Biosynthetic Studies of Marine Lipids. 31.[1] Evidence for a Protonated Cyclopropyl Intermediate in the Biosynthesis of 24-Propylidenecholesterol

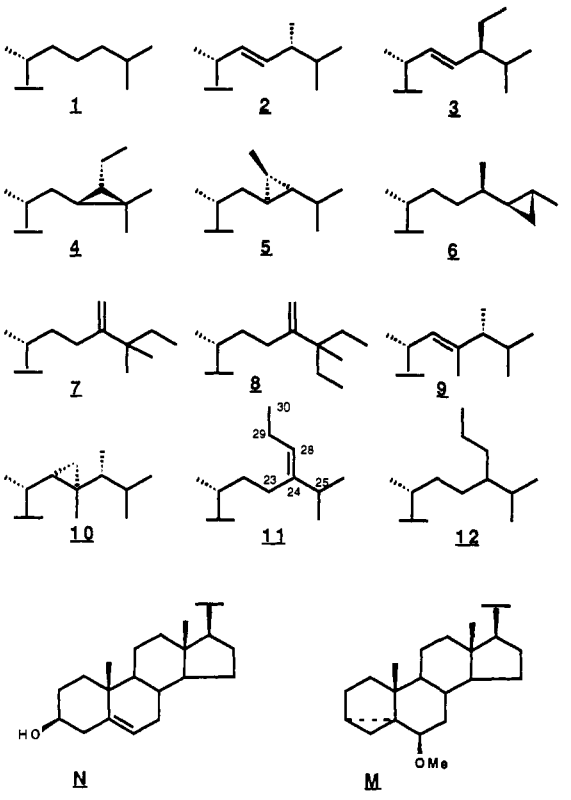
José-Luis Giner and Carl Djerassi*

Contribution from the Department of Chemistry, Stanford University, Stanford, California 94305.
Received August 9, 1990

Abstract: The biosynthesis of 24-propylidenecholesterol (**11-N**) was determined by the use of cell-free extracts from the Chrysophyte alga *Chrysoderma mucosa*. The biosynthetic sequence was shown to proceed via desmosterol (**28-N**), 24-methylenecholesterol (**29-N**), and isofucosterol (**30-N**) to 24-propylidenecholesterol (**11-N**). 24-Vinylcholesterol (**13-N**) was neither a substrate nor a product of the *S*-adenosylmethionine (SAM)-methyltransferase, nor was it detected in cultures grown in the presence of azasterol inhibitors. Chemical degradation of 24-propylidenecholesterol (**11-N**) from enzymatic [^{3}H]SAM methylation of isofucosterol (**30-N**) provided evidence for the protonated cyclopropane intermediate (**27-N**). The mechanism involving such an intermediate is consistent with the structures and stereochemical assignments of trace sterols found in this alga. Evidence for the intermediacy of protonated cyclopropanes in the SAM sterol methyl-transfer reactions leading to isofucosterol (**30-N**), fucosterol (**31-N**), and 24-methylenecholesterol (**29-N**) could not be found.

With the exception of animals and red algae, which contain cholesterol (**1-N**), almost all eukaryotic organisms are capable of adding extra carbon atoms to the side chains of their sterols.[2] These are added enzymatically via an unusual reaction involving the electrophilic addition of the methyl group of a sulfonium salt (*S*-adenosylmethionine[3] (SAM)) to an isolated double bond.[4] Typically, one or two carbon atoms are added at position 24, as illustrated by the side chains of ergosterol (**2**), a typical fungal sterol, and stigmasterol (**3**), a typical plant sterol. In the last 20 years, a large number of sterols bearing unusual side chains have been isolated from marine organisms—chiefly sponges (e.g., **4–8**)[5] and dinoflagellates (e.g., **9–10**).[7] The most unusual feature found in marine sterols is the presence of cyclopropane rings in the side chains.[6] While the great variety of unusual sterols of marine origin probably reflects the great phylogenetic diversity of the marine world, we believe that insight into the chemical mechanisms of sterol side chain biosynthesis can be gained from them.

24-Propylidenecholesterol (**11-N**) occurs in Chrysophyte algae of the order Sarcinochrysidales.[7] These algae are restricted to the marine environment and are believed to be related to the Phaeophytes.[8] Sterols with the saturated version of this side chain (**12**) have been used as geochemical markers for sediments of marine origin.[9] The origin of the linear three-carbon appendage to the side chain is biosynthetically more intriguing than it would appear at first glance. We have proposed two possible pathways



(Figure 1).[7a,10] One route involves a linear extension via 24-vinylcholesterol (**13-N**, Scheme 1); the other involves the rearrangement of a cyclopropane (**14-N**, Scheme 2).

In a painstaking analysis of the minor and trace sterols of *Chrysoderma mucosa*,[11] two isomers of the cyclopropane (**14a-N**, **14b-N**) were found (Figure 2).[10] Several additional sterols bearing three extra carbon atoms in the side chain were found (**15-N–21-N**). All of these can be formally derived from the cyclopropane **14-N** by acid-catalyzed rearrangement. However, when the alga was grown in the presence of methionine-d_3, all of the positions in the propylidene group were labeled with deuterium.[10] Since acid-catalyzed ring opening of the cyclopropane **14-N** is expected to proceed with the addition of a proton to the propylidene group, the route via 24-vinylcholesterol (**13-N**) was fa-

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(11) We thank Dr. Robert A. Andersen (Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, ME) for the identification of the alga.

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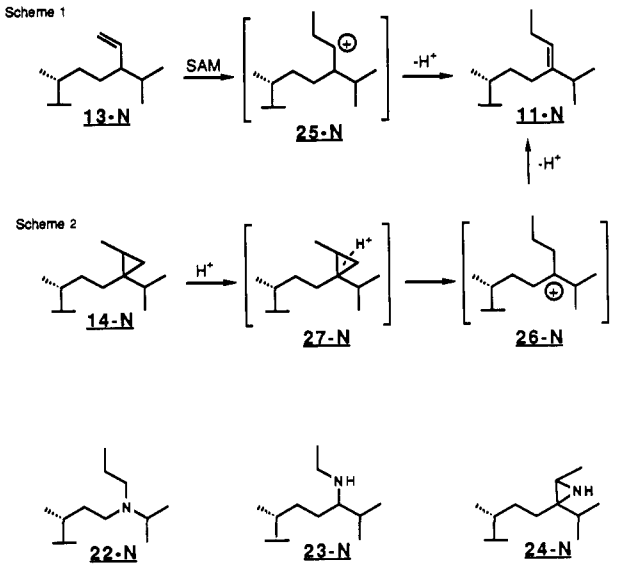

Figure 1. Proposed pathways to 24-propylidenecholesterol (**11-N**)[6a] and "high-energy intermediate analog" inhibitors **22-N**–**24-N** of sterol biosynthesis.

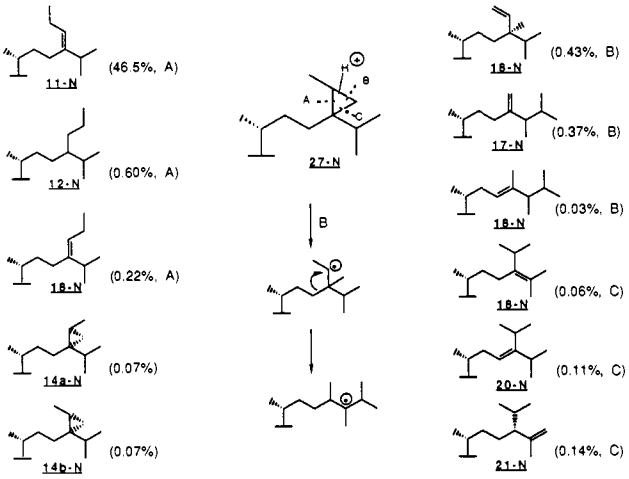

Figure 2. C30 sterols of *C. mucosa*[10] and a hypothetical common intermediate **27-N** (percent sterol mixture, bond broken in intermediate).

vored. Although 24-vinylcholesterol (**13-N**) could not be detected in the sterol mixture, the possibility remained that it was a transient intermediate, present only in very small amounts.

Azasterol Inhibition of Sterol Biosynthesis. In an effort to trap the putative 24-vinylcholesterol (**13-N**) intermediate, possible inhibitors (**22-N**–**24-N**, Figure 1) of the methyl-transfer reaction were designed, constructed, and fed to the alga. Azasterol inhibitors[12] have been used as probes of sterol biosynthesis. They are believed to be "high-energy intermediate analogues" in which the protonated, ammonium form of the molecule mimics the putative carbonium ion intermediate in the methyl-transfer reaction. Perturbation of sterol biosynthesis by such inhibitors has led to insight into the pathways operating in yeast[13] and in higher plants.[14]

Two azasterols (**22-N**, **23-N**) were synthesized as analogues of the hypothetical classical carbonium ion intermediates (**25-N**, **26-N**), and aziridine[15] **24-N** was synthesized as an analogue of

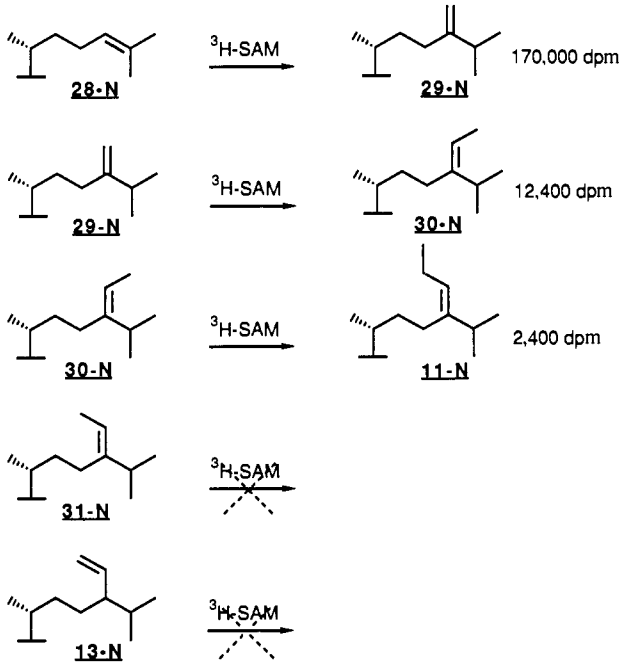

Figure 3. Cell-free extract experiments with *C. mucosa* (25 μCi of [^{3}H]SAM per experiment).

the hypothetical protonated cyclopropane **27-N**. All three azasterols inhibited sterol methylation in *Chrysoderma*, leading to the accumulation of dimethylated (e.g., **3**), monomethylated (e.g., **2**), and, at the highest concentrations, unmethylated (**1**) sterols. No 24-vinylcholesterol (**13-N**), however, could be detected.

Cell-Free Extract Experiments and Degradations. Because the alga does not take up radioactive sterols from the medium, ordinary feeding experiments were not possible and it was necessary to prepare a cell-free extract.[16] Although the mucilaginous cell coat initially caused some difficulties, a cell-free extract of sufficient enzymatic activity was finally prepared with use of techniques developed for higher plants[17] and marine algae.[18] Incubations of this cell-free extract with [^{3}H]SAM and sterol substrates (Figure 3) showed that desmosterol (**28-N**) was converted to 24-methylenecholesterol (**29-N**), 24-methylenecholesterol (**29-N**) to isofucosterol (**20-N**), and isofucosterol (**30-N**) to 24-propylidenecholesterol (**11-N**). The identity of the products was determined by HPLC and coinjection of "cold" carrier and, in the case of 24-propylidenecholesterol (**11-N**), also through derivatization by forming the *i*-methyl ether and the acetate and by hydrogenation. Each intermediate from desmosterol (**28-N**) to 24-propylidenecholesterol (**11-N**) was a worse substrate than its precursor. Perhaps all three alkylations occur at the same enzyme, nonspecific enough to catalyze each interconversion but losing binding affinity with every increment in steric bulk. Neither 24-vinylcholesterol (**13-N**) nor fucosterol (**31-N**) served as substrates.

The direct conversion of isofucosterol (**30-N**) to 24-propylidenecholesterol (**11-N**) rules out the possibility of discrete cyclopropane intermediate **14-N**. Protonated cyclopropane reaction intermediate **27-N**, on the other hand, would be consistent with the trace sterols (Figure 2) as well as with the deuterium-labeling experiment.[10] Bridged carbonium ions have long been drawn to explain aspects of biosynthetic reactions,[19] including the

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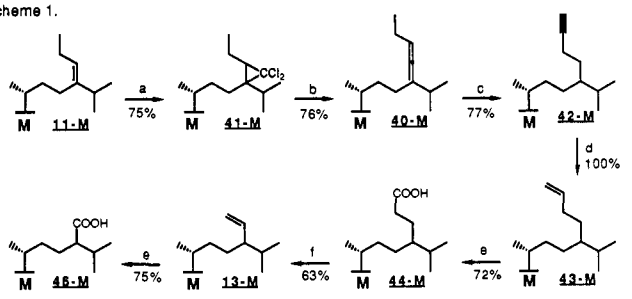
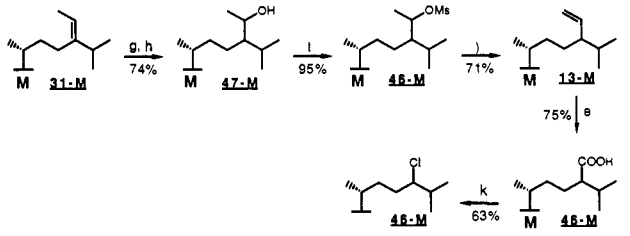
Scheme 1.



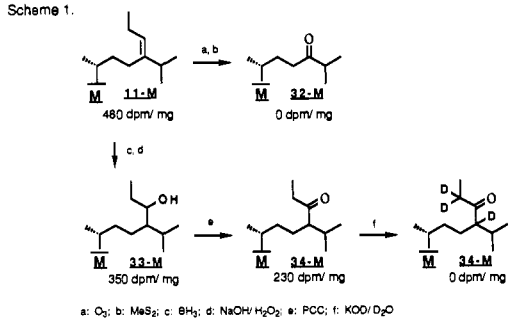
Scheme 2.



a: NaOH/CHCl$_3$; b: n-BuLi; c: KAPA; d: H_2/ Lindlar's catalyst; e: $KMnO_4$/ $NaIO_4$; f: $Pb(OAc)_4$/ $Cu(OAc)_2$; g: BH_3; h: NaOH/ H_2O_2; i: MsCl/ Et_3N; j: KOt-Bu; k: $Pb(OAc)_4$/ LiCl.

Figure 4. Degradation schemes for ^{14}C-labeled 24-propylidenecholesterol (**11-N**) and 24-ethylidenecholesterol (**31-N**).

Scheme 1.



a: O_3; b: MeS_2; c: BH_3; d: NaOH/ H_2O_2; e: PCC; f: KOD/ D_2O

Scheme 2.

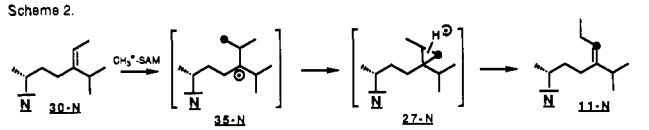


Figure 5. Degradation of tritium-labeled 24-propylidenecholesterol (**11-N**).

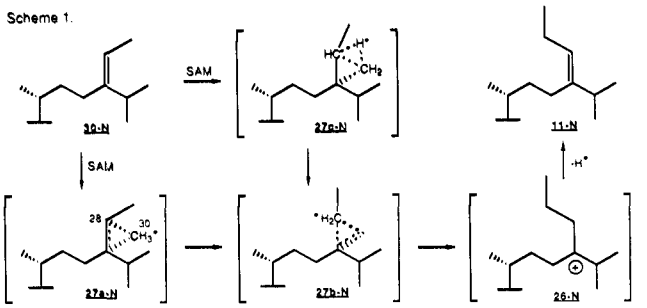
stereochemistry of the SAM sterol methyltransferase of yeast.[20]

If 24-propylidenecholesterol (**11-N**) arises through the rearrangement of the protonated cyclopropane intermediate **27-N**, it would be interesting to know where the carbon atoms in the side chain end up. With this goal, a degradation was developed with unlabeled material by which the propylidene group could be dissected one carbon at a time (Figure 4). However, the low reactivity of the enzyme precluded any experiments with [^{14}C]-SAM because of the low specific activity of ^{14}C or with labeled sterol because of the low affinity of the enzyme for the sterol substrate.

Since we were unable to use a ^{14}C label for technical reasons, we carried out a degradation of ^{3}H-labeled 24-propylidenecholesterol (**11-N**) after protection as the *i*-methyl ether (Figure 5, Scheme 1). Ozonolysis of the 24-propylidene side chain to the ketone **32-M** resulted in complete loss of radioactivity. The sequence hydroboration to the alcohol **33-M**, oxidation to the ketone **34-M**, and exchange of the acidic protons α to the ketone also removed all of the ^{3}H label. If the reaction involves the closure of a highly symmetrical classical carbonium ion **35-N** to the

(20) Arigoni, D. *Ciba Found. Symp.* **1978**, *60*, 243–261.

Scheme 1.



Scheme 2.

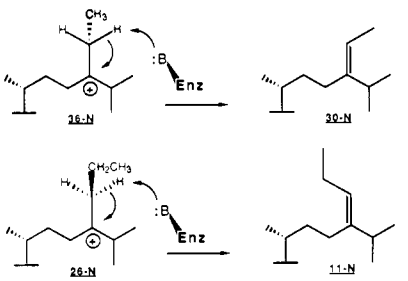


Figure 6. Proposed mechanism of 24-propylidenecholesterol (**11-N**) biosynthesis and origin of double-bond configurations.

hypothetical bridged intermediate **27-N** (Figure 5, Scheme 2), then this result would rule out three of the four possible closures—all of the possible closures but the one that gives the same outcome as direct formation of a bridged carbonium ion **27-N**. It may be a moot point whether there is an open carbonium ion intermediate **35-N** that closes in a stereospecific way under the guidance of the enzyme or whether the bridged species **27-N** is formed directly; the results, however, are fully consistent with the direct formation.

The proposed methyl-transfer reaction is consistent with the stereochemistry of the minor products of the reaction (Figure 2). The stereochemistry of 24-vinyl-24-methylcholesterol (**16-N**) (see Experimental Section for assignment) is consistent with the stereochemistry of the bridged intermediate **27-N** as deduced from the cyclopropanes **14a-N** and **14b-N**.[21] The configuration of 24-isopropenylcholesterol[22] (**21-N**) can result from that of the bridged intermediate **27-N** via a hydride shift that obeys the Ruzicka rule.[19] The stereochemistry of the two cyclopropanes **14a-N** and **14b-N**, however, is puzzling at first glance. While **14a-N** has the correct stereochemistry on the basis of the addition of the methyl group from the β-face[20] of the isofucosterol side chain (**30-N**), its epimer **14b-N** has the opposite stereochemistry at C28. This could be interpreted as the result of the methylation of fucosterol (**31-N**) had it not been shown (Figure 3) that fucosterol (**31-N**) is neither a product nor a substrate of the methyltransferase. An explanation for the isolation of this epimeric cyclopropane **14b-N** can be found in quantum mechanical calculations that show a free rotation of the bridging group in protonated cyclopropane.[23] Rotation of the bridging ethyl group in the protonated cyclopropane resulting from proton migration from C30 to C28 (**27b-N**, Figure 6, Scheme 1) would cause epimerization at that position. An attractive alternative sequence to **27b-N** proceeds via the edge-protonated cyclopropane **27c-N**. This can arise from initial methyl attack at the 24-position to give a secondary carbonium ion at C28 followed by interaction of a methyl C–H bond with the empty π-orbital of the carbonium ion.

The opposite double-bond configurations of the 24-ethylidenecholesterol (**30-N**) and 24-propylidenecholesterol (**11-N**)

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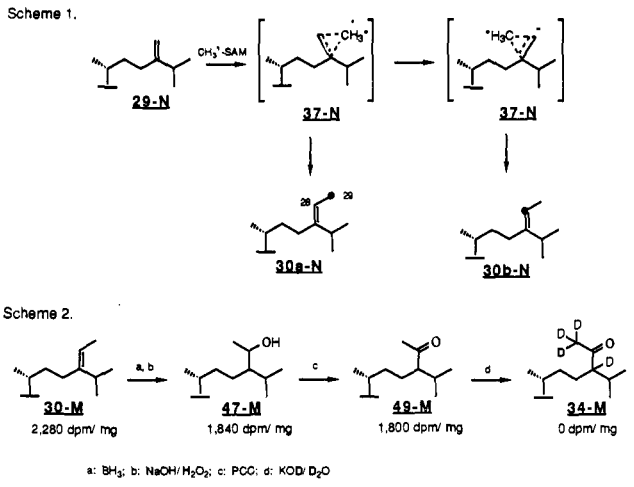

Figure 7. Degradation of tritium-labeled isofucosterol (**30-N**).

found in *Chrysoderma* can be rationalized by the placement of the base at the active site of the enzyme as drawn in Figure 6, Scheme 2. If the base is positioned toward the distal end of the side chain, so that the pro-*S* hydrogen was abstracted in the intermediate leading to isofucosterol (**36-N**) and the pro-*R* hydrogen from that leading to 24-propylidenecholesterol (**26-N**) and if this base is on the side opposite (α-face) to where the methyl group is introduced, then the formation of the double bonds should give the observed configurations.

This mechanism of the formation of the 24-propylidene side chain suggested to us that a similar mechanism may operate in the formation of the 24-ethylidene side chain (Figure 7, Scheme 1). In this case, corner to corner proton migration in the symmetrical bridged intermediate **37-N** could lead to a product in which C28 arises from the second methylation (**30b-N**) equally well as from the first methylation (**30a-N**). The latter case, based on a mechanism involving a classical carbonium ion, has always been tacitly assumed,[2] but it has never been tested experimentally. Since a degradation of ^{14}C-labeled sterol (Figure 4, Scheme 2) was ruled out, a similar degradation to that of the 24-propylidene side chain (**11**) was accomplished (Figure 7, Scheme 2) that showed the label to reside at C29 (**30a-N**) as had been previously assumed.[2] The loss of radioactivity in the hydroboration step may be due to an exchange reaction. We have already shown epimerization due to low-temperature borane migration to occur in this case.[24] Reversible borane migration between C28 and C29 with exchange of the carbon and boron hydrogens could lead to the observed loss of tritium. A similar result was seen in the degradation of 24-propylidenecholesterol (**11-N**, Figure 5).

Experiments to look for evidence of bridged intermediates in the reactions that give rise to 24-methylenecholesterol (**29-N**) and 24-ethylidenecholesterol (**30-N** and **31-N**) were carried out. Such intermediates have been drawn to explain the stereochemistry of the 24-methylene side chain in yeast.[20] If corner to corner proton migration in a protonated cyclopropane intermediate were occurring, we should be able to detect even small amounts with a tritium label (Figure 8, Scheme 1). The energy barrier to this process in a protonated cyclopropane has been calculated to be 1.4 kcal/mol.[23] The tritium isotope effect would probably be in our favor, since the k_H/k_T of deprotonation is probably large, leading to retention of tritium if there were a choice. On the other hand, the k_H/k_T of proton migration is probably small because of the nonlinear transition state in the latter process.[25]

If there were a protonated cyclopropane intermediate to 24-methylenecholesterol (**29-N**), proton migration might lead to tritium label at C25. 24-Methylenecholesterol (**29-N**) (1.8 μCi, prepared with a yeast cell-free extract from the reaction of

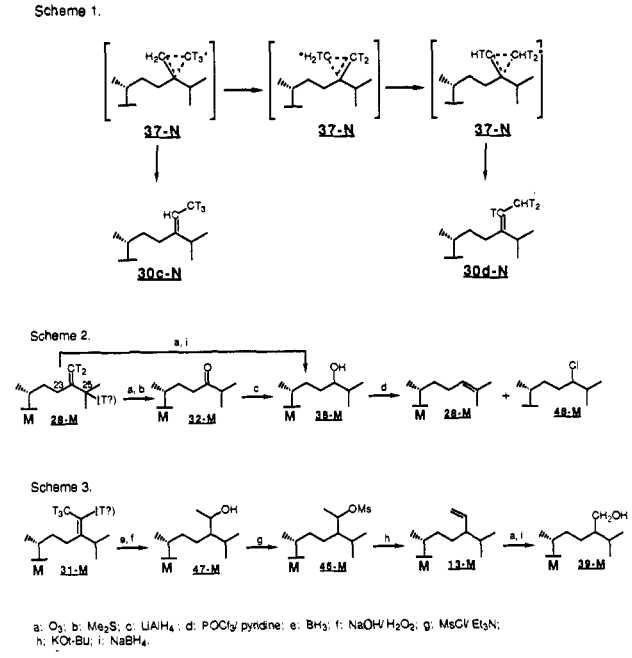

Figure 8. Proton migration in hypothetical protonated cyclopropane intermediates **37-N** and degradations of tritium-labeled 24-methylenecholesterol (**29-N**) and fucosterol (**31-N**).

[^{3}H]SAM and desmosterol (**28-N**)) was protected as the *i*-methyl ether **29-M**[26] and ozonized to the 24-ketone **32-M** (Figure 8, Scheme 2). A small amount of radioactivity remained. However, when the ketone **32-M** was degraded to remove the hydrogen at C25, the radioactivity remained. It was thought that the radioactivity had been introduced at C23 due to the exchange of the acidic hydrogens of the ketone **32-M**. Indeed, when the experiment was repeated in such a way that the ozonide was reduced directly to the alcohol **38-M**, thereby excluding exchange of the acidic hydrogens of the ketone **32-M**, no radioactivity remained.

A similar experiment was carried out with 1.2 μCi of fucosterol (**31-N**), prepared with a cell-free extract prepared from *Macrocystis pyrifera*, a Phaeophyte alga, from the reaction of [^{3}H]SAM and 24-methylenecholesterol (**29-N**). The sterol was protected as the *i*-methyl ether **31-M**,[26] hydroborated, and mesylated (Figure 8, Scheme 3).[27] Elimination gave 24-vinylcholesterol *i*-methyl ether **13-M**. Ozonization of **13-M** (350 000 dpm) followed by $NaBH_4$ gave the 28-alcohol **39-M**. A very small amount of radioactivity remained (300 dpm) as would be expected if ca. 0.3% **30d-N** was formed in addition to **30c-N**, but this could also be a result from borane migration.[24]

Summary

Through a series of degradation experiments made possible by a cell-free extract of *C. mucosa*, we have elucidated the biosynthesis of the marine sterol 24-propylidenecholesterol (**11-N**). The biosynthetic sequence leading to 24-propylidenecholesterol (**11-N**) via enzymatic SAM methyl-transfer reactions was shown to proceed via desmosterol (**28-N**), 24-methylenecholesterol (**29-N**), and isofucosterol (**30-N**) to 24-propylidenecholesterol (**11-N**).

The mechanism of the last biosynthetic reaction (**30-N** → **11-N**) is believed to involve the intermediacy of a protonated cyclopropane **27-N**. We propose that the transfer of the methyl group from SAM to isofucosterol (**30-N**) gives a protonated cyclopropane **27b-N**, which, upon ring opening and deprotonation, leads to 24-propylidenecholesterol (**11-N**, Figure 6, Scheme 1). The *E* configuration of the 24-propylidenecholesterol (**11-N**) and the *Z* configuration of the 24-ethylidenecholesterol (**30-N**) from the alga

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are thought to arise from the placement of the base at the enzyme-active site (Figure 6, Scheme 2). This mechanism is consistent with the results of the degradation experiments, the results from previous feeding experiments with deuterium-labeled methionine,[10] and the structures and stereochemical assignments of trace sterols found in the alga (Figure 2).[10,21]

24-Vinylcholesterol (**13-N**), previously believed[10] to be the precursor to 24-propylidenecholesterol (**11-N**), was neither an intermediate nor a substrate for the SAM sterol methyltransferase, nor was it detected in cultures treated with "high-energy intermediate analogue" inhibitors of sterol biosynthesis.

Although the biosynthesis of a series of cyclopropyl sterols (**5-N–7-N**) from sponges has also been proposed to involve rearrangements of protonated cyclopropane intermediates[28] and supporting evidence has been provided by feeding experiments,[29] there is some doubt whether the protonated cyclopropane intermediates arise via an enzymatic SAM methyl-transfer reaction.[30]

While we have not ruled out the intermediacy of protonated cyclopropanes in enzymatic methyl-transfer reactions leading to 24-methylenecholesterol (**29-N**) or fucosterol (**31-N**, Figure 8), no evidence of corner to corner proton migration was found in degradation experiments.

Experimental Section

Sterols were synthesized as previously described.[31] HPLC chromatography was carried out with use of a Waters Associates HPLC system (M 6000 pump, R403 differential refractometer) equipped with two Altex Ultrasphere ODS 5-μm columns (10 mm (i.d.) × 25 cm) in series.

Algal Growth. *C. mucosa* cultures were maintained in 125-mL Erlenmeyer flasks containing 50 mL or in 1-L Erlenmeyer flasks containing 250 mL of an enriched seawater medium (modified ES medium[32] without added biotin or thiamine) prepared from filtered seawater collected at Hopkins Marine Station, Pacific Grove, CA. The cultures were kept ca. 0.5 m from two 1-m long fluorescent lights at 24 °C. Transfers of 5 mL of culture to 50 mL of fresh medium were made every 2–3 months.

Stereochemical Assignment of 24-Vinyl-24-methylcholesterol (16-N). Reaction of the allylpotassium derived from KO-*t*-Bu/*n*-BuLi deprotonation of 3,4-dimethyl-1-pentene with 23-iodo-6β-methoxy-3α-cyclo-24-norcholane[33] as described previously[31] gave upon separation by reversed-phase HPLC (MeOH, 4 mL/min) 26-methyl-26-isopropyl-27-norcholesta-5,25(*E*)-dien-3β-ol *i*-methyl ether [t_R 71 min, 94%; ^{1}H NMR (400 MHz) ($CDCl_3$) δ 5.125 (t, *J* = 7.0 Hz, 1 H, C25), 3.322 (s, 3 H, OCH_3), 1.563 (s, 3 H, 26-Me), 1.018 (s, 3 H, C19), 0.977 (d, *J* = 6.9 Hz, 6 H, C26-*i*-Pr), 0.905 (d, *J* = 6.7 Hz, 3 H, C21), 0.709 (s, 3 H, C18); low-resolution mass spectrum, *m/z* (relative intensity) 440 (M^+, $C_{31}H_{52}O$, 15), 425 (10), 408 (16), 385 (20), 253 (21), 105 (37), 95 (40), 55 (100)] and 24-vinyl-24-methylcholesterol *i*-methyl ether (**16-M**, t_R 68 min, 6%). The two stereoisomers were separated by reversed-phase HPLC (MeCN/MeOH/EtOAc (3:1:1)) and hydrogenated over PtO_2 in EtOAc to give compounds with the known 24-methyl-24-ethyl side chains.[21]

(24*S*)-24-Vinyl-24-methylcholesterol *i*-methyl ether (16a-M): HPLC (MeCN/MeOH/EtOAc (3:1:1), 4 mL/min) t_R 68 min; ^{1}H NMR (400 MHz) ($CDCl_3$) δ 5.670 (dd, *J* = 17.5, 10.9 Hz, 1 H, C*H*CH_2), 5.000 (dd, *J* = 10.9, 1.7 Hz, 1 H, CHC*H*$_2$), 4.856 (dd, *J* = 17.5, 1.7 Hz, 1 H, CHC*H*$_2$), 3.323 (s, 3 H, OCH_3), 1.017 (s, 3 H, C19), 0.897 (d, *J* = 6.6 Hz, 3 H, C21), 0.844 (s, 3 H, 24-Me), 0.822 (d, *J* = 6.9 Hz, 3 H, C26), 0.793 (d, *J* = 6.8 Hz, 3 H, C27), 0.702 (s, 3 H, C18).

Hydrogenation gave **(24*S*)-24-ethyl-24-methylcholesterol *i*-methyl ether:** ^{1}H NMR (400 MHz) ($CDCl_3$) δ 3.325 (s, 3 H, OCH_3), 1.021 (s, 3 H, C19), 0.916 (d, *J* = 6.4 Hz, 3 H, C21), 0.794 (d, *J* = 7.0 Hz, 3 H, C26), 0.789 (d, *J* = 6.9 Hz, 3 H, C27), 0.752 (t, *J* = 7.5 Hz, 3 H, CH_2CH_3), 0.712 (s, 3 H, 24-Me), 0.671 (s, 3 H, C18); low-resolution mass spectrum, *m/z* (relative intensity) 442 (M^+, $C_{31}H_{54}O$, 18), 427 (13), 410 (22), 387 (22), 107 (40), 105 (43), 95 (40), 57 (100), 55 (63).

(24*R*)-24-Vinyl-24-methylcholesterol *i*-methyl ether (16b-M, the isomer found in the Chrysophyte):[10] HPLC (MeCN/MeOH/EtOAc (3:1:1), 4 mL/min) t_R 67 min; ^{1}H NMR (400 MHz) ($CDCl_3$) δ 5.674 (dd, *J* = 17.6, 10.9 Hz, 1 H, C*H*CH_2), 4.992 (dd, *J* = 10.9 Hz, 1 H, CHC*H*$_2$), 4.858 (d, *J* = 17.6 Hz, 1 H, CHC*H*$_2$), 3.323 (s, 3 H, OCH_3), 1.018 (s, 3 H, C19), 0.898 (d, *J* = 6.5 Hz, 3 H, C21), 0.851 (s, 3 H, 24-Me), 0.824 (d, *J* = 6.8 Hz, 3 H, C26), 0.791 (d, *J* = 6.8 Hz, 3 H, C27), 0.703 (s, 3 H, C18).

Hydrogenation gave **(24*R*)-24-ethyl-24-methylcholesterol *i*-methyl ether:** ^{1}H NMR (400 MHz) ($CDCl_3$) δ 3.325 (s, 3 H, OCH_3), 1.020 (s, 3 H, C19), 0.915 (d, *J* = 6.4 Hz, 3 H, C21), 0.795 (d, *J* = 7.0 Hz, 3 H, C26), 0.784 (d, *J* = 6.8 Hz, 3 H, C27), 0.748 (t, *J* = 7.4 Hz, 3 H, CH_2CH_3), 0.712 (s, 3 H, 24-Me), 0.672 (s, 3 H, C18); low-resolution mass spectrum, *m/z* (relative intensity) 442 (M^+, $C_{31}H_{54}O$, 19), 427 (13), 410 (22), 387 (21), 107 (37), 105 (41), 95 (37), 57 (100), 55 (63).

Synthesis of Azasterols. 24-Aza-24-propylcholest-5-en-3β-ol (22-N). A solution of 20(*R*)-(formylmethyl)-6β-methoxy-3α-cyclopregnane[34] in 30 mL of MeOH/THF (5:1) was treated with 630 mg of *N*-isopropylpropylamine hydrochloride and 165 mg of sodium cyanoborohydride for 6 days at room temperature.[13a,35] The mixture was concentrated by rotary evaporation and extracted with 5% NaOH and ether. Purification by silica gel TLC (eluent, ether saturated with NH_4OH or hexanes/ether (1:1)) gave 20 mg of the alcohol from the reduction of the starting aldehyde and 87 mg of 24-aza-24-propylcholest-5-en-3β-ol *i*-methyl ether (**22-M**, 36%). Deprotection of the i-methyl ether was accomplished by treatment with 50 mg of *p*-toluenesulfonic acid in 5 mL of dioxane/water (9:1) for 2.5 h at 90 °C. The mixture was concentrated and extracted with 5% NaOH and ether. The crude product was purified by silica gel TLC (eluent, hexanes/diethylamine (19:1)) (56 mg, 68%): ^{1}H NMR (400 MHz) ($CDCl_3$) δ 5.354 (m, 1 H, C6), 2.912 (hept, *J* = 6.5 Hz, 1 H, C25), 1.001 (s, 3 H, C19), 0.979 (d, *J* = 6.6 Hz, 3 H, C26 or 27), 0.954 (d, *J* = 6.6 Hz, 3 H, C26 or 27), 0.931 (d, *J* = 6.6 Hz, 3 H, C21), 0.859 (t, *J* = 7.3 Hz, 3 H, $CH_2CH_2CH_3$), 0.671 (s, 3 H, C18); high-resolution mass spectrum, *m/z* (relative intensity) 429.4004 (M^+, $C_{29}H_{51}NO$, 3) (calcd 429.3969), 414 (8), 400 (10), 154 (7), 114 (100), 86 (22).

Synthesis of (24ξ)-*N*-Ethyl-24-aminocholest-5-en-3β-ol (23-N). A solution of 24-oxocholesterol[36] (**32-N**, 260 mg) in 30 mL of MeOH/THF (5:1) was treated with 500 mg of ammonium acetate and 230 mg of sodium cyanoborohydride for 40 h at room temperature. The mixture was concentrated by rotary evaporation and extracted with 5% NaOH and ether. The ether extract was evaporated and the residue acetylated by treatment with acetic anhydride and pyridine in toluene at reflux for 1.5 h. After removal of the solvent by rotary evaporation, the product was purified by silica gel chromatography (eluent, hexanes/ether (19:1, followed by 5:1) and ether) to yield 24-oxocholesteryl acetate (45 mg) and (24ξ)-*N*-acetyl-24-aminocholesteryl acetate (182 mg, 79% based on recovered starting material): ^{1}H NMR (300 MHz) ($CDCl_3$) δ 5.372 (m, 1 H, C6), 5.108 (d, *J* = 9.5 Hz, 1 H, N*H*), 5.065 (d, *J* = 9.5 Hz, 1 H, N*H*), 4.601 (m, 1 H, C3), 3.749 (m, 1 H, C24), 2.033 (s, 3 H, 3-acetate), 1.996 (s, 3 H, *N*-acetyl), 1.013 (s, 3 H, C19), 0.913 and 0.901 (d, *J* = 6.3 Hz, 3 H, C26 or 27), 0.893 (d, *J* = 6.6 Hz, 3 H, C21), 0.871 and 0.860 (d, *J* = 6.5 Hz, 3 H, C26 or 27), 0.670 and 0.661 (s, 3 H, C18); low-resolution mass spectrum, *m/z* (relative intensity) (M^+ – HOAc, $C_{31}H_{51}NO_3 - C_2H_4O$, 71), 382 (15), 255 (46), 213 (91), 170 (70), 72 (99), 60 (100).

N-Acetyl-24-aminocholesteryl acetate (90 mg) was treated with 5 mL of 1 M $LiAlH_4$/THF at reflux for 48 h. Excess reagent was quenched with EtOAc and the mixture extracted with 5% NaOH and ether. Purification by silica gel TLC (eluent, hexanes/diethylamine (19:1)) gave *N*-acetyl-24-aminocholesterol (5 mg) and **23-N** (47 mg, 62% based on recovered starting material): ^{1}H NMR (400 MHz) ($CDCl_3$) δ 5.354 (m, 1 H, C6), 2.609 (m, 1 H, C24), 1.086 (t, *J* = 7.1 Hz, 3 H, CH_2CH_3), 1.004 (s, 3 H, C19), 0.931 and 0.924 (d, *J* = 6.5 Hz, 3 H, C21), 0.882 and 0.875 (d, *J* = 6.6 Hz, 3 H, C26 or 27), 0.862 (d, *J* = 6.6 Hz, 3 H, C26 or 27), 0.677 and 0.673 (s, 3 H, C18); high-resolution mass spectrum, *m/z* (relative intensity) 429.3965 (M^+, $C_{29}H_{51}NO$, 1) (calcd 429.3969), 414 (1), 386 (100), 368 (5), 271 (2), 100 (100), 84 (25), 71 (38), 58 (41).

24,28-Iminostigmast-5-en-3β-ol (24-N). Fucosterol (**31-N**) was converted to the aziridine (presumably a mixture of the 24*R*,28*S* and 24*S*,28*R* isomers) by the literature method and purified as described above.[15]

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Inhibition of Sterol Biosynthesis in *C. mucosa*. Four-day-old cultures of *C. mucosa* (50 mL) were treated with 5 μL of the azasterols **22-N**–**24-N** in ethanolic solution. For each azasterol 10, 1, 0.1, and 0.01% (w/v) dilutions were tested (final concentrations, 24, 2.4, 0.24, and 0.024 μM). The cultures were harvested after 5 weeks. The cells were collected by centrifugation and extracted with $CHCl_3$/MeOH (2:1). The volume of the extracts was brought to 10 mL, and the UV–vis spectra were measured to estimate cell growth. The extracted cells were lyophilized and weighed as another measurement of cell growth. The aziridine **24-N** did not inhibit growth very much, but the amines **22-N** and **23-N** did, with an approximate I_{50} of 2.5 μM. The extracts were evaporated, dissolved in ether, and filtered through Florisil to remove the pigments. After purification of the sterols by silica gel TLC (eluent, hexanes/ether (1:1)) the sterol composition was analyzed by GC–MS (SE-54 coated fused silica capillary column, 0.32 mm (i.d.) × 15 m). All azasterols inhibited the methyltransferase, the aziridine **24-N** only weakly. Only at the lowest concentrations of inhibitors was 24-propylidenecholesterol (**11-N**) detected (ca. 30% of the control). As the concentrations of inhibitors was increased, the degree of side-chain methylation decreased until at the highest levels of **22-N** and **23-N** ca. 40% cholesterol (**1-N**) was measured. Inhibition of the Δ^{22}-dehydrogenase (as shown by the ratios of sterols with Δ^{22} side chain (**2-N**, **3-N**) to those with saturated side chains (24-methyl- and 24-ethylcholesterol)) was observed with **22-N**. No 24-vinylcholesterol (**13-N**) could be detected in any experiments.

***C. mucosa* Cell-Free Extract.** Two-week-old cells from 3 L of cultures (20 g) were harvested by centrifugation and suspended with the aid of a glass–Teflon homogenizer with 50 mL of ice-cold 100 mM NaHEPES (*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid hemisodium salt) buffer (pH 7.5), containing 2 mM DTT (dithiothreitol), 10 mM ascorbic acid, 0.5% (w/v) BSA (bovine serum albumin), and 1% (w/v) PVP 40 (polyvinylpyrrolidone), to which 6 mg of PMSF ((phenylmethyl)sulfonyl fluoride) was added in 0.25 mL of EtOH (buffer A).[17,18] The cells were broken by passage through a French pressure cell at 12000 psi. The broken cells were centrifuged (2000*g*, 10 min) to give a large viscous pellet. The supernatant was centrifuged (35000*g*, 1 h), and the pellets were suspended in 50 mL of buffer A with a glass–Teflon homogenizer and centrifuged again (35000*g*, 1 h). The pellets were suspended in 12 mL of buffer A and frozen in liquid N_2 in 1-mL aliquots.

***M. pyrifera* Cell-Free Extract.** Growing kelp fronds (apical scimitars) were collected in September at the Hopkins Marine Station of Stanford University (Pacific Grove, CA) and were chopped into small pieces with a razor. To remove some of the alginic acids (mucilage), the kelp (100 g) was washed several times with seawater. The material was blotted dry and added to 1 L of ice-cold buffer A containing 1 mM $MgCl_2$ and 5 mM EDTA (buffer A′). PVPP (polyvinylpolypyrrolidone, 100 g) was added, and the mixture was homogenized by a Polytron tissue homogenizer with a fine rotor for a total of 15 min, making sure that the temperature did not exceed 5 °C through the addition of small amounts of liquid N_2. An additional 1 L of ice-cold H_2O was added to decrease the viscosity, and the mixture was centrifuged (2500*g*, 10 min) to remove the large particles. The supernatants were centrifuged (16000*g*, 40 min) and the pellets resuspended with the aid of a glass–Teflon homogenizer in 50 mL of buffer A′ and centrifuged again (17000*g*, 40 min). The pellets were dispersed in 50 mL of buffer A′ again and frozen in liquid nitrogen in 1-mL aliquots.

Yeast Cell-Free Extract. Microsomes from "semi-anaerobically" adapted baker's yeast were depleted of endogenous sterols by preparing an acetone powder.[37] This was suspended in 60 mL of 100 mM Na-HEPES buffer (pH 7.5), 2 mM in $MgSO_4$, 1 mM in DTT, to which a solution of 10 mg of PMSF in 0.3 mL of EtOH was added (buffer B) and frozen in liquid N_2.

SAM Sterol Methyltransferase Assay. Sterol substrates were purified by TLC and HPLC. To solubilize the sterol, Tween 80 (10 mg) was mixed with sterol (0.5 mg) in $CHCl_3$. After the solvent was evaporated, the mixture was dissolved in 1 mL of H_2O.

To disposable 1.5-mL plastic centrifuge tubes were added 30 μl of the *Chryosoderma* cell-free extract, 10 μL of the sterol suspension (or 1% Tween 80 for the blanks), and 10 μL (10 μCi) of [^{3}H]SAM ([^{3}H]-methyl]-*S*-adenosylmethionine, ICN Biomedicals, Inc., 15 Ci/mmol). When more [^{3}H]SAM was required to obtain more radioactivity in the products, the components of the reaction were increased proportionately. The components of the enzymatic reaction were mixed on ice, centrifuged briefly and vortexed to ensure mixing, and incubated at 27 °C in a water bath shaker. After 4 h, the reaction was extracted with 1 mL of EtOAc containing 0.3 mg of cholesterol (as a marker) by shaking with a reciprocating shaker (Mini-Bead Beater) for 10 s. The phases were separated by centrifugation, the aqueous phase was frozen with dry ice, and the organic layer was drawn off. After evaporation of solvent, the extracts were applied in hexanes/ether (9:1) to Pasteur pipette columns containing 5.5 cm (0.75 g) of Florisil and eluted with 4.5 mL of the same solvent mixture. The pipette columns were put into new vials and eluted with 6.5 mL of hexanes/ether (1:1) to elute the sterols. After evaporation of the solvent, the sterol fraction could be counted directly or, after the addition of the appropriate cold carriers, be analyzed by HPLC. All experiments were carried out at least in duplicate. The results are summarized in Figure 3.

Chromatography and Derivatization of the Cell-Free Extract Products. The product of [^{3}H]SAM methylation of desmosterol (**28-N**) was determined by reversed-phase HPLC (MeOH, 3 mL/min) with the aid of cold carriers. The elution order of Δ^5-ergostadienols was as follows: (24ξ)-Δ^{25} (t_R 43 min), $\Delta^{24(28)}$ (t_R 44 min), Δ^{23}-*Z* (t_R 45 min), Δ^{23}-*E* (t_R 46 min), and Δ^{24} (t_R 49 min). The radioactivity of the products of all three cell-free extracts coeluted with the $\Delta^{24(28)}$ isomer (24-methylenecholesterol (**29-N**)).

The product of [^{3}H]SAM methylation of 24-methylenecholesterol (**29-N**) was determined by reversed-phase HPLC (MeOH and MeCN/MeOH/EtOAc (3:1:1) and 50 mM $AgNO_3$ in MeOH/H_2O (49:1)) with the aid of cold carriers. The elution order of Δ^5-stigmastadienols with MeOH (3 mL/min) was as follows: (24ξ)-Δ^{25} (t_R 49 min), Δ^{23}-*E* (t_R 50.5 min), Δ^{23}-*Z* plus $\Delta^{24(28)}$ (t_R 51.5 min), and Δ^{24} (t_R 52.5 min). The elution order with MeCN/EtOAc/MeOH (3:1:1) (3 mL/min) was as follows: (24ξ)-Δ^{25} (t_R 44.5 min), $\Delta^{24(28)}$ plus Δ^{24} (t_R 45.5 min), and Δ^{23} (t_R 46.5 min). The elution order with 50 mM $AgNO_3$ in MeOH/H_2O (49:1) (3 mL/min) was as follows: $\Delta^{24(28)}$-*Z* (t_R 59.5 min) followed by $\Delta^{24(28)}$-*E* (t_R 61 min). The radioactivity from the *Chrysoderma* cell-free extract coeluted with the $\Delta^{24(28)}$-*Z* isomer (isofucosterol (**30-N**)), while that from the *Macrocystis* cell-free extract coeluted with the $\Delta^{24(28)}$-*E* isomer (fucosterol (**31-N**)).

The product of [^{3}H]SAM methylation of isofucosterol (**30-N**) was determined by reversed-phase HPLC (MeOH and MeCN/MeOH/EtOAc (3:1:1)) and by the chromatographic separation of derivatives with the aid of cold carriers. The elution order of the Δ^5-28-methylstigmastadienols **19-N**–**21-N** and 24-propylidenecholesterol (**11-N**) with MeOH was as follows: 24*R*-Δ^{25}, 24*S*-Δ^{25} plus Δ^{23} plus 24-propylidenecholesterol (**11-N**), and Δ^{24}. The elution order with MeCN/EtOAc/MeOH (3:1:1) was as follows: 24*S*-Δ^{25} plus 24-propylidenecholesterol (**11-N**) followed by Δ^{23}. For retention times see ref 21. (24*S*)-28-Methylstigmasta-5,25-dienol (**21b-N**) could be separated from 24-propylidenecholesterol (**11-N**) after conversion to the *i*-methyl ethers (vide infra) by argentic silica gel TLC (eluent, hexanes/ether (79:1)): 24-propylidenecholesterol *i*-methyl ether (**11-M**), R_f = 0.32; (24*S*)-28-methylstigmasta-5,25-dienol *i*-methyl ether (**21b-M**), R_f = 0.42. The two isomers could also be distinguished by hydrogenation over PtO_2 in EtOAc. The elution order of the hydrogenation products on reversed-phase HPLC (MeOH, 4 mL/min) was as follows: 24-propylcholestanol[9,38] (t_R 51 min) followed by 24-isopropylcholestanol[39] (t_R 53 min). The radioactive product was shown to be 24-propylidenecholesterol (**11-N**).

Degradation Scheme for ^{14}C-Labeled 24-Propylidenecholesterol (11-N). 29-Ethyl-6β-methoxy-3α,5-cyclostigmasta-24(28),28-diene (40-M). (*E*)-24-Propylidenecholesterol *i*-methyl ether (**11-M**) (obtained from the Cu^I-modified allylpotassium reaction[31]) was converted to the dichlorocyclopropane **41-M** (75%).[40] To a solution of 43.3 mg of **41-M** in 10 mL of dry THF at 0 °C under Ar was added 0.5 mL of 1.6 M *n*-BuLi/hexane.[41] After 30 min, excess reagent was quenched with a few drops of water and the reaction mixture evaporated under reduced pressure. The residue was purified by silica gel TLC (eluent, hexanes/ether (39:1)) (28.4 mg, 76%): ^{1}H NMR (400 MHz) ($CDCl_3$) δ 5.180 (m, 1 H, C29), 3.321 (s, 3 H, OCH_3), 1.018 (s, 3 H, C19), 1.001 (d, *J* = 6.8 Hz, 3 H, C26 or 27), 0.991 (d, *J* = 6.8 Hz, 3 H, C26 or 27), 0.969 (t, *J* = 7.3 Hz, 3 H, CH_2CH_3), 0.922 (d, *J* = 6.7 Hz, 3 H, C21), 0.711 (s, 3 H, C18); low-resolution mass spectrum, *m/z* (relative intensity) 452 (M^+, $C_{32}H_{52}O$, 1), 437 (1), 420 (2), 328 (5), 313 (11), 296 (11), 253 (100), 227 (12), 124 (23), 109 (28), 95 (20), 81 (26).

(24ξ)-24-(3-Butynyl)-6β-methoxy-3α,5-cyclocholestane (42-M). 29-Ethyl-6β-methoxy-3α,5-cyclostigmasta-24(28),28-diene (**40-M**) was isomerized by the Abrams modification[42] of the "KAPA-zipper" reac-

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tion.[43] To 4.5 mL of dry 2,3-diaminopropane was added 90 mg of lithium wire. After the mixture had turned blue, it was heated under Ar at 70 °C for ca. 2 h until no color remained. The mixture was brought to room temperature, and 1.0 g of KO-*t*-Bu was added. After 10 min, a solution of **40-M** (25.6 mg) in 1.5 mL of dry THF was added. After 15 h, the reaction mixture was extracted with brine and ether, and the ether extracts were filtered through silica gel, concentrated, and purified by silica gel TLC (eluent, hexanes/ether (79:1)) to yield 8.8 mg of partially isomerized material and 12.9 mg of an epimeric mixture of the slightly more polar acetylene **42-M** (77% based on recovered starting material): ^{1}H NMR (400 MHz) ($CDCl_3$) δ 3.323 (s, 3 H, OCH_3), 1.939 (m, 1 H, acetylenic H), 1.018 (s, 3 H, C19), 0.909 (d, J = 6.7 Hz, 3 H, C21 or 26 or 27), 0.845 (d, J = 6.9 Hz, 3 H, 3 H, C21 or 26 or 27), 0.823 and 0.818 (d, J = 6.8 Hz, 3 H, C21 or 26 or 27), 0.710 (s, 3 H, C18); high-resolution mass spectrum, m/z (relative intensity) 452.4029 (M^+, $C_{32}H_{52}O$, 26) (calcd 452.4016), 437 (47), 420 (38), 397 (100), 315 (8), 253 (16), 213 (18), 107 (46), 95 (62), 81 (64).

(24ξ)-24-(3-Butenyl)-6β-methoxy-3α,5-cyclocholestane (43-M). A solution of 12.5 mg of **42-M** was stirred with Lindlar's catalyst (12 mg) under hydrogen in 10 mL of EtOAc containing 2 drops of pyridine. The progress of the reaction was monitored by silica gel TLC (eluent, hexanes/ether (39:1)). After 5 h, the mixture was filtered through silica gel and evaporated (12.5 mg, 100%): ^{1}H NMR (400 MHz) ($CDCl_3$) δ 5.814 (ddt, J = 17.0, 10.2, 6.7 Hz, 1 H, vinyl methine), 4.998 (d, J = 17.0 Hz, 1 H, vinyl methylene), 4.926 (d, J = 10.2 Hz, 1 H, vinyl methylene), 3.324 (s, 3 H, OCH_3), 1.019 (s, 3 H, C19), 0.912 (d, J = 6.5 Hz, 3 H, C21 or 26 or 27), 0.833 (d, J = 6.5 Hz, 3 H, C21 or 26 or 27), 0.815 and 0.807 (d, J = 6.7 Hz, 3 H, C21 or 26 or 27), 0.712 (s, 3 H, C18); high-resolution mass spectrum, m/z (relative intensity) 454.4148 (M^+, $C_{32}H_{54}O$, 63) (calcd 454.4173), 439 (58), 422 (100), 399 (83), 301 (18), 255 (42), 213 (32), 145 (100), 100 (100), 81 (73).

Alternative Synthesis of (24ξ)-24-(3-Butenyl)-6β-methoxy-3α,5-cyclocholestane (43-M). A solution of 340 mg of 6β-methoxy-3α,5-cycloergostan-28-ol mesylate[26b] was heated with 3 g of NaI in refluxing acetone. After 6 h, the reaction mixture was concentrated under reduced pressure and extracted with water and ether. The crude product was purified by silica gel chromatography (eluent, hexanes/ether (9:1)) to give 352 mg of (24ξ)-28-iodo-6β-methoxy-3α,5-cycloergostane (93%): ^{1}H NMR (400 MHz) ($CDCl_3$) δ 3.323 (s, 3 H, OCH_3), 3.288 (m, 1 H, C28), 3.224 (m, 1 H, C28), 1.020 (s, 3 H, C19), 0.944, 0.935, 0.910, 0.901, 0.891, and 0.874 (d, J = 6.6, 9 H, C21, 26 and 27), 0.718 (s, 3 H, C18); low-resolution mass spectrum, m/z (relative intensity) 540 (M^+, $C_{29}H_{49}IO$, 15) 525 (11), 508 (23), 485 (21), 255 (27), 145 (89), 79 (80), 69 (85), 55 (100).

Treatment of 233.5 mg of the iodide with the allylpotassium derived from propene[31] yielded 194.0 mg of **43-M** (99%).

(24ξ)-24-(2-Carboxyethyl)-6β-methoxy-3α,5-cyclocholestane (44-M). A solution of 22.8 mg of **43-M** in 7 mL of *t*-BuOH containing 1 mL of 0.1 M sodium phosphate buffer (pH 7.5) was oxidized with 2 mL of 2.5 mM $KMnO_4$/100 mM $NaIO_4$[44] for 2.5 h. The reaction was stopped by addition of 1 mL of 10% $Na_2S_2O_5$ and 1 mL of 10% hydrochloric acid. The mixture was concentrated under reduced pressure and extracted with brine and ether. The crude product was purified by silica gel TLC (eluent, hexanes/ether (1:1)) (17.2 mg, 72%): ^{1}H NMR (400 MHz) ($CDCl_3$) δ 3.325 (s, 3 H, OCH_3), 2.329 (m, 2 H, CH_2COOH), 1.017 (s, 3 H, C19), 0.910 (d, J = 6.7 Hz, 3 H, C21 or 26 or 27), 0.855 and 0.852 (d, J = 6.5 Hz, 3 H, C21 or 26 or 27), 0.835 and 0.826 (d, J = 6.6 Hz, 3 H, C21 or 26 or 27), 0.708 (s, 3 H, C18); high-resolution mass spectrum, m/z (relative intensity) 472.3927 (M^+, $C_{31}H_{52}O_3$, 50) (calcd 472.3914), 457 (60), 440 (100), 425 (25), 417 (95), 319 (34), 255 (45), 145 (38), 121 (34), 55 (35).

Lead Tetraacetate Decarboxylation[45] of (24ξ)-24-(2-Carboxyethyl)-6β-methoxy-3α,5-cyclocholestane (44-M). A solution of 15.3 mg of **44-M** and 6.0 mg of $Cu(OAc)_2$ in 1 mL of dry benzene containing 5 μL of pyridine was heated with 25 mg of lead tetraacetate in the dark under Ar. Aliquots of 25 mg of lead tetraacetate were added every 2 h for 10 h until only a trace of starting material could be seen by TLC. A drop of ethylene glycol was added, and the mixture was filtered through silica gel. Purification by silica gel chromatography (eluent, hexanes/ether (79:1)) gave 7.9 mg (24ξ)-24-vinylcholesterol *i*-methyl ether[46] (**13-M**) (63%).

(24ξ)-6β-Methoxy-3α,5-cycloergostan-28-oic Acid (45-M). (24ξ)-24-Vinylcholesterol *i*-methyl ether (**13-M**, 21.5 mg) was oxidized with potassium permanganate and sodium periodate in the same way as **43-M**. The reaction was much slower. After 76 h, the reaction was terminated and the products were purified by silica gel TLC (eluent, hexanes/ether (4:1)). The recovered starting material (0.4 mg) was found to be the 24*R*[46] isomer. 24-Oxocholesterol *i*-methyl ether (**32-M**, 1.0 mg) and 5-cholenic acid *i*-methyl ether (1.8 mg) were isolated as well as the desired product **45-M** (16.9 mg, 75%): ^{1}H NMR (400 MHz) ($CDCl_3$) δ 3.322 (s, 3 H, OCH_3), 1.015 (s, 3 H, C19), 0.964 (d, J = 6.7 Hz, 6 H, C26 and 27 or 21), 0.926 and 0.919 (d, J = 6.4 Hz, 3 H, C21 or 27), 0.710 and 0.702 (s, 3 H, C18); high-resolution mass spectrum, m/z (relative intensity) 444.3612 (M^+, $C_{29}H_{48}O_3$, 41) (calcd 444.3602), 429 (63), 412 (65), 389 (100), 291 (24), 255 (26), 213 (25), 145 (36), 95 (47), 81 (33).

Degradation Scheme for ^{14}C-Labeled 24-ethylidenecholesterol (30-N and 31-N). Conversion to 24-Vinylcholesterol *i*-Methyl Ether (13-M). 24-Ethylidenecholesterol (**30-N** and **31-N**) was converted to 6β-methoxy-3α,5-cyclostigmastan-28-ol mesylate (**46-M**) according to the literature procedure.[27] Thus, isofucosterol (**30-N** and **31-N**) was converted to its *i*-methyl ether (72%), hydroborated (74%), and mesylated (95%). The mesylate (**46-M**, 81.6 mg) was treated with 0.4 mg of KO-*t*-Bu in 10 mL of dry THF under Ar at reflux. After 4.5 h, the mixture was concentrated under vacuum, extracted with ether and water, and purified by silica gel chromatography (eluent, hexanes/ether (39:1) followed by (4:1)) to give 9.6 mg of 6β-methoxy-3α,5-cyclostigmastan-28-ol (**47-M**) and 47.5 mg of pure 24-vinylcholesterol *i*-methyl ether[46] (**13-M**) (71%).

(24ξ)-24-chloro-6β-methoxy-3α,5-cyclocholestane (48-M). A solution of 16.9 mg of **45-M** (prepared from 24-vinylcholesterol *i*-methyl ether (**13-M**) as described previously) in 3 mL of dry benzene under N_2 was mixed with 25 mg of lead tetraacetate. After 20 min, 8 mg of LiCl was added and the mixture was heated under reflux. After 2.5 h, another 25 mg of lead tetraacetate and 5 mg of LiCl were added. After 17.5 h, the mixture was filtered through silica gel and purified by silica gel chromatography (eluent, hexanes/ether (39:1)) (10.4 mg, 63%): ^{1}H NMR (400 MHz) ($CDCl_3$) δ 3.780 (m, 1 H, C24), 3.324 (s, 3 H, OCH_3), 1.021 (s, 3 H, C19), 1.013 (d, J = 6.5 Hz, 3 H, C26 or 27), 0.972 and 0.958 (d, J = 6.6 Hz, 3 H, C26 or 27), 0.932 and 0.920 (d, J = 6.5 Hz, 3 H, C21), 0.720 (s, 3 H, C18); high-resolution mass spectrum, m/z (relative intensity) 436.3429 (M^+, $C_{28}H_{47}ClO$, 18) (calcd 436.3284), 434.3315 (M^+, $C_{28}H_{47}ClO$, 48) (calcd 434.3313), 419 (53), 402 (58), 379 (98), 366 (100), 351 (42), 343 (40), 253 (85), 121 (85), 95 (100).

Degradation of ^{3}H-Labeled 24-propylidenecholesterol (11-N). Radiolabeled 24-propylidenecholesterol (**11-N**) was prepared from isofucosterol (**30-N**), [^{3}H]SAM (150 μCi), and *Chrysoderma* cell-free extract (900 μL) as outlined previously. Cold 24-propylidenecholesterol (**11-N**) (3.0 mg) was added to the EtOAc extract of the incubation, purified by HPLC, and converted to the *i*-methyl ether **11-M** as follows: The mixture was evaporated to dryness in a plastic vial, and residual water was removed azeotropically by adding benzene and evaporating under a stream of nitrogen. Toluenesulfonyl chloride (15 mg) and pyridine (100 μL) were added, and the vial was sealed and allowed to stand at room temperature. After 2 days, pyridine (0.5 mL) and MeOH (3 mL) were added, and the mixture was heated under reflux for 6 h.[26] The solvent was evaporated and the residue mixed with water and extracted with ether. The ether extract was purified by silica gel TLC (hexanes/ether (39:1)) and by HPLC with additional unlabeled 24-propylidenecholesterol *i*-methyl ether (**11-M**) (11 000 dpm, 22.8 mg, 480 dpm/mg).

A portion of the protected sterol **11-M** (4.7 mg, 2200 dpm) was ozonized by adding 2 mL of a solution of ozone in CH_2Cl_2/MeOH/pyridine (89:10:1) at −78 °C to a solution of the sterol in 1 mL of the same solvent mixture. After 30 s, dimethyl sulfide (1 mL) was added. After 10 min at −78 °C, the mixture was brought to room temperature. After 2 h, the reaction mixture was evaporated and purified by silica gel TLC (hexanes/ether (9:1)). Further purification by HPLC showed no radioactivity remained in the ketone **32-M**.

A solution of labeled 24-propylidenecholesterol *i*-methyl ether (**11-M**) (16.2 mg, 7,800 dpm) in 5 mL of dry THF at 0 °C was treated with 1 mL of 1 M BH_3/THF under Ar. After 50 min, 2 mL of 10% NaOH was added, followed by 2 mL of 30% H_2O_2. After 7 h, the mixture was extracted with hexanes and ether and filtered through silica gel. Silica gel TLC (eluent, hexanes/ether (2:1)) gave 29-methyl-6β-methoxy-3α,5-cyclostigmastan-28-ol (**33-M**, 6.8 mg, 2400 dpm, 350 dpm/mg). In addition, 29-methyl-6β-methoxy-3α,5-cyclostigmastan-28-one (**34-M**) and a small amount of (24ξ)-24-(1-propenyl)-6β-methoxy-3α,5-cyclocholestane were isolated.

29-Methyl-6β-methoxy-3α,5-cyclostigmastan-28-ol (33-M): ^{1}H NMR (400 MHz) ($CDCl_3$) δ 3.635 (3 isomers) and 3.497 (1 isomer) (m, 1 H, C28), 3.324 (s, 3 H, OCH_3), 1.019 (s, 3 H, C19), 0.96–0.87 (C21, 26, 27 and 30), 0.711 (s, 3 H, C18); high-resolution mass spectrum, m/z (relative intensity) 458.4134 (M^+, $C_{31}H_{54}O_2$, 48) (calcd 458.4144), 443 (61), 426 (100), 403 (97), 255 (72), 213 (51), 161 (72), 145 (80), 121 (70), 95 (80).

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The 28-alcohol **33-M** (5.4 mg, 1900 dpm) was oxidized to the ketone **34-M** with pyridinium chlorochromate (80 mg) in CH_2Cl_2 (5 mL) containing 3 drops of pyridine for 1 h. The mixture was diluted with hexanes (5 mL) and filtered through silica gel. Silica gel TLC (eluent, hexanes/ether (9:1)) gave the ketone **34-M** (5.3 mg, 1200 dpm, 230 dpm/mg).

29-Methyl-6β-methoxy-3α,5-cyclostigmastan-28-one (34-M): ^1H NMR (400 MHz) ($CDCl_3$) δ 3.318 (s, 3 H, OCH_3), 2.392 (m, 2 H, C29), 2.171 (m, 1 H, C24), 1.029 (t, J = 7.2 Hz, 3 H, 29-Me), 1.012 (s, 3 H, C19), 0.896 (d, J = 6.7 Hz, 6 H, C21 and C26), 0.864 (d, J = 6.7 Hz, 3 H, C27), 0.699 and 0.689 (s, 3 H, C18); high-resolution mass spectrum, m/z (relative intensity) 456.3970 (M^+, $C_{31}H_{52}O_2$, 35), (calcd 456.3973), 441 (45), 424 (100), 401 (76), 303 (48), 255 (75), 213 (47), 145 (65), 121 (49), 95 (50).

The acidic protons of the ketone **34-M** (3.5 mg, 800 dpm) were exchanged by heating under reflux in a solution of 5 mL of CH_3OD, 0.75 mL of D_2O, and 0.1 mg of KO-*t*-Bu for 65 h. The mixture was evaporated, extracted with ether, and filtered through silica gel. Silica gel TLC (eluent, hexanes/ether (9:1)) gave 3.0 mg of the ketone. Complete exchange of the C24 and C29 protons was shown by NMR. No radioactivity remained.

Degradation of ^3H-Labeled Isofucosterol (30-N). Radiolabeled isofucosterol (**30-N**) was prepared from 24-methylenecholesterol (**29-N**), [^3H]SAM (50 μCi), and *Chrysoderma* cell-free extract (300 μL) as outlined previously. After addition of cold carrier, it was purified by HPLC and converted to the *i*-methyl ether **30-M** as before (6.5 mg, 14 800 dpm, 2280 dpm/mg).

Hydroboration of the labeled *i*-methyl ether **30-M** (5.8 mg, 13 200 dpm) as described previously gave 6β-methoxy-3α,5-cyclostigmastan-28-ol[27] (**47-M**, 5.7 mg, 10 500 dpm, 1840 dpm/mg).

Pyridinium chlorochromate oxidation of the alcohol **47-M** (4.5 mg, 8200 dpm) as described previously gave 6β-methoxy-3α,5-cyclostigmastan-28-one[26a] (**49-M**, 4.2 mg, 7600 dpm, 1800 dpm/mg).

Exchange of the acidic protons of the ketone **49-M** (2.8 mg, 5000 dpm) was carried out as described previously. The recovered ketone (2.6 mg) had no radioactivity.

Degradation of ^3H-Labeled 24-Methylenecholesterol (29-N). Radiolabeled 24-methylenecholesterol (**29-N**) was prepared from an incubation of 10 μL of desmosterol (**28-N**) suspension (emulsified with Lubrol PX instead of Tween 80), 10 μL of [^3H]SAM (10 μCi), 10 μL of a 1:5 dilution of *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1., from rabbit erythrocytes, 8 units/mL in 24 mM Tris, pH 7.4, containing 1 mM DTT, 1 mM EDTA, and 20% glycerol, Sigma Chemical Co.) with buffer B, and 10 μL of yeast cell-free extract. The product was mixed with cold carrier, converted to the *i*-methyl ether, and purified by HPLC as outlined previously (3 700 000 dpm). Ozonolysis, as described previously, gave 24-oxocholesterol *i*-methyl ether (**32-M**, 7400 dpm). Reduction with $LiAlH_4$ gave 24-hydroxycholesterol *i*-methyl ether (**38-M**).[47] The alcohol **38-M** was treated with 0.1 mL of $POCl_3$ in 1 mL of pyridine for 2 h. The mixture was evaporated and extracted with water and ether. Purification by silica gel TLC (eluent, hexanes/ether (39:1)) gave, after HPLC, 24-chlorocholesterol *i*-methyl ether (**48-M**, 1140 dpm) and desmosterol *i*-methyl ether (**28-M**, 1440 dpm) of approximately equal specific activity.

The experiment was repeated in the same way, but the ozonide was reduced by the addition of a solution of 0.1 g of $NaBH_4$ in 1 mL of MeOH to the reaction mixture instead of Me_2S. The reaction yielded both 24-hydroxycholesterol *i*-methyl ether (**38-M**)[47] and 24-oxocholesterol *i*-methyl ether (**32-M**). This time no radioactivity was found in either product.

Degradation of ^3H-Labeled Fucosterol (31-N). Radiolabeled fucosterol (**31-N**) was prepared from an incubation of 10 μL of 24-methylenecholesterol (**29-N**) suspension (emulsified with sodium cholate instead of Tween 80), 10 μL of [^3H]SAM (10 μCi), and 200 μL of *Macrocystis* cell-free extract as outlined previously. After addition of cold carrier, the product was purified by HPLC and converted to the *i*-methyl ether as before (2 400 000 dpm).

Hydroboration of the labeled *i*-methyl ether as described previously gave 6β-methoxy-3α,5-cyclostigmastan-28-ol (**47-M**)[27] (1 800 000 dpm), which was converted to the mesylate **46-M**[27] (1 200 000 dpm).

The mesylate **46-M** was treated with KO-*t*-Bu in THF as described previously. This time the elimination gave 25% 24-ethylidenecholesterol *i*-methyl ether (**30-M** and **31-M**, 220 000 dpm) as well as 75% of the desired 24-vinylcholesterol *i*-methyl ether (**13-M**) (350 000 dpm). The tritium isotope effect, measured as the ratio of specific activities of the elimination products, was small.

The labeled 24-vinylcholesterol *i*-methyl ether (**13-M**) was ozonized as described previously, with $NaBH_4$ reduction of the ozonide. Purification by silica gel chromatography (eluent, hexanes/ether (2:1)) and HPLC gave 6β-methoxy-3α,5-cycloergostan-28-ol (**39-M**)[27] (300 dpm).

Acknowledgment. Financial support was provided by NIH Grant No. GM-06840. The use of the 400-MHz NMR spectrometer was made possible by NSF Grant CHE-8414329. We thank the University of California, San Francisco Mass Spectrometry Facility (Dr. A. L. Burlingame, Director; NIH Grant No. NIH P-41 RR01614), and the University of Minnesota Mass Spectrometry Service Laboratory (Dr. E. Larka, director) for high-resolution mass spectra. We thank Mr. James Lance (Scripps Institute for Oceanography) for the *C. mucosa* culture; Dr. Donald Weeks (Zoecon Corp.) and Dr. Arthur Grossman (Carnegie Institute of Washington, Stanford, CA) for use of their culturing facilities; Dr. Randall Alberte and Dr. David Epel for use of their laboratory space at the Hopkins Marine Station of Stanford University; Dr. Robert Andersen (Bigelow Laboratory for Ocean Sciences) for the identification of the alga; and Dr. Robert Simoni (Department of Biological Sciences, Stanford University) for the generous use of his French press, centrifuges, and scintillation counter.

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