Phytochemistry, Vol. 21, No. 4, pp. 881–887, 1982.
Printed in Great Britain.

0031-9422/82/040881-07$03.00/0

NOVEL DINOFLAGELLATE 4α-METHYLATED STEROLS FROM FOUR CARIBBEAN GORGONIANS*

W. C. M. C. KOKKE,†‡ LARS BOHLIN,† WILLIAM FENICAL† and CARL DJERASSI‡§

†Institute of Marine Resources, Scripps Institution of Oceanography, La Jolla, CA 92093, U.S.A.; ‡Department of Chemistry, Stanford University, Stanford, CA 94305, U.S.A.

(*Received* 8 *May* 1981)

Key Word Index—*Briareum asbestinum*; *Gorgonia mariae*; *Muriceopsis flavida*; *Pseudoplexaura wagenaari*; *Zooxanthella microadriatica*; Dinophyceae; Gorgonaceae; marine sterols; symbiosis.

Abstract—Fourteen 4α-methyl sterols have been isolated from the gorgonians *Briareum asbestinum, Gorgonia mariae, Muriceopsis flavida* and *Pseudoplexaura wagenaari*, including the following five new sterols: 4α-methyl-24-methylene-5α-cholestan-3β-ol, (24*R*)-4α, 24-dimethyl-5α-cholesta-7,22-dien-3β-ol, 4α,24*S*(or 23ξ)-dimethyl-5α-cholest-7-en-3β-ol, (22*E*, 24*R*)-4α,23,24-trimethyl-5α-cholesta-7,22-dien-3β-ol and (24*R*)-4α,24-dimethyl-5α-cholesta-8(14),22-dien-3β-ol. There is strong evidence that these 4α-methyl sterols are synthesized by the algal (dinoflagellate) symbionts (zooxanthellae) of the gorgonians. It is suggested that analysis of 4Δ-methyl sterol mixtures isolated from a zooxanthellae-bearing invertebrate, collected in several different geographic locations, might give information on the specificity of the symbiotic association between a given animal species and a particular strain of zooxanthellae.

INTRODUCTION

Dinosterol (**1g**) was one of the first new sterols isolated from a cultured, unicellular marine alga [1]. It was found in *Gonyaulax polyedra* (phylum Pyrrophyta), and since then it has been detected in several other species of dinoflagellates [2–7]. It is an unusual sterol in that it has an alkyl substituent in the 23-position and a 4α-methyl group, and its isolation sparked an interest in sterols of marine dinoflagellates. Although only a small number of the *ca* 1000 species of modern dinoflagellates [8] have been investigated, the results certainly justify a continuation of research in this field as several new sterols have been reported with double bonds in unusual positions [4,6,9,10] or with an unprecedented side-chain alkylation pattern [11, 12]. These results also included the observation that 4α-methyl sterols, which are intermediates in sterol biosynthesis in animals and in other divisions of the Plant Kingdom, are often end products of sterol biosynthesis in dinoflagellates [6].

A very careful analysis of the total free sterols of the gorgonian *Plexaura homomalla* (phylum Cnidaria, class Anthozoa, order Gorgonaceae) resulted in the isolation of some 50 sterols [13], including three 4α-methyl sterols, i.e. dinosterol (**1g**) [13, 14], (24*R*)-4α,24-dimethyl-5α-cholest-22-en-3β-ol (**1b**) and (24*S*)-4α,24-dimethyl-5α-cholestan-3β-ol (**1f**) [14]. It was possible of course, that these sterols were of dietary origin, but as *P. homomalla*, like many invertebrates from shallow tropical waters, has dinoflagellate symbionts (zooxanthellae) it was speculated that these 4α-methyl sterols were most likely produced by the symbionts. If this was true it would mean that, in general, animals with dinoflagellate symbionts should be sources of dinoflagellate sterols. We decided to check this hypothesis.

RESULTS

We now report the results of our work on the Caribbean gorgonians *Briareum asbestinum, Gorgonia mariae, Muriceopsis flavida* and *Pseudoplexaura wagenaari*. These animals, which have dinoflagellate symbionts as demonstrated by the presence of peridinin (a dinoflagellate pigment) [15] contain low levels of 4α-methyl sterols including the following new ones: 4α-methyl-24-methylene-5α-cholestan-3β-ol (**1d**), (24*R*)-4α,24-dimethyl-5α-cholesta-7,22-dien-3β-ol (**2b**), 4α,24*S*(or 23ξ)-dimethyl-5α-cholest-7-en-3β-ol (**2f** or **2e**), 7-dehydrodinosterol (**2g**), and (24*R*)-4α,24-dimethyl-5α-cholesta-8(14), 22-dien-3β-ol (**3b**). The sources and relative abundances of the sterol components are listed in Table 1. The isolation of 4α-methylgorgostanol (**1h**) from *B. asbestinum* has been reported earlier [4]. It is also pertinent to note that while 24-methylenelophenol (**2d**) has been isolated from terrestrial sources [16], this is the first record of its isolation from a marine organism.

Structure elucidation of the new sterols

The isolation procedure was aimed at the isolation of 4α-methyl sterols, which were less polar (R_f 0.25) than regular (= 4-desmethyl) sterols (R_f 0.20) on Si gel TLC (E. Merck No. 5775; hexane–ether, 1:1). The ^{1}H NMR spectra of the components of the sterol mixtures confirmed that we had 4α-methyl sterols, as followed from the shift of the proton at C-3 which

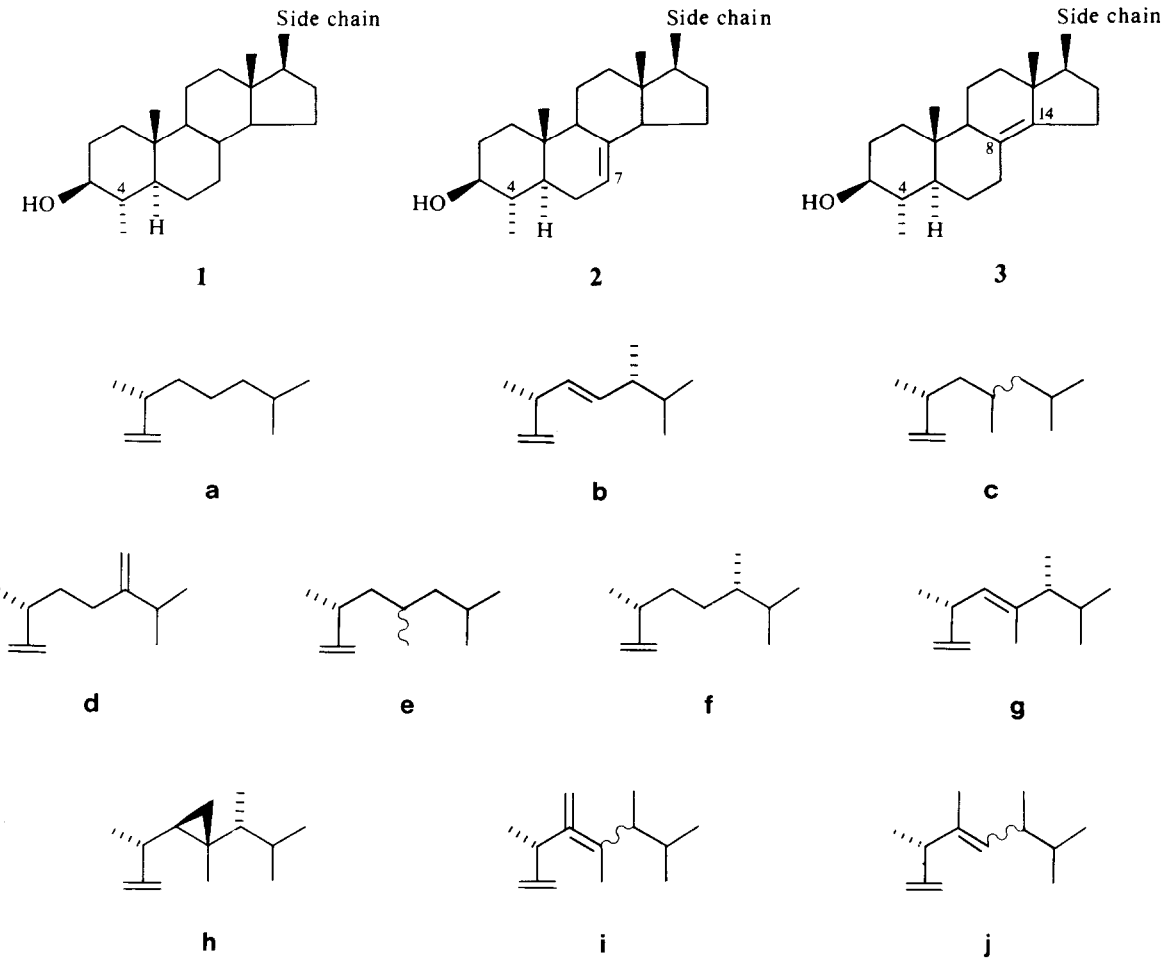
*No. 25 in the Stanford series "Minor and Trace Sterols of Marine Invertebrates"; No. 63 in "Inshore Marine Shallow Water Project Contributions".

§To whom correspondence should be addressed.

Table 1. Abundance (%) of 4α-methyl sterol components of four gorgonians

Trivial or systematic names	MW	*B. asbestinum*	*G. mariae*	*M. flavida*	*P. wagenaari*	*RR*‡
4α-Methyl-5α-cholestan-3β-ol (**1a**)	402	0.3	—	1.7	—	1.16
(24*R*)-4α,24-Dimethyl-5α-cholest-22-en-3β-ol (**1b**)	414	14.5	66.5	16.5	4.4	1.29
4α-Methyl-24-methylene-5α-cholestan-3β-ol (**1d**)	414	8.2	1.4	1.5	1.7	1.51
(24*S*)-4α,24-Dimethyl-5α-cholestan-3β-ol (**1f**)	416	23.8	24.9	1.8	26.8	1.47
(24*R*,22*E*)-4α,23,24-Trimethyl-5α-cholest-22-en-3β-ol (dinosterol) (**1g**)	428	7.2	5.6	59.6	63.8	1.58
(22*R*,23*R*,24*R*)-4α,23,24-Trimethyl-22,23-methylene-5α-cholestan-3β-ol (4α-methylgorgostanol) (**1h**)	442	32.5	—	—	1.1	2.75
(24*R*)-4α,24-Dimethyl-5α-cholesta-7,22-dien-3β-ol (**2b**)	412	5.2	1.3	1.5	0.2	1.57
4α-Methyl-24-methylene-5α-cholest-7-en-3β-ol (24-methylenelophenol) (**2d**)	412	1.0	—	—	—	1.89
4α,24*S*(or 23ξ)-Dimethyl-5α-cholest-7-en-3β-ol (**2f** or **2e**)	414	0.3	—	4.2	0.8	1.77
(24*R*,22*E*)-4α,23,24-Trimethyl-5α-cholesta-7,22-dien-3β-ol (7-dehydrodinosterol) (**2g**)	426	—	—	11.1	1.2	1.86
(24*R*)-4α,24-Dimethyl-5α-cholesta-8(14),22-dien-3β-ol (**3b**)	412	—	—	0.6	—	1.28
4α-Methyl-24-methylene-5α-cholest-8(14)-en-3β-ol (amphisterol) (**3d**)	412	3.6	—	—	—	1.58
(24*S*)-4α,24-Dimethyl-5α-cholest-8(14)-en-3β-ol (**3f**)	414	—	—	1.3	—	1.49
(24*R*,22*E*)-4α,23,24-Trimethyl-5α-cholesta-8(14),22-dien-3β-ol [8(14)-dehydrodinosterol] (**3g**)	426	—	—	0.2	—	1.54

*GLC conditions: 3% OV17, 260°; standard cholesterol.



appears as a characteristic sextet [17] (*ddd*) at δ 3.1 ($CDCl_3$) rather than at δ 3.6 as in the 5α-saturated or at δ 3.5 as in the Δ^5-unsaturated 4-desmethyl analogues. An analysis of the mass spectra indicated that in all cases the C-4 methyl group provided the only additional carbon in the steroid nucleus. Also, in all cases the observed values for the ^{1}H NMR shifts of the angular methyl protons (cf. Table 2) were in good agreement with values for a normal (5α, 14α)-androstane skeleton predicted on the basis of Zürcher's rules [18].

A new sterol with a saturated ring system

Side chain fragmentation in the mass spectrum of a sterol of MW 414 proved that its only degree of unsaturation was in the side chain [19]. Its acetate had a low R_f value on Ag^+–Si gel TLC indicating the presence of a methylene group, which was confirmed by the ^{1}H NMR spectrum (Table 2). The mass spectrum of the free sterol showed a very strong peak at *m/z* 330 (loss of the end of the side chain by a McLafferty rearrangement), thus the methylene was in the 24-position [20]. The structure 4α-methyl-24-methylene-5α-cholestan-3β-ol (**1d**) is completely consistent with the ^{1}H NMR data (Table 2).

New Δ^7-unsaturated sterols

Although mass spectrometry can be used to assign a double bond to the 7-position (the mass spectrum of a Δ^7-unsaturated 4α-methyl sterol should include a diagnostic peak at *m/z* 260) [21], it is more reliable to make this assignment on the basis of the ^{1}H NMR spectrum, since the C-18 methyl signal of Δ^7 sterols occurs at very high field (the calculated shift [18] for the C-18 methyl protons in lathosterol (5α-cholest-7-en-3β-ol) is δ 0.57 ($CDCl_3$) cf. δ 0.68 for cholesterol). Three new sterols of MWs 412, 414 and 426, respectively, (**2b**, **2f** and **2g**) fell into this category.

A $[M-43]^+$ peak in the mass spectrum of the sterol of MW 412 indicated the presence of a Δ^{22}-double bond and of an alkyl substituent in the 24-position. Coupled with the ^{1}H NMR data (Table 2), the most likely structure of this sterol was thus (24*R*)-4α,24-dimethyl-5α-cholesta-7,22-dien-3β-ol (**2b**). The absolute configuration at C-24 was confirmed by hydrogenation of the Δ^{22}-double bond, a process which was accompanied by migration of the nuclear double bond [22], to yield the known sterol (24*S*)-4α-dimethyl-5α-cholest-8(14)-en-3β-ol (**3f**) [6].

The sterol of MW 414 had one degree of unsaturation. In view of the presence of the Δ^7-double bond, the side chain had to be saturated. The proposed structure is (24*S*)-4α-24-dimethyl-5α-cholest-7-en-3β-ol (**2f**); the assigned absolute configuration at C-24 is supported by the near equivalence of one of the isopropyl methyls and the C-28 methyl group (see

Table 2. ^{1}H NMR data of selected 4α-methyl sterols from the four gorgonians (360 MHz, $CDCl_3$, TMS or $CHCl_3$ as int. standard)

	1d	1g	2b	2d	2f*	2g	3b
H-3	3.1 *m*	3.1 *m*	3.1 *m*	3.1 *m*	3.1 *m*	3.1 *m*	3.1 *m*
H-7	—	—	5.19 *m*	5.19 *m*	5.18 *m*	5.17 *m*	—
H-20	—	2.33 *m*	—	—	—	2.33 *m*	—
H-23- and/ or H-22		4.87 *d* $J = 9.6$	5.19 *m* (2 H)	—	—	4.89 *d* $J = 9.8$	5.20 *m* (2 H)
H-25	2.23 *m*	—	—	2.23 *m*	—	—	—
Me-4	0.947 *d* $J = 6.2$	0.946 *d* $J = 6.1$	0.987 *d* $J = 6.3$	0.989 *d* $J = 6.3$	0.988 *d* $J = 6.3$	0.986 *d* $J = 6.3$	0.985 *d* $J = 6.4$
H-18	0.649 *s*	0.680 *s*	0.543 *s*	0.536 *s*	0.529 *s*	0.559 *s*	0.847 *s*
H-19	0.823 *s*	0.827 *s*	0.829 *s*	0.827 *s*	0.825 *s*	0.830 *s*	0.712 *s*
H-21	0.930 *d* $J = 6.2$	0.919 *d* $J = 6.5$	1.016 *d* $J = 6.6$	0.953 *d* $J = 6.5$	0.922 *d* $J = 6.4$	0.938 *d* $J = 5.8$	1.020 *d* $J = 6.6$
H-26,H-27	1.020 *d* $J = 6.9$	0.778 *d* $J = 6.6$	0.820 *d* $J = 6.3$	1.024 *d* $J = 6.8$	0.782 *d*† $J = 6.8$	0.781 *d* $J = 6.6$	0.821 *d* $J = 6.5$
	1.024 *d* $J = 7.0$	0.836 *d* $J = 6.6$	0.837 *d* $J = 5.9$	1.029 *d* $J = 6.9$	0.855 *d* $J = 6.9$	0.839 *d* $J = 6.4$	0.838 *d* $J = 6.1$
H-28	4.65 *s* 4.71 *s*	0.828 *d* $J = 6.6$	0.913 *d* $J = 6.8$	4.66 *s* 4.71 *s*	0.776 *d*† $J = 6.8$	0.933 *d* $J = 6.8$	0.920 *d* $J = 6.9$
H-29	—	1.496 *d* $J = 1.2$	—	—	—	1.502 *s*	—

*Assigned as if this were the right structure; there is an alternate structure (**2e**).
†These assignments might have to be interchanged.

Table 2) (in the ^{1}H NMR spectrum of (24*S*)-24-methylcholest-5-en-3β-ol the doublets of the C-27 and C-28 methyl group occur at δ 0.783 and 0.775, respectively, but there is a much larger difference between the δ values of these doublets in the spectrum of the (24*R*)-epimer: they occur at δ 0.802 and 0.773, respectively)[23]. However, we cannot rule out an alternate structure with a 23-methyl substituent (**2e**) as sterols with a 23-methyl substituent in the side chain (**c**) were recently found in some cultured dinoflagellates [11, 12]. Therefore, the possible existence of the corresponding saturated side chain (**e**) in nature cannot be ignored.

The mass spectrum of the Δ^7 sterol of MW 426 showed the same side chain fragmentation [3] as dinosterol (**1g**) which immediately suggested the structure (22*E*, 24*R*)-trimethyl-5α-cholesta-7,22-dien-3β-ol (7-dehydrodinosterol) (**2b**). Decoupling experiments supported this structure: irradiation of the vinylic proton at δ 2.33 collapsed both the olefinic doublet and one of the almost equivalent methyl doublets at lowest field. For comparison, the ^{1}H NMR data of dinosterol [6] (**1g**) are included in Table 2.

A new $\Delta^{8(14)}$-unsaturated sterol

The ^{1}H NMR spectrum of a second sterol of MW 412 did not show olefinic ring protons, but the MS clearly indicated the presence of one degree of unsaturation in the nucleus (*m/z* 287: loss of side chain; *m/z* 285: loss of side chain +2H, characteristic of sterols containing one double bond in the side chain) [19]. Application of Zürcher's rules [18] and a comparison with the ^{1}H NMR spectra of $\Delta^{8(14)}$-unsaturated sterols indicated the double bond to be in the 8(14)-position. The NMR spectrum proved the presence of a Δ^{22} double bond and of a C-24 methyl substituent. Unfortunately, there was not enough material to correlate this sterol (partial hydrogenation) with a known sterol (either **3f** or its epimer at C-24). However, the most likely structure is (24*R*)-4α,24-dimethyl-5α-cholesta-8(14),22-dien-3β-ol (**3b**), because this configuration at C-24 (24β) is a consistent feature of all known dinoflagellate sterols with a methyl substituent in the 24-position.

DISCUSSION

Elsewhere [6] we reported the isolation from two *Amphidinium* spp. of a sterol of MW 426 to which we assigned the structure 4α,23 (or 22), 24ξ-trimethyl-5α-cholesta-8(14),22-dien-3β-ol (**3i** or **3j**). We could not use analogy to assign the structure of the side chain because dinosterol (**1g**) was not found in these algae. From *M. flavida* we isolated a sterol which was identical by 360 MHz NMR with the above *Amphidinium* sterol. As dinosterol (**1g**) is the main 4α-methyl sterol of *M. flavida* (Table 1) there can be little doubt now that the structure of this *Amphidinium* sterol is 8(14)-dehydrodinosterol (**3g**).

Table 1 shows that there are large differences between the 4α-methyl sterol patterns of the gorgonians. As all animals were collected in about the same location these results support the hypothesis that the sterols are symbiont-derived rather than of dietary origin. This result might have a practical application in problems concerning the specificity of algal symbiosis [24] in invertebrates (*vide infra*).

Schoenberg and Trench [25] have shown that classical taxonomy of dinoflagellates has its limitations. Taxonomists [26–28] consider the dinoflagellate symbionts of many different marine invertebrates (e.g. alcyonarians, zoantharians, some nudibrances, killer clams and jelly fishes) to be one species, *Zooxanthella microadriatica* [29]. Schoenberg and Trench [25] have demonstrated that at the molecular level symbionts isolated from various animals are not necessarily identical: on the basis of electrophoretic

mobilities of proteins from cultured symbionts they could differentiate 12 strains. This result posed the question whether there is an unique association between a certain strain of symbiont and a species of invertebrate host [25, 30, 31]. One way to answer this question would be to isolate the symbionts from animals of the same species collected in several different geographic locations, to bring each of these isolates into culture, and to do electrophoresis of their proteins. This approach, however useful, has several practical disadvantages: isolation is time-consuming, it often fails,* and sometimes an alga, which is not the symbiont, is isolated instead of the symbiont† [32]. Since we have shown [7, 33] (Djerassi C., *et al.*, unpublished results) that there can be large differences in sterol patterns of cultured zooxanthellae, it might be practical to obtain information on the specificity of symbiosis by collecting an invertebrate in various locations and then to analyse its 4α-methyl sterols.

EXPERIMENTAL

General. For purification of sterol mixtures and for separation of their components by HPLC we used Waters Associated HPLC equipment (M6000 or M600A pump, U6K injector, R401 or R403 differential refractometer, two Bondapak μ-C_{18} columns (4 mm × 30 cm) in series (standard eluent MeOH–H_2O, 23:2) and also a Valco model CV-6-HPax valve type injector, a home-made semi-prep. reverse phase column [34] (3/8 inch × 60 cm) built by Dr. Richard Izac at Scripps (eluent MeOH–H_2O, 24:1), and Whatman ODS-2 and ODS-3 columns (9 mm × 50 cm) (eluent MeOH). For monitoring of purification the La Jolla co-authors used Hewlett-Packard 5710A and 402 GLCs with FID for analytical GLC (3% SP2250 column, 260°) and prep. GLC (3% OV25 column, 265°), respectively; a Varian HR 220/Nicolet TT-100 FT-NMR; a LKB 9000 GC/MS (3% SP2250 column, 260°). A Hewlett-Packard 402 GLC was used at Stanford for both analytical GLC (3% OV17 column, 260°) and prep. GLC (3% OV25, 265°); GC/MS at Stanford was done on a MATT-44 (3% OV17 column, 260°) instrument and on a R10–10 Ribermag mass spectrometer for very small samples (SE-54 capillary column). The 360 MHz ^{1}H NMR spectra were run on a Brucker HX360 spectrometer. All shifts are in ppm with respect to TMS. High resolution MS were run on a MAT 711 double focussing spectrometer equipped with a PDP-11/45 computer for data acquisition and reduction.

Collection of the animals and isolation of the 4α-methyl sterols. B. asbestinum, G. mariae, M. flavida and *P. wagenaari* were collected using SCUBA at −10 m, May 1978, at Carrie Bow Cay, Belize, Central America. The freshly collected animals were cut into pieces and stored in PrOH. The usual procedure for making an extract from a sea fan is as follows: The PrOH was decanted; the animal was extracted in the hot with $CHCl_3$–MeOH (typically 2:1). The MeOH–$CHCl_3$ soln was decanted, and the procedure repeated twice. The decantates were combined and taken to dryness. The residue was partitioned between $CHCl_3$ and H_2O, and the $CHCl_3$ layer processed further. Sea whips, such as *B. asbestinum*, were extracted in a Soxhlet ($CHCl_3$–MeOH, 1:1) for several days. The total free sterols were isolated from the extracts CC on Si gel [13]; green pigments were separated from the sterols over a short florisil column (eluent hexane–Et_2O, 1:1). Careful rechromatography over Florisil (eluent hexane–Et_2O, 2:1) was then used to separate the 4α-methyl sterols from the 4-demethyl sterols. Only in the case of *G. mariae* was the 4α-methyl sterol fraction almost free from non-sterols, and the sterol components could be separated directly by reversed phase HPLC. In the case of the other gorgonians the 4α-methyl sterols were accompanied by large amounts of non-sterols. The usual next purification steps were saponification and removal of most of the remaining non-sterols from the neutral unsaponifiables by reversed phase HPLC (home-made column-in which the non-steroidal material was removed first.

B. asbestinum: *separation of the 4α-methyl sterols.* The 4α-methyl sterols (49.2 mg from 62 g extract) were acetylated and used for $AgNO_3$–Si gel TLC [35] (two plates of 20 × 20 cm). Three bands corresponding to fractions 1, 2, and 4 showed up under long-wave UV. 4 fractions were collected as follows (Fraction number, R_f, yield after saponification): 1, 0.43–0.52, 27.7 mg; 2, 0.34–0.43, 13.5 mg; 3, 0.18–0.34, 2.9 mg; 4, 0.09–0.18, 6.5 mg. All fractions were worked up by reverse phase HPLC after saponification (Waters columns, standard eluent). Fractions are discussed in order of increasing retention time in HPLC or GLC.

Fraction 1. Main sterols: (24*S*)-4α,24-dimethylcholestanol (**1f**) and 4α-methylgorgostanol (**1h**). The poor solubility of the latter sterol made use of our standard eluent impractical; a solvent mixture with less H_2O (MeOH–H_2O, 24:1) had to be used to separate the lower MW sterols from this C_{31} sterol. Re-injection of the former mixture afforded three fractions: a mixture of minor components of fraction 1 (4α-methylcholestanol (**1a**), (24*R*)-4α,24-dimethyl-22-dehydrocholestanol (**1b**), an unidentified 4-methyl sterol of MW 414, and (24*S*)24-methyllophenol (**2f**), which were separated by prep. GLC), a mixture of dinosterol (**1g**) and (24*S*)-4α,24-dimethylcholestanol (**1f**), and pure **1f**. Reinjection of the dinosterol-containing fraction afforded a pure sample of this compound.

Fraction 2. Main components: (24*R*)-4α,24-dimethyl-22-dehydrocholestanol (**1b**) and 4α-methylgorgostanol (**1h**). Four fractions were collected containing: (24*R*)-24-methyl-22-dehydrolophenol (**2b**), (24*R*)-4α,24-dimethyl-22-dehydrocholestanol (**1b**), a mixture of dinosterol (**1g**) and (24*S*)-4α,24-dimethylcholestanol (**1f**), and 4α-methylgorgostanol (**1h**).

Fraction 3 consisted largely of (24*R*)-24-methyl-22-dehydrolophenol (**2b**). Using HPLC, minor components (mixture of unidentified components with a shorter retention time, and some (24*R*)-4α,24-dimethyl-22-dehydrocholestanol (**1b**), which has a longer retention time) were removed.

Fraction 4. Two fractions were collected, one containing the minor sterols, the other containing the main sterol, viz. 4α-methyl-24-methylenecholestanol (**1d**). The minor sterols, amphisterol (**3d**) and 24-methylenelophenol (**2d**), were separated by prep. GLC.

G. mariae: *separation of the 4α-methyl sterols.* Yield 98.5 mg from 37.8 g of extract. Non-sterols accounted for about 25% of this mixture. For an initial separation of part of the mixture an ODS-3 column was used. 3 fractions were

*The rate of success in attempts to isolate a zooxanethella and to bring it into culture is about 50% (Lance, J. R., La Jolla, California, personal communication).

†In three cases we cultured algae from private collections, which, according to the donors, were zooxanthellae isolated from invertebrates. They were actually haptophytes (Hibbert, D. J., Cambridge, England, personal communication). No haptophytes are known to occur as symbionts in invertebrates.

collected (fraction number, R_f, wt): 1, 0.83–0.93 (trace sterols), 1.9 mg; 2, 0.93–1.06 (4, 24-dimethyl-22-dehydrocholestanol (**1b**)), 18.0 mg; 3, 1.06–1.22 (4,24-dimethylcholestanol (**1f**) and dinosterol (**1g**)), 9.4 mg. Fraction 2 was pure by 360 MHz ^{1}H NMR. HPLC purification of fraction 1 was repeated using an ODS-2 column; two fractions were collected: fraction A corresponding to the first half of the two partially overlapping main peaks, and fraction B corresponding to the half with the longer retention time in HPLC. Fraction A was re-injected (ODS-2 column) and the tails of the peak were cut. Further work-up by prep. GLC (to remove a small amount of cholesterol) afforded 4, 24-dimethylcholesta-7, 22-dien-3β-ol (**2b**). Fraction B was also re-injected (ODS-2 column) and the front of the peak was cut to remove traces of the main component of fraction A. The main component of the resulting mixture, 4-methyl-24-methylenecholestanol (**1d**) and two unidentified minor components with a shorter retention time in GLC were then separated by prep. GLC. We also used the ODS-2 column to separate the components of fraction 3, and two fractions were collected: fraction C corresponding to the first half of the peak, and fraction D corresponding to the second half. Fraction D was pure 4,24-dimethylcholestanol (**1f**); the main component of fraction C was also **1f**, but fraction C was enriched in dinosterol (**1g**) as compared with fraction 3. Further work-up of fraction C in the same manner eventually produced a sample of **1g** for NMR.

M. flavida: *separation of the 4α-methyl sterols.* Yield 50 mg of 4α-methyl sterols from 11 g extract. This mixture was acetylated and used for $AgNO_3$–Si gel TLC in the manner of Idler [36] (two plates 20 × 20 cm). Two bands showed up under long-wave UV after spraying with berberine hydrochloride. Three fractions were obtained after saponification of the two eluted bands (fraction 1, faster moving band; fraction 3, slower moving band) and the area between (fraction 2). Fraction 1 (R_f, 0.44–0.54) and fraction 2 (0.20–0.44) were combined and subjected to reverse phase HPLC (ODS-2 column). Pure dinosterol (**1g**) was obtained after one run. Several re-injections were needed to obtain pure 4α-methyl-5α-cholestan-3β-ol (**1a**), (24*R*)-4α,24-dimethyl-5α-cholest-22-en-3β-ol (**1b**), 4α,24*S*-dimethyl-5α-cholestan-3β-ol (**1f**), (24*R*)-4α,24-dimethyl-5α-cholesta-7,22-dien-3β-ol (**2b**), 7-dehydrodinosterol (**2g**), (24*R*)-4α,24-dimethyl-5α-cholesta-8(14),22-dien-3β-ol (**3b**), (24*S*)-4α,24-dimethyl-5α-cholest-8(14)-en-3β-ol (**3f**) and 8(14)-dehydrodinosterol (**3g**). To obtain pure 4α,24*S*(or 23ξ)-dimethyl-5α-cholest-7-en-3γ-ol (**2f** or **2e**) prep. GLC was needed. Fraction 3 (R_f, 0.10–0.20) contained one pure sterol, 4α-methyl-24-methylene-5α-cholestan-3β-ol (**1d**).

P. wagenaari: *separation of the 4α-methyl sterols.* Yield 144.9 mg of (impure) 4-methyl sterols from 16.5 mg extract: Non-sterols accounted for about 2/3 of this mixture. It was acetylated and used for $AgNO_3$–Si gel TLC [35] (three plates, 20 × 20 cm). Four fractions were collected,but only two contained sterols: fraction 1 (59 mg after saponification), R_f 0.45–0.60, and fraction 3, R_f 0.19–0.29. Only the acetate of 4-methyl-24-methylenecholestanol (**1d**) ended up in fraction 3 [isolated yield 1.0 mg after saponification and clean-up (Waters columns)]. In order to isolate the trace sterols from fraction 1 the bulk of the main sterols, dinosterol (**1g**) and 4,24-dimethylcholestanol (**1f**), had to be removed. This was done over an ODS-2 column. The minor sterol, 4,24-dimethyl-22-dehydrocholestanol (**1b**) and the trace sterols ended up in the combined front and tails fraction; the first half of the only significant peak was essentially pure dinosterol (**1g**); 4,24-dimethylcholestanol (**1f**) was enriched in the second half of the peak. Some 4-demethyl sterols also ended up in the front-and-tails fraction; they were removed using HPLC (two Waters μ-Porasil columns (4 mm × 30 cm) in series, eluent toluene–EtOAc, 95:5). The cleaned front-and-tails fraction was acetylated. Argentic Si gel TLC followed by saponification afforded two fractions. The minor fraction contained what was left of the main 4-methyl sterols, **1g** and **1f**, and also 4-methylgorgostanol (**1h**). The latter compound was isolated using an ODS-3 column. HPLC (ODS-2 column) of the main fraction gave four fractions (number, R_f): 1, 0.94–1.04; 2, 1.11–1.18; 3, 1.18-1.22; 4, 1.22–1.26 [the main peak was cut at the top and at a shoulder in the downward slope (fractions 2, 3, 4)]. Fraction 1 consisted of 4,24-dimethylcholesta-7,22-dien-3β-ol (**2b**). Fraction 2 was a mixture of 4,24-dimethyl-22-dehydrochol-22-cholestanol (**1b**), the main component of fraction 3, and 7-dehydrodinosterol (**2g**); this mixture was separated by prep. GLC. Fraction 3 consisted of essentially pure 4,24-dimethyl-22-dehydrocholestanol (**1b**). Fraction 4 was a mixture of the main component of fraction 3 (**1b**) and of 4,24-dimethyl-7-dehydrocholestanol (**2f**) which were separated by prep. GLC.

4α-Methyl-24-methylene-5α-cholestan-3β-ol (**1d**). High resolution GC/MS 70 eV, *m/z* (rel. int.): 414.3941 ($C_{29}H_{50}O$) $[M]^+$ (18), 399.3648 $[C_{28}H_{47}O]^+$ (10), 381.3576 $[C_{28}H_{45}]^+$ (2), 330.2945 $[C_{23}H_{38}O]^+$ (100), 316.2783 $[C_{22}H_{36}O]^+$ (14), 315.2724 $[C_{22}H_{35}O]^+$ (20), 288.2420 $[C_{20}H_{32}O]^+$ (19), 287.2393 $[C_{20}H_{31}O]^+$ (51), 286.2289 $[C_{20}H_{30}O]^+$ (8), 269.2269 $[C_{20}H_{29}]^+$ (4), 248.2145 $[C_{17}H_{28}O]^+$ (10), 247.2082 $[C_{17}H_{27}O]^+$ (14), 229.1975 $[C_{17}H_{25}]^+$ (13).

4α-Methylgorgostanol (**1g**). High resolution MS (probe) 70 eV, *m/z* (rel. int.): 442.4166 ($C_{31}H_{54}O$) $[M]^+$ (47), 427.3974 $[C_{30}H_{51}O]^+$ (5), 424.4097 $[C_{31}H_{52}]^+$ (8), 381.3505 $[C_{28}H_{45}]^+$ (3), 371.3380 $[C_{26}H_{43}O]^+$ (10), 353.3205 $[C_{26}H_{41}]^+$ (23), 330.2928 $[C_{23}H_{38}O]^+$ (92), 316.2747 $[C_{22}H_{36}O]^+$ (47), 315.2704 $[C_{22}H_{35}O]^+$ (35), 312.2810 $[C_{23}H_{36}]^+$ (13), 301.2536 $[C_{21}H_{33}O]^+$ (13), 299.2744 $[C_{22}H_{35}]^+$ (42), 297.2604 $[C_{22}H_{33}]^+$ (13), 288.2443 $[C_{20}H_{32}O]^+$ (61), 287.2361 $[C_{20}H_{31}O]^+$ (100), 286.2296 $[C_{20}H_{30}O]^+$ (33), 271.2413 $[C_{20}H_{31}]^+$ (36), 269.2256 $[C_{20}H_{29}]^+$ (20), 247.2047 $[C_{17}H_{27}O]^+$ (13), 245.2246 $[C_{18}H_{29}]^+$ (7), 245.1891 $[C_{17}H_{25}O]^+$ (6), 243.2107 $[C_{18}H_{27}]^+$ (11), 231.2100 $[C_{17}H_{27}]^+$ (15), 231.1771 $[C_{16}H_{23}O]^+$, 229.1949 $[C_{17}H_{25}]^+$ (29), 217.1950 $[C_{16}H_{25}]^+$ (18), 215.1796 $[C_{16}H_{23}]^+$ (13).

(24R)-4α,24-Dimethyl-5α-cholesta-7,22-dien-3β-ol (**2b**). High resolution MS (probe) 70 eV, *m/z* (rel. int.): 412.3693 ($C_{29}H_{48}O$) $[M]^+$ (100), 397.3461 $[C_{28}H_{45}O]^+$ (22), 394.3563 $[C_{29}H_{46}]^+$ (1), 379.3350 $[C_{28}H_{43}]^+$ (1), 369.3139 $[C_{26}H_{41}O]^+$ (6), 314.2595 $[C_{22}H_{34}O]^+$ (16), 287.2382 $[C_{20}H_{31}O]^+$ (30), 285.2221 $[C_{20}H_{29}O]^+$ (66), 269.2249 $[C_{20}H_{29}]^+$ (24), 260.2485 $[C_{19}H_{32}]^+$ (1), 260.2133 $[C_{18}H_{28}O]^+$ (20), 245.1902 $[C_{17}H_{25}O]^+$ (8), 243.2092 $[C_{18}H_{27}]^+$ (15), 243.1789 $[C_{17}H_{23}O]^+$ (1), 227.1803 $[C_{17}H_{23}]^+$ (8), 219.2080 $[C_{16}H_{27}]^+$ (20).

24-Methylenelophenol (**2d**). High resolution GC/MS 70 eV, *m/z* (rel. int.): 412.3686 ($C_{29}H_{48}O$) $[M]^+$ (56), 397.3457 $[C_{28}H_{45}O]^+$ (21), 394.3572 $[C_{29}H_{46}]^+$ (3), 379.3400 $[C_{28}H_{43}]^+$ (4), 328.2745 $[C_{23}H_{36}O]^+$ (56), 314.2590 $[C_{22}H_{24}O]^+$ (10), 313.2538 $[C_{22}H_{33}O]^+$ (8), 287.2325 $[C_{20}H_{31}O]^+$ (4), 286.2257 $[C_{20}H_{30}O]^+$ (28), 285,2199 $[C_{20}H_{29}O]^+$ (100), 269.2291 $[C_{20}H_{29}]^+$ (15), 267.2140 $[C_{20}H_{27}]^+$ (6), 260.2157 $[C_{18}H_{28}O]^+$ (8), 259.2144 $[C_{18}H_{27}O]^+$ (6), 245.1910 $[C_{17}H_{25}O]^+$ (9), 243.2034 $[C_{18}H_{27}]^+$ (6), 241.1954 $[C_{18}H_{25}]^+$ (8), 227.1819 $[C_{17}H_{23}]^+$ (10).

7-Dehydrodinosterol (**2g**). Low resolution GC/MS 70 eV (Ribermag), *m/z* (rel. int.): 426 $[M]^+$ (12), 411 (1), 383 (2), 365

(0.4), 355 (1), 337 (0.4), 315 (3), 314 (12), 313 (3), 299 (3), 287 (8), 286 (12), 285 (49), 281 (1), 271 (2), 269 (10), 267 (3), 260 (3), 245 (1), 243 (1), 241 (1), 229 (1), 227 (3), 69 (100).

4α,24S (or 23ξ)-Dimethyl-5α-cholest-7-en-3β-ol (**2f** or **2e**). Low resolution GC/MS 70 eV (LKB 9000) (high resolution GC/MS data were used to assign the main peaks), *m/z* (rel. int.): 414.3856 ($C_{29}H_{50}O$) $[M]^+$ (100), 399.3623 $[C_{28}H_{47}O]^+$ (27), 396 (4), 381 (10), 287.2426 $[C_{20}H_{31}O]^+$ (21), 269.2258 $[C_{20}H_{29}]^+$ (71), 261 (14), 260 (11), 245.1934 $[C_{17}H_{25}O]^+$ (27), 243.2114 $[C_{18}H_{27}]^+$ (21), 227.1841 $[C_{17}H_{23}]^+$ (33), 215 (10), 213 (9).

Acknowledgements—Financial support was provided by NSF grant No. OCE-76-81737 (to W.F.) and NIH grants No. GM-06840 and GM-28352 (to C.D.). Use of the NMR/MS center at UCSD and of the Stanford 360 MHz ^1H NMR facility (NSF grant No. GP-23633 and NIH grant No. RR-0711) is gratefully acknowledged. Dr. Lois Durham recorded the 360 MHz ^1H NMR spectra, and Annemarie Wegmann the high resolution MS. The gorgonians were identified by Katy Muzik, Smithsonian Institution, Washington D.C. L.B. is grateful to the Fullbright Commission for a travel grant. We wish to thank Professor K. Schreiber, Halle/Saale, East Germany, for a sample of 24-methylenelophenol, and Sally Look and Roy Okuda for technical assistance.

REFERENCES

1. Shimizu, Y., Alam, M. and Kobayashi, A. (1976) *J. Am. Chem. Soc.* **98**, 1059.
2. Withers, N. W., Tuttle, R. C., Holz, G. G., Beach, D. H., Goad, L. J. and Goodwin, T. W. (1978) *Phytochemistry* **17**, 1987.
3. Alam, M., Sansing, T. B., Busby, E. L., Martiniz, D. R. and Ray, S. M. (1979) *Steroids* **33**, 197.
4. Withers, N. W., Kokke, W. C. M. C., Rohmer, M., Fenical, W. and Djerassi, C. (1979) *Tetrahedron Letters*, 3605.
5. Alam, M., Martin, G. E. and Ray, S. M. (1979) *J. Org. Chem.* **44**, 4466.
6. Kokke, W. C. M. C., Fenical, W. and Djerassi, C. (1981) *Phytochemistry* **20**, 127.
7. Kokke, W. C. M. C., Fenical, W., Bohlin, L. and Djerassi, C. (1981) *Comp. Biochem. Physiol. B* **68**, 281.
8. Loeblich Jr., A. R. and Loeblich III, A. R. (1966) *Stud. Trop. Oceanogr.* **3**, 1.
9. Withers, N. W., Goad, L. J. and Goodwin, T. W. (1979) *Phytochemistry* **18**, 899.
10. Swenson, W., Tagle, B., Clardy, J., Withers, N. W., Kokke, W. C. M. C., Fenical, W. and Djerassi, C. (1980) *Tetrahedron Letters* 4663.
11. Alam, M., Schram, K. H. and Ray, S. M. (1978) *Tetrahedron Letters* 3517.
12. Kokke, W. C. M. C., Withers, N. W., Massey, I. J., Fenical, W. and Djerassi, C. (1979) *Tetrahedron Letters*, 3601.
13. Popov, S., Carlson, R. M. K., Wegmann, A. and Djerassi, C. (1976) *Steroids* **28**, 699.
14. Dow, W. C. (1977) M.Sc. Thesis, Stanford University, U.S.A.
15. Johansen, J. E., Svec, W. A., Liaaen-Jensen, S. and Haxo, F. T. (1974) *Phytochemistry* **13**, 2261.
16. Schreiber, K. and Osske, G. (1964) *Tetrahedron* **20**, 2575.
17. Knapp Jr., F. F. and Schroepfer Jr., G. J. (1975) *Steroids* **26**, 339.
18. Zürcher, R. F. (1963) *Helv. Chim. Acta* **46**, 2054.
19. Wyllie, S. G. and Djerassi, C. (1968) *J. Org. Chem.* **33**, 305.
20. Massey, I. J. and Djerassi, C. (1979) *J. Org. Chem.* **44**, 2448.
21. Partridge, L. G., Midgley, I. and Djerassi, C. (1977) *J. Am. Chem. Soc.* **99**, 7686.
22. Wieland, H., Rath, F. and Benend, W. (1941) *Justus Liebigs Ann. Chem.* **548**, 19.
23. Rubinstein, I., Goad, L. J., Clague, A. D. H. and Mulheirn, L. J. (1976) *Phytochemistry* **15**, 195.
24. Trench, R. K. (1979) *Ann. Rev. Plant Physiol.* **30**, 485.
25. Schoenberg, D. A. and Trench, R. K. (1980) *Proc. R. Soc. London Ser. B* **207**, 405.
26. Taylor, D. L. (1974) in *Symbiosis in the Sea* (Vernberg, W. B., ed.) p. 245. University of South Carolina Press, Columbia, S.C.
27. Taylor, D. L. (1973) *Ann. Rev. Microbiol.* **27**, 171.
28. Taylor, D. L. (1975) in *Symposia Soc. Exp. Biol.*, (Jennings, D. H. and Lee, D. L., eds.) No. 29, Symbiosis p. 267. Cambridge University Press, Cambridge.
29. Loeblich III, A. R. and Sherley, J. L. (1979) *J. Mar. Biol. Assoc. U.K.* **59**, 195.
30. Kinzie III, R. A. and Chee, G. S. (1979) *Biol. Bull.* **156**, 315.
31. Schoenberg, D. A. and Trench, R. K. (1980) *Proc. R. Soc. London Ser. B* **207**, 445.
32. Rohmer, M., Kokke, W. C. M. C., Fenical, W. and Djerassi, C. (1980) *Steroids* **35**, 219.
33. Bohlin, L., Kokke, W. C. M. C., Fenical, W. and Djerassi, C. (1981) *Phytochemistry* **20**, 2397.
34. Gilpin, R. K., Korpi, J. A. and Janicki, C. A. (1975) *Analyt. Chem.* **47**, 1498.
35. Kokke, W. C. M. C., Pak, C. S., Fenical, W. and Djerassi, C. (1979) *Helv. Chim. Acta* **62**, 1310.
36. Idler, D. R. and Safe, L. M. (1972) *Steroids* **19**, 315.