

Clusiaxanthone and Tocotrienol Series from *Clusia pernambucensis* and their Antileishmanial Activity

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A análise fitoquímica do extrato acetato de etila da casca do caule de *Clusia pernambucensis* G. Mariz, Clusiaceae, uma espécie do Cerrado brasileiro, conduziu ao isolamento e caracterização completa de uma nova xantona, 1,7-dihidróxi-2-(3-metil-2-butenil)-6',6'-dimetilpirano(2',3':3,4) xantona, denominada clusiaxantona. Quatro tocotrienóis ainda não relatados nesta espécie também foram isolados. Um derivado foi obtido a partir da clusiaxantona, 1-hidróxi,7-metóxi-2-(3-metil-2-butenil)-6',6'-dimetilpirano(2',3':3,4)xantona (7-O-metil-clusiaxantona), e um segundo derivado foi obtido a partir do ácido Z- δ -tocotrienolóico. As estruturas foram estabelecidas com base em dados de ressonância magnética nuclear de ¹H e ¹³C (NMR 1D e 2D), espectrometria de massa com ionização por *electrospray* de alta resolução (HRESIMS) e espectroscopia no infravermelho. No controle da infecção de macrófagos com amastigotas de *Leishmania (Leishmania) amazonensis*, os compostos ativos foram clusiaxantona e seu derivado (CI₅₀ = 66,9 e 57,4 μ M, respectivamente). A citotoxicidade dos compostos foi determinada em macrófagos peritoneais de camundongos BALB/c.

Phytochemical analysis of the ethyl acetate extract from the stem bark of *Clusia pernambucensis* G. Mariz, Clusiaceae, a Brazilian Cerrado species, led to the isolation and full characterization of a new xanthone, 1,7-dihydroxy-2-(3-methyl-2-butenyl)-6',6'-dimethylpyrano(2',3':3,4)xanthone, namely clusiaxanthone. Four previously unreported tocotrienols from this species were also isolated. A derivative was obtained from clusiaxanthone, 1-hydroxy,7-methoxy-2-(3-methyl-2-butenyl)-6',6'-dimethylpyrano(2',3':3,4)xanthone (7-O-methylclusi-xanthone), and an additional derivative was obtained from Z- δ -tocotrienoloic acid. The structures of these compounds were established based on data from ¹H and ¹³C nuclear magnetic resonance (1D and 2D NMR), high resolution electrospray ionization mass spectrometry (HRESIMS) and infrared spectroscopy. The clusi-xanthone and its derivative were able to control macrophage infection by *Leishmania (Leishmania) amazonensis* amastigotes (IC₅₀ = 66.9 and 57.4 μ M, respectively). The cytotoxicity of the compounds was assessed in BALB/c mouse peritoneal macrophages.

Keywords: *Clusia pernambucensis*, Clusiaceae, xanthone, tocotrienol series, *Leishmania (Leishmania) amazonensis*

Introduction

Cutaneous leishmaniasis is a worldwide endemic disease that causes skin lesions usually persisting for several months or years.¹ Patients also suffer because of the toxicity induced by prolonged use of antileishmanial drugs. The clinical symptoms of leishmaniasis are determined by the involved species of *Leishmania*, the parasite virulence and host immune response.² On account of the limited treatment options,³ new therapeutic solutions have been proposed, such as the use of miltefosine and paromomycin⁴ for the past twenty years.

Despite these efforts, the need for new drugs remains. However, progress in the development of new treatments has been slow.^{2,5-7} As previously reported, the screening for compounds from natural product libraries is a key strategy because many plants contain chemical compounds with powerful leishmanicidal activity.^{8,9} In addition, approximately 80% of the population in the Americas use plants to treat various diseases,¹⁰ including leishmaniasis.^{11,12}

Benzophenones, triterpenes and several flavonoids have been isolated from *Clusia pernambucensis*, *C. columnaris*, *C. grandiflora* and *C. spirictu-sanctensis*.^{13,14} These compounds have presented anti-inflammatory, antifungal, antibacterial,¹⁵ anti-HIV,¹⁶ antioxidant and antitumor activities.¹⁷⁻¹⁹ Peraza-Sanchez *et al.*²⁰ demonstrated the activity of the methanol extract of *C. flava* leaves, popularly used for treating wounds and syphilis, in promastigotes of *Leishmania major*. A Peru based research team supported the use of traditional medicine for the treatment of cutaneous leishmaniasis.¹¹ The group observed the activity of *C. amazonica* extracts in *L. amazonensis* axenic amastigotes.¹¹

In this study, we evaluated the potential antileishmanial effects of compounds and derivatives from *Clusia pernambucensis* G. Mariz, Clusiaceae against *Leishmania (Leishmania) amazonensis*, a parasite species that causes cutaneous leishmaniasis.

Results and Discussion

The fractionation of the ethyl acetate extract from the stem bark of *Clusia pernambucensis* led to the isolation of a previously unreported compound, clusiauxanthone (**1**), and six known compounds (**2-7**), δ -tocotrienol (**2**),²¹ δ -tocotrienolic alcohol (**3**),²² *Z*- δ -tocotrienoloic acid (**4**) and δ -tocotrienol methyl ester (**5**),²³ betulinic acid (**6**),^{24,25} and β -sitosterol (**7**)²⁶ (Figure 1). Compounds **2** and **5** were isolated from a natural source for the first time. For *Z*- δ -tocotrienoloic acid (**4**), the chemical shift of the methyl 12a' carbon (δ 12.21 ppm) under the protective effect of the γ -methylene carbon 10'

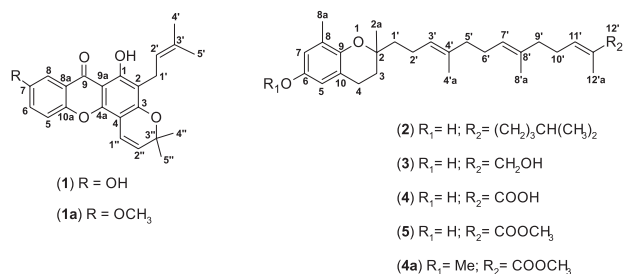


Figure 1. Structures of the isolated compounds **1-5** and semi-synthetic derivatives **1a** and **4a**.

(δ 27.78 ppm) contributed to the stereochemical identification of the carboxyl group. The structures of compounds **2-7** were determined by comparing the experimental data from the ¹H and ¹³C nuclear magnetic resonance (NMR) spectra and high resolution electrospray ionization mass spectrometric (HRESIMS) analyses with previously reported data. This was the first time compounds **2** to **5** were isolated from *C. pernambucensis*.

The methylation of clusiauxanthone and *Z*- δ -tocotrienolic acid resulted in the derivatives **1a**, 1-hydroxy,7-methoxy-2-(3-methyl-2-butenyl)-6',6'-dimethylpyrano(2',3':3,4) xanthone (7-*O*-methylclusiauxanthone), and **4a**, (2*E*,6*E*,10*E*)-methyl 13-(3,4-dihydro-6-methoxy-2,8-dimethyl-2*H*-chromen-2-yl)-2,6,10-trimethyltrideca-2,6,10-trienoate, respectively.

Clusiauxanthone (**1**) was isolated as a yellow solid with $[\alpha]_D -57.4$ (MeOH, *c* 0.11). The molecular formula for clusiauxanthone was determined as C₂₃H₂₂O₅ based on the quasi-molecular ion at *m/z* 379.1540 [M + H]⁺ (1 ppm error) in the HRESIMS spectrum. The vibrational bands at 3325 and 1647 cm⁻¹ in the IR spectrum were consistent with hydroxyl and carbonyl groups, respectively.

The ¹H NMR spectrum of clusiauxanthone (500 MHz, C₃D₅N) showed signals at δ_H 1.74 (s, 3H-4'), 1.96 (s, 3H-5'), 3.62 (d, *J* 7.5 Hz, 2H-1') and 5.49 (t', *J* 7.5 Hz, H-2), and correlations with the carbon signals (¹J_{CH}) at δ_C 18.5 (CH₃-5'), 26.2 (CH₃-4'), 22.3 (CH₂-1') and 123.4 (CH-2') in the HSQC spectrum, respectively, used to characterize the presence of a prenyl moiety. The presence of a 2,2-dimethyl-3,4-dehydropyran was also identified by the signals at δ_H 1.49 (s, 6H-3H4''/3H5''), 5.65 (d, H-2'', 10.0 Hz) and 6.97 (d, H-1'', 10.0 Hz), which showed correlations (¹J_{CH}) with the signals corresponding to carbons C-4''/C-5'' (δ 28.6), C-2'' (δ 116.5) and C-1'' (δ 128.2) in the HSQC spectrum, respectively. The signal at δ_H 13.86 (s) in the ¹H NMR spectrum suggested the presence of a chelatogenic hydroxyl group (HO-1) (Table 1).

The ¹³C NMR spectrum (125 MHz, C₃D₅N) of compound **1**, which was supported by the data from the ¹³C DEPT (distortionless enhancement by polarization)

Table 1. ^1H and ^{13}C NMR data of compounds **1** and **1a**, including the results of the HSQC and HMBC analyses^a

	1 , $\text{C}_5\text{D}_3\text{N}$				1a , $\text{CDCl}_3 + \text{CD}_3\text{OD}$	
	HSQC		HMBC		HSQC	
	$\delta_{\text{C}} / \text{ppm}$	$\delta_{\text{H}} / \text{ppm}$	$^2J_{\text{CH}} / \text{Hz}$	$^3J_{\text{CH}} / \text{Hz}$	$\delta_{\text{C}} / \text{ppm}$	$\delta_{\text{H}} / \text{ppm}$
1	155.2	–		2H-1'	156.0	–
2	107.9	–	2H-1'	H-2'	107.5	–
3	158.7	–		H-1''; 2H-1'	158.5	–
4	104.9	–	H-1''	H-2''	104.8	–
4a	156.6	–		H-1''	157.2	–
5	119.8	7.53 (d, 9.0)			119.7	7.23 (d, 3.0)
6	125.7	7.58 (dd, 9.0, 3.0)		H-8	129.9	7.19 (d, 9.0)
7	156.0	–	H-6; H-8	H-5	165.2	–
8	109.2	8.04 (d, 3.0)			110.7	7.10 (dd, 9.0, 3.0)
8a	121.8	–	H-8	H-5	122.2	–
9	181.9	–		H-8	183.0	–
9a	104.1	–			104.0	–
10a	150.5	–	H-5	H-6; H-8	148.6	–
1'	22.3	3.62 (d, 7.5)			22.2	3.43 (d, 7.4)
2'	123.4	5.49 (t, 7.5)	2H-1'	3H-4'; 3H-5'	123.7	5.23 (t, 7.4)
3'	131.8	–	H-2'; 3H-4'/3H-5'	2H-1'	131.2	–
4'	26.2	1.74 (brs)		H-2'	26.1	1.65 (brs)
5'	18.5	1.96 (brs)		H-2'	18.3	1.86 (brs)
1''	116.5	6.97 (d, 10.0)	H-2''		117.0	6.69 (d, 10.0)
2''	128.2	5.65 (d, 10.0)	H-1''	3H-4''/3H-5''	127.8	5.59 (d, 10.0)
3''	78.8	–	3H-4''; 3H-5''	H-1''	78.0	–
4''	28.6	1.49 (s)		H-2''; 3H-5''	28.7	1.48 (s)
5''	28.6	1.49 (s)		H-2''; 3H-4''	28.7	1.48 (s)
MeO	–	–	–	–	49.2	3.31 (s)
HO-1	–	13.86 (s)				

^aThe number of hydrogens bound to carbon atoms was determined by the comparative analysis of ^1H and DEPT ^{13}C NMR spectra. The chemical shifts and coupling constants (J) were obtained from the 1D ^1H NMR spectrum. A ^1H - ^1H -COSY experiment was also used to make the molecular assignments.

135 analysis, revealed 23 signals corresponding to twelve non-hydrogenated, six methine (all sp^2), one methylene (sp^3) and four methyl carbon atoms. Therefore, the expanded molecular formula $\text{C}_{23}\text{H}_{22}\text{O}_7$ was determined for compound **1** based on these data, and this formula is consistent with the chemical formula of $\text{C}_{23}\text{H}_{22}\text{O}_5$, which was determined by HRESIMS, with the presence of two ether functions.

For compound **1**, a signal at δ_{C} 181.9 was assigned to a carbonyl carbon (C-9), three signals were associated with mono-hydrogenated aromatic carbons at δ_{C} 109.2 (C-8), 119.8 (C-5) and 125.7 (C-6), and eight signals were correlated with non-hydrogenated aromatic carbons at δ_{C} 104.1 (C-9a), 104.7 (C-4a), 107.9 (C-2), 121.8 (C-8a), 155.2 (C-1), 156.6 (C-4a), 156.0 (C-7) and 158.7 (C-3).

The last four carbons represent oxygenated carbons. It was also possible to observe the presence of two olefin carbons at δ_{C} 123.4 (C-2') and 131.8 (C-3') associated with a tri-substituted double bond in the prenyl side chain, and two additional carbons at δ_{C} 116.5 (C-1'') and 128.2 (C-2'') of the pyran substituent. The location of the carbons in the xanthone skeleton and all the unequivocal chemical shift assignments were based on the heteronuclear long-range coupling ($^2J_{\text{CH}}$ and $^3J_{\text{CH}}$) between the hydrogen and carbon atoms as determined by the 2D HMBC spectrum. The location of the prenyl moiety at C-2 was indicated by the HMBC correlations between the methylene protons 2H-1' (δ_{H} 3.62) with both C-2' (δ_{C} 123.4, $^2J_{\text{CH}}$) and C-2 (δ_{C} 107.9, $^2J_{\text{CH}}$) carbons. The location of the prenyl moiety was also confirmed by correlations with both the C-1 (δ_{C} 155.2,

$^3J_{\text{CH}}$) and C-3 (δ_{C} 158.7, $^3J_{\text{CH}}$) carbons. The doublet at δ 6.97 (H-1") showed an HMBC cross-peak to the aromatic C-4 (δ_{C} 104.9, $^2J_{\text{CH}}$), C-4a (δ_{C} 156.6, $^3J_{\text{CH}}$) and C-3 (δ_{C} 158.7, $^3J_{\text{CH}}$) carbons, thus establishing the connectivity of C-1" with C-4. Other similar interactions are summarized in Table 1.

The methylation of compound **1** with an ethereal solution of diazomethane yielded the mono methyl ether **1a** and confirmed the presence of a hydroxyl group at C-7, which formed the methyl ether 7-*O*-Me derivative only. This was determined by its corresponding signal at δ_{C} 165.2 (Table 1). A singlet at δ_{H} 13.86 (*vide supra*) in the ^1H NMR spectrum of **1** (Table 1) indicated the presence of another hydroxyl group at C-1 that chelated the carbonyl carbon at C-9 and interfered with the methylation by diazomethane. Derivative **1a** had a molecular formula of $\text{C}_{24}\text{H}_{25}\text{O}_5$ determined by the protonated peak at m/z 393.1699 ($[\text{M} + \text{H}]^+$ calculated m/z 393.1702) in the HRESIMS spectrum.

Therefore, the structure of the new xanthone isolated from *Clusia pernambucensis* was determined as 1,7-hydroxy-2-(3-methyl-2-butenyl)-6',6'-dimethylpyrano(2',3':3,4) xanthone. This compound was named clusioxanthone (**1**). Unlike other reported related xanthones bearing two hydroxyl groups in the aromatic ring, this compound has one OH group less, similar to that identified in *Garcinia nigrolineata*.²⁷⁻³⁰

The basic core of this chemical class is a tricycle with a symmetrical skeleton. Ring A is formed from the acetate pathway, involving 1-4 carbon atoms, whereas ring B is composed of the 5-8 carbon atoms and is derived from the shikimic acid pathway.³¹

The EtOAc extract from the stem bark of *C. pernambucensis* exhibited an $\text{IC}_{50} = 65.0 \mu\text{g mL}^{-1}$ against *L. (L.) amazonensis* promastigotes. The antileishmanial activity of compounds **1-6** in peritoneal macrophages infected with amastigotes was also determined and indicated that clusioxanthone (**1**) displayed the most significant antileishmanial activity with an $\text{IC}_{50} = 66.9 \mu\text{M}$ (Table 2). The antileishmanial activity of derivative **1a** was similar to that of **1** with an $\text{IC}_{50} = 57.4 \mu\text{M}$.

Azebaze *et al.*³² showed that the activity of synthetic xanthones against axenic *L. amazonensis* amastigotes was increased in the presence of prenyl groups at positions 2 and 4, similar to the activity of the 2-substituted xanthone observed in this study. Hydroxylated and methoxylated xanthones (with structures similar to compound **1a**), which were isolated from *Andrographis paniculata* (Acanthaceae), showed activity against *L. infantum* amastigotes inside peritoneal macrophages.³³ Lipophilic groups play an important role in facilitating the compound

Table 2. Compound activities against *L. (L.) amazonensis* amastigotes IC_{50} , and cytotoxicity in murine macrophages CC_{50}

Compound	$\text{IC}_{50} / \mu\text{M}$	$\text{CC}_{50} / \mu\text{M}$
	<i>L. (L.) amazonensis</i>	murine macrophages
Clusioxanthone (1)	66.9 ± 6.1	45.5 ± 7.7
Compound 1a	57.4 ± 4.1	83.2 ± 8.4
δ -Tocotrienol (2)	> 200.0	> 400.0
δ -Tocotrienolic alcohol (3)	> 200.0	400.0
<i>Z</i> - δ -Tocotrienoloic acid (4)	181.0 ± 8.0	> 400.0
Compound 4a	> 200.0	> 400.0
δ -Tocotrienol-methyl-ester (5)	> 200.0	177.0 ± 16.1
Betulinic acid (6)	> 200.0	> 400.0
Amphotericin B	0.2 ± 0.1	–
Meglumine antimoniate	25.0 ± 2.2	–
Miltefosine	1.3 ± 0.5	–

internalization by macrophages, thereby increasing its availability.^{34,35}

According to the literature, the hydroxy-substituted xanthone nucleus shows potential for the development of active derivatives against *Leishmania* sp., which was observed for the 3,6-bis- ω -diethylaminoalkoxyxanthones tested in *L. mexicana*.³⁶ The results of this study confirm the data obtained in our study.

Monzote *et al.*³⁷ suggested that in *Leishmania* sp. amastigotes, mitochondrial complex III can be a target for compounds, such as tocotrienols, that contain chromanol groups. Among the tocotrienols studied here, only *Z*- δ -tocotrienoloic acid (**4**) showed an $\text{IC}_{50} = 181.0 \mu\text{M}$ against *L. (L.) amazonensis* amastigotes. The derivative compound **4a** did not show increased antileishmanial activity. Therefore, it is possible that the presence of the carboxylic group is beneficial to the antileishmanial activity because the change in substituent groups at C12' in compounds **2**, **3** and **5** failed to improve the antileishmanial activity of tocotrienol.

Conclusions

We isolated and extensively characterized one unreported xanthone (**1**) and a series of four known tocotrienols, δ -tocotrienol (**2**), δ -tocotrienolic alcohol (**3**), *Z*- δ -tocotrienoloic acid (**4**) and δ -tocotrienol methyl ester (**5**), which were previously undescribed in *C. pernambucensis*. Compounds **2** and **5** were isolated from natural sources for the first time, and two compounds, betulinic acid (**6**) and β -sitosterol (**7**), are ubiquitous in plants. Clusioxanthone (**1**) showed moderate activity against *Leishmania (Leishmania) amazonensis*.

Chemical studies to identify derivatives and analogs with lower cytotoxicity and improved antileishmanial activity are desirable. These compounds can be evaluated using an amastigote intramacrophage system. This system is advantageous since it takes into account the pharmacokinetic barriers that all compounds must overcome to reach the target parasite.^{38,39}

Our laboratory is focused on the development of antileishmanial candidates and is currently developing new xanthone derivatives that contain the original hydroxylated xanthone nucleus.

Experimental

General experimental procedures

Optical rotation data were collected on a DIP-370 polarimeter. For infrared analysis, the samples were embedded in KBr pellets under 10,000 kgf, and the experiment was performed with an infrared spectrophotometer (Perkin-Elmer RX-1). Fractionation was performed by column chromatography (7 × 100 cm) on silica gel (0.04–0.063 mm or 230–400 mesh). Thin layer chromatography (TLC) was performed in silica gel plates (60 F₂₅₄ aluminum, Merck).

¹H and ¹³C NMR spectra were recorded at room temperature on a Bruker Avance DRX 500 (500.13 and 125.77 MHz) spectrometer using an inverse detection probe. The chemical shifts are expressed in parts *per* million (ppm) relative to tetramethylsilane (TMS), and the coupling constants (*J* values) are shown in hertz (Hz).

HRESIMS spectra were recorded on Bruker UltrTOF and MicrTOF spectrometers. Electrospray ionization was performed using a time of flight analyzer (ESI-TOF-MS) in both the positive and negative modes.

Analytical high-performance liquid chromatography (HPLC) of the samples was performed on a Waters model 1525 coupled to a UV photodiode array detector (190–800 nm), series 2996, thermostatic oven and Phenomenex silica column (4.6 × 250 mm, 5 μm). The mobile phase consisted of hexane-ethyl acetate pre-filtered through 0.45 μm nylon membranes, vacuum degassed and sonicated for 15 min. Reversed phase-HPLC was performed on a Shimadzu SL6Vp equipment coupled to a UV photodiode array detector (190–800 nm) equipped with a degasser. A Shimadzu octadecylsilane column (4.6 × 250 mm, 5 μm) with an injection volume of 20 μL (2 mg mL⁻¹) and flow rate of 1 mL min⁻¹ was used in the analytical mode. The separation was achieved on a Shimadzu preparative octadecylsilane (20 × 250 mm, 10 μm) column with an injection volume of 1 mL (20 mg mL⁻¹) and a flow rate of 9 mL min⁻¹. The compound detection was monitored at 254 nm.

Plant material

The stem bark of *Clusia pernambucensis* G. Mariz, Clusiaceae, was collected from the gallery forest of the Cerrado biome, Santo Antônio do Descoberto, Goiás State, at an altitude of 1055 metres; south latitude 15°48'35.0"; south longitude 48°20'12.0". The identification of the plant was confirmed by Professor Dr. José Elias de Paula, and a voucher specimen was deposited in the Herbarium of the University of Brasília under the accession number (UB) 3771.

Extraction and isolation

C. pernambucensis extract (22 g) was obtained by macerating 197.11 g of *C. pernambucensis* stem bark with ethyl acetate. From this extract, 14.15 g were fractionated into 312 fractions of 100 mL by column chromatography with a cyclohexane-EtOAc polarity gradient (100:0; 99:1; 98:2; 97:3; 95:5; 93:7; 90:10; 85:15; 80:20; 75:25; 70:30; 60:40; 50:50; 0:100), and sequentially with an EtOAc-MeOH gradient (100:0; 90:10; 85:15; 80:20; 75:25; 70:30; 60:40; 50:50; 0:100). Based on the TLC profile, the fractions were assembled in 21 fractions (CP1-CP21). Fraction CP15 was purified on a silica column to obtain compound **1** (36.2 mg, 0.2 %). The compound detection was monitored at 254 nm. The purification of fraction CP19 was performed using an HPLC Shimadzu, model SL6Vp equipped with an octadecylsilane-C₁₈ (20 × 250 mm, 10 μm) preparative column. The eluent consisted of an isocratic ternary mixture of H₂O:MeOH:MeCN (8:32:60). The samples were injected in a volume of 1 mL (20 mg mL⁻¹) with a flow rate of 9 mL min⁻¹, and the compound detection was monitored at 190, 209 and 254 nm. At the end of this process, four compounds were isolated, δ-tocotrienol (**2**, 1.4 mg, 0.009 %), δ-tocotrienolic alcohol (**3**, 10.2 mg, 0.07%), *Z*-δ-tocotrienoloic acid (**4**, 85.3 mg, 0.6%) and δ-tocotrienol-methyl ester (**5**, 0.6 mg, 0.004%). Betulinic acid (**6**, 100 mg, 0.7%) and β-sitosterol (**7**, 20.2 mg, 0.1%) were also isolated.

A methyl ether derivative (**1a**) of clusiaxanthone (**1**) was produced by methylation of **1** with diazomethane. Compound **4a** was obtained by the methylation of a hydroxyl and terminal carboxyl of *Z*-δ-tocotrienoloic acid (**4**).

1,7-Dihydroxy-2-(3-methyl-2-butenyl)-6',6'-dimethylpyrano(2',3':3,4)xanthone (**1**): yellow solid; [α]_D -57.4 (MeOH, *c* 0.11); IR ν/cm⁻¹ 3324, 2983, 2962, 2909, 2857, 1700, 1647, 1480, 1465, 1357; ¹H and ¹³C NMR (500.13 and 125.77 MHz, C₅D₅N, δ/ppm) see Table 1; HRESIMS (positive mode) *m/z* found: 379.1540 [M + H]⁺; calc *m/z* for [C₂₃H₂₃O₅]⁺ = 379.1545.

1-Hydroxy,7-methoxy-2-(3-methyl-2-butenyl)-6',6'-dimethylpyrano(2',3':3,4)xanthone (**1a**): yellow solid. ^1H and ^{13}C NMR (500.13 and 125.77 MHz, $\text{C}_5\text{D}_5\text{N}$, δ/ppm) see Table 1. HRESIMS (positive mode) m/z found: 393.1699 $[\text{M} + \text{H}]^+$; calc m/z for $[\text{C}_{24}\text{H}_{25}\text{O}_5]^+ = 393.1702$.

Leishmania (Leishmania) amazonensis promastigote culture

C57BL/6 mice were infected with *Leishmania (Leishmania) amazonensis* (strain L(L)a)-MHOM/BR/PH8) and maintained in the biotherium at the Faculty of Health Science/Medicine (University of Brasília, Brazil). Blood was collected by puncture of the hind paw of infected animals. The parasites were incubated in McNeal, Novy and Nicolle (NNN) culture medium pH 7.2 at 22 °C for seven days, and subsequently in Schneider's medium supplemented with 20% fetal bovine serum and 0.25% gentamicin pH 7.2 at 22 °C.

Compound evaluation in *L. (L.) amazonensis* promastigotes

Sterile Schneider's medium (Sigma-Aldrich) was added to each well of a 96 well cell culture plate. Promastigotes (10^5) were then added to the wells and counted in a Neubauer chamber. Serial dilution of the compounds was performed starting at an initial concentration of 300 $\mu\text{mol L}^{-1}$, with 0.1% DMSO (dimethyl sulfoxide) as a negative control. The cell culture plate was incubated for 48 h at 22-26 °C. Next, 100 $\mu\text{g per well}$ of a solution of 5 mg mL^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in PBS (phosphate buffered saline, Sigma-Aldrich) was added to the wells for 4 h at 37 °C. Viability measurements were performed after the addition of 100 μL DMSO to solubilize the formazan crystals.⁴⁰⁻⁴² In this experiment, we used promastigotes in log phase growth to optimize the spectrophotometer reading at 570 nm. The experiments were performed in triplicate, and the IC_{50} values were calculated using Microsoft Excel[®].

Compound evaluation in *L. (L.) amazonensis* amastigotes

Peritoneal macrophages from BALB/c mice were collected and centrifuged in RPMI 1640 (Gibco) medium at 1190 rpm or 300 g. The pellets were recovered and resuspended to count the viable cells by trypan blue (Sigma) staining in a Neubauer chamber. Glass coverslips (13 mm) were placed into a 24 well plate with 4 \times 10^5 macrophages *per well* for 24 h at 37 °C in a 5% CO_2 atmosphere. After the incubation period, the non-adherent

cells were removed and the macrophages were incubated with *L. (L.) amazonensis* promastigotes in stationary phase at a ratio of 10:1 for 4 h at 37 °C in a 5% CO_2 atmosphere. The promastigote-free supernatant was removed, and the infected macrophages were treated with the compounds at initial concentrations of 300 $\mu\text{mol L}^{-1}$ for 48 h. The cells were subsequently harvested, fixed with methanol and stained with Giemsa stain. Miltefosine (Sigma-Aldrich), amphotericin B (Sigma-Aldrich), and meglumine antimoniate (Glucantime[®], Aventis Pharma Brazil; 300 mg mL^{-1} , 81 mg mL^{-1} Sb (V)) at concentrations of 30.0 to 0.056 $\mu\text{mol L}^{-1}$ were used as positive controls, and 0.1% DMSO in a culture medium with amastigotes as a negative control. For each concentration of compound, 200 macrophages were counted using an optical microscope (Olympus). The number of amastigote-infected cells was calculated compared to the negative control.^{43,44} The tests were performed in triplicate.

Evaluation of compound cytotoxicity in murine macrophages

After the peritoneal macrophages were isolated from BALB/c mice, 10^6 cells were placed in each well of a 96 well microplate. The cells were incubated for 12 h at 37 °C in a 5% CO_2 atmosphere. The culture medium was then replaced with the diluted compounds in new culture medium, and the cells were then incubated at 37 °C in a 5% CO_2 atmosphere. A solution of 0.1% DMSO was used as the negative control. After 24 h, cell viability was determined by adding 100 $\mu\text{g per well}$ MTT in PBS (5 mg mL^{-1}) for 4 h. The culture medium was removed and 100 μL DMSO was added. The formazan was quantified in a spectrophotometer at 570 nm.^{45,46}

Supplementary Information

^1H and ^{13}C NMR, HSQC, HMBC and ^1H - ^1H COSY spectra and HRESIMS spectra of clusixanthone and derivative **1a** are available free of charge at <http://jbc.sbbq.org.br> as PDF file.

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