Synthesis of New Conjugates 1*H*-Pyrazolo[3,4-*b*]pyridine-phosphoramidate and Evaluation against *Leishmania amazonensis*

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In this research three series of substituted 1*H*-pyrazolo[3,4-*b*]pyridine phosphoramidates were synthesized and characterized by infrared, ¹H, ¹³C, and ³¹P nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry. The products were obtained in good yields (67-83%) under mild conditions by nucleophilic aromatic substitution reaction of aminoalkylphosphoramidates over 4-chloro-1*H*-pyrazolo[3,4-*b*]pyridines. These compounds were evaluated as antileishmanials against *Leishmania amazonensis* promastigotes *in vitro*. Among all, compounds of a series showed expressive antileishmanial activity. Two of them emerged as the most active, with IC₅₀ values of 6.44 ± 1.49 and 12.25 ± 0.68 μM. The cytotoxicity of this series was assessed on murine cells and presented values similar to the reference drug pentamidine.

Keywords: antileishmanial activity, 1*H*-pyrazolo[3,4-*b*]pyridine, phosphoramidate

Introduction

Leishmaniasis is caused by the protozoan *Leishmania* parasites which are transmitted by the bite of infected female phlebotomine sandflies. The disease affects some of the poorest people on earth, and is associated with malnutrition, population displacement, poor housing, a weak immune system and lack of financial resources. An estimated 900,000-1.3 million new cases and 20,000 to 30,000 deaths occur annually.¹ In the Americas, different dermatropic species that can affect humans are known and *Leishmania amazonensis* is associated with different forms of the disease, including cutaneous, hyperergic mucocutaneous, and the anergic diffuse cutaneous leishmaniasis.² *Leishmania amazonensis* is an important epidemiological species responsible for causing the most

common cutaneous form of the disease and is also able to cause mucosal and visceral forms of leishmaniasis.³ The recommended treatment for leishmaniasis is pentavalent antimony; however, this drug is toxic, causes many side effects and gives rise to drug-resistant parasites when used in high doses, requiring the use of second-line drugs, such as pentamidine and amphotericin B, which are even more toxic. Even with the use of second-line drugs, parasite survival and resistance still occurs.⁴ Miltefosine, an alkyl phospholipid, has emerged as an effective drug against visceral leishmaniasis and was incorporated into the therapeutic routine in South Asia (India, Nepal and Bangladesh). According to the World Health Organization (WHO), strategies to improve leishmaniasis therapy include the search for new drugs, repurposing of existing drugs and combination therapy.⁵ Several reports regarding natural and synthetic new antileishmanial compounds have been described and among them, there are the pyrazolopyridine

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derivatives, which have been revealed as potential new drugs against *Leishmania* infection.^{2,6,7}

1*H*-Pyrazolo[3,4-*b*]pyridine is an example of fused system, which is known to possess remarkable and significant biological and medicinal importance.^{2,8-11} Fused heterocyclic systems containing pyrazole ring are ranked among the most versatile bioactive compounds, and a variety of procedures have been developed for their synthesis.¹²⁻¹⁵

In the past few years, we focused our efforts on the preparation of bioactive heterocycles containing phosphoramidate groups.¹⁶⁻¹⁸ Introduction of a phosphoramidate group essentially changes the physical and chemical properties of the parent molecule, accentuating the polarization and intermolecular bonding characteristics.^{19,20} The P=O group plays a significant role as a strong hydrogen bond acceptor, which is essential for the noncovalent bonding of proteins or other specific ligands to their substrates.²¹ The incorporation of a phosphoramidate group into different heterocyclic systems constitutes an interesting synthetic strategy used in the discovery of new drugs.²²

As part of the program in the rational search for new leishmanicide drugs with greater efficacy and lower toxicity, our laboratory is working on the study of the structure-activity relationship of synthetic compounds. Taking in account the molecular hybridization to improve the pharmacological properties, a series of new 1*H*-pyrazolo[3,4-*b*]pyridine phosphoramidate has been synthesized in our late research. Our research group reported the synthesis of various substances of pyrazole and pyrazolopyridine systems that showed antileishmania activity. A series of 4-arylamino-1*H*-pyrazolo[3,4-*b*]pyridine showed antileishmanial activity at micromolar concentrations.^{2,23-26}

Based on isosteric and group substitutions approaches and development of new drugs that are able to act as multitarget ligands,²⁷⁻²⁹ we report the synthesis of fifteen new 1*H*-pyrazolo[3,4-*b*]pyridine phosphoramidates in search of new compounds with promising biological properties against *L. amazonensis* (Figure 1).

Results and Discussion

Synthesis

The synthetic pathway for the 1*H*-pyrazolo[3,4-*b*]pyridine phosphoramidates (**14a-e**, **15a-e** and **16a-e**) was successfully adapted from our previous research³⁰ and is shown in Schemes 1 and 2.

The intermediate ethyl 4-chloro-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine-5-carboxylate (**10a**), ethyl

Figure 1. Rational approach to the design of new 1*H*-pyrazolo[3,4-*b*]pyridine phosphoramidates.

5-amino-1-phenyl-1*H*-pyrazole-4-carboxylate (**5**) could easily be prepared in 70% yield from the reaction of phenylhydrazine (**1**) and ethyl 2-cyano-3-ethoxyacrylate (**2**), in ethanol under reflux. The decarboxylated derivative 5-amino-1-phenyl-1*H*-pyrazol (**6a**) was obtained in 80% yield upon treatment of (**5**) with phosphoric acid (85%) at 170 °C. The Michael addition of 5-aminopyrazole (**6a**) with diethyl 2-(ethoxymethylene)malonate (**7a**) in ethanol under reflux afforded the 2-(((1-phenyl-1*H*-pyrazol-5-yl)amino)methylene)malonate (**8a**) in 85% yield, which was converted to (**10a**) in 75% yield with phosphorous oxychloride under reflux.^{2,11} The 4-chloropyrazolopyridines **10b,c** were synthesized from β -aminocrotonitrile (**3**) and benzoylacetonitrile (**4**) in a similar sequence.^{2,9} Reaction of 5-aminopyrazoles (**6b,c**) with ethyl 2-cyano-3-ethoxyacrylate (**7b**) in ethanol under reflux afforded the derivatives **8b,c** in 85 and 72% yield, respectively. Cyclization of pyrazoles (**8b,c**) was performed by refluxing downtherm at 250 °C for 40 min and the products isolated by precipitation from hexane in 83% (**9a**) and 79% (**9b**) yield. These derivatives were refluxed with phosphorous oxychloride to produce (**10b,c**) in 62 and 73% yield, respectively.

The aminoalkylphosphoramidates (**13a-e**) were synthesized from diisopropylphosphonate (**11**) and aliphatic diamines (**12a-e**) according to Scheme 2. In order to guarantee monophosphorylation of the diamines, at least 2.5-fold excess of diamine in ethanol were used. Keeping alkaline pH and temperature below 55 °C is required to avoid bis-phosphorylation.³¹

Nucleophilic aromatic substitution by amines has been used as a versatile route to new pyrazolopyridine derivatives.^{2,8,32,33} An halogen atom such as chlorine in the C-4 position as the leaving group and an ester or cyano group in the C-5 position as the withdrawing group propitiate the 1*H*-pyrazolo[3,4-*b*]pyridine nucleus to react readily with a number of nucleophilic compounds representing a good precursor for the synthesis of 4-funcionalized derivatives. With this background, we settled the 1*H*-pyrazolo[3,4-*b*]pyridine (**10a-c**) to react

Scheme 1. (i) EtOH, reflux, 7 h; (ii) H_3PO_4 , 170 °C, 6 h; (iii) EtOH, reflux, 2 h; (iv) ddownthertm, 250 °C, 40 min; (v) $POCl_3$, reflux, 5-8 h.

Scheme 2. (i) CCl_4 , EtOH, $T < 55$ °C, 15 min; (ii) THF, reflux, 24 h.

with excess of the aminoalkylphosphoramidates (**13a-e**) in THF at 90 °C to obtain three series of substituted 1*H*-pyrazolo[3,4-*b*]pyridine phosphoramidates (**14a-e**, **15a-e** and **16a-e**) as solids in 67-83% yield (Scheme 2).

All the synthesized compounds were fully characterized by infrared, 1H , ^{13}C , and ^{31}P nuclear magnetic resonance (NMR) spectroscopy and high-resolution mass spectrometry.

The 1H NMR spectra of compounds **14a-e**, **15a-e** and **16a-e** showed a singlet in the range of 8.26-8.93 ppm attributable to the pyridine ring proton. Compounds **14a-e** showed a quartet and triplet signals related to the ethyl ester group in the ranges 4.24-4.32 ppm and 1.30-1.40 ppm, respectively, with $^3J_{HH}$ ca. 7.1 Hz and a singlet around 8.26 ppm to the pyrazolo ring proton. The resonances of the

isopropyl protons appeared as two doublets at 1.21-1.32 ppm and a doublet of septets around 4.54 ppm with $^3J_{HH}$ ca. 6.0 Hz and $^3J_{PH}$ ca. 6.8 Hz. The NH signal was detected as a broad triplet in the range 9.15-9.39 ppm with $^3J_{HH}$ ca. 4.6 Hz. On the other hand, NHP protons showed coupling with phosphorus and the neighbor methylene group, giving rise to a doublet of triplet around 2.57 ppm with $^3J_{HH}$ ca. 5.5 Hz and $^2J_{PH}$ ca. 7.0 Hz. In the aliphatic region, the unequivocal assignment of the signals for methylene protons was based on COSY (correlation spectroscopy) correlations. The methyl protons signal for compounds **15a-e** showed as a singlet at 2.69-2.80 ppm. Compounds **16a-e** showed signals characteristic for aromatic protons of the phenyl groups around 6.94-8.11 ppm. Typically, the methyne

carbon signal in β position to phosphorus appears as a doublet with $^2J_{PC}$ ca. 5.5 Hz around 70.3 ppm in ^{13}C NMR spectroscopy. In all cases phosphorus and carbon in the aliphatic region showed coupling with $^3J_{PC}$ ca. 4.7 Hz, but no coupling $^2J_{PC}$ was observed. The 1*H*-pyrazolo[3,4-*b*]pyridine phosphoramidates **14a-e**, **15a-e** and **16a-e** showed in their decoupled ^{31}P NMR spectra one signal in the region between 6.93-7.71 ppm, typical for phosphoramidates.^{16-18,30,31,34} Furthermore, infrared spectra exhibited strong absorptions for the P=O at 1249-1188 cm^{-1} , P-O around 1004-972 cm^{-1} , absorptions for the carbonyl group at 1683-1673 cm^{-1} for **14a-e** and absorptions for the nitrile group at 2217-2204 cm^{-1} for **15a-e** and **16a-e**. In the 3429-3193 cm^{-1} region, NH bands were observed.

Antileishmanial activity

For the antileishmanial assays, the 50% growth inhibitory activity value, IC_{50} , of each compound was determined using *L. amazonensis*, in promastigotes evolutive forms (MHOM / BR / 77LTB 0016). The IC_{50} values were determined by linear regression, relating the inhibition percentage and the drug concentration in μM , as shown in Table 1. The results indicate that among the three series **14**, **15** and **16** assayed, compounds containing phenyl substituent at position R_1 of the pyrazole ring (**16**) were found to be the most active against promastigote forms presenting IC_{50} in the range of 6-42 μM . These results observed in series **16** suggest an important role of the phenyl group (R_1) for leishmanicidal activity. The compound **16a**, with the best growth inhibitory activity ($IC_{50} = 6.44 \pm 1.49 \mu M$), possess $n = 2$ (shorter aliphatic chain) whereas **16e**, with $n = 6$ (longer aliphatic chain), presents $IC_{50} = 12.25 \pm 0.68 \mu M$. Interestingly, we observed that spacer length (n) in the three series does not appear to be the major factor that influences growth inhibition of promastigote. The cytotoxicity responses (CC_{50}) of derivatives **16** were assayed on the mice's peritoneal macrophages. The compounds **16a-e** present the SI (selectivity index) values similar to the reference drug pentamidine.

Concerning the cyano derivatives, series **15a-e** ($R_1 = \text{methyl}$), the most active compound was **15e** ($IC_{50} = 9.81 \pm 3.10 \mu M$) with $n = 6$, followed by **15d** ($IC_{50} = 18.89 \pm 2.68 \mu M$) with $n = 5$. Except for **15a** in this series, we can observe an increase of antileishmanial activity with the augment of alkyl chain length, in agreement with literature.³⁵ Meanwhile, the compounds with $R_2 = COOEt$ of series **14** ($R_1 = H$) exhibit lower IC_{50} compared to series **15** and **16** with a cyano group as R_2 . In minor extent, methyl group (R_1 in series **15**) may have also contributed to the antileishmanial activity.

Table 1. Experimental values of IC_{50} for 1*H*-pyrazolo[3,4-*b*]pyridine phosphoramidates **14a-e**, **15a-e** and **16a-e** assayed against *L. amazonensis* promastigotes

Compound	R_1	R_2	n	<i>Leishmania amazonensis</i> promastigotes $IC_{50} / \mu M$
14a	H	CO ₂ Et	2	45.5 $\pm$ 3.53
14b	H	CO ₂ Et	3	64 $\pm$ 1.00
14c	H	CO ₂ Et	4	28 $\pm$ 13.43
14d	H	CO ₂ Et	5	104 $\pm$ 4.24
14e	H	CO ₂ Et	6	224 $\pm$ 7.7
15a	CH ₃	CN	2	37.5 $\pm$ 13.4
15b	CH ₃	CN	3	169 $\pm$ 12.2
15c	CH ₃	CN	4	84.6 $\pm$ 15.1
15d	CH ₃	CN	5	18.89 $\pm$ 2.68
15e	CH ₃	CN	6	9.81 $\pm$ 3.10
16a	C ₆ H ₅	CN	2	6.44 $\pm$ 1.49
16b	C ₆ H ₅	CN	3	21.22 $\pm$ 3.28
16c	C ₆ H ₅	CN	4	23.85 $\pm$ 11.2
16d	C ₆ H ₅	CN	5	41.70 $\pm$ 0.83
16e	C ₆ H ₅	CN	6	12.25 $\pm$ 0.68
Pentamidine				13.00 $\pm$ 0.04

Based in the data regarding activities of the three series, it was chosen to realize the cytotoxicity assay only with the most active series, **16a-e** (Table 2). The cytotoxicity analyses (CC_{50}) of those derivatives were carried out on peritoneal macrophages from BALB/c mice. The selectivity index (SI), which compare the activity of the compounds on the parasite and on the host cell (macrophages), showed that those compounds **16a** and **16e** present the best results (Table 2), being similar to the values of the reference drug, pentamidine.

Conclusions

In summary, 15 new 1*H*-pyrazolo[3,4-*b*]pyridine phosphoramidate derivatives (**14a-e**, **15a-e** and **16a-e**) with different substituents were synthesized in order to evaluate their antileishmanial potential. We have proven that the molecular hybridization involving pyrazolopyridine and phosphoramidate moieties is an important strategy to prepare new bioactive molecules. The compounds

Table 2. Experimental values of cytotoxicity on murine peritoneal macrophages for 1*H*-pyrazolo[3,4-*b*]pyridine phosphoramidates **16a-e**

Compound	R ₁	R ₂	n	Cytotoxicity against murine cell CC ₅₀ / μM	SI (selectivity index) CC ₅₀ /IC ₅₀
16a	C ₆ H ₅	CN	2	56.47 ± 0.16	8.76
16b	C ₆ H ₅	CN	3	77.98 ± 0.17	3.67
16c	C ₆ H ₅	CN	4	128.62 ± 0.19	5.39
16d	C ₆ H ₅	CN	5	87.02 ± 0.17	2.08
16e	C ₆ H ₅	CN	6	100.04 ± 0.23	8.16
Pentamidine				112.94 ± 0.26	8.68

carrying a phenyl group at R₁ and a cyan group at R₂, **16a** and **16e**, presented suitable activity *in vitro* against *L. amazonensis* promastigotes, which makes 5-cyano-3-phenyl-1*H*-pyrazolo[3,4-*b*]pyridine phosphoramidate an interesting class to be explored as new leishmanicidal drugs.

Experimental

Chemistry

Analytical grade reagents and solvents were purchased from commercial sources and used without further purification. Uncorrected melting points were obtained with a Fisher-Johns apparatus. ¹H, ¹³C and ³¹P NMR spectra were recorded on a Varian UP-300 spectrometer at 299.95, 75.42 and 121.42 MHz, respectively, and Varian UP-500 spectrometer at 499.84, 125.69 and 202.34 MHz, respectively. Tetramethylsilane was used as an internal standard or 85% H₃PO₄ as external standard. The chemical shifts (δ) are reported in ppm and the coupling constants (*J*) in hertz. TLC (thin layer chromatography) was carried out using silica gel F-254 Glass Plate (20 × 20 cm). Infrared spectra were recorded on a PerkinElmer Spectrum One FT-IR spectrometer. High resolution mass spectrometry (HRMS) spectra were measured using a LC/MSD-TOF Agilent Technologies instrument. The remaining reagents and solvents that were used were of analytical grade. The Cl-substituted pyrazolo[3,4-*b*]pyridine (**10a-c**)^{2,33,36} and aminoalkylphosphoramidates (**13a-e**)³¹ compounds were prepared as previously reported.

Synthesis

General procedure for 1*H*-pyrazolo[3,4-*b*]pyridine phosphoramidates derivatives (**14a-e**, **15a-e** and **16a-e**)

Cl-Substituted pyrazolo[3,4-*b*]pyridine (**10a-c**) (2.2 mmol) and the aminoalkylphosphoramidate (**13a-e**) (4.4 mmol) were dissolved in THF (10 mL) and the reaction

mixture was heated at reflux until the disappearance of the starting (**10a-c**) (24 h, monitored by TLC). The mixture was poured into ice and the resulting solid was filtered off, washed with distilled water and dried. Recrystallization from ethanol/water (1:3) resulted in pure compounds.

Diisopropyl 2-(5-(ethoxycarbonyl)-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)ethyl phosphoramidate (**14a**)

Yield 83%; solid; mp 101-104 °C; IR (KBr) ν / cm⁻¹ 3161 (νN-H), 1270 (νP=O), 994 (νP-O); ¹H NMR (300 MHz, CDCl₃) δ 1.28 and 1.32 (2d, 12H, *J* 5.7 Hz), 1.37 (t, 3H, *J* 7.1 Hz), 2.85-2.93 (m, 1H), 3.30-3.34 (m, 2H), 3.79 (dt, 2H, *J* 5.7, 7.1 Hz), 3.85 (q, 2H, *J* 7.1 Hz), 4.57 (dhep, 2H, *J* 5.4, 6.3 Hz), 7.30 (t, 1H, *J* 7.4 Hz), 7.49 (t, 2H, *J* 7.9 Hz), 8.10 (d, 2H, *J* 7.5 Hz), 8.25 (s, 1H), 8.91 (s, 1H), 9.37 (t, 1H, *J* 4.9 Hz); ¹³C NMR (125 MHz, CDCl₃) δ 23.5 (d, *J* 4.7 Hz), 40.0, 46.5 (d, *J* 3.4 Hz), 71.1 (d, *J* 5.6 Hz), 100.7, 105.0, 121.3, 123.1, 128.1, 129.3, 135.6, 136.8, 153.1, 167.1; ³¹P{¹H} NMR (202 MHz, CDCl₃) δ 7.21; HRMS (ESI) *m/z*, calcd. for C₂₃H₃₂N₅O₅P: 490.2219 [M + H]⁺, found: 490.2204.

Diisopropyl 3-(5-(ethoxycarbonyl)-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)propyl phosphoramidate (**14b**)

Yield 77%; solid; mp 107-109 °C; IR (KBr) ν / cm⁻¹ 3197 (νN-H), 1265 (νP=O), 986 (νP-O); ¹H NMR (500 MHz, CDCl₃) δ 1.26 and 1.24 (2d, 12H, *J* 5.8 Hz), 1.94-1.97 (m, 2H), 1.33 (t, 3H, *J* 7.1 Hz), 2.65-2.70 (m, 1H), 3.06-3.09 (m, 2H), 3.70-3.74 (m, 2H), 4.26 (q, 2H, *J* 7.0 Hz), 4.51 (dhep, 2H, *J* 6.7, 7.7 Hz), 7.26 (t, 1H, *J* 7.3 Hz), 7.44 (t, 2H, *J* 7.8 Hz), 8.06 (d, 2H, *J* 7.9 Hz), 8.20 (s, 1H), 8.86 (s, 1H), 9.18 (t, 1H, *J* 4.8 Hz); ¹³C NMR (75 MHz, CDCl₃) δ 14.2, 23.8 (d, *J* 5.0 Hz), 31.16 (d, *J* 5.4 Hz), 38.8, 42.9, 60.8, 70.9 (d, *J* 5.6 Hz), 102.2, 104.9, 121.5, 122.5, 129.1, 135.6, 138.3, 152.0, 168.3; ³¹P{¹H} NMR (202 MHz, CDCl₃) δ 7.68 (s); HRMS (ESI) *m/z*, calcd. for C₂₄H₃₄N₅O₅P: 504.2376 [M + H]⁺, found: 504.2383.

Diisopropyl 4-(5-(ethoxycarbonyl)-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)butyl phosphoramidate (14c)

Yield 81%; pale brown solid; mp 97-100 °C; IR (KBr) ν / cm⁻¹ 3197 (νN-H), 1236 (νP=O), 975 (νP-O); ¹H NMR (300 MHz, CDCl₃) δ 1.30 and 1.33 (2d, 12H, *J* 6.1 Hz), 1.40 (t, 3H, *J* 7.1 Hz), 1.66-1.70 (m, 4H), 2.95 (dt, 2H, *J* 6.1, 9.7 Hz), 3.05 (dt, 1H, *J* 5.5, 7.0 Hz), 3.69 (dt, 2H, *J* 4.1, 6.9 Hz), 4.33 (q, 2H, *J* 7.1 Hz), 4.53 (dhep, 2H, *J* 6.0, 7.4 Hz), 7.33 (t, 1H, *J* 7.4 Hz), 7.51 (t, 2H, *J* 7.9 Hz), 8.12 (d, 2H, *J* 7.5 Hz), 8.21 (s, 1H), 8.93 (s, 1H), 9.26 (t, 1H, *J* 4.8 Hz); ¹³C NMR (75 MHz, CDCl₃) δ 14.3, 23.7 (d, *J* 5.2 Hz), 26.2, 29.1 (d, *J* 5.8 Hz), 40.9, 45.7, 61.5, 70.7 (d, *J* 5.5 Hz), 100.7, 105.3, 123.3, 128.3, 129.6, 136.0, 137.3, 149.2, 153.1, 167.6; ³¹P{H} NMR (121 MHz, CDCl₃) δ 7.50 (s); HRMS (ESI) *m/z*, calcd. for C₂₅H₃₆N₅O₅P: 518.2532 [M + H]⁺, found: 518.2506.

Diisopropyl 5-(5-(ethoxycarbonyl)-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)pentyl phosphoramidate (14d)

Yield 76%; pale brown solid; mp 116-119 °C; IR (KBr) ν / cm⁻¹ 3240 (νN-H), 1234 (νP=O), 987 (νP-O); ¹H NMR (500 MHz, CDCl₃) δ 1.30 and 1.27 (2d, 12H, *J* 6.0 Hz), 1.37 (3H, t, *J* 7.1 Hz), 1.54-1.58 (m, 4H), 1.80-1.86 (m, 2H), 2.47-2.52 (m, 1H), 2.90-2.95 (m, 2H), 3.68 (2H, dt, *J* 5.2, 6.6 Hz), 4.30 (q, 2H, *J* 7.1 Hz), 4.52 (dhep, 2H, *J* 6.6, 7.5 Hz), 7.30 (t, 1H, *J* 7.7 Hz), 7.48 (t, 2H, *J* 7.8 Hz), 8.10 (d, 2H, *J* 7.7 Hz), 8.18 (s, 1H), 8.90 (s, 1H), 9.23 (t, 1H, *J* 4.1 Hz); ¹³C NMR (75 MHz, CDCl₃) δ 14.1, 23.7 (d, *J* 5.0 Hz), 28.7, 31.1 (d, *J* 6.3 Hz), 41.0, 45.2, 60.6, 70.4 (d, *J* 5.6 Hz), 100.0, 104.7, 126.8, 122.2, 128.9, 135.2, 138.3, 152.2, 168.3; ³¹P{H} NMR (202 MHz, CDCl₃) δ 7.68 (s); HRMS (ESI) *m/z*, calcd. for C₂₆H₃₈N₅O₅P: 532.2689 [M + H]⁺, found: 532.2668.

Diisopropyl 6-(5-(ethoxycarbonyl)-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)hexyl phosphoramidate (14e)

Yield 72%; pale brown solid; mp 112-114 °C; IR (KBr) ν / cm⁻¹ 3294 (νN-H), 1265 (νP=O), 995 (νP-O); ¹H NMR (500 MHz, CDCl₃) δ 1.21 and 1.23 (2d, 12H, *J* 5.7 Hz), 1.30 (3H, t, *J* 7.1 Hz), 1.45-1.50 (m, 4H), 2.37-2.42 (m, 1H), 2.80-2.85 (m, 2H), 3.59-3.63 (m, 2H), 4.28 (q, 2H, *J* 7.1 Hz), 4.45 (dhep, 2H, *J* 6.1, 7.5 Hz), 7.24 (t, 1H, *J* 7.4 Hz), 7.42 (t, 2H, *J* 7.8 Hz), 8.05 (d, 2H, *J* 7.9 Hz), 8.12 (s, 1H), 8.84 (s, 1H), 9.15 (t, 1H, *J* 5.2 Hz); ¹³C NMR (75 MHz, CDCl₃) δ 14.2, 23.7 (d, *J* 4.4 Hz), 26.2 (d, *J* 6.5 Hz), 29.0, 31.4, 41.2, 60.5, 70.5 (d, *J* 5.5 Hz), 100.0, 104.7, 122.3, 126.6, 129.0, 135.2, 152.2; ³¹P{H} NMR (202 MHz, CDCl₃) δ 7.71 (s); HRMS

(ESI) *m/z*, calcd. for C₂₇H₄₀N₅O₅P: 546.2845 [M + H]⁺, found: 546.2832.

Diisopropyl 2-(5-cyano-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)ethyl phosphoramidate (15a)

Yield 82%; solid; mp 159-162 °C; IR (KBr) ν / cm⁻¹ 3405 (νN-H), 1249 (νP=O), 991 (νP-O), 2217 (νC≡N); ¹H NMR (300 MHz, CDCl₃) δ 1.26 and 1.24 (2d, 12H, *J* 5.3 Hz), 2.69-2.73 (m, 1H), 2.80 (s, 3H), 3.34 (dt, 2H, *J* 6.5, 11.3 Hz), 3.99 (dt, 2H, *J* 5.7, 11.1 Hz), 4.50 (dhep, 2H, *J* 6.2, 7.6 Hz), 6.23 (t, 1H, *J* 5.3 Hz), 7.22-7.25 (m, 3H), 8.02 (d, 2H, *J* 8.7 Hz), 8.65 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 14.5, 23.6 (d, *J* 4.9 Hz), 25.9 (d, *J* 4.2 Hz), 41.1, 70.5 (d, *J* 5.8 Hz), 82.8, 103.6, 119.5, 121.6, 127.0, 129.1, 138.5, 143.6, 151.4; ³¹P{H} NMR (202 MHz, CDCl₃) δ 7.46 (s); HRMS (ESI) *m/z*, calcd. for C₂₂H₂₉N₆O₃P: 457.2117 [M + H]⁺, found: 457.2108.

Diisopropyl 3-(5-cyano-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)propyl phosphoramidate (15b)

Yield 75%; pale brown solid; mp 160-163 °C; IR (KBr) ν / cm⁻¹ 3338 (νN-H), 1232 (νP=O), 989 (νP-O), 2208 (νC≡N); ¹H NMR (300 MHz, CDCl₃) δ 1.28 and 1.25 (2d, 12H, *J* 5.1 Hz), 1.83-1.86 (m, 2H), 2.58-2.61 (m, 1H), 2.76 (s, 3H), 2.90-2.95 (m, 2H), 3.96 (dt, 2H, *J* 5.8, 6.4 Hz), 4.49 (dhep, 2H, *J* 6.1, 7.6 Hz), 6.59 (t, 1H, *J* 6.6 Hz), 7.22-7.25 (m, 1H), 7.40 (t, 2H, *J* 6.2 Hz), 7.97 (d, 2H, *J* 8.7 Hz), 8.26 (s, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 15.2, 23.1 (d, *J* 4.1 Hz), 32.1 (d, *J* 5.9 Hz), 37.2, 39.7, 70.3 (d, *J* 6.0 Hz), 81.8, 104.1, 119.8, 125.9, 128.6, 138.3, 141.9, 150.9, 155.5; ³¹P{H} NMR (202 MHz, CDCl₃) δ 7.41 (s); HRMS (ESI) *m/z*, calcd. for C₂₃H₃₁N₆O₃P: 471.2274 [M + H]⁺, found: 471.2271.

Diisopropyl 4-(5-cyano-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)butyl phosphoramidate (15c)

Yield 81%; pale brown solid; mp 162-165 °C; IR (KBr) ν / cm⁻¹ 3245 (νN-H), 1230 (νP=O), 985 (νP-O), 2208 (νC≡N); ¹H NMR (300 MHz, CDCl₃) δ 1.28 and 1.25 (2d, 12H, *J* 6.1 Hz), 1.56-1.59 (m, 4H), 2.53 (dt, 1H, *J* 7.4, 8.3 Hz), 2.69 (s, 3H), 2.89-2.92 (m, 2H), 3.81 (dt, 2H, *J* 6.1, 7.3 Hz), 4.47 (dhep, 2H, *J* 6.1, 7.6 Hz), 5.48 (t, 1H, *J* 5.8 Hz), 7.26 (t, 1H, *J* 7.4 Hz), 7.44 (t, 2H, *J* 7.6 Hz), 7.99 (d, 2H, *J* 8.7 Hz), 8.30 (s, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 15.6, 23.7 (d, *J* 4.6 Hz), 27.4, 28.4 (d, *J* 6.0 Hz), 40.7, 43.9, 70.7 (d, *J* 5.5 Hz), 82.8, 104.1, 119.6, 126.4, 121.7, 128.9, 138.5, 141.0, 151.3, 155.6; ³¹P{H} NMR (202 MHz, CDCl₃) δ 7.13 (s); HRMS (ESI) *m/z*, calcd. for C₂₄H₃₃N₆O₃P: 485.2430 [M + H]⁺, found: 485.2420.

Diisopropyl 5-(5-cyano-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)pentyl phosphoramidate (**15d**)

Yield 73%; pale brown solid; mp 163-165 °C; IR (KBr) ν / cm⁻¹ 3334 (νN–H), 1232 (νP=O), 987 (νP–O), 2208 (νC≡N); ¹H NMR (500 MHz, CDCl₃) δ 1.28 and 1.27 (2d, 12H, *J* 6.1 Hz), 1.40-1.45 (m, 4H), 1.49-1.54 (m, 2H), 2.36 (dt, 1H, *J* 7.3, 10.8 Hz), 2.70 (s, 3H), 2.88-2.92 (m, 2H), 3.82 (dt, 2H, *J* 6.1, 7.1 Hz), 4.55 (dhep, 2H, *J* 6.2, 7.6 Hz), 5.40 (t, 1H, *J* 5.8 Hz), 7.29 (t, 1H, *J* 7.4 Hz), 7.45 (t, 2H, *J* 8.4 Hz), 8.00 (d, 2H, *J* 7.5 Hz), 8.31 (s, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 15.3, 23.3 (d, *J* 4.5 Hz), 23.0, 29.5, 30.8 (d, *J* 6.2 Hz), 40.7, 43.9, 70.2 (d, *J* 5.8 Hz), 82.5, 103.8, 119.3, 121.4, 126.1, 128.6, 138.2, 140.7, 151.0, 155.3; ³¹P{H} NMR (202 MHz, CDCl₃) δ 7.66 (s); HRMS (ESI) *m/z*, calcd. for C₂₅H₃₅N₆O₃P: 499.2587 [M + H]⁺, found: 499.2573.

Diisopropyl 6-(5-cyano-3-methyl-1-phenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)hexyl phosphoramidate (**15e**)

Yield 72%; pale brown solid; mp 165-168 °C; IR (KBr) ν / cm⁻¹ 3403 (νN–H), 1230 (νP=O), 1004 (νP–O), 2204 (νC≡N); ¹H NMR (500 MHz, CDCl₃) δ 1.30 and 1.32 (2d, 12H, *J* 5.9 Hz), 1.47-1.51 (m, 6H), 1.78-1.81 (m, 2H), 2.46 (dt, 1H, *J* 7.2, 9.0 Hz), 2.73 (s, 3H), 2.91 (dt, 2H, *J* 6.9, 9.2 Hz), 3.82-3.86 (m, 2H), 4.54 (dhep, 2H, *J* 6.2, 7.5 Hz), 5.44 (t, 1H, *J* 5.5 Hz), 7.31 (t, 1H, *J* 7.4 Hz), 7.49 (t, 2H, *J* 8.5 Hz), 8.06 (d, 2H, *J* 8.6 Hz), 8.34 (s, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 15.6, 23.6 (d, *J* 4.5 Hz), 25.9, 26.0, 30.1, 31.3 (d, *J* 6.5 Hz), 41.1, 44.1, 70.4 (d, *J* 5.2 Hz), 82.8, 104.1, 119.5, 121.6, 126.3, 128.9, 138.5, 140.9, 151.3, 155.5; ³¹P{H} NMR (202 MHz, CDCl₃) δ 7.68 (s); HRMS (ESI) *m/z*, calcd. for C₂₆H₃₇N₆O₃P: 513.2743 [M + H]⁺, found: 513.2723.

Diisopropyl 2-(5-cyano-1,3-diphenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)ethyl phosphoramidate (**16a**)

Yield 69%; pale brown solid; mp 108-110 °C; IR (KBr) ν / cm⁻¹ 3411 (νN–H), 1012 (νP=O), 988 (νP–O), 2208 (νC≡N); ¹H NMR (300 MHz, CDCl₃) δ 1.19 and 1.21 (2d, 12H, *J* 6.1 Hz), 2.50 (dt, 1H, *J* 6.9, 8.7 Hz), 3.12 (dt, 2H, *J* 6.4, 12.8 Hz), 3.81 (dt, 2H, *J* 6.2, 8.2 Hz), 4.48 (dhep, 2H, *J* 6.2, 7.5 Hz), 5.75 (t, 1H, *J* 6.2 Hz), 7.33 (t, 1H, *J* 7.7 Hz), 7.50-7.59 (m, 5H), 7.66-7.68 (m, 2H), 8.10 (d, 2H, *J* 7.5 Hz), 8.40 (s, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 23.4 (d, *J* 4.8 Hz), 40.9, 44.4 (d, *J* 4.4 Hz), 70.91 (d, *J* 5.7 Hz), 104.6, 114.6, 119.5, 122.0, 128.0, 129.0, 130.0, 130.5, 138.3, 142.5, 150.7, 151.2; ³¹P{H} NMR (202 MHz, CDCl₃) δ 6.93 (s); HRMS (ESI) *m/z*, calcd. for C₂₇H₃₁N₆O₃P: 519.2274 [M + H]⁺, found: 519.2278.

Diisopropyl 3-(5-cyano-1,3-diphenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)propyl phosphoramidate (**16b**)

Yield 68%; pale brown solid; mp 112-115 °C; IR (KBr) ν / cm⁻¹ 3392 (νN–H), 1230 (νP=O), 985 (νP–O), 2210 (νC≡N); ¹H NMR (500 MHz, CDCl₃) δ 1.28 and 1.29 (2d, 12H, *J* 5.8 Hz), 1.60-1.63 (m, 2H), 2.71-2.74 (m, 1H), 2.93-2.96 (m, 2H), 3.74-3.77 (m, 2H), 4.51 (dhep, 2H, *J* 6.0, 7.5 Hz), 5.48 (t, 1H, *J* 4.8 Hz), 7.31-7.35 (m, 1H), 7.50-7.65 (m, 5H), 8.11 (m, 2H), 8.40 (s, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 23.7 (d, *J* 4.1 Hz), 26.8, 28.3 (d, *J* 6.2 Hz), 40.7, 43.7, 70.6 (d, *J* 5.4 Hz), 82.8, 102.9, 119.5, 122.1, 128.9, 129.2, 129.7, 138.5, 145.1, 150.8, 155.7; ³¹P{H} NMR (202 MHz, CDCl₃) δ 7.43 (s); HRMS (ESI) *m/z*, calcd. for C₂₈H₃₃N₆O₃P: 533.2430 [M + H]⁺, found: 533.2429.

Diisopropyl 4-(5-cyano-1,3-diphenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)butyl phosphoramidate (**16c**)

Yield 70%; pale brown solid; mp 85-89 °C; IR (KBr) ν / cm⁻¹ 3417 (νN–H), 1226 (νP=O), 981 (νP–O), 2210 (νC≡N); ¹H NMR (300 MHz, CDCl₃) δ 1.22 and 1.26 (2d, 12H, *J* 5.8 Hz), 1.33-1.37 (m, 2H), 1.47-1.56 (m, 2H), 2.32 (dt, 1H, *J* 7.2, 9.0 Hz), 2.75-2.80 (m, 2H), 3.63 (dt, 1H, *J* 5.7, 7.0 Hz), 4.46 (dhep, 2H, *J* 6.1, 7.5 Hz), 5.42 (t, 1H, *J* 5.5 Hz), 7.29 (d, 1H, *J* 6.9 Hz), 7.44-7.62 (m, 7H), 8.07 (d, 2H, *J* 7.5 Hz), 8.36 (s, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 23.7 (d, *J* 4.1 Hz), 26.8, 28.3 (d, *J* 6.2 Hz), 40.7, 43.7, 70.6 (d, *J* 5.4 Hz), 82.8, 102.9, 119.5, 122.1, 128.9, 129.2, 129.7, 138.5, 145.1, 150.8, 155.7; ³¹P{H} NMR (202 MHz, CDCl₃) δ 7.43 (s); HRMS (ESI) *m/z*, calcd. for C₂₉H₃₅N₆O₃P: 547.2587 [M + H]⁺, found: 547.2590.

Diisopropyl 5-(5-cyano-1,3-diphenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)pentyl phosphoramidate (**16d**)

Yield 69%; pale brown solid; mp 90-93 °C; IR (KBr) ν / cm⁻¹ 3400 (νN–H), 1232 (νP=O), 985 (νP–O), 2212 (νC≡N); ¹H NMR (300 MHz, CDCl₃) δ 1.27 and 1.29 (2d, 12H, *J* 4.5 Hz), 1.10-1.17 (m, 4H), 1.43-1.48 (m, 2H), 2.20-2.25 (m, 1H), 2.77 (dt, 2H, *J* 7.1, 9.6 Hz), 3.66 (dt, 2H, *J* 5.7, 6.8 Hz), 4.48 (dhep, 2H, *J* 6.1, 7.4 Hz), 5.43 (t, 1H, *J* 5.5 Hz), 6.94 (s, 1H), 7.45-7.56 (m, 6H), 8.09 (m, 2H), 8.37 (s, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 23.6 (d, *J* 4.6 Hz), 27.4, 28.4 (d, *J* 5.8 Hz), 40.7, 41.0, 43.9, 70.7 (d, *J* 5.4 Hz), 82.8, 104.1, 119.6, 121.7, 126.4, 128.9, 138.5, 141.0, 151.3, 155.6; ³¹P{H} NMR (121 MHz, CDCl₃) δ 7.18 (s); HRMS (ESI) *m/z*, calcd. for C₃₀H₃₇N₆O₃P: 561.2743 [M + H]⁺, found: 561.2745.

Diisopropyl 6-(5-cyano-1,3-diphenyl-1*H*-pyrazolo[3,4-*b*]pyridin-4-ylamino)hexyl phosphoramidate (**16e**)

Yield 67%; pale brown solid; mp 83-85 °C; IR (KBr) ν / cm⁻¹ 3404 (νN–H), 1224 (νP=O), 979 (νP–O), 2214

($\nu_{\text{C}\equiv\text{N}}$); ^1H NMR (300 MHz, CDCl_3) δ 1.12 (m, 4H), 1.27 and 1.29 (2d, 12H, J 6.1 Hz), 1.30-1.37 (m, 4H), 2.36 (dt, 1H, J 6.8, 9.0 Hz), 2.77-2.82 (m, 2H), 3.65 (dt, 2H, J 5.5, 6.9 Hz), 4.55 (dhep, 2H, J 5.5, 6.9 Hz), 5.42 (t, 1H, J 5.3 Hz), 7.32 (m, 1H), 7.56-7.62 (m, 5H), 7.63-7.67 (m, 2H), 8.08-8.11 (m, 2H), 8.38 (s, 1H); ^{13}C NMR (125 MHz, CDCl_3) δ 23.6 (d, J 4.4 Hz), 25.8, 26.0, 29.4, 31.3 (d, J 5.9 Hz), 41.1, 43.9, 70.4 (d, J 5.2 Hz), 82.7, 102.9, 119.5, 122.0, 126.7, 128.9, 129.1, 138.5, 145.0, 150.8, 155.7; $^{31}\text{P}\{\text{H}\}$ NMR (121 MHz, CDCl_3) δ 7.65 (s); HRMS (ESI) m/z , calcd. for $\text{C}_{31}\text{H}_{39}\text{N}_6\text{O}_3\text{P}$: 575.2900 $[\text{M} + \text{H}]^+$, found: 575.2902.

Biological evaluation

Antipromastigote activity

L. amazonensis promastigotes in late log-phase in Schneider's medium supplemented with 10% fetal calf serum (FCS), 2 mM *L*-glutamine and antibiotics (100 U mL^{-1} penicillin and 100 $\mu\text{g mL}^{-1}$ streptomycin), were incubated at an average density of 10^7 parasites mL^{-1} in sterile 96-well plates. Compounds to be assayed were added to the culture in a concentration range of 160-1.25 $\mu\text{g mL}^{-1}$ dissolved in DMSO (final concentration less than 0.8% v/v). The assay was carried out in triplicate. Appropriate controls containing DMSO and pentamidine (reference drug) were added to each set of experiments. Parasite viability was assessed by a dye-reduction assay employing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and the absorbance measured in a spectrophotometer at 570 nm.³⁷ Growth inhibitory concentration 50% (IC_{50}) was defined as the concentration of drug required to inhibit by 50% the metabolic activity of *Leishmania* promastigotes compared to the control. $\text{IC}_{50}/24\text{ h}$ were calculated by non-linear regression analysis processed on dose-response curves, using GraphPad Prism 5.0. IC_{50} values represent the mean value calculated from three independent experiments.

Cytotoxic assays on murine peritoneal macrophages

Cytotoxicity was assessed on murine peritoneal macrophages of BALB/c mice by a colorimetric assay based on the mitochondrial reduction in MTT as described previously.³⁷ The cells were isolated from peritoneal cavity of mice with cold RPMI 1640 medium, supplemented with 10% FCS. The 2×10^6 cells well^{-1} were added to microplate and incubated at 37 °C in a humidified 5% CO_2 atmosphere. After 2 h of incubation, no adherent cells were removed and the adhered macrophages were washed with RPMI. Compounds were solubilized in DMSO at concentrations ranging from 320 to 2.5 $\mu\text{g mL}^{-1}$ and added to the cell culture for 72 h of incubation at 37 °C and 5% CO_2 . After

that, culture supernatant was removed. The macrophage viability was measured by adding MTT (0.5 mg mL^{-1} in PBS, 200 $\mu\text{L well}^{-1}$), incubating plates for 2 h at 37 °C, and the colored product formazan was solubilized with DMSO. The results were read in spectrophotometer with wavelength of 570 nm.³⁷

Supplementary Information

Supplementary information (^1H , ^{13}C , ^{31}P NMR and HRMS spectra) is available free of charge at <http://jbcs.sbq.org.br> as PDF file.

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