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Sterols of the Dinoflagellate, *Noctiluca milialis*

Shin-ichi TESHIMA, Akio KANAZAWA and Akio TAGO*

Abstract

The sterols of a dinoflagellate, *Noctiluca milialis*, was investigated. The specimens of *N. milialis* were collected from the sea water where a red water happened. Sterols were isolated and then separated into six fractions by thin-layer chromatography on 10% $AgNO_3$-Kieselgel G. The sterol components of six fractions were identified by a combined gas-chromatography and mass spectrometry. *N. milialis* contained cholesta-5, 22E-dienol (4.8%), 5α-cholestanol (5.6%), 24-methylcholesta-5, 22E-dienol (72.5%), 24-methylenecholesterol (5.9%), and small amounts of other fifteen components. Interestingly, *N. milialis* contained 4α-monomethylsterols such as 4α-methylcholestanol, 4α, 24-dimethylcholest-22-enol, 4α, 24-dimethylcholestanol, 4α-methyl-24-ethylcholest-22-enol, and 4α-methyl-24-ethylcholestanol as minor components (<1%).

Although many new sterols have been isolated from marine invertebrates during the past decade[1-3], the origin of them scarcely has been clarified. Certain marine microorganisms and/or phytoplanktons are generally assumed to be responsible for the formation of new sterols with unusual structures[1]. However, information on the sterols of these organisms occurring in marine environments is only a little. As a part of searching for the sources of unusual sterols, we investigated the sterols of a dinoflagellate, *Noctiluca milialis*, collected from the sea water where a red water happened. This paper deals with the identification of sterol components of *N. milialis*.

Materials and Methods

Specimens of *N. milialis* *N. milialis* was collected in the area off the coast of Tsuyazaki, Fukuoka, Japan, by the members of the Fisheries Experimental Station, Fukuoka Prefecture, February 28, 1977. The size of this plankton is generally about 10 μ diameter, but the specimens analyzed were about 1000 μ diameter and floating near the surface of water as a vermilion-colored layer.

Extraction and Fractionation of Lipids Lipids were extracted from *N. milialis* by using chloroform-methanol according to the procedure of BLIGH and DYER[4]. Lipids (3.487 g) were obtained from 7 liters of the red water and then chromatographed on Kieselgel 60 (100 g) with 0 (500 m*l*), 2 (500 m*l*), 6 (250 m*l*) 10 (250 m*l*), and 15% (250 m*l*) methanol in chloroform. The fractions (1.747 g) eluted

* 鹿児島大学水産学部海洋資源栄養化学研究室 (Lab. of Fisheries Chemistry, Fac. of Fisheries, Univ. of Kagoshima, Kagoshima, Japan)

with 0 and 2% methanol in chloroform were separated into steryl ester and free sterol fractions by column chromatography on Kieselgel 60 (60 g) with 0 (400 m*l*), 2 (500 m*l*), 3 (100 m*l*), 8 (500 m*l*), and 50% (100 m*l*) ethyl acetate in benzene. Steryl ester (0.635 g) and free sterol (0.811 g) fractions were eluted with 0 and 8% ethyl acetate in benzene, respectively. Hydrolysis of the two sterol fractions with 5% potassium hydroxide in ethanol yielded sterols (0.117 g from esterified sterols; 0.229 g from free sterols).

Identification of sterol components Sterols were acetylated with dry pyridine-acetic anhydride (1: 1) and the steryl acetates so obtained were separated into six fractions (fractions *a*, *b*, *c*, *d*, *e*, and *f*) by thin-layer chromatography (TLC) on 10% (w/w) silver nitrate-Kieselgel G with ethanol-free chloroform. Each steryl acetate fraction was subjected to a combined gas-chromatography and mass spectrometry (GLC/Mass) with a column (1 m×2 mm i.d., column temperature 285°C) of 3.0% OV–1 on 60–80 mesh Chromosorb W interfaced with a Japan Electron Optics JEOL–JMS–300 mass spectrometer (ionizing energy, 75 eV)[5]. Analytical GLC was performed on 1.5% OV–17 (column temperature 265°C)[6].

Results

Lipids from *N. milialis* contained 10.1% of sterols, esterified and free sterols. The weight ratio of esterified to free sterols was about 1: 2. The preliminary GLC on 1.5% OV–17 showed that sterols from esterified and free sterol fractions contained almost the same components each other (Table 1). Therefore, the detailed characterization of sterol components was carried out about the sterols from free sterol fraction as mentioned below.

Argentation TLC of the steryl acetates afforded six bands with Rf values of 0.81, 0.75, 0.55, 0.50, 0.30, and 0.10. Each steryl acetate fraction obtained by argentation

Table 1. Sterol composition of the esterified and free sterols isolated from *N. milialis*.

GLC on 1.5% OV–17		Composition (%)*1		Major sterol
Peak	RRT	Esterified sterol	Free sterol	
1	0.67	0.7	0.7	24-Norcholesta-5, 22E-dienol
2	0.93	6.4	5.3	Cholesta-5, 22E-dienol
3	1.00	5.4	5.6	Cholestanol
4	1.15	80.2	81.5	24-Methylcholesta-5, 22E-dienol
5	1.36	6.3	5.9	24-Methylenecholesterol
6	1.58	1.0	1.0	24-Ethylcholest-5-enol

*1 The steryl sulfate fraction eluted with 15% methanol in chloroform by column chromatography on Kieselgel 60 was solvolyzed with 2% acetic acid in dioxane. However, no sterol was detected in the solvolysis product.

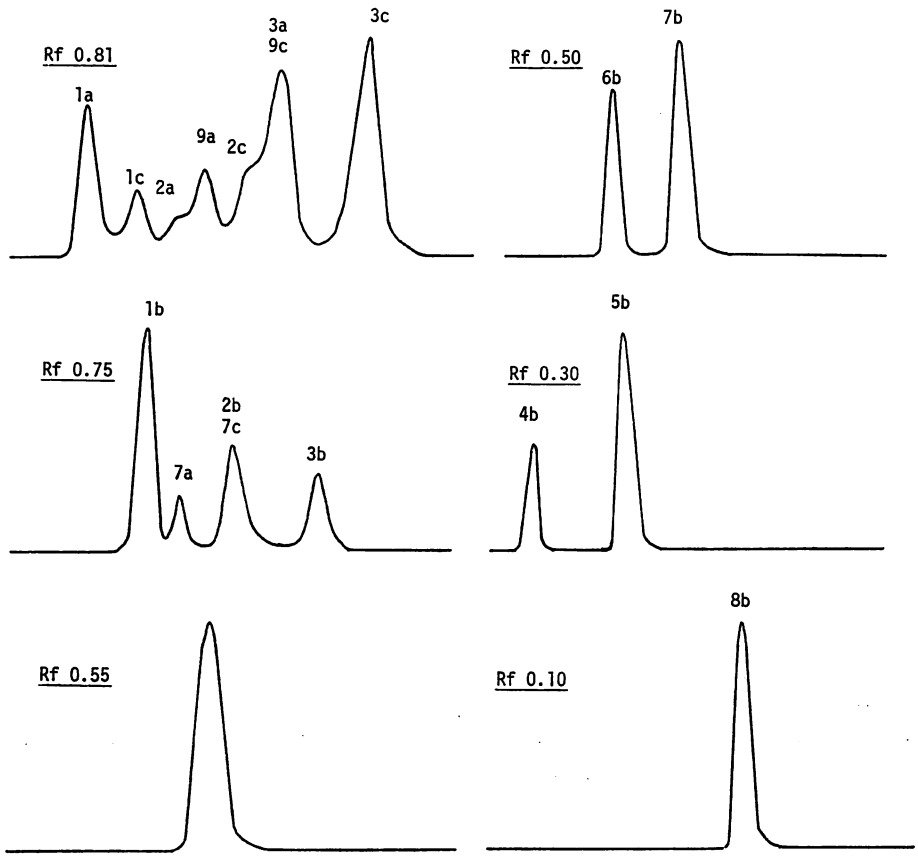

Fig. 1. Gas-chromatogram of the fractions (Rf values, 0.81, 0.75, 0.55, 0.50, 0.30, and 0.10) isolated by argentation TLC. Each fraction was subjected to GLC/Mass.

TLC was revealed to be composed of several components by GLC/Mass (Fig. 1).

Fraction a (Rf 0.81): GLC/Mass showed that this fraction contained at least eight components with RRTs on 3.0% OV-1 of 1.01 (**1a**), 1.14 (**1c**), 1.27 (**2a**), 1.35 (**9a**), 1.48 (**2c**), 1.59 (**3a** and **9c**), and 1.80 (**3c**). The components **1a**, **2a**, and **3a** were identified as 5α-cholestanyl, 24-methylcholestanyl, and 24-ethylcholestanyl acetates, respectively. GLC/Mass: **1a**, m/e 430 (M^+, relative intensity 38%), 415 (10%), 370 (M^+–AcOH, 23%), 355 (26%), 316 (M^+–C–1 to C–4, 5%), 290 (5%), 275 (44%), 257 (14%), 230 (26%), and 215 (M^+–R–42–AcOH, R=side chain, 100%); **2a**, m/e 444 (M^+, 34%), 429 (4%), 384 (M^+–AcOH, 36%), 330 (M^+–C–1 to C–4, 7%), 290 (5%), 276 (6%), 275 (30%), 257 (6%), 230 (40%), and 215 (100%); **3a**, m/e 458 (M^+, 30%), 443 (11%), 398 (M^+–AcOH, 45%), 383 (33%), 344 (M^+–C–1 to C–4, 5%), 290 (29%), 276 (28%), 275 (14%), 257 (12%), 230 (55%), and 215 (100%). The presence of the molecular ion peaks and the intense ions at m/e 215 (base peak), 275, and 257 were characteristic of stanyl acetates[7,8]. The component

9a (RRT 1.35) gave the molecular ion at m/e 456 (9%) corresponding to a C_{29}-monoene steryl acetate and other ions at m/e 413 (M^+–43, 7%), 353 (M^+–43–AcOH, 14%; 43=terminal isopropyl at C–25 to C–27), 344 (M^+–C–22 to C–29–1H, 38%), 329 (m/e 344–CH_3, 11%), 315 (M^+–R–2H, 66%), 284 (m/e 344–AcOH, 5%), 257 (M^+–R–AcOH, 72%), 255 (M^+–R–2H–AcOH, 10%), and 69 (100%). The ions at m/e 315 and 255, together with the ions at m/e 353, 344, 284, and 69 were indicative of the steryl acetate with double bond at C–22[7-9]. The presence of saturated steroid ring was shown by the intense ion at m/e 257. Thus, the component **9a** was identified as 24-ethylcholest-22-enyl acetate.

The components **1c**, **2c**, **9c** (mixture with **3a**), and **3c** were suspected to be 4α-monomethylsteryl acetates. GLC/Mass: **1c**, m/e 444 (M^+, 29%), 429 (M^+–CH_3, 11%), 384 (M^+–AcOH, 43%), 369 (M^+–AcOH–CH_3, 37%), 289 (M^+–R–42, 33%), 262 (M^+–R–AcO, 16%), 244 (M^+–R–27–AcOH, 30%), and 229 (M^+–R–42–AcOH, 100%); **2c**, m/e 458 (M^+, 30%), 443 (M^+–CH_3, 11%), 398 (M^+–AcOH, 45%), 383 (M^+–AcOH–CH_3, 40%), 289 (34%), 244 (29%), and 229 (100%); **3c**, m/e 472 (M^+, 45%), 457 (M^+–CH_3, 15%), 412 (M^+–AcOH, 66%), 397 (M^+–AcOH–CH_3, 56%), 289 (54%), 244 (46%), and 229 (100%). The molecular ions at m/e 444, 458, and 472 and the ion at m/e 289 (M^+–R–42) established that the components **1c**, **2c**, and **3c** had a steroid ring with an extra methyl group and the saturated side chains. The ions at m/e 244 and 229 also supported the presence of extra methyl group in the steroid ring. From the biogenetic grand, the extra methyl group was suspected to be located at C–4α rather than at C–14α. Therefore, the components **1c**, **2c**, and **3c** were identified as 4α-methylcholestanyl, 4α, 24-dimethylcholestanyl, and 4α-methyl-24-ethylcholestanyl acetates, respectively. The GLC/Mass of the peak (RRT 1.59) gave the prominent ions at m/e 470 (M^+, 10%) corresponding to a C_{30} monene steryl acetate, 358 (M^+–C–22 to C–29–1H, 35%), 329 (M^+–R–2H, 58%), and 271 (M^+–R–AcOH, 68%) besides the ions due to 24–ethylcholestanyl acetate (**3a**). The ions at m/e 358 and 329 were indicative of the presence of double bond at C–22[8] and also showed the steroid ring with an extra methyl group, together with the ion at m/e 271. Thus, the component was identified as 4α-methyl-24-ethyl-cholest-22-enyl acetate (**9c**).

Fraction b (Rf 0.75): GLC/Mass showed that this fraction contained at least five components with RRTs of 1.00 (**1b**), 1.11 (**7a**), 1.26 (**2b** and **7c**), and 1.53 (**3b**). The components **1b**, **2b**, and **3b** were identified as cholesteryl, 24-methylcholesteryl, and 24-ethylcholesteryl acetates, respectively. GLC/Mass: **1b**, m/e 368 (M^+–AcOH, 100%), 353 (28%), 260 (M^+–AcOH–108, 35%), 255 (28%), 247 (M^+–AcOH–108, 30%), 228 (5%), and 213 (15%); **2b**, m/e 382 (M^+–AcOH, 100%), 367 (15%), 274 (12%), 261 (13%), 255 (7%), 228 (4%), and 213 (6%); **3b**, m/e 396 (M^+–AcOH, 100%), 381 (10%), 288 (13%), 275 (10%), 255 (7%), and 213 (5%). The component **7a** (RRT 1.11) gave the molecular ion at m/e 442 corresponding to a C_{28} monoene steryl acetate and other ions at m/e 427 (M^+–CH_3, 2%), 344 (M^+–C–22 to C–28–1H, 57%), 329 (m/e 344–CH_3, 18%), 315 (M^+–R–2H, 54%), 284 (m/e

344–AcOH, 14%), 269 (M^+–AcOH, 13%), 257 (M^+–R–AcOH, 100%), 229 (8%), and 215 (8%). The ions at m/e 344, 329, and 285 due to the cleavage of C(20)–C(22) bond[7,8], together with the intense ion at m/e 315 indicated the presence of double bond at C–22. The presence of a saturated steroid ring was shown by the ion at m/e 257 (base peak) and the high molecular ion at m/e 442 (52%). Thus, the component **7a** was identified as 24-methylcholest-22-enyl acetate. The GLC/Mass of the peak (RRT 1.26) yielded the prominent ions at m/e 456 (M^+, 17%) corresponding to a C_{29} monoene steryl acetate, 358 (M^+–C–22 to C–28–1H, 14%), 329 (M^+–R–2H, 13%), and 271 (M^+–R–AcOH, 35%) besides the ions due to 24-methylcholesteryl acetate (**2b**). The ions at m/e 358, 329, and 271 were also observed in the mass spectrum of 4α-methyl-24-ethylcholest-22-enyl acetate (**9c**). Accordingly, the peak (RRT 1.26) was deduced to involve 4α, 24-dimethylcholest-22-enyl acetate (**7c**) besides **2b** as a component.

Fraction c (Rf 0.55): This fraction contained a very small quantity of single component with RRT 1.13. GLC/Mass: m/e 380 (M^+–AcOH, 100%), 365 (M^+–CH_3, 11%), 337 (M^+–43–AcOH, 5%), 282 (M^+–C–22 to C–28–1H–AcOH, 8%), 255 (M^+–R–AcOH, 55%), 228(8%), and 213 (9%). This cracking pattern and its relative intensity were almost similar to those of 24-methylcholesta-5, 22E-dienyl acetate (**7b**) which was detected in fraction d (RRT 0.50) from argentation TLC. The mass spectral data and the mobility in argentation TLC of this component resembled those of 24-methylcholesta-5, 22Z-dienyl acetate[10] from the short-necked

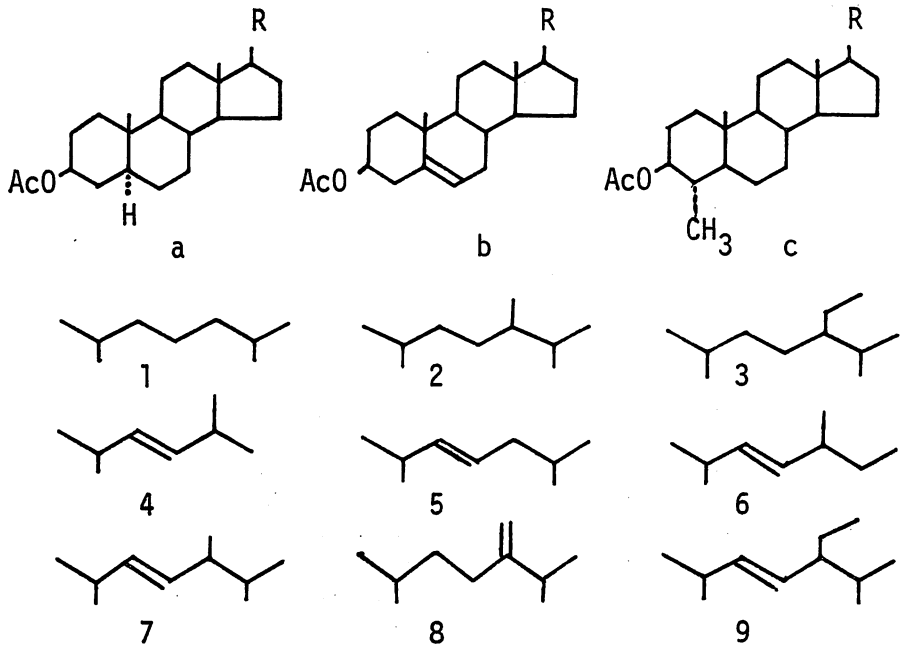

Fig. 2. Sterol components (as acetates) of the dinoflagellate, *N. milialis*.

clam, *Tapes philippinarum*, but the RRT was different with that of 24-methylcholesta-5, 22Z-dienyl acetate (RRT 1.05 in GLC on 1.5% OV–17). Accordingly, this steryl acetate was imagined to have the 27-nor or amurestane-like side chain; namely, 27-nor-24-ethylcholest-22-enyl or 27-nor-23, 24-dimethylcholest-22-enyl acetate. However, further characterization of this component was not carried out in the present study.

Fraction d (Rf 0.50): This fraction contained two components with RRTs of 0.89 (**6b**) and 1.13 (**7b**). The components **6b** and **7b** were identified as occelasteryl (27-nor-24-methylcholesta-5, 22E-dienyl) and 24-methylcholesta-5, 22E-dienyl acetates, respectively. GLC/Mass: **6b**, m/e 366 (M^+–AcOH, 100%), 351 (M^+–AcOH–CH_3, 13%), 337 (M^+–43–AcOH, 2%), 282 (M^+–C–22 to C–28–1H–AcOH, 9%), 255 (55%), 253 (11%), 228 (5%), and 213 (9%); **7b**, m/e 380 (M^+–AcOH, 100%), 365 (9%), 337 (6%), 282 (8%), 255 (58%), 253 (13%), 228 (10%), and 213 (9%). The mass spectrum of **6b** was indistinguishable from that of cholesta-5, 22E-dienyl acetate (**5b**), but **6b** had a shorter RRT in GLC on 1.5% OV–17 and was less polar in argentation TLC than **5b**. Therefore, **6b** was identified as occelasteryl acetate which had first isolated from the annelid, *Pseudopotamilla occelata* by KOBAYASHI and MITSUHASHI[11].

Fraction e (Rf 0.30): This fraction was composed of two components with RRTs

Table 2. Sterol components of free sterol fraction isolated from *N. milialis*.

Component (acetate)	RRT*1 OV–17	RRT*1 OV–1	%	Trivial name
4b	0.67	0.68	0.7	24-Norcholesta-5, 22E-dienyl acetate
6b	0.88	0.89	0.5	Occelasteryl acetate
5b	0.91	0.93	4.8	Cholesta-5, 22E-dienyl acetate
1b	1.00	1.00	0.3	Cholesteryl acetate
1a	1.03	1.01	5.6	5α-Cholestanyl acetate
	1.10	1.13	2.7	Unknown
1c	1.11	1.14	0.2	4α-Methylcholestanyl acetate
7b	1.14	1.13	72.5	24-Methylcholesta-5, 22E-dienyl acetate
7a	1.15	1.11	2.1	24-Methylcholest-22-enyl acetate
2b	1.27	1.26	1.0	24-Methylcholest-5-enyl acetate
7c	1.27	1.26		4α-Methyl-24-methylcholest-22-enyl acetate
2a	1.29	1.27	0.1	24-Methylcholestanyl acetate
8b	1.30	1.31	5.9	24-Methylenecholesteryl acetate
9a	1.36	1.35	2.0	24-Ethylcholest-22-enyl acetate
2c	1.49	1.48	0.4	4α-Methyl-24-methylcholestanyl acetate
3b	1.53	1.53	0.9	24-Ethylcholesteryl acetate
3a	1.58	1.59	0.2	24-Ethylcholestanyl acetate
9c	1.58	1.59		4α-Methyl-24-ethylcholest-22-enyl acetate
3c	1.70	1.80	0.7	4α-Methyl-24-ethylcholestanyl acetate

*1 Acetate derivatives

of 0.68 (**4b**) and 0.93 (**5b**). The components **4b** and **5b** were identified as 24-norcholesta-5, 22E-dienyl and cholesta-5, 22E-dienyl acetates, respectively. GLC/Mass: **4b**, m/e 352 (M^+–AcOH, 100%), 337 (16%), 282 (6%), 255 (63%), 253 (13%), 228 (6%), and 213 (9%); **5b**, 366 (M^+–AcOH, 100%), 351 (10%), 282 (7%), 255 (43%), 253 (6%), 228 (4%), and 213 (6%).

Fraction f (Rf 0.10): This fraction afforded a single component with RRT of 1.31 (**8b**). The component **8b** was identified as 24-methylenecholesteryl acetate. GLC/Mass: **8b**, m/e 380 (M^+–AcOH, 100%), 365 (13%), 296 (M^+–C–23 to C–28–1H–AcOH, 52%), 281 (m/e 296–CH_3, 12%), 253 (21%), 228 (5%), and 213 (7%).

As listed in Table 2, the dinoflagellate, *N. milialis*, was found to contain at least nineteen sterol components, the major sterols being 24-methylcholesta-5, 22E-dienol (72.5% of total sterols), 24-methylenecholesterol (5.9%), 5α-cholestanol (5.6%), and cholesta-5, 22E-dienol (4.8%).

Discussion

Recently, several groups of workers have fixed the attention on the sterols of dinoflagellates in the quest for organisms responsible for the biosynthesis of unusual sterols. SHIMIZU *et al.*[12] have isolated dinosterol (4α, 23, 24-trimethyl-5α-cholest-22-en-3β-ol) from the dinoflagellate, *Gonyaulax tamarensis*. WITHERS *et al.*[13] have also demonstrated the occurrence of dinosterol, 5-dehydrodinosterol, and the 3-oxo derivative (4α, 23, 24-trimethyl-5α-cholestan-3-one) in the non-photosynthetic organism, *Crypthecodinium cohnii*. Moreover, WITHERS *et al.*[14] have shown the incorporation of methionine-CD_3 into the newly synthesized dinosterol, indicating the mechanism for the production of the dinosterol side chain in the dinoflagellate, *C. cohnii*.

The present study shows that the dinoflagellate, *N. milialis*, contained 24-methylcholesta-5, 22E-dienol as the most major sterol. In addition, the results of the present study indicate the occurrence of small amounts of 4α-methylsterols such as 4α-methylcholestanol, 4α, 24-dimethylcholest-22-enol, 4α, 24-dimethylcholestanol, 4α-methyl-24-ethylcholest-22-enol, and 4α-methyl-24-ethylcholestanol. Interestingly, the dinoflagellate, *Amphidinium carterae*, and other *Amphidinium* species have been shown to contain amphisterol (4α-methyl-5α-ergosta-8(14), 24(28)-dien-3β-ol) and other 4-methylsterols more than 4-desmethylsterols.[15] BEASTALL *et al.*[16] have also isolated 4α-methyl-5α-cholest-8(9)-en-3β-ol, 4α, 24-dimethyl-5α-cholesta-8(9), 22-dien-3β-ol, 4α-methyl-24-ethyl-5α-cholest-8(9)-en-3β-ol, and 4α-methyl-24-ethyl-5α-cholestan-3β-ol from the red alga, *Porphyridium cruentum*.

Accumulation of knowledge on the methylsterols occurring in marine planktonic organisms will provide an important information not only on the biosynthetic pathways in planktons but also on the origin of methylsterols in marine invertebrates. STEUDLER *et al.*[17] have detected 4-methylgorgosterol and 4-methylgorgostanol in the zooxanthellae from the gorgonian, *Briareum asbestinum*. The latter C_{31}-sterol was also shown to be present in the snail, *Cyphoma gibbosum*, the predator of *B. asbestinum*. As

for the starfish, *Asterias rubens*, SMITH *et al.*[18] have demonstrated the occurrence of a number of methylsterols which were conceived to be of dietary origin and the possible intermediates in the sterol biosynthesis of *A. rubens*. Quite recently, we have detected 4α-methylcholest-8(14)-en-3β-ol and other 4-methylsterols in the oyster, *Crassostrea virginica*, with suggestion that 4-methylsterols possessing the side chain of C_9 and C_{10} were probably derived from some dietary sources[19].

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