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Note

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Isolation of Arginase Inhibitors from the Bioactivity-Guided Fractionation of *Byrsonima coccolobifolia* Leaves and Stems

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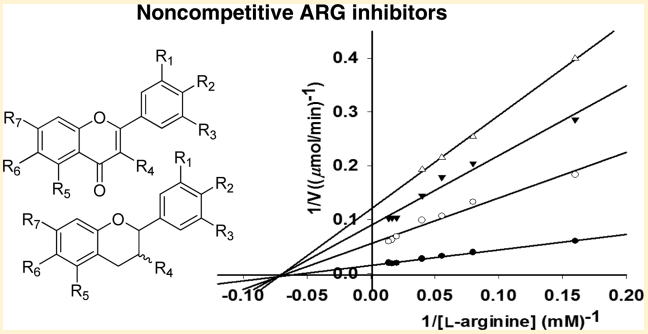
Supporting Information

ABSTRACT: *Byrsonima coccolobifolia* leaf and stem extracts were studied in the search for possible leishmanicidal compounds using arginase (ARG) from *Leishmania amazonensis* as a molecular target. Flavonoids **1b**, **1e**−**1g**, **2a**, **2b**, and **2d**−**2f** showed significant inhibitory activity, with IC_{50} values ranging from 0.9 to 4.8 μM. The kinetics of the most active compounds were determined. Flavonoids **1e**, **1f**, **2a**, **2b**, and **2e** were characterized as noncompetitive inhibitors of ARG with dissociation constants (K_i) ranging from 0.24 to 3.8 μM, demonstrating strong affinity. Structure−activity relationship studies revealed some similarities in the structural features of flavonoids related to ARG activity.



Affecting millions of people per year, leishmaniasis is one of the most neglected tropical diseases and an urgent problem in global public health according to the World Health Organization. To overcome obstacles such as adverse reactions and parasite resistance to available drugs against leishmaniasis, a number of plants have been tested in the search for new front-line drugs.[1,2]

Brazilian cerrado plants, the biodiversity of which is still largely unknown, represent a rich source of new lead compounds.[3] Species such as *Byrsonima coccolobifolia* Kunth. (Malpighiaceae) and *Byrsonima crassa* have been used in folk medicine for the treatment of stomach disorders and gastric ulcers.[4,5] Chemical study of extracts from *B. crassa* with an antiulcerogenic effect has led to the isolation of flavonoids and biflavonoids.[5] Furthermore, crude extracts from the leaves and stems of *Byrsonima crassifolia* and *Byrsonima bucidaefolia* were shown to exhibit good inhibition of the growth of promastigotes of *Leishmania mexicana*. Flavonoids were the principal phytochemicals isolated from *Byrsonima*,[4] which indicates that this genus could contribute to the search for new leishmanicidal compounds because flavonoids have demonstrated antileishmanial activity.[6,7] Recently, some of these phenols were found to be inhibitors of arginase (ARG) from *Leishmania amazonensis* and arginase (ARG-II) from mammals,[8−10] which has motivated the further study of this class of plant secondary metabolites.

Arginase is a metalloenzyme from *L. amazonensis*[11,12] that catalyzes the hydrolysis of L-arginine to L-ornithine and urea, the first reaction in the metabolic pathway of polyamines (PAs), which are essential molecules in most living organisms, including *Leishmania* parasites.[13] Additionally, ARG is associated with the production of nitric oxide (NO) molecules, high concentrations of which could kill the parasites.[14] The relationships of ARG with such molecules (PAs and NO) with distinct properties are explained by the usage of the same substrate, L-arginine, for the enzymatic activities in two biosynthetic pathways.[14]

Studies of mutant *L. mexicana* and *Leishmania major* parasites lacking ARG confirmed that the ARG pathway is essential for parasite viability and infectivity.[13,15,16] Importantly, the X-ray crystal structure of arginase from *L. mexicana* was solved in recent work, which differs only in two amino acids from *L. amazonensis*, making them 99.4% identical.[13,17] The knowledge of leishmanial ARG structure has revealed important features for inhibitor interactions, thus making ARG an attractive target for leishmanicidal drug design.[17]

In a search for new leishmanicidal compounds, extracts from several Brazilian cerrado plants were evaluated against ARG. The crude extracts from the leaves and stems of *B. coccolobifolia* showed the most promising results, with inhibition higher than 60.0% against ARG at a concentration of 31.25 μg/mL. Ethyl acetate extracts from the leaves and stems of *B. coccolobifolia* showed 64% and 65% inhibition of ARG, respectively.

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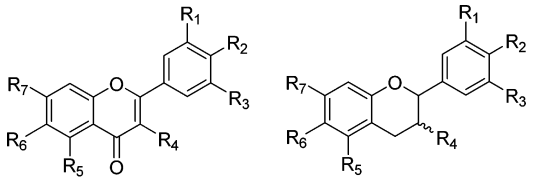
Table 1. Structures and IC_{50} Values of Several Flavonoids as Inhibitors of Arginase



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compound	R_1	R_2	R_3	R_4	R_5	R_6	R_7	IC_{50} (μM)[b]
1a[a]	OH	OH	H	*O*-rha α	OH	H	OH	12.2 ± 1.8
1b	OH	OH	H	glc β	OH	H	OH	2.0 ± 0.1
1c	OH	OCH_3	H	*O*-rha α	OH	H	OH	88.1 ± 9.1
1d	OH	OCH_3	OH	*O*-rha α	OH	H	OH	223.5 ± 15.9
1e	OH	OH	OH	*O*-rha α	OH	H	OH	2.4 ± 0.2
1f	OH	OH	OH	OH	OH	H	OH	2.1 ± 0.2
1g	OH	OH	H	OH	OH	H	OH	4.0 ± 0.5
1h	H	OH	H	OH	OH	H	OH	55.0 ± 4.0
1i	OAc	OAc	H	OAc	OAc	H	OAc	120.8 ± 12.1
1j	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	127.9 ± 9.8
2a	OH	OH	H	OH α	OH	H	OH	1.7 ± 0.1
2b	OH	OH	H	OH β	OH	H	OH	1.6 ± 0.2
2c	H	OH	H	*O*-rha α	OH	H	OH	35.1 ± 3.2
2d	OH	OH	H	OAc α	OH	H	OH	3.7 ± 0.3
2e	OH	OH	H	OAc α	OAc	H	OAc	0.9 ± 0.1
2f	OAc	OAc	H	OAc α	OAc	H	OAc	4.8 ± 0.5

[a]Quercitrin was used as a positive control. [b]The values represent means of individual experiments ± SD.

Phytochemical study of these two EtOAc extracts led to the active known compounds, including flavonoids (**1a**, **1b**, **2a**, **2b**),[8,9,18−20] (+)-syringaresinol (**3**),[21] and trigonostemone (**4**).[22] Compound **1a** was previously isolated from a methanolic extract from the leaves of *B. coccolobifolia*,[23] and compounds **1b**, **2a**, and **2b** have been reported from other species in the genus *Byrsonima*.[4] Furthermore, this is the first report of compounds **3** and **4** in *Byrsonima*.

Isoquercitrin (**1b**), (+)-catechin (**2a**), and (−)-epicatechin (**2b**) showed potent inhibitory activities against ARG, with IC_{50} values ranging from 1.6 to 2.0 μM (Table 1). Quercitrin (**1a**), (+)-syringaresinol (**3**), and trigonostemone (**4**) exhibited moderate inhibitory activity, with IC_{50} values of 12.2 ± 1.8, 13.7 ± 1.5, and 16.6 ± 1.6 μM, respectively. The IC_{50} values of the compounds quercitrin (**1a**), isoquercitrin (**1b**), quercetin (**1g**), and kaempferol (**1h**) were reported previously (10, 3.8, 4.3, and ∼50 μM, respectively).[9,24] Flavonoids **1a** and **1b** were characterized as noncompetitive inhibitors (K_i = 7.20 and 6.90 μM, respectively), and **1g** was characterized as a mixed inhibitor.[24]

In an effort to establish a relationship of the chemical structures of flavonoids with potent ARG inhibitory activity and also to describe the type of inhibition for the potent inhibitors, other flavonoids were also investigated (**1c−j** and **2c−f**). Catechin and quercetin were acetylated (**1i**, **2d−f**) to analyze the effect of substituting hydroxy groups for acetyl groups in the flavonol and flavan-3-ol structural classes on ARG activity. Although this class of natural products has been characterized recently as inhibitors of recombinant ARG from *L. amazonensis*, there are still only a few studies that have explored these compounds for their inhibitory effects.[8,9,24] The present investigation, which searched for new ARG inhibitors, differs from the results of previous reports in that these active flavonoids were identified through a bioactivity-guided approach.

The comparison of quercitrin (**1a**) (IC_{50} = 12.2 ± 1.8 μM) with compound **1e** (IC_{50} = 2.4 ± 0.2 μM) demonstrates an improvement in inhibitory activity when the aromatic ring B of the flavonoid has one extra hydroxy group. The observed increase in the IC_{50} values of flavonoids with the same skeleton as quercitrin (**1a**) (12.2 ± 1.8 μM) and compound **1c** (88.1 ± 9.0 μM) can be related to the presence of a methoxy group instead of a hydroxy group as in the catechol of **1a**. The same trend is seen for the glycoflavonols **1e** (IC_{50} = 2.4 ± 0.2 μM) and **1d** (IC_{50} = 223.5 ± 15.8 μM), which have three substituents in aromatic ring B, in which the replacement of a hydroxy group with a methoxy group decreased the inhibitory activity.

The flavonol aglycones **1f** and quercetin (**1g**) both showed a substantial inhibition of ARG, with IC_{50} values of approximately 2.1 and 4.0 μM, respectively. However, compounds **1h**, which contains only one hydroxy group in aromatic ring B, **1i** (acetylated flavonol), and **1j** (methoxylated flavonol) had less potent effects. These compounds displayed lower activities (IC_{50} = 55.0, 120.8, and 127.9 μM, respectively), and again this observation can be correlated directly with the influence of the functional groups in the B ring. Previous in vitro studies on the leishmania parasite showed that leishmanicidal potential was lowered or completely lost with an increase in the number of methoxy groups in the flavonol structure.[7] Additionally, a recent study showed that galangin, a flavonoid without any substituents in the B ring, exhibited only low inhibition against ARG ($IC_{50} \approx 100$ μM).[24]

IC_{50} values in the range 0.9 to 4.8 μM (Table 1) suggest that flavan-3-ols also have important structural features that allow these substances to bind to the enzyme. The presence of hydroxy groups as substituents on the aromatic ring A and at position 3 of the pyran ring of the flavonoid skeleton has been demonstrated also to be important in relation to ARG inhibition.[24] However, the selective acetylation of (+)-catechin

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(**2a**) at different positions did not show much influence on the enzyme inhibition activities (**2d**–**f**) (Table 1).

In silico studies were performed previously for some flavonoids, demonstrating interactions with ARG enzyme occurring between the ring B moiety and amino acids that are involved with metal bridge Mn_A^{2+}–Mn_B^{2+} coordination in the active site.[9,24] By the present experimental approach the results supported such findings, showing that a catechol group is highly important in reducing ARG activity.

Figure 1 shows Lineweaver–Burk plots for some of the most potent compounds analyzed, providing valuable information

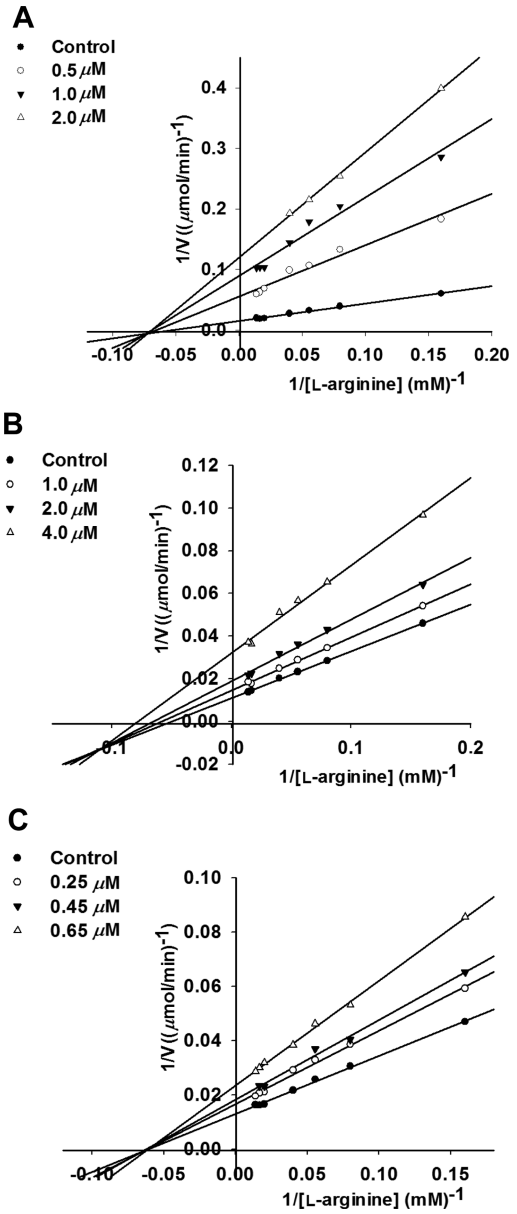

Figure 1. Lineweaver–Burk plots indicate noncompetitive ARG inhibition by (−)-epicatechin (**2b**) (A), myricetin 3-*O*-*α*-L-rhamnoside (**1e**) (B), and 3,5,7-triacetylcatechin (**2e**) (C).

about the mechanism of inhibition. In this investigation, the kinetics of recombinant ARG were measured at pH = 9.6 and afforded a K_M value of 22.6 ± 1.7 mM (R^2 = 0.996), consistent with the data in the literature.[12] The double-reciprocal plots for the evaluated compounds (**1e**, **1f**, **2a**, **2b**, **2e**) showed a decrease in V_{max} values without affecting the affinity (K_M). These results reveal that flavonoids interact with ARG in a noncompetitive manner. Kinetic analysis indicates that these flavonoid interactions can occur in the free enzyme and also in the enzyme–substrate complex at a site that is distinct from the catalytic site, thus inducing changes in the shape of the active site such that the substrate will no longer fit well.

The dissociation constant (K_i) values were obtained using Dixon reciprocal plots of the initial velocities ($1/v$) versus a varying concentration of inhibitor at a constant concentration of L-arginine (Table 2).

Table 2. K_i Values of Some Flavonoids

	compound	K_i (μM)
1a	quercitrin[a]	7.9
1f	myricetin	1.2
2a	(+)-catechin	0.6
2b	(−)-epicatechin	0.2
2e	3,5,7-triacetylcatechin	0.9

[a]Quercitrin was used as a positive control.

Quercitrin (**1a**) was characterized previously as a noncompetitive inhibitor.[24] The double-reciprocal plot of glycoflavonol **1e** showed a noncompetitive inhibitory mechanism. It is possible to deduce that inhibitor **1e** preferentially binds to the ES complex (K_{is} = 1.9 μM); in addition, **1e** also interacts with the free enzyme (K_i = 3.8 μM), as demonstrated by the intersecting lines that converge to the left of the *y*-axis and below the *x*-axis ($\alpha < 1$) in the Lineweaver–Burk plot.

Overall, bioactive flavonoids with potent ARG inhibitory activity were identified by screening the active ethyl acetate extracts of *B. coccolobifolia* leaves and stems, thereby indicating these compounds are responsible for the significant decrease in ARG activity. The type of inhibition for the most active flavonoids (**1e**, **1f**, **2a**, **2b**, **2e**) was determined by kinetic studies showing strong affinity and potency on ARG. Compounds with a flavan-3-ol unit represent a new subclass of noncompetitive flavonoid ARG inhibitor.

Flavonoids **1a**, **2a**, **2b**, **1f**, and **1g** were described previously as inhibitors of the growth of amastigotes of *L. donovani*.[7,25,26] Additionally, quercitrin (**1a**) and quercetin (**1g**) significantly reduced the growth of *L. amazonensis* in an in vivo murine model of cutaneous leishmaniasis.[27] The mechanism of action of quercetin in *L. amazonensis*-infected macrophages was recently related to the increase in reactive oxygen species.[28] Furthermore, the leishmanicidal activities of these flavonoids may also be due to arginase inhibition, confirming these compounds as new lead candidates in the search for leishmanicidal drugs.

EXPERIMENTAL SECTION

General Experimental Procedures. The urea concentration was detected at 600 nm on a Beckman Coulter DU 800 spectrophotometer. The 1D- and 2D-NMR determinations were carried out in acetone-d_6 and MeOH-d_4, using TMS as the internal reference, on a Bruker DRX-400 NMR spectrometer (^{1}H: 400 MHz; ^{13}C: 100 MHz). MS spectra were recorded on a Bruker Daltonics micrOTOF-Q II-ESI-TOF mass spectrometer. Isolation procedures were carried out on silica gel 60 (Merck, 230–700 mesh) and Sephadex LH-20 (Amersham Pharmacia Biotech AB). Thin-layer chromatography (TLC) on precoated aluminum silica 60 F_{254} (Merck) was used to monitor isolation. Compounds were visualized by exposure under $UV_{254/366}$ light and by spraying with sulfuric acid–vanillin solution,

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followed by heating. The solvents used for extract preparation and chromatographic fractionation were obtained from Vetec.

Plant Material. Leaves and stems of *B. coccolobifolia* were collected in July 2011 from the cerrado at the Federal University of São Carlos (UFSCar), São Carlos, SP, Brazil. Voucher specimens (8367) were deposited at the Herbarium of the Botany Laboratory (HUFSCar) at UFSCar after identification by one of the authors (M.I.S.L.).

Extraction and Isolation. Ethanol extracts were prepared by maceration of air-dried and powdered plant parts. The crude extracts were obtained after drying to remove the solvents by rotatory evaporation under reduced pressure at a temperature of 40 °C. Dried extracts were submitted to enzymatic assays against ARG.

The ethanol extracts of the leaves (10.0 g) and stems (30.5 g) of *B. coccolobifolia* exhibited ARG inhibitory activity. These crude extracts were subjected to liquid−liquid partitioning to produce hexane, EtOAc, and hydroalcoholic extracts. The EtOAc extracts from the leaves (2.0 g) and stems (10.0 g) showed significant inhibition of ARG. The leaf EtOAc extract was fractionated on a silica gel 60 column (18.0 × 5.5 cm; 1:9 acetone−hexane), affording 11 fractions (BF1−BF11). Among them, BF11 (1.2 g), with 90.5% ARG inhibition, was chromatographed over silica gel 60 (18.0 × 5.5 cm; 6:4 acetone−hexane), leading to four subfractions (F14I−F14IV). Active subfractions F14III (60.0 mg) and F14IV (128.0 mg), with 65.0% and 95.0% ARG inhibition, respectively, after chromatographic separations by Sephadex L-20 [(F14III: 56.0 × 2.0 cm; MeOH isocratic); (F14IV: 53.0 × 4.0 and 37.0 × 1.4 cm; MeOH isocratic)], afforded flavonoids **1a** (24.9 mg) and **1b** (2.0 mg). Bioactivity-guided fractionation was performed for the stem EtOAc extract, and fraction BC4 (4.0 g), which exhibited the highest ARG inhibition (80.0%), was obtained by elution with 1:9 MeOH−CH_2Cl_2 on silica gel 60 (50.0 × 4.5 cm), yielding 10 subfractions. Flavonols **2a** (12.0 mg) and **2b** (12.0 mg), (+)-syringaresinol (**3**) (0.5 mg), and trigonostemone (**4**) (1.2 mg) were isolated by purification of subfraction 10 (35.0 mg) using a Sephadex LH-20 column (52.0 × 1.5 cm; MeOH isocratic) and monitoring by TLC. The isolated compounds were characterized by NMR (^{1}H and ^{13}C, DEPT-135, HSQC, and HMBC) and mass spectroscopy and compared with data published in the literature.[29−36]

Chemicals. The following natural products were previously isolated in our laboratory: 3,4,6,7,3′,4′,5′-heptamethoxyflavone (**1j**),[29] myricetin 3-*O*-*α*-rhamnoside (**1e**),[30] catechin-3-*O*-*α*-rhamnopyranoside (**2c**),[31] mearnsetin (**1c**), and tamarixetin 3-*O*-*α*-L-rhamnoside (**1d**).[32,33]

Myricetin (**1f**) was obtained by the acid hydrolysis of **1e**.[34] Quercetin (**1g**) was acetylated with the acetic anhydride/pyridine/DMAP method to produce quercetin pentaacetate (**1i**).[35] 3,5,7,3′,4′-Pentaacetylcatechin (**2f**), 3,5,7-triacetylcatechin (**2e**), and 3-acetylcatechin (**2d**) were obtained by acetylation of (+)-catechin with the acetic anhydride/pyridine/DMAP method (**2a**).[35,36] The acetylation of (+)-catechin was monitored by TLC, and the reaction generated partially and completely acetylated compounds, which were purified by chromatography using a silica gel 60 column. All derivatives were fully characterized from their spectroscopic data.

Sigma-Aldrich supplied quercitrin hydrate (**1a**), ≥78% (Sigma Q3001); (+)-catechin hydrate (**2a**), ≥98% (Sigma C1251); (−)-epicatechin (**2b**), ≥90% (Sigma E1753); kaempferol (**1h**), ≥97% (Sigma 60010); quercetin hydrate (**1g**), ≥95% (Aldrich 337951); and quercetin 3-*β*-D-glucoside, ≥90% (Sigma 17793). The enzymatic urea kit was purchased from Biotécnica (Varginha, MG, Brazil).

Expression and Purification of Recombinant ARG. The recombinant enzyme was expressed and purified as described previously,[6] with some modifications. Briefly, the arginase expression plasmid pRSET-Arg was transformed into *E. coli* Rosetta (DE3) pLysS cells, and the culture was grown at 37 °C in SOB medium supplemented with 100 *μ*g/mL ampicillin and 34 *μ*g/mL chloramphenicol until the $OD_{600\ nm}$ reached 0.6−0.8. Arginase expression was induced by adding isopropyl-*β*-D-thiogalactopyranoside to a final concentration of 1 mM. Incubation was extended for an additional 16 h at 30 °C. The cells were harvested by centrifugation (12 × 1000*g* for 10 min at 4 °C) and stored at −80 °C. The cell pellet from 200 mL of medium was thawed at room temperature and resuspended in 30 mL of 2 mM Tris buffer pH 7.0 with 0.3% Triton X-100 (buffer 1). The cellular suspension was submitted to ultrasonic disruption (6 pulses of 1 min with intervals of 30 s, amplitude 20%), and the lysed cells were centrifuged at 12 × 1000*g* for 20 min at 4 °C. The supernatant was applied to a Ni-NTA Superflow column (1 mL), according to the Qiagen protocol. The column was washed with buffer 1 plus 20 mM imidazole (5 mL) and eluted with 250 mM imidazole in buffer 1 (5 mL). The fractions eluted from the column were pooled, dialyzed against buffer 1, and applied to a DEAE Sepharose Fast Flow (HiTrap, 1 mL) anionic column previously equilibrated with column buffer 1 and eluted with a NaCl gradient (0 to 1 M) at a flow rate of 1 mL/min. The protein concentration was determined by the method of Bradford[37] using the protein assay kit (BioRad) and bovine serum albumin as standard.

Kinetic Measurements and Inhibition Assay. The activity of recombinant arginase of *L. amazonensis* was defined as the amount of enzyme that produced 1 *μ*mol of urea in 1 min per mg of protein.[12,38] K_M and V_{max} values were determined based on the rate of the reaction for different L-arginine concentrations (6.25, 12.5, 15.0, 25.0, 50.0, 62.5, 75.0, and 100.0 mM), as determined from Michaelis−Menten kinetic parameters and Lineweaver−Burk analysis. The samples were evaluated against the recombinant ARG at an initial concentration of 100 *μ*M. The IC_{50} values for the active samples were determined by rate measurements for at least 10 inhibitor concentrations (1280, 1024, 512, 256, 128, 64, 16, 4.0, 1.0, and 0.25 *μ*M). For 10 dilutions of inhibitor, mix I was prepared using 50 *μ*L of CHES buffer solution at pH 9.6, 8 *μ*L of arginase solution, and 292 *μ*L of water. A 5 *μ*L sample of each inhibitor was added to 35 *μ*L of mix I, and the reaction mixture was incubated for 10 min at 37 °C. Then, 10 *μ*L of L-arginine solution was added to the reaction and incubated again for 10 min at 37 °C. The final volume of the reaction mixture was 50 *μ*L and contained 50 mM CHES buffer at pH 9.6 and 50 mM of the substrate L-arginine, at pH 9.6. The second reaction was performed using an enzymatic colorimetric assay[39] with a commercially available assay kit (Biotecnica, Brazil). To hydrolyze urea for quantification, 10 *μ*L of the reaction mixture were added to 500 *μ*L of reagent 1 previously prepared (50 mM phosphate buffer, pH 6.7, 60 mM salicylate, 3.2 mM sodium nitroprusside, and 30 000 IU urease) and incubated at 37 °C for 10 min. Then, 500 *μ*L of reagent 2 (140 mM NaOCl and 150 mM NaOH) was added, and the reaction was incubated at the same temperature for a further 10 min. Additionally, control assays were performed without inhibitor as a negative control and in the presence of the known inhibitor quercitrin[9] as a positive control. The enzymatic assay was carried out in duplicate, and the urea concentration was quantified spectrophotometrically at 600 nm. The type of inhibition and K_i values were determined using the same experimental approach with three concentrations of inhibitor and a control under increasing substrate concentrations (6.25, 12.5, 18.0, 25.0, 50.0, 60.0, and 72.0 mM). The kinetics data were analyzed by Lineweaver−Burk plot analysis with the SigmaPlot 12.0 enzyme kinetics module.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The kinetics of recombinant ARG, as represented by Michaelis−Menten and Lineweaver−Burk plots, and Lineweaver−Burk plots for inhibitors (**1a**, **2a**, and **1f**) are 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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