Steroidal Affinity Labels of the Estrogen Receptor. 2. 17α-[(Haloacetamido)alkyl]estradiols

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In a previous study, we described affinity labeling of the lamb uterine estrogen receptor by 17α-[(bromoacetoxy)alkyl/alkynyl]estradiols. However, the intrinsic receptor-alkylating activities of these compounds were probably very hampered by their poor hydrolytic stability in estrogen receptor-containing tissue extracts. Therefore, (i) to develop affinity labels of the receptor not susceptible to hydrolysis and (ii) to specify the structural requirements for 17α-electrophilic estradiol derivatives to be potent affinity labels of the receptor, we prepared four 17α-[(haloacetamido)alkyl]estradiols. Three were bromoacetamides differing at the alkyl substituent (methyl, ethyl, or propyl), and the last was an [(iodoacetamido)propyl]estradiol prepared under both nonradioactive and ^{3}H-labeled forms. Although their affinities for the estrogen receptor were very low (from 0.008% to 0.02% that of estradiol), they appeared to be efficient affinity labels of the receptor due to their irreversible inhibition of [^{3}H]estradiol specific binding in lamb uterine cytosol. The effect of the compounds was time-, pH-, and concentration-dependent, with >50% and >80% estrogen-binding sites inactivated at 0 °C and pH 8.5, for the less active and more active compounds, respectively; the corresponding IC_{50} values varied from ~20 nM to ~10 μM. The order of efficiency was [(bromoacetamido)methyl]estradiol < [(bromoacetamido)ethyl]estradiol ≪ [(bromoacetamido)propyl]estradiol < [(iodoacetamido)-propyl]estradiol. Affinity labeling was directly demonstrated by ethanol-resistant binding of [^{3}H][(iodoacetamido)propyl]estradiol to the receptor. The irreversible inactivation of the hormone-binding site by the four haloacetamides was prevented by treatment of the cytosol with the thiol-specific reagent methyl methanethiosulfonate, suggesting that the target of these compounds was probably the −SH of cysteines. Negative results obtained with other 17α-electrophilic estradiol derivatives suggested that affinity labeling of the receptor by such derivatives required a minimal distance, including at least four C−C or C−N bonds, between the steroid and the electrophilic carbon. We therefore concluded that target cysteines in the hormone-binding site were not in direct contact with the steroid but probably in the immediate neighborhood of the D ring of the bound steroid.

Introduction

The structural organization of the hormone-binding domain of the estrogen receptor is still unknown. It is likely that only parts of the ~250 highly conserved amino acids of this domain[1,2] are directly involved in the formation of the putative hydrophobic pocket, which would perform the hormone-binding function. Affinity labels of the receptor could be useful tools for identifying amino acids of the hormone-binding domain in contact with hormones or antihormones. Such affinity labels could also improve our knowledge on the molecular mechanism of receptor activation and probably could permit new potent and highly selective antiestrogens to be defined. Three series of nonsteroidal electrophilic or photolabeling agents for the estrogen receptor were developed by Katzenellenbogen and co-workers. The first two series only included compounds bearing the electrophilic aziridine function; the first series[3] was related to tamoxifen, a triphenylethylene antiestrogen;[4] compounds of the second series[5] were derived from hexestrol, a potent estrogen.[6] The third series included both electrophilic and photolabeling agents bearing an aryl azide[7] or acyl azide[8] function; they were derived from trioxifen[9] and LY 117,018[10] which are potent antiestrogens. Both types of aziridine affinity labels only react with cysteine 530 of the wild-type human estrogen receptor.[11] However, when cysteine 530 was changed to alanine by *in vitro* mutagenesis, tamoxifen aziridine reacted with cysteine 381.[12] No significant reaction was observed with cysteines 417 and 447, the two other cysteines of the hormone-binding site. The authors concluded that cysteines 381 and 530 are probably closely positioned in the ligand-binding pocket of the receptor.[13]

In a recent study,[14] we found that three estradiol derivatives bearing a 17α, C3−C8 alkyl or alkynyl chain with a terminal bromoacetate function were effective affinity labels of the estrogen receptor. Although displaying both low affinity for the receptor (0.02−0.25% that of estradiol) and low hydrolytic stability in estrogen receptor-containing uterine extracts, these compounds irreversibly inactivated 30−70% of estrogen-binding sites under optimal conditions. Prevention of hormone-binding site inactivation by methyl methanethiosulfonate indicated that compounds alkylated the estrogen receptor at cysteine residue(s). The high instability of the ester function upon incubation with uterine extracts most probably substantially lowered the intrinsic ef-

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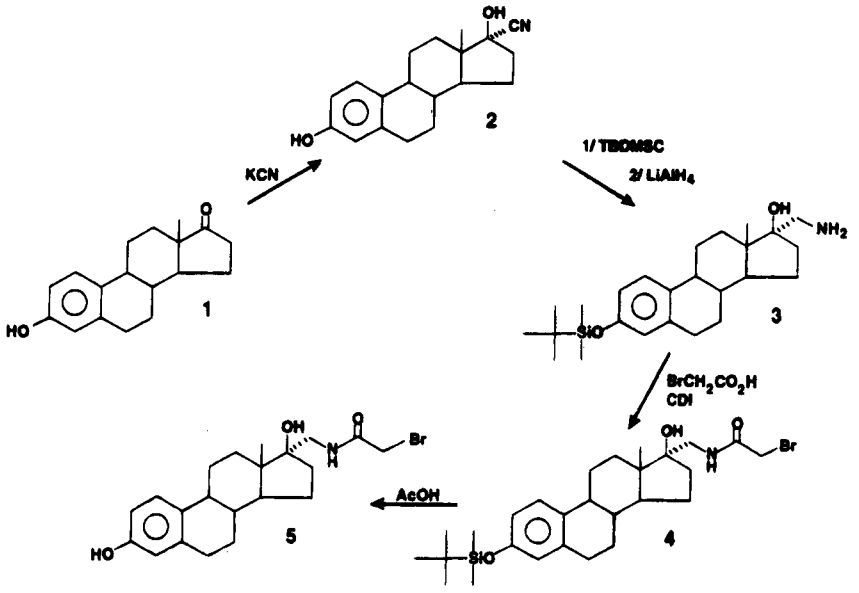
Scheme 1



ficiencies of these bromoacetate derivatives. This is based on the fact that ester hydrolysis decreased the concentration of the compound able to alkylate the receptor and also generated a nonalkylating competitor (whose affinity for the receptor was higher than that of the parent compound) which inhibited interaction of available receptors with intact bromoacetate. Moreover, in these compounds the electrophilic carbon was relatively remote from the steroid nucleus since the linker included from 5 to 10 C−C or C−O bonds, a situation which was not favorable for determining the minimal distance required between the electrophilic carbon and the steroid to make the estradiol derivative an efficient affinity label of the receptor. We therefore undertook to prepare related compounds including (i) the same ($BrCH_2CO$) or a similar (ICH_2CO) electrophilic group, but an amide function instead of the ester function was used for linking the terminal halogen-bearing carbon to the 17α-alkyl substituent of estradiol, and (ii) a shorter *n*-alkyl linker.

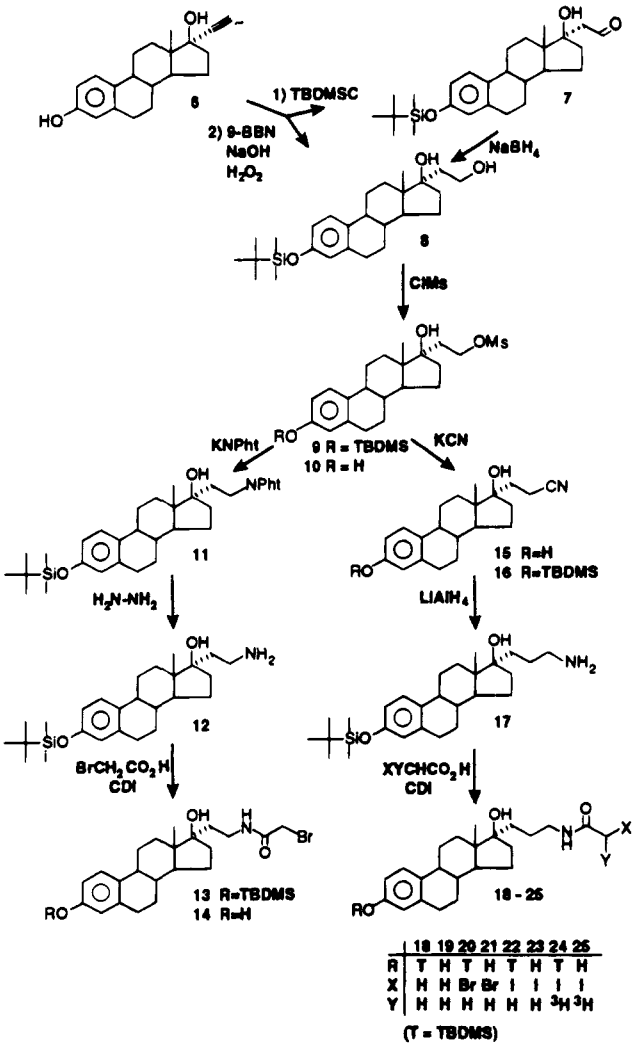
In this paper, we report the synthesis of 17α-[(bromoacetamido)alkyl]estradiols (alkyl = methyl, ethyl, or propyl) and 17α-[(iodoacetamido)propyl]estradiol, the latter under both nonradioactive and ^3H-labeled forms. We then demonstrate that all of these compounds irreversibly and specifically inactivated the hormone-binding site of the estrogen receptor, although their efficiency varied widely according to the type of alkyl arm, with propyl ≫ ethyl > methyl.

Results

Synthesis of Electrophilic 17α-Derivatives of Estradiol. Estrone (**1**) and 17α-ethynylestradiol (**6**) were used as starting materials for preparation of C1−C3, 17α-(haloacetamido)alkyl derivatives of estradiol. 17α-[(Bromoacetamido)methyl]estradiol (**5**) was obtained from estrone in five steps (Scheme 1). Reaction of the ketone function of estrone with KCN mainly afforded cyanhydrin **2**. After protection of the phenolic function as TBDMS ether, compound **2** was reduced by $LiAlH_4$ to primary amine **3**. Condensation with bromoacetic acid in the presence of **1**-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (CDI) afforded bromoacetamide **4**. Removal of the TBDMS group from **4** by AcOH gave 17α-[(bromoacetamido)methyl]estradiol (**5**). C2 and C3 homologs of **5** were both prepared from 17α-ethynylestradiol (**6**) in seven and eight steps, respectively (Scheme 2). After the phenolic function of ethylnylestradiol was protected as TBDMS ether, the alkyne function was submitted to hydroboranation−oxidation conditions (9-BBN and basic hydrogen peroxide) to afford a mixture of aldehyde β-alcohol **7** and corresponding diol **8**. Action of $NaBH_4$ on the mixture converted **7** into **8**. The primary hydroxyl group of **8** was selectively esterified by mesyl chloride to give **9**. Action of potassium phthalimide on mesylate **9** afforded phthalimide **11**, which was hydrolyzed by hydrazine to primary amine **12**. The corresponding bromoacetamide **13** was obtained from condensation with bromoacetic acid in the presence of CDI. Removal of the TBDMS group from **13** by tetrabutylammonium fluoride afforded 17α-[(bromoacetamido)ethyl]estradiol (**14**). Action of KCN on mesylate **9** in DMSO afforded nitrile **15** with deprotected phenol. Reprotection of the phenolic function as TBDMS ether, followed by reduction of nitrile, gave the primary amine **17**. The amine function of **17** was condensed with *N*-acetoxysuccinimide, bromoacetic acid, iodoacetic acid, or [^3H]iodoacetic acid, in the presence of CDI, to give 17α-propylestradiol, bearing the following terminal functions: acetamide (**18**), bromoacetamide (**20**), iodoacetamide (**22**), and [^3H]iodoacetamide (**24**), respectively. Corresponding phenol-free steroids **19**, **21**, **23**, and **25** were obtained from the above-mentioned compounds by the action of tetrabutylammonium fluoride. Mesylate **9** was also deprotected to give **10**.

Hydrolytic Stability. TLC analysis performed after incubation of acetamide **19**, haloacetamides **5**, **14**, **21**, and **23**, and mesylate **10**, at various pH levels (from 7 to 9) in the absence and presence of the lamb uterine cytosol, did not show any modification in the compound R_fs. This was in sharp contrast with the poor stability of corresponding bromoacetate compounds in the uterine cytosol.[14] However, at alkaline pH (pH ≥ 8), the amount of haloacetamides extracted from cytosol progressively decreased according to the incubation time, suggesting

Scheme 2



	18	19	20	21	22	23	24	25
R	T	H	T	H	T	H	T	H
X	H	H	Br	Br	I	I	I	I
Y	H	H	H	H	H	H	^3H	^3H

that these compounds irreversibly reacted with cytosolic components.

Estrogen Receptor-Binding Affinity. Competitive binding radiometric assays, using [^{3}H]estradiol as a tracer, were performed to determine the apparent affinity of synthesized compounds for the cytosolic estrogen receptor. In principle, such an assay is only valid for reversible ligands. To minimize potential irreversible binding of estradiol electrophilic derivatives, the assay was performed at pH 7 since very little irreversible binding occurred at this pH level with the various synthesized electrophiles (cf. following section). At early times (≤3 h), competition at 0 °C indicated that at 1 μM all synthesized estradiol 17α-derivatives were as efficient as 5 nM estradiol for inhibiting the specific binding of [^{3}H]estradiol in cytosol (not shown). This means that in the absence of competing ligand, 1 μM concentration of estradiol derivatives would fully saturate the receptor molecules. A competition equilibrium between [^{3}H]estradiol and the compounds was reached within a few hours at 20 °C (not shown). Table 1 gives the apparent affinity constants of the compounds relative to that of estradiol, calculated from 24 h competition at 20 °C according to Korenman.[15] All of the 17α-(acetamidoalkyl)estradiols **5**, **14**, **19**, **21**, and **23** displayed low affinity for the estrogen receptor; their relative affinity constants ranged from 0.008% to 0.02% that of estradiol. The affinity of bromoacetamides slightly increased with the number of intervening carbons between the steroid and the bromoacetamido function; whereas in the C3 compound, substitution of Br by either H or I slightly decreased the affinity of the compound. The order of decreasing affinity was then **21** > **14** > **19** ~ **23** > **5**. The relative affinity constant for mesylate **10** was about 10-fold higher than those of the haloacetamido derivatives.

Irreversible Inactivation of the Estrogen Receptor Hormone-Binding Site by Haloacetamides. The estrogen receptor-alkylating activities of unlabeled electrophilic estradiol derivatives were evaluated from their ability to irreversibly inhibit estradiol binding, by means of a procedure which allowed us to reveal affinity labeling of the receptor by 17α-[(bromoacetoxy)alkyl/alkynyl]estradiols.[14] Depending on the compound, compound concentration, incubation time, and pH, all four 17α-[(haloacetamido)alkyl]estradiols (**5**, **14**, **21**, and **23**) induced marked irreversible decreases in the capacity of the cytosol to specifically bind estradiol under exchange conditions; whereas in similar conditions mesylate **10** (whose affinity for the receptor was stronger than those of the haloacetamides) and acetamide **19** did not induce any significant decrease. Contrary to 17α-[(bromoacetoxy)alkyl/alkynyl]estradiols, whose effects were not clearly time-dependent,[14] 17α-[(haloacetamido)alkyl]estradiols inhibited estradiol specific binding in cytosol by a time-dependent process. Figure 1 shows the time course of inhibition induced by 1 μM 17α-[(bromoacetamido)propyl]estradiol (**21**) at 0 °C, pH 7.7 (Figure 1A) and pH 8.5 (Figure 1B). The inhibition was more rapid and pronounced at pH 8.5 than at pH 7.7, with a maximal effect of 65% by ~2–4 h and 20% by 4–8 h, respectively. As the results in Figure 1 suggest, the extent of irreversible inhibition of estradiol specific binding in cytosol by bromoacetamides varied greatly according to pH. The results (not shown) were qualitatively similar to those reported for 17α-[(bromoacetoxy)alkyl/alkynyl]estradiols;[14] even at a high concentration (10 μM) the four haloacetamides induced low inhibition (<10%) of estradiol binding at pH 7; at pH 7.5, depending on the compound, some inhibitions were observed which progressively increased until pH 9, reaching ~80% (compounds **21** and **23**), ~60% (compound **14**), and ~50% (compound **5**). At any pH from 7.5 to 9, we observed the same order of efficiency (**5** < **14** < **21** < **23**) for the four compounds. As illustrated in Figure 2, with the two homolog compounds 17α-[(bromoacetamido)propyl]estradiol (**21**) and 17α-[(bromoacetoxy)propyl]estradiol, the efficiencies of haloacetamide derivatives to inhibit estradiol specific binding were much higher than those of bromoacetate derivatives, although the affinities of the haloacetamide derivatives for the estrogen receptor appeared to be lower than those of the bromoacetate derivatives.

Inactivation of the hormone-binding site by haloacetamide derivatives, which required unfilled hormone-binding sites (not shown), varied substantially according to the compound (Figure 3). Both (i) the number of intervening carbons between the steroid and the haloacetamido function and (ii) the type of the halogen appeared to play a crucial role in compound potency. Results obtained with bromoacetamides indicated that from a one-atom to two-atom linkage the compound potency, which can be defined as $1/IC_{50}$, increased by

Table 1. Biochemical Characteristics of Estradiol 17α-Derivatives[a]

compd	17α-alkyl chain	apparent RAC	covalent binding	hormonal activity
estradiol		100		
bromoacetamide **5**	C1	0.0076 ± 0.0010	+	antagonist
mesylate **10**	C2	0.15 ± 0.02	−	agonist
bromoacetamide 14	C2	0.017 ± 0.004	+	agonist/antagonist
acetamide **19**	C3	0.0084 ± 0.0016	−	agonist/antagonist
bromoacetamide **21**	C3	0.019 ± 0.002	+	agonist/antagonist
iodoacetamide **23**	C3	0.0084 ± 0.0012	+	agonist/antagonist

[a] Apparent RCAs (relative affinity constants) of compounds for the cytosolic estrogen receptor were determined from competitive (24 h, 20 °C) radioreceptor assay using [^{3}H]estradiol as tracer, as described in Materials and Methods; data given are means of duplicate determinations in two 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 inactivation). The estrogen agonist and/or antagonist characteristics of compounds were determined from their ability to stimulate or prevent the expression of recombinant estrogen-regulated firefly luciferase gene *in vitro* (M. Pons, et al., unpublished results).

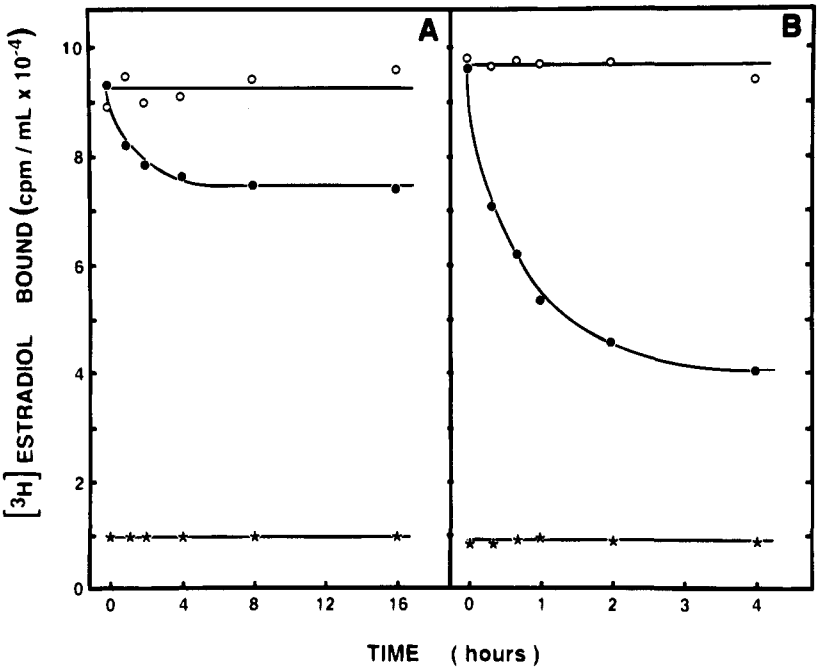


Figure 1. Time course of inactivation of specific estradiol-binding sites in lamb uterine cytosol by a bromoacetamide derivative of estradiol. Uterine cytosol, adjusted to pH 7.7 (A) or 8.5 (B) with NaOH (0.5 M), was incubated at 0 °C with 1 μM acetamide **19** or corresponding bromoacetamide **21**. At the indicated times, sample aliquots were removed and treated for 30 min at 0 °C with an equal volume of charcoal suspension. After centrifugation, the total and nonspecific binding of [^{3}H]estradiol occurring in the supernatants under exchange conditions were determined by the standard indirect irreversible binding assay, as described in Materials and Methods. The total binding of [^{3}H]estradiol in supernatants, corresponding to cytosol incubated with acetamide **19** (○) or bromoacetamide **21** (●), and the nonspecific binding of [^{3}H]estradiol (*), which did not significantly vary according to the compound (either acetamide **19** or bromoacetamide **21**) incubated with cytosol, are represented as functions of the incubation time of cytosol with the steroids. Values are means of duplicate determinations. Experimental variation was under 5%.

6-fold (Table 2), whereas from a two-atom to three-atom linkage the potency increased by 30-fold. The relative potencies of 17α-[(bromoacetamido)propyl]estradiol (**21**) and 17α-[(iodoacetamido)propyl]estradiol (**23**) indicated that bromine substitution by iodine increased the compound potency by 3-fold. Since the relative affinities of the four compounds for the estrogen receptor were very similar, with only a 2.5-fold increase from the lowest (17α-[(bromoacetamido)methyl]estradiol, **5**) to the highest (17α-[(bromoacetamido)propyl]estradiol, **21**) compound (Table 1), affinity for the receptor in this series did not play an important role in compound potency. It is noteworthy that the three-carbon linkage compounds, although displaying affinity for the estrogen receptor 5000- and 12 000-fold lower than that of estradiol, had IC_{50}s in the nanomolar range (Table 2).

Direct Evidence of Affinity Labeling of the Estrogen Receptor by 17α-[(Iodoacetamido)propyl]estradiol. To directly demonstrate that 17α-[(haloacetamido)alkyl]estradiols become covalently attached to the receptor, we prepared **25**, a tritiated form of iodoacetamide **23** which was the most efficient compound. This radioactive compound was incubated with cytosol in the absence and presence of a large excess of unlabeled estradiol to measure its total and nonspecific binding, respectively. First, using a charcoal assay, we compared the total and nonspecific binding of 5 nM [^{3}H]-estradiol (Figure 4A) to those of 25 nM [^{3}H]-17α-[(iodoacetamido)propyl]estradiol (Figure 4B) in cytosol equilibrated at various pH levels. Both total and nonspecific binding of the estradiol derivative showed strong pH dependence with a progressive and marked increase from pH 7.5 to 9. At pH 7 and 7.5, there was very little difference between the two curves, but from pH 8, total binding was clearly higher than the nonspecific binding and the difference between the two curves increased as a function of pH. These results contrasted with those obtained using estradiol, whose nonspecific binding was very low and did not significantly vary according to pH, and also its total binding

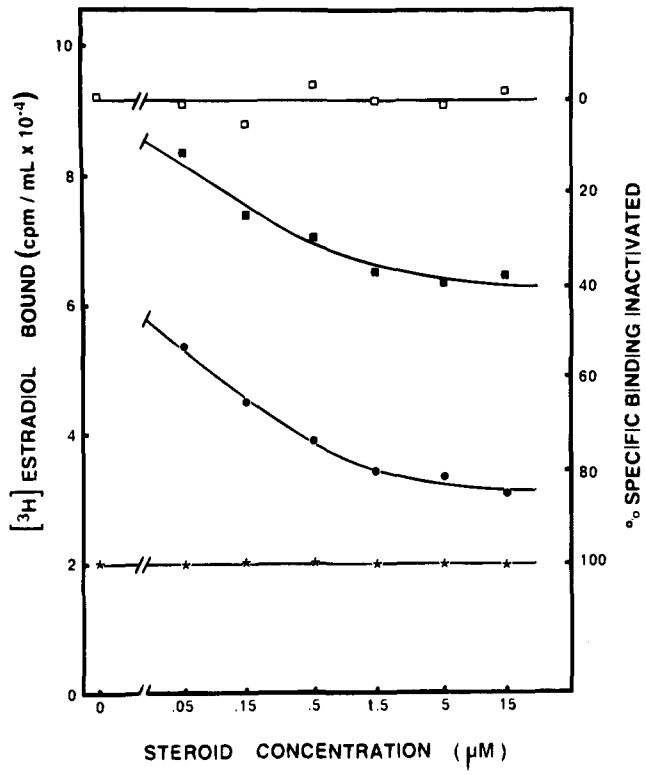


Figure 2. Relative abilities of a bromoacetate derivative of estradiol and the corresponding bromoacetamide derivative to inactivate the hormone-binding site of the estrogen receptor. Uterine cytosol, adjusted to pH 9 with NaOH (0.5 M), was incubated for 4 h at 0 °C without steroid or with increasing concentrations (50 nM−15 μM) of 17α-[3′-(bromoacetoxy)prop-1′-yl]estra-1,3,5(10)-triene-3,17β-diol,[14] corresponding bromoacetamide **21**, or 17α-(3′-hydroxyprop-1′-yl)estra-1,3,5(10)-triene-3,17β-diol,[14] a compound which is rapidly generated from the above bromoacetate upon incubation with cytosol. After charcoal treatment and then centrifugation, supernatant aliquots were incubated under exchange conditions with 20 nM [^{3}H]estradiol in the absence and presence of 5 μM estradiol. Total [^{3}H]estradiol binding corresponding to cytosol incubated with bromoacetamide derivative **21** (●), bromoacetate derivative (■), and corresponding alcohol (□) and nonspecific [^{3}H]-estradiol binding (*), which did not significantly vary according to the steroid incubated with cytosol, are represented as functions of the steroid concentration. Values are means of duplicate determinations. Experimental variation was under 10%. The closed symbols also indicate the percent of specific estradiol-binding sites inactivated (right scale).

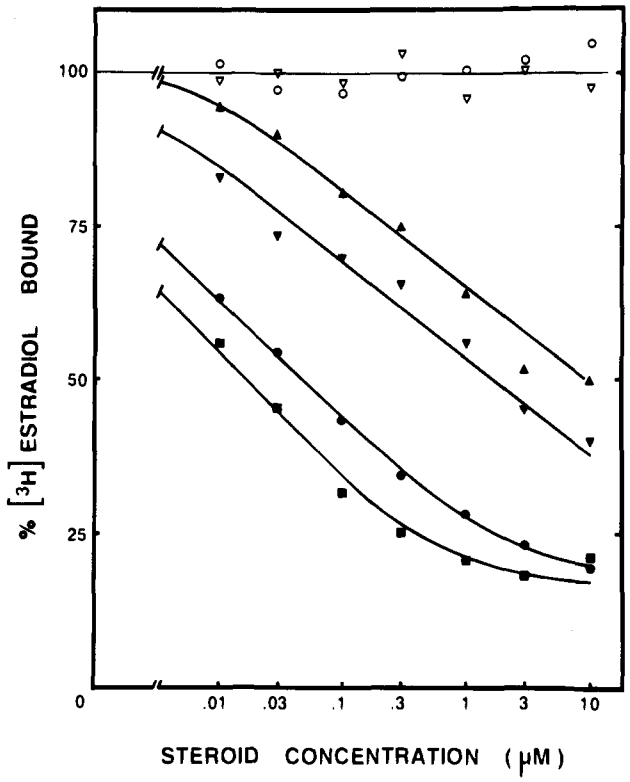


Figure 3. Concentration-dependent inactivation of specific estradiol-binding sites by 17α-[(haloacetamido)alkyl]estradiols. Uterine cytosol, adjusted to pH 8.5 with NaOH (0.5 M), was incubated for 4 h at 0 °C without steroid or with increasing concentrations (10 nM−10 μM) of mesylate **10** (▽), acetamide **19** (○), bromoacetamide **5** (▲), **14** (▼), or **21** (●), or iodoacetamide **23** (■). After charcoal treatment and then centrifugation, supernatant aliquots were incubated under exchange conditions with [^{3}H]estradiol. The specific [^{3}H]estradiol binding in cytosol exposed to the various concentrations of each steroid, expressed as a percent of the specific [^{3}H]estradiol binding of cytosol not exposed to steroid, was plotted against the steroid concentration. Experimental variation between duplicate determinations was less than 10%. The specific binding in the cytosol not exposed to steroid was 143 300 cpm/mL; the nonspecific binding (practically constant regardless of the steroid incubated with cytosol) was 9300 cpm/mL.

decreased slightly with increasing pH above 7.5. Specific binding (estradiol competable) of [^{3}H]-17α-[(iodoacetamido)propyl]estradiol in cytosol, determined according to Blondeau and Robel,[16] was found to be very low at pH 7 and 7.5; thereafter it progressively increased until pH 9, reaching values which corresponded to 25 ± 12% (pH 8.5) and 37 ± 21% (pH 9) of the specific binding of [^{3}H]estradiol measured at these pH levels. These results agreed with those obtained with the unlabeled form of the compound using an exchange assay (Figure 3).

We then used a covalent labeling assay, which involved measuring the residual binding of the ^{3}H-labeled iodoacetamide derivative to cytosolic proteins after treatment of the labeled proteins with hot ethanol. As shown in Figure 5A, total covalent and nonspecific covalent binding of the compound according to pH were very similar to the results obtained with the charcoal assay, although background binding at pH 7 was about

Table 2. Relative Efficiencies of 17α-[(Haloacetamido)alkyl]-estradiols To Inactivate [^{3}H]Estradiol Specific Binding[a]

compd	17α-alkyl chain	IC_{50} (μM)	RE
bromoacetamide **5**	C1	9.7 ± 0.6	0.0019
bromoacetamide **14**	C2	1.6 ± 0.1	0.011
bromoacetamide **21**	C3	0.053 ± 0.004	0.34
iodoacetamide **23**	C3	0.018 ± 0.002	1

[a] The concentrations of 17α-[(haloacetamido)alkyl]estradiols which irreversibly inhibited 50% of the specific binding of [^{3}H]-estradiol (IC_{50}) were calculated from curves similar to those shown in Figure 3; data are means of duplicate determinations in two separate experiments. Relative efficiencies (RE) of compounds to irreversibly inhibit the specific binding of [^{3}H]estradiol, defined as $1/IC_{50}$, were normalized taking the relative efficiency of iodoacetamide **23** as 1.

2-fold lower in the covalent assay than in the charcoal assay. Using the covalent assay, the mean values calculated for specific binding were slightly higher but not significantly different from those determined with the charcoal assay, with values of 42 ± 12% and 81 ± 18% calculated for receptor occupancy at pH 8.5 and 9, respectively. These results indicated that specific binding of the compound, as measured by the charcoal assay, involved covalent binding. Finally, we performed a

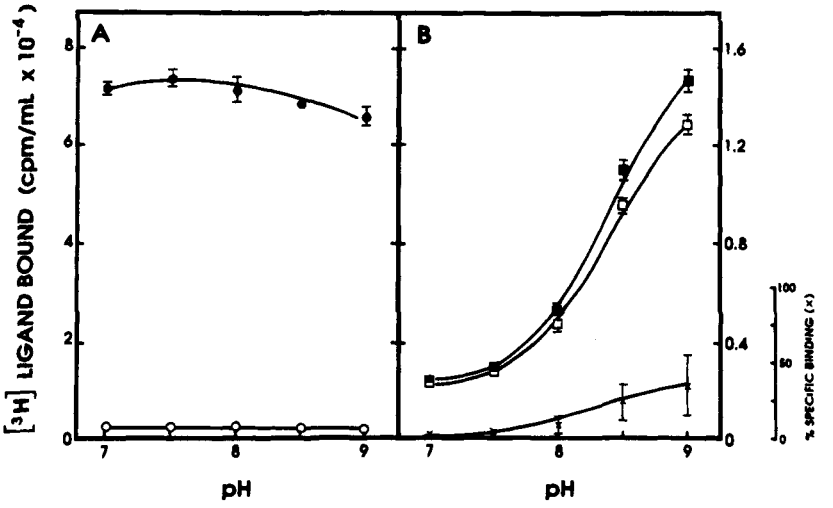


Figure 4. pH-dependent labeling of the estrogen receptor by [^{3}H]estradiol and [^{3}H]-17α-[(iodoacetamido)propyl]estradiol. Aliquots of uterine cytosol, adjusted to pH 7, 7.5, 8, 8.5, and 9 with NaOH (0.5 M), were incubated for 15 h at 0 °C with 5 nM [^{3}H]estradiol or 25 nM [^{3}H]-17α-[(iodoacetamido)propyl]estradiol in the absence and presence of 5 μM unlabeled estradiol. The total and nonspecific binding of [^{3}H]estradiol and [^{3}H]-17α-[(iodoacetamido)propyl]estradiol, determined by charcoal assay, are represented as functions of cytosol pH. (A) Total (●) and nonspecific (○) binding of estradiol. (B) Total (■) and nonspecific (□) binding of 17α-[(iodoacetamido)propyl]estradiol. Values are means of duplicate determinations. The specific binding of 17α-[(iodoacetamido)-propyl]estradiol (×), calculated from its total concentration and its total and nonspecific binding, according to Blondeau and Robel,[16] is plotted as a function of cytosol pH. Symbols (×) also indicate the percent of specific estradiol-binding sites (determined from cytosol equilibrated at pH 8.5) labeled by 17α-[(iodoacetamido)propyl]estradiol (right scale).

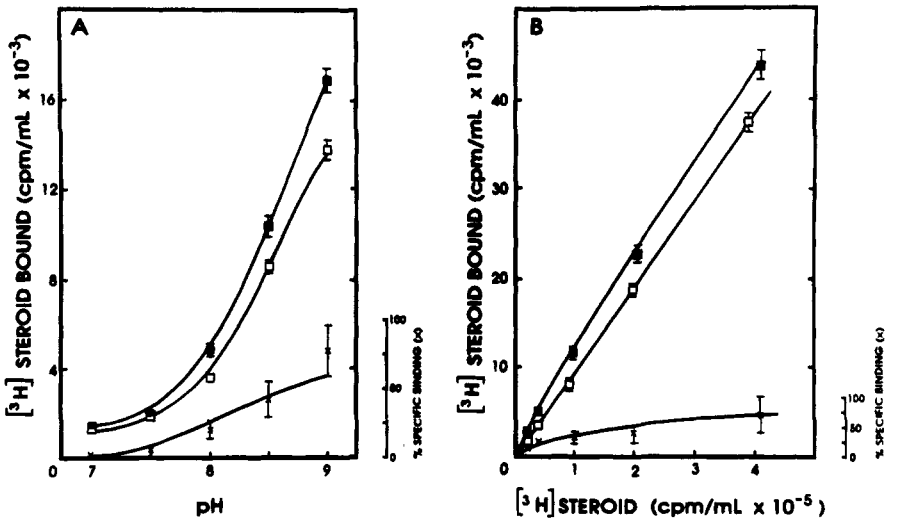


Figure 5. Covalent labeling of the estrogen receptor by [^{3}H]-17α-[(iodoacetamido)propyl]estradiol. (A) Aliquots of uterine cytosol, adjusted to pH 7, 7.5, 8, 8.5, and 9 with NaOH (0.5 M), were incubated for 15 h at 0 °C with 25 nM [^{3}H]-17α-[(iodoacetamido)-propyl]estradiol in the absence and presence of 5 μM estradiol. (B) Cytosol, adjusted to pH 8.5, was incubated for 15 h at 0 °C with various concentrations (5−100 nM) of [^{3}H]-17α-[(iodoacetamido)propyl]estradiol in the absence and presence of 5 μM estradiol. The total (■) and nonspecific (□) binding of the compound, determined by the covalent attachment assay, as described in Materials and Methods, are represented as functions of cytosol pH (A) and compound concentration (B). Values are means of duplicate determinations. The specific binding of 17α-[(iodoacetamido)propyl]estradiol (×), calculated from its total concentration and its total and nonspecific binding, is plotted against cytosol pH or compound concentration. Symbols (×) also indicate the percent of specific estradiol-binding sites (determined from cytosol equilibrated at pH 8.5) labeled by 17α-[(iodoacetamido)propyl]estradiol (right scales).

saturation experiment with cytosol equilibrated at pH 8.5, using a 5−100 nM concentration range for ^{3}H-labeled iodoacetamide derivative (Figure 5B). At all steroid concentrations, total covalent binding was clearly higher than nonspecific covalent binding. The calculated specific covalent binding appeared to be saturable, reaching, with 100 nM iodoacetamide derivative, a value corresponding to 76 ± 32% of the receptor occupancy obtained with 5 nM [^{3}H]estradiol.

Treatment of Cytosol with Methyl Methanethiosulfonate Prevents Inactivation of the Hormone-Binding Site by Haloacetamides. Since inactivation of the hormone-binding site of the receptor by 17α-[(bromoacetoxy)alkyl/alkynyl]estradiols was prevented by pretreatment of the cytosol with methyl methanethiosulfonate,[14] a thiol specific reagent, we tested the ability of this reagent to prevent the effects of haloacetamides. Figure 6 shows that increasing concentrations of the reagent progressively inhibited the effect of the various haloacetamides. This inhibition, detectable from 0.03 mM, was practically complete at 1 mM. Finally, the IC_{50}s of methyl methanethiosulfonate were very similar (0.3−0.5 mM) for all four haloacetamides.

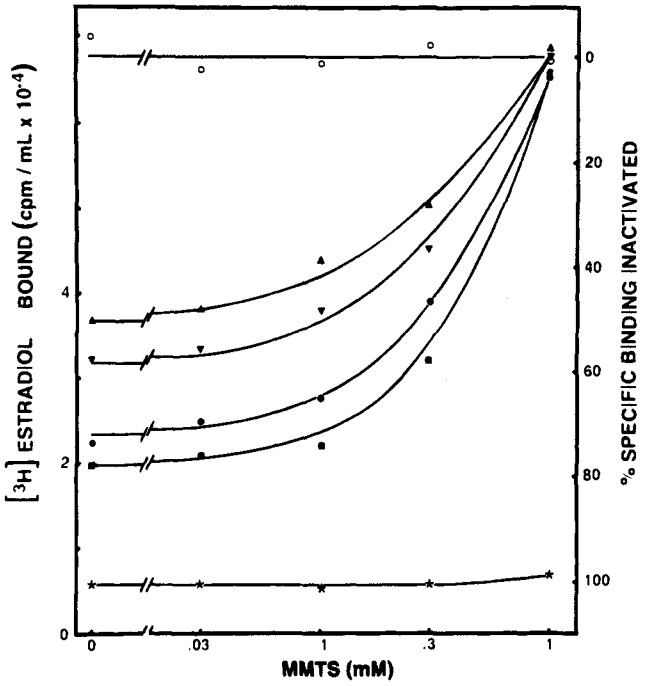


Figure 6. Effect of methyl methanethiosulfonate on the inactivation of specific estradiol-binding sites by haloacetamides. Uterine cytosol (4 mg of protein/mL), adjusted to pH 8.5, was incubated for 2 h at 0 °C with increasing concentrations (0–1 mM) of methyl methanethiosulfonate (MMTS). Aliquots of the various samples were then incubated for 2 h at 0 °C with 5 μM bromoacetamide **5**, **14**, or **21**, iodoacetamide **23**, or acetamide **19** as control. After charcoal treatment and then centrifugation, supernatant aliquots were incubated with [^{3}H]estradiol under exchange conditions. Total binding of [^{3}H]-estradiol in supernatants, corresponding to cytosol incubated with acetamide **19** (○), bromoacetamide **5** (▲), **14** (▼), or **21** (●), or iodoacetamide **23** (■), and nonspecific binding of [^{3}H]-estradiol (*), which did not significantly vary according to the steroid incubated with cytosol, are represented as functions of the MMTS concentration. Experimental variations between duplicate determinations were less than 10%. The closed symbols also indicate the percent of specific estradiol-binding sites inactivated (right scale).

Discussion

In this paper, we describe the synthesis of four 17α-[(haloacetamido)alkyl]estradiols, including three bromoacetamides differing at the alkyl group (methyl, ethyl, or propyl) and one iodoacetamide linked to the steroid through the propyl group. By two different approaches, we demonstrated that all of these compounds were affinity labels of the estrogen receptor. The first method, used with nonradioactive forms of the compounds, was based on time- and pH-dependent irreversible inhibition of [^{3}H]estradiol specific binding in uterine cytosol preexposed to the compounds. We previously validated this method when studying affinity labeling of the receptor by 17α-[(bromoacetoxy)alkyl/alkynyl]estradiols. The second and more direct method was based on the ethanol-resistant saturable labeling of the receptor using [^{3}H]-17α-[(iodoacetamido)propyl]-estradiol. The fact that the results obtained by the two methods for the iodoacetamide compound were in full agreement suggests that the first method allows quantitative determination of receptor affinity labeling by nonradioactive compounds.

According to the same criteria used to determine which type of amino acids at the hormone-binding site were alkylated by bromoacetate derivatives, i.e., pH dependence and methyl methanethiosulfonate prevention of affinity labeling, cysteine residues could be the target of the four 17α-[(haloacetamido)alkyl]estradiols. Affinity labeling of the estrogen receptor by 17α-[(haloacetamido)alkyl]estradiols was a rapid and efficient process since, at 0 °C and pH 8.5, equilibrium was reached within 2 h incubation with up to 80% of the receptor molecules alkylated by micromolar concentrations of the two most active compounds. Moreover, with these two compounds, the ~20 and ~50 nM concentrations required to alkylate 50% of the receptor molecules were surprisingly low. With receptor-rich cytosol and under optimal conditions, i.e., compound concentrations ≤5 nM and pH 8.5, the specific labeling index (ratio of specific covalent labeling to nonspecific covalent labeling) of 17α-[(iodoacetamido)propyl]estradiol was ~0.5. Since both the estrogen receptor titer in cytosol and the affinity of the compound for the receptor were very low, the compound was thus highly selective for target cysteines of the receptor.

17α-[(Haloacetamido)alkyl]estradiols were much more efficient than the related 17α-[(bromoacetoxy)alkyl]-estradiols in specifically alkylating the receptor. These different potencies, in addition to the fact that the haloacetamide effect was time-dependent while that of bromoacetates was not clearly time-dependent, probably resulted from a difference in the hydrolytic stability of the two series of compounds. [(Iodoacetamido)propyl]-estradiol was 3-fold more efficient than its bromo counterpart, although its affinity for the receptor was 2-fold lower. This could have been due to the fact that in the two homolog electrophilic groups, iodine is a better leaving atom than bromide. However, this better mobility of iodine would normally increase the reactivity of the electrophilic carbon for both specific alkylation (affinity labeling of the receptor) and nonspecific alkylation (reaction with cytosolic proteins). Therefore, it was probably not the higher mobility of iodine versus bromine which improved affinity labeling of the receptor. Another explanation is that due to steric hindrance, higher for iodine than for bromine, the location of the electrophilic carbon in the hormone-binding site differed according to the halogen and that in the case of iodine the location was more favorable for the reaction with target cysteines.

The efficiency of the three synthesized bromoacetamides to alkylate receptor molecules, according to either the compound concentration or pH, markedly increased with the length of the electrophilic carbon-bearing arm (3–5 C–C or C–N bonds for **5**, **14**, and **21**, respectively). The increase in efficiency was particularly strong from compound **14** to compound **21**. These great variations in compound efficiencies did not reflect the small variations in compound affinities for the receptor. This contrasts with results obtained with bromoacetoxy derivatives, whose electrophilic carbon-bearing arm included 5, 6, and 10 C–C or C–O bonds, respectively. With these compounds, probably due to compound instability, efficiencies and affinities for the receptor were correlated;[14] thus, in this series the length of the arm, apart from its effect via the compound affinity, did not seem to have a determining role on compound efficiency. It is thus possible that for 17α-electrophilic estradiol derivatives, compound efficiency would strongly

increase with the length of the 17α-arm, for arm lengths ≤ 5 bonds, with further little variation for longer arms. The inability of mesylate **10** and 17α-(epoxypropyl)-estradiols[14] to alkylate the receptor agrees with this hypothesis since these compounds only have a two-carbon arm. This suggests that a minimal "four-bond" distance between the steroid and the electrophilic carbon is required to obtain a reaction of the electrophile with target cysteines at the hormone-binding site. Since in 17α-[(haloacetamido)alkyl]estradiols the steroid moiety is a rigid structure, only the 17α-substituent could confer flexibility. This might be needed to allow binding of the steroid nucleus and contact of the electrophilic carbon with the cysteine SH groups at the hormone-binding site. The electrophile-bearing arm should thus fulfill minimal length conditions, dependent on the relative orientation and distance of the reaction site from the steroid-binding site. Positioning of the covalently bound molecule at the hormone-binding site seems to be quite similar to that of its nonreactive counterpart, as suggested by the identical hormonal activities of 17α-[(haloacetamido)propyl]estradiols **21** and **23** and 17α-(acetamidopropyl)estradiol (**19**) (M. Pons, et al., unpublished results). The fact that at least a three-atom arm seems to be required for reaction of the electrophile with target cysteines at the hormone-binding site suggests that these cysteines are not in direct contact with the steroid but probably in the immediate neighborhood of the D ring of the bound steroid. This is supported by the fact that the presence of each of the four cysteines of the hormone-binding site is not required for high-affinity binding of estradiol.[13]

In conclusion, 17α-[(haloacetamido)propyl]estradiols, although displaying relatively low affinities for the estrogen receptor, efficiently alkylate the receptor hormone-binding site, probably at cysteine residues. Experiments using single mutants (in which the four cysteines were separately changed for alanine) and a double mutant (in which cysteines 381 and 530 were changed for alanines) of the human estrogen receptor[13] are presently in progress in our laboratory to identify, at the hormone-binding site, target cysteines of 17α-[(haloacetamido)alkyl]estradiols.

Materials and Methods

Chemical. Synthesis and Characterization of Estradiol Derivatives. Estrone and 17α-ethynylestradiol were purchased from Sigma. Ammonium [^{3}H]iodoacetate (specific activity 185 TBq/mol, radiochemical purity >98%) used for the preparation of [ICH_2-^{3}H]-17α-[3′-(iodoacetamido)prop-1′-yl]-estra-1,3,5(10)-triene-3,17β-diol was purchased from Isotopchim (Ganagobie-Peyruis, France). Solvents and reagents were purchased from the following commercial sources: Aldrich, Carlo Erba, Fluka, Merck, and Sigma. Analytical TLC was performed with Merck aluminum sheet-baked silica gel (F-254, 0.2 mm). Column chromatography was performed with silical gel (0.063–0.2 mm). Nuclear magnetic resonance spectra were recorded at 32 °C on a AMX WB 360 spectrometer at 360 MHz. For proton spectra, chemical shifts (δ in ppm) were referenced to solvent residual signal ($CDCl_3$, 7.24 ppm; $(CD_3)_2SO$, 2.50 ppm; and CD_3OD, 3.35 ppm). Electron impact (EI) mass spectra were obtained on a LKB 2091 spectrometer, and fast-atom bombardment (FAB) mass spectra, employing a *m*-nitrobenzyl alcohol matrix, were obtained on a Jeol DX 300 spectrometer. For EI mass spectra, the reported data were for an electron energy of 70 eV in the *m/z* form (relative intensity to base peak = 100). Elemental analyses were performed by the microanalytical services of CNRS (Vernaison and Montpellier, France).

Except when otherwise stated, a standard procedure was used for product isolation. This involved quenching of the reaction in water or aqueous solution followed by exhaustive extraction of the mixture with ether or ethyl acetate, washing of extracts with aqueous solutions when necessary, drying of 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 and washing are mentioned in parentheses after "product isolation".

General Procedure I for Phenol Protection as TBDMS Ether. The procedure of Corey and Venkateswarlu[17] was used to protect 3-phenolic steroids. Briefly, a mixture of TBDMS chloride (1.2 equiv) and imidazole (2.4 equiv) in DMF (1 mL/mmol steroid) was added to the steroid (1 equiv). After the mixture was stirred for 1 h at 35 °C, product isolation (H_2O, Et_2O or EtOAc, $NaHCO_3$, Na_2SO_4) afforded the crude 3-(*tert*-butyldimethylsiloxy)steroid.

General Procedure II for TBDMS Group Removal. Procedures used to remove TBDMS from corresponding 3-silyl ether steroids were derived from those described by Corey and Venkateswarlu.[17] Procedure IIa consisted of exposing the silyl ether steroid in THF (5 mL/mmol silyl ether) to 2 equiv of tetra-*n*-butylammonium fluoride for 5 min at 0 °C and then for 15 min at 25 °C. Procedure IIb involved exposing the silyl ether steroid in 1 vol of THF (5 mL/mmol silyl ether) to AcOH/H_2O (2 vol/1 vol) for 2 h at 40 °C. In both cases product isolation (H_2O, Et_2O or EtOAc, Na_2SO_4) afforded the crude 3-hydroxysteroid with high yield.

General Procedure III for Condensation of Bromo- or Iodoacetic Acid with (Aminoalkyl)steroids. Bromo- or iodoacetic acid (2 equiv) and 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (CDI) hydrochloride (2 equiv) were added successively to a solution of the (aminoalkyl)steroid (1 equiv) in THF (10 mL/mmol (aminoalkyl)steroid). The mixture was stirred for 2 h at 25 °C and then poured into Et_2O. Insoluble material was removed by filtration. Product isolation ($NaHCO_3$, H_2O, Na_2SO_4) afforded the crude [(bromo- or iodoacetamido)alkyl]steroid.

17α-Cyanoestra-1,3,5(10)-triene-3,17β-diol (2). Potassium cyanide (7 mL of a 10 M aqueous solution) was added to estrone (600 mg, 2.22 mmol) in 20 mL of dioxane at 0 °C. The mixture was stirred, and AcOH (4.8 mL, 84 mmol) was added dropwise. After stirring for 2 h at 0 °C and then for 15 h at 20 °C, product isolation (H_2O, EtOAc) gave 640 mg of a clear oil.

3-(*tert*-Butyldimethylsiloxy)-17α-[(bromoacetamido)-methyl]estra-1,3,5(10)-trien-17β-ol (4). Cyanhydrin **2** (640 mg of crude compound) was silylated according to general procedure I and afforded 890 mg of 3-O-silylated cyanhydrin. The compound was solubilized in 20 mL of THF, and $LiAlH_4$ (250 mg, 6.59 mmol) was added. The mixture was stirred for 20 h at room temperature. Water was then added dropwise to the mixture until complete decomposition. Product isolation (H_2O, Et_2O, Na_2SO_4) afforded 854 mg of crude primary amine **3** which was condensed with bromoacetic acid according to general procedure III. Chromatography (Et_2O) afforded 495 mg of bromoacetamide **4** (45%): ^{1}H NMR ($CDCl_3$) δ 0.17 (s, 6H, $Si(CH_3)_2$), 0.88 (s, 3H, 18-CH_3), 0.96 (s, 9H, $SiC(CH_3)_3$), 3.30 (m, 1H, 1′-CH_A), 3.57 (m, 1H, 1′-CH_B), 3.91 (s, 2H, CH_2-Br), 6.53 (d, 1H, J = 2.7 Hz, 4-CH), 6.59 (dd, 1H, J_1 = 2.7 Hz, J_2 = 8.4 Hz, 2-CH), 6.93 (m, 1H, NH), 7.07 (d, 1H, J = 8.4 Hz, 1-CH). Anal. ($C_{27}H_{42}NO_3SiBr$) C, H, N, Br.

17α-[(Bromoacetamido)methyl]estra-1,3,5(10)-triene-3,17β-diol (5). Deprotected bromoacetamide **5** was prepared from **4** in 88% yield by general procedure IIb. It was purified by chromatography (Et_2O): ^{1}H NMR ($CDCl_3$) δ 0.85 (s, 3H, 18-CH_3), 3.31 (m, 1H, 1′-CH_A), 3.50 (m, 1H, 1′-CH_B), 3.93 (s, 2H, CH_2Br), 6.55 (d, 1H, J = 2.7 Hz, 4-CH), 6.61 (dd, 1H, J_1 = 2.7 Hz, J_2 = 8.4 Hz, 2-CH), 7.03 (m, 1H, NH), 7.08 (d, 1H, J = 8.4 Hz, 1-CH); FABMS 424 (M^+ + 1, 32), 422 (M^+ + 1, 38), 406 (41), 404 (50), 380 (5), 378 (11), 362 (6), 360 (13), 154 (100). Anal. ($C_{21}H_{28}NO_3Br$) C, H, N, Br.

3-(*tert*-Butyldimethylsiloxy)-17α-(2′-hydroxyethyl)estra-1,3,5(10)-trien-17β-ol (8). The phenol function of 17α-ethynylestradiol (2 g, 6.75 mmol) was protected as TBDMS ether according to general procedure I. Chromatography (Et_2O/

heptane, 1/9) of the obtained compound afforded 2.72 g (6.62 mmol) of 3-(*tert*-butyldimethylsiloxy)-17α-ethynylestradiol. 9-BBN (73.08 mmol, 0.5 M solution in THF) under nitrogen was added dropwise over 5 min to a solution of 2.5 g (6.09 mmol) of 3-(*tert*-butyldimethylsiloxy)-17α-ethynylestradiol in 20 mL of THF at 60 °C. The mixture was stirred for 15 h at room temperature and then cooled to 0 °C; 24 mL of water was added, and stirring was continued for 5 min. Aqueous sodium hydroxide (24 mL, 3 M) was added, and after a further 5 min, 24 mL of hydrogen peroxide (30%) was added dropwise. After 30 min, product isolation ($NaHCO_3$, Et_2O, Na_2SO_4) afforded a mixture of aldehyde **7** and primary alcohol **8**. To transform aldehyde **7** into alcohol **8**, the mixture, dissolved in 50 mL of MeOH, was submitted to the action of $NaBH_4$ (0.461 g, 12.18 mmol) for 1 h at 20 °C. Acetone (5 mL) was added to the medium to quench the reaction. Product isolation (H_2O, EtOAc, Na_2SO_4) and chromatography (Et_2O) afforded 1.75 g of alcohol **8** (67%): ^{1}H NMR ($CDCl_3$) δ 0.17 (s, 6H, $Si(CH_3)_2$), 0.89 (s, 3H, 18-CH_3), 0.96 (s, 9H, $SiC(CH_3)_3$), 3.87 (m, 1H, $J_1 = J_2 = 4.3$ Hz, $J_3 = 10.7$ Hz, 2′-CH_A), 4.04 (m, 1H, $J_1 = J_2 = 2.6$ Hz, $J_3 = 10.7$ Hz, 2′-CH_B), 6.56 (d, 1H, $J = 2.7$ Hz, 4-CH), 6.59 (dd, 1H, $J_1 = 2.7$ Hz, $J_2 = 8.4$ Hz, 2-CH), 7.10 (d, 1H, $J = 8.4$ Hz, 1-CH); EIMS 430 (M^+, 56), 412 (19), 373 (45), 355 (19), 327 (38), 41 (100). Anal. ($C_{26}H_{42}O_3Si$) C, H.

3-(*tert*-Butyldimethylsiloxy)-17α-[2′-(methylsulfonoxy)ethyl]estra-1,3,5(10)-trien-17β-ol (9). Methanesulfonyl chloride (558 mg, 4.87 mmol) was added to a solution of 700 mg (1.63 mmol) of primary alcohol **8** in 7 mL of pyridine. The solution was stirred for 1 h at room temperature. Product isolation (H_2O, Et_2O, Na_2SO_4) and chromatography (EtOAc/heptane, 3/7) afforded 620 mg of mesylate **9** (75%): ^{1}H NMR ($CDCl_3$) δ 0.17 (s, 6H, $Si(CH_3)_2$), 0.88 (s, 3H, 18-CH_3), 0.96 (s, 9H, $SiC(CH_3)_3$), 3.00 (s, 3H, SCH_3), 4.51 (t, 2H, $J_1 = J_2 = 7.4$ Hz, 2′-CH_2), 6.53 (d, 1H, $J = 2.7$ Hz, 4-CH), 6.59 (dd, 1H, $J_1 = 2.7$ Hz, $J_2 = 8.4$ Hz, 2-CH), 7.09 (d, 1H, $J = 8.4$ Hz, 1-CH); EIMS 508 (M^+, 22), 490 (16), 451 (10), 412 (100). Anal. ($C_{27}H_{44}O_5SiS$) C, H.

17α-[2′-(Methylsulfonoxy)ethyl]estra-1,3,5(10)-triene-3,17β-diol (10). The phenol function of mesylate **9** was deprotected according to general procedure IIa for TBDMS group removal. The compound (94%) was purified by chromatography (Et_2O): ^{1}H NMR ($CDCl_3$) δ 0.88 (s, 3H, 18-CH_3), 3.00 (s, 3H, SCH_3), 4.51 (t, 2H, $J_1 = J_2 = 7.4$ Hz, 2′-CH_2), 6.54 (d, 1H, $J = 2.7$ Hz, 4-CH), 6.61 (dd, 1H, $J_1 = 2.7$ Hz, $J_2 = 8.4$ Hz, 2-CH), 7.11 (d, 1H, $J = 8.4$ Hz, 1-CH); EIMS 394 (M^+, 1), 376 (70), 298 (29), 157 (100). Anal. ($C_{21}H_{30}O_5S$) C, H.

3-(*tert*-Butyldimethylsiloxy)-17α-[2′-(*N*-phthalimido)ethyl]estra-1,3,5(10)-trien-17β-ol (11). Potassium phthalimide (556 mg, 3 mmol) was added to a solution of 382 mg (0.75 mmol) of mesylate **9** in 7.5 mL of DMF. The mixture was stirred for 4 h at 45 °C. Product isolation (H_2O, Et_2O, $NaHCO_3$, Na_2SO_4) and then chromatography (EtOAc/heptane, 1/3) afforded 353 mg of phthalimide **11** (84%): ^{1}H NMR ($CDCl_3$) δ 0.16 (s, 6H, $Si(CH_3)_2$), 0.89 (s, 3H, 18-CH_3), 0.95 (s, 9H, $SiC(CH_3)_3$), 3.88 (m, 1H, 2′-CH_A), 4.00 (m, 1H, 2′-CH_B), 6.52 (d, 1H, $J = 2.7$ Hz, 4-CH), 6.57 (dd, 1H, $J_1 = 2.7$ Hz, $J_2 = 8.4$ Hz, 2-CH), 7.07 (d, 1H, $J = 8.4$ Hz, 1-CH), 7.68 (m, 2H, phth), 7.82 (m, 2H, phth); EIMS 559 (M^+, 58), 541 (59), 502 (100), 484 (21), 327 (32). Anal. ($C_{34}H_{45}NO_4Si$) C, H, N.

3-(*tert*-Butyldimethylsiloxy)-17α-(2′-aminoethyl)estra-1,3,5(10)-trien-17β-ol (12). An ethanolic solution (20 mL) of phthalimide **11** (300 mg, 0.54 mmol) and hydrazine hydrate (86 mg of hydrazine, 2.68 mmol) was stirred for 10 h at 30 °C. Product isolation (0.5 M NaOH, Et_2O, Na_2SO_4) afforded 220 mg of crude primary amine **12**.

17α-[2′-(Bromoacetamido)ethyl]estra-1,3,5(10)-triene-3,17β-diol (14). Crude primary amine **12** (200 mg) was condensed with bromoacetic acid according to general procedure III to give compound **13**. Removal of the TBDMS group according to general procedure IIa and then chromatography (Et_2O) afforded 181 mg of bromoacetamide **14** (89%): ^{1}H NMR (CD_3OD) δ 0.94 (s, 3H, 18-CH_3), 3.44 (m, 1H, 2′-CH_A), 3.54 (m, 1H, 2′-CH_B), 4.08 (s, 2H, CH_2Br), 6.51 (d, 1H, $J = 2.7$ Hz, 4-CH), 6.57 (dd, 1H, $J_1 = 2.7$ Hz, $J_2 = 8.4$ Hz, 2-CH), 7.11 (d, 1H, $J = 8.4$ Hz, 1-CH); EIMS 393 (7), 391 (18), 375 (8), 373 (20), 356 (5), 354 (2), 339 (13), 337 (12), 322 (2), 213 (100). Anal. ($C_{22}H_{30}NO_3Br$) C, H, N.

17α-(2′-Cyanoethyl)estra-1,3,5(10)-triene-3,17β-diol (15). Methanesulfonate **9** (460 mg, 0.90 mmol) and NaCN (89 mg, 1.82 mmol) were dissolved in 15 mL of anhydrous DMSO and heated for 15 h at 60 °C. Product isolation (CH_2Cl_2, H_2O, Na_2SO_4) and chromatography (EtOAc/heptane, 3/7) afforded 230 mg of nitrile **15** (78%): ^{1}H NMR ($(CD_3)_2SO$) δ 0.80 (s, 3H, 18-CH_3), 2.53 (m, 2H, 2′-CH_2), 6.43 (d, 1H, $J = 2.7$ Hz, 4-CH), 6.50 (dd, 1H, $J_1 = 2.7$ Hz, $J_2 = 8.4$ Hz, 2-CH), 7.03 (d, 1H, $J = 8.4$ Hz, 1-CH); EIMS 325 (M^+, 61), 310 (34), 298 (12), 293 (11), 159 (100). Anal. ($C_{21}H_{27}NO_2$) C, H.

3-(*tert*-Butyldimethylsiloxy)-17α-(2′-cyanoethyl)estra-1,3,5(10)-trien-17β-ol (16). The phenol function of nitrile **15** was protected as TBDMS ether according to the general procedure I. The compound (92%) was purified by chromatography (EtOAc/heptane, 2/8): ^{1}H NMR ($CDCl_3$) δ 0.17 (s, 6H, $Si(CH_3)_2$), 0.90 (s, 3H, 18-CH_3), 0.96 (s, 9H, $SiC(CH_3)_3$), 2.45 (m, 1H, 2′-CH_A), 2.59 (m, 1H, 2′-CH_B), 6.53 (d, 1H, $J = 2.7$ Hz, 4-CH), 6.60 (dd, 1H, $J_1 = 2.7$ Hz, $J_2 = 8.4$ Hz, 2-CH), 7.09 (d, 1H, $J = 8.4$ Hz, 1-CH); EIMS 439 (M^+, 51), 383 (41), 382 (100). Anal. ($C_{27}H_{41}NO_2Si$) C, H, N.

3-(*tert*-Butyldimethylsiloxy)-17α-(3′-aminoprop-1′-yl)estra-1,3,5(10)-trien-17β-ol (17). $LiAlH_4$ (855 mg, 22.53 mmol) was added to a solution of 1 g (2.27 mmol) of cyanhydrin **16** in 30 mL of THF. The mixture was stirred for 1 h at room temperature, and then the reaction was quenched by cautious addition of EtOAc. Product isolation (H_2O, EtOAc, Na_2SO_4) afforded 890 mg of crude primary amine **17**.

17α-(3′-Acetamidoprop-1′-yl)estra-1,3,5(10)-triene-3,17β-diol (19). $NaHCO_3$ (0.075 mmol, 0.5 M aqueous solution) and 11.8 mg (0.075 mmol) of *N*-acetoxysuccinimide were added to 30 mg (0.068 mmol) of primary amine **17** in 0.68 mL of THF. The mixture was stirred for 15 h at room temperature. Product isolation gave 33 mg of crude acetamide **18**. Phenol deprotection according to general procedure IIa and then chromatography (Et_2O) afforded 19 mg of acetamide **19** (76%): ^{1}H NMR ($CDCl_3$/$(CD_3)_2CO$, 90/10) δ 0.75 (s, 3H, 18-CH_3), 1.82 (s, 3H, $COCH_3$), 3.08 (m, 1H, 3′-CH_A), 3.21 (m, 1H, 3′-CH_B), 6.21 (m, 1H, NH), 6.44 (d, 1H, $J = 2.4$ Hz, 4-CH), 6.50 (dd, 1H, $J_1 = 2.7$ Hz, $J_2 = 8.4$ Hz, 2-CH), 6.97 (d, 1H, $J = 8.4$ Hz, 1-CH); EIMS 371 (M^+, 20), 353 (64), 351 (7), 336 (7), 256 (100). Anal. ($C_{23}H_{33}NO_3$) C, H, N.

17α-[3′-(Bromoacetamido)prop-1′-yl]estra-1,3,5(10)-triene-3,17β-diol (21). Bromoacetamide **21** was prepared from primary amine **17** in 72% yield by condensation with bromoacetic acid followed by phenol deprotection according to general procedures III and IIa, respectively. It was purified by chromatography (Et_2O): ^{1}H NMR ($CDCl_3$) δ 0.87 (s, 3H, 18-CH_3), 3.30 (m, 1H, 3′-CH_A), 3.45 (m, 1H, 3′-CH_B), 4.05 (s, 2H, CH_2Br), 6.54 (d, 1H, $J = 2.7$ Hz, 4-CH), 6.60 (dd, 1H, $J_1 = 2.7$ Hz, $J_2 = 8.4$ Hz, 2-CH), 6.85 (m, 1H, NH), 7.12 (d, 1H, $J = 8.4$ Hz, 1-CH); EIMS 407 (8), 405 (16), 389 (19), 387 (49), 370 (20), 369 (40), 351 (28), 28 (100). Anal. ($C_{23}H_{32}NO_3Br$) C, H, N.

17α-[3′-(Iodoacetamido)prop-1′-yl]estra-1,3,5(10)-triene-3,17β-diol (23). Iodoacetamide **23** was prepared from primary amine **17** in 78% yield by condensation with iodoacetic acid followed by phenol deprotection according to general procedures III and IIa, respectively. It was purified by chromatography (Et_2O): ^{1}H NMR ($CDCl_3$) δ 0.88 (s, 3H, 18-CH_3), 3.31 (m, 1H, 3′-CH_A), 3.41 (m, 1H, 3′-CH_B), 4.04 (s, 2H, CH_2I), 6.54 (d, 1H, $J = 2.7$ Hz, 4-CH), 6.61 (dd, 1H, $J_1 = 2.7$ Hz, $J_2 = 8.4$ Hz, 2-CH), 6.88 (m, 1H, NH), 7.10 (d, 1H, $J = 8.4$ Hz, 1-CH); EIMS 405 (6), 387 (11), 371 (11), 353 (34), 351 (18), 213 (100). Anal. ($C_{23}H_{32}NO_3I$) C, H, N.

^{3}H-Labeled 17α-[3′-(Iodoacetamido)prop-1′-yl]estra-1,3,5(10)-triene-3,17β-diol (25). Ammonium [^{3}H]iodoacetate (37.8 μg, 0.20 μmol; specific activity 185 TBq/mol) was solubilized in 500 μL of THF/H_2O (9/1). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (0.4 mg, 2.09 μmol) and primary amine **17** (3 mg, 6.76 μmol) were added. The mixture was stirred at 40 °C. Formation of iodoacetamide **24** and consumption of [^{3}H]iodoacetic acid were monitored by TLC. After 15 h, iodoacetamide **24** was deprotected by the action of tetra-*n*-butylammonium fluoride (1.3 mg, 4.97 μmol)

for 15 min at 25 °C. Iodoacetamide **25** was purified on an analytical TLC plate. The plate was eluted with EtOAc and then scanned. A major radioactive peak was obtained whose R_f (0.35) was identical to that of unlabeled 17α-[(iodoacetamido)propyl]estradiol. The corresponding plate area was cut out and extracted with 2 × 2.5 mL of EtOH. Aliquots of the ethanolic solution were counted to determine the concentration of the radioactive compound (18.9 μM; 47% yield relative to [^{3}H]iodoacetic acid). Other aliquots were added to unlabeled 17α-[(iodoacetamido)propyl]estradiol solubilized in THF to determine the radiochemical purity of the compound (>95%) by TLC analysis.

Biochemical. Estrogen Receptor Ligands. [6,7-^{3}H]-Estradiol (specific activity 1.96 PBq/mol, radiochemical purity >98%) was purchased from Amersham International (Amersham, England). [ICH_2-^{3}H]-17α-[(Iodoacetamido)propyl]estradiol (specific activity 185 TBq/mol, radiochemical purity >95%) was synthesized as described in the chemical Materials and Methods section. Estradiol and 17α-derivatives of estradiol used for binding studies were solubilized in absolute ethanol. Solutions were stored at −20 °C in the dark. Purity of solubilized compounds was checked before use by TLC.

Cytosolic Estrogen Receptor. Cytosol was prepared in 20 mM Tris-HCl buffer (T_{20}) from immature lamb uteri, as described previously.[14] The protein concentrations (~2 or ~4 mg/mL) determined according to Layne[18] and pH (7−9) of the cytosol are defined in the procedures for each specific experiment.

Competitive Binding Assay: Apparent Relative Affinity Constants. Competition between 5 nM [^{3}H]estradiol and increasing concentrations of nonradioactive estradiol or 17α-derivatives for binding to lamb uterine cytosol (2 mg of protein/mL, pH 7) were performed at 0 and 20 °C, as described previously.[14] Apparent relative affinity constants (RACs) of competitors relative to that of estradiol were calculated according to Korenman,[15] using concentrations of unbound and specifically bound [^{3}H]estradiol at 50% specific binding inhibition and concentrations of unlabeled estradiol and 17α-derivatives which inhibited 50% of the specific binding of [^{3}H]estradiol at 24 h, 20 °C competition.

Labeling of Estrogen Receptor with Radioactive Ligands. Cytosol (2 mg of protein/mL), adjusted to various pH (7−9) with NaOH (0.5 M), was incubated for 15 h at 0 °C with 5 nM [^{3}H]estradiol or various concentrations (5−100 nM) of [^{3}H]-17α-[(iodoacetamido)propyl]estradiol in the absence and presence of 5 μM unlabeled estradiol to measure total and nonspecific [^{3}H]ligand binding, respectively. Binding of [^{3}H]-ligands was determined by a 60 min, 0 °C charcoal assay, as previously described.[14]

Covalent Labeling of the Estrogen Receptor with [^{3}H]-17α-[(Iodoacetamido)propyl]estradiol. Cytosol (2 mg of protein/mL), adjusted to various pH (7−9) with NaOH (0.5 M), was incubated for 15 h at 0 °C with various concentrations (5−100 nM) of [^{3}H]-17α-[(iodoacetamido)propyl]estradiol in the absence and presence of 5 μM estradiol. Aliquots of 100 μL were spotted on GFC discs (Whatman) which, after 10 min of standing, were washed for 1 h in 95% ethanol at 70 °C. Thereafter the discs were dried at 40 °C and counted to determine the total covalent and nonspecific covalent binding of 17α-[(iodoacetamido)propyl]estradiol.

Standard Indirect Irreversible Binding Assay. Cytosol (4 mg of protein/mL), adjusted to various pH (7−9) with NaOH (0.5 M), was first incubated for at least 4 h at 0 °C with estradiol 17α-derivatives or without steroid. Samples were then treated with an equal volume of charcoal suspension 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[14] using, except where otherwise mentioned, 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), whereas the radioactivity of GFC discs was counted in 10 mL of Toluene Scintillator (Packard).

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