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Synthesis and in vitro antiprotozoal evaluation of substituted phenalenone analogues

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ABSTRACT

A set of derivatives encompassing structural modifications on the privileged phenalenone scaffold were assessed for their antiparasitic activities against the most clinically relevant forms of trypanosomiasis and leishmaniasis. Several compounds exhibited leishmanicidal effects at levels comparable or better than the reference drug pentamidine, while the parent phenalenone was shown to have a level of activity against *Trypanosoma cruzi* comparable to the marketed drug benznidazole.

1. Introduction

Trypanosomatids of the order Kinetoplastida are the causative agents of several lethal parasitic diseases, such as Chagas' disease (*Trypanosoma cruzi*), and African sleeping sickness (*Trypanosoma brucei*). The human protozoan parasite Leishmania is the causative agent of leishmaniasis, a disease with a wide variety of clinical manifestations, ranging from self-healing cutaneous lesions (mostly from *Leishmania tropica* and *Leishmania mexicana* complexes) to life-threatening visceral infections caused by different species of the donovani complex (*Leishmania donovani*, *Leishmania infantum*, and *Leishmania chagasi*). The reemergence of trypanosomiasis and leishmaniasis over the last two decades has become a significant threat to human health and the economical development of several developing nations. The drugs which have been most frequently used to treat the leishmaniasis (the pentavalent antimonials, Pentostam, and Glucantime[1]) are however, quite toxic and in some areas resistance can be as high as 40%.[2,3] Likewise those used for trypanosomiasis, such as Nifurtimox (currently discontinued) and Benznidazole are still inadequate due to their undesired side effects.[4] Indeed, no vaccine or recommended drugs are currently available to prevent these diseases. Moreover, once the infection has progressed into its later stages none of the marketed drugs are effective. The emergence of drug-resistant parasites is also becoming an additional and major problem. Further investigations are therefore urgently needed to discover new drugs that are not only effective, but also affordable and readily available for the treatment of these infectious diseases.

Previous work from our group had demonstrated that a set of antifungal phytoalexins,[5,6] based on a phenyl-phenalenone skeleton, possessed leishmanicidal activity.[7] In order to further investigate the effect of electronic and spatial changes on antiprotozoal activity we have synthesized a series of core modified structures,[8–11] as well as a set of new heteroaryl substituted phenalenones. Herein, we report their synthesis and their antiprotozoal activity against two forms of Leishmania (*Leishmania amazonensis*, *L. donovani*) and one form of Trypanosoma (*T. cruzi*).

2. Results and discussion

2.1. Synthesis

The heteroaryl-substituted phenalenones were prepared using a general two step Michael addition-oxidation sequence. The required heteroaryl Grignards for the preparation of compounds **6**, **7**, and **10** were generated in situ via either direct lithiation and metal exchange, or lithium–halogen exchange followed by metal exchange as shown in Table 1 (entries 1–3).

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Table 1
Het-MgBr preparation conditions

Entry	Het-MgBr
1  BuLi, $MgBr_2{\cdot}Et_2O$, THF, -25 °C	 **1**
2  $MgBr_2{\cdot}Et_2O$, THF, -25 °C	 **2**
3  BuLi, $MgBr_2{\cdot}Et_2O$, THF, RT	 **3**

Michael-type addition of 2-furyl Grignard reagent **1** to phenalen-1-one **4** (Scheme 1) furnished the phenolic compound **5** in good yield. Treatment of this intermediate with DDQ gave the desired phenalenone **6** in high yield. In the same manner, addition of the 2-thienyl Grignard reagent **2** to **4** followed by oxidation with DDQ afforded **7**. Addition of the 2-pyridyl Grignard **3** gave the hydroxyl intermediate **9** and the bis-pyridyl adduct **8**. A solution of **9** in chloroform converted cleanly to the oxidized phenalenone system **10**. Attempts to increase the poor yield of **10** by changing reaction conditions or the method of Grignard generation[12–16] were fruitless. The reaction of **4** with sodium cyanide in aqueous DMF introduced a cyano group at the same position as the previous aryls. In situ air oxidation afforded **11** directly.

2.2. Antiprotozoal activity

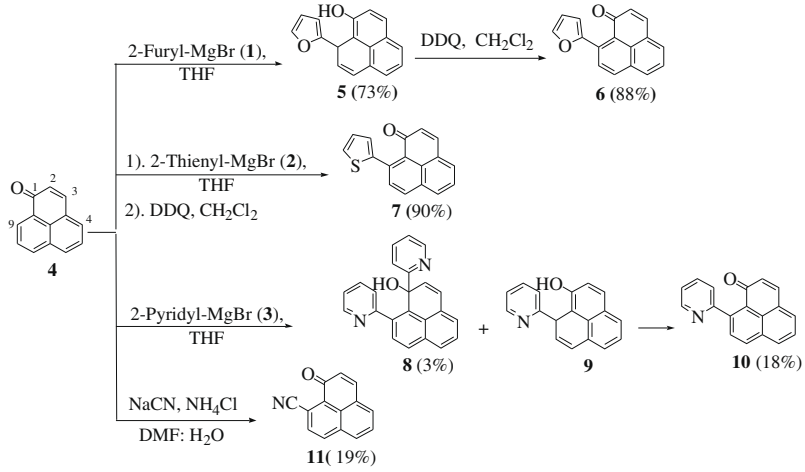
A total of 21 phenalenone-based compounds were investigated for their activities against the clinically relevant forms of trypanosomiasis and leishmaniasis (namely *T. cruzi*, *L. amazonesis*, and *L. donovani*), the results of which are illustrated in Table 2.

While only the parent phenalenone-1-one **4** exhibited significant potency against *T. cruzi* (IC_{50} = 2.6 μg/mL), several of the substituted compounds possessed enhanced activities in a selective manner against *L. amazonensis*. While the addition of either a bulky 2,5-dimethylphenyl (compound **14**), a 2-pyridyl (compound **10)** or an unsubstituted phenyl group (compound **12**) at position 9 proved to be detrimental to activity against *L. amazonensis*, the addition of a 2-furyl (compound **6**) or a 2-thienyl group (compound **7**) afforded compounds with modest IC_{50}'s. The corresponding 4-methoxyphenyl, compound **13**, however, was 30-fold more active than the parent compound **4** and fivefold more active than the positive control, pentamidine. Interestingly, compound **23**, which possesses the 4-methoxyphenyl group on carbon 4, also exhibited activity that was comparable to the positive control, indicating that this change in the relative positions between the carbonyl and the substituted phenyl moiety was tolerated. The addition of the 4-methoxyphenyl group to the β-carbon of the enone system (compound **25**) however, rendered a compound void of activity against any of the parasitic types. Addition of either, a hydroxyl (compound **16**) or methoxy group (compound **19)**, next to the carbonyl of **13** resulted in abolishment of all antiparasitic activity, suggesting that hydrophilic groups were not tolerated in this region of the molecule. Similarly the diol **21** was also found to be void of activity.

Table 2
In vitro antiprotozoal activities of phenalenones (**4**–**25**) and cytotoxicity

Compound	IC_{50} (μg/mL) ± S.D			MCF7[a] %inhibition (10 μM)
	L. amazonensis	*L. donovani*	*T. cruzi*	
4	27.30 ± 1.31	65.07 ± 1.19	2.62 ± 0.86	0
5	—	—	—	nd
6	8.39 ± 1.03	—	—	14
7	14.50 ± 2.5	—	—	4
8	—	—	—	0
10	53.17 ± 1.86	—	—	0
11	3.73 ± 0.04	1.02 ± 0.48	—	44
12	—	—	—	5
13	0.83 ± 0.14	—	—	13
14	42.83 ± 2	27.80 ± 2.12	49.75 ± 0.5	1
15	—	—	—	23
16	—	—	—	19
17	—	—	—	22
18	9.04 ± 1.06	29.90 ± 0.96	48.35 ± 2.1	14
19	—	—	—	15
20	—	—	—	17
21	—	—	—	nd
22	—	—	—	19
23	4.43 ± 0.64	—	11.4 ± 0.99	17
24	—	—	—	12
25	—	—	—	2
Control	5.72[b]	5.72[b]	0.83[c]	90[d]

[a] Cytotoxicity in MCF7 cells. Inhibition of cell growth. Data are expressed as % inhibition = % activity in the presence of vehicle – % activity in the presence of inhibitor.
[b] Reference compound, pentamidine.
[c] Reference compound, benznidazole.
[d] Reference compound, adriamycin; — signifies an IC_{50} >100 μg/mL; nd signifies not determined.



Scheme 1.

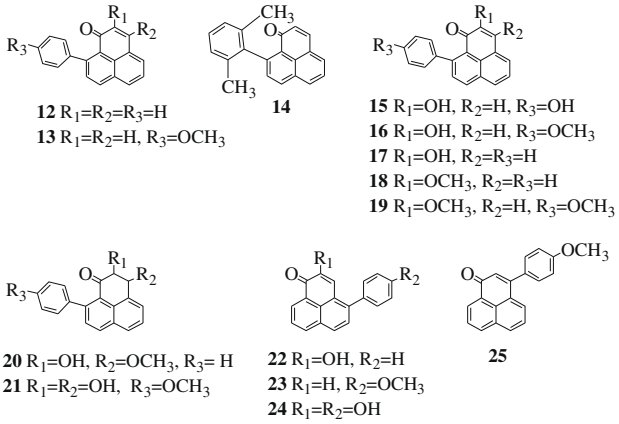


Figure 1. Compounds included in in vitro antiprotozoal study.

Of significant interest was the level of activity displayed by the cyano compound **11** against both forms of Leishmania. It possessed activity equal to that of pentamidine against *L. amazonensis* while being fivefold more active than the control against *L. donovani*, possessing an IC_{50} of 1 μg/mL.

2.3. Cellular cytotoxicity

The ability of these phenalenone-based compounds to inhibit cell growth in a human breast adenocarcinoma cell line (MCF7) was used as a measure of cytotoxicity. In general the compounds possessed only low levels of inhibition (<25% inhibition at 10 μM). The parent phenalenone **4** showed no cytotoxicity at a concentration (0% at 1.8 μg/mL) similar to its IC_{50} against *T. cruzi*. Compound **23** was also non-cytotoxic at a concentration (17% at 2.9 μg/mL) similar to its IC_{50} against *L. amazonensis*, while the more potent and selective antiprotozoal, compound **13**, did not show signs of inhibiting cell growth at a concentration three fold greater (13% at 2.9 μg/mL) than its IC_{50} against *L. amazonensis*. The only compound in this series to show a modest inhibition of cell growth was compound **11**. At a concentration twofold higher than its IC_{50} against *L. donovani* (10 μM or 2.1 μg/mL), it inhibited cell growth by 44% (Fig. 1).

3. Conclusions

Screening of a collection of modified phenalen-1-one based structures has lead to the identification of several new and potent antiprotozoal agents. Interestingly, the majority of the active compounds were more active against *L. amazonensis* than the *L. donovani* strain. Of the active compounds, **23** possessed a level of activity comparable to the positive control pentamidine, while compound **13** was significantly more potent than the control, possessing an IC_{50} <1 μg/mL. Neither compound showed signs of cytotoxicity at these concentrations. Compound **11** was the only compound from this collection that significantly inhibited both forms of Leishmania. It was shown to be more active than the positive control in both strains. Unlike the other compounds in the series, **11** inhibited cell growth in human MCF7 cells at concentrations in the same range as its antiprotozoal activity. Compounds **13** and **23** represent interesting candidates for further development, while further SAR explorations around **11** may lead to compounds with a higher therapeutic window. Given the issues associated with the therapeutic agents currently used for the treatment of *T. cruzi* infections, further development of the parent compound phenalenone-1-one (**4**) is also warranted.

4. Experimental

4.1. Chemistry

4.1.1. General

^1H and ^{13}C NMR spectra were recorded on Bruker AMX400, AMX300 and WP200SY. IR spectra were taken on a Perkin–Elmer 1600 (FTIR) spectrophotometer. High resolution mass spectra were run on a VG-Micromass ZAB-2F at 70 eV. Melting points were recorded in a BÜCHI B-540. Optical rotations were recorded in a polarimeter Perkin–Elmer 343. Organic solvents used were dried by standard methods. All reactions were performed under an atmosphere of argon unless otherwise specified. Commercially obtained reagents were used without further purification. All reactions were monitored by TLC with Merck 60 F_{254} Silica Gel coated plates. Flash column chromatography was carried out using 230–240 mesh silica gel at increased pressure.

4.1.2. Synthetic procedures

4.1.2.1. 9-Furan-2-yl-9*H*-phenalen-1-ol (5). *n*-BuLi (0.33 mL, 0.825 mmol, 2.5 M) in THF was added dropwise to a solution of furan (0.06 mL, 0.825 mmol, 1 M) in THF at −25 °C. After 0.5 h this solution was added to a mixture of $MgBr_2{\cdot}OEt_2$ (156 mg, 0.605 mmol) in THF (5.5 mL) at −25 °C. The reaction mixture was allowed to warm to room temperature and stirring was continued for a further 1 h. A solution of **4** (100 mg, 0.55 mmol) in THF (5.5 mL) was then added and the mixture was heated at reflux for 3 h. The reaction was quenched with saturated aqueous NH_4Cl (10 mL) and extracted with Et_2O (3 × 10 mL). The combined organic layers were washed with water (2 × 10 mL), brine (10 mL) and then dried (Na_2SO_4). Filtration and evaporation of the solvent afforded the crude product. Column chromatography (hexane/EtOAc, 9:1) gave **5** (99.3 mg, 73%) as a yellowish oil. IR ($CHCl_3$) ν_{max} 2348, 1637, 1239, 841 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 7.72–7.68 (m, 2H), 7.41 (br s, 1H), 7.37–7.32 (m, 1H), 7.24–7.20 (m, 2H), 6.83 (d, J = 9.6 Hz, 1H), 6.37 (m, 2H), 6.22 (m, 1H), 6.15 (m, 1H), 5.53 (br s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.1, 152.2, 141.8, 129.5, 129.1, 128.7, 128.3, 127.9, 127.7, 127.4, 124.2, 123.9, 118.8, 116.2, 110.9, 106.3, 36.9. EIMS: *m/z* 246 (100.0), 248 $[M]^+$ (37.7). HRMS: calcd for $C_{17}H_{12}O_2$ 248.0837, found 248.0774.

4.1.2.2. 9-(2-Furyl)-1*H*-phenalen-1-one (6). DDQ (41 mg, 0.18 mmol) was added to a solution of **5** (44.1 mg, 0.18 mmol) in CH_2Cl_2 (1.8 mL) and the reaction mixture was heated at reflux for 3 h. The reaction was quenched with saturated aqueous NH_4Cl (3 mL), and extracted with Et_2O (3 × 3 mL). The combined organic layers were washed with water (2 × 10 mL), brine (10 mL) and then dried (Na_2SO_4). Filtration and evaporation of the solvent afforded the crude product. Column chromatography (hexane/EtOAc, 8:2) gave **6** (39 mg, 88%) as a yellowish oil. IR ($CHCl_3$) ν_{max} 1726, 1237, 843 cm^{-1}. UV–Vis (EtOH) λ_{max} 255, 278, 291, 381 nm. ^1H NMR (300 MHz, $CDCl_3$) δ 8.16 (d, J = 8.5 Hz, 1H), 7.98 (d, J = 8.0 Hz, 1H), 7.97 (d, J = 8.5 Hz, 1H), 7.75 (d, J = 7.0 Hz, 1H), 7.68 (d, J = 9.7 Hz, 1H), 7.62–7.56 (m, 2H), 7.18 (d, J = 3.3 Hz, 1H), 6.71 (d, J = 9.7 Hz, 1H), 6.60 (dd, J = 3.3, 1.5 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.4, 153.1, 143.4, 140.0, 134.5, 134.0, 131.7, 131.4 (×2), 130.2, 129.7, 128.5, 128.4, 126.5, 125.7, 111.9, 111.7. EIMS: *m/z* 246 $[M]^+$ (100.0), 205 (82.6), 189 (91.7). HRMS: calcd for $C_{17}H_{10}O_2$ 246.0688, found 246.0699.

4.1.2.3. 9-(2-Thienyl)-1*H*-phenalen-1-one (7). 2-Thienyllithium (0.825 mL, 0.825 mmol) was added dropwise to a stirring solution of $MgBr_2{\cdot}Et_2O$ (156.2 mg, 0.605 mmol) in THF (1.21 mL) at −25 °C. The reaction mixture was allowed to warm to room temperature. After 1 h a solution of **4** (100 mg, 0.55 mmol) in THF (5.5 mL)

was added and the reaction was heated at reflux for 3 h. The reaction was quenched with saturated aqueous NH_4Cl (10 mL) and extracted with Et_2O (3 × 10 mL). The combined organic layers were washed with water (2 × 10 mL), brine (10 mL) and then dried (Na_2SO_4). Filtration and evaporation of the solvent afforded the intermediate alcohol that was used without further purification. It was dissolved in CH_2Cl_2 (6.18 mL) and DDQ (140.7 mg, 0.618 mmol) was added. The reaction mixture was heated at reflux for 3 h and then quenched with saturated aqueous NH_4Cl (10 mL), and extracted with Et_2O (3 × 10 mL). The combined organic layers were washed water (2 × 10 mL), brine (10 mL) and then dried (Na_2SO_4). Filtration and evaporation of the solvent afforded the crude product. Column chromatography (hexane/EtOAc, 7.5:2.5) gave **7** (130 mg, 90%) as a yellowish oil. IR ($CHCl_3$) ν_{max} 1637, 1238, 835 cm^{-1}. UV–Vis (EtOH) λ_{max} 208, 255, 371 nm. ^1H NMR (300 MHz, $CDCl_3$) δ 8.13 (d, J = 8.3 Hz, 1H), 8.00 (d, J = 8.2 Hz, 1H), 7.77–7.54 (m, 3H), 7.45 (dd, J = 5.0, 1.2 Hz, 1H), 7.20 (dd, J = 3.5, 1.2 Hz, 1H), 7.13 (dd, J = 5.0, 3.5 Hz, 1H), 6.63 (d, J = 9.7 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.7, 143.4, 140.5, 139.9, 133.7, 132.4, 132.0, 131.8 (×2), 130.2, 128.3, 127.1, 126.8, 126.7, 126.6, 126.1 (×2). EIMS: m/z 261 $[M-1]^+$ (100.0), 262 (45.70). HRMS: calcd for $C_{17}H_{10}OS$ 262.0452, found 262.0418.

4.1.2.4. 1, 9-Di-pyridin-2-yl-1*H*-phenalen-1-ol (8) and 9-(2-pyridyl)-1*H*-phenalen-1-one (10). To a solution of EtMgBr (3 mL, 3.00 mmol, 1 M) at room temperature was added 2-iodopyridine (0.23 mL, 2.13 mmol). After 45 min a solution of **4** (500 mg, 2.77 mmol) in THF (27.7 mL) was added and the reaction was heated at reflux for 3 h. The reaction was quenched with saturated aqueous NH_4Cl (5 mL) and extracted with Et_2O (3 × 5 mL). The combined organic layers were washed with water (2 × 10 mL), brine (10 mL) and then dried (Na_2SO_4). Filtration and evaporation of the solvent afforded the crude products. Column chromatography (hexane/EtOAc, 9:1) gave **8** (26.8 mg, 3%) and **9** as a yellowish amorphous solids. A solution of **9** in chloroform in air converted cleanly to **10** (102.3 mg, 18%) as a yellowish amorphous solid. Compound (**8**): IR ($CHCl_3$) ν_{max} 2923, 1585, 1432, 1257, 670 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 8.49 (br d, J = 4.4 Hz, 2H), 7.73–7.67 (m, 3H), 7.61 (dd, J = 8.0, 1.6 Hz, 1H), 7.54 (d, J = 8.2 Hz, 2H), 7.23–7.14 (m, 5H), 6.83 (d, J = 9.7 Hz, 1H), 6.15 (d, J = 9.7 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.2, 156.0, 147.6, 137.3, 131.4, 129.3, 128.6, 128.3, 128.1, 128.0, 126.1, 124.5, 123.8, 122.7, 122.1, 121.3, 117.4, 58.7, 31.7, 29.4, 29.1, 22.5, 13.9. EIMS: m/z 228 (33.4), 256 (39.7), 257 (46.4), 258 (100.0), 336 $[M]^+$ (15.6). HRMS: calcd for $C_{23}H_{16}N_2O$ 336.1263, found 336.1247.

Compound (**9**): ^1H NMR (300 MHz, $CDCl_3$) δ 8.73 (br d, J = 4.5 Hz, 1H), 7.73–7.67 (m, 1H), 7.58–7.53 (m, 2H), 7.43 (d, J = 7.9 Hz, 1H), 7.20–7.13 (m, 4H), 7.00 (d, J = 9.7 Hz, 1H), 6.14 (dd, J = 9.7, 4.7 Hz, 1H), 5.67 (br d, J = 4.7 Hz, 1H).

Compound (**10**): IR ($CHCl_3$) ν_{max} 1638, 1239, 848, 670 cm^{-1}. UV–Vis (EtOH) λ_{max} 212, 251, 315, 361, 394 nm. ^1H NMR (300 MHz, $CDCl_3$) δ 8.72 (br d, J = 4.6 Hz, 1H), 8.23 (d, J = 8.3 Hz, 1H), 8.06 (d, J = 7.6 Hz, 1H), 7.80–7.63 (m, 5H), 7.43 (d, J = 7.9 Hz, 1H), 7.35–7.31 (m, 1H), 6.59 (d, J = 9.7 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 185.4, 160.5, 149.2, 145.1, 140.6, 135.7, 133.9, 131.9, 131.6, 131.5, 130.0, 129.8, 128.3, 127.8, 126.5, 126.2, 123.1, 121.8. EIMS: m/z 256 (100.00), 257 $[M]^+$ (58.09). HRMS: calcd for $C_{18}H_{11}NO$ 257.0841, found 257.0840.

4.1.2.5. 9-Oxo-9*H*-phenalene-1-carbonitrile (11). NaCN (108 mg, 2.20 mmol) was added to a solution of **4** (100 mg, 0.55 mmol) and NH_4Cl (88 mg, 1.65 mmol) in DMF/H_2O (1:1, 5.5 mL) at room temperature. The reaction was heated at reflux for 5 h. The reaction was then extracted with CH_2Cl_2 (3 × 15 mL). The combined organic layers were washed with water (2 × 10 mL), brine (10 mL) and then dried (Na_2SO_4). Filtration and evaporation of the solvent afforded the crude product. Column chromatography (hexane/EtOAc, 9:1) gave **11** (22 mg, 19%) as an orange amorphous solid. IR ($CHCl_3$) ν_{max} 2221, 1639, 843 cm^{-1}. UV–Vis (EtOH) λ_{max} 259, 308, 318, 372, 401 nm. ^1H NMR (300 MHz, $CDCl_3$) δ 8.3 (d, J = 8.3 Hz, 1H), 8.10 (d, J = 8.2 Hz, 1H), 8.00 (d, J = 8.3 Hz, 1H), 7.80 (d, J = 6.9, 1H), 7.70 (d, J = 9.8 Hz, 1H), 7.70 (dd, J = 8.2, 7.0 Hz, 1H), 6.80 (d, J = 9.8 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 182.6, 141.2, 134.1, 133.7, 132.5, 131.4, 131.3, 129.0, 128.9 (×2), 128.1, 127.1, 118.4, 112.2. EIMS: m/z 150 (20.2), 177 (100.0), 205 $[M]^+$ (62.6). HRMS: calcd for $C_{14}H_7NO$ 205.0527, found 205.0500.

4.1.2.6. 9-(2, 6-Dimethyl-phenyl)-1*H*-phenalen-1-one (14). To a stirred solution of **4** (100 mg, 0.55 mmol) in THF (5.55 mL) was added 0.7 mL of 2,6-dimethylphenyl-magnesium bromide (0.71 mmol, 1.0 M solution in THF). The reaction mixture was stirred and heated at reflux for 3 h. The reaction mixture was cooled to room temperature, and quenched with an aqueous saturated solution of NH_4Cl (2 mL) and extracted with EtOAc (3 × 5 mL). The combined organic extracts were washed with water (3 × 5 mL), brine (5 mL) and dried (Na_2SO_4). Removal of solvent under vacuo followed by silica gel column chromatography (EtOAc/hexanes, 1:9) afforded compound **14** (100 mg, 63%) as a yellow solid; mp 154.6–155.1 °C; IR (thin film, NaCl) ν_{max} 1621, 1553, 1351, 1238, 1178, 841, 692, 654, 601 cm^{-1}; UV–Vis (EtOH) λ_{max} 216, 254, 311, 346, 360, 391 nm. ^1H NMR (400 MHz, $CDCl_3$) δ 8.32 (d, J = 8.3 Hz, 1 H), 8.11 (dd, J = 8.2, 1.0 Hz, 1 H), 7.83 (dd, J = 7.0, 1.0 Hz, 1 H), 7.75 (d, J = 9.7 Hz, 1 H), 7.67 (dd, J = 8.2, 7.0 Hz, 1 H), 7.49 (d, J = 8.3 Hz, 1 H), 7.28–7.18 (m, 3 H), 6.60 (d, J = 9.7 Hz, 1 H), 1.93 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 185.5, 146.6, 142.3, 140.5, 134.6, 133.5 (×2), 131.7, 131.6, 131.1, 130.6, 130.0, 128.5, 128.4, 127.4 (×2), 126.7, 126.3 (×2), 20.5 (×2); EIMS: m/z 134.20 (17.36), 239.08 (16.62), 252.08 (26.57), 267.09 (39.77), 269.08 (100.00), 270.10 (39.43), 284.12 $[M]^+$ (14.32); HRMS: calcd for $C_{21}H_{16}O$ 284.1201, found 284.1202.

4.2. Biology/material and methods

4.2.1. Antiparasitic activities. General

For the in vitro studies, samples were dissolved in dimethyl sulphoxide (DMSO; Sigma) to prepare a working solution of 10 mg/mL. Further dilutions were made with RPMI 1640 medium to the final highest concentration of DMSO on 1.5%, which was not toxic to the parasites.

4.2.2. Leishmanicidal activity

L. donovani (MHOM/IN/90/GE1F8R) and *L. amazonensis* (MHOM/BR/77/LTB0016) strains were used in this study. Cultures were handled as previously described.[17] *L. donovani* promastigotes were adapted for culture in RPMI 1640 liquid medium (Gibco-BRL) supplemented with 20% heat inactivated fetal bovine serum, vitamins and amino acids, at 26 °C. Logarithm phase cultures of promastigotes were used for experimental purposes. *L. amazonensis* strain promastigotes, obtained from amastigotes forms isolated from mouse lesions, were maintained at 26 °C in Schneider's medium (Schneider's Insect Medium; Sigma Cell Culture, St. Louis, MO, USA) containing a 10% heat inactivated fetal calf serum, 100 U of penicillin/mL and 100 μg of streptomycin/mL. Subcultures were made in the late-log phase of growth and parasites were used no later than the fifth passage.

The inhibition of promastigotes growth in vitro was assessed using a quantitative colorimetric assay with the oxidation–reduction indicator Alamar Blue® Assay.[18] Briefly, promastigotes were serially diluted in 200 μl RPMI 1640 medium without phenol red and supplemented with 20% (*L. donovani*) or 10% (*L. amazonensis*) heat-inactivated fetal bovine serum in 96-well plates. To these wells were added parasites (10^6/well), and the drug concentration

to be tested. After addition of 10% of Alamar Blue®, the plates were incubated at 26 °C. After 72 h, the plates were analyzed on a Microplate Reader Model 680 (Biorad, Hercules, CA) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Percentage of inhibition and 50% inhibitory concentrations (IC_{50}) were calculated by linear regression analysis with 95% confidence limits. All experiments were performed three times each in duplicate, and the mean values were also calculated. A paired two-tailed *t*-test was used for analysis of the data. Values of $P < 0.05$ were considered significant.

4.2.3. Antitrypanosomal activity

Y strain *T. cruzi* epimastigotes were grown at 28 °C in liver infusion tryptone (LIT) medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum. The inhibition of growth in vitro was assessed as above using an Alamar Blue® Assay (Biosource).

4.2.4. Cytotoxicity activity

MTT proliferation assay: MCF-7 cells were purchased from ATCC and cultured in DMEN containing 10% FBS. For 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Sigma–Aldrich, St. Louis, MO) analysis, cells were plated in 96-well plates at 10,000 cells/well. Twenty-four hours after plating, vehicle (0.1% DMSO, final concentration) or compound was added to cells at indicated doses. Seventy-two hours following compound addition, MTT was added to each well (0.5 mg/mL, final concentration) and plates were incubated for an additional 3 h at 37 °C. Medium was then aspirated and the formazan product was solubilized in SDS–HCl (20% SDS; HCl 0.02 M). The absorbance of each well was measured at 570 nm using a microplate reader.

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