In vitro Evaluation of the Antiparasitic Activity of *p*-Coumaric Acid Prenylated Derivatives

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Dedicated to Prof. Gil Valdo da Silva (*in memorian*)

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Abstract:

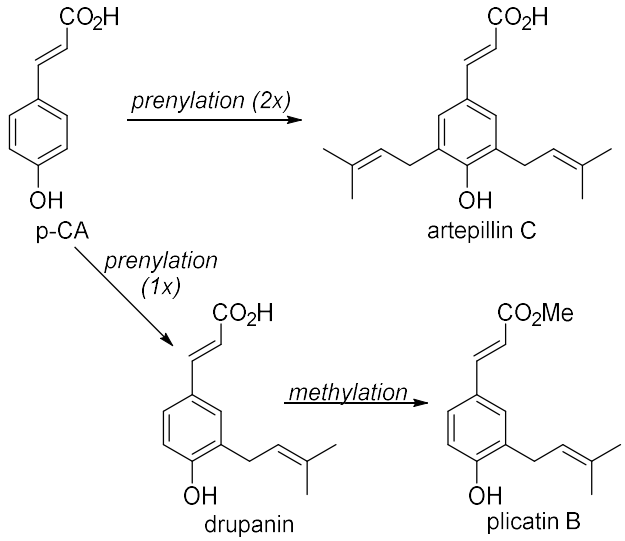
We have evaluated eight *p*-coumaric acid prenylated derivatives *in vitro* for their antileishmanial activity against *Leishmania amazonensis* promastigotes and their antischistosomal activity against *Schistosoma mansoni* adult worms. Compound **7** (methyl 3,5-diprenyl-4-prenyloxycinnamate) was the most active against *L. amazonensis* (IC_{50} = 45.92 µM) and *S. mansoni* (IC_{50} = 64.25 µM). None of the tested compounds killed *S. mansoni* adult worms. Data indicated that the number of prenyl groups, the presence of hydroxyl at C9, and a single bond between C7 and C8 are important structural features for the antileishmanial activity of *p*-coumaric acid prenylated derivatives.

Keywords: artepillin C; *Leishmania amazonensis*; *O*-prenylation; plicatin B; *Schistosoma mansoni*

Introduction

Para-Coumaric acid (*p*-CA), a plant-derived phenolic compound, is widely distributed in the plant kingdom. It is biosynthesized from phenylalanine and tyrosine through the shikimic pathway and may be converted to other phenolic acids of the *p*-hydroxycinnamic acid family (*e.g.*, caffeic acid, ferulic acid, and sinapic acid), flavonoids, lignin precursors, and other secondary metabolites.[1] p-CA can also undergo prenylation reactions in both the nature[2] and the laboratory,[3] to form more complex phenolic acids, such as artepillin C, drupanin, and plicatin B (Scheme 1). Artepillin C, drupanin, and plicatin B occur in the Brazilian green propolis,[4, 5] a resinous product produced by honeybees (*Apis mellifera*) through collection of different parts and exudates of B*accharis dracunculifolia* DC (Asteraceae).[6] Artepillin C and drupanin, the main phenolic acid derivatives in the Brazilian green propolis, underlie many Brazilian green propolis biological activities.[6]

Leishmaniasis is a parasitic Neglected Tropical Disease (NTD) caused by protozoa of the genus *Leishmania*. This disease leads to about 30,000 deaths annually.[7] Its tegumentary (TL) or visceral (VL) manifestations depend on the infecting *Leishmania* species and the mammalian host's immunological and nutritional status. *L. amazonensis* is the main causative agent of TL in the Americas.[8]. TL can cause skin lesions that can be self-healed or culminate in disfiguring scars, accompanied by extensive tissue destruction in nasopharyngeal mucosal tissues.[9] Diagnosing and treating TL early is difficult because the disease evolves slowly and covers large skin areas.[10] Moreover, the drugs available for treating leishmaniasis (*e.g.*, pentavalent antimonials, amphotericin B, pentamidine, miltefosine, and paramomycin) have disadvantages, including toxicity, high cost, and the emergence of parasitic resistance.[11]



Scheme1. Formation of artepillin C, drupanin, and plicatin B by *p*-coumaric acid (p-CA) prenylation.

Schistosomiasis, caused by trematode flatworms of the genus *Schistosoma,* is one of the most significant NTDs in the world.[12] This disease has high morbidity rates and affects over 220 million people worldwide.[12] About 700 million people are at risk of contracting the disease.[13] Praziquantel (PZQ) is the drug of choice to control the disease and to treat schistosomiasis.[14] Nevertheless, PZQ is less effective against juvenile schistosomes,[15] and strains less sensitive to PZQ and diminished efficacy of this drug have been reported.[16-18]

Data on the antileishmanial and antischistosomal activity of prenylated *p*-coumaric acids are scarce.[5, 6, 19] Thus, as part of our interest in the antiparasitic activities of synthetic[20, 21] and natural products,[22, 23] we have investigated the *in vitro* antileishmanial and schistosomal activity of some prenylated derivatives of *p*-coumaric acid against *Leishmania amazonensis* and *Schistosoma mansoni*.

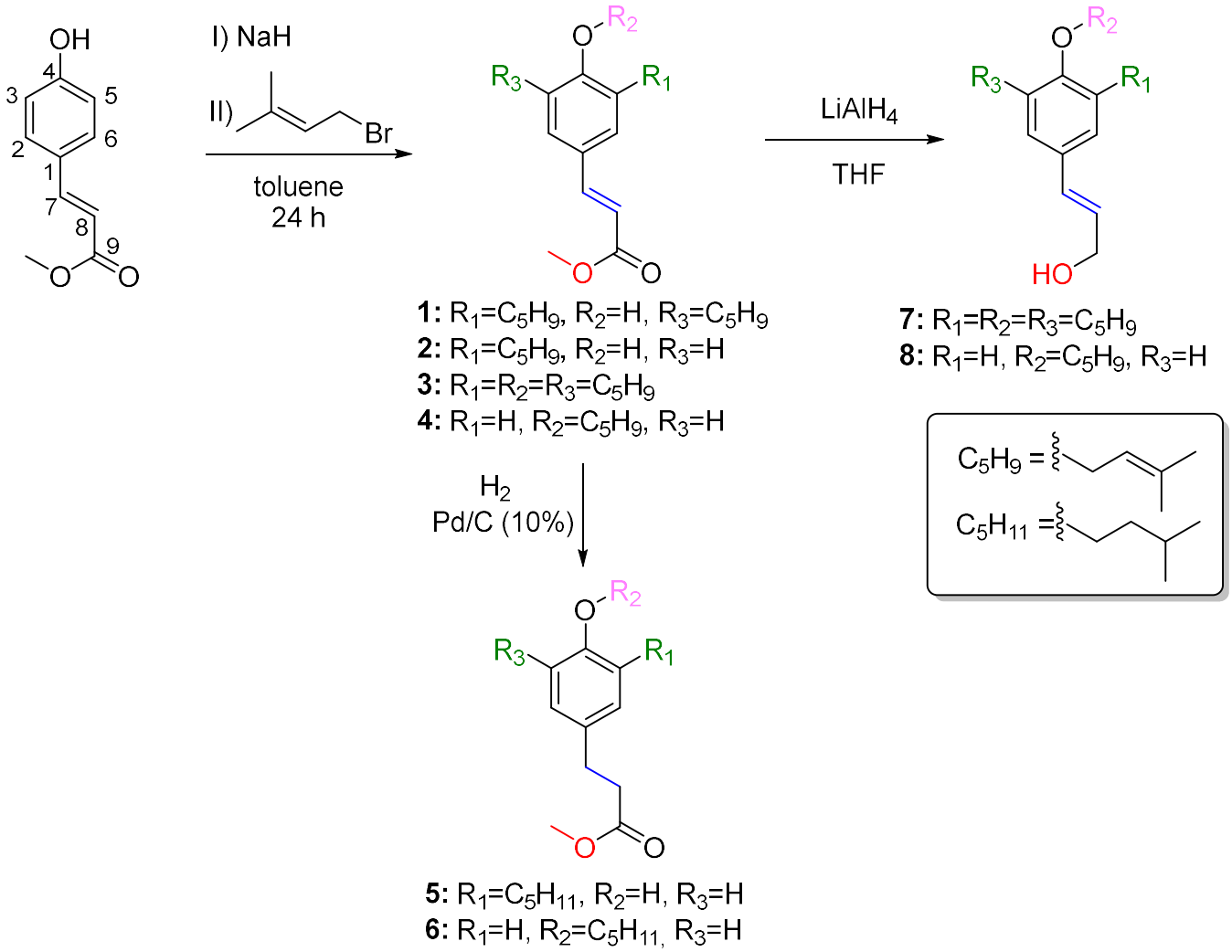
Experimental Section

*Synthesis of Prenylated p-Coumaric Acid Derivatives **1-8***

Methyl *p*-coumarate was synthesized by esterification of *p*-coumaric acid in the presence of sulfuric acid, as catalyst, as previously reported.[21, 24]. Compounds **1–4** were synthesized by alkylating methyl *p*-coumarate according to the methodology employed by Patra and coworkers[3] with modifications (Scheme 1). In this procedure, methyl *p*-coumarate (0.5 mmol) was added to a 25-mL flask equipped with a magnetic stirring bar. Next, 15 mL of toluene was added. The mixture was cooled to 0 °C, and 1.5 mmol NaH was added in portions. After 15 min, 1.5 mmol (153 µL) of prenyl bromide was added dropwise. The reaction progress was monitored through TLC; Hex:EtOAc 8:2 (v/v) solution was used as the eluent. After 24 h, the solvent was removed under reduced pressure in a rotary evaporator, and the reaction mixture was then extracted with EtOAc (3 x 15 mL). The organic phase was washed with saturated NaCl solution (15 mL), dried over $MgSO_4$, and filtered off. The solvent was removed by evaporation at reduced pressure in a rotary evaporator. The compounds were isolated by column chromatography with gradient elution starting with 100% hexane and changing to Hex:EtOAc 9.8:0.2 (v/v) after the first compounds were separated. The resulting solids were dried under vacuum, to yield compounds **1** (32% yield), **2** (18% yield), **3** (15% yield), and **4** (35% yield) (Scheme 1).

Compounds **5** and **6** were obtained from compounds **2** and **4** through catalytic hydrogenation. In this procedure, compounds **2** and **4** and Pd/C (catalyst) were dissolved in HPLC grade EtOAc, transferred to a high-pressure reactor under stirring, and kept under H_2 atmosphere and 400 psi at room temperature for 1-2 h (Scheme 1). The resulting oil was dried under vacuum, to yield compounds **5** and **6** in 100% yield.

Compounds **7** and **8** were synthesized according to the methodology described by Kantee and co-workers[25] with modifications (Scheme 1). In this procedure, $LiAlH_4$ (0.68 mmol) was quickly added to a solution of compounds **3** and **4** (0.34 mmol) in THF (5.5 mL) at 0 °C. The reaction mixture was stirred under N_2 atmosphere at 0 °C for 1 h. After 1 h, the mixture was allowed to stir at room temperature for 9–10 h. The reaction mixture was then added with H_2O, conc. HCl and extracted with EtOAc (x3). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated under vacuum. Purification of the crude residue by column chromatography with isocratic elution with Hex/EtOAc 9.8:0.2 (v/v) gave the corresponding alcohol derivative. The resulting compounds were dried under vacuum, to yield compounds **7** (65% yield) and **8** (89% yield).



Scheme 2. Synthesis of compounds **1-8**.

Antileishmanial Assays

In the beginning of the *Leishmania amazonensis* (MHOM/BR/PH8) growth stationary phase, about 1 x 10^6 promastigotes were cultivated in 96-well plates with RPMI 1640 medium (Gibco).[26] Next, samples of compounds **1–8** were dissolved in DMSO (Synth) and added to the culture at concentrations ranging from 3.12 to 100 µM; amphotericin B (positive control) was added at 1.56 µM. The cultures were incubated in a BOD (Quimis) oven at 25 °C for 24 h. Promastigote viability was determined by parasite growth inhibition, by counting the number of alive promastigotes on a Neubauer (Glass – Porto Alegre, BR) chamber; flagellate motility was considered. RPMI 1640 medium containing 0.1% DMSO was used as negative control. Results are expressed as the inhibition percentage mean compared to the negative control (0.1% DMSO + RPMI 1640).[26] Two independent experiments were performed in three replicates. The maintenance of the life cycle was approved by the Ethics Committee for Animal Care at the University of Franca, under the protocol 010/14.

Antischistosomal Assays

The *S. mansoni* (LE–Luiz Evangelista strain) life cycle is routinely maintained by passage through *Biomphalaria glabrata* snails and BALB/c mice (female, six weeks old, 20—25 g) at the University of Franca. After

5±2 days of infection with 180±10 cercariae, mice were euthanized, and the *S. mansoni* adult worm pairs were recovered under aseptic conditions by perfusion of their livers and mesenteric veins. After that, *Schistosoma mansoni* LE adult worms (Luis Evangelista) were washed in Roswell-Park Memorial Institute (RPMI) 1640 medium (Inlab Diagnostica); transferred to a 24-well plate (two couples per well) containing 2 mL of RPMI 1640 (Inlab, Campinas, SP) buffered with HEPES 20 µM, pH 7.5, and supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% fetal bovine serum (Gibco); and incubated under humid atmosphere with 5% CO_2 at 37 °C for 24 h. Eight worms (four couples) were used for each sample. After incubation, samples of compounds **1-8** were solubilized in dimethylsufoxide (DMSO, Sigma-Aldrich) and applied at 50 µM for the initial screening. Praziquantel (PZQ, Sigma-Aldrich) was previously solubilized in DMSO and used at 1.6 µM, as positive control. RPMI 1640 medium supplemented with 0.1% DMSO was used as negative control.

Adult worm couples were incubated under the same conditions described above and evaluated every 24 h for 72 h under an inverted microscope Primo-Vert (Carl Zeiss 4 – 10 x). The phenotypic changes were classified on the basis of a phenotypic viability scale (0-3), as follows: 3: normal worms, fixed and with good movements; 2: worms with slow movements; 1: worms with severely reduced movements, with or without tegument modifications, and 0: dead worms or with absence of movement after analysis for 2 min.[27] The Ethics Committee for Animal Care of the University of Franca authorized all experiments (Approval number: 5199070417). All animals were handled in agreement with good animal practice guidelines as defined by the University of Franca in accordance with Brazilian law.

Statistical Analysis

All the experiments were performed in triplicate and repeated at least two times. The IC_{50} (the inhibitory concentration that inhibited *L. amazonensis* promastigote or *Schistosoma mansoni* adult viability by 50%) values were calculated by a non-linear regression dose-response inhibition curve. Data were analyzed by repeated measures of two-way analysis of variance followed by Dunnet's comparison. Statistical analyses were performed using GraphPad Prism 5 (GraphdPad Software, San Diego, CA, USA).

Results and Discussion

We conducted *p*-coumaric acid prenylation according to Patra and coworkers.[3] First, the *p*-coumaric acid carboxyl function must be protected through esterification (Scheme 1). After that, the nucleophilic phenolate anion is generated by deprotonating the phenolic hydroxyl in the presence of sodium hydride, as base.[3] Finally, after prenyl bromide is added to the reaction mixture, the *C*-prenylated compounds plicatin B (methyl 3-prenyl-4-hydroxycinnamate, compound **2**) and methyl 3,5-diprenyl-4-hydroxycinnamate (compound **1**) arise as the only products and are further converted to drupanin (Scheme 1) and artepillin C (**5**) after basic hydrolysis. Here, we also verified that compounds **3** and **4** emerged through *O*-prenylation. Formation of these unreported *O*-prenylation products decreased the reaction selectivity, so we obtained compounds **1** and **2**, the synthetic precursors of artepillin C and drupanin in low yields. For this reason, we also investigated the antileishmanial and antischistosomal activities of compounds **3** and **4** and their derivatives.

Compounds that display IC_{50} (half-maximum inhibitory concentration) lower than 10 µM, between 10 and 50 µM, between 50 and 100 µM, and higher than 100 µM are very active, active, moderately active, and inactive, respectively.[26] We found that compounds **5** and **7** are the most active against *L. amazonensis* promastigote (IC_{50} = 49.20 and 45.92 µM, respectively) (Table 1). Plicatin B (compound **2**), a naturally occurring compound in Brazilian propolis,[4] displays moderate activity (IC_{50} = 88.42 µM). Compounds **1**, **4**, **6**, and **8** are also moderately active (50 µM < IC_{50} < 100 µM), whereas compound **3** is inactive (IC_{50} > 100 µM).

We also assessed compounds **1–8** for their *in vitro* antischistosomal activity against *Schistosoma mansoni* adult worms. Initially, we assessed how compounds **1–8** at 50 µg/mL affect *S. mansoni* adult worm mortality (Table 2). Most of the compounds do not kill adult worms (data not shown). Only compounds **3**, **4**, **7**, and **8** affect *S. mansoni* viability. Compounds **4** and **7** are the most active, causing 66.67 ± 0.00 % and 33.30 ± 0.00 % mortality after treatment for 24 h, respectively. We verified that compound **7** recovers 4.15% worm viability after 72 h probably because the worms metabolize compound **7** and excrete compound **8**, to recover their homeostasis.[28]

Table 1. Half-maximum inhibitory concentration (IC_{50}, in µM) of compounds **1–8** against *L. amazonensis* promastigotes after treatment for 24 h.

Sample	IC_{50} ± SD
1	69.62 ± 12.74
2	88.42 ± 14.42
3	111.60 ± 15.66
4	62.44 ± 10.97
5	49.20 ± 18.05
6	82.21 ± 7.96
7	45.92 ± 18.76
8	70.60 ± 15.57
Amphotericin B [b]	100.00 ± 0.00

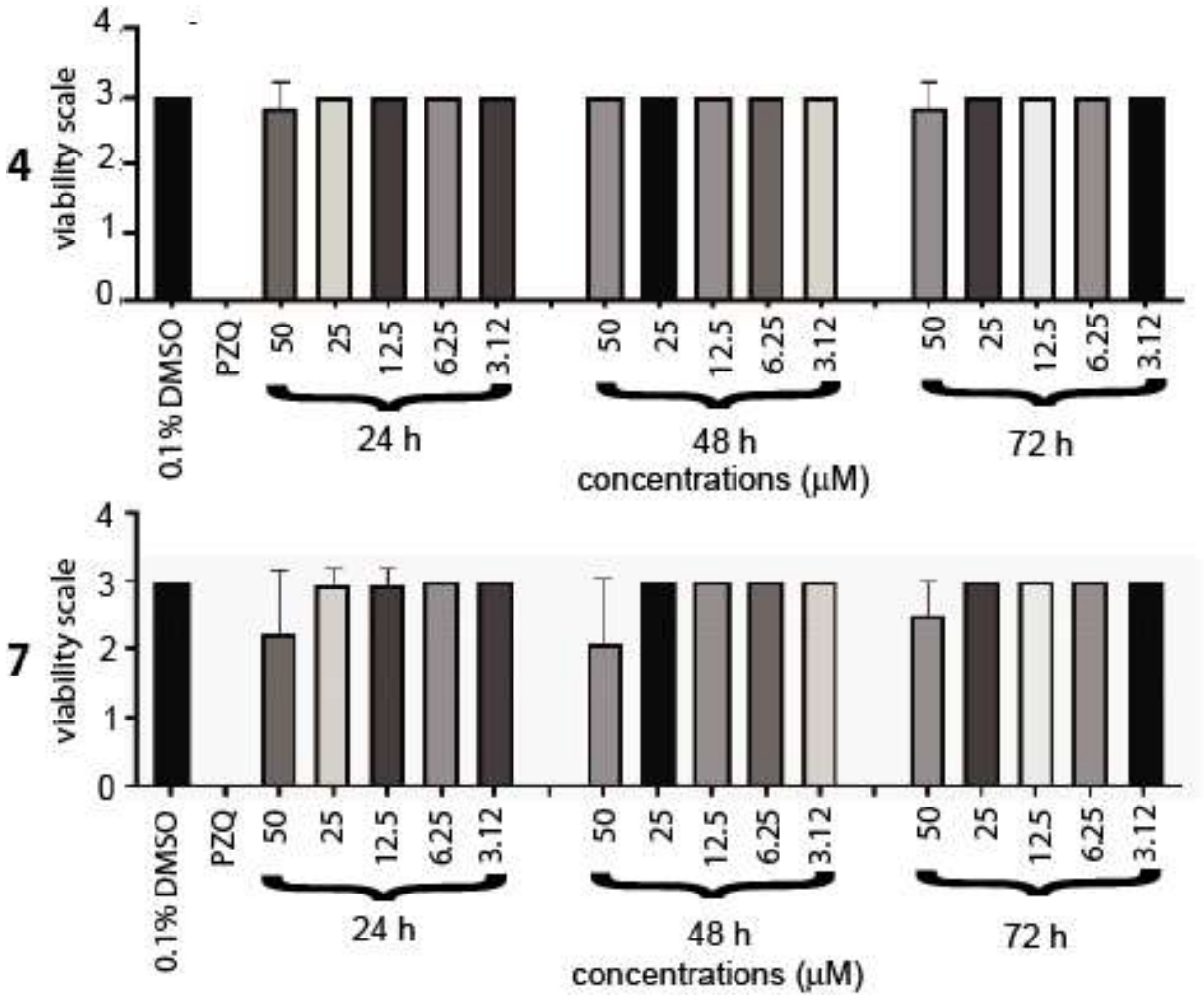
[a] Positive control, tested at 1.56 µM

We also evaluated whether compounds **4** and **7** inhibit *S. mansoni* adult worms at concentrations ranging from 3.1 to 50 µM after treatment for 24, 48, or 72 h (Figure 1). Only at 50 µM does compound **4** display inhibitory effects after treatment for 24 h. Surprisingly, this inhibition is null after treatment for 48 h. After treatment for 72 h, however, compound **4** decreases worm viability. In the case of compound **7**, worm viability decreases to 72.92% and 68.75% after treatment for 24 and 48 h, respectively. Nevertheless, viability increases to 97.92% after treatment for 72 h. Compounds **4** and **7** provide IC_{50} of 69.13 and 52.75 µM after treatment for 72 h, respectively.

Table 2. S. *mansoni* adult worm mortality (%) after treatment with compounds **3, 4, 7,** and **8** at 50 µg/mL for 24 or 72 h and IC_{50} of compounds **4** and **7** after treatment for 24, 48, or 72 h.

Sample[a]	% Mortality[a]		IC_{50} (µM)		
	24 h	72 h[a]	24 h	48 h	72 h
3	33.30 ± 0.00	4.16 ± 4.16			
4	33.30 ± 0.00	33.30 ± 0.00	69.13	69.13	69.13
7	66.67 ± 0.00	58.33 ± 5.45	64.25	51.31	52.75
8	0.00 ± 0.00	12.50 ± 6.09			
PZQ [b]	100.00 ± 0.00	100.00 ± 0.00	0,81	0,81	0,36

[a] Tested with compounds **1–8** at 50 µg/mL. [b] Tested at 1.6 µM.



Scheme 2. *S. mansoni* adult worm viability after incubation with compound **4** or **7**. Data are expressed as mean ± standard deviation.

We compared our data on the antileishmanial activity of compounds **1–8** against *L. amazonensis* promastigotes with the literature,[19] to obtain some structure-activity relationships. For example, comparison between the antileishmanial activity of compounds **1** (di-*C*-prenylated methyl ester) and **2** (mono-*C*-prenylated methyl ester) and **4** (mono-*O*-prenylated methyl ester) and **3** (triprenylated methyl ester) suggested that increasing number of prenyl substituents in the structure of *p*-coumaric acid derivatives increases the lipophilicity and decreases the antileishmanial activity. Indeed, a similar effect of lipophilicity on the antileishmanial activity has been reported for other phenolic compounds (*e.g.*, lindbergins).[29] However, artepillin C (a 3,5-prenylated *p*-coumaric acid derivative) has proven more active against *L. amazonensis* promastigotes (IC_{50} = 17.46 µM) than *p*-coumaric acid (IC_{50} = 48.46 µM).[19] These apparently controversial data indicate that the antileishmanial activity of compounds **1–8** depends on other structural features than the number of prenyl groups, as discussed below.

Comparison between the antileishmanial activity of artepillin C (IC_{50} = 17.46 µM) and its corresponding methyl ester (compound **1**; IC_{50} = 69.62 µM) indicated that the carboxyl group at C9 is essential for the antileishmanial activity. Besides that, the higher antileishmanial activity of compound **7** (triprenylated alcohol; IC_{50} = 40.92 µM) compared to compound **3**, its corresponding methyl ester (IC_{50} = 111.60 µM), reinforced that a hydroxyl group at C9 of a carboxylic acid or alcohol boosts the antileishmanial activity of prenylated *p*-coumaric acid derivatives. On the other hand, compound **8** (*O*-prenylated alcohol; IC_{50} = 70.60 µM) was less active than compound **4**, its corresponding methyl ester (IC_{50} = 62.44 µM), indicating that, alone, the presence of a hydroxyl group at C9 of *p*-coumaric acid derivatives does not ensure good antileishmanial activity.

One of the mechanisms of action of antileishmanial drugs (*e.g.*, amphotericin) is differential binding to ergosterol in *Leishmania* membranes.[30] An antileishmanial drug can target the sterol pathway, mainly sterol from the parasite cell membrane, to form pores or channels on the lipid bilayer membranes of the target host cells. These channels or pores can cause an influx of ions/solutes, killing cells.[31] The hydroxyl group of alcohol (in compounds **7** and **8**), phenol (in compounds **1**, **2**, and **5**), or carboxyl (in artepillin C) are important for the antileishmanial activity because it allows these compounds to target the binding sites of the key enzymes of the ergosterol biosynthetic pathway, which converts ergostatetraenol to ergosterol.[32] In artepillin C, lipophilicity (*i. e.*, two prenyl groups) and hydrophilicity (*i. e.*, a phenolic hydroxyl and a carboxyl) are well balanced, so this compound presents higher antileishmanial activity than compounds **1–8**, prenylated *p*-coumaric acid derivatives.

Compounds **2** (*C*-prenylated) and **4** (*O*-prenylated) are regioisomers that differ in the prenyl group position. Comparison between the IC_{50} of compounds **2** (88.42 µM) and **4** (62.44 µM) revealed that the position of the prenyl group (compound **2** is 3-prenylated and compound **4** is *O*-prenylated) is also important for the antileishmanial activity. In principle, the higher activity of compound **4** compared to compound **2** suggested that *O*-prenylation increases the antileishmanial compared to *C*-prenylation, as previously reported for prenylated chalcone ethers.[30] However, in the case of compounds **4**, **6**, and **8**, *O*-prenylation provides a more linear molecular shape as compared to *C*-prenylated compounds **2** and **6,** which can facilitate permeation through the parasite membrane. Besides that, because of the rotation around the single bond between C7 and

C8, compound **5** can assume more conformations than compound **2**, improving the intermolecular interactions of compound **5** with a potential target. This structural feature of compound **5**, combined with the presence of a phenolic hydroxyl, boosts its antileishmanial activity. Therefore, data from this study suggest that the *in vitro* antileishmanial activity of *p*-coumaric acid derivatives against *L. amazonensis* promastigotes depends on combining the nature of the bond between C7 and C8, the number and position of the prenyl groups, and the molecular shape of compounds **1–8**.

Data on the antischistosomal activity of prenylated compounds are scarce.[33, 34] We have found that only *O*-prenylated compounds affect *S. mansoni* adult worms. Although compounds **4** and **7** are the most active, at 50 µg/mL none of them kill 100% of the worms. On the other hand, these compounds inhibit *S. mansoni* adult worms, with IC_{50} of 69.13 and 64.25 µM after treatment for 24 h, respectively. These IC_{50} indicated that compounds **4** and **7** have higher inhibitory activity against *S. mansoni* adult worms than artepillin C (IC_{50} > 100 µM) after treatment for 24, 48, or 72 h. However, because most of the tested compounds do not inhibit or kill worms, other structure-activity relationships cannot be established from these results only.

Conclusions

Among the tested prenylated *p*-coumaric acid derivatives, only compound **7** displays some antileishmanial and antischistosomal activity. More specifically, this compound displays moderate antileishmanial activity (50 < IC_{50} < 100 µM) against *L. amazonensis* promastigote and moderately inhibits *S. mansoni* adult worms. An increasing number of prenyl groups combined with a hydroxyl at C9, a single bond between C7 and C8, and the molecular shape plays a key role in the antileishmanial activity. However, further studies with diverse prenylated *p*-coumaric acid derivatives must be undertaken to understand the antiparasitic structure-activity relationships of these compounds.

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Author Contribution Statement

T.M.V. and J.G.B synthesized the prenylated p-coumaric acid derivatives; L.A.L.P and P.C.A.F. performed the antileishmanial and antischistosomal assays; L.G.M. supervised the antiparasitic assays; J.K.B. critically read the manuscript and provided financial support; A.E.M.C. designed and drafted the manuscript. All the authors have read the final manuscript and approved its submission.

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