Anti-trypanosomatid benzofuroxans and deoxygenated analogues: Synthesis using polymer-supported triphenylphosphine, biological evaluation and mechanism of action studies

Diego Castro [a], Lucia Boiani [a], Diego Benitez [a], Paola Hernández [a], Alicia Merlino [a], Carmen Gil [b], Claudio Olea-Azar [c], Mercedes González [a,*], Hugo Cerecetto [a,*], Williams Porcal [a,*]

[a] Departamento de Química Orgánica, Facultad de Ciencias-Facultad de Química, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay
[b] Instituto de Química Médica-CSIC, Madrid, Spain
[c] Departamento de Química Inorgánica y Analítica, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Chile

ARTICLE INFO

Keywords:
Hybrid benzofuroxans
Trypanosoma cruzi
Leishmania spp.
Mitochondrial dehydrogenases
Free radicals

ABSTRACT

Hybrid vinylthio-, vinylsulfinyl-, vinylsulfonyl- and vinylketo-benzofuroxans developed as anti-trypanosomatid agents, against *Trypanosoma cruzi* and *Leishmania* spp., have showed low micromolar IC_{50} values. The synthetic route to access to these derivatives was an efficient Wittig reaction performed in mild conditions with polymer-supported triphenylphosphine (PS-TPP). Additionally, the benzofurozan analogues, deoxygenated benzofuroxans, were prepared using PS-TPP as reductive reagent in excellent yields. The trypanosomicidal and leishmanocidal activities of the benzofuroxan derivatives were measured and also some aspects of their mechanism of action studied. In this sense, inhibition of mitochondrial dehydrogenases activities, production of intra-parasite free radicals and cruzipain inhibition were studied as biological target for the anti-trypanosomatid identified compounds. The trypanosomicidal activity could be the result of both the parasite-mitochondrion function interference and production of oxidative stress into the parasite.

1. Introduction

Diseases caused by *Trypanosomatidae*, which share a similar state regarding drug treatment, include Chagas' disease (CD) (*Trypanosoma cruzi*) and Leishmaniasis (*Leishmania* spp.). These trypanosomatids alone are responsible for an infected population of nearly 30 millions and more than 400 millions are at risk. CD is a systemic chronic parasitic infection caused by a hemoflagellate protozoan, *Trypanosoma cruzi* (*T. cruzi*), which is transmitted primarily by species of a blood-feeding triatomine insect known as Vinchuca or Chinche [1,2].

Recently, the World Health Organization (WHO) estimates that 8 million people are infected with CD, and approximately 11,000 deaths are reported annually [3]. Despite this situation, significant scientific progress on the search and pharmacological actions of effective agents to treat CD has been made. In this sense, new structural hits reported in the literature and patents have been developed showing preclinical efficacy in different models [4–9]. While various classes of trypanosomicidal agents have been described in the last years, Nifurtimox (Nfx, Lampit®, Fig. 1) and Benznidazole (Bnz, Rochagan®, Fig. 1), two nitroaromatic compounds, are even today the only clinically available drugs for the chemotherapy of CD [10]. Both nitro-derivatives have exhibited significant activity in the acute phase, with up to 80% of parasitological cures in treated patients, while in the chronic phase, less than 20% of treated patients are parasitologically cured [3]. The bioreduction of the nitro group to unstable nitroanion radicals, which react to produce highly toxic reactive oxygen species (superoxide anion, hydrogen peroxide and hydroxyl radical) and covalent modification of macromolecules by nitroreduction intermediates are the mechanism of action proposed to Nfx and Bnz, respectively [11,12].

Unfortunately, the emergence of *T. cruzi* parasite strains resistant to Nfx and Bzn and severe side effects of these drugs have eroded its efficacies [13–16]. Non selective bioreduction of these trypanosomicidal drugs could be the reason for their side effects in the mammalian host. This fact increases the urgency of

* Corresponding authors. Tel.: +598 2 525 86 18x216; fax: +598 2 525 07 49.
E-mail addresses: megonzal@fq.edu.uy (M. González), hcerecet@fq.edu.uy (H. Cerecetto), wporcal@fq.edu.uy (W. Porcal).

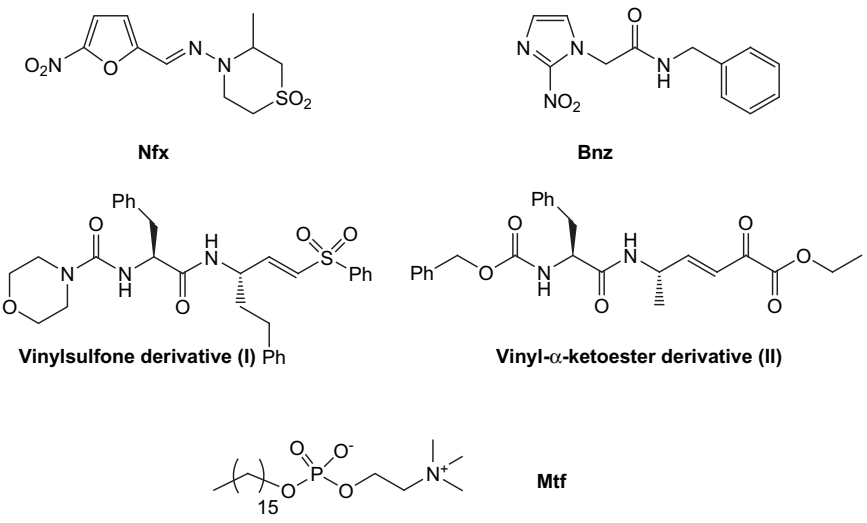
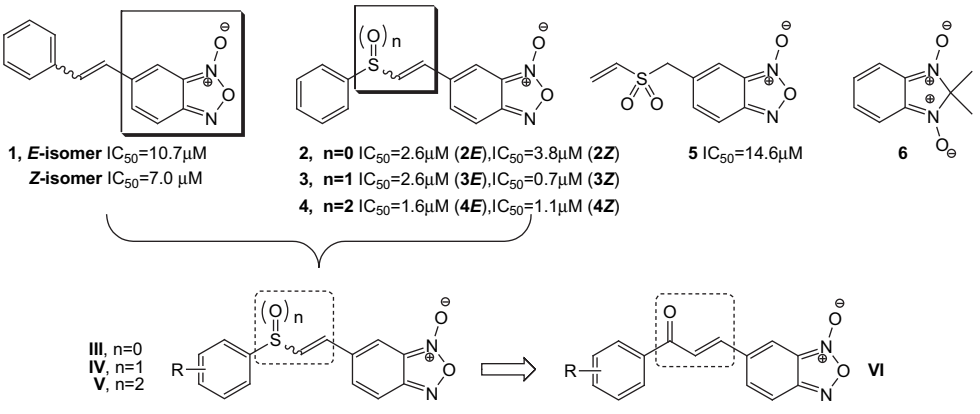


Fig. 1. Drugs used clinically and experimentally as anti-*T. cruzi* and anti-*Leishmania* agents.

the search and development for novel, effective and safer agents to treat this neglected disease. To develop new drugs to combat efficiently this parasitic infection research should be directed toward key differences between the metabolism of the mammal and the parasite. In this sense, several biochemical pathways have been identified as potential differential target such as the enzyme cruzipain, a cysteine protease, characterized by a cysteine-thiol active-site responsible to attack the carbonyl of the amide bond [17–19]. Cruzipain is essential to many biological processes evidences have indicated it is critically required for intracellular parasite survival. Several covalent inhibitors, structurally related to Michael-like systems such as vinylsulfone **I** and vinylketone **II** (Fig. 1), have been reported to inhibit cruzipain preventing parasite intracellular growth and differentiation [20,21]. Also generation of oxidative stress in *T. cruzi*, through selective reduction of drugs by oxidoreductases unique in the parasite, is an attractive target [22,23]. In 1999, the benzo[1,2-*c*]1,2,5-oxadiazole *N*-oxide heterocycle (benzofuroxan, i.e. derivatives **1**–**5** Scheme 1) was reported by our group as *T. cruzi* growth inhibitor [24]. Our initial studies indicated that the *N*-oxide group of this heterocycle system could act as bioreducible group into the parasite generating free radical species [24–26]. On the other hand some studies developed by our group suggested the perturbation of the mitochondrial respiratory chain, inhibiting parasite respiration, and the *N*-oxide moiety were essential for the trypanosomicidal activity [27,28]. After a cluster methodology study, Hansch's series design, 5-phenylethenylbenzofuroxan (**1**, Scheme 1) was revealed as excellent hit for the design of new trypanosomicidal compounds [27]. After that, further studies were performed over this skeleton in order to study the influence of structural modifications on the biological activity [29–31] finding vinylthio-, vinylsulfinyl- and vinylsulfonyl-benzofuroxan derivatives (**2**–**5**, Scheme 1) with remarkable *in vitro* activities against different *T. cruzi* strains and good *in vivo* activities in an acute murine model of Chagas' disease.

The drugs of choice for the treatment of leishmaniasis are sodium stibogluconate (Pentostam®), meglumine antimoniate (Glucantime®), pentamidine and liposomal amphotericin B, but these sometimes meet with failure [32]. Besides, WHO/TDR develops a research program with Miltefosine (Mtf, Fig. 1), a very promising leishmanocidal drug [33,34]. This illness, associated with poverty, does not attract the investment from pharmaceutical companies as a result of the lack of commercial reasons; consequently, efforts to develop new and safer drugs have been carried out mainly by academic institutions. In this sense, we have also



Scheme 1. Previous and new designed anti-trypanosomatid benzofuroxans.

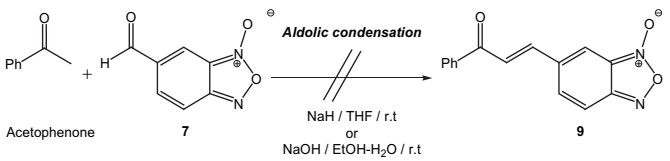
studied new series of structural hits against different leishmania strains [35–38] finding *N*-oxide derivatives as potential anti-leishmania agents (i.e. compound **6**, Scheme 1).

Herein, structural modifications on benzofuroxans **2**–**4** were planned in order to recognize the effects on the anti-*T. cruzi* activity when different substituted-phenyls and Michael-acceptors were used. Therefore, we designed new structures based on the association of benzofuroxan heterocycle with Michael-like systems as for example vinylsulfonyl and the bioisoster vinylketo moiety present in cruzipain inhibitors (Scheme 1, structures **III**–**VI**). We have developed a small library of benzofuroxan derivatives employing a polymer-supported triphenylphosphine (PS-TPP) as reagent. Triphenylphosphine is one of the most widely used phosphorus-containing reagents in organic synthesis for many types of transformations such as Mitsunobu and Wittig reactions. However, the byproduct triphenylphosphine oxide, generated by these reactions, is often difficult to separate from the end-product. The use of commercially available PS-TPP leads to much simpler workups and products isolation [39,40]. So, following the use of a PS-TPP, the triarylphosphine oxide remains attached to the resin (PS-TPPO) and it could be separated only by filtration. The PS-TPP could be regenerated after reduction of the triarylphosphine oxide with trichlorosilane. The designed benzofuroxan derivatives were efficiently synthesized via Wittig reaction using a PS-TPP. Besides, the deoxygenated derivatives, benzofurazans, were prepared by reaction of the corresponding *N*-oxides with PS-TPP. The developed derivatives were examined for antiproliferative *in vitro* activity against *T. cruzi* Tulahuen 2 strain and CL Brener clone, against promastigote form of *Leishmania braziliensis* (MHOM/BR/00/LTB300) and *Leishmania pifanoi* (MHOM/VE/57/LV135) strains and for unspecific cytotoxicity against J774 mouse macrophages. Initial studies of mitochondrial dehydrogenases inhibition, cruzipain inhibition and capacity to produce intra-parasite free radical, through electronic spin resonance (ESR) spectroscopy, were also performed to investigate the mechanisms of action.

2. Chemistry

As occurs with benzofuroxanyl vinylsulfone **4** [30], attempts to obtain benzofuroxanyl chalcone-like derivative **9** (Scheme 2) in solution through classical aldolic conditions were fruitless, causing complete decomposition of the starting heterocycle or reduction products (benzofurazan). This could be explained as the result of the well-known ring opening susceptibility of this heterocycle by nucleophilic attack. Benzofuroxan system reacts very easily with a large number of electron-rich species, as for example enolates, yielding various classes of products such as ring-opening derivatives, other heterocycle derivatives and deoxygenated derivatives [41–43].

Consequently, a solid-phase synthetic strategy was chosen for the synthesis of benzofuroxanyl chalcone-like compounds **9**–**12** using commercially available 2-bromoacetophenones (**8a**–**d**) and PS-TPP, via Wittig conditions (Scheme 3). Treatment of PS-TPP with the 2-bromoacetophenones **8a**–**d** yielded the corresponding phosphonium salt (evaluated by FT-IR for the appearance of new bands near to 1670 cm^{-1} corresponding to the stretching vibration of the C═O bond). Then this phosphonium salts were reacted with the bases NaH or sodium bis(trimethylsilyl)amide (NaHMDS) followed by the condensation with formylbenzofuroxan **7** with the concomitant cleavage of the resin through Wittig reaction resulting in the free chalcone-like and the polymer-supported triphenylphosphine oxide (PS-TPPO, Scheme 3). Filtration of the reaction through a pad of silica yielded benzofuroxanyl chalcone-like compounds **9**–**12** with good overall yields. In these conditions, ^{1}H-NMR showed that chalcone-like compounds **9**–**12** were isolated as the *E* geometric isomers around the olefinic group, the deoxygenated analogues were removed, by chromatography, as secondary products in very low yields.



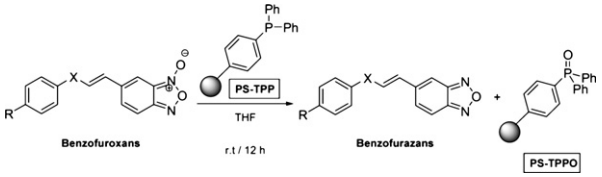
Scheme 2. Attempt to obtain the benzofuroxanyl chalcone-like **9** in solution.

To obtain vinylsulfone, i.e. **21**, the solid-phase synthesis strategy shown in Scheme 4 was first attempted. However, the desired vinylsulfone was not isolated or generated in very low yield. Different oxidation conditions were attempted to generate the resin-attached sulfone. When *m*-CPBA [44] was used the vinylthio-derivative **15*E*/*Z*** (Scheme 5) was obtained as the main product after the Wittig process. This fact could indicate both phosphonium phenyls' steric impediment and bromine-anion's stereo-electronic effects in the proximity of the sulfur atom diminish its susceptibility to oxidant attack. Attempts with oxone or urea-hydrogen peroxide complex (Scheme 4) render little amount, less than 5%, of the desired product, **21**, after the Wittig process.

On the basis of these results, the vinylthio-derivatives, **2**, **14**–**16**, were firstly obtained, as chromatographically separated geometric isomers, through Wittig solid-phase synthesis (Scheme 5). Subsequently, vinylsulfinyl-derivatives, **3**, **17**–**19**, and vinylsulfonyl-derivatives, **4**, **20**–**22**, were selectively obtained in solution using *m*-CPBA at low temperature or H_2O_2 (30%) in acetic acid at reflux, respectively.

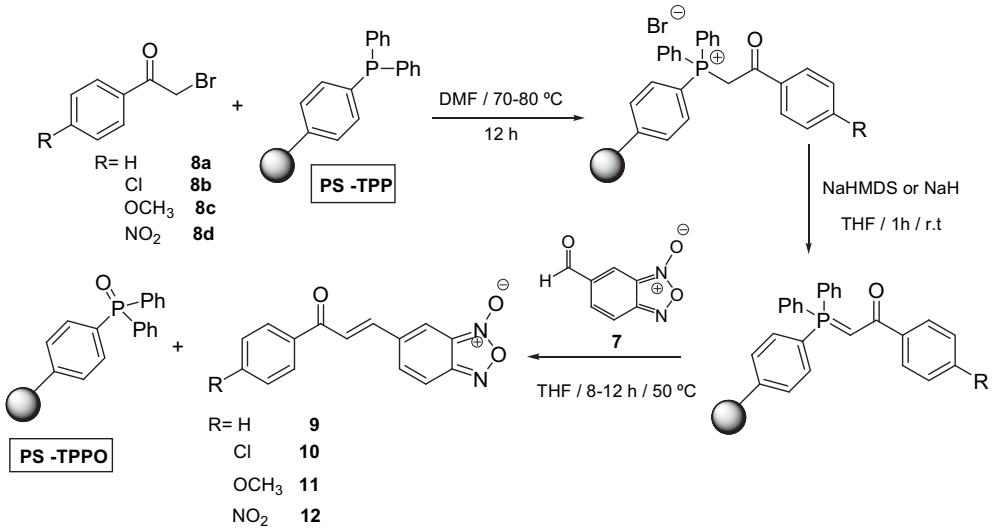
To corroborate the effect of the *N*-oxide moiety in the trypanosomicidal activity of the new hybrid benzofuroxans, some were converted into the corresponding deoxygenated analogues (benzofurazans). Previously, we reported the synthesis of benzofurazans from the corresponding benzofuroxans with triphenylphosphine in boiling ethanol [27]. The byproduct triphenylphosphine oxide generated in this reaction is often hard to separate from the desired product. The use of PS-TPP as reductant agent in mild conditions rendered the benzofurazans **23**–**29** in excellent yields allowing to separate the byproduct, PS-TPPO by a single filtration (Table 1). All of the proposed structures were established by ^{1}H-, ^{13}C-NMR (HMQC, HMBC) spectroscopy and MS. The purity was established by TLC and microanalysis.

Table 1
Synthesis of benzofurazan derivatives using PS-TPP.



Benzofurazan	X	R	Yield (%)[a]	Purity (%)[b]
23	S	H	93	98
24	SO	H	96	96
25	SO_2	H	95	97
26	CO	H	90	98
27	CO	Cl	94	96
28	CO	OCH_3	92	97
29	CO	NO_2	88	96

[a] After filtration and washing (see Experimental Section).
[b] From ^{1}H-NMR analysis.



Scheme 3. Solid-phase synthesis of benzofuroxanyl chalcone-like compounds **9**–**12**.

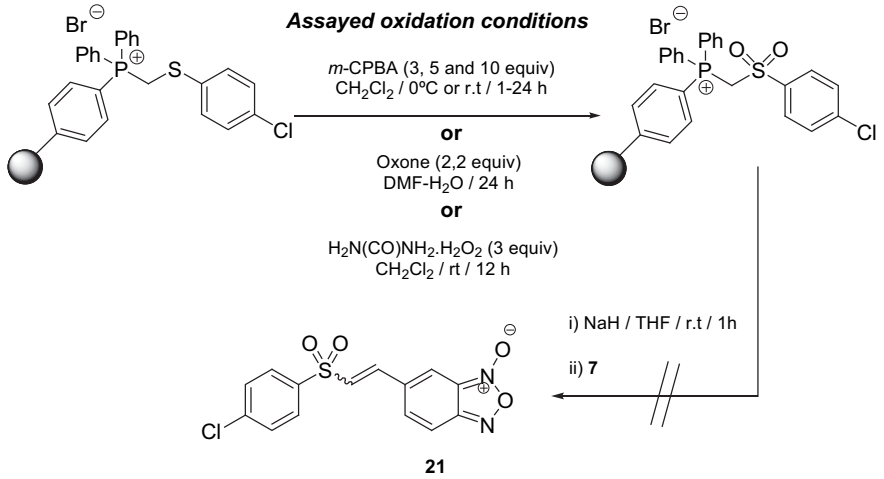
3. Biology

3.1. In vitro trypanosomicidal activity

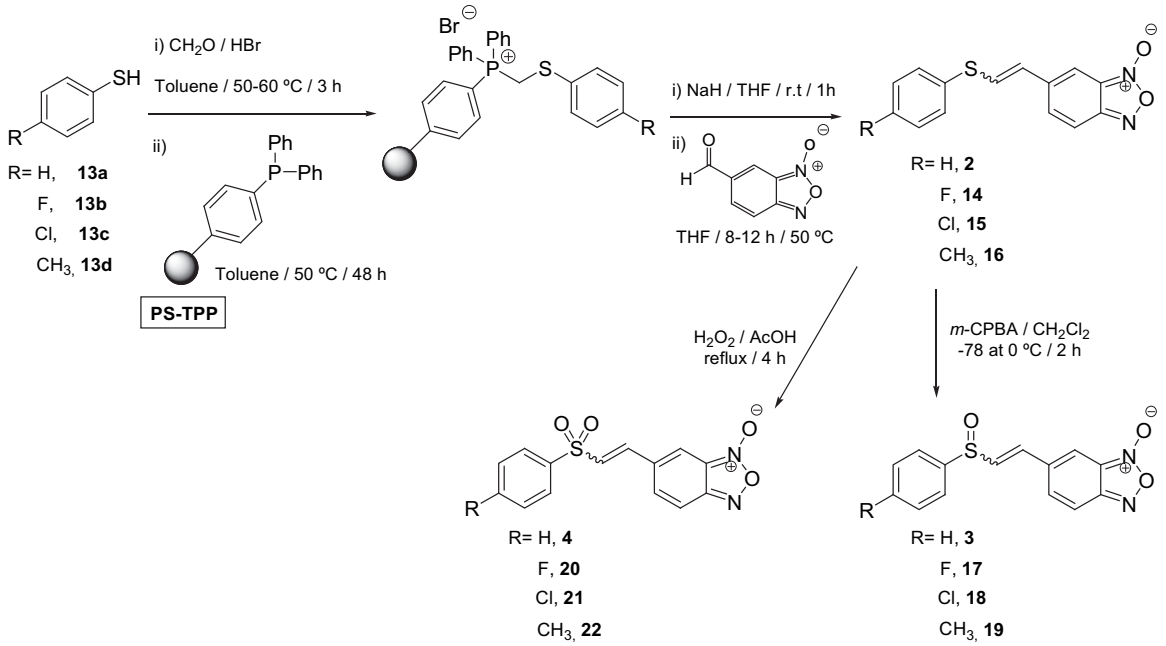
All hybrid benzofuroxans, and some intermediates, were tested *in vitro* against *T. cruzi*, using epimastigote form of Tulahuen 2 strain and CL Brener clone. The existence of the epimastigote form of *T. cruzi* as an obligate mammalian intracellular stage has been revisited and confirmed [45]. The IC_{50} concentration (50% inhibitory growth concentration) for each compound was evaluated in comparison to the control (no drug added to the media). Nfx and Bnz were used as the trypanosomicidal reference drugs. Table 2 shows the effect of developed benzofuroxan derivatives on the growth of *T. cruzi*. Chalcone-like compounds **9**, **11** and **12**, vinylthio **15*E*** and **16*E***, vinylsulfinyl **17*Z*** and **18*Z*** and vinylsulfonyl **20*Z*** and **21*Z*** were the most active compound with IC_{50} between 1.0 and 5.0 μM, all with potencies similar to the reference drugs. The most active derivatives are the vinylthio-benzofuroxan **15*E*** and **17*Z***. Substitution on the phenyl group of the chalcone-like **10**, with chloro group results with moderate trypanosomicidal activity against Tulahuen 2 strain. No clear relationship between geometric isomerism and activity was observed. The deoxygenated derivatives **23**–**29** possess less trypanosomicidal activity than the corresponding *N*-oxide analogues and reference drugs, showing that *N*-oxide moiety is imperative for adequate anti-*T. cruzi* activity. The vinylsulfonyl-derivative **25**, with IC_{50} 25.0 and 18.0 μM for Tulahuen 2 and CL Brener, respectively, possesses higher anti-*T. cruzi* activity than the corresponding vinylthio- and vinylsulfinyl-derivatives, **23** and **24**, respectively, showing that the electrophilic center in this vinylsulfonyl moiety could be important for adequate trypanosomicidal activity. It was not possible to evidence clear different benzofuroxans-activity patterns from Tulahuen 2 strain and CL Brener clone biological data. In general, similar activity-profiles were observed in both parasitic systems, i.e. one of the most active on Tulahuen 2 strain, derivative **15*E***, was the most active against CL Brener, or one of the least active on Tulahuen 2, derivative **19*Z***, was also one of the least active against CL Brener (Table 2).

3.2. In vitro leishmanocidal activity

Initially, the parent compounds **2**–**5**, a phenylvinylthio-, a phenylvinylsulfinyl-, a phenylvinylsulfonyl-, and an



Scheme 4. Attempt to obtain the vinylsulfone **21** through solid-phase synthesis.



Scheme 5. Solid-phase synthesis of vinylthio **2**, **14**–**16** and traditional synthesis of vinylsulfinyl **3**, **17**–**19** and vinylsulfonyl **4**, **20**–**22**.

alkylvinylsulfonyl-derivative were selected to be studied as leishmanocidal agents. According to the results on the leishmanocidal test two of the new phenylvinylthio-derivatives, **14** and **15**, were also studied biologically. They were tested *in vitro* against promastigote form of *L. braziliensis* (MHOM/BR/00/LTB300) and *L. pifanoi* (MHOM/VE/57/LV135) strains. Viability of parasite was assessed colorimetrically using the MTT assay [46,47]. For each derivative, the percentage of cytotoxicity was initially determined at 25 μM as it is indicated in Experimental Section and then in a concentration-response assay, between 0.5 and 50.0 μM, the IC_{50} was calculated and reported in Table 3. Mtf was used as the reference leishmanocidal drug. The most active benzofuroxans are the vinylthio **2** (*Z* and *E* isomers), **14** (*Z* and *E* isomers) and **15** (*E* isomer) and the vinylsulfinyl **3** (*Z* isomer) finding the same profile of activity than in *T. cruzi* parasite. The vinylthio-benzofuroxan **2*Z*** and **15*E*** are at least 10 times more active than reference leishmanocidal drug *in vitro* against promastigote form of *L. braziliensis*. Clearly, in leishmanocidal activity, the *Z*-geometric isomers were more active than the *E*-ones. Benzofuroxans **2*E***, **2*Z***, **14*E***, **14*Z***, and **15*E*** were assayed *in vitro* against both Leishmania strains, and all of them were found to exhibit slightly higher leishmanocidal activity against *L. braziliensis* than against *L. pifanoi*.

Table 2
In vitro anti-*T. cruzi* activity of new benzofuroxan derivatives, Nfx and Bnz.

Compd.	IC_{50} (μM)[a, b]		Compd.	IC_{50} (μM)[a, b]	
	T2[c]	CLB[d]		T2[c]	CLB[d]
9	5.0	nd[e]	**20**	7.5 (*E*)	6.3 (*E*)
				2.7 (*Z*)	2.9 (*Z*)
10	25.0	nd	**21**	4.8 (*E*)	4.8 (*E*)
				1.3 (*Z*)	2.3 (*Z*)
11	4.1	nd	**22**	4.3 (*E*)	9.6 (*E*)
				3.6 (*Z*)	5.1 (*Z*)
12	2.4	nd	**23**	42.0	25.0
14	5.0 (*E*)	5.5 (*E*)	**24**	41.0	32.0
	12.0 (*Z*)	5.9 (*Z*)			
15	1.9 (*E*)	1.0 (*E*)	**25**	25.0	18.0
	4.2 (*Z*)	2.8 (*Z*)			
16	1.9 (*E*)	4.7 (*E*)	**26**	97.0	nd
	4.2 (*Z*)	4.1 (*Z*)			
17	4.5 (*E*)	6.2 (*E*)	**27**	>100.0	nd
	1.6 (*Z*)	3.2 (*Z*)			
18	5.5 (*E*)	6.7 (*E*)	**28**	>100.0	nd
	2.7 (*Z*)	4.7 (*Z*)			
19	4.6 (*E*)	6.0 (*E*)	**29**	>100.0	nd
	6.7 (*Z*)	8.4 (*Z*)			
Nfx	7.7	8.5	**Bnz**	7.4	4.5

[a] IC_{50}: concentration that produces 50% inhibitory effect.
[b] The results are the means of three different experiments with a SD less than 10% in all cases.
[c] T2: Tulahuen 2 strain.
[d] CLB: CL Brener clone.
[e] nd: not determined.

3.3. Unspecific cytotoxicity

Unspecific mammalian cytotoxicity of the developed benzofuroxans was evaluated *in vitro* in the range of 1.0–400.0 μM,

Table 3
IC_{50} Values of selected benzofuroxans against different *Leishmania* spp strains.

Compd.	IC_{50} (μM)[a]	
	L. braziliensis	*L. pifanoi*
	LTB300	LV135
2*E*	1.5	4.3
2*Z*	0.9	4.5
3*E*	31.5	nd[b]
3*Z*	4.6	nd
4*E*	21.5	nd
4*Z*	10.8	nd
5	16.8	nd
14*E*	4.3	4.3
14*Z*	1.7	4.5
15*E*	0.9	4.6
Mtf	9.0	(85.5[c])

[a] The results are the means of three independent experiments with a SD less than 10% in all cases.
[b] nd: not determined.
[c] Compound tested at 5.0 μM, % of growth inhibition is reported.

Table 4
Cytotoxicity of benzofuroxan derivatives against J-774 mouse macrophages.

Compd.	IC_{50} (μM)[a]	SI[b, c]	Compd.	IC_{50} (μM)[a]	SI[b, c]
2*E*/*Z*	30.0	10.0[d]	**14*Z***	43.0	3.6
3*E*	30.0	11.5	**18*E***	18.0	4.0
3*Z*	14.0	20.0	**18*Z***	10.0	6.3
4*E*	16.0	10.0	**22*E***	8.0	1.1
4*Z*	11.0	10.0	**24**	>400.0	>10.0
9	47.0	9.4	**25**	78.0	3.1
10	28.0	1.1	**26**	102.0	1.1
11	31.0	7.6	**27**	251.0	<2.5
12	32.0	13.3	**28**	111.0	<1.1
14*E*	35.0	7.0	**29**	131.0	<1.3

[a] The results are the means of two independent experiments with a SD less than 10% in all cases.
[b] SI: selectivity index.
[c] $SI = IC_{50,\ macrophage}/IC_{50,\ T2,\ epimastigote}$.
[d] Considering the $IC_{50} = 3.0$ μM.

using J774 mouse macrophages as the cellular model (Table 4). The cytotoxicity of the chalcone-like compounds **9**–**12** against macrophages is comparable to that observed for the vinylthio-benzofuroxan **2**, vinylsulfinyl-benzofuroxan **3** and vinylsulfonyl-benzofuroxan **4**. In particular, chalcone-like derivative **9** resulted less toxic against the macrophages than the corresponding vinylsulfone-benzofuroxan bioisoster **4*E*** with similar order of selectivity index (SI). Chalcone-like derivatives **9** and **12** were the most selective among the new studied benzofuroxans (Table 4) being the SI, for Tulahuen 2 strain, near to 10. These results are in agreement with previous reports of selective anti-*T. cruzi* synthetic chalcones [48]. These chalcone-like benzofuroxans could be considered promising agents for further anti-trypanosomal therapy. The deoxygenated chalcone-like compounds **26**–**29** show lower levels of macrophage toxicities than the corresponding *N*-oxide analogues, **9**–**12**, however their SI are worse as result of their lower trypanosomicidal activity. The substitution on the phenyl moiety, by –F, –Cl, or $–CH_3$, of the new vinylthio-, vinylsulfinyl-, and vinylsulfonyl-benzofuroxans do not contribute in the improvement of SI values (compare SI value of unsubstitutedphenyl vinylthio-derivative **2*E*/*Z*** to that of 4-fluorophenyl-vinylthio-ones **14*E*** and **14*Z***, or SI value of unsubstitutedphenyl vinylsulfinyl-derivatives **3*E*** and **3*Z*** to that of 4-chlorophenylvinylsulfinyl-ones **18*E*** and **18*Z***, and SI value of unsubstitutedphenylvinylsulfonyl **4*E*** to that of 4-methylphenylvinylsulfonyl-one **22*E***). No clear relationship between geometric isomerism and activity was observed.

3.4. Mechanism of action studies

In order to determine the mechanism of action the following studies were performed: inhibition of cruzipain (CP), inhibition of the mitochondrial dehydrogenases activity and capability to produce intra-parasite free radicals.

3.4.1. Inhibition of T. cruzi cruzipain

Selected developed benzofuroxans and benzofurazans were studied as inhibitors of *T. cruzi* CP, following a previously described procedure [30]. None of the assayed compounds resulted to be good inhibitors of this enzyme at the studied doses (25.0–100.0 μM) (Table 5) showing these Michael acceptor-system, linked to both heterocycles, were not enable to inhibit this biomolecule.

3.4.2. Inhibition of T. cruzi and Leishmania mitochondrial dehydrogenases

Some of the developed benzofuroxans were tested as possible inhibitors of mitochondrial dehydrogenases in *T. cruzi* and *L. braziliensis*. The percentage of mitochondrial dehydrogenase activities compared to untreated control was assessed using the colorimetric MTT assay performed at very short times, no more than 240 min of incubation, following previously described procedures [37,38,50]. Table 6 shows the results obtained for the studied benzofuroxans as inhibitors of *T. cruzi* mitochondrial dehydrogenases and the comparation with the reference drugs Nfx and Bnz, some relevant features could be observed. The parent compound, vinylsulfone **5** showed the highest inhibition of mitochondrial dehydrogenases with a value of 58.5%. The parent compound, vinylthio **2**, vinylsulfoxide **3*Z*** and vinylsulfones **4*E*** and **20** and chalcone-like derivative **9** show some degree of mitochondrial dehydrogenases inhibition, with values between 14.5 and 25.0%. The result for chalcone-like derivative **9** is in agreement with some previous observations describing chalcones ability to inhibit mitochondrial parasite proteins [49–51]. On the other hand, the rest of the new developed benzofuroxans studied as well as the reference trypanosomicidal drugs, Nfx and Bzn, were not inhibitors of mitochondrial dehydrogenases at the studies time. In order to study the inhibitory effect of benzofuroxan's in *Leishmania* mitochondrial dehydrogenases we selected the most effective inhibitors of *T. cruzi* mitochondrial dehydrogenases, i.e. vinylsulfoxide **3*Z*** and vinylsulfones **4*E*** and **5**, and compare with the leishmanocidal drug Mtf. All the studied compounds exhibited a clear time-dependent inhibitory effect after 30 min of incubation (Fig. 2).

Table 5
Inhibition of *T. cruzi* cruzipain by **2**, **9**, **11**, **20**, **24** and **25**.

Compd.	CP percentage of inhibition (%)[a, b]		
	25 μM	50 μM	100 μM
2	11.0	22.0	30.0
9	0.0	0.0	0.0
11	nd	nd	13.0
20	0.0	0.0	0.0
24	nd	nd	0.0
25	0.0	0.0	0.0

[a] The control assays contained the respective amount of DMSO.
[b] The values are the mean of at least two independent measurements that differed by less than 10%.

3.4.3. Free radical generation: ESR studies

Three of the most *T. cruzi*-active hybrid benzofuroxans, **9, 14** and **20** were studied as intra-parasite free radical producers using ESR spectroscopy and spin-trapping tools. In the spin trapping technique, the cyclic nitrone 5,5-dimethylpyrroline-*N*-oxide (DMPO) is the most used spin trapping compound due to the unique ESR spectrum for trapping hydroxyl and superoxide radical [52].

Table 6
Inhibition of *T. cruzi* mitochondrial dehydrogenases.

Compd.	Percentage of inhibition (%)[a, b]
2*E*/*Z* (1:1)	14.5
3*Z*	25.0[c]
4*E*	25.0[c]
5	58.5
9	21.8
14*E*/*Z* (1:1)	0.0
20*E*/*Z* (1:1)	17.6
21*E*/*Z* (1:1)	4.0
Nfx	4.0[c, d]
Bnz	7.0[c, e]

[a] 120 min of incubation.
[b] Tulahuen 2 strain.
[c] Y strain.
[d] Taken from reference [37].
[e] Taken from reference [38].

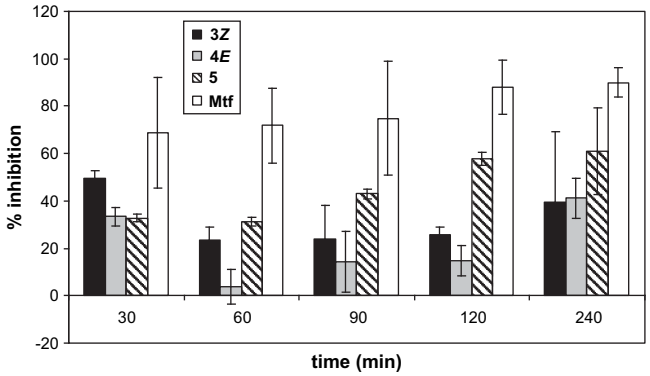


Fig. 2. Inhibition mitochondrial dehydrogenases (%) compared to untreated *L. braziliensis* promastigotes. Mtf activity was taken from reference [38].

Recently, we have developed a group of heteroarylnitrones, especially a furoxanylnitrone derivative (α(*Z*)-(3-methylfuroxan-4-yl)-*N*-*tert*-butylnitrone, FXN), with the ability to directly trap and stabilize oxygen, carbon, and sulfur-centered free radicals [53]. Consequently, the free radical production capacity of these benzofuroxans was assessed on *T. cruzi*-microsomal fraction by ESR using DMPO and FXN for spin trapping. The ESR spectra of the free radical generated by chalcone-like derivative **9** in the *T. cruzi*-microsomal fraction, and trapped by DMPO, showed a pattern consistent with the trapped *N*-oxide free radical together with the well known DMPO-OH$^{\cdot}$ adduct (Fig. 3) [31]. Though the ESR spectra of the free radical generated by the vinylthio-benzofuroxan **14** and vinylsulfonyl-benzofuroxan **20** in the *T. cruzi*-microsomal fraction, and trapped with FXN, showed a pattern consistent with trapped hydroxyl free radical (Fig. 3) [53]. These experiments clearly demonstrated that this family of compounds is able to produce oxygen oxidative species into the parasite which could be one of the modes of action of these hybrid compounds.

4. Conclusions

We have identified new benzofuroxan derivatives as promising anti-trypanosomatid agents. To our knowledge this is the first time that benzofuroxan containing structures are reported also as leishmanocidal compounds. An efficient synthetic route access to the desired benzofuroxans by using Wittig reaction, by a mild method, is achieved involving adequate aldehyde and PS-TPP. Studies developed to determine the mechanism of action showed that trypanosomicidal and leishmanocidal activities of these hybrid benzofuroxans might be result of the interference on the parasite-mitochondrion function and the production of oxygen oxidative species into the parasite. Further structural optimization, QSAR, and *in vivo* activities in an acute murine model of Chagas' disease and Leishmaniosis are currently underway.

5. Experimental

5.1. Chemistry

Compounds **2**–**5** and **7** were prepared according to procedures previously described [24,30]. Melting points were determined with an electrothermal melting point apparatus (Electrothermal 9100) and are uncorrected. Proton and carbon NMR spectra were recorded on a Bruker DPX-400 spectrometer. The chemical shifts values are expressed in ppm relative to tetramethylsilane as internal standard. In ^{13}C-NMR data only narrow peaks were reported. ESR spectra were recorded in the X band (9.85 GHz) using a Bruker ECS 106 spectrometer with a rectangular cavity and 50 kHz field modulation. Mass spectra were determined either on a MSD 5973 Hewlett–Packard or LC/MSD-Serie 100 Hewlett–Packard spectrometers using electronic impact (EI) or electrospray ionization (ESI), respectively. Infrared spectra were recorded on a Perkin–Elmer 1310 apparatus, using potassium bromide tablets for solid and oil products (the frequencies are expressed in cm^{-1}). Microanalyses were performed on a Fisons EA 1108 CHNS-O instrument and were within (0.4% of the calculated compositions). Column chromatography was carried out using Merck silica gel (60–230 mesh). Most chemicals and solvents were analytical grade and used without further purification. All the reactions were carried out in a nitrogen atmosphere.

5.1.1. General procedure for the synthesis of the 5E-[2-(arylcarbonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide derivatives (**9**–**12**)

To a suspension of a polymer-bound triphenylphosphine (0.5 g, 3 mmol/g phosphine) in dry DMF (3.5 mL/mmol) was added 2 equivalents of the corresponding 2-bromoacetophenone. The

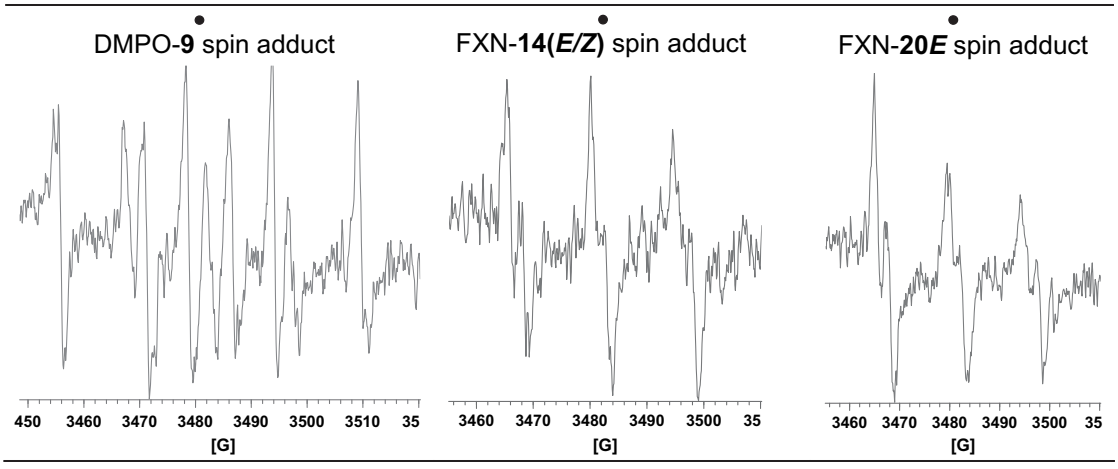


Fig. 3. ESR spectra. DMPO and FXN-benzofuroxan spin adduct obtained with *T. cruzi*-microsomal fraction.

mixture was stirred for 12 h at 70–80 °C. The support was cooled, carefully filtered and extensively washed with dry toluene (2 × 10 mL), dry dichloromethane (2 × 10 mL) and dry diethyl ether (2 × 10 mL). The brown powders were dried at reduced pressure. According to the weight increase the loading was estimated to be in 2.9 mmol phosphonium salt per gram support [54]. To a suspension of polymer-bound phosphonium salt (1 equiv.) in THF was added to 1 M solution of NaHMDS in THF (1.5 equiv.) at room temperature yielding the ylide within 60 min. Excess base was carefully removed by extensive washing of the support with dry THF (30 mL). The support was resuspended in dry THF (3 mL) and the formylbenzofuroxan **7** (0.80 equiv.) was added at room temperature. The mixture was stirred 8–12 h at 50 °C, filtered through a pad of silica and washed with THF (2 × 10 mL), and diethyl ether (2 × 10 mL). The combined organic layers were concentrated at reduced pressure to give the corresponding vinylketobenzofuroxan.

*5.1.1.1. 5E-[2-(phenylcarbonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**9**).* Yellow solid (53%); mp 170.0–171.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.54–7.70 (bs, 3H), 7.58 (d, 1H, J = 15.3 Hz), 7.59 (d, 1H, J = 10.8 Hz), 7.65 (m, 2H), 7.79 (d, 1H, J = 15.6 Hz), 8.06 (d, 2H, J = 7.7 Hz). EI-MS, *m/z* (abundance, %): 266 ($M^{+\cdot}$, 85), 250 (58), 233 (11), 220 (9), 206 (41), 178 (34), 77 (100). Anal. ($C_{15}H_{10}N_2O_3$) C, H, N.

*5.1.1.2. 5E-[2-(4-chlorophenylcarbonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**10**).* Yellow solid (50%); mp 193.5–195.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.50–7.70 (bs, 3H), 7.54 (d, 2H, J = 8.5 Hz), 7.57 (d, 1H, J = 15.7 Hz), 7.80 (d, 1H, J = 15.7 Hz), 8.00 (d, 1H, J = 8.4 Hz). ESI-MS, *m/z*: 301 ($M^{+\cdot}$ + H). Anal. ($C_{15}H_9ClN_2O_3$) C, H, N.

*5.1.1.3. 5E-[2-(4-methoxyphenylcarbonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**11**).* Yellow solid (47%); mp 165.5–167.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.61 (s, 3H), 7.01 (d, 2H, J = 8.6 Hz), 7.44–7.65 (bs, 3H), 7.48 (d, 1H, J = 15.8 Hz), 7.40 (d, 2H, J = 8.9 Hz), 7.67 (d, 1H, J = 15.7 Hz). ESI-MS, *m/z*: 297 ($M^{+\cdot}$ + H). Anal. ($C_{16}H_{12}N_2O_4$) C, H, N.

*5.1.1.4. 5E-[2-(4-nitrophenylcarbonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**12**).* Yellow solid (35%); mp 171.5–173.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.44–7.65 (bs, 3H), 7.50 (d, 1H, J = 15.8 Hz), 7.56 (d, 2H, J = 8.9 Hz), 7.78 (d, 1H, J = 15.7 Hz), 8.24 (d, 1H, J = 8.8 Hz). ESI-MS, *m/z*: 312 ($M^{+\cdot}$ + H). Anal. ($C_{15}H_9N_3O_5$) C, H, N.

*5.1.2. General procedure for the synthesis of the 5(E/Z)-[2-(arylthio)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**2**, **14**–**16**)*

A mixture of paraformaldehyde (1.89 g, 0.063 mmol), toluene (12 mL) and HBr (48%, 35 mL) was heated at 50 °C and treated dropwise within 30 minutes with a solution of corresponding tiophenol (0.04 mmol) in toluene (12 mL). Finally, the mixture was stirred for 60 minutes at 50 °C. The organic layer was washed with cold water, dried with Na_2SO_4 and used in the next reaction without further purification. A suspension of a polymer-bound triphenylphosphine (3.0 g, 3 mmol/g phosphine) in the crude of the bromomethylation process was stirred for 48 h at 50 °C. The support was filtered and washed with dry toluene (2 × 20 mL), dry dichloromethane (2 × 20 mL) and dry diethyl ether (2 × 20 mL). The brown powder was dried at reduced pressure. According to the weight increase the loading was estimated to be in 2.85 mmol phosphonium salt per gram support. To a suspension of polymer-bound phosphonium salt (1 equiv.) in THF was added NaH (1.5 equiv.) at room temperature generating the ylide within 60 min. The excess of the base was carefully removed by extensive washing of the support with dry THF. The support was resuspended in dry THF (6 mL/g resin) and the formylbenzofuroxan **7** (0.80 equiv.) was added at room temperature. The mixture was stirred 8–12 h at 50 °C, filtered through a pad of silica and washed with THF (2 × 10 mL), and diethyl ether (2 × 10 mL). The combined organic layers were concentrated at reduced pressure and the residue purified by column chromatography (SiO_2, petroleum ether/EtOAc (95:5)) to afford spectroscopically pure compounds.

*5.1.2.1. 5E-[2-(4-fluorophenylthio)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**14E**).* Yellow solid (50%); mp 106.0–108.5 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.44 (d, 1H, J = 15.5 Hz), 7.07 (d, 1H, J = 15.5 Hz), 7.15 (t, 2H), 7.36 (bs, 2H), 7.50 (m, 2H), 7.53–7.48 (bs, 1H). EI-MS, *m/z* (abundance, %): 288 ($M^{+\cdot}$, 100), 272 (40), 256 (8), 228 (74), 183 (50), 127 (47). Anal. ($C_{14}H_9FN_2O_2S$) C, H, N, S.

*5.1.2.2. 5Z-[2-(4-fluorophenylthio)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**14Z**).* Yellow solid (25%); mp 95.0–96.5 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.47 (d, J = 10.8 Hz, 1H), 6.75 (d, J = 10.8 Hz, 1H), 7.13 (m, 2H), 7.40 (bs, 1H), 7.48 (m, 2H), 7.54–7.47 (bs, 2H). EI-MS, *m/z* (abundance, %): 288 ($M^{+\cdot}$, 100), 272 (39), 256 (6), 228 (76), 183 (53), 127 (49). Anal. ($C_{14}H_9FN_2O_2S$) C, H, N, S.

*5.1.2.3. 5E-[2-(4-chlorophenylthio)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**15E**).* Yellow solid (39%); mp 110.0–113.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.54 (d, 1H, J = 15.5 Hz), 7.04 (d, 1H, J = 15.6 Hz), 7.41 (m, 4H), 7.45–7.34 (bs, 3H). ESI-MS, *m/z*: 305.0 ($M^{+\cdot}$ + H), 327.0 ($M^{+\cdot}$ + Na). Anal. ($C_{14}H_{10}N_2O_2S$) C, H, N, S.

*5.1.2.4. 5Z-[2-(4-chlorophenyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**15Z**).* Yellow solid (32%), mp 128–130 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.62 (d, J = 10.8 Hz, 1H), 6.77 (d, J = 10.8 Hz, 1H), 7.41 (d, J = 8.1 Hz, 2H), 7.43 (d, J = 8.1 Hz, 2H), 7.54–7.37 (bs, 3H). ESI-MS, *m/z*: 305.0 ($M^{+\cdot}$ + H). Anal. ($C_{14}H_9ClN_2O_2S$) C, H, N, S.

*5.1.2.5. 5E-[2-(4-methylphenylthio)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**16E**).* Yellow solid (53%); mp 129.0–131.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.41 (s, 3H), 7.03 (d, 1H, J = 16.0 Hz), 7.12 (d, 1H, J = 15.3 Hz), 7.25 (d, 2H, J = 7.9 Hz), 7.46 (d, 2H, J = 8.0 Hz), 7.30–7.40(bs, 2H), 7.61 (bs, 1H). EI-MS, *m/z* (abundance, %): 284 ($M^{+\cdot}$, 20), 268 (25), 252 (67), 238 (52), 217 (39), 191 (100). Anal. ($C_{15}H_{12}N_2O_2S$) C, H, N, S

*5.1.2.6. 5Z-[2-(4-methylphenylthio)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**16Z**).* Brown oil (30%); ^{1}H-NMR ($CDCl_3$) δ: 2.40 (s, 3H), 6.55 (d, 1H, J = 10.8 Hz), 6.83 (d, 1H, J = 10.8 Hz), 7.20 (d, 1H, J = 8.1 Hz), 7.51 (d, 1H, J = 8.0 Hz), 7.30–7.45(bs, 2H), 7.63 (bs, 1H). EI-MS, *m/z* (abundance, %): 284 ($M^{+\cdot}$, 100), 268 (59), 252 (34), 238 (15), 217 (56), 191 (58). Anal. ($C_{15}H_{12}N_2O_2S$) C, H, N, S.

*5.1.3. General procedure for the synthesis of the 5(E/Z)-[2-(arylsulfinyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**17**–**19**)*

A solution of the vinylthio-derivative *E* or *Z* (1 equiv.) in CH_2Cl_2 (6.0 mL/mmol) was cooled to −78 °C while a solution of *m*-chloroperbenzoic acid (1 equiv.) in CH_2Cl_2 (2.0 mL/mmol) was added dropwise during 5 min. The mixture was stirred and warmed to room temperature for 2 h and then poured into saturated sodium bicarbonate solution (10.0 mL) and the mixture extracted with CH_2Cl_2 (3 × 10.0 mL). After the workup of the combined organic layers, the residue was purified by column chromatography (SiO_2, petroleum ether: EtOAc (8:2)), yielding spectroscopically pure compounds.

*5.1.3.1. 5E-[2-(4-fluorophenylsulfinyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**17E**).* Yellow solid (51%); mp 117.0–119.0 °C. ^{1}H-NMR

($CDCl_3$): 6.98 (d, 1H, $J = 15.4$ Hz), 7.30 (m, 2H), 7.41 (d, 1H, $J = 15.3$ Hz), 7.55–7.34 (bs, 3H), 7.73 (m, 2H). EI-MS, *m/z* (abundance, %): 304 ($M^{+\cdot}$, 2), 288 (1), 275 (10), 256 (100), 240 (24), 196 (41). Anal. ($C_{14}H_9FN_2O_3S$) C, H, N, S.

*5.1.3.2. 5Z-[2-(4-fluorophenylsulfinyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**17Z**).* Yellow solid (54%); mp 114.0–116.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.69 (d, 1H, $J = 10.7$ Hz), 7.07 (d, 1H, $J = 10.7$ Hz), 7.30 (m, 2H), 7.48–7.62 (bs, 3H), 7.70 (m, 2H). EI-MS, *m/z* (abundance, %): 304 ($M^{+\cdot}$, 21), 288 (6), 275 (31), 256 (100), 240 (13), 215 (27), 196 (59). Anal. ($C_{14}H_9FN_2O_3S$) C, H, N, S.

*5.1.3.3. 5E-[2-(4-chlorophenylsulfinyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**18E**).* Yellow solid (44%); mp 185.0–187.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.98 (d, 1H, $J = 15.4$ Hz), 7.38 (d, 1H, $J = 15.4$ Hz), 7.55–7.34 (bs, 3H), 7.55 (d, 2H, $J = 8.5$ Hz), 7.65 (d, 2H, $J = 8.6$ Hz). ESI-MS, *m/z*: 321.0 ($M^{+\cdot} + H$), 343.0 ($M^{+\cdot} + Na$). Anal. ($C_{14}H_9ClN_2O_3S$) C, H, N, S.

*5.1.3.4. 5Z-[2-(4-chlorophenylsulfinyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**18Z**).* Pale yellow solid (52%); mp 127.0–129.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.67 (d, 1H, $J = 10.7$ Hz), 7.08 (d, 1H, $J = 10.8$ Hz), 7.63–7.53 (bs, 3H), 7.57 (d, 2H, $J = 8.5$ Hz), 7.62 (d, 2H, $J = 8.6$ Hz). ESI-MS, *m/z*: 321.0 ($M^{+\cdot} + H$), 343.0 ($M^{+\cdot} + Na$). Anal. ($C_{14}H_9ClN_2O_3S$) C, H, N, S.

*5.1.3.5. 5E-[2-(4-methylphenylsulfinyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**19E**).* Orange solid (21%); mp 160.0–162.0 °C. ^{1}H-NMR ($CDCl_3$): 2.44 (s, 3H), 6.99 (d, 1H, $J = 15.4$ Hz), 7.35 (d, 1H, $J = 15.4$ Hz), 7.38 (d, 2H, $J = 8.3$ Hz), 7.54–7.42 (bs, 3H), 7.60 (d, 2H, $J = 8.1$ Hz). ESI-MS, *m/z*: 301.0 ($M^{+\cdot} + H$). Anal. ($C_{15}H_{12}N_2O_3S$) C, H, N, S

*5.1.3.6. 5Z-[2-(4-methylphenylsulfinyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**19Z**).* Pale yellow solid (16%); mp 68.0–70.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.72 (d, 1H, $J = 10.9$ Hz), 7.12 (d, 1H, $J = 10.8$ Hz), 7.60–7.50 (bs, 3H), 7.45 (d, 2H, $J = 8.3$ Hz), 7.58 (d, 2H, $J = 8.4$ Hz). ESI-MS, *m/z*: 301.0 ($M^{+\cdot} + H$). Anal. ($C_{15}H_{12}N_2O_3S$) C, H, N, S.

*5.1.4. General procedure for the synthesis of the 5(E/Z)-[2-(arylsulfonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole derivatives (**20**–**22**)*

To a solution of the vinylthio-derivative *E* or *Z* (1 equiv.) in glacial AcOH (4.0 mL/mmol) was slowly added H_2O_2 (30%) (0.4 mL/mmol), and the mixture was heated under reflux for 30 min. The reaction mixture was cooled, neutralized with aqueous $NaHCO_3$, and extracted with EtOAc. After the workup of the combined organic layers, the residue was purified by column chromatography (SiO_2, petroleum ether/EtOAc (8:2)), yielding spectroscopically pure compounds.

*5.1.4.1. 5E-[2-(4-fluorophenylsulfonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**20E**).* Pale yellow solid (56%); mp 134.0–135.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.99 (d, 1H, $J = 15.4$ Hz), 7.28 (t, $J = 8.3$ Hz), 7.35–7.45 (bs, 1H), 7.50–7.70 (bs, 2H), 7.67 (d, 1H, $J = 15.4$ Hz), 8.01 (m, 2H). EI-MS, *m/z* (abundance, %): 320 ($M^{+\cdot}$, 64), 304 (13), 255 (6), 221 (11), 160 (71), 143 (100). Anal.($C_{14}H_9FN_2O_4S$) C, H, N, S.

*5.1.4.2. 5Z-[2-(4-fluorophenylsulfonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**20Z**).* Pale yellow solid (46%); mp 132.5–134.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.72 (d, 1H, $J = 10.9$ Hz), 7.05 (d, 1H, $J = 10.9$ Hz), 7.25 (t, $J = 8.1$ Hz), 7.40–7.65 (bs, 3H), 7.86 (m, 2H). EI-MS, *m/z* (abundance, %): 320 ($M^{+\cdot}$, 51), 304 (4), 255 (11), 221 (18), 160 (45), 143 (68), 95 (100). Anal. ($C_{14}H_9FN_2O_4S$) C, H, N, S.

*5.1.4.3. 5E-[2-(4-chlorophenylsulfonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**21E**).* Pale yellow solid (42%); mp 178.0–180.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.96 (d, 1H, $J = 15.4$ Hz), 7.37 (bs, 1H), 7.45–7.65 (bs, 2H), 7.59 (d, 2H, $J = 8.6$ Hz), 7.67 (d, 1H, $J = 15.4$ Hz), 7.92 (d, 2H, $J = 8.6$ Hz). ESI-MS, *m/z*: 337 ($M^{+\cdot} + H$), 359 ($M^{+\cdot} + H$). Anal. ($C_{14}H_9ClN_2O_4S$) C, H, N, S.

*5.1.4.4. 5Z-[2-(4-chlorophenylsulfonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole (**21Z**).* Pale yellow solid (49%); mp 117.0–118.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 6.67 (d, 1H, $J = 12.0$ Hz), 7.06 (d, 1H, $J = 12.0$ Hz), 7.50–7.55 (bs, 1H), 7.54 (d, 2H, $J = 8.7$ Hz), 7.62–7.58 (bs, 2H), 7.83 (d, 2H, $J = 8.7$ Hz). ESI-MS, *m/z*: 337 ($M^{+\cdot} + H$), 359 ($M^{+\cdot} + H$). Anal. ($C_{14}H_9ClN_2O_4S$) C, H, N, S.

*5.1.4.5. 5E-[2-(4-methylphenylsulfonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**22E**).* Yellow solid (27%); mp 157.0–158.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.43 (s, 3H), 6.98 (d, 1H, $J = 15.4$ Hz), 7.30–7.45 (bs, 1H), 7.41 (d, 2H, $J = 8.2$ Hz), 7.50–7.65 (bs, 2H), 7.64 (d, 1H, $J = 15.4$ Hz), 7.86 (d, 2H, $J = 8.3$ Hz). ESI-MS, *m/z*: 317 ($M^{+\cdot} + H$). Anal. ($C_{15}H_{12}N_2O_4S$) C, H, N, S.

*5.1.4.6. 5Z-[2-(4-methylphenylsulfonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**22Z**).* Pale yellow solid (14%); mp 116.0–118.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 2.42 (s, 3H), 6.71 (d, 1H, $J = 10.9$ Hz), 7.21 (d, 1H, $J = 10.8$ Hz), 7.16 (d, 2H, $J = 8.4$ Hz), 7.40–7.55 (bs, 3H), 7.45 (d, 2H, $J = 8.3$ Hz). ESI-MS, *m/z*: 317 ($M^{+\cdot} + H$). Anal. ($C_{15}H_{12}N_2O_4S$) C, H, N, S.

*5.1.5. General procedure for the synthesis of the deoxygenated analogues (**23**–**29**)*

To a suspension of a polymer-bound triphenylphosphine (2 equiv.) in THF (50 mL/mmol) was added 1 equivalent of the corresponding benzofuroxan derivative. The mixture was stirred for 12 h at room temperature. Then, the support was carefully filtered through a pad of silica and washed with THF and diethyl ether. The combined organic layers were concentrated under reduced pressure to give the corresponding benzofurazan derivatives.

*5.1.5.1. 5E-[2-(phenylthio)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**23**).* Yellow–orange oil (93%); ^{1}H-NMR ($CDCl_3$) δ: 7.04 (d, 1H, $J = 15.6$ Hz), 7.21 (d, 1H, $J = 15.5$ Hz), 7.40 (dd, 1H), 7.45–7.55 (m, 3H), 7.58 (d, 2H), 7.87 (d, 1H), 8.01 (s, 1H). ESI-MS, *m/z*: 255 ($M^{+\cdot} + H$). Anal. ($C_{14}H_{10}N_2OS$) C, H, N, S.

*5.1.5.2. 5E-[2-(phenylsulfonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**24**).* Brown solid (96%); mp 120.5–122.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.04 (d, 1H, $J = 15.4$ Hz), 7.51 (d, 1H, $J = 15.4$ Hz), 7.55 (dd, 1H, $J = 9.4$ Hz, $J = 1.3$ Hz), 7.57 (m, 3H), 7.74 (m, 2H), 7.85 (d, 1H, $J = 9.4$ Hz), 7.89 (s, 1H). ESI-MS, *m/z*: 271 ($M^{+\cdot} + H$). Anal. ($C_{14}H_{10}N_2O_2S$) C, H, N, S.

*5.1.5.3. 5E-[2-(phenylsulfonyl)vinyl]benzo[c]1,2,5-oxadiazole N-oxide (**25**).* Brown oil (95%); ^{1}H -NMR ($CDCl_3$) δ: 7.06 (d, 1H, $J = 15.4$ Hz), 7.53 (dd, 1H, $J = 9.4$ Hz, $J = 1.1$ Hz), 7.58–7.69 (m, 3H), 7.78 (d, 1H, $J = 15.4$ Hz), 7.90 (d, 1H, $J = 9.4$ Hz), 8.00 (m, 2H). ESI-MS, *m/z*: 287 ($M^{+\cdot} + H$). Anal. ($C_{14}H_{10}N_2O_3S$) C, H, N, S.

*5.1.5.4. 5E-[2-(phenylcarbonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole (**26**).* Pale yellow solid (90%); mp 180.5–182.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.56 (t, 2H), 7.66 (m, 1H), 7.69 (d, 1H, $J = 15.8$ Hz), 7.79 (dd, 1H, $J = 1.1$ Hz,

$J = 9.4$ Hz), 7.89 (d, 1H, $J = 15.7$ Hz), 7.95 (d, 2H, $J = 9.4$ Hz), 8.05 (s, 1H), 8.07 (2H, $J = 7.2$ Hz). EI-MS, m/z (abundance, %): 250 ($M^{+\cdot}$, 100), 233 (18), 205 (19), 165 (25), 105 (54), 77 (75). Anal. ($C_{15}H_{10}N_2O_2$) C, H, N.

*5.1.5.5. 5E-[2-(4-chlorophenylcarbonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole (**27**).* Yellow solid (94%); mp 210.0–212.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.69 (d, 2H, $J = 8.0$ Hz), 7.92 (d, 1H, $J = 15.6$ Hz), 8.18 (d, 1H, $J = 10.0$ Hz), 8.22 (d, 1H, $J = 15.6$ Hz), 8.26 (2H, $J = 8.4$ Hz), 8.31 (1H, $J = 9.6$ Hz), 8.54 (s, 1H). ESI-MS, m/z: 285 ($M^{+\cdot} + H$). Anal. ($C_{15}H_9ClN_2O_2$) C, H, N.

*5.1.5.6. 5E-[2-(4-methoxyphenylcarbonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole N-oxide (**28**).* Yellow solid (92%); mp 183.0–185.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 3.60 (s, 3H), 7.70 (d, 2H, $J = 8.4$ Hz), 7.90 (s, 1H, $J = 15.6$ Hz), 8.18 (d, 1H, $J = 10.4$ Hz), 8.22 (d, 1H, $J = 15.6$ Hz), 8.26 (d, 2H, $J = 8.4$ Hz), 8.32 (d, 1H, $J = 9.6$ Hz), 8.55 (s, 1H). ESI-MS, m/z: 281 ($M^{+\cdot} + H$). Anal. ($C_{16}H_{12}N_2O_3$) C, H, N.

*5.1.5.7. 5E-[2-(4-nitrophenylcarbonyl)vinyl]benzo[1,2-c]1,2,5-oxadiazole (**29**).* Yellow solid (88%); mp 205.0–207.0 °C. ^{1}H-NMR ($CDCl_3$) δ: 7.98 (d, 1H, $J = 15.6$ Hz), 8.20 (d, 1H, $J = 9.6$ Hz), 8.25 (d, 1H, $J = 15.6$ Hz), 8.33 (d, 1H, $J = 10.0$ Hz), 8.42 (d, 2H, $J = 9.0$ Hz), 8.45 (d, 2H, $J = 8.8$ Hz), 8.58 (s, 1H). ESI-MS, m/z: 296 ($M^{+\cdot} + H$). Anal. ($C_{15}H_9N_3O_4$) C, H, N.

5.2. Biology

5.2.1. In vitro anti-T. cruzi activity using Tulahuen 2 strain

Trypanosoma cruzi epimastigotes (Tulahuen 2) were grown at 28 °C in an axenic medium (BHI-tryptose) complemented with 5% foetal calf serum. Cells were harvested in the late log phase, resuspended in fresh medium, counted in a Neubauer chamber, and placed in 24-well plates (3×10^6/mL). Cell growth was measured as the absorbance of the culture at 610 nm, which was proved to be proportional to the number of cells present [35,36]. Before inoculation, the media were supplemented with the indicated amount of the studied compound from a stock solution in DMSO. The final concentration of DMSO in the culture media never exceeded 0.8% and the control was run in the presence of 0.8% DMSO and in the absence of any compound. No effect on epimastigote growth was observed in the presence of up to 1% DMSO in the culture medium. The percentage of growth inhibition was calculated as follows: $PGI = \{1 - [(A_p - A_{0p})/(A_c - A_{0c})]\} \times 100$, where $A_p = A_{610}$ of the culture containing the compound at day 5; $A_{0p} = A_{610}$ of the culture containing the compound right after addition of the inocula (day 0); $A_c = A_{610}$ of the culture in the absence of any compound (control) at day 5; $A_{0c} = A_{610}$ in the absence of the compound at day 0. To determine IC_{50} values, 50% inhibitory growth concentrations, parasite growth was followed in the absence (control) and presence of increasing concentrations of the corresponding compound. At day 5, the absorbance of the culture was measured and related to that of the control. The IC_{50} value was taken as the concentration of compound needed to reduce the absorbance ratio to 50%.

5.2.2. Viability of CL Brener clone, LTB300 strain of L. braziliensis and LV135 strain of L. pifanoi

Trypanosoma cruzi epimastigotes (CL Brener clones) were grown as it is indicated above. *L. braziliensis* (MHOM/BR/00/LTB300 strain) and *L. pifanoi* (MHOM/VE/57/LV135) promastigotes were grown at 28 °C in an axenic-RPMI medium supplemented with 5% FBS as previously described [35–38]. Cell-culture plates consisting of 24 wells were filled at 1 mL/well with the corresponding parasite strain culture during its exponential growth in the corresponding medium. BHI-Tryptose medium supplemented with 5% foetal bovine serum (FBS). Different concentration of studied compounds dissolved in DMSO were added and maintained for 5 days. Afterwards, the cells were washed with PBS and incubated (37 °C) with 0.4 mg/mL MTT (Sigma) for 3 h. Then, formazan was dissolved with DMSO (180 μL), and optical densities were measured. Each concentration was assayed three times, and six growth controls were used in each test. Cytotoxicity percentages (PCyt (%)) were determined as follows: $PCyt = [100 - (ODd - ODdm)/(ODc - ODcm)] \times 100$, where ODd is the mean of OD595 of wells with parasites and different concentrations of the compounds, ODdm is the mean of OD595 of wells with different compound concentrations in the medium, ODc is the growth control, and ODcm is the mean of OD595 of wells with medium only. The IC_{50} value was taken as the concentration of drug needed to reduce the absorbance ratio to 50%.

5.2.3. Cytotoxicity to macrophages

J-774 murine macrophage-like cells (ATCC, USA) were maintained by passage in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, and supplemented with 10% heat-inactivated foetal calf serum. J-774 cells were seeded (100.000 cells/well) in 96 well flat bottom microplates (Nunclon) with 200 μL of RPMI 1640 medium supplemented with 20% heat-inactivated foetal calf serum. Cells were allowed to attach for 48 h in a humidified 5% CO_2/95% air atmosphere at 37 °C. Then, cells were exposed to the compounds (1.0–400.0 μM) for 48 h. Afterwards, the cells were washed with PBS and incubated (37 °C) with MTT (0.4 mg/mL) for 3 h. Then, the formazan was dissolved with DMSO (180 μL) and optical densities were measured. Each concentration was assayed three times and six growth controls were used in each test. Cytotoxicity percentages (% C) were determined as follows: $\%\ C = [100 - (ODd - ODdm)/(ODc - ODcm)] \times 100$, where ODd is the mean of OD_{595} of wells with macrophages and different concentrations of the compounds; ODdm is the mean of OD_{595} of wells with different compounds concentration in the medium; ODc is the growth control and ODcm is the mean of OD_{595} of wells with medium only.

5.2.4. Mitochondrial dehydrogenase activities [37,38]

Mitochondrial dehydrogenase activities were measured in 24-well plates. One million of *T. cruzi* epimastigotes (Tulahuen 2 and Y strains) or one million of *L. braziliensis* promastigotes (LTB300) in 500 μL of the corresponding medium were seeded in each well, and 20 μM of studied compounds was added. Two wells with untreated parasites were maintained as controls corresponding to the given time of treatment. The cultures were incubated at 28 °C. At the different time incubations, the parasites were counted, and the colorimetric MTT dye-reduction assay was performed, the tetrazolium salt being converted into purple formazan by mitochondria. Fifty μL of a solution containing 5 mg/mL of MTT in PBS were added to each well, and plates were incubated for an additional 2 h. The reaction was stopped by addition of 500 μL of acidic isopropanol (0.4 mL HCl of 10 N in 100 mL isopropanol). The absorbance was measured at 570 nm. Under our conditions, compounds did not interfere with the reaction mixture. Percentage of mitochondrial dehydrogenase activities (%) was determined using untreated parasites-activities as 100%.

5.2.5. ESR studies

The free radical production capacity of the new benzofuroxan derivatives was assessed on *T. cruzi*-microsomal fraction (4 mg protein/mL) by ESR using DMPO and FXN for spin trapping [25,26,31]. Benzofuroxan derivatives (1 mM, final concentration) were dissolved in DMF (spectroscopy grade) and the solution was added to a reaction medium containing 1 mM NADPH, 1 mM EDTA and 100 mM DMPO or FXN, in 20 mM phosphate buffer, pH = 7.4.

The final mixture was transferred to a 50 μL capillary. All of the spectra were registered in the same scale after 15 scans.

Acknowledgements

Financial support from PDT (Uruguay) is acknowledged. We thank to Dr. J.J. Cazzulo, Instituto de Investigaciones Biotecnológicas, Universidad Nacional de General San Martín-CONICET, Argentina, for performing helpful in the cruzipain isolation and purification. We thank PEDECIBA for scholarship to LB, PEDECIBA-ANII for scholarship to AM and PH, and UdelaR for scholarship to DB.

References

[1] W. De Souza, Pharm. Design 4 (2002) 269–285.
[2] F. Kierszenbaum, Acta Parasitol. 52 (2007) 1–12.
[3] WHO Secretariat, Chagas disease: control and elimination. Sixty-second World Health Assembly A62/17, provisional agenda item 12.12, 03/20/2009.http://apps.who.int/gb/ebwha/pdf_files/A62/A62_17-en.pdf.
[4] www.dndi.org.
[5] M. Corrales, R. Cardozo, M.S. Segura, J.A. Urbina, M.A. Basombrio, Antimicrob. Agents Chemother. 49 (2005) 1556–1560.
[6] P.S. Doyle, Y.M. Zhou, J.C. Engel, J.H. McKerrow, Antimicrob. Agents Chemother. 51 (2007) 3932–3939.
[7] A. Gerpe, A. Merlino, M. Boiani, W. Porcal, P. Fagiolino, M. González, H. Cerecetto, J. Pharm. Biomed. Anal. 47 (2008) 88–94.
[8] L. Boiani, C. Davies, C. Arredondo, W. Porcal, A. Merlino, A. Gerpe, M. Boiani, J.P. Pacheco, M.A. Basombrío, H. Cerecetto, M. González, Eur. J. Med. Chem. 43 (2008) 2229–2237.
[9] M. Boiani, A. Merlino, A. Gerpe, W. Porcal, F. Croce, S. Depaula, M.A. Rodríguez, H. Cerecetto, M. González, Xenobiotica 39 (2008) 236–248.
[10] H. Cerecetto, M. González, Curr. Top. Med. Chem. 2 (2002) 1185–1213.
[11] J.D. Maya, S. Bollo, L.J. Nuñez-Vergara, J.A. Squella, Y. Repetto, A. Morello, J. Perie, G. Chauviere, Biochem. Pharmacol. 65 (2003) 999–1006.
[12] J.D. Maya, B.K. Cassels, P. Iturriaga-Vásquez, J. Ferreira, M. Faúndez, N. Galanti, A. Ferreira, A. Morello, Comp. Biochem. Physiol. A Mol. Integr. Physiol. 146 (2007) 601–620.
[13] S.M.F. Murta, R.T. Gazzinelli, Z. Brener, A.J. Romanha, Mol. Biochem. Parasitol. 93 (1998) 203–214.
[14] A.R.L. Teixeira, R.J. Nascimento, N.R. Sturm, Mem. Inst. Oswaldo Cruz 101 (2006) 463–491.
[15] J.A. Castro, M.M. de Meca, L.C. Bartel, Hum. Exp. Toxicol. 25 (2006) 471–479.
[16] S.R. Wilkinson, M.C. Taylor, D. Horn, J.M. Kelly, I. Cheeseman, Proc. Natl. Acad. Sci. 105 (2008) 5022–5027.
[17] J.H. McKerrow, J.C. Engel, C.R. Cafrey, Bioorg. Med. Chem. 7 (1999) 639–644.
[18] J.J. Cazzulo, Curr. Top. Med. Chem. 2 (2002) 1261–1271.
[19] K. Chibale, C.C. Musonda, Curr. Med. Chem. 10 (2003) 1863–1889.
[20] J.C. Engel, P.S. Doyle, I. Hsieh, J.H. McKerrow, J. Exp. Med. 188 (1998) 725–734.
[21] Y. Choe, L.S. Brinen, M.S. Price, J.C. Engel, M. Lange, C. Grisostomi, S.G. Weston, P.V. Pallai, H. Cheng, L.W. Hardy, D.S. Hartsough, M. McMakin, R.F. Tilton, C.M. Baldinod, C.S. Craika, Bioorg. Med. Chem. 13 (1999) 2141–2156.
[22] J.F. Turrens, Mol. Aspec. Med. 25 (2004) 211–220.
[23] R.L. Krauth-Siegel, R.H. Schirmer, H. Bauer, Angew. Chem. Int. Ed. 44 (2005) 690–715.
[24] H. Cerecetto, R. Di Maio, M. González, M. Risso, P. Saenz, G. Seoane, A. Denicola, G. Peluffo, C. Quijano, C. Olea-Azar, J. Med. Chem. 42 (1999) 1941–1950.
[25] C. Olea-Azar, C. Rigol, F. Mendizábal, R. Briones, H. Cerecetto, R. Di Maio, M. González, W. Porcal, M. Risso, Spectrochim. Acta Part A 59 (2003) 69–74.
[26] C. Olea-Azar, C. Rigol, M. Morillo, J.D. Maya, Y. Repetto, G. Aguirre, H. Cerecetto, R. Di Maio, M. González, W. Porcal, J. Chil. Chem. Soc. 48 (2003) 3–5.
[27] G. Aguirre, L. Boiani, H. Cerecetto, R. Di Maio, M. González, W. Porcal, L. Thomson, V. Tórtora, A. Denicola, M. Möller, Bioorg. Med. Chem. 13 (2005) 6324–6335.
[28] G. Aguirre, L. Boiani, M. Boiani, H. Cerecetto, R. Di Maio, M. González, W. Porcal, A. Denicola, O.E. Piro, E.E. Castellano, M.R. SanÍAnna, E.J. Barreiro, Bioorg. Med. Chem. 13 (2005) 6336–6346.
[29] W. Porcal, P. Hernández, G. Aguirre, L. Boiani, M. Boiani, A. Merlino, A. Ferreira, R. Di Maio, A. Castro, M. González, H. Cerecetto, Bioorg. Med. Chem. 15 (2007) 2768–2781.
[30] W. Porcal, P. Hernández, M. Boiani, G. Aguirre, L. Boiani, A. Chidichimo, J.J. Cazzulo, N.E. Campillo, J.A. Paez, A. Castro, R.L. Krauth-Siegel, C. Davies, M.A. Basombrío, M. González, H. Cerecetto, J. Med. Chem. 50 (2007) 6004–6015.
[31] W. Porcal, P. Hernández, L. Boiani, M. Boiani, A. Ferreira, A. Chidichimo, J.J. Cazzulo, C. Olea-Azar, M. González, H. Cerecetto, Bioorg. Med. Chem. 16 (2008) 6995–7004.
[32] J. Berman, Curr. Opin. Infect. Dis. 16 (2003) 397–401.
[33] P.J. Guerin, P. Olliaro, S. Sundar, M. Boelaert, S.L. Croft, P. Desjeux, K. Wasunna, A.D.M. Bryceson, Lancet Infect. Dis. 2 (2002) 494–501.
[34] E. Rosenthal, P. Marty, J. Postgrad. Med. 49 (2003) 61–68.
[35] A. Gerpe, G. Aguirre, L. Boiani, H. Cerecetto, M. González, C. Olea-Azar, C. Rigol, J.D. Maya, A. Morello, O.E. Piro, V.J. Arán, A. Azqueta, A. López de Ceráin, A. Monge, A. Rojas, G. Yaluff, Bioorg. Med. Chem. 14 (2006) 3467–3480.
[36] M. Boiani, L. Boiani, A. Denicola, S. Torres de Ortiz, E. Serna, N. Vera de Bilbao, L. Sanabria, G. Yaluff, H. Nakayama, A. Rojas de Arias, C. Vega, M. Rolan, A. Gómez-Barrio, H. Cerecetto, M. Gonzalez, J. Med. Chem. 49 (2006) 3215–3220.
[37] L. Boiani, G. Aguirre, M. González, H. Cerecetto, A. Chidichimo, J.J. Cazzulo, M. Bertinaria, S. Guglielmo, Bioorg. Med. Chem. 16 (2008) 7900–7907.
[38] M. Boiani, L. Boiani, A. Merlino, P. Hernández, A. Chidichimo, J.J. Cazzulo, H. Cerecetto, M. González, Eur. J. Med. Chem. 44 (2009) 4426–4433.
[39] L. Boiani, A. Gerpe, V.J. Arán, S. Torres de Ortiz, E. Serna, N. Vera de Bilbao, L. Sanabria, G. Yaluff, H. Nakayama, A. Rojas de Arias, J.D. Maya, J.A. Morello, H. Cerecetto, M. González, Eur. J. Med. Chem. 44 (2009) 1034–1040.
[40] C. Gil, E. Brase, Chem. Eur. J. 11 (2005) 2680–2688.
[41] A.B. Charette, A.A. Boezio, M.K. Janes, Org. Lett. 2 (2002) 3777–3779.
[42] V. Alexanian, M.J. Haddadin, C.H. Issidorides, M.Z. Nazer, Heterocycles 16 (1976) 391–398.
[43] M.Z. Nazer, C.H. Issidorides, M.J. Haddadin, Tetrahedron 35 (1979) 681–685.
[44] N.G. Argyropoulos, J.K. Gallos, D.N. Nicolaides, Tetrahedron 42 (1986) 3631–3636.
[45] F. Zaragoza Dorwald, Organic Synthesis on Solid-Phase, second ed. Wiley-VCH, Weinheim, 2002.
[46] M. Almeida-de-Faria, E. Freymuller, W. Colli, M.J. Alves, Exp. Parasitol. 92 (1999) 263–274.
[47] D. Sereno, J.-L. Lemesre, Parasitol. Res. 83 (1997) 401–403.
[48] A. Dutta, S. Bandyopadhyay, C. Mandal, M. Chatterjee, Parasitol. Int. 54 (2005) 119–122.
[49] J.C. Aponte, M. Verástegui, E. Málaga, M. Zimic, M. Quiliano, A.J. Vaisberg, R.H. Gilman, G.B. Hammond, J. Med. Chem. 51 (2008) 6230–6234.
[50] L. Zhai, M. Chen, J. Blom, T.G. Theander, S.B. Chirstensen, A. Kharazmi, J. Antimicrob. Chemother. 43 (1999) 793–803.
[51] M. Chen, L. Zhai, S.B. Christensen, T.G. Theander, A. Kharazmi, Antimicrob. Agents Chemother. 45 (2001) 2023–2029.
[52] F.A. Villamena, J.L. Zweier, Antimicrobial. Agents Chemother. 6 (2004) 619–629.
[53] W. Porcal, P. Hernández, M. González, A. Ferreira, C. Olea-Azar, H. Cerecetto, A. Castro, J. Med. Chem. 51 (2008) 6150–6159.
[54] M.H. Bolli, S.V. Ley, J. Chem. Soc. Perkin Trans. 1 (15) (1998) 2243–2246.