

known oxidosqualene cyclases. This enzyme family converts oxidosqualene to cyclic triterpene alcohols that are the precursors to membrane sterols. Sequencing revealed that G14J10 could encode a 300 amino acid fragment similar to the N-termini of known oxidosqualene cyclases. In pursuit of a full-length clone, the fragment was radiolabeled with [α - 32 P]-dCTP and was used to screen a λ gt11 *T. cruzi* CL strain epimastigote genomic library⁸ using established protocols.⁹ Although nine of $\sim 6 \times 10^5$ plaques hybridized strongly, no full-length clone was obtained. However, sequencing the longest clone (Genbank accession number AF285827) revealed that it contained the missing 3' sequence, and it lacked only 130 bp from the putative start site. Attempts to PCR-amplify the 5' end of the gene from this genomic DNA library using G14J10 clone-specific primers were unsuccessful, as were attempts to amplify the complete coding sequence from a cDNA pool using spliced leader-specific primers. Fortunately, both fragments share a unique *Bam*H I site, which was used to subclone the two together and reassemble the complete open reading frame.

For complementation studies, the reconstructed coding sequence was subcloned into the galactose-inducible integrative yeast expression vector pRS305GAL¹⁰ and the high-copy derivative pRS426GAL¹¹ to construct pBJ1.21 and pBJ1.22, respectively. Lithium acetate was used to transform⁹ the *Saccharomyces cerevisiae* lanosterol synthase mutant SMY8¹⁰ with both constructs. When plated on expression medium (1% yeast extract, 2% peptone, 2% galactose, 20 mg/L heme) lacking ergosterol, strains expressing the *T. cruzi* oxidosqualene cyclase grew similarly to a positive control (SMY8 expressing native yeast lanosterol synthase from the same plasmid).¹² A negative control (SMY8 with empty vector) required ergosterol to grow. These experiments show that the *T. cruzi* oxidosqualene cyclase gene genetically complements the yeast lanosterol synthase mutant, suggesting that it encodes a lanosterol synthase.

The high-copy construct was used to transform the squalene synthase—lanosterol synthase double mutant LHY4¹¹ to analyze the enzymatic properties in vitro. An extract prepared from a 1-L culture (8 g of yeast) of the LHY4-[pBJ1.22] transformant was incubated¹³ with 20 mg (0.5 mg/mL) of racemic oxidosqualene.¹⁴ After 24 h at 25 °C, the reaction was quenched with 2 volumes of ethanol, and product was isolated essentially as described.¹¹ Column chromatography (5:1 hexane/methylene chloride) provided 8.2 mg of product (82% yield). The control yeast strain containing empty vector did not cyclize oxidosqualene,

indicating that the compound obtained was a product of the recombinant *T. cruzi* oxidosqualene cyclase.

NMR and gas chromatographic studies showed that the product was lanosterol. A 400 MHz 1 H NMR spectrum contained key signals that are within 0.01 ppm of characteristic lanosterol signals:¹⁵ δ 0.688 (C-18, s, 3 H), 0.810 (C-29, s, 3 H), 0.873 (C-30, s, 3 H), 0.911 (C-21, d, $J = 6.4$ Hz, 3 H), 0.981 (C-19, s, 3 H), 1.000 (C-28, s, 3 H), 1.601 (C-27, s, 3 H), 1.681 (C-26, s, 3 H), 3.23 (C-3, dd, $J = 3.8, 11.4$, 3 H), and 5.10 ppm (C-24, m, 1 H). The alcohol product was acetylated with pyridine/acetic anhydride (1:1). Gas chromatography showed a single signal that comigrated with lanosteryl acetate (retention time of standard relative to that of cholesteryl acetate = 1.332; that of sample to cholesteryl acetate = 1.325). Neither cycloartenyl acetate nor parkeyl acetate was observed (<0.5% detection limit).

These in vivo complementation and in vitro product analysis experiments establish that *T. cruzi* encodes a lanosterol synthase (Scheme 1). Animals and fungi biosynthesize sterol from lanosterol, whereas plants biosynthesize structurally similar sterols from cycloartenol. Difficulties in large-scale culture have precluded classical metabolic studies to establish which route *T. cruzi* uses. The existence of a *T. cruzi* lanosterol synthase establishes that lanosterol is a *T. cruzi* metabolite and is consistent with this pathogen using lanosterol as a sterol biosynthetic intermediate. A similar recombinant expression approach established lanosterol as a *T. brucei* metabolite,¹³ and lanosterol biosynthesis has been demonstrated¹⁶ in the kinetoplastid *Crithidia fasciculata*. Thus, kinetoplastids apparently utilize a different initial cyclic intermediate from most other protists (including the amoebae *Acanthamoeba polyphaga*,¹⁷ *Dictyostelium discoideum*,¹⁸ *Naegleria lovaniensis* and *N. gruberi*,¹⁹ and the euglenids *Euglena gracilis*²⁰ and *Astasia longa*²¹), which biosynthesize sterol from cycloartenol as plants do.

The reconstructed 2.7 kbp *T. cruzi* lanosterol synthase gene encodes a predicted 902 amino acid protein that is 67% identical to that of *T. brucei*,¹³ 28–35% identical to other known lanosterol synthases,^{10,12,22} and 30–32% identical to

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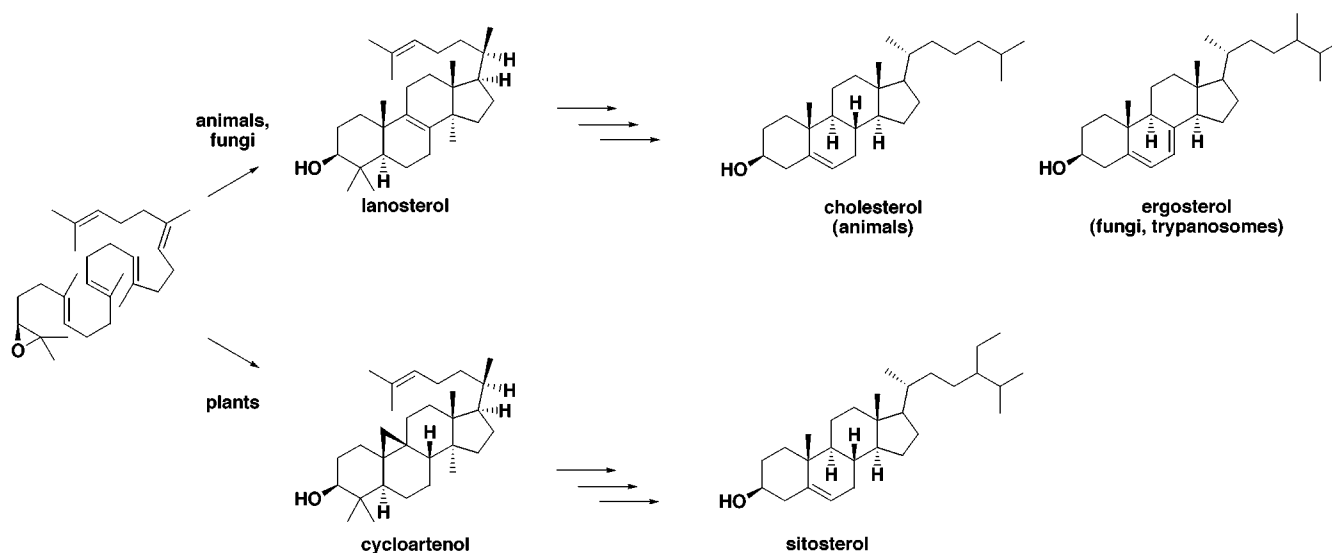
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Scheme 1. Animals and Fungi Cyclize Oxidosqualene to Lanosterol



known cycloartenol synthases.^{18b,23} Interestingly, the *T. cruzi* and *T. brucei* lanosterol synthases have a significant sequence difference (Figure 1) from the mammalian and fungal

cycloartenol synthases.^{18b,23} This threonine/tyrosine dichotomy is catalytically important. The *S. cerevisiae* Thr384Tyr lanosterol synthase mutant is inaccurate,²⁴ producing substantial amounts of parkeol and lanost-24-ene-3 β ,9 α -diol. The converse Tyr410Thr mutant in the *Arabidopsis thaliana* cycloartenol synthase converts oxidosqualene to lanosterol and 9 β -lanosta-7,24-dien-3 β -ol but forms no cycloartenol.²⁵ To abolish parkeol and lanost-24-ene-3 β ,9 α -diol production and maintain accurate lanosterol biosynthesis, the *T. cruzi* lanosterol synthase must have one or more additional catalytically relevant differences from animal and fungal lanosterol synthases. These presumptive compensatory changes are not among the known catalytically relevant lanosterol synthase residues (in *S. cerevisiae* numbering: His146, His234, Val454, and Asp456);^{11,26} the *T. cruzi* lanosterol synthase maintains these residues.

TcrERG7	M	R	V	C	G	Y	N	G	S	Q	L	545
TbrERG7	L	R	M	S	G	Y	N	G	S	Q	L	554
ScERG7	M	T	I	M	G	T	N	G	V	Q	T	389
CaLERG7	M	T	V	M	G	T	N	G	V	Q	V	383
SpoERG7	M	L	M	R	G	T	N	G	L	Q	V	384
RnoERG7	M	K	M	Q	G	T	N	G	S	Q	T	387
HsaERG7	M	K	M	Q	G	T	N	G	S	Q	T	386
DdiCAS1	M	K	M	Q	G	Y	N	G	S	Q	L	368
AthCAS1	M	K	M	Q	G	Y	N	G	S	Q	L	415
PsaCAS1	M	K	M	Q	G	Y	N	G	S	Q	L	415
PgiCAS1	M	K	M	Q	G	Y	N	G	S	Q	L	415
GgLCAS1	M	K	M	Q	G	Y	N	G	S	Q	L	415
LcyCAS1	M	K	M	Q	G	Y	N	G	S	Q	L	423

Figure 1. Animal and fungal lanosterol synthases from *S. cerevisiae*,^{12,22c} *Candida albicans*,^{22a,b} *Schizosaccharomyces pombe*,¹⁰ *Rattus norvegicus*,^{22d,e} and *Homo sapiens*^{22f,g} maintain a tyrosine at a catalytically important residue (at a position corresponding to *S. cerevisiae* Thr384). Cycloartenol synthases from *D. discoideum*,^{18b} *A. thaliana*,^{23a} *Pisum sativum*,^{23b} *Panax ginseng*,^{23c} *Glycyrrhiza glabra*,^{23f} and *Luffa cylindrica*^{23e} maintain tyrosine at the corresponding position, as do the trypanosomatid lanosterol synthases from *T. brucei*¹³ and *T. cruzi* (this work).

lanosterol synthases, which conserve a threonine residue at position 384 (*S. cerevisiae* numbering). The *T. cruzi* lanosterol synthase has a tyrosine at the corresponding position 540, as do the *T. brucei* lanosterol synthase¹³ and the known

The *T. cruzi* lanosterol synthase Tyr540 residue corresponds to the active-site S307 residue in the *Alicyclobacillus acidocaldarius* squalene-hopene cyclase,²⁷ the crystal structure of which has been solved.²⁸ The *T. cruzi* lanosterol synthase Tyr540 and the corresponding threonine in the animal enzymes are therefore probably active-site residues. This active-site difference between human and kinetoplastid lanosterol synthases presents an attractive chemotherapeutic target. Because the tyrosine phenol is more readily ionized

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than the threonine hydroxyl, it is more nucleophilic and it forms stronger hydrogen bonds and ionic interactions. Moreover, the polar functionality in tyrosine is positioned several angstroms farther from the α -carbon than in threonine. It is worth noting that currently available antifungal or antihypercholesterolemic compounds optimized to inhibit human or fungal lanosterol synthases may not inhibit trypanosomatid enzymes efficiently enough to kill trypanosomes. However, effective therapeutics might readily be identified by screening structural analogues of these inhibitors for antikinoplastid activity for their ability to inhibit the growth of the yeast lanosterol synthase mutant expressing *T. cruzi* lanosterol synthase.

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