Potent Inhibition of Steroid Sulfatase Activity by 3-*O*-Sulfamate 17α-Benzyl(or 4′-*tert*-butylbenzyl)estra-1,3,5(10)-trienes: Combination of Two Substituents at Positions C3 and C17α of Estradiol

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Steroid sulfates are precursors of hormones that stimulate androgen- and estrogen-dependent cancers. Thus, steroid sulfatase, the enzyme that catalyzes conversion of DHEAS and E_1S to the corresponding unconjugated steroids DHEA and E_1, appears to be one of the key enzymes regulating the level of active androgenic and estrogenic steroids. Since 17α-substituted benzylestradiols and 3-*O*-sulfamate estrone (EMATE) represent two families of steroid sulfatase inhibitors that probably act through different mechanisms, we synthesized compounds 3-*O*-sulfamate 17α-benzylestradiol (**4**) and 3-*O*-sulfamate 17α-(*tert*-butylbenzyl)estradiol (**5**) that contain two kinds of substituents on the same molecule. In our enzymatic assay using a homogenate of human embryonal (293) cells transfected with steroid sulfatase, compounds **4** and **5** were found to be more potent inhibitors than already known steroid sulfatase inhibitors that have only a C17α-substituent or only a C3-sulfamate group (EMATE). The IC_{50} values of **4** and **5** were, respectively, 0.39 and 0.15 nM for the transformation of E_1S to E_1 and 4.1 and 1.4 nM for the transformation of DHEAS to DHEA. Compound **5** inhibited the steroid sulfatase activity in intact transfected (293) cell culture assays by inactivating the enzyme activity. Compound **5** also inactivates the steroid sulfatase activity at lower concentration than EMATE in microsomes of transfected (293) cells. In this assay, an excess of natural substrate E_1S protects enzyme against inactivation by **5** or EMATE. Furthermore, the unsulfamoylated analogue of **5**, compound **3**, did not inactivate the steroid sulfatase.

Introduction

The enzyme steroid sulfatase is known to catalyze the conversion of dehydroepiandrosterone sulfate (DHEAS) and estrone sulfate (E_1S) to the corresponding unconjugated steroids DHEA and E_1 and may be one of the key enzymes involved in the regulation of the level of active steroids in normal and malignant tissues (Figure 1). Indeed, DHEAS is the main sulfated steroid found in human blood circulation and may thus be a potential source of androgens and estrogens in peripheral tissues.[1] Upon hydrolysis by steroid sulfatase, DHEAS is transformed to DHEA, which is further transformed to 4-androstenedione (Δ^4-dione), testosterone (T), dihydrotestosterone (DHT), 5-androstene-3β,17β-diol (Δ^5-diol), and estradiol (E_2) by steroidogenic enzymes: namely, 3β-hydroxysteroid dehydrogenases, 17β-hydroxysteroid dehydrogenases, 5α-reductases, and aromatase. On the other hand, E_1S is also the most abundant C18-steroid in the circulation and could be transformed to the estrogens E_1 and E_2.[2] These androgens and estrogens are produced locally in target tissues and are probably responsible for the growth of androgen- and estrogen-dependent tissues and associated diseases such as prostate and breast cancers. Interestingly, higher activity of steroid sulfatase has been detected in breast tumor tissues in comparison to normal tissues.[3] Since androgens and estrogens may be synthesized inside cancer cells starting from DHEAS and E_1S available in blood circulation,[1] the use of therapeutic agents that inhibit steroid sulfatase activity may well be a valuable approach to the treatment of hormone-dependent diseases.

Many steroid sulfatase inhibitors have been developed during the past few years.[4] Most of them were obtained from a C3-modification of E_1S, the natural C18-steroid substrate of the enzyme.[4p] In this series of inhibitors, the 3-*O*-sulfamate estrone (**1**, EMATE) is well-known and is one of the most potent inhibitors that has been reported to date (Figure 2).[4p,6] Recently, our group identified for the first time the steroid sulfatase inhibitory effect of an alkyl or substituted benzyl group introduced at position 17α of 17β-estradiol.[5] For this new family of inhibitors, represented by 17α-benzylestradiol (**2**) and 17α-(*tert*-butylbenzyl)estradiol (**3**), we hypothesized that they may inhibit steroid sulfatase activity by a reversible interaction (probably hydrophobic) with a region within the enzymatic site. On the contrary, EMATE (**1**) and its more potent 4-NO_2 analogue, as well as other steroidal or nonsteroidal sulfamates, are active-site-directed irreversible inhibitors of estrone sulfatase.[4,6] Since 17α-benzyl(or *tert*-butylbenzyl)estradiols (**2** or **3**) and 3-*O*-sulfamate estrone (**1**) represent two families of inhibitors with substituents that act at opposite positions on the steroid nucleus, and most likely through different mechanisms, we designed

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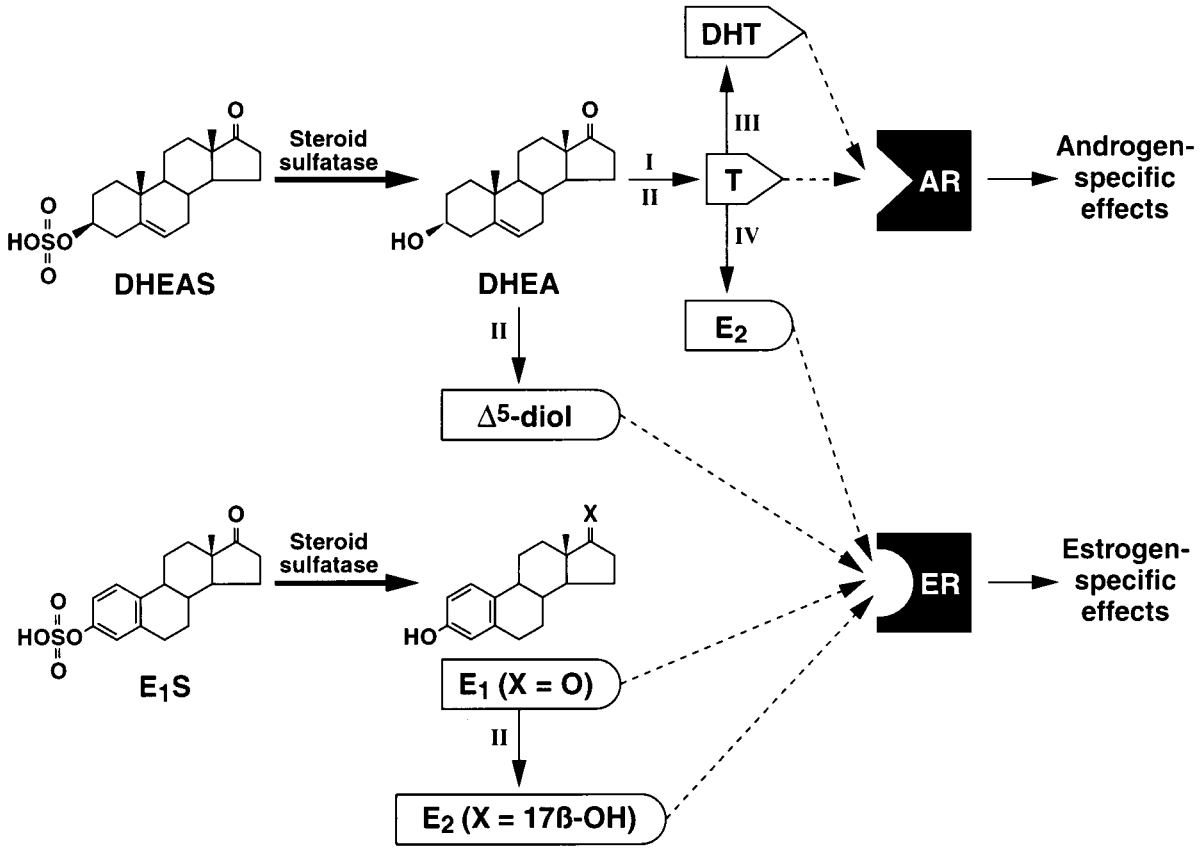
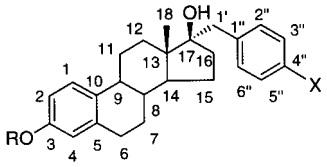


Figure 1. Steroid sulfatase transformation of sulfated steroids DHEAS and E_1S to hormones interacting with androgen (AR) and estrogen (ER) receptors. Other enzymes: 3β-dehydrogenase Δ^5,Δ^4-isomerases (**I**), 17β-hydroxysteroid dehydrogenases (**II**), 5α-reductases (**III**), aromatase (**IV**).

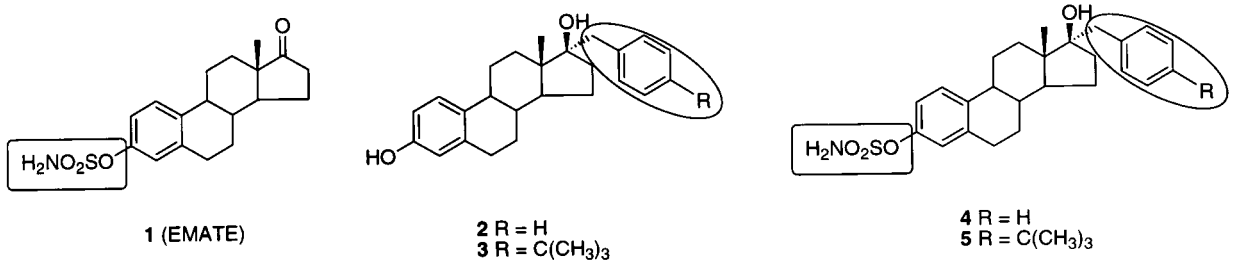
compounds that contain both substituents on the same molecule: namely, 3-*O*-sulfamate 17α-benzylestradiol (**4**) and 3-*O*-sulfamate 17α-(*tert*-butylbenzyl)estradiol (**5**). Herein, we report the chemical synthesis and the inhibitory activity of the first two members (compounds **4** and **5**) of this new series of steroid sulfatase inhibitors. We also report the type of inhibition for the most potent inhibitor, compound **5**, and its unsulfamoylated analogue, compound **3**.

Chemistry

Compounds **4** and **5**, each with two different kinds of substituents that do not act in the same way, were



synthesized by a simple chemical pathway (Scheme 1). The intermediates **2** and **3** were first obtained by a stereoselective addition of Grignard reagents to the C17-carbonyl of E_1. The 18-methyl on the β-face of steroids directs the nucleophile attack on the less hindered α-face, giving the 17α-alkylated compound. An excess of commercially available benzylmagnesium bromide



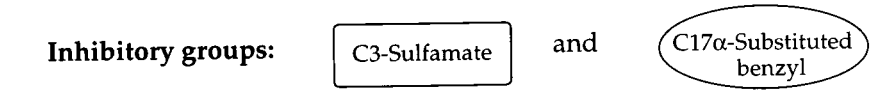
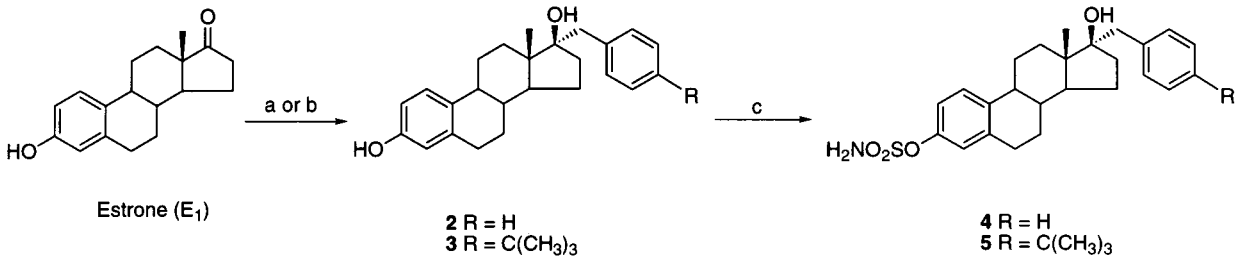


Figure 2. Two families of known inhibitors (**1**−**3**) and newly proposed inhibitors (**4** and **5**) of steroid sulfatase.

Scheme 1[a]



Estrone (E_1)

2 R = H
3 R = $C(CH_3)_3$

4 R = H
5 R = $C(CH_3)_3$

[a] Reagents: (a) BnMgBr, THF, 0 °C−rt, overnight; (b) *tert*-butylbenzyl bromide, Mg, diethyl ether, rt, overnight; (c) H_2NO_2S-Cl, DBMP, CH_2Cl_2, rt, 1.5 h.

(2.0 M in THF) or in situ generated (*tert*-butylbenzyl)-magnesium bromide (*tert*-butylbenzyl bromide, magnesium turnings, diethyl ether, 0 °C) was used for the Grignard reaction (0 °C to room temperature, overnight). In addition to alkylated compound **2** or **3**, unreacted E_1 was obtained because of the low reactivity of such hindered 17-ketosteroids[7] and the low solubility of the phenolate species generated. To facilitate the chromatographic separation of the product (**2** or **3**) from the starting material (E_1), a quantitative carbonyl reduction of E_1 to E_2 was carried out with $NaBH_4$ (MeOH, 1−2 h, 0 °C to room temperature). After silica gel chromatography, compounds **2** and **3** were obtained in yields of 64% and 77%, respectively, in addition to 30% and 17% of E_2. Although higher yields were obtained for the Grignard reaction when the phenolic group was protected as a *tert*-butyldimethylsilyl ether, this protection/deprotection approach added two more steps and was found to be too time-consuming for the improvement of overall yield.

The next step consisted of regioselectively introducing the sulfamoyl group at position C3. The sulfamoyl chloride needed for the sulfamoylation was first synthesized from chlorosulfonyl isocyanate following a modified procedure described by Peterson et al.[8] Thereafter, sulfamoylated derivatives **4** and **5** were obtained in yields of 59% and 79%, respectively, by adding sulfamoyl chloride (6 equiv) to a solution of 3-hydroxysteroids **2** and **3** in dichloromethane and 2,6-di-*tert*-butyl-4-methylpyridine (DBMP).[9] Compounds **4** and **5** were isolated using an aqueous workup and purified by silica gel chromatography with hexane/acetone (75:25) as eluent. Compound **4** was also obtained in a 70% yield by the addition of 5 equiv of sulfamoyl chloride to a solution of 3-hydroxysteroid **2** and THF in the presence of sodium hydride at 0 °C.[10] The inhibitors with mono (**2** and **3**) and bis (**4** and **5**) substituents were fully characterized by IR, ^{1}H NMR, ^{13}C NMR,[11] MS, and elemental analysis.

Results and Discussion

We used a homogenate of human embryonal kidney (293) cells transiently transfected with a sulfatase expression vector as our source of steroid sulfatase activity. As illustrated in Figure 3, the enzyme catalyzed efficiently the transformation of E_1S and DHEAS to E_1 and DHEA, respectively, but E_1 was the preferred substrate. Lineweaver−Burk plot analysis indicates that the V_{max} values for E_1S and DHEAS were 6.25 and 1.5 nmol/mg of protein/min, respectively, while the K_m values were 15 and 19 μM, respectively. Compounds

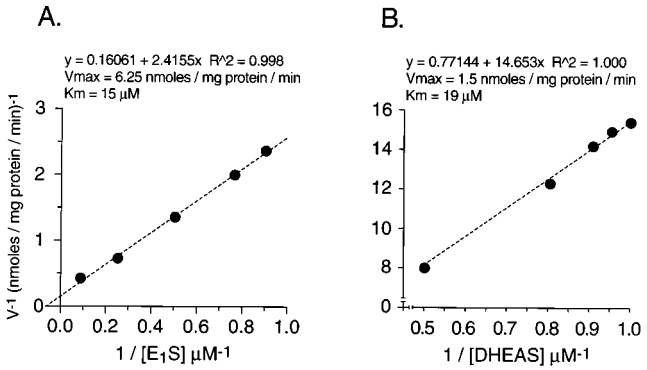


Figure 3. Lineweaver−Burk plots for the transformation of E_1S to E_1 (A) and DHEAS to DHEA (B) by the steroid sulfatase activity of homogenized transfected (293) cells.

2−**5** and reference compound **1** (EMATE) were then tested for their ability to inhibit steroid sulfatase activity transforming E_1S to E_1. As reported in Table 1 and in agreement with the literature, EMATE strongly inhibited the steroid sulfatase activity (IC_{50} = 2.1 nM). On the other hand, compounds **2** and **3**, which contain only a 17α-benzyl or a 17α-*tert*-butylbenzyl group, also strongly inhibited the enzyme activity (IC_{50} = 230 and 8.3 nM, respectively). Interestingly, compounds **4** and **5** (IC_{50} = 0.39 and 0.15 nM, respectively), which bear both inhibiting groups (sulfamate and benzyl/*tert*-butylbenzyl) on the same molecule, were better inhibitors than the preceding compounds **1**−**3** that contain a single inhibitory group. In accordance with the results obtained for compounds **2** and **3**, the *tert*-butylbenzyl analogue **5** was a better inhibitor than the benzyl analogue **4**. When compared to EMATE (**1**), compounds **4** and **5** were found to be 5- and 14-fold more potent for the transformation of E_1S to E_1. Compounds **1**−**5** were also tested for their ability to inhibit the transformation of C19-steroid substrate DHEAS to DHEA by steroid sulfatase (Table 1). The results were similar to those obtained above with E_1S as enzyme substrate. Indeed, compounds **4** and **5** (IC_{50} = 4.1 and 1.4 nM) bearing two inhibiting groups were more potent inhibitors than compounds **2** and **3** (IC_{50} = 325 and 14 nM) that have only a C17α-substituent and EMATE (IC_{50} = 5.6 nM) that has only a C3-sulfamate group. Thus, compounds **4** and **5** were respectively 1.4- and 4-fold more potent than EMATE for the transformation of DHEAS to DHEA. Interestingly, the data in Table 1 suggest an additive-like inhibitory effect of the two kinds of substituents (17α-benzyl or substituted benzyl and 3-*O*-sulfamate) added on the estradiol nucleus.

Because a washing assay is easier using intact transfected cells in culture, this method could be a convenient

Table 1. Inhibition of Steroid Sulfatase Activity by EMATE (**1**) and Newly Synthesized Compounds (**2**−**5**)

compd	substituents	IC_{50} (nM)	
		$E_1S \rightarrow E_1$[a]	DHEAS → DHEA[a]
1 (EMATE)	3-*O*-sulfamate	2.1 ± 0.2	5.6 ± 0.3
2	17α-benzyl	230 ± 56	325 ± 61
3	17α-*tert*-butylbenzyl	8.3 ± 1.6	14 ± 1
4	3-*O*-sulfamate and 17α-benzyl	0.39 ± 0.02 [5][b]	4.1 ± 0.3 [1.4][b]
5	3-*O*-sulfamate and 17α-*tert*-butylbenzyl	0.15 ± 0.01 [14][b]	1.4 ± 0.1 [4][b]

[a] Substrate concentration: [^{3}H]E_1S (100 μM) or [^{14}C]DHEAS (100 μM). [b] The numbers in brackets are the potency of **4** and **5** versus EMATE (**1**).

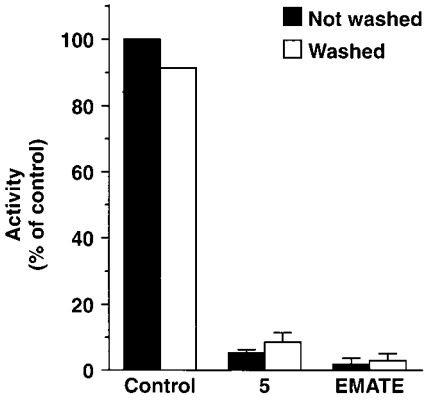


Figure 4. Effect of the new inhibitor **5** and the known inhibitor **1** (EMATE) on steroid sulfatase activity. Inhibitor (5 μM) or control was incubated for 1 h at 37 °C with intact transfected (293) cells (500 000 cells/well) and thereafter washed three times (or not) with DMEM culture medium. The enzymatic activity was then evaluated by incubating [^{3}H]E_1S (650 000 dpm/well) for 18 h and measuring the [^{3}H]E_1 formed. The enzyme activity of the control (100%) was 0.33 nmol/1 000 000 cells/h.

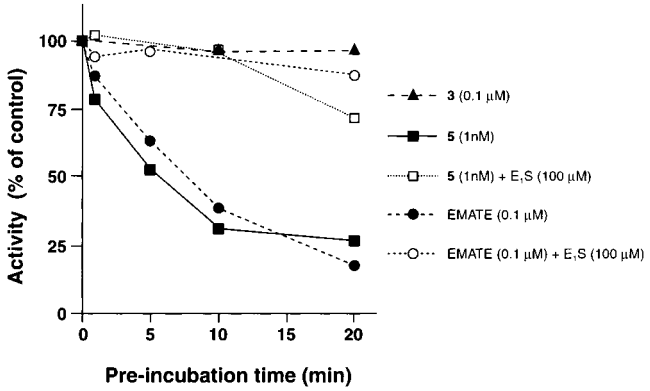


Figure 5. Time-dependent inactivation of steroid sulfatase activity ([^{3}H]E_1S to [^{3}H]E_1) by inhibitors **1** (EMATE), **3**, and **5**, in the absence or presence of a large excess of enzyme substrate E_1S (100 μM). The enzyme activity of the control (100%) was 0.49 nmol/h/mg of protein. The assay was performed as described in the Experimental Section.

way to assess the reversibility/irreversibility of an inhibitor in the case that the inhibitor could be washed out efficiently. As illustrated in Figure 4, compound **5** and EMATE exhibit potent inhibitory effect on steroid sulfatase activity in the experiment using intact cells, and washing does not reverse significantly the inhibitory effect of compound **5** and EMATE. In this assay, however, compound **5** seems to have slightly less potent inhibitory effect than EMATE. This could be due to differential solubility of the compounds or to washing inefficiency. To further analyze the inactivation capability of compound **5** and its unsulfamoylated analogue, compound **3**, we performed a time-dependent inactivation experiment using microsomes of (293) cells transfected with sulfatase expression vector. As illustrated in Figure 5, compound **3**, which does not possess a sulfamoylated group, does not inactivate the sulfatase activity, whereas preincubation with compound **5**, which possesses a sulfamoylated group, inactivates microsomal sulfatase activity at a concentration 100-fold lower than EMATE. Interestingly, the inactivation of both EMATE and compound **5** is reversed by an excess of the substrate E_1S. The result strongly suggests that in the experiment using intact cells (Figure 4) the apparent stronger inhibitory effect of EMATE is probably due to the inefficiency of washing.

In summary, we have developed a new family of potent steroid sulfatase inhibitors by combining the inhibiting actions of two kinds of substituents, a 17α-benzyl (or *tert*-butylbenzyl) and a 3-*O*-sulfamate, on the estradiol nucleus. When compared to EMATE (only a C3-sulfamate group), these compounds, which are represented by **4** and **5**, are 5- and 14-fold more potent inhibitors for the transformation of E_1S to E_1 and 1.4- and 4-fold more potent inhibitors for the transformation of DHEAS to DHEA. Compound **5** also inactivated steroid sulfatase activity in intact (293) transfected cells or in microsomal preparation. We propose that inhibitors such as **4** and **5** act by two different mechanisms, taking into account the nature of the substituents: 3-*O*-sulfamate and 17α-substituted benzyl. After the inhibition of steroid sulfatase by the sulfamate group of **4** and **5**, the released phenolic compounds **2** and **3** were still able to inhibit a neighboring free enzyme molecule. In the situation when the inhibitor concentration is saturating, it is conceivable that the majority of the enzyme molecules will be inactivated by the sulfamate group, since the molecule bearing the sulfamate group (compound **5**) possesses higher affinity than phenolic compound **3** (without sulfamate), and thus the liberated phenolic compound **3** does not have a significant inhibitory effect. In such a case, the 17α-substituent of compound **5** facilitates probably the rate of binding, leading to a more potent inactivation of the enzyme. However, in the situation when the inhibitor concentration is not yet saturating, it is likely that one molecule of **5** could act on two molecules of enzymes, because the hydrolysis of the inactivating sulfamate group generates the compound **3** that exerts its inhibitory effect on the neighboring free enzyme and thus shows an overal better efficacy. An apparent additive inhibitory effect could thus be observed because one molecule of compound **5** could act on two molecules of the enzyme in two different manners: one enzyme molecule is inactivated by covalently binding to the sulfamoyl group and

one is inhibited by the liberated phenolic compound **3**. This situation is unique for inhibitors such as sulfamates **4** and **5**, in which the active group bind covalently to the enzymes generating a free compound (**2** or **3**) that could possesses additional reversible inhibitory effect. Since the C17α-substituent is not limited to a benzyl or a *tert*-butylbenzyl group,[5] the synthesis of other analogues of **4** and **5** can be considered to optimize this new family of promising inhibitors. Furthermore, it is probable that the concept illustrated here of using two kinds of inhibiting substituents on the same molecule can be applied to other steroid sulfatase inhibitors previously reported in the literature.

Experimental Section

A. Chemical Synthesis. Analytical thin-layer chromatography (TLC) was performed on Merck 60 F_{254} silica gel plates (0.20 mm), and compounds were visualized using UV light or ammonium molybdate/sulfuric acid/water (with heating). Flash column chromatography was performed with 230−400 mesh ASTM silica gel 60 (E. Merck). Infrared spectra (IR) are expressed in cm^{-1} and were obtained on a Perkin-Elmer 1600 (series FTIR) spectrophotometer. Nuclear magnetic resonance spectra (NMR) were recorded with a Bruker AC/F 300 spectrometer at 300 MHz (^{1}H) or 75 MHz (^{13}C), and the chemical shifts (δ) are expressed in ppm. Assignment of ^{13}C NMR signals was made easier by distortionless enhancement by polarization transfer (DEPT) experiments, heteronuclear shift correlation (HSC) experiments, and data from the literature.[11] Low-resolution mass spectra (LRMS) were obtained from electron impact (EI) and recorded with a V.G. Micromass 16F spectrometer. High-resolution mass spectra (HRMS) obtained from fast-atom bombardment (FAB) with a NBA matrix were provided by Le Centre Régional de Spectrométrie de Masse (Université de Montréal, Montréal, Canada). Elemental analyses (CHNS) were carried out by Le Laboratoire d'Analyse Élémentaire de l'Université de Montréal (Montréal, Canada).

Synthesis of 3,17β-Dihydroxy-17α-benzylestra-1,3,5-(10)-triene (2). Commercially available estrone (500 mg, 1.85 mmol) in dry THF (50 mL) was stirred under argon atmosphere and treated at 0 °C with benzylmagnesium bromide (2.0 M in THF) (5.55 mL, 11.10 mmol). The reaction mixture was allowed to return to room temperature overnight. Then, a saturated aqueous solution of NH_4Cl was added and the solution extracted with EtOAc. The combined organic layer was washed with brine, dried over $MgSO_4$, and filtered, and solvent was evaporated to dryness. Thereafter, the crude mixture of **2** and remaining estrone was dissolved in MeOH (50 mL), and $NaBH_4$ (140 mg, 3.70 mmol) was added at 0 °C. After complete reduction of estrone to estradiol (1−2 h), the reaction was quenched with H_2O, MeOH was evaporated under vacuum, and the mixture was extracted with EtOAc and treated as above. Purification by chromatography (hexane/EtOAc, 8:2) afforded estradiol (152 mg, 30%) and alkylated compound **2** (428 mg, 64% yield): white solid; IR υ (film) 3415 (OH); ^{1}H NMR δ ($CDCl_3$) 0.97 (s, 3H, 18-CH_3), 2.68 and 2.94 (2d of AB system, J = 13.3 Hz, 2H, CH_2Ph), 2.83 (m, 2H, 6-CH_2), 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, CH_2Ph); ^{13}C NMR δ (acetone-d_6) 15.12 (C18), 23.87 (C15), 27.29 (C11), 28.37 (C7), ∼30 (C6; under solvent peaks), 32.04 (C12), 33.41 (C16), 40.92 (C8), 43.34 (C1′), 44.70 (C9), 47.90 (C13), 50.22 (C14), 83.66 (C17), 113.57 (C2), 115.88 (C4), 126.45 (C4″), 126.99 (C1), 128.29 (C3″ and C5″), 132.05 (C10, C2″, and C6″), 138.43 (C5), 140.38 (C1″), 155.88 (C3); EI-LRMS *m/e* 362 (M^+, 18), 344 ($M^+ - H_2O$, 8.4), 271 ($M^+ - CH_2Ph$, 100), 253 (64), 228 (15), 213 (38), 159 (61), 133 (61), 91 (63). Anal. Calcd for $C_{25}H_{30}O_2$: C, 82.83; H, 8.34. Found: C, 82.40; H, 8.50.

Synthesis of 3,17β-Dihydroxy-17α-(*tert*-butylbenzyl)-estra-1,3,5(10)-triene (3). Magnesium (1.1 g, 45.3 mmol) was added in a dry three-neck flask under argon atmosphere and activated by heating. The system was kept at room temperature, a solution of *tert*-butylbenzyl bromide (2.7 mL, 14.8 mmol) in dry diethyl ether (15 mL) was added dropwise (about 15 min), and the reaction mixture was allowed to stir for 3 h. The in situ generated Grignard reagent was then added slowly to a solution of estrone (400 mg, 1.5 mmol) in dry THF (40 mL) at room temperature, and the reaction was allowed to stir overnight. The reaction mixture was poured into a saturated solution of NH_4Cl, extracted with EtOAc, dried over $MgSO_4$, and evaporated under reduced pressure. The crude mixture of **3** and remaining estrone was dissolved in MeOH (50 mL) and treated at 0 °C with $NaBH_4$ in excess (112 mg) for 30 min. After the usual workup, purification by chromatography (hexane/EtOAc, 9:1) afforded estradiol (68 mg, 17%) and alkylated compound **3** (475 mg, 77% yield): white solid; IR υ (film) 3395 (OH); ^{1}H NMR δ ($CDCl_3$) 0.97 (s, 3H, 18-CH_3), 1.33 (s, 9H, *tert*-butyl), 2.65 and 2.90 (2d of AB system, J = 13.2 Hz, 2H, CH_2Ph-*t*-Bu), 2.84 (m, 2H, 6-CH_2), 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); ^{13}C NMR δ (acetone-d_6) 15.25 (C18), 24.03 (C15), 27.45 (C11), 28.52 (C7), 30.51 (C6; under solvent peaks), 31.87 (C(CH_3)$_3$), 32.21 (C12), 33.61 (C16), 34.92 (C(CH_3)$_3$), 41.11 (C8), 42.92 (C1′), 44.88 (C9), 48.02 (C13), 50.38 (C14), 83.76 (C17), 113.69 (C2), 116.08 (C4), 125.29 (C3″ and C5″), 127.13 (C1), 131.88 (C2″ and C6″), 132.25 (C10), 137.35 (C1″), 138.58 (C5), 149.07 (C4″), 156.05 (C3); EI-LRMS *m/e* 418 (M^+, 1.8), 400 ($M^+ - H_2O$, 18), 385 [$M^+ - (H_2O + CH_3)$, 10], 270 [$M^+ - (CH_2Ph$-*t*-Bu), 100], 253 (56), 159 (54), 147 (82), 133 (83), 57 (75). Anal. Calcd for $C_{29}H_{38}O_2$: C, 83.21; H, 9.15. Found: C, 82.85; H, 9.53.

Synthesis of 3-*O*-Sulfamate 17β-Hydroxy-17α-benzylestra-1,3,5(10)-triene (4). To a stirred solution of 17α-benzylestradiol (**2**) (95 mg, 0.26 mmol) in CH_2Cl_2 (30 mL) was added 2,6-di-*tert*-butyl-4-methylpyridine (DBMP) (160 mg, 0.78 mmol), and the mixture was stirred under argon atmosphere for 15 min at room temperature. Sulfamoyl chloride (182 mg, 1.56 mmol) was then added in portions. After 1.5 h, the solution was washed with water and dried over $MgSO_4$. The solvent was evaporated under vacuum, and the crude mixture was purified by chromatography (hexane/acetone, 75:25) to give unreacted phenol **3** (13 mg, 14%) and sulfamoylated compound **4** (92 mg, 79%): white solid; IR υ (KBr) 3508, 3283 and 3195 (OH and NH_2), 1371 and 1178 (S═O); ^{1}H NMR δ (DMSO-d_6) 0.83 (s, 3H, 18-CH_3), 1.20−1.75 (m, 10H), 1.84 (m, 1H), 2.23 (m, 1H), 2.36 (m, 1H), 2.59 and 2.77 (2d of AB system, J = 13.4 Hz, 2H, CH_2Ph), 2.82 (m, 2H, 6-CH_2), 4.14 (s, OH), 6.96 (d, J = 2.3 Hz, 1H, 4-CH), 7.01 (dd, J_1 = 2.4 Hz and J_2 = 8.4 Hz, 1H, 2-CH), 7.15−7.30 (m, 5H, CH_2Ph), 7.35 (d, J = 8.6 Hz, 1H, 1-CH), 7.89 (s, 2H, NH_2); ^{13}C NMR δ (acetone-d_6) 15.15 (C18), 23.97 (C15), 27.23 (C11), 28.14 (C7), 30.31 (C6; under solvent peaks), 32.13 (C12), 33.52 (C16), 40.61 (C8), 43.44 (C1′), 45.00 (C9), 47.98 (C13), 50.35 (C14), 83.72 (C17), 120.24 (C2), 123.07 (C4), 126.61 (C4″), 127.46 (C1), 128.45 (C3″ and C5″), 132.18 (C2″ and C6″), 139.37 (C10), 139.90 (C5), 140.52 (C1″), 149.45 (C3); FAB-HRMS calcd for $C_{25}H_{30}O_4NS$ (M^+ − H) 440.18954, found 440.19120. Anal. Calcd for $C_{25}H_{31}O_4NS$: C, 68.00; H, 7.08; N, 3.17; S, 7.26. Found: C, 68.43; H, 7.02; N, 3.23; S, 7.81.

Synthesis of 3-*O*-Sulfamate 17β-Hydroxy-17α-(*tert*-butylbenzyl)estra-1,3,5(10)-triene (5). As described above for the synthesis of **4**, 17α-(*tert*-butylbenzyl)estradiol (**3**) (37 mg, 0.088 mmol) was treated in CH_2Cl_2 (30 mL) with DBMP (54 mg, 0.26 mmol) and sulfamoyl chloride (62 mg, 0.53 mmol). After workup, the crude mixture was purified by chromatography (hexane/acetone, 75:25) to afford unreacted phenol **3** (6 mg, 16%) and sulfamoylated compound **5** (26 mg, 59%): white solid; IR υ (KBr) 3538, 3367 and 3196 (OH and NH_2), 1371 and 1186 (S═O); ^{1}H NMR δ (DMSO-d_6) 0.83 (s, 3H, 18-CH_3), 1.26 (s, 9H, *tert*-Butyl), 1.20−1.75 (m, 11H), 1.85 (m, 1H), 2.23 (m, 1H), 2.36 (m, 1H), 2.54 and 2.73 (2d of AB system, J = 13.3 Hz, 2H, CH_2Ph-*t*-Bu), 2.82 (m, 2H, 6-CH_2), 4.11 (s, OH), 6.96 (d, J = 2.0 Hz, 1H, 4-CH), 7.01 (dd, J_1 = 2.2 Hz and J_2 =

8.4 Hz, 1H, 2-CH), 7.20 and 7.26 (2d of AB system, J = 8.2 Hz, 2H, CH_2*Ph-t*-Bu), 7.35 (d, J = 8.6 Hz, 1H, 1-CH), 7.88 (s, NH_2); ^{13}C NMR δ (acetone-d_6) 15.18 (C18), 23.99 (C15), 27.22 (C11), 28.13 (C7), 30.30 (C6; under solvent peaks), 31.85 ($C(CH_3)_3$), 32.13 (C12), 33.58 (C16), 34.90 ($C(CH_3)_3$), 40.90 (C8), 42.89 (C1′), 44.98 (C9), 47.94 (C13), 50.36 (C14), 83.72 (C17), 120.23 (C2), 123.06 (C4), 125.30 (C3″ and C5″), 127.44 (C1), 131.86 (C2″), 137.28 (C1″), 139.36 (C10), 139.91 (C5), 149.10 (C4″), 149.46 (C3); FAB-HRMS calcd for $C_{29}H_{38}O_4NS$ (M^+ + H) 496.25217, found 496.24930. Anal. Calcd $C_{29}H_{39}O_4NS$: C, 69.99; H, 7.90; N, 2.80; S, 6.44. Found: C, 70.31; H, 8.20; N, 2.85; S, 6.51.

B. Steroid Sulfatase Assays. Human embryonal kidney (293) cells (American Type Culture Collection, Rockville, MD), transiently transfected with a sulfatase expression vector (pCMV-sulfa), were used as the source of sulfatase activity. The pCMV-sulfa was constructed by insertion of a cDNA fragment, downstream of 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/10^6 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 10^4 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 of streptomycin sulfate/mL.

Inhibition of Steroid Sulfatase (homogenized cells). Transfected (293) cell homogenate was prepared by repeated freezing (−80 °C) and thawing (five times) and homogenization using a Dounce homogenizer. The reaction was carried out at 37 °C in 1.25 mL of 100 mM Tris-acetate buffer (pH 7.4) containing 5 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 100 μM [^{3}H]E_1S or [^{14}C]DHEAS as substrate, and an ethanolic solution of compound to test (at appropiate 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 2000*g* for 10 min to separate the organic and aqueous phases. Radioactivity in 750 μL of each phase (organic, free steroids; aqueous, sulfated steroids) was determined by liquid scintillating counting with a Beckman LS3801 instrument (Irvine, CA). The IC_{50} values were determined using the DE_{50} program (CHUL Research Center, Québec).

Reversibility or Irreversibility of Inhibitors (intact cells). In a 6-well culture plate containing transfected (293) cells, inhibitor **1** (EMATE) or **5** was added at the concentration of 5 μM to freshly changed culture medium and incubated for 1 h at 37 °C. After incubation, the inhibitor was removed by washing three times with DMEM culture medium. Radiolabeled substrate ([^{3}H]E_1S) was then added to the transfected (293) cell culture and further incubated for 18 h. Thereafter, the unsulfated steroids were extracted from extracellular medium twice with 2 mL of diethyl ether. The organic phases were pooled and evaporated to dryness. The crude extract was solubilized in 50 μL of CH_2Cl_2, applied on a TLC plate of silica gel 60 (Merck, Darmstad, GE), and separated by migration in toluene/acetone (8:2) as eluent. [^{3}H]E_1 was identified by comparison with unlabeled E_1 as reference, revealed by autoradiography, and quantified using a Phosphoimager System (Molecular Dynamics, Sunnyval, CA).

Reversibility or Irreversibility of Inhibitors (homogenized cells). Microsomes of (293) cells transfected with steroid sulfatase were prepared by two successive centrifugations of a sonicated cell suspension at 10000*g* for 15 min and 100000*g* for 30 min. The microsomal fraction (100000*g* pellet) was resuspended in 100 mM phosphate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol. Time-dependent inactivation of the enzyme was performed by preincubation with inhibitor **3** (0.1 μM), **5** (1 nM), or EMATE (0.1 μM) for 0−20 min at 37 °C, followed by incubation with 0.5% dextran-coated charcoal for 15 min. The concentrations indicated have been determined by preliminary experiments. Dextran-coated charcoal was sedimented by centrifugation (2000*g*), and the supernatant was used to determine the steroid sulfatase activity as described above for the incubation with homogenized cells using [^{3}H]E_1S as substrate, except that phosphate buffer was used. In the substrate protection assays, 100 μM E_1S was added along with the inhibitor in the preincubation period.

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