

Structure–Activity Relationships of 17 α -Derivatives of Estradiol as Inhibitors of Steroid Sulfatase

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The steroid sulfatase or steryl sulfatase is a microsomal enzyme widely distributed in human tissues that catalyzes the hydrolysis of sulfated 3-hydroxy steroids to the corresponding free active 3-hydroxy steroids. Since androgens and estrogens may be synthesized inside the cancerous cells starting from dehydroepiandrosterone sulfate (DHEAS) and estrone sulfate (E₁S) available in blood circulation, the use of therapeutic agents that inhibit steroid sulfatase activity may be a rewarding approach to the treatment of androgeno-sensitive and estrogeno-sensitive diseases. In the present study, we report the chemical synthesis and biological evaluation of a new family of steroid sulfatase inhibitors. The inhibitors were designed by adding an alkyl, a phenyl, a benzyl, or a benzyl substituted at position 17 α of estradiol (E₂), a C18-steroid, and enzymatic assays were performed using the steroid sulfatase of homogenized JEG-3 cells or transfected in HEK-293 cells. We observed that a hydrophobic substituent induces powerful inhibition of steroid sulfatase while a hydrophilic one was weak. Although a hydrophobic group at the 17 α -position increased the inhibitory activity, the steric factors contribute to the opposite effect. As exemplified by 17 α -decyl-E₂ and 17 α -dodecyl-E₂, a long flexible side chain prevents adequate fitting into the enzyme catalytic site, thus decreasing capacity to inhibit the steroid sulfatase activity. In the alkyl series, the best compromise between hydrophobicity and steric hindrance was obtained with the octyl group (IC₅₀ = 440 nM), but judicious branching of side chain could improve this further. Benzyl substituted derivatives of estradiol were better inhibitors than alkyl analogues. Among the series of 17 α -(benzyl substituted)-E₂ derivatives studied, the 3'-bromobenzyl, 4'-*tert*-butylbenzyl, 4'-butylbenzyl, and 4'-benzyloxybenzyl groups provided the most potent inhibition of steroid sulfatase transformation of E₁S into E₁ (IC₅₀ = 24, 28, 25, and 22 nM, respectively). As an example, the *tert*-butylbenzyl group increases the ability of the E₂ nucleus to inhibit the steroid sulfatase by 3000-fold, and it also inhibits similarly the steroid sulfatase transformations of both natural substrates, E₁S and DHEAS. Interestingly, the newly reported family of steroid sulfatase inhibitors acts by a reversible mechanism of action that is different from the irreversible mechanism of the known inhibitor estrone sulfamate (EMATE).

Introduction

Both androgen-sensitive prostate cancer¹ and estrogen-sensitive breast cancer² are stimulated by active steroids that are synthesized in the testis, ovaries, or peripheral target tissues such as prostate, breast, and uterine. The regulation of levels of mitogenic steroids aimed at inhibiting the enzymes key to their synthesis is being seriously considered as complementary therapeutic strategy to the blocking of intracellular hormone receptors by androgen or estrogen antagonists and even as a promising alternative. Among the enzymes involved in the steroidogenesis,³ steroid sulfatase or steryl sulfatase (E.C. 3.1.6.2) is a microsomal enzyme that catalyzes the hydrolysis of sulfated 3-hydroxy steroids, the inactive form of steroid hormone or steroid precursor, to the corresponding free active 3-hydroxy steroids. This enzyme is widely distributed in human tissues including the testis,⁴ lung,⁵ chorion,⁶ endometrium,⁷ decidua,^{6b} and brain.⁸ In the Rhesus monkey, steroid sulfatase was

also detected in all 25 tissues examined except the salivary glands.⁹ The best known steroid sulfatase is the human placental enzyme, which has been purified¹⁰ and cloned.¹¹ The purified enzyme possesses high affinity for estrone sulfate (E₁S; K_m = 0.8 μ M) and pregnenolone sulfate (PREGS; K_m = 0.6 μ M), while slightly lower affinity was observed for dehydroepiandrosterone sulfate (DHEAS; K_m = 1.7 μ M) and cholesterol sulfate (CHOLS; K_m = 2.0 μ M).¹⁰

The key role of steroid sulfatase is depicted in Figure 1. After cholesterol, DHEAS is the main steroid found in human blood circulation and represents a potential source of steroidal hormones in peripheral tissues.¹² DHEAS is the precursor of the androgens testosterone (T) and dihydrotestosterone (DHT), both known to stimulate tumor growth in patients with prostate cancer.¹ DHEAS is also the precursor of androst-5-ene-3,17 β -diol (Δ^5 -diol), a C19-steroid with a known estrogenic effect.¹³ E₁S is the most abundant precursor of estrogens estrone (E₁) and estradiol (E₂) in women, and the intense activity of steroid sulfatase has been detected in breast tumor tissues.¹⁴ Since androgens and estrogens may be synthesized inside the cancerous cells

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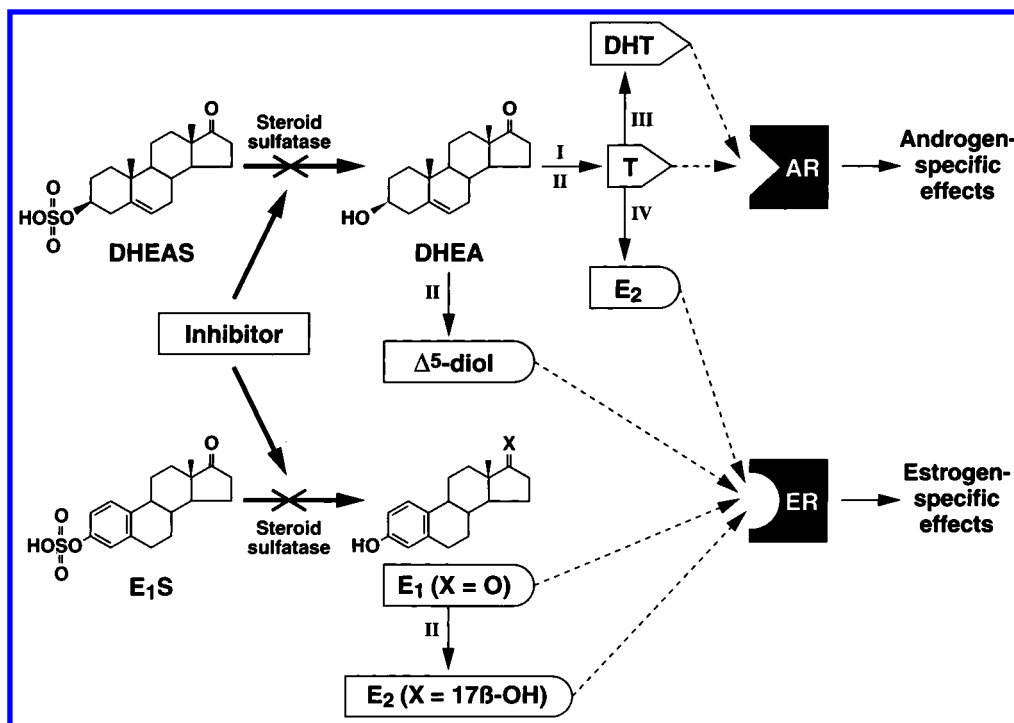


Figure 1. Key role of steroid sulfatase in the transformation of sulfated steroids DHEAS and E₁S into hydroxysteroids DHEA and E₁. Other enzymes: 3 β -dehydrogenase Δ^5, Δ^4 -isomerase (I), 17 β -hydroxysteroid dehydrogenases (II), 5 α -reductases (III), aromatase (IV), androgen receptor (AR), and estrogen receptor (ER).

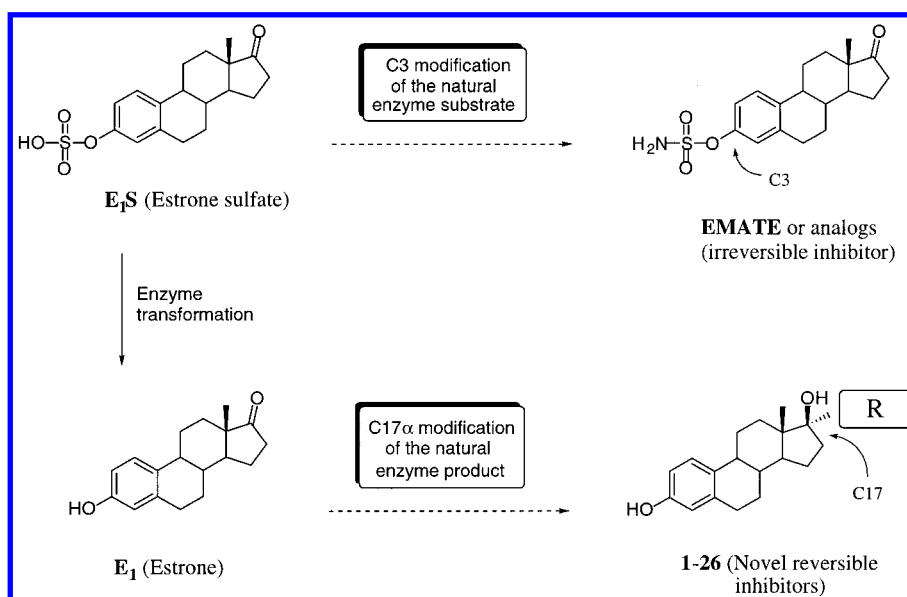


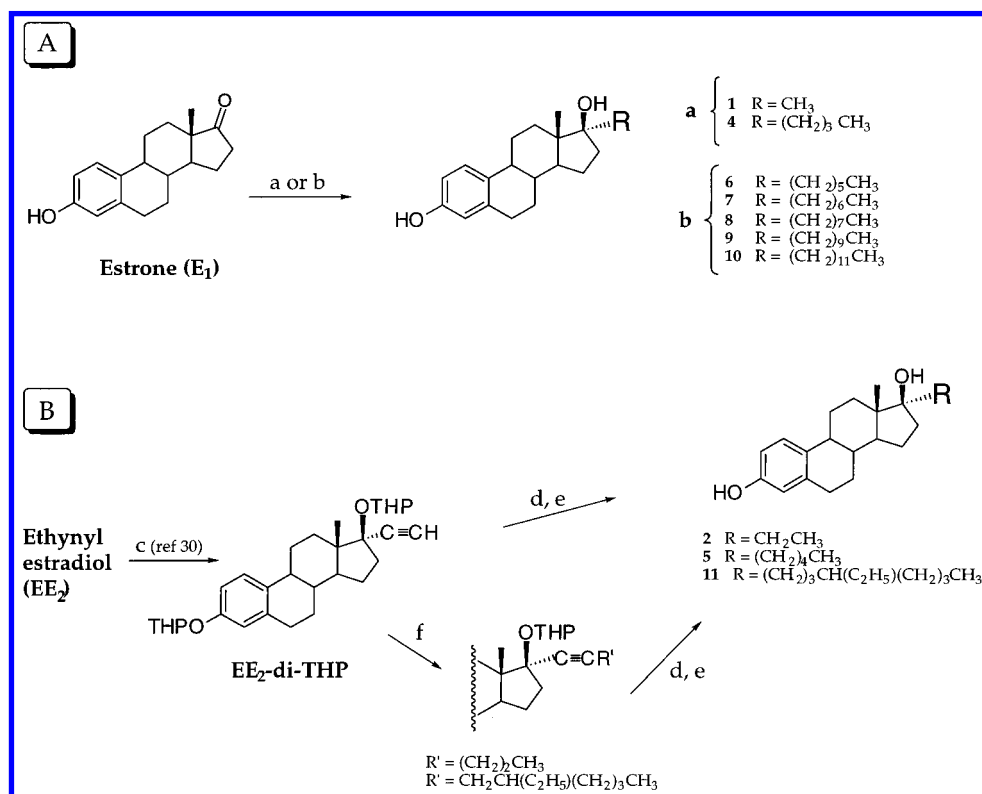
Figure 2. Strategies used to develop two different kinds of steroid sulfatase inhibitors. See Tables 2, 3, and 5 for a representation of R substituents associated with compounds 1–26.

starting from DHEAS and E₁S available in blood circulation,^{12–14a–c,15} the use of therapeutic agents that inhibit steroid sulfatase may prove to be a rewarding approach to the treatment of hormone-sensitive diseases.

Over the past few years, steroidal and nonsteroidal inhibitors of steroid sulfatase have been developed.¹⁶ Most of these inhibitors have in common an aromatic ring substituted at C3 (or pseudo-C3 for nonsteroids) that mimics the phenolic A-ring of the enzyme substrate E₁S (Figure 2). With E₁ as steroid nucleus, a wide variety of chemical groups were introduced at C3 to induce an inhibitory effect, but the most potent was obtained with the sulfamate group, OSO₂NH₂, and

estrone sulfamate (EMATE) was found to efficiently inhibit steroid sulfatase activity in a time-dependent manner.¹⁷ This potent inactivating group was later added to nonsteroidal nuclei such as tetrahydronaphthol,^{17d} coumarin,¹⁸ diethylstilbestrol,^{16b} and *N*-alkanoyl-tyramine¹⁹ and more recently to other steroidal nuclei including equilin,²⁰ equilenin,²⁰ 17 β -(alkylamido)estra-1,3,5(10)-trienes,²¹ 17 β -(*N*-alkylcarbamoyl)estra-1,3,5(10)-trienes,²¹ A-ring-modified estrone,²² and 17 α -benzylestradiol.²³

During our studies on the development of antiestrogens,²⁴ and type 1 17 β -hydroxy steroid dehydrogenase inhibitors,²⁵ we had to synthesize a series of 17 α - and 16 α -derivatives of estradiol. Some of these compounds

Scheme 1^a

^a (a) RLi, THF; (b) *t*-BuLi, RI, *n*-pentane/Et₂O, THF; (c) dihydropyran, *p*-TSA·H₂O, benzene; (d) H₂, Pd/C (10%), EtOH; (e) *p*-TSA·H₂O, MeOH; (f) *n*-BuLi, R'Br, HMPA, THF.

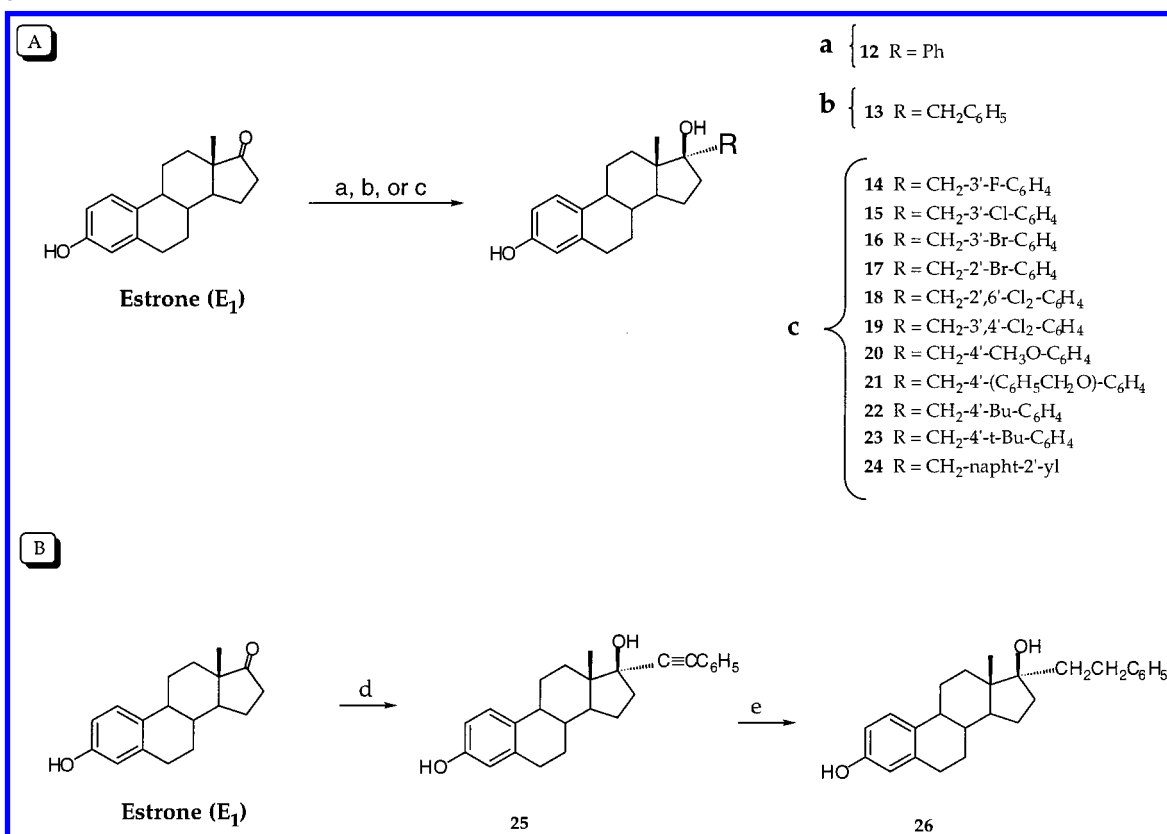
were also tested for their capacity to inhibit the steroid sulfatase. The results of this preliminary SAR study guided our work toward the development of steroid sulfatase inhibitors, and we have reported a preliminary account.²⁶ We now report a full account of the chemical synthesis and the SAR study of this new family of steroid sulfatase inhibitors (Figure 2). In contrast to the known steroid sulfatase inhibitor EMATE that contains an inactivating sulfamate group at position C3 of the estrone A-ring, the newly reported inhibitors have a substituent located at another position (namely, C17 α of the estradiol D-ring) that inhibits reversibly the steroid sulfatase.

Results and Discussion

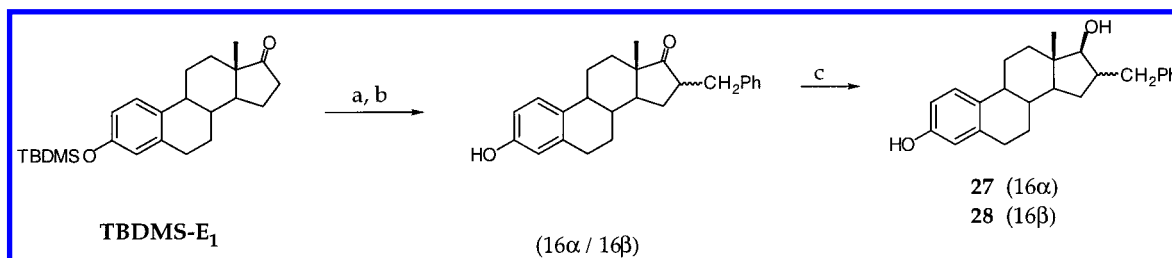
Chemistry. The addition of organomagnesium or organolithium reagents to the C17-carbonyl of E_1 (Schemes 1A and 2) was the main approach used to introduce most of the substituents needed for our SAR study. Since the methyl-18 on the β -face of the steroid directs the nucleophilic attack at the less hindered steroidal α -face, such alkylations of a C17-keto steroid are known to be stereoselective.²⁷ Starting from estrone (E_1), we then obtained almost exclusively the 17 α -alkylation product with unreacted steroid as the only other detectable material. Moderate yields of the alkylated compound ranging from 12% to 91% (average yield = 48%) were obtained, but these yields were not optimized or corrected for the recovery of starting material (E_1). We observed that the low solubility of the phenolate species generated during the reaction prevented the completion of the alkylation process and may be partly responsible for the moderate yields of alkylated products. Higher yields of alkylation (>85%) were

obtained, however, when the phenol was protected as a *tert*-butyldimethylsilyl ether, but this protection/deprotection approach (two more steps) was deemed too time-consuming for the improvement in overall yield obtained. Another pathway (Scheme 1B) was also used to elaborate the C17 α -substituent. In this strategy, the lithium acetylenide generated from di-THP-protected ethynylestradiol ($EE_2\text{-di-THP}$) was added to an alkyl halide to give the corresponding alkyne, which was reduced by catalytic hydrogenation. On the other hand, C16-derivatives of estradiol (Scheme 3) were obtained after enolization of the C17-ketone of TBDMS- E_1 with LDA and addition of benzyl bromide followed by a stereoselective reduction of ketone.

1. Synthesis of 17 α -Alkylestradiols (Scheme 1). The reaction of a hindered ketone, such as C17-keto steroid, with a Grignard reagent having a β -hydrogen (alkyl-MgX) affords mainly the product of carbonyl reduction and generally a slight amount of alkylated product. Although an efficient methodology using Ce(III)Cl₃ and RMgX has been recently described by Li et al.,²⁸ we used the addition of an alkyl lithium reagent (alkyl-Li) for the synthesis of compounds **1**, **4**, and **6–10**. The primary alkyl lithium was generated in situ by the lithium–iodine exchange method (*t*-BuLi, *n*-pentane/diethyl ether) described by Bailey and Punzalan.²⁹ Products **6–10** were obtained from E_1 with alkyl lithiums generated by this methodology, while products **1** and **4** were obtained with commercially available alkyl lithiums (MeLi and *n*-BuLi). The yields of alkylated product range between 12% and 77%, while starting E_1 was the only other detectable product. As above, the yields were not optimized or corrected for the E_1 recovered.

Scheme 2^a

^a (a) C₆H₅Li, THF; (b) C₆H₅CH₂MgCl, THF; (c) RBr, Mg, diethyl ether, THF; (d) RC \equiv CH, *n*-BuLi, HMPA, THF; (e) H₂, Pd/C (10%), EtOH.

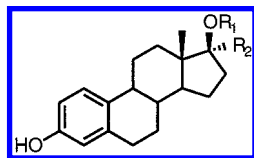
Scheme 3^a

^a (a) LDA, PhCH₂Br, THF; (b) HCl (2%), MeOH; (c) LiAlH₄, THF.

Compounds **2**, **5**, and **11** were synthesized by another approach using the di-THP derivative of 17 α -ethynylestradiol (EE₂-di-THP),³⁰ as starting material. Thus, 17 α -ethyl-E₂ (**2**) was easily obtained by catalytic reduction (H₂, 10% Pd/C) of EE₂-di-THP following by cleavage of THP groups with *p*-toluenesulfonic acid in MeOH in 74% yield for the two steps. Synthesis of compounds **5** and **11** was performed in a three-step sequence where pentyl bromide or 2-ethylhexyl bromide was first added to the lithium acetylide generated from EE₂-di-THP giving the corresponding alkylated product. Thereafter, the triple bond of each compound was reduced as above by catalytic hydrogenation, and THP protecting groups were removed to afford **5** and **11** in overall yields of 59% and 11%, respectively. This indirect three-step approach represents an alternative to the one-step strategy that yields directly 17 α -alkylestradiols.

2. Synthesis of 17 α -(Phenyl, benzyl, or substituted benzyl)estradiols (Scheme 2). The addition of commercially available phenyl-MgX to E₁ (protected or not as TBDMS) failed to afford 17 α -phenylestradiol (**12**).

This compound was alternatively obtained in 68% yield by using a commercially available solution of phenyllithium as described above for compounds **1** and **4**. Contrary to the alkyl series, it was possible to introduce a benzyl or a substituted benzyl group at position 17 α of the hindered keto steroid by a Grignard reaction without formation of reduction product. Except for the benzyl Grignard reagent, which is commercially available, appropriate Grignard reagents were formed in dry diethyl ether by a standard procedure (Mg, RX, heat activation). A solution of E₁ in dry THF was then added at low temperature to excess Grignard reagent generated in situ. Only the alkylated compound and the unreacted E₁ were generally observed at the end of reaction. To facilitate the chromatographic separation of alkylated product from remaining E₁ (closely similar *R_f* on TLC), a quantitative reduction of E₁ to E₂ was carried out with NaBH₄ (MeOH, 0 °C). After this additional step and purification, the yields of 17 α -alkylestradiols **13**–**24** varied between 22% and 91%. Alternatively, compound **25** was obtained by

Table 1. Inhibition of Steroid Sulfatase Activity of JEG Cells by a Series of 17 α -Derivatives of Estradiol (transformation of [³H]E₁S to [³H]E₁)^a

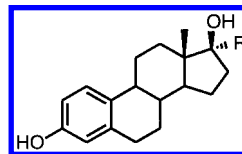
R ₁	R ₂	functional group	% inhib at 20 μ M
H	CH ₂ CH(O)CH ₂	epoxide (more polar on TLC)	27
H	CH ₂ CH(O)CH ₂	epoxide (more polar on TLC)	38
H	(CH ₂) ₃ OH	alcohol	24
H	C \equiv CCH ₂ OH	alcohol	9
H	C \equiv CCH ₂ Br	bromide	59
H	(CH ₂) ₂ CONBuMe	alkylamide	86
H	(CH ₂) ₂ CH ₃ (3)	propyl	75
H	CH ₂ CH=CH ₂	allyl	80
TBDMS ^b	CH ₂ CH=CH ₂	allyl	0
	–CO CH ₂ CH ₂ –	lactone	57
	–CH ₂ CH ₂ CH ₂ –	cycloether	28
E ₁ S	(enzyme substrate)		12
danazol ^c			30

^a Compounds were available in our laboratory and synthesis already reported.^{25b} ^b TBDMS, *tert*-butyldimethylsilyl group or (CH₃)₃CSi(CH₃)₂–. ^c Danazol, 17 α -pregna-2,4-dien-20-yn[2,3-*d*]isoxaz-17-ol.³³

adding to E₁ the lithium acetylide generated from phenylacetylene and *n*-BuLi. The triple bond of **25** was reduced by catalytic hydrogenation (H₂, 10% Pd/C) to give the saturated analogue **26**.

3. Synthesis of 16-Benzylestradiols (Scheme 3). Starting from TBDMS-E₁, the alkylation at position 16 was accomplished through formation of lithium enolate with LDA and addition of benzyl bromide. After cleaving the TBDMS group (2% HCl in MeOH), 16-benzylestrone was obtained as a mixture of two isomers in proportions of 3:1 (16 α : 16 β by NMR).^{25d} Reduction of the carbonyl group with lithium aluminum hydride at –78 °C gave the corresponding alcohols **27** (16 α -benzyl) and **28** (16 β -benzyl), and this mixture was resolved by flash chromatography. As already reported, the reduction was highly stereoselective (by the α -steroidal face) giving the 17 β -orientation of the hydroxy group (17 β -OH).³¹ Since the C17 α -proton and C17-carbon give very characteristic NMR signals according to the orientation of C16-benzyl group,³² the C16 and C17 stereochemistries of compounds **27** and **28** were established unambiguously.

Structure–Activity Relationships. The ability of the synthesized compounds to inhibit steroid sulfatase activity was assayed with homogenized JEG-3 cells by measuring the [³H]E₁ from the enzyme substrate [³H]-E₁S. The results were expressed as the percentage of inhibition (at an inhibitor concentration of 20 μ M) or as IC₅₀ values. A variety of C18-steroids available in our laboratory were first screened for steroid sulfatase inhibition, and some 17 α -estradiol derivatives (Table 1) did show inhibition. In fact, estradiol derivatives such as *N*-methyl-*N*-butylpropanamide, propyl, and allyl (86%, 75%, and 80% of inhibition, respectively), having hydrophobic groups, were better inhibitors of steroid sulfatase than those with hydrophilic groups such as epoxide and alcohol (9–38%). The lower activity of the trialkylsilyl and cyclic ether derivatives (0% and 28%,

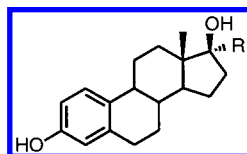
Table 2. Inhibition of Steroid Sulfatase Activity of JEG Cells by 17 α -Alkylestradiols (transformation of [³H]E₁S to [³H]E₁)

compd	R	substituent	IC ₅₀ (nM)
E ₂ (estradiol)	H		84000
1	CH ₃	methyl	19600
2	CH ₂ CH ₃	ethyl	14400
3	(CH ₂) ₂ CH ₃	propyl	5640
4	(CH ₂) ₃ CH ₃	butyl	3490
5	(CH ₂) ₄ CH ₃	pentyl	1980
6	(CH ₂) ₅ CH ₃	hexyl	930
7	(CH ₂) ₆ CH ₃	heptyl	780
8	(CH ₂) ₇ CH ₃	octyl	440
9	(CH ₂) ₉ CH ₃	decyl	≈1000
10	(CH ₂) ₁₁ CH ₃	dodecyl	≈6000
11	(CH ₂) ₃ CH(C ₂ H ₅)(CH ₂) ₃ CH ₃	4-ethyloctyl	520
E ₁ S	(unlabeled enzyme substrate)		7600

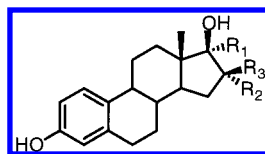
respectively) suggests that the free 17 β -OH is important but equally suggests that a polar substituent or a less bulky group is important. When compared to the first reported steroid sulfatase inhibitor, danazol,³³ and the unlabeled substrate E₁S itself, which gave respectively 30% and 12% of inhibition, estradiol derivatives with a 17 α -hydrophobic side chain were more efficient inhibitors (75–86%) suggesting an interaction between the substituent and the enzyme. It was then decided to optimize these results by preparing a series of hydrophobic derivatives. The optimization of a 17 α -alkylamide derivative of estradiol also resulted in significant inhibition of steroid sulfatase activity and has been reported elsewhere.³⁴

Focusing on alkyl derivatives, a series of 17 α -(saturated alkyl)estradiols **1–10** (Table 2) was prepared to determine the length of the side chain giving the optimal inhibition of steroid sulfatase. The importance of 17 α -chain length and hydrophobicity on steroid sulfatase inhibition is shown by the increased inhibition observed reaching a maximum at C8. To increase the hydrophobicity of the alkyl side chain without increasing its length by too much, we synthesized an estradiol derivative, 17 α -(4'-ethyloctyl)estradiol (**11**), having a branched octyl side chain. With a IC₅₀ of 520 nM, compound **11** produced approximately the same inhibition as the linear octyl analogue **8** (IC₅₀ = 440 nM). However, when considering the entire number of carbons and type of the side chain, compound **11** (10 carbons; branched side chain) was a more efficient inhibitor than compound **9** (10 carbons; linear side chain). These observations brought us to the conclusion that compounds having side chains of eight carbons were the best inhibitors of steroid sulfatase activity (17-fold better than E₁S itself). Moreover, the use of a branched alkyl side chain (**11** vs **9**) increased the ability of the compound to inhibit the steroid sulfatase activity.

In our preliminary screening study (Table 1), we observed that 17 α -allylestradiol also inhibited the steroid sulfatase activity (80% at 20 μ M). We followed this by synthesizing 17 α -benzylestradiol (**13**), knowing that the benzyl group contains an allylic portion and is more hydrophobic and less sterically restrictive with its total of seven carbons than would be a linear heptyl side

Table 3. Inhibition of Steroid Sulfatase Activity of JEG Cells by 17 α -Arylestradiols (transformation of [³H]E₁S to [³H]E₁)

compd	R	substituent	IC ₅₀ (nM)
12	Ph	phenyl	3820
13	CH ₂ Ph	phenylmethyl or benzyl	310
26	CH ₂ CH ₂ Ph	phenylethyl	620
25	C \equiv CPh	phenylethynyl	1020
E ₁ S		(unlabeled enzyme substrate)	7600

Table 4. Inhibition of Steroid Sulfatase Activity of JEG Cells According to the Position of Benzyl Group (transformation of [³H]E₁S to [³H]E₁)

compd (benzyl position)	R ₁	R ₂	R ₃	% inhib at 20 μ M
13 (17 α)	benzyl	H	H	97
27 (16 α)	H	benzyl	H	60
28 (16 β)	H	H	benzyl	62

chain. After obtaining promising results with the benzyl group, we decided to optimize the spacer between the aromatic moiety and the steroidal backbone (Table 3). The benzyloestradiol derivative **13** (IC₅₀ = 310 nM) inhibited the enzyme better than the phenyl analogue **12** (IC₅₀ = 3820 nM) or the phenylethyl analogue **26** (IC₅₀ = 620 nM). A spacer of one methylene (CH₂) between the aromatic group and the steroid was eventually found to give the best inhibition. We also observed that a too rigid spacer such as ethyne (C \equiv C) decreased the inhibiting activity (compare **26** and **25**).

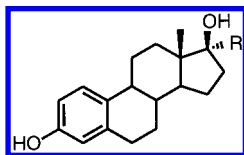
The results of Tables 2 and 3 prompted us to select the benzyl group for further studies. However, before pursuing the optimization, we tested the effect of placing the benzyl group on the neighboring C16-carbon. In Table 4, we can see that the benzyl group at position 17 α gave a higher inhibition of steroid sulfatase than did the benzyl group at position 16; we obtained 60% and 62% of inhibition, respectively, for 16 α -benzyloestradiol (**27**) and 16 β -benzyloestradiol (**28**) compared to 97% for 17 α -benzyloestradiol (**13**). These results confirmed that position 17 α is preferable to position 16 with regard to inhibition of steroid sulfatase.

In the next step, we synthesized a series of estradiol derivatives bearing various substituted benzyls at position 17 α (compounds **14**–**24**) (Table 5). The 17 α -(3-halogenobenzyl)estradiols **14**–**16** gave strong inhibition of steroid sulfatase (IC₅₀ = 130, 110, and 24 nM, respectively for fluoro, chloro, and bromo derivatives), with the 3-bromobenzyl derivative **16** being a 12-fold more potent inhibitor than the lead unsubstituted benzyl derivative **13**. This result suggests that a hydrophobic group can augment the inhibitory activity of 17 α -derivatives of estradiol. It is indeed known that halogen atoms increase the hydrophobicity in the order: bromide

> chloride > fluoride. The 17 α -(2-bromobenzyl)estradiol (**17**) was also synthesized to examine the effect of bromide positioning. It was found that an *ortho* positioning of the bromide atom on the benzyl group inhibited steroid sulfatase activity (IC₅₀ = 840 nM) less than a *meta* bromo substituent (IC₅₀ = 24 nM) or no substituent at all (IC₅₀ = 310 nM). We were unable to synthesize the *para* bromobenzyl analogue because the methodologies using Grignard reagent or SmI₂ failed, although it would have been useful for the sake of comparison. Suspecting the negative steric effect of an *ortho* benzyl substitution and a positive hydrophobic effect of a dihalogenated benzyl derivative, we prepared two dichlorobenzyl derivatives (compounds **18** and **19**). Clearly, a 3,4(*meta,ortho*)-disubstitution was preferable to a 2,6(*di-ortho*)-substitution (IC₅₀ = 80 and 640 nM, for **19** and **18**, respectively). In addition, the 3,4-dichlorobenzyl derivative **19** was a slightly better inhibitor than the monochlorinated analogue **15**.

To optimize the inhibiting effect of the substituted benzyl group, we synthesized five estradiol derivatives (compounds **20**–**24**). A *para* methoxy substitution on benzyl increased the inhibiting potency by only a factor of 3 (IC₅₀ = 110 nM), but this factor increased to 14 (IC₅₀ = 22 nM) when a more hydrophobic substituent such as a *para* benzyloxybenzyl was used. Similar inhibiting potency was also obtained with the addition of either a *para* butylbenzyl or a *para tert*-butylbenzyl at position 17 α of estradiol (IC₅₀ = 25 and 28 nM for **22** and **23**, respectively). Finally, 17 α -(2-naphthylmethyl)estradiol (**24**) exerted good inhibition of steroid sulfatase (IC₅₀ = 120 nM) suggesting that benzyl *meta* and *para* positions can be substituted by an aromatic ring. Compound **24** was however less potent inhibitor than 4-substituted benzyl derivatives **21**–**23** and 3,4-disubstituted analogue **19**.

Inhibitor Potency According to Enzyme Substrate (E₁S or DHEAS). The SAR study reported above was performed with homogenized JEG cells as the source of steroid sulfatase, the transformation of a low concentration (7 nM) of enzyme substrate [³H]E₁S to desulfated product [³H]E₁ serving as the observable reaction. From this study, two compounds representing the new family of steroid sulfatase inhibitors were selected for further enzymatic assays. In addition to 17 α -benzyloestradiol (**13**) and 17 α -*tert*-butylbenzyloestradiol (**23**), the most potent known inhibitor of steroid sulfatase reported at the time of our experiment (EMATE) was included for comparison. The two substrates, [³H]E₁S and [¹⁴C]DHEAS, used at a concentration of 100 μ M and a new source of steroid sulfatase activity (homogenized human embryonic kidney (HEK-293) cells transfected with a sulfatase expression vector) were used in these experiments (Table 6).²³ With labeled E₁S as substrate, the *tert*-butylbenzyl group was found to provide better inhibition (18-fold) than the benzyl group (IC₅₀ = 12 and 220 nM, for **23** and **13**, respectively). Compound **23** was however a 7-fold less potent inhibitor than EMATE. Similarly, when DHEAS was used as substrate, compound **23** was about 5-fold less potent than EMATE. Compound **23** also inhibited the steroid sulfatase transformation of both natural substrates, E₁S and DHEAS, supporting the hypothesis of a unique steroid sulfatase.

Table 5. Inhibition of Steroid Sulfatase Activity of JEG Cells by 17 α -(Substituted benzyl)estradiols (transformation of [³H]E₁S to [³H]E₁)

compd	R	substituent	IC ₅₀ (nM)
13	CH ₂ Ph	benzyl	310
14	CH ₂ Ph-3'-F	3'-fluorobenzyl	130
15	CH ₂ Ph-3'-Cl	3'-chlorobenzyl	110
16	CH ₂ Ph-3'-Br	3'-bromobenzyl	24
17	CH ₂ Ph-2'-Br	2'-bromobenzyl	840
18	CH ₂ Ph-2',6'-Cl ₂	2',6'-dichlorobenzyl	640
19	CH ₂ Ph-3',4'-Cl ₂	3',4'-dichlorobenzyl	80
20	CH ₂ Ph-4'-OCH ₃	4'-methoxybenzyl	110
21	CH ₂ Ph-4'-OCH ₂ Ph	4'-benzyloxybenzyl	22
22	CH ₂ Ph-4'-Bu	4'-butylbenzyl	25
23	CH ₂ Ph-4'- <i>t</i> -Bu	4'- <i>tert</i> -butylbenzyl	28
24	CH ₂ C ₁₀ H ₇	naphth-2'-ylmethyl	120
E ₁ S	(unlabeled enzyme substrate)		7600

Table 6. Inhibition of Steroid Sulfatase Activity of Transfected HEK-293 Cells by Compounds **13**, **23**, and EMATE According to the Labeled Substrate

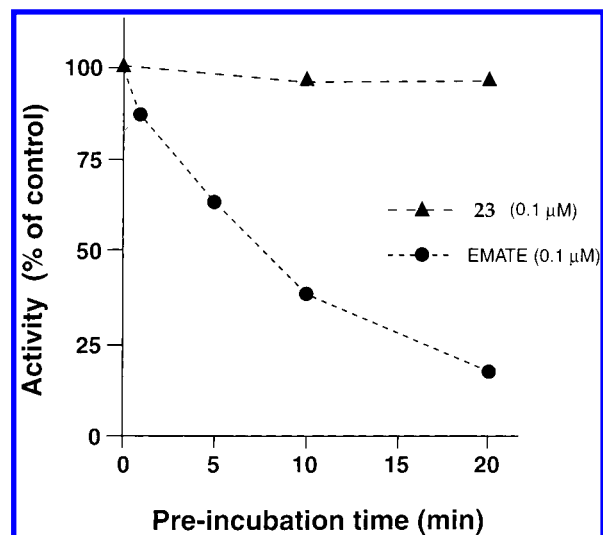
compd	name	IC ₅₀ (nM) ^a	
		[³ H]E ₁ S to [³ H]E ₁	[¹⁴ C]DHEAS to [¹⁴ C]DHEA
13	17 α -benzylestradiol	220 \pm 40	325 \pm 60
23	17 α -(4'- <i>tert</i> -butylbenzyl)-estradiol	12 \pm 3	13 \pm 4
EMATE	3-sulfamoyl estrone	1.6 \pm 0.2	2.4 \pm 0.2

^a The *K_m* values were previously determined and reported as 15 and 19 μ M, respectively, for enzyme substrates E₁S and DHEAS.²³

Mechanism of Inhibition. Using compound **23** as a typical inhibitor of the newly developed steroid sulfatase inhibitors, we wished to verify the nature of the inhibition of such inhibitors. A time-dependent inactivation experiment was then performed using microsomes of HEK-293 cells transfected with a sulfatase expression vector and labeled E₁S as the substrate.²³ As illustrated in Figure 3, the known inactivator EMATE irreversibly inhibited the activity of steroid sulfatase since the enzyme activity could not be restored after the enzyme inhibitor was eliminated through a dextran-coated charcoal treatment. In contrast, 17 α -*tert*-butylbenzylestradiol (**23**) did not inactivate the enzyme because the activity was almost completely restored after the dextran-coated charcoal treatment. Clearly, compound **23** and EMATE do not work in the same manner, suggesting that **23** is a reversible inhibitor of steroid sulfatase. This result concurs with the chemical structure of compound **23** and analogues, which do not permit the formation of a covalent bond with the enzyme. On the other hand, it was known that EMATE can inactivate the enzyme by generating a reactive species originating from the sulfamate group.¹⁷ Thus, compound **23** and related compounds inhibit the steroid sulfatase by a mechanism reversible and different from the irreversible mechanism of EMATE.

Conclusion

Steroid sulfatase inhibitors are showing promise as therapeutic agents against estrogen- and androgen-

**Figure 3.** Time-dependent inactivation of steroid sulfatase activity ([³H]E₁S to [³H]E₁) by known inactivator EMATE and 17 α -(4'-*tert*-butylbenzyl)estradiol (**23**). The enzyme activity of the control (100%) was 0.49 nmol/h/mg of protein. Full details of this experiment were already reported.²³

dependent diseases. Since the report of the first inhibitor, danazol,³³ several steroidal and nonsteroidal inhibitors have been developed,¹⁶ most having the common characteristic of an aromatic ring substituted at C3 (or pseudo-C3 for nonsteroids) that mimics the phenolic A-ring of the enzyme substrate E₁S. We have shifted the focus to an alternative steroidal position and have uncovered a new family of steroid sulfatase inhibitors, easily obtained by adding a substituent at position 17 α of the D-ring estradiol (compounds **1–26**). Indeed, we observed that a hydrophobic substituent induces powerful inhibition of steroid sulfatase while a hydrophilic one was weak. Although a hydrophobic group at the 17 α -position increased the inhibitory activity, the steric factors contribute to the opposite effect. As exemplified by 17 α -decylestradiol (**9**) and 17 α -dodecylestradiol (**10**), a long flexible side chain prevents adequate fitting into the enzyme catalytic site, thus decreasing capacity to inhibit the steroid sulfatase activity. In the alkyl series, the best compromise between hydrophobicity and steric hindrance was obtained with the octyl group (**8**; IC₅₀ of 440 nM), but judicious branching of the side chain could improve this further. Compounds bearing a substituted benzyl group at position 17 α of estradiol were however the most potent inhibitors of steroid sulfatase that we obtained. The inhibitory effect of the benzyl group was increased by adding a halogen, an alkyl, or an alkyloxy directly to the benzyl nucleus. Among the synthesized compounds, strong inhibition was obtained with 17 α -(4'-*tert*-butylbenzyl)estradiol (**23**), 17 α -(4'-butylbenzyl)estradiol (**22**), 17 α -(3'-bromobenzyl)estradiol (**16**), and 17 α -(4'-benzyloxybenzyl)estradiol (**21**) with IC₅₀ values of 28, 25, 24, and 22 nM, respectively. Although these inhibitors are about 100-fold more potent than enzyme substrate E₁S, (used itself as inhibitor), they are still less potent than EMATE. We believe, however, that carefully combining a halogen in the *meta* position and an alkyl or alkyloxy group in the *para* position would produce better inhibitors. Interestingly, the newly reported family of inhibitors acts by a reversible mecha-

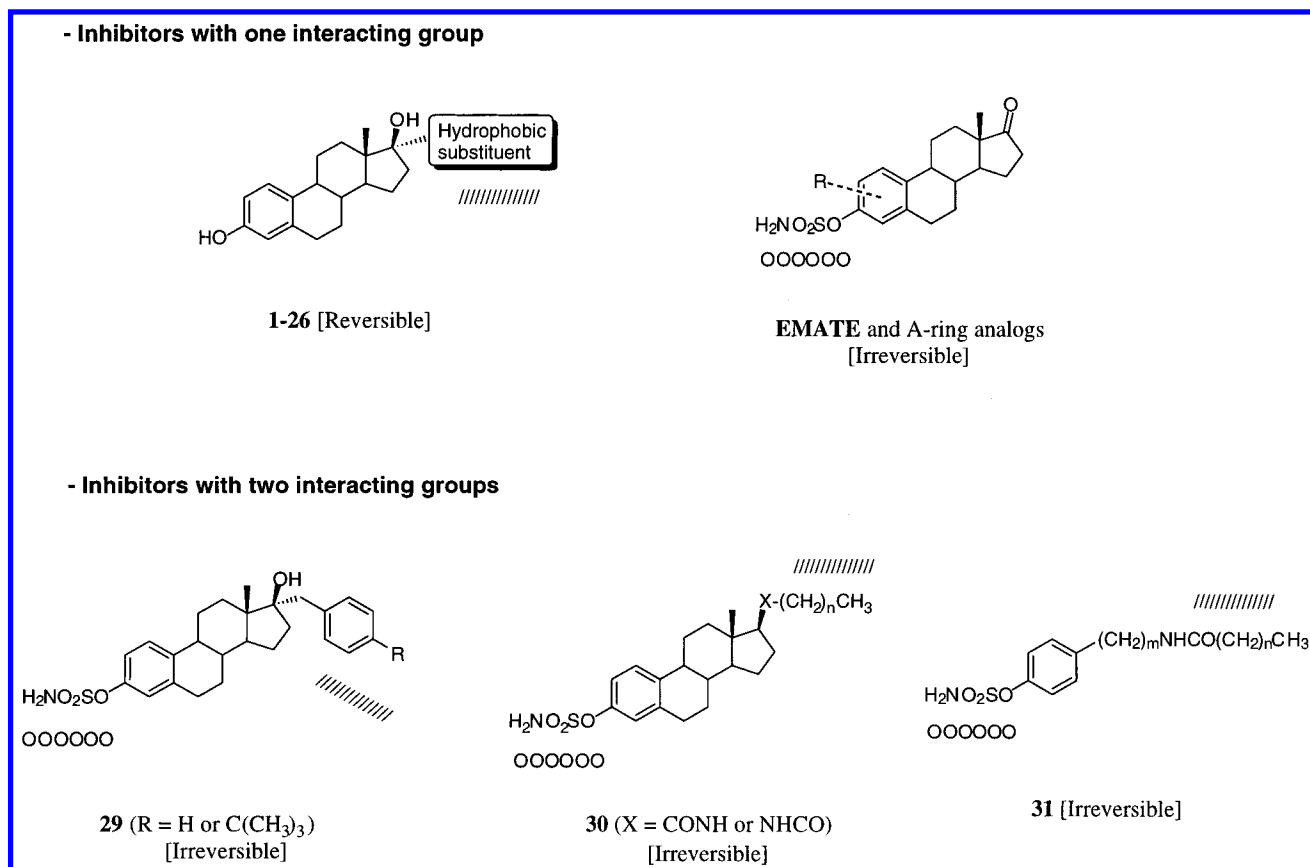


Figure 4. Chemical structures of newly reported inhibitors (**1–26**) and potent known inhibitors (EMATE, **29–31**) of steroid sulfatase indicating their major interacting groups (hydrophobic $////$ or alkylating $OOOOOO$) and their mechanism of action (reversible or irreversible). Other minor interactions with steroid skeleton, carbonyl group, and hydroxyl group are also possible.

nism of action that is different from the irreversible mechanism of EMATE.

Our study has clearly shown the inhibitory effect of a hydrophobic substituent (alkyl side chains or benzyl derivatives) added to position 17α of estradiols (compounds **1–26**). Thus, as exemplified by compound **23**, a *tert*-butylbenzyl group increases the ability of the estradiol nucleus to inhibit reversibly the steroid sulfatase activity by 3000-fold. We took advantage of this fact by preparing estradiol derivatives that contain a 17α -hydrophobic substituent and a 3-sulfamoyl group (compound **29**) (Figure 4).²³ Such compounds were about 1.4–14-fold more potent inhibitors of steroid sulfatase than an estrone analogue having only a sulfamate group at position C3,²³ suggesting a significant role for the 17α -substituent in the enzyme inhibiting process. In addition two families of potent steroid sulfatase inhibitors, recently developed by Li and colleagues,^{19,21} strongly suggest the presence of a potential hydrophobic region in the enzyme neighboring the D-ring of steroid substrates. In the first family of inhibitors, represented by compound **30**,²¹ a hydrophobic side chain can be found at position 17β of estradiol sulfamate. In the second family, potent nonsteroidal inhibitors were obtained by adding a hydrophobic side chain to a sulfamoylated phenol derivative (compound **31**).¹⁹ An aryl sulfamate group is certainly the most important requirement for a potent inhibitor of steroid sulfatase;^{16,35} however, hydrophobic interaction in the region neighboring the D-ring of the steroid could represent an additional factor with regard to the irreversible inhibitors **29–31** and,

of course, the series of reversible inhibitors **1–26** described above.

Experimental Section

A. Chemical Synthesis. 1. General Procedure. Chemical reagents and starting steroids (estrone and ethynylestradiol) were purchased from Aldrich Chemical Co. (Milwaukee, WI), and solvents were obtained from BDH Chemicals (Montréal, Canada). Thin-layer chromatography (TLC) was performed on 0.20-mm silica gel 60 F₂₅₄ plates (E. Merck, Darmstadt, GE), and compounds were visualized by exposure to UV light or with a solution of ammonium molybdate/sulfuric acid/water (with heating). Purification of compounds were performed by flash-column chromatography using 230–400 mesh ASTM silica gel 60 (E. Merck). Infrared spectra (IR) were obtained with a Perkin-Elmer 1600 spectrophotometer and data expressed in cm^{-1} . ^1H and ^{13}C NMR spectra were recorded with a Bruker AC/F 300 spectrometer at 300 and 75 MHz, respectively. The chemical shifts (δ) were expressed in ppm and referenced to chloroform (7.26 and 77.00 ppm), acetone (2.06 and 206.26), and methanol (3.30 and 49.00 ppm), respectively for ^1H and ^{13}C NMR. The ^{13}C NMR signal assignments of several 17α -estradiol derivatives have already been reported by us.³⁶ Low-resolution mass spectra (LRMS) were recorded with a VG Micromass 16F spectrometer or a PE Sciex API-150ex spectrometer. Elemental analyses (C, H, X) were carried out by Le Laboratoire d'Analyse Élémentaire de l'Université de Montréal (Montreal, Canada) or Robertson Microlit Laboratories Inc. (Madison, NJ).

2. Preparation of 17α -Alkylestradiols (Scheme 1). 2.1. General Procedure for Alkylation with Commercially Available Alkylolithium (synthesis of **1 and **4**).** Alkyl-lithium (MeLi or *n*-BuLi) (4.5 or 3.2 equiv) was added dropwise to a stirred solution of **E₁** in dry THF at 0 °C and under an argon atmosphere. After 5 h, the reaction mixture was poured

into water, neutralized with 2 N HCl and extracted with EtOAc. The combined extracts were washed with saturated aqueous NaHCO₃ and brine, dried over MgSO₄, and evaporated to dryness at reduced pressure. The residue was purified by chromatography (hexane/EtOAc, 70:30). The yields were not corrected for unreacted E₁.

3,17 β -Dihydroxy-17 α -methylestra-1,3,5(10)-triene (1): white solid (77% yield); IR ν (film) 3300 (OH); ¹H NMR δ (CDCl₃) 0.90 (s, 3H, 18-CH₃), 1.28 (s, 3H, 17 α -CH₃), 2.83 (m, 2H, 6-CH₂), 4.80 (br, 1H, OH phenol), 6.57 (d, J = 2.4 Hz, 1H, 4-CH), 6.62 (dd, J_1 = 2.7 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 7.16 (d, J = 8.4 Hz, 1H, 1-CH); ¹³C NMR δ (acetone-*d*₆) 14.59, 23.76, 26.57, 27.30, 28.39, ~30 (under solvent peaks), 32.65, 39.59, 40.84, 44.79, 46.68, 50.60, 81.27, 113.59, 115.93, 127.00, 132.16, 138.46, 155.92; LRMS for [M]⁺ 286 *m/z*. Anal. (C₁₉H₂₆O₂) C, H.

3,17 β -Dihydroxy-17 α -butylestra-1,3,5(10)-triene (4): white solid (56% yield); IR ν (film) 3350 (OH); ¹H NMR δ (CDCl₃) 0.91 (s, 3H, 18-CH₃), 0.95 (t, J = 7.0 Hz, 3H, (CH₂)₃CH₃), 2.83 (m, 2H, 6-CH₂), 4.69 (br, 1H, OH phenol), 6.57 (d, J = 2.5 Hz, 1H, 4-CH), 6.62 (dd, J_1 = 3.0 Hz and J_2 = 8.3 Hz, 1H, 2-CH), 7.15 (d, J = 8.4 Hz, 1H, 1-CH); ¹³C NMR δ (acetone-*d*₆) 14.72, 15.15, 24.26, 24.46, 26.87, 27.44, 28.53, ~30 (under solvent peaks), 32.62, 34.69, 37.52, 41.00, 44.86, 47.72, 50.58, 83.31, 113.67, 116.06, 127.10, 132.29, 138.56, 156.03; LRMS for [M]⁺ 328 *m/z*. Anal. (C₂₂H₃₂O₂) C, H.

2.2. General Procedure for Alkylation with in Situ Generated Alkylolithiums (synthesis of 6–10). **2.2.1. Preparation of Alkylolithiums (stock solution \approx 0.1 M).** Alkylolithiums were prepared at –78 °C under an argon atmosphere by addition of *t*-BuLi (2.1 equiv) to iodoalkane dissolved in dry *n*-pentane/diethyl ether, 3:2 (approximatively 0.1 M). The reaction mixture was stirred for 1 h before use.

2.2.2. Addition of Alkylolithiums to E₁. Alkylolithium (10 equiv from stock solution) was added dropwise into a solution of E₁ dissolved in dry THF at 0 °C. The mixture was allowed to slowly return to room temperature overnight. After addition of water and acidification with 1 N HCl (pH 4–5), the aqueous phase was extracted with EtOAc and the combined organic layer was washed with a saturated solution of NaHCO₃ and brine, dried over MgSO₄, and the solvent was evaporated under reduced pressure. The residue was purified by chromatography with an appropriate mixture of hexane/EtOAc as eluent. The yields were not corrected for unreacted E₁.

3,17 β -Dihydroxy-17 α -hexylestra-1,3,5(10)-triene (6): white solid (64% yield); IR ν (film) 3360 (OH); ¹H NMR δ (CDCl₃) 0.91 (t, J = 7.0 Hz, 3H, (CH₂)₅CH₃), 0.92 (s, 3H, 18-CH₃), 2.83 (m, 2H, 6-CH₂), 5.72 (br, 1H, OH phenol), 6.57 (d, J = 2.4 Hz, 1H, 4-CH), 6.63 (dd, J_1 = 2.7 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 7.14 (d, J = 8.4 Hz, 1H, 1-CH); ¹³C NMR δ (acetone-*d*₆) 14.47, 15.12, 23.47, 24.22, 24.51, 27.40, 28.48, ~30 (under solvent peaks), 31.16, 32.57, 32.92, 34.62, 37.76, 40.94, 44.79, 47.65, 50.51, 83.34, 113.63, 116.02, 127.03, 132.20, 138.48, 155.96; LRMS for [M]⁺ 356 *m/z*. Anal. (C₂₄H₃₆O₂) C, H.

3,17 β -Dihydroxy-17 α -heptylestra-1,3,5(10)-triene (7): white solid (12% yield); IR ν (film) 3370 (OH); ¹H NMR δ (CDCl₃) 0.92 (t, J = 6.9 Hz, 3H, (CH₂)₆CH₃), 0.93 (s, 3H, 18-CH₃), 2.82 (m, 2H, 6-CH₂), 6.60 (d, J = 2.3 Hz, 1H, 4-CH), 6.66 (dd, J_1 = 2.5 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 7.15 (d, J = 8.5 Hz, 1H, 1-CH); ¹³C NMR δ (acetone-*d*₆) 14.52, 15.17, 23.47, 24.28, 24.63, 27.46, 28.55, ~30 (2x, under solvent peaks), 31.51, 32.65, 32.84, 34.73, 37.85, 41.02, 44.88, 47.73, 50.60, 83.36, 113.69, 116.08, 127.12, 132.31, 138.57, 156.04; LRMS for [M]⁺ 370 *m/z*. Anal. (C₂₂H₃₈O₂·0.25H₂O) C, H.

3,17 β -Dihydroxy-17 α -octylestra-1,3,5(10)-triene (8): white solid (30% yield of **8** and 44% of E₁); IR ν (film) 3350 (OH); ¹H NMR δ (CDCl₃) 0.89 (t, J = 6.9 Hz, 3H, (CH₂)₇CH₃), 0.91 (s, 3H, 18-CH₃), 2.83 (m, 2H, 6-CH₂), 5.25 (br, 1H, OH phenol), 6.57 (d, J = 2.4 Hz, 1H, 4-CH), 6.63 (dd, J_1 = 2.6 Hz and J_2 = 8.3 Hz, 1H, 2-CH), 7.15 (d, J = 8.4 Hz, 1H, 1-CH); ¹³C NMR δ (CDCl₃) 14.13, 14.41, 22.70, 23.42, 23.66, 26.38, 27.48, 29.37, 29.68, 29.78, 30.58, 31.60, 31.92, 34.34, 36.78, 39.66, 43.79, 46.72, 49.50, 83.81, 112.71, 115.29, 126.45, 132.64, 138.26, 153.48; LRMS for [M]⁺ 384 *m/z*. Anal. (C₂₆H₄₀O₂) C, H.

3,17 β -Dihydroxy-17 α -decylestra-1,3,5(10)-triene (9): white solid (41% yield); IR ν (film) 3380 (OH); ¹H NMR δ (CDCl₃) 0.89 (t, J = 7.1 Hz, 3H, (CH₂)₉CH₃), 0.91 (s, 3H, 18-CH₃), 2.83 (m, 2H, 6-CH₂), 5.39 (br, 1H, OH phenol), 6.57 (d, J = 2.4 Hz, 1H, 4-CH), 6.63 (dd, J_1 = 2.6 Hz and J_2 = 8.3 Hz, 1H, 2-CH), 7.15 (d, J = 8.4 Hz, 1H, 1-CH); ¹³C NMR δ (CDCl₃) 14.13, 14.41, 22.70, 23.42, 23.66, 26.38, 27.48, 29.35, 29.66 (2x), 29.70, 29.83, 30.58, 31.60, 31.93, 34.33, 36.78, 39.66, 43.79, 46.72, 49.51, 83.84, 112.71, 115.29, 126.44, 132.61, 138.23, 153.51; LRMS for [M]⁺ 412 *m/z*. Anal. (C₂₈H₄₄O₂) C, H.

3,17 β -Dihydroxy-17 α -dodecylestra-1,3,5(10)-triene (10): white solid (45% yield); IR ν (film) 3380 (OH); ¹H NMR δ (CDCl₃) 0.88 (t, J = 6.8 Hz, 3H, (CH₂)₁₁CH₃), 0.90 (s, 3H, 18-CH₃), 2.83 (m, 2H, 6-CH₂), 4.68 (br, 1H, OH phenol), 6.56 (d, J = 2.5 Hz, 1H, 4-CH), 6.62 (dd, J_1 = 2.5 Hz and J_2 = 8.2 Hz, 1H, 2-CH), 7.15 (d, J = 8.3 Hz, 1H, 1-CH); ¹³C NMR δ (acetone-*d*₆) 14.52, 15.18, 23.46, 24.28, 24.61, 27.45, 28.55, ~30 (7x, under solvent peaks), 31.55, 32.63, 32.76, 34.69, 37.82, 40.99, 44.85, 47.70, 50.58, 83.42, 113.67, 116.06, 127.14, 132.21, 138.49, 156.00; LRMS for [MH – H₂O]⁺ 423.5 *m/z*. Anal. (C₃₀H₄₈O₂) C, H.

2.3. Synthesis of 3,17 β -Dihydroxy-17 α -ethylestra-1,3,5(10)-triene (2). Di-THP-ethynylestradiol³⁰ (300 mg, 0.65 mmol) was dissolved in EtOAc containing 20 mg of 10% Pd/C. The reaction mixture was then shaken under an atmospheric pressure of hydrogen. After 5 h, the mixture was filtered on Celite and solvent evaporated under vacuum to give the reduction product. Without purification, the crude di-THP derivative of 17 α -ethylestradiol was dissolved in MeOH (30 mL) and *p*-TSA·H₂O (20 mg) was added. The resulting solution was stirred at room temperature for 2 h. Thereafter, water was added, MeOH was partially evaporated under reduced pressure and the residue was extracted with EtOAc. The organic phase was dried over MgSO₄, filtered, and solvent evaporated under vacuum. The crude product was purified by chromatography (hexane/EtOAc, 75:25) to afford 144 mg (74% yield, 2 steps) of 17 α -ethylestradiol (**2**): white solid; IR ν (film) 3320 (OH); ¹H NMR δ (CDCl₃) 0.93 (s, 3H, 18-CH₃), 1.03 (t, J = 7.2 Hz, 3H, CH₂CH₃), 2.83 (m, 2H, 6-CH₂), 5.66 (br, 1H, OH phenol), 6.58 (d, J = 2.6 Hz, 1H, 4-CH), 6.63 (dd, J_1 = 2.4 Hz and J_2 = 8.3 Hz, 1H, 2-CH), 7.14 (d, J = 8.4 Hz, 1H, 1-CH); ¹³C NMR δ (acetone-*d*₆) 8.37, 15.18, 24.17, 27.40, 28.50, ~30 (2 signals under solvent peaks), 32.54, 33.87, 40.95, 44.83, 47.64, 50.53, 83.37, 113.64, 116.03, 127.05, 132.23, 138.52, 156.01; LRMS for [MH – H₂O]⁺ 283.2 *m/z*. Anal. (C₂₀H₂₈O₂) C, H.

2.4. Synthesis of Compounds 5 and 11. (a) Addition of Lithium Acetylide of di-THP-EE₂ to Alkyl Bromide. In a flame-dried flask under an argon atmosphere, di-THP-ethynylestradiol was dissolved in dry THF and hexamethylphosphoramide (HMPA) (2 equiv) and the solution was cooled at –78 °C before addition of *n*-BuLi (2 equiv). After 2 h, bromopentane or 2-ethylhexane (4 equiv) in dry THF was added at –78 °C and the mixture was allowed to return slowly to room temperature overnight. Brine was added and the mixture was extracted with EtOAc. The organic phase was dried over MgSO₄ and the solvent removed under reduced pressure. The alkyne derivative was purified by chromatography with hexane/EtOAc.

(b) Catalytic Hydrogenation of Alkynes and Cleavage of THP Groups. Each alkyne derivative was dissolved in EtOAc containing 20 mg of 10% Pd/C and the reaction mixture was shaken under an atmospheric pressure of hydrogen. After 5 h, the mixture was filtered on Celite and solvent evaporated under vacuum to give the product of reduction. Without purification, the crude di-THP estradiol derivative was dissolved in MeOH (20 mL) and *p*-TSA·H₂O (20 mg) was added. The resulting solution was stirred at room temperature for 2 h. Then water was added, MeOH was partially evaporated under reduced pressure, and the residue was extracted with EtOAc. The organic phase was dried over MgSO₄, filtered, and solvent evaporated under vacuum. The crude product was purified by chromatography (hexane/EtOAc, 70:30). Neither

yield was corrected for the unreacted di-THP-EE₂ remaining from the first step and recovered as EE₂.

3,17β-Dihydroxy-17α-pentylestra-1,3,5(10)-triene (5): white solid (59% yield, 3 steps); IR ν (film) 3380 (OH); ¹H NMR δ (CDCl₃) 0.91 (s, 3H, 18-CH₃), 0.92 (t, J = 6.7 Hz, 3H, (CH₂)₄CH₃), 2.84 (m, 2H, 6-CH₂), 4.83 (br, 1H, OH phenol), 6.56 (d, J = 2.3 Hz, 1H, 4-CH), 6.62 (dd, J_1 = 2.3 Hz and J_2 = 8.3 Hz, 1H, 2-CH), 7.15 (d, J = 8.3 Hz, 1H, 1-CH); ¹³C NMR δ (acetone-*d*₆) 14.55, 15.17, 23.65, 24.27(2x), 27.46, 28.55, ~30 (under solvent peaks), 32.65, 33.78, 34.72, 37.79, 41.03, 44.88, 47.73, 50.58, 83.32, 113.69, 116.08, 127.12, 132.32, 138.60, 156.05; LRMS for [M]⁺ 342 *m/z*. Anal. (C₂₃H₃₄O₂) C, H.

3,17β-Dihydroxy-17α-(4'-ethyloct-1'-yl)estra-1,3,5(10)-triene (11): white solid (11% yield, 3 steps); IR ν (film) 3330 (OH); ¹H NMR δ (CDCl₃) 0.85 and 0.89 (2t, J = 7.3 Hz, 6H, two CH₃ of alkyl chain), 0.90 (s, 3H, 18-CH₃), 2.80 (m, 2H, 6-CH₂), 6.56 (d, J = 2.6 Hz, 1H, 4-CH), 6.62 (dd, J_1 = 2.7 Hz and J_2 = 8.2 Hz, 1H, 2-CH), 7.15 (d, J = 8.4 Hz, 1H, 1-CH); ¹³C NMR δ (CDCl₃) 10.87 (11.07), 14.16, 14.39, 20.81, 23.14, 23.41, 25.91 (25.96), 26.38, 27.48, 28.99 (29.04), 29.66, 31.62, 32.88 (32.94), 34.22, 34.42, 37.24, 39.09, 39.67, 43.81, 46.72, 49.50, 83.78, 112.70, 115.28, 126.42, 132.62, 138.23, 153.50; LRMS for [M + HCO₂]⁻ 457.2 *m/z*.

3. Preparation of 17α-(Phenyl/substituted benzyl)-estradiols 12–24 (Scheme 2). **3.1. Synthesis of 3,17β-Dihydroxy-17α-phenylestra-1,3,5(10)-triene (12).** 17α-Phenylestradiol (**12**) was prepared from commercially available phenyllithium (1.8 M solution in cyclohexane/ether, 70:30) as described for the preparation of **1** and **4**. Purification was performed by flash chromatography with hexane/EtOAc (90:10) as eluent. Unreacted E₁ was however not recovered: white solid (68% yield); IR ν (film) 3380 (OH); ¹H NMR δ (acetone-*d*₆) 0.60 (td, J_1 = 4.2 Hz and J_2 = 12.9 Hz, 1H of 16-CH₂, anisotropic effect), 1.11 (s, 3H, 18-CH₃), 2.74 (m, 2H, 6-CH₂), 6.50 (d, J = 2.2 Hz, 1H, 4-CH), 6.55 (dd, J_1 = 2.5 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 6.98 (d, J = 8.4 Hz, 1H, 1-CH), 7.18 to 7.47 (m, 5H, Ph), 7.94 (s, 1H, OH phenol); ¹³C NMR δ (acetone-*d*₆) 15.49, 24.91, 27.25, 28.42, ~30 (under solvent peaks), 34.62, 39.09, 40.75, 44.54, 47.84, 49.02, 85.94, 113.57, 115.96, 126.99, 127.11, 127.75 (2x), 128.50 (2x), 132.04, 138.42, 148.19, 155.93; LRMS for [M]⁺ 348 *m/z*. Anal. (C₂₄H₂₈O₂·0.5H₂O) C, H.

3.2. Synthesis of 3,17β-Dihydroxy-17α-benzylestra-1,3,5(10)-triene (13). Estrone (500 mg, 1.85 mmol) in dry THF (50 mL) was stirred under an argon atmosphere and treated at 0 °C with commercially available benzylmagnesium chloride (2.0 M in THF) (5.55 mL, 11.10 mmol) at 0 °C. The reaction mixture was allowed to return at room temperature overnight. Then, a saturated solution of NH₄Cl was added and the solution was extracted with EtOAc. The combined organic layer was washed with brine, dried over MgSO₄, filtered, and solvent evaporated to dryness. The crude mixture of **13** and unreacted E₁ was dissolved in MeOH (50 mL) and NaBH₄ (140 mg, 3.70 mmol) was added at 0 °C. After complete reduction of E₁ to E₂ (estradiol) (2 h), the reaction was quenched with H₂O, MeOH was evaporated under vacuum, and the mixture extracted with EtOAc and treated as above. Purification by chromatography (hexane/EtOAc, 80:20) afforded E₂ (152 mg, 30%) and **13** (428 mg, 64%): white solid; IR ν (film) 3415 (OH); ¹H NMR δ (CDCl₃) 0.97 (s, 3H, 18-CH₃), 2.68 and 2.94 (2d, J = 13.3 Hz, 2H, CH₂Ph), 2.83 (m, 2H, 6-CH₂), 4.52 (br, 1H, OH phenol), 6.58 (d, J = 2.3 Hz, 1H, 4-CH), 6.63 (dd, J_1 = 2.5 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 7.18 (d, J = 8.3 Hz, 1H, 1-CH), 7.25 to 7.35 (m, 5H, CH₂Ph); ¹³C NMR δ (acetone-*d*₆) 15.12, 23.87, 27.29, 28.37, ~30 (under solvent peaks), 32.04, 33.41, 40.92, 43.34, 44.70, 47.90, 50.22, 83.66, 113.57, 115.95, 126.45, 126.99, 128.29 (2x), 132.05 (3x), 138.43, 140.38, 155.88; LRMS for [M]⁺ 362 *m/z*. Anal. (C₂₅H₃₀O₂) C, H.

3.3. Preparation of 17α-(Substituted benzyl)estradiols 14–24 (alkylation with in situ generated Grignard reagent). **3.3.1. Preparation of Grignard Reagents (stock solution ≈ 0.5 M).** Magnesium was added in a dry three-neck flask under an argon atmosphere and activated by heat. After addition of diethyl ether, the system was cooled at 0 °C and a solution of substituted benzyl bromide or chloride in dry

diethyl ether was added dropwise (about 15 min). The cooling bath was removed and the reaction mixture was allowed to stir for 2.5 h before its use.

3.3.2. General Procedure for Addition of Substituted Benzylmagnesium Bromide (or chloride) to E₁. A solution of E₁ dissolved in dry THF was added to Grignard reagent (10 equiv) at 0 °C and the reaction was stirred overnight at room temperature. The reaction mixture was poured into a saturated solution of NH₄Cl, extracted with EtOAc, washed with brine, dried over MgSO₄, and evaporated under reduced pressure. The crude materials were purified by chromatography (hexane/EtOAc) to afford a mixture of alkylated compound and unreacted E₁. This mixture was then dissolved in MeOH and treated with NaBH₄ (2 equiv.) at 0 °C. After complete reduction of E₁ to E₂ (~2 h), the reaction was quenched with H₂O, MeOH was evaporated under vacuum, and the products extracted with EtOAc. The organic phase was dried over MgSO₄ and evaporated under vacuum before purification by chromatography (hexane/EtOAc). Only the alkylated compounds were recovered and the yields were not corrected for E₂ (the reduced form of E₁).

3,17β-Dihydroxy-17α-(3'-fluorobenzyl)estra-1,3,5(10)-triene (14): white solid (35% yield); IR ν (film) 3400 and 3210 (OH); ¹H NMR δ (CDCl₃) 0.98 (s, 3H, 18-CH₃), 2.66 and 2.94 (2d, J = 13.3 Hz, 2H, CH₂PhF), 2.84 (m, 2H, 6-CH₂), 4.60 (br, 1H, OH phenol), 6.58 (d, J = 2.7 Hz, 1H, 4-CH), 6.64 (dd, J_1 = 2.7 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 6.95, 7.06 and 7.27 (3m, 4H, CH₂PhF), 7.18 (d, J = 8.3 Hz, 1H, 1-CH); ¹³C NMR δ (acetone-*d*₆) 15.12, 23.93, 27.35, 28.43, ~30 (under solvent peaks), 32.13, 33.60, 41.00, 43.14, 44.76, 48.06, 50.24, 83.76, 113.16 (d, J = 21.1 Hz), 113.63, 116.02, 118.63 (d, J = 21.1 Hz), 127.07, 128.02, 129.82 (d, J = 8.0 Hz), 132.14, 138.51, 143.61 (d, J = 7.5 Hz), 155.97, 163.30 (d, J = 242 Hz); LRMS for [M]⁺ 380 *m/z*. Anal. (C₂₅H₂₉O₂F) C, H, F.

3,17β-Dihydroxy-17α-(3'-chlorobenzyl)estra-1,3,5(10)-triene (15): white solid (49% yield); IR ν (film) 3400 and 3220 (OH); ¹H NMR δ (CDCl₃) 0.96 (s, 3H, 18-CH₃), 2.64 and 2.92 (2d, J = 13.4 Hz, 2H, CH₂PhCl), 2.84 (m, 2H, 6-CH₂), 4.57 (br, 1H, OH phenol), 6.58 (d, J = 2.5 Hz, 1H, 4-CH), 6.64 (dd, J_1 = 2.7 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 7.20 to 7.33 (m, 5H, 1-CH and CH₂PhCl); ¹³C NMR δ (acetone-*d*₆) 15.14, 23.95, 27.35, 28.44, ~30 (under solvent peaks), 32.14, 33.61, 41.00, 43.04, 44.77, 48.06, 50.24, 83.71, 113.63, 116.02, 126.56, 127.04, 129.84, 130.62, 131.96, 132.14, 133.77, 138.50, 143.15, 155.97; LRMS for [M]⁺ 397 *m/z*. Anal. (C₂₅H₂₉O₂Cl) C, H, Cl.

3,17β-Dihydroxy-17α-(3'-bromobenzyl)estra-1,3,5(10)-triene (16): white solid (45% yield); IR ν (film) 3420 (OH); ¹H NMR δ (CDCl₃) 0.96 (s, 3H, 18-CH₃), 2.62 and 2.91 (2d, J = 14.3 Hz, 2H, CH₂PhBr), 2.83 (m, 2H, 6-CH₂), 4.67 (br, 1H, OH phenol), 6.58 (d, J = 2.5 Hz, 1H, 4-CH), 6.64 (dd, J_1 = 2.6 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 7.18 to 7.49 (m, 5H, 1-CH and CH₂PhBr); ¹³C NMR δ (acetone-*d*₆) 15.12, 23.93, 27.35, 28.42, ~30 (under solvent peaks), 32.13, 33.57, 41.01, 42.99, 44.77, 48.06, 50.20, 83.71, 113.62, 116.00, 122.10, 127.05, 129.50, 130.19, 131.06, 132.09, 134.88, 138.49, 143.55, 156.00; LRMS for [M]⁺ 440 *m/z*. Anal. (C₂₅H₂₉O₂Br) C, H, Br.

3,17β-Dihydroxy-17α-(2'-bromobenzyl)estra-1,3,5(10)-triene (17): white solid (25% yield); IR ν (film) 3340 (OH); ¹H NMR δ (acetone-*d*₆) 0.99 (s, 3H, 18-CH₃), 2.86 and 3.26 (2d, J = 13.8 Hz, 2H, CH₂PhBr), 2.80 (m, 2H, 6-CH₂), 6.55 (d_{app}, 1H, 4-CH), 6.61 (dd, J_1 = 2.8 Hz and J_2 = 8.3 Hz, 1H, 2-CH), 7.12 to 7.67 (m, 5H, 1-CH and CH₂PhBr), 7.91 (br, 1H, OH phenol); ¹³C NMR δ (methanol-*d*₄/CDCl₃) 15.56, 24.90, 27.74, 28.95, 30.90, 32.48, 33.32, 41.37, 43.06, 45.24, ~49 (under solvent peaks), 50.84, 84.99, 113.90, 116.36, 127.43, 128.08 (2x), 128.93, 132.94, 133.51, 134.53, 139.15, 139.88, 155.77; LRMS for [MH - H₂O]⁺ 423.2 *m/z*. Anal. (C₂₅H₂₉O₂Br) C, H.

3,17β-Dihydroxy-17α-(2',6'-dichlorobenzyl)estra-1,3,5(10)-triene (18): white solid (22% yield); ¹H NMR δ (DMSO-*d*₆) 0.82 (s, 3H, 18-CH₃), 2.69 (m, 2H, 6-CH₂), 3.04 and 3.15 (2d, J = 13.4 Hz, 2H, CH₂PhCl₂), 6.43 (d, J = 1.9 Hz, 1H, 4-CH), 6.50 (dd, J_1 = 2.0 Hz and J_2 = 8.2 Hz, 1H, 2-CH), 7.05 (d, J = 8.5 Hz, 1H, 1-CH), 7.23 (t_{app}, J = 8.0 Hz, 1H, 4'-CH), 7.42 (d, J = 8.0 Hz, 2H, 3' and 5'-CH), 8.99 (s, 1H, OH); ¹³C

NMR δ (DMSO- d_6) 14.37, 23.38, 26.15, 27.28, 29.15, 30.71, 32.47, 37.30, ~39 (under solvent peaks), 43.29, 47.52, 49.04, 84.45, 112.67, 114.91, 125.97, 128.35, 128.46 (2x), 130.44, 135.65, 136.80 (2x), 137.14, 154.89. Anal. (C₂₅H₂₈O₂Cl₂) C, H, Cl.

3,17 β -Dihydroxy-17 α -(3',4'-dichlorobenzyl)estra-1,3,5-(10)-triene (19): white solid (26% yield); IR ν (film) 3420 (OH); ¹H NMR (acetone- d_6) 0.98 (s, 3H, 18-CH₃), 2.80 (m, 3H, 6-CH₂ and 1H of CH₂PhCl₂), 2.92 (d, J = 13.5 Hz, 1H of CH₂PhCl₂), 6.54 (d, J = 2.7 Hz, 1H, 4-CH), 6.60 (dd, J_1 = 2.7 Hz and J_2 = 8.5 Hz, 1H, 2-CH), 7.12 (d, J = 8.4 Hz, 1H, 1-CH), 7.35 (dd, J_1 = 2.0 Hz and J_2 = 8.4 Hz, 1H, 6'-CH), 7.45 (d, J = 8.1 Hz, 1H, 5'-CH), 7.62 (d, J = 1.9 Hz, 1H, 2'-CH), 7.90 (br, 1H, OH phenol); ¹³C NMR δ (methanol- d_4 /CDCl₃) 15.05, 23.89, 27.26, 28.44, 30.46, 32.06, 32.86, 40.88, 42.88, ~49 (under solvent peaks), 50.56, 83.96, 113.48, 115.48, 126.97, 130.22, 130.56, 131.59, 132.16, 132.33, 133.70, 138.40, 140.73, 155.38; LRMS for [MH - H₂O]⁺ 413.3 m/z . Anal. Calcd for C₂₅H₂₈O₂Cl₂: C, 69.60; H, 6.54; Cl, 16.44. Found: C, 69.06; H, 6.64; Cl, 16.95.

3,17 β -Dihydroxy-17 α -(4'-methoxybenzyl)estra-1,3,5-(10)-triene (20): white solid (28% yield); IR ν (film) 3380 (OH); ¹H NMR δ (CDCl₃) 0.97 (s, 3H, 18-CH₃), 2.64 and 2.88 (2d, J = 14.2 Hz, 2H, CH₂PhOCH₃), 2.83 (m, 2H, 6-CH₂), 3.81 (s, 3H, PhOCH₃), 5.07 (br, 1H, OH phenol), 6.57 (d, J = 2.7 Hz, 1H, 4-CH), 6.64 (dd, J_1 = 2.7 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 6.88 (d, J = 8.6 Hz, 2H, 3' and 5'-CH), 7.18 (d, J = 8.6 Hz, 1H, 1-CH), 7.22 (d, J = 8.6 Hz, 2H, 2' and 6'-CH); ¹³C NMR δ (acetone- d_6) 15.03, 23.76, 27.15, 28.25, ~30 (under solvent peaks), 31.89, 33.29, 40.75, 42.25, 44.52, 47.66, 50.12, 55.19, 83.59, 113.44, 116.67 (2x), 115.83, 126.84, 131.89, 131.98, 132.72 (2x), 138.27, 155.71, 158.78; LRMS for [M]⁺ 392 m/z . Anal. (C₂₆H₃₂O₃) C, H.

3,17 β -Dihydroxy-17 α -(4'-benzyloxybenzyl)estra-1,3,5-(10)-triene (21): white solid (43% yield); IR ν (film) 3330 (OH); ¹H NMR δ (CDCl₃) 0.97 (s, 3H, 18-CH₃), 2.63 and 2.87 (2d, J = 13.3 Hz, 2H, CH₂PhOCH₂Ph), 2.82 (m, 2H, 6-CH₂), 4.63 (br, 1H, OH phenol), 5.06 (s, 2H, CH₂PhOCH₂Ph), 6.57 (d, J = 2.5 Hz, 1H, 4-CH), 6.63 (dd, J_1 = 2.7 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 6.95 (d, J = 8.6 Hz, 2H, 3' and 5'-CH), 7.18 (d, J = 10.4 Hz, 1H, 1-CH), 7.22 (d, J = 8.6 Hz, 2H, 2' and 6'-CH), 7.30 to 7.47 (m, 5H, CH₂PhOCH₂Ph); ¹³C NMR δ (CDCl₃) 14.51, 23.31, 26.33, 27.49, 29.65, 31.37, 33.69, 39.64, 41.45, 43.84, 46.73, 49.53, 70.03, 83.25, 112.72, 114.55 (2x), 115.29, 126.46, 138.19, 153.52, 157.51; LRMS for [M]⁺ 468 m/z . Anal. (C₃₂H₃₆O₃) C, H.

3,17 β -Dihydroxy-17 α -(4'-butylbenzyl)estra-1,3,5-(10)-triene (22): white solid (91% yield); IR ν (film) 3410 (OH); ¹H NMR δ (methanol- d_4 /CDCl₃) 0.90 (t, J = 7.3 Hz, 3H, Ph-(CH₂)₃CH₃), 0.91 (s, 3H, 18-CH₃), 2.54 (t, J = 7.8 Hz, 2H, PhCH₂(CH₂)₂CH₃), 2.60 and 2.82 (2d, J = 13.4 Hz, 2H, CH₂-Ph(CH₂)₃CH₃), 2.78 (m, 2H, 6-CH₂), 6.51 (d, J = 2.5 Hz, 1H, 4-CH), 6.57 (dd, J_1 = 2.6 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 7.05 (d, J = 8.0 Hz, 2H, 3' and 5'-CH), 7.09 (d, J = 10.0 Hz, 1H, 1-CH), 7.16 (d, J = 8.0 Hz, 2H, 2' and 6'-CH); ¹³C NMR δ (CDCl₃/methanol- d_4) 14.25, 15.12, 23.05, 23.87, 27.25, 28.42, 30.44, 31.99, 32.68, 34.55, 35.94, 40.83, 42.88, 44.74, 47.95, 50.52, 84.10, 113.43, 115.86, 126.94, 128.48(2x), 131.76(2x), 132.42, 136.75, 138.61, 141.10, 155.28; LRMS for [M]⁺ 418 m/z . Anal. Calcd for C₂₉H₃₈O₂: C, 83.21; H, 9.15. Found: C, 82.71; H, 9.22.

3,17 β -Dihydroxy-17 α -(4'-tert-butylbenzyl)estra-1,3,5-(10)-triene (23): white solid (77% yield); IR ν (film) 3400 (OH); ¹H NMR δ (CDCl₃) 0.97 (s, 3H, 18-CH₃), 1.33 (s, 9H, *tert*-butyl), 2.65 and 2.90 (2d, J = 13.2 Hz, 2H, CH₂Ph-*t*-Bu), 2.84 (m, 2H, 6-CH₂), 4.68 (br, 1H, OH phenol), 6.58 (d, J = 2.4 Hz, 1H, 4-CH), 6.63 (dd, J_1 = 2.7 Hz and J_2 = 8.3 Hz, 1H, 2-CH), 7.17 (d, J = 8.6 Hz, 1H, 1-CH), 7.22 (d, J = 8.2 Hz, 2H, 2' and 6'-CH), 7.35 (d, J = 8.2 Hz, 2H, 3' and 5'-CH); ¹³C NMR δ (acetone- d_6) 15.25, 24.03, 27.45, 28.52, ~30 (under solvent peaks), 31.87, 32.21, 33.61, 34.92, 41.11, 42.92, 44.88, 48.02, 50.38, 83.76, 113.69, 116.08, 125.29 (2x), 127.13, 131.88 (2x), 132.25, 137.35, 138.58, 149.07, 156.05; LRMS for [M]⁺ 418 m/z . Anal. (C₂₉H₃₈O₂) C, H.

3,17 β -Dihydroxy-17 α -(2'-naphthylmethyl)estra-1,3,5-(10)-triene (24): white solid (59% yield); IR ν (film) 3400 (OH); ¹H NMR δ (acetone- d_6) 1.00 (s, 3H, 18-CH₃), 2.80 (m, 2H, 6-CH₂), 2.90 and 3.09 (2d, J = 13.3 Hz, 2H, CH₂Naphthyl), 6.56 (d, J = 2.2 Hz, 1H, 4-CH), 6.63 (dd, J_1 = 2.6 Hz and J_2 = 8.3 Hz, 1H, 2-CH), 7.14 (d, J = 8.4 Hz, 1H, 1-CH), 7.42 to 7.86 (m, 7H, CH₂Naphthyl), 7.94 (br, 1H, OH phenol); ¹³C NMR δ (acetone- d_6) 15.27, 24.12, 27.51, 28.57, ~30 (under solvent peaks), 32.30, 33.83, 41.16, 43.68, 44.92, 48.19, 50.47, 84.06, 113.76, 116.13, 125.97, 126.52, 127.17, 127.66, 128.42, 128.48, 130.37, 131.21, 132.31, 133.22, 134.50, 138.46, 138.62, 156.10; LRMS for [MH - H₂O]⁺ 395.3 m/z . Anal. (C₂₉H₃₂O₂) C, H.

3.4. Synthesis of Compound 25 (alkylation with phenyllithium acetylide). Phenyllithium acetylide was prepared at -78 °C and under an argon atmosphere by reacting phenylacetylene (5.5 mmol) and *n*-BuLi (5.5 mmol) in dry THF (20 mL) and HMPA (2.0 mL) as cosolvent. A solution of E₁ (300 mg, 1.08 mmol) in dry THF (10 mL) was then added and the reaction mixture was allowed to return slowly to room temperature overnight. After addition of water, the aqueous phase was extracted with EtOAc and the organic layer was washed with brine, dried over MgSO₄, and evaporated to dryness. The crude residue was purified by chromatography (hexane/EtOAc, 80:20) to afford a mixture of alkylated compound **25** and unreacted E₁. This mixture was then dissolved in MeOH (20 mL) and treated with NaBH₄ (2 equiv) at 0 °C. After complete reduction of E₁ to E₂ (2 h), the reaction was quenched with H₂O, MeOH was evaporated under vacuum, and the products extracted with EtOAc. The organic phase was dried over MgSO₄ and evaporated under vacuum before purification by chromatography (hexane/EtOAc, 85:15) to give E₂ (49%) and alkylated compound **25** (45%).

3,17 β -Dihydroxy-17 α -(2'-phenyl-1'-ethyn-1'-yl)estra-1,3,5-(10)-triene (25): white solid (45% yield); IR ν (film) 3420 (OH); ¹H NMR δ (acetone- d_6) 0.95 (s, 3H, 18-CH₃), 2.80 (m, 2H, 6-CH₂), 6.53 (d, J = 2.4 Hz, 1H, 4-CH), 6.60 (dd, J_1 = 2.7 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 7.12 (d, J = 8.4 Hz, 1H, 1-CH), 7.35 (m, 3H, C \equiv CPh), 7.43 (m, 2H, C \equiv CPh), 7.92 (br, 1H, OH phenol); ¹³C NMR δ (acetone- d_6) 13.43, 23.61, 27.43, 28.21, ~30 (under solvent peaks), 34.06, 40.03, 40.66, 44.73, 48.46, 50.69, 80.20, 85.51, 95.15, 113.63, 115.96, 124.49, 127.10, 128.84, 129.28 (2x), 131.94, 132.20 (2x), 138.43, 155.93; LRMS for [MH - H₂O]⁺ 355.3 m/z . Anal. (C₂₆H₃₀O₂) C, H.

3.5. Synthesis of Compound 26 (reduction of triple bond). As reported for compounds **5** and **11**, the catalytic hydrogenation of alkyne **25** afforded the saturated compound **26**, which was purified by chromatography with hexane/EtOAc (85:15).

3,17 β -Dihydroxy-17 α -(2'-phenyl-1'-ethyl)estra-1,3,5-(10)-triene (26): white solid (95% yield); IR ν (film) 3380 (OH); ¹H NMR δ (methanol- d_4) 0.93 (s, 3H, 18-CH₃), 2.75 (m, 4H, 6-CH₂ and CH₂Ph), 6.46 (d, J = 2.5 Hz, 1H, 4-CH), 6.51 (dd, J_1 = 2.6 Hz and J_2 = 8.5 Hz, 1H, 2-CH), 7.04 (d, J = 8.5 Hz, 1H, 1-CH), 7.10 to 7.28 (m, 5H, (CH₂)₂Ph); ¹³C NMR δ (acetone- d_6) 15.00, 24.11, 27.22, 28.28, ~30 (under solvent peaks), 30.92, 32.45, 34.51, 40.30, 40.78, 44.54, 47.63, 50.36, 83.11, 113.47, 115.84, 126.13, 126.86, 128.99 (2x), 129.21 (2x), 132.00, 138.32, 144.61, 155.84; LRMS for [MH - H₂O]⁺ 359.2 m/z . Anal. (C₂₆H₃₂O₂) C, H.

4. Preparation of 16 α - or 16 β -Benzylestradiol 27 or 28 (Scheme 3).
4.1. Alkylation of TBDMS-E₁ and Cleavage of TBDMS Group. Under an argon atmosphere, a solution of lithium diisopropylamide (LDA) was prepared at 0 °C by adding *n*-BuLi (1.17 mmol) to a solution of diisopropylamine (1.34 mmol) in dry THF (25 mL). After 2 h, the mixture was cooled at -78 °C and TBDMS-estrone^{25f} (300 mg, 0.78 mmol) dissolved in dry THF (10 mL) was added dropwise. Benzyl bromide (267 mg, 1.56 mmol) was added after 1 h and the mixture was allowed to warm slowly to room temperature overnight. Then, the mixture was poured into water and extracted with EtOAc. The combined organic phase was washed with brine, dried (MgSO₄), and evaporated to dryness. The crude mixture was purified by chromatography (hexane/EtOAc, 85:15) to give 80% of monobenzyl E₁ and 11% of

dibenzyl E_1 . The TBDMS derivative of monobenzyl compounds (295 mg, 0.62 mmol) was then treated with 2% HCl in MeOH (50 mL) at room temperature to cleave the protecting group. After 6 h, MeOH was partially evaporated, water was added, and the aqueous phase was extracted with EtOAc. The organic phase was dried over $MgSO_4$, filtered, evaporated to dryness, and purified by chromatography (hexane/EtOAc, 80:20) to afford an epimeric mixture of **3-hydroxy-16(α/β)-benzylestra-1,3,5(10)-triene-17-one**: white solid (82% yield; 66% from E_1); IR ν (film) 3400 (OH), 1720 (C=O); 1H NMR δ ($CDCl_3$) 0.73 and 0.96 (2s, 3H, 18- CH_3 , 16 β :16 α /25:75), 2.59 and 2.69 (2dd, J_1 = 10.0 Hz and J_2 = 13.8 Hz, 1H of CH_2Ph , 16 α :16 β /75:25), 2.82 (m, 2H, 6- CH_2), 3.15 and 3.24 (2dd, J_1 = 4.0 Hz and J_2 = 13.8 Hz, 1H of CH_2Ph , 16 α :16 β /75:25), 5.49 (br, 1H, OH phenol), 6.59 (d, J = 2.7 Hz, 1H, 4-CH), 6.65 (dd, J_1 = 2.7 Hz and J_2 = 8.3 Hz, 1H, 2-CH), 7.13 (d, J = 8.5 Hz, 1H, 1-CH), 7.19 to 7.33 (m, 5H, CH_2Ph); LRMS for $[M]^+$ 360 m/z . Anal. ($C_{25}H_{28}O_2$) C, H.

4.2. Reduction of 16 α/β -Benzyl- E_1 (synthesis of 27 and 28). $LiAlH_4$ (1.13 mmol) was added to a solution of 16 α/β -benzyl- E_1 (162 mg, 0.45 mmol) in THF (40 mL) and the mixture was stirred at $-78^\circ C$ for 9 h. Water was then added and the mixture was allowed to stir another 15 min before extraction with EtOAc. The organic phase was washed with brine, dried ($MgSO_4$), and evaporated to dryness. The crude product was purified by chromatography (hexane/EtOAc, 85:15) to give two isomers (16 α - and 16 β -benzyl- E_2).

3,17 β -Dihydroxy-16 α -benzylestra-1,3,5(10)-triene (27): white solid (62% yield); IR ν (film) 3375 (OH); 1H NMR δ ($CDCl_3$) 0.82 (s, 3H, 18- CH_3), 2.70 (dd, J_1 = 8.8 Hz and J_2 = 13.5 Hz, 1H of CH_2Ph), 2.79 (m, 2H, 6- CH_2), 2.90 (dd, J_1 = 6.8 Hz and J_2 = 13.5 Hz, 1H of CH_2Ph), 3.43 (d, J = 7.5 Hz, 1H, 17 α -CH), 4.98 (br, 1H, OH phenol), 6.55 (d, J = 2.6 Hz, 1H, 4-CH), 6.62 (dd, J_1 = 2.6 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 7.14 (d, J = 8.4 Hz, 1H, 1-CH), 7.18 to 7.34 (m, 5H, CH_2Ph); ^{13}C NMR δ ($CDCl_3$) 11.95, 26.21, 27.20, 29.57, 30.02, 36.84, 38.52, 41.60, 44.00, 44.15, 45.50, 48.22, 87.74, 112.71, 115.26, 126.04, 126.46, 128.53 (2x), 128.83 (2x), 132.63, 138.23, 141.26, 153.42; LRMS for $[M]^+$ 362 m/z . Anal. ($C_{25}H_{30}O_2$) C, H.

3,17 β -Dihydroxy-16 β -benzylestra-1,3,5(10)-triene (28): white solid (22% yield); IR ν (film) 3360 (OH); 1H NMR δ ($CDCl_3$) 0.88 (s, 3H, 18- CH_3), 2.43 (q_{app}, J ~ 12 Hz, 1H of CH_2Ph), 2.79 (m, 2H, 6- CH_2), 3.10 (dd, J_1 = 4.8 Hz and J_2 = 13.1 Hz, 1H of CH_2Ph), 3.86 (d, J = 9.6 Hz, 1H, 17 α -CH), 4.78 (br, 1H, OH phenol), 6.54 (d, J = 2.7 Hz, 1H, 4-CH), 6.62 (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), 7.18 to 7.31 (m, 5H, CH_2Ph); ^{13}C NMR δ (acetone- d_6) 13.19, 27.19, 28.28, 30.27 (under solvent peaks), 32.91, 38.61 (2x), 39.41, 43.08, 44.92, 45.14, 49.49, 82.18, 113.56, 115.92, 126.14, 126.97, 128.92 (2x), 129.58 (2x), 132.08, 138.36, 143.70, 155.87; LRMS for $[M]^+$ 362 m/z . Anal. ($C_{25}H_{30}O_2$) C, H.

B. Enzymatic Assays. 1. Homogenized JEG-3 Cells as Source of Steroid Sulfatase Activity (low concentration of E_1S as substrate: Tables 1–5). The JEG-3 cells were purchased from ATCC, on Oct 10, 1989, at passage 127. Cells were grown in DMEM medium (Flow) containing $NaHCO_3$ (3.7 g/L), Hepes (5.96 g/L) and glucose (4.59 g/L). Medium sterilized on a 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/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), 15 min at $37^\circ C$. Enzyme activity was inhibited by diluting the cells in culture medium containing 5% serum. Cells were pelleted, resuspended in culture medium, counted with a haemocytometer and reseeded in culture flasks or harvested for subsequent analysis.

Steroid sulfatase activity was determined by measuring the total labeled estrone ($[^3H]E_1$) formed from labeled estrone sulfate ($[^3H]E_1S$). Homogenate of JEG-3 cells was prepared by allowing the combined cell pellets from several culture flasks to break by repeated (5 times) freezing and thawing processes

followed at 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 $-90^\circ C$.

Assays were carried out in 13- \times 100-mm borosilicate test tubes. The buffer was made of 0.1 M Tris-acetate, 0.005 M EDTA, 10% glycerol, adjusted to pH 7.0. The tubes were immersed in a water and ice bath before adding reagents. To each tube were added 300 μL of cold assay buffer, 7 nM $[^3H]E_1S$, 10 μL of ethanol for the control or 10 μL of a solution of inhibitor 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 controlled temperature water bath for 60 min at $37^\circ C$. Radioactive background was determined by incubating excess (225 μM) of unlabeled E_1S into a tube containing assay buffer, enzyme substrate ($[^3H]E_1S$) and enzyme preparation. The reaction was stopped by shaking the tubes in a water and ice bath and by adding an excess (225 μM) of unlabeled E_1S immediately into each tube. The tubes were shaken once again, then 1.25 mL of xylene was added to each tube and E_1 was extracted by shaking the tubes for 4 min with a centrifugation at 2500 rpm for 10 min to separate the organic and aqueous phases. An aliquot (750 μL) of the organic phase containing $[^3H]E_1$ was counted in 7 mL of scintillation cocktail with a β -counter (Beckman LS3801, Irvine, CA). In our inhibition study, we have chosen to perform the enzymatic assay with a level of enzymatic activity corresponding to approximately 20% of the rate of transformation (0.8 pmol/min) in the linear range with respect to protein and time. The nonenzymatic control (background), which represents about 3%, was subtracted from the measure of enzymatic activity. The results were expressed as the percent of E_1 produced (100% for control without inhibitor) over the concentration of inhibitor. The percents of inhibition were determined at a concentration of 20 μM , while the IC_{50} values were determined using the DE_{50} program (CHUL Research Center, Québec, Canada).

2. Transfected HEK-293 Cells as Source of Steroid Sulfatase Activity (high concentration of E_1S or DHEAS as substrate: Table 6). As previously reported,²³ the enzymatic assays using high concentrations (100 μM) of enzyme substrates ($[^3H]E_1S$ or $[^{14}C]DHEAS$) were performed using human embryonic kidney (HEK)-293 cells (American Type Culture Collection, Rockville, MD) transiently transfected with a sulfatase expression vector (pCMV-sulfa). The pCMV-sulfa was constructed by insertion of a cDNA fragment, downstream the CMV promoter of the pCMV vector, kindly provided by Dr. M. B. Mathews (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). The sulfatase cDNA fragment was obtained by screening of a human placenta cDNA library (Clontech Laboratories Inc., Palo Alto, CA) using the incomplete cDNA fragment kindly provided by Dr. L. J. Shapiro (Howard Hughes Medical Institute, Los Angeles, CA) as probe. Transfection of the expression vector was performed by the calcium phosphate procedure using 10 μg of recombinant plasmid/10⁶ cells (Kingston, R. E.; Chen, C. A.; Okayama, H. In *Current Protocols in Molecular Biology*, Ausubel, E. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K., Eds.; John Wiley and Sons: New York, 1991; pp 9.1.1–9.1.9). The cells were initially plated at 10⁴ cells/ cm^2 in Falcon culture flasks and grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 IU penicillin/mL, and 100 μg streptomycin sulfate/mL.

For the enzymatic assay, the HEK-293 cell homogenate was prepared by repeated freezing ($-80^\circ C$) and thawing (5 times) and homogenization using a Dounce homogenizer. The reaction was carried out at $37^\circ C$ in 1.25 mL of 100 mM Tris-acetate buffer (pH 7.4) containing 5 mM of ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 100 μM of $[^3H]E_1S$ or $[^{14}C]DHEAS$ as substrate, and an ethanolic solution of compound to test (at appropriate concentrations). About 2.2 and 11 mg of protein were used for the transformation of E_1S and DHEAS, respectively. After 2 h of incubation, the reaction was stopped by

addition of 1.25 mL of xylene. The tubes were then shaken and centrifuged at 2000g for 10 min to separate the organic and aqueous phases. Radioactivity in 750 μ L of each phase (organic: free steroids; aqueous: sulfated steroids) was determined by liquid scintillating counting with a Beckman LS3801 (Irvine, CA). The IC₅₀ values were determined using the DE₅₀ program (CHUL Research Center, Québec, Canada).

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Supporting Information Available: Inhibition curves (steroid sulfatase activity vs inhibitor concentration) used for the determination of IC₅₀ values of compounds 1–26. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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