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4α-METHYL-24*S*-ETHYL-5α-CHOLESTAN-3β-OL AND 4α-METHYL-24*S*-ETHYL-5α-CHOLEST-8(14)-EN-3β-OL, TWO NEW STEROLS FROM A CULTURED MARINE DINOFLAGELLATE

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Key Word Index—*Zooxanthella microadriatica*; *Briareum asbestinum*; *Muriceopsis flavida*; Dinophyceae; Gorgonaceae; phytoplankton; marine sterols; 4α-methyl-24*S*-ethyl-5α-cholestan-3β-ol; 4α-methyl-24*S*-ethyl-5α-cholest-8(14)-en-3β-ol.

Abstract—Twenty sterols of the cultured zooxanthellae (dinoflagellate symbionts) originally derived from the Caribbean gorgonian coral *Briareum asbestinum* were identified and found to include two new 4α-methyl sterols, 4α-methyl-24*S*-ethyl-5α-cholestan-3β-ol and 4α-methyl-24*S*-ethyl-5α-cholest-8(14)-en-3β-ol. The assigned structures are based upon 360 MHz ^{1}H NMR and MS analysis of these compounds. The 24*S*-configuration was confirmed by comparison with the 360 MHz ^{1}H NMR spectrum of the partially synthesized 4α-methyl-24*R*-ethyl-5α-cholestan-3β-ol.

INTRODUCTION

Several groups are currently investigating various aspects of the chemistry of cultured, unicellular marine algae (phytoplankton). For instance, carotenoid pigments are being studied by Liaaen-Jensen and her group [1], and their results have already proved to be useful in algal taxonomy. Others are investigating algal species (mainly dinoflagellates) which are known to be primary producers of toxins [2, 3], which are concentrated via the food-chain and which may cause disease or even death in humans who eat filter-feeding marine invertebrates. Our own interests in phytoplankton are based upon the need to define the primary sources of unusual as well as common sterols, which, via dietary accumulation, are concentrated and subsequently also modified by marine invertebrates.

Two groups of algae, the diatoms (class Bacillariophyceae) and the dinoflagellates (class Dinophyceae) are important producers of dietary sterols due to their great abundance in most marine ecosystems [4, 5]. Thus diatom and dinoflagellate sterols, unchanged or modified, should account for a high percentage of the sterols of a filter-feeding animal which does not synthesize its own sterols.

The recent literature includes only one publication concerning the sterols produced by a marine diatom [6], but there are several papers on dinoflagellate sterols [7–15].

RESULTS

In this paper, we wish to report the isolation and structure determination of two new C_{30}-sterols of MWs 430 and 428, respectively, from a cultured dinoflagellate. This dinoflagellate was isolated from the Caribbean gorgonian (phylum Cnidaria, class Anthozoa, order Gorgonaceae) *Briareum asbestinum* in which it was found to live in symbiosis.

The C_{30}-sterols have three more carbon atoms than cholesterol; both new sterols are 4α-methyl sterols as deduced from the presence of a characteristic sextet (*ddd*) at δ 3.1 in the ^{1}H NMR spectra ($CDCl_3$). These spectra also displayed a methyl triplet which indicated the presence of an ethyl substituent. There is no precedent for ethyl substituents in the ring system of sterols. For biosynthetic reasons, we assumed that these substituents were most likely located in the 24-position of the side chains. Indeed, the mass spectra of the two C_{30}-sterols proved that only one of the three additional carbons was located in the skeleton.

The mass spectrum of the sterol of MW 430 resembled that of 5α-cholestan-3β-ol, but two characteristic peaks were found at 14 mμ higher; at *m/z* 247 (M^+ − C-15, C-16, C-17 and side chain) [16] and *m/z* 229 (247 − H_2O), respectively. This is consistent with the presence of a 4α-methyl group. The ^{1}H NMR shifts of the C-18 and C-19 angular methyl groups were very similar to those of 4α-methyl-5α-cholestan-3β-ol, which indicated that the C_{30}-sterols had the normal 5α,14α-androstane skeleton.

The loss of the side chain associated with the peak [17–19] at *m/z* 287 ($C_{20}H_{31}O$) in the mass spectrum of the sterol of MW 428 proved that the only unsaturation was in the ring system, and that two of the three extra carbons had to be attached to the side chain. The absence of olefinic protons in the ^{1}H NMR spectrum limited the possible positions of the double bond to the $\Delta^{8(9)}$- or $\Delta^{8(14)}$-position. The Zürcher values [20] for the C-19 and the C-18 methyl groups in 5α-cholest-8(9)-en-3β-ol and 5α-cholest-8(14)-en-3β-ol are δ 0.933 and 0.567, and

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δ 0.691 and 0.825, respectively. A comparison of these Zürcher values with our experimental values (Table 1) indicates that the sterol of MW 428 has a $\Delta^{8(14)}$-double bond.

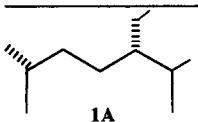
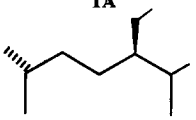
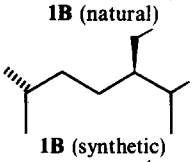
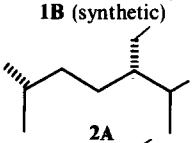
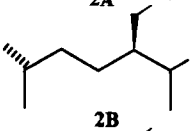
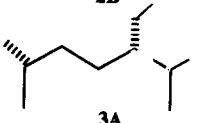
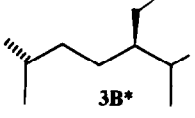
The remaining problem, the configuration at C-24, was solved by comparison of the 360 MHz ^{1}H NMR spectra of the partially synthesized 4α-methyl-24*R*-ethyl-5α-cholestan-3β-ol with **1A** derived from the *B. asbestinum* zooxanthellae and **1B** isolated [15] from a *Glenodinium* species. As shown in Table 1, there is an almost perfect correlation of the chemical shifts for the synthesized compound and **1B**, thus leading to the opposite 24*S*-configuration (**1A**) for the sterol with MW 430. Even though the $\Delta^{8(14)}$-double bond in **2A** and **2B** changes the chemical shifts for the C-18, C-19, C-21 and C-30 protons, sterol **2A** can be assigned the same 24*S*-configuration since it has the same pattern of chemical shifts as **1A** and **3A**. This leads to the structure 4α-methyl-24*S*-ethyl-5α-cholest-8(14)-en-3β-ol (**2A**) for the sterol with MW 428. The 24*S*-assignment for **1A** and **2A** is supported by the fact that clionasterol (**3A**) was also isolated from the same dinoflagellate (Table 3). The saturated sterol **1A**, but not its $\Delta^{8(14)}$-unsaturated analog, was also found in the sterol mixture of the cultured zooxanthellae isolated from the Caribbean gorgonian *Muriceopsis flavida* [21].

DISCUSSION

Previously we reported [15] the isolation and partial elucidation of the structures of 4α-methyl sterols of MWs 430 and 428 from the dinoflagellate *Glenodinium* sp. These sterols were tentatively identified as 4α-methyl-24ξ-ethyl-5α-cholestan-3β-ol (**1A** or **1B**) and 4α-methyl-24ξ-ethyl-5α-cholest-8(14)-en-3β-ol (**2A** or **2B**). We could not use analogy to assign the configuration at C-24 in these sterols because 24ξ-ethyl-cholest-5-en-3β-ol (**3A** or **3B**) was not found in this alga. The new sterols reported in this paper and the above *Glenodinium* sterols are different by 360 MHz ^{1}H NMR (Table 1) which together with a comparison of the ^{1}H NMR spectra to the synthetic compound (**1B**) shows that the *Glenodinium* sterols have the 24*R*-configuration.

As in the present case of the zooxanthellae of *B. asbestinum* (cf. Experimental), 4α-methyl sterols, rather than conventional 4-demethyl sterols, are usually the main components of the sterol mixtures produced by dinoflagellates [7–15]. Because of the abundance of dinoflagellates in phytoplankton—at times even surpassing diatoms in numbers—one must wonder why so few 4α-methyl sterols have been isolated from animals which feed on phytoplankton. The reason could be that these animals, even those unable to synthesize their own

Table 1. 360 MHz ^{1}H NMR data ($CDCl_3$) of three sterols of the zooxanthellae of *B. asbestinum* (**1A**, **2A**, **3A**), of two *Glenodinium* sterols (**1B**, **2B**), of a synthetic compound (**1B**), and of a reference compound (**3B**). (Shifts are δ values; coupling constants in Hz)

Structure Side chain	Nucleus	MW	C18-H	C19-H	C21-H	C26,27-H	C29-H	C30-H
 **1A**	Δ^0	430	0.646 (*s*)	0.823 (*s*)	0.907 (*d*) $J = 6.5$	0.808 (*d*) $J = 7.1$ 0.828 (*d*) $J = 7.0$	0.851 (*t*) $J = 7.4$	0.947 (*d*) $J = 6.4$
 **1B** (natural)	Δ^0	430	0.645 (*s*)	0.821 (*s*)	0.902 (*d*) $J = 6.5$	0.810 (*d*) $J = 7.7$ 0.831 (*d*) $J = 7.2$	0.841 (*t*) $J = 7.5$	0.946 (*d*) $J = 6.4$
 **1B** (synthetic)	Δ^0	430	0.646 (*s*)	0.822 (*s*)	0.902 (*d*) $J = 6.6$	0.811 (*d*) $J = 7.7$ 0.832 (*d*) $J = 7.0$	0.841 (*t*) $J = 7.5$	0.946 (*d*) $J = 6.5$
 **2A**	$\Delta^{8(14)}$	428	0.837 (*s*)	0.714 (*s*)	0.939 (*d*) $J = 6.5$	0.811 (*d*) $J = 6.6$ 0.829 (*d*) $J = 7.2$	0.857 (*t*) $J = 7.4$	0.989 (*d*) $J = 6.3$
 **2B**	$\Delta^{8(14)}$	428	0.838 (*s*)	0.714 (*s*)	0.935 (*d*) $J = 6.6$	0.815 (*d*) $J = 7.7$ 0.836 (*d*) $J = 7.4$	0.846 (*t*) $J = 7.7$	0.989 (*d*) $J = 6.3$
 **3A**	Δ^5	414	0.677 (*s*)	1.008 (*s*)	0.924 (*d*) $J = 6.5$	0.809 (*d*) $J = 7.0$ 0.829 (*d*) $J = 7.1$	0.853 (*t*) $J = 7.3$	—
 **3B***	Δ^5	414	0.676 (*s*)	1.008 (*s*)	0.919 (*d*) $J = 6.5$	0.813 (*d*) $J = 7.5$ 0.833 (*d*) $J = 7.4$	0.843 (*t*) $J = 7.5$	—

*Reference spectrum (360 MHz) was kindly supplied by Dr. M. Rohmer.

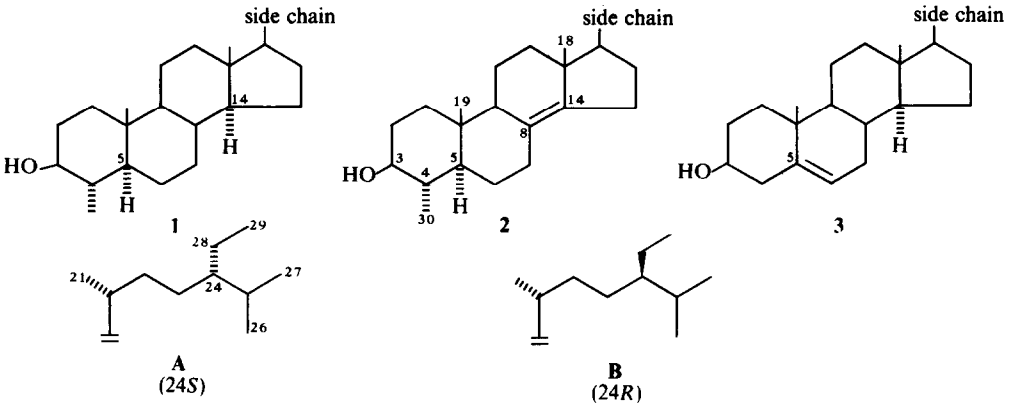
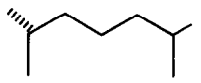
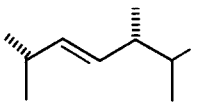
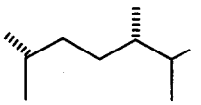
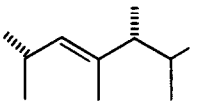
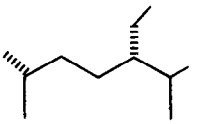
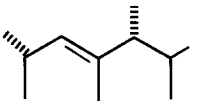
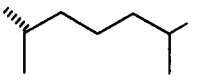
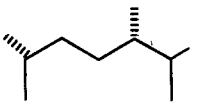
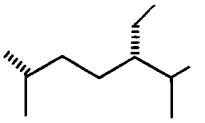


Table 2. Composition* of the free 4α-methyl sterol fraction of the zooxanthellae from *B. asbestinum*

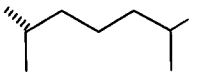
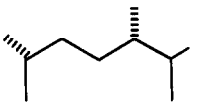
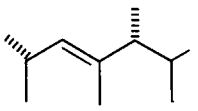
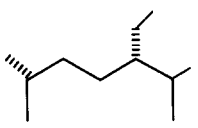
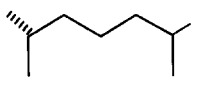
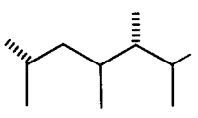
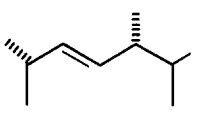
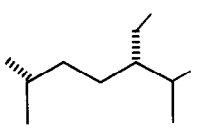
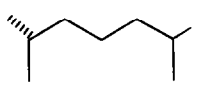
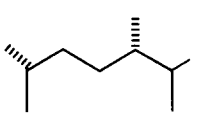
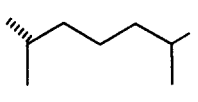
Structure Side chain	Nucleus	Name	MW	%	RR_t†
	Δ^0	4α-Methyl-5α-cholestan-3β-ol‡	402	02.	1.12
	Δ^0	4α,24*R*-Dimethyl-5α-cholest-22-en-3β-ol‡	414	1.0	1.27
	Δ^0	4α,24*S*-Dimethyl-5α-cholestan-3β-ol	416	15.5	1.46
	Δ^0	Dinosterol	428	48.4	1.56
	Δ^0	4α-Methyl-24*S*-ethyl-5α-cholestan-3β-ol	430	14.6	1.83
	Δ^5	5-Dehydrodinosterol	426	6.9	1.61
	$\Delta^{8(14)}$	4α-Methyl-5α-cholest-8(14)-en-3β-ol‡	400	0.5	1.12
	$\Delta^{8(14)}$	4α,24*S*-Dimethyl-5α-cholest-8(14)-en-3β-ol	414	1.8	1.46
	$\Delta^{8(14)}$	4α-Methyl-24*S*-ethyl-5α-cholest-8(14)-en-3β-ol	428	11.1	1.83

*An average of three runs.

†Gas chromatographic conditions: 3% SP-2250, 260°; standard cholesterol.

‡Not isolated, but identified on the basis of its mass spectrum and its RR_t on GC. In applicable cases the configuration at the double bond in the side chain and/or the configuration at C-24 was assigned using analogy.

Table 3. Composition* of the free 4-demethyl sterol fraction of the zooxanthellae from *B. asbestinum*

Structure: Side chain	Structure: Nucleus	Name	MW	%	RR_f†
	Δ^0	5α-Cholestan-3β-ol‡	388	2.1	1.00
	Δ^0	24*S*-Methyl-5α-cholestan-3β-ol‡	402	1.4	1.29
	Δ^0	4-Demethyldinosterol‡	414	0.6	1.37
	Δ^0	24*S*-Ethyl-5α-cholestan-3β-ol‡	416	0.7	1.61
	Δ^5	Cholest-5-en-3β-ol	386	19.1	1.00
	Δ^5	24*S*-Methylcholest-5-en-3β-ol	400	16.1	1.29
	Δ^5	4-Demethyl-5-dehydrodinosterol‡	412	3.7	1.37
	Δ^5	24*S*-Ethylcholest-5-en-3β-ol	414	5.1	1.61
	$\Delta^{5,7}$	Cholesta-5,7-dien-3β-ol	384	35.9	1.17
	$\Delta^{5,7}$	24*S*-Methylcholesta-5,7-dien-3β-ol	398	14.1	1.52
	Δ^7	5α-Cholest-7-en-3β-ol‡	386	1.2	1.16

*An average of three runs.

†Gas chromatographic conditions: 3% SP-2250, 260°; standard cholesterol.

‡Not isolated, but identified on the basis of its mass spectrum and its RR_f on GC. In applicable cases the configuration at the double bond in the side chain and/or the configuration at C-24 was assigned using analogy.

sterols, have the ability to remove the 4-methyl group from dietary sterols.

Although $\Delta^{8(14)}$-unsaturated sterols, such as **2A**, are rare, dinoflagellates are apparently a potential source of this type of sterol. In the dinoflagellates *Amphidinium carterae* and *A. corpulentum* [12,15] they are the end products of sterol biosynthesis.

EXPERIMENTAL

For a description of the instruments used, for details of the isolation and mass-culture of zooxanthellae, and for analytical methods used to separate the sterol components, the reader is referred to another publication [21] in which, *inter alia*, part of our work on this dinoflagellate has been reported. In this previous publication [21] we did not give the abundances of all

the identified components, nor did we assign the configuration at the double bond and/or at the asymmetric centers in the side chain. For this reason detailed information on the sterol composition of the zooxanthellae from *B. asbestinum* follows (see Tables 2 and 3). ^{1}H NMR data of some compounds listed in these tables, viz. 4α,24*S*-dimethyl-5α-cholestan-3β-ol, 4α,24*R*-dimethyl-5α-cholest-22-en-3β-ol (W. C. Dow *et al.*, unpublished results) and 4-demethyldinosterol, (N. W. Withers *et al.*, unpublished results) have not yet been reported in the literature, but they are known compounds.

4α-Methyl sterols are the dominant sterols of the zooxanthella of *B. asbestinum*. The ratio of 4α-methyl sterols to 4-demethyl (= regular) sterols is 88:12. The 4α-methyl sterols were separated by reverse-phase HPLC [22]; in the case of some components, e.g. **2A**, final purification by prep. GLC was necessary (3% OV-25, 265°). The first step in the isolation of the 4-demethyl sterols was $AgNO_3$–Si gel TLC [23] of their acetates to give three fractions: the sterols with a saturated ring system, the sterols with one double bond in the ring, and the 5,7-dienes. Further work-up (reverse-phase HPLC) of the first fraction (after saponification) was not practical because of its small size.

Mass spectra of the new sterols, **1A** and **2A**, and of their epimers **1B** and **2B**, are essentially identical. High resolution mass spectral data of the latter compounds have been published [15]. For 360 MHz ^{1}H NMR data see Table 1.

4α-Methyl-24*S*-ethyl-5α-cholestan-3β-ol (**1A**). Mp 178–181° from MeOH, $[\alpha]_D$ +19° ($CHCl_3$, *c* 0.3). High resolution GC/MS 70 eV, *m/z* (assignment, rel. int.): 430.4123 ($C_{30}H_{54}O$, M^+, 100); 415.3948 ($C_{29}H_{51}O$, 23); 412.4092 ($C_{30}H_{52}$, 7); 397.3806 ($C_{29}H_{49}$, 11); 290.3010 ($C_{21}H_{38}$, 15); 249.2149 ($C_{17}H_{29}O$, 9); 248.2088 ($C_{17}H_{28}O$, 50); 247.2027 ($C_{17}H_{27}O$, 49); 231.2078 ($C_{17}H_{27}$, 13); 230.2004 ($C_{17}H_{26}$, 20); 229.1937 ($C_{17}H_{25}$, 44).

4α-*Methyl*-24*S*-*ethyl*-5α-*cholest*-8(14)-*en*-3β-*ol* (**2A**). High resolution GC/MS 70 eV, *m/z* (assignment, rel. int.): 428.3956 ($C_{30}H_{52}O$, M^+, 100); 413.3687 ($C_{29}H_{49}O$, 19); 410.3899 ($C_{30}H_{50}$, 3); 395.3717 ($C_{29}H_{47}$, 4); 287.2392 ($C_{20}H_{31}O$, 27); 269.2281 ($C_{20}H_{29}$, 10); 261.2202 ($C_{18}H_{29}O$, 4); 260.2134 ($C_{18}H_{28}O$, 6); 245.1941 ($C_{17}H_{25}O$, 11); 243.2065 ($C_{18}H_{27}$, 10); 227.1786 ($C_{17}H_{23}$, 9).

4α-*Methyl*-24R-*ethyl*-5α-*cholestan*-3β-*ol* (**1B**, *synthetic*). Formed by hydrogenation of the partially synthesized (A. Shu, unpublished observation) 4α-methyl-24*S*-ethyl-5α-cholest-22-en-3β-ol (44 mg) over platinum oxide (40 mg) in *n*-hexane–HOAc (4:1.5 ml) at 20° under a slight positive pressure of hydrogen for 12 hr. After recrystallization from MeOH the substance exhibited mp 185–186°, $[\alpha]_D$ +25° ($CHCl_3$, *c* 1.3). High resolution GC/MS 70 eV, *m/z* (assignment, rel. int.): 430.4172 ($C_{30}H_{54}O$, M^+, 100); 415.4001 ($C_{29}H_{51}O$, 19); 412.4079 ($C_{30}H_{52}$, 31); 397.3879 ($C_{29}H_{49}$, 19); 290.2984 ($C_{21}H_{38}$, 9); 271.2414 ($C_{20}H_{31}$, 10); 249.2123 ($C_{17}H_{29}O$, 8); 248.2114 ($C_{17}H_{28}O$, 27); 247.2044 ($C_{17}H_{27}O$, 33); 231.2076 ($C_{17}H_{27}$, 21); 230.2037 ($C_{17}H_{26}$, 17); 229.1940 ($C_{17}H_{25}$, 42); 215.1810 ($C_{16}H_{23}$, 11).

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