

## Kinetic Characterization of Squalene Synthase from *Trypanosoma cruzi*: Selective Inhibition by Quinuclidine Derivatives<sup>∇</sup>

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**The biosynthesis of sterols is a major route for the development of antitrypanosomals. Squalene synthase (SQS) catalyzes the first step committed to the biosynthesis of sterols within the isoprenoid pathway, and several inhibitors of the enzyme have selective antitrypanosomal activity both in vivo and in vitro. The enzyme from *Trypanosoma cruzi* is a 404-amino-acid protein with a clearly identifiable membrane-spanning region. In an effort to generate soluble recombinant enzyme, we have expressed in *Escherichia coli* several truncated versions of *T. cruzi* SQS with a His tag attached to the amino terminus. Deletions of both the amino- and carboxyl-terminal regions generated active and soluble forms of the enzyme. The highest levels of soluble protein were achieved when 24 and 36 amino acids were eliminated from the amino and carboxyl regions, respectively, yielding a protein of 41.67 kDa. The Michaelis-Menten constants of the purified enzyme for farnesyl diphosphate and NAD (NADPH) were 5.25 and 23.34  $\mu\text{M}$ , respectively, whereas the  $V_{\text{max}}$  was 1,428.56  $\text{nmol min}^{-1}\text{mg}^{-1}$ . Several quinuclidine derivatives with antiprotozoal activity in vitro were found to be selective inhibitors of recombinant *T. cruzi* SQS in comparative assays with the human enzyme, with 50% inhibitory concentration values in the nanomolar range. These data suggest that selective inhibition of *T. cruzi* SQS may be an efficient strategy for the development of new antitrypanosomal agents.**

*Trypanosoma (Schizotrypanum) cruzi*, is an intracellular protozoan parasite that infects many wild mammals and humans, being the etiological agent of Chagas' disease, one of the major public health problems in many countries of Central and South America (20). Acute infections can be lethal, but the disease usually evolves into a chronic stage, accompanied in 25 to 30% of cases by severe debilitation and ultimately death due to irreversible lesions of the heart and gastrointestinal tract. It is estimated that 16 to 18 million people are infected with *T. cruzi*, primarily in Central and South America, with 21,000 deaths reported each year (27). Currently available chemotherapy has low efficacy (particularly in chronic infections), frequent toxic side effects, and drug resistance (3, 22). Studies have shown that protozoan parasites such as *T. cruzi* and different species of the *Leishmania* genus require the de novo synthesis of specific endogenous sterols (ergosterol and analogs), which act as essential growth factors for survival (5, 22, 23). These parasites are highly susceptible, in vivo and in vitro, to sterol biosynthesis inhibitors such as antifungal azoles, quinuclidine derivatives, allylamines, statins, and azasterols (5, 26). Indeed, sterol biosynthesis is a major route for intervention in the development of antitrypanosomals.

The enzyme squalene synthase (SQS; EC 2.5.1.21) catalyzes

the condensation of two molecules of farnesyl diphosphate (FPP) to produce squalene, the first committed step of the sterol pathway (Fig. 1). FPP is a major branching point in isoprenoid biosynthesis: it can be converted by SQS to squalene and sterols, or it can be used for the production of other essential isoprenoids, such as dolichols, coenzyme Q, heme, and prenylated proteins. Hence, considerable effort has been devoted to the development of specific inhibitors of SQS, since this should prevent the biosynthesis of sterols while not affecting the production of other essential isoprenoids (14). The inhibition of SQS should also prevent the buildup of sterol intermediates that may occur should later steps of sterol biosynthesis be inhibited.

Published evidence has shown that several inhibitors of mammalian SQS have potent antitrypanosomal activity both in vitro and in animal models (16, 24). Inhibition of parasite growth was associated with a depletion of the parasite's endogenous sterols strongly, suggesting that the main mode of action of these compounds is through the inhibition of SQS. Rational drug design has been utilized in the development of mimetics of several substrates, intermediates, and transition states in the transformation of FPP to squalene (1). One class of compounds of particular interest is the arylquinuclidines, which are protonated at physiological pH and are thought to mimic a high-energy intermediate of the SQS reaction.

Several attempts have been made to express the soluble and active SQS from different organisms in *Escherichia coli*. Different truncated versions of the enzyme have been generated by molecular biology methods (12, 18, 21), based on the previous evidence that a soluble and active form of SQS purified

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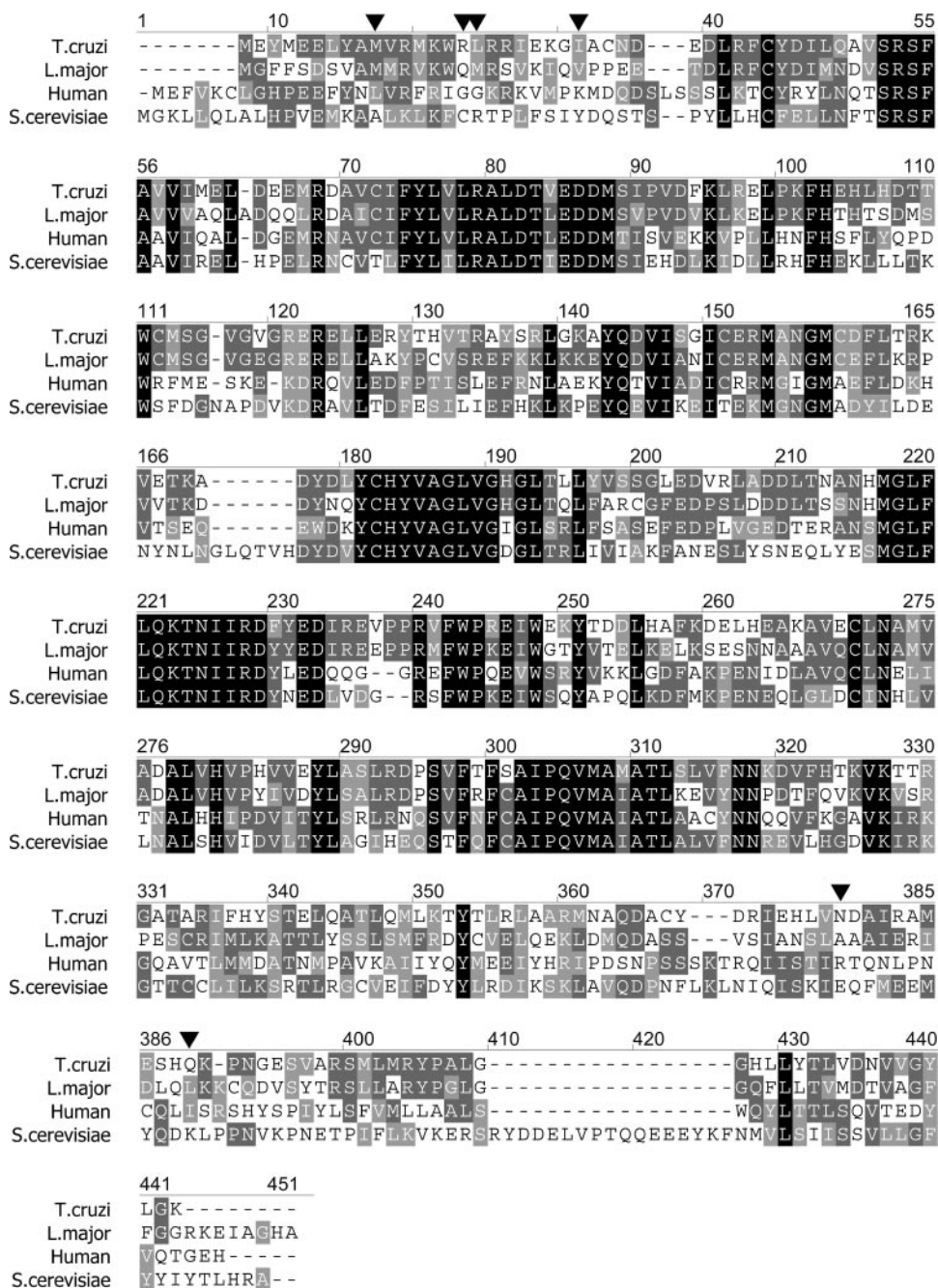


FIG. 2. Alignment of the amino acid sequences of SQS from different organisms: *T. cruzi* (XP806809), *L. major* (CAJ08546), human (I52090), and yeast (CAA42583). Sequences were aligned by using the AlignX module of Vector NTI (Invitrogen). Conserved regions are highlighted. The arrows indicate the site of the different truncations.

to 4 h, was read spectrophotometrically at 540 nm. IC<sub>50</sub> values were calculated from the sigmoidal inhibition curve by using Microsoft Excel.

**Quinuclidine derivatives.** The quinuclidine derivatives used in the present study were synthesized and characterized by nuclear magnetic resonance and mass spectrometry. The full details of their chemical synthesis will be published elsewhere (S. B. Cammerer, C. Jimenez, S. Jones, L. Gros, S. Orenes-Lorente, C. Rodrigues, J. C. F. Rodrigues, A. Caldera, L. M. Ruiz-Perez, W. da Souza, M. Kaiser, R. Brun, J. A. Urbina, D. Gonzalez Pacanowska, and I. H. Gilbert, unpublished data), except for ER119884 and E5700, which were supplied by Tsukuba Research Laboratories, Eisai Co., Ltd., Ibaraki, Japan.

## RESULTS

The amino acid sequence of *T. cruzi* SQS is conserved relative to other eukaryotic SQSs and has 55 to 58% identity and 65 to 73% similarity with other representatives of the *Trypanosomatidae* family (*T. brucei* and *Leishmania major*). As shown in Fig. 2, all of the conserved residues described to be involved in catalysis (17) are present in the *T. cruzi* enzyme such as the aspartate rich motifs involved in substrate binding (<sup>82</sup>DTVED



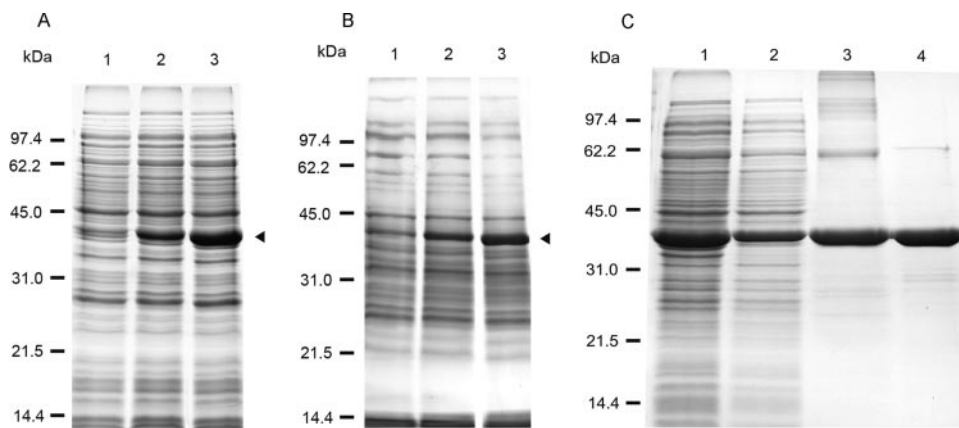


FIG. 3. SDS-PAGE analysis of expression and purification of double-truncated SQS. (A) Soluble fractions of *E. coli* BL21(DE3)RP/pETTcSQS24/36 cells. Lanes 1 to 3 contain the soluble fraction of at 0, 1, and 4 h after induction. (B) Soluble fractions of *E. coli* BL21(DE3)RP/pETTcSQS17/36 cells. Lanes 1 to 3 contain the soluble fraction at 0, 1, and 4 h after induction. The arrows show the position of the *T. cruzi* SQS recombinant protein. (C) Purification of truncated SQS (*TcSQS24/36*). Lane 1, cell extract; lane 2, soluble fraction; lane 3, IMAC; lane 4, after MonoQ HR5/5 chromatography.

and  $^{229}\text{CFYED}$ ). Previous reports have shown the development of SQS expression systems for yeast, rat, and human enzyme (12, 18, 21). For the production of soluble protein, partial deletion of the N-terminal, C-terminal, or both regions was required. Likewise, initial attempts to produce a full-length soluble *T. cruzi* enzyme were unsuccessful. Based on a comparative analysis of other recombinant soluble SQS enzymes several constructs were designed in order to eliminate 13, 16, 17, or 24 amino acids from the amino terminus combined with the elimination of 36 or 46 amino acids from the carboxyl-terminal regions. All of the constructs were cloned into the pET28a(+) expression vector and transformed in *E. coli* BL21(DE3)RP for analysis of the level of expression and solubility on SDS-polyacrylamide gels (data not shown). After analysis for soluble protein and activity measurements, we found that a soluble active double truncated form was only obtained after the elimination of 17 or 24 amino acids from the amino terminus and the elimination of 36 residues from the carboxyl terminus. Panels A and B of Fig. 3 represent the SDS-PAGE analysis after the induction of expression with IPTG for the constructs pETTcSQS24/36 and pETTcSQS17/36, respectively, showing high levels of soluble and active recombinant SQS.

**Purification of truncated soluble *T. cruzi* SQS (*TcSQS24/36*).** The *TcSQS24/36* truncated protein (lacking 24 and 36 amino acids from the amino- and carboxyl-terminal regions, respectively) was the construct that showed the strongest band at approximately 41 kDa and exhibited high SQS activity. The isolation and purification of truncated *T. cruzi* SQS from *E. coli*

cells was accomplished with two purification steps as described in Materials and Methods: IMAC and MonoQ ion-exchange chromatography. The purification data are summarized in Table 1. IMAC analysis yielded a 1.7-fold purification. This material was loaded directly into a MonoQ ion-exchange chromatography column and eluted with a salt gradient. In this step, SQS eluted with a NaCl gradient ranging from 250 to 350 mM NaCl. The MonoQ ion-exchange chromatography gave a further 4.5-fold purification. Pure truncated SQS was stable in buffer A with 40% of glycerol and could be stored at  $-80^\circ\text{C}$  for extended periods of time. The purity of the SQS was determined by SDS-PAGE analysis (Fig. 3C). The enzyme migrates as a single band with an estimated molecular mass for the truncated enzyme of 41.67 kDa. All kinetic experiments were performed with purified recombinant enzyme.

**Kinetic properties of soluble truncated SQS: determination of  $K_m$  and  $k_{\text{cat}}$  values.** Standard procedures were used to determine kinetic parameters.  $K_m$  and  $V_{\text{max}}$  values were obtained by nonlinear regression fit of the data to the Michaelis-Menten equation (Sigmaplot 2002 for Windows, version 8.0). The  $K_m$  and the  $V_{\text{max}}$  for FPP were 5.25  $\mu\text{M}$  and 1,428.56  $\text{nmol min}^{-1} \text{mg}^{-1}$  respectively, and for NADPH these values were 23.34  $\mu\text{M}$  and 1,853.24  $\text{nmol min}^{-1} \text{mg}^{-1}$  (Fig. 4). The calculated  $k_{\text{cat}}$  values were 1.05  $\text{s}^{-1}$  and 1.29  $\text{s}^{-1}$  for FPP and NADPH, respectively. These values are of the same order of magnitude as those described for other truncated recombinant enzymes, as shown in Table 2.

**Inhibition by quinuclidine derivatives.** A series of quinuclidine derivatives were tested against the purified *T. cruzi* re-

TABLE 1. Purification of *TcSQS24/36*<sup>a</sup>

Step	Vol (ml)	Protein concn (mg ml <sup>-1</sup> )	Total protein (mg)	Sp act (nmol min <sup>-1</sup> mg <sup>-1</sup> )	Total U (nmol min <sup>-1</sup> )	% Yield	Fold purification
Soluble extract	8.50	5.70	49.08	100.29	4,922.6	100	1
IMAC-His	11	0.67	7.20	178.26	1,283.5	26	1.7
MonoQ HR5/5	1.50	1.60	2.40	459.97	1,103.9	22	4.5

<sup>a</sup> The truncated protein was purified using a combination of IMAC and MonoQ ion-exchange chromatography.

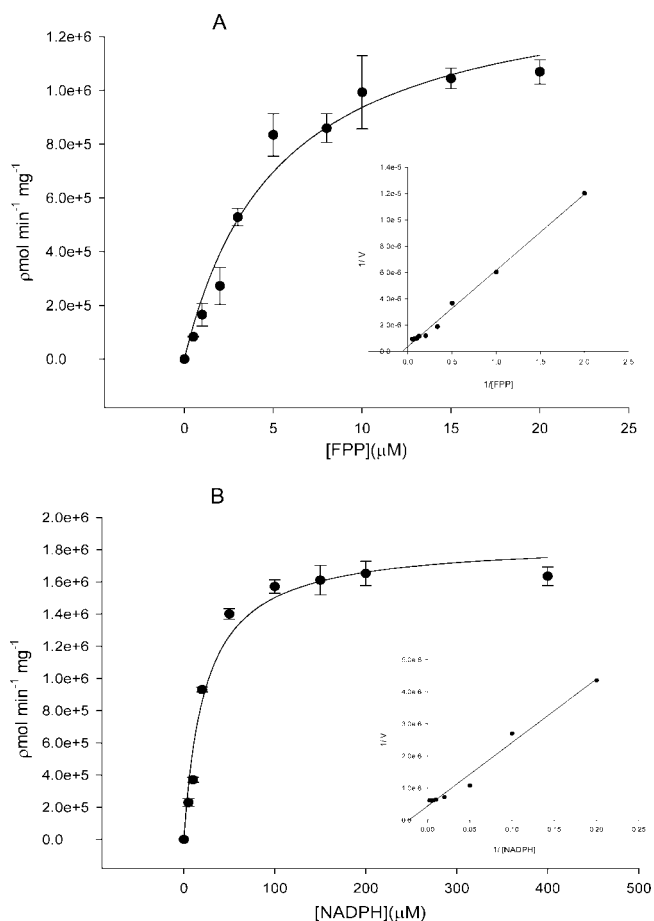


FIG. 4. Michaelis-Menten and Lineweaver-Burk (inset) plots for the calculation of the  $K_m$  and  $V_{max}$  values of *T. cruzi* SQS. (A) Plots for calculation of the  $K_m$  for FPP. The concentration range of FPP was 0.5 to 20  $\mu\text{M}$ . (B) Plots for calculation of the  $K_m$  for NADPH. The concentration range of NADPH was 5 to 400  $\mu\text{M}$ .

combinant enzyme, the human recombinant SQS, and against the intracellular form of the parasite cultured in vitro. When tested against purified recombinant *T. cruzi* enzyme, the analogs gave  $\text{IC}_{50}$  values in the low micromolar or nanomolar range (Table 3). Compound 1 exhibited an  $\text{IC}_{50}$  value of 50 nM, whereas the Eisai compounds E5700 and ER119884 gave

values of 0.84 and 3.52 nM, respectively. We sought to analyze the interaction of some of these compounds with a soluble truncated form of recombinant human SQS. As shown in Table 3, some of the compounds appeared to be less active against the human enzyme and, when  $\text{IC}_{50}$  values were compared, a selectivity index of 140 was obtained in the case of compound 1. Selectivity indexes higher than 27 and 13 were obtained for compounds 2 and 4, respectively. In the case of E5700 and ER-119884, both compounds appear to be equally active on the human and *T. cruzi* enzymes.

When these compounds were assayed against intracellular *T. cruzi* amastigotes in vitro, they exhibited potent and selective antitrypanosomal activity, with  $\text{IC}_{50}$  values in the low micromolar range (Table 3). There was not a direct correlation between inhibition of the enzyme and inhibition of the growth of the parasite, but this may not be totally unexpected, since *T. cruzi* is an intracellular parasite, and hence the activity of the compounds against the parasite will depend not only on the inhibition of the parasite's SQS but also on other factors such as drug penetration through the several permeability barriers involved and other properties of the molecules. However, although there is not a direct correlation between enzyme inhibition and growth inhibition of the intracellular parasites, these studies are proof of principle that inhibitors of *T. cruzi* SQSs also show activity against the clinically relevant form of the parasite. Study of a larger range of analogues will provide more information.

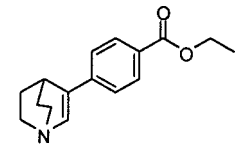
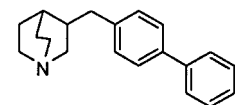
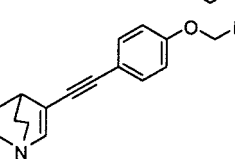
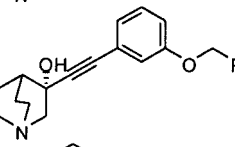
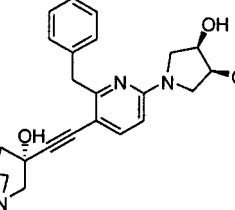
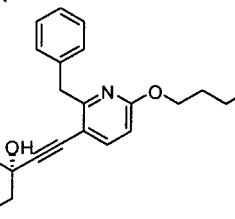
## DISCUSSION

Genes encoding SQS have been isolated from many sources, such as fungi, plants and animals (4, 6–10, 12, 13, 15, 28). The enzyme is monomeric and has been reported to be associated with the endoplasmic reticulum at least in most eukaryotes. The *T. cruzi* enzyme is considerably conserved and a comparative analysis of the amino acid sequences reveals an overall high degree of similarity. The generation of high quantities of soluble enzyme for inhibitor screening was attempted using a strategy that proved to be successful with other eukaryotic SQSs. Thus far, truncated soluble and active recombinant enzymes have been generated for yeast, rat, and human SQSs. In the yeast protein, the 24 carboxyl-terminal residues were removed via genetic manipulation, and a soluble, active enzyme was produced that could account for up to 20% of the total

TABLE 2. Kinetic constants of squalene synthase from different species and sources

Species and/or source <sup>a</sup>	SQS type	FPP		NADPH		$k_{cat}$ ( $\text{s}^{-1}$ )	Reference
		$K_m$ ( $\mu\text{M}$ )	$V_{max}$ ( $\text{nmol min}^{-1} \text{mg}^{-1}$ )	$K_m$ ( $\mu\text{M}$ )	$V_{max}$ ( $\text{nmol min}^{-1} \text{mg}^{-1}$ )		
Human pHSS12/pHSS16	Double-truncated SQS	2.8				1.44	21
<i>T. cruzi</i>	Glycosomal SQS	2.8	0.97	40	0.43		25
<i>T. cruzi</i>	Microsomal/mitochondrial SQS	2.3	1.18	33	0.76		25
<i>L. mexicana</i>	Glycosomal SQS	3.2	0.75	62	0.58		25
<i>L. mexicana</i>	Microsomal/mitochondrial SQS	2.8	0.98	57	0.84		25
Human	Full-length SQS expressed in a baculoviral system	$2.3 \pm 0.5$	$4,800 \pm 460$	$430 \pm 60$	$3,800 \pm 150$		19
Yeast	C-terminal truncated SQS	$2.5 \pm 0.46$		$530 \pm 77$		$0.53 \pm 0.03$	12
<i>T. cruzi</i>							
TcSQS24/36		$5.25 \pm 1.2$	$1,428.56 \pm 317$	$23.34 \pm 4.5$	$1,853.24 \pm 435$	$1.05 \pm 0.16$	

TABLE 3. Interaction of SQS from different sources with quinuclidine analogs<sup>a</sup>

Compound	Structure	IC <sub>50</sub> (μM)		SI <sub>enz</sub>	IC <sub>50</sub> (μM)		SI <sub>growth</sub>
		<i>T. cruzi</i> SQS	Human SQS		<i>T. cruzi</i> amastigote <sup>b</sup>	L6 cells <sup>b</sup>	
1		0.05	7 <sup>b</sup>	140	16.0	265.2	17
2		0.73	>20	>27	4.7	20.5	4.4
3		0.83	0.25 <sup>b</sup>	0.3	3.1	47.2	15
4		1.5	>20	>13	9.6	45	4.7
E5700		0.00084	0.0015	1.8	0.008 <sup>c</sup>	ND <sup>d</sup>	
ER119884		0.00352	0.006	1.7	0.011 <sup>c</sup>	ND	

<sup>a</sup> SI<sub>enz</sub> is the ratio of IC<sub>50</sub> values for human SQS to those for *T. cruzi* SQS, and SI<sub>growth</sub> is the ratio of IC<sub>50</sub> values for growth inhibition against L6 cells to those against intracellular *T. cruzi* amastigotes.

<sup>b</sup> Values to be reported elsewhere (Cammerer et al., unpublished).

<sup>c</sup> Values for inhibition of amastigote growth as reported by Urbina et al. (24).

<sup>d</sup> ND, not determined.

soluble protein when expressed in *E. coli* (12). The production of an active human enzyme was attained after truncation of both the carboxy (47 amino acids) and the amino terminus (30 amino acids) (31 to 371) that yielded a soluble protein with catalytic properties similar to the native enzyme (21). In the present study we generated a soluble enzyme by elimination of 24 amino acids of the amino terminus and 36 amino acids of the carboxyl terminus. The kinetic parameters were compared to those previously reported for preparations of *T. cruzi* glycosomal and microsomal SQS (25) and other recombinant enzymes. The resulting enzyme proved to be catalytically active and exhibited kinetic parameters highly similar to those obtained with the native enzyme in purified glycosomes and mitochondria from *T. cruzi* epimastigotes (25), albeit the  $K_m$  for FPP was slightly higher. Likewise, the  $K_m$  and  $k_{cat}$  values were

highly similar to those obtained for the truncated recombinant enzyme from yeast (12).

The first indication of the antitrypanosomal activity of SQS inhibitors came from studies by Urbina et al. with 3-(biphenyl-4-yl)-3-hydroxyquinuclidine (BPQ-OH), a potent and specific inhibitor of mammalian SQS (25). It was found that BPQ-OH induced a dose-dependent reduction of the proliferation of extracellular stages (epimastigotes) of these parasites with MICs of 30 μM. Growth inhibition and cell lysis induced by BPQ-OH in both parasites was associated with complete depletion of endogenous squalene and sterols. BPQ-OH was able to eradicate intracellular *T. cruzi* amastigotes from Vero cells with an MIC of 30 μM, with no deleterious effects on host cells at up to 100 μM.

Several other analogues have been tested for activity against the *L. major* enzyme and the parasite in vitro (16). An analog

of BPO-OH, where the 3-OH group has been removed by dehydration, leaving a  $\Delta^{2(3)}$  bond, was clearly more potent than BPO-OH, since cell lysis was observed in the presence of 1  $\mu$ M in *L. mexicana* promastigotes and exhibited an  $IC_{50}$  for recombinant SQS from *L. major* of 0.24  $\mu$ M (16).

Recent studies (24) with the compounds E5700 and ER-119884 (Eisai Chemical Company, Tokyo, Japan) showed that they were very potent noncompetitive or mixed-type inhibitors of native *T. cruzi* SQS with  $K_i$  values in the low nanomolar or subnanomolar range in the absence or presence of 20  $\mu$ M inorganic pyrophosphate. Their antiproliferative  $IC_{50}$ s against extracellular epimastigotes and intracellular amastigotes were ca. 10 nM and 0.4 to 1.6 nM, respectively, with no effects on host cells. These compounds are among the most potent anti-trypanosomals ever tested in vitro.

We now show that E5700 and ER-119884 are also highly potent inhibitors of purified recombinant *T. cruzi* SQS, but they have no selectivity toward the parasite's enzyme in comparative assays with the recombinant human enzyme. On the other hand, we have identified analogs such as compound 2, which is a good inhibitor of intracellular amastigote growth in vitro but also a selective inhibitor of *T. cruzi* SQS. Intracellular amastigotes are the clinically relevant form of the parasite. Compound 1 is also selective for the trypanosomal enzyme, although it was less active against the parasite in vitro. These observations offer a new approach for the design of SQS inhibitors with a potential application as antitrypanosomal compounds. Specific inhibitors of the *T. cruzi* enzyme would allow for increased efficacy and the minimization of possible adverse effects due to inhibition of human sterol biosynthesis. Studies are currently under way to determine the structural requirements for specific inhibition.

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