Tetrahedron Vol. 43, No. 10, pp. 2213 to 2222, 1987
Printed in Great Britain.

0040-4020/87 $3.00+.00

BIOSYNTHETIC STUDIES OF MARINE LIPIDS 12.[1]

BIOSYNTHESIS IN MARINE SPONGES OF STEROLS POSSESSING THE $\Delta^{5,7}$-NUCLEUS TYPICAL OF FUNGI AND THE 24-ALKYL SIDE CHAIN CHARACTERISTIC OF PLANTS

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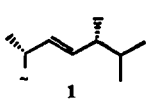
(*Received in USA* 23 *March* 1987)

ABSTRACT: The biosynthesis of sterols with structural patterns common to plant and yeast sterols was studied by incorporation of eleven radiolabeled sterol precursors and mevalonate. The capability of sponges to convert Δ^5- into $\Delta^{5,7}$-sterols, which was as efficient as the conversion of Δ^7- into $\Delta^{5,7}$-sterols, was demonstrated by double labelling experiments. By examining epimeric pairs, stereospecific conversion of codisterol and clerosterol into the corresponding 7-dehydrosterols was established. 24β-24-Alkyl sterols with homo-conjugated 22,25-diene side chains are proposed as actual intermediates in the formation of Δ^{22}-24β-alkylsterols. An alternative pathway via oxidation at C-22 or C-23 with subsequent dehydration of an intermediate 22(R or S)- or C-23(R or S)-hydroxysterol is probably not operative due to lack of incorporation of any of the tritium labeled alcohols. These biosynthetic features, though unexpected for animals, nevertheless are attributed to the sponge because of the absence of symbiotic fungi and algae as shown by electron microscopy.

INTRODUCTION

Sponges are the source of the most diverse number of unconventional sterols with unusual side chains and nuclei[2] known in nature. Examples of nuclei unique to sponges are the A-nor (A)[3] and 19-nor (B)[4] stanols, whose generation has been demonstrated[5,6] to occur by modification of a Δ^5-3β-hydroxy precursor (N). In the context of sponges, 7-dehydro-sterols (C and D), which are often encountered as the major sterols in a number of these animals,[7,8] can also be considered as unusual even though these particular nuclei are well known in terrestrial organisms (in contrast to A and B, which are unique to the marine environment). Such 7-dehydrosterols (C, D) are usually considered typical of yeasts (fungi),[9] despite their vast distribution as minor components in a wide range of organisms, where they perform a key role as pro-vitamin D.[10] It is very common for $\Delta^{5,7}$-sterols to be accompanied by 5α,8α-epidioxides.[8] Isolation of such epidioxides[8] is usually indicative of a $\Delta^{5,7}$-sterol-containing sponge, where the conjugated diene system (D) has been oxidized during storage and/or workup; reinvestigation[8c] of the sterol composition of such freshly lyophilized sponges showed the presence of $\Delta^{5,7}$-sterols and no detectable epidioxides. Of additional interest is the fact that even though sponges belong to the animal kingdom, their 7-dehydrosterols possess 24-alkyl substituents (such as 12 or 13) typical of plant (algal) sterols.[11] The virtually exclusive presence in certain sponges of sterols combining the patterns of yeast and plant sterols suggests that such sterols function as membrane components.[12] This raises interesting questions about their origin and function in this most primitive member of the animal kingdom.

There are four possible sources of these sterols in sponges: (1) dietary origin from exogenous fungi or algae with selective uptake; (2) transformation by the sponge or its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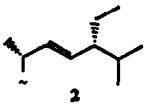
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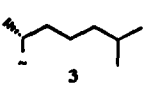
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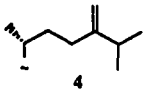
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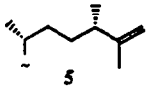
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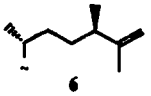
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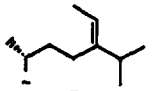
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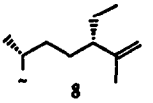
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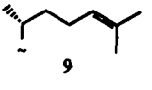
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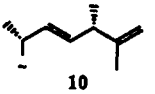
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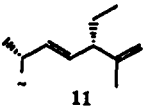
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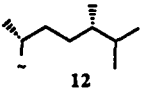
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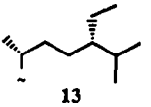
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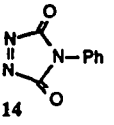
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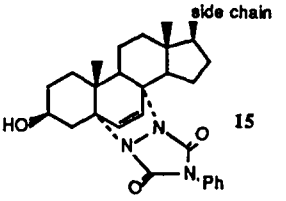
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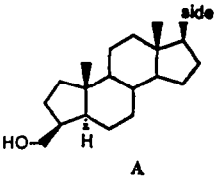
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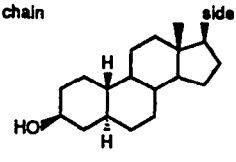
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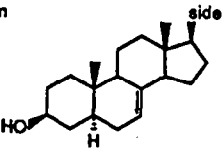
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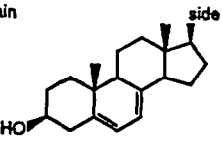
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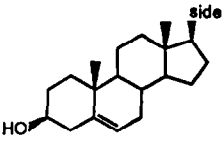
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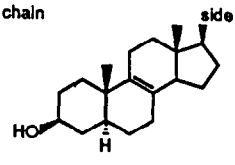
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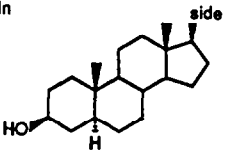
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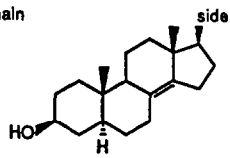
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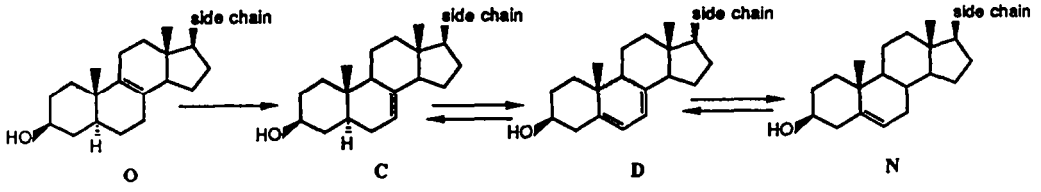
symbionts of other dietary sterols into 7-dehydrosterols; (3) de novo biosynthesis; and (4) synthesis by symbiotic fungi or algae. It was suggested earlier[13a] that these sterols may be derived from a dietary source since both fungi[13b] and some unicellular algae[13b,14] contain $\Delta^{5,7}$-sterols (D). The ability of the sponges to synthesize the sterols by themselves, similar to that of some unicellular protozoans such as Tetrahymena pyriformis,[15] and to convert Δ^{5}-(N) into $\Delta^{5,7}$-sterols (D), was taken into consideration.[13] The possible symbiotic origin of $\Delta^{5,7}$-sterols in dictyoceratid sponges from symbiotic fungi was also reconsidered,[16] since such sterols are known to occur in marine yeasts,[17a,b] but yeasts have never been observed as symbionts in any member of the dictyoceratia.[16] In the event that pathways (2), (3) or (4) are operative in sponges, it would be of interest to determine whether these animals follow the biosynthetic route established[13b] in yeasts,[18] higher plants,[19] or in algae,[20] or whether a unique pathway is employed.

In this paper we present the results of an experimental attempt to answer the above questions. This was done through biosynthetic incorporation studies with radiolabeled sterol precursors as part of our systematic biosynthetic examination[1,21-23] of the origin of unconventional sterols from marine organisms.

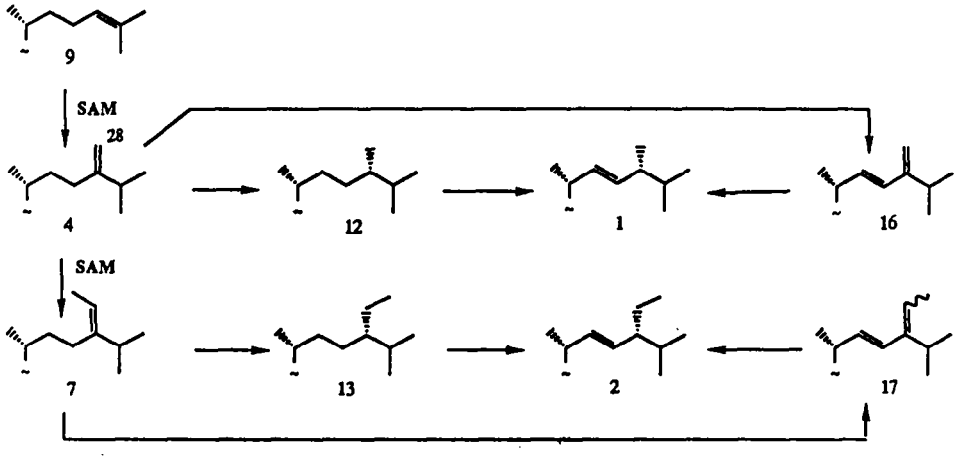
RESULTS AND DISCUSSION

The Australian sponge Pseudaxinyssa sp.[24] and the Papua New Guinea sponge Agelas sp. were ideal candidates for the study of the biosynthesis of 7-dehydrosterols (C and D) in sponges. The former contains a 1:1 mixture of the 24β-methyl- (1D) and 24β-ethyl- (2D) 22-dehydro-homologs of cholesta-5,7-dien-3β-ol (3D) (see Table 1), while Agelas sp. contains the 24β-ethyl- (2C) homolog of cholest-7-en-3β-ol (3C) as its major sterol. According to the generally accepted sequence (Scheme 1) in the de novo biosynthesis of sterols,[13b] Δ^7-stenols (C) are the direct precursors of the $\Delta^{5,7}$-dienes (D). Such Δ^7-stenols are produced by isomerization of Δ^8-stenol precursors (O) and this pathway has been attributed to all eucaryotic cells as a phylogenetic pattern of common origin.[13b]



Scheme 1

In order to determine whether the generally accepted[13b] biosynthetic sequence (C → D) or the reverse conversion (D → C) established in some protozoans,[15] echinoderms[13a] and insects[25] is operative in sponges, radioactive sterol precursors with a Δ^5- (N) and a Δ^7- (C) nucleus were incorporated into the sponge Pseudaxinyssa sp. separately as well as in a double labelling incorporation experiment. Unexpectedly, ergosta-7,24(28)-dien-3β-ol (4C), as well as its Δ^5 isomer, ergosta-5,24(28)-dien-3β-ol (4N), were equally well incorporated into the $\Delta^{5,7}$ sterols 1D and 2D of the sponge (Table 2). This confirms the existence of two alternative paths (C → D and D → C in Scheme 1) and points to the possible transformation of dietary Δ^5 or Δ^7 precursors as the source of $\Delta^{5,7}$-dienes (D) in such sponges. At the same time, our experiments demonstrate the capability of the sponge to introduce alkyl substituents at C-28, a process (Scheme 2) known to operate in plants[13b] via SAM bioalkylation of 24-methylenecholesterol (4N).

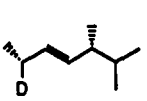
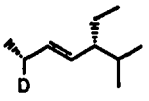
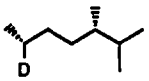
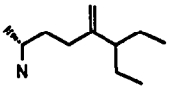
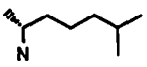
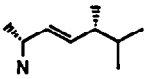
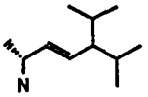
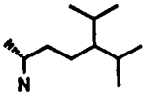
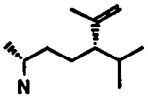
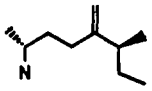
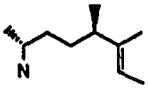
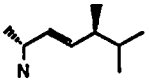
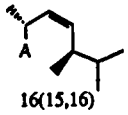
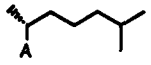
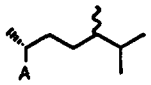
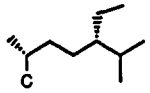
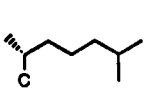
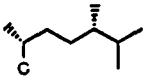
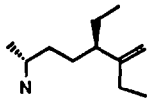
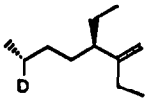


Scheme 2

According to this scheme, and taking into account that the two target sterols (1D and 2D) exist in a ca 1:1 ratio in the sponge (cf. Table 1), a higher incorporation of radioactivity would be expected in the 24-methylsterol 1D, which is produced through fewer biosynthetic steps (4 → 12 → 1, Scheme 1), than in the higher 24-ethyl homolog 2D (4 → 7 → 13 → 2). Surprisingly, the 24-ethyl sterol 2D showed ca 30 times higher incorporation of radioactivity (Table 2). This suggests that 24β-methyl-5,7,22-trien-3β-ol (1D) has a different origin from that (4 → 12 → 1) expected according to Scheme 2 and that another biosynthetic pathway operates in this sponge utilizing different precursors.

Since a precedent of an alternative biosynthesis for 24β-alkyl sterols exists in algae belonging to the class Chlorococcales[26a] (Treboudia sp. and Chlorela sp.), we attempted incorporation experiments with several labeled sterol precursors assumed to be intermediates

TABLE 1. STEROL DISTRIBUTION AMONG SOME SPONGES

SPONGE	MAJOR STEROLS [% COMPOSITION]*		
1. Pseudaxinyssa sp.[24] (AM #4989)**	 40(39,37,39)	 39(40,41,40)	 13(12,11,13)
2. Xestospongia testudinaria	 85(91,90,87)	 8(10,7,9)	 2(2,1,3)
3. Pseudaxinyssa sp.[24] (AM #4988)**	 61(56,58,54)	 38(43,42,45)	 1(0.5,0.7,0.9)
4. Jaspis stellifera	 45(49,51,52)	 8(8,7,6)	 7,(5,9,8)
5. Phakellia aruensis	 16(15,16)	 19(20,19)	 24(R) 10(11,11) 24(S) 8(9,9)
6. Agelas sp.	 71(75)	 18(21)	 6(4)
7. Strongylophora durissima	 89(92,91,94)	 8(9,6,5)	

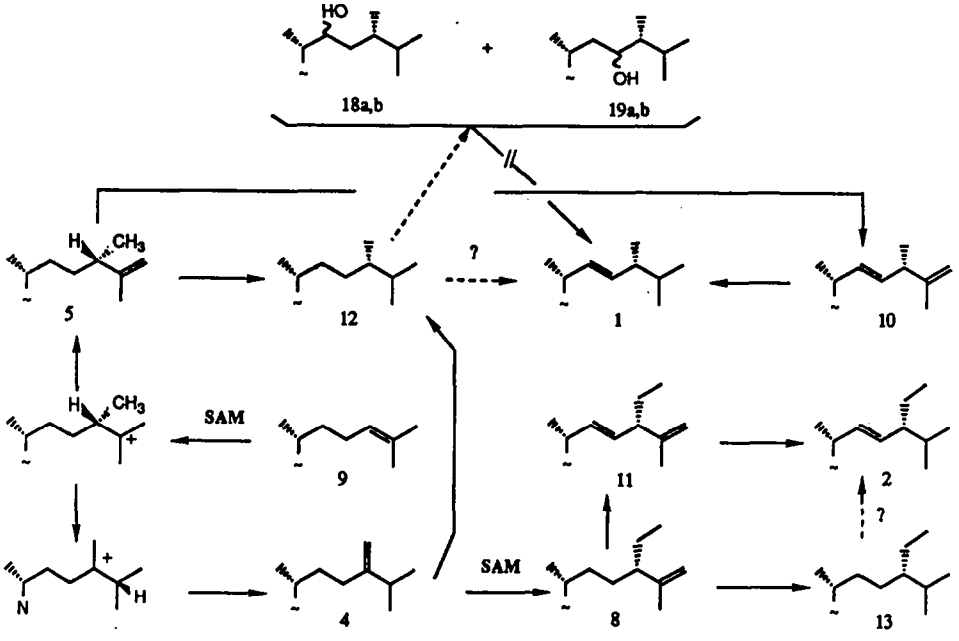
*The % composition of sterols in brackets represents data obtained at different seasons.

**Australian Museum specimen number.

in the biosynthesis of 24β-alkylated algal sterols. Only a Δ^5- nucleus (N) was used in the labeled precursors, since we had already demonstrated (Table 2) that Δ^5-sterols (N) were equally as well incorporated as their Δ^7- isomers (C).

The results of these experiments (Table 2) clearly demonstrate a biosynthetic C-24 and C-28 alkylation sequence (Scheme 3) typical for algae of the class Chlorococcales.[26] While ergosta-5,24(28)-dien-3β-ol (4N) gave only poor incorporation into ergosterol (1D), contrary to such incorporation experiments with fungi and plants, its double bond isomer, codisterol (5N), gave the best incorporation of all the precursors tested. Furthermore, total stereospecificity was established in this conversion of codisterol (5N) into 1D, since epicodisterol (6N) was not transformed (see Table 2) into ergosterol (1D). This proves that no epimerization at C-24 or isomerization into ergosta-5,24(28)-dien-3β-ol (4N) takes place, which is further demonstrated by the lack of incorporation of either codisterol epimer (5N or 6N) into the 24-ethyl homolog 2D — a transformation which has been shown to operate in Cucurbitaceae.[26b] Finally, our results eliminate the possibility of participation of $\Delta^{24(25)}$-intermediates as proposed for some plants.[26b]

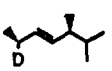
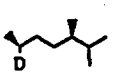
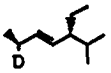
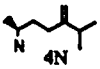
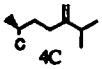
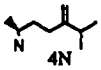
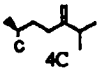
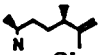
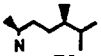
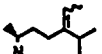
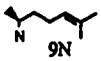
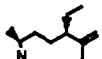
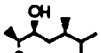
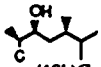
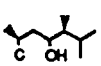
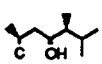
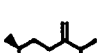
The two alternative paths for the biosynthesis of the 24-ethyl-5,7,22-triene 2D via fucosterol (7N) (Scheme 2) or clerosterol (8N) (Scheme 3) were checked by feeding these labeled sterols to the sponge Pseudaxinyssa sp. The ca 40 times higher efficiency of clerosterol (8N) utilization (Table 2) demonstrates that the biosynthetic sequence established in higher plants[19] is insignificant by comparison to the algal route[20,26] (Scheme 3) in the sponge. As expected, the 24-methyl analogs 1D and 12D remained unlabeled in the incubation experiments with fucosterol (7N) and clerosterol (8N), which excludes the possibility of dealkylation and subsequent alkylation occurring at some intermediate stage as was demonstrated[15] in the protozoan Tetrahymena pyriformis.



Scheme 3

The two principal sponge sterols 1D and 2D thus have parallel individual pathways of biosynthesis (Scheme 3) from a common sterol precursor, such as desmosterol (9N), via the two key intermediates codisterol (5N) and ergosta-5,24(28)-dien-3β-ol (4N). This sequence of the side chain biosynthesis, together with the 24β-stereochemistry of the 24-ethyl substituent 2D, strongly suggests an algal rather than fungal origin for the sponge sterols. However, neither fungi nor algae were detected by electron microscopy; rather the sponge contained only procaryotic symbionts, including some with photosynthetic capabilities, like so many other tropical, flattened sponges.[27] While procaryotes are assumed to be incapable of de novo sterol biosynthesis,[28] analysis of isolated or cultured procaryote symbionts from the host sponges will be necessary to check their possible contribution, since in a few cases low levels of sterols have been detected[29a-c] in some fresh-water cyanobacteria.

TABLE 2. RADIOACTIVE INCORPORATION RESULTS

Sterol Precursor	Labeled at	Amount [μCi] Incubated	Radioactivity* [dpm] Recovered In: 		
 4N	[28-^{14}C]	20	42,000	869,200	1,422,300
 4C	[3-^{3}H]	20	46,000	753,800	1,317,000
 4N	[28-^{14}C]	20	39,324	698,500	1,370,900
 4C	[3-^{3}H]	20	41,625	790,400	1,817,690
 6N	[26-^{14}C]	20	cold	cold	cold
 5N	[26-^{14}C]	20	9,670,800	4,767,800	cold
 7N	[22-^{3}H]	32	cold	cold	174,350
 9N	[24-^{14}C]	16.7	223,625	302,300	422,580
 8N	[26-^{14}C]	20	cold	cold	6,568,700
 (18a)C	[22-^{3}H]	20	cold	cold	cold
 (18b)C	[22-^{3}H]	20	cold	cold	cold
 (19a)C	[23-^{3}H]	20	cold	cold	cold
 (19b)C	[23-^{3}H]	20	cold	cold	cold
 4N	[28-^{14}C]	20	cold	cold	2,760,000**
Mevalonate	[2-^{14}C]	50	Total Sterols = 2,500		

*The actual radioactivity was measured after hydrogenation of the $\Delta^{5,7}$- to the $\Delta^{8,(14)}$- sterols; this step was necessary for effective separation.

**This experiment was done with the sponge Agelas sp. and the radioactivity was counted directly on the free Δ^{5}-sterol.

Since sponges display very little or no de novo biosynthesis[21] (see also last entry in Table 2), but are capable of equally facile Δ^5- and Δ^7-double bond introduction into the sterol nucleus, the question of the sequence in which the second nuclear double bond and the appropriate 24-alkyl substituents are introduced is no longer relevant, as is the case in the de novo biosynthesis of such $\Delta^{5,7}$-dienes (D) in fungi.[18] Therefore three-dimensional diagrams, like the ones used[18a] for yeast biosynthesis, could be applied here as well to represent the parallel biosynthetic sequences taking place in the sponge.

Two major difficulties were encountered during the isolation and purification of the $\Delta^{5,7}$-sterols 1D and 2D: (1) their separation from admixing Δ^5-sterols (N) co-migrating during their HPLC separation on a reverse Altex column and (2) the undesired co-migration of the 24-ethyl-triene 2D and the 24-methyl-diene 12D in a reverse HPLC system.

The first problem was solved by treating $\Delta^{5,7}$-sterols (D) with 4-phenyl-1,2,4-triazoline-3,5-dione (14) to form the adducts 15,[30] which are readily separable by TLC from the unreacted Δ^5-sterols (N). The radioactivity of the adducts 15, corresponding to the peaks of 1D and 2D after HPLC purification, dropped only by 2-2.5%, which is associated with the small amounts of Δ^5-sterols (N) that contaminated the original fraction. The disadvantage of this method was the unsatisfactory recovery of the original $\Delta^{5,7}$-sterols (D) from their adducts (15), probably due to the small quantity of the complex sterol mixture. The above method solved the problem of purification from admixing Δ^5-sterols (N), but the HPLC separation of 2D from the co-migrating 12D could only be achieved after catalytic hydrogenation of the mixture with platinum on activated charcoal. The resulting mixture of ergost-8,14-en-3β-ol (12Q) and stigmast-8(14)-en-3β-ol (13Q) was then readily separable by HPLC.

The isolation of these compounds enabled us to finally establish the operative sequence of the alkylation-(de)saturation steps in the extension of the side chain at C-24. Although ergosta-5,24(28)-dien-3β-ol (4N) was efficiently converted into the 24-methyl-5,7-diene 12D by reduction of the 24(28)-double bond and dehydrogenation at position 7, the subsequent 22(23)-dehydrogenation to ergosta-5,7,22-trien-3β-ol (1D) proceeded very poorly. By contrast, codisterol (5N) gave the best incorporation of all tested precursors into the triene 1D.

This suggests that the dehydrogenation at 22(23) requires the presence of the 25(26)-double bond and that the biosynthesis (Scheme 3) of the trienes 1D and 2D proceeds via the intermediate 22,25-dienes 10 and 11, as was demonstrated for the alga Trebouxia sp.[26a] Sterols with such a diene side chain were not detected in our experimental sponge, probably due to their fast conversion into the major sterols of the sponge, but were encountered as microcomponents in a few other sponges.[31a] Strong evidence for the real intermediacy of such compounds is the recent isolation of the tetraene 10D as the major sterol (>90%) of the Hawaiian sponge Ciocalipta sp.[31b] where it may have accumulated due to the absence or inhibition of the 25-hydrogenase. Additional circumstantial support for the intermediacy of 22,25-diene-sterols is the observed lack of incorporation (Table 2) of any of the epimers of 22- or 23-hydroxy-ergost-7-en-3β-ols (18a,b and 19a,b) (Table 2).

Incorporation of the Δ^5-radiolabeled sterol precursor ergosta-5,24(28)-dien-3β-ol (4N) into Agelas sp. showed similar high incorporation (Table 2) into the typical Δ^7-unsaturated plant sterol, spinasterol (3C), which is the principal sterol of the sponge (Table 1). This additional example, demonstrating the capability of sponges to alkylate at C-24 and C-28 as do plants, raises intriguing questions about the evolutionary relationship between the most primitive and ancient eucaryots — the sponges and plants.

This effective conversion in the sponge of Δ^5- (N) into Δ^7- (C) sterols most probably proceeds via the $\Delta^{5,7}$-sterols (D) by further reduction of their Δ^5-double bond. This capability of sponges to convert Δ^5- (N) into Δ^7-sterols (C) extends this unusual phenomenon, observed earlier in some Mollusca[32] and Echinodermata,[13a] to the Porifera. An earlier hypothesis[33] regarding the taxonomy of chitons (class Amphineura) attributed the abundance of Δ^7-sterols (C) in this most primitive class of the Mollusca to a lack of the $\Delta^7 \rightarrow \Delta^5$-isomerase, in contrast to the more advanced Pelecipoda and Gastropoda of the same order, which contain Δ^5-sterols (N). This assumption, similar to that made for some lower plants and algae containing high amounts of Δ^7-sterols (C), was disproved later when it was demonstrated that mollusks from the class Amphineura can introduce a Δ^7- double bond by converting[32] Δ^5- (N) sterols into Δ^7- (C) analogs via $\Delta^{5,7}$-dienes (D) similar to some Echinoderms,[13a] which can also perform such transformation and produce their Δ^7-sterols from dietary Δ^5-precursors. The reverse transformation ($\Delta^7 \rightarrow \Delta^5$) was shown not to be operative.

The origin and significance of $\Delta^{5,7}$-sterols, present in appreciable quantities (10-20%) in the sterol mixtures of many mollusks, still await elucidation.[13a] There is only one report describing some incorporation of labeled C_{29} sterols (sitosterol) into the $\Delta^{5,7}$-sterol fraction of two mollusks.[34]

In summary, invertebrates containing Δ^7- (C) and $\Delta^{5,7}$-sterols (D) can generate such sterol nuclei via a pathway that is different from that[13b] (O → C → D) in de novo sterol biosynthesis. Therefore evolutionary relationships based on the biosynthetic sequence O → C → D → N is not applicable to these invertebrates.

The efficiency of incorporation of the labeled precursors in our incorporation experiments (Table 2) justifies some additional generalizations. The ratio of the sterols 12D, 1D and 2D remained unchanged through different seasonal collections; this suggests that these sterols are actually synthesized by the sponge Pseudaxinyssa sp. or its symbionts, unless the sponge possesses a complex mechanism for maintaining such a sterol ratio through selective utilization of dietary sterols which would be expected to vary seasonally due to seasonal changes in the abundances of microorganisms. An example would be the ingestion by the sponge of brassicasterol (1N) and poriferasterol (2N), which are known planktonic constituents, followed by introduction of the Δ^7-double bond (Scheme 1). It seems that sponges have evolved mechanisms for fine-tuning and maintaining their constant sterol composition as a species-specific pattern, but it is as yet unknown through which of the discussed possibilities such process is achieved.

Sterol composition in sponges, however, is not simply a reflection of the available planktonic microorganisms (<50 μ) which are unselectively filtered by sponges. In the course of our research, we have moved many sponges with totally different sterol patterns (Table 1) from distant (5 to 30 km) and different ecological sites to two underwater study sites; there their sterol compositions remained essentially unchanged for periods of one month to one year, periods long enough to reflect seasonal changes in the plankton composition. Therefore, we now believe that sponge sterol composition is species-related, rather than a simple reflection of the sterol composition of the available dietary plankton at the collection site. The latter would be possible only if the sponges possessed an extraordinarily selective mechanism for the uptake of specific plankton components present throughout the different seasons regardless of seasonal changes. Therefore, it is very unlikely that even a selective uptake of planktonic microorganisms can provide such a constant composition in sponges with such a wide variety of unusual sterols (see Table 1). The only other conceivable possibility would be the presence of obligate species-specific symbionts, which provide in an unknown manner the sterols for that characteristic and constant sterol pattern or induce their production in the sponge. The last might be related to requirements for efficient functioning of the host-symbiont complex. A closer examination of the symbionts in sponges containing unusual sterol patterns would be neccessary to address this question.

EXPERIMENTAL

General: Waters HPLC equipment (M6000 A and M45 pumps, U6K injector, R401 differential refractometers) as well as Rheodyne model 7120 and a Valco model CV-6-UHPa-N60 injector were used for separation of sterol mixtures. The columns for isolation and further purification were two Altex Ultrasphere ODS columns (10 mm i.d. x 25 cm) connected in series. The purity of HPLC fractions was checked by GC using a Hewlett-Packard model 402 gas chromatograph with FID (3% SP2250 column, 2 mm i.d. x 1.80 m, 260° C). Low resolution mass spectra were recorded with a Hewlett Packard 5995 spectrometer in either DI or GC-MS mode (capillary SE54 column, 15 m, 260° C); 300 MHz ^{1}H NMR spectra were measured on a Nicolet NT 300 WB spectrometer. Radioactivity was determined with a Beckman LZ7500 liquid scintillation counter.

Synthesis of labeled precursors: The synthesis of the labeled sterol precursors has been described elsewhere: [28-^{14}C]-ergosta-5,24(28)-dien-3β-ol,[35] [26-^{14}C]-codisterol,[35] [26-^{14}C]-epicodisterol,[35] [26-^{14}C]-clerosterol,[23] [24-^{14}C]-desmosterol,[36] [22-^{3}H]-fucosterol,[21] [3-^{3}H]-ergosta-7,24(28)-dien-3β-ol,[37] [22-^{3}H]-22(R)-22-hydroxyergosta-7,24(28)-dien-3β-ol and its 22(S)-epimer,[38] [23-^{3}H]-23(R)-23-hydroxy-ergost-7-en-3β-ol and its 23(S)-epimer.[38]

Collection of sponges for the incorporation experiments. Pseudaxinyssa sp. (Australian Museum specimen #Z4989)[24] was collected at depths of 20-25 m from mid-shelf reefs in the central section of the Australian Great Barrier Reef: Lodestone Reef, May 1983; Davies Reef, February 1984; John Brewer Reef, June, 1985, October 1985 and August 1986. Agelas sp. (Chris-

tensen Research Institute specimen #85-026) was collected at 25 m along the outer reef slope near Madang, Papua New New Guinea, in July and October, 1985.

Electron microscopy. For Pseudaxinyssa sp., intact surface (<0.5 mm) and internal tissue was fixed[39] and examined by electron microscopy (3000 X) for the presence of microorganism symbionts.

Incorporation experiments. Specimens were collected and transplanted in situ onto plastic plaques. The precursors were then introduced into the specimens via 10-12 hour aquarium incubations[21,35] and finally returned to their original collection site for approximately one month before collection[40] and analysis. For Pseudaxinyssa sp., dates of experiments for each precursor were: (1) February 1984 for [28-^{14}]-ergosta-5,24(28)-dien-3β-ol; (2) June, 1985 for [28-^{14}]-ergosta-5,24(28)-dien-3β-ol + [3-^{3}H]-ergost-^{7}-en-3β-ol, and [3-^{3}H]-ergost-^{7}-en-3β-ol; (3) October 1985 for [24-^{14}C]-desmosterol, codisterol, epicodisterol, clerosterol, fucosterol, and mevalonate. For Agelas sp., [28-^{14}]-ergosta-5,24(28)-dien-3β-ol was incorporated in October 1985.

ACKNOWLEDGEMENTS

Financial support was provided by NIH grants GM06840 and GM28352. Use of the 300 MHz ^{1}H NMR facility at Stanford University was made possible by NSF grant CHE81-09064. We thank Prof. R.D. Simoni for the use of his liquid scintillation counter; T. Frohm, J. Pierret, B. L. Thompson and C. R. Wilkinson for field assistance, B. Marshall and F. Thomas for electron microscopy, and C. D. Christensen for logistical support. This is contribution number 347 from the Australian Institute of Marine Science and contribution number 3 from the Christensen Research Institute.

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