Biosynthesis of Cyclopropane Rings

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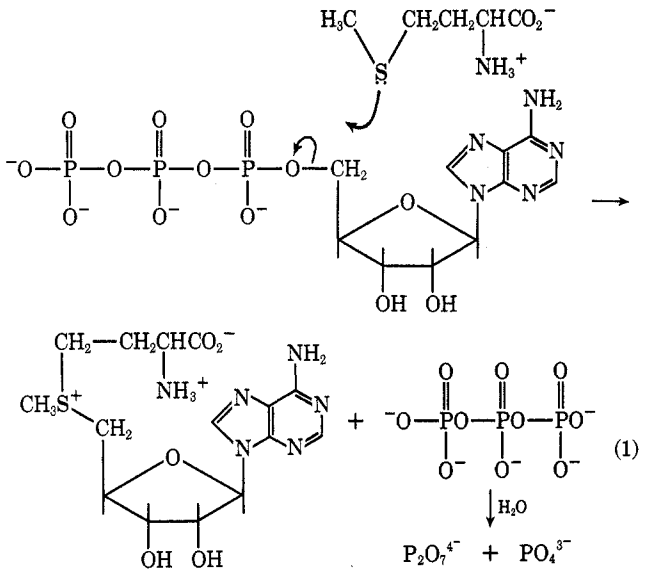
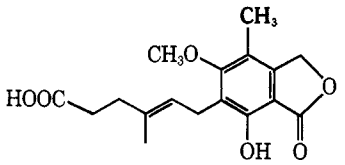
We were led to a study of cyclopropane ring formation in natural products through an interest in biological methylation reactions, the early history of which is contained in a lively account written by Challenger[1] in the 1940's. We now know that the nearly universal biological alkylating agent is an activated derivative of the amino acid methionine. Cantoni[2] showed that this agent, *S*-adenosyl-L-methionine, is formed by the reaction of adenosine triphosphate and methionine.



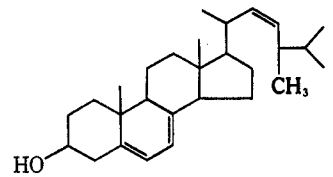
S-adenosyl-L-methionine

In a variety of enzymatic reactions, *S*-adenosylmethionine can serve as an alkylating reagent by donating a methyl group to oxygen, nitrogen, or sulfur atoms. Representatives of all classes of biological compounds can serve as acceptors. Transmethylating enzymes have been discovered which act upon DNA, RNA, proteins, carbohydrates, and lipids and are involved in the formation of such important compounds as antibiotics, hormones, lecithin, transfer RNA, and plant sterols.[3]

In 1954, Birch, Elliott, and Penfold[4] recognized the possibility of enzymatic carbon alkylation processes, and in 1957, Birch, *et al.*,[5] reported that a carbon-bound methyl group in mycophenolic acid arose by alkylation. This paper was followed by the experiments of Alexander and Schwenk,[6,7] which demonstrated the origin of the "extra" methyl group of ergosterol from the methyl group of methionine.



mycophenolic acid (**1**)



ergosterol (**2**)

Hofmann and his colleagues[8-11] established the occurrence of cyclopropane fatty acids in bacteria and indicated that the methylene bridge in the ring arose from a one-carbon unit. O'Leary,[12] and later Chalk and Kodicek[13] and Liu and Hofmann,[14] established that the methylene bridge was derived from the methyl group of methionine.

Several naturally occurring fatty acids with cyclopropane and cyclopropene rings in the chain are now known, as shown in Table I. In most cases the cyclopropane rings have the cis configuration, but trans rings in the mycolic acids have been reported.[15] In no case is the absolute configuration known for the cyclopropane ring of a fatty acid. The simple cyclopropane acids of bacteria occur in the form of esters as a part of phospholipid molecules, while the cyclopropene acids of plants are present principally as components of triglycerides.

Hofmann's isolation of lactobacillic acid from *Lactobacilli* was based in part upon the ability of this and other fatty acids to serve as essential nutrients for this group of organisms under conditions of limited biotin.

(1) F. Challenger, *Chem. Rev.*, **36**, 315 (1945).
(2) G. L. Cantoni, *J. Amer. Chem. Soc.*, **74**, 2942 (1952).
(3) S. H. Mudd and G. L. Cantoni, *Compr. Biochem.*, **15**, 1 (1964).
(4) A. J. Birch, P. Elliott, and A. R. Penfold, *Aust. J. Chem.*, **7**, 169 (1954).
(5) A. J. Birch, R. J. English, R. A. Massy-Westropp, M. Slaytor, and H. Smith, *Proc. Chem. Soc. London*, 204 (1957).
(6) G. J. Alexander, A. N. Gold, and E. Schwenk, *J. Amer. Chem. Soc.*, **79**, 2967 (1957).
(7) G. J. Alexander and E. Schwenk, *J. Biol. Chem.*, **232**, 611 (1958).
(8) K. Hofmann, R. A. Lucas, and S. M. Sax, *ibid.*, **195**, 473 (1952).
(9) K. Hofmann and S. M. Sax, *ibid.*, **205**, 55 (1953).
(10) K. Hofmann, D. B. Henis, and C. Panos, *ibid.*, **228**, 349 (1957).
(11) K. Hofmann, W. M. O'Leary, C. W. Yoho, and T. Y. Liu, *ibid.*, **234**, 1672 (1959).
(12) W. M. O'Leary, *J. Bacteriol.*, **78**, 709 (1959).
(13) K. J. I. Chalk and E. Kodicek, *Biochim. Biophys. Acta*, **50**, 579 (1961).
(14) T. Y. Liu and K. Hofmann, *Biochemistry*, **1**, 189 (1962).
(15) D. E. Minnikin and N. Polgar, *Chem. Commun.*, 1172 (1967).

Table I

Natural Occurrence of Cyclopropane and Cyclopropene Acids[a]

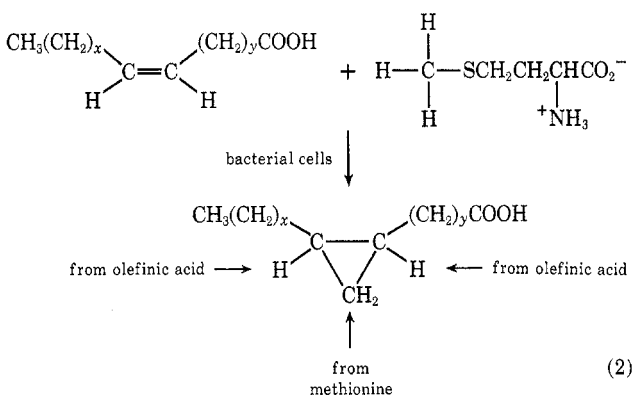
Organism and typical species	Acids
Eubacteria	$CH_3(CH_2)_5$-*c*-$C_3H_4(CH_2)_7COOH$
Lactobacillus	$CH_3(CH_2)_7$-*c*-$C_3H_4(CH_2)_7COOH$ (dihydrosterculic)
Agrobacterium	$CH_3(CH_2)_5$-*c*-$C_3H_4(CH_2)_9COOH$ (lactobacillic)
Escherichia	$CH_3(CH_2)_5$-*c*-$C_3H_4(CH_2)_8CHOHCOOH$
Clostridium, etc.	
Actinomycetales	$CH_3(CH_2)_x$-*c*-$C_3H_4(CH_2)_y$-*c*-$C_3H_4(CH_2)_zCHOHCH(C_{22}H_{45})COOH$
Mycobacterium	(a mycolic acid; *e.g.*, $x = 19$, $y = 14$, $z = 13$, and many similar compounds)
Protozoa	Dihydrosterculic acid
Crithidia	
Plants	$CH_3(CH_2)_7$-*c*-$C_3H_2(CH_2)_7COOH$ (sterculic)
Sterculia	$CH_3(CH_2)_7$-*c*-$C_3H_2(CH_2)_6CHOHCOOH$
Malva	$CH{\equiv}C$-$(CH_2)_7$-*c*-$C_3H_2(CH_2)_6COOH$ (sterculynic)
Hibiscus	$CH_3(CH_2)_7$-*c*-$C_3H_2(CH_2)_6COOH$ (malvalic)
Gossypium, etc.	Dihydrosterculic acid
	Dihydromalvalic acid

[a] For a detailed list of naturally occurring cyclopropane acids and their sources, the reader is referred to an article by W. W. Christie *Top. Lipid Chem.*, **1**, 1 (1970). *c*-C_3H_4 is 1,2-disubstituted cyclopropyl; *c*-C_3H_2 is 1,2-disubstituted cyclopropenyl.

The cells cannot make biotin and are dependent upon it for the carboxylation reactions involved in fatty acid biosynthesis. A supply of fatty acids with appropriate physical properties for the construction of functional membranes will spare the biotin requirements. Olefinic or cyclopropane acids are effective in this respect. This system provides a useful means for studying cyclopropane acid synthesis. Thus Hofmann and Liu[16] were able to administer ^{14}C-labeled *cis*-vaccenic acid (*cis*-octadec-11-enoic acid, the principal olefinic fatty acid of *Lactobacillus* cells) to a culture of *Lactobacillus arabinosus* and observe a substantial conversion to lactobacillic acid. This process obviously consisted of the construction of a methylene bridge across the site of the double bond. Hofmann and Liu[16] at first thought that the "extra" carbon was supplied by formate, but later showed[14] that the methyl group of methionine was a much better source. They verified their hypothesis of double bond alkylenation by degrading the labeled lactobacillic acid derived from labeled methionine and localizing the radioactive carbon in the methylene bridge.

Pohl, Law, and Ryhage[17] showed that methionine labeled with ^2H or ^3H in the methyl group was also incorporated into cyclopropane fatty acids. Quantitation of this process was at first attempted by using doubly labeled substrate with ^3H and ^{14}C in the methionine methyl group. It was expected that the isolated cyclopropane acid would have a $^3H/^{14}C$ ratio 67% of that of the administered methionine, but it was found that the actual ratio was nearly 90%. This could be shown to result from an isotopic discrimination in the cleavage of carbon–hydrogen bonds, since methionine fully labeled with deuterium in the methyl group gave dideuterated cyclopropane fatty acids, as shown by mass spectrometry.

In a similar manner, Polacheck, *et al.*,[18] used oleic-*9,10-d*$_2$ acid to show that the vinyl protons were retained in the conversion of this compound to a cyclopropane fatty acid. Any possibility of double bond isomerization before alkylenation was ruled out by showing that *Lactobacillus* cells convert oleic acid to dihydrosterculic acid, even though they prefer to make the isomeric *cis*-vaccenic acid and lactobacillic acid if they are grown in the presence of biotin. This experiment necessitated the development of a micro method for location of cyclopropane rings, which we accomplished by reductive ring opening and examination of the mass spectrum of the methyl esters of the mixed branched chain acids.[19] The experiments described to this point are summarized in eq 2.



(2)

O'Leary[20] showed that *S*-adenosylmethionine was the preferred precursor of the cyclopropane methylene group by using a mutant strain of *Aerobacter aerogenes* which required this compound. Label from the methyl group of *S*-adenosylmethionine was incorporated into cyclopropane acids, and no isotope dilution was observed when nonisotopic methionine was added.

Analysis of lipids from organisms producing cyclo-

(16) K. Hofmann and T. Y. Liu, *Biochim. Biophys. Acta*, **37**, 364 (1960).

(17) S. Pohl, J. H. Law, and R. Ryhage, *ibid.*, **70**, 583 (1963).

(18) J. W. Polacheck, B. E. Tropp, J. H. Law, and J. A. McCloskey, *J. Biol. Chem.*, **241**, 3362 (1966).

(19) J. A. McCloskey and J. H. Law, *Lipids*, **2**, 225 (1967).

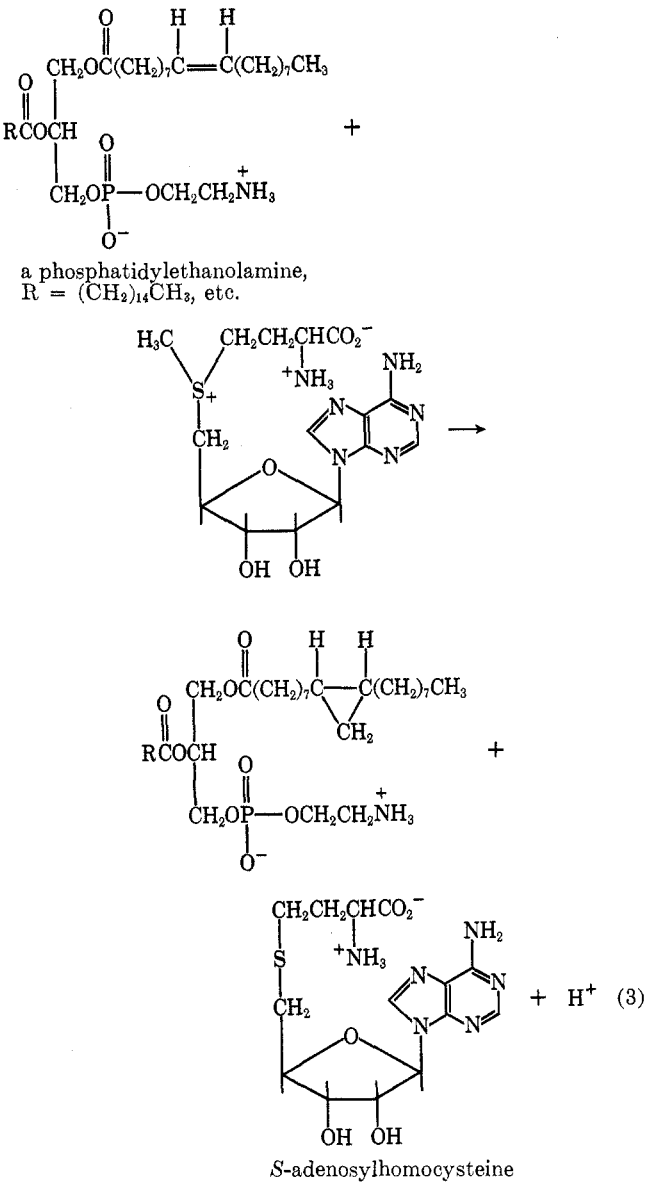
(20) W. M. O'Leary, *J. Bacteriol.*, **84**, 967 (1962).

propane acids[21,22] provided evidence that most of these acids were present in phospholipid molecules and that they were not evenly distributed between the 1 and 2 positions of the glyceride molecules. In *Escherichia coli* 80% were found in the 2 position, while in *Clostridium butyricum* 65% were in the 1 position. This pattern was confused, however, by the fact that the ratio of cyclopropane acid to unsaturated acid precursor varies widely during the growth cycle in batch cultures.[23] Extensive investigations of this phenomenon by Knivett and Cullen[24] have shown that formation of cyclopropane fatty acids by *E. coli* cells is promoted by acid pH, low oxygen tension, high temperature, and Mg^{2+} ions. The low pH and oxygen tension of batch cultures in the stationary phase of growth thus promote formation of these acids.

Another question which can be answered from outside the cell is that of positional isomers. Goldfine and Panos[25] have used Golay capillary gas chromatography to analyze positional isomers of olefinic and cyclopropane acids from *Clostridium butyricum*. Although this organism has a high proportion of hexadec-7-enoic acid compared to hexadec-9-enoic acid (ratio 2:1), the addition of the methylene bridge occurs nearly exclusively at the 9,10 position (ratio of 7,8 isomers to 9,10 isomer 1:19). In the case of the octadecenoic acids, the 11,12 isomer is preferred over the 9,10 isomer by a ratio of 3:1 when approximately equal amounts of olefinic acid are present. The preference would appear, therefore, to be for the double bond seven carbons from the methyl end of the chain.

Studies of biosynthesis from outside the organism, while informative, cannot supply all details about the chemical mechanisms involved in the process. It ultimately becomes necessary to move to the enzyme level where questions about cofactors and active sites can be answered.

We incubated bacterial cell extracts with *S*-adenosylmethionine labeled with ^{14}C in the methyl group and determined the level of radioactivity in fatty acids obtained by saponification of the incubation mixtures. In this way we could demonstrate the enzymatic synthesis of cyclopropane fatty acids.[26] The most promising enzymatic extract was obtained from the anerobe *Clostridium butyricum*, and we were able to purify the enzyme, which we ultimately termed cyclopropane synthetase.[27] Most enzymatic reactions of fatty acids previously investigated involved the coenzyme A esters. We found no evidence for any reaction of the free fatty acids or their coenzyme A esters in the cyclopropane synthetase system. The only fatty acid derivatives which would serve as substrates in the reaction and the only fatty acid containing products that could be found in the unsaponified incubation mixtures were phospholipids. One of the effective substrates was a phosphatidylethanolamine with a high content of olefinic fatty acids. A typical reaction catalyzed by the enzyme is given by eq 3.



a phosphatidylethanolamine, R = $(CH_2)_{14}CH_3$, etc.

S-adenosylhomocysteine

It was possible to imagine that the enzyme might actually remove the acyl chains from the phospholipid molecules to form ester-bound groups at the active site where the alkylenation reaction could take place. This idea was rejected when it was shown that the enzyme could operate on phospholipid derivatives in which the long alkyl chains were bound by ether instead of ester linkages.[28,29]

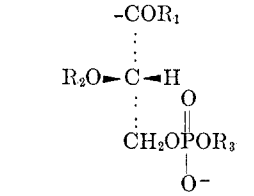
The nature of the substrates for the reaction imposes difficulties, for they are both complicated molecules; furthermore, the phospholipids are water insoluble, while the enzyme and *S*-adenosylmethionine are water soluble. We found that the rate of the reaction was

(21) J. H. Law, H. Zalkin, and T. Kaneshiro, *Biochim. Biophys. Acta*, **70**, 1431 (1963).
(22) J. G. Hildebrand and J. H. Law, *Biochemistry*, **3**, 1304 (1964).
(23) J. A. Croom and J. J. McNeill, *Bacteriol. Proc.*, 170 (1961).
(24) V. A. Knivett and J. Cullen, *Biochem. J.*, **96**, 771 (1965).
(25) H. Goldfine and C. Panos, *J. Lipid Res.*, in press.
(26) H. Zalkin, J. H. Law, and H. Goldfine, *J. Biol. Chem.*, **238**, 1242 (1963).
(27) A. E. Chung and J. H. Law, *Biochem.*, **3**, 967 (1964).
(28) A. E. Chung and H. Goldfine, *Nature*, **206**, 1253 (1965).
(29) P. J. Thomas and J. H. Law, *J. Biol. Chem.*, **241**, 5013 (1966).

very much dependent upon the nature of the phospholipid dispersion[29] and upon the presence of ionic detergents.[27] The reaction was stimulated by anionic detergents and inhibited by cationic ones. The explanation for this was supplied by the work of Bangham and Dawson,[30,31] who showed that phospholipids in solution form large aggregates which can incorporate detergents and other materials and thus take on an altered surface charge. In enzymatic reactions with such lipid aggregates, the rate is dependent to some extent upon charge complementarity between the protein and the substrate aggregates. In the case of cyclopropane synthetase the enzyme must not only approach the lipid aggregate but it must bind individual molecules of phospholipid in such a way that the olefinic alkyl chain is spread out over the active site where alkylenation takes place. The highly aggregated nature of the lipid substrate has so far prevented the determination of good kinetic parameters for the enzymatic reaction because of ambiguity about the effective substrate concentration.

It has been possible, however, to get considerable information about the structural requirements for the phospholipid substrate. By screening a number of natural and synthetic substrates, we could show that, in addition to phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidic acid, and cardiolipin were substrates. This indicated that the enzyme had little preference for what lay opposite to glycerol in the phosphate ester region, provided it did not bear a positive charge, for phosphatidylcholine was not a substrate.

We were able to show that the enzyme was highly specific for the natural L-glycerol phosphatides and that it had a preference for the 1-acyl group[22,29] of the molecule by using the well-defined specificity of snake venom phospholipase A_2. The work of van Deenen and de Haas[32] showed that this enzyme removes only the 2-acyl group of the natural L-phosphatides. We used the phospholipase on a sample of labeled phosphatidylethanolamine which had been obtained by incubating cyclopropane synthetase with DL-dioleylphosphatidylethanolamine and labeled *S*-adenosylmethionine. When the phospholipase reaction had proceeded to completion we separated the free fatty acids, the remaining D-phosphatide, and the L-lysophosphatide by chromatography. The distribution of the labeled cyclopropane acids was approximately 3% in the D-phosphatide, 22% in the liberated fatty acid, and 75% in the lysophosphatide. This showed that the cyclopropane synthetase enzyme reacted nearly exclusively with the L-phosphatide and preferred the 1-acyl group to the 2-acyl group by a factor of 3:1. The essential features of a substrate molecule are summarized as follows.



R_1 = R_2 = alkyl or acyl chain;
R_3 = H, ethanolamine, serine, glycerol, or phosphatidylglycerol

So far we have obtained no details about the intimate mechanism of this unusual reaction. In order to do so it will probably be necessary to prepare large quantities of pure enzyme, a goal which has been frustrated by the fact that the enzyme becomes unstable and loses activity rapidly after approximately 50-fold purification.[27] All efforts to prevent this, to stabilize the enzyme, or to restore lost activity have failed. With crude enzyme preparations we have obtained evidence that the enzyme has a molecular weight of about 80,000 by employing gel filtration techniques.[33]

In the case of the cyclopropene acids of plants, we have no information about the enzymatic processes involved in biosynthesis. Smith and Bu'Lock[34] showed that labeled acetate was incorporated into the chain of cyclopropene acids, but not into the ring. Hooper and Law[35] and Johnson, *et al.*,[36] using *Hibiscus* seedlings, found that methyl-labeled methionine was incorporated predominantly into saturated cyclopropane acids but also into the cyclopropene acid methylene bridge. The fact that the saturated ring acids were heavily labeled led Hooper and Law[35] to favor the proposed[37] desaturation pathway

oleic acid ⟶ dihydrosterculic acid ⟶ sterculic acid

while Smith and Bu'Lock[38] isolated acetylenic acids from *Sterculia* seed oils and favored a pathway involving these compounds

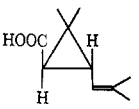
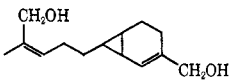
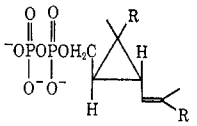
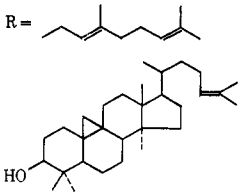
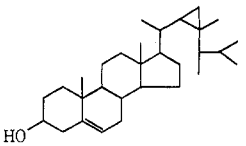
oleic acid ⟶ stearolic acid (octadec-9-ynoic acid) ⟶ sterculic acid

Hooper[39] prepared both labeled stearolic and dihydrosterculic acids, administered them to *Hibiscus* seedlings, isolated the fatty acids, and treated them with methanethiol to prepare the methanethiol addition compounds of the cyclopropene acids. Gas chromatography of the methyl esters of the thiol adducts showed that these were labeled from dihydrosterculic acid, but not from stearolic acid, which confirmed the first pathway.

In addition to the cyclopropane fatty acids, a number of other classes of natural products with cyclopropane rings have been discovered. A few of these are shown

(30) A. D. Bangham and R. M. C. Dawson, *Biochem. J.*, **72**, 486 (1959).
(31) A. D. Bangham and R. M. C. Dawson, *Biochim. Biophys. Acta*, **59**, 103 (1962).
(32) L. L. M. van Deenen and G. H. de Haas, *ibid.*, **70**, 538 (1963).
(33) T. O. Henderson and J. H. Law, unpublished experiments.
(34) G. N. Smith and J. D. Bu'Lock, *Biochem. Biophys. Res. Commun.*, **18**, 426 (1965).
(35) N. K. Hooper and J. H. Law, *ibid.*, **18**, 426 (1965).
(36) A. R. Johnson, J. A. Pearson, F. S. Shenstone, A. C. Fogerty, and J. Giovanelli, *Lipids*, **2**, 225 (1966).
(37) T. L. Wilson, C. R. Smith, and K. L. Mikolajczak, *J. Amer. Oil Chem. Soc.*, **38**, 696 (1961).
(38) G. N. Smith and J. D. Bu'Lock, *Chem. Ind. (London)*, 1840 (1965).
(39) N. K. Hooper, Ph.D. Thesis, Harvard University, 1968.

Table II

Class of compd	Typical structure	Typical source[b]		Ref
Amino acids	$CH_2{=}c\text{-}C_3H_3CH_2CH(NH_3^+)CO_2^-$ Hypoglycin	*Blighia sapida*	Akee fruit	*c*
	$CH_2{=}c\text{-}C_3H_3CH(NH_3^+)CO_2^-$	*Litchi chinensis*	Litchi	*d*
	$CH_2{=}c\text{-}C_3H_3CH(CH_3)CH(NH_3^+)CO_2^-$	*Aesculus californica*	California buckeye	*e*
Terpenes[a]	 Chrysanthemum acids	*Chrysanthemum cinerariaefolium*		*f*
	 Sirenin	*Allomyces*	Water mold	*g*
	 Presqualene pyrophosphate	Probably ubiquitous in sterol-producing organisms		*h*
Sterols[a]	 Cycloartenol	Many plants		*i, j*
Gorgosterol		Sea fans (horny corals)		*k*

[a] The list of cyclopropane terpenes and sterols is a very large one, and the examples given are not adequately representative. The reader is referred to a paper by R. Soman, *J. Sci. Ind. Res.*, **26,** 508 (1967). [b] $c\text{-}C_3H_3$ = 1,2-disubstituted cyclopropyl. [c] E. V. Ellington, C. H. Hassall, J. R. Plimmer, and C. E. Seaforth, *J. Chem. Soc.*, 80 (1959). [d] D. O. Gray and L. Fowden, *Biochem. J.*, **82,** 385 (1962). [e] L. Fowden and A. Smith, *Phytochemistry*, **7,** 809 (1968). [f] L. Crombie and M. Elliott, *Fortsch. Chem. Org. Naturst.*, **19,** 120 (1961). [g] L. Machlis, W. H. Nutting, and H. Rapoport, *J. Amer. Chem. Soc.*, **90,** 1674 (1968). [h] H. Rilling and W. W. Epstein, *ibid.*, **91,** 1041 (1969); *J. Biol. Chem.*, **245,** 4597 (1970). [i] D. H. R. Barton, *J. Chem. Soc.*, 1444 (1951). [j] P. Benveniste, L. Hirth, and G. Ourisson, *Phytochemistry*, **5,** 31 (1966). [k] N. C. Ling, R. L. Hale, and C. Djerassi, *J. Amer. Chem. Soc.*, **92,** 5281 (1970).

in Table II. Among the illustrative examples chosen are compounds with unusual biological activity. The cyclopropane amino acids are hypoglycemic and toxic,[40] the chrysanthemum acids are constituents of the insecticidal pyrethrins,[41] sirenin is the sex pheromone of the water mole *Allomyces*,[42] presqualene pyrophosphate is an important intermediate in the biosynthesis of triterpenes and sterols,[43] and cycloartenol may be a precursor of the phytosterols.[44,45]

For most of these compounds we have little information about the process of ring construction. In the case of presqualene pyrophosphate, the enzymatic synthesis of which has been well studied,[43] two molecules of farnesol pyrophosphate are joined during ring formation. This is most likely an alkylation reaction similar to those involving *S*-adenosylmethionine except that the leaving group is pyrophosphate. We must await further experimental evidence before we can attempt to formulate general principles of ring formation in the remaining natural products.

(40) R. Bressler, C. Corredor, and K. Brendel, *Pharm. Rev.*, **21,** 105 (1969).
(41) See Table II, footnote *f*.
(42) A. W. Barksdale, *Science*, **166,** 831 (1969).
(43) See Table II, footnote *h*.
(44) See Table II, footnote *j*.
(45) L. J. Goad in "Natural Substances Formed Biologically from Mevalonic Acid," T. W. Goodwin, Ed., Academic Press, New York, N. Y., 1970, p 45.