Biosynthesis of Marine Sterol Side Chains

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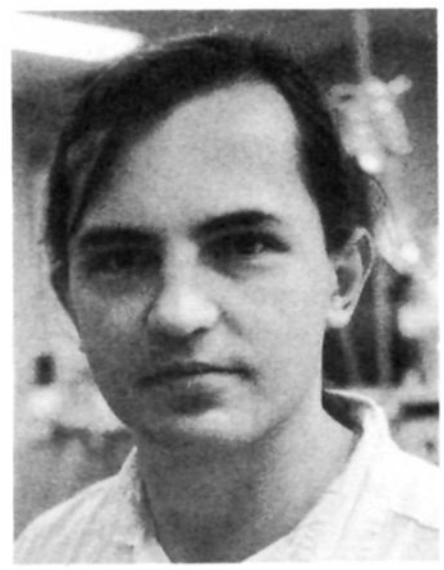
Received December 1, 1992 (Revised Manuscript Received April 27, 1993)

Contents



José-Luis Giner received his B.A. in chemistry from Brandeis University (1979) and continued research on acetylcholinesterase with Prof. Saul G. Cohen to receive his M.A. in 1980. After two years of studying the structure and biosynthesis of neocarzinostatin with Prof. Irving Goldberg at Harvard Medical School he went to Stanford University where he carried out his Ph.D. studies with Prof. Carl Djerassi on "Enzyme-Level Studies of Marine Sterol Biosynthesis" (1990). He is currently a post-doctoral fellow with Prof. Duilio Arigoni at ETH-Zürich where he has been working on stereochemical aspects of squalene cyclization and the use of chiral *tert*-butyl groups in biosynthetic studies.

I. Introduction

Although virtually nonexistent in prokaryotes,[1] sterols are found in the membranes of all eukaryotic organisms. They are considered to be of great evolutionary importance[2] and their biosynthesis is believed to reflect the evolution of their membrane function.[3] In the past 20 years a surprising wealth of sterols with unusual new structures isolated from marine sources has generated much excitement in natural products chemistry. Aside from the 19-nor and A-nor sterols of Axinellid sponges, almost all of the unusual structural features of these sterols are associated with their side chains. Sterol side chains have been isolated with such unusual features as quaternary alkyl groups, cyclopropane and cyclopropene rings, allenes, and acetylenes. Both the mechanisms of their biosynthesis and their ultimate origins in the food chain have been enigmatic. In the last eight years experiments carried out in the laboratory of Carl Djerassi at Stanford have shed light on this often bewildering field.[4] Many questions remain unanswered, but the emerging picture arising from these studies has reached a level of complexity justifying detailed review. In contrast to previous reviews of novel marine sterols,[4-9] the aim of this review is to provide a comprehensive summary and detailed discussion focusing on sterol side chain biosynthesis. Methodological problems will be discussed, and an attempt will be made to point out gaps in the current understanding where further research is likely to be most profitable. It is the author's belief that biosynthesis of unusual marine sterols is related to that of common sterols as variations on a theme. It is hoped that the study of these biosynthetic oddities provides new insights into the biosynthesis of terrestrial plant and fungal sterols as well as some facinating biosynthetic chemistry in its own right.

For purposes of clarity, the sterol nucleus will be omitted in the figures. The side chains will generally be refered to by the trivial name of a sterol bearing that side chain. Limited use will be made of the α–β convention of describing sterol side chain stereochemistry.[10] Thus α refers to the top face of the sterol side chain when drawn in the extended form; β to the bottom face. This stereochemical shorthand, despite its usefulness in describing the stereochemistry at C-24 of phytosterols, suffers from the unfortunate fact that it is opposite to the α–β convention used for the sterol nucleus. This confusion is lamentable. To maintain clarity in stereochemical discussions, the top and bottom face of the side chain as drawn will simply be referred to as such.

II. Dinoflagellate Sterols

The dinoflagellates are a very primitive group of unicellular eukaryotes and their fossil record goes back 250 million years. The geochemical fossils of the 23-methyl sterols typical of dinoflagellates have been used as evidence for the marine origin of geological deposits.[11] However, typical dinoflagellate sterols have also been isolated from freshwater dinoflagellates.[12] Historically dinoflagellate sterols were the first unusual marine sterols to be discovered. Gorgosterol (1) was discovered by Bergman in the 1940's and named after the coral-like animals from which it was isolated.[13] It was later found that gorgosterol is actually produced by zooxan-

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thellae–intracellular photosynthetic dinoflagellate symbionts.[14] The structure of gorgosterol, which bears an unusual C-23 methyl group and a cyclopropane ring in the side chain, was finally elucidated in 1970[15,16] and contributed greatly to the renewed interest in marine sterols.[17]

A. C-23 Methylated Sterols

Because the biosynthesis of 23-methylated sterols seems to be restricted to dinoflagellates, the presence or absence of such sterols in unicellular algae is useful as a chemotaxonomic marker.[18,19] Reports of 23-methyl sterols in the haptophyte *Hymenomonas carterae* casts doubt on the identification of this alga.[20,21] No 23-methyl sterols were found in three other haptophyte algae.[20] A new chemotaxonomic criterion is provided by the recent finding that dinoflagellates, alone of all photosynthetic eukaryotes tested to date, produce lanosterol rather than cycloartenol.[22]

The biosynthesis of the gorgosterol side chain (1) was proposed at the time of the structure proof to involve two successive SAM (*S*-adenosylmethionine) dependent enzymatic methylations of the brassicasterol side chain (Figure 1, 2).[16] The predicted 23-methylated

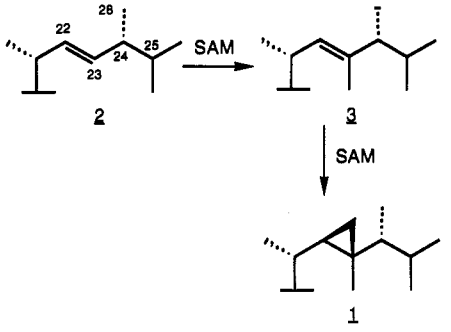

Figure 1.

intermediate (dinosterol, 3) was subsequently isolated from a free-swimming dinoflagellate, *Gonyaulax tamarensis*.[23]

Preliminary evidence supporting this biosynthetic scheme came from feeding experiments in which CD_3-labeled methionine was fed to *Cryptothecodinium cohnii*.[24] Mass spectral analysis of the sterols showed the incorporation of five deuterium atoms into the dinosterol side chain (3), two at C-28 and three in the 23-methyl group. This is consistent with the known biosynthesis of the brassicasterol side chain (2) in yeast, where it has been shown that 24-methylenecholesterol (Figure 2, 4) is an intermediate.[25]

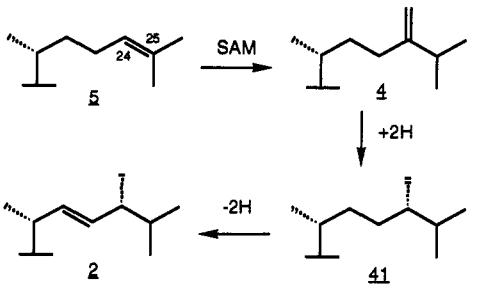

Figure 2.

Because of the difficulties in conducting feeding experiments with these organisms, further research required the preparation of active enzyme extracts.[26] Proof of the above biosynthetic scheme came from experiments with cell-free extracts from *C. cohnii* and *Peridinium foliaceum* using (^{3}H-*methyl*)SAM.[27] An enzyme extract from the former organism catalyzed the enzymatic methylation of the desmosterol side chain (Figure 2, 5) to give the 24-methylenecholesterol side chain (4). Methylation of the brassicasterol side chain (2) gave the dinosterol side chain (3). Enzyme extracts of two other dinoflagellates, *P. foliaceum* and the cultured zooxanthella of *Cassiopoiea xamachana*, also converted the brassicasterol side chain (2) to that of dinosterol (3), but *P. foliaceum* formed peridinosterol, which bears a $\Delta^{17(20)}$ double bond, (Figure 3, 6)[28] as well.

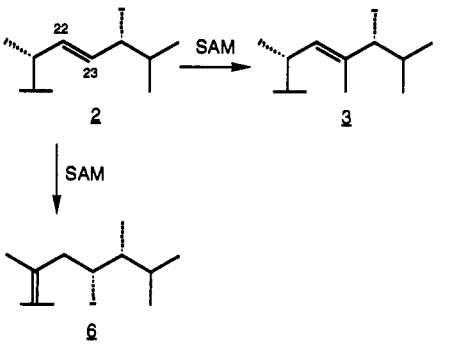

Figure 3.

Different enzymes appear to produce dinosterol (3) and peridinosterol (6) in *P. foliaceum* based on changes in the product ratio under different assay conditions. The *P. foliaceum* system was shown to be capable of forming the gorgosterol side chain 1 from that of dinosterol (3), if in poor yield.

Although gorgosterol is often a major sterol produced by zooxanthellae in the symbiotic state, when these are cultured in the absence of the host animal, gorgosterol is generally no longer formed.[29] It has been suggested that the production of dimethylpropiothetin, which serves as an osmotic regulator in marine algae, competes for SAM and thereby leads to decreased gorgosterol biosynthesis in the aposymbiotic state.[27]

A chromatographic isotope effect was reported for tritium-labeled gorgosterol (1) in the above study. These effects are well known to be of importance with isotopes of hydrogen and must be taken into account, especially as better and better separations are achieved.[30] Such isotope effects are typically small, but a sterol bearing a total of six deuterium and three tritium atoms in the side chain was recently found to elute 2 min before unlabeled material on reverse-phase HPLC.[31] Sterols labeled with tritium in the 3 position have been shown to elute later than unlabeled sterols on normal-phase HPLC.[32] For this reason, as well as for the general purpose of providing better evidence for the identity of a labeled substance, the use of histograms in HPLC analysis of radioactive biosynthetic products is recommended.

Stereochemical aspects of the biosynthesis of gorgosterol (1) remain unresolved. It has been suggested that the biosynthetic enzymes producing dinosterol (3) and peridinosterol (6) share a common intermediate carbonium ion (Figure 4, 7), which, depending on the position of the proton acceptor, leads to different products.[27] It was also argued that the methylation of

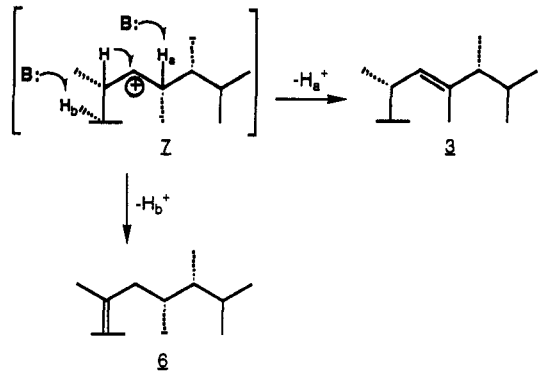

Figure 4.

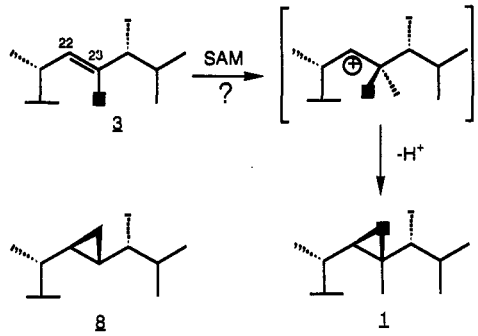

Figure 5.

the dinosterol side chain **3** giving rise to the gorgosterol side chain (**1**) is initiated at the C-23 (Figure 5), as in dinosterol (**3**) and peridinosterol (**6**) biosynthesis rather than at C-22, although the latter would involve the intermediacy of a more stable tertiary carbonium ion.[27] While information regarding the stereochemical course of the 23-methylation is lost in the formation of dinosterol (**3**), attack of the methyl group from the back as drawn is evident in the structure of peridinosterol (**6**). Methylation from the back has been unequivocally demonstrated in the biosynthesis of the 24-methylene side chain (**4**, see Figure 8).[33] On the basis of the structure of gorgosterol (**1**), methylation from the back as drawn would mean that the final methyl group introduced must become the 23-methyl of the product. The structure of 23-norgorgosterol (**8**),[34] however, implies that the cyclopropyl methylene is introduced from the front as drawn.

A sterol resembling gorgosterol, but with the 22,23-methylene bridge on the opposite face (Figure 6, **9**), has

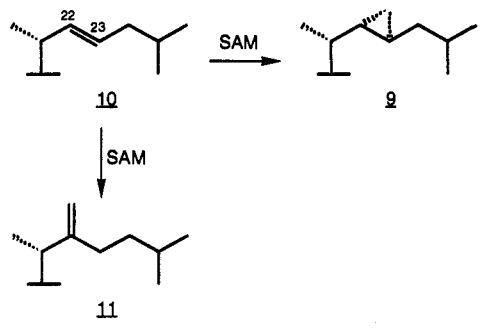

Figure 6.

been isolated in small amounts from various sponges and a soft coral.[35] It presumably originates from the diet of these filter feeders and is likely to be the product of methylation of 22-dehydrocholesterol (**10**). Another sterol of unknown origin that has been isolated in trace amounts from a sponge is 22-methylenecholesterol (**11**).[36] It, too, appears to derive from 22-dehydrocholesterol (**10**). These sterols are likely to come from dinoflagellates, since these are the only organisms known to methylate the Δ^{22} double bond.

B. 27-Norsterols

The 27-norergostane (Figure 7, **12** and **13**) and 24-norcholestane (**14**) side chains present one of the few uninvestigated biosynthetic puzzles in the field of marine sterols. These side chains are frequently

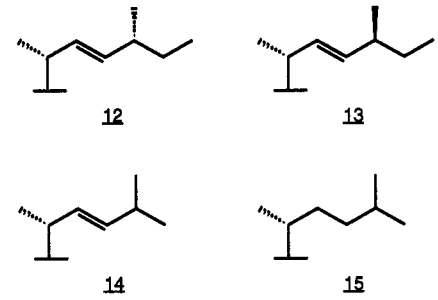

Figure 7.

encountered in trace quantities in sponges where they have been shown to be diet derived.[37] Large quantities (32%) of (24β)-27-norergostenol (**12**) have been isolated from the free-swimming dinoflagellate *Gymnodinium simplex*.[38] Similar amounts (24%) were isolated from a cultured zooxanthella, *Gymnodinium beii*.[39] In both dinoflagellates small amounts of 24-norcholesterol (**15**) were detected, suggesting that these sterols share a common biosynthetic pathway.

The biosynthesis of 27-norsterols (**12-14**) has been suggested to involve the reverse reaction of the SAM methyltransferase.[40,41] A more recent proposal suggests a mechanism of dealkylation analogous to the pathway in insects.[42] The insect pathway of sterol dealkylation has recently been found (in part) in sponges (see below). Clarification of the problem awaits experiments with the dinoflagellates from which these compounds arise.

III. Sponge Sterols

Sponges, a primitive group of multicellular organisms, represent without a doubt, the richest source of bizarre sterols found in nature. While the benefit of these unusual structures to the sponges in which they occur is not understood at present, they have provided biologists with chemotaxonomic markers[43,44] and chemists with some very interesting biosynthetic puzzles. The ground-breaking studies of the Naples group led, in rapid succession, to the discovery of a number of novel sterols: the first extended side chain sterol (aplysterol, **16**, see Figure 27),[45] a cyclopropenyl sterol (calysterol, **17**, see Figure 22),[46] and sterols with the A-nor and 19-nor nuclei,[47,48] thereby establishing marine sponges as sources of unusual sterols. The discovery of the latter group of sterols provided an impetus to the study of sponge sterol biosynthesis because of the importance of 19-nor steroids in oral contraception, but their biosynthesis will not be discussed in this review.[49] The emphasis on isolation and structure determination of new sterols in the Djerassi group in the 1970s, was

gradually replaced by biosynthetic studies in the 1980s. Because sponges incorporate radiolabeled sterols very well, sterol biosynthesis has been extensively studied through feeding experiments. Recently developed methodology for the use of cell-free extracts provides a complementary technique for biosynthetic studies.[50] A review of sponge sterol biosynthesis has recently been published.[4]

A. Common Sterol Side Chains

Although a multitude of unusual sterols have been isolated from sponges, most sponges, in fact, have the mundane sterols found in animals, plants, and fungi., i.e. cholesterol and sterols bearing one or two extra carbon atoms at C-24. The biosynthetic study of such sterols in sponges should be of interest for comparative purposes. However, very few studies have concerned themselves with the biosynthesis of these compounds because they were believed to be diet derived and lack the glamour of the multiply methylated or cyclopropyl sterols.

A recent study systematically surveyed the origins of such sterols in 11 marine sponges.[51] The incorporation of (3-^{3}H) squalene into sterols was determined and time courses of feeding experiments were measured. Because of the symmetry of squalene, the label is expected initially at both the 3 and 24 positions of lanosterol. The label at the C-3, however, will be lost during the removal of the 4-methyl groups via the action of oxidoreductases.[52] On the other hand, the hydride shift from C-24 to C-25 in the conversion of desmosterol (**5**) to 24-methylenecholesterol (Figure 8, **4**) and from C-25

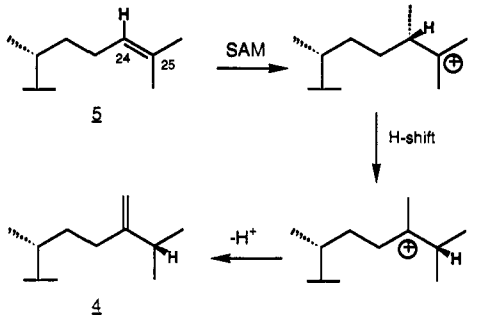

Figure 8.

to C-24 in the reverse process (see dealkylation) does not lead to loss of label from the side chain. In these studies campesterol (**87**) isolated from *Axinella polypoides* was found to be radiolabeled, indicating that this sterol has a different biosynthesis in the sponge—perhaps from 24-methylenecholesterol (Figure 9, **4**) or epicodisterol (**18**)—than in higher plants where the loss

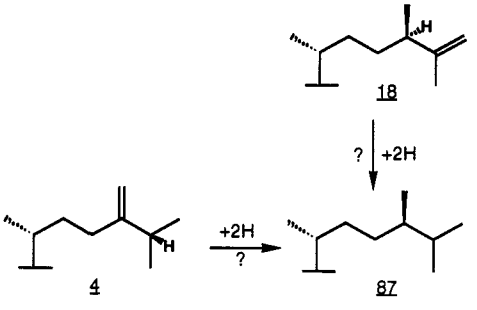

Figure 9.

of the C-24 hydrogen occurs.[53] The sponges in this survey were found to biosynthesize ca. 70% of their sterols, the remainder coming from the diet. An inability to produce Δ^{22} sterols was noted (see cyclopropyl sterols) and sterols with 27-nor side chains (**12**–**14**) and 22-dehydrocholesterol (**10**) were also shown to be of dietary origin.

The biosynthesis of the brassicasterol (**2**) and poriferasterol (**19**) side chains was studied in a sponge of the genus *Pseudoaxinyssa*.[54] These were shown to originate via a biosynthetic sequence resembling that of Chlorophyte algae (Figure 10) rather than that of

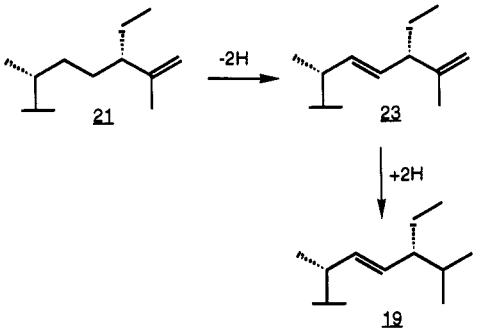

Figure 10.

fungi and higher plants.[55] Thus the codisterol (Figure 28, **20**) and clerosterol (**21**) side chains were better precursors than the 24-methylenecholesterol (**4**) or fucosterol (**22**) side chains. The epicodisterol (**18**) side chain was not metabolized, demonstrating specificity in the enzymes involved. The intermediacy of $\Delta^{22,25}$ sterols (e.g. **23**), in analogy to the situation in Chlorophytes, was proposed but these were not detectable in this sponge. However, this biosynthetic sequence was subsequently demonstrated in detail in another sponge (*Ciocalypta* sp.) that contains >90% of a $\Delta^{22,25}$ sterol.[56]

B. Dealkylation of the Side Chain

An unexpected twist in the relatively straightforward scheme of sterol side chain biosynthesis was discovered when 24-methylenecholesterol (**4**) was shown to be a precursor to cholesterol (**24**) in sponges.[57] This transformation involves the intermediacy of an epoxy sterol (Figure 11, **25**) which is rearranged to desmosterol (**5**).

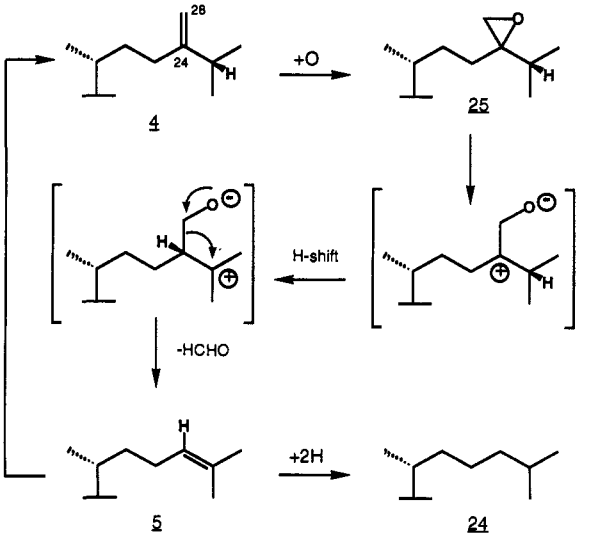

Figure 11.

Such side-chain dealkylations have been well studied in insects which, as a group, are incapable of de novo sterol biosynthesis.[58] Other organisms such as mollusks, crustaceans, and coelenterates also are known to utilize this pathway. (See ref 57 for references.) What makes the finding in sponges so remarkable is that sponges not only are capable of producing their own sterols, but are adept at the reverse process of side chain dealkylation, namely side-chain methylation (see below).

This pathway has been demonstrated in 11 species of sponges and appears to be quite general.[59] The 24-ethylidene side chain (fucosterol (22) and isofucosterol (26)) was shown to be a better substrate than 24-methylenecholesterol (4).[60] Other sponge sterols containing a 24-methylene group such as 24(28)-dehydroaplysterol (Figure 37, 27) have not been examined. Unlike the pathway in insects, 24-methyl and 24-ethyl sterols (87 and 39–41, Figure 18) are not utilized, indicating the inability to introduce the 24(28) double bond. The possibility that this process represents a source of 22-dehydrocholesterol (10) and the 27-norsterols (12–14) (see above) in sponges was ruled out through feeding experiments of brassicasterol (2) and codisterol (20) as well as their epimers. The ability of a completely autotrophic Chlorophyte alga to convert the 24(28)-epoxide (25) to desmosterol (5) has recently been demonstrated, suggesting that side chain dealkylation is more widespread than previously thought.[61]

The discovery of this pathway has practical implications for sponge feeding experiments since it allows the nonspecific incorporation of 24-methylene- and 24-ethylidenecholesterol (4 and 22, 26, respectively) into sterols via the intermediacy of desmosterol (5). In a double-label feeding experiment, using (3-^{3}H,28-^{14}C)-labeled 24-methylenecholesterol (4), it was found, after the usual incubation period, that 90% of the recovered starting material had lost the ^{14}C label via dealkylation and was subsequently realkylated.[60] This could lead to false results with sponges in which the product of desmosterol (5) methylation is not 24-methylenecholesterol (4). This problem has been fortuitously avoided in most of the reported studies through the labeling of 24-methylenecholesterol (4) at the 28 position. In this case, loss of C-28 via dealkylation will also result in the loss of radiolabel.

C. Cyclopropyl and Cyclopropenyl Sterols

Perhaps the most exciting sterols found in sponges are the cyclopropyl and cyclopropenyl sterols.[62] Cyclopropyl sterols can be classified into two groups: those produced by SAM methylation and those arising from a desaturative pathway. The products of the latter pathway are sometimes further desaturated to form cyclopropenyl and acetylenic sterols. The hypothesis that the enzymatic ring opening of cyclopropyl sterols represents a biosynthetic pathway in marine organisms[63] led to detailed mechanistic studies of acid-catalyzed cyclopropane ring opening in the Djerassi lab.[64] Although ring opening of such cyclopropyl sterols no longer seems to be of biosynthetic importance, the carbonium ion chemistry investigated in these studies continues to be of relevance because of the intermediacy of protonated cyclopropanes in their biosynthesis.

A cyclopropyl sterol arising from SAM methylation, sormosterol (28), has recently been isolated from *Lissodendoryx topsenti*.[65] Lederer, in early investigations of SAM-dependent methyl transfer to sterols, considered this compound as a biosynthetic intermediate to 24-methylated sterols.[66] The biosynthesis of sormosterol (28) was shown to proceed via SAM methylation of desmosterol (5) both in feeding experiments and by using cell-free extracts.[65,50] The configuration of sormosterol at C-24 demonstrates methyl transfer from the back as drawn. The radiolabeled form of this sterol was efficiently taken up but not further transformed in feeding experiments, thereby demonstrating that it is a biosynthetic dead end.[65] Because there seems to be a connection between secondary carbonium ions and cyclopropanes (see Figures 5 and 16), a mechanism involving C-25 methylation was proposed (Figure 12).[67] Methylation at C-25 would lead

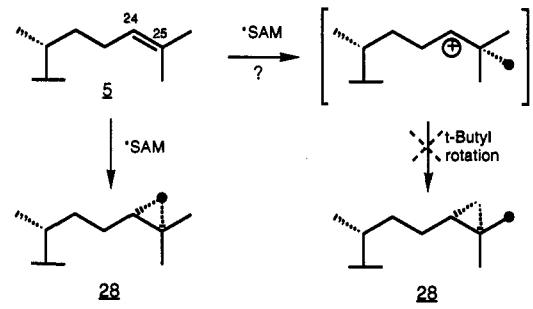

Figure 12.

to a carbonium ion bearing a *tert*-butyl group, the rotation of which could lead to scrambling of the radiolabel. By using cell-free extracts it was shown that the methylene bridge of the cyclopropane derives from SAM, ruling out a mechanism involving both C-25 methylation and rotation of the *tert*-butyl group.

The cyclopropyl and cyclopropenyl sterols of the Haplosclerid sponges have a fascinating biosynthesis. The demonstration that 24-methylenecholesterol (4),[68] but not codisterol (20) or epicodisterol (18), was a precursor to petrosterol (Figure 13, 34) led to the

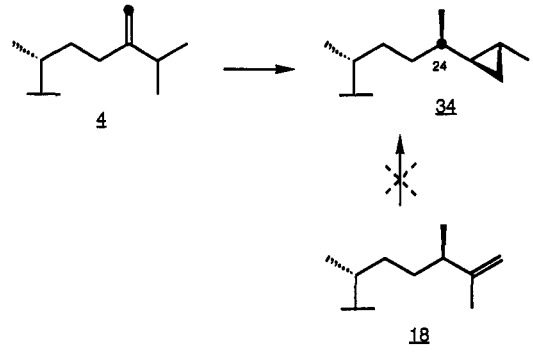

Figure 13.

formulation of a biosynthetic theory named the "cyclopropyl walk" (Figure 14).[69] According to this theory a nonclassical carbonium ion (35), corresponding to the protonated form of dihydrocalysterol (36), arises through ring closure of a 23-carbonium ion 37 and undergoes rearrangement leading to a new cyclopropyl sterol, petrosterol (34). This explains with the co-occurrence of cyclopropyl sterols and unusual acyclic side chains that correspond to ring-opened products. Stereochemical considerations of these rearrangements led to a unified theory of cyclopropyl sterol biosynthesis via

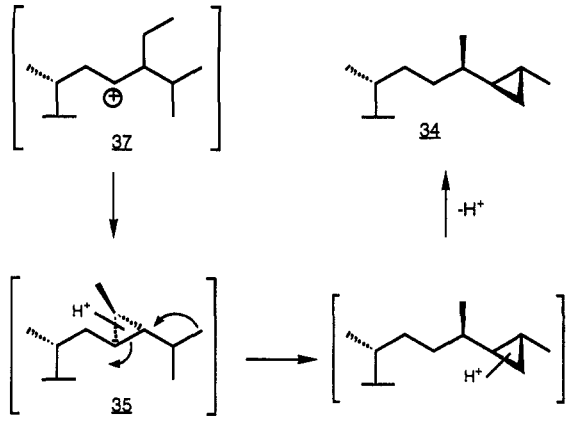

Figure 14.

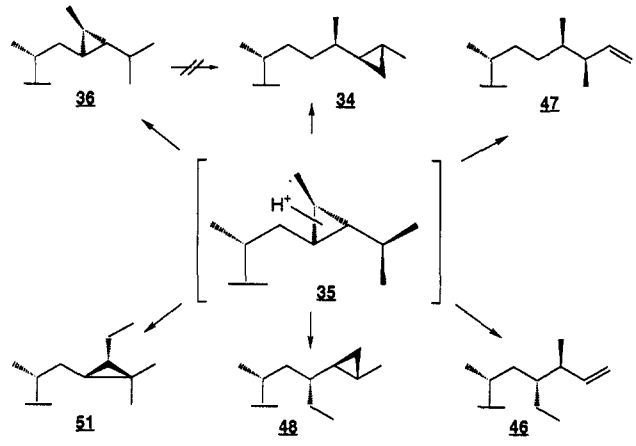

Figure 15.

stereospecific rearrangements of a protonated dihydrocalysterol species (Figure 15, **35**).[70]

Evidence supporting this theory came from chemical degradation of petrosterol (**34**) that had been formed from (28-^{14}C)-24-methylenecholesterol (**4**) by *Petrosia ficiformis*.[68,71] The label was shown to reside in the 24-position (Figure 13), as is consistent with the proposed mechanism. Radiolabeled dihydrocalysterol (**36**) was taken up by the sponge but not converted to other sterols.[71] This is taken to rule out isomerization of dihydrocalysterol (**36**) to petrosterol (**34**) via enzymatic protonation. Since the formation of dihydrocalysterol (**36**) must already pass through such a protonated species (Figure 14), it is argued that dihydrocalysterol (**36**) and petrosterol (**34**) (as well as the other related products) are produced in the same biosynthetic step, through the rearrangement of an enzyme bound carbocationic intermediate. The equal specific activities of the rearranged products from feeding experiments provide further evidence for this novel biosynthetic reaction.[72,73]

Originally the central carbocationic intermediate **35** in this process was proposed to arise via the SAM methylation of 24-methylenecholesterol (Figure 16, **4**).[70,74] It was proposed that the required 23-carbonium ion **37** is generated via a 1,2-hydride shift from a 24-carbonium ion (Figure 16, **38**). Using a cell-free extract prepared from frozen *Petrosia ficiformis* shipped in dry ice from Naples, it was shown that SAM methylation of 24-methylenecholesterol (**4**) gives rise to clerosterol (**21**), a normal sterol, and not the cyclopropyl sterols.[50] This finding led to the proposal that clerosterol (**21**) is

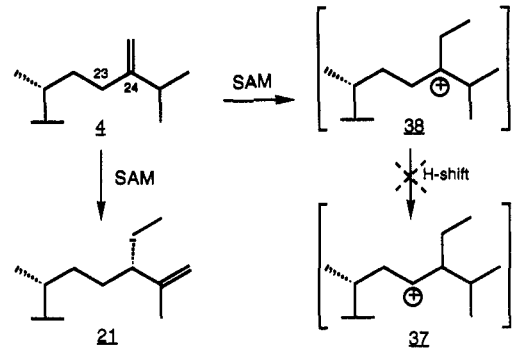

Figure 16.

reduced enzymatically to clionasterol (Figure 17, **39**) and that a faulty variant of enzymatic 22-desaturation gives rise to the unusual sterols. The hypothesis of

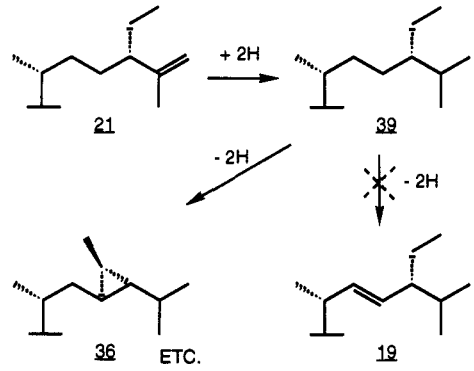

Figure 17.

cyclative desaturation of clionasterol (**39**) was later successfully demonstrated in a feeding experiment with radiolabeled clionasterol (**39**) in *Petrosia ficiformis* (Figure 17).[73] The inability of this substrate to be desaturated normally to the Δ^{22} sterol, poriferasterol (**19**), is taken as evidence that an errant Δ^{22} desaturase is at play. It is ironic that these unusual sterols may arise from a inability of a sponge (phylum Porifera) to produce poriferasterol (**19**).

It is also ironic that sitosterol (**40**), the 24-α isomer of clionasterol (**39**), was tested as a precursor of the cyclopropenyl sterol calysterol (**17**) in the original feeding experiments with *Calyx niceaensis*.[75] In this study fucosterol (Figure 28, **22**) was incorporated, but only poorly. Reduction of fucosterol (**22**) to clionasterol (**39**) or, alternatively, dealkylation to desmosterol (**5**) (Figure 11) provides possible explanations for its incorporation.

The inability of 24-α sterols to serve as substrates was shown in studies of the specificity of this transformation.[72] Feeding experiments in *Petrosia ficiformis* showed neither sitosterol (Figure 18, **40**, 24α-ethylcholesterol) or campesterol (**87**, 24α-methylcholesterol) to be incorporated into cyclopropyl sterols. The feeding of dihydrobrassicasterol (**41**, 24β-methylcholesterol) led to the discovery of a new sterol in this sponge, 29-norhebesterol (**42**). In addition, dihydrobrassicasterol (**41**) was incorporated into 23,24-methylenecholesterol (**43**, 29-nordihydrocalysterol) and norficisterol (**44**).

It is mechanistically interesting that norpetrosterol (**45**) was not formed from dihydrobrassicasterol (**41**), although petrosterol (**34**) is the main product (86%) of

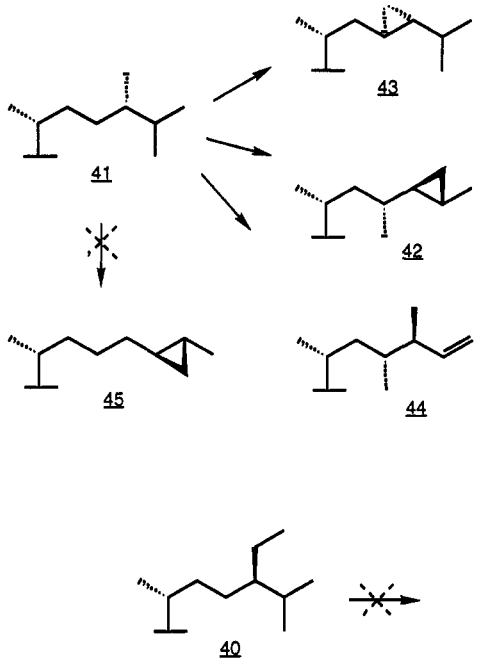

Figure 18.

the desaturative cyclization of clionasterol (39). Norpetrosterol (45) is not known to occur in nature. Conversely, norficisterol (44) represents 44% of the products of dihydrobrassicasterol (41) and its cyclic isomer, 29-norhebesterol (42) 17%, but in the cyclization of clionasterol (39), ficisterol (46) only represented 0.3% of the products. This remarkable reversal in the product ratios upon going from an ethyl to a methyl group at C-24 is likely to be indicative of the destabilization of an intermediate in the reaction mechanism. In terms of protonated cyclopropanes leading directly to new protonated cyclopropanes (Figure 19), the proton

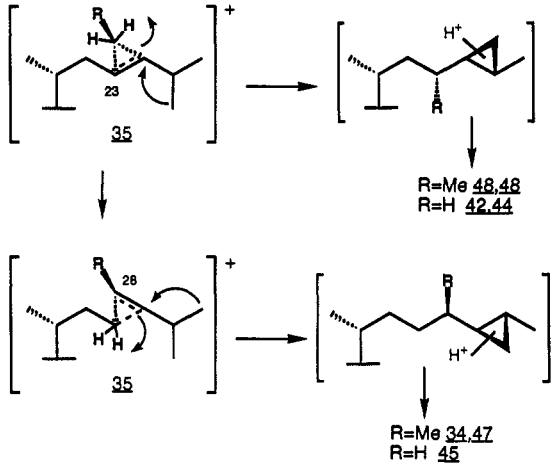

Figure 19.

must reside at C-23 of the protonated cyclopropane (35) to give rise to the petrosterol/26(29)-dehydroaplysterol series (34 and 47); and at C-28 to give rise to the hebesterol/ficisterol series (Figure 15, 48 and 46). While these are both mono-alkyl-substituted corners of the dihydrocalysterol cyclopropane, the C-28 of 23,-24-methylenecholesterol (43) is an unsubstituted corner. Experimental data and calculations shown protonation to be favored at an unsubstituted corner over an alkyl-substituted corner of a cyclopropane.[76]

If considered in terms of classical carbonium ions, the rearrangement that gives rise to petrosterol (34) takes place via a 1,3-hydride shift from C-28 to C-23, followed by a 1,2-alkyl migration of C-23 to C-28 (Figure 20). The stereochemical interrelationships of the

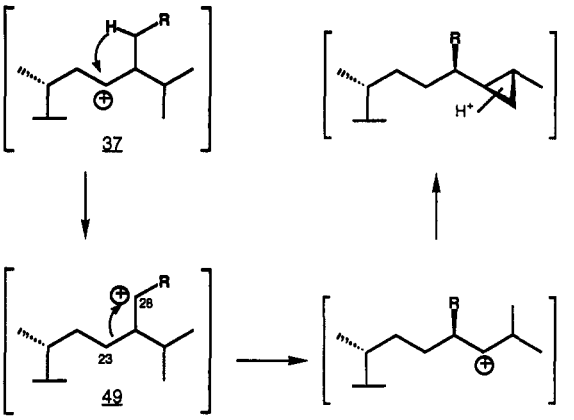

Figure 20.

products which were explained by the intermediacy of nonclassical carbonium ions can also be accounted for by the observance of a least motion condition in a rearrangement involving classical ions. By such a mechanism the route to petrosterol (34) proceeds via secondary carbonium ions, but the formation of norpetrosterol (45) would require the unfavorable rearrangement of a secondary carbonium ion (37) to a primary carbonium ion (49). The route to the hebesterol/ficisterol series (48 and 46), on the other hand, would proceed via an alkyl shift of the 24-alkyl group to C-23 and involves secondary carbonium ions in both cases. Perhaps the difference between these alternative mechanisms of classical or nonclassical ions is merely a question of semantics. The use of the protonated cyclopropane formalism seems to describe the facts somewhat more elegantly.

An amendment to the protonated cyclopropane paradigm has recently been offered with the discovery of the 23-epimer of dihydrocalysterol (Figure 21, 50) as

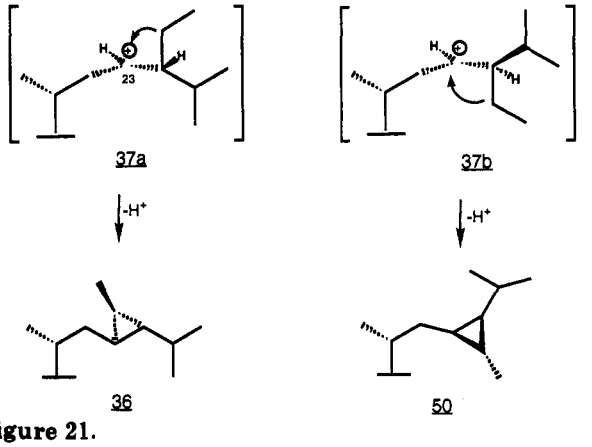

Figure 21.

a minor sterol in *Cribrochalina vasculum*.[77] Although this new sterol may result from isomerization of the protonated from of dihydrocalysterol (35), ring-closure from *Si*-face (opposite face as usual) of the C-23 carbonium ion (37) will lead to the correct stereochemistry directly. *Si*-Face closure of rotamer (37a) also permits the direct formation of nicasterol (Figure 15,

51) from the C-23 carbonium ion (37), without requiring the protonated dihydrocalysterol intermediate (35).[70]

The biosynthesis of cyclopropenyl sterols has been studied in the sponge *Calyx niceaensis*.[78-80] Dihydrocalysterol (36) and 24-methylenecholesterol (4) were shown to be good precursors of the cyclopropenyl sterols, calysterol (Figure 22, 17), (23*R*)-23*H*-isocalysterol (52),

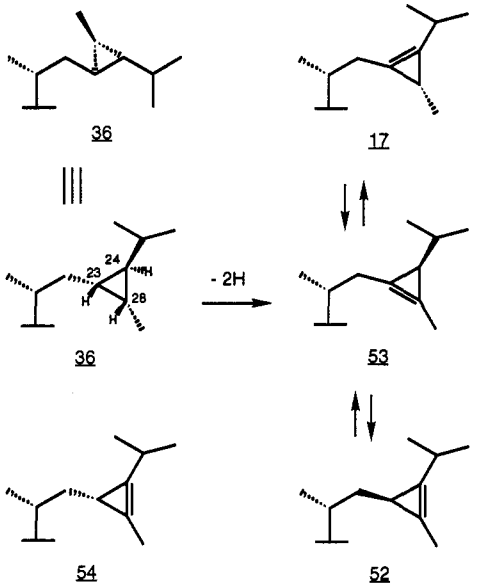

Figure 22.

and (24*S*)-24*H*-isocalysterol (53).[78] The specific activity of (24*S*)-24*H*-isocalysterol (53) was 20-60 times higher than that of the two other cyclopropenyl sterols, suggesting a precursor–product relationship. Desaturation to (24*S*)-24*H*-isocalysterol (53) requires a cis-dehydrogenation, as in the biosynthesis of the cyclopropene fatty acid sterculic acid in higher plants.[81] It has been proposed that the enzyme responsible for the desaturation of dihydrocalysterol (36) is the same desaturase that converts clionasterol (39) to cyclopropyl sterols.[72]

Cyclopropenyl sterols labeled in the sterol nucleus with tritium were shown to be interconverted in the sponge *Calyx niceaensis*.[79] In this experiment another cyclopropenyl sterol, (23*S*)-23*H*-isocalysterol (Figure 22, 54), which is found in the sponge *Calyx podatypa*, was also shown to be converted to the cyclopropenyl sterols. The interconversion of cyclopropenyl sterols supports the hypothesis that (24*S*)-24*H*-isocalysterol (53) is the immediate product of dihydrocalysterol (36) desaturation and is subsequently isomerized. To prove this sequence dihydrocalysterol (36) was prepared labeled with tritium at C-28 and was fed to *Calyx niceaensis* together with (3-^{3}H)dihydrocalysterol (36).[80] The calysterol (17) thus formed was gently oxidized to remove the label at the 3-position of the sterol nucleus. Loss of all radioactivity indicated that loss of the C-28 hydrogen had occurred during biosynthesis, consistent with the intermediacy of (24*S*)-24*H*-isocalysterol (53). Had calysterol (17) been even a minor product of the desaturation reaction, a large tritium isotope effect may have enhanced its production enough to change the apparent course of the reaction. This was not observed.

The cyclopropenyl sterols occur together with acetylenic sterols (Figure 23, 55 and 56) in *Calyx niceaensis*. The acetylenic sterols have been shown to be formed

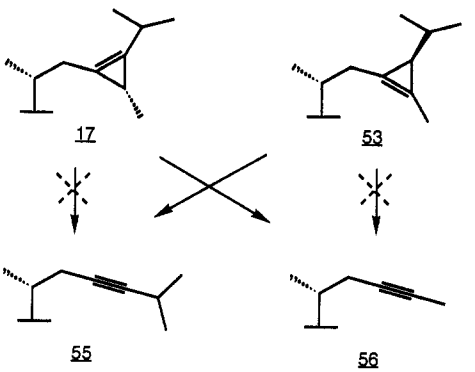

Figure 23.

from the same precursors, 24-methylenecholesterol (4) and dihydrocalysterol (36).[78,79] Cholest-5-en-23-yn-3-ol (55), was formed from dihydrocalysterol (36) with the same specific activity as (24*S*)-24*H*-isocalysterol (53) suggesting that they are products of the same reaction.[78] On the basis of the very high specific activities of the acetylenic sterols formed from radiolabeled cyclopropenyl sterols, only transient intermediates are expected in their formation.[79] The operation of some type of retro-carbene process has been proposed for this transformation on the basis of photochemical studies.[82] However, feeding experiments with labeled cyclopropyl sterols showed that the opposite acetylenes were produced as expected by such a process (Figure 23).[79] A cyclopropyl cationlike intermediate (Figure 24, 57) has been proposed in the desaturation of

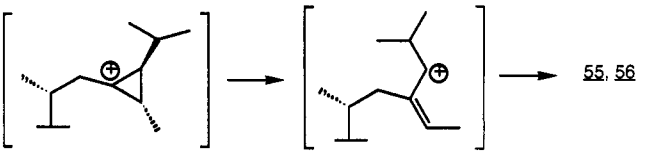

Figure 24.

cyclopropyl to the cyclopropenyl sterols.[72] It is conceivable that electrocyclic rearrangement of this species plays a part in the biosynthesis of the acetylenic sterols.[83]

Acetylenic sterols have also been detected in minute amounts in a higher plant where they seem to represent errors in the functioning of a Δ^{22}-desaturase.[84]

The biosynthesis of two further cyclopropyl sterols isolated from sponges remains to be investigated. A sterol with four extra carbon atoms in the side chain (Figure 25, 29) has been isolated from a *Pseudoaxinyssa*

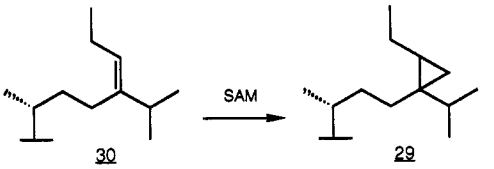

Figure 25.

sp. It probably results from SAM methyl transfer by the sponge to dietary 24-propylidenecholesterol (30) (an algal sterol, see below).[85]

An interesting cyclopropyl sterol with no extra side chain carbon atoms (Figure 26, 31) has been isolated from *Spirastella vagabunda* (3.5%).[86] It also has been found in trace amounts (0.3%) in a deep sea gorgonians (*Pseudothesis* sp.) where it is accompanied by the vinyl cyclopropyl sterol papakusterol (32) (2.7%). The latter

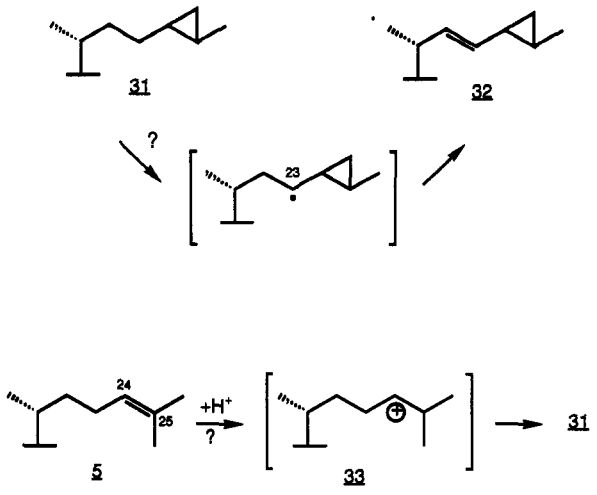

Figure 26.

sterol was isolated from six deep-sea gorgonians (6-16%)[87] and simultaneously from the soft coral *Sarcophytum glaucum* (2.5%) from which it was given the name glaucasterol.[88] The deep-sea gorgonian sterol is a 6:4 mixture of trans stereoisomers, while the soft coral sterol has been shown to be only the 24*S*,25*S* isomer.[89] Since this sterol has been isolated from both coelenterates and sponges, it is probably of planktonic origin.

The cooccurrence of the saturated and unsaturated sterols in the gorgonian raises the possibility that papakusterol (**32**) is produced through desaturation of the cyclopropyl sterol (**31**), perhaps by the gorgonian. This would be of mechanistic interest since the 22-desaturase is thought to initiate double bond formation at C-23.[90] If in this process a radical is generated next to the cyclopropane, one would expect rearrangement with ring opening.[91] An attempt to demonstrate desaturation or ring opening of sormosterol (**28**) have failed with a Δ^{22}-desaturase from yeast.[90]

The biosynthesis of petrosterol, etc. (Figure 14) suggests a possible route to the saturated cyclopropyl sterol (**31**) via the cyclative desaturation of cholesterol. While 24-methyl- and 24-ethylcholesterol served as substrates for this reaction in *Petrosia ficiformis* (see above), cholesterol did not.[72] If this sterol arises via cyclative desaturation, the introduction of the Δ^{22} double bond may occur in another organism since cyclative desaturation is believed to be catalyzed by a faulty Δ^{22}-desaturase.[73] Another possible biosynthetic mechanism could involve anti-Markovnikov enzymatic protonation of desmosterol (**5**) to give a C-24 carbonium ion (Figure 26, **33**), followed by ring closure. Since the enzymatic hydrogenation of desmosterol (**5**) to cholesterol is thought to be initiated by protonation of the double bond,[92] it is possible that a defective Δ^{24}-hydrogenase is at play.

D. Methylation Sequences

Side-chain methylation leads to many of the more unusual structures in sponge sterols and has been well studied. Plant and fungal sterols typically contain one or two extra carbon atoms in the form of a methyl or ethyl group at C-24.[40] In some sponges this process seems to go wild. Sterols with as many as five extra carbons at the terminus of the side chain have been found[93] and many different variations of this process are known. In all cases the extra carbon atoms derive from the biological methyl donor S-adenosylmethionine (SAM) in an unusual enzymatic reaction involving the nucleophilic attack of a double bond on the sulfonium methyl group of SAM.[94] The resulting carbonium ion often rearranges, typically by a 1,2-hydride shift, before loss of a proton gives rise to a new double bond (or in some cases a cyclopropane). Iteration of this process leads in sponges to highly methylated sterol side chains. Because of their great variety of side-chain-methylated sterols, the study of sterol side-chain methylation in marine sponges is well rewarded.

Side-chain extension via methylation at C-26 is characteristic of sponge sterols and of chemotaxonomic importance in Verongid sponges which contain aplysterol (**16**).[45] The biosynthesis of 25-dehydroaplysterol (Figure 27, **58**) in *Aplysina fistularis* was the first

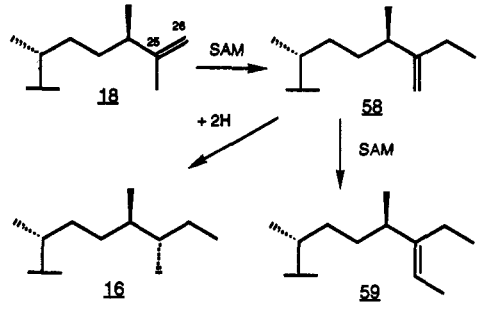

Figure 27.

successful biosynthetic study of sponge sterol side chains.[95] Epicodisterol (**18**), which occurs in this sponge, was transformed into 25-dehydroaplysterol (**58**) and, to a lesser extent, aplysterol (**16**) and verongulasterol (**59**), when fed in radiolabeled form. The lower specific activity of the latter sterols compared to 25-dehydroaplysterol (**58**) is typical for a precursor–product relationship. Codisterol (**20**), on the other hand, was only poorly converted to 25-dehydroaplysterol (**58**) and it was suggested that the observed incorporation was due to a small impurity of epicodisterol (**18**) (3%).

In the same series of experiments 24-methylenecholesterol (Figure 28, **4**) was incorporated into isofucosterol (**26**), 24-ethylcholesterol (**39** and **40**), and 24-isopropenylcholesterol (**57**). The unexpected higher specific activity of 24-ethylcholesterol (**39** and **40**) compared to its probable precursor, isofucosterol (**26**),

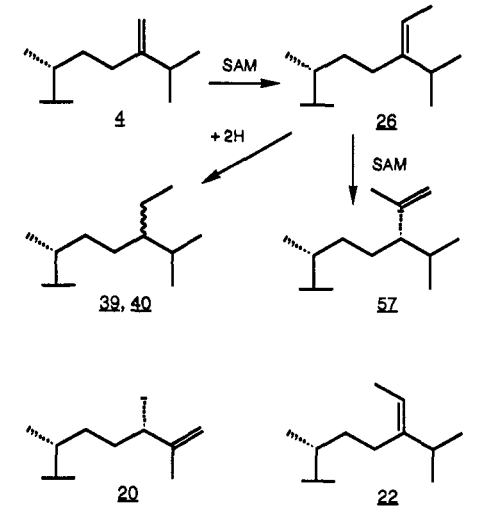

Figure 28.

may be due to the dilution of the latter by dietary fucosterol (22), which was not separated in the analysis. Alternatively, competing dealkylation of isofucosterol (26) (Figure 11) may remove the C-28 radiolabel, thereby diluting the activity. The input of dietary sterols that cannot be separated and competing biosynthetic processes often limit the information available from specific activities. The use and limitations of kinetic arguments in biosynthetic pathways has been reviewed.[96]

A study using a cell-free system of the same sponge confirmed the conversion of epicodisterol (18) to 25-dehydroaplysterol (58) and the nonconversion of codisterol (20).[50] However, clerosterol (Figure 18, 21), not isofucosterol (26), was found to be the product of 24-methylenecholesterol (4) methylation. Use of the cell-free system enabled a dissection of the pathway in the absence of further transformations. Thus the immediate products of desmosterol (Figure 29, 5) methylation

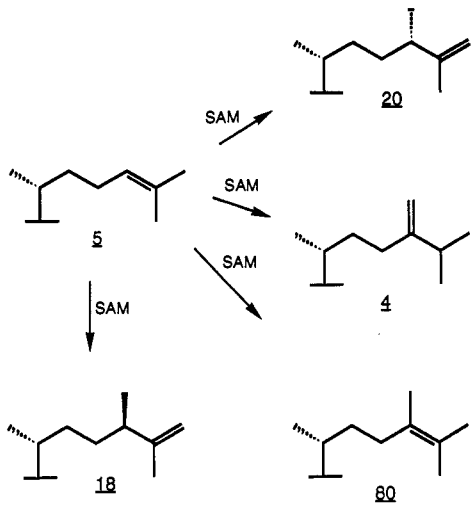

Figure 29.

could be isolated by HPLC after the addition of unlabeled sterols (cold carriers) and were shown to be epicodisterol (18), codisterol (20), 24-methylenecholesterol (4), and 24-methyldesmosterol (60) in a ratio of approximately 12:1:1:1. In feeding experiments the detection of minor products would be more difficult since they could be further converted to even more minor products. A drawback of this approach is that when (^{3}H-*methyl*)SAM is used as a source of radiolabel, the product ratios may be inaccurate due to tritium isotope effects. Advantages of cell-free extracts are that they can be stored at −80 °C for years and the extract from one sponge is enough for dozens of experiments. This eliminates variation that may exist from sponge to sponge or be due to seasonal changes. Because the products of the methylation are typically present in low concentration and SAM-methyltransferases are active in the absence of the cofactors necessary for the oxidoreductase activity, the biosynthetic pathway can often be studied one step at a time.

In plants and fungi the addition of the methyl group to desmosterol (5) has been shown to occur from the back face of the double bond as drawn (see Figure 8).[33] In this respect the stereochemistry at C-24 of epicodisterol (18) presents a puzzle, since the methyl transfer reaction appears to occur from the opposite face as usual. Degradation of epicodisterol (18) showed that the 24-methyl group derives from SAM, thus ruling out a possible carbonium ion rearrangement.[50] An alternative binding arrangement presenting the opposite face of the double bond to the enzyme (e.g. Figure 30) was proposed that would permit opposite face

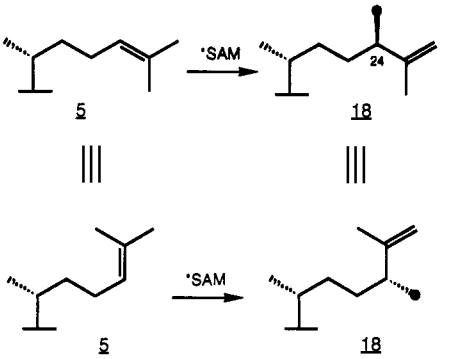

Figure 30.

methylation without changing the relative orientation of the SAM binding site.

The biosynthesis of sterols containing the 24-isopropyl group was studied in a *Pseudoaxinyssa* sp.[97] The sequence 24-methylenecholesterol (Figure 31, 4)

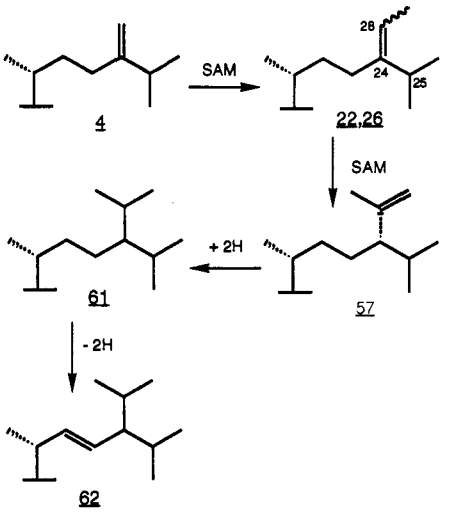

Figure 31.

to 24-ethylidenecholesterol (22 and 26) to (24*S*)-24-isopropenylcholesterol (57) to 24-isopropylcholesterol (61) to Δ^{22}-24-isopropylcholesterol (62) was established. Both isomers of 24-ethylidenecholesterol (22 and 26) were utilized equally. This was considered to be due to nonspecificity of the enzyme, but it may also be a case were the side chain is dealkylated and then realkylated to give the true intermediate (see Figure 11).

The terminal methyl groups of the 24-isopropyl sterols (61 and 62) are diastereotopic: despite the apparent symmetry all four are stereochemically nonequivalent. However, it is no easy task to extract this stereochemical information. In a sequel to the above study, (24*S*)-24-isopropenylcholesterol (57), the precursor to 24-isopropylcholesterol (61), was produced biosynthetically from (26-^{3}H)-24-methylenecholesterol (Figure 32, 4).[98] Chemical degradation showed that the original isopropyl group remains the isopropyl group in the product. This finding is proof of a hydride migration on the front face of the side chain as drawn. Because the authors believed that, in analogy to squalene cyclization,[99] hydride migration must occur

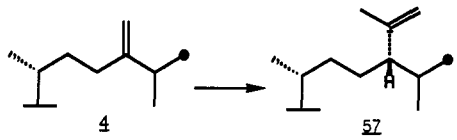

Figure 32.

on the opposite face to the formation of the new bond, this result was taken as proof that 24-ethylidenecholesterol (22 and 26) is methylated from the back face as drawn via intermediate 63a (Figure 33). Opposite-

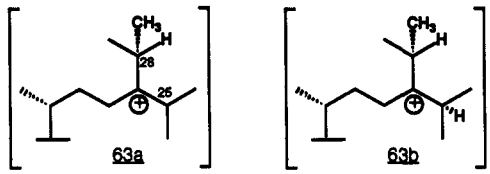

Figure 33.

face hydride migration has been rigorously demonstrated in 24-methylenecholesterol (Figure 8, 4) biosynthesis,[33] and to a lesser extent in 24-ethylidenecholesterol (22 and 26) biosynthesis (see below).[100] However, an opposite-face mechanism need not be necessarily followed. Had the C-25 rather than C-28 hydrogen migrated, the governing factor in producing the correct stereoisomer would be orientation of the C-25 hydrogen, regardless from which side the methyl group was added (e.g. 63b).

The 24-isopropenyl side chain (57) has been encountered in a Chrysophyte alga[101] and an orchid[102] as well as in sponges. For each of the two stereoisomers, the SAM-derived carbon atoms can reside in either the isopropyl or the isopropenyl group. If in the isopropenyl group, the third (final) methyl group to be added can become either the methylene group or the methyl group; if in the isopropyl group, either the *pro-R-* or *pro-S*-methyl group. The same considerations hold for the squalene-derived carbons. Thus there are eight possible biosynthetic routes to each of the two stereoisomers. It would be interesting to know which of these pathways are preferred in these different organisms.

The sponge *Xestospongia testudinaria* contains isofucosterol (26) and fucosterol (22) in a 12:1 ratio. The idea that methyl-group addition and proton loss occurs from opposite faces was studied using *E* and *Z* tritium labeled 24-methylenecholesterol (Figure 34,

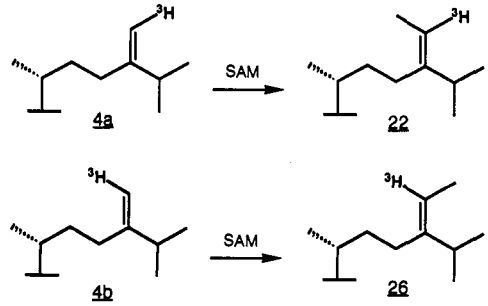

Figure 34.

4a,b).[100] These isotopomers were prepared by decarbonylation of radiolabeled α,β-unsaturated aldehydes.[103] Although this experiment does not address the absolute orientation of the incoming methyl group and the departing proton, the relative orientation can be judged from the radioactivity of the products, fucosterol (22) and isofucosterol (26). Two factors are expected to influence the ratio of the products: (1) a kinetic isotope effect will inhibit the formation of the product arising from loss of tritium and (2) removal of the tritium atom results in the complete loss of radioactivity from that product.

If there are two enzymes, the inhibition of one should not, at saturating levels of sterol substrate, effect the rate of the other. If one enzyme produces two products through the partitioning of a common intermediate, inhibition of the formation of one product via a kinetic isotope effect will enhance the formation of the other.[104] The latter appears to be the case in this sponge, since higher than normal levels of the minor product (22) were formed from the *Z*-isotopomer (4a). However, complete loss of label in one of the products was not seen, as would be expected if an opposite face mechanism was rigidly followed (Figure 35). The product

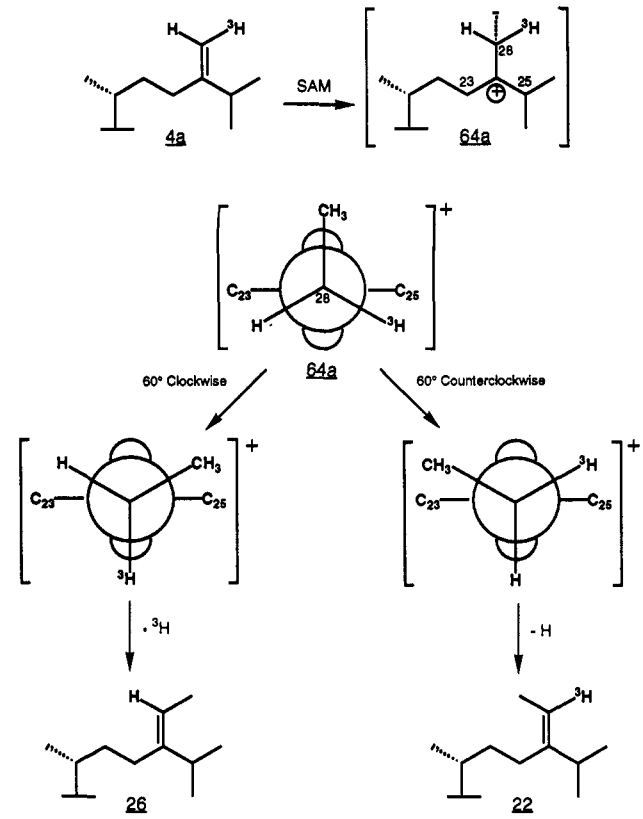

Figure 35.

ratio from the *E*-isotopomeric precursor (4b), where the isotope effect should inhibit the formation of the minor product 22, is the same as the natural ratio. The product ratio from the *Z* isotopomer (4a), where it should inhibit the formation of the major product (26), is nearly 1:1. A complicated argument involving isotope effects and the partitioning into yet another product was put forward. To rule out isomerization, (6-^{3}H)-fucosterol (22) was fed to the sponge; no radiolabel was found in the major isomer, isofucosterol (26). This also rules out interconversion via the dealkylation–realkylation pathway (Figure 11), although such interconversion would not have been detected in the experiments carried out with 28-labeled sterols. Control experiments ruled out incomplete separation of the products and stereochemical impurity of the precursors, which

might have occurred via photoisomerization of the α,β-unsaturated aldehyde intermediates.

Although the authors discuss the results in terms of an opposite face mechanism, an alternative explanation is that proton loss follows a less fixed mechanism governed in part by a least motion principle. Thus, upon generation of the carbonium ion **64** through SAM methylation, the methyl group will be in the plane of the empty p orbital and a rotation by 60° is required before a proton will be properly aligned for departure. If deprotonation occurs at this point, opposite face proton loss will be observed. If this was the only possibility, no radiolabeled isofucosterol (**26**) would result from (*Z*)-(28-^{3}H)-24-methylenecholesterol (Figure 35, **4a**). When deprotonation is hindered due to an isotope effect, rotation to bring another hydrogen into alignment may take two courses, 120° rotation or 60° rotation. The shorter rotation leads now to retention of tritium in the unexpected product (Figure 36). This

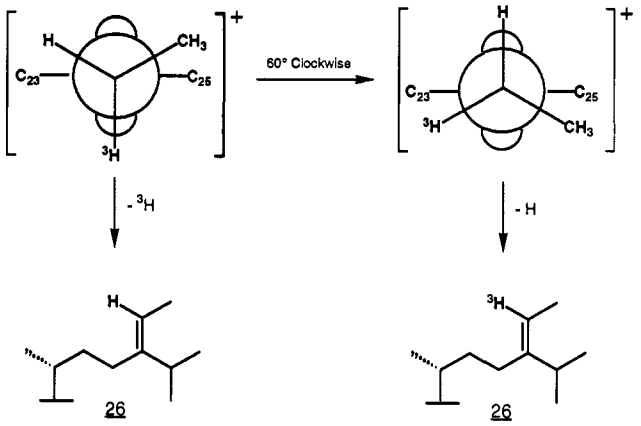

Figure 36.

corresponds to proton loss from the same side as methyl addition. While this requires proton accepting groups to be available on both faces, given the extreme acidity of a carbonium ion, many groups will be able to serve that role. These considerations can account for the anomalous results. It should be stressed that steric factors restricting rotation in a given direction are probably also involved.

The biosynthesis of strongylosterol (Figure 37, **65**)

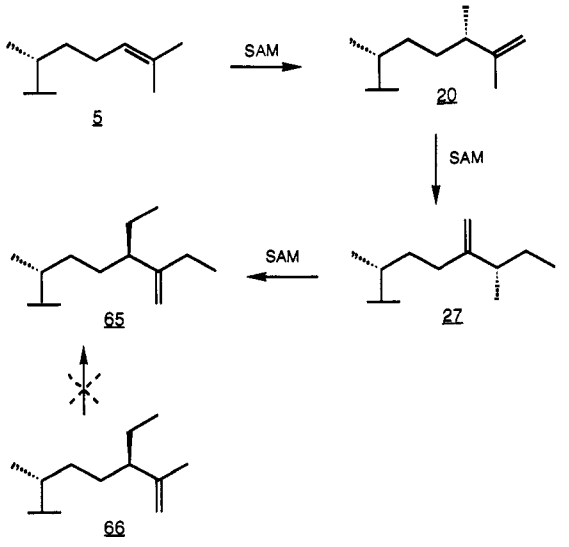

Figure 37.

was studied in the sponge *Strongylophora* durissima.[105] The sequence leading to this sterol was shown to be: desmosterol (**5**) to codisterol (**20**) to 24(28)-dehydroaplysterol (**27**) to stronglyosterol. In analogy to 25-dehydroaplysterol (**58**) biosynthesis in *Aplysina fistularis* (Figure 27),[95] a pathway involving the intermediacy of epiclerosterol (**66**) was considered, although this compound has only recently been encountered in nature in higher plants.[106] However, neither epiclerosterol (**66**) or its isomer (**21**) were incorporated into stronglyosterol (**65**). Epicodisterol (**18**) showed a small amount of incorporation. This may be due to isomeric contamination (see above) by codisterol (**20**). This is an interesting biosynthetic sequence since the chirality at C-24 of the precursor (**20**) is lost, only to reappear with the opposite stereochemistry in the product **65**.

In this study codisterol (**20**) labeled with tritium at C-24 gave rise to strongylosterol (**65**) labeled at C-24, consistent with two 1,2-hydride shifts in the biosynthetic sequence (Figure 38). Note that the inversion of

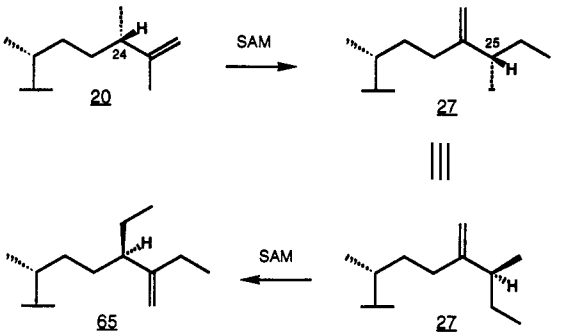

Figure 38.

C-24 stereochemistry requires the involvement of different C-25 rotamers of 24(28)-dehydroaplysterol (**27**) in order that the migrating hydrogen be brought from one face to the other. Both C-25 stereoisomers of 24(28)-dehydroaplysterol (**27**) were equally effective as substrates in the final step of the sequence. Neither sterol has been isolated from this sponge, perhaps due to their efficient conversion. A possibility exists of a dealkylation–realkylation mechanism (see above) interconverting the two 24(28)-dehydroaplysterol (**27**) isomers (Figure 39). The product of dealkylation, 26-

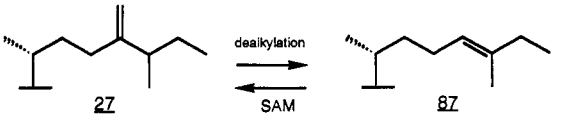

Figure 39.

methyldesmosterol (**67**), is not known in nature, but both (*E*)- and (*Z*)-26-methyldesmosterols (**67**) have been shown to be transformed to 24(28)-dehydroaplysterol (**27**) in a cell-free extract from yeast.[107] Even if both 24(28)-dehydroaplysterol (**27**) isomers are utilized, it may be that only one of the two epimers is the natural precursor. To date only the 25*S* isomer **27** has been found in nature.

Equal incorporation of both codisterol (**20**) and epicodisterol (**18**) was found in the biosynthesis of xestosterol (Figure 40, **68**) in *Xestospongia testudinaria*.[108] Because of this nonspecificity, 25-dehydroaplysterol (**58**) was fed as a mixture of stereoisomers with good incorporation. To probe the degree of

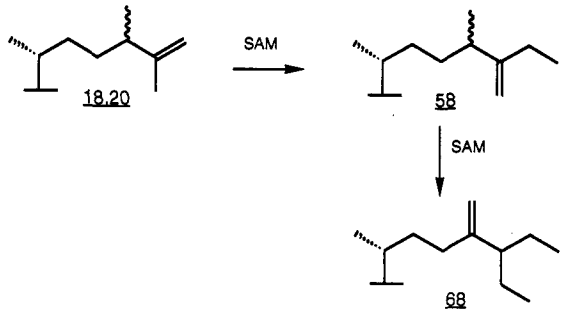

Figure 40.

nonspecificity in this biosynthetic pathway, clerosterol (**21**) and epiclerosterol (**66**) were also fed, but were not utilized. The equal rates of overall transformation of codisterol (**20**) and epicodisterol (**18**) may obscure preferences for opposite C-24 configurations in the individual methylation steps. It remains unknown if the natural sequence is also nonstereospecific; however, to date only the 24*R* isomer of **58** is known in nature.

In another variant of triple side chain methylation, the biosyntheses of stelliferasterol (**69**) and isostelliferasterol (**70**) were investigated in *Jaspis stellifera*,[109] where they represent 5.6% and 1.3% of the total sterols (Figure 41).[110] Clerosterol (**21**) and epiclerosterol (**66**)

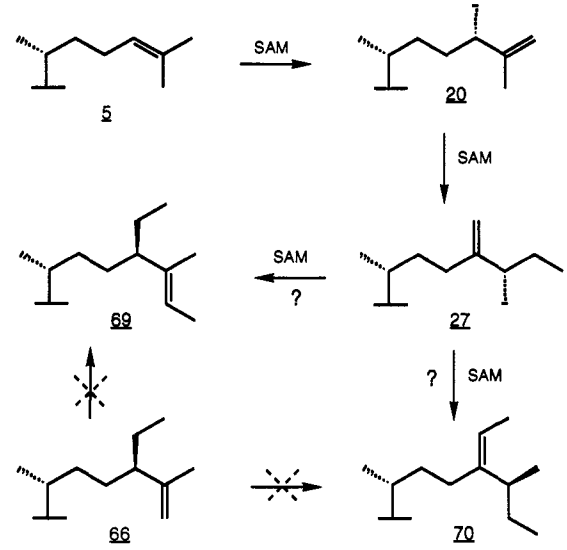

Figure 41.

were ruled out as precursors and 24-methylenecholesterol (**4**) was only incorporated into fucosterol (**22**). Desmosterol (**5**) was converted well into 24(28)-dehydroaplysterol (**27**), which represents 55% of the total sterols, and fucosterol (**22**). Codisterol (**20**) was preferred to epicodisterol (**18**) and was incorporated well into 24(28)-dehydroaplysterol (**27**). Codisterol (**20**) was better incorporated into stelliferasterol (**69**) and isostelliferasterol (**70**) than was desmosterol (**5**). This is expected, since codisterol (**20**) is a more immediate precursor. However, because the expected precursor, 24(28)-dehydroaplysterol (**27**), was more poorly incorporated than codisterol (**20**), the authors dismissed this pathway (Figure 41), although it was the most likely one. It may be worthwhile to repeat this experiment.

The biosynthesis of yet another sterol bearing three extra carbon atoms in the side chain, mutasterol (Figure 42, **71**), was studied in *Xestospongia muta*.[111] This sponge appears to have three distinct sterol compositions, indicating that it is probably not one, but three separate species of sponges. Mutasterol (**71**), is a minor sterol (ca. 3%) in one of these sponges. Its side chain has also been found in a plant triterpene.[112] This sponge also contains 24(28)-dehydroaplysterol (**27**) as ca. 25% of its total sterols and verongulasterol (**59**) (ca. 9%). Radiolabeled desmosterol (**5**) was incorporated well into all three sterols. Codisterol (**20**) was incorporated into 24(28)-dehydroaplysterol (Figure 37, **27**), but much more poorly into mutasterol (**71**) and verongulasterol (**59**) than was desmosterol (**5**). Because of the configuration of C-24, epicodisterol (**18**) is expected to be a precursor of verongulasterol (Figure 27, **59**). It was, however, not greatly preferred to codisterol (**20**). Both isomers of 25-dehydroaplysterol (**58**) resulted in incorporation into verongulasterol (**59**). Because the various stereoisomers of verongulasterol (**59**) are inseparable by HPLC without derivatization, it is not known if these results represent formation of different product isomers when confronted with nonnatural substrates. As mentioned, the 24*S* isomer of 25-dehydroaplysterol (**58**) is not yet known in nature and probably does not represent a natural intermediate. The 25-dehydroaplysterols (**58**) were shown not to isomerize to 24(28)-dehydroaplysterol (**27**), neither did two isomers of 24-dehydroaplysterol (**72**). Feeding experiments with 24(28)-dehydroaplysterol (**27**) were not carried out.

In the above study mutasterol (**71**) was found to be formed from both (*E*)- and (*Z*)-24-dehydroaplysterols (Figure 42, **72**), as had previously been proposed,[63,112]

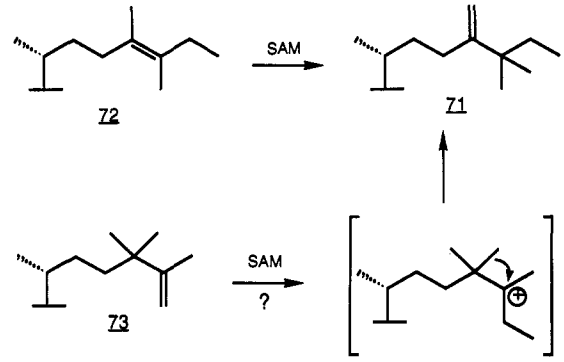

Figure 42.

although in low yield.[111] The possible intermediacy of 24-methylcodisterol (**73**) via mechanism involving a methyl shift (Figure 42) had also been proposed,[63,112] but was not tested. The tetrasubstituted olelfinic 24-dehydroaplysterols (**72**) have not been isolated from the sponge, but, as tetrasubstituted olefins, they are very sensitive to autoxidation (approximately 35 times more than trisubstituted olefins).[113] The trisubstituted olefinic sterols, desmosterol (**5**) and fucosterol (**22**), are well known for their sensitivity to autoxidation.[114,115] Avoidance of light (especially in the presence of photosensitizing pigments) and air should be observed in any attempted isolation. Another tetrasubstituted olefinic sterol, 24-ethyldesmosterol (**74**),[116] has been recently isolated from a sponge and may conceivably lead to mutasterol (**71**) via mechanism involving an ethyl shift (Figure 43).

A possible complication is indicated by the incorporation of (3-^{3}H)squalene into radiolabeled mutasterol

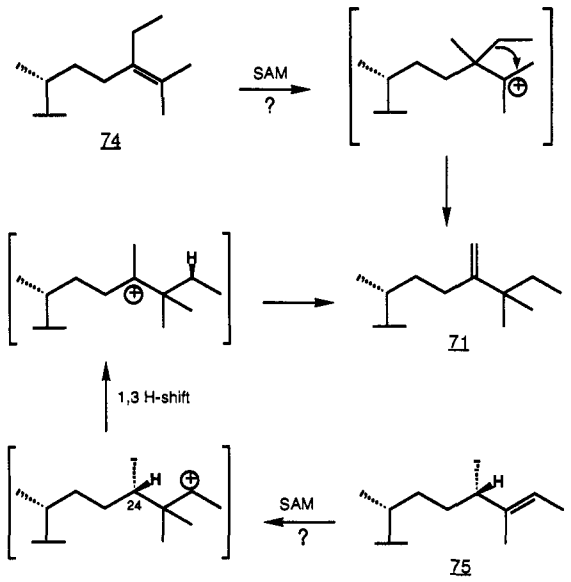

Figure 43.

(71).[111] As discussed above (see common side chains), sterols are only expected to be labeled using (3-^{3}H)-squalene if they retain the hydrogen originally at C-24. A rare case of nonspecific incorporation of (3-^{3}H) label into all sterols via a compartmentalized pool of pyridine dinucleotide has been demonstrated in a sponge,[49] but can be ruled out here since the sterol products were not randomly labeled. All of the routes to mutasterol (71) discussed above proceed with the loss of the C-24 hydrogen. A possible route via jaspisterol (75)[117] involving a 1,3-hydride shift by which the 24-^{3}H label is retained is offered in Figure 43. Xestospongesterol (Figure 44, 76) and 25-methylxestosterol (77), a higher

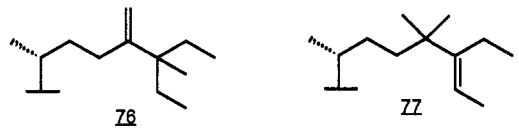

Figure 44.

homolog of mutasterol (71), cooccur in *Xestospongia* sp. and probably have a similar biosynthesis to mutasterol (71).[118]

An analog of mutasterol (71) with a *tert*-butyl instead of a neopentyl group in the side chain (Figure 45, 78)

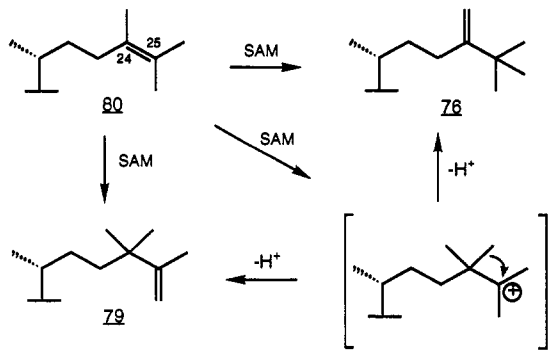

Figure 45.

has been isolated in small amounts from various higher plants.[119,120] Its biosynthesis has been studied using a cell-free extract of *Phaseolus vulgaris* (green beans).[121] It was shown that SAM methylation of 24-methylidesmosterol (60) gives rise to the *tert*-butyl sterol 78 using the bean enzyme as well as the enzymes from yeast and *Chlamydomonas reinhardtii* (an alga), although the *tert*-butyl sterol 78 is not known to occur in the latter two organisms. In yeast it was shown by degradation that the methylation takes place at C-25. In *Chlamydomonas* another product, 24-methylcodisterol (79), is formed concurrently. Although this can be discussed in terms of methylation at C-24 or C-25, it has recently been found that methyl migrations are involved in this reaction, thereby rendering the question of the initial site of methylation a moot point.[122]

IV. Algal Sterols

Algae are the primary producers of biomass in the sea and their sterols are found throughout the food chain. The sterol composition of algae and its chemotaxonomic value has recently been reviewed.[123] While there are important differences between the sterols of different algal groups, they tend to be, with minor variations, the conventional sterols common in plants and fungi. Algal sterol biosynthesis has been included in reviews of phytosterol biosynthesis.[10,124] Very few biosynthetic experiments with sterols have been carried out with marine algae. The permeability barriers of autotrophic algae have limited feeding experiments to substrates such as methionine and mevalonate.[125-127] To solve this problem, crude enzymes systems (cell-free extracts) have been employed.[26,128,129] Because of their affinities to higher plants, the study of algal sterol biosynthesis (mainly with freshwater species) has been important in elucidating phytosterol biosynthesis.[124] New insights are likely to come from the study of the more diverse marine algae.

A. 24-Propylidenecholesterol

24-Propylidenecholesterol (80) was first isolated from scallops in the early 1970s.[130] It has since been shown to occur as the principle sterol in chrysophyte algae of the order Sarcinochrysidales.[131-133] Molecular fossils of this sterol have been of interest in organic geochemistry.[134] The biosynthesis of this compound is surprisingly interesting, since it apparently involves a protonated cyclopropane (Figure 46, 81).

A thorough analysis of the sterols from *Chrysoderma mucosa* cultures succeeded in identifying 30 sterols, several of them new.[101] Among these were two isomeric cyclopropyl sterols 82 and 83 which had been proposed as biosynthetic precursors via cyclopropyl ring opening.[132] Enzyme-catalyzed cyclopropyl ring opening is well known in the biosynthesis of the sterol nucleus in plants[135] and has been considered in the biosynthesis of marine sterol side chains.[63] Several other C_{30} sterols (Figure 46) bearing three additional methyl groups in the side chain were found in support of this theory. On the other hand no 24-vinylcholesterol (Figure 47, 84), which had also been proposed as a precursor,[132] was detected. Feeding experiments with CD_3-labeled methionine and product analysis by mass spectrometry and ^{2}H NMR indicated that all six hydrogen atoms of the 24-propylidene group had been labeled. Because enzymatic acid-catalyzed ring opening of the cyclopropane is expected to proceed with the incorporation of a proton from the medium, this route was ruled out. Also ruled

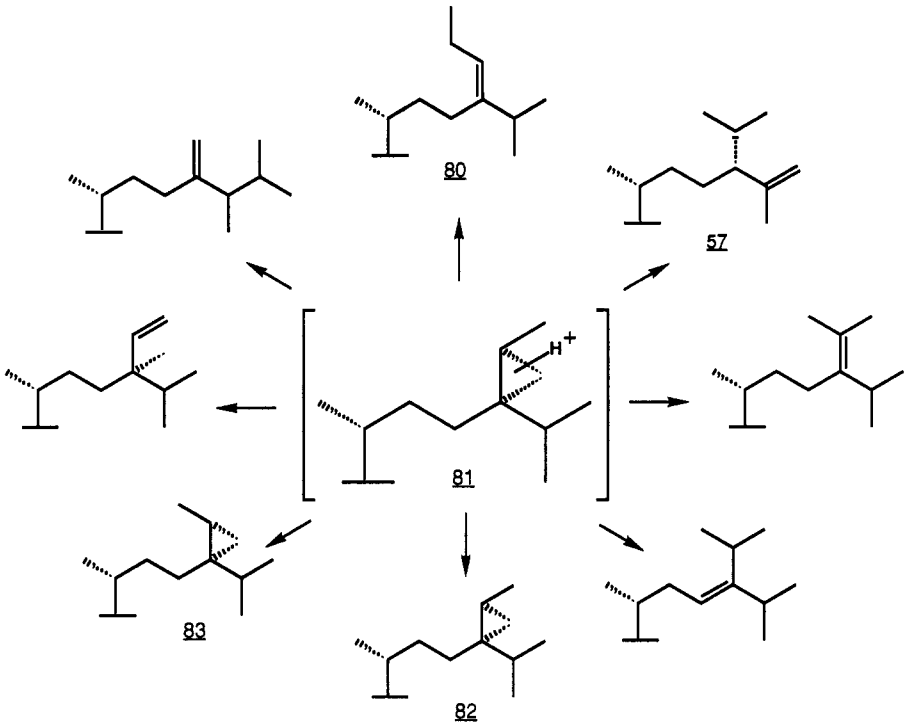

Figure 46.

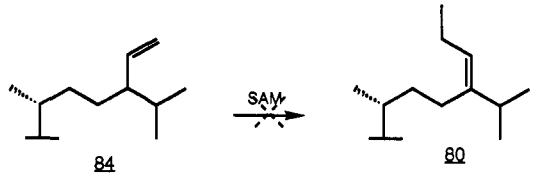

Figure 47.

out through feeding experiments was the intermediacy of ethionine, an analog of methionine which is not known in nature. Since the alga did not take up sterols from the culture medium further experiments were not possible until the production of a cell-free extract.

Experiments with (^{3}H-*methyl*)SAM and a membrane enzyme preparation from *Chrysoderma mucosa* succeeded in demonstrating the biosynthetic sequence (Figure 48).[136] The sequence was shown to be desmo-

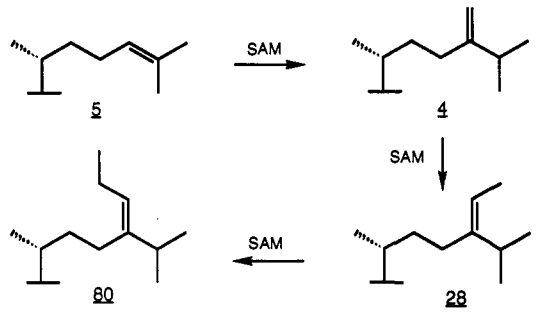

Figure 48.

sterol (**5**) to 24-methylenecholesterol (**4**) to isofucosterol (**26**) to 24-propylidenecholesterol (**80**). Neither fucosterol (**22**) nor 24-vinylcholesterol (**84**) were accepted as substrates. Attempts to detect 24-vinylcholesterol (**84**) through the use of rationally designed enzyme inhibitors[137,138] failed. The interpretation of these results is that a protonated cyclopropane (nonclassical carbonium ion) intermediate (Figure 46, **81**) is formed in the methylation of isofucosterol (**26**), which gives rise to 24-propylidenecholesterol (**80**) [and presumably the other C_{30} products (Figure 46)].

Protonated cyclopropanes have been invoked as enzyme reaction intermediates for oxidosqualene cyclases[99] as well as for SAM-sterol methyltransferases.[33] In this case, the argument for such an intermediate is supported by degradation experiments to localize the radiolabel. Tritium appears to be roughly equally distributed between C-28 and C-29 of the product **80**. This is consistent with a corner to corner (or edge to corner) proton migration, followed by ring opening and proton loss (Figure 49). In comparison, a reaction

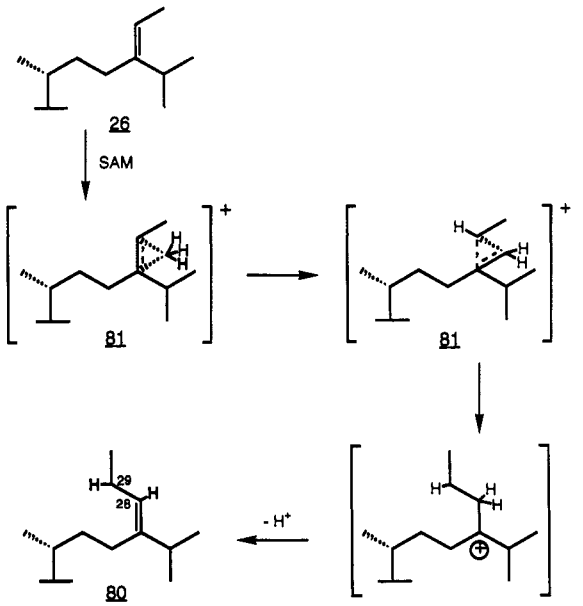

Figure 49.

mechanism involving the intermediacy of classical carbonium ions (Figure 50, **85**) would require a secondary carbonium ion to rearrange to a primary

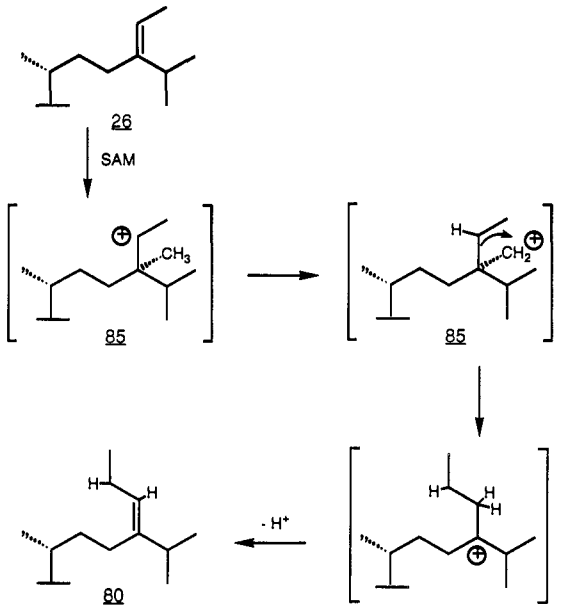

Figure 50.

carbonium ion via 1,3-hydride shift, followed by an alkyl shift. While the intermediate carbonium ions may conceivably be stabilized by the enzyme,[139] they are clearly not stabilized enough to prevent some interesting carbonium ion rearrangements.

Encouraged by the demonstration of a novel biosynthetic mechanism, an attempt was made to demonstrate rearrangements of nonclassical carbonium ions possibly involved in other biological methylation reactions.[136] The reaction that produces the 24-ethylidenecholesterols (22 and 26) may involve the intermediacy of nonclassical carbonium ions having a high degree of symmetry (Figure 51, 86). Scrambling due

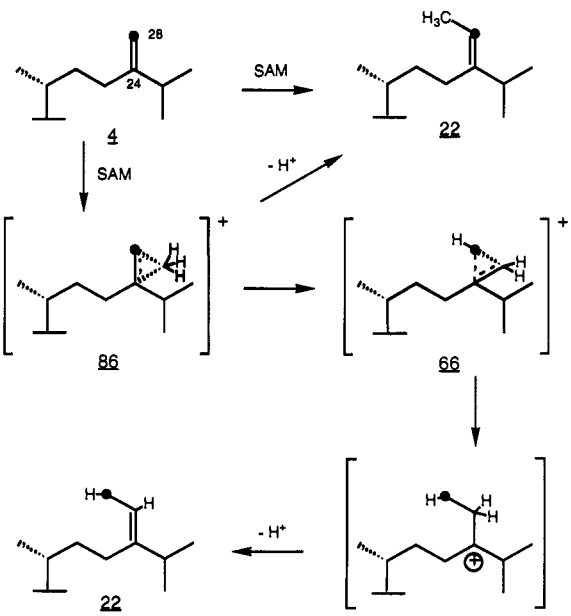

Figure 51.

to isomerization of these nonclassical carbonium ions via corner to corner proton migration was not detected in isofucosterol (26), formed by the cell-free extract of *Chrysoderma mucosa*, nor in fucosterol (22), formed by a cell-free extract of *Macrocystis pyrifera*, a Phaeophyte alga. It is possible that in this experiment the product of scrambling may have been suppressed due to an isotope effect arising from the (^{3}H-*methyl*)SAM used. The tritium isotope effect could have been used to favor the detection of scrambling by using (28-^{3}H)-methylenecholesterol (4) instead of (^{3}H-*methyl*)SAM.

It should be pointed out that the structural similarity of fucosterol (22), the typical Phaeophyte sterol, and 24-propylidenecholesterol (80) should not be considered evidence to ally the Sarcinochrysidales with the Phaeophytes. The biosynthetic precursor to 24-propylidenecholesterol (80) is isofucosterol (26), which has the opposite geometry of the 24(28) double bond.

B. 22-Dehydrocholesterol

22-Dehydrocholesterol (10) is an ubiquitous marine sterol found in small amounts in filter feeding organisms such as sponges. It has been found as the main sterol (82%) in a cultured unicellular red alga (*Porphyridium* sp.),[140] and in two diatoms (*Biddulphia sinensis*, 75%, and *Nitzchia cylindris*, 66%).[141,142] Experiments have not been carried out with any of these organisms, but cholesterol (Figure 52, 24) has been shown to be

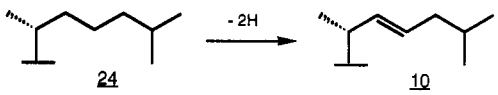

Figure 52.

converted to 22-dehydrocholesterol (10) using a cell-free extract from yeast with Δ^{22}-dehydrogenase activity.[90]

V. Conclusion

Many new and unexpected discoveries have come from the biosynthetic study of marine sterol side chains, e.g. the intricate methylation sequences that give rise to highly alkylated sponge sterols, the concurrent pathways of alkylation and dealkylation in sponges, and the complicated carbonium ion rearrangments leading to 24-propylidenecholesterol (80) and the cyclopropyl sterol petrosterol (34). Although much progress has been made in recent years, many details remain to be worked out and it is expected that the use of enzymatic methods will prove useful for further investigations, especially of methylation sequences. Some interesting uninvestigated problems remain, such as the biosynthesis of the dinoflagellate 27-norsterols and of the C_{27} cyclopropyl sterol papakusterol·glaucasterol (32). Aside from unresolved questions of intimate mechanistic details, further research may shed light on the purpose of these biosynthetic pathways. Do the unusual products of these pathways confer special advantages on the organism? The function of marine sterols in lipid membranes has been investigated, but is not well understood.[143] Steroids with biological activity have been isolated from dinoflagellates and sponges.[21,144] Are the unusual side chains of marine sterols important for the elaboration of chemical defenses or hormones? What does the variety of sterols found in marine organisms say about eukaryotic evolution?

Acknowledgments

The author wishes to thank his mentor Prof. Carl Djerassi and Prof. Duilio Arigoni for his helpful criticism.

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