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Original article

New perspectives on the synthesis and antichagasic activity of 3-alkoxy-1-alkyl-5-nitroindazoles

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ABSTRACT

The synthesis and antiprotozoal activity of some 3-alkoxy-1-alkyl- (**1**, **4**) and 3-alkoxy-1-(ω-aminoalkyl)-5-nitroindazoles (**2**, **3**, **5**–**8**) against different morphological forms of *Trypanosoma cruzi* are reported. These compounds were prepared using simple alkylation reactions and, usually, taking advantage of the reactivity of some indazole-derived betaines previously studied by us. Most indazole derivatives showed *in vitro* activities similar or higher than those of the reference drug benznidazole; this fact, along with low unspecific cytotoxicities against Vero cells shown by some of them, led to very good selectivity indexes (SI). The high efficiency of 5-nitroindazoles **1** and **2** against *T. cruzi* was confirmed by further *in vitro* studies on infection rates and by an additional *in vivo* study in a murine model of acute and chronic Chagas disease. Complementary analyses of the changes in the metabolites excreted by the parasite and on the ultrastructural alterations induced after treatment with indazole derivatives **1** and **2** were also conducted.

1. Introduction

Chagas disease or American trypanosomiasis is a major parasitosis originally endemic to poor rural areas of Latin America, mainly transmitted by hematophagous triatomine insects and caused by the trypanosomatid (Kinetoplastid) protozoan parasite *Trypanosoma cruzi*. At present because of intense international migrations it can also be found in other areas such as Western Europe, USA, Australia, etc, countries in which the infection is transmitted by other routes. Only two drugs, nifurtimox and benznidazole are currently available for the treatment of Chagas disease. This chemotherapy, however, is unsatisfactory because these drugs exhibit limited efficacy in the chronic phase of the disease and also severe toxic side effects. Thus, it is clear that the development of new drugs for the treatment of this parasitosis is urgently needed. Several excellent articles covering different aspects of Chagas disease chemotherapy [1] have been published recently.

In this context, we have reported in the last years the synthesis and antichagasic properties of some 5-nitroindazole derivatives, mainly 1-substituted 3-alkoxy-1*H*-indazoles [2–4], 2-substituted 3-alkoxy-2*H*-indazoles [4] and 1,2-disubstituted indazolin-3-ones [4], as well as those of some 4-substituted and 1,4-disubstituted 7-nitroquinoxalin-2-ones [5,6]. It has been proposed [2–4,7] that the mentioned nitro-group bearing heterocycles act upon intracellular nitro reduction followed by redox cycling leading to reactive oxygen species (ROS) (like nifurtimox) [8] or producing electrophilic metabolites (like benznidazole) [8] able to damage essential biomolecules of parasites. Nevertheless, the classical mechanisms of action proposed for nifurtimox and benznidazole are currently under revision [9] and consequently, that of our nitroheterocycles need to be investigated more thoroughly. Inhibition of trypanothione reductase has also been suggested according to molecular modelling studies carried out with 7-nitroquinoxalin-2-one derivatives [6].

Considering the interest of 3-alkoxy-1-alkyl-5-nitroindazole scaffold [2–4] in the field of antichagasic agents, and that ω-(dialkylamino)alkyl chains are found in some of the most active reported indazoles [2,3] and quinoxalines [6], in this article we describe the synthesis and antichagasic properties of a new family of 5-nitroindazole-derived primary, secondary and tertiary amines

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(**2**, **3**, **5**–**8**); the reported low solubility of some indazole derivatives containing lipophilic substituents [4] led us to introduce in the present compounds an oxaalkyl chain directed to improve their solubility in aqueous media. Compounds **1** and **4** were also prepared and tested as simple models containing the 3-alkoxy-1-alkyl-5-nitroindazole scaffold (Fig. 1).

Antichagasic properties were initially evaluated *in vitro* against epimastigote, amastigote and trypomastigote forms of *T. cruzi*; Vero cells were used in order to determine unspecific cytotoxicity of our compounds. This study was complemented by infectivity assays on Vero cells carried out with products showing highest activity and selectivity index (SI) values, i.e., compounds **1** and **2**. *In vivo* trypanocidal activities of these compounds in a murine model of acute and chronic phases of Chagas disease were also determined.

Furthermore, a ^{1}H NMR study has been conducted in order to observe changes in the nature and percentage of metabolites excretion directed to obtain information about the effect of our compounds on the glycolytic pathway of *T. cruzi* epimastigotes; finally, we have also studied the ultrastructural alterations of parasite epimastigotes treated with our compounds using transmission electron microscopy (TEM).

2. Results and discussion

2.1. Chemistry

Compounds **1** and **4** were prepared by alkylation of 1-methyl-5-nitroindazol-3-ol **9** (Scheme 1). As expected [10,11], mixtures of N_1,*O*- and N_1,N_2-dialkyl derivatives were obtained, but chromatographical separation of 3-alkoxy-1-alkylindazoles (**1**, **4**) from the isomeric 1,2-dialkylindazolinones (**10**, **11**) was very simple.

Compounds **2**, **3**, **5**–**8** were prepared according to the pathway shown in the Scheme 1. The key intermediate is indazolium-3-olate **14**, belonging to a class of betaines which synthesis [12] and reactivity [13] have previously been studied by some of us. These betaines are easily available by cyclization of 2-halogenobenzohydrazides such as **13** which, in turn, was obtained by acylation of 4-aminomorpholine with the corresponding acid chloride **12** [12].

Treatment of betaine **14** with hydrobromic acid [13] yielded ω-(3-hydroxyindazolyl)alkyl bromide **15**. Alkylation of this compound with benzyl bromide or methyl iodide afforded, as expected for 1-substituted indazol-3-ols [10,11], mixtures of *O*- (**16**, **17**) and N_2-alkyl (**18**, **19**) derivatives; partial halogen exchange (Finkelstein reaction), completed by treatment with sodium iodide in acetone, takes also place during the methylation reaction (NMR). ω-(3-Alkoxyindazolyl)alkyl halides **16** and **17**, chromatographically separated from the corresponding 1,2-disubstituted indazolinones **18** and **19**, were then treated with an excess of methylamine in ethanol to afford secondary amines **2** and **5**, respectively. The latter, treated with an additional equivalent of the required halides **16** or **17**, yielded tertiary amines **3** and **6**.

Finally, treatment of iodide **17** with an excess of ammonia in ethanol gave primary amine **7** as the main reaction product, along with a small amount of secondary amine **8**; both compounds were readily separated by chromatography.

Since the control of the reaction of alkyl halides with ammonia or primary amines is difficult, this method is not usually recommended [14] for the preparation of primary or secondary amines, respectively; in our case, however, these processes were very clean, affording primary amine **7** and secondary amines **2** and **5** with excellent yields.

The structure of all compounds has been established on the basis of analytical and spectral data. Hydrazides such as **13** appear in solution (NMR) as mixtures of *Z* and *E* rotamers owing to restricted rotation around the N–CO bond [12]. In this case, the *Z* rotamer/*E* rotamer ratios, determined by ^{1}H NMR as previously reported for related compounds [12], are *ca.* 58:42 and 65:35 in $CDCl_3$ and $(CD_3)_2SO$, respectively.

As previously observed for related betaines [12], the presence on the quaternary nitrogen atom of compound **14** hinders the conformational equilibrium of morpholine ring. Thus the anisochronic NCH_2 protons can be easily distinguished in the ^{1}H NMR spectrum as H_{ax} and H_{eq} by comparison with the spectra of previously described piperidine analogues [12]. A similar effect has been observed for OCH_2 protons but in this case the assignment of the signals is not easy, and they have been distinguished in the spectral description as H_A and H_B.

On the other hand, 3-alkoxy-1-alkylindazoles (**1**, **4**, **16**, **17**) can be easily distinguished by NMR from the isomeric 1,2-disubstituted indazolinones (**10**, **11**, **18**, **19**, respectively) also arising from the alkylation of 1-substituted indazol-3-ols [11].

2.2. In vitro anti-T. cruzi evaluation

In vitro activity of compounds **1**–**8** on epimastigotes, amastigotes and trypomastigotes [15,16] of *T. cruzi*, the unspecific cytotoxicity against Vero cells and the corresponding selectivity indexes (SI) are gathered in Table 1. First we prepared an epimastigotes (extracellular insect vector stage) culture and in a further step we infected Vero cells with metacyclic forms of parasite, which were

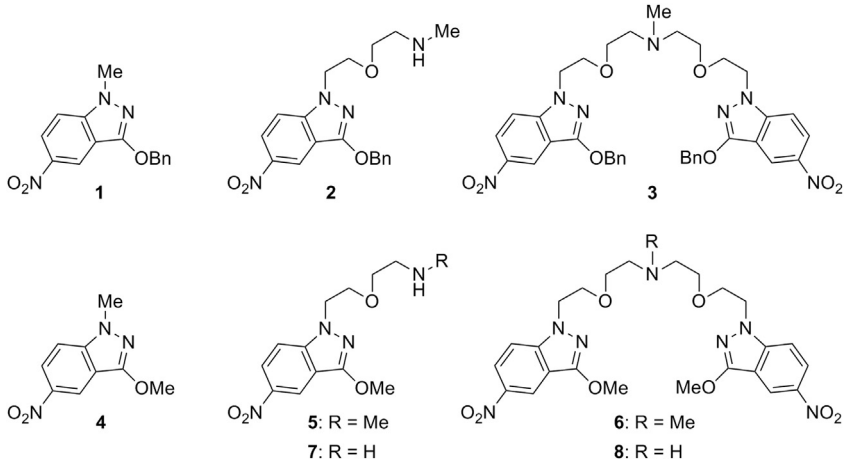
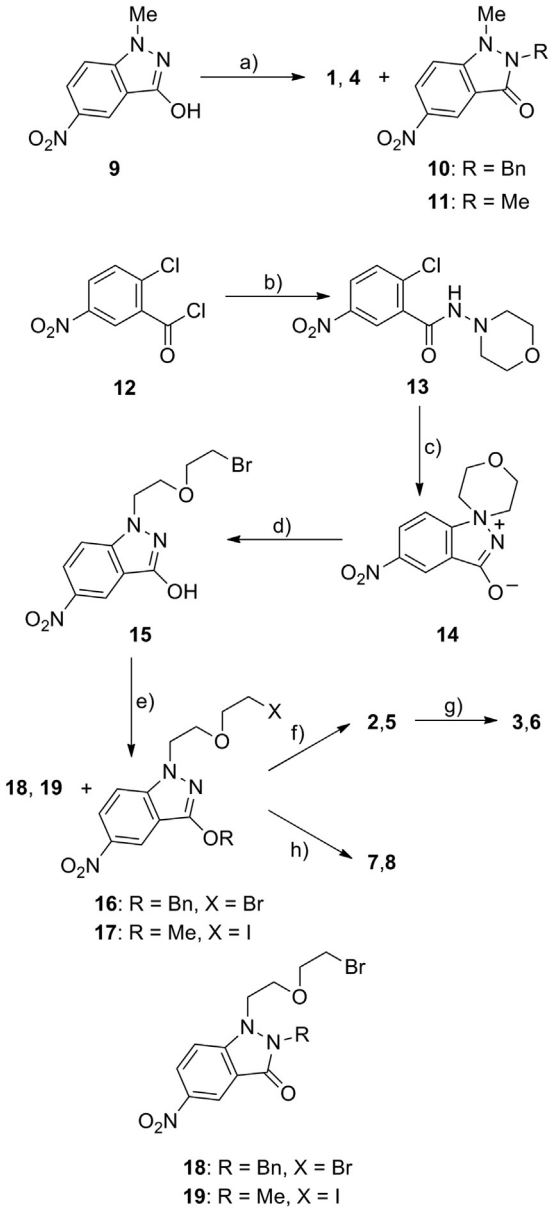


Fig. 1. Chemical structure of indazole derivatives **1**–**8** studied in the present work.



Reagents and conditions: a) for **1** and **10**: BnBr, K_2CO_3, acetone, reflux (5 h), 79% (**1**), 12% (**10**); for **4** and **11**: MeI, K_2CO_3, acetone, rt (24 h) followed by reflux (1 h), 53% (**4**), 45% (**11**); b) 4-aminomorpholine, Et_2O/aq 0.6 M $NaHCO_3$, rt (2 h), 80%; c) $NaHCO_3$, EtOH, reflux (24 h), 95%; d) 48% aq HBr, reflux (3 h), 82%; e) for **16** and **18**: BnBr, K_2CO_3, acetone, rt (24 h) followed by reflux (2 h), 67% (**16**), 10% (**18**); for **17** and **19**: MeI, K_2CO_3, acetone, rt (24 h) followed by reflux (2 h) and then NaI, acetone, reflux (3 h), 50% (**17**), 42% (**19**); f) 33% $MeNH_2$/EtOH, rt (24 h), 92% (**2**), 93% (**5**); g) for **3**: **2**, **16**, K_2CO_3, CH_3CN, reflux (48 h), 85%; for **6**: **5**, **17**, K_2CO_3, CH_3CN, reflux (48 h), 83%; h) **17**, sat NH_3/EtOH, 70 °C (reactor) (72 h), 86% (**7**), 12% (**8**).

Scheme 1. Synthesis of final indazole derivatives **1**–**8**, intermediates **13**–**17** and secondary reaction products **10**, **11**, **18** and **19**.

converted into amastigotes (intracellular mammalian host forms). Activity against trypomastigotes (extracellular peripheral blood mammalian host stage) was determined using infected mice blood collected after infection with the parasite. Compounds **1**–**8** as well as the standard drug benznidazole were assayed at concentrations of 1–100 μM and from the obtained data IC_{50} values shown in Table 1 were calculated.

All compounds show high activities against the three parasite forms, of the same order or slightly lower (up to ca. one half) or higher than those of the reference drug. 3-Benzyloxy derivatives are in general more active than the corresponding 3-methoxy analogues (compare **1** vs **4**, **2** vs **5** and **3** vs **6**). This effect has previously been observed for other 3-alkoxy-1-alkylindazoles [2,3]; since for related 3-BnO and 3-MeO derivatives the reduction potentials corresponding to formation of nitro anion radical in aprotic solvents are similar (ca. −1.2 V) [3,17], we assume, as suggested for 1,2-disubstituted indazolinones [4], that more lipophilic 3-benzyloxy derivatives are better substrates for parasite nitroreductases than 3-methoxy compounds. On the other hand, differences between activities of a primary amine and the corresponding *N*–Me derivative (**7** vs **5**), or between indazole and bisindazole derivatives (**2** vs **3**, **5** vs **6** and **7** vs **8**) are scarce. Great differences are found, however, in the unspecific cytotoxicity against Vero cells and, of course, in the selectivity indexes (SI) resulting from these data; we are not able to find, however, a clear relationship between toxicity and structure. Taking into account all data included in Table 1, compounds **1** and **2**, with SI values exceeding 14–23 times those of benznidazole depending on the parasite stage are the best compounds; on the other hand, compound **8** shows rather good trypanocidal activity but it is also highly toxic for Vero cells, thus resulting SI values ($SI < 1$) so regrettable as those of benznidazole, currently used for the treatment of Chagas disease. Trypanocidal activities of the same order have been published for related 1-[ω-(dialkylamino)alkyl]indazole derivatives; nevertheless, data provided for the latter are heterogeneous (changes in the *T. cruzi* strain and in the model of host cells, differences in the way of expressing trypanocidal activity and unspecific cytotoxicity data, SI values not determined, etc.) [2,3] and a direct comparison with those obtained in this article is difficult. Unfortunately, this situation is rather frequent as has been stressed by some authors [16].

On the other hand, high activities found for the simple indazole derivatives **1** and **4** are very interesting and merit further research; in fact, low activity values against *T. cruzi* epimastigotes have been reported for 1-methyl-3-phenethoxy- and 1-methyl-3-(2-naphthylmethoxy)-5-nitroindazole, closely related analogues of compound **1** [4].

With the aim of obtaining more accurate information about the products showing highest activity and SI values in the previously described assays, compounds **1** and **2**, the spreading of the parasite in Vero cells was studied by measuring the rates of infection and the average number of amastigotes and trypomastigotes present during a 10-days treatment (Fig. 2). Vero cells were infected with metacyclic forms of *T. cruzi* and the gradual conversion of the latter into amastigotes was observed in the invaded cells. During the mentioned period the rate of infection of the host cells gradually increased reaching at the end of experiment 99% of invasion (Fig. 2A).

The test was repeated in the presence of the reference drug and of compounds **1** and **2** at their IC_{25} concentrations. It was found that the rate of infection decreased in all cases in relation to the control, showing compounds **1** and **2**, with reductions in the infection rate of 82% and 78% respectively, a much higher efficiency than benznidazole (19% of decrease).

Concerning the average number of amastigotes per Vero cell (Fig. 2B), the results were consistent with those mentioned above for infection rates. After treatment, compounds **1** and **2** significantly reduced the number of amastigotes per cell in 76% and 63%, respectively, while benznidazole showed only a decrease of 37%.

In relation to the number of trypomastigotes/mL found in the culture medium (Fig. 2C), a maximum was reached in the control on day 10 (ca. 9.5×10^3), but this value was substantially reduced by

Table 1
In vitro activity, unspecific cytotoxicity and selectivity index (SI) found for 5-nitroindazole derivatives **1**–**8** on extra- and intracellular forms of *T. cruzi*.

Compounds	*T. cruzi* IC_{50} (μM)[a]			Vero cells IC_{50} (μM)[a]	SI[b]		
	Epimastigote forms	Intracellular amastigote forms	Trypomastigote forms		Epimastigote forms	Intracellular amastigote forms	Trypomastigote forms
Benznidazole	15.9 ± 1.1	23.3 ± 4.6	16.4 ± 3.2	13.6 ± 0.9	0.8	0.6	0.8
1	17.6 ± 0.7	14.1 ± 1.4	12.0 ± 1.3	193.7 ± 14.9	11.0 (14)	13.7 (23)	16.1 (20)
2	19.1 ± 0.9	16.6 ± 0.9	14.5 ± 0.5	213.6 ± 21.5	11.2 (14)	12.9 (21)	14.7 (18)
3	26.6 ± 1.4	16.5 ± 0.6	16.5 ± 0.8	128.4 ± 8.8	4.8 (6)	7.8 (13)	7.8 (10)
4	24.7 ± 1.8	32.6 ± 2.2	24.8 ± 1.8	183.1 ± 11.4	7.4 (9)	5.6 (9)	7.4 (9)
5	26.7 ± 2.0	23.4 ± 4.0	20.5 ± 1.3	87.6 ± 7.4	3.3 (4)	3.7 (6)	4.3 (5)
6	24.6 ± 0.8	27.9 ± 2.1	20.4 ± 0.7	129.6 ± 9.5	4.1 (5)	4.6 (8)	6.3 (8)
7	27.3 ± 2.4	26.5 ± 1.7	20.7 ± 1.7	87.2 ± 5.5	3.2 (4)	3.3 (5)	4.2 (5)
8	31.2 ± 2.6	25.7 ± 2.0	26.4 ± 2.3	16.9 ± 1.1	0.5 (1)	0.6 (1)	0.6 (1)

[a] IC_{50} = the concentration required to give 50% inhibition, calculated by linear regression analysis from the *Kc* values at concentrations employed (1, 10, 25, 50 and 100 μM).
[b] Selectivity index = IC_{50} Vero cells/IC_{50} extracellular and intracellular forms of parasite. In brackets: number of times that compound SI exceeds the reference drug SI on the different morphological forms of parasite.

the reference drug (41%) and, especially, by compounds **1** and **2** (80% and 75%, respectively). In summary, the results of spreading of parasite in Vero cells are in agreement with those of trypanocidal activity reported in Table 1 for intracellular and extracellular forms of *T. cruzi*.

2.3. In vivo anti-T. cruzi evaluation

Considering the good results obtained in the previously mentioned *in vitro* assays, *in vivo* activity of compounds **1** and **2** was studied on infected female BALB/c mice. Drugs currently used against Chagas disease are effective in the acute phase of the disease, but its efficacy in the chronic phase is very controversial. It was therefore decided to evaluate the activity of our compounds **1** and **2** on murine models of both phases, using benznidazole as the reference drug. For experiments in the acute phase, the first 30 days after infection were considered, while the effect on the chronic phase was studied between 30 and 120 days after infection. Mice were inoculated with metacyclic forms of *T. cruzi* and treatment with test compounds (1 mg/kg/day doses) was initiated by intraperitoneal route 5 days after infection and continued for 5 additional days. The study included different control groups as described in the Experimental section. During the study of the activity in the acute phase, the level of parasitemia was determined every 3–4 days. Fig. 3 shows the number of circulating trypomastigotes/mL of blood vs days elapsed since infection. On the day of maximum parasite load (ca. 14 days after infection) all tested compounds significantly decreased the number of trypomastigotes. On day 30, at the end of the acute phase observation period, compounds **1** and **2** reduced the level of parasitemia by 33% and 39%, respectively, values significantly higher than that found for the reference drug (23%). No mouse died in any of our experiments performed either with the control or with compounds **1** and **2** at the doses used; however, the survival percentage for the mice treated with benznidazole was only about 80%.

In connection with the activity in the chronic phase of the disease, Table 2 shows the differences in the level of anti-*T. cruzi* antibodies between 30 and 120 days after infection followed by the mentioned treatment. As can be seen, compound **1** and especially **2**, were much more effective than benznidazole, reducing significantly the antibodies levels with respect to the control.

2.4. Metabolites excretion study

It is well established that trypanosomatids are unable to completely degrade glucose to CO_2 under aerobic conditions. Their characteristic catabolism is mediated by glycolytic enzymes most of them placed in the organelle called the glycosome [18]. Consequently, they excrete into the medium a considerable part of the hexose skeleton as partially oxidised fragments in the form of fermented metabolites. The nature and percentage of catabolism compounds depend on the pathway used for glucose metabolism by each trypanosomatid species and the life-cycle stage considered, but CO_2, succinate, acetate, L-lactate, pyruvate, L-alanine and ethanol are usually produced [19,20]. Succinate is important because its main role is to maintain the glycosomal redox balance, which allows the reoxidation of NADH produced in the glycolytic pathway. Succinic fermentation has the advantage of requiring only half of the phosphoenolpyruvate produced to maintain the NAD^+/NADH balance, while the remaining pyruvate is converted inside the mitochondrion and the cytosol into acetate, L-lactate, L-alanine or ethanol, according to the degradation pathway followed by each species [20].

To gain information concerning the effect of our indazole derivatives on glucose metabolism in the parasites, the final excretion products were identified. These data were obtained by recording the ^1H NMR spectra of epimastigotes from *T. cruzi* after treatment with the studied compounds at their IC_{25} concentrations. The results were compared with a control of epimastigotes maintained in a cell-free medium for four days after inoculation with the parasite, showing the characteristic signals of CH_3 of ethanol, acetate, L-alanine and L-lactate, and CH_2 signal of succinate.

When *T. cruzi* epimastigotes were treated with compounds **1** and **2**, the succinate excretion appeared clearly decreased (35% and 13%, respectively), probably showing the disruption of glycosomal or mitochondrial enzymes involved in its biosynthesis [20] (Table 3, Fig. S1).

On the other hand, L-lactate, L-alanine and ethanol originate from pyruvate; compound **1** seems to inhibit cytosolic enzymes responsible for the transformation of pyruvate to L-lactate (unknown enzyme) (23% of reduction) and L-alanine (alanine aminotransferase) (19% of reduction) thereby forcing its metabolism to ethanol (pyruvate decarboxylase and NAD-linked alcohol dehydrogenase) (11% of increase) [20]. Among the pyruvate-derived metabolites, compound **2** only affects the lactate levels (17% of decrease), those of alanine and ethanol remaining unchanged. Moreover, acetate excretion does not change after treatment with compounds **1** and **2**, showing the absence of disturbances of enzymes responsible for the production of this metabolite at mitochondrial level [20].

2.5. Ultrastructural alterations

Ultrastructural alterations induced on *T. cruzi* epimastigotes by indazole derivatives **1** and **2** at their IC_{25} concentrations have been studied by transmission electron microscopy (TEM) (Fig. 4). One of

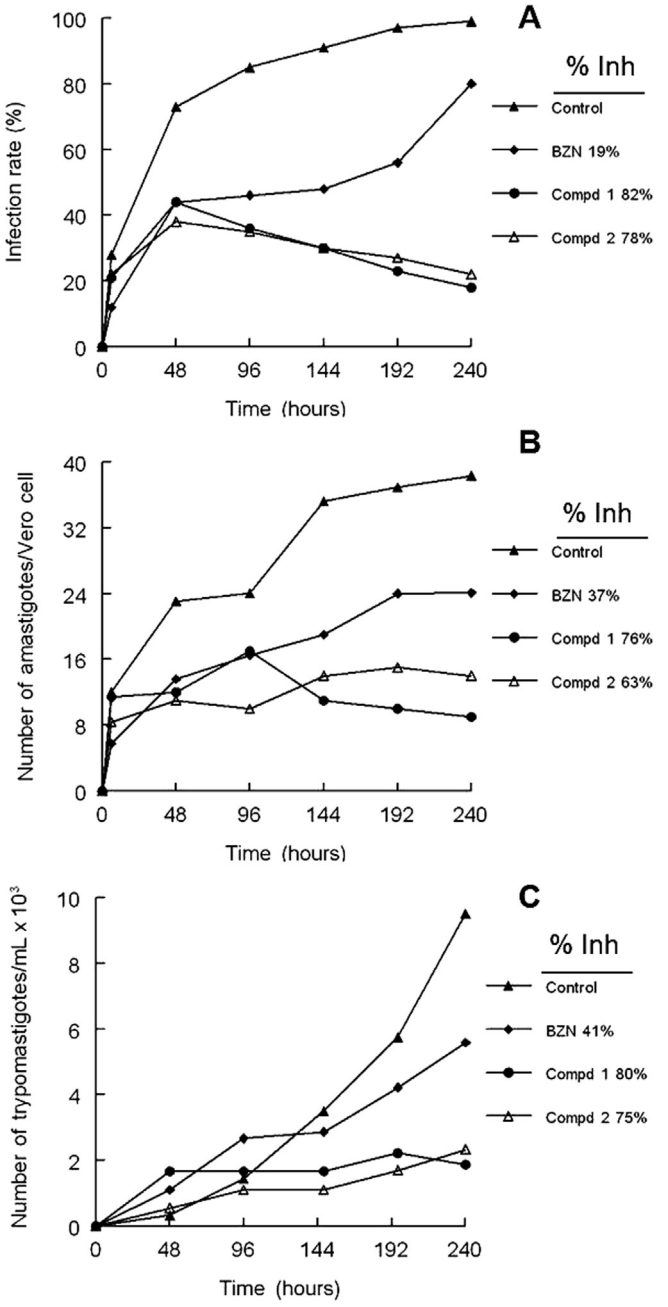


Fig. 2. Effect of benznidazole (BZN) and 5-nitroindazole derivatives **1** and **2** (IC_{25} concentrations) on the infection rate and growth of *T. cruzi*: (A) rate of infection; (B) mean number of amastigotes per infected Vero cell; (C) number of trypomastigotes/mL in the growth medium.

evident morphological changes was the presence of very little electron-dense cytoplasm observed in the majority of treated protozoa; another very significant alteration was a large vacuolization (V), with some lipidic type vacuoles occupying in some cases most of the cytoplasm (figure not shown). Mitochondria (M) and glycosomes (G) were damaged or decreased, in agreement with the metabolic changes described in the previous section. They are also very frequent parasites showing appearance of being dead (DP) and the death of a large number of parasites. These phenomena are also observed when *T. cruzi* is treated with benznidazole, causing disruption of the parasite cytoskeleton with disorganization of the microtubule structure and plasma membrane with scalloped

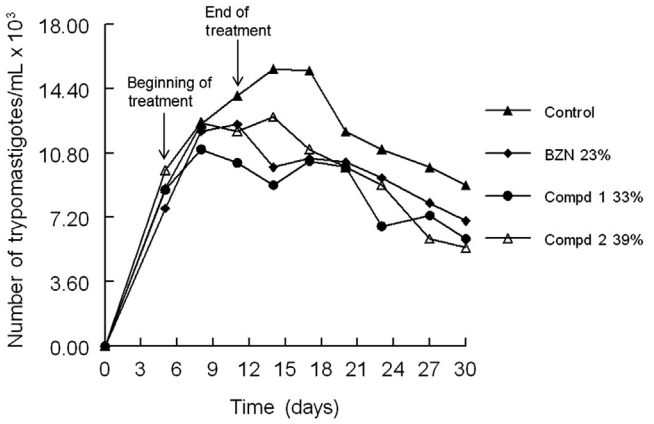


Fig. 3. Parasitemia in the murine model of acute Chagas disease: effect of benznidazole (BZN) and 5-nitroindazole derivatives **1** and **2** (1 mg/kg/day doses during 5 days).

Table 2
Differences in the level of anti-*T. cruzi* antibodies between days 30 and 120 post-infection for benznidazole and for derivatives **1** and **2**.

Compounds[a]	ΔA[b]
Control (untreated)	0.146
Benznidazole	0.110
1	−0.025
2	−0.141

[a] Dose of 1 mg/kg/day for five consecutive days, administered by the intraperitoneal route.
[b] ΔA = absorbance at 490 nm on day 120 post-infection − absorbance at 490 nm on day 30 post-infection (expressed in absorbance units).

appearance, with undulations and discontinuities, perhaps as a result of the mentioned altered cytoskeleton.

3. Conclusions and future outlooks

The biological evaluation has shown that all compounds tested exhibit adequate antichagasic activity against the three studied morphological stages of *T. cruzi*; although the establishment of a clear structure–activity relationship is difficult, the more lipophilic 3-OBn derivatives show, in general, higher activities than the corresponding 3-OMe analogues. Moreover, the large differences observed in the cytotoxicity of the compounds against Vero cells leads to dramatic differences in the values of selectivity indexes in relation to those of the antichagasic standard drug benznidazole; thus compounds **1** and **2** stand out for their high effectiveness, further confirmed through additional infectivity assays and an *in vivo* murine model of Chagas disease. Unfortunately, trypanocidal activity data provided previously for other indazole derivatives are very heterogeneous and a direct comparison with those obtained in the present article cannot be carried out. Nevertheless,

Table 3
Variation in the ^{1}H NMR peaks corresponding to catabolites excreted by *T. cruzi* epimastigotes in the presence of 5-nitroindazole derivatives **1** and **2** with respect to the control test.[a]

Compounds	Lac	Ala	A	S	Eth
1	−23%	−19%	=	−35%	+11%
2	−17%	=	=	−13%	=

(−) peak decrease; (+) peak increase; (=) no difference detected.
[a] Lac, L-lactate; Ala, L-alanine; A, acetate; S, succinate; Eth, ethanol.

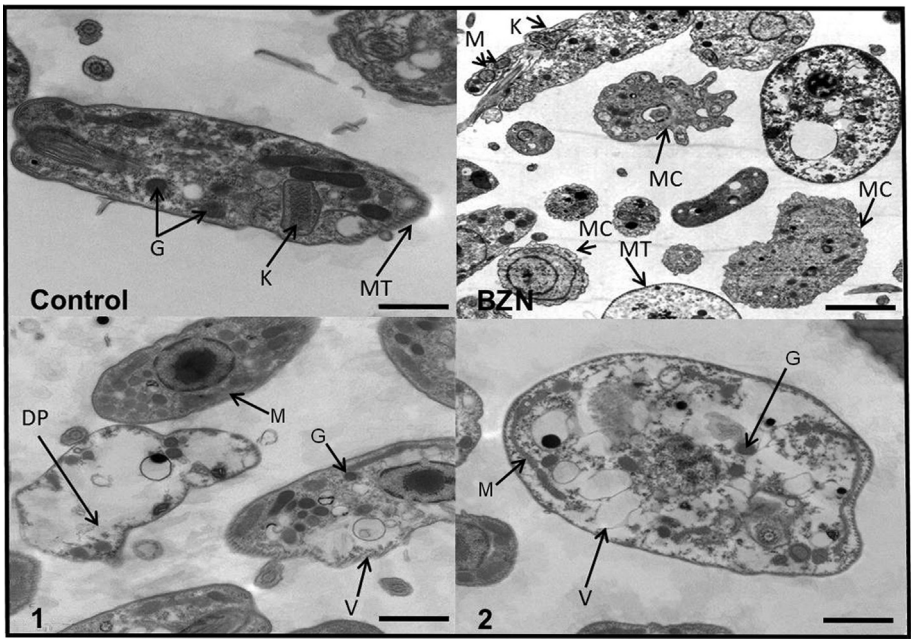


Fig. 4. Ultrastructural alterations observed by TEM in epimastigotes of *T. cruzi* either untreated (control) or treated with 5-nitroindazole derivatives **1** and **2**, showing organelles with their characteristics: kinetoplast (K); vacuoles (V); mitochondrion (M); glycosomes (G); cytoskeleton microtubules (MT); altered cytoplasmic membrane (MC); dead parasites (DP). Control: untreated parasites, bar: 1 μm; BZN: parasites treated with benznidazole, bar: 1.59 μm; 1: parasites treated with compound **1**, bar: 1 μm. 2: parasites treated with compound **2**, bar: 1 μm.

although much work remains to be done in order to increase our knowledge in the field of structure–activity relationships, the potential of indazole scaffold in the development of new antichagasic drugs is confirmed by our present results.

We suggest here, mainly on the basis of metabolic changes caused by indazoles **1** and **2**, that these compounds may interfere with some glycosomal or mitochondrial enzymes involved in the catabolism of *T. cruzi*. For the future, in order to continue our studies, we have planned the synthesis of other 5-nitroindazoles, including fluorescent derivatives that could label the organelles constituting their primary target, as well as the identification of the products arising from the bioreduction of 5-nitroindazoles mediated by trypanosomal nitroreductases. In fact, related studies recently carried out for nifurtimox and benznidazole [9] have greatly advanced the understanding of their mode of action and allowed banishing old assumptions.

4. Experimental section

4.1. Chemistry

4.1.1. General methods

Melting points (mp) were determined in a Stuart Scientific melting point apparatus SMP3. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on a Bruker Avance 300 spectrometer. The chemical shifts are reported in ppm from TMS (δ scale) but were measured against the solvent signal [$(CD_3)_2SO$: δ_H 2.49, δ_C 39.50; $CDCl_3$: δ_H 7.24, δ_C 77.00]. The assignments have been performed by means of different standard homonuclear and heteronuclear correlation experiments (NOE, gHSQC and gHMBC). To simplify the description of the NMR spectra, bis(indazolylalkyl) amines have been numbered as simple compounds. Electron impact (EI) and electrospray (ES^+) mass spectra were obtained on a Hewlett Packard 5973 MSD (70 eV) and on a Hewlett Packard 1100 MSD spectrometer, respectively. DC-Alufolien silica gel 60 PF_{254} (Merck, layer thickness 0.2 mm) was used for TLC, and silica gel 60 (Merck, particle size 0.040–0.063 mm) for flash column chromatography. Solvents and reagents were obtained from different commercial sources and used without further purification. Microanalyses were performed on a Heraeus CHN-O-RAPID analyzer and were within the ±0.3% of the theoretical values.

4.1.2. 3-Benzyloxy-1-methyl-5-nitro-1H-indazole (*1*)

A mixture of 1-methyl-5-nitro-1*H*-indazol-3-ol (**9**) [4,13] (1.93 g, 10 mmol), benzyl bromide (1.88 g, 11 mmol) and potassium carbonate (4.00 g, excess) in acetone (100 mL) was refluxed for 5 h. Inorganic salts were removed by filtration and, after evaporation of acetone and addition of water (100 mL), the crude mixture of benzyl derivatives was extracted with chloroform (3 × 50 mL). This extract was concentrated and applied to the top of a column which was eluted first with chloroform to afford the desired 3-benzyloxy derivative **1** (2.24 g, 79%); mp 130–132 °C (2-propanol) [21]; R_f 0.25 ($CHCl_3$), 0.89 ($CHCl_3$–MeOH 10:1). ^{1}H NMR ($CDCl_3$): δ 8.66 (d, J = 2.1 Hz, 1H, 4-H), 8.21 (dd, J = 9.3, 2.1 Hz, 1H, 6-H), 7.50 (m, 2H, Ph 2-, 6-H), 7.39 (m, 3H, Ph 3-, 4-, 5-H), 7.21 (d, J = 9.3 Hz, 1H, 7-H), 5.41 (s, 2H, OCH_2), 3.92 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$): δ 157.33 (C-3), 142.80 (C-7a), 140.66 (C-5), 136.07 (Ph C-1), 128.51 (Ph C-3, -5), 128.30 (Ph C-4), 128.03 (Ph C-2, -6), 122.41 (C-6), 118.53 (C-4), 111.80 (C-3a), 108.43 (C-7), 70.97 (OCH_2), 35.47 (CH_3); MS (EI): *m*/*z* (%) 283 (30) $[M]^+$, 206 (1), 192 (3), 150 (2), 146 (3), 104 (3), 103 (3), 91 (100).

Further elution of the column with a chloroform–methanol (30:1) mixture afforded the known [11] 2-benzyl-1-methyl-5-nitro-1,2-dihydro-3*H*-indazol-3-one (**10**) (0.34 g, 12%); R_f 0.00 ($CHCl_3$), 0.78 ($CHCl_3$–MeOH 10:1).

4.1.3. 3-Methoxy-1-methyl-5-nitro-1H-indazole (*4*)

A mixture of 1-methyl-5-nitro-1*H*-indazol-3-ol (**9**) [4,13] (1.16 g, 6 mmol), methyl iodide (2 mL, excess) and potassium carbonate (1.93 g, 14 mmol) in acetone (50 mL) was stirred at room temperature for 24 h and then refluxed for 1 h. The reaction mixture was treated as described for compound **1**, affording the known [13]

3-methoxy derivative **4** (0.66 g, 53%) [R_f 0.30 ($CHCl_3$), 0.86 ($CHCl_3$–MeOH 10:1)] and 1,2-dimethyl-5-nitro-1,2-dihydro-3*H*-indazol-3-one (**11**) (0.56 g, 45%) [R_f 0.00 ($CHCl_3$), 0.44 ($CHCl_3$–MeOH 10:1)].

Alternatively, direct alkylation of 5-nitro-1*H*-indazol-3-ol [22] (1.07 g, 6 mmol) with an excess of methyl iodide following the same procedure afforded 0.80 g (64%) of 3-methoxy-1-methylindazole **4** and 0.41 g (33%) of the mentioned 1,2-dimethylindazolinone **11**.

4.1.4. 2-Chloro-N-morpholino-5-nitrobenzamide (**13**)

A solution of 2-chloro-5-nitrobenzoyl chloride (**12**) [12] (14.30 g, 65 mmol) in dry diethyl ether (200 mL) was added at room temperature during 30 min to a well stirred solution of 4-aminomorpholine (6.64 g, 65 mmol) in 0.6 M aq sodium hydrogencarbonate (150 mL). After 2 h the ether was evaporated, and the solid in suspension collected by filtration, washed with water (150 mL) and air-dried (14.86 g, 80%); mp 198–200 °C (decomp.) (ethanol); R_f 0.49 ($CHCl_3$–MeOH 10:1). ^{1}H NMR ($CDCl_3$): δ 8.40 (d, J = 2.7 Hz, 1H, 6-H, *Z* and *E* rot.), 8.21 (*Z* rot.) and 8.15 (*E* rot.) (both dd, J = 8.8, 2.7 Hz) (1H, 4-H), 7.59 (*Z* rot.) and 7.58 (*E* rot.) (both d, J = 8.8 Hz) (1H, 3-H), 6.78 (*Z* rot.) and 6.67 (*E* rot.) (both br s) (1H, NH), 3.86 (m, *Z* rot.) and 3.51 (br m, *E* rot.) (4H, 2′-, 6′-H), 3.00 (m, *Z* rot.) and 2.79 (br m, *E* rot.) (4H, 3′-, 5′-H) (*Z* rot./*E* rot. ratio: 58/42); ^{13}C NMR [$(CD_3)_2SO$, signals of *Z* rot. (65% of the mixture)]: δ 161.57 (CO), 146.03 (C-5), 137.40 (C-2), 136.72 (C-1), 131.24 (C-3), 125.59 (C-4), 123.80 (C-6), 65.85 (C-2′, -6′), 54.60 (C-3′, -5′); MS (EI): *m*/*z* (%) 285 (2) $[M]^+$, 254 (4), 201 (12), 184 (29), 138 (21), 110 (18), 101 (100), 85 (53). Anal. calcd. for $C_{11}H_{12}ClN_3O_4$ (285.68): C 46.25; H 4.23; N 14.71. Found: C 46.38; H 4.10; N 14.48.

4.1.5. 5-Nitro-1H-indazol-1-spiro-4′-morpholinium-3-olate (**14**)

A mixture of hydrazide **13** (4.28 g, 15 mmol) and sodium hydrogencarbonate (3.36 g, 40 mmol) in ethanol (200 mL) was refluxed for 24 h. After cooling, the reaction mixture was absorbed on silica gel and applied to the top of a column of the same material which was eluted with chloroform–methanol mixtures (10:1 to 5:1), affording the title betaine (3.55 g, 95%); mp 234–238 °C (ethanol); R_f 0.12 ($CHCl_3$–MeOH 10:1). ^{1}H NMR [$(CD_3)_2SO$]: δ 8.58 (dd, J = 8.7, 2.1 Hz, 1H, 6-H), 8.51 (dd, J = 8.7, 0.6 Hz, 1H, 7-H), 8.23 (dd, J = 2.1, 0.6 Hz, 1H, 4-H), 4.31 (m, 4H, 2′-, 6′-H_A and 3′-, 5′-H_{ax}), 4.05 (m, 2H, 2′-, 6′-H_B), 3.09 (m, 2H, 3′-, 5′-H_{eq}); ^{13}C NMR [$(CD_3)_2SO$]: δ 169.41 (C-3), 157.39 (C-7a), 149.96 (C-5), 132.70 (C-3a), 126.17 (C-6), 119.11 (C-7), 118.06 (C-4), 63.51 (C-3′, -5′), 62.53 (C-2′, -6′); MS (EI): *m*/*z* (%) 249 (47) $[M]^+$, 219 (36), 218 (37), 191 (100), 161 (52), 146 (33), 145 (45), 133 (21), 117 (32), 103 (23), 90 (55). Anal. calcd. for $C_{11}H_{11}N_3O_4$ (249.22): C 53.01; H 4.45; N 16.86. Found: C 53.08; H 4.49; N 16.79.

4.1.6. 5-(3-Hydroxy-5-nitro-1H-indazol-1-yl)-3-oxapentyl bromide (**15**)

A mixture of betaine **14** (7.73 g, 31 mmol) and 48% aq hydrobromic acid (50 mL) was refluxed for 3 h. After cooling and addition of water (100 mL), the precipitated solid was collected by filtration, washed with plenty water and air-dried (8.39 g, 82%); mp 185–189 °C (ethanol); R_f 0.46 ($CHCl_3$–MeOH 10:1). ^{1}H NMR [$(CD_3)_2SO$]: δ 11.45 (br s, 1H, OH), 8.63 (dd, J = 2.2, 0.5 Hz, 1H, 4′-H), 8.13 (dd, J = 9.3, 2.2 Hz, 1H, 6′-H), 7.65 (dd, J = 9.3, 0.5 Hz, 1H, 7′-H), 4.39 (t, J = 5.3 Hz, 2H, 5-H), 3.81 (t, J = 5.3 Hz, 2H, 4-H), 3.64 (t, J = 5.5 Hz, 2H, 2-H), 3.43 (t, J = 5.5 Hz, 2H, 1-H); ^{13}C NMR [$(CD_3)_2SO$]: δ 156.44 (C-3′), 142.74 (C-7′a), 139.81 (C-5′), 121.44 (C-6′), 118.41 (C-4′), 111.45 (C-3′a), 110.40 (C-7′), 70.12 (C-2), 68.51 (C-4), 48.13 (C-5), 31.99 (C-1); MS (EI): *m*/*z* (%) 331 (20) $[M+2]^+$, 329 (20) $[M]^+$, 285 (1), 249 (16), 218 (3), 206 (22), 192 (100), 176 (14), 160 (6), 146 (50), 107 (7), 91 (6). Anal. calcd. for $C_{11}H_{12}BrN_3O_4$ (330.13): C 40.02; H 3.66; N 12.73. Found: C 39.97; H 3.68; N 12.81.

4.1.7. 5-(3-Benzyloxy-5-nitro-1H-indazol-1-yl)-3-oxapentyl bromide (**16**)

A mixture of bromide **15** (3.30 g, 10 mmol), benzyl bromide (1.88 g, 11 mmol) and potassium carbonate (4.00 g, excess) in acetone (100 mL) was stirred at room temperature for 24 h and then refluxed for 2 h. Inorganic salts were removed by filtration and, after evaporation of acetone and addition of water (100 mL), the crude mixture of benzyl derivatives was extracted with chloroform (3 × 50 mL). This extract was dried ($MgSO_4$), absorbed on silica gel and applied to the top of a column which was eluted first with hexane in order to remove excess of benzyl bromide and then with chloroform to afford the title compound **16** (2.82 g, 67%); mp 91–93 °C (2-propanol); R_f 0.25 ($CHCl_3$), 0.76 ($CHCl_3$–MeOH 50:1). ^{1}H NMR [$(CD_3)_2SO$]: δ 8.51 (d, J = 2.2 Hz, 1H, 4′-H), 8.17 (dd, J = 9.4, 2.2 Hz, 1H, 6′-H), 7.72 (d, J = 9.4 Hz, 1H, 7′-H), 7.56 (m, 2H, Ph 2-, 6-H), 7.39 (m, 3H, Ph 3-, 4-, 5-H), 5.43 (s, 2H, 3′-OCH_2), 4.47 (t, J = 5.0 Hz, 2H, 5-H), 3.83 (t, J = 5.0 Hz, 2H, 4-H), 3.62 (t, J = 5.6 Hz, 2H, 2-H), 3.41 (t, J = 5.6 Hz, 2H, 1-H); ^{13}C NMR [$(CD_3)_2SO$]: δ 156.74 (C-3′), 143.22 (C-7′a), 140.32 (C-5′), 136.28 (Ph C-1), 128.42 (Ph C-3, -5), 128.16 (Ph C-4), 128.12 (Ph C-2, -6), 121.92 (C-6′), 117.39 (C-4′), 110.85 (C-3′a), 110.73 (C-7′), 70.54 (3′-OCH_2), 70.09 (C-2), 68.46 (C-4), 48.43 (C-5), 31.98 (C-1); MS (ES^+): *m*/*z* 444 $[M+2 + Na]^+$, 442 $[M + Na]^+$, 422 $[M+2 + H]^+$, 420 $[M + H]^+$. Anal. calcd. for $C_{18}H_{18}BrN_3O_4$ (420.26): C 51.44; H 4.32; N 10.00. Found: C 51.38; H 4.33; N 10.05.

Further elution of the column with a chloroform–methanol (50:1) mixture yielded several byproducts among which we were able to identify 5-(2-benzyl-5-nitro-3-oxo-1,2-dihydro-3*H*-indazol-1-yl)-3-oxapentyl bromide (**18**) (0.42 g, 10%); mp 106–108 °C (ethanol); R_f 0.00 ($CHCl_3$), 0.26 ($CHCl_3$–MeOH 50:1). ^{1}H NMR ($CDCl_3$): δ 8.81 (d, J = 2.2 Hz, 1H, 4′-H), 8.34 (dd, J = 9.0, 2.2 Hz, 1H, 6′-H), 7.34–7.16 (m, 6H, 7′-H, PhH), 5.19 (s, 2H, 2′-CH_2), 4.02 (t, J = 4.9 Hz, 2H, 5-H), 3.47 (t, J = 5.7 Hz, 2H, 2-H), 3.44 (t, J = 4.9 Hz, 2H, 4-H), 3.15 (t, J = 5.7 Hz, 2H, 1-H); ^{13}C NMR [$(CD_3)_2SO$]: δ 161.09 (C-3′), 149.90 (C-7′a), 141.04 (C-5′), 136.16 (Ph C-1), 128.70 (Ph C-3, -5), 127.78 (Ph C-4), 127.26 (Ph C-2, -6), 126.58 (C-6′), 120.21 (C-4′), 115.17 (C-3′a), 112.67 (C-7′), 69.99 (C-2), 67.03 (C-4), 46.90 (C-5), 44.89 (2′-CH_2), 31.93 (C-1); MS (EI): *m*/*z* (%) 421 (21) $[M+2]^+$, 419 (21) $[M]^+$, 375 (2), 282 (7), 177 (4), 131 (3), 109 (4), 107 (4), 91 (100). Anal. calcd. for $C_{18}H_{18}BrN_3O_4$ (420.26): C 51.44; H 4.32; N 10.00. Found: C 51.24; H 4.57; N 10.12.

4.1.8. 5-(3-Methoxy-5-nitro-1H-indazol-1-yl)-3-oxapentyl iodide (**17**)

A mixture of bromide **15** (1.98 g, 6 mmol), methyl iodide (2 mL, excess) and potassium carbonate (1.93 g, 14 mmol) in acetone (50 mL) was stirred at room temperature for 24 h and then refluxed for 2 h. Inorganic salts were removed by filtration and, after evaporation of acetone and addition of water (100 mL), the mixture of O- and N_2-methyl derivatives was extracted with chloroform (3 × 50 mL). This extract was dried ($MgSO_4$), concentrated and applied to the top of a column which was eluted first with chloroform to afford a 22/78 mixture (^{1}H NMR, molar ratio) of the expected oxapentyl bromide and the corresponding iodide **17**. In order to complete the halogen exchange, this mixture was dissolved in a saturated solution of sodium iodide in acetone (15 mL) and refluxed for 3 h. The solvent was evaporated to dryness, water (50 mL) was added and the solid in suspension was extracted with chloroform (3 × 50 mL). Evaporation of dried ($MgSO_4$) organic layer afforded pure iodide **17** (1.17 g, 50%); mp 86–88 °C (methanol); R_f 0.12 ($CHCl_3$), 0.86 ($CHCl_3$–MeOH 10:1). ^{1}H NMR ($CDCl_3$): δ 8.59 (d, J = 2.0 Hz, 1H, 4′-H), 8.19 (dd, J = 9.3, 2.0 Hz, 1H, 6′-H), 7.35 (d, J = 9.3 Hz, 1H, 7′-H), 4.37 (t, J = 5.1 Hz, 2H, 5-H), 4.08 (s, 3H, OCH_3), 3.86 (t, J = 5.1 Hz, 2H, 4-H), 3.59 (t, J = 6.5 Hz, 2H, 2-H), 3.06 (t, J = 6.5 Hz, 2H, 1-H); ^{13}C NMR [$(CD_3)_2SO$]: δ 157.52 (C-3′), 143.32 (C-

7′a), 140.27 (C-5′), 121.94 (C-6′), 117.36 (C-4′), 110.81 (C-7′), 110.68 (C-3′a), 70.57 (C-2), 68.18 (C-4), 56.52 (OCH_3), 48.44 (C-5), 5.10 (C-1); MS (EI): *m/z* (%) 391 (38) $[M]^+$, 264 (1), 220 (17), 206 (100), 190 (7), 174 (6), 160 (42), 155 (9), 131 (3), 103 (6), 90 (4), 89 (4). Anal. calcd. for $C_{12}H_{14}IN_3O_4$ (391.16): C 36.85; H 3.61; N 10.74. Found: C 36.93; H 3.40; N 10.81.

Further elution of the column with a chloroform–methanol (50:1) mixture yielded N_2-methyl derivatives [22/78 mixture (^{1}H NMR, molar ratio) of oxapentyl bromide and the corresponding iodide], which after treatment with sodium iodide in acetone as described afforded 5-(2-methyl-5-nitro-3-oxo-1,2-dihydro-3*H*-indazol-1-yl)-3-oxapentyl iodide (**19**) (0.98 g, 42%); mp 127–129 °C (2-propanol); R_f 0.00 ($CHCl_3$), 0.55 ($CHCl_3$–MeOH 10:1). ^{1}H NMR ($CDCl_3$): δ 8.72 (d, J = 2.2 Hz, 1H, 4′-H), 8.33 (dd, J = 9.2, 2.2 Hz, 1H, 6′-H), 7.26 (d, J = 9.2 Hz, 1H, 7′-H), 4.15 (t, J = 4.9 Hz, 2H, 5-H), 3.58 (t, J = 4.9 Hz, 2H, 4-H), 3.50 (s, 3H, CH_3), 3.48 (t, J = 6.2 Hz, 2H, 2-H), 2.96 (t, J = 6.2 Hz, 2H, 1-H); ^{13}C NMR [$(CD_3)_2SO$]: δ 160.07 (C-3′), 148.36 (C-7′a), 140.72 (C-5′), 126.29 (C-6′), 120.05 (C-4′), 115.10 (C-3′a), 112.01 (C-7′), 70.55 (C-2), 66.97 (C-4), 46.62 (C-5), 29.06 (CH_3), 4.91 (C-1); MS (EI): *m/z* (%) 391 (87) $[M]^+$, 375 (1), 273 (1), 220 (1), 206 (100), 192 (9), 190 (11), 177 (4), 160 (51), 155 (21), 146 (9), 131 (10), 119 (2), 104 (5), 103 (5), 91 (4). Anal. calcd. for $C_{12}H_{14}IN_3O_4$ (391.16): C 36.85; H 3.61; N 10.74. Found: C 36.83; H 3.52; N 10.63.

Alternatively, halogen exchange can be completed before chromatographic separation of *O*- and N_2-methyl derivatives.

4.1.9. *N-Methyl-5-(3-benzyloxy-5-nitro-1H-indazol-1-yl)-3-oxapentylamine (**2**)*

A mixture of bromide **16** (1.68 g, 4 mmol) and a 33% (ca. 8 M) solution of methylamine in ethanol (50 mL), was stirred at room temperature for 24 h. The mixture was then evaporated to dryness and, after addition of 2 N aq hydrochloric acid (50 mL), extracted with diethyl ether (3 × 50 mL). Solid potassium carbonate was added to the acidic layer until pH 10 and the resulting suspension extracted with chloroform (3 × 50 mL). The organic layer was separated, dried ($MgSO_4$) and evaporated to afford practically pure amine **2** (1.36 g, 92%); mp 84–86 °C (2-propanol); R_f 0.00 ($CHCl_3$), 0.08 ($CHCl_3$–MeOH 10:1). ^{1}H NMR [$(CD_3)_2SO$]: δ 8.52 (d, J = 2.1 Hz, 1H, 4′-H), 8.18 (dd, J = 9.2, 2.1 Hz, 1H, 6′-H), 7.72 (d, J = 9.2 Hz, 1H, 7′-H), 7.55 (m, 2H, Ph 2-, 6-H), 7.40 (m, 3H, Ph 3-, 4-, 5-H), 5.43 (s, 2H, 3′-OCH_2), 4.46 (t, J = 4.9 Hz, 2H, 5-H), 3.75 (t, J = 4.9 Hz, 2H, 4-H), 3.34 (t, J = 5.6 Hz, 2H, 2-H), 2.41 (t, J = 5.6 Hz, 2H, 1-H), 2.10 (s, 3H, CH_3); ^{13}C NMR [$(CD_3)_2SO$]: δ 156.76 (C-3′), 143.27 (C-7′a), 140.32 (C-5′), 136.27 (Ph C-1), 128.44 (Ph C-3, -5), 128.20 (Ph C-4), 128.18 (Ph C-2, -6), 121.89 (C-6′), 117.45 (C-4′), 110.79 (C-3′a), 110.75 (C-7′), 70.56 (3′-OCH_2), 69.67 (C-2), 68.64 (C-4), 50.61 (C-1), 48.59 (C-5), 35.94 (CH_3); MS (ES^+): *m/z* 393 $[M + Na]^+$, 371 $[M + H]^+$. Anal. calcd. for $C_{19}H_{22}N_4O_4$ (370.40): C 61.61; H 5.99; N 15.13. Found: C 61.35; H 6.10; N 15.07.

4.1.10. *N-Methyl-5-(3-methoxy-5-nitro-1H-indazol-1-yl)-3-oxapentylamine (**5**)*

Starting from iodide **17** (0.59 g, 1.5 mmol) and a 33% (ca. 8 M) solution of methylamine in ethanol (30 mL), following the procedure described for compound **2**, pure amine **5** was obtained (0.41 g, 93%); mp 71–73 °C (hexane) [hydrochloride (**5**× HCl), mp 202–204 °C (ethanol)]; R_f 0.04 ($CHCl_3$–MeOH 10:1). ^{1}H NMR ($CDCl_3$) δ 8.59 (d, J = 2.1 Hz, 1H, 4′-H), 8.18 (dd, J = 9.3, 2.1 Hz, 1H, 6′-H), 7.30 (d, J = 9.3 Hz, 1H, 7′-H), 4.35 (t, J = 5.1 Hz, 2H, 5-H), 4.07 (s, 3H, OCH_3), 3.81 (t, J = 5.1 Hz, 2H, 4-H), 3.44 (t, J = 5.1 Hz, 2H, 2-H), 2.56 (t, J = 5.1 Hz, 2H, 1-H), 2.26 (s, 3H, NCH_3); ^{13}C NMR ($CDCl_3$): δ 158.35 (C-3′), 143.60 (C-7′a), 140.78 (C-5′), 122.20 (C-6′), 118.37 (C-4′), 111.83 (C-3′a), 109.23 (C-7′), 70.47 (C-2), 69.44 (C-4), 56.45 (OCH_3), 51.09 (C-1), 49.16 (C-5), 36.17 (NCH_3); MS (EI): *m/z* (%) 295 (5) $[M + H]^+$, 294 (1) $[M]^+$, 263 (8), 251 (7), 237 (7), 219 (100), 206 (29), 193 (49), 174 (18), 160 (20), 149 (7), 131 (4), 117 (5), 103 (14), 89 (8). Anal. calcd. for $C_{13}H_{18}N_4O_4$ (294.31): C 53.05; H 6.16; N 19.04. Found: C 53.19; H 6.01; N 19.15.

4.1.11. *N-Methylbis[5-(3-benzyloxy-5-nitro-1H-indazol-1-yl)-3-oxapentyl]amine (**3**)*

A mixture of amine **2** (0.89 g, 2.4 mmol), bromide **16** (1.01 g, 2.4 mmol) and potassium carbonate (0.35 g, 2.5 mmol) in acetonitrile (100 mL) was refluxed for 48 h. After filtration of salts and evaporation of solvent, the desired product was isolated by column chromatography; elution with chloroform–methanol (100:1 to 50:1) mixtures afforded the title tertiary amine **3** (1.45 g, 85%); R_f 0.00 ($CHCl_3$), 0.36 ($CHCl_3$–MeOH 10:1). Mp (96–98 °C) as well as analytical and some spectral data [^{1}H NMR and MS (ES^+)] have previously been reported [23]. ^{13}C NMR [$(CD_3)_2SO$]: δ 156.70 (C-3′), 143.18 (C-7′a), 140.24 (C-5′), 136.25 (Ph C-1), 128.42 (Ph C-3, -5), 128.17 (Ph C-4), 128.13 (Ph C-2, -6), 121.80 (C-6′), 117.35 (C-4′), 110.75 (C-3′a), 110.72 (C-7′), 70.52 (3′-OCH_2), 68.66 (C-2), 68.53 (C-4), 56.45 (C-1), 48.59 (C-5), 42.40 (CH_3).

4.1.12. *N-Methylbis[5-(3-methoxy-5-nitro-1H-indazol-1-yl)-3-oxapentyl]amine (**6**)*

This compound was prepared as described for 3-benzyloxy analogue **3**, starting from amine **5** (0.53 g, 1.8 mmol), iodide **17** (0.70 g, 1.8 mmol) and potassium carbonate (0.28 g, 2 mmol) in acetonitrile (50 mL); column chromatography, eluted with dichloromethane-methanol mixtures (50:1 to 20:1), afforded the desired compound **6** (0.83 g, 83%); R_f 0.31 ($CHCl_3$–MeOH 10:1). Mp (122–124 °C) as well as analytical and some spectral data [^{1}H NMR and MS (EI)] have previously been reported [23]. ^{13}C NMR ($CDCl_3$) δ 158.31 (C-3′), 143.57 (C-7′a), 140.74 (C-5′), 122.17 (C-6′), 118.28 (C-4′), 111.77 (C-3′a), 109.46 (C-7′), 69.57 (C-2), 69.41 (C-4), 57.12 (C-1), 56.49 (OCH_3), 49.20 (C-5), 42.88 (NCH_3).

4.1.13. *5-(3-Methoxy-5-nitro-1H-indazol-1-yl)-3-oxapentylamine (**7**) and bis[5-(3-methoxy-5-nitro-1H-H-indazol-1-yl)-3-oxapentyl] amine (**8**)*

A stirred suspension of iodide **17** (1.14 g, 2.91 mmol) in a saturated solution of ammonia in ethanol (30 mL) was heated in an autoclave at 70 °C for 72 h. The mixture was then evaporated to dryness and, after addition of 10% aq potassium carbonate (50 mL), extracted with chloroform (3 × 50 mL). The organic layer was dried ($MgSO_4$), concentrated and applied to the top of a flash chromatography column which was eluted with chloroform–methanol mixtures (20:1 to 5:1) to afford, in this elution order, secondary amine **8** (94 mg, 12%) and primary amine **7** (700 mg, 86%).

Compound **7**: mp 70–72 °C (previous sintering) (hexane); R_f 0.06 ($CHCl_3$–MeOH 10:1). ^{1}H NMR [$(CD_3)_2SO$]: δ 8.46 (d, J = 2.0 Hz, 1H, 4′-H), 8.17 (dd, J = 9.5, 2.0 Hz, 1H, 6′-H), 7.70 (d, J = 9.5 Hz, 1H, 7′-H), 4.45 (t, J = 4.9 Hz, 2H, 5-H), 4.04 (s, 3H, OCH_3), 3.76 (t, J = 4.9 Hz, 2H, 4-H), 3.28 (t, J = 5.5 Hz, 2H, 2-H), 2.48 (t, J = 5.5 Hz, 2H, 1-H); ^{13}C NMR [$(CD_3)_2SO$]: δ 157.47 (C-3′), 143.27 (C-7′a), 140.22 (C-5′), 121.88 (C-6′), 117.36 (C-4′), 110.67 (C-7′), 110.55 (C-3′a), 72.83 (C-2), 68.55 (C-4), 56.48 (OCH_3), 48.53 (C-5), 41.17 (C-1); MS (ES^+): *m/z* 303 $[M + Na]^+$, 281 $[M + H]^+$. Anal. calcd. for $C_{12}H_{16}N_4O_4$ (280.28): C 51.42; H 5.75; N 19.99. Found: C 51.57; H 5.89; N 19.72.

Compound **8**: mp 105–107 °C (2-propanol); R_f 0.37 ($CHCl_3$–MeOH 10:1). ^{1}H NMR [$(CD_3)_2SO$]: δ 8.40 (d, J = 2.1 Hz, 2H, 4′-H), 8.08 (dd, J = 9.3, 2.1 Hz, 2H, 6′-H), 7.59 (d, J = 9.3 Hz, 2H, 7′-H), 4.38 (t, J = 4.9 Hz, 4H, 5-H), 4.01 (s, 6H, OCH_3), 3.69 (t, J = 4.9 Hz, 4H, 4-H), 3.24 (t, J = 5.4 Hz, 4H, 2-H), 2.35 (t, J = 5.4 Hz, 4H, 1-H); ^{13}C NMR [$(CD_3)_2SO$]: δ 157.43 (C-3′), 143.17 (C-7′a), 140.12 (C-5′), 121.74 (C-6′), 117.25 (C-4′), 110.57 (C-7′), 110.53 (C-3′a), 70.00 (C-2), 68.59 (C-4), 56.43 (OCH_3), 48.49 (C-5), 48.39 (C-1); MS (ES^+): *m/z* 566

$[M + Na]^+$, 544 $[M + H]^+$. Anal. calcd. for $C_{24}H_{29}N_7O_8$ (543.53): C 53.03; H 5.38; N 18.04. Found: C 52.96; H 5.34; N 17.91.

4.2. Biological evaluation

4.2.1. Parasite strain culture

Epimastigotes of *T. cruzi* SN3 strain (IRHOD/CO/2008/SN3) isolated from domestic *Rhodnius prolixus* from Guajira (Colombia) [24] were cultivated *in vitro* in medium trypanosomes liquid (MTL) with 10% inactivated foetal bovine serum and were kept in an air atmosphere at 28 °C in Roux flasks (Corning, USA) with a surface area of 75 cm^2, according to a previously described methodology [25].

4.2.2. Cell culture and cytotoxicity tests

Vero cells (EACC number 84113001) originally obtained from monkey kidney were grown in RPMI (Gibco), supplemented with 10% inactivated foetal bovine serum in a humidified 95% air, 5% CO_2 atmosphere at 37 °C for two days. The cytotoxicity test for Vero cells was performed according to a previously described methodology [25]. After 72 h of treatment, cell viability was determined by flow cytometry. Thus, 100 μL/well of propidium iodide solution (100 mg/mL) was added and incubated for 10 min at 28 °C in darkness. Afterwards, 100 μL/well of fluorescein diacetate (100 ng/mL) was added and incubated under the same conditions. Finally, the cells were recovered by centrifugation at 400 g for 10 min and the precipitate washed with phosphate buffered saline (PBS). Flow cytometric analysis was performed with a FACSVantage flow cytometer (Becton Dickinson). The percentage viability was calculated in comparison with the control culture. The IC_{50} was calculated using linear regression analysis from the *Kc* values of the concentrations employed (1–100 μM).

4.2.3. In vitro activity: epimastigotes assay (extracellular forms)

According to a reported procedure [25], the obtained compounds and the reference drug (benznidazole) were dissolved in dimethyl sulfoxide, which at a final concentration of 0.01% was shown to be nontoxic and without inhibitory effects on parasite growth. The compounds were added to the culture medium at dosages of 100, 50, 25, 10 and 1 μM. The effects of each compound against *T. cruzi* epimastigotes were tested at 72 h using a Neubauer haemocytometric chamber. The antichagasic effect is expressed as the IC_{50}, i.e. the concentration required to give 50% of growth inhibition, calculated by linear regression analysis from the *Kc* values of the concentrations employed. Results gathered in Table 1 are averages of four separate experiments.

4.2.4. In vitro activity: amastigotes assay (intracellular forms)

Vero cells were grown in RPMI (Gibco) supplemented with 10% inactivated foetal bovine serum and were kept in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were seeded at a density of 1×10^4 cells/well in 24-well microplates (Nunc) with rounded coverslips on the bottom and cultured for 2 days. Afterwards the cells were infected *in vitro* with metacyclic forms [26] of *T. cruzi* at a ratio of 10:1 during 24 h. The non-phagocytosed parasites were removed by washing, and then the drugs (at 100, 50, 25, 10 and 1 μM) were added. Vero cells with the drugs were incubated for 72 h at 37 °C in 5% CO_2. Drug activity was determined on the basis of number of amastigotes in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. The number of amastigotes was determined by analysing 200 host cells distributed in randomly chosen microscopic fields. The antichagasic effect is expressed as the IC_{50}. Results given in Table 1 are averages of four separate experiments.

4.2.5. In vitro activity: trypomastigotes assay (extracellular forms)

Metacyclogenesis was induced by culturing a 5-day-old culture of epimastigotes that was harvested by centrifugation at 7000 g for 10 min at 10 °C. The parasites were then incubated for 2 h at 28 °C at a density of 5×10^8 cells/mL in TAU medium (190 mM NaCl, 17 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 8 mM phosphate buffer, pH 6.0). Thereafter, the parasites were incubated at a 1:100 dilution (final epimastigotes concentration: 5×10^6 cells/mL) for 96 h at 28 °C in TAU3AAG medium (TAU supplemented with 10 mM L-proline, 50 mM sodium L-glutamate, 2 mM sodium L-aspartate and 10 mM D-glucose) in 25 mL culture flasks with a layer of culture medium that was not more than 1 cm in depth [27].

The activity (% of parasites reduction) was compared with the control following a reported methodology [28] with some minor modifications performed in our laboratory. The assay of drug activity against *T. cruzi* was carried out using blood from Balb/c albino mice collected during the parasitemia peak (7th day) after infection with the SN3 strain of *T. cruzi*. The infected blood was diluted with non-infected murine blood to the concentration of 4×10^6 trypomastigotes/mL and then diluted to 1:2 in RPMI 1640 medium–GIBCO (2×10^6 trypomastigotes/mL). Stock solutions of the compounds were prepared in dimethyl sulfoxide. A sample of infected blood and drugs (100, 50, 25, 10 and 1 μM) were added to the well of a 96-microwell plate providing a final volume of 200 μL in order to calculate IC_{50} values. To reproduce the blood bank conditions, plates were incubated at 4–8 °C for 24 h. The experiments were repeated three times. Each solution was examined microscopically for parasite counting using the Neubauer chamber.

4.2.6. In vitro activity: infection assays

Vero cells were grown under the same conditions expressed in the amastigotes assay during two days. Afterwards, the cells were infected *in vitro* with metacyclic forms of *T. cruzi* at a ratio of 10:1. The drugs (IC_{25} concentrations) were added immediately after infection and were incubated for 12 h at 37 °C in 5% CO_2. The non-phagocytosed parasites and the drugs were removed by washing, and then the infected cultures were grown for 10 days in fresh medium. Fresh culture medium was added every 48 h. The drug activity was determined from the percentage of infected cells and the number of amastigotes per infected cell and that of trypomastigotes in the medium was also determined [29], in treated and untreated cultures, in methanol-fixed and Giemsa-stained preparations. The percentage of infected cells and the mean number of amastigotes per infected cell were determined by analysing 200 host cells distributed in randomly chosen microscopic fields. Values represented in Fig. 2 are the means of three separate experiments; in all cases experimental values fall within two standard deviations from the mean.

4.2.7. In vivo trypanocidal activity assay

Groups of three BALB/c female mice (6–8 weeks old, 20–25 g) maintained under standard conditions were infected with 1×10^5 *T. cruzi* metacyclic forms by the intraperitoneal route. The animals were divided into the following groups: (I) group 1: uninfected (not infected and not treated); (II) group 2: untreated (infected with *T. cruzi* but not treated); (III) group 3: uninfected [not infected and treated with 1 mg/kg body weight/day, for five consecutive days (5–10 days postinfection) by the intraperitoneal route] [30] and (IV) group 4: treated [infected and treated for five consecutive days (5–10 days postinfection) with the tested compounds and benznidazole]. This animal experiment was performed with the approval of the ethical committee of the University of Granada.

A blood sample (5 μL) drawn from the mandibular vein of each treated mouse was taken and diluted 1:15 (50 μL of citrate buffer:0.1 M citric acid, 0.1 M sodium citrate and 20 μL of lysis

buffer at pH 7.2:2 M Tris–Cl, $MgCl_2$). The parasites were counted by the Neubauer chamber. The number of bloodstream *T. cruzi* forms was recorded every 3–4 days from 5 to 30 days postinfection. The number of trypomastigote forms was expressed as trypomastigotes/mL. The obtained mean values are gathered in Fig. 3; experimental values lie in each case within two standard deviations from the mean.

Circulating anti-*T. cruzi* antibodies, at days 30 and 120 postinfection, were evaluated quantitatively by an enzyme-linked immunoassay. The blood, diluted to 1:50 in PBS, was reacted with an antigen composed of an excreted Fe–SOD of *T. cruzi* epimastigotes [31]. The results were expressed as the ratio of the absorbance of each sample at 490 nm to the cutoff value. The cutoff for each reaction was the mean of the values determined for the negative controls plus three times the standard deviation.

4.2.8. Metabolites excretion study

Cultures of *T. cruzi* epimastigotes (initial concentration 5×10^5 cells/mL) received IC_{25} concentrations of the compounds (except for control cultures). After incubation for 96 h at 28 °C, the cells were centrifuged at 400 g for 10 min. The supernatants were collected to determine the excreted metabolites using ^1H NMR; chemical shifts were expressed in parts per million (ppm, δ scale), using sodium 2,2-dimethyl-2-silapentane-5-sulphonate as the reference signal. The chemical shifts used to identify the respective metabolites were consistent with those previously described [32].

4.2.9. Ultrastructural alterations

Epimastigotes of *T. cruzi* were cultured at a density of 5×10^5 cells/mL in the corresponding medium containing the compounds tested at their IC_{25} concentrations. After 96 h, these cultures were centrifuged at 400 g for 10 min, and the pellets produced were washed in PBS and then mixed with 2% (v/v) paraformaldehyde/glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 4 h at 4 °C. Following this, the pellets were prepared for transmission electron microscopy (TEM) study using a previously described technique [33].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.ejmech.2013.12.025. These data include MOL files and InChiKeys of the most important compounds described in this article.

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