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Dimeric Flavonoids from *Arrabidaea brachypoda* and Assessment of Their Anti-*Trypanosoma cruzi* Activity

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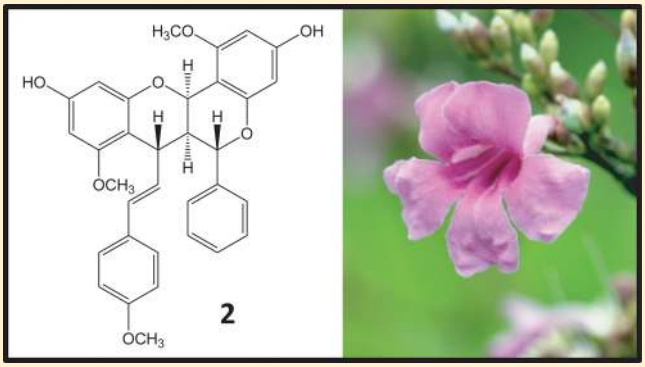
Supporting Information

ABSTRACT: The nonpolar fraction of an aqueous ethanol extract of the roots of *Arrabidaea brachypoda*, a Brazilian medicinal plant, demonstrated significant in vitro activity against *Trypanosoma cruzi*, the parasite responsible for Chagas disease. Targeted isolation of the active constituents led to the isolation of three new dimeric flavonoids (**1**–**3**), and their structures were elucidated using UV, NMR, and HRMS analysis, as well as by chemical derivatization. The anti-*T. cruzi* activity and cytotoxicity toward mammalian cells were determined for these substances. Compound **1** exhibited no activity toward *T. cruzi*, while flavonoids **2** and **3** exhibited selective activity against these trypomastigotes. Compounds **2** and **3** inhibited the parasite invasion process and its intracellular development in host cells with similar potencies to benznidazole. In addition, compound **2** reduced the blood parasitemia of *T. cruzi*-infected mice. This study has revealed that these two dimeric flavonoids represent potential anti-*T. cruzi* lead compounds for further drug development.



The family Bignoniaceae encompasses 120 genera and approximately 800 species of plants that are distributed mostly in tropical and subtropical regions.[1] The genus *Arrabidaea* belongs to the tribe Bignonieae and contains approximately 70 species that occur from Mexico to Argentina. This genus is a large and morphologically diverse clade of Neotropical lianas.[2] Previous phytochemical studies have shown plants in this genus to produce *C*-glucosylxanthones, phenylpropanoids, flavonoids (inclusive of anthocyanidins), allantoin derivatives, and triterpenes.[1,3,4] In traditional medicine, species from the genus *Arrabidaea* are used for different therapeutic purposes and have astringent, anti-inflammatory, antimicrobial, and antitumor properties.[1,4,5] In Brazil, *Arrabidaea brachypoda* Bureau is commonly known as "cervejinha do campo" and is native to the "cerrado" ecosystem. Its traditional uses include the treatment of kidney stones and painful joints (arthritis), while it has also demonstrated significant in vivo anti-inflammatory activity in animal models.[6]

Chagas disease is a parasitic disease caused by the flagellate protozoan *Trypanosoma cruzi*. This disease is transmitted to humans mainly in rural endemic areas through the infected feces of triatomine insects. However, when infected people from rural regions began to migrate to cities, the disease then spread to different, nonendemic areas.[7] The World Health Organization (WHO) reports that globally approximately 10 million people are infected by *T. cruzi*, and more than 25 million people are at risk of infection in endemic countries.[8] Due to population migration, this disease also affects northern countries. In 2007, more than 100 000 *T. cruzi*-infected patients were living in the United States.[9] Chagas disease is treated mainly using benznidazole, even though this compound demonstrates limited effectiveness and severe side effects.[10] As more effective drugs are required, efforts have been devoted to discovering new lead compounds from natural products to combat *T. cruzi* infections.[11,12] The Biota/FAPESP research program for the sustainable use of Brazilian biodiversity (www.biota.org.br) includes the search for new lead compounds for treating Chagas disease within its research program.

Consequently, different plants were screened for their antiparasitic activities, and *A. brachypoda* exhibited promising in vitro and in vivo anti-*T. cruzi* activities. This study describes

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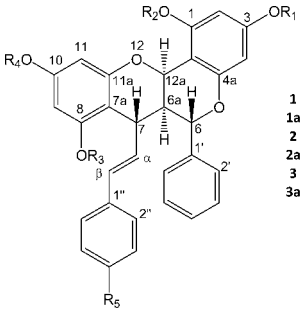
the isolation, structure elucidation, and biological evaluation of three rare dimeric flavonoids (**1**−**3**) from the dichloromethane root extract of this plant.



	R_1	R_2	R_3	R_4	R_5
1	H	CH_3	CH_3	H	OH
1a	$COCH_3$	CH_3	CH_3	$COCH_3$	$OCOCH_3$
2	H	CH_3	CH_3	H	OCH_3
2a	$COCH_3$	CH_3	CH_3	$COCH_3$	OCH_3
3	H	CH_3	CH_3	H	H
3a	$COCH_3$	CH_3	CH_3	$COCH_3$	H

RESULTS AND DISCUSSION

During a preliminary screening procedure for activity against *T. cruzi*, the aqueous ethanol extract of the roots from *A. brachypoda* exhibited an IC_{50} value of 36.4 μg/mL against trypomastigotes; this value is noteworthy for an extract because the reference compound benznidazole gave an IC_{50} value of 11.3 μg/mL. To obtain the active compounds from this extract, the crude aqueous ethanol residue was partitioned with CH_2Cl_2 and a methanol−water (7:3) mixture. The CH_2Cl_2 and aqueous methanol fractions were submitted to biological testing. Antiparasitic activity was identified in the CH_2Cl_2 fraction (IC_{50} 14.0 μg/mL), while the aqueous methanol fraction was inactive. A follow-up investigation of the constituents in the CH_2Cl_2 fraction was undertaken to reveal the constituents active against *T. cruzi*.

HPLC-PDA-ESIMS analysis of the crude extract and fractions revealed three major compounds in the UV trace of the active CH_2Cl_2 fraction (Figure S1, Supporting Information). The PDA-UV spectra revealed the presence of three phenolic compounds with similar UV patterns and the presence of a common chromophore (Figure S1, Supporting Information). The ESIMS of these three peaks exhibited protonated molecular ions at m/z 525.1 $[M + H]^+$ for compound **1**, at m/z 539.2 for **2**, and at m/z 509.1 for **3**. Therefore, these compounds possess unusual structural features compared to substances previously reported in the genus *Arrabidae*.[13] To identify these constituents and assess their biological activities, the extract was fractionated using reversed-phase medium-pressure liquid chromatography (MPLC), thus purifying hundreds of milligrams of each compound (**1**−**3**) in one step (Figure S1, Supporting Information).

Compound **1** was isolated as an amorphous, white solid. The high-resolution (HR)-ESIMS revealed an $[M + H]^+$ ion at m/z 525.1964, corresponding to $C_{32}H_{29}O_7$. The ^{1}H NMR spectrum of **1** exhibited signals for 13 aromatic protons, two ethylene units, two oxygenated methines, two aliphatic methines, and two methoxy groups, which were also confirmed from the HSQC spectrum. The different aromatic signals belonged to four independent rings, named A, B, C, and D. A pair of *meta*-coupled protons at δ_H 6.08 and 5.94 (J = 2.2 Hz) were assigned to the H-2 and H-4 protons in the A ring. The HMBC correlations observed indicated that this ring contains three oxygenated quaternary carbons (C-1, C-3, and C-4a at δ_C 161.1, 161.1, 157.8) and an additional sp^2-hybridized carbon, C-12b, at δ_C 103.8. This last-mentioned carbon was located next to one of the oxygenated methines (δ_H 5.28, H-12a), as demonstrated by the heteronuclear correlations between H-12a and C-1, C-4a, and C-12b. Similar to ring A, ring B exhibited another pair of *meta*-coupled protons at δ_H 6.05 and 5.98 (J = 2.1 Hz), attributed to H-9 and H-11, and was found to be substituted with three oxygenated residues and a quaternary carbon (C-8, C-10, C-11a, and C-7a at δ_C 160.2, 159.1, 155.4, and 101.8). The HMBC correlations between H-7 and C-8, as well as C-11a and C-7a, indicated that this last carbon is linked to the H-7 methine (δ_H 3.17). The H-7 signal also displayed a COSY correlation with the H-6a methine (δ_H 2.22); the latter was linked to the oxygenated H-6 (δ_H 4.78) and H-12a methines (linked to ring A). Therefore, two-fused benzopyran rings were present, as confirmed by the various HMBC correlations observed (Figure 1B).

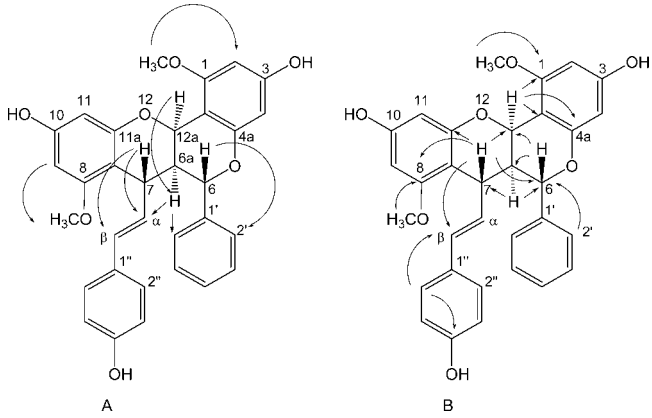


Figure 1. Key NOESY (A) and HMBC (B) correlations for compound **1**.

Two pairs of *ortho*-coupled protons at δ_H 7.05 (H-2″ and H-6″) and 6.64 (H-3″ and H-5″) were assigned to ring C in **1**, while the remaining five aromatic protons corresponded to the unsubstituted ring D at δ_H 7.24 (H-2′ and H-6′) and 7.38 (H-3′, H-4′, and H-5″). Ring C was substituted at C-4″ with a hydroxy group (δ_C 157.7) and at C-1″ with a *trans* double bond (δ_H 5.95 and 5.81, J = 15.9 Hz) that was also attached to the H-7 methine (COSY correlation between H-7 and H-α). However, ring D was shown to be directly attached to C-6 (HMBC between H-2′/H-6′ and C-6). The positions of the two methoxy groups at δ_H 3.81 and 3.62 were assigned using HMBC and NOESY correlations as C-1 (ring A) and C-8 (ring B), respectively (Figure 1). The relative configuration of the fused benzopyran rings was established based on scalar and dipolar couplings (Figure 1). These data were observed as being very similar to those of dependensin, which is a natural product isolated from the plant *Uvaria dependens*,[14] and to those of dimeric flavonoid analogues synthesized from methoxyflavenes under acid catalysis.[15] The X-ray crystal structure of one of these derivatives, (6a,12a-dihydro-1,3,4,8,10,11-hexamethoxy-6-(4′-methoxyphenyl)-7-[(1*E*)-2-(4″-methoxyphenylethenyl)]-6*H*,7*H*-[1]-benzopyrano[4,3-*b*][1]benzopyran), was used to confirmed the relative configuration. While dependensin was isolated as a racemic mixture ($[\alpha]_D$ 0) from *Uvaria dependens*, compound **1** exhibited a negative specific rotation ($[\alpha]^{20}_D$ −108).

To confirm its structure, **1** was acetylated to yield derivative **1a**. The APCIMS of **1a** displayed an $[M + H]^+$ peak at m/z 650, indicating that three acetyl groups were added. This structure was confirmed from its ^{1}H NMR data (three singlets at δ_H 2.25, 2.27, and 2.29). Consequently, **1** was identified as a new dimeric flavonoid and named brachydin A.

The HRESIMS of **2** revealed an $[M + H]^+$ peak at m/z 539.2095, corresponding to $C_{33}H_{31}O_7$. Its NMR spectrum was similar to that of **1** except that **2** was found to contain an

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Table 1. ^{1}H NMR and ^{13}C NMR Data (500 and 125 MHz, CD_3OD, δ in ppm) of Compounds 1−3

	brachydin A (1)		brachydin B (2)		brachydin C (3)	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		161.1, C		160.9, C		161.1, C
2	6.08 d (2.2)	92.8, CH	6.08 d (2.1)	92.7, CH	6.08 d (2.2)	92.8, CH
3		161.1, C		161.1, C		161.1, C
4	5.94 d (2.2)	96.4, CH	5.94 d (2.1)	96.3, CH	5.95 d (2.2)	96.4, CH
4a		157.8, C		157.7, C		157.8, C
6	4.78 d (11.2)	78.2, CH	4.77 d (11.3)	78.1, CH	4.79 d (11.3)	78.2, CH
6a	2.22 dt (11.2, 1.9)	42.4, CH	2.19 dt (11.3, 1.9)	42.3, CH	2.24 dt (11.3, 1.9)	42.2, CH
7	3.17 ddd (5.5, 1.9, 1.6)	34.0, CH	3.17 ddd (5.6, 1.9, 1.6)	34.0, CH	3.21 ddd (5.7, 1.9, 1.7)	34.2, CH
7a		101.8, C		101.6, C		101.5, C
8		160.2, C		160.2, C		160.2, C
9	6.05 d (2.1)	93.0, CH	6.05 d (2.1)	93.0, CH	6.05 d (2.3)	93.0, CH
10		159.1, C		159.2, C		159.2, C
11	5.98 d (2.1)	96.5, CH	5.98 d (2.1)	96.5, CH	5.99 d (2.3)	96.6, CH
11a		155.4, C		155.3, C		155.4, C
12a	5.28 d (2.3)	63.5, CH	5.27 d (2.3)	63.4, CH	5.28 d (2.4)	63.5, CH
12b		103.8, C		103.7, C		103.8, C
1′		140.4, C		140.4, C		140.4, C
2′, 6′	7.24 d (7.6)	128.5, CH	7.23 d (7.0)	128.5, CH	7.24 d (7.6)	128.5, CH
3′, 5′	7.38 m	129.5, CH	7.38 m	129.5, CH	7.38 m	129, CH
4′	7.38 m	129.6, CH	7.38 m	129.5, CH	7.38 m	129.6, CH
α	5.95 dd (15.9, 5.5)	130.7, CH	5.99 dd (15.8, 5.6)	131.5, CH	6.15 dd (15.7, 5.7)	133.8, CH
β	5.81 dd (15.9, 1.6)	131.7, CH	5.83 dd (15.8, 1.6)	131.3, CH	5.91 dd (15.7, 1.7)	132.0, CH
1″		130.4, C		131.1, C		138.6, C
2″, 6″	7.05 d (8.6)	128.4, CH	7.11 d (8.8)	128.2, CH	7.18 m	127.2, CH
3″, 5″	6.64 d (8.6)	116.2, CH	6.75 d (8.8)	114.8, CH	7.18 m	129.4, CH
4″		157.7, C		160.2, C	7.10 m	128.0, C
OMe-1	3.81 s	56.1, CH_3	3.79 s	56.1, CH_3	3.79 s	56.1, CH_3
OMe-8	3.62 s	55.9, CH_3	3.62 s	55.9, CH_3	3.61 s	55.9, CH_3
OMe-4″			3.73 s	55.6, CH_3		

Table 2. Cruzain Inhibitory Activity, Macrophage Cytotoxicity, and Anti-*T. cruzi* Activity of Dimeric Flavonoids (1−3)

			Y strain *T. cruzi*, IC_{50}(μM)[a]	
compound	% cruzain inhibition at 25 μM	host cells LC_{50} (μM)[a]	trypomastigotes	amastigotes
1	0	>20	>20	>20
2	0	15.6 (±0.02)	5.3 (±1.15)	6.0 (±0.33)
3	0	17.3 (±0.84)	6.6 (±0.39)	6.8 (±0.41)
benznidazole		>20	11.3 (±1.84)	14.0 (±0.39)
gentian violet		0.48 (±0.05)		
E-64c	100			

[a]IC_{50} and LC_{50} values are means (±SD) of three independent experiments performed in triplicate.

additional methoxy group. This additional methoxy group was located at C-4″, based on the HMBC and NOESY data. The NMR and MS analysis of the acetylated derivative **2a** was used to confirm the presence of two free hydroxy groups in **2**. Accordingly, this compound **2** (brachydin B) was identified as shown.

Compound **3** (brachydin C) was isolated as an amorphous, white solid. The HRESIMS data for **3** exhibited an $[M + H]^+$ ion at m/z 509.1934 corresponding to $C_{32}H_{29}O_6$. The NMR data for **3** were similar to those obtained for **1** and **2**. Instead of having a free hydroxy group at C-4″, the ^{1}H NMR spectrum displayed a signal at δ_H 7.10 that correlated with a carbon at δ_C 128.0 in the HSQC experiment; these signals were attributed to an aromatic proton at C-4″. The NMR and MS analysis of acetylated derivative **3a** were used to confirm the compound structure. On the basis of these results, **3** was identified as a further new dimeric flavonoid, with the structure shown.

To the best of our knowledge, until now only one compound (dependensin) of this chemical class has been described from *Uvaria dependens*, a plant belonging to the family Annonaceae.[14] The authors indicated that this compound may be artifactual due to a proton-catalyzed dimerization of a flavene unit.[14] In the present study, the negative $[\alpha]_D$ values observed for compounds **1**−**3** were used to confirm that these compounds are original secondary metabolites from *A. brachypoda*. Moreover, no flavenes were detected by LC-PDA-ESIMS analysis, and no acid conditions were used during the solvent extraction process.

Since **1**−**3** were the major components of the active fraction, their anti-*T. cruzi* activity was evaluated. Their activity against extracellular trypomastigotes was determined initially (Table 2). Compound **1** was inactive, while **2** and **3** exhibited potent activity, with IC_{50} values of 5.3 and 6.6 μM, respectively. Under the same assay conditions, the reference drug benznidazole exhibited an IC_{50} value of 11.3 μM. The cytotoxicity of compounds

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1–**3** was assessed subsequently using macrophages. Compound **1** was nonactive at concentrations up to 20 μM, and compounds **2** and **3** exhibited LC_{50} values of 15.6 and 17.3 μM, respectively.

Accordingly, these compounds were several times less cytotoxic than gentian violet (LC_{50} 0.48 μM) used as a reference. Subsequently, the inhibition of the catalytic activity of cruzain for **2** and **3** was assessed to obtain more information regarding their possible mode of action; cruzain is the major trypanosomal protease.[16] As shown in Table 2, none of the isolated compounds inhibited cruzain when tested at 25 μM.

The activities of **2** and **3** were also evaluated against intracellular amastigotes [(IC_{50} 6.0 μM **2** and 6.8 μM **3**)], and an IC_{50} value of 14.0 μM was observed for benznidazole in this assay. Therefore, **2** and **3** might inhibit the parasite intracellularly. Accordingly, their ability to inhibit the parasitic invasion of host cells was examined subsequently. Amphotericin B was used as the positive control for this experiment. As presented in Figure 2,

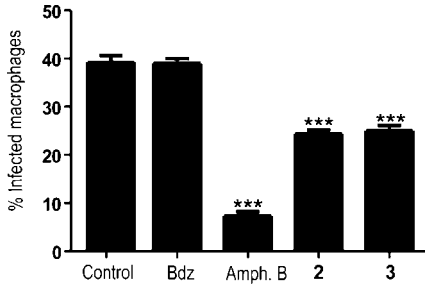

Figure 2. Compounds **2** and **3** inhibited cell invasion. Mouse macrophages were infected with Y strain trypomastigotes and treated with compound **2** or **3**. The percentage of infected macrophages was higher in untreated infected controls than in the cultures treated with compound **2** or **3** at 10 μM (Bdz, benznidazole; Amph. B, amphotericin B). The standard deviations are shown as error bars (***$p < 0.001$).

2 and **3** significantly inhibited parasite invasion relative to untreated cells ($p < 0.001$). In this assay, benznidazole exhibited no observable activity, possibly due to its low cellular penetration effects.[17]

As compound **2** displayed the most potent in vitro anti-*T. cruzi* activities, it was used for an additional in vivo experiment, and its efficacy for treating *T. cruzi*-infected mice was assessed. Consequently, BALB/c mice were infected with Y strain trypomastigotes, and 5 days after infection, 100 mg/kg compound **2** was administered once daily for five consecutive days. During this experiment, an untreated infected group was included, with the mice receiving 100 mg/kg benznidazole. The course of infection was monitored by counting the parasites in the blood samples; the survival of the animals was followed for one month. As revealed in Figure 3, treatment with **2** significantly reduced blood parasitemia and mortality relative to the untreated infected group. No toxicity was apparent during treatment with compound **2**. Compared to the untreated mice, the administration of **2** caused a 92% reduction in peak parasitemia, while a 99% reduction was observed for benznidazole. Therefore, **2** appears to be a promising lead for treating Chagas disease. Further studies to elucidate the mode of action and toxicity of this compound are required.

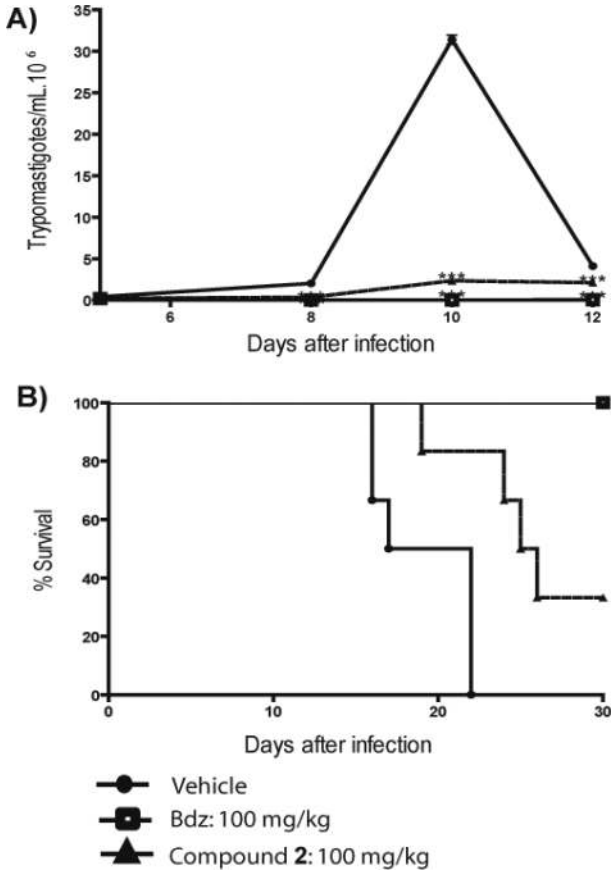

Figure 3. (A) Treatment with compound **2** substantially reduced the parasitemia in infected mice. Female BALB/c mice were infected with 10^4 Y strain trypomastigotes. Five days after infection, the mice were treated orally with compound **2** or benznidazole once daily at 100 mg/kg for five consecutive days. Parasitemia was monitored by counting the trypomastigotes in fresh blood samples. The values represent the means ± SEM of six mice per group [***$p < 0.001$ compared to untreated-infected group (vehicle)]. (B) Treatment with compound **2** increased the survival of the infected mice. The animals were monitored for 30 days after infection. The differences in the curves are statistically significant ($p < 0.05$).

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotations were measured in methanol on a JASCO polarimeter in a 1 cm tube. The UV spectra were measured on a Hach UV–vis DR/4000 instrument. The NMR spectroscopic data were recorded on a 500 MHz Varian Inova NMR spectrometer (Varian, Palo Alto, CA, USA). Chemical shifts are reported in parts per million (δ) using the residual CD_3OD signal (δ_H 3.31; δ_C 49.0) as the internal standard for both ^1H and ^{13}C NMR; the coupling constants (J) are reported in Hz. Complete structural assignments were performed based on 2D experiments (COSY, NOESY, edited-HSQC, and HMBC). HRESIMS data were obtained with a Micromass LCT Premier time-of-flight mass spectrometer using an electrospray ionization (ESI) interface (Waters, Milford, MA, USA). Analytical HPLC was performed using an HP 1100 system equipped with a photodiode array detector (Agilent Technologies, Santa Clara, CA, USA). MPLC was performed using a Büchi 681 pump equipped with a Knauer UV detector and a 460 × 70 mm i.d. column (Büchi) loaded with ZEOprep C_{18} as the stationary phase (15–25 μm) (Zeochem). Semipreparative HPLC was performed using a Shimadzu LC-8A pump equipped with a UV detector and using an X-Bridge C_{18} column (150 × 4.9 mm i.d.) (Waters).

Plant Material. Samples of *A. brachypoda* roots were collected in April 2010 at Sant'Ana da Serra farm João Pinheiro, Minas Gerais, Brazil. The plant was identified at the ICEB of the José Badine Herbarium of the Federal University of Ouro Preto by Prof. Maria Cristina Teixeira Braga Messias. A voucher specimen (no. 17935) has been deposited at the Herbarium of the Federal University of Ouro Preto, Brazil.

Extraction and Isolation. The dried roots (300 g) were extracted successively by percolation, at room temperature, with EtOH–H_2O 70%.

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The crude ethanolic extracts were obtained after filtration and evaporated to dryness under a vacuum at approximately 40 °C, yielding 11.8 g of an aqueous-ethanol extract. Further liquid/liquid extractions on the aqueous-ethanol extract were carried out using CH_2Cl_2 (1 L) and H_2O–MeOH (7:3) (1 L). The crude CH_2Cl_2 and aqueous-methanolic fractions were obtained after decantation and evaporated to dryness under a vacuum at approximately 40 °C, yielding 37.7% (4.44 g) and 62.3% (7.36 g) of the dried fractions, respectively, based on dry mass. The dichloromethane extract (2.5 g) was fractionated initially using MPLC with a Zeoprep C_{18} column as the stationary phase (15–25 μm, 460 × 49 mm i.d.) (Zeochem); MeOH and 0.002% formic acid in water were added using a linear gradient from 5% to 100% MeOH over a 50 h period. The flow rate was 3.5 mL min^{-1}, and the UV absorbance was detected at 217 nm. The MPLC separation generated 235 fractions. Each fraction was analyzed by HPLC-PDA. Fractions 90–98 (149.9 mg), fractions 138–143 (275.2 mg), and fractions 157–171 (298.3 mg) yielded compounds **1**, **2**, and **3**, respectively.

*Brachydin A (**1**):* amorphous, white powder; $[\alpha]^{20}_D$ −108 (*c* 0.1, MeOH); UV λ_{max} (log ε) 266 (4.42) nm; ^{1}H NMR (MeOD, 500 MHz) and ^{13}C NMR (MeOD, 125 MHz), see Table 1; ESIMS *m*/*z* 525.1 $[M + H]^+$; HRMS *m*/*z* 525.1926 $[M + H]^+$ (calcd for $C_{32}H_{28}O_7$, 525.1913).

Preparation of the Acetyl Derivative (1a) of Brachydin A (1). Compound **1** (1.5 mg) was dissolved in pyridine (0.2 mL), and excess acetic anhydride (3 mL) was added before the mixture was stirred for 24 h at room temperature. The mixture was washed with 1% $NaHCO_3$ solution and evaporated in vacuo, yielding **1a** (1.75 mg, 88.7%). Compound **1a**: ^{1}H NMR (CD_3OD, 500 MHz) δ 2.25 (3H, s, $OCOCH_3$), 2.27 (3H, s, $OCOCH_3$), 2.29 (3H, s, $OCOCH_3$), 2.41 (1H, dt, *J* = 11.3, 2.2 Hz, H-6a), 3.35 (1H, dd, *J* = 5.7, 2.2 Hz, H-7), 3.68 (3H, s, OMe-8), 3.86 (3H, s, OMe-1), 4.87 (1H, d, *J* = 11.3 Hz, H-6), 5.42 (1H, d, *J* = 2.2 Hz, H-12a), 5.97 (1H, d, *J* = 15.5 Hz, H-β), 6.19 (1H, dd, *J* = 15.5, 5.7 Hz, H-α), 6.30 (1H, d, *J* = 2.0 Hz, H-2*), 6.32 (1H, d, *J* = 2.1 Hz, H-9*), 6.34 (1H, d, *J* = 2.1 Hz, H-11*), 6.37 (1H, d, *J* = 2.0 Hz, H-4*), 6.96 (2H, d, *J* = 8.7 Hz, H-3″, 5″), 7.28 (2H, m, H-2′, 6′), 7.29 (2H, d, *J* = 8.7 Hz, H-2″, 6″), 7.43 (3H, m, H-3′, 5′, 4′) (*these assignments are interchangeable); APCIMS *m*/*z* 650.8 $[M + H]^+$.

*Brachydin B (**2**):* amorphous, white powder; $[\alpha]^{21}_D$ −119 (*c* 0.1, MeOH); UV λ_{max} (log ε) 254 (4.44) nm; ^{1}H NMR (MeOD, 500 MHz) and ^{13}C NMR (MeOD, 125 MHz), see Table 1; ESIMS *m*/*z* 539.1 $[M + H]^+$; HRMS *m*/*z* 539.2095 $[M + H]^+$ (calcd for $C_{33}H_{30}O_7$, 539.2070).

Preparation of the Acetyl Derivative (2a) of Brachydin B (2). Compound **2** (1.5 mg) was dissolved in pyridine (0.2 mL), and excess acetic anhydride (3 mL) was added before the mixture was stirred for 24 h at room temperature. The mixture was washed with 1% $NaHCO_3$ solution and evaporated in vacuo, yielding **2a** (1.2 mg, 77.5%). Compound **2a**: ^{1}H NMR (CD_3OD, 500 MHz) δ 2.27 (3H, s, $OCOCH_3$), 2.29 (3H, s, $OCOCH_3$), 2.36 (1H, dt, *J* = 11.0, 2.3 Hz, H-6a), 3.32 (1H, m, H-7), 3.68 (3H, s, OMe-8), 3.75 (3H, s, OMe-4), 3.85 (3H, s, OMe-1), 4.87 (1H, d, *J* = 11.0 Hz, H-6), 5.42 (1H, d, *J* = 2.3 Hz, H-12a), 5.92 (1H, d, *J* = 15.9 Hz, H-β), 6.04 (1H, dd, *J* = 15.9, 5.6 Hz, H-α), 6.29 (1H, d, *J* = 2.1 Hz, H-2*), 6.31 (1H, d, *J* = 2.1 Hz, H-4*), 6.32 (1H, d, *J* = 2.0 Hz, H-9*), 6.34 (1H, d, *J* = 2.0 Hz, H-11*), 6.78 (2H, d, *J* = 8.7 Hz, H-3″, 5″), 7.27 (2H, m, H-2′, 6′), 7.41 (3H, m, H-3′, 4′, 5′), 7.45 (2H, d, *J* = 8.7 Hz, H-2″, 6″) (*these assignments are interchangeable); APCIMS *m*/*z* 622.6 $[M + H]^+$.

*Brachydin C (**3**):* amorphous, white powder; $[\alpha]^{20}_D$ −83 (*c* 0.1, MeOH); UV λ_{max} (log ε) 266 (4.22) nm; ^{1}H NMR (MeOD, 500 MHz) and ^{13}C NMR (MeOD, 125 MHz): see Table 1; ESIMS *m*/*z* 509.1 $[M + H]^+$; HRMS *m*/*z* 509.1974 $[M + H]^+$ (calcd for $C_{32}H_{29}O_6$, 509.1964).

Preparation of the Acetyl Derivative 3a of Brachydin C. Compound **3** (1.5 mg) was dissolved in pyridine (0.2 mL), and excess acetic anhydride (3 mL) was added before the mixture was stirred for 24 h at room temperature. The mixture was washed with 1% $NaHCO_3$ solution and evaporated in vacuo, yielding **3a** (2.0 mg, 78.5%). Compound **3a**: ^{1}H NMR (CD_3OD, 500 MHz) δ 2.25 (3H, s, $OCOCH_3$), 2.27 (3H, s, $OCOCH_3$), 2.37 (1H, dt, *J* = 11.5, 2.3 Hz, H-6a), 3.33 (1H, dd, *J* = 5.7, 2.3 Hz, H-7), 3.66 (3H, s, 8-OMe), 3.82 (3H, s, 1OMe), 4.82 (1H, d, *J* = 11.5 Hz, H-6), 5.41 (1H, d, *J* = 2.3 Hz, H-12a), 5.95 (1H, d, *J* = 16.0 Hz, H-β), 6.19 (1H, dd, *J* = 16.0, 5.7 Hz, H-α), 6.29 (1H, d, *J* = 2.0 Hz, H-2*), 6.31 (1H, d, *J* = 2.1 Hz, H-9*), 6.36 (1H, d, *J* = 2.1 Hz, H-11*), 6.39 (1H, d, *J* = 2.0 Hz, H-4*), 7.23 (7H, m, H-2″, 3″, 4″, 5″, 6″, 2′, 6′), 7.39 (3H, m, H-3′, 5′, 4′) (*these assignments are interchangeable); APCIMS *m*/*z* 592.7 $[M + H]^+$.

Mice, Parasites, and Test Compounds. Female BALB/c mice (6–8 weeks old) were supplied by the animal breeding facility at Centro de Pesquisas Gonçalo Moniz (Fundação Oswaldo Cruz, Bahia, Brazil). Y strain trypomastigotes were obtained from the supernatants of previously infected LLC-MK2 cells maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MD, USA) supplemented with 10% fetal bovine serum (FBS; Cultilab, Campinas, Brazil) and 50 μg/mL gentamycin (Novafarma, Anápolis, Brazil) at 37 °C and 5% CO_2. All test compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted in culture medium for use during the assays. Benznidazole (LAFEPE, Recife, Brazil) was used as the positive control for anti-*T. cruzi* activity during the studies. Amphotericin B (Gibco Laboratories, Gaithersburg, MD, USA) was used as a positive control during the invasion assay. Gentian violet (Synth, São Paulo, Brazil) was used as the cytotoxicity control. Female BALB/c mice (6–8 weeks old) were inoculated intraperitoneally with 104 bloodstream trypomastigotes of the Y strain and then divided in three groups (six animals per group). After day 5 of the infection, 100 mg/kg compound **2** was given orally once per day for five consecutive days. For the control group, benznidazole was orally administered at 100 mg/kg. Saline containing 20% DMSO was used as a vehicle. The course of the infection was monitored by counting the number of motile parasites in 5 μL of fresh blood samples drawn from the lateral tail veins, as recommended by standard protocols.[18] Survival was followed every day until the 30th day after infection. All animal experiments and procedures were carried out in accordance with the recommendations of ethical guidelines and were approved by the Animal Ethics Committee of Fundação Oswaldo Cruz, Salvador, BA, Brazil.

Cytotoxicity to Mammalian Cells. Peritoneal exudate macrophages obtained from BALB/c mice were seeded in 96-well plates at 1×10^5 cells per well in RPMI-1640 medium without phenol red and supplemented with 10% FBS and 50 μg/mL gentamycin, and the cells were incubated for 24 h at 37 °C and 5% CO_2. Then, each compound was added at five concentrations ranging from 0.24 to 20 μM in triplicate before being incubated for 72 h. A total of 20 μL/well of AlamarBlue (Invitrogen, Carlsbad, CA, USA) was added to the plates over 10 h. Colorimetric readings were performed at 570 and 600 nm. The LC_{50} values were calculated using the data points gathered from three independent experiments. Gentian violet was used as the positive control at concentrations ranging from 0.04 to 10 μM.

Toxicity against Trypomastigotes. The trypomastigotes collected from the supernatants of LLC-MK2 cells were dispensed into 96-well plates at 4×10^5 cells per well. Extracts from the *Arrabidaea brachypoda* roots were tested in five concentrations ranging from 1.23 to 100 μg/mL. The isolated compounds were tested at five concentrations ranging from 0.24 to 20 μM. The plate was incubated for 24 h at 37 °C and 5% CO_2. Aliquots from each well were collected, and the number of living parasites was counted in a Neubauer chamber for comparison with an untreated parasite culture. This experiment was performed in triplicate, and at least three independent experiments were conducted.

Cruzain Inhibition. Recombinant cruzain was activated in acetate buffer (0.1 M; pH 5.5) containing 5.5 mM DTT (Invitrogen); the protein concentration was adjusted to 0.1 μM. The protein was incubated in phosphate buffer containing 0.01% Triton 100 and transferred to a 96-well plate. After adding the complex, the plate was incubated for 10 min at 35 °C. A solution containing the protease substrate (Sigma-Aldrich) was added and incubated for 10 min, and the plate was read using an EnVision multilabel reader (PerkinElmer, Shelton, CT, USA). The percentage of cruzain inhibition was calculated using the following equation: $100 - (A1/A \times 100)$, where A1 represents the cruzain relative fluorescence unit in the presence of the test inhibitor and A refers to the control RFU (cruzain and substrate only). Compound concentrations were measured in triplicate. (2*S*,3*S*)-*trans*-Epoxysuccinyl-L-leucylamido-3-methylbutane (E-64c) (Sigma-Aldrich) was used as the standard cruzain inhibitor.

In Vitro *T. cruzi* Infection Assay. Peritoneal exudate macrophages were placed at 2×10^5 cells per well in a 24-well plate with round coverslips on the bottom in RPMI supplemented with 10% FBS and incubated for 24 h. The cells were subsequently infected with trypomastigotes at 10 parasites per macrophage. After 2 h, the noninternalized parasites were removed by successive saline washes. The cultures were incubated in complete medium without or with the compounds at five concentrations ranging from 0.12 to 10 μM in triplicate for 6 h. The medium was replaced with fresh medium, and the plate was incubated for 4 days. The coverslips were collected, rinsed in saline solution, and fixed in absolute alcohol before the number of intracellular parasites was manually counted in an optical microscope after hematoxylin and eosin staining (Olympus, Tokyo, Japan). The IC_{50} value was estimated as the concentration that reduced the number of amastigotes per 100 macrophages by 50%.

Invasion Assay. Peritoneal exudate macrophages were cultured at 1×10^5 cells per well in a 24-well plate with rounded coverslips on the bottom in RPMI supplemented with 10% FBS and incubated for 24 h. Cells were infected with trypomastigotes at 100 parasites per macrophage for 2 h and treated with compound **2** or **3** (at 10 μM). Amphotericin B (10 μM) was used as the reference inhibitor. The plate was incubated for 2 h at 37 °C and 5% CO_2 and washed with saline solution to remove any noninternalized parasites. The plates were maintained in RPMI medium supplemented with 10% FBS at 37 °C for 2 h. The cells were fixed in absolute alcohol, and the percentage of infected macrophages was counted manually using an optical microscope after hematoxylin and eosin staining.

Statistical Analysis. To determine the lethal concentration for 50% of the BALB/c mice macrophages (LC_{50}) and the inhibitory concentration for 50% (IC_{50}) of the trypomastigote and amastigote forms of *T. cruzi*, nonlinear regression was used. The one-way ANOVA was followed by Bonferroni's multiple comparison test to determine the statistical significance of the comparisons between the groups during the cell invasion study and in vivo infection experiments. The results were considered statistically significant when $p < 0.05$. All analyses were performed using Graph Pad Prism version 5.01 (Graph Pad Software, San Diego, CA, USA).

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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