Identification of the Free and Conjugated Sterol in a Non-Photosynthetic Diatom, *Nitzschia alba*, as 24-Methylene Cholesterol[1]

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ABSTRACT

Previous studies on the sterol fraction of the nonphotosynthetic marine diatom, *Nitzschia alba*, indicated the major sterol to be either brassicasterol (24*R*-methylcholesta-5,22-dien-3β-ol) or 22-dehydrocampesterol (24*S*-methylcholesta-5,22-dien-3β-ol) on the basis only of gas chromatography-mass spectral analysis. The present studies using nuclear magnetic resonance, infrared, and gas chromatography-mass spectrometry on the free and bound sterol fractions isolated by preparative thin layer chromatography showed the presence in both fractions of a single sterol, with spectral and chromatographic properties identical with those reported for 24-methylenecholesterol (ergosta-5,24(28)-dien-3β-ol). This sterol may be the precursor of 24-methyl sterols found in diatoms. The bound sterol fraction was found to consist of a single compound identified as 24-methylenecholesterol sulfate. No sterol esters or sterol glycosides were detected.

INTRODUCTION

In a previous study (1) on the sterols of the non-photosynthetic marine diatom, *Nitzschia alba*, we reported the major sterol to be either brassicasterol (24*R*-methylcholesta-5,22-dien-3β-ol) or 22-dehydrocampesterol (24*S*-methylcholesta-5,22-dien-3β-ol). This assignment was based only on gas chromatography (GC)-mass spectral analysis which is incapable of distinguishing between these two C-24 epimers (2,3) (see Fig, 1). Recent studies (2-5) have shown that C-24 epimers may be readily distinguished by their 100 or 200 MHz nuclear magnetic resonance (NMR) spectra, thus permitting the assignment of the major sterol in several species of photosynthetic diatoms (4,5) as the 24*S* (or 24-α) isomer, 22-dehydrocampesterol (see reviews [6,7]).

In view of these findings, we undertook a reexamination of free and conjugated sterols of *N. alba* by high resolution NMR as well as by infrared and GC-mass spectrometry. Surprisingly, both the free and bound sterol fractions yielded only a single sterol which was neither of the C-24 epimers, brassicasterol, or 22-dehydrocampesterol, nor was it the isomeric codisterol (Fig. 1) recently identified in the green alga *Codium fragile* (8). The present communication deals with the identification of the *N. alba* sterol as 24-methylenecholesterol (ergosta-5-24(28)-dien-3β-ol), a positional isomer of codisterol and the presumed precursor of the C-24 epimeric sterols (4,6).

MATERIALS AND METHODS

Cells of *N. alba* (Lewin and Lewin strain) were grown at 30 C in a synthetic seawater medium containing Na_2SiO_3 and glucose (9) as described previously (10) and harvested in the late logarithmic growth phase. Total lipids were extracted from the cells (1) and fractionated on a column of Biosil A silicic acid as described elsewhere (11). Neutral lipids (containing free sterols) were eluted with 10 column volumes of chloroform (Fraction I), glycolipids and sulfolipids with 3 column volumes of acetone followed by 3 column volumes of methanol-acetone (1:9, v/v) (Fraction II), and phospholipids with 10 column volumes of methanol (Fraction III). Fraction I (neutral lipids) was further fractionated (11) by chromatography on a column of Biosil A silicic acid using petroleum ether (bp 40-60 C) to elute hydrocarbons, petroleum ether-ethyl ether (9:1, v/v) to elute triglycerides and free fatty acids, petroleum ether-ethyl ether (1:1, v/v) for sterols, and finally ethyl ether and chloroform to remove unidentified polar species of neutral lipids. The pure free sterol component was isolated by preparative thin layer chromatography (TLC) of the petroleum ether-ethyl ether (1:1) fraction on 1 mm thick Silica Gel H layers developed twice in petroleum ether-ethyl ether-acetic acid (50:50:1, v/v); after visualization of the lipid spots with dichlorofluorescein (12), the sterol band was eluted from the plate with chloroform and finally purified by passage through a small column of Biosil A silicic acid eluted with

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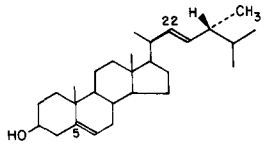
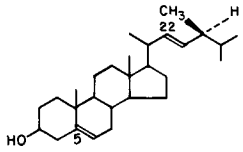
BRASSICASTEROL



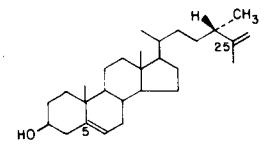
24R-methylcholesta-5,22-dien-3β-ol

22-DEHYDROCAMPESTEROL



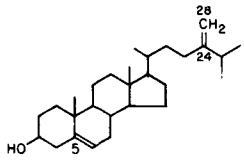
24S-methylcholesta-5,22-dien-3β-ol

CODISTEROL



24S-methylcholesta-5,25-dien-3β-ol

24-METHYLENECHOLESTEROL



ergosta-5,24(28)-dien-3β-ol

FIG. 1. Structures of isomeric diunsaturated C_{28}-sterols: brassicasterol, 22-dehydrocampesterol, codisterol, and 24-methylenecholesterol.

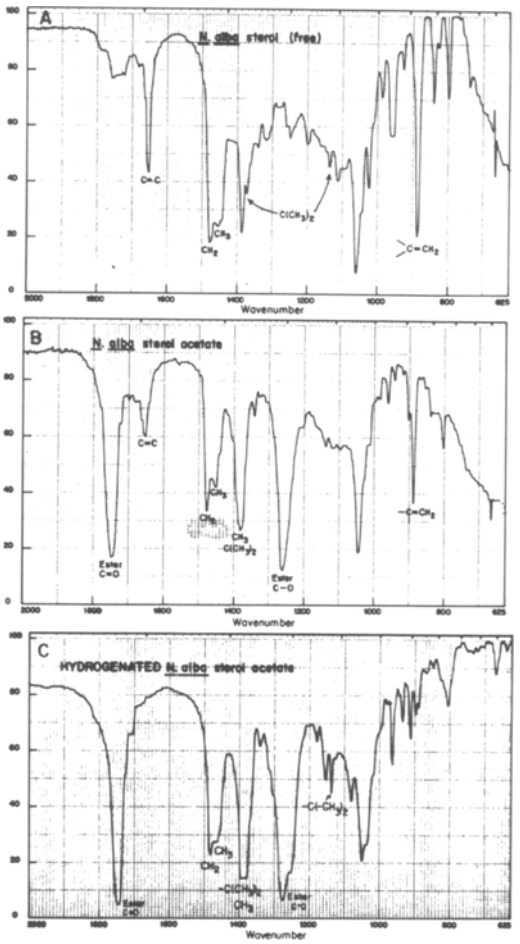


FIG. 2. Infrared spectra (in KBr) of: A, *N. alba* (free) sterol; B, *N. alba* sterol acetate; C, hydrogenated *N. alba* sterol acetate.

petroleum ether to remove extraneous lipid followed by petroleum ether-ethyl ether (1:1) to remove the sterol. The free sterol thus obtained was TLC pure (R_f 0.45) and gave a red color with the sterol spray reagent (12).

The "bound" sterol (sterol sulfate) was isolated from Fraction II (glycolipid + sulfolipid fraction described above) by preparative TLC on Silica Gel H in chloroform-methanol-28% ammonia (65:35:5, v/v) (R_f, 0.67) and precipitated from acetone as the ammonium salt, as described elsewhere (13). The sterol component of the sterol sulfate was obtained in two ways: (a) by hydrolysis with 2.5% methanolic-HCl under reflux for 5 hr (1,12), and (b) by solvolysis in 0.005M HCl in anhydrous tetrahydrofuran at room temperature for 2 hr (13,14). The sterol obtained was purified by preparative TLC as described above.

Authentic sterol samples (brassicasterol, campesterol, stigmasterol, and β-sitosterol) were purchased from Supelco Inc. (Bellefonte, PA); brassicasterol was also isolated from rapeseed oil unsaponifiable material by preparative TLC as described above. 24-Methylenecholesterol was a gift from Dr. D.R. Idler, Marine Sciences Laboratory, St. John's, Newfoundland.

Free sterols were converted to acetate esters by reaction with anhydrous pyridine-acetic anhydride (2:1, v/v) for 16 hr at room temperature as described eleswhere (12). Sterol acetates (10 mg) were hydrogenated in ethyl acetate solution (3 ml) with palladium on carbon catalyst (10 mg) for 4 hr at room temperature and a slightly positive pressure of hydrogen. After centrifugation of the catalyst, the reduced pro-

TABLE I

Gas Liquid and Thin Layer Chromatographic Properties of Sterols

Sterol	GLC relative retention[a]		TLC[b] R_f of acetate	Number of double bonds per molecule
	Free sterol	Acetate		
N. alba sterol	1.31	1.32	0.17	2
Brassicasterol	1.14	1.15	0.46	2
24-Methylenecholesterol[c]	---	1.31	0.17	2
Campesterol	1.36	1.38	0.58	1
Stigmasterol	1.48	1.51	---	2
β-Sitosterol	1.79	1.81	---	1
Cholesterol	1.00	1.00	0.52	1
Cholestanol	---	---	0.62	0
Hydrogenated *N. alba* sterol	---	1.39	0.64	0
Tetrahydrobrassicasterol	---	1.39	0.61	0
Dihydrocampesterol	---	1.39	0.61	0

[a]On a column (46 cm x 0.6 mm) of 3% SE-30 on Chromosorb W at 190C and flow rate of 110 ml/min; relative to cholesterol (ret. time, 20.9 min.) or cholesterol acetate (ret. time, 29.2 min.).

[b]On 10% $AgNO_3$-Silica Gel H plates in solvent system $CHCl_3$-MeOH; 99.6:0.4.

[c]Authentic sample from Dr. D. Idler.

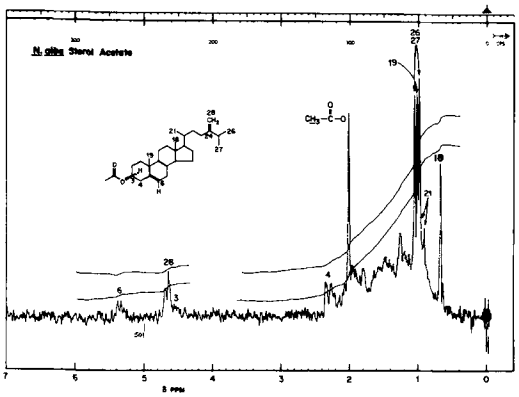


FIG. 3. PMR spectrum (in $CDCl_3$) of *N. alba* sterol acetate.

duct was obtained by evaporation of the solvent and was purified by preparative TLC on Silica Gel H in petroleum ether-ethyl ether-acetic acid (90:10:1, v/v).

Sterols were analyzed as acetates by TLC on 10% $AgNO_3$-Silica Gel H in the solvent system chloroform-methanol (99.6:0.4, v/v) and in both the free form and as acetates by gas liquid chromatography (GLC) on a column (46 cm x 0.6 mm) of 3% SE-30 on Chromosorb W at 190 C.

Infrared spectra were taken in KBr on a Pye-Unicam infrared spectrometer. Proton magnetic resonance spectra were measured with a Varian HA-100 spectrometer in $CDCl_3$ containing 1% tetramethylsilane as internal reference. Mass spectra of the sterol acetates were obtained with a Finnigan 3100D quadrupole mass spectrometer after GLC on a 1.5 m column (2 mm ID) of 3% OV-17 at 240 C; the TMS derivative of the *N. alba* sterol was analyzed on a Shimadzu-LKB 9000 GC-mass spectrometer by Dr. A. Hayashi, Kinki University, Japan.

RESULTS AND DISCUSSION

The free sterol isolated from *N. alba* showed a single component both on GLC and $AgNO_3$-SiO_2 TLC which was well resolved from brassicasterol but had similar chromatographic mobilities to those of 24-methylenecholesterol (Table I). The hydrogenated *N. alba* sterol, however, had GLC retention time and TLC mobility identical with those for tetrahydrobrassicasterol or dihydrocampesterol (Table I).

The acetate of the *N. alba* sterol showed a mass spectrum (Table II) with ion peaks at m/e 380 (M^+-acetate), 365 (M^+-CH_3-acetate), 255 (M^+-side-chain-acetate), and 213 (M^+-side-chain-42-acetate) indicating it was a disunsaturated C_{28} steryl acetate. In addition, an ion peak at m/e 296 (M^+-84-acetate), ascribed to loss of part of the side chain by MacClafferty rearrangement, is characteristic of 24-methylene sterols but not of 25-methylene sterols such as codisterol (8). Brassicasterol acetate had an almost identical mass spectrum (Table II), but that of codisterol differed in several respects (see Table II and Ref. 8). The TMS derivative of the *N. alba* sterol has a mass spectrum identical to that reported for the TMS derivative of 24-methylenecholesterol (15) (Table III). The hydrogenated *N. alba* sterol acetate showed a molecular ion peak at m/e 444 and a spectrum identical to that of tetrahydrobrassi-

TABLE II

Mass Spectral Data[a] for *N. alba* Sterol Acetate and Reference Compounds

	N. alba sterol acetate		24-Methylene-cholesterol acetate[b]		Codisterol acetate[c]		Brassicasterol acetate		Hydrogenated *N. alba* sterol acetate		Tetrahydro-brassicasterol acetate	
Fragment	m/e	R.I.	m/e	R.I.	m/e	R.I.	m/e	R.I.	m/e	R.I.	m/e	R.I.
M^+					440	3			444	0.4	444	0.1
M^+-15									439	0.3	439	0.1
M^+-60	380	2.5	380	100	380	100	380	13	384	2.5	384	0.4
M^+-75	365	0.9	365	24			365	3	369	3.8	369	0.7
M^+-60-30									354	0.1		
M^+-60-42	338	0.1					338	1	342	0.2		
M^+-60-43			337	6			337	2				
M^+-99-15									330	0.2		
M^+-127					313	4						
M^+-129									315	0.3		
M^+-60-83	297	1.2							301	0.2	301	0.1
M^+-60-84	296	4.8	296	63	296	5	296	2	300	0.1		
M^+-154									290	0.4	290	0.2
M^+-60-97	283	0.8					283	2	287	0.1		
M^+-60-98	282	1.1					282	4	286	0.1	286	0.1
M^+-60-99	281	3.3	281	26			281	2	285	0.6	285	0.2
M^+-168	272	0.6	272	10			272	1	276	5.0	276	2.6
M^+-169	271	0.2							275	5.0	275	2.7
M^+-181	259	1.9					259	3				
M^+-60-125	255	2.3			255	6	255	32	259	1.0	259	0.4
M^+-60-127	253	6.5	253	30			253	9	257	4.0	257	2.7
M^+-189	251	0.1					251	1	255	3.0	255	0.5
M^+-212	228	2.7	228	40	228	13	228	7	232	2.0		
M^+-214	226	1					226	2	230	9	230	8
M^+-60-125-42	213	9	213	25	213	13	213	16	217	15	217	13
M^+-60-127-42	211	5					211	6	215	72	215	53
Base peak	81	100					145	100	147	100	107	100

[a]Analyses of acetates carried out here were on a Finnigan 3100D quadrupole mass spectrometer.
[b]Data supplied by Dr. L.J. Goad.
[c]Data from Ref. 8.

TABLE III

Mass Spectral Data[a] for TMS Derivative of *N. alba* Sterol and 24-Methylenecholesterol[b]

Fragmentation	*N. alba* sterol-TMS		24-Methylene-cholesterol-TMS[b]	
	m/e	R.I.	m/e	R.I.
M^+	470	25	470	30
M^+ - 15(CH_3)	455	25	455	23
M^3 - 84(C_{23} to C_{28} + H)[c]	386	51	386	67
M^+ - 90(TMS-OH)	380	57	380	78
M^+ - 99(CH_3 + C_{23} to C_{28} + H)	371	14	371	14
M^+ - 105(CH_3 + TMS-OH)	365	38	365	52
M^+ - 125(side chain)	345	5	345	30
M^+ - 127(side chain + 2H)	343	32	343	42
M^+ - 129(C_1 to C_3 + TMS-O)	341	73	341	100
M^+ - 152(side chain + 27)	318	2	318	3
M^+ - 167(side chain + 42)	303	3	303	---
M^+ - 174(C_{23} to C_{28} + H + TMS-OH)[c]	296	45	296	67
M^+ - 189(CH_3 + C_{23} to C_{28} + H + TMS-OH)	281	25	281	45
M^+ - 211(TMS-OH + 121)	259	17	259	24
M^+ - 213(C_{23} to C_{28} + H + C_1 to C_3 + TMS-O)	257	39	257	60
M^+ - 215(side chain + TMS-OH)	255	17	255	48
M^+ - 217(side chain + 2H + TMS-OH)	253	25	253	52
M^+ - 241(side chain + 27 + TMS-O)	229	9	229	30
M^+ - 243	227	12	---	---
M^+ - 257(side chain + 42 + TMS-OH)	213	23	213	42
M^+ - 259(side chain + 2H + 42 + TMS-OH)	211	11	211	25
M^+ - 341(base peak)	129	100	129	---

[a]Analysis on Shimadzu-LKB 9000 GC-mass spectrometer on a 2% OV-1 column at 250 C.
[b]Data from Ref. 15.
[c]Characteristic peaks for 24-methylenecholesterol (15).

casterol or dihydrocampesterol acetates (Table II), confirming that the *N. alba* sterol contained two double bonds and was probably an isomer of brassicasterol in which the side-chain double bond was in a position other than at C_{22}.

The infrared spectrum of the free *N. alba* sterol (Fig. 2A) showed characteristic bands at 890 cm^{-1} and 1655 cm^{-1} indicative of a $R_1R_2C{=}CH_2$ group and bands at 1390-1375 cm^{-1} (doublet) and 1140 cm^{-1} corresponding to an isopropyl group. The spectrum of the acetate derivative (Fig. 2B), however, did not show the shoulder at 1375 cm^{-1} characteristic of an isopropyl group but only a broad peak at 1380 cm^{-1}. These spectra were identical with those of authentic 24-methylenecholesterol (see Ref. 16,17). In contrast, brassicasterol showed bands at 1680 cm^{-1} and 960 cm^{-1} indicative of a *trans*-double bond, apart from bands at 1395-1380 cm^{-1} (doublet) and 1140 cm^{-1} indicative of an isopropyl group, and codisterol showed bands for a terminal methylene at 890 and 1645 cm^{-1} but lacked any isopropyl bands (8). However, the infrared spectrum of the hydrogenated *N. alba* (Fig. 2C) sterol was identical with that of tetrahydrobrassicasterol or dihydrocampesterol. This evidence is consistent with the presence in the *N. alba* sterol of a methylene group located at C-24.

Confirmation of the terminal double bond at C-24 was obtained by the NMR spectrum (Fig. 3; Table IV) which showed a doublet at δ4.66 and 4.72 due to two protons of a terminal methylene group assigned as C-28, since C-26 methylene protons give a singlet at δ4.66 (see assignments for codisterol in Talbe IV); in brassicasterol, the C-22,23 protons of the Δ^{22} double bond gave a multiplet centered at δ5.2. The second double bond in the *N. alba* sterol was located at the Δ^5 position by the multiplet centered at δ5.36 due to the C-6 proton and by the presence of C-18 and C-19 methyl proton singlets at δ0.68 and 1.04, respectively. The signal at δ1.62 due to the C-27 methyl protons in the spectrum of codisterol (8) was absent from the spectrum of the *N. alba* sterol. The spectrum of the *N. alba* sterol acetate was, in fact, identical with that of an authentic sample of 24-methylenecholesterol acetate.

TABLE IV

NMR Assignments for *N. alba* Sterol (as Acetate)

Group assignment	Chemical shift[a] (δ), ppm				
	N. alba sterol acetate				
	"Free"	From sulfate ester	24-Methylenecholesterol acetate[b]	Codisterol acetate (8)	Brassicasterol acetate (rapeseed)
=C*H*(C-6), multiplet	5.35 - 5.40	5.31 - 5.43	5.3 - 5.4	5.35	5.36 - 5.43
=C*H*(C-28), doublet	4.66 + 4.72	4.66 + 4.71	4.66 + 4.72	---	---
=CH_2(C-26), singlet	---	---	---	4.66	---
O-C-*H*(C-3α), multiplet	4.63	4.63	4.63	4.63	4.60
CH_3-CO-(C-3 acetate), singlet	2.02	2.03	2.02	2.02	2.03
CH_3-C=(C-27), singlet	---	---	---	1.62	---
CH_3(C-19), singlet	1.02	1.03	1.03	1.02	1.02
CH_3(C-28), doublet	---	---	---	0.93 + 1.00	0.89 + 0.95
CH_3(C-21), doublet	0.91 + 0.97	0.91 - 0.98	0.92 + 0.98	0.87 + 0.95	0.98 + 1.05
CH_3(C-18), singlet	0.68	0.68	0.69	0.66	0.69
*H*C=C*H*(C-22,23), multiplet	---	---	---	---	5.18 - 5.25
CH_3(C-26), doublet CH_3(C-27), doublet	1.00 + 1.07	1.00 + 1.07	1.00 + 1.07	---	0.88 + 0.81 0.86 + 0.79

[a]In $CDCl_3$.
[b]Authentic sample from Dr. D. Idler.

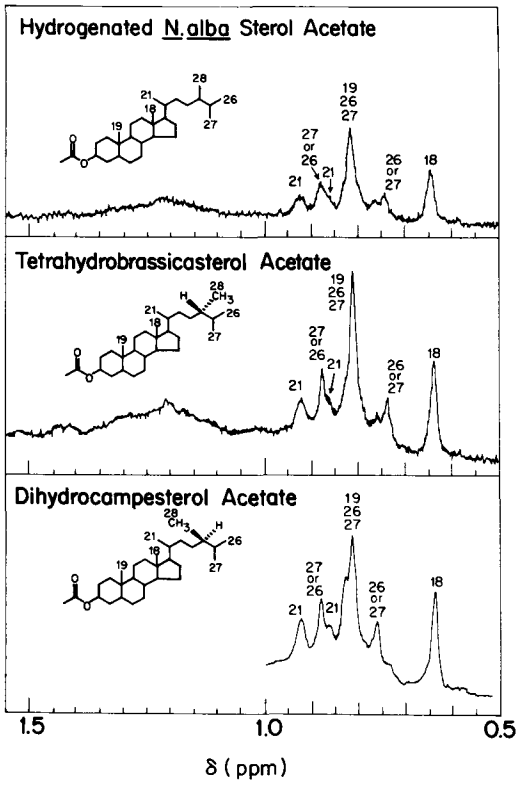


FIG. 4. PMR spectrum (in $CDCl_3$) of acetates of: A, hydrogenated *N. alba* sterol; B. tetrahydrobrassicasterol; and C, dihydrocampesterol.

The tetrahydro derivative of the *N. alba* sterol had an NMR spectrum (Fig. 4) similar to that of a mixture of tetrahydrobrassicasterol and dihydrocampesterol, the two epimeric sterols that would be expected on catalytic hydrogenation of the C-24 methylene group.

The sterol obtained from the sterol sulfate of *N. alba* by mild acid solvolysis (13) showed a single component on GLC and TLC with identical retention time and R_f value, respectively, and identical mass, infrared, and NMR spectra to those of the free sterol in *N. alba*. When strong acid-catalyzed methanolysis was used to cleave the sterol sulfate, a mixture of isomeric sterols and derivatives was obtained as described previously for cholesteryl esters (18).

It may thus be concluded that *N. alba* contains a single sterol, present in both free form and in conjugated form as the sulfate ester, having the structure 24-methylenecholesterol (Fig. 1). The presence in *N. alba* of a minor sterol reported to be clionasterol (1) is in error and is probably attributable to formation of artifacts during strong acid-catalyzed methanolysis of the sulfate ester (see Ref. 18). It should also be mentioned that contrary to our previous suggestion (1), no sterol esters or sterol glycosides have been detected (11,13) on TLC of the neutral lipids (Fraction I) or the glycolipids (Fraction II), respectively.

The finding of 24-methylenecholesterol as the only sterol in *N. alba* is unusual, since all diatoms so far examined appear to contain 22-dehydrocampesterol as principal sterol (4,6,7). However, there is good evidence that 24-methylenecholesterol is the precursor of 22-dehydrocampesterol in the diatom *Phaeodactylem tricornutum* (4,6). Thus, the absence of a 24-methyl sterol in *N. alba* may indicate that this non-photosynthetic diatom lacks the reductase necessary for its formation. 24-Methylenecholesterol occurs in significant amounts in marine invertebrates (16) and is believed to be the precursor of 24-methyl sterols in nature (6,16,17). On the basis of the present findings, 24-methylenecholesterol may have a dietary origin in marine invertebrates.

ACKNOWLEDGMENTS

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