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# 7α,11β-DISUBSTITUTED ESTROGENS: PROBES FOR THE SHAPE OF THE LIGAND BINDING POCKET IN THE ESTROGEN RECEPTOR

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**Abstract:** To investigate whether the estrogen receptor accommodates 7α and 11β substituents on estradiol in two subsites or in a single subsite, we have prepared three sets of ligands bearing single (7α *or* 11β) or double (7α *and* 11β) substituents and measured their binding affinity. The different behavior of each set precludes a definitive choice, but supports a two subsite model with some limitations.© 1997 Elsevier Science Ltd.

The estrogen receptor (ER), a member of the nuclear hormone receptor superfamily, acts as a sequence specific transcription factor whose activity is modulated by the binding of hormonal ligands.[1] Despite the extensive amount of data illustrating how the molecular structure of an estrogen can affect its binding affinity for the receptor as well as its estrogenic activity, details concerning the specific interactions between ligands and the ligand binding domain (LBD) of the ER are scant. X-ray crystal structures of the ER-LBD have not been published, and homology models of this domain, based on crystallographic coordinates of ligand binding domains of other members of the nuclear hormone receptor family, retinoic acid receptor (RAR) [2] and thyroid hormone receptor (TR),[3] are not accurate, because of the modest homology between the LBDs of ER vs. RAR and TR. Thus, at present, it has been difficult to predict exactly how estrogen ligands are oriented in the binding pocket of the ER-LBD, which specific residues interact with the various functional groups and substituents on the ligand, and what conformational changes the receptor may undergo upon binding a specific ligand.

Structure–activity relationship are useful tools for exploring the size and shape of specific regions of the ligand binding pocket of the receptor, provided that the molecular probes constitute a homologous set that can be expected to bind to the receptor in a common orientation. Large, nonpolar substituents on estradiol are tolerated by ER especially well at two sites, 7α and 11β, and numerous estrogens with single and often large substituents at C-7α or C-11β are known to be high affinity ligands for ER and potent agonists and antagonists.[4] Examples of these monosubstituted estrogens are given in Fig. 1.

One issue that has been raised concering 7α and 11β *mono*substituted estrogens is whether there are two independent binding pockets in ER ("two pocket model") to accommodate these substituents independently, as proposed by Leclerq et al.,[5] or whether the 7α or 11β substituent is accommodated within the same pocket by rotation or flipping of the estradiol ligand core around a pseudo $C_2$ symmetric axis that runs roughly through

C-3 and C-17 ("one pocket model") (Fig. 2). The one-pocket model was proposed by Poupaert et al.[6a] and von Angerer[6b] on the basis of the equivalent binding of matched 7α and 11β-monosubstituted estrogens. The two-pocket model is supported by the high binding affinity of some truly $C_2$ symmetrical nonsteroidal ligands bearing identical substituents in positions equivalent to 7α and 11β, such as the tetrahydrochrysene diols **7** and **9**.

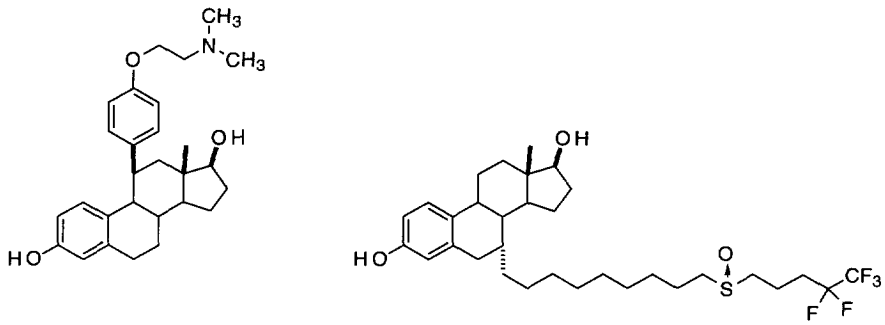
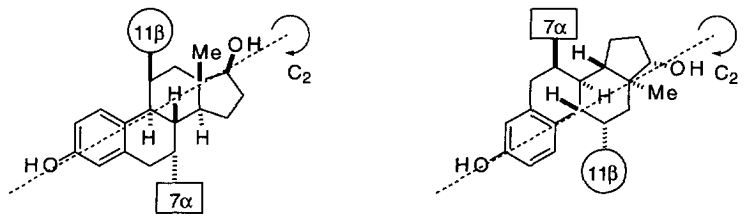



**Figure 1.** Examples of high affinity 7α- and 11β-monosubstituted estrogen ligands for ER.




"normal orientation" "flipped orientation"

**Figure 2.** Possible alternate orientations of 7α- and 11β-substituted estrogens in ER.

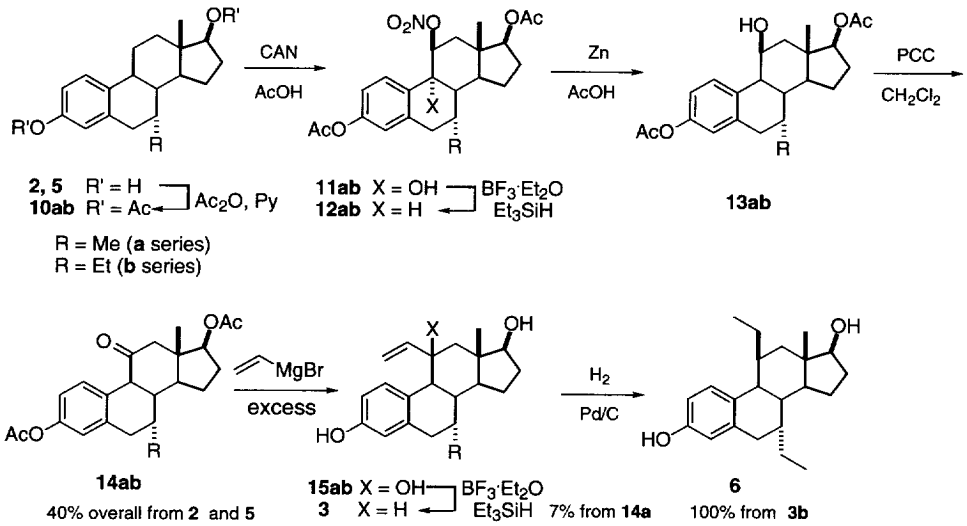
In a recent review,[4] Anstead addressed the issue of substituent accommodation by pockets within the ligand binding site of ER, by analyzing the binding affinity of a variety of 7α- and 11β-monosubstituted estrogens as a function of the size and the polarity of the substituent. He concluded that a preformed pocket of substantial volume (≥44 $Å^3$) exists in ER near the C-11β position, whereas near C-7α, the pocket is rather small, both pockets being deformable beyond these volumes, at least to some degree. To investigate this issue further, we have prepared two series of 7α- and 11β-monosubstituted and disubstituted estradiols (**1–6**, Table 1), as well as a mono- and disubstituted nonsteroidal estrogen of the tetrahydrochrysene class (**7–9**), and measured their binding affinity for ER.

**Synthesis**

The synthetic approach to the two disubstituted estradiol derivatives **3** and **6** is shown in Scheme 1. 7α-Methyl estradiol (**2**) and 7α-ethyl-estradiol (**5**), prepared by alkylation of protected 6-oxoestradiol with methyl

or ethyl iodide, respectively, followed by deoxygenation of the 6-keto group,[7] were transformed after diacetylation of the free hydroxy groups into the 11-keto derivatives **14ab**, following the procedure described by Peters et al.[8] Ceric ammonium nitrate oxidation of the diacetates (**10ab**), followed by two rounds of reduction, gave the 11β-hydroxy estradiols **13ab**, which were oxidized to the corresponding 11-oxoestradiols **14ab**. The introduction of the 11β-vinyl substituent was accomplished by our previously published procedure;[9] the 7α,11β-diethylestradiol (**6**) was obtained by catalytic hydrogenation of **3b** (R = Et).

**Scheme 1**




CAN = Ceric Ammonium Nitrate $(NH_4)_2Ce(NO_3)_6$

The preparation of the nonsteroidal estrogen, the monoethyl tetrahydrochrysene **8**, shown in Scheme 2, parallels our earlier synthesis.[10] Cinnamic acid (**16**) was transformed into the related propanal (**19**) by catalytic hydrogenation, hydride reduction, and partial reoxidation. This aldehyde underwent condensation in the presence of a Stetter thiazolium catalyst,[11] to give the acyloin product (**20**), which was protected as the mixed acetal (**21**). The enolate derived from this intermediate was ethylated. Acid-catalyzed cyclodehydration of **22** gave the tetrahydrochrysene system (**23**), which was cleaved to the free phenol (**8**). The synthesis of the unsubstituted (**7**) and the diethyl (**9**) tetrahydrochrysenes was previously described by one of us.[10]

**Receptor Binding Affinity**

The estrogen receptor (ER) binding affinity was determined in a competitive binding assay using an ER preparation from rat uterus.[12] The relative binding affinities (RBA) values are shown in Table 1. [$^3$H]Estradiol is used as the tracer, and free ligand is removed by adsorption onto dextran coated charcoal. The assays were done at 25 °C to ensure full binding equilibrium.

**Scheme 2**

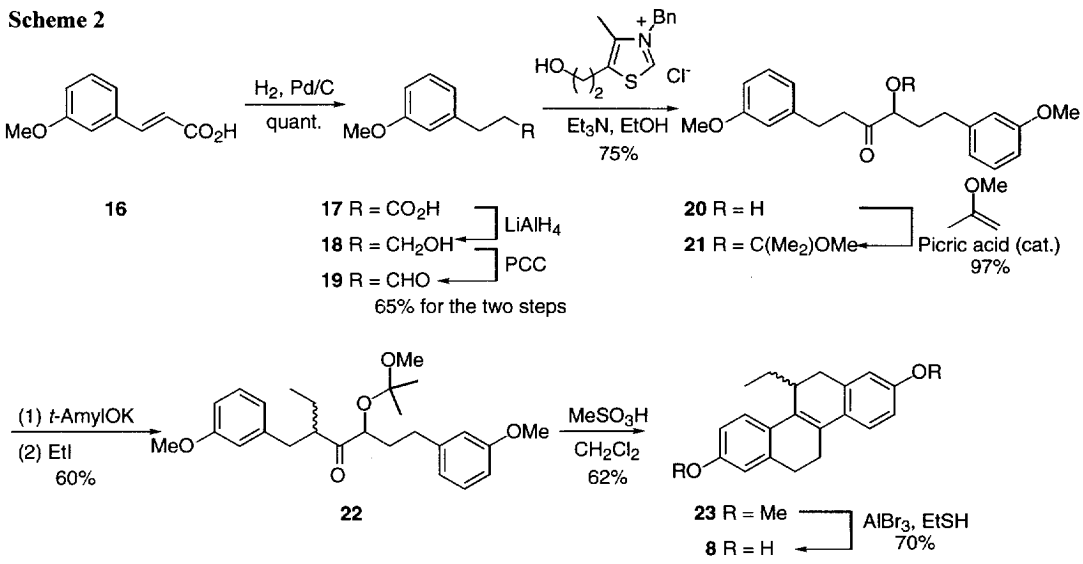
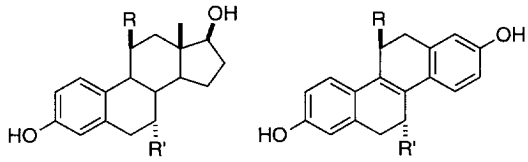



**Table 1**




| Entry | R | R' | RBA[a] 25° C |
|---|---|---|---|
| 1 | $CH_2$=CH- | H- | 1400% |
| 2 | H- | $CH_3$- | 330% |
| 3 | $CH_2$=CH- | $CH_3$- | 760% |
| 4 | $CH_3$-$CH_2$- | H- | 1000% |
| 5 | H- | $CH_3$-$CH_2$- | 59% |
| 6 | $CH_3$-$CH_2$- | $CH_3$-$CH_2$- | 490% |
| 7 | H- | H- | 0.74% |
| 8[b] | H- | $CH_3$-$CH_2$- | 18% |
| 9 | $CH_3$-$CH_2$- | $CH_3$-$CH_2$- | 630% |

[a] RBA: Relative Binding Affinity Estradiol = 100%; [b] racemate

Comparison among the three sets of mono vs. disubstituted ligands, two steroidal and one nonsteroidal, shows three types of behavior. Case A is illustrated in the series **1–3**: here, a single substitution with a vinyl group at 11β (**1**) or a methyl group at 7α (**2**), greatly increases affinity relative to estradiol, whereas double substitution in analog **3** results in a much more modest increase in binding. Case B is illustrated by the other

steroidal series **4–6**, where an 11β-ethyl group (**4**) greatly raises affinity, the 7α-ethyl group (**5**) lowers it (again, relative to the unsubstituted ligand estradiol), and the double substitution (**6**) shows an intermediate value. Case C is seen with the nonsteroidal ligands (**7–9**): Each ethyl substituent makes a positive increment in binding affinity, so that the disubstituted ligand (**9**) is the highest binder.

The divergent effect of double vs. single substitution on ligand binding in the three series does not immediately point to a single model for ER subsites. It helps, however, to consider the *additivity* of binding energies in the three cases.[4] Roughly additive contributions to binding energies would be expected for the two-pocket model, provided that each substituent were having an interaction within its own subsite that was independent of the other substituent-subsite interaction; by contrast, substantial deviations from additivity would be expected for a one-pocket model.

By such an analysis of the data in Table 1, one notes that the Cases B and C (compounds **4-6** and **7-9**, respectively) show additive increments in binding energy for each substituent: With compounds **4-5**, one substituent (11β-ethyl) raises affinity, whereas the other (7α-ethyl) lowers affinity, the disubstituted analog **6**, having a binding affinity (490%) that is nearly exactly that expected from the independent contributions of each substituent (590%, determined from the product of the RBA values of **4** and **5**). Similarly, with compounds **7-9**, the addition of one or two ethyl substituents each raises binding 20-25 fold per substituent. The binding energy additivity shown in both of these cases supports the two-pocket model. The binding energies in Case A, however, deviate from additivity: Each substituent independently raises affinity; however, the disubstituted analog **3**, expected by additivity to have a binding affinity greater than 4,600% (the product of 1400% and 330%), has, in fact, a much lower affinity (760%), being even less than that of the best monosubstituted one (**1**, 1400%). Such non-additivity is more characteristic of a single pocket model.

All of the binding data are consistent with a modified version of the two-pocket model that has two features: The first feature is that the pocket that accommodates the 7α-substituent is smaller that the one that accommodates the 11β-substituent. Thus, the smaller 7α-methyl group raises binding affinity (**2**), whereas the larger 7α-ethyl group lowers it (**5**); by contrast, the larger 11β-vinyl or ethyl groups, that project into the larger pocket, both raise binding affinity (**1** and **4**). The second feature that is critical to this model is that these pockets must be positioned so that the coordinated (i.e., independent and additive) contribution of *two substituents* to the binding energy breaks down, before the energy contribution of a *single substituent* becomes compromised. As a result, the increased binding energies contributed independently by the single substituents in **1** and **2** fail to add in the disubstituted congener **3**. This suggests that ligand repositioning which may be occurring in different ways to best accommodate single substituents, cannot occur optimally when both substituents are there at the same time.

Although the one pocket vs. two pocket question will be definitively resolved only when crystal structures of series of such ligands bound to the ER are available, it is interesting to consider broader issues. From the crystal structures currently available for nuclear hormone receptor ligand complexes, that of the retinoic acid receptor-γ (RAR) complexed with all *trans*-retinoic acid (RA)[2] and of the thyroid hormone receptor (TR) complexed with triiodothyronine ($T_3$),[3] it is evident that the ligand is almost completely enveloped by the receptor; the residues are tightly packed around the ligands, so that it is difficult to see how large substituents could be tolerated by the receptor in this conformation. Motions of residue side chains, together with

movements of the protein backbone, would be needed to provide channels or pockets in the receptor to accommodate substituents.

The situation with ER–ligand complexes may be the same, although there might be, as suggested by Anstead,[4] somewhat more open space available surrounding the interior region of the ligand (above and below the B- and C-rings) than in the RAR-RA and TR-$T_3$ structures. A more general model is one in which the receptor has a certain available space around the ligand (preformed pocket volume)[4] as well as a capacity for deformation in certain directions (deformable pocket volume).[4] Single substituents on estradiol at either C-7$\alpha$ or C-11$\beta$ may fill these voids, moving side chains of the residues lining the binding pocket at these accommodating positions, and perhaps repositioning the core of the ligand slightly (but without a 180° rotation). Beyond a certain point, such as occurs when two substituents together reach a certain size and ligand repositioning can no longer be done optimally, the deformation of the receptor becomes energetically unfavorable. In addition, whereas single substituents of small to modest size may be accommodated in the interior of ER without ligand reorientation (effectively two pockets), the very large substituents (cf. Fig. 1) might still result in ligand flipping (effectively one pocket).

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