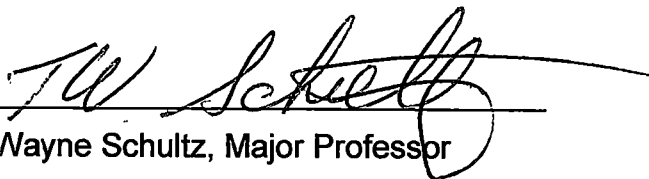


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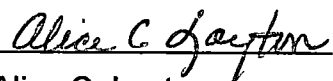
I am submitting herewith a thesis written by Elizabeth Louise Hamblen entitled "Estrogenic Activity of Selected Anilines Evaluated with a Recombinant Yeast Assay." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Ecology and Evolutionary Biology.


T. Wayne Schultz, Major Professor

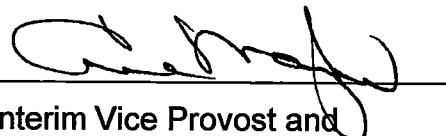

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Interim Vice Provost and
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**ESTROGENIC ACTIVITY OF SELECTED ANILINES EVALUATED
WITH A RECOMBINANT YEAST ASSAY**

**A Thesis
Presented for the
Master of Science Degree
The University of Tennessee, Knoxville**

**Elizabeth Louise Hamblen
May 2001**

DEDICATION

This thesis is dedicated to my parents, Dianne and Sam. Their love and support have meant the world to me. Their sacrifice for my education was selfless and so much appreciated. Their constant encouragement and unconditional love have shaped my life, and their pride in me has meant more than they can ever know. I love them both with all of my heart, and thank them for being the best parents I could ever hope to have.

ACKNOWLEDGEMENTS

The writer wishes to express sincere appreciation to the members of her committee, Dr. Thomas Hallam, Dr. Alice Layton, and especially her major professor, Dr. T. W. Schultz, who so willingly gave of his time and energy in the preparation of this thesis.

Additional appreciation is extended to Glendon Sinks, whose experience and willingness to assist was invaluable in the research, and to Julie Seward for her assistance in reviewing the manuscript. Dr. Mark Cronin's assistance in modeling and descriptors was also extremely helpful.

The author acknowledges the Center of Excellence in Livestock Disease and Human Health and the Department of Ecology & Evolutionary Biology for financial assistance.

Appreciation is extended to Wanda Aycock for assistance with preparing this document.

ABSTRACT

Suspected endocrine disrupters range from natural plant constituents (i.e., phytoestrogens) to industrial organic chemicals (i.e., xenoestrogens), including alkylphenols, bisphenols, polychlorinated biphenyls, and some pesticides. Several structural features are important for estrogenic activity, including a free benzene ring with a polar (e.g., hydroxy) group and a hydrophobic group. The present study was undertaken to explore the estrogenic activity of chemicals containing an amino group on the benzene ring instead of a hydroxy group. There is a great deal of chemical similarity between anilines and phenols, including close proximity in an 8-dimensional hyperspace describing the chemical universe and similar hydrogen-bonding properties. The chemicals chosen for the current study contain a benzene ring with an amino group in the *para*-position. The hydrophobic constituents of the compounds vary in size and shape to explore variations in potency due to the configuration of the hydrophobic group. The study consisted of the following aims: i) generate estrogenic activity and acute toxicity data for a group of 37 substituted anilines utilizing the *Saccharomyces cerevisiae* recombinant yeast assay, ii) calculate hazard indices for each of these chemicals, and iii) compare estrogenic activity between the present group of anilines and a set of previously tested, analogous phenols also tested in the yeast assay. The results indicate that anilines are less estrogenic than phenols, and that acute toxicity often precludes an aniline's ability to induce estrogenic activity. While the natural estrogen 17- β -estradiol is active in the 10^{-10} range, the highest tested aniline had an EC_{50} of 10^{-6} , a difference of four orders of magnitude. Because of their low estrogenicity and acute toxicity, therefore, anilines on average are only very weak environmental estrogens.

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CHAPTER 1

INTRODUCTION

The occurrence of organismal endocrine disruption by xenobiotics is receiving much attention due to the implications in both wildlife and human health. The problem was first reported in the 1970's by authors such as Bitman and Cecil (1970) and Nelson et al. (1978). The 1980's saw increased interest in endocrine disruption (e.g., McLachlan, 1980, McLachlan, 1985), and the 1990's found the focus intensified. During this past decade, many review articles regarding the subject have been written, including Fox (1992), Reijnders and Brasseur (1992), Kavlock et al. (1996), Crain and Guillette (1997), Kavlock (1999), and Taylor and Harrison (1999). Suspected endocrine disrupters include natural plant constituents (i.e., phytoestrogens), industrial organic chemicals (i.e., xenoestrogens), and some pesticides (Waller et al., 1996).

Despite a plethora of circumstantial evidence, a direct link between observed cases and exposure to endocrine-disrupting chemicals has rarely been established (Taylor and Harrison, 1999). In addition, for most cases in which a link does appear to be evident, the specific chemicals and mechanisms involved are unknown (Taylor and Harrison, 1999). The two most established cases for endocrine disruption caused by xenobiotics involve alligators in DDT-polluted Lake Apopka, Florida as reported by Guillette and colleagues (e.g., Guillette et al., 1994), and marine gastropod reproductive effects caused by exposure to tributyltin (see for example Reijnders and Brasseur, 1992).

The main function of the endocrine system is to secrete and regulate hormones in animals and plants (US EPA, 1997). Hormones are regulated through receptors, which are proteins that interact with signaling molecules like hormones to introduce physiological changes. Some functions of receptors include transmitting information between cells, transfer of cellular environmental signals to intracellular targets, and controlled transportation of specific substances.

Endocrine disrupters are chemicals that alter the normal functioning of the endocrine system (Crain and Guillette, 1997). They may act by a variety of mechanisms. For example, (1) endocrine disrupters may bind to receptors meant for endogenous hormones, either activating or blocking them and thus rendering them inactive, (2) they may create additional receptors, which will increase hormonal activity, (3) they may interact with endogenous hormones, altering hormonal messages, and (4) they may affect hormone synthesis, creating an imbalance of circulating hormones (Schultz and Seward, 2000). Endocrine disruption can be described as having two categories of effects: organizational and activational (Crain and Guillette, 1997). A permanent modification of the function of a tissue occurring in a sensitive stage of development is said to be organizational. A temporary alteration of a normally organized tissue is activational (Crain and Guillette, 1997).

Exposure to a chemical is necessary for a toxic response. Important aspects of exposure include duration, route, and bioavailability of the chemical. Disposition, the net result of absorption, distribution, and biotransformation is determined by toxicokinetics. Toxicokinetics determine the probability of reaching the site of action. The toxicodynamic phase, which determines the

scope of the interactions between the toxicant and the site of action, occurs once the site of action has been reached. A biochemical response occurs from the interaction, leading to numerous physiological responses, eventually at the whole-organism level. This interaction can be either continuous or discontinuous, as in the case of receptor-mediated toxicity. Endocrine disrupters operate as receptor-mediated toxicants. Unlike acute toxicity, in which the toxicokinetics is the most important determinant of toxicity, receptor-mediated toxicity is determined by toxicodynamics.

Receptor-mediated toxicity is a "functional toxicity" (McLachlan, 1993). As such, the receptor structure dictates unique requirements for the chemical domain. Instead of physico-chemical properties contributing most to modeling efforts, the structural similarity of a specific toxicophore is of most importance. In receptor-mediated toxicity, interaction of the ligand (i.e., xenobiotic) with the receptor is non-covalent, with a "lock and key" type interface. The compound-of-interest must "fit" into the target receptor in order to create a toxic effect. Binding and activation require specific three-dimensional (3-D) conformations that are controlled by stereoelectronic molecular properties, and which restrict the chemical domain. Receptors bind with a ligand based in part on packing (large constituents next to small constituents and vice versa), ionic interactions (neutralization of charges), hydrophobic interactions, and hydrogen bonding (Creighton, 1993). As such, the structure of the ligand is of utmost importance. Binding can be thought of as a "key" (the ligand) fitting into a "lock" (the binding site on the receptor). The "key" must be of the correct shape in order to fit the "lock." Therefore, the ligand, in this case an environmental estrogen, must have the necessary structural features to fit into the estrogen receptor.

Receptor-mediated toxicity is more complicated than non-receptor-mediated toxicity, particularly as there is potential for multiple mechanisms of action. As such, dealing with mechanism concerns is more crucial for receptor-mediated toxicity than for non-receptor-mediated toxicity. Because of the wide variety of mechanisms of action for endocrine disrupters, the problem is indeed quite complex. Organisms may react differently depending on species, developmental stage, part of the body affected, nutritional health, age, genetics, time of year, and other chemicals with which it has had contact (Schultz and Seward, 2000). Based on all of these factors, testing at the organismal level is ambiguous at best. The inability to determine mechanism of action at the whole-animal level is a major reason that surrogate endpoints are of interest. *In vitro* tests, such as the recombinant yeast assay with *Saccharomyces cerevisiae*, simplify the system with an endpoint that is easily understood (i.e., the estrogen receptor complex is activated by binding of the xenobiotic to the estrogen receptor).

Because of the concern of endocrine disrupters in the environment, there has been a push to test large numbers of industrial chemicals for such effects. The Food Quality Protection Act of 1996 (FQPA) and the Safe Drinking Water Act Amendments of 1996 (SDWA) have requirements that the US EPA develop a program to screen substances for endocrine effects in humans (Kavlock, 1999). This raises the question of how to test so many chemicals quickly and efficiently using appropriate test systems. Whole animal testing (i.e., *in vivo* testing) is expensive and time-consuming. Thus, smaller scale *in vitro* testing is desirable, being less expensive and generally shorter in duration. Often, binding assays are employed, which measure the binding of a substance to a receptor (e.g., a human estrogen receptor). Predictive models are based on such binding data;

however, these data cannot distinguish between receptor antagonists and receptor agonists (Schultz et al., 2000a). Besides binding assays, several other assays have been proposed for screening purposes, including MCF-7 cell proliferation, vitellogenin production in fish, and transfected yeast (Andersen et al., 1999). One such assay involves a yeast strain transfected with the gene coding for the human α -estrogen receptor and a reporter plasmid that encodes for β -galactosidase. Routledge and Sumpter (1996) pioneered the use of such an assay with the Glaxo-Wellcome-derived *S. cerevisiae*. The assay has a 0.08 nM detection limit, a 10,000-fold responsiveness, is short in duration, and is low in cost, all of which make it an excellent candidate for screening (Schultz et al., 2000a). In addition, the recombinant yeast assay allows for determination of acute toxicity as well as receptor-mediated toxicity (Schultz et al., 1998). This assay was utilized in the present study.

Surrogate systems are often useful when multiple mechanisms of action are probable, and are based on measurement endpoints instead of assessment endpoints. Assessment endpoints are the individual physiological responses *in vivo*. Measurement endpoints are responses to a toxicant that measure or describe a property classified by the assessment endpoint (Schultz and Seward, 2000). For example, in the present study, the assessment endpoint is endocrine disruption, while the measurement endpoint is the colorimetric change of chlorophenol red- β -D-galactopyranoside (CPRG) due to the binding and subsequent activation of the estrogen receptor. The measurement endpoint must accurately describe the assessment endpoint with both relevance and consistency (Schultz and Seward, 2000). Relevance is the degree to which a measurement endpoint relates to the assessment endpoint (Schultz and Seward,

2000). The recombinant yeast assay is relevant, in that the CPRG color change is a direct reflection of binding and activation of the estrogen receptor.

Consistency means that the measurement endpoint is exposed to the toxicant in a similar manner as the assessment endpoint (Schultz and Seward, 2000). The recombinant yeast assay is consistent in that the chemicals under study were exposed to and bound with an estrogen receptor in order to elicit a response, just as these substances would bind to a receptor *in vivo*.

The primary event leading to estrogenic activity is binding to the estrogen receptor. The receptor is an intracellular protein found in the nucleus that is inactive in its apoprotein form. Binding to a suitable ligand (e.g., an estrogen) causes conformational changes, which initiates binding to the estrogen response element (ERE) in the DNA, activating transcription. Natural estrogens are generally polycyclic molecules that are rigid and asymmetrical. Figure 1 demonstrates the structure of the native steroidal hormone 17- β -estradiol, including its polarized phenolic A-ring, which has been suggested as the key to estrogenicity (Tabira et al., 1999). Crystal structures of the bound receptor-ligand complex indicate that the A-ring is the part of the hormone that is in most direct contact with the receptor pocket.

In the case of substances which bind to the estrogen receptor, recent studies by Routledge and Sumpter (1997), Routledge et al. (1998), Schultz et al. (1998), and Schultz and Sinks (2001a) have revealed that several structural features are important for interaction with the estrogen receptor (reviewed in Schultz et al., 2000a). First of all, a free benzene ring with a polar (e.g., hydroxy) group is

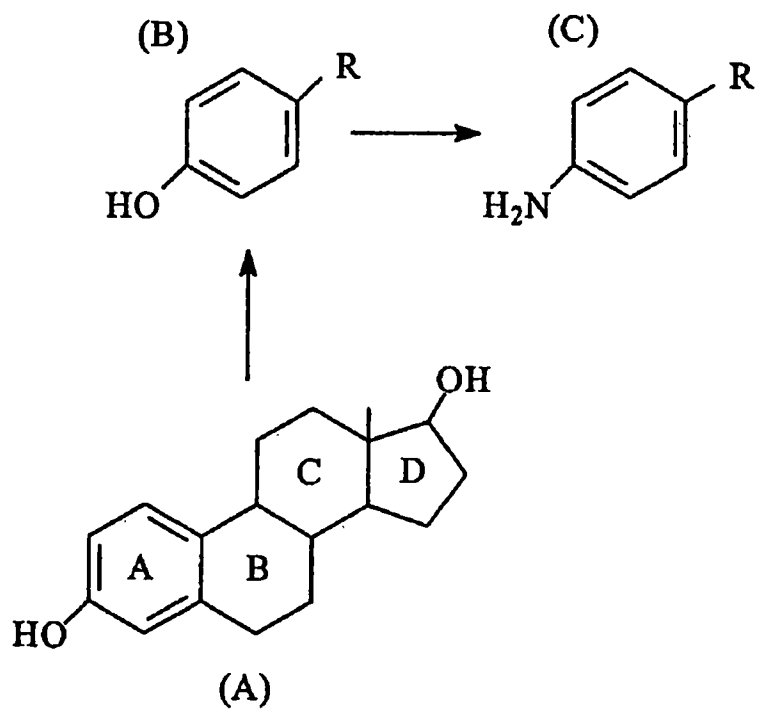


Figure 1: Structural similarities between potential estrogens. (A) 17-β-estradiol, including the A-, B-, C-, and D-rings. (B) Generic phenol structure. (C) Generic aniline structure.

required, with activity increasing as this group moves from the *ortho*- to the *meta*- to the *para*-position. This feature directly corresponds with the phenolic A-ring in 17- β -estradiol. In addition, a bulky hydrophobic group is necessary, with six or seven carbons providing optimal activity. Activity decreases moderately with C5 or C4 groups, decreases dramatically with C3, and is negated with a C2 or C1 group. Activity also decreases for C8 groups and above. Branched groups, especially *tert*-branched groups, increase activity. Therefore, in the case of xenoestrogens, the "key" appears to be a polar group attached to a free benzene ring. This group is essential in order for binding to occur. The remainder of the molecule (i.e., the hydrophobic constituent) determines potency.

The present study was undertaken to explore the estrogenic activity of chemicals containing an amino group on the benzene ring instead of a hydroxy group, as recommended by Schultz et al. (2000a) (see Figure 1). There is a great deal of chemical similarity between anilines and phenols. In an 8-dimensional hyperspace describing the chemical universe, Veith et al. (1988) found anilines and phenols to be immediately proximal, instigating the "guilt by association" and "nearest neighbor" phenomena (R. S. Hunter, personal communication), under which chemicals with similar structures are expected to have similar properties, and therefore behave in a similar manner. Veith et al. (1988) selected a set of 19,972 chemicals of known structure, and calculated molecular connectivity indices for each. Connectivity indices provide a method of using graph theory to quantitate variation in chemical structure (Veith et al., 1988). They then calculated the first 10 orders of indices, using principal component analysis to analyze the data. Eight principal components were found to be significant and were plotted (four at a time) to display the chemical

universe. The end result is the ability to identify analogs by proximity in eight dimensions.

In addition, both hydroxy and amino groups are electron-releasing groups with similar electronegativities (Fessenden and Fessenden, 1994). Both are capable of forming hydrogen bonds, which may be important in receptor binding. The fact that a polar group is required on the benzene ring for binding to the estrogen receptor strongly suggests that hydrogen bonding is a factor in recognition between the ligand and the receptor. It is the strong hydrogen-donor properties of hydroxy groups that contribute most to their hydrogen-bonding capabilities (Fang et al., 2001). Hydroxy groups form stronger hydrogen bonds than do amino groups because -OH is a stronger hydrogen donor than is -NH_2 (Fang et al., 2001). As a result, a benzene with an amino group would be expected to show decreased binding with a receptor dependent upon hydrogen bonding. Therefore, anilines would be expected to have less activity than phenols.

The chemicals chosen for the current study all contain a benzene ring with an amino group in the *para*-position. The hydrophobic constituents of the compounds vary in size and shape to explore variations in potency due to the configuration of the hydrophobic group.

CHAPTER 2

ESTROGENICITY OF ANILINES EVALUATED WITH A RECOMBINANT YEAST ASSAY

Introduction

Steadily increasing attention has been paid to the occurrence of endocrine disruption by xenobiotics over the last several decades, due mostly to its implications in wildlife and human health (Bitman and Cecil, 1970; McLachlan, 1980; Reijnders and Brasseur, 1992; Crain and Guillette, 1997; Kavlock, 1999). Endocrine disrupters can be composed of many different structural components, ranging from natural plant constituents (i.e., phytoestrogens) to industrial organic chemicals (i.e., xenoestrogens), including alkylphenols, bisphenols, and polychlorinated biphenyls (Waller et al., 1996).

Endocrine disrupters operate as receptor-mediated toxicants (Schultz and Seward, 2000). Receptor mediated effects inherently presuppose a ligand-receptor interaction. Such interactions are non-covalent and based in part on packing, ionic interactions, hydrophobic interactions, and hydrogen bonding (Creighton, 1993). The most obvious 2-dimensional (2-D) structural feature required for estrogenic activity is the presence of a polarized phenolic moiety similar to the A-ring of the steroidal estrogen 17- β -estradiol (Tabira et al., 1999; Fang et al., 2001).

Recent legislation has mandated large-scale testing of industrial chemicals for endocrine-disrupting effects. Since testing at the organismal level is ambiguous at best due to the wide variety of mechanisms of action, there is

interest in using *in vitro* assays (Schultz and Seward, 2000). Fang et al. (2000) has quantitatively compared the three major *in vitro* assays for estrogenic activity and found the recombinant yeast-based reporter assay was as good as the estrogen receptor competitive binding and the MCF-7 cell proliferation assays in identifying xenoestrogens. The Glaxo Wellcome-derived *Saccharomyces cerevisiae* strain assay measures estrogenicity as β -galactosidase activity (Routledge and Sumpter, 1996). This is an *in vitro* model that allows for the sensitive, rapid, and inexpensive screening of chemicals for estrogen modulating activity with an easily understood measured endpoint. Investigations using this assay to assess estrogenic activity include studies of alkylphenols (Routledge and Sumpter, 1997; Schultz et al., 2000b).

Steroidal estrogens are polycyclic molecules that are rigid and asymmetrical. Specific 2-D structural features of 17- β -estradiol deemed important for potency include a phenolic A-ring, a D-ring, 7- α - and/or 11- β -substitution, and a hydrophobic backbone (Schultz and Sinks, 2001b; Fang et al., 2001). It appears that any combination of these structural alerts results in a xenoestrogenic activity.

Anilines and phenols contain a number of structural similarities. Veith et al. (1988) constructed an 8-dimensional hyperspace describing the chemical universe. This space used eight descriptors of chemical structure to explain better than 90% of the molecular variability. It is the structure of a molecule that accounts for its properties. Moreover, it is these properties that account for a molecule's toxicity. Molecules with similar structure have similar properties and thus the potential for similar toxicity. In the 8-D space of Veith et al. (1988), the nearest neighbor to phenol is aniline (R. S. Hunter, personal communication).

Routledge and Sumpter (1997) noted that both the position and branching of the substituent affects the estrogenic potency of alkyl-substituted phenolic compounds. Specifically, optimal activity is associated with a tertiary-branched alkyl moiety of 6 to 8 carbon atoms substituted in the *para*-position (Routledge and Sumpter, 1997). Therefore, it is hypothesized that since the nearest neighbor to phenol is aniline, anilines with an alkyl substituent in the *para*-position may also elicit estrogenic effects.

The objective of the current study was to test this hypothesis. Pursuit of this objective consisted of the following aims: i) generate estrogenic activity and acute toxicity data for a group of 37 substituted anilines utilizing the *Saccharomyces cerevisiae* recombinant yeast assay, ii) calculate hazard indices for each of these chemicals, and iii) compare estrogenic activity between the present group of anilines and a set of previously tested, analogous phenols also tested in the yeast assay.

Methods and Materials

Test Strain

The recombinant yeast strain *S. cerevisiae*, developed by Glaxo Wellcome (Routledge and Sumpter, 1996), was used to measure the estrogenicity of a series of anilines. In this strain, the main yeast chromosome containing cDNA of the human α -estrogen receptor (hER- α). In addition, the yeast cells contain the *lac-Z* reporter gene on expression plasmids, preceded by estrogen responsive sequences, which bind to the product of the hER- α . When estrogen or a xenoestrogen bind to the estrogen receptor, transcription of the *lac-Z* reporter gene ensues. The *lac-Z* reporter gene codes for the enzyme β -galactosidase. β -

galactosidase activity is measured colorimetrically as it metabolizes the yellow chlorophenol red- β -D-galactopyranoside (CPRG) into chlorophenol red, which exhibits a maximum absorbance at 540 nm.

Media and Stock Cultures

Minimal media included 13.61 g KH_2PO_4 , 1.98 g $(\text{NH}_4)_2\text{SO}_4$, 4.2 g KOH pellets, 0.2 g MgSO_4 , 1 mL FeSO_4 solution (40 mg / 50 mL H_2O), 50 mg L-leucine, 50 mg L-histidine, 50 mg adenine, 20 mg L-arginine HCl, 20 mg L-methionine, 30 mg L-tyrosine, 30 mg L-isoleucine, 30 mg L-lysine HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 150 mg L-valine, and 375 mg L-serine added to 1 L sterile water. The solution was placed on a heated stirrer to ensure homogenization, and was then sterilized at 121°C for 10 minutes. The minimal media was stored at room temperature.

A solution of 20% w/v D-(+)-glucose was prepared by adding 100 g glucose to 500 mL sterile water. A 4 mg/mL solution of L-aspartic acid was prepared by adding 0.5 g L-aspartic acid to 125 mL sterile water. Moreover, a 24 mg/mL solution of L-threonine was prepared by adding 3.0 g L-threonine to 125 mL sterile water. These solutions were sterilized at 121°C for 10 minutes and stored at room temperature, except for L-threonine, which was stored at 4°C.

A vitamin solution including 8 mg thiamine, 8 mg pyridoxine, 8 mg pantothenic acid, 40 mg inositol, and 20 mL biotin solution (2 mg / 100 mL sterile H_2O) added to 180 mL sterile water was prepared. A 20 mM solution of copper (II) sulfate was prepared by adding 0.200 g copper (II) sulfate to 50 mL sterile water. In addition, a 10 mg/mL solution of CPRG was prepared by adding 0.05 g CPRG to 5.0 mL sterile water. Lastly, a 1% antibiotic solution was prepared by adding 0.1 g ciprofloxacin HCl to 9.9 mL sterile water. These four solutions were

sterilized by filtering through a 0.2 μ m pore size 25 mm disposable filter, and were stored at 4°C except for copper (II) sulfate, which was stored at room temperature.

The growth medium was prepared by adding 2.5 mL glucose solution, 0.5 mL L-aspartic acid solution, 0.25 mL vitamin solution, 0.2 mL L-threonine solution, and 62.5 μ L copper (II) sulfate solution to 22.5 mL minimal medium. Twenty-five μ L of antibiotic solution was added to prevent bacterial growth.

Assay medium was prepared by adding 0.5 mL CPRG to 50 mL fresh growth medium (without antibiotic solution). This medium was seeded by adding 0.4 mL yeast from a 24-hour culture (absorbance at 620 nm of approximately 1.0).

To prepare 10X concentrated yeast stock, 24-hour culture was transferred to a 50 mL centrifuge tube. The cultures were centrifuged at 4°C for 10 minutes at 2,000 g. The supernatant was decanted, and the remaining culture was resuspended in 5 mL of minimal medium with 15% glycerol (1.25 mL of 60% glycerol). Aliquots of 0.5 mL of the 10X concentrated stock culture were transferred to 1.5 mL cryogenic vials.

Yeast stock was prepared by placing growth medium components in a sterile conical flask (final volume of approximately 50 mL) and adding 125 μ L of 10X concentrated yeast stock stored at -20°C. The solution was then incubated at 28°C on an orbital shaker.

Test Chemicals

The compounds tested and used as media components were obtained from Aldrich Chemical Company (Milwaukee, WI, USA), Lancaster Synthesis Inc., (Windham, NH, USA), and Sigma-Aldrich Co. (St. Louis, MO, USA). None were

repurified prior to use. Chemicals tested included alkyl- and alkyloxy-anilines with alkyl-carbons numbering from one to 12, amino-substituted biphenyls, bisanilines, and amino-substituted polycyclic aromatic hydrocarbons. All possessed a common molecular substructure – a polarized anilinic ring.

Analyses

Assays were set-up in a type II laminar flow cabinet. Stock solutions were made in ethanol. Chemicals were serially diluted in absolute ethanol in a 96-well microtiter plate for a total of 12 concentrations. Ten μL aliquots of each concentration were transferred to a second 96-well optically flat bottom microtiter plate. One row was left between each chemical to prevent a “creeping” effect (Beresford et al., 2000). Chemicals were left in the assay plate to evaporate to dryness (approximately 30 minutes). Seeded assay medium containing CPRG was added to the wells in 200 μL aliquots. Each plate contained a 2-fold serial dilution of a positive control of 17- β -estradiol (initial concentration of $2\text{e-}7$) in duplicate. The plates were sealed with Parafilm and were stored in a Fisher Scientific Isotemp Plus CO_2 incubator at $30 \pm 1^\circ\text{C}$. The plates were agitated daily to mix and disperse the growing cells. The absorbance at 540 nm was measured on a Packard SpectraCount plate reader at 3, 5, and 7 days. Positive wells appeared a deep magenta color, accompanied by turbid yeast growth. Clear yellow wells indicated cell lysis caused by chemical toxicity. Yeast growth (cell density) and mortality was measured at 620 nm, and was read at 3, 5, and 7 days. Average cell density of the seeded medium was 4×10^3 cells/ml. This was obtained by diluting 2 mL seeded medium in 50 mL 10% isotone and analyzing on a Culture Counter ZM with sampling stand and channelizer. Each chemical

was evaluated in duplicate, with at least two replicates. Each replicate included 17- β -estradiol as the positive control. A negative control of sterile water was included upon replication for all chemicals displaying estrogenic activity.

EC₅₀ values were determined, representing the molar concentration where observed activity was approximately 50% of the positive control. LC₅₀ values, representing the molar concentration causing 50% lethality in the positive control, were also determined. A Hazard Index was determined for each chemical with an EC₅₀ and LC₅₀ on day 5, and was calculated as the ratio of the EC₅₀ to the LC₅₀. In addition, comparisons were made between aniline estrogenic activity and estrogenic activity of equivalent phenols. Data were obtained from the work of T. W. Schultz.

Molecular Descriptor and Activity Modeling

A value for the hydrophobicity term (logarithm of the 1-octanol/water partition coefficient, log K_{ow}) for each compound was secured from the ClogP for Windows (version 1.0) software (Biobyte Corp., Claremont, CA). To calculate steric, topological, and molecular orbital descriptors, chemical structures were input as 2-D SMILES codes (Weininger, 1988) into the TSAR (version 3.2) molecular spreadsheet (Oxford Molecular Ltd., Oxford, England). The CORINA (version 3.21) program (Oxford Molecular Ltd., Oxford, England) converted the 2-D structures into reasonable 3-D conformations. These 3-D structures were energy minimized using the COSMIC forcefield in TSAR. The 3-D structure of each molecule was subsequently optimized using the VAMP (version 6.5) (Oxford Molecular Ltd., Oxford, England) molecular orbital package utilizing the AM1 Hamiltonian. For each energy-optimized molecule, the substituent was defined such that aniline was the parent molecule.

Molecular connectivities, Kappa indices and assorted topological parameters including Sterimol parameters (Verloop et al., 1976) were calculated by the TSAR spreadsheet from the 2-D structure. Molecular orbital properties were calculated from the VAMP molecular orbital package following energy optimization. Calculation of further steric attributes was undertaken following the energy minimization process in VAMP. The 2-D property values were calculated for the substituent.

Statistical analysis was performed using the MINITAB for Windows (version 12.1) statistical software (MINITAB Inc., State College, PA). Quantitative relationships between the estrogenic activity and lethality and the structural properties were developed using stepwise regression analysis. $\text{Log (EC}_{50}^{-1})$ or $\text{Log (LC}_{50}^{-1})$ in M was taken as the dependent variable, while the structural parameters were used as the independent variables. Model fit was quantified with the r^2 value (coefficient of determination) adjusted for degrees of freedom. The s value (root of the mean square for error) in addition to the F value (Fischer statistic) and the probability greater than the F value ($\text{Pr} > F$) of each model were also reported.

Results

Estrogenicity

Three-, 5-, and 7-day estrogenic activities and ratios to the 17- β -estradiol positive control are presented for the 37 aniline derivatives in Table 1*. Eight chemicals (8, 17, 19, 21, 22, 23, 25, 37) showed an EC_{50} on day 3. Seven

* Tables located in Appendix

additional chemicals (3, 4, 5, 6, 13, 20, 29) were active after 5 days. Additionally, five chemicals (2, 14, 18, 27, 36) were active on day 7. No EC₅₀ was found for seventeen chemicals. The average absorbences at 540 nm for the negative control (i.e., background) were 0.737 +/- 0.043 on day 3, 0.804 +/- 0.040 on day 5, and 0.877 +/- 0.086 on day 7. A "No EC₅₀" notation was assigned to a derivative for which the estrogenic activity was above background but less than the EC₅₀ value. Sixteen compounds at day 5 fell into this group. Moreover, "not active" was assigned for compounds exhibiting activities at or below background levels. Six compounds, including many of the chemicals not substituted in the *para*-position, fell into this group. While activity varied between days 3, 5, and 7, ratios to the positive control were similar (Table 1). Attempts to model estrogenic potency with a variety of molecular descriptors proved unsuccessful.

Lethality

LC₅₀ data ranged from 1.01e-03 for 4-*tert*-butylaniline to 8.13e-05 for 4-pentylaniline (Table 1). No LC₅₀ was found at saturation for the hydrophobic 4-dodecylaniline, 2-aminoanthracene, and 1-adamantanamine as well as 4,4'-diaminobenzophenone. All acute toxicity was attained by day 3, as the LC₅₀ values for day 3, 5, and 7 were the same. With the exception of three significant outliers (4-decylaniline, 9-aminophenanthrene, and 4-aminophenyl disulfide), the LC₅₀ can be modeled [$r^2 = 0.80$, $s = 0.37$, $F = 108.21$, $Pr > F = 0.000$] as follows:

$$\text{Log} (1 / \text{LC}_{50}) = 1.32 + 0.77 \log K_{ow}.$$

Hazard Index

A 3, 5, and 7-day Hazard Index was determined for each chemical with an EC₅₀ on day 5 (Table 2), with the exception of 4-aminodiphenylamine (chemical

25), for which no LC_{50} could be determined due to a color change which interfered with the 620 nm readings. All Hazard Indices were single digits or less except for 2-aminofluorine, which had a Hazard Index of 47.3 on day 5.

Derivative Comparison

A comparison of 5-day estrogenic potency of the amino-substituted compounds and their hydroxy-substituted counterparts are presented in Table 3. Of the 23 derivatives directly comparable, two (4-ethyl and 1-adamantan-substituted) had no EC_{50} as either the phenolic or anilinic homolog, while one derivative (2-adamantan-substituted) was active as the amino-substituted compound but not as its hydroxy-substituted counterpart. Eight derivatives (9, 10, 14, 15, 16, 18, 34, 35) were active as the hydroxylated substance but not active in the amino-isomer. Of these, five (9, 10, 14, 15, 16) have straight-chain substituents of six atoms or greater (carbon and/or oxygen). The three additional chemicals (4-benzyloxy, 6-substituted-quinoline, and 1-substituted-pyrene) also have bulky substituents.

Of the 12 derivatives (4, 5, 6, 8, 13, 17, 19, 20, 21, 22, 25, 29) that were active at day 5 in both the phenolic and anilinic forms, four (4, 5, 21, and 25) had activities in the same order of magnitude. Two of these derivatives (4-*sec*-butyl and 4-*tert*-butyl) are four-carbon, branched substituents. The hydroxy-substituted compound was greater than its amino-substituted counterpart by one order of magnitude for five compounds (6, 13, 17, 19, 22). Three of these (4-pentyl, 4-butoxy, and butyl-4-substituted-benzoate) have a chain of five atoms (carbon and/or oxygen) as part or all of the substituent. The other two chemicals, 4-phenoxy and 4-substituted-biphenyl, have similar bulk to these three, both containing a benzene ring in the substituent. The chemicals 4-cyclohexyl and

4,4'-di-substituted-benzophenone are more active by two orders of magnitude in the phenolic form, both containing a cyclic substituent. Lastly, 2-aminofluorene was four orders of magnitude more active as the hydroxylated analog.

Discussion

Research about the estrogenic activity of groups of organic chemicals, including alkylphenols (Routledge and Sumpter, 1997), biphenyls (Schultz et al., 1998), benzophenones (Schultz et al., 2000a), bisphenols (Schultz and Sinks, 2001a), and polycyclic aromatic hydrocarbons (Schultz and Sinks, 2001b) has led to the development of "rules" for the structural components of a potential xenoestrogen. These "rules" include a ring structure containing a moiety capable of hydrogen-bonding, a hydrogen-bond donor with similar O-O distances as in 17- β -estradiol, a duplication of 7 α - and 11 β -substituents with precise steric hydrophobic centers, and hydrophobicity (Fang et al., 2001). The present study was undertaken to determine if the structurally similar anilines could also activate the estrogen receptor.

Eight chemicals were found to be estrogenically active (i.e., they had an EC₅₀) on day 3. Seven additional chemicals were active on day 5. The activity of chemicals beginning on day 5 supports the findings of Schultz et al. (2000b), who submitted that estrogenic activity should be reported after five days instead of the customary three days, due to delayed activity for some chemicals, which may lead to false negatives. The chemical with the highest activity on day 5 was 4-aminobenzophenone. This activity was four orders of magnitude less than the estrogenic activity of 17- β -estradiol. The graph in Figure 2 compares the

estrogenic activity of 17- β -estradiol and 4-aminobenzophenone, along with the activity of 4-heptyloxyaniline, an aniline with a "No EC₅₀" designation.

There were five chemicals that first showed activity at day 7. These results conflict with the findings of Schultz et al. (2000b), who determined that reading at day 7 was not necessary, as the potency ratios at day 5 and day 7 were the same. For the 15 chemicals in the present study that were active on both days 5 and 7, the potency ratios were indeed the same between the two days. That still leaves the five chemicals (4-ethylaniline, 4-pentyloxyaniline, 4-benzyloxyaniline HCl, 2-aminoanthracene, and 1-adamantanamine) that were first active after day 7, however. One possibility for the delayed activity shown by these compounds is delayed uptake by the yeast cells. There do not appear to be structural signals unique to these five chemicals that explain delayed uptake, however delayed uptake could occur due to the ionization of the amino group in the aniline.

The 2-D structural features outlined by Schultz and Sinks (2001b) and Fang et al. (2001) as important to confer estrogenic activity include the following 17- β -estradiol structural alerts: phenolic A-ring, D-ring, 7- α - and/or 11- β -substitution, and a hydrophobic backbone. It appears that any combination of these will result in estrogenic activity. Examples include alignment of the A- and D-ring in phytoestrogens and alignment of the A- and C-ring in bisphenols and benzophenones (Schultz and Sinks, 2001b).

Rules for phenols exhibiting estrogenic activity (Schultz et al., 2000a) state that a 6- to 8-carbon atom substituent in the *para*-position provides maximal activity, and activity is expected at as little as 3 carbons. We would therefore expect estrogenic activity for the anilines with substituents of 4 through 8 carbons in the *para*-position (4-butylaniline through 4-octylaniline). There is indeed

activity for the three isomers of 4-butaniline and for 4-pentaniline; however, 4-hexylaniline, 4-heptylaniline, and 4-octylaniline are not active. The reason appears to be that the acute toxicity of the anilines causes disruption of cellular function, interfering with β -galactosidase production. The LC_{50} of 4-hexylaniline, 4-heptylaniline, and 4-octylaniline is $e-05$, which is at or above the concentration expected to confer activity; therefore, the anilines with greater than 5 carbon atoms are not active due to acute toxicity. The above trend continues with the alkyloxylanilines. Four-butoxylaniline, which is approximately structurally equivalent to 4-pentaniline, shows activity; however, those chemicals with more carbon atoms (i.e., 4-pentyloxylaniline, 4-hexyloxylaniline, and 4-heptyloxylaniline) do not show activity due to acute toxicity at relatively low concentrations ($e-05$, $e-05$, and $e-04$, respectively). Additionally, 4-benzyloxylaniline and N-methyl-4,4'-methylenedianiline demonstrate acute toxicity which precludes expected activity.

Of the chemicals with a cyclic moiety as part of the substituent, most show moderate estrogenic activity, as expected per Schultz et al. (2000a) and Schultz and Sinks (2001b). Cyclohexylaniline, which contains a bulky cyclohexyl ring, is active at $e-05$. Several chemicals contain a benzene ring as the substituent, including 4-aminobiphenyl, in which the aniline is directly attached to the benzene ring, 4-phenoxyaniline, in which the aniline is connected by an oxygen atom, 4-aminobenzophenone, in which the aniline is connected by a carbonyl group, and 4-aminodiphenylamine, in which the aniline is connected by a nitrogen atom. As expected, all of these show activity resulting from the alignment of the two benzene rings with the A- and C-rings of 17- β -estradiol (Schultz and Sinks, 2001b).

Two additional active chemicals have benzene rings as part of the substituent: 4,4'-diaminobenzophenone and 4,4'-methylenedianiline. Both of these molecules are symmetrical, containing two anilines separated by a carbonyl group (for the benzophenone) or a methyl group (for 4,4'-methylenedianiline). These were expected to have increased activity, because the "key" of the aniline is present at both ends of the molecules (i.e., they can "fit" into the receptor from two different directions). However, both of the anilinic derivatives show reduced estrogenic activity (e-04) on day 5. One possible explanation is that the ionized amino group on the far side of the molecule causes an electrostatic alteration of the ligand-receptor interaction, interfering with binding to the receptor (Schultz and Sinks, 2001b).

Several of the anilines were not active due to an inability to align with the rings of 17- β -estradiol. This includes 4-aminophenyl disulfide and all of the polycyclic amines. As expected, the polycyclic quinolines 3-aminoquinoline and 6-aminoquinoline, which consist of two attached benzene rings with a nitrogen in one ring were not active with the nitrogen skewing the electrons in the ring containing the amino group. One-adamantanamine was not active, as expected due to the bulk of the molecule and the inability to align with any 17- β -estradiol rings. Surprisingly, 2-adamantanamine was active. An explanation for this unexpected activity is lacking at this time.

The Hazard Indices (Table 2) further support the above findings that acute toxicity often precludes an aniline's ability to induce estrogenic activity. With the exception of one chemical on one day, all hazard indices are in the single digits or less, indicating that the LC₅₀'s are on the same order of magnitude or more acutely toxic than the EC₅₀. In order for xenobiotics to exhibit adverse responses

as xenoestrogens, they must exhibit effects at concentrations well below their acute toxicity. When acute toxicity and estrogenic activity are on the same order, the organism will be functionally impaired or die. Thus, expression via the receptor (β -galactosidase production), which includes transcription and translation, will not be exhibited (Layton, personal communication). Because of their acute toxicity and low estrogenicity, anilines on average are not important as environmental endocrine disrupters.

Many basic amines show, based on their log K_{ow} values, a higher than expected partitioning into artificial membranes (Barton et al., 1997). This discrepancy reflects the fact that 1-octanol only supports the partitioning of the neutral form of the amine. However, the phospholipid bilayer of a cell membrane allows for the partitioning of both the neutral and protonated forms of the amine (Austin et al., 1995). The latter is thought to be the result of non-covalent interactions with phospholipids. Specifically, the amines are able to make an energetically favorable electrostatic interaction with the membrane. The molecular structure of primary and secondary amines is complementary to that of the cell membrane. The amines can partition so as to orient their cationic charge with the anionic phosphate head moiety of the lipid bilayer, while the hydrophobic component of the amine aligns with the fatty acyl-chains (Barton et al., 1997). Although amines can partition through membranes in the above manner, however, diffusion is still hindered for ionized amines. Hydrocarbon-substituted anilines, which have pK_a values of approximately 4, are fully ionized at physiological pH, and therefore may show decreased diffusion through membranes. Hydrocarbon-substituted phenols, with pK_a values of approximately 10, are only partially ionized at physiological pH, and therefore experience less of

this phenomenon. If fewer molecules traverse the membrane, then fewer molecules will reach the site of action (i.e., the estrogen receptor), and activity will therefore be diminished at a specific time point.

A comparison of the amino- and hydroxy-substituted derivatives show that, in all cases but one (4-sec-butyl), the phenols are more estrogenically active than the anilines, as expected. The estrogenic activity of 4-sec-butylaniline was approximately equal to the activity of 4-sec-butylphenol. Of the 8 chemicals for which the hydroxy-substituted derivative was active at day 5 while the amino-substituted counterpart was not, all of them have bulky substituents, including 5 with hydrocarbon chains of 6 atoms or greater.

Eight amino-substituted derivatives had the same magnitude or one order difference in estrogenic activity compared to their phenol counterparts. Such a difference in activity was predicted *a priori* based on the work of Schultz et al. (2000a). The reduced activity can be attributed to the difference in electronegativity between aniline and phenol, as well as decreased uptake by the cells due to the ionized amino group of the anilines. Decreased uptake is also hypothesized to be responsible for the reduced activity of the additional anilines from Table 3.

Interpretation of the aniline data is further complicated by the ability of the yeast system to metabolize xenobiotics, as shown by Layton (personal communication). In Phase I metabolism, aniline is transformed to phenylhydroxyamine by N-hydroxylation (Niesink et al., 1996). There is currently no evidence to suggest that the -NHOH group of phenylhydroxyamine is a structural feature related to estrogenicity, however this cannot be ruled out without further experimentation. The degree of biotransformation is also

unknown. High rates of conversion from aniline to phenylhydroxyamine could further explain the decreased estrogenic activity found for the anilinic compounds as compared to the phenolic compounds. Further study is required to investigate the effects of metabolism on aniline estrogenicity.

Chemical recognizability between the ligand (i.e., xenoestrogen) and the receptor is the most important step to induction of estrogenic activity. Binding to the estrogen receptor is imperative for a response to be elicited (Schultz et al., 2000b). It is the difference between the xenobiotic and the ideal ligand that determines the level of activity (Schultz et al., 2000b). The more different the xenoestrogen is from the ideal, the lower the activity will be. The chemical similarities between amino and hydroxy groups are apparently not sufficient to yield equivalent estrogenic activity for the anilines. Therefore, the "nearest neighbor" phenomenon is not adequate, in this case, to describe the "key" to the estrogen receptor.

While several xenobiotics have been shown to bind to endogenous receptors *in vitro*, the *in vivo* consequence of this is unclear. There is mounting evidence suggesting that xenobiotics with weak estrogenic activity in *in vitro* assays, such as the yeast assay used in these studies, do not result in significant effects in more definitive *in vivo* tests (Zacharewski, 1997).

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APPENDIX

APPENDIX

Table 1. Estrogenic activities and ratios to control at 3, 5, and 7 days.

#	Compound	CAS	3d EC ₅₀ Molar	3d ratio	5d EC ₅₀ Molar	5d ratio	7d EC ₅₀ Molar	7d ratio	LC ₅₀ Molar
1	4-methylaniline	106-49-0	No EC ₅₀ *		No EC ₅₀ *		No EC ₅₀ *		9.29E-03
2	4-ethylaniline	589-16-2	No EC ₅₀ *		No EC ₅₀ *		3.30E-03	5.07E+08	4.95E-03
3	4-butaniline	104-13-2	No EC ₅₀ *		1.68E-04	4.28E+06	8.38E-05	8.57E+06	1.54E-04
4	4-sec-butaniline	30273-11-1	No EC ₅₀ *		2.51E-04	1.07E+07	5.24E-05	5.36E+06	2.02E-04
5	4-tert-butaniline	769-92-6	No EC ₅₀ *		5.03E-04	1.86E+07	2.51E-04	2.00E+07	1.01E-03
6	4-pentaniline	33228-44-3	No EC ₅₀ *		2.11E-04	6.21E+06	1.48E-04	7.56E+06	8.13E-05
7	4-hexylaniline (tech)	33228-45-4	not active		No EC ₅₀ *		No EC ₅₀ *		3.53E-05
8	4-cyclohexylaniline	6373-50-8	1.19E-04	1.52E+06	4.16E-05	3.04E+06	2.38E-05	3.35E+06	7.72E-05
9	4-heptylaniline	37529-27-4	No EC ₅₀ *		No EC ₅₀ *		No EC ₅₀ *		2.72E-05
10	4-octylaniline	16245-79-7	No EC ₅₀ *		No EC ₅₀ *		No EC ₅₀ *		2.03E-05
11	4-decylaniline	37529-30-9	No EC ₅₀ *		No EC ₅₀ *		No EC ₅₀ *		4.02E-05
12	4-dodecylaniline	104-42-7	No EC ₅₀ *		No EC ₅₀ *		No EC ₅₀ *		No LC50 at sat.
13	4-butoxylaniline	4344-55-2	not active**		1.51E-04	5.82E+06	1.13E-04	1.94E+07	5.88E-04
14	4-pentyloxylaniline	39905-50-5	not active**		No EC ₅₀ *		3.49E-05	7.15E+06	5.23E-05
15	4-hexyloxylaniline	39905-57-2	not active**		No EC ₅₀ *		No EC ₅₀ *		4.31E-05
16	4-heptyloxylaniline	39905-44-7	not active**		No EC ₅₀ *		No EC ₅₀ *		1.11E-04
17	4-phenoxyaniline	139-59-3	3.94E-05	3.24E+05	1.12E-05	3.60E+05	5.62E-06	5.76E+05	3.60E-04
18	4-benzoyloxylaniline HCl	51388-20-6	not active**		No EC ₅₀ *		1.00E-03	2.06E+08	2.51E-04

* "No EC₅₀" – estrogenic activity above background but less than EC₅₀

** "Not active" – estrogenic activity at or below background

Table 1. (continued)

#	Compound	CAS	3d EC ₅₀ Molar	3d ratio	5d EC ₅₀ Molar	5d ratio	7d EC ₅₀ Molar	7d ratio	LC ₅₀ Molar
19	butyl-4-aminobenzoate	94-25-7	1.94E-04	1.66E+06	4.24E-05	8.78E+05	2.22E-05	1.07E+06	3.45E-04
20	4,4'-diamino- benzophenone	611-98-3	No EC ₅₀ *		3.14E-04	1.41E+07	1.33E-04	1.21E+07	No LC ₅₀ at sat.
21	4-aminobenzophenone	1137-41-3	1.02E-04	4.26E+05	6.27E-06	1.61E+05	2.81E-06	2.82E+05	1.65E-03
22	4-aminobiphenyl	92-67-1	1.02E-04	5.91E+05	6.16E-05	1.58E+06	2.00E-05	1.23E+06	2.83E-04
23	4,4'-methylenedianiline	101-77-9	6.70E-04	5.88E+06	1.89E-04	4.19E+06	9.46E-05	6.57E+06	2.52E-03
24	N-methyl- 4,4'-methylenedianiline	26628-67-1	not active**		No EC ₅₀ *		No EC ₅₀ *		1.18E-04
25	4-aminodiphenylamine	101-54-2	4.75E-04	4.78E+06	1.58E-04	4.35E+06	7.92E-05	6.95E+06	Cannot determine due to color change
26	1-aminoanthracene	610-49-1	not active**		not active**		not active**		1.08E-04
27	2-aminoanthracene	613-13-8	No EC ₅₀ *		No EC ₅₀ *		1.04E-03	2.12E+08	No LC ₅₀ at sat.
28	1-aminofluorine	6344-63-4	not active**		not active**		not active**		1.38E-04
29	2-aminofluorine	153-78-6	not active**		2.99E-03	1.52E+08	2.53E-04	4.83E+07	6.32E-05
30	3-aminofluoranthene	2693-46-1	not active**		not active**		not active**		1.08E-05
31	9-aminophenanthrene	947-73-9	not active**		not active**		not active**		1.46E-05
32	4-aminophenyl disulfide	722-27-0	No EC ₅₀ *		No EC ₅₀ *		No EC ₅₀ *		1.26E-05
33	3-aminoquinoline	580-17-6	No EC ₅₀ *		No EC ₅₀ *		No EC ₅₀ *		2.08E-03
34	6-aminoquinoline	580-15-4	No EC ₅₀ *		not active**		not active**		3.47E-03
35	1-aminopyrene	1606-67-3	not active**		not active**		not active**		1.08E-05
36	1-adamantanamine	768-94-5	No EC ₅₀ *		No EC ₅₀ *		3.97E-03	2.71E+08	No LC ₅₀ at sat.
37	2-adamantanamine HCl	10523-68-9	1.07E-03	1.02E+07	1.33E-03	5.33E+07	3.32E-04	1.92E+07	4.08E-03

Table 2. Hazard Index.

#	Compound	CAS	Hazard Index* Day 3	Hazard Index* Day 5	Hazard Index* Day 7
3	4-butylaniline	104-13-2	No EC ₅₀	1.09E-00	5.46E-01
4	4-sec-butylaniline	30273-11-1	No EC ₅₀	1.24E-00	2.59E-01
5	4-tert-butylaniline	769-92-6	No EC ₅₀	4.99E-01	2.50E-01
6	4-pentylaniline	33228-44-3	No EC ₅₀	2.59E-00	1.82E-00
8	4-cyclohexylaniline	6373-50-8	1.54E-00	5.39E-01	3.08E-01
13	4-butoxyaniline	4344-55-2	not active	2.57E-01	1.93E-01
17	4-phenoxyaniline	139-59-3	1.09E-01	3.12E-02	1.56E-02
19	butyl-4-aminobenzoate	94-25-7	5.62E-01	1.23E-01	6.44E-02
21	4-aminobenzophenone	1137-41-3	6.17E-02	3.81E-03	1.70E-03
22	4-aminobiphenyl	92-67-1	3.59E-01	2.17E-01	7.06E-02
23	4,4'-methylenedianiline	101-77-9	2.66E-01	7.50E-02	3.75E-02
29	2-aminofluorine	153-78-6	not active	4.73E+01	4.00E-00
37	2-adamantanamine HCl	10523-68-9	2.61E-01	3.25E-01	8.15E-02

* Hazard Index = EC₅₀ / LC₅₀

Table 3. Comparisons of amino- and hydroxy-substituted derivatives.

#	Derivative	Amino-sub. 5d EC ₅₀	Hydroxy-sub. 5d EC ₅₀	Amino/Hydroxy
2	4-ethyl	No EC ₅₀	not active	
4	4-sec-butyl	2.51E-04	3.89E-04	6.46E-01
5	4-tert-butyl	5.03E-04	1.58E-04	3.18E-00
6	4-pentyl	2.11E-04	9.50E-06	2.22E+01
8	4-cyclohexyl	4.16E-05	1.39E-07	2.99E+02
9	4-heptyl	No EC ₅₀	9.77E-06	
10	4-octyl	No EC ₅₀	1.89E-06	
13	4-butoxy	1.51E-04	1.88E-05	8.05E-00
14	4-pentyloxy	No EC ₅₀	1.73E-05	
15	4-hexyloxy	No EC ₅₀	4.02E-06	
16	4-heptyloxy	No EC ₅₀	1.88E-06	
17	4-phenoxy	1.12E-05	2.62E-07	4.29E+01
18	4-benzyloxy (HCl)	No EC ₅₀	5.43E-07	
19	butyl-4-substituted-benzoate	4.24E-05	2.01E-06	2.11E+01
20	4,4'-di-substituted-benzophenone	3.14E-04	6.33E-07	4.96E+02
21	4-substituted-benzophenone	6.27E-06	9.84E-07	6.37E-00
22	4-substituted-biphenyl	6.16E-05	1.15E-06	5.35E+01
25	4-aminodiphenylamine/ N-benzyl-4-hydroxyaniline	1.58E-04	6.27E-05	2.52E-00
29	2-substituted-fluorine	2.99E-03	2.68E-07	1.12E+04
34	6-substituted-quinoline	not active	3.44E-04	
35	1-substituted-pyrene	not active	8.93E-07	
36	1-adamantan-substituted	No EC ₅₀	not active	
37	2-adamantan-substituted (HCl)	1.33E-03	not active	

VITA

Elizabeth Louise Hamblen was born in Knoxville, Tennessee on May 1, 1975. She was educated at Clinton Elementary in Clinton, Tennessee, and Woodland Elementary and Jefferson Junior High School in Oak Ridge, Tennessee before attending and graduating from Oak Ridge High School in 1993. In August of that year, she entered the University of North Carolina at Chapel Hill, from which she received a Bachelor of Science in Public Health degree in Environmental Science and Engineering in May of 1996.

She was employed in Durham, North Carolina from 1996 to 1998, when she entered The University of Tennessee at Knoxville pursuing a Master of Science degree in Ecology and Evolutionary Biology with a concentration in Environmental Toxicology. In May of 2001, she completed the requirements for graduation from the University.