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RECENT PROGRESS IN THE MARINE STEROL FIELD

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Abstract - This paper covers recent work - much of it from the authors' laboratory - dealing with novel 3β-hydroxy marine sterols possessing unusual side chains, which have hitherto not been encountered in terrestrial sources. Aside from a description of the proof of structure and stereochemistry of these sterols, attention is also drawn to plausible models of biosynthesis and to the possible biological role in membrane function.

INTRODUCTION

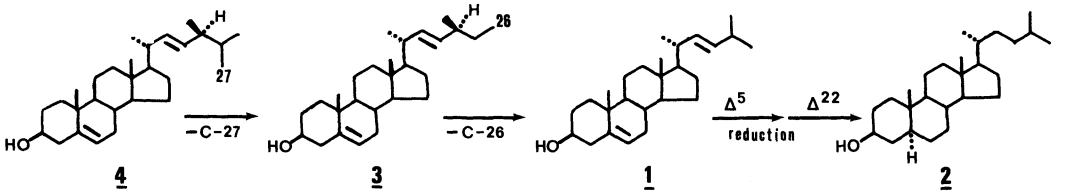
The veritable flood of new steroids encountered in marine sources continues unabated and fully substantiates the prediction made by several investigators (including ourselves using a novel computer program (Ref. 1)) that the marine environment is likely to be an unexcelled source for unusual steroid types, particularly sterols with modified side chains. Two questions remain unanswered - indeed they represent the main rationale for continued work in this field. (1) What are the principal biosynthetic routes leading to these unusual sterols and which organisms in the complicated food web are responsible for the generation of those structural features which have not been encountered in terrestrial species. (2) What is the biological role of these unusual sterols - is it a functional one in membranes, is it a metabolic one or do they serve as starting materials for the synthesis of hormonal or other biologically significant factors? In view of the appearance of several recent reviews (Ref. 2) which have attempted to cover the entire marine steroid chemistry field, the present article will deal primarily with selected topics from the field of marine sterols which are of particular current interest to our research group and which are largely unpublished. For the sake of the reader's convenience, we shall use the same order of presentation as has been employed by Minale and Sodano in their review (Ref. 3) of "non-conventional sterols of marine origin".

C_{26} AND 27-nor-C_{27} STEROLS

The C_{26} sterols are an extremely widespread class of uniquely marine steroids, all of which possess the unprecedented 24-norcholestane skeleton. Four C_{26} sterols have been reported: (i) $\Delta^{5,22E}$ (1), (ii) 5α-Δ^{22E}, (iii) 5α-$\Delta^{7,22E}$ (asterosterol) and (iv) Δ^{5} (halosterol) beginning with the 1970 report by Idler (Ref. 4). We have recently isolated a fifth member of this class, the completely reduced 5α-24-norcholestan-3β-ol (2) (Ref. 5), along with an entire series of C_{27} to C_{29} 5α-stanols from the Hawaiian sponge Terpios zeketi. An early report (Ref. 6) of a possible sixth member of this class, the $\Delta^{5,22Z}$ isomer, in a brachiopod certainly merits further study.

Several perplexing questions concerning this widespread class of sterols remain unanswered. First, despite an extensive and ongoing search notably by Barbier, et al. (Ref. 7), the exact species or class of marine organisms producing the primary C_{26} sterol, probably (1), has not been identified. Sterols of this class have been found in nearly every marine invertebrate phylum (Ref. 3), and several recent reports of C_{26} sterols in sea water (Ref. 8) and marine sediments (Ref. 9) have confirmed the impression that these sterols are truly ubiquitous in the marine environment. Although C_{26} sterols generally are present at very minor percentages in marine sterol mixtures, recently a ctenophore was discovered to contain 30% of its sterol mixture as a C_{26} sterol (Ref. 10). Second, the existence of the 24-nor-C_{26} sterols raises many intriguing and unanswered biosynthetic questions. Obviously the elucidation of the mechanism of the biosynthesis of the C_{26} sterols must await the discovery of the organisms which produce them, but this has not stopped speculation concerning the possible modes of their biosynthesis. Thus the discovery of the three marine 27-nor-24α-methyl cholesterols (typified by occelasterol (3)) by Kobayashi and Mitsuhashi (Ref. 11)

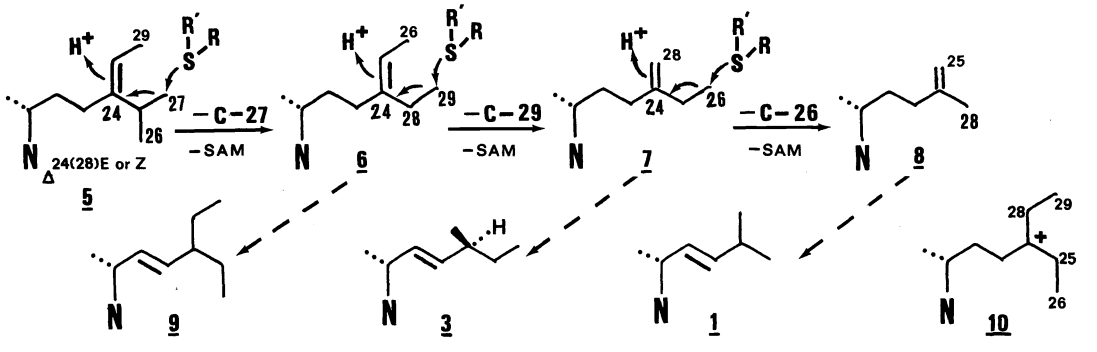
led to their suggestion (shared by others (Ref. 3)) that these novel C_{27} sterols are generated by biological C-27 demethylation of the C_{28} sterol, 24-epibrassicasterol (4) and are themselves intermediates in the biosynthesis of the C_{26} sterols (e.g. 1) through further biodealkylation of C-26, although a specific mode of dealkylation without destruction of configuration at C-24 was not suggested. Another C_{28} sterol of wide occurrence in the seas,



24-methylenecholesterol, has also been suggested to be the precursor of the C_{26} and 27-nor-C_{27} sterols through a reversal of the phytochemical alkylation process (Ref. 12). The dealkylation process involves a carbonium ion intermediate formed by protonation of the 24(28) double bond (Ref. 13) which could alternatively be represented (Ref. 12) by removal of a homoallylically activated methyl group via nucleophylic attack by S-adenosylhomocysteine with expulsion of S-adenosylmethionine (SAM) (Ref. 13). Less likely alternatives involving dealkylation coupled with migration of the C-26 methyl group in a C_{27} sterol (Ref. 13) or cyclization of a non-squalenoid precursor (Ref. 14) have also been suggested.

We shall consider one other possible biosynthetic mechanism for the formation of the C_{26} and 27-nor-C_{27} sterols, and briefly explore the implications of that mechanism. If we consider the abundant and wide-spread 24-ethylidene C_{29} sterols (5) (fucosterol and its geometrical isomer isofucosterol) to be the starting point in the biosynthesis of C_{26} and 27-nor-C_{27} sterols by the reverse alkylation process involving demethylation of a homoallylically activated methyl group discussed above (Ref. 12), then the demethylation sequence (5→6→7→8) giving rise to the 27-nor and C_{26} sterols does not require the postulation of intermediate double bond migrations required for the same sequence commencing with the C_{28} sterols (Ref. 12). Alternatively, if an intermediate carbonium ion (10) is invoked then the terminal carbon atoms on the side chain of the 27-nor-C_{27} sterols could arise either from C-28, C-25 and C-26 or from C-25, C-28 and C-29 of the original C_{29} sterol (5). The final step in each case (6→9, 7→3, and 8→1) would be the introduction of a Δ^{22} double bond by a reduction-dehydrogenation sequence (Ref. 15).

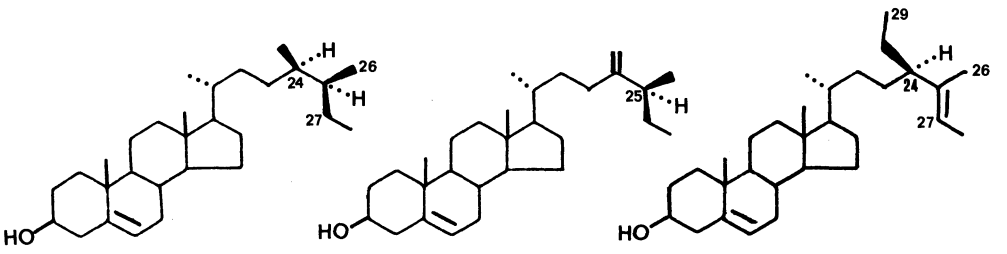
If the C_{29} route to the C_{26} sterols does exist, then novel C_{28} sterols of type 6 or 9 would be expected to occur in nature. Compounds such as 9 would be expected to be extraordinarily difficult to separate from conventional C_{28} sterols which, coupled with their expected minor levels may explain why they have not been identified previously if they do in fact exist.



STEROL SIDE CHAINS MODIFIED BY ALKYLATION OF C-26 AND/OR C-27

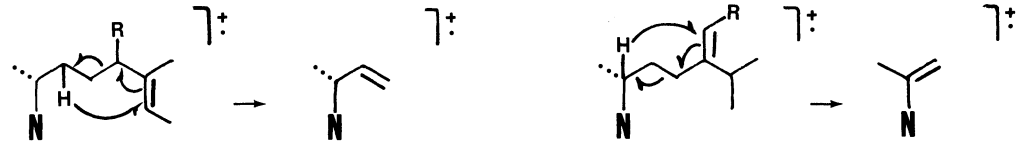
Until recently, the only examples of chain extension by biological alkylation at C-27 were aplysterol (11) and its 24-28 dehydro analog 12, which had been isolated from various Verongia species by Minale and collaborators (Ref. 3). Subsequently, we have encountered these biogenetically intriguing sterols in diverse sponges such as Psammaplysilla purpurea from Hawaii (Ref. 16) and Jaspis stellifera (Ref. 17) from Australia. We now discuss additional examples of such chain extension found in a variety of sponges, which raise important questions with respect to biosynthesis and biological function.

The first new examples of this class are stelliferasterol (13) and isostelliferasterol (21) which we isolated (Ref. 17) together with dehydroaplysterol (12) from Jaspis stellifera. As pointed out earlier (Ref. 17,18), the first indication of an unusual structural feature



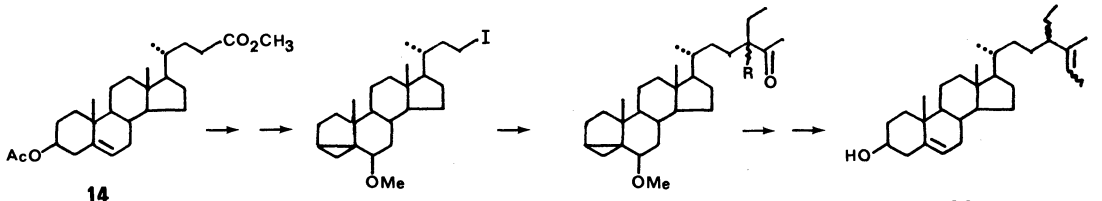
11 (24R,25S) 12 (25S) 13 (24R,25E)

was the intense mass spectral peak at m/e 328 which we attribute to a McLafferty rearrangement from a Δ^{25} double bond, similar to the well known m/e 314 peak arising from McLafferty rearrangement of Δ^{24} steroidal olefins (Ref. 19). The 360 MHz NMR spectrum (Ref. 17) displayed a beautiful resolution of all methyl signals: 0.659 ppm (C-18); 1.003 ppm (C-19); 0.899 ppm (C-21 doublet); 0.748 ppm (C-29 triplet) and two vinyl methyl signals at 1.422 ppm (singlet) and 1.569 ppm (doublet). Given the presence of a Δ^{25}-trisubstituted double bond (m/e 328 mass spectral peak and 5.15 ppm single proton NMR quartet), these two vinyl methyl groups had to be attached to the Δ^{25} double bond with C-26 being the 1.422 ppm singlet and C-30 the 1.569 doublet (which was shown to be coupled to the 5.15 ppm quartet). Structure 13 for stelliferasterol therefore follows automatically - this being the first sterol in which biological methylation at both C-28 and C-27 could be demonstrated.



m/e 328 m/e 314

This structural assignment was confirmed by synthesis (Ref. 17) starting with methyl 3β-acetoxychol-5-enate (14) which was converted by conventional methods into 3α,5-cyclo-6β-methoxy-23-iodonorcholane (15) and then condensed with ethyl 2-ethylacetoacetate to the keto ester 16. Removal of the ethoxycarbonyl group (17), Wittig condensation with triphenylethylphosphonium ylide and acid destruction of the i-ether protecting group afforded a mixture of all four possible isomers of structure 13, which had identical gas chromatographic retention times and mass spectra, but could be distinguished by their 360 MHz proton and ^{13}C NMR spectra. Separation of these isomers was only partially successful in that one isomer (C-24 epimer of natural stelliferasterol) could be isolated in a pure state by reverse phase HPLC. This was sufficient to settle the geometry around the Δ^{25} double bond. Natural stelliferasterol (13) and its pure, synthetic C-24 epimer possess the E stereochemistry since their C-27 olefinic proton signals occurred at 5.15 ppm as compared to 5.30 ppm for the mixture of the two 25Z isomers. Furthermore, the C-26 methyl signal of stelliferasterol (13) and its 24-epimer was found at 1.42 ppm, in contrast to 1.50 ppm for the Z isomers.

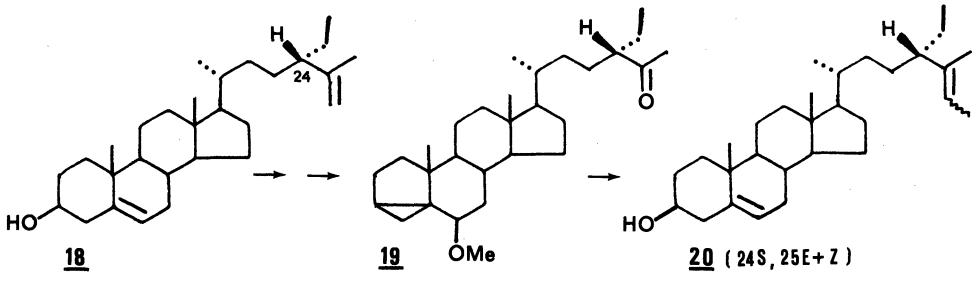


14 15 16 R = CO_2Et 13 + 20

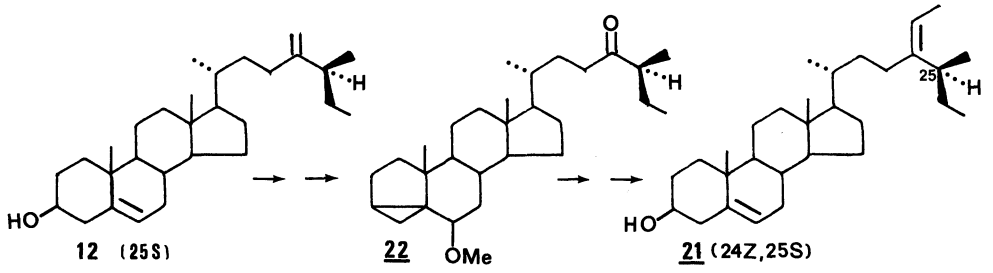
17 R = H

In evaluating possible biosynthetic pathways (vide infra) to stelliferasterol, it was necessary to establish its C-24 stereochemistry and this was accomplished (Ref. 20) by partial synthesis from clerosterol (18) which W. Kokke (Ref. 21) in our Laboratory had isolated from a Mexican Codium species and whose C-24 stereochemistry (24S) had been established earlier by Rubinstein and Goad (Ref. 22). Conversion to the i-methyl ether and ozonolysis provided the key intermediate 19, which upon Wittig condensation and regeneration of the Δ^5-3β-hydroxy moiety led to a readily separable (reverse phase HPLC) mixture of the 25E and 25Z isomers with the 24S configuration (20). The 25E isomer of 20 proved to be epimeric at C-24 from stelliferasterol from which it follows that the natural marine sterol has the 24R,

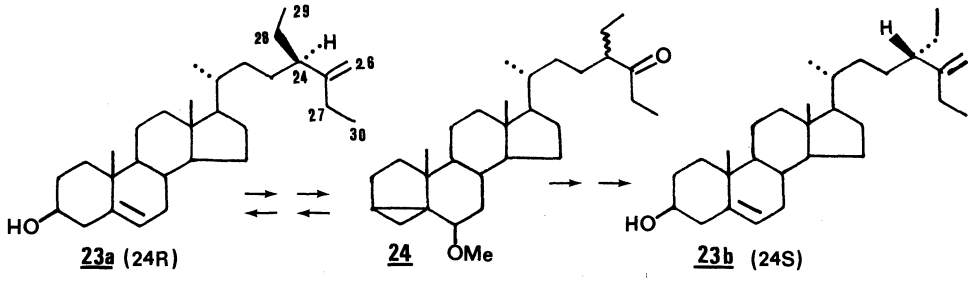
25E stereochemistry (13).



Stelliferasterol (13) was accompanied in the sponge by a structural isomer, which we have named isostelliferasterol (Ref. 17) and which was shown to have the stereostructure 21. The presence of a Δ^{24} and absence of a Δ^{25} double bond was demonstrated by the mass spectrum, which exhibited only an m/e 314, but no m/e 328 peak. Its 360 MHz NMR spectrum (Ref. 17) was again sufficiently well resolved in the methyl region to allow assignment of structure 21, including the Z stereochemistry of the $\Delta^{24(28)}$ double bond. This was based on the chemical shift of the C-25 proton (sextet centered at 2.56 ppm), which was compared to those of isofucosterol (5, Z; C-25 H at 2.8 ppm (Ref. 23)) and fucosterol (5, E; C-25 H at 2.2 ppm (Ref. 23)). The stereochemistry of isostelliferasterol (21) at C-25 (S) could be proved by partial synthesis from dehydroaplysterol (12) where this stereochemical feature had been established (Ref. 3) by X-ray analysis. The reaction sequence was completely analogous to that employed in the synthesis of 24-epistelliferasterol (20) from clerosterol (18), viz. protection of the Δ^5-3β-hydroxy grouping of dehydroaplysterol (12) via its i-methyl ether, ozonolysis to the 24-ketone 22, followed by Wittig condensation and regeneration of the 3β-hydroxy-Δ^5-system.

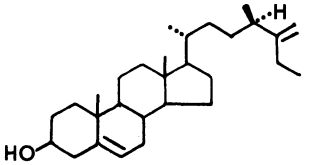


A third sterol in which biological methylation has occurred at C-28 and C-27 is strongylosterol (23), which has recently been encountered by Tursch and his colleagues (Ref. 24) as the sole sterol constituent of the sponge Strongylophora durissima from New Guinea. The Belgian group (Ref. 24) established structure 23 by chemical and spectral means, except for the stereochemistry at C-24. This was settled in our laboratory (Ref. 20) as 24R (23a) by ozonolysis of the i-methyl ether of strongylosterol (23a) to the 26-nor ketone, which was equilibrated with base to the C-24 epimeric mixture 24. Regeneration of the side chain by Wittig condensation and of the Δ^5-3β-hydroxy moiety by acid treatment provided an inseparable mixture of strongylosterol (23a) and its 24-epimer 23b. The absolute configuration at C-24 could then be established by comparison of the relevant NMR chemical shifts in the spectra of the two isomers with those of the synthetic (Ref. 17) stelliferasterol isomers (13,20), thus demonstrating that strongylosterol (23a) is simply the $\Delta^{25(26)}$-double bond isomer of stelliferasterol (13). As expected, the mass spectra of these two sterols (13 and 23a) are qualitatively identical and exhibit only minor quantitative differences.

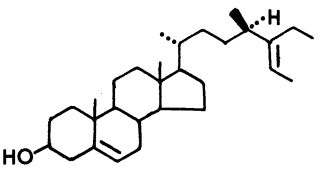


Our recent examination (Ref. 25) of the sterol composition of the Caribbean sponge Verongula cauliformis provided two additional members of this new class of sterols with extended side chains. The first one is the C-24 lower homolog 25 of strongylosterol (23a) and as expected has virtually the same mass spectrum below m/e 350. Its NMR spectrum is fully consistent

with structure 25 and its 24R stereochemistry was established (Ref. 25), in the same manner as described above for strongylosterol (23a). Therefore, the new sterol (25) can be given the trivial name 25(26)-dehydroaplysterol.



25 (24R)



26 (24R)

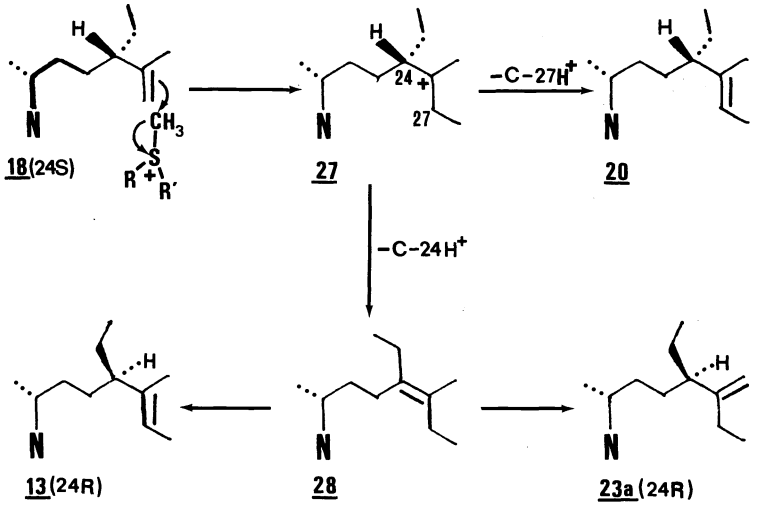
Of considerably greater interest is our isolation (Ref. 25) from the same sponge of a new C_{30} sterol (mol. wght. 426) which we have named verongulasterol. The presence of a Δ^{25} double bond is again demonstrated by the fact that its mass spectrum below m/e 350 closely resembles that of 13, 23 and 25. A detailed 360 MHz NMR spectrum with extensive decoupling has shown that verongulasterol possesses structure 26 and thus represents the first naturally occurring sterol in which biological methylation at C-26 and C-27 has been demonstrated. The structure was verified (Ref. 25) by partial synthesis which was identical in principle to that (Ref. 17) (14→15→16→17→13) used in the stelliferasterol series except for the substitution of ethyl 2-methylpropioacetate for 2-ethylacetoacetate in the alkylation step (cf. 15→16). The stereochemistry at C-24(R) and C-25 (E) was settled by precisely the same means via 360 MHz NMR spectral examination of appropriate synthetic stereoisomers and is in all regards identical with that of stelliferasterol (13). Particularly gratifying is the observation that our "biogenetic computer program" (Ref. 1) has predicted the occurrence of all five novel sterols (13,21,23,25,26) and thus encourages us in our belief that many other biogenetically intriguing sterols predicted by our program (Ref. 1) will still be found in nature.

Both the biogenesis and biological function of these C-26 and C-27 methylated sterols is intriguing. In each instance, this group of sterols represented the chief component of the particular sponge under investigation and in one case - Strongylophora durissima (Ref. 24) - it appeared to be the only sterol. This would suggest a functional role, most likely in the membrane (Ref. 26), in which cholesterol is replaced by these sterols with "extended" side chains. Given the extreme structural specificity of the cholesterol geometry for optimum membrane function (Ref. 27), such a unique replacement carries with it several significant implications. The most obvious one is that the specific structural modifications exhibited by these unusual marine sterols reflect a careful structural adjustment to establish a specific associative compatibility with other membrane constituents. We believe that much can be learned about biological membrane function through a study of such a mutual specificity which has surely evolved over many hundreds of millions of years. Although the function of sterols in biological membranes is currently a very active field of research, most studies have dealt with the role of only a single sterol, cholesterol (Ref. 26). Indeed, recent accounts have suggested (Ref. 27) that 19-nor sterols and major amounts of 4-monomethyl sterols should not exist in any organism. Yet, both 19-nor sterols (Ref. 3) and 4-monomethyl sterols (Ref. 21) have been found in substantical amounts in marine organisms. Furthermore, recent studies (Ref. 28) have shown that synthetic sterols with elongated but less branched side chains than the ones described above, do not function properly in membrane systems in which cholesterol functions well. We feel that careful studies which probe the apparent specificity between the various unusual marine sterols and the membrane systems in which they are found may well offer the key to the resolution of the general role of sterols in biological membranes. All these questions can be answered by appropriately designed experiments and in view of the great current interest in cell membrane structure and function, we intend to undertake such experiments shortly.

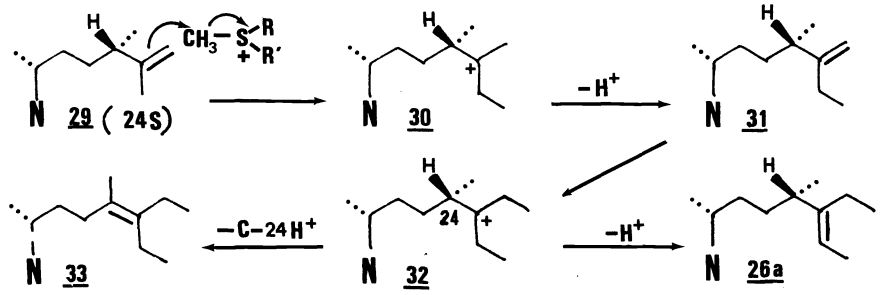
In terms of biosynthesis, no definitive conclusions can so far be derived from the few radioactive labeling experiments that have been performed (Ref. 29) in Verongia species, except to state that de novo sterol biosynthesis does not seem to occur since neither radioactive acetate or mevalonate was incorporated. The lack of incorporation of labeled methionine (Ref. 29) is surprising and would suggest a purely exogenous source for aplysterol (11) and its $\Delta^{24(28)}$-dehydro analog 12 or else an alternate biosynthetic route differing from the usual sterol side chain alkylation mechanism (Ref. 12,30). One such possibility, suggested by Minale and Sodano (Ref. 3), is methyl migration from a C_{29} sterol precursor such as fucosterol.

Taking stelliferasterol (13) (Ref. 17) as an example, standard methylation via S-adenosylmethionine (SAM) of a Δ^{25} intermediate such as clerosterol (18) would lead to the carbonium ion intermediate 27, which through proton loss from C-27 would directly generate the stelliferasterol side chain. However the resulting product would possess the "unnatural" 24S configuration (20) and if such a path were operative, it would first have to proceed through an intermediate in which the C-24 asymmetry was destroyed. This could be accomplished through

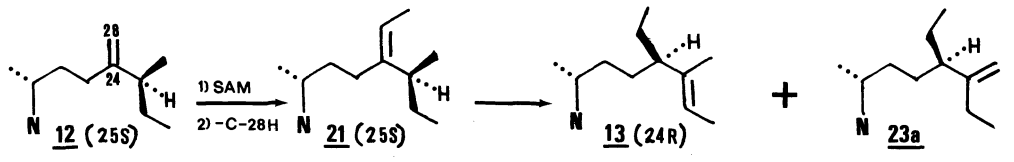
C-24 proton loss to yield the hypothetical intermediate 28, which upon double bond migration could then afford stelliferasterol (13) and strongylosterol (23a). Except for desmosterol (24-dehydrocholesterol), no $\Delta^{24(25)}$ sterol has been isolated from marine sources and the intermediacy of 28 is thus not very convincing. A 24-epiclerosterol precursor (18 with 24R configuration) would eliminate the necessity of postulating an intermediate of type 28, but such a sterol has as yet not been encountered in nature.



An analogous "conventional" route to the doubly alkylated verongulasterol (26) would start with codisterol (29) (Ref. 22) and proceed via 30 to the 24-epimer 31 of $\Delta^{25(26)}$-dehydroaplysterol. Repeated alkylation at the Δ^{25} double bond terminus to the ionic intermediate 32 followed by loss of a proton would then provide the verongulasterol sidechain, with the wrong (24S) stereochemistry (26a). The generation of the 24R configuration from the 24S precursor, codisterol, would require the existence of an intermediate such as 33 (cf. 28 in the stelliferasterol scheme) to be postulated. The entire double alkylation sequence would be particularly attractive if the 24R epimer of codisterol (29) were found in nature and shown to be incorporated into verongulasterol (26).

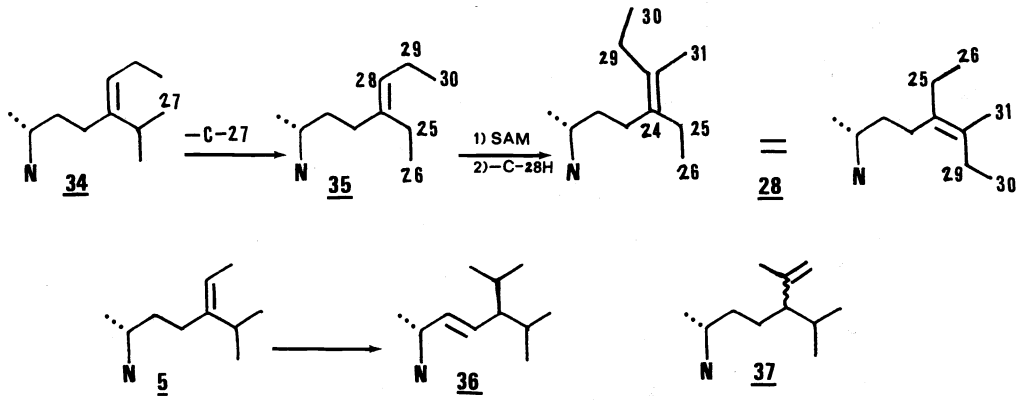


Isostelliferasterol (21) and $\Delta^{24(28)}$-dehydroaplysterol (12) possess the same stereochemistry at C-25 and the former may be derived from the latter by standard bioalkylation at C-28 followed by loss of a C-28 proton. Double bond migration or the well-established hydrogenation-dehydrogenation sequence (Ref. 15) of isostelliferasterol (21) would then represent a second biogenetic route to stelliferasterol (13) and strongylosterol (23a).



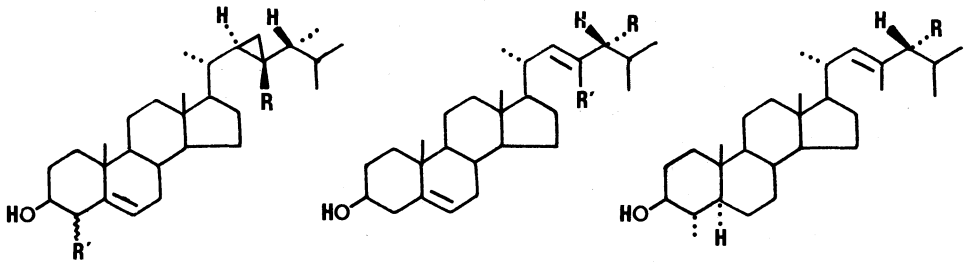
A third and fundamentally different hypothetical biogenetic scheme which we considered (Ref. 17) starts with the naturally occurring 24-propylidenecholesterol (34) (Ref. 31), which is then assumed to undergo homoallylic C-27 demethylation to 35, analogous to the presumed formation of occelasterol (3) and related 27-norergostanes. Biomethylation of 35 at C-28 would

then lead to the intermediate 28, which, as shown above, could be a suitable precursor to stelliferasterol (13) and strongylosterol (23a). In the absence of definite labeling experiments, the eventual isolation of C_{29} and C_{30} sterols with side chains of type 28 or 35 would be presumptive evidence for the operation of such a biosynthetic route. Evidence for the feasibility of an intermediate (28) with a sec-butyl substituent at C-24 exists since 24-isopropyl-22-dehydrocholesterol (36) (Ref. 32) (together with its side chain-saturated analog) (Ref. 33) and probably also 24-isopropenylcholesterol (37) (Ref. 25) have been encountered in sponges; presumably they arise from methylation at C-28 of a fucosterol (5) precursor.



CYCLOPROPYL-CONTAINING STEROLS

Our own interest in marine sterols was prompted by the complete structure elucidation (Refs. 34, 35) of gorgosterol (38), since its structure immediately showed that hitherto unsuspected and unprecedented steps in the biosynthesis of the sterol side chain are feasible. They are the alkylation of positions 23 and 22 (since until that time only alkylation at position 24 had been observed) and the formation of cyclopropane rings - once postulated (Ref. 36) as possible intermediates in the bioalkylation of the sterol side chain from desmosterol. We shall now consider certain aspects of these two unique features of gorgosterol (38), which should be discussed in conjunction with the structures of two of its naturally occurring relatives - 23-demethylgorgosterol (39) (Ref. 37) and 4-methylgorgosterol (40) (Ref. 38).



38 R = Me; R′ = H
39 R = R′ = H
40 R = R′ = Me

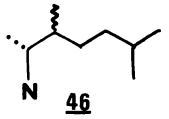
41 R = Me; R′ = H
42 R = R′ = Me (24 ξ)
43 R = R′ = Me; Δ^{23}

44 R = Me
45 R = H

Ciereszko and collaborators (Ref. 38) have shown that gorgosterol (38) and its congeners occur in the zooxanthellae rather than the soft coral hosts with which these symbionts live. They have also isolated trace amounts of 4-methylgorgosterol (40), which raises the as yet unanswered question whether the unique side chain substitution pattern is generated at the stage of lanosterol (or of a partially C-4 and C-14 demethylated lanosterol precursor) with complete ring demethylation being the last step in the biosynthesis of gorgosterol, or whether the 4-methyl analog (40) is simply the end product of a side reaction. In connection with our establishment (Ref. 35) of the complete stereostructure 38 of gorgosterol, we speculated that brassicasterol (41), which frequently co-occurs with gorgosterol (38) and possesses the same C-24 (R) stereochemistry, may be the key biogenetic precursor and that Δ^{22}-23,24-dimethylated precursors might exist in nature. This prediction has since been verified by Kanazawa et al. (Ref. 39) who have encountered 23,24ξ-dimethyl-22-dehydrocholesterol (42) in a variety of coelenterates together with gorgosterol (38) and demethylgorgosterol (39). The same Japanese group (Ref. 40) has also detected the corresponding 23-dehydro analog 43 in a soft coral (Sarcophyta elegans). Another important marine sterol, dinosterol (44) isolated (Ref. 41) from the dinoflagellate Gonyaulax tamarensis, possesses a similar side chain substitution pattern and thus affords indirect evidence that the gorgosterol methylation pattern occurs in the order C-24→C-23→C-22.

However a different order of methylation is not inconceivable, especially since the first C-23 side chain monomethylated sterol, 24-demethyldinosterol (45), has recently been detected (Ref. 42) in another dinoflagellate Gonyaulax diagenesis, which also contained cholesterol and isofucosterol (5). 24-Demethyldinosterol (45) could, of course, have arisen by biological demethylation at C-24 from dinosterol (44), in which case the former (45) sterol is of no major biogenetic significance as far as the path to gorgosterol is concerned. However, it is conceivable that 45 arose by biological methylation of a simple Δ^{22} sterol with the standard C_8 cholesterol side chain, since 22-dehydrocholesterol is known to occur in the marine environment (Ref. 2). The occurrence of such unusual sterols in dinoflagellates is of double significance. They may well be the dietary sources for unusual sterol precursors of coelenterates and other higher marine animals. Even more importantly, they can be grown in an uncontaminated state in cell cultures and thus lend themselves as prime substrates for rigorous biochemical experiments with suitable tracers.

The existence in nature of sterol side chains with 23,24-dimethyl (42-44) or 23-monomethyl (45) substituents raises the question whether 22-monomethyl sterols may not also occur in nature as possible products of biological monoalkylation of a Δ^{22} precursor. In order to facilitate the search for such potential marine natural products, which would be of extraordinary biosynthetic interest, we undertook (Ref. 43) the synthesis of a series of model sterols such as 46-52



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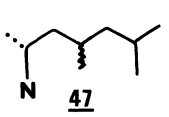

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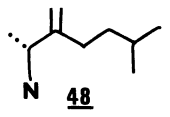

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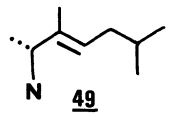

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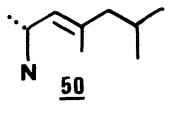

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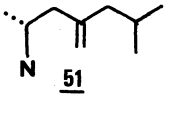

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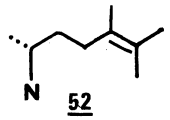

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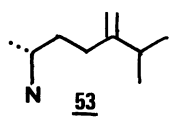


53

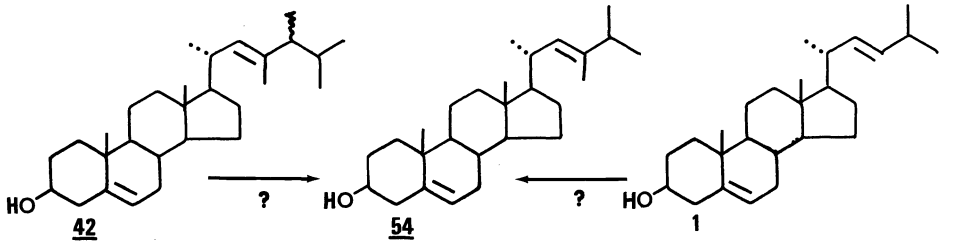
in order to have their spectral properties and gas chromatographic mobility on record. The details will be published elsewhere, but a few comments are appropriate at this stage in order to emphasize the difficulty of recognizing the natural occurrence of 22- and 23-monoalkylated sterols if they are present only in trace quantities. For instance, the gas chromatographic mobility of one of the two synthetic C-22 epimers of 22-methylcholesterol (46) is identical with that of 23ξ-methylcholesterol (47). The main difference is the presence in the mass spectrum of 46 of small peaks (ca. 10% rel. int.) at m/e 301 (fission of 20-22 bond) and m/e 283 (loss of H_2O from m/e 301), which are absent in the mass spectrum of 23-methylcholesterol (47). There are measurable differences in certain NMR methyl signals (e.g. C-21 and C-28 methyls at 0.761 and 0.689 ppm in 46 vs. 0.889 and 0.781 in 47). The NMR and mass spectral differences between 23-methylcholesterol (47) and the very common 24-methylcholesterols are very much smaller and it is quite conceivable that if 23-methylcholesterol (47) is present in nature, it has been mistaken for one of the common 24-methyl isomers.

The situation is somewhat simplified when dealing with side chain-methylated cholesterols which possess additional unsaturation at the site of alkylation. If enough material is available, then ozonolysis provides unambiguous evidence of the side chain structure, as was the case in the structure proof of dinosterol (44) (Ref. 41) and 24-demethyldinosterol (45) (Ref. 42). Furthermore, there are marked mass spectral differences, which will already be noticeable during initial GC-MS screening procedures. For instance, it is a simple matter to distinguish the well known 24-methylenecholesterol (53) from its synthetic (Ref. 43) 22-(48) and 23-(51) methylene counterparts. The base peak in 53 occurs at m/e 314 due to the well studied (Ref. 19) McLafferty rearrangement (transfer of C-20 hydrogen), whereas in the 23-methylene isomer (51) the McLafferty rearrangement is initiated by C-17 hydrogen transfer leading to m/e 300 (100% rel. int.) and in 22-methylenecholesterol (48) by migration of the C-25 hydrogen leading to m/e 342 (100% rel. int.). In each isomer, it is migration of the tertiary hydrogen which is responsible for the dominant McLafferty rearrangement, which is completely consistent with earlier model studies (Ref. 44).

In summary, a concerted search for 22- and 23-monomethyl sterols is warranted, but it should be realized that some may be products of side chain dealkylation and hence may not necessarily imply a direct alkylation of a Δ^{22}-unsaturated sterol. We have already commented upon this point by describing the possible generation of 24-demethyldinosterol (45) (Ref. 42) by biological C-24 demethylation of dinosterol (44) (Ref. 41). A conceivable second example is presented below.

Studies on the origin of marine sediments have included detailed analyses of their sterols and a recent paper (Ref. 9) dealing with the sterol composition of a diatomaceous ooze from Walvis Bay (S.W. Africa) has demonstrated the amazing complexity of such mixtures. Relevant

to our discussion is the detection of gorgosterol (38) and 23,24-dimethyl-22-dehydrocholesterol (42) as well as a C_{27}-$\Delta^{5,22}$-dien-3β-ol of unknown constitution. We suggest that this new sterol may possess the structure 54 and arise from biological dealkylation of an unsaturated 23,24-dimethylcholesterol precursor (e.g. 42) as was already predicted by our computer program (Ref. 1). However, if this structure assignment is correct, then one cannot exclude the hypothetical possibility that 54 is biosynthesized by direct C-23 methylation of 24-norcholesta-5,22-dien-3β-ol (1).

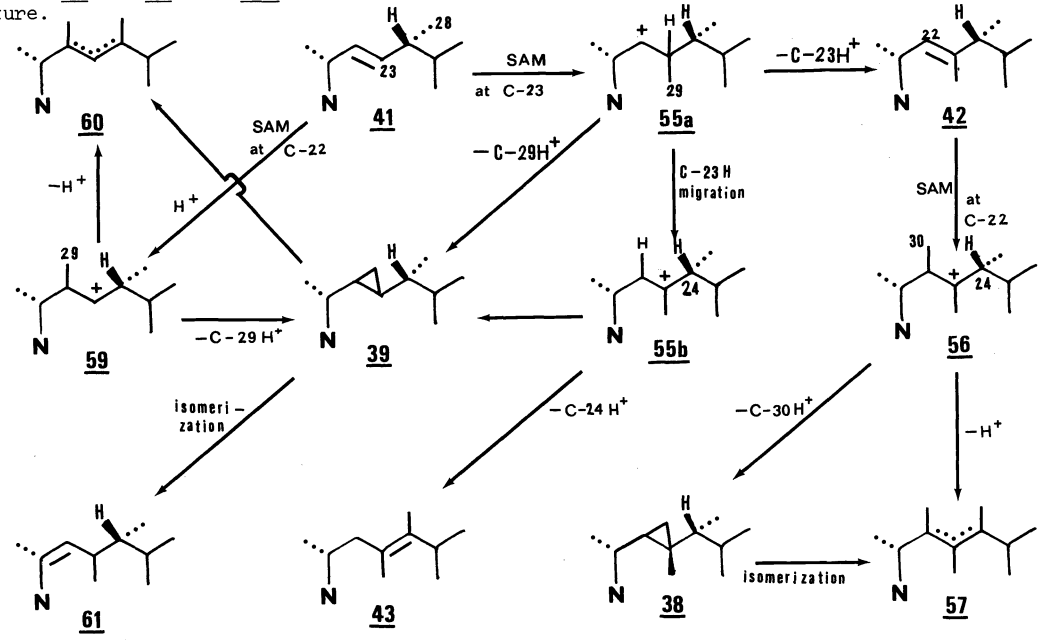


42 54 1

We should now like to conclude with some consideration of the puzzling role of the occurrence of cyclopropane rings in the side chain of marine sterols. The question of whether they play a biological role (membrane or other function) or are metabolic "dead ends" is so far totally unanswered. So is their biosynthesis, but it is likely that this will be settled first and some speculations seem warranted at this time.

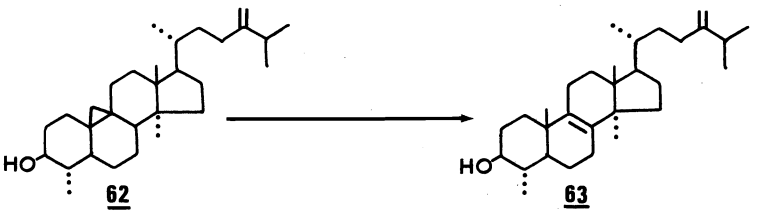
Starting with our original hypothesis (Ref. 35) that the brassicasterol (41) side chain is one of the most likely biogenetic precursors for the gorgosterol (38) alkylation pattern, conventional (Refs. 12,36) alkylation by S-adenosylmethionine (SAM) at C-23 would lead to the key carbonium ion intermediate 55a. Loss of a C-29 proton would then directly generate demethylgorgosterol (39) - there being ample precedent (Refs. 36, 45) for such cyclopropane formation from fatty acid and phytosterol biochemistry. The intermediacy of such an ion 55a is further supported by the fact that simple C-23 proton loss would explain the existence (Ref. 39) in the marine environment of 42 [or of dinosterol (44) (Ref. 41) from an appropriately modified nuclear precursor], while C-23 proton migration to 55b followed by C-24 proton ejection would lead to 43, which has also been encountered (Ref. 40) in nature. A second alkylation of the Δ^{22}-double bond of 42 at C-22 would proceed, via the carbonium ion 56 and C-30 proton expulsion, to gorgosterol (38). The alternative C-22 or C-24 proton loss from 56 could generate a 22,23,24-trimethyl sterol with a Δ^{22} or Δ^{23} double bond and while such a sterol (57) has not yet been encountered in nature, we predict that it will be found in due course.

An alternative biosynthetic route to demethylgorgosterol (39) could be visualized through attack by SAM at C-22 of brassicasterol (41) followed by loss of a C-29 proton from the carbonium ion intermediate 59. This is actually the route considered earlier by Nes (Ref. 12); at this time we consider it less attractive, because the alternative (C-22 or C-24) proton loss from 59 should produce 22,24-dimethyl substituted sterols (60) - by analogy to the production of 42 and 43 from 55a - and such a substitution pattern has not yet been encountered in nature.



60 41 55a 42 59 39 55b 56 61 43 38 57

Lederer (Ref. 36) considered in detail the possibility that cyclopropanes may serve as intermediates for saturated methyl groups through a biochemical reductive opening and rejected it on the basis of negative experimental results which he summarized (Ref. 36). However, there is ample experimental evidence (Refs. 12, 13, 36) in the plant kingdom for the existence of an isomerase, which converts cyclopropanes into allylically unsaturated methyl groups - a typical example being the generation of obtusifoliol (63) from cycloeucalenol (62).



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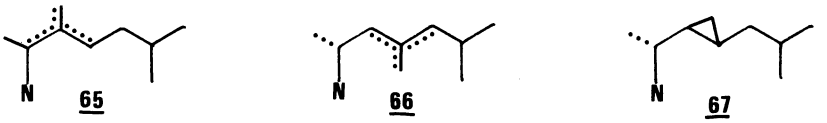
If such an isomerase system could also operate on cyclopropanes of the gorgosterol (38) and demethylgorgosterol (39) types, then it is likely that these cyclopropanes are active intermediates in the biosynthetic introduction of methyl groups at C-22 and C-23. For instance, one product of the action of such an isomerase upon demethylgorgosterol (39) would be the $\Delta^{20(22)}$-23,24-dimethylcholesterol side chain (61), which might then be transformed to the known Δ^{22} (42) and Δ^{23} (43) isomers. If such an isomerase does not exist, then these cyclopropanes are either products of metabolic side reactions (i.e. alternative proton loss from key carbonium intermediates such as 55a and 59) or serve for a particular and as yet undetermined biological function. Appropriate radioactive labeling experiments are required to settle these questions.

Now that alkylation at C-22 and C-23 has been demonstrated through the isolation of a variety of relevant marine sterols, it is not obvious why a 24-methyl group is usually also present. The same type of alkylation and proton elimination steps outlined above with 41 could conceivably proceed from the unsubstituted Δ^{22}-cholesterol side chain (64). Our computer program (Ref. 1) actually includes this eventuality and it is for this reason that the search in the marine environment for sterol side chains of types 65-67 is to be encouraged as indicated above.



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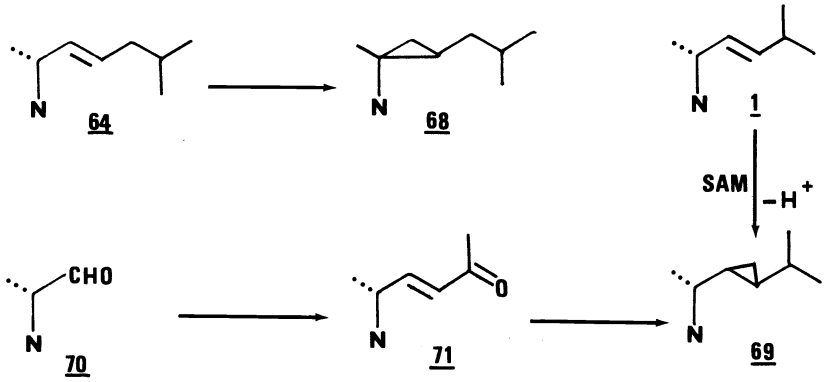
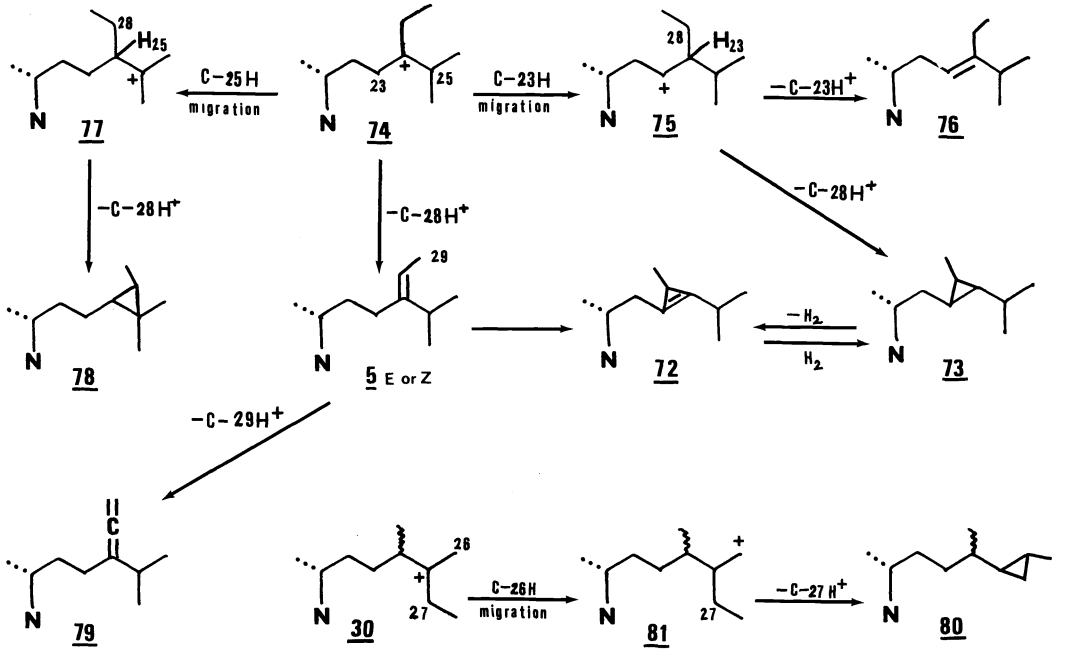
A recent report (Ref. 46) provides circumstantial mass spectral evidence that 20,23-cyclocholesterol (68) may be an algal constituent. If correct, then this would suggest the existence of an isomerase capable of reversing the process typified by 62→63, in other words the production of 20,23-cyclocholesterol (68) from 22-dehydrocholesterol (64). Since the experimental evidence (Ref. 46) did not exclude the isomeric cyclopropane structure 69 - the hypothetical product of the "conventional" cyclopropanation (cf. 41→39) of the ubiquitous marine sterol 24-norcholesta-5,22-dien-3β-ol (1) - we undertook (Ref. 47) its synthesis, which was patterned after our demethylgorgosterol synthesis (Ref. 48) via the key intermediates 70 and 71. In spite of the unknown stereochemistry of the natural product and of 69, the respective mass spectra were so different that there is no doubt that the algal product (Ref. 46) does not have the structure 69. Isolation of additional material so as to permit NMR examination is essential to verify the potentially very important 20,23-cyclocholesterol (68) structure.



Another and biogenetically probably distinct cyclopropane side chain substitution pattern is typified by the sponge sterols calysterol (72) (Ref. 49) and petrosterol (73) (Ref. 50). While it has been demonstrated experimentally (Ref. 51) that labelled fucosterol (5) is biotransformed into calysterol (72), it is not known whether calysterol (72) and petrosterol (73) are components of a common biosynthetic path, and if they are, what their respective relationships are.

One conceptually simple sequence would start with the carbonium ion intermediate 74, which is considered (Refs. 12, 12, 36) to be the initial product of SAM attack upon 24-methylenecholesterol (53) and which leads to (iso)fucosterol (5) upon loss of a C-28 proton. Migration of the C-23 hydrogen in 74 would furnish the isomeric carbonium ion 75, which would yield petrosterol (73) upon ejection of the C-28 proton. Biochemical dehydrogenation of 73 would then offer a simple path to calysterol (72). The postulated C-23 hydrogen migration (74→75) is energetically much less favorable than the alternative C-25 hydrogen shift, which would produce 77 and thence, upon C-28 proton loss, the hitherto unknown isomeric cyclopropane 78. However circumstantial evidence in favor of the intermediacy of a secondary carbonium ion of type 75 is provided by the coexistence (Ref. 52) of calysterol (72) with 24-ethylcholesta-5, 23-dien-3β-ol (76) - one of the very few naturally occurring Δ^{23}-sterols.



Since fucosterol (5) is an intermediate (Ref. 51) in the biosynthesis of calysterol (72), it is conceivable that the cyclopropene ring is produced directly by double bond migration and biochemical dehydrogenation of fucosterol (5), rather than via the cyclopropane petrosterol (73). Indeed if such dehydrogenation should be operative, then petrosterol (73) itself might be a reduction product of calysterol (72) rather than a substrate for further dehydrogenation to 72. The possibility that fucosterol (5) may undergo a formal dehydrogenation via cyclization to a cyclopropene is somewhat strengthened by our recent isolation (Ref. 53) of the first naturally occurring steroidal allene, 24-ethyl-$\Delta^{5,24(28),28}$-cholestatrien-3β-ol (79) from the sponge Callyspongia diffusa, whose principal steroidal constituent is isofucosterol (5).

That petrosterol (73), and hence calysterol (72), are not just oddities, but rather representatives of a new class of steroidal cyclopropanes that are biosynthetically distinct from the gorgosterol (38) type of cyclopropanes, is indicated by the recent encounter (Ref. 54) in a sponge of another methylated cyclopropane, which was shown to have structure 80. In terms of methylation pattern, it clearly falls within the aplysterol (11) group, although nothing is known at this stage about their stereochemical identity at C-24. By analogy to the above postulated biosynthesis of petrosterol (73) from the carbonium ion intermediate 74, a similar sequence (30→81→80) from the related carbonium ion intermediate 30, which we had invoked (vide supra) in the biosynthesis of verongulasterol (26), would lead directly to the cyclopropane 80 (26,27-cycloaplysterol).

C_{19} - C_{25} STEROLS

Very minor or trace levels of sterols with conventional cholesterol nuclei but with biosynthetically unusually short hydrocarbon side chains, e.g. containing less than the eight carbon atoms expected for squalene cyclization products, are present in the extracts of a wide range of marine organisms. In a recent article (Ref. 55), we have considered in detail the structure determination, synthesis, distribution and possible origins of the short side chain marine sterols; therefore, we will discuss this class of compounds only briefly.

Unlike the interesting keto or dihydroxy low molecular weight steroids recently isolated from marine sources (Refs. 56, 57), the C_{19}-C_{25} sterols possess all the structural requirements to be included under the classical definition with that class of structural membrane constituents referred to as sterols. However, recent studies (Refs. 58, 59) have demonstrated that the C_{19}-C_{25} sterols probably cannot function in membrane stabilization as do higher molecular weight sterols. Further, C_{19}-C_{25} sterols have been identified among the autoxidation products of cholesterol (Refs. 60, 61). These findings led us to perform careful experiments with freshly collected material which have now convinced us that C_{19}-C_{25} sterols are present in the tissues of certain marine organisms rather than arising by degradative processes during sample handling or laboratory work-up. Application of established singlet oxygen and biradical autoxidation mechanisms to the set of marine sterol side chains by use of the REACT computer program (Ref. 55) and comparison of the results with experimental short side chain sterol distribution found in marine invertebrates has lent support to the suggested natural environmental or *in vivo* autoxidative formation of the C_{19}-C_{25} marine sterols.

It is significant that autoxidative processes do not account for the existence of the series of 24-nor C_{26} and 27-nor C_{27} marine sterols. Furthermore, the C_{26} and 27-nor C_{27} sterols have side chains of sufficient length to function as sterols in membrane stabilization (Refs. 58, 59). Therefore, the well known C_{26} and 27-nor C_{27} marine sterols must be considered as a class separate from the C_{19}-C_{25} minor marine sterols.

CONCLUSION

The first phase of marine sterol chemistry - the isolation of novel "missing links" in various hypothetical biosynthetic schemes - is by no means over and many important structural types predicted (Ref. 1) earlier are yet to be found in nature. The second phase - biochemical verification through appropriate labeling experiments - has already started, because there are already enough plausible biosynthetic hypotheses available that lend themselves to biochemical verification or refutation. The lower marine organisms (e.g. dinoflagellates) are likely to be better substrates for such tracer studies than the higher animals (e.g. coelenterates, sponges, etc.), because uncertainty about endosymbiont and food chain contributors is minimized. The third and ultimately most important phase - determination of the possible biological function of these uniquely marine sterols - has hardly been touched.

ACKNOWLEDGMENT

The contributions of the various collaborators listed in the bibliography and of Annemarie Wegman (mass spectrometry) as well as Dr. Lois J. Durham (NMR spectroscopy) are gratefully acknowledged. Financial support for the laboratory work was provided by grants No. GM-06840 and AM-04257 from the National Institutes of Health. In addition we acknowledge financial support for the computer and certain mass spectral studies through NIH grant No. RR-00612 and for the use of a 360 MHz NMR spectrometer through NSF grant No. GP-23633 and NIH grant No. RR0711.

Addendum

After completion of this manuscript we were informed by Prof. D. Sica of the University of Naples that the original assignment (Ref. 50) of structure 73 to petrosterol is incorrect, and that it was revised to 80 (24R configuration) by X-ray crystallography (Ref. 62). Direct comparison of petrosterol and 80 isolated in La Jolla (Ref. 54) showed the two to be identical None of the biosynthetic speculations outlined above with respect to calysterol need to be changed except that 73 is now a hypothetical intermediate rather than a substance actually encountered in nature.

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