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STEROLS WITH UNUSUAL NUCLEAR UNSATURATION FROM THREE CULTURED MARINE DINOFLAGELLATES

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Key Word Index—*Amphidinium carterae*; *A. corpulentum*; *Glenodinium* sp.; Dinophyceae; marine sterols; phytoplankton.

Abstract—Several new 4α-methyl sterols with unusual unsaturation in the $\Delta^{8(14)}$- or Δ^{14}-positions, 4α,24*S*-dimethyl-5α-cholest-8(14)-en-3β-ol, 4α-methyl-24ξ-ethyl-5α-cholest-8(14)-en-3β-ol, 4α-methyl-24(*Z*)-ethylidene-5α-cholest-8(14)-en-3β-ol, 4α,23(or 22),24ξ-trimethyl-5α-cholesta-8(14),22-dien-3β-ol, 4α,24*S*(or 23ξ)-dimethyl-5α-cholest-14-en-3β-ol and 14-dehydrodinosterol, have been isolated from extracts of the cultured marine dinoflagellates *Amphidinium carterae*, *A. corpulentum* and *Glenodinium* sp. 4α-Methyl-24ξ-ethyl-5α-cholestan-3β-ol was isolated from the steryl ester fraction of *Glenodinium* sp. The structures of these new sterols are based upon extensive 360 MHz ^{1}H NMR and MS analyses.

INTRODUCTION

The unusually complex sterol mixtures found in many marine invertebrates are known to reflect their efficient dietary accumulation of these compounds. In the gorgonian *Plexaura homomalla*, for example, over 50 of the approximately 100 known marine sterols have been detected [1]. The foundation of the complex marine food web are unicellular algae (phytoplankton) which, while being composed of at least eight plant divisions, are often dominated in nature by certain abundant groups. The frequently observed 'red tides', for example, represent blooms of largely dinoflagellates (Pyrrophyta, Dinophyceae) which discolor seawater to a reddish hue. In attacking the problem of the bio-origin of sterols in various marine animals—especially in an attempt to differentiate between dietary intake and endogenous biosynthesis—one must have knowledge of the sterol distribution in the most likely food sources.

Only limited information is available concerning the sterol components of marine phytoplankton. However, recent reports from this and other laboratories have defined serveral novel sterols with unprecedented side chain alkylation patterns [2–4] and with unsaturation at the $\Delta^{8(9)}$- and $\Delta^{17(20)}$-positions [5–7]. Despite the fact that early intermediates in sterol diagenesis, such as lanosterol (**1**) and obtusifoliol (**2**) have a $\Delta^{8(9)}$-unsaturation, naturally occurring sterols with a double bond in this position are rare [5, 8–12]. Sterols with the $\Delta^{8(14)}$-unsaturation are also rare and only a few terrestrial examples are known [11–15]. Very recently the structure of a new $\Delta^{8(14)}$-unsaturated sterol, amphisterol (**4g**), was reported as the major sterol from the cultured dinoflagellate *Amphidinium carterae* [16].

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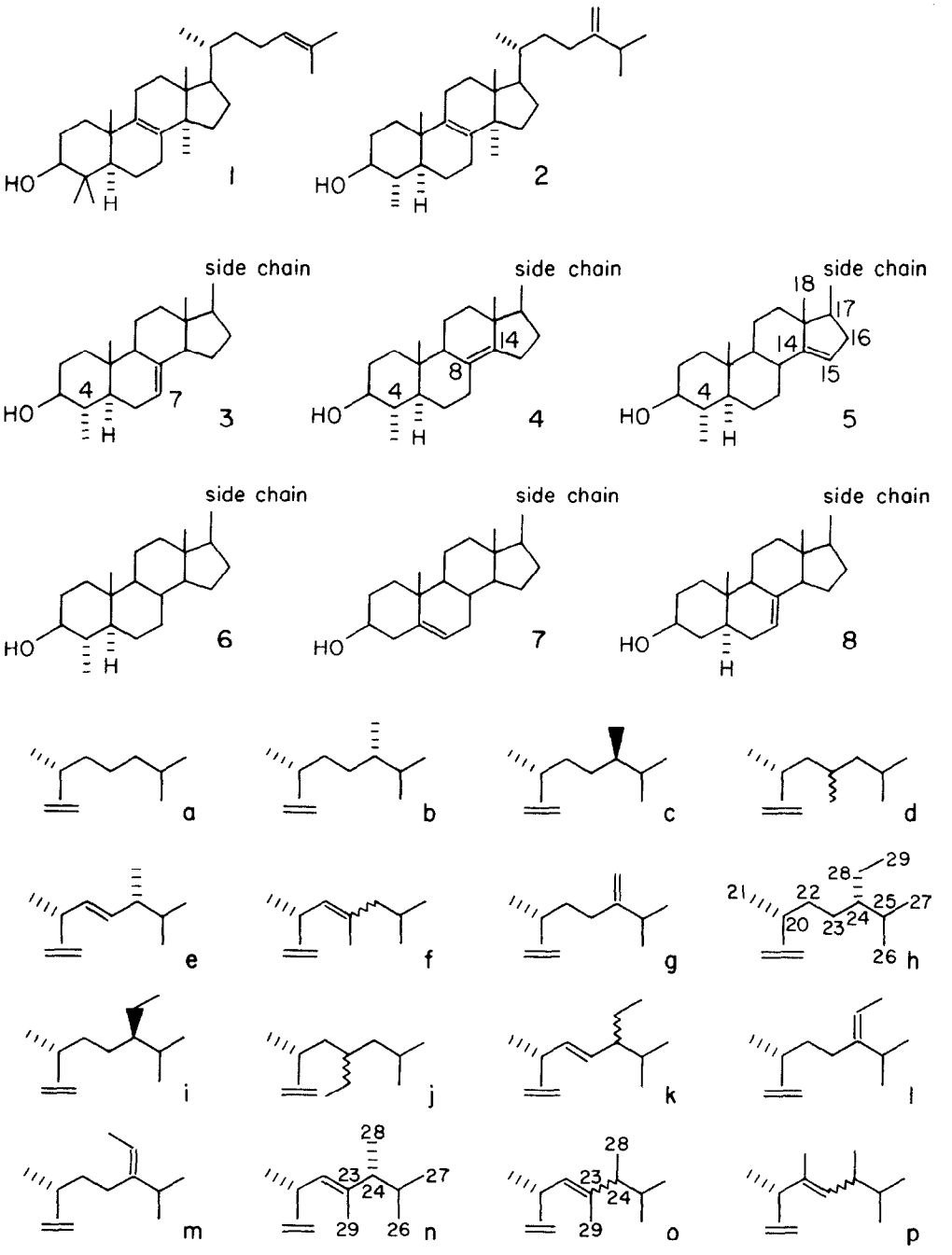
RESULTS

We now wish to report the results of our investigation of the sterol constituents of three cultured marine dinoflagellates, *Amphidinium carterae* Hulburst, *A. corpulentum* Kofoid and Swezy and *Glenodinium* sp. ex Provasoli (1971). These dinoflagellates produce preponderant quantities of 4α-methyl sterols with unique unsaturations in the $\Delta^{8(14)}$- and Δ^{14}-positions and specifically include the new sterols 4α,24*S*-dimethyl-5α-cholest-8(14)-en-3β-ol (**4b**), 4α-methyl-24ξ-ethyl-5α-cholest-8(14)-en-3β-ol (**4h** or **4i**), 4α-methyl-24(*Z*)-ethylidene-5α-cholest-8(14)-en-3β-ol (**4l**), 4α,23(or 22),24ξ-trimethyl-5α-cholesta-8(14),22-dien-3β-ol (**4o** or **4p**), 4α,24*S*(or 23ξ)-dimethyl-5α-cholest-14-en-3β-ol (**5b** or **5d**) and 14-dehydrodinosterol (**5n**). In addition, the dinoflagellate *Glenodinium* sp. produces the unreported saturated sterol 4α-methyl-24ξ-ethyl-5α-cholestan-3β-ol (**6h** or **6i**) in small quantities as a steryl ester component. The sources and abundances of these sterols are listed in Table 1.

Structure elucidation of the new sterols

Dinoflagellates differ from all other groups of marine and terrestrial algae in that their major and often exclusive, sterol components are 4α-methyl sterols ([2–4, 6, 16–18]; W. Fenical *et al.*, unpublished results). These components are readily recognized spectrally by the characteristic ^{1}H NMR signal of the C-3α proton at δ 3.1 ($CDCl_3$) which appears as a predictable sextet [19] and this was the method used to demonstrate the presence of 4α-methyl sterols. The sterols isolated here also possess the normal 5α,14α-androstane skeleton, as illustrated by the close agreement of the measured angular methyl ^{1}H NMR shifts with those calculated for the normal ring system using Zürcher's rules [20].



$\Delta^{8(14)}$-*Unsaturated sterols*

4α-Methyl sterols with unsaturation in the $\Delta^{8(14)}$-position are produced by all three species studied and consisted of sterols with molecular weights of 400, 412, 414, 426 (2 ×) and 428 (C_{28}, C_{29} and C_{30}, respectively). Only the first two, 4α-methyl-5α-cholest-8(14)-en-3β-ol (**4a**) and amphisterol (**4g**), have been reported before [15,16]. These compounds showed MS fragments associated with loss of the side chain at *m/z* 287 or 285, illustrating the presence of either one double bond in the ring, or of two double bonds, one in the ring and the other in the side chain [21]. The 360 MHz ^{1}H NMR spectra of these sterols illustrated only olefinic protons attributable to side chain unsaturation. Hence, the nuclear double bond could only be located at the $\Delta^{8(9)}$- or $\Delta^{8(14)}$-positions. MS fragmentation cannot be used to locate the $\Delta^{8(9)}$-double bond [22], but $\Delta^{8(14)}$-unsaturation usually results in a characteristic peak [23] associated with the loss of the side chain and the nuclear carbons 16 and 17 (*m/z* 261 in the case of the presently discussed sterols). As this peak is present, but observed in low intensity, the $\Delta^{8(14)}$-assignment was reinforced by a definitive ^{1}H NMR study of the C-18 and C-19 methyl groups in comparison to the predicted values for $\Delta^{8(14)}$-unsaturation from Zürcher's rules [20]. For a normal 3β-hydroxy sterol (cholesterol side chain) with a $\Delta^{8(9)}$-double bond, the calculated values for C-18 and C-19 are δ 0.567

Table 1. Abundance (%) of sterol components of three marine dinoflagellates

Trivial or systematic names	*Amphidinium carterae* free sterols	*Amphidinium corpulentum* free sterols	*Glenodinium* sp. free sterols	*Glenodinium* sp. steryl esters	GC: RR_f*
Lophenol (**3a**)	0.9				1.33
4α-Methyl-5α-cholest-8(14)-en-3β-ol (**4a**)	26.0	26.6		4†	1.14
4α,24*S*-Dimethyl-5α-cholest-8(14)-en-3β-ol (**4b**)				18	1.53
Amphisterol (**4g**)	42.7	58.1			1.53
4α-Methyl-24ξ-ethyl-5α-cholest-8(14)-en-3β-ol (**4h** or **4i**)				15	1.91
4α-Methyl-24(*Z*)-ethylidene-5α-cholest-8(14)-en-3β-ol (**4l**)	1.3				2.03
4α,23(or 22),24ξ-Trimethyl-5α-cholesta-8(14),22-dien-3β-ol (**4o** or **4p**)	12.0	4.6			1.56
4α,24*S*(or 23ξ)-Dimethyl-5α-cholest-14-en-3β-ol (**5b** or **5d**)			0.3		1.48
14-Dehydrodinosterol (**5n**)			0.7	6	1.61
4α-Methyl-5α-cholestan-3β-ol (**6a**)			0.6†		1.12
4α,24*S*-Dimethyl-5α-cholestan-3β-ol (**6b**)			15.3	17	1.49
4α,24*R*-Dimethyl-5α-cholest-22-en-3β-ol (**6e**)			1.0	4	1.30
4α-Methyl-24ξ-ethyl-5α-cholestan-3β-ol (**6h** or **6i**)				4	1.84
Dinosterol (**6n**)			79.2	30	1.60
Cholesterol (**7a**)	8.3	4.8		2†	1.00
Other desmethyl sterols (cf. Experimental)	6.3				

* Gas chromatographic conditions: 3% SP2250, 260°; standard cholesterol.
† Not isolated; identified by GC–MS and on the basis of its GLC retention time.

and 0.933, respectively. For $\Delta^{8(14)}$-unsaturation the values are grossly different: C-18, 0.825; C-19, 0.691. As the calculated values for a $\Delta^{8(14)}$-double bond are in close accord with those observed (Table 2), the new sterols are assigned structures **4b**, **4h** or **4i**, **4l** and **4o** or **4p**.

The *Glenodinium*-derived sterol, to which we assign the 24-methyl structure **4b**, could not be differentiated by spectral methods from the 23-methyl isomer **4d**. Structure **4d** had to be taken into consideration because dinoflagellates are known [3, 4] to have the biosynthetic potential to produce sterols alkylated in the 23-position only. Fortunately, it was possible to prepare a reference sample. Hydrogenation of amphisterol (**4g**) yielded a 1:1 mixture of **4b** and **4c** (as determined by ^{1}H NMR, Table 2), which clearly located the methyl substituent at C-24. We assign the 24*S* stereochemistry by analogy to this consistent feature found in 4α,24*S*-dimethyl-5α-cholestan-3β-ol (**6b**) (W. C. Dow *et al.*, unpublished observation) and dinosterol (**6n**) [24] which are the main free sterols of *Glenodininum* sp. (Table 1). This assignment is also supported by the equivalence of the C-28 methyl and one of the isopropyl methyls in the ^{1}H NMR spectrum of this natural product [25].

The *Amphidinium carterae*-derived sterol of MW 426 was assigned structure **4l** based upon its characteristic ^{1}H NMR spectral features. Specifically, an olefinic quartet at δ 5.12 was found for the ethylidene proton, as well as the C-25 allylic isopropyl methine proton at 2.8. This latter shift is known to characterize the 24(*Z*)-isofucosterol (**l**) rather than the 24(*E*)-fucosterol (**m**) side chain [26, 27].

The *Amphidinium*-derived sterol, also of MW 426, was assigned structure **4o** or **4p** (probably **4o**) based upon its ^{1}H NMR and mass spectral features. The characteristic ^{1}H NMR features for the dinosterol side chain (**n**), as defined by spin-decoupling experiments, were clearly present in this molecule (Table 2). Another structure, however, **4p** cannot be rigorously excluded from consideration. The MS fragmentation pattern strongly supports structure **4o** because of its similarity to the fragmentation of dinosterol (**6n**) [18]. In particular, the $(M^+ - 71)$ peak is a prominent feature resulting from C-23–C-24 vinylic cleavage. While fragmentation of this type is not precluded in structure **4p**, the $(M^+ - 71)$ peak is not observed in the MS of sterol **7e**, where the C-23 methyl substituent is absent.

The sterol of MW 428, isolated from the *Glenodinium* sterol ester fraction, is assigned structure **4h** or **4i** based upon ^{1}H NMR and mass spectral analysis. The two additional carbons were clearly established as an ethyl functionality by the observation of a methyl triplet centered at δ 0.846 in the ^{1}H NMR spectrum.

Δ^{14}-*Unsaturated sterols*

Two new sterols possessing unusual Δ^{14}-unsaturation were isolated as minor sterols of *Glenodinium* sp. These sterols had MWs of 414 and 426, respectively, and the acetates of both were found to possess unusually low R_f values on argentic Si gel TLC. While their low R_f values were initially taken to indicate methylene sterols, the ^{1}H NMR spectra of these compounds indicated that they were not methylene sterols but sterols with unusual ring unsaturation. The MS of the MW 414 sterol possessed an intense fragment at *m/z* 287 associated with side chain cleavage. It is known that in Δ^{14}-unsaturated steranes and sterols loss of side chain is facilitated [28] and indeed, the MS of this sterol and that of 4α-methyl-5α-cholest-14-en-3β-ol (**5a**) [29] are identical except for the molecular ion. Also, the ^{1}H NMR shifts for the C-18 and C-19 methyl protons in **5a** (δ 0.90 and 0.85) [30, 31] are in close agreement (Table 2) to those measured here (0.895 and 0.845). As this sterol possesses an additional methyl substituent in the side chain, we suggest the structure 4α,24*S*(or 23ξ)-dimethyl-5α-cholest-14-en-3β-ol (**5b** or **5d**).

The MS of the Δ^{14}-sterol of MW 426 shows the same side chain fragmentation as dinosterol (**6n**) [18]; the ^{1}H NMR spectrum (Table 2) of this new sterol in deuteriochloroform also shows prominent features supporting the dinosterol (**n**) side chain. Unfortunately, decoupling experiments to confirm this assignment were complicated by overlapping

Table 2. 360 MHz ^{1}H NMR spectral data ($CDCl_3$) of selected 4α-methyl sterols from the three algae and of reference compounds (shifts are δ values)

	C3-H	C15-H	C20-H	C22-H	C25-H	C4-Me	C18-H	C19-H	C21-H	C26,27-H	C28-H	C29-H
4a	3.1 (*m*)					0.988 (*d*) *J* = 6.3	0.837 (*s*)	0.714 (*s*)	0.929 (*d*) *J* = 6.5	0.865 (6 H, *d*) *J* = 6.3		
4b (natural)	3.1 (*m*)					0.987 (*d*) *J* = 6.4	0.835 (*s*)	0.713 (*s*)	0.932 (*d*) *J* = 6.8	0.782 (*d*) *J* = 6.8 0.854 (*d*) *J* = 6.8	0.782 (*d*) *J* = 6.8	
4b (hydrogenated **4g**)	3.1 (*m*)					0.988 (*d*) *J* = 6.3	0.836 (*s*)	0.713 (*s*)	0.931 (*d*) *J* = 6.5	0.781 *J* = 6.1 0.849 (*d*) *J* = 9.3	0.781 (*d*) *J* = 6.1	
4c (hydrogenated **4g**)	3.1 (*m*)					0.988 (*d*) *J* = 6.3	0.836 (*s*)	0.713 (*s*)	0.925 (*d*) *J* = 6.6	0.801 (*d*) *J* = 6.2 0.849 (*d*) *J* = 9.3	0.781 (*d*) *J* = 6.1	
4g	3.1 (*m*)				2.2 (*m*)	0.986 (*d*) *J* = 6.4	0.839 (*s*)	0.712 (*s*)	0.961 (*d*) *J* = 6.7	1.021 (*d*) *J* = 6.8 1.026 (*d*) *J* = 6.8	4.66 (*s*) 4.72 (*s*)	
4h or **4i**	3.1 (*m*)					0.989 (*d*) *J* = 6.3	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
4l	3.1 (*m*)				2.83 (*m*)	0.988 (*d*) *J* = 5.6	0.841 (*s*)	0.714 (*s*)	0.958 (*d*) *J* = 5.9	0.976 (6 H, *d*) *J* = 6.6	5.12 (*q*)	1.592 (*d*) *J* = 6.8
4o*	3.1 (*m*)		2.4 (*m*)	4.93 (*d*) *J* = 9.8		0.986 (*d*) *J* = 6.3	0.861 (*s*)	0.715 (*s*)	0.949 (*d*) *J* = 6.6	0.782 (*d*) *J* = 6.6 0.849 (*d*) *J* = 6.9	0.943 (*d*) *J* = 6.8	1.507 (*s*)
5b*	3.1 (*m*)	5.15 (*m*)				0.966 (*d*) *J* = 6.5	0.895 (*s*)	0.845 (*s*)	0.912 (*d*) *J* = 6.4	0.785 (*d*) *J* = 6.2 0.855 (*d*) *J* = 6.9	0.785 *J* = 6.9	
5n	3.1 (*m*)	5.12 (*s*)		4.91 (*d*) *J* = 9.		0.965 (*d*) *J* = 6.4	0.945 (*s*)	0.847 (*s*)	0.937 (*d*)† *J* = 5.7	0.788 (*d*) *J* = 6.5 0.845 (*d*) *J* = 6.5	0.942 (*d*)† *J* = 6.9	1.514 (*d*) *J* = 1.2
6h or **6i**	3.1 (*m*)					0.946 (*d*) *J* = 6.4	0.645 (*s*)	0.821 (*s*)	0.902 (*d*) *J* = 6.4	0.810 (*d*) *J* = 7.7 0.831 (*d*) *J* = 7.2		0.841 (*t*) *J* = 7.5
6n	3.1 (*m*)		2.33 (*m*)	4.87 (*d*) *J* = 9.4		0.946 (*d*) *J* = 6.1	0.680 (*s*)	0.827 (*s*)	0.919 (*d*) *J* = 6.5	0.778 (*d*) *J* = 6.6 0.836 (*d*) *J* = 6.6	0.928 (*d*) *J* = 6.6	1.496 (*d*) *J* = 1.2

* Assigned as if this were the correct structure; there is an alternate structure (cf. text).
† These assignments may have to be interchanged.

Table 3. 360 MHz ^{1}H NMR spectral data (C_6D_6) for 14-dehydrodinosterol (**5n**) and dinosterol (**6n**) (shifts are δ values)

	C3-H	C15-H	C20-H	C22-H	C4-Me	C18-H	C19-H	C21-H	C29-H	C28-H	C26,27-H
5n	2.86 (*m*)	5.25 (*s*)	2.68 (*m*)	5.06 (*d*) $J = 9.7$	0.979 (*d*) $J = 6.4$	1.028 (*s*)	0.725 (*s*)	1.083 (*d*) $J = 6.5$	1.551 (*d*) $J = 1.2$	1.019 (*d*) $J = 6.4$	0.903 (*d*) $J = 6.5$
6n	2.90 (*m*)		2.47 (*m*)	5.04 (*d*) $J = 9.6$	0.999 (*d*) $J = 6.4$	0.750 (*s*) *	0.739 (*s*) *	1.090 (*d*) $J = 6.5$	1.537 (*s*)	1.028 (*d*) $J = 6.7$	0.908 (*d*) $J = 6.8$

* These assignments may have to be interchanged.

bands. However, in deuteriobenzene (Table 3) all bands were observed and each could be interrelated. The Δ^{14}-unsaturation of this sterol is strongly supported by the chromatographic behavior of its acetate, and by spectral data, particularly by the shift (Table 2) of the C-19 methyl group (δ 0.847) which is in good accord with those from **5a** [30] and **5b** (or **5d**). The C-18 methyl signals are far apart (δ 0.945 vs 0.895 for **5b**), but this must be due to shielding by the Δ^{22}-double bond. There is a similar difference in the shift of the C-18 methyl signal in the ^{1}H NMR spectrum of dinosterol (**6n**) (δ 0.680) and of 4α-methyl-5α-cholestan-3β-ol (**6a**) (0.645) [4]. On the basis of these data, we assign the structure 4α,23,24*R*-trimethyl-5α-cholesta-14,22*E*-dien-3β-ol(14-dehydrodinosterol) (**5n**) to this new sterol.

Saturated sterol

One of the new sterols isolated from *Glenodinium* sp. was a saturated sterol of MW 430 (C_{30}). Since it had the usual 4α-methyl sterol nucleus, two additional carbons must be present in the side chain because one of these carbons appeared as a triplet at δ 0.841. On biosynthetic grounds we propose the structure 4α-methyl-24ξ-ethyl-5α-cholestan-3β-ol (**6h** or **6i**) for this compound. The 24*R* epimer (**6h**) is a known sterol [19] but we have been unable to perform a direct comparison of the two specimens.

DISCUSSION

The free sterol composition (Table 1) of *A. carterae* and *A. corpulentum* is qualitatively identical except for a higher content of trace sterols in the former. Our results are in good agreement with the report [16] that amphisterol (**4g**) is the major sterol of *A. carterae* and of other *Amphidinium* species.

It has been pointed out [1] that it might be advantageous to work separately on free sterols and on steryl esters (rather than on the free sterols only, or on the total sterols obtained by saponification of an extract or of a whole organism) because the two fractions might have a different sterol composition. As is illustrated in Table 1, *Glenodinium* sp. is an example where this more careful approach has been beneficial. From the mixture of free sterols, we isolated four components: a main sterol (dinosterol, **6n**), a minor sterol (4α,24*S*-dimethyl-5α-cholestan-3β-ol, **6b**) and two trace sterols, **5b** (or **5d**) and **5n**. Only the trace sterols are new. In contrast, the steryl ester fraction contained four main sterols (including two new ones: **4b** and **4h** or **4i**) and a number of minor sterols (including two new ones: **5n** and **6h** or **6i**).

The $\Delta^{8(14)}$-unsaturated 4α-methyl sterols account for 97% of the identified 4α-methyl sterols and for *ca* 85% of the total free sterols of *A. carterae*. What makes *A. carterae* unusual is the fact that although it has the ability to effect normal sterol biosynthesis—assorted Δ^5-sterols are present (cf. Experimental)—the bulk of the sterols have a $\Delta^{8(14)}$-double bond. Their overwhelming preponderence strongly suggests that they have a biological function, which in turn implies that the $\Delta^{8(14)}$-double bond plays a special role.

As far as the biosynthesis of sterols with a $\Delta^{8(14)}$- or Δ^{14}-double bond is concerned, Schroepfer's [32] suggestion that a $\Delta^{8(14)}$-unsaturated compound might be formed as an intermediate in sterol biosynthesis when demethylation at C-14 takes place may apply in this instance. However, the presence of minor amounts of lophenol (**3a**) [33, 34], identified by ^{1}H NMR, in *A. carterae* raises the question whether $\Delta^{8(14)}$- and perhaps also Δ^{14}-sterols might not have arisen at a later biosynthetic stage from Δ^7-sterols by double bond migration for which there is ample chemical precedent [35].

In connection with our work on other dinoflagellates* the structure and stereochemistry of the side chain of 4α-methyl-24ξ-ethyl-5α-cholestan-3β-ol (**6h** or **6i**) is under investigation because, *a priori*, we cannot rule out the possiblity that the ethyl substituent is actually in the 23-position. Side chains with a 23-ethyl substituent (**j**) were unprecedented but a new sponge sterol (ficisterol) with a C-23 ethyl substituent has recently been isolated [36]. Also some dinoflagellates are known to produce sterols methylated in the 23-position only [3, 4]. Thus it is conceivable that there exist two possible routes for modification of such a side chain (**f**) either by alkylation at C-24 to produce a dinosterol-type side chain (**o**) [4], or further alkylation via a C-23 methylene substituent to generate a side chain with an ethyl group in the 23-position.

There is a remarkable difference in the free sterol concentration of certain dinoflagellates: from *Glenodinium* sp. we isolated 350.8 mg of free sterols/100 g of extracted cell material, whereas in the case of *A. carterae* and *A. corpulentum* only 51.1 and 53.4 mg/100 g of extracted material was obtained. This difference in sterol concentrations raised the question whether *Amphidinium* species contain other compounds which have the same biological function as sterols, e.g. hopanoids (G. Ourisson, P. Albrecht and M. Rohmer, unpublished results). However, our colleague Dr. M. Rohmer failed to detect hopanoids in *A. carterae*.

* 4α-Methyl sterols with an ethyl substituent in the side chain (MW 430) were also found in cultured zooxanthellae isolated from two species of gorgonians. These sterols and the sterol of the same MW reported in this paper are not identical by 360 MHz ^{1}H NMR (W. Fenical *et al.*, unpublished results).

EXPERIMENTAL

General. For isolation and monitoring of the purification we used Waters HPLC equipment (M6000 pump, UK6 injector, R401 differential refractometer, two μC_{18} columns (4 mm i.d. × 30 cm) in series (eluent MeOH–H_2O 92:8), two μ porasil columns (4 mm i.d. × 30 cm) in series (eluent C_6H_6–EtOAc, 95:5), a semi-prep. reverse phase column (3/8" i.d. × 60 cm) built [37] by Dr. Richard Izac at Scripps (eluent MeOH–H_2O, 92:8 or 96:4); a Hewlett–Packard 5710A and 402 gas chromatograph with flame ionization detector; a LKB 9000 GC/MS and a Varian HR220/Nicolet TT100 FT-^{1}H NMR instrument. The 360 MHz ^{1}H NMR spectra were run on a Brucker HX360. 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 aquisition and reduction. RR_fs on GLC were determined using a 3% SP2250 column at 260°. For prep. GLC we used a 3% OV25 column at 265°. Mps were measured on a Fisher–Johns melting point apparatus. Optical rotation was measured on a Perkin–Elmer 142 spectropolarimeter, but in most cases the angle of rotation of a new sterol was not determined because the size of the available sample was too small for a reasonably accurate determination. Cells were mass-cultured [38] in GPM medium [39] and were harvested by continuous centrifugation (Sharples centrifuge). Extraction was made by homogenizing the harvested cells in $CHCl_3$–MeOH, 1:1 (Waring blender). After standing overnight at room temp., the homogenate was filtered and the cell material on the filter was washed with $CHCl_3$–MeOH until the filtrate was almost colorless. The combined filtrate and the washings were taken to dryness and the residue was partitioned between $CHCl_3$ and H_2O. If necessary (if the organics in $CHCl_3$ and H_2O formed an emulsion, or if an appreciable amount of insoluble material was present), filtration through celite preceeded partitioning. The aq. layer was discarded and the extract (the residue of the $CHCl_3$ layer) was worked up by Si gel CC as described elsewhere [1]. The combined free sterol fractions were rechromatographed over florisil (eluents hexane–Et_2O, 2:1 and 1:1) to remove the green pigments.

Amphidinium carterae (PY-1) was cultured at 23° for 3 weeks in 7 batches of 200 l. each. Wet wt of cells: 291 g; yield of extracted cell material (air-dried): 80.2 g; yield of extract: 14.4 g. The 4-methyl sterol fractions of the florisil column afforded crude 4-methyl sterols as a solid. Purification and total or partial separation of the crude sterols was accomplished by reverse-phase HPLC. Three fractions were collected (the maximum of the first sterol peak has R_f 1.00): fr. A, R_f 0.91–1.06/fr. BC, R_f 1.06–1.17/fr. D, R_f 1.17–1.35. Combined yield: 35.0 mg. Fr. A consisted of pure amphisterol (**4g**); fr. BC consisted mainly of 4α-methylcholest-8(14)-en-3β-ol (**4a**), which was further purified by reinjection on HPLC (the front and the tails of the peak were cut). Fr. D was enriched in the minor sterol and in the trace sterols. It was reinjected on HPLC to remove traces of amphisterol (**4g**) which has about the same retention time on GLC as the minor sterol, 4α,23(or 22),24ξ-trimethylcholesta-8(14),22-dien-3β-ol (**4o** or **4p**). The latter sterol and two trace sterols, lophenol (**3a**) and 4α-methyl-8(14)-dehydroisofucostanol (**4l**) were then isolated by prep. GLC. The GLC trace of the latter sample (from which **4o** (or **4p**), **3a** and **4l** were isolated) was used to calculate the percentage of the minor sterol of MW 428 (and hence also of the main sterol of MW 412) in the starting mixture, assuming that the ratio of minor sterol to trace sterols had not changed during the HPLC experiments. The desmethyl sterols were initially overlooked because they were accompanied by large amounts of unsaturated fats and thus they did not show up in the ^{1}H NMR spectra of the corresponding fractions of the florisil column. After 18 months in the freezer, the fractions were rechromatographed (Si gel) to remove decomposed material; the sterols were freed from most of the non-sterols by reverse-phase HPLC (semi-prep. column, MeOH–H_2O, 96:4). Traces of 4-methyl sterols were removed by HPLC (porasil columns). Yield *ca* 6 mg. The GLC trace of this sample indicated the presence of at least nine components, but because of the small size of the sample only the two main components could be isolated (reverse-phase HPLC, prep. GLC) and they were identified by ^{1}H NMR. The desmethyl sterols were cholesterol (**7a**) (56.5%), 24ξ-methyl-22-dehydrocholesterol (**7e** or epimer) and lathosterol (**8a**) (together 4.8%), 24ξ-methylcholesterol (**7b** or **7c**) and 24-methylenecholesterol (**7g**) (together 7.4%), 24ξ-ethyl-22-dehydrocholesterol (**7k**) (6.4%), 24*R*-ethylcholesterol(**7i**, sitosterol) (19%), fucosterol (**7m**) and isofucosterol (**7l**) (together 6.0%). A 360 MHz ^{1}H NMR reference spectrum of **7i** was kindly supplied by Dr. M. Rohmer.

Amphidinium corpulentum (PY-3) was cultured for 13 days at 21° in 3 batches of 200 l. each. Wet wt of cells: 64.2 g; yield of extracted plant material (air-dried) 17.4 g; yield of extract 2.9 g. 220 MHz ^{1}H NMR spectroscopy of the residue of the combined 'sterol fractions' of the florisil column (125 mg) indicated the presence of saponifiables and also that the concentration of sterols was low. Saponification (2 N NaOH, 5 g/95% EtOH, 75 ml/reflux, 2hr) resulted in the formation of polymeric material which was separated from the neutral unsaponifiables by filtration through Si gel (eluent Et_2O). Yield of crude sterols *ca* 40 mg. This mixture was acetylated and used for $AgNO_3$–Si gel TLC [40]. Two bands appeared under long wave UV light: a faster moving band (R_f 0.47–0.63) and a slow moving band (R_f 0.16–0.29). From the latter band, after extraction and saponification, 5.4 mg of amphisterol (**4g**) (pure by GLC and 220 MHz ^{1}H NMR) was obtained. Work-up of the material from the other band afforded 3.9 mg of a mixture of cholesterol (**7a**), 4α-methylcholest-8(14)-en-3β-ol (**4a**) and 4α,23(or 22),24ξ-trimethylcholest-8(14),22-dien-3β-ol (**4o** or **4p**). The desmethyl and 4-methyl sterols of this mixture were separated by HPLC (μ porasil, C_6H_6–EtOAc, 95:5). Reverse-phase HPLC (μC_{18}, MeOH–H_2O, 92:8) of the resulting mixture of **4a** and **4o** (or **4p**) afforded pure **4a** and a mixture enriched in **4o** (or **4p**) from which the latter sterol was isolated by prep. GLC.

Glenodinium *sp.* (PY-33) was cultured for 14 days at 24° in 8 batches of 200 l. each. Wet wt of cells 254.4 g; yield of extracted cell material (air-dried) 74.2 g; yield of extract 12.7 g.

Sterols from steryl esters. The steryl ester fractions of the Si gel column were located using 220 MHz ^{1}H NMR. They were combined and saponified. The sterols were isolated from the saponified material by Si gel CC. Further purification and initial separation of the sterols was accomplished by reverse-phase HPLC (μC_{18}). Four main fractions were collected, each corresponding to one of the main sterols (max. of first sterol peak had R_f 1.00): fr. A, R_f 0.95–1.11/fr. B, R_f 1.11–1.19/fr. C, R_f 1.19–1.26/fr. D, R_f 1.26–1.36. In order not to overlook any minor or trace sterols, which might not give a detectable peak on HPLC, also a front (R_f 0.60–0.95) and a tails fraction (R_f 1.36–1.55) were collected and combined (fr. FT). Combined yield of fr. A–D: 17.0 mg. Fr. A was re-injected on HPLC to give a main fraction and a tails fraction (R_f 1.02–1.17). The main component of the main fraction, 4α,24*S*-dimethylcholest-8(14)-en-3β-ol (**4b**) and the minor component, 14-dehydrodinosterol (**5n**), were separated by prep. GLC. The tails fraction consisted of about equal amounts of **4b** and of 4α,24*R*-dimethyl-22-dehydrocholestanol (**6c**), which were separated by prep. GLC. Fr. B consisted essentially of 4α-methyl-24ξ-ethylcholest-8(14)-en-3β-ol (**4h** or **4i**). It was further purified by reinjection on HPLC (in order to remove the only relevant impurity, viz. the main component of fr. C. Fr. C: dinosterol (**6n**). Fr. D: 4α,24*S*-dimethylcholestanol (**6b**). Three of the minor sterols ended up in fr. FT: cholesterol, 4α-methylcholest-8(14)-en-3β-ol (**4a**) and a sterol of MW 430. The

first two sterols were tentatively identified on the basis of their MS and their GLC R_t; the MW 430 sterol was isolated by prep. GLC and identified as 4α-methyl-24ξ-ethylcholestanol (**6h** or **6i**). The composition of the mixture of sterols from the steryl esters (cf. Table 1) is necessarily inaccurate as the starting mixture (9 components) gave 5 peaks on GLC, two of which were partially overlapping. This GLC trace and that of fr. FT (see above) and the HPLC trace of the starting mixture were used to calculate/estimate the composition of that mixture.

Free sterols. The free sterols were obtained as a semi-solid (262 mg). Fractional crystallization from MeOH afforded 219.9 mg of crystalline material which was acetylated and used for $AgNO_3$–Si gel TLC [40]. Four fractions were collected (#, R_f): 1, 0.61–0.68/2, 0.55–0.61/3, 0.42–0.55/4, 0.03–0.42. Combined yield of fr. 1–3: 203.6 mg. An examination by GC–MS of fr. 1–3 showed them to consist essentially of the acetates of dinosterol (**6n**) (main sterol) and of 4,24-dimethylcholestanol (**6b**);fr. 3 was enriched in the acetate of a (known) minor sterol: 4,24-dimethyl-22-dehydrocholestanol (**6l**) (W. C. Dow *et al.*, unpublished observation). More interesting was fr. 4. After saponification the material was used for reverse-phase HPLC. The HPLC trace showed two well-separated peaks. The peak with the longer R_t corresponded to dinosterol and a low percentage of unidentified minor sterols (yield 1.2 mg); the peak with the shorter R_t corresponded to a mixture of Δ^{14}-sterols (yield 2.2 mg): 14-dehydrodinosterol (**5n**) (86%) and 4α,24*S*(or 23ξ)-dimethylcholest-14-en-3β-ol (**5b** or **5d**) (14%) which were separated by prep. GLC. The mother liquors of the fractional crystallization were taken to dryness; the residue was acetylated (247 mg) and purified by $AgNO_3$–Si gel TLC [40]. Five fractions were collected, but only fr. 2 (R_f 0.43–0.52) and fr. 5 (R_f 0.06–0.14) contained sterols. The mixture from fr. 2 (36.8 mg after saponification) consisted of about equal amounts of dinosterol (**6n**) and 4,24-dimethylcholestanol (**6b**) and a number of minor sterols which could not be isolated: 4α-methylcholestanol (**6a**) (3.7%) and an unidentified sterol of MW 428 (4.5%). The material from fr. 5 (yield 1.8 mg after saponification) consisted of 14-dehydrodinosterol (**5n**) (50%) and 32% of 4α,24*S*(or 23ξ)-dimethylcholest-14-en-3β-ol (**5b** or **5d**) and some unidentified trace sterols. We note that Δ^{14}-sterols behave on $AgNO_3$–Si gel TLC like sterically unhindered methylene sterols.

4α-Methylcholest-8(14)-en-3β-ol (**4a**). Mp 156–158° from MeOH, $[\alpha]_D$ +13° ($CHCl_3$, *c* 0.135). High resolution MS (probe) 70 eV, *m/z* (assignment, rel. int.): 400.3703 ($C_{28}H_{48}O_1$, M^+, 100); 385.3459 ($C_{27}H_{45}O_1$, 16); 382.3584 ($C_{28}H_{46}$, 5); 367.3412 ($C_{27}H_{43}$, 2); 287.2373 ($C_{20}H_{31}O_1$, 8); 269.2262 $C_{20}H_{29}$, 6); 261.2546 ($C_{19}H_{33}$, 1); 261.2243 ($C_{18}H_{29}O_1$, 2); 260.2503 ($C_{19}H_{32}$, 2); 260.2130 ($C_{18}H_{28}O_1$, 4); 245.2245 ($C_{18}H_{29}$, 2); 245.1892 ($C_{17}H_{25}O_1$, 5); 243.2099 ($C_{18}H_{27}$, 7); 227.1807 ($C_{17}H_{23}$, 8).

4α,24S-Dimethylcholest-8(14)-en-3β-ol (**4b**). High resolution GC–MS 70 eV, *m/z* (assignment, rel. int.): 414.3861 ($C_{29}H_{50}O_1$, M^+, 100); 399.3642 ($C_{28}H_{47}O_1$, 23); 396.3789 ($C_{29}H_{48}$, 6); 381.3520 ($C_{28}H_{45}$, 9); 287.2388 ($C_{20}H_{31}O_1$, 86); 269.2266 ($C_{20}H_{29}$, 33); 261.2134 ($C_{18}H_{29}O_1$, 7); 260.2135 ($C_{18}H_{28}O_1$, 10); 245.2217 ($C_{18}H_{29}$, 2); 245.1856 ($C_{17}H_{25}O_1$, 11); 243.2073 ($C_{18}H_{27}$, 19); 227.1821 ($C_{17}H_{23}$, 19).

4α,23(or 22),24ξ-Trimethylcholesta-8(14),22-dien-3β-ol (**4o** *or* **4p**). High resolution GC–MS 70 eV, *m/z* (assignment, rel. int.): 426.3838 ($C_{30}H_{50}O_1$, M^+, 100); 411.3676 ($C_{29}H_{47}O_1$, 11); 408.3787 ($C_{30}H_{48}$, 4); 383.3243 ($C_{27}H_{43}O_1$, 4); 355.3044 ($C_{25}H_{39}O_1$, 6); 337.2915 ($C_{25}H_{37}$, 2); 327.2656 ($C_{23}H_{35}O_1$, 3); 314.2603 ($C_{22}H_{34}O_1$, 6); 313.2550 ($C_{22}H_{33}O_1$, 6); 299.2469 ($C_{21}H_{31}O_1$, 3); 287.2357 ($C_{20}H_{31}O_1$, 37); 286.2272 ($C_{20}H_{30}O_1$, 14); 285.2212 ($C_{20}H_{29}O_1$, 29); 269.2258 ($C_{20}H_{29}$, 19); 261.2198 ($C_{18}H_{29}O_1$, 8); 260.2106 ($C_{18}H_{28}O_1$, 25); 243.2130 ($C_{18}H_{27}$, 11); 227.1812 ($C_{17}H_{23}$, 5). Decoupling was performed in $CDCl_3$ using a 220 MHz spectrometer, but the δ values quoted here were taken from the 360 MHz ^{1}H NMR spectrum (Table 2). Irradiation of the allylic proton at *ca* δ 2.4 caused the collapse of the olefinic doublet and of the methyl doublet at 0.949, thus the latter doublet had to be assigned to the C-21 methyl group. Irradiation at 1.8 affected only one of the methyl doublets (at 0.943), thus we assigned the C-28 methyl group to this doublet.

Amphisterol (**4g**). Mp 138–141° from MeOH, $[\alpha]_D$ +10° ($CHCl_3$, *c* 0.07). High resolution GC–MS 70 eV, *m/z* (assignment, rel. int.): 412.3684 ($C_{29}H_{48}O_1$, M^+, 100); 397.3533 ($C_{28}H_{45}O_1$, 24); 394.3666 ($C_{29}H_{46}$, 4); 379.3358 ($C_{28}H_{43}$, 7); 314.2600 ($C_{22}H_{34}O_1$, 5); 313.2579 ($C_{22}H_{33}O_1$, 5); 285.2252 ($C_{20}H_{29}O_1$, 32); 269.2243 $C_{20}H_{29}$, 5); 261.2243 ($C_{18}H_{29}O_1$, 3); 260.2479 ($C_{19}H_{32}$, 2); 260.2101 ($C_{18}H_{28}O_1$, 9); 245.1964 ($C_{17}H_{25}O_1$, 11); 243.2112 ($C_{18}H_{27}$, 6); 243.1731 ($C_{17}H_{23}O_1$, 2); 241.1969 ($C_{18}H_{25}$, 16); 227.1776 ($C_{17}H_{23}$, 13); Decoupling information (360 MHz ^{1}H NMR, $CDCl_3$). Irradiation at δ 2.25 (H at C-25) caused the collapse of the almost equivalent methyl doublets at lowest field (1.026 and 1.021: the C-26 and C-27 methyl group).

4α-Methyl-24ξ-ethylcholest-8(14)-en-3β-ol (**4h** *or* **4i**). High resolution GC–MS 70 eV, *m/z* (assignment, rel. int.): 428.3956 ($C_{30}H_{52}O_1$, M^+, 100); 413.3686 ($C_{29}H_{49}O_1$, 19); 410.3899 ($C_{30}H_{50}$, 3); 395.3717 ($C_{29}H_{47}$, 4); 287.2393 ($C_{20}H_{31}O_1$, 27); 269.2281 ($C_{20}H_{29}$, 10); 261.2652 ($C_{19}H_{33}$, 1); 261.2202 ($C_{18}H_{29}O_1$, 4); 260.2134 ($C_{18}H_{28}O_1$, 6); 245.1941 ($C_{17}H_{25}O_1$, 11); 243.2065 ($C_{18}H_{27}$, 10); 227.1786 ($C_{17}H_{23}$, 9).

4α-Methyl-8(14)-dehydroisofucostanol (**4l**). Low resolution GC–MS 70 eV, *m/z* (rel. int.): 426 (M^+, 100); 411 (46); 408 (3); 393 (9); 328 (20); 313 (8); 285 (28); 269 (6); 261 (7); 260 (11); 245 (15); 243 (20); 241 (13); 227 (24).

4α,24S (or 23ξ)-Dimethylcholest-14-en-3β-ol (**5b** *or* **5d**). High resolution GC–MS, 70 eV, *m/z* (assignment, rel. int.): 414.3939 ($C_{29}H_{50}O_1$, M^+, 9); 399.3634 ($C_{28}H_{47}O_1$, 3); 287.2412 ($C_{20}H_{31}O_1$, 100); 269.2260 ($C_{20}H_{29}$, 31).

14-Dehydrodinosterol (**5n**). Mp 174–178.5° from CH_2Cl_2. High resolution GC–MS 70 eV, *m/z* (assignment, rel. int.): 426.3892 ($C_{30}H_{50}O_1$, M^+, 28); 411.3657 ($C_{29}H_{47}O_1$, 2); 408.3758 ($C_{30}H_{48}$, 2); 393.3520 ($C_{29}H_{45}$, 1); 383.3335 ($C_{27}H_{43}O_1$, 8); 365.3223 ($C_{27}H_{41}$, 1); 355.3017 ($C_{25}H_{39}O_1$, 16); 337.2884 ($C_{25}H_{37}$, 2); 327.2736 ($C_{23}H_{35}O_1$, 4); 314.2634 ($C_{22}H_{34}O_1$, 29); 313.2543 ($C_{22}H_{33}O_1$, 19); 299.2460 ($C_{21}H_{31}O_1$, 3); 287.2378 ($C_{20}H_{31}O_1$, 41); 286.2304 ($C_{20}H_{30}O_1$, 43); 285.2258 ($C_{20}H_{29}O_1$, 4); 269.2254 ($C_{20}H_{29}$, 20); 247.2063 ($C_{17}H_{27}O_1$, 14); 229.1948 ($C_{17}H_{25}$, 7), 69.0708 (C_5H_9, 100). Probably due to the proximity of the double bonds the above MS has some unusual features. The MS of dinosterol (**6n**) [18] includes a *m/z* 316 peak. Because **5n**, unlike **6n**, has a nuclear unsaturation, the corresponding peak in the MS of **5n** should occur at *m/z* 314. This peak is observed, but it is accompanied by an unexpected peak at *m/z* 313. A sterol with two degrees of unsaturation, one in the nucleus and one in the side chain, is expected [21] to lose its side chain together with two hydrogens on electron impact. We observe the expected peak (at *m/z* 285) but it is low; the 286 and 287 peaks have a much higher intensity. The 360 MHz ^{1}H NMR spectrum of **5n** in $CDCl_3$ (Table 2) is assigned analogous to that of dinosterol (**6n**) (see below and Table 2), because decoupling experiments were inconclusive (the

doublets assigned to the C-21 and C-28 methyl groups, which are the ones expected to collapse on decoupling and one of the angular methyls have almost identical chemical shifts). Decoupling information (360 MHz ^{1}H NMR, C_6D_6). Irradiation of the allylic proton at δ 2.7 collapsed the olefinic doublet and the methyl doublet at lowest field (1.083: C21-H). The other peaks in the methyl region were assigned by analogy.

Dinosterol (**6n**). Decoupling was performed in C_6D_6 using a 220 MHz spectrometer but the δ values quoted here were taken from the 360 MHz ^{1}H NMR spectrum (Table 3). Irradiation of the multiplet at *ca* δ 2.5 collapsed both the olefinic doublet and the methyl doublet at lowest field (1.090). The latter doublet can thus be assigned to the C-21 methyl group. Irradiation at 1.8 caused the collapse of a methyl doublet at 1.028, which must be the doublet of the C-28 methyl group. Decoupling information (360 MHz, $CDCl_3$); Irradiation of the isolated allylic proton at δ 2.35 resulted in the collapse of the olefinic doublet and of the methyl doublet at 0.919, which thus must be caused by the C-21 methyl group. Only the methyl doublet at 0.928 (C-28 methyl group) was affected by irradiation at 1.7.

4α-Methyl-24ξ-ethylcholestan-3β-ol (**6h** *or* **6i**). High resolution GC–MS 70 eV, *m/z* (assignment, rel. int.): 430.4184 ($C_{30}H_{54}O_1$, M^+, 100); 415.3970 ($C_{29}H_{51}O_1$, 25); 412.4078 ($C_{30}H_{52}$, 3); 397.3860 ($C_{29}H_{49}$, 10); 247.2041 ($C_{17}H_{27}O_1$, 53); 229.1960 ($C_{17}H_{25}$, 57).

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