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S-Adenosyl-L-Methionine Inhibitors Δ^{24}-Sterol Methyltransferase and $\Delta^{24(28)}$-Sterol Methylreductase as Possible Agents against *Paracoccidioides brasiliensis*

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We studied the antiproliferative effects of three azasterol analogs [piperidyl-2-yl-5α-pregnan-3β,20(*R*)-diol (AZA-1), 22-piperidin-2-yl-pregnan-22(*S*),3β-diol (AZA-2), and 22-piperidin-3-yl-pregnan-22(*S*),3β-diol (AZA-3)] and their effects on the lipid composition of the pathogenic yeastlike phase of the dimorphic fungus *Paracoccidioides brasiliensis*. Inhibition was 100% for AZA-1 at 5 μM, 62% for AZA-2 at 10 μM, and 100% for AZA-3 at 0.5 μM. The analogs inhibited different stages of the sterol biosynthesis pathway.

Membrane sterol biosynthesis is one of the few areas of difference in primary metabolism between mammals and other eukaryotes, such as plants, fungi, and protozoa. While mammals synthesize C_{27} cholestane-based members of the steroid family, pathogenic fungi, protozoa, and plants require the presence of endogenous sterols (typically ergosterol and 24-alkyl analogs) which act as essential growth factors for these organisms (4, 12). The enzyme responsible for the addition of these alkyl groups to carbon C-24 and for the regulation of carbon flow in the sterol pathway is the Δ^{24}-sterol methyltransferase (SMT) (6). The critical role of this enzyme has stimulated considerable interest in the rational design of SMT inhibitors for potential clinical or agrochemical use as antifungal agents (1, 2, 12). Several sterol analogues behave as antiproliferative agents against fungi, yeast, protozoa, and plants in vitro (1, 2, 8, 11, 12). One sterol analogue, 20-piperidyl-2-yl-5α-pregnan-3β,20(*R*)-diol (AZA-1) has been reported to be an SMT inhibitor in the protozoan species *Trypanosoma cruzi*, *Leishmania donovani*, and the fungus *Pneumocystis carinii* (5, 11, 12). The antiproliferative effect of AZA-1 against these organisms coincided with the depletion of 24-alkyl-sterols and their complete replacement by Δ^{24}-cholesta-type sterols. Recently, Atencio et al. (1) studied the molecular parameters of AZA-1 and its *N*-methyl derivative and established some structure-activity correlation. The information obtained from these compounds and the steric-electric plug model proposed by Nes (6) has allowed the design and synthesis of two new azasterols, 22-piperidin-2-yl-pregnan-22(*S*),3β-diol (AZA-2) and 22-piperidin-3-yl-pregnan-22(*S*),3β-diol (AZA-3) (Fig. 1) (Visbal et al., submitted).

To explore the potential use of these new drugs, we studied the effects of azasterol analogs AZA-1, AZA-2, and AZA-3 on growth and sterol profile in the pathogenic yeastlike (Y) phase of *Paracoccidioides brasiliensis*. This fungus is a thermally dimorphic fungus, the causative agent of paracoccidioidomycosis, a prevalent human systemic mycosis in Latin America where it is geographically constrained. *P. brasiliensis* is sensitive to sterol biosynthesis inhibitors, such as ketoconazole, itraconazole, and saperconazole (9), and is also affected by ajoene, a derivative of allicin, extracted from garlic (8). In the search for new antifungal agents, we chose to test AZA-1, AZA-2, and AZA-3 in *P. brasiliensis* Y phase.

Experiments were performed as previously reported (5, 8, 9). *P. brasiliensis* strain Pb73 (ATCC 32071) was grown at 37°C in 50 ml of PYG liquid medium after inoculation with 10 ml of a seed culture and supplemented with one of the drugs under study. Drugs were dissolved in dimethyl sulfoxide (DMSO) to a final organic solvent concentration in the culture medium that never exceeded 1% (vol/vol). Azasterols were tested in the range of 0.1 to 10 μM. The fungus was grown in the absence of inhibitor (though in the presence of 1% DMSO [vol/vol]) as a control. Cultures were incubated for 5 days at 37°C (Y phase) with continuous shaking on a gyratory shaker operated at 120 rpm. Cell density was measured by turbidimetry in Klett units (9, 10). For lipid analysis, cells were treated as described previously (11, 12), followed by gas-liquid chromatography coupled to mass spectrometry (11, 12).

The antiproliferative effects of azasterols on *P. brasiliensis* are shown in Fig. 2. In the presence of AZA-1, an inhibitor of SMT (1, 4, 11, 12), a dose-dependent inhibition of growth was observed from 0.1 to 5 μM, with 100% growth arrest at 5 μM and higher concentrations. AZA-2 was only able to inhibit growth by 60% at the highest concentration used in these experiments (10 μM). On the other hand, AZA-3 was the most powerful drug, as a concentration of 0.5 μM was able to completely inhibit fungal growth. All three drugs were fungistatic rather than fungicidal, as treated cells resuspended in fresh medium reinitiated normal growth (data not shown), an effect also observed for azoles (3).

To explore which steps in the sterol biosynthesis pathway were blocked, subinhibitory concentrations of drugs were used. *P. brasiliensis* Y cells were grown with 1 μM AZA-1, 2 μM AZA-2, or 0.3 μM AZA-3, as these concentrations yielded a 50% arrest in cell growth at 120 h. The results of lipid analyses

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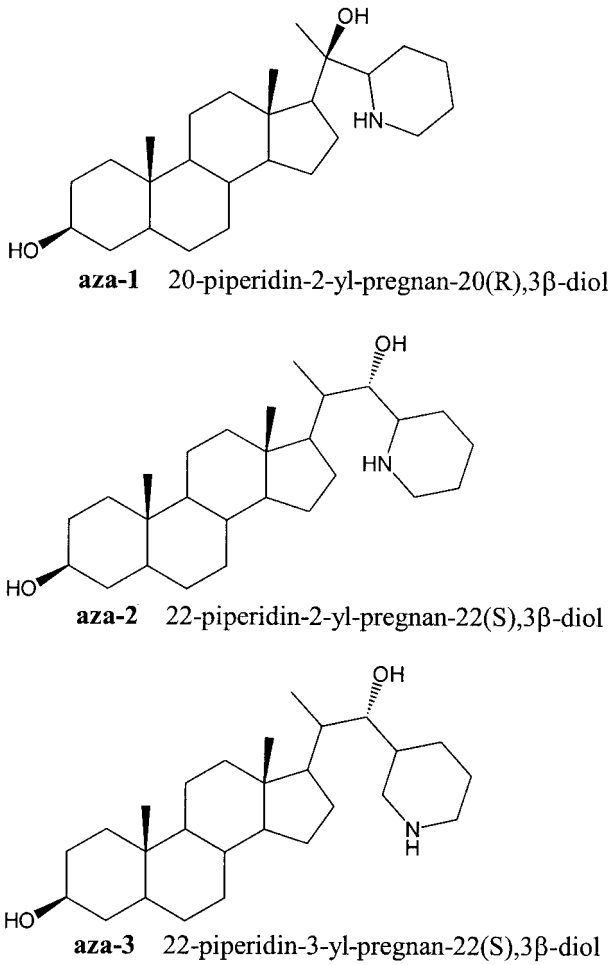


FIG. 1. Chemical structures of AZA-1, AZA-2, and AZA-3.

are shown in Table 1. In control cells, the main sterols were brassicasterol (compound G) (69.1%), ergosterol (compound F) (26.8%), and lanosterol (compound A) (4.1%) (Fig. 3), as reported previously (10). On exposure to AZA-1, ergosta-5,7,24(28)-trien-3β-ol (17.1%) (compound D′) and lanosterol (compound A) (10.8%) accumulated, while exposure to AZA-2 led to an important accumulation of ergosta-5,7,22,24(28)-tetraen-3β-ol (compound E) (50.5%). With AZA-3, an important accumulation of lanosterol (compound A) (34.5%) was observed. Concurrent with the accumulation of these intermediates, final products of the sterol pathway (compounds F and G) decreased substantially (Table 1).

P. brasiliensis (Y phase) was susceptible to the action of azasterols in the following sequence: AZA-3 > AZA-1 > AZA-2. However, they did not act on the same steps in the pathway of sterol biosynthesis. AZA-1 and AZA-2 significantly inhibited the $\Delta^{24\ (28)}$-sterol methylreductase (SMR), the enzyme that catalyzes the saturation of the $\Delta^{24\ (28)}$ double bond during the biosynthesis of compound G (Fig. 3). The inhibition of SMR causes the accumulation of ergosta-5,7,24(28)-trien-3β-ol (compound D′) observed when cells were treated with AZA-1 and the accumulation of ergosta-5,7,22,24(28)-tetraen-3β-ol (compound E) observed when cells were treated with

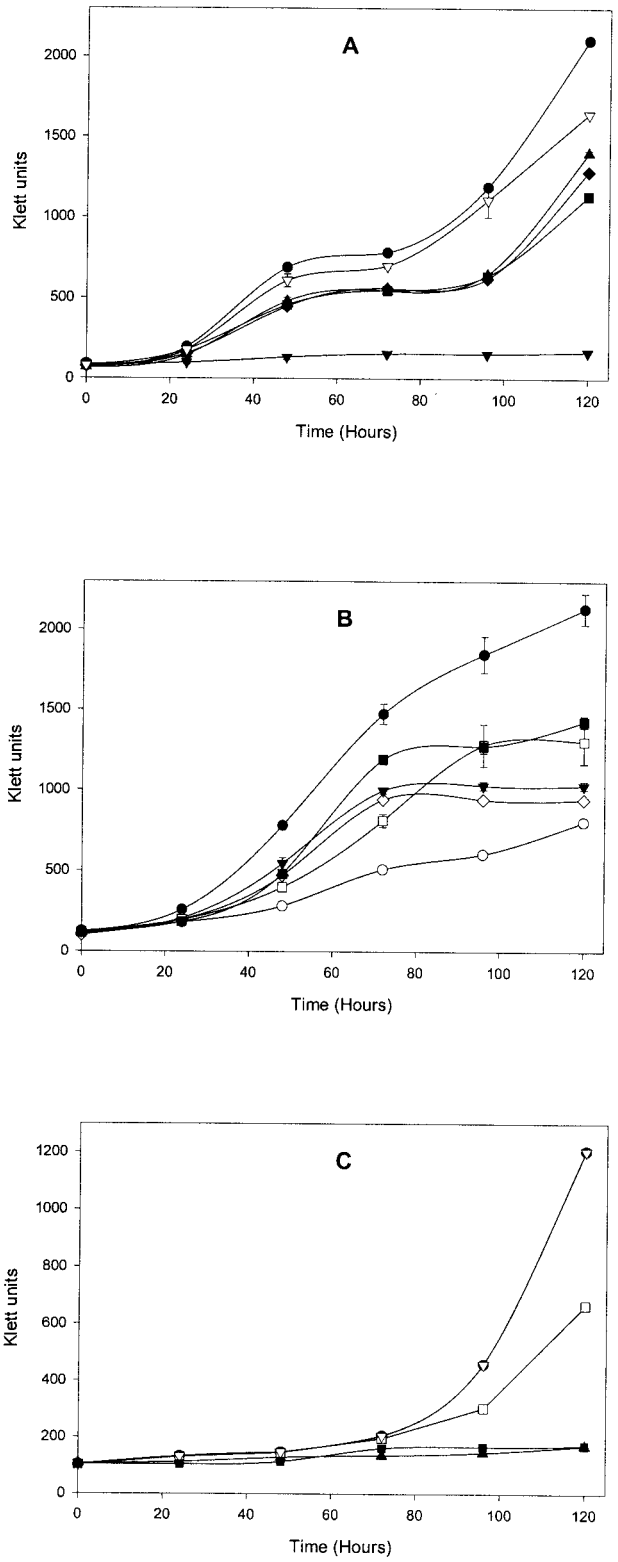
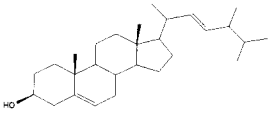
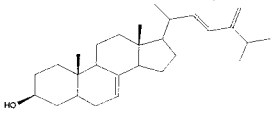
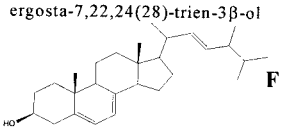
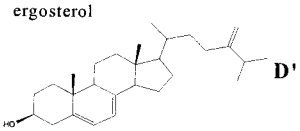
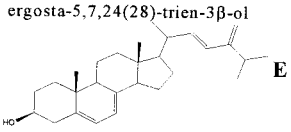
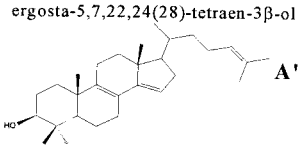
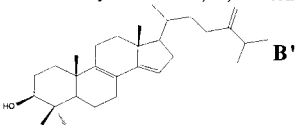
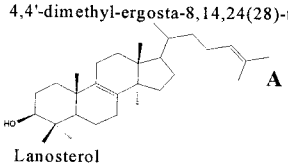
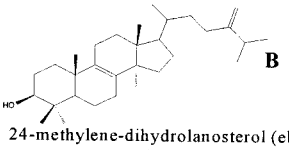
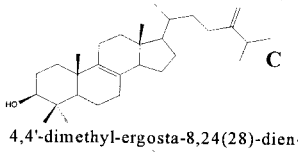
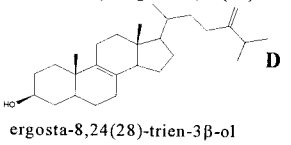


FIG. 2. Antiproliferative effects of AZA-1 (A), AZA-2 (B), and AZA-3 (C) on *P. brasiliensis* (Y phase). Symbols: ●, no azasterol (control); ○, 10 μM; ◇, 8 μM; ▼, 5 μM; △, 2 μM; ■, 1 μM; ◆, 0.8 μM; ▲, 0.5 μM; □, 0.2 μM; ▽, 0.1 μM. The mean values ± standard errors (error bars) for triplicate cultures are shown.

TABLE 1. Free sterols present in *P. brasiliensis* grown in the absence or presence of azasterols

Compound and molecular structure	Retention time (min)	% Composition[a]			
		Control	AZA-1 (1 μM)	AZA-2 (2 μM)	AZA-3 (0.3 μM)
 **G** ergosta-5,22-dien-3β-ol (brassicasterol)	17.6	69.1	54.7	13.5	51.9
 ergosta-7,22,24(28)-trien-3β-ol	18.0	0.0	0.0	23.5	0.0
 **F** ergosterol	18.2	26.8	17.4	0.0	12.2
 **D'** ergosta-5,7,24(28)-trien-3β-ol	18.5	0.0	**17.1**	0.0	0.0
 **E** ergosta-5,7,22,24(28)-tetraen-3β-ol	18.5	0.0	0.0	**50.5**	0.0
 **A'** 4,4'-dimethyl-cholesta-8,14,24-trien-3β-ol	19.2	ND	ND	ND	ND
 **B'** 4,4'-dimethyl-ergosta-8,14,24(28)-trien-3β-c	19.9	ND	ND	ND	ND
 **A** Lanosterol	20.5	4.1	**10.8**	8.2	**34.5**
 **B** 24-methylene-dihydrolanosterol (eburicol)	21.3	0.0	0.0	4.2	1.4
 **C** 4,4'-dimethyl-ergosta-8,24(28)-dien-3β-ol	ND[b]	ND	ND	ND	ND
 **D** ergosta-8,24(28)-trien-3β-ol	ND	ND	ND	ND	ND

[a] Composition as a percentage of the total mass for cells grown in the presence of azasterols or in control cells. The values shown in bold type are discussed in the text.

[b] ND, not detected.

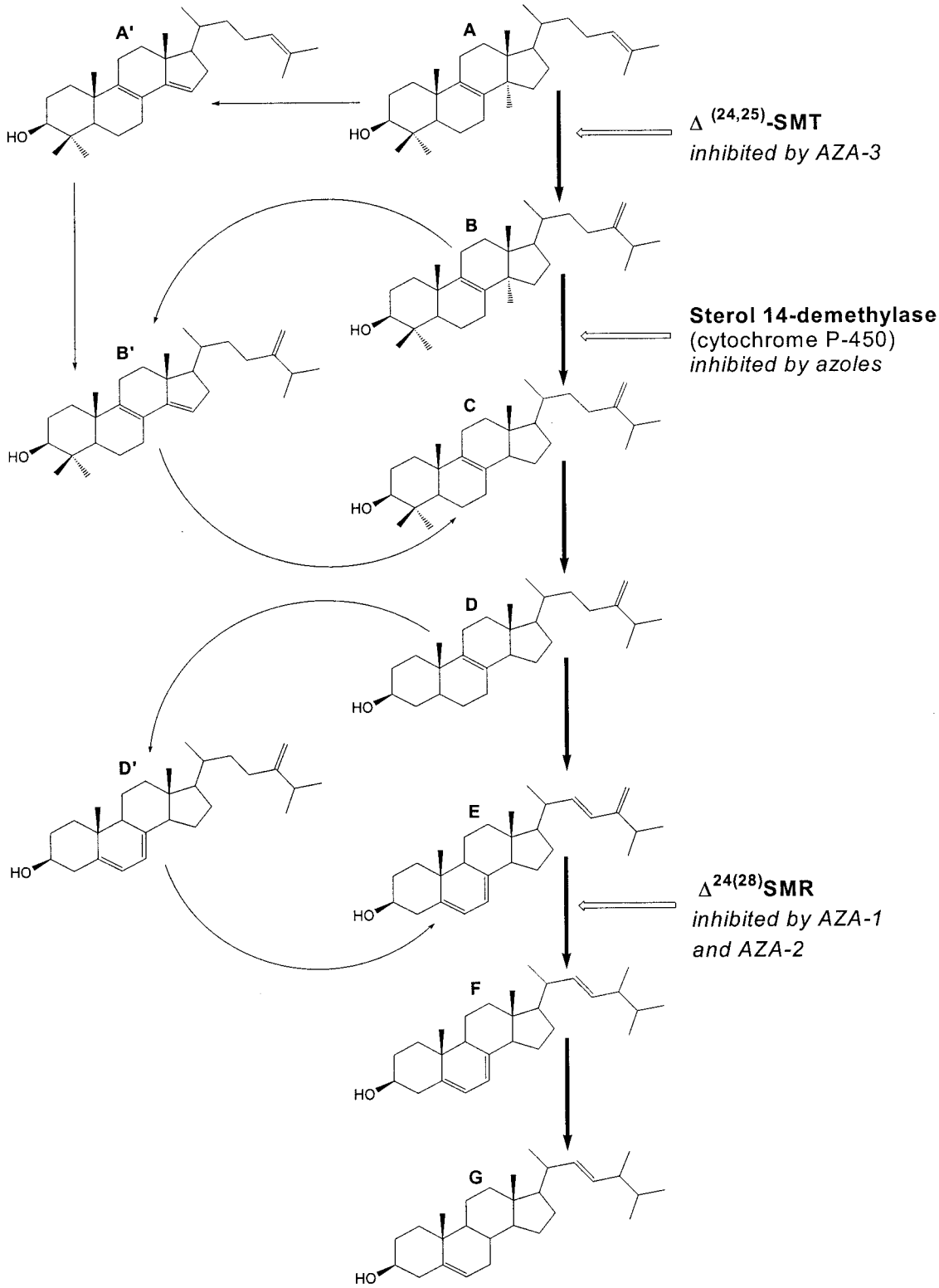


FIG. 3. Simplified pathway of sterol biosynthesis in *P. brasiliensis* and sites of action of the inhibitors used herein. Compound A, lanosterol; compound B, 24-methylene-dihydrolanosterol (eburicol); compound C, 4,4′-dimethyl-ergosta-8,24(28)-dien-3β-ol; compound D, ergosta-8,24(28)-dien-3β-ol; compound E, ergosta-5,7,22,24(28)-tetraen-3β-ol; compound F, ergosterol, the major sterol of most fungi; compound G, ergosta-5,22-dien-3β-ol (brassicasterol), the major sterol in *P. brasiliensis*; compound A′, 4,4′-dimethyl-cholesta-8,14,24(28)-trien-3β-ol; compound B′, 4,4′-ergosta-8,14,24(28)-trien-3β-ol; compound D′, ergosta-5,7,24(28)-trien-3β-ol. The main reaction pathways are indicated by the thick lines.

AZA-2. Therefore, AZA-1 impaired both SMR activity and the introduction of the Δ^{22} bond in the sterol side chain, in agreement with previous studies (4, 11, 12). AZA-2 was much more selective as an SMR inhibitor, due to the accumulation of compound E without interference with the metabolic transformation of the B-C ring system and side chain of the sterol molecule. In addition, a modest accumulation of lanosterol (compound A) suggested that SMT was also slightly affected by AZA-1. AZA-3 produced a major antiproliferative effect on *P. brasiliensis* that was due to SMT inhibition, as deduced from the important accumulation of lanosterol (compound A) recorded in this case. This contrasts with the reported effect of ketoconazole (3) on the accumulation of 24-methylene dihydrolanosterol (compound B) through the inhibition of the cytochrome P-450-dependent C14α-demethylase (3).

Through the rational design of fungal inhibitors of the sterol biosynthesis pathway, some structure-activity correlations can be drawn for the behavior of azasterols against enzymes involved in the biosynthesis of fungal sterols. AZA-3 has a nitrogen atom in a different orientation than that for the other two analogs, and it specifically inhibits the SMT enzyme of *P. brasiliensis*. The nitrogen orientation in AZA-3 and the resulting tertiary amine protonated at physiological pH have a positive charge at a position that is probably near the position occupied by the unstable high-energy intermediate in the active site of this enzyme on *P. brasiliensis*. In addition, the accumulation of lanosterol (compound A) caused by AZA-3 showed that its antiproliferative action is associated with the depletion of 24-methyl sterols. This correlation supports the notion that the presence of an alkyl substituent in position 24 of the sterol molecule is an essential feature for physiological functions of these molecules in *P. brasiliensis*, as shown previously for the protozoan *T. cruzi*, *L. donovani*, and the fungus *P. carinii* (4, 11, 12).

Finally, we propose that the sterol biosynthesis pathway in *P. brasiliensis* goes one step forward in the classical route, ending up in brassicasterol (compound G) instead of ergosterol (compound F) (Fig. 3), a situation similar to that observed in vascular plants where the final products in sterol biosynthesis are Δ^5-24-alkyl sterols, such as stigmasterol, sitosterol, and campesterol (6).

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