Estrogenic Activity of Phenolic Additives Determined By an In Vitro Yeast Bioassay

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We used a recombinant yeast estrogen assay to assess the activity of 73 phenolic additives that are used as sunscreens, preservatives, disinfectants, antioxidants, flavorings, or for perfumery. Forty-one compounds were inactive. The major criteria for activity appear to be the presence of an unhindered phenolic OH group in a para position and a molecular weight of 140–250 Da. Key words: estrogenic activity, phenolic additives, recombinant yeast assay. Environ Health Perspect 109:133–138 (2001). [Online 13 January 2001] http://ehpnet1.niehs.nih.gov/docs/2001/109p133-138miller/abstract.html

There is currently considerable interest worldwide in chemicals that are able to mimic estrogens (i.e., xenoestrogens). Because it is possible that exposure to xenoestrogens may (and only may) lead to adverse physiologic effects in humans and wildlife, intense efforts are under way to identify chemicals that possess estrogenic activity. To date, no large-scale, systematic screening of chemicals for estrogenic activity has been conducted (although one is planned) (1). Instead, the estrogenic activity of some chemicals has been discovered by accident, usually because estrogenic effects have been observed in groups of animals exposed to high doses or concentrations of chemicals (2), or by small-scale screening, for example, alkylphenols (3,4) and phthalates (5,6).

The identification of many structurally diverse chemicals possessing (usually weak) estrogenic activity has allowed structure-activity relationships (SARs) to be developed [e.g., Wailer et al. (7)]. To date, these models have not been used in a predictive sense (i.e., to identify chemicals likely to have estrogenic activity), but they have been useful in identifying the major structural features associated with estrogenic activity. Because receptor binding is based on the hypothesis that all active molecules interact with the receptor site in the same or similar mode, a similar pattern of atoms or functional groups in the compounds activating receptor sites is usually required to facilitate recognition and binding. In the case of the human estrogen receptor site, a ligand with a phenolic group seems to be a common feature of most, but not all, molecules that display a substantial binding activity (8,9). On the basis of this knowledge, we screened a selection of commercially used phenolic additives to assess their estrogenic activity. Phenolic additives are used without modification in widely different end products, and in this respect, they differ from most phenols that are used in the synthesis of other organic chemicals, frequently as components of polymeric materials.

Materials and Methods
Selection of chemicals. We characterized the phenolic additives by searching directories of commercial chemicals displaying chemical structures (10–13). The preliminary search generated a selection of several hundred compounds, but we reduced this number by excluding drugs, dyes, and those phenols used as precursor chemicals for manufacturing other compounds. There were about 140 compounds remaining; we reduced this group to 73 by investigating only compounds that were available in a high-purity form from suppliers. We confirmed the purity of the compounds by HPLC; in the few cases where impurities apparently exceeded 2%, compounds were recrystallized. We believe that the compounds finally tested are a representative, but not comprehensive, selection of phenols that are incorporated without modification into a wide range of commercially important products. They fell into the following classes: UV screening agents, preservatives, disinfectants, antioxidants, and flavors and perfumery components. The chemicals tested are listed in Tables 1–5.

Assessment of estrogenic activity. Details of the yeast estrogenicity assay (including details of the medium components) have been previously described (14). In brief, yeast cells transfected with the human estrogen receptor α (ERα) gene, together with expression plasmids (containing estrogen responsive elements and the lac-Z reporter gene encoding the enzyme β-galactosidase), were incubated in medium containing the test chemical and the chromogenic substrate, chlorophenol red-β-β-d-galactopyranoside (CPRG). Active ligands (which bind to the receptor) induce β-galactosidase (β-gal) expression, and this causes the CPRG (initially yellow) to change into a red product that can be measured by absorbance.

Stock solutions of chemicals (dissolved in ethanol) were serially diluted in ethanol, and 10 µL volumes were transferred to 96-well, flat-bottom plates. After the ethanol was allowed to evaporate to dryness, 200 µL medium containing CPRG and yeast was added to each well. The plates were then incubated at 32°C for 3 days, after which absorbance readings were made at 540 nm using a Spectramax 340 PC plate reader (Molecular Devices, Sunnyvale, CA). We included 17β-estradiol (serially diluted from 1 x 10^-10 M to 4.88 x 10^-12 M) and solvent controls in each assay. Each chemical was tested at least twice. The median effective dose (ED_{50}) for 17β-estradiol was 2.0 x 10^{-10} M ± 0.22 x 10^{-10} M (mean ± SE of 14 experiments).

We determined relative potencies of test chemicals only when the dose–response curves were parallel to that of 17β-estradiol. To do so, the concentration of the test chemical required to produce a half-maximal response (A_{50}) was determined. The median effective dose (ED_{50}) for 17β-estradiol was 2.0 x 10^{-10} M ± 0.22 x 10^{-10} M (mean ± SE of 14 experiments).

The response curves for the additives used as sunscreens or light stabilizers are shown in Figure 1 and are representative of the data obtained from all of the active chemicals. Twenty-two of the test compounds...
produced full dose–response curves that were parallel to that of 17β-estradiol. Ten compounds generated submaximal response curves, a phenomenon that has previously been discussed (15). In some cases, submaximal response curves occurred because the test chemical was toxic to the yeast at high concentrations (e.g., the dose–response curves for benzophenone-3 and benzophenone-7 in Figure 1A), but other chemicals generated shallower dose–response curves than 17β-estradiol for reasons not currently understood (e.g., methyl salicylate, benzyl salicylate, and 2-ethylhexyl salicylate; Figure 1B). Such compounds were designated as having submaximal response in the tabulated results and are detailed in Table 6.

**Discussion**

The results summarized in Tables 1–5 show that activity has been detected in compounds falling into four out of the five usage categories, and only those used in flavoring and perfumery failed to display activity. The group of compounds used to screen UV light contained more estrogenically active compounds than the other groups, and so far, such compounds have not attracted much comment in this context. The structural diversity of the compounds investigated complicates the interpretation of the results, but a slightly clearer picture emerges if the active compounds are ranked in terms of their activity. Figures 2, 3, and 4, respectively, show the structures of phenolic additives with activities of 1/1,000–1/10,000, 1/10,000–1/100,000, and <1/100,000.

The phenolic additives investigated in this study had from one to four nonfused aromatic rings in their structures; in some instances heterocyclic rings were also present. Those compounds found to be estrogenically active had only one or two nonfused aromatic rings in their structures, and with very few exceptions, one of these rings had a phenolic -OH group in a para position to an additional substituent. The structures of the active additives, shown in Figures 2, 3, and 4, make the importance of this configuration more obvious. The substituent may be an alkyl group; a chlorine atom; a methoxy group; or an ester, ketone, or C-C bond linking it to a second aromatic ring. The importance of the para position of the phenolic group is consistent with findings from a study of estrogenic activity of alkylphenolic compounds (4). The phenolic additives that were inactive in this study were chiefly those in which the phenolic -OH group was in an ortho position relative to other substituents, or where the 2,6 positions relative to the phenolic -OH group were occupied by other substituents, for example, additional -OH groups in the case of the gallates and purpurogallin, bromine atoms in the case of tetrabromobisphenol A, or t-butyl groups in the case of 4,4´-methylenebis(2,6-di-t-butyl phenol) (Chemical Abstracts Service (CAS) no. 118-82-1, 2,6-di-t-butyl-4-(dimethylaminomethyl)phenol (CAS no. 88-27-7), octadecyl-3-(3′,5′-di-t-butyl-4-hydroxyphenyl)propionate (CAS no. 2082-79-3), and 2,6 di-t-butylphenol. This suggests that where the hydrophilic phenol

| Table 1. Phenolic additives used as sunscreens or light stabilizers. |
|------------------|------------------|---------------|-------------|
| **Compound** | **CAS registry no.** | **Usage group** | **Estrogenic activity** | **MW** |
| Benzophenone-1 | 131-56-6 | 1.2 | 1/3,000 | 214.2 |
| Benzophenone-2 | 131-55-5 | 1.2 | 1/7,000 | 246.2 |
| Benzophenone-3 | 131-57-7 | 1.2 | Submax | 228.3 |
| Benzophenone-4 | 4065-45-6 | 1 | ND | 308.3 |
| Benzophenone-5 | 121-54-6 | 2 | Submax | 247.3 |
| Benzophenone-7 | 85-19-8 | 2 | Submax | 232.7 |
| Benzophenone-8 | 131-53-3 | 1 | ND | 244.2 |
| Benzophenone-12 | 1843-05-6 | 2 | ND | 326.5 |
| 4,4’-Dihydroxybenzophenone | 611-94-4 | 2 | 1/40,000 | 214.2 |
| Phenyl salicylate | 118-55-8 | 1 | 1/300,000 | 214.4 |
| Benzyl salicylate | 118-58-1 | 1 | Submax | 228.3 |
| M ethyl salicylate | 89-46-3 | 1 | Submax | 274.6 |
| Ethylphenyl salicylate | 118-60-5 | 1 | Submax | 250.9 |
| Triethanolamine salicylate | 2174-16-5 | 1 | ND | 287.3 |
| Resorcinol monobenzoate | 126-76-1 | 2 | 1/80,000 | 214.2 |
| Octozole | 3147-75-9 | 2 | ND | 323.4 |
| 2,4-Di-t-butyl-6-isoo-chloro-2H-benzotioazole-2-ylphenol | 3864-99-1 | 2 | ND | 357.9 |
| 7-Hydroxycoumarin | 93-35-6 | 1 | ND | 162.1 |

Abbreviations: CAS, Chemical Abstracts Service; MW, molecular weight; ND, not detected; Submax, submaximal response curve; usage group 1, cosmetic sunscreen; usage group 2, light stabilizer for polymers. Estrogenic activity suggests the potency related to 17β-estradiol.

| Table 2. Phenolic additives used as preservatives. |
|------------------|------------------|---------------|-------------|
| **Compound** | **CAS registry no.** | **Estrogenic activity** | **MW** |
| Dodecylparaben | 2664-60-0 | ND | 306 |
| Benzylparaben | 94-18-8 | 1/4,000 | 228.2 |
| Butylparaben | 94-26-8 | 1/8,000 | 194.2 |
| Propylparaben | 94-13-3 | 1/30,000 | 180.2 |
| Ethylparaben | 120-47-8 | 1/200,000 | 166.2 |
| M ethylparaben | 99-76-3 | 1/3,000,000 | 152.2 |
| Dichlorophen | 97-23-4 | ND | 269.1 |
| 2-Hydroxybenzophenyl | 90-43-7 | 1/2,000,000 | 170.2 |
| 4-Hydroxybenzophenyl | 92-69-3 | 1/10,000 | 170.2 |
| Salicylic acid | 69-72-7 | ND | 138.1 |

Abbreviations: CAS, Chemical Abstracts Service; MW, molecular weight; ND, not detected.

| Table 3. Phenolic additives used as disinfectants. |
|------------------|------------------|---------------|-------------|
| **Compound** | **CAS registry no.** | **Estrogenic activity** | **MW** |
| Phenol | 108-95-2 | ND | 94.1 |
| 2-Methylphenol | 95-48-7 | ND | 108.1 |
| 4-Methylphenol | 106-44-5 | ND | 108.1 |
| Thymol | 89-83-8 | ND | 150.2 |
| Chlorothymol | 89-68-9 | 1/400,000 | 184.7 |
| 4-t-Amylphenol | 80-46-6 | 1/200,000 | 164.3 |
| Carvacrol | 499-75-2 | ND | 150.2 |
| 4-Chloro-3-methylphenol | 59-50-7 | 1/3,000,000 | 142.6 |
| 4-Chloro-3,5-dimethylphenol | 88-94-0 | 1/900,000 | 156.6 |
| 2,2’-Dihydroxybenzophenyl | 1806-29-7 | ND | 186.2 |
| 8-Hydroxyquinoline | 148-24-3 | ND | 145.2 |

Abbreviations: CAS, Chemical Abstracts Service; MW, molecular weight; ND, not detected.

| Table 4. Phenolic additives used in flavoring and perfumery. |
|------------------|------------------|---------------|-------------|
| **Compound** | **CAS registry no.** | **Estrogenic activity** | **MW** |
| Eugenol | 97-53-0 | ND | 164.2 |
| Isoeugenol | 97-54-1 | ND | 164.2 |
| Vanillin | 121-33-5 | ND | 152.2 |
| Ethyl vanillin | 121-32-4 | ND | 166.2 |
| M ethyl salicylate | 119-36-8 | ND | 152.2 |
| Hexyl salicylate | 6259-76-3 | ND | 222.3 |

Abbreviations: CAS, Chemical Abstracts Service; MW, molecular weight; ND, not detected.
that of the parent compound, but Routledge and Sumpter (4) showed that the estrogenic activity of alkyl phenols peaked with 4-t-octylphenol, which has a molecular weight of 206.4. The series of phenolic additives examined in this study display much more structural variation than the alkyl phenols, but there does seem to be a similar optimum molecular weight. For example, the mean molecular weights of compounds falling within the three potency ranges of 1/1,000–1/10,000, 1/10,000–1/100,000, and <1/100,000 are 208.2, 202.9 and 166.7, respectively. Figure 5, a scatter diagram in which molecular weight is plotted against the negative logarithm of the potency, shows a trend (correlation coefficient –0.74) in which activity diminishes as the molecular weight decreases from an optimum range of about 200–230. Clearly this trend is consistent with the concept of a receptor site that can accommodate molecules of the appropriate size, shape, and charge distribution, but molecular weight is a crucial criterion because it does not take into account any aspect of the shape or charge distribution parameters. Notwithstanding, when the inactive additives are considered, there were nine compounds that had an unhindered phenolic -OH group para to some other substituent; these were all found to have molecular weights of <164 or >302, which suggests that their lack of activity is probably size related (Figure 5). There is no apparent tendency for compounds with molecular weights exceeding about 250 to display a gradually diminishing activity. Instead there seems to be a sharp cutoff between active and inactive compounds, which is what might be expected if a size-exclusion mechanism excludes larger molecules from the estrogen receptor site. Unfortunately, in the group of compounds tested there were no compounds in that crucial molecular weight region with an unhindered phenolic -OH group para to some other substituent.

Thus, the two most important criteria to emerge from this study in relation to phenolic additives displaying estrogenic activity are a) having a phenol with a para configuration and b) having a molecule of appropriate size. Because the structural features of commercially important phenolic additives are so varied, it is only possible to cite a few illustrative examples from the data. For example, 4-hydroxybiphenyl is far more active than 2-hydroxybiphenyl; 4,4'-dihydroxybiphenyl is active and 2,2'-dihydroxybiphenyl is inactive; and insertion of a -CH3- or -CO(CH3)2-group between the two aromatic rings of 4,4'-dihydroxybiphenyl [i.e., to give bis(4-hydroxyphenyl) methane and bisphenol A] makes virtually no difference to the activity of the resulting compounds. Thymol, which has no substituent group in the para position to the phenolic -OH group, is inactive, whereas chlorothymol, which has a chloro group in that position, is active.

The benzophenones present a complex picture; this has been confirmed by Schultz et...
al. (16) in a recent study of the estrogenicity of 18 benzophenone derivatives using a recombinant yeast assay. Schultz et al. (16) attempted to predict the level of estrogenicity using certain structural rules, but few of the compounds they examined are in use as additives. The most active compounds detected in our own work (i.e., benzophenone-1 and benzophenone-2) have phenolic -OH groups in both para and ortho positions, whereas 4,4’-dihydroxybenzophenone, having only para -OH groups, is surprisingly less active. Other benzophenones either produced no response or were classified as slightly estrogenic due to submaximal response curves, but HPLC with UV detection suggests that trace contamination with benzophenone-1 may be responsible for the slight activity observed in some of these compounds. These other compounds only had phenolic -OH groups in the ortho position.

The paraben esters, which have previously been studied in detail using the same yeast assay (17), display a progressive increase in estrogenic activity as the molecular weight increases from 152.2 to 228.2. In general, their activity was substantially greater than the salicylate esters, which is what would be expected because the ester group in the salicylate esters is in an ortho position relative to the phenolic -OH group. The most active of the salicylate esters studied was phenyl salicylate, which with a molecular weight of 214.2 was close to the mean value for the most active group of compounds, whereas its activity level was close to that of ethyl paraben.

This study suggests that a surprisingly large number of chemicals in everyday use may possess weak estrogenic activity, at least in vitro. This contention is supported by a preliminary announcement by Tong et al. (18) of a very intelligent and thorough SAR-based modeling study of the 57,000 chemicals in the database of the U.S. Food and Drug Administration. The authors suggest that over 3,000 of the 57,000 chemicals probably possess weak estrogenic activity (at least in vitro). The identification of chemicals

Table 6. Estrogenic potency values (10% response level) for compounds displaying submaximal responses.

| Compound                  | CAS registry no. | Estrogenic potency |
|---------------------------|-----------------|--------------------|
| Benzophenone-3            | 131-57-7        | 1/100,000          |
| Benzophenone-6            | 131-54-4        | 1/20,000,000       |
| Benzophenone-7            | 85-19-8         | 1/300,000          |
| Benzyl salicylate         | 118-58-1        | 1/600,000          |
| Methyl salicylate         | 89-46-3         | 1/200,000          |
| Ethylhexyl salicylate     | 118-60-5        | 1/2,000,000        |
| Nordihydroguaiaretic acid | 500-38-9        | 1/600,000          |
| Butylated hydroxytoluene  | 128-37-0        | 1/8,000,000        |
| 2,6-Di-t-butylphenol      | 128-39-2        | 1/20,000,000       |
| Butylated hydroxyanisole  | 25013-16-5      | 1/2,000,000        |

CAS, Chemical Abstracts Service.
Conclusions

This study has shown that a substantial number of phenolic additives incorporated in a group of different products display slight estrogenic activity when assessed by an in vitro yeast assay. The strongest activity is chiefly displayed by those compounds in which a phenolic -OH group is in a position to some extent to some other substituents and the molecular weight falls within the range 200–250.

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Figure 4. Chemical structures of compounds with an activity relative to 17β-estradiol of < 1/100,000.

Figure 5. Scatter diagram showing the relationship between molecular weight and estrogenic activity for phenolic additives.
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