Flavonoids. 8.[1] Synthesis and Antifertility and Estrogen Receptor Binding Activities of Coumarins and Δ^3-Isoflavenes

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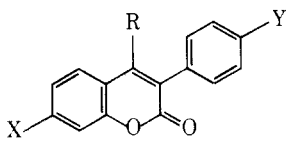
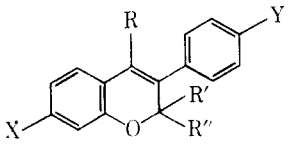
A series of coumarins and Δ^3-isoflavenes was prepared. Although antifertility activity was shown by all of these compounds, the required dosage in mice varied from 13.5 μg/kg/day to 50 mg/kg/day. The most potent compounds were the 2-methyl-4-ethylisoflavenes, two of which (**2a** and **2b**) were about equipotent with DES on a molar basis. They were followed by the 2,2-dimethylisoflavenes, the 2-unsubstituted isoflavene, and the coumarins. The most active compounds possessed an acetoxy group at C-7 and an oxygen function at C-4′. Presence of fluorine at C-4′ or diethylaminoethoxy at C-7 decreased the antifertility activity. The uterotropic activity followed the same trends as the antifertility activity with some evidence for the separation of the two effects in the 2,2-dimethylisoflavene series. Based on a limited study it appears that two phenolic hydroxyl groups are required for the presence of good estrogen receptor binding activity. An apparent lack of correlation between the estrogen binding activity and uterotropic or antifertility effects is probably explained by in vivo metabolism.

In spite of the great advances made in contraception over the past few years, there is still a need for new and better methods of fertility control. In recent years there has been a considerable interest in the development of nonsteroidal antifertility agents because as yet—with the exception of diethylstilbestrol (DES)—no agent of this type is available for human use.

A few years ago the synthesis of a variety of flavonoids, particularly 3-arylcoumarins (**1**) and Δ^3-isoflavenes (**2**), was reported from our laboratory.[1-4] The structural resemblance of these compounds to known antifertility agents possessing the stilbene moiety (e.g., DES, **3**,[5] **4**,[6] and **5**[7]) was obvious. Furthermore they exhibited biological activities (uterotropic[8,9] and antigonadotropic[9]) which are commonly associated with known antifertility compounds. It was therefore considered reasonable to screen compounds of this type for antifertility as well as estrogen receptor binding properties. This paper describes the results of such a study.





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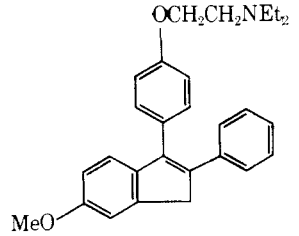
1a, R = Et; X = OH; Y = F
b, R = Et; X = OH; Y = OCH_3
c, R = Et; X = $O(CH_2)_2NEt_2$; Y = F



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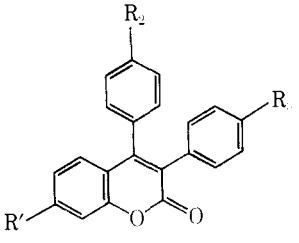

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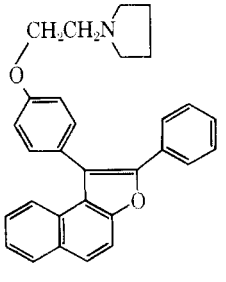

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Chemistry. The synthesis of new coumarins and isoflavenes as well as their derivatives was carried out by following published procedures. Thus the fluorocoumarin **1a** was prepared in three steps[2] starting from *p*-fluorophenylacetonitrile and was converted to the diethylaminoethyl (DEAE) ether **1c** by refluxing with $ClCH_2CH_2NEt_2 \cdot HCl$ and NaOEt in EtOH.[10] These and other, previously prepared, coumarins[2,4] were converted to the isoflavenes **2a–q**[1,2,4] shown in Tables I and II.

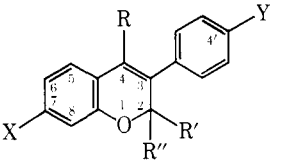
Biological Activity. Table I summarizes the antifertility activity. The antifertility assay used was a general one which should uncover antifertility activity from ovulation through implantation. It consisted of drug administration to female animals before, during, and after caging them with fertile males (see Experimental Section). The treatment regimen allowed observation of the number of fetuses and resorptions, occurrence of fetal malformation, and toxicological and behavioral effects.

Virtually 100% of the control animals (103/104) became pregnant under test conditions. All of the isoflavanoids tested exhibited antifertility activity, and none had any distinctive behavioral, toxicological, or mating effects within their antifertility dose range. The effective contraceptive range varied from 13.5 μg/kg/day for **2a** to greater than 50 mg/kg/day for **2j** and **2k**. Compound **2a** prevented pregnancy at a dose equivalent to DES on a molar basis and was somewhat more potent than ethynylestradiol and mestranol.

As the data in Table I show, the 2-monomethylisoflavenes are much more potent than the corresponding 2,2-dimethylisoflavenes (compare **2a** with **2f** and **2c** with **2h**). The isoflavene without a 2-substituent (**2e**) is intermediate in activity and the coumarins **1b** and **1c** (2-oxo) have low activity. Substitution of a methyl group for the ethyl group at C-4 slightly decreases activity (compare **2b** with **2c**, and **2f** with **2h**). Substitution of methoxy for the acetoxy moiety at C-4′ has little effect on activity (compare **2a** and **2b**); but antifertility activity is decreased by a factor of 740 by the substitution of fluorine at C-4′ (compare **2g** with **2a**). In view of the frequent occurrence of the diethylaminoethyl moiety in stilbene analogs with antifertility activity, it is of interest that diethylaminoethoxy substitution at C-7 markedly reduces activity (compare **2b** with **2d**).

The uterotropic dose given in Table I was obtained by graphic determination of the dose required to yield a uterine weight of 25 g (ca. twice that of controls). Dose–response curves for the most active compounds (for example, **2a** and **2c**) were essentially parallel to that obtained for DES (see Figure 1), suggesting a "true estrogenic" effect. This was confirmed when equivalent results were obtained

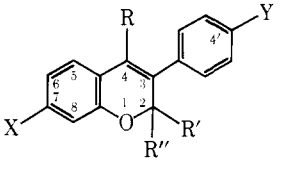
Table I. Antifertility Effects of Coumarins and Δ³-Isoflavenes



Compound	R	R′	R″	X	Y	Ref	Antifertility dose,[a] mg/kg/day	Uterotropic dose,[b] mg/kg/day
DES							0.0053[f]	0.0038
2a	Et	Me	H	OAc	OAc	1	0.0135	0.0052
2b	Et	Me	H	OAc	OMe	1	0.015[g]	0.0107
Mestranol							0.040[h]	0.0082
Ethynyl-estradiol							0.050	
2c	Me	Me	H	OAc	OMe	1	0.053	0.0211
2d	Et	Me	H	ODEAE	OMe	*c*	2.0[h]	0.11
2e	Et	H	H	OAc	OMe	12	2.0[h,i]	>2.0
2f	Et	Me	Me	OAc	OAc	2	3.0[i]	15
2g	Et	Me	H	OAc	F	*c*	10[h]	2.9
2h	Me	Me	Me	OAc	OMe	2	10	13
2i	Et	Me	Me	ODEAE	F	*c*	50[h]	25
1b[d]	Et			OH	OMe	2	50[h]	35
2j	Et	Me	H	ODEAE	F	*c*	>50[j]	>50
1c[d]	Et			ODEAE	F	2	>50	>50
2k[e]	Et	Me	Me	OH	OMe	4	>50[k]	>50
2l	Et	Me	Me	OH	F	*c*		

[a]Dose at which fertility was completely inhibited in eight out of eight animals. Unless otherwise noted there were no fetuses and no resorption sites. Dose based on average initial body weight of 25 g (range 22–27). Dose interval two times unless noted. [b]Dose required to obtain a 25-mg uterine weight (ca. two times controls). (Dose based on idealized weight of 12.5 g/mouse. To convert mg/kg/day to total dose, multiply by 0.0375.) [c]See Experimental Section. [d]R′, R″ = H. [e]*N*-Pyrrolidinomethyl function at C-8. [f]One animal had four resorption sites. [g]Dose interval three times. [h]Dose interval five times. [i]One animal had two resorption sites. [j]Fifty percent of animals had no fetuses or resorption sites on 50 mg/kg/day. [k]Three out of eight animals had no fetuses or resorption sites on 50 mg/kg/day.

Table II. Estrogen Receptor Binding Activity of Δ³-Isoflavenes



Compound	R	R′	R″	X	Y	Estrogen receptor binding (rel act.[a])
DES						100 ± 9.8
2m (**2a**)[b]	Et	Me	H	OH	OH	37 ± 7.5
2n (**2b**)	Et	Me	H	OH	OMe	4 ± 2.3
2o (**2c**)	Me	Me	H	OH	OMe	0.7 ± 0.4
2d	Et	Me	H	ODEAE	OMe	≤0.03
2p (**2f**)	Et	Me	Me	OH	OH	6 ± 3.1
2q (**2g**)	Et	Me	H	OH	F	1.5 ± 1.0

[a]Estradiol = 100. Average of three separate determinations; values reported as mean ± SD. [b]Compounds in parentheses refer to the corresponding acetates reported in Table I.

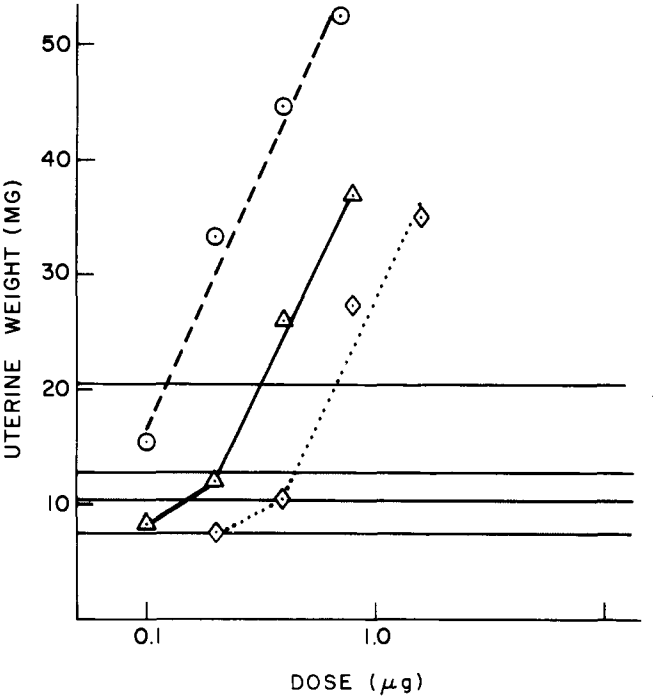


Figure 1. Comparison of uterotropic effects: ⊙ – – ⊙, DES; ▲—▲, **2a**; ◆·····◆, **2c**. The mean and standard deviations for 75 control mice are shown by the line and shaded area.

using the mouse vaginal cornification tests.[11] Results in the uterotropic assay for **2a** and **2e** were close to those reported earlier for these compounds.[8]

Examination of Table I (in which compounds are listed in order of decreasing antifertility activity) indicates that the antifertility activity is very closely correlated with the uterotropic activity for most of the compounds. This was confirmed by the correlation coefficient (r = 0.954) obtained when the regression of logarithm of antifertility dose upon the logarithm of uterotropic dose was examined. The regression equation was ln (antifertility dose) = 0.89 ln

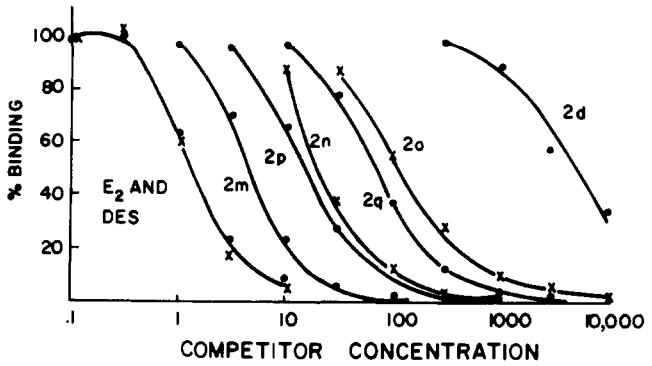


Figure 2. Inhibition of binding of tritiated estradiol to cytosol receptor from immature female rats.

(uterotropic dose) + 0.499. Of the compounds examined only the 2,2-dimethyl analogs **2f** and **2h** (and possibly the 2-unsubstituted analog **2e**) had antifertility doses lower than their uterotropic dose. This suggests that further study in this area should focus on the 2-substituents.

The strong estrogenic activity indicated that the isoflavenes might be acting at least partially as postcoital agents. Therefore, DES and isoflavene **2a** were compared in a postcoital assay. When the compounds were administered daily for five consecutive days after mating, DES prevented pregnancy at 10.6 μg/kg/day, whereas with **2a** two out of the eight mice had become pregnant at 42 μg/kg/day. In a preliminary study on a divided dose of 21 μg/kg of **2a** given twice daily for 5 days, the compound prevented pregnancy in eight out of eight mice. Thus compounds **2a** does exhibit postcoital activity.

Estrogen Receptor Binding. In the in vivo studies, all of the phenolic compounds were administered as the more stable *O*-acetyl derivative. Unpublished work in this laboratory has shown that these acetyl groups are readily cleaved metabolically under in vitro conditions. It is logical to assume that such a situation would obtain in vivo. Thus in vivo tests of compounds **2a–c**, **2f**, and **2g** are measuring the biological effects of the parent phenols **2m–q**. In view of this, studies of binding affinity were carried out using the latter substances. In the following discussion comparisons between the in vivo and in vitro results are made by reference to both the acetates and the parent phenols.

Displacement curves illustrating binding of the isoflavenes to the estrogen receptor from rat uterine cytosol are shown in Figure 2. Although all of the curves essentially parallel those of DES and estradiol, there is a marked variation in relative binding activity. The most potent uterotropic and antifertility compound **2a** (**2m**) also had the greatest relative binding activity (37 compared to estradiol = 100). However, there was not, in general, a direct correlation between estrogen receptor binding activity and uterotropic or antifertility effects. Thus the 4′-methoxy analog of **2b** (**2n**), which was half as effective in the uterotropic assay as **2a** (**2m**) and almost equipotent as an antifertility compound, was bound to the estrogen receptor only one-tenth as strongly as **2a** (**2m**). Likewise **2c** (**2o**) was one-fourth as potent in the uterotropic assay but bound with $\frac{1}{50}$th of the affinity of **2a** (**2m**). In contrast, **2f** (**2p**), which had roughly the same binding affinity as **2b** (**2n**), was <0.005 times as potent an antifertility agent as the latter compound and <0.0007 times as potent in the uterotropic assay.

This apparent discrepancy between relative binding affinity and uterotropic activity is probably explained by in vivo metabolism. The structure of compound **2m** may be optimum for the series tested, providing the best fit with the estradiol receptor site. If interaction with the receptor site is directly related to the uterotropic and antifertility activity, then compound **2a** (→**2m**) should also show the greatest activity in these tests—as is indeed the case. Compound **2n** (**2b**) is not a good binding competitor (in vitro) but could be readily metabolized to the corresponding dihydroxy compound in vivo. Such a conversion would explain its in vivo activity as well as that of methyl ether **2c** and diethylaminoethyl ether **2d**. The requirement of two aromatic phenolic groups to obtain good binding is further illustrated by the relatively low affinity of the 4′-fluoro compound **2q**.

The 2,2-dimethyl analog **2f** (**2p**) cannot be readily converted metabolically to **2m** and thus it is possible that its biological activity more closely reflects the binding data. In this case a simple sixfold reduction in binding affinity appears to produce a 200-fold decrease in antifertility activity and a 2800-fold increase in uterotropic effect. This suggests that some separation of activities is occurring and implies that further study of **2f** may be warranted. It also suggests there may be some threshold limit of binding affinity which must be exceeded to result in a very potent uterotropic compound.

Experimental Section

Chemical Syntheses. Melting points (Kofler hot-stage microscope) and boiling points are uncorrected. Infrared spectra were measured with a Perkin-Elmer 220 spectrophotometer. Ultraviolet spectra were run in methanol on a Cary Model 14 recording spectrophotometer. NMR spectra were recorded on a Varian Model A-100, using tetramethylsilane (Me_4Si) as an internal standard. Chemical shifts are expressed in δ units. GC analyses were carried out using a Varian Model 1400 with column containing 3% SE-30 on Variport. Mass spectra were recorded with an Associated Electrical Industries MS-902 instrument. Where analyses are indicated by mass spectra, the homogeneity of these compounds was established by TLC, GC, and other spectral properties. Microanalyses were carried out by Micro-Tech Laboratories, Skokie, Ill. Analytical results were within ±0.4% of the theoretical values. Each compound had ir, uv, and NMR spectra compatible with its structures.

α-(*p*-Fluorophenyl)-β-ketovaleronitrile. This compound was prepared from *p*-fluorophenylacetonitrile by the reported procedure:[2] bp 115–120° (0.005 mm) (65%). Anal. ($C_{11}H_{10}FNO$) C, H.

Ethyl α-(*p*-Fluorophenyl)-β-ketovalerate. This compound was prepared from the above β-ketonitrile by the reported procedure:[2] bp 95–97° (0.025 mm) (72%). Anal. ($C_{13}H_{15}FO_3$) C, H.

3-(4′-Fluorophenyl)-4-ethyl-7-hydroxycoumarin (1a). This coumarin was prepared by reacting the above β-keto ester with resorcinol in anhydrous HF[2] (64%): mp 257–260° (MeOH). Anal. ($C_{17}H_{12}FO_3$) C, H.

2,2-Dimethyl-4-ethyl-4′-fluoro-7-hydroxy-Δ³-isoflavene (2l). Following a literature procedure,[2] the isoflavene **2l** was prepared from the coumarin **1a** and CH_3MgBr (61%): mp 128–133° (Et_2O–hexane). Anal. ($C_{19}H_{19}FO_2$) C, H.

2-Methyl-4-ethyl-4′-fluoro-7-hydroxy-Δ³-isoflavene (2q). Following a literature procedure,[1] the isoflavene **2q** was prepared from the coumarin **1a** by a successive treatment of the latter with $(Me_3Si)_2NH$ followed by $(i\text{-}Bu)_2AlH$ and finally with excess CH_3MgBr. The crude isoflavene **2q** (85%) was used without purification for the preparation of **2g** and **2j** (vide infra).

2-Methyl-4-ethyl-4′-fluoro-7-acetoxy-Δ³-isoflavene (2g). A solution of the crude isoflavene **2q** (2.27 g) in Ac_2O (20 ml) and pyridine (2 ml) was allowed to stand at room temperature for 0.5 hr. The greenish yellow foam obtained by the removal of Ac_2O and pyridine under vacuum was purified by column chromatography on silica gel (150 g) using PhH–$CHCl_3$ (1:1) and then by preparative TLC [silica gel, PhH–$CHCl_3$ (1:1)] (1.13 g, 29% from **1a**): mp 99–101° (hexane). Anal. ($C_{20}H_{19}FO_3$) C, H.

3-(4′-Fluorophenyl)-4-ethyl-7-[2-(*N,N*-diethylamino)ethoxy]coumarin (1c). To a cooled (5°) solution of **1a** (2 g, 7 mmol) and NaOEt (from 322 mg of Na) in EtOH (115 ml) was added under stirring a solution of $ClCH_2CH_2NEt_2 \cdot HCl$ (1.2 g) in EtOH (25 ml). The mixture was stirred at reflux for 18 hr. The EtOH solution was evaporated under vacuum, treated with H_2O (100 ml), and extracted with $CHCl_3$ (80 ml). The $CHCl_3$ layer was shaken with brine and dried (Na_2SO_4) and the solvent was re-

moved under vacuum. The product was purified by a combination of preparative TLC [silica gel, $CHCl_3$–Me_2CO–MeOH (75:20:5)] and bulb-to-bulb distillation at 207° (0.003 mm) (1.8 g, 69%). Anal. ($C_{23}H_{26}FNO_3$) C, H.

Preparation of DEAE Ethers 2j, 2i, and 2d. These basic ethers were obtained from the corresponding phenols **2q, 2l,** and the acetate **2b** (saponified to the phenol) in exactly the same manner as **1c**. The compound **2j** was purified via its hydrochloride; **2i** and **2d** were purified by preparative TLC [silica gel, $CHCl_3$–Me_2CO–MeOH (80:18:2)] followed by bulb-to-bulb distillation. **2j**·HCl had mp 183–187° (Et_2O–hexane). Anal. ($C_{25}H_{33}FClNO_2$) C, H, Cl. **2j** was yellow oil (71%). Anal. ($C_{25}H_{32}FNO_2$) *m/e* 397.2424. **2i** had bp 160° (0.05 mm) (50%). Anal. ($C_{24}H_{30}FNO_2$) C, H. **2d** had bp 140° (0.15 mm) (47%). Anal. ($C_{25}H_{32}NO_3$) C, H, N.

Preparation of Phenols 2m–q. These phenols were prepared in quantitative yield, from the corresponding acetates **2a–c, 2f,** and **2g** by stirring with excess anhydrous K_2CO_3 in MeOH at room temperature for 18 hr. TLC, GC (Me_4Si ether), and mass spectra indicated these to be essentially pure compounds. Attempts to crystallize **2m, 2o,** and **2q** were abandoned owing to their instability. **2m** was an oil (100% pure, GC). Anal. ($C_{18}H_{18}O_3$) *m/e* 282.1257. **2n** had mp 115–118° (hexane). Anal. ($C_{19}H_{20}O_3$) *m/e* 296.1407. **2o** was an oil (100% pure, GC). Anal. ($C_{18}H_{18}O_3$) *m/e* 282.1257. **2p** had mp 103–107° ($CHCl_3$). Anal. ($C_{19}H_{20}O_3$) *m/e* 296.1410. **2q** was an oil (97% pure, GC). Anal. ($C_{18}H_{17}FO_2$) *m/e* 284.1214.

Biological Procedures. Antifertility Assay. Adult Carworth Farms Swiss–Webster mice were used as the experimental animal. The test compounds, dissolved or suspended in sesame oil, were administered orally at a volume of 0.2 ml per mouse per day for 22 consecutive days to randomized 8-week-old female mice (eight animals/dose). Males of proven fertility were caged with the females (one male/four females) from treatment days 7–19. During this time females were checked for vaginal plugs. In addition, body weights were measured at weekly intervals. The mice were sacrificed on day 26 and the uterine horns examined for the number of fetuses, number of resorption sites, and obvious malformations in the fetuses.

Uterotropic Assay. This assay for estrogenic activity was a slight modification of the original method of Rubin et al.[13] Immature (21-day-old) CF-W female mice were randomized into groups of five mice each. They were treated daily for three consecutive days with 0.1 ml of the sesame oil suspensions (oral route). On the day following treatment, they were sacrificed, and the blotted, lightly compressed uteri were weighed to the nearest 0.1 mg. Ethynylestradiol was used as a standard stimulator for comparison purposes. In addition to the uterine weight, body weights were determined.

Estradiol (E_2) Receptor Binding. Receptor Source. Uteri from immature (22-day-old) female rats (Charles River Labs, Wilmington, Mass.) were homogenized in 8 vol (v/w) of ice-cold TETG buffer (0.05 *M* Tris–HCl), 1 m*M* EDTA, and 0.01 *M* thioglycerol (pH 7.4) using a Polytrol Pt-10 homogenizer (Brinkman Instruments) with 2–3 5-sec bursts to protect against increasing the temperature of the homogenate. The homogenate was centrifuged at 120–140,000g for 1 hr using a 50 Ti rotor in a 12-65B ultracentrifuge (Beckman). The cytosol (supernatant) was removed for testing, and the binding was characterized in a 90-min uptake assay.

Preparation of Test Solutions. The test compounds were dissolved in ethanol, and concentrations were adjusted with TETG buffer to give a stock solution which would result in 10 μM concentration at final incubation volume. In all cases, the final concentration of ethanol in the assay was less than 2%. The competitor solutions were prepared within 1 day of analysis and fresh solutions were used for each test of a given competitor.

Competitive Binding Assay. The method of competitor analysis was based on that of Korenman.[14] The basic assay used a 0.5-ml incubation volume (0.1 ml containing radiolabeled estradiol, 0.1 ml of competitor, and 0.4 ml of cytosol) and was carried out at 4°. The competitor concentration was varied from 0.1 n*M* to 10 μM; radiolabeled E_2 (111 Ci/mmol) was held constant at 6000 cpm. A 40-hr incubation was started by the addition of cytosol to the competitor and radiolabeled E_2. At the end of the incubation the bound E_2-^3H was separated from the free by the addition of 0.5 ml of Dextran coated charcoal (0.5% Norit A with fines removed, 0.05% Dextran T 70). Assay tubes were vortexed and allowed to stand 10 min before centrifugation at 120g. The supernatant was decanted into scintillation vials containing 15 ml of scintillation media [toluene–Triton X-100–Omnifluor (2:1:0.018, v:v:w)]. Counting efficiency was approximately 30%.

Standard competition curves for unlabeled estradiol and DES were included in each assay using five concentrations from 0.1 to 10 n*M*. Five to eight concentrations of each competitor were tested. The molar concentrations of competitors that reduced [^{3}H]estradiol binding by 50% were determined graphically. The effectiveness of the competitor was established using the ratio: unlabeled estradiol concentrations for 50% competition/competitor concentration for 50% competition. This ratio was multiplied by 100 and termed the relative activity (RA).

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