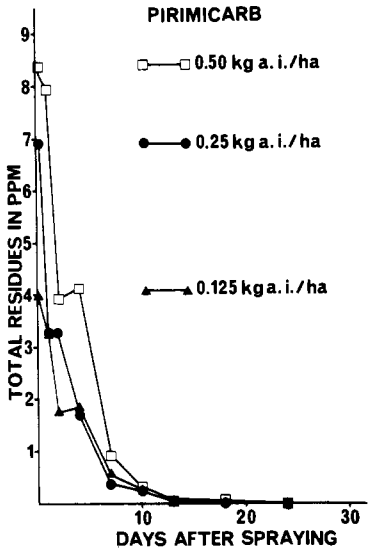


**Figure 2.** Total residues (pirimicarb + (methylamino)pirimicarb and formyl(methylamino)pirimicarb) in asparagus ferns after foliar applications of pirimicarb at 0.125, 0.25, and 0.5 kg AI/ha.

ppm) remained after 24 days. The rapid conversion to toxic metabolites and the disappearance of total residues also occurred in lettuce (Szeto et al., 1984) indicating that pirimicarb is readily degradable in the environment. Spears harvested in the following spring from each field trial were analyzed for residues. None could be detected at the limit of 0.002 ppm.

Based on the results of field trials in 1982 and 1983, it is evident that residues of dimethoate, pirimicarb, and their toxic metabolites degraded readily in asparagus ferns. After insecticide applications, the total residues including toxic metabolites decreased to negligible levels (<0.1 ppm) in 13 days for pirimicarb and in 24 days for dimethoate. The likelihood of finding toxic residues in marketable asparagus spears resulting from using dimethoate and pirimicarb at rates tested in our studies appears to be remote. The facts that contributed to this conclusion are that all insecticide applications are made after harvesting and treated ferns die down in fall, and the demonstration in our studies that the marketable spears harvested in the following spring showed no sign of measureable residues of these insecticides.

**Registry No.** Dimethoate, 60-51-5; pirimicarb, 23103-98-2; dimethoxon, 1113-02-6; (methylamino)pirimicarb, 30614-22-3; formyl(methylamino)pirimicarb, 59333-83-4.

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# Structural–Bioactivity Relationship for Tumor Promotion by Cyclopropenes

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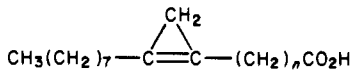
Studies with trout measuring the carcinogenic and promotional activity of various cyclopropenoid compounds show that these componds can be divided into two distinct groups: highly active and completely inactive. 1,2-Dioctylcyclopropene and methyl sterculate show equal and significantly higher activity than methyl malvalate. Methyl dihydrosterculate and 1,2-dialkylcyclopropenes with a chain length of 16 and fewer carbons are completely inactive in this system. Sterculic acid is also more active than malvalic toward depressing cytochrome P-450 levels. It is suggested that expression of activity requires incorporation of the intact cyclopropenoid fatty acid into biomembranes.

Two unusual fatty acids, sterculic and malvalic acids, have a highly strained and reactive cyclopropene ring forming the center of their 18 and 17 carbon chains. The




sterculic acid, $n=7$
malvalic acid, $n=6$

potential intake of these compounds in the human diet may be considerable. Sterculic and malvalic acids are found in the seed lipids from plants of the order Malvales, which includes cotton, kapok, okra, limes, durian, and china chestnuts (Carter and Frampton, 1964). The nut *Pachira aquatica* contains 58% lipid, 26% of which consists of cyclopropene fatty acids (CPFA) (Bohannon and Kleiman, 1978), and is extensively consumed by humans in Brazil and the West Indies. *Sterculia foetida* beans, over half lipid of which 65% is CPFA, are occasionally consumed in India and tropical countries. In addition there are numerous cultivated and uncultivated plants of the order Malvales which have not been surveyed for CPFA. Many of these plants are in the human diet or are consumed by animals in the human food chain. Although human distribution and metabolism of dietary CPFA are unknown, CPFA in the diet of rats are passed through material milk to infant pups (Nixon et al., 1977a).

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The carcinogenic and synergistic activities of CPFA have been examined in only three animal species, trout, rats, and mice. CPFA's have apparent primary heptatocarcinogenic activity in trout (Hendricks et al., 1980) which may be due to their extremely powerful promotional (Hendricks, 1981) ability, acting upon an otherwise undetectably low rate of spontaneous tumors. Tinsley and co-workers (1981 and 1982) observed significant promotional activity of mouse mammary tumors by cottonseed oil as compared with 12 other lipid sources. Tinsley's cottonseed oil was analyzed in this laboratory and found to contain 0.5% CPFA. Several workers (Lee et al., 1969; Wells et al., 1974) have observed a promotional effect for CPFA in rat livers. Nixon and co-workers (Nixon et al., 1974) failed to observe a statistically valid effect in rats. However none of the above rat experiments are conclusive due to excessive variation between groups or very small group sizes (e.g., Wells et al. used 6 rats per group).

Little information exists on the structural requirements for biological activity of cyclopropenes. Shenstone and Vickery (1959) first noted the lesser activity of malvalic acid and inactivity of dihydrosterculic acid with respect to visually estimated pink discoloration of avian eggs. Nordby (1962, 1963) found sterculyl alcohol, sterculyl ether, and 1,2-diocytylcyclopropene (sterculene) to be as active as sterculic acid in pink egg discoloration formation. Deutschman (1964) confirmed the activity of sterculene. Dihydrosterculic acid, a cyclopropane, generally does not affect normal fatty acid metabolism (Shenstone and Vickery, 1959; Wood and Reiser, 1965; Masson, 1957) as does sterculic acid, except, possibly, at high dietary concentrations (Kircher and Heywang, 1966).

Improved methods for the separation (Pawlowski et al., 1981) and analysis (Loveland et al., 1983) of sterculic and malvalic acids now make it possible for us to quantitatively compare the structural requirements necessary for the promotional activity of this group of compounds. Rainbow trout were chosen for this study due to their numerous advantages for the study of carcinogenesis (Hendricks, 1981 and 1982) and their sensitivity to the promotional activity of cyclopropenes.

## EXPERIMENTAL SECTION

Methyl malvalate and sterculate were prepared from *Hibiscus syriacus* and *Sterculia foetida* oils, respectively, by spinning-band distillation (Pawlowski et al., 1981). The 1,2-dialkylcyclopropenes were synthesized by addition of ethyl diazoacetate to the appropriate 1,2-dialkylacetylene followed by decarbonylation and hydride reduction as reported previously (Pawlowski et al., 1972). Methyl dihydrosterculate was prepared by the addition of Simmons–Smith's methylene (LeGoff, 1964) across the double bond of methyl oleate followed by spinning band distillation. NMR spectroscopy shows that the stereochemical integrity of the *cis*-oleate was preserved during methylene addition yielding a *cis*-cyclopropane ring in the methyl dihydrosterculate. Dihydrosterculate isolated from *Sterculia f.* oil and the major rat urinary metabolite both possess their cyclopropane ring in the cis configuration.

Shasta strain rainbow trout (*Salmo gairdneri*) were spawned and hatched in these laboratories as previously described by Hendricks (1981 and 1982) and Sinnhuber et al. (1977). After swimup, fry were divided into duplicate tanks of 100 each and initiated to either control or experimental diets. The cyclopropenoid compounds and/or aflatoxin $B_1$ were incorporated into a semipurified diet of 49.5% casein, 15.6% dextrin, 10% salmon oil, 8.7% gelatin, and mineral and vitamin mixes, which meets all the nutritive requirements of trout. Since one gram of dry diet produces approximately one gram of wet weight trout growth, 50 ppm addition to the diet is roughly equal to 50 mg/Kg dosage at any time. Food consumption was carefully monitored and the entire lot of fish weighed regularly. Samples of the population were taken at 6, 9, and 12 months, and neoplasia diagnosed by gross and histological procedures. Tumors were noted for both number and volume, and histological damage is described in the text.

P-450 studies: Trout were raised on the control diets until three months of age, then placed on diets containing 40, 50, 60, or 70% casein. Beginning at four months of age, 20-day feeding trials were begun in which trout from each diet were fed the same protein level plus a level of cyclopropenoid listed in Table III. Between 30 and 40 trout were used in each trial, and trials were conducted over a period of 5 months. Trout grew from an average weight of 1.5 gm at the beginning to approximately 23 gm at the conclusion of these feeding trials.

On day 21 of the feeding trials, trout were killed by a cranial blow and their livers immediately excised. Livers from trout on the same diet (30–40 livers) were combined and homogenized in 6 volumes (w/v, 1:6) of 0.15 M KCl–0.01 M potassium phosphate buffer, pH 7.4. The homogenates were centrifuged for 15 min at 200*g* and then for 30 min at 9500*g*. The resulting postmitochondrial supernatant was centrifuged at 105 000*g* for 60 min. The microsomal pellet obtained was resuspended in 0.1 M potassium phosphate buffer pH 7.4 so that 6.0 mL contained the equivalent of 1.0 gm of liver.

Protein content of microsomes was determined by the method of Lowry et al. (1951) as modified by Miller (1959). Cytochrome P-450 activity was determined by using a Beckman Acta CIII spectrophotometer with a scattered transmission accessory. The assay and calculations were done according to the procedure described by Mazel (1971).

## RESULTS AND DISCUSSION

All compounds tested can be distinctly classified into one of two categories, highly active or completely inactive. Biologically active cyclopropenes produce abnormal pathology in rainbow trout livers and promote aflatoxin $B_1$-initiated hepatocellular carcinomas. Typical histological damage caused by active cyclopropenes includes pale, fibrous livers with bile duct hyperplasia, large cells with parallel arrays of endoplasmic reticulum resembling striations, necrotic cells, and high mitotic index (Lee et al., 1968; Struthers et al., 1975). The extent of these observed abnormalities are semiquantitative and correlate with the hepatocellular carcinoma incidence reported in Tables I and II.

Methyl sterculate, methyl malvalate, and sterculene show biological activity (Tables I and II). Each of these substances causes extensive liver damage and promotes an increased number of trout hepatocellular carcinomas. We also observed increased tumor volume and early appearance (data not given) proportional to the incidence for these compounds. Methyl malvalate, 17 carbon chain, exhibits significantly less activity than does the 18 carbon chain sterculate, which suggests strict structural requirements for biological activity in this system. As shown by dihydrosterculate (Table II), saturation of the cyclopropene ring leads to total inactivity.

Except for the 1,2-dioctyl compound, all the 1,2-dialkylcyclopropenes tested were totally inactive toward the formation of abnormal histology, tumor promotion, and tumor induction.

1,2-Dioctylcyclopropene exhibits activity equal to methyl sterculate, the most biologically active cyclopropene (Table I). This is not surprising since trout (Eisele et al., 1982),

**Table I. Tumor Incidence in Rainbow Trout Fed Cyclopropenes for 12 Months**

| compound and level | hepatocellular carcinoma no. (%) | hepatocellular carcinoma when fed with 2 ppb aflatoxin $B_1$ no. (%) |
|---|---|---|
| control diet | 0/120 (0) | 33/117 (28) |
| 50 ppm methyl malvalate | 1/116 (1) | 63/119 (53) |
| 200 ppm methyl malvalate | 10/118 (8) | |
| 50 ppm methyl sterculate | 15/118 (13) | 112/112 (100) |
| 50 ppm methyl sterculate | 6/116 (5) | 57/58 (98) |
| 50 ppm 1,2-dioctylcyclopropene[a] | 5/80 (6) | 79/80 (99) |
| 50 ppm 1,2-dioctylcyclopropene[a] | | 106/106 (100) |

[a] Sterculene.

**Table II. Tumor Incidence in Rainbow Trout Fed 1,2-Dialkylcyclopropenes for 12 Months**

| cyclopropene compound fed at 100 ppm | hepatocellular carcinoma no. (%) | hepatocellular carcinoma when fed with 4 ppm aflatoxin $B_1$ no. (%) |
|---|---|---|
| control | 0/80 | 57/80 (71) |
| 1,2-dipropyl- | 0/80 (0) | 64/80 (80) |
| 1,2-dipentyl- | 0/80 (0) | 30/40 (75) |
| 1,2-dihexyl- | 0/80 (0) | 43/80 (54)[d] |
| 1,2-diheptyl- | 0/80 (0) | 47/80 (59)[d] |
| 1,2-dioctyl-[a] | 4/40 (10) | 79/80 (99)[e] |
| dihydrosterculate[b] | 0/79 (0) | 57/80 (71) |
| 1,2-dihexyl-[c] | 0/80 (0) | 66/106 (62) |
| 1,2-diheptyl-[c] | 0/80 (0) | 61/105 (58) |
| control[c] | 0/80 (0) | 65/108 (60) |

[a] Sterculene. [b] A cyclopropane fatty acid. [c] Year 2 of this study. [d] Significantly less than control $P < 0.05$. [e] Significantly greater than control $P < 0.05$.

like most animals (McCarthy, 1964), have an efficient $\omega$-oxidation system. It is quite probable that the animal $\omega$-oxidizes sterculene to sterculate and the observed response is simply a response to sterculic acid. The inactivity of the other compounds demonstrates that the presence of the 1,2-dialkylcyclopropene function alone is insufficient for biological activity.

During the first year of this study 1,2-dihexyl- and 1,2-diheptylcyclopropene did not enhance aflatoxin $B_1$ hepatocarcinogenicity, and in fact appeared so slightly inhibit carcinogenicity (Table II) although these hydrocarbons had no effect upon liver histology. Repeating this study a second year (Table II, bottom) produced results nearly identical with controls.

Presumably, all of the 1,2-dialkylcyclopropenes undergo $\omega$-oxidation by the hepatic microsomes as has been shown for other straight chain hydrocarbons (McCarthy, 1964) and suggested in this study by the activity of sterculene and the high $\omega$-oxidation activity in trout (Eisele, 1982). Their inactivity follows the pattern set by methyl malvalate, i.e., rapidly decreasing activity with decreasing chain length.

Dietary cyclopropenes are reported to significantly decrease the activity of the cytochrome P-450 system in trout (Eisele et al., 1978 and 1983). Here we report that 50 ppm dietary methyl sterculate caused approximately 35% reduction of hepatic cytochrome P-450 levels, while 50 ppm dietary methyl malvalate reduced this system only 18% as compared with control trout (Table III). Apparently, CPFA potency for promotion and for P-450 depression are both dependent on alkyl chain length.

The level of dietary protein was of concern in this study since Lee et al. (1978) reports a decrease in tumor size with decreasing dietary casein for aflatoxin $B_1$ induced hepatoma in trout. The smaller tumors in Lee's (1978) study may be the result of poor growth due to insufficient protein (Hendricks, 1982). Table III also shows that CPFA reduction of the P-450 system in trout is little influenced by level of dietary casein.

The accumulating evidence from CPFA research leads us to assemble the following observations: (*1*) The only identified steps in the metabolism of [$^{14}$C]sterculic acid are ring saturation and $\alpha$-, $\beta$-, and $\omega$-oxidation (Eisele et al., 1977 and 1979; Nixon et al., 1977b). Thus, any metabolism of sterculic acid, beyond $\alpha$-oxidation to malvalate, leads to inactivity, and metabolic activation appears unnecessary. (*2*) Dietary CPFA are incorporated into an animal's tissue (Shenstone and Vickery, 1959; Nixon et al., 1977b; Roehm, 1968) at near the dietary level and these CPFA are also incorporated into the Phospholipids of microsomal and plasma membranes (Einerson, 1982). (3) Preliminary studies with [$^{14}$C]sterculic acid fail to detect any label bound to DNA or any specific membrane protein. (However, binding of a small fraction to DNA below our level of detection could be significant.) (4) Microsomal enzyme activities are depressed by CPFA, suggesting that synergism by CPFA may not require enhanced activation of syncarcinogens. However the dose–response relationships between alteration of liver aflatoxin $B_1$–DNA adduct formation (Bailey et al., 1984), microsomal enzyme depression, and tumor enhancement by CPFA have not been systematically investigated. (5) During the isolation of metabolic products, we have empirically noticed that less malvalic acid relative to sterculic is incorporated into hepatic phospholipids, and this relative difference appears to correlate to their different promotional activities reported here. This leads us to propose that CPFA exhibit their promotional activity via incorporation of the intact cyclopropene ring into lipid membranes. Thus, sterculate is more active than malvalate because of a higher preference for sterculate incorporation into these membranes. The inactive cyclopropenoids probably are not incorporated into membranes at all and this supposition will soon be tested.

We cannot discount the possibility that bioactivity of various CPFAs is related to the distance between the carboxyl group and cyclopropene ring. However, $\omega$-oxidation of diheptylcyclopropene would produce the same amount of separation between the carboxyl and cyclopropene functions as malvalate, yet the diheptyl compound is inactive at comparable dietary levels. The mechanisms by which cyclopropenes incorporated into biomembranes cause promotion of carcinogenesis are currently under investigation.

**Table III. Effect of CPFA on Cytochrome P-450 Content[a] at Different Levels of Dietary Casein**

| level CPFA | casein level 40% | 50% | 60% | 70% | combined protein levels |
|---|---|---|---|---|---|
| 0 CPFA | 0.242 ± 0.134 (3)[b] | 0.265 ± 0.103 (4) | 0.167 ± 0.056 (2) | 0.226 ± 0.185 (4) | 0.204 ± 0.105 (23)[c] |
| 50 ppm malvalate | 0.171 ± 0.074 (2) | 0.202 ± 0.207 (2) | 0.083 | 0.129 ± 0.043 (2) | 0.168 ± 0.111 (7) |
| 50 ppm sterculate | 0.151 ± 0.008 (2) | 0.128 ± 0.054 (2) | 0.129 ± 0.005 (2) | 0.130 ± 0.019 (2) | 0.134 ± 0.024 (8)[c] |

[a] Nanomoles of P-450/mg microsomal protein. [b] Mean ± standard deviation (number of samples, 30–40 livers/sample). [c] Significant difference ($P < 0.05$), $t$ test.

**Registry No.** Sterculic acid, 1089-40-3; methyl steculate, 3220-60-8; methyl malvalate, 5026-66-4; methyl dihydrosterculate, 10152-62-2; cytochrome P-450, 9035-51-2; 1,2-dipentylcyclopropene, 54467-84-4; 1,2-dipropylcyclopropene, 10306-92-0; 1,2-dihexylcyclopropene, 35365-52-7; 1,2-diheptylcyclopropene, 35365-53-8.

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# Carbon Isotope Ratios in Natural and Synthetic Citric Acid as Indicators of Lemon Juice Adulteration

Landis W. Doner

Citric acid from pure lemon juices possess a mean $\delta$ $^{13}C$ value of −24.1‰ (parts per thousand). Commercial citric acid is produced by fermentation of $C_3$ plant, $C_4$ plant, and hydrocarbon sources; its $\delta$ $^{13}C$ value is related predictably to the source. That from $C_4$ sources can be readily detected when added to lemon juice. Only the presence of a high level of the sample of hydrocarbon derived citric acid analyzed in this study could be useful as an indicator of adulteration. Sugars may also be added in the preparation of adulterated juices in order to maintain brix–acid ratios appropriate for pure lemon juice; they can be analyzed for $\delta$ $^{13}C$ to test whether $C_4$ plant derived sugars such as those in high fructose corn syrup or cane syrup are present.

## INTRODUCTION

Single-strength lemon juice contains, on the average, 9.3 g of soluble solids/100 g of juice, of which over 60% is citric acid and over 20% is sugars. Adulteration of lemon juice with citric acid has been a long-standing problem, since lemon juice and concentrates are sold on the basis of titratable acidity, calculated as citric acid (Swisher and Swisher, 1980; Petrus and Vandercook, 1980). Citric acid is relatively inexpensive, being produced by large-scale microbial fermentations of a variety of sources, including beet and cane molasses, corn sugars, and paraffin. Stable

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