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Antiprotozoal Activities of Symmetrical Bishydroxamic Acids

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Abstract—Symmetrical bishydroxamic acids along with their sodium salts containing an alkyl spacer between two aromatic rings were synthesized, and their antiparasitic activities were evaluated. Bishydroxamic acids were conveniently prepared from the alkylation of methyl 4-hydroxybenzoate with various dihalo-alkane, -alkene, and -ether followed by reaction with hydroxylamine. Surprisingly, the bishydroxamic acids and their sodium salts possess strong inhibitory activities against *Plasmodium falciparum* parasites with IC_{50} values in the range of 0.26–3.2 μM. Bishydroxamic acid **3** and its sodium salt **12** also inhibit the growth of *Leishmania donovani*, albeit at higher concentrations. The corresponding biscarboxylic acids and bismethyl esters are inactive. Presumably, the ability of bishydroxamic acids to complex with metallic iron in hemoglobin may be responsible for antimalarial activity of these compounds.

Introduction

Malaria remains the world's most devastating parasitic disease. According to the World Health Organization, at least one million deaths and over 300 million acute illnesses can be attributed to malaria (http://www.who.int/inf-fs/en/InformationSheet01.pdf). Leishmaniasis, which is estimated to cause 59,000 deaths each year (http://www.who.int/tdr/diseases/leish/diseaseinfo.htm), is triggered by another unrelated protozoan parasite. In the search for orally active drug candidates inhibiting the growth of *Plasmodium falciparum*[1] and *Leishmania donovani* parasites,[2] the causative agents of severe malaria and visceral leishmaniasis, we decided to investigate the inhibition potencies of various symmetrical bishydroxamic acids. Although it has been reported that histone deacetylation inhibitors such as suberic acid bisdimethylamide show cytostatic effect against the acute murine malaria *Plasmodium berghei*,[3] only limited biologically active bishydroxamic acids have been reported.[4] Since hydroxamic acids are known[5] to complex with metallic iron and copper in matrix metalloproteinase,[6] it is not surprising that bishydroxamic acids could inhibit malarial parasite growth by affecting hemoglobin through chelation with iron or inhibiting proteases such as methionine aminopeptidase 2,[1] which requires Zn, Co, and Mg. *Leishmania* parasites possess a transferrin receptor[7] and iron chelators reduced the rate of *Leishmania* promastigote growth,[8] so bishydroxamic acids are also worthy of investigation against this parasite.

Results and Discussion

Chemistry

Due to the chelation ability of bishydroxamic acids with metals, three symmetrical bishydroxamic acids **1**–**3**, along with their corresponding sodium salts, methyl esters, and carboxylic acids were synthesized, and their inhibitory activities against *Plasmodium* and *Leishmania* parasites were investigated. As shown in Scheme 1, the syntheses of the methyl esters, carboxylic acids, and hydroxamic acids and their sodium salts were carried out via a typical alkylation of the hydroxy function of methyl 4-hydroxybenzoate (**4**) followed by either reaction with hydroxylamine[9] to provide bishydroxamic

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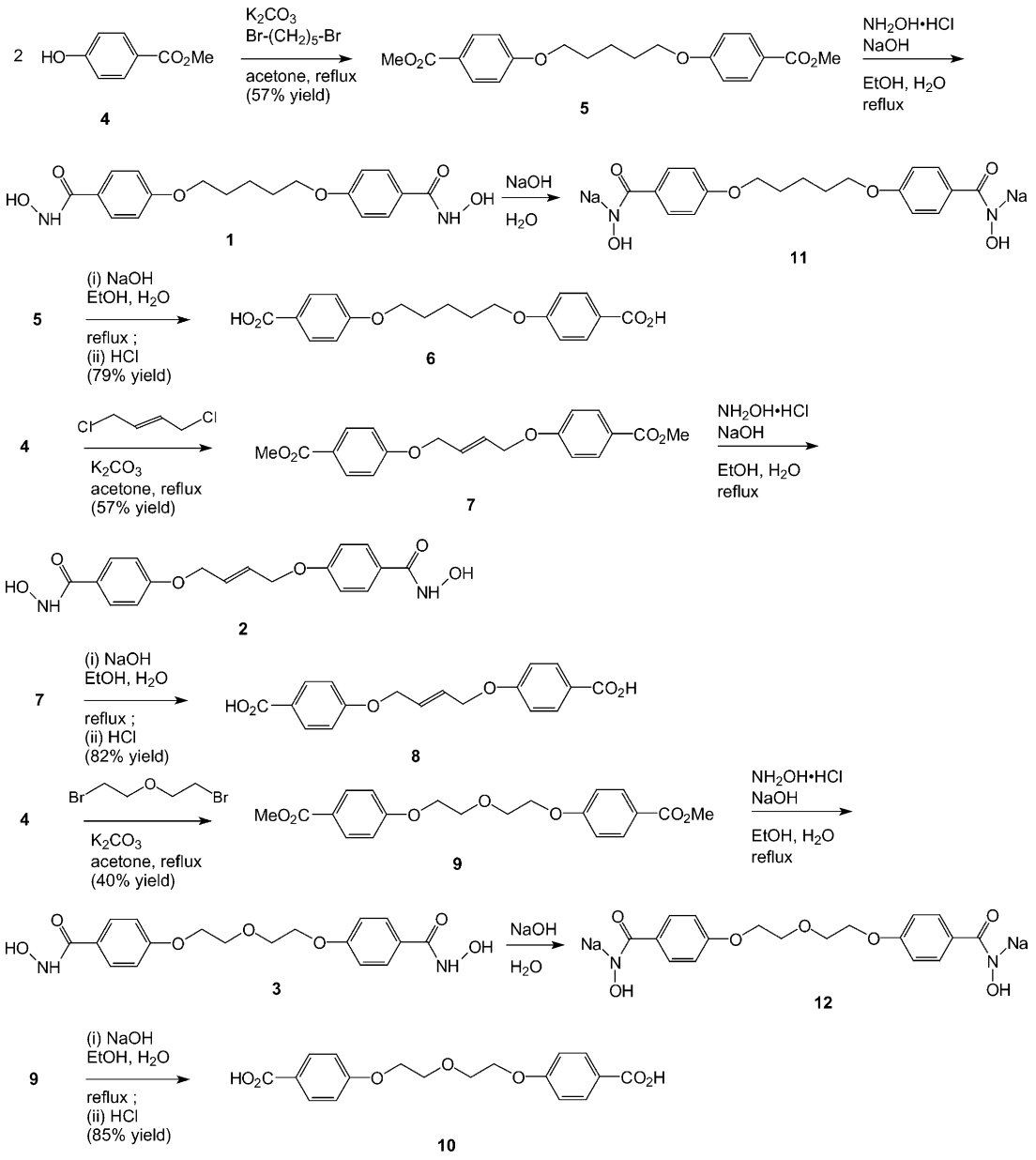

Scheme 1.

acids or with base to give biscarboxylic acids. Hence, treatment of **4** with potassium carbonate and 1,5-dibromopentane, *trans*-1,4-dichloro-2-butene, and bis-2-bromoethyl ether separately in refluxing acetone provided methyl esters **5** (57% yield), **7** (57% yield), and **9** (40% yield), respectively. Heating of esters **5**,[10] **7**, and **9**[11] with hydroxylamine and sodium hydroxide in refluxing ethanol–water produced bishydroxamic acids **1**,[4d] **2**, and **3**, respectively, in good yields. Compounds **2** and **3** have not been reported previously. Carboxylic acids **6**, **8**, and **10** were readily obtained from the basic hydrolysis of esters **5**, **7**, and **9**, respectively, with sodium hydroxide in refluxing ethanol and water, followed by acidification, in 79, 82, and 85% yield, respectively. Sodium salts **11** and **12** were obtained by treating bishydroxamic acids **1** and **3**, respectively, with sodium hydroxide. Carboxylic acids **6**,[12] **8**,[13] and **10**[12a,14] are known compounds, and usages in materials have been reported.

Antiparasitic activity

The analogous methyl esters, **5**, **7**, and **9**, and carboxylic acids, **6**, **8**, and **10**, were used to test whether the bishydroxamic acid function is important for the bioactivities. The sodium salts of **1** and **3**, compounds **11** and **12**, are soluble in water and were also evaluated for their biological activities. Table 1 summarizes data of the inhibition of *P. falciparum* D6 and W2 intraerythrocytic forms and *L. donovani* axenic amastigote-like parasites. As expected, while bisesters **5**, **7**, and **9**, and biscarboxylic acids **6**, **8**, and **10** do not show inhibitory activities, hydroxamic acids **1**–**3** show significant inhibitory activities. Hence, IC_{50} values of hydroxamic acids **1**, **2**, and **3** are 2.2, 0.69, and 0.24 μM, respectively, for D6, and 3.2, 1.05, and 0.35 μM, respectively, for W2. Bishydroxamic acid sodium salt **12** exhibits the strongest activities, with IC_{50} values of 0.26 μM for D6 and

Table 1. In vitro inhibitory activities against *P. falciparum* D6 and W2 and *L. donovani* parasites

Compounds	D6, IC_{50} (μM)	D6, IC_{90} (μM)	W2, IC_{50} (μM)	W2, IC_{90} (μM)	*L. donovani* parasites (μM)
Bisester **5**	>60	NT	>60	NT	NT
Bisester **7**	>60	NT	>60	NT	NT
Bisester **9**	>60	NT	>60	NT	>100
Biscarboxylic acid **6**	>60	NT	>60	NT	NT
Biscarboxylic acid **8**	>60	NT	>60	NT	NT
Biscarboxylic acid **10**	>38	NT	>38	NT	>100
Bishydroxamic acid **1**	2.2	6.2	3.2	NT	NT
Bishydroxamic acid **2**	0.69	1.8	1.05	2.57	NT
Bishydroxamic acid **3**	0.24	0.47	0.35	0.60	18.5±6.0
Bishydroxamic acid sodium salt **11**	1.0	2.0	1.09	NT	NT
Bishydroxamic acid sodium salt **12**	0.26	0.52	0.36	0.60	37.4±14.0
Chloroquine	0.013	0.014	0.45	0.65	NT
Mefloquine	0.013	0.024	8 nM	0.015	NT
Pentamidine	NT	NT	NT	NT	1.19±0.12

NT, not tested.

0.36 μM for W2. Chloroquine and mefloquine were used for comparison, their IC_{50} values being 0.013 μM (both) for D6, and 0.45 μM and 8 nM, respectively, for W2. Moreover, bishydroxamic acid **3** and its sodium salt **12** inhibit *L. donovani* parasites with IC_{50} values of 18.5±6.0 and 37.4±14.0 μM, respectively, with pentamidine used for comparison. It appears that the inhibitory activities against *P. falciparum* D6 and W2 and *L. donovani* parasites of bishydroxamic acid **3** are similar to its sodium salt **12**. The compounds are more active in vitro against *Plasmodium*, perhaps due to the vigorous metabolism of hemoglobin by the parasite. Compound **12** is soluble in water, while compound **3** is soluble in DMSO but only slightly soluble in water. The results demonstrate that bishydroxamic acid functionality is essential for the inhibition of malaria parasites. It is possible that the bishydroxamic acids inhibit hypoxanthine-guanine phosphoribosyltransferases,[15] which lead to antiparasitic activities. The simplicity of the molecular structures of bishydroxamic acids described above may provide a new entry in providing potentially active antimalarial agents that may be used on chloroquine and mefloquine resistant parasites.

Conclusion

Symmetrical bishydroxamic acids and their sodium salts were synthesized from the alkylation of methyl 4-hydroxybenzoate with various dihalogenated alkane, alkene, and ether, followed by reaction with hydroxylamine, and their antiparasitic activities were evaluated. These bishydroxamic acids and their sodium salts inhibit *P. falciparum* with IC_{50} values in the range of 0.26–3.2 μM. Compounds **3** and **12** also inhibit the growth of *L. donovani*, albeit at higher concentrations. Despite their simplicity in structure, the above mentioned bishydroxamic acids show significant inhibitory activities against various parasites. These bishydroxamic acids can easily be modified to provide different spacers by changing the middle alkyl chain to other heteroatom containing chains, and ring systems by changing the aromatic rings to other heterocyclic rings.

Experimental

General methods

Nuclear magnetic resonance spectra were obtained at either 400 or 200 MHz for ^1H and either 100 or 50 MHz for ^{13}C, and reported in ppm. Mass spectra were taken from an IonSpec HiResMALDI mass spectrometer using 2,5-dihydroxybenzoic acid as a matrix.

1,5-Bis-(4-methoxycarbonylphenoxy)pentane (5). A mixture of 11.7 g (0.077 mol) of methyl 4-hydroxybenzoate (**4**), 48.0 g (0.347 mol) of K_2CO_3, and 8.0 g (0.035 mol) of 1,5-dibromopentane in 100 mL of acetone was stirred under reflux for 31 h under argon. After the mixture was cooled to room temperature, the inorganic salts were removed by filtration, and the filtrate was concentrated on a rotary evaporator to remove most of the acetone. The residue was dissolved in 100 mL of dichloromethane, washed with aqueous NaOH (30 mL; 0.5 M solution), dried ($MgSO_4$), and concentrated to dryness to give 7.37 g (57% yield of **5**[10] as a white solid; mp 93–95 °C. Ester **5** was crystallized from ethyl acetate to give white crystals. ^1H NMR ($CDCl_3$) δ 7.98 (d, $J=8.4$ Hz, 4H, Ar), 6.90 (d, $J=8.4$ Hz, 4H, Ar), 4.05 (t, $J=6.4$ Hz, 4H, OCH_2), 3.88 (s, 6H, OCH_3), 1.98–1.60 (m, 6H, CH_2); ^{13}C NMR ($CDCl_3$) δ 166.8, 162.7, 131.5, 122.4, 114.0, 67.8, 51.8, 28.8, 22.6.

***trans*-1,4-Bis-(4-methoxycarbonylphenoxy)-2-butene (7).** By a procedure similar to that above, 3.30 g (26.4 mmol) of *trans*-1,4-dichloro-2-butene, 8.85 g (58.2 mmol) of **4**, 36.5 g (0.264 mol) of K_2CO_3, and 40 mL of acetone gave 3.75 g (40% yield) of **7** as a white solid after crystallization from ethyl acetate. The mother liquor from crystallization was concentrated and column chromatographed on silica gel to give 1.65 g (17% yield; a total of 57% yield) of **7**. Mp 153–155 °C; ^1H NMR ($CDCl_3$) δ 7.98 (d, $J=7$ Hz, 4H, Ar), 6.92 (d, $J=7$ Hz, 4H, Ar), 6.10 (t, $J=1.5$ Hz, 2H, =CH), 4.64 (d, $J=1.5$ Hz, 4H, CH_2), 3.88 (s, 6H, OCH_3); ^{13}C NMR ($CDCl_3$) δ 166.7, 162.1, 131.6, 128.0, 122.9, 114.2, 67.6, 51.8. HRMS calcd for $C_{20}H_{20}O_6Na$: 379.1158, found 379.1154.

2,2′-Bis-(4-methoxycarbonylphenoxy)ethyl ether (9). By a procedure similar to that above, 2.00 g (7.8 mmol) of 2,2′-di(bromoethyl) ether, 2.68 g (17.6 mmol) of **4**, 11.0 g (79.6 mmol) of K_2CO_3, and 15 mL of acetone gave 2.47 g (85% yield) of **9**[11] as a white solid after crystallization from ethyl acetate. Mp 120–123 °C; ^{1}H NMR ($CDCl_3$) δ 7.97 (d, J = 8.5 Hz, 4H, Ar), 6.92 (d, J = 8.5 Hz, 4H, Ar), 4.21 (t, J = 5 Hz, 4H, CH_2), 3.95 (t, J = 5 Hz, 4H, CH_2), 3.88 (s, 6H, OCH_3); ^{13}C NMR ($CDCl_3$) δ 166.7, 162.4, 131.5, 122.8, 114.1, 69.7, 67.5, 51.8.

1,5-Bis-[4-(hydroxyaminocarbonyl)phenoxy]pentane (1). To a mixture of 0.9 g (2.4 mmol) of ester **5** in 24 mL of 0.5 M solution of $NH_2OH{\cdot}HCl$ (12 mmol) was added 4.8 mL (28.8 mmol) of 6 M aqueous NaOH solution. The mixture was refluxed for 5 min, then cooled to room temperature, and 28.8 mL (28.8 mmol) of 1 N HCl was added. The precipitated solids, was collected by filtration and dried thoroughly under vacuum, to weight 0.74 g (82% crude yield). ^{1}H NMR spectrum indicated the presence of **1** and a small amount of biscarboxylic acid **6**. Recrystallization from DMSO–water (10:1) three times gave **1**,[4d] 0.51 g (57% yield): mp 200–202 °C (lit.[4d] 202–203 °C); ^{1}H NMR (DMSO-d_6) δ 7.69 (d, J = 8.8 Hz, 4H, Ar), 6.96 (d, J = 8.8 Hz, 4H, Ar), 4.03 (t, J = 6.4 Hz, 4H, OCH_2), 1.78 (m, 4H, CH_2), 1.55 (m, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ 165.6, 156.7, 131.2, 128.5, 114.1, 67.6, 28.1, 22.0.

***trans*-1,4-Bis[4-(hydroxyaminocarbonyl)phenoxy]-2-butene (2).** A mixture of 0.45 g (1.26 mmol) of ester **7**, 12 mL of 0.5 M solution of $NH_2OH{\cdot}HCl$ (6 mmol), and 1.0 mL (6 mmol) of 6 M aqueous NaOH was treated similarly to that described above for the preparation of **1**. Three crystallizations of the crude product from DMSO–water (10:1) gave **2**, 0.18 g (40% yield): mp 205–210 °C; ^{1}H NMR (DMSO-d_6) δ 7.70 (d, J = 8.8 Hz, 4H, Ar), 6.99 (d, J = 8.8 Hz, 4H, Ar), 6.07 (bs, 2H, =CH), 4.62 (bs, 4H, OCH_2); ^{13}C NMR (DMSO-d_6) δ 166.9, 160.4, 131.3, 128.6, 125.2, 114.3, 67.3. HRMS calcd for $C_{18}H_{18}N_2O_6Na$: 381.1063, found 381.1064.

2,2′-Bis-[(4-hydroxyaminocarbonyl)phenoxy]ethyl ether (3). A mixture of 1.0 g (2.67 mmol) of ester **9**, 27 mL of 0.5 M solution of $NH_2OH{\cdot}HCl$ (13.5 mmol), and 5.3 mL (32 mmol) of 6 M aqueous NaOH was treated similarly to that described above for the preparation of **1**. Two crystallizations of the crude product from DMSO–water (10:1) gave **2**, 0.804 g (80% yield): mp 220–226 °C; ^{1}H NMR (DMSO-d_6) δ 7.68 (d, J = 8 Hz, 4H, Ar), 6.97 (d, J = 8 Hz, 4H, Ar), 4.17 (t, J = 4 Hz, 4H, OCH_2), 3.80 (t, J = 4 Hz, 4H, OCH_2); ^{13}C NMR (DMSO-d_6) δ 164.2, 160.8, 128.8, 125.1, 114.2, 69.0, 67.5. HRMS calcd for $C_{18}H_{20}N_2O_7Na$: 399.1168, found 399.1155.

1,5-Bis-[4-(hydroxycarbonyl)phenoxy]pentane (6). To a solution of 0.30 g (0.81 mmol) of ester **5** in 20 mL of ethanol, 1.6 mL of 6 M aqueous NaOH was added and the solution was stirred under reflux for 10 min. After being cooled to room temperature, the solution was acidified with 1 N HCl to pH 1, and the precipitated white solids were collected by filtration, washed with water, dried under vacuum, and crystallized from ethanol to give 0.22 g (79% yield) of **6**:[12] mp 283–286 °C; ^{1}H NMR (DMSO-d_6) δ 7.86 (d, J = 8.4 Hz, 4H, Ar), 6.99 (d, J = 8.4 Hz, 4H, Ar), 4.06 (t, J = 7 Hz, 4H, OCH_2), 1.79 (pent, J = 7 Hz, 4H, CH_2), 1.56 (pent, J = 7 Hz, 2H, CH_2); ^{13}C NMR (DMSO-d_6) δ 164.0, 160.8, 124.7, 122.8, 114.0, 67.5, 28.2, 22.0.

***trans*-1,4-Bis-[4-(hydroxycarbonyl)phenoxy]-2-butene (8).** A mixture of 0.10 g (0.28 mmol) of ester **7**, 3.0 mL of ethanol, and 2.0 mL (12 mmol) of 6 N NaOH was treated similarly to that described above for the preparation of **6** to give 67 mg (74% yield) of **8**:[13] mp >290 °C; ^{1}H NMR (DMSO-d_6) δ 7.88 (d, J = 9 Hz, 4H, Ar), 7.03 (d, J = 9 Hz, 4H, Ar), 6.09 (bs, 2H, =CH), 4.69 (bs, 4H, CH_2O); ^{13}C NMR (DMSO-d_6) δ 166.9, 161.7, 131.3, 128.2, 123.2, 114.5, 67.4.

2,2′-Bis-[(4-hydroxycarbonyl)phenoxy]ethyl ether (10). A mixture of 0.15 g (0.40 mmol) of **9**, 5.0 mL of ethanol, and 2.7 mL (16.2 mmol) of 6 N NaOH was treated similarly to that described above for the preparation of **6** to give 0.115 g (83% yield) of **10**:[12a,14] mp 308–310 °C; ^{1}H NMR (DMSO-d_6) δ 7.86 (d, J = 7.5 Hz, 4H, Ar), 7.00 (d, J = 7.5 Hz, 4H, Ar), 4.18 (t, J = 3.5 Hz, 4H, OCH_2), 3.83 (t, J = 3.5 Hz, 4H, OCH_2); ^{13}C NMR (DMSO-d_6) δ 166.9, 162.0, 131.3, 123.0, 114.3, 68.9, 67.4.

1,5-Bis-[4-(hydroxy-sodio-aminocarbonyl)phenoxy]pentane (11). To a solution of 0.10 g (0.27 mmol) of **1** in 1 mL of water was added 21.4 mg (0.53 mmol) of NaOH. The solution was concentrated to dryness to give 0.112 g (100% yield) of sodium salt **11**. Compound **11** is soluble in water and ^{1}H NMR is similar to that of **1**.

2,2′-Bis-[(4-hydroxy-sodio-aminocarbonyl)phenoxy]ethyl ether (12). By a procedure similar to that above described for the preparation of **11**, 0.10 g of **3** gave 0.112 g (100% yield) of **12**. Compound **12** is soluble in water and the ^{1}H NMR spectrum is similar to that of **3**.

Inhibition of the growth of *P. falciparum*. A reported method[16] for the inhibition of the growth of *P. falciparum* was followed. The incubation period of the parasites was 66 h and the starting parasitemia was 0.2% with a 1% hematocrit. The medium used was RPMI-1640 culture medium with no folate or *p*-aminobenzoic acid and 10% normal heat-inactivated human plasma, and *P. falciparum* D6 and W2 clones were used. D6 is a clone from the Sierra I/UNC isolates and is susceptible to chloroquine and pyrimethamine but has reduced susceptibilities to mefloquine and halofantrine. W2 is a clone of the Indochina I isolate and is resistant to chloroquine and pyrimethamine but susceptible to mefloquine. Compounds were dissolved in DMSO, diluted 400-fold with complete culture media, and then diluted 2-fold, 11 times, to give a concentration range of 1048-fold by a Biomek 1000 or 2000 liquid handling system into 96-well microtiter plates. The diluted compounds were transferred (25 μL) to test plates, 200 μL of parasitized erythrocytes (0.2% parasitemia and 1% hematocrit) was added, and was incubated at 37 °C in a

controlled environment of 5% CO_2, 5% O_2, and 90% N_2. After 42 h, 25 μL of [^{3}H]-hypoxanthine was added and the plates were incubated for an additional 24 h. The plates were then frozen at −70 °C to lyse the red cells and later thawed and harvested onto glass fiber filter mats by using a 96-well cell harvester. The filter mats were counted in a scintillation counter and the data were downloaded with the custom, automated analysis software developed by WRAIR. For each compound, the concentration–response profile was determined and 50% inhibitory concentrations (IC_{50}) were determined by using a nonlinear, logistic dose–response analysis program.

Inhibition of the growth of *L. donovani* parasites. A reported method[17] for the inhibition of the growth of *L. donovani* parasites was followed. *L. donovani* axenic amastigotes were maintained in modified RPMI medium as previously described.[17] Inhibition of growth by the compounds was measured in a 3-day assay using the tetrazolium dye-based CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI, USA) as previously described.[17] The known anti-leishmaniasis drug, pentamidine, was used as a control.

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