Cholesterol import fails to prevent catalyst-based inhibition of ergosterol synthesis and cell proliferation of *Trypanosoma brucei*

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Abstract *Trypanosoma brucei* (TB) cultured in rat blood, bovine serum, or lipid-depleted serum generated distinct differences in cholesterol availability. Whereas cell proliferation of the parasite was relatively unaffected by cholesterol availability, the ratios of cellular ergostenols to cholesterol varied from close to unity to 3 orders of magnitude different with cholesterol as the major sterol (>99%) of bloodstream form cells. In the procyclic form cultured with lipid-depleted serum, 15 sterols at 52 fg/cell were identified by GC-MS. The structures of these sterols reveal a nonconventional ergosterol pathway consistent with the novel product diversity catalyzed by the recently cloned sterol methyltransferase (SMT). A potent transition state analog of the TB SMT C24 alkylation reaction, 25-azalanosterol (25-AL; inhibition constant Ki = 39 nM), was found to inhibit the growth of the procyclic and bloodstream forms at an IC50 of ~1 μM. This previously unrecognized catalyst-specific inhibition of cell growth was unmasked further using the 25-AL-treated procyclic form, which, compared with control cultures, caused a change in cellular sterol content from ergostenols to cholesterol.** However, growth of the bloodstream form disrupted by 25-AL was not rescued by cholesterol absorption from the host, suggesting an essential role for ergosterol (24-methyl sterol) in cell proliferation and that the SMT can be a new enzyme target for drug design.—Zhou, W., G. A. M. Cross, and W. D. Nes.


Supplementary key words antiparasitic drugs • 25-azalanosterol • enzyme-based inhibitors • protozoa • trypanosomes

*Trypanosoma brucei* (TB), the causative agent for African sleeping sickness, has had its genome sequenced (1) and is reported to be a reemerging disease, with an estimated 50,000 deaths in 2002 (2). The current chemotherapy of human African trypanosomiasis relies on six drugs that display undesirable toxic side effects, and in some cases drug resistance has developed (3). According to Croft, Barrett, and Urbina (4), there is hope to treat some forms of trypanosomiasis using chemotherapy that leads to impaired enzyme action of the parasite sterol pathway. The opportunity to exploit the sterol synthetic pathway in TB for antiparasitic therapy is significant because few enzymes are targeted commercially for chemotherapy (e.g., ornithine decarboxylase) (4, 5).

Although a great deal of research has actually been done with TB, some serious anomalies still exist. The life cycle of TB includes two experimentally accessible developmental forms: one associated with its insect (tsetse) vector (procyclic form), and one from the animal host (bloodstream form). The procyclic form can synthesize C28-phytosterols that typically contain a Δ5,7-diene system, as found in ergosterol normally synthesized by trypanomatid protozoa (6, 7). Consistent with the organism possessing an intact pathway, recent cloning efforts of enzymes of the TB sterol pathway led to the characterization of the lanosterol synthase, 14α-demethylase, and sterol methyltransferase (SMT) enzymes (8–10). Nonetheless, the bloodstream form is thought to lack de novo sterol synthesis; host cholesterol, accumulated by the cell through receptor-mediated endocytosis and metabolism of lipoproteins present in the bloodstream and in the serum supplements added to the culture medium, is used to satisfy the sterol requirements of the cell (6, 11). Yet, based on the 24-methyl sterol pathway and function expected to operate in protozoa, one might expect that, in the absence of a 24-methyl sterol, bloodstream TB would cease growing. Paradoxically, it can survive in the host, presumably by a stoichiometric replacement of the protozoan 24-methyl sterol(s) with human cholesterol (6, 11, 12). This is especially puzzling, because the bloodstream form of the organism is reported to respond quite well to ergosterol biosynthesis inhibitors that target enzymes in the...
postqualene stages of synthesis (13). A further inconsistency is that the ergosterol pathway in the procyclic form of TB is considered to be similar to that of Trypanosoma cruzi and other protozoa (6, 7). However, the recent enzyme-generated product set of the cloned C24 SMT (10) indicates that TB should possess an ergosterol pathway that is atypical for protozoa and similar in many ways to the side chain pathway synthesized by the nonphotosynthetic alga Protothec a wickerhamii (14).

These anomalies, together with our interest in designing taxa-specific, catalyst-based drugs to interfere with SMT activity (14–16), led us to reinvestigate the sterol composition of TB cultured under various conditions of cholesterol availability and in the presence of an inhibitor discovered by us to block catalysis of the cloned TB SMT (10). One reason we have pursued a chemical-biological study of TB is to establish the correct structures and amounts of sterol that occur in the organism at different developmental stages. A more direct rationale for investigating the sterol pathway of TB was to reveal the importance of the C24 methylation reaction in ergosterol synthesis in the bloodstream form. Our observation, consistent with the findings of others (6, 12), that TB cells can accumulate vast amounts of human cholesterol concurrently with a downregulation of the native ergosterol pathway, is intriguing because it implies that the protozoan has developed some degree of sterol auxotrophy and readily will absorb a variety of exogenous sterols to permit sterols to play a dual function in trypanosomes: one structural, which can be satisfied by exogenous sterol, and the other metabolic, for which there are more strict requirements, satisfied only by biosynthesis. In support of this view, we found that the endogenous ergosterol pathway is not eliminated by cholesterol import, nor is the functional importance of hormonal levels of ergosterol discontinued. Thus, as reported here, a transition state analog of the sterol C24 methylation step in ergosterol synthesis is an effective growth inhibitor against both the procyclic and bloodstream forms.

MATERIALS AND METHODS

Parasite and culture procedures

Typically, TB Lister 427 was the strain studied and grown at the Rockefeller University in SDM-79 medium (procyclic form) with 10% bovine serum or 10% “lipid-free serum” (Sigma) at 25°C. The bloodstream form was grown at 37°C in HMI-9 medium containing 10% bovine serum (Sigma) and 10% Serum Plus™ (SAFC Biosciences; includes fetal bovine serum) (17, 18). For one set of experiments with the procyclic form involving inhibitor treatment, TB strain 927 was cultured in Cunningham’s medium (19) supplemented with 10% bovine serum. Bloodstream forms were grown in rats (Sprague Dawley male rats from Charles River Laboratories) fed the standard rat chow (Research Diets). Procyclic forms were grown in rats (Sprague Dawley male rats from Charles River Laboratories) fed the standard rat chow (PicLab Rodent Diet) (20).

For sterol analysis of a bulk preparation of the bloodstream form, parasites were harvested by a standard method that separates the trypanosomes from all blood cells (20). The entire infected blood, harvested by aortic puncture, was centrifuged, the trypanosome layer was separated from most of the red cells by resuspension in Bicine-buffered saline glucose mixture (0.050 M Bicine, 0.077 M glucose, 0.050 M NaCl, 0.005 M KCl, pH 8.0, with NaOH), and the suspension was passed through a DEAE cellulose column to remove residual blood cells and any dead trypanosomes. The trypanosomes in the column flow-through were washed three times in Bicine-buffered saline glucose. Procyclic forms were harvested by centrifugation and washed three times in phosphate-buffered saline. Cell pellets were lyophilized and sent to Texas Tech University for sterol analysis.

Growth inhibition of TB

For inhibitor tests, bloodstream forms were washed and resuspended in fresh medium at a concentration of \(1 \times 10^6\) cells/ml to a final cell density of \(2 \times 10^6\) (monitored daily using a Coulter Counter from Beckman Coulter); for the procyclic form, cultures were seeded with \(1 \times 10^6\) cells/ml and grown to a final cell density of \(5 \times 10^6\) cells/ml. Multiwell plates containing 1 ml aliquots of culture per well were incubated for 72 h in a 5% \(\text{CO}_2\)/95% air mixture. Inhibitors of SMT were added to the culture medium as solutions in dimethyl sulfoxide. An equal amount of dimethyl sulfoxide was added to all cultures in a given series of experiments (not to exceed 0.5%, v/v) and had no effect on parasite proliferation. \(I_{50}\) determinations were undertaken with bloodstream and procyclic form cells assayed with inhibitor concentrations between 0 (control) and 10 \(\mu\)M.

Source and analysis of sterols

All sterols isolated from the parasite except the new compounds described here were prepared and characterized in our earlier publications (21–27). The transition state analog inhibitor, 25-azalanosterol (25-AL), was prepared as described (23). Bloodstream and procyclic form cells, collected after centrifugation, were saponified using 10% KOH in 98% aqueous methanol at reflux temperature for 30 min. The neutral lipids obtained by dilution with water and extraction with n-hexane were analyzed on a GC-MS apparatus with a Hewlett-Packard LS 6500 gas chromatograph interfaced to a 5973 mass spectrometer at 70 eV. GC was performed using an Agilent HP-5 column (30 m × 25 μm in diameter). Film thickness was 0.25 μm, the flow rate of He was set to 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. Single ion monitoring (SIM) was set at the fragment corresponding to \(m/z\) 396 amu, and ergosterol was used as the external reference standard. Identification of sterols was achieved by comparing the fragment pattern of the candidate specimen, ultraviolet light spectrum, and retention time of the sample peak relative to the retention of cholesterol in GC, and the retention time of the sample peak relative to the retention time of cholesterol (αc) in HPLC, with known standards in our sterol collection; cholesterol elutes in GC at 13 min and in HPLC at 20 min. Quantification of the amounts of sterols in the cells and in the medium preparations was accomplished by gas-liquid chromatography against a standard curve for cholesterol; in special cases, quantification was achieved using SIM referenced to ergosterol or HPLC referenced to a standard curve of ergosterol with the detector set at 282 nm. Using GC, cholesterol at 10 ng, or with HPLC, ergosterol at 1 ng, can be detected in the sample preparation. HPLC was performed on an Agilent 1100 series column equipped with a multiple wavelength diode array detector using a C18 reversed-phase analytical column (Phenomenex synergi, 4 μm) eluted with methanol at a flow rate of 1 ml/min. HPLC fractions in the sterol region of the chromatogram (αc, 0.5–2.0) were collected and applied to a thin layer of silica gel G (250 μm) using 15% diethyl ether in benzene as the solvent system. Cho-
lesterol, 31-norlanosterol, and lanosterol were applied to the TLC plate as 4-desmethyl, 4-monomethyl, and 4,4-dimethyl sterol standards, respectively (21).

RESULTS

Sterols of the TB procyclic form

Although ergosterol has long been known as the major sterol synthesized by protozoa, including TB, a more detailed analysis of the sterol composition of these protozoa has not been carried out using modern chromatographic techniques. In the first set of experiments, the total sterols of the nonsaponifiable lipid fraction were isolated from a set of procyclic cells grown to a final volume of 50 ml in lipid-deficient medium, and this mixture was analyzed chromatographically to determine the type and amount of sterols present in the cells. From the GC scan of the total sterols, 11 well-resolved peaks were observed with a major peak, 5(6), eluting in the chromatogram with a retention time coincident with that of an authentic specimen of ergosterol and a set of minor peaks that corresponded to the retention times of several other sterols available to us as reference compounds, including cholesterol and lanosterol (peaks 1 and 15, respectively) (Fig. 1A, Table 1).

In a separate analysis of the total sterol fraction by TLC (Table 1), three bands were evident upon spraying with sulfuric acid-methanol (50:50, v/v) and heating the plate; the band corresponding to cholesterol appeared greenish-blue, characteristic of a sterol (such as ergosterol) with a Δ5,7 system, consistent with that reported earlier for the sterols detected in TB (6). The less polar bands eluting in TLC (Table 1), corresponding to 31-norlanosterol and lanosterol, appeared light orange, as expected for sterols with a lanostenol-type structure (21). The total sterol content was determined by GC at 51 fg/cell.

Determination of the structure of the compounds in Fig. 1 by mass spectrometry, however, suggested to us that the sterol content of the cells was unconventional. The material in the first peak, 1, with molecular mass of M+ 386 amu, matched the mass spectrum of a cholesterol standard (22), but the second peak, 5(6), in GC, with the most mass and the correct retention time for ergosterol, possessed a molecular mass of M+ 382 amu, compared with molecular mass (M+, 396 amu) for that of ergosterol. The fragmentation and the relative intensities of the fragment ions for this compound revealed a 24-dealkyl sterol with three double bonds at positions C5, C7, and C24 (22). The Δ5,7 system generates a greenish-blue color rather a pinkish color for a Δ5-monoene system upon spraying the TLC plate with sulfuric acid-methanol; these results together with the GC-MS data confirm the abundance of cholesta-5,7,24-trienol in the cells. Our limits of detection in GC were such that we could not detect any ergosterol co-eluting with the cholesta-5,7,24-trienol and therefore assumed from the GC that ergosterol was not part of the sterol mixture.

The next set of major peaks in GC, corresponding to peaks 7 and 15, produced mass spectra of exact match to cholessta-7,24-dienol and lanosterol. Peaks 11 and 14 produced mass spectra similar to that of ergosterol, yet these compounds eluted later in GC from ergosterol, suggesting that they were structural isomers of ergosterol not heretofore reported as synthesized by TB. Minor GC peaks, several of which were found to represent pairs of compounds by further analysis using a combination of TLC and HPLC (Table 1), corresponding to peaks 2, 3(4), 6, 9(10), and
TABLE 1. Sterol composition of procyclic form TB cultured with lipid-depleted serum

<table>
<thead>
<tr>
<th>Sterol Structure</th>
<th>GC RRTc</th>
<th>HPLC RRTc</th>
<th>Ultraviolet Light 210 nm</th>
<th>TLC Relative Mobility</th>
<th>Molecular Weight</th>
<th>Percentage of Total Sterol</th>
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<tr>
<td>Cholesterol</td>
<td>1</td>
<td>1.00</td>
<td>210</td>
<td>0.19</td>
<td>386</td>
<td>20.0</td>
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<tr>
<td>Desmosterol</td>
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<td>210</td>
<td>0.19</td>
<td>384</td>
<td>1.0</td>
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<tr>
<td>Zymosterol</td>
<td>3</td>
<td>1.06</td>
<td>282</td>
<td>0.19</td>
<td>384</td>
<td>1.0</td>
</tr>
<tr>
<td>4a-Methylcholesta-8,24-dienol</td>
<td>4</td>
<td>1.06</td>
<td>NAa</td>
<td>210</td>
<td>0.25</td>
<td>398</td>
</tr>
<tr>
<td>Cholesta-5,7,24-trienol</td>
<td>5</td>
<td>1.10</td>
<td>0.71</td>
<td>282</td>
<td>0.19</td>
<td>382</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>6</td>
<td>1.10</td>
<td>282</td>
<td>0.19</td>
<td>396</td>
<td>0.3</td>
</tr>
<tr>
<td>Cholesta-7,24-dienol</td>
<td>7</td>
<td>1.12</td>
<td>0.83</td>
<td>210</td>
<td>0.19</td>
<td>384</td>
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<tr>
<td>Ergosta-8,25(27)-dienol</td>
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<td>1.14</td>
<td>0.80</td>
<td>210</td>
<td>0.19</td>
<td>398</td>
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<tr>
<td>3α-Norlanosterol</td>
<td>9</td>
<td>1.15</td>
<td>NA</td>
<td>0.19</td>
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<td>0.3</td>
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<td>Fecosterol</td>
<td>10</td>
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<td>NA</td>
<td>0.19</td>
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<td>0.74</td>
<td>282</td>
<td>0.19</td>
<td>396</td>
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<td>0.74</td>
<td>282</td>
<td>0.19</td>
<td>396</td>
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<tr>
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<td>1.19</td>
<td>NA</td>
<td>210</td>
<td>0.19</td>
<td>398</td>
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<tr>
<td>Ergosta-5,7,24(25)-trienol</td>
<td>14</td>
<td>1.27</td>
<td>0.83</td>
<td>282</td>
<td>0.19</td>
<td>396</td>
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<tr>
<td>Lanosterol</td>
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<td>1.30</td>
<td>0.94</td>
<td>0.30</td>
<td>426</td>
<td>1.0</td>
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</table>

12(13), were identified as desmosterol, zymosterol, 4-methyl zymosterol, ergosterol, ergosterol isomer (M+ 396), 31-norlanosterol, fecosterol, ergosterol isomer (M+ 396), and fecosterol isomer (M+ 398), by comparison of the mass spectrum of each of these compounds with the mass spectra of authentic compounds (14, 21, 22 and references cited therein). In addition, the character of the ultraviolet light spectrum generated for each compound during HPLC analysis using the multiple wavelength diode array detector can be diagnostic for a Δ5 versus a Δ8 system and, in the case of multiple unsaturation, whether a Δ5,7 system or a Δ8,14 system is present in the nucleus and in the side chain structure or whether a Δ22,24 structure is synthesized (21). Because the initial GC-MS run of the total sterols failed to show any ergosterol, we pursued a SIM analysis of the total sterol fraction; four compounds were detected in the chromatogram, including a compound that co-migrated at trace levels with authentic ergosterol (Fig. 2).

To provide further evidence for ergosterol in the total sterol fraction, HPLC was used with the detector set at 282 nm to highlight the elution pattern for sterols containing a conjugated diene Δ5,7 system. Using this approach, a major peak appeared by HPLC with an αc of 0.71 (first peak in chromatogram A, Fig. 3) that after elution of the sample from the column was found to correspond chromatographically in TLC and gas-liquid chromatography to cholesta-5,7,24-trienol. This compound possessed the same mass spectrum for a standard of cholesta-5,7,24-trienol (22, 27). Three additional peaks were evident by HPLC eluting later than cholesta-5,7,24-trienol at αc 0.74, 0.82, and 0.83. The material at αc 0.82 was vanishingly small, yet the mass spectrum of this compound was taken and found to be an exact match of the spectrum of ergosterol (Fig. 4A). The compounds with αc of 0.74 and 0.83 possessed a mass spectrum similar to that of ergosterol; they generated M+ of 396 amu and fragments for a Δ5,7 system (e.g., m/z 143 and 158 amu) and possessed other diagnostic ions at M+ – Me – H2O (381), M+ – Me – H2O (378), M+ – Me – H2O (363), and M+ side chain (271) of differing intensities typical of an ergostenol structure, with eight carbon atoms in the side chain and a C24-methylated side chain appropriately functionalyzed with a double bond attached to C24 or C25 (25, 26, 28). Based on known retention factors in GC with appropriate reference standards, together with the information on the mass spectra of these compounds (21, 28), we propose the compounds to have the following identities; ergosta-5,7,25(27)-trienol (Fig. 4B), ergosta-5,7,24(28)-trienol (Fig. 4C), and ergosta-5,7,24(25)-sterol...
trienol (Fig. 4D). The side chain pattern of these novel ergostenols is consistent with the side chain structures formed by the cloned TB SMT assayed with zymosterol and Sadenosylmethionine (10), and for the new TB sterols shown in Fig. 4B, C, their mass spectra correspond to mass spectra of authentic specimens isolated from yeast and P. wickerhamii (24, 25).

The occurrence of zymosterol in cells cultured in lipid-deficient medium agrees with the sterol substrate recognition of the cloned TB SMT (10). The detection of lanosterol is consistent with the gene cloning data on the squalene synthase from TB, in which lanosterol is the product of squalene oxide cyclization. The detection of 31-norlanosterol and 4α-methyl zymosterol (cholesta-8,24-dienol) is consistent with the gene-cloning data for the 14α-demethylase enzyme from TB (albeit with a different substrate specificity from the yeast SMT). The large amount of cellular cholesta-5,7,24-trienol appears to be endogenous as well; this compound usually will not accumulate in animal tissues under normal physiological conditions, and possible intermediates such as zymosterol and cholesta-7,24-dienol are synthesized by the cell. These observations, together with the finding of several ergostenols with novel side chain constructions matching that found using zymosterol as substrate for the cloned TB SMT, represent a new observation for the sterol chemistry of this group of microbes.

On the other hand, the desmosterol and perhaps most of the cholesterol in the cells identified by GC-MS (Fig. 1A) may be of dietary origin, because these two sterols are present in significant amounts in the lipid-depleted medium (Fig. 1B). Indeed, the amount of cholesterol in lipid-depleted medium (2.56 μg/ml, or a total of 13 μg of cholesterol for a 50 ml culture) and the amount of cholesterol in the cells (50 ml of culture, which yielded a cell

Fig. 3. Reverse-phase HPLC analysis of sterols from TB procyclic cells cultured in 10% bovine serum (chromatogram A) and from the bloodstream form derived from rat serum (chromatogram B) and authentic ergosterol standard (chromatogram C). Approximately 100 μg of sample was injected into a Phenomenex semi-preparative column as described in Materials and Methods. All chromatograms share the same time axis.

Fig. 4. Mass spectra (abundance on the X-axis is normalized to 100%) and indicated structures associated with compounds isolated by HPLC with ultraviolet light exhibiting the 282 nm fingerprint of ergosterol from procyclic cells cultured in lipid-depleted medium. A: ergosta-5,7,22-trienol. B: ergosta-5,7,25(27)-trienol. C: ergosta-5,7,24(28)-trienol. D: ergosta-5,7,24(25)-trienol.
pellet containing 10 μg of total cholesterol at 51 fg/cell), which show basically the same amounts of cholesterol in the two systems, support the possibility that most of the cellular cholesterol detected in the procyclic cells is derived from the lipid-depleted medium. However, further work is necessary to clarify unambiguously whether and how much cholesterol in these cells is of endogenous origin. The fact that 13 sterols in the protozoan cells were absent from the total sterol composition of the lipid-deficient medium is reason to assume that they are synthesized de novo by the procyclic form.

Sterols of the TB bloodstream form
The total sterols obtained from TB isolated from rat blood were analyzed by GC-MS, and cholesterol was found to predominate (Fig. 5A), accounting for ~96% of the total sterols (Table 2). Minor peaks by GC eluting after cholesterol were associated with the sterols fed to the animal in the form of the commercial rodent diet (Fig. 5B). The origin of the phytosterols present in the bloodstream form are structurally different from those synthesized in the protozoan and were not present in the procyclic cells; therefore, these 24-alkyl sterols appear to be from the diet.

Effects of 25-AL treatment
At a concentration of 1 μM, 25-AL fed to procyclic or bloodstream form cultures caused a 50% inhibition of cell growth. Using a related SMT inhibitor, 25-thialanosterol, and its salt form (in this case, a sulfur atom is used to replace the nitrogen atom in the sterol side chain) prepared and tested as a growth and sterol biosynthesis inhibitor of Candida albicans (15), we found that the procyclic and bloodstream forms responded in a similar manner to that exhibited by C. albicans; that is, TB growth inhibition resulted at an IC50 of ~1 μM in bloodstream forms and slightly higher in procyclic forms (unpublished data).

25-AL was also investigated for its effect on the sterol composition of the procyclic form cultured with 10% serum (Table 3). Cultures were inoculated with identical quantities of cells and grown for 96 h at the same time under the same conditions except for being exposed to different concentrations of 25-AL, after which the sterols were extracted and analyzed quantitatively. In the absence of 25-AL, procyclic cells cultured in medium that included 10% bovine serum had a modified sterol composition compared with that of the procyclic cells cultured in a lipid-depleted medium (Table 1). The control cells with 10% bovine serum contained cholesterol as the major cellular sterol (60% of total sterols), and the amount of 24-alkyl sterol was less than that present in cells cultured in lipid-depleted medium. Zymosterol, which was present in cells grown in lipid-depleted medium, was absent from cells cultured with 10% bovine serum. GC-MS analyses of the sterols from inhibitor-treated cultures of TB revealed a measurable change in the distributions. Compared with the sterol composition of the control, in the presence of 25-AL, ergosterol synthesis was completely suppressed, zymosterol accumulated somewhat, and the amount of cholesterol in the cells increased significantly to a level similar to that in the bloodstream form (Table 3). At the IC50 of 25-AL, there were no detectable 24-alkyl sterols in the cells. The accumulation of zymosterol in the treated cells is consistent with the blockage of SMT activity in vivo and for zymosterol to be the optimal substrate for the enzyme, as reported for the cloned SMT enzyme in vitro. Interestingly, the amount of cholesta-5,7,24-trienol in the treated cells decreased with increased 25-AL added to the medium. The decrease in the endogenously formed

Fig. 5. Gas-liquid chromatographic separation of the sterol fraction derived from the bloodstream form (A) and the rat chow diet (B; the inset in A is the expanded region of the chromatogram corresponding to phytosterol) resolved by GC-MS on an HP-5 capillary column. The chromatograms share the same time axis. Structures of compounds associated with the scan are shown in Fig. 6.
cholesta-5,7,24-trienol by the addition of bovine serum and/or drug treatment may be viewed as diagnostic of an overall downregulation of sterol synthesis in the cells. The amount of total sterol in the treated cells decreased from 82 to 67 fg/cell as the drug concentration in the medium increased.

Commercial BSA and serum obtained from a rat used in these studies were analyzed for sterol. Both of these samples contained primarily cholesterol (99%), and no ergosterols were evident by GC-MS (data not shown). The amount of cholesterol in bovine serum was estimated at 262 μg/ml and in rat serum at 1 mg/ml. In the case of bovine serum, cholesterol availability in procyclic cultures was 100 times that in the lipid-depleted medium. The amount of cholesterol available for absorption into the bloodstream form in rats was four times the amount available to the procyclic cells in culture.

**DISCUSSION**

The nature of the chemical structures presented here (Fig. 6A) coupled with the critical information on the cloned sterol genes suggest that a lanosterol-ergosterol pathway is present in TB and that the route may involve the following order of key transformations from the initially formed 4,4,14-trimethyl sterol intermediate (lanosterol, 15): i) demethylation at C4 (15 converted to 9); ii) demethylation at C14 (9 converted to 4); iii) methylation at C24 (3 converted to 10); iv) rearrangement of the Δ5 bond to the Δ8 bond (10 converted in two steps to 12); v) introduction of the Δ22 bond (intermediate not detected); and vi) reduction of the Δ24 bond (intermediate not detected to 6). There can be no doubt that several of the detected sterols are native to the organism, including sterols common to protozoa, such as lanosterol and zymosterol and the set of ergostenols with unusual side chain construction. Thus, TB can operate the energy-expensive 24-alkyl sterol pathway in a similar manner to other protozoa, making them different from humans biosynthetically.

The occurrence of the final 24-alkyl sterol product ergosterol is problematic. Its concentration in the cells is very low; therefore, one possibility for its origin is a contaminant. However, we failed to detect ergosterol in the medium used to culture TB or in the animal diet, whereas cholesterol and desmosterol (cholesta-5,24-dienol) are present at various levels. Comparison of the genome-annotated sterol synthesis genes from TB and those from Saccharomyces cerevisiae suggest that the last two steps of the ergosterol pathway, corresponding to steps v and vi described above, are absent from TB. In addition, the identifiable set of sterol genes in yeast and TB appear to lack information corresponding to the Δ7 reductase-type enzyme, which is common to animal systems. The absence of genetic information for a Δ7 reductase and a Δ24 reductase from the TB genome might explain the significant amounts of cholessta-5,7,24-trienol in procyclic cells.

However, cholesta-5,7,24-trienol can be an acceptable substrate for the SMT from several sources (10, 16 and references cited therein), yet only small amounts of C24-methylated sterols are formed in vivo, suggesting that cholesta-5,7,24-trienol is unlikely to be a normal substrate for the enzyme under physiological conditions. Another sterol synthesized by TB, zymosterol (cholesta-8,24-dienol), was found to be the optimal substrate of the cloned SMT enzyme. Zymosterol accumulates in procyclic cells after treatment with 25-AL, consistent with it being a preferred substrate of the cloned SMT enzyme. As noted above, total sterol is problematic. Its concentration in the cells is very low; therefore, one possibility for its origin is a contaminant. However, we failed to detect ergosterol in the medium used to culture TB or in the animal diet, whereas cholesterol and desmosterol (cholesta-5,24-dienol) are present at various levels. Comparison of the genome-annotated sterol synthesis genes from TB and those from Saccharomyces cerevisiae suggest that the last two steps of the ergosterol pathway, corresponding to steps v and vi described above, are absent from TB. In addition, the identifiable set of sterol genes in yeast and TB appear to lack information corresponding to the Δ7 reductase-type enzyme, which is common to animal systems. The absence of genetic information for a Δ7 reductase and a Δ24 reductase from the TB genome might explain the significant amounts of cholessta-5,7,24-trienol in procyclic cells.

**TABLE 2. Sterol composition of bloodstream form TB grown in rats**

<table>
<thead>
<tr>
<th>Sterol Composition</th>
<th>RRTc</th>
<th>Molecular Weight</th>
<th>Percentage of Total Sterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>1.00</td>
<td>386</td>
<td>96</td>
</tr>
<tr>
<td>24-Methylenecholesterol</td>
<td>1.13</td>
<td>398</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Campesterol</td>
<td>1.13</td>
<td>400</td>
<td>1</td>
</tr>
<tr>
<td>3-Ketocholesterol</td>
<td>1.16</td>
<td>384</td>
<td>1</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>1.27</td>
<td>414</td>
<td>1</td>
</tr>
<tr>
<td>Isoprenoidol</td>
<td>1.30</td>
<td>412</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Δ5,7 sterols</td>
<td>5/11/12/14</td>
<td>1.10/1.19/1.27</td>
<td>382 and 396</td>
</tr>
</tbody>
</table>

*Sterols with a conjugated Δ5,7 double bond system were detected and quantified by GC and HPLC as shown in Fig. 2. Sterols identified by GC scan (peak number) correspond to the structures shown in Fig. 6.
of other $\Delta^{24}$ reductases reported in the literature. All three olefins $[\Delta^{24}(28), \Delta^{25}(27),$ and $\Delta^{24}(25)]$ are known to be reduced by plant or fungi reductase-type enzymes to give the 24-methyl sterol side chain of ergosterol (28, 29).

The question of whether ergosterol is synthesized by TB was addressed by establishing the general absence of ergostenols in the lipid-depleted medium while alternatively showing that these compounds are present in cells at levels typical of other microbes, that the amounts of ergosterol and the other ergostenols are coordinately downregulated by cholesterol import, and subsequently that cellular ergosterol is effectively eliminated by the addition of 1 $\mu$M 25-AL to the culture medium of procyclic cells. Interestingly, cholesta-5,7,24-trienol content decreased but continued to be detected in cells at the IC$_{50}$ of 25-AL, suggesting that its synthesis, like that of the other sterols, is tightly regulated to cholesterol availability and inhibitor treatment.

We have shown that the amount of ergosterol in cultured TB treated with minimal exogenous cholesterol was 0.2 fg/cell, consistent with that sterol acting in some hormonal or signaling manner. The major amount of sterol in the cell amounted to 50–100 fg/cell, consistent with those sterols acting as bulk membrane sterols (29). Studies on fungi and protozoa indicate that 24-methyl sterols serve a dual role in cells (7, 30–32): one “sparking,” using hormonal levels of sterol to signal cell proliferation, and a second using ~20–100 fg/cell to act as a bulk membrane insert to maintain cell growth. In TB, as in other microbes, ergosterol may spare (partly replace) the bulk sterol (as long as the bulk sterol contains the cholestenol structure) to permit sterol signaling and cell growth.

Elimination of the ability for a biosynthetic renewal of ergosterol through treatment with 25-AL can be reconciled by the hypothesis that 24-alkyl sterols, specifically ergosterol, has a cellular function that is different from that of cholesterol and other sterols that lack a group at C24 in the sterol side chain. In a related study on cholesterol import by Aspergillus fumigatus and its influence on the antifungal potency of ergosterol biosynthesis inhibitors, specifically 14a-demethylase inhibitors, it was concluded that adding serum or cholesterol to the medium partially rescues fungal cells from the drug-induced growth inhibition (33), a finding that is contrary to the observations described here with TB. However, the possibility that cholesterol can serve as an ergosterol surrogate for the cell membrane is entirely consistent with our view (29) and that of others (30–32) that distinct sterol structures are involved with multiple functions.

The general picture that emerges from these studies is one in which 24-methyl sterol biosynthesis and sterol homeostasis (based on the type and amount of cellular sterol) in TB are intimately associated with cholesterol import from the human host. In cases in which cholesterol from the diet is minimal, the ergosterol pathway is upregulated. Alternatively, in cases in which cholesterol availability is significant, the endogenous pathway to ergosterol is downregulated but
not eliminated. This fact, together with the present demonstration that 25-AL can inhibit the growth of both the procyclic and bloodstream forms, and that the mechanism of action is indicated by the accumulation of zymosterol and the loss of 24-methyl sterols from the cells, suggests the possibility that the ergosterol pathway is essential to TB and that SMT may prove to be a potential target for the design of a new type of drug for trypanosomiasis.

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