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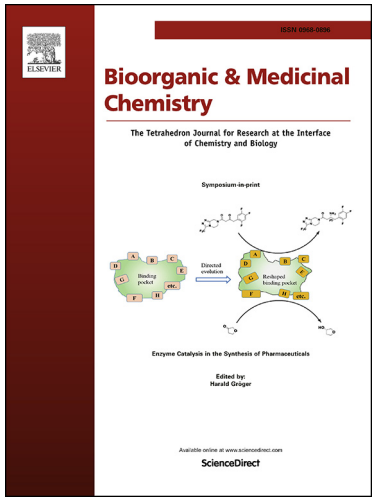

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Phenylhydrazides as inhibitors of *Leishmania amazonensis* arginase and antileishmanial activity

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Abstract: Searching for new substances with antileishmanial activity, we synthesized and evaluated a series of α,α-difluorohydrazide and α,α-difluoramides against *Leishmania amazonensis* arginase (*La*Arg). Four α,α-difluorohydrazide derivatives showed activity against *La*Arg with K_i in the range of 1.3 - 26 μM. The study of the kinetics of *La*Arg inhibition showed that these substances might act via different inhibitory mechanisms or even by a combination of these. The compounds were tested against *L. amazonensis* promastigotes and the best result was obtained to the compound **4 (**EC_{50} of 12.7 ± 0.3 μM). In addition, in order to obtain further insight into the binding mode of such compounds, molecular docking studies were performed to obtain additional validation of experimental results. Considering these results, it is possible to conclude that α,α-difluorohydrazide derivatives are a promising scaffold in the development of new substances against the etiological agent of leishmaniasis by targeting *La*Arg.

Keywords: *Leishmania*, Polyamines, Arginase, difluorohydrazide,

Introduction

Leishmaniasis is a neglected tropical disease that affects 12 million people in the world.[1] Enzymes in the polyamines pathway in trypanosomatids have been studied and proved to be potential antileishmanial targets.[2] Polyamines are involved in the synthesis of trypanothione, a physiologic antioxidant of trypanosomatids that is used to scavenge oxygen (ROS) and nitrogen free radicals (NO) produced by host macrophages.[3,4] In *Leishmania,* the arginase is the first enzyme of polyamine biosynthesis that converts L-arginine into L-ornithine plus urea and is involved in *Leishmania* pathogenesis.[5] The inhibition of the polyamine pathway leads to mitochondrial dysfunction, kDNA disorganization, and parasite death.[6,7] Arginase gene knockout[8] or arginase gene mis-localization mutation showed the importance of arginase in *Leishmania* infection.[9]

Inhibitors of *Leishmania* arginase were first described against *Leishmania mexicana* and *Leishmania amazonensis*.[10,11] Isolated natural compounds also showed high inhibition of *L. amazonensis* arginase.[12–14] Indeed, such inhibitors proved to have a higher potency on parasite arginase in comparison with the mammalian enzyme, which

shows that this target can be selectively modulated.[12] Otherwise, only one synthetic compound (2-(5-methyl-2-(trifluoromethyl)-[1,2,4]triazolo[1,5-a]pyrimidin-7-yl)hydrazinecarbothioamide) was described as a selective *L. amazonensis* arginase inhibitor.[15] Arginase inhibitors first described against human and rat arginases also showed high affinity for *L. mexicana* arginase.[10,16]

The second enzyme of the polyamine pathway is ornithine decarboxylase (ODC), which converts the arginase product L-ornithine into putrescine and carbon dioxide. ODC was the target of eflornithine (Fig. 1), an antiparasitic drug used against *Trypanosoma brucei* species, a trypanosomatid that causes sleeping sickness.[17,18]

Despite the eflornithine being shown as inhibitor of ODC, one important structural aspect of this compound is the presence of a difluoromethyl group, which differentiates it from ornithine. In fact, fluorine is an important substituent in medicinal chemistry due to its capability to make hydrogen bonds, increase the lipophilicity of the compound and alter the electronic aspects of the molecule because of its high electronegativity.[19] Because of these chemical characteristics, and in an attempt to develop new arginase inhibitors, we screened a series of α,α-difluoridrazides (4-9) and α,α-difluoramides (10-12) against *L. amazonensis* arginase. Such substances are structurally related to eflornithine in the presence difluoromethyl groups and nitrogen atoms capable of making electrostatic interactions like drug eflornithine (Fig. 1). Eflornithine is a weak inhibitor of human arginase (Ki = 3.9 mM)[20] and can be a good start to design selective parasite arginase inhibition.

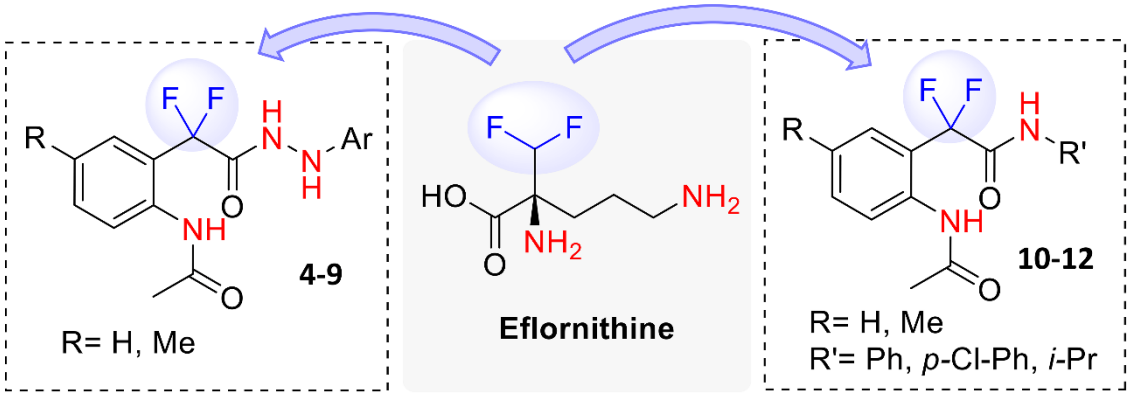
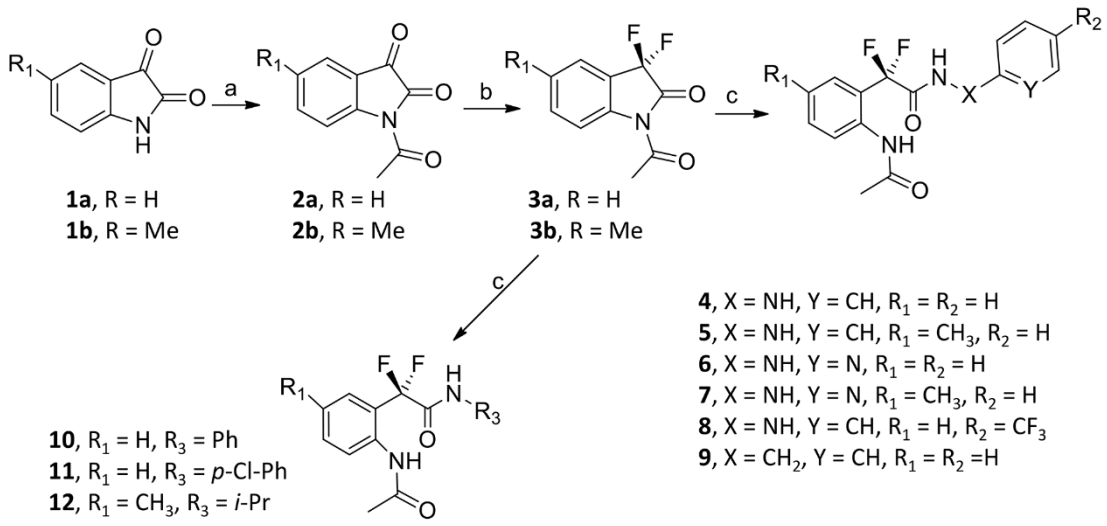


Figure 1. Design of the α,α-difluorohydrazides (4-9) and α,α-difluoramides (10-12) evaluated.

2. Results

2.1 Chemistry

The α,α-difluorohydrazides (**4-9**) and difluoramides (**10-12**) were prepared by acetylation of isatin or 5-methyl isatin with acetic anhydride under reflux and subsequent chemoselective deoxofluorination with (Diethylamino)sulfur trifluoride (DAST) in dichloromethane. Finally, the oxindole ring was opened by a nucleophilic attack of different hydrazides or amines to furnish the final products **4-12** (**Scheme 1**). Compounds **8** and **9** are first described in this study, and **4-7** and **10-12** were previously reported.[21,22]



Scheme 1. Synthesis of compounds **4-12.**

2.2 Kinetics of arginase inhibition and antileishmanial activity

A trial was run with compounds **4-12** at 100 μM against recombinant *L. amazonensis* arginase (*La*Arg) overexpressed in *Escherichia coli*.[11] The compounds **4**, **5**, **8** and **9** showed parasite arginase inhibitory activity (> 70%), compound **6** inhibits only 8%, whereas compounds **10-12** did not inhibit the arginase.

The IC_{50} to the active compounds showed that **4** (IC_{50} = 12 ± 2 μM) and **5** (IC_{50} = 12 ± 3 μM), had better IC_{50} values than those obtained with **8** (IC_{50} = 38 ± 2 μM) and **9** (IC_{50} = 37 ± 6 μM). Kinetic analysis showed that compound **4** inhibited arginase via competition with the substrate while **5, 8** and **9** showed a mixed mechanism (**Fig. 2**). The K_i values of **4** and **5** were, respectively, 5.1 ± 1.4 μM and 1.3 ± 0.8 μM. The promastigote growth inhibition by the arginase inhibitors compounds was determined and only compound **4** showed a dose that kills 50% of the parasite under 100 μM (EC_{50}= 12.7 ± 0.3 μM) **Table 1** summarizes the IC_{50}, Ki and EC_{50} values obtained with the arginase inhibitors.

Antileishmanial activity was determined for the best arginase inhibitor to verify the activity against whole parasite. In a trial performed at 100 μM only compound 4 showed a good inhibition growth. Then, the effective concentration that reduces half of the parasite growth (EC_{50}) was determined for the compound 4 that showed an EC_{50} = 12.7 ± 0.3 μM against *L. amazonensis* promastigotes (Table 1).

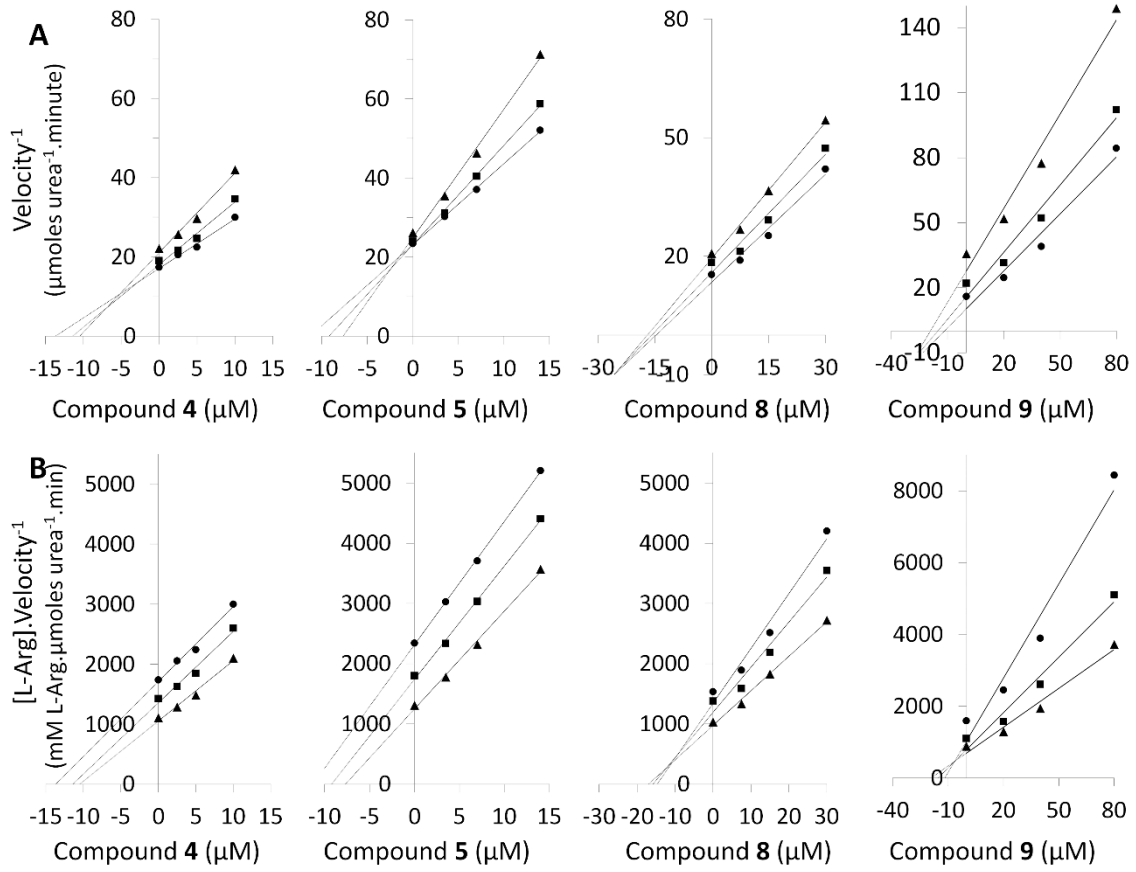
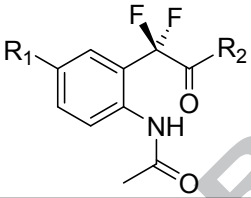
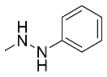
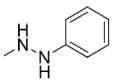
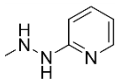
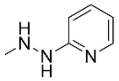
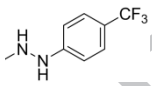
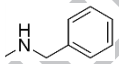
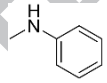
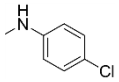


Figure 2. Dixon (A) and Cornish-Bowden plot (B) for arginase inhibitors. The concentrations of L-arginine used were 100 mM (●), 50 mM (■) and 25 mM (▲). Each point drawn represents the mean of three independent experiments (n = 3) performed in duplicate. The difference of slopes between of Cornish-Bowden plot (B) for **4** did not differ ($p=0.33$), while slopes for **5** are different ($p<0.05$).

Table 1. Characterization of arginase inhibition: concentration that inhibit half of enzyme activity (IC_{50}), Ki (dissociation constant for enzyme-inhibitor complex), Kis (dissociation constant for enzyme-substrate-inhibitor complex) and concentration that inhibit half of promastigote growth (EC_{50}) were determined.



Compounds	R1	R2	IC_{50} (µM)	K_i (µM)	K_{is} (µM)	Inhibition mode	Promastigote EC_{50} (µM)
4	H-		12 ± 2	5.1 ± 1.4	-	Competitive	12.7 ± 0.3
5	H_3C-		12 ± 3	1.3 ± 0.8	26 ± 6	Mixed	>100
6	H-		>100	-	-	-	>100
7	H_3C-		> 100	-	-	-	>100
8	H-		38 ± 2 ***, ###	26 ± 1	11 ± 1	Mixed	>100
9	H-		37 ± 6 ***, ###	32 ± 3	6.5 ± 0.2	Mixed	>100
10	H-		inactive	-	-	-	-
11	H-		inactive	-	-	-	-
12	H_3C-		inactive	-	-	-	-
Amphotericin B	-	-	inactive	-	-	-	0.33 ± 0.04

Values of IC_{50}, EC_{50}, Ki and Kis were expressed as a Mean ± SEM (standard error of mean).
***p<0.001 vs compound 4 and ###p<0.001 vs compound 5.
"-"= not determined

2.3 Docking of the competitive inhibitor

To obtain more insight into the binding mode of **4,** molecular docking studies were performed for the prediction of possible interactions to understanding the experimental results. The X-ray crystal structure of *Leishmania mexicana* arginase (*Lm*Arg) determined by D'Antonio et al.[16] was obtained from the Protein Data Bank (PDB Code: 4IU1) and was used for the molecular docking study. Initially, the redocking was performed with *Lm*Arg with the hydroxylated substrate analog nor-N□-hydroxy-L-arginine (nor-NOHA), which is the most effective physiological inhibitor of arginase and exhibited an RMSD value of 1.3 Å.

The L-arginine substrate (**Fig. 3-A, 3-B**) and nor-NOHA inhibitor (**Fig. 3-C, 3-D**) interact with the active site of *Lm*Arg through a hydrogen bond and van der Waals interactions. Redocking the *Lm*Arg-nor-NOHA complex showed that the hydroxyl group of nor-NOHA coordinates to the Mn^{2+} ion; the inhibitor interacts with Asp194, Ser150, Thr257 and Asn143 residues (**Fig. 3-C**, **3-D**); and the L-arginine presented an unfavorable interaction with manganese (**Fig. 3-B**).

The docking analysis indicated that compound **4** interacted with the residues His139, Ala192, and Asp194: the His139 makes a π-π stacking interaction with phenylhydrazine moiety; Ala192 showed a hydrophobic interaction with phenylacetamide while Asp194 makes H-bonds with the two nitrogen atoms in the phenylhydrazine moiety, with the nitrogen of acetamide group, and it also interacts with fluorine (**Fig. 3-E, 3-F**). The ChemScore values calculated for L-arginine, nor-NOHA, and **4** in the complex in the active site of *Lm*Arg were obtained, with -28.21 kJ/mol, -36.43 kJ/mol, and -19.49 kJ/mol, respectively.

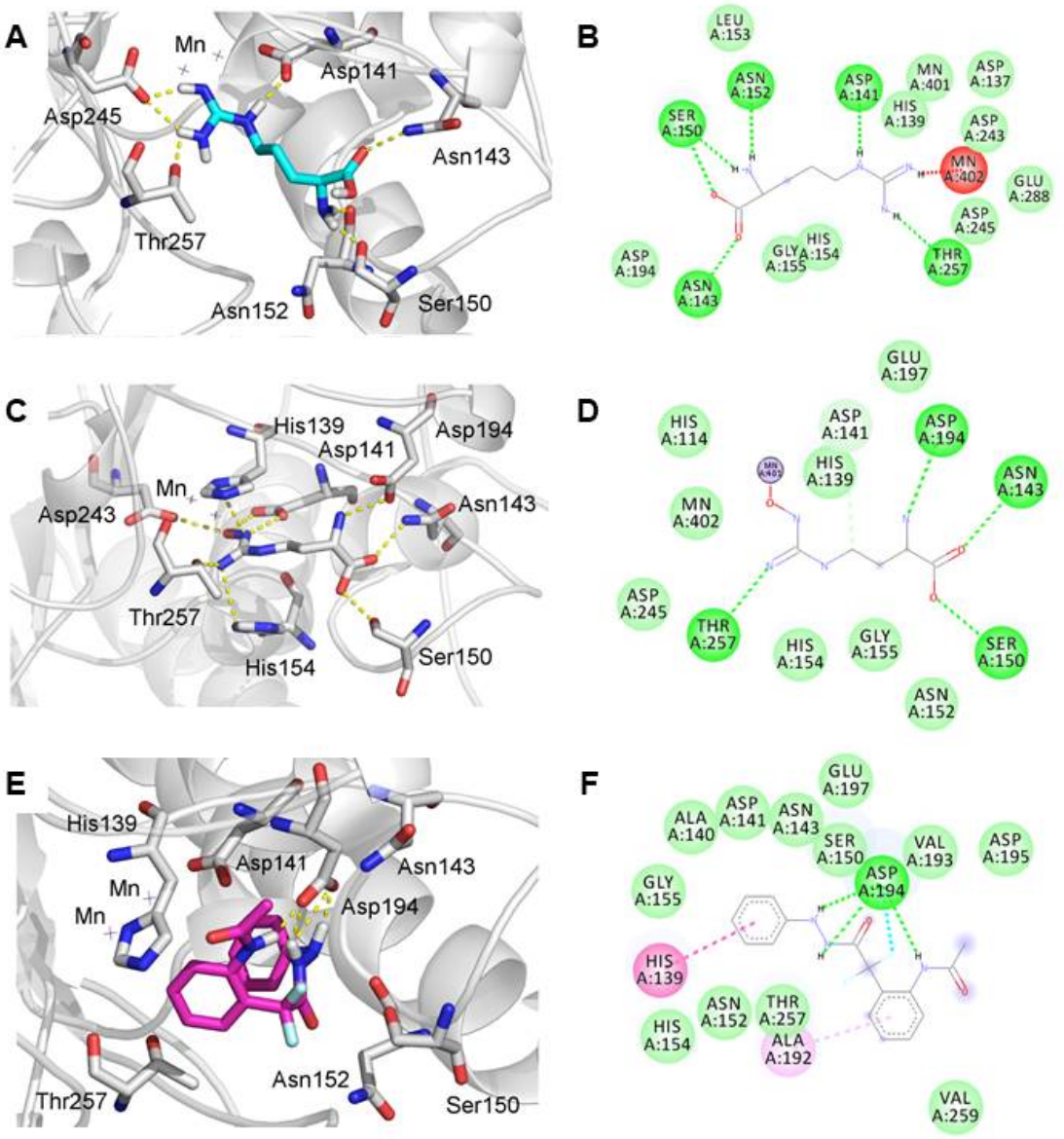


Figure 3: The binding modes and molecular interactions of the (**A**) L-arginine (carbon in cyan), (**C**) substrate analog nor-NOHA (carbon in gray) and (**E**) compound **4** (carbon in magenta) in the active site of *Leishmania mexicana* arginase (PDB Code 4IU1). The binuclear manganese cluster (Mn^{+2}) is represented by a pair of crosses in the monomer. The important residues in the active site of *Lm*Arg and ligands: (**B**) L-arginine, conventional hydrogen bond (green); unfavorable bump (red), van der Walls (green-cyan), (**D**) *Lm*Arg-nor-NOHA, van der Walls (green-cyan), conventional hydrogen bond (green), carbon-hydrogen bond (aquamarine), covalent bond (violet-purple) and (**F**) compound **4,** van der Walls (green-cyan), conventional hydrogen bond (green), halogen/fluorine (cyan), pi-pi stacked (magenta), pi-alkyl (pink)**.** Hydrogen bonds are shown in yellow (**A**, **C** and **E**). The images were generated using PyMOL version 1.7.2.1 and BIOVIA Discovery Studio v.16.1.0.15350.

The docking study was also performed to obtain information about the interaction forms of compound **4** in human arginase-1 (*Hs*Arg) (**Fig. 4)**. The results of the interactions in both enzymes were similar, however, with the human enzyme compound **4** suggests unfavorable interaction. This result can be indicating that compound **4** does not interact with human arginase 1. The sequence was obtained from the Protein Data Bank (PDB Code: 4HWW) and was used for the molecular docking study. The redocking was performed with amino-6-borono-2-(2-(piperidin-1-yl)ethyl) hexanoic acid and exhibited an RMSD value of 1.8 Å. The X-ray crystal structure of *Homo sapiens* arginase (*Hs*Arg) determined by Van Zandt. [23]

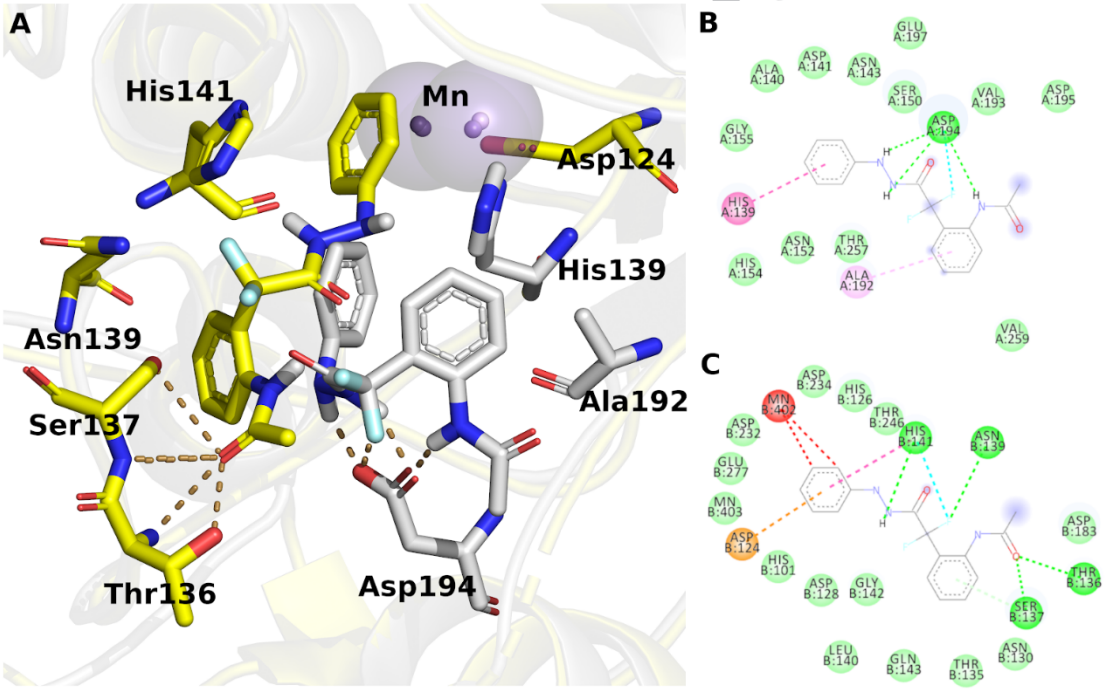


Figure 4: Superposition and molecular interactions of compound **4** in *Leishmania mexicana* and Human Arginase. (A) Superposition of compound **4** (carbono in grey) in the active site of *Leishmania mexicana* Arginase (PDB Code 4IU1) and compound **4** (carbono in yellow) in the active site of *Homo sapiens* Arginase-1 (PDB Code 4HWW). (B) Compound **4** in the active site of *Leishmania mexicana*, van der Walls (dotted lines in light green), conventional hydrogen bond (dotted lines in green), halogen/fluorine (dotted lines in cyan), pi-pi stacked (dotted lines in magenta), pi-alkyl (dotted lines in pink). (C) Compound **4** in the active site of *Homo sapiens*: van der Walls (dotted lines in light green), conventional hydrogen bond (dotted lines in green), halogen/fluorine (dotted lines in cyan), pi-pi stacked (dotted lines in magenta), pi-donor hydrogen bond (dotted lines in light blue), pi-anion (dotted lines in orange) and unfavorable bump (dotted lines in red).The images were generated using PyMOL version 2.4.0a0 (The PyMOL Molecular Graphics System, Schrödinger, LLC) and BIOVIA Discovery

Studio v.16.1.0.15350 (Dassault Systèmes BIOVIA, Discovery Studio Modeling Environment, Release 2017, San Diego: Dassault Systèmes, 2016).

3. Discussion

Leishmania amazonensis arginase inhibitors were described in natural products as polyphenols, which lead to the identification of the catechol as the pharmacophoric group.[11,12,24,25] Subsequently, we have synthesized a compound containing a thiosemicarbazide as the pharmacophoric group needed to inhibit *La*Arg.[15,26] In this study, we have tested a series of α,α-difluorohydrazide derivatives and identified the phenylhydrazide moiety as essential to target *La*Arg in the low micromolar range. Phenylhydrazide is essential for inhibition of *La*Arg arginase as shown for compounds **4**, **5**, and **8**. The compounds **4** and **5** showed the lower IC_{50} (12 μM) that did not differ significantly ($p>0.05$) (**Table 1**). On the other hand, when the *para*-phenyl was replaced by $–CF_3$ (**8**) or the hydrazide group was changed to amino (**9**) the arginase activity decreased (IC_{50} ~38 μM). When phenylhydrazine was replaced with 2-hydrazinopyridine in compounds **6** and **7**, the arginase inhibition deeply decreased (IC_{50} > 100 μM). The substitution of phenylhydrazine to aminophenyl (**10 and 11)** or 2-aminopropane (**12)** led to a complete loss of arginase inhibition.

Despite the structures of compounds **4**, **5**, **8** and **9** being similar, the mechanisms of inhibition were different and may indicate a cleft in the active site that could accommodate compounds **5**, **8**, and **9** together with L-arginine. In particular, **8** and **9** showed lower Kis and could bind together with the substrate L-arginine in the active site. Kis lower than Ki (**Table 2**), indicate that the affinity to the complex enzyme-substrate (ES) is higher than the free enzyme (E). On the other hand, compound **5 (**Ki < Kis), could bind and prevent substrate interaction and it has Kis higher than Ki. This information can be interesting because *Leishmania* can upregulate the transport of L-arginine[27] and increase levels of the arginase substrate and enhance the enzyme inhibition for compound

8 and **9** in a higher level of L-arginine. This result also may indicate that the substrate L-arginine changes the structural conformation of *La*-Arg, decreasing the Kis of compound **9** with complex ES by approximately five times (K_i = 32 μM and K_{is} = 6.5 μM). In spite of the compounds **8** and **9** not having inhibited parasite growth, this result can be used for understanding of the structural changes that can occur with the conformation of the target enzyme in the presence of the inhibitor and substrate. This could be used to design mixed inhibitors with improved activity against the parasite based on the phenylhydrazide moiety.

Previously, we have reported that isomers of natural compounds change the mechanism of *La*Arg inhibition when using chlorogenic and cryptochlorogenic acids that are respectively characterized as competitive and noncompetitive inhibitors. The same results were observed with the antileishmanials verbascoside and isoverbascoside, which are competitive and noncompetitive La-Arg inhibitors, respectively.[14,25]

D'Antonio et al.[16] showed a simulated annealing omit map in which the hydroxyl group of nor-NOHA displaces the metal-bridging hydroxide ion observed in the unliganded enzyme and coordinates with Mn^{2+}. The metal-bridging Mn^{+2}-Mn^{2+} activated a water molecule to generate a hydroxy ion that attaches to the guanidine moiety of substrate L-arginine and when it was used nor-NOHA, a hydroxylated L-arginine, arginase was inhibited.[16] The binuclear manganese cluster is required for stabilization and orientation of the catalytic nucleophile and the dialysis of Mn^{2+} from native arginase I or the substitution of ligands to either manganese in arginase I variants results in up to 20,000-fold reduction in *Kcat* due to the disruption of the metal cluster.[28–30] The binuclear manganese cluster evolved with the optimal structure and chemical properties to orient and activate the metal-bridging hydroxide ion for nucleophilic attack at substrate L-arginine by this enzyme.[30]

The docking analysis to *L. amazonensis* arginase showed that compound **4** interacts with amino acids His139, Ala192 and Asp194. Recently, we have shown that Asp194 and Ser150 were possibly involved in *La*Arg inhibition by caffeic and rosmarinic acids[25] and with the interaction of verbascoside with His139, Asp194 and Ser 150.[14] The amino acids His139 and Asp194 are conserved in arginase family enzymes that are involved in the coordination of the binuclear manganese cluster (Mn^{2+}-Mn^{2+}) in the active site.[31,32] In fact, the amino acids Ser150 and Asp194 were also shown in the crystal structure of *Lm*Arg[16] with synthetic competitive inhibitors[10] ABH (2(*S*)-amino-6-boronohexanoic acid)[33], BEC (*S*-(2-boronoethyl)-L-cysteine)[34] and norNOHA (*N*ω-Hydroxy-nor-l-arginine).[35] However, Ala192, which showed an interaction with phenylacetamide in compound **4**, is not conserved in human arginase: in the equivalent position, human arginase has an aspartic acid. It was also observed that compound **4** showed unfavorable interaction with manganese in the active site of the human arginase. Additionally, the **4** was the only compound that inhibited the growth of promastigote forms. These results can be used as support to design selective drugs to the treatment of leishmaniosis based in the phenylhydrazide group.

4. Conclusion

Four phenylhydrazides (**4**, **5**, **8** and **9**) inhibited arginase in the low micromolar range, while 2-hydrazinopyridine (**6** and **7)** showed weak activity against parasite enzyme. Compounds **8** and **9** showed IC_{50} higher than **4** and **5** because of the presence of a trifluoromethyl substituent in the phenylhydrazine moiety (**8**) or absence of hydrazine (**9**). Docking analysis of compound **4** showed interactions with important amino acids targeted by other arginase inhibitors. Compound **4** showed activity against *L. amazonensis* promastigotes with similar potency that inhibited arginase of the parasite. These results indicate that α,α-difluorophenylhydrazide moiety is a good starting point

for the design of novel *Leishmania* arginase inhibitors with the aim of the development of potential antileishmanial compounds.

5. Experimental section

5.1 Chemistry

5.1.1. N-acetylation of isatins

Compounds **2a** and **2b** were prepared as previously reported.[21]

5.1.2. N-acetylindoline-2,3-diones deoxofluorination

Compounds **3a** and **3b** were prepared as previously reported.[21]

5.1.3. Synthesis of α.α-difluorohydrazides **4-7**

Synthesis of α.α-difluorohydrazides **4-7** was performed as previously reported.[21]

5.1.4. Synthesis of α.α-difluorohydrazides **8**

General procedure. Into a round-bottom flask coupled to a condenser was added 0.6 mmol of the corresponding *N*-acetyl-3,3-difluoroindolin-2-one (**3a** or **3b**) and 3 mL of acetonitrile. The mixture was stirred at room temperature for 5 min. Then, 1 equivalent of neat phenylhydrazine was added to the medium. The flask was then attached to a condenser and the system kept under stirring at reflux for 12 h. At the end of this period, the formed precipitate was vacuum filtered and washed with cold acetonitrile.

5.1.4.1. *N*-(2-(2-(2-(4-(trifluoromethyl)phenyl)hydrazinyl)-1,1-difluoro-2-oxoethyl)phenyl) acetamide **(8)**

^{1}H NMR (DMSO, 400 MHz, δ ppm): 2.04 (s, CH_3CO), 6.77 (d, J = 8.4, 1HAr), 7.37 (t, J = 7.6, 1HAr), 7.47 (d, J = 8.5, 2HAr), 7.58 (t, J = 7.5, 1HAr), 7.64 (d, J = 7.7, 1HAr), 7.73 (d, J = 7.8, 1HAr), 8.61 (s, NH), 9.29 (s, NH), 11.09 (s, NH); **^{19}F NMR** (DMSO, 377 MHz, δ ppm**):** – 59.24, – 100.32; **ESI-MS(–):** *m/z* 386;

CHN: Calc. for $C_{17}H_{14}F_5N_3O_2$: C, 52.72; H, 3.64; N, 10.85; found: C, 52.52; H, 3.63; N, 10.48

5.1.5. Synthesis of 2-(2-acetamidophenyl)-N-benzyl-α,α-difluoroacetamide **9**

To a bound-bottom flask was added the amount of the corresponding *N*-acetyl-3,3-difluoroindolin-2-one and 140 equivalents of acetonitrile. The mixture was allowed to stir at room temperature for 5 minutes. Then, 1 equivalent of benzylamine was added to the medium. The system was kept under stirring overnight. At the end of this period, the formed precipitate was vacuum filtered and washed with cold acetonitrile. The product was air dried and stored under an argon atmosphere with protection from light.
^{1}H NMR (DMSO, 400 MHz, δ ppm): 2.01 (s, CH_3CO), 4.36 (d, J = 9.9, CH_2), 7.22-7.33 (m, 6HAr), 7.52-7.59 (m, 2HAr), 7.76 (d, J = 7.6, 1HAr), 9.48 (s, NH), 9.63 (s, NH); **^{19}F NMR** (DMSO, 377 MHz, δ ppm**): –** 100.51; **ESI-MS(+):** *m/z* 319.1 (48%), 341.0 (100%); **CHN:** Calc. for $C_{17}H_{16}F_2N_2O_2$: C, 64.14; H, 5.07; N, 8.80; found: C, 63.80; H, 5.16; N, 8.70

5.1.6. Synthesis of α.α-difluoramides **10-12**

Synthesis of α.α-difluoramides **10-12** was performed as previously reported.[22]

5.2 Arginase inhibition and kinetics

Recombinant arginase inhibition and kinetics study were performed as previously reported.[11] Briefly, arginase was overexpressed in *E. coli*, and purified.[11] The IC_{50} were determined in a reaction buffer containing 50 mM CHES and 50 mM of L-arginine pH 9,5 with variable concentration of compounds. The activity of arginase was quantified through measure of urea production with colorimetric method of Berthelot.[36]

***5.3 Promastigotes culture* test**

The MHOM/BR/1973/M2269 strain of *L. amazonensis* was used throughout this study. Promastigotes were grown in M199 medium supplemented with 10% fetal bovine serum, 5 ppm of hemin, 50 μg/mL streptomycin and 100Upenicillin. Cells were grown until they reached stationary phase. Initially, 5.0 x10^5 cells were incubated for 72 h with the inhibitors of the parasite arginase that were added to the culture medium in a concentration that ranged from 100 μM to 0.78 μM. Amphotericin B was used as a control for cell growth inhibition in a concentration that ranged from 10 μM to 0.00001. All tests

were performed in 1.5 mL microtubes to a final volume of 1.4 mL. After 72 h of incubation with arginase inhibitors compounds, surviving cells were washed two times with 1 mL of Hanks Balanced Salt Solution (GIBCO® HBSS). Soon after that, the cells were incubated for 13 h with the tetrazolium dye MTT 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) that was added to the culture medium to a final concentration of 0,5 mg/mL. MTT was transformed by the surviving cells producing formazan crystals that were dissolved with 500 µL of DMSO. The concentration of the formazan was determined colorimetrically using the Epoch 2 Microplate Spectrophotometer (Biotech, Instruments, Winooski, Vermont USA) at 570 nm. EC_{50} values were calculated based on a sigmoidal model (Log EC_{50}) using a normalized variable slope in the GraphPad Prism software (version 8 for Windows, San Diego, California, USA). One independent assay was performed six times.

5.5 Molecular docking and bioavailability properties

The structures of the compounds studied were designed, minimized, conformationally analyzed, and optimized, and properties were calculated using the Spartan'10 version 1.0.1. program (Wavefunction, Inc). The calculations were performed for all structures using Conformer Distribution with Molecular Mechanics and MMFF, and 50 conformers were examined for Equilibrium Geometry with Semi-Empirical and RM1, for Energy with Hartree-Fock 6-31G* in the vacuum. The crystal structure of *Leishmania mexicana* arginase (*Lm*Arg) in complex with the hydroxylated substrate analog nor-N□-hydroxy-L-arginine (nor-NOHA), which are the most potent arginase inhibitors, was crystallized and solved by X-ray diffraction.[16] The 3D structure of *Lm*Arg in complex with inhibitor cocrystallized was initially obtained from the Protein Data Bank with PDB Code 4IU1 (resolution of 1.95 Å, method X-ray diffraction).[16] We used the crystal structure of *Lm*Arg instead of a homology model of arginase from *L. amazonensis* since only two residues, not involved in the binding site, were different resulting in a 99.4% amino acid sequence identity. The *pdb* files were prepared, and the water molecules were removed. Subsequently, the molecular docking studies were done using the GOLD software v. 5.5.[37] The Mn^{2+} metal ions are cofactor and have been maintained in coordination with

the metalloenzyme arginase. The parameters for docking were determined by analysis of poses obtained by redocking of the nor-NOHA in *Lm*Arg. The best redocking solutions will be those with the lowest root-mean-square deviation (RMSD) value. Therefore, the docking was performed with Chemscore function with a 20 Å of distance from oxygen (ID 1051) of the catalytic residue Ser150. Automated docking studies were then performed using the genetic algorithm GOLD (Genetic Optimization for Ligand Docking), and twenty GA runs were performed for each molecule. The GOLD score was chosen as fitness function and the standard default settings were used in all calculations. The visual inspection of intermolecular interactions and figures were performed using with PyMOL version 1.7.2.1 (The PyMOL Molecular Graphics System, Schrödinger, LLC) and BIOVIA Discovery Studio v.16.1.0.15350 (Dassault Systèmes BIOVIA, Discovery Studio Modeling Environment, Release 2017, San Diego: Dassault Systèmes, 2016). Default cutoff values of 2.5 Å for hydrogen bonds and 4 Å for Van der Waals interactions were employed.

5.6 Statistical analyses

The data for IC_{50} of the parasite arginase inhibition was evaluated using a one-way analysis of variance (ANOVA) followed by a Tukey post-test. All data was analyzed using GraphPad Prism software (version 8 for Windows, San Diego, California, USA). The differences between the means were considered significant at $p<0.05$.

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References

1. WHO | Leishmaniasis. *WHO*. 2019. https://www.who.int/leishmaniasis/en/.

Accessed June 27, 2019.

2. Colotti G, Ilari A. Polyamine metabolism in Leishmania: from arginine to trypanothione. *Amino Acids*. 2011;40(2):269-285. doi:10.1007/s00726-010-0630-3
3. Bocedi A, Dawood KF, Fabrini R, et al. Trypanothione efficiently intercepts nitric oxide as a harmless iron complex in trypanosomatid parasites. *FASEB J*. 2010;24(4):1035-1042. doi:10.1096/fj.09-146407
4. Fairlamb AH, Cerami A. Metabolism and Functions of Trypanothione in the Kinetoplastida. *Annu Rev Microbiol*. 1992;46(1):695-729. doi:10.1146/annurev.mi.46.100192.003403
5. Balã Na-Fouce R, Calvo-Álvarez E, Álvarez-Velilla R, Prada CF, Pérez-Pertejo Y, Reguera RM. Role of trypanosomatid's arginase in polyamine biosynthesis and pathogenesis. *Mol Biochem Parasitol*. 2011;181:85-93. doi:10.1016/j.molbiopara.2011.10.007
6. Cruz EM, Da Silva ER, Maquiaveli CC, et al. Leishmanicidal activity of Cecropia pachystachya flavonoids: Arginase inhibition and altered mitochondrial DNA arrangement. *Phytochemistry*. 2013;89:71-77. doi:10.1016/j.phytochem.2013.01.014
7. Vannier-Santos MA, Menezes D, Oliveira MF, de Mello FG. The putrescine analogue 1,4-diamino-2-butanone affects polyamine synthesis, transport, ultrastructure and intracellular survival in Leishmania amazonensis. *Microbiology*. 2008. doi:10.1099/mic.0.2007/013896-0
8. Roberts SC, Tancer MJ, Polinsky MR, Gibson KM, Heby O, Ullman B. Arginase Plays a Pivotal Role in Polyamine Precursor Metabolism in Leishmania CHARACTERIZATION OF GENE DELETION MUTANTS*. 2004. doi:10.1074/jbc.M402042200
9. da Silva MFL, Zampieri RA, Muxel SM, Beverley SM, Floeter-Winter LM. Leishmania amazonensis Arginase Compartmentalization in the Glycosome Is Important for Parasite Infectivity. Costa FTM, ed. *PLoS One*. 2012;7(3):e34022. doi:10.1371/journal.pone.0034022
10. Riley E, Roberts SC, Ullman B. Inhibition profile of Leishmania mexicana arginase reveals differences with human arginase I. *Int J Parasitol*. 2011;41(5):545-552. doi:10.1016/j.ijpara.2010.12.006
11. Da Silva ER, Maquiaveli CDC, Magalhães PP. The leishmanicidal flavonols

quercetin and quercitrin target Leishmania (Leishmania) amazonensis arginase. *Exp Parasitol*. 2012. doi:10.1016/j.exppara.2012.01.015

12. Manjolin LC, Dos Reis MBG, Do Carmo Maquiaveli C, Santos-Filho OA, Da Silva ER. Dietary flavonoids fisetin, luteolin and their derived compounds inhibit arginase, a central enzyme in Leishmania (Leishmania) amazonensis infection. *Food Chem*. 2013;141(3):2253-2262. doi:10.1016/j.foodchem.2013.05.025
13. Dos Reis MBG, Manjolin LC, Maquiaveli CC, Santos-Filho OA, Da Silva ER. Inhibition of Leishmania (Leishmania) amazonensis and rat arginases by green tea EGCG, (+)-catechin and (-)-epicatechin: A comparative structural analysis of enzyme-inhibitor interactions. *PLoS One*. 2013;8(11). doi:10.1371/journal.pone.0078387
14. Maquiaveli CC, Lucon-Júnior JF, Brogi S, et al. Verbascoside Inhibits Promastigote Growth and Arginase Activity of Leishmania amazonensis. *J Nat Prod*. 2016;79(5):1459-1463. doi:10.1021/acs.jnatprod.5b00875
15. Da Silva ER, Boechat N, Pinheiro LCS, et al. Novel Selective Inhibitor of Leishmania (Leishmania) amazonensis Arginase. *Chem Biol Drug Des*. 2015;86(5):969-978. doi:10.1111/cbdd.12566
16. D'Antonio EL, Ullman B, Roberts SC, et al. Crystal structure of arginase from Leishmania mexicana and implications for the inhibition of polyamine biosynthesis in parasitic infections. *Arch Biochem Biophys*. 2013;535(2):163-176. doi:10.1016/J.ABB.2013.03.015
17. Bacchi C, Nathan H, Hutner S, McCann P, Sjoerdsma A. Polyamine metabolism: a potential therapeutic target in trypanosomes. *Science (80-)*. 1980;210(4467):332-334. doi:10.1126/SCIENCE.6775372
18. Grishin N V, Osterman AL, Brooks HB, Phillips MA, Goldsmith EJ. X-ray Structure of Ornithine Decarboxylase from Trypanosoma brucei: The Native Structure and the Structure in Complex with α-Difluoromethylornithine,. *Biochemistry*. 1999;38(46):15174-15184. doi:10.1021/bi9915115
19. Cama E, Pethe S, Boucher J-L, et al. Inhibitor Coordination Interactions in the Binuclear Manganese Cluster of Arginase. *Biochemistry*. 2004;43:8987-8999. doi:10.1021/bi0491705
20. Selamnia M, Mayeur C, Robert V, Blachier F. α-Difluoromethylornithine (DFMO) as a potent arginase activity inhibitor in human colon carcinoma cells. *Biochem Pharmacol*. 1998;55(8):1241-1245.

21. Silva Castelo-Branco F, Crizanto De Lima E, Luiz De Oliveira Domingos J, et al. New hydrazides derivatives of isoniazid against Mycobacterium tuberculosis: Higher potency and lower hepatocytotoxicity. *Eur J Med Chem*. 2018;146:529-540. doi:10.1016/j.ejmech.2018.01.071

22. Boechat N, Kover WB, Bastos MM, et al. N-Acyl-3,3-difluoro-2-oxoindoles as versatile intermediates for the preparation of different 2,2-difluorophenylacetic derivatives. *J Braz Chem Soc*. 2008;19(3):445-457. doi:10.1590/S0103-50532008000300011

23. Van Zandt MC, Whitehouse DL, Golebiowski A, et al. Discovery of (R)-2-Amino-6-borono-2-(2-(piperidin-1-yl)ethyl)hexanoic Acid and Congeners As Highly Potent Inhibitors of Human Arginases I and II for Treatment of Myocardial Reperfusion Injury. *J Med Chem*. 2013;56(6):2568-2580. doi:10.1021/jm400014c

24. Dos Reis MBG, Manjolin LC, Maquiaveli CDC, Santos-Filho OA, Da Silva ER. Inhibition of Leishmania (Leishmania) amazonensis and rat arginases by green tea EGCG, (+)-catechin and (-)-epicatechin: A comparative structural analysis of enzyme-inhibitor interactions. *PLoS One*. 2013;8(11). doi:10.1371/journal.pone.0078387

25. da Silva ER, Brogi S, Grillo A, et al. Cinnamic acids derived compounds with antileishmanial activity target *Leishmania amazonensis* arginase. *Chem Biol Drug Des*. 2019;93(2):139-146. doi:10.1111/cbdd.13391

26. Di Costanzo L, Pique ME, Christianson DW. Crystal Structure of Human Arginase I Complexed with Thiosemicarbazide Reveals an Unusual Thiocarbonyl μ-Sulfide Ligand in the Binuclear Manganese Cluster. *J Am Chem Soc*. 2007;129(20):6388-6389. doi:10.1021/ja071567j

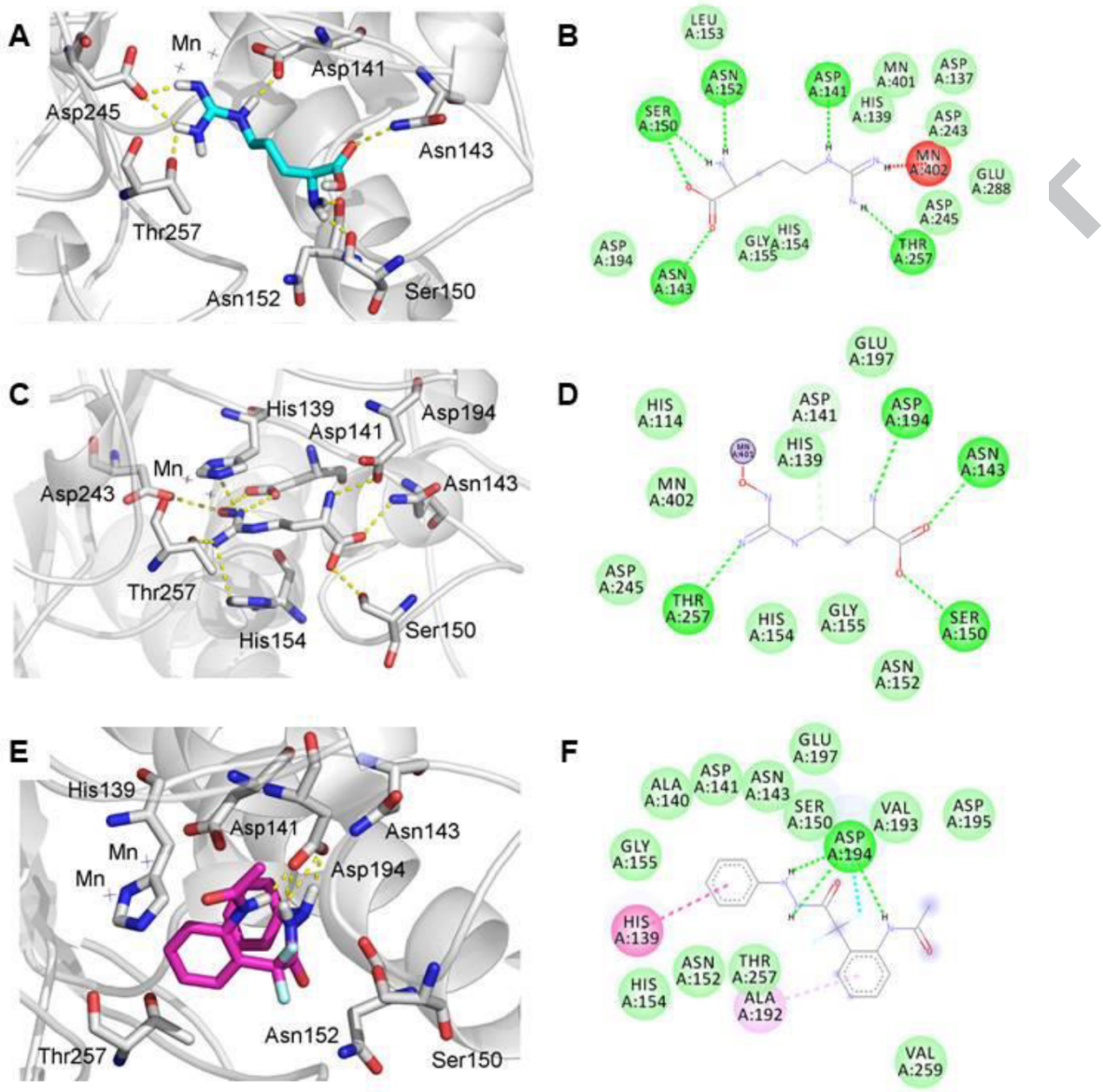
27. Castilho-Martins EA, Laranjeira da Silva MF, dos Santos MG, Muxel SM, Floeter-Winter LM. Axenic Leishmania amazonensis Promastigotes Sense both the External and Internal Arginine Pool Distinctly Regulating the Two Transporter-Coding Genes. El-Sayed NM, ed. *PLoS One*. 2011;6(11):e27818. doi:10.1371/journal.pone.0027818

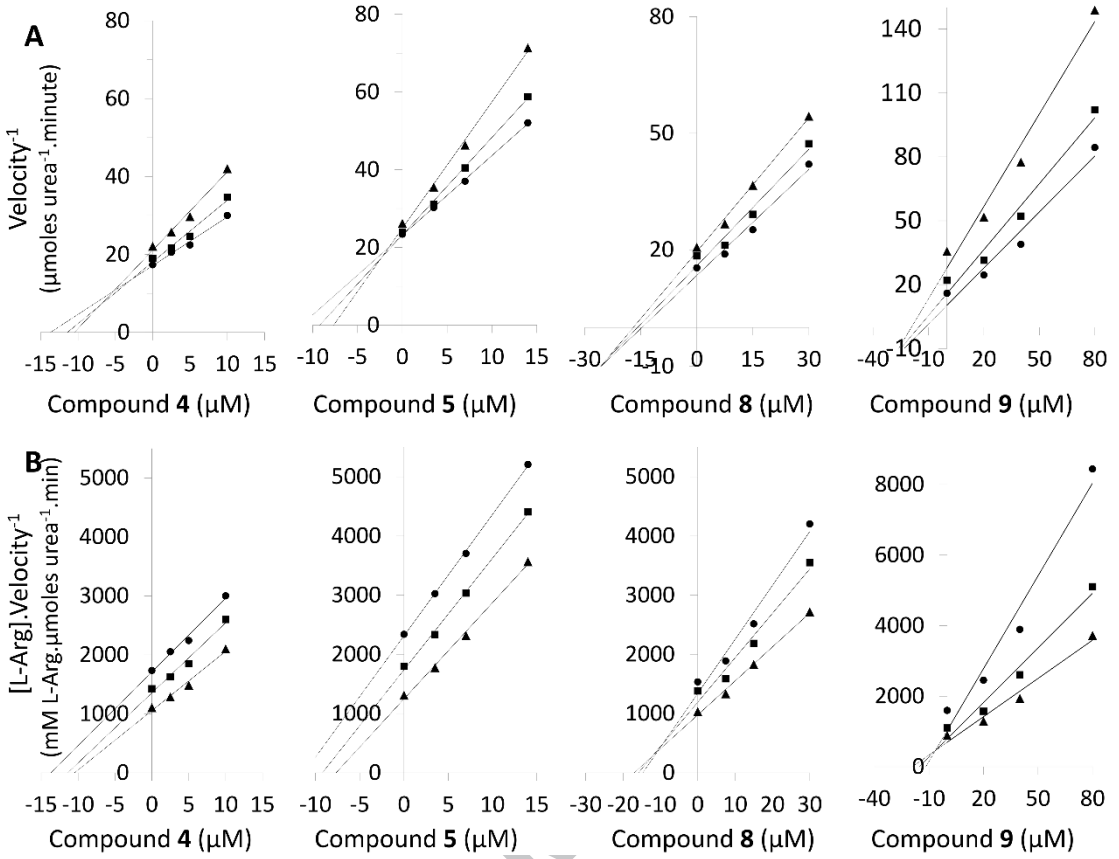
28. Christianson DW. Arginase: Structure, Mechanism, and Physiological Role in Male and Female Sexual Arousal. *Acc Chem Res*. 2005;38(3):191-201. doi:10.1021/ar040183k

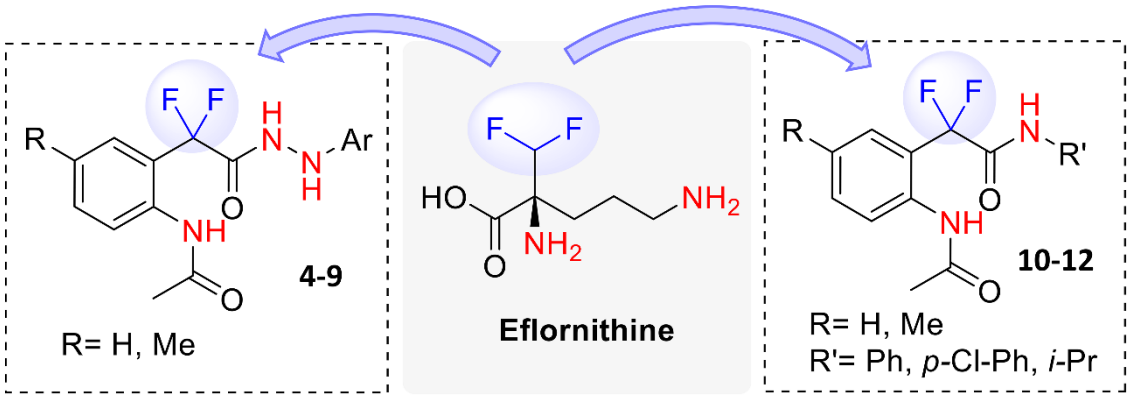
29. Scolnick LR, Kanyo ZF, Cavalli RC, Ash DE, Christianson DW. *Altering the Binuclear Manganese Cluster of Arginase Diminishes Thermostability and*

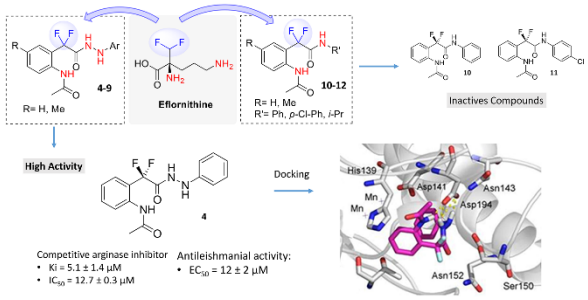
Catalytic Function †.; 1997. https://pubs.acs.org/sharingguidelines. Accessed January 30, 2019.

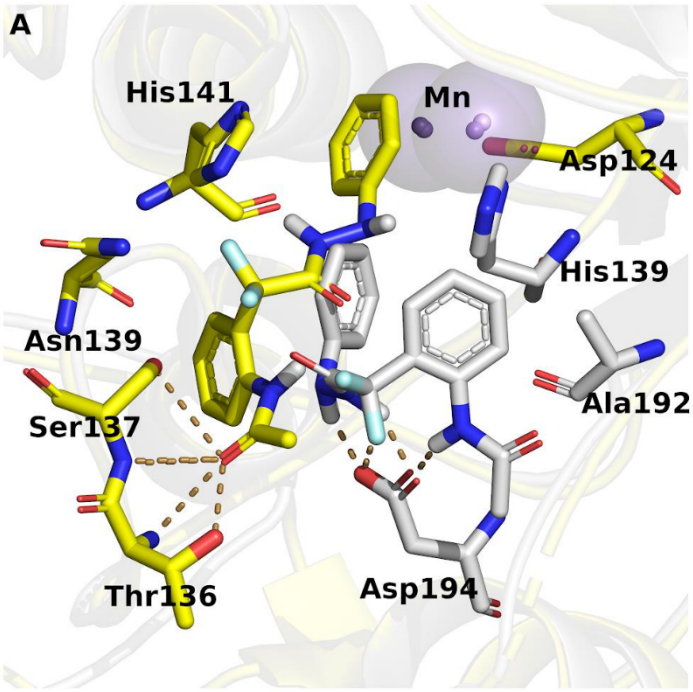
30. Cama E, Emig FA, Ash DE, Christianson DW. Structural and Functional Importance of First-Shell Metal Ligands in the Binuclear Manganese Cluster of Arginase I,. *Biochemistry*. 2003;42(25):7748-7758. doi:10.1021/bi030074y
31. Reczkowski RS, Ash DE. EPR evidence for binuclear manganese(II) centers in rat liver arginase. *J Am Chem Soc*. 1992;114(27):10992-10994. doi:10.1021/ja00053a064
32. Dismukes GC. *Manganese Enzymes with Binuclear Active Sites*. https://pubs.acs.org/sharingguidelines. Accessed January 30, 2019.
33. Baggio R, Elbaum D, Kanyo ZF, et al. Inhibition of Mn2+2-Arginase by Borate Leads to the Design of a Transition State Analogue Inhibitor, 2(S)-Amino-6-boronohexanoic Acid. *J Am Chem Soc*. 1997;119(34):8107-8108. doi:10.1021/ja971312d
34. Kim NN, Cox JD, Baggio RF, et al. Probing Erectile Function: S-(2-Boronoethyl)-l-Cysteine Binds to Arginase as a Transition State Analogue and Enhances Smooth Muscle Relaxation in Human Penile Corpus Cavernosum,. *Biochemistry*. 2001;40(9):2678-2688. doi:10.1021/bi002317h
35. Custot J, Moali C, Brollo M, et al. The New α-Amino Acid Nω-Hydroxy-nor-l-arginine: a High-Affinity Inhibitor of Arginase Well Adapted To Bind to Its Manganese Cluster. *J Am Chem Soc*. 1997;119(17):4086-4087. doi:10.1021/ja970285o
36. FAWCETT JK, SCOTT JE. a Rapid and Precise Method for the Determination of Urea. *J Clin Pathol*. 1960;13(2):156-159. doi:10.1136/jcp.13.2.156
37. Jones G, Willett P, Glen RC, Leach AR, Taylor R. Development and validation of a genetic algorithm for flexible docking. *J Mol Biol*. 1997;267(3):727-748. doi:10.1006/JMBI.1996.0897

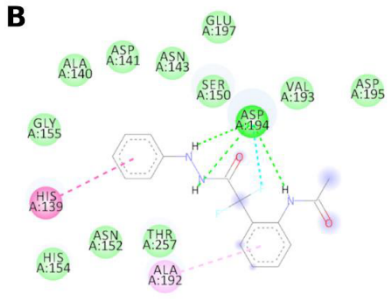


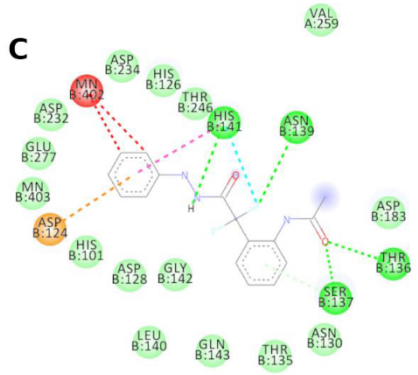


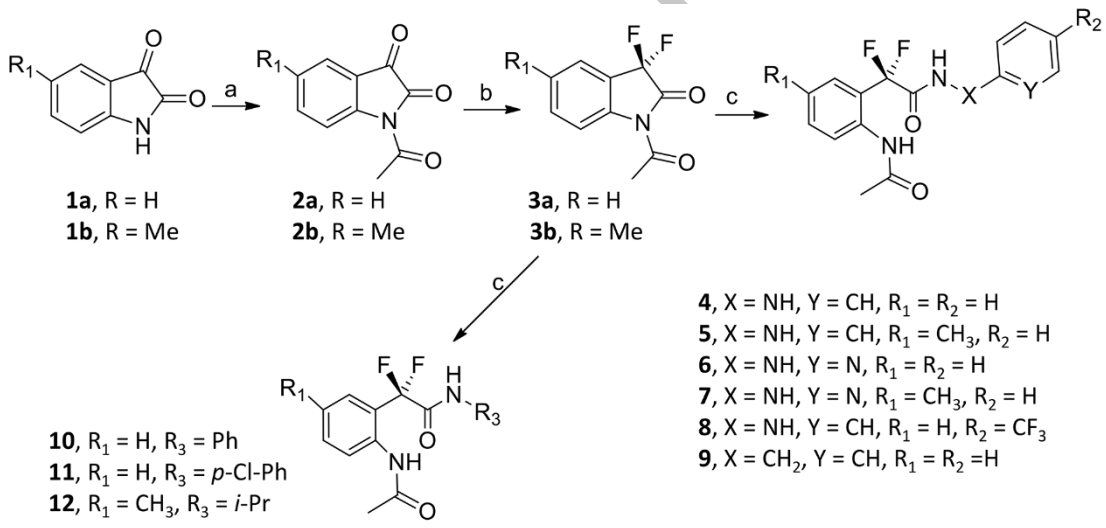












1a, R = H
1b, R = Me

2a, R = H
2b, R = Me

3a, R = H
3b, R = Me

4, X = NH, Y = CH, $R_1 = R_2 = H$
5, X = NH, Y = CH, $R_1 = CH_3$, $R_2 = H$
6, X = NH, Y = N, $R_1 = R_2 = H$
7, X = NH, Y = N, $R_1 = CH_3$, $R_2 = H$
8, X = NH, Y = CH, $R_1 = H$, $R_2 = CF_3$
9, X = CH_2, Y = CH, $R_1 = R_2$ =H

10, $R_1 = H$, R_3 = Ph
11, $R_1 = H$, R_3 = *p*-Cl-Ph
12, $R_1 = CH_3$, R_3 = *i*-Pr

(a) Acetic anhydride, reflux, 4h, 83-89%; (b) DAST, CH_2Cl_2, 16h, r.t., 65-66%; (c) aryl hydrazines (**4-8**) or benzylamine (**9**) or anilines (**10-11**) or isopropylamine (**12**), MeCN, r.t. 50-80%.