23a in 50 mL of methanol was irradiated by a low-pressure mercury lamp for 0.5 h and then evaporated to dryness. HPLC of the residue on silica and elution with 25:1 cyclohexane/ether yielded 8 mg (16%) of crystalline vinylogous urethane **24**: mp 148–150 °C (from MeOH); IR (KBr) NH 3340 (m), C=O, C=C 1650 (s), 1605 (s) cm^{-1}; UV λ_{max} 225 nm (log ϵ 4.10), 297 (4.00), 328 (4.20); ^1H NMR δ 0.95 (t, 3, J = 7 Hz, Me), 1.1–1.3 (m, 2, CH_2), 1.3–2.0 (m, 5, methylenes, methines), 2.00 (dd, 1, J = 16, 13 Hz, H-17β), 2.4–2.6 (m, 2, H-17α, CH), 2.67 (t, 1, J = 13 Hz, H-21β), 2.76 (d, 1, J = 11 Hz, H-3), 3.0–3.1 (m, 2, H-21α, CH), 3.2–3.3 (m, 1, H-5), 3.76 (s, 3, OMe), 6.2–7.5 (m, 4, aromatic Hs); m/e 338 (M^+, base), 180 (20%), 167 (27), 124 (91); exact mass, m/e 338.1992, calcd for $C_{21}H_{26}O_2N_2$, m/e 338.1994.

The second eluate, 6 mg (12%), was starting urethane **23a**. As third eluate there appeared 15 mg (30%) of crystalline imino ester **22b**: mp 135 °C (from MeOH); IR (KBr) C=O 1735 (s), C=C 1605 (w), C=N 1570 (m) cm^{-1}; UV λ_{max} 221 nm (log ϵ 4.15), 266 (3.66); ^1H NMR δ 0.92 (t, 3, J = 7 Hz, Me), 1.1–1.3 (m, 2, CH_2), 1.3–2.0 (m, 6, methylenes, methines), 1.34 (td, 1, J = 13, 6 Hz, H-17β), 2.29 (d, 1, J = 10 Hz, H-3), 2.4–2.5 (m, 1, H-17α), 2.58 (t, 1, J = 14 Hz, H-21β), 3.0–3.1 (m, 1, H-21α), 3.2–3.3 (m, 1, H-6), 3.3–3.4 (m, 1, H-5), 3.75 (s, 3, OMe), 4.11 (dd, 1, J = 6, 2 Hz, H-16), 7.2–7.6 (m, 4, aromatic Hs); m/e 338 (M^+, base), 337 (28%), 194 (20), 180 (25), 124 (78); exact mass, m/e 338.1991, calcd for $C_{21}H_{26}O_2N_2$, m/e 338.1994.

Exposure of the latter compound to glacial acetic acid for 15 min converted it into vinylogous urethane **24**.

(±)-20-Epipseudovincadifformine (3). Photolysis of 50 mg (0.15 mmol) of urethane **23b** under the above conditions (except for the use of a high-pressure lamp and the irradiation lasting 45 min), HPLC of the crude product on alumina, and elution with 9:1 cyclohexane/dichloromethane furnished 12 mg (25%) of crystalline vinylogous urethane **3**: mp 127–128 °C (lit.[6] mp 127–128 °C); spectra identical with those reported[6] [^1H NMR δ 0.91 (t, 3, J = 7 Hz, Me), 1.1–1.3 (m, 2, CH_2), 1.4–2.7 (m, 7, methylenes, methines), 2.14 (t, 1, J = 10 Hz, H-21β), 2.35 (dd, 1, J = 15, 3 Hz, H-17β), 2.58 (dd, 1, J = 15, 12 Hz, H-17α), 2.87 (s, 1, H-3), 2.92 (dd, 1, J = 10, 7 Hz, H-5), 3.18 (dd, 1, J = 11, 4 Hz, H-21α), 3.75 (s, 3, OMe), 6.7–7.3 (m, 4, aromatic Hs)]. As second eluate there was isolated 5 mg (10%) of starting material.

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Registry No. (±)-**2**, 91670-85-8; (±)-**3**, 73836-92-7; **4**, 59936-01-5; **5a**, 42972-47-4; **5a** (ketal), 91670-73-4; **5b**, 91670-74-5; (±)-**6a**, 91670-75-6; (±)-**6b**, 91670-76-7; (±)-**7a**, 91670-77-8; (±)-**7b**, 91670-78-9; (±)-**8a**, 91740-06-6; (±)-**8b**, 91670-80-3; (±)-**8c**, 91670-81-4; (±)-**8e**, 91740-07-7; (±)-**8f**, 91670-83-6; (±)-**9a**, 91670-79-0; (±)-**9b**, 91740-15-7; (±)-**9c**, 91740-08-8; (±)-**9d**, 91686-45-2; (±)-**9e**, 91740-10-2; (±)-**9f**, 91740-09-9; (±)-**10a**, 91740-05-5; (±)-**10d**, 91670-82-5; (±)-**10e**, 91740-11-3; (±)-**10f**, 91670-84-7; (±)-**22a**, 91740-12-4; (±)-**22b**, 91670-87-0; (±)-**23a**, 91670-86-9; (±)-**23b**, 91740-13-5; (±)-**24**, 91740-14-6; (±)-**26a**, 89240-71-1; (±)-**26b**, 91740-18-0; (±)-**26c**, 89300-61-8; (±)-**29a**, 38216-76-1; (±)-**29b**, 89240-70-0; (±)-**29c**, 38216-77-2; (±)-**30a**, 87495-05-4; (±)-**30b**, 91670-96-1; (±)-**30c**, 91670-97-2; (±)-**30d**, 87495-06-5; (±)-**30e**, 91670-93-8; (±)-**30f**, 91670-94-9; (±)-**31a**, 91670-95-0; (±)-**31b**, 87508-89-2; (±)-**31c**, 91740-16-8; (±)-**31d**, 91670-98-3; (±)-**33a**, 89300-59-4; (±)-**33b**, 91670-88-1; (±)-**33c**, 87495-09-8; (±)-**33d**, 91670-88-1; (±)-**33e**, 91670-89-2; (±)-**34a**, 89300-58-3; (±)-**34b**, 91670-90-5; (±)-**34c**, 87508-87-0; (±)-**34d**, 87508-90-5; (±)-**35a**, 61848-78-0; (±)-**35b**, 91740-17-9; (±)-**35c**, 91670-91-6; (±)-**35d**, 87495-10-1; (±)-**35e**, 87495-07-6; (±)-**36a**, 77080-74-1; (±)-**36b**, 73837-57-7; (±)-**36c**, 87508-88-1; (±)-**36d**, 91670-92-7; indoleacetic anhydride, 41547-05-1.

Biosynthetic Studies of Marine Lipids. 4.[1] Mechanism of Side Chain Alkylation in (*E*)-24-Propylidenecholesterol by a Chrysophyte Alga

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A photosynthetic marine Chrysophyte (unicellular alga) was cultured in a medium containing labeled methionine (methyl-^{13}C, methyl-d_3). The ^2H and ^{13}C distribution in the alkyl substituents of the sterols was determined by ^2H and ^{13}C NMR. Although two novel cyclopropyl sterols—(24*R*,28*R*)- [or (24*S*,28*S*)-] and (24*S*,28*R*)-24,28-methylene-5-stigmasten-3β-ol—and their ring opening products were found as trace sterols in the alga, the results of ^2H NMR indicate that the substituent in the side chain of the main sterol ((*E*)-24-propylidenecholesterol) is not formed by ring opening of these cyclopropyl sterols. Instead, the main sterol is most likely formed by further alkylation of a 24-vinyl sterol which has so far not been encountered in nature. Other novel sterols encountered in the Chrysophyte belong to the rare classes of Δ^{23}, 14α-methyl, and $\Delta^{8(14),15}$-diene sterols.

More than two decades ago it was discovered that the methyl group of methionine in the form of *S*-adenosylmethionine (SAM) is used as a carbon source in enzymatic transmethylation reactions.[2] Reactions which SAM is known to undergo are aromatic substitution, methyl ester formation, and alkylation at N,[3] O,[4] and at a double bond.[2,5,6] The primary products of the last reaction are usually olefins, but cyclopropanes can also be formed.[6]

Sterols with side chains having more carbon atoms than the cholesterol side chain are found[7] in plants (phyto-

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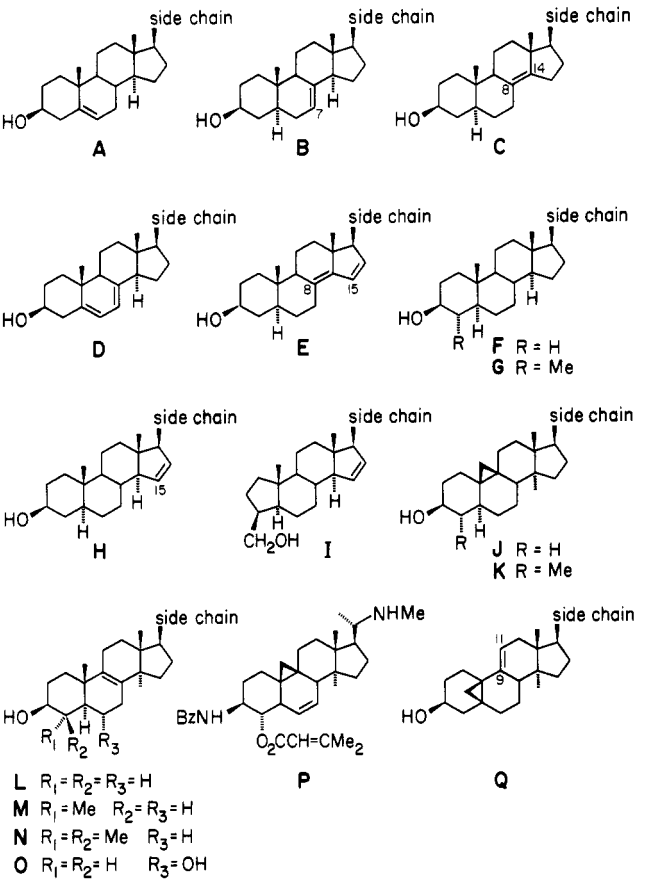
sterols) and in many marine organisms.[8,9] All alkyl substituents in the cholesterol side chain are believed to be derived from methionine. However, this has been demonstrated only in sterols possessing side chains with one or two additional carbon atoms at C24[2,5] and in the case of the dinosterol side chain (**35**).[10] Sterol side chains with many other, different alkylation patterns are known,[8,9] but, with the exception of some sponge sterols, their biosynthesis has not been studied.[1,11]

In the course of our continuing investigation of the sterol composition of cultured, unicellular marine algae we have found several which contain unusual sterols. Examples are a Chrysophyte (phylum Chrysophyta), which is a source of 24(*E*)-propylidenecholesterol (**A21**),[12] (Figure 1) and several dinoflagellates (phylum Pyrrhophyta) whose sterols include 4α-methylgorgostanol (**G36**),[13] gorgosterol (**A36**), and 23-demethylgorgosterol (**A38**).[14] Hence we were in a position to examine the biosynthesis of the side chains of these unusual sterols. Most of our cultured algae are photosynthetic and thus do not need to take up organic compounds from the medium. However, some of them,[15] including the above Chrysophyte were found to have a heterotrophic potential as they readily incorporated methionine making it possible to obtain sterols labeled with stable isotopes (^{2}H, ^{13}C) in the side chain. In this paper we present the results of a study of sterol side chain biosynthesis in the Chrysophyte.

Results and Discussion

Earlier we reported[12] the sterol composition of a still unidentified Chrysophyte which has qualitatively the same sterol composition as *Sarcinochrysis marina* (phylum Chrysophyta, order Ochromonadales, family Sarcinochrysidaceae)[16] and we proposed[12] several possible biosynthetic routes to the main sterol (*E*)-24-propylidenecholesterol (**A21**). One of the routes (route a, Figure 2) was conventional;[17] the other two (routes b and c, Figure 2) were unconventional. The conventional route is just an extrapolation of what is known[5] about how a C_2 substituent is introduced at C24. Route b involving ethionine, the higher homologue of methionine, was based on the assumption that ethionine could conceivably undergo the same reactions as methionine, making it possible to add a two-carbon unit in one step to a substrate. Plants introduce the C19 methyl group in their sterols by enzymatic opening of a three-membered ring in a pentacyclic intermediate[7] as exemplified by the conversion of cycloeucalenol (**K7**) into obtusifoliol (**M7**). It is for this reason that a third route (route c) to (*E*)-24-propylidenecholesterol (**A21**)



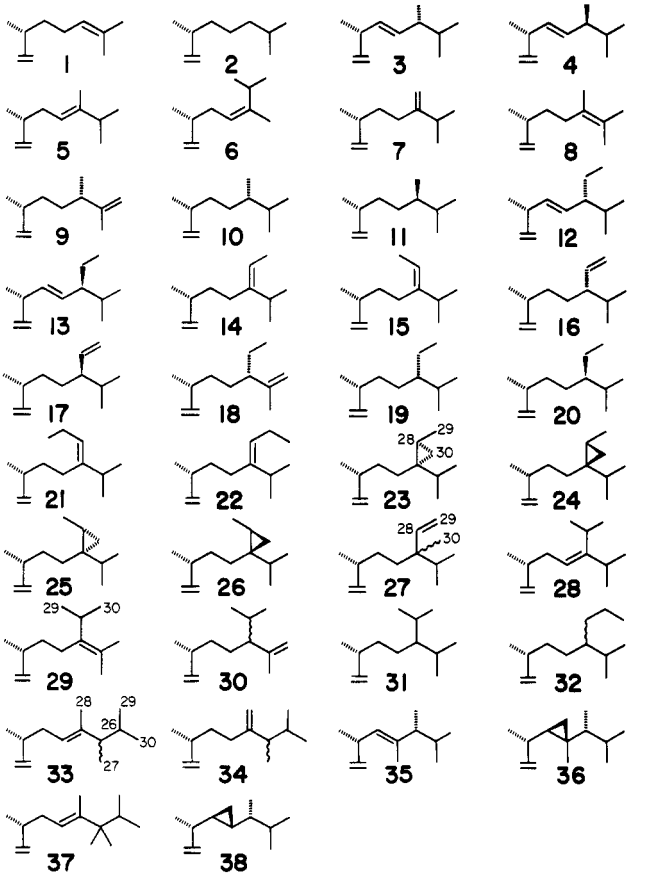


Figure 1. Structures of selected steroids.

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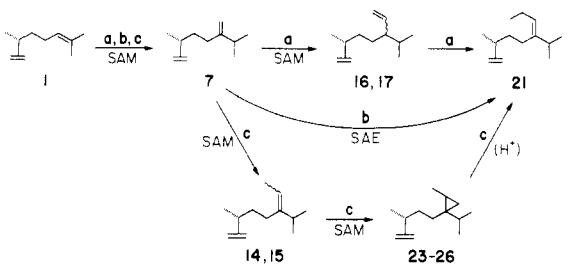
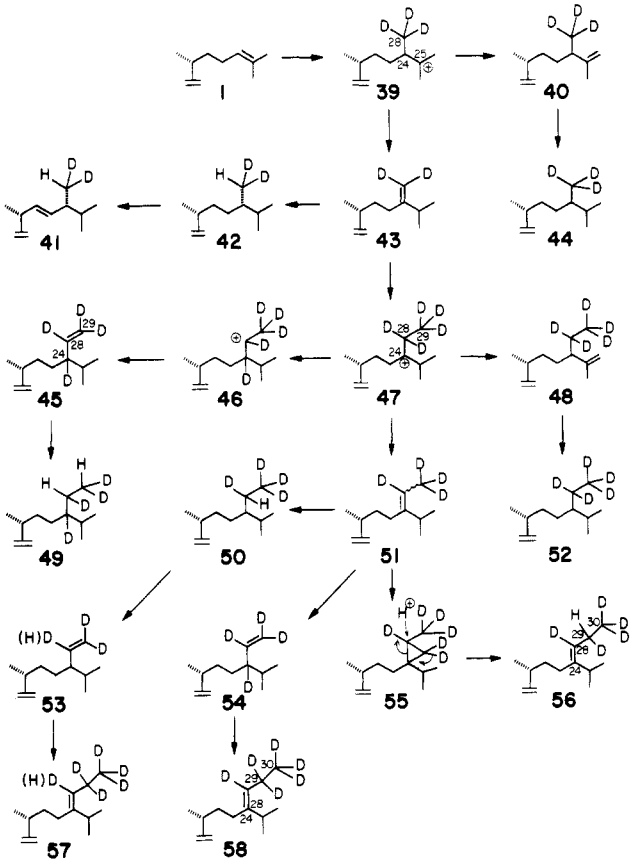


Figure 2. Three possible routes (a, b, c) to (*E*)-24-propylidenecholesterol (**A21**) (SAM, *S*-adenosylmethionine; SAE, *S*-adenosylethionine).

Scheme I. Hypothetical Biosynthetic Schemes via Met-d_3 Incorporation



might be envisioned even though we recognize that route c would involve anti-Markovnikov opening of a three-membered ring.

When we analyzed[12] the sterol mixture of the Chrysophyte by argentic TLC and reverse-phase HPLC eight sterols were isolated. As isolation of any of the postulated intermediates (see Scheme I) might be an argument in favor of the operation of a particular route, we reexamined carefully the free sterol mixture of a larger sample. As before, the sterol acetates were fractionated by argentic TLC and four fractions were collected. After saponifications the sterols from the four fractions were separated by reverse-phase HPLC with methanol and two solvent mixtures. Thirty sterols, including nine new ones, were isolated and identified by NMR and mass spectroscopy. They are listed in Table I together with their abundances and relative retention times (GC and HPLC).

Structure Elucidation of the New Sterols

Two Cyclopropyl Sterols and Their Ring Opening Products. Two minor sterols of M_r 426 with identical GC retention time were isolated from fraction 1 (the sterol mixture with the highest R_f value in argentic TLC). The 360-MHz ^{1}H NMR spectrum showed the presence of a 1:1 mixture of two cyclopropyl sterols with a Δ^5 nuclear unsaturation as deduced from the shift of the angular methyl groups,[18] the presence of eight methyl doublets, and double doublets of cyclopropyl protons at δ 0.416 (1 H), 0.363 (1 H), and −0.243 (2 H). The cyclopropyl sterols could be separated in HPLC with an acetonitrile-based solvent

Table I. Identified Sterols of the Chrysophyte

systematic and trivial names	abundance, %	RRT, HPLC[a]	RRT, GC[b]
5-cholesten-3β-ol (**A2**)	0.78	1.00	1.00
(24*R*)-5,22-ergostadien-3β-ol (brassicasterol) (**A3**)	9.8	0.93	1.12
(24*S*)-5,22-ergostadien-3β-ol (crinosterol) (**A4**)	0.21	0.90	1.12
5,24(28)-ergostadien-3β-ol (**A7**)	0.90	0.85	1.34
(24*R*)-5,25-ergostadien-3β-ol (codisterol) (**A9**)	0.09	0.83	1.31
(24*S*)-5-ergosten-3β-ol (**A10**)	9.5	1.13	1.30
(24*S*)-5,22-stigmastadien-3β-ol (stigmasterol) (**A13**)	4.3	1.08	1.43
(*Z*)-5,24(28)-stigmastadien-3β-ol (isofucosterol) (**A14**)	5.7	0.99	1.79
(*E*)-5,24(28)-stigmastadien-3β-ol (fucosterol) (**A15**)	0.68	0.99	1.70
(24*R*)-5,25-stigmastadien-3β-ol (clerosterol) (**A18**)	0.75	0.94	1.62
(24*S*)-5-stigmasten-3β-ol (clionasterol) (**A19**)	3.0	1.24	1.64
(24*R*)-5-stigmasten-3β-ol (sitosterol) (**A20**)	10.2	1.24	1.64
(*E*)-24-propylidene-5-cholesten-3β-ol (**A21**)	46.5	1.06	1.90
(*Z*)-24-propylidene-5-cholesten-3β-ol (**A22**)	0.22	1.09	2.08
(24*S*,28*S*)- or (24*R*,28*R*)-24,28-methylene-5-stigmasten-3β-ol (**A23** or **A24**)[c]	0.07	1.20	2.15
(24*S*,28*R*)-24,28-methylene-5-stigmasten-3β-ol (**A25**)[c]	0.07	1.20	2.15
(24ξ)-24-methyl-5,28-stigmastadien-3β-ol (**A27**)[d]	0.43	1.12	2.24
24-isopropyl-5,23-cholestadien-3β-ol (**A28**)[c]	0.11	1.06	1.74
24-isopropyl-5,24-cholestadien-3β-ol (**A29**)[c]	0.06	1.15	2.28
(24ξ)-24-isopropyl-5,25-cholestadien-3β-ol (**A30**)	0.14	1.03	1.93
(24ξ)-24-propyl-5-cholesten-3β-ol (**A32**)	0.60	1.36	1.92
(25ξ)-26,26-dimethyl-5,23-ergostadien-3β-ol (**A33**)[c]	0.03	1.11	2.04
(25ξ)-26,26-dimethyl-5,24(28)-ergostadien-3β-ol (**A34**)[c]	0.37	1.09	2.08
5α-ergosta-7,24(28)-dien-3β-ol (**B7**)	0.09	0.83	1.58
5α-ergosta-8(14),24(28)-dien-3β-ol (**C7**)	0.08	0.78	1.34
(24*S*)-5,7-ergostadien-3β-ol (**D10**)	0.10	0.97	1.51
(24*S*)-5α-ergosta-8(14),15-dien-3β-ol (**E10**)[c]	0.09	0.97	1.51
(*E*)-14α-methyl-5α-ergosta-8,23-dien-3β-ol (**L5**)[c]	0.03	0.79	1.38
14α-methyl-5α-ergosta-8,24(28)-dien-3β-ol (**L7**)	0.21	0.79	1.37
4α,14α-dimethyl-5α-ergosta-8,24(28)-dien-3β-ol (obtusifoliol) (**M7**)	0.11	0.91	1.53

[a] RRT = relative retention time. Altex Ultra Sphere ODS columns, MeOH, standard cholesterol. [b] 3% column, 260 °C, standard cholesterol. [c] Hitherto not encountered in nature.

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mixture. The double doublets of cyclopropyl protons are caused by two protons at the same carbon atom which are coupled to only one proton at an adjacent carbon atom. Similar double doublets are observed in the ^{1}H NMR spectra of gorgosterol (**A36**)[19] and two synthetic cyclopropyl sterols,[20] (24*S*,28*R*)- and (24*R*,28*S*)-24,28-methylene-5-stigmasten-3β-ol (**A25** and **A26**). The mass spectra of the cyclopropyl sterols of the Chrysophyte and of the synthetic cyclopropyl sterols **A25** and **A26** were identical. Notably, they include an ($M^+ - 42$) peak which is diagnostic of a methyl-substituted cyclopropyl group in a terminal position.[21] The NMR spectrum (Table II) of one natural product and that of the synthetic (24*S*,28*R*)-cyclopropyl sterol **A25** were identical, suggesting the other natural product to be either (24*S*,28*S*)- or (24*R*,28*R*)-24,28-methylene-5-stigmasten-3β-ol (**A23** or **A24**). This has been confirmed by partial synthesis[22] of the last two compounds from isofucosterol (**A14**). NMR data of the new sterol (**A23** or **A24**) are included in Table II; NMR data of the known cyclopropyl sterol[20] **A25** are added for comparison. It should be noted that only the relative configuration at C24 and C28 of the new sterol has been determined, but not its absolute configuration.

The component of fraction 4 (the sterol mixture with the lowest R_f value in argentic TLC) with the longest retention time in reverse-phase HPLC (MeOH) also had M_r 426. Peaks in the mass spectrum at m/z 314 and 328 (loss of the end of the side chain via a McLafferty rearrangement) indicated the presence of a nuclear unsaturation and of a double bond in the 25-position.[23] The 360-MHz ^{1}H NMR spectrum (Table II) showed the presence of a Δ^5 double bond and of a vinyl substituent at a quaternary carbon atom as three olefinic protons showed up as double doublets at δ 5.674, 4.992, and 4.857, respectively. The methyl region of the NMR spectrum included three methyl singlets (one due to a methyl group at a quaternary carbon in the side chain) and three methyl doublets. The combined MS and NMR evidence indicates that the structure of the new sterol is (24ξ)-24-methyl-5,25-stigmastadien-3β-ol (**A27**). Only one epimer has been isolated.

Three sterols of M_r 426 with isopropyl substituents in the side chain were isolated from fraction 2 of argentic TLC. The isomer with the shortest retention time in reverse-phase HPLC (MeOH) was identified as one of the epimeric 24-isopropyl-5,25-cholestadien-3β-ol (24-isopropenylcholesterol) (**A30**) by comparison of NMR spectra (Table II). This compound has previously been isolated from a sponge.[24]

The NMR spectrum (Table II) of another Δ^5 sterol of M_r 426 showed two sets of equivalent methyl doublets at δ 0.882, and one olefinic proton (dd) at δ 5.09 (in addition to H at C6). This indicated that the most likely structure was 24-isopropyl-5,23-cholestadien-3β-ol (**A28**), which would be the first example of a (rare) Δ^{23}-unsaturated sterol[25] with a C_3 substituent at C24. However, the mass spectrum did not seem to support this structure (**A28**). The mass spectrum of a $\Delta^{5,23}$-unsaturated sterol is expected to include a diagnostic peak at m/z 300 (22% in the case of **A5**)[26] caused by a McLafferty type rearrangement, but the mass spectrum of **A28** displayed a weak one (<3%). To prove that **A28** was the correct structure, the new sterol was hydrogenated and 24-isopropyl-5α-cholestan-3β-ol (**F31**), a known sponge sterol,[27,28] was obtained.

The mass spectrum of the new sterol of M_r 426 with the longest retention time in reverse-phase HPLC (MeOH) showed a base peak at m/z 314 indicative of the presence of a $\Delta^{24(28)}$ double bond.[23] Because of the presence in the NMR spectrum (Table II) of two methyl groups at a double bond, a methyl doublet at δ 0.992, methyl doublets at δ 0.927 and 0.937 (coupled to a methine proton at δ 2.84), and an olefinic proton at δ 5.35, the only possible structure for this new sterol was 24-isopropyl-5,24-cholestadien-3β-ol (24-isopropylidenecholesterol) (**A29**).

It is important to emphasize that the above isopropyl sterols (**A28**, **A29**, **A30**) and 24-methyl-5,28-stigmastadien-3β-ol (**A27**) are not artifacts formed from the cyclopropyl sterols (**A23** (or **A24**), **A25**) during the extraction of the alga. If they were artifacts, both epimers at C24 of **A30** would have been present. Furthermore, the most abundant one (**A27**) of the four ring opened products (see Table I) is at best a very minor product when the synthetic cyclopropyl sterols **A25** and **A26** are exposed to acid.[22]

A New 14-Methyl Sterol. When the components of fraction 4 of argentic TLC were separated by reverse-phase HPLC (MeOH) two compounds of M_r 412 were isolated from a fraction corresponding to the first peak. Their mass spectra were unusual in that the ($M^+ - 15$) peak was the base peak and except for an intense M^+ peak, all other peaks with m/z >200 were of low intensity. In both cases (**L5** and **L7**) the NMR spectrum (Table II) showed the presence of three 3 H singlets in the methyl region, the presence of unsaturation in the side chain, and the absence of olefinic ring protons. This suggested a structure with a Δ^8 double bond and a 14-methyl group, thus placing this substance into the rare class of 14-monomethyl sterols. There was good agreement between NMR data[29] for the quaternary methyls of lanosterol (**N1**) (C18–H δ 0.70, C19–H δ 1.00, and C14–Me δ 0.87) and the NMR data of the two Chrysophyte sterols (**L5** and **L7**, Table II). For comparison NMR data of macdougallin[30] (14α-methyl-5α-cholest-8-ene-3β,5α-diol) (**O2**) are included in Table II. One of the 14-methyl sterols was identified as the known 14α-methyl-5α-ergosta-8,24(28)-dien-3β-ol (**L7**).[31] The new sterol with the same ring system is (*E*)-14α-methyl-5α-ergosta-8,23-dien-3β-ol (**L5**) because the NMR spectrum also showed a methyl at a double bond, an olefinic proton (dd), and three methyl doublets. The shift of the methyl doublets suggested an *E* rather than a *Z* configuration of the double bond.[25]

14-Methyl sterols are rare because they are not on the usual sterol biosynthetic path—demethylation at C14 usually occurring before, rather than after demethylation

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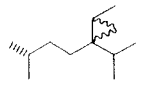
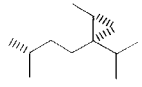
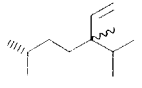
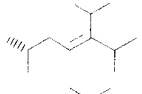
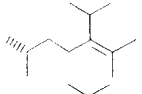
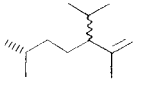
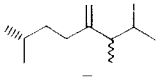
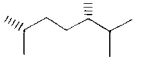
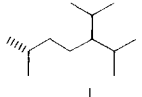
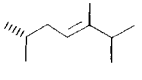
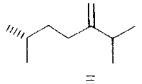
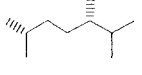
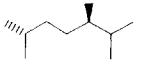
Table II. ^{1}H NMR Data (300 or 360 MHz) of Selected Sterols of the Chrysophyte, of Hydrogenation Products (F31, M10, M11), and of a Reference Compound (O2) (with the Exception of the Last Two Spectra, All Spectra Were Recorded in $CDCl_3$)[a]

compd	side chain	C18–H	C19–H	C21–H	C26–H	C27–H	C28–H	C29–H	C30–H	other
A23 or **A24**[b]		0.664 (s)	1.007 (s)	0.882 (d), J = 6.5	0.956 (d), J = 6.9	0.986 (d), J = 7.0	0.62 (m)	1.079 (d), J = 6.2	0.416 (dd), J = 8.5, 4.1 −0.243 (dd), J = 4, 4	
A25[b]		0.675 (s)	1.007 (s)	0.917 (d), J = 6.5	0.929 (d), J = 6.8	0.935 (d), J = 6.9	0.62 (m)	1.045 (d), J = 6.3	0.363 (dd), J = 8.7, 4.3 −0.243 (dd), J = 4, 4	
A27[b]		0.670 (s)	1.006 (s)	0.907 (d), J = 6.6	0.795 (d), J = 7.2	0.827 (d), J = 6.8	5.674 (dd), J = 17.6, 10.9	4.992 (dd), J = 10.9, 1.6 4.857 (dd), J = 17.6, 1.6	0.853 (s)	
A28[c]		0.689 (s)	1.010 (s)	0.882 (d), J = 6.6	0.975 (d),[d] J = 7	0.975 (d),[d] J = 7	2.8 (m)	0.999 (d),[d] J = 7	0.999 (d),[d] J = 7	C23–H 5.09 (dd), J = 8.2, 6.2
A29[e]		0.686 (s)	1.008 (s)	0.992 (d), J = 7	1.628 (s)	1.646 (s)	2.84 (m)	0.927 (d), J = 7.0	0.937 (d), J = 6.8	
A30[c,e]		0.672 (s)	1.006 (s)	0.901 (d),[d] J = 6.5	4.735 (dd), J = 2.5, 1.2 4.610 (d), J = 2.3	1.560 (s)		0.910 (d),[d] J = 6.2	0.799 (d), J = 6.3	
A34[c]		0.680 (s)	1.008 (s)	0.939 (d), J = 6.9		0.962 (d), J = 7.1	4.707 (s), 4.677 (s)	0.830 (d), J = 6.9	0.854 (d), J = 7.1	
E10[c]		0.890 (s)	0.642 (s)	0.944 (d), J = 6.6	0.781 (d), J = 6.8	0.854 (d), J = 6.8	0.781 (d), J = 6.8			C15–H 5.26 (d), J = 10; C16–H 6.13 (dd), J = 10, 3
F31[b]		0.642 (s)	0.798 (s)	0.921 (d)	0.818 (d),[d] J = 6	0.837 (d),[d] J = 6.5		0.856 (d),[d] J = 7	0.856 (d),[d] J = 7	
L5[b]		0.719 (s)	0.948 (s)	0.875 (d), J = 6.2	0.991 (d), J = 6.8	0.991 (d), J = 6.8	*f*			C14–Me 0.891 (s); C23–H 5.15 (dd), J = 6, 6
L7[c]		0.712 (s)	0.945 (s)	0.929 (d), J = 6.4	1.029 (d), J = 6.8	1.023 (d), J = 6.9	4.713 (s); 4.661 (s)			C14–Me 0.888 (s)
N10[c]		0.703 (s)	0.968 (s)	0.896 (d), J = 6	0.857 (d), J = 6.9	0.781 (d), J = 6.9	0.781 (d), J = 6.9			C14–Me 0.881 (s); C4–Me 0.995 (d), J = 6.1
N11[c]		0.704 (s)	0.968 (s)	0.889 (d), J = 6	0.852 (d), J = 6.9	0.804 (d), J = 7	0.779 (d), J = 7			C14–Me 0.880 (s); C4–Me 0.995 (d), J = 6.2

O2[c]		0.692 (s)	0.966 (s)	0.890 (d), $J = 7$	0.860 (d), $J = 6.7$	0.865 (d), $J = 6.7$				C14–Me 0.904 (s); C3–H 3.60 (m); C6–H 3.76 (m)
A33[c,g]		0.667 (s)	0.932 (s)	1.017 (d), $J = 6.9$		1.053 (d), $J = 6.6$	1.652 (s)	0.921 (d), $J = 6$	0.942 (d), $J = 6$	C23–H 5.279 (dd), $J = 7, 7$
A34[c,g]		0.656 (s)	0.938 (s)	1.010 (d), $J = 6.5$		1.044 (d), $J = 7.0$	4.780 (s); 4.948 (s)	0.878 (d), $J = 6.6$	0.949 (d), $J = 6.4$	

[a] Internal standard $CHCl_3$ (C_6D_6 in the last two spectra); shifts are δ values; splitting constants in Hz; rounded values for J indicate that more accurate values could not be given because of overlapping peaks. [b] 360 MHz. [c] 300 MHz. [d] Interchangable assignments. [e] Published NMR data (ref 24) are not accurate because they were obtained from the spectrum of a mixture of epimers (**A30** was the minor component of that mixture). [f] Shift could not be determined because the signal was hidden under the water peak. [g] Recorded in C_6D_6.

at C4.[32] They have been isolated from a cactus[30] (**O2**), pollen (**J2**),[33] and yeast mutants[34] (**L1**,**L7**), and they have been detected in a mutant of the mold *Ustilago maydis*[35] (**L8**). Larger quantities of such sterols may accumulate in living cells (bramble, *Chlorella sorokiniana*) when the cells are cultured in the presence of certain enzyme inhibitors.[31,36] There is also one *Buxus* alkaloid (**P**) which has a methyl group at C14 and no carbon substituent at C4.[37] Recently, the unprecendented (19,5β)-cyclosterols **Q3**, **Q4**, **Q10**, and **Q11** were isolated from *Nervilia purpurea*, an orchidaceous plant.[38]

Other New Sterols. When (24*S*)-methyl-5,7-ergostadien-3β-ol (**D10**) was isolated from fraction 4 of argentic TLC by reverse-phase HPLC (MeOH) it was obtained as a mixture with an isomeric diene with the same side chain. Both compounds could easily be separated by reverse-phase HPLC with an acetonitrile-based solvent mixture. The NMR spectrum (Table II) of the isomeric diene includes one olefinic proton each at δ 6.13 (dd, J = 10, 3) and δ 5.26 (d, J = 10). The high value for the shift of one of the protons and the large difference in δ values indicates that the double bonds are conjugated and that one proton is bound to carbon number 1 of a 1,3-diene and the other to carbon number 2. Thus, the proposed structure is (24*S*)-methyl-5α-cholesta-8(14),15-dien-3β-ol (**E10**). This structure is consistent with the low R_f value in argentic TLC: we found previously that a sterol with a Δ^{15} double bond (**H2**, **I2**) behaves in argentic TLC like a sterol with a sterically unhindered methylene group (e.g., **A7**) in the side chain.[39] Also, the predicted and observed values for the shifts of the angular methyls of **E10** agree quite well. The reported values for the shift of C18–H and C19–H of the Δ^{15} sterol **H2** are δ 0.731 and 0.822, respectively.[39] The Zürcher additive values[18] for the introduction of a $\Delta^{8(14)}$ double bond are 0.175 for C18–H and −0.117 for C19–H. Thus the predicted values for the shifts of the angular methyls of **E10** are δ 0.906 and 0.605; the observed values are δ 0.890 and 0.643 (see Table II).

A new sterol of M_r 426 from fraction 4 of argentic TLC cochromatographed with (*Z*)-24-propylidene-5-cholestan-3β-ol (**A22**) in reverse-phase HPLC (MeOH). The compounds could be separated by using a THF–MeOH–water mixture. A base peak in the mass spectrum at m/z 314 indicated the presence of a $\Delta^{24(28)}$ double bond[23] and of a nuclear unsaturation. The sterol is a Δ^5 sterol with a methylene group in the side chain. There were only four methyl doublets in the NMR spectrum (Table II). A normal cholesterol side chain (**2**) with a methylene group and two additional methyl substituents would require five methyl doublets. Apparently we had a sterol with an extended side chain. Only one of the methyl groups in the

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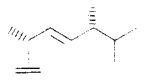
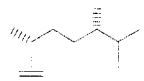
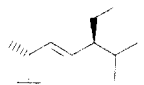
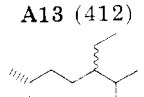
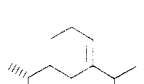
Table III. Incorporation Experiments with Met-d_3 and Met-^{13}C. Abundances (%) of Unlabeled and Isotopically Labeled Species of Each of the Five Main Sterols of the Chrysophyte in Dependence of the Met Concentration[a,b,c]

sterol and side chain (MW)		deuterated species, %								^{13}C-labeled species, %		
		5[e]	10[e]	25[e]	50[e]	100[e]	250[e]	500[e]	1.00[e]		expt. 1[f]	expt. 2[f]
 A3 (398)	d_0	84	83	80	74	84	81	78	70			
	d_1	1		1	7	3	3	4	3			
	d_2	14	16	18	14	13	14	16	24			
	d_3	1	1	1	5		1	2	3			
 A10 (400)	d_0	83	77	80	72	70	73	69	n/d			
	d_1	2	2	2	8	6	8	6				
	d_2	14	21	17	18	20	18	21				
	d_3	1		1	1			1				
 A13 (412)	d_0	94	76	75	74	71	67	65	n/d	$^{13}C_0$	79	91[d]
	d_1	4	11	12	21	19	21	21		$^{13}C_1$	21	9
	d_2	2										
	d_3		11	10	6	9	8	10				
	d_4		2	3		1	3	4				
 A20 + A19 (414)	d_0	80	56	69	64	72	58	51	53[d]	$^{13}C_0$	78	90[d]
	d_1	9	17	16	18	15	18	25	19	$^{13}C_1$	22	10
	d_2	1	2		1	1	8	2	4			
	d_3	8	18	12	15	9	10	15	16			
	d_4	2	7	3	3	2	4	6	6			
	d_5						1		2			
 A21 (426)	d_0	64	55	57	53	49	58	41	39[d]	$^{13}C_0$	78	89[d]
	d_1	9	13	13	15	14	18	18	15	$^{13}C_1$	22	11
	d_2	10	13	11	12	13	8	12	15			
	d_3	13	14	13	14	17	10	15	17			
	d_4	2	2	3	3	3	4	8	7			
	d_5	2	2	2	3	3	1	2	5			
	d_6		1	1	2	1		4	2			

[a] Mass spectra were recorded in the GC/MS mode unless indicated otherwise. [b] Several scans of the mass spectrum of each compound were made, but only one, chosen at random, was used to calculate the ^{2}H label. A comparison of the ^{2}H distribution in, e.g., the two 24-ethyl sterols in the various mixtures demonstrates the inaccuracy of the label determination. [c] ^{13}C labels calculated from 4–8 scans were averaged; doubly labeled species ($^{13}C_2$) were ignored. [d] Heated inlet. [e] The concentration of Met-d_3 in the medium in mg/L. [f] The concentration of Met-^{13}C was 10 mg/L.

side chain was vinylic (δ 0.962). A possible structure consistent with these data is (25ξ)-26,26-dimethyl-5,24-(28)-ergostadien-3β-ol (**A34**).

A sterol which was identified as a double bond isomer of **A34** was isolated from a subfraction (reverse-phase HPLC, MeOH) of fraction 2 of argentic TLC. (24*S*)-Methylcholesterol was the main component of that subfraction. The NMR spectrum (recorded in C_6D_6)[40] was that of a Δ^5 sterol. It showed an olefinic proton (dd), a methyl group attached to a double bond, and four methyl doublets. In view of its molecular weight (426), the sterol had to have an extended side chain. The proposed structure is (25ξ)-26,26-dimethyl-5,23-ergostadien-3β-ol (**A33**). A base peak in the mass spectrum at m/z 69 (vinylic cleavage of the C24–C25 bond) supports the proposed structure.[41] As in the case of 24-isopropyl-5,23-cholestadien-3β-ol (**A28**) (vide supra), the mass spectrum does not show a diagnostic peak at m/z 300 which was expected because we are dealing with a Δ^{23} unsaturated sterol.[26] A trisulfate of a trihydroxy sterol with a structurally related side chain (**37**) has recently been isolated from a sponge from the Arabian Sea.[28c]

Biosynthetic Incorporation Experiments

The most likely precursor (see Figure 2) for 24-propylidenecholesterol (**A21**, **A22**) is a 24-vinyl sterol such as 24-vinylcholesterol (**A16**, **A17**). Both epimers of 5,28-stigmastadien-3β-ol (24-vinylcholesterol) (**A16**, **A17**) were synthesized and their chromatographic properties determined.[42] In reverse-phase HPLC (MeOH) they have a relative retention time of 0.955 and 0.939, respectively (determined by coinjection with cholesterol (**A2**)). If they had been present in the mixture of Chrysophyte sterols they would have ended up in fraction 4 on argentic TLC, and after separation of the components of fraction 4 by reverse-phase HPLC, they would have been obtained as a mixture with clerosterol (**A18**) or with the dienes **D10** and **E10**. The clerosterol sample was pure by NMR; they were not detected by GC in any of the HPLC fractions when the diene mixture was separated.

The fact that we have been unable to detect the 24-vinylcholesterols (**A16**, **A17**) in the Chrysophyte and that we isolated two cyclopropyl sterols (**A23** or **A24**, **A25**) is not a definite argument in favor of route c (Figure 2). It is conceivable that 24-vinylcholesterol (**A16**, **A17**), the intermediate in route a (Figure 2), was not detected because it is very efficiently converted into (*E*)-24-propylidenecholesterol (**A21**) by the Chrysophyte or that another 24-vinyl sterol with a different nucleus serves as precursor.

Incorporation experiments with methionine-*methyl*-^{13}C (Met-^{13}C), methionine-*methyl*-$^2H_3$ (Met-d_3), and ethionine-*ethyl*-$^2H_5$ (Et-d_5) allowed us to determine which route was actually operating.

The composition of the mixture of isotopically labeled species of each of the main sterols for all experiments performed with Met-d_3 and Met-^{13}C is given in Table III. The first experiment with Met-d_3 was similar to an in-

(40) **A33** decomposed before an NMR spectrum in $CDCl_3$ was recorded. That is the reason why C_6D_6 NMR data of **A33** are included in Table II. For comparison C_6D_6 NMR data of **A34** are also given in Table II.

(41) Vinylic cleavage of the side chain is also responsible for a base peak at m/z 69 in the mass spectrum of dinosterol (**G36**) and some analogues with the same side chain (**36**) but a different skeleton. See ref 14 and the following: Kokke, W. C. M. C.; Fenical, W.; Djerassi, C. *Phytochemistry* **1981**, *20*, 127–134.

(42) Catalan, C. A. N.; Kokke, W. C. M. C.; Duque, C.; Djerassi, C. *J. Org. Chem.* **1983**, *48*, 5207–5214.

corporation experiment with the dinoflagellate *Crypthecodinium cohnii*[10] reported by the Liverpool group in that an enormous excess of Met-d_3 (1.0 g/L of medium) was used, apparently in an attempt to completely suppress Met synthesis by the alga. The data in Table I clearly show that Met synthesis was not suppressed as part of the sterol molecules were still unlabeled.

We performed a total of eight runs to determine the dependence of the ^2H incorporation on the Met-d_3 concentration and found that a decrease of the Met-d_3 concentration by a factor 100 (from 1.0 g/L to 10 mg/L) did not significantly influence the results (see Table III).

The data in Table III also indicate that Met-d_3 is much better incorporated than Met-^{13}C under the same conditions. When comparing the percentages of unlabeled sterols (Table III) one has to keep in mind that the methyl group of Met-d_3 was completely labeled (100% d_3), whereas only 90% of the carbon atoms in the methyl group of Met-^{13}C was labeled with ^{13}C.

A comparison of the composition of the mixture of isotopically labeled species of the same sterol for each of the runs with Met-d_3 (Table III) shows that the accuracy of the label distribution by mass spectroscopy is not high. It makes little difference if the sample is run in the GC/MS mode or by using a heated inlet: there is considerable variation in the distribution of the label as calculated from various scans of the same sample. Most of the data in Table III were obtained with a quadrupole type mass spectrometer (Ribermag 10-10); higher accuracy can be obtained with a magnetic type mass spectrometer (e.g., MAT-711, MS-50) if one has the software to average peak intensities of many scans, and if unlabeled samples are run for comparison. A consequence of the uncertainties in the distribution of label determined by mass spectroscopy is that conclusions about biosynthetic reaction mechanisms should not be based solely on mass spectral data as was done in the literature[10] but should be verified by other spectroscopic techniques. Therefore, we resorted to ^2H NMR and ^{13}C NMR analysis.

When the alga was cultured in the presence of Et-d_5 only unlabeled sterols were produced. This negative result did not completely rule out the possibility that ethionine was involved in the synthesis of (*E*)-24-propylidenecholesterol (**A21**) (route b) because the alga might just lack the ability to take up ethionine from the medium. The ^{13}C NMR spectra of (*E*)-24-propylidenecholesterol (**A21**) and of the sitosterol/clionasterol (**A20, A19**) mixture from the Met-^{13}C experiment showed that all carbon atoms of the alkyl substituents in the side chain were labeled with ^{13}C, and that the enrichment in ^{13}C was about the same in the possible positions (C28 and C29 in **A19**, **A20**; C28, C29, and C30 in **A21**). If ethionine had been used to synthesize the propylidene substituent, then two of the three carbon atoms would have been unlabeled.

Figure 3 shows part of the ^{13}C NMR spectrum of 24-(*E*)-propylidenecholesterol (**A21**). The peak of C29 is split because of coupling with C28 or C30 in doubly labeled molecules ($^{13}C_2$). The splitting makes it possible to calculate the abundance of doubly labeled species. This information cannot be obtained from the mass spectrum because the enrichment in ^{13}C is low.

When the ^{13}C NMR spectrum of supposedly pure sitosterol (**A20**)[12] enriched in ^{13}C was recorded some of the peaks of the side chain carbons exhibited satellites. When the ^{13}C NMR data published by Wright et al.[43] were used, the minor component of the mixture could be identified

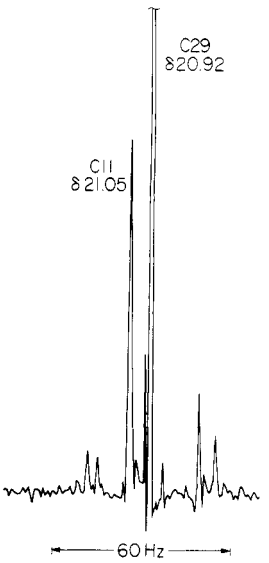


Figure 3. Expansion of part of a resolution-enhanced ^{13}C NMR spectrum (XL-200) of ^{13}C-labeled (*E*)-24-propylidenecholesterol (**A21**) showing the region of the C29 peak. The four low satellite peaks of the C29 peak are part of two AB systems caused by coupling with C30 (J = 34.1 Hz) and C28 (J = 43.1 Hz) in doubly labeled ($^{13}C_2$) molecules.

as clionasterol (**A19**), the C24 epimer of sitosterol (**A20**). The ratio of sitosterol to clionasterol was 3.4:1. Both compounds have the same mobility in GC and reverse-phase HPLC; the presence of the minor component was not detected by 360-MHz ^1H NMR because there is not much difference between the high field ^1H NMR spectra of the epimers.

It is known, mainly through the work of the Liverpool group,[5] that there are two routes (cf. Scheme I) leading to sterols methylated in the 24-position. A carbonium ion (**39**) produced by SAM attack on a desmosterol side chain (**1**) eventually gives an olefin with the double bond in either the 25(27)- or 24(28)-position (**40** or **43**). Subsequent reduction then affords a sterol with a saturated side chain (e.g., **42**) which can be converted into its Δ^{22} analogue (**41**) by enzymatic dehydrogenation. Therefore, an incorporation experiment with Met-d_3 will show which of the routes is actually used by an organism. If there is a 24(28)-unsaturated intermediate (**43**) then only two of the three ^2H atoms of Met-d_3 will be retained at C24 (**42**). No ^2H will be lost if the plant uses the other route to 24-methyl sterols (**44**). Both 24-methylenecholesterol (**A7**) and codisterol (**A9**) are minor sterols of the Chrysophyte (see Table I), but the ^2H distribution in the 24-methyl sterols (see Table III) indicates that the alga uses mainly or exclusively a $\Delta^{24(28)}$ unsaturated intermediate (**43**) to synthesize brassicasterol (**A3**) and 24(*S*)-methylcholesterol (**A10**).

24-Methylenecholesterol (**A7**) is a precursor of 24-ethylcholesterol (**A19, A20**) and its Δ^{22} analogue (**A13**). The results of sterol side chain alkylation studies in the literature[5] indicate that SAM attack on 24-methylenecholesterol (**A7**) will produce a carbonium ion with the positive charge located at C24 (**47**), which again can give either $\Delta^{24(28)}$ or $\Delta^{25(27)}$ unsaturated compounds. However, because 24-vinylcholesterol (**A16, A17**) is a postulated intermediate[12] in one of the proposed routes (route a, Figure 2) to (*E*)-24-propylidenecholesterol (**A22**), we also had to consider the possibility that 24-vinylcholesterol (**A16** and **A17**) or a 24-vinyl sterol with a different nucleus (e.g., Δ^7-4α-methyl) was the precursor of three sterols with a 24-ethyl substituent (**A13, A19** and **A20**) found in the Chrysophyte. 24-Vinylcholesterol (**A16, A17**) could have

(43) Wright, J. L. C.; McInnes, A. G.; Shimizu, S.; Smith, D. G.; Walter, J. A.; Idler, D.; Khalil, W. *Can. J. Chem.* **1978**, *56*, 1898–1903.

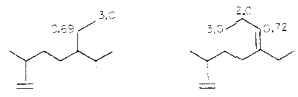


Figure 4. Ratio of ^2H attached to methionine-derived carbons in the side chain of the main sterols as determined by ^2H NMR.

been formed from a carbonium ion with the positive charge at C28 (**46**) by a proton (or deuteron) migration from C28 to C24 (**47** → **46** in Scheme I).

Again, the results of an incorporation experiment with Met-d_3 allowed us to select the most likely intermediate (**45**, **48**, or **51**). The ratio of ^2H at C24, C28, and C29 in the side chain of sterols with a 24-ethyl substituent (**49**, **50**, and **52**) should be 1:1:2 in the case of a Δ^{28} intermediate (**45**), and 0:1:3 and 0:2:3 in the cases of $\Delta^{24(28)}$ and $\Delta^{25(27)}$ unsaturated intermediates (**51** and **48**), respectively. The FT-80A ^2H NMR spectrum of 24-ethylcholesterol-*d* (**A19**, **A20**) produced by the Chrysophyte showed only two peaks (intensity ratio 0.69:3.0, see Figure 4) supporting a $\Delta^{24(28)}$ unsaturated intermediate (**51**). The intensity ratio might differ from the expected 1:3 because part of the ^2H at C28 might have been lost by exchange prior to reduction of the double bond (to give **50**). A different enzyme is probably needed for each alkylation step with SAM.[44] These enzymes ("methyltransferases") might differ in their isotope discrimination against labeled SAM and this might also be a reason why the observed ratio and the predicted ratio are not completely identical.

Clerosterol (**A18**) is a minor sterol of the Chrysophyte (see Table I). Our ^2H NMR measurements of deuterated 24-ethylcholesterol (**A19**, **A20**) (see Figure 4) have ruled out a $\Delta^{25(27)}$ unsaturated intermediate (**48**) in the formation of these 24-ethyl sterols. Therefore, the reason why we obtained these sterols as a mixture of epimers at C24 must have been that the enzymatic reduction of fucosterol (**A15**) and isofucosterol (**A14**) produced different isomers. Alternatively, if only fucosterol (**A15**) or isofucosterol (**A14**) is reduced by the alga, then this reduction is not stereoselective.

Because we have ruled out direct formation of 24-vinylcholesterol (**A12**, **A13**) from the carbonium ion **46** we must assume that this compound, if it is a precursor of (*E*)-24-propylidenecholesterol (**A21**), is formed either from fucosterol (**A15**) or isofucosterol (**A14**) by double bond migration (**51** → **54** in Scheme I) or by dehydrogenation of 24-ethylcholesterol (**50** → **53** in Scheme I).

The expected ratio of the intensities of the peaks of ^2H at C28, C29, and C30 in the ^2H NMR spectrum of (*E*)-24-propylidenecholesterol (**A21**) produced by the Chrysophyte using Met-d_3 is 1:2:3 if the alga makes this sterol via fucosterol or isofucosterol (**A15**, **A14**) by SAM alkylation of 24-vinylcholesterol (**A12**, **A13**) (i.e., **51** → **54** → **58** in Scheme I) and 0.5:2:3 if (*E*)-24-propylidenecholesterol (**A21**) is made by dehydrogenation of 24-ethylcholesterol (**A20**, **A19**) followed by SAM alkylation (i.e., **51** → **50** → **53** → **57** in Scheme I). If it should be formed via a cyclopropyl intermediate (**55** → **56**) the intensity ratio should be 1:1:3. The FT-80A ^2H NMR spectrum of (*E*)-24-propylidenecholesterol (**A21**) showed three peaks with an intensity ratio of 0.72:2:3 (Figure 4). This eliminates the possibility of a cyclopropyl intermediate. The relative intensity of the peak of the olefinic deuteron is lower than expected if 24-vinylcholesterol (**A16**, **A17**), the key intermediate, should be formed by double bond isomerization of fucosterol or isofucosterol (**A15**, **A14**), and higher than expected if it should be formed by dehydrogenation of 24-ethylcholesterol (**A20**, **A19**). Thus the intensity ratio of 0.72:2:3 found by using ^2H NMR (Figure 4) might be interpreted as evidence that (*E*)-24-propylidenecholesterol (**A21**) is formed via the shortest route (i.e., **43** → **47** → **51** → **54** → **58** in Scheme I) if part of the deuterium at C28 should have been lost by exchange. Alternatively, this route and the route via the 24-ethyl intermediate (**50**) (thus **43** → **47** → **51** → **50** → **53** → **57** in Scheme I) might be operating simultaneously.

These experiments constitute the first direct evidence concerning the introduction of three carbons in the cholesterol side chain.

Experiments to obtain additional evidence to support the above conclusion are planned. They will involve sterol side chain transformation experiments with radiolabeled precursors (e.g., 24-methylenecholesterol (**A7**), both epimers of 24-vinylcholesterol (**A16**, **A17**), and 24-ethylcholesterol (**A19**, **A20**)) in a cell-free system prepared from the Chrysophyte. Experiments with cell-free systems are necessary because the Chrysophyte does not take up radiolabeled sterols from the culture medium.

Experimental Section

General Methods. HPLC was performed with Waters equipment (M6000, M6000A, or M45 pump, R401 or R403 differential refractometer), two Altex Ultraspere ODS columns (10 mm ID × 25 cm) in series (eluent absolute MeOH, CH_3CN–MeOH–EtOAc 22:9:9 (mixture B), or THF–MeOH–water 3:3:1 (mixture C)), and one of the following injectors: Waters Model U6K, Rheodyne Model 7120, or Valco Model CV-6-UHPa-N60. Hewlett-Packard Model 402 and 5790A gas chromatographs with FID were used (3% SP2250 column, 2 mm ID × 1.80 m, 260 °C) for sterol analysis. Mass spectra were recorded with a Ribermag 10-10 mass spectrometer (capillary SE54 column, 260 °C). ^2H and ^{13}C labels were calculated by using the tables of Beynon and Williams.[45] ^2H NMR spectra were recorded by using a Varian FT-80A NMR instrument equipped with a microprobe and ^1H NMR spectra by using a Brucker HXS-360 and a Nicolet NMC-300 NMR instrument. The following instruments were used to obtain ^{13}C NMR spectra: Varian FT-80A, XL-200, and XL-300 and Nicolet NMC-300.

Peak Intensities in ^{13}C NMR. After the incorporation experiment with Met-^{13}C it was necessary to ensure that the ratio of ^{13}C derived from endogenous Met to that of ^{13}C derived from Met-^{13}C was about 1:1 at **C28** and **C29** in **A20** and **A19** and about 1:1:1 at **C28**, **C29**, and **C30** in **A21**. This information was obtained from peak intensities in the ^{13}C NMR spectra which were recorded under conditions where the NOE effect is suppressed (proton decoupled spectra, 90° pulse, pulse delay 4–5 s, decoupler off during pulse delay). The same spectra were used to calculate the enrichment in ^{13}C in the substituent carbons relative to the mevalonate-derived carbons. Because the methyl group and the carbonyl group in acetyl-CoA do not have the same $^{13}C/^{12}C$ ratio,[46] the carbons of mevalonate, and thus of terpenes, will not all have the same $^{13}C/^{12}C$ ratio. To calculate the enrichment in ^{13}C in a particular position in the alkyl substituent of the side chain relative to the skeleton, the intensity of the corresponding peak in the ^{13}C NMR spectrum was compared with the average intensity of the peaks of the carbons of an isoprene unit (C23–C27).

Met-d_3, Met-^{13}C, and Et-d_5: Preparation and Analysis. Comar et al.[47] prepared methionine-*methyl*-^{11}C for in vivo studies in mice and humans from homocysteinethiolactone hydrochloride and iodomethane-^{11}C. We used the same thiolactone to prepare labeled Met and Et, iodomethane-d_3 (99$^+$ atom% D, Aldrich, gold

(44) Bansal, S. K.; Knoche, H. W. *Phytochemistry* **1981**, *20*, 1269–1277.

(45) Beynon, J. H.; Williams, A. E. "Mass and Abundance Tables for use in Mass Spectrometry"; Elsevier Publishing Company: Amsterdam, 1963.

(46) DeNiro, M. J.; Epstein, S. *Science* **1977**, *197*, 261–263.

(47) Comar, D.; Cartron, J.-C.; Maziere, M.; Marazano, C. *Eur. J. Nucl. Med.* **1976**, *1*, 11–14.

label), iodoethane-d_5 (99+ atom% D, Merck & Co), and iodomethane-^{13}C (90 atom% ^{13}C, Merck & Co). The following is a typical procedure.

DL-Homocysteine hydrochloride (7.0 g, Aldrich) and iodomethane-d_3 (6.0 g) in 280 mL of an ethanolic NaOH solution (NaOH, 34.2 g, in 95% EtOH, 1200 mL) were stirred overnight. After refluxing for 1 h the solvent was evaporated. The residue, dissolved in water (ca. 40 mL), was introduced into an Amberlite IRA-400(OH^-) (Aldrich) column (bed volume 325 mL) which retained the reaction product. After the inorganics had been eluted with water, Met was eluted with 2 N HOAc. Evaporation of the aqueous HOAc solutions afforded crude Met as a white solid. It was purified by precipitation from a hot aqueous solution (40 mL) with 95% EtOH (40 mL). A second crop of crystals could be obtained from the residue of the mother liquor. Total yield of Met-d_3: 4.5–5.0 g (79–88% based on iodomethane-d_3).

Analysis of labeled Met by GC/MS as the methyl ester was not practical as a mixture of products was obtained when Met was treated with diazomethane: esterification occurs, but also N-alkylation. Trimethylsilylation[48] gave satisfactory results. Thus the labeled amino acid (2 mg), anhydrous CH_3CN (0.5 mL), and bis(trimethylsilyl)acetamide (50–70 μL) (Aldrich) were heated in a reaction vial at 80 °C until the amino acid had gone into solution. After standing overnight at room temperature the mixture was analyzed by GC (3% OV17 column, 130 °C) and GC/MS (capillary SE54 column, 130 °C). The only detectable product was the bis(trimethylsilyl) derivative of the amino acid. The mass spectra showed that no loss of label had occurred during the preparation of the labeled amino acids and that the isotopic purity of labeled iodomethane and iodoethane were in agreement with their manufacturer's specifications: Met-^{13}C bis(trimethylsilyl) ether[49] GC/MS (Ribermag) (70 eV), m/z (assignment, relative intensity) 294 (M^+, 2), 279 (0.1), 263 ($M^+ - CH_3 - {}^{13}CH_3$, 0.6), 251 (6), 232 (2), 219 (10), 218 (6), 202 (4), 177 ($M^+ - OCOSi(CH_3)_3$, 100), 147 ($[(CH_3)_3SiOSi(CH_3)_2]^+$, 15), 128 ($[(CH_3)_3SiOHNC_2]^+$, 56), 75 ($[HOSi(CH_3)_2]^+$, 12), 73 ($[Si(CH_3)_3]^+$, 83), 62 ($[CH_2S^{13}CH_3$, 39).

Incorporation Experiments. The initial runs to determine the dependence of ^2H incorporation on the Met-d_3 concentration in the culture medium were done in 3.5 US gallon bottles containing 10 L of GPM medium to which Met-d_3 had been added. Labeled sterols needed for ^{13}C NMR and ^2H NMR were isolated from algae which had been grown in polyethylene drums in batches of 200 L each. No Met was added to the media in which inoccula were raised. All other conditions for the culture of the algae were the same as reported previously.[12]

Separation of the Sterols. The free sterols were isolated as reported previously.[12] They were acetylated and the acetates were fractionated by argentic silica gel TLC (hexane–benzene 3:2, two developments). Usually three bands showed up under long-wave UV light. Four fractions were collected: fraction 1 corresponded to the band with the highest R_f value, fraction 2 to the band with intermediate R_f value, fraction 3 to the band with the lowest R_f value. The silica gel/$CaSO_4$ from part of the plate between 0.4 cm from the origin and the band with the lowest R_f value was also extracted and afforded fraction 4. Sometimes only two bands showed up under long-wave UV light. If that happened the band with the highest R_f value was divided into three equal zones. After extraction, the two zones with the highest R_f value yielded fraction 1 and the zone with the lowest R_f value yielded fraction 2. The fractions were saponified and the free sterols from each fraction were separated by reverse-phase HPLC. Details of an experiment resulting in the isolation of 30 sterols follow.

Available was ca. 300 mg of a sterol mixture isolated from algae cultured in four batches of 200 L each. Yields (after saponification) of the fractions of argentic TLC (9 plates of 20 × 20 cm):[50] fraction 1 76.2 mg, fraction 2 43.0 mg, fraction 3 142.9 mg, and fraction 4 26.8 mg.

Fraction 1 was injected in HPLC (MeOH, flow 3.5 mL/min, 6.5 mg of sterols/injection, injection volume 3 mL). Seven fractions (a–g) were collected corresponding to each of the six peaks and to a shoulder (e) at the foot of the main peak. Fraction number, retention time (min), relative area (%) (composition): a, 50.5, 4.7 (brassicasterol (**A3**)); b, 54.3, 2.5 (cholesterol (**A2**)); c, 58.8, 12.3 (stigmasterol (**A13**)); d, 61.5, 31.6 (24(*S*)-methylcholesterol (**A10**)); e, 65.1, 0.5 (cyclopropyl sterols (**A25** and **A23** (or **A24**))); f, 67.4, 46.1 (24-ethylcholesterol (**A19**, **A20**)); g, 73.7, 2.3 (24-propylcholesterol (**A32**)). The mixture of cyclopropyl sterols, on reinjection (MeOH) gave only a broad peak, but the mixture could be separated using solvent mixture B. The cyclopropyl sterol with the angular methyl group at highest field (**A23** (or **A24**), Table I) eluted faster (retention time (RT) 54.0 min) than its isomer **A25** (RT 54.7 min) (flow 3.5 mL/min).

Fraction 2 was injected in HPLC (MeOH, flow 3.5 mL/min, 10.8 mg of sterols/injection, injection volume 5 mL). The HPLC trace showed four peaks (a–d). Number, retention time (min), relative area (%) (main component(s)): a, 44.7, 68 (brassicasterol (**A3**)); b, 50.9, 14 (stigmasterol (**A13**) and E-24-propylidenecholesterol (**A21**)); c, 53.0, 11 (24(*S*)-methylcholesterol (**A10**)); d, 57.9, 7 (24-ethylcholesterol (**A19**, **A20**)). The tail fraction of peak a (RT 45.7–48.8 min, main components **A3** and **A2**) was reinjected (mixture B, flow 3.5 mL/min) and afforded brassicasterol (**A3**), cholesterol (**A2**), and impure 24-isopropenylcholesterol (**A30**) with RT 36.15, 38.4, and 39.3 min, respectively. The last compound was further purified by reinjection in MeOH. The front part of peak b (RT 48.8–50.4 min, main component **A21**) was reinjected and five subfractions were collected consisting of brassicasterol (**A3**, RT 36.3 min), an unidentified mixture (RT 39.3 min), (*E*)-24-propylidenecholesterol (**A21**, RT 39.8 min), a mixture of **A21** and 24-isopropyl-5,23-cholestadien-3β-ol (**A28**, RT 40.5 min), and stigmasterol (**A13**, RT 41.9 min). An NMR sample of the new sterol (**A28**) was obtained by reinjection of the corresponding subfraction (same conditions). The main part of peak c (RT 52.0–54.3 min, main component **A10**) was reinjected (mixture B, flow 3.5 mL/min) and four subfractions were collected consisting of an unidentified mixture (RT 37.8–43.2 min), a complicated mixture from which 24,26,26-trimethyl-23-dehydrocholesterol (**A33**) was isolated (vide infra) (RT 43.7 min), stigmasterol (**A12**, RT 44.7 min), and 24(*S*)-methylcholesterol (**A10**, RT 45.9 min). To obtain an NMR sample of the new sterol **A33** the corresponding subfraction was reinjected (mixture C, flow 3.5 mL/min). The HPLC trace showed two peaks. The peak with the shorter RT (47.4 min) corresponded to an inseparable mixture; the other peak (RT 48.9 min) was caused by the new sterol **A33**. The tail fraction of peak c (RT 54.3–57.7 min) was reinjected (mixture B, flow 3.5 mL/min). The first main peak corresponded to 24-isopropylidenecholesterol (**A29**, RT 42.2 min) and the second main peak to 24(*S*)-methylcholesterol (**A10**, RT 44.1 min).

Fraction 3 was injected in HPLC (mixture B, flow 3.5 mL/min, 7.1 mg of mixture/injection, injection volume 2 mL). The HPLC trace showed two peaks. Three fractions (a–c) were collected (the first peak was cut at a shoulder in the upward slope). Fraction number, retention time (min), relative area (%) (components): a, 35.4, 1 (clerosterol (**A18**)); b, 38.5, 10 (brassicasterol (**A3**), fucosterol (**A15**), isofucosterol (**A14**)); c, 40.3 89 ((*E*)-24-propylidenecholesterol (**A21**)). Because we wanted to determine the fucosterol/isofucosterol ratio by NMR, brassicasterol had to be removed from the mixture. This was done by reinjection of fraction b in MeOH (flow 3.5 mL/min). Brassicasterol had RT 53.4 min and fucosterol/isofucosterol had RT 57.0 min.

Fraction 4 was injected in HPLC (MeOH, flow 3.5 mL/min, 5.4 mg of mixture/injection, injection volume 2 mL). The HPLC trace showed nine peaks, ten subfractions were collected (the second peak was cut at a shoulder in the upward slope). Fraction number, retention time (min), relative area (%): a, 35.5, 4.0; b, 40.5, 1.9; c, 41.3, 9.7; d, 44.4, 1.7; e, 45.8, 3.7; f, 47.2, 2.9; g, 49.0, 50.3; h, 51.4, 14.1, i, 53.0, 7.1; j, 54.6, 4.6. Five of the subfractions did not need any further purification. They were fraction c (24-methylenecholesterol (**A7**)), fraction e (clerosterol (**A18**)), fraction g (isofucosterol (**A14**)), fraction h ((*E*)-24-propylidenecholesterol (**A2**)), and fraction j (24-methyl-24-vinylcholesterol (**A27**)). Fraction a was reinjected (MeOH) and the shoulder was cut. After reinjection of the subfraction corresponding to the first part of the peak (mixture C) an NMR sample of 24-methylene-8(14)-dehydrocholestanol (**C7**) was obtained. The subfraction corresponding to the rest of the peak was reinjected (mixture B, flow 3.5 mL/min) and the main component, 14α-methyl-5α-

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ergosta-8,24(28)-dien-3β-ol (**L7**, RT 31.5 min), and a minor component, E-14α-methyl-5α-ergosta-8,23-dien-3β-ol (**L5**, RT 34.2 min), were obtained pure. Fraction b had two components which were inseparable in MeOH, but which could be separated by using mixture B (flow 3.5 mL/min). 5α-Ergosta-7,24(28)-dien-3β-ol (**B7**) had RT 29.7 min and codisterol (**A9**) had RT 30.3 min. Obtusifoliol (**M7**), the main component of fraction d, was purified by reinjection in MeOH. The two main components of fraction e were inseparable in MeOH, but they were easily separated by using mixture B (flow 3.5 mL/min). 24(*S*)-5α-Ergosta-8,15-dien-3β-ol (**E10**) had RT 33.0 min and 24(*S*)-5,7-ergostadien-3β-ol (**D10**) 36.0 min. Fraction i was reinjected in MeOH (to remove material from fractions h and j). The two components were then separated by using mixture C (flow 3.0 mL/min). The major component was (25ξ)-26,26-dimethyl-5,24(28)-ergostadien-3β-ol (**A34**, RT 48.0 min) and the minor component was (*Z*)-24-propylidenecholesterol (**A22**, RT 49.1 min).

(*E*)-24-Propylidenecholesterol (A21): ^{13}C NMR (XL-200, $CDCl_3$) δ (assignment) 145.40 (C24), 140.73 (C5), 123.78 (C28), 121.66 (C6), 71.74 (C3), 56.71 (C14), 55.73 (C17), 50.08 (C9), 42.30 (C13), 42.25 (C4), 39.72 (C16), 37.23 (C1), 36.47 (C10), 36.41 (C25), 35.80 (C20), 34.56 (C22), 31.87 (C7 + C8), 31.61 (C2), 28.22 (C12), 25.97 (C23), 24.30 (C15), 22.30 (C26), 22.18 (C27), 21.05 (C11), 20.92 (C29), 19.38 (C19), 18.73 (C21), 14.75 (C30), 11.82 (C18).

(*Z*)-24-Propylidene-5-cholesten-3β-ol (A22) was first isolated by Idler et al.[51] ^1H NMR (300 MHz) ($CDCl_3$) δ (multiplicity, splitting constant(s) in Hz, assignment) 5.35 (m, C6–H), 5.005 (dd, 7, 7, C28–H), 3.54 (m, C3–H), 2.799 (m, C25–H), 1.009 (s, C19–H), 0.972 [6 H] (d, 7.0, C26–H and C27–H), 0.949 (d, 6.9, C21–H), 0.937 (t, 6.5, C30–H), 0.682 (s, C18–H); ^1H NMR (300 MHz) (C_6D_6) 5.35 (m, C6–H), 5.24 (dd, 7, 7, C28–H), 3.39 (m, C3–H), 2.854 (m, C25–H), 1.059 [6 H] (d, 6.8, C26–H and C27–H), 1.039 (d, 6.6, C21–H), 0.999 (t, 7.6, C29–H), 0.941 (s, C19–H), 0.661 (s, C18–H).

(24ξ)-24-Methyl-5,28-stigmastadien-3β-ol (A27): GC/MS 70eV, *m/z* (relative intensity) 426 (M^+, 72), 411 (17), 408 (16), 393 (13), 383 (14), 365 (23), 341 (11), 328 (47), 314 (50), 313 (4), 310 (4), 299 (28), 296 (6), 281 (14), 273 (13), 271 (56), 255 (23), 253 (9), 245 (6), 241 (10), 239 (5), 231 (17), 229 (16), 227 (8), 215 (8), 213 (22), 211 (8), 55 (100).

24-Isopropyl-5,23-cholestadien-3β-ol (A28). ^1H NMR (300 MHz) (C_6D_6) δ (multiplicity, splitting constant(s) in Hz, assignment) 5.35 (m, C6–H), 5.30 (dd, 8.7, 6.5, C23–H), 1.114 [6 H] and 1.046 [6 H] (d's, 6.8 and 7.0, C26–H, C27–H, C29–H, C30–H), 1.038 (d, 6.6, C21–H), 0.937 (s, C19–H), 0.671 (s, C18–H); GC/MS 70eV, *m/e* (relative intensity) 426 (M^+, 18), 411 (2), 393 (2), 365 (1), 314 (18), 301 (5), 300 (7), 299 (11), 296 (1), 283 (21), 281 (3), 272 (49), 271 (44), 270 (28), 255 (4), 253 (3), 241 (5), 239 (2), 231 (2), 229 (4), 227 (3), 215 (12), 213 (6), 69 (100), 55 (93). To prove the assigned structure **A29** (in EtOAc) was stirred with PtO_2 under hydrogen for a full day. The two reaction products were separated by reverse-phase HPLC (Altex, MeOH). 24-Isopropyl-5α-cholest-23-en-3β-ol (**F28**) had RRT 1.18 in HPLC and 24-isopropyl-5α-cholestan-3β-ol (**F31**) had RRT 1.60. **F28**: ^1H NMR (300 MHz) (C_6D_6) (because of the small sample size (~50 μg) the olefinic proton and the methine proton could not be resolved from the noisy baseline) δ (multiplicity, splitting constant(s) in Hz, assignment) 1.116 [6 H] and 1.043 [6 H] (d's, 6.8 and 7.0, C26–H, C27–H, C29–H, C30–H), 1.043 (d, 7.0, C21–H), 0.698 (s, C19–H), 0.663 (s, C18–H). **F31**: ^1H NMR (300 MHz) (C_6D_6) 1.056 (d, 6.6, C21–H), 0.943 [6 H] and 0.926 and 0.911 (d's, 6.7 and 6.5 and 6.8, C26–H, C27–H, C29–H, C30–H), 0.705 (s, C19–H), 0.665 (s, C18–H).

24-Isopropyl-5,28-cholestadien-3β-ol (A29): GC/MS 70 eV, *m/z* (relative intensity) 426 (M^+, 30), 411 (2), 408 (1), 393 (2), 314 (100), 299 (21), 296 (11), 281 (25), 271 (17), 255 (4), 253 (5), 231 (8), 229 (25), 213 (10), 211 (10), 69 (74), 55 (59); ^1H NMR (300 MHz) (C_6D_6) δ (multiplicity, splitting constant(s) in Hz, assignment) 5.35 (m, C6–H), 3.4 (m, C3–H), 2.925 (m, C28–H), 1.726 and 1.679 (s's, C26–H, C27–H), 1.085 (d, 6.5, C21–H), 1.059 [6 H] (d, 7.0, C29–H, C30–H). Irradiation of the methine proton at δ 2.9 collapsed the equivalent methyl doublets at δ 1.059.

24-Isopropyl-5,25-cholestadien-3β-ol (A30): ^1H NMR (300 MHz) (C_6D_6) 5.35 (m, C6–H), 4.886 and 4.789 (s's, C26–H), 3.4 (m, C3–H), 1.575 (s, C27–H), 1.017 (d, 6.6, C21–H), 0.953 and 0.898 (d's, 6 and 6.5, C29–H, C30–H), 0.943 (s, C19–H), 0.681 (s, C18–H).

(25ξ)-26,26-Dimethyl-5,23-ergostadien-3β-ol (A33): GC/MS (70 eV), *m/z* (relative intensity) 426 (M^+, 10), 411 (2), 408 (4), 393 (1), 383 (1), 314 (6), 301 (4), 300 (7), 299 (7), 296 (1), 283 (21), 272 (24), 271 (50), 270 (25), 255 (4), 253 (6), 241 (4), 239 (2), 231 (2), 229 (3), 227 (3), 215 (11), 213 (8), 81 (84), 69 (100), 55 (88).

(25ξ)-26,26-Dimethyl-5,24(28)-ergostadien-3β-ol (A34): GC/MS (70 eV), *m/z* (relative intensity) 426 (M^+, 17), 411 (5), 393 (2), 314 (100), 299 (27), 296 (11), 281 (24), 271 (33), 255 (6), 253 (7), 245 (3), 243 (2), 241 (3), 239 (3), 231 (8), 229 (25), 215 (6), 213 (13), 211 (10), 81 (33), 69 (47), 55 (47).

(24*S*)-5α-Ergosta-8(14),15-dien-3β-ol (E10): ^1H NMR (300 MHz) (C_6D_6) δ (multiplicity, splitting constant(s) in Hz, assignment 6.26 (dd, 10, 3, C16–H), 5.30 (d, 10, C15–H), 1.008 (d, 6.5, C21–H), 0.916 (d, 6.7, C26–H), 0.914 (s, C18–H), 0.864 (d, 6.9, C28–H), 0.849 (d, 6.7, C27–H), 0.693 (s, C19–H). The compound decomposed before a mass spectrum was recorded.

(*E*)-14α-Methyl-5α-ergosta-8,23-dien-3β-ol (L5): ^1H NMR (360 MHz) (C_6D_6) 5.35 (dd, 6.5, 6.5, C23–H), 1.613 (s, C28–H), 1.047 [9 H] (d's, 7, C21–H, C26–H, C27–H), 0.977 (s, C19–H), 0.884 (s, C14–Me), 0.799 (s, C18–H); GC/MS (70 eV), *m/z* (relative intensity) 412 (M^+, 38), 397 (100), 379 (5), 369 (11), 351 (1), 313 (5), 299 (6), 285 (4), 283 (2), 281 (2), 273 (4), 271 (6), 255 (4), 253 (1), 245 (5), 231 (14), 229 (2), 227 (4), 219 (10), 217 (4), 215 (3), 213 (5), 97 (64), 69 (85), 55 (77).

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