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Original article

Naftifine-analogues as anti-*Trypanosoma cruzi* agents

Alejandra Gerpe [a], Lucía Boiani [a], Paola Hernández [a], Maximiliano Sortino [b], Susana Zacchino [b], Mercedes González [a,*], Hugo Cerecetto [a,*]

[a] *Departamento de Química Orgánica, Facultad de Ciencias-Facultad de Química, Universidad de la República, Iguá 4225, 11400 Montevideo, Uruguay*
[b] *Cátedra de Farmacognosia, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina*

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ABSTRACT

Chagas disease represents a relevant health problem in Central and South America. The first line of treatment is Nifurtimox and Benznidazole which have a great deal of disadvantages that demands the rapid generation of therapeutic alternatives. Based in our research on aza-thiaheterocycles as anti-*Trypanosoma cruzi* agents we identified pharmacophores that act through oxidative stress. Here, we describe the synthesis and the activity of new containing bioactive-heterocycles analogues of naftifine as potential *T. cruzi* membrane sterol biosynthesis inhibitors. Benzimidazole 1,3-dioxides (**11** and **13**) and quinoxaline 1,4-dioxides (**22** and **23**) displayed excellent parasite/mammal selectivity indexes. Analysis of the free sterols from parasite incubated with the compounds showed that any of them are able to accumulate squalene suggesting that in the anti-*T. cruzi* mechanism of action is not involved the inhibition of sterol biosynthesis. Some derivatives were also tested as antifungal agents. The results obtained in the present work open potential therapeutic possibilities of new compounds for these infectious diseases.

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1. Introduction

Chagas disease, or American Trypanosomiasis, remains the major parasitic disease burden in Central and South America, despite recent advances in the control of its vectorial and transfusional transmission [1,2]. Current treatments are based on old and quite unspecific drugs, Nifurtimox (Nfx) and Benznidazole (Bnz) associated with long-term treatments that may give rise to severe side effects [3]. 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 *Trypanosoma cruzi* strains and have significantly low efficacy in long-term chronic infections. Even though the complete genome of *T. cruzi* (*T. cruzi*) CL Brener clone [4] has been sequenced, not new chemotherapeutic agents emerge [5]. However, several metabolic steps essential for parasite survival and for potential use as chemotherapeutic targets have resulted [6,7]. Regarding to the validated metabolic steps used to generate new chemotherapeutic alternatives, membrane sterol biosynthesis is one of the most promising targets [8].

T. cruzi like most pathogenic fungi 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 [9,10]. The *T. cruzi* ergosterol biosynthesis pathway has been chemically validated as a chemotherapeutic target at several steps [11,12], 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 [9,10]. 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 [13,14]. 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 [15,16]. Specifically, the antifungals terbinafine and naftifine (Tbf and Ntf, respectively, Fig. 1) [17–20] proved to be promising anti-*T. cruzi* agents. Recently our group have described a series of 5-nitrofuranes and 5-nitrothiophenes able to accumulate squalene, and potentially inhibit SE, in *T. cruzi* parasite [21,22]. These compounds (**1** and **2**, Fig. 1) have an additional mechanism of action, they are able to produce oxidative stress and consequently they are dual agents. In these sense, we have been working in the research and developing of different oxidative stress producer-heterocycles searching for new anti-*T. cruzi* pharmacophores [23]. Interesting molecular hits were found from its *in vitro* and *in vivo* behaviour (**3**–**7**, Fig. 1). To explore new anti-*T. cruzi* chemical entities we designed hybrid

* Corresponding authors. Tel.: +598 2 5258618; fax: +598 2 5250749.
E-mail addresses: megonzal@fq.edu.uy (M. González), hcerecet@fq.edu.uy (H. Cerecetto).

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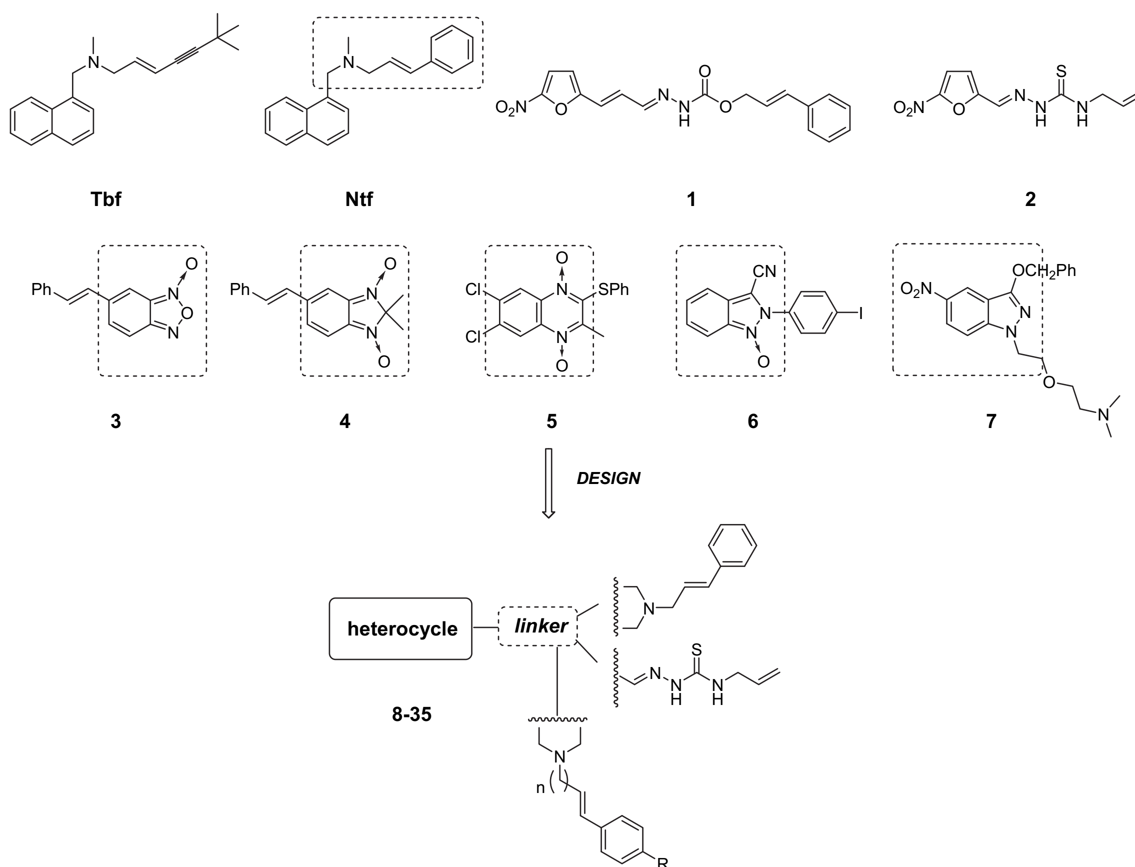

Fig. 1. Anti-*T. cruzi* agents, squalene accumulator-nitrofuranes and design of the hybrid new generation of compounds.

compounds containing bioactive-heterocycles and alkenylamino framework. The rationality was that the bioactive-heterocycles could provide free-radical releaser moities and the alkenylamino the SE-inhibitor pharmacophores. The synthetic efforts were focus on five different heterocycles and three different alkenylamino moieties. Four different *N*-oxides were used, benzofuroxan, benzimidazole 1,3-dioxide, quinoxaline 1,4-dioxide, and indazole *N*-oxide and one nitro-containing system, 5-nitroindazole. The used alkenylamino systems were 3-phenyl-2-propenylamino, framework present in Ntf, N^4-allylthiosemicarbazonyl, moiety contained in parent compound **2**, and homologous of allylamino moiety. Additionally, the biological effects of all the new compounds was analyzed using *T. cruzi* Tulahuen 2 strain as parasitic model, and for three 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 hybrid compounds into the parasite were also studied using HPLC methodology. Furthermore, being some antifungals active against *T. cruzi* [15,16], we proposed to evaluate the designed anti-*T. cruzi* agents for its *in vitro* antifungal properties.

2. Methods and results

2.1. Chemistry

As a first synthetic approach benzofuroxan derivatives **8**–**10** (Scheme 1) were prepared by simple and efficient procedures, using bromide **I** [24] or aldehyde **II** [25] and *N*-methylcinnamylamine (**III**) or commercially available (*E*)-4-(3-phenyl-2-propenyl)piperazine and N^4-allylthiosemicarbazide as starting materials. These compounds were transformed into benzimidazole 1,3-dioxide **11**–**13** (Scheme 1) by reaction with 2-nitropropane in basic milieu [26,27] or into quinoxaline 1,4-dioxide **14**–**23** (Scheme 1) in Beirut conditions [28]. Quinoxaline **24** was obtained from aldehyde **IV** [29]. Quinoxaline 1,4-dioxides **14**–**23** were isolated as a mixture of 6- and 7-isomer. For this family 2- and 3-positions were varied with moieties (–R, –CN, $-NH_2$, $-CO_2R$, –CONHR) with different stereo-electronic properties. The indazole N^1-oxides **25**–**27** (Scheme 1) were prepared by reaction of allyl- or homoallylamines **V**–**VII** [30] and *o*-nitrobenzaldehyde in presence of KCN and basic milieu [31]. The 5-nitroindazoles **28**–**34** (Scheme 1) were prepared by nucleophilic substitution using halides **VIII**–**X** [32] and **III**, 4-(3-phenyl-2-propenyl)piperazine or (*E*/*Z*)-4-pentenylamine (**XI**, [30]). Being **XI** a primary amine, product **35** (Scheme 1) was also obtained in this reaction as result of a di-substitution process.

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, **8**–**35**, 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 [33,34]. The compounds were incorporated into the media at 25 μM and their

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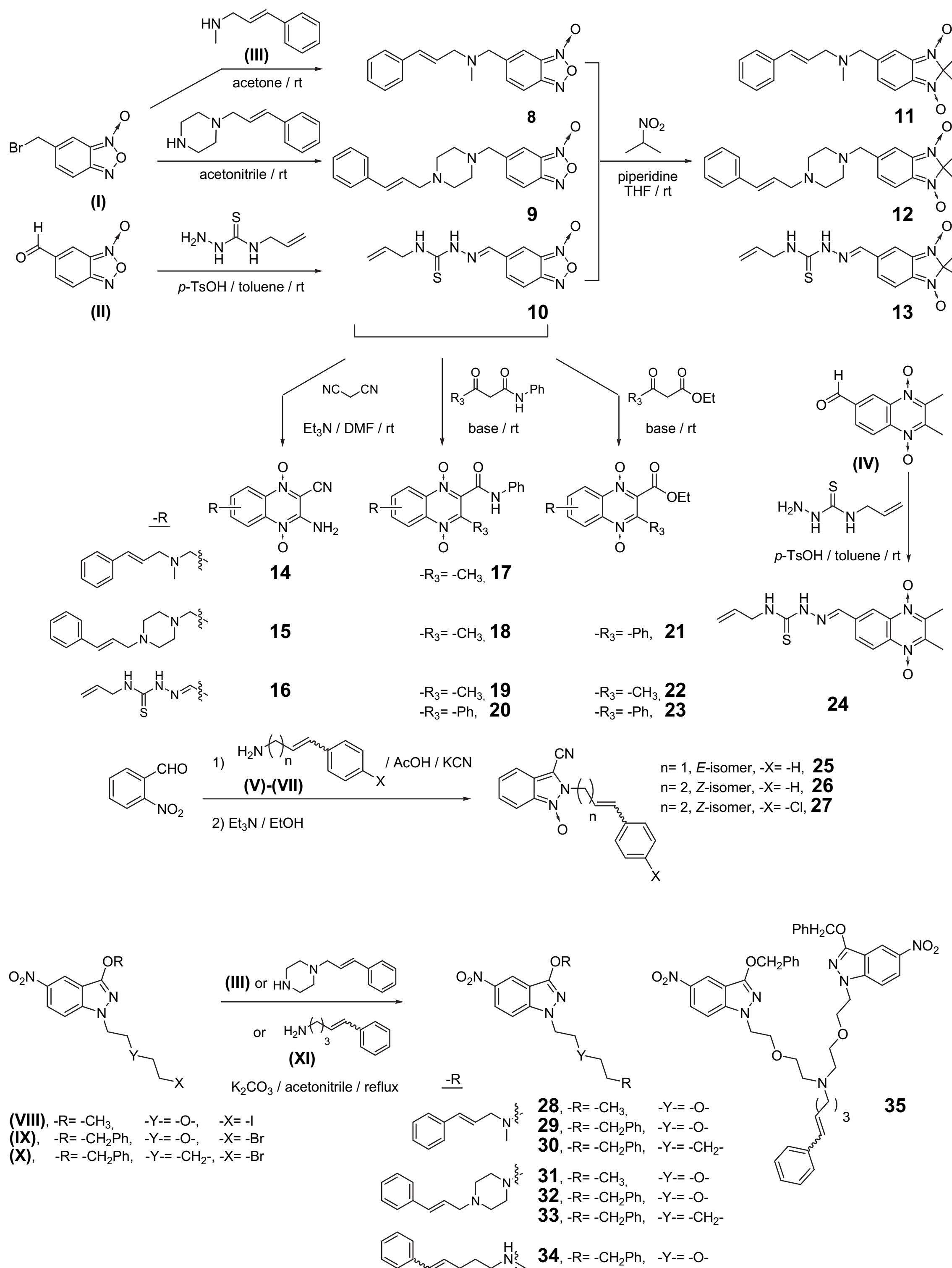

Scheme 1. Synthetic procedures used to prepare the Naftifine-analogues.

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. Some derivatives were also studied against the high virulent [35,36] CL Brener clone (Table 1).

5-Nitroindazoles **31** and **34** were the most active anti-*T. cruzi* (Tulahuen 2 strain) derivatives, with $ID_{50} = 3.8$ and 4.5 μM, near to 2 and 1.5 times more active than the reference drugs Nfx and Bnz. While the benzimidazole 1,3-dioxide **13**, the quinoxaline 1,4-dioxides **21**–**23**, and the 5-nitroindazoles **29**, **30**, **32**, and **33** displayed ID_{50} values between 6.5 and 12.0 μM, which also resulted as active as the references (Nfx, Bnz, Tbf, and Ktz) (Table 1). No

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Table 1
Biological Characterization of Naftifine-analogues Against *T. cruzi.*

Compd.	ID50[a](μM)	Compd.	ID50[a](μM)	Compd.	ID50[a](μM)
Activity against Tulahuen 2 strain					
8	22.6	**14**	(0.0)[b]	**25**	(28.0)
9	(14.0)	**15**	(0.0)	**26**	(30.0)
10	(33.0)	**16**	(6.0)	**27**	(20.0)
11	22.7	**17**	(34.0)	**28**	23.1
12	25.0	**18**	25.4	**29**	10.0
13	7.2	**19**	(0.0)	**30**	7.8
		20	(18.0)	**31**	3.8
Nfx	7.7[c]	**21**	9.5	**32**	6.5
Bnz	7.4[c]	**22**	12.0	**33**	10.6
Tbf	17.0[c]	**23**	7.5	**34**	4.5
Ktz	10.0[c]	**24**	(21.0)	**35**	(0.0)
Activity against CL Brener clone					
8	16.9	**Nfx**	8.5[c]	**Tbf**	42.0[c]
31	7.9	**Bnz**	4.5[c]	**Ktz**	5.0[c]
33	4.5				

[a] The results are the means of three independent experiments with an SD less than 10% in all cases.
[b] Values in parenthesis correspond to percentage of *T. cruzi* growth inhibition at 25.0 μM.
[c] From [37].

susceptibility differences between both strains, with the studied compounds, were observed.

2.2.2. *In vitro unspecific cytotoxicity*

Mammal cytotoxicity of selected new compounds 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* (25.0 μM) (Table 2) [37]. These derivatives were selected regarding its *in vitro* anti-*T. cruzi* activities and its widespread structural motives at the heterocycle level (Scheme 1). The selectivity indexes, SI, were expressed as the ratio between ID_{50} in macrophages and ID_{50} in *T. cruzi* (Tulahuen 2 strain). The benzimidazole 1,3-dioxide **11**, and the quinoxalines 1,4-dioxides **22** and **23** have similar or better SI values than the biosynthesis-membrane-sterol inhibitors (Tbf and Ktz), displaying **22** at least 1.3-fold the selectivity of Nfx (Table 2). Clear structural exigencies could be observed regarding the toxic effects being all the studied 5-nitroindazoles toxics against this mammal system, independently of the kind of alkenylamino appendage (see toxicity of **29**, **32** and **34**). For the same alkenylamino moiety some considerations could be extracted, the benzimidazole 1,3-dioxide is preferable to the benzofuroxan system (compare toxicity of **8** and **11**), and ester containing quinoxaline 1,4-dioxide is preferable to the benzimidazole 1,3-dioxide system (compare toxicity of **13** and **22** and **23**).

Table 2
Biological Characterization of New Derivatives Against Mammal Macrophages.

Compd.	ID_{50}[a] (μM) J-774 macrophages	SI[b]		ID_{50}[a] (μM) J-774 macrophages	SI[b]
8	<100.0	<4.4	**28**	<100.0	<4.3
11	>400.0	>17.6	**29**	<100.0	<10.0
12	109.0	4.4	**30**	<100.0	<12.8
13	104.0	14.4	**31**	<100.0	<26.3
21	<100.0	<10.5	**32**	<100.0	<15.4
22	>400.0	>53.3	**33**	<100.0	<9.4
23	>400.0	>33.3	**34**	<100.0	<22.2
Nfx	316.0	41.0	**Ktz**	<100.0	<10.0
			Tbf	339.0	19.9

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

2.2.3. *In vitro antifungal activity [38,39]*

Due to some antifungals are actives against *T. cruzi* [15,16] 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 en Micología (CEREMIC) from Facultad de Ciencias Bioquímicas y Farmacéuticas (Rosario, 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 [40] and filamentous fungi [41]. For this study we selected some relevant derivatives with structural modifications at the heterocycle level. As antifungal reference compounds Tbf, Ktz, and amphotericin B (AnfB) were used. Although the studied compounds were less active than the reference compounds the indazole derivatives, i.e. **25**, **31**, and **33**, displayed moderate activities mainly against the dermatophytes *Microsporum gypseum*, *Trichophyton rubrum* and *Trichophyton mentagrophytes* that transform them in molecular hits. Derivative **33** showed the best activity against *Cryptococcus neoformans* with MIC = 31.2 μg/mL, an interesting result considering that *C. neoformans* continues to be an important infection in immunocompromised patients, for which nearly 50% is lethal [42].

2.3. *Squalene accumulation analysis*

Since the steroids from the lipid fractions of *T. cruzi* epimastigotes have been previously described [43], 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) [22,44], the controls (untreated, Tbf- and Nfx-treated) and derivative-treated parasites were collected and the total lipids were extracted and analyzed as described previously [12,22,43,45,46]. Quantitative analyses of squalene (for untreated parasite and Tbf) from sterol fractions were done by HPLC. None of the studied compounds were able to accumulate squalene (see Table S1 in Supplementary Content section). These results demonstrate that the presence, in the developed derivatives, of the selected alkenylamino moieties is not enough to guarantee *T. cruzi* sterol biosynthesis inhibition.

3. Discussion

We report the synthesis of twenty-eight new hybrid compounds and their activity against *T. cruzi*, macrophages and a wide panel of fungi. These compounds were designed combining the alkenylamino moieties present in Ntf and compound **2** and a bioactive heterocycle (benzofuroxan, benzimidazole 1,3-dioxide, quinoxaline 1,4-dioxide, indazole *N*-oxide, and 5-nitroindazole).

The most interesting derivatives, against *T. cruzi* (Tulahuen 2 strain and CL Brener clone) and fungi (*C. neoformans*, *M. gypseum*, *T. rubrum*, and *T. mentagrophytes*), were the 5-nitroindazole derivatives **31** and **33** (Table 1 and Table 3). However, they were, like Ktz, toxic against macrophages at the assayed doses (Table 2). According to the selectivity indexes benzimidazole 1,3-dioxides **11** and **13** and quinoxaline 1,4-dioxides **22** and **23** could be considered for further biological studies. Specially, derivative **22** showed better SI than Nfx that convert it in a lead compound.

It is clear that the mechanisms of anti-*T. cruzi* or the modest antifungal activity are not related to the squalene accumulation. The structural modifications from the parent compounds, Ntf and **2**,

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Table 3
Antifungal Activity of Naftifine-analogues Against Filamentous Fungi and Yeasts, Expressed as Minimum Inhibitory Concentration (MIC)/Minimum Fungicidal Concentration (MFC).

Compd.	MIC/MFC (μg/mL)[a,b]									
	Ca	Ct	Sc	Cn	Afu	Afl	Ani	Mg	Tr	Tm
10	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
13	>250	>250	>250	>250	>250	>250	>250	250/250	250/250	250/250
16	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
18	>250	>250	>250	>250	>250	>250	>250	125/>250	125/>250	125/>250
19	>250	>250	>250	250/>250	>250	>250	>250	250/250	250/250	250/250
21	250/>250	250/>250	125/250	125/250	>250	>250	>250	250/>250	125/250	125/250
24	>250	>250	>250	>250	>250	>250	>250	>250	>250	>250
25	>250	>250	>250	>250	>250	>250	>250	62.5/125	62.5/125	62.5/125
31	125/125	125/125	62.5/62.5	62.5/62.5	>250	>250	>250	62.5/125	62.5/125	62.5/125
32	>250	>250	>250	>250	>250	>250	>250	125/>250	125/>250	125/>250
33	>250	>250	>250	31.2/62.5	>250	>250	>250	62.5/125	62.5/125	62.5/125
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 = minimum inhibitory concentration, MFC = minimum 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: amphotericin B.

allowed us to generate compounds with better anti-trypanosomal activities, but not sharing the mechanism of squalene accumulation.

Structurally talking, the substitution of naphtyl, in Nft, or 5-nitrofuryl moieties, in compound **2**, by the different assayed heterocycles carried out to different anti-*T. cruzi* profiles (Fig. 2). In example the benzofuroxan system leads to poorly actives derivatives (see **8** and **10**, Fig. 2) while benzimidazole 1,3-dioxide heterocycle displays compounds with good trypanosomicidal behaviours (compare compound **2** and **13** activities, Fig. 2). Among the quinoxaline 1,4-dioxides, the biological behaviour is not uniform. Quinoxalines **14**, **17**, **16**, **19**, **20**, and **24** (Fig. 2) were completely inactive in the assayed conditions. This lack of activity of 2-ciano-3-amino-derivatives **14** and **16** could be explained for compounds' poor solubilities in the biological milieu [47]. While, in compounds **17**, **19**, **20** and **24**, without apparent solubility problems, the lack of activity could be related to the electronic characteristics on the 2- and 3-substituents (amide or alkyl moieties). Otherwise, the 2-ester-substituted derivatives, **22** and **23**, display the best

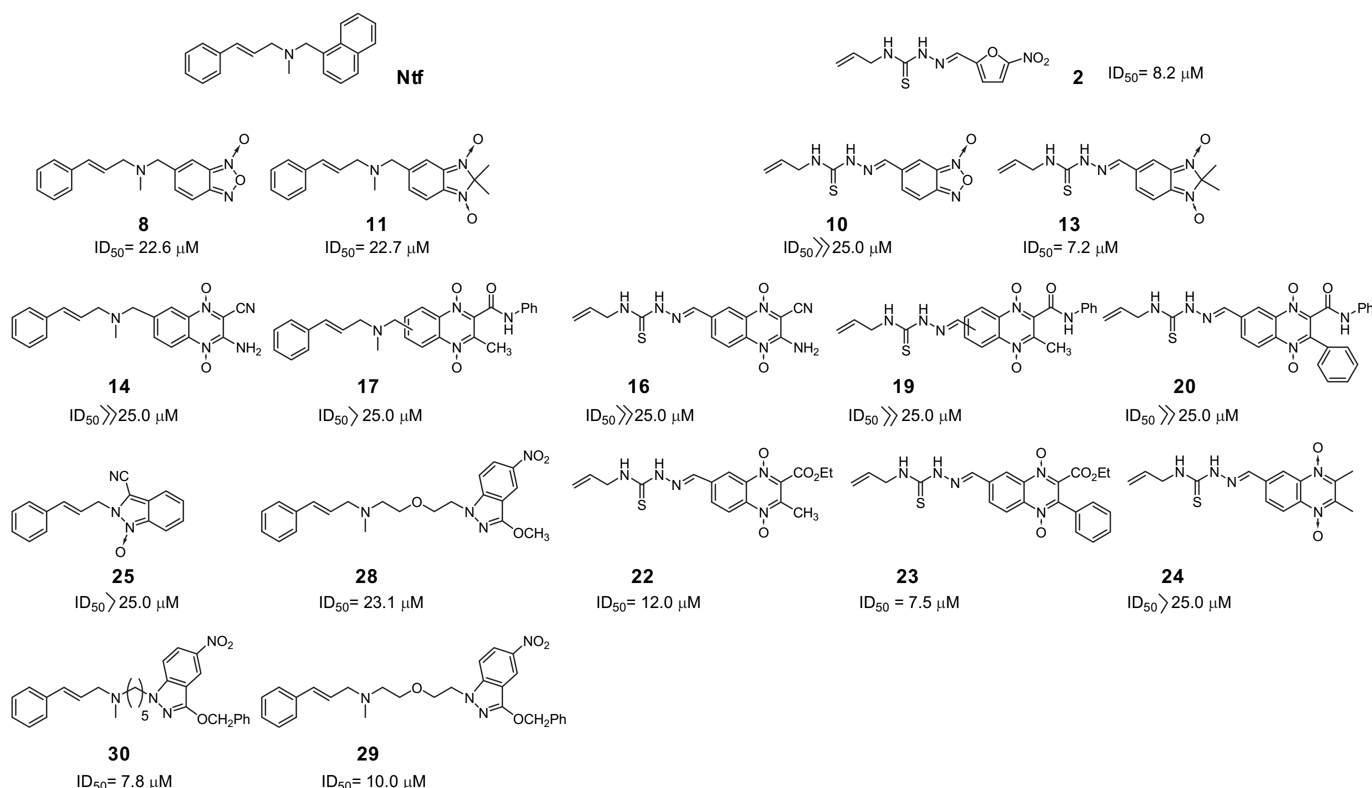

Fig. 2. Structural-anti-*T. cruzi* activity for the Naftifine-analogues. The ID_{50} refer to Tulahuen 2 strain.

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biological profile, being this feature in accordance to our previous QSAR [47]. The change by an indazole *N*-oxide heterocycle promotes less active compounds in contrast to 5-nitroindazolyl moiety which leads to very active Nft analogues.

4. Conclusions

We have developed and identified new trypanosomicidal agents, of which benzimidazole and quinoxaline containing *N*-oxide high selectivity indexes. This transforms them in molecular lead for further structural modifications and further biological studies, especially *in vivo* evaluations.

5. Experimental

Some 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 **I**, **II**, and **IV**–**XI** and ethyl benzoylacetate were prepared following synthetic procedures previously reported [24,25,29,30,32,48]. For the synthetic procedures used to prepare intermediate **III**, *N*-phenylbenzoylacetamide and *N*-phenylacetoacetamide see Supplementary Content section. 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 an 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. *(E)-5-[(N-Methyl-3-phenyl-2-propenylamino) methyl]benzofuroxan (**8**)*

A mixture of (**I**) (108 mg, 0.5 mmol) and (**III**) (70 mg, 0.5 mmol) in acetone (50.0 mL) was stirred at room temperature for 24 h. The solvent was evaporated *in vacuo* and the residue was purified by column chromatography (SiO_2, petroleum ether:ethyl acetate, 8:2). Red oil, 31 mg (23%). ^1H NMR ($CDCl_3$) δ_H: 2.30 (3H, s), 3.26 (2H, d, $J = 6.4$ Hz), 3.56 (2H, s), 6.30 (1H, dt, $J = 6.4$, 15.6 Hz), 6.58 (1H, d, $J = 15.6$ Hz), 7.39 (8H, m). ^{13}C NMR ($CDCl_3$) δ_C: 42.7, 60.5, 61.4, 126.7, 127.0, 128.1, 129.0, 133.6, 137.2, the benzofuroxan carbons were not detected at room temperature. EI-MS, *m*/*z* (abundance, %): 295 (M^+, 8), 278 ($M^+ - 17$, 15), 174 (33), 144 (33), 177 (75), 105 (100), 91 (44), 77 (54). IR, ν: 714, 1019, 1115, 1267, 1364, 1451, 1489, 1536, 1598, 1624, 1725.

5.2. *(E)-5-[4-(3-Phenyl-2-propenyl)piperazine-1-yl methyl]benzofuroxan (**9**)*

A mixture of (**I**) (103 mg, 0.5 mmol) and (*E*)-4-(3-phenyl-2-propenyl)piperazine (136 mg, 0.7 mmol) in CH_3CN (30.0 mL) was stirred at room temperature for 12 h. The solvent was evaporated *in vacuo* and the residue was purified by column chromatography (SiO_2, petroleum ether:ethyl acetate, 1:1). Yellow oil, 140 mg (89%). ^1H NMR ($CDCl_3$) δ_H: 2.66 (4H, bs), 2.75 (4H, bs), 3.27 (2H, d, $J = 6.8$ Hz), 4.49 (2H, s), 6.29 (1H, dt, $J = 6.8$, 15.6 Hz), 6.57 (1H, d, $J = 15.6$ Hz), 7.30 (8H, m). ^{13}C NMR ($CDCl_3$) δ_C: 50.1, 52.9, 59.8, 60.9, 124.0, 126.9, 128.3, 129.0, 135.0, 142.0, the benzofuroxan carbons were not detected at room temperature. EI-MS, *m*/*z* (abundance, %): 350 (M^+, 1), 272 (17), 214 (31), 201 (18), 177 (24), 158 (24), 149 (100), 117 (35), 55 (47). IR, ν: 749, 822, 1184, 1246, 1293, 1400, 1563, 1705.

5.3. *4-Allyl-1-(N[1]-oxide-benzo[1,2-c][1,2,5] oxadiazole-5-yl)-methylidenethiosemicarbazide (**10**)*

A mixture of (**II**) (263 mg, 1.6 mmol), 4-allylthiosemicarbazide (210 mg, 1.6 mmol) and *p*-TsOH (catalytic amount) in toluene (50.0 mL) was stirred at room temperature for 24 h. The precipitate was filtered and washed with petroleum ether. Yellow solid, 433 mg (98%); mp 172.0–173.0 °C. ^1H NMR (acetone-d_6) δ_H: 4.37 (2H, m), 5.24 (1H, d, $J = 10.3$ Hz), 5.30 (1H, d, $J = 16.0$ Hz), 5.98 (1H, s), 7.62 (1H, bs), 7.74 (1H, bs), 8.14 (1H, bs), 8.26 (1H, s), 8.64 (1H, bs), 10.76 (1H, bs). ^{13}C NMR (acetone-d_6) δ_C: 46.7, 115.8, 134.9, 139.6, 179.1, the benzofuroxan carbons were not detected at room temperature. EI-MS, *m*/*z* (abundance, %): 277 (M^+, 4), 259 ($M^+ - 18$, 4), 231 (62), 185 (30), 163 (28), 148 (41), 115 (100), 105 (78), 81 (28), 56 (85). IR, ν: 615, 812, 936, 1107, 1221, 1287, 1358, 1518, 1613, 1701.

5.4. *(E)-2,2-Dimethyl-5-[(N-methyl-3-phenyl-2-propenylamino)methyl]-2H-benzimidazole 1,3-dioxide (**11**)*

A mixture of **8** (148 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 4 days. The solvent was evaporated *in vacuo* and the residue was purified by column chromatography (SiO_2, ethyl acetate). Red oil, 126 mg (75%). ^1H NMR ($CDCl_3$) δ_H: 1.72 (6H, s), 2.28 (3H, s), 3.23 (2H, d, $J = 6.8$ Hz), 3.37 (2H, s), 6.27 (1H, dt, $J = 6.8$, 16.0 Hz), 6.56 (1H, d, $J = 16.0$ Hz), 7.01 (1H, d, $J = 9.6$ Hz), 7.16 (1H, s), 7.20 (1H, d, $J = 9.6$ Hz), 7.25 (1H, d, $J = 7.6$ Hz), 7.34 (2H, t, $J = 7.6$ Hz), 7.40 (2H, d, $J = 7.6$ Hz). ^{13}C NMR ($CDCl_3$) δ_C: 24.6, 42.8, 60.5, 61.4, 97.7, 114.1, 115.8, 126.7, 127.3, 128.0, 129.0, 133.4, 133.5, 136.0 (two carbons), 137.2, 144.3. EI-MS, *m*/*z* (abundance, %): 337 (M^+, 7), 321 ($M^+ - 16$, 17), 304 ($M^+ - 16$-17, 8), 279 (29), 215 (26), 192 (18), 175 (42), 159 (32), 146 (43), 144 (71), 117 (100), 91 (39), 77 (19). IR, ν: 695, 747, 970, 1026, 1096, 1181, 1235, 1368, 1404, 1453, 1516, 2934.

5.5. *(E)-2,2-Dimethyl-5-[4-(3-phenyl-2-propenyl) piperazine-1-yl-methyl]-2H-benzimidazole 1,3-dioxide (**12**)*

A mixture of **9** (120 mg, 0.3 mmol), 2-nitropropane (0.04 mL, 0.4 mmol) and piperidine (0.04 mL, 0.4 mmol) in THF (20.0 mL) was stirred at room temperature for 4 days. The solvent was evaporated *in vacuo* and the residue was purified by column chromatography (SiO_2, ethyl acetate). Red solid, 47 mg (35%); mp 154.0–156.5 °C. ^1H NMR ($CDCl_3$) δ_H: 1.72 (6H, s), 2.56 (8H, bs), 3.23 (2H, d, $J = 6.8$ Hz), 3.35 (2H, s), 6.29 (1H, dt, $J = 6.5$, 15.6 Hz), 6.55 (1H, d, $J = 15.6$ Hz), 6.98 (1H, d, $J = 9.8$ Hz), 7.16 (1H, bs), 7.19 (1H, d, $J = 9.8$ Hz), 7.26 (1H, d, $J = 7.2$ Hz), 7.32 (2H, t, $J = 7.2$ Hz), 7.39 (2H, d, $J = 7.2$ Hz). ^{13}C NMR ($CDCl_3$) δ_C: 24.6, 53.3, 53.3, 61.2, 62.5, 97.7, 114.3, 115.8, 126.2, 126.8, 128.0, 129.0, 133.3, 134.0, 136.6, 136.8, 137.2, 143.3. EI-MS, *m*/*z* (abundance, %): 376 ($M^+ - 16$, 6), 259 (16), 243 (11), 201 (100), 159 (20), 144 (18), 117 (61), 91 (18). IR, ν: 695, 745, 970, 1007, 1140, 1260, 1385, 1400, 1650, 2811, 2934, 3450.

5.6. *4-Allyl-1-(2,2-dimethyl-1,3-dioxide-2H-benzimidazole-5-yl)-methylidenethiosemicarbazide (**13**)*

A mixture of **10** (200 mg, 0.7 mmol), 2-nitropropane (0.08 mL, 0.9 mmol) and piperidine (0.09 mL, 0.9 mmol) in THF (50.0 mL) was stirred at room temperature for 24 h. The solvent was evaporated *in vacuo* and the residue was purified by column chromatography (SiO_2, petroleum ether:ethyl acetate, 1:1). Red solid, 77 mg (34%); mp >300.0 °C. ^1H NMR ($CDCl_3$) δ_H: 1.75 (6H, s), 4.41 (2H, d,

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J = 4.0 Hz), 5.29 (2H, m), 5.98 (1H, m), 7.30 (2H, s), 7.32 (1H, d, J = 9.0 Hz), 7.38 (1H, d, J = 9.0 Hz), 7.45 (1H, bs), 7.73 (1H, s), 9.87 (1H, bs). ^{13}C NMR ($CDCl_3$) δ_C: 24.7, 47.4, 98.6, 116.2, 116.8, 118.0, 128.2, 133.4, 136.2, 136.6, 136.8, 139.7, 178.0. EI-MS, m/z (abundance, %): 319 (M^+, 4), 303 ($M^+ - 16$, 3), 286 (M^+ – 16-17, 6), 189 (100), 174 (44), 132 (27), 115 (36), 56 (43).

5.7. General procedure for the preparation of derivatives **14–16**

A mixture of benzofuroxan (**8–10**) (0.4 mmol) and malononitrile (26 mg, 0.4 mmol) was stirred at 0 °C for 10 min. Then, two drops of Et_3N in DMF (0.5 mL) were added and the reaction was stirred at room temperature for 24–48 h. The desired product was filtered and washed with petroleum ether.

5.8. (E)-3-Amino-7-[(N-methyl-3-phenyl-2-propenylamino)methyl]quinoxaline-2-carbonitrile 1,4-dioxide (**14**)

Red solid, 114 mg (79%); mp 149.0–151.1 °C. ^{1}H NMR (DMSO-d_6) δ_H: 2.25 (3H, s), 3.29 (2H, d, J = 6.8 Hz), 3.41 (2H, bs), 3.81 (2H, s), 6.39 (1H, dt, J = 6.8, 16.0 Hz), 6.59 (1H, d, J = 16.0 Hz), 7.25 (1H, d, J = 7.4 Hz), 7.33 (1H, t, J = 7.4 Hz), 7.45 (1H, d, J = 7.4 Hz), 8.02 (1H, s), 8.26 (2H, m). ^{13}C NMR (DMSO-d_6) δ_C: 60.0, 62.0, 111.4, 118.9 (two carbons), 121.2, 127.1, 127.3, 128.4, 129.4, 133.0 (two carbons), 134.0, 137.0, 134.4 (two carbons), 146.6. EI-MS, m/z (abundance, %): 345 ($M^+ - 16$, 13), 328 (M^+ – 16-17, 42), 254 (60), 238 (46), 199 (70), 183 (87), 146 (67), 117 (100), 115 (75), 91 (46), 77 (23). IR, ν: 691, 745, 963, 1036, 1117, 1206, 1333, 1617.

5.9. (E)-3-Amino-7(6)-[4-(3-phenyl-2-propenyl) piperazine-1-yl-methyl]quinoxaline-2-carbonitrile 1,4-dioxide (**15**)

As equimolecular mixture of 6- and 7-positional isomers. Red solid, 83 mg (50%). ^{1}H NMR (DMSO-d_6) δ_H: 2.51 (8H, bs), 3.10 (2H, d, J = 6.0 Hz), 3.67/3.71 (2H, s), 6.30 (1H, m), 6.51 (1H, d, J = 15.6 Hz), 7.23 (1H, t, J = 7.6 Hz), 7.32 (2H, t, J = 7.6 Hz), 7.42 (2H, t, J = 7.6 Hz), 8.00 (2H, bs), 8.18 (1H, s), 8.25 (2H, m). ^{13}C NMR (DMSO-d_6) δ_C: 53.5, 53.6, 61.0, 61.1/61.3, 109.0/109.4, 118.9, 119.4, 120.6, 127.1, 128.0, 128.2, 129.4, 132.4, 132.8, 135.8, 136.9, 137.5, 137.6, 146.6/147.0. EI-MS, m/z (abundance, %): 400 ($M^+ - 16$, 2), 384 (M^+ – 16-16, 10), 267 (19), 201 (100), 183 (51), 117 (63), 91 (17), 77 (9).

5.10. 4-Allyl-1-(3-amino-2-cyano-1,4-dioxide quinoxaline-7-yl)-methylidenethiosemicarbazide (**16**)

Red solid, 133 mg (97%); mp >400.0 °C. ^{1}H NMR (DMSO-d_6) δ_H: 4.03 (2H, bs), 4.26 (2H, m), 5.18 (2H, m), 5.93 (1H, m), 8.27 (3H, m), 8.57 (1H, d, J = 5.2 Hz), 8.93/8.99 (1H, bs), 11.73/11.84 (1H, bs). ^{13}C NMR (DMSO-d_6) δ_C: 46.7, 109.6, 116.5, 118.9, 119.6, 120.7, 132.0, 134.5, 135.8, 137.9 (two carbons), 140.6, 147.1, 178.3. EI-MS, m/z (abundance, %): 342 ($M^+ + H$, 5), 102 (100). IR, ν: 835, 922, 1105, 1188, 1335, 1509, 1624.

5.11. General procedure for the preparation of derivatives **17**, **18**, **20**, **21**, and **22**

A mixture of the corresponding benzofuroxan (**8–10**) (1.0 mmol), the corresponding amide or ester (*N*-phenylacetoacetamide, *N*-phenylbenzoylacetamide, ethyl benzoylacetate, or ethyl acetoacetate) (1.0 mmol), anhydrous K_2CO_3 (138 mg, 1.0 mmol) in acetone (50.0 mL) was stirred at room temperature for 48 h. Then, water (40.0 mL) was added and the precipitate was filtered and washed with water.

5.12. (E)-3-Methyl-7(6)-[(N-methyl-3-phenyl-2-propenylamino)methyl]-N-phenylquinoxaline-2-carboxamide 1,4-dioxide (**17**)

As equimolecular mixture of 6- and 7-positional isomers. Yellow solid, 350 mg (77%). ^{1}H NMR (DMSO-d_6) δ_H: 2.22 (3H, s), 2.51 (3H, s), 3.24 (2H, d, J = 6.0 Hz), 3.70/3.82 (2H, s), 6.41 (1H, dt, J = 6.0, 16.0 Hz), 6.60 (1H, d, J = 16.0 Hz), 7.24–8.02 (11H, m), 8.46 (1H, s), 8.50 (1H, d, J = 8.8 Hz), 11.06 (1H, bs). ^{13}C NMR (DMSO-d_6) δ_C: 15.1, 48.9, 60.0, 60.6, 120.4 (two carbons), 120.8, 125.6, 127.1, 128.0, 128.3, 129.5 (two carbons), 130.0, 134.4, 138.9 (two carbons), 139.2, 140.0 (two carbons), 142.2, 145.6, 158.8. EI-MS, m/z (abundance, %): 438 ($M^+ - 16$, 1), 421 (M^+ – 16-17, 2), 254 (11), 146 (34), 117 (80), 115 (100), 91 (78), 77 (65), 65 (48), 51 (30). IR, ν: 693, 752, 970, 1223, 1269, 1331, 1447, 1497, 1563, 1599, 1622, 1680, 2789.

5.13. (E)-3-Methyl-N-phenyl-7-[4-(3-phenyl-2-propenyl)piperazine-1-ylmethyl]quinoxaline-2-carboxamide 1,4-dioxide (**18**)

Yellow solid, 199 mg (39%); mp 164.2–165.7 °C. ^{1}H NMR (DMSO-d_6) δ_H: 2.50 (11H, m), 3.12 (2H, d, J = 5.6 Hz), 3.77 (2H, s), 6.32 (1H, dt, J = 5.6, 16.4 Hz), 6.54 (1H, d, J = 16.4 Hz), 7.30–7.45 (8H, m), 7.68 (2H, d, J = 8.0 Hz), 7.96 (1H, d, J = 9.2 Hz), 8.43 (1H, s), 8.50 (1H, d, J = 9.2 Hz), 10.94 (1H, bs). ^{13}C NMR (DMSO-d_6) δ_C: 15.0, 53.5 (two carbons), 61.0, 61.8, 119.6, 120.0, 120.5, 125.6, 127.1, 127.9, 128.3, 129.4, 130.0, 132.9, 134.2, 137.2, 137.3, 138.6, 138.8 (two carbons), 139.4, 144.2, 158.1. EI-MS, m/z (abundance, %): 493 ($M^+ - 16$, 1), 477 (M^+ – 16-16, 1), 360 (6), 276 (12), 201 (73), 117 (100), 91 (28), 77 (17), 56 (16). IR, ν: 691, 749, 967, 1007, 1055, 1154, 1264, 1320, 1445, 1495, 1555, 1599, 1682, 2814.

5.14. 4-Allyl-1-[1,4-dioxide-3-phenyl-2-(N-phenyl carbamoyl)quinoxaline-7-yl]methylidene thiosemicarbazide (**20**)

Yellow solid, 268 mg (54%); mp 241.5–243.1 °C. ^{1}H NMR (DMSO-d_6) δ_H: 4.27 (2H, bs), 5.15 (2H, dd, J = 10.0, 18.0 Hz), 5.92 (1H, m), 7.10 (1H, dd, J = 7.2, 7.6 Hz), 7.30 (2H, dd, J = 7.6, 8.0 Hz), 7.38 (2H, d, J = 8.0 Hz), 7.49 (3H, m), 7.60 (2H, m), 8.34 (1H, s), 8.52 (1H, d, J = 8.8 Hz), 8.67 (1H, s), 8.74 (1H, d, J = 8.8 Hz), 9.07 (1H, bs), 10.81 (1H, bs), 11.91 (1H, bs). ^{13}C NMR (DMSO-d_6) δ_C: 46.4, 116.1, 119.5, 119.8, 121.0, 125.0, 128.7, 129.0 (three carbons), 129.5, 135.3, 137.9, 138.8 (three carbons), 139.9 (three carbons), 156.9, 178.0. EI-MS, m/z (abundance, %): 480 ($M^+ - 17$, 1), 367 (19), 350 (100), 230 (86), 77 (78), 56 (33). IR, ν: 695, 943, 1094, 1196, 1287, 1329, 1512, 1700.

5.15. Ethyl (E)-3-phenyl-7(6)-[4-(3-phenyl-2-propenyl)piperazine-1-ylmethyl]quinoxaline-2-carboxylate 1,4-dioxide (**21**)

As equimolecular mixture of 6- and 7-positional isomers. Yellow solid, 293 mg (56%). ^{1}H NMR (DMSO-d_6) δ_H: 0.96/0.97 (3H, t, J = 7.2 Hz), 2.51 (8H, m), 3.12 (2H, d, J = 5.2 Hz), 3.79 (2H, s), 4.14/4.16 (2H, q, J = 7.2 Hz), 6.31 (1H, dt, J = 5.2, 15.6 Hz), 6.54 (1H, d, J = 15.6 Hz), 7.24 (1H, d, J = 7.2 Hz), 7.32 (2H, dd, J = 7.2, 7.6 Hz), 7.44 (2H, d, J = 7.6 Hz), 7.56 (5H, m), 7.98 (1H, d, J = 8.8 Hz), 8.41 (1H, s), 8.46 (1H, d, J = 8.8 Hz). ^{13}C NMR (DMSO-d_6) δ_C: 14.2, 53.5 (two carbons), 60.9, 61.8, 63.5, 119.5, 121.2, 127.1, 127.8, 128.3, 129.3, 129.5 (two carbons), 130.7, 131.3, 133.0, 134.5, 137.5, 137.7, 138.3, 139.5, 145.0, 145.5, 160.0. EI-MS, m/z (abundance, %): 508 ($M^+ - 16$, 1), 491 (M^+ – 16-16, 1), 291 (14), 233 (11), 219 (21), 201 (100), 117 (82), 91 (17), 77 (17), 56 (10). IR, ν: 695, 743, 849, 1007, 1094, 1152, 1235, 1331, 1443, 1740, 2803.

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*5.16. 4-Allyl-1-(2-ethoxycarbonyl-3-methyl-1,4-dioxidequinoxaline-7-yl)methylidene thiosemicarbazide (**22**)*

Yellow solid, 167 mg (43%); mp 195.1–197.3 °C. ^{1}H NMR (DMSO-d_6) δ_H: 1.36 (3H, t, J = 7.2 Hz), 2.43 (3H, s), 4.25 (2H, bs), 4.50 (2H, q, J = 7.2 Hz), 5.14 (2H, dd, J = 10.4, 17.2 Hz), 5.92 (1H, m), 8.06 (1H, d, J = 9.2 Hz), 8.18 (1H, s), 8.26 (1H, s), 8.45 (1H, d, J = 9.2 Hz), 9.02 (1H, bs), 11.88 (1H, bs). ^{13}C NMR (DMSO-d_6) δ_C: 14.3, 14.6, 46.4, 63.8, 116.1, 116.6, 131.2, 135.3 (two carbons), 139.9, 140.0, 140.1, 149.6, 149.8, 149.8, 160.1, 178.0. EI-MS, m/z (abundance, %): 389 (M^+, 1), 372 (M^+ − 17, 18), 356 (M^+ − 17-16, 13), 260 (29), 170 (39), 115 (100), 56 (66). IR, ν: 621, 760, 928, 1007, 1061, 1109, 1202, 1296, 1329, 1422, 1477, 1534, 1738, 2359.

*5.17. General procedure for the preparation of derivatives 19, and **23***

A mixture of **8** (1.0 mmol) and the corresponding amide or ester (*N*-phenylacetoacetamide, or ethyl benzoylacetate) (1.0 mmol) was stirred at 0 °C for 10 min. Then, four drops of Et_3N in DMF (1.0 mL) were added and the reaction was stirred at room temperature for 5–10 days. The desired product was filtered and washed with petroleum ether.

*5.18. 4-Allyl-1-[3-methyl-2-(N-phenylcarbamoyl)-1,4-dioxidequinoxaline-7(6)-yl]methylidene thiosemicarbazide (**19**)*

As equimolecular mixture of 6- and 7-positional isomers. Orange solid, 144 mg (33%). ^{1}H NMR (DMSO-d_6) δ_H: 2.51 (3H, s), 4.27 (2H, bs), 5.16 (2H, dd, J = 10.4, 17.2 Hz), 5.94 (1H, m), 7.21 (1H, t, J = 7.6 Hz), 7.43 (2H, t, J = 7.6 Hz), 7.68 (2H, t, J = 7.6 Hz), 8.32/8.33 (1H, s), 8.49 (1H, m), 8.66 (2H, m), 9.00 (1H, bs), 10.96 (1H, bs), 11.85 (1H, bs). ^{13}C NMR (DMSO-d_6) δ_C: 15.2, 46.8, 116.5, 119.6/119.9, 120.4, 120.8/121.1, 125.6, 129.8, 130.0, 135.7, 138.0, 137.8, 138.6, 138.7, 139.0, 140.5, 141.0, 157.9, 178.5. EI-MS, m/z (abundance, %): 402 (M^+ − 17-17, 20), 304 (21), 288 (59), 185 (24), 168 (100), 131 (44), 115 (44), 91 (42), 77 (42), 56 (72). IR, ν: 741, 920, 1192, 1333, 1507, 1538, 1659.

*5.19. 4-Allyl-1-(2-ethoxycarbonyl-1,4-dioxide-3-phenylquinoxaline-7-yl)methylidene thiosemicarbazide (**23**)*

Orange solid, 23 mg (5%); mp 171.2–172.6 °C. ^{1}H NMR ($CDCl_3$) δ_H: 1.21 (3H, t, J = 3.6 Hz), 4.36 (2H, q, J = 3.6 Hz), 4.45 (2H, m), 5.33 (2H, m), 6.03 (1H, m), 7.54 (3H, m), 7.63 (1H, bs), 7.78 (2H, m), 8.12 (1H, s), 8.21 (2H, m), 8.34 (1H, d, J = 9.4 Hz), 10.05 (1H, bs). ^{13}C NMR ($CDCl_3$) δ_C: 14.2, 47.4, 63.0, 117.9, 129.0, 129.0, 129.1, 129.2, 130.3, 130.6, 133.5, 136.0, 137.1, 137.8, 141.1, 142.8, 143.7, 166.8, 178.2. EI-MS, m/z (abundance, %): 417 (M^+ − 17-17, 6), 319 (14), 306 (69), 290 (18), 276 (27), 233 (100), 204 (44), 129 (24), 115 (100), 77 (62), 56 (65).

*5.20. 4-Allyl-1-(2,3-dimethyl-1,4-dioxide quinoxaline-6-yl)methylidenethiosemicarbazide (**24**)*

A mixture of (**IV**) (436 mg, 2.0 mmol), 4-allylthiosemicarbazide (262 mg, 2.0 mmol) and *p*-TsOH (catalytic amount) in toluene (50.0 mL) was stirred at room temperature for 20 h. The precipitate was filtered and washed with petroleum ether. Yellow solid (from EtOH), 543 mg (82%); mp 244.5–247.0 °C. ^{1}H NMR (DMSO-d_6) δ_H: 2.76 (6H, s), 4.26 (2H, bs), 5.16 (2H, dd, J = 12.0, 16.0 Hz), 5.91 (1H, m), 8.28 (1H, s), 8.42 (1H, d, J = 8.0 Hz), 8.53 (1H, d, J = 8.0 Hz), 8.62 (1H, s), 9.01 (1H, bs), 11.75 (1H, bs). ^{13}C NMR (DMSO-d_6) δ_C: 15.0, 46.4, 116.1, 119.4, 120.4, 128.6, 135.4, 136.7, 136.8, 137.4, 140.4, 142.3, 142.5, 178.0. EI-MS, m/z (abundance, %): 331 (M^+, 1), 314 (M^+ − 17, 14), 298 (M^+ − 17-16, 14), 217 (34), 201 (79), 184 (74), 169 (38), 115 (100), 56 (63). IR, ν: 677, 938, 1098, 1200, 1318, 1503, 1659, 3195.

*5.21. (E)-2-(3-Phenyl-2-propenyl)-2H-indazole-3-carbonitrile N-oxide (**25**)*

A mixture of 2-nitrobenzaldehyde (166 mg, 1.1 mmol) and (*E*)-3-phenyl-2-propenylamine (**V**) (146 mg, 1.1 mmol) in acetic acid (20.0 mL) was stirred at room temperature for 24 h. Then, KCN (143 mg, 2.2 mmol) was added and the reaction was stirred for 48 h. After that, water (20.0 mL) was added and the intermediate was extracted with CH_2Cl_2 (3 × 20.0 mL), the organic layer was dried with anhydrous Na_2SO_4 and the solvent was evaporated *in vacuo*. Five drops of Et_3N in EtOH (10.0 mL) was added and the reaction was stirred at room temperature for 48 h. The solvent was evaporated *in vacuo*, and the residue was fractioned between water (50.0 mL) and EtOAc (3 × 20.0 mL). The organic layer was dried with anhydrous Na_2SO_4 and the solvent was evaporated *in vacuo*. The residue was purified by column chromatography (SiO_2, petroleum ether:ethyl acetate, 1:1). Yellow solid, 61 mg (20%); mp 120.1–122.3 °C. ^{1}H NMR ($CDCl_3$) δ_H: 5.36 (2H, t, J = 6.8 Hz), 6.89 (1H, d, J = 15.6 Hz), 7.32 (7H, m), 7.70 (1H, d, J = 7.2 Hz), 7.86 (1H, d, J = 7.2 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 47.8, 91.1, 110.5, 114.3, 118.8, 118.9, 122.3, 128.8–129.9 (phenyl carbons), 135.2, 137.6, 139.5. EI-MS, m/z (abundance, %): 275 (M^+, 2), 259 (M^+ − 16, 1), 117 (100), 115 (37), 91 (15). IR, ν: 586, 750, 962, 1279, 1341, 1439, 1491, 2203.

*5.22. (Z)-2-(4-Phenyl-3-butenyl)-2H-indazole-3-carbonitrile N-oxide (**26**)*

A mixture of 2-nitrobenzaldehyde (166 mg, 1.1 mmol) and (*Z*)-4-phenyl-3-butenylamine (**VI**) (162 mg, 1.1 mmol) in acetic acid (20.0 mL) was stirred at room temperature for 24 h. Then, KCN (143 mg, 2.2 mmol) was added and the reaction was stirred for 5 days. After that, water (20.0 mL) was added and the intermediate was extracted with CH_2Cl_2 (3 × 20.0 mL), the organic layer was dried with anhydrous Na_2SO_4 and the solvent was evaporated *in vacuo*. Five drops of Et_3N in EtOH (10.0 mL) was added and the reaction was stirred at room temperature for 48 h. The solvent was evaporated *in vacuo*, and the residue was fractioned between water (50.0 mL) and EtOAc (3 × 20.0 mL). The organic layer was dried with anhydrous Na_2SO_4 and the solvent was evaporated *in vacuo*. The residue was purified by column chromatography (SiO_2, petroleum ether:ethyl acetate, 7:3). Orange oil, 140 mg (44%). ^{1}H NMR ($CDCl_3$) δ_H: 3.04 (2H, m), 4.68 (2H, t, J = 7.0 Hz), 5.73 (2H, m), 6.63 (1H, d, J = 11.2 Hz), 7.09–7.24 (5H, m), 7.39 (2H, m), 7.64 (1H, d, J = 7.6 Hz), 7.76 (1H, d, J = 8.4 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 27.1, 46.0, 96.0, 110.6, 114.2, 118.7, 122.0, 125.1, 127.1, 127.2, 128.2–129.0 (phenyl carbons), 129.1, 133.7, 136.0. EI-MS, m/z (abundance, %): 289 (M^+, 1), 274 (M^+ − 16, 7), 183 (34), 155 (40), 131 (87), 115 (87), 91 (100), 77 (20). IR, ν: 700, 747, 1057, 1206, 1352, 1445, 1493, 1528, 1701, 2923.

*5.23. (Z)-2-[4-(4-Chlorophenyl-3-butenyl)]-2H-indazole-3-carbonitrile N-oxide (**27**)*

A mixture of 2-nitrobenzaldehyde (166 mg, 1.1 mmol) and (*Z*)-4-(4-chlorophenyl)-3-butenylamine (**VII**) (200 mg, 1.1 mmol) in acetic acid (20.0 mL) was stirred at room temperature for 24 h. Then, KCN (143 mg, 2.2 mmol) was added and the reaction was stirred for 48 h. After that, water (20.0 mL) was added and the intermediate was extracted with CH_2Cl_2 (3 × 20.0 mL), the organic layer was dried with anhydrous Na_2SO_4 and the solvent was evaporated *in vacuo*. Five drops of Et_3N in EtOH (10.0 mL) was added and the reaction was stirred at room temperature for 48 h.

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The solvent was evaporated *in vacuo*, and the residue was fractioned between water (50.0 mL) and EtOAc (3 × 20.0 mL). The organic layer was dried with anhydrous Na_2SO_4 and the solvent was evaporated *in vacuo*. The residue was purified by column chromatography (SiO_2, petroleum ether:ethyl acetate, 1:1). Yellow oil, 38 mg (11%). ^{1}H NMR ($CDCl_3$) δ_H: 3.01 (2H, m), 4.66 (2H, t, J = 6.8 Hz), 5.78 (1H, m), 6.56 (1H, d, J = 11.6 Hz), 7.02 (2H, d, J = 8.4 Hz), 7.18 (2H, d, J = 8.4 Hz), 7.40 (2H, m), 7.63 (1H, d, J = 8.4 Hz), 7.75 (1H, d, J = 8.0 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 27.2, 46.2, 92.1, 110.9, 114.5, 119.0, 122.4, 126.1, 127.6, 128.7, 128.8, 129.6, 129.9, 133.0, 133.4, 134.8. EI-MS, m/z (abundance, %): 323 (M^+, 1), 308 ($M^+ - 16$, 4), 183 (38), 165 (31), 155 (44), 129 (100), 115 (68). IR, ν: 586, 747, 841, 1013, 1092, 1244, 1293, 1358, 1443, 1493, 2207, 2926.

5.24. General procedure for the preparation of derivatives **28–35**

A mixture of the indazole reactant (**VIII**, **IX** or **X**) (1.0 equiv.), amines ((**III**), (*E*)-4-(3-phenyl-2-propenyl)piperazine or (**XI**)) (1.0 equiv.), K_2CO_3 (1.0 equiv.), and KI (catalytic amount) (in the case of **IX** or **X**) in acetonitrile (15.0 mL/0.1 mmol) was heated at reflux for 10–38 h. The solvent was evaporated *in vacuo* and the residue was fractioned by column chromatography (SiO_2, ethyl acetate or CH_2Cl_2 for **31**–**33**).

5.25. (E)-N-[5-(3-Methoxy-5-nitro-1H-indazole-1-yl)-3-oxapentyl]-N-methyl-3-phenyl-2-propenamine (**28**)

Orange oil, 77 mg (94%). ^{1}H NMR ($CDCl_3$) δ_H: 2.21 (3H, s), 2.53 (2H, t, J = 5.6 Hz), 3.11 (2H, dd, J = 1.2, 6.8 Hz), 3.52 (2H, t, J = 5.6 Hz), 3.86 (2H, t, J = 5.2 Hz), 4.10 (3H, s), 4.38 (2H, t, J = 5.2 Hz), 6.18 (1H, dt, J = 6.8, 16.0 Hz), 6.45 (1H, d, J = 16.0 Hz), 7.38 (6H, m), 8.15 (1H, dd, J = 2.0, 9.2 Hz), 8.58 (1H, d, J = 2.0 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 31.0, 49.3, 56.2, 56.6, 60.6, 69.5, 69.7, 109.4, 111.9, 118.4, 122.4 (two carbons), 126.3, 127.6, 128.6, 133.2, 136.8, 140.9, 143.6, 158.4. EI-MS, m/z (abundance, %): 410 (M^+, 3), 393 ($M^+ - 17$, 7), 160 (38), 146 (9), 117 (100), 91 (7). IR, ν: 741, 1142, 1208, 1331, 1487, 1547, 1617.

5.26. (E)-N-[5-(3-Benzyloxy-5-nitro-1H-indazole-1-yl)-3-oxapentyl]-N-methyl-3-phenyl-2-propenamine (**29**)

Yellow oil, 52 mg (53%). ^{1}H NMR ($CDCl_3$) δ_H: 2.28 (3H, s), 2.63 (2H, t, J = 5.6 Hz), 3.20 (2H, d, J = 6.8 Hz), 3.59 (2H, t, J = 5.6 Hz), 3.90 (2H, t, J = 5.2 Hz), 4.43 (2H, t, J = 5.2 Hz), 5.45 (2H, s), 6.21 (1H, dt, J = 6.8, 16.0 Hz), 6.49 (1H, d, J = 16.0 Hz), 7.40 (11H, m), 8.17 (1H, dd, J = 1.6, 9.2 Hz), 8.64 (1H, d, J = 1.6 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 42.7, 49.7, 56.4, 60.7, 69.5, 69.9, 71.5, 109.9, 112.4, 118.8, 122.8, 125.2, 126.8–129.0 (phenyl carbons), 134.7, 136.7, 136.9, 141.3, 144.0, 158.1. EI-MS, m/z (abundance, %): 486 (M^+, 3), 395 (11), 160 (32), 146 (7), 117 (100), 91 (42). IR, ν: 700, 741, 804, 970, 1142, 1329, 1483, 1539, 1615, 2938.

5.27. (E)-N-[5-(3-Benzyloxy-5-nitro-1H-indazole-1-yl)pentyl]-N-methyl-3-phenyl-2-propenamine (**30**)

Orange oil, 96 mg (99%). ^{1}H NMR ($CDCl_3$) δ_H: 1.34 (2H, m), 1.56 (2H, m), 1.92 (2H, m), 2.27 (3H, s), 3.15 (2H, t, J = 6.4 Hz), 3.41 (2H, d, J = 6.0 Hz), 4.23 (2H, t, J = 6.8 Hz), 5.45 (2H, s), 6.28 (1H, m), 6.50 (1H, d, J = 16.4 Hz), 7.35 (11H, m), 8.21 (1H, dd, J = 2.0, 9.2 Hz), 8.68 (1H, d, J = 2.0 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 24.7, 27.0, 29.5, 42.2, 48.9, 53.5, 60.3, 71.1, 108.5, 111.9, 118.8, 122.5, 126.3, 126.3, 127.5–128.6 (phenyl carbons), 132.7, 136.3, 137.0, 140.8, 142.6, 157.5. EI-MS, m/z (abundance, %): 484 (M^+, 4), 467 ($M^+ - 17$, 7), 393 (18), 246 (7), 160 (19), 146 (20), 117 (100), 91 (51). IR, ν: 695, 741, 1140, 1194, 1329, 1453, 1485, 1539, 1615, 2938.

5.28. (E)-3-Methoxy-5-nitro-1-{5-[4-(3-phenyl-2-propenyl)piperazine-1-yl]-3-oxapentyl}-1H-indazole (**31**)

Orange solid, 182 mg (98%); mp 86.4–87.7 °C. ^{1}H NMR ($CDCl_3$) δ_H: 2.47 (10H, m), 3.13 (2H, d, J = 6.8 Hz), 3.50 (2H, t, J = 5.2 Hz), 3.82 (2H, t, J = 5.2 Hz), 4.07 (3H, s), 4.34 (2H, t, J = 5.2 Hz), 6.24 (1H, dt, J = 6.8, 15.6 Hz), 6.50 (1H, d, J = 15.6 Hz), 7.27 (6H, m), 8.15 (1H, dd, J = 2.0, 9.4 Hz), 8.54 (1H, d, J = 2.0 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 49.6, 53.0, 53.5, 56.9, 57.9, 61.1, 69.4, 69.8, 109.9, 112.2, 118.6, 122.6, 125.9, 126.8, 128.0, 128.9, 134.1, 137.1, 141.2, 143.9, 158.7. EI-MS, m/z (abundance, %): 465 (M^+, 10), 448 ($M^+ - 17$, 22), 293 (13), 229 (13), 215 (100), 172 (18), 117 (72). IR, ν: 741, 806, 1011, 1142, 1329, 1487, 1549, 1617, 1713, 2811.

5.29. (E)-3-Benzyloxy-5-nitro-1-{5-[4-(3-phenyl-2-propenyl)piperazine-1-yl]-3-oxapentyl}-1H-indazole (**32**)

Yellow solid (EtOH:H_2O, 1:1), 86 mg (62%); mp 98.3–99.6 °C. ^{1}H NMR ($CDCl_3$) δ_H: 2.46 (10H, m), 3.13 (2H, d, J = 6.8 Hz), 3.50 (2H, t, J = 5.6 Hz), 3.86 (2H, t, J = 5.2 Hz), 4.40 (2H, t, J = 5.2 Hz), 5.46 (2H, s), 6.26 (1H, m), 6.52 (1H, d, J = 15.6 Hz), 7.40 (10H, m), 8.23 (1H, dd, J = 1.8, 6.8 Hz), 8.67 (1H, d, J = 1.8 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 49.7, 53.4, 53.9, 58.1, 61.4, 69.7, 69.9, 71.5, 109.9, 112.4, 118.9, 122.8, 126.7–129.0 (phenyl carbons), 133.6, 136.6, 137.3, 141.3, 144.0, 158.0. EI-MS, m/z (abundance, %): 541 (M^+, 6), 524 ($M^+ - 17$, 14), 450 (29), 369 (10), 229 (12), 215 (100), 172 (16), 117 (81), 91 (73). IR, ν: 617, 739, 1009, 1121, 1327, 1485, 1539, 1615, 2807.

5.30. (E)-3-Benzyloxy-5-nitro-1-{5-[4-(3-phenyl-2-propenyl)piperazine-1-yl]pentyl}-1H-indazole (**33**)

Yellow solid, 158 mg (98%); mp 215.2–216.6 °C. ^{1}H NMR ($CDCl_3$) δ_H: 1.40 (2H, m), 1.96 (4H, m), 3.04 (2H, t, J = 5.6 Hz), 3.59 (4H, m), 3.87 (2H, d, J = 7.2 Hz), 3.99 (4H, bs), 4.26 (2H, t, J = 6.4 Hz), 5.46 (2H, s), 6.39 (1H, dt, J = 7.2, 15.6 Hz), 6.84 (1H, d, J = 15.6 Hz), 7.43 (11H, m), 8.25 (1H, dd, J = 2.0, 9.2 Hz), 8.69 (1H, d, J = 2.0 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 23.6, 24.1, 29.1, 48.0, 48.5, 48.6, 57.2, 59.7, 71.5, 109.0, 122.4, 115.0, 119.1, 123.1, 127.6–130.1 (phenyl carbons), 134.7, 136.7, 141.4, 142.0, 143.1, 158.0. EI-MS, m/z (abundance, %): 539 (M^+, 4), 522 ($M^+ - 17$, 27), 448 (23), 367 (10), 246 (13), 215 (21), 172 (15), 117 (56), 91 (100). IR, ν: 696, 739, 808, 953, 1026, 1140, 1190, 1329, 1375, 1451, 1485, 1541, 1615, 2384, 2942.

5.31. (E/Z)-N-[5-(3-Benzyloxy-5-nitro-1H-indazole-1-yl)-3-oxapentyl]-5-phenyl-4-pentenamine (**34**)

Yellow oil, 27 mg (7%). *E*/*Z* proportion: 22:78. *Isomer E.* ^{1}H NMR ($CDCl_3$) δ_H: 1.51 (2H, m), 2.16 (2H, m), 2.51 (2H, t, J = 7.4 Hz), 2.63 (2H, t, J = 5.2 Hz), 3.46 (2H, t, J = 5.2 Hz), 3.86 (2H, t, J = 5.2 Hz), 4.39 (2H, t, J = 5.2 Hz), 5.45 (2H, s), 6.20 (1H, m), 6.37 (1H, d, J = 16.0 Hz), 7.41 (11H, m), 8.21 (1H, dd, J = 2.0, 9.4 Hz), 8.67 (1H, d, J = 2.0 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 30.0, 31.1, 49.5, 49.7, 49.7, 69.9, 71.1, 71.5, 109.7, 112.5, 118.9, 122.8, 123.8–129.1 (phenyl carbons), 130.6, 134.0, 136.6, 138.0, 141.4, 144.0, 158.1. *Isomer Z.* ^{1}H NMR ($CDCl_3$) δ_H: 1.51 (2H, m), 2.31 (2H, m), 2.51 (2H, t, J = 7.4 Hz), 2.63 (2H, t, J = 5.2 Hz), 3.46 (2H, t, J = 5.2 Hz), 3.86 (2H, t, J = 5.2 Hz), 4.39 (2H, t, J = 5.2 Hz), 5.45 (2H, s), 5.61 (1H, m), 6.43 (1H, d, J = 11.6 Hz), 7.41 (11H, m), 8.21 (1H, dd, J = 2.0, 9.4 Hz), 8.67 (1H, d, J = 2.0 Hz). ^{13}C NMR ($CDCl_3$) δ_C: 26.6, 30.6, 49.5, 49.7, 49.7, 69.9, 71.1, 71.5, 109.7, 112.5, 118.9, 122.8, 123.8–129.1 (phenyl carbons), 129.7, 132.6, 136.6, 138.0, 141.4, 144.0, 158.1. EI-MS, m/z (abundance, %): 500 (M^+, 9), 409 (8), 369 (23), 295 (6), 279 (11), 205 (7), 174 (72), 144 (15), 117 (14), 91 (100). IR, ν: 700, 739, 1140, 1329, 1485, 1539, 1615, 2936.

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5.32. (E/Z)-N,N-Bis[5-(3-benzyloxy-5-nitro-1H-indazole-1-yl)-3-oxapentyl]-5-phenyl-4-pentenamine (**35**)

Yellow oil, 242 mg (67%). *E*/*Z* proportion: 36:64. *Isomer E.* ^{1}H NMR ($CDCl_3$) δ_H: 1.42 (2H, m), 2.06 (2H, m), 2.33 (2H, t, $J = 6.8$ Hz), 2.45 (4H, t, $J = 5.6$ Hz), 3.31 (4H, t, $J = 5.6$ Hz), 3.77 (4H, t, $J = 5.6$ Hz), 4.32 (4H, t, $J = 5.6$ Hz), 5.45 (4H, s), 5.56 (1H, m), 6.34 (1H, d, $J = 15.6$ Hz), 7.41 (22H, m), 8.16 (1H, dd, $J = 2.0$, 9.4 Hz), 8.64 (1H, d, $J = 2.0$ Hz). ^{13}C NMR ($CDCl_3$) δ_C: 27.3, 30.9, 49.7, 54.4, 55.0, 69.9, 70.4, 71.5, 109.9, 112.4, 118.8, 122.7, 126.3–129.1 (phenyl carbons), 130.6, 136.6, 138.0, 138.0, 141.3, 143.9, 158.0. *Isomer Z.* ^{1}H NMR ($CDCl_3$) δ_H: 1.42 (2H, m), 2.22 (2H, m), 2.33 (2H, t, $J = 6.8$ Hz), 2.45 (4H, t, $J = 5.6$ Hz), 3.31 (4H, t, $J = 5.6$ Hz), 3.77 (4H, t, $J = 5.6$ Hz), 4.32 (4H, t, $J = 5.6$ Hz), 5.45 (4H, s), 6.14 (1H, m), 6.41 (1H, d, $J = 11.6$ Hz), 7.41 (22H, m), 8.16 (1H, dd, $J = 2.0$, 9.4 Hz), 8.64 (1H, d, $J = 2.0$ Hz). ^{13}C NMR ($CDCl_3$) δ_C: 26.6, 28.0, 49.7, 54.4, 55.0, 69.9, 70.4, 71.5, 109.9, 112.4, 118.8, 122.7, 126.3–129.1 (phenyl carbons), 129.6, 132.7, 136.6, 138.0, 141.3, 143.9, 158.0. EI-MS, *m*/*z* (abundance, %): 841 ($M^+ + 2H$, 50), 840 ($M^+ + H$, 100), 501 (19), 345 (27). IR, ν: 700, 754, 806, 967, 1142, 1198, 1329, 1451, 1485, 1539, 1615, 2938.

5.33. Biology

5.33.1. Anti-T. cruzi in vitro test using epimastigotes of Tulahuen 2 strain or CL Brener clone

T. cruzi epimastigotes (Tulahuen 2 strain or CL Brener clone) were grown at 28 °C in an axenic medium (BHI-Tryptose) as previously described [31,32,37], 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. Compounds **34** and **35** were studied as mixtures of *E* and *Z* geometric isomers (for proportions see synthetic procedures). 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)]} × 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%.

5.33.2. Unspecific mammalian cytotoxicity [22,32]

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.

5.33.3. 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 (Argentina) were used: *Candida albicans* ATCC 10231, *Candida tropicalis* C 131, *Saccharomyces cerevisiae* ATCC 9763, *C. neoformans* ATCC 32264, *Aspergillus flavus* ATCC 9170, *Aspergillus fumigatus* ATTC 26934, *Aspergillus niger* ATCC 9029, *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. 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 [49,50].

5.33.4. Antifungal susceptibility testing

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 [49,50] 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 °C for yeasts and *Aspergillus* spp. 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 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.

5.33.5. Squalene level analysis

Epimastigote forms of *T. cruzi* (Tulahuen 2 strain) 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

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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.

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Appendix. Supporting information

Supplementary content about synthesis and lipid analyses (three pages) associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2010.01.052.

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