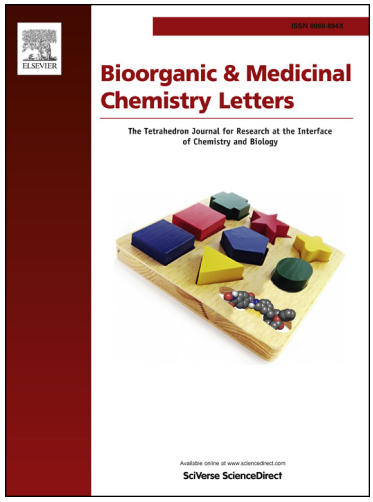

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Synthesis and trypanocidal activity of novel benzimidazole derivatives

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Keywords

Benzimidazole derivatives; Chagas disease; Trypanosoma cruzi; Triosephosphate isomerase

Abstract

The present work reports the synthesis and biological activity of a series of 14 benzimidazole derivatives designed to act on the enzyme triosephosphate isomerase of *Trypanosoma cruzi* (TcTIM). This enzyme is involved in the metabolism of glucose, the only source of energy for the parasite. In this study, we found four compounds that inhibit TcTIM moderately and lack inhibitory activity against human TIM (HsTIM). *In vitro* studies against *T. cruzi* epimastigotes showed two compounds that were more active than the reference drug nifurtimox, and these presented a low cytotoxic effect in mouse macrophages (J744 cell line).

Chagas disease (CD), also called American trypanosomiasis, is caused by the protozoan parasite *Trypanosoma cruzi*. CD is a life threatening illness considered a Neglected Tropical Disease (NTD) by the World Health Organization (WHO).[1] The parasite is transmitted to humans through the infected feces of a blood-sucking triatomine bug, which breaks through skin or mucous membranes during the insect´s bite. CD is also transmitted by the ingestion of contaminated food, infected blood transfusions, congenital transmission, or organ transplantation.[2] Actually, CD causes more deaths in Latin America than any other parasitic infection, affecting approximately eight million people, of whom 30-40% have or will develop cardiomyopathy, digestive megasyndromes, or both.[3,4] In spite of this problem, no immediate prospects for vaccines have been developed. Drug therapy is the only method currently available for the treatment of Chagas disease.[5] In this sense, only nifurtimox (NFX) and benznidazole (BZ) are widely used as treatment. However, neither of these drugs are ideal due to their side effects and variable efficacy; depending on the phase of the disease, they are only effective in the acute and early chronic phases of the infection.[6] At the chronic phases, more than 80% of the treated patients with these drugs do not get parasitological cure. Thus, it is crucial to continue the search for new and better drugs to treat Chagas disease chemotherapy.[7,8]

Recently, there has been considerable interest in the inhibition of the parasite's only energy supply: the glycolytic pathway, in which triosephosphate isomerase (TIM) plays a crucial role.[9,10] This homodimeric enzyme catalyzes the interconversion of dihydroxyacetone-phosphate (DHAP) to glyceraldehyde-3-phosphate (G3P).[11] Since TIM is present in both *T. cruzi* (TcTIM) and human (HsTIM) cells, and it contains a highly conserved catalytic site, the design of new drugs intended to inhibit the catalytic site must be ruled out. An alternative, extremely hydrophobic, and non-conserved site at the homodimeric interface of the enzyme has been proposed to inhibit its activity. In TcTIM, this region is constituted by aromatic residues, whereas aliphatic and aromatic residues are present in the interface of HsTIM.[12] Several efforts have been made to find a selective inhibitor of TcTIM, in which heterocyclic systems such as phenazine dioxides, thiadiazines and thiazoles have been identified as candidates by high-throughput screening.[13] A group of amphiphilic benzothiazole derivatives were also identified by in silico studies.[14,15] These works

demonstrate the affinity of the TcTIM interface for molecules with aromatic heterocyclic systems.

Molecular modifications, such as isosteric replacement, represent a useful strategy to find new biologically active compounds and help achieve better pharmacokinetic and pharmacodynamic properties. Ligand-based drug design is one of the most important molecular modification tools based on the knowledge of other molecules that bind to the biological target of interest.[16] In this context, our research group has synthesized a large number of benzimidazole derivatives with antiparasitic activities.[17,18] Some of these compounds present activity as TcTIM inhibitors, such as derivatives (**I**) and (**II**) shown in **Figure 1**. These compounds presented 52% and 48% inhibition at a concentration of 200 µM, respectively.[19]

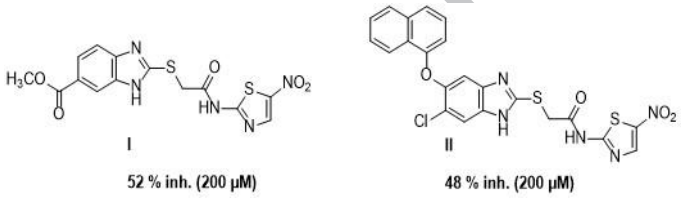
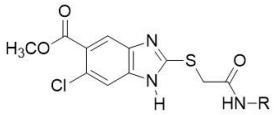


Figure 1. Benzimidazole derivatives and the inhibition presented on TcTIM.

Based on the above study, a set of molecules designed by the isosteric replacement of the thiazole group by pyrazine, pyrimidine, substituted pyridines and substituted anilines (Table 1) are proposed in this work. Herein, we present the synthesis, characterization and TcTIM inhibition activities of these compounds. Furthermore, we selected the compounds which showed a significant inhibition of TcTIM to describe their anti-*T cruzi* activity and cytotoxicity assays.

Table 1.

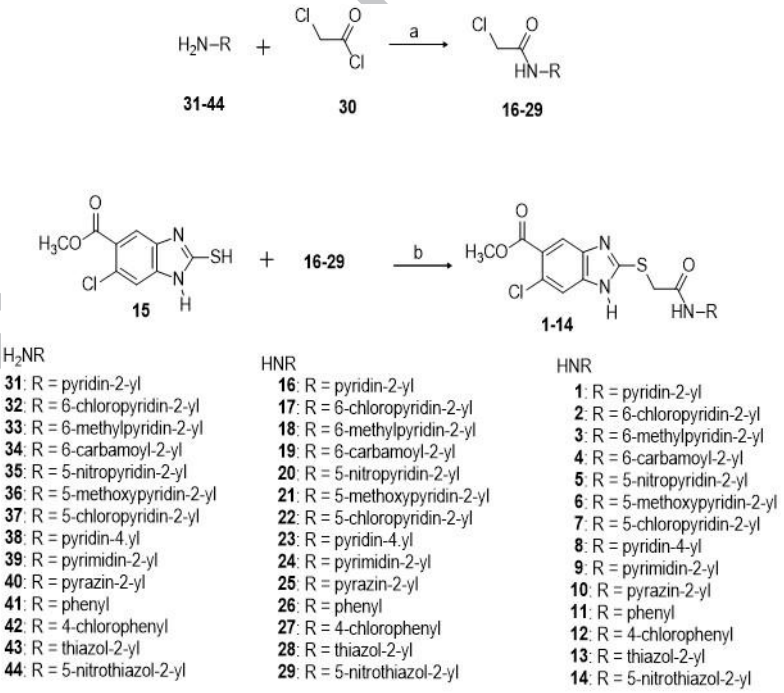
Chemical structure of the benzimidazole derivates **1–14**



Compound	R	Compound	R

1	pyridin-2-yl	**8**	pyridin-4-yl
2	6-chloropyridin-2-yl	**9**	pyrimidin-2-yl
3	6-methylpyridin-2-yl	**10**	pyrazin-2-yl
4	6-carbamoylpyridin-2-yl	**11**	phenyl
5	5-nitropyridin-2-yl	**12**	4-chlorophenyl
6	5-methoxypyridin-2-yl	**13**	thiazol-2-yl
7	5-chloropyridin-2-yl	**14**	5-nitrothiazol-2-yl

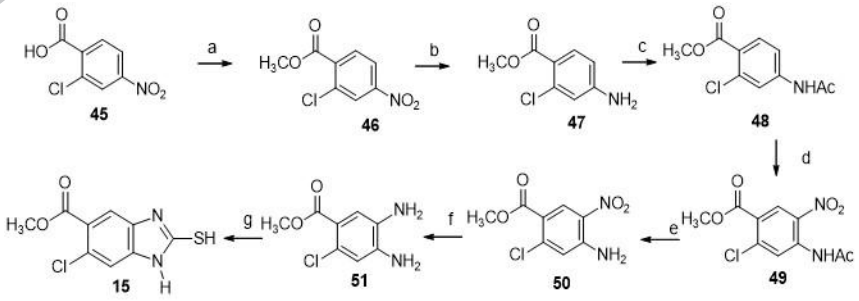
Reaction methyl 6-chloro-2-mercapto-1*H*-benzimidazole-5-carboxylate (**15**) with the appropriate 2-chloroacetamides (**16–29**) by a straightforward substitution reaction afforded the corresponding final product **1–14** as shown in Scheme 1. Acetone was used as the solvent and potassium carbonate as a base. The *S*-methylation reactions were carried out at low temperatures to avoid *N*-methylation. The solubility of the compounds obtained and the electronic effect of aromatic amines in the 2-chloroacetamide were decisive in the yields obtained.



Scheme 1. Reagents and conditions: (a) NEt_3, $CHCl_3$, 0 °C; (b) K_2CO_3, Acetone, 0 °C.

In turn, 2-chloroacetamides (**16–29**) were previously synthetized by condensing 2-chloroacetyl chloride (**30**) with the appropriate aromatic amines (**31–44**). Chloroform and triethylamine were used under a N_2 atmosphere.

On the other hand, the synthesis of the common precursor **15** was carried out following the reactions outlined in Scheme 2. Compound **15** was prepared by methods previously described by our research group.[20] The synthesis started with commercially available 2-chloro-4-nitrobenzoic acid (**45**), which was treated with dimethyl sulfate to obtain methyl 2-chloro-4-nitrobenzoate (**46**). The catalytic reduction of the nitro group with H_2 and Raney nickel at room temperature afforded methyl 4-amino-2-chlorobenzoate (**47**), which upon treatment with Ac_2O led to methyl 4-(acetylamino)-2-chlorobenzoate (**48**). Then, **48** was treated with HNO_3/H_2SO_4 at 0-5°C to give a mixture of two isomers. The required methyl 4-(acetylamino)-2-chloro-5-nitrobenzoate (**49**) was isolated from the reaction mixture by crystallization. Subsequently, the acetylamino group was hydrolyzed with KOH in EtOH, and methyl 4-amino-2-chloro-5-nitrobenzoate (**50**) was obtained. It is noteworthy that following this procedure, no hydrolysis of the methyl esters was observed. Compound (**50**) was reduced in a hydrogenation apparatus with H_2 in the presence of Raney nickel to give *o*-phenylenediamine (**51**), than upon cyclocondensation with ethyl xanthate, generated *in situ*, yield methyl 6-chloro-2-mercapto-1*H*-benzimidazole-5-carboxylate (**15**). The nitration and cyclocondensation reactions are the steps that reduce the total yield of this synthesis. The former gives the 4-(acetylamino)-2-chloro-3-nitrobenzoate isomer as a by-product, and the latter gives relative low yields of **15** when the xanthate is generated *in situ*. The best yields were obtained when the xanthate was previously formed, and then added to the reaction mixture.



Scheme 2. Reagents and conditions: (a) $(Me)_2SO_4$, $NaHCO_3$, DMF; (b) H_2, Raney-Ni, MeOH; (c) Ac_2O, AcOH; (d) HNO_3, H_2SO_4, 0 - 5 ºC; (e) MeOH, KOH: (f) H_2, Raney-Ni, MeOH, AcOEt; (g) CS_2, KOH, EtOH.|

***In vitro* inhibition experiments on TIMs**

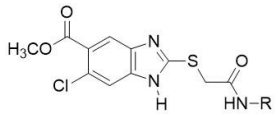
Previously, it has been observed that benzimidazole derivatives **I** and **II** exhibit moderate inhibitory activity on TcTIM.[21] In this research, these compounds were used as templates to make structural changes to determine the influence of the substituents on the inhibitory activity. Results obtained from the *in vitro* evaluation on recombinant TcTIM are summarized in **Table 2.**

The results indicate some influence of the substituents on the inhibitory activity of TcTIM. The presence of a chlorine atom at position 6(5) of the benzimidazole nucleus in compound **14** increases the inhibitory activity on the enzyme (69%) when compared with its analog compound **I** (52%). None of the substituents at position 2 of the benzimidazole nucleus was better than that with a 5-nitrothiazol-2-yl (**14**), although results obtained from the inhibition with **10** (pirazin-2-yl) and **12** (4-chlorophenyl) are considered.

The compounds with the highest inhibition of the enzyme are those with a 4-chlorophenyl group (**12**) and the heterocyclic substituents pyrazine (**10**) and nitrothiazole (**14**); this last one being the most active inhibitor of the enzyme. Compounds having pyridines (**1**,**8**) and aniline (**11)** show low to moderate activity. Compounds **3–5**, with a 6-methylpyridin-2-yl, 6-carbamoylpyridin-2-yl, 6-nitropyridin-2-yl or pyrimidin-2-yl group (**9**), have zero inhibitory activity on the enzyme, while **2** and **7**, with chlorine attached to the pyridine ring, have moderate activity. It is worth mentioning that this group of compounds, except **9**, had low solubilities at 200 µM in DMSO. Hence the study was conducted at a lower concentration of 100 µM.

Table 2.

In vitro inhibitory activity of compounds (**1–14**) on TcTIM.



Compound	R	Concentration (μM)	% Inhibition TcTIM[a]
1	pyridin-2-yl	200	10
2	6-chloropyridin-2-yl	100	21
3	6-methylpyridin-2-yl	100	0
4	6-carbamoylpyridin-2-yl	100	0
5	5-nitropyridin-2-yl	100	0
6	5-methoxypyridin-2-yl	100	4
7	5-chloropyridin-2-yl	100	41
8	pyridin-4-yl	200	33
9	pyrimidin-2-yl	200	0
10	pyrazin-2-yl	200	68
11	phenyl	200	33
12	4-chlorophenyl	100	65
13	thiazol-2-yl	200	59
14	5-nitrothiazol-2-yl	200	69

[a]Inhibition of TcTIM activity was measured indirectly quantifying the amount of NADH consumed by the reduction of DHAP by α-glycerolphosphate dehydrogenase.[22]

Figure 3 shows the curves of TcTIM inactivation by compounds **10** and **14**, while Table 3 shows the TcTIM and HsTIM inhibitory activity for compounds **7**, **10**, **12**, **13** and **14**. These results confirm that compound **14** has a slightly better capacity for TcTIM inhibition than compound **10**. However, compound **10** has a higher selectivity towards TcTIM than HsTIM; therefore, this compound is proposed as a potential selective TcTIM inhibitor. Although **7** and **12** have moderate to good TcTIM inhibitory activities and no activity against HsTIM, it was not possible to calculate the IC_{50} because of their low solubilities.

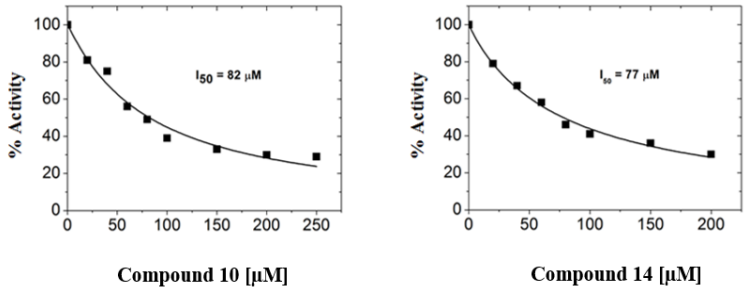


Figure 3. Inactivating curves of compounds **10** and **14**. The IC_{50} for each of the compounds tested in the inactivation of TcTIM was calculated according to reference.[23]

Table 3.

In vitro inhibitory activity on HsTIM and TcTIM IC_{50} values.

Compunds	% Inhibition TcTIM	IC_{50} TcTIM (μM)	% Inhibition HsTIM[a]
7	41 (100 μM)	---	0 (100 μM)
10	68 (200 μM)	82	6 (200 μM)
12	65 (100 μM)	---	0 (100 μM)
13	59 (200 μM)	223	0 (200 μM)
14	69 (200 μM)	77	15 (200 μM)

[a]These experiments were done exactly as those carried out for the TcTIM inhibition described in Ref. 22.

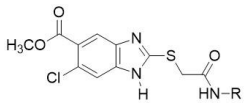
***In vitro* trypanocidal activity**

In vitro trypanocidal assays were performed with compounds **7**, **10**, **12**, **13** and **14** since these presented the highest inhibitory action on TcTIM. The selected group of compounds was tested against two different strains of *T. cruzi:* INC-5 and NINOA isolated from Instituto Nacional de Cardiología and from a patient with Chagas disease in Oaxaca, México, respectively[24] These compounds were tested against the epimastigote forms of *T. cruzi* (Table 4). Although the epimastigote is not the infective form of the parasite, the

trypanocidal activity assay against this form is considered valid since several works demonstrated that the most active compounds against the epimastigote frequently correspond to the most active compounds against the infective trypomastigote.[25,26]

Table 4.

In vitro susceptibility of bloodstream epimastigote to compounds **7**, **10, 12–14** and NFX.



Compound	R	IC_{50} INC-5[a] (µM)	IC_{50} NINOA[a] (µM)	CC_{50}[b] (µM)
7	5-chloropyridin-2-yl	95.049 ± 1.749	123.756 ± 2.786	82.732 ± 1.609
10	pyrazin-2-yl	28.672 ± 0.602	98.799 ± 1.990	134.580 ± 1.995
12	4-chloro phenyl	186.230 ± 4.103	56.967 ± 0.961	90.436 ± 1.426
13	thiazol-2-yl	48.912 ± 0.787	119.530 ± 2.151	104.443 ± 1.326
14	5-nitrothiazol-2-yl	42.516 ± 0.800	95.363 ± 2.143	84.895 ± 1.425
Nfx	---	50.750 ± 0.839	89.804 ± 1.138	131.503 ± 0.490

[a]The in vitro trypanocidal assays were carried out according to the method previosly reported, with slight modifications.[27]
[b]CC_{50}: concentration that produces the cytotoxicity of 50% J774 cells.[28]

As shown in Table 4, compound **10** has the best IC_{50} against the *T. cruzi* epimastigote INC-5 strain, better than that of the reference drug, NFX. Compounds **7** and **12** were the least effective against the INC-5 strain. For this strain, the presence of the pyrazine ring in **10** increases the inhibition of the parasite considerably with respect to compounds with pyridine (**7**), aniline (**12**) and thiazole (**13**, **14**). The results obtained for the inhibition rate of TcTIM for compounds **10**, **13** and **14**, and the data obtained in the test with the parasitic INC-5 strain, it is evident that for all three compounds, there is a significant correlation between the inhibition of *T. cruzi* epimastigotes and TcTIM.

On the other hand, the NINOA strain is more resistant to NFX than the INC-5 strain. Compound **12** (with 4-chloroaniline) has the best IC_{50} against the NINOA strain, better than that of the reference drug, NFX. Substituted compounds **7** (with pyridine), **10** (with pyrazine) and **13** and **14** (with thiazole) were not better than the reference drug.

Another important criterion in the search for molecules with antiprotozoal activity is their toxicity to mammalian cells. Hence, the *in vitro* cytotoxicity of **7**, **10**, and **12-14** was determined using mouse macrophages from the J744 cell line. As observed in Table 4, compound **10** presents a better cytotoxicity profile than NFX and the best profile in the series. The other compounds (**7**, **12–14**) present slightly higher cytotoxicity than the reference drug.

In summary, the final compounds (**1–14**) were obtained in moderate to good yields. Only four compounds (**10, 12, 13, 14**) inhibited TcTIM significantly with compound **10** being distinguished from the others since it presented a good inhibition of TcTIM and a very low activity against HsTIM. Compound **10** also showed better inhibitory activity than NFX against *T. cruzi* epimastigotes of the INC-5 strain, and a more favorable cytotoxicity profile in mammalian cells.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found in the online version, at:

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22. *In vitro inhibition TIM experiments:* recombinant TcTIM or HsTIM at a concentration of 5 µg/mL were incubated at 36 °C in a buffer containing 100 mM triethanolamine, 10 mM EDTA, pH 7.4 and the compound to be tested (**1-14**) were dissolved in 10 % of dimethyl sulfoxide (DMSO). After 2 h, 1 µL was withdrawn and added to 1 mL of the reaction mixture for the activity assay. Inhibition of TcTIMs activity was measured indirectly quantifying the amount of NADH consumed by the reduction of DHAP by α-glycerolphosphate dehydrogenase. Controls were performed using enzyme alone, substrate alone, and enzyme with DMSO.

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27. *Biological assays against T. cruzi:* Epimastigotes forms of the NINOA and INC-5 strains (isolated from a patient with Chagas disease in Oaxaca, Mexico and Instituto Nacional de Cardiología) of *T. cruzi* were used in this study. *T. cruzi* epimastigotes were grown at 28 °C in a liquid medium containing 0.3% yeast extract, 0.9%

tryptose, 0.4% dextrose, 1% disodium phosphate 2-hydrate, 0.36% sodium chloride, 0.04% potassium chloride, 0.15% powdered beef liver, 0.5% brain heart infusion, and 0.5 to 1.0 mg/100 ml hemin. Stock solutions (10 mg/mL) in DMSO of each test compound and reference drug were prepared and subsequent dilutions were done with sterile distilled water. Experiments were carried out using 96-well microplates containing 1×10^5 epimastigotes/ml. The compounds were dissolved in dimethyl sulfoxide (DMSO; final solvent concentration not greater than 1%) and were evaluated at 100, 50, 10, and 5 μg/mL. For each experiment there were controls of parasites growing in the presence of DMSO. The different mixtures and their corresponding concentrations were added to the wells, and the plates were incubated at 28 °C for 24 h. All assays were performed in duplicate. The activity was evaluated using the resarzurin. The plates were read at 535 nm em, 590 nm ex, on a multiwell plate spectrophotometer. The activity is expressed as IC_{50} (μg/mL).

28. *Citotoxicity test:* mouse macrophages from J744 cell line (J744 cells) was used to assess the cytotoxic effects on mammalian cells. The cells were cultured in standard culture medium (DMEM with 10 % Fetal Bovine Serum). The cultures were incubated for 24 h at 37 °C, 5 % CO_2 and 95 % relative humidity with different concentrations of test compounds and reference drug. The final concentration of DMSO, used as solvent, remained below 1 % in the culture medium. Untreated cells were included as controls. The cytotoxicity was determined by the resazurin method. Briefly, resazurin solution were added to each well and the plates were incubated for 3 h. The fluorescence emission was measured as indicated above, (535 nm em, 590 nm ex), on a multiwall plate spectrophotometer. Cytotoxicity was scored as a percent of metabolic reduction of treated cultures versus untreated control cells.

Graphical abstract