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Benzo[1,2-*c*]1,2,5-oxadiazole *N*-oxide derivatives as potential antitrypanosomal drugs. Part 3: Substituents-clustering methodology in the search for new active compounds☆

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Abstract—The results of a study on the use of Hansch's series design, cluster methodology, for the generation of new benzo[1,2-*c*]1,2,5-oxadiazole *N*-oxide derivatives as antitrypanosomal compounds are described. In vitro activity of these compounds was tested against Tulahuen 2 strain of *Trypanosoma cruzi*. Clearly, the Hansch methodology allowed identifying two cluster-substituents suitable for further structural modifications. The most effective drugs, derivatives **11**, **18**, and **21**, with 50% inhibitory concentration (IC_{50}) of the same order as that of the reference drug, represent an excellent structural point of chemical modifications for the design of future drugs. Preliminary results from the study of the mechanism of action of these benzofuroxans point to perturbation of the mitochondrial electron chain, inhibiting parasite respiration.
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1. Introduction

Chagas' disease (CD) or American trypanosomiasis is produced by several strains of the protozoan parasite of the order Kinetoplastida *Trypanosoma cruzi*.[1] This disease represents a serious public health problem in the countries and zones where it is endemic (21 countries in Central and South America) because there are no effective methods of immunoprophylaxis or chemotherapy. Currently, 16–18 million persons are infected and 100 million are at risk.[2] Cases have also been reported in the United States.[3] In humans, *T. cruzi* induces an acute phase followed by a life-long chronic phase. The acute infections are commonly asymptomatic stages.

In some cases, especially in children, fever, swelling of the lymph glands, enlargement of the liver and spleen, or local inflammation at the site of infection have been evidenced. Patients with chronic infections are affected by heart lesions or pathological dilations of the digestive tract (megacolon and megaesophagus) and disorders of nerve conduction of these organs. Patients with severe chronic disease become progressively more ill and ultimately die of heart failure.[4] CD, and their consequences, in immunocompromised patients has been reported as an opportunistic infection that potentially produces morbidity and loss of the patients' quality of life.[5] The forms of *T. cruzi* present in the human host are the bloodstream trypomastigote and the intracellular replicative amastigote form. The existence of the epimastigote form as an obligate mammalian intracellular stage has been revisited[6] and confirmed recently.[7] The wide tissue distribution of intracellular *T. cruzi* amastigotes during both acute and chronic phases makes CD a more difficult subject for drug targeting in comparison to leishmaniasis where amastigotes are restricted to macrophages and

Keywords: Benzofuroxan; *T. cruzi*; Hansch's series design; Mitochondrial parasite respiration.

☆ Part of this research is presented in the Uruguayan patent of invention: Cerecetto, H.; Di Maio, R.; González, M.; Porcal, W.; Denicola, A. *UR Patent No. 28.019*, 2003: Derivados de 5-etenilbenzofuroxano, procedimiento de preparación y utilización.

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human African trypanosomiasis is characterized in the second stage of the disease by the presence of trypanosomes in the central nervous system and cerebrospinal fluid. Since vaccinations against trypanosomatic infections are still under development,[8] the need for improving chemoprophylactic/chemotherapeutic approaches in the control of these pathogens is indisputable. The current chemotherapy against CD is still inadequate.[9] The main drugs in use are Nifurtimox® (Nfx) and Benznidazole®; however, they have undesirable side effects and are yet inefficient to treat chronic CD, which can be regarded as a disease with no cure. Furthermore, the production and commercialization of Nfx have been discontinued by the pharmaceutical company.

We have previously reported a series of benzo[1,2-*c*]-1,2,5-oxadiazole *N*-oxide derivatives (benzofuroxans) with anti-*T. cruzi* activity.[10] Derivatives **1–3** (Fig. 1) produced the best in vitro trypanosome inhibitors. These compounds were designed as new antitrypanosomal agents with the hypothesis that the *N*-oxide moiety would be responsible for generating toxic radical species. For some of these benzofuroxans, free radical generation was proved and the radical species characterized using ESR spectroscopy.[11] Lipophilic–hydrophilic balance of the compounds also played an important role in their effectiveness as antichagasic drugs. In addition, the cytotoxicity of these compounds against mammalian fibroblasts was comparable to that of Nfx.[10a]

Since these benzofuroxans were developed as analogues of previously described nitrofuran derivatives,[12] systematic structural modifications were not used in the design process.

Herein, we report the design of new benzofuroxans using substituents-clustering tools previously described by Hansch and co-workers.[13] The new structures were designed initially based on the five substituent-clusters shown in Chart 1. Hansch and co-workers established these clusters using the substituent variables π, $\mathscr{F}$, $\mathscr{R}$, MR, and the capacity to establish hydrogen bonds as acceptor and donor.[13b] The use of five substituent-clusters in the design of the new benzofuroxans enabled us to identify lead compounds, so it was unnecessary to study the superior number of Hansch clusters (i.e., 10, 20, or 60). To corroborate the biological relevance of the *N*-oxide group some derivatives were converted into the corresponding deoxygenated analogues. In addition, the heme-reduction products of some derivatives (i.e., *o*-nitroanilines) were prepared to investigate their activity and to compare with the parent compounds.

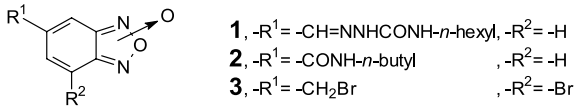
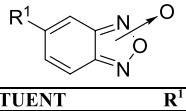


Figure 1. Benzofuroxan derivatives with anti-*T. cruzi* activity.



CLUSTER	SUBSTITUENT	R^1 (Derivative)
I	$-NO_2$ (**4**), -CHO (**5**), -CN (**8**), $-CH=C(CN)_2$ (**10**), -*E*-$CH=CHNO_2$ (**11**)	
II	$-CO_2H$ (**6**), -CH=NOH (**7**), $-CH_2OH$ (**9**)	
III	-H (**14**), $-CH_3$ (**15**), -Cl (**16**), -Br (**17**), $-CH_2Cl$ (**18**)	
IV	$-CH_2I$ (**20**), -*E*-CH=CHPh (**21**), -Ph (**22**)	
V	$-OCH_3$ (**25**), $-N(CH_3)_2$ (**26**)	

Chart 1. Clusters and benzofuroxan substituents selected from Hansch's series design methodology.

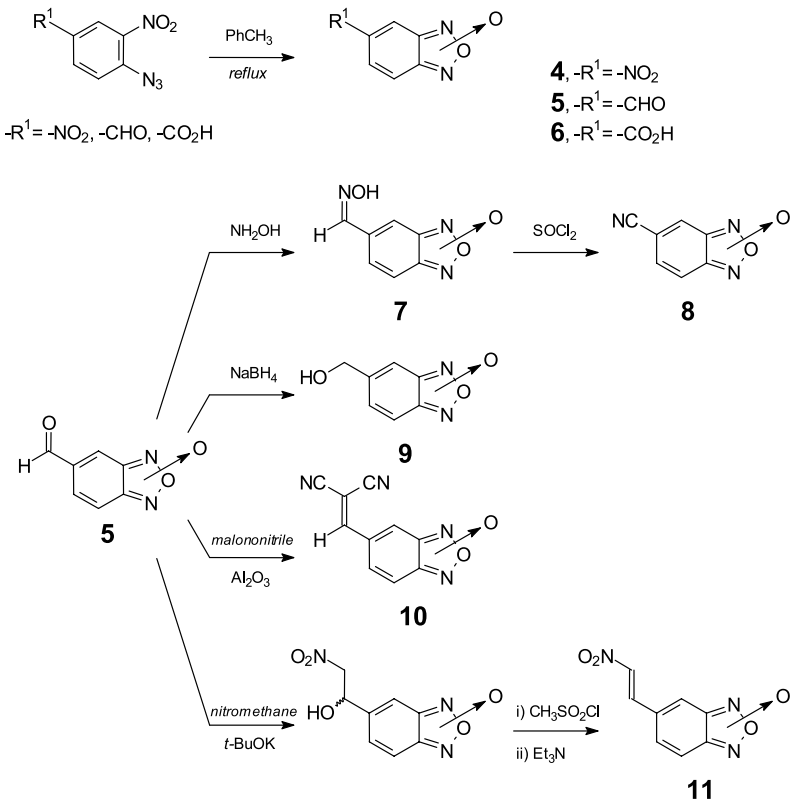
The biological activity was evaluated in vitro against Tulahuen 2 strain of *T. cruzi*. Finally, in order to gain insight into the mechanism of action, the benzofuroxans' capacity to produce reactive nitrogen and oxygen species was studied. Thus, their NO production capacity, their parasite's respiration inhibition capacity, and their potential production of superoxide were analyzed.

2. Methods and results

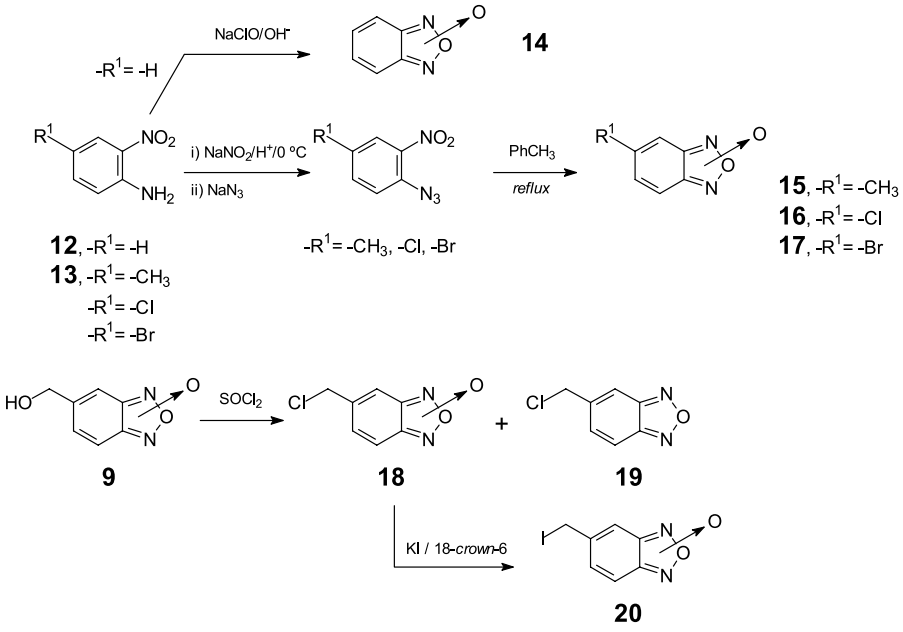
2.1. Synthesis

Derivatives **4**, **5**, and **6** were prepared, using the appropriate nitrophenyl azides reactants, by cyclocondensation in boiling toluene (Scheme 1).[14] Derivative **5** was converted into nitrile **8** via *cluster II*-derivative **7**.[15] Alcohol **9** was obtained as previously reported from aldehyde **5**.[10a] *Cluster I*-derivatives **10** and **11** were also obtained using derivative **5**. Thus, derivative **10** was obtained via a free solvent-Knoevenagel process between aldehyde **5** and malononitrile in the presence of basic-Al_2O_3.[16] The Beirut reaction between benzofuroxan and malononitrile to obtain quinoxaline dioxide was minimized in these conditions.

When derivative **5** was reacted at low temperature with nitromethane and potassium *t*-butoxide followed by treatment with methanesulfonyl chloride and triethylamine,[17] derivative **11** was obtained with a moderate global yield (11% in two steps). The *E*-stereochemistry of derivative **11** was established using the corresponding ^{1}H NMR coupling constant between olefinic protons (J = 13.7 Hz). Under the conditions used for the first step (i.e., nitroaldolic process), formation of the corresponding 1-hydroxybenzimidazole 3-oxide from benzofuroxan and nitromethane was not detected.[18] The *cluster III*-benzofuroxan derivatives **14–18** were prepared as shown in Scheme 2.[10a,14c,19] Derivative **14** was prepared from *o*-nitroaniline by reaction with sodium hypochlorite in basic medium while derivatives **15–17** were prepared, using the appropriate nitrophenyl azide reactants by cyclocondensation in boiling toluene. Chloromethyl derivative **18** was prepared as previously described from alcohol 9.[10a] In this process deoxygenated derivative **19** was obtained as a secondary product. From the reaction between chloride **18** and excess of potassium iodide, in the presence of crown ether 18-crown-6, was obtained *cluster IV*-derivative **20** (Scheme 2). The reaction was difficult to follow by TLC (SiO_2, diethyl ether/ethyl acetate (10%)), because of the very close R_f values of reactant **18** and product **20**.

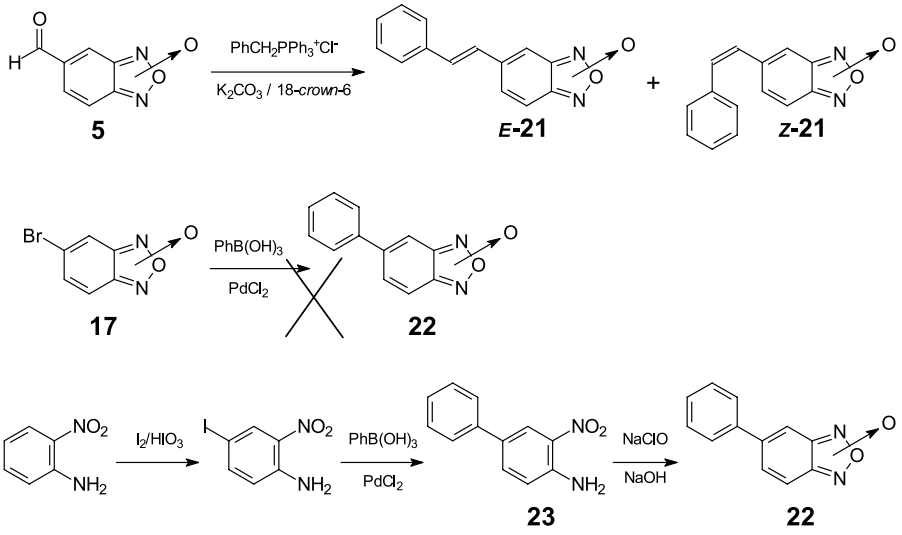


Scheme 1. Synthesis of *cluster I*- and *cluster II*-benzofuroxan derivatives.

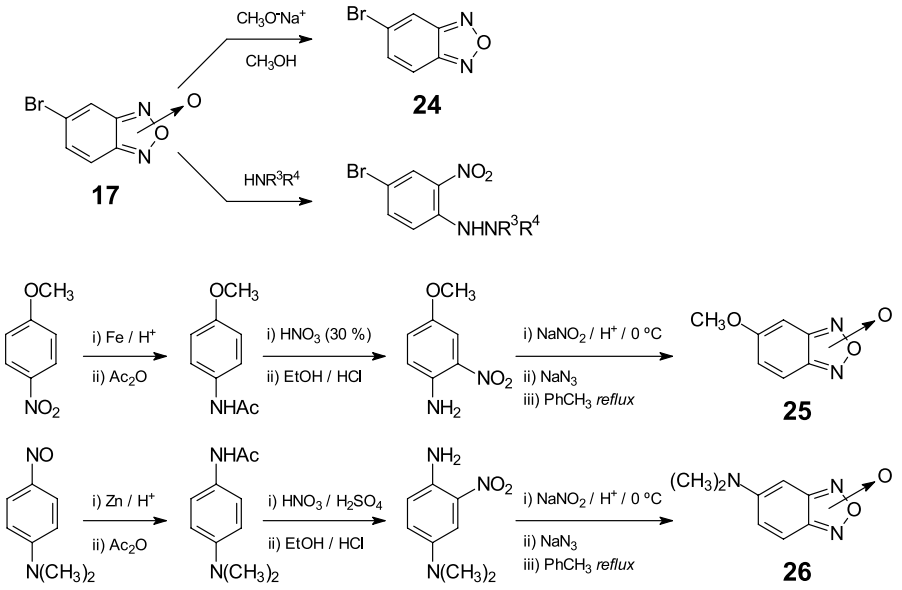


Scheme 2. Synthesis of *cluster III*- and *cluster IV*-benzofuroxan derivatives.

Bodens–Wittig methodology, depicted in Scheme 3, was used to prepare the olefin **21**.[20] In this process both geometric isomers, *Z*-**21** and *E*-**21**, were generated and isolated independently using column chromatography (SiO_2, petroleum ether/ethyl acetate (10%)). Attempts to obtain phenyl derivative **22** from bromo derivative **17**, using Suzuki methodology, were fruitless. Bromobenzofuroxan, **17**, did not react under any of the assayed conditions.[21] Derivative **22** was obtained from *o*-nitroaniline following the sequence shown in Scheme 3 with a good global yield (42% from *o*-nitroaniline). To prepare *cluster V*-derivatives (compounds **25** and **26**, Scheme 4) nucleophilic substitution processes were assayed using bromo derivative **17** as reactant.[22]



Scheme 3. Synthesis of derivatives **21** and **22** from *cluster IV*.



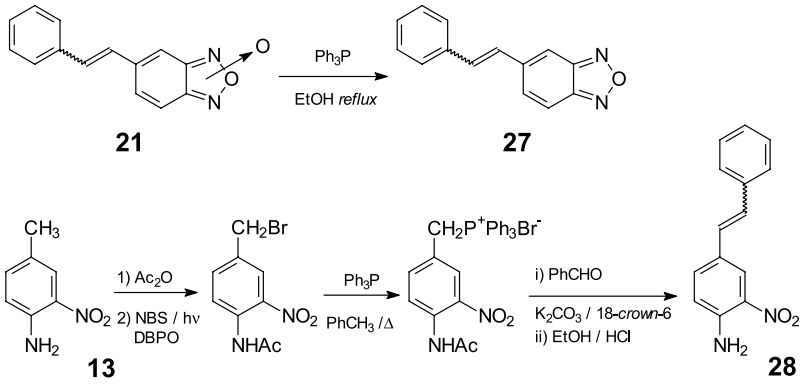
Scheme 4. Synthesis of *cluster V*-benzofuroxan derivatives.

However, it was not possible to obtain the desired products. In the case of reaction with sodium methoxide the deoxygenated derivative **24** was obtained as the main product, whereas when bromo-derivative **17** was treated, under different conditions, with secondary amines (i.e., morpholine), *o*-nitrophenylhydrazine derivatives were obtained.[23] Finally, derivatives **25** and **26** were obtained from the corresponding *o*-nitroaniline as shown in Scheme 4.[14c,24]

In order to corroborate the effect of the *N*-oxide moiety in the displayed activity, besides the sub-products **19** (Scheme 2) and **24** (Scheme 3), we developed the **21**-deoxygenated analogue **27** (Scheme 5). The reaction between *E*/*Z*-**21** with triphenylphosphine (Ph_3P), in boiling ethanol, yielded derivative *E*/*Z*-**27**.[25] Otherwise, to study the effect of the possible ferrous reduction-metabolic product in the biological response, besides commercial products **12** and **13** (Scheme 2) and intermediate **23** (Scheme 3), the **21**-deriving *o*-nitroaniline was developed. Compound **28** (an inseparable mixture of *Z*- and *E*-isomers) was prepared following the sequence shown in Scheme 5.

Benzofuroxan derivatives exist as a mixture of isomers at room temperature (**A** and **B**, Fig. 2); equilibrium concentrations are dependent on a number of factors (i.e., the nature and the position of the substituents at the ring).[26] The room temperature (303 K) ^1H and ^{13}C NMR spectra of the benzofuroxans show benzo-protons and -carbons as broad peaks, indicating extensive benzofuroxan tautomerism. The spectra (^1H, ^{13}C NMR, and HMQC, HMBC, and COSY experiments) were simplified at low temperature, from 263 to 230 K, where it was observed that one of these isomers predominated.[27]

All new compounds were identified by IR, MS, ^1H NMR, ^{13}C NMR, COSY, and HETCOR experiments,



Scheme 5. Synthesis of deoxy derivative **27** and *o*-nitroaniline **28**.

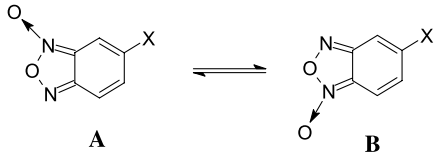


Figure 2. Benzofuroxan tautomeric equilibria at room temperature.

and their purity was established by TLC and microanalysis.

2.2. Biological characterization

2.2.1. Antitrypanosomal activities. All compounds were tested in vitro against *T. cruzi*. Epimastigote forms of *T. cruzi*, Tulahuen 2 strain, were grown in axenic media as described in Section 5. The compounds were incorporated into the media at 25 μM and their ability to inhibit the parasite growth was evaluated in comparison to the control (deprived of drug). Nfx was used as the reference trypanocidal drug. Growth of the parasite was followed by measuring the increase in absorbance at 600 nm, which was proved to be proportional to the number of cells present.[10,12] The percentage of inhibition, summarized in Table 1, was calculated as follows: $\% = \{1 - [(A_p - A_{0p})/(A_c - A_{0c})]\} \times 100$, where $A_p = A_{600}$ of the culture containing the drug at day 5; $A_{0p} = A_{600}$ of the culture containing the drug right after addition of the inocula (day 0); $A_c = A_{600}$ of the culture in the absence of any drug (control) at day 5; $A_{0c} = A_{600}$ in the absence of the drug at day 0. The IC_{50} concentration (50% inhibitory concentration) was assessed for compounds presenting higher trypanocidal activity. Readings were done on day 5 of growth, and IC_{50} values determined as the drug concentration required to reduce by half, the absorbance of that of the control (without drug) (Fig. 3).

2.3. Approaches to the study of mechanism of action

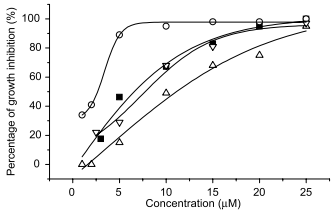
In order to gain insight into the molecular mechanism of antitrypanosomal activity of benzofuroxans, we developed some preliminary studies on a selected series of derivatives. We selected for the studies three benzofuroxans with high antitrypanosomal activity, derivatives **11**, **18**, and *E*-**21**, one benzofuroxan with medium activity, derivative **5**, and two benzofuroxans with low activity, derivatives **8** and **25**. The capacity to act as nitric oxide (NO)-releasing agents, effects on mitochondria-dependent superoxide production and parasite respiration[28] were studied for selected benzofuroxans.

Table 1. In vitro activity of benzofuroxan derivatives and Nfx on *T. cruzi* (Tulahuen strain) at 25 μM

Compound	Percentage of growth inhibition (%)[a,b]
Nfx	100.0
2	90.0
Cluster I	
4	89.0
5	67.0
8	9.0
10	66.0
11	92.0
Cluster II	
6	1.0
7	38.0
9	21.0
Cluster III	
14	35.0
15	22.0
16	2.0
17	5.0
18	97.0
Cluster IV	
20	93.0
E-**21**	93.0
Z-**21**	100.0
E-**21**:*Z*-**21** (50:50)	97.0
22	31.0
Cluster V	
25	10.0
26	26.0

[a] Inhibition of epimastigotes growth, concentration = 25 μM.
[b] The results are the means of three different experiments with SD less than 10% in all cases.

2.3.1. Evaluation of benzofuroxans as NO donors. According to recent descriptions, some benzofuroxan derivatives are capable of acting as NO-releasing compounds.[15] NO is an important messenger implicated in the regulation of numerous biological processes.[29] In



Compd.	IC_{50} (μM)[c]
Nfx	6.7
2	10.0[a]
4	22.5
5	20.0
11	2.5
18	2. 4
20	10.9
***E*-21**	10.8
***Z*-21**	7.0
***E*-21:*Z*-21** (50:50)	7. 7

a Taken from literature [10a]

Figure 3. Dose–response curves as anti-*T. cruzi* for selected benzofuroxan derivatives, **11** (○), *E*-**21** (△), *E*-**21**:*Z*-**21** (50:50) (▽), and Nfx (■).

particular, it plays a crucial role in the immune response by its cytotoxic action on macrophages and leukocytes. Thus, benzofuroxan-trypanocidal activities could be related to their potential NO release capacity. Griess methodology was used as a primary screening of NO-donor capacities, measuring nitrite yields generated by benzofuroxans incubated either with or without 20 mM cysteine (1 h at 25 or 37 °C). Most derivatives tested did not release ·NO, even at high concentrations (400 μM) and in the presence of excess of cysteine. Only derivative **11** was able to yield nitrite both in the absence (10% nitrite yield after 1 h at 37 °C) and in the presence of thiol (15% nitrite yield after 1 h at 37 °C) (Fig. 4).

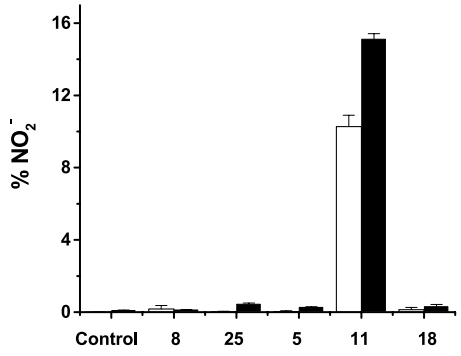


Figure 4. Nitrite yield by benzofuroxan derivatives after 1 h at pH 7.4 and 37 °C, in the absence (white columns) or in the presence (black columns) of 50-fold excess of cysteine.

2.3.2. Oxygen uptake and cyanide effect. The six selected benzofuroxans and Nfx were studied as described in Section 5, investigating their effect on parasite respiration.[30] Figure 5A shows time-dependent inhibition of *T. cruzi* respiration by selected benzofuroxan derivatives. Clearly, it was seen that the most active benzofuroxans did not increase oxygen uptake but rather significantly inhibited cellular respiration. Figure 5B shows the effect of cyanide on parasite respiration on incubation with the benzofuroxans. Fifty micromolar of NaCN was able to inhibit respiration up to 95% in the control run, thus, cyanide-insensitive respiration only 5%; whereas incubation with Nfx significantly increased it to 50%. On the other hand, the effect of the benzofuroxan derivatives on cyanide-insensitive oxygen uptake was minor, except for derivatives **5**, **11**, and *E*-**21** (Fig. 5B). However, the observed result with derivative **5** could be an artifact due to the special reactivity of this compound. Thus, in an independent experiment it was demonstrated, using TLC, that derivative **5** (25 μM) rapidly reacted with NaCN (50 μM) dramatically reducing cyanide concentration in the medium.

3. Discussion

Some of the benzofuroxan derivatives investigated show excellent antitrypanosomal activity, similar to that of Nfx, at 25 μM. In particular, derivatives **11** and **18** resulted in the most active benzofuroxans described until now. The Hansch methodology proved, in this case, to be an excellent tool in the search for new anti-*T. cruzi* agents. Clearly, it was observed that substituents contained in clusters I and IV yielded the most active compounds. The mesomeric electron-withdrawing properties of cluster I-substituents could be used to explain the activity and in the case of cluster IV-substituents the volume should be taken into account to explain this fact.[27]

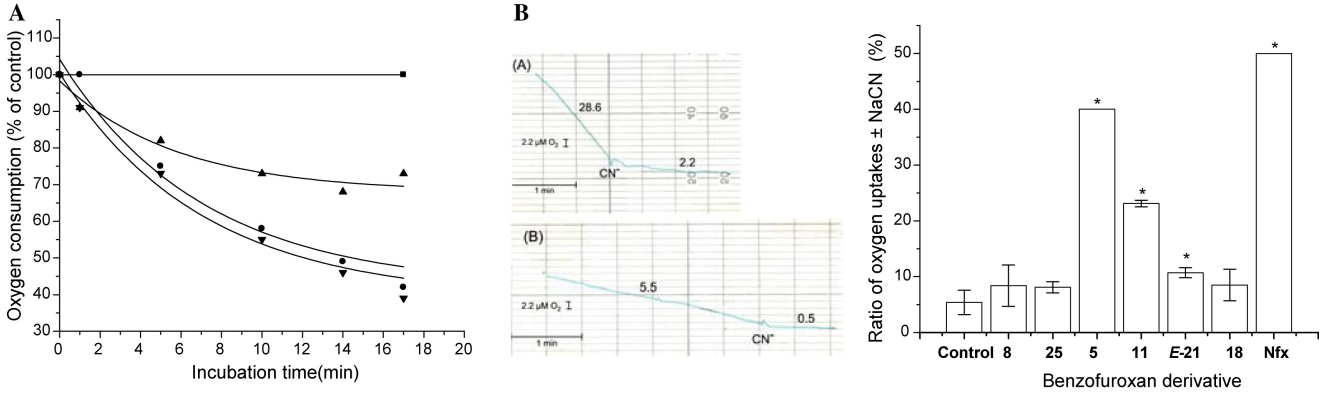


Figure 5. (A) Time-dependent inhibition of *T. cruzi* respiration in the presence of 25 μM of benzofuroxan derivatives **11** (▼), **18** (●), and *E*-**21** (▲). Control was run with no drug and 0.5% DMSO (■). (B) Effect of cyanide (NaCN, 50 μM) on respiration. Left: Oxygen consumption by *T. cruzi* (1 mg protein) incubated for 5 days in the absence (A) or presence (B) of 25 μM of derivative *E*-**21**. The numbers represent rates of oxygen consumption (in μmol min^{-1} mg $protein^{-1}$). Right: NaCN-insensitive oxygen uptake. For each benzofuroxans (25 μM) and control (no drug), the ratio of rates of oxygen consumption before and after addition of NaCN was calculated as percentage, as mean ± SD of two independent experiments (*statistical difference with control for Student's *t* test, $p < 0.05$).

On the other hand, it is possible to observe that substituents contained in clusters II, III (except for derivative **18**), and V yielded the least active compounds. The substituents acting as hydrogen bond donors belong to cluster II and mesomeric electron-releasing substituents to cluster V. These properties could be playing some role in the observed deficient trypanocidal activity.[27] Four out of five derivatives in cluster III were inactive. However, derivative **18** of cluster III is one of the most active developed products. In this case the substituent chloromethyl could be relocated in another cluster, or the Hansch methodology used with a superior number of clusters (10, 20 or 60). Nevertheless, in our opinion, derivative **18** was especially active because it shares particular structural characteristics that enhance its activity: the benzylic-like electrophilic center (chloromethyl substituent). This good electrophile center, such as in derivative **20** or in parent compound **3**, could react with biological nucleophiles (amines and phosphates from DNA, thiols, alcohols, and amines from proteins) enhancing its cytotoxicity. Derivatives **20** and **21** (as *Z*-isomer or *E*:*Z*-mixture) showed IC_{50} values similar to the parent compound **2** and of the same order as Nfx. However, derivative **21** resulted in a more attractive lead compound because its structure allows further synthetic modifications. For the different geometric isomers of derivative **21** we could observe that the spatial distribution did not significantly affect the activity. The 50:50 mixture of *Z*- and *E*-isomers showed similar IC_{50} to that of the *Z*-isomer.

As observed previously,[10a,31] the absence of the *N*-oxide moiety produced a total loss of activity, confirming that this group plays a role in the mechanism of the benzofuroxans' trypanosome toxicity (compare activity of derivative **21** with activity of deoxy-derivative **27**, Table 2). In the same manner, it was possible to observe the dramatic decay in the activity of derivative **18** when it was deoxygenated (**19**). In the case of derivative **17** and the deoxy-analogue **24** the difference in activity was not too clear because derivative **17** itself was poorly active.

Recently, the capacity of ferrous salts and oxyhemoglobin to reduce benzofuroxan derivatives to the corresponding *o*-nitroanilines was described,[32] suggesting that blood is a possible site for metabolizing benzofuroxans. Because hemine is incorporated into the *T. cruzi* culture medium in the in vitro tests, we evaluated some benzofuroxan metabolic products in order to investigate whether the benzofuroxan bio-activities were the result of the hemine-reduction products (*o*-nitroanilines). Clearly, it was demonstrated that the trypanocidal activity is not due to the corresponding ferrous-reduced derivatives (Table 2). When the ferrous-reduced metabolite (**28**) of the most active derivative **21** was evaluated in vitro, a complete lack of activity was observed. The same behavior was observed for the ferrous-reduced metabolite of derivative **15**, compound **13**. However, *o*-nitroanilines **12** and **23** presented similar activities as the corresponding benzofuroxans, **14** and **22**, respectively, but this result could be due to the toxicity of anilines.

Table 2. In vitro activity on *T. cruzi* of deoxygenated and ferrous-reduced derivatives at 25 μM

Percentage of growth inhibition (%)[a,b]		
Parent compound	Deoxygenated analogue	Ferrous-reduced derivative
14, 35.0	—	**12**, 26.0
15, 22.0	—	**13**, 0.0
17, 5.0	**24**, 0.0	—
18, 97.0	**19**, 28.0	—
E-**21**:*Z*-**21** (50:50), 97.0	**27**, 10.0	**28**, 0.0
22, 31.0	—	**23**, 41.0

[a] Inhibition of epimastigotes growth, concentration = 25 μM.
[b] The results are the means of three different experiments with SD less than 10% in all cases.

In order to gain insight into the metabolism of benzofuroxans, the generation of metabolite **28** (as *Z* and *E* mixture) in the *T. cruzi* culture treated with compound **21** (as *Z* and *E* mixture) was investigated. Using TLC analysis, it was possible to observe the formation of a new metabolic product after 4 h of treatment. This compound did not correspond to the *o*-nitroaniline **28**. The structure of this metabolite was not further characterized but could be like the mammalian microsomal-metabolite recently described for benzofuroxan.[33]

The benzofuroxan-trypanocidal activity is not the result of their NO-release capacity, since only the active nitroalkene **11** yielded nitrite but at a concentration 200-fold superior to the corresponding IC_{50}. This capacity could be related to the substituent (2-nitroalkenyl) rather than the benzofuroxan system. Also, it was observed that the benzofuroxans' trypanocidal activity is related to their capacity to inhibit cellular respiration. In the absence of drugs, cyanide-insensitive respiration is minor (close to 5%), suggesting that cytochrome aa_3 is the main *T. cruzi* terminal oxidase as observed before.[30] Treatment with Nfx increased the cyanide-insensitive respiration to more than 50%, suggestive of superoxide/H_2O_2 production. This is in agreement with previous ESR results consistent with the trapping of hydroxyl radical (DMPO–OH adduct) when incubating an Nfx analogue with *T. cruzi* homogenates and the spin trap.[11b] However, treatment with the most active benzofuroxans did not increase the cyanide-insensitive respiration to such high levels (up to 22% for derivative **11**), suggesting a different mechanism of action than nitrofuranes like Nfx.

On the other hand, if we consider the potential use of benzofuroxan derivatives as drugs, these should present adequate behavior in vivo according to Lipinski's rules and their topological polar surface area (TPSA).[34] Lipinski described the desired ranges for certain properties thought to be important for pharmacokinetics and drug development. They are: C log $P < 5$, number of hydrogen bond donors $\leqslant 5$, number of hydrogen bond acceptors $\leqslant 10$, and molecular weight <500. A compound that fulfills at least three of these criteria adheres to Lipinski's rule. Another very helpful parameter for the prediction of absorption is the polar surface area (PSA), defined as the sum of surfaces of polar atoms

Table 3. Nfx and the properties of benzofuroxans to fulfill Lipinski rule

Ref	Lipinski rule					TPSA[b,c]
	Clog P[a]	No. of H-bond donors[b]	No. of H-bond acceptors[b]	Mol wt	No. of criteria met	
Rule	<5	⩽5	⩽10	<500	At least 3	
4	3.92	0	7	181.0	All	97.3
5	2.07	0	5	164.0	All	68.6
11	4.36	0	7	207.0	All	97.3
18	2.88	0	4	184.5	All	51.5
20	2.95[d]	0	4	276.0	All	51.5
21	4.76	0	4	238.0	All	51.5
Nfx	8.51	0	8	287.0	3	108.7

[a] Theoretical Log P (Clog P) was calculated using Villar method, at AMI semiempirical method, implemented in Spartan'04, 1.0.1 version, suite of programs.[36]

[b] http://www.molinspiration.com/cgi-bin/properties.

[c] TPSA, topological polar surface area.

[d] This value was obtained from http://www.molinspiration.com/cgi-bin/properties because parameters for iodine atom are not available into Villar method.

in a molecule, which provides good correlation with experimental transport data (intestinal absorption, Caco-2 monolayers penetration, and blood–brain barrier crossing).[35] Table 3 lists such physicochemical properties for the trypanocidal benzofuroxan derivatives. All of the potent antiparasitic benzofuroxans presented herein are fully compatible with Lipinski's rule and possess better topological polar surface area (TPSA) than Nifurtimox.

4. Conclusions

A new series of benzo[1,2-*c*]1,2,5-oxadiazole *N*-oxide was synthesized using a facile methodology. The preliminary antitrypanosomal studies of these derivatives showed that some of them exhibit significant in vitro activity. The *N*-oxide moiety of these compounds participates in the pharmacophore, whereas the ferrous-reduced analogues (potential metabolites using heme from the culture) are not responsible for the antitrypanosomal activity observed. The excellent in vitro activities suggest that compounds like **11**, **18**, **20**, and **21** (as a mixture of geometric isomers), and to a lesser extent derivatives **4** and **5**, represent a new class of anti-*T. cruzi* agents. Further structural optimization and in vivo studies to investigate the ability of these drugs to decrease the parasitemia of infected mice and the toxicity are currently under way.

5. Experimental

5.1. Chemistry

All starting materials were commercially available research-grade chemicals and were used without further purification. Compounds **4–9**, **14–18**, **25**, and **26** were prepared according to the literature.[10,15,19–21] All solvents were dried and distilled prior to use. All the reactions were carried out in a nitrogen atmosphere. The typical work-up included washing with brine and drying the organic layer with sodium sulfate. Melting points were determined using a Leitz Microscope Heating Stage Model 350 apparatus and are uncorrected. Elemental analyses were obtained from vacuum-dried samples (over phosphorous pentoxide at 3–4 mmHg, 24 h at room temperature) and performed on a Fisons EA 1108 CHNS-O analyzer, and were within ±0.4% of theoretical values. 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}). ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX-400 (at 400 and 100 MHz) instrument, with tetramethylsilane as the internal reference and in the indicated solvent; the chemical shifts are reported in ppm. The ^1H and ^{13}C NMR signals reported were obtained at room temperature. In ^{13}C NMR data only narrow peaks were reported. Bruker software was used to perform NOE, COSY, HMQC, and HMBC experiments. Mass spectra were recorded on a Shimadzu GC-MS QP 1100 EX instrument, for electronic impact at 70 and 20 eV, or chemically ionized with methane.

5.1.1. 5-(2,2-Dicyanoethenyl)-N^1-oxidebenzo[1,2-*c*]1,2,5-oxadiazole (10). A mixture of aldehyde **5** (300 mg, 1.8 mmol), Al_2O_3 (typ T, 550 mg), malononitrile (120 mg, 1.8 mmol), and dried CH_2Cl_2 (3 mL) was stirred at room temperature for 24 h. Then, the Al_2O_3 was filtered and washed with excess of CH_2Cl_2. The organic solvent was evaporated in vacuo and the residue was purified by crystallization from $CHCl_3$, brown needles (230 mg, 40%); mp 135.0–136.5 °C; IR ν_{max} 3073, 2228, 1605, 1570, 1485 cm^{-1}; ^1H NMR (acetone-d_6, 400 MHz) δ: 7.93 (br s, 1H), 8.03 (br s, 1H), 8.24 (br s, 1H), 8.44 (s, 1H); ^{13}C NMR (acetone-d_6, 100 MHz) δ: 86.51, 112.72, 113.75, 134.50; MS, *m*/*z* (abundance): 212 ($[M]^{+\cdot}$, 100%), 196 (27%), 166 (17%), 152 (27%). Anal. ($C_{10}H_4N_4O_2$) C, H, N.

5.1.2. 5-(2-Nitroethenyl)-N^1-oxidebenzo[1,2-*c*]1,2,5-oxadiazole (11)

5.1.2.1. 5-(1-Hydroxy-2-nitroethyl)-N^1-oxidebenzo[1,2-*c*]1,2,5-oxadiazole. A mixture of CH_3NO_2 (160 mg, 2.6 mmol), *t*-BuOK (32 mg, 0.3 mmol), and THF:*t*-BuOH (1:1, 26 mL) as solvent was cooled at 0 °C. Then, a solution of aldehyde **5** (400 mg, 2.4 mmol) in THF (12 mL) was added slowly. The final mixture was stirred during 24 h

at 0 °C. The solvent was evaporated in vacuo and the residue was treated with aqueous HCl (5%) (20 mL) and extracted with AcOEt (3 × 10 mL). After the work-up the organic solvent was evaporated in vacuo. The product was obtained as an oil (300 mg, 55%) and was used in the next reaction without further purification; ^{1}H NMR (acetone-d_6, 400 MHz) δ: 2.95 (br s, 1H), 4.73 (dd, J_1 = 9.3 Hz, J_2 = 13.0 Hz, 1H), 4.95 (dd, J_1 = 3.2 Hz, J_2 = 13.0 Hz, 1H), 5.69 (dd, J_1 = 2.8 Hz, J_2 = 9.4 Hz, 1H), 7.61 (br s, 2H), 7.70 (br s, 1H).

5.1.2.2. 5-(2-Nitroethenyl)-N^1-oxidebenzo[1,2-*c*]1,2,5-oxadiazole (11). A mixture of 5-(1-hydroxy-2-nitroethyl)-N^1-oxidebenzo[1,2-*c*]1,2,5-oxadiazole (300 mg, 1.3 mmol), $MeSO_2Cl$ (152 mg, 1.3 mmol) and CH_2Cl_2 as solvent (1.4 mL) was stirred at 0 °C for 2 h. Et_3N (0.25 mL) was added and the mixture was stirred for 30 min. Then EtOAc (30 mL) was added and the organic layer was washed with H_2O (10 mL), aqueous HCl (10%) (10 mL), and brine (10 mL). After the work-up the organic solvent was evaporated in vacuo. The residue was purified by column chromatography (SiO_2, petroleum ether/EtOAc (9:1)) and the product was crystallized from EtOH, orange solid (100 mg, 20%); mp 141.5–143.0 °C; IR ν_{max} 3100, 1635, 1608, 1533 cm^{-1}; ^{1}H NMR (acetone-d_6, 400 MHz) δ: 7.76 (br s, 1H), 7.86 (br s, 1H), 8.17 (br s + d, J = 13.7 Hz, 2H), 8.18 (d, J = 13.7 Hz, 1H); ^{13}C NMR (acetone-d_6, 100 MHz) δ: 118.29 (br s), 136.29, 140.87; MS, *m*/*z* (abundance): 207 ([M]$^{+\cdot}$, 100%), 191 (12%), 166 (17%), 160 (55%), 144 (21%). Anal. ($C_8H_5N_3O_4$) C, H, N.

5.1.3. 5-Chloromethylbenzo[1,2-*c*]1,2,5-oxadiazole (19). A mixture of alcohol **9** (1.0 g, 6.0 mmol), $SOCl_2$ (1.3 mL, 18.0 mmol), and DMF (0.15 mL) was stirred at reflux during 3 h. The mixture was poured onto aqueous $NaHCO_3$–ice and the aqueous mixture was extracted with EtOAc (3 × 30 mL). The combined extracts were dried and evaporated in vacuo. The residue was purified by column chromatography (SiO_2, petroleum ether/EtOAc (9:1)), yielding derivative **18** as a colorless oil (440 mg, 40%) (R_f = 0.40, SiO_2, petroleum ether/EtOAc (8:2)) and derivative **19** as a colorless oil (110 mg, 10%) (R_f = 0.45, SiO_2, petroleum ether/EtOAc (8:2)); **19**: IR ν_{max} 1600, 1540, 1450 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 4.66 (s, 2H), 7.46 (dd, J_1 = 1.4 Hz, J_2 = 9.3 Hz, 1H), 7.84 (d, J = 1.4 Hz, 1H), 7.88 (d, J = 9.5 Hz, 1H); MS, *m*/*z* (abundance): 168 ([M]$^{+\cdot}$, 60%), 133 (70%), 117 (20%). Anal. ($C_7H_5ClN_2O$) C, H, N.

5.1.4. 5-Iodomethyl-N^1-oxidebenzo [1,2-*c*]1,2,5-oxadiazole (20). A mixture of chloride **18** (100 mg, 0.54 mmol), KI (450 mg, 2.7 mmol), 18-crown-6 (680 mg, 2.7 mmol), and DMF as solvent (2 mL) was stirred at room temperature for 4 h. Then H_2O (20 mL) was added and extracted with EtOAc (3 × 20 mL). After the work-up the organic solvent was evaporated in vacuo. The residue was purified by column chromatography (SiO_2, petroleum ether/EtOAc (95:5)), colorless oil that crystallized at 4 °C (45 mg, 30%); IR ν_{max} 2957, 1620, 1593, 1537, 1480 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 4.42 (s, 2H), 7.5 (br s, 3H); MS, *m*/*z* (abundance): 276 ([M]$^{+\cdot}$, 12%), 149 (100%), 133 (9%), 89 (54%). Anal. ($C_7H_5IN_2O_2$) C, H, N.

5.1.5. 5-(2*E*/*Z*-Phenylethenyl)-N^1-oxidebenzo[1,2-*c*]1,2,5-oxadiazole (21). A mixture of aldehyde **5** (100 mg, 0.6 mmol), benzyltriphenylphosphonium chloride (280 mg, 0.7 mmol), K_2CO_3 (100 mg, 0.7 mmol), 18-crown-6 (1.8 mg), and THF (3 mL) as solvent was stirred at reflux during 15 min. The solvent was evaporated in vacuo, the residue was treated with brine (5 mL) and extracted with EtOAc (3 × 10 mL). After the work-up the organic layer was evaporated in vacuo. The residue was purified by column chromatography (SiO_2, petroleum ether/EtOAc (9:1)), yielding derivative *E*-**21** as a yellow solid (73 mg, 50%) (R_f = 0.75, SiO_2, petroleum ether/EtOAc (8:2)) and derivative *Z*-**21** as a yellow solid (19 mg, 13%) (R_f = 0.65, SiO_2, petroleum ether/EtOAc (8:2)); *E*-**21**: mp 143.8–145.5 °C; IR ν_{max} 1614, 1572, 1525, 1485, 962, 758, 735 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.09 (d, J = 16.3 Hz, 1H), 7.21 (d, J = 16.3 Hz, 1H), 7.34 (t, J = 7.2 Hz, 1H), 7.40 (t, J = 7.6 Hz, 2H), 7.54 (d, J = 7.3 Hz, 2H), 7.60 (br s, 3H); ^{13}C NMR (acetone-d_6, 100 MHz) δ: 109.00 (br s), 114.00 (br s), 119.00 (br s), 126.52, 127.46, 129.36, 129.43, 133.61, 136.32; MS, *m*/*z* (abundance): 238 ([M]$^{+\cdot}$, 90%), 222 (16%), 192 (16%), 178 (100%), 149 (3%). Anal. ($C_{14}H_{10}N_2O_2$) C, H, N. *Z*-**21**: mp 61.5–62.9 °C; IR ν_{max} 1610, 1570, 1520, 1480 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 6.74 (d, J = 12.2 Hz, 1H), 6.94 (d, J = 12.2 Hz, 1H), 7.18 (br s, 1H), 7.33 (br s, 5H), 7.42 (br s, 2H); MS, *m*/*z* (abundance): 238 ([M]$^{+\cdot}$, 3%), 222 (30%), 192 (25%), 178 (26%), 149 (40%). Anal. ($C_{14}H_{10}N_2O_2$) C, H, N.

5.1.6. 5-Phenyl-N^1-oxidebenzo[1,2-*c*]1,2,5-oxadiazole (22)

5.1.6.1. 4-Iodo-2-nitroaniline. A mixture of *o*-nitroaniline (1.0 g, 7.3 mmol), I_2 (910 mg, 3.6 mmol), HIO_3 (640 mg, 3.6 mmol), and acetic acid (10 mL) was stirred at 50 °C during 3 h. The solvent was evaporated in vacuo, the residue was treated with H_2O (20 mL) and neutralized with aqueous NaOH (6 N). The orange precipitate was filtered and dried (1.80 g, 94%) and was used in the next reaction without further purification; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 6.11 (br s, 2H), 6.62 (d, J = 8.7 Hz, 1H), 7.58 (dd, J_1 = 2.0 Hz, J_2 = 8.7 Hz, 1H), 8.44 (d, J = 2.0 Hz, 1H); MS, *m*/*z* (abundance): 264 ([M]$^{+\cdot}$, 100%), 218 (26%), 91 (17%).

5.1.6.2. 4-Phenyl-2-nitroaniline (23). A mixture of 4-iodo-2-nitroaniline (132 mg, 0.5 mmol), NaOH (80 mg, 2 mmol), phenylboronic acid (67 mg, 0.55 mmol), $PdCl_2$ (2 mg), and H_2O (1 mL) and MeOH (2 mL) as solvent was stirred at room temperature during 24 h. Then, the reaction was heated at 100 °C during 2 h. The solvent was evaporated in vacuo and the residue was treated with aqueous HCl (5%), pH neuter, and extracted with EtOAc (3 × 10 mL). After the work-up the organic layer was evaporated in vacuo. The residue, brown solid, was the product (80 mg, 75%) and was used in the next reaction without further purification; ^{1}H NMR (acetone-d_6, 400 MHz) δ: 7.07 (br s, 2H), 7.20 (d, J = 8.8 Hz, 1H), 7.35 (t, J = 8.0 Hz, 1H), 7.46 (t, J = 8.0 Hz, 2H), 7.66 (d, J = 7.5 Hz, 2H), 7.78

(dd, J_1 = 2.2 Hz, J_2 = 8.7 Hz, 1H), 8.33 (d, J = 2.2 Hz, 1H); ^{13}C NMR (acetone-d_6, 100 MHz) δ: 120.27, 123.32, 126.38, 127.23, 127.44, 129.19, 129.33, 134.56, 139.37, 145.62; MS, *m*/*z* (abundance): 214 ([M]$^{+\cdot}$, 100%), 184 (4%), 168 (32%).

5.1.6.3. 5-Phenyl-*N*^{1}-oxidebenzo[1,2-*c*]1,2,5-oxadiazole (22). NaOH (8 mg) was dissolved in EtOH 95% (5 mL) with heating. Then, **23** (34 mg, 0.16 mmol) was added to the hot ethanolic solution and an intense red solution was obtained. The mixture was cooled at 0 °C, aqueous NaClO (2.8 M) (0.2 mL) was added dropwise, and the reaction was stirred during 20 min. The solvent was evaporated in vacuo and the residue was neutralized with aqueous HCl (10%) and extracted with EtOAc (3 × 10 mL). After the work-up the organic layer was evaporated in vacuo. The residue was purified by column chromatography (SiO_2, petroleum ether/EtOAc (9:1)), red-brown solid (20 mg, 59%); mp 122.0–123.5 °C; IR ν_{max} 1610, 1566, 1525, 756, 715 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.4 (m, 5H), 7.5 (br s, 3H); MS, *m*/*z* (abundance): 212 ([M]$^{+\cdot}$, 81%), 196 (2%), 166 (2%), 152 (58%). Anal. ($C_{12}H_8N_2O_2$) C, H, N.

5.1.7. 5-(2*E*/*Z*-Phenylethenyl)benzo[1,2-*c*]1,2,5-oxadiazole (27). A mixture of *E*/*Z*-**21** (100 mg, 0.4 mmol), Ph_3P (110 mg, 0.4 mmol) and EtOH (30 mL) as solvent was heated at reflux for 2 h. The EtOH was evaporated in vacuo. The residue was washed with petroleum ether to eliminate the unreacted Ph_3P and then purified by column chromatography (SiO_2, petroleum ether/EtOAc (0–10%)), yellow oil (70 mg, 75%); IR ν_{max} 3050, 1624, 1541, 962, 694 cm^{-1}. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 6.68 (d, J = 12.0 Hz, 0.5H), 6.89 (d, J = 12.0 Hz, 0.5H), 7.20 (d, J = 16.0 Hz, 0.5H), 7.28 (m, 3.5H), 7.36 (t, J = 7.3 Hz, 0.5H), 7.43 (t, J = 7.2 Hz, 1H), 7.59 (d + d + s, J = 7.9 Hz, J = 16.0 Hz, 1.5H), 7.69 (s, 0.5H), 7.76 (d, J = 8.8 Hz, 0.5H), 7.77 (d, J = 8.1 Hz, 0.5H), 7.84 (d, J = 9.7 Hz, 0.5H); MS, *m*/*z* (abundance): 222 ([M]$^{+\cdot}$, 32%), 205 (82%), 191 (45%), 165 (100%). Anal. ($C_{14}H_{10}N_2O$) C, H, N.

5.1.8. 4-(2*E*/*Z*-Phenylethenyl)-2-nitroaniline (28)

5.1.8.1. 4-Bromomethyl-2-nitroacetanilide. A mixture of 4-methyl-2-nitroacetanilide (10.0 g, 52.0 mmol), NBS (7.3 g, 41.0 mmol), PDBO (0.5 g, 2.1 mmol), and CCl_4 (100 mL) as solvent was heated under reflux during 5 h. Then, the precipitate, succinimide, was filtered and washed with CCl_4. The organic phase was evaporated in vacuo and the residue corresponding to the product, pure by TLC, was used without further purification (13.2 g, 95%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.31 (s 3H), 4.49 (s, 2H), 7.68 (dd, J_1 = 2.1 Hz, J_2 = 8.8 Hz, 1H), 8.25 (d, J = 2.1 Hz, 1H), 8.79 (d, J = 8.8 Hz, 1H), 10.34 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 25.99, 31.34, 123.05, 126.32, 133.50, 135.14, 136.36, 136.82, 169.38; MS, *m*/*z* (abundance): 272/274 ([M]$^{+\cdot}$, 3/3%), 193 (45%), 151 (100%).

5.1.8.2. (4-Acetylamino-3-nitrophenyl)methyl triphenyl phosphonium bromide. A mixture of 4-bromomethyl-2-nitroacetanilide (13.2 g, 48 mmol), Ph_3P (12.6 g, 48 mmol) in anhydrous toluene (100 mL) was heated at reflux for 8 h. The precipitate, corresponding with the desired product, was filtered and washed with dry toluene (white needles (11.4 g, 62%)). The salt was used without further purification.

5.1.8.3. 4-(2*E*/*Z*-Phenylethenyl)-2-nitroaniline (28). A mixture of benzaldehyde (150 mg, 1.4 mmol) (4-acetylamino-3-nitrophenyl)methyltriphenyl phosphonium bromide (760 mg, 1.4 mmol), K_2CO_3 (200 mg, 1.4 mmol), 18-crown-6 (20.0 mg), and THF (10 mL) as solvent was stirred at reflux during 8 h. The solvent was evaporated in vacuo, the residue was treated with brine (5 mL) and extracted with EtOAc (3 × 10 mL). After the work-up the organic layer was evaporated in vacuo. The residue was purified by column chromatography (SiO_2, petroleum ether/EtOAc (9:1)), yielding 4-(2*E*/*Z*-phenylethenyl)-2-nitroacetanilide as an orange oil (332 mg, 84%). A mixture of this product (236 mg, 0.8 mmol), concentrated hydrochloride acid (0.3 mL), and ethanol (10 mL) as solvent was heated at reflux during 1 h. The solvent was evaporated in vacuo and the residue was neutralized with aqueous saturated $NaHCO_3$ and extracted with EtOAc (3 × 10 mL). After the work-up the organic layer was evaporated in vacuo and the residue corresponding to the product, pure by TLC, was used without further purification (oil, 188 mg, 98%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 6.14 (br s, 2H), 6.83 (dd, J_1 = 3.0 Hz, J_2 = 8.7 Hz, 1H), 7.01 (s, 2H), 7.27 (br t, J = 7.7 Hz, 1H), 7.37 (br t, J = 7.3 Hz, 2H), 7.50 (br d, J = 6.6 Hz, 2H), 7.61 (d, J = 8.7 Hz, 1H), 8.24 (s, 1H); MS, *m*/*z* (abundance): 240 ([M]$^{+\cdot}$, 75%), 224 (9%).

5.1.8.4. 5-(2*E*/*Z*-Phenylethenyl)-*N*^{1}-oxidebenzo[1,2-*c*]-1,2,5-oxadiazole (21). The crude of the hydrolysis process (above reaction) was neutralized with NaOH (150 mg) and then NaOH (150 mg) was added. The mixture was cooled at 0 °C, aqueous NaClO (2.8 M) (1.5 mL) was added dropwise and the reaction was stirred during 30 min. Aqueous NaClO (2.8 M) (1.5 mL) was added again and the mixture was stirred during 30 min. The solvent was evaporated in vacuo and the residue was neutralized with aqueous HCl (10%) and extracted with EtOAc (3 × 10 mL). After the work-up the organic layer was evaporated in vacuo. The residue, brown solid (75%), was chromatographically (TLC) identical with *E*/*Z*-**21** obtained by the other procedure.

5.2. Biology

Trypanosoma cruzi epimastigotes (Tulahuen 2 strain) were grown at 28 °C in an axenic medium (BHI-Tryptose) as previously described,[10,12,37] complemented with 5% fetal calf serum. Cells from a 10-day-old culture (stationary phase) were inoculated into 50 mL of fresh culture medium to give an initial concentration of 1×10^6 cells/mL. Cell growth was followed by measuring everyday the absorbance of the culture at 600 nm. Before inoculation, the media were supplemented with the indicated amount of the drug from a stock solution in DMSO. The final concentration of DMSO in the culture media never exceeded 0.4% and the control was run in the presence of 0.4% DMSO and in the absence of any

drug. No effect on epimastigotes growth was observed by the presence of up to 1% DMSO in the culture media. To determine IC_{50} values, 50% inhibitory concentrations, parasite growth was followed in the absence (control) and presence of increasing concentrations of the corresponding drug. At day 5, the absorbance of the culture was measured and related to the control. The IC_{50} value was taken as the concentration of drug needed to reduce the absorbance ratio to 50%.

5.3. Studies of mechanism of action

5.3.1. Screening of benzofuroxans as ·NO donors. Nitric oxide (·NO) release was assessed by determining the main aerobic decomposition product of ·NO, nitrite.[38a] The benzofuroxans were assayed at a final 400 μM concentration in 0.1 M phosphate buffer, pH 7.4, either with or without 20 mM cysteine for 1 h at 25 or 37 °C. All assays contained 8% DMSO. Nitrite content was then determined by Griess reaction.[38b] Derivative *E*-**21** was not assayed due to poor solubility.

5.3.2. Oxygen uptake.[37] *Trypanosoma cruzi* epimastigotes (Tulahen 2 strain) from 5 days of growth were harvested by centrifugation (815 g for 15 min), washed and resuspended in 0.14 M NaCl, 2.7 mM KCl, 8 mM Na_2HPO_3, 15 mM KH_2PO_4, pH 7.4 (medium A). Protein concentration was determined by Biuret assay in the presence of 0.2% deoxycolate. *T. cruzi* cells (1 mg protein/mL) were incubated at 28 °C with or without 25 μM of benzofuroxan derivatives, and aliquots were taken with time to measure respiration (oxygen uptake). Oxygen consumption was determined with a water-jacketed Clark electrode (YSI Model 5300) using 1.5 mL reaction mixtures in medium A at 28 °C. The electrode was calibrated with oxygen-saturated medium A at 28 °C (220 μM O_2). The amount of parasites used in each assay was always the equivalent of 1 mg of protein/mL.

5.3.3. NaCN-insensitive oxygen uptake. Under sterile conditions, *T. cruzi* cells (late exponential phase) were inoculated into culture medium (1 mg protein/mL) with and without 25 μM of benzofuroxan derivatives. After 5 days of growth at 28 °C, cells were harvested by centrifugation and oxygen uptake determined as described above, before and after addition of 50 μM sodium cyanide. Control was run in the absence of drug and with 0.1% DMSO. Results are expressed as a percentage of control.

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