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DEHYDRODINOSTEROL, DINOSTERONE AND RELATED STEROLS OF A NON-PHOTOSYNTHETIC DINOFLAGELLATE, *CRYPTHECODINIUM COHNII*

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Key Word Index—*Crypthecodinium cohnii*; dinoflagellate; algae; sterols; dinosterol; dehydrodinosterol; dinosterone.

Abstract—The heterotrophic dinoflagellate *Crypthecodinium cohnii* contained the 4α-methyl sterols, dinosterol, dehydrodinosterol (4α,23,24-trimethylcholesta-5,22-dien-3β-ol) and the tentatively identified 4α,24-dimethylcholestan-3β-ol and 4α,24-dimethylcholest-5-en-3β-ol. The major 4-demethyl sterol was cholesta-5,7-dien-3β-ol which was accompanied by a smaller amount of cholesterol and traces of several other C_{27},C_{28} and C_{29} sterols. In addition, a 3-oxo-steroid fraction was isolated and the major component identified as dinosterone (4α,23,24-trimethylcholest-22-en-3-one). The possible biosynthetic relationships of these compounds are discussed.

INTRODUCTION

The heterotrophic dinoflagellate, *Crypthecodinium cohnii*, contains unusually high concentrations of a polyunsaturated, 22:6(n-3), fatty acid [1] and this contrasts with photosynthetic dinoflagellates which also contain shorter chain di-, tri-, tetra- and pentaenoic acids [2]. The nature of the triglycerides and phospholipids of *C. cohnii* has been investigated and the effects of environmental conditions on their composition reported [3]. In the course of these studies an unidentified sterol fraction was obtained (Beach, D. H. and Holtz, G. G., unpublished observations) which upon further examination was found to contain predominantly a complex mixture of unknown 4α-methyl sterols with only a relatively small amount of the 4-demethyl sterols, cholesterol and cholesta-5,7-dien-3β-ol (Goad, L. J. and Goodwin, T. W., unpublished work). Whilst our studies were in progress, Shimizu *et al.* [4] reported the isolation and characterisation of a novel 4α-methyl sterol together with cholesterol from the photosynthetic dinoflagellate *Gonyaulax tamarensis*. The unique 4α-methylsterol, dinosterol (1), has a saturated ring system and an unusual side chain alkylation pattern, the configuration of which was determined by X-ray analysis [5]. We now report that one of the major sterols from *C. cohnii* is also dinosterol (1) but it is accompanied by the corresponding Δ^5-compound (2), smaller amounts of other 4α-methyl sterols and by dinosterone (3).

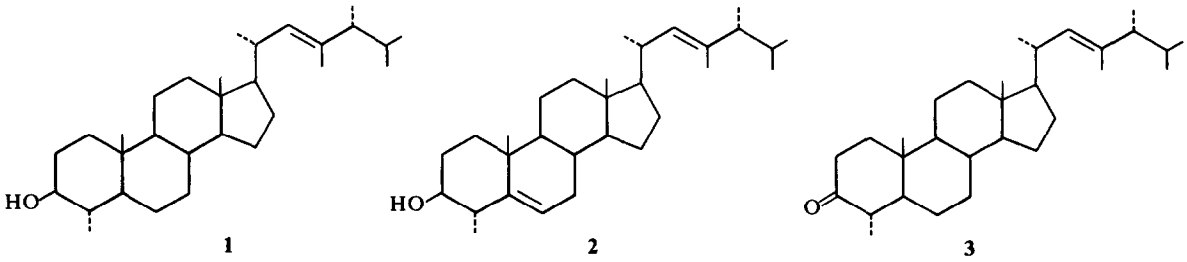
RESULTS AND DISCUSSION

TLC of the non-saponifiable lipids of *C. cohnii* showed four steroid components with R_f values corresponding to 3-oxo-steroid (4.3 mg/g dry wt of cells), 4,4-dimethylsterols (2 mg/g dry wt), 4-monomethylsterols (19 mg/g dry wt) and 4-demethylsterols (5.1 mg/g dry wt). The 4α-methylsterols, which constituted *ca* 60% of the total steroids, were analysed by GLC and found to contain a major component (~ 66%) with a RR_t (1.53 on 3% OV-17) which indicated it to be dinosterol (1) [4]. Acetylation of the 4α-methylsterols followed by TLC on $AgNO_3$–Si gel gave two major steryl acetate fractions together with more minor components. The least polar steryl acetate was crystallized from $CHCl_3$–MeOH and identified by MS and PMR spectroscopy as dinosteryl acetate. The MS of the acetate showed a M^+ at *m/e* 470 with fragmentation ions at *m/e* 398, 358, 339, 329, 271, and 229. For the free sterol the M^+ was at *m/e* 428 with ions at *m/e* 385, 367, 357, 316 (80%) 287 (100%) and 271 (60%) in accord with the published data for dinosterol (1) [4]. The PMR spectrum of the acetate (Table 1) was

Table 1. PMR chemical shifts of dinosteryl acetate, dehydrodinosteryl acetate and dinosterone obtained from *Crypthecodinium cohnii**

	C-18 *s*	C-19 *s*	C-21 *d*, *J* = 6 Hz	C-26 *d*, *J* = 6 Hz	C-27 *d*, *J* = 6 Hz	C-28 *d*, *J* = 6 Hz	C-31 *d*, *J* = 7 Hz	C-33 *d*, *J* = 1 Hz	C-3 *m*	C-6 *m*	C-22 *m*
Dinosteryl acetate	0.678	0.840	0.927	0.776	0.836	0.918	0.800	1.491	4.368	—	4.879
Dehydrodinosteryl acetate	0.709	1.040	0.930	0.776	0.836	0.930	0.977	1.491	4.400	5.352	4.878
Dinosterone	0.709	1.070	0.930	0.775	0.835	0.918	0.968	1.491	—	—	4.878

* Spectra were recorded at 220 MHz in $CDCl_3$ with TMS as the internal standard.



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also essentially the same as that reported for the dinosterol (1) isolated from *G. tamarensis* [4].

A second major 4α-methyl steryl acetate obtained from *C. cohnii* had a MS with a very weak M^+ at *m/e* 468 and with fragmentation ions at *m/e* 408 (M^+ − acetate, 100%), 365 (*m/e* 408 − part of side chain by C-24, C-25 cleavage), 357 (M^+ − part of side chain by C-20, C-22 cleavage), 337 (*m/e* 408 − part side chain by C-23, C-24 cleavage), 329 (M^+ − side chain), 297 (*m/e* 357 − acetate), 296 (*m/e* 408 − part side chain by C-20, C-22 cleavage-H), 269 (*m/e* 329 − acetate) and 267 (*m/e* 408 − side chain). For the 3β-hydroxysterol MS, ions were recorded at *m/e* 426 (M^+), 383, 355 (M^+ − part side chain by C-23, C-24, cleavage), 314 (M^+ − part side chain by C-20, C-22 cleavage), 285 (M^+ side chain − 2H) and 269 (M^+ − side chain − H_2O). These fragmentations indicated a similar structure to dinosterol (1) but with an additional double bond located in the ring system. The TMSi ether of the sterol showed a parent ion at *m/e* 498 with a fragmentation ion at *m/e* 369 (M^+ − 129, 41%) strongly indicative of a Δ^5-sterol [6]. We assigned the trivial name dehydrodinosterol (2) to this compound. Treatment of the free sterol (2) with Collin's reagent [7] yielded a ketone, with M^+ at *m/e* 424, which rearranged after addition of HCl to the 4-ene-3-one (4), M^+ at *m/e* 424 and λ_{max} 250 nm similar to that of 4-methylstigmast-4-en-3-one [8]. The PMR spectrum (Table 1) of dehydrodinosteryl acetate revealed an olefinic proton signal at δ 5.352 which together with characteristic shifts for the C-18 and C-19 methyl protons [9], located the double bond at the C-5, C-6 position. Thus the structure of dehydrodinosterol (2) was established as 4α,23,24-trimethylcholesta-5,22-dien-3β-ol.

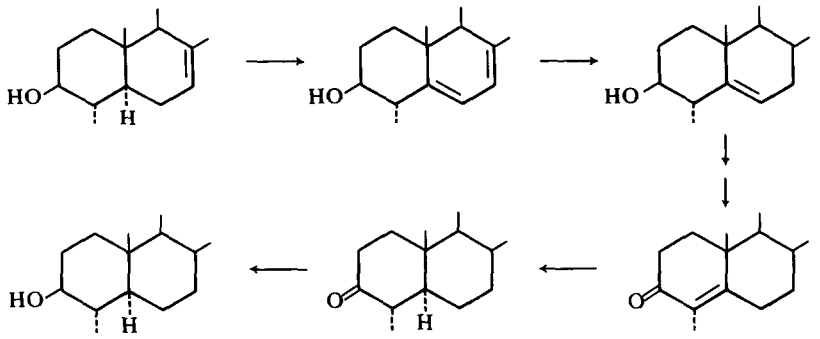
The minor 4α-methylsterols detected in *C. cohnii* included one with M^+ at *m/e* 416. The acetate of this sterol was slightly less polar than dinosteryl acetate on $AgNO_3$–Si gel TLC. The MS with ions at *m/e* 458 (M^+) and *m/e* 271 (M^+ − side chain-acetate) indicated a saturated ring system such as that of dinosterol (1) but with a saturated C_9 side chain. The provisional structure 4α,24-dimethylcholestan-3β-ol is assigned to this compound but it must be regarded as tentative, particularly in relation to the location of the side chain methyl group at C-24. A second minor monomethyl steryl acetate with a polarity similar to dehydrodinosteryl acetate on $AgNO_3$–Si gel TLC had a small ion in its MS at *m/e* 456 but the ion at *m/e* 396 for loss of the acetate was particularly prominent and this fact coupled with an ion at *m/e* 269 (M^+ − side chain-acetate) suggests that this compound is the Δ^5 analog of the previously described sterol, i.e. 4α,24-dimethyl-cholest-5-en-3β-ol.

The 4-demethyl sterols accounted for 16% of the total *C. cohnii* steroids. The main components were cholesterol and cholesta-5,7-dien-3β-ol in a ratio of *ca* 1:7. The identities of these compounds were confirmed by acetylation of the mixture and separation by TLC on $AgNO_3$–Si gel. The cholesteryl acetate was identical to authentic material by TLC, GLC and MS (ions at *m/e* 368, 353, 255 and 213). The cholesta-5,7-dien-3β-yl acetate was identified by TLC, GLC, UV (λ_{max} 295, 282, 272 and 262 nm), MS (*m/e* 366, 351, 253) and PMR (δ 0.63, *s*, C-18; 0.95, *s*, C-19; 5.36, *m*, C-6; 5.52, *m*, C-7).

Several minor 4-demethyl sterols were tentatively identified on the basis of $AgNO_3$–Si gel TLC of their acetates, GLC R_t and MS as cholestan-3β-ol (*m/e* 388 and 215), cholest-7-en-3β-ol (acetate *m/e* 428), cholesta-7,22-dien-3β-ol (acetate *m/e* 426), a diunsaturated C_{29} sterol (acetate *m/e* 454), 24 (or 23)-methylcholesta-5,7-dien-3β-ol (acetate *m/e* 440) and a C_{29}-5,7-diene sterol (acetate *m/e* 454, UV λ_{max} 295, 282, 272 and 262).

In addition to the 3β-hydroxysterols, a 3-oxo steroid fraction was obtained which accounted for 14% of the total steroids isolated from *C. cohnii*. GLC analysis indicated one major component with a RR_t of 1.66 on 3% OV-17. The MS had a M^+ at *m/e* 426 and major fragmentation ions at *m/e* 383 (22%), 314 (88%) and 285 (100%) and was similar to that reported [4] for the Jones' oxidation product (3) of dinosterol (1). The IR spectrum exhibited a strong absorption at 1725 cm^{-1} characteristic of a ketone. The PMR spectrum (Table 1) confirmed a structure based upon the dinosterol skeleton. There was no signal for a C-3 proton but the shifts for the C-18 and C-19 methyl group protons, compared to the corresponding shifts of dinosteryl acetate, were characteristic of a 3-one system [10]. Potassium borohydride reduction of the sterone yielded a mixture containing mainly dinosterol (1), identified by GLC, TLC and MS, and a small amount of the 3α-hydroxy isomer of 1. The structure of the sterone was therefore confirmed as (22*E*,24*R*)-4α,23,24-trimethylcholest-22-en-3-one (3). MS of the sterone fraction revealed other minor M^+ ions at *m/e* 412, 414 and 440 whilst GLC gave minor peaks at RR_t s 1.37 and 1.88 in addition to the dinosterone (3) peak. These other components, which amounted to *ca* 10% of the total sterone mixture remain to be identified.

The sterol composition reported here for the heterotrophic dinoflagellate *C. cohnii* differs from that reported [4] for the photosynthetic species *G. tamarensis* in its greater complexity. In addition to dinosterol (1), *C. cohnii* produced a novel 4α-methylsterol, dehydrodinosterol (2) as a major constituent. The presence of this Δ^5-sterol (2) together with dinosterone (3) suggests a possible biosynthetic route for the novel 4α-methyl saturated ring system of dinosterol (1) which may be by an analogous route to that producing 5α-cholestanol in animal tissues [11, 12], namely 3β-hydroxy-Δ^7 → 3β-hydroxy-$\Delta^{5,7}$- → 3β-hydroxy-Δ^5 → 3-oxo-Δ^5- → 3-oxo-Δ^4- → 3-oxo-5α- → 3β-hydroxy-5α- as shown in Scheme 1. However, 3-oxo-steroids are also



Scheme 1. Possible route for the saturation of the sterol ring system in dinosterol production.

intermediates in the demethylation reactions responsible for loss of the 4α- and 4β-methyl groups in 4-demethyl sterol elaboration in both plants and animals [13, 14] and a similar role in *C. cohnii* would account for the presence of **3** in this dinoflagellate. It is striking that although the major sterol of *C. cohnii* is a 4α-methyl ring saturated compound with additional methyl groups at C-23 and C-24 of the side chain, i.e. dinosterol (**1**), the principal 4-demethyl sterol is the diene cholesta-5,7-diene-3β-ol which has an unsubstituted side chain. Moreover only a trace amount of 4-demethyl stanol (5α-cholestanol) was present. This seems to indicate a dichotomy in the sterol biosynthetic pathways, one branch apparently leading to dinosterol (**1**) which accumulates and the other route, in which side chain transmethylation is relatively unimportant, leading to the 4-demethyl sterols. In this respect it will be of interest to ascertain if the traces of C_{29} 4-demethyl sterol detected in *C. cohnii* have the dinosterol type of side chain or the more conventional phytosterol structure with a C-24 ethyl group. With regard to dinosterol (**1**) production, it should be noted that the introduction of a Δ^{22} bond into 4α-methyl sterols is apparently not common in nature although Δ^{22}-4α-methyl sterols have been reported in red algae [15]. However, from our present knowledge of sterol side chain alkylation mechanisms it is reasonable to conclude that Δ^{22} introduction is a necessary prerequisite for the C-23 alkylation reaction.

EXPERIMENTAL

General procedures. GLC was on a 1.5 m × 6 mm glass column of 3% OV-17, N_2 carrier flow rate 60 ml/min and at a temp. of 240° for free sterols and 260° for steryl acetates or sterones. MS were at 70 eV at 180° by direct probe. IR spectra were recorded for KBr discs and UV spectra in EtOH. 220 MHz PMR spectra were recorded in $CDCl_3$ with TMS as the internal standard by the Physico Chemical Measurements Unit, Harwell, U.K.

Culture of algae and isolation of sterols. Crypthecodinium cohnii (Seligo) Chatton in Grassé (Woods Hole Strain d) [16] was grown at 27° in MLH medium [17] and harvested at the stationary phase of growth after 12–14 days, lyophilised and stored at − 20°. Cells (5 g) were saponified 18 hr at 20° with 8% KOH in MeOH with 2% pyrogallol followed by 1.5 hr reflux. The unsaponifiable lipid was extracted with Et_2O in the usual manner. TLC on Si gel developed with $CHCl_3$–EtOH (49:1) gave fractions containing hydrocarbons (R_f 0.9), sterones (R_f 0.7) 4,4-dimethylsterols (R_f 0.6), 4α-methylsterols (R_f 0.5) and 4-demethylsterols (R_f 0.3). Steryl acetates were prepared by treatment with C_5H_5N–Ac_2O (1:1) and separated by TLC on $AgNO_3$–Si gel (1:9) developed with pure $CHCl_3$. Approximate R_f values were dinosteryl acetate 0.41, dehydrodinosteryl acetate 0.35, cholesteryl acetate 0.35, cholesta-5,7-dien-3β-yl acetate 0.2.

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REFERENCES

1. Harrington, G. W. and Holtz, G. G. (1968) *Biochim. Biophys. Acta* **164**, 137.
2. Harrington, G. W., Beach, D. H. and Holtz, G. G. (1970) *J. Protozool.* **17**, 213.
3. Beach, D. H. and Holtz, G. G. (1973) *Biochim. Biophys Acta* **316**, 56.
4. Shimizu, Y., Alam, M. and Kobayashi, A. (1976) *J. Am. Chem. Soc.* **98**, 1059.
5. Finer, J., Hirotza, K. and Clardy, J. (1977) in *Marine Natural Products* (Faulkner, D. J. and Fenical, W. H., eds.) p. 147. Plenum Press, New York.
6. Brooks, C. L. W., Horning, E. C. and Young, C. S. (1968) *Lipids* **3**, 391.
7. Collins, J. C. (1968) *Tetrahedron Letters*, 3363.
8. Mazur, Y., Weizmann, A. and Sondheimer, F. (1958) *J. Am. Chem. Soc.* **80**, 6293.
9. Cohen, A. I. and Rock, S. (1974) *Steroids* **24**, 243.
10. Zurcher, R. F. (1963) *Helv. Chim. Acta* **46**, 2054.
11. Shefer, S., Milch, S. and Mosbach, E. H. (1965) *J. Lipid Res.* **6**, 33.
12. Smith, A. G. and Goad, L. J. (1975) *Biochem. J.* **146**, 35.
13. Miller, W. L. and Gaylor, J. L. (1970) *J. Biol. Chem.* **245**, 5375.
14. Mulheirn, L. J. and Ramm, P. J. (1972) *Chem. Soc. Rev.* **1**, 259.
15. Beastall, G. H., Tyndall, A. M., Rees, H. H. and Goodwin, T. W. (1974) *Eur. J. Biochem.* **41**, 301.
16. Provasoli, L. and Gold, K. (1962) *Arch. Microbiol.* **42**, 196.
17 Tuttle, R. C. and Loeblich, A. R., III (1975) *Phycologia* **14**, 1.