New trypanocidal hybrid compounds from the association of hydrazone moieties and benzofuroxan heterocycle

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A B S T R A C T

Hybrid compounds containing hydrazones and benzofuroxan pharmacophores were designed as potential *Trypanosoma cruzi*-enzyme inhibitors. The majority of the designed compounds was successfully synthesized and biologically evaluated displaying remarkable in vitro activity against different strains of *T. cruzi*. Unspecific cytotoxicity was evaluated using mouse macrophages, displaying isothiosemicarbazone **10** and thiosemicarbazone **12** selectivity indexes (macrophage/parasite) of 21 and 27, respectively. In addition, the mode of anti-trypanosomal action of the derivatives was investigated. Some of these derivatives were moderate inhibitors of cysteinyl active site enzymes of *T. cruzi*, cruzipain and trypanothione reductase. ESR experiments using *T. cruzi* microsomal fraction suggest that the main mechanism of action of the trypanocidal effects is the production of oxidative stress into the parasite.

Keywords:
Benzofuroxan
Trypanosoma cruzi
ESR
Cruzipain
Trypanothione reductase

1. Introduction

Chagas‘ Disease (CD) is the third largest disease burden in Latin America after malaria and schistosomiasis, all considered as the so-called neglected diseases. CD is caused by *Trypanosoma cruzi* (*T. cruzi*), a hemoflagellate protozoan (family Trypanosomatidae, order Kinetoplastida).[1,2] This parasitic disease represents a real health public problem in South America, affecting at least 15 million people with more than 25 million at the risk of infection.[3] The absence of an efficient chemotherapeutic approach to treat CD requires urgent attention.[4] The only clinically available drugs for the chemotherapy of CD are two nitroaromatic compounds, Nifurtimox (Nfx, Lampit®, Fig. 1) and Benznidazole (Bnz, Rochagan®, Fig. 1).[5,6] Both drugs have important disadvantages such as severe side effects, strain resistance, and variable efficacy.[7–11] For these reasons the development of more safe and efficient drugs against CD is urgent.[12] The mode of action for these drugs is not fully understood. However, it has been proposed in several studies that Nfx could act by generation of free radical toxic species through a redox-cycling process and Bnz by reaction with macromolecules as DNA, lipids, and proteins of the parasite, both by bioreduction of the nitro group during their metabolization.[13,14] Non-selective bioreduction of these trypanocidal drugs could be the reason of its toxic effects in the mammalian host. Other compounds have been studied as anti-*T. cruzi* drugs, for example the commercially available anti-fungal agents Ketoconazole® (Ktz, Fig. 1) and Terbinafine® (Tbf, Fig. 1), both sterol-membrane biosynthesis inhibitors.[15,16] These anti-fungal agents have been shown to have potent effects against cultures of *T. cruzi*.

The search for new compounds able to generate oxidative stress in *T. cruzi* through selective reduction by oxidoreductases unique in the parasite is an attractive target in the chemotherapy of CD.[17–21] In this sense, we investigated the capability of the *N*-oxide moiety as trypanocidal pharmacophore having the hypothesis that the *N*-oxide moiety would act as bioreducible group.[22] In previous works, we found that benzofuroxan (benzo[1,2-*c*]1,2,5-oxadiazole *N*-oxide) derivatives displayed excellent in vitro and in vivo anti-*T. cruzi* activities, 5-arylethenylbenzofuroxans and vinylsulfonylbenzofuroxans (**I** and **II**, respectively, Scheme 1) being the best ones.[23,24] Studies undertaken by our group suggested the perturbation of the mitochondrial electron chain, inhibiting parasite respiration, and that the *N*-oxide moiety is essential for the trypanocidal activity.[25] A new target recently explored for the chemotherapy of CD is cruzipain (CP).[26] CP is the major

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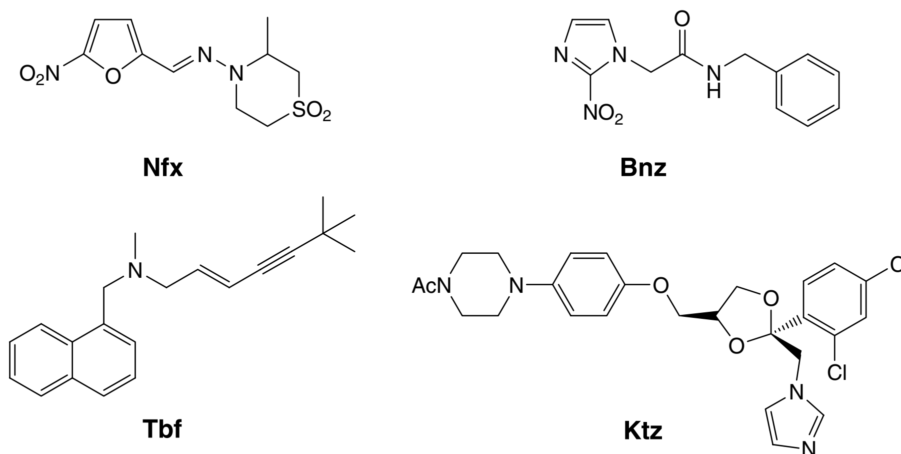
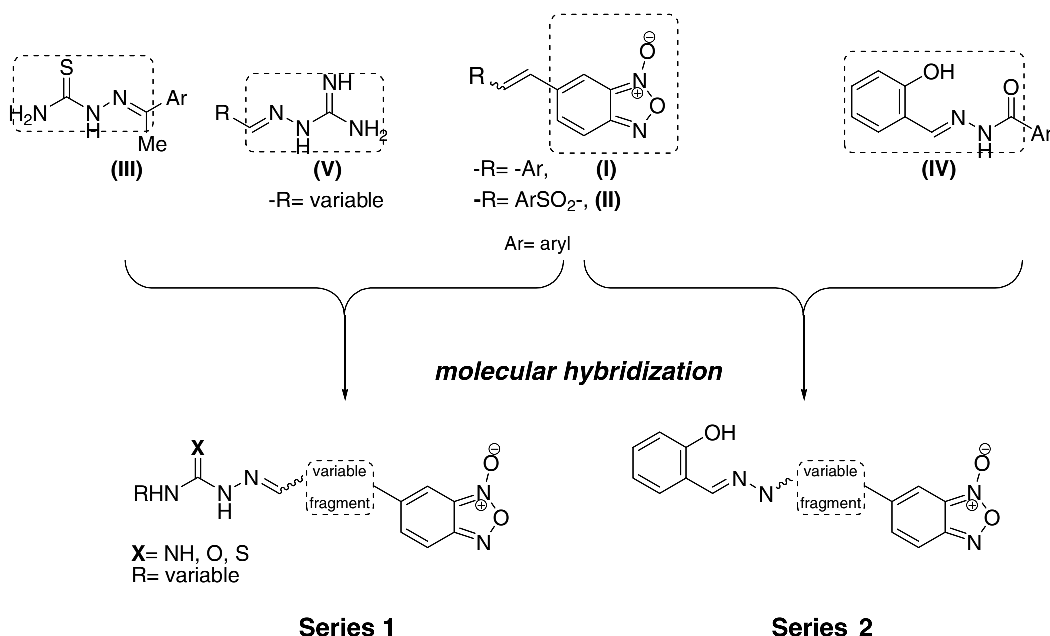


Figure 1. Drugs used clinically and experimentally as anti-*T. cruzi* agents.



Scheme 1. Design of hybrid compounds planned from the association of hydrazone moiety and trypanocidal benzofuroxan system.

lysosomal proteinase of *T. cruzi*, which plays a prominent role in nutrition of the parasite and in the host/parasite interplay.[27,28] In recent years, several families of compounds such as vinylsulfones, thioureas, and hydrazone derivatives have been reported as inhibitors of CP with in vitro and in vivo trypanocidal activity.[29–32] Particularly, thiosemicarbazone derivatives (**III**, Scheme 1) were revealed as a privileged scaffold for the selective inhibition of this enzyme showing good activity at non-cytotoxic concentrations in in vitro assays with mammalian cells.[33] Some *N*-acylhydrazones, like salicylaldehyde *N*-acylhydrazones **IV** (Scheme 1), showed inhibitory activity against the *P. falciparum* cysteine protease, falcipain, and CP.[30]

Another of the most studied biomolecules as key targets in *T. cruzi* is trypanothione reductase (TR).[34] All trypanosomatids have a unique thiol metabolism in which the ubiquitous glutathione reductase (GR: GSSG + NADPH + H^+ rightarrow 2 GSH + $NADP^+$) is replaced by TR. TR is an NADPH-dependent flavoenzyme responsible for maintaining the reducing intracellular milieu and thus protecting the parasite against oxidative stressors. Trypanothione—N^1, N^8-bis(glutathionyl)spermidine—is a low molecular weight thiol exclusively found in parasitic protozoa of the order Kinetoplastida. The absence of trypanothione in the mammalian host together with the sensitivity of trypanosomatids to oxidative stress renders the enzymes of this parasite-specific thiol metabolism attractive as drug target molecules. A large number of TR inhibitors have been studied, identifying some that bind to TR reversibly, some that act as subversive substrates, and also some that are irreversible inhibitors. The first subversive substrates of TR described were amidinohydrazone containing nitrofuranes and naphthoquinones like **V** (Scheme 1) synthesized by Henderson.[35] Subversive substrates are reduced by the flavoenzyme in a single-electron step to the respective radical which then reacts with molecular oxygen to yield superoxide anion radicals.

In our effort to develop selective and novel trypanocidal compounds, we decided to proceed with a molecular hybridization strategy. Thus, we combined the hydrazone pharmacophore fragment with the benzofuroxan system aiming to produce novel templates for the generation of compounds with anti-chagasic activity

Scheme 2. Synthesis of thiosemicarbazone-benzofuroxan hybrid derivatives **2–4**.

(Series 1 and 2, Scheme 1). The developed derivatives were examined for anti-proliferative in vitro activity against two stocks of *T. cruzi*, the Tulahuen 2 strain and the CL Brener clone, for the unspecific cytotoxicity against J774 mouse macrophages, and the inhibition of CP and TR. Studies of the capacity of intraparasite-free radical production, through ESR spectroscopy, were also performed.

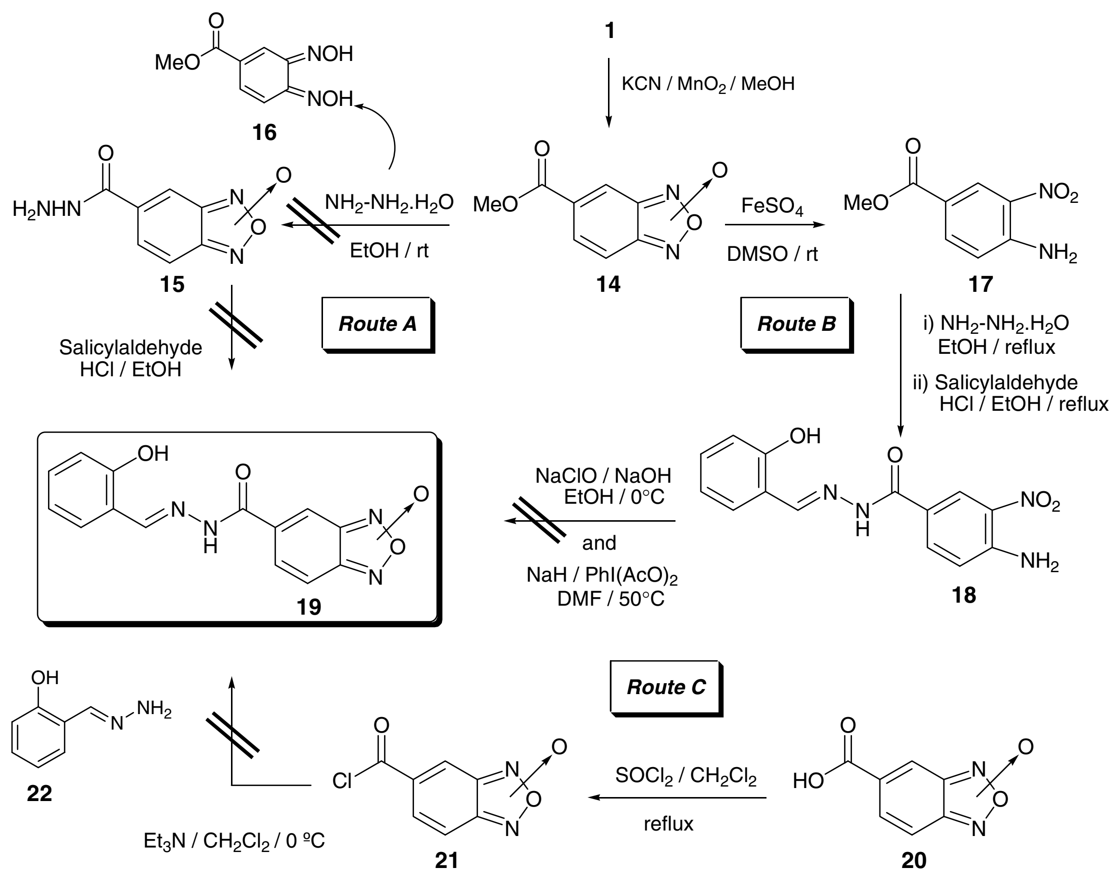
2. Methods and results

2.1. Chemistry

Thiosemicarbazones belonging to Series 1 (Scheme 1) possessing different lateral chains (hydrogen, allyl, and phenyl), **2–4** (Scheme 2), were synthesized by traditional condensation of commercially available thiosemicarbazides with 5-formylbenzofuroxan **1** (Scheme 2). Aldehyde **1** was obtained as previously reported from 4-chloro-3-nitrobenzaldehyde in a one-pot process, via S_NAr with azide and subsequent cyclization in DMSO as a result of the pyrolysis of the *o*-nitrophenylazide intermediate.[36]

Another designed thiosemicarbazone involved a phenoxy-spacer between the heterocyclic system and the hydrazone moiety (derivative **12**, Scheme 3). Together with derivative **12** the semicarbazone and amidinohydrazone analogues, **11** and **13**, respectively, were designed and nucleophilic displacements of bromine, in derivative **8**, by the phenols **5–7** (Scheme 3) were assayed. In these reaction conditions, only the semicarbazone **11** was obtained adequately, while the desired products **12** and **13** were not generated. In the reaction with thiosemicarbazone containing phenol **5** the isothiosemicarbazone **10** was obtained as main product as a result of the better nucleophilic capability of the sulfur atom. The chemical structure of this product was established using NMR (^{1}H-, ^{13}C-), NOE-diff experiments, and two-dimensional experiments such as HMQC and HMBC. To obtain the desired products, **12** and **13**, the order of reactions was changed (Scheme 3). Consequently, the thiosemicarbazone **12** and amidinohydrazone **13** were obtained in a two-step process from condensation of thiosemicarbazide and aminoguanidine with aldehyde **9**, which was synthesized by reaction of the corresponding bromide derivative **8** with

Scheme 3. Synthesis of semicarbazone-, thiosemicarbazone-, and amidinohydrazone-benzofuroxan hybrid derivatives.



Scheme 4. Attempts to obtain *N*-acylhydrazone-benzofuroxan hybrid derivative **19**.

the phenolate carbanion, *p*-hydroxybenzaldehyde, generated in basic medium.

For the preparation of the *N*-acylhydrazone **19**, belonging to Series 2 (Scheme 1), several synthetic routes were attempted (Routes A–C, Scheme 4). In the synthetic route A (Scheme 4) it was planned to prepare the *N*-acylhydrazone derivative via the condensation between hydrazide and aldehyde. However, when methyl ester **14**, prepared from aldehyde **1** (Scheme 2), was treated with hydrazine to obtain the intermediate hydrazide **15** the ring-opening *o*-benzoquinone dioxime **16** was isolated as the main reaction product (Scheme 4). This dioxime could be the result of the reductive capability of the hydrazine reactant.[37] We proposed as an alternative synthetic process the synthesis of the *N*-acylhydrazone moiety previous to the cyclization step to benzofuroxan system (Route B). So, the *N*-acylhydrazone **18** was obtained by substitution/condensation from 3-amino-4-nitrobenzoic acid methyl ester, which was synthesized by reduction of the benzofuroxan **14**. After that, some attempts to obtain *N*-acylhydrazone **19** by oxidative cyclization, NaOCl or $PhI(Ac_2)O$, were unsuccessful. Finally, as the third approach Route C (Scheme 4) was assayed. In this procedure acyl chloride **21** was reacted with hydrazone **22** yielding a mixture of products, where the desired one was not identified. Currently, we are working in other synthetic strategies to generate the desired *N*-acylhydrazone-benzofuroxan hybrids.

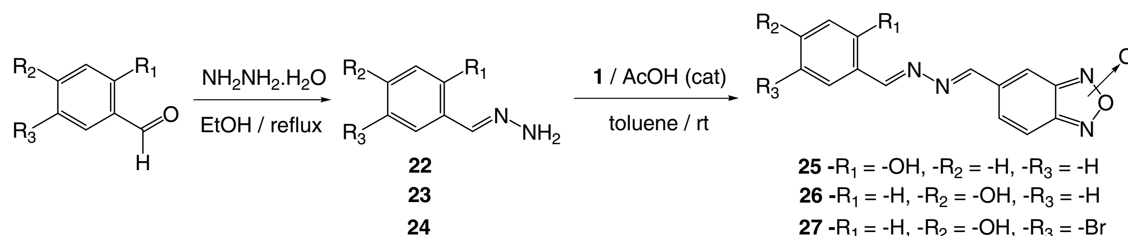
Unsymmetrical-substituted aldazines **25–27** (Scheme 5), belonging to Series 2, were synthesized by condensation of aldehyde **1** and hydrazone derivatives **22**–**24** in moderate to good yields.

All of the proposed structures were established by NMR (^1H, ^{13}C, HMQC, and HMBC experiments), IR, and MS. The purity was established by TLC and microanalysis. ^1H and ^{13}C NMR spectra of the benzofuroxans showed broad signals at room temperature due to the rapid tautomeric equilibrium.[23a,25c,38] This phenomenon is observed at room temperature, but is well resolved at low temperature.[23a] The tautomerism in benzofuroxan derivatives depends on solvent, temperature, and both nature and position of substituents.

2.2. Biology

2.2.1. In vitro anti-*T. cruzi* activity

All hybrid derivatives, and some intermediates, were initially tested in vitro against the epimastigote form of *T. cruzi*, Tulahuen 2 strain.[22–25] Recently, the existence of the epimastigote form of



Scheme 5. Synthesis of hydrazone-benzofuroxan hybrid derivatives **25**–**27**.

Table 1
In vitro anti-*T. cruzi* activity of new benzofuroxan derivatives, Nfx, Bnz, Ktz, and Tbf against Tulahuen 2 strain

Derivative	PGI[a]	IC_{50} (μM)[b,c]
2	5	≫25.0
3	35	>25.0
4	93	8.9
9	98	8.7
10	100	6.8
11	25	>25.0
12	70	15.0
13	40	>25.0
25	17	≫25.0
26	52	~25.0
27	48	~25.0
Nfx	100	7.7
Bnz	100	7.4
Ktz	100	10.0
Tbf	100	17.1

[a] PGI: percentage of growth inhibition at 25 μM.
[b] IC_{50}: concentration that produces 50% inhibitory effect.
[c] The results are the means of three independent experiments with a SD less than 10% in all cases.

T. cruzi as an obligate mammalian intracellular stage has been revisited and confirmed.[39] The compounds were incorporated into the media at 25 μM and its ability to inhibit parasite growth was evaluated in comparison to the control (no drug added to the media) (Table 1). Nfx, Bnz, Ktz, and Tbf were used as the reference trypanocidal drugs. Besides, the IC_{50} concentrations (50% inhibitory dose) were determined for the most active derivatives (Table 1). Thiosemicarbazones **4** and **12**, aldehyde **9** and isothiosemicarbazone **10** were the most active derivatives with IC_{50} between 6.8 and 15.0 μM, all of them with IC_{50} values similar to those of the reference drugs (IC_{50} between 7.4 and 17.1 μM). Unsubstituted or allyl-substituted-thiosemicarbazones **2** and **3** were poor parasite growth inhibitors. However, derivative **4**, the phenyl-substituted thiosemicarbazone, presented excellent activity showing the relevance of the aromatic moiety in the anti-parasite activity. The semicarbazone **11** and amidinohydrazone **13** exhibited poor activity compares to thiosemicarbazone **12**, indicating in this case the relevance of the thiocarbonyl moiety. This fact could be explained in terms of our previous results, where the hydrophilicity and the presence of a hydrogen-bond-donor moiety in the benzofuroxan lateral chain decrease the activity.[23a] On the other hand, aldazines **25**–**27** were less active against *T. cruzi* than the reference drugs. The hybrid designed derivatives phenylthiosemicarbazone **4**, thiosemicarbazone **12**, and amidinohydrazone **13**, with high, moderate and poor trypanocidal activity, respectively, against the Tulahuen 2 strain were selected to study against the CL Brener clone (Table 2). According to these results, it was not possible to observe differential susceptibilities in the two studied strains, the biological behavior of the selected benzofuroxans being similar in both parasite populations.

Table 2
In vitro anti-*T. cruzi* activity of selected new benzofuroxan derivatives, Nfx, Bnz, Ktz, and Tbf against CL Brener

Derivative	IC_{50} (μM)[a,b]
4	2.6
12	19.8
13	38.2
Nfx	8.5
Bnz	4.5
Ktz	5.0
Tbf	42.0

[a] IC_{50}: concentration that produces 50% inhibitory effect.
[b] The results are the means of three independent experiments with a SD less than 10% in all cases.

2.2.2. Unspecific cytotoxicity

Mammal cytotoxicity of the most effective compounds was studied in vitro using J-774 mouse macrophages as the cellular model with doses (50–400 μM) at least two times higher than the doses used for *T. cruzi* (25 μM) (Table 3).[23b] Ktz and Tbf were included in the study as trypanocidal references. The thiosemicarbazone **4** and amidinohydrazone **3** were the most toxic of the studied derivatives. The cytotoxicity of these two derivatives against macrophages is comparable to that observed for the reference drug Ktz. According to the toxic effects against *T. cruzi* amidinohydrazone **13** and azine **27** were the least selective derivatives with selectivity indexes (SI) lower than 3.4 (Table 3). Aldehyde **9** has a similar selectivity to that observed for Tbf, while derivatives **10** and **12** were the least toxic against mammal and the most selective among the studied compounds with SI, for Tulahuen 2 strain, higher than 20.9.

2.3. Mechanism of action studies

In order to confirm or discard some possible mechanisms of action the following studies were performed with the hybrid derivatives: inhibition of CP, inhibition of TR, and capability of the developed derivatives to produce intra-parasite free radicals.

2.3.1. Inhibition of cruzipain

Some of the developed benzofuroxans were tested as possible inhibitors of *T. cruzi* CP, following a previously described procedure.[24] The assayed derivatives displayed variable capability of CP inhibition in the assay conditions used (Table 4). They showed IC_{50} values between 32 and 100 μM, except for the semicarbazone **11** and the thiosemicarbazones **2** and **3**, where almost complete lack of inhibition was observed in the studied doses. Thiosemicarbazone **12** and amidinohydrazone **13**, with IC_{50} of 43 and 32 μM, respectively, emerge as CP-inhibitor lead structures for future chemical optimization through SAR investigation. Clearly, some structural exigencies for CP-inhibition could be inferred with this kind of derivatives. On the one hand, as it has been previously described, thiosemicarbazone moiety-containing derivatives are better inhibitors than semicarbazone analogues (compare the inhibitions by derivatives **11** and **12**).[33] In this sense, we identified, to our knowledge for the first time, the amidinohydrazone moiety as the best thiosemicarbazone analogue CP-inhibitor. On the other hand, analyzing azine derivatives **25–27** it was observed, as previously described,[30] that the *o*-hydroxyl motive is preferential to the *p*-hydroxyl one for CP inhibition (compare *ortho*-derivatives **25** and **27** to *para*-hydroxyl **26**). Comparing these results with the growth

Table 3
Cytotoxicity of benzofuroxan derivatives against J-774 mouse macrophages

Derivative	$IC_{50,macrophage}$[a]	SI[b,c]
4	<50.0	<5.6
9	60.0	6.9
10	142.0	20.9
12	400.0	26.7
13	<50.0	<2.0
27	84.0	<3.4
Ktz	<50.0	<5.0
Tbf	88.0	5.1

[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 = $IC_{50,macrophage}/IC_{50,T2,epimastigote}$.

Table 4
Inhibition of CP and TR activities of some selected hybrid compounds

Derivative	Findings in enzymatic studies	
	CP, IC_{50} (μM)[a]	% of TR inhibition (doses, μM)[a]
2	>100[b]	19 (100) 9 (40)
3	>100[c]	9 (100) 13 (40)
4	78	0 (100) 0 (20)[d]
11	>100[e]	0 (100) 0 (20)[f]
12	43	0 (100) 0 (20)[f]
13	32	50 (100) 30 (40)
25	68	–[g]
26	100	–
27	73	–

[a] The results are the means of two independent experiments with a SD less than 10% in all cases.
[b] 7% of inhibition at 100 μM.
[c] 36% of inhibition at 100 μM.
[d] At ⩾40 μM the compound starts to precipitate in the assayed buffer.
[e] 23% of inhibition at 100 μM.
[f] At ⩾20 μM the compound starts to precipitate in the assayed buffer.
[g] '–', not determined.

inhibition properties of benzofuroxans, it could be proposed that the main mode of action of these compounds is not the inhibition of CP.

2.3.2. Inhibition of *T. cruzi* trypanothione reductase

Hybrid derivatives **2**–**4** and **11**–**12** were studied as reversible inhibitors of *T. cruzi* TR, following a previously described procedure.[24] The compounds resulted poor TR inhibitors at the doses tested (Table 4). Some problems with the solubility in the assay buffer of thiosemicarbazones **4** and **12** and semicarbazone **11** probably promoted the lack of enzymatic inhibition capability. Amidinohydrazone **13** showed the best TR-inhibition capability, with a IC_{50} of 100 μM. As it was previously reported,[35,40] the amidinohydrazone moiety could interact positively in the enzymatic site. Comparing these results with the growth inhibition properties of benzofuroxans, it could be proposed that the main mode of action of these compounds is not the inhibition of TR.

2.4. Free radical generation: ESR studies

In order to study the benzofuroxan's intraparasite-free-radical producer ability we selected hybrid compounds, thiosemicarbazones **4** and **12**. They were selected due to their *T. cruzi* growth inhibition capability and their lower CP and TR inhibition properties. For this purpose we used ESR spectroscopy and spin-trapping tools.[41] According to these experiments both thiosemicarbazones were able to produce free radicals in presence of *T. cruzi*-microsomal fraction. The ESR spectra showed a hyperfine pattern consistent with the *N*-oxide free radical DMPO-trapped (Fig. 2).[25b] Additionally, the most potent anti-*T. cruzi* hybrid derivative, **4**, also showed in the ESR spectra the characteristic signals of DMPO-hydroxyl radical spin adduct (marked with * in Fig. 2, $a_N = a_H = 15.2$ G). This could confirm that this compound was able to produce oxidative stress into the parasite. In the case of the least potent derivative **12**, the presence of hydroxyl radical was not clearly observed.

3. Conclusions

Our results showed that the novel hybrid compounds, like **12**, have relevant selective trypanocidal activities. The biological studies indicated that these derivatives could act mainly by production of free radical species through the parasite metabolism identifying both *N*-oxide and hydroxyl free radical as biological intermediates. On the other hand, the observed inhibition of cysteinyl active site enzymes of *T. cruzi*, like CP and TP, by these compounds was negligible, concluding that these biochemical pathways are not involved in the mode of action of these hybrid compounds. Furthermore, derivative **10**, obtained as a secondary product, showed excellent biological profile. Deep studies on this non-designed derivative are being performed. In vivo studies to investigate the ability of these drugs to decrease the parasitemia of infected mice are currently underway.

4. Experimental

Compounds **1**,[36] **8**,[25c] **21**[22a], and **22**–**24**[42] were prepared according to literature procedures. Melting points were determined with an electrothermal melting point apparatus (Electrothermal 9100) and were uncorrected. Proton and carbon NMR spectra were recorded on a Bruker DPX-400 spectrometer. The chemical shift values are expressed in ppm relative to tetramethylsilane as internal standard. In ^{13}C NMR 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 modula-

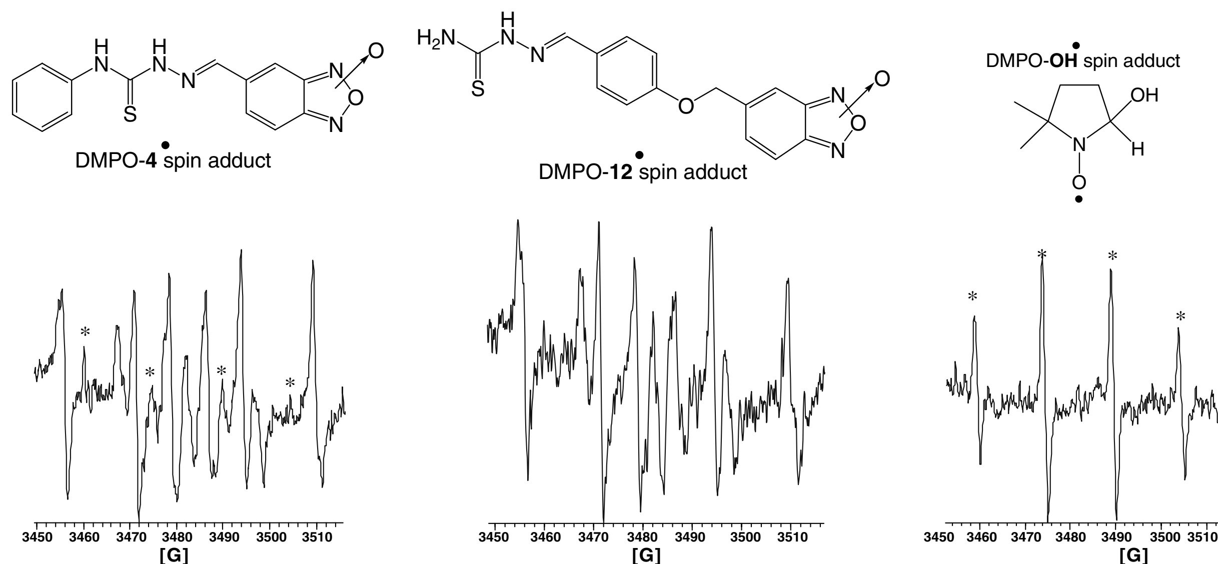


Figure 2. ESR spectra. *Left and centre*: DMPO-benzofuroxan spin adduct obtained with *T. cruzi* microsomal fraction. *Right*: pattern of DMPO-hydroxyl radical spin adduct.

tion. 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 and the frequencies were expressed in cm^{-1}. Elemental analyses were carried out 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 of analytical grade and used without further purification. All the reactions were carried out in a nitrogen atmosphere.

4.1. General procedure for the synthesis of thiosemicarbazone-benzofuroxan hybrid derivatives (2–4)

A mixture of **1** (1.0 equiv), the corresponding thiosemicarbazide (1.0 equiv) and AcOH (catalytic amounts) in dry toluene (12 mL/mmol) as solvent was stirred at room temperature until the carbonyl compound was not present (checked by TLC). The solid product was collected and washed with toluene, Et_2O and purified as it is indicated.

4.2. Benzofuroxan-5-carboxaldehyde thiosemicarbazone (2)

Purified by crystallization from EtOH; yellow solid (92%); mp 205.0–206.0 °C. ^{1}H NMR ($CDCl_3$) δ: 7.86–7.71 (br s, 2H), 8.09 (s, 1H), 8.26 (br s, 1H), 8.30 (s, 1H), 8.37 (s, 1H), 11.72 (s, 1H). ^{13}C NMR (DMSO-d_6) δ: 179.4 (C=S), 140.3 (CH=N). EI-MS, *m/z* (abundance,%): 237 ($M^{+\cdot}$, 5), 221 (15), 185 (45), 105 (100). Anal. Calcd for ($C_8H_7N_5O_2S$): C, 40.50; H, 2.97; N, 29.52. Found: C, 40.37; H, 2.88; N, 29.35.

4.3. *N*⁴-Allyl benzofuroxan-5-carboxaldehyde thiosemicarbazone (3)

Purified by crystallization from petroleum ether/EtOAc (8:2); yellow solid (50%); mp 172.0–173.0 °C. ^{1}H NMR (acetone-d_6) δ: 4.38 (m, 2H), 5.13 (d, 1H, *J* = 10.3 Hz), 5.25 (d, 1H, *J* = 17.2 Hz), 6.02 (m, 1H), 7.74–7.62 (br s, 2H), 8.13 (br s, 1H), 8.26 (s, 1H), 8.64 (br s, 1H), 10.76 (br s, 1H). ^{13}C NMR (acetone-d_6) δ: 179.0 (C=S), 139.5 (CH=N), 134.8 (CH_2–*C*H=CH_2), 115.7 (CH_2–CH=*C*H_2), 46.7 (*C*H_2–CH=CH_2). EI-MS, *m/z* (abundance, %): 277 ($M^{+\cdot}$, 8), 259 (10), 231 (55), 185 (36), 148 (43), 115 (100), 105 (89). Anal. Calcd for ($C_{11}H_{11}N_5O_2S$): C, 47.64; H, 4.00; N, 25.26. Found: C, 47.40; H, 3.89; N, 25.01.

4.4. *N*⁴-Phenyl benzofuroxan-5-carboxaldehyde thiosemicarbazone (4)

Purified by crystallization from petroleum ether/EtOAc (8:2); yellow solid (63%); mp 166.0–167.0 °C. ^{1}H NMR (acetone-d_6) δ: 7.25 (t, 1H, *J* = 7.7 Hz), 7.40 (t, 2H, *J* = 7.7 Hz), 7.64 (br s, 1H), 7.75 (d, 2H, *J* = 7.7 Hz), 7.82 (br s, 1H), 8.27 (br s, 1H), 8.35 (s, 1H), 10.03 (br s, 1H), 10.98 (br s, 1H). ^{13}C NMR (acetone-d_6) δ: 177.4 (C=S), 140.1 (CH=N), 139.9 (C1), 128.4 (C3), 125.9 (C2), 125.4 (C4). EI-MS, *m/z* (abundance,%): 313 ($M^{+\cdot}$, 18), 295 (41), 231 (30), 151 (50), 93 (100). Anal. Calcd for ($C_{14}H_{11}N_5O_2S$): C, 53.66; H, 3.54; N, 22.35. Found: C, 53.39; H, 3.30; N, 22.21.

4.5. 4-Hydroxybenzaldehyde thiosemicarbazone (5)[43]

A mixture of *p*-hydroxybenzaldehyde (300 mg, 2.46 mmol), thiosemicarbazide (220 mg, 2.46 mmol), and *p*-TsOH (catalytic amounts) in dry toluene (5.0 mL) as solvent was stirred at room temperature for 24 h. The precipitated solid was collected and washed with toluene and was used without further purification. Brown solid, 420 mg (88%); ^{1}H NMR (acetone-d_6) δ: 6.91 (d, 2H,*J* = 9.4 Hz), 7.32 (br s, 1H), 7.67 (d, 2H,*J* = 9.4 Hz), 7.80 (br s, 1H), 8.10 (s, 1H), 10.25 (br s, 1H). ESI-MS, *m/z*: 196 ($M^{+\cdot}$+H).

4.6. 4-Hydroxybenzaldehyde semicarbazone (6)[44]

To a mixture of *p*-hydroxybenzaldehyde (300 mg, 2.46 mmol) in EtOH (5.0 mL), a solution of semicarbazide chlorhydrate (274 mg, 2.46 mmol) and NaOAc (200 mg, 2.45 mmol) in water (2 mL) was added. The mixture was stirred at room temperature for 24 h. The precipitated solid was collected and washed with toluene, and was used without further purification. Pale yellow solid, 320 mg (73%); ^{1}H NMR (acetone-d_6) δ: 6.16 (br s, 2H), 6.89 (d, 2H, *J* = 8.7 Hz), 7.67 (d, 2H,*J* = 8.8 Hz), 7.88 (s, 1H), 8.76 (br s, 1H), 9.55 (br s, 1H). ESI-MS, *m/z*: 180 ($M^{+\cdot}$+H).

4.7. 4-Hydroxybenzaldehyde amidinohydrazone (7)[45]

A solution of *p*-hydroxybenzaldehyde (300 mg, 2.46 mmol) in ethanol (7.0 mL) was treated with an aqueous solution of aminoguanidine bicarbonate (30–40%) (430 mg, 3.20 mmol) and with catalytic amounts of HCl (c). The reaction mixture was heated at reflux for 4 h. The solvent was evaporated in vacuo and the residue was neutralized with aqueous saturated $NaHCO_3$ and extracted with EtOAc (3 $\times$ 20 mL). After the work-up the organic layer was evaporated in vacuo and the residue corresponding to the product, purified by TLC, was used without further purification. Pale yellow solid, 200 mg (50%); ^{1}H NMR (CD_3OD:D_2O) δ: 6.78 (d, 2H,*J* = 8.6 Hz), 7.53 (d, 2H,*J* = 8.6 Hz), 7.96 (s, 1H). ESI-MS, *m/z*: 179 ($M^{+\cdot}$+H).

4.8. 5-[(4-Formylfenoxy)methyl]benzofuroxan (9)

A mixture of **8** (1.0 g, 4.4 mmol), *p*-hydroxybenzaldehyde (530 mg, 4.4 mmol), K_2CO_3 (600 mg, 4.4 mmol), KI (30 mg, 0.022 mmol), and 18-crown-6 (10 mg, 0.044 mmol) in acetone (50.0 mL) was stirred at room temperature for 4 h. The organic solvent was evaporated in vacuo and the residue was treated with aqueous NaOH 20% (50.0 mL) and extracted with EtOAc (3 $\times$ 40.0 mL). The organic layer was washed with saturated sodium chloride solution (40.0 mL) and dried with Na_2SO_4. Then the organic solvent was evaporated in vacuo and the residue purified by column chromatography (SiO_2, petroleum ether/EtOAc (8:2)), yielding derivative **9** as a pale yellow solid (1.1 g, 91%); mp 136.0–138.0 °C. ^{1}H NMR ($CDCl_3$) δ: 5.18 (s, 2H), 7.13 (d, 2H, *J* = 8.7 Hz), 7.32 (br s, 1H), 7.57 (br s, 2H), 7.91 (d, 2H, *J* = 8.7 Hz), 9.93 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 183.7 (C=O), 162.8 (C4), 129.1 (C2), 128.9 (C1), 116.4 (C3), 69.2 (CH_2). EI-MS, *m/z* (abundance,%): 270 ($M^{+\cdot}$, 5), 254 (13), 149 (34), 133 (100). IR (KBr) ν: 3045, 2870, 1681, 1633, 1600, 1537, 1488, 1373, 1257, 1164, 1112, 1047, 1016, 856, 796, 671, 648, 572. Anal. Calcd for ($C_{14}H_{10}N_2O_4$): C, 62.22; H, 3.73; N, 10.37. Found: C, 62.03; H, 3.56; N, 10.12.

4.9. 1-(4-Hydroxyphenylmethylidene)-*S*-(benzofuroxan-5-ylmethyl)isothiosemicarbazone (10)

A mixture of **5** (170 mg, 0.87 mmol), **8** (200 mg, 0.87 mmol), K_2CO_3 (120 mg, 0.87 mmol), KI (8 mg, 0.022 mmol), and 18-crown-6 (2 mg, 0.044 mmol) in acetone (10.0 mL) was stirred at room temperature for 2 h. The organic solvent was evaporated in vacuo and the residue purified by column chromatography (SiO_2, petroleum ether/EtOAc (7:3, 1:1)). Derivative **10** was obtained as a reddish oil that crystallized at 4 °C (80 mg, 27%); ^{1}H NMR ($CDCl_3$) δ: 4.34 (s, 2H), 5.44 (br s, 2H), 6.88 (d, 2H, *J* = 8.6 Hz), 7.40 (br s, 3H), 7.64 (d, 2H, *J* = 8.6 Hz), 8.32 (s, 1H). ^{13}C NMR (acetone-d_6) δ: 159.6 (C4), 159.3 (S—C=NH), 153.7 (N—C=NH), 129.7 (C2), 127.6 (C1),

115.8 (C3), 32.8 (CH_2). EI-MS, *m/z* (abundance,%): 343 ($M^{+\cdot}$, 5), 325 (20), 308 (15), 133 (75), 120 (100). IR (KBr) ν: 3000–2500, 1701, 1655, 1600, 1520, 1232, 1167, 1105, 1010, 879, 839, 748, 526. Anal. Calcd for ($C_{15}H_{13}N_5O_3S$): C, 52.47; H, 3.82; N, 20.40. Found: C, 52.24; H, 3.60; N, 20.24.

4.10. 4-(Benzofuroxan-5-ylmethyloxy)benzaldehyde semicarbazone (11)

A mixture of **6** (195 mg, 1.10 mmol), **8** (250 mg, 1.10 mmol), K_2CO_3 (151 mg, 1.10 mmol), KI (9 mg, 0.06 mmol), and 18-crown-6 (3 mg, 0.01 mmol) in acetone (10.0 mL) was stirred at room temperature for 4 h. The precipitated solid was collected and purified by crystallization from EtOH. Pale yellow solid 50 mg (42%); mp 218.0–219.0 °C. ^1H NMR (DMSO-d_6) δ: 5.23 (s, 2H), 6.40 (br s, 2H), 7.09 (d, 2H, *J* = 8.8 Hz), 7.49 (br s, 1H), 7.69 (d, 2H, *J* = 8.8 Hz), 7.70–7.78 (br s, 2H), 7.79 (s, 1H), 10.08 (s, 1H). ^{13}C NMR (DMSO-d_6) δ: 159.3 (C4), 157.6 (C=O), 139.8 (C=NH), 129.0 (C2), 128.9 (C1), 115.8 (C3), 68.9 (CH_2). EI-MS, *m/z* (abundance,%): 327 ($M^{+\cdot}$, 1), 311 (6), 278 (8), 238 (11), 133 (100). IR (KBr) ν: 3476, 3273, 2926, 1700, 1655, 1541, 1369, 1259, 1170, 1059, 1014, 946, 854, 789, 553. Anal. Calcd for ($C_{15}H_{13}N_5O_4$): C, 55.05; H, 4.00; N, 21.40. Found: C, 54.88; H, 3.89; N, 21.29.

4.11. 4-(Benzofuroxan-5-ylmethyloxy)benzaldehyde thiosemicarbazone (12)

A mixture of aldehyde **9** (150 mg, 0.56 mmol), thiosemicarbazide (50 mg, 0.56 mmol), and *p*-TsOH (catalytic amounts) in dry toluene (10 mL/mmol) as solvent was stirred at room temperature until the carbonyl compound was not present (checked by TLC). The solid product was collected, washed with EtOH, acetone and purified by crystallization from EtOH. Yellow pale solid 72 mg (38%); mp 207.0–209.0 °C. ^1H NMR (DMSO-d_6) δ: 5.25 (s, 2H), 7.10 (d, 2H, *J* = 8.8 Hz), 7.49 (br s, 1H), 7.77 (br s, 2H), 7.78 (d, 2H, *J* = 8.8 Hz), 7.90 (br s, 1H), 8.01 (s, 1H), 8.08 (br s, 1H), 11.30 (s, 1H). ^{13}C NMR (DMSO-d_6) δ: 178.6 (C=S), 160.0 (C4), 142.8 (C=NH), 129.8 (C2), 128.3 (C1), 115.9 (C3), 68.9 (CH_2). EI-MS, *m/z* (abundance,%): 341 ($M^{+\cdot}$−2, 2), 269 (3), 254 (11),149 (16), 133 (100). IR (KBr) ν: 3280, 2926, 1727, 1645, 1530, 1334, 1267, 1145, 1059, 1015, 956, 8576, 784, 570. Anal. Calcd for ($C_{15}H_{13}N_5O_3S$): C, 52.47; H, 3.82; N, 20.40. Found: C, 52.21; H, 3.72; N, 20.19.

4.12. 4-(Benzofuroxan-5-ylmethyloxy)benzaldehyde amidinohydrazone (13)

A mixture of **9** (100 mg, 0.37 mmol), aminoguanidine bicarbonate (50 mg, 0.37 mmol), NaOAc (61 mg, 0.74 mmol) and catalytic amount of HCl (c) in EtOH (3.0 mL) was heated at reflux for 6 h. Then the mixture was allowed to cool to room temperature, and the precipitated solid was collected and crystallized from EtOH. Pale yellow solid 90 mg (75%); mp 215.0–217.0 °C. ^1H NMR (DMSO-d_6) δ: 5.27 (s, 2H), 7.15 (d, 2H, *J* = 8.9 Hz), 7.4–7.8 (br s, 6H), 7.84 (d, 2H, *J* = 8.8 Hz), 8.12 (s, 1H), 11.67 (br s, 1H). ^{13}C NMR (DMSO-d_6) δ: 160.4 (C4), 156.3 (N—C=NH), 147.2 (C=NH), 130.1 (C2), 127.7 (C1), 115.9 (C3), 69.0 (CH_2). EI-MS, *m/z* (abundance,%): 310 ($M^{+\cdot}$−16, 28), 177 (100), 133 (73). IR (KBr) ν: 3400–2800, 1650, 1610, 1590, 1456, 1385, 1307, 1250, 1047, 1010, 871, 613, 534. Anal. Calcd for ($C_{15}H_{14}N_6O_3$): C, 55.21; H, 4.32; N, 25.75. Found: C, 55.03; H, 4.12; N, 25.59.

4.13. Methyl benzofuroxan-5-carboxylate (14)

To a mixture of **1** (200 mg, 1.22 mmol) in MeOH (30.0 mL) was added KCN (90 mg, 1.34 mmol) and subsequently MnO_2 (850 mg, 9.76 mmol). The final mixture was stirred for 6 h at room temperature. The resulting dispersion was filtered through a short pad of Celite, the organic phase was concentrated in vacuo, and the residue was used in the next reaction without further purification. Brown solid (210 mg, 89%). ^1H NMR (DMSO-d_6) δ: 4.00 (s, 3H), 7.59 (br s, 1H), 7.88 (br s, 1H), 8.25 (br s, 1H). EI-MS, *m/z* (abundance,%): 194 ($M^{+\cdot}$, 100), 178 (59),167 (20), 103 (59).

4.14. Methyl 4-amino-3-nitrobenzoate (17)

A mixture of **14** (370 mg, 1.89 mmol) and $FeSO_4.7H_2O$ (3.15 g, 12.0 mmol) in DMSO (10.0 mL) as solvent was stirred for 1 h at room temperature. The mixture was treated with water (50.0 mL) and extracted with EtOAc (3 × 10.0 mL). The organic layer was washed with brine (40.0 mL) and dried over Na_2SO_4. Then the organic solvent was evaporated in vacuo and the residue was used in the next reaction without further purification. Yellow solid (190 mg, 44%). ^1H NMR (DMSO-d_6) δ: 3.93 (s, 3H), 6.42 (br s, 2H), 6.86 (d, 1H, *J* = 8.7 Hz), 8.03 (dd, 1H, *J* = 8.9 Hz, *J* = 1.9 Hz), 8.87 (d, 1H, *J* = 2.0 Hz). EI-MS, *m/z* (abundance,%): 196 ($M^{+\cdot}$, 100), 165 (38),135 (34).

4.15. 4-Amino-2′-(2-hydroxybenzylidene)-3-nitrobenzodrazide (18)

A solution of the methyl ester **17** (205 mg, 1.05 mmol) and hydrazine monohydrate (55%) (2.25 mL, 72 mmol) in absolute EtOH (7.0 mL) was stirred at reflux for 4 h. The solvent was concentrated in vacuo, and the reaction mixture was diluted with water and extracted with EtOAc. The organic layer was dried over Na_2SO_4 and evaporated in vacuo. 4-Amino-3-nitrobenzohydrazide was obtained as an orange solid (190 mg, 93%) and was used in the next reaction without further purification. ^1H NMR (acetone-d_6) δ: 4.42 (s, 2H), 7.03 (d, 1H, *J* = 8.9 Hz), 7.72 (s, 2H), 7.84 (dd, 1H, *J* = 8.8 Hz, *J* = 1.8 Hz), 8.52 (d, 1H, *J* = 1.7 Hz), 9.6 (br s,1H). A solution of 2-hydroxybenzaldehyde (0.05 mL, 0.51 mmol) in absolute EtOH (2.0 mL) was added to a mixture of 4-amino-3-nitrobenzohydrazide (100 mg, 0.51 mmol) and catalytic amount of HCl (c) in absolute EtOH (10.0 mL). The final mixture was stirred at room temperature for 1 h. Next, the solvent was partially concentrated at reduced pressure and the resulting mixture was poured into cold water. After neutralization with aqueous $NaHCO_3$ (10%), the precipitate was collected and washed with petroleum ether. Orange solid (120 mg, 78%). ^1H NMR (acetone-d_6) δ: 6.94 (m, 2H), 7.12 (d, 1H, *J* = 8.9 Hz), 7.31 (m, 1H), 7.53 (d, 1H, *J* = 7.4 Hz), 7.87 (s, 2H), 7.97 (dd, 1H, *J* = 8.9 Hz, *J* = 1.9 Hz), 8.63 (s, 1H), 8.71 (d, 1H, *J* = 1.8 Hz), 11.4 (br s, 1H), 11.9 (br s, 1H). EI-MS, *m/z* (abundance,%): 300 ($M^{+\cdot}$, 19), 181 (36),165 (100), 119 (38).

4.16. General procedure for the synthesis of unsymmetrical azines (25–27)

A mixture of **1** (1.0 equiv), the corresponding hydrazone (1.0 equiv), and AcOH (catalytic amounts) in dry toluene (12 mL/mmol) as solvent was stirred at room temperature until the carbonyl compound was not present (checked by TLC). The solid product was collected and washed with toluene, Et_2O and purified by column chromatography (SiO_2, petroleum ether/EtOAc (8:2)).

4.17. 2-[(Benzofuroxan-5-yl)methylene]-1-(2-hydroxybenzylidene)hydrazine (25)

Yellow solid (37%); mp 216.0–217.0 °C. ^1H NMR (acetone-d_6) δ: 7.00 (m, 2H), 7.45 (t, 1H, *J* = 7.7 Hz), 7.76 (d, 1H, *J* = 8.8 Hz), 7.88 (br s, 1H), 8.05 (br s, 2H), 8.80 (s, 1H), 9.00 (s, 1H), 11.08 (br s, 1H). ^{13}C NMR (acetone-d_6) δ: 164.1 (CH=N), 161.4 (CH=N), 160.3 (C2),

135.3 (C4), 132.0 (C6), 121.2 (C3), 119.2 (C1), 117.6 (C5). EI-MS, *m/z* (abundance,%): 282 ($M^{+\cdot}$, 14), 266 (8), 256 (55), 213 (39), 129 (56). Anal. Calcd for ($C_{14}H_{10}N_4O_3$): C, 59.57; H, 3.57; N, 19.85. Found: C, 59.40; H, 3.41; N, 19.72.

4.18. 2-[(Benzofuroxan-5-yl)methylene]-1-(4-hydroxybenzylidene)hydrazine (26)

Yellow solid (59%); mp 219.0–220.0 °C. ^{1}H NMR (acetone-d_6) δ: 6.97 (d, 2H, J = 8.8 Hz), 7.77 (d, 2H, J = 8.8 Hz), 7.86 (br s, 1H), 8.21 (br s, 2H), 8.75 (s, 1H), 8.81 (s, 1H), 8.90 (br s, 1H). ^{13}C NMR (acetone-d_6) δ: 164.1 (CH=N), 161.4 (CH=N), 161.8 (C4), 132.3 (C2), 125.5 (C1), 119.8 (C3). EI-MS, *m/z* (abundance,%): 282 ($M^{+\cdot}$, 20), 266 (12), 256 (67), 213 (43), 129 (71). Anal. Calcd for ($C_{14}H_{10}N_4O_3$): C, 59.57; H, 3.57; N, 19.85. Found: C, 59.44; H, 3.43; N, 19.78.

4.19. 2-[(Benzofuroxan-5-yl)methylene]-1-(5-bromo-2-hydroxybenzylidene)hydrazine (27)

Yellow solid (75%); mp 201.0–202.0 °C. ^{1}H NMR (acetone-d_6) δ: 6.98 (d, 2H, J = 8.7 Hz), 7.57 (dd, 1H, J = 8.8 Hz, J = 2.5 Hz), 7.85 (br s, 1H), 7.95 (d, 1H, J = 2.4 Hz), 8.03 (br s, 2H), 8.86 (s, 1H), 8.93 (br s, 1H), 11.16 (br s, 1H). ^{13}C NMR (acetone-d_6) δ: 163.8 (CH=N), 162.5 (CH=N), 158.5 (C2), 137.1 (C4), 133.5 (C6), 122.1 (C1), 120.2 (C3), 118.6 (C5). EI-MS, *m/z* (abundance,%): 360/362 ($M^{+\cdot}$, 75), 345 (24), 301 (32), 200 (100). Anal. Calcd for ($C_{14}H_9BrN_4O_3$): C, 46.56; H, 2.51; N, 15.51. Found: C, 46.39; H, 2.38; N, 15.41.

4.20. In vitro anti-*T. cruzi* activity

Trypanosoma cruzi epimastigotes (Tulahuen 2 strain or CL Brener clone) were grown at 28 °C in an axenic medium (BHI–ryptose) 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.[46] 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 $A_p = A_{600}$ of the culture containing the compound at day 5; $A_{0p} = A_{600}$ of the culture containing the compound right after addition of the inocula (day 0); $A_c = A_{600}$ of the culture in the absence of any compound (control) at day 5; $A_{0c} = A_{600}$ in the absence of the compound at day 0. To determine IC_{50} values, 50% inhibitory 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%.

4.21. Cytotoxicity to mouse macrophages

J-774 mouse macrophages were seeded (100,000 cells/well) in 96-well flat bottomed microplates (Nunclon) with 200 μL of RPMI 1640 medium supplemented with 20% heat-inactivated fetal 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 (25–1000 μM) for 48 h. Afterwards, the cells were washed with PBS and incubated (37 °C) with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) 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: $\% C = [100 - (OD_d - OD_{dm})/(OD_c - OD_{cm})] \times 100$, where OD_d is the mean of OD_{595} of wells with macrophages and different concentrations of the compounds; OD_{dm} is the mean of OD_{595} of wells with different compound concentrations in the medium; OD_c is the growth control and OD_{cm} is the mean of OD_{595} of wells with medium only.

4.22. *T. cruzi* CP inhibition assays

CP was purified to homogeneity from epimastigotes of the Tulahuen 2 strain by ConA–Sepharose affinity chromatography, as previously described,[47] and its activity was assayed in a reaction mixture (1 mL) containing (final concentration) 50 mM Tris-acetate buffer, pH 8.0, 0.3 mM Bz–Pro–Phe–Arg–pNA, and 10 mM β-mercaptoethanol. Absorbance at 410 nm was followed at 30 °C in a Beckman Model 25 recording spectrophotometer. The derivatives were added as solutions in DMSO, and the controls contained the same solvent concentration. E-64 was used as a positive control of inhibition.

4.23. *T. cruzi* TR inhibition assays

Recombinant *T. cruzi* TR was prepared according to a published procedure.[48] Trypanothione disulfide was purchased from Bachem, Heidelberg, Germany. TR activity was measured spectrophotometrically at 25 °C in TR assay buffer (40 mM Hepes, 1 mM EDTA, pH 7.5) as described.[40] Stock solutions of the compounds were prepared in DMSO. The assay mixtures (1 mL) contained in TR assay buffer 100 μM NADPH and 105 or 93 μM trypanothione disulfide (TS_2) and varying concentrations of the inhibitor. NADPH, enzyme, and inhibitor were mixed and the reaction was started by adding TS_2. The absorption decrease at 340 nm due to NADPH consumption was followed. Control assays contained the respective amount of DMSO instead of inhibitor.

4.24. ESR studies

The free radical production capacity of the new benzofuroxan derivatives was assessed on *T. cruzi*-microsomal fraction (4 mg protein/mL) by Electronic Spin Resonance (ESR) using 5,5-dimethyl-1-pirroline-*N*-oxide (DMPO) for spin trapping.[25] 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, 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.

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References and notes

1. De Souza, W. *Pharm. Des.* **2002**, *4*, 269.
2. Teixeira, A. R. L.; Nitz, N.; Guimaro, M. C.; Gomes, C.; Santos-Buch, C. A. *Postgrad. Med. J.* **2006**, *82*, 788.
3. www.who.int/tdr.
4. Pink, R.; Hudson, A.; Mouriès, M. A.; Bendig, M. *Nat. Rev. Drugs Disc.* **2005**, *4*, 727.
5. Rodrigues Coura, J.; Castro, S. L. *Mem. Inst. Oswaldo Cruz* **2002**, *97*, 3.
6. Schofield, C. J.; Jannin, J.; Salvatella, R. *Trends Parasitol.* **2006**, *22*, 583.
7. Murta, S. M. F.; Gazzinelli, R. T.; Brener, Z.; Romanha, A. J. *Mol. Biochem. Parasitol.* **1998**, *93*, 203.
8. Cerecetto, H.; González, M. *Curr. Top. Med. Chem.* **2002**, *2*, 1185.
9. de Mecca, M. M.; Diaz, E. G.; Castro, J. A. *Toxicol. Lett.* **2002**, *136*, 1.
10. Kanes Hima, E. N.; Castro-Prado, M. A. *Mem. Inst. Oswaldo Cruz* **2005**, *100*, 325.
11. Castro, J. A.; de Meca, M. M.; Bartel, L. C. *Hum. Exp. Toxicol.* **2006**, *25*, 471.
12. Nwaka, S.; Ridley, R. G. *Nat. Rev. Drug. Disc.* **2003**, *2*, 919.
13. Maya, J. D.; Bollo, S.; Nuñez-Vergara, L. J.; Squella, J. A.; Repetto, Y.; Morello, A.; Perie, J.; Chauviere, G. *Biochem. Pharmacol.* **2003**, *65*, 999.
14. Maya, J. D.; Cassels, B. K.; Iturriaga-Vásquez, P.; Ferreira, J.; Faúndez, M.; Galanti, N.; Ferreira, A.; Morello, A. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **2007**, *146*, 601.
15. Lazardi, K.; Urbina, J. A.; de Souza, W. *Antimicrob. Agents Chemother.* **1990**, *34*, 2097.

16!. Maldonado, R. A.; Molina, J.; Payares, G.; Urbina, J. A. *Antimicrob. Agents Chemother.* **1993**, *37*, 1353.

17. Steenkamp, D. J. *Antioxid. Redox Signal.* **2002**, *4*, 105.
18. Kubata, B. K.; Kabututu, Z.; Nozaki, T.; Munday, C. J.; Fukuzumi, S.; Ohkubo, K.; Lazarus, M.; Maruyama, T.; Martin, S. K.; Duszenko, M.; Urade, Y. *J. Exp. Med.* **2002**, *196*, 1241.
19. Turrens, J. F. *Mol. Aspects Med.* **2004**, *25*, 211.
20. Krauth-Siegel, R. L.; Schirmer, R. H.; Bauer, H. *Angew. Chem. Int. Ed.* **2005**, *44*, 690.
21. Maya, J. D.; Morello, A. *J. Biol. Sci.* **2005**, *5*, 847.
22. (a) Cerecetto, H.; Di Maio, R.; González, M.; Risso, M.; Saenz, P.; Seoane, G.; Denicola, A.; Peluffo, G.; Quijano, C.; Olea-Azar, C. *J. Med. Chem.* **1999**, *42*, 1941; (b) Aguirre, G.; Cerecetto, H.; Di Maio, R.; González, M.; Porcal, W.; Seoane, G.; Denicola, A.; Ortega, M. A.; Aldana, I.; Monge-Vega, A. *Arch. Pharm.* **2002**, *335*, 15.
23. (a) Aguirre, G.; Boiani, L.; Boiani, M.; Cerecetto, H.; Di Maio, R.; González, M.; Porcal, W.; Denicola, A.; Piro, O. E.; Castellano, E. E.; Sant'Anna, C. M. R.; Barreiro, E. J. *Bioorg. Med. Chem.* **2005**, *13*, 6336; (b) Porcal, W.; Hernández, P.; Aguirre, G.; Boiani, L.; Boiani, M.; Merlino, A.; Ferreira, A.; Di Maio, R.; Castro, A.; González, M.; Cerecetto, H. *Bioorg. Med. Chem.* **2007**, *15*, 2768; (c) Boiani, L.; Davies, C.; Arredondo, C.; Porcal, W.; Merlino, A.; Gerpe, A.; Boiani, M.; Pacheco, J. P.; Basombrío, M. A.; González, M.; Cerecetto, H. *Eur. J. Med. Chem.* **2008**, doi:10.1016/j.ejmech.2007.12.016.
24. Porcal, W.; Hernández, P.; Boiani, M.; Aguirre, G.; Boiani, L.; Chidichimo, A.; Cazzulo, J. J.; Campillo, N. E.; Paez, J. A.; Castro, A.; Krauth-Siegel, R. L.; Davies, C.; Basombrío, M. A.; González, M.; Cerecetto, H. *J. Med. Chem.* **2007**, *50*, 6004.
25. (a) Olea-Azar, C.; Rigol, C.; Mendizabal, F.; Briones, R.; Cerecetto, H.; Di Maio, R.; González, M.; Porcal, W.; Risso, M. *Spectrochim. Acta, Part A* **2003**, *59*, 69; (b) Olea-Azar, C.; Rigol, C.; Opazo, L.; Morello, A.; Maya, J. D.; Repetto, Y.; Aguirre, G.; Cerecetto, H.; Di Maio, R.; González, M.; Porcal, W. *J. Chil. Chem. Soc.* **2003**, *48*, 77; (c) Olea-Azar, C.; Rigol; Mendizábal, F.; Cerecetto, H.; Di Maio, R.; González, M.; Porcal, W.; Morello, A.; Repetto, Y.; Maya, J. D. *Lett. Drug Des. Dev.* **2005**, *2*, 294; (d) Aguirre, G.; Boiani, L.; Cerecetto, H.; Di Maio, R.; González, M.; Porcal, W.; Thomson, L.; Tórtora, V.; Denicola, A.; Möller, M. *Bioorg. Med. Chem.* **2005**, *13*, 6324.
26. McKerrow, J. H.; Engel, J. C.; Cafrey, C. R. *Bioorg. Med. Chem.* **1999**, *7*, 639.
27. Cazzulo, J. J. *Curr. Top. Med. Chem.* **2002**, *2*, 1261.
28. Aparicio, I. M.; Scharfstein, J.; Lima, A. P. *Infect. Immun.* **2004**, *72*, 5892.
29. Roush, W. R.; Gwaltney, S. L.; Cheng, J.; Scheidt, K. A.; McKerrow, J. H.; Hansell, E. *J. Am. Chem. Soc.* **1998**, *120*, 10994.
30. Ifa, D. R.; Rodrigues, C. R.; de Alencastro, R. B.; Fraga, C. A. M.; Barreiro, E. J. *J. Mol. Struct. Theochem.* **2000**, *505*, 11.
31. Du, X.; Hansell, E.; Engel, J. C.; Caffrey, C. R.; Cohen, F. E.; McKerrow, J. H. *Chem. Biol.* **2000**, *7*, 733.
32. Doyle, P. S.; Zhou, Y. M.; Engel, J. C.; McKerrow, J. H. *Antimicrob. Agents Chemother.* **2007**, *51*, 3932.
33. Du, X.; Guo, C.; Hansell, E.; Doyle, P. S.; Caffrey, C. R.; Holler, T. P.; McKerrow, J. H.; Cohen, F. E. *J. Med. Chem.* **2002**, *45*, 2695.
34. Krauth-Siegel, R. L.; Inhoff, O. *Parasitol. Res.* **2003**, *90*, S77.
35. Henderson, G. B.; Ulrich, P.; Fairlamb, A. H.; Rosenmerg, I.; Pereira, M.; Sela, M.; Cerami, A. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 5374.
36. Porcal, W.; Boiani, M.; Merlino, A.; Gerpe, A.; González, M.; Cerecetto, H. *Org. Proc. Res. Develop.* **2008**, *12*, 156.
37. Alexanian, V.; Haddadin, M. J.; Issidorides, C. H.; Nazer, M. Z. *Heterocycles* **1981**, *16*, 391.
38. (a) Gasco, A.; Boulton, A. J.. In *Advances in Heterocycles Chemistry*; Katritzky, A. R., Boulton, A. J., Eds.; Wiley: New York, 1981; Vol. 29, pp 251–340; (b) Visentin, S.; Amiel, P.; Fruttero, R.; Boschi, D.; Roussel, C.; Giusta, L.; Carbone, E.; Gasco, A. *J. Med. Chem.* **1999**, *42*, 1422; (c) Ermondi, G.; Visentin, S.; Boschi, D.; Fruttero, R.; Gasco, A. *J. Mol. Struct.* **2000**, *523*, 149.
39. Almeida-de-Faria, M.; Freymuller, E.; Colli, W.; Alves, M. J. *Exp. Parasitol.* **1999**, *92*, 263.
40. Jockers-Scherübl, M. C.; Schirmer, R. H.; Krauth-Siegel, R. L. *Eur. J. Biochem.* **1989**, *180*, 267.
41. Olea-Azar, C.; Rigol, C.; Mendizábal, F.; Briones, R. *Mini-Rev. Med. Chem.* **2006**, *6*, 211.
42. (a) Dabideen, D. R.; Cheng, K. F.; Aljabari, B.; Miller, E. J.; Pavlov, V. A.; Al-Abed, Y. *J. Med. Chem.* **2007**, *50*, 1993; (b) Sousa, C.; Freire, C.; de Castro, B. *Molecules* **2003**, *8*, 894; (c) Jain, M. P.; Kumar, S. *Talanta* **1979**, *26*, 909.
43. Hakki, E.; Cihat, S.; Mevlut, E.; Nuram, Y. *J. Indian Chem. Soc.* **1989**, *66*, 45.
44. Vieites, M.; Buccino, P.; Otero, L.; González, M.; Piro, O. E.; Sánchez Delgado, R.; Sant' Anna, C. M. R.; Barreiro, E. J.; Cerecetto, H.; Gambino, D. *Inorg. Chim. Acta* **2005**, *358*, 3065.
45. Messeder, J. C.; Tinoco, L. W.; Figueroa-Villar, J. D.; Souza, E. M.; Santa Rita, R.; de Castro, S. L. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 3079.
46. Denicola, A.; Rubbo, H.; Rodriguez, D.; Radi, R. *Arch. Biochem. Biophys.* **1993**, *304*, 279.
47. Labriola, C.; Sousa, M.; Cazzulo, J. J. *Biol. Res.* **1993**, *26*, 101.
48. Sullivan, F. X.; Walsh, C. T. *Mol. Biochem. Parasitol.* **1991**, *44*, 145.