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Heteroallyl-containing 5-nitrofuranes as new anti-*Trypanosoma cruzi* agents with a dual mechanism of action

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Abstract—New heteroallyl-containing 5-nitrofuranes were synthesized as potential anti-*Trypanosoma cruzi* agents with a dual mechanism of action, oxidative stress and inhibition of membrane sterol biosynthesis. Some of the derivatives were found to have high and selective activity against the proliferative stages of the parasite, with IC_{50} values against the clinically relevant intracellular amastigote forms in the low micromolar to sub-micromolar range. Oxidative stress was verified measuring cyanide dependent respiration. Inhibition of the de novo sterol biosynthesis at the level of squalene epoxidase was confirmed, using high-resolution gas–liquid chromatography coupled to mass spectrometry, by the disappearance of the parasite's mature sterols and the concomitant accumulation of squalene. The in vitro activities of these novel compounds were superior to that of nifurtimox, a nitrofuran currently used in the treatment of human Chagas' disease, and terbinafine, a commercially available allylamine-based squalene epoxidase inhibitor. The results support further in vivo studies of some of these nitrofuran derivatives.

1. Introduction

Chagas' disease remains the major parasitic disease burden in Latin America, despite recent advances in the control of its vectorial and transfusional transmission.[1–3] Chemotherapy to control this parasitic infection remains unsatisfactory, despite significant progress in our understanding of the biochemistry and physiology of its etiological agent, *Trypanosoma cruzi* (*T. cruzi*),[4,5] which has resulted in the validation of several metabolic steps essential for parasite survival as potential chemotherapeutic targets.[5,6] Current specific treatments are based on old and quite unspecific drugs, associated with long-term treatments that may give rise to severe side effects. In fact, although nifurtimox (Nfx, Fig. 1) and Benznidazole (Bnz), the only two drugs available for clinical treatment of this disease, are able to eliminate patent parasitemia and to reduce serological titers in acute and early chronic infections, they are not active against all *T. cruzi* strains and have significantly lower efficacy in long-term chronic infections.[5,6]

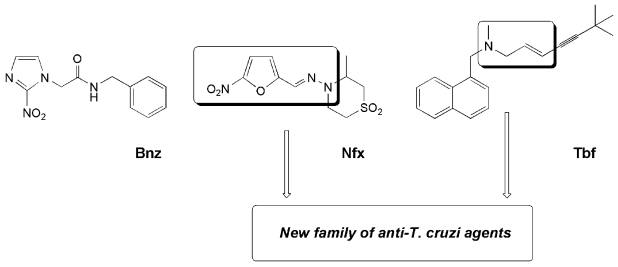

Figure 1. Benznidazole, nifurtimox and terbinafine. Structural targets used in the design of the new series of molecules.

Both drugs act via the reduction of the nitro group. In the case of Nfx, reduction generates an unstable nitro anion radical which produces highly toxic reduced oxygen species, whereas Bnz involves covalent modification of macromolecules by nitro reduction intermediates.[7] The side effects of these drugs result from the oxidative

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or reductive damage in the host's tissues and are thus inextricably linked to its anti-parasitic activity. Despite these limitations, some studies involving newer nitroimidazole derivatives have been recently described[8] and we have recently shown that novel 5-nitrofuryl derivatives possess high and selective anti-*T. cruzi* activity in vitro and in vivo.[9–15] Moreover, we have demonstrated that the main mechanism of action of this family of compounds is the induction of oxidative stress in the parasite.[16–19]

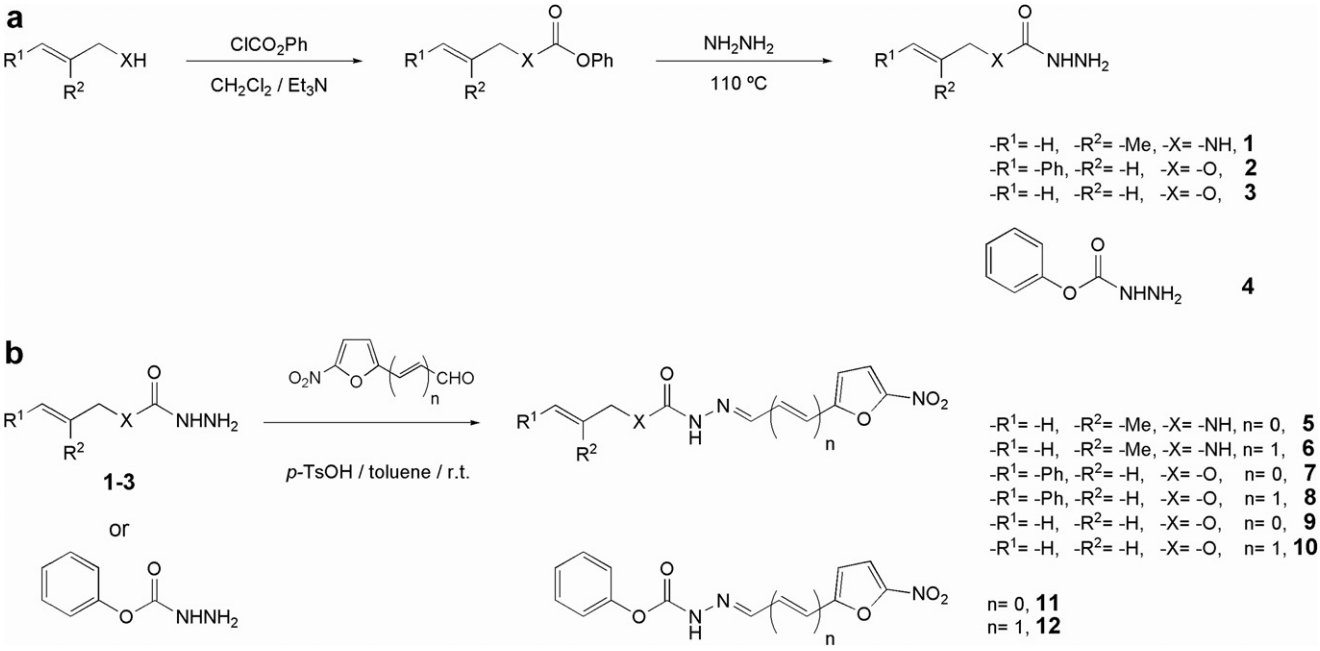
On the other hand, ergosterol biosynthesis inhibitors are among the most promising approaches for the development of more potent and safer anti-*T. cruzi* drugs as this parasite, like most pathogenic fungi and yeasts, requires specific 24-alkyl sterols for cell viability and proliferation in all stages of its life cycle and cannot use the abundant supply of cholesterol present in its mammalian hosts.[20] The *T. cruzi* ergosterol biosynthesis pathway has been chemically validated as a chemotherapeutic target at several steps,[21] including squalene epoxidase (SE; EC 1.14.99.7), a mono-oxygenase that catalyzes the conversion of squalene to 2,3-oxidosqualene.[20] SE is essential for the synthesis of cholesterol in mammals and ergosterol in fungi and is potently inhibited by allylamines, which have been successfully used as antifungal agents.[22,23] Allylamine derivatives have also been shown to be potent in vitro and in vivo *T. cruzi* growth inhibitors, acting by a selective reduction of the parasite's endogenous membrane sterol levels.[24,25] Specifically, terbinafine (Tbf, Fig. 1)[26–29] proved to be a promising anti-*T. cruzi* agent.

In this paper, we describe the synthesis and characterization of novel heteroallyl containing 5-nitrofuranes (**5**–**10**, Scheme 1) and secondary products (**11** and **12**, Scheme 1) that were shown to act against *T. cruzi* proliferative stages (extracellular epimastigotes and intracellular amastigotes) by dual mechanism of action, involving both oxidative stress and sterol biosynthesis inhibition at the level of SE. The results indicate that such derivatives are good candidates for in vivo chemotherapeutic studies in appropriate animal models for Chagas' disease.

2. Methods and results

2.1. Synthesis

The basic strategy of the synthetic efforts is delineated in Scheme 1 and was aimed at combining in a single molecule a nitrofuran moiety with potential for the generation of free radicals and redox cycling with heteroallyl groups that could inhibit the parasite's SE. The heteroallyl containing 5-nitrofuranes were prepared, with adequate yields, using the typical procedure (Scheme 1b) consisted in the reaction, at room temperature, between 5-nitrofurfural or 3-(5-nitrofuryl)acroleine (1 equiv) and the corresponding semicarbazide o carbazate (**1**–**4**, 1 equiv), in the presence of catalytic amounts of *p*-toluenesulphonic acid (*p*-TsOH) and dried toluene as solvent. After toluene evaporation in vacuo, the product was purified by crystallization or chromatographic column. The semicarbazide and carbazate reactants (**1**–**3**) were prepared using the synthetic route shown in Scheme 1a.[30] In the carbazate **3** preparation, hydrazinolysis step, the secondary product **4** was obtained due to an allylic alcohol loss. All new compounds, derivatives **5**–**12**, were characterized by NMR (^{1}H, ^{13}C, NOE-diff, COSY, and HMQC and HMBC experiments) and mass spectrometry. Purity was established by TLC and microanalysis (C, H, N).



Scheme 1. Synthetic procedure used for the development of 5-nitrofuryl derivatives.

2.2. Biological characterization

2.2.1. *Trypanosoma cruzi* growth inhibition experiments. All compounds had a dose-dependent effect on the growth of extracellular *T. cruzi* epimastigotes in LIT (EP strain) or BHI-tryptose (Y strain) medium at 28 °C, with comparable results. For the EP strain (Fig. 2), minimal concentrations to induce full growth arrest after 96 h (MIC) were in the range of 3 to >30 μM, the potency rank being **12 >8 ≈ 5 ≈ 11 ≈ 6 > 7 > 9 > 10** (Table 1). Against the intracellular stages, grown in Vero cells at 37 °C, a similar order of activity was observed (**12 > 8 ≈ 5 > 11**) with IC_{50} values in the low micromolar to sub-micromolar values and IC_{99} from 3 to 30 μM (Fig. 3 and Table 1). The selectivity index ($IC_{50(ama)}$/ $IC_{50(Vero)}$) for these compounds ranged from 8.4 to 35.7 (Table 1). On the other hand, compounds **6**, **7**, **9**, and **10** proved to be toxic to the host cells at concentrations comparable or lower than those required to affect the amastigote burden (not shown).

2.3. Mechanism of action studies

2.3.1. Sterol biosynthesis inhibition. In control epimastigotes ergosterol, its 24-ethyl analog (24-ethyl-5,7,22-cholestatrien-3b-ol) and precursors were the endogenous sterols, as reported before.[31,32] All 5-nitrofuran derivatives induced a dose-dependent reduction of the levels of parasite's sterols endogenous and a concomitant accumulation of squalene, indicating a blockade of the de novo sterol biosynthesis at the level of SE (Table 2). The order of activity to induce these effects correlated very well with that for growth inhibition: in all cases

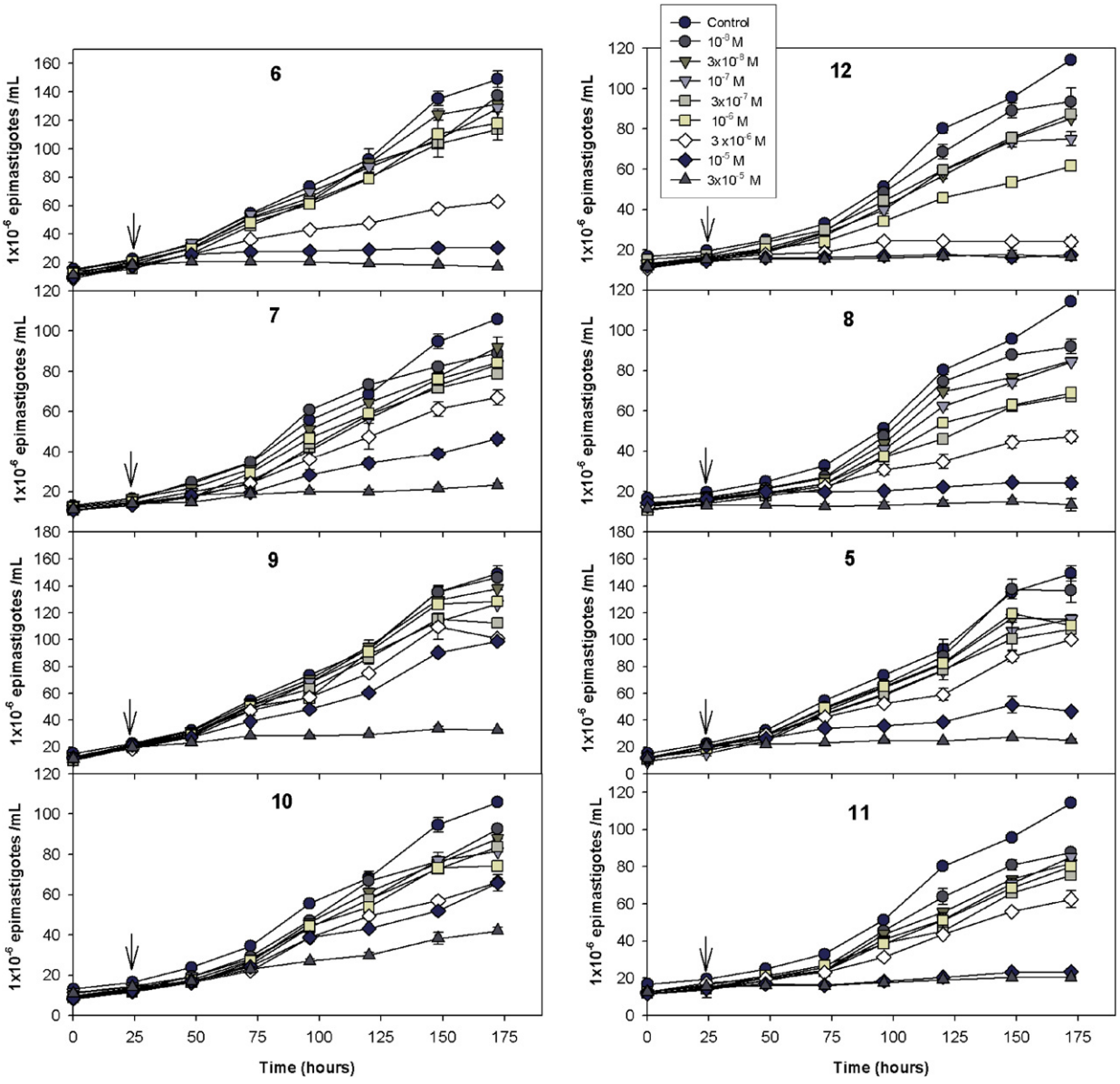


Figure 2. Effects of 5-nitrofuryl derivatives **6**–**12** on the proliferation of *T. cruzi* epimastigotes. Epimastigotes were cultured in liver infusion-tryptose medium at 28 °C, with agitation, as described in Experimental section. Arrow indicates the time of addition of the compound, at the indicated concentrations. Experiments were carried out in triplicate and each bar represents one standard deviation.

Table 1. Antiproliferative activities of 5-nitrofuryl derivatives against *T. cruzi* and Vero cells in vitro

Compound	MIC_{epi}[a] (μM)	$(IC_{50})_{ama}$ (μM)	MIC_{ama}[b] (μM)	$(IC_{50})_{Vero}$ (μM)	SI[c]
12	3	0.31	3	9.1	29.4
8	10	0.65	10	11.1	17.1
5	10	0.90	10	32.1	35.7
11	10	2.3	30	19.4	8.4
6	10		Toxic[d]		—
7	30		Toxic[d]		—
9	30		Toxic[d]		—
10	>30		Toxic[d]		—

[a] Minimal concentration to induce full growth arrest of epimastigotes (EP strain) after 96 h in LIT medium.
[b] Minimal concentration required to reduce by >99% amastigote infection (EP strain) in Vero cells after 96 h.
[c] Selectivity index = $(IC_{50})Vero/(IC_{50})_{ama}$.
[d] Significant toxicity against host cells at concentrations below those required to reduce amastigote infection.

complete growth arrest (at MIC) was associated with the disappearance of the parasite's endogenous sterols (compare Tables 1 and 2), strongly suggesting a causal relationship between these effects. The concentrations of the most potent compounds (**12**, **5**, **8**, **11**, and **6**) required to induce full growth arrest and endogenous sterol's depletion in *T. cruzi* epimastigotes, were 10- to 3-fold lower than that of terbinafine, obtained under identical conditions in a previous study using the same parasite strain;[33] against the intracellular epimastigotes the IC_{50} and IC_{99} values were of the same order of magnitude.[33] These results indicate that a primary mechanism of action of the 5-nitrofuryl derivatives is a blockade of the parasite's endogenous sterol synthesis at the level of SE, probably associated to the presence of the heteroallyl moiety.

2.3.2. Redox cycling. A second mechanism of action of the 5-nitrofuryl derivatives could be oxidative stress due to the reduction of the 5-nitrofuran moiety, the subsequent generation of reduced oxygen metabolites and redox cycling, which should lead to an increase of the parasite's oxygen consumption.[34–37] To test this hypothesis, oxygen uptake experiments were performed in *T. cruzi* Y strain epimastigotes, with and without inhibition of respiration with cyanide.[38,39] Some of the assayed derivatives (**5**, **8**, and **11**) increased oxygen consumption in absence of cyanide, between 2% and 60% with respect to untreated control cells. The effect was also observed after inhibition of the parasite mitochondrial respiration by cyanide (Table 3). In the absence of drugs, cyanide-insensitive respiration is almost undetectable suggesting that cytochrome aa_3 is the main *T. cruzi* terminal oxidase, as previously reported.[35] However, treatment with the 5-nitrofuryl derivatives **5**, **8**, and **11** and, to less extent, **12**, induced an increase in cyanide-insensitive respiration to relatively high levels, i.e. between 20% and 46% of the control cells' basal respiration for the first three ones and near to 10% for the last one. This fact is suggestive of superoxide/H_2O_2 production.

3. Discussion

The results indicated that the in vitro activity of the new heteroallyl containing 5-nitrofuran derivatives against *T. cruzi* epimastigotes and amastigotes is superior in most cases to that of Nfx, the nitrofuran currently used

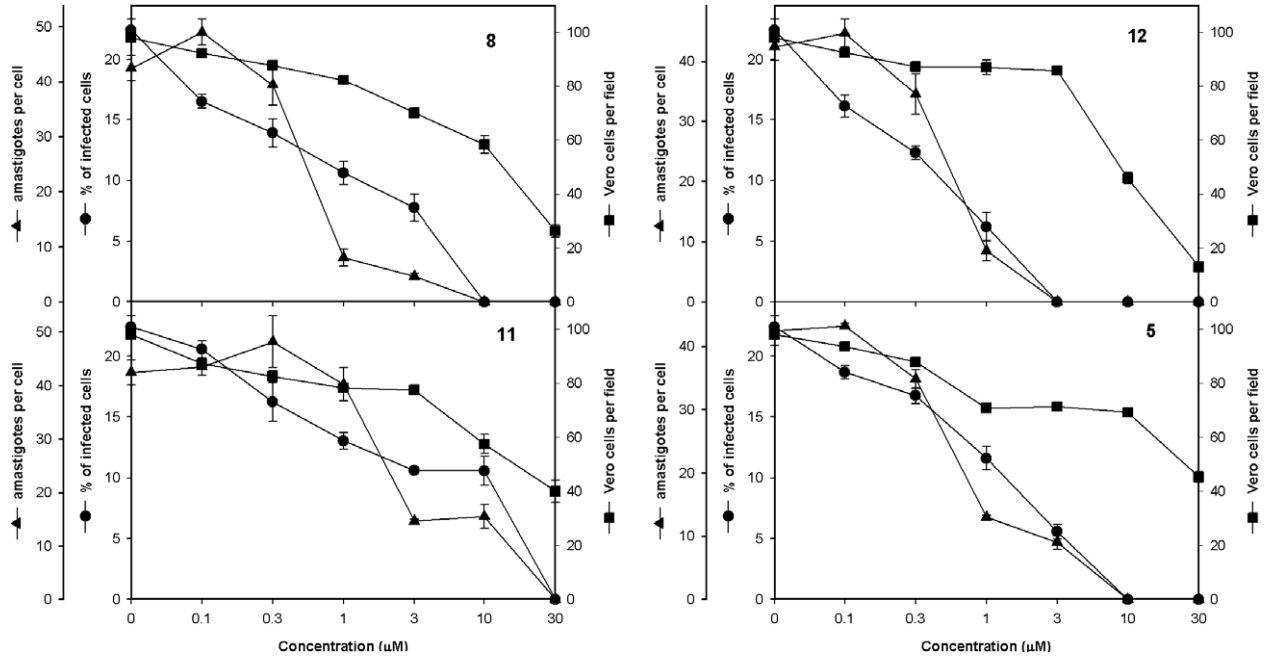


Figure 3. Concentration dependence of the effects of 5-nitrofuryl derivatives **5**, **8**, **11**, and **12** on the proliferation of *T.cruzi* amastigotes cultured in Vero cells at 37 °C. Shown are the percentage of infected cells (●), the number of amastigotes per cell (▲), and the number of Vero cells per field (■) after 96 h of incubation, as a function of the drug concentration. Vero cells were infected with *T. cruzi*, as described in Section 5. Experiments were carried out in quadruplicate and each bar represents one standard deviation.

Table 2. Free sterols and precursors present in *T. cruzi* epimastigotes (EP stock) grown in the presence or absence of 5-nitrofuryl derivatives **5–12**[a]

Sterol[b]	Control	**12**		**8**		**5**	**11**		Control	**6**		**7**		**9**	**10**
		3 μM	10 μM	3 μM	10 μM	10 μM	3 μM	10 μM		3 μM	10 μM	10 μM	30 μM	30 μM	30 μM
Exogenous cholesterol	55.7	46.9	8.1	33.9	6.7	31.9	32.3	35.0	48.8	20.3	9.2	52.5	22.0	53.1	29.4
Endogenous squalene	6.6	45.2	91.9	19.0	93.3	68.1	39.1	60.6	3.7	44.4	90.8	16.0	64.4	33.1	36.4
24-Methyl-5,7,22-cholestatrien-3β-ol (ergosterol)	11.6	7.9	n.d.	21.0	n.d.	n.d.	7.6	4.4	13.5	18.4	n.d.	10.4	5.8	7.0	15.0
24-Ethyl-5,7,22-cholestatrien-3β-ol	12.2	n.d.	n.d.	16.0	n.d.	n.d.	9.0	n.d.	14.6	10.8	n.d.	13.8	7.8	6.8	10.6
Ergosta-5,7-24(24[1])-trien-3β-ol	6.5	n.d.	n.d.	2.1	n.d.	n.d.	2.4	n.d.	6.0	n.d.	n.d.	2.3	n.d.	n.d.	4.0
Ergosta-5,7-dien-3β-ol	3.0	n.d.	n.d.	8.0	n.d.	n.d.	6.0	n.d.	6.2	6.1	n.d.	5.0	n.d.	n.d.	4.6
24-Ethyl-5,7-cholestadien-3β-ol	4.4	n.d.	n.d.	n.d.	6.7	n.d.	3.6	n.d.	5.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ergosta-7,24(24[1])-dien-3β-ol	—	—	—	—	—	—	—	—	1.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d., not detected.
—, not studied.
[a] Two independent experiments were performed.
[b] Sterols were extracted from *T. cruzi* epimastigotes cultured in LIT medium for 120 h in the presence or absence of the indicated concentrations of the experimental compounds; they were separated from polar lipids by silicic acid column chromatography and analyzed by quantitative capillary gas–liquid chromatography and mass spectrometry, as described in Section 5. Results are expressed as mass percent.

for the treatment of this disease, as well as that of the commercially available SE inhibitor terbinafine (see Table 3). In particular, derivatives **5**, **8**, **11**, and **12**, the last two compounds generated as secondary products in the synthetic procedures, showed relatively high potency against the intracellular amastigote form (low micromolar to sub-micromolar IC_{50} values), with appreciable selectivity indexes (see Table 1). Derivatives **5**, **8**, and **11** acted through a dual molecular mechanism, producing oxidative stress and inhibiting membrane sterol biosynthesis at the level of SE, while derivative **12** acted primarily as a SE inhibitor of remarkable potency. Oxidative stress was verified measuring cyanide dependent respiration. Inhibition of the de novo sterol biosynthesis at the level of squalene epoxidase was confirmed, using high-resolution gas–liquid chromatography coupled to mass spectrometry, by the disappearance of the parasite's mature sterols and the concomitant accumulation of squalene.

4. Conclusions

In conclusion, we have developed a new series of nitrofuranes with relevant activity against *T. cruzi*. These compounds may serve as leads for further exploration of the structural requirements of these antiparasitic activities and the promising in vitro results warrant further in vivo studies in animal models of Chagas' disease.

5. Experimental

5.1. Chemistry

All starting materials were commercially available research-grade chemicals and used without further purification. All solvents were dried and distilled prior to use. All the reactions were carried out in a nitrogen atmosphere. Intermediates **1**–**4** were prepared applying previously described methodology[30] and its purities were established by ^{1}H NMR analysis. The typical workup included washing with brine and drying the organic layer with sodium sulfate before concentration in vacuo. Melting points were determined with a Gallenkamp melting point apparatus and are uncorrected. Elemental analyses were obtained from vacuum-dried samples (over phosphorous pentoxide at 3–4 mm Hg, 24 h at room temperature) and performed on a Fisons EA 1108 CHNS-O analyzer. ^{1}H NMR spectra were recorded on a Bruker DPX-400 (at 400 MHz) instrument, with tetramethylsilane as the internal reference and in the indicated solvent; the chemical shifts are reported in ppm. *J* values are given in Hz. The ^{1}H NMR and ^{13}C NMR signals reported were obtained at 303 K. Mass spectra were recorded on a Shimadzu GC-MS QP 1100 EX instrument, using electronic impact at 70 eV.

5.2. General procedure for the synthesis of 5–12

A mixture of the corresponding aldehyde (5-nitro-2-furaldehyde or 3-(5-nitrofuryl)acroleine) (1 equiv), the corresponding semicarbazide or carbazate reactant (**1**–**4**, 1

Table 3. Effects of 5-nitrofuryl derivatives on oxygen uptake in *T. cruzi* epimastigotes (Y strain)

Compound	IC_{50}[a] (μM)	Concentration in the assay (μM)	Oxygen uptake[a,b] (%)	Oxygen redox cycling[a,c] (%)
5	3.8	80.0	110 ± 20	33 ± 6
		120.0	148 ± 30	27 ± 2
8	3.7	80.0	89 ± 5	38 ± 8
		120.0	160 ± 30	40 ± 10
11	10.6	60.0[d]	105 ± 10	31 ± 20
12	6.7	166.0	88 ± 11	8 ± 5
Tbf	44.7[e]	—	—	—
Nfx	6.5	180.0	117	80 ± 10
Control	—	—	100[f]	0

[a] For details, see Section 5.
[b] Percent rate of oxygen consumption compared with that of control cells.
[c] Percent rate of oxygen consumption in presence of KCN (13 μM).
[d] Solubility problems were observed at higher doses.
[e] From Ref. 40.
[f] Control respiration was 32.2 nanoatoms of oxygen per min and per mg of protein.

equiv), *p*-TsOH (catalytic amounts), and toluene as solvent was stirred at room temperature until the carbonyl compound was not present (SiO_2, 1% MeOH in CH_2Cl_2). Each product was purified as it is indicated.

5.2.1. 4-(2-Methyl-2-propen-1-yl)-1-(5-nitrofurfurylidene)-semicarbazide (5). Crystallization from toluene:ethyl acetate (twice); yield: 14%; brown–yellow solid, mp 155.0–157.0 °C; found: C, 47.4; H, 4.4; N, 22.3 ($C_{10}H_{12}N_4O_4$ requires C, 47.6; H, 4.8; N, 22.2); δ_H (DMSO-d_6) 1.68 (3H, s, $-CH_3$), 3.70 (2H, d, $-CH_2$), 4.78 (2H, s, $-CH_2$=), 7.12 (1H, bs, NH), 7.17 (1H, d, furan-H), 7.79 (1H, d, furan-H), 7.82 (1H, s, –CH=N), 10.90 (1H, bs, NH); δ_C (DMSO-d_6) (HMQC, HMBC) 20.9, 45.3, 110.3, 113.4, 116.0, 128.5, 144.0, 152.2, 153.8, 155.6; EI/MS *m*/*z* (abundance,%) 252 ($M^{\cdot+}$, 7), 235 (11), 155 (100), 125 (4).

5.2.2. 4-(2-Methyl-2-propen-1-yl)-1-[3-(5-nitro-2-furyl)-2-propenylidene]semicarbazide (6). Purification: SiO_2, petroleum ether:ethyl acetate (0–40%); yield: 16%; brown solid, mp 172.0–174.0 °C; found: C, 51.9; H, 5.1; N, 19.9 ($C_{12}H_{14}N_4O_4$ requires C, 51.8; H, 5.1; N, 20.1); δ_H (DMSO-d_6) 1.67 (3H, s, $-CH_3$), 3.66 (2H, d, $-CH_2$), 4.76 (2H, s, $-CH_2$=), 6.89 (1H, d, –CH=), 6.98–7.05 (3H, m, NH, furan-H, –CH=), 7.68–7.73 (2H, m, furan-H, –CH=N), 10.60 (1H, bs, NH); EI/MS *m*/*z* (abundance,%) 278 ($M^{\cdot+}$, 33), 261 (7), 181 (100) and 135 (61).

5.2.3. 1-(5-Nitrofurfurylidene)-4-(3-phenyl-2-propen-1-yl)-carbazate (7). Purification: SiO_2, petroleum ether:ethyl acetate (0–30%); yield: 30%; brown solid, mp 146.0–148.0 °C; found: C, 57.1; H, 3.9; N, 13.4 ($C_{15}H_{13}N_3O_5$ requires C, 57.1; H, 4.2; N, 13.3); δ_H (acetone-d_6) 4.87 (2H, d, $-CH_2$), 6.44 (1H, dd, –CH=), 6.79 (1H, d, –CH=), 7.10 (1H, d, furan-H), 7.26–7.31 (3H, m, phenyl-H), 7.48–7.50 (2H, m, phenyl-H), 7.59 (1H, d, furan-H), 8.18 (1H, s, –CH=N), 10.53 (1H, bs, NH); δ_C (acetone-d_6) (HMQC, HMBC) 66.0, 113.2, 113.9, 124.0, 127.5, 128.7, 129.0, 132.6, 134.2, 136.8, 152.6, 152.8, 164.0; EI/MS *m*/*z* (abundance,%) 315 ($M^{\cdot+}$, 10).

5.2.4. 1-[3-(5-Nitro-2-furyl)-2-propenylidene]-4-(3-phenyl-2-propen-1-yl)carbazate (8). Purification: SiO_2, petroleum ether:ethyl acetate (0–30%); yield: 35%; yellow solid, 210.0 °C (d); found: C, 59.9; H, 4.3; N, 12.1 ($C_{17}H_{15}N_3O_5$ requires C, 59.8; H, 4.4; N, 12.3); δ_H (acetone-d_6) 4.84 (2H, d, $-CH_2$), 6.45 (1H, dd, –CH=), 6.75 (1H, d, –CH=), 6.89 (1H, d, –CH=), 6.92 (1H, d, furan-H), 7.00–7.13 (1H, m, –CH=), 7.26–7.48 (5H, m, phenyl-H), 7.56 (1H, d, furan-H), 8.41 (1H, d, –CH=N), 10.20 (1H, bs, NH); δ_C (acetone-d_6) (HMQC, HMBC) 65.8, 112.8, 114.5, 122.6, 124.2, 125.0, 126.2, 127.7, 130.9, 134.0, 136.9, 144.6, 152.2, 153.0, 155.3; EI/MS *m*/*z* (abundance,%) 341 ($M^{\cdot+}$, 25).

5.2.5. 1-(5-Nitrofurfurylidene)-4-(2-propen-1-yl)carbazate (9). Purification: preparative-TLC, SiO_2, CH_2Cl_2:ethyl ether (73:27); yield: 20%; yellow solid, mp 168.0–170.0 °C; found: C, 45.0; H, 3.6; N, 17.4 ($C_9H_9N_3O_5$ requires C, 45.2; H, 3.8; N, 17.6); δ_H (acetone-d_6) 4.70 (2H, d, $-CH_2$), 5.25 (1H, dd, –CH=), 5.38 (1H, dd, –CH=), 6.02 (1H, ddd, –CH=), 7.10 (1H, d, furan-H), 7.59 (1H, d, furan-H), 8.17 (1H, s, –CH=N), 10.52 (1H, bs, NH); δ_C (acetone-d_6) (HMQC, HMBC) 66.0, 113.1, 113.9, 117.7, 132.0, 133.2, 151.0, 152.0, 152.8; EI/MS *m*/*z* (abundance,%) 239 ($M^{\cdot+}$, 18).

5.2.6. 1-[3-(5-Nitro-2-furyl)-2-propenylidene]-4-(2-propen-1-yl)carbazate (10). Purification: preparative-TLC, SiO_2, CH_2Cl_2:ethyl ether (73:27); yield: 40%; brown–orange solid, 130.0 °C (d); found: C, 49.8; H, 4.0; N, 15.9 ($C_{11}H_{11}N_3O_5$ requires C, 49.8; H, 4.2; N, 15.8); δ_H (acetone-d_6) 4.67 (2H, d, $-CH_2$), 5.21 (1H, dd, –CH=), 5.36 (1H, dd, –CH=), 5.99 (1H, ddd, –CH=), 6.89 (1H, d, –CH=), 6.99 (1H, d, furan-H), 7.08 (1H, dd, –CH=), 7.57 (1H, d, furan-H), 7.97 (1H, d, –CH=N), 10.19 (1H, bs, NH); δ_C (acetone-d_6) (HMQC, HMBC) 66.0, 113.9–145.3 (7 carbons), 152.1, 153.9, 155.9; EI/MS *m*/*z* (abundance,%) 265 ($M^{\cdot+}$, 30).

5.2.7. 1-(5-Nitrofurfurylidene)-4-phenylcarbazate (11). Purification: preparative-TLC, SiO_2, CH_2Cl_2:ethyl ether (73:27); yield: 35%; yellow solid, 120.0 °C (d); found: C, 52.1; H, 3.0; N, 14.9 ($C_{12}H_9N_3O_5$ requires C, 52.4; H, 3.3; N, 15.3); δ_H (acetone-d_6) 7.16 (1H, d, furan-H),

7.24–7.30 (3H, m, phenyl-H), 7.43–7.47 (2H, m, phenyl-H), 7.62 (1H, d, furan-H), 8.27 (1H, s, –CH=N), 10.95 (1H, bs, NH); EI/MS *m/z* (abundance,%) 275 ($M^{\cdot+}$, 36).

5.2.8. 1-[3-(5-Nitro-2-furyl)-2-propenylidene]-4-phenylcarbazate (12). Purification: preparative-TLC, SiO_2, CH_2Cl_2:ethyl ether (73:27); yield: 45%; brown solid, 140.0 °C (d); found: C, 55.5; H, 3.4; N, 13.6 ($C_{14}H_{11}N_3O_5$ requires C, 55.8; H, 3.7; N, 14.0); δ_H (acetone-d_6) 6.97 (1H, d, –CH=), 7.02 (1H, d, furan-H), 7.13 (1H, dd, –CH=), 7.21–7.28 (3H, m, phenyl-H), 7.43–7.45 (2H, m, phenyl-H), 7.58 (1H, d, furan-H), 7.98 (1H, d, –CH=N), 10.61 (1H, bs, NH); EI/MS *m/z* (abundance,%) 301 ($M^{\cdot+}$, 48).

5.3. Biology

5.3.1. Anti-*T. cruzi* in vitro evaluation. *Parasite.* The EP[41] and Y[42] strain of *T. cruzi* were used in this study. Handling of live *T. cruzi* was done according to established guidelines.[43]

Studies of in vitro antiproliferative activity. The epimastigote form of the EP strain of the parasite was cultivated in liver infusion tryptose medium,[41] supplemented with 10% newborn calf serum (Gibco) at 28 °C with strong agitation (120 rpm). The cultures were initiated with a cell density of 2×10^6 epimastigotes per mL and the experimental compounds were added at a cell density of $0.5–1.0 \times 10^7$ epimastigotes per mL. Cell densities were measured with a electronic particle counter (model ZBI; Coulter Electronics Inc., Hielah, Fla.) and by direct counting with an hemocytometer. Cell viability was followed by Trypan blue exclusion using light microscopy.

Y strain *T. cruzi* epimastigotes, used in the oxygen uptake experiments, were grown at 28 °C in an axenic medium (BHI-tryptose medium, 100 mg of streptomycin/mL, and 100 U of penicillin/mL) supplemented with 5% (v/v) heat-inactivated fetal calf serum (FCS). Cells were harvested in the late log phase, resuspended in fresh medium, counted in Neubauer's chamber and adjusted to a concentration of 8×10^6/mL. Compounds with selective anti-*T. cruzi* activity (**5**, **8**, **11** and **12**) were studied, trying to correlate their capacity to inhibit growth and to increase the parasite's oxygen consumption. The compounds were added to the epimastigote cultures, 400 mL/well, dissolved in dimethylsulfoxide (DMSO), and incubated at 28 °C. The highest used concentration of DMSO was 1% (v/v) and did no effect by itself on the proliferation of the parasites.

To assess viability, parasites incubated for 48 h with the experimental compounds were washed with phosphate-buffered saline (PBS) and incubated in PBS–glucose (0.99 g/L) (28 °C) with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL) for 4 h. Parasites and formazan crystals were dissolved with 400 mL of isopropanol 50% plus 10% (w/v) sodium dodecyl sulphate (SDS), incubated 30 min at room temperature, and read at 570 nm. The percent of cell viability was calculated as follows: $\%C = [(OD_p - Bl)/(OD_c - Bl)] \times 100$, where OD_p represents the mean OD_{570} value recorded for culture containing different doses of products; OD_c represents the mean OD_{570} value recorded for the control wells; and Bl represents the mean OD_{570} value for the blank wells (PBS–glucose and MTT solution without parasites). The IC_{50} values were determined by sigmoidal regression analysis from this inhibition percentage using statistic error up to 10%.

Amastigotes (EP strain) were cultured in Vero cells maintained in minimal essential medium supplemented with 1% fetal calf serum in a humidified 95% air–5% CO_2 atmosphere at 37 °C as previously described.[32,44–46] Briefly, the cells were infected with 10 tissue culture-derived trypomastigotes per cell for 2 h and then washed three times with phosphate-buffered saline (PBS) to remove non adherent parasites; fresh medium with and without drugs was added and the cells were incubated for 96 h with a medium change at 48 h. Quantification of the number of infected cells, the number of parasites per cell by use of light microscopy and statistical analysis of the results was carried out as described.[32,44–46]

5.3.2. Studies of lipid composition. For the analysis of the effects of drugs on the lipid composition of the epimastigotes, total lipids from control and drug-treated cells were extracted and fractionated into neutral and polar lipid fractions by silicic acid column chromatography and gas–liquid chromatography.[45,46] The neutral lipid fractions were first analyzed by thin layer chromatography (on Merck 5721 silica gel plates with heptane-isopropyl ether-glacial acetic acid [60:40:4] as developing solvent) and conventional gas–liquid chromatography (isothermal separation in a 4-m glass column packed with 3% OV-1 on Chromosorb 100/200 mesh, with nitrogen as carrier gas at 24 mL/min and flame ionization detection in a Varian 3700 gas chromatograph). For quantitative analysis and structural assignments, the neutral lipids were separated in a capillary high resolution column (25 m × 0.20 mm i.d. Ultra-2 column, 5% phenyl-methyl-siloxane, 0.33 μm film thickness) in a Hewlett-Packard 6890 Plus gas chromatograph equipped with a HP5973A mass sensitive detector. The lipids were dissolved in chloroform and injected; the column was kept a 50 °C for 1 min, then the temperature was increased to 270 °C at a rate of 25 °C min^{-1}, and finally to 300 °C at a rate of 1°C min^{-1}. The carrier gas (He) flow was kept constant at 0.5 mL min^{-1}. Injector temperature was 250 °C and the detector was kept at 280 °C.

5.3.3. Oxygen uptake experiments. Y strain *T. cruzi* epimastigotes were harvested by 3000 rpm (15 min) centrifugation, followed by washing and re-suspension in 0.05 M sodium phosphate buffer (pH 7.4) containing glucose (5.5 mM). Respiration measurements were carried out polarographically with a Clark No. 5331 electrode (yellow Springs Instruments) in a 5300 YSI model (Simpson Electric Co.).[17,38,39] The assays were performed in a chamber maintained at 28 °C. The amount of parasites used was equivalent to 0.7 mg of protein per mL. In order to evaluate redox cycling, mitochondrial respiration was inhibited with 13 μM

potassium cyanide. The final concentration of the experimental compounds used in the oxygen uptake experiments was the IC_{50} obtained from the growth inhibition experiments as described above, corrected to maintain the parasite mass–drug ratio. This correction was made taking into account that the IC_{50}s (growth experiments) were determined using an initial cell density of 3×10^6 parasites/mL (equivalent to 0.038 mg protein/mL), while in the oxygen uptake experiments a cell density of 6×10^7 parasites/mL was used (equivalent to 0.70 mg protein mL^{-1}). Thus, the original IC_{50}s were corrected by a mean factor of 27-fold (range: 18–37) to account for the parasite mass increase in the oxygen uptake experiments. Values in Table 3 are expressed as mean for two independent experiments, three runs in each experiment. The studied compounds were dissolved in DMSO. Results were corrected according to the observed effect produced by DMSO (1%). Nfx was included as reference.

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