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Phytosterol biosynthesis pathway in *Mortierella alpina*

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Dedicated to Professor Rod Croteau on the occasion of his 60th birthday.

Abstract

The Zygomycetes fungus *Mortierella alpina* was cultured to growth arrest to assess the phytosterol biosynthesis pathway in a less-advanced fungus. The mycelium was found to produce 13 sterols, but no ergosterol. The sterol fractions were purified to homogeneity by HPLC and their identifies determined by a combination of GC–MS and ^{1}H NMR spectroscopy. The principal sterol of the mycelium was cholesta-5, 24-dienol (desmosterol) (83%), with lesser amounts of 24β-methyl-cholesta-5,25(27)-dienol (codisterol) (2%), 24-methyl-desmosterol (6%), 24(28)-methylene cholesterol (3%) and lanosterol (3%) and several other minor compounds (3%). The total sterol accounted for approximately 0.07% of the mycelial dry wt. Mycelium fed methionine-*methyl*-$^{2}H_3$ for 6 days, generated 3 ^{2}H-24-methyl(ene) sterols, [C28-$^{2}H_2$]24(28)-methylenecholesterol, [C28-$^{2}H_3$]24-methylcholesta-5,24-dienol and [C28-$^{2}H_3$]24β-methyl-cholesta-5,25(27)-dienol. The formation of the 24-methyl sterols seems to be catalyzed by the direct methylation of a common Δ^{24}-acceptor sterol thereby bypassing the intermediacy of an isomerization step for rearrangement of the $\Delta^{24(28)}$-bond to $\Delta^{25(25)}$-position as operates in Ascomycetes fungi and all plants.

Keywords: Sterol methyltransferase; Cholesterol; Ergosterol biosynthesis; Isotope labeling; *Mortierella alpina*; Fungal evolution

1. Introduction

The 24-methylation pattern of the sterol side-chain that gives rise to sterol diversity is determined by the enzymatic properties and number of sterol methyltransferase (SMTs) in individual organisms, whereas the type and amount of sterols that accumulate as end products are governed by developmental and functional constraints (Nes, 2003). In sterol-based chemosystematics, the size and direction of the C24-alkylation is phylogenetically significant as well as the formation of either cycloartenol (plant) or lanosterol (fungal) from the cyclization of squalene-oxide (Nes et al., 1990; Nes, 2000). Primitive organisms generally fail to methylate the sterol Δ^{24}-bond (Volkman, 2005), yet with increasing advancement the pattern of the fungal sterol composition will change from one that lacks 24-methyl sterols to one that contains mostly 24β-methyl sterol or one for plants that contains mostly 24α-ethyl sterols. Ergosterol (24β-methyl cholesta-5,7,22*E*-trienol), a C_{28} sterol, is considered to be the typical fungal sterol of Ascomycetes and Basidiomycetes and is present at greater than 50% and as much as 85–96% of the total sterol of most more-advanced fungi (Weete, 1989). Alternatively, cholesterol, a C_{27}-sterol, often is the major sterol of less-advanced Chytridiomycetes fungi (Nes, 1987; Weete et al., 1989).

The sterol pathway of Zygomycetes fungi, positioned between the Chytridiomycetes and Ascomycetes on an evolutionary scale, differs from other fungi with some Zygomycetes synthesizing primarily C_{28} sterols, but not necessarily synthesizing ergosterol, and others synthesizing primarily C_{27} sterols (Weete and Gandhi, 1997). Among these are members [*Mortierella alpina*] of the family Mortierellaceae in which the occurrence of desmosterol (**12**) (ca. 86% total sterol) was reported as the most abundant sterol together with minor amounts of 24(28)-methylene

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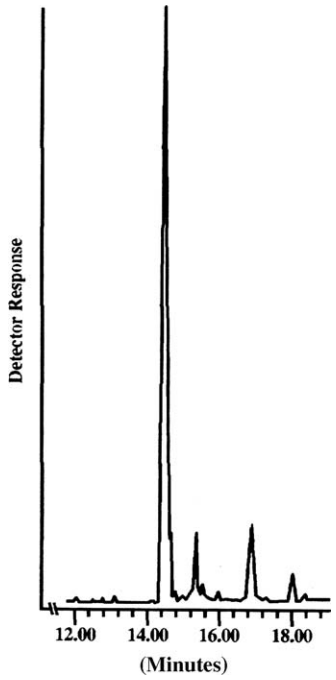

Fig. 1. DB-5 capillary GC–MS analysis of the neutral lipids from extracted saponified growth-arrested *M. alpina* mycelium.

cholesterol (**14**), 24-methyl cholesta-5,24-dienol and 24β-methyl cholesta-5, 25(27)-dienol (Shimizu et al., 1992) (see Fig. 1 for structures). However, the work showing unconventional phytosterols could not be repeated and in that study desmosterol (**12**) and 24(28)-methylene cholesterol (**14**) were cited to make up the fungal sterol mixture in a 84/16 ratio (Weete and Gandhi, 1997). In none of these analyses was a detailed work-up of the sterol composition undertaken nor have the biosynthetic ambiguities for the existence of several 24-methyl sterols been adequately addressed.

In the course of our continuing investigations of the sterol composition of plants and fungi, we found that fungi can synthesize as many as 38 sterols and plants can synthesize as many as 60 sterols during their life history (Nes et al., 1988; Nes, 1990; Guo et al., 1995). All of the 24-methyl(ethyl) in the sterol side chain from these organisms appears to originate via *C*-methylation of a Δ^{24}-acceptor sterol catalyzed by sterol methyltransferase (SMT) (Nes, 2005). Biogenetic schemes for the formation of 24-methyl sterols invoke an ionic mechanism that limits the outcome to $\Delta^{23(24)}$-, $\Delta^{24(28)}$- or $\Delta^{25(27)}$-olefins (Arigoni, 1978). Consequently, the formation of a 24-methyl $\Delta^{24(25)}$-sterol is assumed to follow the formation of a $\Delta^{24(28)}$-sterol (Goodwin, 1981).

In support of this view, incorporation experiments performed with ^{3}H-labeled 24-methyl(ene) lanosterols and methionine-*methyl*-$^{2}H_3$ [Met-d_3] to the more-advanced Ascomycetes *Gibberella fujikori* point to a *C*-methylation pathway that proceeds from a Δ^{24}-sterol acceptor to a $\Delta^{24(28)}$-sterol product. The fungus will either reduce the olefin product directly or isomerize it to a $\Delta^{24(25)}$-sterol prior to reduction to generate the 24β-methyl sterol side chain (Nes and Le, 1990). In similar fashion, a $\Delta^{24(28)}$- to $\Delta^{24(25)}$-pathway is well-documented in the biosynthesis of 24-alkyl sterols in vascular plants (Fujimoto et al., 1997; McKean and Nes, 1977). In contradistinction to the accepted pathway for 24-methyl cholesta-5,24(25)-dienol, we recently found that a cloned protozoan SMT can generate three 24-methyl(ene) Δ^{8}-sterols – 24-methylcholesta-8,24(25)-dienol, 24(28)-methylene cholest-8-enol and 24-methyl-cholesta-8,25(27)-dienol, from zymosterol (cholesta-8,24-dienol) (Zhou et al., 2006). In this paper, we describe the isolation and characterization of *M. alpina* sterols from a preparative scale growth experiment which allowed minor sterols in the mycelium to be identified. In addition, the mycelium was incubated with Met-d_3 to assess the *C*-methylation pathway to phytosterols. The results are largely consistent with information on lanosterol-based pathways to Δ^{5}-sterols and reveal the SMT effectiveness to generate a product set of unique fungal sterols.

2. Results and discussion

Mycelium at different stages of *M. alpina* growth were analyzed for changes in sterol composition and found to exhibit the same relative pattern of compounds from an initial sterol profiling of the non-saponifiable lipid fraction by GC–MS (Fig. 1). The sterol content of the mycelium was found to be ≈0.07% of the mycelium dry wt. which is about the same amount of total sterol produced in the mycelium of the more-advanced ascomycetous fungi, ca. 0.1% (Nes and Heupel, 1986). Five major sterols were detected eluting in the GLC chromatogram around 14.5, 14.8, 15, 17 and 18 min. corresponding to authentic specimens of desmosterol (**12**), codisterol (**16**), 24(28)-methylene cholesterol (**15**), 24-methyl cholest-5,24-dienol (**14**) and lanosterol (**1**) (Fig. 2). The accumulation of Δ^{5}-sterols by *M. alpina* is similar to other fungi. The possibility that the 24-methyl sterols were derived from the growth medium led us to extract the individual medium components, a mixture of 1% yeast extract-2% peptone-2% anhydrous dextrose. The yeast extract was found to contain trace amounts of ergosterol at ca. 100 μg/100 g as reported by Nes (1987). None of the medium components contained the unusual sterols detected in the mycelium of *M. alpina*. Therefore, we confirmed that the three 24-methyl sterols (**12**, **14** and **15**) previously identified in *M. alpina* (Shimizu et al., 1992) are endogenously formed by the fungus. However, in our study we could not detect any 24,25-methylene cholesterol [contains the cyclopropane ring in the sterol side chain] as reported by Shimizu et al. (1992). For reference purposes, we prepared a similar compound in the lanosterol and ergosterol series (Venkatramesh et al., 1996a,b).

In order to establish the biosynthetic pathway to desmosterol (**12**) and the 24-methyl sterols, a large amount of mycelium was prepared. The amount of mycelium cultured

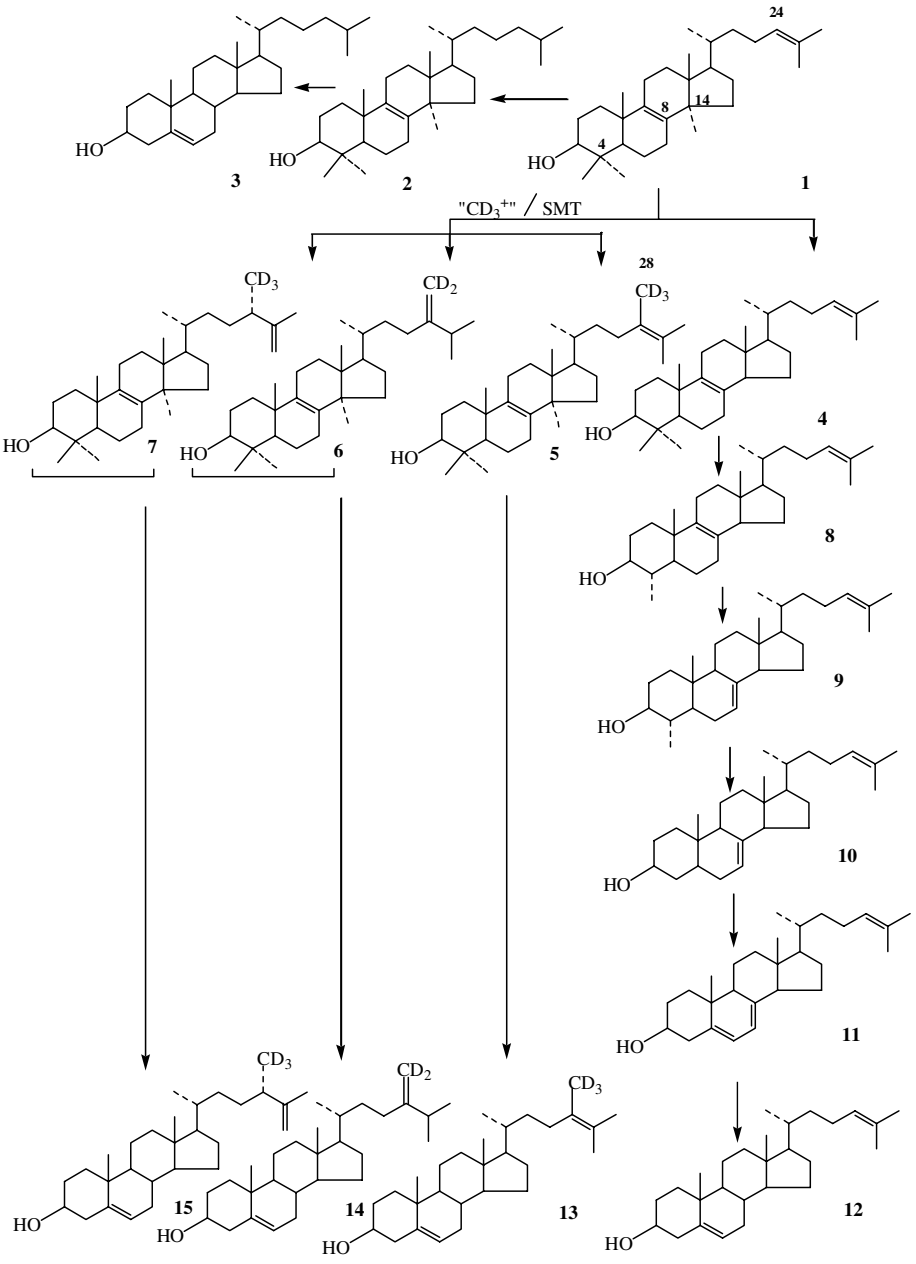

Fig. 2. Hypothetical *M. alpina* pathways for the conversion of lanosterol to Δ^5-24-dealkyl and 24-alkyl sterols; incorporation of ^2H from Met-d_3 into the sterol side chain is indicated in the figure. Compounds **6** and **7** were not detected in the mycelium but are included in the biosynthetic pathway to illustrate their putative intermediate status.

was ten times the amount of mycelium that other investigators cultured for sterol analysis. From the combined mycelium approximately 63 g dry wt. of mycelium (dry wt. determined by demonstrating the fresh mycelium contained 82% water) and 600 mg of non-saponifiable lipid fraction was obtained. The sterol mixture of the non-saponifiable lipids was first subjected to separation by preparative silica gel thin layer chromatography into C4-desmethyl, C4-methyl and C4-dimethyl sterol compounds followed by HPLC using a Zorbax C_{18} semipreparative column to generate pure compounds. The structures of the compounds were tentatively identified by their movement in chromatography and mass spectroscopic fragmentation pattern relative to similar data for reference compounds. From the C4-desmethyl sterol zone following HPLC fractionation, six sterols were isolated: desmosterol (**12**), cholesterol (**3**), cholesta-5,7,24-trienol (**11**), codisterol (**15**), 24(28)-methylene cholesterol (**14**) and 24-methyl cholesterol (**13**). The three sterols eluting in GLC at 1.12, 1.15 and 1.24 considered to possess a 24-methyl side chain exhibit similar molecular weights of M^+ 398 amu and each one contains a base peak or a significant peak in the mass spectrum at *m/z* 314 amu indicating the presence of a $\Delta^{24(28)}$–$\Delta^{24(25)}$- or $\Delta^{25(27)}$-double bond and of a nuclear unsaturation (Rahier and Benveniste, 1988). From the C4-monomethyl sterol zone following HPLC fractionation, two sterols were iso-

lated: 4α-methyl cholesta-8,24-dienol (**8**) and 4α-methyl cholesta-7,24-dienol (**9**). From the C4-dimethyl sterol zone following HPLC fractionation, four sterols were isolated: 24,25-dihydrolanosterol (**2**), lanosterol (**1**), 14-desmethyl lanosterol (**4**) and 24-methyl lanosterol (**5**). When the mycelium was incubated with $^{2}H_3$-methionine, the sterols cited above were detected as well as a trace of cholesta-7,24-dienol (**10**). The abundance of individual sterols in the *M. alpina* mycelium is reported in Table 1. The structure of the HPLC pure sterols were analyzed further by ^{1}H NMR spectroscopy (data not shown), and in each case the spectroscopic data matched the ^{1}H NMR spectra of authentic specimens from our steroid collection (Nes and Le, 1990; Guo et al., 1995; Venkatramesh et al., 1996a,b; Nes et al., 1997), thereby confirming the identity of each of the sterols reported in Table 1.

Our efforts were next directed to establish the endogenous synthesis of the 24-methyl sterols by treating mycelium of *M. alpina* with Met-d_3. We found that addition of 50 mg of Met-d_3 to 250 mL of medium was adequate to yield ^{2}H-labeled 24-methyl sterols without significant alteration of the sterol composition. From 1.1 g dry wt. mycelium treated with Met-d_3, we obtained 2.5 mg of non-saponifiable lipids. The sterols in the non-saponifiable lipid fraction were separated by HPLC. The compounds eluting from the column were examined by GC–MS. In the mass spectrum of these compounds, a shift in 2 mass units was detected in the control sample versus the isotopically labeled 24(28)-methylene cholesterol (**14**) sample (data not shown) and a shift in 3 mass units was detected in the control sample versus the isotopically labeled codisterol sample (Fig. 3). However, the sample of ^{2}H-24-methyl cholesta-5,24(25)-dienol unexpectedly contained a 3 mass unit increase compared to the control (Fig. 4). If the sterol were to arise by the isomerization of the $\Delta^{24(28)}$-sterol precursor, then the isotopically labeled sterol should contain 2 ^{2}H-atoms in the

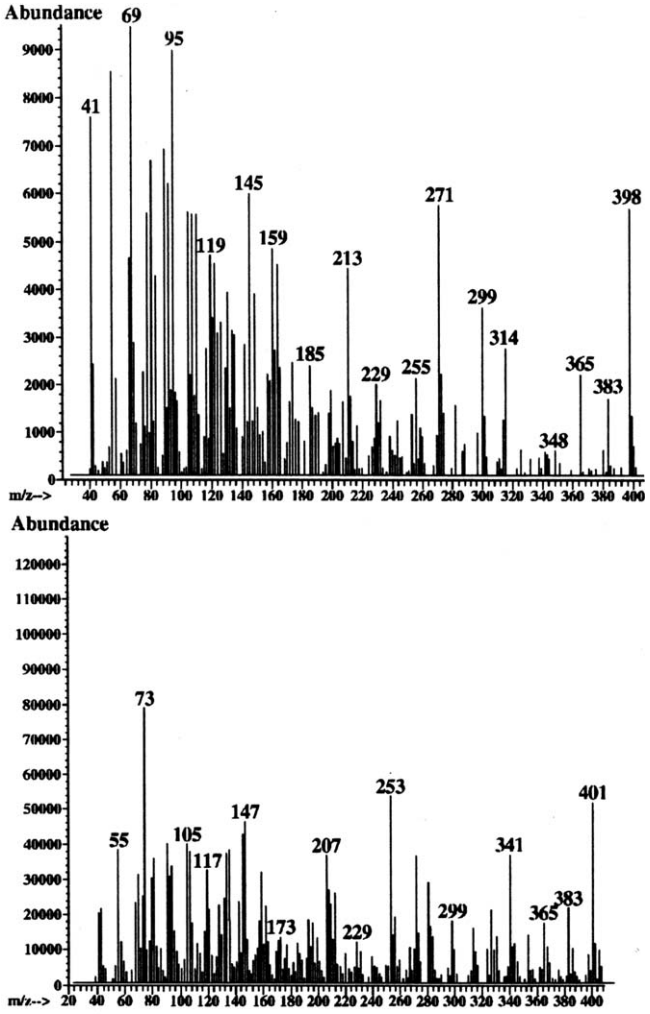

Fig. 3. Mass spectra of $\Delta^{5,25(27)}$-24-methyl sterols isolated from the mycelium. The upper mass spectrum is of codisterol (**15**) from control mycelium and the lower mass spectrum is of codisterol (**15**) originating from the Met-d_3-incubation.

Table 1
Relative composition, chromatographic and mass spectral properties of C4-desmethyl, C4-monomethyl and C4-dimethyl sterols isolated from mycelium of *Mortierella alpina*[a]

Sterol	[Structure]	R_f [TLC]	RRT_c [GLC]	α_c [HPLC]	M^+ [MS]	% of total
Lanosterol	**1**	0.47	1.31	1.00	426	3
14-Desmethyl lanosterol	**4**	0.47	1.32	1.05	412	0.1
24-Methyl lanosterol	**5**	0.47	1.55	1.12	440	0.1
24,25-Dihydrolanosterol	**2**	0.47	1.24	1.16	428	0.1
4α-Methyl cholesta-8,24-dienol	**8**	0.39	1.16	0.91	398	1.1
4α-Methyl cholesta-7,24-dienol	**9**	0.39	1.21	0.93	398	0.3
Cholesterol	**3**	0.31	1.00	1.00	386	0.2
Desmosterol	**12**	0.31	1.05	0.85	384	83.0
Cholesta-5,7,24-trienol	**11**	0.31	1.06	0.80	382	1.0
Cholesta-7,24-dienol	**10**	0.31	1.10	0.90	384	0.1
24-Methyl desmosterol	**13**	0.31	1.24	1.05	398	6.0
24(28)-Methylene cholesterol	**14**	0.31	1.15	0.98	398	3.0
Codisterol	**15**	0.31	1.12	0.97	398	2.0

[a] R_f value is the distance run by the sterol sample relative to the distance run by the mobile phase using silica gel G plates developed twice in benzene/diethyl ether; RRT_c and α_c refer to the retention time of the sterol sample relative to the retention time to cholesterol; mass spectroscopy was performed using the HP GC–MS and the molecular ion for the relevant compounds is reported in atomic mass units. Structures of compounds are shown in Fig. 2.

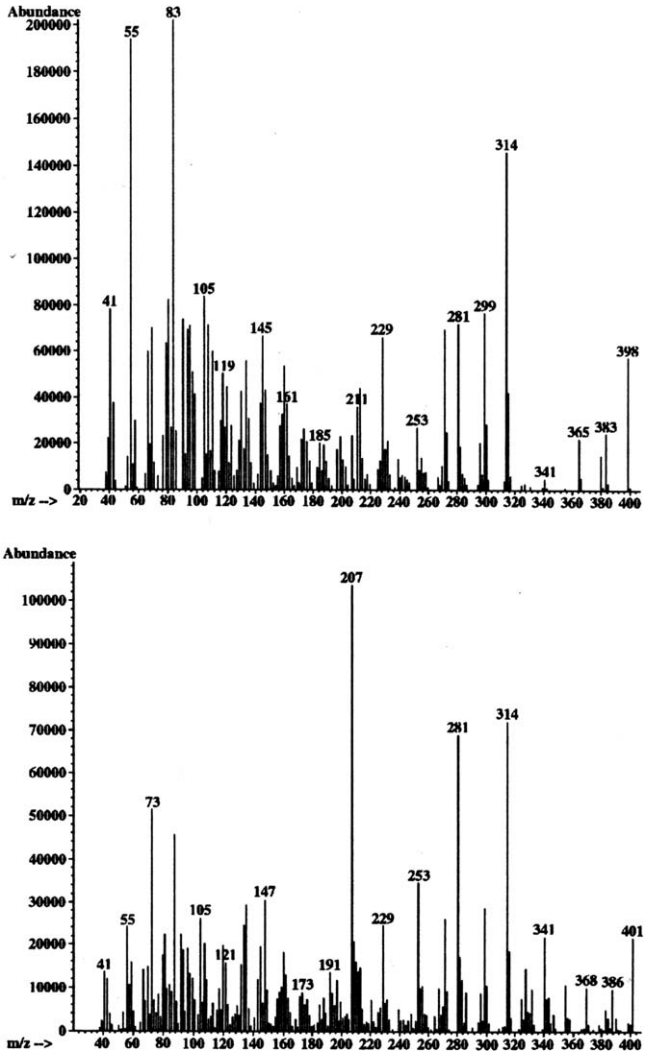

Fig. 4. Mass spectra of $\Delta^{5,24(25)}$-24-methyl sterols isolated from the mycelium. The upper mass spectrum is of 24-methyl cholesta-5,24-dienol from control mycelium and the lower mass spectrum is of 24-methyl cholesta-5,24-dienol originating from the Met-d_3-incubation.

side chain. By comparison of the ^{1}H NMR spectra of different ^{2}H and ^{13}C isotopically labeled species of ergosterol (Zhou et al., 1996), we observed that the signal for C28 derived from methyl of [$^2H_3$-*methyl*]AdoMet decreased relative to the signal for the control sample. The HPLC pure [28-^{2}H]24-methyl cholesta-5,24-dienol (**13**) (24-methyl desmosterol) was taken for ^{1}H NMR spectroscopic analysis and the spectrum of the compound was identical to the non-labeled specimen except the signal for C28 bearing the ^{2}H-atoms was decreased (data not shown) consistent with the methyl addition directed exclusively at C-24 of the sterol side chain.

3. Concluding remarks

In a recent report, we demonstrated that the cloned protozoan SMT is capable to catalyze the formation of multiple products with the $\Delta^{24(28)}$-, $\Delta^{25(27)}$- and $\Delta^{24(25)}$-24-methyl side chains from a zymosterol substrate (Zhou et al., 2006). In *M. alpina*, 24-methyl lanosterol (**5**) was detected, but other 24-methyl lanosterols were not found perhaps due to a high rate of turnover of these compounds. With this new information, we surmise that the 24-methyl desmosterol as well as the other two 24-methyl(ene) sterols synthesized by *M. alpina* are derived by direct SMT catalyzed *C*-methylation of lanosterol and the resulting 24-methyl lanosterols are the native intermediates to Δ^5-24-methyl sterols. Thus, with the possibility of alternate pathways operating to generate 24-dealkyl and 24-alkyl sterols, desmosterol is likely an end product to serve the bulk membrane insert role and 24-methyl sterols likely act as signal molecules, in analogy to individual sterol functions played in other fungi to control growth and maturation (Rodriquez et al., 1985; Nes and Heupel, 1986; Bloch, 1988).

4. Experimental

M. alpina CBS 210.32 was kindly provided by the Japanese group (Shimizu et al., 1992). Methionine-*methyl*-$^2H_3$ with 99.3% D_2 enrichment was purchased from MSD-Isotopes (Montreal Canada). The methods for culturing fungi statically (without shaking) at room temperature in 250 mL flasks containing 50 mL medium (1% yeast extract-2% peptone-2% anhydrous dextrose [YPD]) for 8 days were as described (Nes and Le, 1990). In a typical experiment, from an inoculum of ca. 0.02 mg dry wt. mycelium/flask generated a growth arrested mycelium of 0.28 mg dry wt./flask. To obtain the amount of mycelium considered necessary to begin the sterol analysis, many flasks were cultured over a three month period and the combined mycelium from each harvest stored at −4 °C.

The neutral lipids from the mycelium were extracted after saponification as previously described (Nes and Le, 1990), and were then monitored by GC–MS on a Hewlett-Packard 6890 GC interfaced to a 5793 mass spectrometer at 70 eV with on column injection using a DB-5 column (30 m × 25 m with 0.25-μm film thickness) (J&W Scientific, Folsom, CA) and He as the carrier (1.2 mL/min) (Guo et al., 1995), or chromatographed on thin layer plates (0.25 and 1.00 mm thick) coated with silica gel G. The plates were developed 2× in Et_2O–C_6H_6 (15:85) and the separated components into C4-dimethyl sterols, C4-monomethyl sterols and C4-dimethyl sterols were further fractionated into pure compounds by HPLC using a C_{18}-Zorbax semipreparative column eluted with 100% methanol at ambient temperature (Guo et al., 1995). NMR spectroscopy was performed at ambient temperature on a Varian Unity Inova 500 MHz spectrometer in deuterochloroform with tetramethylsilane as internal standard. Reference sterols characterized in stereochemical detail by MS and ^{1}H NMR were reported in our earlier papers (Nes and Le, 1990; Guo et al., 1995; Venkatramesh et al., 1996a,b; Nes et al., 1997).

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