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17α-Alkan (or alkyn) amide derivatives of estradiol as inhibitors of steroid-sulfatase activity

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Abstract

To develop inhibitors of steroid sulfatase without residual estrogenic activity, we have designed a series of estradiol (E_2) derivatives bearing an alkan (or alkyn) amide side chain at position 17α. A hydrophobic alkyl group was selected from our previous study where 17α-octyl-E_2 was found to inhibit strongly the steroid-sulfatase activity. Furthermore, it is known that an alkylamide side chain blocks the estrogen-receptor activation. Starting from ethynylestradiol, the chemical synthesis of target compounds was short and efficient with overall yields of 22–42% (3 or 4 steps). Among these compounds, *N*-octyl,*N*-methyl-3-(3′,17′β-dihydroxy-1′,3′,5′(10′)-estratrien-17′α-yl)-propanamide (**15**) was the most potent inhibitor, with an IC_{50} value of 0.08 μM for the transformation of estrone sulfate (E_1S) to estrone (E_1) by homogenated JEG-3 cells. *N*-butyl, *N*-hexyl, and *N*,*N*-dioctyl propanamide derivatives of E_2 (IC_{50} values of 6.4, 2.8, and >20 μM, respectively) were less potent inhibitors than *N*-octyl analog **15**. Furthermore, the unsaturated propynamide analog of **15** gave lower inhibition (four times) than the saturated compound. Compound **15** is also about 100-fold more effective in interacting with the enzyme than substrate E_1S itself. The ability of target compounds to bind the estrogen receptor, to stimulate the proliferation of estrogen-sensitive ZR-75-1 cells, or to inhibit the E_2-stimulation of ZR-75-1 cells was also evaluated. Although a mixed estrogenic/anti-estrogenic activity was obtained for tested compounds at 1 μM, no estrogenic activity was observed at 0.03 μM for **15**. In conclusion, a promising inhibitor of steroid-sulfatase activity was obtained by introducing a hydrophobic octyl group in a 17α-propanamide side chain of E_2, but further structure-activity relationships (SAR) studies are necessary to minimize the residual estrogenic activity. © 1999 Elsevier Science Inc. All rights reserved.

Keywords: Steroid sulfatase; Inhibitor; Enzyme; Steroid; Estradiol; Alkylamide; Cancer

1. Introduction

Estradiol (E_2) is known to be involved in both the etiology and maintenance of growth of breast cancer [1]. The use of an anti-estrogen to block the interaction of E_2 (or other estrogens) with the estrogen receptor (ER) is thus a logical approach to therapeutic treatment of estrogen-dependent diseases [1–4]. The well-known anti-estrogen tamoxifen has been widely used in the treatment of breast cancer [4,5]. Tamoxifen is known, however, to possess mixed estrogenic/anti-estrogenic activities that are highly species-, tissue-, cell-, and gene-specific [6–10]. Recently, a nonsteroidal compound named EM-800 was reported as the most potent anti-estrogen described so far [11]. This compound is a pure anti-estrogen (i.e. without residual estrogenic activity) and is devoid of the estrogenic activity observed in human endometrial cancer Ishikawa cells for other nonsteroidal anti-estrogens, namely, raloxifene, droloxifene, 4-OH-toremifene, and 4-OH-tamoxifen [12]. Furthermore, steroidal compounds have also been described as pure anti-estrogens. These compounds, exemplified by ICI 164384 [13,14], EM-139 [15], and RU 51625 [16], contain a methylbutylalkanamide side chain at the 7α- or 11β-position of E_2, whereas ICI 182780 [17] contains a pentafluoropentylsulfinyl alkane side chain at the 7α-position of E_2.

Another approach to the treatment of estrogen-dependent cancers is the inhibition of enzymes involved in the formation (steroidogenesis) of active steroidal hormones (Fig. 1). Conversion of androstenedione (Δ^4-dione) to estrone (E_1) by aromatase, estrone sulfate (E_1S) to E_1 by steroid sulfatase, and E_1 to E_2 by 17β-hydroxysteroid dehydrogenase (17β-HSD type

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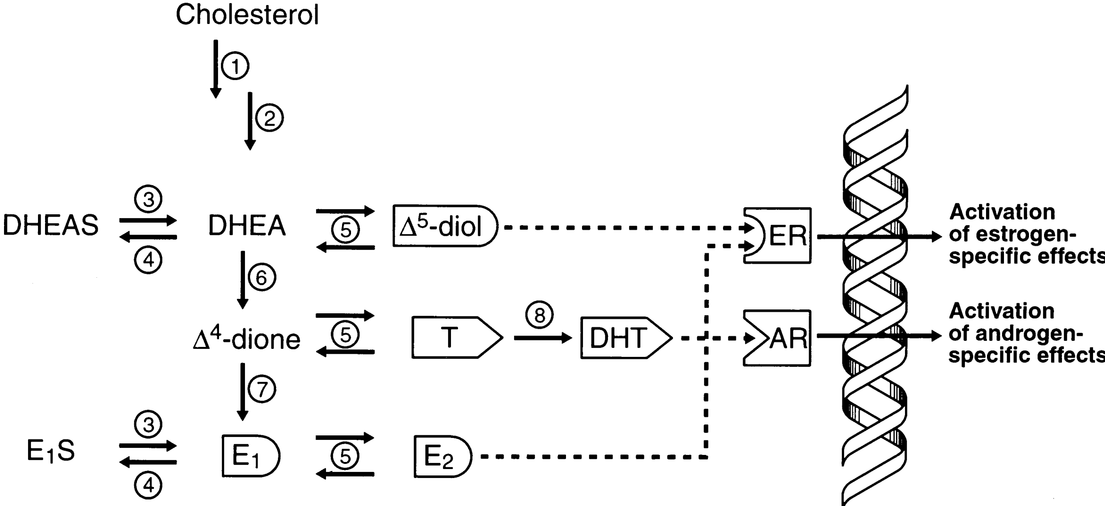

Fig. 1. The enzymatic steps involved in the biosynthesis of the active estrogens E_2, E_1, and 5-androsten-3β,17β-diol (Δ^5-diol), as well as the androgens dihydrotestosterone (DHT) and testosterone (T). The enzymes are: 1) P450 side-chain cleavage, 2) P450 17α-hydroxylase / P450 17,20-lyase, 3) steroid sulfatase, 4) steroid sulfotransferase, 5) 17β-hydroxysteroid dehydrogenases, 6) 3β-hydroxysteroid dehydrogenase / Δ^5-Δ^4 isomerase, 7) aromatase, and 8) 5α-reductases. ER: estrogen receptor, AR: androgen receptor.

1) are important enzymatic pathways that are thought to occur in cancer cells and may explain the high concentration of estrogens in breast tumors. The above-mentioned steroidogenic enzymes are three attractive targets to block the formation of estrogens and potentially reduce their levels. Although aromatase inhibitors have been widely studied in the past [18–20], the development of 17β-HSD inhibitors [21–24] and steroid-sulfatase inhibitors [25–30] has been reported more recently.

We previously reported that 17α-alkyl-estradiols, such as 17α-octyl-estradiol (**1**; IC_{50} = 440 nM) and 17α-(substituted benzyl)-estradiols (IC_{50} = 22–298 nM), inhibit strongly the hydrolysis of E_1S to E_1 by steroid-sulfatase activity found in JEG-3 cells [30]. Unfortunately, these compounds have a proliferative effect on estrogen-sensitive ZR-75-1 cells [31], which is unsuitable for further therapeutic purpose. We reported, however, that it is possible to combine two inhibitory effects (inhibition of 17β-HSD type 1 and anti-estrogenic activity) in the same alkanamide side chain added at position 16α of an estradiol nucleus [22]. The same strategy will be used herein to obtain a nonestrogenic inhibitor of steroid sulfatase. Considering that a hydrophobic alkane side chain (octyl) at position 17α of estradiol inhibits strongly the steroid-sulfatase activity [30] and that an alkanamide side chain could remove or diminish the unsuitable estrogenic activity of the estradiol nucleus [13–16], we synthesized a series of estradiol derivatives (compounds **9–16**) with these two requirements (Fig. 2). In addition to the chemical synthesis, we report their ability to inhibit the transformation of E_1S to E_1 by JEG-3 homogenated cells, their binding affinity to the ER, as well as their ability to inhibit estrogenic activity and to exhibit anti-estrogenic activity in estrogen-sensitive ZR-75-1 human breast cancer cells.

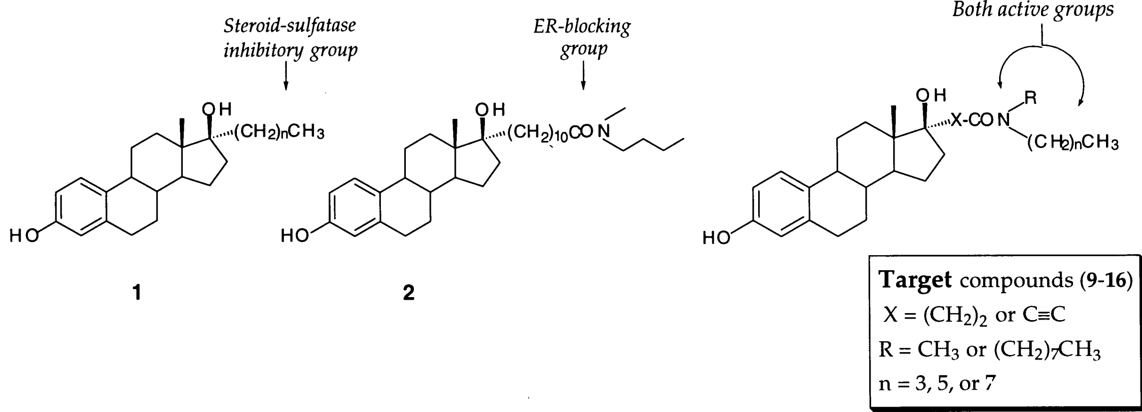

Fig. 2. New estradiol derivatives designed to contain two inhibitory groups in the same 17α-alkylamide side chain (compounds **9–16**).

2. Experimental

2.1. Chemical synthesis

Chemical reagents and starting material were purchased from Aldrich (Milwaukee, WI, USA) or Sigma (St. Louis, MO, USA). Solvents were obtained from Baker (Montréal, QC, Canada) or Fischer (Montréal, QC, Canada). TLC was performed on 0.20-mm silica gel 60 F_{254} plates (E. Merck, Darmstadt, Germany), and 230–400-mesh ASTM silica gel 60 (E. Merck) was used for flash-column chromatography. IR spectra were obtained on a Perkin–Elmer 1600 (FT-IR series) spectrophotometer (Norwalk, CT, USA) and expressed in cm^{-1}. NMR spectra were recorded on a Bruker AC/F 300 spectrometer (Bruker, Billerica, MA, USA). The chemical shifts (δ in ppm) were referenced to chloroform (7.26 for ^{1}H and 77.0 ppm for ^{13}C). Only specific signals were reported in ^{1}H NMR, whereas all signals were listed in ^{13}C NMR. As previously reported [32,33], several ^{13}C NMR signals of compounds bearing an amide group on the side chain are duplicated. Low-resolution mass spectra (MS) were recorded with a V.G. Micromass 16F (Manchester, UK) or Hewlett–Packard (Palo Alto, CA, USA) spectrometers. High-resolution mass spectra (HRMS) were provided by the Centre Régional de Spectrométrie de Masse (Université de Montréal, Montréal, QC, Canada). The purity of tested compounds was determined by HPLC (Waters Associates, Milford, MA, USA) by using an ultraviolet detector (215 nm).

*2.1.1. General procedure for amide formation (synthesis of **5–8**)*

Tributylamine (3.5 equiv.) was added under anhydrous conditions to carboxylic acid derivative **4** [34] dissolved in dry CH_2Cl_2 and this mixture was cooled to −10°C. Isobutyl chloroformate (3.0 equiv.) was added and the mixture was allowed to react for 30 min. Dialkylamine (*N*-methylbutylamine, *N*-methylhexylamine, *N*-methyloctylamine or dioctylamine) was then added in excess (10 equiv.) and the cooling bath was removed. After 3 h, CH_2Cl_2 was added and the organic phase was washed with aqueous 1 N HCl and dried over $MgSO_4$. The solvent was removed under reduced pressure and the crude oil was purified by flash-column chromatography (hexane/EtOAc, 4:6 or 5:5) to give the corresponding amides **5–8**.

*2.1.1.1. N-Butyl,N-methyl-3-[3′-(i-butyloxy-carbonyloxy)-17′β-hydroxy-1′,3′,5′(10′)-estratrien-17′α-yl]-2-propynamide (**5**) [35].* Colorless oil (69% yield); IR υ (neat) 3340 (OH, alcohol), 2210 (C≡C), 1750 (C=O, conjugated carbonate), 1610 (C=O, amide); ^{1}H NMR δ ($CDCl_3$) 0.91 (s, 3H, 18′-CH_3), 0.94 (m, 3H, CH_3 of butyl), 1.00 (d, J = 7.0 Hz, 6H, $(CH_3)_2$CH), 2.88 (m, 2H, 6′-CH_2), 2.96 and 3.18 (2s, 3H, $CONCH_3$, two conformers, 62:38, respectively), 3.40 and 3.55 (2t, J = 7.5 Hz, 2H, $CONCH_2CH_2$), 4.03 (d, J = 6.6 Hz, 2H, *i*-Pr CH_2O), 6.89 (d, J = 2.6 Hz, 1H, 4′-CH), 6.94 (dd, J_1 = 2.6 Hz and J_2 = 8.1 Hz, 1H, 2′-CH), 7.28 (d, J = 8.1 Hz, 1H, 1′-CH); MS m/e 509 (M^+, 14), 438 (M^+-[*i*-BuOCO], 2.9), 370 (M^+-[C≡CCONMeBu], 5.9), 270 (M^+-[C≡CCONMeBu + *i*-BuOCO], 100).

*2.1.1.2. N-Hexyl,N-methyl-3-[3′-(i-butyloxy-carbonyloxy)-17′β-hydroxy-1′,3′,5′(10′)-estratrien-17′α-yl]-2-propynamide (**6**).* Colorless oil (56% yield); IR υ (neat) 3360 (OH, alcohol), 2220 (C≡C), 1760 (C=O, conjugated carbonate), 1615 (C=O, amide); ^{1}H NMR δ ($CDCl_3$) 0.87 (m, 3H, CH_3 of hexyl), 0.89 (s, 3H, 18′-CH_3), 0.98 (d, J = 6.9 Hz, 6H, $(CH_3)_2$CH), 2.84 (m, 2H, 6′-CH_2), 2.93 and 3.16 (2s, 3H, $CONCH_3$, two conformers, 62:38, respectively), 3.36 and 3.52 (2t, J = 7.5 Hz, 2H, $CONCH_2CH_2$), 4.00 (d, J = 6.7 Hz, 2H, *i*-Pr CH_2O), 6.87 (d, J = 1.9 Hz, 1H, 4′-CH), 6.91 (dd, J_1 = 2.2 Hz and J_2 = 8.4 Hz, 1H, 2′-CH), 7.26 (d, J = 8.4 Hz, 1H, 1′-CH); MS m/e 537 (M^+, 15), 437 (M^+-[*i*-BuOCO], 5.4), 370 (M^+-[C≡CCONMeHe], 6.9), 270 (M^+-[C≡CCONMeHe + *i*-BuOCO], 100), 96 (73).

*2.1.1.3. N-Octyl,N-methyl-3-[3′-(i-butyloxy-carbonyloxy)-17′β-hydroxy-1′,3′,5′(10′)-estratrien-17′α-yl]-2-propynamide (**7**).* Colorless oil (84% yield); IR υ (neat) 3365 (OH, alcohol), 2225 (C≡C), 1758 (C=O, conjugated carbonate), 1617 (C=O, amide); ^{1}H NMR δ ($CDCl_3$) 0.88 (m, 3H, CH_3 of octyl), 0.90 (s, 3H, 18′-CH_3), 0.99 (d, J = 6.6 Hz, 6H, $(CH_3)_2$CH), 2.85 (m, 2H, 6′-CH_2), 2.94 and 3.16 (2s, 3H, $CONCH_3$, two conformers, 70:30, respectively), 3.37 and 3.52 (2t, J = 7.5 Hz, 2H, $CONCH_2CH_2$), 4.01 (d, J = 6.6 Hz, 2H, *i*-Pr CH_2O), 6.87 (d, J = 1.9 Hz, 1H, 4′-CH), 6.91 (dd, J_1 = 2.2 Hz and J_2 = 8.4 Hz, 1H, 2′-CH), 7.26 (d, J = 8.4 Hz, 1H, 1′-CH); MS m/e 565 (M^+, 9.1), 370 (M^+-[C≡CCONMeOc], 7.6), 270 (M^+-[C≡CCONMeOc + *i*-BuOCO], 100), 96 (99).

*2.1.1.4 N,N-Dioctyl-3-[3′-(i-butyloxy-carbonyloxy)-17′β-hydroxy-1′,3′,5′(10′)-estratrien-17′α-yl]-2-propynamide (**8**).* Colorless oil (65% yield); IR υ (neat) 3360 (OH, alcohol), 2220 (C≡C), 1764 (C=O, conjugated carbonate), 1615 (C=O, amide); ^{1}H NMR δ ($CDCl_3$) 0.83 and 0.85 (2t, 6H, CH_3 of octyls), 0.89 (s, 3H, 18′-CH_3), 0.98 (d, J = 6.9 Hz, 6H, $(CH_3)_2$CH), 2.84 (m, 2H, 6′-CH_2), 3.30 and 3.47 (2t, J = 8.0 Hz, 4H, 2× $CONCH_2CH_2$), 3.75 (br, 1H, 17′β-OH), 4.00 (d, J = 6.6 Hz, 2H, *i*-Pr CH_2O), 6.86 (d, J = 2.3 Hz, 1H, 4′-CH), 6.90 (dd, J_1 = 2.4 Hz and J_2 = 8.4 Hz, 1H, 2′-CH), 7.25 (d, J = 8.4 Hz, 1H, 1′-CH); MS m/e 665 (M^+, 6.9), 371 (M^+-[C≡CCONOcOc], 7.4), 270 (M^+-[C≡CCONOcOc + *i*-BuOCO], 100), 96 (100).

*2.1.2. General procedure for cleavage of i-butyl-carbonate group (synthesis of **9–12**)*

The carbonate derivatives **5–8** were dissolved in MeOH and a solution of K_2CO_3 (1%, w/v) in H_2O/MeOH (25:75, v/v) was added. The resulting solution was stirred at room temperature for 3 h. The reaction mixture was acidified with aqueous 1 N HCl and MeOH was evaporated under reduced

pressure. The residue was extracted with EtOAc and the organic phase was dried ($MgSO_4$) and evaporated. The crude oils were purified by flash-column chromatography (hexane/EtOAc, 6:4) to give compounds **9–12**.

*2.1.2.1. N-Butyl,N-methyl-3-(3′,17′β-dihydroxy-1′,3′,5′(10′)-estratrien-17′α-yl)-2-propynamide (**9**) [35].* Amorphous white solid (91% yield); IR υ (neat) 3320 (OH, alcohol and phenol), 2205 (C≡C), 1600 (C=O, amide); ^{1}H NMR δ ($CDCl_3$) 0.85 (s, 3H, 18′-CH_3), 0.97 (t, J = 7.0 Hz, 3H, CH_3 of butyl), 2.74 (m, 2H, 6′-CH_2), 2.99 and 3.22 (2s, 3H, $CONCH_3$, two conformers, 63:37, respectively), 3.44 and 3.59 (2t, J = 6.8 Hz, 2H, $CONCH_2CH_2$), 6.56 (s_{app}, 1H, 4′-CH), 6.65 (dd, J_1 = 2.2 Hz and J_2 = 8.4 Hz, 1H, 2′-CH), 7.00 (d, J = 8.4 Hz, 1H, 1′-CH), 7.40 (br, 1H, OH phenol); ^{13}C NMR δ ($CDCl_3$) 12.69, 13.82, 19.82, 19.85, 22.84, 26.13, 27.12, 28.90, 29.56, 30.31, 32.48, 32.94, 36.59, 38.60, 39.12, 42.74, 42.93, 46.70, 47.73, 49.78, 49.90, 51.24, 78.36, 78.49, 80.01, 95.70, 96.21, 112.75, 115.12, 126.30, 131.27, 137.51, 154.43, 154.82; MS m/e 409 (M^+, 46), 394 (3.0), 391 (3.3), 376 (4.3), 338 (15), 270 (M^+-[C≡CCONMeBu], 100); HRMS calculated for $C_{26}H_{35}O_3N$: 409.2617, found 409.2608; HPLC purity = 95.3% (C-18 NovaPak column (Waters Associates, Milford, MA, USA); MeCN:MeOH:H_2O/35:15:50).

*2.1.2.2. N-Hexyl,N-methyl-3-(3′,17′β-dihydroxy-1′,3′,5′(10′)-estratrien-17′α-yl)-2-propynamide (**10**).* Colorless oil (90%); IR υ (neat) 3330 (OH, alcohol and phenol), 2220 (C≡C), 1614 (C=O, amide); ^{1}H NMR δ ($CDCl_3$) 0.86 (s, 3H, 18′-CH_3), 0.88 (t, J = 6.8 Hz, 3H, CH_3 of hexyl), 2.74 (m, 2H, 6′-CH_2), 2.98 and 3.21 (2s, 3H, $CONCH_3$, two conformers, 69:31, respectively), 3.42 and 3.56 (2t, J = 7.4 Hz, 2H, $CONCH_2CH_2$), 6.55 (d, J = 2.4 Hz, 1H, 4′-CH), 6.63 (dd, J_1 = 2.5 Hz and J_2 = 8.4 Hz, 1H, 2′-CH), 7.01 (d, J = 8.5 Hz, 1H, 1′-CH); ^{13}C NMR δ ($CDCl_3$) 12.64, 13.89, 22.43, 22.48, 22.79, 26.11, 26.29, 26.71, 27.11, 28.21, 29.49, 31.46, 32.40, 32.94, 36.53, 38.53, 39.11, 42.78, 43.01, 46.92, 47.67, 49.75, 49.90, 51.47, 79.87, 79.94, 95.82, 96.33, 112.73, 115.12, 126.16, 131.12, 137.42, 154.37, 154.84; MS m/e 437 (M^+, 33), 366 (12), 270 (M^+-[C≡CCONMeHe], 68), 210 (50), 96 (100); HRMS calculated for $C_{28}H_{39}O_3N$: 437.2930, found 437.2927; HPLC purity = 98.9% (C-18 NovaPak column; MeCN: MeOH:H_2O/40:20:40).

*2.1.2.3. N-Octyl,N-methyl-3-(3′,17′β-dihydroxy-1′,3′,5′(10′)-estratrien-17′α-yl)-2-propynamide (**11**).* Colorless oil (81%); IR υ (neat) 3330 (OH, alcohol and phenol), 2223 (C≡C), 1614 (C=O, amide); ^{1}H NMR δ ($CDCl_3$) 0.85 (s, 3H, 18′-CH_3), 0.87 (t, J = 6.4 Hz, 3H, CH_3 of octyl), 2.71 (m, 2H, 6′-CH_2), 2.98 and 3.21 (2s, 3H, $CONCH_3$, two conformers, 65:35, respectively), 3.42 and 3.57 (2t, J = 7.4 Hz, 2H, $CONCH_2CH_2$), 6.55 (d, J = 2.4 Hz, 1H, 4′-CH), 6.64 (dd, J_1 = 2.3 Hz and J_2 = 8.3 Hz, 1H, 2′-CH), 6.99 (d, J = 8.4 Hz, 1H, 1′-CH), 7.50 (br, 1H, OH phenol); ^{13}C NMR δ ($CDCl_3$) 12.69, 14.04, 22.60, 22.88, 26.13, 26.77, 27.16, 28.37, 29.15, 29.26, 29.40, 29.57, 31.75, 32.44, 32.97, 36.59, 38.70, 39.14, 42.73, 42.95, 46.99, 47.74, 49.81, 49.93, 51.52, 78.56, 78.65, 80.07, 80.16, 95.35, 95.91, 112.71, 115.11, 126.31, 131.37, 137.55, 154.37, 154.44, 154.78; MS m/e 465 (M^+, 12), 394 (3.3), 270 (M^+-[C≡CCONMeOc], 66), 96 (100); HRMS calculated for $C_{30}H_{43}O_3N$: 465.3243, found 465.3216; HPLC purity = 98.3% (C-18 NovaPak column; MeCN: MeOH:H_2O/45:20:35).

*2.1.2.4. N,N-Dioctyl-3-(3′,17′β-dihydroxy-1′,3′,5′(10′)-estratrien-17′α-yl)-2-propynamide (**12**).* Colorless oil (94%); IR υ (neat) 3350 (OH, alcohol and phenol), 2220 (C≡C), 1608 (C=O, amide); ^{1}H NMR δ ($CDCl_3$) 0.85 (s, 3H, 18′-CH_3), 0.87 (t, J = 6.3 Hz, 6H, CH_3 of octyls), 2.73 (m, 2H, 6′-CH_2), 3.36 and 3.51 (2t, J = 7.4 Hz, 4H, 2× $CONCH_2CH_2$), 6.54 (d, J = 2.6 Hz, 1H, 4′-CH), 6.63 (dd, J_1 = 2.6 Hz and J_2 = 8.4 Hz, 1H, 2′-CH), 6.92 (br, 1H, OH phenol), 7.00 (d, J = 8.4 Hz, 1H, 1′-CH); ^{13}C NMR δ ($CDCl_3$) 12.65, 13.98, 22.54, 22.81, 26.24, 26.89, 27.15, 27.37, 29.04, 29.10, 29.22, 29.40, 29.52, 31.70, 32.97, 38.56, 39.14, 43.00, 45.19, 47.67, 49.53, 49.91, 78.70, 79.93, 95.39, 112.78, 115.15, 126.18, 131.10, 137.39, 154.44, 154.73; MS m/e 563 (M^+, 20), 492 (2.2), 336 (13), 270 (M^+-[C≡CCONOcOc], 50), 194 (46), 96 (100); HRMS calculated for $C_{37}H_{57}O_3N$: 563.4338, found 563.4313; HPLC purity = 97.1% (C-8 Zorbax column (Chromatographic Specialities, Inc., Brokville, ON, Canada); MeOH:H_2O/85:15).

*2.1.3. General procedure for catalytic hydrogenation of a triple bond (synthesis of **13–16**)*

Alkynamide derivatives **9–12** were dissolved in EtOAc containing a catalytic amount of Pd/C (10% content). The reaction mixtures were then shaken overnight under atmospheric pressure of hydrogen, filtered on celite, and the solvent was evaporated under reduced pressure. The crude oils were purified by flash-column chromatography (hexane/EtOAc, 6:4) to give the saturated compounds **13–16**.

*2.1.3.1. N-Butyl,N-methyl-3-(3′,17′β-dihydroxy-1′,3′,5′(10′)-estratrien-17′α-yl)-propanamide (**13**) [36].* White foam (65% yield); IR υ (film) 3250 (OH, alcohol and phenol), 1615 (C=O, amide); ^{1}H NMR δ ($CDCl_3$) 0.83 (s, 3H, 18′-CH_3), 0.93 and 0.95 (2t, J = 7.5 Hz, 3H, CH_3 of butyl), 2.72 (m, 2H, 6′-CH_2), 2.96 and 3.02 (2s, 3H, $CONCH_3$, two conformers, 49:51, respectively), 3.32 and 3.40 (2 m, 2H, $CONCH_2CH_2$), 6.57 (d, J = 2.3 Hz, 1H, 4′-CH), 6.68 (dd, J_1 = 2.4 Hz and J_2 = 8.4 Hz, 1H, 2′-CH), 7.03 (d, J = 8.4 Hz, 1H, 1′-CH), 7.95 (br, 1H, OH phenol); ^{13}C NMR δ ($CDCl_3$) 13.86, 14.30, 19.97, 20.09, 23.16, 26.09, 27.36, 28.17, 29.41, 29.67, 30.51, 30.91, 31.33, 33.84, 34.39, 35.47, 39.37, 43.24, 46.72, 47.99, 49.20, 49.92, 82.89, 112.84, 115.26, 126.14, 132.08, 137.92, 154.09, 174.53, 174.62; HRMS calculated for $C_{26}H_{39}O_3N$: 413.2930, found

413.2930; HPLC purity = 99.6% (C-18 NovaPak column; MeCN:MeOH:H_2O/30:20:50).

*2.1.3.2. N-Hexyl,N-methyl-3-(3′,17′β-dihydroxy-1′,3′,5′(10′)-estratrien-17′α-yl)-propanamide (**14**).* White foam (52%); IR υ (film) 3290 (OH, alcohol and phenol), 1615 (C=O, amide); ^{1}H NMR δ ($CDCl_3$) 0.82 (s, 3H, 18′-CH_3), 0.87 (m, 3H, CH_3 of hexyl), 2.70 (m, 2H, 6′-CH_2), 2.94 and 3.00 (2s, 3H, $CONCH_3$, two conformers, 45:55, respectively), 3.30 (m, 2H, $CONCH_2CH_2$), 6.55 (d, J = 2.3 Hz, 1H, 4′-CH), 6.66 (dd, J_1 = 2.3 Hz and J_2 = 8.4 Hz, 1H, 2′-CH), 7.01 (d, J = 8.5 Hz, 1H, 1′-CH), 8.40 (br, 1H, OH phenol); ^{13}C NMR δ ($CDCl_3$) 13.87, 14.20, 22.41, 23.01, 25.91, 26.27, 26.39, 27.08, 27.22, 28.03, 28.21, 29.54, 31.09, 31.41, 33.75, 33.93, 35.40, 39.17, 42.95, 46.58, 48.14, 49.01, 50.09, 82.85, 112.84, 115.21, 125.77, 131.30, 137.49, 154.53, 174.63, 174.75; MS m/e 441 (M^+, 12), 423 (M^+-H_2O, 27), 408 (M^+-[H_2O + CH_3], 7.5), 326 (M^+-[NMeHe], 58), 226 (100), 213 (66), 170 (96); HRMS calculated for $C_{28}H_{43}O_3N$: 441.3243, found 441.3256; HPLC purity = 99.6% (C-18 NovaPak column; MeCN:MeOH:H_2O/40:20:40).

*2.1.3.3. N-Octyl,N-methyl-3-(3′,17′β-dihydroxy-1′,3′,5′(10′)-estratrien-17′α-yl)-propanamide (**15**).* White foam (77%); IR υ (film) 3280 (OH, alcohol and phenol), 1617 (C=O, amide); ^{1}H NMR δ ($CDCl_3$) 0.85 (s, 3H, 18′-CH_3), 0.87 (m, 3H, CH_3 of octyl), 2.74 (m, 2H, 6′-CH_2), 2.96 and 3.03 (2s, 3H, $CONCH_3$, two conformers, 44:56, respectively), 3.35 (m, 2H, $CONCH_2CH_2$), 6.56 (d, J = 2.4 Hz, 1H, 4′-CH), 6.67 (dd, J_1 = 2.6 Hz and J_2 = 8.4 Hz, 1H, 2′-CH), 6.93 (br, 1H, OH phenol), 7.07 (d, J = 8.4 Hz, 1H, 1′-CH); ^{13}C NMR δ ($CDCl_3$) 14.03, 14.21, 22.58, 23.08, 25.95, 26.85, 27.03, 27.23, 27.58, 27.69, 28.95, 29.14, 29.24, 29.33, 29.65, 31.15, 31.59, 31.71, 31.75, 34.13, 39.21, 42.96, 46.44, 46.62, 48.21, 48.99, 82.99, 112.91, 115.31, 125.80, 131.39, 137.62, 154.64, 174.53; MS m/e 469 (M^+, 15), 451 (M^+-H_2O, 47), 436 (M^+-[H_2O + CH_3], 12), 326 (M^+-[NMeOc], 100), 254 (69), 213 (55), 198 (100), 170 (63); HRMS calculated for $C_{30}H_{47}O_3N$: 469.3556, found 469.3568; HPLC purity = 99.3% (C-18 NovaPak column; MeCN: MeOH:H_2O/55:20:25).

*2.1.3.4. N,N-Dioctyl-3-(3′,17′β-dihydroxy-1′,3′,5′(10′)-estratrien-17′α-yl)-propanamide (**16**).* White foam (52%); IR υ (film) 3280 (OH, alcohol and phenol), 1615 (C=O, amide); ^{1}H NMR δ ($CDCl_3$) 0.85 (s, 3H, 18′-CH_3), 0.88 (m, 6H, CH_3 of octyls), 2.75 (m, 2H, 6′-CH_2), 3.33 (m, 4H, 2× $CONCH_2CH_2$), 6.56 (d, J = 2.4 Hz, 1H, 4′-CH), 6.66 (dd, J_1 = 2.5 Hz and J_2 = 8.4 Hz, 1H, 2′-CH), 6.85 (br, 1H, OH phenol), 7.07 (d, J = 8.5 Hz, 1H, 1′-CH); ^{13}C NMR δ ($CDCl_3$) 13.99, 14.21, 22.54, 23.05, 25.95, 26.82, 26.98, 27.22, 27.57, 27.66, 28.92, 29.11, 29.17, 29.22, 29.28, 29.62, 31.17, 31.54, 31.69, 34.09, 39.23, 42.99, 46.42, 46.62, 48.18, 49.02, 82.93, 112.88, 115.29, 125.77, 131.31, 137.57, 154.64, 174.50; MS m/e 567 (M^+, 7.0), 549 (M^+-H_2O, 14), 352 (M^+-[$C_{15}H_{32}$], 33), 326 (M^+-[NOcOc], 20), 296 (41), 268 (25), 142 (100); HRMS calculated for $C_{37}H_{61}O_3N$: 567.4652, found 567.4658; HPLC purity = 99.8% (C-18 NovaPak column; MeCN:MeOH:H_2O/70:20:10).

2.2. Biological evaluation of compounds

2.2.1. Inhibition of steroid-sulfatase activity (^3H-E_1S to ^3H-E_1)

2.2.1.1. Source of enzyme activity. The JEG-3 cells used as the source of steroid-sulfatase activity were purchased from American Type Culture Collection (Rockville, MD, USA) on October 10, 1989, at passage 127. Cells were grown in DMEM medium (Life Technologies, Burlington, ON, Canada) containing $NaHCO_3$ (3.7 g/l), HEPES (5.96 g/l), and glucose (4.59 g/l). Medium sterilized on Millipore 0.22-μm membrane was supplemented with fetal bovine serum or bovine calf serum (5%), L-glutamine (1%), penicillin (100 IU/ml), and streptomycin (50 μg/ml). Cells were grown in 175-cm^2 flasks seeded at a density of 500 000 cells per flask. 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) for 15 min at 37°C. Enzyme activity was inhibited by diluting the cells in culture medium containing 5% serum. Cells were pelleted, resuspended in culture medium, counted with a hemocytometer, and reseeded in culture flasks or harvested for subsequent analysis.

2.2.1.2. Enzymatic assay. Cell homogenate was prepared by allowing the combined cell pellets from several culture flasks to break by a repeated (five times) freezing and thawing process followed each time by homogenization with a hand tissue grinder. Homogenized cells were aliquoted at 10 million/ml and were kept up to 1 year at −90°C. The steroid-sulfatase assay was performed in 13 × 100-mm borosilicate test tubes. The assay buffer was made of 0.1 M Tris-acetate, 0.005 M EDTA, 10% glycerol, adjusted at pH 7.0. The tubes were immersed in a water and ice bath before adding reagents. To each tube was added 300 μl of cold assay buffer, 7 nM ^3H-E_1S, and 10 μl of ethanol for the control or 10 μl of an increasing amount of tested compounds **9–16** diluted in ethanol. The sample rack was shaken and the reaction was started by the addition of 20 000 homogenized cells/100 μl of assay buffer. The tubes were shaken by hand and immediately immersed in a thermostated water bath for 60 min at 37°C. Background was determined by incubating excess (225 μM) of unlabeled E_1S into a tube containing assay buffer, radioactivity, and enzyme. The reaction was stopped by shaking the tubes in a water and ice bath. Excess (225 μM) of unlabeled E_1S was added immediately to each tube. The tubes were shaken once again, then 1.25 ml of xylene was added to each tube and total E_1 was extracted by shaking the tubes for 4 min. The tubes were centrifuged at 2 500 rev./min for 10 min. A portion of the organic phase (750 μl) was counted with 7 ml of scintillation cocktail in a β-counter (Beckman LS 3801, Irvine, CA, USA) adjusted for tritium (^3H). Data were expressed as

3 (Ethynyl-estradiol)

4

5 (R_1 = Me, R_2 = *n*-Bu)
6 (R_1 = Me, R_2 = *n*-He)
7 (R_1 = Me, R_2 = *n*-Oc)
8 (R_1 = *n*-Oc, R_2 = *n*-Oc)

13 (R_1 = Me, R_2 = *n*-Bu)
14 (R_1 = Me, R_2 = *n*-He)
15 (R_1 = Me, R_2 = *n*-Oc)
16 (R_1 = *n*-Oc, R_2 = *n*-Oc)

9 (R_1 = Me, R_2 = *n*-Bu)
10 (R_1 = Me, R_2 = *n*-He)
11 (R_1 = Me, R_2 = *n*-Oc)
12 (R_1 = *n*-Oc, R_2 = *n*-Oc)

Fig. 3. Chemical synthesis of estradiol derivatives **9**–**16**. The reagents are: (a) *n*-BuLi, CO_2, THF; (b) 1. ClCOO *i*-Bu, $N(Bu)_3$, CH_2Cl_2 2. HNR_1R_2; (c) K_2CO_3, H_2O, MeOH; (d) H_2, Pd/C (10%), EtOAc. [Me: methyl (CH_3); *n*-Bu: *n*-butyl (-$(CH_2)_3CH_3$); *n*-He: *n*-hexyl (-$(CH_2)_5CH_3$); *n*-Oc: *n*-octyl (-$(CH_2)_7CH_3$)].

percentage of ^{3}H-E_1 produced (100% for control without inhibitor) versus the concentration of tested compound. The IC_{50} value was calculated by using the DE_{50} program (CHUL Research Center, Sainte-Foy, QC, Canada).

2.2.2. ER binding affinity screening

The affinity binding assay on the ER from rat uterine was performed under the standard procedure established in our laboratory [37].

2.2.3. Proliferative and antiproliferative activities

An estrogen-sensitive human breast cancer cell line (ZR-75-1) was used in our in vitro assay to evaluate the ability of tested compounds to stimulate the proliferation of ZR-75-1 cells (proliferative activity) and the ability of tested compounds to inhibit the 0.1 nM estradiol-induced proliferation (antiproliferative activity). Compounds were tested at two concentrations: 0.03 and 1 μM. Results were expressed as proliferative activity or antiproliferative activity in percent (%) of control (100% for 0.1 nM of E_2). The procedure of this assay has been described previously by our group [38].

3. Results and discussion

3.1. Chemistry

The chemical synthesis of estradiol derivatives with a propynamide (**9**–**12**) or a propanamide (**13**–**16**) side chain is shown in Fig. 3. The carboxylic acid **4** was first obtained in 80% yield by adding carbon dioxide to the corresponding lithium acetylide of ethynylestradiol (**3**) [34]. The intermediate compound **4** was then transformed to amides **5**–**8** by treatment with isobutylchloroformate and an appropriate secondary amine (methylbutylamine, methylhexylamine, methyloctylamine, or dioctylamine). Isobutylchloroformate first reacted with carboxylic acid to give a mixed anhydride (activated species), which was transformed to the amide by adding a secondary amine. The carbonate group of compounds **5**–**8** was easily hydrolyzed with K_2CO_3 in aqueous MeOH to afford phenolic compounds **9**–**12** in excellent yields. The saturated alkanamides **13**–**16** were thereafter obtained by catalytic hydrogenation (H_2, Pd/C) of the corresponding alkyne derivatives **9**–**12**. Starting from ethynylestradiol (**3**), the overall unoptimized yields for the formation of alkanamides **13**–**16** varied from 22% to 42%. This sequence of three or four steps enabled us to obtain our target compounds efficiently with a 17α-alkan (or alkyn) amide side chain containing a butyl, hexyl, octyl, or dioctyl group.

3.2. Biological results

One of our goals in this study was to develop steroid-sulfatase inhibitors without residual estrogenic activity. Our strategy consisted of using an alkan (or alkyn) amide side chain at position 17α of E_2 to help us to reduce the estrogenic activity of the E_2 nucleus. Indeed, the introduction of an alkynamide side chain at this position was reported to

Table 1
Inhibition of steroid-sulfatase activity transforming E_1S to E_1

Compounds	Substituents (R)	% at 20 μM	IC_{50} (μM)
1 [30]		88	0.44
10	C≡C–C(=O)–N	81	1.3
11	C≡C–C(=O)–N	84	0.35
12	C≡C–C(=O)–N	14	>20
13	$(CH_2)_2$–C(=O)–N	46	6.4
14	$(CH_2)_2$–C(=O)–N	62	2.8
15	$(CH_2)_2$–C(=O)–N	91	0.08
16	$(CH_2)_2$–C(=O)–N	18	>20
E_1S (enzyme substrate)		38	7.6

markedly decrease the ER binding affinity [35], whereas no uterotrophic activity or stimulation of mice uterine weight (ER-sensitive tissues) was observed for a 17α-methylbutyl-undecanamide derivative of estradiol (compound **2**) [39]. In addition, we tried to optimize the steroid-sulfatase inhibitory potency of an alkanamide side chain by increasing the hydrophobicity. Eight compounds (**9–16**) with different alkynamide or alkanamide side chain at 17α-position of E_2 were then synthesized to verify our hypothesis.

The inhibition of steroid-sulfatase activity was assayed by measuring the total ^{3}H-E_1 formed from ^{3}H-E_1S with homogenized JEG-3 cells (Table 1). Among the tested compounds **10–16**, we observed that *N*-methyl,*N*-octyl-propanamide-E_2 (**15**) gave the best inhibition of steroid-sulfatase activity with an IC_{50} value of 0.08 μM. This compound, which has an octyl group, possessed higher hydrophobicity than analog compounds **14** and **13** that have a hexyl group or a butyl group (IC_{50} = 2.8 or 6.4 μM, respectively). In the unsaturated series, the same tendency was observed and compound **11** with an octyl group was a better inhibitor of steroid sulfatase than the analog with the hexyl group (IC_{50} = 0.35 and 1.3 μM for **11** and **10**, respectively). Because a higher hydrophobic group could increase the enzyme inhibition, it was not expected that *N*,*N*-dioctyl-propanamide-E_2 (**16**) and *N*,*N*-dioctyl-propynamide-E_2 (**12**), which each have two hydrophobic octyl groups, would give such poor inhibition of steroid-sulfatase activity (IC_{50} >20 μM). However, we previously reported [30] that a too-long alkane side chain at position 17α of E_2 (17α-[decyl or dodecyl]-E_2, IC_{50} values of 1 μM and 6 μM, respectively) decreased the potency of such compounds to inhibit steroid sulfatase. The steric hindrance of a large substituent (like a dioctylamide group of compounds **12** and **16**) was responsible for lowering the inhibitory activity. Given these results, it was not necessary for us to synthesize alkanamide derivatives with a higher length of *N*-alkyl substituent than *N*-octyl.

The side-chain hydrophobicity and steric hindrance are two factors used to explain, in a same series of amides (saturated or unsaturated), the following observed order of inhibitory activity: *N*-octyl (nC_8) > *N*-hexyl (nC_6) > *N*-butyl (nC_4) ≫ *N*,*N*-dioctyl (2× nC_8). Furthermore, the ratio of two amide conformers evaluated by NMR was about 1 to 1 for saturated amides **13–16** (without a triple bond) and about 1 to 3 for unsaturated amides **10–12** (with a triple bond). Thus, the presence or absence of the triple bond influenced the orientation of amide moiety (conformer ratio) and may be a factor that could explain, at least partially, the different results between both series of amides (saturated and unsaturated). From all tested amides **10–16**, compound **15**, with a saturated methyloctylpropanamide side chain, was the most potent inhibitor in the present study, about 5-fold better than 17α-octyl-E_2 (**1**) (IC_{50} = 0.44 μM). Compound **15** was also about 100-fold more effective in interacting with the enzyme than substrate E_1S itself.

The other important point of this study is the estrogenic and anti-estrogenic activities of the newly synthesized compounds. The ER-binding affinity was determined at a concentration of 1 μM (Table 2). With the exception of the bulky *N*,*N*-dioctyl propanamide **16**, which showed no ER binding, the propynamide and propanamide derivatives of E_2 moderately bound the ER and, consequently, it was difficult for them to compete with E_2 for binding the receptor. Thereafter, the estrogenic activity was evaluated by the ability of these compounds to stimulate the proliferation of estrogen-sensitive ZR-75-1 cells (ER^+) and the anti-estrogenic activity was evaluated by their ability to inhibit an E_2 (0.1 nM)-stimulated proliferation of ZR-75-1 cells (Table 2). According to a standard protocol established in our laboratory [38], two high concentrations (0.03 and 1 μM) of the tested compounds were used for this latter study. Such concentrations, respectively 300 and 10 000-fold higher than reference E_2 (0.1 nM) concentration, were selected to be sure to detect residual estrogenic activity. As previously mentioned, 17α-octyl-E_2 (**1**) stimulated fully the ZR-75-1 cell proliferation at 1 μM and no antiproliferative activity was observed. On the contrary, the pure anti-estrogen ICI 164384 induced no stimulation of ER^+ cell proliferation (estrogenicity), but exhibited a maximal (100%) antiproliferative (anti-estrogenicity) activity at both concentrations used. Unfortunately, all of the 17α-alkan (or alkyn) amide-E_2 derivatives **10–16** possessed an estrogenic or a mixed estrogenic/anti-estrogenic activity at different levels. Compounds **10–12**, with a triple bond, exhibited strong estrogenic activities at 1 μM (62–90%) and did not have the ability to inhibit E_2-stimulated proliferation of cells at 0.03 and 1 μM. These results are in accordance to those we obtained previously [35], where all synthesized compounds (methylbutyl alkynamide derivatives of E_2 with different spacers between the ethynyl

Table 2
ER-binding affinity and proliferative/antiproliferative activities on estrogen-sensitive ZR-75-1 cells

Compounds	Substituents (R)	ER binding (%) 1 μM	Proliferative activity (%)*		Antiproliferative activity (%)	
			0.03 μM	1 μM	0.03 μM	1 μM
1 [30]		—	23	100	0	0
10	C≡C–C(O)–N	52	17	62	0	0
11	C≡C–C(O)–N	24	0	80	0	0
12	C≡C–C(O)–N	9	20	90	0	19
13	$(CH_2)_2$–C(O)–N	—	0	27	0	29
14	$(CH_2)_2$–C(O)–N	23	0	52	0	42
15	$(CH_2)_2$–C(O)–N	22	0	29	0	50
16	$(CH_2)_2$–C(O)–N	0	0	8	0	23
ICI 164384 (pure anti-estrogen) [13,14]		—	0	0	100	100

* The proliferation of 0.1 nM of E_2 was fixed at 100%.

moiety and the amide groups) stimulated ZR-75-1 cell proliferation at a high concentration (1 μM) and did not exert anti-estrogenic activity. On the other hand, none of the propanamide derivatives **13–16** stimulated the proliferation of ZR-75-1 cells at 0.03 μM, but they did stimulate cells at 1 μM (8–52%). Moreover, they inhibited the E_2-stimulated proliferation of ZR-75-1 cells at 1 μM. For example, compound **15** inhibited 50% of E_2-stimulated proliferation of cells at 1 μM and had the lowest estrogenic activity (0 and 29%) at both concentrations studied. Despite a weak mixed estrogenic/anti-estrogenic activity, we concluded that compound **15** represents a compromise for a nonestrogenic inhibitor of steroid sulfatase.

4. Conclusion

A series of 17α-propanamide (or propynamide) derivatives of E_2 (compounds **9–16**) was efficiently synthesized in three to four steps with overall unoptimized yields ranging between 22% and 42%. Each compound was designed to contain in the same side chain a hydrophobic alkyl group (butyl, hexyl, or octyl) to inhibit the steroid-sulfatase activity and an amide group to block the activation of the ER (estrogenic activity). In our enzymatic assay, for the transformation of E_1S (the substrate) to E_1 by homogenized JEG-3 cells, the propynamide derivatives **10–12** and propanamide derivatives **13–16** inhibited the enzyme in the following order: *N*-octyl (nC_8) > *N*-hexyl (nC_6) > *N*-butyl (nC_4) ≫ *N*,*N*-dioctyl ($2\times nC_8$). Except for bulky *N*,*N*-dioctyl derivatives **12** and **16**, all compounds were better inhibitors of steroid sulfatase than substrate E_1S itself (100-fold for compound **15**). Although a high concentration of 1 μM was used in our ER-binding assay, compounds **10–16** were found to bind weakly the ER (0 to 52%). Unfortunately, their ability to bind the ER was correlated with their ability to stimulate the proliferation of estrogen-sensitive ZR-75-1 cells. Indeed, most of the compounds studied possessed a mixed estrogenic/anti-estrogenic activity at the higher concentration of 1 μM. However, compound **15** (*N*-octyl,*N*-methyl-3-(3′,17′β-dihydroxy-1′,3′,5′(10′)-estratrien-17′α-yl)-propanamide) represents a good compromise between inhibition of steroid-sulfatase activity (IC_{50} = 0.08 μM) and estrogenic activity (only 29% at 1 μM). Further studies are necessary to optimize the inhibitory activity on steroid sulfatase and to minimize the residual estrogenic activity of this series of 17α-alkanamide derivatives of E_2.

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