The Addition of Mercaptans to Methyl Sterculate and Sterculene: An Hypothesis Concerning the Nature of the Biological Activity Exhibited by Cyclopropene Derivatives

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Abstract

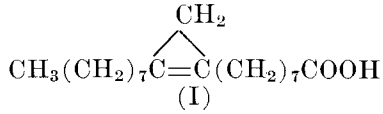
Lipids that contain a cyclopropene ring have been found to be biologically active when incorporated into the diet of laying hens. Methyl sterculate and sterculene (1,2-di-n-octylcyclopropene) are examples of these compounds. When added to dilute solutions of methyl mercaptan and β-mercaptopropionic acid, the sulfhydryl group added to the double bond of the cyclopropene ring. The cyclopropyl methyl and 2-carboxyethyl derivatives were isolated and their structure established. This reaction of sulfhydryl groups with the cyclopropene ring may have its counterpart in the animal's body, and could be the cause of the physiological effects that are observed when cyclopropene derivatives are fed to laying hens.

Introduction

During the last decade of the 19th century the Halphen test was developed for the detection of cottonseed oil (5). In 1928 Sherwood observed that the incorporation of this oil into the diet of laying hens caused their eggs to discolor on storage; the whites turned pink and the yolks a salmon-orange color (19). Lorenz et al. (7a,b,c) found that the yolk fat from pink eggs gave a positive Halphen test; they suggested that the substance in cottonseed oil giving the Halphen test was responsible for the pink discoloration.

Schaible and Bandemer (13) found that the pink color was caused by diffusion of the conalbumin into the yolk of the egg, and formation of a colored iron conalbumin complex. They suggested that ingestion of cottonseed oil by the hen caused an increase in the permeability of the vitellin membrane surrounding the yolk, thereby allowing diffusion of yolk and white constituents to occur.

In 1952 Nunn (11) established the structure of the major fatty acid component of *Sterculia foetida* oil, the one that is responsible for the Halphen test, as ω-(2-n-octyl-cycloprop-1-enyl) octanoic acid, which he named sterculic acid (I).



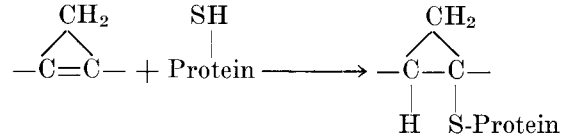
(I)

This acid, together with larger amounts of a C_{18} cyclopropenoid fatty acid, ω-(2-n-octyl-cycloprop-1-enyl) heptanoic acid, called malvalic acid (18,20), are also found in very small quantities in the glycerides of cottonseed oil, and cause the latter to give the Halphen test.

The biological activity of sterculic acid has been shown to reside in the cyclopropene ring. Masson et al. (8) demonstrated that 25 mg/day of this acid in the rations of laying hens caused pink egg discoloration. When the cyclopropene ring was destroyed by mild hydrogenation (8), or with gaseous sulfur dioxide or hydrogen chloride (3), both cottonseed oil and *S. foetida* oil no longer gave the Halphen test and pink egg discoloration was eliminated. That the acid or ester group was not required for biological activity was shown by Nordby et al. (10). Sterculyl methyl ether and sterculene (compound 11, see below) were prepared and fed to hens (18 mg/day). After one month in storage the eggs from these hens had turned pink.

Effects other than pink egg formation have been noted by Schneider et al. (14,15,16). A daily dietary intake of 100 mg of *S. foetida* oil by the hen caused 100% embryo mortality in fertile eggs by the 19th day of incubation; an intake of 200 mg of the oil caused the hens to stop laying. They also observed that feeding the oil to sexually maturing pullets retarded comb, ovary and oviduct development, and caused a lack of egg production, enlargement of the gall bladder and liver, and a decrease in the iodine number of the chicken's body fat.

A possible mechanism to account for these diverse biological effects is the reaction of the cyclopropene ring with the sulfhydryl groups present in physiologically active proteins that are intimately associated with lipids. Examples of these are the proteins in membranes, hormones and enzymes, or co-factors involved in fatty acid metabolism. The enhanced reactivity of the double bond in the cyclopropene ring over that of the double bonds present in the ordinary unsaturated fatty acids may cause the former to add sulfhydryl groups readily under physiological conditions; conditions under which the latter are unreactive. This irreversible addition of protein sulfhydryl groups to the cyclopropene ring



would greatly alter the physical and biochemical properties of the protein molecule, and by this alteration produce the gross physiological changes that are observed *in vivo*.

The inhibition of sulfhydryl groups on membrane permeability has recently been demonstrated by Fong et al. (4,17). They showed that the attachment of the disulfide hormone vasopressin to a kidney membrane by formation of a new disulfide bond disrupted the normal disulfide-sulfhydryl interchange (6) in the membrane and increased its permeability. This reversible disulfide formation differs, however, from sulfhydryl inhibition by cyclopropenes; in the latter case a stable sulfide linkage would be formed.

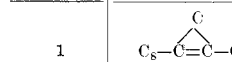
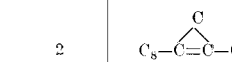
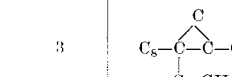
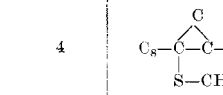
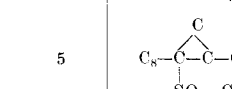
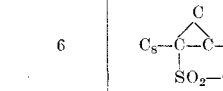
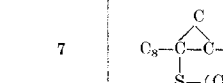
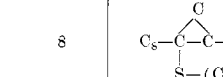
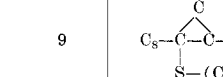
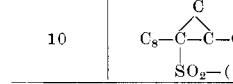
The experiments reported here were designed to test the hypothesis *in vitro*.

Experimental

GLC was performed with the Aerograph A-90-C and Research Specialties Co. instruments, the refrac-

TABLE I

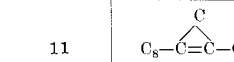
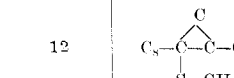
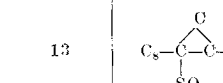
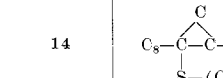
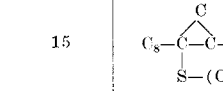
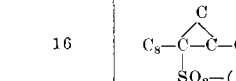
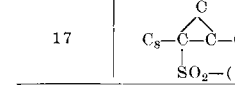
Methyl Sterculate and Its Derivatives

Cmpd. No.	Structure	bp °C/mm	n_D^{20}	d_{20}^{20}	Sapn. Eq.	Neut. Eq.	Calc.	Analysis	
								Calc.	Found
1		125/.05	1.4575	0.8899	305		309	77.86 C 11.76 H	77.80 11.49
2			1.4643	0.9002		294.5	294.5		
3		150/.05	1.4729	0.9370	350		357	70.73 C 11.31 H 8.99 S	71.38 11.31 9.22
4			1.4801	0.9476		343	343		
5		185/.05	1.4738	1.0149	382		389	64.91 C 10.38 H 8.25 S	65.25 10.49 8.06
6			1.4797	1.0290		374	375		
7			1.4818	1.0034	206	412	207 415	66.66 C 10.21 H 7.74 S	66.70 10.15 7.56
8		195/.03	1.4744	0.9762	212		214	67.30 C 10.35 H 7.48 S	67.46 10.17 7.18
9			1.4884	1.0205		206	201		
10			1.4870			211	217		

[a] In the tables, the hydrogen atoms are omitted from the cyclopropene and cyclopropane rings for simplicity; C_8- is $CH_3-(CH_2)_7-$ and $-C_7-$ is $-(CH_2)_7-$.

TABLE II

Sterculene and Its Derivatives

Cmpd. No.	Structure	bp °C/mm	n_D^{20}	d_{20}^{20}	Sapn. Eq.	Neut. Eq.	Calc.	Analysis	
								Calc.	Found
11		100/.05	1.4532	0.8132				86.16 C 13.72 H	86.46 13.53
12		123/.05	1.4690	0.8652				76.82 C 12.90 H 10.26 S	77.03 13.11 10.31
13		155/.06	1.4668	0.9407				69.69 C 11.70 H 9.30 S	71.34 11.73 9.55
14			1.4784	0.9405		374	370		
15		150/.08	1.4712	0.9211	386		384	71.90 C 11.53 H 8.34 S	72.03 11.54 8.51
16			1.4777	1.0130		432	402		
17		212/.17	1.4684	0.9682					

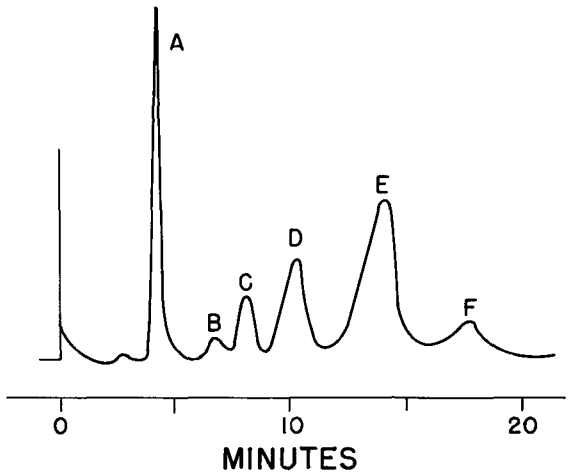


FIG. 1. Separation diagram showing the methyl esters obtained by methanolysis of *Sterculia foetida* oil. (A) Palmitate, (B) stearate, (C) oleate, (D) linoleate, (E,F) sterculate. Five foot ¼ inch diam 20% diethyleneglycol succinate polyester column, 200C, 2 atm He pressure. Aerograph-A90-C, Katharometer detector.

tive indices determined with an Abbe type Zeiss refractometer, the IR spectra obtained with the Perkin-Elmer Infracord 137B, the n.m.r. spectra with the Varian A-60, and the elemental analyses were done by Schwartzkopf Microanalytical Laboratory, Woodside, N. Y. The *Sterculia foetida* nuts were obtained from the Depart. of Forestry, Republic of the Philippines, and the β-mercaptopropionic acid from Evans Chemetics, Inc. Methyl oleate was purified by urea adduction and fractional distillation through a spinning band column.

The physical and chemical properties of all of the compounds prepared in this study are given in Tables I and II.

Methyl Sterculate (Compound 1, Table I): *S. foetida* oil (827 g) was trans-esterified in dry methanol (7.5 liters) with sodium methoxide (prepared from 26 g sodium) for 3 days at room temp. Glacial acetic acid (68 ml) was added, the solution diluted to 15 liters with methanol and urea (1 kg) was added and dissolved at room temp. The solution was cooled to −16C in a freezer, the liquid filtered from the solid and then cooled successively to −30C and −45C in a dry ice-acetone bath. Precipitates from the latter two low temp crystallizations were combined, washed with water to remove urea, and redissolved in methanol (5 liters). When this solution was cooled to −45C it precipitated pure methyl sterculate (206 g). The fractionation procedure was followed by gas chromatography. Although methyl sterculate always rearranged and gave two peaks on the separation diagrams (9), the peaks were readily distinguished from those of the methyl esters of the other fatty acids of *S. foetida* oil (Fig. 1).

Sterculic Acid. (Compound 2, Table I). Freshly distilled methyl sterculate (32.08 g) was saponified at room temp overnight with KOH (15 g) in ethanol (150 ml). Water was added, followed by dilute H_2SO_4. The sterculic acid was extracted with petroleum ether and dried *in vacuo* in a tared flask. The yield was 29.9 g, mp 18.2–19.5C: lit. 18.2C (11), equivalent wt 294.5 (theoretical E. W. 294.5).

Sterculene. (Compound 11, Table II). The procedure of Nordby et al. (10) was modified and scaled up. Methyl sterculate (50 g) in dry ether (500 ml) was reduced with $LiAlH_4$ (6 g). The sterculyl alcohol (45.5 g) was dissolved in dry pyridine (100 ml),

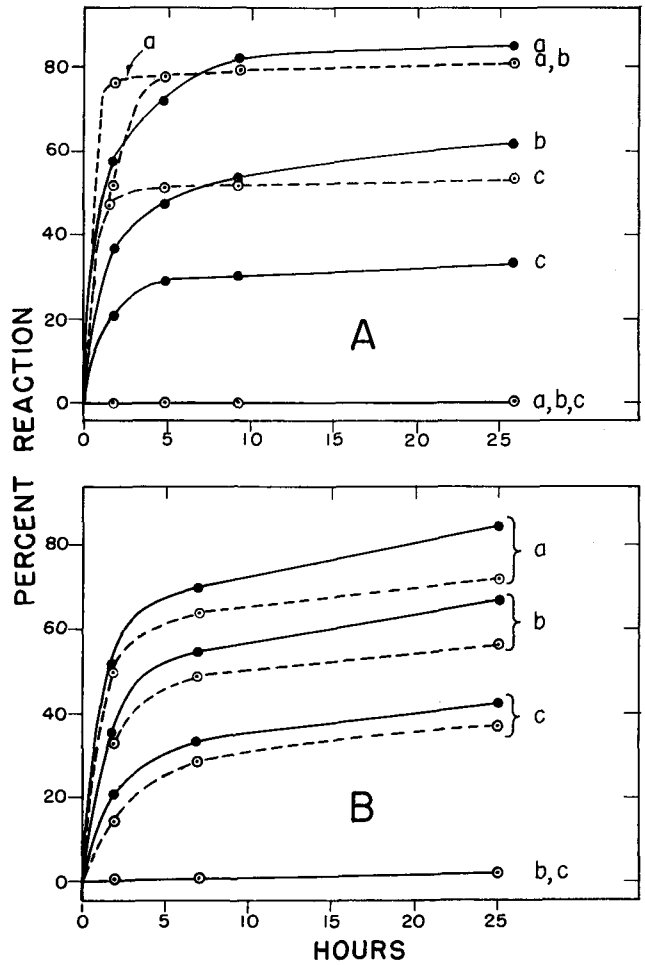


FIG. 2. Reactions of methyl sterculate (——●——), sterculene (– – –⊙– – –), and methyl oleate (——⊙——) in benzene at room temp in the dark with (A) methyl mercaptan and (B) β-mercaptopropionic acid. Concentrations: (a) 0.2 M mercaptans, 0.1 M olefins: (b) 0.1 M mercaptans, 0.2 M olefins; (c) 0.1 M mercaptans, 0.1 M olefins.

cooled on an ice bath, and p-toluenesulfonyl chloride (35 g) was added in four increments over ½ hr. The mixture was stirred and the temp maintained below 20C for 2 hr after which the reaction mixture was set into the ice chest overnight. The solid-liquid mixture was poured into a separatory funnel and mixed with petroleum ether (350 ml) and water (10 ml). The mixture was shaken, the two lower layers discarded and the upper layer washed with water. After drying (sodium sulfate) and removal of solvent, the sterculyl tosylate (72 g) was reduced with $LiAlH_4$ (6 g) in refluxing tetrahydrofuran. Wet ether was added to decompose excess hydride, the organic layer filtered from the inorganic salts and evaporated. The crude sterulene (42 g) was distilled (100C/0.05 mm) to yield 35 g of product.

Rate Studies. Sterculene, methyl sterculate, and methyl oleate were dissolved in benzene or absolute ethanol to form 0.4 molar solutions. Aliquots were mixed with aliquots of standardized solutions of methyl mercaptan or β-mercaptopropionic acid, and of solvent, to give 60 ml reaction mixtures. Samples (10 ml) of the reaction mixtures and of a blank were removed periodically, added to water (20 ml), and their sulfyhydryl concentration determined by titration with 0.1*N* iodine in ethanol to a visual endpoint. Representative results are shown in Figures 2 and 3.

Derivatives from Methyl Mercaptan. Compounds 3,4,5,6,12,13, Tables I and II. Methyl sterculate or

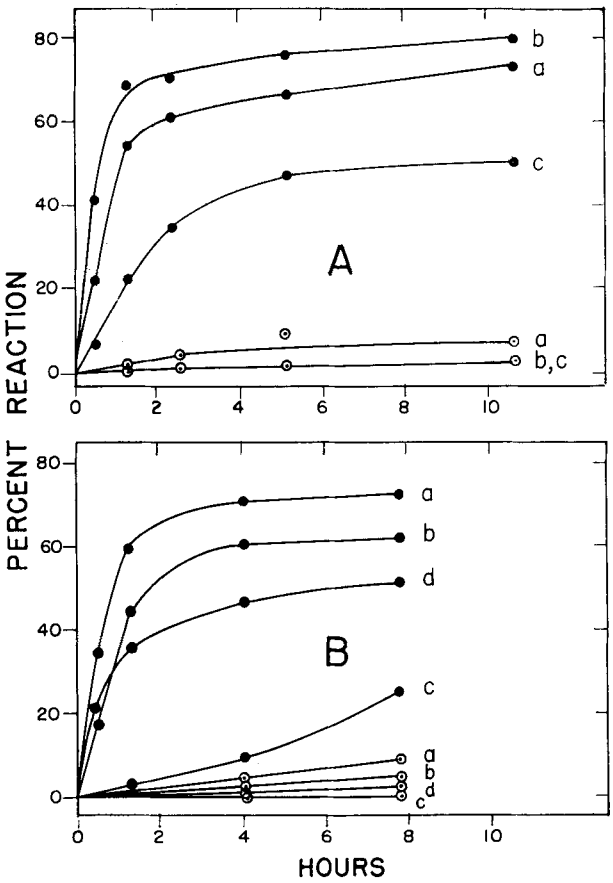


FIG. 3. Reactions of 0.13 M methyl sterculate (——●——) and methyl oleate (——⊙——) in ethanol at room temp with (A) 0.13 M methyl mercaptan and (B) 0.10 M β-mercaptopropionic acid.
Conditions: (a) sunlight; (b) sunlight, 0.006 M benzoyl peroxide; (c) dark; (d) dark, 0.006 M sodium β-mercaptopropionate.

sterculene (10 g) was mixed with methyl mercaptan (5 g) in benzene (100 ml). The reactions were slightly exothermic. After standing overnight at room temp, solvent and excess mercaptan were removed *in vacuo* and the sulfides distilled.

Portions of the products were dissolved in 10–15 volumes glacial acetic acid and oxidized with a threefold excess of 30% hydrogen peroxide at 80C for 1 hr. Water was added and the sulfones extracted with petroleum ether.

Neither the sulfides nor the sulfones rapidly decolorized bromine or permanganate solutions.

Saponification equivalents were determined by hydrolysis of the esters with 1*N* KOH in ethanol, and back titration with 0.5*N* HCl. The acids so obtained were dissolved in ethanol and their neutralization equivalents obtained with 0.5*N* NaOH and a pH meter.

Derivatives from β-Mercaptopropionic Acid. (Compounds 7,8,9,10,14,15,16,17; Tables I and II. Methyl sterculate or sterculene (10 g) was mixed with β-mercaptopropionic acid (10 g) in benzene (100 ml). After standing overnight at room temp, solvent was removed *in vacuo* and the residue taken up in petroleum ether. Excess mercaptan was washed out with water until the aqueous phase was free of sulfhydryl groups as measured by iodine titration. The sulfides were then extracted from the p.e. phase with a small excess of alkali in 50% ethanol. It was necessary to use 50% ethanol rather than water alone because of the stable emulsion that formed when compound 14 was prepared. The sodium salt of this acid was soluble in p.e. The aqueous phases were then acidified and the products extracted with p.e. Not being distillable, they were dried *in vacuo* for analysis.

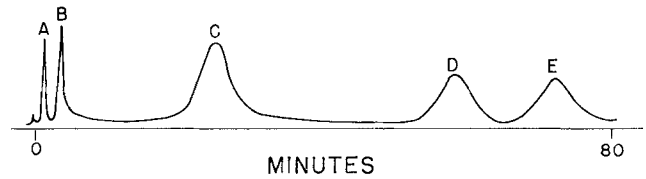


FIG. 4. Separation diagram showing (A) sterculene, (B) compound 12, (C) compound 13, (D) compound 15 (2-carbomethoxyethyl 1,2-di-n-octylcyclopropyl sulfide) and (E) compound 17 (2-carbomethoxyethyl 1,2-di-n-octylcyclopropyl sulfone). Six foot ¼ in. diam 15% diethyleneglycol succinate polyester column, 200C, 195 ml Argon/min. RSCo Instrument, Sr^{90} detector.

The acids were esterified by dissolving them in 10 volumes of methanol containing 7% by weight of boron trifluoride and allowing the reaction to proceed at room temp overnight. The esters were isolated by extraction with p.e. and were distilled. The oxidation to the sulfones and determinations of the saponification and neutralization equivalents were performed as before. The saponification equivalents of the sulfone esters (compound 17 and the dimethyl ester of compound 10) could not be obtained. The sulfones were cleaved by the alkali during the saponifications.

Chromatography of the Derivatives

Gas-Liquid. Compounds 3,11,12,13,15 and 17 passed through diethyleneglycol succinate polyester columns and gave single peaks. After the reaction of sterculene with the mercaptans, a small peak having the same retention time as the starting material was observed. This was probably an impurity in the sterculene; methyl sterculate showed no unreacted material. A separation diagram of sterculene and its volatile derivatives is shown in Figure 4.

Paper Partition. The acids were chromatographed as their ethylamine salts with water saturated butanol and ethylamine vapor in the tank (2). The papers were sprayed with 1% potassium permanganate or 0.2% bromcresol green solutions. A list of the R_f values is given in Table III.

Thin-Layer. The acids and esters were chromatographed on Silica Gel G, the former with a solvent composed of 10% acetic acid, 10% ether, and 80% p.e. (Solvent I); and the latter with 1% acetic acid, 20% ether, and 79% p.e. (Solvent II). The plates were sprayed with 50% H_2SO_4 and the spots developed by heating in a 110C oven for 5 min.

A list of the R_f values of the derivatives on these systems is given in Table III.

Results

In all cases, reactions of the cyclopropene derivatives with mercaptans consumed the sulfhydryl groups and methyl oleate was essentially unreactive. The reactions in dilute solution were accelerated by light, peroxides, and small amounts of base (Fig. 3). Because of this ability to proceed by both free radical and ionic mechanisms, the reactions were erratic and difficult to reproduce. They proceeded toward completion more readily with an excess of mercaptan than with an excess of the cyclopropene derivative and more readily with methyl mercaptan than with β-mercaptopropionic acid (Fig. 2).

The products of the reactions of the two cyclo-

TABLE III

R_f Values of the Compounds by Paper and TLC

Paper		Thin-Layer			
		Solvent I		Solvent II	
Compd. No.	R_f	Compd. No.	R_f	Compd. No.	R_f
7	0.90	2	0.81	1	0.79
14	0.88	14	0.76	15	0.78
2	0.86	4	0.74	3	0.71
4	0.85	7	0.47	8	0.48
6	0.82	9	0.44	17	0.36
16	0.82	16	0.35	7	0.28
9	0.68	6	0.26	5	0.23
10	0.59	10	0.18		

propenes with the two mercaptans were isolated from larger reaction mixtures. They were high-boiling or non-volatile liquids that could be oxidized to sulfones with hydrogen peroxide in acetic acid. The derivatives from β-mercaptopropionic acid could be esterified, those from methyl sterculate saponified without disturbing the sulfide linkage.

The addition of R–SH to the double bond of methyl sterculate can give two positional isomers, the 9 or 10 sulfide. These could not be separated and were treated as a single compound. Because of the symmetry of the sterculene molecule, only a single isomer is possible, and this was attested by the symmetrical curves obtained by GLC of the derivatives of sterculene (Fig. 4).

The IR spectra showed that in all cases after reaction with the mercaptans, sterculene and methyl sterculate lost their characteristic cyclopropene absorption bands at 5.38 and 9.92 μ. The sulfides exhibited a weak band at 9.7 μ (cyclopropane) and in the sulfones this band shifted to 9.5 μ. The latter gave the typical strong sulfone absorptions at 7.7 and 8.85 μ. The spectra of three representative compounds are shown in Figure 5.

The n.m.r. spectra of methyl 1,2-di-n-octylcyclopropyl sulfide (compound 12) and methyl 1,2-di-n-octylcyclopropyl sulfone (compound 13), were run in CCl_4 with tetramethylsilane as an internal standard. The sulfide showed proton absorption peaks at $\tau = 8.03$ (CH_3 —S—), $\tau = 8.53$ (methylene next to the cyclopropane ring), $\tau = 8.72$ (—CH_2—), $\tau = 9.18$ (CH_3—) and $\tau = 9.8$ (cyclopropane hydrogens). The absorption bands of the sulfone were at $\tau = 7.23$ (CH_3 —SO_2—), $\tau = 8.47$ (methylene next to cyclopropane), $\tau = 8.72$ (—CH_2—), $\tau = 9.18$ (CH_3—) and $\tau = 9.72$ (cyclopropane hydrogens). No peaks were observed in the region of olefinic protons.

Discussion

The absence of unsaturation as determined by the bromine and permanganate tests, together with the spectroscopic and analytical data, showed that simple addition of one sulfhydryl group to the double bond of the cyclopropene ring was taking place without rearrangement or ring opening. These reactions to give cyclopropyl sulfides are in contrast to the reactions of the cyclopropene ring with carboxylic acids (12) and hydrogen halides (1) where ring opening with the formation of allylic derivatives has been observed. The mercaptan reactions also proceed under much milder conditions and are primarily free radical rather than ionic in character.

The observed stability of the sulfide derivatives to alkali and acid is useful for the characterization of more complicated systems. Work on the addition of the sulfhydryl group of cysteine to the cyclopropene ring is in progress and work with glutathione and reduced insulin is envisioned. The isolation and characterization of lipid-amino acid fragments from the hydrolysis of animal tissue that correspond to the sterculic acid-cysteine reaction product will be good evidence that the cyclopropene compounds do indeed exert their biological activity *in vivo* by reaction with the sulfhydryl groups of the proteins in the organism, be it the chicken or the egg.

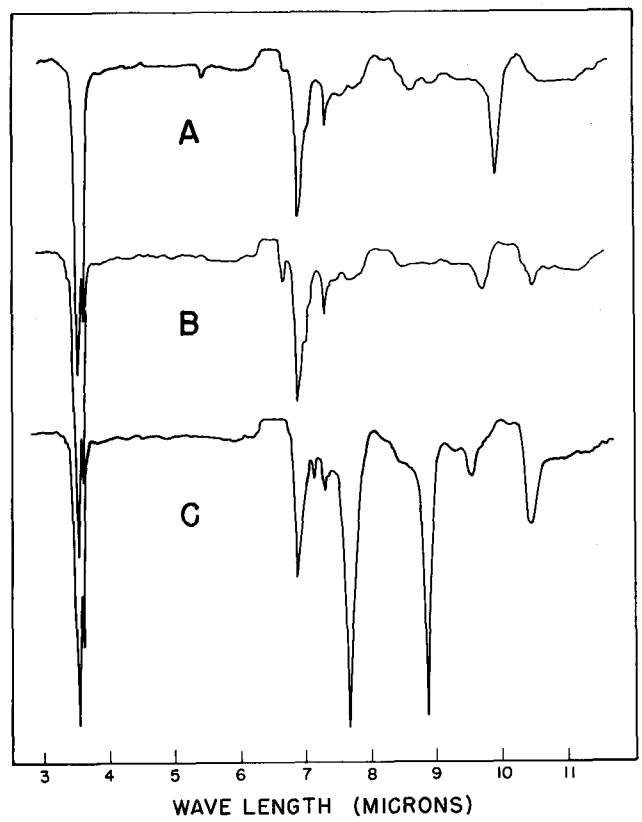


FIG. 5. Infrared spectra of (A) sterculene (1,2-di-n-octylcyclopropene), (B) compound 12 (methyl 1,2-di-n-octylcyclopropyl sulfide) and (C) compound 13 (methyl 1,2-di-n-octylcyclopropyl sulfone). 1.4% solutions in CCl_4, 0.5 mm cells.

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