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Sterols of *Saccharomyces cerevisiae* *erg6* Knockout Mutant Expressing the *Pneumocystis carinii* S-Adenosylmethionine:Sterol C-24 Methyltransferase (SAM:SMT)

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Abstract

The AIDS-associated lung pathogen *Pneumocystis* is classified as a fungus although *Pneumocystis* has several distinct features such as the absence of ergosterol, the major sterol of most fungi. The *P. carinii* S-adenosylmethionine:sterol C24-methyltransferase (SAM:SMT) enzyme, coded by the *erg6* gene, transfers either one or two methyl groups to the C-24 position of the sterol side chain producing both C₂₈ and C₂₉ 24-alkylsterols in approximately the same proportions whereas most fungal SAM:SMT transfer only one methyl group to the side chain. The sterol compositions of wild type *Saccharomyces cerevisiae*, the *erg6* knockout mutant (*erg6*^Δ), and *erg6* expressing the *P. carinii* or the *S. cerevisiae* *erg6* gene were analyzed by a variety of chromatographic and spectroscopic procedures to examine functional complementation in the yeast expression system. Detailed sterol analyses were obtained using high performance liquid chromatography (HPLC) and proton nuclear magnetic resonance spectroscopy (¹H-NMR). The *P. carinii* SAM:SMT in the *erg6* restored its ability to produce the C₂₈ sterol ergosterol as the major sterol, and also resulted in low levels of C₂₉ sterols. This indicates that while the *P. carinii* SAM:SMT in the yeast *erg6* cells was able to transfer a second methyl group to the side chain, the action of C₂₄(28)-sterol reductase (coded by the *erg4* gene) in the yeast cells prevented the formation and accumulation of as many C₂₉ sterols as that found in *P. carinii*.

Keywords

24-Alkylsterols; complementation; ergosterol; fungi; gas-liquid chromatography; high performance liquid chromatography; immunodeficiency-dependent disease; nuclear magnetic resonance spectroscopy; transformation

Pneumocystis (Pc), can occur in mammalian hosts as a transient commensal in healthy hosts or a pathogenic lung opportunistic infectious agent of those who are immunodeficient. The organism can cause a pneumonia (PcP) in AIDS patients and other individuals such as those

undergoing chemotherapy and other immunosuppressive treatment. The organism grows rapidly in the lungs of AIDS patients which is a major immediate cause of death in these people. The genus *Pneumocystis* is classified as an Ascomycetous fungus. It is comprised of species with broad genetic diversity and is known to have several unusual characteristics unlike those of most fungi (Stringer 1993; Kaneshiro 2004; Walzer and Cushion 2005). Species of *Pneumocystis* have been only found among mammals and are host species-specific (e.g., *P. jirovecii* infect humans; *P. carinii* laboratory rats; *P. murina* mice). The major hindrance to studying this important lung pathogen is the absence of an in vitro culture system that can provide indefinite passaging, hence molecular genetics approaches are not feasible. The state of art in *Pneumocystis* cultivation also prevents production of mass numbers of organisms for direct biochemical analyses. Recovery of organisms from infected laboratory animal lungs is expensive and labor-intensive but most biochemical studies thus far have been performed on *P. carinii* isolated and purified from laboratory rats or *P. murina* from mice.

Ergosterol and its biosynthesis are currently targets of most antifungals used clinically today (Kaneshiro 1998, 2004). Unlike most other fungi *P. carinii* lacks ergosterol, Amphotericin B binds membrane 24-alkylsterols leading to the dissipation of ion gradients and transmembrane potentials and the triazole drugs such as fluconazole block sterol 14 α -demethylase action during sterol biosynthesis. Another attractive potential drug target for pathogens with 24-alkylsterols such as *Trypanosoma*, *Leishmania* and *Pneumocystis* is the enzyme *S*-adenosylmethionine:C-24 sterol methyltransferase (SAM:SMT, AdoMet) as mammals do not have 24-alkylsterols and SAM:SMT hence these drugs might have less side effects on the host than other antifungal agents. Blocking *P. carinii* SAM:SMT by an azasterol compound inhibited the proliferation of these cells in primary cultures (Urbina et al. 1997) suggesting that this enzyme is a target for future drug development.

Most fungal SAM:SMT enzymes transfer only one methyl group to the sterol side chain producing the C₂₈ sterol ergosterol as the dominant sterol. In contrast, it was discovered that the recombinant *P. carinii* SAM:SMT expressed in *Escherichia coli*, a bacterium that does not synthesize sterols, had unusual properties (Kaneshiro et al. 2001, 2002). This enzyme preferred lanosterol and 24-dehydrolanosterol over zymosterol as sterol substrates and it catalyzed the transfer of one or two methyl groups to the C-24 position of the sterol side chain, similar to some plants (Bouvier-Nave et al., 1998; Nes 2000). Figure 1 shows the designation of carbon positions in sterols illustrated by those in pneumocyststerol (Fig. 1).

In an earlier study (Nkinin et al. 2011) we used recombinant system approaches to better understand *P. carinii* sterol biosynthesis and employed a eukaryotic fungal expression system *Saccharomyces cerevisiae*, which produces its own sterols. Functional complementation was demonstrated for the *P. carinii* sterol 14 α -demethylase enzyme (14DM, coded by the *erg11* gene) in *S. cerevisiae*. Identification and quantification of sterols in the wild type yeast, the *erg11* knockout mutant (*erg11*) transformed with the *S. cerevisiae* *erg11* gene as positive control, and *erg11* transformed with the *P. carinii* *erg11* gene demonstrated that the *P. carinii* enzyme was fully functional in the *erg11* mutant. In the present study, we examined the ability for the *P. carinii* SAM:SMT to complement the lack of this enzyme in the yeast *erg6* knockout mutant.

MATERIALS AND METHODS

Saccharomyces cerevisiae strains

The sterols of four *S. cerevisiae* strains were compared in this study: wild type, a *erg6* mutant, the *erg6* mutant transformed with the *P. carinii erg6* gene (*Sc erg6_{Pcerg6}*), and the positive control *erg6* mutant transformed with the *S. cerevisiae* wild type *erg6* gene (*Sc erg6_{Scerg6}*).

Wild type and erg6 knockout of S. cerevisiae. *Saccharomyces cerevisiae* wild type strain BY4742 (genotype (MATa, leu2-0, his3-1, lys2-0, ura3-0) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The *S. cerevisiae erg6* knockout strain (*erg6*) YML008C (genotype MATalpha his3delta1 leu2delta0 ura3delta0 deltaERG6) was also obtained from ATCC. Both the wild type (BY4742) and the *erg6* knockout mutant, (*Sc erg6*) were grown in SC-URA/G418 medium, a chemically defined medium lacking uracil and containing 200 µg/L geneticin (G418), Gibco, Grand Island, NY) supplemented with 20 mg/L uracil.

Transformations with the P. carinii erg6 gene. SAM:SMT is not essential for *Saccharomyces cerevisiae* hence its *erg6* mutant is viable and proliferates in culture even though it lacks 24-alkylsterols (Bard et al. 1977). Hence, transformations were performed on haploid organisms.

The *P. carinii erg6* template DNA was obtained from *E. coli* transformed with plasmids containing the target gene (Nicole Worsham, Akhelish Pandey, unpublished). Plasmid DNA was extracted using the QIAprep® Spin Miniprep Kit (Qiagen Sciences, Valencia, CA) and the sequence of the *P. carinii erg6* gene on the plasmid was verified. The target sequence was amplified by PCR using *Pcerg6F* primer 5' ATGTCTTTTGAAGTATAGAGAAG 3' and *NSerg6R* primer 5' AACCAAAGGTTTTCTACAAACCCATAATTG 3'. Primers were designed so that the C-terminal fusion tag would be translated in-frame with the recombinant protein. PCR was performed using a commercial kit (Promega, Madison, WI) with an optimized annealing temperature of 50 °C. The PCR product was cloned according to the pYES2.1 Expression Kit (Invitrogen, Carlsbad, CA), transformed into TOP 10 One Shot® Chemically Competent *E. coli* (Invitrogen) and grown on Luria Broth Agar (LBA) plates containing ampicillin (100 µg/mL) overnight at 37 °C. Colonies were then transferred into selective liquid broth media (Luria Broth/Amp 100µg/mL) and grown overnight at 37 °C with shaking. The cells were harvested and plasmid DNA isolated and screened by PCR for the *erg6* insert. Clones producing appropriate-sized PCR products for the *P. carinii erg6* gene on the plasmid were sequence-verified for the correct *erg6* sequence.

Transformation of the pYES2.1*erg6* plasmid containing the *P. carinii erg6* gene into the *S. cerevisiae erg6* strain YML008C was accomplished by electroporation (one pulse at 10 µF × 10 capacitance, 480 ohms, 1.5 kV of resistance) (GenePulser II, Bio-Rad Laboratories, Philadelphia, PA). Treated yeast cells were grown on agar plates containing SC-URA/G418 at 30 °C.

Plasmid DNA from selected yeast colonies were screened by PCR and sequenced for verification of the correct *erg6* nucleotide sequence. The yeast clones were also screened for the presence and location of the *erg6* knockout construct using the primer YML008C-A: 5' CTGTTGCCGATAACTTCTTCATTCC 3' that binds upstream of the *erg6* locus and KAN-B: 5' CTGCAGCGAGGAGCCGTAAT 3', which binds in the knockout cassette yielding a 508-bp PCR product. The colonies were then transferred into liquid medium (SC-URA/G418) and grown in a shaking incubator (Beckman, Fullerton, CA) set at 30 °C and 150 rpm. Alternatively, mass cultures were grown at room temperature (22 – 25°C) on a shaker (Belly dancer, Stovall Life Science Inc, Greensboro, NC, USA).

Transformation of the S. cerevisiae erg6 knockout mutant with wild type S. cerevisiae erg6 gene (control). Wild type strain BY4742 was used to obtain the yeast's *erg6* gene for transformation into the yeast *erg6* strain as a positive control. Protocols for this transformation were similar to those described above for transformation with the *P. carinii erg6* gene.

Expression and detection of the recombinant SMT

Expression of the recombinant SMT was under the control of a galactose-inducible promoter. Cells were first grown in SC-URA/G418) with glucose as the carbon source, then transferred to SC-URA/G418 with raffinose to remove the glucose repression of the recombinant *erg6* gene followed by growth in SC-URA/G418 with galactose to induce expression of the recombinant gene.

Cells were homogenized in a Mini-Bead Beater™ (BioSpec Products, Bartlesville, OK), subjected to centrifugation, and an aliquot of the supernate was spectrophotometrically analyzed for protein content (Nano Drop® ND-1000 spectrophotometer, Thermo Scientific, Asheville, NC) using the RC DC™ Protein Assay (Bio-Rad Laboratories, Hercules, CA) prior to analysis by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). ColorBurst™ pre-dyed molecular weight marker (Sigma Aldrich, St. Louis, MO) was run in a separate lane. The gels were then subjected to Western blot analyses using anti-V₅-HRP-conjugated antibodies and visualized with stabilized TMB (3,3',5,5'-tetramethylbenzidine) substrate for horseradish peroxidase (Promega, Madison, WI).

Sterol analyses

Sterols were extracted from *S. cerevisiae* cells, purified by biphasic partitioning, and neutral lipid fraction was obtained using silicic acid column chromatography as previously described (Kaneshiro et al. 1994; Giner et al. 2002, 2004; Nkinin et al. 2011). The CHCl₃ eluate containing the sterols and steryl esters was saponified (Kaneshiro et al. 1994; Giner et al., 2006, 2004; Nkinin et al. 2011) to obtain the total sterol fraction.

GLC Total sterols for GLC analysis were first converted to their silyl derivatives with BSTFA (Supelco, Bellefonte, PA) to enhance volatilization and detection. Analysis was performed on a Hewlett Packard 5890 GC (Agilent Technologies, Palo Alto, CA) equipped with a 30 m × 0.32 mm i.d. SPB-5 capillary column coated with 0.25 µm film (Supelco). In some analyses, sterol samples were co-injected into the GLC column with authentic

cholestane as an internal standard. Oven temperature for isothermal analyses was set at 250 °C; injector and the flame ionization detector were both set at 290 °C. Helium was the carrier gas and column head pressure was set at 155 kPa. In other analyses, a temperature program was used with oven temperature held at 225 °C for 2 min, then increased to 290 °C at 2 °C/min, and then held isothermal at the final temperature for 10 min.

Isolation and purification of sterols—Individual sterols were isolated from total sterol samples (>500 µg) by a combination of thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) (Giner et al. 2004, 2006; Nkinin et al. 2011) (Fig. 2). The HPLC system was comprised of a Waters 6000A pump, Waters 410 differential refractometer, and two Altex 10×250-mm columns in series, both packed with 5-µm particle size Ultrasphere ODS. The mobile phase was methanol (MeOH; 3 ml/min) or, in selected cases, acetonitrile/MeOH/ethanol (MeCN/MeOH/EtOAc; 11:4:4). All extraction and purification steps were carried out under low light conditions to minimize autoxidation.

Nuclear Magnetic Resonance (NMR) spectroscopy—The solvent of the individual fractions isolated by HPLC was evaporated with N₂, and the residues were dissolved in CDCl₃ for ¹H-NMR analysis. Spectra were acquired using a Bruker Avance-600 instrument with a TXI probe at 30 °C. Calibration was by the residual solvent signal (7.26 ppm). Sterols were identified by comparison of their spectra with authentic standards and published values.

RESULTS

Transformation

S. cerevisiae strains transformed with the *P. carinii* or *S. cerevisiae erg6* gene were shown to express the recombinant SAM:SMT. Western blot analysis indicated a protein product (~43 kDa) that bound to the anti-V5 tag had a molecular mass similar to that of SAM:SMT (Fig. 3).

Sterol analysis by GLC

GLC analysis of total sterols in the four strains of *S. cerevisiae* resolved 6–13 sterol components (4–6 samples of each strain). Earlier GLC and GC-MS data showed that ergosterol comprised 71.6% of total sterols in the wild type *S. cerevisiae* strain BY4742 (Nkinin et al., 2011). In the present study, ergosterol was not detected in the *erg6* mutant YML008C by GLC analysis; the major peak found in this strain was one that eluted with authentic zymosterol (Fig. 4, A). On the other hand ergosterol was the major sterol in the positive control, which was the *erg6* mutant transformed with the *S. cerevisiae erg6* gene (*Sc erg6_{Scerg6}*) where it accounted for 86.7% of the total sterols. The yeast *erg6* knockout mutant YML008C transformed with the *P. carinii erg6* gene (*Sc erg6_{Pcerg6}*) also contained ergosterol as the major sterol component comprising 75.2% of total sterols (Fig. 4, B).

Structural identities determined by NMR

Sterol fractions isolated by preparative HPLC are shown for the combined 4,4-dimethyl and 4α-methyl sterols of strains analyzed in this study (Fig. 2). Definitive structural

identifications of a total of 37 sterols among the *S. cerevisiae* strains analyzed were obtained in this study by NMR (Table 1, Fig. 5). Spectral data of several sterols observed in the present study were previously described (Giner et al., 2002; Giner et al., 2004; Nkinin et al., 2011). Of the remaining sterols, all but one, are known.

The novel 22Z isomer of $5,7,22,24$ cholestatetraenol (Fig. 5, Q) was identified by comparison of its NMR spectrum with those previously reported for other 22Z $22,24$ steroids (Hutchins et al. 1970; Haage et al. 1988). (22Z)-Cholesta-5,7,22,24-tetraenol (Fig. 5, Q): $^1\text{H-NMR}$ (600 MHz, CDCl_3): δ 6.086 (1H, d, $J = 11.5$ Hz), 6.026 (1H, dd, $J = 11.5$, 10.9 Hz), 5.575 (1H, m), 5.590 (1H, m), 5.127 (1H, t, $J = 10.3$ Hz), 3.640 (1H, m), 1.812 (3H, s).

DISCUSSION

This is the first report of a *S. cerevisiae* *erg6* knockout mutant transformed with the *P. carinii* *erg6* gene. Biochemical evidence for complementation demonstrated that the recombinant *P. carinii* SAM:SMT was functional in sterol biosynthesis of transformed *S. cerevisiae*. Complementation by *erg6* originating from both yeast and *P. carinii* resulted in sterol compositions which contained approximately 70% ergosterol (Fig. 5, BB), which was a higher percentage than the 54% found in the wild type. This is thought to be due to higher expression of the SAM:SMT enzyme proteins in the transformed mutants.

The sterols of other *S. cerevisiae* *erg6* mutants, including ones not produced by gene knockout procedures, have previously been examined and generally agree with our analysis (Barton et al. 1974; Bard et al. 1977; Valachovic et al. 2006). The present study on the YML008C *erg6* knockout mutant is the first time that the 22Z isomer of cholesta-5,7,22,24-tetraenol was detected in *erg6* mutant yeasts. Apparently, the fidelity of the yeast 22-desaturase depends on the presence of an alkyl group at C24.

In our HPLC-NMR analysis, there was less zymosterol than found previously in a *S. cerevisiae* *erg6* mutant (Valachovic et al. 2006). It is likely that in that study the GLC analyses did not resolve between several sterols present and that cholestane sterols and their stereoisomers co-eluted as a single GLC peak. We conclude that the large peak in the GLC of *S. cerevisiae* *erg6* sterols (Fig. 4A) that had the same elution time from GLC columns as zymosterol very likely also contained several cholestane sterols, and that we were able to resolve and identified these in the present study by a combination of preparative HPLC and NMR.

The *Pneumocystis* SAM:SMT (coded by the *erg6* gene) generates C28 (ergostane) and C29 (stigmastane) sterols, whereas the yeast SMT can only generate C28 sterols (Fig. 6). When complemented with *Pneumocystis* *erg6* gene, stigmastane sterols were detected (7.6% of the total sterols), mainly as the 4,4-dimethyl sterol, pneumocysterol (3.5%). Based on the higher proportions of alkylated 4,4-dimethyl sterols in the yeast mutant expressing the *P. carinii* *erg6* gene, the *Pneumocystis* SAM:SMT catalyzes the alkylation of 4,4-dimethyl sterols better than the yeast SMT. The relatively low amounts of stigmastanes in *P. carinii* *erg6* transformed yeast is due to the presence of the native yeast $24(28)$ -sterol reductase (coded

by the *erg4* gene), which depletes the $^{24(28)}$ -sterols (Fig. 6, 2) needed for production of C29 sterols.

The yeast $^{24(28)}$ -sterol reductase (coded by the *erg4* gene) reduces the 24Z-24-ethylidene side chain (Fig. 6, 3) with the same stereochemistry as the 24-methylene side chain, yielding only 24 β -alkylsterols (Fig. 6, 4 and 5) and no 24 α -alkylsterols but appears to be less active, based on the proportion of unreduced sterols in *Pneumocystis* (Giner et al. 2002). Thus, among the desmethylsterols, the ratio of unreduced to reduced ergostanes (24-methylene- to 24 β -methyl sterols (Fig. 6, 2 and 4) was 2:5 in *Pneumocystis carinii* (Giner et al., 2002), while in wild type yeast it was 1:4, if one includes sterols further processed by the yeast 22-desaturase (coded by *erg5*) (Fig. 6, 6). The difference becomes greater still if one includes C29 sterols, which arise from unreduced ergostanes, bringing the ratio in *Pneumocystis* to 8:5. On the other hand, the ratios of unreduced to reduced stigmastanes (24Z-14-ethylene- to 24 β -ethyl sterols (Fig. 6, 3/5 + 7) were very similar, 2:1, in both *P. carinii* (Giner et al. 2002) and in the yeast mutant expressing *P. carinii erg6*. Based on the greater ratio of unreduced vs. reduced stigmastanes (Fig. 6, 3/5+7 = 2.1) relative to the same ratio for ergostanes (Fig 6, 2/4 + 6 = 1:40) in the yeast transformed with *P. carinii erg6*, the 24-methylene sterol side chain (Fig. 6, 2) is a better substrate than for the yeast $^{24(28)}$ -sterol reductase (Fig. 6, 3). The $^{24(28)}$ -sterol reductase does not appear to act on 4,4-dimethyl- or 4 α -methylsterols in either yeast or *P. carinii*, which accounts for the predominance of pneumocysterol (Fig. 5, C) in *Pneumocystis jirovecii* blocked in 14 α -desmethylase activity (coded by the *erg11* gene) (Giner et al, 2004).

The 24-ethyl side chain is a substrate for the yeast 22-desaturase (coded by the *erg5* gene) (Fig. 6). However, based on the product ratios, it is not a preferred substrate compared to the natural 24-methyl side chain. This has been shown experimentally using cell-free extracts (Giner and Djerrassi 1990). The yeast (<http://www.yeastgenome.org/#>) has the *erg6*, *erg4* and *erg5* genes but apparently, *P. carinii* has only the *erg6* and *erg4* genes. There is currently no evidence that *P. carinii* has the *erg5* gene (pgp.cchmc.org/html/geno_data_gdb.html) nor that it is capable of forming 22 sterols (Giner et al. 2002).

Sterols of *P. carinii* compared to those of *S. cerevisiae*

The sterols produced by *P. carinii* were comprised of roughly equal proportions of ergostane and stigmastane 24-alkyl sterols (Table 2). In contrast, the *S. cerevisiae erg6* knock out transformed with the *P. carinii erg6* gene had much less sterols with stigmastane side chains. Both *P. carinii* and *S. cerevisiae* strains (native and transformed) were shown to have SAM:SMT activity and both have the *erg4* gene that codes for the enzyme C-24(28) sterol reductase. In the yeast, the *P. carinii* SAM:SMT appears to be less able to add a second methyl group at the methylene function. It is not known if the C-24(28) sterol reductase in the yeast has higher activity than its counterpart in *P. carinii* that reduces substrate availability for the second methyl transfer by the *P. carinii* SAM:SMT; whether the flux of newly synthesized ergostane sterols is particularly high toward membrane synthesis in the yeast cell; or due to other differences between the yeast and *P. carinii*. Thus, it cannot be ruled out that other differences in cellular architecture, biochemistry, or

physiology between these cells might play important roles in the accumulation and hence the distinct compositions of the two eukaryote species.

This report provided definitive biochemical data that a *S. cerevisiae* mutant lacking a functional *erg6* gene was transformed with the *P. carinii erg6* gene leading to the production in the yeast cell of a functional SAM:SMT enzyme protein. Complementation was demonstrated.

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Fig. 1.
Sterol carbon positions. An example of carbon numbers is shown for the sterol pneumocysteroles.

Fig. 2.
HPLC traces of the combined 4,4-dimethyl and 4 α -methyl sterols of the yeast strains analyzed in this study. The identities of the sterols are given with the letter codes above the peaks (see Figure 5 for names and structures). Asterisks indicate the stigmastane sterols produced by the *P. carinii* sterol methyltransferase.

Fig. 3. Western blot analysis using α -V5-HRP antibodies. Lanes 1 & 2. Two separate cultures of *S. cerevisiae erg6* expressing the *P. carinii* S-adenosylmethionine:sterol C-24 methyltransferase (SAM:SMT) protein. The α -V5 antibodies bind to the V5-epitope tag on the SAM:SMT protein demonstrating that the recombinant *P. carinii* SAM:SMT is expressed and is of the expected size (MW ~43 kDa) (bold arrow). Lane 3. ColorBurst™ molecular mass ladder.

Fig. 4. Gas-liquid chromatograms of sterols from *S. cerevisiae* *erg6* knockout mutant strain YML008C (*erg6*) (A) and mutant transformed with the *P. carinii* *erg6* gene (B). The major peaks in the wild type yeast and the *erg6* strain transformed with the *P. carinii* or the *S. cerevisiae* (not shown) *erg6* gene was ergosterol. The major peak in the *erg6* mutant yeast contained zymosterol and other cholestane sterols.

Fig. 5. Structures of sterols identified in *S. cerevisiae* strains. **A.** Lanosterol. **B.** 24-Methylenelanosterol. **C.** (24Z)-24-Ethylidenelanosterol (Pneumocysterol). **D.** (24S)-24-Ethyllanosta-8,25-dienol. **E.** 14-Desmethylanosterol. **F.** 14-Desmethyl-24-methylenelanosterol. **G.** (24Z)-24-Ethylidene-14-desmethyllanosterol. **H.** 4 α ,14 α -Dimethylcholesta-8,24-dienol. **I.** 4 α ,14 α -Dimethylergosta-8,24(28)-dienol (Obtusifoliol). **J.** 4 α -Methylcholesta-8,24-dienol. **K.** 4 α -Methylergosta-8,24(28)-dienol. **L.** (24Z)-4 α -Methylstigmasta-8,24(28)-dienol. **M.** Cholesta-8,24-dienol (Zymosterol). **N.** Cholesta-7,24-

dienol **O**. Cholesta-5,7,24-trienol. **P**. (22*E*)-Cholesta-5,7,22,24-tetraenol. **Q**. (22*Z*)-Cholesta-5,7,22,24-tetraenol. **R**. Cholesta-5,8,24-trienol. **S**. Cholesta-5,7,9(11),24-tetraenol. **T**. (Ergosta-8,24(28)-dienol (Fecosterol). **U**. (24*S*)-Ergost-8-enol. **V**. Ergosta-7,24(28)-dienol (Episterol). **W**. Ergosta-7,22,24(28)-trienol. **X**. (24*S*)-Ergost-7-enol (Fungisterol). **Y**. (24*R*)-Ergosta-7,22-dienol. **Z**. Ergosta-5,7,24(28)-trienol. **AA**. (24*S*)-Ergosta-5,7-dienol. **BB**. (24*R*)-Ergosta-5,7,22-trienol (Ergosterol). **CC**. (24*S*)-Ergosta-5,8-dienol. **DD**. (24*R*)-Ergosta-5,8,22-trienol. **EE**. (24*S*)-Ergosta-5,7,9(11)-trienol. **FF**. (24*R*)-Ergosta-5,7,9(11),22-tetraenol. **GG**. (24*Z*)-Stigmasta-8,24(28)-dienol. **HH**. (24*Z*)-Stigmasta-7,24(28)-dienol. **II**. (24*Z*)-Stigmasta-5,7,24(28)-trienol. **JJ**. (24*S*)-Stigmasta-5,7-dienol. **KK**. (24*R*)-Stigmasta-5,7,22-trienol.

Fig. 6.
Schematic showing formation of 14-alkyl side chains in *Saccharomyces cerevisiae* (*S.c.*)
and *Pneumocystis carinii* (*P.c.*).

Table 1

Quantification of sterols in wild-type *S. cerevisiae*, *S. cerevisiae* *erg6* knockout mutant, and the mutant transformed with the *S. cerevisiae* or the *P. carinii* *erg6* gene. Definitive structural assignments were determined by NMR of sterols isolated by TLC and HPLC. Bolded numbers indicate sterols found in only the strain transformed with the *P. carinii* *erg6* (*Sc erg6_{Pcerg6}*) in the present study.

Sterol ^a	% of Total			
	WT ^b	Sc <i>erg6</i> ^c	Sc <i>erg6_{Scerg6}</i> ^d	Sc <i>erg6_{Pcerg6}</i> ^e
Lanosterol (A) ^{f,g,h}	13.2	5.1	8.6	5.0
24-Methylenlanosterol (B) ^{g,h}	ND ⁱ	ND	0.9	2.5
(24Z)-24-Ethylidenlanosterol (Pneumocysterol) (C) ^{g,h}	ND	ND	ND	3.5
(24S)-24-Ethyllanosta-8,25-dienol (D) ^g	ND	ND	ND	0.2
14-Desmethylanosterol (E) ^{f,h}	1.5	1.2	1.8	0.8
14-Desmethyl-24-methylenlanosterol (F) ^g	ND	ND	0.6	0.6
(24Z)-24-Ethylidene-14-desmethyllanosterol (G) ^{g,h}	ND	ND	ND	0.3
4α,14α-Dimethylcholesta-8,24-dienol (H) ^f	0.5	ND	ND	0.2
4α,14α-Dimethylergosta-8,24(28)-dienol (Obtusifoliol) (I) ^{f,g}	0.4	ND	ND	0.1
4α-Methylcholesta-8,24-dienol (J) ^{f,h}	0.6	0.6	0.6	0.4
4α-Methylergosta-8,24(28)-dienol (K) ^{f,h}	0.8	ND	1.6	0.4
(24Z)-4α-Methylstigmasta-8,24(28)-dienol (L) ^h	ND	ND	ND	0.2
Cholesta-8,24-dienol (Zymosterol) (M) ^{f,h}	2.7	14.8	0.3	0.7
Cholesta-7,24-dienol (N)	ND	2.6	ND	ND
Cholesta-5,7,24-trienol (O)	ND	39.4	ND	ND
(22E)-Cholesta-5,7,22,24-tetraenol (P)	ND	30.2	ND	ND
(22Z)-Cholesta-5,7,22,24-tetraenol (Q)	ND	2.8	ND	ND
Cholesta-5,8,24-trienol (R)	ND	2.6	ND	ND
Cholesta-5,7,9(11),24-tetraenol (S)	ND	0.7	ND	ND
Ergosta-8,24(28)-dienol (Fecosterol) (T) ^{f,h}	5.6	ND	ND	ND
(24S)-Ergost-8-enol (U) ^{f,h}	0.4	ND	0.3	0.1
Ergosta-7,24(28)-dienol (Episterol) (V) ^f	8.0	ND	2.6	1.9
Ergosta-7,22,24(28)-trienol (W) ^f	0.3	ND	ND	ND
(24S)-Ergost-7-enol (Fungisterol) (X) ^{f,h}	1.0	ND	1.7	0.9
(24R)-Ergosta-7,22-dienol (Y) ^f	0.5	ND	1.1	0.7
Ergosta-5,7,24(28)-trienol (Z) ^f	1.4	ND	0.4	0.3
(24S)-Ergosta-5,7-dienol (AA) ^f	7.6	ND	7.2	6.7
(24R)-Ergosta-5,7,22-trienol (Ergosterol) (BB) ^f	54.1	ND	70.5	69.2
(24S)-Ergosta-5,8-dienol (CC) ^f	0.2	ND	ND	ND
(24R)-Ergosta-5,8,22-trienol (DD) ^f	ND	ND	0.2	0.2

Sterol ^a	% of Total			
	WT ^b	Sc <i>erg6</i> ^c	Sc <i>erg6</i> _{Sc<i>erg6</i>} ^d	Sc <i>erg6</i> _{P<i>erg6</i>} ^e
(24 <i>S</i>)-Ergosta-5,7,9(11)-trienol (EE) ^f	ND	ND	0.2	0.2
(24 <i>R</i>)-Ergosta-5,7,9(11),22-tetraenol (FF) ^f	0.5	ND	1.4	1.5
(24 <i>Z</i>)-Stigmasta-8,24(28)-dienol (GG)	ND	ND	ND	0.3
(24 <i>Z</i>)-Stigmasta-7,24(28)-dienol (HH)	ND	ND	ND	1.2
(24 <i>Z</i>)-Stigmasta-5,7,24(28)-trienol (II)	ND	ND	ND	0.6
(24 <i>S</i>)-Stigmasta-5,7-dienol (JJ)	ND	ND	ND	0.3
(24 <i>R</i>)-Stigmasta-5,7,22-trienol (KK)	ND	ND	ND	1.0

^a Capital letters in parentheses indicate their structures as shown in Fig. 2. All sterols had the 3-β configuration and all without a 5 double bond are 5α-sterols.

^b Data for *S. cerevisiae* WT parental strain BY4742 from Nkinin et al. 2011.

^c *S. cerevisiae* *erg6* knockout strain YML008C derived from WT parental strain BY4742.

^d *S. cerevisiae* *erg6* knockout strain YML008C ((*erg6*) transformed with the *S. cerevisiae* *erg6* gene (control)

^e *S. cerevisiae* *erg6* knockout strain YML008C transformed with the *P. carinii* *erg6* gene.

^f NMR spectral data shown in Nkinin et al., 2011

^g NMR spectral data shown in Giner et al., 2004

^h NMR spectral data shown in Giner et al., 2002

ⁱ ND, not detected.

^j Tentative identification.

Table 2

Pneumocystis carinii 24-alkylsterols.

Sterol	% of total ^a	Adjusted % of total ^b
<u>Ergostane Sterols:</u>		
24-Methylenlanosterol (B) ^c	0.29	2.04
14-Desmethyl-24-methylenlanosterol (F)	0.21	1.47
4 α -Methylergosta-8,24(28)-dienol (K)	0.27	1.89
4 α -Methylergosta-8(14),24(28)-dienol	0.05	0.35
Ergosta-7,24(28)-dienol (Episterol) (V)	1.16	8.14
(24S)-Ergost-7-enol (Fungisterol) (X)	2.51	17.61
Ergosta-5,24(28)-dienol (24-methylenecholesterol)	0.65	4.56
(24S)-Ergost-5-enol	1.92	13.47
25-Methylergosta-5,24(28)-dienol	0.02	0.14
Ergosta-8,24(28)-dienol (Fecosterol) (T)	0.11	0.35
(24S)-Ergost-8-enol (U)	0.17	1.19
Ergosta-8(14),24(28)-dienol	0.05	0.35
(24S)-Ergost-8(14)-enol	0.48	3.37
(24S)-5 β -Ergostanol	0.15	0.89
Sum		56.40
<u>Stigmastane Sterols:</u>		
(24Z)-24-Ethylidenlanosterol (Pneumocysterol) (C)	0.31	2.18
(24Z)-24-Ethylidene-14-desmethyllanosterol (G)	0.10	0.70
(24Z)-4 α -Methylstigmasta-8,24(28)-dienol (L)	0.04	0.28
(24Z)-Stigmasta-7,24(28)-dienol (HH)	2.60	18.25
(24S)-Stigmast-7-enol	1.30	9.12
(24S)-Stigmasta-7,25-dienol	0.06	0.42
(24Z)-Stigmasta-5,24(28)-dienol	0.68	4.77
(24S)-Stigmast-5-enol	0.35	2.46
(24S)-Stigmasta-5,25-dienol	0.04	0.28
(24Z)-Stigmasta-8,24(28)-dienol (GG)	0.15	1.05
(24Z)-Stigmasta-8(14),24(28)-dienol	0.29	2.04
(24S)-Stigmast-8(14)-enol	0.24	1.68
Sum		43.23

^aFrom Giner et al. 2002. Sterol analyses were performed on purified preparations of *Pneumocystis carinii* isolated from the lungs of corticosteroid-immunosuppressed rats infected with *P. carinii* (Kaneshiro et al., 1993).

^bCalculated without cholesterol (synthesized by the rat) and 24 α -alkyl and ²²phytosterols present in rat diets. These are regarded as sterols scavenged by *P. carinii* from its environment in the rat lung (Giner et al., 2002).

^cLetters in parentheses indicate structures shown in figure 3.