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HYDROXYLATED TRIPHENYLACRYLONITRILES ADOPT A UNIQUE ORIENTATION WITHIN THE BINDING SITE OF THE ESTROGEN RECEPTOR*

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Summary—The relative binding affinities of a series of twelve *para*-hydroxylated triphenylethylenes (TPEs) for the estradiol receptor (ER) of calf uterus cytosol were measured by a competition method. The results obtained under equilibrium conditions support the hypothesis of the additivity of the energies corresponding to each of the hydrogen-bond type interactions of di- or tri-hydroxylated TPEs with the estradiol binding site of ER and strongly suggest that, whichever ring is hydroxylated, the orientation of the TPE in the steroid binding site is always the same. A hydroxyl group in a given position always interacts with the same location within the site.

Mono-hydroxylation of the highly hydrophobic non-substituted TPE skeleton led to a large increase in relative binding affinity for ER which could be explained by a dual mechanism whereby the interaction specific to the hydroxyl is accompanied by a temperature- or time-dependent binding process that is not related to the hydroxylation position.

INTRODUCTION

In the course of studies on the estrogen and/or antiestrogen action of triphenylethylenes (TPEs) of the triphenylacrylonitrile series [1–5], we have investigated the correlations among several response parameters: stimulation of MCF-7 cell growth, cytotoxicity in MCF-7 and BT_{20} cells, induction of progesterone receptor and relative binding affinity (RBA) for the estradiol receptor (ER). In the present report, we analyse in detail the results obtained in the ER binding experiments performed under different incubation conditions on calf uterus cytosol for twelve of these hydroxylated TPEs in order to establish whether the molecules adopt a preferential orientation within their binding site.

In general, the binding of ligand to steroid receptor depends upon the configuration of the steroid or non-steroid skeleton of the ligand, steric hindrance from substituents neighbouring the functional groups of the ligand, van der Waals, aromatic–aromatic or electrostatic interactions... and the, as yet unknown, flexibility of the protein. It is assumed that substrate specificity is largely determined by hydrogen bonds (H-bonds) whilst the other forces stabilize the ligand–macromolecule complex. Many studies have indicated that the complexes formed by certain anti-estrogens with ER are different from the estradiol–receptor complex as regards their physico-chemical properties and conformation [6–11].

The conformation of the TPE skeleton can be described as a propeller with a double bond as the hub. The spatial arrangement of the α, α' and β-phenyl rings (Fig. 1) resembles three blades situated nearly symmetrically around a shaft constituted by a perpendicular to the central double bond [1]. Whereas published studies have principally focussed on the configuration of a single hydroxy derivative of a TPE, usually 3 or 4-hydroxy-tamoxifen, where a reactive basic side-chain implicated in anti-estrogenic activity is retained [13–16], we have compared several *para*-hydroxylated derivatives without a basic side-chain in order to analyse H-bonding with ER under different conditions of complex stability. One or more *para*-hydroxy groups were permutated around the TPE skeleton. Indeed, as illustrated in Fig. 2, the TPE could theoretically be superimposed in six ways with an estradiol (E_2) molecule. A comparison of the binding stabilities of the TPE–ER complexes should determine the most likely superpositions(s) and indicate whether the TPEs adopt unique positions within the binding site.

All the hydroxylated TPEs we tested are biologically active. They induce the progesterone receptor to maximal levels but at different concentrations and promote with varying efficacy the growth of an ER-positive cell-line [3–5].

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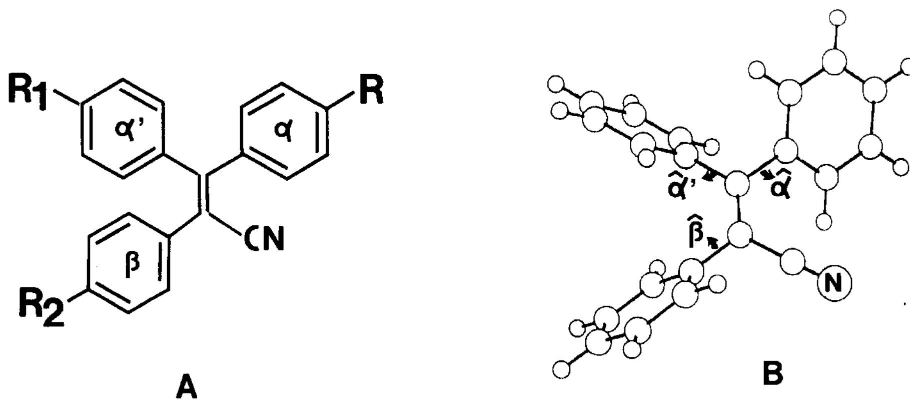

Fig. 1. (A) General structure of tested TPEs. (B) Propeller-like configurations of TPEs. Molecular geometry calculations on the unsubstituted TPE by the program SCRIPT [12] indicate angles of 50° ($\hat{\beta}$), 51° ($\hat{\alpha}$, $\hat{\alpha}'$). According to crystallography studies [1], angle values range from 45–55° for α, 38–47° for α′, and 45–58° for β.

MATERIALS AND METHODS

Chemicals and materials

The synthesis, chemical characterization, and analytical purity of the triphenylacrylonitrile derivatives have been described previously [1, 5]. Solutions of the test compounds in 95% ethanol were stored at 2°C in the dark and checked before use by HPLC [5].

Relative binding affinities (RBAs) for the estrogen receptor (ER)

Calf uteri (20–40 g) were excised and divided into 1 g fractions which were stored at −70°C until use. The fractions were homogenized in an ice-cooled glass–Teflon Potter in TED buffer (10 mM Tris–HCl, 1.5 mM EDTA, 1 mM dithiothreitol, pH 7.4) and the homogenate was centrifuged at 0–4°C for 1 h at 180,000 *g* to obtain cytosol. Cytosol aliquots were incubated either for 2 h at 0°C or 5 h at 25°C with 1 nM [6, 7 ^{3}H]estradiol and increasing concentrations (0.3 nM–10 μM) of unlabelled competitor (final volume 250 μl containing 1% ethanol). The incubated cytosol was stirred for 30 s at 0°C with 50 μl of DCC (0.6% dextran T70, 6% charcoal Norit A) and then centrifuged for 10 min at 4000 *g*. The radioactivity of a 200 μl-supernatant sample was measured by liquid

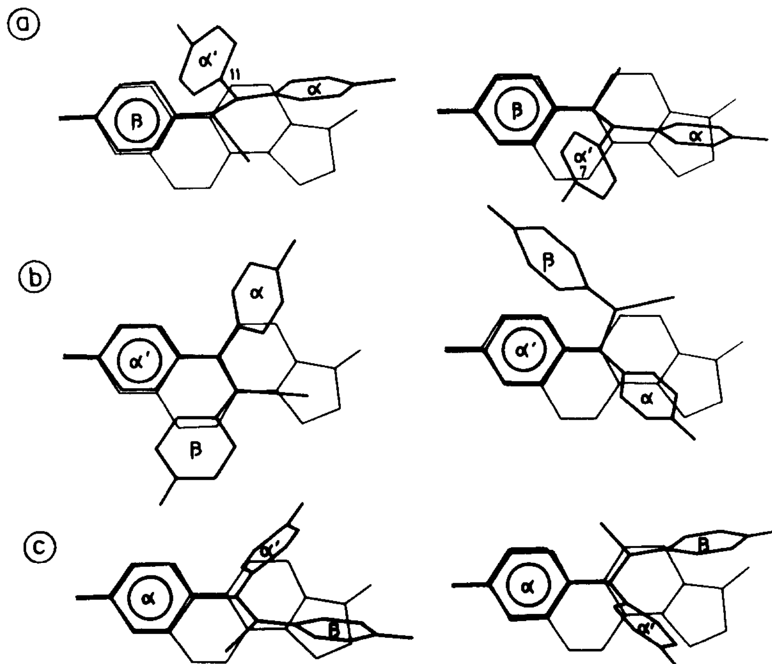

Fig. 2. Theoretical superpositions of TPEs with estradiol. Superposition on ring A of estradiol of the (a) β-phenyl ring, (b) α′-phenyl ring, (c) α-phenyl ring of TPEs. The left and right-hand superpositions differ by a 180° rotation around the bond linking the α-phenyl ring to the central double-bond. On the LHS, the α or α′-phenyl ring is close to position C-11 of estradiol, on the RHS close to positions C-7,8.

scintillation. Relative binding affinities (RBAs) were deduced from competition curves by determining the ratio of the molar concentrations (IC_{50}s) of unlabelled competitor to that of estradiol, that reduced specific labelled estradiol binding by 50%. Each IC_{50} was calculated mathematically from the straight line between the two experimental points on either side of the 50% specific binding level. RBAs were expressed as a percentage relative to an RBA value of 100 for estradiol. The sensitivity of the assay was very high (lower limit 0.01 for the RBA). Each experiment was repeated at least 4 times.

RESULTS

The RBAs of the twelve hydroxylated TPEs are given in Table 1 and are compared to the RBA of the unsubstituted TPE 1. The RBAs were measured under two sets of incubation conditions, namely, 2 h at 0°C and 5 h at 25°C, that were chosen as a function of the interaction kinetics of estradiol with the uterine cytosol estrogen receptor. The first set of incubation conditions (2 h at 0°C) reflects differences in association rates and indicates whether an interaction between the ligand and receptor is feasible; the second set (5 h at 25°C) reflects differences in dissociation rates and, by comparison with the first set of values, gives an idea of the stability of the receptor complex [17, 18]. An increase in RBA on increasing time and temperature indicates that the complex formed by the test compound is more stable than the estradiol–receptor complex; a decrease in RBA that it is less stable.

The compounds in Table 1 have been classified into three groups. The first group comprises those with RBAs that remain stable or increase when incubation time and temperature are increased. They are all structurally related by the presence of a p-OH group on ring α which therefore seems to be important for binding to ER and essential for the stability of this binding. The compounds of the second group have RBAs at 0°C that do not differ substantially from those of the first group. However, a marked decrease in RBA is recorded at 25°C and would thus seem to indicate that these compounds with a β-OH group on ring β but not on ring α can bind effectively to ER but that this binding is unstable after incubation at the higher temperature and longer incubation time. The third group is constituted of only two TPEs with very low RBAs that furthermore decrease with time and temperature. This suggests that compounds with a p-OH group on ring α' only cannot bind effectively to ER.

The overall analysis reveals a strong hierarchy in the relative importance of the position of the p-OH group on a TPE skeleton insofar as the binding to ER and/or the stability of this binding are concerned. First, the presence of a p-OH on ring α is conducive to binding and essential for the stability of the complex. Second, a p-OH on ring β (in the absence of a p-OH on ring α) is conducive to binding only and not stability. Third, a p-OH on ring α' (in the absence of a p-OH on rings α and/or β) does not seem to have any particular relevance.

More detailed conclusions can be drawn from a more quantitative analysis. In Table 2 the effect of hydroxylation in a specific position on binding at 25°C has been expressed as an RBA ratio. After 5 h incubation at 25°C, the increase in RBA for hydroxylation of any given ring is virtually the same whatever the starting molecule as long as this molecule already possesses a hydroxy group. The RBA increases by factors of 26, 1.9 and 2.8 after hydroxylation of rings α, α', and β respectively. Hydroxylation of the highly hydrophobic unsubstituted TPE 1 increases RBA by a virtually identical factor multiplied by a constant value of about 14 since we obtained the following factors:

—400 ($= 28.5 \times 14$) after hydroxylation of ring α
—24 ($= 1.7 \times 14$) after hydroxylation of ring α'
—37 ($= 2.6 \times 14$) after hydroxylation of ring β.

Table 1. Relative binding affinities (RBAs)[a] of TPEs for ER binding in calf uterus cytosol according to the position of the hydroxy group (on ring α, ring β, ring α')

				Competition for $[^3H]E_2$ binding	
	R(α)	$R_1(\alpha')$	$R_2(\beta)$	RBA_1 (2 h at 0°C)	RBA_2 (5 h at 25°C)
1	H	H	H	0.04 ± 0.01	0.09 ± 0.04
2Z	OH	H	H	40 ± 8	36 ± 11
7Z	OH	CH_3	H	29 ± 4	28 ± 5
4	OH	OH	H	28 ± 4	62 ± 11
8	OH	OH	CH_3	49 ± 9	93 ± 17
5Z	OH	H	OH	27 ± 5	74 ± 20
9Z	OH	CH_3	OH	36 ± 9	78 ± 1
6	OH	OH	OH	41 ± 6	166 ± 7
3	H	H	OH	29 ± 4	3.3 ± 0.7
5E	H	OH	OH	19 ± 3	6.1 ± 1.5
9E	CH_3	OH	OH	28 ± 9	9.1 ± 1.6
2E	H	OH	H	3.7 ± 0.9	2.2 ± 0.8
7E	CH_3	OH	H	8.1 ± 0.8	2.5 ± 0.6
E_2				100	100

[a]RBAs, determined under two sets of incubation conditions, are the means (± SEM) of at least 4 experimental values.

Table 2. Influence of hydroxylation on different TPE rings on RBAs measured at 25°C

a → b	TPEa	TPEb	RBA of TPEa	RBA of TPEb	RBAb/RBAa	
	1	2Z	0.09	36	400	
α: H → OH	2E	4	2.2	62	28	
	5E	6	6.1	166	27	26
	3	5Z	3.3	74	22.4	
	1	2E	0.09	2.2	24	
α′: H → OH	2Z	4	36	62	1.7	
	5Z	6	74	166	2.2	1.9
	3	5E	3.3	6.1	1.8	
	1	3	0.09	3.3	37	
	2Z	5Z	36	74	2.1	
β: H → OH	2E	5E	2.2	6.1	2.8	
	7Z	9Z	28	78	2.8	2.8
	7E	9E	2.5	9.1	3.6	
	4	6	62	166	2.7	

This increase in RBA on monohydroxylation of TPE 1 could be the resultant of two phenomena: (1) the 28.5-, 1.7- or 2.6-fold increase could be due to the same interaction of the α, α′ or β hydroxy groups respectively with the receptor binding site as in TPEs that are already hydroxylated (see above); (2) the 14-fold increase that occurs regardless of hydroxylation position could be due to: (a) formation by TPE 1 of an ER complex (ERC) of different conformation (ERC_I) than that formed by hydroxylated TPEs (ERC_{II}), (b) different solvation of the more hydrophobic TPE 1, should the presence of water molecule(s) contribute to the structure of the steroid binding site, (c) more marked interaction of TPE 1 with non-specific binding proteins.

All these results at 25°C are summarized in the regular parallelepiped of Fig. 3 whose sides represent the incremental increase in RBA due to hydroxylation on a given ring. The supplementary increment due to the first hydroxylation is indicated by the fact that TPE 1 is outside this geometric figure. The results obtained with two pairs of available methylated compounds (7E → 9E and 7Z → 9Z), also shown in Fig. 3, provide further support for the model. According to this figure, the affinity of the trihydroxylated TPE 6, for example, can be written as follows:

$$K_6 = K_1 \times k_C \times k_\alpha \times k_{\alpha'} \times k_\beta$$

where K_6 and K_1 represent the association constants of TPEs 6 and 1, respectively, for ER and where k_C, k_α, $k_{\alpha'}$ and k_β represent the incremental increases in affinity resulting from the first hydroxylation, then from hydroxylations in positions α, α′ and β. The corresponding binding energy is:

$$E_6 = E_1 + E_C + E_\alpha + E_{\alpha'} + E_\beta .$$

This relation illustrates the additivity of the parameters influencing affinity and, in particular, of the interactions of the α, α′ and β hydroxy groups with the binding site. Whatever the nature of the starting molecule, the constancy of the k_α, $k_{\alpha'}$ and k_β values and, consequently, of the E_α, $E_{\alpha'}$ and E_β values suggests that these interactions are independent of each other, i.e. the molecule adopts a unique position in the binding site, where each hydroxyl group, when present, may interact at specific and independent locations of the hormone-binding domain.

Additivity of the contributions of various substituents to overall binding affinity has already been demonstrated for binding to other steroid hormone receptors and to plasma proteins [19–22] although combinations of certain substitutions may, in some cases, induce long distance effects.

Analysis of the RBAs obtained after incubation at 0°C for 2 h did not lead to conclusions that were as clear-cut as the above. However, since mono- or di-hydroxylation on only rings α or β but not α′ led to comparable RBA values (compare values 40/29/27 corresponding to compounds 2Z/3/5Z respectively or values 28/19/41 corresponding to compounds 4/5E/6

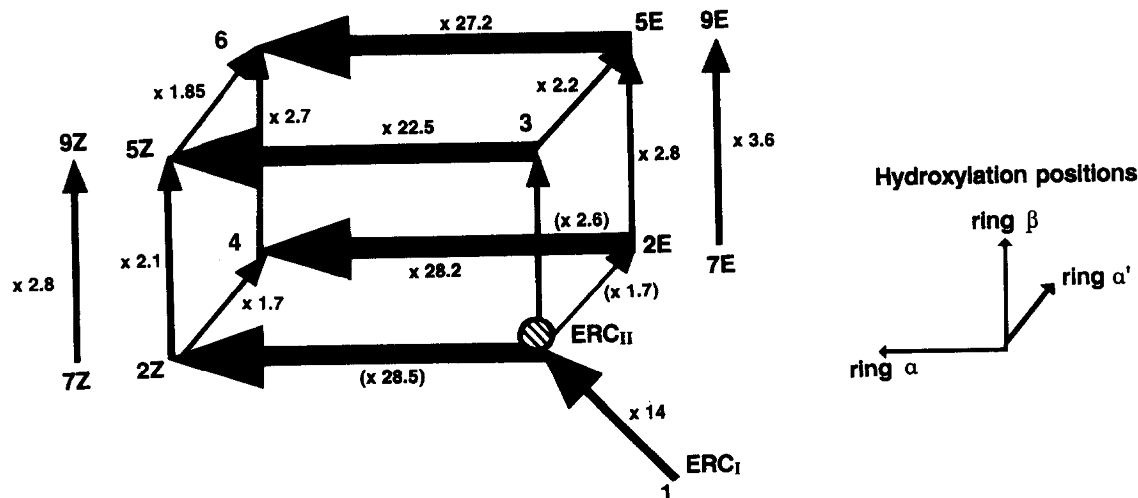

Fig. 3. Representation of the influence of *p*-hydroxylation of the α, α′, β rings of TPEs on RBAs for ER measured at 25°C. The size of the arrows indicates the intensity of the effect. ERC_I and ERC_{II} indicate the hypothetical conformations of the ligand–receptor complexes.

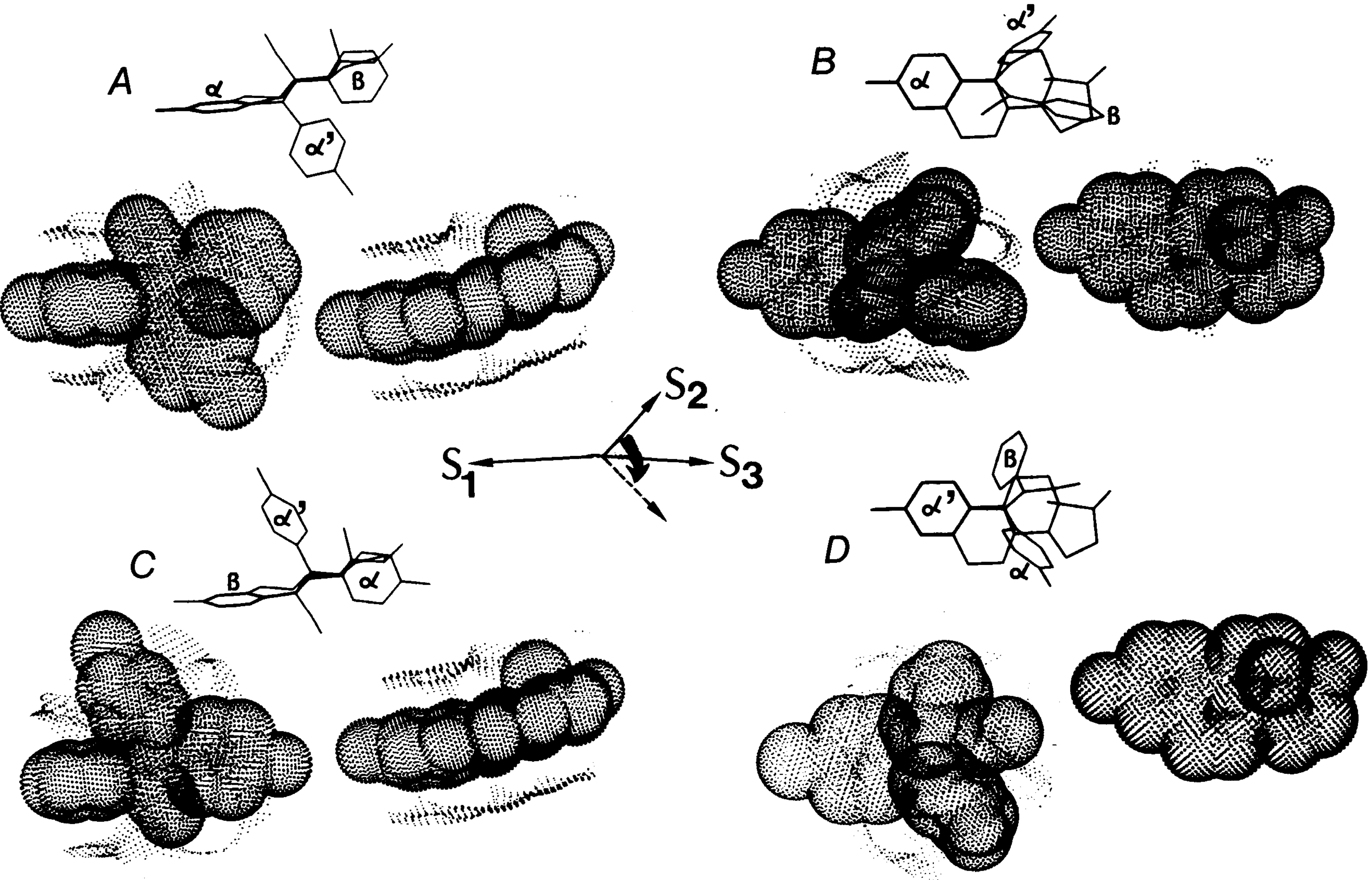

Fig. 4. Superpositions of the phenyl rings of TPE 7Z with the A-ring of estradiol. (A) and (B) α-Phenol ring, (C) β-phenyl ring, (D) α′-phenyl ring. Beneath each superposition are given the atomic surface (spheres) and van der Waals signature (dotted contours) of 7Z (LHS) and E_2 (RHS). The graphic representation of the molecules was performed with the software MANOSK [24].

respectively) (Table 1), it is likely that either of these phenolic groups could interact with the relevant H-bonding zone of ER. This suggests that at 0°C a TPE might gain access to the site in two different orientations but that only one of them is stable enough to be found at equilibrium (by increasing incubation time or temperature) as indicated above by the data obtained after 5 h incubation at 25°C.

In order to determine which, among the six superimpositions shown in Fig. 2, might be the most likely, we superimposed the known crystallographic structures of TPE 7Z (1) and E_2 (Fig. 4) and compared their van der Waals signatures [23] using the software MANOSK [24]. The least favorable fit (Fig. 4D) corresponds to the superposition of the α'-ring of the TPE with the E_2 A-ring. Superposition of either the α- or β-ring of the TPE with the E_2 A-ring are equally satisfactory (Figs 4 A–C). A slight preference for a position where the α'-ring overlaps C-7 rather than C-11 was noted for the superposition of the α-ring on the A-ring (A compared to B), whereas the reverse was true (α'-ring corresponding to C-11) for the superposition of the β-ring on the A-ring (C). The van der Waals signature shows that the E_2 molecule would best fit into a sheath with interactions above and below the plane of the molecule that are situated outside the atomic volume. These interactions cover a broader area in the case of the TPE.

CONCLUSION

It was demonstrated long ago that the two hydroxy groups of E_2 are inequivalent in their contribution to the overall binding affinity of E_2 to ER [25, 26]. The hydroxy group in C3 is considered to be involved in the first recognition step and acts as a H-bond donor. It is this H-bond donor rather than the conformation of the steroid that would foremost govern the interaction with ER (for review, see Ref. [27]).

In a recent study of the binding of 2,3-diarylindenes to ER, Katzenellenbogen's team concluded that these molecules also adopt a unique position in the ER steroid binding site [28]. The present study, however, suggests that a TPE could adopt two orientations at the incubation temperature they used (0°C), i.e. under experimental conditions that probably do not reflect an equilibrium state but rather the relative association rates of the ligand with ER. This may not be unusual since binding of substrates in reverse positions to an active site has already been reported, e.g. for 3 (or 17)β-hydroxysteroid: NAD oxydoreductase [29, 30]. On the other hand, under conditions approaching equilibrium (25°C), TPEs as well as diarylindenes would indeed appear to adopt a unique orientation. Because of the strong contribution of α-hydroxylation to the affinity of a TPE for ER at 25°C, the main anchorage point of the TPEs (S_1) would probably be the α-phenolic ring which could mimic the E_2 A-ring in its interaction with ER leading to the activated form of the receptor that initiates the physiological response. If this is effectively the case, analysis of the crystal structures of TPEs (Fig. 4) suggests that the α'-phenolic ring becomes oriented in the C7 or C-11 direction towards a location S_2 with which it interacts less markedly whereas the β-phenolic ring interacts with a location S3 close to the zone of interaction of the estradiol D-ring. The "perfect" parallelepiped we recorded in Fig. 3 indeed defines by triangulation the positions of the H-bonds which increase and stabilize the interactions of TPEs with the ER binding site. The distances separating the $\alpha\alpha'$, $\alpha\beta$ and $\alpha'\beta$ oxygen atoms ($\sim$ 10, 13 and 7Å, respectively) pinpoint the spatial disposition of the amino-acids able to act as appropriate donors or acceptors of protons within the steroid binding site. Prediction of the 3D-structure of the hormone-binding domain of ER is still in its very preliminary stages [31–33] but binding experiments with organometallic estradiol derivatives have suggested that vicinal space positioning of a cysteine (possibly Cys 530) and lysine (or arginine) residues is needed for estradiol D-ring binding [34, 35]. Cys 530 of human ER has also recently been identified as a site of reaction with the affinity labels ketononestrol aziridine, an estrogen, and tamoxifen aziridine, an antiestrogen [36].

Finally, since affinities of TPEs for ER from different species (rat, mouse or calf) were found to be correlated [5], the amino-acids involved are probably located in a highly conserved region of the receptor protein in spite of the presumed slight variations in the hormone binding domains of ER from these species.

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