Donor–Acceptor Tetrahydrochrysenes, Inherently Fluorescent, High-Affinity Ligands for the Estrogen Receptor: Binding and Fluorescence Characteristics and Fluorometric Assay of Receptor†

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ABSTRACT: We have examined the binding behavior and fluorescence characteristics of a series of novel ligands for the estrogen receptor (ER). These ligands are derivatives of 5,6,11,12-tetrahydrochrysene (THC), a structure that embodies a stilbene chromophore, found in many nonsteroidal estrogens, within a rigid tetracyclic system where it cannot easily be distorted from planarity, thus providing the conjugation and rigidity required for efficient fluorescence. Additional steric bulk, as trans-disposed ethyl substituents at the internal C-5 and C-11 positions, is required for the highest relative binding affinity (RBA), and the *trans*-5,11-diethyl-2,8-dihydroxy-THC derivative binds to ER with an affinity greater than that of estradiol. The replacement of one of the phenolic hydroxyl groups of this THC derivative with an electron-withdrawing group (COMe, COOMe, $CONH_2$, CN, or NO_2) yields unsymmetrical THCs with binding affinities 15–40% that of estradiol (E_2). The fluorescence emission shifts from about 380 nm for the dihydroxy THC to 475–688 nm for the donor–acceptor THCs. The emission of these donor–acceptor THCs is highly solvatochromic and shifts to longer wavelengths as the solvent polarity increases. In ethanol, the fluorescence quantum yield of the first four of these compounds is high (Φ_f = 0.43–0.69), but the fifth compound, the nitro-THC, is almost nonemissive in protic solvents. When they are incubated with protein solutions containing ER ($\sim 10^{-9}$ M), the emission from the donor–acceptor THCs bound specifically to ER is in the 500–570-nm range, whereas fluorescence from non-receptor-bound fluorophores is in the 425–460-nm range. Thus, fluorescence from these probes bound specifically to ER could be measured under equilibrium conditions as well as after the removal of free and non-receptor-bound material by treatment with charcoal–dextran. This is one of the first demonstrations of ligands whose fluorescence is distinctly different when free, when bound to ER, or when bound to non-receptor proteins. It is also the first demonstration of ER assay by fluorescence under equilibrium conditions.

The estrogen receptor (ER)[1] is a member of the superfamily of nuclear receptor proteins [for reviews see Evans (1988), O'Malley (1990), and Wahli and Martinez (1991)], whose transcriptional activity is modulated by the binding of agonist and antagonist ligands (Katzenellenbogen et al., 1985). ER is of clinical importance in the management of breast cancer, as the presence of ERs in breast cancer cells has proven to be a useful prognostic indicator for the success of hormonal therapy (Thorpe, 1988; Osborne et al., 1980; DeSombre et al., 1979; McGuire et al., 1975). In general, the absence of estrogen receptor correlates with a poorer prognosis (Allegra et al., 1979; Osborne et al., 1980; Paridaens et al., 1980).

Traditionally, the presence of estrogen receptors has been determined by utilizing their ability to bind a variety of ligands, both steroidal and nonsteroidal, with nanomolar affinity (Toft et al., 1967; Katzenellenbogen et al., 1978). These assays for ER are done by radiometric binding methods, using ligands labeled to high specific activity with tritium (Glasscock & Hoekstra, 1959; Jensen & Jacobsen, 1960; Jordan et al., 1981; Katzenellenbogen et al., 1983) or iodine-125 (Hochberg & Rosner, 1980). Generally, these assays require a nonequilibrium step (i.e., charcoal adsorption, filtration) to separate bound ligand from free. More recently, immunoassay techniques utilizing monoclonal antibodies to the ER have been developed (Greene & Jensen, 1982; King & Green, 1984; McCarty et al., 1985). These afford greater sensitivities than the ligand binding assay but depend on a structural feature (epitope recognition) rather than a functional feature (e.g., ligand binding). An immunologically recognizable receptor may not necessarily be biochemically active.

It would be very desirable to have a fluorescent ligand for the estrogen receptor. With such a probe, the kinetics of ligand–receptor association and dissociation could be studied in real time. Also, if sufficiently selective and sensitive, such a molecule might be used to assay breast cancer cells for their ER content, either by flow cytometry on suspended (viable) cells or by fluorescence microscopy on cells or tissue preparations, thereby characterizing not only the ER content but also its population or spatial distribution. Such a characterization of the heterogeneity of ER distribution in breast tumor samples may provide a more accurate prognostic indicator for therapy.

To be useful, a fluorescent estrogen must satisfy certain criteria, both biochemical and photophysical: It needs to have high affinity for ER coupled with modest non-receptor (i.e., nonspecific) binding; furthermore, it needs to be strongly fluorescent (high absorption and high quantum yield), and its emission needs to be well separated from background fluo-

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[1] Abbreviations: ER, estrogen receptor; THC, tetrahydrochrysene (chrysene is another name of benzo[*c*]phenanthrene); RBA, relative binding affinity; BSA, bovine serum albumin; NS, nonspecific binding; E_2, estradiol [1,3,5(10)-estratriene-3,17β-diol]; cps, fluorescence photon counts (counts per second); DES, diethylstilbestrol [(*E*)-3,4-bis(4-hydroxyphenyl)-3-hexene].

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rescence (protein fluorescence and cell autofluorescence). Ideally, the fluorescence signal from the probe bound to ER should be distinguishable in intensity, emission wavelength, lifetime, or polarization from free or nonspecifically bound probe.

There have been numerous reports on the development of fluorescent probes for ER. The agents prepared to date can be divided into three classes: conjugates (consisting of distinct ligand and fluorophore components attached together) (Fevig et al., 1987; Carlson et al., 1989; Rau et al., 1980; Lee et al., 1977; Lee, 1980; Fisher et al., 1982; Joyce et al., 1982), inherently fluorescent ligands (in which the fluorophore is embodied within the structure of the ligand) (Anstead & Katzenellenbogen, 1988; Katzenellenbogen et al., 1986; Nelson et al., 1984; Lee et al., 1977), and photofluorogenic agents (in which a high-affinity nonfluorescent precursor is converted photochemically into a fluorescent but generally low-affinity fluorochrome) (Bindal & Katzenellenbogen, 1986). While there are inherent advantages and disadvantages of all three types of fluorescent estrogens, in most cases the binding or fluorescence behavior of the compounds investigated to date has not proved adequate for them to be useful in routine assays of ER.

Recently, we have described the synthesis of a set of inherently fluorescent estrogens that are the first to combine high receptor-binding affinity with good fluorescence characteristics (Hwang et al., 1992). These are the donor–acceptor tetrahydrochrysenes. In this report, we describe the ER binding affinity of the tetrahydrochrysenes and their fluorescence characteristics, and we present experiments in which their binding to ER can be assayed directly through their fluorescence properties, under both equilibrium and nonequilibrium conditions.

MATERIALS AND METHODS

Materials. The tetrahydrochrysenes were synthesized and characterized as reported (Hwang et al., 1992).

The following compounds were obtained from the sources indicated: tritium-labeled estradiol ([6,7-^{3}H]E_2) [estra-1,3,5-(10)-trien-3,17β-diol], 51 Ci/mmol, from Amersham Corp; 4,4′-dihydroxystilbene, Spectrum Chemical Co.; 4-methoxybutyrophenone, Pfaltz and Bauer, Inc.; diethylstilbestrol (DES), unlabeled estradiol, activated charcoal, Trizma base, and bovine serum albumin (BSA) from Sigma Chemical Co.; coumarin I and ethylenedinitrilotetraacetic acid tetrasodium salt (EDTA) from Eastman Kodak Co.; dextran, grade C, from Schwarz/Mann; Triton X-114 from Chem Central—Indianapolis; sodium azide, 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP), boron trifluoride·dimethyl sulfide, titanium trichloride, 2,2,2-trifluoroethanol, and spectrophotometric grade acetonitrile, from Aldrich Chemical Co.; 2,5-diphenyloxazole (PPO) from Research Products International Corp; dimethylformamide (DMF) from EM Science; spectrophotometric grade cyclohexane and ethyl acetate from Mallinckrodt, Inc.; diethyl ether, 2-propanol and spectrophotometric-grade acetone from Fisher Chemical; and absolute ethanol from Midwest Grain Products Co.

Lamb uterine cytosol was prepared and stored as previously described (Carlson et al., 1977). ER expressed in yeast was obtained as a gift from Karo Bio. Before use in fluorescence experiments, it was dialyzed at ~1:500 (v/v) against TEA buffer overnight at 4 °C to remove fluorescent impurities.

All experiments were done at 0–4 °C in TEA buffer (0.01 M Tris-HCl, 0.0015 M EDTA, and 0.02% NaN_3, pH 7.4 at 25 °C). Free ligand was removed with charcoal–dextran, prepared as previously reported (Katzenellenbogen et al., 1973) and used at 1 part to 10 parts of protein solution.

Molecular mechanics calculations, using X-ray crystallographic data as reported in Hwang et al. (1992), were performed with the MAXIMIN option of the SYBYL molecular modeling system (Version 5.41, Tripos Associates, St. Louis, MO). Molecular superpositions were also performed with this system using the "fit" command.

Synthesis of (E)-4,5-Bis(4-hydroxyphenyl)-oct-4-ene (**4d**). To prepare di-(*n*-propyl)stilbestrol, 4-methoxybutyrophenone was first coupled ($TiCl_3$, $LiAlH_4$, and THF; McMurray, 1989) to give the stilbestrol dimethyl ether as the cis and trans isomers (42%, predominantly cis). A portion of the cis compound was isolated and characterized. The cis/trans mixture of the stilbestrol dimethyl ether was deprotected in $BF_3{\cdot}S(CH_3)_2$ (Williard & Fryhle, 1980); recrystallization from benzene (Winkler et al., 1971) afforded the trans isomer of di-(*n*-propyl)stilbestrol (**4d**; Dodds et al., 1939). The trans stereochemical assignment was based on the allylic methylene signal at 2.08 ppm ($CDCl_3$). In the corresponding cis isomer, the signal for this group is observed at 2.45 ppm. The assignments are consistent with the work of Leimner and Weyerstahl (1982). Di-(*n*-propyl)stilbestrol (**4d**) had the following properties: mp 143–146 °C; ^{1}H NMR ($CDCl_3$) δ 7.05 (d, 4 H, J = 8.4 Hz, Ar*H* meta to -OH), 6.82 (d, 4 H, J = 8.3 Hz, Ar*H* ortho to -OH), 4.59 (br s, 2 H, ArO*H*), 2.08 (t, 4 H, J = 7.5 Hz, $CH_2CH_2CH_3$), 1.16 (m, 4 H, CH_2CH_2-CH_3), 0.64 (t, 6 H, J = 7.3 Hz, $CH_2CH_2CH_3$); MS (electron impact, 10 eV) m/z 296 (100, M^+), 267 (22, $M - C_2H_5$), 253 (7, $M - C_3H_7$), 173 (5), 149 (5), 107 (14). Anal. Calcd for $C_{20}H_{24}O_2$: C, 81.04; H, 8.16. Found: C, 81.03; H, 8.22.

Relative Binding Affinity. Relative binding measurements were performed as previously reported (Katzenellenbogen et al., 1973) using yeast expressed ER or lamb uterine cytosol, diluted to ~1.5 nM receptor. The protein solution was incubated with buffer or several concentrations of unlabeled competitor together with 10 nM ^{3}H-E_2 at 0 °C for 18–24 h. The unlabeled competitor was diluted in 1:1 dimethylformamide/TEA buffer to ensure solubility. All data is reported relative to E_2 = 100%.

Radioactivity was determined in a Nuclear Chicago Isocap 300 liquid scintillation counter using a xylene-based scintillation fluor (Katzenellenbogen et al., 1974).

Spectrophotometric Assays. UV measurements were determined on a Hewlett-Packard 8451A diode array spectrophotometer. Spectrofluorometric analysis was determined on a Spex Fluorolog 2 (Model IIIC) spectrofluorometer equipped for photon counting. Fluorescence intensities were measured at the maximum of the emission band. All solvents were spectrophotometric grade. Fluorescence quantum yields (Φ_f) were obtained from lamp-corrected spectra and were calculated following the procedure of Pesce (1971) using coumarin I in ethanol (Φ_f = 0.64) as the standard (Olmsted, 1979).

To conserve the ER preparations, fluorescence spectra and assays were performed in a microcuvette (7 × 7 × 33 mm, 0.6 mL). Under typical ER assay conditions (2.5-mm slits, 9.0-nm resolution), a 1 nM solution of bound ligand gave a photon counting rate of (5–15) × 10^4 cps at the emission maximum. Typical detection limits were 0.01–0.1 nM in organic solvents and 0.1 nM in ER preparations from the yeast expression system. Because of higher fluorescence background in ER preparations from lamb uterus, detection levels are ca. 0.5 nM.

Scatchard Analysis. Lamb uterine cytosol or ER expressed in yeast was diluted to 2–3 nM receptor and incubated at 0 °C for 2–3 h with various concentrations of fluorescent ligand or ^{3}H-E_2 in the absence or presence of 3 μM unlabeled E_2. Aliquots of the incubation solution were removed to determine the total concentration of ligand present (determined radiometrically for ^{3}H-E_2 or spectrofluorometrically for the fluorescent ligand). The incubations with the fluorescent ligand could be scanned at equilibrium (without removing free and nonspecifically bound ligand) and the specific binding to ER seen as the difference between the spectrum of total binding (in the absence of unlabeled E_2) and that of the nonspecific binding (in the presence of unlabeled E_2).

The incubation solutions were then treated with charcoal–dextran and the bound ligand was determined. Fluorometric measurements of ligand–protein interactions were done with the cuvette holder cooled to ~4 °C. A protein incubation with no ligand was diluted and processed in parallel as a background. Data were processed according to the method of Scatchard (1949) to give a direct measure of binding affinities.

Ethyl Acetate Extraction. Samples were extracted twice with ethyl acetate, and the extracts were combined and evaporated to dryness under a stream of nitrogen. They were redissolved in ethanol and their fluorescence was measured. Comparison with a standard curve prepared in ethanol permitted conversion of fluorescence intensity to nanomolar concentrations.

Log $P_{o/w}$. Estimates of the octanol–water partition coefficient (log $P_{o/w}$) were obtained as previously reported (Pomper et al., 1990) using the reversed-phase method of Minick (Minick et al., 1988).

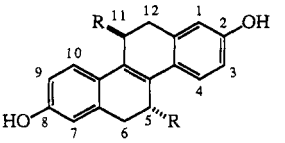
RESULTS

Structure–Affinity Relationships of the Tetrahydrochrysene Estrogens for the Estrogen Receptor. The structures of the tetrahydrochrysenes (THCs) that we have studied (**1–3**) are given in Chart I. All of these systems embody a stilbene chromophore, such as is found in the high-affinity nonsteroidal estrogen diethylstilbestrol (**4c**, DES), within a rigid tetracyclic system. The binding affinities for ER of the THCs, together with those of several other nonsteroidal estrogens which have structural similarities to the THCs, are given in Table I.

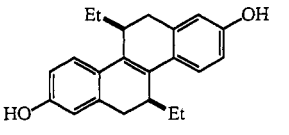
The first structure–affinity relationship that we investigated was the alkyl substitution pattern in the bisphenol THC series (**1a–d**). While the unsubstituted bisphenol THC (**1a**) has relatively low affinity for ER, the binding increases with the introduction of alkyl substituents at positions 5 and 11 (**1b–d**); the binding reaches a maximum with ethyl substituents and then declines with propyl. Further analysis of the diethyl-substituted THC showed that the trans isomer (**1c**) has a higher affinity for ER than the cis (**2**); in fact, the affinity of the trans isomer **1c** exceeds that of estradiol. Thus, we elected to use the *trans*-5,11-diethyl-substituted tetrahydrochrysene system as the base structure for subsequent investigations.

The stilbestrols (**4a–d**) show a similar increase and decline in ER binding affinity with internal alkyl substitution, so that DES (**4c**) and the diethyl-THC (**1c**) have roughly comparable binding affinities. A structural overlay of these two compounds (structure A, Chart II) indicates similarities and differences: By projecting above and below the plane of the linking ethylene unit, the ethyl groups in both DES and diethyl-THC occupy roughly comparable regions of space, although the extent of projection is greater with the diethyl-THC system, because there is an extra atom contributed by the ring carbons C5 or C11. It is of note that these regions are roughly congruent to the 7α and 11β positions on the steroidal estrogen estradiol (see superposition of diethyl-THC and 7α,11β-diethylestradiol, structure B, Chart II); both of these sites are known to be very tolerant of substantial alkyl substitution (Bowler et al., 1989; Gabbard & Segaloff, 1983; Belanger et al., 1981). Other differences between DES and diethyl-THC are discussed below.

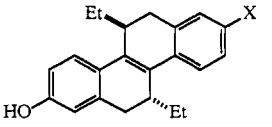
Chart I: Structures of Tetrahydrochrysene Estrogens (**1–3**) and Related Stilbestrol (**4**) and Hexestrol (**5**) Systems



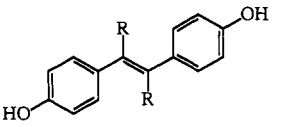
1a (R = H)
1b (R = Me)
1c (R = Et)
1d (R = Pr)



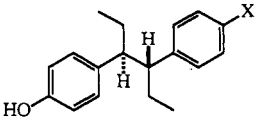
2 (cis)



3a (X = -CO-CH_3)
3b (X = -CO-OCH_3)
3c (X = -CO-NH_2)
3d (X = -C≡N)
3e (X = -NO_2)



4a (R = H)
4b (R = Me)
4c (R = Et) DES
4d (R = *n*-Pr)



5a (X = OH) Hexestrol)
5b (X = -CO-CH_3)
5c (X = -C≡N)
5d (X = -NO_2)

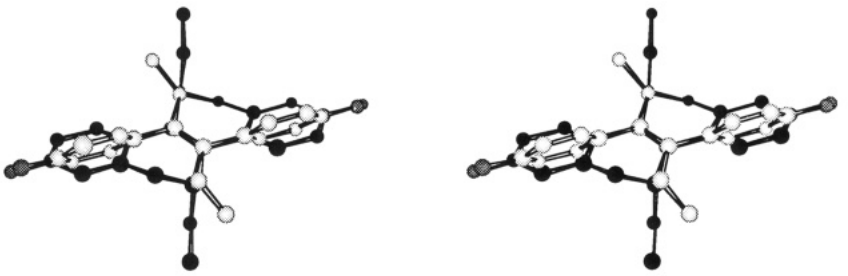
Table I: Relative Binding Affinity of Tetrahydrochrysene and Other Nonsteroidal Estrogens for the Estrogen Receptor[a]

cmpd class	cmpd no.	alkyl subst at C-5, 11 (R)	function at C-2 (X)	RBA[a]	θ[b] (deg)
bisphenol THC	**1a**	H	-OH	8	
	1b	Me	-OH	8	
	1c	Et	-OH	126	26[c]
	1d	Pr	-OH	48	
	2	Et (cis)	-OH	13	
unsymmetrical THC	**3a**	Et	-CO-CH_3	40 ± 9	
	3b	Et	-CO-OCH_3	24 ± 9	
	3c	Et	-CO-NH_2	15 ± 3	
	3d	Et	-C≡N	16 ± 6	
	3e	Et	-NO_2	4.4	
stilbestrol	**4a**	H	-OH	0.37 ± 0.04	5.2[d]
	4b	Me	-OH	28 ± 16	
	4c (DES)	Et	-OH	99 ± 5	63[e]
	4d	Pr	-OH	81.5 ± 7.9	
hexestrol[f]	**5a**	Et	-OH	300	
	5b	Et	-CO-CH_3	4.1	
	5c	Et	-C≡N	5.2	
	5d	Et	-NO_2	11	

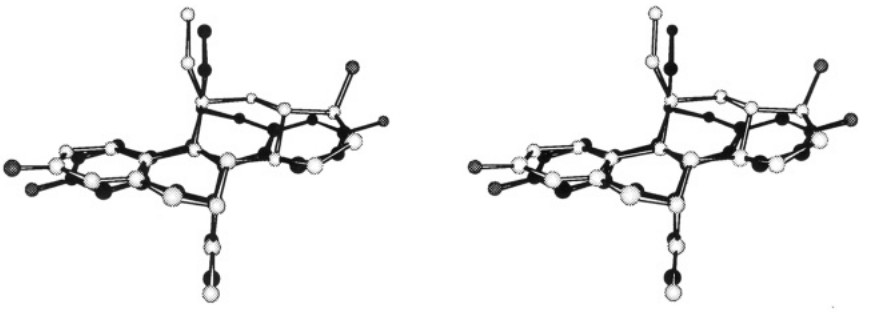
[a] Data are relative binding affinity (RBA) values with reference to estradiol as the standard (RBA = 100). [b] Dihedral angle of the phenyl group with respect to the ethylene carbons. [c] Dihedral angle obtained from X-ray structure (Hwang et al., 1992). [d] Based on the X-ray crystal structure of *trans*-stilbene (Hoekstra et al., 1975). [e] Based on the X-ray crystal structure of DES (Weeks et al., 1970). [f] Data are from Katzenellenbogen et al. (1981).

Chart II: Structural Overlays and Dihedral Relationships of Stilbene Chromophore Estrogens

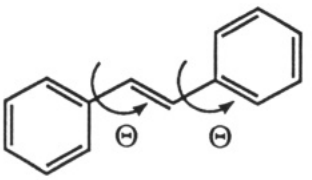
(A) DES (**4c**) and Diethyl-THC (**1c**) Overlay in Relaxed Stereo[a]



(B) Diethyl-THC (**1c**) and 7α,11β-Diethylestradiol Overlay in Relaxed Stereo



(C) Torsion Angles in a Stilbene Chrompore



[a] The diethyl-THC structure is from X-ray crystallography (Hwang et al., 1992). The diethylstilbestrol and 7α,11β-diethylestradiol structures were constructed and minimized with the SYBYL molecular modeling system (Tripos Associates, St. Louis, MO).

Another interesting comparison between the THCs and stilbestrols concerns the geometry of the stilbene chromophore and its perturbation by substituents. The geometry of the stilbene chromophore can be characterized by the dihedral angles between the aryl groups and the ethylene linker (see structure C, Chart II). From the X-ray crystallographic structure of the diethyl THC, this angle is 26° (Hwang et al., 1992) (see Table I), whereas in X-ray structures of diethylstilbestrol (**4c**), a much greater dihedral angle is observed (62.8°) (Weeks et al., 1970). The tetracyclic nature of the THC system is rigid, so that the planarity of its stilbene chromophore is not substantially altered when alkyl substituents are added at the C-5 and C-11 positions.

If one uses the simple $\cos^2\theta$ relationship for the dependence of conjugation between double bonds on the dihedral angle (Dewar, 1952) [which occurs twice in the stilbene system, hence a $(\cos^2\theta)^2$ relationship], one would predict in the highest affinity diethyl-THC (**1c**; $\theta = 26°$) that 65% of the stilbene chromophore persists, whereas in diethylstilbestrol (**4c**; $\theta = 63°$), that only 4% of the stilbene chromophore remains. Figure 1 shows the UV spectrum of diethylstilbestrol (**4c**), stilbestrol (**4a**), and *trans*-diethyl-THC (**1c**) in cyclohexane. Diethylstilbestrol shows no characteristic stilbene absorption at 300 nm. Stilbestrol, assumed to retain the conformation of its parent, *trans*-stilbene, is thus expected to be nearly planar ($\theta = 5.2°$; Hoekstra et al., 1975), and shows an absorption at 302 nm ($\epsilon = 18\,700$). In *trans*-diethyl-THC, the rigidity of the tetracyclic framework and the additional substitution produces three effects in the UV spectrum relative to stilbestrol: a bathochromic shift in the absorption band to 334 nm, a hyperchromic effect ($\epsilon = 24\,000$), and the appearance of vibrational fine structure.

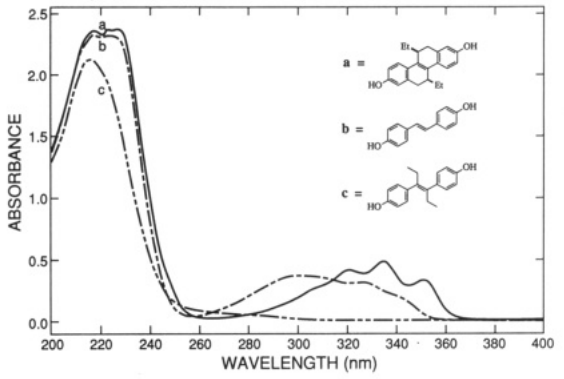


FIGURE 1: UV spectra of diethylstilbestrol (**4c**), stilbestrol (**4a**), and *trans*-diethyl-THC (**1c**) in cyclohexane. All concentrations are 20 μM.

We have studied in some detail the contrary effect that alkyl substitution has on the ER binding affinity and on the stilbene conjugation required for effective fluorescence, as manifest in other acyclic or monocyclic nonsteroidal estrogen series [viz., triarylethylenes, 1,2-diaryl-3,4-dihydronaphthalenes, and 2,3-diarylindenes (Anstead et al., 1988)]. One can make the generalization that when the conjugation in the stilbene system can be interrupted by twisting about one or more aryl ethylene bonds, then substituents that raise receptor binding affinity will also tend to diminish conjugation, because they will increase this twisting; they will thereby also reduce the potential for building an effective fluorochrome (Anstead

Table II: Absorption and Emission Properties of THC Derivatives **1c** and **3a–e**

cmpd no. (X-substituent)	λ_{max}, ab (nm, EtOH)	ϵ	λ_{max}, em (nm, EtOH)	Stokes shift (cm^{-1}, EtOH)	Φ_f[a] (EtOH)	Φ_f (TEA)[b]
1c (-OH)	337	35106	382	3500		
3a (-$COCH_3$)	380	22801	525	7300	0.430	0.041
3b (-COOMe)	364	42400	482	6700	0.691	0.102
3c (-$CONH_2$)	350	18608	472	7400	0.580	0.457
3d (-CN)	366	28300	456	5400	0.691	0.165
3e (-NO_2)[c]	414	15300	688	9620	0.0038[d]	0.00024[d]

[a] Determined relative to coumarin I in ethanol (Φ_f = 0.64) (Olmsted, 1979). Excitation was at 360 nm. [b] Tris–EDTA–azide buffer. [c] From Anstead et al. (1992). [d] Determined relative to acridine yellow in ethanol (Φ_f = 0.45) (Anstead et al., 1992).

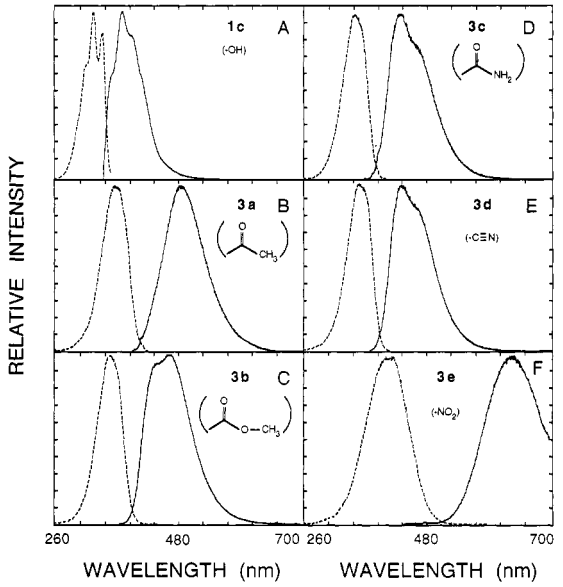


FIGURE 2: Excitation–emission spectra of the tetrahydrochrysenes **1c** and **3a–e**. The spectra of the chrysenes in acetonitrile (approximately 0.1 μM) were scanned using 0.5-mm slits (1.8-nm resolution) and peak maxima were set at 100% relative intensity (peak maxima ranges were approximately 10^5 cps). All of the emission spectra (solid lines) were uncorrected for photomultiplier response. All of the excitation spectra (dashed lines) are uncorrected for the lamp spectrum except for the nitrotetrahydrochrysene (**3e**, panel F), where the excitation spectrum extends to sufficiently long wavelength that spikes in the lamp spectrum appear in the uncorrected excitation trace. [Second-order spikes in the emission spectrum of diol chrysene **1c** (panel A) and in the excitation spectrum of the nitrochrysene **3e** (panel F) were deleted.]

et al., 1990). The tetracyclic structure of the THC system enforces rigidity upon the stilbene chromophore, regardless of alkyl substitution. Thus, the THCs escape the ironic reciprocal relationship between ER binding and fluorescence encountered in the noncyclic stilbestrols.

While the bisphenol **1c** has the highest ER binding affinity, its fluorescence emission is at a relatively short wavelength (cf. Table II; Figure 2A). To increase the wavelength of fluorescence emission, we synthesized THC systems in which one of the phenolic hydroxyl groups was replaced with an electron acceptor system (**3a–e**) (Hwang et al., 1992). Since the remaining phenolic hydroxyl group can act as an electron donor, this set can be considered donor–acceptor THCs.

The structural similarity between hexestrol (**5a**) and diethyl-THC (**1c**) suggests that substituents replacing the second hydroxyl group in both compounds might have a similar effect on their binding affinity. These replacements in both the hexestrol series (**5b–d**; Katzenellenbogen et al., 1981) and the unsymmetrical THCs (**3a–e**) do result in a lowering of their binding affinities to the estrogen receptor to less than that of the symmetrical parent compound (**5a** or **1c**) (Table I), presumably either because of their increased steric bulk or because of differences in their orientation or capacity for hydrogen-bond donation or acceptance. However, as seen in Table I, these substituents have a greater effect on binding in the hexestrol series than in the unsymmetrical THC series, and the binding affinity of comparable compounds in both series shows little similarity. In the unsymmetrical THC series, the relative binding affinity of the first four members **3a–d** follows the order ketone > ester > nitrile > amide, but all are sufficiently high (15–40%) to warrant further study as fluorescent probes for the estrogen receptor. The affinity of the corresponding nitro compound **3e** is lower; this compound is, however, unsuitable as a fluorescent probe for the estrogen receptor for other reasons (see below).

Absorbance and Fluorescence Characteristics. The UV absorption and fluorescence emission of the THC derivatives were measured in ethanol and are summarized in Table II. The excitation–emission spectra in acetonitrile are shown in Figure 2. Both the absorption and emission of the donor–acceptor chromophore systems in **3a–e** were observed at far longer wavelengths (Figure 2B–F) than that of the diol THC **1c**, containing only donor groups (Figure 2A). The short wavelength emission of **1c** (ca. 380 nm) makes it only marginally useful as a fluorescent probe in biological systems. The donor–acceptor THCs, designed to have improved fluorescence characteristics, do indeed have longer wavelength emission maxima (ca. 450–680 nm), beyond the range of most cellular autofluorescence. They exhibit high molar absorptivity, with absorption maxima at wavelengths that will cause little photodamage to biological systems.

From the emission spectra of the donor–acceptor systems shown in Figure 2B–F, it is clear that the position of the emission maximum and the magnitude of the Stokes shift reflect the electron-accepting ability of the variable substituent. Also, two emission bands are apparent in the spectra of the ester, amide, and nitrile donor–acceptor systems in acetonitrile (cf. Figure 2C–E). It is very unlikely that the dual emission is due to fluorescent impurities, since the shape of the emission spectrum does not change when the excitation wavelength is shifted from the peak to halfway down either side of the excitation spectrum; also, all of these compounds appeared to be homogeneous by HPLC analysis under the various conditions used to determine their octanol–water partition coefficients (see below). Dual fluorescence emission is frequently observed when there is emission from two distinct excited-state species whose interconversion takes place at a rate comparable to their rates of fluorescence (Ireland & Wyatt, 1976). We have seen this phenomenon in a different series of fluorescent ligands for the estrogen receptor (Haroutounian & Katzenellenbogen, 1988).

The quantum yields of the donor–acceptor THCs **3a–e** were measured by an indirect method, using coumarin I in ethanol (Φ_f = 0.64) as a standard (Olmsted, 1979). (Acridine yellow G was used as the standard for compound **3e**; Anstead et al., 1992.) The quantum yield of compounds **3a–e** in ethanol was

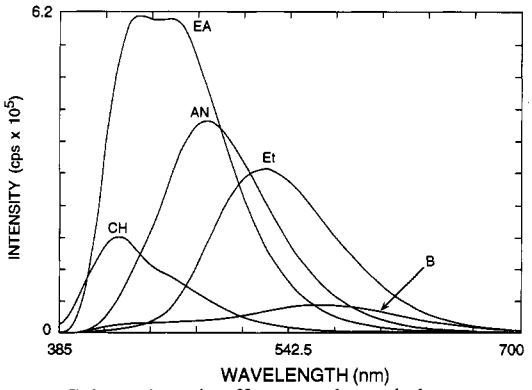


FIGURE 3: Solvatochromic effects on the emission spectra of the ketone tetrahydrochrysene (**3a**). Emission spectra of a 100 nM solution of the ketone tetrahydrochrysene (**3a**) were measured in various solvents: cyclohexane (CH), ethyl acetate (EA), acetonitrile (AN), ethanol (Et), and TEA buffer (B). Excitation was at 377 nm and slit width was 1.25 mm (4.5-nm resolution). The most intense peak (in ethyl acetate) (6.2×10^5 cps) was set at 100%; the intensities of the other emission peaks are shown to scale.

Table III: Fluorescence Solvatochromism of Donor–Acceptor THCs (**3a–d**)

solvent	E_T[a] (kcal/mol)	λ_{max}^{em}(relative intensity) X = COMe **3a**	X = COOMe **3b**	X = $CONH_2$ **3c**	X = CN **3d**
cyclohexane	31.2	424 (5.3)[b]	404 (20.3)	403 (7.1)	408 (12.7)
EtOAc	38.1	435 (12.3)	425 (20.3)	420 (9.6)	425 (14.7)
CH_3CN	46.0	483 (8.1)	463 (13.2)	428 (5.7)	433 (11.7)
EtOH	51.9	525 (5.8)	482 (15.3)	472 (6.1)	456 (13.0)
TEA[c]	63.1	593 (0.7)	515 (3.1)	504 (3.1)	483 (6.9)

[a] Empirical solvent polarity parameter (Reichardt, 1965). [b] Numbers in parentheses represent the relative intensity of emission ($\times 10^4$ cps) at λ_{max}^{em}. [c] TEA buffer; see Materials and Methods.

much higher than in aqueous (TEA) buffer, except in the case of the amide **3c**; emission efficiency of amide **3c** in TEA buffer was exceptionally high ($\Phi_f = 0.46$), unlike the other derivatives. The nitro-THC **3e** is poorly emissive in protic solvents, with a quantum yield in ethanol of 0.0038 and estimated in water to be 0.00024 (Anstead et al., 1992). The nitro-THC is, however, quite emissive in aprotic solvents of intermediate polarity (e.g., diethyl ether and ethyl acetate). We are presenting elsewhere a more complete study of the fluorescence of this interesting compound (Anstead et al., 1992).

Solvent Effect on the Emission of Donor–Acceptor THCs **3a–d**. The utility of fluorescent probes for biological systems often depends upon the extent to which their fluorescence properties are sensitive to their environment. As a model for this, we have measured for the donor–acceptor THCs the sensitivity of their fluorescence emission maximum and intensity to solvent polarity, i.e., their fluorescence solvatochromism. The results are exemplified in Figure 3 and summarized in Table III and Figure 4.

The fluorescence properties of the THCs **3a–d** are greatly affected by the solvent polarity. These changes are illustrated nicely with the ketone THC **3a** (cf. Figure 3): As the polarity increases, the λ_{max} of emission becomes shifted to a longer wavelength and the fluorescence intensity varies; in a solvent of intermediate polarity (EtOAc), the fluorescence intensities were higher than in either a nonpolar solvent (cyclohexane), a more polar solvent (acetonitrile), or a protic solvent (ethanol, TEA buffer). Dual fluorescence with this compound is apparent in cyclohexane and ethyl acetate. Very similar changes are noted with the other three donor acceptor systems,

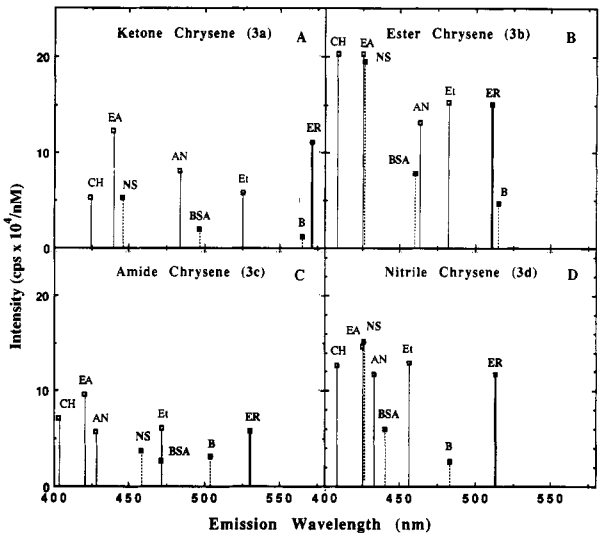


FIGURE 4: Fluorescence properties of ketone chrysene **3a**, ester chrysene **3b**, amide chrysene **3c**, and nitrile chrysene **3d**; the height of each line represents the relative intensity of the emission and the positioning along the *x*-axis the wavelength of emission. Fluorescence was measured in cyclohexane (CH), ethyl acetate (EA), acetonitrile (AN), ethanol (Et), bovine serum albumin (BSA), and TEA buffer (B). Receptor solutions with the ER blocked by E_2 were used to measure fluorescence due to nonspecific binding (NS), while the difference between receptor solutions with the ER blocked or unblocked gave the fluorescence due to specific binding to ER (ER). Excitation was at 360 nm except for the ketone chrysene **3a**, which was at 377 nm.

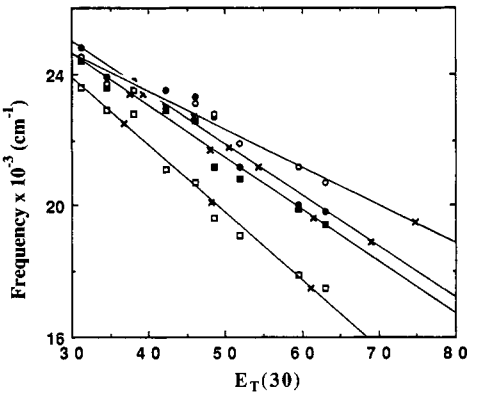


FIGURE 5: Fluorescence emission (wavenumber, cm^{-1}) compared to an empirical solvent polarity scale [$E_T(30)$] (Reichardt, 1965, 1979). Ketone **3a** (□), ester **3b** (■), amide **3c** (●), and nitrile **3d** (○) were measured in solvents of increasing polarity: cyclohexane, diethyl ether, ethyl acetate, acetone, acetonitrile, 2-propanol, ethanol, 2,2,2-trifluoroethanol, and water. Fluorescence emission in protein solutions for each chrysene are noted by × and show increasing polarity NS < BSA < ER.

the ester **3b**, amide **3c**, and nitrile **3d**. Figure 4 provides a convenient display of the emission wavelengths and relative intensities of all of the four systems **3a–d**: the position along the *x*-axis represents the emission wavelength, and the *y*-axis, the relative emission intensity.

We have attempted to relate the increasing emission wavelength of all of the donor–acceptor THCs with the increasing polarity of the solvent. When compared to an empirical solvent polarity parameter E_T (Reichardt, 1965, 1979), the emission maxima showed a linear relationship with a correlation coefficient (r^2) of –0.98 or greater for the solvents (in order of increasing E_T values) cyclohexane, diethyl ether, ethyl acetate, acetone, acetonitrile, 2-propanol, ethanol, 2,2,2-trifluoroethanol, and water (Figure 5). The order of solvent

sensitivity was ketone (**3a**) > amide (**3c**) ~ ester (**3b**) > nitrile (**3d**).

Addition of acid (HCl, pH = 3) did not affect the fluorescence emission of ester **3b** or amide **3c**. In contrast, the fluorescence of ester **3b** and amide **3c** were completely quenched in basic ethanol (0.001 N NaOH). Thus, we conclude that deprotonation of the phenolic hydroxyl group of the THC derivatives **3b** and **3c** results in the loss of their fluorescence, in contrast to another phenol-acceptor fluorophore (Katzenellenbogen et al., 1986).

Fluorescence of Donor–Acceptor THCs in Estrogen Receptor Preparations: Nonspecific versus Specific Binding. The fluorescence properties of the donor–acceptor THCs **3a–d** were measured in a buffer solution (TEA), in a solution of bovine serum albumin (BSA, a model for nonspecific binding), and in preparations of ER from lamb uterine cytosol or ER expressed in yeast. For measurement of "nonspecific fluorescence" (NS; i.e., fluorescence from fluorophore bound to non-receptor proteins), the same preparations of ER were used except that the ER was blocked with an excess of estradiol.

Standard curves of fluorescence intensity versus concentration could easily be generated in pure solutions (TEA, ethanol). However, in protein solutions the ligand is distributed between the bound and free states, and the fluorescence exhibits an intermediate wavelength and intensity (Deranleau et al., 1980). To force the binding equilibrium toward the fully bound state, increasing amounts of protein were added to a constant concentration of ligand until the fluorescence intensity and wavelength reached a plateau. These constant fluorescence values were considered to represent ligand bound to either BSA or non-receptor proteins (NS) (Lloyd, 1984).

The fluorescence properties of the donor–acceptor THCs in estrogen receptor preparations are shown in Figure 4, along with their properties in pure solvents. The fluorescence intensity of all the emissive THCs is greater when bound to NS, a mixture of proteins with hydrophobic binding sites, than it is when bound to the pure protein BSA. The emission wavelength, due to the solvatochromism of the dyes, suggests a less polar environment for NS binding than for BSA binding (Turner & Brand, 1968). Although steroid binding to both protein classes is considered to be a low-affinity, nonspecific interaction, the microenvironment of the binding sites appears to be quite different.

The specific, high-affinity binding to ER can be blocked by excess estradiol. The difference in the fluorescence spectrum seen between a blocked and unblocked sample is considered to be that bound specifically to ER (Figure 6). This can be seen in yeast-expressed ER preparations both in classical, nonequilibrium conditions (after charcoal–dextran treatment to remove free and NS; Figure 6B) and also under equilibrium conditions (Figure 6A). The fluorescence pattern seen with the emissive THCs when bound to receptor preparations represents the first example of a binding measurement with ER under equilibrium conditions where NS binding can be distinguished from specific binding. (This experiment is discussed further in the section below.)

The wavelength of the fluorescence, when compared to a scale of solvent polarity, can be used to estimate the binding site polarity in proteins (Turner & Brand, 1968). The fluorescence from specific ER binding suggests that the ER binding site has a very polar microenvironment as polar as or more polar than water (Figure 5) (see Discusssion).

Direct Fluorometric Measurement of Estrogen Receptor Binding Affinity and Capacity. For the fluorometric assay of receptor binding, the donor–acceptor THCs **3a–d** were

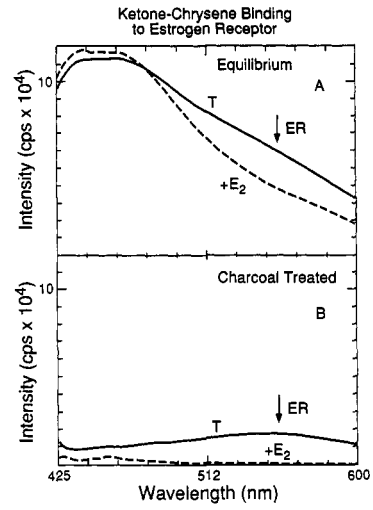


FIGURE 6: Fluorescence emission spectra of ketone chrysene **3a** bound to receptor solutions where the ER was unblocked (T) or blocked by E_2 (+E_2), under equilibrium conditions (panel A) and after treatment with carcoal–dextran (panel B). Fluorophore bound to nonspecific proteins emits with a maximum at ~445 nm, while that bound to ER emits at ~570 nm (arrow). Excitation for the ketone chrysene was 377 nm.

incubated at various concentrations (0.5–30 nM) with a preparation of ER, in the absence and presence of a 100-fold excess of estradiol. A background incubation, to measure the autofluorescence of the cytosol, was included with each experiment. The fluorescence spectra of 5 nM ketone chrysene bound to a protein preparation containing 2 nM yeast-expressed ER under equilibrium conditions is presented in Figure 6A. The most intense fluorescence at ~450 nm results from the ketone bound to non-receptor proteins (nonspecific binding). When a 100-fold excess of estradiol is added to block the estrogen receptor (+E_2) there is a loss of fluorescence at ~570 nm representing loss of binding to ER and a rise in fluorescence at 450 nm. Displacing 2 nM ketone from the receptor frees it to bind to non-receptor proteins so the nonspecific fluorescence at ~450 nm actually increases when the ER is blocked. There is too much overlap in the spectrum to discern a change in the proportion of free ligand (565 nm) when the ER is blocked or unblocked.

The difference spectra for these two incubations gives a measure of the ketone bound to ER. Using the appropriate standard curves to convert fluorescence intensity to nanomolar concentration, we could construct a binding curve (Figure 7A). No attempt was made to quantitate the total and nonspecific binding in this equilibrium binding assay.

The same incubations were then treated with charcoal–dextran to remove the free ligand and most of the nonspecific binding (Figures 6B and 7B). The fluorescence from the nonspecific binding (~450 nm) drops dramatically while that from the specific remains relatively unchanged. Despite precautions (rapid measurement at 4 °C), after charcoal treatment, some dissociation of the specifically bound ligand was already occurring as the system attempted to reestablish an equilibrium state. The wavelength of the fluorescence of the specifically bound ketone chrysene after charcoal treatment (difference spectra of T and + E_2 in Figure 6B) has shifted from ~570 to ~550 nm. Using several concentrations of ligand and the appropriate standard curves to convert intensity to concentration, we could construct a binding curve (Figure 7B).

The same samples were then extracted with ethyl acetate, and the extracts were evaporated under a stream of nitrogen

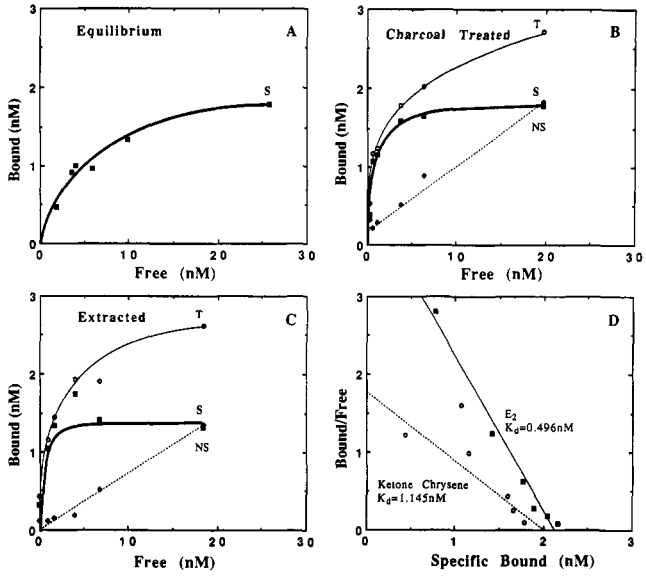


FIGURE 7: Binding curves of ketone chrysene **3a** under equilibrium conditions (panel A), after charcoal treatment (panel B), and after extraction (panel C). The data from the specific binding curve shown in panel B are presented as a Scatchard plot in panel D along with those of ^{3}H-E_2. ER expressed in yeast was incubated with several concentrations of ligand in the absence (total, T) or presence (nonspecific, NS) of an excess of estradiol at 0 °C for 3 h. The concentration of bound ligand was determined by fluorometry. The specific binding to ER (S) is the difference between total and NS. When the data are processed according to Scatchard (1949), the ketone chrysene binds to 100% of the sites bound by ^{3}H-E_2 with a K_d relative to E_2 of 43%.

and resuspended in ethanol. Now, in a uniform environment (ethanol), the fluorescence signal could easily be quantitated and plotted as a binding curve (Figure 7C). A comparable binding curve was generated using the same protein solution and ^{3}H-E_2 (data not shown).

Scatchard transformation (Scatchard, 1949) of these data (Figure 7D) gave direct binding affinities (dissociation constant, K_d) as well as the number of binding sites for both ^{3}H-E_2 and the ketone **3a**. For clarity, only the data points from the charcoal-treated samples are shown. The data from the extracted samples result in essentially the same line. The straight line of the Scatchard plot shows that the ligands are binding to only one site. Comparison of the K_d of the ketone **3a**, measured by fluorescence, with that of estradiol shows its affinity to be 43%. This agrees very well with the value of 40% ± 9% determined in the indirect RBA assay.

Similar experiments with the other donor–acceptor THCs showed high-affinity binding (K_d 2–6 nM) and full saturation of ER sites (91–102% of the sites measured by ^{3}H-E_2). Comparison of these affinities with that of estradiol, measured in the same protein preparation, gives a relative affinity from these direct measurements. The K_d of estradiol in these experiments was 0.4–0.7 nM. Although the fluorescence of the THCs varied depending on the environment, quantitative measurements could be obtained by using the proper standards. The relative affinities obtained by the direct binding in the fluorometric assay and the competitive binding of the radiometric assay are quite comparable (Table IV).

The ester showed very high nonspecific binding in the binding experiments, so that ~30% of the total ligand was bound nonspecifically in ER preparations expressed in yeast and ~80% was bound nonspecifically in lamb cytosol preparations. The amide had the least nonspecific binding of the emissive THCs, 2% in yeast and ~40% in lamb cytosol. The nonspecific binding of estradiol is even lower.

Table IV: Relative Binding Affinities and Lipophilicity of Donor–Acceptor THCs **3a–d**

cmpd no. (X = substituent)	relative binding affinities[a]		log $P_{o/w}$
	fluorometric	radiometric	
3a (-COMe)	43	40 ± 9[b]	5.2
3b (-COOMe)	57	24 ● 9	6.0
3c (-$CONH_2$)	20	15 ± 3	4.0
3d (-CN)	17	16 ± 6	5.4

[a] Relative to E_2 = 100%. [b] ± range, n = 2.

Nonspecific binding depends mostly on the lipophilicity of the molecule. We have found that nonspecific binding of steroids correlates well with their octanol/water partition coefficients (log $P_{o/w}$) (Katzenellenbogen et al., 1982), which can be estimated readily by a reversed-phase HPLC method (Minick et al., 1988; Pomper et al., 1990). The log $P_{o/w}$ values for the donor–acceptor THCs are given in Table IV. These show the amide to be the least lipophilic, the ester to be the most lipophilic, and the ketone and nitrile to be of intermediate lipophilicity. This correlates well with the binding of these ligands to both BSA and NS proteins, as measured in the fluorescence assays.

DISCUSSION

We have recently described the synthesis of a series of novel nonsteroidal estrogens, the tetrahydrochrysenes, in which a stilbene chromophore, commonly found in other nonsteroidal estrogen classes, is held to near planarity in a rigid tetracyclic system (Hwang et al., 1992). This rigid system can be adorned at internal positions with alkyl substituents that provide the steric bulk required for high-affinity binding to ER without being distorted from planarity. Thus, it retains the through-conjugation required for the development of efficient fluoresence. In the basic bisphenol system with trans-disposed ethyl groups at positions 5 and 11, one of the hydroxyl groups can be replaced by a series of electron acceptors to provide the donor–acceptor substitution pattern found in many fluorophores. Four of the five donor–acceptor THCs that we have prepared have intense, long-wavelength fluorescence emission that is quite sensitive to solvent polarity, and they also retain good affinity for the ER. Thus, they represent the first inherently fluorescent estrogens that have good affinity for ER and useful fluorescence. With these donor–acceptor THCs, we can demonstrate ER binding by fluorescence; their emission is sufficiently solvatochromic that the emission of receptor-bound fluorophore is red-shifted from the emission of nonspecifically bound material by ca. 100 nm. As a result, we can measure ER binding under equilibrium as well as nonequilibrium conditions (i.e., after adsorption of free and nonspecifically bound material by charcoal–dextran treatment). The ER binding capacity and affinity that we measure by fluorometric titration is comparable to that obtained by radiometric and competitive binding methods.

There have been many reports of the preparation and use of fluorescent estrogens in the assay of ER, both in solution and by fluorescence microscopy and flow cytometry (see introduction for references). Many of these are initial reports that have not been followed up substantially; they often do not provide adequate chemical characterization of the fluorescent species nor rigorous, well-controlled demonstrations of ER binding. We have chosen to be cautious in our development of fluorescent estrogens and have dealt only with rigorously characterized materials and with fluorescence assays that include appropriate controls for nonspecific binding and

background fluorescence. We also recognize the sensitivity problems that derive from the limited binding capacity of the ER preparations. Thus, in earlier reports we have described the preparation and use of 12-oxo-9(11)-dehydroestradiol (Katzenellenbogen et al., 1986; Martin et al., 1983), a compound with high affinity for ER but whose fluorescence is intense only in alkaline medium, thus making the direct assay of ER by this compound difficult. Similarly, we prepared some ligand–fluorophore conjugates with both estrogens (Martin et al., 1983; Fevig et al., 1987) and progestins (Carlson et al., 1989) but found that the fluorophore nitrobenzoxadiazole was quenched upon binding to the respective receptors. Therefore, we were gratified to find that the donor–acceptor THC systems described here afforded both high ER binding affinity and useful fluorescence properties, with which one can clearly assay ER by fluorescence.

It is the high solvatochromism of the four donor–acceptor THCs that permits the specific binding to ER to be measured under equilibrium conditions. Thus, comparison of fluorescence spectra of samples in the absence and presence of estradiol (for total and nonspecific determinations) shows an excess of emission from total vs nonspecific samples in the long-wavelength region (corresponding to the emisssion of ER-bound fluorphore), whereas the reverse is true in the shorter wavelength region (corresponding to fluorophore bound nonspecifically). Here, there is actually a greater emission from the $+E_2$ sample, since the fluorophore displaced by E_2 from ER becomes largely nonspecifically bound. A more definitive assay of specifically bound material is seen after excess free and loosely bound fluorophore is removed by adsorption onto charcoal–dextran. In fluorescence titration experiments, we obtain estimates of ER binding affinity and capacity that are similar to those determined radiometrically using binding and competition assays with ^3H-E_2.

One unexpected observation from these studies is that the donor–acceptor THCs, when bound to ER, emit at very long wavelengths. Their emission maxima, in fact, are similar to that of the free fluorophore in water. This suggests that the ligand binding environment of ER is quite unusual or is exceptionally well matched electronically to the THCs. It is known that the ligand binding site of ER has a preference for hydrophobic character in the internal region (the four-ringed carbocycle) but prefers polar hydroxyl groups at the periphery (3 and 17β hydroxyls in estradiol). Since the donor–acceptor THCs mimic this hydrophobic interior–polar periphery quite nicely, it may be that the ligand binding site in ER is able to afford exceptionally good stabilization of the excited state of these fluorophores and thus effect a very large Stokes shift. These issues will be interesting to investigate when more detailed structural information on the ligand binding site of ER becomes available.

We are currently extending our studies of these donor–acceptor THC fluorescent estrogens to their interaction with ER in cells, to direct measurements of the kinetics of ER ligand association and dissociation, and to estimates of the nature of the microenvironment of the ER binding site.

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