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Analogs of Squalene and Oxidosqualene Inhibit Oxidosqualene Cyclase of *Trypanosoma cruzi* Expressed in *Saccharomyces cerevisiae*

Simonetta Oliaro-Bosso, Maurizio Ceruti, Gianni Balliano, Paola Milla, Flavio Rocco, and Franca Viola*

Dipartimento di Scienza e Tecnologia del Farmaco, Università di Torino, 10125 Turin, Italy

ABSTRACT: Recently, a number of inhibitors of the enzyme oxidosqualene cyclase (OSC; EC 5.4.99.7), a key enzyme in sterol biosynthesis, were shown to inhibit in mammalian cells the multiplication of *Trypanosoma cruzi*, the parasite agent of Chagas' disease. The gene coding for the OSC of *T. cruzi* has been cloned and expressed in *Saccharomyces cerevisiae*. The expression in yeast cells could be a safe and easy model for studying the activity and the selectivity of the potential inhibitors of *T. cruzi* OSC. Using a homogenate of *S. cerevisiae* cells expressing *T. cruzi* OSC, we have tested 19 inhibitors: aza, methylidene, vinyl sulfide, and conjugated vinyl sulfide derivatives of oxidosqualene and squalene, selected as representative of different classes of substrate analog inhibitors of OSC. The IC_{50} values of inhibition (the compound concentration at which the enzyme is inhibited by 50%) are compared with the values obtained using OSC of pig liver and *S. cerevisiae*. Many inhibitors of pig liver and *S. cerevisiae* OSC show comparable IC_{50} for *T. cruzi* OSC, but some phenylthiovinyl derivatives are 10–100 times more effective on the *T. cruzi* enzyme than on the pig or *S. cerevisiae* enzymes. The expression of proteins of pathogenic organisms in yeast seems very promising for preliminary screening of compounds that have potential therapeutic activity.

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Sterols are eukaryotic membrane components that are necessary to support cell growth and differentiation. Sterol biosynthesis is a well-established chemotherapeutic target in pathogenic eukaryotes, such as fungi (1,2). The final product of the sterol biosynthetic pathway in fungi is the 24-alkyl sterol ergosterol. Ergosterol or similar 24-alkyl sterols are also the final products of the sterol synthetic pathway of some pathogenic protozoa including *Trypanosoma* species. These organisms cannot completely substitute these sterols with the cholesterol biosynthesized in host mammalian cells and need to synthesize at least some of their own distinctive sterols (3–5). This dependence on sterols suggests that sterol biosynthesis inhibitors may be useful antiprotozoal drugs.

The sterol biosynthetic pathway (Fig. 1) offers many potential targets for therapeutic purposes. Many effective antifungal drugs already in use act by inhibiting different enzymes of sterol biosynthesis; the allylamine antifungal drug terbinafine inhibits squalene epoxidase (6), and azole drugs such as ketoconazole and itraconazole are inhibitors of lanosterol C_{14}-demethylase (7). The activity of these inhibitors can be explained as a consequence either of depletion of the ergosterol or of accumulation of toxic intermediates or side products. In addition to the antifungal activity, some of these compounds

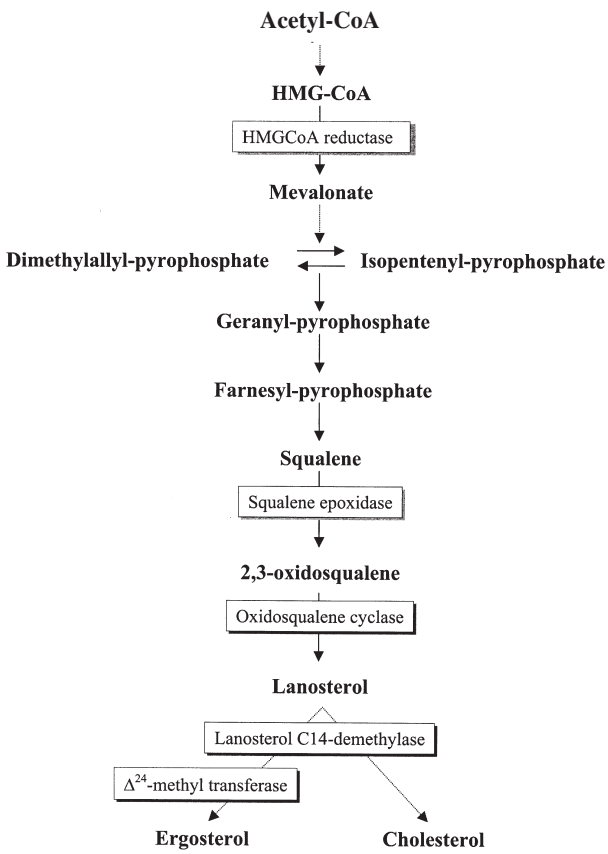

FIG. 1. Schematic representation of sterol biosynthetic pathways. The end products (ergosterol and cholesterol) are typical for fungi and animals, respectively. In the boxes are shown the enzymes cited in the text as targets for antimicrobial drugs.

*To whom correspondence should be addressed at Dipartimento di Scienza e Tecnologia del Farmaco, Via P. Giuria, 9-10125 Torino, Italy.
E-mail: franca.viola@unito.it

Abbreviations: IC_{50}, inhibitor concentration reducing enzymatic activity by 50%; OSC, oxidosqualene cyclase.

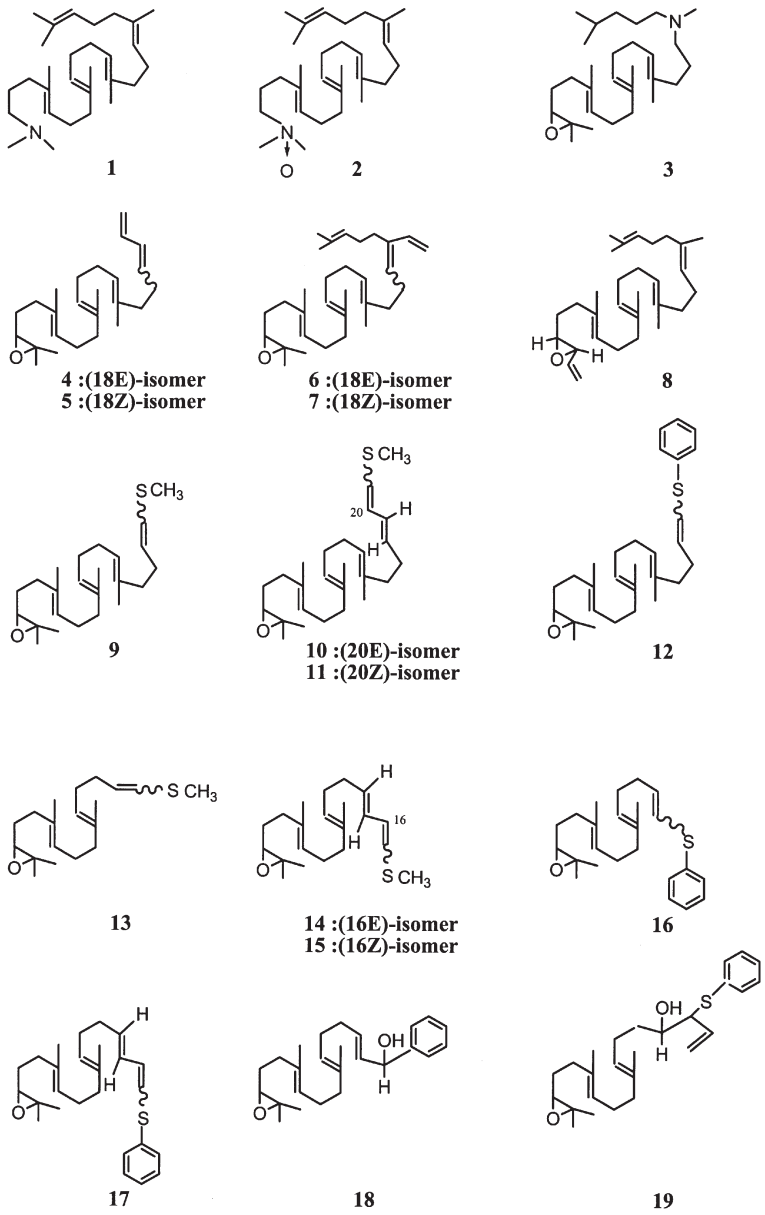

FIG. 2. Squalene and 2,3-oxidosqualene derivatives tested as inhibitors of *Trypanosoma cruzi* oxidosqualene cyclase.

were shown to inhibit the growth of *Trypanosoma* and other kinetoplastid parasites (8). Kinetoplastid parasites have also been shown recently to be susceptible to inhibitors of squalene synthase (9,10) and oxidosqualene cyclase (11,12).

The enzyme oxidosqualene cyclase (OSC; EC 5.4.99.7), which catalyzes the formation of the first cyclic precursor of sterols, is considered a good target for the inhibition of sterol biosynthesis in both humans and fungi. Many inhibitors, designed on the basis of the complex cyclization mechanism of OSC (13), are active on the enzyme and have been investigated as potential cholesterol-lowering or antifungal drugs (1,2,13). The recent crystallization of human OSC as a complex with the potent inhibitor RO48–8071 opens the way to new structure-based studies of the cyclization mechanism (14).

We have designed and tested against mammalian and fungal OSC many acyclic substrate analogs modified either to mimic the high-energy carbocationic intermediates (compounds **1**–**3** of Fig. 2) or to bear reactive functions able to interact with the active site residues, as the methylidene (compounds **4**–**8**), vinyl sulfide (compounds **9**, **12**, **13**, **16**), conjugated vinyl sulfide (compounds **10**, **11**, **14**, **15**, **17**), hydroxysulfide (compound **19**), or hydroxyphenyl derivatives (compound **18**) (15–20). OSC of different origins can have significantly different susceptibilities to the inhibitors, showing

the possibility of selective inhibition (19,20). Therefore, it would be worth testing the activity of these inhibitors on the OSC of the pathogen protozoon *Trypanosoma cruzi*, which recently has been shown to be susceptible to pyridinium ion-based inhibitors and to some umbelliferone aminoalkyl derivatives (11,12). *Trypanosoma cruzi*, like other pathogenic protozoa, is difficult to culture for an extensive, preliminary screening of the many available, effective inhibitors of OSC. Recently, the gene coding for the OSC of *T. cruzi*, a lanosterol synthase, has been cloned and expressed in *Saccharomyces cerevisiae* (21). The enzyme expressed in the yeast cells could be a safe and easy model for a preliminary study of the activity and the selectivity of the potential inhibitors of *T. cruzi* OSC.

In this paper we report the activity of different types of substrate analogs as inhibitors of the *T. cruzi* OSC expressed in *S. cerevisiae* cells. Basically, three types of inhibitors were studied: analogs of the carbocationic intermediates, as the azasqualenes (15,18); oxidosqualene derivatives bearing a conjugated methylidene group, designed as affinity-labeling inhibitors (16,22); and vinyl sulfide, and conjugated vinyl sulfide derivatives of oxidosqualene, very effective inhibitors of yeast OSC (17,19,20), (Fig. 2).

MATERIALS AND METHODS

Materials, substrates, and test compounds. All the components of buffers and culture broths were obtained from Sigma-Aldrich (Milan, Italy) unless otherwise specified.

The substrate of OSC, 2,3-oxidosqualene, was synthesized as previously described (23). The labeled [^{14}C]-(3*S*)2,3-oxidosqualene was obtained through biological synthesis by incubating a pig liver S_{10} supernatant with *R,S*[2-^{14}C]mevalonic acid (55 mCi/mmol, 2.04 GBq/mmol) (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom), in the presence of the OSC inhibitor U-14266A (24), as previously described (19). [2-^{14}C]Acetate (50 mCi/mol) was obtained from Amersham Pharmacia Biotech. The synthesis of the inhibitors tested (compounds **1**–**19** of Fig. 2) has been described elsewhere (16,17,19,20,23,25,26). The identity and purity of inhibitors was checked by TLC, ^{1}H NMR and mass spectra before testing the inhibition of *T. cruzi* OSC.

Strains of S. cerevisiae and culture conditions. SMY8pBJ1.21 strain, expressing the OSC of *T. cruzi*; SMY8pSM61.21, expressing the wild-type yeast OSC; and SMY8pSM60.21, expressing the *Arabidopsis thaliana* OSC obtained by transformation of lanosterol synthase mutant strain of *S. cerevisiae* SMY8 (*MATα erg7::HIS3 hem1::TRP1 ura3–52-trpl-Δ63 leu2–3.112 his3-Δ200 ade2* Gal$^+$) were kindly provided by Professor S.P.T. Matsuda [Department of Chemistry and Biochemistry and Cell Biology, Rice University, Houston, TX (21,27,28)]. All strains were grown in a YPD culture broth (yeast extract 1%, peptone 2%, dextrose 2%) containing hemin (0.013 mg/mL) and ergosterol (0.02 mg/mL). Hemin had to be added to the growth medium since the SMY8 strains contains a mutation (hem1::TRP1) affecting heme biosynthesis. The presence of a heme mutant background is necessary for the viability of lanosterol synthase mutants in aerobic conditions (27). OSC expression was induced in YPG broth (yeast extract 1%, peptone 2%, galactose 2%) containing hemin (0.013 mg/mL). All strains were preserved in 40% glycerol at –80°C.

Enzymatic assays. The activity of OSC was assayed in cell-free homogenates obtained from cultures grown to late exponential phase in YPG at 30°C. The cells were centrifuged at 3000 × *g* for 10 min and the homogenates prepared by lysing the cell wall with lyticase (2 mg/g wet cells in 1.2 M sorbitol, 0.02 M KH_2PO_4, pH 7.4, for 60 min at 30°C). The spheroplasts obtained after lysis were sedimented at 3000 × *g* for 10 min, washed twice with 1.2 M sorbitol, 0.02 M KH_2PO_4 and homogenized with a Potter device in 10 mM MES/TRIS buffer, containing 0.2 mM EDTA and 1 mM PMSF, pH 6.9. Proteins in the homogenate were quantified with the Protein Assay Kit SIGMA, based on the method of Lowry, modified by Peterson (29). The homogenates could be used fresh or after storage at –80°C for several months. The OSC activity was assayed by incubating the homogenate in the presence of the labeled [^{14}C]-(3*S*)-2,3-oxidosqualene (1000 cpm) diluted with unlabeled (*R,S*)2,3-oxidosqualene to a final concentration of 25 μM. Cold and labeled substrates and inhibitors, when present, were added as solutions in $CHCl_3$ to test tubes in the presence of Tween-80 (0.2 mg/mL of final volume) and Triton X-100 (1 mg/mL of final volume). The solvent was evaporated under nitrogen. The substrate and inhibitors were dissolved in 50 μL of MES/TRIS buffer, containing 0.2 mM EDTA, pH 6.9 and the amount of homogenate necessary to obtain a 20% substrate transformation was added and diluted to a final volume of 250 μL buffer. After 30 min of incubation at 35°C in plugged tubes, the enzymatic reaction was stopped by adding 1 mL of KOH in methanol (10% wt/vol) and heating at 80°C for 30 min in a water bath. After extracting with 2 mL of petroleum ether, the solvent was evaporated and the extract was dissolved in a small amount of CH_2Cl_2 and spotted on TLC plates (Alufolien Kieselgel 60F254; Merck, Darmstadt, Germany) using *n*-hexane/ethyl acetate (85:15, vol/vol) as developing solvent. The conversion of the labeled substrate into labeled lanosterol was analyzed by a System 200 Imaging Scanner (Hewlett-Packard, Palo Alto, CA); the counts were collected for 5 min, and the percentage of transformation was calculated by integration. For the K_m determinations different final concentrations of substrate (1–200 μM) were used and the incubation times arranged to obtain percentages of transformation not exceeding 10% (to keep safely within the range of linear relationship between product formation and time of reaction). The values of the enzymatic rate obtained at the different substrate concentrations were fitted by a nonlinear regression method to the Michaelis–Menten equation to calculate the K_m (30).

IC_{50} values (the concentration of inhibitor that reduces the enzymatic conversion by 50%) were calculated by nonlinear regression analysis of the residual activity vs. the log of concentrations of inhibitors. The values in the table are the means of two separate experiments each in duplicate.

RESULTS

Inhibition of T. cruzi OSC in cell-free homogenate of SMY8pBJ1.21 strain. To screen the inhibitors we used a homogenate of cells of the SMY8pBJ1.21 strain grown to late exponential phase. The *T. cruzi* OSC, known to partition between the cytosolic lipid bodies and the microsomes (31), was highly expressed in these cells. Detergents are needed to ensure the access of the apolar substrate and inhibitors to the enzyme in the lipid bodies and in the membranes and were used according to previous experiences (15,16,32). The enzyme activity was evaluated by radiometric scanning of thin-layer chromatograms of the organic extract of incubation mixtures. In our experimental conditions (plugged test tubes with no NADPH added), the only labeled peaks detected corresponded to cold standards of 2,3-oxidosqualene and lanosterol. No label was found in the 4-desmethylsterol region.

The specific activity, expressed as nmol of lanosterol formed, was 4 nmol/h/mg of proteins. The value of K_m, calculated by a nonlinear regression method, was 66 ± 13.5 μM.

To check the reliability and suitability of this homogenate for inhibition studies, we determined that the K_m values of the *S. cerevisiae* and *A. thaliana* enzymes expressed in the same host were, respectively, 30 ± 14.2 and 91 ± 29.6 μM, in agreement with the data reported for these enzymes using different enzymatic preparations (33,34). Therefore, the OSC expressed in transformed SMY8 cells have the same affinity for the substrate previously determined and seem suitable for testing inhibitors and comparing the results with those previously obtained with OSC of different origin.

All the inhibitors assayed, illustrated in Figure 2 and listed in Table 1, are substrate analogs already tested on mammalian and fungal OSC and were chosen as the more representative of different classes of compounds designed to inhibit OSC by different mechanisms. Prior to testing the inhibition of *T. cruzi* OSC, compound **1** was tested with the *S. cerevisiae* OSC expressed in yeast, compounds **12** and **16** were tested with pig liver enzyme. The IC_{50} values determined and found to be the same as those reported in References 18 and 20, respectively.

Table 1 shows the IC_{50} values obtained with some aza and methylidene derivatives of the substrate and with a series of vinyl and conjugated sulfide derivatives of truncated oxidosqualene. The activities are compared with those obtained previously by using detergent-solubilized microsomes prepared from pig liver and *S. cerevisiae* cells. The azasqualene derivatives are the 2-aza-2,3-dihydrosqualene **1**, its N-oxide **2**, and the 19-azasqualene **3** (25,26). The OSC of *T. cruzi* and of pig liver are inhibited similarly by 2-aza derivatives **1** and **2**, but not by 19-aza derivative **3**, which is a 30 times less effective inhibitor of *T. cruzi* enzyme. The methylidene derivatives **4**, **6**, **7**, and **8** show comparable activities against the *T. cruzi* and pig liver enzyme whereas compound **5** is five times less effective on *T. cruzi* OSC. As already observed for mammalian enzyme, the isomers of inhibitor with the same configuration as the substrate are at least 10 times more effective (16). The presence of a vinyl function in position 2 as in the *trans* 2-vinyl-2-oxido-1,1-bisnorsqualene **8**, as already observed in pig and *S. cerevisiae* OSC, is not effective for inhibition (18).

TABLE 1
Inhibition of *Trypanosoma cruzi* OSC Expressed in SMY8pBJ1.21 Yeast Strain by Aza, Methylidene, and Vinyl and Dienic Sulfide Derivatives of Substrate Compared with IC_{50} Previously Obtained with OSC from Pig Liver and *Saccharomyces cerevisiae*

Compound[a]	IC_{50} (μM)[b] OSC *T. cruzi*	OSC Pig liver[c]	OSC *S. cerevisiae*[c]
1	0.8	0.15	10.0
2	4.0	3.3	16.0
3	50.0	1.7	35.0
4	5.5	3.5	1.5
5	100.0	20.0	15.0
6	2.0	4.0	5.0
7	0.2	0.4	1.0
8	100.0	100.0	50.0
9	1.7	1.0	0.05
10	>100	1.2	0.4
11	>100	1.0	0.9
12	0.09	7.0	1.0
13	18.0	5.0	1.5
14	12.0	3.6	6.0
15	10.0	2.6	2.0
16	0.07	7.0	10.0
17	0.6	20.0	12.0
18	0.15	9.0	>100
19	0.5	7.5	40.0

[a]For structures of these compounds see Figure 2.
[b]Values are the means of two separate experiments, each with duplicate incubations. The maximal deviations from the mean were less than 10%.
[c]See References 16–20. OSC, oxidosqualene cyclase; IC_{50}, inhibitor concentration reducing enzymatic activity by 50%.

Compounds **9**–**19** of Table 1 are vinyl and conjugated sulfide derivatives of truncated oxidosqualene (17,19,20). The *T. cruzi* OSC seems specifically inhibited by all the phenyl derivatives tested, i.e., compounds **12**, **16**, **17**, **18**, and **19**. The IC_{50} values for these derivatives are in the range 70–600 nM, or 10 to 100 times lower than the IC_{50} found in pig liver OSC. Furthermore, the corresponding methyl derivatives **9**, **13**, **14**, and **15**, which are very active in pig and *S. cerevisiae* (19,20), are less effective on *T. cruzi* OSC. The major difference is observed with compound **9**, showing, with *T.cruzi,* a 30 times larger IC_{50} than with *S. cerevisiae*. Compounds **13** and **16** were also tested with the OSC of *A. thaliana*, expressed in the yeast strain SMY8pSM60.21. The IC_{50} of both compounds were similar, 4.5 and 2 μM, respectively. IC_{50} of phenyl derivatives **12**, **16**, **17** and **19**, tested with the OSC of *S. cerevisiae* expressed in a SMY8-derived strain (SMY8pSM61.21) were in complete accordance with the data shown in Table 1, obtained previously (18,20) with a wild-type *S. cerevisiae* (33).

DISCUSSION

We used a mutant strain of *S. cerevisiae* expressing the OSC of the pathogen protozoon *T. cruzi* to test a series of OSC inhibitors, with the aim of determining the susceptibility of this

enzyme to different types of OSC inhibitors and possibly identifying specific inhibitors. OSC can be considered a promising target to inhibit the multiplication of pathogenic microbes, but, unlike some targets for sterol inhibition, such as Δ^{24} methyl transferase, OSC also is present in the mammalian cells. Specificity for the pathogen enzyme is consequently an important prerequisite for inhibitors.

We have tested 19 inhibitors, selected as representative of different classes of inhibitors. The azasqualene derivatives, which were designed as analogs of the high-energy carbocationic intermediates generated at C-2 after the opening of the oxiranic ring, or at C-19, after the closure of the fourth ring, have been well studied as inhibitors of mammalian and fungal OSC (15,25,26). From the comparison of the IC_{50} obtained with the *T. cruzi* and with the pig and *S. cerevisiae* OSC, these compounds do not show specificity for the *T. cruzi* enzyme, probably because the mechanism of the initial step of the cyclization is very conserved in all the OSC (13). The closure of the last ring and the rearrangement are probably differently controlled in different OSC, as the 19-aza-derivative is very active only against pig liver OSC. The 29-methylidene derivatives **4**–**7**, previously shown to be time-dependent inhibitors (16,22), similarly lack specificity.

The most selective inhibition of *T. cruzi* OSC was found in the series of vinyl-thio-derivatives. The design of these compounds was based on the hypothesis that a partial cyclization of these compounds in the active site of the enzyme, because of the excellent properties of the sulfur in stabilizing the electron-deficient α-carbon, owing to its good π- and σ-donor properties, could generate carbocationic intermediates that were more stable and more able to interact strongly with nucleophilic amino acid residues. Our previous results showed that a methyl sulfide derivative, such as compound **9**, is a very effective inhibitor of *S. cerevisiae* OSC and a time-dependent inhibitor of pig OSC, whereas phenyl sulfide derivatives, such as **16**, **17**, and **19**, are poor inhibitors of both pig and yeast OSC (17,19,20). The *T. cruzi* OSC seems to be specifically inhibited by all the phenyl derivatives tested (compounds **12**, **16**–**19**). On the basis of these results, we initially speculated that the inhibition could be ascribed to specific π-π interaction of the aromatic derivatives with the Tyr540 of *T. cruzi* (21), which in the other lanosterol synthases is substituted for a conserved threonine residue. A tyrosine is conserved at this position in cycloartenol synthase (35,36), the plant enzyme converting oxidosqualene into cycloartenol, the precursor of plant sterols. The type of amino acid present in this position seems catalytically relevant for determining the final product of cyclization. Mutagenesis experiments have shown that the T/Y substitution causes a loss of accuracy in determining the final product of cyclization. A *S. cerevisiae* T384Y mutant produces substantial amounts of parkeol and lanost-24-ene-3β,9α-diol in addition to lanosterol (35), and the Y410T mutant of *A. thaliana* does not form cycloartenol, but rather lanosterol and 9β-lanosta-7,24-dien-3β-ol (36). To test the hypothesis that Tyr540 determines inhibitor specificity, we studied the inhibition of compounds **13** and **16** using a homogenate of a SMY8pSM60.21 strain expressing the OSC of *A. thaliana*. In contrast to our hypothesis, both the methyl and the phenyl derivatives showed similar IC_{50}, respectively, 4.5 and 2 μM. Therefore, the specificity of the aromatic derivatives for the *T. cruzi* OSC may result from more complex differences between the two active sites, allowing different interactions with the inhibitors and possibly relating to the mechanism of control of the rearrangement and of the formation of the final product (37).

In conclusion, our results show that the homogenate of a yeast strain expressing enzymes of pathogenic organisms can be used for a preliminary screening of compounds potentially active against eukaryotic pathogenic organisms in totally safe conditions. The aromatic vinyl-thio-derivatives of the substrate of OSC are very promising candidates for the inhibition of sterol biosynthesis in *T. cruzi* and possibly related pathogens, and could also be considered as models for the design of new inhibitors.

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REFERENCES

1. Mercer, E.I. (1991) Sterol Synthesis Inhibitors: Their Current Status and Modes of Action, *Lipids 26*, 584–597.
2. Abe, I., and Prestwich, G.D. (1999) Squalene Epoxidase and Oxidosqualene Lanosterol Cyclase—Key Enzymes in Cholesterol Biosynthesis, in *Comprehensive Natural Products Chemistry. Isoprenoid Including Carotenoids and Steroids* (Cane, D., ed.), Vol. 2, pp. 267–298, Elsevier, Oxford, United Kingdom.
3. Urbina, J.A. (1997) Lipid Biosynthesis Pathways as Chemotherapeutic Targets in Kinetoplastid Parasites, *Parasitology 114*, S91–S99.
4. Coppens, I., and Courtoy, P.J. (1995) Exogenous and Endogenous Sources of Sterols in the Culture-Adapted Procyclic Trypomastigotes of *Trypanosoma brucei*, *Mol. Biochem. Parasitol. 73*, 179–188.
5. Contreras, L.M., Vivas, J., and Urbina, J.A. (1997) Altered Lipid Composition and Enzyme Activities of Plasma Membranes from *Trypanosoma (Schizotrypanum) cruzi* Epimastigotes Grown in the Presence of Sterol Biosynthesis Inhibitors, *Biochem. Pharmacol. 53*, 697–704.
6. Ryder, N.S., Stuetz, A., and Nussbaumer, P. (1992) Squalene Epoxidase Inhibitors: Structural Determinants for Activity and Selectivity of Allylamines and Related Compounds, in *Regulation of Isopentenoid Metabolism* (Nes, W.D., Parish, E.J., and Trzaskos, J.M., eds.), pp. 192–204, American Chemical Society, Washington, DC.
7. Vanden Bossche, H., Marichal, P., Coene, M.-C., Willemsens, G., Le Jeune, L., Cools, W., and Verhoeven, H. (1992) Cytochrome P450-dependent 14α-Demethylase: Target for Antifungal Agents and Herbicides, in *Regulation of Isopentenoid Metabolism* (Nes, W.D., Parish, E.J., and Trzaskos, J.M., eds.), pp. 219–230, American Chemical Society, Washington, DC.
8. Docampo, R., and Schmuñis, G.A. (1997) Sterol Biosynthesis Inhibitors: Potential Chemotherapeutics Against Chagas Disease, *Parasitol. Today 13*, 129–130.
9. Urbina, J.A., Concepcion, J.L., Rangel, S., Visbal, G., and Lira,

R. (2002) Squalene Synthase as Chemotherapeutic Target in *Trypanosoma cruzi* and *Leishmania mexicana*, *Mol. Biochem. Parasitol. 125*, 35–45.
10. Orene Lorentes, S., Gomez, R., Jimenez, C., Cammerer, S., Yardley, V., de Luca-Fradley, K., Croft, S.L., Ruiz-Perez, L.M., Urbina, J., Gonzalez Pacanowska, D., *et al*. (2005) Biphenylquinuclidines as Inhibitors of Squalene Synthase and Growth of Parasitic Protozoa, *Bioorg. Med. Chem. 13*, 3519–3529.
11. Buckner, F.S., Griffin, J.H., Wilson, A.J., and Van Voorhis, W.C. (2001) Potent Anti-*Trypanosoma cruzi* Activities of Oxidosqualene Cyclase Inhibitors, *Antimicrob. Agents Chemother. 45*, 1210–1215.
12. Oliaro-Bosso, S., Viola, F., Matsuda, S., Cravotto, G., Tagliapietra, S., and Balliano, G. (2004) Umbelliferone Aminoalkyl Derivative as Inhibitors of Oxidosqualene Cyclase from *Saccharomyces cerevisiae, Trypanosoma cruzi,* and *Pneumocystis carinii*, *Lipids 39*, 1007–1012.
13. Abe, I., Rohmer, M., and Prestwich, G.D. (1993) Enzymatic Cyclization of Squalene and Oxidosqualene to Sterol and Triterpenes, *Chem. Rev. 93*, 2189–2206.
14. Thoma, R., Schulz-Gasch, T., D'Arcy, B., Benz, J., Aebi, J., Dehmlow, H., Hennig, M., Stihle, M., and Ruf, A. (2004) Insight into Steroid Scaffold Formation from the Structure of Human Oxidosqualene Cyclase, *Nature 432*, 118–122.
15. Viola, F., Brusa, P., Balliano, G., Ceruti, M., Boutaud, O., Schuber, F., and Cattel, L. (1995) Inhibition of 2,3-Oxidosqualene Cyclase and Sterol Biosynthesis by 10- and 19-Azasqualene Derivatives, *Biochem. Pharmacol. 50*, 787–796.
16. Ceruti, M., Rocco, F., Viola, F., Balliano, G., Milla, P., Arpicco, S., and Cattel, L. (1998) 29-Methylidene-2,3-oxidosqualene Derivatives as Stereospecific Mechanism-Based Inhibitors of Liver and Yeast Oxidosqualene Cyclase, *J. Med. Chem. 41*, 540–554.
17. Viola, F., Balliano, G., Milla, P., Cattel, L., Rocco, F., and Ceruti, M. (2000) Stereospecific Synthesis of *trans*-Vinyldioxidosqualene and β-Hydroxysulfide Derivatives, as Potent and Time-Dependent 2,3-Oxidosqualene Cyclase Inhibitors, *Bioorg. Med. Chem. 8*, 223–232.
18. Viola, F., Ceruti, M., Cattel, L., Milla, P., Poralla, K., and Balliano, G. (2000) Rationally Designed Inhibitors as Tools for Comparing the Mechanism of Squalene-Hopene Cyclase with Oxidosqualene Cyclase, *Lipids 35*, 297–303.
19. Ceruti, M., Balliano, G., Rocco, F., Milla, P., Arpicco, S., Cattel, L., and Viola, F. (2001) Vinyl Sulfide Derivatives of Truncated Oxidosqualene as Selective Inhibitors of Oxidosqualene and Squalene Hopene Cyclases, *Lipids 36*, 629–636.
20. Rocco, F., Oliaro-Bosso, S., Viola, F., Milla, P., Roma, G., Grossi, G., and Ceruti, M. (2003) Conjugated Methyl Sulfide and Phenyl Sulfide Derivatives of Oxidosqualene as Inhibitors of Oxidosqualene and Squalene-Hopene Cyclases, *Lipids 38*, 201–207.
21. Joubert, B.M., Buckner, F.S., and Matsuda, S.P.T. (2001) Trypanosome and Animal Lanosterol Synthases Use Different Catalytic Motifs, *Org. Lett. 3*, 1957–1960.
22. Xiao, X., and Prestwich, G.D. (1991) 29-Methylidene-2,3-oxidosqualene: A Potent Mechanism-Based Inactivator of Oxidosqualene Cyclase, *J. Am. Chem. Soc. 113*, 9673–9678.
23. Ceruti, M., Balliano, G., Viola, F., Cattel, L., Gerst, N., and Schuber, F. (1987) Synthesis and Biological Activity of Azasqualenes, bis-Azasqualenes and Derivatives, *Eur. J. Med. Chem. 22*, 199–208.
24. Field, R.B., Holmund, C.E., and Whittaker, N.F. (1979) The Effects of the Hypocholesteremic Compound 3 β-(β-dimethylaminoethoxy)-androst-5-en-17-one on the Sterol and Steryl Ester Composition of *Saccharomyces cerevisiae*, *Lipids 14*, 741–747.
25. Duriatti, A., Bouvier-Navé, P., Benveniste, P., Schuber, F., Delprino, L., Balliano, G., and Cattel, L. (1985) *In vitro* Inhibition of Animal and Higher Plants 2,3-Oxidosqualene-Sterol Cyclases by 2-Aza-2,3-dihydrosqualene and Derivatives, and by Other Ammonium-Containing Molecules, *Biochem. Pharmacol. 34*, 2765–2777.
26. Ceruti, M., Rocco, F., Viola, F., Balliano, G., Grosa, G., Dosio, F., and Cattel, L. (1993) Synthesis and Biological Activity of 19-Azasqualene 2,3-Epoxide as Inhibitor of 2,3-Oxidosqualene Cyclase, *Eur. J. Med. Chem. 28*, 675–682.
27. Corey, E.J., Matsuda, S.P.T., Baker, C.H., Ting, A.Y., and Cheng, H. (1996) Molecular Cloning of a *Schizosaccharomyces pombe* cDNA Encoding Lanosterol Synthase and Investigation of Conserved Tryptophan Residues, *Biochem. Biophys. Res. Commun. 219*, 327–331.
28. Hart, E.A., Hua, L., Darr, L.B., Wilson, W.K., Pang, J., and Matsuda, S.P.T. (1999) Directed Evolution to Investigate Steric Control of Enzymatic Oxidosqualene Cyclization. An Isoleucine-to-Valine Mutation in Cycloartenol Synthase Allows Lanosterol and Parkeol Biosynthesis, *J. Am. Chem. Soc. 121*, 9887–9888.
29. Peterson, G.L. (1977) A Simplification of the Protein Assay Method of Lowry *et al.* Which Is More Generally Applicable, *Anal. Biochem. 83*, 346–356.
30. Duggleby, R.G. (1981) A Nonlinear Regression Program for Small Computers, *Anal. Biochem. 110*, 9–18.
31. Milla, P., Viola, F., Oliaro-Bosso, S., Rocco, F., Cattel, L., Joubert, B.M., LeClair, R.J., Matsuda, S.P.T., and Balliano, G. (2002) Subcellular Localization of Oxidosqualene Cyclases from *Arabidopsis thaliana, Trypanosoma cruzi*, and *Pneumocystis carinii* Expressed in Yeast, *Lipids 37*, 1171–1176.
32. Milla, P., Athenstaedt, K., Viola, F., Oliaro-Bosso, S., Kohlwein, S.D., Daum, G., and Balliano, G. (2002) Yeast Oxidosqualene Cyclase (Erg7p) Is a Major Component of Lipid Particles, *J. Biol. Chem. 277*, 2406–2412.
33. Balliano, G., Viola, F., Ceruti, M., and Cattel, L. (1988) Inhibition of Sterol Biosynthesis in *Saccharomyces cerevisiae* by *N,N*-Diethylazasqualene and Derivatives, *Biochim. Biophys. Acta 959*, 9–19.
34. Taton, M., Benveniste, P., and Rahier, A. (1992) Inhibition of 2,3-Oxidosqualene Cyclases, *Biochemistry 31*, 7892–7898.
35. Meyer, M.M., Segura, M.J.R., Wilson, W.K., and Matsuda, S.P.T. (2000) Oxidosqualene Cyclase Residues That Promote Formation of Cycloartenol, Lanosterol, and Parkeol, *Angew. Chem. Int. 39*, 4090–4092.
36. Herrera, J.B.R., Wilson, W.K., and Matsuda, S.P.T. (2000) A Tyrosine-to-Threonine Mutation Converts Cycloartenol Synthase to an Oxidosqualene Cyclase That Forms Lanosterol as Its Major Product, *J. Am. Chem. Soc. 122*, 6765–6766.
37. Segura, M.J.R., Lodeiro, S., Meyer, M.M., Patel, A.K., and Matsuda, S.P.T. (2002) Directed Evolution Experiments Reveal Mutation at Cycloartenol Synthase Residue His477 That Dramatically Alter Catalysis, *Org. Lett. 4*, 4459–4462.

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