DINOFLAGELLATE STEROLS IV: Isolation and structure of 4α,23ξ,24ξ-trimethylcholestanol from the dinoflagellate Glenodinium hallii.

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Received 4-16-81

ABSTRACT

The dinoflagellate Glenodinium hallii was investigated for its sterol composition. Five of the six sterols were isolated and identified as cholest-5-en-3β-ol, (24ξ)-24-methylcholest-5-en-3β-ol, stigmasta-5,22-dien-3β-ol, (22E,24R)-4α,23,24-trimethyl-5α-cholest-22-en-3β-ol, and 4α,23ξ,24ξ-trimethyl-5α-cholestan-3β-ol.

INTRODUCTION

Dinoflagellates are unicellular microorganisms which are unique in the sense that they have characteristics of both plants and animals. They are ubiquitous in the marine environments in which they form the primary food chain. A number of dinoflagellates produce extremely toxic compounds (e.g. saxitoxin and its C-11 hydroxy and N-hydroxy derivatives) and thus cause serious public health problems. Dinoflagellates have also been reported (1-5) to produce a number of sterols with highly substituted side chains.

In search of unusual sterols with possible biosynthetic importance, we investigated the sterol composition of a relatively primitive dinoflagellate Glenodinium hallii. In this communication we report the isolation and structure of a hitherto unknown sterol of G. hallii.

EXPERIMENTAL

The melting points were recorded on a Fisher-John hot stage melting point apparatus and were uncorrected. The NMR spectra were recorded on a FT-80A or 220 MHz nmr spectrometer in $CDCl_3$. The mass spectra and g.c.-m.s. were recorded on a HP 5930A GC-MS spectrometer equipped with a HP 5933A data system at an ionizing voltage of 70 eV. Analytical gas liquid chromatography (glc) was performed on a Varian Model 2400 gas chromatograph, equipped with a 0.125" x 6' glass column packed with 2.5% SE-30 on gas Chrom Q, column temp. 250°C and carrier gas (N_2) flow rate of 30ml/min. TMS-ether of the sterols were prepared by adding about 3 drops of Tri Sil Z into a vial containing 0.1 mg of the sterol. The reverse phase high pressure liquid chromatography was performed on a Waters HPLC System equipped with a differential refractometer and a Radial Compression Module (RCM 100) containing a Radial-Pak C18 cartridge (Waters Associates). The operating pressure was 1000 psi and methanol was the eluting solvent. The optical activity was determined on a Perkin-Elmer 141 Polarimeter.

The dinoflagellate (6) was cultured in 5 Gal solution bottles (15 L volume) in Guillard F Medium (7). The cell cultures were directly extracted with $CHCl_3$ (1 L/10 L of culture) at the peak of the growth curve (15 x 10^6 cells/L). The residue from the chloroform extract was saponified with 10% KOH in 80% MeOH and the non-saponifiable lipids were extracted with ether. The glc of the ether extract showed the presence of six sterols (Fig. 1).

The residue from the ether extract was chromatographed on a silica gel 60 (230-400 mesh, E. Merk) column (0.6 x 60 cm). The column was eluted with CH_2Cl_2 at approximately 40 psi. Fractions of 4 ml volume were collected. Fractions were combined on the basis of TLC [silica gel 60, F254, E. Merk, solvent system $CHCl_3$:MeOH (98:2%)] to yield two sterol containing fractions, sterol mixture A (Rf 0.66) and sterol mixture B (Rf 0.55, similar to cholesterol).

The sterol mixture A (11.7 mg) on analytical glc showed the presence of three sterols (sterol I, II and III) with a RRT (relative retention time to cholesterol) of 1.48, 1.52, and 1.82 respectively. Sterol mixture A was separated into three fractions by reverse-phase

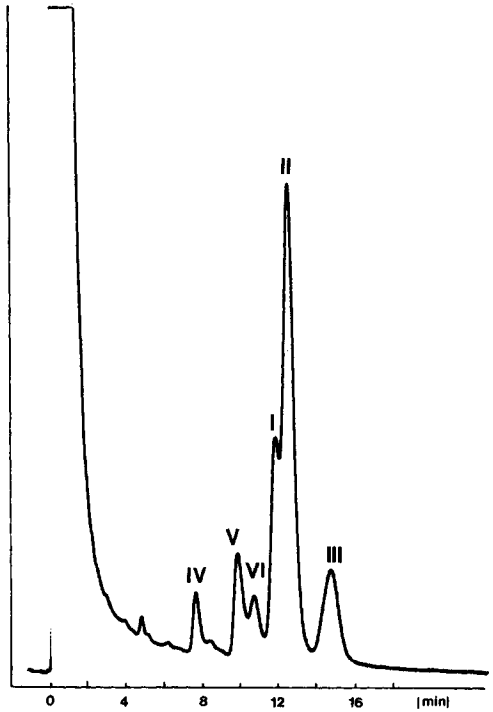
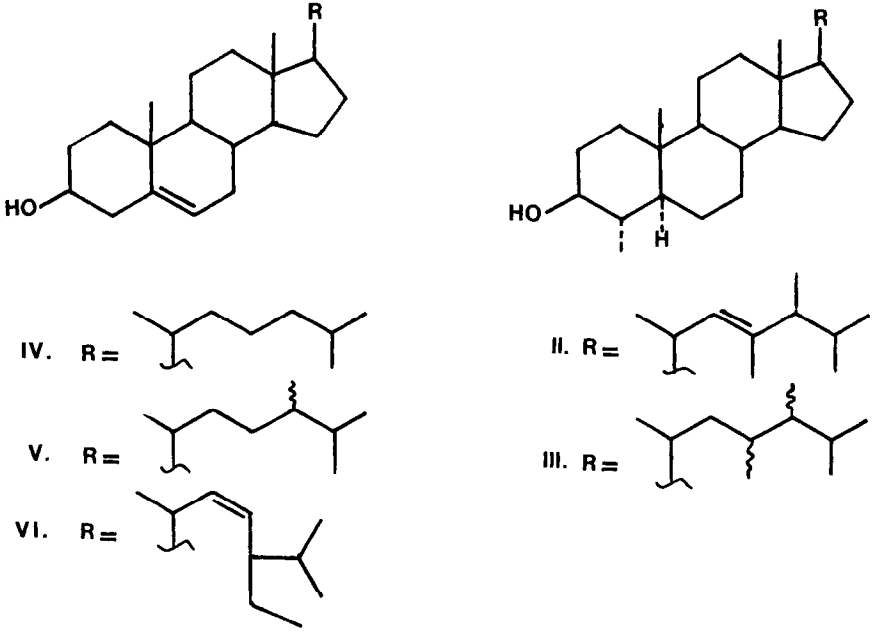


Figure 1. Gas chromatogram of the sterol mixture from G. hallii.



(RP) HPLC. Fraction I was found, by glc, to contain sterol I and II in almost equal quantity. The other two fractions contained pure sterols II and III.

Fraction II on crystallization gave needles of m.p. 221°C [^{1}H-nmr (80 MHz), δ0.7 (3H,S), 0.81(3H,d,J=6.2 Hz), 0.83 (3H,s), 0.85 (3H,d, J=7.2 Hz), 0.94 (6H,d,J=6 Hz), 0.95 (3H,d,J=7 Hz), and 1.49 (3H,d,J= 1.5 Hz); m.s.428 (M^+), 316 (78%), 287 (100%), 271 (60%) and 229 (37%)] and was identified as dinosterol on the basis of its mp, nmr, ms and co-gas chromatography of the free sterol and its TMS-derivative.

Fraction III gave colorless needles, m.p. 182-183°C, $[\alpha]_D$ = -13° (C. 0.1733, $CHCl_3$), and M^+ at 430.420 (100%, $C_{30}H_{54}O$). Other major fragments were at m/z 415 (26%, M^+-CH_3) 397 (19.5%, M^+-CH_3-H_2O), 247 (28%, M^+-$C_{13}H_{27}$) and 229 (37%, M^+-$C_{13}H_{29}O$). The mass spectral fragmentation pattern was similar to that which has been reported for 24-methylcholestanol and 24-ethylcholestanol (8). The 220 MHz ^{1}H-nmr spectrum of the isolated sterol confirmed the presence of a saturated nucleus and eight methyl groups in the molecule [δ 0.643(3H,s), 0.772 (3H,d,J=6.9 Hz), 0.822(3H,s), 0.781(3H,d,J=6.5 Hz), 0.855(3H,d,J=6.0 Hz), 0.905(3H,d,J=6.9 Hz), 0.915(3H,d,J=6.5 Hz), and 0.945(3H,d,J=6.7 Hz)]. The C-3 proton was present around δ 3.1 as a multiplet. No vinylic proton (δ 4.85-5.1) absorption was present in the nmr spectrum.

Sterol mixture B (4.6 mg) showed the presence of three sterols on glc [RRT 1.0, 1.29 and 1.38] and was separated into two pure and one enriched fractions by RP-HPLC. Fraction I gave colorless needles, m.p. 146°C, M^+at m/z 386 and was identified as cholesterol [IV] on the basis of its m.p., mass spectrum and glc comparison with an authentic sample. Fraction 2 was found to be a mixture containing about 25% cholesterol and 75% of sterol V. The major sterol in fraction 2 was tentatively identified as 24-methylcholesterol [V] on the basis of g.c.-m.s. [m/z 400 (M^+, 100%), 382, 315, 289 and 255] and co-glc of the free sterol and its TMS-ether with an authentic sample. Fraction 3 on glc showed the presence of all three [IV, V and VI] sterols, but sterol VI was the major constituent (60%). The major sterol in fraction 3 had a molecular ion at m/z 412 (100%), and other fragments at 351, 300, 271, 255, 159, 145, 133, 97, 83. The major sterol of fraction

3 was purified by repeated RP-HPLC and was tentatively identified as stigmasterol on the basis of m.p. and co-glc, of the free sterol and its TMS-ether, with an authentic sample.

Hydrogenation of Dinosterol

Dinosterol (3 mg) was dissolved in 5 ml of ethyl acetate and 6 mg of 10% Pd on charcoal was added. The solution was stirred under hydrogen for 24 hours at room temperature. The hydrogenation product was separated by filtration and recrystallized from $CHCl_3$:MeOH, mp 183°, M^+ 430 (100%, 430.042), ^{1}H-nmr ($CDCl_3$, 220 MHz) δ 0.643(3H,s), 0.771(3H,d, J=6.8 Hz), 0.822(3H,s), 0.781(3H,d,J=6.8 Hz), 0.855(3H,d,J=6.5 Hz), 0.895 (3H,d,J=7 Hz), 0.915(3H,d,J=6.5 Hz), 0.945(3H,d,J=6.7 Hz). The hydrogenated product was identified as 4α,23ξ,24ξ-trimethylcholestanol by its ^{1}H-nmr and m.s. data.

RESULTS AND DISCUSSION

The chloroform extract from 100 L of the G. hallii culture gave 365.6 mg of a residue, which after saponification gave 73.7 mg (25.1%) of non-saponifiable lipids. The column chromatography of non-saponifiable lipids on silica gel 60 gave a dinosterol containing fraction (sterol mixture A) and a cholesterol containing fraction (sterol mixture B).

The sterol mixture A constituted about 71 percent of the total sterols of G. hallii, and was separated into three fractions by RP-HPLC. Fraction I was enriched with sterol I (19% of the total sterols), which could not be totally separated from dinosterol (sterol II). Fraction II upon crystallization gave (22E,24R)-4α,23, 24-trimethyl-5α-cholest-22-en-3β-ol [dinosterol (sterol II)] which was identified by its nmr, m.s. and m.p. Dinosterol constituted about 55% of the total sterols of G. hallii.

Fraction III upon evaporation and crystallization gave sterol III

(12.5% of the total sterols). The ^{1}H-nmr spectrum of the sterol III showed the presence of a saturated nucleus and eight methyl groups in the molecule (see experimental section). The presence of five methyl groups in the side chain was supported by the mass spectral fragment at m/z 247 indicating ring D cleavage with a side chain. The presence of an extra methyl group on the nucleus was indicated by the fragments at m/z 229 (37%) which have been reported (1-4) to be present in dinosterol (37%), and 4α-methylgorgostanol (39%). The presence of a methyl group at C-4 was also indicated by the fragments m/z 180 and 125 [$C_{12}H_{20}O$, ring A and B intact + CH_3 and $C_8H_{13}O$, ring A + CH_3 respectively] (9).

On the basis of ^{1}H-nmr and mass spectral data the new sterol (III) was identified as 4α,23ξ,24ξ-trimethyl-5α-cholestan-3β-ol. An authentic sample of 4α,23ξ,24ξ-trimethyl-5α-cholestan-3β-ol was prepared by the hydrogenation of dinosterol. The glc, ms and 220 MHz ^{1}H-nmr spectrum of the synthetic compound were identical to those of sterol III. The difference in absorptions at 0.895 (synthetic) and 0.905 ppm (natural) could be attributed to the different epimeric forms.

The sterol mixture B (4.6 mg) was separated into three fractions by RP-HPLC. Fraction 1 contained a sterol which was identified as cholest-5-en-3β-ol [cholesterol (sterol IV)] by the m.p., g.c.-m.s., and the glc of the free sterol and its TMS-ether. Fraction 2 on glc showed the presence of two sterols. The major sterol in this fraction was identified as (24ξ)24-methylcholest-5-en-3β-ol [24-methylcholesterol (sterol V)] on the basis of g.c.-m.s. and co-glc with an authentic sample of the free sterol and its TMS-ether. The third fraction upon repeated chromatography afforded sterol VI, identified as stigmasta-5,22-

dien-3β-ol [stigmasterol (sterol VI)] on the basis of its m.p., g.c.-m.s. and co-glc with an authentic sample of the free sterol and its TMS-ether.

In conclusion, the dinoflagellate G. hallii produces six major sterols, five of which were identified as cholesterol (1%), 24-methylcholesterol (6%), stigmasterol (5%), dinosterol (55%), and 4α,23ξ,24ξ-trimethylcholestan-3β-ol(12.5%)(10). The sixth sterol(sterol I, 19% of the total sterols) could not be isolated in a pure state. In fact, the retention time of sterol I and dinosterol on 1% OV-17 column was so close that initially sterol I escaped detection and appeared as an insignificant shoulder in its TMS-ether form. However, a better separation of the TMS-ethers of dinosterol and sterol I was achieved on a 2.5% SE-30 column. The mass spectral pattern of the TMS-ether of sterol I [m/z 488(M^+, 16%); 473($M^+ - CH_3$, 34%); 398($M^+ - C_4H_{12}OSi$, 66%); 383($M^+ - C_5H_{15}OSi$); 229($M^+ - C_{16}H_{37}OSi$, 66%)] was similar to the mass spectrum of the TMS-ether of sterol III(11), with the exception that the peaks were separated by 14 mass units. On the basis of the mass spectral fragmentaion pattern it is evident that sterol I is a stanol with a 4α-methyl group and a methyl group either at C-22, 23 or 24 of the side-chain.

Similar to other dinoflagellates, G. hallii produces dinosterol which constitutes about 55% of the total sterols. However, in contrast to other dinoflagellates this primitive dinoflagellate produces only a very small amount (1% of the total sterols) of cholesterol. Similarly no trace of 22,23-methylene-4α,23,24-trimethyl-5α-cholestan-3β-ol (4α-methylgorgostanol) was detected in the sterols of the dinoflagellate; however, 4α-methylgorgostanol has been reported to be one of the major sterols of another dinoflagellate of this genus (G. foliaceum) (4).

The biosynthesis of the newly isolated sterol could be envisioned as quenching of the carbonium ion formed in the biosynthesis of dinosterol as proposed by Goad, et al. (12). The significance of these unusual sterols in dinoflagellates is still unclear.

ACKNOWLEDGMENTS

This work was supported by a grant (E-745) from the Robert A. Welch Foundation. We thank Dr. C.-H. Niu, NIADDK, Bathesda, Md. for recording the 220 MHz ^{1}H-nmr spectrum and Mr. Howie James, Waters Associates, Inc. Houston, for loaning us the RCM 100 module and a Radial Pak-C18 cartridge.

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