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RECENT STUDIES IN THE MARINE STEROL FIELD

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Abstract - Recent work in the marine sterol field, notably from our own laboratory, is reviewed with major emphasis on novel structural types, possible biosynthetic origin, probable biological function and prognosis for the future.

INTRODUCTION

More is known about the intimate details of the biosynthesis of cholesterol (Ref. 1) and of plant sterols substituted in the side chain (Ref. 1 & 2) than probably any other naturally occurring substance. Similarly, an enormous literature exists on the biological function of cholesterol (Ref. 3) and on the basic role of sterols in maintaining plasma membrane integrity (Ref. 4). Less than a decade ago, one could have concluded that the overall picture with respect to chemical structure (i.e. isolation of novel structural types), biosynthesis and biological function of sterols had been drawn and that only minor details had to be filled in. This conclusion was severely shaken by the isolation in recent years of a bewildering variety of new sterols from marine organisms. Not only do these marine sterols possess structural variations unprecedented in terrestrial sources, but they also raise new questions concerning their biosynthesis and biological function. Progress has been so rapid that even some of the most recent reviews (Ref. 5-7) have become outdated. The purpose of the present paper is to summarize some of the most recent results from our own laboratory and to delineate the directions in which our research on marine sterols is progressing.

The occurrence of sterols in marine organisms can be discussed in various ways. Schmitz in his review (Ref. 5) simply used a chemical approach by discussing the sterol structures in terms of carbon content. While convenient from a chemical standpoint, such a presentation has no bearing on biosynthesis or biological function and none was intended in that review. Goad (Ref. 6) used a taxonomic approach starting at the bottom of the evolutionary tree with algae and fungi, and then proceeding via sponges, coelenterate animals and other intermediate phyla to the chordates. In addition to emphasizing the chemotaxonomic potential of marine sterol analyses - demonstrated in a striking fashion in sponges by Bergquist (Ref. 8) - this approach offers important clues to the possible origin of certain sterols in the food chain. The taxonomic approach offers some assistance in designing appropriate experiments for biosynthesic studies, which are much more complicated than in terrestrial organisms. To paraphrase an infamous limerick, when wondering about the origin of marine sterols we usually do not know "who is doing what, with which, and to whom." As Goad correctly pointed out (Ref. 6), the existence of a given sterol in a specific marine organism may be due to one or more of the following four processes:

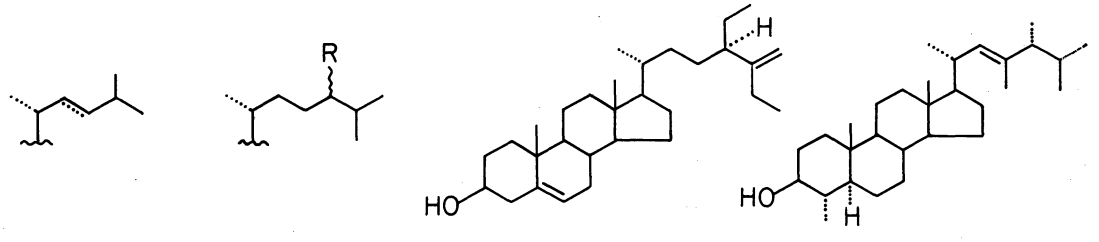
(1) *De novo* sterol biosynthesis via acetate, mevalonate and squalene.
(2) Dietary origin without further chemical modification.
(3) Dietary origin of sterol(s) followed by chemical modification.
(4) Result of symbiotic relationship between host and symbiont (e.g. algae, fungi, bacteria).

In terrestrial animals and plants, it is usually simple to differentiate *de novo* biosynthesis from other factors, especially since the dietary intake generally is either known or can be controlled. In most marine invertebrates, this is not the case. Indeed, it is this uncertainty which until now has been the chief experimental and conceptual impediment to more rapid biosynthetic studies with isotopically labelled precursors. As a result, many of the present biosynthetic generalizations are speculative and rest on an approach, which had proved highly successful in the sixties in the field of indole alkaloid plant biosynthesis prior to successful tracer incorporation experiments: By isolating and structurally characterizing all members of a given chemical class (in the present instance, sterols) - within one organism, in related species and, most importantly, in potential food chain contributors - one eventually amasses a large body of related chemical structures, which frequently fit into a consistent hypothetical biosynthetic scheme. Recent examples of this approach will

be offered below. In depending upon such an approach, it is important not to overlook trace constituents, which may be rapid turnover intermediates in a multi-step sequence to a final (predominating) sterol.

When examining the by now extraordinarily voluminous literature on marine sterols (Ref. 5-7) one is struck by the following qualitative picture. In terms of chemical structure one encounters three situations:

(a) The most common is the isolation of a wide variety of sterols - with most of the structural variations occurring in the side chain. By the use of sensitive separation (high performance liquid and gas chromatography, as well as argentic thin layer chromatography) and detection (mass spectrometry coupled with gas chromatography, and high magnetic field NMR spectroscopy) methods, nearly fifty sterols could be detected in a single marine invertebrate (Ref. 9); mixtures of ten to thirty sterols are very common (Ref. 10). It is likely that the bulk of these sterols are accumulated unchanged from the food; some of these dietary sterols have very characteristic structural features associated with specific food web contributors. An example are sterols with the 24-norcholesterol side chain (1) (Ref. 5, 11), which are almost certainly of phytoplanktonic origin, although nobody has yet been able to verify experimentally which of the proposed biosynthetic schemes (Ref. 7) is operative in nature. By contrast to the unique 24-norcholesterol types (1), isolation of sterols with the unsubstituted cholesterol or 24-alkylated (one or two carbon atoms) cholesterol side chain (2) offers only occasionally useful information with respect to possible dietary origin.



1 2 3 4

R=H; CH_3; C_2H_5

(b) Less common, but more significant, are instances in which a complex mixture of sterols is encountered - usually with all conceivable common and bizarre side chains - which possess a unique nuclear structural feature. These represent the most persuasive evidence for the occurrence of subsequent chemical transformation of dietary sterols and some recent pertinent examples will be presented below.

(c) Of greatest interest to us are the rare examples where one organism contains only a single or very few sterols with unique structural features. An example is the Indo-pacific sponge *Strongylophora durissima*, which appears to contain only a single sterol, strongylosterol (3), with an extended side chain (Ref. 12) never encountered in terrestrial sterols. The predominant or exclusive presence of a structurally unique sterol not only narrows the biosynthetic options, but also suggests a unique biological role.

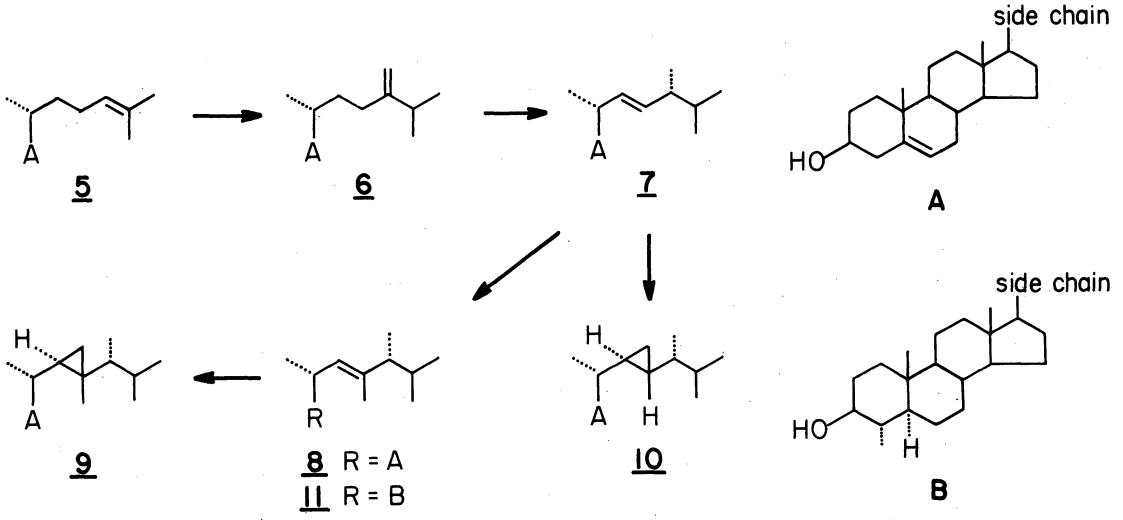
In the absence of conventional sterols, the conclusion is almost inevitable (Ref. 13) that such novel sterols replace cholesterol and its simple analogs (2) as plasma membrane components. Since the extra substitution and/or branching centers on the side chain (compare 3 with 2), we are assuming as a working hypothesis that this should reflect itself in corresponding changes in the lipophilic portion of the phospholipids, i. e. the fatty acid chain and that novel fatty acids, therefore, should be encountered. Preliminary experiments in our laboratory (Ref. 14) suggest that this approach merits serious experimental scrutiny as does the preparation of model membranes with such phospholipids followed by comparing the membrane-stabilizing properties of different sterols. Unfortunately, our preliminary experiments with the heterotropic marine dinoflagellate *Crypthecodinium cohnii*, using the fluorescence polarization technique with 1,6-diphenyl-1,3,5-hexatriene as a probe, and comparing cholesterol with dinosterol (4) (Ref. 15), were equivocal (Ref. 16). Our initial choice of *C. cohnii* was based on the report by Withers *et al.* (Ref. 17) that dinosterol (4) represented the principal sterol of this dinoflagellate. Furthermore, the Liverpool group (Ref. 18) had provided some concrete experimental evidence for the biosynthetic elaboration of the dinosterol side chain. Unfortunately, the dinosterol content of this organism varies and our particular specimen - though containing significant amounts of dinosterol (4) - was rich in cholesterol (*ca.* 50%). A similar observation has been recorded by Alam's group (Ref. 19) in their investigation of the sterol content of dinoflagellates of the genus *Gonyaulax*. To obtain unambiguous results with model membranes, one needs to deal with an organism which is essentially devoid of cholesterol or other conventional sterols.

Such experiments are now in progress in our laboratory. The main experimental barrier to such work has been the availability of fresh material to permit rapid isolation of undecomposed phospholipids. Unfortunately, most of the ideal condidate organisms (e. g. *Strongylophora durissima*) (Ref. 12) live in inaccessible regions, where rapid phospholipid isolation cannot be performed.

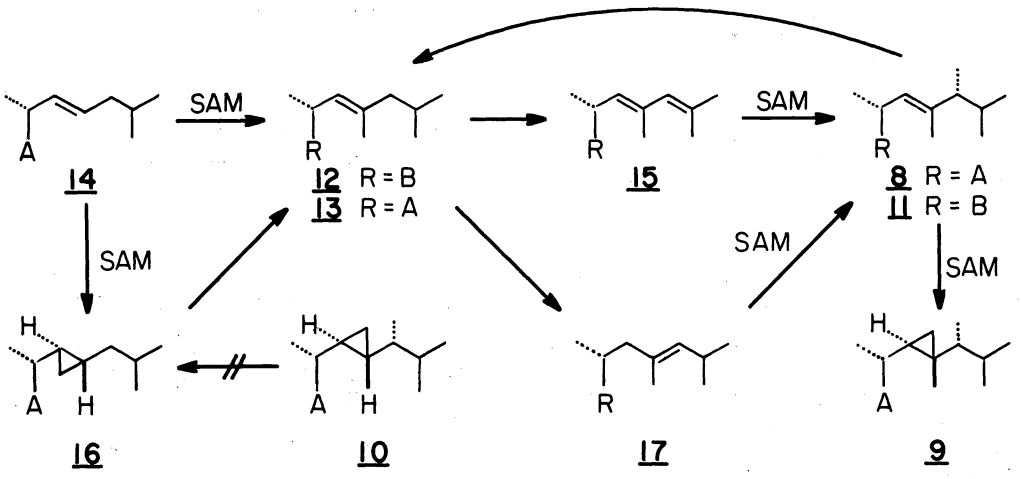
I would now like to turn to a description of recent experiments dealing with the isolation and structural identification of novel marine sterols. As will be shown, they either raise the possibility of novel biosynthetic pathways or else extend the scope of existing ones.

POSSIBLE ORIGIN OF 23-METHYLATED STEROLS

Our own interest in a systematic search for novel sterols from marine sources was prompted by the structure elucidation of gorgosterol (9) (Ref. 20) and subsequently of 23-demethylgorgosterol (10) (Ref. 21) - two sterols with the hitherto unknown feature of carbon substitution in positions 22 and 23 of the cholesterol side chain. We speculated (Ref. 7, 20) that the gorgosterol side chain (9) was generated from brassicasterol (7), which in turn is produced by the generally accepted S-adenosylmethionine (SAM) biomethylation (Ref. 1, 2, 6) of desmosterol (5). Repeated SAM bioalkylation of 7 would then lead to an intermediate Δ^{22}-23,24-dimethyl sterol (8), whose further transformation to gorgosterol (9) would be unexceptional. The lower homolog, 23-demethylgorgosterol (10), could be the product of direct SAM bioalkylation of the Δ^{22}-double bond of brassicasterol (7) and the demonstration (Ref. 20, 21) by X-ray analysis that all three sterols (7, 9, 10) possess the same absolute configuration of the relevant asymmetric centers in the side chain is consistent with such a common origin. Further indirect support for our postulated gorgosterol biosynthetic scheme was provided six years later by the isolation of two Δ^{22}-23,24-dimethyl sterols, 11 (dinosterol) (Ref. 15), and 8 (23,24-dimethyl-22-dehydrocholesterol) (Ref. 22). Except for the biosynthesis of dinosterol (11) through d_3-methionine incorporation (Ref. 18) in the dinoflagellate *C. cohnii*, this biosynthetic scheme has not yet been validated through biochemical tracer experiments, but reasonable indirect evidence (e. g. identical C-24 stereochemistry of 7, 4 and 9; natural existence of Δ^{22}-23,24-dimethyl sterols) is available.



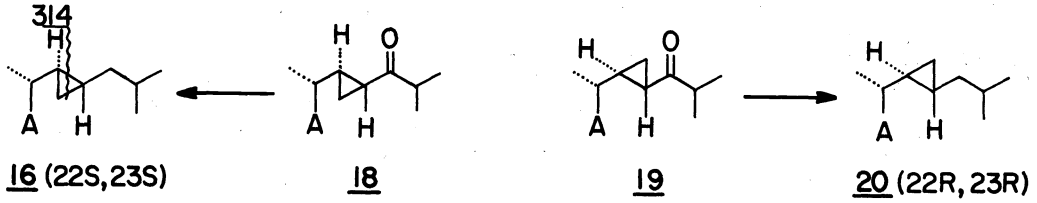
Recently, two 23-monomethyl sterols have been isolated from marine organisms - 24-demethyldinosterol (12) (Ref. 23) and 23-methyl-22-dehydrocholesterol (13) (Ref. 24 & 25). The latter has been isolated from a unicellular alga (Ref. 24) as well as from a soft coral (Ref. 25), thus providing a possible lead to a dietary origin in the soft coral. The demonstration of the natural occurrence of sterols with only a single methyl group at C-23 in the side chain raises new and interesting biosynthetic questions. One possibility is that these sterols arise by biodealkylation of the allylic C-24 methyl substituent of 8 and 11 and are thus simply side products on the main path to gorgosterol (9). Precedents for such biodealkylations in the sterol side chain are provided by the isolation of 27-nor-24-methyl sterols (Ref. 26), which presumably arise from 24-methyl sterols (e. g. 7), and of 24-nor sterols (e. g. 1), which may be the products of two such biodealkylation steps.



However, we have already drawn attention (Ref. 24) to a second possible bio-origin for such Δ^{22}-23-monomethyl sterols (12, 13), namely the direct SAM biomethylation of an isolated Δ^{22}-double bond, which is widely distributed (Ref. 6) in the marine environment (e.g. 22-dehydrocholesterol (14)). If such a process occurs, then one must also take into consideration the possibility of further biological dehydrogenation to a 23-methyl-$\Delta^{22,24}$-diene (15), which could then undergo repeated SAM biomethylation at C-24 to lead to the dinosterol side chain (8, 11) and thence to gorgosterol (9). Such a path is also consistent with the d_3-methionine incorporation experiments of Withers *et al.* (Ref. 18). Another variant (not applicable to *C. cohnii* (Ref. 18) because a d_6 rather than d_5 species would be expected with d_3 -methionine) would be double bond migration of 12 and 13 to the hitherto unknown Δ^{23}-23-methyl sterols (17), which would lead directly to the dinosterol (11) side chain upon SAM biomethylation. In other words, which of the methyl groups is introduced first in the biosynthesis of sterols bearing methyl groups in both the C-23 and C-24 position?

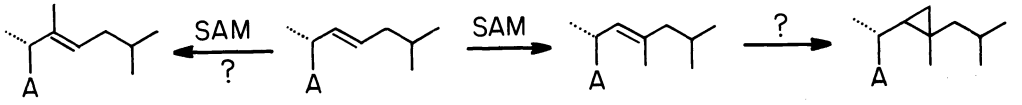
We have now been able to provide significant evidence (Ref. 27) that the direct bioalkylation of a Δ^{22}-double bond is feasible in the absence of a C-24 substituent through the observation that 22S,23S-methylenecholesterol (16) and its 5α-dihydro analog are widely distributed marine sterols, albeit in trace quantities. The reason that 22S,23S-methylenecholesterol (16) has been overlooked until now is that its gas chromatographic mobility is nearly identical with that of the common marine sterol 24-methylenecholesterol (6). Furthermore, their respective mass spectra are virtually identical and are characterized by a base peak at m/z 314. Such a peak is generally associated (Ref. 28) with a McLafferty rearrangement of $\Delta^{24(28)}$-double bonds (e. g. 6), but it is also characteristic (Ref. 20 & 21) of two bond fissions in a cyclopropane ring as indicated schematically by the wavy line in 16. The new sterol (16) was first detected in our laboratory when it was noted that whereas its gas chromatographic mobility was essentially identical with that of 24-methylenecholesterol (6), it differed dramatically in its HPLC mobility (much longer retention time). The presence of four cyclopropane signals in the 360 MHz NMR spectrum coupled with the characteristic (Ref. 20 & 21) m/z 314 mass spectral fragmentation peak immediately pointed to 22,23-methylenecholesterol as the most likely structural candidate.

The structure and absolute configuration was established by Wolff-Kishner reduction of the synthetic ketones 18 (Ref. 29) and 19 (Ref. 30), whose absolute configurations were known, because of interrelationship with standards determined by X-ray analysis. The naturally occurring 22S,23S-isomer 16 showed identical gas chromatographic mobility with the synthetic 22R,23R-isomer 20, but the NMR spectra (notably the C-21 methyl signal) exhibited characteristic differences.



The determination of the absolute configuration was important, because it proved to be opposite to that (22R,23R) of 23-demethylgorgosterol (10) (Ref. 21). Consequently naturally occurring 22S,23S-methylenecholesterol (16) could not have arisen by biodealkylation of the C-24 methyl substituent of 23-demethylgorgosterol (10) and presumably was produced by direct SAM biomethylation of 22-dehydrocholesterol (14). If such a biological process is feasible, then it is equally conceivable that Δ^{22}-23-methyl sterols (e. g. 13) are generated from 22-dehydrocholesterol (14) rather than by C-28 demethylation from 8. This in turn makes the hypothetical question raised earlier a real one: Does the gorgosterol (9) biosynthesis proceed only from "right to left" (i. e. 5→7→8→9) or can it also proceed from "left to right" (14→13→8→9)?

A definite answer will only be provided by detailed biochemical tracer experiments, but a search for potential "missing links" is clearly indicated with the hitherto unknown sterols 15 and 17 being prime candidates. As a result of the isolation of Δ^{22}-23-methyl (e. g. 13) and 22,23-methylene (e. g. 16) sterols, the search for related, but as yet, unknown sterols is justified. For instance, if 23-methyl-22-dehydrocholesterol (13) arises directly from 22-dehydrocholesterol (14), is it possible that such SAM biomethylation produces also 22-methyl-22-dehydrocholesterol (21)? In view of the existence of 23-methyl-Δ^{22}-sterols (12, 13), is it not possible that cyclopropanation of that double bond might have occurred in the marine environment to provide the as yet unknown 24-demethylgorgosterol (22)?



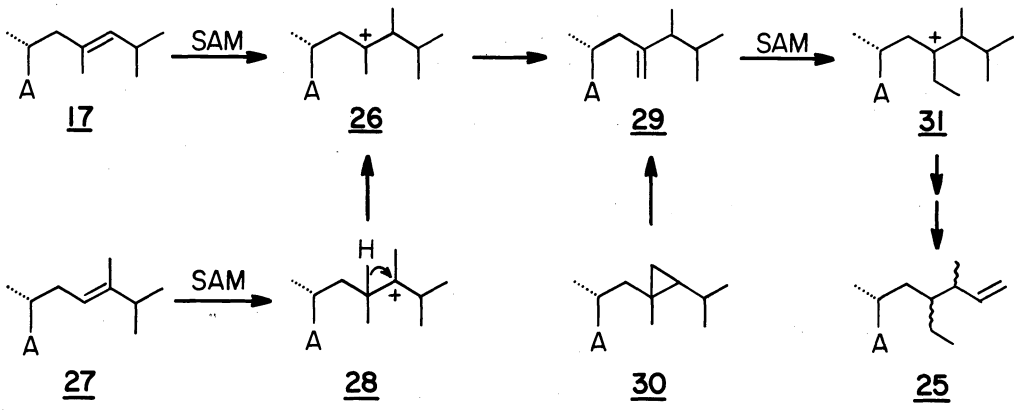
21 14 13 22

Our isolation (Ref. 27) of 22S,23S-methylenecholesterol (16) raises again the question of the biochemical role of the cyclopropane ring in the sterol side chain, which has only been encountered in marine, but not terrestrial sterols. Are they metabolic dead ends or do they represent biosynthetic intermediates? Even prior to the isolation of such sterols, Lederer (Ref. 2) had already speculated that they may be intermediates in the generation of saturated methylated side chains, whereas we have suggested (Ref. 7) that an isomerase (of the type known (Ref. 1 & 2) to exist in the plant kingdom in the conversion of the steroidal 9,10-methylene grouping into allylically unsaturated methyl groups) might also operate in the sterol side chain. If such an isomerase exists, then it is conceivable that 22,23-methylenecholesterol (16) may be a precursor of Δ^{22}-23-methyl sterols (e. g. 13), possibly via a $\Delta^{20(22)}$-23-methyl intermediate. Some circumstancial evidence supporting the existence of such an isomerase is provided by our recent study (Ref. 10) of the minor sterols of the Mediterranean sponge *Petrosia ficiformis*. Its principal sterol (60 %) is the novel cyclopropane-containing petrosterol (23)(Ref 31), but from the accompanying multi-component (32 sterols) mixture, there was isolated a new sterol, 26(29)-dehydroaplysterol (24). Its structure is precisely the one expected from enzymatic isomerization of petrosterol (23); successful incorporation of radioactive petrosterol into the sponge would be required to determine whether the isolation of 24 is only fortuitous or of real biogenetic significance.

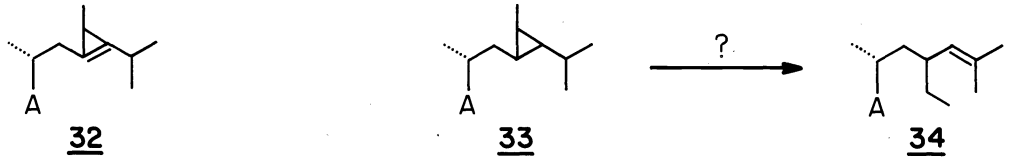
23 24

Our detailed study (Ref. 10) of the minor sterols in *Petrosia ficiformis* led to the unexpected observation that 23-ethylated sterols are also produced in nature. The first representative of this class was named ficisterol (25)(Ref. 32) and its unusual structure was confirmed by synthesis (Ref. 33). The absence of a C-27 methyl group, though noteworthy, is not unique, since this structural feature is present in various 27-nor-24-methyl sterols of marine origin (Ref. 26). However, the presence of an ethyl substituent at C-23 is unique

and points towards the occurrence in nature of 23-methylene intermediates (e. g. 29), which could then serve as substrates for SAM biomethylation in the standard manner (Ref. 1, 2, 6) to afford a 23-ethyl precursor 31 for eventual biotransformation to ficisterol (25). Theoretically, the most straightforward route to such a 24-methyl-23-methylene intermediate 29 would be via the carbonium ion 26, which in turn could originate from two hitherto unknown precursors, namely a Δ^{23}-23-methyl (17) or a Δ^{23}-24-methyl (27) sterol. Isomerization of the cyclopropane 30 offers another possible path to the 23-methylene sterol 29. We are currently synthesizing such sterols in order to facilitate their possible recognition from natural sources.



Still another conceivable route to ficisterol may be via ring opening of a substituted 23-24 cyclopropyl ring. A related cyclopropene, calysterol (32) is known (Ref. 34) to undergo chemical ring opening to various oxygenated 23-ethyl analogs; if the saturated analog 33 of calysterol (32) exists in nature, it is conceivable that it may isomerize to a Δ^{24}-23-ethyl sterol (34), whose subsequent biotransformation to ficisterol (25) would be relatively straightforward. It remains to be seen whether any of these speculative schemes can be supported by experimental evidence.



ISOLATION AND POSSIBLE BIOSYNTHESIS OF NOVEL C_{30} AND C_{31} STEROLS

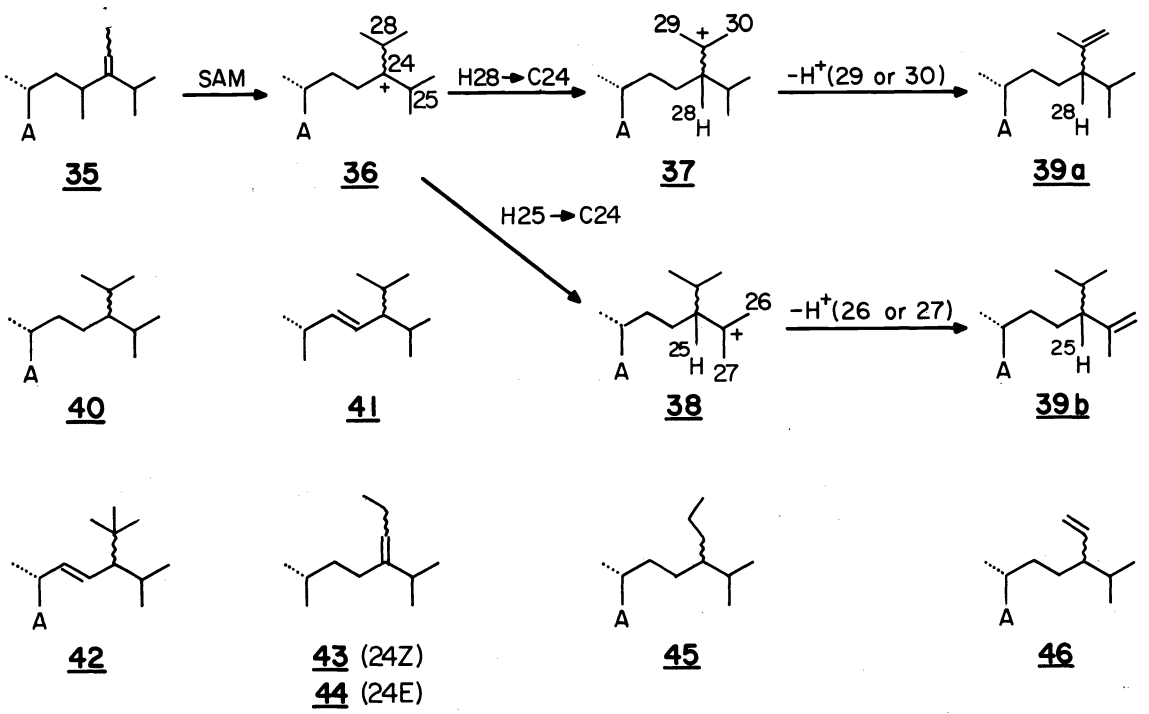
The occurrence of bioalkylation of the sterol side chain in positions 22 and 23 is one unique feature of marine sterols. As outlined in the preceeding section, most of our current views on their biosynthesis rests on analogy to well documented steps from the biosynthesis of "conventional" C_{28} and C_{29} sterols (i. e. 2) arising from a single or two biomethylation steps, and on the isolation of "missing links" in hypothetical biogenetic schemes. A second unique feature of marine sterols deals with C_{30} and C_{31} sterols with a conventional nucleus but unusually long side chains arising from triple or even quadruple biomethylations. Aside from the successful incorporation (Ref. 35) of d_3-methionine into 24E-propylidenecholesterol (44) produced by a chrysophyte (Ref. 36), no hard biochemical evidence is as yet available on their biosynthesis. However, as will be shown below, highly plausible hypothetical biosynthetic schemes can now be envisaged because of the ever increasing number of novel, but closely related, structures which have been encountered recently in our laboratory. As a result, it is now possible to predict with a reasonable degree of certainty which additional novel sterols are likely to be found.

From a biosynthetic viewpoint, all of the C_{30} and C_{31} sterols to be discussed below can be divided into two subgroups depending upon whether they are derived by further bioalkylation of a $\Delta^{24(28)}$- or a Δ^{25}-precursor.

C_{30} AND C_{31} STEROLS WITH CONVENTIONAL NUCLEI DERIVABLE FROM $\Delta^{24(28)}$PRECURSORS

Biosynthetically the most easily rationalized C_{30} sterols are the recently described 24-isopropyl and isopropenyl cholesterol derivatives (Ref. 37 & 38), since their side chain carbon skeleton is almost certainly generated by SAM biomethylation at C-28 of the very common marine sterols fucosterol or isofucosterol (35) to provide the key intermediate 36. It is interesting that both 24R- and 24S-isopropenylcholesterol (39) were isolated (Ref. 38) from the same sponge (*Verongia cauliformis*) and we believe that one explanation for the co-occurrence of both isomers is the operation of two equally plausible competitive hydrogen migrations in the intermediate 36 - migrations which have ample precedent in the biotransformation (Ref. 1 & 2) of desmosterol (5) to 24-methylenecholesterol (6). Migration of the C-28 proton of 36 to C-24 would lead to 37 and thence, upon loss of either the C-29 or C-30 proton to 24-isopropenylcholesterol (39a). Alternatively, hydrogen migration could originate from the other tertiary center at C-25 yielding intermediate 38, which upon proton loss from C-26 or C-27 would generate 25-dehydro-24-isopropylcholesterol (39b). Depending upon the stereochemical course of these hydrogen migrations, one could have resulted in a 24R and the other in a 24S enantiomer. The subsequent bioreduction to 24-isopropylcholesterol (40) and 24-isopropyl-22-dehydrocholesterol (41) involves well-trodden biosynthetic steps and it is interesting to note that these two new sterols are the only two constituents of the sterol mixture of a Pacific *Pseudoaxinyssa* species (Ref. 8, 37). The absence of the usual diverse sterol mixture, including conventional sterols, in this sponge suggests that 40 and 41 are modified dietary sterols.

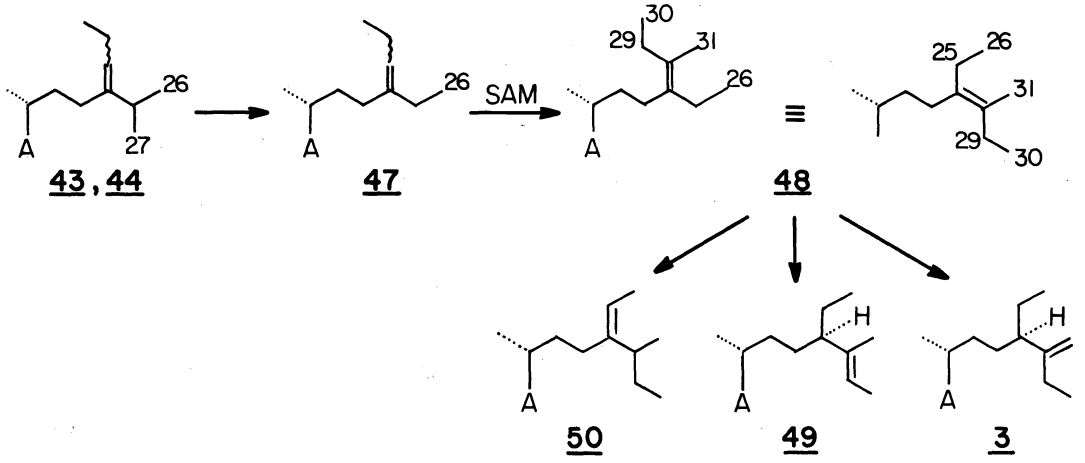
A related example is the Pacific sponge *Trachyopsis* which has been reported (Ref. 8) to contain two principal sterols - 24-isopropyl-22-dehydrocholesterol (41) and a C_{31} sterol which has been assigned tentatively the structure of 24-tert.butyl-22-dehydrocholesterol (42). If this structure assignment is correct, then it would represent the first example of quadruple biomethylation, presumably via SAM alkylation of 24-isopropenylcholesterol (39). *A priori*, such bioalkylation of a tertiary center would appear unprecedented; however, two well-documented examples have now been encountered in our laboratory as described below.



Another related group of marine sterols are 24Z-propylidenecholesterol (43), first encountered in a scallop (Ref. 40) and subsequently in other marine organisms (Ref. 41), and its 24E-isomer 44, which proved to be the principal constituent of a chrysophyte (Ref. 36). The saturated 24-n-propylcholesterol (45) was also present as a trace constituent of that alga, which we were able to culture in the laboratory. Fortunately this organism incorporates (Ref. 35) d_3-methionine to the extent of approximately 25%. Work is now in progress in our

laboratory to determine whether the propyl side chain is generated through a cyclopropyl intermediate (Ref. 35) or via three single SAM biomethylation steps starting with desmosterol (5) and proceeding *via* 24-methylenecholesterol (6) and 24-vinylcholesterol (46). This sterol, although available synthetically (Ref. 42), has so far not been encountered in nature and a search for it is now under way in our laboratory.

The natural occurrence of 24-propylidenecholesterol (43, 44) and the demonstration, through the isolation of 39, 40 and 41, that SAM alkylation can lead to branched alkyl chains (35→36), introduces the theoretical possibility that such propylidene sterols may be biosynthetic intermediates for another class of C_{30} sterols, which under ordinary circumstances would be assumed to arise from Δ^{25}-precursors (see next section). As has already been pointed out by us (Ref. 7), if 24-propylidenecholesterol (43, 44) underwent biodemethylation of the allylic C-26 or C-27 substituent, the resulting 27-nor sterol 47 upon SAM alkylation at C-28 - by analogy to the transformation of 35→36 - would yield the branched sterol 48. The latter has the carbon skeleton of three recently isolated C_{30} sponge sterols, strongylosterol (3)(Ref. 12), stelliferasterol (49)(Ref. 43) and isostelliferasterol (50)(Ref. 43) which could all be derived by double bond migration (or hydrogenation followed by dehydrogenation (Ref. 39)) of intermediate 48.



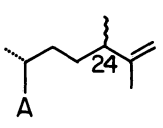
C_{30} AND C_{31} STEROLS WITH CONVENTIONAL NUCLEI DERIVABLE FROM Δ^{25}-PRECURSORS

Structurally, this is the more interesting subgroup, since it encompasses all of the recently isolated sterols with side chains longer than those existing in conventional sterols of the cholesterol type (2). Their common feature is that they have undergone side chain elongation through bioalkylation of the two termini at C-26 and/or C-27. The obvious biosynthetic precursors would be codisterol (51) and clerosterol (53), both of which are known algal constituents (Ref. 6). Both of them have the 24S configuration, which is opposite to that (24R) found in those marine sterols with "extended" side chains (e.g. 3, 49 and aplysterol (Ref. 44)) in which this stereochemical feature has been established. Fortunately, we have recently demonstrated (Ref. 38) the natural occurrence of the 24R isomer 52 of codisterol, thus indicating that both C-24 epimers of sterols with Δ^{25}-double bonds can be considered plausible biosynthetic starting materials.

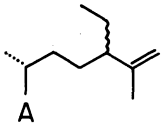
The isolation and structure elucidation of the first two C_{30} sterols possessing terminal ethyl, rather than methyl substituents - verongulasterol (55)(Ref. 45) and xestosterol (57) (Ref. 46) - have already been reported from our laboratory. Recently (Ref. 47), we have also encountered the 25Z isomer 56 of verongulasterol in some *Xestospongia* species. Whereas the E (55) and Z (56) isomers of verongulasterol are only trace constituents, xestosterol (57) may be of biosynthetic as well as biological significance, since it represents over 70% of the sterol mixture of that sponge. We have now established the structure of three additional trace sterols from *Xestospongia* species which shed considerable light on the range and site of possible biomethylations.

The first is a C_{30} sterol, named mutasterol (from *Xestospongia muta*), which was shown by spectroscopic and synthetic means to be 24-methylene-25-ethylcholesterol (58)(Ref. 48). The

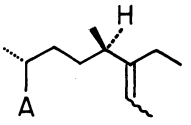
other two sterols (Ref. 49), 59 and 60, are related to mutasterol (58) in that they are also derived from a *Xestospongia* species and that they contain the structurally unusual feature of an acyclic, quaternary carbon in the sterol side chain. These two new sterols (59, 60) are the first authenticated C_{31} sterols with a C_{12} side chain, in other words, products of quadruple biomethylation. If the tentatively assigned (Ref. 8) 24-tert. butyl-22-dehydrocholesterol structure (42) should prove to be correct, then this would be a third example of such massive bioalkylation.



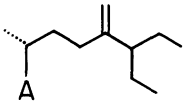
51 (24S)
52 (24R)



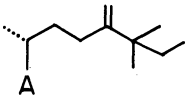
53 (24S)
54 (24R)



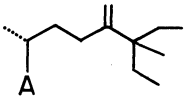
55 (25E)
56 (25Z)



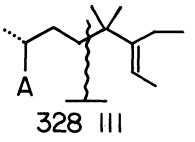
57



58



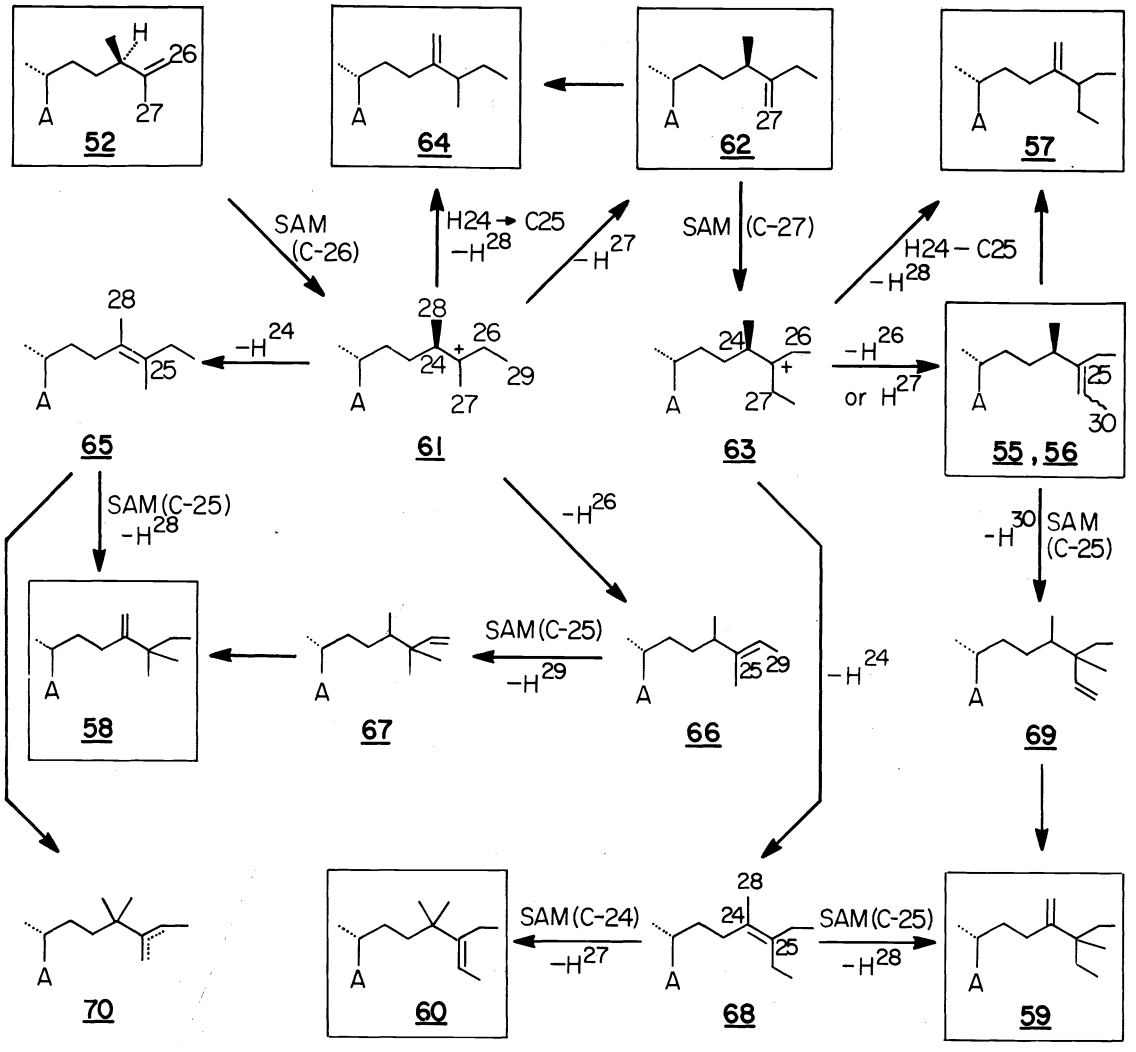
59



60

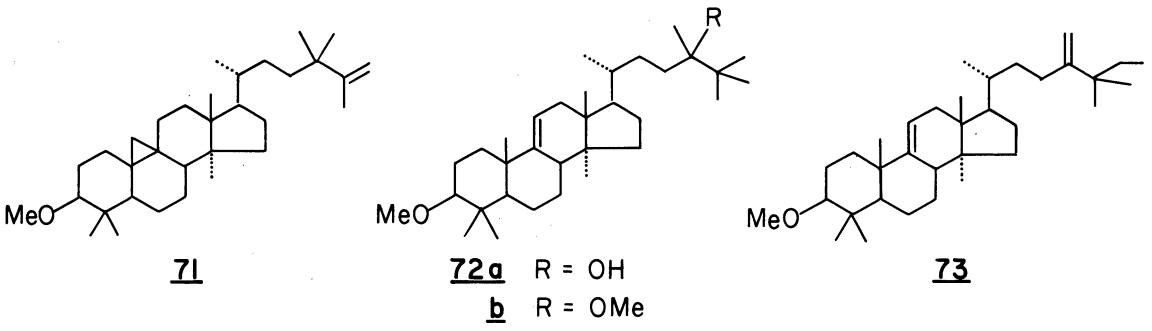
Mutasterol (58), 25-methylxestosterol (59) and xestospongesterol (60) offer beautiful illustrations of how our detailed knowledge of NMR and mass spectral behavior can lead rapidly to structure assignments with sub-milligram quantities of material. Each of these sterols displayed well differentiated methyl and olefinic signals in their 360 MHz spectra, especially when the measurements were performed in both $CDCl_3$ and C_6D_6, which could readily be interpreted. Equally significant was the use of earlier accumulated (Ref. 28, 50) generalizations on the mass spectral fragmentation of sterols with unsaturated side chains. Mutasterol (58) and 25-methylxestosterol (59) displayed their base peak at m/z 314 due to a McLafferty rearrangement (Ref. 28) associated with the $\Delta^{24(28)}$-double bond. Xestospongesterol (60), on the other hand, had its two most intense peaks at m/z 328 and 111 as indicated schematically in structure 60. We had earlier called attention (Ref. 50) to the fact that Δ^{25}-double bonds in sterols such as 51 - 56 exhibit peaks at m/z 328 (associated with McLafferty rearrangement of the Δ^{25}-double bond) as well as at m/z 314 (due to electron-impact induced rearrangement to a Δ^{24}-isomer, which then undergoes its own diagnostic McLafferty rearrangement). The virtual absence of such an m/z 314 peak in the xestospongesterol (60) spectrum can be attributed to the presence of a quaternary center at C-24 which precludes rearrangement of the Δ^{25}-double bond. The base peak at m/z 111 (see wavy line in 60) is clearly due to fission at the highly labilized allylic quaternary center at C-24.

The isolation of this group of C_{30} and C_{31} sterols from the same or related sponges fits into a consistent biosynthetic scheme starting with epicodisterol (52). Those structures enclosed in boxes refer to sterols, whose presence in nature has been demonstrated; all but one of these (64)(Ref. 44) originate from our laboratory. The following scheme is self-explanatory, since it consists only of four conventional processes for which there exist ample precedent (Ref. 1, 2, 6): (a) one-step SAM biomethylation of a double bond (e. g. 52→61); (b) proton elimination from a carbon adjacent to the resulting carbonium ion (e. g. 61→62); (c) 1,2-migration of a tertiary hydrogen to a tertiary carbonium center followed by proton loss (e. g. 61→64); (d) double bond migration (e. g. 62→64), presumably via a biochemical hydrogenation - dehydrogenation sequence (Ref. 39).



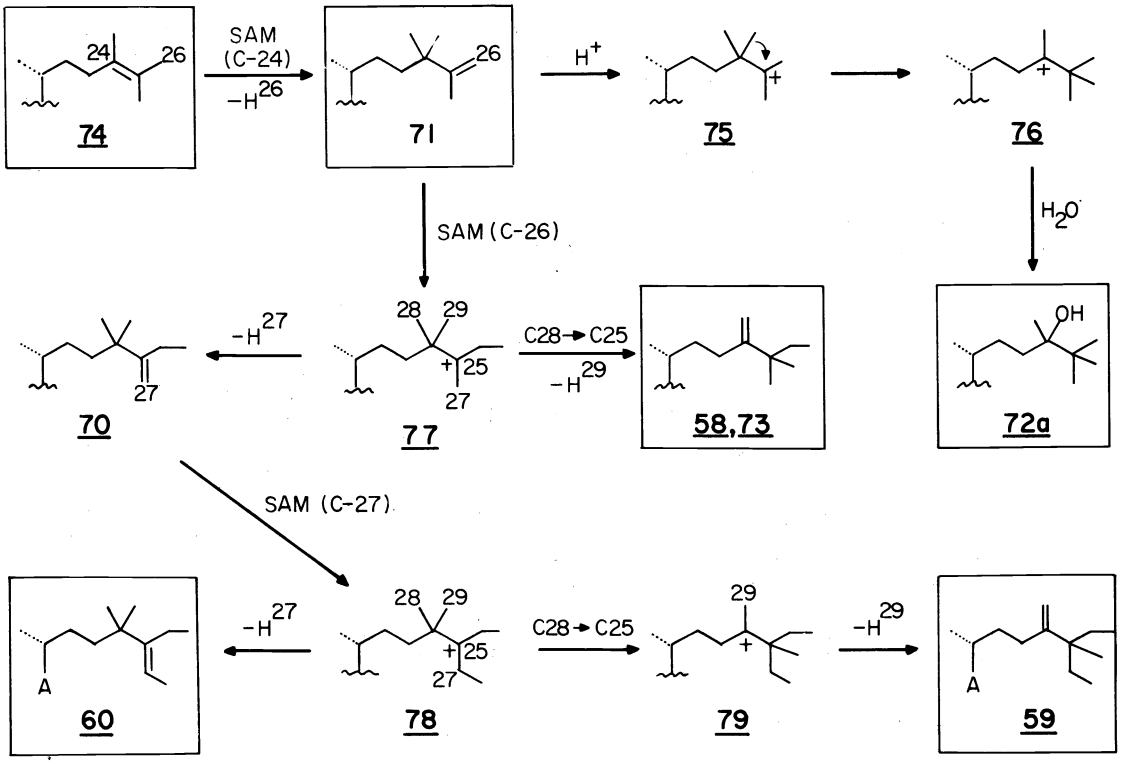
The most noteworthy feature is the series of quaternary alkylations, hitherto unknown in sterol side chain biogenesis (Ref. 1, 2, 6), which suggest that several highly alkylated desmosterol (5) analogs (e. g. 65 and 68) should exist in nature. The circumstantial evidence for 24,26,27-trimethyldesmosterol (68) is particularly strong, since both 25-methylxestosterol (59) and xestospongesterol (60) would be formed from it by SAM alkylation at either C-24 or C-25. Mutasterol (58) could arise from SAM alkylation at C-25 of either 65 or 66 (in the latter via the intermediate 67), both of which are so far unknown. If the lower homolog 70 of xestospongesterol (60) is eventually found in nature, then it would afford strong indirect evidence in favor of a 24,26-dimethyldesmosterol (65) intermediate, just as the co-occurrence of 59 and 60 strongly point toward the existence of 24,26,27-trimethyldesmosterol (68) in nature. Clearly, an active search for such "missing links" (e. g. 65 - 70) is justified and, judging from our recent experience, the prognosis for encountering at least one or two of them is good. Recognition of 65 and 66 would be especially facile, since these two sterols have already been synthesized (Ref. 51).

While no terrestrial sterols with quaternary carbons in the side chain are known, a few such examples have been encountered among terrestrial tetracyclic triterpenes (4,4,14-trimethyl steroids). The pertinent examples come from two genera of the Rutaceae plant family, *Neolitsea* (containing cycloneolitsin (71)(Ref. 52) and several close analogs (Ref. 53) including the two 24-oxygenated triterpenes 72a and 72b) and *Bosistoa* (containing (Ref. 54) bosistoin (73)). The latter has the identical side chain substitution pattern as our marine sterol mutasterol (58).



71 72a R = OH b R = OMe 73

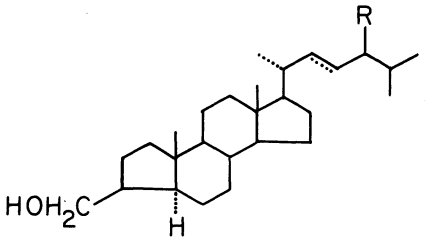
In addition to the type of sterol side chain biosynthesis envisaged above, both the Chinese (Ref. 53) and Australian (Ref. 54) groups considered also methyl migration alternatives (e. g. 77→73; 78→79). If such processes do indeed occur - at present they are purely speculative - then they must also be considered viable hypotheses for the biosynthesis of mutasterol (58) and 25-methylxestosterol (59), as indicated in the following scheme (boxed structures refer to side chains encountered in terrestrial plants or marine invertebrates).



SIGNIFICANCE OF 3β-HYDROXYMETHYL-A-NOR STERANES

Whereas the various sterol structures outlined in the preceding section all fit into a consistent (albeit largely hypothetical) sterol side chain biosynthetic scheme, in most instances (notably sponge sterols) it is not known whether such sterols are of dietary origin, are products of modification subsequent to ingestion, or arise through some symbiotic relationship. *De novo* biosynthesis from acetate or mevalonate seems unlikely (Ref. 6, 55). In this section will be considered the most convincing evidence for the occurrence in sponges

of chemical modification subsequent to dietary intake: a unique class of nuclearly modified sterols, the 3β-hydroxymethyl-A-nor steranes.



80a R = H
b R = Me
c R = Et

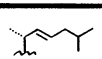
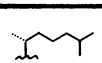
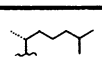
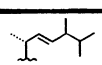
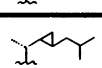
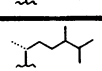
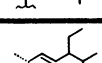
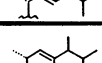
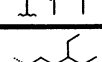
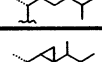
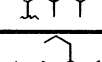
Minale and Sodano (Ref. 56) were the first to record the isolation and structure elucidation of a group of sterols (80) from the Mediterranean sponge *Axinella verrucosa*, which contained conventional side chains (with and without a Δ^{22}-double bond) but the hitherto unknown 3β-hydroxymethyl-A-nor nucleus. In a series of subsequent studies (Ref. 57), the Italian workers were able to demonstrate through radioactive tracer incorporation experiments, that cholesterol, via Δ^{4}-cholesten-3-one, is efficiently converted by the sponge into 3β-hydroxymethyl-A-norcholestane (80a). Noteworthy is the absence of sterols with conventional nuclei in that sponge. No further publications on the isolation of such A-nor sterols appeared in the literature until 1979, when Kanazawa *et al.* (Ref. 58) reported that the Japanese sponge *Hymeniacidon perlevis* contained the same six A-nor sterols (80), isolated earlier by Minale and Sodano (Ref. 56), as well as ten trace sterols of unknown structure. In the meanwhile, we have encountered (Ref. 59-61) fourteen A-nor sterols (see Table 1) from sponges living in as diverse localities as the Red Sea, the Pacific and the Caribbean. While it is very likely that additional members of this uniquely marine class of sterols will be discovered in the future, the following four generalizations can already be made based on the material collected in Table 1.

(1) The five sponges (Table 1) belonging to the family Axinellidae possess, either exclusively or overwhelmingly, A-nor sterols, whereas the two members of the Hymeniacidonidae family studied so far also contained substantial amounts of conventional Δ^{5}- or saturated 5α-sterols. These observations may be of considerable taxonomic utility once more representatives of these sponge families have been examined.

(2) Almost every major class of conventional and unconventional side chain has already been uncovered among these sponge A-nor sterols. Noteworthy are the 24-norcholestane side chain 81, indicative of planktonic organisms, and its presumed biosynthetic precursor 82; the 22, 23-methylene cholesterol side chain (84), which, as mentioned in an earlier section of this paper, can be considered *prima facie* evidence for direct bioalkylation of an isolated Δ^{22}-double bond (which was also found in the form of 80a); and finally the dinosterol side chain (85), which may be the precursor of the gorgosterol side chain (86), typical of zooxanthellae-containing coelenterates. Even in the absence of the elegant tracer experiments of the Italian group (Ref. 57), the existence of such a variety of side chains together with the unique 3β-hydroxymethyl-A-nor nucleus in sponges growing in totally different geographical locations (see Table 1) demonstrates that the sponge possesses an efficient enzyme system which can transform sterols originating from many potential members of the food web to a common sterol type. This raises the obvious question as to what function this interesting molecular change serves. The total absence of conventional sterol nuclei in some of the Axinellidae suggests that the A-nor sterols replace sterols with the cholesterol nucleus as membrane constituents. If true, then this might reflect itself in some peculiarity of the head groups of their plasma phospholipids rather than in the fatty acid component, since the predominant side chains of these A-nor sterols are of the conventional type (cf. 80). Therefore, a detailed analysis of the phospholipids of some members of the Axinellidae is clearly warranted.

(3) Nuclear saturation is very common, but not obligatory. The presence in trace quantities of a Δ^{7}-unsaturated A-nor sterol (82a) points to some contribution by asteroids in that sponge's food chain, since Δ^{7}-nuclear unsaturation is typical of star fish. Particularly noteworthy is the occurrence in substantial amounts in two sponges of a new sterol, which we have shown (Ref. 61) to have the structure of 3β-hydroxymethyl-A-norcholest-15-ene (83). The presence of a 15-16 double bond in steroids with the usual C/D trans hydrindane stereochemistry creates considerable strain and it is not surprising that Δ^{15}-unsaturated steroids are the least known among all nuclear-unsaturated steroids. While a few Δ^{15}-17-ketones and related compounds have been synthesized (Ref. 63), no Δ^{15}-sterol has ever been prepared. Indeed, only one naturally occurring representative, the plant sterol vernosterol (88), has

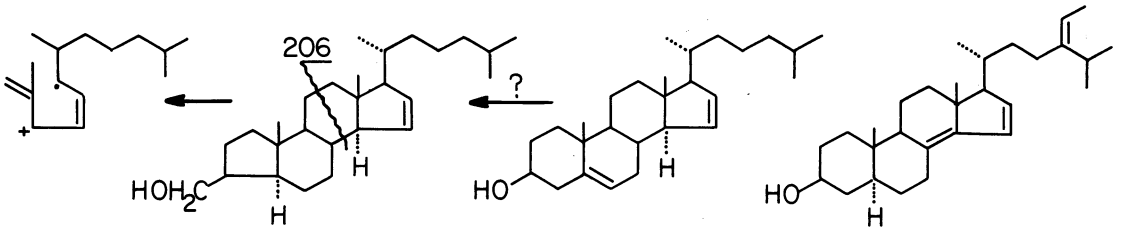
TABLE 1. 3β-Hydroxy-A-nor steranes from sponges (Class: *Demospongiae*)

Structure Side Chain Nucleus	M^+	Subclass: *Tetractinomorpha* Order: *Axinellida* Family: *Axinellidae*					*Ceractinomorpha* *Halichondria* *Hymeniacidonidae*		
		Axinella verrucosa (56) Mediterranean	*Axinella* Species (59) Pacific	*Homaxinella trachys* (61) Pacific	*Phakellia aruensis* (8) Pacific	*Teichaxinella morchella* (62) Caribbean	*Stylotella agminata* (59) Pacific	*Hymeniacidon perlevis* (58) Pacific	Unidentified Sponge (62) Red Sea
81  Δ^0	372	-	-	-	-	-	-	- *	1
82a  Δ^7	384	-	-	-	-	-	-	-	T
82b  Δ^0	386	-	-	-	-	1	-	-	3
80a  Δ^0	386	4	3	-	11	4	1 **	3 *	5
83  $\Delta^{15(16)}$	386	-	-	8	-	-	-	-	6
80a  Δ^0	388	49	28	33 *	12	26	26	26 *	22
80b  Δ^0	400	13	- **	13	23	26	- **	14 *	30
84  Δ^0	400	-	-	-	-	-	-	-	3
80b  Δ^0	402	8	29	25	22	12	31	29 *	9
80c  Δ^0	414	8	11	-	11	13	10	8	5
85  Δ^0	414	-	-	13	-	1	-	-	-
80c  Δ^0	416	8	17	6	21	18	10	16 *	16
86  Δ^0	428	-	-	-	-	-	2 **	-	1
87  Δ^0	430	-	-	-	-	-	-	-	T

* Normal Δ^5-sterol nucleus

** Normal 5α-H sterol nucleus

apparently such a structural feature, which in vernosterol is stabilized through conjugation with an additional $\Delta^{8(14)}$-double bond.



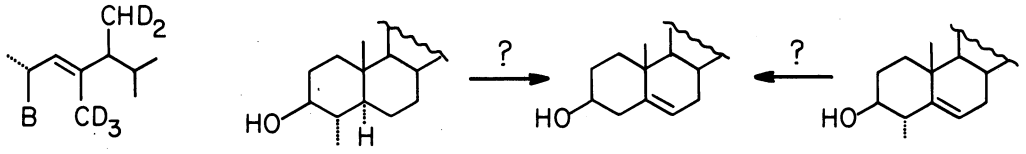
89 83 90 88

The overall structure of 83 was confirmed (Ref. 61) by homogeneous catalytic hydrogenation to the known (Ref. 56) 3β-hydroxymethyl-A-nor-5α-cholestane (80a), while the location of the double bond rested on extensive ^{1}H NMR, ^{13}C NMR and mass spectral studies. Thus, the two most intense mass spectral fragmentation peaks corresponded to loss of the allylically labilized side chain and to an ion of mass 206 ($C_{15}H_{26}$), which has never been observed in other marine sterols. Schematically (see wavy line in 83), it can be visualized as resulting from fission of the 11-12 and 8-14 bonds. The ion's high abundance is, undoubtedly, due to the fact that it represents an ionized, conjugated triene (e. g. 89). Among various NMR parameters (e. g. olefinic proton signals) pointing to a Δ^{15}-double bond, a particularly characteristic one is the downfield shift of the C-18 angular methyl signal (δ 0.736 in 83 vs. 0.647 in 80a), whereas the C-19 methyl signal is only slightly affected (δ 0.757 in 83 vs. 0.737 in 80a).

(4) The ability of the Axinellidae sponges to modify ingested sterols in a unique manner, coupled with our increasing knowledge of the origin of certain characteristic sterol side chains, offers an interesting vehicle for studying the food chain of such sponges. In addition, the structure elucidation of such A-nor sterols can point to the existence of as yet undiscovered sterols with conventional nuclei, which must exist somewhere in the marine environment. A particularly interesting example is the as yet unknown 15-dehydrocholesterol (90), which almost certainly must be the dietary precursor of the A-nor sterol 83. The search for organisms containing 15-dehydrocholesterol (90) should focus on geographic habitats close to those of the sponges that contain the A-nor sterol 83.

STEROL SYNTHESIS VIA SYMBIONTS

In the preceding sections, direct or indirect evidence has been presented for the operation of at least two processes - i. e. dietary intake with and without further chemical modification - to account for the diverse sterol structures encountered in many marine invertebrates. A major effort is currently under way in our laboratory (Ref. 24, 65, 66) as well as in others (Ref. 67) to investigate the sterol content of various potential food chain contributors, notably those that can be cultured, with primary focus on those organisms (Ref. 65, 67) which produce some of the more esoteric sterols (e. g. gorgosterol (9)). The purpose of our studies is to select appropriate substrates for biosynthetic experiments with isotopic tracers - experiments that should be much easier to conduct with cultured unicellular organisms. An example is the successful incorporation by the Liverpool group (Ref. 18) of d_3-methionine into *Crypthecodinium cohnii* - the resulting deuterium distribution in the labeled dinosterol (91) being consistent with the standard sterol side chain alkylation biosynthesis (5→6→7→8).



91 B A C

The third major route to invertebrate sterols - via symbionts - lends itself to the same type of methodological approach. We are particularly interested in this third alternative, because most marine organisms (coelenterates), rich in gorgosterol (9) and related cyclopropane-containing sterols, are of a type that contain endosymbiotic algae, known as zooxanthellae and classified as dinoflagellates. Ciereszko and collaborators (Ref. 68) have mechanically separated the zooxanthellae from several Caribbean gorgonians, rich in gorgosterol (9), and succeeded in isolating gorgosterol and related sterols with the same side chain from the zooxanthellae. Since gorgonians, lacking endosymbiotic zooxanthellae, do not contain gorgosterol, it has been assumed that the zooxanthellae are the real source of gorgosterol and related sterols isolated from gorgonians. If true, then separation of such zooxanthellae, followed by culturing in the laboratory, should provide the ideal experimental model for detailed biosynthetic studies with isotopically labelled precursors. Experiments conducted along those lines in our laboratory (Ref. 69) have shown that the situation is considerably more complex.

We selected three taxonomically dinstinct gorgonians, *Briareum asbestinum*, *Muriceopsis flavida*, and *Gorgonia mariae*, and confirmed that they contained significant amounts of gorgosterol (9) and 23-demethylgorgosterol (10). We succeeded in culturing single colony-derived zooxanthellae from these three gorgonians and found that none of them contained either gorgosterol (9) or 23-demethylgorgosterol (10). Even more surprising was the observation (Ref. 69) that the whole animals (host plus symbiont) contained predominantly Δ^5-3β-hydroxy sterols (A), whereas the cultured zooxanthellae derived from them contained almost exclusively 4α-methyl-3β-hydroxy 5α-sterols (B). There are several possible explanations for this unexpected anomaly:

(1) The earlier reported (Ref. 68) isolation of gorgosterol (9) from zooxanthellae by the Oklahoma group was due to contamination by animal debris. Alternatively, the lipophilic cell surface of the mechanically separated zooxanthellae retained adsorbed sterols from the host.

(2) The metabolism of zooxanthellae cultured *in vitro* is qualitatively different from that of the wild growing algae. While it is known (Ref. 70, 71) that the primary productivity of many zooxanthellae is increased in the presence of host animal homogenates, no evidence exists that such changes would affect qualitatively primary sterol synthesis.

(3) Gorgosterol (9) and related sterols are not synthesized by the zooxanthellae, but are products of animal metabolism. One aspect of such host animal metabolism could involve the last steps in side chain alkylation, since the cultured zooxanthellae contained substantial amounts (nearly 50% of total sterols) of dinosterol (4). However, if dinosterol (4) from the zooxanthellae is a precursor of gorgosterol (9), then the host must also transform the saturated 4α-methyl-3β-hydroxy nucleus B into a Δ^5-3β-hydroxy sterol A. While C-4 demethylation is well known in sterol metabolism (Ref. 1), this would be the first instance in sterol biosynthesis, where the introduction of the Δ^5-double bond occurs in a saturated 4α-methyl precursor (B). It is conceivable that this unprecedented nuclear change (B→A) need not be obligatory, since we have also encountered (Ref. 69) in the cultured zooxanthellae small amounts of 4α-methyl-Δ^5-3β-hydroxy sterols (C) possessing the dinosterol (4) side chain. It is thus conceivable that C rather than B is the nuclear precursor, if the host does indeed produce A from a precursor synthesized by the zooxanthellae.

(4) Perhaps the most intriguing alternative, which is not excluded by our experimental results (Ref. 69), is that zooxanthellae synthesize different sterols, depending whether they live alone or symbiotically with a host. The functional reason for such qualitative differences in sterol biosynthesis may be associated with membrane permeability (Ref. 13). In the host-symbiont combination, the zooxanthellae generate simple carbon sources (e.g. glucose, glycerol, alanine, etc.) which must pass through the cell membrane of the symbiont to the host. Such a "leaking" membrane may not be required by the cultured zooxanthellae, who, therefore, synthesize different sterols in the absence of the appropriate stimulus from the host.

Obviously, much additional experimentation is required before the role of the symbiont in marine sterol biosynthesis is clarified, but it is clear that this is a fruitful field of study, which we plan to explore.

CONCLUSION

The prognosis for the future of marine sterol chemistry is excellent. It is quite likely that additional novel structures will be encountered, which will serve to delineate even further the range (in terms of carbon content) and variety of substitution patterns possible in the side chain. Unique nuclear variations seem less abundant, but when they are found, they are likely to be of considerable taxonomic significance. Of the two unique types - 19-nor sterols (Ref. 72, 73) and 3β-hydroxymethyl-A-nor sterols (*vide supra*) - much more is known about the distribution and biosynthesis of the latter. More detailed knowledge of the biosynthesis of marine 19-nor sterols (Ref. 74) is not only desirable for its intrinsic interest, but also because of the potential economic importance of 19-nor steroids (oral con-

traceptives).

The ongoing isolation of different structural types, notably in terms of side chain variations, offers important strategic information about how to plan eventual biosynthetic experiments, i. e. what labeled precursor to feed and what product to look for. The main problem is how to feed such precursors and to whom? Of particular relevance is the progress made in our and other laboratories on the sterol composition of unicellular organisms capable of laboratory culture; several of them appear to be very promising substrates for biosynthetic studies.

The question of the biological function of the more unusual marine sterols - notably those which represent the predominant or exclusive sterol constituent of certain marine organisms - remains the most important one. As indicated earlier (Ref. 13), membrane function may be one of the keys to that puzzle and complementary studies on the phospholipid components of those marine organisms, which contain exclusively unusual sterols, are urgently needed.

Finally, continuing studies of marine sterols are likely to shed considerable light on certain geochemical and ecological questions; some of the unusual sterols could serve as geochemical tracers in marine sediments; they could help in a variety of problems dealing with food chain relationships; and finally, they could simplify the study of the numerous symbiotic relationships which are so common among lower marine invertebrates.

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