Steroidal Affinity Labels of the Estrogen Receptor α. 4. Electrophilic 11*β*-Aryl Derivatives of Estradiol

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Ten electrophilic estradiol 11*β*-aryl derivatives were synthesized, with three different types of 11*β*-substituent: (i) *p*ØO$(CH_2)_2$X (compounds: **6**, X = OSO_2CH_3; **7**, X = I; **13**, X = $NHCOCH_2$-Cl; **15**, X = $N(CH_3)COCH_2Br$; and **16**, X = $N(CH_3)COCH_2Cl$); (ii) *p*ØO$(CH_2)_5$X (compounds: **17**, X = I; **20**, X = $NHCOCH_2Br$; and **22**, X = $N(CH_3)COCH_2Br$); and (iii) *p*ØC≡CCH_2X (compounds: **27**, X = $NHCOCH_2Cl$; and **29**, X = $N(CH_3)COCH_2Cl$). The range of their apparent affinity constants for binding the lamb uterine estrogen receptor α (ERα) was 3−40% that of estradiol. Six electrophiles, chloroacetamides **13**, **16**, **27,** and **29**, iodide **17,** and bromoacetamide **20** (whose arm linking the electrophilic carbon to the 11*β*-phenyl group includes at least six bonds), were able to irreversibly inhibit the binding of [^{3}H]estradiol to ER (25−60% decrease in binding sites), with the following compound effectiveness order: **17** < **13** < **16** ∼ **20** ∼ **27** ∼ **29**. Mesylate **6**, iodide **7** (whose linking arm includes only three bonds), and bromoacetamides **15** and **22** (which differ from **16** by the Cl to Br change and from **20** by the NH to NCH_3 change, respectively) were much less effective (<10% decrease in binding sites, if any). The fact that the inactivation of estradiol-binding sites by the six electrophiles was totally prevented by estradiol indicated that they were ER affinity labeling agents. When ER was modified by methyl methanethiosulfonate, an SH-specific reagent, the different compounds led to very contrasting results in ER affinity labeling. With modified ER, iodide **17** and chloroacetamides **27** and **29** were practically inactive, chloroacetamides **13** and **16** and bromoacetamide **20** were still active but less effective than on the native ER, whereas tertiary bromoacetamides **15** and **22**, found to be practically inactive on native ER, became the most effective electrophiles (∼45% and ∼65% binding sites inactivated, respectively). The results indicate that in the steroid-filled hormone-binding pocket: (i) nucleophilic residues are localized on the *β*-side but relatively remote from the steroid nucleus (distance from C-11 > "seven bonds"); (ii) relatively discrete changes in the electrophilic functionality, such as Cl to Br or NH to NCH_3 of haloacetamido compounds, can markedly modify the positioning of the electrophilic center which could no longer react with the nucleophilic residues; and (iii) cysteine residues (probably homologues of human ERα cysteine 381 and/or cysteine 530) are, at least partly, the covalent attachment sites of the electrophiles. Moreover, modification of cysteine residues by methyl methanethiosulfonate changes the structure of the hormone-binding pocket, whose labeling by the various electrophiles is profoundly altered.

Introduction

The biological effects of estrogens in target cells are mediated by nuclear receptors. Until 1996, it was generally accepted that only one type of estrogen receptor (ER), cloned in 1985[1,2] and now termed ERα, existed. Recently, cDNA clones encoding another subtype of ER, termed ER*β*, were obtained from various tissues from rat,[3] mouse,[4] and human.[5] Interestingly, the ER*β* mRNA was found in organs such as prostate[6] and testis,[7] which are not considered to be major estrogen target organs, whereas it was much lower than the ERα mRNA in the uterus,[6] which is the major estrogen target organ in mammals.

Recently, the crystal structures of the hormone-binding domain of the human ERα bound to various estrogens or antiestrogens were established. Tanenbaum et al.[8] determined the structure of covalently dimerized domains bound to estradiol, whereas the structure of *S*-carboxymethylated domain bound: (i) to estradiol or raloxifene (a benzothiophene partial antiestrogen) or (ii) to diethylstilbestrol (a diphenylethylene estrogen) or 4-hydroxytamoxifen (a triphenylethylene partial antiestrogen) was determined by Brzozowski et al.[9] and Shiau et al.,[10] respectively. The overall architecture of the ERα ligand-binding domain was similar to those seen in the crystal structure of other nuclear receptor hormone-binding domains.[11−16] The ERα hormone-binding domain appears to comprise 12 α-helices and 2 *β*-strands. This domain is folded into a three-layer structure with three internal helices sandwiched between the most external helices. The ligand-binding pocket is predominantly formed by hydrophobic residues

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located in six distinct structural elements, whereas the polar amino acids E353 and R394 are directly involved in hydrogen bonds with the phenolic hydroxyl of the estradiol A-ring (or diethylstilbestrol, raloxifene, or 4-hydroxytamoxifen counterpart) and H524 is involved in a single hydrogen bond with the 17β-hydroxyl of the estradiol D-ring (or diethylstilbestrol or raloxifene counterpart). Agonists and antagonists bind at the same site within the core of the ligand-binding domain but demonstrate different binding modes.[9,10]

Affinity labeling of receptors enables direct identification of nucleophilic amino acid residues of the hormone-binding site which are in contact or in close proximity to electrophilic ligands. This approach, much more versatile than the crystallization approach, can be used to demonstrate, from the whole ER in its soluble state, the different positions of various hormone agonists and antagonists in the ER hormone-binding pocket. Using both wild-type and various cysteine to alanine mutants of the human ERα, three out of the four cysteines (C381, C417, C447, C530) of the hormone-binding domain were found to be covalent attachment sites of electrophilic nonsteroidal and steroidal ligands of ERα. In wild-type and C530A mutant ERαs, C530 and C381 were respectively the covalent attachment sites of an aziridine derivative of tamoxifen,[17,18] whereas C417 and C530 were alkylated by 17α-[(haloacetamido)alkyl]estradiols,[19−21] compounds which displayed more agonistic characters than the former. The results are in agreement with the crystal structure established for the ERα ligand-binding domain, since all three cysteines are located within or in very close proximity to structural elements involved in delineation of the hormone-binding pocket.[9]

With the aim of gaining further insight into the positioning of ligands in the hormone-binding pocket, we undertook to synthesize and then evaluate electrophilic 11β-estradiol derivatives as ERα affinity labeling agents. Recently, we reported on the synthesis and properties of six new 11β-estradiol derivatives in which an 11β-ethyl, 11β-butyl, or 11β-decyl chain was substituted with various electrophilic functionalities: bromide, (methylsulfonyl)oxy, (*p*-tolylsulfonyl)oxy, or bromoacetamido.[22] In these six electrophilic compounds, only 11β-[(tosyloxy)decyl]estradiol displayed significant ER affinity labeling activity. The covalent attachment site of the 11β-decyl derivative was not identified. It did not seem to occur at cysteine residues since the ER alkylation process was not prevented by the thiol-specific reagent methyl methanethiosulfonate (MMTS). Moreover, due to the length and mobility of the *n*-decyl chain, the relative location of the electrophilic carbon from the steroid nucleus was not clear. This reduced the interest of the compound for analysis of the ER binding pocket.

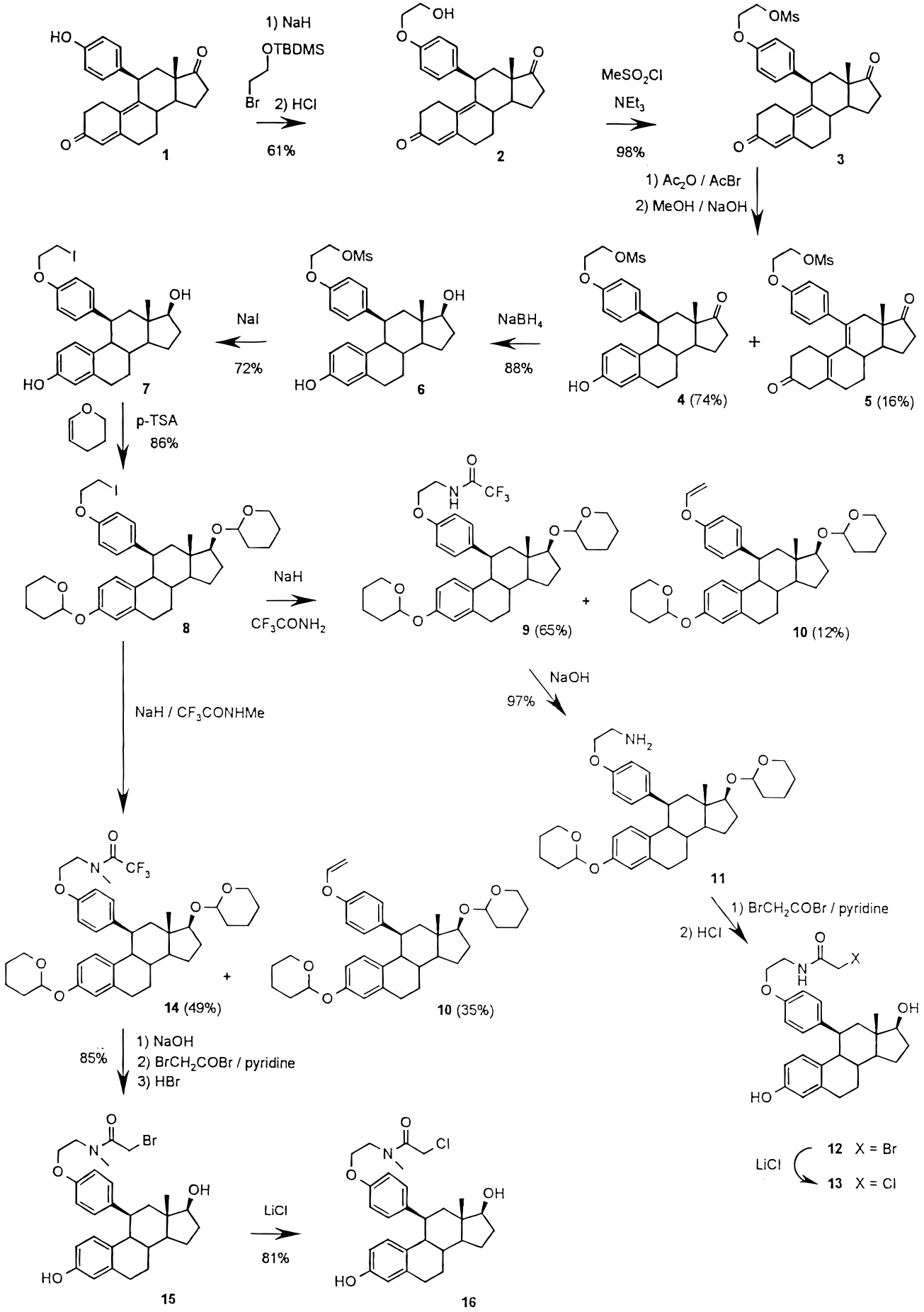
To overcome this drawback, we decided to synthesize and then evaluate a series of electrophilic 11β-aryl derivatives of estradiol in which the 11β-substituent included: (i) a phenyl ring, to confer rigidity from C-11 (contrary to an 11β-aliphatic arm) to the axial substituent and to restrict localization of the electrophilic carbon to the β-side of the steroid nucleus; (ii) on the phenyl ring, various linear *para* chains with differing lengths and mobilities in order to determine from ER-bound electrophiles the remoteness of potential ER covalent attachment sites; and (iii) various terminal electrophilic functionalities in order to favor ER affinity labeling by the compounds. Ten electrophilic 11β-arylestradiol derivatives were prepared in which various electrophilic functionalities, e.g. iodide, (methylsulfonyl)oxy, haloacetamido, or *N*-methylhaloacetamido, are linked to the steroid by an 11β-*p*-ethoxyphenyl, -*p*-pentoxyphenyl, or -*p*-propynylphenyl group. Note that since several of these compounds are related to steroidal pure antiestrogens,[23,24] and since there is presently no model for the structure of ER bound to pure antiestrogens, affinity labeling of ER by the compounds could give useful information to define the binding mode of such compounds. In this paper, we report: (i) the synthesis of these 10 electrophilic 11β-estradiol derivatives; (ii) their affinity for the lamb ERα; and (iii) their ability to become irreversibly bound to lamb and human ERαs, either native or modified by MMTS.

Results

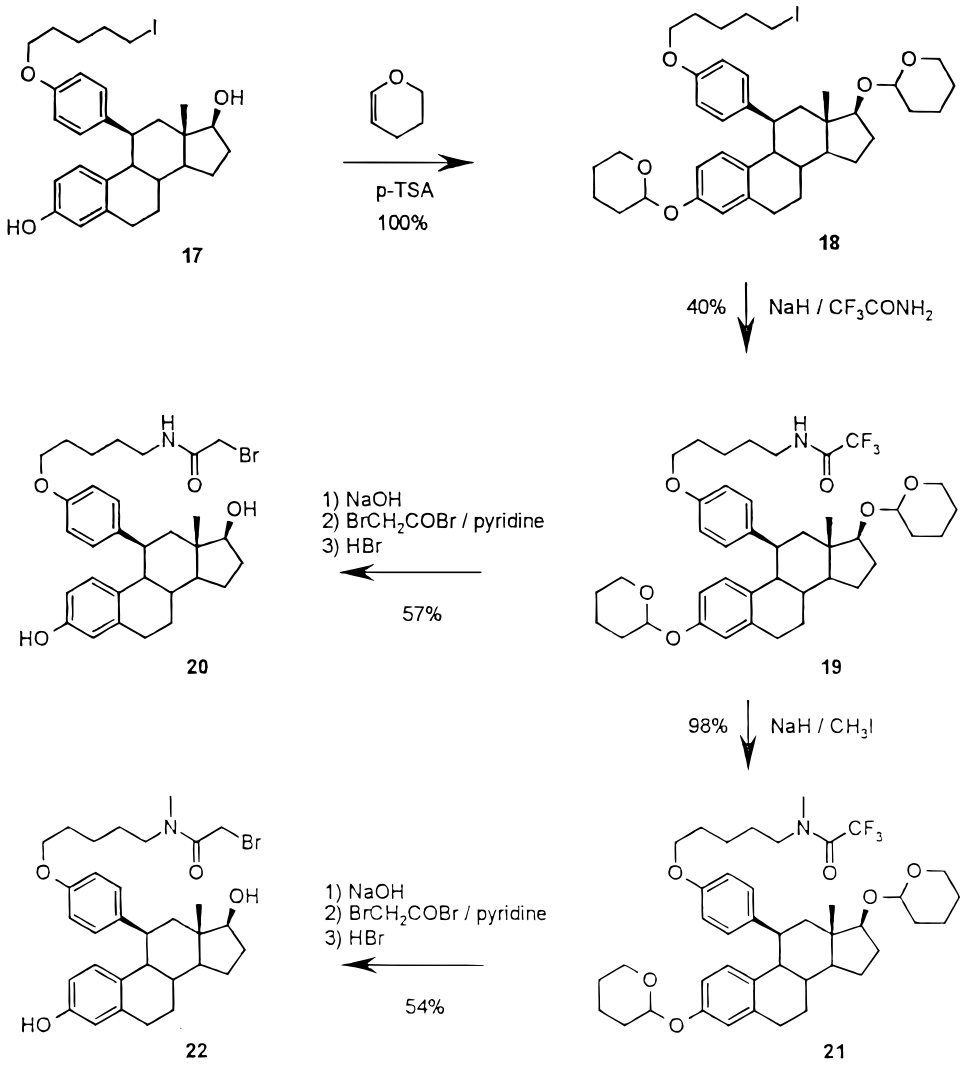
Synthesis of Electrophilic 11β-Estradiol Derivatives. All of the electrophilic estradiol derivatives described here were prepared according to a common strategy starting from the appropriate hydroxy- or iodine-substituted 11β-(alkoxyphenyl)- or 11β-(alkynylphenyl)estrane derivative.

Scheme 1 exemplifies the synthesis of estradiol 11β-(*p*-ethoxyphenyl) derivatives. The already reported 11β-(4-hydroxyphenyl)estradienedione **(1)**[23] was used as starting material. The hydroxyethyl chain was introduced in **1** through alkylation of the phenol function by bromoethanol protected as its *tert*-butyldimethylsilyl (TBDMS) ether. Acidic hydrolysis of the TBDMS group afforded the primary alcohol **2** (61%), which was esterified by methanesulfonyl chloride to give mesylate **3** (98%). The A-ring in **3** was aromatized with acetyl bromide and acetic anhydride, through formation of the 2-en-3-ol acetate, and alkaline hydrolysis of the ester[24,25] gave estratrienolone **4** (74%). This reaction also gave rise to the formation of the nonconjugated estra-5(10),9-(11)-dien-3-one **5** (16%) resulting from abstraction of the 11α proton instead of a proton on C-2, in α position to the 3-keto function. Borohydride reduction of the 17-keto function of mesylate **4** provided mesylate **6** (88%), which was converted to iodide **7** (72%) by the action of sodium iodide. The latter was used as a template to prepare bromoacetamido (**12** and **15**) and chloroacetamido (**13** and **16**) derivatives, respectively. Protection of the two hydroxyls of **7** as tetrahydropyranyl (THP) ethers gave **8** (mixture of stereoisomers). Substitution of the iodine atom in **8** by the trifluoroacetamido group provided secondary trifluoroacetamide **9** (65%) along with a small amount of vinyl ether **10** (12%), resulting from dehydroiodination of **8**. Alkaline hydrolysis of **9** yielded primary amine **11** (97%), which was acylated by bromoacetyl bromide. Deprotection of the 3- and 17β-hydroxyls by hydrochloric acid in a mixture of methanol and THF led to a partial halogen exchange, yielding a mixture (70:30) of the expected secondary bromoacetamide **12** together with secondary chloroacetamide **13**. The presence of **12** and **13** in the obtained product was observed by NMR spectroscopy: two singlets corresponding to CH_2-Br and CH_2-Cl appeared at 3.86 and

Scheme 1. Synthesis of Estradiol 11β-Ethoxyphenyl Derivatives



Scheme 2. Synthesis of Estradiol 11β-Pentoxyphenyl Derivatives

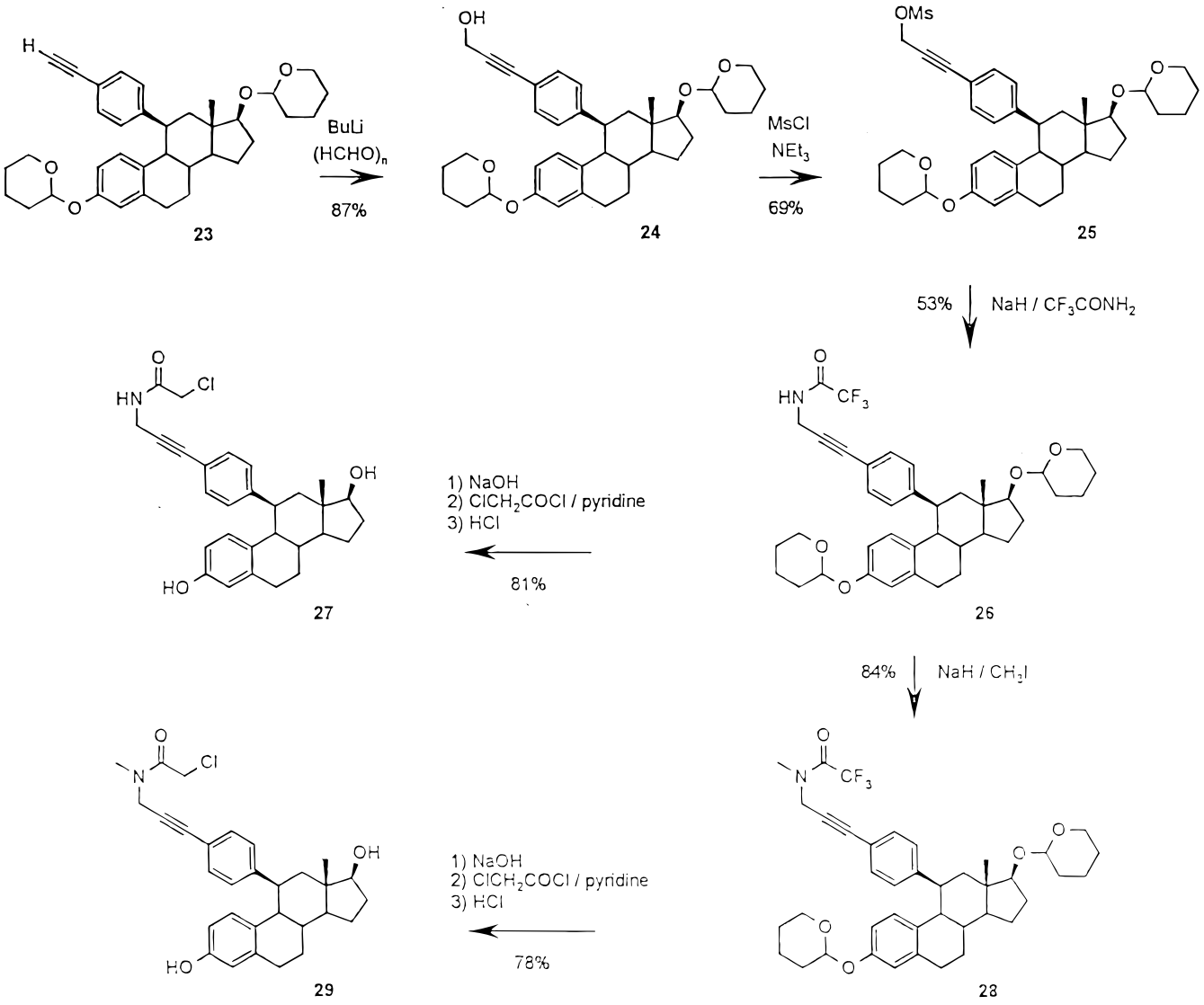


4.06 ppm, respectively. As the two compounds were unseparable by chromatography, bromoacetamide **12** was converted to chloroacetamide **13** by the action of lithium chloride. The *N*-methyl homologues **15** and **16** of **12** and **13**, respectively, were prepared analogously by substitution of the iodine atom of **8** by the *N*-methyltrifluoroacetamido group. Due to the higher basicity/nucleophilicity ratio of the secondary vs the primary amide anion, a higher amount of the vinyl compound **10** was produced in this case (35% vs 12%). The yield of tertiary trifluoroacetamide **14** (49%) was lower than that obtained for **9** in the unsubstituted series (65%). Alkaline hydrolysis of the amide bond of **14** gave the corresponding *N*-methyl secondary amine which was acylated with bromoacetyl bromide. Hydrobromic acid was used in order to avoid any halogen exchange during deprotection of THP ethers. Moreover, brine washing of the organic extracts during the workup was omitted. The tertiary *N*-methylbromoacetamide **15** was thus prepared in 85% yield. As above, the corresponding *N*-methylchloroacetamide **16** (81%) was obtained by substitution of the bromine atom of **15** with chlorine.

The synthesis of electrophilic estradiol 11β-(*p*-pentyloxyphenyl) derivatives is shown in Scheme 2. Preparation of 11β-[4-(5-iodopentyl)phenyl]estradiol **(17)** from **1** was previously described.[24] Iodide **17** was used as starting material for the preparation of bromoacetamides **20** and **22**. Protection of the two hydroxyls of **17** as THP ethers gave **18** (mixture of stereoisomers). Substitution of the iodine atom in **18** by the trifluoroacetamido group yielded the secondary trifluoroacetamide **19** (40%). In this case, no dehydroiodinated product was noticed, but untransformed starting material (20%) was recovered. Alkaline hydrolysis of the amide bond of **19** yielded the corresponding primary amine, which was acylated with bromoacetyl bromide. Hydrobromic acid was used to deprotect the 3- and 17β-hydroxyls, producing bromoacetamide **20** (57%). Since substitution of the iodine atom of **18** by the *N*-methyltrifluoroacetamido group, to give the tertiary trifluoroacetamide **21**, was even less efficient than in the previous *p*-ethoxyphenyl series, **21** was nicely prepared (98%) by *N*-methylation of the secondary trifluoroacetamide **19**. The tertiary *N*-methylbromoacetamide **22** was prepared (54%) by the same method as that used to obtain **20**.

Scheme 3 illustrates the synthesis of electrophilic estradiol 11β-(*p*-propynylphenyl) derivatives. The propargyl alcohol **24** was prepared in 87% yield by hydroxyalkylation (butyllithium, paraformaldehyde) of the previously described, bis-protected 11β-(4-ethy-

Scheme 3. Synthesis of Estradiol 11β-Propynylphenyl Derivatives



nylphenyl)estradiol **(23)**;[26] it was further converted to mesylate **25** (69%). The secondary trifluoroacetamide **26** was obtained (53%) from **25** by substitution of the methanesulfonoxy group with the trifluoroacetamido group. *N*-Methylation of **26** afforded the tertiary trifluoroacetamide **28** (84%). In that *p*-propynylphenyl series, chloroacetamides **27** and **29** were prepared directly through alkaline hydrolysis of **26** and **28**, respectively, followed by acylation with chloroacetyl chloride and deprotection of the 3- and 17β-hydroxyls (81% and 78% yields, respectively).

For all secondary haloacetamides (**13**, **20,** and **27**), in DMSO, the NMR signal of the CH_2Cl/Br group appeared as a singlet, whereas two singlets (~1:1 ratio) were obtained for each of the CH_2Cl/Br and NCH_3 groups of tertiary haloacetamides (**15**, **16**, **22,** and **29**). These results suggested that, at least in DMSO at 20 °C, secondary haloacetamides displayed a single conformation (presumably the *Z* conformation) whereas, due to hindered rotation about the N−CO bond, tertiary haloacetamides displayed both the *E* and *Z* conformations.

ER Binding Affinity. The apparent relative affinities of electrophiles were determined by competitive binding between increasing concentrations of compounds and a constant [^{3}H]estradiol concentration, using lamb uterine cytosol as an ERα source. The fact that electrophiles could irreversibly bind ER and cytosol components (which would tend to overestimate and underestimate, respectively, the affinity of the compound) could markedly alter the compound's affinity value. Therefore, to minimize potential irreversible binding of compounds to ER and to cytosolic components, binding experiments were performed at pH 7.0, since the reactivity of nucleophilic amino acid residues generally increases with pH. Table 1 gives the apparent affinity constants of the compounds for the lamb uterine ER, relative to that of estradiol (100%), which were calculated according to Korenman.[27] There was no clear relationship between the compound affinity, which according to compound ranged from 3% to 40%, and the size of the 11β-chain since the above extreme values were obtained for chloroacetamide **13** and mesylate **6** whose 11β-substituent lengths are similar, whereas compounds with either a shorter chain such as iodide **7** or a longer chain such as iodide **17** and bromoacetamides **20** and **22** showed intermediate values. In the haloacetamide series, methylation of the nitrogen in the amide function or the Cl to Br substitution did not

Table 1. Structural and Binding Characteristics of Electrophilic 11β-Estradiol Derivatives to ER

		electrophile-bearing chain[a]				
compd	11β-substituent	class	length	degree of freedom	apparent RAC[b]	covalent binding[c]
estradiol					100	
6	$\text{ØO(CH}_2\text{)}_2\text{OSO}_2\text{CH}_3$	1	3	2	39.9 ± 8.4	−
7	$\text{ØO(CH}_2\text{)}_2\text{I}$				10.6 ± 2.0	−
13	$\text{ØO(CH}_2\text{)}_2\text{NHCOCH}_2\text{Cl}$	2	6	4	3.30 ± 0.66	+
15	$\text{ØO(CH}_2\text{)}_2\text{N(CH}_3\text{)COCH}_2\text{Br}$				4.22 ± 0.69	−
16	$\text{ØO(CH}_2\text{)}_2\text{N(CH}_3\text{)COCH}_2\text{Cl}$				6.96 ± 0.52	+
17	$\text{ØO(CH}_2\text{)}_5\text{I}$	3	6	5	8.23 ± 1.54	+
20	$\text{ØO(CH}_2\text{)}_5\text{NHCOCH}_2\text{Br}$	4	9	7	17.1 ± 5.9	+
22	$\text{ØO(CH}_2\text{)}_5\text{N(CH}_3\text{)COCH}_2\text{Br}$				9.70 ± 2.49	−
27	$\text{ØC}\equiv\text{CCH}_2\text{NHCOCH}_2\text{Cl}$	5	6	2	14.5 ± 2.8	+
29	$\text{ØC}\equiv\text{CCH}_2\text{N(CH}_3\text{)COCH}_2\text{Cl}$				19.4 ± 0.2	+

[a] The 10 electrophiles were classified according to the type of the electrophile-bearing chain. Five different classes were defined, which are characterized: (i) by the remoteness of the electrophilic carbon from the 11β-phenyl ring (the length of the linking arm was evaluated according to the number of bonds); and (ii) by the degree of freedom of the chain. [b] Apparent RACs (relative affinity constants) of compounds for the cytosolic lamb uterine ER were determined by competitive (20 h, 20 °C) radioreceptor assay using [^{3}H]estradiol as tracer, as described in Materials and Methods. Data are means of duplicate determinations ± SD, in two to five separate experiments. [c] Covalent binding of compounds to ER was established from their ability to irreversibly inhibit binding of [^{3}H]estradiol (cf. Results, Irreversible Binding to ER). The symbol (−) means that the decrease in estradiol-binding sites induced by the compound, if any, was <10%.

markedly change the affinity of the 11β-derivatives. Therefore in each of the three haloacetamide series, the apparent affinities of the compounds were similar (compare **13**/**15**/**16**, **20**/**22**, and **27**/**29**). They were however lower in the ethoxy series than in the pentoxy or propynyl series. The apparent affinities of the 10 electrophiles were roughly in the same range as previously found for electrophilic estradiol 11β-alkyl derivatives.[22] Compared to the affinities of chemically inert 11β-estradiol derivatives, the compound affinities appeared slightly lower than those of pure antiestrogens bearing a bulky amidoalkyl[28] or amidoalkoxyphenyl[23] 11β-substituent and much lower than those of estrogens derived from estradiol by introducing various short aliphatic chains at the 11β-position.[25,29]

Irreversible Binding to ER. Affinity labeling receptors is usually a concentration- and time-dependent process which, depending on the type of electrophile, could be influenced by temperature and pH.[19,20] Specific ER alkylating activities of the 10 electrophiles were evaluated from their ability to irreversibly inhibit specific estradiol binding in cytosol by means of a procedure which was validated previously.[20] This procedure includes three steps: (i) incubation of cytosol with the electrophile to allow the ER affinity labeling; (ii) removal of unbound electrophile by charcoal absorption; and (iii) measurement of the residual concentration of specific [^{3}H]estradiol-binding sites in cytosol, under exchange conditions to allow displacement of the noncovalently ER-bound electrophile. With high-affinity electrophiles, such as those evaluated in this study, it could be difficult to evaluate their ER affinity labeling ability since incomplete [^{3}H]estradiol labeling of ERα noncovalently occupied by the electrophile would result in a false positive ER affinity labeling assessment. Two criteria, which distinguish between covalent and noncovalent binding of the electrophile to ER, can be used to avoid such misinterpretations. The effect of electrophile on the estradiol-binding site concentration in cytosol can be determined either: (a) according to the time of cytosol exposure to the electrophile;[20] or (b) using increasing concentrations of [^{3}H]estradiol at the exchange step.[19]

(a) Affinity labeling of receptors is usually a slower process than classical noncovalent binding since it first requires conventional binding and then involves a time-dependent chemical reaction. Therefore, if according to increasing times of cytosol exposure to the compounds the decrease in [^{3}H]estradiol-binding sites is much more prolonged for the electrophile than for control compounds (such as a chemically inert analogue of the electrophile or unlabeled estradiol), then the electrophile likely alkylated ER.

(b) Contrary to the classical binding of a chemically inert ligand to ER, the covalent binding of a reactive ligand cannot be reversed by an increase in the amount of [^{3}H]estradiol used for displacement of noncovalently bound ligand. Therefore, an irreversible decrease in specific [^{3}H]estradiol binding in cytosol preexposed to an electrophilic ligand (decrease evaluated from the specific binding in cytosol samples, incubated without ligand or with control compounds) probably signals an ER affinity labeling process. This approach could, however, be limited by the increase of nonspecific [^{3}H]-estradiol binding (proportional to the applied concentration of [^{3}H]estradiol) to cytosol components, which restricts the accuracy of the measurements.

Another criterion could be used to determine the specificity of the alkylation process. Occupation of the hormone-binding pocket by a ligand such as estradiol must totally inhibit specific covalent attachment of the electrophile to ER.[19]

To determine the alkylation level of ER, the cytosol was thus incubated for increasing times with various electrophile concentrations at either 0 or 25 °C and at either pH 7.0 or 8.5. Cytosol was concomitantly incubated without steroid or with 100 nM unlabeled estradiol to obtain reference values for the specific estradiol-binding site concentration. Six out of the 10 electrophiles elicited marked decreases in the estradiol-binding site concentration. Figure 1 shows the results of a representative experiment in which cytosol was incubated for 16 h at 0 °C, pH 8.5, without steroid, with 100 nM estradiol or with 200 nM 11β-estradiol derivatives. Mesylate **6** and iodide **7**, whose structures include the ethoxyphenyl group (Table 1), did not have any significant effect on the binding site concentration. Among the three haloacetamido homologues of **6** and **7**, the two chloroacetamides **13** and **16** induced a marked effect (40−50% decrease in the binding site concentration

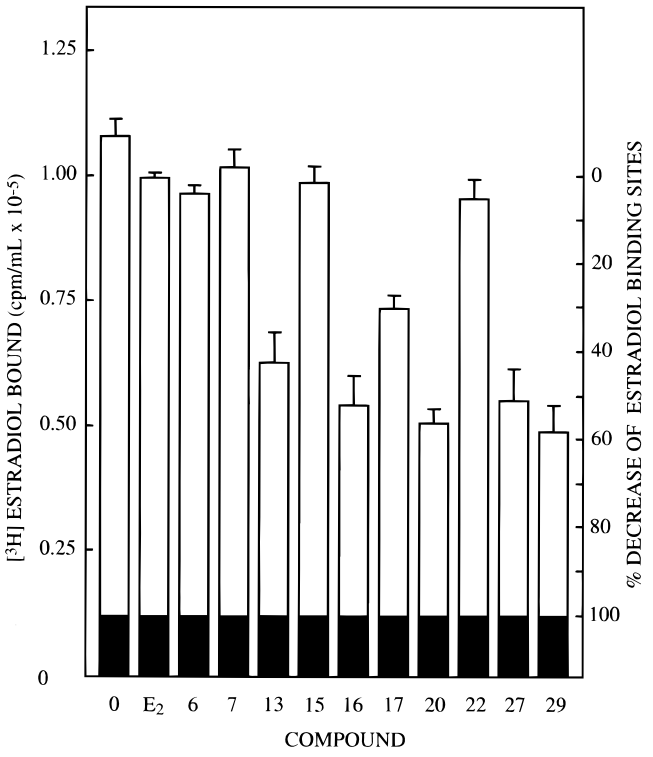


Figure 1. Inactivation of specific estradiol-binding sites in lamb uterine cytosol by 11β-estradiol derivatives. Uterine cytosol (4 mg protein/mL, pH 8.5) was incubated for 16 h at 0 °C, without steroid (0), with 100 nM estradiol (E_2), or with 200 nM of the synthesized electrophilic 11β-estradiol derivatives. Samples were then charcoal treated. Charcoal was pelleted by centrifugation, and supernatant aliquots were incubated under exchange conditions with 20 nM [^{3}H]estradiol in the absence and presence of 5 μM unlabeled estradiol. Total binding and nonspecific binding of [^{3}H]estradiol were determined as described in Materials and Methods. Total binding (unfilled bars) and nonspecific binding (filled bars), which did not significantly vary according to the compound incubated with cytosol, are represented. Values are means of duplicate determinations. The top of the unfilled bars also indicates the percentage decrease in the concentration of specific estradiol-binding sites (right scale), using the concentration of binding sites measured in cytosol exposed to estradiol as the reference binding site concentration.

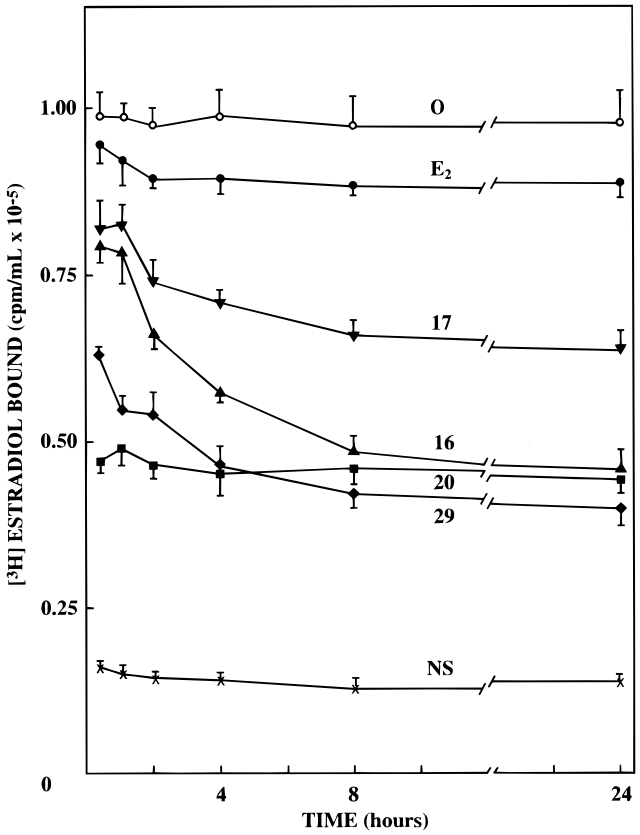


Figure 2. Time-course of inactivation of specific estradiol-binding sites. Uterine cytosol was incubated at 0 °C, pH 8.5, without steroid, with 100 nM estradiol, or with 200 nM chloroacetamide **16** or **29**, iodide **17,** or bromoacetamide **20**. After various periods of time, aliquots were removed and immediately charcoal treated. After sample centrifugation, the total binding and nonspecific binding of [^{3}H]estradiol (20 nM) in supernatants occurring under exchange conditions were determined. The total binding of [^{3}H]estradiol in supernatants corresponding to cytosol incubated without steroid (0) or with estradiol (●, E_2), chloroacetamide **16** (▲) or **29** (◆), iodide **17** (▼), or bromoacetamide **20** (■) and the nonspecific binding of [^{3}H]estradiol (X, NS), 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.

relative to that measured in cytosol exposed to estradiol), whereas the tertiary bromoacetamide **15**, which is directly derived from **16** by the Cl to Br substitution, had no effect. The iodopentyloxyphenyl derivative **17** induced a 30% effect. Of the two bromoacetamido homologues of **17**, the secondary amide **20** was effective (∼55%), whereas the tertiary amide **22** was not. Finally, the secondary and tertiary chloroacetamides **27** and **29**, whose structures include the propynylphenyl group, induced a 50−60% effect. The effect of compounds depended on the compound concentration (not shown); however, it did not markedly increase for compound concentrations > 100 nM (cf Figure 3). Depending on the ER/protein ratio in cytosol (0.9−2.2 pmol ER/mg protein in the various experiments performed), the percentage decreases in binding site concentration varied markedly, i.e., the higher the relative ER titer, the higher the decrease. However, the same qualitative results were obtained in the various experiments performed, i.e., compounds **6**, **7**, **15**, and **22** did not induce a significant decrease (which would be ≥10%) in the binding site concentration, whereas for the other compounds the order for increasing effectiveness was always: **17** < **13** < **16** ∼ **20** ∼ **27** ∼ **29**. With compound concentration ≤ 300 nM, regardless of the conditions used (temperature, pH, incubation time), the maximum decrease in the binding site concentration never exceeded 70%. Results almost identical to those shown in Figure 1 were obtained when incubation of cytosol with 11β-estradiol derivatives was performed at 25 °C instead of at 0 °C (not shown). The major difference between the two series of results concerned the binding site concentration measured from control cytosol incubated without compound. Due to the unprotected hormone-binding sites, at 25 °C there was a slight time-dependent decrease in the binding site concentration, which was not observed at 0 °C. When exposure of cytosol to the 11β-estradiol derivatives was performed at pH 7.0 instead of at pH 8.5, decreases in binding sites, although qualitatively similar to those shown in Figure 1, were less marked. Finally, similar qualitative results were obtained with compounds **13**, **16**, **17**, **20**, **27,** and **29**

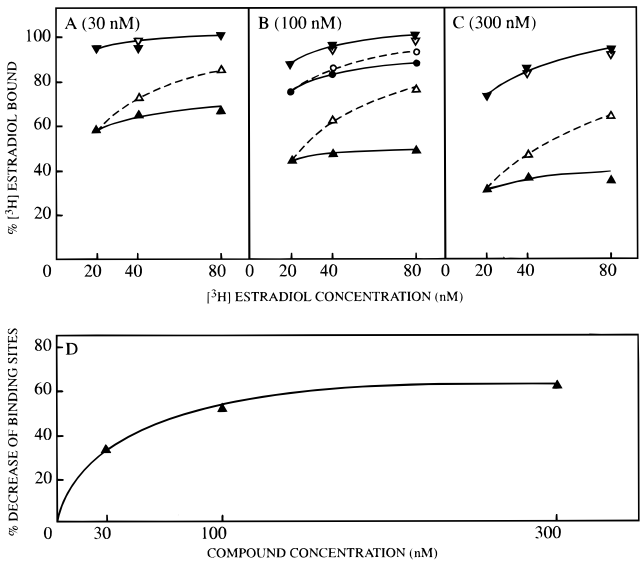


Figure 3. Irreversible inactivation of specific estradiol-binding sites by bromoacetamide **20**. Uterine cytosol was incubated for 16 h at 0 °C, pH 8.5, without steroid, with 100 nM estradiol, or with various concentrations (30, 100, or 300 nM) of bromoacetamide **20** or **22**. After charcoal treatment and then centrifugation, supernatant aliquots were incubated for 16 h at 20 °C with 20, 40, or 80 nM [^{3}H]estradiol in the absence and presence of 10 μM unlabeled estradiol. Total binding and nonspecific binding of [^{3}H]estradiol in the aliquots were determined. (A−C) Specific [^{3}H]estradiol-binding site concentrations determined from cytosol incubated with 30 nM (A), 100 nM (B), or 300 nM (C) estradiol (●) or bromoacetamide **20** (▲) or **22** (▼) are expressed as percentages of the specific [^{3}H]estradiol-binding site concentration measured from cytosol incubated without steroid. Percentage inhibitions of [^{3}H]-estradiol binding elicited by the various concentrations of the three steroids at 20 nM [^{3}H]estradiol concentration were used to determine theoretical binding levels which would be obtained at 40 and 80 nM [^{3}H]estradiol if decreases in specific binding measured at 20 nM [^{3}H]estradiol were due exclusively to classical competitive inhibition of the compounds; cytosol incubated with estradiol (○) or bromoacetamide **20** (△) or **22** (▽). (D) Deficit in estradiol-binding site concentration induced by bromoacetamide **20** and determined using 80 nM [^{3}H]-estradiol is plotted against the concentration of **20**. Values are means of duplicate determinations; experimental variation was under 10%.

using wild-type human ERα[30] expressed in COS cells; the effects of the compounds (60−85% decrease in the binding site concentration according to the compounds) were more pronounced than those obtained with the lamb uterine ER, possibly due to the higher ER/protein ratio in COS extracts than in uterine cytosols. Further studies of ER affinity labeling by 11β-aryl derivatives of estradiol were then performed with the lamb uterine cytosol at 0 °C, pH 8.5, using compounds at 100−300 nM.

Kinetic studies were performed to determine whether the observed decreases in [^{3}H]estradiol-binding site concentration following cytosol exposure to certain of the electrophiles were due to affinity labeling of ER or to classical binding of electrophiles. Figure 2 shows the time-course variation of the estradiol-binding site concentration in cytosol incubated without steroid, with unlabeled estradiol or with electrophile **16**, **17**, **20,** or **29**. For control cytosol incubated without steroid, according to the incubation time there was no variation in the binding site concentration. Estradiol elicited a slight decrease which was stabilized after only 2 h (effect which probably reflected completion of the formation of the ER:estradiol complex). Electrophiles **16**, **17,** and **29** induced much more gradual and pronounced decreases than that induced by estradiol, since at least 8 h was required for binding stabilization. A similar result was obtained with electrophiles **13** and **27** (not shown). Bromoacetamide **20** induced a marked decrease, which did not show any time dependence.

Kinetic studies would not be suitable in some instances to reveal an affinity labeling process[19] (cf. Discussion). Therefore, to potentially distinguish between conventional binding and covalent attachment of **20** to the ER hormone-binding site, experiments were performed in which the hormone-binding site concentration was determined using increasing amounts of [^{3}H]estradiol. Figure 3 shows the binding site concentration in cytosol preincubated with 30 nM (Figure 3A), 100 nM (Figure 3B), or 300 nM (Figure 3C) bromoacetamide **20** or its *N*-methyl homologue **22** or 100 nM unlabeled estradiol, as a function of the amount of [^{3}H]-estradiol (20, 40, or 80 nM) used for exchange. Under equilibrium conditions, the deficit in binding sites measured with 20 nM [^{3}H]estradiol in cytosol preincubated with unlabeled estradiol or with **22** appeared to result from conventional binding of residual steroid, since this deficit was progressively (at the expected ratios) resorbed when 40 and 80 nM [^{3}H]estradiol, instead 20 nM, were used. This was not observed with cytosol preincubated with **20**. There were substantial differences between the [^{3}H]estradiol binding measured with 40 and 80 nM [^{3}H]estradiol and those calculated assuming that the deficit measured with 20 nM [^{3}H]-estradiol would result only from conventional occupancy of ER by bromoacetamide **20**. Following incubation of cytosol with compound **20**, the deficit in binding sites measured with 80 nM [^{3}H]estradiol differed only slightly from that measured with 20 nM. Figure 3D shows the variation in this deficit measured with 80 nM [^{3}H]-estradiol, according to the concentration of **20**; relative to the deficit in binding sites obtained for 300 nM, the decrease was >50% and >80% for 30 and 100 nM **20**, respectively.

According to the kinetics or the exchange criterion, the six electrophiles appeared to bind covalently to ER. To check whether this covalent binding occurred at or very close to the hormone-binding site, the effects of the compounds were then determined using the estradiol-filled ER. In this case, none of the six compounds was able to significantly decrease the hormone-binding site concentration (Figure 4). We therefore concluded that electrophiles **13**, **16**, **17**, **20**, **27,** and **29** were indeed affinity labels of ERα.

Since the ER covalent attachment sites of reactive ligands identified so far are exclusively cysteines, we then examined the possible involvement of cysteine residues in the alkylation process of ERα by 11β-estradiol derivatives. The effects of the compounds were determined using cytosol treated by MMTS, a thiol-specific reagent which transforms SH into S−S−CH_3. Eight out of the 10 electrophiles were able to significantly decrease the binding site concentration in MMTS-treated cytosol (Figure 5). Of the four electrophiles found that had no significant effect on the native ER, mesylate

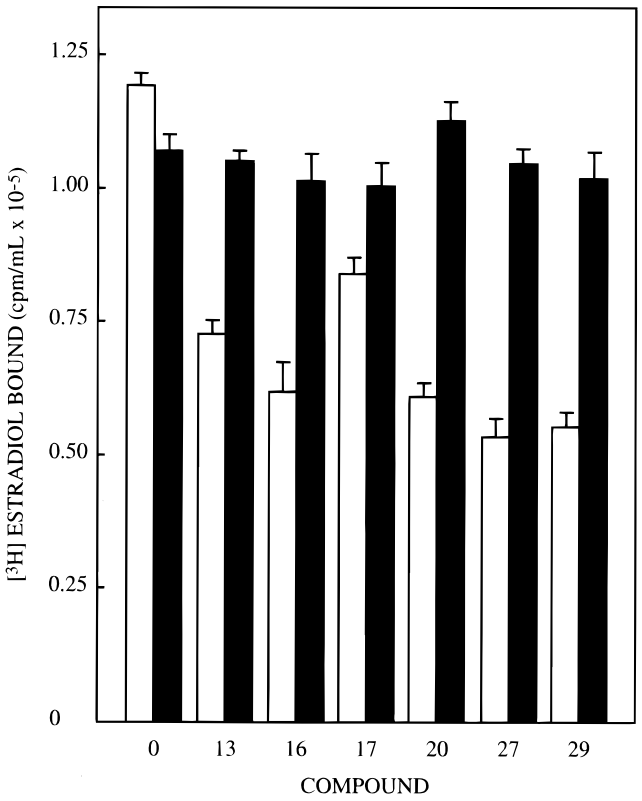


Figure 4. Inability of electrophilic 11β-estradiol derivatives to inactive estradiol-filled hormone-binding site of ER. Uterine cytosol was preincubated for 4 h at 0 °C, pH 8.5, with or without 100 nM estradiol. Aliquots of the two samples were then incubated for 16 h at 0 °C without steroid (0) or with 200 nM chloroacetamide **13**, **16**, **27,** or **29**, iodide **17,** or bromoacetamide **20**. After charcoal removal of steroid, the total binding and nonspecific binding of [^{3}H]estradiol (30 nM) occurring under exchange conditions in the various samples were determined. The specific [^{3}H]estradiol-binding site concentration in samples corresponding to cytosol preincubated without (unfilled bars) or with (filled bars) estradiol is represented. Values are means of duplicate determinations.

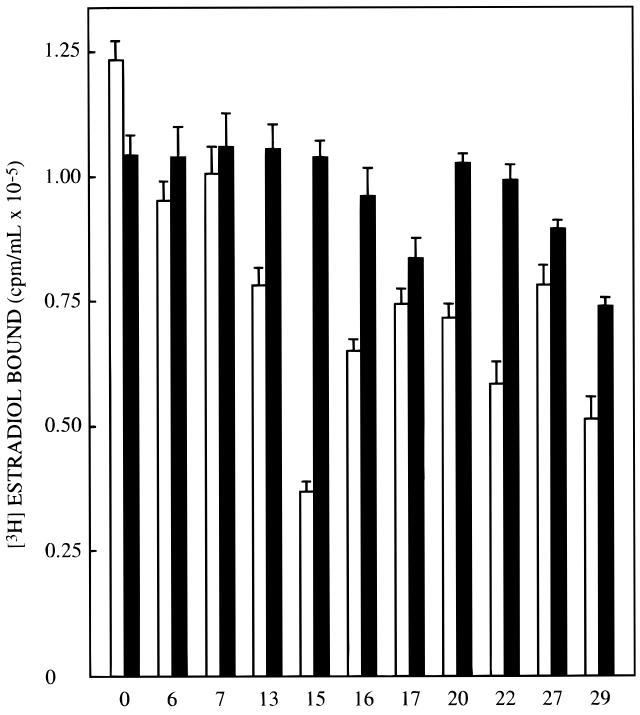


Figure 5. Inactivation of specific estradiol-binding sites in MMTS-treated cytosol by 11β-estradiol derivatives. Effect of binding site occupancy by estradiol. Uterine cytosol was preincubated for 4 h at 0 °C, pH 8.5, with 2 mM MMTS and then for an additional 4 h with or without 100 nM estradiol. Aliquots of the two samples were incubated for 16 h at 0 °C without steroid (0) or with 200 nM of each of the 10 synthesized electrophilic estradiol derivatives (**6**−**29**). The total binding and nonspecific binding of [^{3}H]estradiol (30 nM) occurring under exchange conditions were determined. The specific [^{3}H]-estradiol-binding site concentration in samples corresponding to cytosol preincubated without (unfilled bars) or with (filled bars) estradiol is represented. Values are means of duplicate determinations.

6 and iodide **7** were still inactive, whereas tertiary bromoacetamides **15** and **22** became very effective (∼45% and ∼65% effectiveness, respectively). The six other electrophiles induced decreases in the binding site concentration which were less pronounced than those obtained with the native ER (compare Figure 5 with Figure 1). For chloro- and bromoacetamides **13**, **16,** and **20**, the decreases (25−35%) appeared to reflect a specific effect, since they were totally prevented by estradiol occupancy of the hormone-binding site, whereas hormone-binding site occupancy by estradiol only partially prevented the decrease in the binding site concentration induced by iodide **17** and chloroacetamides **27** and **29** (30−50%). This latter result suggested that after charcoal treatment of cytosol, the non-ER-bound residual concentration of these particular electrophiles or derivatives was sufficient to reduce the specific binding of [^{3}H]-estradiol measured in cytosol exposed to each of the other electrophiles. In the absence of estradiol, due to complexing by ER and then protection from charcoal absorption, the concentration of residual electrophile would be higher than in the presence of estradiol. This difference between residual concentrations of electrophile could explain, via classical competitive binding with [^{3}H]estradiol, the relatively minor difference in the binding site concentrations measured between unfilled and estradiol-filled ER samples exposed to **17**, **27**, or **29**. In this case, the deficit in binding site concentration resulting from exposure of cytosol to the compounds would essentially result from conventional binding and not from ER affinity labeling. This was confirmed using increasing amounts of [^{3}H]estradiol for the exchange step. The deficit in binding sites resulting from cytosol exposure to **17**, **27**, or **29** progressively disappeared as the [^{3}H]estradiol concentration increased (not shown). We concluded that cysteine blockade by MMTS had very contrasted effects on the ability of electrophiles to covalently interact with the hormone-binding site of ERα: (i) iodide **17** and chloroacetamides **27** and **29**, which were initially effective, became practically inactive; (ii) chloroacetamides **13** and **16** and bromoacetamide **20** were still active but with lower effectivenesses; and (iii) tertiary bromoacetamides **15** and **22**, first inactive, became the most effective compounds.

Discussion

The 10 high-affinity steroidal electrophiles synthesized and evaluated as affinity labeling agents of ERα in this study are closely related; i.e., they are derived from 11β-phenylestradiol by introducing on the 11β-

phenyl group, in the *para* position, a linear substituent that includes a terminal electrophilic functionality. Five different classes of compounds can be defined according to the 11β-substituent type (Table 1): (1) ØO$(CH_2)_2$X (**6** and **7**), (2) ØO$(CH_2)_2$N(H/CH_3)COCH_2X (**13**, **15,** and **16**), (3) ØO$(CH_2)_5$X (**17**), (4) ØO$(CH_2)_5$N(H/CH_3)COCH_2X (**20** and **22**), and (5) ØC≡CCH_2N(H/CH_3)COCH_2X (**27** and **29**), where X = Cl, Br, I, or OSO_2CH_3. However, three parameters, i.e., the type of electrophilic functionality and the length and flexibility of the arm linking the electrophilic carbon to the 11β-phenyl group, should largely account for the reactivity of these molecules in the ERα hormone-binding pocket. There are four different types of electrophilic functionality in these molecules, with the following chemical reactivity order: methanesulfonate < iodide < chloroacetamide < bromoacetamide. According to the compound, the electrophilic center is three (**6** and **7**), six (**13**, **15**−**17**, **27,** and **29**), or nine (**20** and **22**) bonds away from the 11β-phenyl group, whereas the degree of freedom of the linking arm is two (**6**, **7**, **27,** and **29**), four (**13**, **15,** and **16**), five (**17**), or seven (**20** and **22**).

On the basis of results obtained in kinetic and binding displacement experiments with lamb uterine cytosol, 6 out of the 10 electrophiles appeared to be effective affinity labeling agents of native ERα, since: (i) ERα is the major ER form expressed in the uterus; and (ii) very similar results were obtained using extracts from cells transfected with a human ERα expression vector. The two class 1 compounds **6** and **7**, which include the shorter linking arm (comprising three bonds), did not alkylate the ER hormone-binding site, whereas with the unique exception of the two tertiary bromoacetamides **15** and **22**, all members of classes 2−5 (including one iodide and five haloacetamides, whose linking arm comprises six or nine bonds) were able to effectively alkylate the ERα hormone-binding site. The inefficacy of **15** and **22**, which only differ from active compounds **16** and **20** by a CH_2Cl to CH_2Br and a NH to NCH_3 change in the haloacetamido function, respectively, was astonishing. The effect of the NH to NCH_3 change found with class 4 bromoacetamides was not observed with classes 2 and 5 chloroacetamides, since **16** and **29** were found to be at least as effective as **13** and **27**, respectively, for alkylating ER. These results indicate that, depending on the compound class, the positioning of the compound within the hormone-binding pocket would be strongly influenced by discrete compound modifications. For classes 2 and 5 chloride compounds, the NH to NCH_3 change would have no marked effect on the compound positioning; whereas the Cl to Br change for the class 2 tertiary chloroacetamide **16** and the NH to NCH_3 change for the class 4 bromoacetamide **20** would modify the compound positioning in the hormone-binding pocket. The results suggest that starting from the 11β-phenylestradiol structure:

(i) A three-bond linking arm is insufficient for the electrophilic center to be localized near a reactive nucleophilic amino acid residue in the hormone-binding pocket. The absence of ERα affinity labeling by class 1 compounds **6** and **7** probably resulted from the short linking arm in these compounds and not from their type of electrophilic functionality, since 11β-(bromoacetamidobutyl)estradiol, whose distance between the electrophilic center and C-11 was quite similar to the corresponding distance in **6** and **7**, although including a strong electrophilic carbon, was unable to alkylate the ERα hormone-binding site.[22] Conversely, compound **17**, whose electrophilic center is identical but more remote from C-11 than that of **7**, was an effective ER affinity labeling agent.

(ii) A six- or nine-bond linking arm allows the electrophilic center to be localized in the neighborhood of a reactive nucleophilic amino acid residue in the hormone-binding pocket. However, with this type of linking arm, relatively discrete changes in the arm (such as NH to NCH_3 change) or in the electrophilic functionality (such as CH_2Cl to CH_2Br change) modify the positioning of the electrophilic center, which can no longer be attacked by the nucleophilic amino acid residue of the hormone-binding pocket.

Similar variations in ER affinity labeling efficiency were previously reported for a series of closely related electrophilic hexestrol derivatives;[31] i.e., the activity of the compounds markedly varied according to the length and type of the chain linking an aziridine function to the hexestrol nucleus. The inability of class 1 compounds to alkylate ER suggests that the covalent attachment sites of electrophilic 11β-estradiol derivatives are relatively remote (at least "seven bonds" away) from the steroid nucleus when the derivatives are bound to ER. This assumption agrees with previous results obtained with other electrophilic 11β-estradiol derivatives, since compounds whose linear arm was at most seven bonds long were ineffective for alkylating ERα, whereas a 10-bond linking arm compound was very effective.[22] Moreover, with the exception of class 4 compounds (in which nine bonds separate the electrophilic center from the 11β-phenyl ring), for all other 11β-derivatives the direct branching of the phenyl ring (with an axial configuration) on the steroid nucleus restricts the location of the electrophilic center to the β-side of the steroid. Therefore, when these 11β-derivatives are in the hormone-binding pocket, relative to the steroid nucleus, the target amino acid residues are localized on the β-side.

ERα was modified by MMTS with the aim of determining the potential involvement of cysteine residues of the hormone-binding pocket in the ER affinity labeling process. This approach was successfully used to highlight that the covalent attachment of tamoxifen aziridine and of electrophilic 17α-estradiol derivatives to ERα occurred at cysteine residues, since: contrary to native or wild-type ERα, MMTS-modified ER[19,20] and the double (C381A/C530A) and quadruple (C381A/C417A/C447A/C530A) cysteine to alanine ERα mutants[18,21] were no longer alkylated by the electrophiles. The effects of 11β-estradiol derivatives on MMTS-modified ER were striking: (i) class 1 compounds were still ineffective; (ii) compounds **15** and **22**, found to be ineffective on native ER, became the most effective compounds; and (iii) the activity of the other classes 2−5 compounds decreased, i.e., classes 2 and 4 compounds were still active but at a slight lesser extent, whereas classes 3 and 5 compounds displayed very little, if any, activity. These results can be interpreted as follows: (i) for iodide **17** and chloroacetamides **27** and **29**, cysteinyl residues in native ER would be quasi-exclusive covalent attachment sites without any site in MMTS-modified

ER; (ii) for bromoacetamides **15** and **22**, there would be no covalent attachment site in native ER, whereas in MMTS-modified ER non-cysteinyl residues would provide such sites; and (iii) for chloroacetamides **13** and **16** and bromoacetamide **20,** both cysteinyl residues and non-cysteinyl residues in native ER would be the covalent attachment sites, or cysteinyl residues in native ER and non-cysteinyl residues in MMTS-modified ER would be the covalent attachment sites. Since the lamb ERα hormone-binding domain has >95% identity with its human counterpart[32] with only one nonconservative change (R503 in lamb ER is changed to Q502 in human ERα) and perfect conservation of the four cysteines (C382, C418, C448, and C531 in lamb ERα), both the crystal structure and the results of the human ERα affinity labeling studies with tamoxifen aziridine could likely be extended to the lamb ER. If we assume that the binding mode of electrophilic 11β-estradiol derivatives to ERαs would be similar to those of raloxifene[9] and 4-hydroxytamoxifen[10] with the 11β-substituent acting as the aminoethoxyphenyl group of the two nonsteroidal antiestrogens, then the cysteinyl residues involved in the covalent attachment sites of 11β-estradiol derivatives would probably be homologues of human ERα C381 and/or C530. This was deduced from the fact that these two residues: (i) were located in structural elements of the hormone-binding domain (helix 5/helix 6 and helix 11/helix 12 loop, respectively) which contact the aminoethoxy chain of nonsteroidal antiestrogens;[9] and (ii) constituted the exclusive covalent attachment sites of tamoxifen aziridine.[17,18] The lamb homologues of human ERα C447 and C417 did not seem to be involved since C447, located at the core of the hormone-binding domain, was not accessible to iodoacetic acid,[33] a very small thiol reagent, and since C417 was found to be a covalent attachment site only for reactive estrogenic 17α-estradiol derivatives.[21] The assumption that the homologues of human ERα C381 and/or C530 would probably be the covalent attachment sites of the electrophilic 11β-estradiol derivatives to lamb ERα is in accordance with the fact that human ERα C530 was recently found to be the major covalent attachment site of two aziridine ligands directly obtained from iodides **7** and **17** and closely related to haloacetamides **13**, **15,** and **16** and to bromoacetamides **20** and **22**, respectively.[34] The presence of many potential non-cysteinyl attachment sites for the electrophiles, e.g., 12 conserved histidyl residues and 9 conserved lysyl residues in both lamb and human ERα hormone-binding domains, makes it difficult to determine which of these residues would constitute the non-cysteinyl attachment sites; some of these nucleophilic residues located close to C381 and C530 may be the potential sites.

As revealed by the striking changes in the ER alkylating properties of bromoacetamides **15** and **22**, methylthiolation of cysteine residues (presumably all except the C447 homologue) changed the accessibility of nucleophilic amino acids in the hormone-binding pocket. Presently we do not know whether the modification of cysteines induced only local or remote changes in the ER structure. Nevertheless, this effect suggests that models of the ERα hormone-binding domain bound to estrogen or antiestrogen, which were established from *S*-carboxymethylated crystallized domain, may differ from those that would be obtained using unmodified hormone-binding domain.

In conclusion, we were successful in preparing a series of electrophilic 11β-arylestradiol derivatives which are ERα affinity labeling agents. The covalent attachment sites of these electrophiles have not yet been identified. However, both cysteinyl (probably homologues of human ERα C381 and/or C530) and non-cysteinyl residues located on the β-side and remote from C-11 of the steroid (distance > "seven bonds") appear to be the electrophile covalent attachment sites. Studies involving the expression of recombinant ERα hormone-binding domain are under way to identify, by mass spectrometry analysis, the covalent attachment sites of the 17α- and 11β-estradiol derivatives we developed. The expected results could be used, in conjunction with the three-dimensional model of the human ERα hormone-binding domain, established from crystallized ER fragments, to model the interaction of 17α- and 11β-derivatives with ERα. Such studies should enhance the knowledge of ERα/ligand interactions, which result in full or defective activation of ERα, thus accounting for the estrogen or antiestrogen activity of ER ligands.

Materials and Methods

Chemical Synthesis and Characterization of 11β-Substituted Estradiol Derivatives. All solvents and reagents were used as received from commercial sources. Reaction progress was monitored using Merck silica gel 60 F_{254} TLC plates (thickness 0.5 mm). Column chromatography was carried out on Merck silica gel 60H (particle size < 60 μm). Melting points (mp) were determined on a Kofler apparatus and were uncorrected. Infrared (IR) spectra of compounds were recorded on either a Nicolet 5SX or a Perkin-Elmer 580 spectrophotometer as chloroform solutions or Nujol suspensions. Ultraviolet (UV) spectra were recorded on a Perkin-Elmer lambda 9 or on a Varian Cary 2200 spectrophotometer. Proton nuclear magnetic resonance (NMR) spectra were recorded at 300 MHz on a Bruker AC300 spectrometer. Unless otherwise mentioned, compounds were dissolved in deuteriochloroform (with 1−2 drops of deuteriopyridine in a few cases). Chemical shifts (δ) were expressed downfield from tetramethylsilane (used as internal standard). Mass spectra were obtained on the following mass spectrometers: Finnigan 4500 (EI, 70 eV); Autospec E (LSIMS) or ZAB-HFQ (FAB) (Micromass Ldt). Combustion analyses were carried out by the Analytical Department of Hœchst Marion Roussel (Romainville) and are within ±0.4%, unless otherwise noted. The usual workup involved dilution of the reaction mixture in an aqueous solution (generally 2 M sodium bicarbonate, unless otherwise stated) followed by extraction with an organic solvent (ethyl acetate, unless otherwise mentioned), washing with small portions of brine, and drying over magnesium sulfate. After filtration, the solvent was evaporated to dryness under reduced pressure.

11β-[4-(2-Hydroxyethoxy)phenyl]estra-4,9-diene-3,17-dione (2). Under an argon atmosphere, a 50% oily suspension of sodium hydride (2.78 g, 57.93 mmol) was added to a suspension of phenolic compound **1**[23] (15 g, 41.38 mmol) in DMF (200 mL). After 25 min stirring, a brown solution was obtained. (2-Bromoethoxy)-*tert*-butyldimethylsilane (19.8 g, 82.76 mmol) in DMF (110 mL) was added to the latter and the mixture was stirred for 22 h at room temperature; 6 N hydrochloric acid (50 mL) was added and the resulting solution was stirred for an additional hour. The usual workup and then chromatography (EtOAc−cyclohexane, 8:2) afforded 10.3 g of pure primary alcohol **2** (61%) as an amorphous solid, which was crystallized (CH_2Cl_2−isopropyl ether) to yield 9.51 g (57%) of whitish crystals: mp 201 °C; IR 3605, 1735, 1658, 1609, 1582, 1509 cm^{-1}; NMR 0.56 (s, 3H, CH_3-18), 2.04 (t, 1H, OH),

3.96 (m, 2H, CH_2−OH), 4.05 (d, 2H, CH_2−OΦ), 4.39 (d, 1H, H-11), 5.80 (s, 1H, H-4), 6.84−7.10 (2d, 4H, C_6H_4) ppm.

2-[4-(3,17-Dioxoestra-4,9-dien-11*β*-yl)phenoxy]ethyl Methanesulfonate (3). Methanesulfonyl chloride (2.8 mL, 36.17 mmol) was added to an ice-cooled solution of **2** (9.49 g, 23.34 mmol) in dichloromethane (200 mL) containing triethylamine (5 mL, 36.1 mmol) and 4-(dimethylamino)pyridine (200 mg, 1.6 mmol). The mixture was then stirred for 2 h at 0 °C. The usual workup and then recrystallization of the crude product (CH_2Cl_2−isopropyl ether) gave 11.12 g of pure mesylate **3** (98%) as whitish crystals: mp 164 °C; IR 1736, 1659, 1609, 1584, 1509, 1360, 1176 cm^{-1}; NMR 0.56 (s, 3H, CH_3-18), 3.09 (s, 3H, CH_3−SO_2), 4.22 and 4.56 (2m, 4H, CH_2−OΦ and CH_2−OSO_2), 4.39 (d, 1H, H-11), 5.80 (s, 1H, H-4), 6.82−7.11 (2d, 4H, C_6H_4) ppm.

2-[4-(3-Hydroxy-17-oxoestra-1,3,5(10)-trien-11*β*-yl)phenoxy]ethyl Methanesulfonate (4). A mixture of acetyl bromide (5.3 mL, 71 mmol) and acetic anhydride (10.6 mL, 112 mmol) was added to an ice-cooled solution of compound **3** (10.63 g, 21.94 mmol) in dichloromethane (100 mL). The reaction mixture was stirred for 1.5 h, while allowing it to reach room temperature, and then the solvents were removed under reduced pressure. The remaining oil was solubilized in THF (100 mL); the solution was cooled to 0 °C and methanol (100 mL) was slowly added followed by 2 N sodium hydroxide (74.5 mL). The mixture was stirred for 45 min at room temperature, and then 6 N hydrochloric acid (31 mL) was added. After concentration under reduced pressure, the usual workup and then chromatography (CH_2Cl_2−EtOAc, 9:1) afforded first 1.75 g of **5** (16%) and then 7.87 g of pure mesylate **4** (74%) as amorphous solids. Compound **4**: IR 3598, 1733, 1611, 1584, 1512, 1359, 1175 cm^{-1}; NMR 0.44 (s, 3H, CH_3-18), 3.04 (s, 3H, CH_3−SO_2), 4.00 (t, 1H, H-11), 4.13 and 4.51 (2m, 4H, CH_2−OΦ and CH_2−OSO_2), 6.49 (dd, 1H, H-2), 6.67 (d, 1H, H-4), 6.79 (d, 1H, H-1), 6.63−7.02 (2d, 4H, C_6H_4) ppm. Compound **5**: IR 1733, 1713, 1607, 1572, 1508, 1360, 1175 cm^{-1}; NMR 1.03 (s, 3H, CH_3-18), 2.83 (m, 2H, H-4), 3.11 (s, 3H, CH_3−SO_2), 4.24 and 4.59 (2m, 4H, CH_2−OΦ and CH_2−OSO_2), 6.82−7.11 (2d, 4H, C_6H_4) ppm.

2-[4-(3,17*β*-Dihydroxyestra-1,3,5(10)-trien-11*β*-yl)phenoxy]ethyl Methanesulfonate (6). Sodium borohydride (638 mg, 16.87 mmol) was added to an ice-cooled solution of compound **4** (8.18 g, 16.88 mmol) in methanol (81 mL) and THF (81 mL). After 1 h stirring at 0 °C, acetone (6 mL) was added, followed 5 min later by 0.5 N hydrochloric acid (until to reach pH 2). The usual workup afforded 7.7 g of crude product, which was purified by crystallization: the crude product was solubilized in a hot mixture of methanol and dichloromethane (1:1, 200 mL); isopropyl ether (300 mL) was added and the resulting solution was concentrated under reduced pressure, then cooled in an ice bath. Pure mesylate **6** (7.25 g, 88%) was obtained as white crystals: mp 211 °C; IR (Nujol) 3374, 1620, 1609, 1580, 1510, 1497, 1348, 1175 cm^{-1}; NMR 0.34 (s, 3H, CH_3-18), 3.04 (s, 3H, CH_3−SO_2), 3.70 (t, 1H, H-17), 3.93 (t, 1H, H-11), 4.12 and 4.50 (2m, 4H, CH_2−OΦ and CH_2−OSO_2), 6.48 (dd, 1H, H-2), 6.66 (d, 1H, H-4), 6.78 (d, 1H, H-1), 6.62−7.01 (2d, 4H, C_6H_4) ppm; UV (EtOH) 228 (*ϵ* 17400), 281 (*ϵ* 3500), 286 (*ϵ* 3650) nm, (EtOH/NaOH 0.1 N) 228 (*ϵ* 16900), 280 (*ϵ* 3200), 287 (*ϵ* 3300), 302 (*ϵ* 3100) nm. Anal. ($C_{27}H_{34}O_6S$) C, H, S.

11*β*-[4-(2-Iodoethoxy)phenyl]estra-1,3,5(10)-triene-3,-17*β*-diol (7). Sodium iodide (4.94 g, 32.95 mmol) and mesylate **6** (6.41 g, 13.17 mmol) were dissolved in 2-butanone (214 mL). The mixture was stirred under reflux for 17 h, then the solvent was evaporated under vacuum, and the residue was dissolved in a mixture of ethyl acetate and DMF (9:1, 1.5 L). The usual workup ($Na_2S_2O_3$) and then crystallization (EtOH) of the crude product afforded 4.94 g of pure iodide **7** (72%) as white crystals: mp 242 °C; IR (Nujol) 3343, 1611, 1579, 1507 cm^{-1}; NMR 0.34 (s, 3H, CH_3-18), 3.34 (t, 2H, CH_2I), 3.72 (t, 1H, H-17), 3.94 (t, 1H, H-11), 4.13 (t, 2H, CH_2−OΦ), 6.49 (dd, 1H, H-2), 6.66 (d, 1H, H-4), 6.79 (d, 1H, H-1), 6.62−7.00 (2d, 4H, C_6H_4) ppm; UV (EtOH) 230 (*ϵ* 16400), 281 (*ϵ* 3500), 286 (*ϵ* 3500) nm, (EtOH/NaOH 0.1 N) 228 (*ϵ* 16900), 280 (*ϵ* 3100), 287 (*ϵ* 3100), 301 (*ϵ* 3000) nm. Anal. ($C_{26}H_{31}O_3$ I) C, H, I.

3,17*β*-Bis[tetrahydro(2*H*)-2-pyranyloxy]-11*β*-[4-(2-iodoethoxy)phenyl]estra-1,3,5(10)-triene (8). *p*-Toluenesulfonic acid (510 mg, 2.96 mmol) was added to iodide **7** (4.39 g, 8.47 mmol) in THF (86 mL) and distilled dihydropyran (26 mL) and the mixture was stirred for 1.5 h; then triethylamine (8.5 mL) was added. The usual workup and then chromatography (cyclohexane−EtOAc−NEt_3, 90:10:0.1) gave 5.01 g of pure compound **8** (86%) as a white amorphous solid: IR 1609, 1580, 1512, 1500 cm^{-1}; NMR 0.35 (s, 3H, CH_3-18), 3.35 (t, 2H, CH_2I), 3.40−3.70 and 3.90 (m, 6H, H-11, H-17 and 2 CH_2−OCO), 4.14 (t, 2H, CH_2−OΦ), 4.58 (t) − 4.68 (t) − 4.82 (m) − 4.85 (m) (1H, OCHO-17), 5.32 (t, 1H, OCHO-3), 6.62 (m, 1H, H-2), 6.79 (m, 1H, H-4), 6.84 (m, 1H, H-1), 6.62−6.98 (2d, 4H, C_6H_4) ppm.

***N*-[2-[4-[3,17*β*-Bis[tetrahydro(2*H*)-2-pyranyloxy]estra-1,3,5(10)-trien-11*β*-yl]phenoxy]ethyl]trifluoroacetamide (9).** Under an argon atmosphere, a 50% oily suspension of sodium hydride (230 mg, 4.8 mmol) was added to trifluoroacetamide (494 mg, 4.36 mmol) in THF (12 mL). After 20 min stirring, iodide **8** (1.5 g, 2.18 mmol) in DMF (12 mL) was added and the mixture was stirred for 1.5 h at 65 °C. The usual workup (NH_4Cl) and then chromatography (cyclohexane−EtOAc−NEt_3, 75:25:01) gave 148 mg of the vinyl ether **10** (12%) derived from dehydroiodination of **8** and 956 mg of pure trifluoroacetamide **9** (65%) as a white amorphous solid. Compound **9**: IR (Nujol) 1721, 1613, 1574, 1561, 1512, 1500 cm^{-1}; NMR 0.36−0.37 (2s, 3H, CH_3-18), 3.40−3.95 (m, 6H, H-11, H-17 and 2 CH_2−OCO), 3.72 (q, (t after D_2O addition), 2H, CH_2−N), 4.00 (t, 2H, CH_2−OΦ), 4.58−4.68 (2t, 1H, OCHO-17), 5.31 (t, 1H, OCHO-3), 6.61 (m, 1H, H-2), 6.79 (m, 1H, H-4), 6.83 (m, 1H, H-1), 6.61−7.00 (2d, 4H, C_6H_4) ppm. Compound **10**: IR 1645, 1622, 1605, 1580, 1574, 1507, 1499 cm^{-1}; NMR 0.35 (s, 3H, CH_3-18), 3.45−3.95 (m, 6H, H-11, H-17 and 2 CH_2−OCO), 4.33 and 4.58−4.68 (d, 1H and m, 2H, OCHO-17 and vinyl CH_2), 4.96−5.31 (2t, 1H, OCHO-3), 6.56 (m, 2H, H-2 and CH−OΦ), 6.79 (m, 2H, H-1 and H-4), 6.72−7.02 (2d, 4H, C_6H_4) ppm; UV (EtOH) 233 (*ϵ* 21200), 279 (*ϵ* 3000), 285 (*ϵ* 2600) nm.

3,17*β*-Bis[tetrahydro(2*H*)-2-pyranyloxy]-11*β*-[4-(2-aminoethoxy)phenyl]estra-1,3,5(10)-triene (11). Trifluoroacetamide **9** (1.06 g, 1.58 mmol) in methanol (5 mL) and 1 N sodium hydroxide (3.1 mL) were stirred for 3 h at room temperature. The usual workup (NH_4Cl) yielded 886 mg of primary amine **11** (97%) as a white amorphous solid: IR 3385, 1610, 1581, 1512, 1498 cm^{-1}; NMR 0.36 (s, 3H, CH_3-18), 3.42−3.90 (m, 6H, H-11, H-17 and 2 CH_2−OCO), 3.90 (t, 2H, CH_2−OΦ), 4.59−4.68 (2t, 1H, OCHO-17), 5.31 (t, 1H, OCHO-3), 6.60 (m, 1H, H-2), 6.78 (m, 1H, H-4), 6.84 (m, 1H, H-1), 6.63−6.97 (2d, 4H, C_6H_4) ppm.

2-Chloro-*N*-[2-[4-(3,17*β*-dihydroxyestra-1,3,5(10)-trien-11*β*-yl)phenoxy]ethyl]acetamide (13). Bromoacetyl bromide (64 *μ*L, 0.74 mmol) in dichloromethane (4.5 mL) was added over a 5-min period to primary amine **11** (406 mg, 0.705 mmol) in dichloromethane (4.5 mL) containing pyridine (62 *μ*L, 0.785 mmol). After 1 h stirring at 0 °C, the usual workup gave 488 mg of bromoacetamide, whose 3- and 17*β*-hydroxyls were immediately deprotected: the crude product was solubilized in methanol (3.2 mL) and THF (3.2 mL); 2 N hydrochloric acid (1 mL) was added and the mixture was stirred for 1.5 h at room temperature. The usual workup and then chromatography (CH_2Cl_2−MeOH−NH_4OH, 95:5:0.25) gave 220 mg of an amorphous solid containing a mixture (70:30) of bromoacetamide **12** and chloroacetamide **13:** NMR (DMSO-d_6) 3.86 (s, CH_2Br) and 4.06 (s, CH_2Cl); MS (LSIMS) 528(MH^+), 527(M^+) and 484(MH^+), 483(M^+), respectively. Conversion of bromoacetamide **12** to chloroacetamide **13** was performed as follows: a mixture of **12** and **13** (200 mg) in DMF (4.8 mL) was stirred for 20 h at room temperature with lithium chloride (160 mg, 3.78 mmol). Evaporation of the solvent under reduced pressure and then chromatography afforded 110 mg of pure chloroacetamide **13** (35%) as a white amorphous solid: IR (Nujol) ≈3550, 1658, 1608, 1578, 1538, 1510 cm^{-1}; NMR (DMSO-d_6) 0.18 (s, 3H, CH_3-18), 3.43 (m, 3H, H-17 and CH_2N),

3.89 (m, 3H, H-11 and CH_2−OΦ), 4.06 (s, 2H, CH_2Cl), 4.40 (d, 1H, OH-17), 6.29 (dd, 1H, H-2), 6.45 (m, 1H, H-4), 6.66 (m, 1H, H-1), 6.60−6.95 (2d, 4H, C_6H_4), 8.41 (t, 1H, NH), 8.89 (s, 1H, OH-3) ppm. Anal. ($C_{28}H_{34}NO_4Cl$) H, N, Cl; C: calcd 69.48, found 68.6.

***N*-[2-[4-[3,17*β*-Bis[tetrahydro(2*H*)-2-pyranyloxy]estra-1,3,5(10)-trien-11*β*-yl]phenoxy]ethyl]-*N*-methyltrifluoroacetamide (14).** Under an argon atmosphere, a 50% oily suspension of sodium hydride (38 mg, 0.8 mmol) was added to *N*-methyltrifluoroacetamide (139 mg, 1.09 mmol) in THF (2 mL). After 20 min stirring, iodide **8** (0.5 g, 0.728 mmol) in DMF (2.5 mL) was added and the mixture was stirred for 2 h at 80 °C. The usual workup (NH_4Cl) and then chromatography (cyclohexane−EtOAc−NEt_3, 80:20:0.1) gave 141 mg of vinyl ether **10** (35%) derived from dehydroiodination of compound **8** and 245 mg of pure amide **14** (49%) as a white amorphous solid. Compound **14**: IR 1692, 1610, 1582, 1576, 1512, 1498 cm^{-1}; NMR 0.35−0.36 (2s, 3H, CH_3-18), 3.13−3.14−3.25−3.26 (4s, 3H, CH_3−N), 3.43−4.15 (m, 10H, H-11, H-17, 2 CH_2−OCO, CH_2−N and CH_2−OΦ), 4.58−4.68 (2t, 1H, OCHO-17), 5.31 (t, 1H, OCHO-3), 6.60 (m, 1H, H-2), 6.78 (m, 1H, H-4), 6.84 (m, 1H, H-1), 6.60−6.98 (2d, 4H, C_6H_4) ppm.

2-Bromo-*N*-[2-[4-(3,17*β*-dihydroxyestra-1,3,5(10)-trien-11*β*-yl)phenoxy]ethyl]-*N*-methylacetamide (15). Trifluoroacetamide **14** (652 mg, 0.951 mmol) in THF (7.5 mL) and 1 N sodium hydroxide (1.22 mL) were stirred for 1.5 h at room temperature. The usual workup (NH_4Cl) yielded 561 mg of secondary amine (quantitative yield) as an oil, which was immediately bromoacetylated: the crude product was solubilized in dichloromethane (6 mL) containing pyridine (85 μL, 1.06 mmol) and bromoacetyl bromide (87 μL, 1.0 mmol) in dichloromethane (6 mL) was added over a 5-min period. After 1 h stirring at 0 °C, the usual workup gave 636 mg of bromoacetamide (94%), whose 3- and 17*β*-hydroxyls were immediately deprotected: the crude product was solubilized in methanol (4 mL) and THF (4 mL); 2 N hydrobromic acid (2 mL) was added and the mixture was stirred for 1.5 h at room temperature. The usual workup (no brine washing) and then chromatography (CH_2Cl_2−MeOH−NH_4OH, 95:5:0.25) gave 438 mg of pure bromoacetamide **15** (85%) as an amorphous solid. Crystallization (CH_2Cl_2−isopropyl ether) gave 352 mg of compound **15** (68%) as white crystals: mp 158 °C; IR (Nujol) ≈3550, 1612, 1584, 1512, 1502 cm^{-1}; NMR (DMSO-d_6) 0.18 (s, 3H, CH_3-18), 2.88−3.08 (2s, 3H, CH_3N), 3.47 (m, 3H, H-17), 3.59−3.67 (2t, 2H, CH_2N), 3.86 (t, 1H, H-11), 3.95−4.04 (2t, 2H, CH_2−OΦ), 4.11−4.16 (2s, 2H, CH_2Br), 4.41 (d, 1H, OH-17), 6.29 (dd, 1H, H-2), 6.45 (d, 1H, H-4), 6.67 (m, 1H, H-1), 6.67−6.95 (2d, 4H, C_6H_4), 8.89 (s, 1H, OH-3) ppm; UV (EtOH) 230 (ε 19300), 281 (ε 3500), 286 (ε 3500) nm, (EtOH/NaOH 0.1 N) 230 (ε 18500), 280 (ε 3000), 287 (ε 3100), 302 (ε 2900) nm; MS (EI) 541(M^+). Anal. ($C_{29}H_{36}NO_4Br$) C, H, N, Br.

2-Chloro-*N*-[2-[4-(3,17*β*-dihydroxyestra-1,3,5(10)-trien-11*β*-yl)phenoxy]ethyl]-*N*-methylacetamide (16). A solution of bromoacetamide **15** (165 mg, 0.304 mmol) and lithium chloride (129 mg, 3.05 mmol) in DMF (4 mL) was stirred for 20 h at room temperature and then the solvent was removed under reduced pressure. The usual workup and then chromatography (CH_2Cl_2−MeOH−NH_4OH, 95:5:0.25) afforded 135 mg of pure chloroacetamide **16** (89%) as a white amorphous solid. Crystallization (CH_2Cl_2−isopropyl ether) gave 123 mg of compound **16** (81%) as white crystals: mp 229 °C; IR (Nujol) ≈3550, 1640, 1612, 1582, 1510 cm^{-1}; NMR (DMSO-d_6) 0.18 (s, 3H, CH_3-18), 2.88−3.05 (2s, 3H, CH_3N), 3.48 (m, 3H, H-17), 3.60−3.67 (2t, 2H, CH_2N), 3.95−4.02 (2t, 2H, CH_2−OΦ), 3.87 (m, 1H, H-11), 4.36−4.40 (2s, 2H, CH_2Cl), 4.40 (d, 1H, OH-17), 6.29 (dd, 1H, H-2), 6.45 (d, 1H, H-4), 6.66 (d, 1H, H-1), 6.66−6.96 (2d, 4H, C_6H_4), 8.88 (s, 1H, OH-3) ppm; UV (EtOH) 228 (ε 18000), 280 (ε 3400), 286 (ε 3500) nm, (EtOH/NaOH 0.1 N) 229 (ε 18000), 280 (ε 3000), 287 (ε 3100), 301 (ε 2900) nm. Anal. ($C_{29}H_{36}NO_4Cl$) H, N, Cl; C: calcd 69.93, found 69.4.

3,17*β*-Bis[tetrahydro(2*H*)-2-pyranyloxy]-11*β*-[4-[(5-iodopentyl)oxy]phenyl]estra-1,3,5(10)-triene (18). *p*-Toluenesulfonic acid (338 mg, 1.78 mmol) was added to iodide **17**[24] (3.55 g, 6.33 mmol) in THF (34 mL) and distilled dihydropyran (17 mL) and the mixture was stirred for 1.5 h at room temperature; triethylamine (6.4 mL) was then added. The usual workup and then chromatography (cyclohexane−EtOAc−NEt_3, 90:10:0.1) gave 5.1 g of crude iodide **18** (quantitative yield) as a colorless oil: IR 1610, 1581, 1512, 1498 cm^{-1}; NMR 0.37 (s, 3H, CH_3-18), 3.19 (t, 2H, CH_2I), 3.40−3.70 and 3.80−4.05 (m, 5H, H-11 and 2 CH_2−OCO), 3.67 (m, 1H, H-17), 3.85 (t, 2H, CH_2−OΦ), 4.59−4.68 (2m, 1H, OCHO-17), 5.31 (m, 1H, OCHO-3), 6.61 (m, 1H, H-2), 6.77 (m, 1H, H-4), 6.85 (m, 1H, H-1), 6.61−6.96 (2d, 4H, C_6H_4) ppm.

***N*-[5-[4-[3,17*β*-Bis[tetrahydro(2*H*)-2-pyranyloxy]estra-1,3,5(10)-trien-11*β*-yl]phenoxy]pentyl]trifluoroacetamide (19).** Under an argon atmosphere, a 50% oily suspension of sodium hydride (37 mg, 0.77 mmol) was added to trifluoroacetamide (118 mg, 1.05 mmol) in THF (2 mL). After 20 min stirring, iodide **18** (535 mg, 0.734 mmol) in DMF (2 mL) was added and the mixture was stirred for 5.5 h at room temperature. The usual workup (NH_4Cl) and then chromatography (hexanes−EtOAc, 7:3) gave 107 mg of unreacted iodide **18** (20%) along with 210 mg of pure trifluoroacetamide **19** (40%) as a white amorphous solid: IR 3340, 1727, 1610, 1576, 1512, 1498, 1180 cm^{-1}; NMR 0.37 (s, 3H, CH_3-18), 3.70−4.00 (m, 10H, H-11, H-17, 2 CH_2−OCO, CH_2−N and CH_2−OΦ), 4.59−4.67 (m, 1H, OCHO-17), 5.31 (m, 1H, OCHO-3), 6.31 (s, 1H, NH), 6.61 (m, 1H, H-2), 6.70−6.90 (m, 2H, H-1 and H-4), 6.61−6.97 (2d, 4H, C_6H_4) ppm.

2-Bromo-*N*-[5-[4-(3,17*β*-dihydroxyestra-1,3,5(10)-trien-11*β*-yl)phenoxy]pentyl]acetamide (20). Trifluoroacetamide **19** (550 mg, 0.77 mmol) solubilized in THF (5 mL) and methanol (3 mL) was stirred for 2.5 h at room temperature with 1 N sodium hydroxide (1.54 mL). The usual workup (NH_4Cl) yielded 480 mg of primary amine (quantitative yield) as a white amorphous solid. Bromoacetyl bromide (45 μL, 0.52 mmol) in dichloromethane (4 mL) was added over a 5-min period to primary amine (306 mg, 0.495 mmol) in dichloromethane (4 mL) containing pyridine (45 μL, 0.551 mmol). After 1 h stirring at 0 °C, the usual workup (no brine washing) gave 328 mg of bromoacetamide whose 3- and 17*β*-hydroxyls were immediately deprotected: the crude product was solubilized in methanol (2.5 mL) and THF (2.5 mL); 2 N hydrobromic acid (0.66 mL) was added and the mixture was stirred for 1.5 h at room temperature. The usual workup (no brine washing) and then chromatography (EtOAc−hexanes−MeOH−NH_4OH, 80:20:2.5:0.25) gave 160 mg of pure bromoacetamide **20** (57%) as an amorphous solid: IR (Nujol) ≈3550, 1658, 1612, 1580, 1543, 1511, 1505 cm^{-1}; NMR (DMSO-d_6) 0.18 (s, 3H, CH_3-18), 3.07 (q, 2H, CH_2N), 3.46 (m, 1H, H-17), 3.81 (s, 2H, CH_2Br), 3.83 (m, 3H, H-11 and CH_2−OΦ), 4.40 (d, 1H, OH-17), 6.29 (dd, 1H, H-2), 6.45 (d, 1H, H-4), 6.70 (d, 1H, H-1), 6.62−6.94 (2d, 4H, C_6H_4), 8.24 (t, 1H, NH), 8.89 (s, 1H, OH-3) ppm; UV (EtOH) 230 (ε 17400), 282 (ε 3700), 288 (ε 3600) nm, (EtOH/NaOH 0.1 N) 230 (ε 17000), 281 (ε 3100), 288 (ε 3200), 301 (ε 2900) nm. Anal. ($C_{31}H_{40}NO_4Br$) H, N; C: calcd 65.26, found 64.8; Br: calcd 14.00, found 13.0.

***N*-[5-[4-[3,17*β*-Bis[tetrahydro(2*H*)-2-pyranyloxy]estra-1,3,5(10)-trien-11*β*-yl]phenoxy]pentyl]-*N*-methyltrifluoroacetamide (21).** Under an argon atmosphere, a 50% oily suspension of sodium hydride (108 mg, 2.25 mmol) was added to trifluoroacetamide **19** (1.42 g, 1.99 mmol) in DMF (34 mL). After 20 min stirring at room temperature, methyl iodide (243 μL, 3.9 mmol) in THF (2 mL) was added and the mixture was stirred for 1 h at room temperature. The usual workup (NH_4Cl) and then chromatography (cyclohexane−EtOAc−NEt_3, 75:25:0.1) gave 1.423 g of pure trifluoroacetamide **21** (98%) as a white amorphous solid: IR 1691, 1610, 1580, 1512, 1498 cm^{-1}; NMR 0.36−0.37 (CH_3-18), 3.00−3.10 (2s, 3H, CH_3−N), 3.30−3.95 (m, 10H, H-11, H-17, 2 CH_2−OCO, CH_2−N and CH_2−OΦ), 4.58−4.67 (2t, 1H, OCHO-17), 5.31 (t, 1H, OCHO-3), 6.60 (m, 1H, H-2), 6.78 (m, 1H, H-4), 6.85 (m, 1H, H-1), 6.60−6.96 (2d, 4H, C_6H_4) ppm.

2-Bromo-*N*-[5-[4-(3,17*β*-dihydroxyestra-1,3,5(10)-trien-11*β*-yl)phenoxy]pentyl]-*N*-methylacetamide (22). Trifluo-

roacetamide **21** (464 mg, 0.637 mmol) solubilized in THF (3 mL) and methanol (3 mL) was stirred for 2.5 h at room temperature with 1 N sodium hydroxide (0.9 mL). The usual workup (NH_4Cl) yielded 431 mg of secondary amine (quantitative yield) as a white amorphous solid. Bromoacetyl bromide (58 μL, 0.67 mmol) in dichloromethane (5 mL) was added over a 5-min period to the crude secondary amine (431 mg) in dichloromethane (5 mL) containing pyridine (57 μL, 0.7 mmol) and the mixture was stirred for 1 h at 0 °C. The usual workup (no brine washing) gave 405 mg of bromoacetamide whose 3- and 17β-hydroxyls were immediately deprotected: the crude product was solubilized in methanol (3 mL) and THF (3 mL); 2 N hydrobromic acid (0.8 mL) was added and the mixture was stirred for 1.5 h at room temperature. The usual workup (no brine washing) and then chromatography (EtOAc−hexanes−MeOH−NH_4OH, 80:20:2.5:0.25) gave 201 mg of pure bromoacetamide **22** (54%) as an amorphous solid: IR (Nujol) ≈3550, 1635, 1611, 1580, 1510, 1502 cm^{-1}; NMR (DMSO-d_6) 0.19 (s, 3H, CH_3-18), 2.81−2.99 (2s, 3H, CH_3−N), 3.20−3.50 (m, 3H, CH_2N and H-17), 3.84 (m, 3H, H-11 and CH_2−OΦ), 4.07−4.10 (2s, 2H, CH_2Br), 4.34−4.39 (2d, 1H, OH-17), 6.29 (dd, 1H, H-2), 6.45 (d, 1H, H-4), 6.69 (d, 1H, H-1), 6.62−6.94 (2d, 4H, C_6H_4), 8.89 (s, 1H, OH-3) ppm; UV (EtOH) 230 (ϵ 19900), 282 (ϵ 3800), 288 (ϵ 3700) nm, (EtOH/NaOH 0.1 N) 230 (ϵ 19500), 281 (ϵ 3200), 288 (ϵ 3300), 302 (ϵ 2900) nm; MS (FAB) 583(M^+), 503(M^+ − Br). Anal. (C_{32} H_{42} NO_4 Br) C, H, N; Br: calcd 13.67, found 13.1.

3-[4-[3,17β-Bis[tetrahydro(2*H*)-2-pyranyloxy]estra-1,3,5-(10)-trien-11β-yl]phenyl]-2-propyn-1-ol (24). Butyllithium in hexanes (11.8 mL, 1.6 M) was slowly added to a stirred solution of ethynylphenyl steroid **23**[26] (7.2 g, 13.32 mmol) in THF (60 mL) at −30 °C. Five min after the addition was complete, paraformaldehyde (662 mg, 22 mmol) was added and the mixture was allowed to reach room temperature, while stirring for 2 h. The usual workup (NH_4Cl) and then chromatography (cyclohexane−EtOAc, 8:2) gave 6.64 g of propargyl alcohol **24** (87%) as a white solid. Crystallization (Et_2O) afforded 5.06 g of **24** (67%) as white crystals: mp 234 °C; IR 3608, 1607, 1574, 1555, 1506, 1498 cm^{-1}; NMR 0.31−0.33 (2s, 3H, CH_3-18), 3.40−3.60 and 3.80−4.00 (2m, 4H, 2 CH_2−OCO), 3.68 (dt, 1H, H-17), 4.43 (s, 2H, CH_2−OH), 4.58−4.67 (2m, 1H, OCHO-17), 5.32 (m, 1H, OCHO-3), 6.60 (dd, 1H, H-2), 6.79 (m, 2H, H-1 and H-4), 7.04−7.16 (2d, 4H, C_6H_4) ppm.

3-[4-[3,17β-Bis[tetrahydro(2*H*)-2-pyranyloxy]estra-1,3,5-(10)-trien-11β-yl]phenyl]-2-propyn-1-yl Methanesulfonate (25). Triethylamine (0.19 mL, 1.39 mmol) and methanesulfonyl chloride (0.11 mL, 1.39 mmol) were added to an ice-cooled solution of propargyl alcohol **24** (508 mg, 0.89 mmol) in dichloromethane (8 mL) containing 4-(dimethylamino)pyridine (7 mg, 0.06 mmol) and the mixture was then stirred for 1 h at 0 °C. The usual workup and then chromatography (cyclohexane−EtOAc−NEt_3, 70:30:0.1) gave 400 mg of pure mesylate **25** (69%) as a white amorphous solid: IR 2220, 1606, 1575, 1507, 1498, 1366, 1175 cm^{-1}; NMR 0.33 (s, 3H, CH_3-18), 3.14 (s, 3H, CH_3−SO_2), 3.55 and 3.93 (2m, 5H, H-11 and $2CH_2$−OCO), 3.69 (t, 1H, H-17), 4.57 and 4.66 (2m, 1H, OCHO-17), 5.31 (m, 1H, OCHO-3), 6.60 (dd, 1H, H-2), 6.80 (m, 2H, H-1 and H-4), 7.08−7.18 (2d, 4H, C_6H_4) ppm.

***N*-[3-[4-[3,17β-Bis[tetrahydro(2*H*)-2-pyranyloxy]estra-1,3,5(10)-trien-11β-yl]phenyl]-2-propyn-1-yl]trifluoroacetamide (26).** Under an argon atmosphere, a 50% oily suspension of sodium hydride (364 mg, 7.59 mmol) was added to trifluoroacetamide (780 mg, 6.9 mmol) in THF (18 mL). After 20 min stirring, mesylate **25** (2.27 g, 3.5 mmol) in DMF (18.5 mL) was added and the mixture was stirred for 1 h at 60 °C. The usual workup (NH_4Cl) and then chromatography (cyclohexane−EtOAc−NEt_3, 70:30:0.1) gave 1.24 g of pure secondary trifluoroacetamide **26** (53%) as a whitish amorphous solid: IR 3440, 1730, 1606, 1575, 1539, 1534, 1507, 1498 cm^{-1}; NMR 0.34 (s, 3H, CH_3-18), 3.45−3.60 and 3.85−4.00 (2m, 5H, H-11, and 2 CH_2−OCO), 3.68 (t, 1H, H-17), 4.33 (d, 2H, CH_2−N), 4.58−4.67 (2m, 1H, OCHO-17), 5.31 (m, 1H, OCHO-3), 6.59 (dd, 1H, H-2), 6.80 (m, 2H, H-1 and H-4), 7.06−7.16 (2d, 4H, C_6H_4) ppm.

2-Chloro-*N*-[3-[4-(3,17β-dihydroxyestra-1,3,5(10)-trien-11β-yl)phenyl]-2-propyn-1-yl]acetamide (27). Secondary trifluoroacetamide **26** (504 mg, 0.757 mmol) in THF (5 mL) and methanol (5 mL) was stirred for 4.5 h at room temperature with 1 N sodium hydroxide (2.25 mL). The usual workup (NH_4-Cl) yielded 422 mg of secondary amine (98%) as a yellow amorphous solid. Chloroacetyl chloride (37 μL, 0.457 mmol) in dichloromethane (4 mL) was added over a 5-min period to an ice-cooled solution of secondary amine (248 mg, 0.435 mmol) in dichloromethane (4 mL) containing pyridine (10 μL, 0.484 mmol). After 1 h stirring at 0 °C, the usual workup gave 276 mg of chloroacetamide (98%) whose 3- and 17β-hydroxyls were immediately deprotected: the crude product was solubilized in methanol (2.5 mL) and THF (2.5 mL); 2 N hydrochloric acid (1 mL) was added and the mixture was stirred for 1.5 h at room temperature. The usual workup and then chromatography (CH_2Cl_2−MeOH−NH_4OH, 95:5:0.5; then EtOAc−hexanes−MeOH−NH_4OH, 80:20:2.5:0.25) gave 172 mg of pure chloroacetamide **27** (81% from **26**) as an amorphous solid. Crystallization (CH_2Cl_2−isopropyl ether) afforded 156 mg of **27** (74%) as white crystals: mp 168 °C; IR (Nujol) ≈3550, 1663, 1611, 1583, 1535, 1506 cm^{-1}; NMR (DMSO-d_6) 0.15 (s, 3H, CH_3-18), 3.49 (m, 1H, H-17), 3.94 (t, 1H, H-11), 4.09 (s, 2H, CH_2Cl), 4.11 (d, 2H, CH_2N), 4.43 (d, 1H, OH-17), 6.28 (dd, 1H, H-2), 6.46 (d, 1H, H-4), 6.67 (d, 1H, H-1), 7.05−7.13 (2d, 4H, C_6H_4), 8.71 (t, 1H, NH), 8.91 (s, 1H, OH-3) ppm; UV (EtOH) 250 (ϵ 23500), 261 (ϵ 21800), 278 (ϵ 2800), 284 (ϵ 2700), 290 (ϵ 2400) nm, (EtOH/NaOH 0.1 N) 250 (ϵ 29700), 260 (ϵ 25000), 302 (ϵ 2700) nm; MS (El) 477(MH^+), 442(M^+ − Cl). Anal. ($C_{29}H_{32}NO_3Cl$) H, N, Cl; C: calcd 72.87, found 72.0.

***N*-[3-[4-[3,17β-Bis[tetrahydro(2*H*)-2-pyranyloxy]estra-1,3,5(10)-trien-11β-yl]phenyl]-2-propyn-1-yl]-*N*-methyltrifluoroacetamide (28).** Under an argon atmosphere, a 50% oily suspension of sodium hydride (52 mg, 1.09 mmol) was added to secondary trifluoroacetamide **26** (660 mg, 0.991 mmol) in DMF (15 mL). After 10 min stirring, methyl iodide (123 μL, 1.98 mmol) in THF (1.2 mL) was added and the mixture was stirred for 1 h at room temperature. The usual workup (NH_4Cl) and then chromatography (cyclohexane−EtOAc−NEt_3, 80:20:0.1) gave 569 mg of pure tertiary trifluoroacetamide **28** (84%) as a white amorphous solid: IR 2235, 2215, 1696, 1617, 1607, 1575, 1555, 1507, 1498 cm^{-1}; NMR 0.34 (s, 3H, CH_3-18), 3.14−3.22 (2s, 3H, CH_3N), 3.45−3.65 and 3.85−4.00 (2m, 5H, H-11, and $2CH_2$−OCO), 3.68 (m, 1H, H-17), 4.36−4.45 (2s, 2H, CH_2N), 4.58−4.66 (2m, 1H, OCHO-17), 5.31 (m, 1H, OCHO-3), 6.59 (dd, 1H, H-2), 6.80 (m, 2H, H-1 and H-4), 7.06−7.16 (2d, 4H, C_6H_4) ppm.

2-Chloro-*N*-[3-[4-(3,17β-dihydroxyestra-1,3,5(10)-trien-11β-yl)phenyl]-2-propyn-1-yl]-*N*-methylacetamide (29). Tertiary trifluoroacetamide **28** (626 mg, 0.921 mmol) in THF (5.5 mL) and methanol (5.5 mL) was stirred for 1 h at room temperature with 1 N sodium hydroxide (1.85 mL). The usual workup (NH_4Cl) yielded 525 mg of secondary amine (98%) as a yellow amorphous solid. Chloroacetyl chloride (44 μL, 0.57 mmol) in dichloromethane (4 mL) was added over a 5-min period to an ice-cooled solution of secondary amine (317 mg, 0.543 mmol) in dichloromethane (4 mL) containing pyridine (84 μL, 0.604 mmol) and the mixture was stirred for 1 h at 0 °C. The usual workup gave 347 mg of chloroacetamide (97%) whose 3- and 17β-hydroxyls were immediately deprotected: the crude product was solubilized in methanol (2.5 mL) and THF (2.5 mL); 2 N hydrochloric acid (1.2 mL) was added and the mixture was stirred for 2 h at room temperature. The usual workup and then chromatography (CH_2Cl_2−MeOH−NH_4OH, 95:5:0.25) gave 213 mg of pure chloroacetamide **29** (78% from **28**) as an amorphous solid. Crystallization (CH_2Cl_2−isopropyl ether) afforded 203 mg of chloroacetamide **29** (74%) as white crystals: mp 240 °C; IR (Nujol) ≈3580, ≈3520, 1645, 1620, 1580, 1501 cm^{-1}; NMR (DMSO-d_6) 0.16 (s, 3H, CH_3-18), 2.92−3.06 (2s, 3H, CH_3N), 3.49 (m, 1H, H-17), 3.93 (t, 1H, H-11), 4.36−4.43 (2s, 5H, CH_2Cl, CH_2N and OH-17), 6.28 (dd, 1H, H-2), 6.46 (d, 1H, H-4), 6.66 (d, 1H, H-1), 7.06−7.14 (2d, 4H, C_6H_4), 8.71 (t, 1H, NH), 8.91 (s, 1H, OH-3) ppm; UV (EtOH) 251 (ϵ 26400), 261 (ϵ 23900), 277 (ϵ 3200), 284 (ϵ 3100), 289 (ϵ

2700) nm, (EtOH/NaOH 0.1 N) 250 (ϵ 32900), 260 (ϵ 27400), 302 (ϵ 3000) nm. Anal. ($C_{30}H_{34}NO_3Cl$) C, H, N, Cl.

Biological Materials. [6,7-^{3}H]Estradiol (specific activity 2.00 PBq/mol, radiochemical purity > 98%) was purchased from Amersham International (Amersham, England). Estradiol and 11β-derivatives used for binding studies 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.

Cytosolic ER. Cytosol was prepared in Tris-HCl buffer (T_{20}), pH 7 (competitive binding experiments) or 8.5 (irreversible binding experiments), from immature lamb uteri as described previously.[19] The protein concentration, determined according to Layne,[35] was adjusted to 2 mg/mL (competitive binding experiments) or 4 mg/mL (irreversible binding experiments).

Competitive Binding Assay: Apparent Relative Affinity Constants. Competition assays between 5 nM [^{3}H]-estradiol and increasing concentrations of nonradioactive estradiol or 11β-derivatives for binding the lamb uterine cytosol (2 mg protein/mL, pH 7) were performed for 20 h at 20 °C, as described previously.[19] Apparent relative affinity constants (RACs) of competitors were calculated according to Korenman[27] using concentrations of unbound and specifically bound [^{3}H]estradiol at 50% specific binding inhibition, as well as the concentration of unlabeled estradiol and 11β-derivatives which inhibited 50% of the specific binding of [^{3}H]estradiol.[19]

Standard Irreversible Binding Assay. Cytosol (4 mg protein/mL, pH 8.5) was first incubated for 16 h at 0 °C with estradiol, with 11β-derivatives, or without any steroid. Samples were then treated with an equal volume of charcoal suspension (1% charcoal, 0.1% dextran T70 in T_{20}, 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 described previously[19] using, except where otherwise stated, 20 nM [^{3}H]estradiol in the absence and presence of 5 μM nonradioactive estradiol.

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

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