

Antileishmanial Constituents of the Panamanian Endophytic Fungus *Edenia* sp.

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Bioassay-directed fractionation of extracts from the fermentation broth and mycelium of the fungus *Edenia* sp. led to the isolation of five antileishmanial compounds, preussomerin EG1 (**1**), palmarumycin CP<sub>2</sub> (**2**), palmarumycin CP<sub>17</sub> (**3**), palmarumycin CP<sub>18</sub> (**4**), and CJ-12,371 (**5**). Compounds **3** and **4** are new natural products, and this is only the second report of compound **1**. The structures of compounds **1–5** were established by spectroscopic analyses (HRMS and NMR). All metabolites caused significant inhibition of the growth of *Leishmania donovani* in the amastigote form, with IC<sub>50</sub> values of 0.12, 3.93, 1.34, 0.62, and 8.40 μM, respectively. Compounds **1–5** were inactive when tested against *Plasmodium falciparum* or *Trypanosoma cruzi* at a concentration of 10 μg/mL, indicating that they have selective activity against *Leishmania* parasites. Compounds **1–5** showed weak cytotoxicity to Vero cells (IC<sub>50</sub> of 9, 162, 174, 152, and 150 μM, respectively); however, the therapeutic window of these compounds is quite significant with 75, 41, 130, 245, and 18 times (respectively) more antileishmanial activity than cytotoxicity.

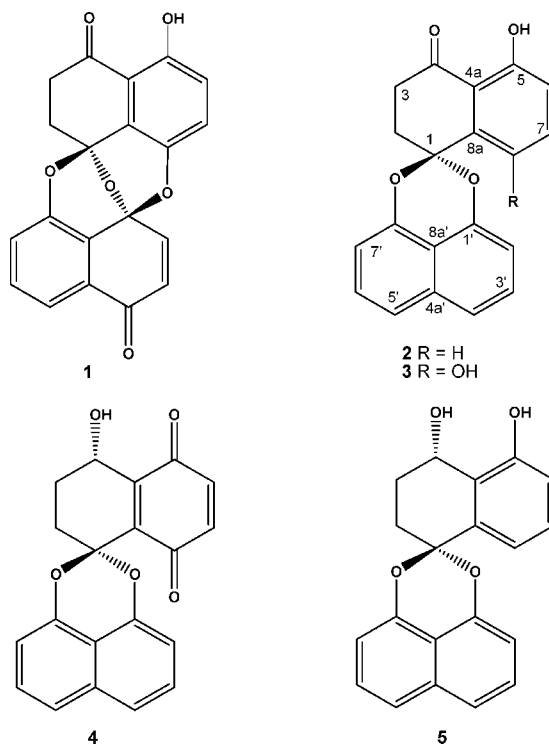
Leishmaniasis is a major tropical disease that largely affects populations of the developing world. According to the World Health Organization (WHO), leishmaniasis can be classified into four main forms: visceral leishmaniasis (the most dangerous because it can be mortal), cutaneous leishmaniasis (the most common form, which causes a variety of skin lesions), mucocutaneous leishmaniasis (which begins with skin ulcers that can spread, causing tissue destruction, mainly of the nose and mouth), and diffuse cutaneous leishmaniasis (produces chronic skin lesions that are very difficult to cure).<sup>1</sup> Current treatment against leishmaniasis is based on toxic chemotherapeutic compounds, such as sodium stibogluconate and meglumine antimonate, that have several serious side effects, which themselves can be fatal to patients.<sup>1,2</sup> Moreover, these agents are expensive and therapies are required for relatively long durations, two characteristics that combine to exclude many patients from having access to any treatment.<sup>1,2</sup> While approximately 600 000 infections are officially reported each year, it is estimated that 2 million new cases occur annually and that 12 million people are currently infected worldwide.<sup>1,2</sup>

As a part of the ongoing research activities of the Panamanian International Cooperative Biodiversity Group (ICBG) program,<sup>3,4</sup> we have been searching for new antiparasitic agents from plants,<sup>5,6</sup> marine organisms,<sup>7,8</sup> and, more recently, endophytic fungi. In this paper we describe the isolation, structural elucidation, and biological activity of the major compounds from the endophytic fungus *Edenia* sp.

## Results and Discussion

The endophytic fungus *Edenia* sp. (mitosporic Ascomycota) was isolated from a mature leaf of *Petrea volubilis* L. (Verbenaceae) collected from the Coiba National Park in Panama. The culture broth was extracted sequentially with organic solvents (hexanes, CHCl<sub>3</sub>, and EtOAc). The hexanes extract was active against

*Leishmania donovani* (IC<sub>50</sub> = 0.06 μg/mL) and was fractionated by gravity column chromatography to give 18 fractions (F1–F18). Compounds **1** and **2** were obtained by crystallization and NP-HPLC purification of early eluting fractions (F3 and F4), respectively, and identified by comparing their NMR and MS data with reported data.<sup>9,10</sup> Compounds **3** and **4** were obtained by recrystallization from fractions F5 and F8, respectively. Finally, the known compound **5** was isolated from a relatively polar fraction, F9 (eluted with hexanes–CH<sub>2</sub>Cl<sub>2</sub>, 3:7), and this colorless solid was purified by recrystallization using MeOH. The physical data of **5** were identical to reported data.<sup>11</sup>



Compound **3** was isolated as a white powder. The molecular formula was determined as C<sub>20</sub>H<sub>14</sub>O<sub>5</sub> by APCI-HR-MS in positive

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**Table 1.** NMR Data for Compounds **3** and **4** in CDCl<sub>3</sub> (<sup>1</sup>H, 400 MHz and <sup>13</sup>C, 100 MHz)

position	<b>3</b>			<b>4</b>		
	$\delta_C$	$\delta_H$ ( $J_{HH}$ in Hz)	HMBC	$\delta_C$	$\delta_H$ ( $J_{HH}$ in Hz)	HMBC
1	102.0			98.5		
2	29.0	2.51 t, (6.7)	1, 3, 4, 8a	25.4	2.29 m, 2.05 m	1, 3, 4, 8a
3	33.8	2.75 t, (6.7)	1, 2, 4, 4a	27.5	2.07 m, 1.97 m	1, 2, 4, 4a
4	202.0			62.8	4.89 m	2, 4a, 8a
4a	119.8			135.8		
5	157.2			189.2		
6	128.9	7.26 d, (9.3)	4a, 5, 7, 8	135.1	6.84 d, (10.2)	5, 7, 4a
7	121.9	7.07 d, (9.3)	5, 6, 8a	138.3	6.80 d, (10.2)	5, 6, 8a
8	147.6			183.6		
8a	114.5			144.1		
1'	146.3			146.4		
2'	110.5	7.05 d, (7.8)	1', 3', 4', 8a'	120.5	7.48 brd, (8.3)	1', 3', 4', 8a'
3'	127.6	7.49 dd, (8.7, 7.8)	1', 2', 3', 4a'	127.2	7.39 dd, (8.3, 7.3)	1', 2', 3', 4a'
4'	121.9	7.61 d, (8.7)	2', 3', 8a'	109.1	6.85 dd, (7.3, 1)	2', 3', 8a'
4a'	134.1			134.2		
5'	121.9	7.61 d, (8.7)	6', 7', 8a'	109.3	6.90 dd, (7.3, 1)	6', 7', 8a'
6'	127.6	7.38 dd, (8.7, 7.8)	4a', 6', 7', 8'	127.4	7.41 dd, (8.3, 7.3)	4a', 6', 7', 8'
7'	110.5	7.05 d, (7.8)	5', 6', 8', 8a'	120.6	7.48 brd, (8.3)	5', 6', 8', 8a'
8'	146.3			146.6		
8a'	113.5			112.9		
OH-4					3.12 d, (3.0)	2, 3
OH-5		12.37 brs	5, 7, 8a			
OH-8		5.29 s	6, 4a			

ion mode, which showed a protonated parent ion peak at  $m/z$  335.1013  $[M + H]^+$ . The formula was consistent with the number of protons and carbons observed by NMR spectra. The <sup>1</sup>H NMR spectra (Table 1) showed two doublets at  $\delta_H$  7.26,  $J = 9.3$  Hz (H-6) and 7.07,  $J = 9.3$  Hz (H-7), which indicated the existence of a 1,2,3,4-tetrasubstituted aromatic ring. Two doublets of doublets with  $J$  values of 8.7 and 7.8 Hz and two broad doublets mutually coupled with  $J$  values of 8.7 and 7.8 Hz, respectively, confirmed the presence of a 1,8-dioxynaphthalene moiety. Signals at  $\delta_H$  2.51 (t, 6.7 Hz, H<sub>2</sub>-2) and 2.75 (t, 6.7 Hz, H<sub>2</sub>-3) indicated the presence of two adjacent methylene groups, and signals at  $\delta_H$  5.29 (s, OH-8) and 12.37 (brs, OH-5) suggested two hydroxy groups, one of which was involved in a hydrogen bond (OH-5). The <sup>13</sup>C NMR spectrum showed 20 resonances (Table 1), interpreted from multiplicity-edited HSQC data as 10 quaternary, two methylene, and eight methine carbons. This spectrum also had resonances for a cyclic ketone ( $\delta_C$  202.0, C-4) and a naphthalene ring system [ $\delta_C$  146.3 (C-1'); 110.5 (C-2'); 127.6 (C-3'); 121.9 (C-4'); 134.1 (C-4a'); 121.9 (C-5'); 127.6 (C-6'); 110.5 (C-7'); 146.3 (C-8'); 113.5 (C-8a')]. HMBC correlations supported these assignments, as did data comparisons with known compounds **2** and **5**. The chemical shift of the two-carbon resonance at  $\delta_C$  146.3 was consistent with the placement of a dioxin bridge to C-1' and C-8' of the naphthalene core. Finally, the chemical shifts of the signals at  $\delta_C$  157.2 and 147.6 were consistent with the positioning of phenolic hydroxy groups at C-5 and C-8. HMBC correlations to these carbon signals from H-6 and H-7 further confirmed the positioning of these two functional groups. This new compound, given the trivial name palmarumycin CP<sub>17</sub> (**3**), has the same overall molecular arrangement as observed in CJ-12,372<sup>11</sup> and cladospirone B<sup>12</sup> with variations occurring only in the nature of the substituents at C-2 and C-4.

Compound **4** was isolated as a reddish-brown solid. An APCI-HR-MS analysis gave a molecular ion at  $m/z$  335.1112, which was consistent with the molecular formula C<sub>20</sub>H<sub>14</sub>O<sub>5</sub>, indicating 14 degrees of unsaturation. The aromatic region of the <sup>1</sup>H NMR spectrum of **4** showed six proton resonances. Integration and proton-proton coupling revealed the presence of two ABC spin systems ( $\delta_H$  7.48, brd,  $J = 8.3$  Hz, H-2' or H-7'; 7.41, dd,  $J = 8.3, 7.3$  Hz, H-3' or H-6'; 6.90, dd,  $J = 7.3, 1.0$  Hz, H-4' or H-5';  $\delta_H$  7.48, brd,  $J = 8.3$  Hz, H-2' or H-7'; 7.39, dd,  $J = 8.3, 7.3$  Hz, H-3' or H-6'; 6.85, dd,  $J = 7.3, 1.0$  Hz, H-4' or H-5'). These aromatic protons were consistent with a 1,8-disubstituted naphthalenyl aromatic ring. Also evident were the resonances of an AB system

at  $\delta_H$  6.84 (d,  $J = 10.2$  Hz, H-6) and 6.80 (d,  $J = 10.2$  Hz, H-7) and the resonances of two vicinal methylene groups with diastereotopic protons [ $\delta_H$  2.29 (m, H-2a), 2.05 (m, H-2b) and 2.07 (m, H-3a), 1.97 (m, H-3b)] connected by COSY to a deshielded methine group [ $\delta_H$  4.89 (m, H-4)]. The <sup>13</sup>C NMR spectrum of **4** (Table 1) confirmed the presence of 20 carbon resonances, with their multiplicities determined from a DEPT spectrum as nine quaternary, nine methine, and two methylene carbon atoms. Four quaternary carbons and two aromatic methines were consistent with the presence of a benzoquinone core [ $\delta_C$  135.8 (C-4a); 189.2 (C-5); 135.1 (C-6); 138.3 (C-7); 183.6 (C-8); and 144.1 (C-8a)]. Additionally, compound **4** showed the same signals as compound **3** for a 1,8-disubstituted naphthalene group. The position of the functional groups in compound **4** was confirmed by analyses of COSY, HMBC, and NOESY data. On the basis of these data the structure of **4** was elucidated as 4(*S*)-hydroxy-2,3-dihydro-2(*H*)-spiro[naphthalene-1(4*H*),2'-naphtho[1,8-*de*][1,3]dioxin]-5,8-dione and was given the trivial name palmarumycin CP<sub>18</sub>.

Although this is the first report of **4** as a natural product, Sakemi and co-workers in 1992<sup>13</sup> described the synthesis of **4** by means of oxidation of the *p*-hydroquinone moiety of compound CJ-12,372 using 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ). The absolute configuration of the single stereogenic center (C-4) of **4** was determined as *S* based on comparison of its specific rotation with that of synthetic 4(*S*)-hydroxy-2,3-dihydro-2(*H*)-spiro[naphthalene-1(4*H*),2'-naphtho[1,8-*de*][1,3]dioxin]-5,8-dione.<sup>13</sup> The occurrence of the hydroquinone CJ-12,371 (**5**) in *Edenia* sp., also of 4*S*-configuration, as well as from the unidentified fungus (N983-46),<sup>11</sup> is consistent with it being a biosynthetic precursor to metabolite **4**, or vice versa.

Compounds **3** and **4** are new members of the palmarumycins family, a group of bioactive natural products based on a 1,8-dihydroxynaphthalene-derived spiroketal unit linked to a second oxidized naphthalene unit. These metabolites are characteristic of endophytic fungi from the Sphaeriodaceae family. They were first isolated from *Coniothyrium palmarum*,<sup>10</sup> and compounds **1** and **2** were recently isolated from another endophytic fungus, *Edenia gomezpompae* (Pleosporaceae),<sup>9</sup> while compound **5** was isolated from an unidentified endophytic fungus (N983-46).<sup>11</sup>

Compounds **1**–**5** were tested against three parasites (*L. donovani*, *T. cruzi*, and *P. falciparum*) and showed strong and selective activity against *Leishmania* (Table 2). Compound **1** (IC<sub>50</sub> = 0.12  $\mu$ M) was the most active and inhibited growth of *L. donovani* amastigotes

**Table 2.** Biological Activity of **1–5** against Tropical Parasites and Mammalian Cell Lines

compound	IC <sub>50</sub> (μM)				TW
	<i>L. donovani</i>	<i>P. falciparum</i>	<i>T. cruzi</i>	Vero cells	
<b>1</b>	0.12	16.5	>10	9	75
<b>2</b>	3.93	>10	18.5	162	41
<b>3</b>	1.34	>10	>10	174	130
<b>4</b>	0.62	>10	>10	152	245
<b>5</b>	8.40	>10	26.5	150	18

with a similar potency to amphotericin B (IC<sub>50</sub> = 0.09 μM), which was used as a positive control. This compound showed a marked cytotoxicity to mammalian Vero cells (IC<sub>50</sub> = 9 μM), the host cell line for the antileishmania assays, although it was 75-fold more active against *L. donovani* amastigotes (Table 2).<sup>14</sup> Compounds **3** (1.34 μM) and **4** (0.62 μM) were less active than compound **1**, but they also showed less cytotoxicity to mammalian Vero cells (174 and 152 μM, respectively), thus giving therapeutic windows of 130 and 245, respectively (Table 2).

Structures similar to those discussed here were recently reported from an unidentified freshwater-derived fungus. These bisnaphthospiroketal-containing compounds showed moderate nematocidal activity to *Bursaphelenchus xylophilus*.<sup>15</sup> A consideration of the structural features necessary for antileishmania activity in our series of compounds suggests that the naphthalene core is an essential component. Previous work with related compounds from the freshwater-derived fungus found that potency was dependent on the presence of a bis-spirobisnaphthalene core structure.<sup>15</sup> However, further analysis is needed to verify these hypotheses and identify additional functional groups that may be important to the biological properties of this class of metabolites.

The antiparasitic effects of compounds **1–5** were also comparable to those of other natural products such as the dimeric naphthylisoquinoline alkaloids isolated from a plant in the genus *Ancistrocladus*.<sup>16–18</sup> Within this group of about 20 metabolites, ancistrocladinium A,<sup>17</sup> ancistrocladinium B,<sup>17</sup> ancistrotanzanine A,<sup>16</sup> and ancistrotanzanine B<sup>16</sup> showed potent antileishmanial activity (IC<sub>50</sub> values of 0.722, 1.1, 1.8, and 1.6 μg/mL, respectively). The protoberberine alkaloids are another group of aromatic-type compounds with antileishmanial activity.<sup>18–20</sup> Berberine analogues<sup>19</sup> possess significant activity both *in vitro* and *in vivo* against several species of *Leishmania*. Both of these alkaloid classes have components with a planar structure, similar to the new compounds reported herein. Thus, it is possible that these compounds may interact at the same target site. However, it is important to point out that the alkaloids mentioned above are not as potent or selective as compounds **1–5**. Furthermore, other natural product classes such as quinones, alkaloids, terpenes, saponins, phenolic derivatives, and other metabolites have been reported to have potent antileishmanial activity; however, most are not selective and would be predicted to have toxic side effects.<sup>18,20,21</sup> Thus, the new metabolites from *Edenia* sp. appear to be promising drug leads.

## Experimental Section

**General Experimental Procedures.** Melting point measurements were carried out on an Electrothermal apparatus and are uncorrected. Optical rotations were measured with a Rudolf Research Analytical Autopol III 6971 automatic polarimeter. IR spectra were recorded on a Perkin-Elmer FT-IR RXI spectrophotometer. NMR spectra including COSY, NOESY, HMBC, and HMQC experiments were recorded in CDCl<sub>3</sub> on a JEOL Eclipse 400 MHz spectrometer at 400 MHz (<sup>1</sup>H) or 100 MHz (<sup>13</sup>C) NMR, using TMS as an internal chemical shift reference. APCI-HR-MS were acquired on a JEOL LC-mate mass spectrometer (INDICASAT). HPLC was carried out on a Waters LC system, including a 600 pump and a 996 photodiode array detector, and a YMC-Pack SIL (150–10 mm) NP-HPLC column. Column chromatography used silica gel 60 (70–230 mesh, Merck), and TLC (analytical and preparative) was performed on precoated silica gel 60

F254 plates (Merck). All solvents were HPLC grade and were used without further purification.

**Fungal Material.** The fungus *Edenia* sp. was isolated from mature leaves of *Petrea volubilis* L., collected in Coiba National Park, Veraguas, Panama, in 2004 by Alicia Ibañez (STRI, Panama City). Identification of the fungi was done on the basis of the DNA sequence of the nuclear ribosomal internal transcribed spacer region and the first 600bp of the nuclear ribosomal large subunit by Dr. Elizabeth Arnold of the University of Arizona. A voucher was deposited at the Smithsonian Tropical Research Institute (voucher number 349B1).

Eight 1 L Erlenmeyer flasks, each containing 0.5 L of malt extract media (Scharlau Chemie), were individually inoculated with a 1 cm<sup>2</sup> agar plug taken from a stock culture of *Edenia* sp. and placed on an orbital shaker at 200 rpm for 15 days.

**Extraction and Isolation.** After incubation, all flask contents were combined and filtered. The combined culture filtrate (4 L) was extracted exhaustively with hexanes. The organic phase was concentrated under vacuum to give a dark brown solid (1 g). The extract was subjected to Si gel (400 g) open column chromatography eluting with a stepwise gradient of 100% hexanes to 100% CH<sub>2</sub>Cl<sub>2</sub> to 1:1 CH<sub>2</sub>Cl<sub>2</sub>–MeOH to yield 18 major fractions (F1–F18). From fraction F3, eluted with hexanes–CH<sub>2</sub>Cl<sub>2</sub> (8:2), a colorless solid precipitated, which was purified by recrystallization from MeOH to yield preussomerin EG1 (1 mg, **1**). Fraction F4, eluted with hexanes–CH<sub>2</sub>Cl<sub>2</sub> (7:3), was purified by NP-HPLC (YMC-Pack SIL 150 × 10 mm NP-HPLC column, 80% hexanes–20% CHCl<sub>3</sub>, 254 nm, 0.6 mL/min) to afford pure palmarumycin CP<sub>2</sub> (3 mg, **2**) as a colorless solid. Similarly, a colorless solid precipitated from fraction F5, eluted with hexanes–CH<sub>2</sub>Cl<sub>2</sub> (6:4), which was purified by recrystallization from MeOH to yield palmarumycin CP<sub>17</sub> (3 mg, **3**). Fraction F8 (eluted with hexanes–CH<sub>2</sub>Cl<sub>2</sub>, 4:6) contained a reddish-brown solid (10 mg, **4**). Finally, CJ-12,371 (**5**) was isolated from fraction F9 (eluted with hexanes–CH<sub>2</sub>Cl<sub>2</sub>, 3:7). This colorless solid was purified by recrystallization from MeOH (5 mg).

**Preussomerin EG1 (1):** yellow solid, mp 216–217 °C (dec); [α]<sub>D</sub><sup>25</sup> –46 (c 0.13, MeOH); IR ν<sub>max</sub> (KBr) 3077, 1678, 1642, 1591, 1297 cm<sup>–1</sup>; APCI-HR-MS *m/z* 349.0632 [M + H]<sup>+</sup> (calcd for C<sub>20</sub>H<sub>15</sub>O<sub>6</sub>, 349.0712).

**Palmarumycin CP<sub>2</sub> (2):** colorless solid, mp 169–171 °C (dec); IR ν<sub>max</sub> (KBr) 3398, 3061, 1643, 1609, 1269 cm<sup>–1</sup>; APCI-MS *m/z* 319.0963 [M + H]<sup>+</sup> (calcd for C<sub>20</sub>H<sub>15</sub>O<sub>4</sub>, 319.0970).

**Palmarumycin CP<sub>17</sub> (3):** colorless powder, mp 152–153 °C (dec); IR ν<sub>max</sub> (KBr) 3371, 3063, 1645, 1607, 1265 cm<sup>–1</sup>; <sup>1</sup>H NMR and <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>), see Table 1; APCI-MS *m/z* 335.1013 [M + H]<sup>+</sup> (calcd for C<sub>20</sub>H<sub>15</sub>O<sub>5</sub>, 335.0919).

**Palmarumycin CP<sub>18</sub> (4):** reddish-brown solid, mp 112–114 °C (dec); [α]<sub>D</sub><sup>25</sup> +260 (c 0.7, CHCl<sub>3</sub>); IR ν<sub>max</sub> (KBr) 3417, 1660, 1607 cm<sup>–1</sup>; <sup>1</sup>H NMR and <sup>13</sup>C NMR (400 MHz, CDCl<sub>3</sub>), see Table 1; APCI-HR-MS *m/z* 335.1112 [M + H]<sup>+</sup> (calcd for C<sub>20</sub>H<sub>15</sub>O<sub>5</sub>, 335.0919).

**CJ-12,371 (5):** colorless solid, mp 237–239 °C (dec); [α]<sub>D</sub><sup>25</sup> –26 (c 0.14, CHCl<sub>3</sub>); IR ν<sub>max</sub> (KBr) 3440, 3140, 1609, 1261 cm<sup>–1</sup>; APCI-MS *m/z* 321.1114 [M + H]<sup>+</sup> (calcd for C<sub>20</sub>H<sub>17</sub>O<sub>4</sub>, 321.1127).

**Bioassays.** All bioassays employed axenically grown (i.e., cell free) amastigotes of *L. donovani* (LD-1S/MHOM/SD/00-strain 1S). *L. donovani* is employed, as it is the species responsible for the visceral and lethal form of the disease. The assay measures parasite growth and survival and employs PicoGreen. Samples for screening were tested in duplicate at a single concentration of 10 μg/mL. Results were expressed as percentage of parasite growth (% G) compared to control. Samples that showed 50% G or less were considered active and were then assayed at six concentrations (0.00032, 0.0016, 0.08, 0.4, 2, and 10 μg/mL) to determine IC<sub>50</sub> values. The therapeutic window (TW) was determined as the ratio of the IC<sub>50</sub> for the Vero cell line (cytotoxicity) compared to that for *L. donovani*. Amphotericin B was used as a positive control, and the typical IC<sub>50</sub> response of *L. donovani* to this drug is between 70 and 120 ng/μL.<sup>14</sup>

To determine selectivity, the compounds were also tested in other bioassays. Malaria bioassays were performed as previously reported by us, using chloroquine as a positive control (IC<sub>50</sub> = 80–100 nM).<sup>22</sup> Chagas disease bioassays were performed following the protocol of Buckner et al. and using nifurtimox as a positive control (IC<sub>50</sub> = 3–5 μg/mL).<sup>23</sup> Cytotoxicity bioassays were performed using MTT and green monkey Vero kidney cells.<sup>24</sup> All bioassays were performed in duplicate, testing at 10, 2, 0.4, 0.08, and 0.016 μg/mL.

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