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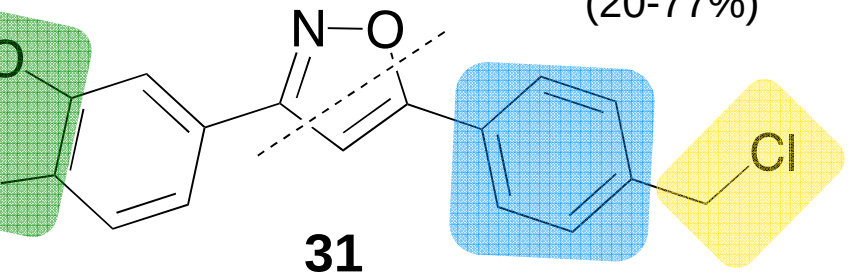
ndisin and veraguensin isoxazole derivatives

Synthesized via microwave-assisted
1,3-dipolar cycloaddition
(20-77%)



31

Most active compound

IC_{50}= 1.13 µM (*T. cruzi*)
IC_{50}= 5.08 µM (*L. amazonensis*)
SI= 36-161

-CH_3
-CH_2OH
-CH_2OAc
-H

↓ *Selectivity, trypanocidal and leishmanicidal activity*

Ring increases *Trypanocidal activity*

Group increases *Trypanocidal activity*

Design and synthesis of a new series of 3,5-disubstituted isoxazoles active against *Trypanosoma cruzi* and *Leishmania amazonensis*

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Abstract: Chagas disease and leishmaniasis are neglected tropical diseases (NTDs) endemic in developing countries. Although there are drugs available for their treatment, efforts on finding new efficacious therapies are continuous. The natural lignans grandisin (**1**) and veraguensin (**2**) show activity against trypomastigote *T. cruzi* and their scaffold has been used as inspiration to design new derivatives with improved potency and chemical properties. We describe here the planning and microwave-irradiated synthesis of 26 isoxazole derivatives based on the structure of the lignans **1** and **2**. In addition, the *in vitro* evaluation against culture trypomastigotes and intracellular amastigotes of *T. cruzi* and intracellular amastigotes of *L. amazonensis* and *L. infantum* is reported. Among the synthesized derivatives, compounds **17** (IC_{50}= 5.26 μM for *T. cruzi*), **29** (IC_{50}= 1.74 μM for *T. cruzi*) and **31** (IC_{50}= 1.13 μM for *T. cruzi* and IC_{50}= 5.08 μM for *L. amazonensis*) were the most active and were also evaluated against recombinant trypanothione reductase of *T. cruzi* in a preliminary study of their mechanism of action.

Keywords: natural product analogues; drug design; isoxazole; *Trypanosoma cruzi*; *Leishmania amazonensis*; trypanothione reductase.

Abbreviations: %GI, percentage of growth inhibition; AB, amphotericin B; BZN, benznidazol; logP, partition coefficient octanol/water; MW, microwave; ND, not determined due to absence of trypanocidal or leishmanicidal activity; NTD, neglected tropical disease; SI, selectivity index; (r)TR, (recombinant) trypanothione reductase.

1. Introduction

Chagas disease and leishmaniasis are neglected tropical diseases that affect more than 15 million people worldwide, representing a major public health issue especially in Latin American countries [1–3]. Benznidazole and nifurtimox are the only drugs available for treatment of Chagas disease, while pentavalent antimonials, amphotericin B, miltefosine, paromomycin and pentamidine are drugs for leishmaniasis treatment. There are drawbacks associated with these drugs, such as high cost, toxicity and limited efficacy resulting in high levels of treatment interruption. Resistant strains of *T. cruzi* and *Leishmania* spp. are also emerging and might compromise even more the limited therapeutic options [4–8].

Through the centuries, biodiversity has been a useful source of medicinal therapies. There are reports of traditional use of plants dating from more than two thousand years ago and, currently, about 39% of the drugs on the market are natural products or have a chemical structure directly based on natural products [9–12]. Several natural products as flavonoids, terpenes, quinones and lignans have been tested against trypanosomatid parasites [13–15]. In spite of some identified hits, there are still limitations associated to them, especially regarding the isolation of larger quantities to conduct further biological investigations [16]. On the other hand, secondary metabolites obtained from natural sources represent a library of diverse chemical features that can give insights on the development of new chemical entities to treat parasitic diseases [16,17].

The development of efficient drugs for treatment of Chagas disease and leishmaniasis depends on the identification of new lead compounds [18–20]. Grandisin (**1**) and veraguensin (**2**) are two tetrahydrofuran lignans isolated from *Virola surinamensis* twigs and *Piper solmsianum* inflorescences that have been described as having promising trypanocidal activity (IC_{50}= 3.7 µM and 2.3 µM, respectively) against the *T. cruzi* trypomastigote stage (Figure 1) [21,22].

Attempts on the total synthesis of lignans **1** and **2** were successful and exemplify the importance of organic synthesis to the field of natural products [23–26]. Likewise, the design of analogues applying distinct molecular modification strategies is a valuable tool, and can be used to overcome problems in isolation, physicochemical properties or to optimize the bioactivity of known natural product hits [27–31]. Our research group has been working on different compounds based on the grandisin and veraguensin scaffold. Between them, non-rigid 1,4-diaryl-1,4-diol analogues were synthesized with the same methoxylation pattern of compounds **1** and **2**, and displayed IC_{50} values as low as 10 µM on *T. cruzi* trypomastigotes [32]. In another work, cyclic

derivatives obtained through Michael addition-carbocyclization were planned aiming to increase water solubility and also yielded compounds with good trypanocidal activity [33]. These results confirm the potential of the tetrahydrofuran lignans scaffold and encourage us to continue our research for bioactive compounds related to their structure.

In this work we describe the planning, synthesis and a preliminary evaluation of the structure-activity relationship of a set of isoxazole derivatives structurally related to the lignans **1** and **2** (Figure 1). The choice of the isoxazole ring as a bioisosteric replacement is justified by its higher water solubility, what can improve biological and chemical properties. The design of the compounds also relied on molecular simplification, once the chiral tetrahydrofuran ring in compounds **1** and **2** gives place to the aromatic isoxazole ring. Both strategies have been widely used in medicinal chemistry on the study of molecules, affording new hits.

As the synthesis of compounds containing different substituents linked to the isoxazole ring is feasible and straightforward, we prepared compounds substituted with one and two aromatic rings bearing different R groups via a microwave-assisted methodology.

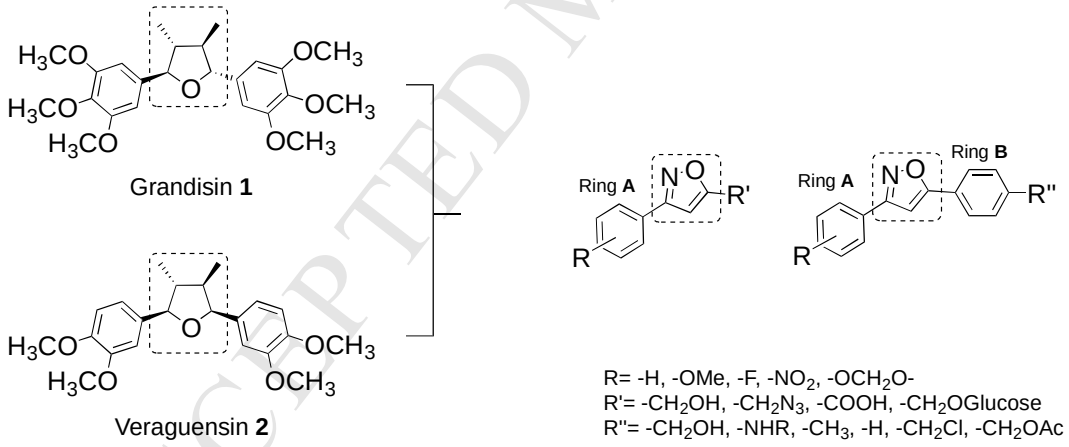


Fig 1. Structure of trypanocidal tetrahydrofuran lignans grandisin (**1**), veraguensin (**2**) and proposed derivatives.

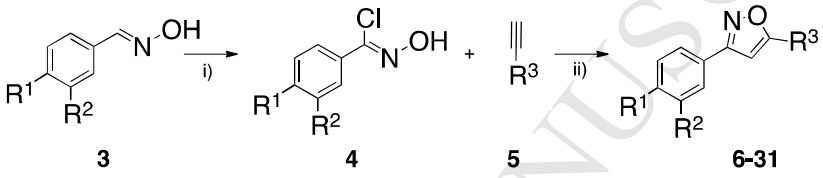
Thereafter, the derivatives were assessed against *T. cruzi* and *L. amazonensis* intracellular amastigotes. The chemical variability present in the first synthesized series allowed us to identify sites for punctual modifications that enhanced bioactivity and selectivity, providing hints on the structure-activity relationship. The inhibitory potential of the compounds on recombinant trypanothione reductase (rTR) was also evaluated, due to its relevance as a target for anti-trypanosomatid drug design and its presence in both genera *Trypanosoma* and *Leishmania*.

2. Results and Discussion

2.1. Chemistry

Over the last years, microwave irradiation has been proved a powerful tool in synthetic organic chemistry, allowing the preparation of compounds more efficiently in comparison to traditional methods. Although there is a range of different applications for its use, one of the main advantages of microwave irradiation is the possibility of preparing potentially bioactive molecules within minutes [34–37].

Based on the methodology described by Himo et al. (2005) [38], we focused on synthesizing 3,5-disubstituted isoxazole derivatives via 1,3-dipolar cycloaddition, under microwave irradiation in order to increase the speed of the synthesis (Scheme 1).



R^1 = -H, -OMe, -F, $-NO_2$, $-OCH_2O-$
R^2 = -H, -OMe, $-OCH_2O-$
R^3 = $-CH_2OH$, $-CH_2N_3$, -COOH, $-CH_2O$Glucose, -Ph, -Ph(4-CH_2OH), -Ph(4-NHR), -Ph(4-CH_3), -Ph(4-CH_2Cl), -Ph(4-CH_2OAc)

Scheme 1. Reagents and conditions: i) NCS, DMF, MW: 30 ˚C, 150 W, 1 min; ii) $CuSO_4$, sodium ascorbate, $NaHCO_3$, MW: 30 ˚C, 150 W, 10 min.

Our initial efforts synthesizing 5-hydroxymethyl-3-phenylisoxazole (**6**) have shown that after 1 minute of irradiation (at 30 °C) it was possible to visualize by TLC analysis the total consumption of the starting material, leading to a more apolar product suggested to be the imidoyl chloride (Scheme 1, intermediate **4**). Subsequently, when the imidoyl chloride was irradiated with an alkyne and the final product was purified, we could observe that the yield depends on the total time of irradiation (Table 1).

Table 1. Time-based yield optimization for 5-hydroxymethyl-3-phenylisoxazole (**6**) synthesis.

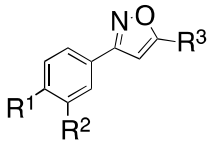
Entry	R^1	R^2	Time[a] (min)	Yield (%)
i	-H	$-CH_2OH$	1	37
ii			3	35
iii			10	77
iv			15	74

a. After addition of the alkyne. All the experiments were performed at 30 °C and the maximum potency was set to 150 W.

The highest yield was obtained when the reaction was further irradiated for 10 minutes (Table 1, entry 6iii). As irradiation for 15 minutes did not enhance the yield of

the desired product, 10 minutes was standardized for the synthesis of a series of isoxazole derivatives **6**-**20** (Table 2). Furthermore, we compared the yield of the products obtained via the microwave-assisted reaction (20-77%) with those obtained via the reaction under conventional conditions [38] (18-72%) and it was not possible to observe much difference besides a significant decrease in reaction time.

Table 2. Isoxazole derivatives initially synthetized and their yields (%)



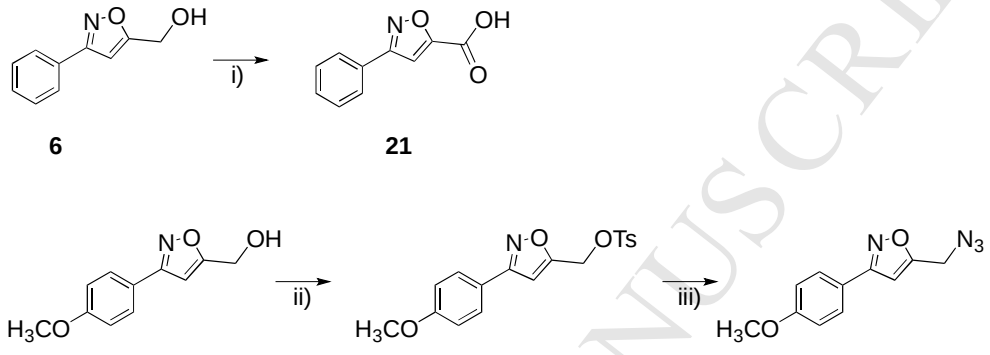
Compound	R^1	R^2	R^3	Yield (%)[a]	Yield MW[b] (%)
6	-H	-H	$-CH_2OH$	72	77
7	$-OCH_3$	-H	$-CH_2OH$	52	56
8	$-OCH_3$	$-OCH_3$	$-CH_2OH$	50	55
9	-F	-H	$-CH_2OH$	47	38
10	$-NO_2$	-H	$-CH_2OH$	36	32
11	$-OCH_2O-$		$-CH_2OH$	63	56
12	-H	-H	$-C_6H_4$-4-CH_2OH	47	52
13	$-OCH_3$	-H	$-C_6H_4$-4-CH_2OH	45	52
14	$-OCH_3$	$-OCH_3$	$-C_6H_4$-4-CH_2OH	51	51
15	-F	-H	$-C_6H_4$-4-CH_2OH	42	40
16	$-NO_2$	-H	$-C_6H_4$-4-CH_2OH	22	20
17	$-OCH_2O-$		$-C_6H_4$-4-CH_2OH	18	22
18	-H	-H	$-C_6H_4$-4-NH_2	49	50
19	-H	-H	(2,3,4,6-tetracetyl-α-D-glucopyranose-1-yl)methyl	40	54
20	$-OCH_2O-$		(2,3,4,6-tetracetyl-α-D-glucopyranose-1-yl)methyl	46	48

a. Yield obtained in a non-irradiated reaction; i) NCS, DFM, rt.; ii) $CuSO_4$, sodium ascorbate, $NaHCO_3$, alkyne, H_2O/tBuOH, rt.

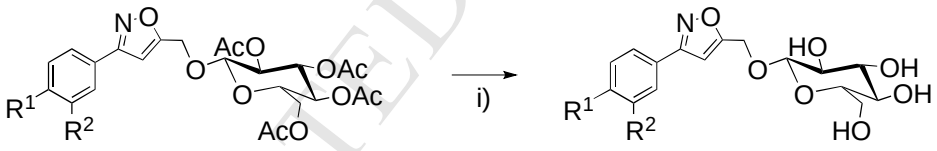
b. All microwave experiments were performed at 30 °C and the maximum potency was set to 150 W.

Additionally, compound **6** was oxidized with Jones reagent, yielding compound **21** (Scheme 2), and the derivative **22** was synthesized from previously obtained compound **7** through protection with tosyl chloride (compound **7a**) followed by reaction with sodium azide. Also, compounds **19** and **20** were synthesized from a glycosidic alkyne, and further deprotected leading to **23** and **24** (Scheme 3) [39]. These further

modifications were planned to increase the diversity related to functional groups and physicochemical properties. The ionized moieties in compounds **21** and **22** might be subject to ionic interactions with molecular targets while the monosaccharide unit present in derivatives **23** and **24** led to the most hydro soluble compounds in the series. Carbohydrates are also known for acting on cellular signaling in both parasites and humans [40–43], and glycosides for being responsible for different biological activities, such as antiviral [44,45], antiparasitic [46,47], antibacterial [48–50] and cytotoxic [51,52].



Scheme 2. Reagents and conditions: i) Jones reagent 8N, acetone, 30 min, 0 °C; ii) TsCl, Et_3N, K_2CO_3, H_2O/CH_2Cl_2, 2 h, rt.; iii) NaN_3, DMF, MW, 7 min, 70 °C.



19 and **20** — **23** and **24**

19. R^1 = -H, R^2 = -H
20. R^1 = $-OCH_2O-$ = R^2

23. R^1 = -H, R^2 = -H
24. R^1 = $-OCH_2O-$ = R^2

Scheme 3. Reagents and conditions: i) NaOMe, MeOH, pH= 9, 15 min, rt.

All synthetized compounds were purified by column chromatography and characterized by nuclear magnetic resonance (^1H, ^{13}C NMR) and mass spectrometry (ESI-TOF). In addition, IR spectroscopy was employed in the characterization when necessary. The spectral data is available as supplementary material.

2.2. Biologic investigation and structural optimization.

2.2.1. Trypanocidal evaluation

T. cruzi presents a complex life cycle and a wide range of mammalian hosts. Its trypomastigote stage is the infective form both for triatomine vectors and mammals, while its intracellular amastigote stage is the main form responsible for the disease [53].

Considering the importance of the amastigote stage for the pathogenesis of Chagas disease, compounds **6**-**24** were firstly screened (at 100 µM) against this life stage of the parasite (Tulahuen strain). The compounds that led to a percentage of growth inhibition (%GI) >50% were assayed in different concentrations to determine the half maximal inhibitory concentration (IC_{50}). The cytotoxicity to THP-1 cells (CC_{50}) was also assessed to calculate the selectivity index (SI).

The results showed that most of the compounds were active against intracellular amastigotes to some extent, except for compounds **8** and **21** (Table 3). These results allowed us to set the synthetic direction for obtaining new improved derivatives that will be discussed in the session 2.2.2.

Table 3. Trypanocidal activity of compounds **6**-**24** against intracellular amastigotes

Compound	%GI (100 μM)	IC_{50} (μM)	CC_{50} (μM)	SI
6	7.57 (±0.06)	>100	ND	-
7	9.32 (±0.49)	>100	ND	-
8	0.00 (±0.00)	ND	ND	-
9	8.27 (±0.54)	>100	ND	-
10	12.17 (±0.86)	>100	ND	-
11	1.95 (±0.06)	>100	ND	-
12	7.01 (±1.62)	>100	ND	-
13	5.77 (±0.06)	>100	ND	-
14	14.15 (±1,02)	>100	ND	-
15	10.57 (±1.41)	>100	ND	-
16	15.86 (±1.49)	>100	ND	-
17	65.04(±4.55)	5.26 (±0.96)	70.18 (±14.94)	13.3
18	72.97 (±2.58)	90.39 (±14.49)	407.2 (±88.2)	4.5
19	1.16 (±1.00)	>100	ND	-
20	3.15 (±1.13)	>100	ND	-
21	0.00 (±0.00)	ND	ND	-
22	60.35 (±4.64)	ND	<15.6	-
23	7.30 (±0.40)	>100	ND	-
24	2.93 (±0.32)	>100	ND	-
BZN 20 μM	85.94 (±1.29)	10.18 (±0.3)	> 500	> 49.1

The results are averages ± SD of triplicates. Compounds **17-18** were tested in triplicate in two independent experiments.

In the series substituted with only one aromatic ring (ring A, compounds **6**-**11**), a *para* substitution on the aromatic ring led to a slight increase in activity when compared with the unsubstituted compound **6**. On the other hand, a 3,4-substitution on the A ring led to a loss of activity (compound **8**) or to a 3-fold decrease in activity (compound **11**) in this series.

In the series substituted with two aromatic rings (rings A and B, compounds **12**-**18**), except for compound **13**, a substituent group on the A ring seems to contribute to an increase in biological activity in comparison with compound **12**. When the methylenehydroxy group in the B ring is replaced by a primary amine (compound **18**), the activity also increases. These findings are summarized in Figure 2. Compounds **17**

and **18** were the most promising identified among compounds **6**-**24**, with IC_{50} values of 5.26 μM and 90.39 μM, respectively.

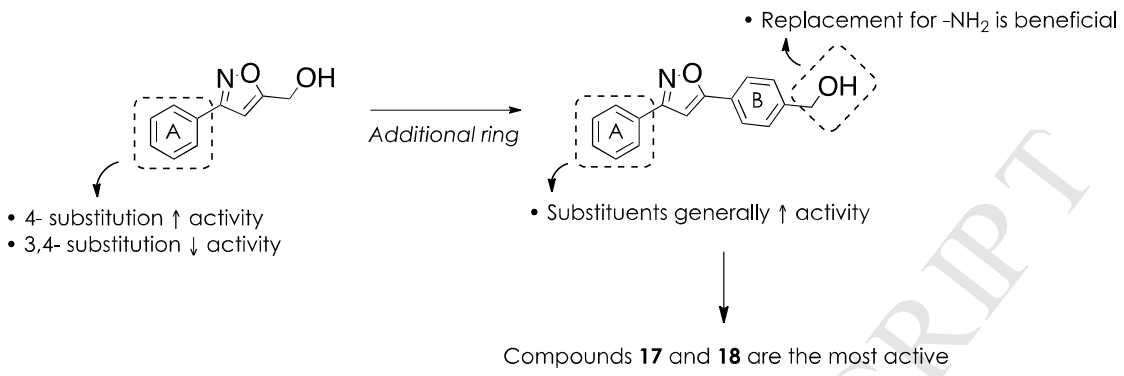


Fig 2. Preliminary SAR for derivatives **6**-**24**.

Regarding compound **17**, the methylenedioxy group linked to the aromatic ring A seems to be important for the activity. It was the A ring substituent that led to a higher increase in activity in comparison with other substituents in compounds **12**-**16**. Also, the replacement of the methylenedioxy group in compound **17** by two methoxy groups (compound **14**), which maintains similar chemical properties, modifies the conformation of the molecule due to a steric clash between the aromatic substituents, and this might be the cause of the lower activity of compound **14** (Figure 3).

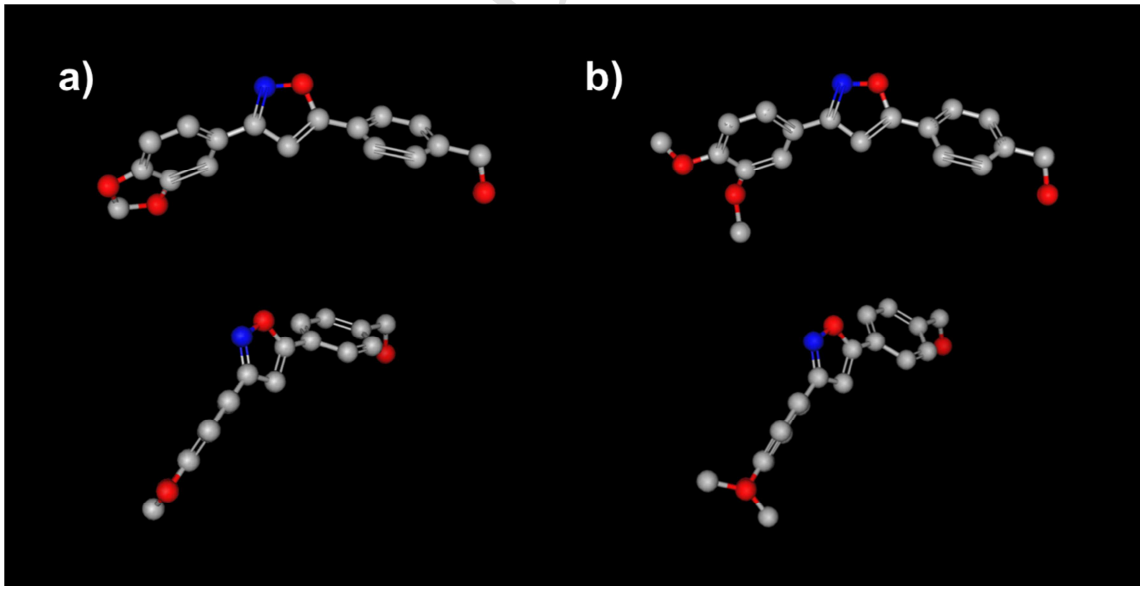


Fig 3. 3D shape of compounds **17** (a) and **14** (b) generated using Marvin Suite (version 16.4.25.0, 2016, http://chemaxon.com/).

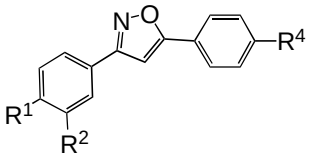
Compounds **19**-**20** and **23**-**24** displayed only a small inhibition of growth of *T. cruzi*. At the first experiment, compound **22** showed a percentage of growth inhibition similar to compound **17**, but when it was assayed again one week later, the activity was lost. Mass spectrometry analysis (HRMS ESI+/TOF) of the stock solution (Supporting Information, Figure S1) revealed the absence of a peak relative to the cationized molecule or other adduct, suggesting that compound **22** degraded in the test solution. However, two relevant peaks are observed at m/z 247.1470 and m/z 188.0713. The first one might be relative to the oxidized compound **22** (calculated for $C_{11}H_{11}N_4O_3^+$: 247.0826) and the second peak shows that oxidation is likely to have occurred at the azide moiety, since it can be attributed to the fragment 4-methoxyphenylisoxazolylmethylene (calculated for $C_{11}H_{10}NO_2^+$: 188.0706).

2.2.2. Synthesis of new derivatives

For a better understanding of the properties responsible for the activity of compounds **17** and **18**, derivatives **25**-**30** were synthetized using the methodology already described in session 2.1. Otherwise, compound **31** was obtained directly from **17** through reaction with thionyl chloride, under N_2 atmosphere, in excellent yield (94%).

Compounds **25**-**27** were planned to verify the importance of the amine group for compound **18** activity. Moreover, compounds **28**-**31** were synthesized to understand the role of the *para*-substituted ring B for activity. The structure of derivatives **25**-**31** together with results of the trypanocidal evaluation are shown in Table 4.

Table 4. Trypanocidal activity of compounds **25**-**31** against intracellular amastigotes.



Compound	R^1	R^2	R^4	%GI (100 μM)	IC_{50} (μM)	CC_{50} (μM)	SI
25	-H	-H	-H	29.40 (±2.21)	>100	ND	-
26	-H	-H	-NHAc	0.00 (±0.00)	ND	ND	-
27	-H	-H	-NHBoc	0.00 (±0.00)	ND	ND	-
28	$-OCH_2O-$		$-CH_2OAc$	47.59 (±4.00)	23.21 (±3.19)	>500	>21.5
29	$-OCH_2O-$		$-CH_3$	81.65 (±3.53)	1.74 (±0.41)	81.32 (±9.74)	46.7
30	$-OCH_2O-$		-H	64.77 (±3.54)	23.02 (±6.94)	>500	>21.7
31	$-OCH_2O-$		$-CH_2Cl$	95.75 (±0.31)	1.13 (±0.39)	181.8 (±25.9)	160.9
BZN 20 μM				85.94 (±1.29)	10.18 (±0.3)	> 500	> 49.1

The results are averages ± SD of two independent experiments run in triplicate.

Considering compound **18**, acetylation and carbamatization of the primary amine lead to a loss of trypanocidal activity in compounds **26** and **27**, respectively. In both derivatives, the nitrogen lone pair is delocalized into a carbonyl, resulting in a lack of basicity. Besides, a reduction in activity is observed when the amine group is removed (compound **25**), suggesting therefore that a basic group might play an important role for the bioactivity of compound **18** in *T. cruzi* (Figure 4).

When compound **17** was acetylated (compound **28**) or had its methylenehydroxy group removed (compound **30**) the activity had a fourfold decrease. However, an improvement of activity and selectivity was observed in derivatives where the hydroxyl was removed (compound **29**) or replaced by a chlorine atom (compound **31**) (Figure 4). The calculation of some chemical properties using Marvin (version 16.4.25.0, 2016, http://chemaxon.com/, Supporting Information, Table S1) showed that compounds **17**, **29** and **31** have a similar surface area, while compound **28** has a larger and compound **30** a smaller surface area. Previously, we observed that the presence of two benzene rings linked to the isoxazole ring favored the inhibition of *T. cruzi* growth, and now our results indicate that there is an ideal range of molecular size in order to optimize bioactivity in this class of compounds.

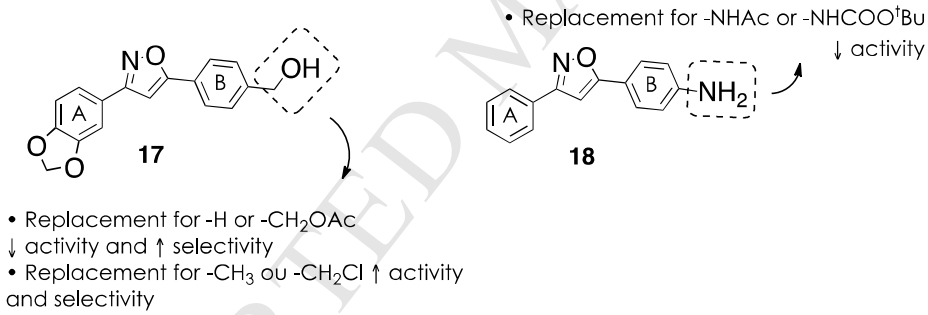


Fig 4. Compounds **25**-**30** contribution for SAR of 3,5-disubstituted isoxazoles.

An important aspect of compounds **17**, **29** and **31** is their potency compared to the reference drug benznidazole (Figure 5). The amastigote stage of *T. cruzi* is predominately found during the chronic course of Chagas disease, which cannot be effectively treated by benznidazole or other drugs. Therefore, these hits will be further investigated aiming to design new efficacious and selective molecules to treat and control the mortality and morbidity of Chagas disease.

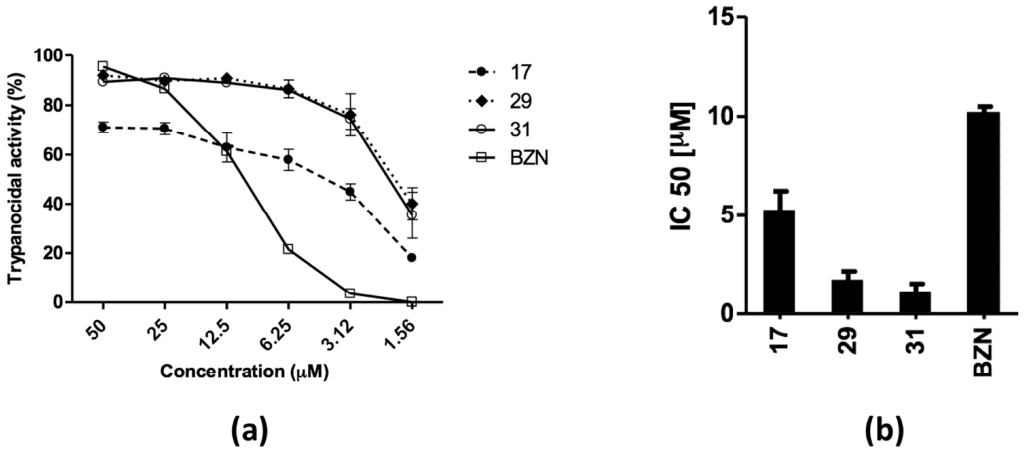


Fig 5. Inhibition curves (a) of compounds **17**, **29**, **31** and comparison of their IC_{50} values with BZN IC_{50} (b).

Finally, although 6 compounds had high activity against *T. cruzi* amastigotes (IC_{50}<100 μM, compounds **17**, **18**, **28**-**31**), none of them were active against the trypomastigote stage of the parasite (Tulahuen) at the tested concentration. It is not uncommon for compounds to have different potencies against different *T. cruzi* life stages [54–56], implying that morphological changes experienced by the parasite during its life cycle could affect its interactions with micromolecules.

2.2.3. Leishmanicidal evaluation

Alongside Chagas disease, leishmaniasis is also a NTD that affects millions of people and lacks options for efficient treatment. In the New World, the *Leishmania mexicana* and *Leishmania braziliensis* complexes comprise 7 different species, whereas *L. braziliensis* and *L. guyanensis* are the main species responsible for mucocutaneous leishmaniasis; *L. mexicana*, *L. amazonensis*, *L. venezuelensis*, *L. panamensis* and *L. peruviana* are causative agents for cutaneous leishmaniasis. Other 5 species are commonly found in the Old World, being *L. donovani* and *L. infantum*, responsible for causing visceral leishmaniasis and *L. major*, *L. tropica* and *L. aethiopica* etiological agents of cutaneous leishmaniasis [57]. As we obtained molecules with an interesting bioactivity against *T. cruzi*, we found valuable to test compounds **6**-**31** also against intracellular amastigotes of *L. amazonensis* (MHOM/BR/77/LTB0016). Similarly to the trypanocidal screening, the IC_{50} and CC_{50} (THP-1 cells) values were only determined for compounds that led to a percentage of growth inhibition >50% (%GI). The results are summarized in Table 5.

Table 5. Leishmanicidal activity of compounds **9**, **10**, **19**, **20**, **23**-**25**, **28**, **29** and **31** against *L. amazonensis* intracellular amastigotes.

Compound	%GI (100 μM)	IC_{50} (μM)	CC_{50} (μM)	SI
9	3.35 (±0.61)	>100	ND	-
10	4.98 (±0.09)	>100	ND	-
19	2.06 (±0.02)	>100	ND	-
20	2.05 (±1.09)	>100	ND	-
23	4.57 (±0.18)	>100	ND	-
24	3.82 (±0.51)	>100	ND	-
25	6.03 (±1.97)	>100	ND	-
28	15.65 (±8.29)	>100	ND	-
29	28.12 (±1.51)	>100	ND	-
31	57.67 (±1.85)	5.08 (±1.31)	181.8 (±25.9)	35.8
AB 2 μM	81.62 (±0.43)	0.14 (±0.02)	27.86 (± 0.98)	199

The results are averages ± SD of triplicates. Compound **31** was tested in triplicate in two independent experiments.

While most of the compounds inhibited the growth of *T. cruzi* to some degree, in this assay only derivatives **9**, **10**, **19**, **20**, **23**-**25**, **28**, **29** and **31** presented activity at 100 μM against *L. amazonensis*. Overall, it is not possible to observe a clear correlation between their structure and leishmanicidal activity. However, regarding the derivatives based on the structure of compound **17** (compounds **28**, **29**, **30** and **31**), lipophilicity is a factor that might be related with percentage of growth inhibition. While compound **17** does not show any activity against *L. amazonensis*, compounds **28**, **29** and **31** are increasingly more potent as their lipophilicity, indicated by a higher logP value, also increases (Figure 6). Lipophilic compounds are more permeable to cellular membranes, what could justify this high *in vitro* activity. Additionally, a *para*-substituted B ring might also be important for activity, as compound **30** showed no growth inhibition at 100 μM, even though its logP value is 3.56. From an electronic point of view, the different substituents in compounds **17**, **28**, **29** and **31** modify the electron distribution on the aromatic ring B and this characteristic, together with lipophilicity, could favor the activity for these compounds.

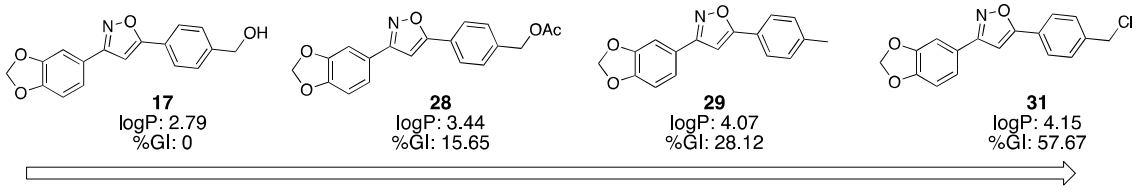


Fig 6. Positive correlation between logP and leishmanicidal activity for compounds **17**, **28**, **29** and **31.**

In view of the promising leishmanicidal property of compound **31**, we also tested its activity against intracellular amastigotes of *L. infantum* (MHOM/BR/74/PP75) to gain an insight into the spectrum of action of the compound. Despite the high activity observed against *L. amazonensis*, compound **31** had only a weak activity against *L. infantum* (%GI= 9.01 ± 0.75 at 50 μM).

In conclusion, compound **31** was found to be the most active one against intracellular amastigotes of both *T. cruzi* and *L. amazonensis*.

2.2.4. Enzymatic assay

Since the essential role of trypanothione reductase (TR) to the redox metabolism of trypanosomatids was established, it has become one of the main exploited targets in *T. cruzi* and *Leishmania* spp [58–61]. Different inhibitors have been described in literature, but up to date, none of them proceeded to the further step of drug development [62–66].

In order to investigate potential molecular targets for the most promising synthesized molecules (IC_{50}< 10 μM), the rTR inhibitory activity of compounds **17**, **29** and **31** was assessed. All compounds evaluated have IC_{50} values >100 μM suggesting that TR is not the main target involved on their antiparasitic property. Consequently, additional studies are necessary to elucidate the mechanism of action of the compounds presented herein. Once a mechanism of cell death or molecular target is identified, further chemical modifications might be planned to obtain isoxazole derivatives with an optimized activity.

3. Concluding remarks

By using bioisosterism and molecular simplification strategies, 26 isoxazole derivatives analogues of grandisin and veraguensin were designed and synthesized. The screening of their trypanocidal, leishmanicidal (intracellular amastigotes) and cytotoxic activity (THP-1 cells) afforded 6 non-cytotoxic derivatives highly active against *T. cruzi* (compounds **17**, **18**, **28**, **29**, **30** and **31**) and one non-cytotoxic derivative highly active against *L. amazonensis* (compound **31**).

Different criteria for hit identification are described in the literature and include, for example, IC_{50} values <1 μg/mL and SI>50 for Chagas disease [18,20,67]. Among our compounds, three have IC_{50} values lower than the IC_{50} for benznidazole (compounds **17**, **29** and **31**), and two have IC_{50}<1 μg/mL (compounds **29** and **31**). Concerning selectivity, compound **31** has a SI=160.9 for cells infected by *T. cruzi* and a SI=35.8 for cells infected by *L. amazonensis*, making it the most promising compound described herein for satisfying all the hit selection criteria. Compound **31**, as well as other compounds in this study, also fits drug-likeness requirements related to Lipinski's rule of five (Supporting Information, Table S1) suggesting potential for further development and biological investigations, especially due to its action on both parasites [68].

4. Supporting Information

Representative copies of ^{1}H and ^{13}C spectra are available online.

5. Experimental section

5.1. Chemistry

Melting points were measured in a melting point apparatus MQAPF- 301 and are reported uncorrected. All ^{1}H and ^{13}C NMR spectra were obtained in Nuclear Bruker Advance DPX 400 MHz, Bruker Fourier 300 MHz and Varian Oxford AS-400 using TMS as internal standard, unless indicated otherwise. Mass spectra were performed in ESI-TOF Bruker micrOTOF Q II and Waters Xevo G2-S QTOF. Reactions under microwave irradiation were conducted in a Discovery - CEM Explorer microwave reactor with cooling, pressure and gas addition systems. Solvents and reagents, purchased from Sigma Aldrich, were treated and purified when necessary, according to literature [69]. Finally, thin layer chromatography was performed on silica G60 gel layers SILICYCLE® with fluorescence indicator F-254 and column chromatography was performed using silica gel with particle size 40-63 and 63-200 μm (Sigma Aldrich) and hexane:ethyl acetate (Tedia) as eluent.

5.1.1. General procedure for preparation of isoxazoles **6**-**31** under microwave irradiation.

To a solution of an aldoxime (0.1 mmol) in DMF (0.3 mL) in a microwave tube, *N*-chloro succinimide (0.105 mmol) was slowly added to avoid overheating. The tube was sealed and submitted to microwave irradiation (30 °C, 150 W) for 1 min, and then TLC analysis showed the complete consumption of the starting material. An alkyne (0.105 mmol), copper (II) sulphate (2 mol%), sodium ascorbate (10 mol%), sodium bicarbonate (0.4 mmol) and water (0.3 mL) were added and the tube was further irradiated for 10 min (30 °C, 150 W). The solution was diluted with 15 mL of brine, extracted with ethyl acetate (3 x 10 mL), dried (Na_2SO_4) and concentrated under vacuum. The crude extract was purified by flash column chromatography on silica (hexane:ethyl acetate 6:4) yielding the expected product.

5.1.2. General procedure for preparation of isoxazoles **6**-**20**.

The compounds were synthesised based on procedure reported in the literature [38]. For the preparation of the imidoyl chloride, N-chloro succinimide (0.1 mmol) was slowly added to a solution of an aldoxime (0.105 mmol) in DMF (1 mL) and the reaction was stirred until the starting material was not visible on the TLC analysis. After, the reaction was diluted with brine (15 mL), extracted with ethyl ether (3x 10 mL), dried over Na_2SO_4, concentrated under vacuum and utilized without any purification in the next step. Following, propargylic alcohol (0.105 mmol), copper (II) sulphate (2 mol%), sodium ascorbate (10 mol%), sodium bicarbonate (0.4 mmol) and 4 mL of H2O:t-BuOH were added to the product obtained in the first part, and the reaction was further stirred for 4 hours. Next, the reaction was diluted with brine (15 mL), extracted with ethyl acetate (3x 10 mL), dried over Na_2SO_4, concentrated under vacuum and the crude extract was purified by flash column chromatography on silica (hexane:ethyl acetate 6:4) yielding the expected product. After purification, they were compared via TLC analysis to the respecting compounds synthesized under microwave irradiation, when this comparison was desired, and characterized by NMR and mass spectrometry.

5.1.3. Structural characterization of compounds **6**-**31**

5.1.3.1. 5-hydroxymethyl-3-phenylisoxazole (**6**)

Yield: 72%. Yield MW: 77%. Mp: 48.8-49.5 °C. ^{1}H NMR (300 MHz, Methanol-d_4): δ = 7.78-7.85 (m, 2H, Ar), 7.43-7.50 (m, 3H, Ar), 6.76 (s, 1H, Isoxazole), 4.71 (s, 2H, CH_2). ^{13}C NMR (75 MHz, Methanol-d_4): δ = 171.7, 162.5, 130.1, 128.9, 128.8, 126.8, 100.0, 56.7. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{10}H_{10}NO_2$ 176.0712; Found 176.0699.

5.1.3.2. 5-hydroxymethyl-3-(4-methoxyphenyl)isoxazole (**7**)

Yield: 52%. Yield MW: 56%. Mp: 90.1-92.0 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.74 (d, *J*= 9.1 Hz, 2H, Ar), 6.98 (d, *J*= 9.1 Hz, 2H, Ar), 6.52 (s, 1H, Isoxazole), 4.82 (s, 2H, CH_2), 3.86 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.4, 162.1, 161.0, 128.2, 121.3, 114.3, 99.8, 56.7, 55.4. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{11}H_{12}NO_3$ 206.0817; Found 206.0815.

5.1.3.3. 5-hydroxymethyl-3-(3,4-dimethoxyphenyl)isoxazole (**8**)

Yield: 50%. Yield MW: 55%. Mp: <25 °C. ^{1}H NMR (400 MHz, Methanol-d_4): δ = 7.42 (d, *J*= 2.0 Hz, 1H, Ar), 7.39 (dd, *J*= 2.0, 8.3 Hz, 1H, Ar), 7.04 (d, *J*= 8.3 Hz, 1H, Ar), 6.73 (t, *J*= 0.8 Hz, 1H, Isoxazole), 4.70 (d, *J*= 0.8 Hz, 2H, CH_2), 3.89 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, Methanol-d_4): δ = 174.4, 163.6, 152.3, 150.9, 122.9, 121.3, 112.9, 110.9, 100.8, 56.5 (x2), 56.4. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{12}H_{14}NO_4$ 236.0923; Found 236.0922.

5.1.3.4. 3-(4-fluorophenyl)-5-hydroxymethylisoxazole (**9**)

Yield: 47%. Yield MW: 38%. Mp: 78.8-81.8 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.79 (dd, *J*= 5.3, 8.6 Hz, 2H, Ar), 7.15 (t, *J*= 8.6 Hz, 2H, Ar), 6.54 (s, 1H, Isoxazole), 4.83 (s, 2H, CH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.9, 163.8 (d, $^{1}J_{CF}$= 249 Hz), 161.6, 128.7 (d, $^{3}J_{CF}$= 8 Hz), 125.0, 116.1 (d, $^{2}J_{CF}$= 22 Hz), 99.9, 56.6. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{10}H_9FNO_2$ 194.0617; Found 194.0600.

5.1.3.5. 5-hydroxymethyl-3-(4-nitrophenyl)isoxazole (**10**)

Yield: 36%. Yield MW: 32%. Mp: 156.0-157.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.00 (d, *J*= 9.0 Hz, 2H, Ar), 8.33 (d, *J*= 9.0 Hz, 2H, Ar), 6.67 (s, 1H, Isoxazole), 4.88 (s, 2H, CH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 172.9, 160.7, 148.7, 134.9, 127.7, 124.3, 100.1, 56.6. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{10}H_9N_2O_4$ 221.0562; Found 221.0555.

5.1.3.6. 3-(3,4-benzodioxole)-5-hydroxymethylisoxazole (**11**)

Yield: 63%. Yield MW: 56%. Mp: 76.0-77.7 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.28 (d, *J*= 1.6 Hz, 1H, Ar), 7.24 (dd, *J*= 8.0, 1.6 Hz, 1H, Ar), 6.86 (d, *J*= 8.0 Hz, 1H, Ar), 6.47 (s, 1H, Isoxazole), 6.01 (s, 2H, OCH_2O), 4.78 (s, 2H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 171.8, 162.1, 149.2, 148.2, 122.7, 121.2, 108.6, 106.9, 101.5, 99.9, 56.5. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{11}H_{10}NO_4$ 220.0610; Found 220.0598.

5.1.3.7. 5-(4-hydroxymethylphenyl)-3-phenylisoxazole (**12**)

Yield: 47%. Yield MW: 52%. Mp: 168.8-169.3 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.83-7.89 (m, 4H, Ar), 7.45-7.52 (m, 5H, Ar), 6.84 (s, 1H, Isoxazole), 4.78 (s, 2H, CH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.1, 163.0, 143.1, 130.0, 129.1, 128.9, 127.4, 126.8, 126.7, 126.0, 97.5, 64.8. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{16}H_{14}NO_2$ 252.1025; Found 252.0987.

5.1.3.8. 5-(4-hydroxymethylphenyl)-3-(4-methoxyphenyl)isoxazole (**13**)

Yield: 45%. Yield MW: 52%. Mp: 157.0-157.9 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.82 (d, *J*= 6.6 Hz, 2H, Ar), 7.80 (d, *J*= 6.6 Hz, 2H, Ar), 7.49 (d, *J*= 8.4 Hz, 2H, Ar), 7.00 (d, *J*= 8.4 Hz, 2H, Ar), 6.78 (s, 1H, Isoxazole), 4.78 (s, 2H, CH_2), 3.87 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 169.9, 162.6, 161.0, 143.0, 128.2, 127.3, 126.8, 126.0, 121.6, 114.3, 97.2, 64.8, 55.4. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{17}H_{16}NO_3$ 282.1130; Found 282.1139.

5.1.3.9. 5-(4-hydroxymethylphenyl)-3-(3,4-dimethoxyphenyl)isoxazole (**14**)

Yield: 51%. Yield MW: 51%. Mp: 142.6-145.5 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.83 (d, *J*= 8.4 Hz, 2H, Ar), 7.48 (d, *J*= 8.4 Hz, 2H, Ar), 7.47 (d, *J*= 2.0 Hz, 1H, Ar), 7.36 (dd, *J*= 2.0, 8.4 Hz, 1H, Ar), 6.95 (d, *J*= 8.4 Hz, 1H, Ar), 6.79 (s, 1H, Isoxazole), 4.77 (s, 2H, CH_2), 3.98 (s, 3H, OCH_3), 3.95 (s, 3H, OCH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.0, 162.7, 150.6, 149.3, 143.1, 127.3, 126.7, 126.0, 121.8, 119.9, 111.0, 109.3, 97.3, 64.8, 56.0 (x2). HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{18}H_{18}NO_4$ 312.1236; Found 312.1236.

5.1.3.10. 3-(4-fluorophenyl)-5-(4-hydroxymethylphenyl)isoxazole (**15**)

Yield: 42%. Yield MW: 40%. Mp: 179.0-179.6 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.82-7.89 (m, 4H, Ar), 7.51 (d, *J*= 8.6 Hz, 2H, Ar), 7.18 (t, *J*= 8.6 Hz, 2H, Ar), 6.80 (s, 1H, Isoxazole), 4.79 (s, 2H, CH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.4, 162.1, 143.2, 128.7 (d, $^{3}J_{CF}$= 8 Hz), 127.4, 126.1, 116.1 (d, $^{2}J_{CF}$= 22 Hz), 97.3, 64.8. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{16}H_{13}FNO_2$ 270.0930; Found 270.0922.

5.1.3.11. 5-(4-hydroxymethylphenyl)-3-(4-nitrophenyl)isoxazole (**16**)

Yield: 22%. Yield MW: 20%. Mp: 189.3-191.2 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.28 (d, *J*= 9.0 Hz, 2H, Ar), 7.99 (d, *J*= 9.0 Hz, 2H, Ar), 7.77 (d, *J*= 8.4 Hz, 2H, Ar), 7.45 (d, *J*= 8.4 Hz, 2H, Ar), 6.89 (s, 1H, Isoxazole), 4.65 (s, 2H, CH_2). ^{13}C NMR (100 MHz,

$CDCl_3$): δ = 171.5, 161.4, 148.8, 144.3, 135.3, 127.8, 127.5, 126.1, 125.9, 124.4, 97.6, 64.0. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{16}H_{13}N_2O_4$ 297.0875; Found 297.0900.

5.1.3.12. 3-(3,4-benzodioxole)-5-(4-hydroxymethylphenyl)isoxazole (**17**)

Yield: 18%. Yield MW: 22%. Mp: 180.1-181.7 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.83 (d, *J*= 8.5 Hz, 2H, Ar), 7.50 (d, *J*= 8.5 Hz, 2H, Ar), 7.39 (d, *J*= 1.8 Hz, 1H, Ar), 7.34 (dd, *J*= 1.8, 8.1 Hz, 1H, Ar), 6.91 (d, *J*= 8.1, 1H, Ar), 6.75 (s, 1H, Isoxazole), 6.04 (s, 2H, OCH_2O), 4.78 (s, 2H, CH_2). ^{13}C NMR (100 MHz, Methanol-d_4): δ = 171.5, 164.1, 150.8, 149.7, 145.4, 128.4, 127.5, 126.8, 124.0, 122.4, 109.6, 107.6, 102.9, 98.6, 64.6. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{17}H_{14}NO_4$ 296.0923; Found 296.0891.

5.1.3.13. 5-(4-aminophenyl)-3-phenylisoxazole (**18**)

Yield: 49%. Yield MW: 50%. Mp: 130.6-132.4 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.80-7.87 (m, 2H, Ar), 7.65 (d, *J*= 8.7 Hz, 2H, Ar), 7.74-7.50 (m, 3H, Ar), 6.75 (d, *J*= 8.7 Hz, 2H, Ar), 6.64 (s, 1H, Isoxazole), 3.95 (bs, 2H, NH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.9, 162.8, 148.3, 129.8, 129.4, 128.9, 127.4, 126.8, 117.9, 114.9, 95.1. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{15}H_{13}N_2O$ 237.1028; Found 237.0993.

5.1.3.14. 5-[(2,3,4,6-tetraacetyl-α-D-glucopyranose-1-yl)methyl]-3-phenylisoxazole (**19**)

Yield: 40%. Yield MW: 54%. Mp: 111.1-113.6 °C. ^{1}H NMR (400 MHz, Methanol-d4): δ = 7.81-7.86 (m, 2H, Ar), 7.46-7.51 (m, 3H, Ar), 6.85 (s, 1H, Isoxazole), 5.28 (t, *J*= 9.6 Hz, 1H, H3'), 5.05 (t, *J*= 9.6 Hz, 1H, H4'), 4.95 (t, *J*= 8.4 Hz, 1H, H2'), 4.94 (d, *J*= 13.9 Hz, 1H, CH), 4.87 (d, *J*= 13.9 Hz, 1H, CH), 4.86 (t, *J*= 8.4 Hz, 1H, H1'), 4.29 (dd, *J*= 4.6, 12.4 Hz, 1H, H6'), 4.16 (dd, *J*= 2.5, 12.4 Hz, 1H, H6'), 3.92 (m, 1H, H5'), 2.04 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 2.00 (s, 3H, CH_3), 1.96 (s, 3H, CH_3). ^{13}C NMR (100 MHz, Methanol-d4): δ = 170.6, 170.2, 169.4, 168.3, 130.2, 129.0, 126.8, 101.6, 99.9, 72.6, 72.1, 71.0, 68.2, 61.74, 61.72, 29.7, 20.73, 20.71, 20.6. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{24}H_{28}NO_{11}$ 506.1618; Found 506.1655.

5.1.3.15. 5-[(2,3,4,6-tetraacetyl-α-D-glucopyranose-1-yl)methyl]-3-(3,4-benzodioxole)isoxazole (**20**)

Yield: 46%. Yield MW: 48%. Mp: 110.4-112.0 °C. ^{1}H NMR (400 MHz, Methanol-d4): δ = 7.37-7.31 (m, 2H, Ar), 6.93 (d, *J*= 8.3 Hz, 1H, Ar), 6.77 (s, 1H, Isoxazole), 6.03 (s, 2H, OCH_2O), 5.28 (t, *J*= 9.3 Hz, 1H, H3'), 5.05 (t, *J*= 9.3 Hz, 1H, H4'), 4.94 (t, *J*= 9.1 Hz, 1H, H2'), 4.93 (t, *J*= 13.4 Hz, 1H, CH), 4.84 (d, *J*= 9.1 Hz, 1H, H1'), 4.83 (d, *J*= 13.4 Hz,

1H, CH), 4.29 (dd, *J*= 4.6, 12.4 Hz, 1H, H6'), 4.16 (dd, *J*= 2.3, 12.4 Hz, 1H, H6'), 3.92 (m, 1H, H5'), 2.05 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 2.00 (s, 3H, CH_3), 1.96 (s, 3H, CH_3). ^{13}C NMR (100 MHz, Methanol-d4): δ = 172.4, 171.2, 171.6, 171.3, 170.3, 163.5, 150.9, 149.8, 123.9, 122.4, 109.7, 107.6, 103.0, 102.9, 101.3, 74.1, 73.0, 72.7, 69.7, 63.0, 62.7, 20.6, 20.59, 20.56, 20.54. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{25}H_{28}NO_{13}$ 550.1561; Found 550.1522.

5.1.3.16. (3,5-diphenyl)isoxazole (**25**)

Yield: 42%. Mp: 141.2-143.8 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.83-7.89 (m, 4H, Ar), 7.47-7.51 (m, 6H, Ar), 6.84 (s, 1H, Isoxazole). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 170.3, 162.9, 130.2, 130.0, 129.0, 128.9, 127.4, 126.8, 125.8, 97.4. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{15}H_{12}NO$ 222.0913; Found 222.0912.

5.1.3.17. 5-(4-acetamidephenyl)-3-phenylisoxazole (**26**)

Yield: 19%. Mp: 179.0-182.0 °C. ^{1}H NMR (300 MHz, Methanol-d_4): δ = 7.85-7.91 (m, 2H, Ar), 7.84 (d, *J*= 8.8 Hz, 2H, Ar), 7.73 (d, *J*= 8.8 Hz, 2H, Ar), 7.46-7.52 (m, 3H, Ar), 7.10 (s, 1H, Isoxazole), 2.17 (s, 3H, CH_3). ^{13}C NMR (75 MHz, Methanol-d_4): δ = 170.4, 170.1, 163.0, 140.5, 129.9, 128.7, 126.3, 126.1, 122.6, 119.6, 96.7, 23.3. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{17}H_{15}N_2O_2$ 279.1134; Found 279.1139.

5.1.3.18. 5-(tert-butylcarbamatephenyl)-3-phenylisoxazole (**27**)

Yield: 48%. Mp: 188.3-192.9 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.84-7.90 (m, 2H, Ar), 7.77 (d, *J*= 8.9 Hz, 2H, Ar), 7.50 (d, *J*= 8.9 Hz, 2H, Ar), 7.45-7.50 (m, 3H, Ar), 6.76 (s, 1H, Isoxazole), 6.65 (bs, 1H, NH), 1.54 (s, 9H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.0, 162.6, 140.2, 130.2, 130.0, 128.9, 126.8, 122.5, 118.4, 96.6, 53.5, 28.3. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{20}H_{21}N_2O_3$ 337.1552; Found 337.1559.

5.1.3.19. 5-(4-acetoxymethylphenyl)-3-(3,4-benzodioxole)isoxazole (**28**)

Yield: 28%. Mp: 164.2-168.0 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.83 (d, *J*= 8.3 Hz, 2H, Ar), 7.48 (d, *J*= 8.3 Hz, 2H, Ar), 7.38 (d, *J*= 1.5 Hz, 1H, Ar), 7.35 (dd, *J*= 1.5, 8.1 Hz, 1H, Ar), 6.91 (d, *J*= 8.1 Hz, 1H, Ar), 6.76 (s, 1H, Isoxazole), 6.04 (s, 2H, CH_2), 2.14 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.8, 169.8, 162.6, 149.2, 148.3, 138.1, 128.7, 127.3, 126.0, 123.0, 121.2, 108.7, 107.0, 101.5, 97.6, 65.7, 21.0. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{19}H_{16}NO_5$ 338.1028; Found 338.1028.

5.1.3.20. 3-(3,4-benzodioxole)-5-(4-methylphenyl)isoxazole (**29**)

Yield: 26 %. Mp: 141.9-144.9 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.71 (d, *J*= 8.2 Hz, 2H, Ar), 7.37 (d, *J*= 1.7 Hz, 1H, Ar), 7.33 (dd, *J*= 1.7, 8.0 Hz, 1H, Ar), 7.28 (d, *J*= 8.2 Hz, 2H, Ar), 6.91 (d, *J*= 8.0 Hz, 1H, Ar), 6.69 (s, 1H, Isoxazole), 6.03 (s, 2H, CH_2), 2.41 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.4, 162.5, 149.1, 148.2, 140.5, 129.7, 125.7, 124.8, 123.2, 121.1, 106.6, 107.0, 101.5, 96.7, 21.5. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{17}H_{14}NO_3$ 280.0974; Found 280.0975.

5.1.3.21. 3-(3,4-benzodioxole)-5-phenylisoxazole (**30**)

Yield: 29%. Mp: 125.2-127.9 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.83 (dd, *J*= 1.9, 7.7 Hz, 2H, Ar), 7.52 – 7.44 (m, 2H, Ar), 7.39 (d, *J*= 1.70 Hz, 1H, Ar), 7.34 (dd, *J*= 1.7, 8.0 Hz, 1H, Ar), 6.90 (d, *J*= 8.0 Hz, 1H, Ar), 6.75 (s, 1H, Isoxazole), 6.04 (s, 2H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.2, 162.6, 149.1, 148.2, 130.2, 129.0, 125.8, 123.1, 121.2, 108.6, 107.0, 101.5, 97.3. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{16}H_{12}NO_3$ 266.0817; Found 266.0831.

5.1.4. General procedure for tosylation

The compounds were synthesized from primary alcohols following a methodology described in literature [70]. Purification were done by column chromatography on silica (hexane:ethyl acetate 8:2) yielding the desired products.

5.1.4.1. 3-(4-methoxyphenyl)-5-[(4-methylbenzenesulfonate)methylphenyl)isoxazole (**7a**)

Yield: 76%. Mp: 81-82 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.81 (d, *J*= 8.3 Hz, 2H, Ar), 7.67 (d, *J*= 8,8 Hz, 2H, Ar), 6.98 (d, *J*= 8,3 Hz, 2H, Ar), 6.98 (d, *J*= 8,8 Hz, 2H, Ar), 6.50 (s, 1H, Isoxazole), 5.17 (s, 2H, CH_2), 3.85 (s, 3H, OCH_3), 2.41 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 164.5, 162.1, 161.2, 145.5, 132.5, 130.0, 128.2, 128.0, 120.8, 114.4, 102.9, 61.2, 55.4, 21.7.

5.1.5. 3-phenylisoxazole-5-carboxylic acid (**21**)

Jones reagent 8 N (0.3 mmol) was added dropwise to a solution of compound **7** (0.1 mmol) in acetone (4 mL) in an ice bath. The solution was stirred for 30 minutes when TLC analysis showed the total consumption of the starting material. The solution was diluted in acetone (15 mL) filtered through celite and evapored under vacuum. The crude extract was purified by flash column chromatography on silica (hexane:ethyl acetate 3:7) affording compound **23** as a white solid. Yield: 69%. Mp: 155.0-57.4 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.78-7.90 (m, 2H, Ar), 7.45-7.55 (m, 3H, Ar), 7.25 (s, 1H, Isoxazole). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 166.9, 165.5, 162.3, 134.4, 133.0, 132.8, 131.9, 130.7, 111.3. HRMS (ESI-TOF) m/z: $[M-H]^-$ Calcd for $C_{10}H_6NO_3$ 188.0353; Found 188.0321.

5.1.6. 5-(azidomethyl)-3-(4-methoxyphenyl)isoxazole (**22**)

DMF (0.3 mL), sodium azide (0.15 mmol) and compound **8a** (0.1 mmol) were added to a microwave tube and submitted to irradiation for 7 minutes (150 W, 70 ºC). At the end, the solution was diluted with 10 mL of brine, extracted with ethyl acetate (3 x 10 mL), dried ($NaSO_4$) and concentrated under vacuum. The product (compound **22**) was obtained quantitatively (20 mg, 100%). Mp: 51.9-53.0 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.75 (d, *J*= 9.0 Hz, 2H, Ar), 6.99 (d, *J*= 9.0 Hz, 2H, Ar), 6.55 (s, 1H, Isoxazole), 4.50 (s, 2H, CH_2), 3.87 (s, 3H, OCH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 166.7, 161.2, 128.2, 121.0, 114.4, 101.1, 55.4, 45.5. IR (KBr): 2100 (N_3) cm^{-1}. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{11}H_{11}N_4O_2$ 231.0882; Found 231.0872.

5.1.7. General procedure for deacetylation of 2,3,4,6-tetracetyl-α-D-glucopyranosydes (**19** and **20**)

The protected glucoside (compound **19** or **20**, 0.1 mmol) was solubilized in methanol (1 mL) and NaOMe (1 M) was added until the pH of the solution reached 9. The solution was stirred for 15 min, cooled to 0 ºC and then the resin Dowex 50WX-200 was added until pH dropped to 7. The solution was filtered and the product (**23** or **24**) was obtained in good yields [39].

5.1.7.1. 5-[(2,3,4,6-tetrahydroxy-α-D-glucopyranose-1-yl)methyl]-3-phenylisoxazole (**23**)

Yield: 95%. Mp: 149.0-152.0°C. HRMS (ESI-TOF) m/z: $[M+Na]^+$ Calcd for $C_{16}H_{19}NO_7Na$ 360.1054; Found 360.1056.

5.1.7.2. 3-(3,4-benzodioxole)-5-[(2,3,4,6-tetrahydroxy-α-D-glucopyranose-1-yl)methyl]isoxazole (**24**)

Yield: 80%. Mp: 186.3-188.5 °C. HRMS (ESI-TOF) m/z: $[M+Na]^+$ Calcd for $C_{17}H_{19}NO_9Na$ 404.0952; Found 404.0941.

5.1.8. 3-(3,4-benzodioxole)-5-(4-chloromethylphenyl)isoxazole (**31**)

A solution of compound **17** (0.1 mmol) in thionyl chloride (100 μl) was stirred under a nitrogen atmosphere for 1 hour when TLC analysis showed total consumption of the starting material. After, the reaction was cooled down to 0 °C, quenched with water and extracted with CH_2Cl_2 (3x 10 mL). The combined organic layers were then washed with brine and dried under vacuum affording compound **31** as a pale yellow powder (30 mg, 94%). Mp: 162.1-163.2 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.82 (d, *J*= 8.4 Hz, 2H, Ar), 7.51 (d, *J*= 8.4 Hz, 2H, Ar), 7.38 (d, *J*= 1.7 Hz, 1H, Ar), 7.34 (dd, *J*= 8.0, 1.7 Hz, 1H, Ar), 6.90 (d, *J*= 8.0 Hz, 1H, Ar), 6.76 (s, 1H, Isoxazole), 6.04 (s, 2H, CH_2), 4.63 (s, 2H, CH_2). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 169.6, 163.2, 149.2, 148.2, 139.4, 129.2, 127.4, 122.9, 121.2, 106.6, 101.5, 97.7, 46.6. HRMS (ESI-TOF) m/z: $[M+H]^+$ Calcd for $C_{17}H_{13}ClNO_3$ 314.0584; Found 314.0576.

5.2. Biologic assays

5.2.1. Trypanocidal and Leishmanicidal screening in intracellular amastigotes.

THP-1 cells (ATCC TIB202) were grown in RPMI-1640 without phenol red (Sigma-Aldrich, CO. St. Louis, MO, USA) supplemented with 10% FBS (Life Technologies, USA), 12.5 mM HEPES, penicillin (100 U/ml), streptomycin (100 μg/ml) and Glutamax (2 mM) (37 °C, 5% CO_2).

L. amazonensis MHOM/BR/77/LTB0016 and *L. infantum* MHOM/BR/74/PP75 promastigotes, expressing β-galactosidase, were grown in Schneider's insect medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 5% of heat inactivated FBS and 2% of human urine (26 °C, 5% CO_2).

T. cruzi Tulahuen trypomastigotes (raised from infected L929 cell line), expressing β-galactosidase, were provided by the Laboratory of Cellular and Molecular Parasitology, Centro de Pesquisas René Rachou, FIOCRUZ, Belo Horizonte [71].

Afterwards, THP-1 cells were cultivated in 96 well plates (4.0×10^4 cells/well) in supplemented RPMI-1640 and treated with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) for 72 h (37 °C, 5% CO_2) to allow cell differentiation into non-dividing macrophages [72].

Four days culture promastigotes of *L. amazonensis* (4.0×10^6 parasites/ml) and *L. infantum* (4.0×10^6 parasites/ml) were washed with phosphate buffered saline (PBS, pH 7.4) and incubated in RPMI-1640 supplemented with 10% of heat-inactivated human B+ serum for 1 h (34 °C) to parasite opsonization. THP-1 cells were then incubated with a parasite/cell ratio of 10:1 for 3 h (34 °C, 5% CO_2) for *L. amazonensis* and *L. infantum*, and with a parasite/cell ratio of 2:1 overnight (37 °C, 5% CO_2) for *T.*

cruzi. After that, non-adherent parasites were removed by washing with PBS and infected cells were incubated with 180 µl of full supplemented RPMI-1640 medium for another 24 h (34 °C and 37 °C, 5% CO_2) to allow the transformation of promastigotes/trypomastigotes into intracellular amastigotes.

The infected cells were treated with 20 µl of each compound (2 µM of DMSO-diluted stock solution in 18 µM of RPMI-1640) in triplicate, followed by incubation for 48 h (34 °C and 37 °C, 5% CO_2). Subsequently, cells were carefully washed with PBS and incubated for 16 h (34 °C, 5% CO_2) with 250 µl of chlorophenolred-ß-d-galactopyranoside (Sigma–Aldrich Co., St. Louis, MO, USA) (CPRG) at 100 µM and Nonidet P-40 (Amresco Inc, Solon, Ohio, USA) (NP-40) 0.1%. Optical density was read at 570/630 nm in an Infinite M200 TECAN, Austria. Amphotericin B (Bristol-Myers, Squibb) and benznidazole (Sigma Aldrich) were used as positive control and DMSO 1% as negative control.

5.2.2. Trypanocidal screening in trypomastigotes

Culture *T. cruzi* Tulahuen trypomastigotes (raised from infected L929 cell line), were cultivated in 96 well plates (1.5×10^6 parasites/well) and treated with the compounds (2 µM of DMSO-diluted stock solution in 18 µM of RPMI-1640) serially diluted in concentrations ranging from 250 to 3.9 µM. After incubation for 72 h (37°C, 5% CO_2), cell viability was assessed by the MTT assay, which consists in the colorimetric measurement of the metabolization of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan by viable cells. DMSO 1% was used as negative control, and benznidazole (Sigma Aldrich) as positive control. The optical density was read at 540 nm in a Infinite M200 TECAN microplate reader immediately after the dissolution of formazan crystals with DMSO [73,74].

5.2.3. Cytotoxicity assay (MTT)

THP-1 cells were grown and cultivated in 96 well plates (4.0×10^6 cells/well), as described in 5.2.1., treated with the compounds (2 µM of DMSO-diluted stock solution in 18 µM of RPMI-1640) serially diluted in concentrations ranging from 15.6 µM to 500 µM and incubated for 72 h (37 °C, 5% CO_2). Cell viability was assessed as described in 5.2.2.

5.2.4. Trypanothione reductase enzyme assay

Recombinant trypanothione reductase from *T. cruzi* (TcTR), was expressed in *Escherichia coli* BL21DE3 and purified by affinity chromatography. TR assays were performed as described by Hamilton et al. (2003) [75]. In 96 well micro plates (final volume= 240 μL), TcTR (1 m-unit), HEPES (40 mM, pH 7.5), NADPH (0.15 mM), DTNB (25 μM) and EDTA (1 mM) were incubated for 5 min (27 °C) before $T(S)_2$ (1 μM) and the tested compound (diluted in DMSO) were added. Compounds and controls were pre-incubated at 27 ^{o}C for 30 min and 10 μL of DTNB was added to the reaction mixture. Absorbance at 412 nm was measured for 30 min to determine the enzymatic activity. DMSO 1% was used as negative control and clomipramine as positive control.

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Design and synthesis of a new series of 3,5-disubstituted isoxazoles active against *Trypanosoma cruzi* and *Leishmania amazonensis*

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Highlights

- NP-based analogues were planned using bioisosterism and simplification strategies.
- 26 isoxazole derivatives were synthesized.
- Their trypanocidal and leishmanicidal activities were evaluated.
- 22 compounds were active against *T. cruzi* and 10 against *L. amazonensis.*