Bioorganic Chemistry 89 (2019) 102996

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Evaluation of the antitrypanosoma activity and SAR study of novel LINS03 derivatives

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ARTICLE INFO

Keywords:
Anti-*Trypanosoma cruzi*
Antiparasitic compounds
Hydrophilic lipophilic balance
Natural product analogues
SAR analysis

ABSTRACT

Chagas' disease is a parasitic infection caused by *Trypanosoma cruzi* that is still treated by old and toxic drugs. In the search for novel alternatives, natural sources are an important source for new drug prototypes against *T. cruzi* to further structural exploitation. A set of natural-based compounds (LINS03) was designed, showing promising antitrypanosoma activity and low cytotoxicity to host cells. In this paper, nine novel LINS03 derivatives were evaluated against *T. cruzi* trypomastigotes and amastigotes. The selectivity was assessed through cytotoxicity assays using NCTC mammalian cells and calculating the CC_{50}/IC_{50} ratio. The results showed that compounds **2d** and **4c** are noteworthy, due their high activity against amastigotes (IC_{50} 13.9 and 5.8 μM) and low cytotoxicity (CC_{50} 107.7 μM and > 200 μM, respectively). These compounds did not showed alteration on plasma membrane permeability in a Sytox green model. SAR analysis suggested an ideal balance between hydrosolubility and lipophilicity is necessary to improve the activity, and that insertion of a *meta*-substituent is detrimental to the activity of the amine derivatives but not to the neutral derivatives, suggesting different mechanisms of actions. The results presented herein are valuable for designing novel compounds with improved activity and selectivity to be applied in future studies.

1. Introduction

Chagas' disease (CD) is a neglected tropical parasitic infection caused by the trypanosomatid parasite *Trypanosoma cruzi*. The natural life cycle of *T. cruzi* depends on an invertebrate host, most commonly *Triatoma* spp. which transmits the parasite through the infected feces eliminated alongside the blood feeding. The transmission can also occur via blood transfusion, organ transplant and contaminated food [1]. This alternative transmission led to a disease spreading to non-endemic regions (such as Europe, US, Canada and Japan) due to the migration of infected individuals [2]. Despite this, no drug has recently been approved for the treatment of CD and only two drugs are available, benznidazole and nifurtimox, which are nitroheterocyclic molecules that are frequently associated with many harmful side effects that impair the patient's compliance to the treatment.

Natural sources are frequently a great inspiration for new medicinal entities. The interest on such sources resides on the fact that natural molecules come from several different metabolic pathways from a variety of plants, animals and microorganisms providing a great deal of chemical diversity which no chemical library is capable of generating [3]. These molecules can reach the market as found *in natura*, such as paclitaxel, morphine and digoxin, or they can even be used as prototypes for designing new and improved molecules, such as captopril (inspired from a peptide isolated from the venom of *Bothrops jararaca*) [4].

To obtain active compounds against *T. cruzi*, a series of analogues were designed from gibbilimbols A and B (Fig. 1), natural molecules with simple motifs isolated from the leaves of *Piper malacophyllum* [5–7]. Previous results showed that the insertion of a functional group into the side chain led to important changes in the antitrypanosoma and cytotoxic activity [5]. while the substitution of the phenol for a benzene or methoxybenzene lowered the cytotoxic profile [6]. Recently, an exploratory data analysis using several molecular descriptors was performed in order to better understand which characteristics play the role in the biological activity and selectivity profile [7]. The results showed that there are two different subsets of compounds: a group of basic

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https://doi.org/10.1016/j.bioorg.2019.102996
Received 27 November 2018; Received in revised form 25 March 2019; Accepted 17 May 2019
Available online 18 May 2019

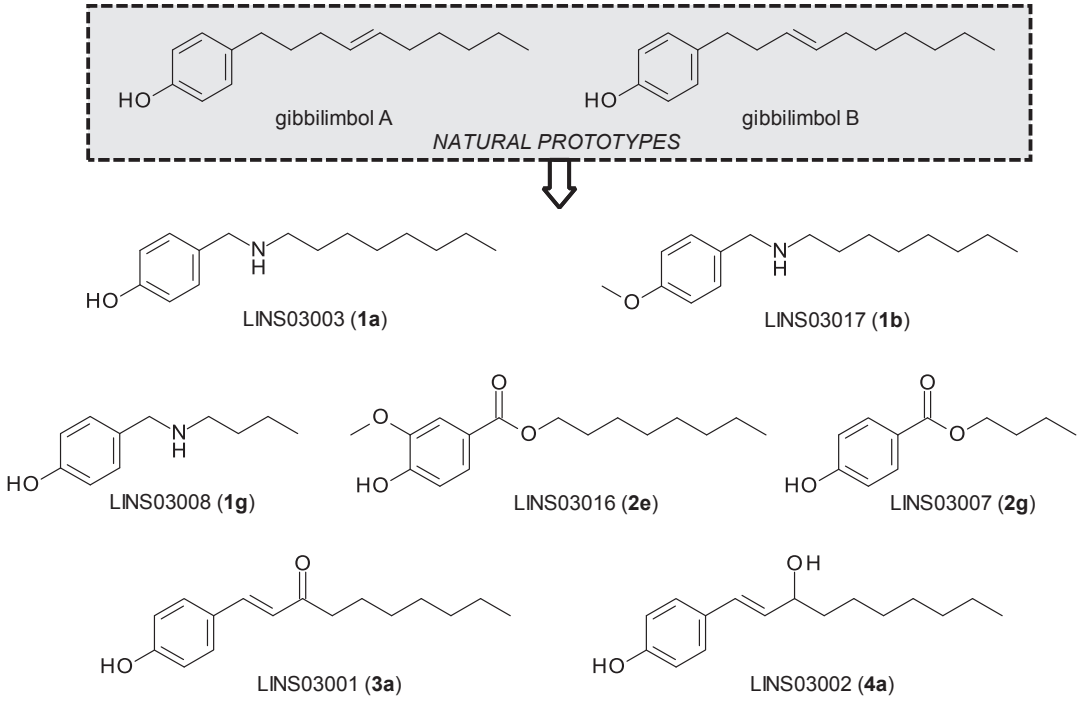


Fig. 1. Molecular structures of the natural prototypes and the previously tested analogues from the LINS03 series.

amines (1), where ionization (represented by distribution coefficient at pH 5.0, log$D_{5.0}$) is associated to the activity and neutral compounds (**2–4**) in which hydrophilic lipophilic balance (HLB) is the most important feature to increase the activity.

Among the promising compounds previously reported, the vanillic acid ester (LINS03016, **2e**) was the first with additional substituent in the *meta* position of the aromatic ring, showing good activity against both trypomastigotes and amastigotes forms of *T. cruzi* [7], when compared to the standard drug benznidazole. In addition, analogues with shorter side chain showed reduced activity against *T. cruzi* in comparison to the longer analogues. Only the ester LINS03007 (**2g**) presented moderate activity against the amastigote form [5], reinforcing the hypothesis of different mechanisms of action between basic and neutral analogues. Additionally, none of the compounds showed important interference on plasma membrane permeability assessed through Sytox Green method.

Considering the progress made so far, this work proposes a new series of analogues (Fig. 2) exploiting different properties of these molecules: (1) the addition of another substituent in the aromatic ring on *meta* position (**1d–f** and **2d**) in order to improve hydrosolubility and volume, both associated to high activity; (2) the modification of the substituent of the α,β-unsaturated alcohols (**4b** and **4c**); (3) three novel α,β-unsaturated ketones with different aromatic rings and side chain lengths (**3c**, **3g** and **3h**) to understand the impact of the side chain length in these neutral analogues on biological activity.

2. Materials and methods

2.1. Chemicals and equipment

All the reagents and solvents were acquired from commercial sources in adequate purity to be used without further purification. The progress of the reactions was monitored through TLC in silica gel plates with fluorescence indicator and visualized in UV at 254 nm. The hydrogen (^{1}H) and carbon (^{13}C) NMR data were acquired on a Bruker Advance 300, operating at 300 MHz (^{1}H) and 75 MHz (^{13}C) in $CDCl_3$. Chemical shifts (δ, in ppm) were measured using TMS as internal standard. Coupling constants are reported in hertz (Hz), if applicable. Purity of the tested compounds was determined using gas chromatography coupled with a mass spectrometer (GC-LRMS) on a Shimadzu QP2010 Plus model with helium as carrier gas and silica gel capilar column (RESTEK, model Rtx-5 ms, 30 m × 0.25 mm × 0.25 μM). Compounds with > 95% purity were considered pure for biological testing.

2.2. Synthesis of compounds 1

In a flask, 2 mmol of the corresponding aldehyde and 2 mmol (0.258 g) of 1-octylamine were dissolved in 15 mL of MeOH and stirred for **3h** (Scheme 1). Afterwards 6 mmol (0.227 g) of sodium borohydride were added and the reaction was kept at 25 °C overnight. The solvent was then evaporated, and the residue was dissolved in 15 mL of EtOAc and washed with 3 × 15 mL of distilled H_2O. The organic layer was dried over anhydrous Na_2SO_4 and evaporated. The crude material was purified through silica gel chromatography, using hexane:EtOAc (1:3) as eluent.

4-[(Octylamino)methyl]benzene-1,2-diol ***(1d)***. 0.2773 g of protocatechuic aldehyde yielded 0.2411 g (0.96 mmol, 48%) of **1d** as greenish solid (mp 85–87 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.64 (d, J = 8.0 Hz, 1H), 6.54 (d, J = 7.0 Hz, 2H), 4.47 (br.s, 3H), 3.57 (s, 2H), 2.67 (t, J = 7.4 Hz, 2H), 1.60–1.45 (m, 2H), 1.35–1.15 (m, 10H), 0.87 (t, J = 6.6 Hz, 3H).^{13}C NMR (75 MHz, DMSO): δ = 145.40, 144.35, 131.96, 119.24, 115.99, 115.58, 53.17, 48.97, 31.73, 29.78, 29.46, 29.17, 27.34, 22.57, 14.42.

4-[(Octylamino)methyl]-2-methoxyphenol ***(1e)***. 0.3475 g of vanillin yielded 0.2852 g (1.1 mmol, 53%) of **1e** as white solid (mp 68–70 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.85 (d, J = 8.6 Hz, 2H), 6.78 (d, J = 8.0 Hz, 1H), 3.89 (s, 3H), 3.71 (s, 2H), 2.62 (t, J = 7.2 Hz, 2H), 1.56–1.44 (m, 2H), 1.38–1.14 (m, 10*H*), 0.88 (t, J = 6.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 146.64, 144.70, 132.27, 121.00, 114.20, 110.85, 55.88, 53.99, 49.47, 31.84, 30.00, 29.54, 29.28, 27.40, 22.67, 14.11.

[(3,4-Dimethoxyphenyl)methyl]octylamine ***(1f)***. 0.3401 g of veratraldehyde yielded 0.240 g (0.86 mmol, 43%) of **1f** as orange oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.83–6.71 (m, 3H), 3.81 (s, 3H), 3.79 (s, 3H), 3.66 (s, 2H), 2.55 (t, J = 7.2 Hz, 2H), 1.50–1.40 (m, 2H),

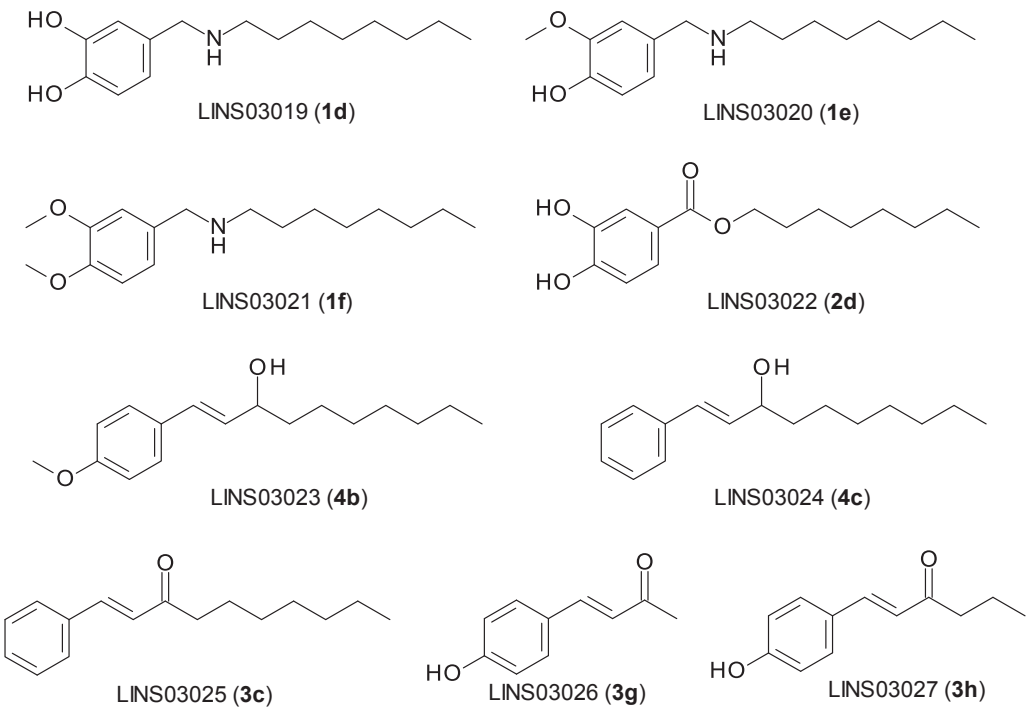


Fig. 2. The new set of LINS03 analogues.

1.37–1.16 (m, 10*H*), 0.80 (t, J = 6.6 Hz, 3H).^{13}C NMR (75 MHz, $CDCl_3$): δ = 148.95, 147.96, 133.17, 120.19, 111.37, 110.98, 55.91, 55.85, 53.89, 49.50, 31.84, 30.10, 29.54, 29.28, 27.40, 22.66, 14.10.

2.3. Synthesis of compound 2d

In a microwave flask, 1.5 mmol of 3,4-dihydroxybenzoic acid and 1 mmol of 1-octanol were dissolved in 3 mL of tetrahydrofuran and then 0.2 mL of concentrated sulfuric acid was added (Scheme 2). The reaction proceeded in a microwave reactor (Discovery, CEM Inc.) for 1 h at 100 °C. Solvent was removed and the residue dissolved in 15 mL of CH_2Cl_2 and washed with 5x15 mL of $NaHCO_3$ saturated solution. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to give the product **2d** with adequate purity.

*Octyl 3,4-dihydroxybenzoate (**2d**)*. 0.231 g of 3,4-dihydroxybenzoic acid and 0.1301 g of 1-octanol yielded 55% of the ester as a white powder (mp 94–96 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.67 (d, J = 1.7 Hz, 1H), 7.57 (dd, J = 8.3, 1.7 Hz, 1H), 6.91 (d, J = 8.3 Hz, 1H), 6.21 (br.s, 1H), 6.05 (br.s, 1H), 4.28 (t, J = 6.6 Hz, 2H), 184–164 (m, 2H), 1.47–1.18 (m, 10*H*), 0.88 (t, J = 6.7 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 167.22, 148.90, 143.20, 123.71, 122.70, 116.67, 114.81, 65.36, 31.80, 29.30, 29.20, 28.69, 26.04, 22.65, 14.09.

2.4. Synthesis of compounds 4

To a solution of 1 mmol of the corresponding ketone in MeOH, 3 mmol of sodium borohydride were added (Scheme 3). The mixture was stirred at 25 °C for 3 h and the solvent was evaporated. The residue was dissolved in 15 mL of EtOAc and washed with 3x15 mL of distilled water. The organic layer was then dried over anhydrous Na_2SO_4 and evaporated. The crude material was purified through silica gel chromatography, using hexane:EtOAc (9:1) as eluent.

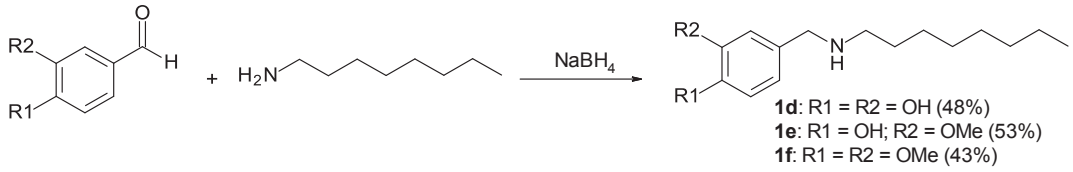
*(1E)-1-(4-Methoxyphenyl)dec-1-en-3-ol (**4b**)*. 0.2604 g of (1*E*)-1-(4-methoxyphenyl)dec-1-en-3-oneyielded 0.2151 g (0.81 mmol, 82%) of **4b** as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.34–7.28 (m, 2H), 6.88–6.81 (m, 2H), 6.50 (d, J = 15.9 Hz, 1H), 6.07 (d, J = 15.9 Hz, 1H), 4.23 (q, J = 7.1 Hz, 1H), 3.80 (s, 3H), 1.68–1.50 (m, 2H), 1.48–1.20 (m, 10*H*), 0.88 (t, J = 6.7 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 159.26, 130.46, 129.81, 129.48, 127.64, 114.00, 73.35, 55.31, 37.45, 31.83, 29.57, 29.27, 25.51, 22.67, 14.10.

*(1E)-Phenyldec-1-en-3-ol (**4c**)*. 0.2301 g of (1*E*)-phenyldec-1-en-3-one (**7**) yielded 0.2092 g (0.9 mmol, 90%) of **4c** as colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.44–7.17 (m, 5H), 6.57 (d, J = 15.9 Hz, 1H), 6.25 (d, J = 15.9 Hz, 1H), 4.28 (q, J = 7.2 Hz, 1H), 1.72–1.51 (m, 2H), 1.49–1.15 (m, 10*H*), 0.88 (t, J = 6.7 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 136.76, 132.62, 130.21, 128.57, 127.60, 126.45, 73.15, 37.39, 31.81, 29.55, 29.25, 25.46, 22.65, 14.09.

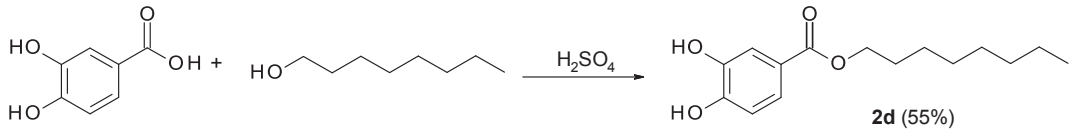
2.5. Synthesis of compound 3c

In a mortar, 2 mmol of benzaldehyde and 2 mmol of 2-nonanone and 2 mmol of solid NaOH were grinded (Scheme 4) for 15 min and afterwards neutralized with 1 M HCl. The crude material was recrystallized from EtOH:water giving the product **7** with adequate purity.

*(1E)-phenyldec-1-en-3-one (**3c**)*. 0.2130 g of benzaldehyde and 0.2845 g of 2-nonanone yielded 0.2074 g (1.8 mmol, 90%) of **3c** as a white powder (mp 50–52 °C). ^{1}H NMR (300 MHz, $CDCl_3$):



Scheme 1. Synthetic scheme for the preparation of the analogues **1d-f**.



Scheme 2. Preparation of the ester derivative **2d**.

δ = 7.62–7.50 (m, 3H), 7.43–7.37 (m, 2H), 6.75 (d, J = 16.3 Hz, 1H), 2.66 (t, J = 7.4 Hz, 2H), 1.71–1.60 (m, 2H), 1.42–1.10 (m, 10*H*), 0.87 (t, J = 6.6 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.58, 147.61, 142.31, 134.64, 128.95, 128.26, 126.30, 41.00, 31.72, 29.32, 27.44, 24.42, 22.64, 14.10.

2.6. Synthesis of compounds *3g* and *3h*

In a flask, 5 mmol of 4-hydroxybenzaldehyde were dissolved in 10 mL of the corresponding ketone (acetone or 2-pentanone) and 5 mL of EtOH (Scheme 5). Afterwards, 2 mL of a 40% NaOH solution to the stirred solution and reacted for 2 h. The solvent was evaporated, and the crude material was recrystallized from EtOH:water giving the products with adequate purity.

*(1E)-1-(4-Hydroxyphenyl)but-1-en-3-one (**3g**).* 0.6272 g of 4-hydroxybenzaldehyde yielded 0.502 g (3.1 mmol, 62%) of **3g** as yellowish solid (mp 109–112 °C).^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.55–7.36 (m, 3H), 6.87 (d, J = 8.7 Hz, 2H), 6.61 (d, J = 16.1 Hz, 1H), 5.55 (br. s, 1H), 2.37 (s, 3H).^{13}C NMR (75 MHz, $CDCl_3$): δ = 199.38, 158.51, 144.08, 130.35, 126.81, 124.69, 116.13, 27.33.

*(1E)-1-(4-Hydroxyphenyl)hex-1-en-3-one (**3h**).* 0.6305 g of 4-hydroxybenzaldehyde yielded 0.542 g (2.85 mmol, 57%) of **3h** as yellowish solid (mp 116–121 °C).^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.58–7.40 (m, 3H), 6.86 (d, J = 8.62 Hz, 2H), 6.63 (d, J = 16.1 Hz, 1H), 5.26 (br. s, 1H), 2.63 (t, J = 7.3 Hz, 2H), 1.81–1.61 (m, 2H), 0.98 (m, 3H).^{13}C NMR (75 MHz, $CDCl_3$): δ = 201.20, 158.06, 142.54, 130.24, 127.21, 124.01, 116.02, 42.70, 18.02, 13.89.

2.7. Biological assays

BALB/c mice were obtained from the animal breeding facility at the Instituto Adolfo Lutz-SP, Brazil. The animals were maintained in sterilized cages under a controlled environment, and received water and food *ad libitum*. Animal procedures were performed with the approval of the Research Ethics Commission, in agreement with the Guide for the Care and Use of Laboratory Animals from the National Academy of Sciences.

2.8. Trypanosoma cruzi parasites and mammalian cell maintenance

Trypomastigotes of *T. cruzi* (Y strain) were maintained in Rhesus monkey kidney cells (LLC-MK2-ATCC CCL 7), cultivated in RPMI-1640 medium supplemented with 2% FBS at 37 °C in a 5% CO_2-humidified incubator. Macrophages were collected from the peritoneal cavity of BALB/c mice by washing them with RPMI-1640 medium supplemented with 10% FBS and were maintained at 37 °C in a 5% CO_2-humidified incubator. The murine conjunctive cells (NCTC clone 929, ATCC) and LLC-MK2 were maintained in RPMI-1640 supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2.

2.9. Determination of the 50% inhibitory concentration (IC_{50})

Extracellular trypomastigotes. Trypomastigotes were counted in a hemocytometer chamber and seeded at 1x10^6 cells/well in 96-well microplates. The compounds were diluted in RPMI-1640 medium and incubated for 24 h at 37 °C in a 5% CO_2 humidified incubator. The trypomastigote viability was determined by the colorimetric resazurin assay (0.011% in PBS) in a spectrophotometer microplate reader (FilterMax F5, Molecular Devices, USA) at 570 nm. Benznidazole was used as a standard drug. Compounds were tested to the highest concentration of 150 μM and were reported as not active when the IC_{50} value was above this concentration [8].

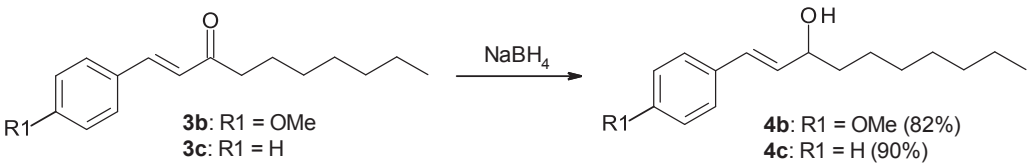
Intracellular amastigotes. Peritoneal macrophages were seeded at 1x10^5/well in 16-well chamber slides (NUNC, Thermo, USA) and maintained for 24 h in the same medium at 37 °C in a 5% CO_2 humidified incubator for attachment. Non-adherent cells were removed by two step washings with medium. After 24 h, these cells were infected with 1x10^6 culture trypomastigote forms for 2 h. Subsequently, infected cells were incubated with the compounds for 48 h. Finally, the slides were fixed with MeOH, stained with Giemsa, and observed in light microscopy. The parasite load was defined by counting 200 macrophages/well by evaluating the infection index defined by the equation (number of infected macrophages)x(number of intracellular amastigotes)/number of total macrophages (out of 200 macrophages). Benznidazole was used as the standard drug [8].

2.10. Cytotoxicity in mammalian cells

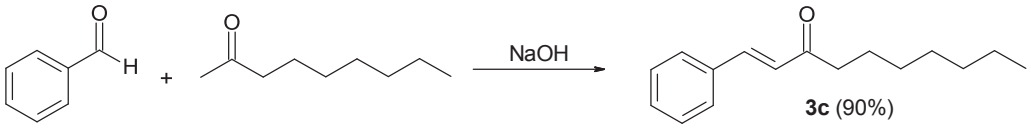
NCTC cells were counted in a hemocytometer chamber, seeded at 6 × 10^4/well and incubated in with the compounds (200–1.56 μM) for 48 h at 37 °C in a 5% CO_2-humidified incubator. The cell viability was determined using the MTT assay [9]. Benznidazole was used as a standard drug. The selectivity index (SI) was determined using the relationship between CC_{50} against NCTC cells/IC_{50} against parasites.

2.11. Evaluation of plasma membrane permeability

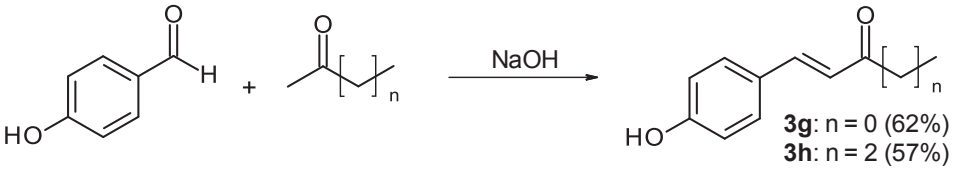
Compounds **4** and **6** were incubated with trypomastigotes of *T. cruzi* aiming to study the plasma membrane permeability using the fluorescent probe Sytox Green (Molecular Probes Inc.). Sytox Green is a high-affinity nucleic acid stain that penetrates cells with compromised plasma membranes and enhances its fluorescence by more than 500-fold upon nucleic acid binding. The parasite cells were washed in phosphate-buffered saline, suspended at 2x10^6 cells/well and incubated for 15 min with 1 μM of Sytox Green in Hanks' balanced salts solution (HBSS; Sigma-Aldrich) supplemented with 10 mM D-Glucose (HBSS + Glu) as described [10]. The compounds were added at the previously determined IC_{50} and the fluorescence was measured every 10 min for a total period of 120 min. Maximum fluorescence was determined by the addition of 0.5% of the non-ionic surfactant Triton X-100 (positive control). The control group consisted of untreated



Scheme 3. Preparation of the α,β-unsaturated alcohols **4b** and **4c.**



Scheme 4. Preparation of the α,β-unsaturated ketone **3c**.



Scheme 5. Preparation of the α,β-unsaturated ketones **3g** and **3h**.

trypomastigotes. Fluorescence was measured using a fluorimetric microplate reader (FilterMax F5, Molecular Devices, USA) with excitation and emission wavelengths of 485 and 520 nm, respectively [10,11].

2.12. Group efficiency analysis

Group efficiency (GE) values were calculated using the Eq. (1):

$$GE = (1.37 \times \Delta pIC_{50})/\Delta HA \quad (1)$$

where ΔpIC_{50} is the variation of the negative logarithm of IC_{50} value in molar between the substituted and non-substituted compound and ΔHA is the variation of heavy (non-hydrogen) atoms in the specific substituent [12].

2.13. Descriptor calculations

Molecular surface area (MSA), log$D_{5.0}$ and HLB descriptors were calculated using Marvin Sketch 16.1.8.0 (ChemAxon Inc.) using the default configuration in the software [7].

3. Results and discussion

3.1. Chemistry

The amines **1d–1f** were prepared through reductive amination method, i.e. by the formation of the imine *in situ* that is further reduced to its amine analogue, without isolation of the imine [7]. This led to moderate yields possibly due to competition with the reduction of the aldehyde to its corresponding alcohol. Ester **2d** was prepared through classic Fischer esterification adapted to a microwave reactor. This adaptation was done due to the possible oxidation of the catechol moiety, especially when high concentration of the catalyst is employed. By using the microwave instead of the conventional method (using Dean-Stark apparatus and reflux) previously reported [7], it was possible to keep a more uniform and stable temperature (100 °C) which facilitates the dehydration step and thus less acid may be used [13]. This led to better yield than the previous report.

The α,β-unsaturated alcohols **4b** and **4c** were obtained from the reduction of their corresponding carbonyl analogues **3** by using sodium borohydride as reductive agent, giving excellent yields [5]. The analysis of the NMR spectra showed that only the *trans* isomer is obtained and that the reduction was selective to the carbonyl group (1,2-reduction). This may be explained by the stabilization of the C=C bond by conjugation to the aromatic ring, making the 1,4-reduction less feasible. The α,β-unsaturated ketones **3** were synthesized following base-catalyzed aldol condensation between and aromatic aldehyde and alkyl ketone using sodium hydroxide as a basic catalyst. Compound **3c** was not isolated when the conventional methodology was used (i.e. with the reagents dissolved in ethanol). As alternative, a solvent-free reaction was carried out by grinding the reactants benzaldehyde and 2-nonanone with solid sodium hydroxide (1 equivalent each) in a mortar. This alternative methodology furnished a solid mixture, that after neutralization with HCl, afforded **3c** with high yield and purity [14]. This reaction is a nice example of a green chemistry approach, since no solvent, heating or electric energy were necessary.

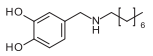
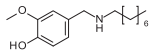
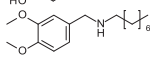
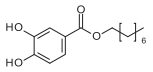
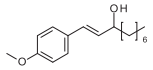
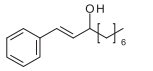
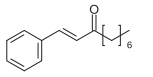
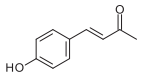
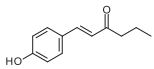
3.2. Biological evaluation

The antiparasitic activity of the compounds **1–4** was determined *in vitro* against trypomastigote (extracellular) and amastigote (intracellular) forms of *T. cruzi* (IC_{50}) and mammalian NCTC cells in order to determine cytotoxicity (CC_{50}) and calculate the selectivity index of the analogues, following the same methodology described in Varela et al. [7] (Table 1).

As can be noted in Table 1, all the long side chain compounds (**a-f**) showed activity against the trypomastigote forms of *T. cruzi;* compound **1d** was the most potent (IC_{50} 4.8 μM), followed by compounds **2d** and **4b** (IC_{50} 5.3 and 9.4 μM, respectively). Two compounds were active against the replicative form (amastigotes) of *T. cruzi* (**2b** and **4c**), showing IC_{50} values of 13.9 μM and 5.8 μM, respectively. Additionally, the α,β-unsaturated alcohol **4c** showed the highest SI (34.5) among the 27 analogues in the LINS03 series tested so far considering the amastigote form of the parasite [5–7]. Intracellular amastigotes are the clinical relevant form of the parasite and persist in the chronic phase of the disease [15]. Additionally, amastigotes are responsible for the amplification of the parasitic load and thus the anti-amastigote activity could reduce the parasitic burden in chronic patients. This compound also showed an IC_{50} value of 9.8 μM against the trypomastigotes. Trypomastigotes can also be found in humans, causing an elevated parasitemia in the acute phase of the disease; drugs lacking efficacy against this parasite form, may leave trypomastigotes alive, resulting in the recrudescence of the disease after treatment [16]. The results obtained for compound **4c** are comparable to the standard drug benznidazole. The promising results in both parasite forms suggest that this compound should be further evaluates as lead compound. Additionally, compounds with antitrypomastigote activity can help avoiding the spread of the infection [17]. The selectivity of **4c** against both forms of the parasite were at least 20-fold higher than the cytotoxicity to mammalian cells, fulfilling the requirements of the DNDi for a hit compound against CD [16].

Interestingly, the 4-methoxybenzenic α,β-unsaturated alcohol, **4b** was inactive against the amastigotes of the parasite but it showed activity against the trypomastigote form, similarly to its non-substituted analogue **6**. The 4-hydroxy analogue of these structures (**4a**) was also active against trypomastigotes with an IC_{50} value of 6.1 μM but was inactive against the amastigotes [5]. This suggests for these reduced analogues, the absence of a substituent in the aromatic ring favors the antiparasitic activity for both forms of the parasite. Moreover, the substitution of the hydroxyl group for a methoxyl or hydrogen improved significantly the selectivity towards the parasitic cells in comparison to the NCTC cells. On the other hand, this profile seems opposite to the observed activity of the α,β-unsaturated ketones. Compounds **3c**, **3g** and **3h** were inactive against amastigotes and only compound **3c** showed comparable activity (IC_{50} 16.5 μM, SI 12.1) to its

Table 1
Antiparasitic activity of the compounds against trypomastigote and amastigote forms of *T. cruzi* and cytotoxicity against mammalian NCTC cells.

Code	Structure	*T. cruzi* IC_{50} μM (± SD)		CC_{50} μM (± SD)	SI	
		Trypomastigotes (TCT)	Amastigotes (TCA)	NCTC	TCT	TCA
1d		4.8 ± 2.4	> 100	9.9 ± 0.6	2.1	–
1e		16.4 ± 0.3	> 100	53.0 ± 4.6	3.2	–
1f		12.2 ± 2.8	> 100	21.7 ± 16.1	1.8	–
2d		5.3 ± 3.8	13.9 ± 3.2	107.7 ± 7.0	20.3	7.7
4b		9.4 ± 1.7	> 100	> 200	> 21.3	–
4c		9.8 ± 1.5	5.8 ± 0.1	> 200	> 20.4	> 34.5
3c		16.5 ± 3.7	> 100	> 200	> 12.2	–
3g		> 100	> 100	> 200	–	–
3h		> 100	> 100	> 200	–	–
BZN		16.4 ± 4.1	5.2 ± 3.1	> 200	> 12.2	> 38.5

BZN: benznidazole; ND: not determined; IC_{50}: 50% effective concentration; CC_{50}: 50% cytotoxic concentration; SI: Selectivity Index calculated from CC_{50}/IC_{50}.

phenolic analogue **3a** (IC_{50} 11.8 μM, SI 7.0), but with higher selectivity. However, the 4-hydroxy and 4-methoxy analogues (**3a** and **3b**) presented moderate activity against amastigotes, suggesting that the presence of these polar groups in the aromatic ring increased the activity against this intracellular form.

A recent work [7] reported that the 3-methoxy-4-hydroxy substitution pattern on the aromatic ring led to improvement on the anti-trypanosoma activity (IC_{50} 4.4 μM and 6.8 μM for TCT and TCA, respectively) and selectivity (SI 28.0 and 18.8, respectively) for the ester derivative **2e**. In the present work, it was found that the 3,4-dihydroxy substitution pattern of compound **2d** gives comparable results against the trypomastigote form (IC_{50} 5.3 μM, SI 20.3) but it led to lowered activity in a 2-fold order against the amastigote form (IC_{50} 13.9 μM) and decreased selectivity (SI 7.8). The 3-methoxy-4-hydroxy substitution pattern also led to lowered lipophilicity, leading to the improvement the biological activity and reduced cytotoxicity as well, which is corroborated by Varela et al. [7]. This also reinforces that the phenolic hydroxyl group is intrinsically linked to cytotoxicity as well, whereas compound **2d** is the most cytotoxic among the neutral compounds in this series.

The first publication with these compounds [5] reported that lower homologues **1g** and the **2g**, both containing a side chain with 6-atom length, showed poor activity against *T. cruzi*. Only the compound **2g** presented moderate activity against the amastigotes of the parasite (IC_{50} 26.4 μM), while **1g** did not showed significant activity. Compounds **3g** and **3h** were tested to determine whether the length of the side chain could affect the antiparasitic activity and cytotoxicity in the α,β-unsaturated series. This modification produced again inactive analogues against both forms of the parasite (Table 1). However, they also showed low cytotoxicity to the mammalian cells. This may be due to the lower lipophilicity of these compounds, which impairs the permeation through the cell membrane. Given this, it seems that the reduction of the side chain length is detrimental to the activity of such compounds, corroborating to the hypothesis that there is an optimal lipophilicity to achieve highly active compounds.

The first amine derivative evaluated by our group was the 4-hydroxy derivative **1a**, which was considerably active against amastigotes (IC_{50} 5.5 μM) but was also cytotoxic to mammalian cells (CC_{50} 23.5 μM) [5]. Since the 4-methoxy and the non-substituted analogues showed lower cytotoxicity [6,7], the higher toxicity of **1a** was correlated to the presence of the known toxicophoric phenol group [18,19]. In fact, all the phenolic analogues showed higher cytotoxicity than the non-phenolic, corroborating to the hypothesis. However, all the amine derivatives previously reported also showed high cytotoxicity that could be associated with the characteristic of this functional group [7], although they presented high activity against *T. cruzi* amastigotes indeed. Considering that the HLB is a key factor to achieve high activity with lower cytotoxicity, the analogues **1d**-**1f** were designed to detect if an additional substitution in *meta*-position would improve the activity against the parasite without increasing the cytotoxicity.

Curiously, this behavior was not observed for these amine derivatives. The insertion of a *meta*-substituent led to inactivity against the amastigote form of the parasite, but retained the activity against trypomastigote forms. Accordingly, the high cytotoxicity of such compounds virtually confirm the toxicophoric characteristic of both phenol and amine groups. As can be noted in the compound **1d**, the insertion of an additional hydroxyl in the 3-position increased significantly the cytotoxicity to mammalian cells, while the presence of the 3-methoxy group in compound **1e** led to lower cytotoxic activity than the **1a** [5]. On the other hand, the insertion of this 3-methoxy group in compound **1b** (CC_{50} 31.9 μM) [7] resulted in compound **1f** which present increased cytotoxicity (CC_{50} 21.7 μM). This suggests that the insertion of the additional substituent in the 3-position of the amine derivatives is detrimental to the activity against TCA and decreases the selectivity of such compounds. Moreover, this also reinforces the hypothesis that the amine derivatives may act through a different mechanism of action from the non-ionizable molecules **2**–**4** [7].

3.3. Membrane disruption activity

Oliveira et al. [11] reported that the natural product gibbilimbol B had a membrane disruption activity, possibly due to the structural

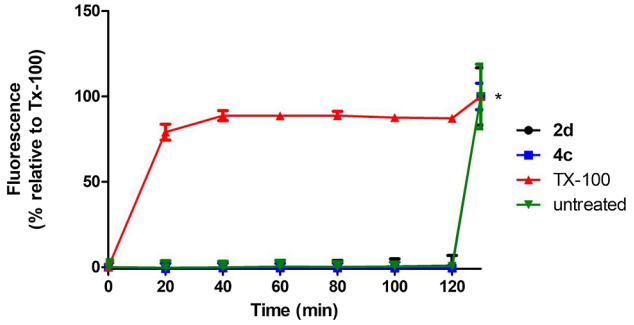


Fig. 3. Effects on plasma membrane permeabilization of compounds **2d** and **4c** in trypomastigotes of *T. cruzi*. Sytox Green dye (1 μM) fluorescence was spectrofluorometrically measured every 10 min. Minimum (untreated) and maximum permeabilization (TX-100) were obtained. Fluorescence was quantified by calculating the mean percentages of untreated (0%) and TX-100%-treated (100%) parasites.

similarity between this alkylphenol and the membrane phospholipids (polar head and apolar tail). This surfactant-like characteristic could to favor the interaction between the gibbilimbol and the ergosterol of the membrane giving to this molecule the capacity of forming a pore, like amphotericin B for *Leishmania* (*L.*) *infantum* [20], a parasite of the same family of *T. cruzi*. The assay uses the Sytox Green dye, which is not able to cross intact plasma membrane. If the membranes' stability is compromised, this dye becomes able to enter into the cells and bind to the nucleic acid, becoming fluorescent. Previous work[7] reported the evaluation of the most active compounds in this model and only two (**1b** and **2e**, amine and ester, respectively) showed to promote a small increase (< 20%) in the intracellular fluorescence, suggesting that these compounds may led to some alteration in the plasma membrane integrity. This behavior could also justify the cytotoxicity to mammalian cells, since the mammalian plasma membrane could also be affected as well.

The two active derivatives of this set (**2d** and **4c**) were submitted to the plasma membrane assay for a period of 120 min incubation (Fig. 3). None of the both compounds increased significantly ($p > 0.05$) the fluorescence levels when compared to positive control (TX-100) in a similar manner than observed with the former derivatives [7] (Table 2). This indicates that the lethal action of both compounds is other than causing a membrane disruption and should be evaluated for future optimization studies.

3.4. Ionization and hydrophilic lipophilic balance

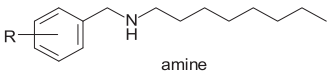
Even though the amines of this set proved to be inactive against amastigotes after the addition of a substituent in *meta*-position of the aromatic ring, the most active derivative against this form is still **1b** (methoxy group in the *para*-position) [7]. This conducted to a more systematic investigation on why the amines presented herein showed low activity against the amastigotes.

It was observed that the ionization capability and the HLB play important roles in the activity of these amine analogues [7]. Regarding the ionization, the log$D_{5.0}$ showed important correlation to the activity of such basic compounds, possibly indicating that these molecules should penetrate the macrophages in the unionized form, and when reaching the more acidic pH in the intracellular environment they become protonated, accumulating inside this compartment and exerting their activity against the amastigotes. The compounds **1d-1f** are considerably more hydrophilic than the former amine analogues due to the presence of the oxygenated substituents, which lead the log$D_{5.0}$ < 1.1 (Table 3). This data was confirmed by the lower values of the calculated log$D_{5.0}$ for these compounds.

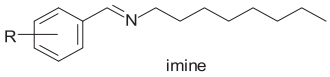
Table 2
Relative permeability to the Sytox Green probe in *T. cruzi* trypomastigotes.

Compound	Relative permeability to TX-100 (mean ± SD, n = 3)
2d	0.8 ± 2.4
4c	−0.5 ± 0.6
untreated	0.5 ± 0.8

Table 3
Calculated values of log$D_{5.0}$ and HLB for the basic analogues.



amine



imine

Compound	Chemotype	R	log$D_{5.0}$	HLB	HLB/log$D_{5.0}$	IC_{50} TCA (μM)
1a	Amine	4-OH	1.11	8.75	7.88	5.5[a]
1b	Amine	4-OMe	1.26	8.82	7.00	1.33[b]
1c	Amine	H	1.41	7.14	5.06	13.3[c]
1d	Amine	3,4-di-OH	0.84	10.30	12.26	> 100
1e	Amine	3-OMe-4-OH	0.96	10.31	10.74	> 100
1f	Amine	3,4-di-OMe	1.11	10.25	9.23	> 100
5a	Imine	4-OH	1.39	3.34	2.40	> 100[a]
5b	Imine	4-OMe	1.53	3.43	2.24	> 100[c]
5c	Imine	H	1.65	1.72	1.04	50[c]

[a] Available from Varela et al. [5].
[b] Available from Varela et al. [7].
[c] Available from Varela et al. [6].

However, the corresponding imines are also basic, with similar log$D_{5.0}$ values to the amines and unfortunately they showed poor activity against the amastigote forms of *T. cruzi*. The explanation to this different activity profile may be correlated to the HLB values. The imines present lower HLB values (< 4.0) than the amines (> 7.0), suggesting that the hydrophilicity may also be a key factor related to the activity. On the other hand, the compounds **1d–1f** are more hydrophilic than the mono-substituted amines (such as **1b**) as expressed by the higher HLB values, which can difficult their penetration in the parasite cells and their antiparasitic activity indeed.

When the information given by these two descriptors are putted together, it can be suggested that there is an optimal value for the log$D_{5.0}$ and HLB values, which can be described as the HLB/log$D_{5.0}$ ratio. The amines with anti-amastigote activity present HLB/log$D_{5.0}$ ratio values between 5 and 8, while the more lipophilic imines present HLB/log$D_{5.0}$ ratio value < 3. The di-substituted amines, in counterpart, present HLB/log$D_{5.0}$ ratio values > 9. Considering this, further amine analogues should be designed considering the HLB/log$D_{5.0}$ ratio in this ideal range. Possibly this range indicates the optimal lipophilicity value to reach the intracellular environment adequately.

3.5. Group efficiency

To evaluate the efficiency of the inserted groups in the amine derivatives **1**, group efficiency (GE) analysis was carried out. The GE analysis is a measurement of the binding efficiency for an added functional group in the parent molecule [12]. Accordingly, it represents how much the added group contributed to overall affinity of the molecule, considering the number of heavy atoms [21]. This only makes sense when a given set of compounds act binding in the same molecular target and accordingly it was assumed that these amine derivatives act through the same mechanism of action [22].

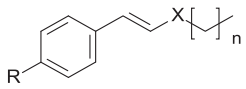
The GE analysis (Table 4) indicated that the insertion of substituents on the 3-position of the aromatic ring play different roles in the activities against trypomastigotes, amastigotes and mammalian cells. Considering the activity against trypomastigotes, the insertion of the 3-OH group led to important improvement against the parasite, with a GE

Table 4
GE analysis of the substituent in the 3-position of the amine analogues (1) calculated using Eq. (1).

3-R	GE pIC_{50} TCT	GE pIC_{50} TCA	GE pCC_{50}
OH	0.75[a]	< −1.73[a]	0.51[a]
OMe	0.01[a]	< −0.86[a]	−0.24[a]
	−0.31[b]	< −1.29[b]	0.11[b]

[a] Calculated based on compound **1a**.
[b] Calculated based on compound **1b**.

Table 5
Lipophilicity and volume descriptors calculated for the α,β-unsaturated compounds.



Compounds	X	n	R	$\log D_{5.0}$	MSA	MSA/$\log D_{5.0}$	IC_{50} TCA (μM)
3a	C=O	6	OH	5.09	422.33	82.97	22.5[a]
3b	C=O	6	OMe	5.23	459.81	87.92	26.2[b]
3c	C=O	6	H	5.39	411.53	76.35	> 100
3g	C=O	0	OH	2.16	236.60	109.54	> 100
3h	C=O	2	OH	3.31	299.77	90.56	> 100
4a	CHOH	6	OH	4.67	432.51	92.61	> 100[a]
4b	CHOH	6	OMe	4.82	470.11	97.53	> 100
4c	CHOH	6	H	4.98	421.99	84.73	5.8

[a] Available from Varela et al. [5].
[b] Available from Varela et al. [6].

value of 0.75. The insertion of the 3-OMe group did not contribute or even worsen the efficacy of the compounds **1e** and **1f** in comparison to the prototypes **1a** and **1b**. Curiously, the analysis indicates that this role is totally different regarding the anti-amastigote activity. The insertion of any substituent in the 3-position led to loss of efficacy against the intracellular form of *T. cruzi* (negative GE values). However, the results suggest that the role of the 3-substituents is more important in the cytotoxicity than in the antiparasitic activity. The insertion of the 3-OH group led to increased cytotoxicity with considerable efficiency, suggesting that this group contributes more significantly to the cytotoxicity of compound **1d** instead to increase the antitrypanosomal activity and corroborating to the hypothesis that the phenol is a toxicophore [18]. Accordingly, the insertion of the 3-OMe group did not contribute to the cytotoxicity or even decreased this effect, as can be verified by the small or negative GE values for the 3-OMe group.

3.6. Neutral compounds

Previous report with the neutral LINS03 compounds **2–4** [7] suggested that bigger molecules may be more effective against amastigotes as indicated by the molecular surface area (MSA) value. As can be seen in Table 5, the ketones **3a** and **3b** are more active than the alcohol analogues **4a** and **4b**. However, compound **4c** showed improved activity against both forms of the parasite, with low cytotoxicity to mammalian cells, leading to very high SI values.

Analyzing the descriptors in Table 5, it is possible to verify that the biological activity of the neutral compounds is highly associated to lipophilicity and molecular size. The conversion of the ketones to the corresponding alcohols leads to increased MSA values, even though the activity did not improve as well. Additionally, it also decreases lipophilicity (as described by the $\log D_{5.0}$values). However, no direct correlation can be seen between the presence of functional groups in the aromatic ring and the antiparasitic activity. The presence of hydroxyl or methoxyl in the aromatic rings seems to improve the activity of the ketones, but not of the corresponding alcohols.

As can be seen in Table 5, the active analogues **4c**, **3a** and **3b** have $\log D_{5.0}$ values ranging from 4.98 to 5.23. This suggests that the optimal $\log D_{5.0}$ value seems around 5.00. More hydrophilic compounds (such as **4a**, **4b**, **3g** and **3h**) and more lipophilic compounds (**3c**) showed to be inactive. However, this data alone could not explain the role of the substituents in the aromatic ring.

The presence of substituents increases the MSA values, but as can be seen in Table 5, there is no correlation between MSA and IC_{50} values. By weighting the MSA values with the $\log D_{5.0}$, it can be seen that a MSA/$\log D_{5.0}$ ratio around 85 seems optimal. This suggests that the insertion of a hydroxyl or methoxyl groups (**a** and **b**, respectively) leads to higher MSA values, but is also decreases the $\log D_{5.0}$, impairing the biological activity of the alcohols **4** (which are more hydrophilic) and improving the activity of the ketones **3** (which are more lipophilic). In summary, the substituents on the aromatic ring seem to contribute to optimize the lipophilicity of the molecule instead of performing a specific interaction with the biological target.

3.7. Conclusions

In summary, nine LINS03 analogues with antitrypanosoma activity were reported, and compounds **2d** and **4c** showed a promising effect due to their potency against *T. cruzi* amastigotes with high selectivity. Additionally, none of these compounds induced alteration of plasma membrane of *T. cruzi*. The balance between lipophilicity and hydrosolubility showed to be the key factors associated with the activity of the analogues, and must be considered in a novel set of compounds. These SAR results will certainly aid the design of future analogues of the LINS03 series with improved activity.

Declaration of Competing Interest

The authors declare none.

Acknowledgements

The authors are grateful to São Paulo Research Foundation (FAPESP) [Grants 2016/25028-3; 2018/03918-2; 2018/07885-1, 2015/23403-9] and to National Council for Scientific and Technological Development (CNPq) [Grant 455411/2014-0] for financial support to this work and to the Scientific award to JHGL, AGT and JPSF [Grant 306355/2018-3].

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