activity in ER$^+$ Tissues)

Estrogenic activity of tested compounds was investigated *in vivo* using the OVX mouse model by measuring the weight of the uterus (Fig. **6**) and the vagina (Fig. **7**), two estrogen-sensitive (ER$^+$) tissues. As observed in Fig. (**6**), E1 and E2 increase the weight of the uterus. For the OVX mice control group, we have noticed a 66 % reduction of the weight of the uterus compared with the group of mice intact; which is perfectly logical because this control group of mice has lowered the endogenous source of estrogens provided by the ovaries. When administrated s.c. to OVX mice, E1 (0.06 μg) is converted into E2 by the 17β-HSD1 and we observed an 7.5-time increase of the uterine weight compared to the control group (OVX-CTR). We also administered s.c. E2 (0.02 μg) to the OVX mice with an 6.5-time increase of uterine weight compared to the control group (OVX-CTR). When we tested **5a**, we could see that at a 10 μg dose uterine growth is stimulated passing from 20 mg for the OVX-CTR group to 100 mg for the OVX-**5a** group. This result reflects the fact already observed by us that this inhibitor of 17β-HSD1 stimulates the ER$^+$ cell line ZR-75-1 (data not shown) and MCF-7 (see above). In counterpart, at a 50 μg injection dose, **5a** weakly stimulates the growth of the uterus passing from 20 mg for the OVX-CTR group to 39 mg for the OVX-**5a** group. One hypothesis to explain this unexpected result at the higher dose of **5a** is that we have an ER-independent mechanism as discussed above, which is responsible for growth inhibition. In counterpart, at the low dose, we have an ER-dependent mechanism and cell stimulation by ER is favored.

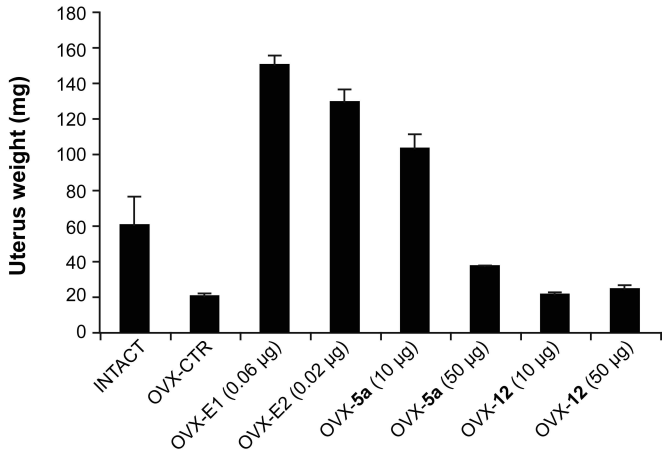


Fig. (6). Effect of E1, E2, **5a** and **12** on the weight of estrogen-sensitive tissue (uterus) of ovariectomized Balb/c mice. CTR, control group (vehicle only); OVX, ovariectomized mice; E1, estrone; E2, estradiol.

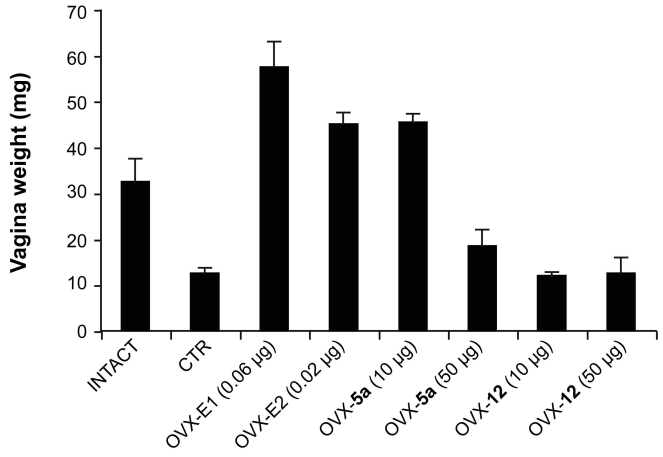


Fig. (7). Effect of E1, E2, **5a** and **12** on the weight of estrogen-sensitive tissue (vagina) of ovariectomized Balb/c mice. CTR, control group (vehicle only); OVX, ovariectomized mice; E1, estrone; E2, estradiol.

Finally, when we tested the 2-MeO analogue of **5a**, compound **12**, at two doses of 10 μg and 50 μg, we obtained no weight stimulation of the uterus reflecting the non-estrogenic character of **12** on the uterus at these two doses. Furthermore, the non-estrogenic character of **12** had been evaluated by measuring the weight of the vagina instead of the weight of the uterus (Fig. **7**). Clearly, measuring of the vagina demonstrated the same tendency as previously observed with the uterus.

CONCLUSIONS

The 2-MeO-E2 nucleus was used as a strategy to reduce the estrogenicity of a series of inhibitors of 17β-HSD1. The presence of the 2-MeO group clearly decreased the potency of these inhibitors in HEK-293 cells overexpressing 17β-HSD1 and did not fully eliminate their proliferative estrogenic activity on T-47D ER^+ cells. However, no stimulation of estrogen-sensitive tissues (uterus and vagina) was observed with compound **12**, when tested *in vivo* at two concentrations of 10 and 50 μg/day. Although this last result is interesting, additional work will be needed to increase the inhibitory potency of compound **12** on 17β-HSD1 as well as to reduce its ER-binding affinity in order to obtain an inhibitor of 17β-HSD1 acting in a range of concentrations without producing an estrogenic effect. On the other hand, this inhibitor could be alternatively used at high concentrations, thus providing an ER-independent cytotoxic effect on breast cancer cells and potential antiangiogenic effects. Considering the presence of the 2-MeO-E2 nucleus, it would be also interesting to perform other biological studies to measure the potential of compound **12** as angiogenesis inhibitor and its effectiveness upon the inhibition on tubulin polymerization.

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