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Original article

Activity of a hydroxybibenzyl bryophyte constituent against *Leishmania* spp. and *Trypanosoma cruzi*: In silico, in vitro and in vivo activity studies

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Abstract

The synthesis and potent antiprotozoal activity of 14-hydroxylunularin, a natural hydroxybibenzyl bryophyte constituent is reported. 14-Hydroxylunularin was highly active in vitro assays against culture and intracellular forms of *Leishmania* spp. *and Trypanosoma. cruzi*, in absence of cytotoxicity against mammalian cells. Preliminary structure–activity relationship studies showed that the reported bioactivity depends on hybridization at the carbon–carbon bridge, position and number of free hydroxy group on the aromatic rings. The reported results were also in agreement with the in silico prediction using Non-Stochastic Quadratic Fingerprints-based algorithms. The same compound also showed antiprotozoal activity in *Leishmania* spp. infected mice by oral and subcutaneous administration routes, with an optimal treatment of a daily subcutaneous administration of 10 mg/kg of body weight for 15 days. This study suggested that 14-hydroxylunularin may be chosen as a new candidate in the development of leishmanicidal therapy.

Keywords: *Leishmania*; *Trypanosoma cruzi*; Bryophytes; Hydroxybibenzyl constituents; TOMOCOMD software

1. Introduction

The discovery and development of essential drugs for neglected diseases such as Chagas' and Leishmaniasis is a major concern in the pharmaceutical world. Recent reviews on the chemotherapy of Chagas' disease and American cutaneous leishmaniasis stress the deficiencies of the currently available therapeutic agents and the urgent need for new candidates [1,2]. Leishmaniasis is a group of tropical diseases caused by parasites of about 20 species of the genus *Leishmania*, which were transmitted by a group of 50 species and subspecies of phebotomine insects [3,4]. Official data show that there are 12 million infected people around the world, 350 million at risk of acquiring the disease, and 1.5–2 million that will be infected annually [5,6]. Leishmaniasis showed a complex and diverse clinical manifestations and epidemiology [7].

Currently used drugs for treatment of human cutaneous leishmaniasis are quite toxic and cause severe side effects

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such as pancreatitis and cardiac toxicity that have therefore limited their use. Conventional therapy consists in the parenteral administration of pentavalent antimonium [sodium stibogluconate or Pentostam® (GSK) and meglumine antimoniate or Glucantime® (Aventis)] during 28 days, generally under strict medical supervision, making the treatment unaffordable for most patients.

The emergence of antimony-resistant strains of *Leishmania donovani* is alarming. For example, up to 60% of the patients in Bihar state (India) do not respond to antimonials [8,9].

The most significant recent advance in leishmaniasis therapy came from the oral treatment with Miltefosine® (Zentaris), although its use is restricted due to teratogenicity. This drug is currently in Phase IV clinical trials for visceral and cutaneous leishmaniasis [10].

On the other hand, the World Health Organization has identified Chagas' disease as a major and increasing problem in public health, particularly in Latin America [6,11], where 18–20 million people in 17 countries are infected, and almost 40 million are exposed to the infection of *Trypanosoma cruzi* transmitted by triatomine vectors, blood transfusion or congenital. In the past the pattern of the disease was almost exclusively rural, nevertheless, the rising levels of urbanization and emigration extended the disease into an urban environment. Medication for Chagas' disease is usually effective only when given during the acute stage of infection. Once the disease has progressed to later stages, no medication has consistently proven to be effective. As happens in *Leishmania*, the current synthetic drugs such as nifurtimox (a nitrofuran derivative) and benznidazole (a nitroimidazole derivative), are associated to severe side effects, including cardiac and/or renal toxicity, which accounts for the need to search new effective chemotherapeutic and chemo prophylactic agents against *T. cruzi* [12,13].

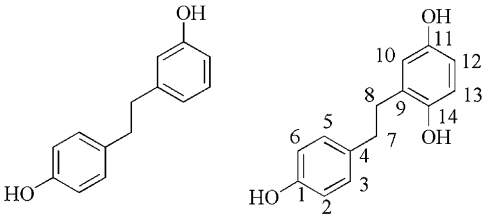
The handling and screening of large databases to find reduced sets of potential new active compounds is possible [14,15]. Thus, the development of computational approaches based on discrimination functions plays an important role, allowing the previous in silico identification from large chemical libraries of structural subsystems responsible for a property or biological activity with a considerable reduction of costs, time and efforts to select a new drug candidate.

The purpose of this work is to demonstrate the in vitro and in vivo activity of a hydroxybibenzyl bryophyte constituent against *Leishmania* spp. and *T. cruzi* predicted through in silico studies.

2. Chemistry

Hydroxybibenzyl derivatives are endowed with a large variety of biological effects including: antifungal, antimicrobial, and cytotoxic activities, among others [16–20]. In particular, liverworts are a rich source of hydroxybibenzyl compounds [21,22]; such as lunularin (**1**) [23,24] and 14-hydroxylunularin (**2**) [25] (Fig. 1).

As a part of our work in the synthesis and biological evaluation of natural compounds from bryophytes [26,27],



Lunularin (**1**) 14-Hydroxylunularin (**2**)

Fig. 1. Hydroxybibenzyl constituent of bryophytes.

compound **2** was prepared following a described methodology [28] based on a Wittig olefination with a high to excellent yield. The olefination reaction was carried out according to Boden's protocol [29,30] in order to improve the final yield of compound **4**, required for a multigram scale synthesis (Scheme 1). The reaction proceeds straightforward using K_2CO_3 (2 eq) with 18-crown-6 ether as catalyst in refluxing toluene, giving **4** in 88% yield. Under these conditions, (2,5-dimetoxybenzyl)triphenylphosphonium chloride (**3**) reacted with *p*-anisaldehyde to give the stilbene **4** exclusively as the *E*-isomer. Stilbene **4** was hydrogenated to **5** and further deprotected to obtain 2,5,4′-trihydroxybibenzyl (**2**) at an overall yield of 56% in three steps. Finally, all compounds were characterized by ^1H and ^{13}C NMR studies, and mass spectrometry.

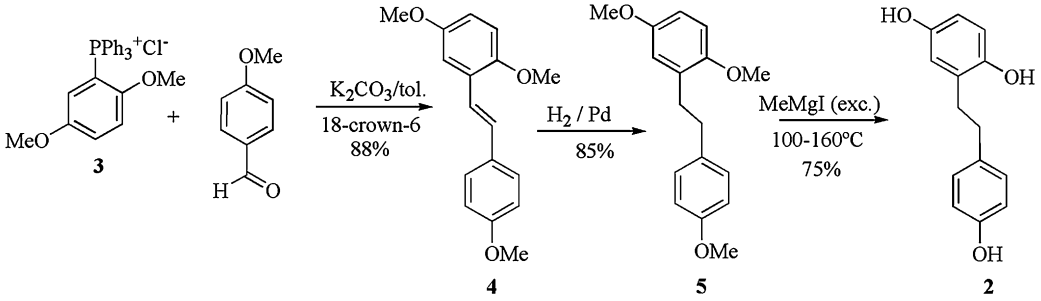
Resveratrol (*trans*-3,5,4′-trihydroxystilbene) and lunularin (**1**), required as a control compounds for the in vitro test, were synthesized according to Yus et al. [31] and Huneck [32], respectively.

3. Computational analysis

A novel strategy to generate molecular fingerprints-based on the application of discrete mathematics and linear algebra theory has been recently developed. The approach affords to carry out rational in silico molecular design (selection/identification) and QSAR/QSPR studies. This algorithm has been applied to the prediction of several physical, physicochemical, chemical, pharmacokinetical parameters as well as biological properties.

In the current study, 14-hydroxylunularin (**2**) was in silico evaluated by the TOMOCOMD strategy [33,34] in order to predict antitrypanosomal and antileishmanial activities. Biological assays were also performed to confirm in vitro activities on both *Leishmania* spp. and *T. cruzi* proliferative forms as well as cytotoxicity on mammalian cells. In addition, the in vivo assays against *Leishmania amazonensis* are here reported, resulting this compound as a new and promising leishmanicidal drug candidate.

In silico predictions were conducted for antitrypanosomal activity using the discriminant function previously reported by some of the present authors [35]. A new model [Eq. (1)] to predict antileishmanial activity of 14-hydroxylunularin (**2**) was generated considering a data set of 168 organic-chemicals (see Section 5).



Scheme 1. Preparation of compound **2**.

$$\text{Class} = -4.49 + 1.37\times10^{-5}\,{}^{H}q_{7}(x) - 0.17\,q_{1\text{L}}(x_{\text{E}}) + 6.72\times10^{-2}\,q_{0\text{L}}(x_{\text{E}}) + 1.44\times10^{-3}\,q_{5\text{L}}(x_{\text{E}}) - 0.27\times10^{-8}\,{}^{H}q_{15\text{L}}(x_{\text{E}}) \quad (1)$$

$N = 120$, $\lambda = 0.316$, $D^2 = 8.49$, $F(5,115) = 49.644$, $p < 0.0001$.

Model 1, generated by Eq. (1), allowed the correct classification of 96.66% of chemicals included in the active training set (58 from 60 compounds) and 90.16% of the inactive group (55 from 61). An overall 93.39% of correct classification was observed for the whole group. Both models (for antitrypanosomal and antileishmanial activities, respectively) predicted the activity of **2** showing a positive expectancy of being active ($\Delta P = 98.33\%$ for antileishmanial and $\Delta P = 28.06\%$ for antitrypanosomal activity). Positive values of $\Delta P\%$ indicate a positive probability of being active and vice versa. Compounds **1**, **5** and resveratrol were also evaluated using Model 1 and the results are compiled in Table 1.

4. Biological results and discussion

According to in silico results, accuracy of the prediction was tested by assaying compound **2** in vitro against epimastigote and amastigote forms of *T. cruzi*, and on promastigote and amastigote stages of *Leishmania* spp. Promastigotes and epimastigotes are the extracellular dividing forms inside the insect vectors of *Leishmania* spp. and *T. cruzi*, respectively. The easily culturable and drug sensitivity of these forms make these models an excellent choice for preliminary in vitro screening [34,36,37]. The results for inhibition of in vitro *Leishmania* proliferation after 72 h of incubation with compound **2** are shown in Table 2. Interestingly, compound **2** showed a similar IC_{50} (1.1 μM) when was tested against both *Leishmania braziliensis* and *L. amazonesis* promastigote strains but a lower IC_{50} value (0.5 μM) when tested on *L. donovani* promastigotes. To note, compound **2** tested in a range of (0.43–434 μM) showed a higher leishmanicidal activity when compared with pentamidine ($IC_{50} = 9.8$ μM), taken as a reference drug. Regardless of the *Leishmania* strain tested, no live parasites could observe beyond 13.03 μM (data not shown).

Table 1
Classification of compounds **1**,**2**,**5** and resveratrol using Model 1 (prediction for antileishmanial activity)

Compounds	$\Delta P\%$[a]
1	97.42
2	98.33
5	62.68
Resveratrol	99.14

[a] $\Delta P\% = [P(\text{active}) - P(\text{inactive})] \times 100$.

Likewise, the in vitro results on *T. cruzi* epimastigote forms evidenced an important trypanocidal activity ($IC_{50} = 5.8$ μM), higher than that obtained with the reference drug benznidazole (54.7 μM). Nevertheless altogether the leishmanicidal activity of compound **2** was consistently higher than that on *T. cruzi* epimastigotes.

Multiplicative forms of the *Leishmania* and *T. cruzi* that appears in humans are amastigotes. For this reason, more selective methods are required to determine the activity of this compound [38]. The antiamastigote assays were performed to determine the activity of **2**, pentamidine and benznidazole against *Leishmania* amastigotes infecting peritoneal macrophages and *T. cruzi* amastigotes infecting NCTC-929 fibroblasts, respectively. Our results (Table 2) showed that compound **2** ($IC_{50} = 1.5$ μM) on intracellular amastigotes of *L. amazonensis* is less potent than the reference drug pentamidine ($IC_{50} = 0.9$ μM) with full disappearance of intracellular amastigotes at concentration higher than 4.34 μM (data not shown). On *T. cruzi* amastigotes, the ranking was reversed, being the IC_{50} of compound **2** more active than the reference drug benznidazol $IC_{50} = 17.3$ μM and 192.1 μM, respectively.

In order to establish a preliminary structure–activity relationship we tested three bibenzyl derivates of **2** against promastigotes form of *Leishmania* spp. as compiled in Table 3. Compounds **1**, **5** and resveratrol showed less activity than 14-hydroxylunularin (**2**) at 100 μg/mL. The results suggest that a chemical structure with sp^3 hybridization at the carbon–carbon bridge and 2,5,4′-substitution with free hydroxy groups on the aromatic rings increased the leishmanicidal and trypanocidal activities in agreement with the recently results from Kedzierski et al. [39].

An important criterion in the search of active compounds with antiprotozoal activity is their toxicity on mammalian host cells [40]. For this purpose, the cytotoxicity was determined using two different cells lines and peritoneal macrophage concentrations ranging from 2.17 to 21.70 μM (Table 4). As observed in the resazurin tests [41], compound **2** showed

Table 2
In vitro activity of 14-hydroxylunularin (**2**) and reference drugs towards extracellular and intracellular forms of *Leishmania* spp. and *T. cruzi*

Extracellular forms					Intracellular forms	
Compounds	Leishmanicidal activity on promastigotes[a]			Antiepimastigote activity of *T. cruzi*	Antiamastigote activity of *Leishmania* spp.	Antiamastigote activity of *T. cruzi*
	L. donovani (PP75)	*L. braziliensis* (M2903)	*L. amazonensis* (PH8)	Clone CL-B5	*L. amazonensis* (PH8)	Clone CL-B5
14-Hydroxylunularin (**2**) (IC_{50},μM)	0.5	1.1	1.1	5.8	1.5	17.3
Pentamidine (IC_{50},μM)	9.8	9.8	9.8	–	0.9	–
Benznidazole (IC_{50},μM)	–	–	–	54.7	–	192.1

[a] Control test was included.

no toxicity at the highest concentration assayed, whereas in the trypan blue exclusion assay, a low toxicity was observed on peritoneal murine macrophages at 21.70 μM with 18.88% of cell taking the dye, reduced to 2.11% at 4.34 μM. Noteworthy the toxicity is very low on logarithmically growing mammalian host cells, within the range of high antiparasitic activity. Due this low value observed for compound **2** at these concentrations, we suggest that the activity described on parasites was the result of a specific toxicity.

Taken into consideration the high potency of compound **2** on promastigote and amastigote forms of *Leishmania* and the absence of cytotoxicity in the different models assayed, the in vivo assays were performed as previously described [42–44]. The outcome of the treatment with intravenous Glucantime and oral and subcutaneous 14-hydroxylunularin (**2**) on *L. amazonensis* infected BALB/c mice are presented in Table 5.

The subcutaneous treatment with antimonial drug Glucantime at 100 mg/kg along 15 days reduced the lesion weight by 72.1% ($P < 0.05$) and the parasite load by 98.2% ($P < 0.05$) compared with the untreated mice. When **2** was subcutaneously administrated on a daily basis at 25 mg/kg for 15 days or orally in a single dose for 15 days, we observed a potent antiparasite effect. In this case, the weight of the lesion decreased by 98.3% ($P < 0.01$) and 80.9% ($P < 0.01$), respectively. Surprisingly, treatment with **2** at 10 mg/kg for 15 days was sufficient to decrease the lesion weight and the parasite load either by the subcutaneous or the oral route. This specific treatment caused the lesion weight to decrease by 95.9% and the parasite load by 93% (subcutaneous route). Also oral administrations at 10 mg/kg of **2** caused a decrease of 90% and 68.6%. Particularly, the oral route of administration will be optimal regarding a potential commercialization of this drug. The results showed that the evaluated compound exhibited a strong antiproliferative activity on all developmental stages of the parasites, *Leishmania* spp.

In summary, the TOMOCOMD approaches allowed the assignment of the antitrypanosomal and antileishmanial potentialities of the novel compound **2** by a preliminary in silico study. These predictions were also confirmed by in vitro and in vivo assays. Based on this preliminary study of structure–activity relationship we suggest that the antiparasitic activity of bibenzyl phenolic compounds is strongly dependent on the position and number of free hydroxy group on the aromatic ring. Furthermore, the presence of a saturated carbon bridge linking both aromatic groups is another important factor for biological activity. In all, 14-hydroxylunularin bearing 2,5,4′-hydroxy substituent on a scaffold consisting of two phenyl groups linked through carbon–carbon [CH_2] bridge showed the highest leishmanicidal and trypanocidal activity among the set of hydroxybibenzyl derivatives assayed. To our knowledge, this is the first study to show these activities for secondary metabolites from bryophytes. Although the mode of action of compound **2** is still unknown, we concluded that this compound provides a new perspective in the treatment against leishmaniasis.

5. Experimental protocols

5.1. Experimental procedure for the chemical synthesis

5.1.1. E-2,5,4′-Trimethoxystilbene (*4*)

p-Anisaldehyde (1.0 eq) and (2,5-dimethoxybenzyl) triphenylphosphonium chloride (1.1 eq) were dissolved in the minimum amount of toluene, and K_2CO_3 (2.0 eq) and a trace

Table 3
In vitro activity of bibenzyl derivatives against promastigotes of three *Leishmania* spp. strain

Compound (100 μg/mL)	Percentual lysis of *Leishmanial* promastigotes		
	L. donovani (PP75)	*L. braziliensis* (M2903)	*L. amazonensis* (PH8)
1	N/R[a]	<70	<70
2	100	100	100
5	N/R	<70	<70
Resveratrol	<70	80	<70

[a] N/R = not reported.

Table 4
Cytotoxicity effects of compound **2** on mammalian cells

Compound 14-Hydroxylunularin (**2**) (μM)	NCTC-Clone 929 fibroblast[a] (%C ± SD[c])	J 774 Murine macrophage[a] (%C ± SD)	Peritoneal macrophage[b] (%C ± SD)
21.7	7.91 ± 2.89	18.88 ± 3.25	1.40 ± 0.9
8.6	2.93 ± 0.42	4.89 ± 4.56	0
4.3	0.66 ± 2.04	2.11 ± 2.36	0
2.1	0	0.76 ± 1.18	0

[a] According to resazurin test.
[b] According to trypan blue exclusion method.
[c] Percentage of cytotoxicity ± standard deviation.

Table 5
Effect of treatment with 14-hydroxylunularin (**2**) by oral and subcutaneous (SC) route on *L. amazonensis*-infected BALB/c mice

Compound **2** (mg/kg)	No. of mice	Route of adminst.	Lesion weight (g) ± SD	Supp. of weight lesion (%)	Supp. of parasite load in lesion (%)	Mean parasite quantitation in lesion ($\times 10^6$)
10	11	Oral	0.0350 ± 0.0364	−90.0[b]	−68.6[b]	1.34
10	12	SC	0.0144 ± 0.0151	−95.9[c]	−93.0[b]	0.29
25	10	Oral	0.0067 ± 0.0123	−80.9[c]	−93.1[b]	0.29
25	10	SC	0.0058 ± 0.0045	−98.3[c]	−97.0[b]	0.12
Glu[a]	13	SC	0.0980 ± 0.0857	−72.1[b]	−98.2[b]	0.76
None (control)	13	Oral	0.3520 ± 0.1534	–	–	4.28

Supp = suppresion.
[a] Glucantime 100 mg/kg.
[b] $P < 0.05$ (treated mice *vs* control).
[c] $P < 0.01$ (treated mice *vs* control).

amount of 18-crown-6 ether were added at r.t. The reaction mixture was then heated to reflux for 18 h. The solvent was evaporated under vacuum and the residue was purified by chromatographic column on silica gel (88% yield). In particular, for this reaction the ratio of (*E*/*Z*) isomers is heavily dependent on the solvent and base, ranging from 70/30 (*E*/*Z*) to exclusively the *E*-isomer in toluene and K_2CO_3 as base, yielding compound **4** in 88%.

White solid, mp: 61.9–62.7 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.46 (d, 2H, $J = 8.4$ Hz, Ar–H), 7.31 (d, 1H, $J = 16.2$, CH=C), 7.10 (d, 1H, $J = 2.7$ Hz, Ar–H), 7.04 (d, 1H, $J = 16.2$ Hz, CH=C), 6.89 (d, 2H, $J = 8.7$ Hz, Ar–H), 6.82 (d, 1H, $J = 8.7$ Hz, Ar–H), 6.76 (dd, 1H, $J_1 = 3$ Hz, $J_2 = 9$ Hz, Ar–H), 3.83 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3). ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ: 55.2, 55.7, 56.2, 107.4, 111.4, 112.2, 113.2, 114.0, 121.1, 127.6, 127.7, 128.8, 130.1, 130.6, 151.2, 151.7, 153.7. EM: *m*/*z* (%) 270 (100), 255 (11), 240(3), 227 (43).

5.1.2. 2,5,4′-Trimethoxybibenzyl (*5*)

Compound **4** (1 mmol) was dissolved in ethyl acetate, palladium on activated carbon (5% Pd) was added (50 mg/mmol) and hydrogenation performed at 4 bar hydrogen pressure. The catalyst was filtered off and the solvent removed in vacuum. The crude material was purified by chromatographic column (SiO_2; CH_2Cl_2), yielding compound **5** in 85%.

White solid, mp: 164.9–166.2 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.18 (d, 2H, $J = 8.3$ Hz, Ar–H), 6.88 (d, 2H, $J = 8.3$ Hz, Ar–H), 6.83–6.85 (m, 1H, Ar–H), 6.75–6.77 (m, 2H, Ar–H), 3.83 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 2.87–2.94 (m, 4H, CH_2–CH_2). ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ: 33.2, 35.7, 55.6, 56.0, 56.3, 111.5, 111.7, 114.1, 116.7, 129.7, 132.0, 134.8, 152.2, 153.9, 158.2. EM: *m*/*z* (%) 272 (47.5), 151 (25.3), 121 (100).

5.1.3. 2,5,4′-Trihydrohybibenzyl (14-hydroxylunularin) (*2*)

Methyl iodide (87.8 mmol) and magnesium (23.4 mmol) were stirred together in 24 mL of dry diethyl ether until the initial exothermic reaction had subsided. A solution containing 1 mmol of **5** in 24 mL of dry diethyl ether was added dropwise and the solution was concentrated under reduced pressure. The residue was heated to 100 °C while still under vacuum and was then heated at 160 °C for 15 min under argon. The cooled reaction mixture treated slowly with 20 mL of 10% aqueous NH_4Cl and extracted with three 20 mL portions of ether. The combined organic extract was washed with 20 mL of water and 20 mL of brine. The dried ($MgSO_4$) organic phase was concentrated under reduced pressure. The residue was purified by flash chromatography on a silica gel column yielding compound **2** in 75%.

White solid, mp: 145.7–146.1 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 7.04 (d, 2H, $J = 8.4$ Hz, Ar–H), 6.72 (d, 2H, $J = 8.4$ Hz, Ar–H), 6.65 (d, 1H, $J = 8.6$ Hz, Ar–H), 6.58 (d, 1H, $J = 3$ Hz, Ar–H), 6.48 (dd, 1H, $J_1 = 3$ Hz, $J_2 = 8.7$ Hz, Ar–H), 2.77 (br s, 4H, Ar–CH_2=CH_2–Ar). ^{13}C NMR ($CDCl_3$, 100.5 MHz) δ: 33.7, 35.9, 113.8, 115.8, 116.3, 117.4, 117.5, 129.9, 130.0 (×2), 134.0, 148.7, 151.0, 156.2. EM: *m*/*z* (%) 230 (26.2), 136 (6), 107 (100). Anal. $C_{14}H_{14}O_3$ (230): calcd (C 73.04, H 6.08); found (C 72. 99, H 5.98).

5.2. Biological assays

5.2.1. In vitro activity on Leishmania spp. promastigote

Cultures of *Leishmania* spp. were obtained from IICS (Instituto de Investigaciones en Ciencias de la Salud, Asunción, Paraguay) and identified by isoenzyme analysis. The maintenance, cultivation and isolation of promastigote parasites were performed as usual. Promastigotes inhibition studies were performed on *L. amazonensis* (IFLA/BR/67/PH8), *L. braziliensis* (MHOM/BR/75/M2903) and *L. donovani* (MHOM/BR/74/PP75) grown at 22 °C in Schneider's *Drosophila* medium containing 20% foetal bovine serum. Promastigotes cultures in the logarithmic phase were transferred at a concentration of 10^6 cells per milliliter. Compounds (1 mg) were dissolved in 40 μL of dimethyl sulphoxide (DMSO) and added to 1 mL of the medium from which aliquots are drawn. From this stock solution 200 μL were dissolved in 800 μL of medium. 100 microlitres of this solution was mixed with 100 μL of parasite culture reaching a concentration of the compound of 100 μg/mL and 0.4% of DMSO, and transferred into microtitre plates by triplicates. The activity of compounds was evaluated after 72 h by optical counting of the cells after appropriate dilutions and referred to the untreated control cells. IC_{50} was determined

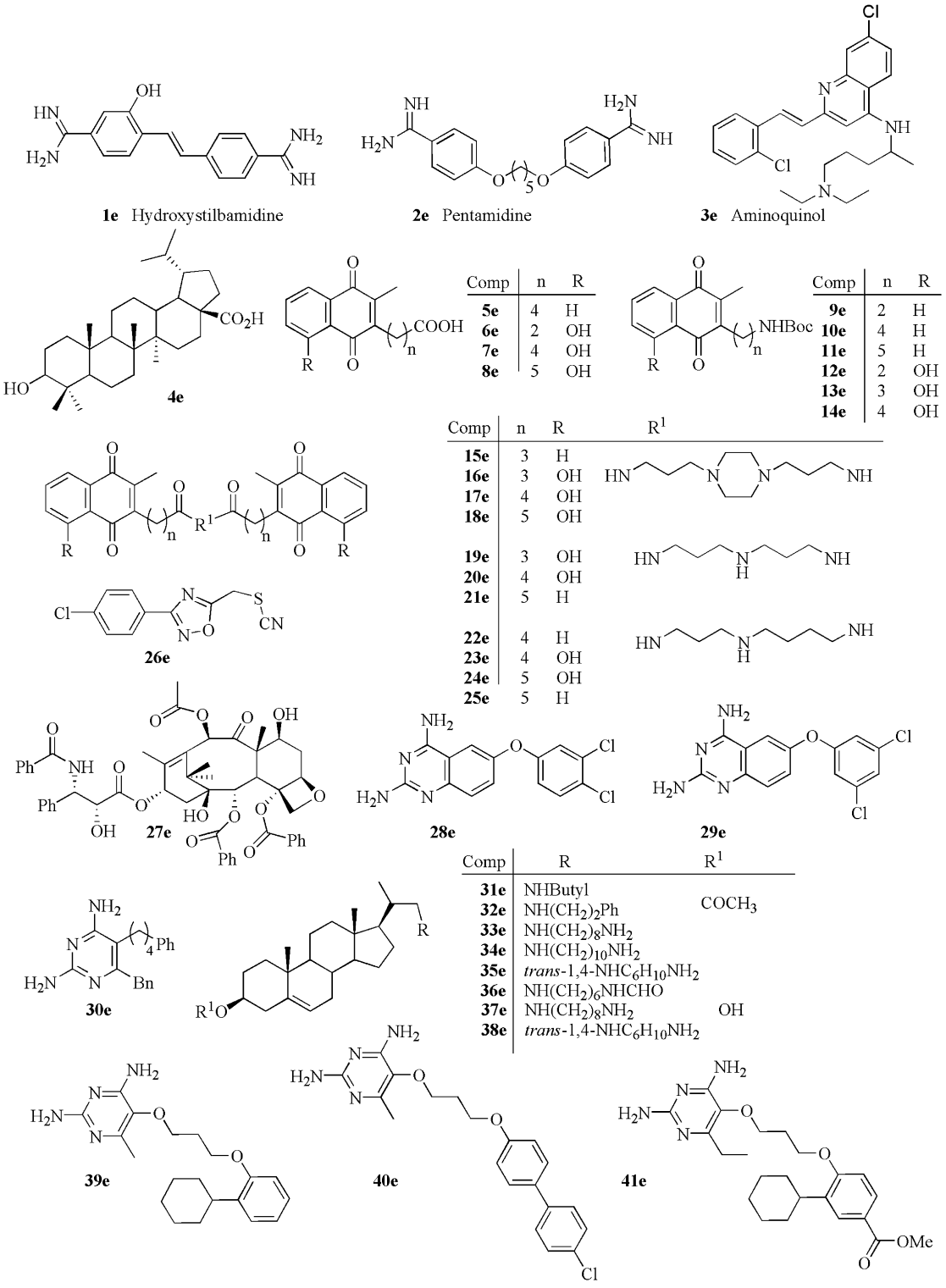


Fig. 2. Active compounds included in the training set.

in three independent assays. Pentamidine was used as a reference drug.

5.2.2. T. cruzi epimastigote susceptibility assay

For in vitro studies, the clone CL-B5 of *T. cruzi* stably transfected with *Escherichia coli* β-galactosidase gene (lacZ), was used. The epimastigotes were grown at 28 °C in liver infusion tryptose broth (LIT) with 10% foetal bovine serum (FBS), penicillin and streptomycin and harvested at the exponential growth phase. The screening assay was performed in 96-well microplates (Sarstedt, Sarstedt, Inc.) with cultures that had not reached the stationary phase. Briefly, epimastigotes were seeded at 1×10^5 parasites/mL in 200 μL growth media. The plates were then incubated with the respective drugs at 28 °C

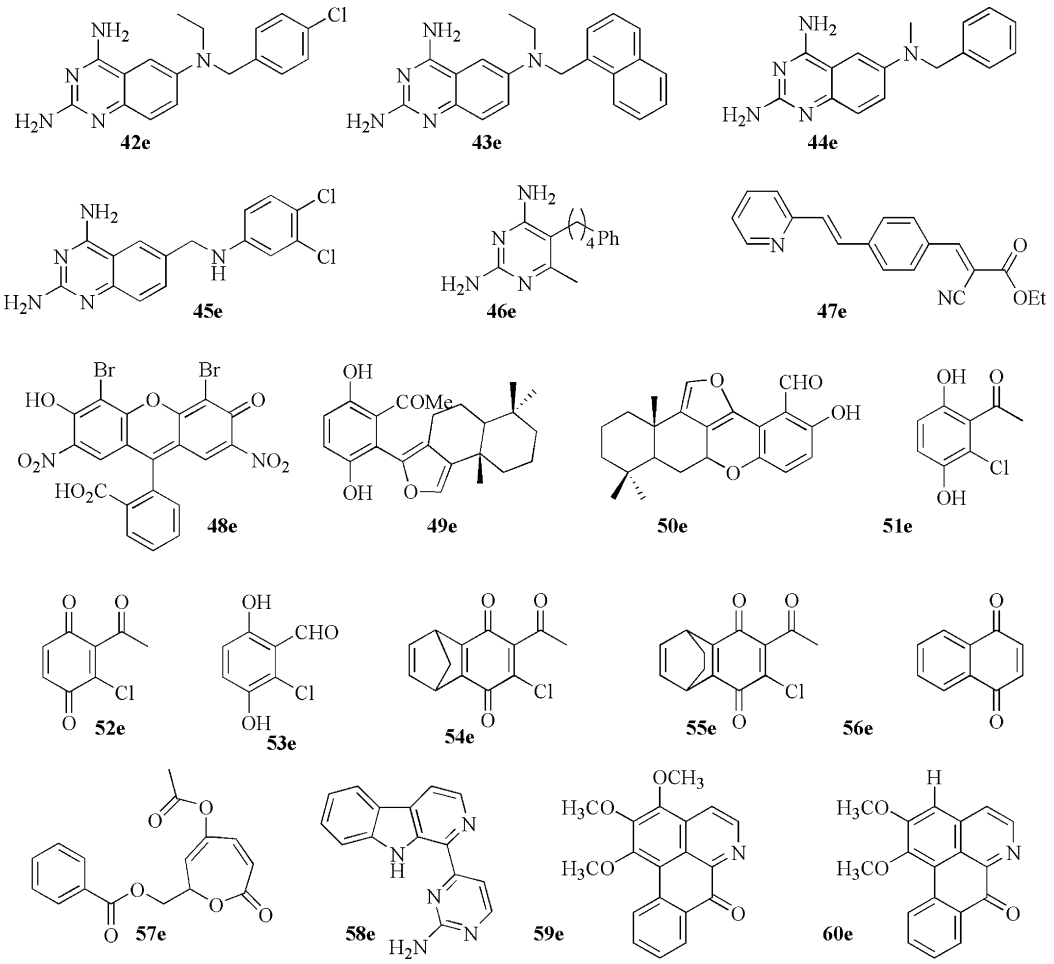


Fig. 2. (*continued*).

for 72 h, afterwards 50 μL of CPRG solution was added resulting in a final concentration of 200 μM. The plates were incubated at 37 °C for an additional 6 h and then read at 595 nm. Each concentration was tested in triplicate. The efficacy of each compound was estimated by calculating the IC_{50} values. These values were calculated by the sigmoidal dose–response curve adjustment using the statistical software program GraphPad Prims 3.0. Benznidazole was used as reference drug.

5.2.3. In vitro cytotoxicity on peritoneal macrophages

Healthy BALB/c mice (18–20 g) fasted for 12 h were used. Macrotitration plates with 24 wells with round slides were impregnated with nitric acid for better macrophage adherence. The procedure was carried out in conditions of total sterility using GELAIRE® BSB_{4A} Flow Laboratories Laminar Flow. Mice peritoneum was open and 6 mL of cold PBS was inoculated and mixed inside the animal cavity, the content was taken with a syringe and centrifuged to 1200 rpm during 10 mm, resuspended in RPMI® and counted in a Neubauer chamber; finally was placed inside the wells (1.10^5 macrophages/mL). 14-Hydroxylunularin (**2**) was weighed and DMSO (MERCK®) was used as solvent. Dilutions of the product at 10, 5 and 1 μg/mL were tested. Macrowell was incubated at 37 °C (5% CO_2) for 48 h and for the evaluation an OLYMPUS® inverted microscope was used and the reading was taken with trypan blue to 0.4%. One hundred macrophages were counted and the frequency of live and dead, according to trypan blue exclusion method, was determined. The assays with the evaluated compound, its dilutions and controls were made in triplicate. Pentamidine was used as reference drug.

5.2.4. In vitro cytotoxicity in cellular lines

The cell lines used were NCTC clone 929 and murine J774 macrophages, which were grown in Minimal Essential Medium (SIGMA®) and in RPMI 1640 medium (SIGMA®), respectively. Both media were supplemented with 10% heat-inactivated FBS, penicillin G (100 U/mL) and streptomycin (100 μg/mL). Cell cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. The procedure for cell viability measurement was evaluated with resazurin by a colorimetric method.

Macrophages. J774 macrophages were seeded (5×10^4 cells/well) in 96-well flat-bottomed microplates with 100 μL of RPMI 1640 medium. The cells were allowed to attach for 24 h at 37 °C, 5% CO_2, then the medium was replaced by different concentrations of the drugs in 200 μL of medium, and exposed for another 24 h. Growth controls were also included. Afterwards, a 20 μL of 1 mM resazurin solution was added

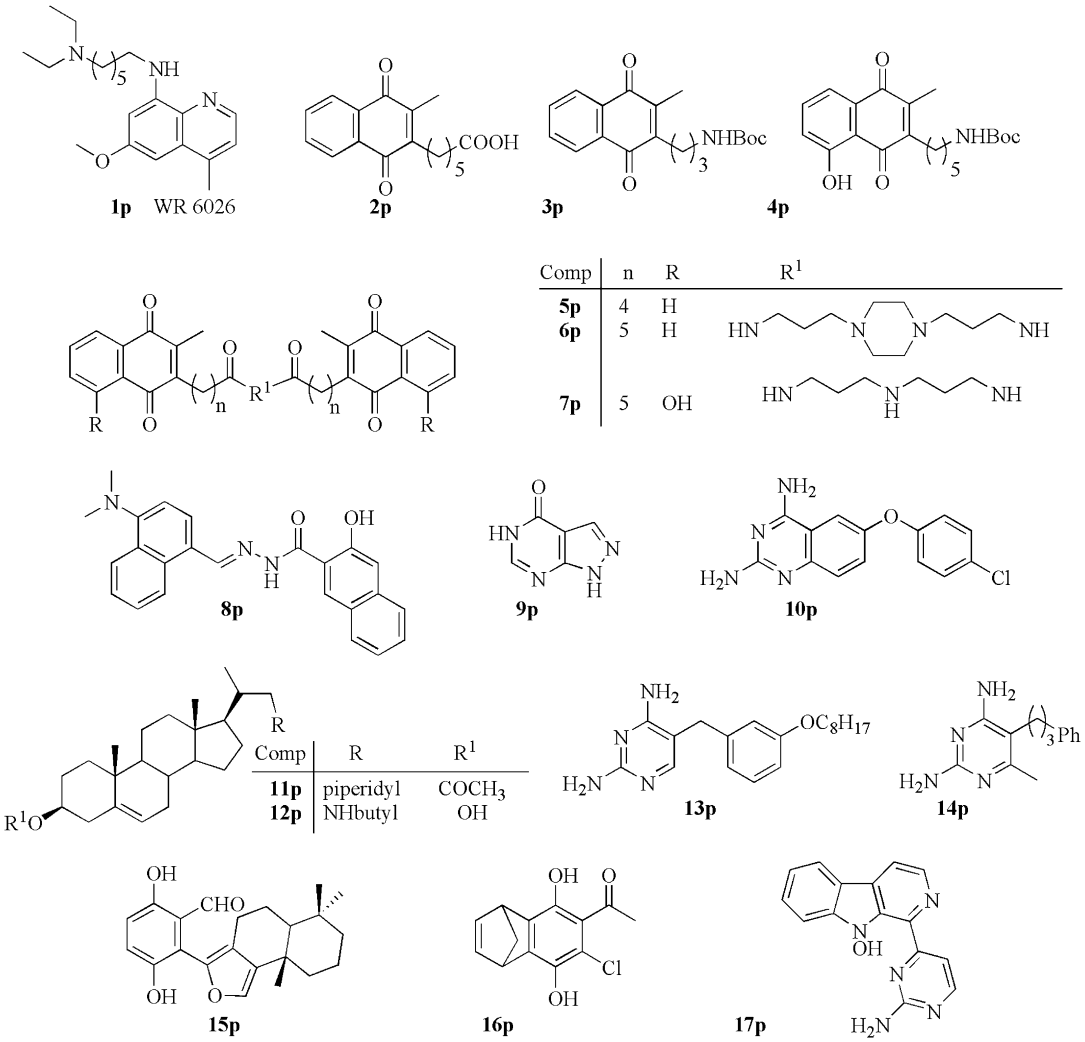


Fig. 3. Active compounds included in the test set.

and plates were returned to incubator for another 3 h to evaluate cell viability. *Fibroblasts*. NCTC clone 929 cells were plated in 96-microtitre plates at 3×10^4 cells per well in 100 μL growth medium. The cells were grown overnight at 37 °C, 5% CO_2. Thereafter the medium was removed and the compounds were added in 200 μL medium for 24 h. After incubation, 20 μL of 2 mM resazurin solution was added to each well. The plates were incubated for 3 h to allow optimal oxidation–reduction. The reduction of resazurin was determined by dual wavelength absorbance measurement at 490 and 595 nm. Background was subtracted. Each concentration was assayed three times. Medium and drug controls were used in each test as blanks.

5.2.5. Activity in Leishmania amastigotes

Intracellular forms (amastigotes) of *L. amazonensis* (PH8) maintained in Golden hamsters (*Mesocricetus auratus*) by successive passages every two months were used. Briefly, macrophages were obtained from mice as aforementioned in cytotoxicity section. The granuloma, containing the parasites, was removed from hamster feet, grinded and centrifuged to 500 rpm for 10 min to remove the remaining tissues. The supernatant, containing amastigotes, was washed with RPMI®, and centrifuged again at 3000 rpm for 10 min; amastigotes in the pellet were counted in a Neubauer chamber. Macrophages in the macrotitration plates allowed to adhere for 3 h were infected with the hamster amastigotes of *L. amazonensis* (PH8) at a 1:5 macrophage:amastigotes ratio and incubated at 37 °C (5% CO_2) for 2 h. A stock of 14-hydroxylunularin (**2**) was weighed and diluted in DMSO (Merck) at 1% in the culture media to a concentration of 1 μg/mL (4.34 μM). Dilution of 0.5, 0.3, 0.1 μg/mL and controls were prepared in triplicate. Then, they were incubated again at 37 °C during 48 h and reading was made in an OLYMPUS® inverted microscope. Percentages of infected macrophages in each assay were determined microscopically at magnification of 1000×.

5.2.6. T. cruzi amastigote susceptibility assay

The activity was evaluated by colorimetric method using CPRG, NCTC-929 fibroblasts were seeded in 24-well tissue culture plates at an optimal concentration of 2.5×10^3 cells/well, previously determined. NCTC-929-derived trypomastigotes

Table 6
Classification of active and inactive compounds included in the training set using Model 1

Compound	$\Delta P\%$[a]	Class	Compound	$\Delta P\%$[a]	Class
Training active group					
1e	98.89	+	**31e**	49.93	+
2e	98.21	+	**32e**	98	+
3e	98.44	+	**33e**	61.61	+
4e	98.32	+	**34e**	73.14	+
5e	91.2	+	**35e**	70.38	+
6e	89.4	+	**36e**	25.56	+
7e	93.64	+	**37e**	88.62	+
8e	94.91	+	**38e**	91.54	+
9e	59.54	+	**39e**	96	+
10e	73.09	+	**40e**	99.59	+
11e	78.04	+	**41e**	93.44	+
12e	69.28	+	**42e**	91.25	+
13e	75.27	+	**43e**	99.75	+
14e	80.02	+	**44e**	87.26	+
15e	99.46	+	**45e**	96.93	+
16e	99.72	+	**46e**	94.08	+
17e	99.84	+	**47e**	96.92	+
18e	99.90	+	**48e**	52.83	+
19e	99.97	+	**49e**	99.88	+
20e	99.98	+	**50e**	99.54	+
21e	99.98	+	**51e**	48.79	+
22e	99.97	+	**52e**	2.561	+
23e	99.99	+	**53e**	18.05	+
24e	99.99	+	**54e**	94.81	+
25e	99.98	+	**55e**	98.41	+
26e	−33.3	−	**56e**	67.53	+
27e	100	+	**57e**	−35	−
28e	97.37	+	**58e**	57.74	+
29e	96.84	+	**59e**	96.27	+
30e	99.88	+	**60e**	98	+
Training inactive group					
3-Episiostatin B	−99.70	−	Ganglefene	−82.80	−
Thiacetazone	−98.00	−	Metadiphenil bromidum	22.93	+
TBHQ	55.71	+	Quateron	−83.80	−
Cloral betaine	−99.40	−	Pancuronium	−91.30	−
Vernelan	−99.00	−	Ethylene	−96.60	−
Cetohexazine	−94.10	−	Dioxychlorane	−99.90	−
Carbavin	−96.60	−	Aliflurane	−62.50	−
Phenacemide	−86.30	−	Vinyl ether	−98.30	−
Tetharbital	−95.60	−	Tiouracilo	−89.40	−
Brofoxine	−51.10	−	Thiamazol methyl iodide	−98.50	−
Norantoin	−73.10	−	Diclofutime mesilate	−65.30	−
Orotonsan Fe	−96.20	−	Percloroetane	−99.20	−
Ferrocholinate	−99.90	−	Lindane	−98.10	−
Ferrosi ascorbas	−64.20	−	Nitrodan	−99.90	−
Arecoline	−99.20	−	Ascaridole	−98.80	−
Butanolum	−97.80	−	Pyrantel tartrate	−91.40	−
Etamsylate	−90.40	−	Fentanilo	50.09	+
Sango-Stop	−99.40	−	Tenalidine tartrate	−97.60	−
Besunide	−50.80	−	Dioxoprometazine	−99.80	−
Spironolactone	42.40	+	*N*-Hidroxymetil-*N*-metilurea	−99.90	−
Glycerol	−99.20	−	2,4,5-Triclofenol	28.40	+
Propamin soviet	−98.80	−	Norgamem	−99.90	−
Cystamine	−99.90	−	Furtrethonium iodide	−99.80	−
Amifostine	−100	−	Isofenefrine	−99.20	−
Adeturon	−100	−	Phenylethanolamine	−49.20	−
Glisolamide	−90.00	−	Cefalexin	−43.10	−
Glibutimine	−96.50	−	Streptomycin	63.60	+
Ag 307	−99.30	−	Azirinomycin	−100	−
Bromcholine	−99.90	−	Gentamicin A1	−95.60	−
Mebetide	−99.50	−	(2-Hidroxypropyl) trimetilamonium hydroxide	−99.90	−
Minoxidil	−96.40	−			

[a] Results of the classification of compounds obtained from Eq. (1) (using non-stochastic quadratic indices): $\Delta P\% = [P(\text{active}) - P(\text{inactive})] \times 100$.

were added to the monolayers at a parasite:cell ratio of 5:1 and incubated for 24 h at 33 °C with 5% CO_2. The infected cells were then washed twice with PBS to remove the extracellular trypomastigotes. The drugs were added in triplicate to give a final volume of 900 μL/well. The plates were incubated for 7 days at 33 °C. At this time, 100 μL of CPRG solution (final concentration 400 μM) in 0.3% Triton X-100 was added. Following 4 h of incubation at 37 °C, the colorimetric reaction was quantified by measuring at optical densities (OD) of 595 nm. Background controls (only NCTC-929 cells) were subtracted from all values. The results were expressed as IC_{50} values.

Table 7
Classification of active and inactive compounds included in test series using Model 1

Compound	$\Delta P\%$[a]	Class	Compound	$\Delta P\%$[a]	Class
Test active set					
1p	−60.50	−	**10p**	95.58	+
2p	92.94	+	**11p**	33.37	+
3p	67.00	+	**12p**	84.37	+
4p	83.82	+	**13p**	84.02	+
5p	99.68	+	**14p**	92.52	+
6p	99.80	+	**15p**	99.76	+
7p	99.99	+	**16p**	97.91	+
8p	99.81	+	**17p**	10.43	+
9p	−98.10	−			
Test inactive set					
Amantadine	−60.70	−	Cyclopropane	−95.90	−
Mizoribine	−90.20	−	Basedol	−98.30	−
Triclofos	−100	−	Mipimazole	−99.80	−
Nitroinosite	−100	−	Didym levulinate	−99.10	−
Methenamine	−100	−	Metriponate	−99.90	−
Cobalti glutamas	−99.00	−	Prasterone	73.42	+
Cobalti besilas	−94.20	−	Febensamin	−99.30	−
Canrenone	91.98	+	Guanazole	−100	−
Urea	−99.60	−	Fluorembichin	−99.90	−
Pallirad	−99.80	−	Mitoguazone	−100	−
Quimbosan	−90.90	−	Acetylcholine	−100	−
Glicondamide	−87.50	−	Methacholine chloride	−100	−
RMI 11894	−65.30	−	Dopamine	5.41	+
Barbismetylii iodidum	−100	−	Ampicillin	−8.03	−
Frigen 113	−98.30	−	Kanamycin A	−99.7	−

[a] Results of the classification of compounds obtained from Eq. (1) (using non-stochastic quadratic indices): $\Delta P\% = [P(\text{active}) - P(\text{inactive})] \times 100$.

5.2.7. In vivo assays with L. amazonensis

5.2.7.1. Animals. Male and female BALB/c mice bred in the animal colony at IICS (Asunción, Paraguay) were used. Six groups of 10–13 animals of 6–8 weeks (approx. 18–20 g) were inoculated in the right hind footpad with 2×10^6 amastigotes obtained from donor hamsters. The parasites were delivered in 100 μL of phosphate buffered saline (PBS). Lesion development was monitored by serial measurements of footpad thickness and expressed as the difference between the infected and uninfected footpad. The six groups were distributed as follows: Group I: infected control. Group II: reference drug; *N*-methylglucamine antimoniate (Glucantime® Aventis France) with pentavalent antimony Sb^v was dissolved in 50 μL of PBS and administered by subcutaneous route (100 mg/kg/d). Group III: compound **2** was made up in 50 μL of PBS and 5 μL of polysorbate (Tween 80, OSI France) and administered by oral route (10 mg/kg/d). Group IV: compound **2** was administered by oral route (25 mg/kg/d). Group V: compound **2** was administered by subcutaneous route (10 mg/kg/d). Group VI: compound **2** was administered by subcutaneous route (25 mg/kg/d).

5.2.7.2. Treatment. The treatment started four weeks after inoculation once the infection was well-established and lesion was apparent and carried pit for 15 days. The animals were killed one week after the end of the treatment to assess parasitological burden in the infected footpad. Granulomas were extracted, weighed and prepared as usual. The number of amastigotes per host lesion cell nucleus was counted. Percentage of parasite suppression was calculated from the ratio of the counts of drug-treated mice and the infected untreated control mice multiplied by 100. A corrected parasite suppression index was calculated by the following formula: 100-mean parasites treated mice/mean parasites untreated mice × 100.

5.3. Statistical analysis

For the statistical analysis in biological assays, means ± standard deviation (SD) were used and calculated by the Microsoft Office Excel 2003 software, unless indicated otherwise. Multiple comparisons between groups were made with a one-way analysis of variance (ANOVA). Paired comparisons between groups were carried out by Student's *t*-test. *P* values of <0.05 were considered significant (two tailed).

5.4. Topological molecular computer design (TOMOCOMD) approach

A Non-Stochastic Quadratic Fingerprints-based approach is introduced to classify and to identify, in a rational way, new antileishmanial compounds. A data set of 168 organic-chemicals [45], 77 with antileishmanial activity and 91 having other clinical uses, was divided at random in training and test data sets. Making use of the LDA technique implemented in the STATISTICA software [46], the linear model (Eq. (1)) was obtained. An external validation procedure was conducted in order to test the predictive ability of the obtained model. In this case the system was able to perform well in 89.36% of the cases. Only two from 17 active compounds (88.23%) and three from 30 inactive ones (90.00%) were misclassified.

Active compounds against Leishmania, which were considered in this study, are representatives of families with diverse structural patterns. Figs. 2 and 3 show the whole active set (training and test groups) collected from the literature. The selected inactive group included antivirals, sedative/hypnotics, diuretics, anticonvulsivants, haemostatics, oral hypoglycaemics, antihypertensives, antihelminthics, anticancer compounds as well as some other kinds of drugs, ensure a large structural variability.

Classification of compounds in both, training and active groups are shown in Tables 6 and 7.

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