

New Promising Compounds with in Vitro Nanomolar Activity against *Trypanosoma cruzi*

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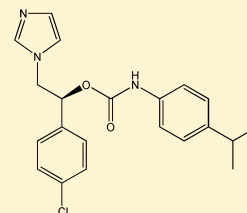
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Supporting Information

ABSTRACT: The antiparasitic activity of azole and new 4-aminopyridine derivatives has been investigated. The imidazoles **1** and **3–5** showed a potent in vitro antichagasic activity with IC₅₀ values in the low nanomolar concentration range. The (*S*)-**1**, (*S*)-**3**, and (*S*)-**5** enantiomers showed (up to) a thousand-fold higher activity than the reference drug benznidazole and furthermore low cytotoxicity on rat myogenic L6 cells.



KEYWORDS: *Trypanosoma cruzi*, CYP51, antiparasitic activity, Chagas disease, azole, aminopyridine

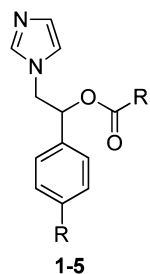
Chagas disease (CD) is one of the most important neglected tropical disorders, caused by the Kinetoplastid parasite *Trypanosoma cruzi*,¹ and approximately 10 million people worldwide are estimated to be infected.² CD is a complex anthroponosis transmitted to humans by blood-sucking insects of the Triatominae subfamily (mainly *Triatoma infestans*, *Rhodnius prolixus*, and *Triatoma dimidiatae*).³ Currently, the treatment of CD is restricted to nifurtimox and benznidazole used only in the acute phase. The clinical efficacy in chronic patients is limited and controversial, and the undesirable side effects of both drugs are a major drawback in their use.^{4,5} To date, posaconazole and E1224, a pro-drug of ravuconazole, are in phase 2 of clinical trials for chronic Chagas disease treatment.⁶ However, the development of new effective and safe drugs is urgent because of the lack of vaccines, the inadequate chemotherapy, the large number of infected people, and their migration toward nonendemic countries.

Similar to fungi and yeasts, Kinetoplastids are strictly dependent on endogenously produced sterols, essential cellular components, that modulate membrane fluidity/permeability and also play multiple regulatory functions related to cell division, growth, and developmental processes. Sterol 14 α -demethylase (CYP51) is an essential enzyme in sterol biosynthesis and its inhibition causes the block of sterol production and the accumulation of toxic methylated sterol precursors followed by pathogen growth arrest and death.^{7,8}

For these reasons, CYP51 of *T. cruzi* (CYP51_{Tc}) is a highly drug-targetable enzyme for the rational design of new antitrypanosomal drugs. Currently, antifungal azoles (i.e.,

ketoconazole, fluconazole, posaconazole, and voriconazole) are known as CYP51_{Tc} inhibitors due to the presence of an imidazole or triazole ring able to coordinate the heme-iron.^{9,10} Moreover, recent studies demonstrated that *N*-heterocyclic rings can be effectively replaced by 3-pyridyl or 4-pyridyl moieties.^{11–13}

We have selected eight compounds available from our laboratory library: racemic 1-imidazolyl-2-phenylethanol derivatives¹⁴ (**1–5**) (Figure 1) and new 4-aminopyridines (**6–8**) (Scheme 1). Compounds **1–5** belonging to azole class have been chosen for their structural similarity with CYP51_{Tc} inhibitors; the new 4-aminopyridines (**6–8**), second generation



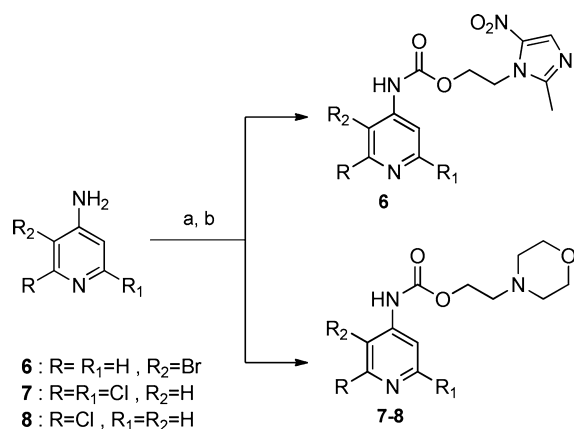
- 1**: R=H, R₁=4-biphenyl
2: R=F, R₁=phenyloxymethyl
3: R=Cl, R₁=4-(propan-2-yl)-phenylamino
4: R=F, R₁=4-(propan-2-yl)-phenylamino
5: R=Cl, R₁=2,6-dichloropyridin-4-yl-amino

Figure 1. Compounds **1–5** described in this study.

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Scheme 1. Synthesis of Compounds 6–8^a

^a(a) TEA, triphosgene, anhydrous benzene, reflux, 5 h; (b) metronidazole for compound 6, 12 h, r.t. (yield 68%); 2-(morpholin-4-yl)ethanol for compounds 7 (yield 70%) and 8 (yield 60%), 12 h at r.t.

compounds, have been selected for the presence of the *N*-4-pyridyl moiety able to interact with the CYP51_{Tc} active site, as previously reported.¹² Compounds 1–8 have a nitrogen atom for the heme-iron coordination and hydrophobic moieties able to interact with lipophilic tunnel in the CYP51_{Tc} active site. Here, we report the evaluation of their antiparasitic activity.

Compounds 1–5 have been prepared as previously reported.¹⁴ Compound 6 was obtained by reaction of metronidazole with 3-bromo-4-aminopyridine previously activated with triphosgene, in anhydrous benzene; 7 and 8 were similarly obtained by 2-(morpholin-4-yl)ethanol with 2,6-dichloro-4-aminopyridine and 2-chloro-4-aminopyridine, respectively (Scheme 1).

The biological activity of the selected compounds has been evaluated as reported elsewhere¹⁵ against different parasites: *T. brucei rhodesiense* STIB 900 trypanomastigote stage; *T. cruzi* Tulahuen C2C4 amastigote stage; *L. donovani* MHOM-ET-67/L82 amastigote stage; *P. falciparum* K1 erythrocytic stages. Furthermore, the cytotoxic activity against cultured rat L6 myogenic cells was determined (Table 1). The reported data

Table 1. In Vitro Antiparasitic Activity and Cytotoxicity of 1–8 and Reference Drugs; Data Represent IC₅₀ Values Given in μM

compd	Tb ^a	Tc ^b	Ld ^c	Pf ^d	L6 ^e	SI ^f
1	42.1	0.014	5.4	2.2	23.6	1686
2	293.8	24.4	17.3	36.7	182.5	7.5
3	18.2	0.005	7.0	1.8	13.0	2600
4	19.1	0.005	15.0	4.6	16.8	3360
5	34.0	0.036	11.4	1.5	27.2	756
6	138.9	48.4	85.1	60.2	222.1	4.6
7	117.4	133.1	122.7	23.1	79.3	0.6
8	230.3	175.3	190.4	79.8	237.6	1.3
ref drug ^g	MEL	BNZ	MIL	CHL	PDT	
	0.0075	1.671	0.366	0.328	0.001	

^a*T. brucei rhodesiense* STIB 900 trypanomastigote stage. ^b*T. cruzi* Tulahuen C2C4 amastigote stage. ^c*L. donovani* MHOM-ET-67/L82 amastigote stage. ^d*P. falciparum* K1 erythrocytic stages. ^eRat myogenic L6 cells. ^fSI = selectivity index, IC₅₀L6/IC₅₀Tc. ^gMEL = melarsoprol; BNZ = benznidazole; MIL = miltefosine; CHL = chloroquine; PDT = podophyllotoxin.

showed that 1 and 3–5 possess high activity toward *T. cruzi* with IC₅₀ values from 5.0 to 36.0 nM; 3 and 4 turned out to be the most active compounds with an IC₅₀ of 5.0 nM. Furthermore, 1 and 3–5 proved to be highly selective against *T. cruzi*, as evidenced by low IC₅₀ values compared to the other studied parasites, characterized by low cytotoxicity with IC₅₀ values ranging from 13.0 to 27.2 μM. The new 4-aminopyridine derivatives 6–8 showed lower antiparasitic activity than the imidazoles 1–5.

The high activity of the racemic azole derivatives prompted us to define the differences in the antitrypanosomal activity of the single enantiomers.

Compounds 1, 3, and 5 have been selected and pure (*R*)-1, (*S*)-1, (*R*)-3, (*S*)-3, (*R*)-5, and (*S*)-5 have been prepared as previously described for racemic compounds¹⁴ from the corresponding enantiomeric 1-(phenyl)-2-(1*H*-imidazol-1-yl)-ethanols obtained by modification of a reported procedure.¹⁶ The following enantiomeric purity was observed: (*R*)-1 98.0% ee; (*S*)-1 98.0% ee; (*R*)-3 98.5% ee; (*S*)-3 98.5% ee; (*R*)-5 98.8% ee; (*S*)-5 98.8% ee (see Supporting Information).

The antiparasitic activity of pure enantiomers is reported in Table 2 and clearly indicate that both the (*R*) and (*S*)

Table 2. In Vitro Activity of Compounds 1, 3 and 5 and Their Pure Enantiomers against *T. cruzi*; the Cytotoxic Activity on L6 Cells Is Also Given; Data Represent IC₅₀ Values in μM with Standard Deviation (SD)

compd	Tc ^a	SD	L6 ^b	SD	SI ^c
1	0.0149	0.0109	23.59	5.92	1583
(<i>R</i>)-1	0.1927	0.1167	27.50	4.89	143
(<i>S</i>)-1	0.0038	0.0030	18.24	1.82	4800
3	0.0052	0.0031	12.97	0.73	2494
(<i>R</i>)-3	0.1120	0.0469	13.96	1.77	125
(<i>S</i>)-3	0.0018	0.0008	13.44	2.63	7467
5	0.0364	0.0170	27.25	3.33	749
(<i>R</i>)-5	0.0972	0.0340	15.38	4.83	158
(<i>S</i>)-5	0.0236	0.0143	31.65	5.00	1341

^a*T. cruzi* Tulahuen C2C4 amastigote stage. ^bRat myogenic L6 cells. ^cSI = selectivity index, IC₅₀L6/IC₅₀Tc.

enantiomers are highly active and that the biological activity is mainly due to the (*S*) enantiomers, indeed (*S*)-1 and (*S*)-3 show IC₅₀ values 50–60 times lower than the corresponding (*R*) enantiomers. The carbamate (*S*)-3 was found to be the most active compound with an IC₅₀ of 1.8 nM vs *T. cruzi*, resulting in a molar basis approximately a thousand-fold more active than benznidazole. It is noteworthy that all compounds showed a low toxicity toward L6 cells, including the most active azole derivatives, 1 and 3–5, and the corresponding pure enantiomers.

Furthermore, on the basis of the structural similarity between the studied compounds and the current CYP51_{Tc} inhibitors, a docking study of compounds 1–8 against the energy minimized structure of CYP51_{Tc} (PDB ID: 2WX2), CYP51_{Tb} (PDB ID: 3GW9), and CYP51_{Li} (PDB ID: 3LAD; *L. infantum* belonging to *L. donovani* complex) was performed. Proteins pretreatment was performed by means of the *Protein Preparation Wizard* tool of Maestro 9.2 suite¹⁷ and the energy minimization using MacroModel¹⁸ with the OPLS2005 force field. Docking calculations were performed by means of GOLD5.1¹⁹ using the ASP scoring function because it was the one that best reproduced the crystallographic pose of

cocrystallized ligands. In the CYP51_{Tc} active site, **1**, **3**, and **5** are mostly stabilized by the coordination of the nitrogen in position 3 of the imidazolic ring to heme-iron and by hydrophobic interactions in the pocket formed by Val102, Tyr103, Met106, Phe110, Tyr116, Leu127, Ala287, Ala288, Met289, Phe290, Ala291, Met360, and Met460. Compound **2** belongs to the same class of compounds **1**, **3**, and **5**, coordinating the heme-iron with the N3 of the imidazolic ring. The lack of its activity could be ascribed to the loss of most of the hydrophobic interactions that stabilize the other active molecules in the pocket described just above. The difference in the activity between (*R*) and (*S*) enantiomers of the most active compounds could be ascribed to the better coordination of the imidazolic ring to the heme-iron of (*S*) enantiomers than that of (*R*) ones. From our docking calculations, the N3 of the imidazolic ring in all (*S*) enantiomers coordinate the heme-iron in about 1.9 Å distance, while in (*R*) enantiomers, the slight difference in the conformation and the consequent rotation of the N3 cause a less favored coordination (e.g., 2.0 Å for compound (*R*)-**5**, 2.2 Å for compound (*R*)-**1**, and 2.3 Å compound (*R*)-**3** approximately) (Figures 2–4). The high

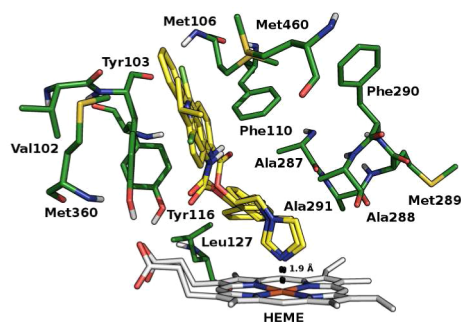


Figure 2. Binding mode of (*S*) enantiomers. The protein and the heme prosthetic group (CYP51_{Tc}) are represented in green and gray, respectively; compounds **1**, **3**, and **5** in yellow. The distance between the N3 of the imidazolic ring and the heme-iron is represented in black dots.

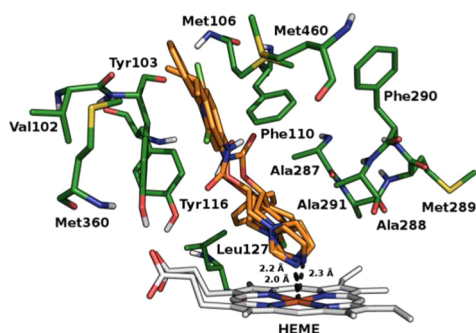


Figure 3. Binding mode of (*R*) enantiomers. The protein and the heme prosthetic group (CYP51_{Tc}) are represented in green and gray, respectively; compounds **1**, **3**, and **5** in orange. The distance between the N3 of the imidazolic ring and the heme-iron is represented in black dots.

selectivity of compounds **1**, **3**, and **5** toward *T. cruzi* over *T. brucei* and *L. donovani* is probably due to the active site volume and surface area smaller in *T. brucei* and *L. donovani* than in *T. cruzi* that could favor their access in the active site.²⁰ The aminopyridines **6**–**8** do not show an interesting CYP51_{Tc}

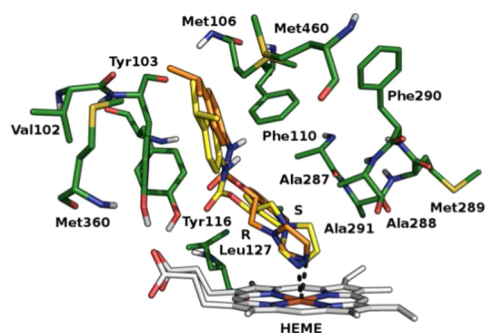


Figure 4. Different conformations in the CYP51_{Tc} active site of the compound **3**; (*R*)-**3** shown in orange and (*S*)-**3** in yellow.

inhibitory activity, probably because of the lack of the heme-iron coordination (see Figure S4 in Supporting Information).

A preliminary study on hepatic metabolism has been carried out on the most active (*S*)-**1** and (*S*)-**3**. The results have demonstrated that (*S*)-**3** is oxidized but not hydrolyzed by the CYP-dependent metabolism. On the contrary, the ester moiety of (*S*)-**1** is hydrolyzed by the CYP enzyme family, and the product is eliminated through the phase 1 metabolism. It has been previously demonstrated that easy metabolized molecules are not CYP inhibitors nor suicide substrate.^{21–23} Hence, those compounds could be considered not to be toxic for the human liver.

The reported results have evidenced that the azole compounds **1** and **3**–**5** exert a potent *in vitro* antichagasic activity showing IC₅₀ values in the low nanomolar concentration range.

The (*S*) enantiomers are more active than the corresponding (*R*); in addition, (*S*)-**3** showed an activity a thousand times higher than the reference drug benznidazole. Furthermore, all compounds showed low toxic effects on rat myogenic L6 cells.

Moreover, the docking studies are consistent with the hypothesis of CYP51_{Tc} inhibition, and they agree with the higher activity of (*S*) enantiomers over (*R*) enantiomers and the high selectivity vs *T. cruzi*. These molecules represent an excellent starting point in the development of cheap, easy workup and highly active antitrypanosomal drugs.

■ ASSOCIATED CONTENT

Supporting Information

All synthetic procedures, molecular docking, and biological assay details. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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