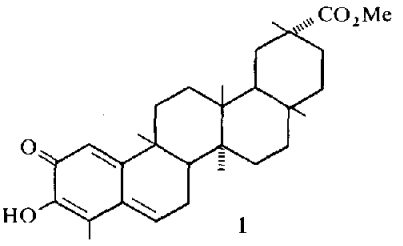
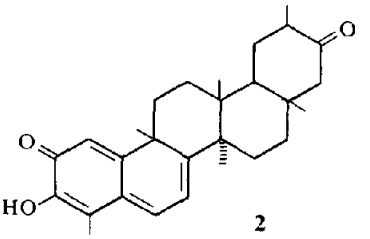


1



2

to a fine powder which was successively extracted in a Soxhlet with petrol and ethanol. On preliminary chemical screening of each extract [6], no alkaloid or flavonoids could be detected. The reddish petrol extract (16 g) was refluxed 1 hr with 160 ml MeOH and the suspension was filtered. The methanolic filtrate was evapd and 3 g of the residue were chromatographed on a Si gel column, affording sitosterol (195 mg), epifriedelanol, epifriedelanyl acetate, pristimerin and tingenone. All five compounds were identified by direct comparison with authentic specimens. From the ethanolic extract only dulcitol (1.8 g) was isolated and its identity was ascertained by IR, NMR, mmp and co-TLC, and from the properties of its hexacetate.

Acknowledgements—To the World Health Organization for a research grant No. 77051 and to Syntex de Mexico for financial help. We wish to thank Prof. Dr. G. B. Marini-Bettolo, from Italy and A. González González from Spain, for supplying us with authentic samples of celastrol, pristimerin and tingenone, and Ing. H. Gómez Junco and Dr. J. Treviño Abrego for MS.

REFERENCES

1. Marini-Bettolo, G. B. (1974) *Il. Farmaco, Ed. Sci.* **29**, 550.
2. Kupchan, S. M. and Smith, R. M. (1977) *J. Org. Chem.* **42**, 115.
3. Kupchan, S. M., Hintz, H. P. J., Smith, R. M., Karim, A., Cass, N. W., Court, W. A. and Yatagai, M. (1977) *J. Org. Chem.* **42**, 3660.
4. Domínguez, X. A., Franco, R., Cano, G., García, S. and Peña, V. (1978) *Rev. Latinoam. Quim* **9**, 33.
5. Domínguez, X. A., Rovalo, M., Rojas, G. M. and Espinosa, G., unpublished results.
6. Dominguez, X. A. (1973) *Métodos de Investigación Fitoquímica*. Limusa-Wiley, México.

Phytochemistry, 1979, Vol. 18, pp. 899–901. © Pergamon Press Ltd. Printed in England. 0031-9422/79/0501-0899 $02.00/0

A NEW STEROL, 4α-METHYL-5α-ERGOSTA-8(14),24(28)-DIEN-3β-OL, FROM THE MARINE DINOFLAGELLATE *AMPHIDINIUM CARTERAE*

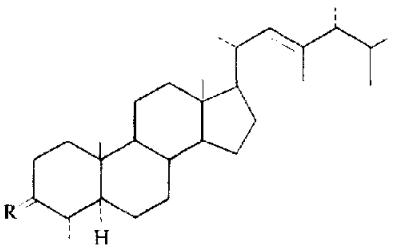
NANCY W. WITHERS, L. JOHN GOAD and TREVOR W. GOODWIN
Department of Biochemistry, University of Liverpool, P.O. Box 147, Liverpool, L69 3BX, U.K.

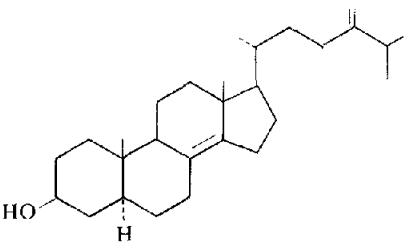
(*Received* 26 *October* 1978)

Key Word Index—*Amphidinium carterae*; *Amphidinium* species; dinoflagellates; algae; sterols; amphisterol; 4α-methyl-5α-ergosta-8(14),24(28)-dien-3β-ol; cholesterol.

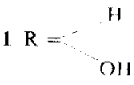
Many novel sterols have been isolated from marine invertebrate organisms [1–4] and it is considered that at least some of these may be derived in the animals diet from algal sources. In the quest for the algae responsible for the biosynthesis of unusual sterols, attention has recently been focused upon dinoflagellate species. Shimizu *et al.* [5] reported the isolation of an unusual 4α-methylsterol, dinosterol (**1**) and cholesterol from the photosynthetic dinoflagellate *Gonyaulax tamarensis*. In an examination of the non-photosynthetic organism *Crypthecodinium cohnii*, we [6] also found dinosterol (**1**) as the major sterol but in addition identified the corresponding Δ^5 compound, dehydrodinosterol (**2**), the 3-oxo derivative (**3**) and a small amount of cholesta-5,7-dien-3β-ol. We have also reported studies on the mechanism of methylation of the dinosterol (**1**) side chain [7]. In our continuing studies of the dinoflagellate sterols, we now report the characterization of another new sterol, 4α-methyl-5α-ergosta-8(14),24(28)-dien-3β-ol (**4**), for which we suggest the trivial name amphisterol, from *Amphidinium carterae* and other *Amphidinium* species.

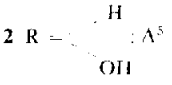
Amphidinium carterae (Py-1) was grown [8] at 18°,





4

1 R = 

2 R = 

3 R = O

harvested by continuous centrifugation, and lyophilized to yield 11.3 g of dry cells. The non-saponifiable lipid (~98 mg) was obtained [6] and separated by PLC on Si gel into the 4-demethylsterol (5 mg) and 4α-methylsterol (10 mg). The 4-demethylsterol was rerun on PLC to yield a small amount of cholesterol (< 1 mg) identified by GLC and MS.

GLC analysis (OV-1) of the 4α-methylsterols revealed four components with retention times relative to cholesterol of 1.18 (29%), 1.55 (62%) 1.67 (3%) and 1.99 (6%). The MS of the mixture had molecular ions at *m/e* (rel. int.) 426 (20), 412 (100) and 400 (55) indicating the presence of a C_{29} diunsaturated sterol as the major component, accompanied by a C_{28} monounsaturated sterol and a small amount of a C_{30} diunsaturated sterol.

The 4α-methylsterol mixture was acetylated and subjected to PLC on $AgNO_3$-Si gel to yield a major component at R_f 0.15 which was pure by GLC. MS showed fragmentations similar to those observed in the MS of 24-methylenelophenyl acetate [9] but with striking differences in the intensities of the ions. The intense molecular ion was at *m/e* 454 confirming a C_{29} diunsaturated steryl acetate structure. Fragment ions at *m/e* 327 (M-side chain-2H), 269 (M-side chain-acetate), 287 (M-side chain-42) and 227 (*m/e* 287-acetate) located one double bond in the side chain and the other in the ring system. A very weak ion at *m/e* 370 indicated loss of 84 mu from the side chain by the McClafferty rearrangement, which is a characteristic fragmentation leading to an intense ion in 24-methylenesterols with a Δ^5 or Δ^7 bond [9], although it is not a prominent fragmentation in the MS of 24-methylenesterols with a $\Delta^{8(9)}$ bond or a 14α-methyl group [9–12]. The MS evidence thus indicated that amphisterol possibly had a 24-methylene group and a nuclear double bond at a position other than Δ^7.

High resolution (360 MHz) ^{1}H NMR spectroscopy verified the identification of a methylene group by the appearance of signals of equal intensity at δ 4.63 and 4.70 integrating for two protons. Doublets at δ 1.024 (J = 7 Hz) and 1.019 (J = 7 Hz) for the non-equivalent C-26 and C-27 methyl protons and at 0.958 (J = 6.5 Hz) for the C-21 methyl protons located the methylene group at the C-24 position. The absence of olefinic proton signals, other than those at C-28, revealed that the nuclear double bond was tetrasubstituted and therefore must be at either the Δ^3, Δ^4, $\Delta^{8(9)}$ or $\Delta^{8(14)}$ position. The Δ^3 and Δ^4 locations were eliminated by the presence in the ^{1}H NMR spectrum of a signal for a 3α-proton (4.40, *br m*) and a doublet at 0.844 (J = 6.5 Hz) for the 4α-methyl group [13]. Of the remaining possibilities, a $\Delta^{8(14)}$ bond was identified by means of the chemical shifts of the C-18 and C-19 methyl protons at 0.837 and 0.724, respectively, which were in excellent agreement with those reported for 4α-methyl-5α-cholest-8(14)-en-3β-ol [14] and other $\Delta^{8(14)}$ sterols [15, 16]. By contrast, the chemical shifts for the C-18 and C-19 protons of a $\Delta^{8(9)}$ sterol are reported at δ 0.62 and 0.99, respectively [15].

From the foregoing evidence, amphisterol was therefore identified as 4α-methyl-5α-ergosta-8(14),24(28)-dien-3β-ol (**4**). Amphisterol was also identified by GLC and MS as the major sterol in *A. carterae* (Py-2), *A. carterae* (= *hoefleri*) (Py-450), *A. klebsii* (Py-43); *A. rhynchocephalum* (Py-4) and *A. corpulentum* (Py-3), but it was not detected in significant quantities in fourteen other species of dinoflagellate examined in our laboratory.

Other $\Delta^{8(14)}$ sterols have been reported in nature. 4α-Methyl-5α-cholesta-8(14),24-dien-3β-ol was identified as a minor constituent of yeast sterols [15] and 5β-stigmast-8(14)-en-3α-ol and 5α-stigmasta-8(14),22-dien-3β-ol have been reported from higher plants [17, 18]. 4,4-Dimethyl- and 4α-methyl-5α-cholest-8(14)-en-3β-ol and 4,4-dimethyl- and 4α-methyl-5α-cholest-8(14),24-dien-3β-ol have been identified in the bacterium *Methylococcus capsulatus* [19].

It is noteworthy that a $\Delta^{8(14)}$ sterol has been suggested as an intermediate arising in sterol biosynthesis as a consequence of the C-14 demethylation step [20]. The reason for the accumulation of amphisterol (**4**) and its possible phylogenetic significance in *Amphidinium* species remains a topic for interesting speculation.

EXPERIMENTAL

MS were determined by direct probe at 70 eV. ^{1}H NMR spectra were measured at 360 MHz using $CDCl_3$ solns of the

steryl acetate with TMS as the internal standard. GLC was on a 20 m OV-1 (WCOT) capillary column at 243°.

Dinoflagellate culture and sterol isolation. Amphidinium carterae (Py-1) was cultured at 18° in 200 l. of GPM medium [8]. The cells were harvested, lyophilized (11.3 g dry wt) and the non-saponifiable lipid (98 mg) was obtained after reflux with 8% NaOH in 90% EtOH and petrol extraction. PLC on Si gel developed with $CHCl_3$–EtOH (98:2) gave the 4α-methylsterols (~10 mg) and 4-demethylsterols (~5 mg), and the latter were repurified on this PLC system to give ~1 mg 4-demethyl sterol. The 4α-methylsterols were treated with Py-Ac_2O and the 4α-methyl steryl acetate (~7 mg) separated by PLC on 10% $AgNO_3$-Si gel developed with pure $CHCl_3$ to give amphisteryl acetate (2 mg).

MS *m/e* (rel. int.): 454 (M^+, 78) 438(18), 394(8), 379(8), 370(3), 327(14), 313(5), 269(10), 243(15), 241(20), 227(22), 55(100). ^{1}H NMR ($CDCl_3$): δ 0.724 (3H, *s*, C-19), 0.837 (3H, *s*, C-18), 0.844 (3H, *d*, J = 6.5 Hz, C-30), 0.958 (3H, *d*, J = 6.5 Hz, C-21), 1.019 (3H, *d*, J = 7 Hz, C-26 or C-27) 1.024 (3H, *d* J = 7 Hz, C-26 or C-27) 2.047 (3H, *s*, $COCH_3$), 4.40 (1H, *brm*, C-3α), 4.63 and 4.70 (1H each, C-28).

Acknowledgements—We are deeply indebted to Professor F. T. Haxo, Scripps Institution of Oceanography, La Jolla, California for cultures of dinoflagellates and Dr. L. J. Mulheirn, Shell Biosciences Laboratory, Sittingbourne, U.K. for ^{1}H NMR spectra. N.W.W. gratefully acknowledges support of a Leverhulme Visiting Fellowship (1976–77).

REFERENCES

1. Bergman, W. (1962) in *Comparative Biochemistry* (Florkin, M. and Mason, H. S., eds.) Vol. 3, p. 103. Academic Press, London.
2. Schmitz, F. J. (1978) in *Marine Natural Products* (Scheuer, P., ed.) Vol. 1. Academic Press, New York.
3. Minale, L. and Sodano, G. (1977) in *Marine Natural Products Chemistry* (Faulkner, D. J. and Fenical, W. H., eds.) p. 87. Plenum Press, New York.
4. Goad, L. J. (1976) in *Biochemical and Biophysical Perspectives in Marine Biology* (Malins, D. C. and Sargent, J. R., eds.) Vol. 3, p. 213. Academic Press, New York.
5. Shimizu, Y., Alan, M. and Kobayashi, A. (1976) *J. Am. Chem. Soc.* **98**, 1059.
6. Withers, N. W., Tuttle, R. C., Holz, G. G., Beach, D. H., Goad, L. J. and Goodwin, T. W. (1978) *Phytochemistry* **17** 1987.
7. Withers, N. W., Tuttle, R. C., Goad, L. J. and Goodwin, T. W. (1979) *Phytochemistry* **18**, 71.
8. Siegelman, H. W. and Guillard, R. R. L. (1971) *Methods Enzymol.* **23A**, 110.
9. Knights, B. A. (1967) *J. Gas Chromatogr.* 273.
10. Goad, L. J., Williams, B. L. and Goodwin, T. W. (1967) *Eur. J. Biochem.* **3**, 232.
11. Goulston, G., Goad, L. J. and Mercer, E. I. (1975) *Phytochemistry* **14**, 457.
12. Fryberg, M., Oehlschlager, A. C. and Unrau, A. M. (1975) *Arch. Biochem. Biophys.* **173**, 171.
13. Sucrow, W., Littmann, W. and Räduchel, B. (1977) *Chem. Ber.* **110**, 1523.
14. Iida, T., Tamura, T., Miura, N. and Matsumoto, T. (1977) *Steroids* **29**, 453.
15. Barton, D. H. R., Harrison, D. M., Moss, G. P. and Widdowson, D. A. (1970) *J. Chem. Soc. C* 775.
16. Rubinstein, I., Goad, L. J., Clague, A. D. H. and Mulheirn, L. J. (1976) *Phytochemistry* **15**, 195.
17. Rakhit, S., Dhar, M. M., Anad, H. and Dhar, M. L. (1959) *J. Sci. Indian Res. (India)* **18B**, 422.
18. Zalkow, L. H., Cabat, G. A., Chetty, G. L., Ghosal, M. and Keen, G. (1968) *Tetrnhedron Letters* 5727.
19. Bouvier, P., Rohmer, M., Benveniste, P. and Ourisson, G. (1976) *Biochem. J.* **159**, 267.
20. Schroepfer, G. J., Lutsky, B. N., Martin, J. A., Huntoon, S., Fowcans, B., Lee, W. H. and Varmilion, J. (1972) *Proc. Roy. Soc. London Ser. B.* **180**, 125.