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The impact of structural and metabolic variation on the toxicity and carcinogenicity of hydroxy- and alkoxy- substituted allyl- and propenylbenzenes.

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Abstract

The metabolic fate of a compound is determined by numerous factors including its chemical structure. Although the metabolic options for a variety of functional groups are well understood, and can often provide a rationale for comparison of toxicity based on structural analogy, at times, quite minor structural variations may have major consequences for metabolic outcomes and toxicity. In this paper, the effects of structural variations on metabolic outcomes is detailed for a group of related hydroxy- and alkoxy- substituted allyl- and propenylbenzenes. These classes of compounds are naturally occurring constituents of a variety of botanical based food items. The classes vary from one another by the presence or absence of alkylation of their *para*-hydroxyl substituents and/or the position of the double bond in the alkyl side chain. We provide an overview of how these subtle structural variations alter the metabolism of these important food-borne compounds, ultimately influencing their toxicity, particularly their DNA reactivity and carcinogenic potential. The data reveal that detailed knowledge of the consequences of subtle structural variations for metabolism is essential for adequate comparison of structurally related chemicals. Taken together, it is concluded that predictions in toxicological risk assessment should not be performed on the basis of structural analogy only, but should include analogy of metabolic pathways across compounds and species.

Keywords: safrole, estragole, methyleugenol, eugenol, chavicol, anethole, isoeugenol, isoeugenol methyl ether, structure-activity relationship

Introduction

The toxicity of chemical compounds is a function of their structure as well as the structure of the main metabolic products. The metabolic fate of any compound in a biological system is, in turn, primarily determined by its chemical structure and specifically the presence of functional groups and their transformation. Additional factors that play a critical role in determining the specific enzymes involved and the kinetics of the metabolic processes include, to various extents, the dose of the compound, species, stran, sex, co-exposures and genetic factors. A chemical structure is defined by not only the overall molecular skeleton, e.g. aromatic or alicyclic compounds, but also by the type and relative position of functional groups. Minor differences in chemical structure may result in significant differences in either the biological properties of the parent compound or in its metabolic fate, and may have major consequences for the biological activity and therefore toxicity of a compound. In the first case, minor differences in chemical structure may influence the affinity for a molecular target and thus impact the biological activity of a compound. In the second case, apparently minor variations in either the type or the relative position of functional groups of similar compounds may dramatically alter their properties as substrates for metabolic enzymes and may result in major differences in the metabolic products. Consequently, while the metabolic options for a variety of functional groups are well understood, and can often provide a rationale for comparison of metabolic fate and toxicity based on structural analogy, the predictions may not always apply.

The organocyanides (nitriles) provide an illustration of the impact of structural differences on the toxicity of compounds carrying the same functional group. The type of molecular skeleton to

which the nitrile is attached determines the degree of toxicity subsequent to different metabolic outcomes. Specifically, nitrile attached to a simple aliphatic skeleton is metabolised to release toxic hydrogen cyanide, and thus aliphatic nitriles are highly toxic.[1-3] However, a nitrile function directly attached to an aromatic ring is metabolically stable and such organonitriles have low toxicity.[4] A similar example is found in the ability of aliphatic and aromatic carboxylic acids to undergo conjugation reactions. Benzoic acid, in which the carbon of the carboxylic acid is directly attached to the aromatic ring, is conjugated with glycine to form hippuric acid in humans.[5,6] In contrast, phenylacetic acid, the homologue in which the carboxylic acid is attached on the ring via a spacer of one aliphatic carbon, is conjugated with glutamine to form phenylacetylglutamine.[7] More interestingly, the presence of an alpha-methyl function (hydratropic acid) on phenylacetic acid results in complete loss of the carboxylic acid's capacity to form amino acid conjugates, and it undergoes metabolism by conjugation with glucuronic acid instead.[8]

The influence of subtle structural variations on the biological properties of chemical compounds is intentionally explored in a variety of chemical applications and in the design of biologically active, novel molecules with applications in therapeutics, where structure is modified according to desired pharmacological activity combined with absence of toxicity. Structurally related substances with different properties can be found as naturally occurring components of plants, including edible ones, in the form of pharmacologically active principles or flavouring substances. For example, 2-hydroxybenzoic acid (salicylic acid) is commonly used for the treatment of acne and warts, while 3-hydroxybenzoic acid is used as a fragrance ingredient.

In this paper the impact of subtle structural variations on metabolism and resulting toxicity is evaluated for a group of structurally related hydroxy- and alkoxy-substituted allyl- and propenylbenzenes (Figure 1), which are present naturally as constituents of a variety of edible plants and are added as flavouring substances in a range of food items. The nine members of the group examined in this paper are naturally occurring flavouring substances that differ in two specific structural features: a) the position of the double bond on the alkyl side chain; and b) the number and alkylation state of the hydroxyl groups on the aromatic ring (Figure 1). All nine structures bear an alkyl side chain and a hydroxy or methoxy (alkoxy) moiety on the *para*-position relative to the alkyl side chain. In four of the nine structures an additional methoxy-moiety is present on the meta-position relative to the alkyl side chain.

A number of food compounds belong to each of these classes: allylalkoxybenzenes include estragole, methyleugenol and safrole; propenyl alkoxybenzenes include anethole (4-methoxypropenylbenzene) and isoeugenol methyl ether (3,4-dimethoxypropenylbenzene); allyl hydroxybenzenes include chavicol (4-hydroxyallylbenzene) and eugenol (4-hydroxy-3-methoxyallylbenzene); and propenyl hydroxybenzenes include isochavicol and isoeugenol (4-hydroxy-3-methoxypropenylbenzene). An overview of the natural occurrence of these compounds in foods is presented in Table 1. It is clear from this compilation that plants and herbs can contain several of these compounds simultaneously and that each compound is found in several different plants. Thus, the allylalkoxybenzenes safrole, methyleugenol and estragole are constituents of nutmeg, cinnamon, anise star, tarragon, sweet basil, and sweet fennel (Table 1), and they are present in the essential oils as well as in food products derived from these plants (e.g. basil pesto sauce). The members of hydroxy- and alkoxy-substituted allyl- and

propenylbenzenes are structural analogues that differ in the number of alkylated hydroxyl moieties and/or the position of the double bond in the alkyl side chain. Interestingly, distinct toxicity has been reported for some but not all of these compounds (Table 2). Thus, safrole, methyleugenol and estragole have been reported to have DNA reactivity and show hepatotoxicity leading to liver tumors in rodents at high dose levels[9-15], while the related compounds eugenol, chavicol, isoeugenol, isochavicol, isoeugenol methyl ether and anethole do not show DNA reactivity (Table 2). An overview of the chemical mechanisms responsible for the differential metabolic fate and subsequent biological properties among these structural analogues is presented here as a means to understand the ultimate hazard potential of these flavour substances.

A. Toxicity of hydroxy- and alkoxy-substituted allyl- and propenylbenzenes

The differences in the reported toxicity profiles among the members of these structurally related groups of hydroxy- and alkoxy-substituted allyl- and propenylbenzenes are intriguing given their structural similarity and may seem puzzling before their metabolic fate is examined. A brief description of their respective toxicity profiles is presented in the following sections.

A.1. Allylalkoxybenzenes: safrole, methyleugenol and estragole

There is ample evidence for the carcinogenicity of allylalkoxybenzenes in rodents. Clear evidence of hepatocellular adenomas, hepatocellular carcinomas and hepatoblastomas has been

shown in National Toxicology Program (NTP) studies of methyleugenol, dosing 37, 75, or 150 mg/kg bw/day to B6C3F1 male and female mice by gavage.[16] Similar responses were found in F344/N rats, including increased incidences of hepatocellular adenomas and carcinomas in both males and females, following administration of 37, 75, 150, and 300 mg/kg bw/day of methyleugenol by gavage. Furthermore, the incidences of hepatic cholangiomas and hepatic cholangiocarcinomas were also increased at the two highest dose levels in male rats.[16] These effects have been attributed to the formation of DNA adducts of reactive metabolites, specifically the 1'-sulfoxy metabolites. Similarly, the hepatocarcinogenicity of safrole and estragole has been correlated to the formation of their respective 1'-sulfoxy metabolites (Figure 2).[9,17-21] The role of the 1′-sulfoxymetabolite in hepatotoxicity and hepatocarcinogenicity of alkenylbenzenes has been revealed in experiments by co-administration of the specific sulfotransferase inhibitor pentachlorophenol (PCP) to mice, which resulted in potent inhibition of hepatic tumour induction upon long-term dietary administration of safrole or its metabolite, 1'-hydroxysafrole.[11,22]

The involvement of sulfotransferases in the generation of DNA reactive metabolites was also demonstrated in genotoxicity studies of 1'-hydroxymethyleugenol using *Salmonella typhimurium* TA100 strains expressing human sulfotransferases (SULT), particularly the SULT1A1 and SULT1C2 isoforms.[23] Herrmann et al.[24] also studied the role of SULT enzymes in the formation of hepatic DNA adducts by methyleugenol in vivo. Using FVB/N mice [wild-type (wt)] and genetically modified strains including a Sult1a1 knockout (ko) strain, a strain transgenic for human SULT1A1/2 (tg) and for the combination of both modifications (ko-tg), the authors showed that the adduct formation by both methyleugenol and 1'-hydroxymethyleugenol was almost completely dependent on the presence of SULT1A enzymes, with human SULT1A1/2

producing stronger effects than mouse Sult1a1. Further support for the role of SULTs in the formation of the DNA adducts of the allylalkoxybenzenes comes from studies reporting reduced formation of estragole DNA adducts in both in vitro as well as in vivo rat models by the SULT inhibitor nevadensin, an important flavonoid present in basil.[25-27]

A.2. Propenyl alkoxybenzenes: anethole and isoeugenol methyl ether

The liver is the target organ for anethole toxicity. Anethole induces liver carcinogenicity in laboratory animals (female Sprague Dawley rats) at high dose levels in the diet.[28] Unlike the case of methyleugenol, it was shown that a non-genotoxic mechanism was responsible for the neoplastic effects of anethole, involving continuous elevated tissue concentrations and resulting in hepatotoxicity of its metabolite, anethole epoxide, that in turn leads to regenerative hyperplasia.[29-40] Thus, the liver carcinogenicity of anethole in laboratory animals at high dose levels is likely due to a non-genotoxic mode of action including cytotoxicity followed by regeneration of damaged tissue. Data on the toxicity of isoeugenol methyl ether are limited.

Additional support for a non-genotoxic mechanism for anethole is derived from consistently negative results for genotoxicity in several *in vivo* and *in vitro* studies. When tested *in vitro*, *trans*-anethole was negative for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and TA1538, either in the presence or in the absence of metabolic activation with S-9 at concentrations up to 25,000 µg/plate.[29-31,41,42] Similarly, absence of mutagenicity was reported for anethole in a rec assay in *B. subtilis* and in a point mutation assay in *E. coli*[43], as well as in a mutagenicity assay in *S. cerevisiae*.[44] In addition, no increase in chromosomal

aberrations was observed in Chinese hamster ovary cells treated with anethole, either with or without S-9 metabolic activation.[32] Furthermore, no increase in unscheduled DNA synthesis was found in studies using primary rat hepatocytes isolated from female SD-CD rats treated with anethole or anethole-1,2-diol at concentrations of 10^{-2} to 10^{-6} M, or up to the highest dose tested (0.3 µl/ml).[33-37,45,46] When assessed in a mouse lymphoma forward mutation assay, anethole was not mutagenic in the absence of metabolic activation. However, in the presence of metabolic activation, anethole induced forward mutations at the thymidine kinase locus of L5178Y $TK^{+/-}$ mouse lymphoma cells, although this seems to be the only incidence of mutagenic activity reported for anethole.[30]

Lack of genotoxicity was also reported when anethole was tested *in vivo*, including in three independent mouse micronucleus assays. Anethole did not induce either sister chromatid exchanges (SCE) or chromosomal aberrations in bone marrow cells of B6C3F1 mice injected intraperitoneally at doses ranging from 2.5 to 2000 mg/kg bw.[40,47,48] In a different study, treatment of Swiss albino mice with 250, 500 or 1000 mg anethole/kg bw/day for 7 days did not result in an increased number of micronucleated cells in femoral bone marrow cells, even though target tissue cytotoxicity was documented, as judged by an altered ratio of polychromatic to normochromatic erythrocytes.[49]

Consistent with the lack of genotoxicity, there was no significant increase in DNA adducts in bone marrow cells in B6C3F1 or CD-1 mice treated with 0.7 to 10 mg anethole as a single intraperitoneal injection.[38,39]

A.3. Allyl hydroxybenzenes: chavicol and eugenol

No carcinogenicity has been observed in studies with eugenol in F344/N rats.[10,50,51] No significant dose related increase in the incidences of hepatocellular neoplasms were observed in either male or female B6C3F1 mice in a 2-year bioassay study.[50,51] Eugenol is not genotoxic at concentrations that do not result in cellular toxicity[52-54] and short term toxicity tests did not reveal hepatotoxicity in rats.[53,55,56] This lack of genotoxicity and carcinogencity of eugenol is reflected in the establishment of an Acceptable Daily Intake (ADI) of 0-2.5 mg/kg bw per day by JECFA[52,53] or of 1.0 mg/kg bw per day by EFSA.[54] Data on the toxicity of chavicol are limited.

A.4. Propenyl hydroxybenzenes: isochavicol and isoeugenol

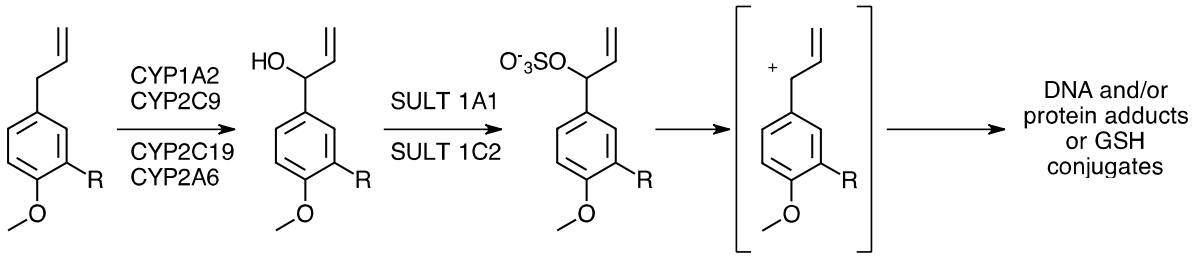
In a 2-year NTP bioassay of isoeugenol in B6C3F1 mice some evidence of increased incidences of hepatocellular adenoma, hepatocellular carcinoma, and hepatocellular adenoma or carcinoma (combined) were reported.[57] However, these responses were not dose-related and were only reported in male B6C3F1 mice, a sex and species with historically high levels of background hepatocellular neoplasms.[51] Hepatotoxic or hepatocarcinogenic effects were not observed in female mice or in either rat sex. Isoeugenol tested negative in various genotoxicity assays[57] and short term toxicity tests in rats did not reveal hepatotoxicity.[55] Data on the toxicity of isochavicol are limited.

B. Metabolic pathways

B.1. Allylalkoxybenzenes: safrole, methyleugenol and estragole

For this group of structural analogues the 1'-sulfoxy metabolites have been identified as the ultimate DNA-reactive metabolites responsible for the carcinogenicity. These metabolites are the products of sulfotransferase-mediated conjugation of the 1'-hydroxy metabolites, formed upon 1'-hydroxylation of the allylalkoxybenzenes by cytochromes P450 (Scheme 1).[9,17-21] Whether the 1'-sulfoxy metabolite decomposes to give rise to an unstable reactive carbocation that subsequently reacts with nucleophilic sites in macromolecules including DNA (Scheme 1), or whether the 1'-sulfoxy metabolite reacts directly with the nucleophilic sites remains to be elucidated.

Scheme 1:

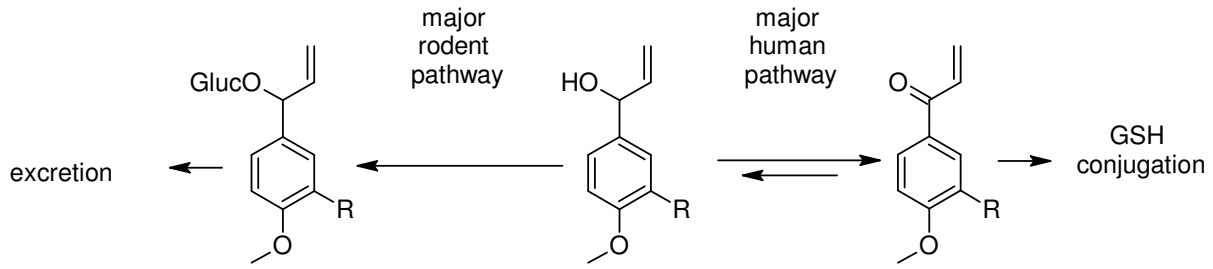


Formation of the 1'-hydroxy metabolites of allylalkoxybenzenes was demonstrated using human liver microsomes. Cytochromes P450 1A2, 2C9 and 2C19 were shown to be the primary enzymes catalyzing the 1'-hydroxylation of methyleugenol[58], whereas P450 2C9 and 2A6 were

identified as the enzymes catalyzing 1'-hydroxylation of safrole[59], and P450 1A2 and 2A6 were shown to be the principle enzymes for 1'-hydroxylation of estragole.[60]

Once formed, the 1'-hydroxy metabolites of allylalkoxybenzenes undergo conjugation reactions. In rats, conjugation with glucuronic acid catalysed by UDP-glucuronosyltransferases (UGTs) appears to be the major detoxification pathway and results in a stable metabolite which is excreted in the urine (Scheme 2).[61-64] Oxidation of 1'-hydroxy metabolites to the corresponding ketone has been also identified as an alternative but minor metabolic pathway in rats, whereas it appeared a major pathway in humans.[65-67] In addition, while glucuronidation is the main conjugation pathway for the 1'-hydroxy metabolites, the keto-metabolite is efficiently conjugated with GSH.[65] Reduction of the keto metabolite back to 1'-hydroxyestragole has also been observed (Scheme 2).

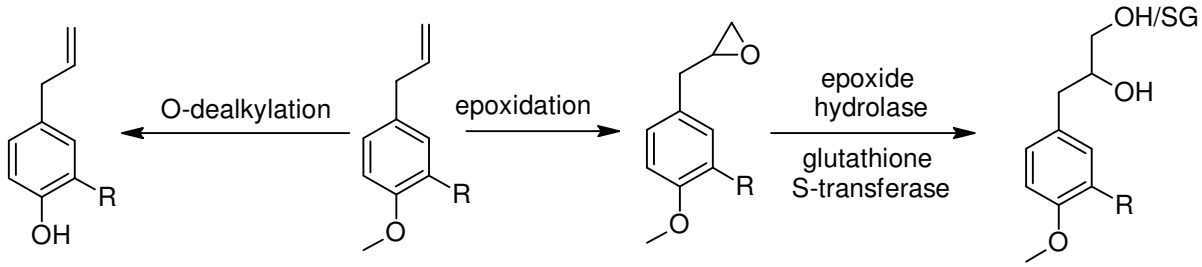
Scheme 2:



While the 1'-hydroxylation can lead to downstream bioactivation of safrole, methyleugenol and estragole, two competing metabolic pathways have been described for this group of compounds. In the first alternative pathway, *O*-dealkylation (Scheme 3) competes favourably with 1'-hydroxylation and liberates a free phenolic functional group that provides an efficient

detoxification option for conjugation and elimination.[64,68,69] Epoxidation of the allyl double bond is the second alternative pathway (Scheme 3). The epoxides of the allylalkoxybenzenes have been shown to be DNA-reactive and form adducts *in vitro*. However, these epoxides were also shown to be rapidly detoxified by epoxide hydrolases and/or glutathione S-transferases *in vivo*.[70-74] Therefore, accumulation of these adducts *in vivo* is considered.[71]

Scheme 3:



Taken together, of three possible metabolic pathways only one leads to the bioactivation of safrole, methyleugenol and estragole, through the formation of DNA reactive 1'-sulfoxy metabolites (Scheme 1). This pathway starts with 1'-hydroxylation, followed by sulfate conjugation, and results in what are currently believed to be the ultimate carcinogenic metabolites for this class of compounds.[11,20,38,39] These electrophiles may react readily with DNA, RNA and proteins but can also be detoxified through reaction with H_2O or conjugation with glutathione.[39,76-78] Therefore, only a fraction of the 1'-sulfoxy metabolite is expected to form DNA adducts.

The types of DNA adducts formed have been examined for estragole *in vitro* (Figure 2). The major adduct formed was with the guanine base, specifically the N^2-(*trans*-isoestragol-3'-yl)-

deoxyguanosine (E-3'-N^2-dG). Other adducts included N^2-(estragol-1'-yl) deoxyguanosine (E-1'-N^2-dG), 7-(*trans*-isoestragol-3'-yl)-deoxyguanosine (E-3'-7-dG), and 8-(*trans*-isoestragol-3'-yl)-deoxyguanosine (E-3'-8-dG).[75,79] Adducts of estragole with deoxyadeninosine, such as N^6-(*trans*-isoestragol-3'-yl)-deoxyadenosine (E-3'-N^6-dA), are also formed to a significant extent in the liver of male rats (F344) exposed to 600 mg/kg bw of estragole for 4 weeks.[78] The structure of these adducts is consistent with initial formation of the 1'-sulfoxy metabolite of estragole, and the loss of the sulfate moiety which is facilitated by resonance stabilisation via isomerisation of the allylic double bond from the C2'-C3 to the C1'-C2' position, and the presence of the *para*-methoxy substituent on the phenyl ring.[80]

Herrmann et al.[81] reported the detection of similar adducts of methyleugenol in surgical human liver samples from 30 subjects. Using isotope-dilution ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), they detected N^2-(*trans*-methylisoeugenol-3'-yl)-2'-deoxyguanosine adducts (Figure 3) in 29 liver samples as the major adduct and N^6-(*trans*-methylisoeugenol-3'-yl)-2'-deoxyadenosine (Figure 3) at lower levels in most samples as well. The maximal and median levels of both adducts combined were 37 and 13 adducts per 10^8 nucleosides.

The biotransformation of these substances and the likely formation of DNA adducts is both dose-dependent and species-specific. Conversion by *O*-dealkylation to metabolites with a free phenolic functional group that can be readily excreted with or without conjugation is the predominant route of biotransformation for this group of compounds at low doses in rodents and humans.[64,68,69,82,83] In contrast, oxidation of the allyl side chain that generates 1'-hydroxy

metabolites and alkene epoxides occurs at higher dose levels.[64,68] Rodent toxicity and carcinogenicity studies typically employ high dose levels, thus favouring the latter metabolic pathways. This is demonstrated by the results of studies reporting that the metabolite profile of safrole changed in a dose-dependent fashion over a range of oral dose levels of 0.9, 60 or 600 mg/kg bw. As predicted, a shift from mainly *O*-dealkylated metabolites detected at lower dose levels to 1'-hydroxylation products on the allyl side chain and/or epoxide formation was observed that was more prominent when the dose increased from 60 to 600 mg/kg bw.[62] A similar shift in metabolic profile of estragole was reported in another study in mice and rats. As the dose increased (0.05, 5, 50, 500,1 000 mg/kg bw), the relative extent of *O*-demethylation decreased with a parallel increase in 1'-hydroxylation, the latter amounting to 1.3, 2.1, 5.2, 7.8 and 9.4% of the dose at increasing dose levels in the mouse study.[64,68] This dose-dependent metabolic shift from *O*-demethylation to 1'-hydroxylation was consistent with the results of pharmacokinetic modeling using a physiologically based kinetic (PBK) model developed for estragole bioactivation and detoxification in the male rat (Punt et al., 2008). The main metabolic pathway of estragole at low doses was shown to be *O*-demethylation. Saturation of the *O*-demethylation pathway was shown to occur at higher doses of estragole leading to increased relative formation of 1'-hydroxyestragole.

In contrast to this, a study on hepatic DNA adduct formation in mice exposed to methyleugenol did not provide an indication of a dose dependent metabolic shift.[24] Also, Gupta et al.[85] reported a nearly linear dose response curve for hepatic DNA adduct formation upon exposure of mice to a dose range of safrole varying from 0.001 to 10 mg per mouse. This linear dose response behaviour is also in line with results from physiologically based kinetic (PBK) modelling, which predicted that formation of the reactive sulfoxy metabolites and the resulting DNA adducts

formed upon exposure to methyleugenol, estragole or safrole would be linear with the dose at dose levels as high as the ones causing tumors in rodent bioassays down to dose levels in the range of realistic human exposure.[66,67,86,88] It might be that the metabolic shift occurs especially at dose levels higher than the ones causing tumors in rodent bioassays.

Overall, these studies demonstrate that a reactive intermediate carbocation is the ultimate carcinogenic metabolite by virtue of DNA adduct formation. The generation of the carbocation in this group of related *para*-allylalkoxyalkylbenzenes is the result of a bioactivation pathway involving cytochrome P450-mediated allyl side chain 1'-hydroxylation followed by sulfate conjugation of the hydroxyl moiety to form the 1'-sulfoxy metabolite. The presence of a 2,3-unsaturated allyl side chain provides the activated carbon (C1), which is both allylic and benzylic and serves as the required electrophilic moiety. The overview also suggests that *O*-dealkylation that liberates a hydroxyl moiety is an efficient detoxification pathway that facilitates excretion. Several species differences have been identified in the relative contribution and efficiency of metabolic pathways involved in the biotransformation of allylalkoxybenzenes. Among the three main pathways, namely *O*-dealkylation, allyl chain epoxidation and allyl chain 1'-hydroxylation, evidence has shown that *O*-dealkylation appears to be more favoured in the mouse and human than in the rat at comparable doses.[64,68,69] Consistent with this difference, the metabolic shift from *O*-dealkylation to 1'-hydroxylation of the allyl side chain with increasing dose is more significant and readily observed in rats compared to mice.[10,20,75] In addition, in rats, a clear dose-dependent increase in 1'-hydroxysafrole or its downstream metabolites is observed in the urine, whereas no evidence was found of such metabolites occurring in humans at low dose (1.66 mg; ~0.03 mg/kg bw).[62] In in vitro incubations the metabolic fate of the 1'-hydroxyl moiety toward

oxidation or conjugation appeared to be species dependent. Oxidation to the ketone, which is efficiently scavenged by GSH, appeared to be a minor pathway for 1'-hydroxyestragole conversion inincubations with rat liver microsomes, compared to 1'-hydroxyl glucuronidation[84], but appears to be the major pathway for conversion of 1'-hydroxyestragole in incubations with human liver microsomes.[65] In spite of this species difference, the formation of 1'-sulfoxyestragole was found to vary less than 2-fold between rat and human.[65] Studies with 1'-hydroxymethyleugenol and 1'-hydroxysafrole have also revealed the same species-specific differences between glucuronidation and oxidation routes of metabolism as well as a similar relatively small difference in the formation of the 1'-sulfoxy metabolite between rat and human.[66,67]

B.2. Propenyl alkoxybenzenes: anethole and isoeugenol methyl ether

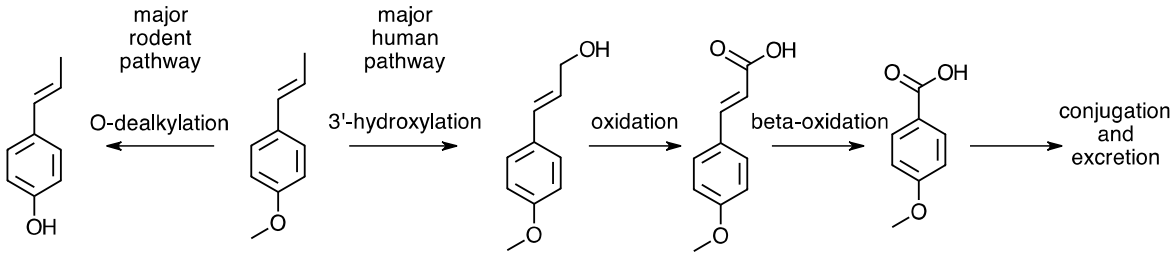
Anethole and isoeugenol are the propenyl relatives of estragole and methyleugenol, respectively, as a result of isomerisation of the double bond in the allyl side chain (Figure 1). Anethole epoxide has been determined to be the ultimate hepatotoxic metabolite of anethole based on extensive biochemical and toxicological data.[88] The target organ for anethole toxicity is the liver where anethole epoxide has been shown to be 10 times more potent and exhibit hepatocellular cytotoxicity at concentrations 10 times lower compared to those of anethole itself.[33-37,89] The primary role of the epoxide is also corroborated by the increase in the hepatotoxic effects of anethole that are observed in freshly isolated rat hepatocytes in the presence of inhibitors of enzymes responsible for anethole epoxide detoxification, such as epoxide hydrolase and glutathione S-transferase.[35] Furthermore, from data based on species and sex differences it can

be seen that the higher the rate of daily production of anethole epoxide, i.e. the more efficient the formation of the active metabolite, the lower the dose levels of anethole that result in signs of hepatotoxicity.[88,90] A correlation between the rate of conversion of anethole to anethole epoxide and the hepatotoxic effects observed in short-term and long-term rodent studies have been quantitatively demonstrated.[28]

Efficient detoxification of anethole epoxide in vivo, by epoxide hydrolase and/or glutathione S-transferases, is likely at low doses and maintains the epoxide metabolite at non-hepatotoxic levels. Similar observations have been reported in the case of the epoxides of the allylalkoxybenzenes[70-74], such that the epoxide DNA adducts shown to form *in vitro*, are considered unlikely to accumulate *in vivo* at low doses.[71]

An alternative metabolic pathway for propenyl alkoxybenzenes is the 3'-hydroxylation of the side chain (Scheme 4) and this is the main route in humans at low dose levels.[91] Following 3'-hydroxylation, *trans*-anethole is efficiently detoxified via subsequent β-oxidation that yields *para*-methoxy benzoic acid (Scheme 4), followed in turn by rapid conjugation with glycine and elimination in the urine.[82,91] This pathway competes effectively with epoxidation at low dose levels, making bioactivation and toxicity less likely. Therefore, at low dose levels, epoxidation is a minor pathway and is quantitatively similar among mice, rats, and humans albeit slightly more prominent in the rat compared to the mouse or human.

Scheme 4:



The shift between 1'-hydroxylation and 3'-hydroxylation of the side chain is an illustration of the metabolic and toxicological impact that results from a small structural difference between isomers, comparing those with an allyl double bond and those with a propenyl double bond in the side chain. The change in the location of the double bond results in a different regioselectivity of the cytochrome P450 catalysed reaction resulting in the formation of a non-genotoxic metabolic intermediate (3'-hydroxy-) instead of the proximate carcinogenic metabolite (1'-hydroxy-). By extension, the location of the double bond shifts the toxicological profile from that of DNA adduct-forming genotoxic compounds to that of non-genotoxic ones which are readily conjugated and rapidly excreted in the urine.

Additional metabolic options for detoxification are available in compounds with an additional methoxy substituent at the meta-position, as in the case of isoeugenol methyl ether. The additional methoxy group increases the probability of detoxification via *O*-demethylation and subsequent conjugation of the hydroxyl group with a concomitant reduction in the formation of the corresponding epoxide.[92] The fraction of metabolites resulting from the epoxidation pathway account for less than 1% of the urinary metabolites detected in male Wistar rats administered a single oral dose of 200 or 400 mg isoeugenol methyl ether/kg bw. The majority of urinary

metabolites, greater than 77%, were products of 3- and 4-*O*-demethylation and 3'-hydroxylation. The extent of hepatotoxic effects reported for isoeugenyl methyl ether is directly correlated to the levels of formation of the corresponding epoxide. Taken together, exposure to anethole and isoeugenol methyl ether at dose levels relevant for human dietary intake is consistent with absence of toxicity given the efficient detoxification that occurs at similar levels in rodents and humans. It is only in the context of chronic exposure to high dose levels in female rats that the continuous daily production of hepatotoxic concentrations of anethole epoxide has been shown to occur which in turn results in a continuum of cytotoxicity, cell death, regenerative cell proliferation, and following prolonged exposure, liver tumors. The relationship between high-dose anethole exposure, epoxide formation and hepatotoxicity has been also assessed quantitatively in a 2-year study using female rats.[28] A higher fraction of anethole was converted to the epoxide with higher dose of parent compound. A dose of 200 mg/kg bw per day of anethole resulted in 15% epoxide metabolites, with an estimated epoxide dose of 30 mg/kg bw per day, whereas a dose of 550 mg/kg bw per day of anethole resulted in 23% epoxide metabolites, with an estimated epoxide dose of 120 mg/kg bw per day and a proportional increase in the incidence and severity of hepatotoxicity.[88] A clear threshold of anethole hepatoxicity can be determined, as shown by the absence of hepatotoxicity at a dietary dose of 120 mg/kg bw per day of anethole and an estimated epoxide dose of approximately 22 mg /kg bw per day. Female rats exhibited both hepatic toxicity and hepatic tumors at the highest dietary dose of anethole (550 mg/kg bw per day). By comparison, human exposure to anethole is estimated to be a daily intake of 0.002 mg/kg bw, orders of magnitude below the no-effect dose in rodents. Clearly, a large margin of safety exists between dose levels causing hepatotoxicity in experimental animals and levels expected to occur in realistic human exposure scenarios.

Thus, comparison of the metabolic profile of anethole and isoeugenyl methyl ether to that of the related allylalkoxybenzenes safrole, methyleugenol and estragole, reveals that a small structural difference such as the isomerisation of the double bond in the allyl side chain results in distinct metabolic pathways that favor detoxification.

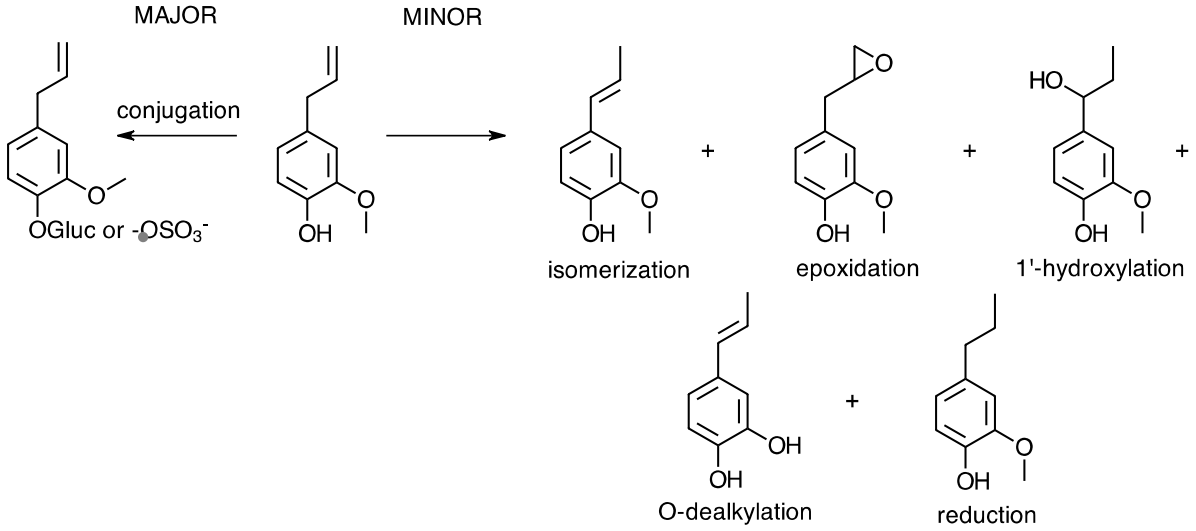
Metabolic studies have shown that at low dose levels (<5 mg/kg bw/day) anethole is rapidly absorbed and detoxified in rodents primarily by *O*-demethylation and in humans primarily by 3'-hydroxylation (scheme 4).[82,89,91,93]

B.3. Allyl hydroxybenzenes: chavicol and eugenol

Chavicol and eugenol are allyl hydroxybenzenes and the structural analogues of methyleugenol and estragole, respectively, lacking the methoxy substituent but containing a hydroxyl moiety *para*- to the allyl side chain (Figure 1). Thus, the presence of a free hydroxyl moiety on the ring accounts for the difference in toxicity of eugenol and chavicol compared to that of methyleugenol and estragole. The free phenolic function allows conjugation to become a primary mode of metabolism and excretion (Scheme 5). Additional metabolic options for eugenol include: a) isomerization to yield isoeugenol, with the subsequent paths to epoxidation of the allyl double bond yielding an epoxide that hydrolyzes to the corresponding diol; b) hydroxylation at the allyl position to yield 1'-hydroxyeugenol; c) *O*-dealkylation at position 3' of the ring to yield 3,4-dihydroxypropenylbenzene; or d) reduction of the double bond of the side chain to yield 3-methoxy-4-hydroxypropylbenzene (Scheme 5). Compared to the primary

metabolic pathway of *para*-hydroxyl conjugation, these alternatives all yield minor metabolites that are not of toxicological concern.

Scheme 5



Conjugation with glucuronic acid or sulfate is efficient for all of these metabolites, which possess a free phenolic hydroxyl group or other polar oxygenated functional groups and results in rapid renal elimination.[94] The metabolic fate of eugenol has been demonstrated in fasted humans (n=2), where 94 and 103% of a dose of approximately 6.4 µg/kg bw [^{14}C]-eugenol was excreted in conjugated form in the 24-hour urine. Of these urinary conjugates, over 85% was accounted for by predominantly glucuronic acid conjugates of eugenol, followed by sulfate conjugates.[95] In a separate trial in human volunteers (n=8), who received a single dose of 150 mg of eugenol (approximately 2.5 mg/kg bw) in gelatin tablets after consumption of a normal breakfast, more than 55% of the eugenol dose was excreted in the urine as the glucuronic acid or sulfate conjugates within 24 hours. An additional 40% of the dose was accounted for by other

conjugated metabolites of isomerized, epoxidized, or reduced forms of eugenol. In total, conjugated eugenol and its conjugated metabolites accounted for >95% of the administered dose, while unconjugated eugenol accounted for less than 0.1%.[94] The authors concluded that eugenol undergoes rapid first pass conjugation and rapid elimination.[94]

Even though eugenol and chavicol share the allyl side chain with methyleugenol and the allylalkoxybenzenes, the free phenolic hydroxyl moiety is a major structural difference that increases possibilities for swift conjugation and excretion, which likely explains the absence of DNA reactivity and carcinogenicity for eugenol compared to methyleugenol.[16,51] The metabolic fate of eugenol in rodents is reported to be similar to that in humans with respect to rapid conjugation and elimination.[95,96] However, sulfate conjugates appear to be the primary metabolites, while glucuronic acid conjugates seem to form as a result of saturation of sulfate conjugation pathways. Thus, at least in rodents, the type of conjugates also seems to be dose-dependent, with sulfate conjugates being the principle metabolites at low doses (0.5, 5, and 50 mg/kg bw), and glucuronic acid conjugates predominating at the highest dose (1000 mg/kg bw).

B.4. Propenyl hydroxybenzenes: isochavicol and isoeugenol

This group, represented by isochavicol and isoeugenol, combines two structural differences relative to the allylalkoxybenzenes, namely isomerisation of the allyl side chain and replacement of the *para*-methoxy substituent by a *para*-hydroxyl moiety. Both changes convert methyleugenol to isoeugenol, while the isomerisation of the allyl side chain distinguishes isoeugenol from eugenol (Figure 1). The equivalent changes distinguish isochavicol from

estragole and chavicol, respectively. Anethole and isochavicol share the propenyl side chain and differ only in the presence of the free phenolic group. A dramatically different toxicity profile is also observed for the propenyl hydroxybenzenes compared to the allylalkoxybenzene relatives as a result of different metabolic fates associated with the presence of a hydroxyl substituent at the *para*-position in combination with the shift in the double bond in the allyl side chain. As expected, the predominant detoxification pathway for isoeugenol is the conjugation of the free phenolic function with sulfate and glucuronic acid. Indeed, greater than 85% of [^{14}C]-isoeugenol administered at a dose of 156 mg/kg bw by gavage to male Fischer-344 rats is excreted in the urine within 72 hours as the glucuronic acid and sulfate conjugates.[97] Intravenous administration results in a similar metabolic and elimination pattern with 82% of a 15.6 mg/kg bw dose of [^{14}C]-isoeugenol excreted as the same urinary conjugates within 72 hours. Regardless of administration route, the remaining dose was accounted for with approximately 10% excreted in the feces, up to 0.1% recovered as expired CO_2, and less than 0.25% remaining in selected tissues. The conclusion that isoeugenol does not present a carcinogenic hazard is consistent with the efficient detoxification of isoeugenol, due to its free phenolic hydroxyl, similar to the group of allyl hydroxybenzenes.

Conclusions

A mechanism-based approach has been taken to illustrate how minor structural changes may result in different metabolic pathways of either bioactivation or detoxification with a significant impact on the toxicological outcome. The impact of small structural differences on toxicity is important to consider when using structural analogy as a rationale for predictions in toxicity

assessment. The structural similarities and differences among allylalkoxybenzenes, propenyl alkoxybenzenes and their allyl and propenyl hydroxybenzene relatives were presented in this paper as examples. When viewed in the context of their metabolic fate, it is clear that structural features alone are not adequate as the basis for comparative toxicity assessment. Comparative approaches are more effective in assessing toxicity when based on a combination of both structural analogy and metabolic analogy as has been demonstrated here by comparing the metabolic fate and toxicity outcomes of structurally related substances naturally occurring in foods that differ only in the position of the double bond of the alkenyl side chain and/or in the presence of a *para*-hydroxy or *para*-methoxy functional group. The relationship between chemical structure and ADME (absorption, distribution, metabolism and excretion) characteristics determines whether detoxification or bioactivation of the parent compound may occur and whether effective elimination of metabolites may follow. The impact of subtle structural changes on the metabolic fate for the four types of substances demonstrates that a mode-of-action-based approach that includes both parent compound and metabolites can more accurately evaluate the potential toxicity, DNA reactivity and carcinogenicity of these related food constituents. It is concluded that isomerisation of the double bond in the allyl side chain and/or the presence of a free phenolic hydroxyl moiety shifts the metabolism in favour of detoxification and eliminates the possibilities for formation of a DNA reactive carcinogenic metabolite. It is with this insight that the remarkable differences in the toxicity of these related hydroxy- and alkoxy-substituted allyl-and propenyl-benzenes can be explained. It also illustrates how subtle structural changes among parent compounds that do not seem critical in and of themselves can have significant effects on toxicity simply due to critical divergence in the relevant metabolic products. Furthermore, species differences in relative metabolic pathway

contributions to biotransformation of a compound must also be taken into account, as illustrated in the case of the allylalkoxybenzenes by the preferential formation of the 1'-hydroxy metabolite and its glucuronidated product in rodents but an oxo-metabolite and its glutathione conjugate in humans. It is concluded that comparisons in toxicological risk assessment should be done on the basis of structural analogy, not only of the parent compounds but also for the metabolic products with due consideration to species-specific metabolic pathways.

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Table 1. Occurrence of allylalkoxybenzenes in the modern food chain.[98]

Substance	Natural occurrence in Food
Anethole	Anise hyssop, fresh apple, fennel, buckwheat, caraway, cheese, cherimoya, clove, coriander seed, dill seed, elder glower, ginger, green tea, juniper berry, lemon balm, licorice, mastic, mustard, myrtle berry, basil, ouzo, passion fruit, pastis, pepper, peppermint oil, piper betle, rhubarb, star anise, sweet grass oil, sweet marjoram
Chavicol	Apple brandy, cassia leaf, chervil, cider, cloudberry, clove bud, clove stem, galangal, basil, passion fruit juice, pimento berry and leaf, pineapple, piper betle, rosemary, sour cherry, sweet marjoram, wheaten bread
Estragole	Anise, anise hyssop, fresh apple, apple juice, bilberry, fennel, nutmeg, chervil, cider, cinnamonium, cinnamon bark, clove bud and leaf, coriander seed, elder flower, Korean mint, laurel, lemon balm, licorice, mastic, mustard, myrtle berry and leaf, basil, orange juice, passion fruit, pimento berry and leaf, piper betle, rosemary, sage, star anise, sweet fennel, sweet grass oil, sweet marjoram, tarragon, grapes
Eugenol	Honey, acerola, American cranberry, anise, anise hyssop, apple brandy, fresh apple, apricot, arctic bramble, Armagnac, Ashanti pepper, banana, beer, stone apple, bilberry, black choke berry, black currents, blackberry, porcino mushrooms, botrytised wine, bourbon vanilla, bourbon whiskey,

	buchu oil, buckwheat, calamus, Canadian whiskey, bell pepper, carrot, cassia leaf, celery seed oil, chervil, Chinese quince, cider, cinnamon bark, leaf and root bark, clary sage, cloudberry, clove bud, leaf and stem, cocoa, coffee, cognac, crispbread, cuttlefish, dill herb and seed, elder flower, elderberry juice, European cranberry, smoked fatty fish, fenugreek, fig, ginger, globe artichoke, grape brandy, grapefruit oil, green mate, guava fruit, blueberry, Indian dill seed, Irish whiskey, Japanese whiskey, dried bonito, Korean mint, kumquat oil, lamb's lettuce, laurel, smoked lean fish, lemon peel oil, licorice, lime oil, lingonberry, macadamia nut, mace, malt whiskey, mango, mastic, mate, melon, myrtle leaf, basil, okra, orange juice, orange oil, oregano, ouzo, passion fruit, pennyroyal oil, pepper, peppermint oil, pimento berry and leaf, piper betle, plum, plum brandy, plum wine, pickled plum, quince, raspberry, red wine, rhubarb, rose wine, rosemary, rum, sage, Scotch blended whiskey, Scotch spearmint oil, sea buckthorn, sherry, sour cherry, sage, spearmint oil, strawberry, savory, sweet cherry, sweet grass oil, sweet marjoram, tamarind, Caja fruit, tarragon, tequila, thyme, fresh tomato, tomato juice and paste, wax jambu, white wine, yuzu oil
Ischavicol	Apple cider
Isoeugenol	Apple brandy, beer, bilberry, blackberry, Chinese quince, cinnamon leaf, clove bud and stem, coffee, cuttlefish, dill seed, elder flower, smoked fatty fish, grapefruit juice, green tea, guava fruit, blueberry, dried bonito, mace,

	malt, mate, mushroom, nutmeg, pimento leaf, piper betle, plum, rum, thyme, fresh tomato, wort , yuzu oil
Methyl eugenol	Anise hyssop, apple brandy, fresh apple, arctic bramble, Ashanti pepper, banana, bilberry, black tea, blackberry, buchu oil, buckwheat, calamus, cardamom, carrot, chervil, Chinese quince, cinnamomum, clove bud and leaf, cognac, lemon grass oil, eucalyptus oil, grapefruit juice, galangal, hog plum, kaboso oil, dried bonito, Korean mint, kumquat oil, laurel, lemon balm, lovage seed, mace, mastic, myrtle berry and leaf, nutmeg, basil, orange juice and oil, parsnip, peach, pepper, pimento berry and leaf, piper betle, plum, prune, quince, rosemary, sapodilla fruit, sherry, star anise, strawberry guava, sweet grass oil, tarragon, grapes
Isoeugenol methyl ether	Calamus, ginger, dried bonito, mastic, orange oil, citrus fruits, nutmeg, tarragon, calamus, dill, soursop
Safrole	Acerola, Ashanti pepper, banana, cinnamomum, cinnamon bark, leaf and root bark, cocoa, coriander seed, dill herb, green mate, dried bonito, kumquat oil, lemon balm, macadamia nut, mace, nutmeg, basil, pepper, piper betle, star anise, tamarind

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Table 2. Summary of hepatotoxicity, genotoxicity and carcinogenicity of the hydroxy- and alkoxy-substituted allyl- and propenylbenzenes.

compounds	hepatotoxicity	genotoxicity	carcinogenicity
Allylalkoxybenzenes *(estragole, methyleugenol, safrole)*			
	+	+	+
Propenyl alkoxybenzenes *(anethole, isoeugenol methylether)*			
	+	-	+
Allyl hydroxybenzenes *(chavicol, eugenol)*			
	-	-	-
Propenyl hydroxybenzenes *(isochavicol, isoeugenol)*			
	-	-	-

Figure legends

Figure 1. Structural formulas of the hydroxy- and alkoxy- substituted alkyl- and propenylbenzenes included in the present structure activity analysis.

Figure 2. Schematic presentations of DNA adducts formed with allylalkoxybenzenes using estragole as the model compound: N^2-(*trans*-isoestragol-3'-yl)-deoxyguanosine (E-3'-N^2-dG), N^2-(estragol-1'-yl)-deoxyguanosine (E-1'-N^2-dG), 7-(*trans*-isoestragol-3'-yl)-deoxyguanosine (E-3'-7-dG), 8-(*trans*-isoestragol-3'-yl)-deoxyguanosine (E-3'-8-dG), and N^6-(*trans*-isoestragole-3'-yl)-deoxyadenosine (E-3'-N^6-dA).[75,79]

Figure 3. Schematic presentations of methyleugenol DNA adducts detected in surgical human liver samples: N^2-(*trans*-methylisoeugenol-3'-yl)-2'-deoxyguanosine (ME-3'-N^2-dG) and N^6-(*trans*-methylisoeugenol-3'-yl)-2'-deoxyadenosine (ME-3'-N^6-dA).[81]

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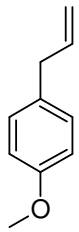
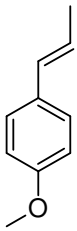
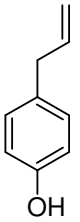
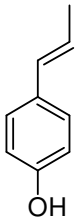
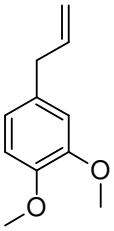
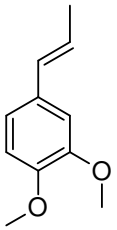
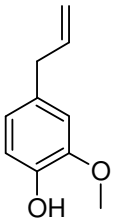
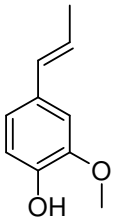
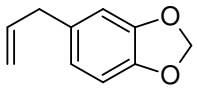
Allyl alkoxybenzenes	***Propenyl alkoxybenzenes***	***Allyl hydroxybenzenes***	***Propenyl hydroxybenzenes***
			
estragole	anethole	chavicol	isochavicol
			
methyl eugenol	isoeugenolmethyl ether	eugenol	isoeugenol
			
safrole			

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931 **Figure 1.**

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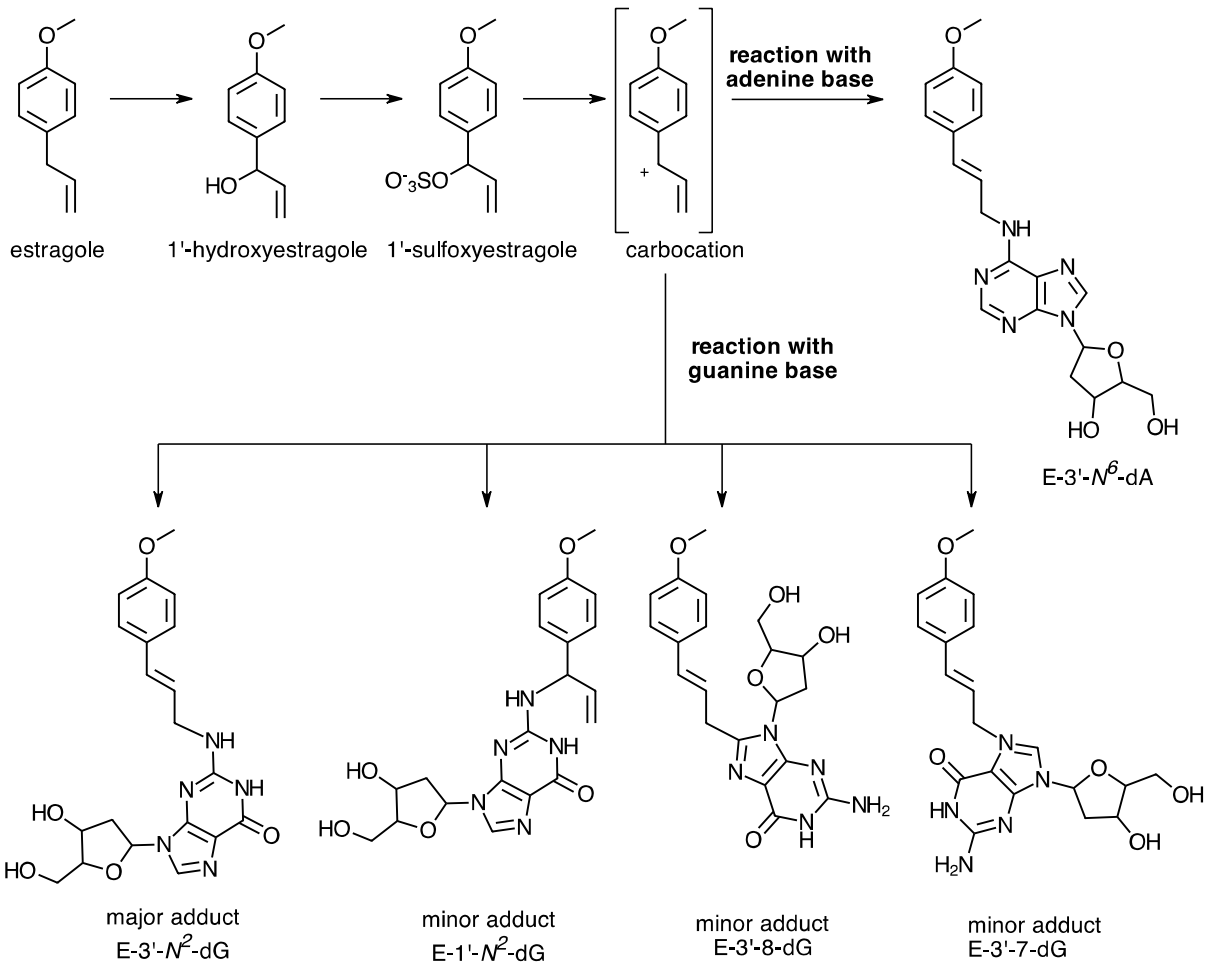

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934 **Figure 2.**

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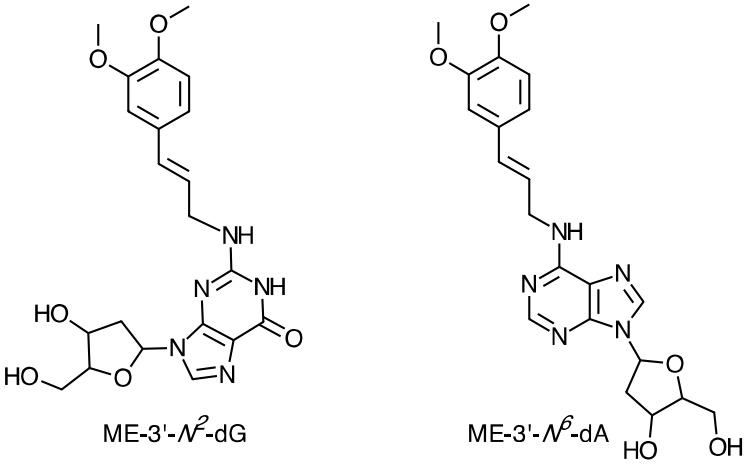

Figure 3.