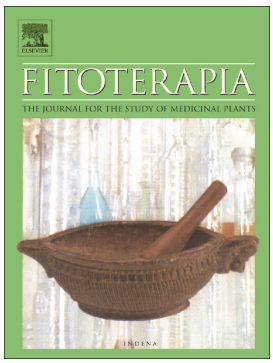

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An unprecedented chlorine-containing piperamide from *Piper pseudoarboreum* as potential leishmanicidal agent

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Abstract

A phytochemical investigation of the ethanolic extract of leaves from *Piper pseudoarboreum* led to the isolation of 3-chlorosintenpyridone **1**, an unprecedented chlorinated piperamide, together with the known compounds **2-12**. Their structures were established based on 1D and 2D (COSY, ROESY, HMQC, and HMBC) NMR spectroscopy, in addition to high resolution mass spectrometry. The proposed biosynthetic pathway of compound **1** is discussed. Compounds **1-12** were tested *in vitro* for their leishmanicidal potential against promastigote stages of *Leishmania amazonensis*, *L braziliensis*, *L. guyanensis* and *L. infantum*. Two compounds from this series, the alkamide **1** (IC_{50} 3.4-5.2 μM) and the fatty acid **9** (IC_{50} 18.7-29.6 μM) displayed higher or similar potency to Miltefosine, used as the reference drug.

Keywords: *Piper pseudoarboreum*, chlorinated-piperamide, biosynthetic pathway, leishmanicidal.

1. Introduction

Piperaceae family belongs to the Piperales order, comprising five genera: *Piper, Peperomia Manekia, Zippelia,* and *Verhuellia* [1]. *Piper* is the largest genus in this family, with nearly 2000 species of herbs, shrubs, or smalls trees, distributed throughout the tropical and sub-tropical forests, mainly in Central and South America, the Caribbean, Africa, Asia, and Pacific Islands [1,2]. Species of this genus have demonstrated to be of economic, ecological and medicinal value. In fact, these plants have been commonly used in traditional Chinese medicine, Indian Ayurvedic system and folk medicine of Latin America [3-5]. Furthermore, members of the genus *Piper* play an important role as source of therapeutic agents [6,7], biosynthesizing great chemical diversity [2,8], including alkaloids [9], flavonoids [10], terpenoids [11], phenylpropanoids [12], phenolic acids [13] and lignans [14]. These constituents have been reported to possess antioxidant [10], anti-inflammatory [15], antitumor [16] and antiparasitic properties [13,14,17].

As part of an intensive investigation into potential leishmanicidal agents from *Piper* species, we report herein on the isolation and structure elucidation of a new piperamide (**1**), along with eleven known compounds (**2**-**12**) from the leaves of *P. pseudoarboreum*. Their chemical structures were identified on the basis of their spectrometric and spectroscopic data, including 1D and 2D NMR experiments, and comparison with those reported in the literature. The proposed biosynthesis of **1** from precursors **2** and **12** is discussed. All natural compounds (**1**-**12**) have been evaluated for their antileishmanial activity against a panel of *Leishmania* sp. promastigote stages.

2. Material and methods

2.1. General

Optical rotations were determined on a Perkin-Elmer 241 automatic polarimeter. UV spectra were obtained in absolute MeOH on a JASCO V-560 spectrometer. IR (film) spectra were

measured on a Bruker IFS 55 spectrometer. The 1D and 2D spectra were recorded on Bruker Avance 400 and 600 spectrometers; the chemical shifts are given in δ (ppm) and were referred to the residual solvent signal ($CDCl_3$: δ_H 7.26, δ_C 77.36; C_6D_6: δ_H 7.15, δ_C 128.62), with TMS as internal reference. EIMS and HREIMS were recorded on a Micromass Autospec spectrometer. Silica gel 60 (15-40 mm) for column chromatography, silica gel 60 F254 for TLC were purchased from Panreac, and Sephadex LH-20 was obtained from Pharmacia Biotech. Centrifugal planar chromatography (CPC) was performed using a Chromatotron instrument (model 7924T) Harrison Research Inc., Palo Alto, CA, USA) on manually coated silica gel 60 GF_{254} (Merck) using 1, 2 or 4 mm plates. The developed TLC plates were visualized by UV light, and then by spraying with staining system of H_2O-H_2SO_4-AcOH (1:4:20) followed by heating of silica gel plates to approximately 150°C. All solvents used were analytical grade from Panreac.

2.2. Plant material and extraction

Leaves of *Piper pseudoarboreum* Yunker were collected in Iquitos, Maynas Province, Department of Loreto, Perú in November 2009. The plant material was identified by botanist Juan Celedonio Ruiz Macedo, and a voucher specimen (AMZ 11114) was deposited at the Amazonense Herbarium of the Universidad Nacional de la Amazonia Peruana, Iquitos, Perú.

2.3. Extraction and isolation

The air-dried powdered leaves of *P. pseudoarboreum* (200.3 g) were extracted with 96% ethanol by Soxhlet (48 h x 5 L). The extract was concentrated *in vacuo* to afford 42.9 g (21.4%) of crude extract, which was suspended in water and partitioned successively, with dichloromethane (DCM) and ethyl acetate (EtOAc). The organic fractions were concentrated under reduced pressure, whereas the aqueous residue was lyophilized, providing 9.2, 1.2 and 8.9 g of extract, respectively.

The DCM fraction (9.2 g) was chromatographed on silica gel column, using mixtures of hexanes/EtOAc of increasing polarity (10:0 to 0:10 *v*/*v*) to yield fractions 1-7. Fr.5 (1.1 g) was chromatographied by Sephadex LH-20 column, using a mixture of $CHCl_3$/MeOH (1:1, *v*/*v*) as eluent to afford 14 fractions, which were combined on the basis of their TLC profiles in four fractions (Fr.5A to Fr.5D). Sub-fraction Fr.5C (218.8 mg) was chromatographed by CPC, using mixtures of hexanes/EtOAc of increasing polarity (8:2 to 2:8) to give 36 fractions, which were combined in eight fractions (Fr.5C1 to Fr.5C8). Fractions Fr.5C4 (26.6 mg) and Fr.5C5 (25.2 mg) were purified by preparative TLC, using CH_2Cl_2/Et_2O (95:5, *v*/*v*) as eluent to yield compounds **1** (1.6 mg, 0.0008 %) and **3** (4.8 mg, 0.002 %), respectively. Fr.5C6 afforded compound **4** (17.6 mg, 0.009 %), whereas Fr.5C7 (9.8 mg) yielded compound **5** (1.1 mg, 0.0006 %) after being purified by preparative TLC with hexanes/2-propanol (8:2, *v*/*v*). Fr.6 (1489 mg) was subjected to column chromatography on Sephadex LH-20, using a mixture of $CHCl_3$/MeOH (1:1, *v*/*v*) as eluent to afford 15 fractions, which were combined based on their TLC profiles in six sub-fractions (Fr.6A to Fr.6F). Sub-fraction Fr.6D (276.6 mg) was further purified by CPC, using a hexanes/EtOAc system (6:4 to 4:6, *v*/*v*) to yield 57 fractions, which were combined into eleven sub-fractions (Fr.6D1 to Fr.6D11) on the basis of their TLC profiles. Sub-fraction Fr.6D1 afforded compound **9** (11.7 mg, 0.006 %), and preparative TLC of Fr.6D2 (14.2 mg) with CH_2Cl_2/Et_2O (9:1, *v*/*v*) yielded compound **11** (1.0 mg, 0.0005 %). Compound **10** (1.0 mg, 0.0005 %) was obtained from Fr.6E (12.4 mg) after purification on preparative TLC, using mixtures of hexanes/EtOAc (1:1, *v*/*v*) as eluent. Fr.7 (624 mg) was chromatographied on Sephadex LH-20, using mixtures of $CHCl_3$/MeOH (1:1, *v*/*v*) as eluent to provide 12 sub-fractions, which were grouped according to their TLC profiles in seven fractions (Fr.7A to Fr.7G). Sub-fraction Fr.7C yielded compound **12** (21.3 mg, 0.01 %). Fr.7D (77 mg) was chromatographed on a silica gel column eluted in a step gradient manner with CH_2Cl_2/Me_2CO (9:1 to 7:3, *v*/*v*) to provide four final fractions, Fr.7D1 to Fr.7D4. Compound **2** (1.1 mg, 0.0006 %) was obtained from Fr.7D1. Fr.7D3 (14 mg) afforded

compounds **7** (1.4 mg, 0.0007 %) and **8** (1.2 mg, 0.0006 %) after preparative TLC with hexanes/2-propanol (9:1, *v*/*v*), double development, whereas Fr.7E (9.3 mg) yielded compound **6** (2.5 mg, 0.001 %) by preparative TLC eluted with CH_2Cl_2/Me_2CO (8:2, *v*/*v*). The structures of the compounds were identified by one and two-dimensional ^1H and ^{13}C NMR spectroscopy and mass spectrometry, and comparison with data reported in the literature.

2.3.1. N-(3,4-Dimethoxy-3-phenyl-propanoyl)-3-chloro-Δ^3-pyridin-2-one (**1**)

Brownish oil; UV λ_{max} (MeOH) (log ε) 226 (3.9) 276 (3.4) nm; IR ν_{max} (film) 1696, 1514, 1462, 1160, 759 cm^{-1}; ^1H and ^{13}C NMR see table 1; EIMS *m/z* 325 $[M + 2]^+$ (10), 323 $[M^+]$ (27), 192 (26), 164 (100), 151 (49), 132 (6), 91 (8); HREIMS *m/z* 323.0916 (calcd for $C_{16}H_{18}NO_4$ Cl, 323.0924).

2.3.2. 3-Chloro-5,6-dihydropyridin-2(1H)-one (**2**)

Brownish oil; UV λ_{max} (MeOH) (log ε) 276 (3.3); IR (film) ν_{max} 3392, 1656, 1182 cm^{-1}; ^1H NMR (δ, $CDCl_3$): 2.50 (2H, dt, *J* = 4.6, 7.0 Hz, H-5); 3.48 (2H, t, *J* = 7.0 Hz, H-6), 5.83 (1H, s, NH), 6.79 (1H, t, *J* = 4.6 Hz, H-4); ^{13}C NMR (δ, $CDCl_3$): 25.2 (CH_2-5), 39.9 (CH_2-6), 127.4 (s, C-3), 137.1 (CH-4), 161.1 (s, C-2); EIMS *m/z* 133 $[M + 2]^+$ (66), 131 $[M]^+$ (100), 103 (57), 101 (94), 76 (21), 74 (56); HREIMS *m/z* 131.0137 (calcd for C_5H_6NOCl, 131.0138).

2.4. In vitro promastigotes susceptibility assay

Autochthonous isolate of *Leishmania infantum* (MCAN/ES/92/BCN83) obtained from an asymptomatic dog from the Priorat region (Catalunya, Spain) were kindly given by Prof. Montserrat Portús (Universidad de Barcelona). *L. braziliensis* (2903), *L. amazonensis* (MHOM/Br/79/Maria) and *L. guyanensis* (141/93) were kindly provided by Prof. Alfredo Toraño (Instituto Salud Carlos III, Madrid). The promastigotes were grown *in vitro* in a Schneider's Insect medium supplemented with 20% heat-inactivated fetal bovine serum

(FBS), and 100 U/mL of penicillin plus 100 µg/mL of streptomycin at 26 °C in 25 mL in tissue culture flasks. The assay of *in vitro* antileishmanial activity was performed using a method described elsewhere [18]. Briefly, promastigotes (2.5×10^5 parasites/well) were cultured in 96-well plastic plates. Compounds were dissolved in dimethylsulfoxide (DMSO). Different dilutions of the compounds (a first screening using 100 µg/mL, and then 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 µg/mL for active compounds) up to 200 µL final volume were added. The final DMSO concentration never exceeded 0.1% (v/v) with no effect on the parasites proliferation or morphology. After 48 h at 26 °C, 20 µL of 2.5 mM resazurin solution was added, and the fluorescence emission (535_{ex}–590_{em} nm) was determined with a fluorometer Infinite 200 (Tecan i-Control) to calculate growth inhibition rate (%). All tests were carried out in triplicate. Miltefosine was used as reference drug. The efficacy of each compound was estimated by calculating the IC_{50} (concentration of the compound that produced a 50% reduction in parasites) and GI% (percentage of growth inhibitory).

2.5. Cytotoxicity assay on macrophages

J774 murine macrophages were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, penicillin G (100 U/mL) and streptomycin (100 µg/mL). Cell cultures were maintained in a humidified 5% CO_2 air atmosphere at 37 °C. The assay was carried out according methods previously described [18]. J774 macrophages cell line was seeded (5×10^4 cells/well) in 96-well flat-bottom plates with 100 µL of RPMI 1640 medium. The cells were allowed to attach for 2 h at 37 °C, 5% CO_2, and then was added 100 µL of RPMI-1640 medium with test compound at different concentrations (100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 µg/mL to the final volume) and exposed for another 48 h. Growth controls and signal-to-noise were also included. Afterwards, a volume of 20 µL of 2.5 mM resazurin solution in PBS was added, and plates were returned to the incubator for another 3 h to evaluate cell viability. The reduction of resazurin was determined by fluorometry as in the

promastigote assay. Each concentration was assayed by triplicate. Cytotoxicity effect of compounds was defined as the 50% reduction of cell viability of treated culture cells with respect to untreated culture (CC_{50}).

2.6. Statistical and data analysis for in vitro assays

All values were expressed as means ± standard error of the mean (S.E.M.). Statistically significant differences between treated and control samples were tested by one-way analysis of variance (ANOVA). The efficacy against parasite (IC_{50}) and cytotoxicity effect (CC_{50}) of compounds were calculated from Probit analysis using SPSS v15.0 software.

3. Results and discussion

The dichloromethane fraction obtained from the ethanolic extract from leaves of *P. pseudoarboreum* was subjected to multiple chromatographic steps, involving column chromatography on silica gel, Sephadex LH-20 and preparative TLC to yield one new piperamide (**1**), and eleven known compounds (**2**-**12**) (see Fig. 1).

The structures of the known compounds were elucidated on the basis of spectroscopic data, including homo and heteronuclear correlation NMR experiments, and comparison with data reported in the literature as 3-chloro-5,6-dihydropyridin-2(1*H*)-one (**2**) [19], piplaroxide (**3**) [20], 4,5-dihydropiperlonguminine (**4**) [21], 4-hydroxi-*N*-[2-(4-methoxy-phenyl)-ethyl] benzamide (**5**) [22], (*E*)-*N*-*p*-coumaroyltyramine (**6**) [23], (*E*)-*N*-feruloyltyramine (**7**) [24], (*Z*)-*N*-feruloyltyramine (**8**) [25], (*S*)-13-hydroxy-octadeca-(9*Z*,11*E*,15*Z*)-trienoic acid (**9**) [26], cinnamic acid (**10**) [27], ferulic acid (**11**) [28], and 4-methoxy-dihydroferulic acid (1**2**) [29].

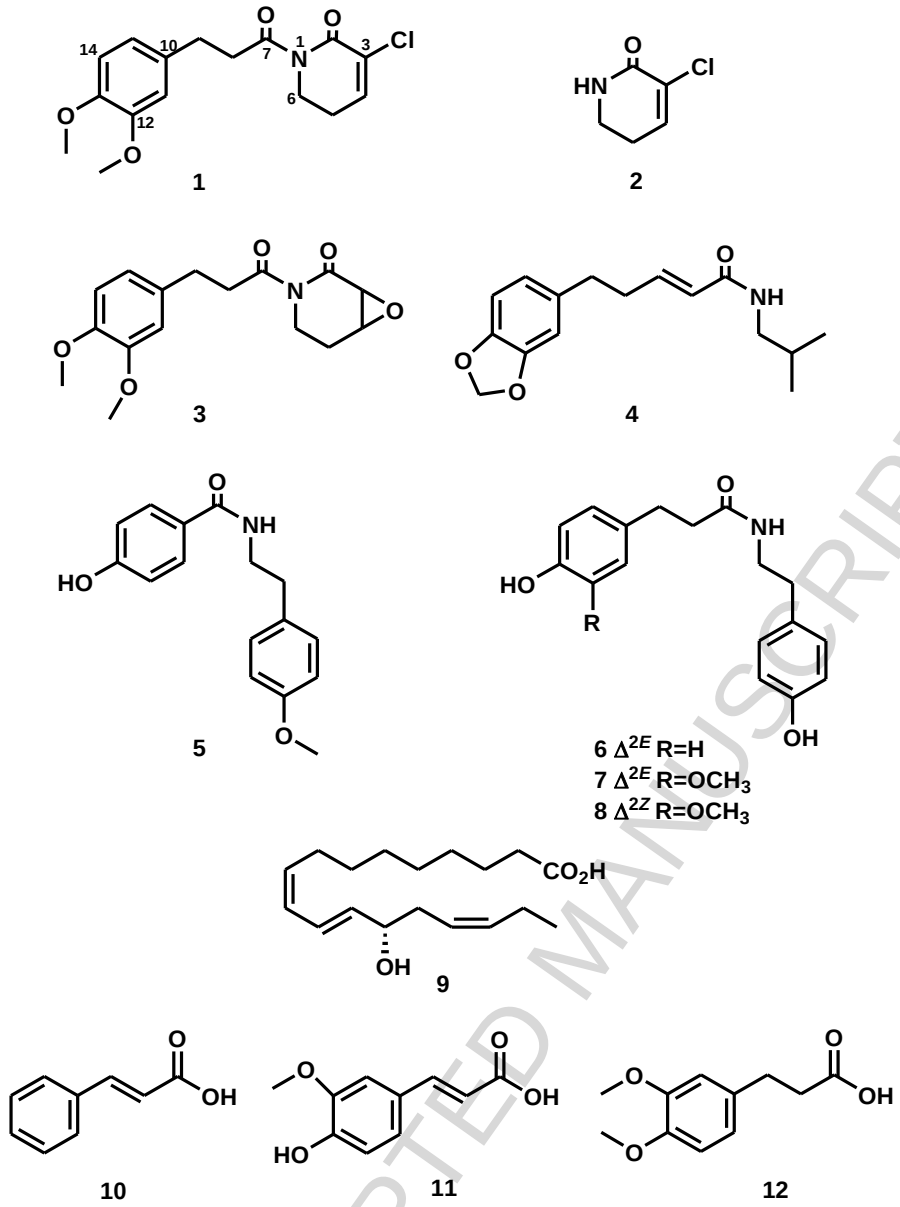


Figure 1. Chemical structure of compounds isolated from *Piper pseudoarboreum*

The structure of the new piperamide **1** was elucidated as follows. Compound **1** was obtained as brownish oil. Its molecular formula was determined to be $C_{16}H_{18}NO_4Cl$ by HREIMS, which showed the molecular ion peak at *m/z* 323.0916 $[M]^+$ (calcd for 323.0924), consistent with eight degrees of unsaturation. The EIMS gave a series of characteristic isotopic fragment peaks with the ratio of 3:1 at *m/z* 323/325, suggesting the presence of a chlorine atom. The UV absorption maximum at 226 and 276 nm indicated the presence of α,β-unsaturated carbonyl and aromatic chromophores in the molecule. The latter was also supported by the

absorption bands observed in the IR spectrum, which are characteristic of a conjugated carbonyl (1696 cm^{-1}) and an aromatic ring (1514, 1462, and 759 cm^{-1}). Two spin systems were identified by combined analysis of the $CDCl_3$ ^1H NMR spectrum (Table 1) and ^1H-^1H COSY experiment. The first one was identified as an A_2B_2 spin system for the C-8 and C-9 methylenes at δ_H 3.26 and δ_H 2.95 (each 2H, t, J = 7.6 Hz), whereas the second one was an A_2M_2X spin system with signals at δ_H 2.47 (2H, dt, J = 6.6, 4.6 Hz, H-5), 4.02 (2H, t, J = 6.6 Hz, H-6), and 7.03 (1H, t, J = 4.6 Hz, H-4). In addition, resonances for two methoxyl groups at δ_H 3.85 (3H, s) and 3.87 (3H, s) were observed in the ^1H NMR spectrum.

Table 1. ^1H and ^{13}C NMR data for compound **1** (δ, J are given in parentheses)

Position	δ_H [a]	δ_H [c]	δ_C [b]		**HMBC (H-C)**
2			161.0	C	
3			128.2	C	
4	7.03 (t, 4.6)	5.93 (t, 4.5)	140.9	CH	C-2, C-3, C-5, C-6
5	2.47 (td, 6.6, 4.6)	1.16 (qd, 6.2,4.5)	25.1	CH_2	C-3, C-4, C-6
6	4.02 (t, 6.6)	3.30 (t, 6.2)	41.1	CH_2	C-2, C-4, C-5, C-7
7			175.3	C	
8	3.26 (t, 7.6)	3.32 (t, 7.5)	40.9	CH_2	C-7, C-9, C-10
9	2.95 (t, 7.6)	3.03 (t, 7.5)	30.6	CH_2	C-7, C-8, C-10, C-11, C-15
10			133.4	C	
11	6.77 s	6.78 s	111.8	CH	C-9, C-10, C-12, C-13, C-15
12			147.3	C	
13			148.8	C	
14	6.78 *	6.59 (d, 8.4)	111.1	CH	C-10, C-12, C-13, C-15
15	6.78 *	6.79 (d, 8.4)	120.3	CH	C-9, C-10, C-11, C-13, C-14
12-OMe	3.85 s	3.40 s	55.9	CH_3	C-12
13-OMe	3.87 s	3.42 s	55.8	CH_3	C-13

[a] Measured at 600 MHz in $CDCl_3$. [b] Measured at 150 MHz in $CDCl_3$.
[c] Measured at 600 MHz in C_6D_6. *Overlapping signals.

To determine the substitution pattern of the aromatic ring, the ^{1}H NMR spectrum of compound **1** was recorded in C_6D_6. The spectrum displayed a typical ABC spin system at δ_H 6.59 (1H, d, *J* = 8.4 Hz, H-14), 6.78 (1H, s, H-11), and 6.79 (1H, d, *J* = 8.4 Hz, H-15), suggesting the presence of a 1,3,4-trisubstituted benzene ring. Its ^{13}C NMR (Table 1) spectrum displayed 16 carbon signals, and along with the HSQC experiment, provided resonances for a 1,3,4 trisubstituted aromatic ring at δ_C 148.8 (C-13), 147.3 (C-12), 133.4 (C-10), 120.3 (CH-15), 111.8 (CH-11) and 111.1 (CH-14); a trisubstituted double bond at δ_C 140.9 (CH-4) and 128.2 (C-3); and two carboxyl carbons at δ_C 175.3 (C-7) and 161.0 (C-2). In addition, the ^{13}C NMR spectrum showed resonances for two methoxy groups at δ_C 55.9 and 55.8, and four methylene groups at δ_C 41.1, 40.9, 30.6 and 25.1.

These data indicate that compound **1** is an alkamide with a piperine skeleton, containing one propionate group, two methoxy groups, one aromatic ring, and one lactam moiety. The spectroscopic data closely resembled those reported for the known compound sintenpyridone [30]. Consequently, the structure elucidation of this molecule was greatly aided by comparison of their spectroscopic data. Even so, it should be noted that a complete set of 1D and 2D NMR (COSY, ROESY, HSQC and HMBC) was acquired for **1** in order to gain the complete and unambiguous assignment of ^{1}H and ^{13}C NMR resonances as listed in Table 1. The placement of the functional groups in compound **1** was assigned based on the HMBC experiment. Thus, the cinnamoyl unit was established by the ^{3}J correlations between the proton signal at δ 2.95 (H_2-9) and the resonances at δ_C 175.3 (C-7), 133.4 (C-10), 111.8 (C-11) and 120.3 (C-15). The α,β-unsaturated δ-lactam was established by long-range correlations between the carboxyl resonance at δ_C 161.0 (C-2) and the signals at δ_H 7.03 (H-4) and δ_H 4.02 (H_2-6). Cinnamoyl and lactam units are linked through an amide function between the carboxyl carbon C-7 of the phenolic moiety and the nitrogen of the lactam unit. This attachment was defined by the observed cross-peak from the carboxyl signal at δ_C 175.3 (C-7) to the resonance at δ_H 4.02 (H_2-6), whereas the methyl signals of the methoxyl groups at

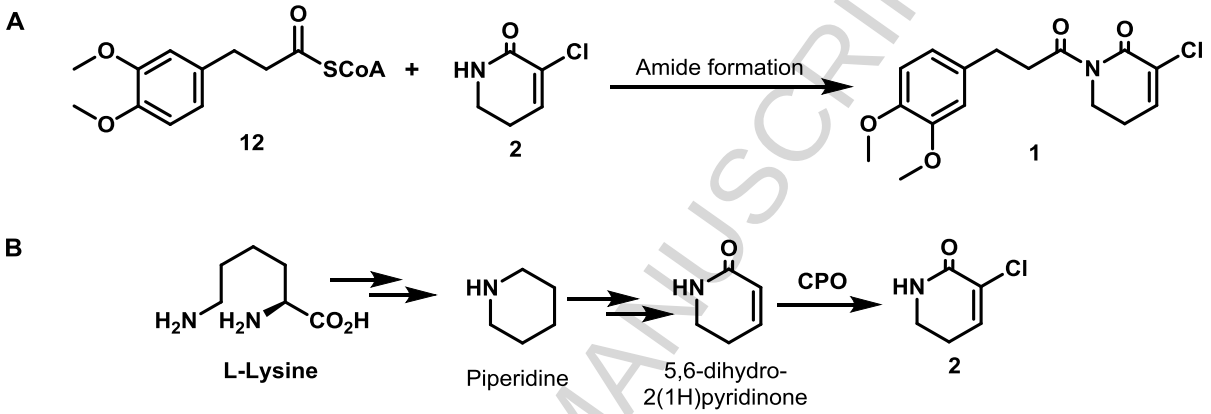
δ_H 3.85 and δ_H 3.87 were linked to the resonances at δ_C 147.3 (C-12) and δ_C 148.8 (C-13). All of these data, and comparison with those found in the literature for sintenpyridone [30], established the structure of compound **1** as *N*-(3,4-dimethoxy-3-phenyl-propanoyl)-3-chloro-Δ^3-pyridin-2-one, to which we have given the name 3-chlorosintenpyridone. Compound **1** is the first example for a naturally occurring chlorinated piperamide.

Compound **2** was isolated for the first time from a natural source, and is the second reported lactam-type chlorinated metabolite from *Piper* species [31]. The detailed ^{13}C NMR assignments of **2** [19], which have not been previously reported, were achieved by 1D and 2D techniques, including HMBC, HSQC and COSY experiments.

The distinctive feature of alkamides isolated from Piperaceae family is the presence of an aliphatic, cyclic or aromatic amine residue linked to a straight chain acid moiety with or without aromatic terminus, which is often cinnamic acid [32]. Moreover, the biosynthesis of natural piperine-type of amides isolated from Piperaceae species has been proposed to have L-lysine as basic precursor, whereas phenylalanine is the precursor of the acid moiety [33]. Based on this hypothesis, the biosynthetic pathway of compound **1** is proposed through an amide formation, as shown in Scheme 1. Thus, the tertiary amide unit in 3-chlorosintenpyridone (**1**) could be formed by reaction between the halogenated monocyclic lactam **2** and the 4-methoxy-dihydroferulic acid-coenzyme A ester derived from **12**. This is supported by the fact that precursors **2** and **12** are also isolated from this phytochemical study. Compound **2** is biogenetically derived from L-lysine, which is converted to piperidine, precursor of piperidone, and further catalysis by chloroperoxidase (CPO) in the last step of its biosynthesis yield **2**. Moreover, compound **12** could be derived from L-phenylalanine by the typical of shikimic acid pathway through cinnamic acid (**10**), which is the precursor of ferulic acid (**11**). Precursors **10** and **11** were also isolated from the plant (Scheme 1).

Furthermore, halogenated natural products have mainly been isolated from marine environments and microorganisms, and they are rare in terrestrial higher plants. Accordingly,

a diverse collection of enzyme catalysts to install and remove halogens from organic scaffolds has evolved in nature [34]. The unprecedented structure of organochlorine alkamide **1** suggests a biosynthetic halogenation to generate the chlorine-containing lactam **2** by regioselective chlorination, involving a halogenase as a biocatalyst [35]. This consideration may have some biogenetic implications and leads us to suggest that the enzymatic system in *P. pseudoarboreum* is quite different from other *Piper* species.



Scheme 1. (A) Proposed biogenesis of compound **1** from compounds **2** (B) and **12**.

Leishmaniasis is the second most widespread neglected tropical diseases, affecting millions of people worldwide [36]. In recent years, the situation has deteriorated in many ways, which together with current chemotherapy drawbacks, and the absence of vaccines mean that efforts are necessary to identify new classes of leishmanicidal agents. Consequently, there has been a recent increase in the use of plant derived natural products as source of leishmanicidal agents [37]. Compounds isolated from *P. pseudoarboreum* are closely related to those reported from *Piper tuberculatum*, which exhibit interesting antileishmanial profiles [38]. Furthermore, it has been suggested that the structural characteristic of alkamides confer on them specific biological activities [17]. This has encouraged us to explore the biological potential of the isolated compounds as leishmanicidal agents.

In this study, compounds **1-12** were evaluated for their growth inhibition activity against four promastigotes of *Leishmania* species: *L. amazonensis*, *L. braziliensis*, *L. guyanensis*, and *L. infantum*, as well as for cytotoxicity against J774 murine macrophages in order to determine possible toxicity (Table 2). Piperamide **1** was found to be the most active compound, with IC_{50} values of 3.4 and 3.7 µM against *L. braziliensis* and *L. guyanensis*, respectively, 6-fold more potent than Miltefosine (IC_{50} 17.7 and 19.4 µM, respectively) used as a reference drug. Also notable was the activity against *L. amazonensis*, which was 9-fold higher than the reference drug (IC_{50} 3.4 µM *vs* 30.7 µM). By contrast, its proposed precursors, **2** and **12** are inactive (IC_{50} > 50 µM), indicating that the assembly of both units in compound **1** impact positively on the antileshmanial activity.

Table 2. Leishmanicidal and cytotoxic activity of isolated compounds [a] from *Piper pseudoarboreum*.

Compounds	*L. amazonensis*		*L. braziliensis*		*L. guyanensis*		*L. infantum*		*J774*
	IC_{50} [b]	**SI** [d]	**IC_{50}**	**SI**	**IC_{50}**	**SI**	**IC_{50}**	**SI**	**CC_{50}** [c]
1	3.4±0.3	2.3	3.4±0.3	2.3	3.7±0.3	2.1	5.2±0.3	1.5	2.5±0.0
3	71.1±0.6	0.9	63.6±0.4	1.0	52.1±0.09	1.2	53.8±0.5	1.1	60.7±3.7
4	107.3±0.2	0.6	113.5±0.2	0.5	84.4±5.8	0.7	92.4±0.6	0.6	59.6±2.4
9	21.1±0.2	4.0	18.7±0.01	4.5	21.8±1.7	3.8	29.6±0.2	2.8	83.3±2.4
M [e]	30.7±0.9	4.4	17.7±0.4	7.7	19.4±1.2	7.0	17.7±1.8	7.7	136±10

[a] Compounds not include in the table were inactive (IC_{50} >100 µM for all *Leishmania* species). [b] IC_{50} (µM): concentrations able to inhibit 50% of the cells. [c] CC_{50} (µM): concentrations able to eliminate 50% of murine macrophages. [d] SI: selectivity index (CC_{50}/IC_{50}). [e] M: Miltefosine was used as a positive control.

Furthermore, the activity of compound **9** against the four promastigotes of *Leishmania* species (IC_{50} 18.7-29.6 µM) was close to that of Miltefosine (IC_{50} 17.7-30.7 µM). In addition, compounds **3** and **4** displayed weak activity, with IC_{50} values ranging from 52.1 µM to 113.5 µM against all the assayed *Leishmania* species. These findings highlight compound **1** and **9** and deserve further investigation against the clinically relevant amastigote forms. These results provide experimental evidence of the leishmanicidal potential of piperamides, which is

consistent with previous works showing that this type of natural compounds, such as piperine and piplartine, are potential leishmanicidal agents [39,40].

In summary, one new piperamide alkaloid with a unique chlorinated alkamide skeleton, and eleven known compounds were identified from *P. pseudoarboreum*. The putative biosynthetic formation of **1** from **2** and **12** is supported by the fact that these precursors are also present in the plant. Evaluation of the isolated compounds against a panel of promastigote stages of *Leishmania* revealed that compounds **1** and **9** are promising antileishmanial agents, showing higher to similar effectiveness than the known leishmanicidal drug Miltefosine. Thus, these experimental data reinforce the potential of *Piper* species as source of lead compounds and chemical diversity. In particular, alkamides could be a therapeutic alternative against neglected tropical diseases caused by protozoan parasites belonging to the genus *Leishmania*. Moreover, chlorine carbon skeletons in natural products from terrestrial plants are very rare, and, therefore, their isolation may be indicative of alternative pathways for piperamides' biosynthesis.

Conflict of interest

The authors declare no conflicts of interest

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Appendix A. Supplementary data

IR, 1D and 2D NMR spectra of compound **1**; EIMS and HREIMS spectra of compounds **1** and **2**. Supplementary data to this article can be found in the online version.

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