Structural Features of Alkylphenolic Chemicals Associated with Estrogenic Activity*

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The ability of certain man-made chemicals to mimic the effects of natural steroid hormones and their potential to disrupt the delicate balance of the endocrine system in animals are of increasing concern. The growing list of reported hormone-mimics includes the alkylphenolic (AP) compounds, a small number of which have been reported to be weakly estrogenic. In their most basic form, APs are composed of an alkyl group, which can vary in size, branching, and position, joined to a phenolic ring. The aim of this project was to identify the important structural features responsible for the estrogenic activity of AP chemicals. This was achieved by incubating APs with different structural features in a medium containing a previously described estrogen-inducible strain of yeast (Saccharomyces cerevisiae) expressing the human estrogen receptor and comparing their activity spectrophotometrically by the resulting color change of the medium. The results were compared to the effects of the main natural estrogen 17β-estradiol.

The data indicate that both the position (para > meta > ortho) and branching (tertiary > secondary > normal) of the alkyl group affect estrogenicity. Optimal estrogenic activity requires a single tertiary branched alkyl group composed of between 6 and 8 carbons located at the para position on an otherwise unhindered phenol ring. The results are discussed in relation to the purity and composition of the chemicals tested.

In the early 1930s, scientists began synthesizing compounds based on the phenanthrene nucleus of steroidal estrogens in an attempt to produce substances with similar properties and considerable clinical value. Estrogenic activity was assessed by subcutaneous administration of chemicals (dissolved in sesame oil) to ovariectomized rats that were then observed for the onset of estrus (1). It soon became apparent that the phenanthrene condensed ring structure was not required for estrogenic activity, with the discovery that diphenyl and diphenyl methane derivatives (2) and stilbene derivatives (3–6) containing two hydroxyl groups in the para positions (e.g. 4,4′-dihydroxy diphenyl) were also active. The substituted derivative of stilbene (4,4′-dihydroxy stilbene) was renamed stilbestrol and was used as the parent compound in the production of another series of estrogenic compounds, including 4,4′-dihydroxy diethylstilbene (later known as diethylstilbestrol), which is still one of the most potent man-made estrogens. During this period, the first references to active compounds containing only one benzene ring, such as para-hydroxy propenyl benzene (anol) (7) and some of the alkylphenolic compounds, are found (8). It seems that this information was long forgotten, until it recently resurfaced within the context of endocrine disruption.

Some members of a surprisingly large group of natural and man-made chemicals are estrogenic (9–17). The unforeseen biological activity and exposure to man-made xenoestrogens have been implicated in the increased incidence of certain cancers and also with various disorders of the male reproductive system, including reduced testicular size and sperm production in humans and wildlife (18, 19), through both direct (receptor-mediated) and indirect actions on the endocrine system. For example, dioxins produce physiological responses in vitro similar to those seen by administration of potent synthetic estrogens and antiestrogens, without interacting directly with the estrogen receptor (17). In receptor binding studies and transfected chicken embryo fibroblast (CEF) cells, alkylphenols have been shown to interact with the estrogen receptor directly (20) and act in an identical way to 17β-estradiol in stimulating receptor transcription (21).

One pathway via which exposure of the developing male fetus to xenoestrogens could result in reduced testicular size and sperm production in adult life has been proposed. Ultimate testicular size and fertility of adult animals is determined shortly after testicular differentiation by the final number of Sertoli cells present. Exposure of the fetus to estrogens at this critical time point could reduce the number of Sertoli cells. Recent studies with rats (22) and rainbow trout (23) have demonstrated an inhibition of testicular growth after exposure to alkylphenolic chemicals. These findings support the contention that exposure to low levels of weakly estrogenic environmental chemicals, including alkylphenols, could affect gonadal development in the fetus. In view of this, it is important to isolate the structural features responsible for the activity of xenoestrogens, so that chemicals with similar features can be identified.

In this study we have screened alkylphenolic compounds of known structure for estrogenicity using a previously described recombinant yeast screen (24). Shifts in potency derived from specific structural changes are used to identify the important structural features responsible for their activity.

MATERIALS AND METHODS

The Recombinant Yeast—Details of the estrogen-inducible expression system in yeast and preparation of the medium components have been described previously (24). In brief, the DNA sequence of the human estrogen receptor was integrated into the yeast genome, which also contained expression plasmids carrying estrogen-responsive sequences controlling the expression of the reporter gene Lac-Z (encoding the enzyme β-galactosidase). Thus, in the presence of estrogens, β-galactosidase is synthesized and secreted into the medium, where it breaks down the chromogenic substrate chlorophenol red-β-n-galactopyranoside (CPRG),1 which is initially yellow, into a red product that can be measured by absorbance.

1 The abbreviations used are: CPRG, chlorophenol red-β-n-galactopyranoside; AP, alkylphenolic; APE, ethoxylated alkylphenolic compound.

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Assay Procedure—As described previously in Routledge and Sumpter (24), stock solutions of alkylphenolic chemicals and the estradiol standard were serially diluted in ethanol, and 10-μl aliquots of each concentration were transferred in duplicate to optically flat 96-well microtiter plates and allowed to evaporate to dryness. Aliquots (200 μl) of the assay medium containing recombinant yeast and the chromogenic substrate CPRG were then dispensed to each sample well. Each plate contained at least one row of blanks (assay medium only). Possible spurious results resulting from conversion of the CPRG by the chemicals alone was addressed by incubating identical plates in assay medium without yeast. The plates were sealed with autoclave tape and shaken vigorously for 2 min on a titer plate shaker before incubation at 32 °C. After an 84-h incubation, the color of the medium was read at an absorbance of 540 nm (using a Titertek Multiskan MCC/340 plate reader). At this stage, control wells (blanks) appeared light orange in color, due to background expression of β-galactosidase, and turbid, due to growth of the yeast. Positive wells were indicated by a deep red color with turbid yeast growth. Clear yellow wells indicated lysis (a toxic response), and the color may vary. All data were corrected for turbidity using a second reading at 620 nm. The plates were left at room temperature for an additional 7 days and observed for possible enzyme-inhibiting action, indicated by responses below background.

Assay Validation—The specificity of the screen was determined using steroids purchased from Sigma. Stock solutions (2 × 10^{-6} M) were made in ethanol, and serial dilutions were carried out in ethanol to achieve final concentrations of 1 × 10^{-8} to 5 × 10^{-12} M in the microtiter wells. The ability of the yeast screen to detect known environmental estrogens has been described previously in Routledge and Sumpter (24). To demonstrate that the chemicals tested were acting through the estrogen receptor, the ability of tamoxifen, an estrogen antagonist known to act via the estrogen receptor, to inhibit the activity of the alkylphenolic chemicals was investigated. A 40 μl stock solution of tamoxifen (Aldrich) was serially diluted in ethanol, and 10-μl aliquots of each concentration were transferred in duplicate to optically flat 96-well microtiter plates and allowed to evaporate to dryness. Aliquots (200 μl) of the assay medium containing recombinant yeast, the chromogenic substrate CPRG, and 17β-estradiol (or alkylphenolic chemical) were then dispensed to each sample well. The concentration of 17β-estradiol added to the medium was sufficient to produce an obvious, but not maximal, response. Controls were assay medium alone (largest attainable response) and assay medium without the addition of estradiol or alkylphenols (normal background expression), which was also incubated concurrently with tamoxifen. The plates were sealed with autoclave tape and shaken vigorously for 2 min on a titer plate shaker before incubation at 32 °C. After an 84-h incubation, the color of the medium was read at an absorbance of 540 nm. At this stage, medium without 17β-estradiol or alkylphenolic chemicals appeared light orange in color, due to background expression of β-galactosidase, and turbid, due to growth of the yeast. Wells containing only 17β-estradiol or alkylphenolic chemicals appeared red in color, due to increased synthesis of β-galactosidase. The antiestrogenic activity of tamoxifen was observed as a dose-dependent inhibition of the color change of the medium from yellow to red.

Chemicals Tested—Alkylphenolic chemicals with particular structural features were obtained from commercial and industrial sources and assessed for estrogenic activity. Chemicals tested were chosen on the basis of purity and knowledge of their composition. The activities of alkylphenolic chemicals were compared to the effects of the main natural estrogen 17β-estradiol (purchased from Sigma). A 200 μM stock of 17β-estradiol was prepared in ethanol, after which serial dilutions were carried out to produce final concentrations of 10 nM to 5 μM in the wells. The alkylphenolic compounds 4-methylphenol (99% pure), 4-ethylphenol (99% pure), 4-propylphenol (99% pure), 3-tert-butylphenol (99% pure), 4-tert-butylphenol (99% pure), and 4-tert-amylphenol (99% pure) were purchased from Aldrich. 2-phenylphenol (99% pure), 3-phenylphenol (99% pure), 4-phenylphenol (99% pure), and 4-phenylphenol (99% pure) were purchased from MTM (Lancashire, United Kingdom). 2-tert-butylphenol (99% pure), 2,2'-sec-butylphenol (99% pure), 4,4'-sec-butylphenol (98% pure), 4-α-pentylphenol (98% pure), 4-tert-hexylphenol (99% pure), 4-tert-heptylphenol (99% pure), 2,4'-di-nonylphenol (98% pure), 4,4'-di-nonylphenol (98% pure), 4-nonylphenol (95% pure), 2,2'-di-nonylphenol (98% pure), 2,4'-di-nonylphenol (99% pure), 2,4-dimethylphenol (97% pure), 2,4-di-tert-butylphenol (97% pure), 4-nonylphenol (95% pure), 4-methyl-2-nonylphenol (95% pure), 4-methyl-2,6-dimethylphenol (95% pure), 4-methyl-2,6-dimethylphenol (95% pure), 4-methyl-2,4-dimethylphenol (95% pure), and 2,2'-di-nonylphenol were purchased from Sigma. A 1000 μg/liter stock of 4-nonylphenol dipropoxylate (4-DPP + 2PO; 95% pure) was supplied by Witco (Houston, TX). Stock solutions of the chemicals were made up in ethanol, after which serial dilutions were carried out to achieve final concentrations of between 5 nm and 60 μM in the wells.

Relative Potencies—The estrogenic activity of alkylphenolic chemicals was assessed by dividing the concentration of the chemical producing a half-maximal response by the concentration of 17β-estradiol required to produce the same response. The data presented in this paper were the product of a single assay to ensure a fair representation of the potencies of the chemicals relative to one another.

RESULTS

Validation of the Recombinant Yeast Screen—The specificity of the screen was assessed by the ability of a range of steroids and steroid metabolites, diluted from 1 × 10^{-8} M, to stimulate synthesis of β-galactosidase in the yeast. The data presented in Fig. 1 show that the strongest response was seen in the wells containing 17β-estradiol. The next most potent steroids were estrone, the man-made estrogen diethylstilbestrol, 17α-estradiol, and estriol, which had potencies approximately 2, 4, 40, and 300 times less than that of 17β-estradiol, respectively. All the other steroids failed to induce β-galactosidase activity. Sensitivity and reproducibility were assessed by measuring the response of the screen to triplicate dilutions of 17β-estradiol, over a concentration of 3072 ng/liter to 1.5 ng/liter, compared to triplicates with assay medium only. Variance did not exceed 0.5% of each mean value at each point across the dilution range, and a detectable increase in β-galactosidase production was consistently produced with 2 ng of 17β-estradiol/liter.

Assay Response to Known Environmental Estrogens—The response of the screen to genistein (phytoestrogen), bisphenol-A (epoxy resin precursor), and α, p'-DDT (pesticide) tested over a concentration range of 10 μg/liter to 50 μg/liter has been previously in Routledge and Sumpter (24). All produced a dose-dependent elevation in β-galactosidase production. Thus, the assay is capable of detecting known xenoestrogens.

Inhibition of 17β-estradiol and Alkylphenolic Chemicals by Tamoxifen—The ability of the antiestrogen tamoxifen to inhibit the effect of 17β-estradiol and alkylphenolic chemicals was
used to illustrate the requirement of these compounds to interact with the estrogen receptor to stimulate the synthesis of β-galactosidase. Yeast cells were incubated in the presence and absence of 17β-estradiol, 4-sec-butylphenol, 4-n-heptylphenol, and 4-tert-octylphenol at concentrations required to produce a distinct, but not maximal, synthesis of β-galactosidase and concurrently with varying concentrations of tamoxifen. Fig. 2 illustrates that in the absence of 17β-estradiol or alkylphenolic compounds, the final background absorbance of the medium \( (A_{540}) \) remained at approximately 0.9. In the presence of 17β-estradiol or alkylphenolic chemicals, the increased synthesis of β-galactosidase elevated the absorbance of the medium to approximately 2.0 during the same incubation period. Wells in which the chemicals were incubated together with tamoxifen all showed a dose-dependent inhibition of expression of β-galactosidase, indicating that all the compounds are acting via the estrogen receptor. Tamoxifen on its own behaved as a weak partial agonist, producing a maximal stimulatory response (approximately half that produced by 17β-estradiol) at a concentration 4 orders of magnitude greater than that of 17β-estradiol.

**Estrogenicity of Alkylphenolic Chemicals**—After having previously assessed the response of the screen to a variety of natural and synthetic estrogens, we proceeded to test alkylphenolic chemicals with different structural features for estrogenic activity. All the compounds were tested over a concentration range of 5 mM down to 60 nM. There was no indication that any of the chemicals reacted with the CPRG or affected the performance of the enzyme. Different structural features of alkylphenolic chemicals were assessed for their ability to confer estrogenic activity.

**Size of the Alkyl Group**—Fig. 3 shows the effect of increasing alkyl group size of 4-tert substituted alkylphenolic chemicals on estrogenic activity. The small alkyl group size of 4-methylphenol and 4-ethylphenol (1 and 2 carbons, respectively) means these exist only as normal branching forms. 4-propylphenol (3 carbons) exists only as normal and secondary branched forms. 4-methylphenol and 4-ethylphenol did not stimulate β-galactosidase production in the yeast; however, 4-propylphenol (3 carbons in the alkyl group) produced a weak response (20,000,000

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2 E. J. Routledge and J. P. Sumpter, unpublished result.
times less potent than estradiol). The response increased with each additional carbon, up to a maximum of 8 carbon atoms, but all were much less potent than 17β-estradiol: 4-tert-butylphenol (4 carbons; 1,500,000-fold less potent), 4-tert-amylphenol (5 carbons; 100,000-fold less potent), 4-tert-hexylphenol (6 carbons; 6,000-fold less potent), 4-tert-heptylphenol (7 carbons; 3,000-fold less potent), and 4-tert-octylphenol (8 carbons; 1,000-fold less potent). Activity seems to decrease when the carbon number exceeds 8 because 4-nonylphenol (9 carbons; 1,000-fold less potent) and 4-nonylphenol (10 carbons; 6,000-fold less potent) are much less potent than estradiol and 25 times more potent than its secondary branched equivalent (Fig. 4B). 4-tert-octylphenol was approximately 30,000 times less potent than estradiol and 25 times more potent than its normal branched equivalent (Fig. 5B).

Fig. 6 summarizes the relative potency of all the 4-branched APs tested compared to 17β-estradiol. In general, 4-tertiary branching structures containing 6–8 carbons are approximately 30 times more potent than 4-normal and 4-secondary equivalents, which are similar in potency in vitro. Estrogenicity increases with alkyl group size irrespective of the type of branching. 4-tert-octylphenol was 1,500 times more potent than 4-tert-butylphenol, whereas 4-sec-octylphenol was only 65 times more potent than 4-sec-butylphenol, indicating that secondary branched structures have a more limited potency range in vitro compared to 4-tert equivalents.

Fig. 5 compares 4-normal (unbranched) alkylphenolic chemicals to their 4-tert branched equivalents. Chemicals with both types of branching were active, but in this case the trend was not so well defined. Of the normal branched structures available, the most potent was 4-n-amylylphenol, which was approximately 30,000 times less potent than estradiol and about 3 times more potent than its 4-tert equivalent (Fig. 5A). The addition of 2 carbons to the alkyl group reversed this pattern because 4-tert-heptylphenol was approximately 3,000 times less potent than estradiol and 25 times more potent than its normal branched equivalent (Fig. 5B).

Position of the Alkyl Group on the Phenol Ring—Fig. 7 shows the effect of positional changes of alkylation on estrogenic
potency. Fig. 7A shows that 2 (ortho)- and 3 (meta)-tert-butylphenol did not stimulate β-galactosidase production in the screen and therefore possess no detectable estrogenic activity. 4-tert-butylphenol produced a dose-dependent response from $5 \times 10^{-3}$ M, making it approximately 1,500,000 times less potent than 17β-estradiol. 4-tert-octylphenol was 1,000 times less potent than 17β-estradiol and 10,000 times more potent than the 2-tert equivalent (Fig. 7B). 4-sec-decylphenol was 100,000 times less potent than estradiol (Fig. 7C), whereas no observable activity was detected with 2-sec-decylphenol (>100,000K times less potent than 17β-estradiol). This indicates that the alkyl group must be at the para or 4th position on the phenol ring to be active in the estrogenic assay.

Effect of Methylation—Fig. 8 illustrates the effect of adding a small nonestrogenic methyl group (see Fig. 6) onto the phenolic ring of an existing AP. Addition of a methyl group to the second (ortho) position on the phenolic ring of 4-nonylphenol did not affect the activity because 2-methyl 4-nonylphenol and 4-nonylphenol were both approximately 30,000 times less potent than 17β-estradiol. If the alkyl groups change position so that the methyl group is in the 4th position and the nonyl group is in the 2nd position, there is a 33-fold reduction in activity. 2-nonylphenol is about 3 times less potent than 4-nonylphenol (data not shown), so the addition of the methyl group in the para position was responsible for an additional 11-fold reduction in activity not attributable to the positional change of the nonyl (9-carbon alkyl) group alone. Moreover, 3-methyl 2-nonylphenol (in which the methyl group is at the meta or 3rd position) and 2-nonylphenol are equipotent (data not shown). This indicates that the para or 4th position (opposite the hydroxyl group on the phenolic ring) is an important feature for estrogenicity because activity is reduced only when this position, rather than another position, is methylated.

Effect of Di-Substitution—Fig. 9 illustrates the effect of di-substitution on estrogenic activity. However, interpretation of these results is complicated by the fact that the 2,4-di-nonylphenol was not pure; it contained about 25% “free” nonylphenol. The response produced by the 2,4-di-nonylphenol may be entirely attributable to the nonylphenol in the sample. It is possible, therefore, that 2,4-di-nonylphenol has no intrinsic activity. 2,4-di-butylphenol (99% pure) and 2,6-di-butylphenol (99% pure) both failed to stimulate β-galactosidase production in the assay (data not shown). It therefore seems likely that di-substitution (or addition of another alkyl group larger than a methyl group) greatly reduces or completely abolishes estrogenic activity.

Effect of the Ethoxylate Chain Length—Fig. 10 shows the effect of ethoxylate chain length and branching on estrogenic potency of 4-substituted alkylphenolic compounds. The short chain ethoxylates are particularly relevant because they are some of the main breakdown products of nonionic surfactants. Fig. 10A shows that the addition of 2 ethoxylates to 4-nonylphenol results in a 165-fold reduction in activity, indicating that a free ring hydroxyl group is required for optimal activity. Moreover, addition of a branched propylene oxide group results in at least a 35,000-fold reduction in activity because no β-galactosidase production was observed. Fig. 10B indicates that the same is true for secondary

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**Fig. 5.** Response of the estrogen screen to 4-substituted alkylphenolic compounds that vary in their alkyl group branching. The yeast cells were incubated with (A) 4-n-amylphenol (■) and 4-tert-amylphenol (▲). B. 4-tert-heptylphenol (▲) and 4-n-heptylphenol (▲) at concentrations ranging from 5 molar to 60 nanomolar.

**Fig. 6.** Relative potency of 4-substituted alkylphenolic compounds with varying alkyl group size and branching compared to the main natural estrogen 17β-estradiol.

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**Fig. 7.** A. Relative potency (x-axis) at 5 mM, 50 mM, and 500 mM, which corresponds to 5, 50, and 500 nM, respectively (y-axis).

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**Fig. 8.** Effect of adding a small nonestrogenic methyl group (see Fig. 6) onto the phenolic ring of an existing AP. Addition of a methyl group to the second (ortho) position on the phenolic ring of 4-nonylphenol did not affect the activity because 2-methyl 4-nonylphenol and 4-nonylphenol were both approximately 30,000 times less potent than 17β-estradiol. If the alkyl groups change position so that the methyl group is in the 4th position and the nonyl group is in the 2nd position, there is a 33-fold reduction in activity. 2-nonylphenol is about 3 times less potent than 4-nonylphenol (data not shown), so the addition of the methyl group in the para position was responsible for an additional 11-fold reduction in activity not attributable to the positional change of the nonyl (9-carbon alkyl) group alone. Moreover, 3-methyl 2-nonylphenol (in which the methyl group is at the meta or 3rd position) and 2-nonylphenol are equipotent (data not shown). This indicates that the para or 4th position (opposite the hydroxyl group on the phenolic ring) is an important feature for estrogenicity because activity is reduced only when this position, rather than another position, is methylated.

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**Fig. 10.** Effect of altering the size and branching of the ethoxylate side chain. A. Relative potency of 4-substituted alkylphenolic compounds with varying alkyl group size and branching compared to the main natural estrogen 17β-estradiol. B. Relative potency of 4-substituted alkylphenolic compounds with varying alkyl group size and branching compared to the main natural estrogen 17β-estradiol.

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**Estrogenic Activity of APs**

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branched alkylphenolic chemicals because the addition of two propylene oxide groups to 4-sec-dodecylphenol results in a complete loss of activity over the concentration range tested.

Fig. 7. The effect of positional changes in alkylation on the estrogenic potency of alkylphenolic compounds in the yeast screen. The yeast cells were incubated with (A) 4-tert-butylphenol (○), 3-tert-butyphenol (●), and 2-tert-butyphenol (▲). B, 4-tert-octylphenol (●) and 2-tert-octylphenol (▲). C, 4-sec-decylphenol (○) and 2-sec-decylphenol (□) at concentrations ranging from 5 mM to 60 nM.

Fig. 8. The effect of a single point ring methylation on the estrogenic potency of alkylphenolic compounds in the yeast screen. The yeast cells were incubated with 4-nonylphenol (x), 2-methyl 4-nonylphenol (▲), and 4-methyl 2-nonylphenol (○) at concentrations ranging from 5 mM to 60 nM.

Fig. 9. The effect of di-substitution on the estrogenic potency of alkylphenolic compounds in the yeast screen. The yeast cells were incubated with 4-nonylphenol (●) and 2,4 di-nonylphenol (□) at concentrations ranging from 5 mM to 60 nM.

Estrogenic Activity of Different Isomers of Phenylphenol—Many of the features required for estrogenic activity of alkylphenolic chemicals may apply to other groups of chemicals, such as those composed of two benzene rings. Fig. 11 again illustrates the importance of positional changes on estrogenicity in three phenylphenol derivatives. Analogous to the situation observed with alkylphenolic chemicals, the most active derivative (4-phenylphenol) is 20,000 times less potent than 17β-estradiol and bears the phenyl group opposite to the hydroxyl group. Each successive movement of the phenyl group around the phenolic ring results in an approximately 7-fold reduction in activity.

DISCUSSION
A large number of test systems now exist to identify estrogenic activity. These include traditional bioassays relying on relatively complex biological responses, such as stimulation of water inhibition, uterine growth and vaginal cornification in ovariectomized laboratory rodents (16, 25), and the expression of the egg yolk precursor vitellogenin in male oviparous verte-
brates (26, 27), in vitro assays including hepatocyte cell cultures (11), and proliferation of MCF-7 breast cancer cell lines (28) and some crystallographic and computer modeling studies (29, 30). More recently, the advancement of molecular biology has allowed the production of recombinant systems. In these systems, the DNA sequence of the estrogen receptor is stably integrated and expressed in host cells that also contain reporter genes that monitor the activity of the receptor by the production of easily quantifiable endpoints, such as the expression of an enzyme or the production of light by luciferase (31–33).

In vitro studies using a range of different cell lines and transfection assays have shown that certain xenoestrogens can produce biological responses similar to 17β-estradiol itself, albeit only when present at a 1,000- to 100 million-fold greater concentration. The actual discovery of many weak xenoestrogens in vitro relies on the simple fact that these systems combine extreme sensitivity with the ability to operate over an extremely large concentration range (34).

The information available on the estrogenic activity of alkylphenolic chemicals in vitro and in vivo is surprisingly consistent, despite species differences in affinity of receptors for 17β-estradiol (35) and variations in uptake, bioavailability, and metabolism in different systems. In accordance with our findings (Fig. 3), early experiments by Dodds and Lawson (8) indicated that a single 100-mg dose of 4-tert-amylphenol (4-tert-pentylphenol) was more potent than 4-tert-butylphenol. Contrary to our results, however, none of the 4-normal (unbranched) alkylphenolic compounds (except the propyl derivatives) were active at the same dose, possibly due to metabolic conversion to nonestrogenic derivatives in vivo. The method of administration (the vehicle and route of administration) may also affect the response to certain xenoestrogens (8).

Data derived from fish (hepatocyte culture), avian (CEF cells), and mammalian (MCF-7) systems (21, 28) are also concordant with our findings. All agree that 4-tert-octylphenol is approximately 10-fold more potent than 4-nonylphenol and between 103 and 104 times less potent than 17β-estradiol. This suggests that species differences in the amino acid sequence of the estrogen receptor are likely to occur in regions that are not important for AP binding. The discoveries that 4-ethylphenol was inactive, but 4-propylphenol produced a weak proliferative response in MCF-7 cell culture, and that 4-sec and 4-tert-butyphenol were equipotent and over 10 times less potent than 4-nonylphenol (28) also correspond to our findings (see Figs. 3 and 4A).

The basic molecular mechanisms of hormone-dependent transcription activation by the human estrogen receptor in mammalian cells (including DNA binding, hormone binding, nuclear localization, and transcriptional enhancement of a gene bearing estrogen-responsive sequences) have been shown to operate in yeast (36). Yeast-based systems have, therefore, been widely used to establish the mechanisms of ligand binding, as well as dissecting receptor function and transcriptional activation using site-directed mutagenesis (37–39). In vitro tests, however, represent only a part of the metabolic system present in entire animals and may not illustrate the effect of bioconcentration, both of which may affect estrogenic potency. Thus, the potency of a chemical in vitro may not be relevant to an in vivo situation. Chemicals identified as being estrogenic in vitro should therefore be tested in in vivo studies retrospectively. The advantages and limitations of yeast-based systems are discussed in Routledge and Sumpter (24).

The prerequisite of alkylphenolic compounds to interact with the estrogen receptor to stimulate the production of β-galactosidase in this system was tested by incubating the yeast cells in
the absence or presence of alkylphenols together with the antiestrogen tamoxifen. Tamoxifen has been shown to be a competitive inhibitor of estradiol binding to estrogen receptors (40) and is used in the treatment of hormone-dependent breast cancer. Although tamoxifen is able to inhibit the action of estradiol, it itself exhibits slight estrogenic activity and acts as a partial agonist (41, 42), which is in agreement with our findings. Fig. 2 illustrates the ability of tamoxifen to inhibit the response of the screen to both 17β-estradiol and alkylphenolic chemicals in a dose-dependent manner. This substantiates the requirement for both the estrogen receptor and an active ligand to stimulate the production of β-galactosidase.

Certain structural motifs are now realized to be important for estrogenicity. Xenoestrogens are generally fat-soluble mono or diphenolic chemicals in which hydrophobicity is achieved by either chlorination (for example o, p′-DDT, o, p′-DDE, and some polychlorinated biphenyls are all weakly estrogenic) or by the addition of a bulky hydrocarbon side chain to the meta (3rd carbon) or para (4th carbon) position of an otherwise unhindered ring (such as alkylphenols). The present study indicates that the size and degree of branching of the alkyl group, as well as its position relative to the hydroxyl group on the phenol ring, are also important features for estrogenic activity of APs and illustrates the effectiveness of this yeast-based system in identifying structure-activity relationships of xenoestrogens. However, it should always be borne in mind that the chemical structures illustrated within the figures represent only a two-dimensional image. It is likely that certain aspects of the structures such as charge distribution and “twist” may produce novel structural features that could only be revealed in three-dimensional images and that may also be important in determining the estrogenic potency of this class of chemicals.

Estrogenicity requires that the alkyl group is composed of 3 or more carbons. The addition of each carbon increases the hydrophobicity of the molecule, which may explain, in part, why short chain alkyl groups (containing 3 or 4 carbons) are active. A stepwise increase in activity then occurs with the addition of each carbon (Fig. 3), but divergent activities occur between different branching groups (Figs. 4–6). Only a limited number of 4-secondary and 4-normal branching structures were available to test, but the results indicate that they are likely to be similar in potency. This is not surprising, given the structural similarity of their alkyl groups. The 4-tert branching structures, however, were generally much more potent, indicating that the branching on the α-carbon (the carbon attached to the phenol ring) may be an important feature for estrogenicity. This observation is further supported by the fact that the 30-fold reduction in activity seen between 4-tert- octylphenol and 4-nonylphenol may be due in part to the fact that branching also occurs in places outside the α-carbon in the case of 4-nonylphenol (43).

Activity was greatest with 4-tert-octylphenol (8 carbons), in which 1,000 molecules produced a response similar to that obtained by 1 molecule of 17β-estradiol, but 4-nonylphenol (9 carbons) was 30 times less active (Fig. 3). This drop in activity only occurs in 4-tert-branched structures and therefore may be due to the isomeric heterogeneity of 4-nonylphenol compared with that of some other 4-alkylphenolic compounds. Recent high-resolution gas chromatographic analyses of para-nonylphenol have identified 22 para-isomers (43). Isomeric purity of alkylphenolic chemicals is largely dependent on the nature of the starting olefin. It is possible that due to the augmentation of possible branching forms (44), 4-nonylphenol contains isomeric branching structures that are both greater in activity and relatively inactive compared to 4-tert-octylphenol, resulting in an overall reduction in observed effect.

The position of alkylation on the phenolic ring (irrespective of branching form) also affects estrogenicity, possibly because this affects the alignment of the alkylphenol in the receptor. The potent ortho-para directing influence of the hydroxyl group on the phenol ring does not favor the formation of meta-isomers. However, results from those meta-substituted alkylphenols that were available, along with the data obtained from the phenylphenols (Fig. 11), indicate that estrogenicity increases as the alkyl group is moved from ortho to meta to para, respectively (Fig. 7). The importance of the para position was further illustrated by substitution of an existing alkylphenol with a small inactive methyl group (Fig. 3). Replacing places of a methyl and a nonyl (9-carbon) group situated on the 2nd (ortho) and 4th (para) position on the ring results in a reduction in activity (Fig. 8) that is not entirely accounted for by the movement of the active nonyl group away from the para position alone. It seems, therefore, that the size and type of branching, as well as the position of the alkyl group on the phenolic ring, are key features in the estrogenicity of AP compounds.

Di-substitution with an alkyl group of 4 carbons or greater greatly reduces or completely abolishes activity, which emphasizes the importance of an unhindered ring structure (Fig. 9). Because no decrease in activity was observed between 4-nonylphenol and 2-methyl-4-nonylphenol, it seems that the alkyl groups must be similar in size for this effect to be noticeable.

Substitution of the hydroxyl group also affects estrogenicity of APs. The compounds tested in Fig. 10 are more relevant to the biodegradation of alkylphenolic surfactants, such as nonylphenol polyethoxylates (NPnEO, where n = 6–40). The parent surfactant itself is not active, but during sewage treatment, APnEO are biodegraded via shortening of the ethoxylation chain to short-chain ethoxylates and carboxylic acid derivatives and finally APs (45), which are all estrogenic (24). Fig. 10 indicates that short-chain ethoxylates are less active than the unsubstituted equivalent, but substitution of the hydroxyl group with a similar-sized propylene oxide derivative results in a much greater reduction in activity. As biodegradation of the primary branched ethoxylated alkylphenols results in the production of estrogenic metabolites, but secondary hydroxyl groups cannot be oxidized to carboxylates by bacteriological activity, the biodegradation of propoxylated alkylphenols is therefore less likely to result in the formation of estrogenic intermediates.

Ethoxylated alkylphenolic compounds (APEs) comprise one of the largest volume surfactants in production. Approximately 450 million pounds of APE surfactants were sold in the United States in 1988 alone (46). APEs are used in both household and institutional cleaning products (47). The four largest industrial uses of APEs are in plastics (12) and elastomers, textiles (cleaning, spinning, weaving, finishing), agricultural chemicals (wetters and emulsifiers), and paper (pulping). In the household market the main reported uses of APEs are in laundry detergents and hard-surface cleaners. In addition, nonylphenol polyethoxylates are widely used as a spermicide in contraceptive creams and jellies and in prophylactics. APEs have also been found in hair-care products, including shampoos, hair colorings, and hair styling aids, and certain APs are used in the manufacture of flavors and fragrances, all of which are possible routes for human exposure.

The potential impact of AP compounds and other xenoestrogens on humans and wildlife is unclear but may be influenced by factors such as the level and route of exposure, metabolism, bioconcentration, lifestyle, and stage of development. Unlike natural steroidal estrogens, many man-made xenoestrogens are lipophilic and may bioaccumulate; in fish, an average

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*3 C. Green, personal communication.*
bioconcentration factor for nonylphenol would seem to be approximately 300 (48). Moreover, circulating xenoestrogenic chemicals may not be recognized and sequestered by plasma steroid-binding proteins such as sex hormone binding globulin and α-feto protein (49), which are critical in preventing early estrogen exposure of the fetus, and thus unmodulated action of APs on target cells and tissues may occur. All these questions must be addressed if the potential risk from exposure to xenoestrogens is to be determined with any accuracy.

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