New Limonene-Hybrid Derivatives with Anti-*T. cruzi* Activity

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Abstract: The development of hybrid compounds containing limonene- and recognized anti-*T. cruzi*-heterocycle-frameworks is described. The six new compounds displayed broad antitrypanosomal activities having 5-nitrofuran and 5-nitroindazole derivatives, the best profiles. In addition, a 5-nitroindazole derivative evaluated against a panel of fungi exhibited relevant activities. Knowing that free-radical-production operates as one of the mechanisms of action on these heterocycles, we studied a potential extra-mechanism, membrane-sterols changes. Non-relevant *T. cruzi* squalene accumulation was observed for any of the tested hybrid-limonene derivatives.

Keywords: Limonene derivatives, Anti-*T. cruzi* agents, Antifungal, Lipinski rule, 5-nitrofuran, 5-nitroindazole.

INTRODUCTION

Parasitic diseases are the cause of much suffering and deaths throughout the world, mainly in developing areas like Africa, Asia and Latin America. In these regions, the economic and social impact caused by these sicknesses is very high because the parasites infect millions of people. Among these parasites, the protozoa genera are responsible for the principal sicknesses, such as *Trypanosoma cruzi* (Chagas' disease, CD). CD is endemic in South and Central America, Mexico and the southern United States, being considered one of the most serious parasite diseases in tropical regions [1]. There are currently 8 to 10 million people infected and a further 28 million, mainly in Latin America, are at risk of infection [2]. Typically, 30-40% of the chagasic individuals show clinical symptoms of the chronic phase associated with cardiac and digestive dysfunctions, and at least 12.500 die every year [3]. Despite this, the therapeutic arsenal available to treat this illness is deficient, most of the drugs present several side-effects, are antiques, or even nonexistent. Nifurtimox (Nfx) and Benznidazole (Bnz) are still the first-choice drugs for CD, both developed about 30 years ago, active against the acute stage of the illness and with strong side effects [4]. Unfortunately, these treatments have significant drawbacks in terms of route of administration, length of treatment, toxicity and cost, which limit their use in endemic areas. These facts make the search for new trypanosomicidal agents a priority [5]. The monoterpene limonene (**1**, Fig. (**1**)) is the main constituent of the essential oils of citric plants and has been used as a chiral auxiliary in asymmetric synthesis [6] and as a building block in organic synthesis [7,8] due to its natural abundance and synthetic versatility. In this sense, we have described the synthesis of some limonene aminoalcohols derivatives and their activity as acaricidal and antileishmanial [9,10]. More recently, we have reported the synthesis and the anti-*T. cruzi* activity of several limonene-amino derivatives [11,12]. Among the studied compounds, derivative **2** (Fig. (**1**)) showed trypanosomicidal activity in contrast to the inactive Limonene [12]. Besides, we identified some kinds of heterocycles (i.e. derivatives **3-6**, Fig. (**1**)) with excellent anti-*T. cruzi* [13] properties, with a suggested mode of action related to free-radical production [14].

Herein, we planned the generation of new anti-*T. cruzi* molecular hits hybridizing the limonene framework with bioactive heterocycles, i.e. benzofuroxan, benzimidazole dioxide, 5-nitrofuran, and 5-nitroindazole. We have selected the amine **2** as the nucleophilic entity to prepare the new designed compounds (Fig. (**1**)). Additionally, the biological effects of the new hybrid compounds were evaluated against *T. cruzi* Tulahuen 2 strain as the parasitic model. Due to the limonene-analogues possessing an alkenylamino group that could potentially modify the parasitic membrane sterols profile [15], we also analyzed the squalene and ergosterol level-changes produced by the hybrid derivatives into the parasite, using TLC and HPLC methodologies. Furthermore, being some antifungals active against *T. cruzi* [16], we have evaluated one of the most active anti-*T. cruzi* agent for its *in vitro* antifungal properties.

RESULTS AND DISCUSSION

In order to prepare the designed benzofuroxan **7**, we carried out the nucleophilic reaction between bromomethyl derivative (**I**) [17] and amine **2**, as diasteromeric mixture, in moderate yield (Scheme **1**). The benzofuroxan system is able to suffer opening-heterocycle-processes in the presence of nucleophiles, to produce *o*-nitroaniline-derivatives, consequently derivative **7** was obtained, in 27% of yield, from a

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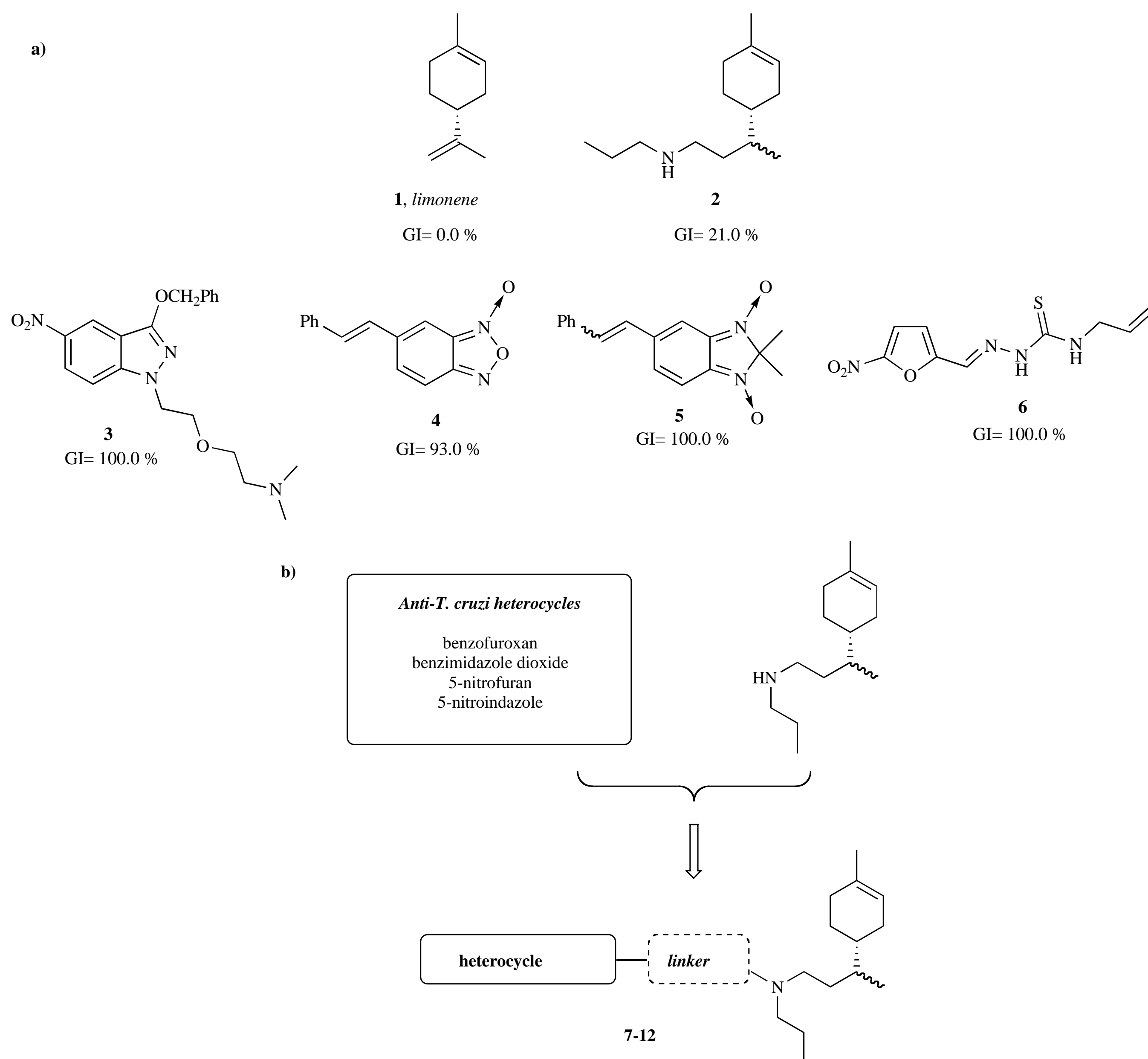
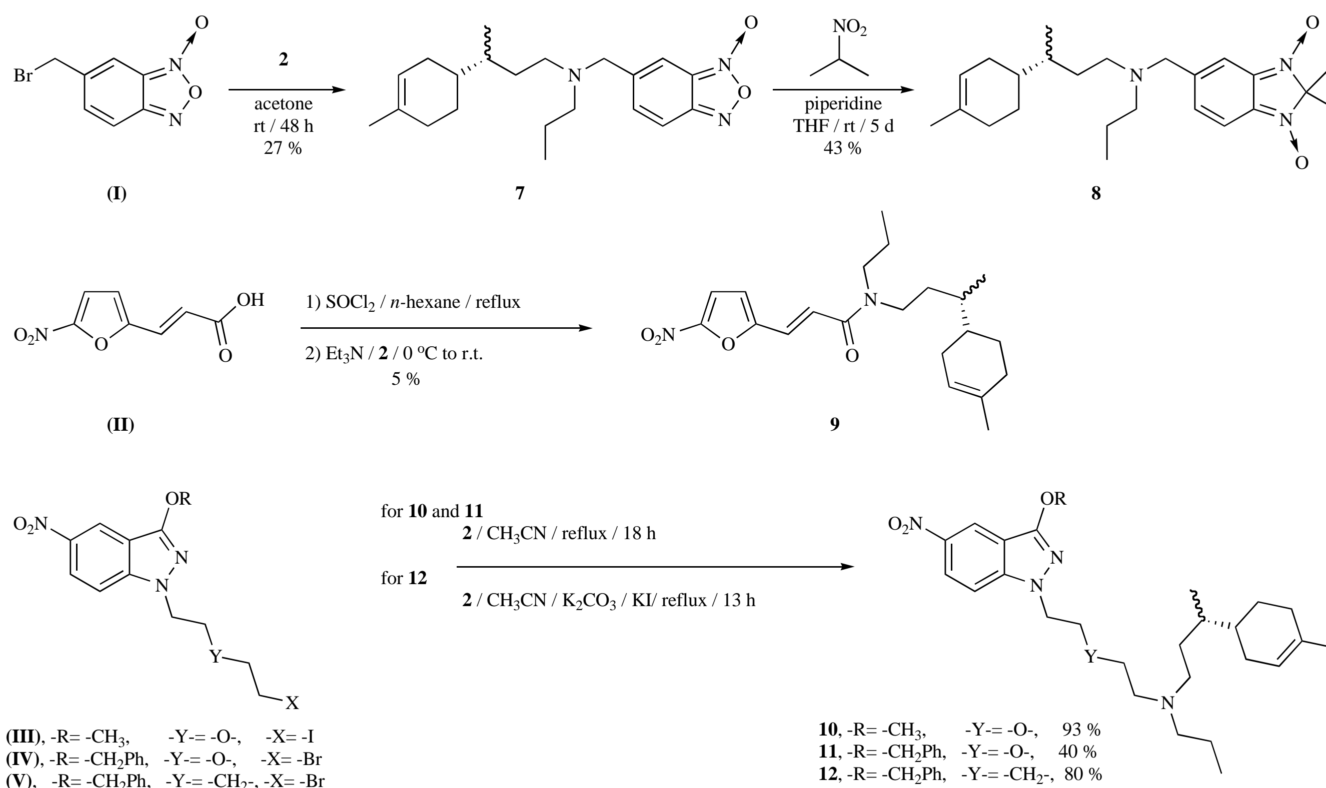


Fig. (1). a) Limonene (**1**), limonene derivative (**2**) and heterocycle derivatives previously evaluated as antitrypanosomal agents. GI means percentage of Tulahuen 2 epimastigotes *T. cruzi* growth inhibition at 25 μM. **b**) Design of new hybrid compounds using limonene framework.

complex mixture of products. The benzofuroxan **7** was converted into the benzimidazole dioxide **8** using 2-nitropropane in a Beirut-like reaction [18] (Scheme **1**). For the preparation of derivative **9**, an amidation process was used employing the acid **(II)**, as acyl chloride [15b], and the amine **2** (Scheme **1**). In the activation process, the corresponding acyl chloride results scantily stable that conduct to a very low yield of the desired product **9**. Attempts to improve the yield of this reaction, with mild activation-conditions (i.e. DCC/NHS), were unsuccessful due to the tedious exhaustive chromatographic process to eliminate the dicyclohexylurea achieving to similar yield (data not shown).

Three different 5-nitroindazole derivatives, **10-12**, were designed varying the stereo-characteristic of the substituent on C-3 and the stereoelectronic-characteristic of the lateral chain in 1-position. For this purpose, starting materials **(III)-(V)** [19] were submitted to a nucleophilic reaction with amine **2** generating the desired products in good to excellent yields (Scheme **1**).

All the compounds (**7-12**) were obtained, characterized and biologically evaluated as a non separable diasteromeric-mixture, because the diasteromers could not be isolated either by crystallization or by chromatographic methods using normal support phases. All the proposed structures for the new derivatives, **7-12**, were established by ^{1}H-, ^{13}C-NMR (HMQC, HMBC) spectroscopy and MS. The purity was analyzed and established by TLC and microanalysis, respectively.



Scheme 1. Synthetic procedures used to prepare derivatives **7-12**. The indicated yields correspond to products after purification processes.

To evaluate the anti-*T. cruzi* activity, derivatives **7-12** were 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 [20]. 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, terbinafine (Tbf) and ketoconazole (Ktz) were used as the reference trypanosomicidal agents. The hybrid *N*-oxide derivatives **7** and **8** were less active than the reference drugs and than the parent compounds **4** and **5**. However, the hybrid nitro-derivatives **9-12** displayed similar activities that Nfx and Bnz had and better than Tbf, Ktz and the parent compounds, 5-nitrofuran **6** and 5-nitroindazole **3**. These compounds could be considered as chemical starting points for further modifications to generate new anti-*T. cruzi* agents.

Due to the fact that some antifungal compounds are also active against *T. cruzi* [16], we decided to evaluate derivative **12** 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 Micologia (CEREMIC) from Facultad de Ciencias Bioquímicas y Farmacéuticas (Rosario, Argentine) or Control Lab from Río de Janeiro (Brazil) were used (Table **2**). Minimal inhibitory concentration (MIC) was determined by using broth microdilution techniques for yeasts [23] and filamentous fungi [24]. Tbf, Ktz, and amphotericin B (AnfB) were used as antifungal reference compounds and the parent compound **6** (Fig. (**1**)), with capability to accumulate squalene into *T. cruzi* [15b], was also included in the assay. Results showed that derivative **12** displayed moderate activities against *Candida albicans, Candida tropicalis,* and the dermatophytes *Microscoporum gypseum,*

Table 1. Biological Characterization of New Derivatives Against *T. cruzi* Tulahuen 2 strain.

Compd.	ID_{50}[a] (µM)
7	> 25.0 (38.0 %)[b]
8	20.0
9	5.6
10	7.7
11	5.5
12	6.3
3	16.5[c]
4	10.7[d]
5	3.4[e]
6	8.2[f]
Nfx	7.7
Bnz	7.4
Tbf	17.0
Ktz	10.0

[a]Results are the means of three independent experiments with a SD less than 10% in all cases. [b]Value in parenthesis correspond to percentages of *T. cruzi* growth inhibition respect to control, at 25 µM. [c]Against CL Brener clone. From reference [17]. [d]From reference [21]. [e]From reference [22]. [f]From reference [15b].

Table 2. Antifungal Activity of 12 Against Filamentous Fungi and Yeasts, Expressed as Minimal Inhibitory Concentration and Minimal Fungicidal Concentration

Compd.	MIC / MFC (µg/mL)[a,b]									
	Ca	Ct	Sc	Cn	Afu	Afl	Ani	Mg	Tr	Tm
12	62.5/62.5	62.5/62.5	15.6/15.6	15.6/15.6	>250	>250	>250	62.5/125	62.5/125	62.5/125
6[c]	> 250	> 250	> 250	> 250	>250	>250	>250	> 250	> 250	> 250
AnfB[d]	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* C 113, Tm: *Trichophyton mentagrophytes* ATCC 9972. [c]From reference [15b]. [d]AnfB: amphotericin B.

Trichophyton rubrum and *Trichophyton mentagrophytes* (MICs= 62.5 µg/mL). In contrast, it showed high activities (although lower than reference compounds) against *Saccharomyces cerevisiae,* and *Cryptococcus neoformans* (MICs= 15.6 µg/mL) turning **12** into a good molecular hit for the development of new antifungal agents. The high activity of compound **12** against *C. neoformans* is particularly interesting, since this fungus is an ubiquitous encapsulated yeast which produce morbidity and many times mortality in at-risk patients such as long-term glucocorticosteroid users, leukaemic patients solid organ transplant recipients and patients having AIDS [25]. Although there has been a decline in the AIDS-associated cryptococcal infections in the last five years [26], *C. neoformans* continues to be an important infection in immunocompromised patients, 50 % of which is nearly lethal.

In addition, **12** was fungicide rather than fungistatic at a low concentration (MFC = 15.6 µg/mL), which is a very appreciated attribute for an antifungal compound.

Since the steroids from the lipid fractions of *T. cruzi* epimastigotes have been previously described [27], this form was selected (Tulahuen 2 strain) to study the effects of the new derivatives on ergosterol biosynthesis. After a pre-established protocol ($1 \times ID_{50}$ per 8×10^6 cells/mL, 120 h of incubation) [15b], the controls (untreated, Tbf- and Nfx-treated) and active-derivative-treated parasites were collected and the total lipids were extracted and analyzed as previously described [15b,27]. Qualitative analyses of neutral lipid fractions were carried out using TLC. Then, quantitative analyses of ergosterol and squalene from sterol fractions were determined by using HPLC (Table **3**).

In the assayed experimental conditions, the limonene-hybrid derivatives were unable to accumulate squalene and deplete ergosterol like Tbf or the parent compound **6** (Table **3**). Fig. (**2**) shows the qualitative TLC-study of *T. cruzi* squalene accumulation obtained in different experimental conditions. The presence of an alkenylamine moiety in the studied compounds, like the anti-*T. cruzi* Tbf, does not assure the squalene-accumulation ability.

In terms of hybrid-compounds druglikeness capabilities, their addition to Lipinski's rule criteria was studied [30]. In this sense, we determined some properties, i.e. Log *P*, number of hydrogen bond donors and acceptors, and molecular weight (Table **4**) [31], which determine whether these derivatives are similar to the known drugs. In spite of the excellent *in vitro* activities, derivatives **11** and **12** do not adscribe to the Lipinski's rule (two violations) consequently some provision could be taken when they will evaluate *in vivo*. Furthermore, all derivatives have adequate topological polar surface areas (TPSA [32], Table **4**) turning some of them into promising candidates for further drug development.

Table 3. Squalene and Ergosterol Composition of *T. cruzi* Epimastigotes Treated with Compounds 8-12, 6, Nfx and Tbf after 120 h

Compd.	$C_{squalene}$ (µg/mL)	$C_{ergosterol}$ (µg/mL)	Ratio[a]
8	< 0.6[b]	ns[c]	-
9	tlcd	ns	-
10	0.6[b] - 2.1[d,e]	ns	-
11	< 0.6	ns	-
12	< 0.6	ns	-
6[f]	15.9 ± 0.9	5.5 ± 1.9	2.9 ± 1.0
Nfx[f]	0.6 - 2.1	ns	-
Tbf[f]	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, in HPLC studies. [c]ns: not studied. [d]tlcd= detected by TLC. [e]Limit of quantification 2.1 µg/mL, in HPLC studies. [f]From reference [15b].

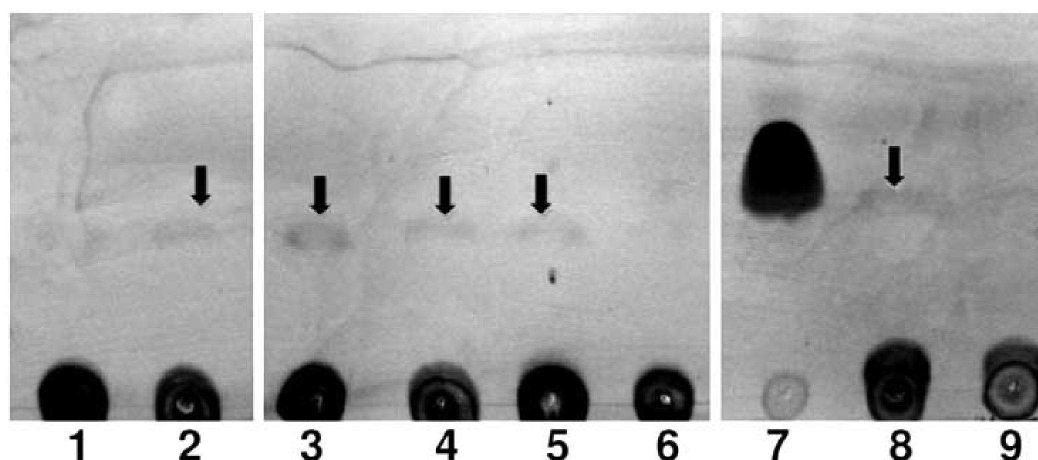


Fig. (2). Effects on squalene accumulation of limonene-hybrid derivatives. *T. cruzi* was treated according to reference [15b]; then lipids were extracted and TLC was performed (for details see Experimental Section). Lanes: 1- DMSO-treated parasite; 2- **9** ($1 \times ID_{50}$ per 8×10^6 cells/mL); 3- Tbf ($2 \times ID_{50}$ per 8×10^6 cells/mL); 4- Tbf ($1 \times ID_{50}$ per 8×10^6 cells/mL); 5- **6** ($2 \times ID_{50}$ per 8×10^6 cells/mL); 6- untreated parasite; 7- squalene; 8- **6** ($1 \times ID_{50}$ per 8×10^6 cells/mL); 9- Nfx ($1 \times ID_{50}$ per 8×10^6 cells/mL).

Table 4. Estimated Properties of Studied Compounds[a]

Compd.	miLog*P*	nON	nOHNH	MW	nviolations	TPSA (Å^2)
rule	≤ 5.00	< 10	< 5	< 500.00	≤ 1	-
1	3.62	0	0	136.24	0	0.00
2	3.75	1	1	209.38	0	12.03
3	3.42	8	0	384.44	0	85.36
4	4.39	4	0	238.25	0	51.49
5	3.92	4	0	280.33	0	57.51
6	2.24	7	2	254.27	0	95.38
7	5.64	5	0	357.50	1	54.73
8	5.17	5	0	399.58	1	60.74
9	5.18	6	0	374.48	1	79.27
10	5.83	8	0	472.63	1	85.36
11	7.42	8	0	548.73	2	85.36
12	8.40	7	0	546.76	2	76.12
Nfx	0.71	8	0	287.30	0	108.71
Tbf	5.72	1	0	291.44	1	3.23

[a]miLog*P*, logarithm of compound partition coefficient between *n*-octanol and water; nON, number of hydrogen bond acceptors; nOHNH, number of hydrogen bond donors; MW, molecular weight; TPSA, topological polar surface area.

EXPERIMENTAL

All starting materials were commercially available research-grade chemicals and were used without further purification. All solvents were dried and distilled prior to use. All the reactions were carried out in a nitrogen atmosphere. Reactants **2**, **(I)**-**(V)** were prepared following synthetic procedures previously reported [11,17,19]. Amine **2** was used as diasteromeric mixture [11]. Proton and carbon NMR spectra were recorded on a Bruker DPX-400 spectrometer in $CDCl_3$ at 400 and 100 MHz, respectively. For derivative **7** in ^{13}C NMR only narrow peaks were reported. The chemical shifts values, δ, 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 and 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-{*N*-[3-(4-Methyl-3-cyclohexenyl)butyl]-*N*-propylamino}methylbenzofuroxan (7)

A mixture of **13** (108 mg, 0.5 mmol) and **2** (105 mg, 0.5 mmol) in acetone (50.0 mL) was stirred at room temperature

for 48 h. After that, the solvent was evaporated *in vacuo* and the residue was fractioned by column chromatography (SiO_2, petroleum ether:ethyl acetate, 8:2), yielding the desired product as a yellow oil (48 mg, 27%).

IR: 801, 1015, 1075, 1260, 1377, 1456, 1489, 1536, 1595, 1622, 2926. ^{1}H-NMR: 0.81/0.83 (3H, d, J = 6.0 Hz), 0.89 (3H, t, J =7.2 Hz), 1.27-1.95 (15H, m), 2.41-2.47 (4H, bs), 3.54 (2H, m), 5.37 (1H, bs), 7.36 (3H, bs). ^{13}C-NMR: 12.2, 16.4/16.8, 20.7, 23.8, 27.9, 29.8, 31.2/31.3, 31.5/31.8, 35.4/35.7, 38.7/38.9, 52.7, 56.5, 59.0, 121.2/121.3, 134.4. MS *m/z* (%): 357 ($M^{+\cdot}$, 4), 340 ($M^{+\cdot}$-17, 11), 328 (11), 260 (23), 312 (7), 244 (46), 220 (68), 204 (100), 176 (29), 160 (45), 149 (51), 89 (46).

Anal. Calcd. For $C_{21}H_{31}N_3O_2$. C(70.6), H(8.7), N(11.8). Found C(70.4), H(8.3), N(11.7).

2,2-Dimethyl-5-{*N*-[3-(4-methyl-3-ciclohexenyl)butyl]-*N*-propylamino}methyl-2*H*-benzimidazole 1,3-dioxide (8)

A mixture of **7** (179 mg, 0.5 mmol), 2-nitropropane (0.05 mL, 0.5 mmol), and piperidine (0.05 mL, 0.5 mmol) in THF (20.0 mL) was stirred at room temperature for 5 days. After that, the solvent was evaporated *in vacuo* and the residue was fractioned by column chromatography (SiO_2, ethyl acetate), yielding the desired product as red oil (86 mg, 43%).

IR: 799, 1098, 1181, 1237, 1372, 1453, 1559, 1622, 2930. ^{1}H-NMR: 0.82/0.84 (3H, d, J = 4.8 Hz), 0.88 (3H, t, J = 7.2 Hz), 1.17 (3H, s), 1.27-1.54 (8H, m), 1.72 (6H, s), 1.94-2.46 (8H, m), 3.36 (2H, m), 5.37 (1H, bs), 7.00 (1H, d, J = 9.4 Hz), 7.12 (1H, s), 7.18 (1H, d, J = 9.4 Hz). ^{13}C-NMR: 12.3, 16.4/16.8, 20.7, 23.8, 24.6, 25.8/27.5, 27.9/29.8, 31.3/31.4, 31.6/31.8, 35.5/35.7, 38.7/39.0, 52.7, 56.6, 58.9, 97.6, 113.6, 115.5, 121.3/121.4, 133.6, 134.4, 136.7, 136.8, 145.7. MS *m/z* (%): 399 ($M^{+\cdot}$, 2), 382 (15), 366 (4), 286 (18), 246 (43), 208 (83), 191 (20), 175 (100), 159 (24), 144 (52), 137 (34), 112 (21), 72 (22).

Anal. Calcd. For $C_{24}H_{37}N_3O_2$. C(72.1), H(9.3), N(10.5). Found C(71.9), H(9.0), N(10.1).

(E)-*N*-[3-(4-Methyl-3-ciclohexenyl)butyl]-3-(5-nitrofuran-2-yl)-*N*-propylpropenamide (9)

A mixture of **(II)** (1 mmol), and $SOCl_2$ (2 mmol) in dry hexane (20.0 mL) was heated at reflux for 1.5 h, under nitrogen atmosphere. After that, the reaction was cooled at 0 ºC, and Et_3N (2.5 mmol) and **2** (1.2 mmol) were added. The mixture was stirred at room temperature for 1.5 h. The mixture was fractioned between ethyl acetate:aqueous $NaHCO_3$ (5 %), the organic layer was washed with aqueous HCl (1M) and dried with anhydrous Na_2SO_4. The solvent was evaporated *in vacuo* and the residue was fractioned by column chromatography (Al_2O_3, petroleum ether:ethyl acetate, 0 to 60 %), yielding the desired product as brown oil (5%).

^{1}H-NMR: 0.95 (3H, m), 1.00 (3H, m), 1.65-196 (8H, m), 1.97 (3H, bs), 2.00 (4H, bs), 3.42 (4H, m) 5.38 (1H, bs), 6.70 (1H, two d, J = 3.8 Hz), 7.08 (1H, two d, J = 15.4 Hz), 7.36 (1H, two d, J = 3.8 Hz), 7.45 (1H, two d, J = 15.4 Hz); ^{13}C-RMN (from the HSQC and HMBC experiments): 11.0, 16.0, 24.0, 25.0, 29.0, 29.5, 31.0, 31.5, 38.0, 38.5, 46.0, 49.0, 113.0, 114.0, 121.0, 122.0, 126.0, 134.0, 153.0, 153.5, 164.0; MS *m/z* (%): 374 ($M^{+\cdot}$, 1.5), 206 ($M^{+\cdot}$ – $C_7H_4NO_4$ – H_2, 13), 166 ($M^{+\cdot}$ - $C_{14}H_{26}N$, 67).

Anal. Calcd. For $C_{21}H_{30}N_2O_4$. C(67.4), H(8.1), N(7.5). Found C(67.6), H(7.9), N(7.2).

General Procedure for the Synthesis of Derivatives 10 and 11

A mixture of the indazole reactant (**(III)** or **(IV)**, 1.0 equiv.), **2** (1.0 equiv.), in acetonitrile (15.0 mL/0.1 mmol) was heated at reflux for 18 h. The solvent was evaporated *in vacuo* and the residue was fractioned by column chromatography (SiO_2, ethyl acetate).

3-(4-Methyl-3-ciclohexenyl)-N-[5-(3-methoxy-5-nitro-1H-indazole-1-yl)-3-oxapentyl]-N-propylbutaneamine (10)

Yellow oil (93%). IR: 806, 1142, 1208, 1331, 1487, 1522, 1549, 1617, 2926. ^{1}H-NMR: 0.82 (6H, m), 1.17-1.94 (15H, m), 2.32-2.52 (6H, m), 3.45 (2H, t, J = 6.2 Hz), 3.86 (2H, t, J = 5.2 Hz), 4.12 (3H, s), 4.38 (2H, t, J = 5.2 Hz), 5.38 (1H, bs), 7.36 (1H, d, J = 9.2 Hz), 8.21 (1H, dd, J = 2.0, 9.2 Hz), 8.63 (1H, d, J = 2.0 Hz). ^{13}C-NMR: 12.2, 16.4/16.8, 20.5, 23.8, 27.4/28.0, 29.7, 31.2/31.3, 31.4/31.6, 35.8/35.9, 38.8/39.1, 49.8, 53.5, 53.9, 56.9, 57.1, 69.9, 70.5, 109.9, 112.3, 118.8, 121.3/121.4, 122.7, 134.4, 141.3, 144.0, 158.8. MS *m/z* (%): 472 ($M^{+\cdot}$, 14), 375 (14), 222 (100), 86 (70).

Anal. Calcd. For $C_{26}H_{40}N_4O_4$. C(66.1), H(8.5), N(11.9). Found C(65.8), H(8.8), N(11.6).

N-[5-(3-Benzyloxy-5-nitro-1*H*-indazole-1-yl)-3-oxapentyl]-3-(4-methyl-3-ciclohexenyl)-*N*-propylbutaneamine (11)

Yellow oil (40%). IR: 700, 739, 1142, 1331, 1451, 1485, 1539, 1615. ^{1}H-NMR: 0.82 (3H, d, J = 6.4 Hz), 0.83 (3H, t, J = 7.4 Hz), 1.37 (6H, m), 1.65 (3H, s), 1.96 (6H, m), 2.50 (6H, m), 3.44 (2H, t, J = 6.2 Hz), 3.87 (2H, t, J = 5.2 Hz), 4.40 (2H, t, J = 5.2 Hz), 5.38 (1H, bs), 5.46 (2H, s), 7.41 (6H, m), 8.22 (1H, dd, J = 2.0, 9.2 Hz), 8.67 (1H, d, J = 2.0 Hz). ^{13}C-NMR: 12.2, 16.4/16.8, 20.6, 23.8, 27.4/28.0, 31.3/31.4, 31.5/31.7, 35.8/35.9, 38.9, 39.1, 49.8, 53.5, 53.9, 57.2, 69.9, 70.6, 71.5, 109.9, 112.5, 118.8, 121.3/121.4, 122.7, 128.5, 128.7, 129.0, 134.4, 136.7, 141.3, 144.0, 158.0. MS *m/z* (%): 548 ($M^{+\cdot}$, 10), 451 (11), 411 (22), 222 (100), 91 (32), 86 (41).

Anal. Calcd. For $C_{32}H_{44}N_4O_4$. C(70.0), H(8.1), N(10.2). Found C(69.8), H(7.9), N(9.9).

N-[5-(3-Benzyloxy-5-nitro-1*H*-indazole-1-yl)pentyl]-3-(4-methyl-3-ciclohexenyl)-*N*-propylbutaneamine (12)

A mixture of **(V)** (100 mg, 0.2 mmol), **2** (50 mg, 0.2 mmol), K_2CO_3 (33 mg, 0.2 mmol) and KI (catalytic amount) in acetonitrile (30.0 mL) was heated at reflux for 13 h. After that, the solvent was evaporated *in vacuo* and the residue was treated with water (50.0 mL) and extracted with CH_2Cl_2 (3 × 20.0 mL). The organic layer was dried with anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue was fractioned by column chromatography (SiO_2, petroleum ether:ethyl acetate, 8:2), yielding the desired product as yellow oil (104 mg, 80%).

^{1}H-NMR: 0.85 (3H, d, J = 6.4 Hz), 0.89 (3H, t, J = 7.4 Hz), 1.26-1.48 (10H, m), 1.65 (3H, s), 1.70 (2H, m), 1.95 (6H, m), 2.42 (6H, m), 4.23 (2H, t, J = 6.8 Hz), 5.38 (1H, bs), 5.46 (2H, s), 7.26 (1H, d, J = 9.2 Hz), 7.42 (3H, m), 7.54 (2H, d, J = 6.8 Hz), 8.23 (1H, dd, J = 2.0, 9.2 Hz), 8.69 (1H, d, J = 2.0 Hz). ^{13}C-NMR: 12.3, 16.4/16.8, 20.3, 23.8, 25.2, 25.9, 26.9, 28.0, 29.8, 29.9, 31.2/31.4, 35.9/36.1, 38.9/39.1, 49.3, 52.7, 54.2, 56.4, 71.5, 108.9, 112.3, 119.1, 121.3/121.4, 122.8, 128.5, 128.7, 128.9, 134.4, 136.7, 141.2, 143.0, 157.9. MS m/z (%): 546 ($M^{+\cdot}$, 20), 517 (19), 449 (53), 409 (100), 222 (42), 91 (89).

Anal. Calcd. For $C_{33}H_{46}N_4O_3$. C(72.5), H(8.5), N(10.3). Found C(72.7), H(8.6), N(10.0).

Anti-*T. cruzi In Vitro* Test Using Epimastigotes of Tulahuen 2 Strain

Trypanosoma cruzi epimastigotes, Tulahuen 2 strain, were grown at 28 ºC in an axenic medium (BHI-Tryptose) as previously described [17-19, 20-21], supplemented with 5% fetal bovine serum. 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 was never exceeded from 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 was calculated as follows: GI (%) = $\{1 - [(Ap - A0p)/(Ac - A0c)]\} \times 100$, 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 compared to the control. The ID_{50} value was taken as the concentration of drug needed to reduce the absorbance ratio to 50%.

Antifungal Susceptibility Testing

Microorganisms and Media

For the antifungal evaluation, strains from the American Type Culture Collection (ATCC), Rockville, MD, USA and CEREMIC (C), Centro de Referencia en Micología, Facultad de Ciencias Bioquímicas y Farmacéuticas, Suipacha 531-(2000)-Rosario (Argentine) were used: *Candida albicans* ATCC 10231, *Candida tropicalis* C 131, *Saccharomyces cerevisiae* ATCC 9763, *Cryptococcus neoformans* ATCC 32264, A*spergillus flavus* ATCC 9170, *A. fumigatus* ATTC 26934, *A. niger* ATCC 9029, *Trichophyton 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 ^{o}C, maintained on slopes of Sabouraud-dextrose agar (SDA, Oxoid) and were subcultured every 15 days to prevent pleomorphic transformations. Inocula of cells or spore suspensions were obtained according to reported procedures and adjusted to 10^3 cells/spores with colony forming units (CFU)/mL [33].

Antifungal Test

Minimal Inhibitory Concentrations (MIC) of each 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 and Laboratory Standards [33] 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. Microtiter trays were incubated at 35 ^{o}C for yeasts and *Aspergillus* spp. and at 28-30 ^{o}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 solution of each compound was two-fold diluted with RPMI 1640 from 250-0.98 μg/mL (final volume = 100 μL) and a final DMSO concentration ≤ 1%. A volume of 100 μL of inoculum suspension was added to each well, with the exception of the sterility control where sterile water was added to the well instead. 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.

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 ^{o}C with strong aeration. The studied compound was added at a concentration equivalent to 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 3,000 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 3,000 rpm during 15 min. The pellets were treated with chloroform:methanol (2:1) during 12 h at 4 ^{o}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 system's petroleum ether:ethyl acetate or petroleum ether (to see squalene) as eluents. The chromatograms were obtained by vaporizing the plates with iodine and heating them at 100 ^{o}C. Quantitative analyses of squalene from sterol fractions were 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% acetonitrile 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 were done by using the same column and equipment measuring at 250 nm. The mobile phase consisted of acetonitrile: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.

Compounds Properties Calculation

Polar surface area (TPSA), miLog*P*, and violations of Lipinski's rule of five, were calculated using Molinspiration online property calculation toolkit [31].

CONCLUDING REMARKS

New limonene-framework containing heterocycles with anti-*T. cruzi* activities were identified. Especially the hybrids 5-nitrofuran **9** and the 5-nitroindazoles **10-12** showed excellent trypanosomicidal profile with higher anti-*T. cruzi* activity than the parent compounds, **3** and **6**. On the other hand, derivative **12** was identified as a good molecular hit for its antifungal activity. The mechanism of anti-*T. cruzi* action of **9-12** could be related to the recognized oxidative stress production capability of these nitro-compounds [28,29]. The limonene substructure could modify the lipophilicity of these heterocycles, promoting adequate parasite penetration (Table **4**), more than forward a mechanism related to membrane-sterol modification as a result of the presence of the alkenylamino moiety.

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