In Vivo Anti-Chagas Vinylthio-, Vinylsulfinyl-, and Vinylsulfonylbenzofuroxan Derivatives‡

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New benzofuroxans were developed and studied as antiproliferative *Trypanosoma cruzi* agents. Compounds displayed remarkable *in vitro* activities against different strains, Tulahuen 2, CL Brener and Y. Its unspecific cytotoxicity was evaluated using human macrophages being not toxic at a concentration at least 8 times, and until 250 times, that of its *T. cruzi* IC_{50}. Some biochemical pathways were studied, namely parasite respiration, cysteinyl active site enzymes and reaction with glutathione, as target for the mechanism of action. Not only *T. cruzi* respiration but also Cruzipain or trypanothione reductase were not affected, however the most active derivatives, the vinylsulfinyl- and vinylsulfonyl-containing benzofuroxans, react with glutathione in a redox pathway. Furthermore, the compounds showed good *in vivo* activities when they were studied in an acute murine model of Chagas' disease. The compounds were able to reduce the parasite loads of animals with fully established *T. cruzi* infections.

Introduction

Parasitic diseases affect hundreds of millions people around the world, mainly in underdeveloped countries. Since parasitic protozoa are eukaryotic, they share many common features with its mammalian host, making the development of effective and selective drugs a hard task. Despite the great effort that has been made in the discovery of unique targets that afford selectivity, many of the drugs used today to treat this parasitosis have serious side effects. Diseases caused by *Trypanosomatidae*, which share a similar state regarding drug treatment, include Chagas disease, the causative agent of which is *Trypanosoma cruzi* (*T. cruzi*[a]).[1] This trypanosomatid alone is responsible for an infected population of nearly 20 millions, and more than 200 million are at risk.[2] Although sequencing of the *T. cruzi* genome was recently completed,[3] no new drugs have been described yet. Drugs currently used in the treatment of Chagas disease are two nitroaromatic heterocyles, Nifurtimox (4-(5-nitrofurfurylindenamino)-3-methylthiomorpholine 1,1-dioxide) (Nfx) produced only in El Salvador by Bayer, and Benznidazole (*N*-benzyl-2-(2-nitro-1*H*-imidazol-1-yl)acetamide) (Bnz) produced by Roche. They were introduced empirically over three decades ago.[4] Both drugs are active in the acute phase of the disease, but its efficacies are very low in the established chronic phase. What is more, differences in drug susceptibility among different *T. cruzi* strains lead to varied parasitological cure rates according to the geographical area. Extensive work, in the last two decades, has helped to understand the molecular basis of the antichagasic activity of both drugs currently used in the clinic.[5] Nfx acts via the reduction of the nitro group to a nitroanion radical that in term reacts with oxygen to produce superoxide, a highly toxic metabolite, in a process known as redox-cycling. The mechanism of action of Bnz also involves nitro reduction, but reduced intermediates act covalently modifying bio-macromolecules.[6] Most frequent side effects of these drugs include anorexia, vomiting, peripheral polyneuropathy, and allergic dermopathy that are probably a result of oxidative or reductive damage to the host's tissue and are thus inextricably linked to its antiparasitic activity.[1] Other drugs have been analyzed as anti-*T. cruzi* agents in the last years, among them the antifungal Ketoconazole (1-[4-[4-(2*R*,4*S*)-2-(2,4-dichlorophenyl)-2-(1-imidazol-1-ylmethyl)-1,3-dioxolan-4 -yl]methoxy]-phenyl-1-piperazinylethanone) (Ktz) and Terbinafine (*N*-[(2*E*)-6,6-dimethyl-2-hepten-4-yn-1-yl]-*N*-methyl-1-naphthalenemethanamine) (Tbf) have been demonstrated to act as *T. cruzi* membrane sterol inhibitors.[7] These antifungal agents were found as excellent inhibitors of the sterol-membrane biosynthesis.[4a] It has been pointed out that drugs that produce oxidative stress by redox-cycling may be selective, as long as they are selectively reduced by oxidoreductases that are unique to the parasite.[8] The same could be said for drugs that produce reductive damage such as Bnz. Following this reasoning, our group has been looking for less toxic and more selective antichagasic drugs by using an *N*-oxide moiety as the bioreductive group.[9] Thus, benzofuroxan derivatives (benzo[1,2-*c*]1,2,5-oxadizole *N*-oxide) were described for the first time as *in vitro* anti-*T. cruzi* agents,[10] displaying activities similar or higher than the reference drugs (Chart 1).[11−14] Some of these studies demonstrated that the *N*-oxide moiety is essential for activity; additionally it was found

‡ 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 28,019, 2003: Derivados de 5-etenilbenzofuroxano, procedimiento de preparación y utilización.

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[a] Abbreviations: *T. cruzi*, *Trypanosoma cruzi*; Nfx, Nifurtimox; Bnz, Benznidazole; Ktz, Ketoconazole; Tbf, Terbinafine; CP, cruzipain; TR, trypanothione reductase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione oxidate; MCPBA, m-chloroperbenzoic acid; pi, postinfection; HB, hydrogen bond.

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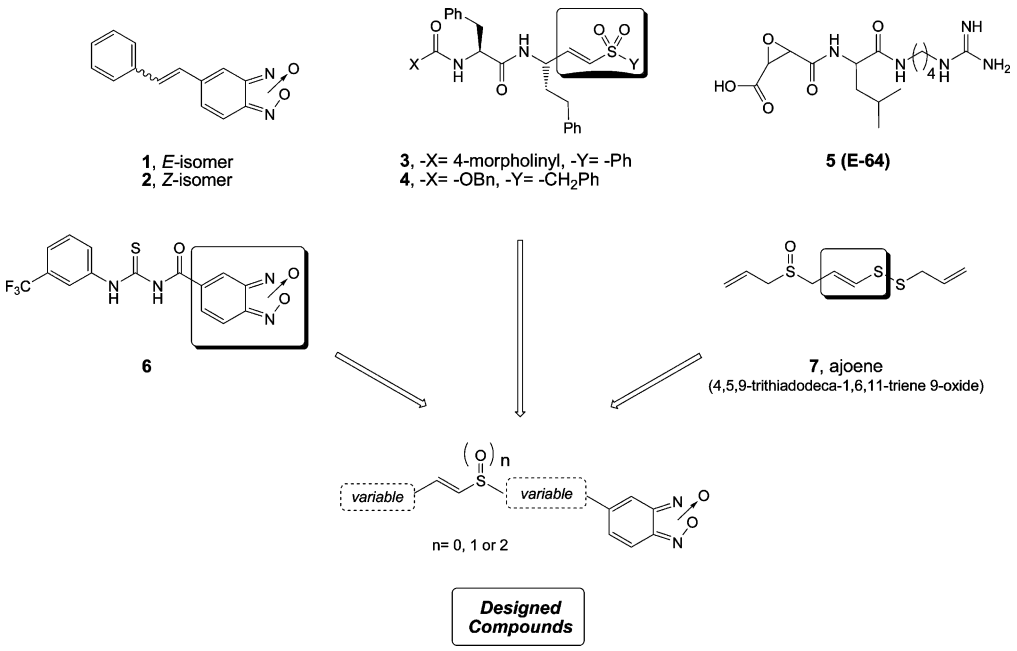
Chart 1. Experimentally Active Compounds against *T. cruzi* and Strategy to Design Novelty Structures



that some structural motives are important for optimal activity. Specially, derivatives **1** and **2** (Chart 1) porting a phenylethenyl moiety were some of the best anti-*T. cruzi* benzofuroxans developed by us.[15] Furthermore, our studies proved that some benzofuroxans generate free radicals in biological medium[16,17] and some others inhibit parasite respiration.[13]

In the field of developing effective agents acting on key targets in *T. cruzi* two of the most studied biomolecules are Cruzipain (CP) and trypanothione reductase (TR). CP is an endoproteinase able to digest proteins such as casein, bovine serum albumin, and denatured hemoglobin.[18] It is the major lysosomal proteinase of *T. cruzi* and can thus be expected to play a prominent role in nutrition of the parasite. In addition, other roles have been proposed for the enzyme, i.e., CP may be involved in the penetration of trypomastigote into the mammalian cell[19,20] and in the differentiation steps of the parasite's life cycle.[21] Engel and co-workers[22] demonstrated that vinylsulfonyl derivative **3** (Chart 1), among others, arrested growth of epimastigotes of *T. cruzi* at concentrations as low as 10 μM and caused cell death after 72 h. Others irreversible inhibitors have been described (**4**−**6**, Chart 1).[23] Benzofuroxan **6**, identified from a virtual screening using a comprehensive compound library, was one of the most interesting inhibitors[23c] and, according to the authors, presents the additional advantage that it adheres to Lipinski's "rule of 5" for becoming a potential drug.

On the other hand, 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.[24] Direct precursors for the biosynthesis of trypanothione are the tripeptide glutathione (GSH) and the polyamine spermidine (Figure 1). The absence of trypanothione from 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.[25] A large number of TR inhibitors have been studied, identifying those that bind to TR reversibly,[26] that act as subversive substrates (turncoat/sabotage inhibitors),[27] or that are irreversible inhibitors. Only a few irreversible TR inhibitors are known.[28] One is ajoene (**7**, Chart 1), the spontaneous degradation product of allicin, the major sulfur component of garlic. Ajeone is a subversive substrate and a covalent inhibitor of both reductases, and the modified enzymes show increased oxidase activity.[29] Recently, we described a family of hybrid metallic complexes which possess as the main mechanism of action the production of oxidative stress and the irreversible inhibition of TR.[28b]

Using compounds **1**−**7** (Chart 1) as a template for the design of new active anti-*T. cruzi* agents, we have planned the development of molecules that incorporate these recognized pharmacophores. In the present work, vinylthio-, vinylsulfinyl-, and vinylsulfonyl-containing benzofuroxans (Chart 1) were synthesized as entities that conjugate two different pharmacophores, namely the benzofuroxan system and the vinylthio moieties. The derivatives were examined for antiproliferative *in vitro* activity against three different *T. cruzi* strains, Tulahuen 2, CL Brener, and Y. Unspecific cytotoxicity of these derivatives was evaluated *in vitro* against human macrophages, THP-1 cells. For the best derivatives, biochemical studies were performed to get an insight into its mode of action. Effects on oxygen uptake, inhibition of two essential cysteinyl active site enzymes, CP and TR, and chemical reaction with GSH were analyzed. Docking studies were used to explain the compounds' lack of CP inhibition activity. Experimental chemotherapy studies were also performed using a murine model of Chagas disease with Tulahuen 2 infection, evaluating the blood trypomastigote and antibody levels.

Results

Chemistry. Two different approaches were used to obtain the two-pharmacophore-containing derivatives, the condensation/oxidation procedure to generate phenylthio-, phenylsulfinyl-, and phenylsulfonylvinyl derivatives, and substitution/elimination step to generate the vinylsulfonylmethyl derivative. In the first approach (Table 1), attempts to obtain sulfone **17** or **18** were performed through the condensation of sulfone **9**[30] with aldehyde

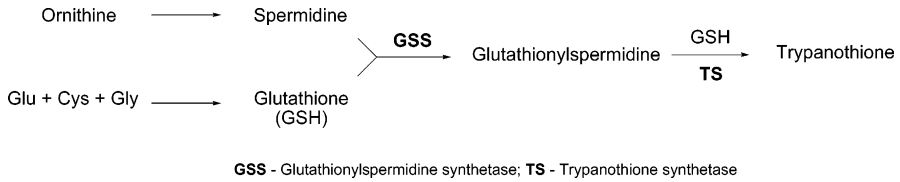
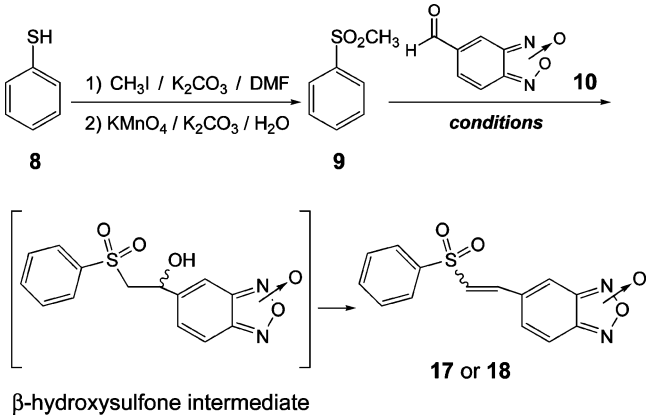


Figure 1. *De novo* trypanothione synthesis from glutathione and spermidine.

Table 1. Attempt to Obtain Derivatives **17**/**18** from Sulfone **9**



conditions	results
n-BuLi/THF/0 °C	5-hydroxymethylbenzofuroxan, 5-hydroxymethylbenzofurazan[a]
t-BuOK/THF/r.t. (several days)	no reaction
NaOH (50%)/CH_2Cl_2-H_2O/ r.t.	decomposition

[a] Reduction products.

10;[11] however, in the different assayed reaction conditions the desired products were not observed. When *n*-butyllithium (*n*-BuLi) was used as base, the formyl group was reduced to the corresponding alcohol, yielding 5-(hydroxymethyl)benzofuroxan, as well as the deoxygenated benzofuroxan system (benzofurazan derivative) as a secondary product. Besides, attempts to obtain the intermediate *β*-hydroxysulfone or sulfones **17**/**18** using *t*-BuOK or NaOH as base, were fruitless (see Table 1).

Consequently, the Boden−Wittig process[31] was assayed using phosphonium salt **12**[32] and aldehyde **10** (condition c, Scheme 1), generating the vinylthio derivatives **13** and **14**, as chromatographically separated geometric isomers, in good yields. Deoxygenated analogues, benzofurazans, were marginally generated in these olefination reactions even though Boden's mild conditions were used. Selective oxidation of sulfides **13** and **14** were performed using classical conditions. Vinylsulfinyl derivatives **15** and **16** were obtained using MCPBA at low temperature (−78 °C) and vinylsulfonyl derivatives **17** and **18** using H_2O_2 in acetic acid at reflux.[33] In the MCPBA oxidation, small amounts of derivatives **17**/**18** were obtained, less than 5%. In both conditions (MCPBA and H_2O_2), no olefin oxidation was observed. In the second chemical approach, a vinylsulfonylmethyl derivative was prepared (Scheme 2). For this, bromide **20**[12] was reacted with 2-mercaptoethanol, producing derivative **21** in good yield which was converted successively in sulfones **22** and **23** as it is shown in Scheme 2.[34]

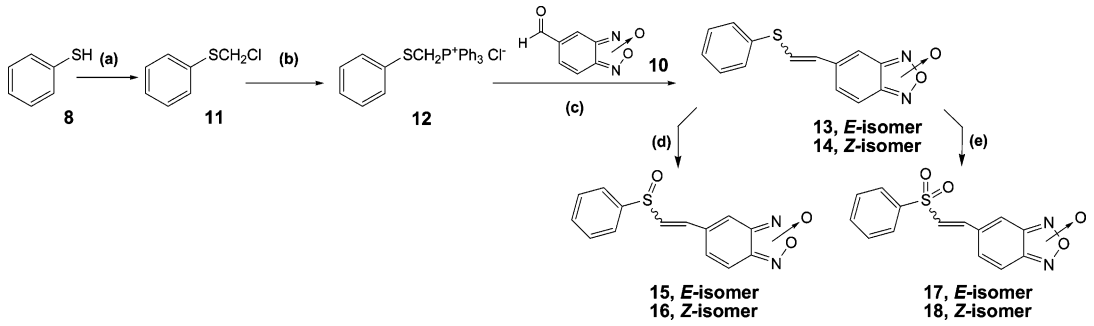
All of the proposed structures were established by NMR (^{1}H, ^{13}C, COSY, HMQC, and HMBC experiments), IR, and MS. The purity was established by TLC and microanalysis. The stereochemistry around the olefinic carbon−carbon bond was established using the corresponding ^{1}H NMR coupling constant. It is well-known that benzofuroxan derivatives exist at room temperature as a mixture of tautomers. The benzo substituent could occupy the 5- or 6-position, and the proportion of both tautomers in the equilibrium depends on the electronic characteristic of the substituent.[35] At 303 K, ^{1}H and ^{13}C NMR spectra of the benzofuroxans showed broad signals due to the rapid tautomeric equilibrium. When NMR experiments were carried out at low temperature, the aromatic region showed narrow peaks corresponding to both tautomers.[14,16,36,37]

Biology. *In Vitro* Antitrypanosomatid Activity. The existence of the epimastigote form of *T. cruzi* as an obligate mammalian intracellular stage has been revisited[38,39] and confirmed recently. Therefore, compounds were tested *in vitro* against the epimastigote form of the parasite.[40] As a first screening, the ability of developed derivatives to inhibit the growth of the epimastigote form of *T. cruzi* (Tulahuen 2, CL Brener ,and Y strains) was evaluated at 25 μM, and the IC_{50} was determined for the most active compounds (Table 2). This family of derivatives was evaluated against the *in vivo* susceptible Tulahuen 2 strain and CL Brener clone and the *in vivo* partially Nfx- and Bnz-resistant Y strain.[41] Parasites were grown in the presence of the compound for 5 days, and the percentage of growth inhibition was determined against control (no drug added to the medium) as explained in the Experimental Section.[42] Apolar benzofuroxans, **13**−**18** and **23**, showed good activity at 25 μM against the epimastigote form of the three different strains, and the IC_{50} was determined. The lower activity of derivative **22**, the most polar developed benzofuroxan, could be explained in term of our previous results[11,15] being the consequence of **22** hydrophilicity and the presence of a hydrogen bond donor moiety in the benzofuroxan lateral chain.

Unspecific Cytotoxicity. Cytotoxicity of the studied compounds against mammalian cells was evaluated *in vitro* at 25−1000 μM, using THP-1 human macrophages as the cellular model (Table 3).[14,43] In the study, Ktz and Tbf were included as trypanocidal references. *Z* stereoisomers, **2**, **16**, and **18**, were more toxic against human macrophages than the *E* analogues, maybe as consequence of better solubilities in the assay's biological medium. The new derivatives were less or as toxic as the parent compounds. Remarkably, phenylsulfonylvinyl derivative **17** showed the best selectivity indexes, being poorly toxic against the mammalian system at concentrations that are at least 250, 50,, and 80 times that of its IC_{50} against *T. cruzi* epimastigotes, Tulahuen 2, CL Brener, and Y strains, respectively.

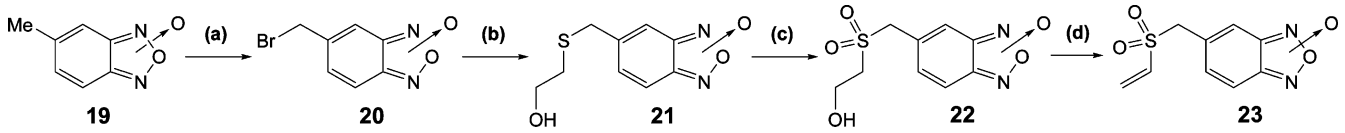
***In Vivo* Anti-*T. cruzi* Evaluation.**[44−46] The best anti-*T. cruzi* agents *in vitro* against Tulahuen 2 strain, compounds **13**−**18** as equimolecular mixtures of geometric isomers, **13**:**14** (1:1), **15**:**16** (1:1) and **17**:**18** (1:1), were evaluated *in vivo* in a murine model of acute Chagas disease. In this preliminary study, female Swiss mice were inoculated intraperitoneally with 2000 blood trypomastigotes, and treatment began 5 days post-infection with oral administration of 60 mg/kg/day of each compound during 30 days. The administration was done using a saline:Tween 80 (95:5) (vehicle) solution. A group treated in the same manner with vehicle (control) was included. The level of parasitemia was determined weekly[47] (Figure 2), the mortality was observed daily, and serological tests were performed 60 and 90 days post-infection (Table 4). None of the animals treated with benzofuroxans died during the treatment while in the control group the survival fraction was 75%.

Scheme 1[a]



[a] Reagents and conditions: (a) CH_2O/HCl (concd)/toluene/50 °C. (b) PPh_3/toluene/reflux. (c) K_2CO_3/18-crown-6/toluene/reflux. (d) MCPBA/CH_2Cl_2/−78 °C to rt. (e) H_2O_2 (30%)/AcOH/reflux.

Scheme 2[a]



[a] Reagents and conditions: (a) NBS/DBPO/CCl_4/reflux. (b) 2-mercaptoethanol/*t*-BuOK/THF/−10 °C. (c) MCPBA/CH_2Cl_2/ 0 °C to rt. (d) MsCl/Et_3N/CH_2Cl_2/0°.

Table 2. *In Vitro* Anti-*T. cruzi* Activity

	IC$_{50}$ (μM)[a,b]		
compd	T2[c]	CLB[d]	Y
1	10.8	7.5	6.2
2	7.0	15.7	9.0
13	2.6	4.7	7.6
14	3.8	3.5	10.0
15	2.6	15.1	8.8
16	0.7	5.0	1.5
17	1.6	7.6	5.0
18	1.1	6.1	2.1
22	>25.0	-[e]	-
23	14.6	9.1	12.3
Nfx	7.7	8.5	6.5
Bnz	7.4	4.5	3.8
Ktz	10.0	5.0	9.8
Tbf	17.1	42.0	44.7

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

Table 3. Cytotoxicity of Benzofuroxan Derivatives to THP-1 Human Macrophages

		SI[c]		
compd	THP-1 IC$_{50}$ (μM)[a,b]	T2[d]	CLB[e]	Y
1	109.9	10.2	14.7	17.7
2	62.6	8.9	4.0	7.0
15	124.0	47.7	8.2	14.1
16	105.0	150.0	21.0	70.0
17	409.0	255.6	53.8	81.8
18	109.0	99.1	17.9	51.9
22	300.0	< 12.0	-[f]	-
23	274.0	18.8	30.1	22.3
Ktz	44.0	4.4	8.8	4.5
Tbf	329.3	19.3	7.8	7.4

[a] IC$_{50}$: concentration that produces 50% inhibitory effect. [b] The results are the means of two different experiments with a SD less than 10% in all cases. [c] SI: selectivity index = IC$_{50,macrophage}$/IC$_{50,epimastigote}$. [d] T2: Tulahuen 2. [e] CLB: CL Brener strain. [f] Not determined.

The three compound mixtures were able to diminish the trypomastigote on the day of the maximum parasitic charge, day 26 post-infection (pi) (compare parasites' charge for untreated animals, 716 tryp/100 fields, to **17**:**18**-treated animals, 76 tryp/100 fields, **15**:**16**-treated animals, 160 tryp/100 fields, and to **13**:**14**-treated animals, 334 tryp/100 fields). For the benzofuroxan-treated animals, complete and permanent suppression of parasitemia was observed from day 41 pi that followed near zero parasitemia from day 35 pi. Benzofuroxan parasitemia levels, during all the studied days, were in agreement with the *in vitro* benzofuroxan behavior, showing *in vivo* activities, in general, in the order **17**:**18** > **15**:**16** > **13**:**14**. No signs of toxicity were observed during the animals' treatment with the equimolecular mixtures **13**:**14**, **15**:**16**, or **17**:**18**. All the

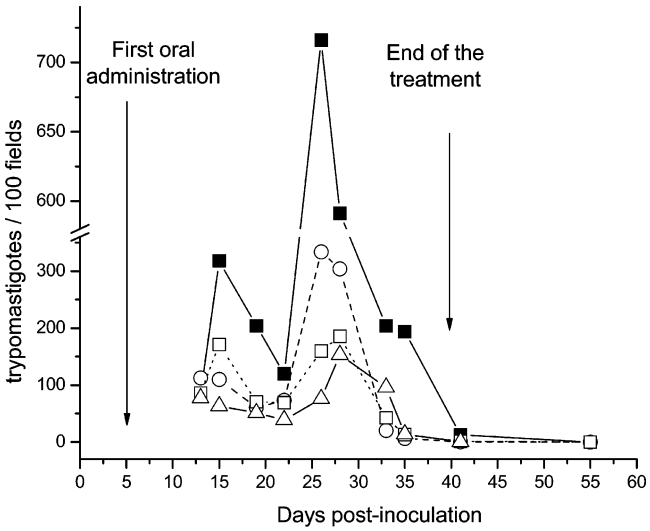


Figure 2. Parasitemia in the murine model of acute Chagas disease. Control (untreated) animals (■) and those receiving 60 mg/kg/d of **13**:**14** (1:1) (○), **15**:**16** (1:1) (□), or **17**:**18** (1:1) (Δ).

Table 4. Differences in the Level of Anti-*T. cruzi* Antibodies, Expressed in Absorbance Units (abs), between days 60th and 90th Postinfection for the Five Studied Groups

treatment	ΔA[a]
vehicle	0.028
Bnz[b]	0.071
13:**14** (1:1)	0.051
15:**16** (1:1)	−0.096
17:**18** (1:1)	−0.052

[a] ΔA: Absorbance at 490 nm, day 90 pi − Absorbance at 490 nm, day 60 pi. [b] Bnz treatment: 100 mg/kg/day, orally administered during 30 days (see Experimental Section).

Table 5. Inhibition of *T. cruzi* Cruzipain by **1**, **2**, **13**, **15**−**18**

compd	inhibitor concn (μM)	inhibition (%)[a,b]
1	10	12
	20	0
	50	43
	100	22[c]
2	10	19
	20	11
	50	20
	100	44
1:**2** (1:1)	10	0
	25	23
	50	42
13	10	0
	25	15
	50	25
15	10	2
	20	0
	50	24[d]
	100	12
16	10	0
	20	23
	50	8[d]
	100	3
17	10	0
	20	0
	50	35[d]
	100	0
18	10	2
	20	3
	50	24[d]
	100	19
E-64[e]	10	100

[a] The control assays contained the respective amount of DMSO. [b] The values are the mean of at least two independent measurements that differed by less than 10%. [c] Solubility problems. [d] At 50 μM concentration or higher the compounds were not completely soluble. [e] For structure see Chart 1.

animals survived until the end of the parasitemia study (day 55). None of the animals treated with the benzofuroxans or Bnz, used as control in the antibodies studies, showed negative anti-*T. cruzi* serology. However, equimolecular mixtures of sulfoxides, **15**:**16**, and sulfones, **17**:**18**, decreased antibodies levels between day 60 and 90 (Table 4), showing higher performance than Bnz in this assay. Differences in the level of anti-*T. cruzi* antibodies are in agreement with the parasitemia findings.

Mechanism of Action Studies. In order to confirm or exclude some possible mechanisms of action, the following studies were performed: effect on the parasite respiration, inhibition of CP and TR, and reaction with glutathione.

Oxygen Uptake Effect. The ability of benzofuroxans to modify parasite respiration was studied. Derivatives **17** and **18** were studied as described in Experimental Section, investigating its effect on parasite respiration.[48] None of them inhibit parasite respiration or increase the oxygen consumption as result of oxygen redox-cycling.

Inhibition of *T. cruzi* Cruzipain Studies. Some of the new benzofuroxans together with the parent compounds and methylbenzofuroxan, **19**, were studied as inhibitors of *T. cruzi* CP at 10−100 μM. None of the assayed derivatives resulted in good inhibitors of CP at the studied doses. Only the parent compounds, **1** and **2**, or as the equimolecular mixture (**1**:**2**, 1:1), caused nearly 50% inhibition at 50 and 100 μM, respectively (Table 5). Derivative **19** did not produce CP inhibition in the assayed conditions. Consequently, it could be said that the benzofuroxan system is not a CP inhibitor. On the other hand, the studied vinylthio-, vinylsulfinyl-, and vinylsulfonyl-containing benzofuroxans, **13** and **15**−**18**, were only weak inhibitors under the experimental conditions. The benzofuroxans' lack of CP inhibition was explained by docking studies.[49−51] In Table 6 are shown the residues in the binding site involved in ligand−CP complex formation. Ligands **17** and **18** were compared to inhibitor **3** as a reference structure. The vinylsulfonyl inhibitor **3** forms a covalent irreversible attachment to the active-site cysteine residue Cys25 of CP with the vinyl carbon adjacent to the Phe moiety. The Phe moiety of the inhibitor **3** binds in the S2 pocket of CP through a hydrogen bond (HB) between the nitrogen (N^a, see Figure 3a) to the C═O of Gly66. In the CP complex structure, HBs are formed between a SO_2 oxygen and hydrogen-donor side chains of the enzyme. Thus, one of the SO_2 oxygens has HBs with Gln19 (Nϵ2) and His159 (Nδ1). The other SO_2 oxygen makes HBs with Gln19 (Nϵ2) and Trp177 (Nϵ1) of the CP. In addition, compound **3** could establish a HB with Asp158 as well as aromatic interactions with Trp177. The interaction data predicted with the LPC program[52] reveal that the benzofuroxans **17** and **18** covalently bound to CP (Cys25) show one hydrogen bond between the vinylsulfonyl oxygen and His159 (Nδ1) and aromatic interaction with Trp177. However, the hydrogen bonds with Asp158, Gln19, and Gly66 do not exist, these interactions being crucial for inhibition (Table 6). The ΔG_{bind} and K_d of each complex calculated with the STC program[53] and the experimental IC_{50} (μM) values are shown in Table 6, showing that the predicted values of ΔG_{bind} are in agreement with the experimental results.

Inhibition of *T. cruzi* Trypanothione Reductase Studies. Derivatives **15**−**18** were first studied as reversible inhibitors of *T. cruzi* TR. The assays contained NADPH, TR, and 100 μM of each inhibitor and trypanothione disulfide (TS_2) which corresponds to a substrate concentration of 6 × K_m.[27b] The parent compound **1** was assayed at 40 μM because at higher concentrations it precipitated in the buffer. Under these conditions, none of the benzofuroxans proved to be inhibitors of TR. Only compound **15** showed a slight inhibition of 13%. An interesting observation was made when compounds **15**−**18** were assayed. The color of the assay solutions changed from slight yellow at the beginning to light-brown at the end of the assay. This was a first indication that reduced trypanothione [$T(SH)_2$] generated during catalysis reacted with compounds **15**−**18** (see following section). In a second series of kinetics, derivatives **15**−**18** were studied for its ability to inactivate reduced TR in a time-dependent fashion. None of the compounds caused more than 15% inactivation, the vinylsulfinyl derivative **16** and vinylsulfonyl derivative **18** being the best inactivators at 100 μM (Table 7). These results indicate that any of the studied benzofuroxan derivatives possess TR irreversible inhibition as the main anti-*T. cruzi* mode of action.

Table 6. Cruzipain (CP) Residues in Contact with the Studied Compounds, **3**, **17** and **18**, and Theoretical ΔGBind and K*d* and Experimental IC_{50} Values of the Studied Complexes

complex	Gly66 (O)	Gln19 (Nϵ2)	His 159 (Nδ1)	Trp177	Asp158 (O)	ΔG_{bind} (kcal/mol)	$K_{d\ (kcal/mol)}$	IC_{50} (μM)
1f29[a]	HB[b] (Na^c)	HB (**O**═S═**O**)	HB (S═**O**)	aromatic (Ph) HB (**O**═S)	HB (Nb^c)	−8.33	7.8×10^{-7}	0.008[d]
CP−**17**	-[e]	-	HB (S═**O**)	aromatic (Ph)	-	−4.45	0.5×10^{-3}	> 50.0
CP−**18**	-	-	HB (S═**O**)	aromatic (Ph)	-	−4.01	1.1×10^{-3}	> 50.0

[a] 1f29: complex CP−**3**, according to PDB code. [b] HB: hydrogen bond. [c] According to Figure 3a. [d] From reference 54. [e] No interaction.

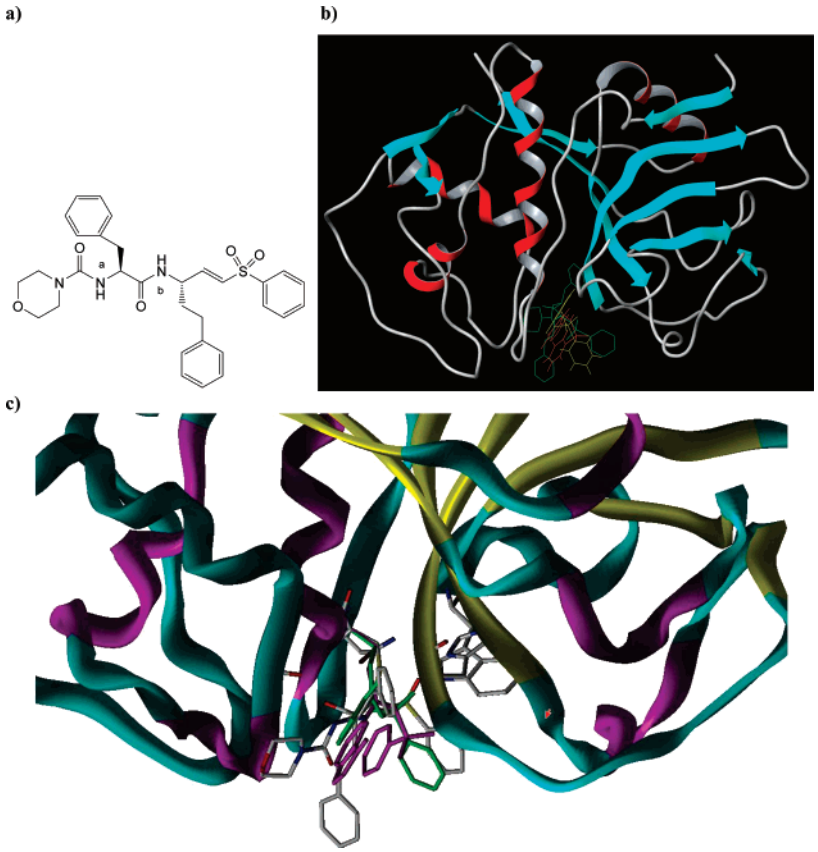


Figure 3. (a) Inhibitor **3**. (b) Structural superposition of **1f29** (inhibitor **3** in green) and the model complex with compounds **17** (yellow) and **18** (red). (c) Zoom of the structural superposition of **1f29** (inhibitor **3** in standard colors) and the model complex with compounds **17** (green) and **18** (purple).

Table 7. Time-Dependent Inactivation of Reduced *T. cruzi* Trypanothione Reductase (TR) by **15**−**18**

	volume activity (Δ*A*/min) at preincubation time (h)				
reaction mixture[a]	0 h	1 h	3 h	4 h	24 h
NADPH, TR, buffer, DMSO (control 1)	0.104	0.105	-[b]	0.104	-
15, TR, buffer (control 2)	0.104	0.086	-	0.091	-
15, NADPH, TR, buffer	0.103	0.105	-	0.105	-
NADPH, TR, buffer, DMSO (control 1)	0.112	0.108	0.118	-	0.095
16, TR, buffer (control 2)	0.101	0.107	-	-	0.108
16, NADPH, TR, buffer	0.107	0.107	0.090	-	0.081
NADPH, TR, buffer, DMSO (control 1)	0.104	0.105	-	0.104	-
17, TR, buffer (control 2)	0.102	0.110	-	0.106	-
17, NADPH, TR, buffer	0.101	0.099	-	0.098	-
NADPH, TR, buffer, DMSO (control 1)	0.104	0.105	-	0.104	-
18, TR, buffer (control 2)	0.104	0.102	-	-	-
18, NADPH, TR, buffer	0.097	0.084	-	0.088	-

[a] For conditions see Experimental Section. [b] Not determined.

Reactivity with Glutathione. In order to confirm the capacity of the new benzofuroxans to react with thiols in the reductive metabolism of the parasite, GSH was used as a model compound. The reactions between benzofuroxans, **2**, **14**, **16**, or **18**, and GSH were followed spectroscopically in the UV−visible region. The *Z*-isomers were selected because of their high solubility under the assay conditions and for their optimum activity compared to the *E*-isomers. The results are shown in Figure 4. Clearly, it was confirmed that the vinylsulfinyl and vinylsulfonyl derivatives, **16** and **18**, react with this biological thiol faster than the parent compound **2** and the vinylthio derivative **14**. The first compounds were able to react with GSH, at 28 °C, after 2 min of incubation. After 48 h, parent compound **2** was unable to react with GSH. Taking into account the UV-signal shifts, the GSH reacted mainly via a redox pathway, producing GSSG and the corresponding vinylthio- and vinylsulfinyl derivatives from compound **18** and the vinylthio-containing benzofuroxan from **16** (Figure 4c). These results were confirmed by isolation and characterization of the redox products. Kinetic studies were not performed; however, it was qualitatively observed that compound **16** reacted with GSH faster than compound **18**. Oxadiazole reduction product formation (the corresponding dioxime; see Figure 4c) was not observed in the UV spectrum (data not shown).

Discussion

The new benzofuroxan derivatives, **13**−**18**, **22**, and **23**, are efficiently obtained using condensation/oxidation or substitution/oxidation procedures and chromatographically isolated as pure stereoisomeric forms. Except derivative **22**, the new benzofuroxans maintain or improve the parent compounds' activities *in vitro* against parasite *T. cruzi*; for example, derivatives **16** or **18** are at least 3 times more active than parent compound **2** *in vitro*. Furthermore, these new benzofuroxans have low mammalian cytotoxicity; in our *in vitro* model the new compounds are less or as toxic as the parent compounds, derivatives **16**−**18** having the best selectivity index (expressed as $IC_{50,macrophage}/IC_{50,epimastigote}$). The activity does not relate to inhibition of cellular respiration, CP, or TR. According to docking studies, the lack of CP inhibition is the result of structural motive deficiency in the developed compounds (see Table 5 and Figure 3). In the case of TR, molecular details using docking were not studied, but according to our results, it is possible to summarize

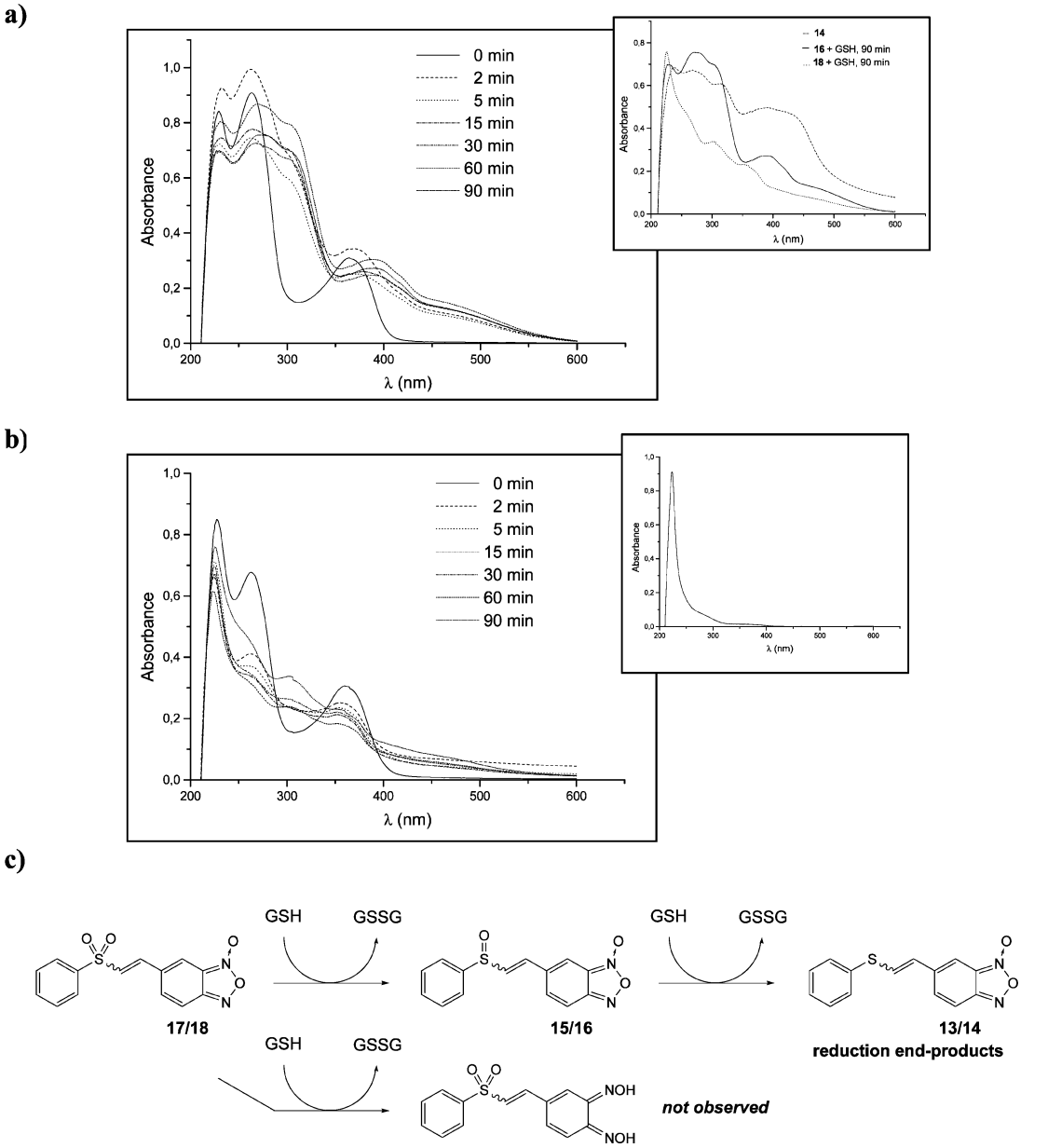


Figure 4. Studies of the reaction between benzofuroxans **14**, **16**, and **18** and GSH using UV−visible spectroscopy. (a) Spectra changes with time for compound **16** treated with GSH. Inset: spectrum of derivative **14** (**16**'s and **18**'s reduction end-product) and **16** and **18** after GSH treatment. (b) Spectra changes with time for compound **18** treated with GSH. Inset: spectrum of GSH. (c) Proposed redox pathway for derivatives **15/16** and **17/18**.

that only presence of sulfoxide or vinylthio moieties, as in ajoene (Chart 1), is not enough for TR inhibition capacity. Any of the studied compounds were able to inactivate reduced TR in a time-dependent manner.

The mammal/parasite selectivity for these compounds could be explained in terms of the vinylsulfinyl and vinylsulfonyl derivatives' capability to react with GSH in a redox process. First, it is well-known that GSH is one of the essential precursors of TSH synthesis in the parasite cells (Figure 1). Second, *T. cruzi* has much lower GSH levels than those of the mammalian host. Therefore, in parasites the depletion of GSH levels, and consequently TSH levels, could be more dangerous than the GSH diminution in mammals. While in mammals GSH synthesis can be inhibited in up to 80−90% without evidence of toxicity, in *T. cruzi* this situation aggravates the precarious defense against oxidative stress.[55] Accordingly, in mammalian cells the effects of derivatives **15**−**18** could be precluded by the enzyme GR maintaining the GSH level (see upper pathway in Figure 5). On the contrary, in the parasitic cells compounds **15**−**18** could decrease GSH levels irreversibly because TR is unable to reduce GSSG effectively (see lower pathways in Figure 5), although another possibility is that GSSG could deplete TSH, tryparedoxin (Tpx), and/or TR as a result of chemical[55,56] or biochemical[24,57] pathways (Figure 5, lower pathways). This proposal is in agreement with the differential *in vitro* cytotoxicity of studied sulfoxides and sulfones (compare selectivity index values, Table 3, for *Z*-derivatives **16** and **18** with the *Z*-parent compound **2**).

Furthermore, the oral treatments of Tulahuen 2-infected mice at 60 mg/kg/day with the most *in vitro* active benzofuroxans, for a total of 30 doses, produce parasitological eradication and complete animal survival and in some cases decrease the anti-*T. cruzi* antibody levels at the end of the experimental protocol. According to these findings, the equimolecular mixture of

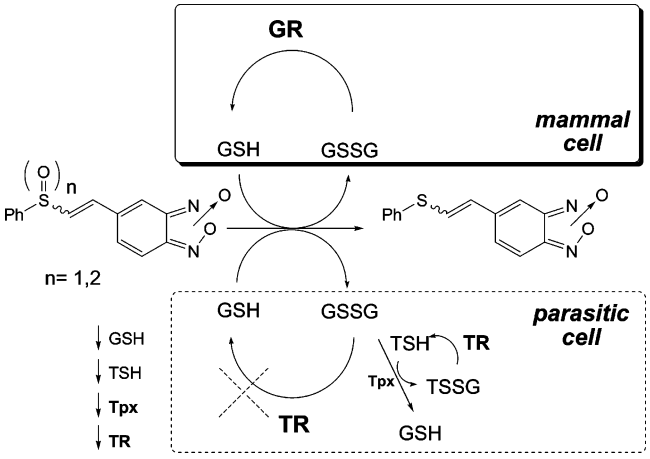


Figure 5. Speculative mechanisms of vinylsulfinyl- or vinylsulfonylbenzofuroxan selective mammal cytotoxicities.

vinylsulfinyl derivatives **15** and **16** has the best *in vivo* activity, the *Z*-isomer **16** being the best *in vitro* anti-*T. cruzi* agent against Tulahuen 2 and the Nfx, Bnz-partially resistant Y strains. Furthermore, no signs of toxicity in the studied animals, during the treatment, were observed.

Conclusions

The new benzofuroxan derivatives developed in this study were *in vitro* active against different strains of the protozoa *T. cruzi*. In general, parasite toxic effects were not associated with mammal cytotoxicity in macrophages. Derivative **17**, with a phenylsulfonylvinyl substituent, possesses the best $IC_{50,macrophage}/IC_{50,epimastigote}$ ratio (more than 200 in the case of Tulahuen 2 strain). The rest of the new derivatives did not possess unspecific cytotoxicity in human macrophages at a concentration at least 8 times that of its IC_{50} against *T. cruzi* (i.e., derivative **15**, with a phenylthiovinyl substituent). The structural motives included in these compounds, the vinylsulfinyl and vinylsulfonyl moieties, could act as thiol-depleting entities. The inhibition of cysteinyl active site enzymes, CP and TR, was excluded as the main mechanism of action. Additionally, after 30 day treatments, these vinylthio, vinylsulfinyl, and vinylsulfonyl derivatives were able to reduce the parasitemia of acute infected animals with Tulahuen 2 strain without toxicity signs. The effects of the sulfoxides and sulfones were also demonstrated with the anti-*T. cruzi* antibody level modifications. In summary, a new family of antiparasitic agents was developed that displays promising *in vivo* activity, deserving further study. The synthesis of new derivatives in order to complete QSAR analysis and preclinical studies, such as doses, schedule, strains, and toxicological studies, is currently in progress.

Experimental Section

Compounds **1**, **2**, **10**, **11**, **19**, and **20** were prepared according to literature procedures.[11,12,15,32] Melting points were determined with an electrothermal melting point apparatus (Electrothermal 9100) and are uncorrected. Proton and carbon NMR spectra were recorded on a Bruker DPX-400 spectrometer. The chemical shifts values are expressed in ppm relative to tetramethylsilane as internal standard. 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. Microanalyses were performed on a Fisons EA 1108 CHNS-O instrument and were within ±0.4% of the calculated compositions. Column chromatography was carried out using Merck silica gel (60−230 mesh). Most chemicals and solvents were analytical grade and used without further purification. All the reactions were carried out in a nitrogen atmosphere. The typical workup included washing with brine and drying the organic layer with sodium sulfate before concentration.

Triphenyl(phenylthiomethyl)phosphonium Chloride (12). A solution of **11** (1.0 g, 6.3 mmol) and Ph_3P (1.65 g, 6.3 mmol) in dry toluene (10.0 mL) was heated at reflux for 12 h. Then the mixture was allowed to cool to room temperature, and the white crystalline solid was collected and washed with petroleum ether and ethyl ether. This procedure was repeated until absence of PPh_3 in the organic solvent (checked by TLC). White solid, 1.8 g (68%). mp 225.0−227.0 °C; ^{1}H NMR ($CDCl_3$) δ 5.51 (d, J = 9.6 Hz, 2H), 7.22 (m, 3H), 7.34 (m, 2H), 7.65 (m, 2H), 7.76 (m, 7H), 7.88 (m, 6H); ESI-MS, *m/z*: 385.1 ($M^{+\cdot}$ − Cl); Anal. ($C_{25}H_{22}ClPS$) C, H, N, S.

5(*E* and *Z*)-[2-(Phenylthio)vinyl]benzo[1,2-*c*]1,2,5-oxadiazole *N*^{1}-Oxide (13 and 14). A mixture of **10** (0.5 g, 3 mmol), phosphonium salt **12** (1.3 g, 3 mmol), K_2CO_3 (0.4 g, 3 mmol), 18-crown-6 (10 mg, 0.03 mmol), and dry toluene as solvent was stirred at reflux during 2.5 h. Then the mixture was allowed to cool to room temperature, the brown solid was collected and washed with toluene, and the organic solvent was evaporated *in vacuo*. The residue was purified by column chromatography (SiO_2, petroleum ether:EtOAc (95:5 to 90:10)), yielding derivative **13** as an orange-yellow solid (0.37 g, 45%) and derivative **14** as an orange solid (0.26 g, 32%). **13**: mp 79.0−80.0 °C; ^{1}H NMR ($CDCl_3$): 6.97 (d, 1H, J = 15.4 Hz), 7.40 (bs, 1H), 7.50−7.60 (bs, 2H), 7.61 (d, 1H, J = 15.2 Hz), 7.62 (m, 2H), 7.68 (d, 1H, J = 7.8 Hz), 8.00 (d, 2H, J = 7.4 Hz); EI-MS, *m/z* (abundance, %): 270 ($M^{+\cdot}$, 100), 254 (94), 238 (14), 223 (47), 210 (100), 165 (62); Anal. ($C_{14}H_{10}N_2O_2S$) C, H, N, S. **14**: mp 89.0−90.0 °C; ^{1}H NMR ($CDCl_3$): 6.48 (d, J = 10.8 Hz, 1H), 6.85 (d, J = 10.8 Hz, 1H), 7.50−7.60 (bs, 3H), 7.41 (m, 3H), 7.51 (d, J = 7.5 Hz, 1H); EI-MS, *m/z* (abundance, %): 270 ($M^{+\cdot}$, 100), 254 (87), 238 (9), 223 (38), 210 (100), 165 (33); Anal. ($C_{14}H_{10}N_2O_2S$) C, H, N, S.

5(*E* and *Z*)-[2-(Phenylsulfinyl)vinyl]benzo[1,2-*c*]1,2,5-oxadiazole *N*^{1}-Oxide (15 and 16). A solution of **13** or **14** (0.25 g, 0.93 mmol) in CH_2Cl_2 (6.0 mL) was cooled to − 78 °C while a solution of *m*-chloroperbenzoic acid (0.16 g, 0.93 mmol) in CH_2Cl_2 (2.0 mL) was added dropwise during 5 min. The mixture was stirred and warmed to room temperature for 2 h and then poured into saturated sodium bicarbonate solution (10.0 mL) and the mixture extracted with CH_2Cl_2 (3 × 10.0 mL). After the workup of the combined organic layers, the residue was purified by column chromatography (SiO_2, petroleum ether:EtOAc (8:2)), yielding derivative **15** as a yellow solid (0.12 g, 45%) or derivative **16** as a brown solid (0.11 g, 42%). **15**: mp 120.0−122.0 °C; ^{1}H NMR ($CDCl_3$): 6.99 (d, 1H, J = 15.4 Hz), 7.38 (d, 1H, J = 15.5 Hz), 7.40−7.60 (bs + m, 6H), 7.70 (m, 2H); EI-MS, *m/z* (abundance, %): 286 ($M^{+\cdot}$, 8), 270 (5), 254 (41), 224 (23), 125 (100); Anal. ($C_{14}H_{10}N_2O_3S$) C, H, N, S. **16**: mp 106.0−107.0 °C; ^{1}H NMR ($CDCl_3$): 6.75 (d, 1H, J = 10.8 Hz), 7.32 (d, 1H, J = 10.8 Hz), 7.60 (m, 3H), 7.75 (bs + m, 5H); EI-MS, *m/z* (abundance, %): 286 ($M^{+\cdot}$, 4), 270 (3), 224 (45), 125 (100); Anal. ($C_{14}H_{10}N_2O_3S$) C, H, N, S.

5(*E* and *Z*)-[2-(Phenylsulfonyl)vinyl]benzo[1,2-*c*]1,2,5-oxadiazole *N*^{1}-Oxide (17 and 18). To a stirred solution of **13** or **14** (0.2 g, 0.74 mmol) in glacial AcOH (3.0 mL) was slowly added H_2O_2 (30%) (0.3 mL), and the mixture was heated at reflux for 20 min. The reaction mixture was cooled, neutralized with aqueous $NaHCO_3$, and extracted with EtOAc. After the workup of the combined organic layers, the residue was purified by column chromatography (SiO_2, petroleum ether/EtOAc (8:2)), yielding derivative **17** as a yellow solid (0.19 g, 86%) or derivative **18** as a yellow solid (0.13 g, 58%) and derivative. **17**: mp 168.0−170.0 °C; ^{1}H NMR ($CDCl_3$): 6.97 (d, 1H, J = 15.4 Hz), 7.40 (bs, 1H), 7.50−7.60 (bs, 2H), 7.61 (d, 1H, J = 15.2 Hz), 7.62 (m, 2H), 7.68 (d, 1H, J = 7.8 Hz), 8.00 (d, 2H, J = 7.4 Hz); EI-MS, *m/z* (abundance, %): 302 ($M^{+\cdot}$, 5), 286 (15), 203 (33), 125 (100); Anal. ($C_{14}H_{10}N_2O_4S$) C, H, N, S. **18**: mp 150.0−152.0 °C; ^{1}H NMR ($CDCl_3$): 6.70 (d, 1H, J = 12.0 Hz), 7.03 (d, 1H, J = 12.0 Hz), 7.40−7.69 (bs+t, 6H), 7.87 (d, 2H, J = 7.4 Hz); EI-MS, *m/z*

(abundance, %): 302 ($M^{+\cdot}$, 2), 286 (8), 203 (18),125 (100); Anal. ($C_{14}H_{10}N_2O_4S$) C, H, N, S.

5-(2-Hydroxyethylthiomethyl)benzo[1,2-*c*]1,2,5-oxadiazole *N*^{1}-Oxide (21). A solution of *t*-BuOK (0.15 g, 1.32 mmol) and 2-mercaptoethanol (0.10 mL, 1.32 mmol) in dry THF (10.0 mL) was added dropwise to a solution of **20** (0.30 g, 1.32 mmol) in dry THF (10.0 mL). The temperature was maintained at −10 °C for 30 min and then warmed to room temperature for 2 h. The solvent was removed *in vacuo* and then poured into water (30.0 mL) and extracted with AcOEt (3 × 10.0 mL). After the workup of the combined organic layers, the residue was purified by column chromatography (SiO_2, petroleum ether/EtOAc (7:3)), yielding derivative **21** as a pale yellow oil (0.12 g, 40%). ^{1}H NMR ($CDCl_3$): 2.67 (t, J = 5.9 Hz, 2H), 3.76 (s, 2H), 3.80 (t, J = 5.8 Hz, 2H), 3.94 (m, 1H), 7.33 (bs, 1H), 7.55 (bs, 2H); ESI-MS, *m/z*: 227 ($M^{+\cdot}$ + H); Anal. ($C_9H_{10}N_2O_3S$) C, H, N, S.

5-(2-Hydroxyethylsulfonylmethyl)benzo[1,2-*c*]1,2,5-oxadiazole *N*^{1}-Oxide (22). A solution of **21** (0.11 g, 0.49 mmol) in CH_2Cl_2 (4.0 mL) was cooled at 0 °C while a solution of *m*-chloroperbenzoic acid (0.21 g, 1.22 mmol) in CH_2Cl_2 (2.0 mL) was added dropwise. The mixture was warmed to room temperature until derivative **21** was not present (SiO_2, petroleum ether:EtOAc (8:2)). After the workup, the residue was purified by column chromatography (SiO_2, petroleum ether:EtOAc (100 to 90:10)), white solid (0.79 g, 63%). mp 128.0−130.0 °C; ^{1}H NMR ($CDCl_3$): 3.30 (t, J = 5.5 Hz, 2H), 4.11 (m, 2H), 4.49 (m, 1H), 4.64 (s, 2H), 7.55 (bs, 1H), 7.71 (bs, 2H); ESI-MS, *m/z*: 259 ($M^{+\cdot}$ + H); Anal. ($C_9H_{10}N_2O_5S$) C, H, N, S.

5-Vinylsulfonylmethylbenzo[1,2-*c*]1,2,5-oxadiazole *N*^{1}-Oxide (23). A solution of **22** (55 mg, 0.21 mmol) in CH_2Cl_2 (5.0 mL), Et_3N (75 μL, 0.53 mmol), and methanesulfonyl chloride (0.20 μL, 0.23 mmol) was stirred at 0 °C for 20 min. The mixture was washed with a saturated solution of NH_4Cl (5.0 mL), dried, concentrated *in vacuo*, and purified by column chromatography (SiO_2, petroleum ether/EtOAc (8:2)), yielding **23** (25 mg, 49%) as a pale yellow solid. mp 134.0−136.0 °C; ^{1}H NMR ($CDCl_3$): 4.62 (s, 2H), 6.25 (d, J = 9.2 Hz, 1H), 6.28 (d, J = 2.5 Hz, 1H), 6.97 (dd, J = 9.9 Hz, J = 6.7 Hz, 1H), 7.52 (bs, 1H), 7.67 (bs, 2H); ESI-MS, *m/z*: 241 ($M^{+\cdot}$ + H); Anal. ($C_9H_8N_2O_4S$) C, H, N, S.

***In Vitro* Anti-*T. cruzi* Activity.** *Trypanosoma cruzi* epimastigotes (Tulahuen 2 strain, Brener strain, Y strain) were grown at 28 °C in an axenic medium (BHI-tryptose) complemented with 5% fetal calf serum. Cells from 5-day-old culture (stationary phase) were inoculated to 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 medium was supplemented with the indicated amount of the studied compound from a stock solution in DMSO. The final concentration of DMSO in the culture medium never exceeded 0.4%, and the control was run in the presence of 0.4% DMSO and in the absence of compound. No effect on epimastigote growth was observed in the presence of up to 1% DMSO in the culture medium. The percentage of inhibition was calculated as follows: $\% = \{1 - [(A_p - A_{0p})/(A_c - A_{0c})]\}100$, where $A_p = A_{600}$ of the culture containing the drug at day 5; $A_{0p} = A_{600}$ of the culture containing the compound just after the 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 the control. The IC_{50} value was taken as the concentration of compound needed to reduce the absorbance ratio to 50%.

Cytotoxicity to Human Macrophages. THP-1 human macrophages were seeded (100000 cells/well) in 96-well flat bottom 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. Afterward, the cells were washed with PBS and incubated (37 °C) with 0.4 mg/mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma) for 3 h. Then, formazan was dissolved with DMSO (180 μL), and optical densities were measured. Each concentration was assayed three times, and six growth controls were used in each test. Cytotoxicity percentages (%*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.

***In Vivo* Anti-*T. cruzi* Activity (acute model)**. Swiss female mice (45 days old, 25−30 g) were infected by intraperitoneal injection of 2000 blood trypomastigotes. One group of four animals was used as control, treated with vehicle (saline:Tween 80, 95:5), and three groups of five animals were treated with the three mixtures of studied benzofuroxans. For serologic studies, one group of four animals treated with Bnz was included. Treatments were started 5 days after animal infection. Compounds were administered orally as a vehicle suspension (0.1 mL/mouse), at a dose of 60 mg/kg/day for benzofuroxan derivatives and 100 mg/kg/day for Bnz, during 30 days (6 day treatment, 1 day rest, until 30 doses completed). The level of parasitemia was checked weekly by counting in a Neubauer chamber the number of parasites in 5 μL of blood drawn from the tail of the mice and diluted 1:10 in ammonium chloride solution. Quantitative evaluation of circulating anti-*T. cruzi* antibodies, at days 60 and 90 postinfection, was carried out by the use of an enzyme-linked immunospot assay. The sera diluted to 1:100 was reacted with an antigen constituted by a soluble homogenate of *T. cruzi* epimastigotes.[45] The results are expressed as the ratio of the absorbance (*A*) of each serum sample at 490 nm to the cutoff value. The cutoff for each reaction was the mean of the values determined for the negative controls plus three times the standard deviation.

Animals. Animals were housed in wire mesh cages at 20 ± 2 °C with natural light−dark cycles. The animals were allowed to feed "ad libitum" to a standard pellet diet and water and were used after a minimum of 3 days acclimation to the housing conditions.[58] The experimental protocols with animals were evaluated and supervised by the local Ethics Committee, and the research adhered to the Principles of Laboratory Animal Care.[59] Animals were evaluated by supervision of international protocols, and they were sacrificed in a humane way in accordance with recognized guidelines on experimentation. At the end of experiments they were anesthetized with ethyl ether and sacrificed by cervical dislocation.

Oxygen Uptake.[17] *T. cruzi* epimastigotes (Tulahuen 2 strain) from 5 days of growth were harvested by centrifugation (800*g* for 10 min), washed with a solution of NaCl (0.14 M), KCl (2.7 mM), Na_2HPO_3 (8 mM), KH_2PO_4 (15 mM), pH 7.4 (medium A), and then resuspended in medium A−glucose (5.5 mM glucose). Protein concentration was determined by the biscinchoninic acid assay. Oxygen consumption was determined with a water-jacketed Clark electrode (YSI Model 5300) using a 2 mL reaction mixture in medium A−glucose 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 to 0.2 mg of protein/mL (40 × 10^6 parasites/ mL). Drugs were added at a 200 μM final concentration in DMSO. Control was run in the absence of drug and with 0.1% DMSO. Cyanide-insensitive respiration was determined after addition of KCN at a final concentration of 20 mM.

***T. cruzi* Cruzipain (CP) Inhibition Assays. Experimental Studies.** Cruzipain was purified to homogeneity from epimastigotes of the Tulahuen 2 strain by ConA-Sepharose affinity chromatography, as previously described,[60] 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 on a Beckman Model 25 recording spectrophotometer. The inhibitors were added as solutions in DMSO, and the controls contained the same solvent concentration. E-64 was used as reference.

Theoretical Studies. CP-docking studies were first performed on a set of well-known ligands covalently bound to the active-site Cys25 of CP[54] (for example, compound **3** in Table 6). In this study the high-resolution crystal structure of CP bound with phenyl-containing vinylsulfone inhibitor **3** (pdb code 1f29[54]) was used as a reference structure, adding protein hydrogen atoms and calculating partial charges with the MMFF94 procedure[51] implemented in the Sybyl 6.9 program.[50] The molecular docking studies with **17** and **18** were carried out using Flexidock,[49] implemented in Sybyl 6.9, minimizing by use of the MMFF94 force field and the MMFF94 charges. The structures of the ligands **17** and **18** were built with standard bond lengths and angles using Sybyl, and their energies were minimized using the Powel method with a conjugated gradient of <0.001 kcal/mol convergent criteria provided by the MMFF94 force field and electrostatic charges based on the same method. An initial model of the complex CP−inhibitor for compounds **17** and **18** was obtained by transference of system coordinates of vinylsulfonyl derivatives **17** and **18** to the structure of the CP complex (pdb code 1f2A) using the option Fit Monomer of Sybyl, and the covalent bond between Cys 25 and the vinyl carbon at the β position of SO_2 group of **17** and **18** was created. Once the ligand was situated manually in the active site of the protein, both side chains and the ligand were minimized using the MMFF94 force field and the MMFF94 charges while leaving the backbone rigid using the Powel method with a conjugated gradient of <0.03 kcal/mol convergent criteria. These complexes represent the entry for the docking studies. A molecular docking approach was employed to identify the more favorable interaction of **17** and **18** in the binding site of the CP receptor. During the flexible docking analysis, the protein was considered rigid except the residues involved in the binding site and the ligands were considered flexible. The default FlexiDock parameters were utilized in all cases, with iterations set to 30000, obtaining a series of model complexes. Analysis of the receptor−ligand complex models generated was based on the hydrogen bond and aromatic and hydrophobic interactions predicted with the LPC program[52] and the free energy and dissociation constants obtained from the difference accessible surface area obtained using the STC program.[53]

***T. cruzi* Trypanothione Reductase (TR) Inhibition Assays.** Recombinant *T. cruzi* TR was prepared according to a published procedure.[61] 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.[27b] Stock solutions of the compounds were prepared in DMSO. The assay mixtures (1 mL) contained TR assay buffer, 100 μM NADPH, 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.

Test of Time-Dependent Inactivation of Reduced TR. In a total volume of 50 μL of TR assay buffer (40 mM HEPES, 1 mM EDTA, pH 7.5), 170 mU TR was incubated at 25 °C with 100 μM inhibitor in the presence of 160 μM NADPH. At different times between 0 and 24 h, 5 μL aliquots were removed, and the remaining activity was measured in a standard TR assay. Because of the 200-fold dilution, reversible inhibition was not recorded under these conditions. Two control series contained buffer, TR, and NADPH or buffer, TR, and inhibitor.

Reactivity with Glutathione. Solution A: GSH (100 mM) in phosphate buffer (0.1 M Na_2HPO_4, 1.5 mM EDTA, pH 7.4). Solution B: studied compounds (20 mM) in anhydrous EtOH. The reaction was started by mixing solution A (5 μL) (GSH final concentration 1 mM) with solution B (5 μL) (studied compound final concentration 200 μM) in phosphate buffer (490 μL). The reaction mixture was maintained at 28 °C. At variable times (0, 2, 5, 15, 30, 60, and 90 min) the reactions were quenched by cooling, at 0 °C, and the UV−visible spectra (Shimadzu UV-1603) was acquired between $\lambda = 200$ and 600 nm. As negative controls, GSH and each compound were incubated at 28 °C in phosphate buffer.

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Supporting Information Available: Elemental analysis results for benzo[1,2-*c*]1,2,5-oxadiazole N^1-oxide derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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