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Short communication

Synthesis and evaluation of the antiparasitic activity of bis-(arylmethylidene) cycloalkanones

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ABSTRACT

A series of bis-(arylmethylidene)-cycloalkanones was synthesized by cross-aldol condensation. The activity of the compounds was evaluated against amastigotes forms of *Trypanosoma cruzi* and promastigotes forms of *Leishmania amazonensis*. The cytotoxicity of the active compounds on uninfected fibroblasts or macrophages was established *in vitro* to evaluate the selectivity of their antiparasitic effects. Six compounds displayed trypanocidal activity against amastigotes intracellular forms of *T. cruzi* with IC_{50} values ranging from 7.0 to 249 μM. Besides these six compounds, eight other molecules exhibited significant leishmanicidal activity (IC_{50} values ranging from 0.6 to 110.4 μM). Two compounds can be considered as promising antiparasitic lead molecules because they showed IC_{50} values in the low-micromolar range (≤1.2 μM) with an adequate SI (≥19.9). To understand the mechanism of action of these compounds, two possible molecular targets were investigated: trypanothione reductase (TR) and cruzain.

1. Introduction

Chagas' disease and Leishmaniasis are endemic protozoan diseases recognized as epidemiological priorities by the World Health Organization (WHO). Chagas' disease is found mainly in Latin America and it is estimated to affect 12 to 14 million people. Leishmaniasis affects 12 million people in 88 countries, 72 of which are developing countries [1]. Chagas' disease and Leishmaniasis are caused by the trypanosomatid parasites *Trypanosoma cruzi* and *Leishmania* sp, respectively. There are limited drugs available for the treatment of both diseases, and the currently used chemotherapeutic agents are still inadequate due to their extreme toxicity. In addition, drug resistant parasite strains have become more prevalent [2–6]. The search for more effective and less toxic drugs to treat both diseases have became mandatory.

Bis-(arylmethylidene)-cycloalkanones have been found to display a range of biological effects including antineoplastic [7], antioxidant [8–10], antitubercular [11] and antifertility activities [12]. It has been proposed that the activity of this class of compounds is related to their ability to act as Michael acceptors with nucleophilic biological macromolecules, especially nucleic acids and enzymes [13].

To the best of our knowledge, there is no report on the application of this class of compounds for the treatment of parasitic infections. Therefore, we decided to evaluate the *in vitro* antileishmanial and antitrypanosomal activities of a series of bis-(arylmethylidene)cycloalkanones. The cytotoxicity of compounds to non-infected cells was also evaluated.

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To understand the mechanism of action of these compounds, two possible molecular targets were investigated: trypanothione reductase (TR) and cruzain.

Trypanothione Reductase (TR) [14,15] is a vital enzyme for the antioxidant defenses in trypanosomatids. TR exerts a key role in combating oxidative stress, regenerating the main antioxidant present in these protozoa, trypanothione [16]. TR is involved in the reduction of trypanothione disulfite $T[S]_2$ to the dithiol T[SH]. This enzyme also is considered an attractive due to its high conservation between *Leishmania* and trypanosomes [17–19], since it is expected that a TR inhibitor will be potentially active against both *Trypanosoma* and *Leishmania* parasites.

Besides participating in an essential pathway for parasite survival, the remarkable structural differences with respect to the human enzyme, Glutathione Reductase (GR), makes TR as potential target for the development of selective inhibitors. The main differences between TR and GR are related to the size, charge and distribution of hydrophilic/hydrophobic regions in the active site of the enzymes. The active site of TR is larger than that of GR and is negatively charged with surrounding hydrophobic residues to accommodate the positively charged spermidine portion of $T[S]_2$ [20]. In contrast, the GR active site has three positively charged arginine residues (Arg37, Arg38 and Arg347), required for interaction with its substrate, GSSG, which has a formal charge of −2 at physiological pH [21].

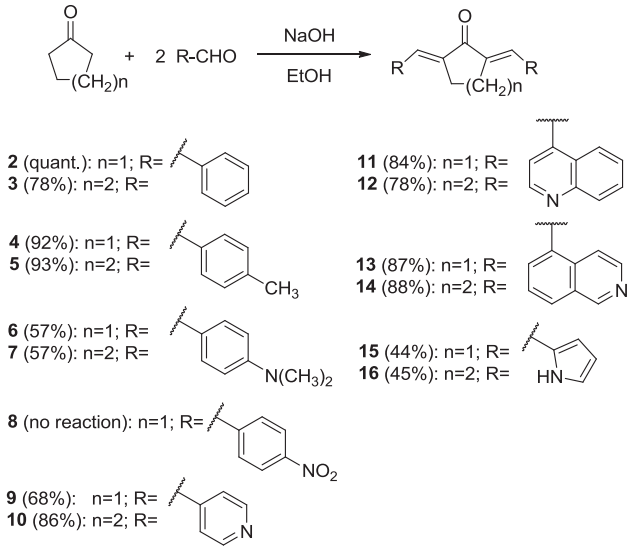
We hypothesized that bis-(arylmethylidene)-cycloalkanones could interact strongly with the active site of TR due to the presence of bulky and hydrophobic substituents. As human GR has a hydrophilic and smaller binding site, low affinity would be expected between these compounds and GR. To confirm this hypothesis, enzyme inhibition assays were carried out against TR and GR.

Cruzain, the major cysteine protease from *T. cruzi*, which is among the most studied therapeutic targets for Chagas' disease [22–24], was also evaluated as a possible mechanism of trypanocidal activity. Cysteine proteases play a key role at various stages in the life cycle of the parasites and are involved in host protein degradation by the parasite, facilitating the evasion of host defense mechanism, invasion cellular, replication and differentiation of the parasite [23,25]. These enzymes contain a catalytic cysteine residue in its active site responsible for the hydrolysis of peptide bonds [26,27].

There are several classes of cruzain inhibitors [24,28,29], but typically cysteine protease inhibitors possess an electrophilic α,β-unsaturated moiety, such as, vinyl ketone, vinyl sulfones, vinyl amide, vinyl ester and vinyl nitrile, acting as a Michael acceptor for the nucleophilic sulfhydryl groups of catalytic cysteine residue [30,31]. Based on the potential of the bis-(arylmethylidene)-cycloalkanones to act as Michael acceptor, the evaluation of the ability of these compounds to inhibit cruzain is of interest.

2. Chemistry

The bis-(arylmethylidene)cycloalkanones **2**–**16** were synthesized by cross-aldol condensation of cycloalkanones (cyclopentanone or cyclohexanone) with a variety of commercially available aldehydes as outlined in Scheme 1. The main advantages of this synthesis are that the compounds can be obtained by a simple and rapid one-step procedure in good yield from readily available starting materials. The only exception was the synthesis of the dinitro derivative **8**, which cannot be obtained using the usual method under basic conditions [32]. The synthesis of **8** was repeated several times varying the reaction temperature and time and the ratio of the reagents, but in all cases, only the unreacted starting materials were recovered. Then, the reaction was performed under microwave irradiation in solvent-free condition [33] and the product was obtained in low yield (17%), even after optimization attempts (Scheme 2).

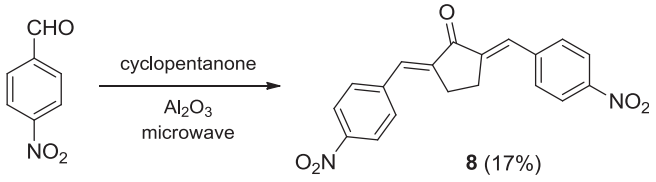


Scheme 1. General synthetic route to bis-(arylmethylidene)cycloalkanones.

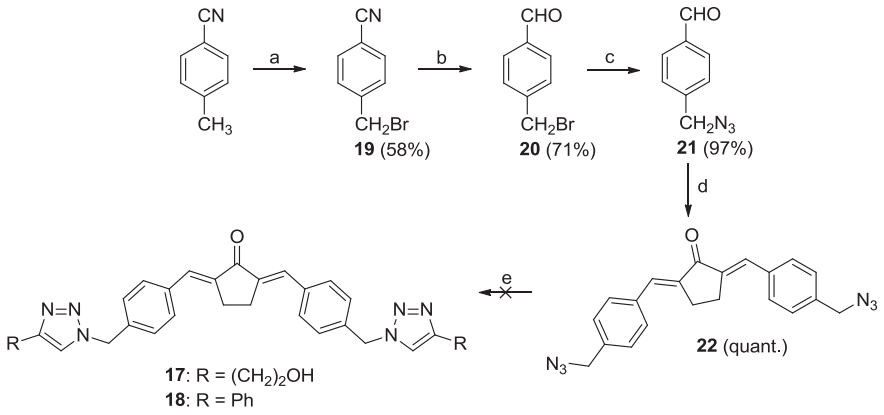
The first attempt to obtain compounds **17** and **18** (Scheme 3) consisted of a five-step procedure. Initially, reaction of *p*-tolunitrile with *N*-bromosuccinimide (NBS) in the presence of radical initiator $(PhCOO)_2$ provided the bromo derivative **19** in 58% yield [34]. Reduction of the nitrile group of **19** with diisobutylaluminum hydride (DIBALH) followed by displacement of the derived benzylic bromide **20** with sodium azide afforded 4-(azidomethyl)benzaldehyde **21** in 97% yield [34,35]. Cross-aldol condensation of **21** with cyclopentanone led to diazide derivative **22** in quantitative yield. However, the attempted copper-catalyzed 1,3-dipolar cycloadditions between azide derivative **22** and 3-butyn-1-ol or phenylacetylene were unsuccessful, probably because of the low solubility of the **21** in the reaction medium. To overcome this problem, we decided to repeat the reaction replacing azide **22** by azide **21**, considerably more soluble in organic solvents. Thus, in the presence of the sodium ascorbate, $CuSO_4 \cdot 5H_2O$ and terminal alkyne, the azide **21** was completely consumed, and the 1,4-disubstituted triazole products **23** and **24** were formed in 61 and 86% yield, respectively. Finally, the last step consisted in the aldol condensation of the aldehydes **23** and **24** with cyclopentanone, resulting in the formation of the desired products **17** and **18** (Scheme 4).

3. Biological assays

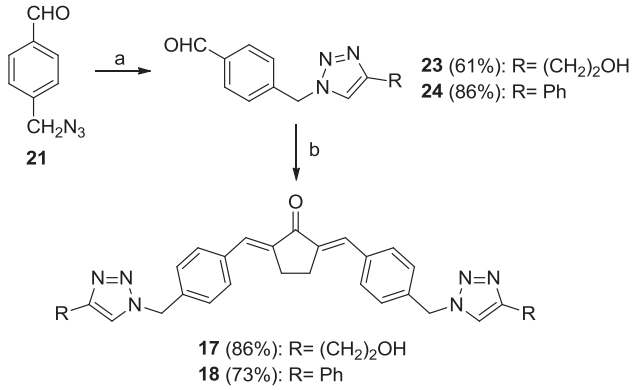
The compounds were evaluated *in vitro* for their ability to inhibit recombinant *T. cruzi* TR using the colorimetric assay described by Hamilton et al. (2003) [36]. Inhibition of human GR activity was carried out in parallel according to Carlberg and Mannervik (1985) [37]. Cruzain inhibition was measured based on a fluorimetric



Scheme 2. Synthetic route for the preparation of **8**.



Scheme 3. Synthetic route for the preparation of compounds **17** and **18**. Reagents and conditions: (a) benzoyl peroxide, NBS, benzene, reflux, 4 h. (b) (i) DIBALH, toluene, 0 °C, N_2 atm, 2 h. (ii) HCl 10%, $CHCl_3$, rt, 1 h. (c) NaN_3, THF/water, reflux, 24 h. (d) NaOH 0.2 M, EtOH 95%, rt, 2 h. (e) Terminal alkyne, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, CH_2Cl_2/water, rt, 24 h.



Scheme 4. Alternative route for the preparation of **17** and **18**. Reagents and conditions: (a) terminal alkyne, $CuSO_4 \cdot 5H_2O$, sodium ascorbate, CH_2Cl_2/water, r.t., 24 h. (b) NaOH 0.2 M, EtOH 95%, r.t., 4–20 h.

assay, as previously described [38]. The compounds were also assayed *in vitro* against promastigotes forms of *Leishmania* (*L.*) *amazonensis* [39] and amastigotes forms of *T. cruzi* [40,41]. Results are expressed as percentage of inhibition of parasite growth after treatment with each compound. Cell viability test was performed to evaluate the cytotoxicity of the compounds against normal cell, the murine fibroblast L-929 and murine peritoneal macrophages [39,42]. The concentrations that caused 50% inhibition of the enzymes activity and parasite growth (IC_{50}) were evaluated for the more potent compounds (those presenting more than 70% inhibition in the initial screening at 100 μM).

4. Results and discussion

Initially, the *in vitro* antiprotozoal activities of the synthesized compounds were assayed against *T. cruzi* and *L. amazonensis*. IC_{50} values are summarized in Table 1.

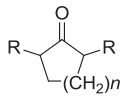
Six compounds (**2**, **5**, **11**, **13**, **14** and **16**) displayed *in vitro* trypanocidal activity with IC_{50} values ranging from 7.0 to 249 μM (Table 1). There was a good correlation between leishmanicidal and trypanocidal activity. All compounds active against intracellular

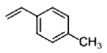
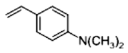
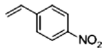
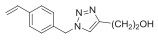
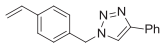
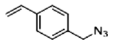
Table 1
Biological data for bis-(arylmethylidene)cycloalkanones.

Compound	IC_{50} *T. cruzi* (μM)	CC_{50} fibroblast (μM)	SI[a]	IC_{50} *L. amazonensis* (μM)	CC_{50} macrophage (μM)	SI[b]
2	73 ± 18	375 ± 177	5.1	46.6 ± 4.3	76.2 ± 6.9	1.6
3	>250	–	–	8.4 ± 0.3	50.6 ± 8.0	6
4	>250	–	–	52.6 ± 0.7	39.4 ± 4.2	0.7
5	230.5 ± 27.6	250 ± 0	1.1	110.4 ± 1.6	19.7 ± 3.3	0.2
6	>250	–	–	44.1 ± 5.5	17.6 ± 1.6	0.4
7	>250	–	–	18.6 ± 1.1	20.8 ± 3.8	1.1
8	>250	–	–	ND[c]	–	
9	>250	–	–	ND[c]	–	
10	>250	–	–	1.2 ± 0.0	23.9 ± 9.6	19.9
11	7.0 ± 2.1	25.3 ± 12.2	3.6	4.9 ± 0.9	3.7 ± 0.9	0.8
12	>250	–	–	0.6 ± 0.1	0.6 ± 0.5	1
13	31.3 ± 22.2	500 ± 0	16	0.9 ± 0.2	21.4 ± 5.3	23.8
14	68.7 ± 8.8	187.5 ± 88.4	2.7	8.4 ± 2.7	78.1 ± 9.9	9.3
15	>250	–	–	14.7 ± 0.9	55.8 ± 5.0	3.8
16	249 ± 104	375 ± 177	1.5	5.3 ± 1.8	83.2 ± 3.3	15.7
17	>250	–	–	50.9 ± 13.3	60.4 ± 3.8	1.2
18	>250	–	–	ND[c]	–	–
22	>250	–	–	ND[c]	–	–
Amphotericin B[d]	–	–	–	0.08 ± 0.03	0.7 ± 0.21	8.8
Benznidazole[d]	3.8 ± 0.8	>1000	>262	–	–	–

[a] Selectivity index (SI) is the ratio of murine fibroblast viability (CC_{50}) to the IC_{50} on *T. cruzi*.
[b] SI is the ratio of murine macrophage viability (CC_{50}) to the IC_{50} on *L. amazonensis*.
[c] ND = not determined due to low solubility.
[d] Positive control.

Table 2
Inhibitory activities of bis-(arylmethylidene)cycloalkanones against *T. cruzi* TR and human GR.



Compound	*n*	R	TR % inhibition at 100 μM	IC_{50} TR (μM)	IC_{50} GR (μM)
2	1		0	>100	>100
3	2		75.3	45.8 ± 11.2	>100
4	1		0	>100	>100
5	2		0	>100	>100
6	1		0	>100	>100
7	2		0.7	>100	>100
8	1		0	>100	>100
9	1		2.2	>100	>100
10	2		97.0	14.7 ± 4.2	>100
11	1		18.6	>100	>100
12	2		18.5	>100	>100
13	1		9	>100	>100
14	2		17.4	>100	>100
15	1		0	>100	>100
16	2		0	>100	>100
17	1		0	>100	>100
18	1		0	>100	>100
22	1		2.5	>100	228 ± 11.2
Clomipramine[a]	–	–	–	12.3 ± 2.3	–
Carmustine[a]	–	–	–	–	8.1 ± 1.9

[a] Positive control.

forms of *T. cruzi* were also active against extracellular promastigote forms of *L. amazonensis*. However, all compounds were more potent against *L. amazonensis* (IC_{50} values ranging from 0.6 to 110.4 μM) than against *T. cruzi*. Besides these six compounds, eight other molecules (**3**, **4**, **6**, **7**, **10**, **12**, **15** and **17**) exhibited significant leishmanicidal activity, demonstrating that the differences in susceptibility can be associated with the species parasites life stage.

The cytotoxicity of the active compounds on uninfected fibroblasts or macrophages was established *in vitro* to evaluate the selectivity of their antiparasitic effects. The selectivity index (SI) was calculated as the ratio of the CC_{50} value determined on the uninfected cells (cytotoxicity) to IC_{50} determined on parasite cells. Among the active compounds, compound **13** was identified as the promising compound with high SI (≥16) and remarkable activity against both parasites (Table 1).

In an attempt to investigate a possible mechanism of action, all the compounds were first screened at 100 μM for TR, GR inhibitory activity. The compounds **3** and **10** were able to inhibit more than 75% TR activity and then their IC_{50} values were determined as 45.8 and 14.7 μM, respectively. These results suggest that the presence of cyclohexanone ring is essential for activity, since the corresponding cyclopentyl analogs **2** and **9** were inactive (Table 2). A possible explanation for the difference in the activity between **3**–**2** and **10**–**9** analogs may be related to solubility differences. The cyclohexanone derivatives are always more soluble than corresponding cyclopentanone. All compounds exhibited weak inhibitory activity against GR, which is a good evidence of the selectivity of the compounds active against TR. The results are summarized in Table 2. These results are consistent since the active site of GR is narrow and cannot accommodate bulky hydrophobic substrates.

Compounds **3** and **10** exhibit good association between TR inhibitory activity and antileishmanial potency (extracellular forms of the parasite promastigotes), while showing almost no activity against amastigote forms of *T. cruzi*. The low trypanocidal activity of these two compounds may be due to their inability to cross the fibroblast and parasite's cell membranes and to reach their intracellular target. In an intracellular assay, any active compound must enter the host cell and the parasite in sufficient concentration to inhibit TR.

Compound **10**, as well as compound **13**, can be considered as a promising antileishmanial lead candidate because it showed IC_{50} values in the low-micromolar range (1.2 μM) with an adequate SI (19.9).

Given that several trypanocidal compounds were not TR inhibitors, their activity against cruzain was evaluated as an alternative mechanism of action. Due to the low solubility of most compounds in the assay buffer, only eight were evaluated against this enzyme and none showed significant activity at 100 μM (Table 3). We speculate that, although these compounds are potential Michael acceptors, they have low affinity for the active site of the enzyme. In other words, despite their potential as electrophiles, these compounds probably show low chemical complementarity to the enzyme's active site. There results show that the antiparasitic activity of the molecules described in this study is not due to cruzain inhibition, and further studies are necessary to determine their mechanism of action.

5. Conclusions

A new class of antiparasitic compounds has been discovered in this study, including molecules active at low micromolar concentrations against the parasites *T. cruzi* and *Leishmania* (*L.*) *amazonensis*. Based on its antiparasitic activity and low toxicity to mammalian cells, compounds **10** and **13** were identified as the best candidates for further studies for the development of novel antiparasitic drugs. A clear correlation between enzyme inhibition (TR or cruzain) and the antiparasitic activity was not observed to the active compounds of this series, except for compound **10**, which probably acts by inhibiting the enzyme TR. Further studies are needed to establish the mechanism of action of other active compounds.

Table 3
Inhibitory activities of bis-(arylmethylidene)cycloalkanones against cruzain.

Compound	Cruzain % inhibition at 100 μM
3	31 ± 2
5	32 ± 3
8	35 ± 3
9	23 ± 5
10	66 ± 2
12	42 ± 5
14	19 ± 5
17	16 ± 3

6. Experimental

6.1. General information

All reagents of analytical grade were obtained from commercial suppliers and used without further purification. 4-(Azidomethyl) benzaldehyde was synthesized in three stages from 4-benzonitrile according to previously reported procedures [34,35]. Reactions were monitored by TLC using silica gel coated plates and different solvents solutions as the mobile phase. Melting points were determined on a Microquímica MQAPF 301 apparatus and are uncorrected. FT-IR spectra were recorded using a PerkinElmer Spectrum One infrared spectrometer and absorptions are reported as wave numbers (cm-1). All NMR spectra were recorded on a Bruker Avance DPX 200 spectrometer (200 MHz). Chemical shifts are expressed in ppm relative to residual peaks of chloroform (δ = 7.27 ppm, 77.23 ppm), dimethyl sulfoxide (δ = 2.50 ppm, 39.50 ppm) or pyridine (δ = 7.22, 7.58, 8.74 ppm, 123.90, 135.90, 150.4 ppm) for ^{1}H and ^{13}C, respectively and multiplicity designated as follows: s, singlet; br, broad singlet; d, doublet; t, triplet; q, quintet; m, multiplet. Coupling constants (*J*) are given in hertz. Experimental data for compounds **2** [43,44], **3** [44,45], **4** [46,47], **5** [47], **6** [43,45], **7** [45,47], **8** [45,48], **9**–**10** [43], **11**–**12** [49], **19**–**21** [34,35] were previously described in the literature.

6.2. Synthesis

6.2.1. General procedure for the preparation of bis(arylmethylidene) cycloalkanones

The correspondent aldehyde (2 mmol) and ketone (1 mmol) were dissolved in a round-bottom flask containing 2 mL of EtOH 95%. Further, 1.5 mL of a solution of NaOH 0.2 M was added dropwise and the reaction stirred at room temperature until completion. The course of the reaction was monitored by TLC. After completion of the reaction, the precipitated product was filtered and washed with cold water.

*6.2.1.1. Synthesis of (2E,5E)-2,5-bis(isoquinolin-5-ylmethylene)cyclopentanone (**13**).* Obtained from the general procedure as a green solid, yield 87%; mp 239.0–240.0 °C; IR (neat, cm^{-1}) 3068, 2908, 1680, 1621, 1594, 1568, 1490, 1443; ^{1}H NMR (DMSO-d_6 + TFA) δ 9.96 (s, 2H), 8.76 (d, 2H, *J* = 6.6), 8.68 (d, 2H, *J* = 6.6), 8.57 (d, 2H, *J* = 8.2), 8.39 (d, 2H, *J* = 8.2), 8.16 (s, 2H), 8.08 (t, 2H, *J* = 8.2), 3.06 (s, 4H); ^{13}C NMR (DMSO-d_6 + TFA) δ 194.6, 148.6, 142.8, 137.3, 136.6, 133.2, 132.3, 131.9, 130.5, 127.9, 126.7, 121.9, 26.6.

*6.2.1.2. Synthesis of (2E,6E)-2,6-bis(isoquinolin-5-ylmethylene) cyclohexanone (**14**).* Obtained from the general procedure as a green solid, yield 88%; mp 192.1–193.3 °C; IR (neat, cm^{-1}) 3030, 2931, 2918, 2860, 2842, 1660, 1615, 1603, 1579, 1567, 1488; ^{1}H NMR (DMSO-d_6 + TFA) δ 9.96 (s, 2H), 8.73 (d, 2H, *J* = 6.4), 8.54 (d, 2H, *J* = 8.0), 8.41 (d, 2H, *J* = 6.4), 8.17–8.14 (m, 4H), 8.04 (t, 2H, *J* = 8.0), 2.71 (br, 4H), 1.63 (br, 2H); ^{13}C NMR (DMSO-d_6 + TFA) δ 188.5, 148.5, 141.0, 137.1, 136.9, 133.1, 133.0, 131.3, 131.2, 130.4, 127.9, 122.4, 28.2, 22.8.

*6.2.1.3. Synthesis of (2E,5E)-2,5-bis((1H-pyrrol-2-yl)methylene) cyclopentanone (**15**).* Obtained from the general procedure as a red solid, yield 44%; mp 265.2–267.8 °C; IR (neat, cm^{-1}) 3261, 3101, 2962, 2914, 2837, 1670, 1604, 1583, 1538, 1454; ^{1}H NMR (pyridine-d_5) δ 12.53 (s, 2H), 8.00 (s, 2H), 7.30 (br, 2H), 6.84 (br, 2H), 6.59–6.58 (m, 2H), 3.00 (s, 4H); ^{13}C NMR (pyridine-d_5) δ 194.7, 133.8, 131.2, 123.6, 122.9, 115.0, 112.3, 27.0.

*6.2.1.4. Synthesis of (2E,6E)-2,6-bis((1H-pyrrol-2-yl)methylene) cyclohexanone (**16**).* Obtained from the general procedure as a red solid, yield 45%; mp 204.1–205.8 °C; IR (neat, cm^{-1}) 3254, 3093, 2954, 2930, 2911, 2854, 1644, 1588, 1558, 1490, 1449; ^{1}H NMR (DMSO-d_6) δ 11.41 (s, 2H), 7.61 (s, 2H), 7.05 (br, 2H), 6.52 (br, 2H), 6.25 (br, 2H), 2.75–2.73 (m, 4H), 1.77 (br, 2H); ^{13}C NMR (DMSO-d_6) δ 186.9, 129.4, 129.0, 125.9, 122.2, 113.2, 110.8, 27.8, 21.6.

*6.2.1.5. Synthesis of (2E,5E)-2,5-bis(4-(azidomethyl)benzylidene) cyclopentanone (**22**).* Obtained from the general procedure as a yellow solid, yield 100%; mp 155.2–158.8 °C; IR (neat, cm^{-1}) 3030, 2912, 2848, 2093, 1698, 1615, 1600, 1561, 1509; ^{1}H NMR ($CDCl_3$) δ 7.64–7.60 (m, 6H), 7.40 (d, 4H, *J* = 8.0), 4.39 (s, 4H), 3.13 (s, 4H); ^{13}C NMR ($CDCl_3$) δ 196.3, 137.8, 136.9, 135.9, 133.4, 131.3, 128.7, 54.6, 26.7.

*6.2.1.6. Synthesis of (2E,5E)-2,5-bis(4-((4-(2-hydroxyethyl)-1H-1,2,3-triazol-1-yl)methyl)-benzylidene)cyclopentanone (**17**).* Obtained from the general procedure as a yellow solid, yield 86%; mp 170.0 °C dec; IR (neat, cm^{-1}) 3273, 3136, 2917, 1691, 1625, 1598, 1565, 1511, 1050; ^{1}H NMR (DMSO-d_6) δ 7.94 (s, 2H), 7.68 (d, 4H, *J* = 8.0), 7.40–7.36 (m, 6H), 5.60 (s, 4H), 4.72 (s, 2H), 3.63–3.61 (m, 4H), 3.06 (s, 4H), 2.76 (t, 4H, *J* = 6.7); ^{13}C NMR (DMSO-d_6) δ 195.3, 144.8, 138.1, 137.6, 135.0, 132.0, 131.0, 128.3, 122.7, 60.3, 52.3, 29.2, 26.0.

*6.2.1.7. Synthesis of (2E,5E)-2,5-bis(4-((4-phenyl-1H-1,2,3-triazol-1-yl)methyl)-benzylidene)cyclopentanone (**18**).* Obtained from the general procedure as a yellow solid, yield 73%; mp 301.7 °C dec; IR (neat, cm^{-1}) 3125, 3093, 2949, 1695, 1628, 1602, 1566, 1512.

6.2.2. General procedure for the copper-catalyzed 1,3-dipolar cycloadditions

To a suspension of 3-butyn-1-ol or phenylacetylene (1.24 mmol), azide **21** (1.24 mmol), $CuSO_4 \cdot 5H_2O$ (0.12 mmol) in CH_2Cl_2 (4 mL) was added a solution of ascorbic acid (0.37 mmol) and sodium bicarbonate (0.37 mmol) in H_2O (4 mL). The mixture was stirred for 20 h. It was then diluted with H_2O (3 mL), extracted with CH_2Cl_2 (3 × 5 mL) and the combined organic layers washed with water (10 mL), dried over $MgSO_4$, filtered and evaporated to dryness under reduced pressure.

*6.2.2.1. Synthesis of 4-((4-(2-hydroxyethyl)-1H-1,2,3-triazol-1-yl) methyl)benzaldehyde (**23**).* Obtained from the general procedure as a pale yellow solid, yield 61%; mp 88.3–89.7 °C; IR (neat, cm^{-1}) 3454, 3144, 2953, 2859, 2747, 1686, 1606, 1578, 1553, 1042; ^{1}H NMR ($CDCl_3$) δ 9.99 (s, 1H), 7.86 (d, 2H, *J* = 8.0), 7.41–7.37 (m, 3H), 5.58 (s, 2H), 3.91 (t, 2H, *J* = 5.9), 2.93 (t, 2H, *J* = 5.9), 2.90 (br, 1H); ^{13}C NMR ($CDCl_3$) δ 191.7, 146.4, 141.4, 136.6, 130.5, 128.5, 122.0, 61.6, 53.7, 28.9.

*6.2.2.2. Synthesis of 4-((4-phenyl-1H-1,2,3-triazol-1-yl)methyl) benzaldehyde (**24**).* Obtained from the general procedure as a pale yellow solid, yield 86%; mp 125.3–127.0 °C; IR (neat, cm^{-1}) 3082, 2828, 2741, 1691, 1607, 1578; ^1H NMR ($CDCl_3$) δ 10.01 (s, 1H), 7.88 (d, 2H, $J = 7.8$), 7.83–7.76 (m, 3H), 7.45–7.32 (m, 5H), 5.66 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 191.6, 141.4, 136.6, 130.6, 130.4, 129.0, 128.55, 128.51, 125.9, 53.9.

6.3. Biology

6.3.1. Trypanothione reductase enzyme assay

Recombinant trypanothione reductase from *T. cruzi* (*Tc*TR), was expressed in *Escherichia coli* BL21DE3 and purified by affinity chromatography. The method is based on the reduction of oxidized trypanothione by TcTR (T[S]2 → T[SH]2) and its *in situ* regeneration by 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (T[SH]2 → T[S]2). TR assays were performed as described by Hamilton et al. (2003) [36] in 96 well micro plates containing 40 mM HEPES (pH7.5), 1 mM EDTA, 1 μM oxidized trypanothione (T[S]$_2$ Bachem®), 150 μM NADPH and 37 ng of TcTR in a final volume of 240 μL per well, compounds and controls were pre-incubated at 27 °C for 30 min, when 10 μL of DTNB was added to the reaction mixture. Absorbance was read at λ412 nm for measuring the mean curve slope (δAbs/δt) during 30 min with 5 min interval at 27 °C in a TECAN® Infinite M2000 microplate reader. The percentage of inhibition was determined as (1 − (δAbs)compound)/((δAbs)negative control) × 100. DMSO 1% (v/v) and Clomipramine (Novartis) were used as negative and positive inhibition control respectively. All experiments were done in triplicate and repeated at least three times.

6.3.2. Human Glutathione Reductase enzyme assay

The colorimetric assay was performed as described by Carlberg and Mannervik (1985) [37] in 96 well plates containing 100 mM Potassium Phosphate Buffer (pH 7.0), 1 mM EDTA, 400 μM NADPH, and 10 mU of recombinant human glutathione reductase (Sigma Aldrich, St. Louis) in a volume of 240 μL per well. Compounds and controls were pre-incubated at 27 °C for 30 min. The reaction was started with the addition of 10 μL of 1 mM oxidized glutathione. Absorbance was read at λ340 nm for measuring the mean curve slope (δAbs/δt) during 5 min with 30 s interval at 30 °C in a TECAN® Infinite M200 microplate reader. The percentual inhibition was calculated as (1 − (δAbs)compound)/((δAbs)negative control) × 100. DMSO 1% and Carmustine (Bristol-Myers Squibb, New York) were used as negative and positive inhibition control respectively.

6.3.3. Cruzain enzyme assay

Recombinant cruzain was gently provided by Dr. Anna Tochowicz and Dr. James McKerrow, from the University of California San Francisco. Cruzain activity was measured based on the cleavage of the fluorogenic substrate Z-Phe-Argaminomethylcoumarin (Z-FR-AMC) in a Synergy 2 (Biotek), from the Center of Flow Cytometry and Fluorimetry at the Biochemistry and Immunology Department (UFMG), using filters of 340 nm for excitation and 440 nm for emission. All assays were performed in sodium acetate 0.1 M pH 5.5 and in the presence of 5 mM dithiothreitol (DTT) and 0.01% Triton X-100. The final concentration of cruzain was 0.5 nM, and the substrate concentration was 2.5 μM (Km = 1 μM). Compounds were initially screened at 100 μM, without pre-incubation with the enzyme, and the data here reported refers to this assay condition. To verify if the inhibition observed was due to artifacts the percentages of inhibition for the most active compounds were was measured in the absence of Triton and in the presence of 0.1% [50,51]. To evaluate time-dependence, compounds were assayed after a 10-min pre-incubation with enzyme. The compounds were neither time-dependent nor sensitive to Triton concentration. All assays were performed in triplicates, followed for 5 min, and activity was calculated based on compared to a DMSO control.

6.3.4. Biological evaluation in L. amazonensis

Parasites – L. amazonensis (IFLA/BR/1967/PH-8) was used in this study. Parasites were grown at 24 °C in Schneider's medium (Sigma, St. Louis, MO, USA), supplemented with 20% heat-inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at pH 7.4. Stationary-phase promastigotes were prepared as described [52].

Antileishmanial in vitro activity – Inhibition of cell growth was assessed *in vitro* by cultivating stationary-phase promastigote (5×10^5) of *L. amazonensis* in the presence of different individual concentrations of the compounds in 96-well culture plates (Corning Life Sciences, Corning, NY, USA), for 48 h at 24 °C. Cell viability was assessed by measuring the cleavage of 2 mg/ml of MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide] (Sigma). Absorbances were measured by using a multi-well scanning spectrophotometer (LABTRADE, model 660) at a wavelength of 570 nm. Analyses were performed in triplicate, and results were expressed as the mean percentage reduction of the parasites compared to non-treated control wells. The 50% inhibitory concentration (IC_{50}) was determined by applying the sigmoidal regression of the concentration–response curves. Data shown are representative of three different experiments, performed in triplicate, which presented similar results.

6.3.5. Cytotoxicity in murine macrophages

Cytotoxicity was evaluated by cultivating murine peritoneal macrophages (5×10^5) with different concentrations of the compounds in 96-well plates, for 24 h. Cell viability was assessed by the MTT assay, and results were expressed as the percentage of cell lyses compared to cultures treated with amphotericin B (1 μg/mL).

6.3.6. Biological evaluation in T. cruzi

In vitro antitrypanosomal activity – The *in vitro* antitrypanosomal activity in amastigote forms of *T. cruzi* was evaluated by colorimetric beta-galactosidase assay developed by Buckner et al. (1996) [53] and modified by Romanha et al. (2010) [41]. *T. cruzi* (Tulahuen strain) expressing the *E. coli* beta-galactosidase gene were grown on monolayer of mouse L-929 fibroblasts. Cultures assayed for beta-galactosidase activity were grown in RPMI 1640 medium without phenol red plus 10% fetal bovine serum and glutamine. Ninety-six-well tissue culture micro plates were seeded with L-929 fibroblasts at 4.0×10^3 per well in 80 μL and incubated overnight at 37 °C, 5% CO_2. Beta-galactosidase- expressing trypomastigotes were then added at 4.0×10^4 per well in 20 μL. After 2 h, the medium with trypomastigotes that have not penetrated in the cells was discarded and replaced by 200 μL of fresh medium. After 48 h, the medium was discarded again and replaced by 180 μL of fresh medium and 20 μL of test compounds. Each compound was tested in tetraplicate. After 7 days culture development, chlorophenol red beta-D-galactopyranoside at 100 μM and Nonidet P-40 at 0.1% were added to the plates and incubated overnight, at 37 °C. The absorbance was measured at 570 nm in an automated microplate reader. Benznidazole at its IC_{50} (1 μg/mL = 3.81 μM) was used as positive control. The results are expressed as percentage of parasite growth inhibition.

6.3.7. Cytotoxicity in human cells

The active compounds were tested *in vitro* for determination of cytotoxic over L-929 cells using the alamarBlue® dye. Were used the same cell number, time of the cell development and time of compound exposure used for the beta-galactosidase assay. The cells

were exposed to compounds at crescents concentrations starting at IC_{50} value of the *T. cruzi*. The compounds were tested in tetraplicate. After 96 h of compounds exposure the alamarBlue® was added and the absorbance at 570 and 600 nm measured after 4–6 h. The cell viability was expressed as the percentage of difference in the reduction between treated and untreated cells [41]. IC_{50} values were calculated by linear interpolation and the Selectivity Index (SI) was determined based on the ratio between CC_{50} and IC_{50} values.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at HYPERLINK "doi:doi:10.1016/j.ejmech.2013.11.011. These data include MOL files and InChiKeys of the most important compounds described in this article.

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