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5-Nitrofuranes and 5-nitrothiophenes with anti-*Trypanosoma cruzi* activity and ability to accumulate squalene

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ABSTRACT

Chagas disease represents a serious public health problem in South America. The first line of treatment is Nifurtimox and Benznidazole which generate toxic effects in treated patients. We have recently shown that a number of 5-nitrofuranes possess activity against *Trypanosoma cruzi* through oxidative stress and inhibition of parasite ergosterol biosynthesis, specifically at the level of squalene epoxidase. Here, we identify new 5-nitrofuranes and the thia-analogues with excellent effects on the viability of *T. cruzi* and adequate parasite/mammal selectivity indexes. Analysis of the free sterols from parasite incubated, during 120 h, with the compounds showed that some of them accumulated squalene suggesting the squalene epoxidase activity inhibition of the parasite. Nifurtimox was able to accumulate squalene only at lower incubation times. Due to this fact some derivatives were also tested as antifungal agents. Quantitative structure–activity relationship studies were also performed showing relevant features for further new derivatives design. Taken together, the results obtained in the present work point to a more general effect of 5-nitrofuranes and 5-nitrothiophenes in trypanosomatids, opening potential therapeutic possibilities of them for these infectious diseases.

1. Introduction

Chagas disease, or American Trypanosomiasis, remains the major parasitic disease burden in Latin America, despite recent advances in the control of its vectorial and transfusional transmission.[1–4] Even though the complete genome of *Trypanosoma cruzi* (*T. cruzi*) CL Brener clone[5] has been sequenced, the chemotherapy to control this parasitic infection remains unsatisfactory.[3,6] However, significant progress in understanding the biochemistry and physiology of *T. cruzi* has resulted in the validation of several metabolic steps essential for parasite survival and for potential use as chemotherapeutic targets.[7–9] Current specific treatments are based on old and quite unspecific drugs, Nifurtimox (Nfx, Fig. 1) and Benznidazole (Bnz) associated with long-term treatments that may give rise to severe side effects. In fact, although Nfx and Bnz 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 low efficacy in long-term chronic infections.[5,6]

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.[9] The side effects of this drug results from the oxidative damage in the host's tissues and is thus inextricably linked to its anti-parasitic activity. Despite these limitations, we have recently shown that novel 5-nitrofuryl derivatives posses high and selective anti-*T. cruzi* activity in vitro and in vivo.[10–18] Moreover, we have demonstrated that one of the mechanism of action of this family of compounds is the induction of oxidative stress in the parasite.[19–22]

Regarding to the validated metabolic steps used to generate new chemotherapeutic alternatives, membrane sterol biosynthesis is one of the most promising targets. *T. cruzi* 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.[23] The *T. cruzi* ergosterol biosynthesis pathway has been chemically validated as a chemotherapeutic target at several steps,[24] including squalene epoxidase (SE; EC 1.14.99.7) a microsomal mono-oxygenase that catalyzes the conversion of squalene to 2,3-oxidosqualene using molecular oxygen.[23] This conversion is the first oxygen-dependent step in the middle stage of the sterol biosynthetic pathway. SE employees NADPH, and FAD to catalyze the conversion of

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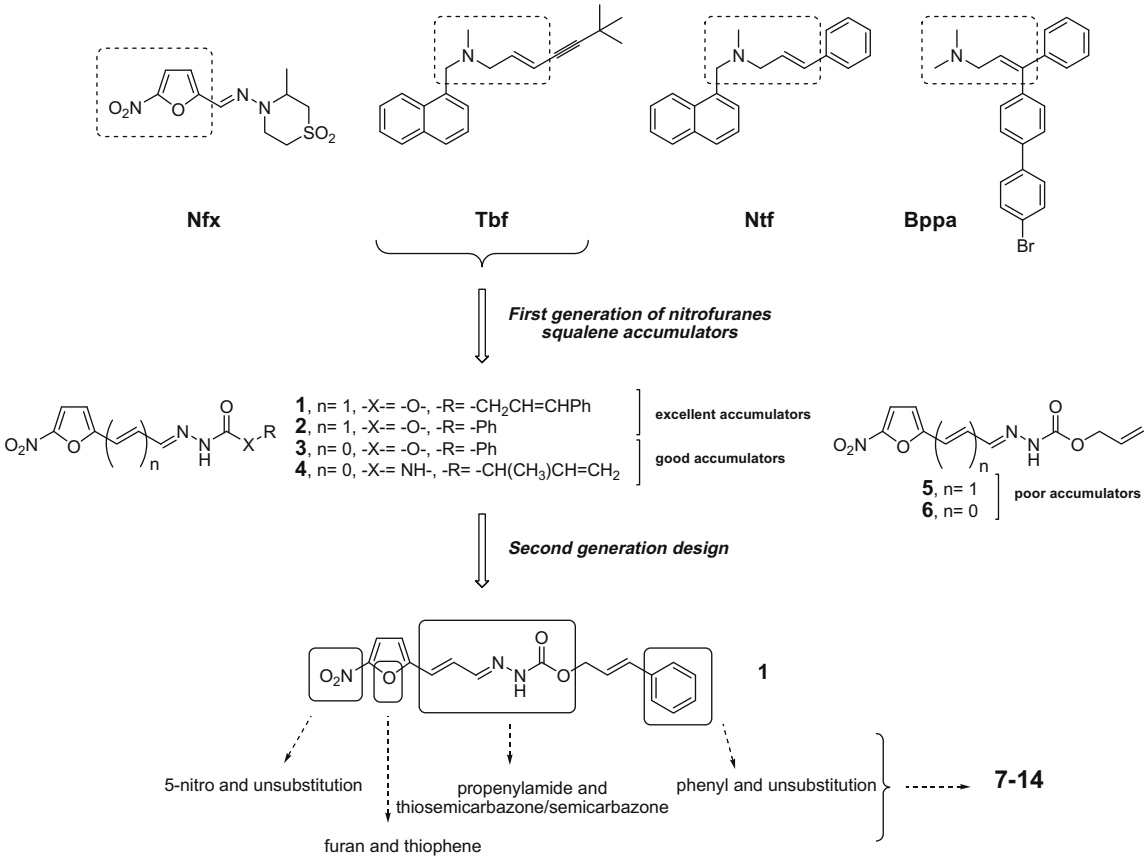


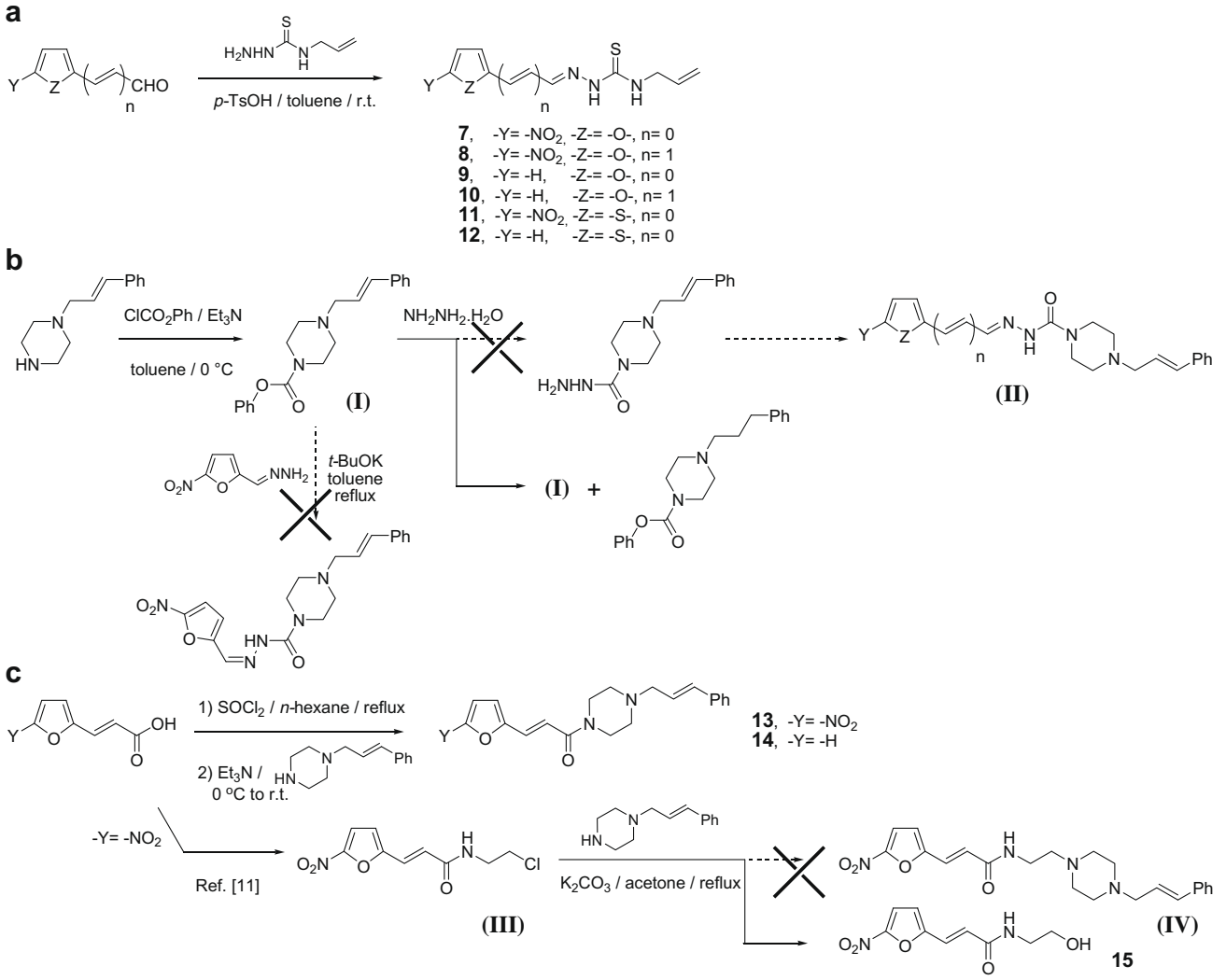
Figure 1. Well-known anti-*T. cruzi* agents, first generation of squalene accumulator-nitrofuranes and design of the second generation.

squalene to 2,3-oxidosqualene, receiving electrons from NADPH via NADPH-cytochrome P450 reductase (Fig. S1, Supplementary data). 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.[25,26] Allylamine derivatives have also 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.[27,28] Specifically, the antifungals terbinafine and naftifine (Tbf and Ntf, respectively, Fig. 1)[29–32] proved to be promising anti-*T. cruzi* agents. Also, the synthetic compound *N*,*N*-dimethyl-3-[4-(4-bromophenyl)phenyl]-3-phenyl-2-propenamine (Bppa, Fig. 1) showed excellent in vitro anti-*T. cruzi* profile[33] and in vivo for some assayed Bppa derivatives.[33–35] In this sense, recently our group have described a series of 5-nitrofuranes able to accumulate squalene, and potentially inhibit SE, in *T. cruzi* parasite.[18] These nitrofuranes (**1**–**4**, Fig. 1) have an additional mechanism of action, they are able to produce oxidative stress and consequently, they are dual agents.[18] From a chemical point of view besides the 5-nitrofuran moiety, these compounds were designed as heteroallyl-derivatives in order to simulate the framework allylamine present in Tbf. From a biological point of view, and according to our results,[18] this framework is not responsible for the squalene accumulation because some of the previously evaluated derivatives, parent compounds **2** and **3** (Fig. 1), were as good as squalene-accumulators as the heteroallyl-derivatives, that is, **1** and **4** (Fig. 1), or others heteroallyl-containing nitrofuranes (i.e., **5** and **6**, Fig. 1) were enable to accumulate this biomolecule.[18] In order to complete the structure–activity information new SE inhibitors were designed, derivatives **7**–**14** (Fig. 1). The synthetic efforts were focus on three different structural modifications. Modifications on the 5-nitrofuran system were analysed studying 5-nitrothiophene-derivatives and unsubstituted ones (Fig. 1). Also, the heteroallyl-system was maintained but in two different stereo-electronic forms, as allylamide and (3-phenyl)allylamine moieties (like Ntf, Fig. 1), preserving the heterocycle ring using as the connection via thiosemicarbazone, semicarbazone, or amide linkers (Fig. 1). Additionally, the biological effects of all the new compounds was analyzed, **7**–**14** and the secondary product **15** (Scheme 1), using *T. cruzi* Tulahuen 2 strain as parasitic model, and for two compounds the CL Brener clone. The selectivity towards the parasite was studied by determining the toxicity of the most active anti-*T. cruzi* compounds against mammal-J-774 cells. The sterols level-changes produced by the nitrocompounds into the parasite were also studied using TLC and HPLC methodologies. Furthermore, being some antifungals active against *T. cruzi*,[27,28] we proposed to evaluate the designed anti-*T. cruzi* agents for its in vitro antifungal properties. Quantitative structure–activity relationship studies allowed identifying some of the structural requirements for squalene accumulation.

2. Methods and results

2.1. Chemistry

A simple and efficient procedure to generate thiosemicarbazone derivatives, **7**–**12**, was employed (Scheme 1a). Commercially available N^4-allylthiosemicarbazide was reacted with different aldehydes in toluene as solvent, generating the desired compounds, **7**–**12**, in high yields.[12] Previously developed methodology[36] used in our group (carbamates synthesis followed by hydrazinolysis),[10,11,18] was attempted to prepare semicarbazone derivatives containing (3-phenyl)allylamino framework (structures **(II)**, Scheme 1b). However, in the hydrazinolysis of the intermediate



Scheme 1. Synthetic procedures used to prepare the new studied derivatives.

(I), reduction of the allylic-double bond took place as the main process without generation of the desired semicarbazide (see Supplementary data), being in our hands impossible to separate the desired alkene reactant from the mixture of alkene and alkane. Another effort to generate the desired semicarbazone derivatives was performed using the carbamate intermediate **(I)** and the hydrazone of 5-nitrofurfural as reactant (see Scheme 1b), but this procedure was unsuccessful in our hands. Finally, the amide-linked derivatives, **13** and **14** (Scheme 1c), were obtained following previously described methodologies with adequate yield.[10,11] The generation of the 5-nitrofuryl derivative **IV** was endeavoured using chloroethylamide **III**[11] and commercially available substituted-piperazine (Scheme 1c). In this process we were enabled to obtain the desired product **IV**, generating in this condition the alcohol **15** as the main product. All the proposed structures were established by ^{1}H, ^{13}C NMR (HMQC, HMBC) spectroscopy and MS. The purity was analyzed and established by TLC and microanalysis, respectively.

2.2. Biological characterization

2.2.1. In vitro anti-*T. cruzi* activity

The new derivatives, **7**–**15**, were initially tested in vitro against the epimastigote form of *T. cruzi*, Tulahuen 2 strain. The existence of the epimastigote form of *T. cruzi* as an obligate mammalian intracellular stage has been revisited and confirmed.[37,38] The compounds were incorporated into the media at 25 μM and their ability to inhibit the parasite growth was evaluated in comparison to the control (no drug added to the media) at day 5. The ID_{50} doses (50% inhibitory dose) were determined for all of them (Table 1). Nfx, Bnz, Tbf and ketoconazole (Ktz) were used as the reference trypanosomicidal agents. The thiosemicarbazones **7** and **10** were also studied against the high virulent[39,40] CL Brener clone (Table 1).

5-Nitrofuranes **8** and **13** were the most active anti-*T. cruzi* derivatives, with ID_{50} = 1.9 and 1.0 μM, near to 4 and 7 times more active than the reference drugs Nfx and Bnz. While the 5-nitrothiophene **11**, the 5-unsubstituted analogue **12** and the secondary product **15** displayed ID_{50} values between 4.2 and 6.4 μM, which also resulted more active than the references (Nfx, Bnz, Tbf and Ktz) (Table 1). The other 5-unsubstituted derivatives, **9**, **10** and **14**, were less or equally active than the reference drugs. No susceptibility differences between both strains, with the studied compounds, were observed.

2.2.2. In vitro unspecific cytotoxicity

Mammal cytotoxicity of some of the new compounds, **7**, **8**, **10**–**12**, was studied in vitro using J-774 mouse macrophages as the cellular model with doses (100.0–400.0 μM) at least four times higher than the doses used for *T. cruzi* (25 μM) (Table 2).[41] These derivatives were selected regarding its in vitro anti-*T. cruzi* activities and its widespread structural motives at the heterocycle level

Table 1
Biological characterization of new derivatives against *T. cruzi* tulahuen 2 strain

Compd	ID_{50}[a] (μM)
7	8.2 (6.4)[b]
8	1.9
9	>25.0
10	16.0 (9.6)
11	4.2
12	6.4
13	1.0
14	>25.0
15	5.0
Nfx	7.7 (8.5)[c]
Bnz	7.4 (4.5)[c]
Tbf	17.0 (42.0)[c]
Ktz	10.0 (5.0)[c]

[a] The results are the means of three independent experiments with a SD less than 10% in all cases.
[b] Values in parenthesis correspond to CL Brener clone activity.
[c] From Ref. 41.

(Scheme 1). The selectivity indexes, SI, were expressed as the ratio between ID_{50} in macrophages and ID_{50} in *T. cruzi*. Derivatives **7**, **10** and **12** have better SI values than the biosynthesis-membrane-sterol inhibitors Tbf and Ktz, having thiophene-derivative **12** 1.5-fold the selectivity of Nfx (Table 2). No structural exigencies could be observed regarding the toxic effects.

2.2.3. In vitro antifungal activity[42,43]

Due to some antifungals are actives against *T. cruzi*[27,28] we decided to evaluate the designed anti-*T. cruzi* agents for its in vitro antifungal properties. To determine the antifungal activity, microorganisms from the *American Type Culture Collection* (ATCC) or clinical isolates provided by the Centro de Referencia Micobiológica (CEREMIC) from Facultad de Ciencias Bioquímicas y Farmacéuticas (Argentina) or Control Lab from Río de Janeiro (Brazil) were used (Table 3). Minimal inhibitory concentration (MIC) of each compound was determined by using broth microdilution techniques for yeasts[44] and filamentous fungi.[45] For this study we selected derivatives **7**–**12** with structural modifications at the heterocycle level and with widespread squalene-accumulation profiles (see below, Table 4). As antifungal reference compounds Tbf, Ktz and amphotericin B (AnfB) were used. Although the studied compounds, 5-nitrofuranes, 5-nitrothiophenes and the 5-unsubstituted analogues, were less active than the reference compounds derivative **10** showed moderate activities against *Cryptococcus neoformans*, *Microsporum gypseum*, *Trichophyton rubrum* and *Trichophyton mentagrophytes*.

Table 2
Biological characterization of new derivatives against mammal macrophages

Compd	D_{50}[a] (μM)	
	J-774 macrophages	SI[b]
7	200.0	24.4
8	<100.0	<52.6
10	>400.0	>25.0
11	<100.0	<23.8
12	>400.0	>62.5
Nfx	316.0	41.0
Tbf	339.0	19.9
Ktz	<100.0	<10.0

[a] The results are the means of three independent experiments with a SD less than 10% in all cases.
[b] SI: selectivity index, $ID_{50,macrophage}/ID_{50,T.\ cruzi}$.

2.3. Squalene and ergosterol accumulation analysis

Since the steroids from the lipid fractions of *T. cruzi* epimastigotes has been previously described,[33] this form was selected (Tulahuen 2 strain) to study the effects on new derivatives acting on ergosterol biosynthesis. After a pre-established protocol ($1 \times ID_{50}$ per 8×10^6 cells/mL, 120 h of incubation),[46] the controls (untreated, Tbf- and Nfx-treated) and derivative-treated parasites were collected and the total lipids were extracted and analysed as described previously.[33,47,24b] Qualitative analyses of neutral lipid fractions were carried out using TLC (see Supplementary data). Then, quantitative analyses of ergosterol and squalene from sterol fractions were done by HPLC. Derivatives **7**, **8** and **11** were able to accumulate squalene in different degrees with the concomitant decrease of ergosterol (Table 4, Fig. 2). The most squalene-accumulator was the 5-nitrofuran derivative **7** with similar capability to Tbf while the thia-analogue **11** showed good squalene-accumulation level. The 5-nitrofuran homologue, derivative **8**, was discrete accumulator. In the three cases ergosterol concentrations were lower than untreated-parasite values however the ergosterol depletion promoted by Tbf was the best. This fact could be indicating some differences on the mechanism of action between Tbf and the studied furyl/thienyl derivatives. For derivates **10**, **12**–**15** and Nfx we were unable to detect squalene accumulation, using HPLC analysis, after 120 h of incubation (Table 4). However, at lower incubation times (72 h) derivatives **13**, **15** and Nfx showed, using TLC analysis, some degree of squalene accumulation however lesser than Tbf or derivative **7** (Fig. 2c). We were able to detect squalene accumulation for 5-unsubstituted derivative **14** at $4 \times ID_{50}$ doses in the same experimental conditions.

Table 3
Antifungal activity of new compounds against filamentous and yeast, expressed as minimal inhibitory concentration and minimal fungicidal concentration

Compd	MIC/MFC[a,b] (μg/mL)									
	Ca	Ct	Sc	Cn	Afu	Afl	Ani	Mg	Tr	Tm
7	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
8	>250	>250	>250	>250	>250	>250	>250	250/250	250/250	250/250
9	>250	>250	>250	250/>250	>250	>250	>250	>250	>250	>250
10	>250	>250	>250	125/>250	>250	>250	>250	125/250	125/250	125/250
11	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
12	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
AnfB[c]	0.78	1.56	0.50	0.25	0.50	0.50	0.50	0.125	0.075	0.075
Tbf	1.56	1.56	3.12	0.39	0.78	0.78	1.56	0.04	0.01	0.025
Ktz	0.5	0.50	0.5	0.25	0.125	0.50	0.25	0.05	0.025	0.025

[a] MIC = minimal inhibitory concentration, MFC = minimal fungicidal concentration.
[b] Ca: *Candida albicans* ATCC 10231, Ct: *Candida tropicalis* C 131 2000, Sc: *Saccharomyces cerevisiae* ATCC 9763, Cn: *Cryptococcus neoformans* ATCC 32264; An: *Aspergillus niger* ATCC 9029, Afu: *Aspergillus fumigatus* ATCC 26934; Afl: *Aspergillus flavus* ATCC 9170, Mg: *Microsporum gypseum* C 115; Tr: *Trichophyton rubrum* C113, Tm: *Trichophyton mentagrophytes* ATCC 9972.
[c] AnfB: anphotericin B.

Table 4
Squalene and ergosterol composition of *T. cruzi* epimastigotes treated with compounds **7**, **8**; **10**–**15**; Nfx and Tbf after 120 h

Compd	$C_{squalene}$ (μg/mL)	$C_{ergosterol}$ (μg/mL)	Ratio[a]
7	15.9 ± 0.9	5.5 ± 1.9	2.9 ± 1.0
8	4.6 ± 0.9	4.5 ± 0.9	1.0 ± 0.4
10	0.6[b]–2.1[c]	—	—
11	7.4 ± 0.4	5.0 ± 0.9	1.5 ± 0.3
12	0.6–2.1	—	—
13	0.6–2.1	—	—
14	0.6–2.1	—	—
15	0.6–2.1	—	—
Nfx	0.6–2.1	—	—
Tbf	12.0 ± 1.0	2.7 ± 0.5	4.4 ± 1.0
Untreated	0.6–2.1	6.8 ± 0.5	0.1–0.3

[a] Ratio: $C_{squalene}/C_{ergosterol}$.
[b] Limit of detection 0.6 μg/mL.
[c] Limit of quantification 2.1 μg/mL.

2.4. Structure–activity relationship studies

Initially 17 compounds were used in the structure–activity relationship studies, the parent compounds **1**–**6**, the new derivatives **7**, **8**, **10**–**15** and the references compounds Nfx, Tbf and Bppa. The ability to accumulate squalene (aasq) into the parasite was used as the biological activity. Due to the different bibliographic origin[18,33] of the dependent variable, squalene accumulation value, this was used as categorical values. When the compounds are excellent squalene accumulators (more than 8-fold of squalene level comparing to the untreated control), derivatives **1**, and **2**, the activity was categorized as 2; when the compounds are good/discrete squalene accumulators (2–8-fold of squalene level comparing to the untreated control), derivatives **3**, **4**, **7**, **8**, **11**, Bppa and Tbf, the activity was categorized as 1; and when the compounds are poor or not squalene accumulators (same squalene level that the untreated control), derivatives **5**, **6**, **10**, **12**–**15** and Nfx, the activity was categorized as 0.

As the independent variables were employed physicochemical parameters extracted from molecular modelling calculations. Molecular modelling studies were performed by calculating compounds stereoelectronic properties, using DFT calculations.[48,49] A detailed conformational search for each of the molecules was performed, using MM methods, to find the minimum energy and highest abundance conformer. The molecules with amine-protonable group were studied as cationic forms, derivatives **13**, **14**, Tbf and Bppa. The geometry of this conformer was fully optimized by applying B3LYP/6-31G*//PM3 in the gas phase that allows to obtain acute results with low time of computational calculi. Then, single point B3LYP/6-31G* calculation was performed.[50–52] The properties determined and examined in this study were total energy, magnitude of dipolar moment, HOMO's and LUMO's energies, gap (E_LUMO–E_HOMO), hardness (η) [(E_LUMO–E_HOMO)/2], electronegativity (Elect) [(-E_LUMO–E_HOMO)/2], electrophilic index (EI) [$(Elect)^2/(2\ \eta)$], volume, area, and the logarithm of the partition coefficient of the non-ionized molecules (Log *P*) (see Supplementary data). Theoretical Log *P* (*c*Log *P*) was calculated using the Villar method, implemented in PC SPARTAN 04 package[53] at

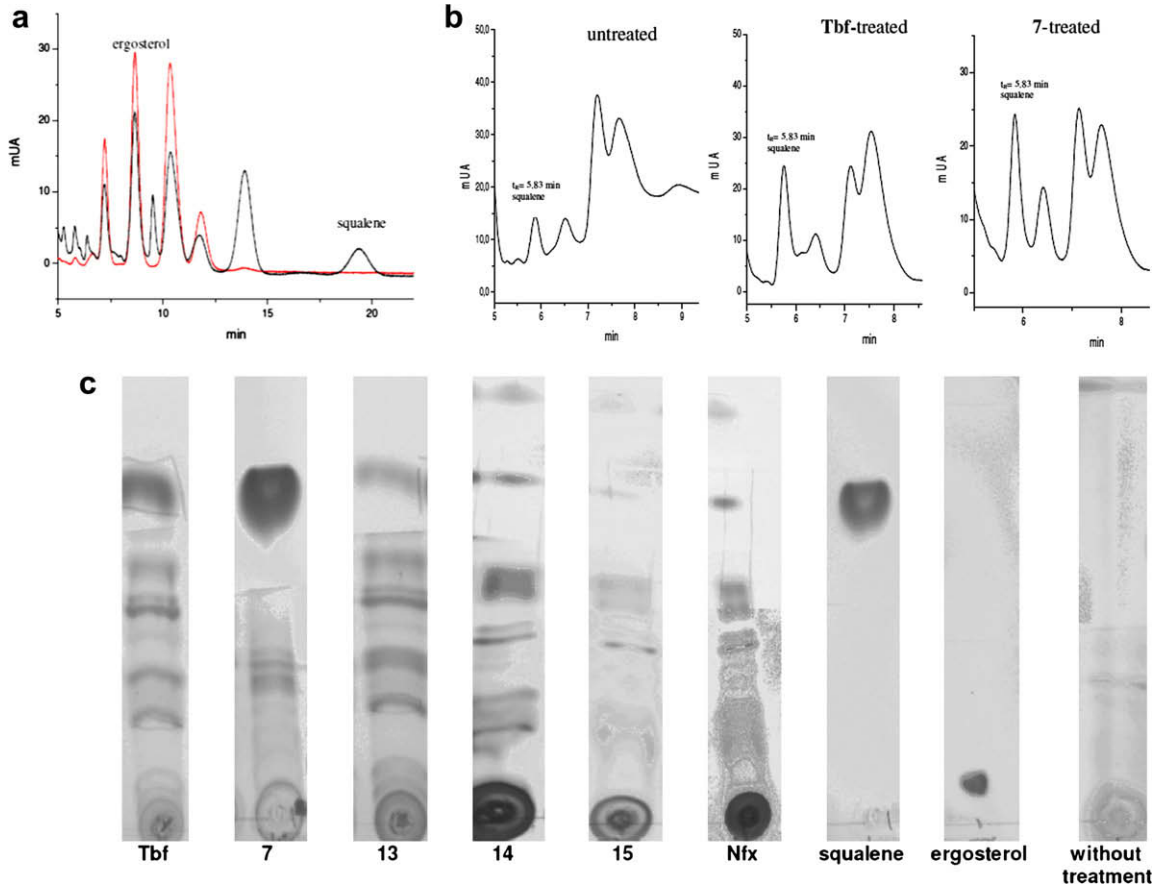


Figure 2. (a) HPLC-lipid extracts profiles from untreated epimastigote *T. cruzi* using two different UV lambda detection (210, black, and 250 nm, red), acetonitrile/water (9:1), flux rate: 0.8 mL/min. (b) HPLC-chromatograms obtained from the analysis of the lipid extracts from epimastigotes, untreated (left), Tbf-treated (centre) and **7**-treated (right) (acetonitrile, flux rate: 0.8 mL/min, detection 210 nm). (c) TLC-analysis (see Section 5 for details) of lipid extracts from epimastigote *T. cruzi* treated with Tbf, **7**, **13**, **15** and Nfx and with **14** (at 4 × ID_{50} doses per 8 × 10^6 cells/mL) during 72 h.

the AM1 semiempirical level. Besides, the correlation matrix for the used physicochemical descriptors was performed and cross-correlations between the descriptors were not obtained (see Supplementary data). Thus, these parameters are orthogonal, allowing its safely use in the multilinear regression relationship.[54,55]

Non-statistical significant correlations were obtained when the different theoretical descriptors were studied as independent variable, but it was observed a clear tendency between activities and *c*Log *P*, as it could expect the greatest squalene accumulators are positioned in the maximum of *c*Log *P*. Consequently, multivariable regressions between the aasq and the physicochemical descriptors were studied. When the complete compound-population was studied adequate correlations were found between aasq and EI, *c*Log *P*, and $(c\text{Log}\,P)^2$ ($r^2 = 0.7802$) or aasq and E_LUMO, *c*Log *P*, and $(c\text{Log}\,P)^2$ ($r^2 = 0.7486$). When Bppa or Tbf were excluded from the analyses the statistics of these correlations were improved ($r^2 = 0.8402$ and $r^2 = 0.7790$, or $r^2 = 0.7872$ and $r^2 = 0.8023$, respectively). When the homogeneous population of furyl/thienyl derivatives were analyzed, compounds **1**–**8**, and **10**–**15**, the best statistic results, Eqs. 1 and 2, were obtained. These equations show that aasq is correlated with E_LUMO, or EI, and, in a quadratic manner, with the *c*Log *P*.

$$\begin{aligned}\text{aasq} = &-0.4\ (\pm0.3) + 0.9\ (\pm0.1)\text{E\_LUMO} + 2.1\ (\pm0.2)\ c\text{Log}P \\ &- 0.21\ (\pm0.03)\ (c\text{Log}P)^2 \\ &n = 15,\ r^2 = 0.8711,\ r^2_{\text{adj}} = 0.8360,\ F = 24.8, \\ &p < 0.0001 \end{aligned} \quad (1)$$

$$\begin{aligned}\text{aasq} = &-1.3\ (\pm0.3) - 0.22\ (\pm0.03)\ \text{EI} + 2.1\ (\pm0.2)\ c\text{Log}P \\ &- 0.21\ (\pm0.03)\ (c\text{Log}P)^2 \\ &n = 15,\ r^2 = 0.8667,\ r^2_{\text{adj}} = 0.8304,\ F = 23.8, \\ &p < 0.0001 \end{aligned} \quad (2)$$

These equations show the relevance of the compounds lipophilicity in the ability to accumulate squalene, showing that the typical quadratic behaviour corresponding to an enzymatic inhibition process could be involved. On the other hand, according to the obtained QSARs the electronic properties of compounds, expressed by LUMO energy or electrophilic capability, also govern the bio-activity. Less negative E_LUMO or less electrophile-capability of compounds promote better ability to accumulate squalene. In order to identify the LUMO most contributor atomic centre we mapped the LUMO orbitals and the electronic density maps (Fig. 3). Between the 5-nitro-substituted series, the 5-positions of the furanes are the most contributor centre.

Taking this in mind, we re-studied new correlations that involved the atoms near to 5-position of the furan ring, specifically the nitro nitrogen and 5-furan carbon Mulliken atomic charges (q_N and q_C, respectively, Eqs. 3 and 4). Clearly, in the 5-nitrofuran series the ability to accumulate squalene is correlated to less positive Mulliken charges of the nitro nitrogen and 5-furan carbon atoms being Eqs. 3 and 4 completely in agreement with the descriptors resulted in QSARs equations 1 and 2.

$$\begin{aligned}\text{aasq} = &\ 46\ (\pm14) - 141\ (\pm43)\ q_N + 1.8\ (\pm0.4)\ c\text{Log}P \\ &- 0.19\ (\pm0.04)\ (c\text{Log}P)^2 \\ &n = 11,\ r^2 = 0.7790,\ r^2_{\text{adj}} = 0.6843,\ F = 8.23, \\ &p = 0.0107 \end{aligned} \quad (3)$$

$$\begin{aligned}\text{aasq} = &\ 70\ (\pm20) - 151\ (\pm41)\ q_C + 1.4\ (\pm0.4)\ c\text{Log}P \\ &- 0.15\ (\pm0.04)\ (c\text{Log}P)^2 \\ &n = 11,\ r^2 = 0.8058,\ r^2_{\text{adj}} = 0.7226,\ F = 9.7, \\ &p = 0.0069 \end{aligned} \quad (4)$$

These equations also indicate that the electronic properties of the studied compounds could play a role in the ability to accumulate squalene. Probably, the compounds could act through their redox-nitro moiety ability (see discussion below).

3. Discussion

We report the development of a second generation of 5-nitrofuranes and their thia-analogues with anti-*T. cruzi* activity and squalene accumulation capability.

In the assay against Tulahuen 2 strain, 5-nitrofuran derivatives **8** and **13** were the most active while **7**, **15** and the thiophenes **11**, and **12** were equal or more active than Nfx, Bnz, Tbf and Ktz (Table 1). Regarding the cytotoxicity against macrophages and comparing to reference compounds (Nfx, Tbf, Ktz, Table 2) we found 5-nitrofuran **7** and thiophene **12** with good SI being derivative **12** the best one. The nitro-substituted derivatives are more toxic than the unsubstituted analogues (compare cytotoxicities of **8** and **10**, or of **11** and **12**) this in accordance with the well known characteristic of nitrocompounds. However, it must be pointed out the nitro-moiety is relevant for the squalene-accumulation properties (see below). The 5-unsubstituted furan **10** possessed similar ID_{50}

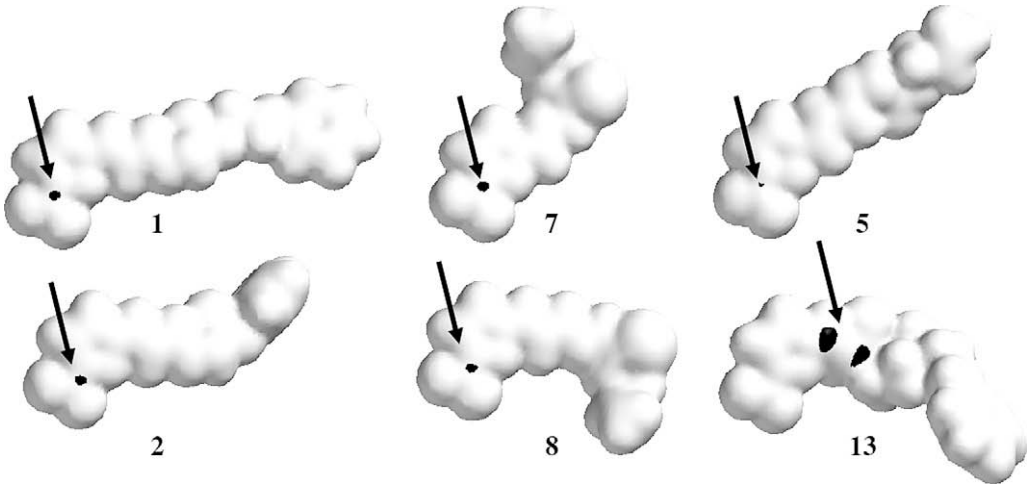


Figure 3. Electron density (solid white, isovalue 0.02) and LUMO (solid black, isovalue 0.018) isosurfaces for good (left), medium (centre), and bad (right) 5-nitrofuran-squalene accumulators. The narrows indicate the E_LUMO map.

against *T. cruzi*-Tulahuen 2 strain to Tbf (16.0 and 17.0 μM, respectively) however derivative **10** has better activity against CL Brener clone (ID_{50}s of 9.6 and 42.0 μM, respectively) and SI (>25.0 and 19.9, respectively) than the antifungal reference Tbf. On the other hand, furan **10** was the only studied compound with some level of activities against the studied fungus (Table 3). These results open the therapeutic potential of 5-unsubstituted furanes and thiophenes, that is, **10** and **12**, that in general are badly considered as pharmacophore in the neglected diseases, like trypanosomatids-associated illness.

In the squalene accumulation changes studies, some relevant features could be observed. On the one hand, unlike Nfx, 5-nitrofuranes **7**, **8**, and the 5-nitrothiophene **11** were able to accumulate squalene in the studied experimental conditions (Table 4) resulting derivatives **7** and **11** as squalene-accumulators as Tbf. In these cases ergosterol was concomitantly depleted however the ergosterol-depletions for these were lower than the observed value for Tbf showing an alternative ergosterol biosynthetic pathway probably operating for these derivatives. On the other hand, chemical-structural exigencies could be extracted from this study, for example the necessity of an allyl-moiety is not essential for the accumulation of squalene, like it was observed with the parent compounds **1** and **2** (Fig. 1), being allyl-derivatives **10**, and **12**–**14** unable to accumulate squalene when were tested at 120 h of incubation. Moreover, due to 5-unsubstituted derivatives **10**, **12** and **14** are not squalene-accumulators it seems to indicate the presence of the 5-nitro-moiety is relevant for the ability to accumulate this biological precursor.

These structural questions could be answered analyzing the QSARs results. On the one hand, as it is discussed above the compounds lipophilic property clearly affect the compounds ability to accumulate squalene in a quadratic manner characteristic of enzyme–drug interaction validating the hypothesis of an enzymatic inhibition. On the other hand, in the cases of 5-nitro-substituted furanes the QSARs seem to show the nitro-nitrogen should exhibit a particular electrophile character to accumulate squalene. Taking into account SE starts its catalytic cycle, terminating with the production of 2,3-oxidosqualene, as the enzyme-reduced form[56] reacting with molecular oxygen (Fig. S1, Supplementary data) it could be speculated that the studied nitro-derivatives disturb this process as electron-scavenger entities. At least two different pathways could be affected by the nitro-derivatives. Firstly, as NADPH-cytochrome P450 reductase electron-acceptor, being this enzyme the responsible of maintaining adequate levels of squalene epoxidase in its reduced form, the nitro-derivatives could be interfering in this process as redox partner of NADPH or redox substrate of NADPH-cytochrome P450 reductase (pathways (a) or (b) Fig. S2a, Supplementary data). As it was previously probed,[18–22] the nitro-derivatives studied here are able to suffer reduction by one electron, generating concomitantly the nitro-anion free radical (Fig. S2b, Supplementary data). Secondly, as molecular oxygen scavenger, being the squalene epoxidation an oxygen-dependent pathway the nitro-derivatives could be reacting with oxygen promoting the decrease of this entity (pathway (c) Fig. S2a, Supplementary data). This kind of process has been previously used as a manner to probe the oxidative stress mechanism of action of anti-trypanosomatids 5-nitrofuranes,[18–20,57] known as redox cycling studies. However, being this process operative in most of the 5-nitrofuranes in the case study herein the speculated consumption of molecular oxygen seems to need a special redox potential.

4. Conclusions

We have developed and identified new furanes and thiophenes as anti-*T. cruzi* agents. Observing high selectivity indexes and new manner of action for some of them, we could propose them as molecular lead for further structural modifications and further biological studies, especially in vivo evaluations.

5. Experimental

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. 3-(5-Nitrofuryl)propenoic acid, 3-furylpropenoic acid, and intermediate **III** were prepared following synthetic procedures previously reported.[10,11] Melting points were determined with an electrothermal melting point apparatus (Electrothermal 9100) and were uncorrected. Proton and carbon NMR spectra were recorded on a Bruker DPX-400 spectrometer. The chemical shifts values are expressed in ppm relative to tetramethylsilane as internal standard. Mass spectra were determined on a MSD 5973 Hewlett–Packard spectrometer using electronic impact at 70 eV (EI). Infrared spectra were recorded on a Perkin–Elmer 1310 apparatus, using potassium bromide tablets the frequencies were expressed in cm^{-1}. Microanalyses were performed on a Fisons EA 1108 CHNS-O instrument and were within ±0.4% of the calculated compositions. Column chromatography was carried out using Merck silica gel (60–230 mesh).

5.1. General procedure for the preparation of thiosemicarbazones 7–12

The corresponding aldehyde (5-nitrofurfural, 3-(5-nitrofuryl)acroleine, furfural, 5-nitrothiophene-2-carbaldehyde, or thiophene-2-carbaldehyde, 1.0 mmol), 4-allylthiosemicarbazone (1.0 mmol), *p*-TsOH (catalytic amount) and toluene (10.0 mL) were stirred at room temperature until the aldehyde was not present. The solid was collected by filtration, crystallized from toluene, washed with *n*-hexane and dried.

5.1.1. 4-Allyl-1-(5-nitrofurfurilydene)thiosemicarbazide (7)

Yellow solid, 221 mg (87%); mp 179.0–181.0 °C. ^{1}H NMR (DMSO-d_6) δ_H: 4.24 (2H, t, J = 5.7 Hz), 5.13 (2H, m), 5.91 (1H, m), 7.37 (1H, d, J = 4.0 Hz), 7.81 (1H, d, J = 4.0 Hz), 8.00 (1H, s), 8.70 (1H, t, J = 5.7 Hz), 11.94 (1H, s). ^{13}C NMR (DMSO-d_6) δ_C: 46.8, 114.5, 116.0, 116.7, 130.6, 135.4, 153.3, 156.0, 178.3. EI-MS, *m/z* (abundance, %): 254 ($M^{+\cdot}$, 1), 237 ($M^{+\cdot}-17$, 8), 209 ($M^{+\cdot}-NO_2$, 4), 197 (7), 141 (100), 115 (35), 81 (24), 56 (32). IR, ν: 619, 806, 1096, 1215, 1279, 1356, 1509, 1551, 3343. Anal. Calcd for $C_9H_{10}N_4O_3S$.

5.1.2. 4-Allyl-1-[3-(5-nitro-2-furyl)-2-propenylidene] thiosemicarbazide (8)

Yellow solid, 188 mg (67%); mp 175.4–178.0 °C. ^{1}H NMR (DMSO-d_6) δ_H: 4.19 (2H, t, J = 5.6 Hz), 5.11 (2H, m), 5.89 (1H, m), 7.03 (3H, m), 7.75 (1H, d, J = 4.0 Hz), 7.90 (1H, d, J = 8.0 Hz), 8.61 (1H, t, J = 5.6 Hz), 11.70 (1H, s). ^{13}C NMR (DMSO-d_6) δ_C: 46.6, 114.5, 116.2, 116.6, 123.6, 131.0, 135.5, 142.6, 153.0, 155.6, 178.1. EI-MS, *m/z* (abundance, %): 280 ($M^{+\cdot}$, 6), 263 ($M^{+\cdot}-17$, 17), 234 ($M^{+\cdot}-NO_2$, 14), 167 (61), 115 (100), 92 (27), 81 (21), 56 (64). IR, ν: 799, 1098, 1219, 1356, 1462, 1526, 3300. Anal. Calcd for $C_{11}H_{12}N_4O_3S$.

5.1.3. 4-Allyl-1-furfurylidenethiosemicarbazide (9)

Yellow solid, 127 mg (61%); mp 128.0–129.0 °C. ^{1}H NMR ($CDCl_3$) δ_H: 4.39 (2H, m), 5.30 (2H, m), 5.98 (1H, m), 6.54 (1H, dd, J = 1.6, 3.2 Hz), 6.74 (1H, d, J = 3.2 Hz), 7.54 (1H, s), 7.68 (1H, br s), 7.77 (1H, br s), 9.85 (1H, br s). ^{13}C NMR ($CDCl_3$) δ_C: 47.3, 112.5, 114.5, 117.5, 132.4, 133.7, 145.2, 149.1, 177.2. EI-MS, *m/z*

(abundance, %): 209 ($M^{+\cdot}$+H, 2), 182 (4), 115 (100), 109 (9), 96 (63), 81 (19), 56 (27). IR, *v*: 586, 745, 920, 1011, 1221, 1287, 1526, 3154. Anal. Calcd for $C_9H_{11}N_3OS$.

5.1.4. 4-Allyl-1-(3-furyl-2-propenylidene)thiosemicarbazide (10)

Orange solid, 181 (77%); mp 130.0–132.0 °C. ^{1}H NMR ($CDCl_3$) δ_H: 4.38 (2H, m), 5.30 (2H, m), 5.96 (1H, m), 6.47 (2H, m), 6.74 (2H, m), 7.47 (2H, br s), 7.56 (1H, d, *J* = 8.0 Hz), 9.29 (1H, br s). ^{13}C NMR ($CDCl_3$) δ_C: 47.3, 112.2, 112.6, 117.6, 122.7, 127.2, 133.7, 144.1, 144.2, 152.4, 177.7. EI-MS, *m/z* (abundance, %): 235 ($M^{+\cdot}$, 5), 135 (16), 121 (63), 115 (100), 93 (79), 80 (23), 67 (37), 56 (35). IR, *v*: 583, 623, 739, 920, 957, 1007, 1075, 1136, 1221, 1285, 1545, 3152. Anal. Calcd for $C_{11}H_{13}N_3OS$.

5.1.5. 4-Allyl-1-(5-nitrothenylidene)thiosemicarbazide (11)

Orange solid, 267 mg (99%); mp 201.7–202.6 °C. ^{1}H NMR (DMSO-d_6) δ_H: 4.23 (2H, m), 5.14 (2H, m), 5.90 (1H, m), 7.53 (1H, d, *J* = 4.4 Hz), 8.08 (1H, d, *J* = 4.4 Hz), 8.23 (1H, s), 8.73 (1H, t, *J* = 5.6 Hz), 11.89 (1H, br s). ^{13}C NMR (DMSO-d_6) δ_C: 46.7, 116.6, 130.0, 131.3, 135.4, 136.2, 147.4, 151.5, 178.0. EI-MS, *m/z* (abundance, %): 270 ($M^{+\cdot}$, 2), 244 (6), 157 (81), 115 (100), 95 (25), 81 (39), 56 (63). IR, *v*: 617, 733, 814, 930, 1107, 1235, 1298, 1356, 1439, 1491, 1539, 3135. Anal. Calcd for $C_9H_{10}N_4O_2S_2$.

5.1.6. 4-Allyl-1-thenylidenethiosemicarbazide (12)

White solid, 198 mg (88%); mp 161.3–162.8 °C. ^{1}H NMR ($CDCl_3$) δ_H: 4.40 (2H, br s), 5.28 (2H, dd, *J* = 10.4, 17.2 Hz), 5.99 (1H, m), 7.08 (1H, dd, *J* = 4.0, 5.0 Hz), 7.30 (1H, d, *J* = 4.0 Hz), 7.40 (1H, d, *J* = 5.0 Hz), 7.45 (1H, br s), 8.08 (1H, s), 9.90 (1H, br s). ^{13}C NMR ($CDCl_3$) δ_C: 47.2, 117.4, 128.2, 128.9, 131.2, 133.7, 137.7, 138.4, 177.6. EI-MS, *m/z* (abundance, %): 225 ($M^{+\cdot}$, 3), 125 (9), 115 (100), 56 (40). IR, *v*: 594, 700, 914, 1036, 1221, 1287, 1552, 3146. Anal. Calcd for $C_9H_{11}N_3S_2$.

5.1.7. 4-(3-Phenyl-2-propenyl)piperazine-1-ylamide of 3-(5-nitro2-furyl)propenoic acid (13)

A mixture of 3-(5-nitrofuryl)propenoic acid (1.0 mmol), $SOCl_2$ (2.0 mmol), and dry *n*-hexane (25.0 mL) was heated at reflux for 1.5 h. Then a mixture of 4-(3-phenyl-2-propenyl)piperazine (1.2 mmol) and Et_3N (2.5 mmol) was added at 0 °C and the mixture was stirred for 1.5 h at room temperature. The organic mixture was treated EtOAc (30.0 mL) and washed successively with aqueous saturated solution of $NaHCO_3$ (10.0 mL), aqueous solution of HCl (1 M, 10.0 mL). After dried with Na_2SO_4 the organic layer was evaporated in vacuo and the crude was purified by column chromatography (Al_2O_3, petroleum ether/EtOAc (0–60%)). Brown-orange solid (15%); mp 136.0–139.0 °C. ^{1}H NMR ($CDCl_3$) δ_H: 2.53 (4H, m), 3.23 (2H, d, *J* = 6.8 Hz), 3.73 (4H, m), 6.29 (1H, d, *J* = 15.4 Hz), 6.57 (1H, d, *J* = 15.4 Hz), 6.71 (1H, d, *J* = 3.3 Hz), 7.17 (1H, d, *J* = 15.3 Hz), 7.27 (1H, t, *J* = 7.7 Hz), 7.36 (2H, m), 7.37 (1H, d, *J* = 3.3 Hz), 7.40 (2H, m), 7.47 (1H, d, *J* = 15.3 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 45.0, 53.0, 61.0, 113.0, 115.0, 122.0, 126.0, 126.5, 128.0, 128.5, 134.0, 153.0, 164.0. EI-MS, *m/z* (abundance, %): 367 ($M^{+\cdot}$, 1), 350 ($M^{+\cdot}$−17, 13), 172 (17), 117 (100). UV (acetonitrile, 0.01 mg/mL), λ_{max} = 246 (ε = 99 mL mg^{-1} cm^{-1}) and 360 nm. Anal. Calcd for $C_{20}H_{21}N_3O_4$.

5.1.8. 4-(3-Phenyl-2-propenyl)piperazine-1-ylamide of 3-(2-furyl)propenoic acid (14)

Following the procedure described for derivative **13**. Column chromatography (Al_2O_3, petroleum ether/EtOAc (0–60%)). Yellow solid (25%); mp 111.0–113.0 °C. ^{1}H NMR ($CDCl_3$) δ_H: 2.51 (4H, br s), 3.19 (2H, dd, *J* = 6.6, 1.5 Hz), 3.70 (4H, br s), 6.34 (1H, dd, *J* = 15.8, 6.9 Hz), 6.56 (1H, dd, *J* = 3.4, 1.9 Hz), 6.61 (1H, dd, *J* = 15.8 Hz), 6.76 (1H, d, *J* = 3.4 Hz), 6.93 (1H, d, *J* = 15.2 Hz), 7.25 (1H, t, *J* = 7.3 Hz), 7.34 (2H, t, *J* = 7.6 Hz), 7.41 (1H, d, *J* = 15.2 Hz), 7.46 (2H, d, *J* = 7.8 Hz), 7.66 (1H, d, *J* = 1.5 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 44.0, 53.0, 61.0, 112.0, 114.0, 116.0, 126.0, 127.0, 129.0, 129.5, 133.0, 144.0, 152.0, 164.0. EI-MS, *m/z* (abundance, %): 322 ($M^{+\cdot}$, 20), 172 (52), 117 (100). UV (acetonitrile, 0.01 mg/mL), λ_{max} = 255 and 303 (ε = 143 mL mg^{-1} cm^{-1}) nm. Anal. Calcd for $C_{20}H_{22}N_2O_2$.

5.1.9. *N*-(2-Hydroxyethyl)-3-(5-nitro-2-furyl)propenamide (15)

A mixture of intermediate **III** (1.0 mmol), 4-(3-phenyl-2-propenyl)piperazine (1.0 mmol), K_2CO_3 (2.0 mmol), and acetone (10.0 mL) was heated at reflux during 3.0 h. The acetone was evaporated in vacuo and the crude of reaction was treated EtOAc (30.0 mL) and washed successively with aqueous saturated solution of $NaHCO_3$ (10.0 mL), aqueous solution of HCl (1 M, 10.0 mL). After dried with Na_2SO_4 the organic layer was evaporated in vacuo and the crude was purified by column chromatography (Al_2O_3, petroleum ether/EtOAc (0–60%)). Brown oil (10%). ^{1}H NMR ($CDCl_3$) δ_H: 2.19 (1H, m), 4.05 (2H, d, *J* = 9.4 Hz), 4.38 (2H, d, *J* = 9.4 Hz), 6.69 (1H, d, *J* = 3.8 Hz), 6.86 (1H, d, *J* = 15.9 Hz), 7.12 (1H, d, *J* = 15.9 Hz), 7.35 (1H, t, *J* = 3.8 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 55.0, 68.0, 113.0, 114.0, 120.0, 124.0, 154.0, 163.0. EI-MS, *m/z* (abundance, %): 226 ($M^{+\cdot}$, 3), 208 ($M^{+\cdot}-H_2O$, 42), 162 ($M^{+\cdot}-NO_2$, 100). Anal. Calcd for $C_9H_{10}N_2O_5$.

5.2. Biology

5.2.1. Anti-*T. cruzi* in vitro test using epimastigotes of Tulahuen 2 strain or CL Brener clon

Trypanosoma cruzi epimastigotes (Tulahuen 2 strain or CL Brener clon) were grown at 28 °C in an axenic medium (BHI-Tryptose) as previously described,[10–12] supplemented with 5% fetal bovine serum (FBS). Cells from a 10-day-old culture (stationary phase) were inoculated into 50 mL of fresh culture medium to give an initial concentration of 8×10^6 cells/mL. Cell growth was followed by measuring everyday the absorbance of the culture at 600 nm. Before inoculation, the media were supplemented with the indicated amount of the drug from a stock solution in DMSO. The final concentration of DMSO in the culture media never exceeded 0.4% and the control was run in the presence of 0.4% DMSO and in the absence of any drug. No effect on epimastigote growth was observed by the presence of up to 1% DMSO in the culture media. The percentage of growth inhibition (PGI) was calculated as follows: PGI (%) = $\{1 - [(Ap - A0p)/(Ac - A0c)]\} \times 100$, where Ap = A_{600} of the culture containing the drug at day 5; A0p = A_{600} of the culture containing the drug just after addition of the inocula (day 0); Ac = A_{600} of the culture in the absence of any drug (control) at day 5; A0c = A_{600} in the absence of the drug at day 0. To determine ID_{50} values, 50% inhibitory concentrations, parasite growth was followed in the absence (control) and presence of increasing concentrations of the corresponding drug. At day 5, the absorbance of the culture was measured and related to the control. The ID_{50} value was taken as the concentration of drug needed to reduce the absorbance ratio to 50%.

5.2.2. Unspecific mammalian cytotoxicity[41]

J-774 murine macrophage-like cells (ATCC, USA) were maintained by passage in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, and supplemented with 10% heat-inactivated fetal calf serum. J-774 cells were seeded (1×10^5 cells/well) in 96 well microplates with 200 μL of RPMI 1640 medium supplemented with 20% heat inactivated fetal calf serum. Cells were allowed to attach for 48 h in a humidified 5% CO_2/95% air atmosphere at 37 °C and, then, exposed to compounds (100.0–400.0 μM) for 48 h. Afterwards, cell viability was assessed by measuring the mitochondrial-dependent reduction of MTT (Sigma) to formazan. For that purpose, MTT was added to cells to a final concentration 0.4 mg/mL and cells were incubated at 37 °C for 3 h.

After removing the media, formazan crystals were dissolved in DMSO (180 μL), and the absorbance at 595 nm was read using a microplate spectrophotometer. Results are expressed as ID_{50} (compound concentration that reduce 50% control absorbance at 595 nm). Every ID_{50} is the average of three different experiments.

5.2.3. Antifungal in vitro test

5.2.3.1. Microorganisms and media. For the antifungal evaluation, strains from the American Type Culture Collection (ATCC), Rockville, MD, USA and CEREMIC (C), Centro de Referencia Micológica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531-(2000)-Rosario (Argentina) were used: *Microsporum canis C* 112, *Epidermophyton floccosum C* 114, *T. rubrum* C 110, *T. mentagrophytes* ATCC 9972 and *M. gypseum* C 115. Strains were grown on Sabouraud-chloramphenicol agar slants for 48 h at 30 °C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and subcultured every 15 days to prevent pleomorphic transformations. Inoculate of cell or spore suspensions were obtained according to reported procedures and adjusted to 10^3 cells/spores with colony forming units (CFU) /mL.

5.2.3.2. Antifungal susceptibility testing. Minimal Inhibitory Concentration (MIC) of each studied compound was determined by using broth microdilution techniques according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) formely National Committee for Clinical Laboratory Standards for yeasts (M27-A2) and for filamentous fungi (M 38 A). MIC values were determined in RPMI 1640 buffered to pH 7.0 with MOPS. The starting inocules were 1×10^5–5×10^5 CFU/mL. Microtiter trays were incubated at 35 °C for yeasts and hialohyphomycetes and at 28–30 °C for dermatophyte strains in a moist, dark chamber, and MICs were visually recorded at 48 h for yeasts, and at a time according to the control fungus growth, for the rest of fungi. For the assay, stock solutions of compounds were twofold diluted with RPMI 1000-1 μg/mL (final volume = 100 μL) and a final DMSO concentration ⩽ 1%. A volume of 100 μL of inocule suspension was added to each well with the exception of the sterility control where sterile water was added to the well instead. Pure compounds were tested from 100 to 1 μg/mL. MIC was defined as the minimum inhibitory concentration of compound which resulted in total inhibition of the fungal growth. Ktz, Tbf and AnfB were used as positive controls.

5.3. Squalene and ergosterol level analysis

Epimastigote forms of *T. cruzi* (Tulahuen 2 strain) (16×10^6 cells/mL) were maintained in an axenic medium (BHI-Tryptose) for analysis of the effects of the studied compounds on sterol biosynthesis modifications. The experiments were carried out in cultures at 28 °C and with strong aeration. The studied compound was added at a concentration equivalent of ID_{50} per 8×10^6 cells/mL as DMSO solution. Control samples received only the vehicle. Parasites were exposed to the treatment by 120 h. They were counted by optical microscopy using a Neubauer chamber. The control and drug-treated parasites were centrifugated at 3000 rpm during 15 min, and then the pellets were collected and washed with buffer phosphate (10.0 mL, 0.05 M, pH 7.4) and centrifugated at 3000 rpm during 15 min. The pellets were treated with chloroform/methanol (2:1) during 12 h at 4 °C. Then the organic phases were evaporated with nitrogen and the residues were treated with acetonitrile (AcCN, HPLC quality) (600 μL) during 1 min and the AcCN solutions were filtered through a cellulose-RC (0.45 μm, Sartorius) filter. TLC analyses of neutral lipid fractions were carried out using silica-gel plates (Merck 5538-7) employing the systems petroleum ether/EtOAc or petroleum ether (to see squalene) as eluents. The chromatograms were obtained by vaporizing the plates with iodine and heating them at 100 °C. Quantitative analyses of squalene from sterol fractions was done by HPLC using a C-18 Chromosorb column (25 cm × 0.4 cm internal diameter, 10 μm particle size) in a Perkin–Elmer LC-135C/LC-235C Diode Array Detector, Series 410 LC BIO PUMP, with the UV detector set at 210 nm. The mobile phase consisted of 100% AcCN and was kept constant at a flow-rate of 0.8 mL/min. The calibration curve of squalene was constructed (using cholesterol as an internal standard) for quantification of this sterol in the lipid extracts from *T. cruzi* and it is the following: $C_{squalene}$ (μg/mL) = 4.42×10^{-5} ($\pm 0.16 \times 10^{-5}$) $A - 2.77$ (±2.03), where A corresponds to the area of the peak at 5.70–5.85 min. Quantitative analyses of ergosterol from sterol fractions was done using the same column and equipment measuring at 250 nm. The mobile phase consisted of AcCN/water (9:1) and was kept constant at a flow-rate of 0.8 mL/min. The calibration curve of ergosterol was constructed (using cholesterol as an internal standard) for quantification of this sterol in the lipid extracts from *T. cruzi* and it is the following: $C_{ergosterol}$ (μg/mL) = 1.52×10^{-4} ($\pm 0.14 \times 10^{-4}$) $A' - 2.02$ (±2.30), where A' corresponds to the area of the peak at 8.50–8.75 min.

5.4. Quantitative structure–activity relationship studies

The molecular structures of the studied compounds, **1**–**8**, **10**–**15**, Nfx, Tbf and Bppa were subjected to complete geometry optimization, in gas phase, using PC SPARTAN 04 package[53] as follows: conformational search using MMFF conformer module, after that from the most stable conformer fully optimization by applying B3LYP/6-31G*//PM3. Structural, geometrical, electronic and hydrophobic descriptors were extracted from the calculus. Theoretical Log *P* was calculated using the Villar method at the AM1 semiempirical level, using the structures generated in the fully geometrical optimization. The relationships between the selected descriptors and the ability to accumulate squalene (aasq) were quantified by the use of multiple linear regressions. In the equations n represents the number of data points, r^2 is the correlation coefficient, r^2_{adj} is the adjusted correlation coefficient, and F value is related to the F-statistic analysis (Fischer test).

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Supplementary data

Supplementary data (synthesis, lipid analyses and QSAR data) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.09.013.

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