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Oxidosqualene cyclase from *Saccharomyces cerevisiae*, *Trypanosoma cruzi*, *Pneumocystis carinii* and *Arabidopsis thaliana* expressed in yeast: A model for the development of novel antiparasitic agents

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ABSTRACT

A series of 25 compounds, some of which previously were described as inhibitors of human liver microsomal oxidosqualene cyclase (OSC), were tested as inhibitors of *Saccharomyces cerevisiae, Trypanosoma cruzi*, *Pneumocystis carinii* and *Arabidopsis thaliana* OSCs expressed in an OSC-defective strain of *S. cerevisiae*. The screening identified three derivatives particularly promising for the development of novel anti-Trypanosoma agents and eight derivatives for the development of novel anti-Pneumocystis agents.

Enzymes of the last steps of sterol biosynthesis have been regarded for a long time as good targets for treatment of fungal diseases. Squalene epoxidase and 14α-demethylase, for instance, are the targets of the most effective antifungal drugs currently used, but oxidosqualene cyclase (OSC), 24-methyltransferase and $\Delta^{24(28)}$ sterolmethylreductase are also considered as good target candidates.[1]

Some of the enzymes listed above have been recently addressed as good targets for development of novel antiparasitic drugs. Several inhibitors of sterol 24-methyltransferase or sterol 14α-demethylase, for instance, are good candidates for the treatment of Chagas disease, a chronic infection caused by the protozoan parasite *Trypanosoma cruzi*.[2]

In the last few years, a variety of OSC inhibitors have also been tested on *T. cruzi*, *Trypanosoma brucei*, *Pneumocystis carinii* and *Leishmania mexicana* with the aim of developing new agents against these microorganisms.[1c,1d,3]

The diseases caused by protozoan parasites are becoming an urgent research topic, not only in tropical regions, but also in once safer countries.[4] These diseases cause high rates of mortality and morbidity, and few drugs are currently available for their treatment.[5] Therefore, the identification of new derivatives endowed with a selective action on protozoan OSC is a hot topic in medicinal chemistry.

In this study, we were aiming at identifying new starting points for the development of antiparasitic agents. Therefore a set of compounds, previously described as effective inhibitors of human oxidosqualene cyclase,[6] were tested for their ability to inhibit oxidosqualene lanosterol cyclases from *T. cruzi* and *P. carinii* expressed in an OSC-defective *Saccharomyces cerevisiae* strain.[7a] As a comparison, the study was extended to two further OSCs expressed in the same OSC-defective strain, namely OS lanosterol cyclase from *S. cerevisiae*,[7a] and OS cycloartenol synthase from *Arabidopsis thaliana*.[7a] A preliminary attempt at evaluating the human versus parasite selectivity was done by testing compounds against human OSC expressed in the same OSC-defective yeast strain.[7b] The structures of the compounds evaluated are depicted in Figure 1.

The preparations of the compounds **1** (Ro 48-8071), **2–9, 10** (BIB 515) and **13** have been previously described.[6,8] The syntheses of benzo[*d*]isothiazole OSC inhibitors are outlined in Schemes 1 and 2. Alkylation of benzo[*d*]isothiazole **26**[6] with an excess of 1,4-dibromobutane gave the bromide **27** and its amination provided the final amines **11** and **12**, as well as the intermediate **28**. Acylation of **28** under basic conditions thus yielded derivative **18**. The compounds **14–17**, containing a carbon based linker, were obtained by converting **26** to triflate **29**, which was subjected to a

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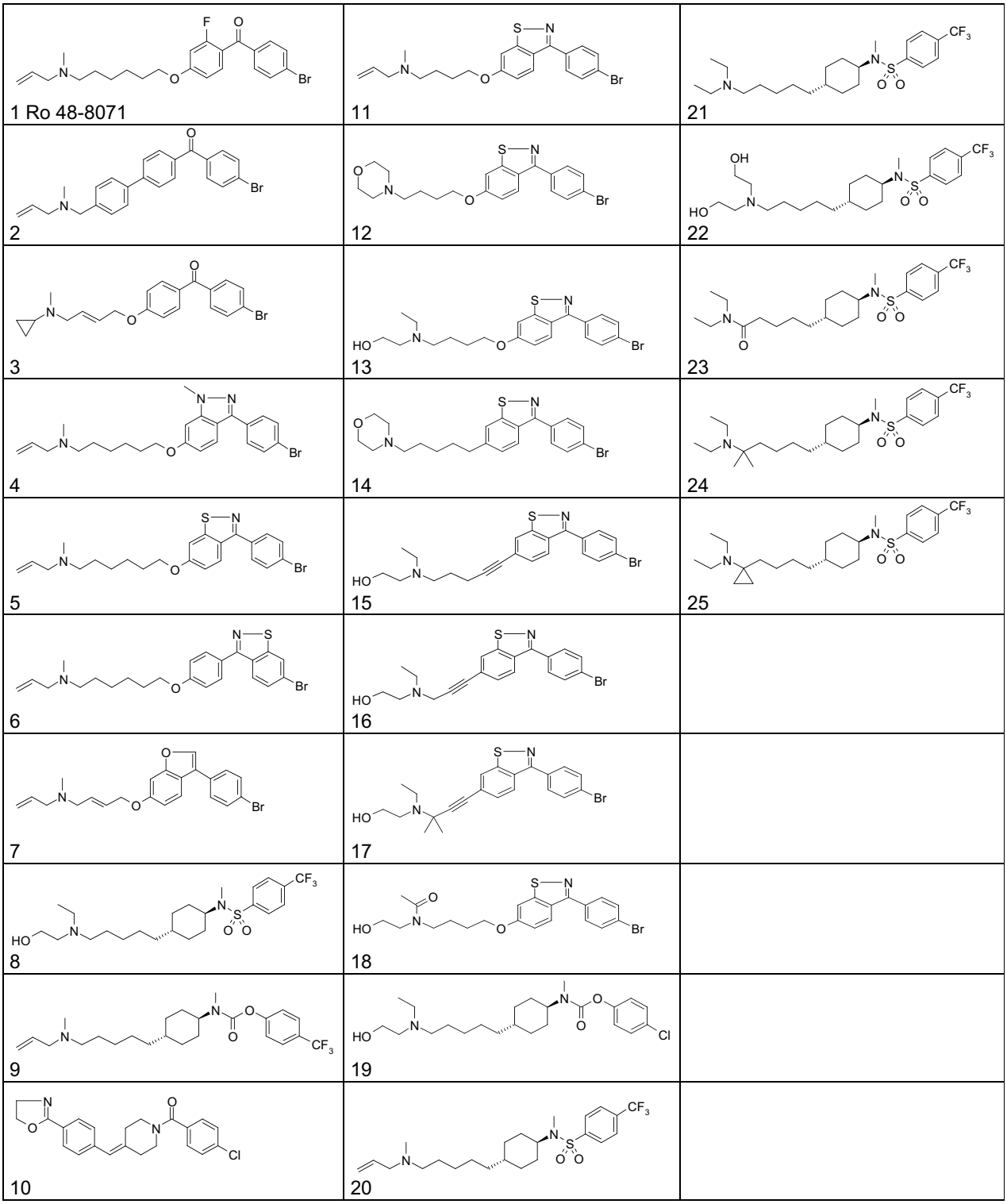
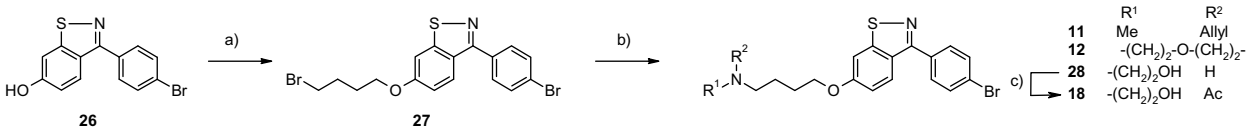


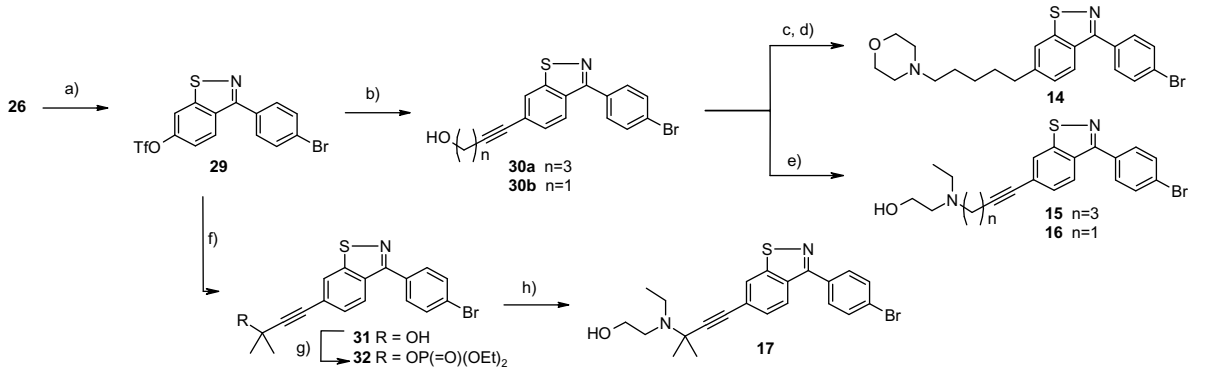
Figure 1. Chemical structures of investigated compounds.

Sonogashira reaction to give the intermediates **30a/b** and **31**, respectively. Hydrogenation of **30a**, followed by in situ formation of the triflate and amination, provided amine **14** in three steps. Direct mesylation of the alcohols **30a/b** and subsequent amination yielded the products **15** and **16**, respectively. Amine **17** was derived from alkynol **31** via phosphonate **32**.

The syntheses of aminocyclohexanes with carbon-linked spacers are outlined in Schemes 3 and 4. LAH reduction of **34**,[8c] mesylation and cleavage of the BOC-protecting group yielded the building block **35** as a TFA salt. Treatment of **35** with the appropriate chloroformate or sulfonyl chloride, followed by amination provided the carbamate derivative **19** or the sulfonamides **20–22**. Starting from **34**, ester hydrolysis, amide formation under standard conditions, followed by BOC deprotection gave the intermediate amine **36** as an HCl salt. Sulfonamide formation provided the desired amide **23**. For the preparation of the α,α-branched amines **24** and **25** modified Bouveault reaction conditions were used.[9]



Scheme 1. Synthesis of benzo[*d*]isothiazole OSC inhibitors **11**, **12** and **18**. Reagents and conditions: (a) 1,4-dibromobutane, K_2CO_3, acetone, 50 °C (71%); (b) **11**: MeNHallyl, DMA, rt (quant.), **12** HBr: morpholine, DMF, *i*Pr_2NEt, rt (50%), **28**: 1—$HOCH_2CH_2NH_2$, DMA, rt; 2—HCl, MeOH, rt (70%); (c) NaOH, Ac_2O, *i*PrOH, rt (86%).



Scheme 2. Synthesis of benzo[*d*]isothiazole OSC inhibitors **14**–**17**. Reagents and conditions: (a) $(TfO)_2O$, pyridine, 0 °C to rt (98%); (b) **30a**: $PdCl_2(PPh_3)_2$, PPh_3, CuI, $HCC(CH_2)_3OH$, NEt_3, rt to 50 °C (72%), **30b**: $Pd(PPh_3)_4$, CuI, $HCCCH_2OH$, piperidine, rt to 80 °C (60%); (c) **14**: H_2, PtO_2, EtOH, rt (93%); (d) 1—$(TfO)_2O$, *i*Pr_2NEt, CH_2Cl_2, 0 °C to rt; 2—morpholine, CH_2Cl_2, rt (86%); (e) **15**: 1—$MeSO_2Cl$, DMAP, pyridine, CH_2Cl_2, 0° to rt; 2—$NH(Et)(CH_2CH_2OH)$, DMA, rt (54%, 2 steps), **16**: 1—$MeSO_2Cl$, DMAP, pyridine, CH_2Cl_2, rt; 2—$NH(Et)(CH_2CH_2OH)$, DMA, rt (31%, 2 steps); (f) $PdCl_2(PPh_3)_2$, PPh_3, CuI, $HCCC(Me)_2OH$, NEt_3, rt to 50 °C (61%); (g) *n*BuLi, ClP(=O)$(OEt)_2$, NEt_3,THF, −78 °C to 0 °C (33%); (h) $NH(Et)(CH_2CH_2OH)$, $Pd(PPh_3)_4$, THF, 50 °C (8%).

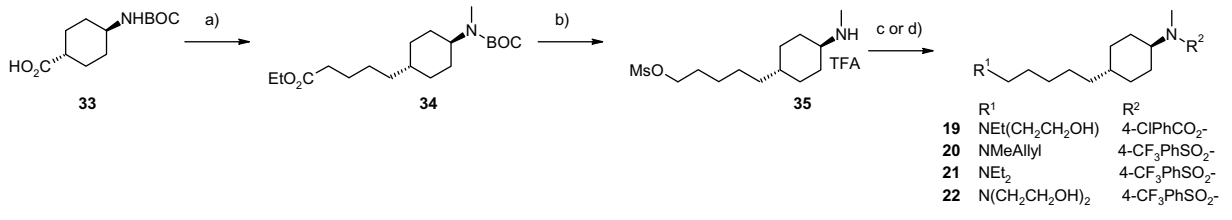
A preliminary screening of 10 compounds from different chemical classes, carried out in cell free homogenates,[10] identified three lead compounds, **5, 8** and **9** (Table 1). Compound **5** was one of the most potent inhibitors of OSC from *P. carinii*, slightly more potent then **1** (Ro 48-8071). Furthermore, it belongs to a new heterocyclic series—the phenyl-benzo[*d*]isothiazoles, not yet explored in this context. Compound **9**, a substituted cyclohexylamine, was the most potent inhibitor of OSC from *T. cruzi*, and one of the most active against OSC from *P. carinii*. In addition, this compound is in a different chemical class from compounds **1** (Ro 48-8071) and **5**. Compound **8**, in the same chemical class as **9**, was less potent than **5** and **9** for inhibition of OSC from *P. carinii* and *T. cruzi*. This notwithstanding, compound **8** was used as a lead compound because it had potency not too different from **9** on human OSC, 2-fold less in human liver,[8c] 3-fold less when tested on homogenates from yeast recombinant strain SMY8.

For the next round of testing we wanted to investigate the influence of both spacer unit and basicity of the amine in the two distinct series. Therefore, eight analogs of compound **5** (i.e. heterocyclic analogs of Ro 48-8071: compounds **11–18**) and seven analogs of the cyclohexylamines **8** and **9** (compounds **19–25**) were selected (Table 2).

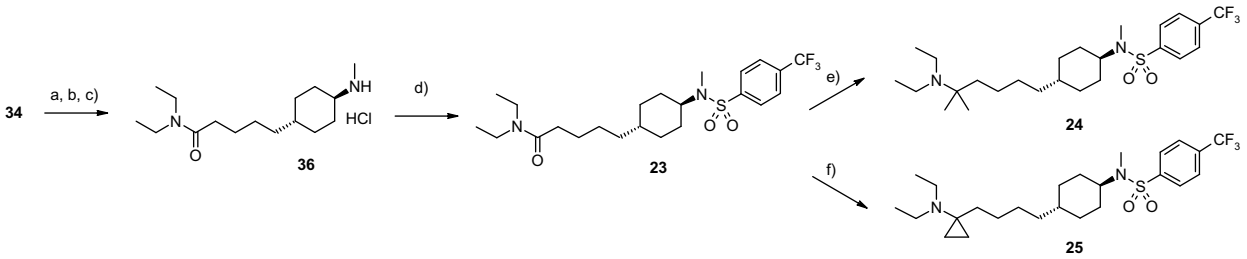
In the heterocyclic series, the reduction of the chain length from *n*-hexyl to *n*-butyl, as demonstrated for compounds **5** and **11**, did not influence the potency of the inhibitors for *P. carinii* and *T. cruzi* OSCs. For the inhibition of OS lanosterol cyclase from *S. cerevisiae*, a 10-fold drop in activity was seen, whereas a significant increase in potency was found for OS cycloartenol cyclase from *A. thaliana*.

An increase in inhibitory effect on OSC from *P. carinii* was seen when the methylallylamine was replaced by a morpholine or *N*-hydroxyethylethylamine residue (**11** vs **12** and **13**). These changes did not affect the activity of the inhibitors against *S. cerevisiae*, whereas a decrease in activity was observed for *T. cruzi* and *A. thaliana.*

For OSCs from *P. carinii* and *S. cerevisiae*, the replacement of the amine moiety by an amide residue (as in compound **18**) was detrimental to the activity of the inhibitors. This is similar to the effects previously observed for the inhibition of *human* liver OSC.[8c] However, the inhibition of OS lanosterol cyclase from *T. cruzi* was hardly affected and that of OS cycloartenol cyclase from *A. thaliana* remained submicromolar.



Scheme 3. Synthesis of aminocyclohexane derivatives **19**–**22**. Reagents and conditions: (a) 1—carbonyldiimidazol, MeOH, rt (99%); 2—NaH, MeI, DMF, 0 °C to rt; 3—$LiBH_4$, THF, reflux; 4—oxalylchloride, DMSO, NEt_3, CH_2Cl_2, −78 °C to rt; 5—triethyl-phosphono acetate, EtOH, NaOMe, rt (86%, 4 steps); 6—Pd/C, H_2, MeOH, rt (94%); 7—$LiBH_4$, THF, reflux, (73%, 2 steps); 8—oxalylchloride, DMSO, NEt_3, CH_2Cl_2, −78 °C to rt; 9—triethyl-phosphono acetate, EtOH, NaOMe, rt (88%, 2 steps); 10—Pd/C, H_2, MeOH, rt (94%); (b) 1—LAH, THF, rt (97%); 2—$MeSO_2Cl$, NEt_3, CH_2Cl_2, rt; 3—TFA, CH_2Cl_2, rt (73%, 2 steps); (c) **19** HCl: 1—4-Cl-PhOCOCl, *i*Pr_2NEt, CH_2Cl_2, rt (82%); 2—$NH(Et)(CH_2CH_2OH)$, MeOH, 60 °C; 3—HCl in EtOAc (56%, 2 steps); (d) 1—4-CF_3-$PhSO_2Cl$, *i*Pr_2NEt, CH_2Cl_2, rt (74%); 2—**20**: NHMeAllyl, MeOH, 60 °C (56%); **21**: Et_2NH, MeOH, 60 °C (73%); **22**: $NH(CH_2CH_2OH)_2$, MeOH, 60 °C (60%).



Scheme 4. Synthesis of aminocyclohexane derivatives **23–25**. Reagents and conditions: (a) aq LiOH, THF; $KHSO_4$, EtOAc (quant.); (b) EDCI, HOBt, $NHEt_2$, NMM, CH_2Cl_2, rt (86%); (c) HCl, dioxane, rt (quant.); (d) 4-CF_3-$PhSO_2Cl$, iPr_2NEt, DMAP, CH_2Cl_2, 0 °C to rt (85%); (e) MeMgBr, $ZrCl_4$, THF, −10 °C to rt (63%); (f) EtMgBr, $ZrCl_4$, THF, −10 °C to rt (58%).

Table 1
Effect of inhibitors (first series) on oxidosqualene cyclase activity of homogenates prepared from yeast recombinant strains SMY8 expressing *S. cerevisiae*, *T. cruzi*, *P. carinii*, *A. thaliana* and *H. sapiens* OSCs[7]

Compound	IC_{50}[a] (μM)				
	OSC[b] *S. cerevisiae*	OSC[b] *T. cruzi*	OSC[b] *P. carinii*	OSC[c] *A. thaliana*	OSC[b] *H. sapiens*
1 fumarate	0.21	0.90	0.34	1.75	0.17
2 HCl	0.32	0.60	0.05	0.25	0.25
3 HCl	0.18	1.50	0.20	0.26	0.27
4 fumarate	0.29	3.00–5.00	≫5	2.60	2.30
5 fumarate	0.07	0.34	0.23	0.95	0.16
6 fumarate	0.28	≫5	0.55	10.00	20.34
7 fumarate	0.21	3.00–5.00	1.20	3.00	90.00
8 fumarate	0.27	1.60	3.00	3.00	0.45
9 citrate	0.05	0.07	0.34	1.00	0.14
10	0.67	≫5	≫5	2.5	1.15

[a] Values are the means of two separate experiments with duplicate incubations, each. The maximum deviations from the mean were less than 10%.
[b] Lanosterol cyclase.
[c] Cycloartenol cyclase.

Table 2
Effect of the second series of inhibitors on oxidosqualene cyclase activity of homogenates prepared from yeast recombinant strains SMY8 expressing *S. cerevisiae*, *T. cruzi*, *P. carinii*, *A. thaliana* and *H. sapiens* OSCs[7]

Compound	IC_{50}[a] (μM)				
	OSC[b] *S. cerevisiae*	OSC[b] *T. cruzi*	OSC[b] *P. carinii*	OSC[c] *A. thaliana*	OSC[b] *H. sapiens*
11	0.70	0.25	0.20	0.07	0.26
12 HBr	0.60	1.10	0.07	0.48	0.12
13 HCl	0.70	6.50	0.05	2.30	0.52
14	0.40	1.10	0.05	0.65	0.10
15	5.00	>10	2.25	1.70	0.23
16	0.30	2.30	0.80	0.55	4.60
17	2.20	4.20	1.00	1.25	11.5
18	100	5.50	100	0.80	>1
19 HCl	0.10	2.00	2.50	0.70	4.12
20	0.30	0.30	0.12	0.44	0.1
21	3.60	2.40	0.60	1.00	2.44
22	1.50	2.70	0.40	1.50	0.72
23	>100	>100	>100	2.00	>10
24	7.00	>100	1.00	4.10	4.93
25	0.50	0.40	1.30	0.32	0.44

[a] Values are the means of two separate experiments with duplicate incubations, each. The maximum deviations from the mean were less than 10%.
[b] Lanosterol cyclase.
[c] Cycloartenol cyclase.

Modification of the linker of the side chain from oxygen to carbon (**12** vs **14**) did not influence the potency of the inhibitors for all OSCs tested.

Introducing rigidity in the spacer unit caused a drop in the activity against the lanosterol cyclases, when comparing compounds **13** and **15**. This effect seemed to be most pronounced for the inhibition of OSC from *P. carinii*. Some of the activity could be recovered by shortening the spacer unit from pentynyl to propynyl (**15** vs **16**). This was most apparent for the activity on *S. cerevisiae*, but also visible on *T. cruzi*.

The activity of the compounds was not changed when introducing substituents as branching points alpha to the amine (**16** vs **17**) for OSC from *P. carinii*, whereas it dropped 2- to 7-fold for the other OSCs.

In the series of cyclohexylamines, changes both in the spacer region and at the amine part of the molecules were also investigated. In the both subseries, the carbamates (**9** vs **19**) and the sulfonamides (**8** vs **20**), the allylmethyl amine residue was the most promising group. In the sulfonamide subseries, modifications at the amine did not significantly influence the activity against OS cycloartenol cyclase (compounds **8, 20–22**). The amide **23** was not an inhibitor of the lanosterol cyclases, but it is still effective against the cycloartenol cyclase. Disubstitution by methyl residues alpha to the amino group led to a 2-fold drop in activity against lanosterol cyclase from *P. carinii* and *S. cerevisiae* and a detrimental drop in the activity against *T. cruzi* enzyme (**24** vs **21**). An improved potency was observed for the cyclopropyl derivative **25** for all OSCs with the exception of lanosterol cyclase of *P. carinii*.

Altogether, while most of the inhibitors showed identical or comparable activity against both *T. cruzi* and *P. carinii* OSCs, five compounds (**2, 12, 13, 14** and **24**) proved to be specific for *P. carinii* enzyme showing at least a 10-fold selectivity, but even more than 100-fold for the best compounds (**13** and **24**). On the other hand inhibitor **9**, the most effective on *T. cruzi* enzyme (IC_{50} 0.07 μM), was only 5-fold more active than on the *P. carinii* enzyme. Compound **11** was the only inhibitor of *A. thaliana* OSC with an $IC_{50} < 0.1$ μM, but it showed only a weak selectivity. Surprisingly enough, the amides **18** and **23** inhibited *A. thaliana* OSC, whereas they were inactive or only slightly active against the other OSCs.

All the compounds were also tested as inhibitors of human OSC expressed in the same defective strain of *S. cerevisiae*, in order to gain a preliminary evaluation of the human versus parasite selectivity (Table 1). The compounds showed a poor human versus *T. cruzi* selectivity. The IC_{50} values on human enzyme were comparable or even lower than those on the parasite OSC. Similar results were obtained with *P. carinii* enzyme, with the exception of compounds **2** and **13**, which proved 5 and 10 times as effective against parasite enzyme, respectively. A higher selectivity (40–80 times) was seen for the less active compounds **6** and **7**.

The effect of some derivatives on oxidosqualene cyclases from *T. cruzi* and *P. carinii* was also tested on spheroplasts[11] prepared from recombinant yeast cells expressing OSCs from these pathogens (Tables 3 and 4). As recently observed,[11] spheroplasts from these recombinant yeast strains can make sterols, although the post-squalene section of the biosynthetic pathway appears rather depressed as indicated by the higher squalene/sterols ratios

Table 3
Effect of the most representative inhibitors on sterol biosynthesis in spheroplasts of *S. cerevisiae* expressing *T. cruzi* OSC[11]: % radioactivity incorporated into non-saponifiable lipids separated on TLC

Compound	% Radioactivity incorporated into non-saponifiable lipids[a]				
	Squalene	Oxido squalene	Lanosterol	Mono methylsterols	Ergosterol
Control	50.22	6.10	18.01	3.37	22.30
1 fumarate					
0.1 μM	52.35	6.59	26.20	1.75	13.11
1 μM	42.59	38.31	11.09	1.94	6.07
2 HCl					
0.1 μM	44.89	9.88	22.12	2.47	20.64
1 μM	47.22	34.59	6.77	2.66	8.76
3 HCl					
0.1 μM	48.34	8.69	22.99	2.72	17.26
1 μM	40.90	33.39	12.38	2.29	11.04
5 fumarate					
0.1 μM	44.47	33.26	10.29	1.32	10.66
1 μM	52.38	33.37	5.08	1.62	7.55
9 citrate					
0.1 μM	52.31	37.42	3.33	1.43	5.51
1 μM	54.58	38.65	1.71	1.29	3.77
11					
1 μM	42.41	28.74	7.03	3.19	18.63
10 μM	46.47	24.37	3.20	8.19	17.77
14					
1 μM	45.04	15.86	19.33	2.01	17.76
10 μM	46.17	24.76	4.65	7.52	16.90
20					
0.1 μM	54.01	34.86	4.15	1.40	5.58
1 μM	56.42	37.51	2.40	1.01	2.66

[a] Values are the means of two separate experiments with duplicate incubations, each. The maximum deviations from the mean were less than 10%.

Table 4
Effect of the most representative inhibitors on sterol biosynthesis in spheroplasts of *S. cerevisiae* expressing *P. carinii* OSC[11]: % radioactivity incorporated into non-saponifiable lipids separated on TLC

Compound	% Radioactivity incorporated into non-saponifiable lipids[a]				
	Squalene	Oxido squalene	Lanosterol	Mono methylsterols	Ergosterol
Control	70.33	1.72	10.42	2.82	14.71
1 fumarate					
0.1 μM	71.45	11.06	9.46	1.06	6.97
1 μM	60.54	30.96	3.36	1.54	3.60
2 HCl					
0.1 μM	72.47	1.80	13.01	2.20	10.52
1 μM	62.92	27.80	5.16	1.12	3.00
3 HCl					
0.1 μM	62.92	2.50	17.43	3.25	13.90
1 μM	64.27	16.7	10.21	1.99	6.83
5 fumarate					
0.1 μM	66.59	8.23	10.78	2.91	11.49
1 μM	63.37	24.96	5.20	1.39	5.08
6 fumarate					
0.1 μM	65.98	1.17	14.49	2.90	15.46
1 μM	67.66	1.44	14.29	3.44	13.17
9 citrate					
0.1 μM	70.38	9.03	8.89	1.81	9.89
1 μM	67.53	24.95	2.39	1.06	4.07
12 HBr					
0.1 μM	71.04	0.68	9.55	3.57	15.16
1 μM	69.63	1.72	15.78	2.82	10.05
13 HCl					
0.1 μM	71.03	2.39	9.93	3.76	12.89
1 μM	69.90	15.72	5.81	2.80	5.77
14					
0.1 μM	69.01	1.30	12.55	4.02	13.12
1 μM	69.62	5.44	9.84	3.91	11.19

[a] Values are the means of two separate experiments with duplicate incubations, each. The maximum deviations from the mean were less than 10%.

compared with intact recombinant cells. Results confirmed data from experiments with homogenates: all the compounds caused a dose-dependent increase of the ratio between radioactive oxidosqualene and sterols extracted from treated cells, thus indicating that OSC was inhibited. The tested compounds did not substantially affect the fraction of radioactive squalene, suggesting that squalene epoxidase was not affected.

In conclusion, the screening of 25 compounds as inhibitors of *P. carinii* and *T. cruzi* OSCs has identified several promising derivatives as starting points for the development of novel antiparasitic agents. The compounds **9, 11** and **20**, which showed an activity $\leqslant$0.3 μM, are particularly promising as novel anti-Trypanosoma agents, while the compounds **2, 3, 5, 11, 12, 13, 14** and **20** show promise as novel anti-Pneumocystis agents. The derivatives **11** and **20** showed similar potency against both parasital OSCs. As a rule, the *P. carinii* enzyme was more susceptible than *T. cruzi* enzyme to the action of the inhibitors. While three out of the four most active compounds against *P. carinii* OSC were analogs of compound **5**, the most active compound against the *T. cruzi* enzyme was the cyclohexylamine derivative **9**, the only compound more active against *T. cruzi* than against *P. carinii* OSC.

Homology models built taking advantage of the established structure of human OSC[12] could be useful to relate the structure and activity of compounds to the differences in the active site of target enzymes. In silico studies are in progress with the aim of finding novel active molecules and improving the parasite versus human selectivity of the most active compounds of the present series.

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References and notes

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