

Structure–Activity Relationships for a Large Diverse Set of Natural, Synthetic, and Environmental Estrogens

Hong Fang,[†] Weida Tong,^{*,†} Leming M. Shi,^{†,‡} Robert Blair,[§] Roger Perkins,[†]
William Branham,[§] Bruce S. Hass,[§] Qian Xie,[†] Stacy L. Dial,[§]
Carrie L. Moland,[§] and Daniel M. Sheehan[§]

*R.O.W. Sciences, Inc., 3900 NCTR Road, MC 910, Jefferson, Arkansas 72079, and
Division of Genetic and Reproductive Toxicology, National Center for Toxicological Research,
Jefferson, Arkansas 72079*

Received October 3, 2000

Understanding structural requirements for a chemical to exhibit estrogen receptor (ER) binding has been important in various fields. This knowledge has been directly and indirectly applied to design drugs for human estrogen replacement therapy, and to identify estrogenic endocrine disruptors. This paper reports structure–activity relationships (SARs) based on a total of 230 chemicals, including both natural and xenoestrogens. Activities were generated using a validated ER competitive binding assay, which covers a 10^6 -fold range. This study is focused on identification of structural commonalities among diverse ER ligands. It provides an overall picture of how xenoestrogens structurally resemble endogenous 17β -estradiol (E_2) and the synthetic estrogen diethylstilbestrol (DES). On the basis of SAR analysis, five distinguishing criteria were found to be essential for xenoestrogen activity, using E_2 as a template: (1) H-bonding ability of the phenolic ring mimicking the 3-OH, (2) H-bond donor mimicking the 17β -OH and O–O distance between 3- and 17β -OH, (3) precise steric hydrophobic centers mimicking steric 7α - and 11β -substituents, (4) hydrophobicity, and (5) a ring structure. The 3-position H-bonding ability of phenols is a significant requirement for ER binding. This contributes as both a H-bond donor and acceptor, although predominantly as a donor. However, the 17β -OH contributes as a H-bond donor only. The precise space (the size and orientation) of steric hydrophobic bulk groups is as important as a 17β -OH. Where a direct comparison can be made, strong estrogens tend to be more hydrophobic. A rigid ring structure favors ER binding. The knowledge derived from this study is rationalized into a set of hierarchical rules that will be useful in guidance for identification of potential estrogens.

Introduction

There is a growing body of evidence that some man-made chemicals, now called endocrine-disrupting chemicals (EDCs),¹ have the potential to disrupt the endocrine system by mimicking endogenous hormones such as estrogens and androgens (1). Recent legislation mandates that the Environmental Protection Agency develop a screening and testing program for potential EDCs, of which xenoestrogens figure predominately (2). Xenoestrogens contain a number of chemical classes that display a broad range of structural diversity (3). For example, DES, DDTs, polychlorinated biphenyls (PCBs), alkylphenols, phthalates, and parabens have been found to be estrogenic. It has long been an enigma why such struc-

turally diverse chemicals have estrogenic activity like the endogenous hormone estradiol.

SARs for estrogens date back more than six decades to the early work of Dodds et al. (4, 5). The succeeding two decades saw the discovery of nonsteroidal estrogens, such as DES, based on understanding of the important structural features governing potency for steroidal estrogens. Recently, a number of SAR studies have been reported for steroidal estrogens (6) and nonsteroidal estrogens (7). These are generally focused on identification of structural characteristics for chemicals within similar two-dimensional (2D) structural frameworks, including E_2 derivatives (6), DES derivatives (8), PCBs (9), phytoestrogens (10), alkylphenols (11), raloxifenes (12), and others. Modern computer-based tools have enabled the development of quantitative structure–activity relationship (QSAR) models for identifying steric and electrostatic features of a molecule in three-dimensional (3D) space for estrogenic activity (8, 13–19). Recent crystallographic structures of the human ER α subtype (hER α) with a number of ligands, including E_2 , DES, raloxifene, and 4-OH-tamoxifene, have also been reported (20, 21). By aligning these four ligands on the basis of the superposition of their ER binding sites, we have been able to demonstrate the common binding characteristics among these ligands (22).

* To whom correspondence should be addressed: R.O.W. Sciences, Inc., 3900 NCTR Road, MC 910, Jefferson, AR 72079. Telephone: (870) 543-7142. Fax: (870) 543-7382. E-mail: wtong@nctr.fda.gov.

[†] R.O.W. Sciences, Inc.

[‡] Current address: BASF Corp., P.O. Box 400, Princeton, NJ 08543.

[§] National Center for Toxicological Research.

¹ Abbreviations: SAR, structure–activity relationship; QSAR, quantitative structure–activity relationship; ER, estrogen receptor; hER α , human estrogen receptor α subtype; EDCs, endocrine disrupting chemicals; RBA, relative binding affinity; NA, not active; NCTR, National Center for Toxicological Research; 2D, two-dimensional; 3D, three-dimensional; E_2 , 17β -estradiol; E_1 , estrone; E_3 , estriol; EE, ethynylestradiol; DES, diethylstilbestrol; DMS, dimethylstilbestrol; DDTs, 1,1,1-trichloro-2,2'-bischlorophenylethane derivatives; PCBs, polychlorinated biphenyls; log *P*, hydrophobicity.

In principle, chemicals with similar biological activity share common structural features. This implies that structurally diverse estrogens possess a certain degree of structural commonality essential to eliciting estrogenic activity. Although a number of chemical classes are known to be estrogenic, little thorough structural evaluation has been presented for their structural resemblance to the E₂ or the strong synthetic estrogen DES. Moreover, only limited efforts have explored the structural similarities between different chemical classes of xenoestrogens. To better understand the structural requirements for ER binding, it is important to have a reliable data set, obtained with consistent assay design, covering a broad range of chemical classes. Recently, we reported ER binding activity data for a large number of chemicals, including natural, synthetic, and environmental estrogens, using a validated rat ER competitive binding assay (23, 24). This data set, called the NCTR data set, currently contains 230 chemicals. It was designed a priori to cover broad structural diversity and a wide range of binding activities for elucidating the structural characteristics of xenoestrogens and natural estrogens.

The rat uterine cytosol ER competitive binding assay is the gold standard for in vitro ER assays. When our results are compared to results from other ER binding assays, there is a general consistency between relative ER activities across different assay methods and species (25). For example, we found a high linear correlation for ER binding affinities among a diverse group of chemicals assayed with the ER from rat uterine cytosol and hER α .² Further, we also found that ER assay results correlated very well with those from a yeast-based reporter gene assay and a MCF-7 cell proliferation assay. These findings demonstrate that ER binding is the major determinant across three levels of biological complexity (receptor binding, a yeast reporter gene response, and cell proliferation) of estrogen action. Moreover, chemicals positive in uterotrophic responses (in vivo estrogenic activity) are also positive in the ER binding assay, indicating that binding affinity is a good predictor of in vivo activity with few false negatives observed (26). Therefore, understanding the structural requirements for ER binding provides strong guidance in identifying potential in vivo estrogenic EDCs.

The NCTR data set covers most known estrogenic chemical classes as well as some new estrogenic chemicals. A careful SAR examination of the NCTR data set in conjunction with knowledge of the recently reported ligand-ER crystal structures should allow a better understanding of the general structural requirements of a chemical binding to the ER in a quantitative manner. In turn, the knowledge can be used to develop a predictive toxicology model for rapidly identifying potential estrogenic EDCs.

Materials and Methods

ER Binding Activities. The ER binding affinities of chemicals were determined by using a competitive receptor binding assay described previously (23). Briefly, a chemical's binding activity was determined by competing with radiolabeled [³H]-E₂ for the ER in rat uterine cytosol. The IC₅₀ (50% inhibition of [³H]E₂ binding) for each competitor was determined. The relative binding affinity (RBA) for each competitor was calculated by dividing the IC₅₀ of E₂ by the IC₅₀ of the competitor

and multiplying by 100 (E₂ RBA = 100). The validated assay incubation conditions were 20 h at 4 °C using 17 mg of uterine tissue/mL (B_{max} = 0.22 nM) with 1 nM [³H]E₂. The competing chemical concentrations ranged from 1 nM to 1 mM. Chemicals that failed to compete for [³H]E₂ binding to the ER were designated as "not active" (NA). Chemicals that exhibited binding, but did not reach 50% inhibition in the designed concentration range, were designated as "slight binders". All assays were repeated at least twice; the IC₅₀ values of positive chemicals are the means of the replicate values. The standard deviation of IC₅₀ for each chemical was reported (23), and only the mean RBA value was used for this study. The purity of chemicals as well as their effect on RBAs was also studied by Blair et al. (23). The largest fold difference (~10-fold) was found for nonylphenol from different commercial sources due to the impurity of the sample.

Molecular Modeling. The crystal structures of E₂, DES, raloxifene, and 4-OH-tamoxifen bound to the ER were obtained from the Protein Data Bank (PDB) as entries 1A52, 3ERD, 1ERR, and 3ERT, respectively. The alignment of these four ligands, based on root-mean-square (RMS) fitting of their receptor coordinates, was performed using the InsightII software package (Molecular Simulations, Inc., San Diego, CA). Log *P* was calculated using the atom/fragment contribution method (27). Pharmacophore searching was performed with the CATALYST package (Molecular Simulations, Inc.). The energy differences for E₂ and DES between the conformation in their binding modes and that in the minimum conformation of the free ligands were calculated using the AM1 model Hamiltonian of the AMPAC/MOPAC module in InsightII (Molecular Simulations, Inc.). The atom-atom distance was also measured using InsightII (Molecular Simulations, Inc.).

Results

We determined the ER RBA of 230 chemicals, of which 130 were active and 100 inactive. To the best of our knowledge, this is the largest published ER competitive binding data set. This NCTR data set has been extensively used to build and validate a series of computational models proposed for priority setting of potential estrogenic EDCs (22).

For the convenience of analysis and presentation, the NCTR data set is divided into seven major categories according to the chemical structural characteristics. The chemical names and structures, as well as RBA values, are shown in Figures 1-7 for 130 active chemicals and a few of the selected inactive chemicals. Steroids are chemicals with a steroidal backbone. E₂ derivatives have a phenolic A ring (Figure 1A), whereas the others lack a phenolic A ring (Figure 1B). The common structural feature of DES-like chemicals is two benzene rings separated by two carbons that are connected by a double bond in DES derivatives (Figure 2A) and by a single bond in hexestrol derivatives (Figure 2B). In this group of chemicals, triphenylethylene derivatives (Figure 2C) are structurally similar to DES derivatives, but have an additional phenyl group attached to the ethylene bridge group. Most synthetic antiestrogens contain this structural feature. Phytoestrogens contain four major structurally distinct chemical classes: flavonoids, coumestans, chalconoids, and mycoestrogens. Flavonoids are the largest class, containing flavones (Figure 3A), flavanones (Figure 3B), and isoflavones (Figure 3C). They share a benzene ring directly connected to a chromone or 4-chromanone. Coumestans (Figure 3D) contain the smallest number of chemicals, of which coumestrol is the most rigid and flat molecule of all the phytoestrogens used. In contrast, chalconoids (Figure 3E) are the most structurally flexible

² H. Fang, W. Tong, and D. Sheehan, unpublished results.

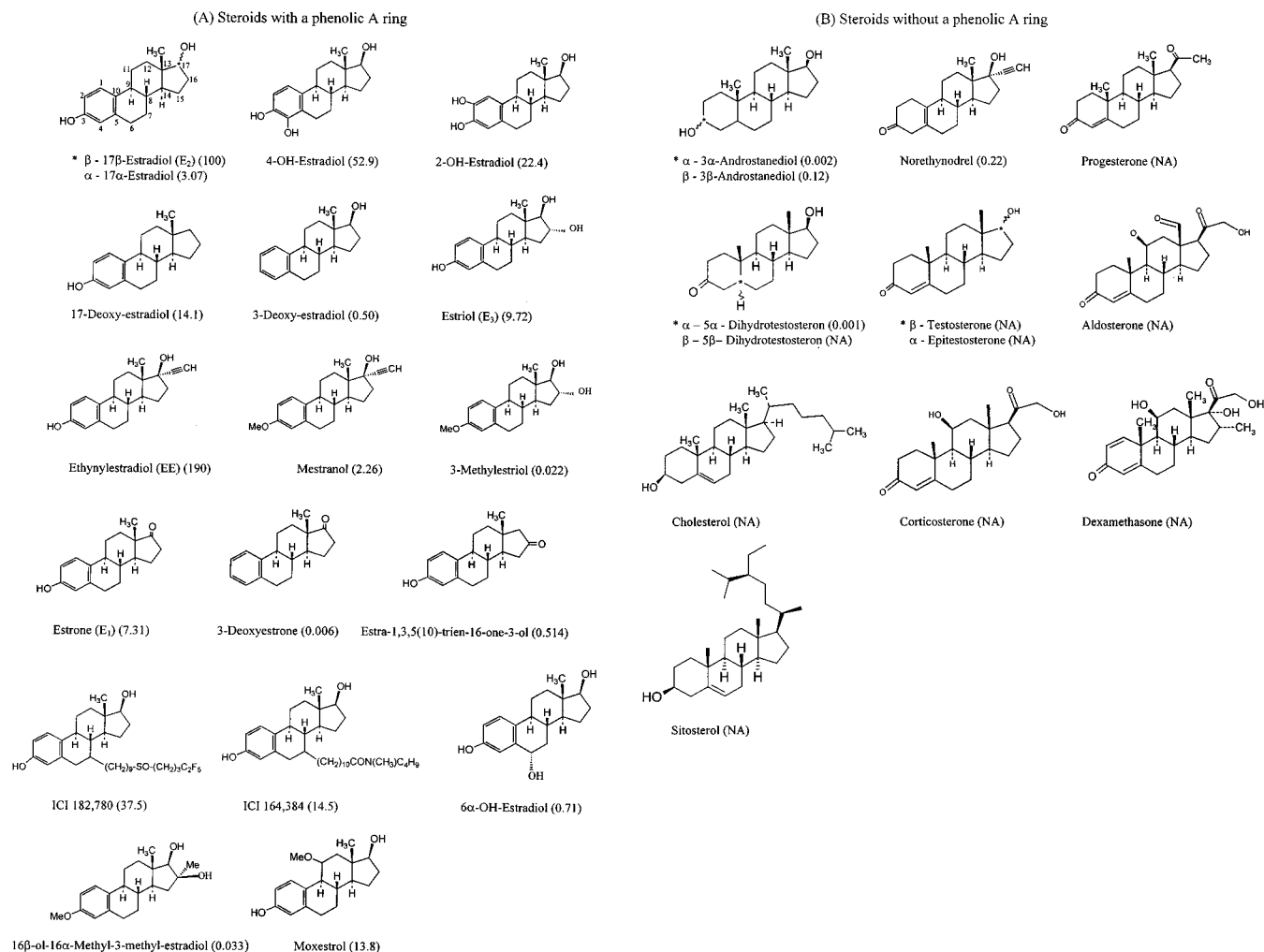


Figure 1. Steroids with (A) and without (B) a phenolic ring.

chemicals of the phytoestrogens. The generic structure of chalconoids is two benzene rings separated by three carbons. Mycoestrogens (Figure 3F) have a large ring fused with a benzene ring that contains two OH groups at positions 14 and 16. Chemicals with two benzene rings connected by one carbon atom are classified as diphenylmethanes, including diphenolalkanes (Figure 4A), benzophenones (Figure 4B), and DDTs (Figure 4C). Biphenyls are chemicals with two phenyl rings directly connected to each other. They can be chlorinated as PCBs (Figure 5A) or nonchlorinated (Figure 5B). Alkylphenols (Figure 6A), parabens (Figure 6B), and alkyloxyphenols (Figure 6C) are categorized as phenols. They all contain a single phenolic ring. Most chemicals in this group have a long alkyl chain substituted at the para position. The chemicals that do not belong to any of these groups are found in miscellaneous (Figure 7).

Chemicals that exhibit ER binding have a broad range of structural diversity and RBAs. A common structural feature for steroids, DES-like chemicals, and most phytoestrogens is the presence of two rings (one of them usually a phenolic ring) separated by two carbons. Chemicals either with two rings separated by one carbon atom (diphenylmethanes), connected directly (PCBs), or possessing only one ring (alkylphenols, phthalates, and kepone) typically have relatively lower binding affinities than chemicals with two rings separated by two atoms (25), although there is overlap in RBAs among some

chemical classes. Table 1 shows the mean RBA values for each chemical class. Steroids and DES-like chemicals have the strongest binding activities. The RBAs of the rest of the chemical classes follow this order: phytoestrogens > diphenylmethanes > biphenyls > phenols. There are only a few inactive chemicals in the DES-like chemical and phenol classes. The mean RBA of DES-like chemicals is 2.63, which is the highest of the groups that were examined, while phenols (mean RBA = 0.0015) has the lowest mean RBA. These observations suggest that although both classes are likely to bind to the ER, their binding specificity is different due to the nature of their structures.

Steroids. E_2 , with an RBA of 100, is one of the most active estrogens. It has a hydrophobic backbone with OH groups at each end of the molecule. Such a molecular configuration was considered as the most important structural features for ER binding (6). The recently reported crystal structure of the ER- E_2 complex reveals that the 3- and 17 β -OH groups primarily serve as H-bond donors and acceptors in interacting with the receptor binding site (20). The elimination or modification of either of these two OH groups significantly reduces a chemical's binding affinity for the receptor, as shown in Table 2. This impact is more dramatic at the 3-position than at the 17 β -position. For example, the loss of binding activity of 3-deoxy- E_2 is 28-fold greater than the loss for 17-deoxy- E_2 , indicating that the 3-OH is more important than the

Table 1. General Information for the ER Ligand Categories, Including the Number of Chemicals with the Ratio of Active and Inactive Ligands, Mean RBA Values,^a and Representative Chemicals with Key Structural Features^b

ER Ligand Categories	# of cmpds	Active / Inactive	Mean RBA ^a	Representative Chemicals (RBA)	Key Structural Features ^b				
					Phenolic Ring	17 β -OH	7 α or 11 β steric bulks	Additional ring ^c	logP
DES-LIKE CHEMICALS	20	19 / 1	2.63	DES (400) 	1	1	1	1	5.64
STEROIDS	31	23 / 8	2.45	E ₂ (100) 	1	1	0	1	3.94
PHYTOESTROGENS	46	34 / 12	0.069	Genistein (0.45) 	1	1	0	1	2.84
DIPHENYLMETHANES	29	18 / 11	0.01	Bisphenol A (0.008) 	1	0	0	1	3.64
BIPHENYLS	12	8 / 4	0.0042	2',3',4',5'-Tetrachloro-4-biphenylol (0.23) 	1	0	0	1	3.94
PHENOLS	25	23 / 2	0.0015	Nonylphenol (0.03) 	1	0	0	0	6.0

^a Mean RBA values are calculated for active ligands only. ^b The presence and absence of a structural feature are represented as 1 and 0, respectively. ^c A ring mimics one of the B, C, and D rings of E₂.

Table 2. Effects of Elimination and Modification of the 3- and 17 β -OH on RBAs

elimination or modification	from (RBA)	to (RBA)	fold difference
3-OH	E ₂ (100)	3-deoxy-E ₂ (0.5)	200
	E ₁ (7.31)	3-deoxy-E ₁ (0.006)	1218
	EE (190)	mestranol (2.26)	84
	E ₃ (9.72)	3-methylestriol	442
17 β -OH	E ₂ (100)	17-deoxy-E ₂ (14.1)	7
	E ₂ (100)	E ₁ (7.31)	14

17 β -OH in ER binding. This is consistent with the findings from the ER-E₂ crystal structure that the 3-OH has H-bonding interactions with Glu 353, Arg 394, and a water molecule, whereas 17 β -OH only forms one H-bond with His 524.

The elimination of 3-OH caused a greater reduction in the activity for estrone (E₁) than for E₂ (Table 2). A similar result was also observed for 3-OH methylation of estriol (E₃) and ethynylestradiol (EE). In both cases, the fold reduction in activity for stronger estrogens (E₂ and EE) was about 6 times lower than the fold reduction for relatively weak estrogens (E₁ and E₃). This indicates that the phenolic ring is more critical for weak estrogens than strong ones.

The lack of a phenolic ring is a likely explanation for why most other steroids such as androgens, progesterones, and cholesterol were inactive in ER binding, or exhibited very low activity, such as dihydrotestosterone (Figure 1B). Clearly, the precise distance (d_{O-O}) between the 3- and 17 β -OH groups of steroids, as well as their orientation, governs binding affinity. For examples, because the two OH groups had the same distance and orientation as those of E₂ (d_{O-O} = 11.0 Å), 3 β -androstane-diol (RBA = 0.12, d_{O-O} = 11.3 Å) was one of the most active steroids that does not contain a phenolic ring. Also, 17 α -E₂ (RBA = 3.07, d_{O-O} = 10.4 Å) has a RBA 33-fold lower than that of E₂, and the RBA of 3 α -androstane-diol

(RBA = 0.002, d_{O-O} = 9.95 Å) is 60-fold lower than that of its β -isomer.

The RBA of 17-deoxy-E₂ (RBA = 14.1) was slightly larger than that of estrone (RBA = 7.31), indicating that the 17-ketone group might not contribute significantly to the binding as a H-bond acceptor. If the ketone group of E₁ was moved from the 17-position to the 16-position to form estra-1,3,5(10)-trien-16-one-3-ol, the RBA was decreased 16-fold. A similar activity reduction was also observed with introduction of an OH group at the 16 α -position of E₂, resulting in a relatively weak estrogen, E₃. This demonstrates that a small polar group at the 16-position reduces a chemical's activity.

Systematic studies on the influences of substituents at various positions of E₂ revealed that for most positions the introduction of substituents results in a loss of binding affinity (17, 28). For example, the E₂ metabolites 2-OH-E₂ (RBA = 22.4) and 4-OH-E₂ (RBA = 52.9), 6 α -OH-E₂ (RBA = 0.71), and E₃ (RBA = 9.72) were less active than E₂ with 2–140-fold differences. The only positions where introduction of a substituent does not interfere with binding are 7 α , 11 β , and 17 α (θ). The degree of increase or decrease in activity is strongly dependent on the substituents in those positions. The 7 α - and 11 β -positions are structurally equivalent for steroids and can bear large substituents. Small steric substituents introduced at these positions generally increase activity (29). Large substituents reduce the RBA and give rise to antiestrogenic activity, such as ICI 182,780 (RBA = 37.5) and ICI 164,384 (RBA = 14.5). A 17 α -ethynyl substituent is favorable for ER binding. EE is a stronger estrogen (RBA = 190) than E₂ (RBA = 100). Norethynodrel (RBA = 0.22) is a progesterone derivative with a 17 α -ethynyl substitution, and is a popular oral contraceptive drug. It is a moderately strong binder (454-fold weaker than E₂).

DES-like Chemicals. DES (RBA = 400) is one of the highest-affinity synthetic estrogens. Its activity is 4 times

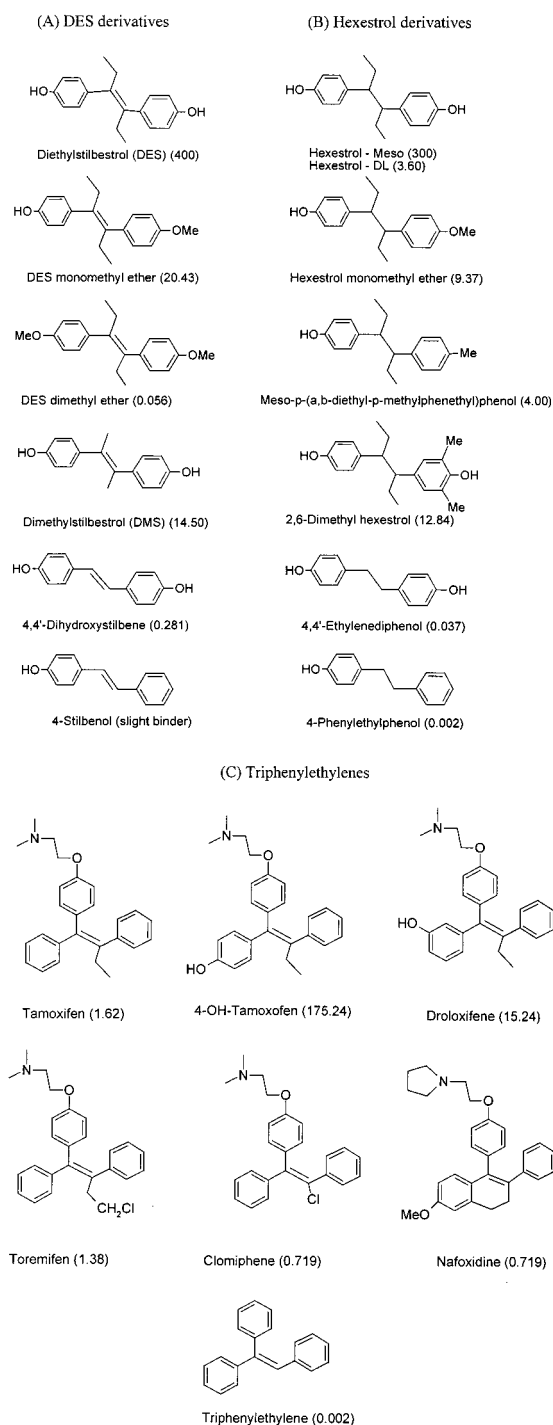


Figure 2. (A) DES, (B) hexestrol, and (C) triphenylethylene derivatives.

higher than that of E_2 . The symmetry of the molecule, the distance between the two OH groups, and the two ethyl side chains provide precisely correct spacing for hydrophobic and H-bond interactions (21).

To efficiently bind to a receptor, the ligand tends to adopt a conformation that generally is not the one with the lowest energy. The free ligand which is in a stable conformation adjusts to a less stable conformation to achieve optimal binding. Nevertheless, ligand binding to the ER will release energy in general. The energy difference of a molecule, between the conformation in its binding mode and that in the minimum conformation of the free ligand, contributes to the binding affinity in a

negative way. The less energy a molecule sacrifices during the binding, the stronger the binding affinity will be. E_2 has a much larger energy difference ($\Delta H_f = 40.5$ kcal/mol) than DES ($\Delta H_f = 17.8$ kcal/mol), which might help explain why DES is a better binder.

The O–O distance is 1 Å larger in DES ($d_{O-O} = 12.1$ Å) than in E_2 and functionally resembles the 3- and 17 β -OH's of E_2 . The loss of the H-bonding capability of one OH group reduces the RBA. For example, if one of the OH groups is converted to its methyl ether, DES monomethyl ether (RBA = 20.43), the RBA decreases ~20-fold (Figure 2A). A similar activity reduction was also observed for hexestrol (RBA = 300) (Figure 2B); replacing one of the OH groups with a methoxy group resulted in a 32-fold less active derivative, hexestrol monomethyl ether (RBA = 9.37). The reduction in activity is much more significant when both OH groups of DES are methylated; the RBA decreases more than 7000-fold for DES dimethyl ether (RBA = 0.056). This demonstrates that while the loss of one H-bond donor will reduce the binding affinity by ~20-fold, the loss of the second H-bond donors diminishes the activity by a further 350-fold.

4-Methyl-substituted hexestrol [meso-*p*-(α,β -diethyl-*p*-methylphenethyl)phenol, RBA = 4.00] and hexestrol monomethyl ether (RBA = 9.37) have comparable binding affinities. This is consistent with the observation that 17-deoxy- E_2 has an RBA close to that of E_1 . In both cases, the chemicals with or without a H-bond acceptor exhibit similar RBAs. It suggests that with a phenolic ring at one end, the contribution of the OH group at the other end is mainly through H-bond donor interaction with the receptor.

The degree of contributions to the binding activity of the two ethyl groups of DES is as significant as its OH groups. DES (RBA = 400), dimethylstilbestrol (DMS, RBA = 14.50), and 4,4'-dihydroxystilbene (RBA = 0.28) are in sequence one carbon atom shorter at the two ethylene side chains. The RBAs of these latter two chemicals were 28- and 1423-fold lower than that of DES. The ethyl groups may contribute to ER binding in three distinct ways. First, they increase the molecule's hydrophobicity. The hydrophobicity of DES ($\log P = 5.64$) is larger than that of E_2 ($\log P = 3.94$). The two ethyl groups contribute more than one-third of the hydrophobicity of the overall structure. Second, they maintain the rigid binding conformation of DES. The decrease in the length of the side chains introduces more flexibility for both phenolic rings. Third, probably the most important contribution of the ethyl groups to binding is to occupy the precise space that interacts with the receptor binding pocket site, which resembles the 11 β - and 7 α -substituents of E_2 , and therefore increase its RBA. It is evident that the attachment of small alkyl substituents to the 11 β -position can lead to a considerable increase in binding affinity for E_2 , such as 11 β -chloromethyl-, ethyl-, and vinyl- E_2 (28) with RBA values in the range of 120–230. On the basis of the recent crystal structure of the E_2 - and DES-ER complexes, both ligands could be aligned together by overlaying their receptor coordinates. By modifying the ethyl group to the 11 β -position of the E_2 binding conformation, we have been able to compare the relative position of the ethyl groups from 11 β -ethyl- E_2 with that of DES. As shown in Figure 8, the ethyl groups from both molecules are positioned extremely close to each other, and fit well in the hydrophobic binding pocket. This observation also suggests that the specific

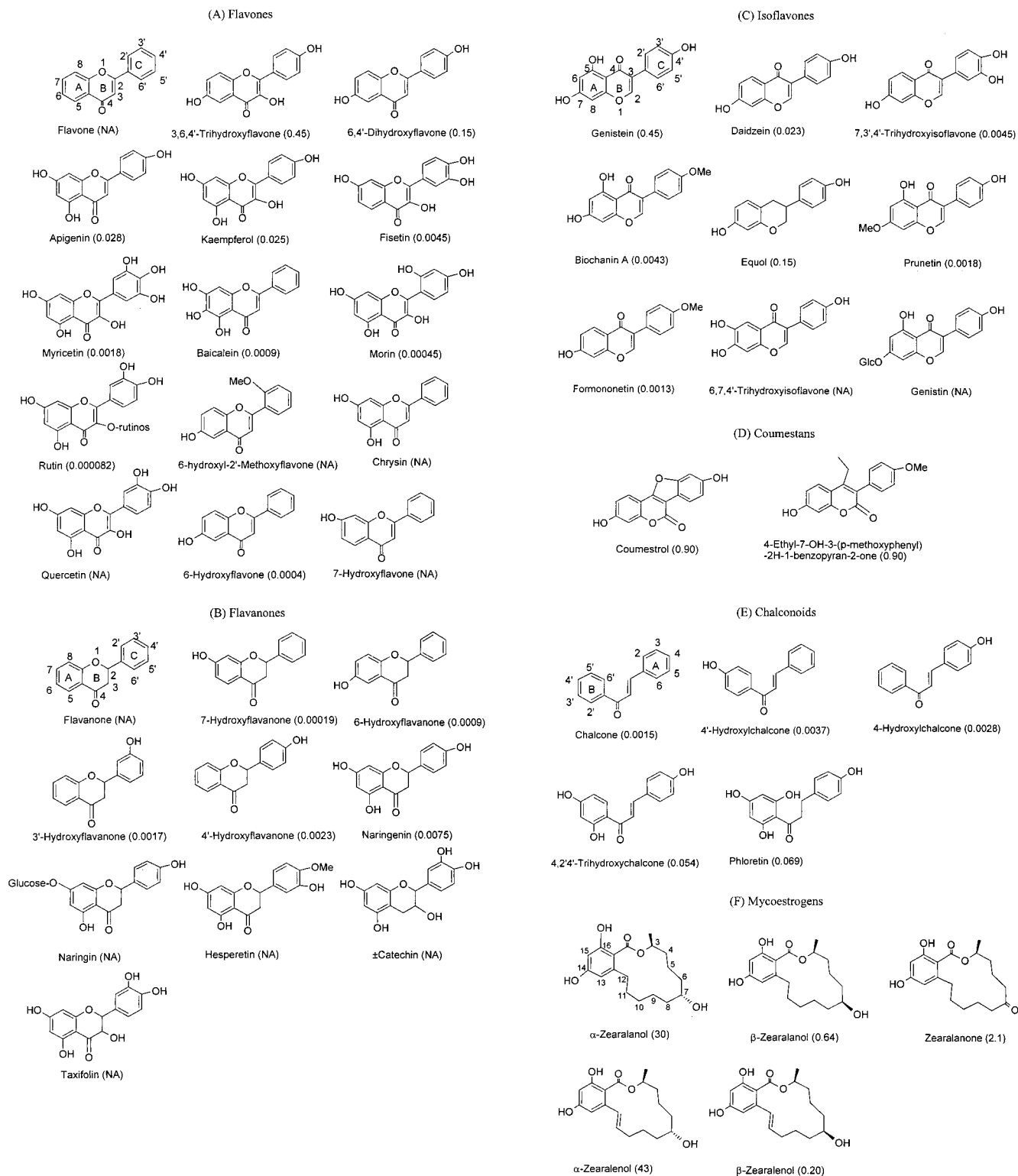


Figure 3. (A) Flavones, (B) flavanones, (C) isoflavones, (D) coumestans, (E) chalconoids, and (F) mycoestrogens.

orientation of the two ethyl groups of DES is critical to its function. That may explain why meso-hexestrol (RBA = 300) was 80-fold stronger than DL-hexestrol (RBA = 3.60).

In general, DES derivatives were stronger binders than hexestrol derivatives, because the former has a rigid double bond connecting the two benzene rings. This is evident from the RBA comparison of three pairs of chemicals between these two chemical classes: DES versus hexestrol, DES monoether versus hexestrol mono-

ether, and 4,4'-dihydroxystilbene versus 4,4'-ethylene-diphenol. However, 4-phenylethylphenol (RBA = 0.002) with single phenol group was more active than 4-stilbenol (slight binder). These observations indicate that when both OH groups contribute to the binding, a rigid structure is critical for a better fit to the ER. However, when a chemical contains only one phenolic ring, binding is dependent on how well the rest of the structure fits into the binding pocket and the binding activity is more favorable to a chemical with certain flexibility.

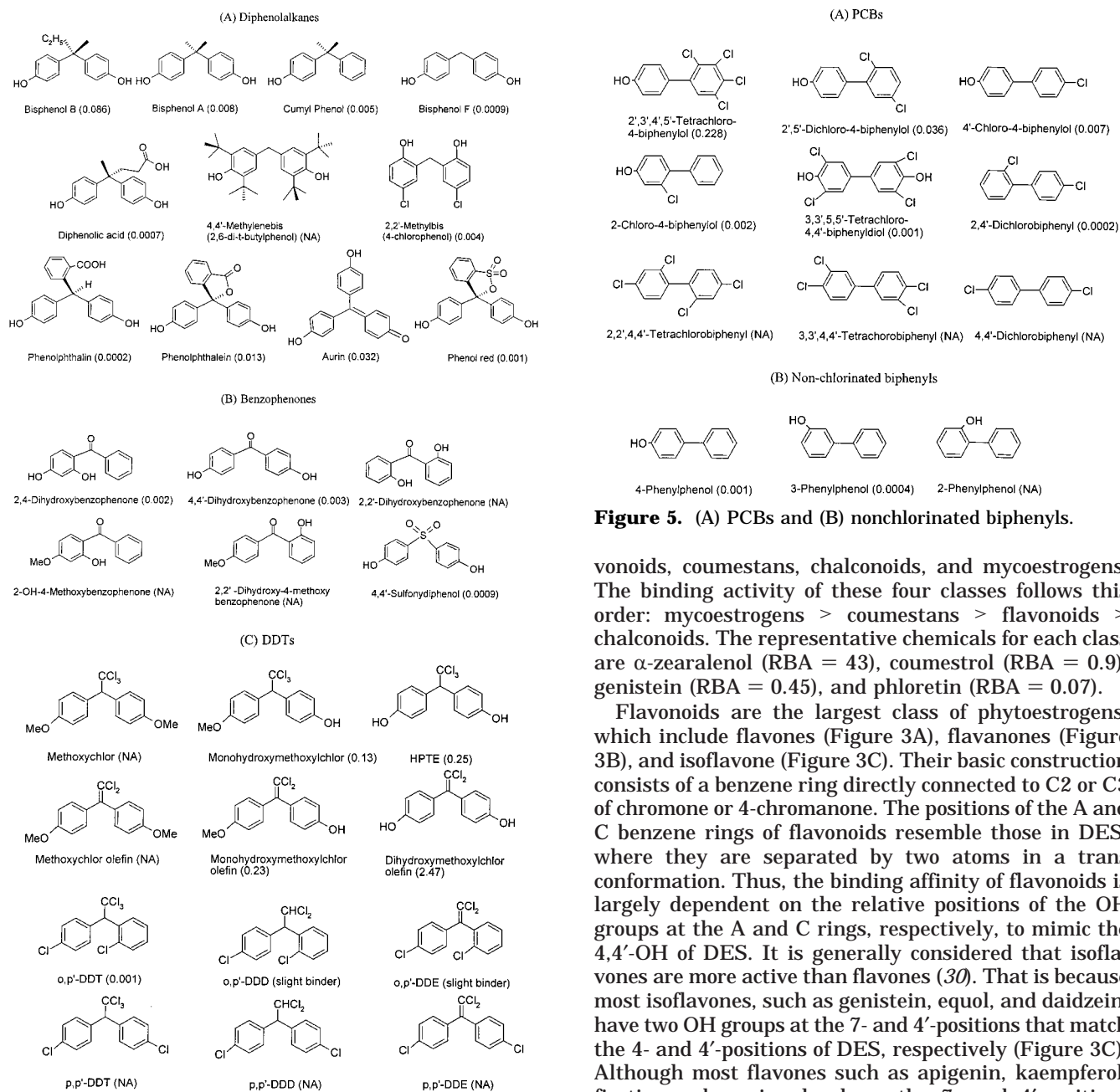


Figure 4. (A) Diphenolalkanes, (B) benzophenones, and (C) DDTs.

Triphenylethylenes (Figure 2C), which act as anti-estrogens, are structurally similar to DES, but have an additional phenyl group attached at the ethylene moiety. The RBA value, presented here, cannot distinguish agonist from antagonist activity. 4-OH-tamoxifen (RBA = 175.24) binds to the ER 100 times stronger than tamoxifen (RBA = 1.62) and 10 times stronger than droloxifene (RBA = 15.24) because its phenolic ring resembles the A ring of E_2 . If its OH group is eliminated (tamoxifen, RBA = 1.62) or its position is changed (droloxifene, RBA = 15.24), the binding affinity is reduced. For clomiphene, a chloro substituent at the ethylene end, and for toremifene, a chloro substituent at the ethyl side chain both produce binding affinities similar to that of tamoxifen.

Phytoestrogens. There are four major structurally distinct chemical classes of phytoestrogens (10): fla-

Figure 5. (A) PCBs and (B) nonchlorinated biphenyls.

vonoids, coumestans, chalconoids, and mycoestrogens. The binding activity of these four classes follows this order: mycoestrogens > coumestans > flavonoids > chalconoids. The representative chemicals for each class are α -zearalenol (RBA = 43), coumestrol (RBA = 0.9), genistein (RBA = 0.45), and phloretin (RBA = 0.07).

Flavonoids are the largest class of phytoestrogens, which include flavones (Figure 3A), flavanones (Figure 3B), and isoflavone (Figure 3C). Their basic construction consists of a benzene ring directly connected to C2 or C3 of chromone or 4-chromanone. The positions of the A and C benzene rings of flavonoids resemble those in DES, where they are separated by two atoms in a trans conformation. Thus, the binding affinity of flavonoids is largely dependent on the relative positions of the OH groups at the A and C rings, respectively, to mimic the 4,4'-OH of DES. It is generally considered that isoflavones are more active than flavones (30). That is because most isoflavones, such as genistein, equol, and daidzein, have two OH groups at the 7- and 4'-positions that match the 4- and 4'-positions of DES, respectively (Figure 3C). Although most flavones such as apigenin, kaempferol, fisetin, and morin also have the 7- and 4'-positions occupied by OH groups, these two positions correspond to the 3- and 4'-positions of DES (Figure 3A). Actually, the 6- and 4'-positions of flavones match the 4- and 4'-positions of DES, respectively. This was evident because 3,6,4'-trihydroxyflavone (RBA = 0.45) and 6,4'-dihydroxyflavone (RBA = 0.15) have RBAs comparable to those of isoflavones.

Like the 4- and 4'-OH groups of DES, the 6- and 4'-OH groups of flavonoids (7- and 4'-OH in isoflavones) play a similar role in binding. Although a small difference (~2.5-fold) in RBA values was observed for the biochanin A (RBA = 0.0043) and prunetin (RBA = 0.0018) pair and the 6-hydroxyflavanone (RBA = 0.0009) and 4'-hydroxyflavanone (RBA = 0.0023) pair, it is difficult to eliminate the possibility that both phenolic rings (A and C) of flavonoids can functionally mimic the phenolic A ring of E_2 . This suggests the possibility that flavonoids may bind to the ER in either of two orientations that differ by 180°. The recent description of the crystal structure for the genistein-ER β complex revealed that its phenolic C ring

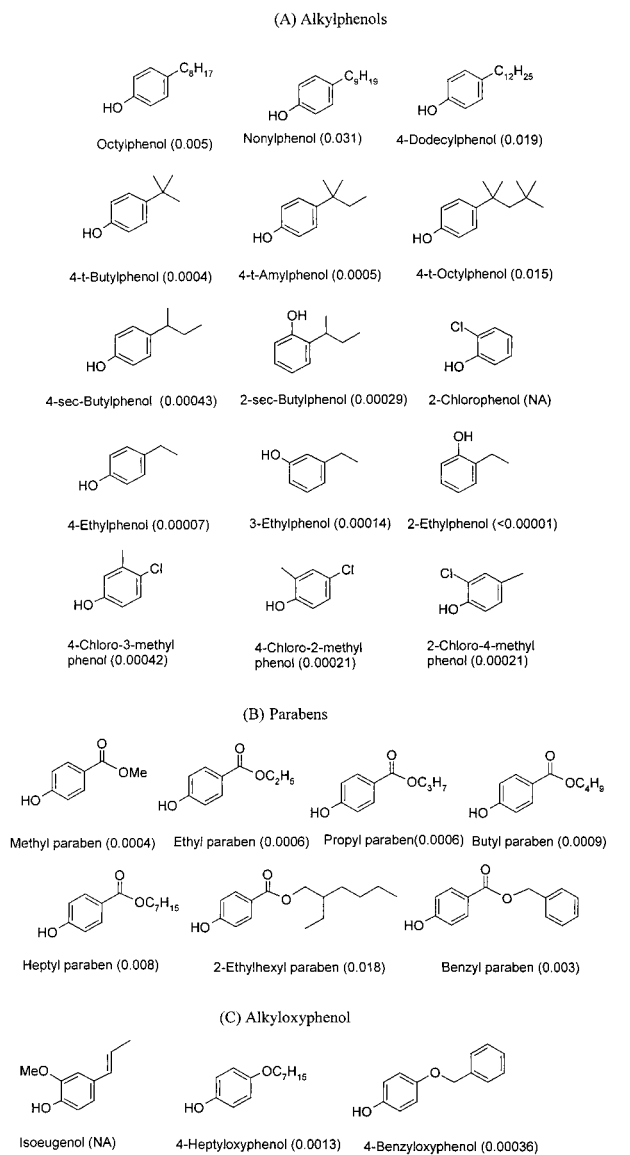


Figure 6. (A) Alkylphenols, (B) parabens, and (C) alkyloxyphenols.

served the same function as the A ring of E₂ to form the H-bond interaction with the receptor (31).

Like DES derivatives that have larger RBAs than hexestrol derivatives, flavones are slightly better binders than flavanones because their benzene rings are attached to the double bond of the B ring of chromone, which leads to a more rigid and flat structure. Within the same structural frame, the flavone apigenin (RBA = 0.028) has an RBA 4 times higher than that of the flavanone naringenin (RBA = 0.0075). Similarly, genistein was more potent than 2,3-dihydroxygenistein (32). However, the result is only applicable for flavonoids containing both OH groups at the A and C rings. The relative activity is reversed between 6-hydroxyflavanone (RBA = 0.0009) and 6-hydroxyflavone (RBA = 0.0004), which are consistent with observations on DES-like chemicals.

Generally, a large steric hindrance will reduce activity. Genistein and naringin are inactive compared to genistein (RBA = 0.45) and naringenin (RBA = 0.0075). However, compared to quercetin (NA), rutin exhibited a very low activity. This indicates that the 3-position of flavones is equivalent to the 7 α -position of E₂ where a large group can be fitted in the binding pocket.

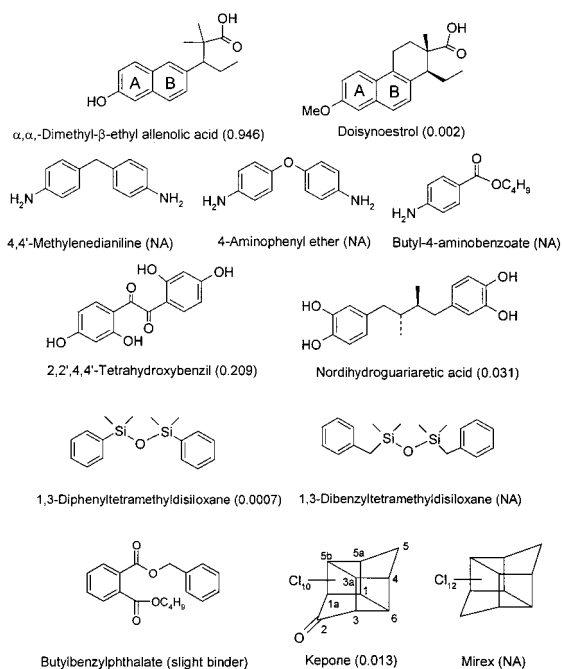


Figure 7. Miscellaneous chemicals.

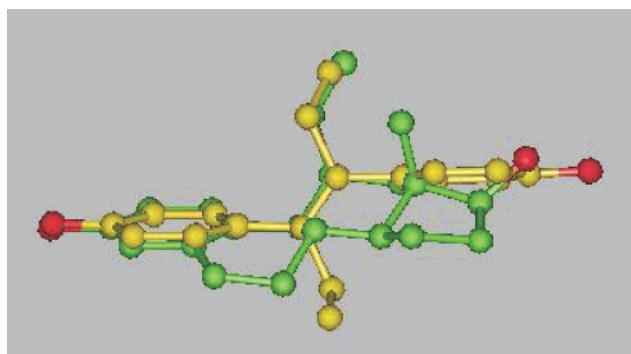


Figure 8. Superimposed structures of 11 β -ethyl-E₂ (green) and DES (yellow). E₂ and DES are first aligned on the basis of the superposition of the receptor coordinates of the crystal structures between the E₂- and DES-ER complexes. The 11 β -position of the E₂ is then modified by adding an ethyl group to form 11 β -ethylestradiol. The ethyl groups of 11 β -ethyl-E₂ and DES are positioned closely in a hydrophobic binding pocket.

Converting one of the OH groups to a methyl ether significantly reduced the RBA of isoflavones. Prunetin (RBA = 0.0018) and biochanin A (RBA = 0.0043) have 250- and 100-fold lower RBAs, respectively, than their parent compound, genistein (RBA = 0.45), while the RBA of formononetin (RBA = 0.0013) is 17-fold lower than that of daizein.

Unlike the fact that 1-OH-E₂ is less potent than E₂ (33), an additional OH group in position 5 of isoflavones, which resembles the 1-position of E₂, increases estrogenic activity (10, 34). Specifically, genistein is 20-fold more active than daizein, and biochanin A is 4-fold more active than formononetin. This may be explained by the formation of an intramolecular H-bond between the 5-OH and the carbonyl groups which enhances the electron withdrawal of the carbonyl group and lead to a better 7-OH H-bond donor.

Two coumestans were assayed, and both had RBAs ~100-fold lower than that of E₂ (Figure 3D). While coumestrol has a relatively rigid and flat structure that is similar to E₂, 4-ethyl-7-hydroxy-3-(*p*-methoxyphenyl)-

Table 3. Relationship between the O–O Distance and the RBA

mycoestrogen	RBA	d_{O-O} (Å)	mycoestrogen	RBA	d_{O-O} (Å)
α -zearalanol	30	11.00	β -zearalanol	0.20	9.85
α -zearalenol	43	11.28	zearalanone	2.1	9.77
β -zearalanol	0.64	9.23			

2H-1-benzopyran-2-one has an ethyl group that is functionally similar to that of DES. The latter had the same RBA as coumestrol because its ethyl group favors binding which compensates for the reduced RBA from converting one of the OH groups to a methyl ether.

The generic structure of chalconoids is two benzene rings connected by three carbons (Figure 3E). The O–O distance between the two OH groups in the B ring and the one in the A ring is ~ 11 and ~ 12 Å, respectively, which is comparable to those of DES and E_2 . Since their flexible molecular structures do not favor binding, chalconoids have RBAs that are 1000-fold lower than that of E_2 . The elimination of one of the OH groups on one side, such as 4-hydroxychalcone and 4'-hydroxychalcone, caused a loss in activity of ~ 20 -fold. The results further demonstrate that two OH groups within a precise distance play an important role in ER binding.

Mycoestrogens are the most active chemicals in phytoestrogens (Figure 3F). As shown in Table 3, α -zearalenol and α -zearalanol had RBAs 2 orders of magnitude higher than those of their β -isomers. The activity of zearalanone was between those of its α - and β -isomers. The order of their RBAs was consistent with that of their O–O distances. In α -zearalenol and α -zearalanol, these were about 11.0 Å, the same distance found between the 3- and 17 β -OH groups of E_2 ; β -zearalanol and β -zearalenol are 1 Å shorter (Table 3). This indicates that their RBAs are dependent on one of the critical structural features, the O–O distance, when chemicals have the same structural frameworks.

Diphenylmethanes. The generic structure for this chemical class is two benzene rings separated by one carbon (or other atoms) (Figure 4). The 4-OH substituent is critical for binding, which is supported by a number of observations:

All chemicals with a 4-OH substituent exhibit binding activity, except 4,4'-methylenebis(2,6-di-*tert*-butylphenol) that contains four steric hindered *tert*-butyl groups ortho to the 4- and 4'-OH groups. These prevent H-bond interactions between the phenolic rings and the receptor.

Two benzophenones (Figure 4B), 2,4-dihydroxybenzophenone and 4,4'-dihydroxybenzophenone, both containing a 4-OH group, exhibit weak binding activity. However, 2,2'-dihydroxybenzophenone is inactive due to the absence of a 4-OH group. Similarly, 2,2'-dihydroxy-4-methoxybenzophenone and 2-OH-4-methoxybenzophenone are inactive because of the methylation of the 4-OH.

Among the 12 DDTs (Figure 4C), the four with 4-OH substitution show much stronger binding than the others. Methoxychlor and methoxychlor olefin are inactive because of methylation of their OH groups.

Bisphenol A (RBA = 0.008) and cumyl phenol (RBA = 0.005) have comparable binding affinities, as do monohydroxymethoxychlor (RBA = 0.13) and HPTE (RBA = 0.25). It suggests that only one phenolic ring is critical in binding for chemicals with the sp^3 hybrid bridged carbon. However, this is not the case for chemicals with the sp^2 hybrid bridged carbon. Dihydroxymethoxychlor

Table 4. Hydrophobicity (log *P*) of DDTs

DDT	log <i>P</i>	DDT	log <i>P</i>	DDT	log <i>P</i>
<i>o,p'</i> -DDT	6.79	<i>o,p'</i> -DDD	5.87	<i>o,p'</i> -DDE	6.00
<i>p,p'</i> -DDT	6.91	<i>p,p'</i> -DDD	6.02	<i>p,p'</i> -DDE	6.51

olefin (RBA = 2.47) binds 10 times stronger than monohydroxymethoxychlor olefin (RBA = 0.23). This suggests that the second phenolic ring of methoxychlor olefin derivatives contributes to the binding, which may be analogous to the 17 β -OH of E_2 . The crystal structures of four ligands with the ER reveal that a considerable flexibility of the His 524 residue of the receptor permits H-bond interactions with ligands in a wide range of relative positions. Because of the sp^2 hybridization, the angle between the two phenyls in olefins (120° with sp^2) are larger than that in the non-olefin methoxychlor (109° with sp^3). The distance between the two O atoms for olefin derivatives is ~ 9.7 Å, and that for the non-olefins is ~ 9.3 Å. Therefore, the O–O distance for olefin methoxychlor is closer to that in E_2 and more favorable for binding.

For diphenolalkanes, changes in chain length at the bridging carbon lead to the RBA changes: bisphenol B (RBA = 0.086) > bisphenol A (RBA = 0.008) > bisphenol F (RBA = 0.0009). The longer the side chain, the greater the binding affinity for the ER (35). Common pharmacophore identification indicated that the optimal structural superposition between bisphenol A and E_2 is one in which the two benzene rings of bisphenol A are positioned over the A and C rings of E_2 , with the bridge carbon overlaying the B ring (Figure 9). Thus, the effect of steric substituents on the bridge atom of diphenylmethanes on ER binding is analogous to the effect of 7 α -substituents on E_2 binding.

DDT, DDD, and DDE isomers have structural frameworks similar to that of bisphenol A (Figure 4C). The *o,p'*-isomers are active in binding, while *p,p'*-isomers are not. The orthochlorine of *o,p'*-isomers mimics the steric 11 β -substituent of E_2 and increases structural rigidity, which favors binding. DDT isomers have greater hydrophobicity (log *P*) than either DDD or DDE isomers (Table 4), which might explain why *o,p'*-DDT is the strongest binder among the six DDT derivatives that were evaluated.

Biphenyls. 4-OH-PCBs tend to be good binders (Figure 5A), which is consistent with observations in other chemical classes. As the number of chloro substitutions at the nonphenolic ring increases, more electron withdrawal is found in the phenolic ring, which results in higher pK_a values (36) and a better H-bond donor. 2',3',4',5'-Tetrachloro-4-biphenylol and 2',5'-dichloro-4-biphenylol are the strongest binders in the group. Both chemicals have an orthochlorine substitution at the nonphenolic ring. Korach et al. (9) reported that PCB compounds with the strongest affinities possess either single- or multi-orthochlorine substitution. The ortho substitution restricts the conformational flexibility of PCBs, which favors binding. However, our data show that a considerable improvement of binding is only associated with the orthochlorine substitution at the nonphenolic ring. For example, 2-chloro-4-biphenylol (RBA = 0.002) has binding affinity similar to that of 4'-chloro-4-biphenylol (RBA = 0.007), but 28 times lower than that of 2',5'-dichloro-4-biphenylol (RBA = 0.036). Analyzing common pharmacophores between E_2 and 2',3',4',5'-tetrachloro-4-biphenylol (Figure 9), we found

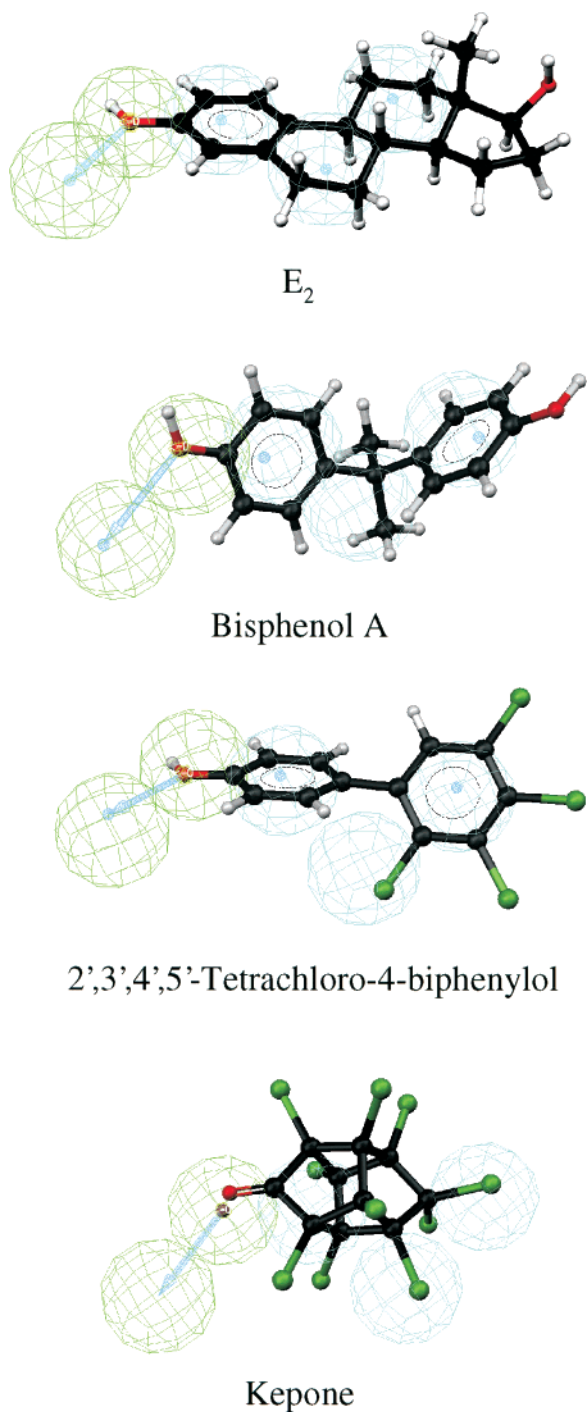


Figure 9. Four-point pharmacophore of E_2 , the 3-OH and the centers of rings A, B, and C, identified three structurally diverse xenoestrogens, bisphenol A, 2',3',4',5'-tetrachloro-4-biphenylol, and ketone.

that the two benzene rings of PCBs match the A and C ring of E_2 very well while the orthochlorine occupies the B ring position. For PCBs having two orthochlorine substitutions at the nonphenolic ring, the second orthochlorine is most likely to overlay the 11β -position of E_2 where the introduction of small steric substituents normally improves binding. Thus, these types of PCBs are better binders. It is evident that 2',4',6'-trichloro-4-biphenylol is more active than 2',3',4',5'-tetrachloro-4-biphenylol (9). It is safe to conclude that the contributions of orthochlorine substitution to binding are associated not only with the restriction of conformational flexibility but

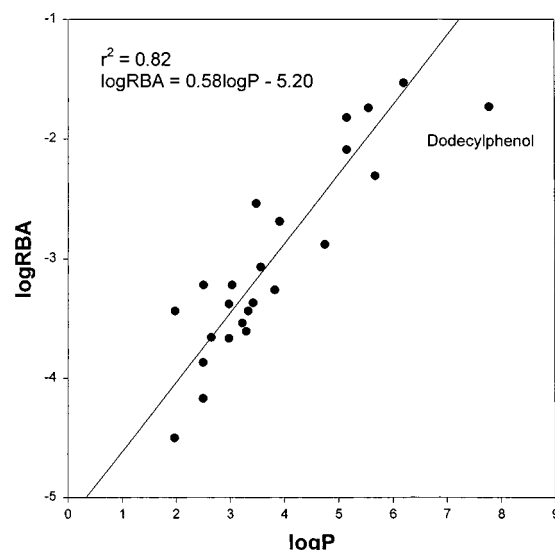


Figure 10. Correlation of log RBA with log P for phenols.

also with possibly acting as a hydrophobic group resembling the B ring and/or 11β -substituent of E_2 .

The phenylphenols (Figure 5B) are relatively lower affinity binders than the 4-OH-PCBs. With the change of OH position from 4 to 3 and to 2, the RBA decreases in the following order due to the decrease in the accessibility of the OH group for H-bond interaction: 4-phenylphenol > 3-phenylphenol > 2-phenylphenol.

Phenols. Alkylphenols (Figure 6A), parabens (Figure 6B), and alkyloxyphenols (Figure 6C) are contained in this class; nonylphenol has the highest RBA (0.031). The activities of the phenols are largely dependent on the alkyl chain length at the para position. 2-Ethylhexylparaben (RBA = 0.018) has an RBA close to that of 4-*tert*-octylphenol (RBA = 0.015), and a chain length with the same number of carbons (eight). The activity of parabens follows this order: 2-ethylhexyl > heptyl > benzyl > butyl > propyl = ethyl > methyl. A similar trend is also observed in alkylphenols (11, 23). The relationship of ER binding activity (log RBA) with hydrophobicity (log P) is linearly correlated for this class ($r^2 = 0.82$, $\log RBA = 0.58 \log P - 5.20$; Figure 10). Each unit of log P change will cause 0.58 unit of logRBA change, demonstrating the importance of hydrophobicity for binding. However, dodecylphenol is an outlier, indicating that this linear correlation is only valid within a certain range.

Miscellaneous. A number of miscellaneous chemicals in the NCTR data set were identified to be active in ER binding (Figure 7). Some have similar structural frameworks as previously discussed structural classes, while others do not.

Doisyonic and allenolic acids are nonsteroidal estrogens. Their binding affinities for the ER are normally 100-fold lower than that of E_2 , but their *in vivo* potencies are high because of a long duration of action (37). Two of their derivatives, α,α -dimethyl- β -ethylallenolic acid and doisyonoestrol, were tested; both exhibited an ability to compete E_2 for ER binding. Their OH and acid groups as well as their naphthalene substructures match the 3- and 17β -OH and A and B rings of E_2 , respectively, very well in 3D space.³ Because of the methylation of its OH

³ X. Qian, H. Fang, H. Hong, W. Tong, R. Perkins, and D. M. Sheehan, unpublished results.

group, doisynoestrol has activity ~ 100 -fold lower than that of doisylnolic acid, which is consistent with the observations for steroids.

4,4'-Methylenedianiline and 4-aminophenyl ether structurally resemble bisphenol F, where the OH groups are replaced with the amino groups. Both chemicals are inactive in binding. A similar result is also observed for butyl-2-aminobenzoate, which is structurally similar to butylparaben. This evidence reinforces the importance of the phenolic ring in binding, particularly its essential role for weak estrogens.

Benzil is a selective inducer and a potent *in vitro* activator of microsomal epoxide hydrolase (38). It is also used as a photoinitiator for the visible light polymerizing resin system that is widely applied in modern dentistry (39). Benzil has a structural resemblance to stilbene and has been studied as a new type of inducer of drug-metabolizing enzyme along with stilbene oxide (40). The 4,4'-hydroxylated benzils share a similar structural construction with the 4,4'-hydroxylated stilbenes: two OH groups separated by two benzene rings with two sp^2 (carbonyl) carbons. Their close structural similarity leads to similar binding affinities found for 2,2',4,4'-tetrahydroxybenzil (RBA = 0.209) (Figure 7) and 4,4'-dihydroxystilbene (RBA = 0.281) (Figure 2A).

Nordihydroguariaretic acid is an antioxidant for fats and oils in foods. It is a flexible molecule with two catechols separated by four carbons. According to our recent study on common pharmacophore identification of estrogens,³ nordihydroguariatic acid shares structural commonalities with E_2 : its two catechol groups mimic E_2 's A and D rings, while its bridged carbons fold in such a way to occupy the space recognized by 7α -substituents of E_2 . Normally, hydroxylation of phenol to create catechols reduces estrogenic activity (34), and the flexible structure is not favorable for binding. Therefore, even though nordihydroguariatic acid shares the key pharmacophores of E_2 , its RBA is much lower.

Siloxanes are widely used for industrial and consumer product applications (41). A single subcutaneous injection in mice using samples from breast implant or poly-(dimethylsiloxane) results in the wide distribution of the low-molecular weight siloxanes throughout the body (42). Studies by Bennett et al. (43) have shown that a series of low-molecular weight siloxanes alter male reproductive function in a number of mammalian species. An estrogenic effect on the immature rat uterus has also been demonstrated for several organosiloxane compounds (44). Two linear disiloxanes were included for this study. Neither chemical possesses H-bonding capability at either end of the structure which is normally considered to be essential for a chemical to bind to the ER. However, 1,3-diphenyltetramethyldisiloxane exhibits marginal activity. Chalcone (RBA = 0.0015), 1,3-diphenyltetramethyldisiloxane (RBA = 0.0007), and butylbenzylphthalate (slight binder) share similar structural frameworks: two benzene rings (hydrophobic centers) separated by three atoms. Their activities are proportional to the rigidity of their backbone, suggesting that the precise distance of two hydrophobic centers with a rigid linkage favors binding.

Of the variety of estrogens whose structures have little resemblance to E_2 , kepone is of particular interest for many reasons. Not only does it produce a variety of "estrogen-like" effects on the female reproductive system, but it also causes other toxic effects, including in the

neuromuscular system, liver, etc. (45). Kepone (RBA = 0.013) was found to be 10 000 times weaker than E_2 in its affinity for the ER. How its structural features relate to E_2 in binding is still puzzling. Kepone contains four cyclopentanes in the left, right, top, and bottom sides fused together to form a cagelike structure (Figure 7). It appears that the carbonyl group at the C_2 -position is essential because mirex, its analogue that has the carbonyl group replaced with two chlorine atoms, is inactive in binding. Common pharmacophore identification indicates that kepone is similar to E_2 in various structural features: the carbonyl group along with the hydrophobic center of the 2,3,3a,5b,1a-cyclopentane ring, C_4 -Cl, and the hydrophobic center of the two Cl atoms at the C_5 -position mimics the function of the phenolic A, B, and C rings in E_2 (Figure 9). It is important to point out that kepone can rapidly pick up moisture to form kepone hydrate that should be more active for the ER (46).

Discussion

In principle, the biological activity of a chemical is determined by its structure. The chemical structure can be represented in three different general ways: 2D substructures, 3D pharmacophores, and physicochemical properties. A 2D substructure is a structural fragment of a molecule, which often can be used as a strong indicator of a particular activity, such as the phenolic ring for the ER. A 3D pharmacophore is a portion of a chemical's 3D structure that is considered essential in eliciting the biological activity of interest, such as the precise O—O distance, the orientation of the OH group, and the location of the hydrophobic center. A physicochemical property of a molecule is a measure of one property of a whole molecule. For example, $\log P$ measures a chemical's hydrophobicity. ER binding activity relates to all these structural features. The SAR studies for ER binding by individual chemical classes indicate that some features may well represent binding dependencies for one structural class, while other features may better represent binding dependencies for a different structural class. These structural features are inherently related, suggesting that structural commonality exists among structurally diverse estrogens.

It is well-accepted that the precise spacing of two OH groups at either end of an essential planar and primarily hydrophobic molecule is considered the structural basis for ER binding (47). The E_2 -ER H-bonding network (Figure 11) demonstrates the critical role of H-bonding ability for a chemical to bind to the ER (20). Although the "anchor-like" H-bonding network at the A ring of E_2 imposes an absolute requirement that an effective ligand must contain a phenolic ring, the remainder of the binding pocket can accept a number of different hydrophobic groups. By comparing various estrogen classes through SAR studies, we can summarize the general structural requirements relevant to the template E_2 structure: (1) H-bonding ability of the phenolic ring mimicking the 3-OH, (2) H-bond donor mimicking the 17β -OH and O—O distance between 3- and 17β -OH, (3) precise steric hydrophobic centers mimicking steric 7α - and 11β -substituents, (4) hydrophobicity, and (5) a ring structure. These important features provide the structural basis for a xenoestrogen to exhibit binding activity.

H-Bonding Ability of the Phenolic Ring Mimicking the 3-OH. The importance of H-bonding has been

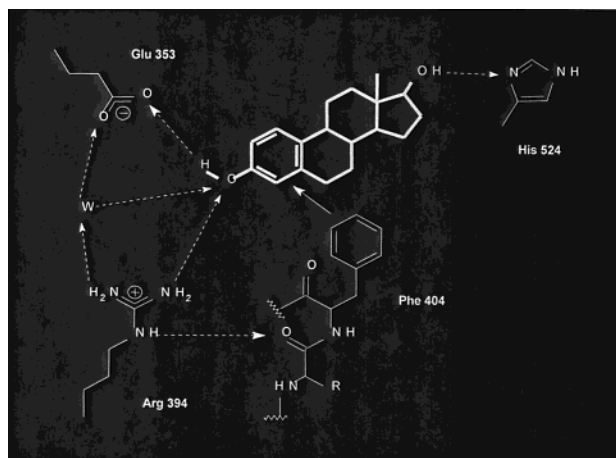


Figure 11. ER–E₂ hydrogen-bonding network (52).

Table 5. H-Bond Distances between Two OH Groups of Four Ligands and the Receptor

ER ligand	3-OH			17-OH
	Glu 353 [d _{O–O} (Å)]	Arg 394 [d _{O–N} (Å)]	water [d _{O–O} (Å)]	His 524 [d _{O–N} (Å)]
E ₂	2.37	3.08	2.82	2.77
DES	2.51	3.33	3.19	2.67
raloxifene	2.38	2.93	2.97	2.62
4-OH-tamoxifen	2.45	3.04	3.07	

recognized in most biological systems. The O atom is one of the most important heteroatoms directly associated with H-bonding. Of the 130 active chemicals in the NCTR data set, 125 chemicals contain an O atom and 3 chemicals contain a Cl atom that may serve as a weak H-bond acceptor. Even though the degree of specificity is reduced because of two active chemicals without H-bonding ability that produce specific steric effects, it is difficult to avoid the conclusion that the requirement of H-bonding ability is the most important characteristic for estrogen.

It has been long understood that the phenolic ring is normally associated with estrogenic activity. One hundred eight of 130 active chemicals (83%) in the NCTR data set contain a phenolic ring. The contribution of the phenolic ring in binding is much more significant than any other structural feature. The current crystal structures of four ligand–ER complexes reveal that the phenolic rings of all four ligands are closely positioned at the same location to allow H-bond interactions with Glu 353, Arg 394 of the receptor, and a water molecule. The distance of the phenolic O atom of these four ligands to Glu 353, Arg 394, and the water molecule is summarized in Table 5. It appears that the H-bonding of the phenolic OH is much stronger with Glu 353 than Arg 394 and the water molecule because of the shorter distance. Thus, the phenolic OH contributes to binding largely through its H-bond donor ability because of the H-bond acceptor properties of Glu 353. The phenolic OH is a better H-bond donor than an acceptor. Replacing OH with the weak H-bond donor NH₂ reduces activity for E₂ (17), and diminishes activity for bisphenol F and butylparaben (4-aminophenyl ether and butyl-4-aminobenzoate, Figure 7). It is important to point out that the H-bond donor ability of the phenolic OH is dependent on a number of factors, particularly the nature of ortho substituents that affect the OH accessibility. The H-bond donor ability for several ortho-substituted phenols follows this trend:

phenol > 2-methylphenol = 2-*tert*-butylphenol > 2,6-dimethylphenol > 2,6-di-*tert*-butylphenol (48), in which 2,6-di-*tert*-butylphenol is not a H-bond donor. This is consistent with the lack of binding activity observed for 4,4'-methylenebis(2,6-di-*tert*-butylphenol) (Figure 4A). In conclusion, a good H-bond donor mimicking the 3-OH of E₂ may be more important than a H-bond acceptor for xenoestrogen.

H-Bond Donor Mimicking the 17β-OH and O–O Distance between 3- and 17β-OH. Chemicals could be strong estrogens if they possess a phenolic ring and an additional OH group within a certain distance range. Chemicals containing only one phenolic group are most likely to be weak to medium ER ligands. The 17β-OH of E₂ makes a single H-bond as a donor with His 524, because the two N atoms of imidazole act as an H-bond acceptor in most cases. This is consistent with the observation of similar RBAs for the E₁ and 17-deoxy-E₂ (Figure 1A) pair and the meso-*p*-(α,β-diethyl-*p*-methylphenethyl)phenol and hexestrol monomethyl ether pair (Figure 2B). Therefore, a stronger H-bond donor, such as the phenolic OH, favors binding at the position that resembles the 17β-OH of E₂.

The imidazole of His 524 is not only able to rotate (because of the alkyl chain), but its two equivalent imidazole N atoms could compensate for the change in the oxygen position of 17β-OH and maintain a favorable H-binding position. This was recognized in the E₂– and raloxifene–ER crystal structure. The phenolic A rings of both molecules overlap quite well, whereas the 17β-OH group of E₂ and the OH group of raloxifene are 5 Å apart. However, these two OH groups have a similar distance to the N atom of the imidazole (Table 5). It suggests that the flexible His 524 is more tolerant of the second OH groups of a chemical in forming an effective H-bond interaction. Although the H-bonding strength is dependent on the relative positions (angle and distance) between the imidazole of His 524 and the OH group, the O–O distance could provide a fair estimation of the H-bonding ability of the second OH group analogous to the 17β-OH of E₂.

For purposes of analysis, we have divided the NCTR data set into four activity categories: strong (RBA > 1), medium (1 > RBA > 0.01), weak (0.01 > RBA > detectable activity), and inactive. As shown in Figure 12, most strong to medium ER ligands contain two OH groups with an O–O distance ranging from 9.7 to 12.3 Å. The chemicals with an O–O distance not in this range are highly likely to be weak estrogens.

Precise Steric Hydrophobic Centers Mimicking Steric 7α- and 11β-Substituents. The volume of the ER binding pocket (450 Å³) is about twice that of E₂ (245 Å³) (20). The length and breadth of the E₂ skeleton are well matched by the receptor, but there are large unoccupied cavities at the 7α- and 11β-positions of E₂. The positions of these cavities allow steric groups of certain sizes to fit, and are of great importance for xenoestrogens, including DES-like chemicals, diphenylmethanes, and biphenyls. For example, the binding affinities of DES, DMS, and 4,4'-dihydroxystilbene change dramatically with changes in the side chain length on the C–C double bond. This trend was also observed (4) in *in vivo* activity; 4,4'-dihydroxystilbene had 250-fold lower potency than DMS and 16700-fold lower potency than DES. Even triphenylethylene (RBA = 0.002) (Figure 2C) that has no H-bonding ability still exhibits weak

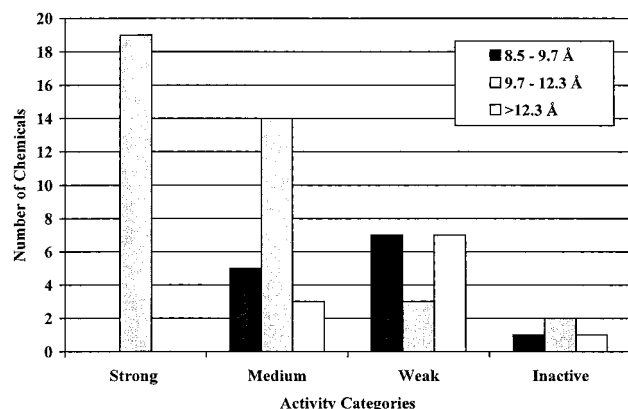


Figure 12. Effect of the range of the O–O distance on RBA. The activity categories were arbitrarily defined as follows. For strong estrogens, RBA > 1. For medium estrogens, the RBA is between 0.01 and 1. For the weak estrogens, RBA is between 0.01 and a detectable activity. Inactive chemicals show no detectable activity in the assay. The RBA for E₂ is set to 100.

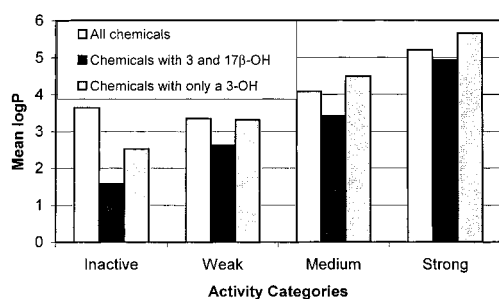


Figure 13. Relationship of the mean log *P* values with binding activity. The activity categories are defined in the legend of Figure 12.

binding. Clearly, the precise location of steric bulk in the region of the 7 α - and/or 11 β -positions of E₂ is critical for binding. It is evident that DL-hexestrol's RBA activity is about 100-fold lower than that of mesohexestrol. The two isomers only differ in the direction of the two ethyl substituents. Similarly, indenestrol A was found to have higher affinity for the ER (49) than the enantiomers of indenestrol B (50).

Hydrophobicity. The ER ligand-binding pocket has a 3D "cross"-like shape; the center and vertical ends are mainly hydrophobic, whereas the polar functions are located at opposite ends of the horizontal cavity. An effect of hydrophobicity in binding was observed for phthalates (51). Hydrophobicity (log *P*), expressed as a ratio of solubility between octanol and water, is important for many biological end points. Log *P* values too high or too low can be associated with poor transport characteristics. Unlike the aforementioned three estrogen-related structural features that are critical in binding only by interacting with specific amino acids of the receptor, hydrophobicity is a physicochemical property.

Although the mean log *P* values for strong, medium, and weak estrogens exhibited a positive trend (Figure 13), the inactive chemicals also have a wide range of log *P* values. This suggests that better binders tend to have larger log *P* values but not vice versa. We plotted log RBA versus log *P* for individual chemical classes; the only linear correlation was for phenols (Figure 10). Phenols consist of only a phenolic ring serving as a H-bond anchor and an alkyl chain for hydrophobicity. The linear correlation between log RBA and log *P* for phenols

suggests that log *P* is only important in binding when the key pharmacophores are in place. This can be further demonstrated by plotting mean log *P* values versus activity categories for chemicals containing two OH groups with distances between 9.7 and 12.3 Å (mimicking the 3- and 17 β -OH of E₂) and only one 3-OH group. As shown in Figure 13, the positive effect of hydrophobicity on binding is much more apparent for chemicals containing the 3- and 17 β -OH, and a 3-OH. Interestingly, for each activity category, the mean log *P* value of chemicals with one OH group is larger by almost the same amount compared to that of chemicals containing two OH groups mimicking the 3- and 17 β -OH of E₂. This indicates that chemicals lacking the effective O–O distance require greater hydrophobicity to reach binding activities similar to those of chemicals with the 3- and 17 β -OH groups.

Ring Structure. Effective ER ligands must possess a ring structure. A literature survey of more than 2000 unique chemicals that were tested by various in vitro and/or in vivo assays for estrogenic activity reveals that no estrogenic chemical has been found that does not possess a ring structure. The ring construction increases the rigidity of both the structure and the steric center, which favors ER binding. It appears that the flat aromatic ring, which fits better to the narrow "tunnel-like" arrangement in the receptor for the phenolic A ring, is more important than other ring structures. The majority of xenoestrogens contain at least one aromatic ring. Only five chemicals without aromatic rings were found to be active, of which four are steroids (Figure 1B) in addition to kepone. All five chemicals possess H-bond capability with a rigid hydrophobic backbone that matches the A, B, and C rings of E₂ very well. It suggests that nonaromatic estrogens require several key structural features to exhibit binding activity, at least including a strong electronegative atom (O, S, N, etc.) for H-bond interaction and a rigid hydrophobic backbone.

There are several substructures inherently associated with ER binding activity. The common structural framework for most estrogens contains an aromatic ring, normally a phenolic ring, separated from a hydrophobic center that mimics one of the centers of E₂'s B, C, or D ring. PCBs have two benzene rings connected directly, which resemble the A and C rings. The ortho substituents of PCBs match the B ring and/or 11 β -substituents of E₂ and increase binding affinity. Bisphenol A and DDT-like chemicals have two benzene rings separated by one atom, whereas two rings mimic the A and C ring and the substituents on the bridge atom match the B ring. The two benzene rings of DES derivatives resemble the A and D rings. The binding of DES derivatives is mainly governed by the presence or absence of two OH groups at the 4,4'-position and two ethyl groups to mimic the 7 α - and 11 β -substituents of E₂. It seems that two benzene rings separated by three atoms provide an optimal steric interaction with the receptor, which is demonstrated by three weak estrogens, chalcone, 1,3-diphenyltetramethyldisiloxane, and butylbenzylphthalate, which contain no H-bonding capability mimicking either the 3- or 17 β -OH of E₂.

Summary. The SAR studies on 230 diverse chemicals reveal five structural features that are most important for chemical binding to ER. The representative chemical for each structural category listed in Table 1 demonstrates that the more key structural features a chemical contains, the more active it is. These findings can be

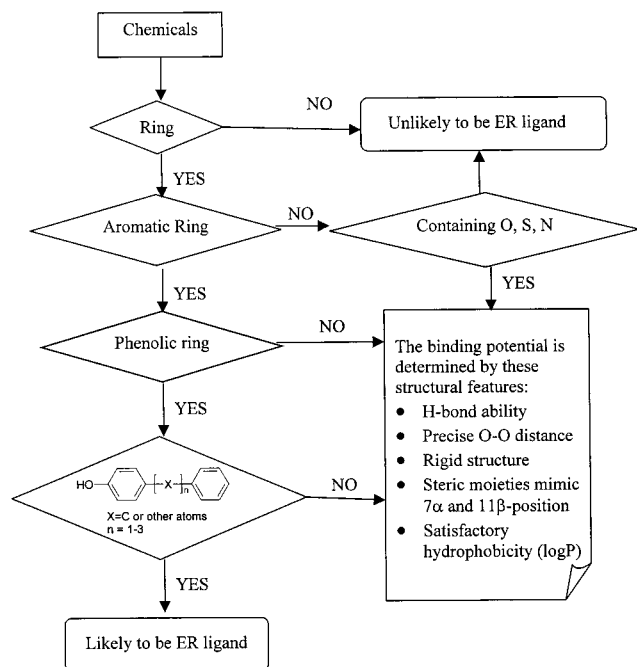


Figure 14. Flowchart for identification of ER ligands.

generalized as a set of “if-then” rules for guidance in identifying potential ER ligands, which is depicted in Figure 14:

(1) If a chemical contains no ring structure, then it is unlikely to be an ER ligand.

(2) If a chemical has a nonaromatic ring structure, then it is unlikely to be an ER ligand if it does not contain an O, S, N, or other heteroatom for H-bonding. Otherwise, its binding potential is dependent on the existence of the key structural features. Kepone, dihydrotestosterone, norethynodrel, and 3 α - and 3 β -androstenediol are active ER ligands that fall into this category.

(3) If a chemical has a non-OH aromatic structure, then its binding potential is dependent on the existence of the key structural features. A total of 16 chemicals in the NCTR data set, including *o,p'*-DDT, 1,3-diphenyltetramethyldisiloxane, 3-deoxyl-E₂, mestranol, and others, fall into this category.

(4) If a chemical contains a phenolic ring, then it tends to be an ER ligand if it contains any additional key structural features. For the chemicals containing a phenolic ring separated from another benzene ring with the number of bridge atoms ranging from none to three, it will most likely be an ER ligand.

Conclusion

The goal of the study presented here was to explore the change in ER binding activity with the change of structure based on the NCTR data set, which to the best of our knowledge is so far the largest published structurally diverse data set with a consistent quantitative binding activity measurement. The data set conveys information far beyond the scope of this paper. The knowledge derived from this study enhances our understanding of the important ER binding-related structural features, which in turn should be important in guiding the development of predictive toxicology models for rapid identification of xenoestrogens using computational approaches. No matter what computational approaches are

used, the primary step is to identify ER-related structural characteristics. The knowledge then could be used to design alignment for 3D QSAR, to select ER-related structural descriptors for QSAR, and to identify key structural features for pharmacophore models. All this tells us that structure–activity relationship analyses of xenoestrogens are essential.

Acknowledgment. We gratefully acknowledge the U.S. Department of Energy and the U.S. Food and Drug Administration (FDA) for postdoctoral support through the Oak Ridge Institute for Science and Education. This work was partially supported by the FDA's Office of Women's Health and the Chemical Manufacturer's Association.

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