Steroidal Affinity Labels of the Estrogen Receptor. 3. Estradiol 11β-*n*-Alkyl Derivatives Bearing a Terminal Electrophilic Group: Antiestrogenic and Cytotoxic Properties

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With the aim of developing a new series of steroidal affinity labels of the estrogen receptor, six electrophilic 11β-ethyl (C_2), 11β-butyl (C_4), or 11β-decyl (C_{10}) derivatives of estradiol bearing an 11β-terminal electrophilic functionality, i.e. bromine (C_4), (methylsulfonyl)oxy (C_2 and C_4), bromoacetamido (C_2 and C_4), and (*p*-tolylsulfonyl)oxy (C_{10}), were synthesized. The range of their affinity constants for binding the estrogen receptor was 0.4−37% that of estradiol; the order of increasing affinity (i) relative to the 11β-alkyl arm was ethyl < butyl and (ii) relative to the electrophilic functionality was bromoacetamido < bromine < (methylsulfonyl)oxy. Regardless of the conditions used, including prolonged exposure of the receptor to various pH levels (7−9) and temperatures (0−25 °C), the extent of receptor affinity labeling by the 11β-ethyl and 11β-butyl compounds, if any, was under 10%. This was in sharp contrast to results obtained using 11β-((tosyloxy)decyl)estradiol which labeled from 60% to 90% of the receptor hormone-binding sites with an EC_{50} of ∼10 nM. Estrogenic and antiestrogenic activities of the compounds were determined using the MVLN cell line, which was established from the estrogen-responsive mammary tumor MCF-7 cells by stable transfection of a recombinant estrogen-responsive luciferase gene. The two 11β-ethyl compounds were mainly estrogenic, whereas the three 11β-butyl and the 11β-decyl compounds essentially showed antiestrogenic activity. The fact that the chemical reactivities of 11β-ethyl and 11β-butyl compounds were not compromised by interaction with the estrogen receptor made the synthesized high-affinity compounds potential cytotoxic agents which might be able to exert either (i) a specific action on estrogen-regulated genes or (ii) a more general action in estrogen-target cells. Therefore the ability of the compounds (1) to irreversibly abolish estrogen-dependent expression of the luciferase gene and (2) to affect the proliferation of MVLN cells were determined. All electrophiles were able to irreversibly suppress expression of the luciferase gene; the antiestrogenic electrophiles were more potent than the estrogenic ones but less efficient than 4-hydroxytamoxifen, a classical and chemically inert triphenylethylene antiestrogen. Only the antiestrogenic electrophiles decreased cell proliferation; however, they were less potent than 4-hydroxytamoxifen. In conclusion, the synthesized electrophilic estradiol 11β-ethyl and 11β-butyl derivatives (i) were not efficient affinity labels of the estrogen receptor and (ii) did not display significant cytotoxicity in estrogen-sensitive mammary tumor cells. However, since these derivatives displayed high affinity for the estrogen receptor, they could be used to prepare potential cytotoxic agents which might be selective for tumors affecting estrogen-target tissues, by coupling them with a toxic moiety.

Introduction

The nuclear receptor superfamily comprises[1,2] more than 100 structurally related proteins which act as transcriptional regulators. Members of this family possess two main functional domains: the DNA-binding domain and the hormone-binding domain which include ∼70 amino acids and ∼250 amino acids, respectively, and are the most conserved sequences in these proteins. The DNA-binding domain structures of estrogen and glucocorticoid receptors were established in the early 1990s on the basis of NMR[3,4] and crystallographic studies.[5] Structures of hormone-binding domains of human unliganded retinoic X receptor α (RXRα),[6] liganded retinoic receptor γ (RARγ),[7] and rat thyroid hormone receptor α_1 ($TR\alpha_1$)[8] were recently proposed from crystallographic data. Using information obtained from the crystal structure of RARγ, and through a general sequence alignment of ligand-binding domains of nuclear receptors, Wurtz *et al.*[9] inferred a putative general structure of the ligand-binding pocket of nuclear receptors.

Affinity labeling of receptors provides a means to directly identify amino acids of the hormone-binding pocket which are in contact with or in close proximity to the ligands. Concerning the estrogen receptor, such an approach was first used by J. Katzenellenbogen's group who developed several series of nonsteroidal affinity- and photoaffinity-labeling agents of the receptor.[10−14] Recently, we described affinity labeling of the estrogen receptor by electrophilic estradiol 17α-derivatives.[15,16] Covalent attachment sites of tamoxifen

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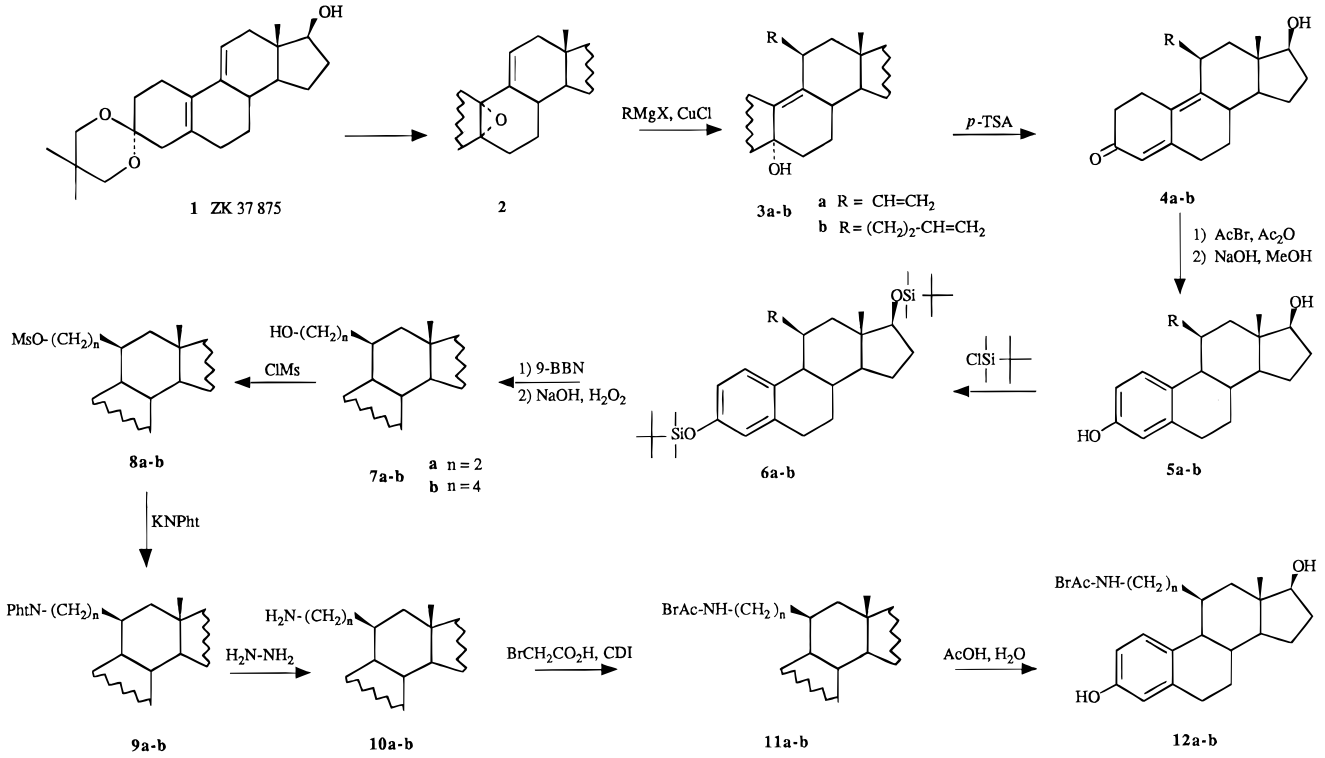
Scheme 1



aziridine, a nonsteroidal antiestrogen, and 17α-[(haloacetamido)alkyl]estradiols were identified in the human estrogen receptor. Three out of the four invariant cysteines (C381, C417, C447, and C530 in the human estrogen receptor) of the hormone-binding domain were found to be the covalent attachment sites of these electrophiles: C381 and C530 were alkylated by tamoxifen aziridine,[17,18] whereas C417 and C530 were alkylated by the estradiol 17α-derivatives.[19] These results were in agreement with the general structure proposed for the ligand-binding pocket of nuclear receptors since the three cysteines were located at the extreme border or in structural elements involved in delineation of the hormone-binding pocket. Moreover, they suggested that the conformation of the antiestrogen-liganded receptor differs from those of unfilled and estrogen-filled receptors. Finally, they enabled development of a selective mode of superimposition of tamoxifen-class antiestrogens with estradiol, which can account for the relative positioning of the two types of ligands in the hormone-binding pocket.[19]

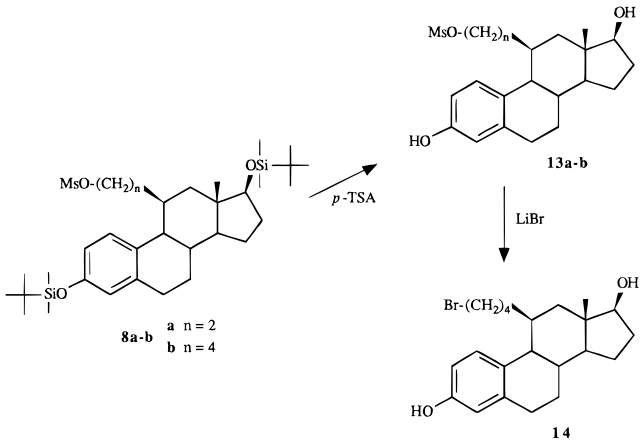
Studies performed by Belanger et al.[20] evinced, for the first time, the very high affinity of 11β-aliphatic or aromatic derivatives of 17α-ethynyl estradiol for the estrogen receptor. These results suggested that large 11β-substituents of steroidal ligands are tolerated by the hormone-binding pocket of the estrogen receptor. Therefore, with the aim of developing 11β-derived steroidal affinity labels which display high affinity for the estrogen receptor and would be useful for gaining further information concerning the positioning of ligands in the hormone-binding pocket of the receptor, we prepared six new estradiol 11β-derivatives in which an 11β-ethyl, 11β-butyl, or 11β-decyl chain was substituted with various electrophilic functionalities: bromine, mesyloxy, tosyloxy, or bromoacetamido. We then determined their binding affinity and ability to become irreversibly bound to the receptor. Finally, we report their hormonal activity, their ability to irreversibly abolish the expression of a recombinant estrogen-responsive gene, and to affect the growth of estrogen-sensitive mammary tumor cells.

Results

Synthesis of Electrophilic Estradiol 11β-Derivatives. To obtain the projected estradiol 11β-derivatives, we used the regio- and stereospecific synthesis pathway first described by Belanger *et al.*,[20] whereby a 5α,10α-epoxyestr-9(11)-ene is submitted to the action of Grignard reagents in the presence of a catalytic amount of copper chloride. 3,3-[(2,2-dimethyltrimethylene)dioxy]-estra-5(10),9(11)-dien-17β-ol (**1**) and 3,3-(ethylenedioxy)-estra-5(10),9(11)-dien-17-one (**15**) were used as starting materials for the preparation of electrophilic estradiol derivatives, including bromoacetamido compounds **12a** and **12b** (Scheme 1), (methylsulfonyl)oxy compounds **13a** and **13b**, bromobutyl compound **14** (Scheme 2), and tosylate **20** (Scheme 3). Preparation of epoxy 5α,10α-derivatives **2** and **16** from **1** and **15** were previously described.[21,22] In the presence of a catalytic amount of copper chloride, the addition of vinyl- or butenylmagnesium chloride to epoxide **2** only afforded the 11β-substituted steroid **3a** or **3b** (Scheme 1). The action of *p*-toluenesulfonic acid, through simultaneous deketalization and dehydration of the compound, converted **3a,b** into dienone **4a,b**. The A ring of the compound was then aromatized by treatment with a mixture of acetyl bromide and acetic anhydride. This treatment acylated the phenol function, which was regenerated by saponification.

The phenolic and 17β-hydroxy functions of **5a,b** were protected as TBDMS ethers to give **6a,b**. The terminal vinyl or butenyl function was submitted to hydroboration−oxidation conditions (9-BBN and basic hydrogen peroxide) to afford the primary alcohol **7a,b**. The primary hydroxyl group was esterified by methanesulfo-

Scheme 2



nyl chloride to give **8a,b**. Action of potassium phthalimide on mesylate **8a,b** afforded phthalimide **9a,b**, which was hydrolyzed into primary amine **10a,b**. The corresponding bromoacetamide **11a,b** was obtained by condensation with bromoacetic acid in the presence of carbodiimide. Removal of the TBDMS group from **11a,b** with a mixture of acetic acid and water in THF afforded 11β-[(bromoacetamido)alkyl]estradiol **12a,b**. Removal of the TDMS groups from **8a,b** gave 11β-[[(methylsulfonyl)oxy]alkyl]estradiol **13a,b** (Scheme 2). Action of LiBr on **13b** gave 11β-(bromobutyl)estradiol **14**.

In the presence of copper chloride, addition of a TBDMS derivative of (hydroxydecyl)magnesium bromide to epoxide **16** afforded the silylated 11β-hydroxydecyl steroid **17** (Scheme 3). The sequential action of $NaBH_4$ and acetic anhydride converted the 17-keto function of compound **17** into a 17β-acetoxy function, whereas action of hydrochloric acid through simultaneous deprotection of the primary alcohol and elimination of the tertiary alcohol gave dienone **18**. The primary alcohol was esterified with *p*-toluenesulfonyl chloride, and the A ring of the compound was aromatized with a mixture of acetic bromide and acetic anhydride to give **19**. Tosylate **20** with free 3- and 17β-hydroxy functions was obtained from **19** by saponification.

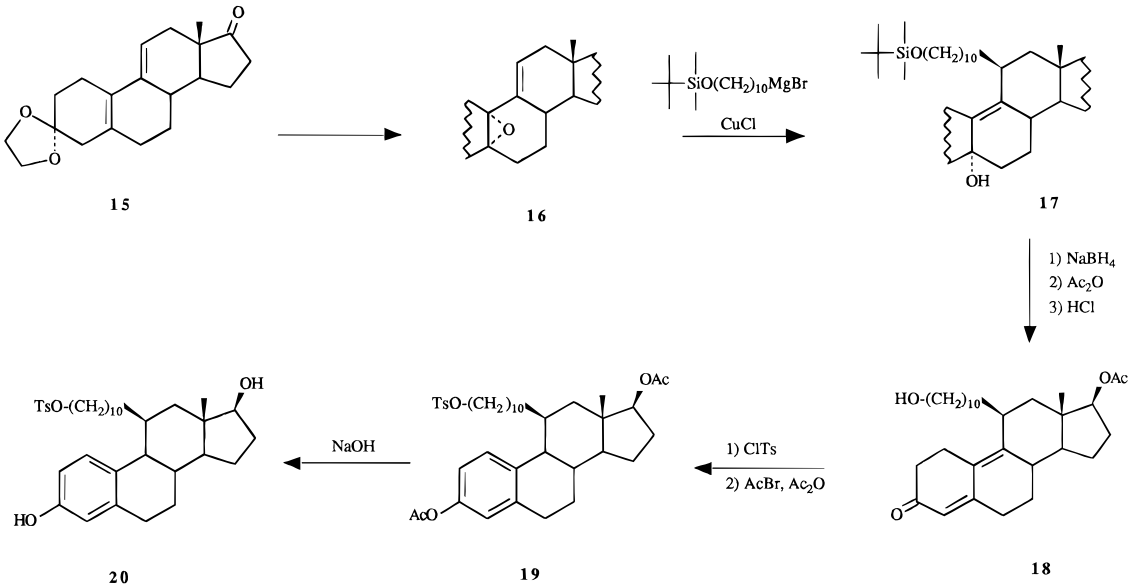
Table 1. Reversible and Irreversible Binding of Electrophilic Estradiol 11β-Derivatives[a] to the Estrogen Receptor

compound	11β-alkyl chain	apparent RAC	covalent binding
estradiol		100	
butene **5b**	C_4	38.60 ± 0.80	
bromoacetamide **12a**	C_2	0.38 ± 0.05	−
bromoacetamide **12b**	C_4	1.64 ± 0.28	−
mesylate **13a**	C_2	8.15 ± 0.10	−
mesylate **13b**	C_4	36.80 ± 0.98	−
bromide **14**	C_4	11.00 ± 3.70	−
tosylate **20**	C_{10}	2.59 ± 0.12	+

[a] Apparent RACs (relative affinity constants) of compounds for the cytosolic estrogen receptor were determined by competitive binding (20 h, 20 °C) radiometric assay using [^{3}H]estradiol as tracer, as described in the Materials and Methods; data given are means of duplicate determinations ± SD in two to four separate experiments. Covalent binding of compounds to the estrogen receptor was established from their ability to irreversibly inhibit binding of [^{3}H]estradiol (cf. Results, irreversible binding to the estrogen receptor) symbol (−) means that the decrease in estradiol-binding sites inactivated by the compound, if any, was <10%.

Estrogen Receptor Binding Affinity. The apparent affinity of synthesized compounds for the lamb estrogen receptor was determined by competitive binding radiometric assay using [^{3}H]estradiol as tracer. At 20 °C the competition level between [^{3}H]estradiol and the compounds did not significantly change after 20 h incubation, suggesting that binding equilibrium was reached under these conditions. Table 1 gives the corresponding apparent affinity constants of the compounds, relative to that of estradiol (100%), which were calculated according to Korenman.[23] Affinities of the compounds (from 0.4% to 37%) were much lower than those reported for other 11β-derivatives of estradiol including both small-group-substituted, e.g. chloromethyl,[24] vinyl,[25] or ethyl[25,26] molecules, or large-group-substituted, e.g. *N*-propyl- or *N*-butylundecanamide, molecules.[27] Affinities of the electrophilic estradiol derivatives markedly varied according to the length of the 11β-*n*-alkyl arm and to the substituent on the ϵ carbon of the alkyl chain. Affinities increased when the ethyl chain was changed to the *n*-butyl chain (compare **12a** with **12b** and **13a** with **13b**) and when the $NHCOCH_2Br$ group was changed to Br and then to the

Scheme 3



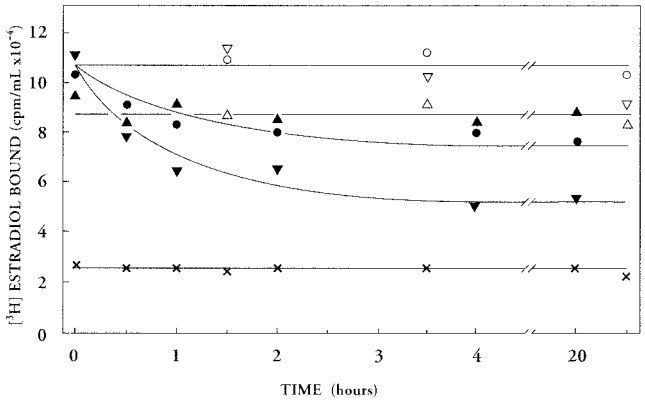


Figure 1. Time course of inactivation of specific estradiol-binding sites in lamb uterine cytosol by 11β-[(tosyloxy)decyl]-estradiol. Uterine cytosol (4 mg of protein/mL, pH 8.5) was incubated at 0 or 25 °C without steroid, with 50 nM estradiol or 50 nM tosylate **20**. After various periods of time, aliquots were removed and treated for 30 min at 0 °C with an equal volume of charcoal suspension. Charcoal was pelleted by centrifugation, then the total and nonspecific binding of [^{3}H]-estradiol occurring in the supernatant under exchange conditions were determined by the standard irreversible binding assay, as described in the Materials and Methods. The total binding of [^{3}H]estradiol in supernatants corresponding to cytosol incubated at 0 °C (open symbols) or 25 °C (close symbols) without steroid (○, ●), with estradiol (△, ▲), or tosylate **20** (▽, ▼) and the nonspecific binding of [^{3}H]estradiol (×) which did not significantly vary according to the compound incubated with cytosol, are represented as functions of the incubation time. Values are means of duplicate determinations; experimental variation was under 10%.

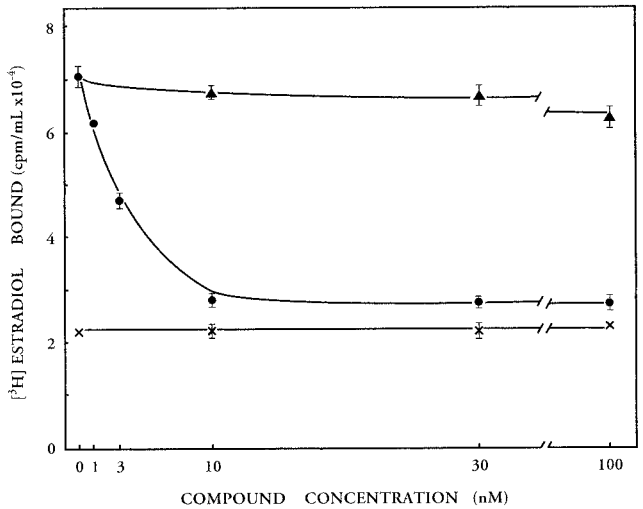


Figure 2. Concentration-dependent inactivation of specific estradiol-binding sites by 11β-[(tosyloxy)decyl]estradiol. Uterine cytosol was incubated for 4 h at 25 °C without steroid or with increasing concentrations of estradiol or tosylate **20**. After charcoal treatment and then centrifugation, supernatant aliquots were incubated under exchange conditions with [^{3}H]-estradiol. The total binding of [^{3}H]estradiol in supernatant corresponding to cytosol incubated with estradiol (▲) or tosylate **20** (●) and the nonspecific binding of [^{3}H]estradiol (×) which did not significantly vary according to the steroid incubated with the cytosol, are represented as functions of the steroid concentration. Values are means of triplicate determinations; error bars indicate standard deviations.

OSO_2CH_3 group (compare **12b**, **13b**, and **14**). Due to irreversible binding of the compound to the estrogen receptor (cf. following section), the 2.6% value determined for the 11β-decyl derivative **20** affinity was probably an overestimate of the true affinity constant of the compound.

Irreversible Binding to the Estrogen Receptor. The estrogen receptor-alkylating activities of electrophilic estradiol derivatives were evaluated from their ability to irreversibly inhibit estradiol specific binding in cytosol.[15] Affinity labeling of the receptor is usually a concentration- and time-dependent process which, depending of the type of electrophile, could be influenced by temperature and pH. To determine the alkylation level of the receptor, the cytosol was thus incubated at various pH values (from 7 to 9) and at either 0 or 25 °C for increasing times with the electrophilic estradiol 11β-derivatives, at a concentration usually equivalent to 20 nM estradiol; excess compound was removed and the concentration of estrogen binding sites was determined by incubating the cytosol with [^{3}H]estradiol under exchange conditions. Cytosol was concomittantly incubated without steroid, with unlabeled estradiol or with the high-affinity, but chemically inert, compound **5b**, to obtain reference values for the concentration of estrogen binding sites in cytosol. Any decrease from these values elicited by one of the electrophiles would characterize an estrogen receptor affinity labeling agent.[15] Results obtained with tosylate **20** (Scheme 3) indicated that at 25 °C, but not at 0 °C, this compound was an efficient affinity labeling agent of the receptor. Figure 1 shows variations in the concentration of binding sites for [^{3}H]estradiol in cytosol incubated for increasing times at pH 8.5 and 0 or 25 °C with 50 nM tosylate **20**. At 0 °C, the compound did not elicit any marked time-dependent decrease in the capacity of cytosol to bind [^{3}H]estradiol. However, a substantial decrease occurred at 25 °C, and the concentration of binding sites reached a plateau from 4 h, which accounted for 50% and 40% of the concentration of binding sites in the controls (cytosol incubated without steroid, in which unprotected binding sites were progressively heat-inactivated at 25 °C, and also cytosol incubated with 50 nM unlabeled estradiol, in which protected binding sites were stable at 25 °C). The effect of compound **20**, not dependent on the cytosol pH, increased with the compound concentration, reaching a quasi-optimal level (70−90% of binding sites inactivated) for 10 nM **20** (Figure 2). Moreover, inactivation of estrogen-binding sites by compound **20** was not prevented by methyl methanethiosulfonate, an SH group specific reagent, suggesting that the covalent attachment site of the compound was not a cysteine residue. Conversely, none of the other electrophilic estradiol 11β-derivatives (at a concentration equivalent to 20 nM estradiol) was able to induce, at 0 or 25 °C and within the 7−9 pH range, a marked decrease in the capacity of cytosol to bind estradiol, even after a prolonged exposure (20 h) of cytosol to the compound. The potential decrease in the concentration of binding sites, if any, was always under 10% (Table 1). Therefore, in the estradiol 11β-alkyl-substituted series, only compounds with a long electrophile-bearing arm appeared to be able to specifically alkylate the estrogen receptor.

Estrogenic and Antiestrogenic Properties. To determine the estrogen agonist/antagonist activity of estradiol 11β-derivatives, we used the MVLN cell line,[28] which was derived from estrogen-responsive MCF_7 cells by stable transfection of the p-Vit-tk-Luc plasmid. For a large series of estrogenic or antiestrogenic compounds, it was previously shown that the bioluminescent response of these cells was qualitatively similar to natural

Table 2. Estrogenic/Antiestrogenic and Antiproliferative Activities of Electrophilic Estradiol 11β-Derivatives

compound	11β-alkyl chain	estrogen agonism[a] %	estrogen agonism[a] EC_{50} (nM)	estrogen antagonism[a] %	estrogen antagonism[a] IC_{50} (nM)	luciferase inactivation[b] $t^{1/2}$ (days)	antiproliferative activity[c] (%)
butene **5b**	C_4	80	<10			>30	20
bromoacetamide **12a**	C_2	90	110			>30	5
bromoacetamide **12b**	C_4			90	320	20	90
mesylate **13a**	C_2	95	6			>30	10
mesylate **13b**	C_4			95	6	22	90
bromide **14**	C_4			85	3	26	80
tosylate **20**	C_{10}			95	4	13	85
4-hydroxytamoxifen				100	7	10	100

[a] The estrogen agonism or antagonism of compounds was determined from experiments similar to those described in the Figure 3 legend. For estrogen agonists, the percent value indicates the maximal luciferase specific activity induced by the compound relative to that induced by 0.1 nM estradiol (100%); the EC_{50} value is the compound concentration required to induce a luciferase specific activity equal to 50% of that induced by 0.1 nM estradiol. For estrogen antagonists, the percent value indicates the maximal inhibition of luciferase specific activity induced by 0.1 nM estradiol; the IC_{50} value is the compound concentration required to inhibit by 50% the luciferase specific activity induced by 0.1 nM estradiol. Values are means of two independent determinations; experimental variation was under 5% (percent values) or 30% (EC_{50} or IC_{50} values). [b] Experiments similar to those described in the Figure 4 legend were performed for 7, 14, 21, and 28 days of treatment to determine the kinetics of luciferase irreversible inhibition of expression. The time for half-irreversible inhibition was obtained by interpolation or extrapolation of experimental curves (logarithm of luciferase specific activity percent plotted against the treatment time); experimental variation was under 30%. [c] MVLN cells were grown for 7 days without compound, in the presence of 0.1 nM estradiol or in the presence of 100 nM estradiol 11β-derivatives or 4-hydroxytamoxifen. Cells were processed as described in the Materials and Methods. Antiproliferative activity was calculated from the difference between DNA amounts measured in wells exposed to estradiol and in wells exposed to estradiol 11β-derivatives. It was expressed as a percentage of antiproliferative activity determined for 4-hydroxytamoxifen. Values are means of triplicate determinations; experimental variation was under 10%.

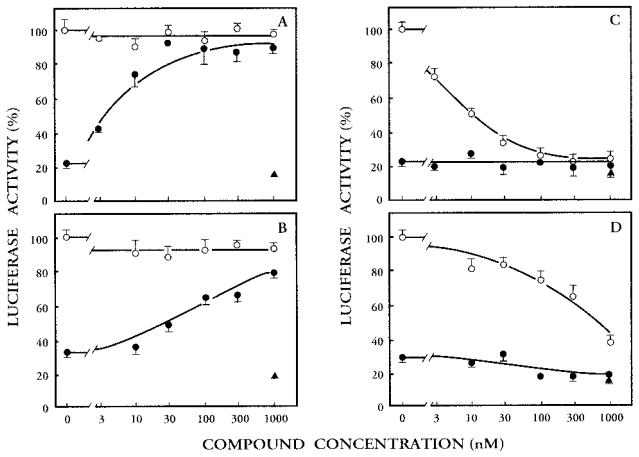


Figure 3. Estrogenic and antiestrogenic activities of electrophilic estradiol 11β-derivatives in MVLN cells. MVLN cells were incubated for 24 h in FCS/C medium with 1 μM 4-hydroxytamoxifen (▲) or with increasing concentrations of mesylate **13a** (A), bromoacetamide **12a** (B), mesylate **13b** (C) or bromoacetamide **12b** (D) in the absence (●) and presence (○) of 0.1 nM estradiol. Luciferase activity and protein concentration of cell homogenates were then determined. Specific luciferase activity, expressed as a percentage of the specific luciferase activity corresponding to cells incubated with estradiol alone, is plotted against the compound concentrations. Values are means of duplicate determinations; error bars indicate standard deviations.

estrogenic responses such as progesterone receptor induction.[29]

Estrogenic and antiestrogenic activities of compounds were determined separately by incubating the cells for 24 h with increasing concentrations of compounds in the absence and in the presence of 0.1 nM estradiol, respectively. Activity of the electrophilic compounds (Figure 3, Table 2) was not correlated with the whole 11β-substituent, but rather with the alkyl part of the substituent, since the two estradiol 11β-ethyl derivatives **12a** and **13a** mainly displayed estrogenic activity, whereas the three estradiol 11β-butyl derivatives **12b**, **13b**, and **14** and the 11β-decyl derivative **20** showed almost pure antiestrogenic activity. The relative efficiency of compounds to induce estrogenic expression of the luciferase gene was in line with their relative affinities for the estrogen receptor, whereas the efficiency of antiestrogenic compounds to inhibit expression of the gene was not correlated with their affinity for the receptor (compare affinities and activities of **12b** and **20** for instance).

Irreversible Inactivation of the Luciferase Gene. Long-term treatment of MVLN cells with antiestrogens was shown to progressively abolish the ability of cells to express the luciferase gene in response to estrogens.[30] This irreversible effect, which seems to result from an epigenetic mechanism, such as methylation or chromatin remodeling rather than gene mutation (Badia *et al.*, submitted), progressively affected the whole cell population and occurred by a first-order process, with a half-inactivation time of ~10 days in the case of 4-hydroxytamoxifen. Since the electrophilic estradiol 11β-derivatives **12a**−**14** displayed high affinity and did not react *in vitro* with the estrogen receptor, they could be concentrated (via the receptor) in the nucleus of estrogen target cells where, by virtue of their chemical reactivity, they could exert general cytotoxicity or targetted cytotoxicity toward for instance estrogen-regulated genes involved in cellular proliferation (i.e. genes coding for growth factors, growth factor receptors, protooncogenes, etc.). To clarify this possibility, we first investigated the ability of the compounds to inactivate the luciferase gene of MVLN cells. All of the electrophilic estradiol 11β-derivatives decreased the ability of cells to express luciferase in response to estradiol. During a 7-day cell treatment, only the antiestrogenic compounds **12b**, **13b**, and **14** elicited a significant loss (≥20%) in luciferase inducibility (not shown); whereas no marked inactivation was observed with the estrogenic compounds **5b, 12a**, and **13a**. During a 21-day treatment, all of the compounds were found to be efficient; there was 20−75% less luciferase inducibility depending on the compound (Figure 4). The results indicated that (i) the compounds were less efficient than 4-hydroxytamoxifen (Table 2) and (ii) estrogen antagonism or the electro-

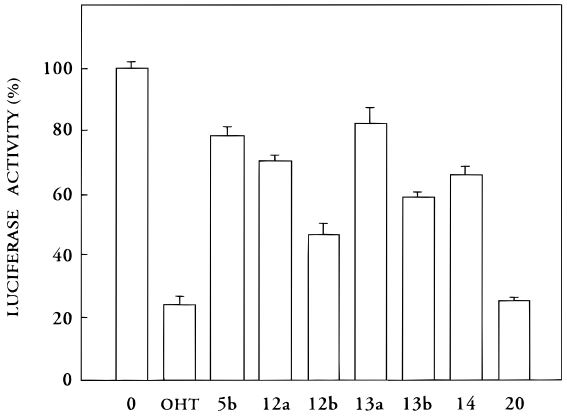


Figure 4. Irreversible inhibition of estrogen-induced expression of luciferase in MVLN cells by estradiol 11β-derivatives. MVLN cells were grown for 21 days in FCS/C medium in the absence of compounds (0) or in the presence of 100 nM 4-hydroxytamoxifen (OHT), butene **5b**, bromoacetamide **12a** or **12b**, mesylate **13a** or **13b**, bromide **14**, or tosylate **20**. Cells were then rinsed and incubated for 7 days in FCS/C medium containing 100 nM estradiol. Luciferase activity and protein concentration of cell homogenates were then determined. Specific luciferase activities (expressed as percents of the specific luciferase activity corresponding to cells not exposed to 4-hydroxytamoxifen or estradiol 11β-derivatives) are represented. Values are means of duplicate determinations; error bars indicate standard deviations.

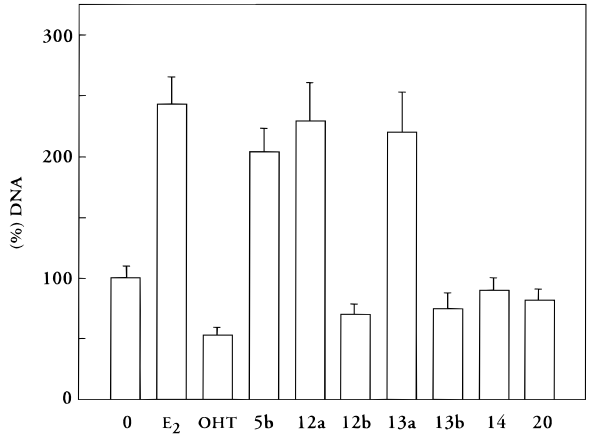


Figure 5. Proliferative and antiproliferative activities of estradiol 11β-derivatives. MVLN cells were grown for 7 days in FCS/C medium in the absence of compounds (0) or in the presence of 0.1 nM estradiol (E_2), 100 nM 4-hydroxytamoxifen (OHT), butene **5b**, bromoacetamide **12a** or **12b,** mesylate **13a** or **13b**, bromide **14**, or tosylate **20**. The well DNA contents were then determined. Values are means of triplicate determinations; error bars indicate standard deviations.

philic character of the compound was not required to induce the inactivation process since the mainly estrogenic and nonelectrophilic compound **5b** was able to inactivate the gene. We then tested the electrophilic compounds using the ETL cell line, obtained from MCF-7 cells by stable transfection of a plasmid including an ERE-tk-Luc sequence (P. Balaguer *et al.*, unpublished results) instead of the p-Vit-tk-Luc sequence used to generate the MVLN cell line. Contrary to MVLN, expression of the luciferase gene of ETL cells was not profoundly suppressed by antiestrogens such as 4-hydroxytamoxifen; therefore this cell line could be useful for determining whether estrogen receptor-targetted, chemically reactive molecules could inactivate estrogen-regulated genes. After a 14-day treatment with the different electrophiles, the ability of ETL cells to express luciferase in response to estradiol was not markedly changed (not shown). We therefore concluded that estrogen receptor ligands bearing a single electrophilic group on a 11β-alkyl substituent of estradiol-related molecules did not seem to be able to inactivate estrogen target genes.

Antiproliferative Activity. The proliferative or antiproliferative activity of compounds was evaluated from their ability to promote or prevent DNA accumulation from MVLN cells grown for 7 days in "steroid-free" medium. Contrary to estradiol, the four antiestrogenic derivatives **12b**, **13b**, **14**, and **20** did not promote MVLN cell proliferation (Figure 5); however, the decrease in DNA accumulation they elicited was significantly lower than that induced by 4-hydroxytamoxifen. Conversely, the three estrogenic derivatives **5b**, **12a,** and **13a** were almost as efficient as estradiol in stimulating cell proliferation.

Discussion

Despite a much higher affinity for the estrogen receptor than estradiol 17α-electrophilic derivatives,[15,16] all five new synthesized steroidal electrophiles, related either to 11β-ethylestradiol or 11β-butylestradiol, displayed no significant estrogen receptor affinity labeling activity, since any of these compounds was able to elicit a significant decrease in the concentration of estradiol binding sites in cytosol. Conversely tosylate **20** induced a strong decrease in the estradiol-binding site concentration. This effect very probably reflected the covalent attachment of the compound in the receptor hormone-binding site. Indeed, another 11β-derivative, i.e. 11β-(chloromethyl)estradiol was initially thought to be an affinity-labeling agent of the receptor[31−33] based on its apparent inability to be displaced by estradiol. It was later demonstrated that this compound was in fact a reversible ligand;[24] however, due to its very high affinity for the receptor, 10−30-fold higher than that of estradiol, its dissociation rate and therefore exchange rate under typical assay conditions were negligible. In contrast with the high affinity of such ligands as 11β-(chloromethyl)estradiol, the binding affinity constant of tosylate **20** appeared at least 38-fold lower than that of estradiol. Since the value of the affinity constant mainly reflects the compound dissociation rate from receptor, the decrease of estradiol binding-site concentration following incubation of the receptor with tosylate **20** very probably did not result from low dissociation rate of noncovalently receptor-bound compound but rather from covalent attachment of the compound in the receptor hormone-binding pocket. Therefore we concluded that tosylate **20,** like electrophilic estradiol 17α-derivatives, was a very efficient receptor affinity labeling agent. The fact that none of the five compounds **12a**−**14**, whose electrophilic carbon is two to seven C−C or C−N bonds away from C-11, was able to alkylate the receptor suggests that there was no reactive nucleophilic amino acid residue in the close vicinity of the C ring when the steroidal ligands were in the hormone-binding pocket. Conversely, affinity labeling of the receptor by compound **20,** whose electrophilic centre is 10 C−C bonds away from C-11, suggests that at least one nucleophilic amino acid residue was in the remote vicinity of the C ring. The length of the decyl substituent in compound **20** allowed the electrophilic carbon to

potentially scan a large volume centered at C-11. When compound **20** was in the receptor hormone-binding pocket, the location of the electrophilic carbon relative to the steroid nucleus therefore could not be *a priori* ascertained. For instance, although the original orientation of the substituent at C-11 is above and roughly perpendicular to the median plane of the steroid, the terminal carbon of the decyl chain could still be located under the median plane of the steroid. The uncertainty concerning the relative location of the electrophilic carbon reduces the interest of the compound for analysis of the hormone-binding pocket of the receptor. Nevertheless, the inability of methyl methanethiosulfonate to prevent affinity labeling of the receptor and pH-independence of the process suggest that, contrary to tamoxifen aziridine, ketononestrol aziridine, and 17α-[(bromoacetamido)alkyl]estradiols, the covalent attachment site(s) of tosylate **20** did not occur at cysteine residues. In the hormone-binding pocket of the receptor, the environment of the electrophilic carbon of compound **20** thus differed from that of electrophilic centers of tamoxifen aziridine, ketononestrol aziridine, and 17α-[(bromoacetamido)alkyl]estradiols.

The estradiol electrophilic 11β-derivatives proved to be either estrogenic (11β-ethyl compounds) or antiestrogenic (11β-butyl and 11β-decyl compounds). The fact that the type of hormonal/antihormonal activity was related to the alkyl part of the 11β-substituent but not to the size of the whole substituent (which was for estrogenic compounds **12a** and **13a** similar to or higher than that of antiestrogenic compound **14**) or to the type of terminal electronegative group (**12a** and **12b** on one hand and **13a** and **13b** on the other displayed opposite hormonal activities, even though they had the same terminal function) was astonishing. This could result (i) from intrinsic but still unexplained properties of the compounds or (ii) from hydrolysis of the compounds in MVLN cells. In cytosolic extracts, all electrophiles appeared to be fairly stable, with only a decrease in the amount of extractible compounds according to time or temperature, which was probably due to reaction of the compounds with cytosolic nucleophiles. However, since no such radioactive compounds were available, it was difficult to study their potential metabolism or hydrolysis within MVLN cells at the concentrations used. Note that hydrolysis could convert the bulky 11β-substituents of **12a** and **13a** into smaller ones, i.e. aminoethyl and hydroxyethyl, respectively. For homologous compounds **12b** and **13b,** the relative decrease in size resulting from hydrolysis would be less pronounced. In any case, with estradiol 11β-substituted derivatives, it appears that the threshold which separates estrogenic from antiestrogenic compounds is rapidly reached when the size of the 11β-alkyl chain increases, with a threshold between C-2 and C-4.

According to the two criteria used, i.e. inactivation of the luciferase gene and inhibition of MVLN cell growth, the six electrophiles did not appear to exert cytotoxic activity in these cells. All electrophiles, whether they were estrogen agonists or antagonists, abolished expression of the luciferase gene, and those displaying antiestrogenic activity also decreased cell proliferation. However, to induce these two effects, the compounds were less efficient than 4-hydroxytamoxifen, a chemically inert compound whose effects are probably due to its antiestrogenicity. It is thus difficult to determine whether part of the observed effects were due to the chemical reactivity of the compounds. The fact that luciferase gene expression in ETL cells was not markedly abolished by the compounds is another argument favoring the absence of specific cytotoxicity of the compounds. Nevertheless, the two interesting characteristics of 11β-ethyl- or 11β-butylestradiol derivatives, i.e. high affinity for the estrogen receptor and lack of neutralizing chemical reactivity by the receptor, make them a choice support for grafting a cytotoxic moiety such as nitrosoureas, nitrogen mustards, intercalating drugs, platinum complexes, or enediyne structures, which are used in antitumoral therapy but whose general toxicity is high due to their lack of selectivity. Such hybrid estradiol-cytotoxic molecules, which would display both high affinity for the estrogen receptor and strong cytotoxic activity, might be useful agents for the treatment of estrogen receptor-containing mammary tumors. Work is presently in progress in our laboratory to develop such potential selective antitumor agents.

Materials and Methods

Chemical. Synthesis and Characterization of Estradiol Derivatives. Starting materials were 3,3-[(2,2-dimethyltrimethylene)dioxy]estra-5(10),9(11)-dien-17β-ol (**1**, ZK 37 875) (obtained from Professor Wiechert of Schering) and 3,3-(ethylenedioxy)estra-5(10),9(11)-dien-17-one (**15**).[21] Starting reactions were carried out under an argon atmosphere with dry solvents used under anhydrous conditions. Melting points (mp) were determined on a Bioblock melting point apparatus and were uncorrected. Infared spectra (IR) were recorded on a Perkin-Elmer 983 spectrophotometer by means of potassium disks. Proton magnetic resonance (^{1}H NMR) spectra were recorded at 32 °C on a Bruker WM 360 WB spectrometer. Except for compounds **5a** and **12b** (which were solubilized in DMSO-d_6), compounds were dissolved in $CDCl_3$ from which the residual signal was taken as reference at 7.24 ppm for ^{1}H. Except when otherwise stated, column chromatography was performed with silica gel (0.063−0.2 mm). Thin-layer chromatography (TLC) was performed using 0.25 mm silica gel plates with F-254 indicator. Elemental analyses were determined for C, H, and O and were within 0.4% of theoretical values. Standard product isolation involved quenching the reaction mixture in water or aqueous solution, followed by exhaustive extraction with ether or ethyl acetate, washing extracts with aqueous solutions when necessary, drying organic extracts over Na_2SO_4, filtration, and evaporation of the solvent under reduced pressure. The solvents and the aqueous solutions used for quenching the reaction are mentioned in parentheses after "product isolation".

[3,3-(2,2-Dimethyltrimethylene)dioxy]-11β-vinylestr-9-ene-5α,17β-diol (3a). A 1.73 M solution of vinylmagnesium chloride (8.8 mL, 15.2 mmol) in THF was cooled to −30 °C, and a solution of copper(I) chloride (80 mg, 0.8 mmol) in 6 mL of THF was added. The reaction mixture was stirred for 0.5 h, a solution of epoxide **2** (0.95 g, 2.33 mmol) in 3 mL of THF was added dropwise, and the mixture was stirred for 3 h while the temperature rose from −30 to −10 °C. The reaction mixture was poured into cold, saturated aqueous ammonium chloride. Product isolation (EtOAc, NaCl, Na_2SO_4) and then chromatography on aluminum 90 basic oxide (EtOAc−hexane, 2:8) gave 850 mg (84%) of **3a** which was recrystallized from diethyl ether: mp 121−123 °C; IR 3451, 1638 cm^{-1}; NMR ($CDCl_3$) 0.87 (s, 3H, ketal-CH_3), 0.95 (s, 3H, 18-CH_3), 1.06 (s, 3H, ketal-CH_3), 3.55 (m, 4H, $2CH_2O$), 3.67 (t, 1H, J = 6 Hz, 17-CH), 3.69 (m, 1H, 11-CH), 4.76−4.96 (dd, 2H, J_1 = 10.3 Hz, J_2 = 17.3 Hz, 2′-CH_2), 5.86 (m, 1H, 1′-CH) ppm. Anal. ($C_{25}H_{38}O_4$) C, H, O.

[3,3-(2,2-Dimethyltrimethylene)dioxy]-11β-(3′-butenyl)-estr-9-ene-5α,17β-diol (3b). The 11β-butenyl derivative **3b** was prepared from epoxide **2** by the procedure described for

compound **3a** using butenylmagnesium chloride instead of vinylmagnesium chloride. Compound **3b** (85%) was purified by chromatography on aluminium 90 basic oxide (EtOAc−hexane, 4:6): mp 153.3−154.4 °C; IR 3419, 1736, 1655 cm^{-1}; NMR ($CDCl_3$) 0.90 (s, 6H, ketal-2CH_3), 0.95 (s, 3H, 18-CH_3), 3.45 (m, 4H, 2CH_2O), 3.65 (t, 1H, J = 6.1 Hz, 17-CH), 4.98 (dd, 2H, J_1 = 10.3 Hz, J_2 = 17 Hz, 4′-CH_2), 5.76 (m, 1H, 3′-CH) ppm. Anal. ($C_{27}H_{42}O_4$) C, H, O.

11β-Vinyl-17β-hydroxyestra-4,9-dien-3-one (4a). A solution of 410 mg (1.02 mmol) of **3a** and 20 mg (0.11 mmol) of *p*-toluenesulfonic acid in acetone (20 mL) was stirred for 2 h at room temperature. Product isolation (EtOAc, $NaHCO_3$, Na_2SO_4) and then chromatography (EtOAc−hexane, 5:5) gave 270 mg (90%) of **4a** which was recrystallized from diethyl ether: mp 143.1−145.3 °C; IR 3419, 1736, 1655 cm^{-1}; NMR ($CDCl_3$) 0.85 (s, 3H, 18-CH_3), 3.61 (t, 1H, J = 6 Hz, 17-CH), 3.72 (m, 1H, 11-CH), 4.80−4.96 (m, 2H, 2′-CH_2), 5.66 (s, 1H, 4-CH), 5.90 (m, 1H, 1′-CH) ppm. Anal. ($C_{20}H_{26}O_2$) C, H, O.

11β-(3′-Butenyl)-17β-hydroxyestra-4,9-dien-3-one (4b). The derivative **4b** was prepared from **3b** by the procedure described for compound **4a**. Compound **4b** (84%) was purified by chromatography (EtOAc−CH_2Cl_2, 2:8) as a colorless oil: IR 3427, 1710, 1655 cm^{-1}; NMR ($CDCl_3$) 0.85 (s, 3H, 18-CH_3), 3.49 (t, 1H, J = 6 Hz, 17-CH), 4.85 (dd, 2H, J_1 = 10.3 Hz, J_2 = 17.3 Hz, 4′-CH_2), 5.52 (s, 1H, 4-CH), 5.63 (m, 1H, 3′-CH) ppm. Anal. ($C_{22}H_{30}O_2$) C, H, O.

11β-Vinylestra-1,3,5(10)-triene-3,17β-diol (5a). Acetic anhydride (2.4 mL, 25.4 mmol) and acetyl bromide (1.3 mL, 7.6 mmol) were added, with stirring, to an ice-cooled solution of compound **4a** (1.91 g, 6.4 mmol) in methylene chloride (15 mL). After the mixture was stirred for 2 h at 0 °C, a saturated aqueous sodium bicarbonate solution was added. Extraction with methylene chloride, followed by the usual workup, gave the phenolic acetate which was treated with 10 N sodium hydroxide in methanol (120 mL) for 4 h at room temperature. After acidification with 0.5 N hydrochloric acid, product isolation (EtOAc, Na_2SO_4) and then chromatography (EtOAc−hexane, 3:7) gave 1.63 g of **5a** (85%): mp 204.9−206.1 °C (lit.[25] 196−198 °C); IR 3380, 1620, 1586 cm^{-1}; NMR (DMSO-d_6) 0.70 (s, 3H, 18-CH_3), 3.22 (m, 1H, 11-CH), 3.50 (t, 1H, J = 6 Hz, 17-CH), 4.86−4.96 (dd, 2H, J_1 = 10.3 Hz, J_2 = 17.3 Hz, 2′-CH_2), 5.67 (m, 1H, 1′-CH), 6.40 (d, 1H, J = 2.6 Hz, 4-CH), 6.46 (m, 1H, 2-CH), 6.85 (d, 1H, J = 8.2 Hz, 1-CH) ppm. Anal. ($C_{20}H_{26}O_2$) C, H, O.

11β-(3′-Butenyl)estra-1,3,5(10)-triene-3,17β-diol (5b). Compound **5b** was prepared from **4b** by the procedure described for compound **5a**. The product (85%) was purified by chromatography (EtOAc−hexane, 3:7): mp 182.4-183.8 °C; IR 3410, 1625, 1590 cm^{-1}; NMR ($CDCl_3$) 0.92 (s, 3H, 18-CH_3), 3.7 (dd, 1H, J_1 = 7.3 Hz, J_2 = 8.2 Hz, 11-CH), 4.91 (m, 2H, 4′-CH_2), 5.71 (t, 1H, J = 6 Hz, 3′-CH), 6.52 (d, 1H, J = 2.6 Hz, 4-CH), 6.62 (dd, 1H, J_1 = 2.7 Hz, J_2 = 8.2 Hz, 2-CH), 6.98 (d, 1H, J = 8.2 Hz, 1-CH) ppm. Anal. ($C_{22}H_{30}O_2$) C, H, O.

3,17β-Bis[(*tert*-butyldimethylsilyl)oxy]-11β-vinylestra-1,3,5(10)-triene (6a). A mixture of 500 mg (3.3 mmol) of TBDMS chloride and 400 mg (5.9 mmol) of imidazole in 1.3 mL of DMF was added to 300 mg (1.01 mmol) of **5a**. After the mixture was stirred for 2 h at room temperature, product isolation (CH_2Cl_2, $NaHCO_3$, Na_2SO_4) and recrystallization from ethyl acetate gave 510 mg of **6a** (96%): mp 204.9−206.1 °C; IR 1609,1571 cm^{-1}; NMR ($CDCl_3$) 0.10−0.15 (2s, 12H, 2Si-(CH_3)$_2$), 0.75 (s, 3H, 18-CH_3), 0.87−0.95 (2s, 18H, 2SiC(CH_3)$_3$), 3.26 (m, 1H, 11-CH), 3.60 (t, 1H, J = 6.1 Hz, 17-CH), 4.90−4.97 (dd, 2H, J_1 = 10.3 Hz, J_2 = 17.3 Hz, 2′-CH_2), 5.70 (m, 1H, 1′-CH), 6.50 (d, 1H, J = 2.6 Hz, 4-CH), 6.56 (m, 1H, 2-CH), 6.92 (m, 1H, 1-CH) ppm. Anal. ($C_{32}H_{54}O_2Si_2$) C, H, O.

3,17β-Bis[(*tert*-butyldimethylsilyl)oxy]-11β-(3′-butenyl)-estra-1,3,5(10)-triene (6b). Compound **6b** was prepared from **5b** by the procedure described for compound **6a**. The product (77%) was purified by recrystallization from ethyl acetate: mp 109.8−111.2 °C; IR 1630, 1585 cm^{-1}; NMR ($CDCl_3$) 0.05−0.17 (2s, 12H, 2Si(CH_3)$_2$), 0.86 (s, 3H, 18-CH_3), 0.89−0.96 (2s, 18H, 2SiC(CH_3)$_3$), 3.60 (t, 1H, J = 6.1 Hz, 17-CH), 4.91 (m, 2H, 4′-CH_2), 5.72 (m, 2H, 3′-CH), 6.51 (d, 1H, 4-CH), 6.60 (m, 1H, 2-CH), 6.96 (d, 1H, J = 8.2 Hz, 1-CH) ppm. Anal. ($C_{34}H_{58}O_2Si_2$) C, H, O.

3,17β-Bis[(*tert*-butyldimethylsilyl)oxy]-11β-(2′-hydroxyethyl)estra-1,3,5(10)-triene (7a). 9-BBN (0.5 M solution in THF, 10.22 mmol) under argon was added dropwise to a solution of 770 mg (1.46 mmol) of **6a** in 5 mL of THF at 60 °C. After 1 h, the solution was cooled to −5 °C, 1.5 mL of water was added, and stirring was continued for 5 min. Sodium hydroxide (3 M, 1.5 mL) was added, and after an additional 10 min, 1.5 mL of hydrogen peroxide (30%) was added dropwise. After 30 min of stirring at 0 °C, product isolation ($NaHCO_3$, EtOAc, Na_2SO_4) and then chromatography (EtOAc−hexane, 5:95) afforded 750 mg of the primary alcohol **7a** (94%): mp 148.1−149.9 °C; IR 3437, 1570 cm^{-1}; NMR ($CDCl_3$) 0.03−0.06 (2s, 12H, 2Si(CH_3)$_2$), 0.84 (s, 3H, 18-CH_3), 0.87−0.94 (2s, 18H, 2SiC(CH_3)$_3$), 3.40 (m, 2H, 2′-CH_2), 4.00 (m, 1H, 11-CH), 6.50 (d, 1H, J = 2.6 Hz, 4-CH), 6.70 (m, 1H, 2-CH), 7.02 (d, 1H, J = 8.2 Hz, 1-CH) ppm. Anal. ($C_{32}H_{56}O_3Si_2$) C, H, O.

3,17β-Bis[(*tert*-butyldimethylsilyl)oxy]-11β-(4′-hydroxybutyl)estra-1,3,5(10)-triene (7b). The 11β-hydroxybutyl derivative **7b** was prepared from **6b** by the procedure described for compound **7a**. Product **7b** (97%) was purified by chromatography (EtOAc−hexane, 1:3) and recrystallizated from pentane: mp 114.1−115.2 °C; IR 3455, 1585 cm^{-1}; NMR ($CDCl_3$) 0.04−0.17 (2s, 12H, 2Si(CH_3)$_2$), 0.84 (s, 3H, 18-CH_3), 0.88−0.94 (2s, 18H, 2SiC(CH_3)$_3$), 3.58 (t, 1H, J = 6.1 Hz, 17-CH), 6.51 (d, 1H, J = 2.6 Hz, 4-CH), 6.59 (m, 1H, 2-CH), 6.95 (d, 1H, J = 8.2 Hz, 1-CH) ppm. Anal. ($C_{34}H_{60}O_3Si_2$) C, H, O.

3,17β-Bis[(*tert*-butyldimethylsilyl)oxy]-11β-[2′-[(methylsulfonyl)oxy]ethyl]estra-1,3,5(10)-triene (8a). Methanesulfonyl chloride (0.4 mL, 5 mmol) was added to a solution of 600 mg (1.1 mmol) of primary alcohol **7a** in 1.5 mL of triethylamine. The solution was stirred for 1 h at room temperature. Product isolation ($NaHCO_3$, EtOAc, Na_2SO_4) and chromatography (EtOAc−hexane, 1:9) afforded 550 mg of **8a** (80%): mp 148.9−150.3 °C; IR 1608, 1550 cm^{-1}; NMR ($CDCl_3$) 0.03−0.07 (2s, 12H, 2Si(CH_3)$_2$), 0.85 (s, 3H, 18-CH_3), 0.88−0.92 (2s, 18H, 2SiC(CH_3)$_3$), 2.90 (s, 3H, SCH_3), 3.45 (m, 2H, 2′-CH), 3.95 (m, 1H, 11-CH), 6.45 (d, 1H, J = 2.6 Hz, 4-CH), 6.70 (m, 1H, 2-CH), 6.95 (d, 1H, J = 8.2 Hz, 1-CH) ppm. Anal. ($C_{33}H_{58}O_5Si_2S$) C, H, O.

3,17β-Bis[(*tert*-butyldimethylsilyl)oxy]-11β-[4′-[(methylsulfonyl)oxy]butyl]estra-1,3,5(10)-triene (8b). The 11β-(mesyloxy)butyl derivative **8b** was prepared from **7b** by the procedure described for compound **8a**. The product (90%) was purified by chromatography (EtOAc−hexane, 1:9): mp 76.1−77.1 °C; IR 1608, 1570 cm^{-1}; NMR ($CDCl_3$) 0.03−0.10 (2s, 12H, 2Si(CH_3)$_2$), 0.84 (s, 3H, 18-CH_3), 0.88−0.96 (2s, 18H, 2SiC-(CH_3)$_3$), 3.60 (t, 1H, J = 6.1 Hz, 17-CH), 4.14 (m, 2H, 4′-CH), 6.51 (d, 1H, J = 2.6 Hz, 4-CH), 6.60 (m, 1H, 2-CH), 6.94 (d, 1H, J = 8.2 Hz, 1-CH) ppm. Anal. ($C_{35}H_{62}O_5Si_2S$) C, H, O.

11β-[2′-(Bromoacetamido)ethyl]estra-1,3,5(10)-triene-3,17β-diol (12a). Potassium phthalimide (490 mg, 2.64 mmol) was added to a solution of 1.41 g (2.26 mmol) of mesylate **8a** in 20 mL of DMF. The mixture was stirred for 20 h at 45 °C. Product isolation (H_2O, $NaHCO_3$, EtOAc, Na_2SO_4) and recrystallization from ethanol gave phthalimide **9a** (52%), mp 110−111.5 °C. A solution of 200 mg (0.29 mmol) of **9a** and 80.8 μL of hydrazine hydrate (1.63 mmol) in 12 mL of EtOH was stirred for 10 h at 30 °C. Product isolation (EtOAc, 0.5 N NaOH, Na_2SO_4) afforded 150 mg of crude primary amine **10a** (90%). Bromoacetic acid (99 mg, 0.78 mmol) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (CDI) hydrochloride (149 mg, 0.78 mmol) were added to a solution of **10a** (195 mg, 0.36 mmol) in THF (3 mL). The mixture was stirred for 5 min, and then 6.5 μL of pyridine was added. After 30 min of stirring at 40 °C, the mixture was poured in H_2O. Product isolation (H_2O, $NaHCO_3$, EtOAc, Na_2SO_4) afforded the crude 11β-(bromoacetamido)ethyl steroid **11a**. Removal of the TBDMS group was performed by stirring a solution of **11a** in 30 mL of THF, 10 mL of AcOH, and 10 mL of H_2O for 20 h at room temperature. Product isolation (H_2O, $NaHCO_3$, EtOAc, Na_2SO_4) then chromatography (CH_2Cl_2−AcOEt, 3:7) and recrystallization from ethyl ether afforded 80 mg (51%) of **12a** as an amorphous solid: mp 170−172 °C; IR 3520, 1685 cm^{-1}; NMR ($CDCl_3$) 0.85 (s, 3H, 18-CH_3), 3.20 (m, 2H, 2′-CH_2), 3.50 (m, 1H, 17-CH), 4.00 (s, 2H, CH_2Br), 6.45 (d, 1H, J = 2.6 Hz,

4-CH), 6.50 (m, 1H, 2-CH), 6.95 (m, 1H, 1-CH) ppm. Anal. ($C_{22}H_{30}NO_3Br$) C, H, O.

11β-[4′-(Bromoacetamido)butyl]estra-1,3,5(10)-triene-3,17β-diol (12b). The 11β-(bromoacetamido)butyl derivative **12b** was prepared from **8b** by the procedure described for compound **12a**. The product (54%) was purified by chromatography (CH_2Cl_2−AcOEt, 2:3) and recrystallized from ethyl ether: mp 240 °C dec; IR 3535, 1675 cm^{-1}; NMR (DMSO-d_6) 0.85 (s, 3H, 18-CH_3), 2.95 (m, 2H, 4′-CH_2), 3.50 (t, 1H, J = 6 Hz, 17-CH), 3.80 (s, 2H, CH_2-Br), 6.50 (d, 1H, J = 2.6 Hz, 4-CH), 6.60 (m, 1H, 2-CH), 6.95 (m, 1H, 1-CH) ppm. Anal. ($C_{24}H_{34}NO_3Br$) C, H, O.

11β-[2′-[(Methylsulfonyl)oxy]ethyl]estra-1,3,5(10)-triene-3,17β-diol (13a). Silylated 3- and 17β-hydroxy groups of mesylate **8a** were deprotected by exposing the compound (530 mg, 0.85 mmol) solubilized in AcOEt (4 mL) to *p*-toluenesulfonic acid (72 mg, 0.4 mmol). The mixture was stirred for 1 h at 40 °C. Product isolation (H_2O, EtOAc, Na_2SO_4) and then chromatography (EtOAc−CH_2Cl_2, 2:8) afforded 335 mg of **13a** (65%): mp 159.4−162.1 °C; IR 3385, 1580 cm^{-1}; NMR ($CDCl_3$) 0.85 (s, 3H, 18-CH_3), 2.85 (s, 3H, SCH_3), 3.45 (m, 2H, 2′-CH_2), 3.85 (m, 1H, 11-CH), 6.50 (d, 1H, J = 2.6 Hz, 4-CH), 6.65 (dd, 1H, J_1 = 2.7 Hz, J_2 = 8.2 Hz, 2-CH), 7.00 (d, 1H, J = 8.2 Hz, 1-CH) ppm. Anal. ($C_{21}H_{30}O_5S$) C, H, O.

11β-[4′-[(Methylsulfonyl)oxy]butyl]estra-1,3,5(10)-triene-3,17β-diol (13b). Compound **8b** (550 mg, 0.84 mmol) was deprotected by the procedure described for compound **8a**. Product isolation and then chromatography (EtOAc−CH_2Cl_2, 2:8) afforded 180 mg of **13b** (50%): mp 145.1−146.5 °C; IR 3385, 1580 cm^{-1}; NMR ($CDCl_3$) 0.90 (s, 3H, 18-CH_3), 2.95 (s, 3H, S-CH_3), 3.70 (t, 1H, J = 6 Hz, 17-CH), 4.41 (m, 2H, 4′-CH_2), 6.53 (m, 1H, 4-CH), 6.61 (m, 1H, 2-CH), 6.96 (d, 1H, J = 8.2 Hz, 1-CH) ppm. Anal. ($C_{23}H_{34}O_5S$) C, H, O.

11β-(4′-Bromobutyl)estra-1,3,5(10)-triene-3,17β-diol (14). A mixture of 260 mg (3 mmol) of LiBr and 120 mg (0.28 mmol) of mesylate **13b** in 15 mL of acetone was heated to reflux for 5 h. Product isolation (H_2O, EtOAc, Na_2SO_4) and then chromatography (EtOAc−CH_2Cl_2, 2:8) afforded 90 mg (80%) of the 11β-bromobutyl derivative **14**: mp 117.1−118.5 °C; IR 3400, 1575 cm^{-1}; NMR ($CDCl_3$) 0.90 (s, 3H, 18-CH_3), 3.40 (m, 2H, 4′-CH_2), 3.65 (t, 1H, J = 6 Hz, 17-CH), 6.50 (d, 1H, J = 2.6 Hz, 4-CH), 6.58 (m, 1H, 2-CH), 6.90 (d, 1H, J = 8.2, Hz, 1-CH) ppm. Anal. ($C_{22}H_{31}O_2Br$) C, H, O.

3,3-(Ethylenedioxy)-5α-hydroxy-11β-[10′-[(*tert*-butyldimethylsilyl)oxy]decyl]estr-9-en-17-one (17). A 0.59 M suspension of the Grignard reagent prepared from 31.2 g (88.8 mmol) of 10-bromodecanol, protected as TBDMS ether, and 2.6 g (107 mmol) magnesium in 70 mL of THF was cooled in an ice bath, Copper(I) chloride (875 mg, 8.85 mmol) was added, and then the mixture was cooled to −20 °C. A solution of 19.5 g (59 mmol) of epoxide **16**[21] in 100 mL of THF was added dropwise over 15 min; the temperature rose to −15 °C. The mixture was stirred for 1 h at that temperature; it was then poured in cold, saturated aqueous ammonium chloride solution. Product isolation (AcOEt, Na_2SO_4) followed by chromatography (AcOEt−cyclohexane, 3:7) gave 18.5 g (52%) of ketone **17** as an oil: IR 3508, 1733 cm^{-1}; NMR ($CDCl_3$) 0.05 (s, 6H, $Si(CH_3)_2$), 0.89 (s, 9H, $SiC(CH_3)_3$), 1.01 (s, 3H, 18-CH_3), 1.25 (wide s, 18H, 11-$C(CH_2)_9$), 3.60 (t, 2H, J = 6.5 Hz, CH_2OSi), 3.94−4.02 (m, 4H, 2 CH_2O) ppm. A 7.4 g (30%) sample of starting material was also recovered.

11β-(10′-Hydroxydecyl)-17β-acetoxyestra-4,9-dien-3-one (18). Sodium borohydride (1.45 g, 38.3 mmol) was slowly added to an ice-cooled solution of 23.1 g (38.3 mmol) of compound **17** in 285 mL of methanol. The mixture was stirred for 45 min at 0 °C, acetone (30 mL) was added, and the solution was concentrated under vacuum. Product isolation (NaCl, AcOEt, Na_2SO_4) yielded 22.3 g (96%) of an oil which was immediately dissolved in pyridine (45 mL). Acetic anhydride (22.5 mL) was added, and the solution was left at room temperature for 72 h. Product isolation (NH_4Cl, AcOEt, Na_2SO_4) gave an oil (23.7 g) which was dissolved in methanol (200 mL) and 2 N hydrochloric acid (100 mL). After 1 h of stirring at room temperature, product isolation ($NaHCO_3$, AcOEt, Na_2SO_4) followed by chromatography (AcOEt−cyclohexane, 1:1) afforded 15.2 g (84%) of dienone **18** as an oil: IR 3624, 1729, 1654, 1601 cm^{-1}; NMR ($CDCl_3$), 1.00 (s, 3H, 18-CH_3), 1.27 (wide s, 18H, 11-$C(CH_2)_9$), 2.07 (s, 3H, CH_3CO), 2.96 (q, 1H, J = 7 Hz, 11-CH), 3.63 (t, 2H, J = 6.5 Hz, CH_2-OH), 4.57 (dd, 1H, J_1 = 7.5 Hz, J_2 = 9 Hz, 17-CH), 5.68 (s, 1H, 4-CH) ppm.

3,17β-Diacetoxy-11β-[10′-[[(4-methylphenyl)sulfonyl]-oxy]decyl]estra-1,3,5(10)-triene (19). 4-Methylbenzenesulfonyl chloride (2.55 g, 12.4 mmol) was added to a solution of 2.53 g (5.36 mmol) of dienone **18** in pyridine (12 mL). The suspension obtained was stirred for 2 h at room temperature. Product isolation ($NaHCO_3$, AcOEt, Na_2SO_4) gave 3.41 g of an oil which was dissolved in methylene chloride (32 mL) and cooled in an ice bath. A mixture of acetic anhydride (3.2 mL, 33.8 mmol) and acetyl bromide (1.6 mL, 21.6 mmol) was slowly added under argon while maintaining the flask in ice. The solution was then stirred at room temperature for 1.5 h. Methanol (5 mL) was cautiously added, and then product isolation ($NaHCO_3$, CH_2Cl_2, Na_2SO_4) followed by chromatography (AcOEt−cyclohexane, 2:8) afforded 1.82 g (56%) of diacetate **19** as an oil: IR 1747, 1727, 1630, 1611, 1609, 1599, 1581, 1499, 1118 cm^{-1}; NMR ($CDCl_3$) 0.95 (s, 3H, 18-CH_3), 1.18 (wide s, 18H, 11-$C(CH_2)_9$), 2.07 (s, 3H, 17-$COCOCH_3$), 2.28 (s, 3H, 3-$COCOCH_3$), 2.44 (s, 3H, Ph-CH_3), 4.01 (t, 2H, J = 6.5 Hz, CH_2OSO_2), 4.63 (dd, 1H, J_1 = 7 Hz, J_2 = 9 Hz, 17-CH), 6.78 (d, 1H, J = 2.5 Hz, 4-CH), 6.84 (dd, 1H, J_1 = 2.5 Hz, J_2 = 8.5 Hz, 2-CH), 7.13 (d, 1H, J = 8.5 Hz, 1-CH), 7.34 and 7.79 (AA′BB′, 4H, C_6H_4) ppm. A small amount of the corresponding 10-chlorodecyl derivative (0.42 g, 16%) was also isolated.

11β-[10′-[[(4-Methylphenyl)sulfonyl]oxy]decyl]estra-1,3,5(10)-triene-3,17β-diol (20). A solution of 379 mg (0.57 mmol) of diacetate **19** in methanol (9 mL) and 2 N sodium hydroxyde (3.5 mL) was stirred for 2 h at room temperature. Hydrochloric acid (2 N, 3.7 mL) was then added. Product isolation ($NaHCO_3$, AcOEt, Na_2SO_4) followed by chromatography on silica gel (AcOEt−cyclohexane, 3:7) and then on Lichrosorb RP 18 (water−methanol, 10:90) afforded 114 mg (34%) of tosylate **20** as an off-white amorphous powder: IR 3602, 1621, 1607, 1599, 1581, 1497, 1358, 1189, 1170 cm^{-1}; NMR ($CDCl_3$) 0.92 (s, 3H, 18-CH_3), 1.25 (wide s, 11-$C(CH_2)_9$), 2.45 (s, 3H, Ph-CH_3), 3.71 (t, 1H, J = 8 Hz, 17-CH), 4.02 (t, 2H, J = 6.5 Hz, CH_2OSO_2), 6.55 (d, 1H, J = 2.5 Hz, 4-CH), 6.64 (dd, 1H, J_1 = 2.5 Hz, J_2 = 8 Hz, 2-CH), 7.00 (d, 1H, J = 8 Hz, 1-CH), 7.34 and 7.79 (AA′BB′, 4H, C_6H_4) ppm. Anal. ($C_{35}H_{50}O_5S$) C, H, S.

Biological. Materials. [6,7-^{3}H]Estradiol (specific activity 1.89 PBq/mol, radiochemical purity >98%), was purchased from Amersham International (Amersham, England). Estradiol, luciferin, and 4′,6-diamidino-2-phenylindole were purchased from Sigma Chemical Co. (St. Louis, MO). 4-Hydroxytamoxifen was given by Dr. A. E. Wakeling (Zeneca, Macclesfield, England). Estradiol, 4-hydroxytamoxifen, and estradiol 11β-derivatives used for binding studies and cell experiments were solubilized in absolute ethanol. Solutions were stored at −20 °C in the dark. The purity of solubilized compounds was checked before use by TLC. Cell culture materials came from Life-Technologies (Cergy-Pontoise, France).

Cytosolic Estrogen Receptor. Cytosol was prepared in 20 mM Tris-HCl buffer (T20), pH 7 (competitive binding experiments) or 8.5 (irreversible binding experiments), from immature lamb uteri as described previously.[15] The protein concentration, determined according to Layne,[34] was adjusted to 2 mg/mL (competitive binding experiments) or 4 mg/mL (irreversible binding experiments).

Competitive Binding Assay: Apparent Relative Affinity Constants. Competition between 5 nM [^{3}H]estradiol and increasing concentrations of nonradioactive estradiol or 11β-derivatives for binding to lamb uterine cytosol (2 mg protein/mL, pH 7) were performed for 20 h at 20 °C, as described previously.[15] Apparent relative affinity constants (RACs) of competitors were calculated according to Korenman[23] using concentrations of unbound and specifically bound [^{3}H]estradiol at 50% specific binding inhibition and concentrations of unlabeled estradiol and 11β-derivatives which inhibited 50% of the specific binding of [^{3}H]estradiol.[15]

Standard Indirect Irreversible Binding Assay. Cytosol (4 mg of protein/mL, pH 8.5) was first incubated for 20 h at 25 °C with estradiol, 11β-derivatives, or without any steroid.

Samples were then treated with an equal volume of charcoal suspension (1% charcoal, 0.1% dextran T70 in T20, pH 7) for 30 min at 0 °C, and charcoal was removed by centrifugation. The total and nonspecific [^{3}H]estradiol binding formed under exchange conditions (16 h, 20 °C) in supernatant aliquots were determined by charcoal assay, as previously described,[15] using 10 nM [^{3}H]estradiol in the absence and presence of 5 μM nonradioactive estradiol.

Radioactivity Determinations. The radioactivity of the various cytosol samples (100 or 200 μL) was counted in 4 mL of Emulsifier Safe (Packard).

Cell Lines and Cell Culture Conditions. MVLN cells were established[28] by transfecting MCF-7 cells (mammary tumor cells expressing the estrogen receptor and whose growth and expression of specific genes are stimulated by estrogens) with the pVit-tk-Luc plasmid. In this reporter gene, the fragment -331/-87 of the 5′ flanking region of *Xenopus* vitellogenin A_2 gene, which contains a palindromic estrogen response element (ERE), was inserted in front of the Herpes simplex virus promoter for thymidine kinase as previously reported for pVit-tk-CAT.[35] This regulatory part controls expression of the firefly luciferase structural gene. Cells were cultured either in Dulbecco's modified Eagle's medium (DMEM) supplemented with 3% fetal calf serum (FCS medium) or in DMEM without phenol red supplemented with 3% of a "steroid-free", dextran-coated, charcoal-treated fetal calf serum (FCS/C medium). Medium was replaced every other day.

Luciferase Assay. Cells were rinsed twice in luminescence buffer (1.5 mM potassium phosphate, 8 mM $MgCl_2$, pH 7.4), suspended in 0.5 mL of luminescence buffer containing 2 mM ATP and kept frozen until the luciferase assay was performed, as described elsewhere,[28] in the presence of 0.1 mM luciferin, 0.1 mM CoA, and 0.3% Triton X-100, using an LKB Wallac 1251 luminometer (Sundyberg, Sweden). The protein assay was performed on 0.1 mL of cellular homogenate according to Lowry's method.[36] The experimental values were expressed as arbitrary luminescence units per milligram of protein.

Estrogenic and Antiestrogenic Assay. Cells grown in FCS/C medium for at least 3 days were seeded in 6-well tissue culture plates (10^5 cells/well). One day later, increasing concentrations of 4-hydroxytamoxifen or estradiol 11β-derivatives in ethanol (0.1% final concentration) without or with 0.1 nM estradiol were added to the medium. One day later, cells were rinsed twice with medium alone and then processed for luciferase activity determination.

Luciferase Gene Inactivation Assay. Cells grown in FCS/C medium for at least 3 days were seeded in T25 tissue culture flasks ($\sim 5 \times 10^5$ cells/flask). One day later, medium was supplemented with 0.1% ethanol with or without 100 μM 4-hydroxytamoxifen or estradiol 11β-derivatives. Medium was replaced every other day, and cells were grown for various periods of time (to prevent confluency, when necessary cells were trypsinized and seeded at lower concentration in new wells). Cells were rinsed twice with medium alone, followed by the addition of medium with or without 100 nM estradiol. Cells were grown for 7 days, rinsed twice with medium alone, and then processed for luciferase activity determination.

Cell Proliferation Assay. Cells grown in FCS/C medium for at least 3 days were seeded in 24-well tissue culture plates (5×10^3 cells/well). One day later, medium was supplemented with 0.1% ethanol containing or not estradiol (100 nM), 4-hydroxytamoxifen, or estradiol 11β-derivatives (100 μM). Cells were grown in triplicate for 7 days. The DNA contents of the wells were determined according to a method using fluorescence enhancement of 4′,6-diamidino-2-phenylindole complexed with DNA.[37] Doubling time was determined by semilogarithmic analysis of DNA content.

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