

# Natural and anthropogenic environmental oestrogens: the scientific basis for risk assessment\*

## Structure/activity relationships

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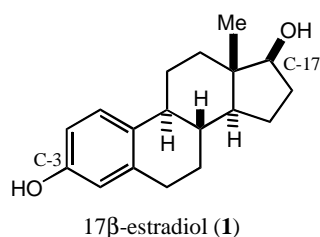
*Abstract.* Accumulated structure-activity data on environmental oestrogens has focused on several structural sub-categories within this general family of chemical agents. Of these compounds, the most extensively studied include phyto-oestrogens, macrolactones, alkylphenols and arylphenols. For phyto-oestrogens, binding to the oestrogen receptor (ER) is observed for the flavone and isoflavone nucleus particularly when the stilbene-like core is appropriately functionalized with hydroxylation at the 4' and/or 7 positions. Simple alkyl phenols behave as weak oestrogens when substituted at the *para* position with a branched aliphatic group such as found in *p*-octyl and *p*-nonyl phenol. In the case of polychlorinated biphenyls (PCBs), quantitative structure-activity studies (QSAR) indicate that the electron donating properties of the hydroxyl moiety and the aromatic component correlate highly with affinity to the ER. Further studies have shown that the phenolic ring of PCBs favour orientation over the oestradiol D-ring with the hydroxyl group of the PCB in close proximity to the 17-hydroxyl of oestradiol while maximizing the hydrophobic overlap in the central regions. Structure-activity studies on diphenylethanes, such as dichlorodiphenyltrichloroethane (DDT), demonstrate the significance of the substituents present on both the aryl rings and the side-chain. Other structural classifications include polyhalogenated carbocycles.

## INTRODUCTION

Endogenous oestrogens, such as 17 $\beta$ -oestradiol, have long been recognized as the primary hormones involved in the development and maintenance of the female sex organs, mammary glands, and other sexual characteristics. More recently, their involvement in the growth and/or function of a number of other tissues, such as the skeleton, the cardiovascular system, and the central nervous system has been recognized. These natural steroids are derived from a common platform represented by the 18-carbon oestrogen ring system (**1**). Critical structural features of this steroid class include a phenol at the C-3 position of the aromatic A-ring, a relatively flat and rigid hydrocarbon core, and a ketone or alcohol functionality at the C-17 position. A detailed pharmacophore model postulates the contribution of the two hydroxyl groups of 17 $\beta$ -oestradiol to receptor binding, with the C-3 hydroxyl acting primarily as a hydrogen bond donor and contributing approximately 1.9 kcal/mol to the binding free energy, while the 17 $\beta$ -hydroxy functions primarily as a hydrogen bond acceptor and contributes 0.6 kcal/mol (ref. 1). This model is supported by recent X-ray crystallographic data on 17 $\beta$ -oestradiol bound in the ligand binding domain of the ER (ref. 2).

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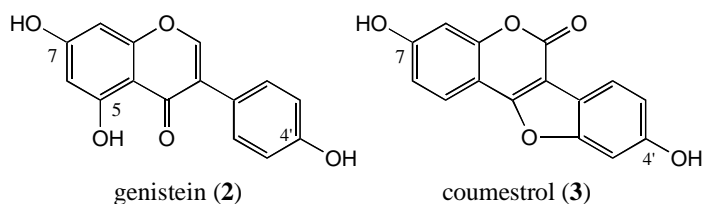
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The discovery that compounds which structurally deviate from the oestrogen platform can mimic the pharmacology of the natural steroid has proven of great significance in the field. Pioneering observations in the 1930s (ref. 3) that subcutaneous administration of non-steroidal derivatives cause the onset of oestrus in ovariectomized rats has, over the years, led to the identification of an enormous and structurally diverse array of compounds which can act as oestrogen agonists and/or antagonists. One such group, environmental oestrogens, consist of naturally occurring compounds, or commercially produced chemicals that are derived from a variety of relatively common and abundant sources such as pesticides, plastics, combustion by-products, plants (phyto-oestrogens), and agricultural products, among others. In this context, we wish to review non-steroidal environmental oestrogens with particular focus on underlying structural themes among this unique class of chemical entities.

### PHYTO-OESTROGENS

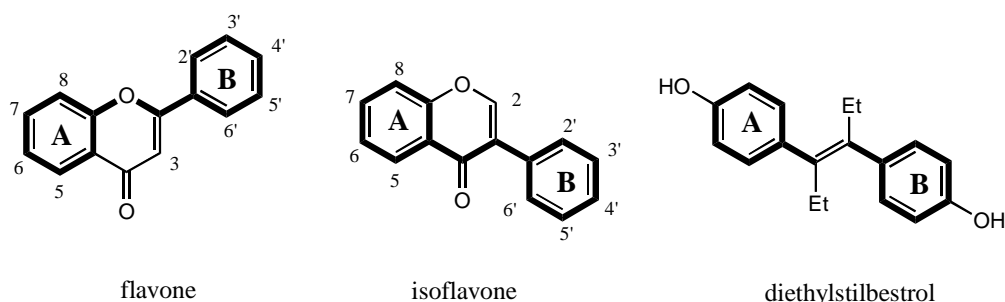
Almost 200 different naturally occurring phyto-oestrogens with some degree of biological activity have been identified from plant sources to date. Of these, the most studied include genistein (**2**, Figure 1) and coumestrol (**3**, Figure 1). Genistein is a weak oestrogen which competes with 17β-oestradiol in receptor binding assays (ref. 4) and has been shown to have oestrogenic activity on the uterus, mammary and hypothalamic axis in rats (ref. 5). Coumestrol binds effectively to the ER, stimulates MCF-7 breast cancer cell proliferation, and has demonstrated a number of oestrogen agonist activities including effects on the uterus and bone (ref. 6). Structural studies on coumestrol have revealed the importance of the phenolic hydroxyl groups in regulating the uterotrophic activity for this class of compounds, i.e., blocking of these groups results in a significant decrease in uterine weight gain after administration to rats (ref. 7). Methylation of the 4'-hydroxyl group diminishes the oestrogenic response to a greater extent than methylation of the corresponding 7-hydroxyl group, although neither of these point-modifications are as oestrogenic as coumestrol. Replacement of the 7 or 4' hydroxyl group with hydrogen diminishes activity by approximately 1/6 while replacement of both hydroxyl groups eliminates oestrogenic activity altogether. Other modifications that decrease oestrogenicity include opening of the furan ring, formation of the *o*-methoxycinnamic acid derivative, and the presence of additional hydroxyl groups.



**Fig. 1.** Common phyto-oestrogens.

A recent systematic study of the structural requirements for flavones and isoflavones reveal several molecular features which regulate the pharmacological characteristics of these compounds (ref. 8). Structural comparisons with diethylstilbestrol (DES), a potent non-steroidal oestrogen, reveal a common stilbene-like core imbedded in both DES and the flavonoid nucleus (see Figure 2). The hydroxylation pattern on the A,B-diaryl core plays a significant role in determining the affinity of a given compound for the ER. Miksicek has examined a wide range of hydroxyl flavonoids for their relative binding affinities to the ER (ref. 8). These results show that 4',5-dihydroxyflavone, 4',7-dihydroxyflavone, and 4',7-dihydroxyisoflavone compete with 17β-oestradiol for binding to the ER, but only when present in a 1,000- to 10,000-fold molar excess relative the steroid. Higher relative affinity for the ER is displayed by

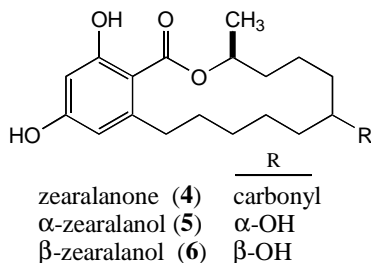
4',6-dihydroxyflavone whereas 3',6-dihydroxyflavone, 4'-hydroxyflavone, 6-hydroxyflavone, or 7-hydroxyflavone are significantly less potent. Examination of these agents in a transient transfection assay measuring the transcriptional activation of the human ER indicate that the most important structural features for functional oestrogenic activity include a stilbene-like diaryl core containing a minimum of one hydroxyl group on each of these aryl rings. In addition, hydroxyl substituents in the 4' and 7 of the flavone or isoflavone framework result in transcriptional activation, data which correlates well with the ER binding affinity observed for these agents. An additional hydroxyl group at the 5-position on these nuclei are well-tolerated and, in some cases, increase oestrogenicity (ref. 8). The hydroxylation pattern of the A ring indicate that mono-hydroxylation at the 5-, 6-, or 7-position result in oestrogenicity when the 4'-position is concomitantly hydroxylated. Such flexibility is not apparent in the B-ring, where transposition of a hydroxyl group from the 4' to 3' greatly diminishes transcriptional activation. Vicinal hydroxylation patterns that create catechols, such as 7,8 and 3',4' flavones, appear to abolish oestrogenic activity. In addition, 4'-methoxylation significantly lowers the oestrogenicity *in vitro* setting although demethoxylation to the more active hydroxyl metabolite may occur *in vivo*. (ref. 8).



**Fig. 2.** Structures and conventional numbering systems for the flavone and isoflavone nucleus as compared to diethylstilbestrol.

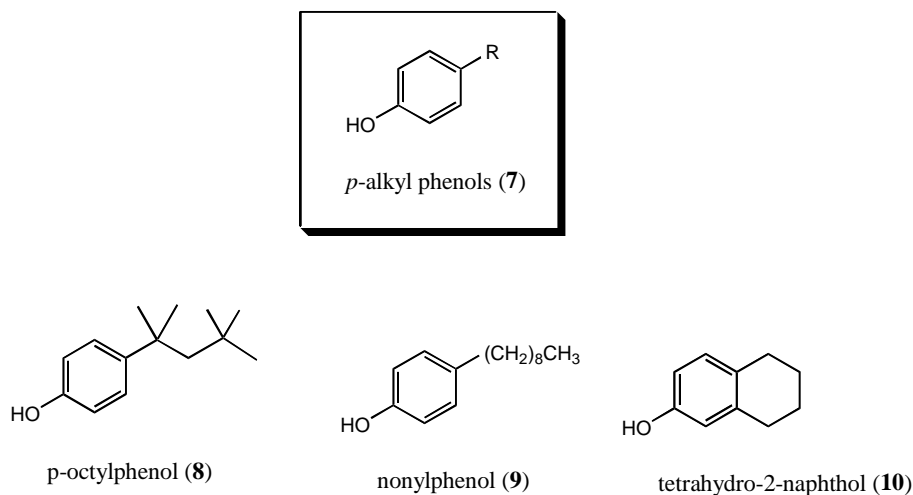
## MACROLACTONES

A oestrogenic potential of a series of 14-membered ring was originally identified as a result of observed hyper-oestrogenicity in swine following the ingestion of mould-infected corn. These mycotoxins, exemplified by zearalenone (4), have been extensively studied for their oestrogen agonist behavior (ref. 9). Competitive receptor binding experiments against oestradiol have shown zearalenone and analogues bind to the ER with a relative binding order of  $\alpha$ -zearalenol (5) > zearalenone,  $\beta$ -zearalenol (6). *In vivo* studies indicate  $\alpha$ -zearalenol is uterotrophic in rats. This compound has also been used to promote growth in cattle (ref. 10) and relieve post-menopausal stress in women (ref. 11). Despite their apparent structural dissimilarity with oestrogens, these lactones possess a phenolic hydroxyl group which is implicated as a potential site for interaction with the ER in a manner similar to the 3-OH group of oestrogens. A model to rationalize the ER binding of trans-zearalenone has been proposed (ref. 13).



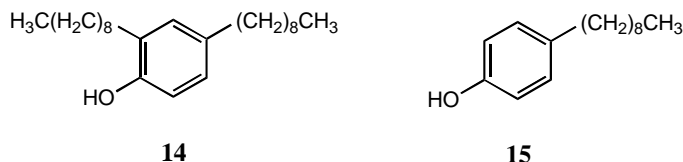
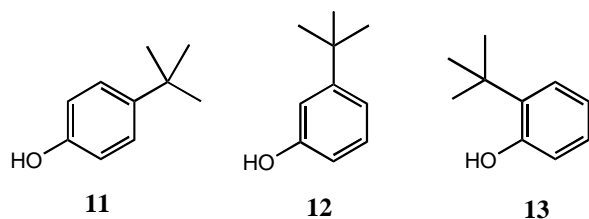
### ALKYLPHENOLS AND ARYLPHENOLS

Soon after the identification of DES as a potent non-steroidal oestrogen, other substituted phenols were also found have oestrogenic activity (ref. 3). Subsequent work has shown a phenolic hydroxyl group with an alkyl substituent at the *para* position, such as **7**, is a common pharmacophore among many oestrogenic compounds. The most studied of these agents includes *p*-octyl phenol (**8**) and *p*-nonyl phenol (**9**) which have shown uterotrophic activity *in vitro* (refs 13, 14) and *in vivo* (ref. 15). When tested in human breast cancer cells (MCF-7), the most potent of the alkyl phenols is *p*-octyl phenol which produces biological responses similar to 17 $\beta$ -oestradiol, although at 1000-fold greater concentration (ref. 14). The oestrogenic activity of bicyclic structures having a phenolic substituent, such as 1-, and 2-naphthol (**10**), has also been described (ref. 13).

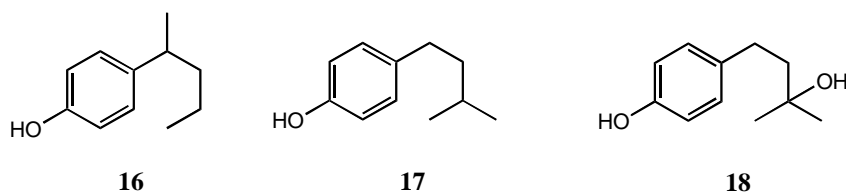


**Fig. 3.** Structures of common alkyl phenols.

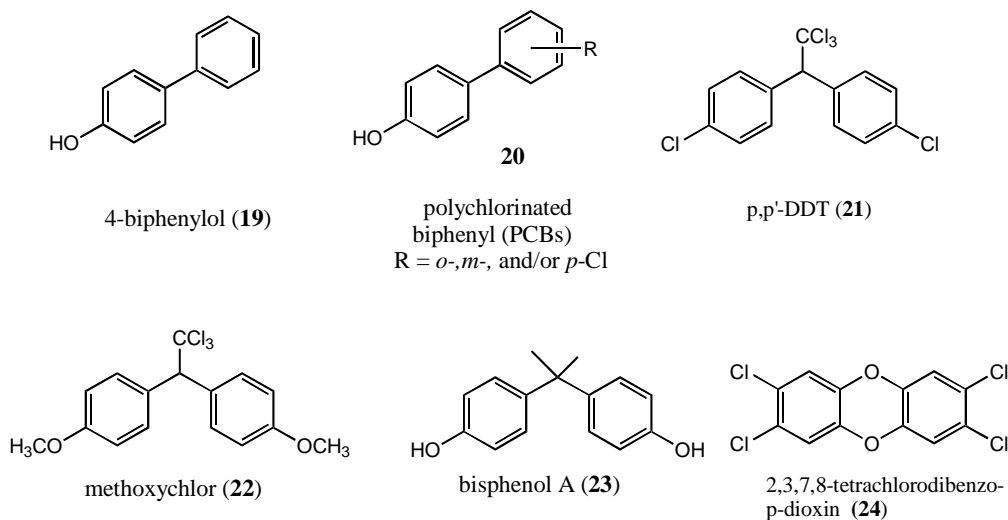
A comprehensive examination of the structural requirements for alkyl phenols has recently been published which indicates that both the position and nature of the alkyl group dramatically effects the oestrogenic character of these compounds (ref. 16). Although significantly less potent than 17 $\beta$ -oestradiol, oestrogen-like activity in a yeast screen is observed for compounds such as **7** in which the *para* alkyl group R contains three or more carbon atoms (ref. 16). In addition, tertiary alkyl chains are generally the most oestrogenic indicating that the branching at the benzylic position is an important feature. For example, *p*-tert-butylphenol (**11**) is approximately 30 fold more active than *p*-nonyl **9**. The position of the alkyl substituent on the aromatic ring also plays an important role. Oestrogenicity decreases as the alkyl group is moved from *para* (**11**) to *meta* (**12**) to *ortho* (**13**), respectively. Di-substitution with large alkyl groups (> 3 carbons/substituent) greatly diminishes activity, e.g., **14** is significantly less potent than the 2,4-disubstituted analogue **15**.



A series of alkyl phenols has been studied for their ability to inhibit binding of  $^3\text{H}$ -17 $\beta$ -oestradiol to the ER (ref. 13). This data suggests that size, position, and the hydrophobic character of the alkyl group dictates receptor affinity. A four carbon substituent *para* to the phenolic hydroxyl group provides enhanced binding properties. Introduction of branching in the benzylic position, e.g., **16** vs. **17**, increases the affinity to the receptor whereas the inclusion of hydrophilic functionality, such as the hydroxyl group in **18**, decreases binding.



In addition to aliphatic functionality on a phenol (**7**, R = alkyl), the analogous aromatic substituents (**7**, R = aryl) are also oestrogenic. Compounds in this class include: (i) biphenyls (**19**) and polychlorinated biphenyls (PCBs, **20**); (ii) diphenylmethanes such as p,p'-DDT (**21**), methoxychlor (**22**) and bisphenol-A (**23**); and (iii) tricyclic polynuclear aromatics, most commonly represented by dioxin (**24**). The position of the aryl group relative to the phenol plays a significant role, i.e., movement of the phenyl group from *para* to *meta* to *ortho* position results in 7-fold reduction in activity for each successive repositioning (ref. 16). In the case of PCBs, such as **20**, quantitative structure-activity studies (QSAR) indicate that the electron donating properties of the hydroxyl moiety and the aromatic component correlate highly with ER binding affinity (ref. 17). In addition, conformational modeling of a sub-set of PCB's indicate that the most polarizable conformers are most closely associated with receptor affinity. Other studies have shown using comparative field analysis (CoMFA) that the ER binding affinity of polychlorinated biphenyls could be related to a steric field (ref. 18). In this study, conformational restriction imparted by *ortho* substitution was critical for ER binding affinity whereas a hydroxyl group on the aromatic A ring was not essential for binding. Further studies have shown that the phenolic ring of PCBs favour orientation over the oestradiol D-ring with the hydroxyl group of the PCB in close proximity to the 17-hydroxyl of oestradiol while maximizing the hydrophobic overlap in the central B- and C-rings (ref. 19).



For DDT (**21**) and analogues, structural analysis has identified geometric similarities between these compounds and  $17\beta$ -oestradiol in which the internuclear distances of relevant functional groups are overlapping (ref. 20). A diverse array of DDT analogues have been examined for their oestrogenic potential using a glycogen response in the uterus of immature rats. Many diphenylmethane, (**25**) diphenylethane (**26**) and triphenylmethane (**27**) compounds were oestrogen-like when the *p*- or *p'*-position is substituted with a hydrogen, hydroxyl, or methoxy functionality whereas similar substitution with halide or alkyl groups result in diminished activity. For the diphenylethane (**26**) series, a R'' group such as trichloroethane or vinyl halide was found to be necessary for oestrogenic activity whereas more polar groups, such as alcohol, aldehyde or carboxylic acid, are inactive. Using such cumulative data, a model has been proposed in which the active sites of diphenylethanes are 9–11 angstroms apart, a distance which is similar to both natural and synthetic steroids (ref. 20).

The substitution pattern on the biphenyl ring of DDT and analogues plays a major role in regulating interactions of these compounds with the ER. For example, *o,p'*-DDD analogue **28** binds with considerably higher affinity to the ER than analogues **29** (*m,p'*) and **30** (*p,p'*) (ref. 21). Similar trends in binding affinity are also observed for the *o,p'* vs *p,p'* analogues of DDT and DDE. The halogenated side-chain R'' of diphenylethane **26** also affects the ER binding properties of these molecules with the following trends in receptor affinity being observed for *o,p'*-halogen substituted derivatives:  $\text{CCl}_3$  (DDT) >  $\text{CHCl}_2$  (DDD)  $\gg$   $=\text{C}(\text{Cl})_2$  (DDE) (ref. 21). Inhibition of  $^3\text{H}$ - $17\beta$ -oestradiol binding by DDT and analogues has been shown to correlate with increases in uterine weight gain after administration to rats (ref. 21). The metabolic profile of the substituents present on the biphenyl core is also important. Extensive metabolism studies with methoxychlor (**22**) indicate this compound is progressively demethylated to the corresponding mono- and bis-phenol derivatives. Binding studies with methoxychlor indicate that the demethylated metabolites have a higher affinity for the oestrogen receptor (ref. 21).

In general, DDT and analogues have oestrogenic activity which is several orders of magnitude less potent than  $17\beta$ -oestradiol. In a recent study, a large number of environmental chemicals were tested for oestrogenic activity in a yeast-based ER receptor assay and compared with  $17\beta$ -oestradiol. These data indicate the *o,p'*-DDT, *o,p'*-DDD, and *o,p'*-DDE were 8, 15, and 24 million times less potent than  $17\beta$ -oestradiol (ref. 22). Other studies have shown that in MCF-7 cells, *o,p'*-DDT has approximately one millionth the activity of  $17\beta$ -oestradiol (ref. 23).

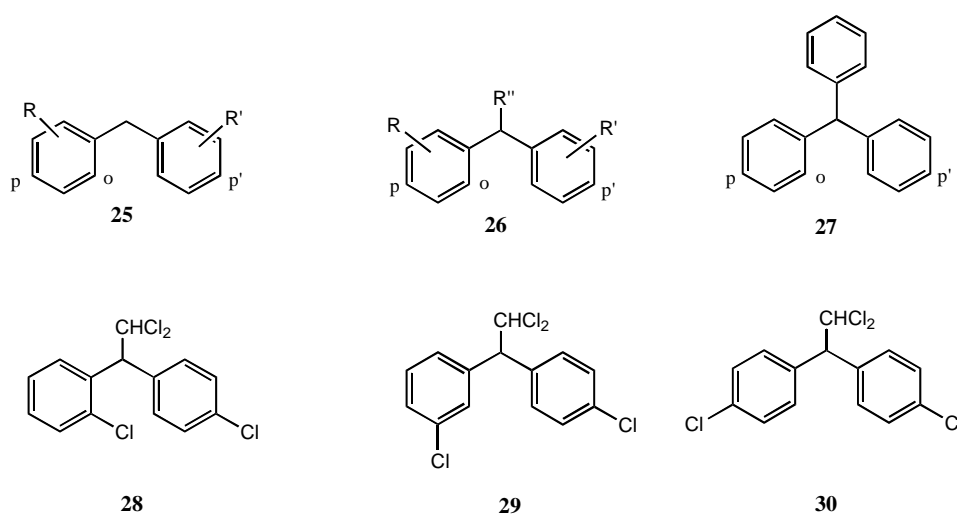


Fig. 4. DDT and analogues.

### NON-AROMATIC OESTROGENS

Although the majority of non-steroidal oestrogens possess an aromatic ring, a number of interesting compounds have been identified which lack this pharmacophore. The majority of these agents are xenoestrogens derived from commercial sources, such as pesticides, and include halogenated carbocycles such as hexachlorocyclohexane (**31**), chlordecone (kepone, **32**), and toxaphene, dieldren, endosulfan (**33**), and chlordan. Studies with hexachlorocyclohexane (**31**) indicate that it has oestrogen agonist activity in MCF-7 cells although it does not bind to the oestrogen receptor (ref. 24). Comparative molecular field analysis of chlordecone and oestradiol indicate the optimal structural alignment is one in which the bulk of the chlordecone nucleus is positioned over the B-, C-, and D-ring of oestradiol with the carbonyl moiety pointing toward the 3-hydroxyl position of the steroid (ref. 19). Although the origin(s) of the oestrogen agonist behaviour exhibited by this class of compounds is not entirely clear, the mode of action for this class of compounds may occur via non-classical pathways (ref. 24).

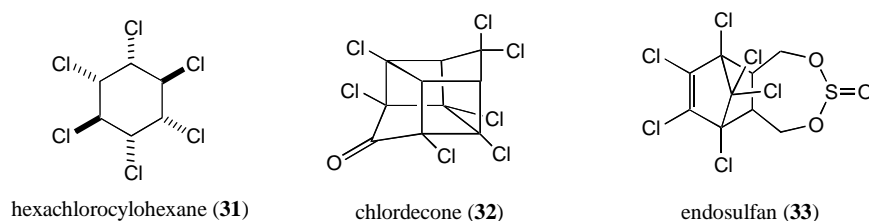
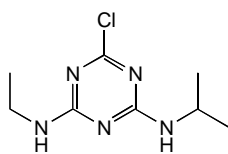


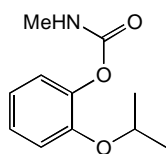
Fig. 5. Structures of environmental oestrogens that lack an aromatic ring pharmacophore.

### MISCELLANEOUS TEMPLATES

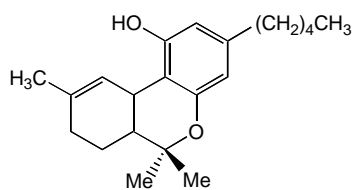
A variety of additional structural frameworks have been identified as being oestrogenic in some context. These include: (i) atrazine (**34**) (ref. 25); (ii) insecticidal carbamate insecticides (ref. 26) such as aldicarb, propoxur (**35**), bendocarb, carbaryl, methamomyl and oxamyl; (iii) delta-9-tetrahydrocannabinol (**36**) (ref. 27); (iv) phenol red and analogues (**37**) (ref. 28); and (v) phthalates (**38**) (ref. 29).



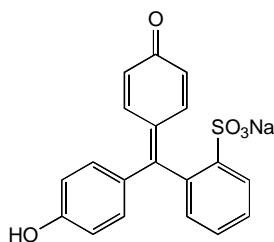
atrazine (34)



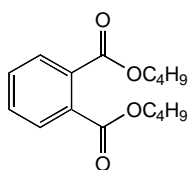
propoxur (35)



tetrahydrocannabinol (36)



Phenol Red (37)



di-n-butylphthalate (38)

## CONCLUSIONS

Since the initial observations in the 1930s that non-steroidal compounds can elicit similar biological responses to that of  $17\beta$ -oestradiol, a diverse array of non-steroidal structural platforms have been identified. On such group, environmental oestrogens, consists of a diverse group of structures including flavones/isoflavones, macrolactones, aryl/alkyl phenols, and halogenated carbocycles, and others. A pharmacophore model which has emerged from this vast body of structural information defines several molecular determinants of oestrogen agonism. These include a phenolic moiety, thought to be critical for favourable interactions with the ER, and a hydrophobic alkyl or aryl group at the *para* position relative to the phenol. Although a variety of mechanisms which are independent of the ER have been advanced to explain the actions of oestrogen agonists and antagonists (refs 24, 30), it is generally accepted that the majority of their biological effects are mediated via interaction with this receptor. The sequence of events which allows individual ligands to manifest profiles of gene activation which are similar to, or different than, the natural ligand is incompletely understood and, consequently, the subject of much research and debate (refs 31, 32). The recent discovery of a second ER, ER $\beta$ , adds a further layer of complexity in understanding the structural requirements for oestrogenicity, particularly since little is known with regards to the interactions of non-steroidal oestrogens to this ER isoform.

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