EVIDENCE ON THE CARCINOGENICITY OF

ESTRAGOLE

DRAFT

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Reproductive and Cancer Hazard Assessment Section
Office of Environmental Health Hazard Assessment
California Environmental Protection Agency

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by

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PREFACE

The Safe Drinking Water and Toxic Enforcement Act of 1986 (Proposition 65, California Health and Safety Code 25249.5 *et seq*.) requires that the Governor cause to be published a list of those chemicals "known to the state" to cause cancer or reproductive toxicity. The Act specifies that "a chemical is known to the state to cause cancer or reproductive toxicity...if in the opinion of the state's qualified experts the chemical has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer or reproductive toxicity." The lead agency for implementing Proposition 65 is the Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency. The "state's qualified experts" regarding findings of carcinogenicity are identified as the members of the Carcinogen Identification Committee of the OEHHA Science Advisory Board (22 CCR 12301).

Estragole was assigned a final priority of 'high' carcinogenicity concern and placed on the Final Candidate list of chemicals for Committee review on June 12, 1998. A public request for information relevant to the assessment of the evidence on the carcinogenicity of this chemical was announced in the *California Regulatory Notice Register,* also on June 12, 1998. No information was received as a result of this request.

This draft document *Evidence on the Carcinogenicity of Estragole* was developed to provide the Committee with relevant information for use in its deliberations, and reviews the available scientific evidence on the carcinogenic potential of estragole. A public meeting of the Committee to discuss this evidence is scheduled for October 7, 1999**.** At this meeting it is expected that the Committee will render an opinion on whether estragole has been clearly shown to cause cancer. Written public comment on the document should be submitted to OEHHA by September 14, 1999**,** in order to be considered by the Committee in advance of the meeting. During the October 1999 meeting, the public will have an opportunity to present verbal comments to the Committee.

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1 EXECUTIVE SUMMARY

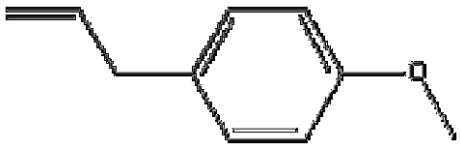
Estragole occurs naturally in many culinary herbs, including anise, star anise, basil, bay, tarragon, fennel, and marjoram. Widespread human exposure to estragole occurs through the consumption of these herbs and through the use of the oils derived from them as flavors and fragrances in numerous foods, cosmetics, and other consumer products. Estragole is a constituent of turpentine oil, and indoor air exposure may result from the use of turpentine oil in furniture and other wood treatments.

Estragole or its metabolites administered to adult or newborn mice of different strains, through different routes of administration, produced malignant liver tumors. Carcinogenicity of estragole has not been adequately studied in the rat. One subcutaneous injection study of derivatives of estragole in adult male rats did not observe any treatment-related increase in tumors. Regarding other relevant data, estragole produced genotoxic effects in *Salmonella typhimurium,* yeast, and mammalian cells. Several DNA adducts have been characterized. Further strong supporting evidence of carcinogenicity comes from comparisons of compounds structurally similar to estragole (e.g., safrole, methyleugenol) which produce liver tumors and tumors at other sites in rodents.

The mode of action for estragole carcinogenicity has been well characterized and proceeds through a genotoxic mechanism. Estragole is metabolized by the liver to 1'-hydroxyestragole and several epoxide compounds. 1'-Hydroxyestragole is further conjugated with sulfate to form a sulfuric acid ester compound that readily binds to DNA and is responsible for most, if not all, of estragole's hepatocellular carcinogenicity in mice. Metabolism of estragole through this pathway appears to be quantitatively consistent among humans and rodents.

2 INTRODUCTION

2.1 Identity of Estragole



Molecular Formula: $C_{10}H_{12}O$
Molecular Weight : 148.20
CAS Registry No.: 140-67-0
Chemical Class: Alkenylbenzenes

Synonyms: 1-allyl-4-methoxybenzene, 3-(*p*-methoxyphenyl)propane, 4-allyl-1-methoxybenzene, 4-allylanisole, 4-allylmethoxybenzene, 4-methoxyallylbenzene, *p*-allyl anisole, 1-methoxy-4-(2-propenyl)benzene, chavicol methyl ether, chavicyl methyl ether, esdragol, esdragole, esdragon, estragol, isoanethole, methyl chavicol, *o*-methyl chavicol, NCI-C60946, *p*-allylanisole, *p*-methoxyallylbenzene, tarragon.

Estragole is a colorless liquid, insoluble in water (<0.1 g/100 mL), with an anise odor. It has a specific gravity of 0.965 g/L, a boiling point of 215-216°C, and a flash point of 81°C.

2.2 Occurrence and Use

Estragole occurs naturally in many common plants including anise, star anise, basil, bay, tarragon, fennel, marjoram, and is also present in American wood turpentine oil (Guenther and Althausen, 1972; Leung, 1980). Estragole is present in considerably higher concentrations in volatile oils derived from these plants and trees. Flavor and fragrances containing estragole are used in numerous foods and food products, perfumes, soaps, and detergents. The Flavor and Extract Manufacturer's Association (FEMA, 1978), noting a wide range of exposures, estimated several years ago that daily per capita consumption of estragole in the U.S. was 70 μg. More recent estimates were not identified. The U.S. Department of Food and Agriculture (U.S. FDA, 1996) lists the status of estragole along with many essential oils containing estragole (e.g., extracts of anise, basil, bay leaves, tarragon and fennel) as "generally recognized as safe" (GRAS) for food use. Estragole is listed by the Organisation for Economic Co-operation and Development (OECD, 1997) as a high production volume chemical. U.S. production of estragole in 1990 was estimated by the U.S. Environmental Protection Agency (U.S. EPA, 1995) to range from <1 million to 3.4 million pounds. Estragole may occur in indoor air because of wood treated with turpentine oil, and because it is a minor constituent of tobacco smoke (EDF, 1998; Thelestam *et al.*, 1980).

The biological function of estragole in plants is not clearly understood; however, estragole and estragole-containing essential oils have been reported to possess insecticidal, anti-viral, and anti-bacterial properties (Leung, 1980; Okunade and Olaifa, 1987). Potential human exposures to estragole from estragole-containing plants and plant extracts are described in more detail below.

Anise and star anise (also called anise seed, sweet cumin, illicium, Chinese anise, Chinese star anise) contains about 1% to 4% volatile oil of which estragole is a primary component (Leung, 1980). Anise oil and star anise oil are used interchangeably in the U.S. Anise and star anise oils are used in pharmaceutical and cosmetic product formulations. They are used as stimulants and expectorants in cough mixtures or lozenges. They are also used to expel gas from the alimentary canal. Star anise has been used in Chinese medicine for hundreds of years for these same properties (Leung, 1980). Both oils are used for their fragrant properties in toothpastes, perfumes, soaps, detergents, creams and lotions. Maximum use levels in perfumes are 0.25% for anise oil and 0.4% for star anise oil (Leung, 1980). Anise, star anise and their oils are used as food flavorings in alcoholic beverages (bitters, brandies, and liquers (e.g., anisette)), other non-alcoholic beverages, frozen desserts, candy (e.g., licorice candies), baked goods, gelatins, puddings, meats, and meat products (Leung, 1980). Both anise and star anise are used widely as domestic spices where anise predominates in Western dishes and star anise is primarily used in Chinese foods.

Basil (sweet basil and common basil) contains about 0.8% volatile oil of which approximately 70% is estragole, although the amount varies with the source of basil (Leung, 1980). Sweet basil oil (about 55% estragole) has been used in fragrances since the 1900s with concentrations in some creams and lotions at 0.0025% (usual) and 0.01% (maximum) and in perfume at 0.09% (usual) and 0.4% (maximal) (Opdyke, 1973). Other product uses include toothpastes, hair dressings and mouth washes. Basil is used as a food, a spice, and is included in some liquers. Italian foods (e.g., pesto) in particular use large quantities of basil. Basil oil has been used as a folk remedy as a cure for warts, worms, and other ailments (Leung, 1980). Basil oil has been used in Chinese medicine for hundreds of years for the control of stomach spasms, kidney ailments and to promote blood circulation (Leung, 1980).

Bay leaves (i.e., West Indian Bay) are distilled to yield an essential oil (3.9% by weight, called Myrcia oil) of which estragole is a lesser constituent. Two additional varieties of volatile oils, an anise-scented and a lemon-scented oil, are also commonly derived from bay leaves. The anise-scented variety contains estragole (32%) and methyleugenol (43%) whereas the lemon-scented variety contains mostly citral (>80%). Bay oil is used for its fragrant properties in bay rum, and is also used in creams, after shave lotions, soaps, detergents, and perfumes (Leung, 1980). Bay oil is use in foods as a flavor ingredient in alcoholic and non-alcoholic beverages, frozen desserts, candy, baked goods, gelatins and puddings, meats, meat products, and condiments and relishes, usually at low concentrations (<0.01%).

Chervil (also called salad chervil) contains a volatile oil in the herb (0.03%) and in the fruit (0.9%) of which estragole is one of two major constituents (Leung, 1980). Chervil is uses in foods as a flavor ingredient in non-alcoholic beverages, frozen desserts, candy, meat and meat products, and condiments and relishes; concentrations of the herb may reach 0.1% in these foods. Chervil leaves are used as a domestic spice in soups, salads, vinegars, omelets, and other dishes.

Tarragon (sometimes called estragon) contains an essential oil (0.25% to 1%) which consists mainly of estragole (about 70%) (Leung, 1980). Oil of tarragon has been used in product formulations since the 1920s with concentrations in soaps, creams and lotions at 0.01% (usual) and 0.1% (maximum), detergents at 0.001% (usual) and 0.01% (maximum), in perfume at 0.04% (usual) and 0.4% (maximum) (Opdyke, 1974). Tarragon is commonly used as a domestic herb. It is extensively used as a flavor component in numerous food products including non-alcoholic beverages, candies, meats, meat-products, condiments and relishes, flavored vinegars and oils, and gravies (Leung, 1980). Other minor food uses include liquers, frozen dairy products, baked goods, gelatins and puddings, with the highest reported usage of 0.04% in baked goods.

Fennel contains about 2% to 6% volatile oil which contains mostly *trans*-anethole; estragole is present in smaller amounts (Leung, 1980). Fennel and its essential oil have been used as pharmaceuticals for their stimulant property and their ability to relieve alimentary gas. Bitter (common) fennel is used as a food flavoring agent in alcoholic beverages, baked goods, meat products, fats and oils, snack foods, and gravies with typical uses as high as about 0.1% in meat products (Leung, 1980). Sweet fennel is used in non-alcoholic beverages, candy, baked goods, meat products, condiments and relishes, gravies, and processed vegetables with typical concentrations as high as about 0.3% in meat products (Leung, 1980). Fennel has been commonly used in both folk and Chinese medicine.

Marjoram (sweet, pot and wild marjoram) contains up to 3% volatile oil of which estragole is one of many constituents. Marjoram oil has been used for its fragrant properties in soaps, detergents, creams, lotions, and perfumes (Leung, 1980). Marjoram and marjoram oil are used in numerous foods and food products, including beverages, desserts, baked goods, meat products, soups, condiments and processed vegetables (Leung, 1980). Marjoram has been reported to be used as a folk medicine (Leung, 1980).

Other estragole-containing essential oils have been reported in the literature. The essential oil of *Artemisia dracunculus L*. (77.5% estragole), also called "Piemontese," is extensively used as a flavoring agent in foods and drinks (Zani *et al.*, 1991). Estragole has been reported as a constituent of the glucoside, lusitanicoside, and a constituent of the oil distilled from the fruit and leaves of Manchurina *Fagara mantshurica* Honda (Guenther and Althausen, 1972). Mexican avocado leaves have been steam distilled to yield an essential oil (3.1% of the leaf by weight) which contains 95% estragole (Leung, 1980). No information was located on the current uses of this oil. The volatile oil of the leaves of *Clausena anisata* contains estragole as its major component. The oil from these

leaves has been used to repel ticks, and the leaves of this plant are burned in some parts of Africa and the Philippines to repel mosquitoes (Okunade and Olaifa, 1987).

3 DATA ON ESTRAGOLE CARCINOGENICITY

Three publications (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987) describe numerous long-term rodent carcinogenicity studies of estragole, structurally similar alkenylbenzenes, and their metabolites or synthetic derivatives. (A comparison of the chemical structures of these compounds can be found in Figure 3, page 33) Other relevant data include genetic toxicity studies of estragole in bacteria, yeast, and mammalian cells. Additional evidence comes from comparisons of compounds structurally similar to that of estragole.

3.1 Epidemiological Studies of Carcinogenicity

No studies on the long-term health effects of human exposure to estragole have been reported.

3.2 Carcinogenicity Studies in Animals

Eight bioassays of estragole have been conducted in mice. Three studies employed oral administration of estragole: one in female adult CD-1 mice, one in newborn male CD-1 mice, and one in newborn female CD-1 mice. Four carcinogenicity studies administered estragole or its metabolites by intraperitoneal injection: two in newborn male $B6C3F_1$ mice, one in newborn male CD-1 mice, and one in adult female A/J mice. One bioassay administered estragole by subcutaneous injection to newborn CD-1 male mice. Several additional carcinogenicity studies which administered metabolites or derivatives of estragole, but not estragole itself, were conducted in both mice and rats, through various routes of administration.

3.2.1 Oral Exposure Studies

Newborn Mouse Gavage Study: Miller et al., 1983

Groups of newborn CD-1 mice (approximately 50 to 60 animals/sex for each test compound) were administered 25 mmol/kg body weight of estragole, safrole, eugenol, or anethole dissolved in trioctanoin via oral intubation, twice per week beginning at day 4 of life, for a total of 10 doses. Additional groups of male and female mice were administered 10 doses of 5.0 μmol/kg body weight anethole. Groups of trioctanoin-treated male and female mice served as vehicle controls. All animals were sacrificed at 14 months. Statistically significant increases of hepatocellular carcinomas were reported in male mice (36/49, $p < 0.001$) treated with estragole compared with control mice (14/24). No increased incidence of liver tumors was observed in estragole-treated female mice (4/44) relative to controls. Increased incidences of hepatocellular carcinoma were

also observed for male mice treated with safrole, but not with eugenol or anethole (Table 1). No increased tumor incidences were observed in female mice receiving safrole, eugenol or anethole. However, it should be noted that this study utilized a less-than-lifetime exposure regimen which may have the reduced the ability to detect tumorigenicity.

Table 1. Hepatocellular carcinomas in newborn CD-1 mice administered estragole and other alkenylbenzenes via oral gavage (Miller *et al*., 1983).

Treatment	Total dose (mmol/kg body weight)	Sex	Incidence of hepatocellular carcinoma	Average number of hepatocellular carcinomas/ mouse[a]
Trioctanoin (vehicle)	0	M	14/59	0.6
	0	F	1/47	0.02
Estragole	25	M	36/49[b]	3.5[b]
	25	F	4/44	0.1
Safrole	25	M	30/49[b]	3.0[b]
	25	F	6/53	0.2
Eugenol	25	M	13/51	0.5
	25	F	0/52	0
Anethole	25	M	9/51	0.3
	25	F	1/56	0.02
Anethole	50	M	18/59	0.6
	50	F	2/50	0.04

[a] Hepatocellular carcinomas ≥ 2 mm in diameter were tabulated.

[b] Significantly greater than controls ($p<0.001$).

Mouse Dietary Exposure: Miller et al., 1983

Groups of eight-week-old female CD-1 mice were fed diet containing estragole, safrole, or 1'-hydroxyestragole (Table 2). Mice received target concentrations in the diet of 0 (control), 0.23% estragole, 0.45% estragole, 0.25% safrole, 0.50% safrole, or 0.25% 1'-hydroxyestragole; however, to prevent early morbidity the animals were given ¼ of the target concentration for the first 10 days of dosing and ½ of the target concentration from day 10 through day 20 of dosing. After the first 20 days, dosing at the target concentration was administered for 12 months. The mice were sacrificed at 20 months of age. Survival to 10 months was high (≥86%). Increased incidences of hepatocellular carcinomas ($p < 0.001$) were reported for all test groups relative to the controls (Table 2).

A significant dose-response trend ($p<0.001$) in the incidence of hepatocellular carcinomas was observed for estragole-treated groups.

Table 2. Hepatocellular carcinoma in CD-1 female mice fed diets containing estragole, safrole, or 1'-hydroxyestragole (Miller *et al.*, 1983).

Treatment	%	Number of mice			Hepatocellular carcinomas
	in diet	At start	10 months	20 months	Number *(%)*[a]
Untreated controls	0	50	50	39	0 *(0)*
Estragole	0.23	50	48	35	27 *(56)*[b]
Estragole	0.46	50	49	34	35 *(71)*[b]
Safrole	0.25	50	47	25	34 *(72)*[b]
Safrole	0.50	50	49	26	39 *(80)*[b]
1'-Hydroxyestragole[c]	0.25	50	43	0	24 *(56)*[b]

[a] Percentages based on 10-month survival, as reported by Miller *et al.* (1983).

[b] Significantly greater than controls ($p<0.001$).

[c] Metabolite of estragole.

3.2.2 Intraperitoneal Injection Studies

Newborn Mouse Intraperitoneal Injection Study: Miller et al., 1983

Groups of 50 to 60 newborn male CD-1 mice were given four intraperitoneal injections totaling 9.45 mmol of the test compound/kg body weight per mouse (Table 3). Doses were administered on days 1, 8, 15, and 22 of life in the following proportions of total dose 1:2:4:8, respectively. Alkenylbenzenes tested were estragole, safrole, eugenol, and anethole, and specific metabolites or derivatives, namely estragole-2',3'-oxide, 1'-hydroxysafrole, safrole-2',3'-oxide, 1'-acetoxysafrole-2',3'-oxide, and eugenol-2',3'-oxide. A vehicle control group (trioctanoin) and an untreated group were also included in the study. All mice were sacrificed at 12 months. Increased incidence of hepatocellular carcinomas were observed for mice treated with estragole (30/46, $p < 0.001$), safrole (32/48, $p < 0.001$), safrole-2',3'-oxide (30/46, $p < 0.001$), and 1'-hydroxysafrole-2',3'-oxide (28/51, $p < 0.05$) relative to the incidence for the vehicle controls (11/42) (Table 3). Miller *et al.* (1983) reported the tumor data as percentage of hepatocellular carcinoma-bearing mice. Incidence data have been estimated from the reported percentages.

Table 3. Hepatocellular carcinomas in newborn male CD-1 mice administered intraperitoneal injections of estragole, other alkenylbenzenes, and their derivatives (Miller *et al.*, 1983).

Treatment	Total dose (mmol/kg)	Incidence of hepatocellular carcinoma	Average number of hepatocellular carcinomas/ mouse[a]
Trioctanoin (vehicle)	0	11/42	0.5
Untreated	0	7/46	0.2
Estragole	9.45	30/46[b]	1.7[b]
Safrole	9.45	32/48[b]	1.9[b]
Eugenol	9.45	11/45	0.6
Anethole	9.45	14/42	0.5
Estragole-2',3'-oxide[e]	9.45	19/48	0.6
1'-Hydroxysafrole	4.72	30/46[b]	2.7[b]
Safrole-2',3'-oxide	9.45	6/44	0.3
1'-Hydroxysafrole-2',3'-oxide	9.45	28/51[c]	1.0[d]
1'-Acetoxysafrole-2',3'-oxide	9.45	16/41	0.5
Eugenol-2',3'-oxide	9.45	15/49	0.5

[a] Only hepatocellular carcinomas ≥ 2 mm in diameter were tabulated.

[b] Significantly greater than vehicle controls ($p<0.001$).

[c] Significantly greater than vehicle controls ($p<0.05$)

[d] Significantly greater than vehicle controls ($p<0.01$)

[e] Metabolite of estragole.

Newborn Mouse Intraperitoneal Injections Study: Miller et al., 1983

Groups of approximately 50 to 60 newborn male B6C3F$_1$ mice were given four intraperitoneal injections of one of the following test compounds: estragole, 1'-hydroxyestragole, 1'-hydroxy-2',3'-dehydroestragole, elemicin, myristicin, anethole, dill apiol, parsley apiol, methyleugenol, 1'-hydroxyelemicin, 3'-hydroxyanethole, and 1'-

hydroxymethyleugenol (Table 4). The doses given for each compound are listed in Table 4 and were administered on days 1, 8, 15, and 22 of life with fractions of the total dose corresponding to the ratio 1:2:4:12 (i.e., 0.25, 0.5, 1.0 and 3.0 mg), respectively. In the groups of mice administered 1'-hydroxyestragole and 1'-hydroxy-2',3'-dehydroestragole, over 50% of the mice died within one week of the first injection. For these two compounds the experiment was repeated using doses in proportions of the total dose of

Table 4. Hepatocellular carcinomas in newborn male B6C3F$_1$ mice administered intraperitoneal injections of estragole, other alkenylbenzenes, and their derivatives (Miller *et al.*, 1983).

Treatment	Total dose (μmol)	Incidence of hepatocellular carcinoma	Average number of hepatocellular carcinomas/ mouse[a]
Trioctanoin (vehicle)	0	24/58	0.5
Untreated	0	23/82	0.5
Estragole	4.75	33/41[b]	2.4[b]
1'-Hydroxyestragole[c]	1.90	59/60[b]	5.6[b]
1'-Hydroxyestragole[c]	2.85	40/40[b]	5.1[b]
1'-Hydroxyestragole[c]	4.65	45/46[b]	5.8[b]
1'-Hydroxy-2',3'-dehydroestragole[c]	1.86	29/30[b]	9.4[b]
Elemicin	4.75	15/44	0.4
Myristicin	4.75	14/45	0.4
Anethole	4.75	15/47	0.4
Dill apiol	4.75	12/52	0.4
Parsley apiol	4.75	11/55	0.2
Methyleugenol	4.75	56/58[b]	3.2[b]
1'-Hydroxyelemicin	4.75	26/63	0.6
3'-Hydroxyanethole	4.75	10/53	0.3
1'-Hydroxymethyleugenol	2.85	41/44[b]	3.5[b]

[a] Only hepatocellular carcinomas ≥ 2 mm in diameter were tabulated.

[b] Significantly greater than vehicle controls ($p<0.001$).

[c] Metabolites of estragole.

the ratio 0.6:2:4:12 on days 1, 8, 15, and 22, respectively. A vehicle control group (trioctanoin) and an untreated group were also included in the study. Surviving mice were sacrificed at 18 months. Increased incidences of hepatocellular carcinomas ($p < 0.001$) were observed for mice treated with estragole, methyleugenol, 1'-hydroxyestragole, 1'-hydroxymethyleugenol, and 1'-hydroxy-2',3'-dehydroestragole relative to the incidence for the vehicle controls (Table 4). Miller *et al.* (1983) reported the tumor data as percentage of hepatocellular carcinoma-bearing mice. Incidence data in Table 4 were derived from the reported percentages.

Newborn Mouse Intraperitoneal Injection Study: Wiseman et al., 1987

Sulfate-conjugated metabolites of 1'-hydroxyestragole have been strongly implicated as the major, ultimate electrophilic and carcinogenic metabolite of estragole (reviewed in Wiseman *et al.*, 1987). To test this hypothesis, Wiseman *et al.* (1987) pretreated mice with pentachlorophenol, a potent sulfotransferase inhibitor, prior to administration of estragole or other alkenylbenzenes. Groups of 12-day old male B6C3F$_1$ mice (18 to 59 animals per group) were divided into two separate groups for each test compound. One group was given a single intraperitoneal injection of the test compound. The other group was administered the first compound and a single injection of pentachlorophenol (0.04 mmol/kg body weight) 45 minutes prior to its administration. Doses are listed in Table 5. Separate groups of mice were injected with trioctanoin (vehicle) or trioctanoin plus pentachlorophenol as controls. The first appearance of hepatocellular carcinoma in the treated mice was at nine months, and the experiment was terminated at ten months. In estragole only-treated mice, high incidences of hepatocellular carcinoma were observed (95%) relative to solvent controls (17%) (Table 5). In mice administered both estragole and pentachlorophenol, the increase in hepatocellular carcinoma (18%) relative to solvent controls (9%) was not statistically significant, indicating that sulfotransferase activity is an important factor in the carcinogenic potential of estragole. Inhibition of mouse liver carcinogenicity by pentachlorophenol was previously observed for safrole (Boberg *et al.*, 1983). The carcinogenic activities of *cis*- and *trans*-asarone were not inhibited by pentachlorophenol, indicating that these alkenylbenzenes are activated by a metabolic route that does not involve sulfate conjugation.

Table 5. Effect of the sulfotransferase inhibitor, pentachlorophenol (PCP), on the development of hepatocellular carcinomas in B6C3F$_1$ mice given an intraperitoneal injection of estragole or other alkenylbenzenes (Wiseman *et al.*, 1987).

Treatment	Total dose (mmol/ kg)	PCP	Incidence of hepatocellular carcinoma	Average number of hepatocellular carcinomas/ mouse [a]
Trioctanoin (vehicle)	0	-	10/59	0.2
Trioctanoin (vehicle)	0	+	4/45	0.1
Estragole	0.75	-	38/40[b,c]	6.6[b,c]
Estragole	0.75	+	7/39	0.2
trans-Asarone	0.75	-	33/39[b]	2.0[b]
trans-Asarone	0.75	+	32/37[b]	1.8[b]
cis-Asarone	0.25	-	21/34[b]	1.1[b]
cis-Asarone	0.50	-	17/18[b]	2.1[b]
cis-Asarone	0.25	+	30/38[b]	1.5[b]

[a] Only hepatocellular carcinomas ≥ 2 mm in diameter were tabulated.

[b] Significantly greater than vehicle controls with or without pretreatment with PCP ($p<0.001$).

[c] Significantly greater than estragole-treated group treated with PCP ($p<0.001$).

A/J Mouse Intraperitoneal Injection Study: Miller et al., 1983

Miller *et al.* (1983) conducted experiments in strain A/J mice, a strain sensitive to the development of lung tumors (Table 6). Groups of eight-week-old female A/J mice (25 animals/group) were given intraperitoneal injections of estragole, other alkenylbenzenes, or their metabolites, two days/week for 12 weeks. Doses of the test compounds, dissolved in trioctanoin, were either 0.5 or 1.0 mmole/kg body weight. The mice were sacrificed eight months after the first injection. Separate groups of mice were used as vehicle and untreated controls. An additional group of mice was given a single injection of ethyl carbamate as a positive control. No increases in lung tumors were observed in mice treated with the parent compounds, estragole, safrole, or anethole. However, statistically significant increases in lung tumors were reported for several epoxide metabolites of estragole, estragole-2',3'-oxide and 1'-hydroxyestragole-2',3'-oxide, as well as the positive control, ethyl carbamate (Table 6).

Table 6. Lung adenomas in female A/J mice treated with estragole, safrole and selected metabolites (Miller *et al.*, 1983).

Treatment	Total dose (mmol/kg)	Incidence of lung tumors	Average number of adenomas/ mouse
Vehicle control (trioctanoin)	0	3/24	0.13
Untreated control	0	1/25	0.04
Estragole	24	1/18	0.06
Safrole	24	1/19	0.05
Anethole	24	3/17	0.24
1'-Hydroxyestragole[d]	12	5/22	0.23
1'-Hydroxysafrole	12	2/21	0.10
1'-Hydroxyanethole	12	1/16	0.06
Estragole-2',3'-oxide[d]	24	1/17	0.06
Safrole-2',3'-oxide	24	2/19	0.11
1'-Hydroxyestragole-2',3'-oxide[d]	12	5/19	0.32
1'-Hydroxyestragole-2',3'-oxide[d]	24	9/19[b]	0.79[b]
1'-Hydroxysafrole-2',3'-oxide	12	5/18	0.33
1'-Hydroxysafrole-2',3'-oxide	24	9/20[a]	0.65[b]
Ethyl carbamate (positive control)	5.6	10/10[c]	7.2[c]

[a] Significantly greater than vehicle controls ($p<0.05$).

[b] Significantly greater than vehicle controls ($p<0.01$).

[c] Significantly greater than vehicle controls ($p<0.001$).

[d] Metabolites of estragole.

3.2.3 Subcutaneous Injection Studies

Newborn Mouse Subcutaneous Injection Study: Drinkwater et al., 1976

Groups of newborn male CD-1 mice (initial number of mice not reported) were administered four subcutaneous injections at the nape of the neck containing estragole, 1'-hydroxyestragole, or 1'-hydroxysafrole dissolved in heat-sterilized trioctanoin as a vehicle. The dosing regimen was as follows. Each group of mice received 0.17, 0.47, 0.95 and 2.84 μmol of the test compound (in trioctanoin) on days 1, 8, 15 and 22 of life, respectively (total dose = 4.43 μmol). The body weights were not reported for the carcinogenicity experiment; however, weights for 21-day old mice used in the metabolism studies of this report averaged 16 g. Surviving male mice, 60 to 79 per test group, were weaned at 22 days and observed for 15 months. Another group of male mice received higher doses of estragole: 0.35, 0.69, 1.38 and 2.77 μmol (in trioctanoin) on days 1, 8, 15 and 22 of life, respectively (total dose = 5.19 μmol). Surviving male mice were weaned at 22 days and observed for 15 months. Sixty-six male mice, injected with trioctanoin on days 1, 8, 15 and 22, served as controls. All mice were sacrificed at 15 months of age.

Table 7 summarizes the observations of survival and tumor formation in male mice receiving estragole, 1'-hydroxyestragole, 1'-hydroxysafrole, or vehicle (trioctanoin) among mice that survived dosing as newborns. For the low- and high-dose groups of estragole-treated mice, 76% and 95% were alive at 12 months, and 59% and 89% were alive at 15 months, respectively. For the 1'-hydroxyestragole-treated mice, 75% were alive at 12 months, and 55% were alive at 15 months. The authors noted that these survival incidences were consistent with previous reports for CD-1 mice. The first liver tumors were observed at 12 months; with the majority observed at autopsy (i.e., 15 months). Increased incidences of hepatocellular carcinomas were observed in estragole-treated animals in both the low-dose group (14/60, $0.05 < p < 0.1$) and high-dose group (7/18, $p < 0.02$)), as compared with the vehicle controls (6/51). These tumors were also observed in animals administered 1'-hydroxyestragole (35/50, $p < 0.001$) and 1'-hydroxysafrole (30/51, $p < 0.001$).

Table 7. Hepatocellular carcinomas in newborn male CD-1 mice treated by subcutaneous injection with estragole, 1'-hydroxyestragole, or 1'-hydroxysafrole (Drinkwater *et al.*, 1976).

Treatment	Total dose (μmol)	Number of mice			Hepatocellular carcinoma	
		weaned	12 months	15 months	Number (%)[a]	Multiple tumors number (%)[a]
Trioctanoin (vehicle)	0	66	51	44	6 (*12*)	0 (*0*)
Estragole	4.4	79	60	47	14 (*23*)	3 (*5*)
Estragole	5.2	19	18	17	7 (*39*)[b]	5 (*28*)
1'-Hydroxyestragole[d]	4.4	67	51	43	35 (*70*)[c]	32 (*64*)[c]
1'-Hydroxysafrole	4.4	60	51	44	30 (*59*)[c]	20 (*39*)[c]

[a] As reported by Drinkwater *et al.* (1976), the percentages are based on number of animals alive at 12 months, the time the first tumor was observed.

[b] Significantly different from controls ($p<0.05$).

[c] Significantly different from controls ($p<0.001$).

[d] Metabolite of estragole.

3.2.4 Carcinogenicity Studies of Estragole Metabolites and Derivatives

Newborn Mouse Intraperitoneal Injection Study: Miller et al., 1983

Groups of approximately 50 to 60 male newborn $B6C3F_1$ mice were given four intraperitoneal injections of one of the following alkenylbenzene derivatives: 1'-hydroxyestragole, 1'-hydroxysafrole, 1-allyl-1'-hydroxy-4-methoxynaphthalene, 1'-hydroxyallylbenzene, benzyl alcohol, anisyl alcohol, and myristicin (Table 8) (Miller *et al.*, 1983). Doses of 1.87 mmol/kg body weight for 1'-hydroxyestragol and 3.75 mmol/kg body weight for the other test compounds were administered on days 1, 8, 15, and 22 of life with fractions of the total dose corresponding to the ratio 1:2:4:8, respectively. A vehicle control group (trioctanoin) and an untreated group were also included in the study. All mice were sacrificed at 12 months. Increased incidence of hepatocellular carcinomas were observed for mice treated with 1'-hydroxyestragole (25/27, $p < 0.001$), 1'-hydroxysafrole (24/26, $p < 0.001$), 1-allyl-1'-hydroxy-4-methoxynaphthalene (22/34, $p < 0.001$), and 1'-hydroxysafrole-2',3'-oxide (28/51, $p < 0.001$) relative to the incidence

for the vehicle controls (5/32). Miller *et al.* (1983) reported the tumor data as percentage of hepatocellular carcinoma-bearing mice. For the purpose of this report, since incidence data were not presented, they were derived from the reported percentages.

Table 8. Hepatocellular carcinomas in newborn male B6C3F$_1$ mice administered intraperitoneal injections of estragole, other alkenylbenzenes, and their derivatives (Miller *et al.*, 1983).

Treatment	Total dose (mmol/kg)	Incidence of hepatocellular carcinoma	Average number of hepatocellular carcinomas/ mouse[a]
Trioctanoin (vehicle)	0	5/32	0.2
Untreated	0	5/32	0.1
1'-Hydroxyestragole[c]	1.87	25/27[b]	2.7[b]
1'-Hydroxysafrole	3.75	24/26[b]	2.7[b]
1-Allyl-1'-hydroxy-4-methoxynaphthalene	3.75	22/34[b]	1.1[b]
1'-Hydroxyallylbenzene	3.75	4/32	0.1
Benzyl alcohol	3.75	3/30	0.1
Anisyl alcohol	3.75	5/32	0.2
Myristicin	3.75	7/33	0.2

[a] Only hepatocellular carcinomas ≥ 2 mm in diameter were tabulated.

[b] Significantly greater than vehicle controls ($p<0.001$).

[c] Metabolite of estragole.

Newborn Mouse Intraperitoneal Injection Studies: Wiseman et al., 1987

Wiseman *et al.* (1987) treated groups of male and female C3H/HeJ and C57BL/6J mice (33 to 50 per group) with four intraperitoneal injections of 1'-hydroxyestragole or 1'-hydroxysafrole, primary metabolites of estragole and safrole, respectively. The test compounds were dissolved in trioctanoin and administered on days 1, 8, 15 and 22 after birth at doses of 0.1, 0.04, 0.04 and 0.08 mmol/kg body weight, respectively. Separate groups of animals given only trioctanoin served as vehicle controls. The experiment was terminated at 14 months; the first tumor-bearing mouse was observed at 10 months. Male

C3H/He mice treated with 1'-hydroxyestragole exhibited statistically significant increases in the incidence of hepatocellular carcinomas relative to vehicle controls (Table 9). Male and female C57BL/6J and female C3H/He mice treated with 1'-hydroxyestragole did not have significantly elevated incidences of liver tumors compared to controls. Male mice of both strains treated with 1'-hydroxysafrole exhibited statistically significant increases in the incidence of hepatocellular carcinomas relative to vehicle controls; whereas, results in females were negative. The authors reported the tumor data as percentages of hepatocellular carcinoma-bearing mice. Incidence data were derived from the reported percentages.

Table 9. Hepatocellular carcinomas in newborn CeH/HeJ and C57BL/6J mice administered intraperitoneal injections of metabolites of estragole or safrole (Wiseman *et al.*, 1987).

Strain/Treatment	Sex	Incidence of hepatocellular carcinomas	Average number of hepatocellular carcinomas/ mouse[a]
CeH/HeJ			
Trioctanoin (vehicle)	M	8/31	0.3
1'-Hydroxyestragole[c]	M	26/34[b]	3.0[b]
1'-Hydroxysafrole	M	28/41[b]	1.6[b]
Trioctanoin (vehicle)	F	0/32	0
1'-Hydroxyestragole[c]	F	2/34	0.06
1'-Hydroxysafrole	F	1/33	0.03
B57BL/6J			
Trioctanoin (vehicle)	M	2/42	0.07
1'-Hydroxyestragole[c]	M	5/36	0.3
1'-Hydroxysafrole	M	10/30[b]	0.4[b]
Trioctanoin (vehicle)	F	0/32	0
1'-Hydroxyestragole[c]	F	0/33	0
1'-Hydroxysafrole	F	0/37	0

[a] Only hepatocellular carcinomas ≥ 2 mm in diameter were tabulated.

[b] Significantly greater than vehicle controls of same sex and strain ($p<0.005$).

[c] Metabolites of estragole.

Newborn Mouse Intraperitoneal Injection Studies: Wiseman et al., 1987

Wiseman *et al.* (1987) also investigated the effect of age and dose by comparing the relative induction of hepatocellular carcinomas in mice dosed with 1'-hydroxyestragole at day one versus day 12 of birth. Two separate groups of male $B6C3F_1$ mice (35-45 animals per group) were administered a single intraperitoneal injection of 1'-hydroxyestragole at concentrations of 0 (vehicle, trioctanoin), 0.05, 0.10 or 0.15 mmol/kg body weight on day one after birth or day 12 after birth (Table 10). The animals were sacrificed at 14 months. Statistically significant increased incidences of hepatocellular carcinomas ($p<0.001$) were observed in all 1'-hydroxyestragole-treated mice relative to controls. A stronger dose-related response was observed for mice treated at day 12 of life compared to those treated at day one of life.

Table 10. Hepatocellular carcinomas in $B6C3F_1$ mice administered a single intraperitoneal injection of 1'-hydroxyestragole at day one or day 12 of life (Wiseman *et al.*, 1987).

Dose (mmol/kg body weight)	Age at treatment (day)	Incidence of hepatocellular carcinomas	Average number of hepatocellular carcinomas/ mouse[a]
0 (vehicle)	1	2/39	0.08
0.05	1	23/35[b]	0.9[b]
0.10	1	26/45[b]	1.2[b]
0.15	1	25/40[b]	1.8[b]
0 (vehicle)	12	3/37	0.08
0.05	12	29/39[b]	1.9[b]
0.10	12	41/47[b]	3.8[b]
0.15	12	40/45[b]	4.5[b]

[a] Only hepatocellular carcinomas ≥ 2 mm in diameter were tabulated.

[b] Significantly greater than vehicle controls of same age at treatment ($p<0.001$).

Newborn Mouse Intraperitoneal Injection Study: Wiseman et al., 1987

Groups of male $B6C3F_1$ mice were administered four intraperitoneal injections of 1'-hydroxyestragole or other structurally related compounds. Test compounds (dissolved in trioctanoin) were given on days 1, 8, 15 and 22 after birth with fractions of the total dose

corresponding to 1:2:4:12, respectively. Total doses of each test compound are listed in Table 11. Animals were sacrificed at 13 months; the first hepatocellular carcinoma appeared at nine months. Increased incidences of hepatocellular carcinoma were observed for mice treated with 1'-hydroxy-2',3'-dehydroestragole, 1'-hydroxyestragole, 1'-acetoxyestragole, 1'-hydroxyelemicin, 1'-acetoxyelemicin, 3'-bromo-*trans*-anethole (high dose only), *cis*-asarone and *trans*-asarone compared to vehicle controls ($p<0.001$). A dose-related trend in the incidences of hepatocellular carcinoma, as well as the average number of liver tumors per mouse, was observed for mice treated with 0.4 and 1.9 μmol 1'-hydroxyestragole.

Table 11. Hepatocellular carcinomas in newborn B6C3F$_1$ mice administered intraperitoneal injections of derivatives of estragole and other alkenylbenzenes (Wiseman *et al.*, 1987).

Treatment	Total dose (μmol)	Incidence of hepatocellular carcinoma	Average number of hepatocellular carcinomas/ mouse[a]
Trioctanoin (vehicle)	0	3/31	0.1
1'-Hydroxy-2',3'-dehydroestragole[d]	0.4	39/41[b]	7.0[b]
1'-Hydroxyestragole[d]	0.4	24/47[b]	1.0[b]
1'-Hydroxyestragole[d]	1.9	40/42[b]	5.2[b]
1'-Acetoxyestragole[d]	1.9	37/42[b]	3.5[b]
1'-Hydroxyelemicin	9.5	23/45[b]	0.8[b]
1'-Acetoxyelemicin	9.5	25/48[b]	0.8[b]
1'-Oxoestragole[d]	1.9	9/21	0.7[c]
1'-Oxoestragole[d]	1.4	7/31	0.3
3'-Bromo-*trans*-anethole	1.9	25/31[b]	1.8[b]
3'-Bromo-*trans*-anethole	1.4	9/44	0.2
cis-Asarone	4.8	34/41[b]	2.3[b]
trans-Asarone	4.8	39/44[b]	1.8[b]

[a] Only hepatocellular carcinomas ≥ 2 mm in diameter were tabulated.

[b] Significantly greater than vehicle controls ($p<0.001$).

[c] Significantly greater than vehicle controls ($p<0.01$).

[d] Metabolites or derivatives of estragole.

Newborn Mouse Intraperitoneal Injection Studies: Wiseman et al., 1987

Groups of male B6C3F$_1$ mice (approximately 40 animals per group) were given a single intraperitoneal injection of metabolites of estragole, safrole or related compounds at 12 days of age. Test compounds were dissolved in trioctanoin. Doses ranged from 0.01 to 2.5 mmol/kg depending on the test compound (Table 12). Mice were sacrificed at 12

Table 12. Hepatocellular carcinomas in B6C3F$_1$ mice administered a single intraperitoneal injection of derivatives of estragole and other alkenylbenzenes on day 12 of life (Wiseman *et al.*, 1987).

Treatment	Total dose (mmol/kg)	Incidence of hepatocellular carcinoma	Average number of hepatocellular carcinomas/ mouse[a]
Trioctanoin (vehicle)	0	9/40	0.2
1'-Hydroxy-2',3'-dehydroestragole[d]	0.01	34/36[b]	2.4[b]
1'-Hydroxy-2',3'-dehydroestragole[d]	0.05	39/39[b]	12.3[b]
1'-Hydroxy-2',3'-dehydroestragole[d]	0.1	32/33[b]	11.1[b]
1'-Hydroxy-2',3'-dehydrosafrole	0.01	18/39[c]	0.7[c]
1'-Hydroxy-2',3'-dehydrosafrole	0.1	37/37[b]	10.7[b]
1'-Hydroxyestragole[d]	0.01	8/37	0.2
1'-Hydroxyestragole[d]	0.1	36/38[b]	4.6[b]
1'-Acetoxyestragole[d]	0.1	38/38[b]	4.4[b]
1'-Hydroxysafrole	0.1	26/38[b]	1.4[b]
1'-Acetoxysafrole	0.1	30/38[b]	1.5[b]
1'-Hydroxyelemicin	0.1	7/36	0.3
1'-Hydroxyelemicin	0.25	14/43	0.4
3'-Hydroxy-*trans*-anethole	0.1	5/39	0.2
3'-Hydroxy-*trans*-anethole	2.5	15/41	0.4

[a] Only hepatocellular carcinomas ≥ 2 mm in diameter were tabulated.

[b] Significantly greater than vehicle controls ($p<0.001$).

[c] Significantly greater than vehicle controls ($p<0.05$).

[d] Metabolites or derivatives of estragole.

months of age, with the first appearance of hepatocellular carcinoma at 7.5 months. High incidences of hepatocellular carcinomas relative to controls were observed for all doses of derivatives and metabolites of estragole, except for the group given the lowest dose of 1'-hydroxyestragole (Table 12).

A/J Mouse Intraperitoneal Injection Studies: Wiseman et al., 1987

Wiseman *et al.* (1987) tested derivatives of estragole in preweanling A/J mice, a strain sensitive to the induction of lung tumors. Groups of A/J mice were given intraperitoneal injections (0.05 mmol/kg body weight) of 1'-acetoxy-2',3'-dehydroestragole or 1'-acetoxyestragole, either in a single injection on day 12 of life or in a pair of injections on day 8 and day 12 of life (Table 13). Each group contained equal numbers of male and female mice. Since lung tumor incidences were not different between males and females, the authors reported the results as combined incidences. One group of mice administered trioctanoin served as vehicle controls and a separate group, given N-ethyl-N-nitrosourea (0.2 mmol/kg), served as positive controls. Animals were sacrificed at nine months. All tumors were reported as lung adenomas. Statistically significant increases in lung tumor incidences were reported for mice treated with 1'-acetoxy-2',3'-dehydroestragole relative to controls ($p<0.001$). Marginal increased incidences of lung tumors were observed in mice treated with 1'-acetoxyestragole either on day 12 ($p=0.06$) or on days 8 and 12 ($p=0.06$) compared to controls.

Table 13. Lung adenomas in A/J mice administered derivatives of estragole by intraperitoneal injection (Wiseman *et al.*, 1987).

Treatment	Dose (mmol /kg)	Treated on days	Incidence of lung adenomas	Average number of lung adenomas/ mouse [a]
Trioctanoin (vehicle control)	0	12	5/40	0.15
1'-Acetoxy-2',3'-dehydroestragole[b]	0.05	12	19/47[a]	0.57[a]
1'-Acetoxy-2',3'-dehydroestragole[b]	0.05	8 & 12	20/52[a]	0.42[a]
1'-Acetoxyestragole[b]	0.05	12	13/45	0.31
1'-Acetoxyestragole[b]	0.05	8 & 12	12/42	0.40

N-ethyl-N-nitrosourea (positive control)	0.2	12	22/24[a]	6.7[a]

[a] Significantly greater than vehicle controls ($p<0.01$).

[b] Derivatives of estragole.

Mouse Skin Painting Study: Miller et al., 1983

In an initiation/promotion assay, groups of 40 female eight-week old CD-1 mice were treated topically with alkenylbenzene epoxides, followed by repeated application of croton oil (Table 14). Test compounds were estragole-2,3-oxide, safrole-2,3-oxide, eugenol-2,3-oxide, 1'-hydroxyestragole-2',3'-oxide, and 1'-hydroxysafrole-2',3'-oxide. Test compounds (11.2 μmol each) were dissolved in 0.15 mL acetone and applied to the shaved skin of the mice, four days/week for six weeks. One week after the last dose of test compound, 0.15 mL of a 0.6% solution of croton oil in acetone was applied topically. The experiment was terminated at 40 weeks. Increased incidences of benign skin tumors ($p < 0.005$) were observed for all test groups relative to the controls (Table 14). Tumors were diagnosed as epidermal papillomas and keratoacanthomas. Miller *et al.* (1983) reported the tumor data as percentages of tumor-bearing mice. Incidence data were derived from the reported percentages.

Table 14. Benign skin tumors in female CD-1 mice treated topically with epoxides of estragole, safrole, and eugenol (Miller *et al.*, 1983).

Treatment	Total dose (μmol)	Incidence of skin tumors	Average number of tumors/mouse
Acetone only (vehicle)	0	3/40	0.1
Estragole-2,3-oxide[d]	269	13/40[a,b]	0.6[c]
Safrole-2,3-oxide,	269	14/40[a]	0.7[c]
Eugenol-2,3-oxide	269	16/40[a]	0.9[c]
1'-Hydroxy-2',3'-dehydroestragole[d]	269	18/40[a]	1.1[c]
1'-Hydroxysafrole-2',3'-oxide	269	33/40[a]	2.7[c]

[a] Significantly greater than vehicle controls ($p<0.001$).

[b] In addition, one mouse had a fibromyosarcoma of the skin.

[c] Significantly different from vehicle controls ($p<0.005$).

[d] Metabolites or derivatives of estragole.

Rat Subcutaneous Injection Study: Miller et al., 1983

Miller *et al.* (1983) also administered various derivatives of estragole and other alkenylbenzenes to groups of 20 male Fischer rats by subcutaneous injection. A total of 20 injections (two per week) were administered to the same location on the hind leg of the animals. Test compounds, listed in Table 15, were dissolved in 0.2 mL of trioctanoin. Survival was good, as 16 to 19 animals of each group survived to 20 months. The experiment was terminated at 24 months. Tumor incidences are shown in Table 15; no significant increases in tumor incidence were observed for derivatives of estragole.

Table 15. Tumors in male Fischer rats treated with repeated subcutaneous injections of metabolites of estragole and other alkenylbenzenes (Miller *et al.*, 1983).

Treatment	Total dose (mmol)	Injection site sarcomas	Incidence of liver carcinoma	Tumors at other sites[a]
Trioctanoin (vehicle)	0	0/20	0/20	1 leiomyosarcoma (abdominal cavity) 1 subcutaneous fibrosarcoma 1 pulmonary adenoma
1'-Hydroxyestragole[d]	1	3/20	1/20	1 subcutaneous fibroliposarcoma 1 hemangioendotheliosarcoma (spleen) 1 lymphoma (spleen, liver, lymph nodes) 1 papillary mesothelioma (mesentery)
1'-Acetoxysafrole	0.6	4/20[b]	1/20	1 subcutaneous sarcoma 1 renal cell carcinoma 1 mammary fibroadenoma
1'-Hydroxysafrole	2	0/20	11/20[c]	2 subcutaneous fibromas 1 subcutaneous hemangioendotheliosarcoma
Estragole-2',3'-oxide[d]	2	1/20	0/20	1 basal cell carcinoma (lip) 1 adenocarcinoma (duodenum)
Safrole-2',3'-oxide	2	1/20	0/20	
Eugenol-2',3'-oxide	2	2/20	0/20	1 keratoacanthoma (skin) 1 sebaceous gland carcinoma (skin, near injection site) 1 fibromyosarcoma
1'-Hydroxyestragole-2',3'-oxide[d]	2	0/20	1/20	2 subcutaneous fibromas 1 epidermal carcinoma 1 gastric adenoma 1 subcutaneous sarcoma 1 islet cell adenoma (pancreas)
1'-Hydroxysafrole-2',3'-oxide	2	4/20[b]	0/20	2 subcutaneous sarcomas 1 subcutaneous fibroma

[a] In addition to the tumors listed, the majority of rats that survived at least 18 months exhibited unilateral or bilateral interstitial cell tumors of the testes.

[b] Significantly different from vehicle controls ($p<0.05$).

[c] Significantly different from vehicle controls ($p<0.001$).

[d] Metabolites of estragole.

3.3 Other Relevant Data

Along with the reported animal bioassays, additional evidence related to the carcinogenicity of estragole is available. This includes studies of genetic toxicity, observations of the pharmacokinetics and metabolism, and structure-activity comparisons.

3.3.1 Genetic Toxicology

Estragole produced mixed results in *Salmonella typhimurium* reverse mutation and other bacterial-based assays with and without metabolic activation. Several studies demonstrated that estragole caused increases in unscheduled DNA synthesis (UDS) in human and rat cell lines as well as *ex vivo* in livers of rats treated with oral doses of estragole. Several DNA adducts of estragole have been characterized. Additional genotoxicity evidence comes from studies on estragole-containing essential oils and on estragole metabolites or related derivatives.

Genotoxicity Tests in Bacteria and Yeast

In the reverse mutation assay, To *et al.* (1982) reported negative results for estragole in *Salmonella* mutagenicity tests, with or without exogenous activation, in strains TA98, TA100, TA1537, TA1538 and equivocal results for strain TA1535. However, with the addition of the sulfation cofactor PAPS (3'-phosphoadenosine-5'-phosphosulfate) which likely important in the carcinogenic mode of action of estragole (see page 36), the mutagenicity of estragole in strain TA1535 was significantly increased in the presence of exogenous activation enzymes (S9, from Aroclor-induced rats). In another study, estragole was not mutagenic in *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537 in the absence or presence of hamster or rat liver S9 (Zeiger *et al.*, 1987). The essential oil "Piemontese" (77.5% estragole) was negative in the *Salmonella typhimurium* reverse mutation assay in strains TA98, TA100, TA1535, or TA1537 with or without metabolic activation (Zani *et al.*, 1991).

Estragole was negative in a DNA-repair test in *Bacillus subtilis* (Rec assay) without S9, and negative in the *Escherichia coli* WP2 *uvr* A reversion test with or without S9 (Sekizawa and Shibamoto, 1982). However, in a separate study in *Bacillus subtilis* (Rec-assay), estragole was positive as was the essential oil "Piemontese" (77.5% estragole) which elicited dose-related increases in DNA damaging activity (rec+ inhibition) equivalent to that of estragole alone (Zani *et al.*, 1991).

The essential oils of tarragon (60% estragole) and of basil (16.5% estragole) were tested for genotoxic potential in the yeast, *Saccharomyces cerevisiae* D7. Oil of tarragon induced a 8-fold increase in nuclear reverse mutations and a 10-fold increase in gene conversions relative to controls; oil of basil was negative for these endpoints (Bianchi *et al.*, 1989 abstract).

Metabolites and derivatives of estragole have also been tested for mutagenic potential in bacteria. Drinkwater *et al.* (1976) showed dose-related mutagenicity for 1'-acetoxyestragole in *Salmonella typhimurium* strain TA100, but not in strain TA98. 1'-Hydroxyestragole did not significantly increase the number of mutants in TA98 or TA100 with or without addition of Aroclor-treated rat liver S-13 fraction. Addition of the sulfation cofactor PAPS had no effect on mutation rates. Swanson *et al.* (1979) reported positive, but weak activity, for estragole and 1'-hydroxyestragole in strain TA100 without exogenous activation, and increased mutagenicity for each compound in the presence of NADPH-fortified rat liver microsomes. Neither compound was mutagenic in strain TA98. Epoxide metabolites of estragole, namely estragole-2',3'-oxide and 1'-hydroxyestragole-2',3'-oxide, produced a strong dose-related increase in mutation frequency in strain TA1535 with or without exogenous activation (Swanson *et al.*, 1979). Phillips *et al.* (1981) observed significant, dose-related increases in mutagenicity of 1'-hydroxyestragole-2',3'-oxide and 1'-acetoxyestragole in *Salmonella typhimurium*, strain TA100. Sekizawa and Shibamoto (1982) reported estragole to be negative for mutagenicity in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 or TA1538, with or without exogenous activation.

Genotoxicity in Mammalian Cells

Howes *et al.* (1990) investigated the ability of estragole and other structurally related alkenylbenzenes to induce UDS in rat hepatocytes. The authors reported excellent correlation between UDS induction and results from rodent hepatocarcinogenicity studies. Estragole, safrole and methyleugenol, having "known rodent hepatocarcinogenicity" all induced UDS; whereas, anethole, isosafrole, and allylbenzene, chemicals for which hepatocarcinogenicity evidence is "equivocal or negative", did not induce UDS. Chan and Caldwell (1992) demonstrated that both estragole and 1'-hydroxyestragole induced UDS in rat hepatocytes, with 1'-hydroxyestragole being more potent that estragole. Similarly, Müller *et al.* (1994) reported that estragole or basil oil (88% estragole) induced DNA repair (UDS) in rat hepatocytes and *ex vivo* in livers from rats treated with oral doses of 0.5, 1.0 or 2.0 g/kg body weight. Estragole did not increase chromosomal aberrations in V79 cells with or without activation using rat liver S9 or rat hepatocytes (Müller *et al.*, 1994).

Metabolites and derivatives have also been tested for genotoxic potential in mammalian cells. Francis *et al.* (1981) reported that estragole, 1'-hydroxyestragole, and estragole-2',3'-oxide caused increases in UDS in a bromodeoxyuridine (BrdU) assay in human normal and xeroderma cell lines.

DNA adducts and abasic sites

Drinkwater *et al.* (1980) observed the formation of abasic sites in supercoiled SV40 DNA treated with 1'-acetoxyestragole (used as a model reactive compound to look at DNA binding of estragole). Phillips and Hanawalt (1982) observed alkali-labile sites in DNA of human cells treated with 1'-acetoxyestragole.

Numerous investigators have studied the mechanism of estragole carcinogenesis by examining DNA binding and characterizing DNA adducts formed by estragole and its reactive metabolites. Randerath *et al.* (1984) utilized ^{32}P-postlabeling methods to analyze DNA adduct formation in the livers of adult female CD-1 mice administered intraperitoneal injections of estragole, safrole and other alkenylbenzenes. Estragole and safrole exhibited the strongest binding to mouse liver DNA (200-300 pmol adduct/mg DNA, from a 10 mg dose). Two major guanine adducts of estragole were detected. In a companion article, Phillips *et al.* (1984) utilized ^{32}P-postlabeling methods to analyze DNA adduct formation in the livers of newborn male B6C3F$_1$ mice administered intraperitoneal injections of estragole, safrole and other alkenylbenzenes. Doses administered were 0.25, 0.5, 1.0 and 3.0 mmol/kg body weight on days 1, 8, 15 and 22 of life, respectively. The highest levels of adduct formation were observed with estragole, safrole, and methyleugenol treatments. DNA adducts levels observed in estragole-treated mice were 30.0, 14.8 and 9.4 pmol/mg DNA on days 23, 29 and 43, respectively.

Phillips *et al.* (1981) reported observing two major and two minor liver DNA adducts in adult female CD-1 mice and preweanling B6C3F$_1$ mice given an intraperitoneal injection of 1'-[2',3'-^{3}H]hydroxyestragole. Adducts produced *in vivo* co-eluted in an HPLC system against those produced via reactions of ^{14}C-labeled nucleosides with 1'-acetoxyestragole, but not with 1'-hydroxyestragole-2'-3'-oxide or 1'-oxo-estragole. These four adducts were assigned the following proposed structures, based upon characterization by NMR: (I) N^2-(trans-isoestragol-3'-yl)dG, (II) N^6-(trans-isoestragol-3'-yl)dA, (III) N^2-(estragol-1'-yl)dG, and (IV) N^2-(cis-isoestragol-3'-yl)dG. Later work from this laboratory (Wiseman *et al.*, 1985) investigated *in vivo* adduct formation in 12-day old mice dosed with 1'-hydroxyestragole and confirmed three of these adducts, but the cis isomer (i.e., IV) was not observed. However, two additional adducts of guanine at positions C-8 (V)and N-7 (VI) were observed and characterized. It should be noted that analogous DNA adducts (I-III, V-VI)) are formed *in vivo* by 1'-hydroxyestragole or 1'-hydroxysafrole or *in vitro* by 1'-acetoxyestragole or 1'-acetoxysafrole (Phillips, 1994).

Although estragole-2',3'-oxide and 1'-hydroxyestragole-2',3'-oxide have been shown to be hepatocarcinogenic (Miller *et al.*, 1983; Wiseman *et al.*, 1987) and produce DNA adducts *in vitro* (Swanson *et al.*, 1981; Qato and Guenthner, 1995; Luo and Guenthner, 1996), adducts of these epoxides were not among the major adducts characterized in mouse liver following *in vivo* administration of estragole (reviewed in Luo and Guenthner, 1996). The absence of these adducts *in vivo* is likely due to the efficient removal of estragole-2',3'-epoxide (and related epoxides) by glutathione S-transferases and epoxide hydrolase enzymes (Luo and Guenthner, 1994; 1996).

3.3.2 Pharmacokinetics and Metabolism

Metabolism and distribution studies of estragole have been conducted in rats, mice and humans. It appears that there are no major qualitative differences between species in the metabolites formed, just in the relative proportions of the metabolites produced. Estragole is readily absorbed through the gut. As shown in Figure 1, mixed function oxidases in the liver metabolize the allylic side chain of estragole to one of three

Figure 1. Metabolism of estragole and pathways related to carcinogenesis

[insert metabolism figure here]

compounds, namely, 1'-hydroxyestragole, 4-methoxycinnamyl alcohol, and estragole-2',3'-oxide. 1'-Hydroxyestragole is either conjugated with glucuronide or sulfate; sulfate conjugates (sulfuric acid esters) in aqueous media have considerable delocalization of charge (Miller, 1994) and easily react with DNA and other cellular nucleophiles. 4-Methoxycinnamyl alcohol is further oxidized to 4-methoxycinnamic acid (or conjugates) which in turn can undergo further oxidation to 4-methoxybenzoic acid (or conjugates). Estragole-2',3'-oxide can be acted upon by epoxide hydrolase (or presumably non-enzymatic hydration) to form 2',3'-dihydroxy-4-propyanisole which can undergo further oxidation to 4-methoxyphenyllactic acid and 4-methoxyphenylacetic acid. Alternately, a major route of metabolism/detoxification involves conversion of estragole to hydroxy-allylbenzene by O-demethylation and ultimately metabolized to CO_2.

Studies of metabolism

Solheim and Scheline (1973) conducted some of the initial studies of metabolism of estragole in the rat. Male albino rats were administered a dose of 100 mg estragole/kg body weight by gavage or by intraperitoneal injection. Urinary, biliary and fecal metabolites were collected, extracted and analyzed by gas chromatography with flame ionization detection. A major urinary metabolite was the O-demethylation product, 4-hydroxy-allylbenzene, which following hydrolysis by β-glucuronidase and sulfatase constituted 39% and 46% of the dose for oral and intraperitoneal routes, respectively. Other major urinary metabolites included 4-methoxyphenyllactic acid (% of dose: 9% oral and 17% i.p.), 4-methoxybenzoic acid (% of dose: 2% oral and 2% i.p.), and 4-hippuric acid (the glycine conjugate of 4-methoxybenzoic acid) (% of dose: 6% oral and 12% i.p.).

Drinkwater *et al.* (1976) compared the formation of 1'-hydroxyestragole in 21-day old versus adult CD-1 male mice following administration of estragole. Animals (total numbers not reported) were administered 185 mmol estragole/kg body weight by intraperitoneal injection. Other animals were administered injections of trioctanoin as vehicle controls. Urine was collected after 24 hours, treated with β-glucuronidase, solvent extracted, and analyzed for 1'-hydroxyestragole using an HPLC-based assay. Adult and 21-day-old mice were not different in their relative production of 1'-hydroxyestragole. The percentage of the total dose of estragole recovered as urinary 1'-hydroxyestragole (or conjugates) was 22.2% and 23.5% for the adult and 21-day-old mice, respectively.

Delaforge *et al.* (1980) studied epoxide and diol-epoxide formation of estragole and other alkenylbenzenes *in vivo* and in rat liver microsomes *in vitro*. Male Wistar rats were given a single intraperitoneal injection of 200 mg estragole/kg body weight (in corn oil). Urine was collected every two hours. Animals were sacrificed after 24 hours. In addition, estragole metabolism was studied using liver microsomal preparations. Samples of urine or microsomal reactions were extracted and derivatized for analysis by gas chromatography-mass spectrometry. The authors reported detecting estragole epoxide and estragole diolepoxide in urine from estragole treated rats and in rat liver microsomal

preparations incubated with estragole. No quantitative data on the levels of epoxides present in urine or microsomal preparations were reported.

Swanson *et al.* (1981) studied the oxidation of side chains of estragole and other alkenylbenzenes by rat and mouse liver microsomes *in vitro*. Estragole was converted by rat and mouse microsomes to 1'-hydroxyestragole and estragole-2',3'-oxide. Microsomal oxidation of either 1'-hydroxyestragole or estragole-2',3'-oxide yielded 1'-hydroxyestragole-2',3'-oxide. Formation of these metabolites was dependent on cytochrome P-450 and a NADPH-generating system. The high efficiency of epoxide hydrolase in hydrolyzing the epoxide moiety of estragole-2',3'-oxide or 1'-hydroxyestragole-2',3'-oxide was demonstrated by comparing reactions with and without

Table 16. Metabolism of [^{14}C]estragole administered as a single 50 mg/kg dose, to rats (by gavage) and mice (by intraperitoneal injection) (Anthony *et al.*, 1987).

Metabolite	[^{14}C] excretion **% of dose**	
	Rat	Mouse
Urinary metabolites[a]		
Estragole	Not detected	Not detected
1'-Hydroxyestragole	5.4	5.2
4-Methoxycinnamyl alcohol	2.9	1.5
2',3'-Dihydroxy-4-propylanisole	<0.1	<0.1
4-Methoxyphenyllactic acid	4.5	3.0
4-Methoxycinnamic acid	0.1	0.1
4-Methoxycinnamoylglycine	0.6	0.7
4-Methoxyphenylacetic acid	<0.1	<0.1
4-Methoxyphenaceturic acid	1.2	3.3
4-Methoxybenzoic acid	0.1	0.1
4-Methoxyhippuric acid	8.2	6.7
Total recovery of ^{14}C in 24 hr urine	37.1	36.3
Exhaled $^{14}CO_2$	54.4	41.7

[a] Urine samples (collected over 24 hours) were analyzed after treatment with β-glucuronidase.

the addition of an epoxide hydrolase inhibitor, trichloropropylene oxide. Twenty-minute incubations of estragole-2',3'-oxide in the presence of the inhibitor yielded recoveries of

estragole-2',3'-oxide of about 50% in mouse microsomes and about 80% in rat microsomes, whereas in the absence of the inhibitor yields were less than 5% and 1%, respectively.

Anthony *et al.* (1987) studied the metabolism of estragole in rats and mice over a wide range of doses, paying particular attention to the formation of the putative carcinogen, 1'-hydroxyestragole. Female Wistar rats were given a single dose of [^{14}C]estragole by oral intubation in trioctanoin, equivalent to either 0.05, 0.5, 5, 50, 100, 500 or 1000 mg/kg body weight. Mice (sex and strain not reported) were administered single doses of [^{14}C]estragole in trioctanoin by intraperitoneal injection equivalent to 0.05, 1, 5, 50, 100, 250, 500 or 1000 mg/kg body weight. Exhaled $^{14}CO_2$, urine, and feces were collected and analyzed. Measurements of ^{14}C in exhaled air, urine and feces indicated that elimination of administered estragole was essentially complete within 24 hours, except for the two highest doses in which significant excretion was observed during the 24-48 hour period following dosing. For both rats and mice, the ^{14}C label was eliminated primarily through exhaled air and urinary excretion. Elimination via the feces was minimal (in most cases less than 0.5% of the dose). The proportion of the dose recovered in the urine increased with administered dose, while the proportion of exhaled $^{14}CO_2$ decreased with increasing dose. Anthony *et al.* (1987) reported that their results confirmed the importance of the O-demethylation pathway which leads to the exhalation of $^{14}CO_2$. However, Anthony *et al.* (1987) did not assay for 4-hydroxy-allylbenzene, the first product of O-demethylation and a major urinary product found by Solheim and Scheline (1973). Chromatography and mass spectral analysis of the urinary metabolites from the 50 mg/kg dose groups are indicated in Table 17. One of the key findings of the Anthony *et al.* study was that the proportion of the administered dose excreted as 1'-hydroxyestragole increased with increasing dose of estragole (Table 17, Figure 2).

Table 17. Excretion of 1'-hydroxyestragole (as glucuronide conjugate) in the urine of rats and mice given [^{14}C]estragole (Anthony *et al.*, 1987).

Dose (mg/kg body weight)	[^{14}C]1'-Hydroxyestragole **% of dose (range)** [a]	
	Rat	Mouse
0.05	1.3 (0.4-2.1)	1.3 (1.2-1.4)
5	3.0 (1.4-6.0)	2.1 (0.6-3.8)
50	5.4 (4.5-7.2)	5.2 (3.4-6.5)
500	11.4 (10.5-12.3)	7.8 (6.4-8.6)
1000	13.7 (12.5-15.6)	9.4 (5.6-14.1)

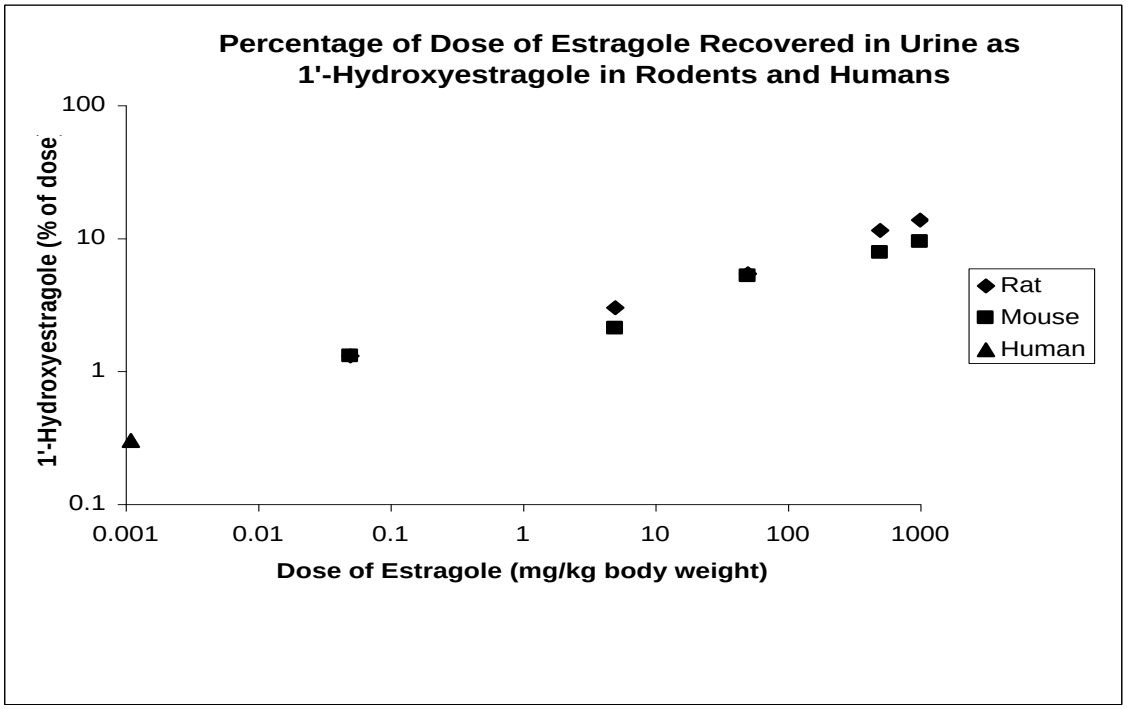
[a] Values are means (N=4 or more) with the range of values indicated in parentheses.

Sangster *et al.* (1987) studied the metabolism of estragole in human volunteers who ingested small amounts of radiolabeled estragole. Two healthy male volunteers, ages 35 and 47, ingested a gelatin capsule containing 100 μg of [methoxy-^{14}C]estragole (equivalent to 0.0011 mg/kg body weight for each individual) on two separate occasions (at least six months apart). The dose administered is roughly equivalent to a dose typically encountered in the diet. Urine was collected at eight hour intervals for 48 hours. Exhaled air samples were collected every 30 minutes for eight hours. Based on the low level of elimination in the feces of rats and mice given estragole, fecal samples from the volunteers were not collected. Approximately 40% of the dose was excreted in the urine within 10 hours and about 60% of the total dose was excreted in the urine by 48 hours. Cumulative excretion of exhaled $^{14}CO_2$ accounted for about 12% of the dose at eight hours. The collection of excreted radiolabel accounted for only 65% to 70% of the dose of estragole. The authors felt this incomplete recovery of radiolabel was most likely due to an inadequate monitoring period for $^{14}CO_2$ in exhaled air.

Analysis of urinary metabolites indicated the presence of 4-methoxyhippuric acid (12%), 4-methoxyphenyllactic acid (4%), 4-methoxycinnamoylglycine (0.8%), and 4-methoxyphenylacetic acid (0.5%). Concentrations of 1'-hydroxyestragole, the putative proximate toxic metabolite of estragole, were measured as 0.2% and 0.4% of the total dose for the two subjects. The authors compared the metabolism and distribution results from the human data to those from earlier animal studies and concluded that the qualitative aspects of estragole metabolism are similar for mice, rats and humans. Sangster *et al.* (1987) noted "most importantly the formation of 1'-hydroxyestragole, resemble those seen in rats and mice at very low doses."

Figure 2 is a graph of the percentage of total dose of estragole recovered in the urine as 1'-hydroxyestragole, the putative carcinogenic metabolite, for mice and rats (Anthony *et al.*, 1987) and for humans (Sangster *et al.*, 1987) compared to the dose of estragole administered. The observed trend appears to be quantitatively consistent for humans and rodents. Thus, based on the mechanism of action of estragole carcinogenesis in rodents, the observation that 1'-hydroxyestragole is produced in humans following ingestion of estragole at concentrations typically found in the diet adds to the public health concern regarding this agent.

Figure 2. 1'-Hydroxyestragole produced as a function of dose of estragole [a]



[a] Data points represent means of four or more observations in rats and mice (Anthony *et al.*, 1987) and a mean of two observations in humans (Sangster *et al.*, 1987).

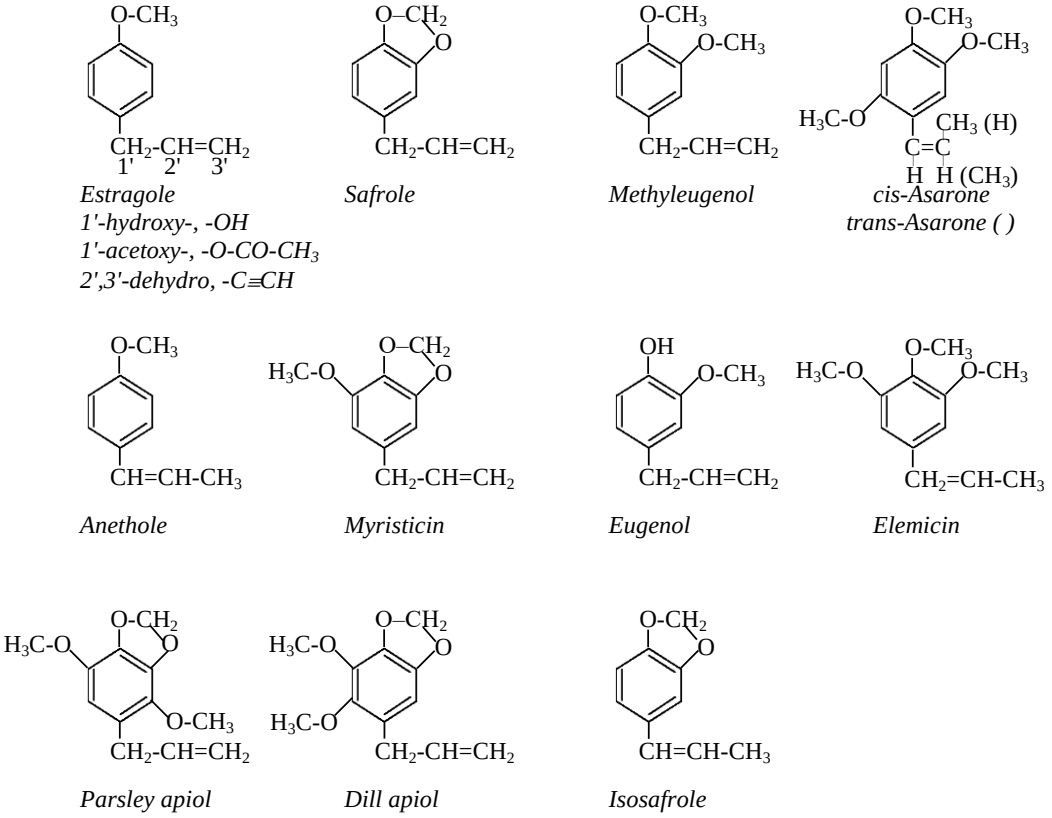
3.3.3 Structure-Activity Comparisons

Estragole and other structurally-similar alkenylbenzene compounds are shown in Figure 3. Several of these, namely safrole, methyleugenol, *cis*-asarone, and *trans*-asarone, produced increased incidences of liver tumors and tumors at other sites in rats and mice. Additionally, synthetic derivatives of estragole such as 1'-hydroxy-2',3'-dehydroestragole and 1'-acetoxyestragole, and of safrole, namely 1'-hydroxy-2',3'-dehydrosafrole and 1'-acetoxysafrole, were highly carcinogenic in newborn mice. Eugenol and elemicin produced mixed or suggestive results in cancer studies while myristicin, parsley apiol, dill apiol, and trans-anethole produced negative results. DNA adduct levels in the livers of newborn mice treated via intraperitoneal injection with various alkenylbenzenes correlated well with tumor incidences.

Safrole (1-allyl-3,4-methylenedioxybenzene, CAS No. 94-59-7) was the first of this class to be discovered to have carcinogenic properties (Miller *et al.*, 1982). Chronic administration of safrole in the diet at 0.5 to 1.0% to adult rodents for a year or more was required to induce appreciable increases in liver tumors in adult rats or mice; however, relatively small total doses of 0.5 to 1.0 mg administered by intraperitoneal injection or by gavage to newborn mice induced high incidences of liver tumors within one year

(Epstein *et al.*, 1970; Borchert *et al.*, 1973; Miller *et al.*, 1983; Wiseman *et al.*, 1987). Safrole is also a transplacental and lactational carcinogen (Vesselinovitch *et al.*, 1979). Additionally, administration of safrole to pregnant mice resulted in the formation of DNA adducts in a wide number of parental and fetal tissues (Lu *et al.*, 1986). The carcinogenicity of safrole metabolites, namely 1'-hydroxysafrole, safrole-2',3'-oxide, and 1'-hydroxysafrole-2',3'-oxide, was also clearly demonstrated (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987). Safrole is classified as a carcinogen by the International Agency for Research on Cancer (group 2B) and is listed on the California "Proposition 65" list of compounds "known to the state" to cause cancer.

Figure 3. Structures of estragole and related alkenylbenzenes



Methyleugenol (CAS No 93-15-2, a component of sweet bay, lemongrass and cloves) and its metabolite, 1'-hydroxymethyleugenol, induced a high incidences of liver tumors in male newborn mice following intraperitoneal administration (Miller *et al.*, 1983, see Table 4). Recently, the National Toxicology Program reported that there was clear evidence that methyleugenol was carcinogenic in male and female mice and rats from two-year gavage studies (NTP, 1998). In male and female rats, positive dose-related liver neoplasms occurred in all dosed groups of rats. Dose-related neoplasms of the glandular stomach included neuroendocrine tumors in male and female rats at the higher dosages. Also renal tube hyperplasia and adenoma were observed for the higher doses in male rats. In mice, chemical-related increases in the incidences of liver neoplasms were also observed.

Cis-asarone (β-asarone, cis-1-propenyl-2,4,5-trimethoxybenzene, CAS No. 2883-86-9), a major component of oil of calamus, a bitters flavor, induced mesenchymal tumors of the small intestine in rats administered high levels in the diet (Gross *et al.*, 1967). *Cis*-asarone induced hepatomas in $B6C3F_1$ mice administered four intraperitoneal doses (4.75 mmol/kg body weight total dose) during the preweanling period (Table 11) and following a single dose of 0.25 or 0.50 mmol/kg body weight at day 12 of life (Table 5) (Wiseman *et al.*, 1987). Considerable hepatocarcinogenic activity was also observed in mice treated with *trans*-asarone (Wiseman *et al.*, 1987, Table 5, Table 11).

Chemical derivatives of estragole and safrole were highly carcinogenic in newborn mice. These compounds included 1'-hydroxy-2',3'-dehydroestragole (Table 4, Table 11, Table 12, Table 14), 1'-acetoxyestragole (Table 11, Table 12), 1'-acetoxy-2',3'-dehydroestragole (Table 13), 1'-hydroxy-2',3'-dehydrosafrole (Table 12), and 1'-acetoxysafrole (Table 12) (Miller *et al.*, 1983; Wiseman *et al.*, 1987). Fennell *et al.* (1985) studied DNA adduct formation of 1'-hydroxy-2',3'-dehydroestragole in infant male $B6C3F_1$ mice. Intraperitoneal administration of this derivative to mice resulted in extensive binding to DNA, RNA and protein. A single adduct was observed and co-migrated on a HPLC system with a previously characterized of adduct of guanine. Inhibition (>95%) of cytosolic sulfotransferase was demonstrated by administration of pentachlorophenol to mice. Pretreatment of 12-day old mice with pentachlorophenol before dosing inhibited 1'-hydroxy-2',3'-dehydroestragole DNA adduct formation by 87% to 97%, providing strong support for the 1'-sulfoöxy-containing metabolite as the ultimate carcinogenic metabolite of estragole-like compounds. Wiseman *et al.* (1986) reported the presence of activating mutations in the *c-Ha*-ras protooncogene in hepatocellular carcinomas by 1'-hydroxy-2',3'-dehydroestragole.

Other structurally similar alkenylbenzenes (Figure 3) and their metabolites have exhibited mixed results in rodent cancer bioassays. *trans*-Anethole (found in anise, fennel, avocado) did not induce tumors in newborn mice by intraperitoneal injection or gavage (Miller *et al.*, 1983, Table 1, Table 3, Table 4). However, 3'-bromo-*trans*-anethole did induce liver tumors in mice (Wiseman *et al.*, 1987, Table 11). Eugenol (found in cloves, allspice, artichoke) did not induce tumors in newborn mice by intraperitoneal injection or gavage (Table 1, Table 3); whereas, eugenol-2,3-oxide did induce skin tumors in mice by

topical application (Table 14) but not in rats when administered by subcutaneous injection (Table 15) (Miller *et al.*, 1983). NTP (1983) reported equivocal evidence for liver tumors in mice fed 0.3 and 0.6% eugenol for two years, and negative findings in rats. Myristicin (found in nutmeg, mace, carrots, bananas) was negative in male newborn mice by intraperitoneal injection (Miller *et al.*, 1983, Table 4, Table 8). Elemicin (found in nutmeg, sassafras) was negative in newborn mice by intraperitoneal injection (Miller *et al.*, 1983, Table 4). However, the metabolite 1'-hydroxyelemicin produced increased liver tumors in newborn mice at higher doses (Wiseman *et al.*, 1987, Table 11), but not at lower total doses (Table 4, Table 12) (Miller *et al.*, 1983; Wiseman *et al.*, 1987). A related derivative, 1'-acetoxyelemicin, produced increased liver tumors in newborn mice given four intraperitoneal injections (Wiseman *et al.*, 1987, Table 11). Parsley apiol and dill apiol exhibited negative results in newborn mice given four intraperitoneal injections (Miller *et al.*, 1983, Table 4).

Phillips *et al.* (1984) compared the DNA adducts formed in the livers of newborn mice following administration of four intraperitoneal injections of estragole, safrole, anethole, eugenol, methyleugenol, elemicin, myristicin, dill apiol or parsley apiol using the same experimental protocol that was used by Miller *et al.* (1983) for the cancer bioassays. DNA binding correlated well with reported liver tumor incidences. The highest levels of binding were detected for methyleugenol (73 pmol/mg DNA), estragole (30), and safrole (17.5); and most adducts were still present 20 days following the last administration of the test compound. Significant adduct levels were observed for myristicin (7.8 pmol/mg DNA), elemicin (3.7) and lower levels for anethole (<1), dill apiol (1.4), and parsley apiol (<1). No binding was detected for eugenol.

Several investigators have tried to explain the relative carcinogenic potentials of the alkenylbenzenes with respect to their structure and metabolism. Miller *et al.* (1983) hypothesized that the reason dill apiol, parsley apiol, myristicin, elemicin, and eugenol are less potent or inactive relative to estragole, methyl eugenol, and safrole is due to stereochemical hindrance produced by substitution at positions 3, 4, or 5 of the benzene ring, which may alter metabolism. Tsai *et al.* (1994) conducted quantum mechanical calculations and reported that the carbonium ions (i.e., DNA reactive species) of estragole, methyleugenol, safrole, *cis*-asarone, *trans*-asarone, and elemicin are predicted to be considerably more stable than those of anethole, myristicin, allylbenzene and isosafrole, predictions which correlate well with observed genotoxicity and carcinogenicity findings. In the case of eugenol, the authors hypothesized, based on quantum chemical considerations, that the carbonium ion of eugenol may be rapidly stabilized by deprotonation to form a quinone methide, thus reducing its reactivity with DNA. An equally plausible explanation is that the phenolic group of eugenol is readily conjugated, and thus rapidly detoxified.

3.3.4 Pathology

The liver tumors observed in the carcinogenicity studies in rodents (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987) were reported as hepatomas (i.e., hepatocellular carcinomas) and were diagnosed by standard criteria. The hepatomas were

diagnosed as Type A, Type B, or mixed Type A and B based on the morphological criteria of Jones and Butler (1975). Type A hepatomas rarely metastasize; type B metastasize more frequently.

3.4 Mechanism

The mode of action for estragole carcinogenicity has been characterized, and proceeds through a genotoxic mechanism (Phillips, 1994). This mechanism involves the metabolism of estragole by liver cytochrome P450 to 1'-hydroxyestragole and several DNA-reactive epoxides. 1'-Hydroxyestragole is further conjugated with sulfate to form a sulfuric acid ester compound, 1'-sulfoöxyestragole (Figure 1) that readily binds to DNA and is responsible for most, if not all, of estragole's hepatocellular carcinogenicity (Wiseman *et al.* 1987).

The primary evidence for this mechanism of action comes from genotoxicity and carcinogenicity studies of estragole, and structurally-similar compounds, especially safrole. One primary observation is that 1'-hydroxyestragole and 1'-hydroxysafrole are more carcinogenic than their parent compounds, estragole and safrole (Drinkwater *et al.*, 1976; Miller *et al.*, 1983). The importance of sulfation in the mechanism of carcinogenesis has been demonstrated in a number of studies. Boberg *et al.* (1983) demonstrated that safrole-induced liver carcinogenicity in mice was inhibited by pretreatment with pentachlorophenol, a potent sulfotransferase inhibitor, and that brachymorphic mice (deficient in liver synthesis of the sulfotransferase co-factor, PAPS) did not develop liver tumors from exposure to safrole. In similar experiments, pretreatment with pentachlorophenol inhibited tumor induction in mice exposed to estragole (Wiseman *et al.*, 1987, Table 5) or 1'-hydroxysafrole (Boberg *et al.*, 1987). Similar involvement of sulfation has been demonstrated with the estragole derivative, 1'-hydroxy-2',3'-dehydroestragole (Fennell *et al.*, 1985). Randerath *et al.* (1984) studied DNA adduct formation by safrole in the livers of mice pretreated with pentachlorophenol. Significant reductions in DNA adduct formation were associated with pretreatment with moderate to high levels of pentachlorophenol.

Epoxides of estragole, namely estragole-2',3'-oxide and 1'-hydroxyestragole-2',3'-oxide, have also been shown to be hepatocarcinogenic (Miller *et al.*, 1983, Wiseman *et al.*, 1987), and to produce DNA adducts *in vitro* (Swanson *et al.*, 1981; Qato and Guenthner, 1995; Luo and Guenthner, 1996). These epoxides are likely to play only a minor role, if any, in mediating estragole's tumorigenicity, since DNA adducts associated with these epoxides have not been detected following *in vivo* administration of estragole (Luo and Guenthner, 1996), and removal by glutathione S-transferases and epoxide hydrolase enzymes is predicted to be rapid and efficient (Luo and Guenthner, 1994; 1996).

4 SUMMARY AND CONCLUSIONS

4.1 Summary of Evidence

No studies of the long-term health effects of human exposure to estragole were reported; however, several studies have demonstrated the carcinogenic effects of estragole in mice. Estragole or its metabolites administered to adult or newborn mice of different strains, through different routes of administration, produced malignant liver tumors. Administration of estragole to adult female CD-1 mice via the diet for 12 months induced increased incidences of hepatocellular carcinomas compared with control mice (Miller *et al.*, 1983). Administration of ten doses of estragole by oral intubation to newborn CD-1 mice produced increased incidences of liver tumors in males, but not females (Miller *et al.*, 1983). Estragole administered by multiple intraperitoneal or subcutaneous injections to newborn male CD-1 mice or by multiple intraperitoneal injections to male $B6C3F_1$ mice resulted in high incidences of hepatocellular carcinoma (Drinkwater *et al.*, 1976; Miller *et al.*, 1983). A single intraperitoneal dose of estragole administered to newborn male $B6C3F_1$ mice was also found to be sufficient to induce a high incidence of liver cancer (Wiseman *et al.*, 1987). 1'-Hydroxyestragole, the putative proximate toxic metabolite of estragole, also induced high incidences of liver tumors when administered by subcutaneous injection to newborn male CD-1 mice or via intraperitoneal injection to newborn male CD-1, $B6C3F_1$, CeH/HeJ, or C57Bl/6J mice (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987), or in the diet for 12 months to adult female CD-1 mice (Miller *et al.*, 1983). Other metabolites of estragole (i.e., estragole-2',3'-oxide and 1'-hydroxyestragole-2',3'-oxide) and synthetic derivatives (i.e., 1'-acetoxyestragole, 1'-hydroxy-2',3'-dehydroestragole, and 1'-acetoxy-2',3'-dehydroestragole) were also potent carcinogens in mice (Drinkwater *et al.*, 1976; Miller *et al.*, 1983; Wiseman *et al.*, 1987). The carcinogenicity of estragole has not been investigated in the rat, although one subcutaneous injection study of derivatives of estragole in male rats did not observe any treatment-related increases in tumors.

Estragole and its metabolites produced genotoxic effects in bacteria, yeast, and mammalian cells. Results of mutagenicity testing of estragole in *Salmonella typhimurium* were generally negative (Sekizawa and Shibamoto, 1982; Zeiger *et al.*, 1987), likely due to the complex metabolism required for bioactivation *in vivo*. Positive results were reported for estragole in strain TA1535 with the addition of the sulfation cofactor PAPS (To *et al.*, 1982). The putative toxic metabolites of estragole, namely 1'-hydroxyestragole and epoxides of estragole, were generally positive in mutagenicity assays with or without exogenous activation (Swanson *et al.*, 1979; Phillips *et al.*, 1981). Estragole produced mixed results in a DNA repair test (Rec assay), exhibiting dose-related DNA damage in *Bacillus subtilis* in one study (Zani *et al.*, 1991) and exhibiting negative results in *B. subtilis* and *E. coli* in another (Sekizawa and Shibamoto, 1982). Estragole and its metabolites induced UDS in several studies in human and rat cell lines (Francis *et al.*, 1981; Howes *et al.*, 1990; Chan and Caldwell, 1992) or *ex vivo* in the livers of rats treated orally with estragole (Müller *et al.*, 1994). Estragole or its metabolite, 1'-hydroxyestragole, administered to mice binds readily to DNA; several DNA adducts have

been characterized (Phillips, 1994). The level of binding and the adducts formed are equivalent to those produced by safrole, a structurally related carcinogen.

Strong supporting evidence of estragole's carcinogenic potential comes from comparisons to structurally similar compounds (e.g., safrole, methyleugenol) which produced liver tumors and tumors at other sites in rodents. There is an extensive body of evidence to show that safrole induces hepatocellular carcinomas in rats and mice and functions through a genotoxic mechanism identical to that of estragole (Miller *et al.*, 1982; Phillips, 1994). Methyleugenol also induced hepatocellular carcinomas in newborn mice (Miller *et al.*, 1983) and tumors at multiple sites in rats and mice in two-year feeding studies (NTP, 1998). Other compounds structurally similar to estragole or its metabolites, including *cis*-asarone, *trans*-asarone, 1'-hydroxy-2',3'-dehydroestragole, 1'-acetoxyestragole, 1'-hydroxy-2',3'-dehydrosafrole, 1'-acetoxysafrole, 1'-hydroxyelemicin, and 1'-acetoxyelemicin, produced increased incidences of liver tumors and tumors at other sites in rodents (reviewed in Section 3.3.3). The magnitude of DNA adduct formation in newborn mice following administration of estragole and structurally-related compounds correlated well with liver tumor incidences (Phillips *et al.*, 1984).

The mode of action for estragole carcinogenicity has been well characterized and proceeds through a genotoxic mechanism identical to that of safrole (Miller *et al.*, 1982; Phillips, 1994). Estragole is metabolized by the liver to 1'-hydroxyestragole and several epoxide species. 1'-Hydroxyestragole is further conjugated with sulfate to form a sulfuric acid ester compound that readily binds to DNA and is responsible for most, if not all, of estragole's hepatocellular carcinogenicity (Boberg *et al.*, 1983, 1987; Randerath *et al.*, 1984; Wiseman *et al.*, 1987). Metabolism of estragole through this metabolic pathway appears to be quantitatively consistent among humans and rodents (Anthony *et al.*, 1987; Sangster *et al.*, 1987) (Figure 3). These observations raise the public health concern regarding human exposure to estragole and closely related alkenylbenzenes.

4.2 Conclusion

There is convincing evidence from carcinogenicity studies that estragole induces cancer in mice. Estragole induced liver cancer in multiple strains and both sexes of mice exposed by several different routes of administration. Furthermore, although estragole has not been adequately tested in rats, two closely-related compounds, safrole and methyleugenol, both caused cancer in rats. Further evidence of estragole's carcinogenic potential includes observations of genotoxicity in several short-term tests, DNA adduct formation *in vivo* and *in vitro*, chemical-structural analogies with recognized carcinogens, and a relatively clear understanding of the carcinogenic mode of action.

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