Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 16 (2008) 2984–2991
Bioorganic & Medicinal Chemistry

Novel 1,3,4-thiadiazolium-2-phenylamine chlorides derived from natural piperine as trypanocidal agents: Chemical and biological studies

Welisson da Silva Ferreira,[a] Leonardo Freire-de-Lima,[b] Víctor Barbosa Saraiva,[b] Frederico Alisson-Silva,[b] Lucia Mendonça-Previato,[b] José Osvaldo Previato,[b] Aurea Echevarria[a] and Marco Edilson Freire de Lima[a,*]

[a]*Universidade Federal Rural do Rio de Janeiro, Instituto de Ciências Exatas, Departamento de Química, Rodovia BR 465, Km 7, CEP 23.890-000 Seropédica, RJ, Brazil*
[b]*Universidade Federal do Rio de Janeiro, Centro de Ciências da Saúde, Instituto de Biofísica Carlos Chagas Filho-Ilha da Cidade Universitária, CEP 21.990-400 Rio de Janeiro, RJ, Brazil*

Received 29 August 2007; revised 15 December 2007; accepted 20 December 2007
Available online 25 December 2007

Abstract—We herein describe the synthesis and characterization of nine new 1,3,4-thiadiazolium-2-phenylamine chlorides derived from natural piperine. We evaluate their toxic effects against the different evolutive forms of *Trypanosoma cruzi*, and the host cell (murine macrophages). The results obtained show that mesoionic hydrochloride **MI** possesses the best activity profile. Compound **MI** may be a prototype for use in the development of a new chemotherapeutic agent with high efficiency, which may be employed in the treatment of Chagas' disease.

1. Introduction

Chagas' disease, or American trypanosomiasis, is an endemic parasitic illness widespread in Latin America, from northern Mexico to Argentina.[1,2] Together with malaria, leishmaniasis, and African trypanosomiasis, Chagas' disease is a major parasitic cause of death and hardship, especially in developing countries.[3] According to the World Health Organization, there are 16–18 million people already infected and some 120 million at risk of becoming infected, with ~200,000 new cases and more than 14,000 deaths every year.[1,2]

The etiologic agent of Chagas' disease is the flagellated protozoan *Trypanosoma cruzi*, which is transmitted to humans either by transfusion of infected blood, from an infected mother to her child, or by hematophagous Reduviid vectors.[3] Contagion usually occurs by contact of insect feces with eyes, mouth, or open skin lesions. The disease has two phases: acute and chronic. After the initial acute phase, which is asymptomatic in most cases, a chronic condition establishes which can lead in ~40% of cases to irreversible lesions in gastrointestinal tract and heart.[3] The efforts in insect control and in the quality of blood for transfusion have reduced the impact of this parasitic illness, but the treatment of people already infected, most of them in the chronic phase of the disease, is still a challenge.[1–4] Currently, the chemotherapeutic agents available are unsatisfactory for chronic cases and there is neither a vaccine nor other preventive treatments. There are only two drugs available, the nitroheterocyclic compounds nifurtimox and benznidazole (**1** and **2**, Fig. 1).[5] Both compounds have low efficacy and produce severe side effects. Chagas' disease is *now* considered a neglected disease, among other parasitic illnesses, due to the fact that the pharmaceutical industry does not regard as cost effective the investment on drugs to fight these illnesses. Therefore, it is necessary to find new therapeutic alternatives to treat chagasic patients.[6]

Kapil described the results of an investigation of the activity of natural piperine **3** (Fig. 1) against promasti-

Keywords: Chagas' disease; Mesoionic compounds; 1,3,4-Thiadiazolium-2-phenylamine; Piperine; *Trypanosoma cruzi*.
* Corresponding author. Tel.: +55 2126821872; fax: +55 2126822807; e-mail: marco@ufrrj.br

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.12.049

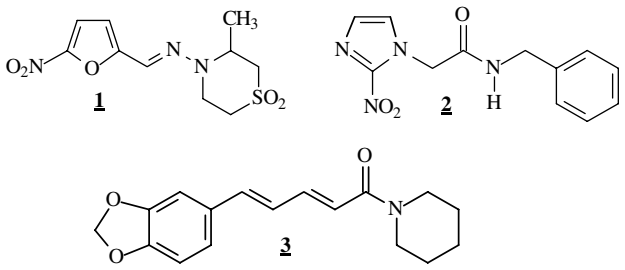


Figure 1. Structures of nifurtimox **1**, benznidazole **2**, and piperine **3**.

gote forms of *Leishmania donovani*.[7] More recently, Raay and co-workers described encouraging data obtained for the activity of piperine intercalated into mannose-coated liposomes with hamsters infected with *L. donovani*.[8] Based on these results, we investigated the activity of this natural amide and of a series of derivatives and analogues on *T. cruzi*.[9] The natural amide **3** is the main secondary metabolite in *Piper nigrum*, occurring mainly in the fruits. *P. nigrum* (popularly known as black pepper) is the most common spice worldwide. It is a perennial vine, widely used in folk medicine in India, where it originates. Piperine **3** is very abundant in the plant, being extracted from the dry fruits with a yield of 3–7%.[10,11] Various biological activities have been attributed to piperine including insecticidal,[12–14] inhibition of liver metabolism,[15] stimulation of melanocyte proliferation,[16] and anti-tumoral activity.[17]

Recently we described the synthesis and evaluation of toxicity of a series of 1,3,4-thiadiazolium-2-phenylamine salts derived from different substituted cinnamic acids against promastigote and amastigote forms of *Leishmania amazonensis*, which afforded very promising results.[18] Since protozoa from genera *Leishmania* and *Trypanosoma* belong to the same family (Trypanosomatidae), we devised the preparation of new mesoionic 1,3,4-thiadiazolium-2-phenylamine chlorides derived from piperine, thus combining the mesoionic and the natural amide moieties, having in mind the concept of molecular hybridization, which has proven to be a very useful tool in the design of new bioactive compounds.[19]

Mesoionic systems are dipolar five-membered heterocyclic rings in which both the negative and positive charges are delocalized throughout endo- and exocyclic atoms.[20,21] Mesoionic rings are present in the structure of numerous compounds with useful and wide-ranging biological activities, including anti-bacterial,[22,23] inhibition of platelet aggregation,[24] and anti-tumoral effects.[25,26]

2. Results and discussion

2.1. Chemistry

Nine new mesoionic 1,3,4-thiadiazolium-2-phenylamine chlorides (**MI**–**MIX**), derivatives and analogues of natural piperine **3**, were synthesized following two experimental procedures found in the literature, employing acyl chlorides[26,27] or aromatic aldehydes[28] as synthetic precursors.

Scheme 1 shows the synthesis of precursors obtained directly from natural product **3**. Piperic acid **4** was obtained from **3** in excellent yield through basic hydrolysis.[9,11] The saturated derivative **5** was prepared from **4** by catalytic hydrogenation of the conjugated double bonds[9] while the nitro-derivative **6** was prepared from **5** by nitration under acidic condition.[29]

The derivatives of the cinnamic series (Scheme 2) were obtained employing piperonal **7** as starting material, through classical reactions such as Knoevenagel's methodology (**8** and **12**),[18] catalytic hydrogenation (**9**),[9] and nitration (**10** and **11**).[29]

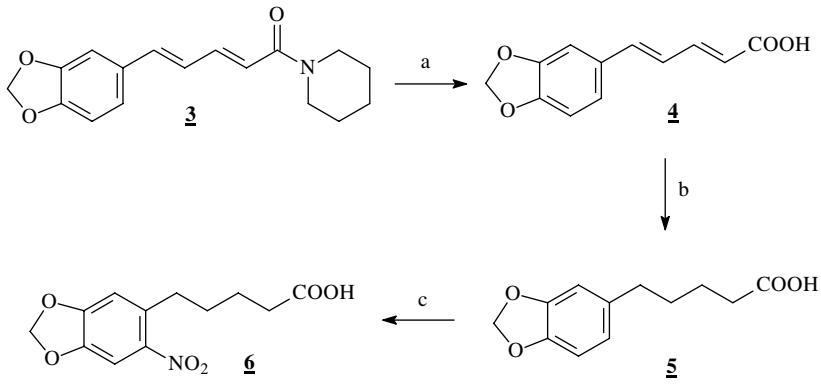
The main reagent for the synthesis of all these mesoionic hydrochlorides, 1,4-diphenylthiosemicarbazide ($C_6H_5NHNHCSNHC_6H_5$), is commercially available or can be easily prepared by a known method.[30] Scheme 3 shows the synthesis of mesoionic hydrochlorides (**MI**–**IX**) employing the respective acyl chlorides and 1,4-diphenylthiosemicarbazide in dry 1,4-dioxane,[18,27] affording the mesoionic compounds (**MI**–**VII**) in moderate to high yields (34–83%, Entries A and B). The aromatic aldehydes **7** and **11** (Scheme 3, Entry C) reacted with 1,4-diphenylthiosemicarbazide, in the presence of trimethylsilyl chloride in dry dimethylformamide,[28] generating the mesoionic salts **MVIII** (68%) and **MIX** (40%). Thus, we were able to prepare three series of compounds (Scheme 3, Entries A, B, and C) having a saturated or unsaturated side chain with none, two or four carbon atoms as spacers between the methylenedioxyphenyl and mesoionic moieties.

We planned the preparation of nitro derivatives of the three series of mesoionic salts (Scheme 3, Entries A–C), since the nitro group present in the structures of nifurtimox (**1**) and benznidazole (**2**) is pharmacophoric to the trypanocidal activity showed by these compounds. The toxic effects of nitro derivatives are due to the formation of various free radical intermediates and/or electrophilic metabolites from the nitroreductases, that act on the nitro group of R-NO_2-type molecules.[5]

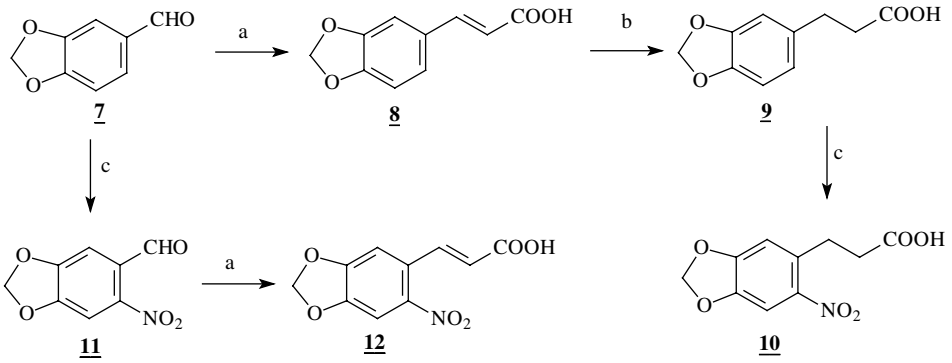
The structures of all mesoionic salts **MI**–**IX** (Scheme 3), derivatives and analogues of the natural piperine, were fully characterized by IR, MS, ^{1}H and ^{13}C NMR spectral data, and microanalyses. The spectral assignments were based on literature data[9,18,27] and are consistent with the structures described.

2.2. Biological activity

The toxic effects of mesoionic derivatives were evaluated against the different evolutive forms of *T. cruzi* (Y strain) and also against the host cell (murine macrophages). The results obtained are depicted in Table 1. Firstly, we evaluated the anti-proliferative effects of nine derivatives on epimastigotes of *T. cruzi* obtaining IC_{50} values ranging from 0.64 to 113.06 μM. The four more active derivatives (**MI**, **MII**, **MIII**, and



Scheme 1. Preparation of acid precursors from piperine **3**. Reagents and conditions: (a) KOH, ethanol, reflux, 24 h, then HCl (aq), 0 °C (93%); (b) ethyl acetate, Pd/C, H_2, 3 h (76%); (c) CH_3COOH (glacial), HNO_3 (concd) 3 h (77%).



Scheme 2. Preparation of the precursors of the cinnamic series (**8**, **9**, **10**, and **12**). Reagents and conditions: (a) malonic acid, pyridine, piperidine, reflux, 8 h, then HCl (aq), 0 °C (75–93%); (b) ethyl acetate, Pd/C, H_2, 3 h (80%); (c) CH_3COOH (glacial), HNO_3 (concd) 3 h (62–82%).

MIX), which showed the lowest IC_{50} values on epimastigotes, were evaluated against murine macrophages and the results show a better selectivity profile for **MI** (Table 1). The higher toxicity on epimastigotes shown by **MI**, **MII**, and **MIII** derivatives suggests that the conjugation between the mesoionic and methylenedioxyphenyl rings is an important structural feature for the activity of these compounds. Derivatives **MII**, **MIII**, and **MIX** showed significant toxic effects on epimastigotes of *T. cruzi*, but they were also found to be toxic for host cells (murine macrophages). Furthermore, **MI** was evaluated against trypomastigote and amastigote forms of *T. cruzi*, showing an interesting activity profile, better than its precursor, the natural amide piperine (**3**), and being more active against the intracellular amastigote form than the reference drug benznidazole (**2**, Table 1).

3. Conclusions

We were able to prepare a new series of mesoionic 1,3,4-thiadiazolium-2-phenylamine chlorides which are derivatives or analogues of the natural amide piperine. The results obtained in the synthesis and biological evaluation of the new compounds highlight the potential use of natural piperine as a precursor for new molecules which may be employed in the treatment of Chagas' disease. Compound **MI** showed the best activity profile.

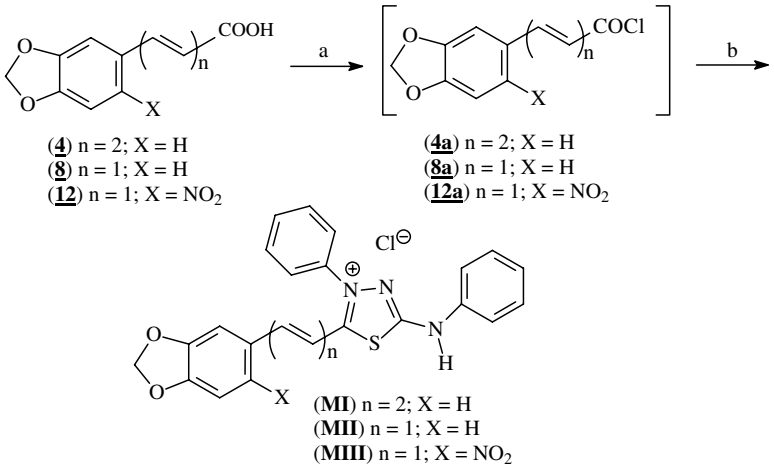
4. Experimental protocols

4.1. Chemistry

Melting points were determined in a capillary with a Melt-Temp. ^1H and ^{13}C NMR were recorded at room temperature using Bruker-AC 200 MHz, Bruker-DRX 600 MHz, and Bruker-DRX 400 MHz spectrometers, employing tetramethylsilane as internal reference. The chemical shifts (δ) are given in ppm and coupling constants (J) in Hertz. Mass spectra were obtained at 70 eV on a Shimadzu-CG/MS QP 2000A equipped with a solid probe. Fragments were described as m/z ratio. IR spectra were recorded on a Perkin-Elmer 5987A spectrometer in KBr pellets. The microanalyses were carried out with elemental apparatus CHNS-O EA-1110 of Carlo Erba Instruments. Thin layer chromatography (TLC) was carried out on silica gel plates with a fluorescence indicator F_{254} (0.2 mm, E. Merck); the spots were visualized in UV light (254 nm). Column chromatography was performed on silica using Kieselgel 60 (230–400 mesh, E. Merck). All solvents and reagents used in the present study were of analytical grade.

4.1.1. General procedure for the preparation of derivatives 1,3,4-thiadiazolium-2-phenylamine chlorides (MI–MVII). These compounds were synthesized by the coupling of the corresponding acyl chlorides (1.37 mmol) which were added to a stirred solution of 1,4-diphenylthiose-

Entry A (unsaturated series):

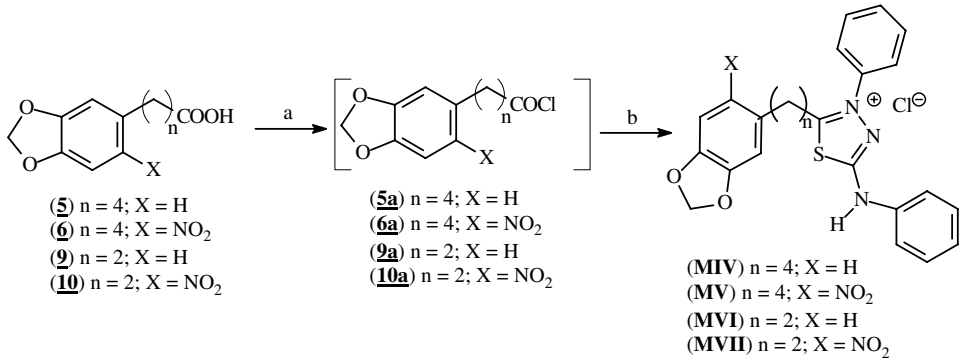


(**4**) n = 2; X = H
(**8**) n = 1; X = H
(**12**) n = 1; X = NO_2

(**4a**) n = 2; X = H
(**8a**) n = 1; X = H
(**12a**) n = 1; X = NO_2

(**MI**) n = 2; X = H
(**MII**) n = 1; X = H
(**MIII**) n = 1; X = NO_2

Entry B (saturated series):

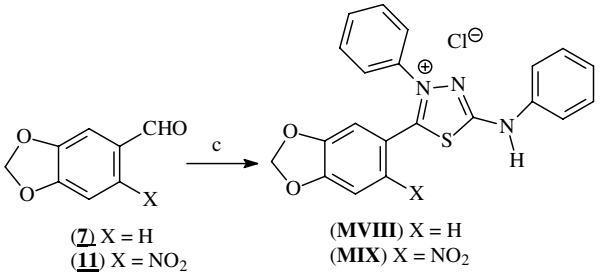


(**5**) n = 4; X = H
(**6**) n = 4; X = NO_2
(**9**) n = 2; X = H
(**10**) n = 2; X = NO_2

(**5a**) n = 4; X = H
(**6a**) n = 4; X = NO_2
(**9a**) n = 2; X = H
(**10a**) n = 2; X = NO_2

(**MIV**) n = 4; X = H
(**MV**) n = 4; X = NO_2
(**MVI**) n = 2; X = H
(**MVII**) n = 2; X = NO_2

Entry C (from aromatic aldehydes):



(**7**) X = H
(**11**) X = NO_2

(**MVIII**) X = H
(**MIX**) X = NO_2

Scheme 3. Preparation of mesoionic hydrochlorides (**MI**–**MIX**). Reagents and conditions: (a) $(COCl)_2$, 25 °C, 2 h (100%); (b) 1,4-diphenylthiosemicarbazide, 1,4-dioxane, 25 °C, 24–48 h (34–83%); (c) 1,4-diphenylthiosemicarbazide, TMSCl, DMF, 25 °C, 24 h (40–68%).

micarbazide (1.37 mmol) in dry 1,4-dioxane (2 mL) at room temperature.[22,23] After 48 h standing, the products were separated by vacuum filtration and washed with a sequence of solvents (1,4-dioxane, toluene, and ethyl ether, respectively). In this way, the mesoionic compounds **MI**–**MVII** were prepared.

In order to facilitate the assignment of NMR data, we have opted for numbering the structures according to the example given below (Fig. 2).

4.1.1.1. 4-Phenyl-5-[4-(3,4-methylenedioxyphenyl)-1(*E*)-3(*E*)-butadienyl]-1,3,4-thiadiazolium-2-phenylamine chloride (MI). Yield 57%; mp 155 °C (decomposed); IR (KBr, cm^{-1}) 3449, 2926, 1600, 1565, 1361, 1256, 758, 692; ^1H NMR (DMSO-d_6, 400 MHz) δ 12.39 (s, NH), 7.78 (m, H2″), 7.73 (m, H3″ and 4″), 7.54 (m, Hβ), 7.46 (s, H2′), 7.38 (t, H3′, J = 7.5 Hz), 7.22 (s, H2‴), 7.18 (m, Hγ and δ), 7.09 (t, H4′, J = 7.3 Hz), 7.04 (d, H6‴, J = 8.0 Hz), 6.94 (d, H5‴, J = 8.0 Hz), 6.52 (d, Hα, J = 14.8 Hz) 6.06 (s, OCH_2O); ^{13}C NMR (DMSO-d_6, 400 MHz) 159.80 (C2), 149,01 (C3‴ and C4‴), 138,00 (Cδ), 132.52 (C4″), 131.50 (C1‴), 131.20 (C3′), 130.61 (C3″), 127.33 (C6‴ and 2″), 125.61 (Cγ), 124.70 (C4′), 120.30 (C2′), 114.52 (Cα), 109.91 (C5‴), 107.01 (C2‴), 102.83 (OCH_2O); MS (70 eV, *m/z*) (%) 426 (5), 284 (15), 177 (100), 161 (65), 149 (31), 127 (22), 77 (10). Anal. Calcd for $C_{25}H_{20}N_3SO_2Cl$: C, 65.00; H, 4.36; N, 9.10. Found: C, 65.08; H, 4.27; N, 9.16.

Table 1. In vitro activities of mesoionic compounds **MI**–**MIX** against three evolutive forms of *Trypanosoma cruzi* and its cytotoxicity on murine macrophages

Compound	IC_{50} (μM)			LD_{50} (μM)
	Epimastigotes	Trypomastigotes	Amastigotes	Cytotoxicity[a]
Piperine	7.31 ± 1.5	>[ ]$_{max}$[b]	4.91 ± 1.1	20.01 ± 3.35
MI	10.83 ± 2.2	6.70 ± 1.7	1.35 ± 0.95	38.56 ± 4.6
MII	4.13 ± 1.2	>[ ]$_{max}$	NT[c]	1.95 ± 0.5
MIII	0.64 ± 0.14	>[ ]$_{max}$	NT	1.08 ± 0.23
MIV	31.50 ± 2.0	NT	NT	NT
MV	45.24 ± 5.5	NT	NT	NT
MVI	83.42 ± 6.6	>[ ]$_{max}$	NT	NT
MVII	40.84 ± 4.1	NT	NT	NT
MVIII	113.06 ± 10.09	NT	NT	NT
MIX	13.42 ± 3.0	>[ ]$_{max}$	NT	6.62 ± 2.3
Benznidazole[d]	2.21 ± 0.85	6.61 ± 2.4	2.51 ± 0.7	—

[a] Against murine macrophages.
[b] IC_{50} values highest than the maximum concentration allowed.
[c] Not tested.
[d] Reference drug.

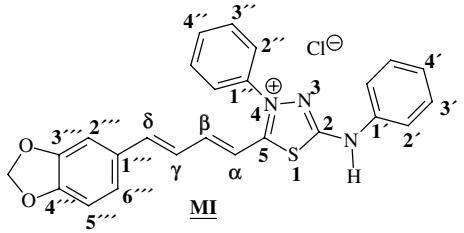


Figure 2. Chemical structure of the mesoionic salt **MI**. The skeleton numbering is only for NMR assignments.

4.1.1.2. 4-Phenyl-5-[2-(3,4-methylenedioxyphenyl)-(*E*)-ethenyl]-1,3,4-thiadiazolium-2-phenylamine chloride (MII). Yield 34%; mp 294–296 °C; IR (KBr, cm^{-1}) 3425, 2900, 2674, 1600, 1567, 1362, 1259, 754, 690; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.24 (s, NH), 7.87 (d, Hβ, J = 15.5 Hz), 7.81 (m, H2″) 7.74 (s, H3″ and 4″), 7.56 (d, H2′, J = 7,6 Hz), 7.41 (t, H3′, J = 7.5 Hz), 7.41 (s, H2‴), 7.32 (d, H6‴, J = 8.0 Hz), 7.14 (t, H4′, J = 7.1 Hz), 7.01 (d, H5‴, J = 7.8 Hz), 6.95 (d, Hα, J = 15.9 Hz), 6.10 (s, OCH_2O); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 166.03 (C5), 161.71 (C2), 152.98 (Cβ), 149.53 (C4‴ and C3‴), 132.71 (C4″), 131.35 (C2″), 130.68 (C3″), 128.13 (C5‴), 127.31 (C3′), 125.28 (C4′), 119.98 (C2′), 110.58 (Cα), 110.06 (C2‴), 103.31 (OCH_2O); MS (70 eV, *m*/*z*) (%) 399 (100), 283 (45), 250 (20), 191 (65), 161 (46), 118 (32), 77 (25). Anal. Calcd for $C_{23}H_{18}N_3SO_2Cl$: C, 63.37; H, 4.16; N, 9.64. Found: C, 63.25; H, 4.27; N, 9.72.

4.1.1.3. 4-Phenyl-5-[2-(6-nitro-3,4-methylenedioxyphenyl)-(*E*)-ethenyl]-1,3,4-thiadiazolium-2-phenylamine chloride (MIII). Yield 37%; mp 274–276 °C; IR (KBr, cm^{-1}) 3426, 3052, 2922, 2736, 1615, 1565, 1385, 1330, 1260, 757, 692; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.69 (s, NH), 8.09 (d, Hβ, J = 15.8 Hz), 7.83 (m, H2″), 7.74 (m, H3″ and 4″), 7.71 (s, H5‴), 7.59 (d, H2′, J = 7.5 Hz), 7.43 (t, H3′, J = 7.9 Hz), 7.43 (s, H2‴), 7.18 (t, H4′, J = 7.4 Hz), 7.07 (d, Hα, J = 15.6 Hz), 6.27 (s, OCH_2O); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 168.52 (C5), 162.20 (C2), 150.87 (C6‴), 149.13 (C3‴ and 4‴), 143.87 (Cβ), 138.77 (C1″), 137.77 (C1′), 132.72 (C4″), 131.10 (C3′), 130.44 (C3″), 126.90 (C2″), 125.46 (C4′), 119.97 (C2′), 115.49 (Cα), 108.57 (C2‴), 106.52 (C5‴), 105.11 (OCH_2O); MS (70 eV, *m*/*z*) (%) 444 (11), 368 (10), 269 (77), 208 (25), 135 (95), 118 (35), 91 (65), 77 (100). Anal. Calcd for $C_{23}H_{17}N_4SO_4Cl$: C, 57.44; H, 3.56; N, 11.65. Found: C, 57.49; H, 3.63; N, 11.58.

4.1.1.4. 4-Phenyl-5-[4-(3,4-methylenedioxyphenyl)-butyl]-1,3,4-thiadiazolium-2-phenylamine chloride (MIV). Yield 51%; mp 234–236 °C; IR (KBr, cm^{-1}) 3443, 3026, 2924, 1600, 1567, 1320, 1247, 759, 693; ^{1}H NMR ($CDCl_3$, 200 MHz) δ 12.61 (s, NH), 7.78 (m, H2″), 7.64 (m, H3″ and 4″), 7.52 (d, H2′, J = 7.8 Hz), 7.14 (t, H3′, J = 7.8 Hz), 6.96 (t, H4′, J = 7.4 Hz), 6.67 (d, H5‴, J = 7.8 Hz), 6.50 (dd, H6‴, J = 6.0 and 1.6 Hz), 6.45 (d, H2‴, J = 1.6 Hz), 5.90 (s, OCH_2O), 3.19 (t, Hδ, J = 7.2 Hz), 2.39 (t, Hα, J = 7.2 Hz), 1.72 (m, Hβ), 1.56 (m, Hγ); ^{13}C NMR ($CDCl_3$, 200 MHz) δ 169.70 (C5), 159.71 (C2), 147.41 (C3‴), 145.50 (C4‴), 138.32 (C1″), 136.90 (C1′), 131.40 (C4″), 129.81 (C3′), 128.60 (C3″), 125.51 (C4′), 123.42 (C2″), 120.81 (C6‴), 118.50 (C2′), 108.40 (C5‴), 107.90 (C2‴), 100.61 (OCH_2O), 34.41 (Cα), 30.63 (Cδ), 28.51 (Cβ), 24.80 (Cγ); MS (70 eV, *m*/*z*) (%) 429 (35), 280 (100), 135 (23), 77 (15). Anal. Calcd for $C_{25}H_{24}N_3SO_2Cl$: C, 69.74; H, 5.62; N, 9.76. Found: C, 69.68; H, 5.59; N, 9.81.

4.1.1.5. 4-Phenyl-5-[4-(6-nitro-3,4-methylenedioxyphenyl)-butyl]-1,3,4-thiadiazolium-2-phenylamine chloride (MV). Yield 50%; mp 226–228 °C; IR (KBr, cm^{-1}) 3444, 3052, 2923, 2774, 1609, 1567, 1380, 1330, 1252, 758, 694; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 12.69 (s, NH), 7.71 (m, H2″), 7.66 (m, H3″ and 4″), 7.59 (d, H2′, J = 7.9 Hz), 7.21 (t, H3′, J = 7.6 Hz), 7.01 (t, H4′, J = 7.2 Hz), 6.64 (s, H2‴), 5.90 (s, OCH_2O), 3.19 (t, Hδ, J = 7.6 Hz), 2,74 (t, Hα, J = 7.6 Hz), 1.85 (qui, Hβ, J = 7.6 Hz), 1.64 (qui, Hγ, J = 7.5 Hz); ^{13}C NMR (DMSO-d_6, 200 MHz) δ 170.30 (C5), 160.31 (C2), 151.70 (C6‴), 146.13 (C4‴), 142.20 (C3‴), 138.65 (C1″), 137.04 (C1′), 133.25 (C1‴), 131.63 (C4″), 130.07 (C3′), 129.31 (C3″), 125.70 (C2″), 123.70 (C4′), 118.10

(C2′), 110.30 (C5‴), 104.92 (C2‴), 103.10 (OCH_2O), 31.73 (Cδ), 29.21 (Cγ), 28.43 (Cβ), 28.09 (Cα); MS (70 eV, *m*/*z*) (%) 474 (15), 228 (12), 280 (75), 135 (100), 118 (64), 91 (48), 77 (85). Anal. Calcd for $C_{25}H_{23}N_4SO_4Cl$: C, 58.76; H, 4.54; N, 10.96. Found: C, 58.83; H, 4.78; N, 11.05.

4.1.1.6. 4-Phenyl-5-[2-(3,4-methylenedioxyphenyl)-ethyl]-1,3,4-thiadiazolium-2-phenylamine chloride (MVI). Yield 83%; mp 272–274 °C; IR (KBr, cm^{-1}) 3423, 3023, 2897, 2769, 1600, 1570, 1321, 1242, 752, 690; ^1H NMR ($CDCl_3$, 400 MHz) δ 12.26 (s, NH), 7.70 (m, H2″, 3″ and 4″), 7.56 (d, H2′, *J* = 8.5 Hz), 7.36 (t, H3′, *J* = 7.9 Hz), 7.09 (t, H4′, *J* = 7.3 Hz), 6.78 (d, H5‴, *J* = 8.0 Hz), 6.75 (d, H2‴, *J* = 1.5 Hz), 6.5 (dd, H6‴, *J* = 7.9 and 1.7 Hz), 5.98 (s, OCH_2O), 3.38 (t, Hα, *J* = 7.3 Hz), 2.94 (m, Hβ); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 149.70 (C3‴), 148.60 (C4‴), 139.72 (C1″), 138.41 (C1′), 133.10 (C4″), 132.52 (C1‴), 131.34 (C3′), 130.53 (C3″), 126.61 (C2″), 126.03 (C4′), 123.02 (C6‴), 120.20 (C2′), 109.91 (C5‴), 109.53 (C2‴), 102.50 (OCH_2O), 34.91 (Cα), 34.91 (Cβ); MS (70 eV, *m*/*z*) (%) 401 (100), 280 (15), 211 (93), 135 (50), 118 (20), 91 (10), 77 (23). Anal. Calcd for $C_{23}H_{20}N_3SO_2Cl$: C, 63.08; H, 4.60; N, 9.59. Found: C, 63.15; H, 4.48; N, 9.64.

4.1.1.7. 4-Phenyl-5-[2-(6-nitro-3,4-methylenedioxyphenyl)-ethyl]-1,3,4-thiadiazolium-2-phenylamine chloride (MVII). Yield 42%; mp 248–250 °C; IR (KBr, cm^{-1}) 3426, 3052, 2932, 2732, 1615, 1565, 1385, 1330, 757, 692; ^1H NMR (DMSO-d_6, 200 MHz) δ 12.34 (s, NH), 7.70 (m, H2″, 3″ and 4″), 7.57 (m, H2′), 7.52 (s, H5‴), 7.40 (t, H3′, *J* = 7.8 Hz), 7.09 (m, H4′), 7.09 (s, H2‴), 6.21 (s, OCH_2O), 3.47 (m, Hα), 3.15 (m, Hβ); ^{13}C NMR (DMSO-d_6, 200 MHz) δ 170.32 (C5), 160.31 (C2), 151.72 (C6‴), 146.12 (C4‴), 142.24 (C3‴), 138.60 (C1″), 137.03 (C1′), 133.21 (C1‴), 131.61 (C4″), 130.01 (C3′), 129.33 (C3″), 125.71 (C2″), 123.70 (C4′), 118.12 (C2′), 110.31 (C5‴), 104.91 (C2‴), 103.10 (OCH_2O), 28.40 (Cβ), 28.02 (Cα), MS (70 eV, *m*/*z*) (%) 446 (5), 282 (12), 269 (100), 208 (28), 177 (40), 136 (32), 91 (43), 77 (46). Anal. Calcd for $C_{23}H_{19}N_4SO_4Cl$: C, 57.20; H, 3.97; N, 11.60. Found: C, 57.39; H, 3.82; N, 11.42.

4.1.2. General procedure for the preparation of derivatives 1,3,4-thiadiazolium-2-phenylamine chlorides (MVIII) and (MIX). To a solution of 1,4-diphenylthiosemicarbazide (0.25 mmol) in dry DMF (1 mL) was added TMS-Cl (0.8 mmol), followed by aldehydes (0.62 mmol). The mixture was stirred for 24 h at room temperature,[28] the products were separated by vacuum filtration and washed with a sequence of solvents (1,4-dioxane, toluene, and ether ethylic, respectively). In this way, the following compounds (**MVIII**–**MIX**) were prepared.

4.1.2.1. 4-Phenyl-5-(3,4-methylenedioxyphenyl)-1,3,4-thiadiazolium-2-phenylamine chloride (MVIII). Yield 68%; mp 286–287 °C; IR (KBr, cm^{-1}) 3455, 3054, 2993, 2645, 1600, 1565, 1314, 1245, 761, 693; ^1H NMR (CD_3OD, 400 MHz) δ 12.37 (s, NH), 7.70 (m, H2″), 7.62 (m, H3″ and 4″), 7.59 (d, H2′, *J* = 7.8 Hz), 7.43 (t, H3′, *J* = 7.7 Hz), 7.15 (t, H4′, *J*= 7.6 Hz), 7.11 (dd, H6‴, *J* = 8.1 and 1.7 Hz), 7.06 (d, H5‴, *J* = 8.2 Hz), 6.94 (d, H2‴, *J* = 1.6 Hz), 6.13 (s, OCH_2O); ^{13}C NMR (CD_3OD, 400 MHz) δ 165.13 (C5), 161.47 (C2), 152.72 (C3‴), 149.05 (C4‴), 139.68 (C1″), 139.04 (C1′), 132.60 (C4″), 131.18 (C3′), 130.70 (C3″), 127.31 (C6‴), 127.27 (C2″), 125.30 (C4′), 119.70 (C2′), 117.28 (C1‴), 110.50 (C2‴), 110.38 (C5‴), 103.91 (OCH_2O); MS (70 eV, *m*/*z*) 373 (82), 257 (24), 224 (27), 165 (100), 118 (18), 91 (10), 77 (14). Anal. Calcd for $C_{21}H_{16}N_3SO_2Cl$: C, 61.54; H, 3.93; N, 10.25. Found: C, 61.47; H, 4.02; N, 10.37.

4.1.2.2. 4-Phenyl-5-(6-nitro-3,4-methylenedioxyphenyl)-1,3,4-thiadiazolium-2-phenylamine chloride (MIX). Yield 40%; mp 264–266 °C; IR (KBr, cm^{-1}) 3437, 3010, 2777, 1610, 1566, 1329, 1267, 754, 690; ^1H NMR (DMSO-d_6, 200 MHz) δ 12.58 (s, NH), 7.92 (s, H5‴), 7.63 (m, H2″), 7.57 (m, H3″ and 4″), 7.55 (m, H2′), 7.45 (t, H3′, *J* = 7.5 Hz), 7.45 (sl, H2‴), 7.18 (t, H4′, *J* = 7.4 Hz), 6.36 (s, OCH_2O); ^{13}C NMR (DMSO-d_6, 400 MHz) δ 162.7 (C5), 152.4 (C2), 151.2 (C6‴), 146.4 (C4‴), 142.3 (C3‴), 138.4 (C1″), 137.0 (C1′), 134.3 (C1‴), 131.7 (C4″), 130.0 (C3′), 129.5 (C3″), 125.1 (C2″), 124.2 (C4′), 118.6 (C2′), 110.8 (C5‴), 106.0 (C2‴), 105.1 (OCH_2O); MS (70 eV, *m*/*z*) (%) 418 (20), 298 (52), 206 (28), 178 (64), 135 (75), 91 (18), 77 (100). Anal. Calcd for $C_{21}H_{15}N_4SO_4Cl$: C, 55.45; H, 3.32; N, 12.32. Found: C, 55.57; H, 3.28; N, 12.57.

4.2. Biological assays

4.2.1. Parasites. *Trypanosoma cruzi* (Y strain) was obtained from the Fundação Oswaldo Cruz (Rio de Janeiro, Brazil) culture collection. The epimastigote forms were cultured in brain heart infusion (BHI) supplemented with 10 mg of hemin and 20 mg of folic acid $liter^{-1}$ and 5% heat-inactivated fetal calf serum (FCS) (BHI-FCS medium) at 28 °C.[31] Tissue culture-derived trypomastigotes were obtained after infection of confluent monolayers of Vero cells with blood trypomastigotes (Y strain) to establish the intracellular cycle and maintained in RPMI 1640 medium containing 10% FCS under an atmosphere of 5% CO_2 at 37 °C.[32] These tissue culture-derived trypomastigotes were used to infect murine peritoneal macrophages in vitro to evaluate the toxic effect of the drugs.

4.2.2. Anti-epimastigote effect. The toxic effect of the drugs on epimastigotes was evaluated as previously described.[9] The drugs were stored as 1.0 mg mL^{-1} stock solution in DMSO (Sigma) and were used in serial dilution (1:2) in BHI-FCS medium before use. Drug-free control medium contained comparable final concentration of DMSO (0.05%). Epimastigotes (1.10^5 cells) were incubated in BHI-FCS medium with or without drugs in a final volume of 1.0 mL in 24-well plates (TPP). After 7 days of treatment, the toxic effect of the drugs was quantified by the direct count of the live epimastigotes in a Neubauer chamber.

4.2.3. Cytotoxicity to macrophages. The evaluation of the toxic effects of the compounds was carried out as previously described.[33] Murine peritoneal macrophages

were seeded (1.10^6 cells/well) in 24-well plates (TPP) with 1 mL of RPMI medium containing 10% FCS. The cells were allowed to attach for 24 h at 37 °C and then exposed to the active compounds dissolved in DMSO against epimastigotes (maximum final concentration of solvent was 0.05%) for 72 h. Afterwards, the cells were washed with PBS, and RPMI was added to the culture before the addition of vital dye trypan blue in a final concentration of 0.01%. The toxic effect of the drugs was monitored by the count of 200 cells in a Neubauer chamber where plasma membrane permeability was evaluated.

4.2.4. Anti-trypomastigote activity. The anti-trypomastigote activity of the drugs was monitored at concentrations which were found non-toxic to macrophages. The parasites were harvested from infected cultures as described above,[33,34] and the motility of the trypomastigotes was quantified 24 h after the treatment, by the direct count of the live trypomastigotes in a Neubauer chamber.

4.2.5. Anti-amastigote effect. Resident peritoneal cells from non-infected BALB/c mice were harvested and cultured as previously described.[34] Adhered macrophages were infected with *T. cruzi* metacyclic trypomastigotes at a 10:1 parasite/macrophage ratio and incubated at 37 °C under 5% CO_2 for 1 h. Subsequently, non-infective cells were removed by extensive washing with PBS, and the infected macrophage cultures were treated with increasing concentrations (0.5, 1.0, 2.5, 5.0, and 10 μg/mL) of **MI** per 72 h. After that, monolayers were washed with PBS at 37 °C, fixed in methanol, and stained with Giemsa. The amastigote survival was determined by the counting of 200 cells in triplicate, where the percentage of infected cells was analyzed, as well as the number of amastigotes per macrophage and the endocytic index of these infected cells.[34]

4.2.6. Statistical analysis. The 50% inhibitory concentrations (IC_{50}) values shown in the Table 1 represent the mean of experiments carried out in triplicate. The IC_{50} of all compounds were determined by linear regression analysis using the program IGOR Pro 2.03 (Lake Oswego, Oregon - USA).

Acknowledgments

Authors acknowledge financial supports from FAPERJ, CNPq, and CAPES (PROCAD). Fellowships from CNPq to Welisson da Silva Ferreira and V.B. Saraiva and from Capes to L. Freire-de-Lima are gratefully acknowledged. Authors are grateful to the National Center for Nuclear Magnetic Resonance of Macromolecules (UFRJ) for acquisition of NMR spectra at 400 and 600 MHz.

References and notes

1. World Health Organization: Chagas disease information. The UNICEF-UNDP Program on TDR [http://www.who.int/tdr/diseases/chagas/].
2. The Pan American Health Organization: Chagas Disease (American Trypanosomiasis) [http://www.paho.org/english/as/dpc/cd/chagas.htm].
3. Schmuñis, G. A. A Tripanossomíase americana e seu impacto na saúde pública das Américas. In *Trypanosoma cruzi e Doença de Chagas*; Brener, Z., et al., Eds.; Guanabara Koogan: Rio de Janeiro, 2000; pp. 1–15.
4. Vinhaes, M. C.; Schofield, C. J. *Trends Parasitol.* **2003**, *19*, 112.
5. Maya, J. D.; Cassels, B. K.; Iturriaga-Vásquez, P.; Ferreira, J.; Faúndez, M.; Galanti, A.; Morello, A. *Comp. Biochem. Physiol. A* **2007**, 601.
6. Trouiller, P.; Olliaro, P.; Torreele, E.; Orbinski, J.; Laing, L.; Ford, N. *The Lancet* **2002**, *359*, 2188.
7. Kapil, A. *Planta Med.* **1993**, *59*, 474.
8. Raay, B.; Medda, S.; Mukhopadhyay, S.; Basu, M. K. *Indian J. Biochem. Biophys.* **1999**, *36*, 248.
9. Ribeiro, T. S.; Freire-de-Lima, L.; Previato, J. O.; Mendonça-Previato, L.; Heise, N.; Lima, M. E. F. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 3555.
10. Semler, U.; Gross, G. G. *Phytochemistry* **1988**, *27*, 1566.
11. Ikan, R. In *Natural Products: A Laboratory Guide*, 2nd ed.; Academic Press, 1991; pp 233–238.
12. Su, H. C. F.; Horvat, R. *J. Agric. Food Chem.* **1981**, *29*, 115.
13. de Paula, V. F.; Barbosa, L. C. D.; Demuner, A. J.; Pilo-Veloso, D.; Picanco, M. C. *Pest Manag. Sci.* **2000**, *56*, 168.
14. Pridgeon, J. W.; Meepagala, K. M.; Becnel, J. J.; Clark, G. G.; Pereira, R. M.; Linthicum, K. J. *J. Med. Entomol.* **2007**, *44*, 263.
15. Koul, S.; Koul, J. L.; Taneja, S. C.; Dhar, K. L.; Jamwal, D. S.; Singh, K.; Reen, R. K.; Sing, J. *Bioorg. Med. Chem.* **2000**, *8*, 251.
16. Lin, Z. X.; Hoult, J. R. S.; Bennet, D. C.; Raman, A. *Planta Med.* **1999**, *65*, 600.
17. Sunila, E. S.; Kuttan, G. *J. Ethnopharm.* **2004**, *90*, 339.
18. Silva, E. F.; Canto-Cavalheiro, M. M.; Braz, V. R.; Cysne-Finkelstein, L.; Leon, L. L.; Echevarria, A. *Eur. J. Med. Chem.* **2002**, *37*, 979.
19. Viegas-Junior, C.; Danuello, A.; Bolzani, V. S.; Barreiro, E. J.; Fraga, C. A. M. *Curr. Med. Chem.* **2007**, *14*, 1829.
20. Ollis, W. D.; Stanforth, S. P.; Ramsden, C. A. *Tetrahedron* **1985**, *41*, 2242.
21. Athayde, P. F.; Miller, J.; Simas, A. M. *Synthesis-Stuttgart* **2000**, *11*, 1565.
22. Stewart, T. G.; Kier, L. B. *J. Pharm. Sci.* **1965**, *54*, 731.
23. Montanari, C. A.; Amaral, A. T.; Giesbrecht, A. M. *Pharm. Sci.* **1997**, *3*, 565.
24. Hellberg, M.; Stubbins, J. F.; Glennon, R. A. *Bioorg. Med. Chem.* **2000**, *8*, 1917.
25. Senff-Ribeiro, A.; Echevarria, A.; Silva, E. F.; Franco, C. R.; Viegas, S. S.; Oliveira, M. B. *Br. J. Cancer* **2004**, *19*, 297.
26. Senff-Ribeiro, A.; Echevarria, A.; Silva, E. F.; Veiga, S. S.; Oliveira, M. B. *Anticancer Drugs* **2004**, *15*, 269.
27. Santos, A. C. S.; Echevarria, A. *Magn. Reson. Chem.* **2001**, *30*, 182.
28. Kappel, J. C.; Yokum, T. S.; Barany, G. *J. Comb. Chem.* **2004**, *6*, 746.
29. Murphy, B. P. *J. Org. Chem.* **1985**, *50*, 5873.
30. Marckwald, W. *Chem. Ber.* **1892**, *25*, 3098.
31. Previato, J. O.; Jones, C.; Gonçalves, L. P. B.; Wait, R.; Travassos, L. R.; Mendonça-Previato, L. *Biochem. J.* **1994**, *301*, 151.
32. Andrews, N. W.; Alves, M. J. M.; Schumacher, R. I.; Colli, W. *Exp. Parasitol.* **1985**, *60*, 255.

33. Delorenzi, J. C.; Freire-de-Lima, L.; Gattass, C. R.; Andrade-Costa, D.; He, L.; Kuehne, M. E.; Saraiva, E. M. *Antimicrob. Agents Chemother.* **2002**, *46*, 2111.

34. Saraiva, V. B.; Gibaldi, D.; Previato, J. O.; Mendonça-Previato, L.; Bozza, M. T.; Freire-De-Lima, C. G.; Heise, N. *Antimicrob. Agents Chemother.* **2002**, *46*, 3472.