Supplemental Material: S1

Title*:* *In vitro* and *In vivo* Trypanosomicidal Action of Novel Arylimidamides Against *Trypanosoma cruzi*

Authors: Guedes-da-Silva, Francisca Hildemagna,[1]; Batista, Denise Gama Jaén[1]; Meuser, Marcos Batista[1]; Fulco, Tatiana[1]; Araújo, Julianna Silva[1]; Da Silva, Patrícia Bernardino[1]; Da Silva Cristiane França[1]; Donald A. Patrick[2]; Svetlana M. Bakunova[2]; Stanislav A. Bakunov[2]; Richard R. Tidwell[2]; Oliveira, Gabriel Melo[1]; Britto, Constança[3]; Moreira, Otacílio[3] and Maria de Nazaré C. Soeiro[1]

Authors' addresses: [1]Laboratório de Biologia Celular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil, [2] University of North Carolina, Chapel Hill, North Carolina, USA, [3]Laboratório de Biologia Molecular e Doenças Endêmicas Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil

Running title: *In vitro* and *in vivo* trypanosomicidal effect of arylimidamides

*Correspondent footnote

E-mail: soeiro@ioc.fiocruz.br

Phone Number: 55 21 25621368

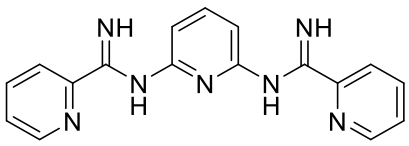
Fax Number: 55 21 25984469

General experimental

Uncorrected melting points were measured on a Thomas–Hoover Capillary melting point apparatus. ^{1}H NMR spectra were recorded in DMSO-d_6 on a Varian Gemini 2000 300 MHz or a Varian Inova 400 MHz spectrometer. Organic starting materials were prepared by established procedures as noted, or purchased from Aldrich Chemical Co., Milwaukee, WI, as were anhydrous solvents in Sure-seal® containers, which were used without further purification. Reaction mixtures were monitored by reverse phase HPLC. Organic layers of extraction mixtures were neutralized as necessary with acidic or basic washes, washed with saturated NaCl solution and dried over $MgSO_4$ or Na_2SO_4 before being evaporated under reduced pressure. Final products were dried under high vacuum over P_2O_5. Analytical HPLC chromatograms were recorded on an Agilent 1200 chromatograph using a Zorbax Rx C8 column (4.6 × 75 mm, 3.5 µm) maintained at 40 °C and UV photodiode array detection at 230, 254, 265, 290, and 320 nm. Area % values are reported at the wavelengths where the strongest signals of the products were observed. Mobile phases consisted of mixtures of acetonitrile (0–75%) or methanol (0–95%) in water containing formic acid (80 mM), ammonium formate (20 mM) and triethylamine (15 mM). Samples were eluted at appropriate gradients at a flow rate of 1.5 mL/min. Preparative reverse phase HPLC was performed on a Varian ProStar Chromatography Workstation configured with two PS-215 pumps fitted with 50 mL pump heads, a Dynamax Microsorb C18 (60 Å) column (41.4 × 25 cm, 8 µm), PS-320 variable wavelength UV–Vis detector, and a PS-701 fraction collector. Mobile phases consisted of mixtures of acetonitrile (0–75%) in water containing a buffer (formic acid, 40 mM and ammonium formate, 10 mM) or 0.1% trifluoroacetic acid. Flow rates were maintained at 40 mL/min. Select fractions

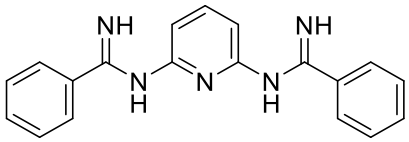
were analyzed for purity by analytical HPLC as described above. Pooled purified fractions were evaporated under reduced pressure, reconstituted in water, and lyophilized (3 cycles) on a VirTis BenchTop or 6K lyophilizer. Low resolution ESI mass spectra were recorded on an Agilent Technologies 1100 Series LC/MSD Trap mass spectrometer, or on a Thermo Electron Corporation Finnigan TSQ Quantum Discovery Max mass spectrometer at the North Carolina State University Mass Spectrometry Facility located in the Department of Chemistry. Elemental analyses were performed by Atlantic Microlab, Norcross, GA, and were within ± 0.4% of calculated values. The compounds reported as salts frequently analyzed correctly for fractional moles of water and/or other solvents; in each case ^{1}H NMR spectra was consistent with the analysis.

General procedure for 19SAB003, 19SAB005, and 19SAB007. Sodium bis(trimethylsilyl)amide (2M solution in tetrahydrofuran, 10 mL, 20 mmol) was added to a stirred solution of the diaminopyridine (10 mmol) in dry tetrahydrofuran (20 mL) under argon atmosphere at ambient temperature.The mixture turned dark blue and a precipitate began to form. After 30 minutes, the aromatic nitrile (20 mmol) was added and the mixture was stirred overnight. The mixture was poured over ice and the crude free base was obtained by filtration of the precipitate or extraction with dichloromethane. An aliquot of the base was converted to the hydrochloride salt using aqueous HCl.

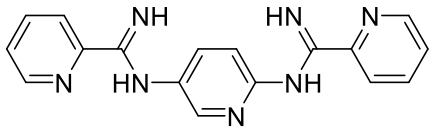


N,N″-(Pyridine-2,6-diyl)dipicolinimidamide dihydrochloride (19SAB003) was prepared by the general procedure from 2,6-diaminopyridine and 2-cyanopyridine. Recrystallization

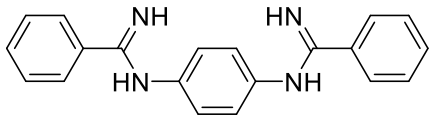
of the crude product gave 2.00 g (63 %) of the pure free base (mp 171-173 °C). An aliquot (0.5622 g) was converted to the hydrochloride salt by dissolving in 1M HCl followed by lyophilization to give a solid (0.5647 g): mp > 195 °C (decomposes); ^{1}H NMR (300 MHz) δ 12.67 (s, 1H), 11.06 (s, 2H), 10.62 (s, 1H), 8.95 (d, *J* = 4.6 Hz, 2H), 8.95 (d, *J* = 8.0 Hz, 2H), 8.28-8.22 (m, 3H), 7.90 (dd, *J* = 7.6 and 4.6 Hz, 2H), 7.73 (d, *J* = 8.0 Hz, 2H); ESI-MS *m/z* 318.04 ($[M + 1]^+$); HPLC 100 area % (230 nm). Anal. Calcd. for $C_{17}H_{15}N_7 \cdot 2.3HCl \cdot 2.3H_2O$: C, 46.13; H, 4.99; N, 22.15; Cl, 18.42. Found C, 45.98; H, 4.86; N, 21.96; Cl, 18.51.



N,N″-(Pyridine-2,6-diyl)dibenzimidamide dihydrochloride (19SAB005) was prepared by the general procedure from 2,5-diaminopyridine and benzonitrile. The crude free base was obtained by extraction into dichloromethane followed by recrystallization from acetonitrile: yield 2.23 g (71%), mp 175-177 °C. An aliquot (1.00 g) was recrystallized from aqueous HCl to give the hydrochloride salt as a solid (0.8285 g): mp > 235 °C (dec); ^{1}H NMR (400 MHz) δ 12.48 (s, 2H), 10.74 (s, 2H), 10.41 (s, 2H), 8.18 (t, *J* = 8.0 Hz, 1H), 8.00 – 7.92 (m, 4H), 7.86 – 7.76 (m, 2H), 7.74 – 7.65 (m, 4H), 7.52 (d, *J* = 8.1 Hz, 2H); ESI-MS *m/z* 316.03 ($[M + 1]^+$); HPLC 100 area % (230 nm). Anal. Calcd for $C_{19}H_{17}N_5 \cdot 2HCl \cdot H_2O$: C, 56.16; H, 5.21; N, 17.24; Cl, 17.45. Found C, 56.24; H, 5.20; N, 17.17; Cl, 17.26.



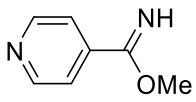
N,N″-(Pyridine-2,5-diyl)dibenzimidamide dihydrochloride (19SAB007) was prepared by the general procedure from 2,5-diaminopyridine and 2-cyanopyridine. The free base was purified by preparative HPLC and was then converted to the hydrochloride salt using 1M HCl followed by lyophilization to give a solid (1.26 g, 32 %): mp 271-273 °C (dec); ^{1}H NMR (400 MHz) δ 12.59 (s, 1H), 12.14 (s, 1H), 11.76 (s, 1H), 11.30 (s, 1H), 10.25 (s, 1H), 9.57 (s, 1H), 8.98 – 8.88 (m, 2H), 8.65 (d, *J* = 8.3 Hz, 2H), 8.59 (d, *J* = 8.0 Hz, 1H), 8.25 (tdd, *J* = 7.9, 4.7, 1.7 Hz, 2H), 8.19 – 8.12 (m, 1H), 8.04 (d, *J* = 8.7 Hz, 1H), 7.88 (ddt, *J* = 9.4, 5.6, 2.8 Hz, 2H); ESI-ESI-MS *m/z* 318.01 ([M + 1]$^{+}$); HPLC 96 area % (230 nm). Anal. Calcd for $C_{17}H_{15}N_7 \cdot 2HCl \cdot 2.2H_2O$: C, 47.49; H, 5.02; N, 22.81; Cl, 16.49. Found C, 47.65; H, 4.99; N, 22.80; Cl, 16.21.



N,N″-(1,4-Phenylene)dibenzimidamide dihydrochloride (28SMB008) was prepared as above from 1,4-phenylenediamine (2.00 g, 18.49 mmol), benzonitrile (4.79 g, 46.45 mmol) and sodium bis(trimethylsilyl)amide (2 M solution in tetrahydrofuran, 21 mL, 20 mmol) in tetrahydrofuran (60 mL total). After stirring overnight, the reaction mixture was diluted with water. The precipitated product was filtered off and recrystallized from 1M HCl to give the hydrochloride salt (2.20 g, 31%): mp 318-320 °C; ^{1}H NMR (300 m Hz) δ 11.96 (s, 1H)), 9.92 (s, 2H), 9.34 (s, 2H), 8.00 (d, *J* = 6.8 Hz, 4H), 7.91 (t, *J* = 7.0 Hz,

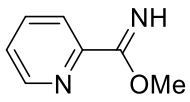
2H), 7.70-7.64 (m, 8H); ESI-MS *m/z* 315.7 ([M + 1]$^+$); HPLC 100 area %. Anal. Calcd for $C_{20}H_{18}N_4 \cdot 2HCl \cdot 1.2H_2O$: C, 58.74; H, 5.52; N, 13.70; Cl, 17.34. Found C, 58.9; H, 5.44; N, 13.69; Cl, 17.12.

Imidate derivatives of 2- and 4-cyanopyridine



Methyl isonicotinimidate

A suspension of 4-cyanopyridine (20.3 g, 195 mmol) in dry MeOH (210 mL) was treated with sodium methoxide (0.5 M solution in MeOH, 50 mL, 25 mmol). The mixture was stirred for 1 week, and aliquots of this solution (0.75 M) were used directly in the next step.



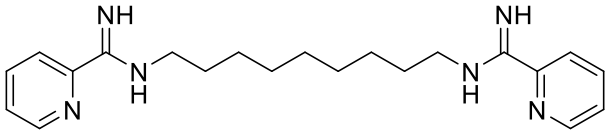
Methyl picolinimidate

The title compound was prepared analogously from 2-cyanopyridine to give stock solutions of 0.667 M or 0.75 M.

General procedure for 23SMB046, 23SMB050, 26SMB060, and 27SMB005

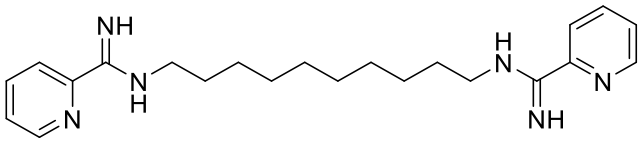
To a solution of the appropriate diamine (1 equivalent) in MeOH (10 mL) was added HCl/dioxane (4 M solution, 1 equivalent) followed by the imidate solution (2

equivalents). The reaction mixture was stirred at room temperature until complete by HPLC. All products were purified by preparative HPLC except for 23SMB050.



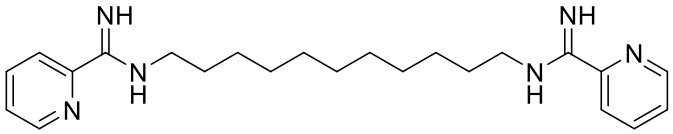
N,N″-(Nonane-1,9-diyl)dipicolinimidamide trihydrochloride (23SMB046)

The title compound was prepared from methyl picolinimidate (13.3 mmol) and 1,9-diaminononane (6.65 mmol) by the general procedure. The lyophilized product obtained after preparative HPLC was dissolved in 1M aqueous HCl and lyophilized. The residue was dissolved in EtOH and precipitated out by addition of 4M HCl/dioxane. Yield, 2.25 g (77 %): mp 175-178 °C; ^{1}H NMR (300 m Hz) δ 10.19 (s, 2H), 9.85 (s, 2H), 9.54 (s, 2H), 8.82 (dt, J = 4.6 and 0.7 Hz), 1H), 8.43 (d, J = 8.0 Hz), 8.17 (td, J = 7.8 and 1.7 Hz, 2H), 7.80-7.76 (m, 2H), 3.51 (q, J = 7.0 Hz, 4H), 1.64-1.61 (m, 4H), 1.31 (s, 10H); ESI-MS m/z 387.3 ([M + 1]$^+$); HPLC 100 area % (230 nm). Anal. Calcd for $C_{21}H_{30}N_6 \cdot 3.2HCl \cdot H_2O$: C, 50.32; H, 7.08; N, 16.77; Cl, 22.64. Found C, 50.31; H, 6.98; N, 16.85; Cl, 22.33.



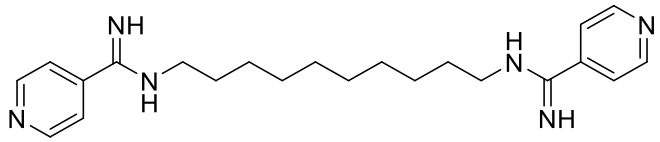
N,N″-(Decane-1,10-diyl)dipicolinimidamide dihydrochloride (23SMB050)

The title compound was prepared from methyl picolinimidate (13.3 mmol) and 1,10-diaminodecane (6.65 mmol) by the general procedure. After 1 week, the precipitated product was filtered off and was recrystallized directly from 1 M HCl. Yield 2.00 g (66 %): mp 250-252 °C; ^{1}H NMR (300 m Hz) δ 10.14 (s, 2H), 9.80 (s, 2H), 9.48 (s, 2H), 8.82 (dt, *J* = 4.8 and 0.8 Hz), 1H), 8.37 (dd, *J* = 8.0 and 0.9 Hz), 8.15 (td, *J* = 7.8 and 1.7 Hz, 2H), 7.81-7.77 (m, 2H), 3.49 (q, *J* = 7.0 Hz, 4H), 1.66-1.61 (m, 4H), 1.31-12.9 (m, 12H); ESI-MS *m/z* 381.4 ([M + 1]$^+$); HPLC 100 area % (230 nm). Anal. Calcd for $C_{22}H_{32}N_6 \cdot 2HCl$: C, 58.27; H, 7.56; N, 18.53; Cl, 15.64. Found C, 57.97; H, 7.69; N, 18.32; Cl, 15.42.



N,N″-(Dodecane-1,11-diyl)dipicolinimidamide dihydrochloride (26SMB060)

The title compound was prepared from methyl picolinimidate (15 mmol) and 1,12-diaminododecane (7.5 mmol) by the general procedure. The lyophilized product obtained after preparative HPLC was dissolved in 1M aqueous HCl, and lyophilized (3 cycles). Yield 2.20 g (61 %): mp 226-228 °C; ^{1}H NMR (300 MHz) δ 10.20 (s, 2H), 9.86 (s, 2H), 9.56 (s, 2H), 8.83-8.82 (m, 2H), 8.45 (d, *J* = 4.9Hz), 2H), 8.20-8.14 (m, 2H), 7.82-7.77 (m, 2H), 3.52-3.40 (m, 4H), 1.64 (s, 4H), 1.26 (s, 12H); ESI-MS *m/z* 409.3 ([M + 1]$^+$); HPLC 100 area % (265 nm). Anal. Calcd for $C_{24}H_{36}N_6 \cdot 2HCl$: C, 59.87; H, 7.95; N, 17.15; Cl, 14.73. Found C, 59.58; H, 7.96; N, 17.29; Cl, 14.67.



N,N″-(Decane-1,10-diyl)diisonicotinimidamide tetrahydrochloride (27SMB005)

The title compound was prepared from methyl isonicotinimidate (15 mmol) and 1,10-diaminodecane (7.5 mmol) by the general procedure. The lyophilized product obtained after preparative HPLC was converted to its HCl salt as immediately above. Yield 1.72 g (51 %): mp 239-241 °C; ^{1}H NMR (300 MHz) δ10.42 (s, 2H), 9.93 (s, 2H), 9.66 (s, 2H), 8.96-8.94 (m, 4H), 7.95-7.93 (m, 4H), 3.48-3.46 (m, 4H), 1.66 (s, 4H), 1.32 (s, 12H); ESI-MS *m/z* 381.3 ([M + 1]$^{+}$); HPLC 100 area % (230 nm). Anal. Calcd for $C_{22}H_{32}N_6 \cdot 4.8HCl \cdot 2H_2O$: C, 44.67; H, 6.95; N, 14.21; Cl, 27.72. Found C, 44.38; H, 7.00; N, 13.99; Cl, 28.68.

Supplemental Material: S2

Title*:* *In vitro* and *In vivo* Trypanosomicidal Action of Novel Arylimidamides Against *Trypanosoma cruzi*

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Authors' addresses: [1]Laboratório de Biologia Celular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil, [2] University of North Carolina, Chapel Hill, North Carolina, USA, [3]Laboratório de Biologia Molecular e Doenças Endêmicas Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brazil

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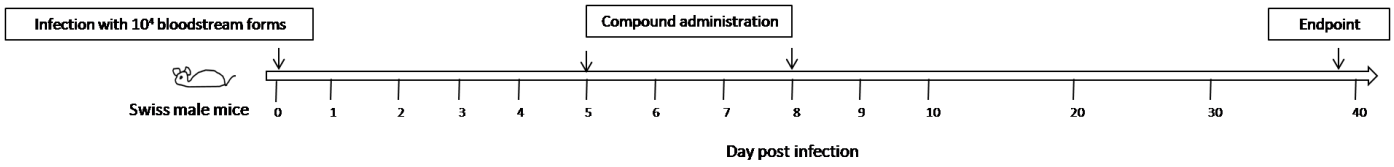
*Correspondent footnote
E-mail: soeiro@ioc.fiocruz.br
Phone Number: 55 21 25621368
Fax Number: 55 21 25984469

Effect of 35DAP073 (20-5 mg/kg/day) and benznidazole (100 mg/kg/day) given at the 5 and at the 8 dpi on mouse experimental model of *T. cruzi* infection (Y strain)



Effect of 35DAP073 and benznidazole using monotherapy (5 mg/kg/day and 100 mg/kg/day) and combinatory schemes (0.5 mg/kg/day 35DAP073 + Bz 100 mg/kg/day) given for 10 consecutive days on mouse experimental model of *T. cruzi* infection (Colombiana strain)