Evolutionarily conserved $\Delta^{25(27)}$-olefin ergosterol biosynthesis pathway in the alga *Chlamydomonas reinhardtii*

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Abstract **Ergosterol is the predominant sterol of fungi and green algae. Although the biosynthetic pathway for sterol synthesis in fungi is well established and is known to use C24-methylation-C24 (28)-reduction ($\Delta^{24(28)}$-olefin pathway) steps, little is known about the sterol pathway in green algae. Previous work has raised the possibility that these algae might use a novel pathway because the green alga *Chlamydomonas reinhardtii* was shown to possess a mevalonate-independent methylerythritol 4-phosphate not present in fungi. Here, we report that *C. reinhardtii* synthesizes the protosterol cycloartenol and converts it to ergosterol (C24β-methyl) and 7-dehydroporiferasterol (C24β-ethyl) through a highly conserved sterol C24- methylation-C25-reduction ($\Delta^{25(27)}$-olefin) pathway that is distinct from the well-described acetate-mevalonate pathway to fungal lanosterol and its conversion to ergosterol by the $\Delta^{24(28)}$-olefin pathway. We isolated and characterized 23 sterols by a combination of GC-MS and proton nuclear magnetic resonance spectroscopy analysis from a set of mutant, wild-type, and 25-thialanosterol-treated cells. The structure and stereochemistry of the final C24-alkyl sterol side chains possessed different combinations of 24β-methyl/ethyl groups and $\Delta^{22(23)}$*E* and $\Delta^{25(27)}$-double bond constructions. When incubated with [methyl-$^2H_3$]methionine, cells incorporated three (into ergosterol) or five (into 7-dehydroporiferasterol) deuterium atoms into the newly biosynthesized 24β-alkyl sterols, consistent only with a $\Delta^{25(27)}$-olefin pathway. Thus, our findings demonstrate that two separate isoprenoid-24-alkyl sterol pathways evolved in fungi and green algae, both of which converge to yield a common membrane insert ergosterol.**—Miller, M. B., B. A. Haubrich, Q. Wang, W. J. Snell, and W. D. Nes. **Evolutionarily conserved $\Delta^{25(27)}$-olefin ergosterol biosynthesis pathway in the alga *Chlamydomonas reinhardtii*. *J. Lipid Res.* 2012.** 53: **1636–1645.**

Supplementary key words green algae • sterol evolution • cycloartenol • membranes • sterol C24-methyl transferase • sterol C25-reductase

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The defining feature of eukaryote membranes, other than animals that possess the C_{27} cholesterol, is the presence of C_{28}- to C_{30}-steroidal compounds of varied side-chain constructions characterized by a C24-alkyl group. The addition of C_1 to C_3 side chains is derived by transmethylation reactions requiring S-adenosyl-L-methionine as the methyl donor and catalyzed by the sterol C24-methyltransferase (24-SMT) family of enzymes (1, 2). Using differences in the C24-alkyl group size, stereochemistry, and complexity in further transalkylations at C22, C23, C27, and C28 as taxonomic traits, and linking them to steroidogenesis, we are able to reason the grouping of 24-alkyl sterol- containing organisms into more or less primitive and advanced forms of life (3). Fossil steranes identified from their diagenetic remains in sedimentary rocks confirm the evolution of 24-alkyl sterol diversity in Eukarya noted in the chemotaxonomy studies and further suggest an ancient origin (< 2.7 billion years ago) of the sterol frame (4–6).

In accordance with the shifting geneome-metabolome congruence in sterol evolution, three general pathways of isoprenoid-sterol biosynthesis are often considered: one in animals that yields cholesterol (C24-H), one in fungi that yields ergosterol (C24β-methyl), and one in land plants that yields stigmasterol (C24α-ethyl) (7–9). These compounds can be assembled modularly in three stages: In module I, synthesis of the basic C_5-unit, isopentenyl diphosphate (Δ^3-IPP) from glucose, can originate from two independent and nonhomologous metabolic pathways, namely the acetate-mevalonate or the mevalonate-independent 2-C-methyl-D-erythritol 4-phosphate pathways (**Fig. 1**) (1, 10–12). In module II, Δ^3-IPP is converted to the protosterols, lanosterol (nonphotosynthetic lineage), or cycloartenol (photosynthetic lineage) (2, 3, 13, 14). In module III, protosterols are converted to Δ^5-sterols via a C24-reduction or coupled C24-alkylation-reduction pathway that yields the exquisite 24-alkyl sterol patterns observed throughout nature (1, 7).

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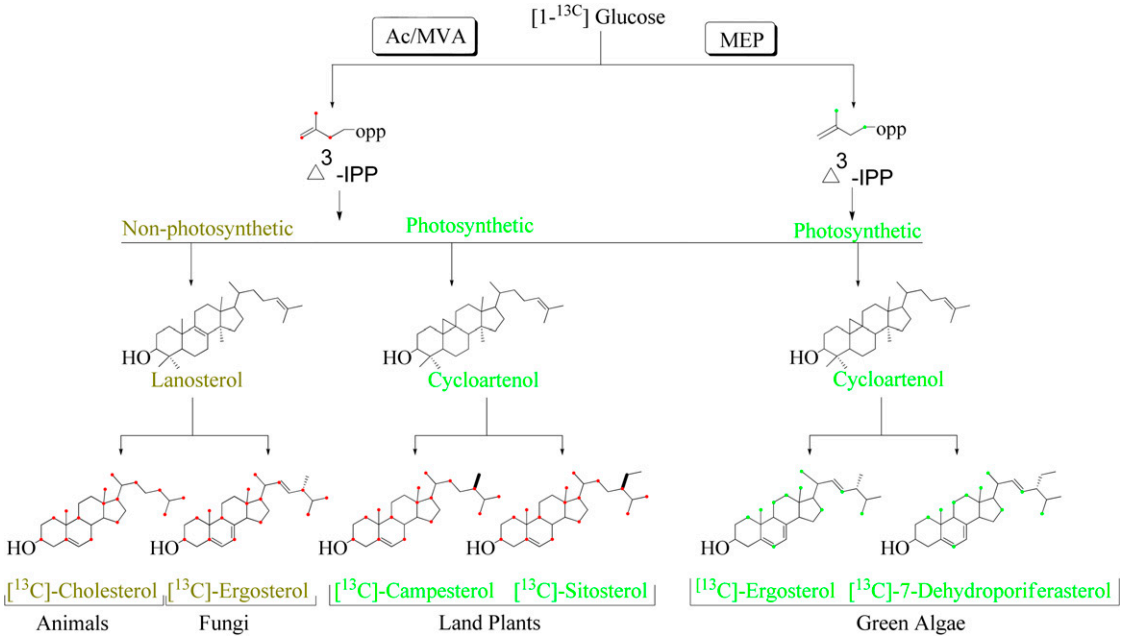

Fig. 1. Pattern of ^{13}C-incorporation into isopentenyl diphosphate and sterols from incubation of [1-^{13}C]glucose. Ac/MVA, acetate-mevalonate pathway; MEP, mevalonate-independent methylerythritol 4-phosphate pathway.

A striking finding to emerge from in vivo isotopically labeling, sterol biosynthesis inhibitor-treatments, and cell-free 24-SMT enzyme studies among phylogenetically diverse algae that include the green, brown, and golden brown algae (15–18) that is not necessarily obvious by current bioinformatic analyses (19) is that ergosterol biosynthesis in the green algae may use a different set of enzymatic reactions in module III from fungi. A crucial

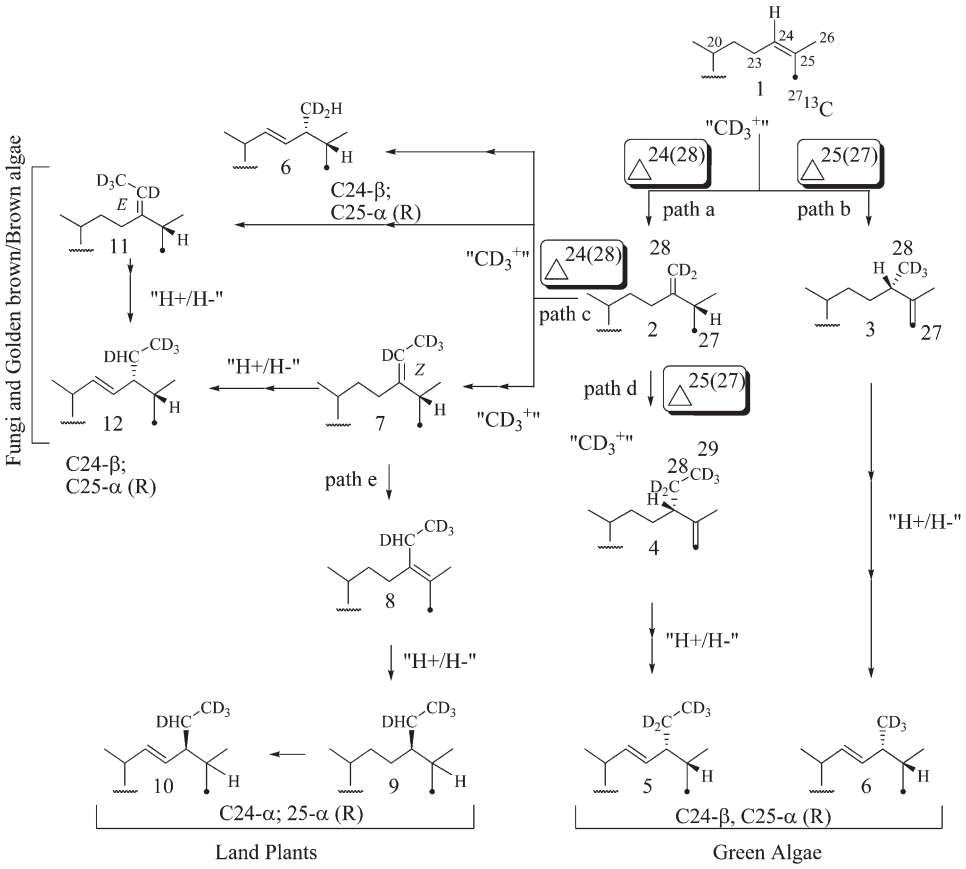

Fig. 2. Sterol C24-alkylation-reduction pathways to 24-alkyl sterol products. The incorporation sites at C24 from incubation with [methyl-$^{2}H_3$]methionine are shown; ^{13}C27 label is shown as a dot.

difference between ergosterol biosynthesis in these organisms involves the sterol C24-methyl transferase activities and side-chain reductase specificities involved in generating the final ergosterol side chain. Thus, green algae express a 24-SMT that recognizes cycloartenol (16) and a sterol C25(27)-reductase (25-SR) of unknown substrate preference that operate in tandem to introduce stereoselectively the methyl cation at C24 and the hydride ion at C25 from S-adenosyl-L-methionine and NADPH, respectively (**Fig. 2**). Alternatively, fungi synthesize a 24-SMT that prefers substrates zymosterol or lanosterol, depending on the organism, and a sterol C24(28)-reductase the prefers ergosta-5,7,24(28)-trienol as substrate (1). In both sterol C-methylation-reduction pathways, the resulting ergosterol contains the same stereochemistry at C24 and C25 (1).

Therefore, it was surprising that several investigations on sterol biosynthesis in the green alga *Chlamydomonas reinhardtii* reported that 24-alkyl(idene) sterol biosynthesis followed the fungal $\Delta^{24(28)}$-olefin pathway (20, 21). To deduce generalities for a $\Delta^{25(27)}$-olefin pathway in the synthesis of algal ergosterol and its 24-ethyl homolog, we have examined the ability of *C. reinhardtii* to synthesize sterol intermediates in the presence and absence of an inhibitor of the sterol C24-methylation reaction and after genetic manipulation to induce intermediates to accumulate in the cell. In a painstaking analysis of the minor and trace compounds of mutant and treated cells, 23 different sterols were detected, many of which contained a $\Delta^{25(27)}$ bond consistent with a $\Delta^{25(27)}$-olefin pathway to ergosterol. Moreover, the sterol profiles of these cells failed to show C24(28)-ethylidene derivatives required in the synthesis of 24β-ethyl(idene) sterols synthesized in golden brown or brown algae or which can serve as precursor of land plant sitosterol (22, 23). Analysis of isotopically labeled ergosterol and 7-dehydroporiferasterol isolated from *C. reinhar*dtii grown in the presence of [methyl-$^2H_3$]methionine provided further verification for the $\Delta^{25(27)}$-olefin pathway to $\Delta^{5,7}$-C24β-alkyl sterols, which ultimately become the architectural components of algal cell membranes.

MATERIALS AND METHODS

C. reinhardtii strains and culture conditions

C. reinhardtii wild-type strains 21gr (mt+; CC-1690 and 6145c; CC-1691) and ergosterol mutants KD7 and KD21 (24), obtained from the Chlamydomonas Genetics Center, Duke University (Durham, NC), were grown at 23°C on a 13:11 h light:dark cycle with aeration in medium I or medium II of Sager and Granick (25) as previously described (26). The KD7PY mutant was a product of a cross between 6145c and KD7 and was selected due its its ability to grow on agar plates in medium I containing nystatin (2 mM). For the inhibitor studies, cells (1×10^6/ml) were cultured for 3 days in medium I containing 1 μM 25-thialanosterol iodide salt. Cell number was determined using a hemocytometer.

Source and analysis of sterols

Sterol analysis was performed as described previously (27). Briefly, algal cells at approximately 1×10^7 cells/ml were harvested by centrifugation and saponified in 10% aqueous methanolic KOH (10% w/v) at reflux for 30 min to give hexane-soluble neutral lipids. The neutral lipids were routinely examined by GC-MS (30 m HP-5 capillary column coupled to a HP 6890 gas chromatograph interfaced to a 5973 mass spectrometer at 70 eV; GC flow rate of He was set at 1.2 ml/min, injector port was 250°C, and the initial temperature was set at 170°C, held for 1 min, and increased at 20°C/min to 280°C) and HPLC equipped with a photodiode array detector used to provide UV spectra relevant to double bond character in the molecule. In several cases, sterols isolated from the nonsaponifiable lipid fraction and purified by HPLC (analytical Phenomenex Luna column, ODS-100A, eluted with methanol at 20°C at 1 ml/min or analytical TOSOHAAS TSK gel column, ODS-120A with acetonitrile/isopropanol [65/35, v/v] at 35°C at 1 ml/min) were examined by proton nuclear magnetic resonance spectroscopy (^{1}HNMR) (spectra measured in deuterochloroform solutions on a Varian Unity Inova 500 MHz spectrometer with the chemical shifts referenced to chloroform resonating at 7.265 ppm and reported as δ in ppm, ppm) to confirm structure and stereochemistry of the side-chain C24-alkyl group. Authentic reference specimens for comparative GC-MS and ^{1}HNMR analyses are taken from our sterol collection reported in references 27–31 and from literature values (32). Sterols are referenced to the retention time of cholesterol in capillary GC at 13.8 min (old column) or 14.5 min (new column) and

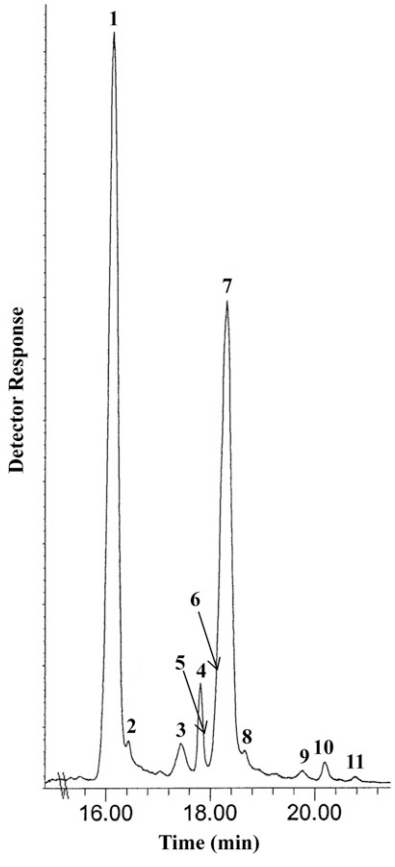

Fig. 3. Gas-liquid chromatograph separation of sterols isolated from wild-type *C. reinhardtii*. Peaks in the total ion current (TIC) chromatogram correspond to peak 1, ergosta-5,7,22-trienol (ergosterol); peak 2, ergosta-8,25(27)-dienol; peak 3, ergosta-7,25(27)-dienol; peak 4, ergost-7-enol; peak 5, 4α,14α- dimethylergosta-8,25(27)-dienol; peak 6, 4α,14α-dimethylergosta-8,24(28)- dienol; peak 7, porifersta-5,7,22-trienol (7-dehydroporifersterol); peak 8, porifersta-8,25(27)-dienol; peak 9, porifersta-7,25(27)-dienol; peak 10, poriferst-7-enol; and peak 11, cycloart-24(25)-enol (cycloartenol).

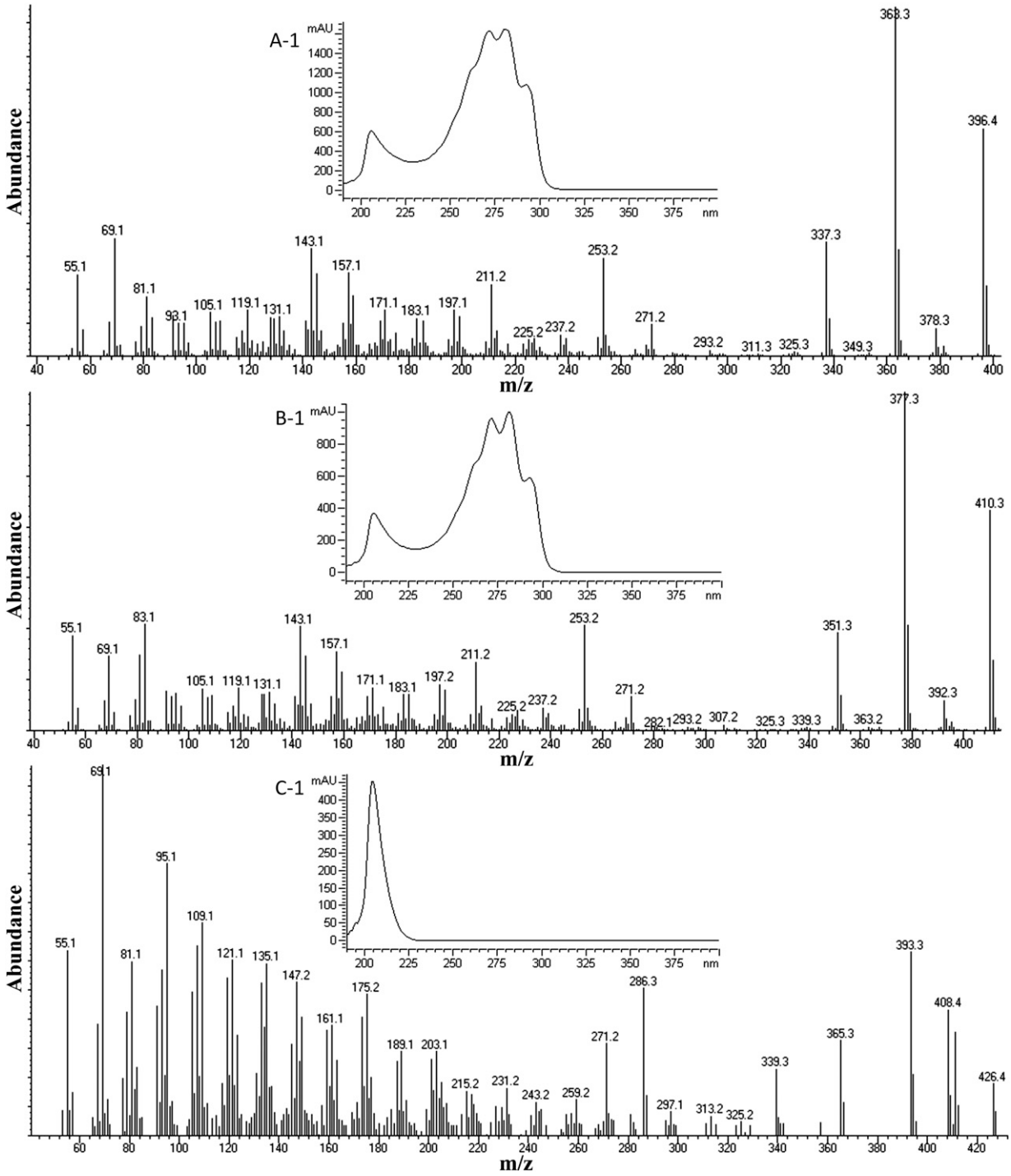

Fig. 4. Mass spectra and UV spectra (inset) for select sterols from control *C. reinhardtii* cells analyzed by GC-MS shown in Figure 1. A: GC peak 1. B: Peak 7. C: Peak 11.

in HPLC at 16.5 min (Luna column) or 26.8 min (TSK gel) affording the relative retention times to cholesterol in GC as the RRTc or in HPLC as the α_c values.

Feeding of [methyl-$^2H_3$]methionine to *C. reinhardtii*

L-[methyl-$^2H_3$]methionine (98 atom % of ^2H) (Sigma, St. Louis) was administered to wild-type *C. reinhardtii* cultures inoculated with 1×10^7 cells/ml at 1 mg/ml. After 3 days inoculation under light, the cells were harvested by centrifugation, and the total sterols from the cultures were examined by GC-MS.

RESULTS AND DISCUSSION

Total sterols of wild-type strain

When this work was undertaken, little information was available on the sterol composition of Chlamydomonas. Several recent reports documented that this alga synthesizes two major sterol products, ergosterol and 7-dehydroporiferasterol, and variably a minor compound, ergost-7-enol (20, 21, 33–35). Moreover, [3-^{3}H]squalene-2,3-oxide incubated

TABLE 1. Chromatographic and spectral properties of sterols from *Chlamydomonas reinhardtii*

Systematic Name	Structure[a]	GC (RRTc)	UV (λmax)	MW (M^+)
Cycloart-24(25)-enol	1	1.43	EA	426
24β-Methyl cycloart-25(27)-enol	2	1.57	EA	440
Cycloart-24(28)-enol	3	1.59	EA	440
4α, 14α-Dimethyl ergosta-8,25(27)-dienol	4	1.23	EA	426
4α, 14α-Dimethyl ergosta-8,24(28)-dienol	5	1.25	EA	426
Ergosta-8,25(27)-dienol	6	1.15	EA	398
4α-,14α-Dimethyl porifersta-8,25(27)-dienol	7	1.40	EA	440
Ergosta-7,25(27)-dienol	8	1.17	EA	398
Porifersta-8,25(27)-dienol	9	1.29	EA	412
Ergost-7-enol	10	1.23	EA	400
Porifersta-7,25(27)-dienol	11	1.34	EA	412
Ergosta-5,7-dienol	12	1.2	282	398
Poriferst-7-enol	13	1.39	EA	414
Ergosta-5,7,22-trienol	14	1.10	282	396
Poriferata-5,7-dienol	15	1.35	282	412
Porifersta-5,7,22-trienol	16	1.28	282	410
Lanosta-8,24-dienol [b]	17	1.33	EA	426
4α-,14α-Dimethyl cholesta-8,24-dienol	18	1.15	EA	412
14α-Methyl cholesta-8,24-dienol	19	1.07	EA	398
Cholesta-7,24-dienol	20	1.12	EA	384
Ergosta-5,7,25(27)-trienol	21	1.18	282	396
Ergosta-5,7,22,25(27)-tetraenol	22	1.12	282	394
Porifersta-5,7,25(27)-trienol	23	1.33	282	410
Porifersta-5,7,22,25(27)-tetraenol	24	1.26	282	408

[a] Structures of sterols are shown in Fig. 7.
[b] Lanosterol was not detected in the cells and is given for reference purpose only.

in a microsomal enzyme preparation converts exclusively to cycloartenol (36). On the other hand, information is lacking about the types and amount of C4 methyl- or C24(28)-ethylidene intermediates involved in the sequence of chemicals to ergosterol or its 24-ethyl homolog from which a reliable sterol biosynthesis pathway could be constructed.

In the present study, the total sterol fraction of *C. reinhardtii* was analyzed by GC-MS, and 11 sterols were detected (**Fig. 3**), including ergosterol, 7-dehydroporiferasterol, and cycloartenol, which possessed mass and UV spectra similar to authentic specimens (**Fig. 4**). The major sterols in the sterol composition, ergosterol and 7-dehydroporiferasterol, were purified by HPLC, and ^{1}HNMR analysis confirmed their structure and C24β-methyl/ethyl group stereochemistry (supplementary Table I). Minor compounds detected in the GC chromatogram were identified according to GC

TABLE 2. Sterol composition of *Chlamydomonas reinhardtii* cells

Sterol[a]	WT[b]	KD7[c]	KD21[c]	25-TL[d]	KD7PY[c]
1	0.3		0.7	2.5	1.1
2					0.3
3					0.3
4	tr[e]	tr		tr	0.9
5	tr	tr	tr	tr	tr
6	2.7				
7					tr
8	2.6	14.9		8.1	21.4
9	1.6				
10	3.5		3	0.5	
11	0.5	6.4			10.6
12			21.3		
13	0.9		4.6		
14	50.8			36.6	
15			70.4		
16	37.2			27.5	1.5
17					
18				15.5	
19				0.5	
20				8.8	
21		22.6			21.5
22		19.4			12.7
23		1.7			2.9
24		35			26.8

[a] Structures of sterols are shown in Fig. 7.
[b] WT, wild-type cells.
[c] Mutant cell lines.
[d] 25TL, 25-thialanosterol salt treated cells.
[e] tr, trace amount of sterol at less than 0.3%; blank refers to no sterol detected in cells.

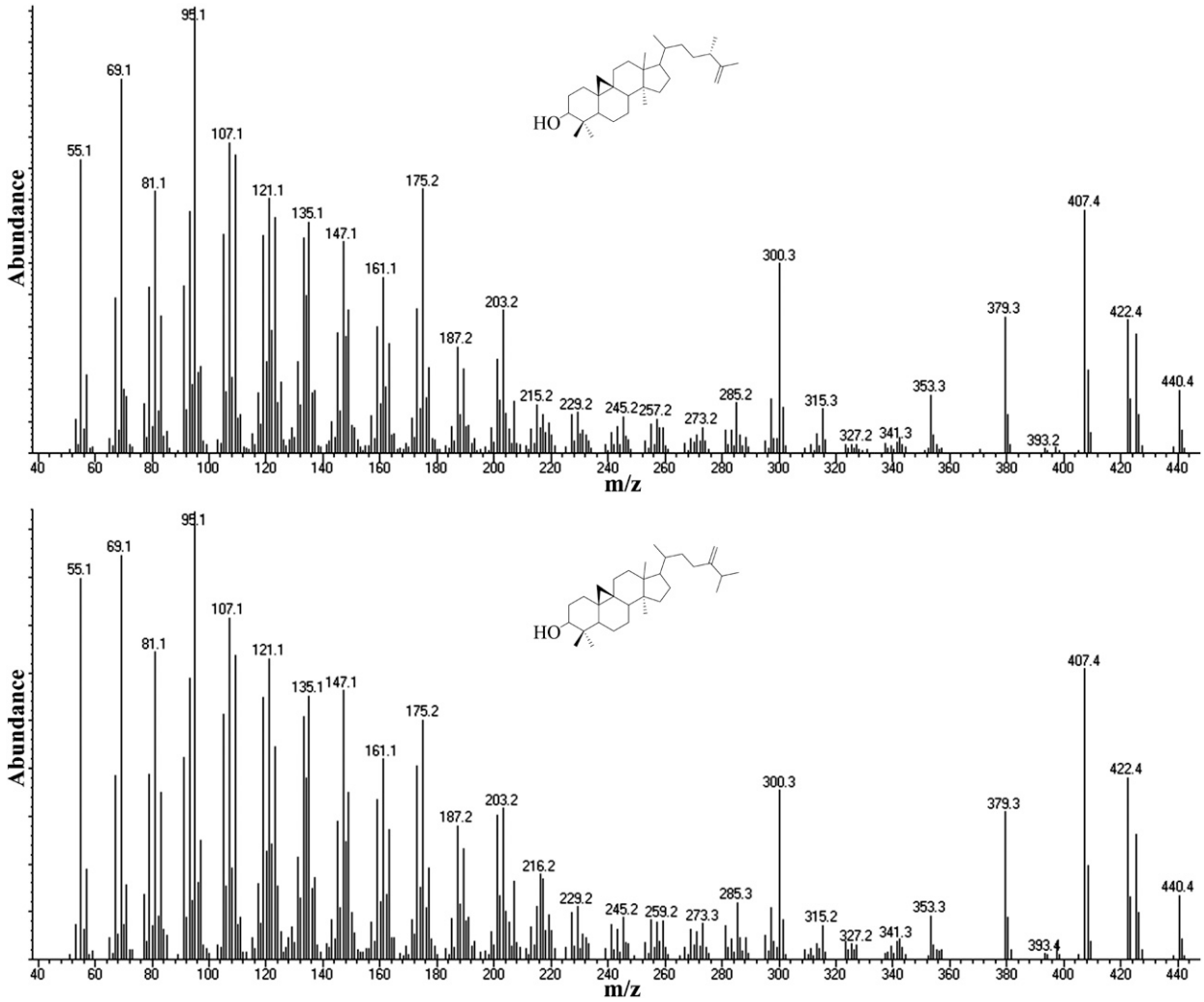

Fig. 5. Mass spectra of cyclolaudenol and 24(28)-methylenecycloartanol.

retention times and mass spectra relative to standards corresponding to cycloartenol, 4α,14α-dimethylergosta-8,25(27)-dienol, 4α,14α-dimethylergosta-8,24(28)-dienol (obtusifoliol) ergosta-7,25(27)-dienol, ergosta-8,25(27)-dienol, ergost-7-enol, poriferst-7,25(27)-dienol, poriferst-8,25(27)-dienol, and poriferst-7-enol (**Tables 1 and 2**). The finding of a set of C4-methyl intermediates, including cycloartenol, in the sterol composition of wild-type cells was significant (Fig. 4C) because it confirmed the "photosynthetic lineage" of sterol biosynthesis in this alga. Moreover, the natural occurrence of $\Delta^{25(27)}$-sterols in cells was consistent with ergosterol formation proceeding from a $\Delta^{25(27)}$-olefin pathway.

Induced accumulation of sterol intermediates

To generate a more robust sterol profile from which a sterol biosynthesis pathway for *C. reinhardtii* could be established, we next examined the sterol composition of cells engineered to produce modified sterol compositions using mutant strains generated previously by Bard et al. (24), one mutant created by us from the Bard strains, and mutants after inhibitor treatment of wild-type cells. The sterol composition of KD7 was examined first because this mutant strain was reported to accumulate six unconventional C_{28} and C_{29} $\Delta^{25(27)}$-sterol products, specifically a C_{28}-7,25(27)-diene, C_{28}-5,7,25(27)-triene, and C_{28}-5,7,22,25(27)-tetraene and the corresponding C_{29}-ethyl homologs (37); two of them, ergosta-7,25(27)-dienol and poriferst-7,25(27)-dienol, were detected in our analysis of the sterol composition of wild-type cells (Table 2), suggesting that the $\Delta^{25(27)}$-sterol pathway might be operational in *C. reinhardtii*. In our investigation of the sterols from KD7, we detected eight sterols, including the six reported by Bard et al. (24) and two minor C4-methyl sterols that were detected in the wild-type cells, 4α,14α-dimethylergosta-8,25(27)-dienol and obtusifoliol (Table 2). In similar fashion, we investigated the sterol composition of KD21 reported previously to synthesize C_{28}-7-ene, C_{28}-5,7-diene, C_{29}-7-ene, and C_{29}-5,7-diene sterols, and in our studies we detected the same major sterols as well as two minor sterols obtusifoliol and cycloartenol, which were detected in wild-type cells (Table 2). Using HPLC, we purified four sterols from KD7 and two sterols from KD21. The structures of these compounds were confirmed by ^{1}HNMR (supplementary Table I). Based on spectra of reference materials (31, 37, 38), the sterols from the different cell types possessed one or more functional groups of a Δ^{22}*E* double bond,

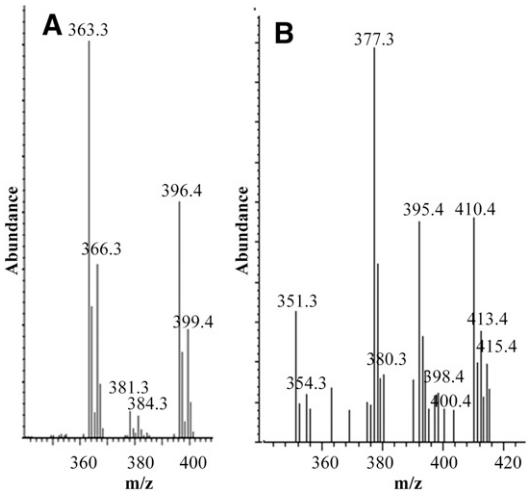

Fig. 6. Mass spectra of GC peaks corresponding to ergosterol (A) and 7-dehydroporiferasterol (B) from cells administered [methyl-$^2H_3$]methionine.

a C24β-methyl/ethyl stereochemistry, and $\Delta^{25(27)}$-double bond in the sterol side chain. In addition, the combination of UV, MS, and ^{1}HNMR analyses shows sterols from KD7 and KD21 to be populated by Δ^7 and $\Delta^{5,7}$ nuclei.

Analysis of a strain (KD7PY) derived by selection on nystatin of cells produced in a cross of KD7 and wild-type *C. reinhardtii* yielded 13 cometabolites, of which three were new C4-sterol intermediates not detected in wild-type, KD7, or KD21 cells. KD7PY cells accumulated trace amounts of cycloladenol and 24(28)-methylenecycloartanol along with minor amounts of 4α,14α-dimethylporifersta-8,25(27)-dienol (Table 2). The mass spectra of the structural isomers cycloladenol and 24(28)-methylenecycloartanol are almost identical to each other and, depending on the nature of the GC column, they coelute as we reported previously (13) (**Fig. 5**). Indeed, Bard et al. (24) also noted the coelution of $\Delta^{25(27)}$- and $\Delta^{24(28)}$-sterols in their investigation of KD7 sterols, making structure identification equivocal based on GC-MS analysis. Our use of capillary GC column allows us to separate these structural isomers such that the $\Delta^{25(27)}$-olefin elutes before the $\Delta^{24(28)}$-olefin by a retention factor $\Delta^{25(27)}/\Delta^{24(28)}$ of approximately 0.99 (Table 1); this chromatographic technique was developed from studies on 24-SMT action (37, 38).

A final incubation of *C. reinhardtii* with 25-thialanosterol salt, designed to block sterol C24-methyltransferase activity (27, 39), led to growth inhibition after progressive addition of 25-thialanosterol to the medium (1–10 μM). At 1 μM, 10 sterols were detected by GC-MS analysis (Table 2); three of them were previously unidentified in the other cells studied and were determined to be 4α,14α-dimethylcholesta-8,24-dienol (31-norlanosterol), 14α-methylcholesta-8,24-dienol (14α-methylzymosterol), and cholesta-7,24-dienol, all sterols lacking a C24-alkyl group in the side chain.

Incorporation of [methyl-$^2H_3$]methionine

The wild-type *C. reinhardtii* strain contains two major sterols, ergosterol and 7-dehydroporiferasterol. The corresponding mass spectra for these 24β-alkyl sterols in the high mass end revealed ions at M^+, M^+-CH_3, M^+-H_2O, and M^+-CH_3-H_2O. Relevant ions for ergosterol appeared at *m/-* 396, 381, 378, and 363 amu and for 7-dehydroporiferasterol at *m/z* 410, 395, 392, and 377 amu (Fig. 3). When the alga was administered [methyl-$^2H_3$]methionine, three deuterium atoms were incorporated into the side chain of ergosterol (M^+ *m/z* 399), and three or five atoms were present in the 7-dehydroporiferasterol (M^+ *m/z* 413 and 415) (**Fig. 6**). These experiments with [methyl-$^2H_3$]methionine established that the C24β-methyl/ethyl group in *C. reinhardtii* sterols is introduced via a $\Delta^{25(27)}$-olefin intermediate, analogous to 24β-methyl sterols in other green algae (Chlorella and Trebouxia; 22, 40) and in the nonphotosynthetic, Chlorella-like alga Prototheca (13, 41). Our results rule out the $\Delta^{24(28)}$-olefin pathway used in fungal ergosterol biosynthesis (39, 42) because, in the fungal pathway, the methyl groups in 24-methyl and 24-ethyl sterols incorporate two and four deuterium atoms, respectively (Fig. 2).

General consideration for ergosterol biosynthesis in *C. reinhardtii*

In all, 23 different sterols have been detected in *C. reinhardtii* vegetative cells, many of them $\Delta^{25(27)}$-sterols. In assembling the intermediates from the different cell types and treatments into a committed ergosterol pathway for the wild-type strain, several compounds were considered to be by-products. Thus, on the basis of our present understanding of biosynthetic relationships that posit that cycloartenol is a key branch point intermediate of phytosterol metabolism because it can serve as the $\Delta^{24(25)}$-protosterol precursor of $\Delta^{24(28)}$-sterols as well as $\Delta^{25(27)}$-sterols, the following pathway is suggested: cycloartenol → cycloladenol → ergosta-8,25(27)-dienol → ergost-7-enol → ergosta-5,7-dienol → C_{28} ergosterol (**Fig. 7**). The proposed cycloartenol-ergosterol sequence eliminates the typical intermediates in fungal ergosterol biosynthesis of zymosterol and fecosterol [ergosta-8,24(28)-dienol], which convert to ergost-7-enol through the action of a sterol 8-7 isomerase and sterol 24(28)-reductase enzyme (supplementary Fig. I1); a parallel pathway exists in the formation of C_{29} sterols, which requires the intermediacy of a $\Delta^{24(28)}$-derivative. In this case, the C_1-transfer product 24(28)-methylenecycloartanol converts to obtusifoliol, which can serve as substrate for the second C_1-transfer reaction catalyzed by 24-SMT to form 4α,14α-dimethylporifersta-8,25(27)-dienol. The latter sterol then converts to 7-dehydroporiferasterol (Fig. 7). Accumulation of 14α-methylzymosterol and 4α,14α-dimethylporifersta-8,25(27)-dienol, intermediates in mutant or treated cells, is consistent with complete demethylation at C4 occurring before C14-demethylation in the biosynthesis of 24-alkyl sterols in *C. reinhardti.* Although 24(28)-methylenelophenol is considered a substrate for $\Delta^{24(28)}$-ethylidene products and a branch point intermediate in the biosynthesis of 24-methyl and 24-ethyl sterols in plants (7, 43), the proposed removal of both C4 methyl groups before the elimination of the C14 methyl group rules out this compound as intermediate to 7-dehydroporiferasterol in *C. reinhardtii.*

In defining evolutionary characters, data from several sources (1, 2), including natural product profiling, bioinformatic

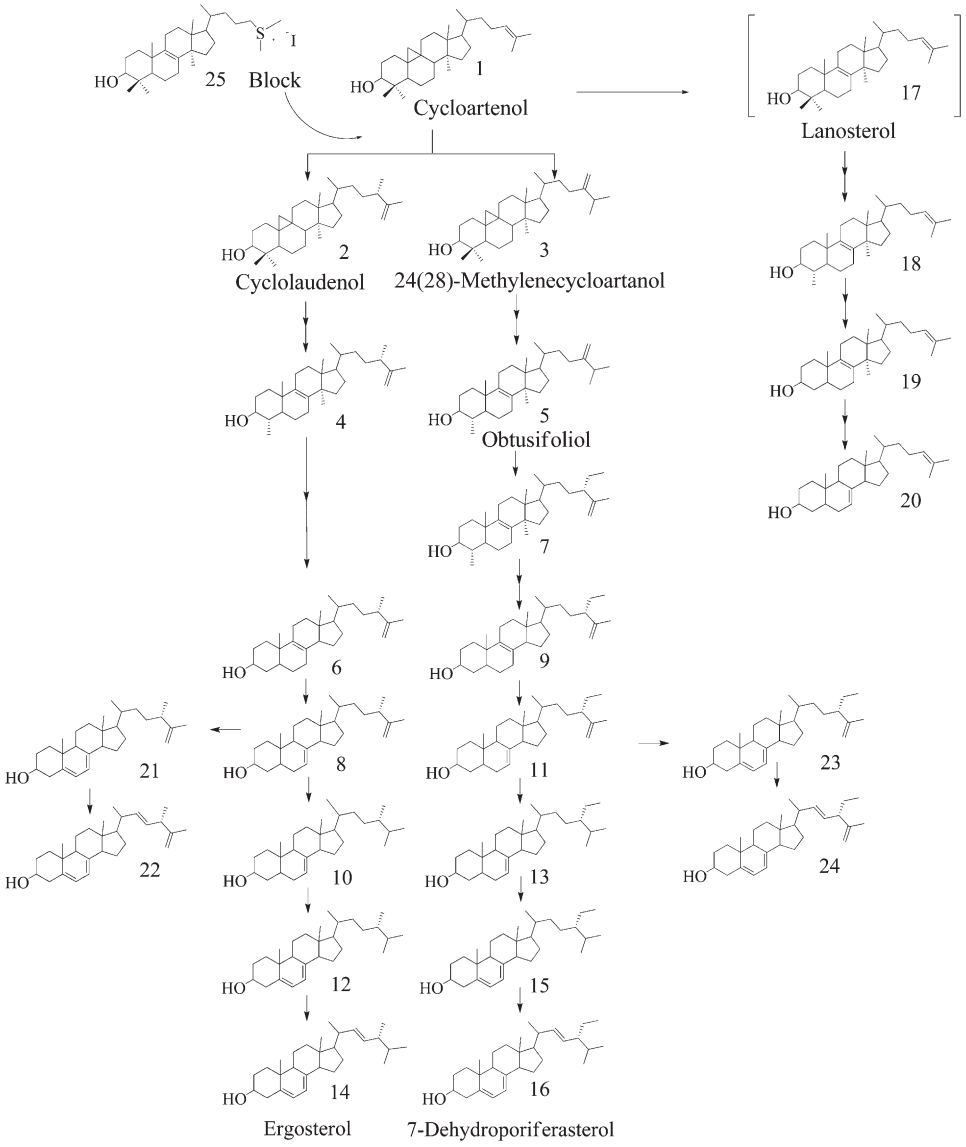

Fig. 7. Proposed sterol biosynthesis pathway from cycloartenol to ergosterol and 7-dehydroporiferasterol in *C. reinhardtii*; inhibition of sterol C24-methyltransferase action from incubation with 25-thialanosterol salt is shown. In brackets is shown lanosterol, which was not detected in cells but is presumed to be an intermediate to cholesta-7,24-dienol. Systematic naming of sterols are reported in Tables 1 and 2.

analyses, and isotopic labeling studies (15, 19), point toward the existence of a mevalonate-independent pathway to sterols in Chlorophyta, including green algae, and diatoms (44–46). Alternatively, other algal groups associated with the Streptophyta operate the acetate-mevalonate pathway to sterols, such as the Klebsormidales Spirogyra aligned with land plants (45) in addition to all fungal and animal systems (1, 40). Our work confirms that the chemistry of the C24-alkylation-reduction reactions provides an independent set of characters. Thus, the cycloartenol -$\Delta^{25(27)}$-olefin pathway to ergosterol/7-dehydroporiferasterol defines sterol biosynthesis in green algae, and the cycloartenol-$\Delta^{24(28)}$-olefin pathway to 24β-methyl/ethyl sterols or to 24α-methyl sterols defines sterol biosynthesis in brown and golden brown algae or in diatoms, respectively (2, 47). In dinoflagellates and choanflagellates, respectively, the lanosterol -$\Delta^{24(28)}$ pathway to uncommon C_{30}-phytosterols (gorgosterol) or to ergosterol defines the sterol pathway (18, 32, 48).

In support of the phyla-specific differences reported in module III, the cloned 24-SMT from the ascomyetous fungus *Paracoccidiodes brasiliensis* (49) recognizes lanosterol and cycloartenol (as expected for conformational reasons [50]) and converts them to a single $\Delta^{24(28)}$ product. Only the lanosterol-based product, however, is further converted to ergosterol because fungi lack enzymes required to open the 9β,19-cyclopropane ring system (1, 2). Future studies in progress to determine whether the *C. reinhardtii* 24-SMT can catalyze lanosterol and cycloartenol to the same set of $\Delta^{25(27)}$- and $\Delta^{24\,(28)}$-C_1 transfer products should further illuminate the evolution of these pathways.

Although the information is incomplete, three competing phylogenies constructed from these characters reasonably describe the separate evolutions of ergosterol biosynthesis.

We surmise that in green algae the ergosterol pathway starts with (i) the mevalonate-independent methylerythritol 4-phosphate pathway to Δ^3-IPP, followed by (ii) the isoprenoid pathway to cycloartenol, which becomes (iii) the $\Delta^{25(27)}$-sterol pathway. In contrast, the three modules acting successively in ergosterol biosynthesis in fungi are: (i) the acetate-mevalonate pathway to Δ^3-IPP, (ii) the isoprenoid pathway to lanosterol, and (iii) the $\Delta^{24(28)}$-olefin pathway. Thus, there is considerable diversity with respect to pathways and to the formation of ergosterol, a likely result of convergent evolution in the biosynthesis of membrane inserts.

It is possible that green algae and fungi had a common ancestor and that the fungi lost the ability to make cycloartenol as a result of mutational divergence in which the cycloartenol synthase underwent "channel switching" in the catalytic reaction path to form lanosterol (1). In such a model in which the acetate-mevalonate pathway was common to algae and fungi, in module II, cycloartenol formation would precede lanosterol for thermodynamic reasons (3), and in module III, the sterol C24-methyltransferase reductases were recruited into a patchwork assembly of enzymatic reactions that recognize the same sterol template for catalysis. The presence of lanosterol synthases in some algae and land plants (36, 51–53) and the $\Delta^{25(27)}$-olefin pathway as the source of C_{30} sterols of elongated side chains extending from C27 in dinoflagellates (32) is consistent with this proposal.

In similar fashion, channel switching in sterol C24-methyl transferase activities may have been an evolutionary event that redirected the reaction path from a single product to one or more 24-alkyl(idene) product(s) that therefore is partially responsible for the chemical traits of phylogenetic significance in ergosterol of algae or fungi and sitosterol of higher plants. Although these catalysts may have evolved by duplication and functional divergence (54), the way in which the enzymatic reactions within modules and the modules themselves have been organized to yield end products remains enigmatic. A combination of the organic/enzymatic approach and amino acid sequence alignments and X-ray structures of the relevant enzymes in more organisms across Domains may lead to a better understanding of sterol evolution.

Note added in proof

The original article appeared online with three tables in text. Table 1 was subsequently moved to supplementary data online and the other tables renumbered.

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