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Estradiol and estrone C-16 derivatives as inhibitors of type 1 17β-hydroxysteroid dehydrogenase: Blocking of ER$^+$ breast cancer cell proliferation induced by estrone

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Abstract—Estrogens play an important role in the development of breast cancer. Inhibiting 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1)—the enzyme responsible for the last step in the biosynthesis of the most potent estrogen, estradiol (E_2)—would thus allow hindering the growth of estrogen-sensitive tumors. Based on a previous study identifying 16β-benzyl-E_2 (**1**) as a lead compound for developing inhibitors of the transformation of estrone (E_1) into E_2, we modified the benzyl group of **1** to improve its inhibitory activity. Three strategies were also devised to produce compounds with less residual estrogenic activity: (1) replacing the hydroxy group by a hydrogen at position 3 (C3); (2) adding a methoxy at C2; and (3) adding an alkylamide chain known to be antiestrogenic at C7. In order to test the inhibitory potency of the new compounds, we used the human breast cancer cell line T-47D, which exerts a strong endogenous 17β-HSD1 activity. In this intact cell model, 16β-*m*-carbamoylbenzyl-E_2 (**4m**) emerged as a potent inhibitor of 17β-HSD1 with an IC_{50} value of 44 nM for the transformation of [^{14}C]-E_1 (60 nM) into [^{14}C]-E_2 (24-h incubation). In another assay aimed at assessing the unwanted estrogenic activity, a 10-day treatment with **4m** at a concentration of 0.5 μM induced some proliferation (38%) of T-47D estrogen-sensitive (ER$^+$) breast cancer cells. Interestingly, when **4m** (0.5 μM) was given with E_1 (0.1 nM) in a 10-day treatment, it blocked 62% of the T-47D cell proliferation induced by E_1 after its reduction to E_2 by 17β-HSD1. Thus, in addition to generating useful structure–activity relationships for the development of 17β-HSD1 inhibitors, our study demonstrates that using such inhibitors is a valuable strategy for reducing the level of E_2 and consequently its proliferative effect in T-47D ER$^+$ breast cancer cells.
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1. Introduction

It is now well recognized that estrogenic hormones are important stimulators of most types of breast cancers.[1,2] Estrogens are synthesized from circulating inactive steroids by enzymes such as human 17β-hydroxysteroid dehydrogenases (17β-HSDs).[3] These regulate the level of active steroids by catalyzing the interconversion of oxidized (17-ketone) and reduced (17β-hydroxy) forms.[4–6] There are 14 known isoforms in this family; each has a specific tissue distribution and displays selective substrate affinity.[7–10] Type 1 (17β-HSD1) is responsible for the last enzymatic step in the synthesis of potent estrogen E_2, which is the reduction of estrone (E_1) (Fig. 1). Activation of the estrogen receptor (ER) by

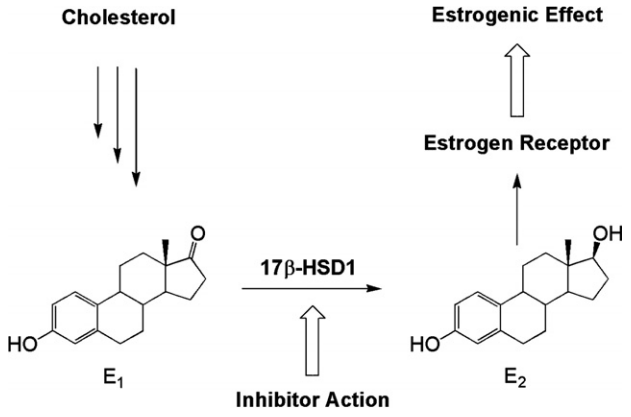

Figure 1. Role of 17β-HSD1 in the synthesis of E_2.

Keywords: 17β-hydroxysteroid dehydrogenase; Enzyme; Inhibitor; Steroid; Cancer; Estrogen; T-47D cells; MCF-7 cells; Medicinal chemistry.
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binding of E_2 is crucial to the growth and development of many mammary tumors.[11] There are literature reports indicating that 17β-HSD1 is expressed in a large proportion of breast cancer tissues (61–100%),[12–15] whereas others indicate the opposite;[16,17] a high 17β-HSD1 expression is nonetheless associated with a poor prognosis.[16] Inhibiting 17β-HSD1 activity could thus constitute a valuable way of reducing E_2 level,[18–20] with the aim of shrinking breast tumors. Indeed, it was recently reported that, in immunodeficient mice inoculated with MCF-7 breast cancer cells stably expressing 17β-HSD1, an inhibitor of 17β-HSD1 was able to reduce tumor size by 86% after 28 days.[21,22] Such inhibition of 17β-HSD1 activity could notably complement the use of an antiestrogen in the treatment of breast cancer.

Our group has previously reported the synthesis of a number of E_2 derivatives modified at position 16 (C16).[23–28] In one of these studies, compound **1** (Fig. 2) was identified as a good inhibitor of 17β-HSD1 with an IC_{50} value of 0.79–1.0 μM using purified enzyme.[23] This study also suggested that the 16β-phenyl group interacts with residues Leu96 and Val196 located near the nicotinamide residue of the NADPH cofactor. Based on these results, we introduced different modifications at C3, C16, and C17 of compound **1** (Fig. 2; compounds **4m**,**p**–**7m**,**p**) in an attempt to modulate interaction with important amino acids belonging to the catalytic site. Another desirable characteristic of an inhibitor of 17β-HSD1 is that it must be devoid of estrogenic activity. Thus, with the aim of reducing such activity, we modified the E_2 nucleus of inhibitor **4m** using three strategies (Fig. 2; compounds **17**–**19**): (1) replacing the hydroxyl group by a hydrogen at C3; (2) adding a methoxy group at C2; and (3) adding an alkylamide side chain at C7α. We now report the chemical synthesis, inhibitory potency on 17β-HSD1, and proliferative activity on ER^+ breast cancer cell lines of a series of new C16 derivatives of E_2 and E_1.

2. Results and discussion

2.1. Chemical synthesis

Compounds **4m** and **4p** were easily obtained from E_1 (Scheme 1) following the sequence of three reactions used previously for the synthesis of **1**.[23] First, an aldol condensation using *meta*-amido or *para*-amidobenzaldehyde[29] afforded **2m** and **2p**, respectively. Second, the carbonyl of **2m** and **2p** was stereoselectively reduced into the 17β-OH derivatives **3m** and **3p**. Third, the double bond at C16 was stereoselectively reduced by a catalytic hydrogenation to give the saturated E_2 derivatives **4m** and **4p**, respectively, bearing a 16β-oriented 3-carbamoylbenzyl and 4-carbamoylbenzyl group. To investigate the E_1 scaffold as well, alcohols **4m** and **4p** were oxidized with Jones' reagent to give the ketones **5m** and **5p**. We alternatively tried performing a catalytic hydrogenation with palladium on carbon to transform in one step the α,β-unsaturated ketones **2m** and **2p** directly into **5m** and **5p**, but a mixture of 16α- and 16β-oriented side chain as well as C17 alcohol and ketone was obtained. The 3-OH group of **4m** and **4p** was also methylated using cesium carbonate and methyl iodide to obtain the methoxy derivatives **6m** and **6p**. The latter compounds were then oxidized at C17 with Jones' reagent to afford **7m** and **7p**. The C16β/C17β-stereochemistry of final alcohols **4m**, **4p**, **6m**, and **6p** was easily determined using the characteristic ^1H NMR (~3.8 ppm) and ^{13}C NMR (~83 ppm) signals for position 17. These two signals are clearly characteristic of a C16β/C17β-stereochemistry as previously published for 16β-allyl-E_2.[30] The C16β-stereochemistry of final ketones **5m**, **5p**, **7m**, and **7p** was confirmed by 2D NMR experiments showing nuclear Overhauser effects between 16α-CH and three characteristic protons (14α-CH, 15α-CH, and one proton of CH_2Ph).

As a next step, in order to reduce the estrogenic effects associated with the E_2 nucleus of **4m** (See Section 2.3), we tested three strategies, resulting in the synthesis of alcohols **17**–**19** (Scheme 2). In the first strategy, we removed the hydroxyl group at C3. The phenol of E_1 was thus converted into a triflate derivative, which was used for the palladium-mediated reduction giving 3-deoxy-E_1 (**8**).[31] The latter was submitted to the sequence of three reactions described above (aldolization with *meta*-amidobenzaldehyde, reduction of C-17 ketone, and catalytic hydrogenation), to afford **11**, **14**, and **17**, respectively, with a 33% yield for three steps. In the second strategy, a methoxy group was added at C2. The starting 3-*O*-benzyl-2-methoxy-E_1 (**9**) was synthesized as reported in the literature.[32] Therefore the aldolization

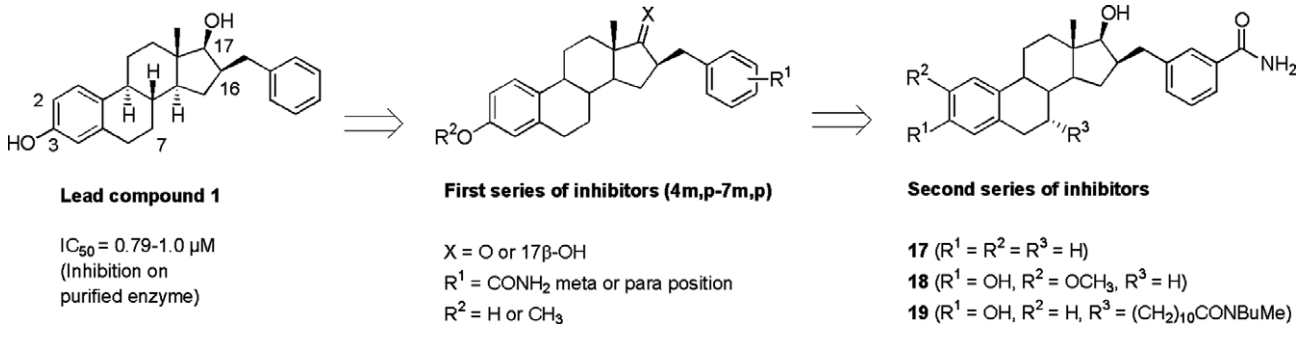
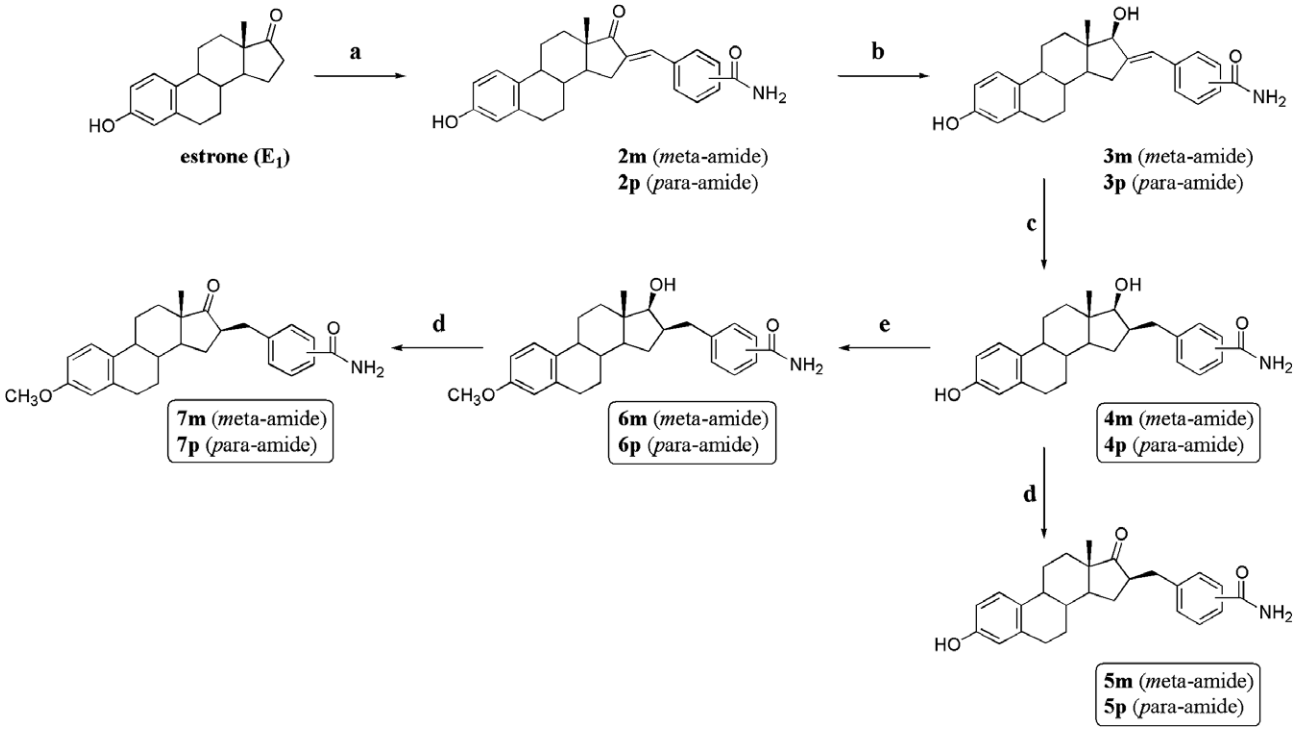
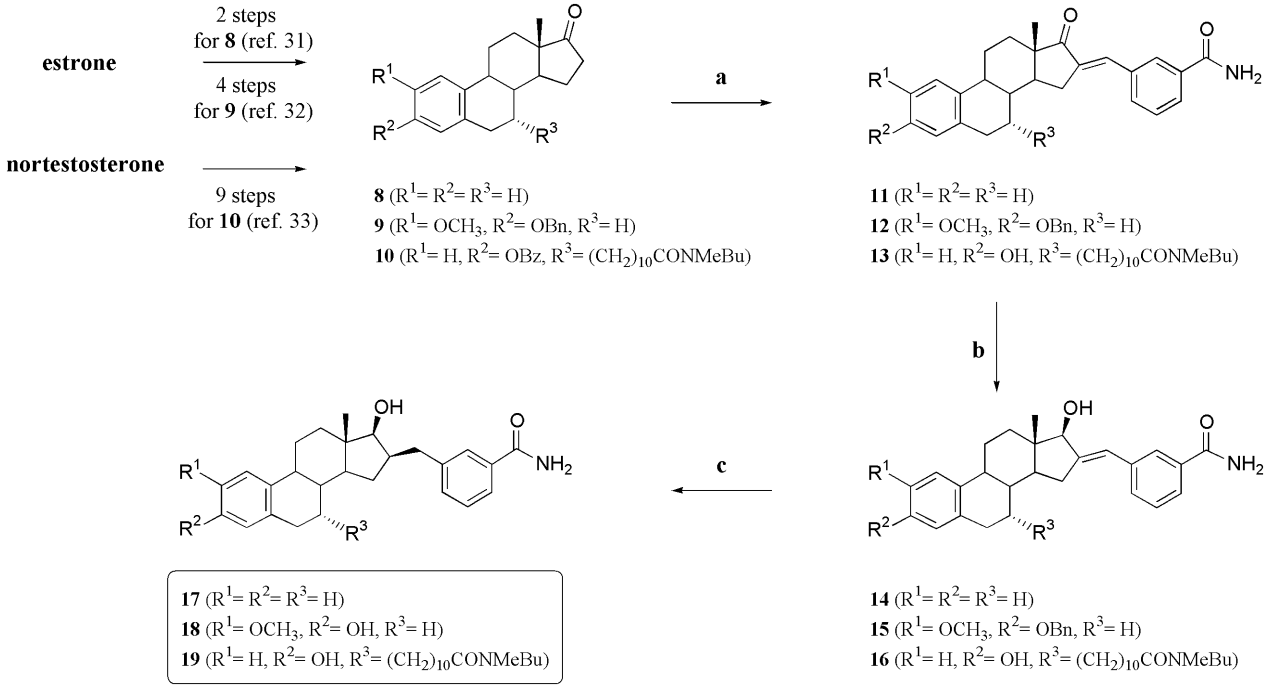


Figure 2. Inhibitors of 17β-HSD1. The stereogenic centers are illustrated only for steroid **1**, but they are the same for all other steroid derivatives reported in this paper. Partial numbering of carbons is represented on steroid **1**.



Scheme 1. Chemical synthesis of the first series of inhibitors, **4m**,**p**–**7m**,**p**. Reagents and conditions: (a) *meta*- or *para*-amidobenzaldehyde, KOH 10%, EtOH, 110 °C, 0.5 h; (b) $NaBH_4$, CH_2Cl_2, MeOH, 0 °C, 2–4 h; (c) H_2, Pd/C 10%, EtOH, rt, 12 h; (d) Jones' reagent (2.7 M), acetone, rt, 5 min; (e) Cs_2CO_3, MeI, acetone, 70 °C, 1 h.



Scheme 2. Chemical synthesis of the second series of inhibitors, **17**–**19**. Reagents and conditions: (a) *meta*-amidobenzaldehyde, KOH 10%, EtOH, 110 °C, 0.5 h; (b) $NaBH_4$, CH_2Cl_2, MeOH, 0 °C, 2–4 h; (c) H_2, Pd/C 10%, EtOH, rt, 12 h.

of **9** with *meta*-amidobenzaldehyde and its reduction with sodium borohydride was performed in the presence of benzylether, which was removed during the catalytic hydrogenation step. Then the 2-methoxy-E_2 C-16 derivative **18** was obtained with a 57% yield for the three steps. In the third strategy, the E_2 derivative **19** having two functionalized side chains—a 16β-(*meta*-carbamoylbenzyl) and a 7α-(*N*-methyl,*N*-butyl undecanamide)—was synthesized from the ketone **10**,[33] which was previously obtained from 19-nortestosterone as

reported in the literature.[34,35] The aldolization of **10** with *meta*-amidobenzaldehyde and a 10% KOH solution, the reduction of the 17-ketone, and the catalytic hydrogenation afforded the desired functionalized product **19** in good overall yield (58%). The C16β/C17β-stereochemistry of alcohols **17**–**19** was established by NMR spectroscopy as discussed above.

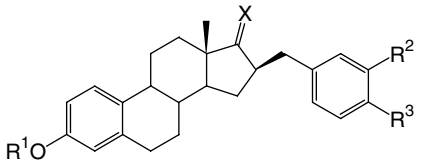
2.2. 17β-HSD1 inhibition studies

The first series of compounds were tested for their ability to inhibit the transformation of labeled E_1 (60 nM) into E_2 catalyzed by 17β-HSD1 in intact breast cancer T-47D cells, which are known to express the enzyme.[36] All inhibitors were tested at three concentrations (0.1, 1, and 10 μM) and results are reported in Table 1.

At a concentration of 10 μM, **4m**, **5m**, and **6m** show more than 90% of inhibition, **7m**, **4p**, and **5p** show around 75% of inhibition and **6p** and **7p** are weaker inhibitors (38% and 56%, respectively). A better discrimination between compounds was obtained at lower concentrations: **4m** and **5m** clearly constitute the best inhibitors with 77% and 51% inhibition, respectively, at 0.1 μM. Furthermore, only **4m** and **5m** are better inhibitors than unlabeled E_1 at all three concentrations. These results also indicate that, at position 17, a β-hydroxyl group is better than a ketone for the inhibition of 17β-HSD1 (see compounds **4m** and **6m** versus **5m** and **7m**). This result is in contradiction with the known affinity data of E_1 (ketone) and E_2 (17β-OH) for 17β-HSD1, which clearly favor E_1 (K_m = 0.03 μM) over E_2 (K_m = 4.6 μM).[37] However, the tendency observed with **4m**–**7m** was reversed with the weaker inhibitors **4p**–**7p**, which have a $CONH_2$ in para position on the 16β-aryl group. The positioning of the amido ($CONH_2$) group has a drastic effect on enzyme inhibition since compounds **4m**–**7m**, with a *meta*-amido group, are clearly better inhibitors than compounds **4p**–**7p** bearing a *para*-amido group. Taken together, the above results indicate that the carbamoylphenyl moiety is involved in key interactions with amino acids within the enzyme catalytic site. It is also known that a 17β-OH and a 17-ketone induce two different orientations of the phenyl ring and consequently of the amide group. Preliminary results not presented herein also indicate that a longer spacer between the 16β-steroidal carbon and the phenyl ring—two or three CH_2 groups instead of one for **4m**—reduces the inhibitory potency. Thus, the 17β-OH/*meta*-carbamoylbenzyl constitutes the best arrangement for inhibition of 17β-HSD1. In our study, we also tested the effect of a methoxy group at position 3 instead of the hydroxyl group: it considerably decreases the inhibitory potency, from 77% for compound **4m** to 16% for compound **6m** at 0.1 μM. This observation was also made for compounds with a 17β-OH or a 17-ketone as well as *meta*- or *para*-amide positioning. In fact, these results are in agreement with the known role of phenolic OH group at C3 of E_2, which is known to interact with His221.[38–40] For the most active inhibitors **4m** and **5m**, we determined an IC_{50} value of 44 nM and 171 nM (Table 1). These results were found to be quite satisfactory considering the great improvement of the inhibitory potency when compared to compound **1** (IC_{50} = 0.79–1.0 μM).[23] Furthermore, we also determined that compound **4m** did not inhibit at a concentration of 10 μM the oxidative transformation of E_2 (60 nM) into E_1 catalyzed by 17β-HSD2 overexpressed in intact HEK-293 cells (data not shown). This is an interesting characteristic of **4m** because an inhibitor of 17β-HSD1 should preferably not inhibit the 17β-HSD2 activity that inactivates the potent estrogen E_2.

A 17β-HSD1 inhibitor must be devoid of estrogenic activity to be considered useful in the treatment of breast cancer. Given its E_2 nucleus, and based on our previous work on E_2 derivatives modified at C6, C16, and C17,[28,31,32] inhibitor **4m** was expected to display some estrogenic activity. Three strategies were then applied to reduce this unwanted activity. First, we removed

Table 1. Inhibitors of 17β-HSD1 (first series) and their inhibitory potency in T-47D intact cells (transformation of [^{14}C]-E_1 into [^{14}C]-E_2)



Compound	R^1	R^2	R^3	X 17β-OH or O	Inhibition[a] (%) 0.1 μM/1 μM/10 μM	IC_{50}[b] (nM)
4m	H	$CONH_2$	H	OH	77/94/96	44 ± 7
5m	H	$CONH_2$	H	O	51/88/95	171 ± 22
6m	CH_3	$CONH_2$	H	OH	16/57/90	—
7m	CH_3	$CONH_2$	H	O	10/28/76	—
4p	H	H	$CONH_2$	OH	8/20/74	—
5p	H	H	$CONH_2$	O	8/39/76	—
6p	CH_3	H	$CONH_2$	OH	0/10/38	—
7p	CH_3	H	$CONH_2$	O	5/19/56	—
E_1	—	—	—	—	37/85/94	218 ± 20

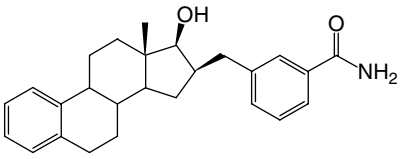
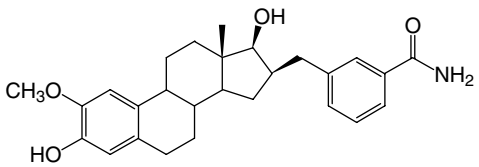
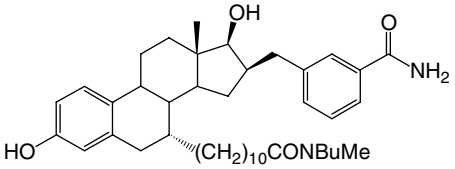
[a] The experiment was performed in triplicate. SD < ±5%. The inhibitors were tested at three concentrations of 0.1, 1, and 10 μM.
[b] Mean ± SD of an experiment performed in triplicate.

the hydroxyl group at position 3. Second, we introduced a methoxy group at position 2. This strategy rises from the use of 2-MeO-E_2, Panzem®, as a potential chemotherapeutic agent for treatment of cancer. Previous reports demonstrated that 2-MeO-E_2 can reduce growth of many cancer cell lines.[41–43] The last strategy was to introduce a 7α-side chain known for its antiestrogenic property.[44] The chemical structure and inhibitory potency of compounds **17**–**19** are reported in Table 2. Results indicate that none of these compounds is a more efficient inhibitor of 17β-HSD1 activity than **4m** in T-47D cells. In fact, removing the hydroxyl group at position 3 and introducing a pure antiestrogen side chain at position 7α decreases significantly the inhibitory potency, down to 24% and 4%, respectively, for **17** and **19** at 0.1 μM. Nevertheless, compound **18** bearing a methoxy group at position 2 shows the best inhibition in this second series of inhibitors (81%, 69%, and 37% at 10, 1, and 0.1 μM, respectively). It however remains less potent than **4m**. The presence of a 2-MeO group has also been reported to reduce the inhibitory activity of a potent 17β-HSD1 inhibitor, an E_2 derivative, by 10-fold.[40]

2.3. Proliferative activity on ER^+ cell lines

To detect any undesirable estrogenic activity in the new compounds, cell proliferative assays were carried out on T-47D and MCF-7 cell lines which are known to express the estrogen receptor (ER^+). Proliferative activities of compounds **4m**, **5m**, **17**, **18**, and **19** were evaluated at 0.01, 0.1, and 1 μM (Fig. 3). From the data collected, it is clear that compounds **4m** and **5m** exert the most important proliferative activities at 1 and 0.1 μM but none at 0.01 μM in both cell lines. As expected, the E_2-derivative **4m** induces cell proliferation somewhat more strongly than E_1-derivative **5m**. This result is in agreement with the stronger binding affinity of E_2 for ER.[45,46] Compounds **17** and **18** exert a same pattern of proliferative activity at all three concentrations and in both cell lines. In T-47D cells, removing the hydroxyl at C3 (compound **17**) and introducing a methoxy at C2 (compound **18**) significantly reduce the proliferative activity of our lead compound **4m** by 47% and 41% at 1 μM, respectively, whereas no proliferative activity was observed at 0.1 and 0.01 μM. In MCF-7 cells, compounds **17** and **18** exert 31% and 21% less estrogenic activity than compound **4m** at 0.1 μM. Moreover, compound **19**, having the characteristic side chain of pure antiestrogens EM-139[34] and ICI 164384,[35] totally inhibits growth at 0.01 μM and even brings cell density under that of the control level at 1 and 0.1 μM in T-47D cells. Even better results were obtained with MCF-7 cells in which compound **19** brought cell density under that of the control at all three concentrations tested. This compound acts more like an antiestrogen possessing a weak inhibitory activity toward 17β-HSD1. Indeed, among the compounds resulting from the three strategies for reducing estrogenic activity, compound **19** exerts the weakest inhibition for the transformation of E_1 into E_2 (Table 2). Such results are in agreement with our previous work using an alkylamide chain at C7α and a short bromobutyl chain at C16α of E_2,[28] which indicated that 17β-HSD1 does not tolerate well a long alkylamide side chain at C7α.

Table 2. Inhibitors of 17β-HSD1 (second series) and their inhibitory potency in T-47D intact cells (transformation of [^{14}C]-E_1 into [^{14}C]-E_2)

Compound	Chemical structure	Inhibition[a] (%) 0.1 μM/1 μM/10 μM
17		24/44/79
18		37/69/81
19		4/45/61

[a] The experiment was performed in triplicate. SD < ±5%. The inhibitors were tested at three concentrations of 0.1, 1, and 10 μM.

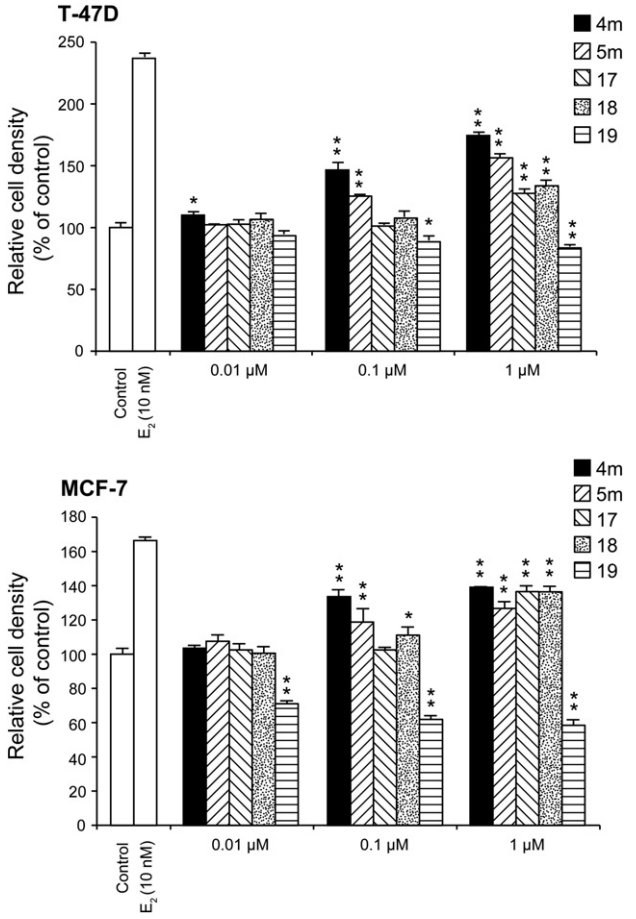


Figure 3. Effects of selected inhibitors on the growth of estrogen-starved T-47D and MCF-7 cells after 8 days of treatment. Control is fixed as 100%. Results are expressed as means ± SEM of triplicate. $^{*}P \leqslant 0.05$ versus control. $^{**}P \leqslant 0.01$ versus control.

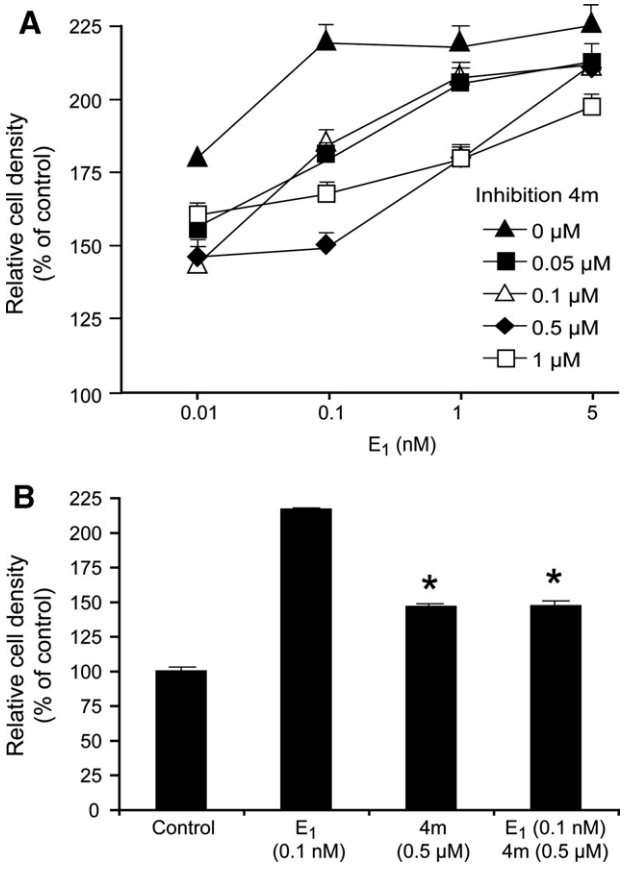


Figure 4. (A) Cell growth of T-47D cells induced by E_1 (0.01 to 5 nM) in the presence of inhibitor **4m** (0 to 1 μM) after 8 days of incubation. The cell proliferation without E_1 (control) is fixed as 100%. (B) Growth of T-47D cells induced by a physiologic concentration of E_1 (0.1 nM) in the presence or absence of inhibitor **4m** (0.5 μM). Results are expressed as means ± SEM of triplicate. $^{*}P \leqslant 0.01$ versus control and versus E_1 (0.1 nM).

2.4. Inhibition of E_1-induced cell proliferation by inhibitor 4m

Even though our inhibitors exert some estrogenic effects when tested in the absence of E_1, we investigated the possibility that such compounds could block the proliferative effect induced by E_1 in breast cancer ER^+ cell line T-47D. We tested the ability of the most potent inhibitor **4m** to inhibit the cell growth induced by the transformation of E_1 (0.01–5 nM) into potent estrogen E_2 (Fig. 4A). We did not select compounds **5m**, **17**, **18**, and **19** for this assay, even though they are less estrogenic, because they are also less potent inhibitors of the 17β-HSD1 activity.

Results indicated that inhibitor **4m** is able to inhibit the proliferative effect induced by the conversion of E_1 into E_2 by 17β-HSD1 activity in a concentration-dependent manner. The stronger cell growth inhibition, was, however, obtained at a concentration of 0.5 μM rather than 1 μM. At the latter concentration, the inhibitory effect of **4m** is probably counterbalanced by its residual estrogen-like proliferation effect on ER^+ cells. In another experiment (Fig. 4B), the optimal concentration (0.5 μM) of inhibitor **4m** reduced 62% of the cell growth induced by 0.1 nM of E_1. This concentration is close to the intracellular concentration of E_1 in breast cancer cells.[47] Although **4m** was successful in reducing the cell growth from 220% to ~150%, it is not possible to reduce the proliferation at the basal level (100%) because of the proliferative activity of **4m** itself (150%). Given the fact that inhibitor **4m** stimulated by itself cell growth to ~150% over the basal level—the same level of cell proliferation obtained with a combination of E_1 and **4m**—the proliferative activity induced by E_1 in T-47D appears indeed to be the result of the conversion of E_1 into E_2 by 17β-HSD1 activity. Thus, although E_1 binds the estrogen receptor, the results shown in Figure 4B suggest that E_2 is the only steroid that promotes the ER^+ cell growth observed when using E_1.

The reduction of E_1-induced cell proliferation obtained when using inhibitor **4m** could also be the result of an antiestrogenic activity of this E_2 derivative. Indeed, an antiestrogenic compound will block the proliferative (estrogenic) effect of E_2 mediated by their action on the estrogen receptor. As illustrated in Figure 5, the enzyme inhibitor **4m** does not reverse the proliferative effect on ER^+ cells of E_2 (0.1 nM) as the pure

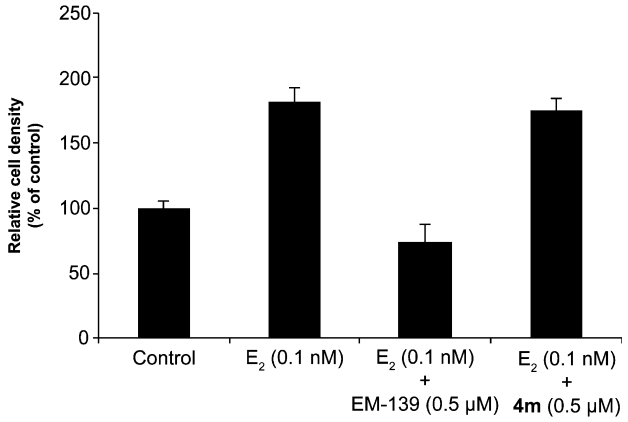


Figure 5. Effect of inhibitor **4m** and the pure antiestrogen EM-139[34] on the inhibition of E_2 (0.1 nM)-induced proliferation (antiestrogenic activity) of estrogen-sensitive human breast cancer MCF-7 cells. Two days after plating, the cells were incubated for 8 days with the indicated concentration of compounds. Media was changed every second day. Results are expressed as means ± SEM of triplicate.

antiestrogen EM-139 does. This result suggests that **4m** does not work as an antiestrogenic compound, but acts instead as an inhibitor of E_1-into-E_2 transformation (inhibitor of 17β-HSD1).

3. Conclusion

In this report, we extended results of previous studies of E_1 and E_2 derivatives at C16, aimed at developing 17β-HSD1 inhibitors. Among the eleven inhibitors that were synthesized, **4m** was the most potent inhibitor with an IC_{50} value of 44 nM for the transformation of E_1 into E_2 in intact T-47D cells. A *meta*-carbamoylbenzyl group, which probably mimics the nicotinamide side chain of cofactor NADPH, appears to be an important characteristic of this new 17β-HSD1 inhibitor. Interestingly, the proliferative activity induced by a physiologic concentration of E_1 (0.1 nM) in T-47D ER^+ cells was reduced by 62% by **4m**. The cell growth reduction was not 100% because a weak (38%) estrogenic activity was induced by **4m** itself, an E_2 derivative. Three strategies were thus tested to decrease the undesirable estrogenic activity. Although the resulting inhibitors **17**–**19** were less estrogenic than our lead compound **4m**, they exhibited an important drop in inhibitory activity on 17β-HSD1.

By emerging as a potent inhibitor of 17β-HSD1, **4m** warrants further investigations. Since a crystallization study has generated an enzyme/inhibitor complex appropriate for X-ray analysis, we are confident to report soon the key interactions established between the potent inhibitor and the enzyme residues.

4. Experimental

4.1. Chemistry

Chemical reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Flash chromatography was performed on Silicycle 60 230–400-mesh silica gel (Québec, Québec, Canada). Thin-layer chromatography (TLC) was performed on Whatman 0.25-mm silica gel 60 F_{254} plates (Fisher Scientific, Nepean, Ontario, Canada) and compounds were visualized by exposure to UV light (254 nm), a solution of ammonium molybdate/sulphuric acid/water, and/or a solution of *para*-anisaldehyde/sulphuric acid/acetic acid/ethanol (plus heating). Infrared (IR) spectra were recorded on a Perkin-Elmer 1600 (Norwalk, CT, USA) and obtained from a thin film of the solubilized compound on NaCl pellets (usually in CH_2Cl_2) or in KBr pellets containing the solid compound. Only significant bands are reported (in cm^{-1}). ^1H and ^{13}C NMR spectra were recorded, respectively, at 300 (^1H) and 75.5 (^{13}C) MHz or at 400 (^1H) and 100 (^{13}C) MHz using a Bruker AC/F 300 or a Bruker AVANCE 400 spectrometer (Billerica, MA, USA). The chemical shifts (δ) are expressed in ppm and referenced to chloroform (7.26 and 77.0 ppm), methanol (3.31 and 49.0 ppm), or acetone (2.05 and 29.8 ppm) for ^1H and ^{13}C, respectively (Fig. 6). Low-resolution mass spectra (LRMS) were recorded with an atmospheric pressure chemical ionization (APCI) source on positive mode. The purity of tested compounds **4m**,**p**–**7m**,**p** and **17**–**19** was determined by high-performance liquid chromatography (HPLC). The analyses were performed using a Waters 600E pump, a Water 717 plus Autosampler, and a Waters 996 Photodiode Array Detector (Milford, MA, USA). A Waters Nova-Pack C18 column (150 × 3.9 mm, 4 μm, 60 Å) was used with the following eluent system (1 mL/min flow rate): (A) a gradient of 10% MeOH ca 20 mM of $AcONH_4$ and 90% of H_2O ca 20 mM of $AcONH_4$ to 100% MeOH ca 20 mM of $AcONH_4$ (from 0 to 30 min); (B) 100% MeOH ca 20 mM of $AcONH_4$ (from 30 to 45 min); and (C) 10% MeOH ca 20 mM of $AcONH_4$ and 90% of H_2O ca 20 mM of $AcONH_4$ (from 45 to 62 min).

4.1.1. General procedure for aldolization at the 16-position of E_1 template. To a stirred solution of E_1, **8**,[31] **9**,[32] or **10**[33] (0.5 mmol) in EtOH (5.6 mL) were added *meta*- or *para*-amidobenzaldehyde[29] (1 mmol) and a

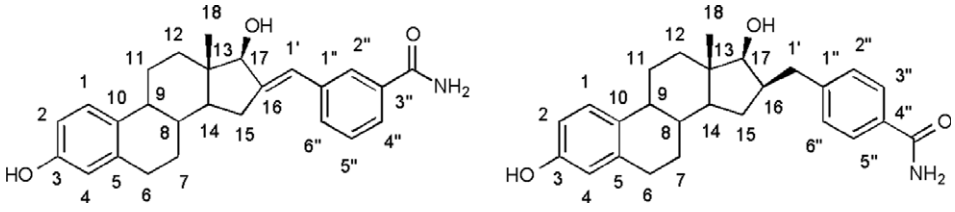


Figure 6. Carbon numbering used for the assignment of representative ^1H NMR signals.

KOH aq solution (10% w/w, 1.13 mL). The reaction mixture was stirred for 0.5 h at 130 °C, then H_2O (2 mL) was added and EtOH evaporated under reduced pressure. The aqueous phase was extracted with EtOAc and the organic phase was dried over $MgSO_4$ and evaporated under vacuum. Purification by flash chromatography (hexanes/EtOAc) afforded products **2m**, **2p**, **11**, **12**, or **13**.

4.1.1.1. 3-(3-Hydroxy-17-oxo-estra-1,3,5(10)-trien-16-ylidenemethyl)-benzamide (2m). Yellow amorphous solid (80% yield); hexanes/EtOAc: 50/50 for chromatography. IR (film) ν 3304 (OH and NH_2), 1706 (C=O, ketone), 1652 (C=O, amide); ^{1}H NMR (400 MHz, MeOH-d_4) δ 1.00 (s, 18-CH_3), 1.30–2.50 (m, unassigned CH and CH_2), 2.66 and 3.00 (2m, 15-CH_2), 2.85 (m, 6-CH_2), 6.50 (d, J = 2.4 Hz, 4-CH), 6.55 (dd, J_2 = 8.4 Hz, J_1 = 2.5 Hz, 2-CH), 7.09 (d, J = 8.4 Hz, 1-CH), 7.44 (s, 1′-CH), 7.55 (t, J = 7.7 Hz, 5″-CH), 7.78 (d, J = 7.8 Hz, 6″-CH), 7.88 (d, J = 7.8 Hz, 4″-CH), 8.11 (s, 2″-CH); ^{13}C NMR (100 MHz, MeOH-d_4) δ 15.0, 27.1, 27.9, 30.0, 30.5, 32.8, 39.5, 45.3, 49.2, 49.8, 113.8, 116.1, 127.1, 129.3, 130.0, 130.4, 132.0, 133.3, 134.5, 135.6, 137.2, 138.7, 138.8, 156.1, 171.7, 211.7; LRMS calcd for $C_{26}H_{28}NO_3$ $[M+H]^+$ 402.2.

4.1.1.2. 4-(3-Hydroxy-17-oxo-estra-1,3,5(10)-trien-16-ylidenemethyl)-benzamide (2p). Yellow amorphous solid; hexanes/EtOAc: 40/60–30/70 for chromatography. IR (film) ν 3353 (OH and NH_2), 1715 (C=O, ketone), 1660 (C=O, amide); ^{1}H NMR (400 MHz, MeOH-d_4) δ 1.02 (s, 18-CH_3), 1.30–2.50 (m, unassigned CH and CH_2), 2.67 and 3.00 (2m, 15-CH_2), 2.86 (m, 6-CH_2), 6.51 (d, J = 2.4 Hz, 4-CH), 6.57 (dd, J_2 = 8.4 Hz, J_1 = 2.5 Hz, 2-CH), 7.11 (d, J = 8.4 Hz, 1-CH), 7.46 (s, 1′-CH), 7.71 (d, J = 8.3 Hz, 2″-CH and 6″-CH), 7.95 (d, J = 8.3 Hz, 3″-CH and 5″-CH); ^{13}C NMR (75 MHz, MeOH-d_4) δ 15.0, 27.1, 27.9, 30.1, 30.5, 32.8, 39.5, 45.3, 49.0, 49.8, 113.8, 116.1, 127.2, 129.1 (2C), 131.4 (2C), 132.0, 133.0, 135.3, 138.7, 139.5, 140.1, 156.1, 171.6, 211.7; LRMS calcd for $C_{26}H_{28}NO_3$ $[M+H]^+$ 402.2.

4.1.1.3. 3-(17-Oxo-estra-1,3,5(10)-trien-16-ylidenemethyl)-benzamide (11). Yellow amorphous solid (47% yield); hexanes/EtOAc: 40/60–30/70 for chromatography. IR (film) ν 3348 and 3192 (NH_2), 1710 (C=O, ketone), 1670 (C=O, amide); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.02 (s, 18-CH_3), 1.40–2.55 (m, unassigned CH and CH_2), 2.62 and 3.00 (2m, 15-CH_2), 2.97 (m, 6-CH_2), 5.84 and 6.18 (2bs, NH_2), 7.14 (m, 3H aryl), 7.32 (d, J = 6.5 Hz, 1H aryl), 7.49 (d, J = 3.5 Hz, 1′-CH), 7.52 (d, J = 7.7 Hz, 5″-CH), 7.71 (d, J = 7.8 Hz, 6″-CH), 7.77 (d, J = 7.7 Hz, 4″-CH), 8.04 (s, 2″-CH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.5, 25.7, 26.8, 29.1, 29.3, 31.7, 37.6, 44.5, 47.9, 48.6, 125.2, 125.8, 125.9, 127.5, 129.0, 129.1, 131.9, 133.5, 133.8, 136.3, 136.4, 137.4, 139.6, 168.8, 209.3; LRMS calcd for $C_{26}H_{28}NO_2$ $[M+H]^+$ 386.2.

4.1.1.4. 3-(3-Benzyloxy-2-methoxy-17-oxo-estra-1,3,5(10)-trien-16-ylidenemethyl)-benzamide (12). Yellow oil (93% yield); hexanes/EtOAc: 50/50–30/70 for chromatography. IR (film) ν 3366 and 3163 (NH_2), 1704 (C=O, ketone), 1673 (C=O, amide); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.01 (s, 18-CH_3), 1.20–2.45 (m, unassigned CH and CH_2), 2.60 and 2.96 (2m, 15-CH_2), 2.80 (m, 6-CH_2), 3.87 (s, OCH_3), 5.11 (s, OCH_2), 5.88 and 6.25 (2bs, NH_2), 6.65 (s, 1-CH), 6.85 (s, 4-CH), 7.40 (m, CH of BnO, 1′-CH and 5″-CH), 7.70 (d, J = 7.7 Hz, 6″-CH), 7.77 (d, J = 7.7 Hz, 4″-CH), 8.03 (s, 2″-CH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.5, 26.1, 26.9, 28.9, 29.0, 31.6, 37.8, 44.3, 47.9, 48.4, 56.2, 71.0, 109.5, 114.5, 127.2 (2C), 127.6, 127.7, 128.5 (3C), 129.0, 129.1, 131.9, 132.1, 133.5, 133.8, 136.2, 137.3, 137.4, 146.4, 147.6, 168.8, 209.3; LRMS calcd for $C_{34}H_{36}NO_4$ $[M+H]^+$ 522.1.

4.1.1.5. *N*-butyl-*N*-methyl-11-[16-(3-carbamoylbenzylidene)-3-hydroxy-17-oxo-estra-1,3,5(10)-trien-7α-yl]-undecanamide (13). Yellow oil (80% yield); hexanes/EtOAc: 20/80–0/100 for chromatography. IR (film) ν 3350 and 3194 (OH and NH_2), 1713 (C=O, ketone), 1670 and 1624 (C=O, amides); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.98 (s, 18-CH_3), 0.92 and 0.95 (2t, J = 7.4 Hz, CH_2CH_3), 0.90–3.00 (m, unassigned CH and CH_2), 2.94 and 2.98 (2s, NCH_3), 3.26 and 3.38 (2t, J = 7.6 Hz, NCH_2), 5.95 and 6.80 (2bs, NH_2), 6.65 (d, J = 2.3 Hz, 4-CH), 6.68 (dd, J_2 = 8.4 Hz, J_1 = 2.4 Hz, 2-CH), 7.13 (d, J = 8.5 Hz, 1-CH), 7.47 (s, 1′-CH), 7.50 (d, J = 7.7 Hz, 5″-CH), 7.69 (d, J = 7.7 Hz, 6″-CH), 7.84 (d, J = 7.6 Hz, 4″-CH), 8.07 (s, 2″-CH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.8, 14.5, 19.9 (20.0), 25.0 (25.5), 25.1, 26.8, 27.1, 28.6, 28.8, 28.9, 29.0, 29.1, 29.3 (2C), 29.3 (30.5), 31.8, 32.9 (33.5), 33.6 (35.5), 34.5 (2C), 38.2, 41.2, 45.1, 47.7 (50.0), 48.0, 113.2, 116.2, 126.8, 127.8, 128.9, 129.4, 130.3, 132.0, 133.3 (2C), 136.1, 136.5, 137.2, 154.5, 169.0, 173.7, 209.5; LRMS calcd for $C_{42}H_{57}N_2O_4$ $[M-H]^+$ 653.3.

4.1.2. General procedure for the reduction of the 17-ketone group on the E_1 template. $NaBH_4$ (0.85 mmol) was added to a cooled (0 °C) solution of **2m**, **2p**, **11**, **12**, or **13** (0.5 mmol) in MeOH (12.7 mL) and CH_2Cl_2 (2.5 mL). After the mixture was stirred for 3–4 h at 0 °C, the reaction was quenched by addition of H_2O and the extraction was performed with CH_2Cl_2. The organic phase was dried over Na_2SO_4, and evaporated to dryness. The crude product was purified by flash chromatography (hexanes/EtOAc) to afford **3m**, **3p**, **14**, **15**, or **16**.

4.1.2.1. 3-(3,17β-Dihydroxy-estra-1,3,5(10)-trien-16-ylidenemethyl)-benzamide (3m). Yellow amorphous solid (86% yield); hexanes/EtOAc: 30/70 for chromatography. IR (film) ν 3344 (OH and NH_2), 1654 (C=O, amide); ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.72 (s, 18-CH_3), 1.20–2.40 (m, unassigned CH and CH_2), 2.78 (m, 6-CH_2 and 1 H of 15-CH_2), 4.06 (s, 17α-CH), 6.48 (d, J = 2.4 Hz, 4-CH), 6.54 (m, 2-CH and 1′-CH), 7.06 (d, J = 8.5 Hz, 1-CH), 7.40 (t, J = 7.7 Hz, 5″-CH), 7.55 (d, J = 7.8 Hz, 6″-CH), 7.66 (d, J = 7.7 Hz, 4″-CH), 7.92 (s, 2″-CH); ^{13}C NMR (100 MHz, MeOH-d_4) δ 11.9, 27.6, 28.6, 30.7, 31.6, 37.6, 39.9, 44.4, 45.4, 48.9, 85.7, 113.8, 116.2, 123.3, 126.2, 127.2, 128.5, 129.6, 132.5, 132.6, 135.1, 138.8, 140.0, 148.5, 156.0, 172.6; LRMS calcd for $C_{26}H_{30}NO_3$ $[M+H]^+$ 404.3.

4.1.2.2. 4-(3,17β-Dihydroxy-estra-1,3,5(10)-trien-16-ylidenemethyl)-benzamide (3p). Yellow amorphous solid; hexanes/EtOAc: 35/65 for chromatography. IR (film) ν 3352 and 3196 (OH and NH_2), 1652 (C=O, amide); ^{1}H NMR (400 MHz, acetone-d_6) δ 0.74 (s, 18-CH_3), 1.30–2.40 (m, unassigned CH and CH_2), 2.85 (m, 6-CH_2 and 1 H of 15-CH_2), 4.14 (s, 17α-CH), 6.55 (d, J = 2.5 Hz, 4-CH), 6.61 (m, 2-CH and 1′-CH), 7.12 (d, J = 8.4 Hz, 1-CH), 7.51 (d, J = 8.3 Hz, 2″-CH and 6″-CH), 7.93 (d, J = 8.3 Hz, 3″-CH and 5″-CH); ^{13}C NMR (75 MHz, acetone-d_6) δ 11.7, 27.2, 28.2, 30.3, 31.4, 37.2, 39.4, 44.0, 45.0, 48.4, 85.2, 113.6, 115.9, 122.6, 127.0, 128.5 (2C), 128.7 (2C), 131.9, 132.5, 138.4, 142.2, 149.8, 155.9, 168.7; LRMS calcd for $C_{26}H_{30}NO_3$ $[M+H]^+$ 404.1.

4.1.2.3. 3-(17β-Hydroxy-estra-1,3,5(10)-trien-16-ylidenemethyl)-benzamide (14). Yellow amorphous solid (95% yield); hexanes/EtOAc: 40/60–30/70 for chromatography. IR (film) ν 3385 and 3180 (OH and NH_2), 1670 (C=O, amide); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.75 (s, 18-CH_3), 1.30–2.50 (m, unassigned CH and CH_2), 2.79 (dd, J_2 = 16.8 Hz, J_1 = 6.7 Hz, 1 H of 15-CH_2), 2.92 (m, 6-CH_2), 4.17 (s, 17α-CH), 5.98 and 6.19 (2bs, NH_2), 6.59 (d, J = 1.8 Hz, 1′-CH), 7.14 (m, 3H aryl), 7.32 (d, J = 6.9 Hz, 1H aryl), 7.42 (t, J = 7.6 Hz, 5″-CH), 7.57 (d, J = 7.7 Hz, 6″-CH), 7.61 (d, J = 7.5 Hz, 4″-CH), 7.87 (s, 2″-CH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.1, 26.0, 27.4, 29.4, 30.6, 36.3, 37.9, 43.3, 44.5, 47.7, 84.9, 122.2, 124.9, 125.2, 125.6 (2C), 125.7, 127.3, 128.6, 129.1, 131.6, 136.5, 138.4, 140.0, 147.7, 169.7; LRMS calcd for $C_{26}H_{30}NO_2$ $[M+H]^+$ 388.2.

4.1.2.4. 3-(3-Benzyloxy-17β-hydroxy-2-methoxy-estra-1,3,5(10)-trien-16-ylidenemethyl)-benzamide (15). Yellow oil (90% yield); hexanes/EtOAc: 40/60–30/70 for chromatography. IR (film) ν 3348 and 3198 (OH and NH_2), 1667 (C=O, amide); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.75 (s, 18-CH_3), 1.30–2.40 (m, unassigned CH and CH_2), 2.70 (m, 6-CH_2 and 1 H of 15-CH_2), 3.87 (s, OCH_3), 4.16 (s, 17α-CH), 5.11 (s, OCH_2Ph), 5.88 and 6.22 (2bs, NH_2), 6.59 (d, J = 2.0 Hz, 1′-CH), 6.64 (s, 1-CH), 6.86 (s, 4-CH), 7.36 (m, CH of OCH_2*Ph* and 5″-CH), 7.56 (d, J = 7.8 Hz, 6″-CH), 7.60 (d, J = 7.7 Hz, 4″-CH), 7.87 (s, 2″-CH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.1, 26.4, 27.5, 29.0, 30.5, 36.3, 38.1, 43.3, 44.3, 47.5, 56.3, 71.0, 84.9, 109.6, 114.5, 122.3, 124.9, 127.2 (3C), 127.7 (2C), 128.5 (2C), 128.6, 131.6, 132.5, 133.3, 137.3, 138.4, 146.4, 147.6, 147.7, 169.4; LRMS calcd for $C_{34}H_{38}NO_4$ $[M+H]^+$ 524.1.

4.1.2.5. *N*-butyl-*N*-methyl-11-[16-(3-carbamoylbenzylidene)-3,17-dihydroxy-estra-1,3,5(10)-trien-7α-yl]-undecanamide (16). Yellow oil (75% yield); hexanes/EtOAc: 20/80–0/100 for chromatography. IR (film) ν 3360 (OH and NH_2), 1667 and 1621 (C=O, amides); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.74 (s, 18-CH_3), 0.92 and 0.94 (2t, J = 7.5 Hz, CH_2CH_3), 0.90–3.00 (m, unassigned CH and CH_2), 2.93 and 2.97 (2s, NCH_3), 3.26 and 3.37 (2m, NCH_2), 4.16 (s, 17α-CH), 5.94 and 6.52 (2bs, NH_2), 6.59 (d, J = 1.7 Hz, 1′-CH), 6.61 (d, J = 2.4 Hz, 4-CH), 6.68 (dd, J_2 = 8.4 Hz, J_1 = 2.5 Hz, 2-CH), 7.14 (d, J = 8.5 Hz, 1-CH), 7.41 (t, J = 7.7 Hz, 5″-CH), 7.55 (d, J = 7.8 Hz, 6″-CH), 7.64 (d, J = 7.7 Hz, 4″-CH), 7.87 (s, 2″-CH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 11.2, 13.8, 20.0, 25.0, 25.0 (25.5), 27.1, 27.3, 28.8, 29.0, 29.2 (2C), 29.3, 29.4, 29.4 (30.6), 30.2, 32.9 (33.3), 33.6 (35.5), 34.6 (2C), 36.5, 38.2, 41.4, 43.5, 44.0, 47.6 (49.9), 85.0, 113.1, 116.3, 122.4, 125.1, 126.8, 127.5, 128.6, 130.7, 131.4, 133.4, 136.7, 138.4, 147.7, 154.3, 169.7, 173.6; LRMS calcd for $C_{42}H_{61}N_2O_4$ $[M+H]^+$ 657.3.

4.1.3. General procedure for the hydrogenation of the benzylidene double bond at the 16-position of E_2 template. A mixture of **3m**, **3p**, **14**, **15**, or **16** (0.2 mmol) and palladium on charcoal (15% in weight) in anhydrous EtOH (3.8 mL) under hydrogen atmosphere was stirred 12 h at rt. Then the palladium reagent was removed by filtration on celite, washed with MeOH, and the filtrate was concentrated under reduced pressure. Purification by flash chromatography (hexanes/EtOAc) afforded **4m**, **4p**, **17**, **18**, or **19**.

4.1.3.1. 3-(3,17β-Dihydroxy-estra-1,3,5(10)-trien-16β-ylmethyl)-benzamide (4m). White amorphous solid (98% yield); hexanes/EtOAc: 20/80–0/100 for chromatography. IR (film) ν 3330 (OH and NH_2), 1652 (C=O, amide); ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.89 (s, 18-CH_3), 1.10–2.60 (m, unassigned CH and CH_2), 2.72 (m, 6-CH_2), 3.14 (d, J = 9.5 Hz, 16α-CH), 3.81 (d, J = 9.3 Hz, 17α-CH), 6.45 (d, J = 2.5 Hz, 4-CH), 6.52 (dd, J_2 = 8.4 Hz, J_1 = 2.6 Hz, 2-CH), 7.07 (d, J = 8.4 Hz, 1-CH), 7.37 (m, 5″-CH and 6″-CH), 7.67 (dt, J_2 = 7.4 Hz, J_1 = 1.5 Hz, 4″-CH), 7.73 (s, 2″-CH); ^{13}C NMR (100 MHz, MeOH-d_4) δ 13.3, 27.5, 28.6, 30.6, 33.0, 38.8, 38.9, 39.8, 43.3, 45.3, 45.4, 49.8, 83.0, 113.7, 116.0, 125.9, 127.1, 129.1, 129.4, 132.6, 133.5, 134.7, 138.7, 144.3, 155.8, 172.7; LRMS calcd for $C_{26}H_{32}NO_3$ $[M+H]^+$ 406.1. HPLC purity = 98.6%, rt = 26.6 min, λ = 218 nm.

4.1.3.2. 4-(3,17β-Dihydroxy-estra-1,3,5(10)-trien-16β-ylmethyl)-benzamide (4p). White amorphous solid (75% yield, 3 steps from E_1); hexanes/EtOAc: 30/70 for chromatography. IR (film) ν 3353 (OH and NH_2), 1663 (C=O, amide); ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.88 (s, 18-CH_3), 1.10–2.55 (m, unassigned CH and CH_2), 2.72 (m, 6-CH_2), 3.14 (d, J = 9.5 Hz, 16α-CH), 3.80 (d, J = 9.3 Hz, 17α-CH), 6.45 (d, J = 2.5 Hz, 4-CH), 6.53 (dd, J_2 = 8.4 Hz, J_1 = 2.6 Hz, 2-CH), 7.07 (d, J = 8.4 Hz, 1-CH), 7.30 (d, J = 8.2 Hz, 2″-CH and 6″-CH), 7.78 (d, J = 8.2 Hz, 3″-CH and 5″-CH); ^{13}C NMR (75 MHz, MeOH-d_4) δ 13.3, 27.5, 28.7, 30.7, 33.1, 38.9 (2C), 39.9, 43.3, 45.4 (2C), 49.9, 83.0, 113.7, 116.0, 127.2, 128.7 (2C), 130.0 (2C), 132.2, 132.6, 138.8, 148.5, 155.9, 172.5; LRMS calcd for $C_{26}H_{32}NO_3$ $[M+H]^+$ 406.2. HPLC purity = 93.5%, rt = 27.2 min, λ 209 nm.

4.1.3.3. 3-(17β-Hydroxy-estra-1,3,5(10)-trien-16β-ylmethyl)-benzamide (17). White amorphous solid (75% yield); hexanes/EtOAc: 40/60–30/70 for chromatography. IR (film) ν 3346 and 3180 (OH and NH_2), 1666 (C=O, amide); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (s, 18-CH_3), 1.10–2.65 (m, unassigned CH and CH_2), 2.84 (m, 6-CH_2), 3.16 (dd, J_2 = 12.9 Hz, J_1 = 4.3 Hz, 16α-

CH), 3.87 (d, J = 9.5 Hz, 17α-CH), 5.90 and 6.24 (2bs, NH_2), 7.10 (m, 3H aryl), 7.30 (d, J = 7.1 Hz, 1H aryl), 7.38 (m, 5″-CH and 6″-CH), 7.61 (d, J = 6.6 Hz, 4″-CH), 7.72 (s, 2″-CH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.6, 25.9, 27.3, 29.4, 32.1, 37.5, 37.6, 37.9, 41.7, 44.4, 44.5, 48.7, 82.1, 124.5, 125.3, 125.6 (2C), 127.9, 128.5, 129.0, 132.6, 133.2, 136.6, 140.2, 142.9, 169.7; LRMS calcd for $C_{26}H_{32}NO_2$ $[M+H]^+$ 390.1. HPLC purity = 93.8%, rt = 34.4 min, λ = 209 nm.

4.1.3.4. 3-(3,17β-Dihydroxy-2-methoxy-estra-1,3,5(10)-trien-16β-ylmethyl)-benzamide (18). Yellow amorphous solid (68% yield); hexanes/EtOAc: 40/60–30/70 for chromatography. IR (film) ν 3352 and 3204 (OH and NH_2), 1664 (C═O, amide); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (s, 18-CH_3), 1.10–2.60 (m, unassigned CH and CH_2), 2.73 (m, 6-CH_2), 3.15 (dd, J_2 = 12.8 Hz, J_1 = 4.3 Hz, 16α-CH), 3.85 (s, OCH_3), 3.87 (d, J = 9.8 Hz, 17α-CH), 5.88 and 6.22 (2bs, NH_2), 6.63 (s, 1-CH), 6.78 (s, 4-CH), 7.38 (m, 5″-CH and 6″-CH), 7.60 (d, J = 7.2 Hz, 4″-CH), 7.71 (s, 2″-CH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.6, 26.5, 27.5, 28.9, 32.1, 37.5, 37.7, 38.2, 41.7, 44.2, 44.4, 48.6, 56.0, 82.1, 108.1, 114.6, 124.5, 128.0, 128.5, 129.5, 131.7, 132.6, 133.2, 142.9, 143.5, 144.6, 169.7; LRMS calcd for $C_{27}H_{34}NO_4$ $[M+H]^+$ 436.1. HPLC purity = 97.5%, rt = 27.1 min, λ = 213 nm.

4.1.3.5. *N*-butyl-*N*-methyl-11-[16β-(3-carbamoylbenzyl)-3,17-dihydroxy-estra-1,3,5(10)-trien-7α-yl]-undecanamide (19). White amorphous solid (96% yield); hexanes/EtOAc: 10/90–0/100 for chromatography. IR (film) ν 3322 (OH and NH_2), 1667 and 1618 (C═O, amides); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87 (s, 18-CH_3), 0.92 and 0.95 (2t, J = 7.4 Hz, CH_2CH_3), 0.90–2.90 (m, unassigned CH and CH_2), 2.93 and 2.98 (2s, NCH_3), 3.16 (dd, J_2 = 13.1 Hz, J_1 = 4.1 Hz, 16α-CH), 3.26 and 3.35 (2m, NCH_2), 3.86 (d, J = 9.6 Hz, 17α-CH), 5.90 and 6.40 (2bs, NH_2), 6.59 (d, J = 2.2 Hz, 4-CH), 6.65 (dd, J_2 = 8.3 Hz, J_1 = 2.2 Hz, 2-CH), 7.11 (d, J = 8.5 Hz, 1-CH), 7.38 (m, 5″-CH and 6″-CH), 7.63 (d, J = 7.4 Hz, 4″-CH), 7.73 (s, 2″-CH); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.6, 13.8, 20.0, 24.9 (25.5), 25.1, 27.1, 27.3, 28.8, 28.9, 29.0, 29.1, 29.2, 29.3, 29.4 (30.6), 31.6, 33.0 (33.7), 33.4, 33.6 (35.5), 34.6, 37.6, 37.8, 38.2, 41.5, 41.7, 44.5, 44.9, 47.6 (49.9), 82.2, 113.0, 116.2, 124.6, 126.8, 128.1, 128.5, 131.0, 132.7, 133.0, 136.8, 143.0, 154.2, 169.8, 173.6; LRMS calcd for $C_{42}H_{63}N_2O_4$ $[M+H]^+$ 659.5. HPLC purity = 95.7%, rt = 35.2 min, λ = 220 nm.

4.1.4. General procedure for Jones' oxidation of the 17β-hydroxy group of the E_2 template. To a solution of **4m**, **4p**, **6m**, or **6p** (0.120 mmol) in acetone (15 mL) was added a solution of Jones' reagent (0.127 mmol) at 0 °C. After the mixture was stirred for 5 min at room temperature, the reaction was quenched by addition of isopropanol (0.8 mL) and acetone was evaporated under reduced pressure. H_2O (6 mL) was added to the slurry mixture and the aqueous phase was extracted with EtOAc. Then the combined organic layer was dried over $MgSO_4$ and evaporated. The crude product was purified by chromatography (hexanes/EtOAc) to afford **5m**, **5p**, **7m**, or **7p**.

4.1.4.1. 3-(3-Hydroxy-17-oxo-estra-1,3,5(10)-trien-16β-ylmethyl)-benzamide (5m). White amorphous solid (49% yield); hexanes/EtOAc: 40/60 for chromatography. IR (film) ν 3454, 3328, 3288 and 3231 (OH and NH_2), 1732 (C═O, ketone), 1658 (C═O, amide); ^{1}H NMR (300 MHz, MeOH-d_4 + acetone-d_6) δ 0.73 (s, 18-CH_3), 1.20–2.65 (m, unassigned CH and CH_2), 2.78 (m, 6-CH_2 and 1 H of CH_2-Aryl), 3.20 (dd, J_2 = 13.6 Hz, J_1 = 4.2 Hz, 16α-CH), 6.47 (d, J = 2.4 Hz, 4-CH), 6.54 (dd, J_2 = 8.4 Hz, J_1 = 2.5 Hz, 2-CH), 7.07 (d, J = 8.5 Hz, 1-CH), 7.41 (m, 5″-CH and 6″-CH), 7.75 (m, 4″-CH and 2″-CH); ^{13}C NMR (75 MHz, MeOH-d_4) δ 14.1, 27.1, 27.9, 28.7, 30.5, 33.1, 38.1, 39.4, 45.4, 50.0 (2C), 52.3, 113.8, 116.1, 126.7, 127.1, 129.3, 129.6, 132.0, 133.8, 135.1, 138.7, 141.8, 156.1, 172.4, 223.7; LRMS calcd for $C_{26}H_{30}NO_3$ $[M+H]^+$ 404.2. HPLC purity = 93.8%, rt = 28.6 min, λ = 208 nm.

4.1.4.2. 4-(3-Hydroxy-17-oxo-estra-1,3,5(10)-trien-16β-ylmethyl)-benzamide (5p). White amorphous solid (50% yield); hexanes/EtOAc: 45/55 for chromatography. IR (film) ν 3421, 3350, 3184 (OH and NH_2), 1729 (C═O, ketone), 1660 (C═O, amide); ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.70 (s, 18-CH_3), 1.30–2.65 (m, unassigned CH and CH_2), 2.79 (m, 6-CH_2 and 1 H of CH_2-Aryl), 3.18 (dd, J_2 = 13.5 Hz, J_1 = 4.3 Hz, 16α-CH), 6.47 (d, J = 2.5 Hz, 4-CH), 6.53 (dd, J_2 = 8.4 Hz, J_1 = 2.6 Hz, 2-CH), 7.06 (d, J = 8.4 Hz, 1-CH), 7.31 (d, J = 8.2 Hz, 2″-CH and 6″-CH), 7.80 (d, J = 8.2 Hz, 3″-CH and 5″-CH); ^{13}C NMR (75 MHz, MeOH-d_4) δ 14.1, 27.0, 27.9, 28.7, 30.5, 33.1, 37.9, 39.3, 45.4, 50.0 (2C), 52.2, 113.8, 116.1, 127.1, 128.8 (2C), 130.3 (2C), 132.0, 132.9, 138.7, 145.6, 156.1, 172.2, 223.5; LRMS calcd for $C_{26}H_{30}NO_3$ $[M+H]^+$ 404.1. HPLC purity = 98.9%, rt = 28.3 min, λ = 209 nm.

4.1.4.3. 3-(3-Methoxy-17-oxo-estra-1,3,5(10)-trien-16β-ylmethyl)-benzamide (7m). White amorphous solid (69% yield); hexanes/EtOAc: 40/60 for chromatography. IR (film) ν 3339 and 3191 (NH_2), 1734 (C═O, ketone), 1660 (C═O, amide); ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.70 (s, 18-CH_3), 1.25–2.60 (m, unassigned CH and CH_2), 2.80 (m, 6-CH_2 and 1 H of CH_2-Aryl), 3.20 (dd, J_2 = 13.6 Hz, J_1 = 4.2 Hz, 16α-CH), 3.71 (s, OCH_3), 6.57 (d, J = 2.6 Hz, 1-CH), 6.64 (dd, J_2 = 8.6 Hz, J_1 = 2.7 Hz, 2-CH), 7.14 (d, J = 8.7 Hz, 4-CH), 7.40 (m, 5″-CH and 6″-CH), 7.71 (m, 4″-CH and 2″-CH); ^{13}C NMR (100 MHz, MeOH-d_4) δ 14.1, 27.0, 27.9, 28.8, 30.6, 33.1, 38.1, 39.3, 45.4, 49.8, 50.0, 52.3, 55.5, 112.5, 114.6, 126.7, 127.2, 129.3, 129.6, 133.1, 133.8, 135.1, 138.8, 141.8, 159.0, 172.3, 223.6; LRMS calcd for $C_{27}H_{32}NO_3$ $[M+H]^+$ 418.1. HPLC purity = 92.4%, rt = 33.2 min, λ = 210 nm.

4.1.4.4. 4-(3-Methoxy-17-oxo-estra-1,3,5(10)-trien-16β-ylmethyl)-benzamide (7p). White amorphous solid (64% yield); hexanes/EtOAc: 50/50 for chromatography. IR (film) ν 3449, 3350 and 3195 (NH_2), 1732 (C═O, ketone), 1662 (C═O, amide); ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.68 (s, 18-CH_3), 1.30–2.60 (m, unassigned

CH and CH_2), 2.81 (6-CH_2 and 1 H of CH_2-Aryl), 3.17 (dd, J_2 = 13.5 Hz, J_1 = 4.2 Hz, 16α-CH), 3.71 (s, OCH_3), 6.58 (d, J = 2.4 Hz, 4-CH), 6.64 (dd, J_2 = 8.6 Hz, J_1 = 2.6 Hz, 2-CH), 7.13 (d, J = 8.6 Hz, 1-CH), 7.31 (d, J = 8.2 Hz, 2″-CH and 6″-CH), 7.80 (d, J = 8.2 Hz, 3″-CH and 5″-CH); ^{13}C NMR (75 MHz, MeOH-d_4) δ 14.1, 27.0, 27.9, 28.7, 30.6, 33.1, 38.0, 39.2, 45.4, 49.8, 50.0, 52.2, 55.5, 112.5, 114.7, 127.2, 128.8 (2C), 130.3 (2C), 132.9, 133.1, 138.7, 145.6, 159.0, 172.2, 223.4; LRMS calcd for $C_{27}H_{32}NO_3$ $[M+H]^+$ 418.2. HPLC purity = 99%, rt = 33.0 min, λ = 232 nm.

4.1.5. General procedure for the methylation of the hydroxyl group at the 3-position of E_2 template. A solution of **4m** or **4p** (0.34 mmol) in acetone (18 mL) was treated with cesium carbonate (0.51 mmol) and iodomethane (4.8 mmol). The resulting mixture was stirred at reflux for 1 h, then filtered on Celite and washed with CH_2Cl_2. The filtrate was evaporated and the crude product was purified by chromatography (hexanes/EtOAc) to afford **6m** or **6p**.

4.1.5.1. 3-(17β-Hydroxy-3-methoxy-estra-1,3,5(10)-trien-16β-ylmethyl)-benzamide (6m). White amorphous solid (76% yield); hexanes/EtOAc: 45/55 for chromatography. IR (film) ν 3348 and 3198 (OH and NH_2), 1654 (C=O, amide); ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.88 (s, 18-CH_3), 1.10–2.60 (m, unassigned CH and CH_2), 2.78 (6-CH_2), 3.15 (d, J = 10.8 Hz, 16α-CH), 3.71 (s, OCH_3), 3.81 (d, J = 9.3 Hz, 17α-CH), 6.56 (d, J = 2.2 Hz, 4-CH), 6.64 (dd, J_2 = 8.6 Hz, J_1 = 2.5 Hz, 2-CH), 7.15 (d, J = 8.6 Hz, 1-CH), 7.37 (m, 5″-CH and 6″-CH), 7.67 (d, J = 7.4 Hz, 4″-CH), 7.73 (s, 2″-CH); ^{13}C NMR (75 MHz, MeOH-d_4) δ 13.3, 27.5, 28.6, 30.7, 33.0, 38.8, 38.9, 39.8, 43.3, 45.3, 45.4, 49.9, 55.5, 83.0, 112.4, 114.5, 125.9, 127.1, 129.1, 129.4, 133.5, 133.7, 134.7, 138.8, 144.3, 158.8, 172.6; LRMS calcd for $C_{27}H_{34}NO_3$ $[M+H]^+$ 420.2. HPLC purity = 96.7%, rt = 33.6 min, λ = 212 nm.

4.1.5.2. 4-(17β-Hydroxy-3-methoxy-estra-1,3,5(10)-trien-16β-ylmethyl)-benzamide (6p). White amorphous solid (79% yield); hexanes/EtOAc: 45/55 for chromatography. IR (film) ν 3336 and 3182 (OH and NH_2), 1670 (C=O, amide); ^{1}H NMR (400 MHz, MeOH-d_4) δ 0.87 (s, 18-CH_3), 1.10–2.60 (m, unassigned CH and CH_2), 2.76 (m, 6-CH_2), 3.14 (d, J = 9.5 Hz, 16α-CH), 3.71 (s, OCH_3), 3.81 (d, J = 9.3 Hz, 17α-CH), 6.56 (d, J = 2.4 Hz, 4-CH), 6.64 (dd, J_2 = 8.6 Hz, J_1 = 2.6 Hz, 2-CH), 7.15 (d, J = 8.6 Hz, 1-CH), 7.30 (d, J = 8.2 Hz, 2″-CH and 6″-CH), 7.78 (d, J = 8.2 Hz, 3″-CH and 5″-CH); ^{13}C NMR (100 MHz, MeOH-d_4) δ 13.3, 27.5, 28.6, 30.7, 33.0, 38.8, 38.9, 39.8, 43.3, 45.3, 45.4, 49.9, 55.4, 82.9, 112.4, 114.5, 127.1, 128.6 (2C), 129.9 (2C), 132.2, 133.7, 138.8, 148.4, 158.9, 172.5; LRMS calcd for $C_{27}H_{34}NO_3$ $[M+H]^+$ 420.1. HPLC purity = 98.9%, rt = 33.1 min, λ = 232 nm.

4.2. Biological assays

4.2.1. Cell culture. Two ER-positive breast cancer cell lines, T-47D and MCF-7, were obtained from the American Type Culture Collection (ATCC) and maintained in 75 cm^2 culture flasks at 37 °C under 5% CO_2 humidified atmosphere. The T-47D cells were grown in RPMI medium (Sigma–Aldrich, Oakville, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 μg/mL) and estradiol (1 nM). The MCF-7 cells were propagated in Dulbecco's modified Eagle's medium containing a nutrient mixture F-12 ham (DME-F12) (Sigma–Aldrich, Ontario, Canada) and supplemented with 5% FBS, glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 μg/mL) and estradiol (1 nM). Both medium were prepared without phenol red.

4.2.2. Type 1 17β-HSD inhibition. T-47D cells were seeded in 24-well plates at 3×10^4 cells/well in proliferation assay medium (see Section 4.2.3). After 48 h, 60 nM of [4-^{14}C]-estrone (American Radiolabeled Chemicals Inc., St. Louis, MO, USA) and an ethanolic solution of inhibitor (0.5%, v/v) at concentrations of 0.1, 1, and 10 μM (0.1 nM to 100 μM for IC_{50}) were added to freshly changed culture medium and the cells were incubated for 24 h. Each inhibitor was assessed in triplicate. After incubation, labeled steroids (E_1 and E_2) were extracted and quantified according to an established procedure.[31,48]

4.2.3. Cell proliferation assay. Quantification of cell growth was determined using CellTitter 96® Aqueous Solution Cell Proliferation Assay (Promega, Nepean, Ontario, Canada) following the manufacturer's instructions. T-47D and MCF-7 cells were resuspended in their respective medium (RPMI or DME-F12) supplemented with insulin (50 ng/mL) and 5% dextran-coated charcoal treated FBS to remove the remaining estrogen present in the serum and medium. Aliquots (100 μL) of the cell suspension were seeded in 96-well plates (3000 cells/well). After 48 h, the medium was changed with appropriate dilution of the different inhibitors and reference compounds in growth medium and was replaced every 2 days until 8 days of treatment.

To determine the proliferative (estrogenic) activity, the estrogen-sensitive T-47D and MCF-7 cells were grown in absence (control fixed as 100%) or presence of an inhibitor (**4m**, **5m**, **17**, **18**, or **19** at 0.01, 0.1, and 1 μM) or estrogen E_2 at 10 nM. To determine the inhibition of E_1-induced cell proliferation, the T-47D (ER^+) cells were grown in the presence of E_1 (0.01, 0.1, 1, or 5 nM) and inhibitor **4m** (0.05, 0.1, 0.5, or 1 μM). The cell proliferation without E_1 and inhibitor **4m** (control) was fixed as 100%. To determine the potential antiestrogenic activity of inhibitor **4m**, the MCF-7 (ER^+) cells were grown in the presence of estrogen E_2 (0.1 nM) and pure antiestrogen EM-139[34] (0.5 μM) or inhibitor **4m** (0.5 μM). The cell proliferation without E_2 and tested compounds (control) was fixed as 100%.

4.2.4. Statistical analysis. Statistical significance was determined according to the Duncan–Kramer multiple range test.[49]

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