CURRENT AWARENESS DOCUMENT

SAFROLE AND ITS ALKENYLBENZENE CONGENERS
SAFROLE, ESTRAGOLE, AND RELATED COMPOUNDS

CARCINOGENICITY AND STRUCTURE ACTIVITY
RELATIONSHIPS. OTHER BIOLOGICAL PROPERTIES.
METABOLISM. ENVIRONMENTAL SIGNIFICANCE.

Prepared by

Yin-Tak Woo, Ph.D., D.A.B.T.
David Y. Lai, Ph.D.

Science Applications Internation Corporation
8400 Westpark Drive
McLean, Virginia 22102

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EPA Project Officer and Scientific Editor
Joseph C. Arcos, D.Sc.

Extradivisional Scientific Editor
Mary F. Argus, Ph.D.

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5 3 2 4 Safrole, Estragole and Related Compounds

5 3 2 4 1 Introduction

Safrole (3,4-methylendioxy-1-allylbenzene), estragole (4-methoxy-1-allylbenzene) and related compounds constitute a relatively new class (alkenylbenzene congeners) of carcinogens of considerable environmental importance A variety of ring-substituted derivatives of allylbenzene* and propenylbenzene* are found in many edible plants and spices and were or are still used as components of pesticides, food additives and pharmaceutical preparations. The types of edible plants or spices which contain naturally occurring alkenylbenzene compounds include sassafras, tarragon, sweet basil, cloves, anise, nutmeg, sweet bay, parsley, parsnip, carrots, bananas, black pepper and processed tobacco (see Section 5 3 2 4 5) The tendency of modern cuisine to use spice flavors in the more concentrated form of oleoresins, extracts and essential oils (volatile oils obtained by steam distillation and/or solvent extraction) is of some concern because of the much higher concentration of alkenylbenzene compounds in these preparations Besides natural occurrence, there is also evidence that treatment of oranges with several abscission agents** (e g , cycloheximide, glyoxal diamine) can cause the appearance of six alkenylbenzene compounds (e g , eugenol, elemicin) in orange juices and essential oil (see Section 5 3 2 4 5) Cinnamyl compounds are closely related to alkenylbenzene compounds, they may arise from the metabolism of both allylbenzene and propenylbenzene compounds A number of cinnamyl compounds

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*In this section, the term "propenylbenzene" is used exclusively to indicate "1-propenylbenzene " The term "allylbenzene" is used to denote "2-propenylbenzene" in order to avoid confusion

**For explanation of the use of abscission agents, see Section 5 3 2 4 5

(e g , cinnamaldehyde, coniferaldehyde, cinnamyl cinnamate) occur naturally in edible plants and in wood Several synthetic cinnamyl compounds are used as food additives and fragrances

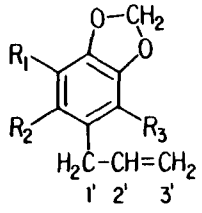
The carcinogenicity of safrole was first discovered in 1960-1961 when three independent studies by the U S Food and Drug Administration (1), Homburger et al (2) and Abbott et al (3) presented evidence of tumor induction or preneoplastic changes in the liver of rats receiving high doses of safrole in the diet The discovery immediately led to the banning of its use as a flavoring agent in soft drinks (e g , root beers) and as a food additive Since these initial reports, safrole has been established to be a relatively weak hepatocarcinogen in adult animals and a moderately active hepatocarcinogen in preweanling male mice Several closely related compounds (e g , dihydrosafrole, β-asarone) have also been found to be carcinogenic by the U S Food and Drug Administration (4-6) Since 1973, an extensive series of systematic studies on alkenylbenzene compounds has been carried out by the Millers and their associates at the University of Wisconsin Close to 50 metabolites, derivatives or analogs of safrole and estragole have been tested for carcinogenic and/or mutagenic activity These studies have not only identified several additional naturally occurring carcinogenic alkenylbenzene compounds, but have also led to a better understanding of the structure-activity relationships of these compounds and the role of metabolic activation to electrophilic intermediates in the mechanism of chemical carcinogenesis

5 3 2 4 2 Physicochemical Properties and Biological Effects

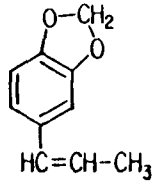
5 3 2 4 2 1 PHYSICAL AND CHEMICAL PROPERTIES

The physical and chemical properties of safrole and related naturally occurring and synthetic compounds (7-17) and cinnamyl compounds (18) have been

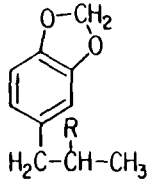
described in various publications The structural formulas of the alkenylbenzene and cinnamyl compounds tested for genotoxicity, and the physical properties of some of these compounds, are depicted and summarized in Tables LVII and LVIII, respectively Derivatives of propenylbenzene exist in two geometric isomeric forms Naturally occurring anethole and isosafrole are predominantly in the trans isomeric form, whereas asarone is mainly in the cis (β-) isomeric form Safrole and related naturally occurring alkenylbenzene compounds are colorless or slightly yellow, oily liquids with the characteristic odor of the plants from which they have been extracted They are practically insoluble in water, but are miscible with most organic solvents Safrole may be chemically oxidized to yield piperonyl acrolein (3,4-methylenedioxycinnamaldehyde) as the major product, 1'-oxosafrole as a minor product, and traces of piperonal (3,4-methylenedioxybenzaldehyde) Chemical oxidation of isosafrole yields piperonal as the major product, piperonyl acrolein as the minor product and traces of 1'-oxosafrole (20) Cinnamyl alcohol (3-phenyl-2-propen-1-ol) usually occurs in nature only in the form of esters, such as cinnamyl cinnamate Purified free cinnamyl alcohol forms colorless needles with an odor resembling hyacinths It is slightly soluble in water and very soluble in alcohol and ether Cinnamyl alcohol is oxidized by mild oxidizing agents to cinnamaldehyde and by strong oxidizing agents to cinnamic acid and/or benzaldehyde Cinnamaldehyde (3-phenyl-2-propenal) is a yellow liquid with cinnamon odor at room temperature It is slightly soluble in water, reacts typically as an aldehyde and may be easily oxidized to cinnamic acid, benzaldehyde and/or benzoic acid Cinnamic acid (3-phenyl-2-propenoic acid) occurs usually in trans form as pale yellow to off-white crystals, with a characteristic aromatic odor It undergoes reactions typical of a carboxyl group, an olefinic double bond, and the benzene nucleus (18)



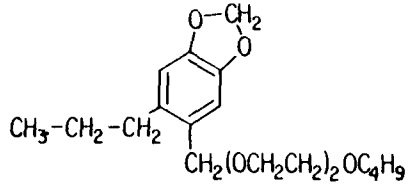
Safrole* (R_1=R_2=R_3=H-)
Myristicin* (R_1=CH_3O-, R_2=R_3=H-)
Dill apiol* (R_1=R_2=CH_3O-, R_3=H-)
Parsley apiol* (R_1=R_3=CH_3O-, R_2=H-)



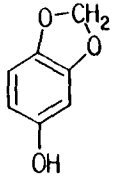
Isosafrole*



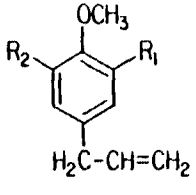
Dihydrosafrole (R=H-)
Piperonyl sulfoxide (R=n-C_8H_{17}-S(=O)-)



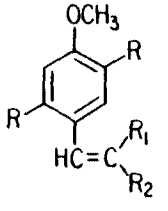
Piperonyl butoxide



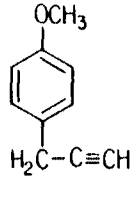
Sesamol*



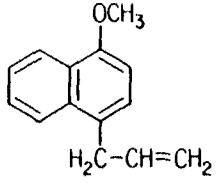
Estragole* (R_1=R_2=H-)
Methyleugenol* (R_1=CH_3O-, R_2=H-)
Elemicin* (R_1=R_2=CH_3O-)



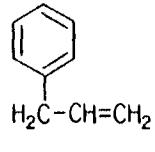
trans Anethole* (R=R_1=H-, R_2=CH_3-)
α-Asarone* (R=CH_3O-, R_1=H-, R_2=CH_3-)
β-Asarone* (R=CH_3O-, R_1=CH_3-, R_2=H-)



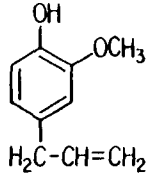
2',3'-Dehydroestragole



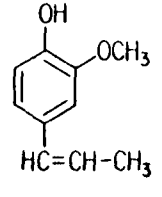
1-Allyl-4 methoxynaphthalene



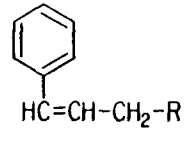
1-Allylbenzene



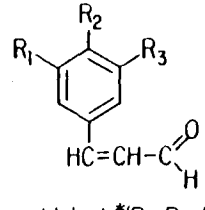
Eugenol*



Isoeugenol*



Cinnamyl alcohol (R=HO-)
Cinnamyl anthranilate (R=2 aminobenzoate)



Cinnamaldehyde* (R_1=R_2=R_3=H-)
Coniferaldehyde* (R_1=CH_3O-, R_2=HO-, R_3-H-)
Sinapaldehyde* (R_1=R_3=CH_3O-, R_2=HO-)
3,4,5-Trimethoxycinnamaldehyde (R_1=R_2-R_3=CH_3O-)

* *Naturally occurring compounds*

Table LVII

Structural Formulas of Safrole and Related Compounds

Table LVIII
Physico-Chemical Properties of Safrole and Related Alkenylbenzene Compounds

Compound	m.p	b p	Density	Refractive index	UV absorptivity λ_{max} (ϵ_M)	Reference
Safrole	11°C	232-234°C	d_4^{20} = 1 096	1 5383 (20°C)	236 (257), 285 (234)	(7)
Isosafrole	6 8°C	253°C	d_4^{20} = 1 122	1 5782 (20°C)	267 (716), 305 (329)	(7)
Dihydrosafrole		228°C	d_4^{20} = 1 0695	1 5187 (25°C)		(7)
Piperonyl butoxide		180°C (1 mm Hg)	d_4^{25} = 1 06	1 50 (20°C)		(19)
Estragole		216°C	d_4^{21} = 0 9645	1 5230 (17 5°C)		(11)
trans-Anethole	21 4°C	81°C (2 3 mm Hg)	d_4^{20} = 0 9883	1 5614 (20°C)	259 (22,300)	(11)
β-Asarone	62°C	296°C		1 5719 (11°C)		(11)
Eugenol	-9 2°C	255°C	d_4^{20} = 1 0664	1 5410 (20°C)		(11)
Cinnamyl alcohol	33°C	257°C	d_{35}^{35} = 1 0397	1 5758 (33°C)		(18)
Cinnamaldehyde	-7 5°C	252°C	d_{20}^{20} = 1 1102	1 6195 (20°C)		(18)
trans-Cinnamic acid	133°C	300°C	d_4^4 = 1 2475			(18)

The alkylating activity of a number of alkenylbenzene and cinnamyl compounds and their derivatives has been extensively studied in view of the well known role of alkylation in mutagenesis and carcinogenesis. Eder et al (21) found safrole, isosafrole, eugenol and trans-cinnamyl alcohol inactive as alkylating agent using 4-(p-nitrobenzyl)pyridine (NBP) as the nucleophilic acceptor Miller and Miller and their associates (12, 14, 15, 17, 22-25) have studied the in vitro reaction of various metabolites and derivatives of safrole and related compounds with nucleophiles, such as nucleosides and sulfhydryl compounds Using guanosine as the nucleophile, the Millers' group found that the relative electrophilicity of seven safrole metabolites and derivatives follows the order 1'-oxo- >> 1'-acetoxy- > 1'-acetoxy-2',3'-epoxy- > 1'-hydroxy-2',3'-epoxy- > 2',3'-epoxy- $\geq$ 1'-oxo-2',3'-epoxy- > (inactive) 1'-hydroxy- (see Table LIX) A similar ranking was observed for the four estragole derivatives tested Judging from the time course of reaction, the 1'-oxo- and 1'-acetoxy- derivatives are fast-acting with most of the reaction completed within a few hours, whereas the epoxide derivatives react slowly and steadily and may not reach the maximum level even after 96-97 hours Both the 1'-oxo- and 1'-acetoxy derivatives of safrole and estragole are highly unstable, the $t_{1/2}$ of 1'-acetoxysafrole and 1'-acetoxyestragole in aqueous solution is 1 8 min. (26). A comparative study using glutathione as the nucleophile indicates that 1'-oxosafrole is a "soft" electrophile while 1'-acetoxysafrole is a "hard" electrophile (23) 1'-Oxosafrole is expected to prefer reacting with "soft" nucleophiles (e g , GSH and other mercapto compounds) before reacting with "hard" nucleophile (e.g., oxygen and amino groups of purine and pyrimidine bases of DNA)

Table LIX
Electrophilic Reactivities of Derivatives of Safrole and Related Compounds with Guanosine[a]

Compound	Incubation time			
	2 hr	17 hr	21-24 hr	96-97 hr
1'-Oxosafrole	~55	~80	80	
1'-Acetoxysafrole	11 8, ~20	20	25	
1'-Acetoxysafrole-2',3'-oxide	2		15	44
1'-Hydroxysafrole-2',3'-oxide	2		10	20
Safrole-2',3'-oxide	~0 5		5 8	20
1'-Oxosafrole-2',3'-oxide	~0 5		4 8	13
1'-Hydroxysafrole	<0.1			
3'-Acetoxyisosafrole	0 2			
3'-Hydroxyisosafrole	<0 1			
1'-Oxoestragole	~16		~80	90
1'-Acetoxyestragole	~20	20	~25	25
1'-Hydroxyestragole-2',3'-oxide	<1			15, 27
Estragole-2',3'-oxide				20
1'-Acetoxy-1-allyl-4-methoxy-naphthalene		n.t [b]		
1'-Acetoxyallylbenzene		0.1		

[a] Summarized from the data of Borchert et al. (12), Drinkwater et al (14), Wislocki et al. (15), Miller et al. (22) and Phillips et al. (17) The numbers shown are the percentage of nucleosides (^{14}C-labeled) that have reacted with the compound specified.

[b] Not detected using guanosine However, in another comparative test using inosine as the nucleophile, 1'-acetoxy-1-allyl-4-methoxynaphthalene is a more active electrophilic reactant than 1'-acetoxysafrole and 1'-acetoxyestragole

5 3 2 4 2 2 BIOLOGICAL EFFECTS OTHER THAN CARCINOGENICITY

Toxicity The basic acute and subacute pharmacological and pathological effects of safrole and isosafrole in animals were first reported in 1894 and 1895 by Heffter (27, 28) Several cases of nonfatal human poisoning by safrole and oil of sassafras (which is 80% safrole) were recorded in the literature (29, 30) The early toxicity data on safrole and related compounds in humans and animals were reviewed by Jacob (31) and Leidy (32) in 1958 Beginning in the late 1950's and early 1960's, the U S Food and Drug Administration undertook an extensive series of toxicity studies (4, 33-36) on various food additives and fragrances which included many alkenylbenzene and cinnamyl compounds The acute oral LD_{50} data of some of these compounds are summarized in Table LX There appears to be few clearly discernible structure-toxicity relationships among these compounds One consistent observation is that the saturated propylbenzene congeners (e g , dihydrosafrole, dihydroanethole, propylbenzene) are less toxic than the corresponding unsaturated allylbenzene (e g , safrole, estragole, allylbenzene) and propenylbenzene (e g , isosafrole, anethole, propenylbenzene) compounds (35) For propenylbenzene congeners (which exist in two geometric isomeric forms), there is some evidence for a substantial positional effect on the acute toxicity of the compounds The cis-isomer of anethole, for example, is about 10-30 times more toxic than the trans-isomer when administered intraperitoneally to rodents (39, see also 11, 40) The most common acute toxic sign of the alkenylbenzene compounds is CNS depression (35) A number of alkenylbenzene compounds appears to be capable of producing psychoactive effects (41, 42) and it is believed that myristicin and/or elemicin may be responsible for the hallucinogenic effect of nutmeg The liver is the most affected organ in subacute and chronic studies, the pathological changes observed include hepatic cell enlar-

Table LX

Acute Toxicity of Safrole and Related Compounds by Oral Administration

Compound	Species	LD_{50} (mg/kg)	Reference
Safrole	Mouse	2,350	(35, 36)
	Rat	1,950	(35, 36)
Isosafrole	Mouse	2,470	(36)
	Rat	1,340	(36)
Dihydrosafrole	Mouse	3,700	(36)
	Rat	2,260	(36)
Piperonyl butoxide	Mouse	8,300	(37)
	Rat	11,500	(37)
Estragole	Mouse	1,250	(35)
	Rat	1,820	(35)
Anethole	Mouse	3,050	(35)
	Rat	2,160	(35)
	Guinea pig	2,090	(35)
Calamus oil (β-asarone)	Rat	777	(35)
Eugenol	Mouse	3,000	(35, 36)
	Rat	2,680	(35, 36)
	Guinea pig	2,130	(35, 36)
Cinnamaldehyde	Rat	2,220	(35)
	Guinea pig	1,160	(35)
Cinnamyl anthranilate	Rat	>5,000	(38)

gement and various degrees of focal fatty metamorphosis, bile duct proliferation and architectural irregulatory, and for some of the compounds, focal necrosis and fibrosis, hyperplasia and neoplasia (4, 36)

Effects on microsomal mixed-function oxidases Safrole, isosafrole, dihydrosafrole, piperonyl butoxide and several other methylenedioxyphenyl (MDP) compounds have a dual effect on microsomal mixed-function oxidases The immediate effect is interaction with cytochrome P-450 resulting in inhibition of mixed function oxidases, whereas the delayed effect is the induction of new synthesis of cytochrome P-448 or P-450 leading to an increase in mixed function oxidase activity Owing to its pharmacological and toxicological significance, this subject has been extensively studied. Readers are referred to reviews by Hodgson and Philpot (43) and Ioannides et al (44) for details The acute inhibition of mixed function oxidases was the basis for the development of MDP compounds (e g , piperonyl butoxide, piperonyl sulfoxide) as synergists for insecticides (45, 46) The mechanism of inhibition is believed to involve metabolic activation of MDP compounds to reactive intermediate(s) which form a ligand complex with the heme group or bind covalently to the protein moiety of cytochrome P-450 Various reactive intermediates (e g , benzodioxolium ion, free radical, carbanion, carbene) have been postulated, at present, the carbene intermediate (see Section 5 3 2 4 4 2) is the most widely accepted Paradoxically, the formation of metabolite-cytochrome P-450 complex (most likely carbene ligand complex) appears to be the initiating event for trigerring the induction of new cytochrome P-450 synthesis (47) Induction of mixed-function oxidases by MDP compounds may occur in the liver, as well as in extrahepatic tissues such as intestines and kidney The types of cytochrome P-450 induced are similar to those induced by phenobarbital and 3-methylcholanthrene, there is also evidence for the induction of a novel type of

cytochrome P-450 by isosafrole (48-50) A recent structure-activity relationship study by Cook and Hodgson (51) showed that the methylene carbon (of the methylenedioxy group) plays an important role in cytochrome P-450 induction, substitution of one or both methylene hydrogen with methyl group(s) completely abolishes metabolite-cytochrome P-450 complex formation and subsequent cytochrome P-450 induction

Mutagenicity The *mutagenicity of safrole and related compounds has been* studied in a variety of test organisms ranging from phages, bacteria, and yeasts, through higher plants and drosophila, to mammalian cells Safrole was one of the compounds selected by an International Collaborative Program for testing in over 20 different short-term tests for carcinogenicity, the results of these studies have been published in a recent monograph (52) The following discussion focuses only on mutagenicity studies using the Ames Salmonella test

Close to 50 *derivatives and structural analogs of safrole and estragole* have been assayed in the Ames Salmonella test The results of these studies are summarized in Table LXI About half of these compounds were shown to be mutagenic in one or more studies, however, for some of these compounds, conflicting results were reported by different investigators In some cases, the discrepancy may be due to the use of different methods (plate incorporation vs liquid suspension) or criteria for considering positive results Virtually all the mutagenic compounds are base-pair substitution mutagens (i e., positive in tester strains TA100, TA1530 or TA1535), with a few compounds (1'-acetoxysafrole, 1'-oxosafrole-2,'3,-oxide, 1'-acetoxyestragole and 1'-acetoxy-1-allyl-4-methoxynaphthalene) displaying weak frameshift activity (14, 16) As the data in Table LXI indicate, compounds with saturated side chain (e g , 2',3'-dihydro-2',3'-dihydroxysafrole, dihydrosafrole, piperonyl

Table LXI
Mutagenicity of Safrole and Related Alkenylbenzene Compounds in the Ames Test

Compound[a]	Without activation		With activation		Carcinogenicity
	Plate incorporation	Liquid suspension[b]	Plate incorporation	Liquid suspension[b]	
Safrole	- (16, 53-59)	- (21, 56, 60)	- (16, 53, 55-59)	- (16, 21, 59, 60) + (56, 61)	+
1'-Hydroxysafrole	- (14, 53, 55, 57) + (16)	- (60)	- (14, 53, 55, 57) + (16)	- (60)	+
1'-Acetoxysafrole	- (57) + (14, 53, 55)	+ (60)	- (55, 57)		+
1'-Oxosafrole	- (55)		- (55)		-, ±
Safrole-2',3'-oxide	+ (16, 55, 56)		+ (16, 55)[c]		-[e]
1'-Hydroxysafrole-2',3'-oxide	+ (16, 55, 56)		+ (16, 55)[c]		+
1'-Acetoxysafrole-2',3'-oxide	+ (16, 55)		+ (16, 55)[c]		-
1'-Oxosafrole-2',3'-oxide	+ (16, 55)		+ (16, 55)[c]		n t
Safrole metabolite I[d]	- (56)		- (56)	- (56)	n t
Safrole metabolite II[d]	- (56)		- (56)	+ (56)	n t
Safrole metabolite III[d]	- (56)		- (56)	- (56)	n t
2',3'-Dihydrodihydroxysafrole	- (54)				n t

Table LXI (continued)

Compound[a]	Without activation		With activation		Carcinogenicity
	Plate incorporation	Liquid suspension[b]	Plate incorporation	Liquid suspension[b]	
Myristicin	- (62)		- (62)		-
Isosafrole	- (54, 55, 59)	- (21)	- (55)	- (21, 59)	-
3'-Hydroxyisosafrole	- (55)		- (55)		-
3'-Acetoxyisosafrole	- (55)		- (55)		±
Dihydrosafrole	- (55)		- (55)		+
Piperonyl sulfoxide		- (63)		- (63)	+
Piperonyl butoxide		- (63, 64)		- (63, 64)	-
Estragole	- (54, 58, 59) w+ (16)		- (58) w+ (16)		+
1'-Hydroxyestragole	- (14) + (16)		- (14) + (16)		+
1'-Acetoxyestragole	+ (14)				n t
Estragole-2',3'-oxide	+ (16, 54)		+ (16)[c]		-[e]
1'-Hydroxyestragole-2',3'-oxide	+ (16)		+ (16)[c]		-[e]
Methyleugenol	- (54, 59)	- (65)		- (59, 65)	+
Methyleugenol-2',3'-oxide	+ (54)				n t

Table LXI (continued)

Compound[a]	Without activation		With activation		Carcinogenicity
	Plate incorporation	Liquid suspension[b]	Plate incorporation	Liquid suspension[b]	
trans-Anethole	- (58, 59) w+ (16)		- (58) w+ (16)	w+ (59, 66)	-
3'-Hydroxy-trans-anethole	- (16)		+ (16)		-
α-Asarone	- (66)		- (66)		n t
β-Asarone	- (66, 67)		- (66) + (67)		+
Eugenol	- (16, 54, 56, 58, 59)	- (21, 64, 68)	- (16, 56, 58)	- (21, 56, 69, 64, 68)	-
Eugenol-2',3'-oxide	+ (16, 54)		+ (16)[c]		±, -[e]
Eugenol metabolite II[d]	- (56)		- (56)	- (56)	n t
Eugenol metabolite III[d]	- (56)		- (56)	- (56)	n t
Isoeugenol	- (59, 66)		- (66)	- (59)	n.t
Allylbenzene	- (54)	- (21)		- (21)	n t
Allylbenzene-2',3'-oxide	+ (54)				n t.
1'-Acetoxyallylbenzene	w+ (14)				n t
1'-Acetoxy-1-allyl-4-methoxynaphthalene	+ (14)				n t
Cinnamyl alcohol		- (21, 69)		- (21, 69)	n t

Table LXI (continued)

Compound[a]	Without activation		With activation		Carcinogenicity
	Plate incorporation	Liquid suspension[b]	Plate incorporation	Liquid suspension[b]	
Coniferyl alcohol	- (70)		- (70)		n t
Cinnamaldehyde		- (21, 69, 71, 72) + (64)		- (21, 69, 71, 72) + (64)[c]	-
α-Chlorocinnamaldehyde		+ (71)		+ (71)[c]	n.t
α-Bromocinnamaldehyde		+ (71)		+ (71)[c]	n t.
α-Methylcinnamaldehyde		- (71)		- (71)	
Cinnamic acid	- (70)		- (70)		n t
Cinnamyl anthranilate			- (73)	- (73)	-
Deoxypodophyllotoxin	- (70)		- (70)		n t

[a] See Table LVII for structural formulas, w+ = weakly active

[b] Modified Ames test Compounds are preincubated for 20 minutes with the bacteria in liquid media before plating on agar plate.

[c] Mutagenicity of the compound is reduced in the presence of metabolic activation system

[d] The chemical names for these compounds are "Safrole metabolite I," 3-N,N-dimethylamino-1-(3',4'-methylenedioxyphenyl)-1-propanone, "Safrole metabolite II," 3-piperidyl-1-(3',4'-methylenedioxyphenyl)-1-propanone, "Safrole metabolite III," 3-pyrrolidinyl-1-(3',4'-methylenedioxyphenyl)-1-propanone, "Eugenol metabolite II," 3-piperidyl-1-(3'-methoxy-4'-hydroxyphenyl)-1-propanone, "Eugenol metabolite III," 3-pyrrolidinyl-1-(3'-methoxy-4'-hydroxyphenyl)-1-propanone

[e] Active as tumor initiator

butoxide, piperonyl sulfoxide) are all inactive Most alkenylbenzene congeners are inactive as such with some of them weakly mutagenic after metabolic activation In contrast, all epoxides of the alkenylbenzene congeners tested are moderately active direct-acting mutagens The relative mutagenic potency of 2',3'-epoxides follows the order safrole-2',3'-oxide > estragole-2',3'-oxide > eugenol-2',3'-oxide > methyleugenol-2',3'-oxide = allylbenzene-2',3'-oxide (54, 55) Among the 1'-oxidized derivatives of allylbenzene congeners, only the 1'-acetoxy derivatives display direct-acting mutagenic activity, their relative mutagenic potency follows the order 1'-acetoxy-1-allyl-4-methoxynaphthalene $\geq$ 1'-acetoxyestragole $\geq$ 1'-acetoxysafrole >> 1'-acetoxyallylbenzene (14) 3'-Acetoxyisosafrole, an isomer of 1'-acetoxysafrole, is not mutagenic 1'-Hydroxy derivatives of allylbenzene congeners are either inactive or weakly mutagenic, but may be further activated by S-9 mix The only 1'-oxo derivative (1'-oxosafrole) tested is nonmutagenic A number of 1'-oxidized derivatives of safrole-2',3'-oxide and estragole-2',3'-oxide are also direct-acting mutagens, but their potency is substantially lower than that of unoxidized 2',3'-oxides (16, 55) A comparison of the direct-acting mutagenic activity of epoxides and 1'-oxidized derivatives of safrole, estragole and related compounds with their electrophilic reactivity toward guanosine or inosine (see Table LIX) indicates a very good correlation, with the notable exception of 1'-oxosafrole (which is highly reactive but nonmutagenic) As discussed in Section 5 3 2 4 2 1, 1'-oxosafrole is a very short-lived "soft" electrophile It is probably degraded or reacts with noncritical cellular nucleophiles before it can reach DNA

Among the various individual compounds, safrole has been consistently shown to be nonmutagenic in the plate incorporation assay with or without metabolic activation In modified Ames test, in which the chemical is pre-

incubated in liquid suspension for 20 minutes before plating, two separate studies (56, 61) showed positive results, however, these findings were not confirmed by other investigators (see Table LXI) 3-Piperidyl-1-(3',4'-methylenedioxyphenyl)-1-propanone ("safrole metabolite II") was also shown to be positive in the liquid suspension assay system, but negative in the plate incorporation system (56), no confirmatory data are available Estragole and trans-anethole are the only two alkenylbenzene compounds that were reported to be mutagenic without activation in one study (16), however, the weak direct-acting activity was not detected in several other studies (54, 58, 59) With metabolic activation, trans-anethole is weakly or marginally active, a clear-cut dose-response relationship was demonstrated using a liquid suspension assay (59) β-Asarone was shown to be inactive in one study (66) but clearly mutagenic in TA100 after metabolic activation with S-9 in another study (67) In the latter study, three commercial calamus-containing drugs, known to contain β-asarone, were also mutagenic in TA100 after activation

Among cinnamyl compounds tested, cinnamaldehyde is the only compound which displays potential mutagenic activity Cinnamaldehyde has been shown to be a direct-acting mutagen in TA100 in one study (64), however, this activity was not observed in several earlier studies (21, 69, 71, 72) Whereas the mutagenicity of cinnamaldehyde may be debatable, its α-bromo- and α-chloro-derivarives are potent direct-acting mutagens for TA100 (71) The greatly enhanced mutagenicity is attributed to an increase in the electrophilicity of the carbon at the β-position to the aldehyde function, as a result of substitution with electron-withdrawing (-I) halogen at the α-carbon As may be expected, α-substitution with an electron-donating (+I) methyl group yields a nonmutagenic compound (71)

A comparison of the mutagenicity of safrole and related compounds with their carcinogenicity shows a notable number of discrepancies in the correlation (see Table LXI) Several carcinogenic compounds (e g , safrole, dihydrosafrole, piperonyl sulfoxide, methyleugenol, β-asarone) are either inactive or not consistently mutagenic On the other hand, some compounds which are clearly mutagenic, such as safrole-2',3'-oxide, 1'-acetoxysafrole-2',3'-oxide, estragole-2',3'-oxide, 1'-hydroxyestragole-2',3'-oxide and eugenol-2',3'-oxide are either not carcinogenic or active only as tumor-initiators The lack of correlation may be due, at least in part, to the requirement of sulfatation in the metabolic activation of allylbenzene congeners to ultimate carcinogens (see Section 5 3 2 4 4 1) The requirement of cytosol for the metabolic activation of α,β-unsaturated carbonyl compounds and their corresponding allylic alcohols to mutagens (69) may also be a factor in explaining the inconsistency between the results yielded by the plate incorporation and the liquid suspension assay systems for some of the compounds The existence of mutagenic noncarcinogens may be related to the inability of reactive epoxides to reach target macromolecules Alternatively, these results may suggest that somatic cell mutation alone is insufficient to bring to completion the process of carcinogenesis

Teratogenicity Very little information is available on the teratogenicity of safrole and related compounds Technical grade piperonyl butoxide has no significant teratogenic effects in rats given daily oral doses of up to 500 mg/kg body weight of the compound from day 6 to 15 of gestation (74, 75) In a three-generation reproduction study, the progeny of rats fed diets containing 100 or 1,000 mg/kg body weight technical grade piperonyl butoxide showed no adverse effects However, higher doses of 10,000 and 25,000 mg/kg caused marked reduction in pregnancies and complete infertility, respectively

Several cinnamyl compounds used as food or fragrance additives, have been tested in chick embryos as a screening test for teratogenicity Verrett et al. (76) found both cinnamyl anthranilate and α-methylcinnamaldehyde to be not teratogenic in developing chick embryos at doses of up to 10 mg/egg A related compound, methyl anthranilate, was teratogenic, causing skeletal anomalies which included micromelia and phocomelia Abramovici and Rachmuth-Roizman (77) tested a variety of α,β-unsaturated aldehydes and alcohols, including cinnamaldehyde and cinnamyl alcohol, in chick embryos. Both cinnamyl compounds were teratogenic The optimal teratogenic dose was 0 5 μmol/embryo for cinnamaldehyde and 5 μmol/embryo for cinnamyl alcohol, 58 2% and 23 1% of the embryos had malformations (mainly limbs and skeleton) at the respective dose In the same study, a number of other α,β-unsaturated aldehydes (e g , citral, farnesal, benzaldehyde) are also teratogenic, whereas other unsaturated alcohols and saturated aldehydes are either considerably less active or inactive The authors (77) suggested that α,β-unsaturated aldehydes, particularly those with shorter linear chains, are potential teratogens Interaction between the liposoluble unsaturated aldehydes and some key lipid constituents of the embryonic cell membrane was postulated to be a possible mechanism of the teratogenic action of these compounds

5 3 2 4.3 Carcinogenicity and Structure-Activity Relationships

5 3 2 4 3.1 OVERVIEW

Since the first report in 1961 on the carcinogenicity of safrole in rats, close to 50 derivatives and related compounds have been tested for carcinogenic activity These compounds include metabolites, synthetic derivatives and structural analogs of safrole, estragole and eugenol The major findings of these studies are summarized in Table LXII, the vast majority of these

Table LXII
Summary of Comparative Carcinogenesis Bioassay Data on Safrole, Related Compounds, and Their Metabolites[a]

Compound	Oral		i p	s c.		Skin tumor initiation	Pulmonary adenoma	References
	Rat	Mouse	Mouse	Rat	Mouse			
Safrole	+ (L)	+ (L)	+ (L)	-	+ (L)	-	-	(13, 55, 78, 79)
1'-Hydroxysafrole	+ (L,F)	+ (L,I)	+ (L)	+ (L,S)	+ (L)	-	-	(13, 14, 55, 78, 79)
1'-Acetoxysafrole	+ (F)	-[b]		+ (S)	+ (L)	-		(13, 55, 79)
1'-Oxosafrole	-			± (S)	-	-		(55)
1'-Methoxysafrole						-		(13)
Safrole-2',3'-oxide			-	-		-, +	-	(55, 79)
1'-Hydroxysafrole-2',3'-oxide			+ (L)	+ (S)		+	-, +	(55, 79)
1'-Acetoxysafrole-2',3'-oxide			-			-		(55, 79)
Myristicin			-					(79)
Dill apiol			-					(79)
Parsley apiol			-					(79)
Isosafrole	+ (L)[c]	± (L)[c]		-				(13)
3'-Hydroxyisosafrole				-				(13)
3'-Acetoxyisosafrole				± (L)				(13)
3'-Methoxyisosafrole				-				(13)
3'-Bromoisisafrole				+ (S)				(13)
Dihydrosafrole	+ (E)[c]	+ (F)[c]				-		(13)
1'-Hydroxydihydrosafrole						-		(13)
1'-Acetoxydihydrosafrole						-		(13)
1'-Methoxydihydrosafrole						-		(13)

Table LXII (continued)

Compound	Oral		i p	s c		Skin tumor initiation	Pulmonary adenoma	References
	Rat	Mouse	Mouse	Rat	Mouse			
Estragole		+ (L)	+ (L)		+ (L)		-	(14, 79)
1'-Hydroxyestragole		+ (L)	+ (L)	± (S)	+ (L)		-	(14, 79)
Estragole-2',3'-oxide			-	-		+	-	(79)
1'-Hydroxyestragole-2',3'-oxide				-		+	+	(79)
1'-Hydroxy-2',3'-dehydroestragole				+ (L)				(79)
Methyleugenol			+ (L)					(79)
1'-Hydroxymetheugenol			+ (L)					(79)
Elemicin			-					(79)
1'-Hydroxyelemicin			-					(79)
trans-Anethole		-	-				-	(79)
3'-Hydroxy-trans-anethole			-				-	(79)
Eugenol		-	-					(79)
Eugenol-2',3'-oxide			-	-		+		(79)
1'-Hydroxyallylbenzene			-					(79)
1'-Hydroxy-1-allyl-4-methoxynaphthalene			+ (L)					(79)

[a] Except where otherwise noted, most of these studies were carried out in the laboratory of J A and E C Miller under standardized bioassay conditions Abbreviations for target organs· L, liver, F, forestomach, I, interscapular s c tissue, S, local sarcoma, E, esophagus See Table LVII for structural formulas of most of the compounds

[b] Inconclusive due to high mortality in one study and low dose in another study

[c] U S National Cancer Institute (NCI) or Food and Drug Administration (FDA) data, see Table LXIII

studies were carried out in the laboratory of J A and E C Miller under standarized bioassay conditions thus permitting direct comparison of relative potencies. These data, along with those discussed in Sections 5 3 2 4 3 2, 5 3 2 4 3 3 and 5 3 2 4 3 4, suggest the following structure-activity relationships

Ring substitution with methoxy groups (the methylenedioxy group in safrole may be considered as two methoxy groups) is an essential feature of carcinogenic alkenylbenzene congeners The optimal number of methoxy substitutions is two (e.g , safrole, methyleugenol), one of which being in the p-position relative to the alkenyl side chain A methoxy group in the p-position may contribute to carcinogenicity by stabilizing (through resonance) the electrophilic intermediate generated during metabolic activation of the alkenyl side chain Extensive ring substitutions (e g , as in myristicin, dill apiol, parsley apiol, elemicin) yield inactive compounds possibly because of steric hindrance Annelation to an additional aromatic ring, however, appears to give rise to a potential carcinogen, 1-allyl-4-methoxynaphthalene, as indicated by the carcinogenicity of its 1'-hydroxy derivative (see Table LXII) and the potent mutagenicity of its 1'-acetoxy derivative (see Table LXI) Apparently, instead of creating steric hindrance, the introduction of an additional aromatic ring provides a planar bicyclic compound with a more favorable molecular geometry possibly for intercalation into DNA O-Demethylation of the p-methoxy group yields inactive compounds (e g., eugenol) probably because of less favorable resonance stabilization as well as easier excretion of phenolic compounds.

Modification of the alkenyl side chain can have a significant effect on the carcinogencity of the alkenylbenzene congeners Analysis of the available data suggests the following structure-activity relationships

a) Allylbenzene congeners (e g , safrole, estragole) are generally more carcinogenic than their propenylbenzene (β-methylvinylbenzene) isomers (e g , isosafrole, trans-anethole) The lower carcinogenic activity of propenylbenzene congeners may be related to their greater tendency to be oxidized to cinnamic metabolites (see Section 5.3 2 4 4), which have lower genotoxic potential

b) With the exception of 1'-oxosafrole, 1'-oxidized derivatives of allylbenzene congeners are more genotoxic than their parent compounds The 1'-hydroxy derivatives of safrole, estragole, methyleugenol (and possibly, 1-allyl-4-methoxynaphthalene) are all more potent as carcinogens, and they show evidence for direct-acting carcinogenicity 1'-Acetoxysafrole is a potent direct-acting carcinogen, whereas the 1'-acetoxy derivatives of a variety of allylbenzene congeners are all direct-acting mutagens in the Ames test (see Table LXI) These are findings which form the basis for the Millers' conclusion (e g , 79) that 1'-hydroxylation followed by esterification (with sulfate) represents the major metabolic activation pathway of safrole and related compounds The lack of genotoxicity of 1'-oxosafrole may be attributed to its high instability and reactivity, making it unlikely to reach target DNA molecules in a significant amount before being degraded or reacting with noncritical cellular nucleophiles

c) In contrast to 1'-oxidized derivatives of allylbenzene congeners, 3'-oxidized derivatives (both hydroxy and acetoxy) of propenylbenzene congeners (e.g , isosafrole, trans-anethole) show questionable or no carcinogenic activity As mentioned in (a),

further oxidation to cinnamic metabolites may be a factor in limiting the carcinogenic potential of these derivatives The only carcinogenic 3'-substituted derivative is 3'-bromoisosafrole, which is expected to be a direct-acting alkylating agent because of the good leaving tendency of bromine group

d) The 2',3'-epoxide derivatives of a variety of allylbenzene congeners are all direct-acting mutagens (see Table LXI) However, they (e g , safrole-2',3'-oxide, estragole-2',3'-oxide, eugenol-2'3,'-oxide) are inactive as "complete" carcinogens and are active only as tumor initiators Apparently these 2',3'-epoxy derivatives lack tumorigenesis promoting activity 2',3'-Epoxidation alone appears to be insufficient as a metabolic pathway to generate electrophilic intermediates which have "complete" carcinogenic activity

e) Saturation of the 2',3'-double bond of the allyl side chain does not necessarily abolish the carcinogenicity of safrole In fact, dihydrosafrole and piperonyl sulfoxide are carcinogenic Piperonyl butoxide, another saturated derivative of safrole (but with more extensive ring substitution), is not carcinogenic.

f) Further unsaturation of the 2',3'-double bond of the allyl side chain may have a potential to enhance the carcinogenicity of the compound Indeed, 1'-hydroxy-2',3'-dehydroestragole is a potent hepatic carcinogen, with a potency higher than any of the safrole and estragole derivatives tested (see Section 5 3 2 4 3 4)

5 3 2 4 3 2 CARCINOGENICITY OF SAFROLE AND RELATED COMPOUNDS

The carcinogenicity of safrole was discovered in 1960-1961 when three separate chronic studies by the U.S Food and Drug Administration (1), Homburger et al (2), and Abbott et al (3) all showed the induction of hepatomas and preneoplastic changes in rats administered high concentrations (0 5-1%) of safrole in the diet The discovery immediately led to the banning of its use as food additive Since the initial reports, more than ten additional studies were reported confirming the carcinogenicity of safrole in various strains of rats and mice (see Table LXIII) The compound is only weakly hepatocarcinogenic when fed to adult rats or mice, but is a moderately strong carcinogen when administered to preweanling male mice. Only liver adenomas were found in male Carworth Farms CFN rats fed 1% (10,000 ppm) safrole in the diet for 1 year, starting at the age of 6-10 weeks No tumors were detected in rats given a dietary level of 0 1% safrole (2). In groups of 25 male and 25 female 3-week-old weanling Osborne-Mendel rats maintained on diets containing 0, 100, 500, 1,000 and 5,000 ppm safrole for 2 years, the respective liver adenoma incidences were 3/50, 1/50, 2/50, 8/50 and 19/50 Fourteen of the rats in the 5,000 ppm group, two in the 500-ppm group and two in the control group bore malignant liver tumors The increase in tumor incidence was significant only in the highest dose group (34) Of 18 adult Charles River CD random-bred rats (average initial weight, 252 g) fed 5,000 ppm safrole for 22 months, only 3 developed hepatic carcinomas, no such tumors were found in controls (55). No significant carcinogenic effects were noted in adult CD rats 18 months after receiving 20 twice-weekly s c. injections of 18 6 μmole (3 mg) safrole (13) In adult CD-1 mice, dietary administration of 0 4-0 5% safrole for 12-13 months induced liver tumors in only 12-25% of male mice at 16-17 months after the commencement of treatment. Adult female CD-1

Table LXIII
Carcinogenicity of Safrole and Related Compounds[a]

Compound[b]	Species and strain	Route	Principal organs affected	References
Safrole	Mouse, CD-1	oral, i p or s c	Liver	(13, 55, 78, 79)
		topical[c]	None	(55)
	Mouse, BALB/c, $B6C3F_1$ or $B6AKF_1$	oral	Liver	(80-84)
	Mouse, $B6C3F_1$	oral, transplacental and/or lactational	Liver	(85)
	Mouse, A/He or A/J	i p	None[d]	(79, 86)
	Mouse, ICR/Ha	s c	Liver, lung	(87)
	Rat, Osborne-Mendel, CFN or CD	oral	Liver	(1, 2, 13, 34, 36, 55)
	Rat, CD	s c	None	(13)
	Dog, --	oral	Skin, tongue	(Cited in 81)
Myristicin	Mouse, $B6C3F_1$	i p	None	(79)
Dill apiol	Mouse, $B6C3F_1$	i p	None	(79)
Parsley apiol	Mouse, $B6C3F_1$	i p	None	(79)
Isosafrole	Mouse, $B6C3F_1$	oral	Liver (marginal)	(80, 81)
	Mouse, $B6AKF_1$	oral	None	(80, 81)
	Rat, Osborne-Mendel	oral	Liver	(4, 36)
	Rat, CD	s c	None	(13)
Dihydrosafrole	Mouse, $B6C3F_1$ or $B6AKF_1$	oral	Forestomach, liver	(80, 81)
	Mouse, CD-1	topical[c]	None	(13)
	Rat, Osborne-Mendel	oral	Esophagus	(4, 33, 36)

Table LXIII (continued)

Compound[b]	Species and strain	Route	Principal organs affected	References
Piperonyl sulfoxide	Mouse, $B6C3F_1$ or $B6AKF_1$	oral	None	(80)
	Mouse, $B6C3F_1$	oral	Liver	(88)
	Rat, F344	oral	None	(88)
Piperonyl butoxide	Mouse, $B6C3F_1$ or $B6AKF_1$	oral	None	(80)
	Mouse, $B6C3F_1$	oral	None	(89)
	Mouse, ICR/Ha	s c	None	(90)
	Rat, F344	oral	None	(89)
Sesamol	Rat, --	oral	Various organs (mostly benign)	(91)

[a] See also Tables LXII and LXV for carcinogenesis studies of metabolites of safrole

[b] See Table LVII for structural formulas

[c] Skin painting with promotion by croton oil

[d] Limited (24-week) bioassay only, pulmonary adenoma assay Negative results are considered inconclusive evidence of noncarcinogenicity

[e] Categorized as compounds requiring additional testing due to increased, but not significant, incidences of tumors

mice appeared to be more susceptible with 46-70% bearing hepatomas after feeding of 0.13-0.5% safrole (13, 55, 78) Similar sex difference has also been observed in adult $B6C3F_1$ mice given 180 twice-weekly intragastric administration of 120 mg safrole/kg body weight, the liver tumor incidences were 11% for males and 61% for females (85). Repeated topical treatment of adult CD-1 mice with safrole (5 x 3 μmol/wk for 6 wk) produced no significant carcinogenic effects after 30 additional weeks of promotion with croton oil (13)

In contrast to adults, male infant or preweanling mice are highly susceptible to the hepatocarcinogenic effect of safrole A high incidence (50-58%) of liver tumors was observed in male Swiss albino ICR/Ha mice one year after receiving four s c injections of safrole, totaling only 0 66 or 6 6 mg, on days 1, 7, 14 and 21 after birth A slight increase in the incidence of pulmonary tumors was also noted (87) Similar treatment of infant or preweanling CD-1 mice (total dose 9 45 μmole or 1 5 mg) led to a significant increase in the incidence of liver tumors in the males (14/35 treated vs 3/36 controls), however, no liver tumors developed in the 27 treated females after 16 months (13) Besides subcutaneous administration, intraperitoneal injections (total dose 9 45 μmole) and oral administration (via stomach tube, 10 twice-weekly doses of 2 5 μmole/g body weight starting at day 4 after birth) of low doses of safrole are equally effective in inducing liver tumors (61-67% treated vs 24-26% controls) in male CD-1 mice (79).

In other studies, $B6C3F_1$ and $B6AKF_1$ mice were given 464 mg/kg body weight safrole by gavage from days 7-28 after birth and subsequently 1,112 ppm in the diet for up to 82 weeks Liver tumors occurred in 11/17 male and 16/16 female mice of the first strain, 3/17 male and 16/17 female mice of the second strain, these incidences were significantly higher than those of the controls when the data in both sexes were combined (80, 81) Lipsky et al (82-84)

described the sequential histopathological and biochemical changes during safrole-induced carcinogenesis in BALB/c mice Vesselinovitch et al (85) studied transplacental and lactational carcinogenesis by safrole in $B6C3F_1$ mice Pregnant mice, nursing mothers or offspring were given oral doses of 120 mg safrole/kg body weight via gavage No significant increases in liver tumors were observed in offspring exposed in utero on days 12, 14, 16 and 18 of gestation, however, renal epithelial tumors (not seen in controls) developed in 7% of females Male (but not female) offspring nursed by safrole-treated mothers had a significantly higher incidence of liver tumors (34% treated vs 3% controls) A combination of transplacental, lactational and post-weanling exposure brought about high incidences of liver tumors (51% males, 80% females) in mice of both sexes. Besides rodents, dogs were reported to be susceptible, carcinomas of the skin and tongue were seen in dogs receiving safrole for 6 years (unpublished FDA data, cited in 81)

Besides safrole, a variety of related compounds have been tested for carcinogenic activity Myristicin, dill apiol and parsley apiol, three naturally occurring substances that are closely related to safrole (differing only by one or two additional methoxy group(s) on the ring), have been found to be noncarcinogenic when injected into preweanling male $B6C3F_1$ mice on days 1, 8, 15 and 22 after birth at a total dose of 4 75 μmoles (79). It appears that extensive ring substitution reduces or abolishes the carcinogenicity of safrole, possibly because of steric hindrance

Isosafrole, an isomer of safrole, has marginal or no carcinogenic activity in mice and is hepatocarcinogenic in rats, with a potency substantially lower than that of safrole In $B6C3F_1$ mice, oral administration of isosafrole (215 mg/kg body weight by i p injection on days 7-28 after birth followed by 517 ppm in the diet for up to 82 weeks) led to a small, marginally significant

increase (5/18 treated males and 1/16 treated females vs 8/79 and 0/87 controls, P = 0 05 with combined data) in the incidence of liver tumors The same treatment had no carcinogenic effect in B6AKF$_1$ mice (80, 81) In young adult Osborne-Mendel rats, dietary administration of 5,000 ppm for 2 years induced liver tumors in 5/50 rats (including 3 hepatocellular carcinomas) compared to 19/50 (14 malignant) safrole-treated rats A higher dose (10,000 ppm) was acutely toxic whereas lower doses (2,000 and 1,000 ppm) were not carcinogenic (4, 36) No significant carcinogenicity was noted in adult CD rats, 18 months after receiving 20 twice weekly s c injections of 18 6 μmole isosafrole (13). Thus, a change of the side chain from allyl to propenyl reduces the carcinogenic activity of the compound.

Of the three side chain-saturated derivatives of safrole that have been tested for carcinogenic activity, two (dihydrosafrole and piperonyl sulfoxide) are carcinogenic in at least one animal species suggesting that saturation of the side chain does not necessarily abolish the carcinogenicity of the compound Dihydrosafrole has been shown to induce tumors of the forestomach and the liver in mice and tumors of the esophagus in rats Oral administration of dihydrosafrole (464 mg/kg body weight by i p injection on days 7-28 after birth followed by 1,400 ppm in the diet for up to 82 weeks) to B6C3F$_1$ and B6AKF$_1$ mice brought about significant increase in the incidences of hyperplasia and tumors of the forestomach Fifteen of 17 (88%) treated female B6C3F$_1$ mice and 14 of 18 (78%) treated female B6AKF$_1$ mice had forestomach tumors (including 3 malignant) compared to 5/17 (28%)* and 0/15 female

*The incidence of stomach tumors in strain B6C3F$_1$ control mice (23% males, 28% females) reported in this study were substantially higher than historical controls No explanation was given for this unusual finding

controls Male $B6AKF_1$ mice also had a higher incidence (7/17 treated vs 0/18 controls) of stomach tumors (81) Besides stomach tumors, carcinomas of the liver developed in 10/17 (60%) male $B6C3F_1$ mice and 7/17 (41%) male $B6AKF_1$ mice in significantly higher incidences than controls No such increases were seen in female mice (80, 81) A clear-cut dose-response relationship in the induction of esophageal tumors was seen in Osborne-Mendel rats maintained on diets containing 2,500, 5,000 or 10,000 ppm dihydrosafrole for 2 years The respective tumor incidences were 20%, 74% and 75% Of these esophageal tumors, 5%, 32% and 50% were malignant No tumors were observed in rats fed 1,000 ppm of the compound (4, 33, 36) Besides rodents, there is some evidence that dogs given dihydrosafrole for 2 years had hyperplasia of the esophagus (FDA unpublished data, cited in 81)

The potential carcinogenicity of piperonyl sulfoxide was first investigated by Innes et al (80) as a part of a large-scale test of industrial and agricultural chemicals Two strains ($B6C3F_1$ and $B6AKF_1$) of mice were given 46 4 mg/kg body weight of the compound by stomach tube on days 7-28 after birth and 111 ppm in the diet for up to 82 weeks The compound was classified as suspect carcinogen requiring additional testing because an increased, though not significant incidence of tumors (reticulum cell sarcoma) was observed (80) A subsequent study by the U S National Cancer Insitute (88) showed that technical grade piperonyl sulfoxide (88% pure with 12% related compounds) is carcinogenic toward male $B6C3F_1$ mice producing an increased incidence of hepatocellular carcinoma The compound is not carcinogenic toward female mice and Fischer 344 rats of both sexes The compound was administered for 105 weeks in the diet at doses of 1,500 or 3,000 ppm for male mice, 3,000 or 6,000 ppm for female mice, 350 or 700 ppm for male rats and 700 or 1,400 ppm for female mice with the high doses being the maximum tolerated

dose The incidences of hepatocellular carcinoma in male mice were 6/18, 31/50 and 46/50 for control, low-dose and high-dose groups, respectively (88). Technical grade piperonyl butoxide was also categorized as a compound requiring additional testing in the study of Innes et al (80) However, a subsequent National Cancer Institute bioassay (89) showed no evidence of carcinogenicity of the compound in either $B6C3F_1$ mice or Fischer 344 rats, fed maximum tolerated doses in the diet for 2 years Piperonyl butoxide is also noncarcinogenic in Swiss albino ICR/Ha mice one year after receiving repeated s c injections (totaling 30 mg) 1-19 days after birth (90) There is evidence, however, that the compound acts synergistically with Freon 112 or 113 (see Section 5 3 2 4 3 6)

Besides the above-mentioned compounds, there is indication that sesamol (1-hydroxy-3,4-methylenedioxybenzene), a naturally occurring minor constituent of sesame oil, may be weakly carcinogenic Ambrose et al (91) found a total of 20 "proliferative lesions" in 134 rats fed diets containing 0 016 to 1 0% sesamol for 400-634 days Sixteen of these lesions (which include adenomas, papillomatous foci, polyps and nodules) were benign while four were either malignant (1 fibrosarcoma of the ovary) or undetermined Most of the lesions were distributed in the mammary and adrenal glands, bladder, uterus and ovary No such lesions were found in the controls, nor in rats receiving the lowest doses (0 008%) of the compounds

5 3 2 4 3 3 CARCINOGENICITY OF ESTRAGOLE AND RELATED COMPOUNDS

Estragole, a naturally occurring substance stucturally related to safrole, is hepatocarcinogenic in CD-1 or $B6C3F_1$ mice by three different routes of administration (see Table LXIV). Repeated subcutaneous injections of estragole totaling 4 4 or 5 2 μmoles between the days 1 and 22 after birth

Table LXIV
Carcinogenicity of Estragole and Related Compounds[a]

Compound[b]	Species and strain	Route	Principal organs affected	References
Estragole	Mouse, CD-1 or $B6C3F_1$	oral, i p or s c	Liver	(14, 79)
	Mouse, A/J	i p	None[c]	(79)
Methyleugenol	Mouse, $B6C3F_1$	i p	Liver	(79)
Elemicin	Mouse, $B6C3F_1$	i.p	None	(79)
Eugenol	Mouse, $B6C3F_1$	oral	Liver (equivocal)	(92)
	Mouse, CD-1	oral or i.p	None	(79)
	Mouse, ICR/Ha	skin painting	None	(93)
	Rat, F344	oral	None	(92)
trans-Anethole (isoestragole)	Mouse, CD-1 or $B6C3F_1$	oral or i p	None	(79)
	Mouse, A/He or A/J	i p	None[c]	(79, 86)
Calamus oil[d] (β-Asarone)	Rat, --	oral	Intestines	(5, 6)

[a]See also Tables LXII and LXV for carcinogenesis studies of metabolites and derivatives of estragole.

[b]See Table LVII for structural formulas

[c]Limited (24-week) bioassay only, pulmonary adenoma assay Negative results are considered inconclusive evidence of noncarcinogenicity

[d]Oil of calamus obtained from Jammu, India It consists of the following compounds; β-asarone, 75 8%, calamene, 3.84%, calamol, 3 2%, α-asarone, 1.32%, camphene, 0 92%, β-pinene, 0 56%, and asaronaldehyde, 0 2%

induced, after 15 months, hepatocellular carcinomas in 23% and 39% of male CD-1 mice, respectively (14) Some 73% of male CD-1 mice developed hepatomas 14 months after receiving 10 twice-weekly intragastric administrations of 2 5 μmole/g body weight of the compound starting on day 4 after birth Like with safrole, preweanling female mice are refractory to the carcinogenic effect of estragole Repeated intraperitoneal injections to preweanling male CD-1 mice (total dose, 9 45 μmole) or $B6C3F_1$ mice (total dose, 4 75 μmole) led to hepatoma incidences of 65% (after 12 months) and 83% (after 18 months), respectively (79) The carcinogenic potency of estragole is approximately equal to that of safrole in preweanling male mice

Two ring-substituted higher homologs of estragole have been tested for carcinogenic activity in male preweanling $B6C3F_1$ mice by Miller et al (79) The introduction of one additional methoxy group enhances the carcinogenicity of the compound as indicated by the higher tumor incidence and higher average number of hepatomas per mouse in preweanling male mice given methyleugenol (96%, 3 2) than in those given equimolar doses of estragole (83%, 2 4) However, further substitution with a methoxy group (yielding elemicin) abolishes the carcinogenic activity of the compound Interestingly, these structure-activity relationships parallel those of safrole in which the optimal number of methoxy substitutions (considering methylenedioxy group as two methoxy groups) appears to be two (see Section 5 3 2 4 3 2)

Ring hydroxylation and shift of the methoxy group from the para to the meta position (yielding eugenol), together, reduce or virtually abolish the carcinogenic activity of estragole Thus, eugenol was found completely non-carcinogenic in the study of Miller et al (79), in which a total dose of 25 μmole/g body weight was administered orally or a total dose of 9 45 μmole/ mouse was injected intraperitoneally to preweanling male CD-1 mice As dis-

cussed in Section 5 2 2 5 3 2 (Vol IIIA), there is some evidence that eugenol increases the incidence of hepatocellular carcinomas in B6C3F$_1$ mice but the evidence is equivocal due to the absence of dose-dependency In Fischer 344 rats, eugenol is clearly noncarcinogenic (92) The lack of (or lower) carcinogenicity of eugenol may be due to poorer absorption, to the generally easier excretion of phenolic compounds, and to shift of the methoxy group from para to meta which is a less effective position for resonance stabilization of metabolically generated electrophilic intermediate(s)

Also in accord with the greater carcinogenic potency of safrole than isosafrole, trans-anethole (isoestragole) is inactive under conditions in which estragole was carcinogenic In fact, trans-anethole showed no significant carcinogenicity in preweanling male CD-1 mice given a total dose (50 μmole/g body weight) twice that of estragole by gavage or an equimolar dose by intraperitoenal injection (79). trans-Anethole is also inactive in the pulmonary adenoma assay using strain A/He mice (79, 86)

In contrast to the lack of carcinogenicity of trans-anethole, there is some evidence that its close analog, β-asarone, may be carcinogenic Oil of calamus, a volatile oil present in the rhizomes (about 1%) of the plant Acorus calamus (commonly called sweet flag in U S), was found carcinogenic in rats (5, 6). In this study, groups of rats were fed diets containing 0, 500, 1,000, 2,500 or 5,000 ppm oil of calamus Malignant mesenchymal tumors of the small intestines were found in treated rats at all levels, and the incidence was dose-related The tumors were first detected after 59 weeks of feeding The particular variety of oil of calamus (from Jammu, India) used in this study consists of 75 8% β-asarone, 3 84% calamene, 3 2% calamol, 1 32% α-asarone, 0 92% camphene, 0 56% β-pinene and 0 2% asaronaldehyde Owing to its abundance in oil of calamus, the carcinogenic activity of the oil is generally attributed to β-asarone

5 3 2 4 3 4 CARCINOGENICITY OF METABOLITES OF SAFROLE, ESTRAGOLE AND RELATED COMPOUNDS

In an attempt to elucidate the metabolic activation pathways of safrole, estragole and related compounds, Millers and associates have synthesized a variety of metabolites and derivatives and tested their carcinogenic activity The major findings of these studies are summarized in Table LXII For comparison of relative potency, the carcinogenicity data (by i p administration to preweanling mice) of some of these compounds are summarized in Table LXV

Six metabolites of safrole have been tested for carcinogenic activity by various routes of administration By oral administration, 1'-hydroxysafrole (5,500 ppm in diet) induces a high incidence (89-92%) of hepatocellular carcinomas in adult male rats A few papillomas of the forestomach have also been observed Under similar conditions, safrole (5,000 ppm in diet) induces only a low incidence (6-16%) of liver tumors Fed at a lower dietary level (4,100 ppm), 1'-acetoxysafrole is not hepatocarcinogenic but induces a high incidence (64%) of multiple papilloma (with a few squamous cell carcinomas) of the forestomach (13, 55) At an even lower dietary level (2,500 ppm), 1'-oxosafrole is not carcinogenic (55) Subcutaneous injections of 1'-acetoxysafrole consistently led to the induction of injection-site sarcomas in 20-30% of the rats tested (13, 79) 1'-Hydroxysafrole-2',3'-oxide is approximately equipotent to 1'-acetoxysafrole in the induction of local sarcomas (79) 1'-Hydroxysafrole induces much fewer local sarcomas (13) or no local sarcomas (79), but is hepatocarcinogenic 1'-Oxosafrole has little or no local carcinogenic activity (55) whereas safrole and safrole-2',3'-oxide are both inactive by the s c route (79) These results, along with mutagenicity data and metabolic studies, led Millers and associates to conclude that 1'-hydroxy-

Table LXV

Relative Carcinogenicity of Metabolites of Safrole, Estragole and Related Compounds in the Induction of Liver Tumors in Male Infant Mice by Intraperitoneal Administration[a]

Compound	CD-1 mice[b]			$B6C3F_1$ mice[c]			
	Total dose (umol)	% Incidence at 12 months	Av no hepatoma per mouse	Total dose (umol)	% Indicence at 12 months	% Indicence at 13-18 months	Av no hepatoma per mouse
Safrole	9 45	67	1 9				
1'-Hydroxysafrole	4 72	65	2 7	3 75	92		2 7
1'-Hydroxysafrole-2',3'-oxide	9 45	55	1 0				
Estragole	9 45	65	1 7	4 75		83	2 4
1'-Hydroxyestragole				1 87	93		2 7
				1 9		98	5 6
1'-Hydroxy-2',3'-dehydroestragole				1.86		97	9 4
Methyleugenol				4 75		96	3 2
1'-Hydroxymethyleugenol				2 85		93	3 5
1'-Hydroxy-1-allyl-4-methoxynaphthalene				3.75	65		1 1

[a] Summarized from E C Miller, A B Swanson, D H Phillips, T L Fletcher, A Lein and J A Miller [Cancer Res 43, 1124 (1983)]

[b] Compound given in 10 doses during the first 4 or 5 weeks after birth

[c] Compound given in 4 doses prior to weaning at 22 days of age

safrole is a proximate carcinogen of safrole 1'-Acetoxysafrole is a potential ultimate carcinogen, however, there is no evidence that esterification of 1'-hydroxysafrole with acetic acid occurs to any significant extent Both 1'-hydroxysafrole-2',3'-oxide and 1'-oxosafrole possibly also represent ultimate carcinogens The evaluation of the degree of carcinogenic activity of the latter is limited by its instability and high toxicity

Various metabolites of safrole have also been assayed in mice The results support the conclusion that 1'-hydroxysafrole is a proximate carcinogen of safrole 1'-Hydroxysafrole is more potent than the parent compound in male preweanling mice given i.p. (see Table LXV) or s.c. (55) injections of the compounds In adult female mice, 1'-hydroxysafrole induces slightly fewer hepatomas than does the parent compound, but the decrease may be due to the induction, by 1'-hydroxysafrole, of a large number of angiosarcomas in interscapular subcutaneous tissue, shortening the lifespan of the animals (79) 1'-Acetoxysafrole is more hepatocarcinogenic than safrole after s c injection, however, the compound lacks local carcinogenic activity in mice (55) As in rats, 1'-oxosafrole and safrole-2',3'-oxide are inactive whereas 1'-hydroxysafrole-2',3'-oxide is carcinogenic in mice Another putative electrophilic metabolite, 1'-acetoxysafrole-2',3'-oxide, also fails to induce any significant carcinogenic effect in mice. There is strong evidence that 1'-sulfooxysafrole (the sulfate ester of 1'-hydroxysafrole) is the major ultimate electrophilic and carcinogenic metabolite of 1'-hydroxysafrole Boberg et al (78) recently showed that the carcinogenic effect of 1'-hydroxysafrole in female adult CD-1 mice can be virtually eliminated by treating the animals with pentachlorophenol, a sulfotransferase inhibitor. Brachymorphic mice, which lack the enzyme system for the synthesis of 3'-phosphoadenosine-5'-phosophosulfate (PAPS, activated sulfate), are much less responsive than their

phenotypically normal littermates to the induction of liver tumors by 1'-hydroxysafrole Attempts to synthesize 1'-sulfooxysafrole have thus far been unsuccessful because of its high instability and reactivity

In contrast to enhancement of carcinogenicity of safrole by 1'-oxidation of the allyl side chain, there is little or no evidence that oxidation at the corresponding position of isosafrole brings about activation of the compound (13) By subcutaneous injection to adult male rats, 3'-hydroxyisosafrole fails to show any carcinogenic effect 3'-Acetoxyisosafrole is also inactive for inducing local tumors at the injection site, but may have a marginal or weak hepatocarcinogenic effect. Repeated s.c. injections (2 times/week for 10 weeks) of 18 6 μmoles of the compound led to the induction of one liver carcinoma among 18 rats, the effect was considered to be probably a direct consequence of the treatment because of the extremely low spontaneous incidence of liver carcinomas in Fischer rats Of the two other tested 3'-substituted derivatives of isosafrole, 3'-methoxyisosafrole is inactive while 3'-bromoisosafrole displays local carcinogenic activity, inducing sarcomas at the injection site of 2/18 rats These results suggest that the role of 3'-oxidation in the metabolic activation of isosafrole is questionable and remains to be elucidated Further studies by oral administration are needed The local carcinogenic effect of 3'-bromoisosafrole is probably due to the good leaving tendency of the bromine group (see Section 5.2 1 1 2 1, Vol IIIA) generating the electrophilic carbonium ion on the allyl moiety.

As with safrole, the 1'-hydroxy derivative of estragole is substantially more potent than its parent compound by i p. injection (see Table LXV) to preweanling male mice. 1'-Hydroxyestragole is approximately equipotent to estragole by dietary administration to adule female CD-1 mice. The compound exhibits marginal local carcinogenic activity after s c. injection, inducing

local sarcomas in only 3/20 rats (79) Like safrole-2',3'-oxide, estragole-2',3'-oxide is also noncarcinogenic after i p or s c injection to preweanling male mice, and only shows some tumor-initiatory activity The results indicate that 2',3'-epoxidation is less important than 1'-hydroxylation and subsequent esterification in the metabolic activation of estragole This conclusion is further supported by the finding that 1'-hydroxy-2',3'-dehydroestragole is a strong hepatocarcinogen In fact, the compound is more potent than any of the estragole and safrole derivatives thus far tested (see Table LXV) The replacement of the double bond by a triple bond is expected to eliminate the possibility of epoxidation, leaving 1'-hydroxylation as the sole metabolic pathway of side chain oxidation. The mechanism of enhancement of carcinogenic activity by further unsaturation of the 2',3'-bond is not clear It is possible that the acetylenic bond may contribute to carcinogenic activity by stabilizing the electrophilic intermediate (such as carbonium ion) giving it a greater chance to reach target DNA molecules.

Among other carcinogenic congeners, 1'-hydroxymethyleugenol is at least as carcinogenic or possibly more potent than its parent compound (see Table LXV) 1'-Hydroxy-1-allyl-4-methoxynaphthalene is an active hepatocarcinogen when administered intraperitoneally to preweanling male mice (see Table LXV), while its 1'-acetoxy derivative is a direct-acting mutagen (see section 5.3 2.4.2 2). These results indicate that 1'-hydroxylation followed by esterification is a common metabolic activation for all carcinogenic allylarene congeners

Several metabolites of noncarcinogenic analogs or of trans-anethole (a noncarcinogenic isomer) of estragole have been tested for carcinogenic activity. 1'-Hydroxyelemicin is inactive after i p injection to preweanling mice (see Table LXV), indicating that the lack of carcinogenicity of elemicin

is not due to inability to undergo side chain oxidation but possibly to excessive substitution of the aromatic ring Like 3'-hydroxyisosafrole, 3'-hydroxy-trans-anethole (3'-hydroxyisoestragole) is also inactive In agreement with the results of safrole-2',3'-oxide and estragole-2',3'-oxide, eugenol-2',3'-oxide has little or no "complete" carcinogenic activity but is active as an initiator in skin tumorigenesis

5 3 2 4 3 5 CARCINOGENICITY OF CINNAMYL (CINNAMIC) COMPOUNDS

Cinnamyl (cinnamic) compounds have been used widely as food additives, fragrances or flavoring agents Some cinnamyl compounds are found in wood products or smoke They are structurally related to safrole, estragole and their isomers, 3'-hydroxyisosafrole, for example, is actually a ring substituted derivative of cinnamyl alcohol. Several cinnamyl compounds have been tested for carcinogenic activity (see Table LXVI). In the pulmonary adenoma assay by Stoner et al (86), both cinnamyl alcohol and cinnamaldehyde are inactive in A/He mice given i.p injections of maximally tolerated doses of the compounds, 3 times a week for 8 weeks and observed for another 16 weeks It should be noted, however, that owing to the nature of the study (short duration of only 24 weeks, insensitivity to many hepatocarcinogens), the negative results are suggestive but not conclusive evidence for the lack of carcinogenicity of the compounds

Schoental and Gibbard (95) showed that 3,4,5-trimethoxycinnamaldehyde is carcinogenic in rats. In a small scale experiment in which two doses of the compound (150 mg/kg body weight, i p , as a 20% suspension in aqueous ethanol, followed within one week by a s c dose of 100 mg/kg in dimethylformamide) were given to 6 young adult rats, 4 of 4 rats which survived longer than 17 months developed tumors These consisted of a sarcoma in the peritoneal

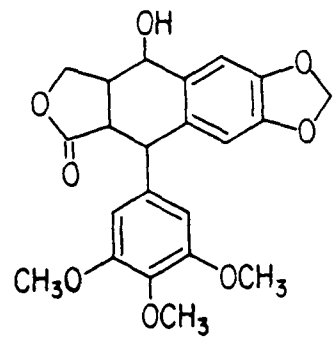
Table LXVI
Carcinogenicity of Cinnamyl Compounds

Compound[a]	Species and strain	Route	Principal organs affected	References
Cinnamyl alcohol	Mouse, A/He	i p	None[c]	(86)
Cinnamyl aldehyde	Mouse, A/He	i p	None[c]	(86)
	Rat, --	i p / s c	None	(Schoental and Gibbard, cited in 94)
3,4,5-Trimethoxy-cinnamaldehyde	Rat, --	i p / s c	Nasal cavity, peritoneum, testis	(95)
Cinnamyl anthranilate	Mouse, B6C3F$_1$	oral	Liver	(96)
	Mouse, A/He	i p	Lung	(86)
	Rat, F344	oral	Pancreas, kidney	(96)
Methyl anthranilate	Mouse, A/He	i p	None[c]	(86)
Anthranilic acid	Mouse, B6C3F$_1$	oral	None	(97)
	Rat, F344	oral	None	(97)

[a]See Table LVII for structural formula

cavity, a mesothelioma of the tunica albuginea of the testis, and two nasal squamous carcinomas. The latent period for these tumors ranged from 20 to 25 months In view of the extremely low spontaneous incidence of nasal carcinoma in rats, the results are of particular importance in the light of several reports of increased incidences of nasal tumors among woodworkers and population groups exposed to wood smoke (see Section 5 3 2.4 5) Whereas it is not known whether 3,4,5-trimethoxycinnamaldehyde as such is present in wood lignins, several closely related compounds have been found in wood (see Section 5 3 2 4 5). In contrast to its 3,4,5-trimethoxy derivative, unsubstituted cinnamaldehyde is not carcinogenic (unpublished data by Schoental and Gibbard, cited in 94) Apparently, the methoxy groups, particularly the p-methoxy group, confer carcinogenic activity to the compound.

Of relevance to the above finding is an interesting report by Sabine et al. (98) that C3H A^{vy} mice, which have a high "spontaneous" incidence of mammary and hepatic tumors in the laboratories of the National Cancer Institute in United States, developed much fewer "spontaneous" tumors when tested in Australia. The discrepancy has been attributed to the use in the NCI laboratory of red cedar (Juniperus virginiana) wood bedding which is known to contain methylenedioxy- and polymethoxylignins such as podophyllotoxin It has been suggested that podophyllotoxin may account for some of the carcinogenic effects of red cedar wood bedding From the structural point of view, podophyllotoxin may be considered as consisting of one molecule of a safrole derivative and one molecule of 3,4,5-trimethoxyphenol derivative and it is possible that the compound may in fact yield these derivatives in the course of its metabolic or microbial degradation (94, 95).



Podophyllotoxin

Cinnamyl anthranilate, a synthetic flavoring agent, was first found to be carcinogenic in the pulmonary adenoma assay by Stoner et al. (86) The compound produced a statistically significant increase in the incidence and multiplicity (average number/mouse) of lung adenomas in strain A/He mice which received i p injections of maximally tolerated doses (up to 500 mg/kg body weight) three times a week for 8 weeks and survived for an additional 16 weeks The carcinogenicity of the compound has been confirmed in a carcinogenesis bioassay by U S National Cancer Institute and National Toxicology Program (96) Groups of 50 Fischer 344 rats and $B6C3F_1$ mice of each sex were fed diets containing 15,000 or 30,000 ppm (maximally tolerated dose) cinnamyl anthranilate for 103 weeks and then observed for an additional 2 or 3 weeks Significant, dose-related increases in the incidences of liver tumors (carcinomas or adenomas) were observed in dosed mice (males control 47%, low dose 60%, high dose 79%, females control 14%, low dose 41%, high dose 67%) The chemical is also carcinogenic in male (but not female) rats, inducing low incidences of acinar-cell carcinomas or adenomas of the pancreas (a rare type of tumor) and tumors of the renal cortex It is particularly interesting to note that when tested separately, neither the cinnamyl alcohol moiety nor the anthranilic acid moiety of cinnamyl anthranilate are active in the pulmonary adenoma assay. Anthranilic acid (2-aminobenzoic acid) is also noncarcinogenic in $B6C3F_1$ mice and Fischer 344 rats in a 2-year feeding study using maximally tolerated doses (97).

5 3 2 4 3 6 MODIFICATION OF CARCINOGENESIS BY SAFROLE AND RELATED COMPOUNDS

As for virtually all carcinogens, the carcinogenicity of safrole and related compounds is modified by a number of host and environmental factors The modification studies provided important clues to the understanding of the

activating metabolic pathways of the carcinogens Wislocki et al (55) showed that phenobarbital (PB, 0 1% in drinking water), a well known inducer of microsomal mixed-function oxidases, enhances the hepatocarcinogenicity of safrole (0 5% in diet) in CD rats The incidence of hepatocellular carcinomas in rats receiving both PB and safrole was 67%, substantially higher than that of rats given safrole (17%) or PB (6%) alone The enhancing effect was attributed to the increased oxidation of safrole to the proximate carcinogen, 1'-hydroxysafrole As much as 10 times more 1'-hydroxysafrole was detected in the urine of rats pretreated with PB before safrole administration (12) Boberg et al (78) demonstrated that chronic administration of a nontoxic level (0 05%) of pentachlorophenol, a potent inhibitor of cytosolic sulfotransferase (100, 101), in the diet of adult female CD-1 mice strongly inhibits (to the extent of 82-100%) the hepatocarcinogenic effect of both safrole (0 13 or 0 25% in diet) and 1'-hydroxysafrole (0.14 or 0 27% in diet) Induction of hepatic tumors by a single i.p injection of 1'-hydroxysafrole to preweanling $B6C3F_1$ mice is also inhibited by prior treatment with pentachlorophenol Brachymorphic* mice, which are deficient in enzymes for synthesis of 3'-phosphoadenosine-5'-phosphosulfate (PAPS, the sulfated coenzyme also known as "activated sulfate," needed for the sulfotransferase), develop much fewer liver tumors than their phenotypically normal littermates when exposed to 1'-hydroxysafrole (dietary administration to adult females or i.p. to preweanling males). These results, along with metabolism and DNA

*Brachymorphism, a recessive trait in mice, is phenotypically characterized by disproportionately short stature as a result of undersulfation of the glycosaminoglycans in the cartilage (102) Homozygotic brachymorphic (bm/bm) mice have reduced capacity to synthesize PAPS because of defect in one or both of the enzymes involved in the synthesis of PAPS from ATP and sulfate ion (103, 104) The heterozygotic (+/bm) and wild type (+/+) mice are phenotypically indistinguishable

binding studies (see Section 5 3 2 4 4), provide convincing evidence that 1'-hydroxylation by cytochrome P-450-dependent microsomal mixed-function oxidase followed by sulfation by cytosolic sulfotransferase represents the principal metabolic pathway for activation of safrole to its ultimate carcinogenic form, 1'-sulfooxysafrole

An unusual case of syncarcinogenesis was noted by Epstein et al (90) in a study involving piperonyl butoxide and Freon 112 or 113 (see Section 5 2.1 3 8, Vol IIIB) When administered singly, neither fluoroalkane nor piperonyl butoxide is carcinogenic in neonatal mice However, combined treatment of piperonyl butoxide with either Freon 112 or 113 induce liver tumors in male mice The mechanism of synergism is unclear and is hypothesized to involve modification of metabolism of Freon by piperonyl butoxide. In this respect, it is relevant to note that safrole, isosafrole and related methylenedioxybenzene compounds (including piperonyl butoxide) are inducers of microsomal mixed-function oxidases (see Section 5 3 2.4 2 2), have been shown to modify the metabolism of carcinogens (e g , 105), and may be expected to be possible modifiers of carcinogenesis

5 3 2 4 4 Metabolism and Mechanism of Action

5 3 2 4.4 1 METABOLISM

Metabolism of Safrole The metabolism of safrole has been extensively studied and was the subject of several comprehensive reviews between 1977 and 1983 (44, 106-109) The initial metabolism of safrole involves three principal types of reactions (a) 1'-hydroxylation of the allyl side chain, (b) epoxidation of the 2',3'-double bond of the allyl side chain, and (c) oxidation ("demethylenation") of the methylenedioxy group. The resulting metabolites are further metabolized by a variety of pathways, giving rise to a large

number of metabolites (see Fig 13) The relative importance of each of these pathways which contribute, to some extent, to the carcinogenicity of safrole, is discussed below

There is ample evidence that 1'-hydroxylation is the predominant metabolic activation pathway of safrole. 1'-Hydroxysafrole (compound II in Fig 13) has been detected in the liver, plasma, urine (mainly conjugated as glucuronide) and bile of several species of animals given safrole (12, 13, 78, 110-113). Human volunteers given low doses of safrole did not excrete 1'-hydroxysafrole in their urine (112). Pretreatment with typical inducers of microsomal mixed-function oxidases (e.g., phenobarbital, 3-methylcholanthrene) increases the urinary excretion of conjugated 1'-hydroxysafrole by rats or mice, but not by hamsters and guinea pigs (12). 1'-Hydroxysafrole is regarded to be the proximate carcinogen of safrole because (a) it is more carcinogenic (see Section 5.3 2.4 3.4) and mutagenic (see Section 5.3.2.4 2.2) than the parent compound, (b) pretreatment of rats with phenobarbital enhances the carcinogenicity of safrole (see Section 5 3 2.4.3.6), and (c) it can be further metabolized to electrophilic, ultimate carcinogens. At least three of these metabolites -- 1'-sulfooxysafrole (compound III in Fig 13), 1'-oxosafrole (VI) and 1'-hydroxysafrole-2',3'-oxide (IV) -- have been considered to be possible candidates for ultimate carcinogen

The evidence that 1'-sulfooxysafrole may be an ultimate carcinogen of safrole was first obtained by Wislocki et al. (15) who demonstrated that in vitro incubation of 1'-hydroxysafrole with mouse or rat liver cytosol in the presence of 3'-phosphoadenosine-5'-phosphosulfate (PAPS, the sulfated coenzyme also known as "activated sulfate," needed for sulfotransferase) generates reactive intermediates which bind covalently to nucleic acids. 1'-Acetoxy-

LEGEND TO FIGURE 13

Fig. 13. The principal metabolic pathways of safrole (I). The chemical names of the metabolites are II, 1'-hydroxysafrole, III, 1'-sulfooxysafrole, IV, 1'-hydroxysafrole-2',3'-oxide; V, 1',2',3'-trihydroxy-2',3'-dihydrosafrole; VI, 1'-oxosafrole; VIIa, 3'-(glutathion-S-yl)-1'-oxo-2',3'-dihydrosafrole (SR = glutathionyl group), VIIb, 3'-(N-acetylcystein-S-yl)-1'-oxosafrole (SR = N-acetylcysteinyl group); VIIIa, 3-N,N-dimethylamino-1-(3',4'-methylenedioxyphenyl)-1-propanone (R = methyl group), VIIIb, 3-piperidyl-1-(3',4'-methylenedioxyphenyl)-1-propanone (NR_2 = piperidyl group), VIIIc, 3-pyrrolidinyl-1-(3',4'-methylenedioxyphenyl)-1-propanone (NR_2 = pyrrolidinyl group), IX, 3'-hydroxyisosafrole; X, 3,4-methylenedioxybenzoylglycine, XI, safrole-2',3'-oxide, XII, 2',3'-dihydroxy-2',3'-dihydrosafrole; XIII, 2-hydroxy-3-(3',4'-methylenedioxyphenyl)propionic acid; XIV, 3,4-dihydroxy-1-allylbenzene (allylcatechol), XVa, eugenol; XVb, 3-hydroxy-4-methoxy-1-allylbenzene, XVI, 2',3'-epoxypropylcatechol, XVII, 2',3'-dihydroxypropylcatechol. Virtually all hydroxylated metabolites can be further conjugated with glucuronic acid whereas carboxylated metabolites can be conjugated with glycine. PAPS = 3'-phosphoadenosine-5'-phosphosulfate ("activated sulfate")

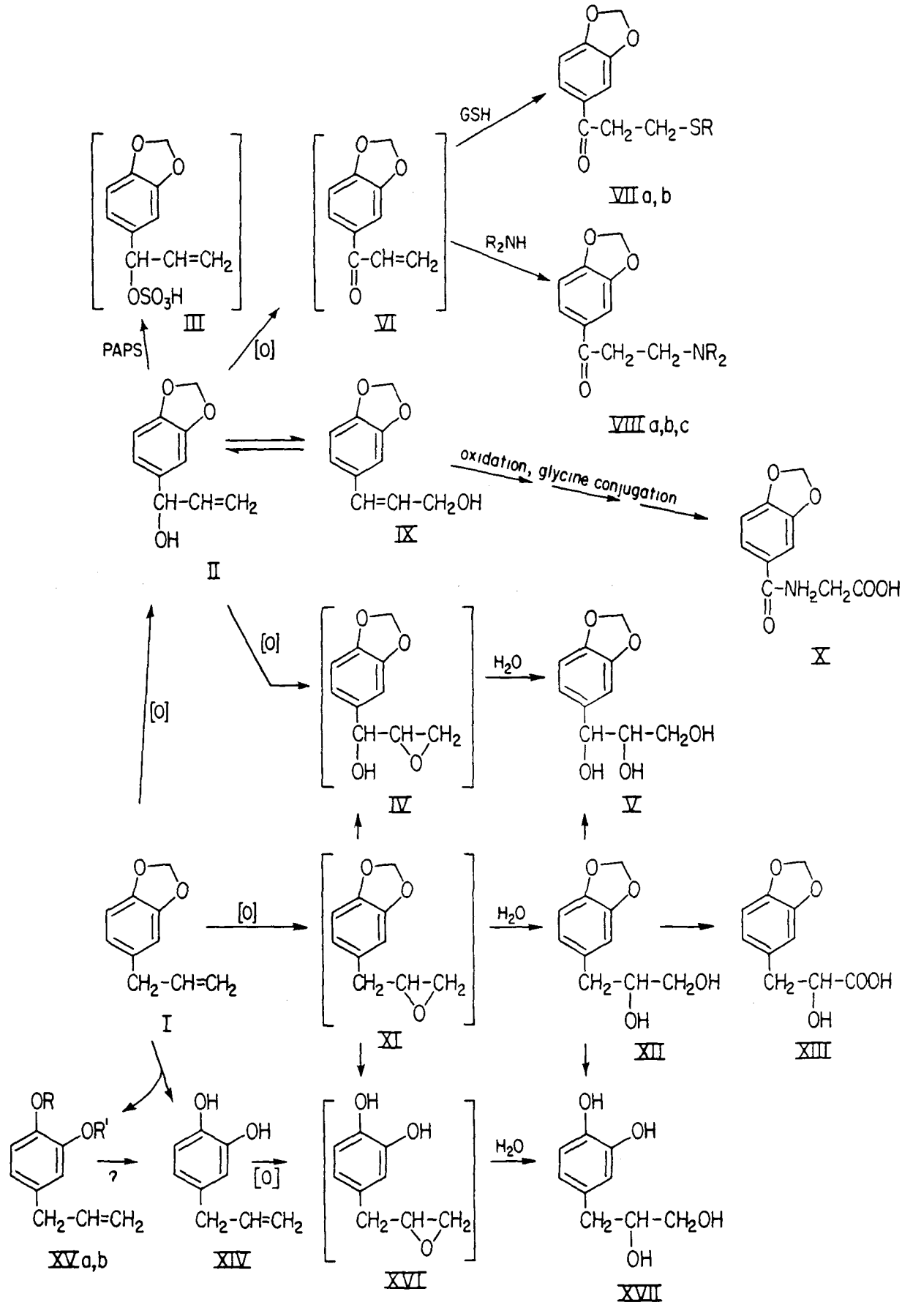

GSH
R2NH
PAPS
[O]
oxidation, glycine conjugation
H2O
III
VI
VII a, b
VIII a,b,c
II
IX
X
IV
V
I
XI
XII
XIII
XV a,b
XIV
XVI
XVII

Fig. 13

safrole*, a chemically synthesized model 1'-ester derivative of 1'-hydroxysafrole, is a reactive electrophile (see Section 5 3 2 4 2 1) and a direct-acting carcinogen (see Section 5 3 2 4.3.4) and mutagen (see section 5 3 2 4 2.2) Strong evidence for an important role of 1'-sulfooxysafrole in the hepatocarcinogenesis by safrole or 1'-hydroxysafrole in the mouse has recently been provided by Boberg et al (78) They showed that chronic administration of a nontoxic level of pentachlorophenol, a potent inhibitor of sulfotransferase, strongly inhibits (by as much as 82-100%) the hepatocarcinogenic activity of safrole and 1'-hydroxysafrole. Brachymorphic mice, which lack competent enzymes for synthesis of PAPS, develop much fewer liver tumors than normal mice in response to 1'-hydroxysafrole treatment (see Section 5.3.2.4.3.6). In both cases, reduced levels of covalent binding of 1'-hydroxysafrole to DNA and RNA were observed These results, together with the finding that the major DNA adducts of 1'-hydroxysafrole in the mouse liver are formed via an ester of 1'-hydroxysafrole (114, see also Section 5 3.2.4.4.2), led to the conclusion (78, 109) that 1'-sulfooxysafrole is the major ultimate electrophilic metabolite, responsible for the DNA binding and carcinogenicity of 1'-hydroxysafrole (and safrole) in the mouse liver.

The presence of 1'-oxosafrole as a metabolite of safrole was first deduced from the study of Oswald et al (115) who detected three nitrogen-containing metabolites in the urine of rats and guinea pigs given safrole These metabolites were identified as 3-N,N-dimethylamino-1-(3',4'-methylenedioxyphenyl)-1-propanone (compound VIIIa in Fig. 13), 3-piperidyl-1-(3',4'-methylenedioxyphenyl)-1-propanone (compound VIIIb) and 3-pyrrolidinyl-1-(3',4'-methylenedioxyphenyl)-1-propanone (compound VIIIc). All three of these

*Attempts to demonstrate in vitro formation of 1'-acetoxysafrole (by substituting PAPS with acetyl-CoA) were unsuccessful (15).

β-aminoketones ("Mannich base") may decompose to yield 1'-oxosafrole upon heating and are believed to be the condensation (Michael addition) products of the vinylketone (1'-oxosafrole) with the secondary amines (dimethylamine, piperidine, pyrrolidine) present in body fluids (20, 115) The possibility that 1'-oxosafrole may be a potential ultimate carcinogen of safrole was raised by Wislocki et al (15) who demonstrated the strong electrophilic reactivity of the compound (see Section 5 3 2 4 2 1) However, attempts to demonstrate the carcinogenic or mutagenic activity of synthetic 1'-oxosafrole have thus far been unsuccessful (55). The lack of genotoxicity of 1'-oxosafrole has been attributed to its extremely high reactivity and instability (55) and to the "soft" nature of its electrophilicity (23) In rats and mice given a single i p dose of 1'-oxosafrole, the two major biliary and urinary metabolites detected were the GSH-conjugates, 3'-(glutathion-S-yl)-1'-oxo-2',3'-dihydrosafrole (compound VIIa) and 3'-(N-acetylcystein-S-yl)-1'-oxo-2',3'-dihydrosafrole (compound VIIb) Apparently, in contrast to 1'-sulfooxysafrole (a "hard" electrophile), 1'-oxosafrole (a "soft" electrophile) is extensively detoxified by glutathione (a "soft" nucleophile) and must first deplete cellular GSH before it can react with the oxygen atoms or amino groups ("hard" nucleophiles) of nucleic acid bases (23)

1'-Hydroxysafrole-2',3'-oxide is an in vitro metabolite of 1'-hydroxysafrole (15) The reaction is catalyzed by rat or mouse liver microsomes and is dependent on NADPH. The yield is substantially higher when an inhibitor of epoxide hydrase is included in the incubation medium (55). Trace amounts of 1'-hydroxysafrole-2',3'-oxide (as glucuronide) and 1',2',3'-trihydroxy-2',3'-dihydrosafrole (compound V) have also been found in the urine of rats given safrole (110, 111). 1'-Hydroxysafrole-2',3'-oxide is a relatively long-lived electrophilic metabolite (15, see also Section 5 3 2 4 2.1) It is a direct-

acting mutagen and carcinogen and is also active as a tumor initiator (55, 79) The metabolite is considered to be a possible ultimate carcinogen of safrole, although its contribution to overall DNA binding and the carcinogenicity of safrole still remains to be investigated

A fourth metabolic pathway of 1'-hydroxysafrole is isomerization to 3'-hydroxyisosafrole (or 3,4-methylenedioxycinnamyl alcohol, compound IX in Fig 13) This pathway is called "cinnamoyl pathway" by some investigators The mechanism has been postulated to involve protonation of the hydroxy group of 1'-hydroxysafrole, loss of H_2O to form allylic cation, isomerization of the allylic cation and rehydration to form 3'-hydroxyisosafrole (116) The equilibrium strongly favors the formation of 3'-hydroxyisosafrole (116) 3'-Hydroxyisosafrole is further metabolized by oxidation and conjugation with glycine to yield the hippuric acid derivative, 3,4-methylenedioxybenzoylglycine (compound X) as the major urinary metabolite (110, 117). Owing to the lack of carcinogenicity and mutagenicity of 3'-hydroxyisosafrole and 3'-acetoxyisosafrole, the cinnamoyl pathway is generally considered to represent the detoxication of safrole It should be noted, however, that one of the possible intermediates in this pathway, 3,4-methylenedioxycinnamaldehyde, is potentially carcinogenic because a closely related compound, 3,4,5-trimethoxycinnamaldehyde, is in fact carcinogenic (see Section 5.3 2 4 3 5).

Direct epoxidation of the 2',3'-double bond of the allyl side chain is the second principal initial metabolic pathway of safrole This pathway is called the "epoxide-diol pathway" by some investigators. Trace amounts of safrole-2',3'-oxide (compound XI in Fig. 13) have been found in the urine of rats and guinea pigs given safrole (110) and in in vitro studies using rat hepatocytes (118) Its presence can also be deduced from the detection of its dihydrodiol, 2',3'-dihydroxy-2',3'-dihdyrosafrole (compound XII) and further

oxidation product, 2-hydroxy-3-(3,4-methylenedioxyphenyl)propionic acid (compound XIII), as urinary metabolites (110, 119) Safrole-2',3'-oxide is a relatively long-lived electrophile It is capable of directly reacting with nucleosides (15, see also Section 5 3 2 4 2 1) and is a direct-acting mutagen (Section 5 3 2 4 2 2) However, safrole-2',3'-oxide appears to play a limited role in carcinogenesis by safrole It is inactive as a "complete" carcinogen and is active only as a tumorigenesis initiator (see Sections 5 3 2 4 3 1 and 5 3 2.4 3 4)

Oxidation of the methylenedioxy group of safrole is the third and the major initial metabolic pathway of the compound This pathway is called the "demethylenation pathway" by some investigators The predominant urinary metabolite in animals (110, 112, 113) or humans (112) exposed to safrole is allylcatechol (3,4-dihydroxy-1-allylbenzene, compound XIV in Fig 13) Small amounts of eugenol (XVa) and its isomer, 3-hydroxy-4-methoxy-1-allylbenzene (XVb) and some monohydroxy metabolites have also been detected The intact allyl side chain of the above metabolites may be further oxidized to yield epoxides (e g , 2',3'-epoxypropylcatechol, compound XVI) which in turn can be hydrated to diol metabolites (e g , 2',3'-dihydroxypropylcatechol, compound XVII) and further oxidized to corresponding propanoic acids (110, 120) The demethylenation pathway is generally considered to represent detoxification because the resulting metabolites lack the resonance-stabilizing p-methoxy group, are much more hydrophilic, and are expected to be more readily excreted However, there is some suggestive evidence that reactive intermediate(s) (e g , carbene) may be generated during demethylenation of the methylenedioxy group and interact with cytochrome P-450 and the endoplasmic reticulum to contribute to carcinogenesis by epigenetic mechanism(s) (see further discussion in Section 5 3 2 4.4 2) Formaldehyde is a possible

metabolite in the demethylation pathway, however, there is no experimental evidence for this so far

Metabolism of Myristicin, Isosafrole and Dihydrosafrole Very little information is available on the metabolism of myristicin. β-Aminopropiophenones (compounds VIIIa,b,c in Fig 13) corresponding to those reported for safrole have been detected in the urine of animals given myristicin (121) suggesting that 1'-oxomyristicin may be a metabolic intermediate of myristicin Myristicin binds covalently to DNA following metabolic activation (see Section 5 3 2 4 4 2), the nature of the DNA adduct is not known The metabolism of isosafrole and dihydrosafrole has recently been studied by Klungsøyr and Scheline (117) Demethylenation of the methylenedioxy group is by far the most predominant metabolic pathway, accounting for 92% and 95% of the total metabolism of isosafrole and dihydrosafrole, with 4-propenylcatechol (1,2-dihydroxy-4-propenylbenzene) and 4-(1-propyl)-catechol as the major metabolite, respectively For isosafrole, the other metabolites (e g , 3'-hydroxyisosafrole, 3,4-methylenedioxycinnamic acid, 1',2'-dihydroxydihydrosafrole, 3,4-methylenedioxybenzoyl glycine) are attributable to metabolism via the epoxide-diol and the cinnamoyl pathways Trace amounts of 1'-hydroxysafrole have been detected in the urine of rats given 3'-hydroxyisosafrole (116). Besides demethylenation, dihydrosafrole is also metabolized by ring hydroxylation and 1'- as well as 2'-hydroxylation Interestingly, 1',2'-dihydroxydihydrosafrole is also a metabolite (albeit very minor, accounting for 0 2% of the total dose) of dihydrosafrole, despite the lack of double bond in the side chain

Metabolism of Estragole and Related Compounds. The metabolism of estragole and related compounds bears a close resemblance to safrole and related compounds, with the exception that the demethylenation step is replaced by the

O-demethylation step Solheim and Scheline (122) showed that estragole is metabolized in the rat by (a) the O-demethylation pathway to yield 4-hydroxy-1-allylbenzene (approx 39-46% of the dose), (b) the epoxide-diol pathway to yield 2',3'-epoxide, 2',3'-dihydrodiol and eventually 2-hydroxy-3-(4-methoxyphenyl)propionic acid and 4-methoxybenzoyl glycine (approx 17-31% of the dose), and (c) 1'-hydroxylation to yield 1'-hydroxyestragole (approx 5-10% of the dose) which in turn undergoes isomerization to 3'-hydroxyisoestragole and further metabolism by the cinnamoyl pathway A metabolic study by Drinkwater et al (14) found that 23% of an intraperitoneal dose (1 85 mmol/kg body weight) of estragole may be recovered as 1'-hydroxyestragole (mostly as conjugates) in the urine of preweanling mice (which are highly susceptible to the hepatocarcinogenic effect of estragole) Zangouras et al (123) showed that the proportion of the dose converted to 1'-hydroxyestragole in rodents is nonlinearly dose-dependent increasing from 1% at 0 05 mg/kg to 12% at 1,500 mg/kg. DNA binding studies (see Section 5 3 2 4 4 2) led the Millers and their associates (109, 114) to suggest that, as with safrole, 1'-sulfooxyestragole may be the principal electrophilic metabolite responsible for the DNA binding and carcinogenicity of estragole The metabolism of two higher homologs (methyleugenol and elemicin) of estragole has also been studied by Solheim and Scheline (124, 125) The most significant change is the substantial decrease in the importance of the O-demethylation pathway with the increase of ring substitution with methoxy group(s). For both compounds, the cinnamoyl and the epoxide-diol pathways are prominent. Large amounts of 1'-hydroxy derivatives of methyleugenol and elemicin have been found in the bile (124, 125). Some of the 1'-hydroxy metabolites appear to be oxidized to 1'-oxo metabolites, as suggested by the detection of β-aminopropiophenones as urinary metabolites (124, 126).

As with the methoxyallylbenzene congeners, the extent of ring substitution can have a dramatic effect on the metabolism of methoxypropenylbenzene congeners O-Demethylation is the predominant metabolic pathway in the metabolism of trans-anethole (127, 128), but this pathway is only a very minor one for its dimethoxy and trimethoxy homologs, isomethyleugenol and isoelemicin (124, 125) The cinnamoyl pathway and, to a lesser extent, the epoxide-diol pathway account for most of the metabolism of isomethyleugenol and isoelemicin in the rat (124, 125). The metabolism of trans-anethole displays significant dose-dependence and species difference At low doses, as much as 56-72% of the compound is metabolized by the O-demethylation pathway. At high doses, however, the O-demethylation pathway appears to be saturated and the cinnamoyl and epoxide-diol pathways dominate A species comparison study indicates that rats favor the epoxide-diol pathway while mice favor the cinnamoyl pathway (128)

5 3.2 4 4 2 MECHANISM OF ACTION

Safrole and several of its congeners are genotoxic carcinogens Covalent binding to DNA of safrole and its proximate carcinogen, 1'-hydroxysafrole has been convincingly demonstrated by Boberg et al (78) Modifying factors (e g., pentachlorophenol, brachymorphism), which inhibit the hepatocarcinogenicity of these compounds in mice by 82-100% (see Section 5.3 2 4.3 6), also reduce their covalent binding to liver DNA by 85-89% There is, moreover, a reasonably good correlation between DNA-binding activity and carcinogenicity of congeners of safrole Randerath et al (129) studied the in vivo covalent binding of 10 alkenylbenzene compounds to mouse liver DNA The "covalent binding indexes" of the compounds follow the order methyleugenol (36 0) > safrole (28 4) = estragole (28 4) > myristicin (10 7) > dill apiol (7 7) > parsley apiol (3 0) > elemicin (2.3) > anethole (0.16) > allylbenzene (0 16) >

eugenol (no binding) The three compounds (methyleugenol, safrole and estragole) that exhibit the highest DNA-binding activities are carcinogenic, their relative carcinogenic potencies (see Table LXV) correlate with their covalent binding indexes The seven compounds that show a lower level or lack of DNA-binding activities are all noncarcinogenic (see Section 5 3 2 4 3) The covalent binding indexes of some of these compounds (e g , myristicin, dill apiol) appear to suggest a greater genotoxic potential than the carcinogenicity data would indicate It would be interesting to investigate whether the nature of DNA adducts and the repair efficiency of these adducts of carcinogenic compounds differ from those of noncarcinogenic compounds Consistent with carcinogenicity data, the covalent binding index of estragole is substantially higher than that of anethole (isoestragole) Another in vivo DNA binding study by Fennell et al (24) showed high levels of covalent binding of 1'-hydroxy-2',3'-dehydroestragole, a very potent hepatocarcinogen (see Table LXV), to mouse liver DNA

The nature of DNA-adducts formed in mouse liver following administration of the 1'-hydroxy derivative (the proximate carcinogen) of safrole, estragole and 2',3'-dehydroestragole has been studied by the Millers and associates (17, 24, 25) Four or five major nucleoside adducts have been found in the hepatic DNA of mice given 1'-hydroxyestragole (25, 114). They were identified as N^2-(estragol-1'-yl)deoxyguanosine (two diastereomers), N^2-(trans-isoestragol-3'-yl)deoxyguanosine, N^2-(cis-isoestragol-3'-yl)deoxyguanosine and N^6-(trans-isoestragol-3'-yl)deoxyadenosine These adducts arise as the result of the reaction of an ester of 1'-hydroxyestragole with purine bases in DNA by either S_N1, S_N2 or a modified S_N2 (S_N2') mechanisms (see Fig 14). Five analogous nucleoside adducts have been found in mice given 1'-hydroxysafrole (25, 114, 130) These results, coupled with the demonstration of the critical role of

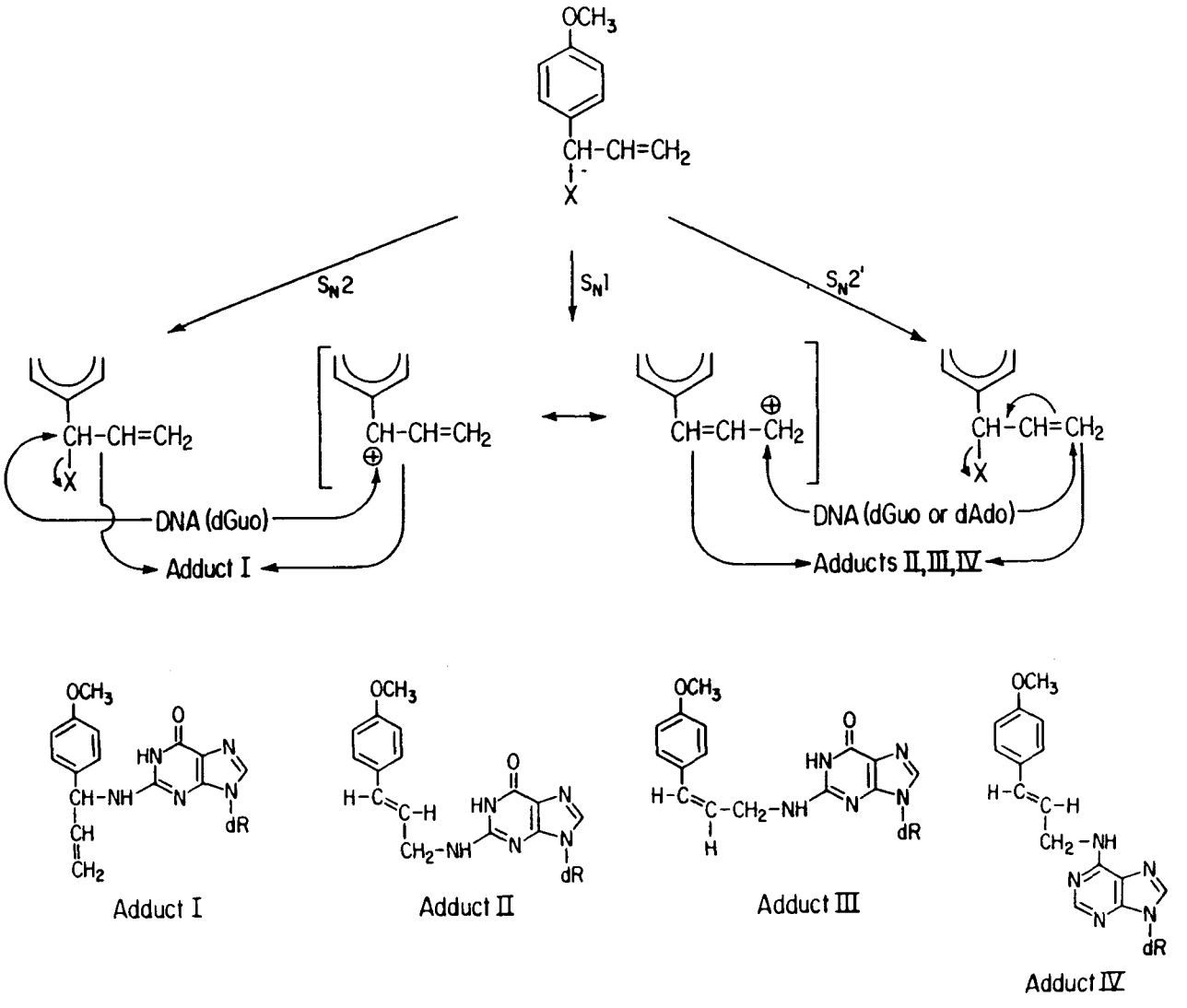


Fig. 14. Proposed mechanisms by which an ester of 1'-hydroxyestragole can react with purine bases in DNA to yield the adducts found in mouse liver DNA *in vivo*. In the formulas, X = $-OSO_3^-$ or $-OCOCH_3$, dR = deoxyribose. The chemical names of the adducts are: Adduct I, N^2-(estragol-1'-yl)deoxyguanosine (two diastereomers), Adduct II, N^2-(*trans*-isoestragol-3'-yl)deoxyguanosine, Adduct III, N^2-(*cis*-isoestragol-3'-yl)deoxyguanosine, Adduct IV, N^6-(*trans*-isoestragol-3'-yl)deoxyadenosine [Modified from D.H. Phillips, J. A Miller, E. C. Miller and B. Adams. *Cancer Res.* 41, 176 (1981)].

sulfation in carcinogenesis by safrole or 1'-hydroxysafrole (78), suggest that 1'-hydroxylation followed by sulfation is the predominant metabolic activation pathway for both safrole and estragole Only a single nucleoside adduct, which comigrates on high performance liquid chromatography (HPLC) with N^2-(2',3'-dehydroestragol-1'-yl)deoxyguanosine (obtained by *in vitro* reaction of 1'-acetoxy-2',3'-dehydroestragole with dGMP), has been detected in hepatic DNA of mice given 1'-hydroxy-2',3'-dehydroestragole (24) suggesting that an electrophilic 1'-ester is the ultimate carcinogen of the compound

The molecular mechanism of carcinogenesis after the initial covalent binding of the carcinogen to DNA is not clearly understood. Most of the N^2-guanine and N^6-adenine adducts are removed quite rapidly from mouse liver DNA through the repair mechanism(s) Nonetheless, a significant fraction of each adduct persists for up to 20 days after treatment (17) It has been postulated that the formation of adduct at the N^6-position of adenine may lead to mutation by causing mispairing, between deoxycytidine and the imino tautomeric form of deoxyadenosine, during DNA replication (131). Adduct formation can facilitate the cleavage of the sugar-phosphate backbone in DNA leading to apurinic/apyrimidinic sites which could, under certain special conditions (see Section 5 3 1 1 4 2), lead to mutation. Alternatively, chemically induced DNA repair is often error prone and can lead to infidelity of DNA replication. Cultured human cells exposed to 1'-acetoxysafrole or 1'-acetoxyestragole undergo DNA repair replication shortly (4-11 hours) after the treatment (26) DNA damage, consistent with the presence of apurinic/apyrimidinic sites, have been demonstrated in a small fraction of these cells (132) However, a comparative study by Drinkwater *et al.* (133) show that the capacity of a variety of structurally different types of carcinogens (including 1'-acetoxyestragole) to produce apurinic/apyrimidinic sites in supercoiled DNA

does not correlate well with their mutagenic activity in the Ames test Also, a number of mutagenic, electrophilic metabolites of alkenylbenzene compounds (e g , safrole-2',3'-oxide, estragole-2',3'-oxide) are inactive as "complete" carcinogens (see Sections 5 3 2 4 3 1 and 5 3 2.4 3 4) suggesting that somatic cell mutation alone may not be sufficient to explain the complete process of carcinogenesis The elucidation of the mechanism of carcinogenesis by safrole, estragole and related compounds awaits further studies

In addition to the genotoxic mechanisms described above, there is suggestive evidence that the methylenedioxy group may contribute to the overall carcinogenicity of safrole and related compounds through epigenetic mechanisms Studies by various investigators (rev in 44) show ligand complexing and covalent binding of reactive intermediates (most likely carbene intermediate, see Fig. 15) of safrole with the heme and protein moieties, respectively, of cytochrome P-450. Such binding may cause structural and functional changes in the endoplasmic reticulum resulting in loss of ribosomes ("degranulation") known indeed to occur following treatment with safrole or other carcinogens (134) It has been suggested (134) that loss of ribosomes could lead to impairment of glycoprotein synthesis and contribute to the process of malignant transformation by epigenetic mechanisms The simultaneous loss of cytochrome P-450 activities through ligand complexing and covalent binding and, on the other hand, increase in cytochrome P-448 activities through enzyme induction by safrole may also be contributory factors because such changes have been found to be often conducive to chemical carcinogenesis (134)

Another theoretically possible way by which the methylenedioxy group may contribute to carcinogenicity is through the release of formaldehyde Being a cyclic acetal, the methylenedioxy group may, under acidic conditions, be degraded to diol and formaldehyde (which is reactive and carcinogenic, see

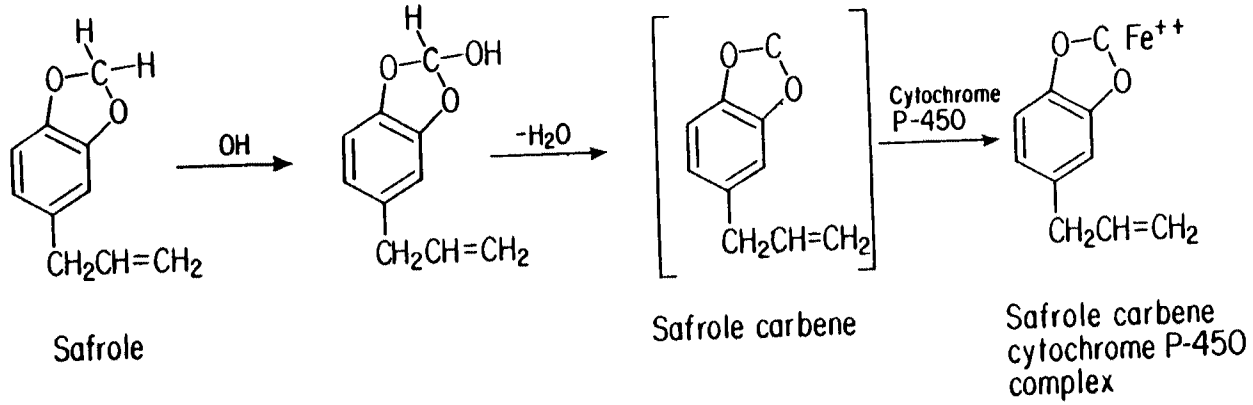


Fig. 15. Proposed possible mechanism of formation of a safrole carbene cytochrome P-450 complex [Adapted from C. Ioannides, M. Delaforge and D. V. Parke: Food Cosmet. Toxicol. 19, 657 (1981)].

Section 5 2 1 7 1) The induction of forestomach tumors in mice by dihydrosafrole lends some support to this hypothesis The *in situ* release of reactive compound at or near the target site is expected to be more hazardous than the administration of the compound at a distant site However, this hypothesis does not explain why other methylenedioxy derivatives discussed in Section 5 3 2 4.1 2 are incapable of inducing stomach tumors.

5 3 2 4 5 Environmental Significance

Safrole and its congeners including the cinnamyl compounds may occur in the environment as naturally occurring constituents of food materials of plant origin, of spices and herbal medicines, as synthetic food additives, as cosmetics and toiletry ingredients, or as pesticide residues. The natural occurrence, economic production and uses of a number of these compounds are summarized in Table LXVII In particular, the essential oils, extracted by steam distillation or solvent extraction from parts of plants (e g., root, rhizome, seed, bark), contain high concentrations of alkenylbenzene and related compounds. For example, as much as 93% of a sample of Brazilian oil of sassafras is safrole (149) Plants from different parts of the world may contain widely different quantities of alkenylbenzene compounds. The oil of calamus extracted from Indian Acorus plants contains about 80% β-asarone (5, 145), whereas that of European variety contains only 5% β-asarone (146) An analysis of myristicin content in essential oils extracted from roots of 24 different varieties of cultivated parsnip showed a wide range of 18.3 to 66.2% (138) A number of essential oils were or still are extensively used as food flavoring agents or as cosmetics ingredients Besides natural occurrence, there is some evidence that treatment of oranges with certain abscission agents* (such as cycloheximide, 5-chloro-3-methyl-4-nitro-1H-pyrazol, glyoxal diamine) may cause the appearance of eugenol, methyleugenol, *cis*- and *trans*-

* for footnote, see p 383

methylisoeugenol, elemicin and isoelemicin in orange juices at levels of 4-40 ppb and in essential oils (150) The mechanism of enhancement of this chemically induced environmental formation of alkenylbenzene compounds is unknown Several methylenedioxy compounds structurally related to safrole (e g., piperonyl butoxide and sulfoxide), are used as insecticides or insecticide synergists. There is little information on the extent of food contamination by residues of these pesticide synergists A study of the environmental fate of methyleugenol, an insect attractant useful in control of fruit fly, showed $t_{1/2}$ of 16 hours in soil and 24 hours in water at 22°C When topically applied to the surface of tomatoes, about 3.8% of the dose was still present after 24 hours, none was detected after 5 days (151).

Human exposure to alkenylbenzne and cinnamyl compounds occurs mainly through ingestion of foodstuffs or drugs to which naturally occurring or synthetic flavor additives are added. Safrole, isosafrole and dihydrosafrole were used as flavoring agent in root beer prior to the banning of their use in foods by the U S. Food and Drug Administration (FDA) in 1961 To some extent, sassafras bark is still being used as herbal tea or as an ingredient thereof, and in folk medicine (152). According to Segelman et al (153), a single herbal tea bag sold by some producers may contain 2 5 mg sassafras bark equivalent to 200 mg safrole. If completely absorbed, the consumption of such tea could lead to exposure to a potentially hazardous dose of 3.0 mg/kg body

*Abscission agents are synthetic plant-growth regulating chemicals which promote the separation or shedding of a plant part (e g , leaf, flower, fruit or stem) from the parent plant They are used (a) to thin (shed) fruits in trees with too many fruits, so that the size and quality of the remaining fruits may be improved, (b) to shed leaves just before mechanical harvesting of crops such as cotton, and (c) to promote separation of mature fruits from tree branches for easier picking.

weight (a total subcutaneous dose of approximately 66 mg/kg body weight is carcinogenic in infant mice). It is interesting to note that safrole is detected in the expired air of a group of 62 nonsmoking volunteers with no known exposure to safrole suggesting that the compound is being bioaccumulated involuntarily in the general population (154) β-Asarone was at one time used as a flavoring agent (confering bitter flavor) in liqueurs and vermouth at levels of up to 10-30 ppm (147) before it was banned by FDA in 1967 Its use is still permitted in some countries (137). Calamus drugs containing β-asarone are being used in Europe Several commercial calamus drugs were shown to be mutagenic in the Ames test (67), long-term use of these drugs may represent a carcinogenicity risk. Estragole, the major constituent of oils of tarragon and basil, has been used as a flavoring agent in gourmet types of vinegar (155) as well as in a variety of food products (candy, chewing gum, ice cream) at levels of 2-50 ppm (135) Cinnamyl anthranilate has been used as a synthetic flavoring agent (to imitate grape or cherry flavor) in the United States and in Europe. It is added to a variety of food products (e g , chewing gums, ice cream, baked goods, gelatin, beverages) at levels of 1 7 to 730 ppm (135). The banning of this compound was reported to be under consideration by FDA (156). The World Health Organization (157) recommended that no acceptable daily intake (ADI) should be allocated to any of the above food additives

Among alkenylbenzene and cinnamyl compounds with equivocal or no evidence for carcinogenicity, trans-anethole is the most widely used food additive It is reported to be used in a variety of food products such as nonalcoholic beverages (11 ppm), alcoholic beverages (1,400 ppm), ice cream (26 ppm), candy (340 ppm), baked goods (150 ppm), and chewing gums (1,500 ppm) to impart the popular aniseed flavor (135). The estimated annual consumption of the com-

pound as a food additive in the United States is 70 tons, which represents an average daily intake of 60 ug per person (144) In France, as much as 200 tons of the compound is used annually because of the popularity of alcoholic and non-alcoholic aniseed beverages (40). Cinnamaldehyde is another popular food flavoring (cinnamon) agent used in food products at levels ranging from 7 7 to 4,900 ppm (135) The World Health Organization (157) recommended that the acceptable daily intake for humans should not exceed 0.7 mg/kg body weight. Myristicin is the principal physiologically important ingredient of oils of nutmeg and mace, which enjoyed high esteem in the early Middle Ages in the Arab world and in India as almost a panacea for treatment of a wide variety of ailments such as toothache, dysentery, cholera, rheumatism, halitosis and skin diseases (137) The medicinal use of these spices declined sharply during the 19th century when their narcotic, toxic and hallucinogenic properties were discovered Myristicin is also found in many edible plants and in black pepper (see Table LXVII), its concentration in parsnip root is particularly high (138)

The literature on the potential carcinogenicity risk of human exposure to naturally occurring alkenylbenzene compounds is rather scanty Morton (152, 158, 159) reported that cancer of the esophagus was the most common type of tumor among male cancer patients in South Carolina (U S.A). She suggested that induction of esophageal cancer may be associated with the common use of sassafras tea (which contains safrole) as a folk medicine for cure of fever, pneumonia, bronchitis and mumps among the native residents, particularly by the black population. In this respect, it is important to note that dihydrosafrole, a synthetic derivative of safrole, induces indeed esophageal cancer in rats. Although safrole itself is predominantly a hepatocarcinogen in rodents, its potential to induce esophageal cancer should not be discounted

Table LXVII
Environmental Occurrence, Production and Economic Uses of Safrole and Related Compounds

Compound	Natural Occurrence/ Economic Production	Uses	References
Safrole[a]	Sassafras[b], sweet basil, cinnamon, nutmeg, mace, ginger, black pepper	Flavoring agent in root beer[c], tea, folk medicine	(7, 135)
Myristicin	Nutmeg[b], mace[b], parsnip[b], black pepper, carrot, parsley, celery, dill	Flavoring agent, herbal medicine	(136-140)
Dill apiol	Dill, Indian dill	Flavoring agent	(79)
Parsley apiol	Parsley, fennel, sassafras	Flavoring agent	(79)
Isosafrole[a]	Ylang ylang	Flavoring agent[c], fragrance, production of piperonyl butoxide	(7, 10, 135)
Dihydrosafrole[a]	Synthetic, U.S production/ import volume in 1977 4 million lb	Flavoring agent[c], production of piperonyl butoxide	(7, 141)
Piperonyl butoxide	Synthetic, U S production volume in 1978 334,000 kg	Insecticide synergist	(8, 142)
Piperonyl sulfoxide	Synthetic	Insecticide synergist	(88, 142)
Estragole[a]	Tarragon[b], sweet basil[b], anise, U S production volume in 1977 >1,360 kg	Flavoring agent, fragrance	(135, 142)

Table LXVII (continued)

Compound	Natural Occurrence/ Economic Production	Uses	References
Methyleugenol[a]	Sweet bay, cloves, lemongrass, black pepper, U S production volume in 1977 >1,800 kg	Flavoring agent, fragrance, insect attractant	(79, 136, 143)
Elemicin	Nutmeg, elemi gum, sassafras	Flavoring agent	(79)
trans-Anethole	Anise[b], fennel[b], coriander, U S production in 1977 1 1 million kg	Flavoring (aniseed) agent, fragrance	(10, 135, 143)
β -Asarone[a]	Calamus (Acornus calamus) plants[b], plants of Asarum and Asiasarum genera	Flavoring agent[c] in bitters, liqueurs and vermouths, herbal medicine	(5, 137, 145-148)
Eugenol	Cloves[b], allspice, artichoke, pimenta, bayleaf, cinnamon	Flavoring agent, fragrance, ingredient in temporary denture fillings	(135)
Cinnamaldehyde	Cinnamon[b], also produced synthetically, U S production volumes in 1977 910 kg	Flavoring (cinnamon) agent for foods, beverages, pharmaceuticals and liqueurs	(18, 135)
Cinnamyl anthranilate[a]	Synthetic, U S production/ import volume in 1977 454 kg	Flavoring (grape, cherry) agent[d], cosmetics fragrance	(9, 135)

[a]Clear evidence of carcinogenicity demonstrated, World Health Organization recommends that no acceptable daily intake should be allocated

[b]The compound is a (the) major constituent in essential oil derived from the plant

[c]Use now banned in U S by the Food and Drug Administration

[d]Under consideration for banning

Besides safrole, sassafras and other herbal teas may contain other carcinogenic substances, such as tannin (see Section 5 3.2 6 3) The possibility of syncarcinogenesis among these food components should be investigated

In contrast to the scanty epidemiologic literature on alkenylbenzene compounds, there is ample epidemiologic evidence to indicate excess cancer (particularly nasal adenocarcinoma) risk among woodworkers exposed to wood dust (especially furniture and cabinet makers) throughout the world (rev. in 160). In addition, the high incidence of nasopharyngeal cancer among the Southern Chinese and the Highland Kenyans was postulated to be associated, in part, with chronic exposure to wood smoke (161) Although most of the above mentioned population groups at risk are often simultaneously exposed to other industrial or environmental chemicals or agents (e.g , formaldehyde, benzene, herbicides, paint solvents, nitrosamines, Epstein-Barr virus) with carcinogenic potential, it is believed that at least part (if not most) of the carcinogenic effects is contributed by naturally occurring chemicals present in wood dust or wood smoke Despite some early work, the search for naturally occurring carcinogens in woods is still in the exploration stage (94, 162) The potential carcinogens include phenolic and flavonoid compounds (see Sections 5 3 2 6 2 and 5 3 2 6.3), substituted cinnamyl compounds and podophyllotoxin. Sinapaldehyde (3,5-dimethoxy-4-hydroxycinnamaldehyde) and related compounds have been detected in the smoke of Chinese incense derived from sandal wood, in eucalyptus wood abundant in Kenya and in other angiospermous woods (94, 163-165). A closely related compounds, 3,4,5-trimethoxycinnamaldehyde, has been shown to be a nasal carcinogen in rats (see Section 5.3.2 4.3.5) Podophyllotoxin is suspected to account for the apparent carcinogenic effect of red cedar (Juniper virginiana) wood bedding in the induction of "spontaneous" tumors in C3HA^{vy} mice (see Section 5 3 2.4.3 5)

Whether these compounds could contribute to the apparent human carcinogenicity of wood dust or wood smoke remains to be elucidated.

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