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Leishmanicidal Activities of Novel Synthetic Furoxan and Benzofuroxan Derivatives

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A novel series of furoxan (1,2,5-oxadiazole 2-oxide) (compounds 3, 4a and -b, 13a and -b, and 14a to -f) and benzofuroxan (benzo[c][1,2,5]oxadiazole 1-oxide) (compounds 7 and 8a to -c) derivatives were synthesized, characterized, and evaluated for *in vitro* activity against promastigote and intracellular amastigote forms of *Leishmania amazonensis*. The furoxan derivatives exhibited the ability to generate nitric oxide at different levels (7.8% to 27.4%). The benzofuroxan derivative 8a was able to increase nitrite production in medium supernatant from murine macrophages infected with *L. amazonensis* at 0.75 mM after 48 h. Furoxan and benzofuroxan derivatives showed remarkable leishmanicidal activity against both promastigote and intracellular amastigote forms. Compounds 8a, 14a and -b, and 14d exerted selective leishmanicidal activities superior to those of amphotericin B and pentamidine. *In vitro* studies at pH 5.4 reveal that compound 8a is stable until 8 h and that compound 14a behaves as a prodrug, releasing the active aldehyde 13a. These compounds have emerged as promising novel drug candidates for the treatment of leishmaniasis.

Leishmaniasis is caused by more than 20 species of the protozoan parasite *Leishmania* and is transmitted to humans by the bite of infected female phlebotomine sand flies (1). The disease is widely distributed in 98 tropical and subtropical countries and poses a major public health problem and a risk for people living in or traveling to areas of endemicity. It has an annual estimated worldwide incidence of 600,000 and a prevalence of 12 million cases (2). Increased rates in patients coinfected by HIV and the development of resistance to current drugs have transformed leishmaniasis into a serious public health problem (3, 4).

This parasitic disease presents a wide range of clinical symptoms, characterized by cutaneous, mucocutaneous, or visceral leishmaniasis which differs in immunopathologies and degrees of morbidity and mortality. In humans, cutaneous leishmaniasis has shown a wide spectrum of clinical manifestations which can vary from localized cutaneous leishmaniasis to serious clinical forms such as diffuse cutaneous leishmaniasis and mucocutaneous leishmaniasis (5). In the New World, mainly Latin America, *Leishmania amazonensis* is one of the main common infectious agents responsible for these clinical manifestations (6, 7).

Despite current advances in understanding the molecular biology and biochemistry of the parasite, the first-choice treatment of several forms of leishmaniasis remains focused on the use of the obsolete pentavalent antimonials, such as sodium stibogluconate and meglumine antimoniate (8). Pentavalent antimonials are toxic and frequently ineffective, and their administration requires medical supervision (9, 10). In addition, antimony resistance has become common during leishmaniasis treatment (11, 12). On this issue, in the last few decades efforts have been made to discover alternative treatments. Amphotericin B (and its liposomal formulations), pentamidine, paromomycin, and miltefosine are currently being used in leishmaniasis treatment as second choices. However, these drugs are expensive and may be even more toxic than antimonials (8, 13, 14).

Furoxan (1,2,5-oxadiazole 2-oxide) and benzofuroxan (benzo[c][1,2,5]oxadiazole 1-oxide) derivatives have been explored as pharmacophores to design leishmanicidal drug candidates (15, 16). Hernández and coworkers have identified a series of furoxan and benzofuroxan derivatives active against *L. amazonensis*. The compounds (*E*)-*N*′-(5-benzofuroxanylmethylidene)benzo[d][1,3]dioxole-5-carbohydrazide and (*E*)-*N*′-(4-hydroxy-3-methoxyphenylmethylidene)-3-methylfuroxan-4-carbohydrazide were less active than amphotericin B; however, they were, respectively, 5- to 50-fold more selective than the reference drug in cytotoxicity studies against murine macrophages (17) (Fig. 1). Boiani and coworkers have described the evaluation of alkylnitrates and furoxan derivatives against two promastigote forms of two *Leishmania* species: *Leishmania braziliensis* and *Leishmania pifanoi* (18). Benzofuroxan derivatives containing vinylthio, vinylsulfinyl, vinylsulfonyl, and vinylketo subunits have also demonstrated leishmanicidal activity superior to that of miltefosine against *L. braziliensis* and *L. pifanoi*. However, despite the excellent activity, the benzofuroxan derivatives were not able to act as nitric oxide (NO) donors, and these compounds have shown high cytotoxicity against J-774 mouse macrophage cells (18).

The mechanism of action of benzofuroxan derivatives is not totally understood. It was hypothesized that benzofuroxan derivatives could produce oxygen/nitrogen reactive species in the parasite and inhibit mitochondrial dehydrogenases (15–18). On the other hand, for furoxan derivatives it has been proposed that the

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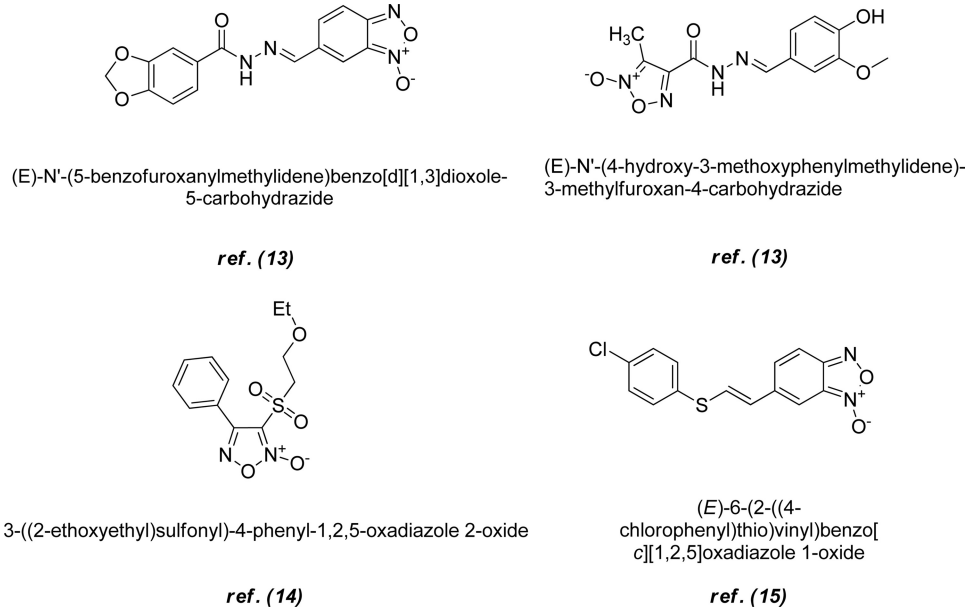

FIG 1 Chemical structures of some reported furoxan and benzofuroxan derivatives active against *Leishmania* species (13–15).

N-oxide group seems to act as a bioreducible group in the parasite, generating free radical species such as nitric oxide (NO). The leishmanicidal activity of furoxan derivatives seems to be related to the increase of nitric oxide levels, and the loss of activity by furoxan derivatives without an *N*-oxide subunit is described elsewhere (16–18). NO is a potent antimicrobial agent which helps to eliminate intracellular pathogens. The *Leishmania* species, during their different life stages, have variable sensibilities to reactive oxygen species (ROS). After the recognition of the parasite, macrophages are activated to "effector cells" and trigger several oxidative and immune responses to destroy the unwanted guest (19). Moreover, NO is able to interrupt the *Leishmania* life cycle by inactivating parasite enzymes such as cysteine proteinases. Specifically, NO mediates chemical modification of the cysteine residue present in the site of proteinases of *Leishmania* spp., blocking this enzyme activity (20).

The *N*-acyl hydrazone (NAH) subunit has been described as a privileged structure to design new compounds with different pharmacological effects, including antiparasitic activity (17). Several studies have demonstrated that this subunit is the pharmacophoric group to develop cysteinyl protease inhibitors and that it could contribute to improving the biological activity of the new furoxan and benzofuroxan derivatives (21–23). Therefore, in a continuing effort to develop new candidate drugs to treat leishmaniasis, we report here the synthesis, NO donor ability, leishmanicidal activity, and cytotoxic effect on murine peritoneal macrophages of 15 furoxan (compounds 3, 4a and -b, 13a and -b, and 14a to -f) and benzofuroxan (compounds 7 and 8a to -c) derivatives containing an *N*-acyl hydrazone subunit designed as antiparasitic agents to treat this neglected disease.

MATERIALS AND METHODS

Chemistry. Melting points (mp) were measured with an electrothermal melting point apparatus (SMP3; Bibby Stuart Scientific) in open capillary tubes and are uncorrected. Infrared (IR) spectra (KBr disc) were produced on an FTIR-8300 Shimadzu spectrometer, and the frequencies are expressed per cm. ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR spectra were scanned on a Bruker DRX-400 (400-MHz) NMR spectrometer using $CDCl_3$, dimethyl sulfoxide (DMSO)-d_6, and acetone$_{d6}$ as the solvents. Chemical shifts were expressed in parts per million (ppm) relative to tetramethylsilane. The coupling constants are reported in hertz (Hz), and signal multiplicities are reported as singlet (s), doublet (d), doublet of doublet (dd), and multiplet (m). Mass spectrometry of all compounds was performed with a model micrOTOF electrospray ionization-time of flight (ESI-TOF) (Bruker Daltonics) spectrometer, and the spectra were accumulated for 60 s. The compounds were injected at a flow rate of 300 μl/h, a capillary voltage of 4.5 kV, a cone voltage of 120 V, and a desolvation temperature at 180°C. The spectra were obtained in positive mode and acquired in the range of 200 to 800 *m/z*. Elemental analyses (C, H, and N) were performed on a PerkinElmer model 240C analyzer, and the data were within ±0.4% of the theoretical values. High-pressure liquid chromatography (HPLC) analysis was performed on a Shimadzu LC-10AD chromatograph equipped with a model SPD-10A UV-visible (UV-Vis) detector (Shimadzu). All compounds were analyzed by HPLC, and their purity was confirmed to be greater than 98.5%. The compounds were separated on a reversed-phase C_{18} (5-μm particle, 250-mm by 4.6-mm inside diameter [i.d.]) Shimadzu Shim-Pack CLC-ODS (M) column. HPLC-grade solvents (acetonitrile, methanol, acetic acid, and toluene) were used in the analyses and were bought from a local supplier. The progress of all reactions was monitored by thin-layer chromatography (TLC), which was performed on 2.0- by 6.0-cm^2 aluminum sheets precoated with silica gel 60 (HF-254; Merck) to a thickness of 0.25 mm. The developed chromatograms were viewed under UV light (265 nm) and treated with iodine vapor. Merck silica gel (70/230 mesh) was used for preparative column chromatography. Reagents and solvents were purchased from commercial suppliers and used as received.

Compounds 2, 7, and 12 were synthesized according to a previously described methodology (24–27). The compounds 2-, 3-, or 4-aminobenzohydrazide and 2-, 3-, or 4-hydroxybenzohydrazide were purchased commercially.

Preparation of compound 3. A mixture of (2-oxido-4-phenyl-1,2,5-oxadiazol-3-yl)methanol (compound 2) (0.4 g, 1.68 mmol), pyridinium chlorochromate (0.37 g, 1.71 mmol), and 20 ml of dry dichloromethane was stirred under nitrogen at 25°C for 24 h. Compound 3 was isolated by the addition of 30 ml of water. Then, the aqueous phase was extracted with

chloroform (4 times, 40 ml each). The organic phase was dried with sodium sulfate or magnesium sulfate. After filtration, the organic phase was concentrated under reduced pressure to produce brown oil. The samples were further purified with silica gel column chromatography, using hexane-ethyl acetate (6:4) as the mobile phase, producing compound 3 as an oil.

Data for 4-phenyl-1,2,5-oxadiazol-3-carbaldehyde 2-oxide (compound 3) are as follows: yield, 45%; infrared (IR) V_{max} (cm^{-1}; KBr pellets), 3,059 (C-H aromatic), 2,827 (C-$H_{aldehyde}$), 1,696 (C═O $imide_{aldehyde}$), 1,620 and 1,548 (C═N furoxan), 1,600 and 1,460 (C═C aromatic), 1,358 (N-O). ^{1}H NMR (400 MHz, $acetone_{d6}$) δ: 10.1 (1H; s), 7.97 (2H; m), 7.62 (3H; m) ppm. ^{13}C NMR (100 MHz, $acetone_{d6}$) δ: 115.78, 126.94, 128.32, 129.98, 131.95, 157.8, and 178 ppm. Analysis calculated for $C_9H_6N_2O_3$: C, 56.85; H, 3.18; N, 14.73. Found: C, 56.3; H, 3.19; N, 14.5. Low-resolution mass spectrometry (LRMS): *m/z* 191.05 $[M + H]^+$.

General procedure for the synthesis of compounds 4a and b and 8a to c. A mixture of 6-formyl-2,1,3-benzoxadiazol 1-oxide (compound 7) (0.37 g, 2.25 mmol) or 4-phenyl-1,2,5-oxadiazol-3-carbaldehyde 2-oxide (compound 3) (0.43 g, 2.25 mmol); 2-, 3-, or 4-aminobenzohydrazide (0.32 g, 2.15 mmol); and 8 ml of anhydrous ethanol containing 5 drops of glacial acetic acid was stirred at room temperature while protected from light for 15 h. The reaction was monitored by TLC using hexane-ethyl acetate (1:1) as the mobile phase. The compounds 4a and b or 8a to c were isolated by filtration and purified by crystallization using ethanol to produce yellow solids.

Data for 2-amino-*N*′-[(1*E*)-(2-oxido-4-phenyl-1,2,5-oxadiazol-3-yl) methylene]benzohydrazide (compound 4a) are as follows: yield, 48%; melting point (mp), 190 to 192°C. R_f, 0.07 (1:1 ethyl acetate-hexane). IR V_{max} (cm^{-1}; KBr pellets): 3,369 (N-H amine), 3,079 (C-H aromatic), 1,647 (C═O amide), 1,606 (C═N imine), 1,600 and 1,460 (C═C aromatic), 1,450 (N-O furoxan). ^{1}H NMR (300 MHz, $DMSO_{d6}$) δ: 11.97 (1H; s; N-H), 8.44 (1H; s; C-H_{imine}), 7.76 (2H; dd; J_{ortho} = 7.8 Hz and J_{meta} = 1.5 Hz), 7.61 (2H; d; J_{ortho} = 7.8 Hz), 7.56 (1H; dt; J_{ortho} = 7.8 Hz and J_{meta} = 1.5 Hz), 7.48 (1H; d; J_{ortho} = 7.9 Hz), 7.22 (1H; dd; J_{ortho} = 7.9 Hz and J_{meta} = 1.9 Hz), 6.77 (1H; dd; J_{ortho} = 7.9 Hz and J_{meta} = 1.9 Hz), 6.58 (1H; d; J_{ortho} = 7.9 Hz), 6.45 (2H; s; N-H_{amine}) ppm. ^{13}C NMR (75 MHz, $DMSO_{d6}$) δ: 165.2, 156.58, 148.7, 132.90, 131.76, 129.62, 129.31, 128.30, 126.90, 124.92, 121.7, 118.82, 116.40, 113.7 ppm. Analysis calculated for $C_{16}H_{13}N_5O_3$: C, 59.44; H, 4.05; N, 21.66. Found: C, 59.38; H, 4.27; N, 21.53. LRMS: *m/z* 324.10 $[M + H]^+$.

Data for 4-amino-*N*′-[(1*E*)-(2-oxido-4-phenyl-1,2,5-oxadiazol-3-yl) methylene]benzohydrazide (compound 4b) are as follows: yield, 50%; mp, 209 to 212°C. R_f, 0.3 (1:1 ethyl acetate-hexane). IR V_{max} (cm^{-1}; KBr pellets): 3,470 (N-H amine), 3,082 (C-H aromatic), 1,635 (C═O amide), 1,602 (C═N imine), 1,597 and 1,458 (C═C aromatic), 1,450 (N-O furoxan). ^{1}H NMR (300 MHz, $DMSO_{d6}$) δ: 11.98 (1H; s; N-H), 8.31 (1H; s; C-H_{imine}), 7.77 (2H; dd; J_{ortho} = 7.9 Hz and J_{meta} = 2 Hz), 7.60 (2H; d; J_{ortho} = 7.9 Hz), 7.56 (1H; dt; J_{ortho} = 7.9 Hz and J_{meta} = 2 Hz), 7.22 (2H; dd; J_{ortho} = 8.2 Hz), 6.59 (2H; dd; J_{ortho} = 8.2 Hz), 5.89 (2H; s; N-H_{amine}) ppm. ^{13}C NMR (75 MHz, $DMSO_{d6}$) δ: 164.70, 157.21, 149.87, 131.02, 130.07, 129.70, 129.42, 126.92, 125.02, 121.8, 115.33, 113.41 ppm. Analysis calculated for $C_{16}H_{13}N_5O_3$: C, 59.44; H, 4.05; N, 21.66. Found: C, 59.70; H, 4.19; N, 21.85. LRMS: *m/z* 324.10 $[M + H]^+$.

Data for 2-amino-*N*′-[(1*E*)-(3-oxido-2,1,3-benzoxadiazol-5-yl) methylene]benzohydrazide (compound 8a) are as follows: yield, 96%; mp, 202 to 203°C. R_f 0.57 (1:1 ethyl acetate-hexane). IR V_{max} (cm^{-1}; KBr pellets): 3,421 (N-H aromatic), 3,078 (C-H aromatic), 1,622 (C═O amide), 1,612 (C═N imine), 1,560 and 1,464 (C═C aromatic), 1,450 (N-O furoxan). ^{1}H NMR (300 MHz, $DMSO_{d6}$) δ: 10.97 (1H; s; N-H), 8.44 (1H; s; C-H_{imine}), 7.66 to 8.22 (3H; m), 7.58 (1H; d; J_{ortho} = 8 Hz), 7.22 (1H; dd; J_{ortho} = 8 Hz/J_{meta} = 2 Hz), 6.78 (1H; d; J_{ortho} = 8 Hz), 6.58 (1H; dd; J_{ortho} = 8 Hz/J_{meta} = 2Hz), 6.45 (2H; s; N-H_{amine}). ^{13}C NMR (75 MHz, $DMSO_{d6}$) δ: 165.2, 149.1, 147.2, 133.0, 132.7, 131.0, 129.7, 129.4, 129.2, 128.5, 119.6, 116.7, 115.2, 114.4 ppm. Analysis calculated for $C_{14}H_{11}N_5O_3$: C, 56.56; H, 3.73; N, 23.56. Found: C, 56.77; H, 3.52; N, 23.80. LRMS: *m/z* 298.09 $[M + H]^+$.

Data for 3-amino-*N*′-[(1*E*)-(3-oxido-2,1,3-benzoxadiazol-5-yl) methylene]benzohydrazide (compound 8b) are as follows: yield, 70%; mp, 211 to 213°C. R_f, 0.76 (1:1 ethyl acetate-hexane). IR V_{max} (cm^{-1}; KBr pellets): 3,431 (N-H aromatic), 3,082 (C-H aromatic), 1,654 (C═O amide), 1,610 (C═N imine), 1,556 and 1,467 (C═C aromatic), 1,450 (N-O furoxan). ^{1}H NMR (300 MHz, $DMSO_{d6}$) δ: 10.01 (1H; s; N-H), 8.82 (1H; s; C-H_{imine}), 7.65 to 8.13 (6H; m), 6.73 to 7.15 (3H; m; N-H_{amine}). ^{13}C NMR (75 MHz, $DMSO_{d6}$) δ: 164.7, 148.9, 147.2, 133.8, 133.3, 130.7, 130.2, 129.7, 129.4, 118.6, 117.9, 115.2, 114.4, 112.1 ppm. Analysis calculated for $C_{14}H_{11}N_5O_3$: C, 56.56; H, 3.73; N, 23.56. Found: C, 56.33; H, 3.59; N, 23.64. LRMS: *m/z* 298.09 $[M + H]^+$.

Data for 4-amino-*N*′-[(1*E*)-(3-oxido-2,1,3-benzoxadiazol-5-yl) methylene]benzohydrazide (compound 8c) are as follows: yield, 98%; mp, 225 to 227°C. R_f 0.46 (1:1 ethyl acetate-hexane). IR V_{max} (cm^{-1}; KBr pellets): 3,419 (N-H aromatic), 3,080 (C-H aromatic), 1,654 (C═O amide), 1,627 (C═N imine), 1,550 and 1,461 (C═C aromatic), 1,450 (N-O furoxan). ^{1}H NMR (300 MHz, $DMSO_{d6}$) δ: 11.80 (1H; s; N-H), 8.45 (1H; s; C-H_{imine}), 7.72 to 8.03 (3H; m), 7.69 (2H; dd; J_{ortho} = 7.8 Hz/J_{meta} = 1.9 Hz), 6.50 (2H; dd; J_{ortho} = 7.8 Hz/J_{meta} = 1.9 Hz), 5.87 (2H; s; N-H_{amine}). ^{13}C NMR (75 MHz, $DMSO_{d6}$) δ: 163.7, 158.9, 152.1, 142.7, 137.9, 129.8, 128.8, 119.4, 115.7, 113.3, 112.4 ppm. Analysis calculated for $C_{14}H_{11}N_5O_3$: C, 56.56; H, 3.73; N, 23.56. Found: C, 56.19; H, 3.88; N, 23.71. LRMS: *m/z* 298.09 $[M + H]^+$.

General procedure for the synthesis of compounds 13a and -b. A mixture of 3-hydroxybenzaldehyde or 4-hydroxybenzaldehyde (0.4 g, 3.3 mmol), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU; 0.5 ml, 3.3 mmol), and 10 ml of dichloromethane was stirred under nitrogen at room temperature for 20 min. Then, 3,4-bis(phenylsulfonyl)-1,2,5-oxadiazole 2-*N*-oxide (compound 12) (1.0 g, 2,73 mmol) was slowly added to the reaction mixture. The reaction mixture was stirred under nitrogen at room temperature for 2 to 2.5 h and monitored by TLC (1:1, dichloromethane-hexane). The compounds 13a and -b were isolated by the addition of 50 ml of dichloromethane. Then, the organic phase was washed with saturated potassium carbonate (5 times, 30 ml). The organic phase was dried with sodium sulfate. After filtration, the organic phase was concentrated under reduced pressure to produce a yellow solid. The samples were further purified with silica gel column chromatography, using dichloromethane-hexane (5:4) as the mobile phase to give the compounds 13a and -b as white powder.

Data for 3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy} benzaldehyde (compound 13a) are as follows: yield, 57%; mp, 102 to 104°C. R_f, 0.1 (1:1 dichloromethane-hexane). IR V_{max} (cm^{-1}; KBr pellets): 3,081 (C-H aromatic), 2,762 (C-H aldehyde), 1,697 (C═O aldehyde), 1,598 (C═N furoxan), 1,442 (N-O furoxan), 1,560 to 1,450 (C═C aromatic), 1,357 and 1,165 (S═O sulfone). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 10.03 (1H; s; C-$H_{aldehyde}$), 8.10 (2H; d, J_{ortho} = 8.0 Hz), 7.86 (1H; m), 7.83 (3H; t), 7.66 (3H; m) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ: 190.50, 157.80, 153.30, 138.20, 137.82, 135.87, 130.93, 129.87, 128.60, 128,32, 125.76, 119.82, 110.69 ppm. Analysis calculated for $C_{15}H_{10}N_2O_6S$: C, 52.02; H, 2.91; N, 8.09. Found: C, 52.06; H, 3.22; N, 7.91. LRMS: *m/z* 347.03 $[M + H]^+$.

Data for 4-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy} benzaldehyde (compound 13b) are as follows: yield, 33%; mp, 118 to 120°C. R_f, 0.2 (1:1 dichloromethane-hexane). IR V_{max} (cm^{-1}; KBr pellets): 3,078 (C-H aromatic), 2,744 (C-H aldehyde), 1,707 (C═O aldehyde), 1,599 (C═N furoxan), 1,450 (N-O furoxan), 1,537 to 1,450 (C═C aromatic), 1,357 and 1,161 (S═O sulfone). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 10.03 (1H; s; C-$H_{aldehyde}$), 8.03 (4H; m), 7.92 (1H; tt; J_{ortho} = 9 Hz and J_{meta} = 2.9 Hz), 7.75 (2H; tt; J_{ortho} = 9 Hz), 7.66 (2H; d; J_{ortho} = 9 Hz) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ: 193.50, 158.90, 158.65, 138.20, 137.80, 135.60, 133.20, 131.60, 130.10, 121.30, 112.90 ppm. Analysis calculated for $C_{15}H_{10}N_2O_6S$: C, 52.02; H, 2.91; N, 8.09. Found: C, 51.88; H, 2.73; N, 8.27. LRMS: *m/z* 347.03 $[M + H]^+$.

General procedure for the synthesis of compounds 14a to -f. A solution of 3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}benzaldehyde (compound 13a) (0.3 g, 0.87 mmol) or 4-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}benzaldehyde (compound 13b) (0.3 g, 0.87 mmol) in 15 ml ethanol containing 5 drops of glacial acid acetic was stirred at room temperature for 15 min. Later, 2-, 3-, or 4-hydroxybenzaldehyde (0.106 g, 0.87 mmol) was added, and the reaction mixture was stirred under nitrogen at room temperature for 12 h and monitored by TLC (1:1, ethyl acetate-hexane). The solvent was concentrated under reduced pressure, and ice-water was added in order to precipitate the desired products. If necessary, the samples could be further purified with silica gel column chromatography, using ethyl acetate-hexane (1:1) as the mobile phase to give the compounds 14a to -f with variable yields (60 to 90%).

Data for 2-hydroxy-*N*′-[(1*E*)-(3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}phenyl)methylene]benzohydrazide (compound 14a) are as follows: yield, 90%; mp, 230 to 233°C. R_f, 0.1 (1:1 ethyl acetate-hexane). IR V_{max} (cm^{-1}; KBr pellets): 3,448 (O-H), 3,250 (N-H), 3,082 (C-H aromatic), 1,631 (C═O amide), 1,537 (C═N imine), 1,450 (N-O furoxan), 1,456 and 1,163 (S═O sulfone). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 11.92 (1H; s; O-H), 11.72 (1H; s), 8.48 (1H; s; C-H_{imine}), 8.08 (2H; d; J_{ortho} = 9 Hz), 7.93 (1H; tt; J_{ortho} = 9 Hz/J_{meta} = 1.2 Hz), 7.81 (4H; m), 7.70 (1H; d; J_{ortho} = 9 Hz), 7.60 (1H; t; J_{ortho} = 9 Hz), 7.51 (1H; dd; J_{meta} = 1.2 Hz), 7.45 (1H; t; J_{ortho} = 9 Hz/J_{meta} = 1.2 Hz), 6.97 (2H; t; J_{ortho} = 9 Hz) ppm. ^{13}C NMR (75 MHz, $CDCl_3$) δ: 164.7, 158.6, 158.5, 152.9, 147.3, 147.1, 136.9, 136.4, 136.3, 130.8, 130.1, 128.8, 128.6, 126.1, 121.5, 119.1, 117.7, 117.3, 116.2, 111.3 ppm. Analysis calculated for $C_{22}H_{16}N_4O_7S$: C, 55.0; H, 3.36; N, 11.66. Found: C, 54.6; H, 3.18; N, 11.29. LRMS: *m/z* 481.08 $[M + H]^+$.

Data for 3-hydroxy-*N*′-[(1*E*)-(3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}phenyl)methylene]benzohydrazide (compound 14b) are as follows: yield, 85%; mp, 120 to 124°C. R_f, 0.1 (1:1 ethyl acetate-hexane). IR V_{max} (cm^{-1}; KBr pellets): 3,327 (O-H), 3,218 (N-H), 3,080 (C-H aromatic), 1,629 (C═O amide), 1,537 (C═N imine), 1,450 (N-O furoxan), 1,446 and 1,163 (S═O sulfone). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 11.88 (1H; s; O-H), 9.77 (1H; s; N-H), 8.46 (1H; s; C-H_{imine}), 8.07 (2H; dd; J_{ortho} = 9 Hz/J_{meta} = 2 Hz), 7.93 (1H; tt; J_{ortho} = 9 Hz/J_{meta} = 2 Hz), 7.78 (3H; m), 7.67 (1H; d; J_{ortho} = 9 Hz), 7.58 (1H; t; J_{ortho} = 9 Hz), 7.51 (1H; d; J_{meta} = 1.2 Hz), 7.32 (3H; m), 6.99 (1H; dd; J_{meta} = 2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 163.4, 158.5, 157.4, 152.8, 146.2, 136.9, 136.7, 136.2, 134.6, 130.7, 130.0, 129.6, 128.5, 125.8, 121.3, 118.8, 118.2, 117.5, 114.4, 111.2 ppm. Analysis calculated for $C_{22}H_{16}N_4O_7S$: C, 55.0; H, 3.36; N, 11.66. Found: C, 55.3; H, 3.62; N, 11.72. LRMS: *m/z* 481.08 $[M + H]^+$.

Data for 4-hydroxy-*N*′-[(1*E*)-(3-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}phenyl)methylene]benzohydrazide (compound 14c) are as follows: yield, 60%; mp, 206 to 210°C. R_f, 0.1 (1:1 ethyl acetate-hexane). IR V_{max} (cm^{-1}; KBr pellets): 3,427 (O-H), 3,224 (N-H), 3,081 (C-H aromatic), 1,653 (C═O amide), 1,537 (C═N imine), 1,450 (N-O furoxan), 1,454 and 1,165 (S═O sulfone). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 11.70 (1H; s; O-H), 10.16 (1H; s; N-H), 8.44 (1H; s; C-H_{imine}), 8.07 (2H; dd; J_{ortho} = 9 Hz/J_{meta} = 2 Hz), 7.92 (1H; tt; J_{ortho} = 9 Hz/J_{meta} = 2 Hz), 7.80 (5H; m), 7.65 (1H; d; J_{ortho} = 9 Hz), 7.56 (1H; t; J_{ortho} = 9 Hz), 7.49 (1H; d; J_{meta} = 2 Hz), 6.87 (2H; d; J_{ortho} = 9 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 160.8, 158.5, 152.8, 149.3, 145.3, 136.8, 135.2, 135.1, 130.6, 130.0, 128.5, 125.7, 123.7, 121.1, 117.4, 115.0, 111.2 ppm. Analysis calculated for $C_{22}H_{16}N_4O_7S$: C, 55.0; H, 3.36; N, 11.66. Found: C, 55.2; H, 3.44; N, 11.29. LRMS: *m/z* 481.08 $[M + H]^+$.

Data for 2-hydroxy-*N*′-[(1*E*)-(4-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}phenyl)methylene]benzohydrazide (compound 14d) are as follows: yield, 90%; mp, 186 to 189°C. R_f, 0.1 (1:1 ethyl acetate-hexane). IR V_{max} (cm^{-1}; KBr pellets): 3,448 (O-H), 3,251 (N-H), 3,083 (C-H aromatic), 1,622 (C═O amide), 1,537 (C═N imine), 1,450 (N-O furoxan), 1,446 and 1,163 (S═O sulfone). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 11.97 (1H; s; O-H), 10.2 (1H; s; N-H), 8.5 (1H; s; C-H_{imine}), 8.06 (2H; dd; J_{ortho} = 8 Hz/J_{meta} = 2 Hz), 7.92 (1H; tt; J_{ortho} = 8 Hz/J_{meta} = 2 Hz), 7.90 (3H; m), 7.77 (2H; t; J_{ortho} = 8 Hz), 7.54 (2H; d; J_{ortho} = 9 Hz), 7.45 (1H; dd; J_{ortho} = 8 Hz/J_{meta} = 2 Hz), 6.97 (2H; dd; J_{ortho} = 8 Hz/J_{meta} = 2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 164.7, 158.9, 153.8, 143.3, 136.8, 136.1, 133.8, 132.4, 130.0, 128.9, 128.7, 128.5, 120.0, 119.0, 117.2, 116.0, 111.3 ppm. Analysis calculated for $C_{22}H_{16}N_4O_7S$: C, 55.0; H, 3.36; N, 11.66. Found: C, 54.7; H, 3.11; N, 11.38. LRMS: *m/z* 481.08 $[M + H]^+$.

Data for 3-hydroxy-*N*′-[(1*E*)-(4-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}phenyl)methylene]benzohydrazide (compound 14e) are as follows: yield, 60%; mp, 201 to 204°C. R_f, 0.1 (1:1 ethyl acetate-hexane). IR V_{max} (cm^{-1}; KBr pellets): 3,410 (O-H), 3,253 (N-H), 3,080 (C-H aromatic), 1,625 (C═O amide), 1,539 (C═N imine), 1,450 (N-O furoxan), 1,415 and 1,168 (S═O sulfone). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 11.83 (1H; s; O-H), 9.78 (1H; s; N-H), 8.47 (1H; s; C-H_{imine}), 8.06 (2H; dd; J_{ortho} = 8 Hz/J_{meta} = 2 Hz), 7.90 (1H; t; J_{ortho} = 8 Hz/J_{meta} = 2 Hz), 7.80 (4H; m), 7.52 (2H; d; J_{ortho} = 8 Hz), 7.32 (3H; m), 6.98 (1H; dd; J_{meta} = 2 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 163.3, 158.1, 157.4, 153.7, 146.4, 136.9, 136.2, 135.1, 132.7, 130.2, 129.6, 128.5, 128.2, 120.1, 118.8, 118.1, 114.5, 111.3 ppm. Analysis calculated for $C_{22}H_{16}N_4O_7S$: C, 55.0; H, 3.36; N, 11.66. Found: C, 55.2; H, 3.21; N, 11.51. LRMS: *m/z* 481.08 $[M + H]^+$.

Data for 4-hydroxy-*N*′-[(1*E*)-(4-{[5-oxido-4-(phenylsulfonyl)-1,2,5-oxadiazol-3-yl]oxy}phenyl)methylene]benzohydrazide (compound 14f) are as follows: yield, 90%; mp, 142 to 145°C. R_f, 0.1 (1:1 ethyl acetate-hexane). IR V_{max} (cm^{-1}; KBr pellets): 3,412 (O-H), 3,253 (N-H), 3,082 (C-H aromatic), 1,626 (C═O amide), 1,539 (C═N imine), 1,451 (N-O furoxan), 1,415 and 1,168 (S═O sulfone). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 11.69 (1H; s; O-H), 10.12 (1H; s; N-H), 8.44 (1H; s; C-H_{imine}), 8.05 (2H; dd; J_{ortho} = 9 Hz/J_{meta} = 1.2 Hz), 7.92 (1H; t; J_{ortho} = 9 Hz/J_{meta} = 1.2 Hz), 7.82 (6H; m), 7.51 (2H; d; J_{ortho} = 9 Hz), 6.86 (2H; d; J_{ortho} = 9 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) δ: 162.9, 160.7, 158.1, 152.5, 145.9, 136.9, 136.2, 135.2, 132.9, 130.0, 128.6, 128.5, 120.0, 115.0, 111.3 ppm. Analysis calculated for $C_{22}H_{16}N_4O_7S$: C, 55.0; H, 3.36; N, 11.66. Found: C, 55.32; H, 3.44; N, 11.71. LRMS: *m/z* 481.08 $[M + H]^+$.

Leishmanicidal activity. (i) Animals. Adult male Swiss albino mice (20 to 35 g) were used in the experiments. They were housed in single-sex cages under a 12-h light/12-h dark cycle (lights on at 06:00) in a controlled-temperature room (22 ± 2°C). The mice had free access to food and water. Groups of two animals were used in each test group. The experiments were performed after the protocol was approved by the local Institutional Ethics Committee (protocol number CEUA/FCF/Car no. 53/2012). All experiments were performed in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals.

(ii) Parasite culture. Promastigotes of *L. amazonensis* (MPRO/BR/1972/M1841-LV-79) recently isolated from mice were maintained at 28°C in liver-infusion tryptose (LIT) supplemented with 10% fetal bovine serum (FBS), penicillin (Sigma-Aldrich), and streptomycin (Sigma-Aldrich).

(iii) Promastigotes. Cultured promastigotes of *L. amazonensis* at the end of the exponential growth phase were seeded at 8×10^6 parasites/ml in 96-well flat-bottom plates (TPP; Sigma-Aldrich). Compounds, amphotericin B, and pentamidine (Sigma-Aldrich) were dissolved in DMSO (the highest concentration was 1.4%, which was not hazardous to the parasites, as previously determined), added to the parasite suspension to final concentrations between 0.5 μM and 100.0 μM, and incubated at 28°C for 72 h. The assays were carried out in triplicate. Leishmanicidal effects were assessed by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) method (28, 29). Absorbances were read at 490 nm. The drug concentration corresponding to 50% parasite growth inhibition was expressed as the inhibitory concentration (IC_{50}) in μM (28).

(iv) Cytotoxicity using murine macrophages. To determine the cytotoxicity, thioglycolate-stimulated mice were used to collect peritoneal macrophages. Murine peritoneal macrophages were seeded in 96-well flat-bottom plates (TPP) at a density of 1×10^5 cells/well (100 μl/well) in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 25

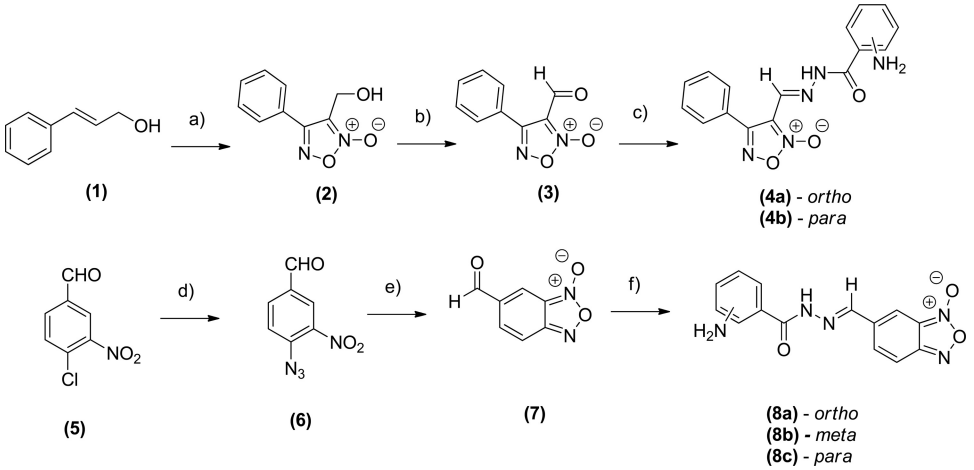

FIG 2 Reagents and conditions: (a) $NaNO_2$, CH_3COOH, 0°C, 24 h, 90%; (b) pyridinium chlorochromate, CH_2Cl_2, 25°C, 24 h, 45%; (c) aminobenzohydrazide derivative, ethanol, H^+_{cat}, 25°C, 2 to 4 h, 85 to 98%; (d) NaN_3, DMSO, 75°C, 1 h, 99%; (e) toluene, 120°C, 1.5 h, 78%; (f) aminobenzohydrazide derivative, ethanol, H^+_{cat}, 25°C, 2 to 4 h, 85 to 98%.

mM HEPES, and 2 mM L-glutamine and incubated for 4 h at 37°C in a 5% CO_2-air mixture. The medium was removed, and then new medium was added to the cells, which were treated with different concentrations of compounds, amphotericin B, and pentamidine (Sigma-Aldrich). Cells without drugs were used as a negative control. After that, plates were incubated for 24 h at 37°C in a 5% CO_2-air mixture. Subsequently, the MTT colorimetric assay was carried out as described above. Absorbance was read in a 96-well plate reader (Robonik) at 595 nm. The drug concentration corresponding to 50% of cell growth inhibition was expressed as the inhibitory concentration (CC_{50}) (29).

(v) Amastigotes. Murine peritoneal macrophages were plated at 3×10^5 cells/well on coverslips (13-mm diameter) previously arranged in a 24-well plate in RPMI 1640 medium supplemented with 10% inactivated FBS and allowed to adhere for 4 h at 37°C in 5% CO_2. Adherent macrophages were infected with promastigotes in the stationary growth phase using a ratio of 5:1 *L. amazonensis* parasites per cell at 37°C in 5% CO_2 for 4 h. After that time, the noninternalized parasites were removed by washing, and infected cultures were incubated in RPMI 1640 medium for 24 h at 37°C in 5% CO_2 to allow parasite multiplication. Then, infected cells were treated with different concentrations of the compounds, amphotericin B (Sigma-Aldrich), and pentamidine (Sigma-Aldrich) for 24 h. The cells were then fixed in a methanol solution and stained with Giemsa stain. The number of amastigotes/100 macrophage cells and the percent infected cells were determined. The concentration that caused a 50% decrease of growth inhibition compared to the control was determined by regression analysis and expressed as the inhibitory concentration (IC_{50}) in μM (29).

Nitric oxide production. (i) *In vitro* studies. A solution of the appropriate compound (20 μl) in DMSO was added to 2 ml of a mixture of 50 mM phosphate buffer (pH 7.4) and methanol (1:1, vol/vol), containing 5 mM L-cysteine. The final concentration of the compound was 10^{-4} M. After 1 h at 37°C, 1 ml of the reaction mixture was treated with 250 μl of Griess reagent (4 g of sulfanilamide, 0.2 g of *N*-naphthylethylenediamine dihydrochloride, 85% phosphoric acid [10 ml] in distilled water [final volume, 100 ml]). After 10 min at room temperature, the absorbance was measured at 540 nm using a Shimadzu UV-2501PC spectrophotometer. Standard sodium nitrite solutions (10 to 80 nmol/ml) were used to construct the calibration curve. The yields of nitrite are expressed as % NO_2^- (mol/mol). No production of nitrite was observed in the absence of L-cysteine (30–32).

(ii) Production in macrophage medium. Culture supernatants from murine peritoneal macrophages were infected with *L. amazonensis* and treated with the compounds 8a and 14a at 17 μM and 10.4 μM, respectively. Culture supernatants were collected after 48 h, and nitrite production was determined using Griess reagent. One hundred microliters of culture supernatants and 100 μl of Griess reagent were pipetted into 96-well plates and incubated at room temperature for 10 min. Then, absorbance was read in a 96-well plate reader (Robonik) at 540 nm (33). The results, expressed as millimolar concentrations, were determined after extrapolation of values obtained from a standard curve made with sodium nitrite ($NaNO_2$). Macrophages were used as a negative control while macrophages infected and not infected with *L. amazonensis* and treated with 5 μg/ml of lipopolysaccharide (LPS) were used as positive controls. All assays were performed in triplicate ($n = 3$ experiments).

***In vitro* stability study.** *In vitro* hydrolysis was performed using the ultrahigh-performance liquid chromatography (UPLC) method. The equipment used was a model Acquity H class UPLC (Waters) equipped with a UV-visible (UV-Vis) detector. The compounds 8a and 14a were separated in a BEH reverse-phase C_{18} column (1.7-μm particle, 2.1 by 50 mm). The gradient flow was 30:70 (water-acetonitrile) to 10:10:80 (methanol-water-acetonitrile) in 3 min for compound 8a. The gradient flow was 50:50 (water-acetonitrile) to 70:30 (water-acetonitrile) in 3 min for compound 14a. The flow rate for both methods was 0.4 ml/min over 6 min and detection at 254 nm. The calibration curve was linear for compound 8a ($r^2 = 0.9990$, $n = 6$, 10 to 30 μg/ml) and 14a ($r^2 = 1$, $n = 6$, 20 to 80 μg/ml).

For hydrolysis, an appropriate solution of compounds 8a and 14a was diluted in acetonitrile at 100 μg/ml. Then, these solutions were diluted in buffer (pH 5.4) and water (pH 7.0) to 15 μg/ml for compound 8a and 50 μg/ml for compound 14a. The samples were subjected to constant agitation in a shaker (137 rpm) at 37°C during the entire assay. The samples were obtained at the following times: 0, 4, 6, 8, 18, and 24 h. All analyses were conducted in triplicate, and the results are expressed as the averages of the concentrations in percentages (± standard error of the mean [SEM]). The data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons among groups using the software GraphPad Prism (version 5.01).

RESULTS

Chemistry. The synthetic routes for the preparation of the furoxan (compounds 3, 4a and -b, 13a and -b, and 14a to -f) and benzofuroxan (compounds 7 and 8a to -c) derivatives are summarized in Fig. 2 and 3.

The compounds 4a and 4b were obtained in three synthetic steps with an overall yield of 39 to 45%. Cinnamic alcohol was reacted with sodium nitrite in acetic acid medium to give (2-

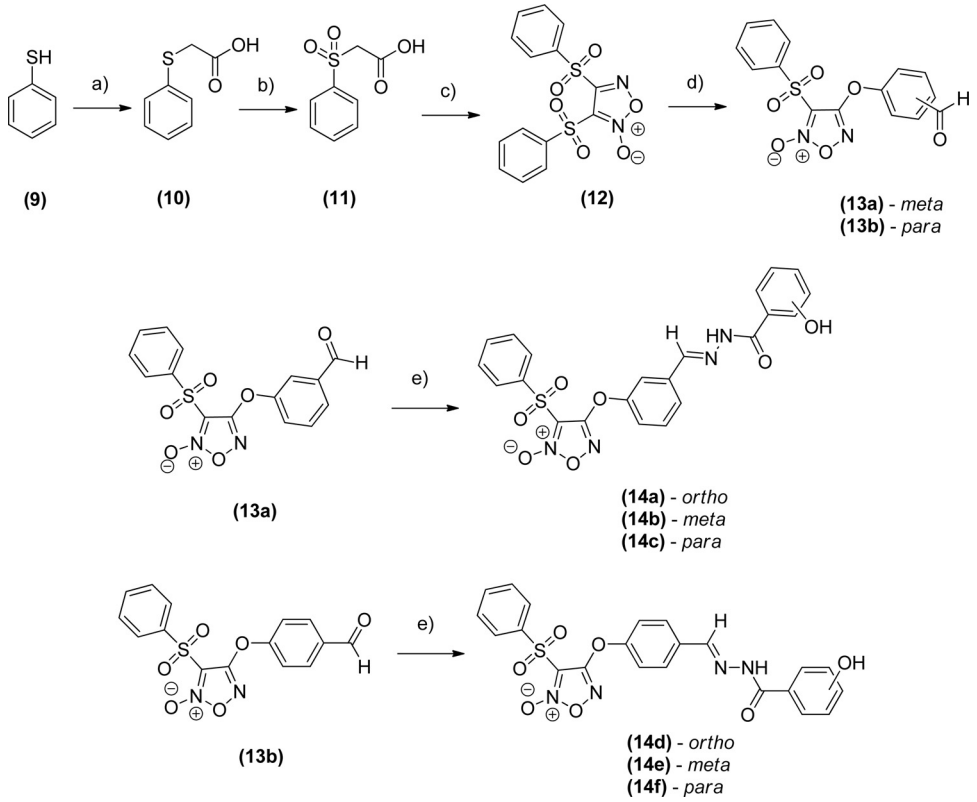

FIG 3 Reagents and conditions: (a) $ClCH_2COOH$, NaOH, H_2O, 120°C, 1 h, 95%; (b) acetic acid, H_2O_2 30%, 25°C, 24 h, 90%; (c) HNO_3 red fuming 99%, acetic acid, 0°C to 110°C, 45 min, 45%; (d) hydroxybenzaldehyde, DBU, CH_2Cl_2, 25°C, 2 to 4 h, 46 to 57%; (e) aminobenzohydrazide derivative, ethanol, H^+_{cat}, 25°C, 12 h, 60 to 90%.

oxido-4-phenyl-1,2,5-oxadiazol-3-yl)methanol (step 1). The alcohol function was oxidized to aldehyde (step 2) using pyridinium chlorochromate in dichloromethane medium with a yield of 51% (Fig. 2) (34).

The benzofuroxan (compound 7) and bis-aryl sulfonylfuroxan (compound 12) derivatives were prepared according to the procedures previously described (24–27, 33). The 3- or 4-hydroxybenzaldehyde was reacted with bis-aryl sulfonylfuroxan (compound 12), using 1,8-diazabicyclo[5.4.0]undec-7-ene as a base, to give compound 13a or 13b in yields varying between 46 and 57% (Fig. 3).

The last step to obtain *N*-acyl hydrazones 4a and -b, 8a to -c, and 14a to -f involves the coupling reaction between the aldehyde function and previously selected benzohydrazides (step 3) to obtain the target compounds in excellent yields varying between 85 and 98% (Fig. 2 and 3). The structures of all the compounds were established by mass spectroscopy, elemental analysis, IR spectroscopy, and ^1H and ^{13}C NMR. All compounds were analyzed by HPLC, and their purity was confirmed to be over 98.5%. The analysis of ^1H NMR spectra of all acyl hydrazone derivatives (compounds 4a and -b, 8a to -c, and 14a to -f) has shown a single signal referring to ylidenic hydrogen attributed to the *E*-diastereomer (35, 36).

Determination of the IC_{50}. (i) Promastigotes. The leishmanicidal activity of the furoxan (compounds 3, 4a and -b, 13a and -b, and 14a to -f) and benzofuroxan (compounds 7 and 8a to -c) derivatives was initially determined against *L. amazonensis* promastigote forms. The compounds were biologically active against the parasite in a dose-dependent manner. The aldehyde intermediates (compounds 3, 13a and -b, and 7) were very active against the promastigote form; the IC_{50}s for these compounds ranged from 0.79 to 4.29 μM. However, the selectivity index 1 (SI^1) for the intermediates ranged from 0.38 to 3.61, suggesting that these aldehydes (compounds 3, 13a and -b, and 7) could cause toxic effects against macrophage cells. Compounds 8a and 14a to -e were at least 1.6-fold more active than pentamidine used as a control against promastigote forms. A remarkable antiprotozoan effect was observed for compounds 8a and 14a (IC_{50}, <2.97 μM), which showed higher activity against *L. amazonensis* than did the standard drug amphotericin B (IC_{50}, 3.22 μM). Compound 14b has demonstrated activity comparable to that of amphotericin B. Cytotoxicity studies using murine macrophages have demonstrated that the intermediate compounds (3, 13a and -b, and 7) were not selective for the parasite, and the SI^1 ranged from 0.38 to 3.61. The selectivity index 1 (SI^1) represents the ratio between CC_{50} and IC_{50} for promastigotes. The compounds 8a and 14a to -e were more selective for the parasite than for mammalian cells (SIs ranging from 7.5 to 38.6). It is worth highlighting that these results are better than those for the control drugs pentamidine (SI^1, 3.5) and amphotericin B (SI^1, 7.16) (Table 1).

(ii) Intracellular amastigotes. Based on the previously obtained data for promastigotes (Table 1), compounds 8a and 14a to -e, which presented higher SI values than those of the control drugs pentamidine and amphotericin B, were further evaluated against intracellular amastigotes. *L. amazonensis*-infected macrophages were maintained in medium containing the compounds in

TABLE 1 Biological activity of compounds, amphotericin B, and pentamidine against promastigotes and amastigotes of *L. amazonensis* (IC_{50}); inhibition of macrophages (CC_{50}); selectivity index (SI); and NO release data[c]

Compound	IC_{50} (μg/ml) for promastigotes[a]	CC_{50} (μg/ml) for macrophages[a]	SI^1	IC_{50} (μg/ml) for amastigotes[a]	SI^2	% NO_2^- (mol/mol),[a,b] L-Cys, 50×10^{-4} M
3	1.48 ± 0.11	5.35 ± 0.07	3.61	ND	ND	12.3 ± 0.7
4a	67.98 ± 0.34	152.17 ± 2.20	2.24	ND	ND	8.2 ± 0.5
4b	95.83 ± 0.65	180.12 ± 25.83	1.88	ND	ND	7.8 ± 0.6
7	4.29 ± 0.21	1.62 ± 0.16	0.38	ND	ND	0
8a	2.09 ± 0.17	63.18 ± 0.61	30.22	2.16 ± 0.16	29.25	0
8b	163.81 ± 0.77	248.31 ± 2.83	1.52	ND	ND	0
8c	40.20 ± 0.33	200.33 ± 2.39	4.98	ND	ND	0
13a	0.79 ± 0.01	1.68 ± 0.02	2.13	ND	ND	28.2 ± 1.1
13b	0.82 ± 0.05	2.03 ± 0.83	2.47	ND	ND	27.8 ± 1.5
14a	2.97 ± 0.18	114.6 ± 4.1	38.58	2.12 ± 0.1	54.05	25.3 ± 1.3
14b	3.21 ± 0.23	32.10 ± 5.83	10.0	4.3 ± 0.52	7.47	24.1 ± 2.6
14c	4.77 ± 0.39	35.83 ± 1.62	7.51	6.33 ± 0.40	5.66	27.4 ± 3.1
14d	4.60 ± 0.37	45.62 ± 1.47	9.91	4.64 ± 0.10	9.83	24.5 ± 2.3
14e	6.43 ± 0.54	208.33 ± 0.17	32.40	8.22 ± 0.27	25.34	26.2 ± 2.7
14f	12.43 ± 0.65	23.54 ± 2.22	1.89	ND	ND	25.8 ± 2.4
AmpB	3.22 ± 0.03	23.10 ± 2.52	7.16	4.92 ± 0.14	4.69	0
Pent	10.19 ± 0.85	35.69 ± 6.84	3.50	6.25 ± 0.58	3.27	0
DNS	ND	ND	ND	ND	ND	11.2 ± 0.8

[a] Mean ± standard error of the mean.
[b] Determined by Griess reaction, after incubation for 1 h at 37°C in pH 7.4 buffered water-methanol mixture, in the presence of a 1:50 molar excess of L-cysteine.
[c] Abbreviations: DNS, isosorbide dinitrate (DNS possesses two ONO_2 groups that may release NO); SI^1, ratio between CC_{50} and IC_{50} for promastigotes; SI^2, ratio between CC_{50} and IC_{50} for amastigotes; AmpB, amphotericin B (reference drug); Pent, pentamidine (reference drug); ND, not determined.

a different range of concentrations (20 to 0.5 μM) for a 24-h period. The compounds 8a, 14a and -b, and 14d were more active against intracellular amastigotes than was amphotericin B. Compounds 8a and 14a (IC_{50}, <2.16 μM) showed more potent antiparasitic effects than did amphotericin B and pentamidine (IC_{50}s of 4.92 μM and 6.25 μM, respectively). The selectivity index 2 (SI^2) represents the ratio between CC_{50} and IC_{50} for amastigotes. Furthermore, the SI^2s of compounds 8a, 14a and -b, and 14d (SI^2, >7.47) showed relevant selectivity for the parasite, compounds 8a and 14a being at least 6.23-fold less cytotoxic to the mammalian cells (SI^2 values of 29.25 and 54.05, respectively) than were pentamidine (SI^2, 3.27) and amphotericin B (SI^2, 4.69).

Nitric oxide production. (i) *In vitro* studies. The nitrite production from the oxidative reaction of nitric oxide, oxygen, and water for all compounds (compounds 3, 4a and -b, 7, 8a to -c, 13a and -b, and 14a to -f) was measured using the Griess reaction procedure (30–32). This procedure is based on the chemical diazotization reaction, which uses sulfonamide and *N*-1-naphthylenediamine dihydrochloride under acid conditions. The extent of thiol-induced NO generation was determined after 1 h of incubation in the presence of a large excess of L-cysteine (1:50). The results, expressed as percentages of nitrite (NO_2^-; mol/mol), are summarized in Table 1. Isosorbide dinitrate (DNS), used as a control, induced 11.2% of nitrite formation. All furoxan compounds (compounds 4a, 4b, and 14a to -f) were capable of inducing nitrite formation in a range between 7.8% and 27.4%. The benzofuroxan derivatives (compounds 8a to -c) were not able to generate nitrite in the medium.

(ii) Production in macrophage medium. Culture supernatants from murine *L. amazonensis*-infected peritoneal macrophages treated with 17 μM and 10.4 μM concentrations of the compounds 8a and 14a, respectively, were collected after 48 h of incubation, and the nitrite content was determined by the Griess reaction procedure (26–28). Our results showed that both compounds (8a and 14a) were able to increase nitrite in the medium at 0.75 mM and 1.0 mM (Fig. 4).

***In vitro* stability study.** In order to characterize stability, the chemical hydrolysis of compounds 8a and 14a was carried out in aqueous buffer solution (pH 5.4) and water (pH 7.0). The compound 8a has demonstrated stability until 18 h at pH 7.0. In the acid medium (pH 5.4), this compound was stable until 8 h (Fig. 5). During the experiment, we did not observe the hydrolysis of compound 8a to its parental aldehyde (compound 7). On the other hand, the furoxan 14a was less stable than was the benzofuroxan derivative 8a. During the first 4 h, at pH 7.0, the concentration of compound 14a was reduced by 40%. Compound 14a was immediately cleaved in acid medium (pH 5.4), and the data obtained

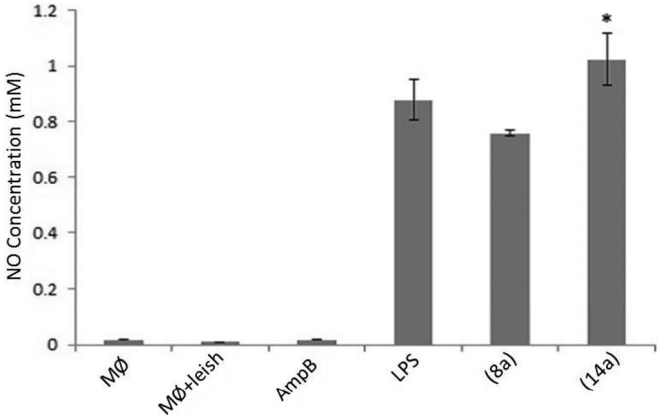

FIG 4 NO production by *Leishmania*-infected macrophages treated with LPS, amphotericin B (AmpB), and compounds 8a and 14a at 17 μM and 10.4 μM, respectively, compared to control macrophages and macrophages infected with *Leishmania*. *, $P < 0.05$ (Tukey's test) compared with LPS.

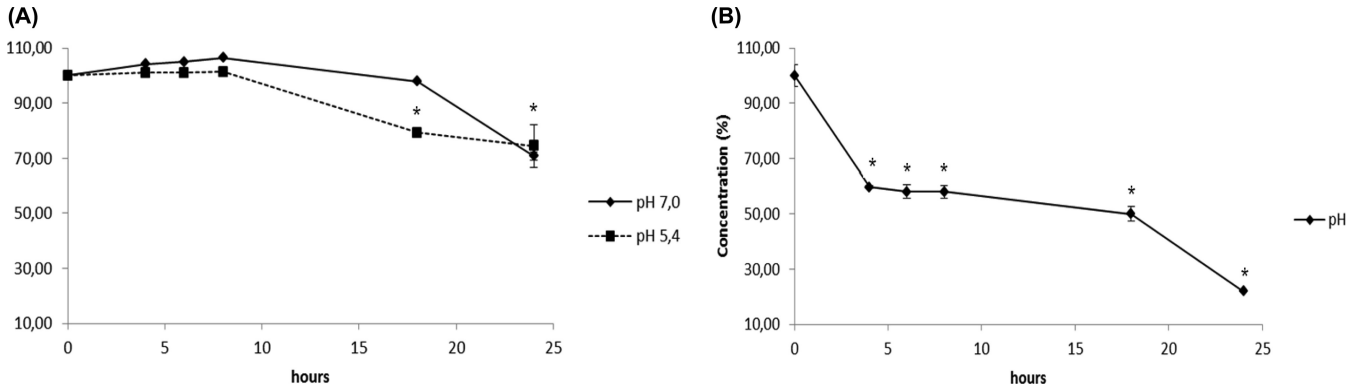

FIG 5 *In vitro* stability study. (A) Hydrolytic profile of compound 8a in water (pH 7.0) and buffer (pH 5.4). (B) Hydrolytic profile of compound 14a in water (pH 7.0) (data are represented as means ± SEMs and expressed as %).

suggested that total conversion to its parental aldehyde 13a occurred in the first hour (results not shown).

DISCUSSION

The discovery of new, safe, and effective leishmanicidal agents is urgently necessary since most of the drugs currently in use demonstrate problems that make treatment difficult, such as (i) variable efficacy, (ii) undesirable and serious side effects, (iii) the presence or induction of resistance to drugs, (iv) the need to be used during long-term therapy, and (v) high costs (37). Thus, we have described here the synthesis and leishmanicidal activity of novel furoxan and benzofuroxan derivatives.

Benzofuroxan and furoxan derivatives have been characterized as an interesting pharmacophore to antiparasitic activity against *Schistosoma*, *Mycobacterium*, *Plasmodium*, and *Leishmania* parasites (15–18, 38–41). Specifically, the antiparasitic activity of these derivatives has been associated with their ability to release NO (furoxan) or to cause oxidative stress (benzofuroxan).

Promastigote and intracellular amastigote forms of *Leishmania* are both susceptible to NO effects. Exposure of *Leishmania infantum* promastigotes to exogenous NO donor during log phase diminished their infectivity and viability to 75% of the starting values (42). As *Leishmania* parasites reside in the form of amastigotes inside macrophage mammalian cells, the innate and adaptive immune responses are responsible for controlling the infection (43). The increase in levels of cytokines, such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α), activates inducible nitric oxide synthase (iNOS) to produce NO in a micromolar concentration, which kills the parasite (44).

However, as a defense mechanism, phagolysosomal amastigotes decrease NO production in infected macrophages by inhibition of iNOS (45, 46). The iNOS has an important role in *Leishmania* infection. It was demonstrated previously that iNOS mutant mouse strains were highly susceptible to *Leishmania* infection (47). Since NO levels are reduced in infected macrophages, compounds with NO donor ability could help to eliminate intracellular amastigote forms of the parasite.

In fact, NO donors such as *S*-nitrosothiols have shown leishmanicidal activity useful in treating cutaneous leishmaniasis. *S*-Nitrosoglutathione, for instance, was able to inhibit the growth of *L. amazonensis* promastigotes forms with an IC_{50} of 68.9 μM (48). Moreover, *S*-nitrosoglutathione demonstrated activity against intracellular amastigotes and showed a healing effect on localized cutaneous leishmaniasis lesions caused by *L. major* and *L. braziliensis* in mice (49). Topical treatment of cutaneous leishmaniasis lesions by NO donors seems to improve healing and reduce the number of parasites; however, this beneficial effect is directly related to the chemical stability of the NO donor compound (49, 50).

The molecular mechanism whereby NO exerts its cytotoxic activity is not completely understood. Some studies have proposed that NO effects are due to the inhibition of mitochondrial respiration, glycolysis, peroxidation of membrane lipids, disruption of Fe-S clusters and zinc fingers, inactivation of peroxidases and arginases, mutation of DNA, and inhibition of DNA synthesis and repair (51–53). In addition, S-nitrosylation of some essential parasite enzymes by NO donors such as *S*-nitrosoacetylpenicillamine inhibited parasite cysteine proteinase *in vivo* (54, 55).

The novel furoxan (compounds 4a and -b and 14a to -f) and benzofuroxan (8a to -c) derivatives presented in this work have shown leishmanicidal activity against *L. amazonensis* promastigotes and intracellular amastigotes. In promastigote studies, compounds 3, 7, 8a, 13a and -b, and 14a to -e were more active than the standard drug pentamidine. However, compounds 3, 7, and 13a and -b have demonstrated high cytotoxicity to macrophage cells. Outstanding leishmanicidal activity was observed for compounds 8a and 14a (IC_{50}, <2.97 μM), which present SI^1s 4.22 times higher than that of amphotericin B. However, leishmanicidal activity against promastigotes does not guarantee activity against intracellular amastigotes, the clinically relevant form of *Leishmania* species. Then, we performed intracellular amastigote studies in which we have found that compounds 8a, 14a and -b, and 14d were more active against amastigote forms than were amphotericin B and pentamidine. All compounds presented good selectivity to the parasite, with SI^2 values greater than 7.47. Notably, compounds 8a and 14a showed SI^2 values of 29 and 54, respectively.

The leishmanicidal activity observed here for furoxan derivatives seems to be related in part to the NO donor ability of these compounds. *In vitro* studies for evaluation of NO donor ability were carried out using the Griess reaction after incubation of all compounds with a large excess of L-cysteine. The results have demonstrated that all furoxan compounds were capable of inducing nitrite formation, between 7.8% and 27.4%. The NO donor ability of furoxan derivatives is directly related to the substitution in the carbon atom at the 3 position neighboring the N-oxide

subunit. Furoxan derivatives containing an arylsulfonyl substitution (compounds 14a to -f) were able to generate nitric oxide at higher levels than were phenylfuroxan (compounds 4a and -b) and benzofuroxan (compounds 8a to -c) derivatives, respectively. The results of furoxan derivatives confirm our previous proposal that derivatives with an electron-withdrawing substituent at the 3 position, i.e., 3-phenylsulfonyl-substituted derivatives, were better NO-releasing compounds than were 3-aryl-substituted furoxans (34). Benzofuroxan derivatives (compounds 8a to -c) were not able to act as NO-releasing compounds in this assay; however, these compounds seem to induce NO production in the parasite. It has been reported that some compounds are able to induce nitric oxide production in infected macrophages, although this mechanism is not completely understood (56, 57).

Structure-activity relationship studies reveal that in general bis-aryl sulfonyl derivatives (compounds 14a to -e) are more active than phenylfuroxan (compounds 4a and -b) and benzofuroxan (compounds 8b and -c). Also, the pattern of substitution of the benzoyl moiety seems to influence the leishmanicidal activity. We have observed that *ortho* substitution (2-hydroxy or 2-aminobenzoyl) is more potent than *meta* substitution (3-hydroxybenzoyl), which is more potent than *para* substitution (4-hydroxy or 4-aminobenzoyl). Considering the same NO donor levels during *in vitro* studies, these results suggest that not only NO release is involved in the leishmanicidal activity but also other mechanisms that need to be further investigated.

It has been described that the *N*-acyl hydrazone subunit could be unstable in acid media (58, 59). So, in order to characterize the chemical stability of the most active compounds, 8a and 14a, we have performed chemical hydrolysis *in vitro*. We have studied the stability at two pH values, 5.4 and 7.0. pH 5.4 was studied due to its correlation with the pH value of phagolysosome, and pH 7.0 was studied to characterize the stability of the compounds at neutral pH. The benzofuroxan derivative 8a has been shown to be stable for 18 h at pH 7.0 and 8 h at pH 5.4. On the other hand, the concentration of furoxan 14a at 4 h at pH 7.0 was found to be 60%. Compound 14a was very unstable in acidic media, and it was immediately hydrolyzed at pH 5.4. Interestingly, for this compound we have identified the aldehyde 13a by the UPLC method. This result suggested that compound 14a could act as a prodrug, "masking" the aldehyde cytotoxicity toward the macrophage and facilitating drug permeation across the macrophages and parasite membranes. Then, in acidic media containing the phagolysosome, the compound 14a could be chemically converted, releasing the aldehyde 13a.

In conclusion, a novel series of furoxan (compounds 4a and -b and 14a to -f) and benzofuroxan (compounds 8a to -c) derivatives was synthesized and characterized. The furoxan derivatives (compounds 4a and -b and 14a to -f) have demonstrated nitric oxide donor properties. The benzofuroxan derivative 8a is able to increase nitrite production in culture supernatants from murine macrophages infected with *L. amazonensis*. Among all the compounds that show some selectivity to the promastigote parasite; the derivatives 8a and 14a to -e have demonstrated good activity against promastigote forms as well as good selectivity to the parasite. So, these compounds were selected for further evaluation against intracellular amastigote forms of *L. amazonensis*. The results have shown that compounds 8a, 14a and -b, and 14d were more effective than amphotericin B. The SI^2 values of these compounds were greater than 7.47, while pentamidine and amphotericin B have shown values of 3.27 and 4.69, respectively. Bis-aryl sulfonyl derivatives (compounds 14a to -e) are more active than phenylfuroxan (compounds 4a and -b) and benzofuroxan (compounds 8b and -c) derivatives against *L. amazonensis* amastigotes. *In vitro* hydrolysis studies have shown that compound 8a is stable at pH 5.4 and 7.0 until 8 h and 18 h, respectively. The compound 14a was hydrolyzed 40% in 4 h at pH 7.0. This furoxan derivative (compound 14a) is unstable at pH 5.4, being immediately hydrolyzed. The results presented here highlight the compounds 8a, 14a and -b, and 14d as novel lead drug candidates for the treatment of leishmaniasis.

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