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BIOSYNTHETIC STUDIES OF MARINE LIPIDS—3[1]

EXPERIMENTAL DEMONSTRATION OF THE COURSE OF SIDE CHAIN EXTENSION IN MARINE STEROLS*

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Abstract—The Pacific sponge *Aplysina fistularis* was fed cholesterol-[4-^{14}C], (24*R*)-methyl-25-dehydrocholesterol-[26-^{14}C] (epicodisterol-[26-^{14}C]), (24*S*)-methyl-25-dehydrocholesterol-[26-^{14}C] (codisterol-[26-^{14}C]), and 24-methylenecholesterol-[28-^{14}C]. Only epicodisterol, which has the same stereochemistry at C-24 as (24*R*,25*S*)-24,26-dimethylcholesterol (aplysterol), was converted with high efficiency into (24*R*)-24,27-dimethyl-25-dehydrocholesterol (25-dehydroaplysterol). Further side chain extension [to *E*-(24*R*)-24,26,27-trimethyl-25-dehydrocholesterol (verongulasterol)] could also be demonstrated.

INTRODUCTION

With only very few exceptions, such as the protozoan *Tetrahymena pyriformis*,[2] eukaryotic life is impossible without sterols which are an essential part of their cell membranes. Cholesterol has evolved as the specific sterol required by higher animals.[3] Higher plants,[4] instead, have a preference for sterols alkylated in the 24-position of the side chain. In contrast, many lower invertebrates, especially those which are known, or thought to be unable, to synthesize sterols *de novo*,[5] have apparently not developed a need for a specific sterol. Complex mixtures of sterols are often isolated from individual species of filter and suspension feeders (e.g. sponges[6] and gorgonians[7]). Where cholesterol, the most common sterol in nature, is the main component of the marine organism's sterol mixture, it is likely that those sterols are simply derived from planktonic food that constitutes the diet of these animals.[8] Where algal symbionts (zooxanthellae) are present in the tissues of these invertebrates,[9] algal sterol such as gorgosterol[10] (**1v**), (24*S*)-methylcholesterol[11] (**1i**), or 24-methylenecholesterol[12] (**1f**) often predominate and are produced by the algal symbiont.[10,12,13]

Some sponges contain large quantities of $\Delta^{5,7}$ (**3**) and $\Delta^{5,7,9(11)}$ (**4**) sterols,[5,6] suggesting that these sponges need sterols with such unsaturated nuclei. Other sponges that seem to require sterols with uncommon skeletons are members of the family Axinellidae,[14] which convert dietary Δ^5 sterols (**1**) into A-norsterols (**5**),[15,16] *Axinella polypoides*, which has only 19-norsterols[17] (**6**) and a *Dysidea* sp. from the Black Sea, which has some normal sterols, but which has about a 20× higher level of sterol epidioxides (**7**).[18] In almost every instance, the major sterol type is characterized by a single or at best a few nuclei, but a plethora of side chains.

In contrast to the above sponges which seem to need sterols with a particular skeleton—irrespective of the structure of the side chain—there are now many examples known of sponges that have evolved a need for a main sterol with an unusual side chain, but with a normal (i.e. Δ^5-3β-hydroxyandrostene) skeleton. The first reported example was aplysterol (**1q**) ([24*R*,25*S*]-24,26-dimethylcholesterol)[19] which was found to be the main sterol of all sponges of the genus *Aplysina* (= *Verongia*) examined.[20–22] Aplysterol (**1q**) is unusual in that it is alkylated at C-26. Sterols with alkylation at one terminus or both termini (C-26, C-27) of the cholesterol side chain (e.g. **1s**) have been found only in sponges.[22,23]

The Italian group, which discovered aplysterol (**1q**), also attempted to determine its origin. Radiolabeled acetate and mevalonate were taken up by the sponge *Aplysina* (= *Verongia*) *aerophoba*, but no radioactivity ended up in the sterol mixture,[21] suggesting that the sponge is incapable of *de novo* synthesis of the sterol and hence, that aplysterol (**1q**) is most likely a modified dietary sterol.

The stereochemistry at the asymmetric centers in the side chain of aplysterol (**1q**) has been determined by X-ray analysis.[19] It has been suggested[24] that aplysterol (**1q**) is formed via alkylation at C-26 of epicodisterol ([24*R*]-methyl-25-dehydrocholesterol) (**1h**)—which has the same stereochemistry at C-24 as aplysterol (**1q**)—followed by reduction of the double bond in the side chain. This suggestion was supported by the isolation[22] from *Aplysina* (= *Verongia*) *cauliformis* of a compound which could be the primary product of alkylation of epicodisterol (**1h**), viz. (24*R*)-24,27-dimethyl-25-dehydrocholesterol (25-dehydroaplysterol)[22] (**1n**).

The postulated intermediacy[24] of epicodisterol (**1h**) in the biosynthesis of aplysterol (**1q**) creates another problem, viz. the problem of the origin of epicodisterol (**1h**) itself as there is no known planktonic source of this sterol. It has only been reported[25] to occur in *Aplysina* (= *Verongia*) *cauliformis* as a mixture with its (24*S*)-epimer, codisterol (**1g**), which is a well-known algal constituent.[26] Conceivably, a mixture of codisterol (**1g**)

* This paper is dedicated to one of the pioneers of sterol biosynthesis, Professor Edgar Lederer, on the occasion of his seventy-fifth birthday.

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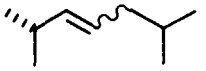
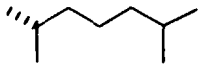
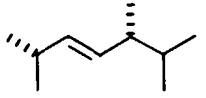
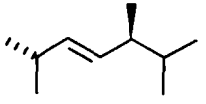
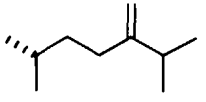
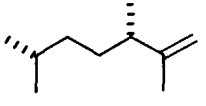
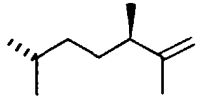
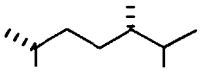
Table 1. Identified sterols of *Aplysina fistularis*

Systematic and trivial names[a]	Side chain	MW	Abundance (%)	RRT[b] HPLC	RRT[c] GC
E/Z-Cholesta-5,22-dien-3β-ol (**1a**)		384	2.5	0.76	0.93
Cholest-5-en-3β-ol (**1c**)		386	10.0	1.00	1.00
E-(24*R*)-Methylcholesta-5,22-dien-3β-ol (brassicasterol, **1d**)		398	3.0	0.91	1.13
E-(24*S*)-Methylcholesta-5,22-dien-3β-ol (crinosterol, **1e**)		398	2.5	0.87	1.13
24-Methylenecholesterol (**1f**)		398[d]	0.1	0.85	1.33
(24*S*)-Methylcholesta-5,25-dien-3β-ol (codisterol, **1g**)		398[d]	0.4	0.79	1.25
(24*R*)-Methylcholesta-5,25-dien-3β-ol (epicodisterol, **1h**)		398[d]	0.4	0.79	1.25
(24*S*)-Methylcholest-5-en-3β-ol (**1i**)		400[d]	3.0	1.08	1.33

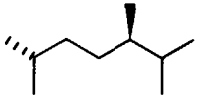
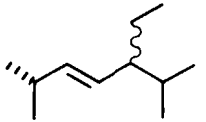
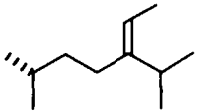
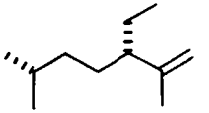
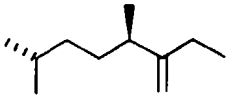
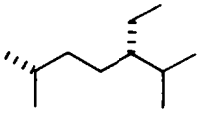
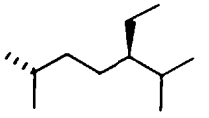
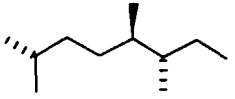
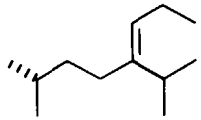
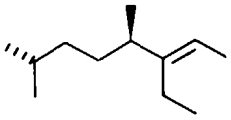
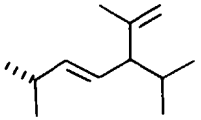
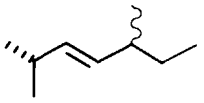
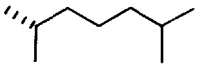
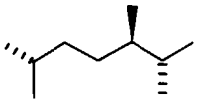
(24*R*)-Methylcholest-5-en-3β-ol (**1j**)		400[d]	2.0	1.08	1.33
(24*S*)-Ethylcholesta-5,22-dien-3β-ol (**1k**)		412	1.0	1.05	1.42
E-24-Ethylidenecholest-5-en-3β-ol (isofucosterol, **1l**)		412[d]	1.0	0.96	1.75
(24*S*)-Ethylcholesta-5,25-dien-3β-ol (clerosterol, **1m**)		412	0.5	0.89	1.60
(24*R*)-24,27-Dimethylcholesta-5,25-dien-3β-ol (25-dehydroaplysterol, **1n**)		412[d]	39.0	0.94	1.70
(24*S*)-Ethylcholest-5-en-3β-ol (**1o**)		414[d]	3.0	1.16	1.65
(24*R*)-Ethylcholest-5-en-3β-ol (**1p**)		414[d]	2.5	1.16	1.65
(24*R*,25*S*)-24,26-dimethylcholest-5-en-3β-ol (aplysterol) (**1q**)		414[d]	23.0	1.22	1.69

Table 1—*continued.*

Systematic and trivial names[a]	Side chain	MW	Abundance (%)	RRT:[b] HPLC	RRT:[c] GC
Z-24-n-Propylidenecholest-5-en-3β-ol (**1r**)		426	trace	1.03	1.97
E-(24*R*)-24,26,27-trimethylcholesta-5,25-dien-3β-ol (verongulasterol, **1s**)		426[d]	3.5	1.05	2.13
(24*S* [or *R*])-isopropenylcholesta-5,22-dien-3β-ol (**1t**)		426[d]	0.5	1.02	1.85
E-(24ξ)-27-Nor-24-methyl-5α-cholest-22-en-3β-ol (**2b**)		386	0.1	0.85	0.93
5α-Cholestan-3β-ol (**2c**)		388	1.5	1.08	1.00
(24*R*,25*S*)-24,26-Dimethylcholesta-5,7-dien-3β-ol (7-dehydroaplysterol, **3q**)		412[d]	0.5	1.06	2.00

[a] See Fig. 1 for structures of the nuclei (**1**, **2** and **3**).
[b] Whatman M9 10/50 ODS-2 column, eluent abs. MeOH, standard cholesterol.
[c] 3% SP2250 column, 260°, standard cholesterol.
[d] Structure checked by 360 MHz NMR; the other compounds were identified using their mass spectra and their RRT's in GC and HPLC.

and 24-epicodisterol (**1h**) might be formed by *in vivo* double bond migration of the common marine sterol 24-methylenecholesterol (**1f**).[27]

In this paper we report our work on the biosynthesis of aplysterol (**1q**) in the sponge *Aplysina fistularis* (= *Verongia thiona*) collected at one intertidal site in La Jolla, California. Four precursors were fed to the sponge: epicodisterol (**1h**), codisterol (**1g**), 24-methylenecholesterol (**1f**) and cholesterol (**1c**). As mentioned above, **1f** and **1h** are likely precursors of aplysterol (**1q**). Codisterol (**1g**) was included in order to determine whether the sponge is capable of epimerizing it into **1h**. Cholesterol was included to answer the question as to why there is relatively little cholesterol (**1c**) in *Aplysina* spp. Can all dietary sterols, including cholesterol (**1c**), be converted into aplysterol (**1q**)?

RESULTS

Before doing any feeding experiment a procedure for the isolation of pure sterols from the mixture using reverse phase HPLC (two different columns and different solvent mixtures) was developed (Experimental). The results of a complete analysis of the sterols of *Aplysina fistularis* are given in Table 1 and differ in two important respects from literature reports[20,28] of the sterol composition of *A. fistularis* (= *Verongia thiona*). First, we found that 25-dehydroaplysterol (**1n**), and not aplysterol (**1q**) was the main sterol of *A. fistularis*. One possible explanation for this different result might be that we worked with small (i.e. young) sponges (30–80 g wet weight). Second, we found that an important sterol had been misidentified:[20,28] *A. fistularis* from La Jolla, California, does not contain 24(28)-dehydroaplysterol (**1w**) but rather 25-dehydroaplysterol (**1n**). If one does not use NMR for identification it is easy to confuse these two sterols as they have exactly the same mobility in GC and reverse phase HPLC. The difference is only a peak of low intensity at *m/z* 328 in the mass spectrum of 25-dehydroaplysterol (**1n**), indicative of a Δ^{25} double bond.[24]

In addition, a novel sterol of MW 412 was isolated from *A. fistularis*. An $M^+ - 59$ peak (corresponding to loss of part of ring A)[29] in its mass spectrum indicated that the two degrees of unsaturation were in the ring system as a $\Delta^{5,7}$ diene chromophore.[29] The presence of the diene system was confirmed by two 1H multiplets in the NMR spectrum (δ 5.389, 5.576). The Me region of the NMR spectrum included three Me doublets and a Me triplet. Because of the source of the sterol this immediately suggested an aplysterol side chain (**q**). As shown in Table 2, there is good agreement between the shifts of the two Me doublets at highest field and the Me triplet in the spectra of aplysterol (**1q**) and the new sterol (**3q**). Thus the new sterol is (24*R*,25*S*)-24, 26-dimethylcholesta-5,7-dien-3β-ol (7-dehydroaplysterol, **3q**).

Kokke *et al.*[25] isolated from *Aplysina* (= *Verongia*) *cauliformis* 24-isopropenylcholesterol (**1t**) as a mixture of epimers at C-24. The epimers have very similar NMR spectra; the only difference is the shift of the C-18 Me group (δ 0.666 vs 0.672). We have now identified 24-isopropenylcholesterol (**1t**) as a minor sterol component in *Aplysina fistularis* but found it to be sterically pure (single signal at δ 0.666 for C-18 Me group).

The synthesis of the radiolabeled precursors for the feeding experiments was straightforward and essentially a variation of our earlier synthesis[22] of the C-24 epimers of 25-dehydroaplysterol (**1n**). Codisterol-[26-^{14}C] (**1g**) and epicodisterol-[26-^{14}C][30] (**1h**) were synthesized in the following manner. Ethyl 2-methylacetoacetate was alkylated with the *i*-methyl ether (**8x**) of 23-iodo-24-norcholest-5-en-3β-ol to give the protected keto-ester **8y**. Saponification followed by decarboxylation afforded the *i*-ether of 27-nor-ergost-5-en-3β-ol-25-one as a mixture of epimers at C-24 (**8za** + **8zb**). The radiolabel was then introduced by a Wittig reaction of these ketones (**8za**, **8zb**) with ^{14}C-labeled methylenetriphenylphosphorane. The resulting mixture of ^{14}C-labeled *i*-ethers (**8g**, **8h**) of codisterol and epicodisterol was separated by reverse phase HPLC. NMR data of codisterol (**1g**), epicodisterol (**1h**) and some synthetic intermediates are included in Table 1. 24-Methylenecholesterol-[28-^{14}C] was made by a Wittig reaction of the *i*-ether (**8zc**) of cholest-5-en-3β-ol-24-one with ^{14}C-labeled methylenetriphenylphosphorane followed by deprotection.

The results of the feeding experiments are given in Table 3. It should be mentioned that we did not try to purify labeled sterols with a shorter RT in HPLC than 25-dehydroaplysterol (**1n**) (see Table 1 and Experimental) because it would have been very difficult to obtain them completely free from recovered labeled precursors (**1f**, **1g**, or **1h**) which are also eluted faster than 25-dehydroaplysterol (**1n**).

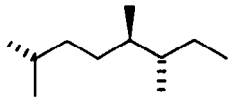
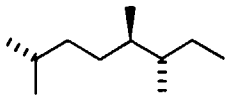
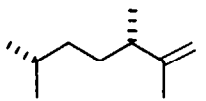
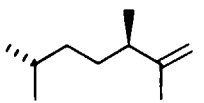
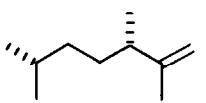
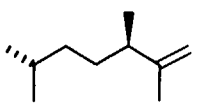
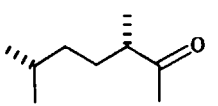
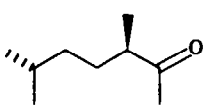
DISCUSSION

The incorporation experiments of sponges with radiolabeled sterols, carried out by the Naples group,[15–17,31] give a good idea of the percentages of recovery of radioactivity, and of the conversion of a precursor that constitutes a successful experiment. Thus they recovered 20.4% of the radioactivity after a feeding experiment[15] of *Axinella verrucosa* with cholesterol-[4-^{14}C] (**1c**); 66% of the recovered radioactivity was in 3β-hydroxymethyl-A-nor-5α-cholestane (**5c**). In this manner they obtained convincing evidence that *A. verrucosa* converts Δ^5 sterols (**1**) into A-norsterols (**5**). In contrast, when fucosterol-[7,7-$^3H_2$] (**1u**) was fed[31] to *Calyx niceaensis*, 56% of the radioactivity was recovered, but only 2.7% of the recovered radioactivity was in the target compound calysterol (**1ze**), suggesting that fucosterol (**1u**) is not a good precursor of calysterol (**1ze**), or that sterol metabolism in *C. niceaeneis* is slow, or both.

The data in Table 3 clearly demonstrate that epicodisterol (**1h**) is an excellent precursor of 25-dehydroaplysterol (**1n**) since 76.8% of the recovered radioactivity was found in 25-dehydroaplysterol (**1n**) after an incorporation period of 30 days. The specific activities of aplysterol (**1q**) and verongulasterol (**1s**) of 12,800 and 19,600 dpm/mg, respectively, indicate that further alkylation of 25-dehydroaplysterol (**1n**) to give verongulasterol (**1s**) proceeds slightly faster than conversion of 25-dehydroaplysterol (**1n**) into aplysterol (**1q**). A side reaction is biological reduction of epicodisterol (**1h**) to 24-methylcholesterol (**1i, j**).

The results of the feeding experiments with codisterol (**1g**) (Table 3) indicate that *A. fistularis* is unable to epimerize codisterol (**1g**) at C-24. It is true that a low percentage (2.3%) of the recovered radioactivity was found in the sample of 25-dehydroaplysterol (**1n**), but

Table 2. 360 MHz ^{1}H-NMR data for aplysterol (**1q**), 7-dehydroaplysterol (**3q**), codisterol (**1g**), epicodisterol (**1h**), and some synthetic intermediates (**8g**, **h**, **za**, **zb**) ($CDCl_3$, internal standard $CDCl_3$, shifts are δ values, splitting constants in Hz)

Compound	Side chain	C18-H	C19-H	C21-H	C26-H	C27-H	C28-H	C29-H
1q		0.681 (s)	1.008 (s)	0.909 (d) J 6.5	—	0.798 (d)[a] J 6.6	0.812 (d)[a] J 6.7	0.861 (t) J 7.3
3q		0.620 (s)	0.944 (s)	0.933 (d) J 6.5	—	0.803 (d)[a] J 6.6	0.813 (d)[a] J 6.6	0.862 (t) J 7.3
1g		0.670 (s)	1.004 (s)	0.909 (d) J 6.6	4.658 (bs)	1.635 (s)	0.990 (d) J 6.9	—
1h		0.672 (s)	1.005 (s)	0.914 (d) J 6.5	4.654 (bs)	1.649 (s)	0.984 (d) J 6.9	—
8g		0.705 (s)	1.017 (s)	0.903 (d) J 6.5	4.659 (bs)	1.635 (bs)	0.991 (d) J 6.9	—
8h		0.708 (s)	1.018 (s)	0.907 (d) J 6.5	4.655 (bs)	1.651 (bs)	0.985 (d) J 6.9	—
8za		0.703 (s)	1.015 (s)	0.915 (d) J 6.5	2.121 (s)	—	1.074 (d) J 6.9	—
8zb		0.705 (s)	1.015 (s)	0.912 (d) J 6.5	2.126 (s)	—	1.062 (d) J 6.9	—

[a] These assignments may be interchanged.

this radioactive material almost certainly originated from epicodisterol-[26-^{14}C] (**1h**) which was present in codisterol-[26-^{14}C] (**1g**) as an impurity (*ca* 3%). This also explains the low specific activity of the aplysterol (**1q**) and verongulasterol (**1s**) samples. Thus, the only reaction which codisterol (**1g**) undergoes is reduction of the 25(26)-double bond since 14.8% of the recovered radioactivity was found in the 24-methylcholesterol (**1i, j**) sample.

The specific activities of sterol samples isolated after an incorporation experiment with 24-methylene-cholesterol-[28-^{14}C] (**1f**) indicate that double bond isomerization to give a codisterol/epicodisterol (**1g, h**) mixture followed by alkylation does not occur to a significant extent as the 25-dehydroaplysterol (**1n**) sample is almost cold (1200 dpm/mg). Reduction of the 24(28) double bond does occur. However, from a comparison of the specific activities it follows that alkylation at C-28 to give fucosterol (**1l**) and subsequent further alkylation to 24-isopropenylcholesterol (**1t**) or reduction to 24-ethylcholesterol (**1o, p**) proceed faster than the formation of 24-methylcholesterol (**1i, j**).

Cholesterol-[4-^{14}C] (**1c**) is not a precursor of aplysterol (**1q**); *A. fistularis* only converts this compound into 5α-cholestanol (**3c**). A filter or suspension feeder that is free of algal symbionts and that does not synthesize sterols or modify dietary sterols should have cholesterol (**1c**) as its main sterol. *Lophogorgia* spp. from Pacific Mexico are typical examples.[8] But there is relatively little cholesterol (**1c**) in *A. fistularis* (Table 1). Perhaps this sponge has a mechanism to selectively eliminate specific sterols, including cholesterol (**1c**), as recent work suggests that *A. fistularis* is capable of exuding some natural products into sea water.[32,33]

Our results leave the problem of the origin of epicodisterol (**1h**) unsolved. Might it be that symbiotic micro-organisms living in the sponge[34] produce desmosterol (**1zd**) or epicodisterol (**1h**) which the sponge then converts into 25-dehydroaplysterol (**1n**)? This explanation is in conflict with the published observation[21] that mevalonate fed to a *Verongia* (= *Aplysina*) sp. is not converted into sterols.

EXPERIMENTAL

General. For separation of sterol mixtures we used Waters HPLC equipment (M6000, M6000A and M45 pumps, U6K injector, R401 and R403 differential refractometers) and also a Rheodyne model 7120 and a Valco model CV-6-UHPa-N60

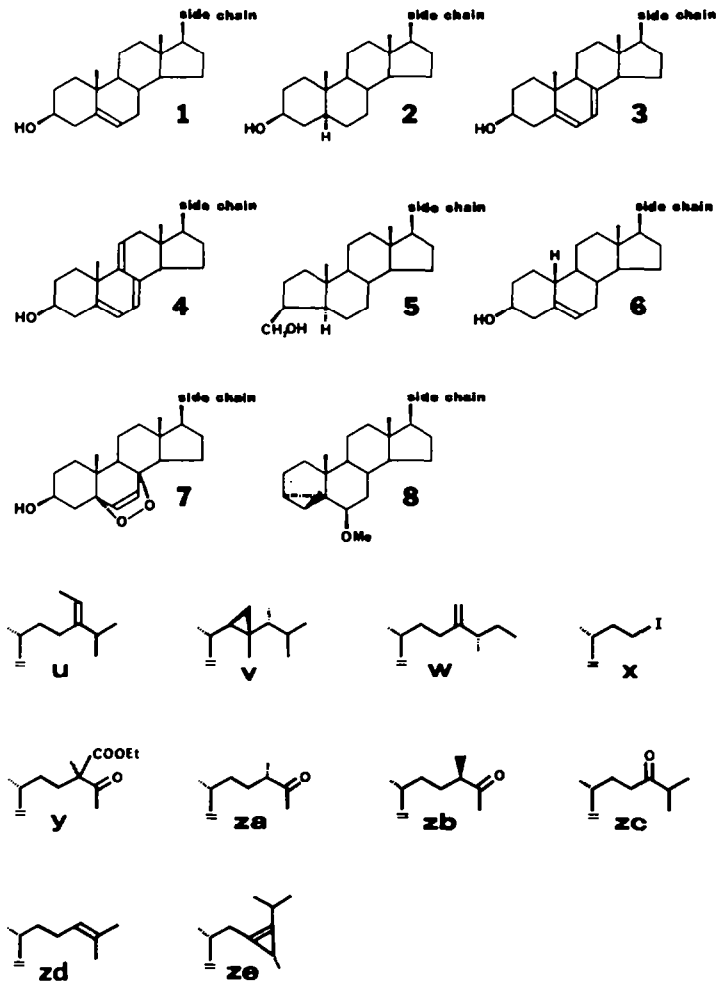
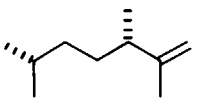
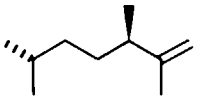
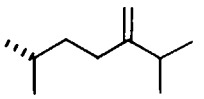
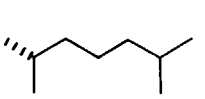
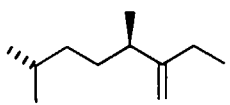
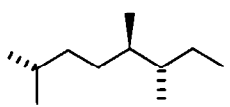
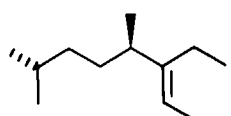


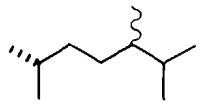
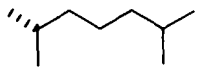
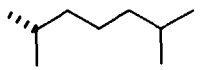
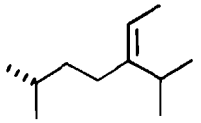
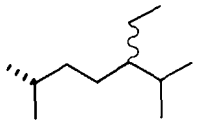
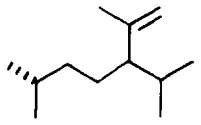
Fig. 1. Structures of the sterol nuclei and of selected side chains. The structures of side chains a–t are given in Table 1.

Table 3. Feeding experiments with *Apylsina fistularis*

Precursor	 Codisterol-[26-^{14}C] (**1g**)		 Epicodisterol-[26-^{14}C] (**1h**)		 24-Methylenecholesterol-[28-^{14}C] (**1f**)	 Cholesterol-[4-^{14}C] (**1c**)
Incubation period (days)	10	30	10	30	30	30
Sponge, wet weight (g)	30	36	32	83	45	45
Total fed (dpm × 10^{-6})	41.4	42.2	38.4	46.2	46.2	110.0
Isolated total free sterols	20.6	40.2	21.0	95.8	51.0	32.4
(mg, dpm/mg × 10^{-5})	1.68	1.10	1.87	0.67	1.01	6.70
Recovered radioactivity (% of total fed)	8.4	10.5	10.2	13.9	11.2	19.7

The following lines in this table give incorporation (in dpm/mg, % of recovered radioactivity, and % of radioactivity fed) in the sterols whose side chain is shown in the first column.

 **1n**	1600[b] 0.4 0.03	6400[b] 2.3 0.24	6000 12.5 1.28	132,000 76.8 10.7	1200 0.5 0.06	cold
 **1g**	cold	800[b] 0.2 0.02	1200 0.2 0.02	12,800 4.4 0.6	cold	cold
 **1s**	—	900[b] 0.03 0.003	2000 0.04 0.004	19,600 1.1 0.15	1100 0.04 0.004	—

 **1i, j**	58,000 1.7 0.15	325,000 14.8 1.55	15,000 0.4 0.04	40,000 3.0 0.4	12,000 0.6 0.07		cold
 **1c**	cold	cold	cold	cold	cold		6.3×10^6 [c]
 **2c**	cold	cold	cold	cold	cold		900,000 2.0 0.4
 **1l**	—	cold	—	cold	25,000 0.3 0.03		—
 **1o, p**	cold	cold	cold	cold	96,500 5.3 0.6		cold
 **1t**	—	—	—	cold	13,500 0.07 0.008		—

[a] "Cold", dpm not significantly different from background radiation; —, not determined.
[b] Probably caused by an alkylation product of epicodisterol (**1**) which was present as an impurity (*ca* 3%) in precursor.
[c] Recovered starting material.

injector. The columns were Whatman M9 10/50 ODS-2 or Whatman M40 10/50 ODS-2 for the first separation step, and two Altex Ultrasphere ODS columns (5 μm, 10 mm i.d. × 25 cm) for further purification. Note that the point of injection, and not the beginning of the solvent peak, was used to calculate (relative) retention times in HPLC. 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. × 1.80 m, 260°). Preparative GC was performed with the same instrument using a 3% OV25 column (6 mm i.d. × 1.80 m, 265°) and an effluent splitter. Low resolution mass spectra were recorded with a Varian MAT-44 mass spectrometer (probe or capillary SE54 column, 9 m, 260°) or with a MAT-711 mass spectrometer (probe); 360 MHz NMR spectra on a Brucker HXS-360 NMR instrument; and 300 MHz NMR spectra on an instrument consisting of a Nicolet NIC 1180 data system and an Oxford Instruments magnet. Radioactivity was determined with a Beckman LS7500 liquid scintillation counter.

Collection of sponge. Aplysina fistularis Pallas (1766) (= *Verongia thiona, V. aurea, Spongia fulva*) was collected intertidally at Casa Cove, La Jolla, California, at 0 to −5 m (depths corrected for tidal height). *A. fistularis* occurs in at least two forms which differ in their natural products chemistry.[35] We used the shallow water form (< 5 m) which contains a 9 : 1 mixture of aerothionin and homoaerothionin.[35] Specimens were collected not more than 2 hr before the start of an experiment, and were transferred to the La Jolla laboratory in cool, aerated sea water.

Incorporation experiments. The radiolabeled precursor was dissolved in a minimum amount (< 1 ml) of ether and then poured into a 1 gal glass jar containing 2 l of unfiltered sea water. The contents of the jar were continuously aerated using a standard aquarium pump with a sintered glass inlet and maintained at ambient ocean surface temp (~ 13°) in a cold room with a 12 hr light cycle. A single sponge, weighing approx. 30–40 g (wet weight), which was still attached to its rock substratum, was transferred to the jar after 1 hr and maintained for 2 days.[36] After this time a continuous flow of surface sea water was added to the jar for up to 28 days. All sponges harvested after being maintained in the lab for 30 days looked very healthy. Two experiments (with **1g** and **1h**) had to be terminated early (after 12 days, Table 3) because of deterioration of the sponges caused by problems with the running sea water supply. Incorporation experiments were terminated by placing the sponges in polyethylene containers with EtOH in which they were shipped to Stanford University.

Isolation of sterols. The alcohol was decanted and the sponge was homogenized in 1 : 1 $CHCl_3$–MeOH in a Waring Blender. The homogenate was filtered, the material on the filter was again homogenized in $CHCl_3$–MeOH and then filtered, and the process was repeated. The filtrates and the decantate were combined, the solvent evaporated, and the residue partitioned between $CHCl_3$ and water. Sterols were isolated from the residue of the $CHCl_3$ layer by silica gel column chromatography using hexane–ether mixtures. The crude, free sterols were saponified and the neutral unsaponifiables further purified over a silica gel column. In a representative experiment, a sponge of 83 g wet weight gave 1.1 g of extract from which 95.8 mg of pure sterols were obtained.

Separation and identification of the free sterols of A. fistularis. Most of the components of the mixture (Table 1) were identified using 360 MHz NMR; the remaining compounds were identified on the basis of their mass spectra and their relative retention times (RRT) both in GC and reverse phase HPLC.

Fractionation of the total free sterols of *A. fistularis* by HPLC using an ODS-2 column and abs MeOH afforded eight fractions as shown in Fig. 2. For further purification Altex columns were used together with abs MeOH, or CH_3CN–MeOH–EtOAc 22 : 9 : 9 ("solvent mixture B"), or THF–MeOH–water 3 : 3 : 1 ("solvent mixture C"). It should be stated that the purification of any radioactive compound was

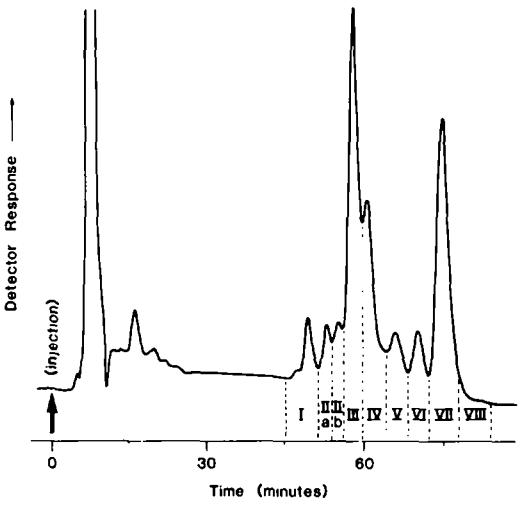


Fig. 2. HPLC trace of the free sterols of *A. fistularis* (Whatman M9 10/50 ODS-2 column, absolute MeOH, flow 3.5 ml/min, injection volume 2.5 ml (2 mg of sterol mixture/ml)).

continued until its specific radioactivity remained constant after at least three successive purifications with different solvents, after it was pure by GC and HPLC. Purification was also stopped when a fraction was cold, i.e. when its specific activity was less than 250 dpm/mg (= 20 × background). In the sequel we will describe in detail the work-up of the above eight ODS-2 fractions (Fig. 2).

Fraction I showed two peaks in GC. The component with the shorter RT was **1a**; after separation by preparative GC the second peak was shown by 360 MHz NMR to be a 1 : 1 mixture of **1g** and **1h**.

Fraction IIa contained **1e** and traces of **1f**.

Fraction IIb contained **1d, 1m,** and also some **1n** because of contamination with the next fraction.

Fraction III. **1n** was the main component; minor components were **1c** and **1l**. This fraction was re-chromatographed over the ODS-2 column (MeOH) and the tail of the peak was collected separately. The first part of the peak was practically pure **1n**, which was further purified by repeated rechromatography over Altex columns: (i) MeOH, flow 3.0 ml/min, RT 52.2 min (cholesterol : RT 53.0 min); (ii) mixture B, flow 3.0 ml/min, RT 46 min (cholesterol : RT 50.0 min); (iii) MeOH. The above tail fraction was passed through Altex columns (mixture B, flow 3.0 ml/min) to separate cholesterol (RT 50 min) and a small amount of **1t** (RT 51.2 min) from the main peak (RT 46.5 min) caused by a mixture of **1n** and **1l** (ratio of **1n** : **1l** = 3.5 : 1). Isofucosterol (**1l**) was isolated from this mixture by means of two successive runs through Altex columns (MeOH, flow 2.5 ml/min; **1l**: RT 72 min; **1n**: RT 70.2 min). Further purification of **1l** using Altex columns and solvent mixture C, and then again MeOH was done after the feeding experiment with **1f**. It should be noted that **1u** and **1l** cannot be separated using Altex columns with MeOH, or solvent mixture B or C. We have shown by 360 MHz NMR that *A. fistularis* contained only **1l**.

Fraction IV consisted mainly of **1c** with minor amounts of **1n, 1t, 1k** and **1s**. HPLC over Altex columns (MeOH, flow 3.0 ml/min) divided Fraction IV into five subfractions: IVa (**1n**, RT 52.2 min), IVb (**1c**, RT 53 min), IVc (**1t**, RT 54.5 min), IVd (**1k**, RT 56.5 min) and IVe (**1s**, RT 58.5 min). Only after the feeding experiment with cholesterol-[4-^{14}C] was the **1c** fraction IVb further purified using Altex columns with (i) mixture B, (ii) mixture C, and (iii) MeOH. The material of subfraction IVc (95% pure **1t**) was rechromatographed over Altex columns following the sequence (i) MeOH, (ii) mixture B, and (iii) mixture C. Subfractions IVd and IVe were worked up in the same manner as subfraction IVc.

Fraction V of the ODS-2 separation gave four peaks in GC with RRT 1.0 (cholestanol [2c]), 1.33 (1i, j), 2.00 (3q) and 2.13 (1o). Their ratio was 2.9:1.8. These sterols could not be separated over Altex columns when MeOH was used as eluent, but a baseline separation was obtained with solvent mixture B (flow 3.0 ml/min). The compounds were eluted in the order 3q (RT 52 min), 1o (RT 55 min), 1i, j (RT 57.5 min), 2c (RT 61 min). Each compound was further rechromatographed over Altex columns using again solvent mixture B, then mixture C, and finally MeOH.

Fraction VI contained 1o, p as an epimeric mixture. It was purified to constant specific activity using Altex columns and (i) MeOH, (ii) solvent mixture B, and (iii) solvent mixture C.

Fraction VII. Aplysterol (1q) was essentially the only constituent. It was purified in the same manner as 1o, p (Fraction VI).

Preparation of ketoester 8y. NaOH (0.24 g, 50% in oil, 5 mmol) was placed in a 3-necked flask and the oil was removed by washing the suspension twice with anhyd ether. The base was suspended in 25 ml of anhyd DMF and 0.72 ml (5 mmol) of ethyl 2-methylacetoacetate were added dropwise to the stirred suspension. After cessation of H_2 evolution, 800 mg (1.7 mmol) of the iodide[37] 8x dissolved in ether (3 ml) was slowly dropped into the flask and the mixture heated in an oil bath at 100–110° for 20 min. After cooling, the mixture was poured into 100 ml of ice–water and extracted with ether (4 × 20 ml). The organic phase was washed with water, 1% HCl, water, 1% $NaHCO_3$ and water. After drying ($MgSO_4$) and evaporation of the solvent the crude 8y was purified over a silica gel column with hexane–ether 8:1 as an eluent to yield 585 mg (71%) of it as an oil. MS (MAT-44), 70 eV (probe), *m/z* (rel. int.): 486 (M^+, 8), 471 (9), 454 (12), 431 (15), 255 (11), 213 (15), 55 (100).

3α,5-Cyclo-6β-methoxy-24R-methyl-27-norcholestan-25-one (8zb) *and its 24S epimer* 8za. The ketoester 8y (500 mg) was saponified and decarboxylated by refluxing 1.5 hr with 100 ml of EtOH and 50 ml of 6 N KOH. The mixture was concentrated to one-half of its volume, 50 ml of water was added and it was then extracted with ether (4 × 25 ml). The organic layer was washed with water, 2% HCl, water, 1% $NaHCO_3$ and water. After drying and evaporation of the solvent 440 mg (88%) of a 50:50 mixture of the epimeric ketones 8za and 8zb was obtained which could be separated by HPLC on Altex columns using MeOH with 8% water as an eluent. The NMR data for both ketones are included in Table 3. MS (MAT-711), 70 eV (probe), *m/z* (rel. int.): 414 (M^+, 73), 399 (39), 382 (100), 359 (58), 261 (17), 255 (23), 213 (16), 43 (44).

Preparation of methyl-[^{14}C]-triphenylphosphonium iodide. Six break-seal ampoules, each containing 1 mCi of [^{14}C]-methyl iodide (specific activity 58 mCi/mmol) were cooled in Dry Ice, the seals broken and 15 mg of triphenylphosphine dissolved in 0.3 ml of anhyd benzene quickly introduced into each vial. After sealing, the ampoules were kept at room temp for 3 days, the supernatant was pipetted off and the crystals were washed with benzene and then taken out of the vials dissolved in absolute MeOH (3 × 0.5 ml). The methanolic solns from the six ampoules were combined and the solvent blown off with anhyd N_2. The residue was kept in a vacuum desiccator for 24 hr, the vitreous material was covered with benzene which after occasional stirring induced the crystallization of the salt. The supernatant benzene was decanted and the solid washed twice with anhyd ether and kept in the desiccator under vacuum 48 hr. Yield 46.4 mg (97% based on MeI), specific activity 56 mCi/mmol).

Codisterol-[26-^{14}C] and epicodisterol-[26-^{14}C] 3-methyl ethers (8g *and* 8h). [$Ph_3P^{14}CH_3$]I (13.9 mg, 34.2 μmol) was placed in a 4 ml vial with stirring bar and then sealed with a serum stopper. After flushing the vial with anhyd N_2 by means of inlet and outlet needles, 0.5 ml of ether (freshly distilled from LAH and kept over Na) followed by 28 μl (67 μmol) of 2.4 M soln of n-BuLi in hexane were introduced. After 3 hr at room temp 30.9 mg (74 μmol) of the epimeric mixture of 8za and 8zb (1:1) dissolved in ether (1.0 ml) was introduced into the reaction vial which was kept at room temp for 3 days with continuous magnetic stirring. The solvent was then blown off and the residue transferred to a small silica gel column (4 g) with hexane–ether 9:1 (3 × 1 ml); the same solvent mixture was used to elute the ethers 8g and 8h. This epimeric mixture could be separated by reverse phase HPLC [Altex columns, MeOH–water 98:2, flow 3.0 ml/min, injection volume 0.5 ml (3–4 mg/ml)]. Codisterol-[26-^{14}C] 3-methyl ether (8g) has RT 144 min, epicodisterol-[26-^{14}C] 3-methyl ether (8h) has RT 146 min.

By rechromatographing each peak, 8g and 8h were obtained 97% pure as determined in a parallel separation using "cold" material (Purity calculated from the 18-Me signal ratio in the 360 MHz NMR spectrum (C_6D_6) of the free sterols, epicodisterol (1g) C-18 δ 0.655, codisterol (1h) C-18 δ 0.668 ppm).

Codisterol-[26-^{14}C] (1g). To 8g (2.96 mg) dissolved in dioxane (2 ml) was added 0.4 ml of 5% aqueous soln of *p*-toluenesulfonic acid and the mixture was refluxed for 1.5 hr. After cooling, the soln was diluted with ether (15 ml), washed with water (2 × 2 ml), 2% $NaHCO_3$ (3 ml), water and dried ($MgSO_4$). After solvent evaporation, the residue was dissolved in MeOH (1 ml) and chromatographed through Altex columns with MeOH as eluent (flow rate 3.0 ml/min, RT 44.5 min) to yield 2.5 mg of labeled sterol (18% based on the phosphonium salt). Specific activity 56 mCi/mmol. 300 MHz NMR (C_6D_6) (assignment δ [multiplicity] *J* in Hz): C18-H 0.668 (s), C19-H 0.945 (s), C21-H 0.987 (d) 6.5, C27-H 1.634 (br s), C28-H 1.035 (d) 6.9, C26-H 4.818 (d) 1.35 and 4.831 (br s).

Epicodisterol-[26-^{14}C] (1h). By the same procedure as above and from 3.28 mg of 8h, 2.7 mg of labeled 1h was obtained after HPLC purification (flow rate 3.0 ml/min, RT 45 min). Yield 20%. Specific activity 57 mCi/mmol. 300 MHz NMR (C_6D_6) (assignment δ [multiplicity] *J* in Hz): C18-H 0.655 (s), C19-H 0.944 (s), C21-H 0.991 (d) 6.6, C27-H 1.647 (br s), C28-H 1.042 (d) 6.9; C-26-H 4.785 (d) 1.2 and 4.798 (br s).

24-Ketocholesterol 3-methyl ether (8ac). Natural 1f isolated by preparative argentic TLC[38] of the acetates of the sterol mixture from soft corals from Palau where it is the main sterol[1,2] was treated with tosyl chloride in pyridine followed by KOAc in anhyd MeOH in the usual fashion[39] to yield 8f as an oil. MS of 8f (MAT-44), 70 eV (probe), *m/z* (rel. int.): 412 (M^+, 1.5), 397 (2), 380 (2), 357 (5), 328 (3), 313 (4), 296 (4), 285 (6), 253 (11), 227 (5), 213 (6), 201 (4), 55 (100). The protected olefin 8f (60 mg) was dissolved in CH_2Cl_2 (10 ml), two drops of pyridine were added and the solution was ozonized at −70°. After the usual work up, the ketone 8ac was purified by column chromatography on silica gel using hexane–ethyl acetate 93:7 as eluent and further purified by HPLC (ODS-2, MeOH) to yield 33 mg (55%) of pure 8ac, m.p. 93–95°. 300 MHz NMR ($CDCl_3$) (δ [multiplicity, number of protons, *J*]): 3.320 (s, 3H), 2.768 (t, 1H, 2.8), 1.089 (d, 6H, 6.8), 1.017 (s, 3H), 0.907 (d, 3H, 6.5), 0.710 (s, 3H), 0.644 (t, 1H, 5.0) and 0.428 (dd, 1H, 8.0 and 5.0). MS (MAT-44), 70 eV, probe, *m/z* (rel. int.): 414 (M^+, 17), 399 (12), 382 (23), 359 (25), 261 (5), 255 (16), 213 (19), 71 (100).

24-Methylenecholesterol-[28-^{14}C] (1f). Using the procedure described for preparing labeled 1g but with 8ac instead of 8za and 8zb for the Wittig reaction, 7.0 mg of the 8f was obtained from 29 mg of 8ac. After removing the protecting group and final purification of the free sterol by HPLC (Altex, MeOH, flow rate 3 ml/min, RT 46.3 min), 6.0 mg of labeled 1f were obtained. Yield 45% (based on the amount of phosphonium salt employed). Specific activity 54 mCi/mmol.

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REFERENCES AND NOTES

[1] For their first two publications in this series, see: Y. M. Sheikh and C. Djerassi, *J. Chem. Soc. Chem. Commun.* 1057 (1976); Y. M. Sheikh and C. Djerassi, *Tetrahedron Lett.* 3111 (1977).

[2] R. L. Conner, J. R. Landrey, J. M. Joseph and W. R. Nes, *Lipids* **13**, 692 (1978) and refs therein.

[3] W. R. Nes and W. D. Nes, *Lipids in Evolution*. Plenum Press, New York (1980).

[4] W. R. Nes, *Advances in Lipid Research* (Edited by R. Paoletti and D. Kritchevsky), Vol. 15, p. 233 (1977); L. J. Goad, *Lipids and Lipid Polymers in Higher Plants* (Edited by M. Tevini and H. K. Lichtenthaler), p. 146. Springer, Berlin (1977).

[5] L. J. Goad, *Pure Appl. Chem.* **53**, 837 (1981).

[6] As many as 74 sterols have been isolated from a single sponge. See: T. Itoh, D. Sica and C. Djerassi, *J. Chem. Soc. Perkin Trans. 1* 147 (1983).

[7] S. Popov, R. M. K. Carlson, A. Wegmann and C. Djerassi, *Steroids* **28**, 699 (1976).

[8] W. C. M. C. Kokke, W. Fenical, L. Bohlin and C. Djerassi, *Comp. Biochem. Physiol.* **68B**, 281 (1981).

[9] D. L. Taylor, *Symbiosis in the Sea* (Edited by W. B. Vernberg), p. 245. University of South Carolina Press, Columbia (1972); D. L. Taylor, *Ann. Rev. Microbiol.* **27**, 171 (1973); D. L. Taylor, *Symp. Soc. Exp. Biol.* **29**, 267 (1975); R. K. Trench, *Ann. Rev. Plant Physiol.* **30**, 485 (1979).

[10] N. W. Withers, W. C. M. C. Kokke, W. Fenical and C. Djerassi, *Proc. Nat. Acad. Sci. U.S.A.* **79**, 3764, 6390 (1982).

[11] A. Kelecom and A. M. Solé-Cava, *Comp. Biochem. Physiol.* **72B**, 677 (1982).

[12] W. C. M. C. Kokke, unpublished results.

[13] C. Duque, National University of Colombia, Bogota, unpublished results.

[14] For references, see: M. L. Eggersdorfer, W. C. M. C. Kokke, C. W. Crandell, J. E. Hochlowski and C. Djerassi, *J. Org. Chem.* **47**, 5304 (1982); L. Bohlin, U. Sjöstrand, G. Sodano and C. Djerassi, *J. Org. Chem.* **47**, 5309 (1982).

[15] M. De Rosa, L. Minale and G. Sodano, *Experientia* **31**, 408 (1975).

[16] M. De Rosa, L. Minale and G. Sodano, *Experientia* **32**, 1112 (1976); L. Bibolino, L. Minale and G. Sodano, *J. Chem. Soc. Chem. Commun.* 524 (1978); A. De Stefano and G. Sodano, *Experientia* **36**, 630 (1980).

[17] L. Minale and G. Sodano, *J. Chem. Soc. Perkin Trans 1* 1888 (1977); M. De Rosa, L. Minale and G. Sodano, *Experientia* **31**, 758 (1975); L. Minale, D. Perrico and G. Sodano, *Experientia* **35**, 296 (1979).

[18] S. Popov and N. L. Marekov, Institute of Organic Chemistry, Bulgarian Academy of Sciences, Sofia, private communication.

[19] P. De Luca, M. De Rosa, L. Minale and G. Sodano, *J. Chem. Soc. Trans 1* **17**, 2123 (1977); P. De Luca, M. De Rosa, L., Minale, R. Puliti and G. Sodano, *J. Chem. Soc. Chem. Commun.* 825 (1973).

[20] M. De Rosa, L. Minale and G. Sodano, *Comp. Biochem. Physiol.* **46B**, 823 (1973).

[21] L. Minale and G. Sodano, *Marine Natural Products Chemistry* (Edited by D. J. Faulkner and W. H. Fenical), p. 87. Plenum Press, New York (1977).

[22] W. C. M. C. Kokke, W. H. Fenical, C. S. Pak and C. Djerassi, *Tetrahedron Lett.* 4373 (1978).

[23] For examples see: C. Djerassi, *Pure Appl. Chem.* **53**, 873 (1981); L. N. Li and C. Djerassi, *J. Am. Chem. Soc.* **103**, 3606 (1981); L. N. Li, U. Sjöstrand and C. Djerassi, *J. Org. Chem.* **46**, 3867 (1981); L. N. Li and C. Djerassi, *Tetrahedron Lett.* 4639 (1981).

[24] C. Djerassi, N. Theobald, W. C. M. C. Kokke, C. S. Pak and R. M. K. Carlson, *Pure Appl. Chem.* **51**, 1815 (1979).

[25] W. C. M. C. Kokke, C. S. Pak, W. Fenical and C. Djerassi, *Helv. Chim. Acta* **62**, 1310 (1979).

[26] I. Rubinstein and L. J. Goad, *Phytochemistry* **13**, 481 (1974).

[27] 24-Methylenecholesterol is often a major sterol of an ecologically important group of unicellular marine algae, the diatoms. See: M. Kates, P. Tremblay, R. Anderson and B. E. Volcani, *Lipids* **13**, 34 (1978); J. L. Boutry, M. Barbier and M. Ricard, *J. Exp. Mar. Biol. Ecol.* **21**, 69 (1976).

[28] R. D. Walkup, G. C. Jamieson, M. R. Ratcliff and C. Djerassi, *Lipids* **16**, 631 (1981).

[29] A. A. L. Gunatilaka, Y. Gopichand, F. J. Schmitz and C. Djerassi, *J. Org. Chem.* **46**, 3860 (1981).

[30] S. Maruyama, Y. Fujimoto, M. Morasaki and N. Ikekawa, *Tetrahedron Lett.* 1701 (1982), reported the synthesis of a mixture of codisterol and epicodisterol, but they did not separate these compounds.

[31] L. Minale, R. Riccio, O. Scalona, G. Sodano, E. Fattorusso, S. Magno, L. Mayol and C. Santacroce, *Experientia* **33**, 1550 (1977).

[32] R. P. Walker, Ph.D. Thesis, University of California, San Diego (1981); J. E. Thompson, Ph.D. Thesis, University of California, San Diego (1983).

[33] J. E. Thompson, K. D. Barrow and D. J. Faulkner, *Acta Zoologica* (Stockholm) **64**, 199 (1983).

[34] P. R. Bergquist, *Sponges*, pp. 197, 198. University of California Press, Berkeley and Los Angeles (1978).

[35] R. J. Andersen and D. J. Faulkner, *Tetrahedron Lett.* 1175 (1973).

[36] A. A. Tymiak and K. L. Rinehart, *J. Am. Chem. Soc.* **103**, 6763 (1981).

[37] N. Theobald, R. J. Wells and C. Djerassi, *J. Am. Chem. Soc.* **100**, 7677 (1978).

[38] Ref. 25 (note 10).