Using Three-Dimensional Quantitative Structure-Activity Relationships to Examine Estrogen Receptor Binding Affinities of Polychlorinated Hydroxybiphenyls

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Certain phenyl-substituted hydrocarbons of environmental concern have the potential to disrupt the endocrine system of animals, apparently in association with their estrogenic properties. Competition with natural estrogens for the estrogen receptor is a possible mechanism by which such effects could occur. We used comparative molecular field analysis (CoMFA), a three-dimensional quantitative structure-activity relationship (QSAR) paradigm, to examine the underlying structural properties of *ortho*-chlorinated hydroxybiphenyl analogs known to bind to the estrogen receptor. The cross-validated and conventional statistical results indicate a high degree of internal predictability for the molecules included in the training data set. In addition to the phenolic (A) ring system, conformational restriction of the overall structure appears to play an important role in estrogen receptor binding affinity. Hydrophobic character as assessed using hydropathic interaction fields also contributes in a positive way to binding affinity. The CoMFA-derived QSARs may be useful in examining the estrogenic activity of a wider range of phenyl-substituted hydrocarbons of environmental concern. *Key words:* comparative molecular field analysis, estradiol equivalents, estrogen receptor, polychlorinated biphenyls, quantitative structure-activity relationships. *Environ Health Perspect* 103:702–707 (1995)

There is a growing concern (*1,2*) that a number of chemicals released into the environment can disrupt the endocrine system of animals, including humans. Among these chemicals are the persistent, bioaccumulative organochlorine compounds that include some industrial chemicals such as the polychlorinated biphenyls (PCBs). The ability to mimic natural hormones such as estradiol is one mechanism by which such disruption could occur. Certain halogenated aromatic compounds have been shown (*3*) to elicit estrogenic hormonal activity. Mixtures of PCBs were used as commercial insulators due to their favorable dielectric properties (*4,5*). However, these products have been banned in the United States since the 1970s (*6,7*). The highly chlorinated and lipophilic nature of these compounds underlie their persistence as environmental contaminants, and they are known to cause adverse reproductive effects in males (*8*) and females (*8, 9*).

Modified mixtures of PCBs, which are lower in overall chlorine content yet rich in *ortho*-chlorinated biphenyls, have been proposed as alternatives to the highly chlorinated PCB mixtures. The lower chlorine content favors increased metabolism in biological systems. Upon metabolic hydroxylation at vacant *para* positions, the more highly *ortho*-chlorinated biphenyls can be converted to possible metabolites capable of binding to the estrogen receptor (*10*). Bioaccumulation of these metabolites is not expected to be a problem as long as the enzymes responsible for conjugation and elimination of such polar compounds are not saturated and remain functional; however, continuous exposure to PCBs may result in an overall increase in steady-state concentrations of these metabolites (*11*).

In addition to the PCB family of chemicals, other phenyl-substituted hydrocarbons such as the biphenyl ethanes and ethylenes related to diethylstilbestrol (DES) (*12*) and triphenylethanes (*13*), including tamoxifen and nafoxidine, have been shown to be ligands of the estrogen receptor. A common substructure of many of these chemicals is a phenolic ring system, with an obvious relationship to the phenolic A ring in estradiol (Fig. 1). However, it is not clear if in all cases the hydroxyl group has unique properties as a substituent or if any similar-sized (isosteric) group, such as chlorine, could replace it. Such a substructure serves as a unique way to align the molecules for comparing the overall structures of otherwise structurally diverse chemicals. In this way it may be possible to determine their estradiol equivalency (*14*) in a manner analogous to determining the dioxin equivalency (*15*) of structurally related chlorinated aromatic hydrocarbons of environmental health concern. We used comparative molecular field analysis (CoMFA) (*16*), a three-dimensional quantitative structure–activity relationship (QSAR) paradigm, to examine the unique physicochemical properties of polychlorinated hydroxybiphenyls (Table 1) underlying their estrogen receptor binding affinities and thus their potential estradiol equivalency.

Methods

The SYBYL molecular modeling software package (version 6.0, Tripos Associates, Inc., St. Louis, Missouri) was used to do all molecular modeling. The coordinates for estradiol and DES were retrieved from the Cambridge Structural Database. The remaining molecules were constructed in SYBYL using the sketch option. All molecules were minimized using the standard Tripos force field (*17*) to an energy change convergence criterion of 0.001 kcal/mol. The SYBYL geometries were used as starting coordinates for full-geometry optimization using MOPAC 5.0 (Quantum Chemistry Program Exchange, Indiana University, Bloomington, Indiana) with the AM1 (*18*) model Hamiltonian.

For each biphenyl molecule (including DES), using the MOPAC/AM1 optimized structure as a starting point, the conformational space about the twist bond(s) connecting the two ring systems was explored. This bond was systematically searched at 5° increments. The lowest energy conformer obtained from the search was then subjected to full MOPAC/AM1 optimization. This structure was then aligned relative to estradiol by root-mean-squares (RMS) fit of the hydroxyphenyl ring (A) carbons and the corresponding carbons of the *para*-substituted ring of the molecule to be fitted. Due to structural symmetry of the biphenyls, several alignments with estradiol are possible. It was therefore necessary to devise a logical process for the selection of the "active conformation" to be used in the analyses.

The procedure adopted may be summarized as follows. First, all possible orientations (RMS-fitted superpositions with estradiol) of an individual molecule to be aligned were saved as separate files. Each of these molecules was then subjected to a field-fit (template forcing) minimization procedure using estradiol as the template. Simply put, the field-fit algorithm forces a test molecule to assume a conformation and charge distribution similar as possible to that of a template molecule at the expense of an increase in the internal potential ener-
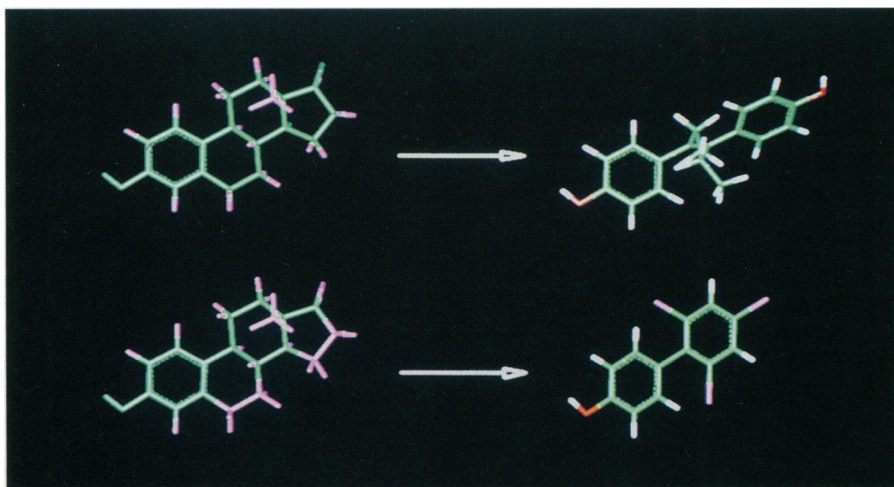
Figure 1. Comparison of the chemical structure of estradiol with a diethylstilbestrol derivative (top) and an *ortho*-substituted biphenyl derivative (bottom). Green-colored atoms and bonds designate substructural features of estradiol which are similar, if not identical, in size and shape to the comparison molecules.

gy of the molecular system. Two additional penalty terms are added to the Tripos molecular mechanics force field equation to achieve this: a steric field fit energy term and a electrostatic field fit energy term. The orientation of the test molecule displaying the lowest energy penalty in order to mimic the template molecule estradiol was then optimized using full geometry and selected as the "active" conformer in the "active" alignment. This process is repeatable and assures the selection of a low-energy conformer most closely structurally related to the template.

The binding affinities of the compounds included in the training set are reported in terms of the concentration (molar equivalents, Meq) of competitor required to displace 50% of the bound [³H]estradiol from the estrogen receptor. The values for all compounds were determined using uterine cytosol from ovariectomized mice as reported by Korach et al.(*10*). These values are reported as the negative log of the concentration necessary to displace 50% of bound [³H]estradiol from the estrogen receptor and are designated as pC_{50}.

The steric and electrostatic interaction energies were calculated using an sp^3 hybridized carbon probe atom with a charge of +1.0 and a distance-dependent dielectric function at all intersections of a regularly-spaced (1.0 Å) grid of dimensions 20 Å × 17 Å × 16 Å. The cutoff value for all interactions was set at ±30.0 kcal/mol.

Hydrophobicity field calculations were performed according to the method of Kellogg et al. (*19*) as employed in hydropathic interaction (HINT). The hydropathic interaction energies were determined at intersection points on the same grid as used for the CoMFA interaction energies. At each grid intersection point, the net sum of the following empirical

equation was evaluated over all the atoms for a given molecule:

$$A_t = \Sigma \, s_i \, a_i \, R_{it} \qquad (1)$$

where s_i = solvent accessible surface area for atom i; a_i = hydrophobic atom constant for atom i; and $R_{it} = e^{-r}$ (r = distance between atom i and test point t). These values were imported into the QSAR molecular spreadsheet (MSS) and treated as a single-field (electrostatic) CoMFA-type column.

All statistical analyses were performed using the partial-least-squares (PLS) methodology (*20*) as employed in the QSAR module of SYBYL 6.0 running on Silicon Graphics Indigo and Onyx workstations. All initial analyses were performed using the leave-one-out (LOO) cross-validation (*21*) technique and 10 principal components (PCs). The cross-validated r^2 (referred to as q^2 here) was computed as follows:

$$q^2 = (SD - PRESS)/SD \qquad (2)$$

where SD is the sum of the squared deviations between the measured and mean binding affinities of the training set molecules, and PRESS is the sum of the squared deviations between the predicted and measured binding affinities for every molecule.

To increase the signal-to-noise ratio in the CoMFA analyses, steric and electrostatic columns with a standard deviation of less than 2.0 kcal/mol ("minimum σ") were not included in the cross-validated analysis. A minimum σ of 0.5 kcal/mol was implemented in the analyses using hydropathic fields. The optimal number of components to be used in the subsequent non-cross-validated analysis was determined as that which yielded the highest q^2 value and the lowest standard error of cross-validated predictions (SEP) for training set molecules

determined during the LOO procedure. For the non-cross-validated analyses, the minimum σ was reduced to 0.0 kcal/mol (all columns were included in the analyses). The results of all conventional and three-dimensional QSAR analyses are summarized in Table 2.

Results

Often the biological potency of highly lipophilic molecules such as the phenyl-substituted hydrocarbons is driven by their ability to cross biological membranes *in vivo* (*22*) and, on a more pragmatic level, their solubility in the assay medium *in vitro*. In the absence of direct experimental data, the conventional computational manner in which partitioning/solubility is incorporated into QSAR analyses is to estimate the octanol:water partition coefficient using an additive technique (*23–25*). Additive approaches have proven to be inadequate for estimating partition coefficients for positional isomers (*26*). To further illustrate the limitations of the additive fragment (atom) methods with respect to positional isomers, the HINT-calculated logP (HINT logP) value was used as a regressor. This resulted in a cross-validated correlation coefficient (q^2) of -0.003 ($s = 1.434$) indicating that all of the internal, cross-validated predictions were outside the range of the standard deviation of the data set. This analysis is useless. The low statistical significance is also represented in a conventional correlation coefficient (r^2) of 0.221 (SEP = 1.264).

To overcome this phenomenon in the analyses presented here, we used an empirically derived, three-dimensional field representing the hydrophobicity of the molecules. The use of these fields as determinants of the hydropathic character of molecules of this type has been validated in analyses using structural isomers and experimental RP-HPLC retention time, or generator column, data (*26*). The analysis based on HINT fields as the sole regressor was less internally consistent yet more statistically robust than the unit-dimensional HINT logP-based analysis ($q^2 = -0.302$ using 1 PC with SEP = 1.634, $r^2 = 0.625$ with $s = 0.877$).
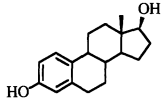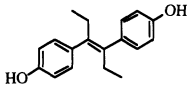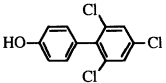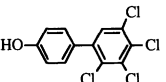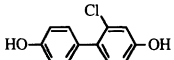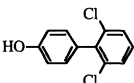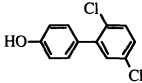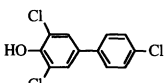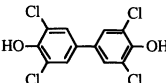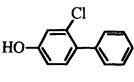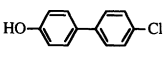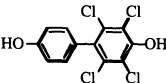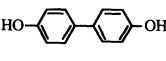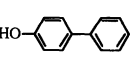
Using this 14-molecule training set and the CoMFA steric and electrostatic fields as regressors, a cross-validated correlation coefficient of 0.276 using 2 PCs with a standard error of cross-validated predictions (SEP) of 1.273 was generated. This value indicates a low degree of internal consistency, or predictability, for molecules included in the training set. The non-cross-validated analysis yielded a statistically robust correlation coefficient of 0.957 with a standard error of 0.310. The model based on the steric field alone was more predictive ($q^2 = 0.345$ using 2 PCs with a

SEP of 1.211 with similar non-cross-validated results (r^2 = 0.960; s = 0.301). The electrostatic field model was much less predictive (q^2 = -0.058 using 3 PCs; SEP = 1.614), yet statistically robust (r^2 = 0.979; s = 0.229). The combination of steric and hydropathic fields resulted in a compromised model displaying a cross-validated coefficient (q^2 = 0.083 using 1 PC) with a decreased SEP (1.371). The non-cross-validated analysis yielded a correlation coefficient (r^2) of 0.835 (s = 0.582).

Table 1. Physical properties of compounds in the quantitative structure–activity relationship analysis (training set)

Nomenclature	Compound/structure	HINT log P	2-1-1'-2' Angle	pC₅₀(Meq) Actual	pC₅₀(Meq) Predicted[a]
Estradiol		4.87	NA	0.000	-0.059
Diethylstilbestrol		5.24	NA	0.398	0.415
2,4,6-Trichloro-4'-biphenylol		5.22	-90.1	-1.623	-1.666
2,3,4,5-Tetrachloro-4'-biphenylol		5.81	-60.4	-1.978	-2.076
2-Chloro-4,4'-biphenyldiol		3.37	60.4	-1.954	-2.145
2,6-Dichloro-4'-biphenylol		4.63	90.0	-2.589	-2.646
2,5-Dichloro-4'-biphenylol		4.63	119.7	-2.704	-2.324
3,4',5-Trichloro-4-biphenylol		5.22	40.1	-3.000	NA
3,3',5,5'-Tetrachloro-4,4'-biphenyldiol		5.14	40.3	-3.132	NA
2-Chloro-4-biphenylol		4.04	59.9	-3.398	-3.415
4'-Chloro-4-biphenylol		4.04	40.0	-3.591	-3.901
2,3,5,6-Tetrachloro-4,4'-biphenyldiol		3.33	-89.8	-3.699	NA
4,4'-Biphenyldiol		2.66	42.4	-4.000	-4.137
4-Biphenylol		3.33	-57.6	-4.000	-3.484

Abbreviations: HINT, hydropathic interaction; NA, not applicable.
[a]Predictions taken from non-cross-validated analysis of steric field three-dimensional quantitative structure–activity relationship model without ionizable compounds (8,9, and 12).

Table 2. Summary of comparative molecular field analysis statistical results

Parameter	logP	Hydropathic field	Steric field	Steric[a] field	Electrostatic field	Steric and electrostatic fields	Steric and hydropathic fields
q^2	-0.003 (1)[b]	-0.302 (1)	0.345 (2)	0.544 (2)	-0.058 (3)	0.276 (2)	0.083 (1)
SEP	1.434	1.634	1.211	1.124	1.614	1.273	1.317
r^2	0.221	0.625	0.960	0.974	0.979	0.957	0.835
s	1.264	0.877	0.301	0.270	0.229	0.310	0.582
F	3.405	19.983	130.526	148.464	152.528	122.319	60.688
p	0.090	0.001	0.000	0.000	0.000	0.000	0.000
Relative field contributions							
Steric	NA	100%	100%	NA	55%	65%	
Electrostatic	NA	NA	NA	100%	45%	NA	
Hydropathic	100%	NA	NA	NA	NA	35%	

Abbreviations: SEP, standard error of cross-validated predictions; NA, not applicable.
[a]Three dimensional quantitative structure–activity relationship model without ionizable compounds (8,9, and 12).
[b]Number of principal components in parentheses.

Three PCB derivatives (8, 9, and 12) in the training set possess chlorine substituents adjacent to the *para*-hydroxyl group. The pK_a of the hydroxyl group in these compounds is calculated by manufacturer (CompuDrug Chemistry Ltd, Budapest) to be lower (6.7, 6.2, and 5.8, respectively) than the pH of the assay medium (7.6). Therefore, it is expected that these compounds would be ionized and behave much differently from the remaining nonionized compounds expressing much higher pK_a values (greater than 9.0 in all cases). A subsequent analysis using only steric fields as regressors and the remaining 11 compounds as observations yielded a much more predictive (q^2 = 0.544 using 2 PCs) and robust (r^2 = 0.974) model.

To demonstrate the utility of this three-dimensional QSAR model, the estrogen receptor binding affinities of several PCBs (Fig. 2) of environmental concern [3,4,3′,4′-tetrachlorobiphenyl (PCB77), 2,5,2′,5′-tetrachlorobiphenyl (PCB52), and 2,4,5,2′4′,5′-hexachlorobiphenyl (PCB153)] were predicted using the 11-compound model described above. To our knowledge, these three PCBs have not been screened for the estrogen receptor binding affinity. Our predictions illustrate the importance of conformational restriction about the twist bond. PCB77, the least conformationally restricted of the test set, was predicted to have a binding affinity (predicted pC_{50}) of -2.447. The *ortho*-substituted (conformationally restricted) and non-*para*-substituted PCB52 was predicted to be a weaker binder (predicted pC_{50} = -3.104). The putative biologically active (estrogenic) metabolite of PCB52, 2,2′,5,5′-tetrachloro-4-biphenylol, was predicted to be slightly more potent than the parent (pC_{50} = -3.011). The conformationally restricted and *para*-substituted analog PCB153 was predicted to be much more potent than PCB52 with a predicted pC_{50} value of -2.613.

Discussion

Results from *in vivo* uterine weight assays indicate that the *ortho*-substituted PCB52 acts as an estrogenic compound (27). This activity may be manifest through a putative hydroxylated metabolite binding competitively to the estrogen receptor. PCB77, a dioxinlike coplanar biphenyl, was discovered to produce antiestrogenic responses (27). In this case, the antiestrogenicity may be mediated by induction of estrogen metabolizing enzymes (28), through down-regulation of estrogen receptors (29), or decreased affinity of the ligand:receptor complex for the DNA estrogen response element (30), rather than competitive inhibition at the estrogen receptor. This non-*ortho*-substituted PCB was modeled in the non-coplanar conformation which possibly accounts for the relatively "active" predicted value. The low-energy barrier to rotation about the twist bond assures the existence of the coplanar conformation of this molecule *in vivo*. As such, this molecule is a competitive ligand for the Ah (dioxin) receptor, and it is therefore suggested that the predicted estrogen receptor affinity is an overestimate and not truly reflective of actual estrogenic or antiestrogenic potency of the compound.

The low binding affinity prediction for PCB52, the conformationally restricted yet non-*para*-substituted test set molecule, is presumably due to the lack of a *para*-substituent. The increase in the predicted affinity value for the *para*-hydroxylated derivative (2,2,′,5,5′-tetrachloro-4-biphenylol) supports this conclusion. It is important to note that none of the training set molecules possesses a single chlorine substituent adjacent to the hydroxyl moiety of the phenol ring. This may have implications regarding the predictive power of the model with respect to compounds exhibiting this substitution pattern. It has, however, been shown that halogenation of the phenolic (A) ring of estradiol in the corre-
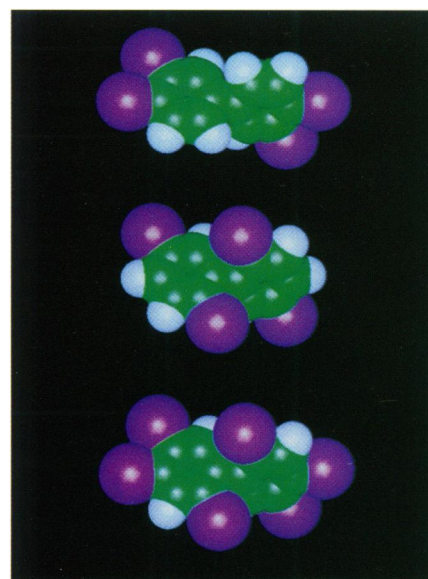

Figure 2. PCB analogs included in test set. Top: PCB77; middle: PCB52; bottom: PCB153.

sponding positions (2 or 4 substituted) does not diminish, and in certain cases enhances, the estrogen receptor binding ability of the ligand (31). In summary, the model suggests that biphenyls possessing both *ortho*- and *para*-substituents, characteristics of environmentally persistent PCB residues, may be competitive ligands of the estrogen receptor.

The model confirms the use of the phenolic (A) ring alignment in comparing various potential estrogen receptor ligands of environmental origin. This approach could be particularly useful when applied to additional hydroxylated and halogenated metabolites or transformation products of certain phenyl-substituted chemicals of environmental concern (32). As previously reported (33) and supported here, the phenolic ring moiety is not a necessary requirement for estrogen receptor affinity. Further binding studies, ideally on the nonhydroxylated PCBs predicted in this report, are needed to assess the unique role and contribution of the hydroxyl group. Additionally, work is needed to assess the role and importance of solubility in the assay medium and molecular hydrophobic properties in estrogenic activity of such chemicals, especially in comparing *in vitro* and *in vivo* results. In some cases the *in vitro* binding results may be complicated by desolvation energy differences associated with the hydroxyl group and its hydrogen bonding properties (34). If the hydroxyl group is shown not to be a unique requirement in such structures, it may be possible to extend this approach to a more structurally diverse range of conformationally restricted phenyl-substituted chemicals, such as the chlorinated diphenylethers.

It is also important to acknowledge

that the available training data set is heavily weighted toward unsymmetrical chlorination patterns. The environmental/biological relevance of several of these compounds is questionable (35,36). In the final model, only two compounds (5 and 10) possessed a chlorine substituent on the phenolic ring. Studies are currently underway in our laboratory to explore the effects of substitution patterns on the phenolic ring. Special emphasis is being placed on the effects of chlorine adjacent to the hydroxy group. The more highly chlorinated compounds of this type are biologically persistent due to increased fat solubility (relative to congeners possessing fewer chlorines) and possibly resistance to metabolic transformations (e.g., glucoronide conjugation) due to steric blocking (11).

While the approach presented here is a useful tool for predicting the estrogen receptor binding affinity of unknown compounds, it does have a caveat in the form of the limited availability of compatible binding assay data. Further refinement of the model will continue as the data become available. Ultimately, it is anticipated that the model will assume one of two forms. Using binding data of the form used in the development of the model (C_{50} values for displacement of estradiol from the estrogen receptor) coupled with data indicating whether the given ligand induces either estrogenic or antiestrogenic effects *in vitro* or *in vivo*, the model could be logically divided into two models. One model would be constructed for estrogenic compounds (agonists), another for compounds that demonstrate antiestrogenic responses through competitive antagonism at the estrogen receptor. Clearly, the existence of mixed agonists/antagonists must be considered in these refined models. It is possible to include compounds displaying this pharmacological profile in both, or neither, models. The underlying hypothesis is that mixed agonists/antagonists possess structural features distinct from or common to the pure agonists/antagonists structures which would be identified and quantified by the CoMFA QSAR model. The results of these models, in the form of the three-dimensional CoMFA contour plots, would be compared to highlight structural differences of the binding domain of the receptor which are induced upon complexation with estrogenic and antiestrogenic compounds. These results will be used in conjunction with protein homology modeling studies to aid in the refinement and validation of models of the hormone binding domain of the estrogen receptor (37).

In conclusion, PCBs and related compounds are ubiquitous environmental contaminants. Traditionally, the most significant toxicological action of members of these classes of compounds has been attributed to their resistance to metabolic degradation and their ability to achieve coplanar conformations. The present study suggests that the non-coplanar PCBs and related compounds should be considered as potential environmental toxicants due to their interaction with hormone receptors. Non-coplanarity, manifest through *ortho*-substitution, increases the steroidlike (more rigid) structural nature of the PCBs, contributes to the overall hydrophobic bulk structure, and possibly serves to inhibit any competitive coplanar-type binding activity (dioxin-like). The technique described here has been proven to be a useful tool for the prediction of the estrogen receptor binding affinities for a variety of structurally related chemical compounds. Currently underway in our laboratory is the development of a CoMFA model including less structurally related molecules. It is anticipated that models of this type may provide the foundation for the description of the toxicological activity of ostensibly structurally diverse, phenyl-substituted hydrocarbons of environmental concern in terms of estradiol equivalents.

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Society of Toxicology
Reproductive and Developmental Toxicology Subsection
Graduate/Postdoctoral Student Award

We announce our intention to make awards of recognition for the best platform and/or poster presentation by graduate students or postdoctoral fellows in the areas of reproductive and developmental toxicology at the 1996 Annual Meeting of the Society of Toxicology, which will be held in Anaheim, California on March 10–14. General areas of research can include female or male reproductive toxicology, reproductive endocrine toxicology, teratology/developmental toxicology, and/or postnatal functional assessment. Candidates for these awards should send to the address listed below, by November 1, 1995, a copy of the abstract that is being submitted to the Society for this meeting. An outline of the talk or a copy of the poster material should also be included if possible, to assist the judges.

The abstracts and posters should describe original research which may include applied studies, investigations of mechanisms of toxic response, or studies of basic biochemical, physiologic, or genetic mechanisms of action. Interested individuals may request Society information and abstract forms from the address below. All submitted material will be treated as confidential. The winning presentations will be announced at the Annual Meeting of the Specialty Subsection in Anaheim. For further information, please contact:

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