

Aurapten, a coumarin with growth inhibition against *Leishmania major* promastigotes

H.B. Napolitano¹, M. Silva¹,
J. Ellena¹, B.D.G. Rodrigues¹,
A.L.C. Almeida², P.C. Vieira²,
G. Oliva¹ and
O.H. Thiemann¹

¹Laboratório de Proteína, Cristalografia e Biologia Estrutural,
Instituto de Física de São Carlos, Universidade de São Paulo, São Carlos,
SP, Brasil
²Departamento de Química, Universidade Federal de São Carlos, São Carlos,
SP, Brasil

Abstract

Correspondence

O.H. Thiemann
Laboratório de Proteína,
Cristalografia e Biologia Estrutural
Instituto de Física de São Carlos
USP
Av. Trabalhador São-carlense, 400
13566-590 São Carlos, SP
Brasil
Fax: +55-16-273-9881
E-mail: thiemann@if.sc.usp.br

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Several natural compounds have been identified for the treatment of leishmaniasis. Among them are some alkaloids, chalcones, lactones, tetralones, and saponins. The new compound reported here, 7-geranyloxycoumarin, called aurapten, belongs to the chemical class of the coumarins and has a molecular weight of 298.37. The compound was extracted from the Rutaceae species *Esenbeckia febrifuga* and was purified from a hexane extract starting from 407.7 g of dried leaves and followed by four silica gel chromatographic fractionation steps using different solvents as the mobile phase. The resulting compound (47 mg) shows significant growth inhibition with an LD₅₀ of 30 µM against the tropical parasite *Leishmania major*, which causes severe clinical manifestations in humans and is endemic in the tropical and subtropical regions. In the present study, we investigated the atomic structure of aurapten in order to determine the existence of common structural motifs that might be related to other coumarins and potentially to other identified inhibitors of *Leishmania* growth and viability. This compound has a comparable inhibitory activity of other isolated molecules. The aurapten is a planar molecule constituted of an aromatic system with electron delocalization. A hydrophobic side chain consisting of ten carbon atoms with two double bonds and negative density has been identified and may be relevant for further compound synthesis.

Key words

- Coumarin
- Aurapten (7-geranyloxycoumarin)
- X-rays
- *Leishmania*
- Inhibitor

Introduction

Leishmaniasis is a disease caused by a protozoan parasite of the order Kinetoplastida. According to the World Health Organization (1), 12 million infected people exist in 88 countries, with approximately 350 million people being at risk. In view of the lack of safe drugs and the serious secondary effects caused by available chemotherapy (2), there is a need

for new drugs for the treatment of leishmaniasis infections. Therefore, the discovery of novel classes of inhibitors can be an important step which contributes to overcoming the drug resistance of *Leishmania* (3).

The use of phytotherapy to treat human diseases has its roots in pre-historical times and most of the effective drugs currently available are obtained from plants (4). The biodiversity existing in Brazilian plants is a poten-

tial source of many new bioactive molecules to be studied and explored (5).

In a search for new bioactive substances potentially useful against leishmaniasis, we used *Leishmania major* Friedlin (WHO MHOM/IL/80/Friedlin) cells as a model system to screen small molecule compounds obtained from Brazilian plants. The screening was done using an *in vivo* culture assay in the presence or absence of the purified compound and the parasite growth rate was monitored microscopically. This approach permitted us to identify a new compound which was subsequently shown to be 7-geranyloxy coumarin (aurapten). The compound belongs to the class of coumarins and shows significant growth inhibition of *L. major* promastigotes at concentrations of micrograms per ml.

Material and Methods

Extraction of the plant inhibitor

The leaves of *Esenbeckia febrifuga* (Rutaceae) were collected in Piracicaba, SP, Brazil, by Prof. Ricardo Ribeiro Rodrigues (Escola Superior de Agricultura Luiz de Queiroz, ESALQ, Code No. 84295) in February 2000 and extracted according to the procedure illustrated in Figure 1 and described below.

Ground leaves (407.7 g) were submitted to a first extraction step with 4.5 liters hexane, resulting in 9.5 g of extract after the solvent was removed. The extract was fractionated by silica gel chromatography through a 30 cm high column measuring 5 cm in diameter using a hexane/ethyl acetate (100:0, 9:1, 8:2, 7:3, 1:1, 0:100) and an ethyl acetate/methanol (100:0, 9:1, 7.5:2.5, 6:4, 1:1, 0:100) gradient system. From this purification, 30 fractions of 25 ml each were collected. Fraction 14 (hexane:ethyl acetate, 8:2) was further fractionated on a 3 x 26 cm silica gel column using a gradient system with hexane, methylene chloride and methanol (hexane, 100%; hexane/methylene chloride, 100:0, 90:10, 80:20, 70:30, 1:1; methylene chloride/methanol, 100:0,

90:10, 70:30, 1:1, 0:100). The 74 fractions (25 ml each) were collected and, based on thin-layer chromatography (TLC) on silica gel (0.25-mm thick silica gel, Merck PF254 (Darmstadt, Germany), hexane/methylene chloride/acetone, 3:1:0.5), and detected with vanillic acid, UV light at 254 and 360 nm and Dragendorff reagent, eight fractions were pooled. Fraction four was submitted to chromatographic separation on a 2 x 31 cm silica gel column eluted isocratically with hexane, methylene chloride and acetone (3:1:0.5). This step resulted in 87 fractions (25 ml each), twelve of which were pooled after TLC analysis as above. Fraction two was submitted to chromatographic separation on a 1 x 20 cm silica gel column using methylene chloride as the mobile phase and resulted in 24 fractions (25 ml each), four of which were pooled after TLC analysis. Fraction four resulted in an amorphous yellow solid (47 mg), which is 7-geranyloxy coumarin (aurapten), according to NMR ^1H , ^{13}C spectra (named after the standard chemical nomenclature according to IUPAC rules) and GC/MS, that was crystallized by vapor diffusion, at room temperature, from hexane/methylene chloride (2:1). The purity of the compound was confirmed by TLC (0.25-mm thick silica gel), NMR and GC/MS.

Parasite assays

Stock solutions of aurapten were prepared in dimethyl sulfoxide (DMSO) at 5.0 mg/ml. Further dilutions of aurapten were made directly in the *L. major* culture medium immediately before use. In all experiments the final concentration of DMSO was less than 0.5% (v/v), a concentration that does not affect parasite growth rate, mobility or morphology (6).

L. major-Friedlin promastigotes were grown to a concentration of 1.5×10^4 cells/ml in M199 (Gibco Laboratories, New York, NY, USA) supplemented with 10% fetal bovine serum, 10 mM adenine, 10 mg/ml penicillin, 10 mg/ml streptomycin, 0.25% hemin chlo-

ride, 0.1% biotin, and 1.0 M HEPES, pH 7.5. The parasite cultures were prepared with or without aurapten at final concentrations of 2.0, 5.0, 7.0, 10.0, and 15.0 $\mu\text{g/ml}$. The effect of aurapten on parasite growth was monitored by counting in triplicate in a Neubauer chamber after 48-h incubation at 26°C.

Crystallographic characterization of aurapten

Aurapten was crystallized from hexane/methylene chloride (2:1) by vapor diffusion at room temperature. The typical crystal was yellow and was needle shaped. X-ray diffraction was carried out using a Nonius KappaCCD diffractometer at room temperature. The structure was solved by direct methods using the SHELXL97 software (7) and was refined anisotropically with full-matrix least-squares on F^2 using SHELXL97 (7). The hydrogen atoms were placed at the calculated position, except for those involved in H-bonds, found on difference maps and further refined. Final indices were: $R_1(F_o) = 0.0895$ and $wR_2(F^2) = 0.2723$ for 165 refined parameters. The crystallographic parameters for $\text{C}_{19}\text{H}_{22}\text{O}_3$ are: molecular weight = 298.37, orthorhombic, space group Pnma [No. 62], $Z = 4$, $a = 6.557$ (1) Å, $b = 7.117$ (2) Å, $c = 35.650$ (2) Å, $V = 1664.0$ (9) Å³, $d_c = 1.191$ Mg.m⁻³, λ (Mo K α) = 0.71073 Å, $\mu = 0.104$ mm⁻¹, 3205 measured reflections, 1616 unique ($R_{\text{int}} = 0.0301$), 1579 of which were considered for calculation purposes as observed with an $I \geq 2\sigma$ (I). The crystallographic data were deposited in the Cambridge Crystallographic Data Centre (deposit@ccdc.cam.ac.uk) under accession number 232201.

Results and Discussion

We investigated and characterized the anti-proliferative activity of the coumarin (7-geranyloxycoumarin), or aurapten, with a molecular weight of 298.37 against *L. major* Friedlin promastigotes in axenic cultures. As shown in Figure 2, *L. major* promastigote growth was significantly inhibited by aurapten,

with an LD_{50} of 30 μM . This value represents a significant inhibitory activity against *L. major* and is similar to those obtained for other inhibitors isolated from other plants (8). The negative control, corresponding to the parasite in the presence of DMSO, the aurapten sol-

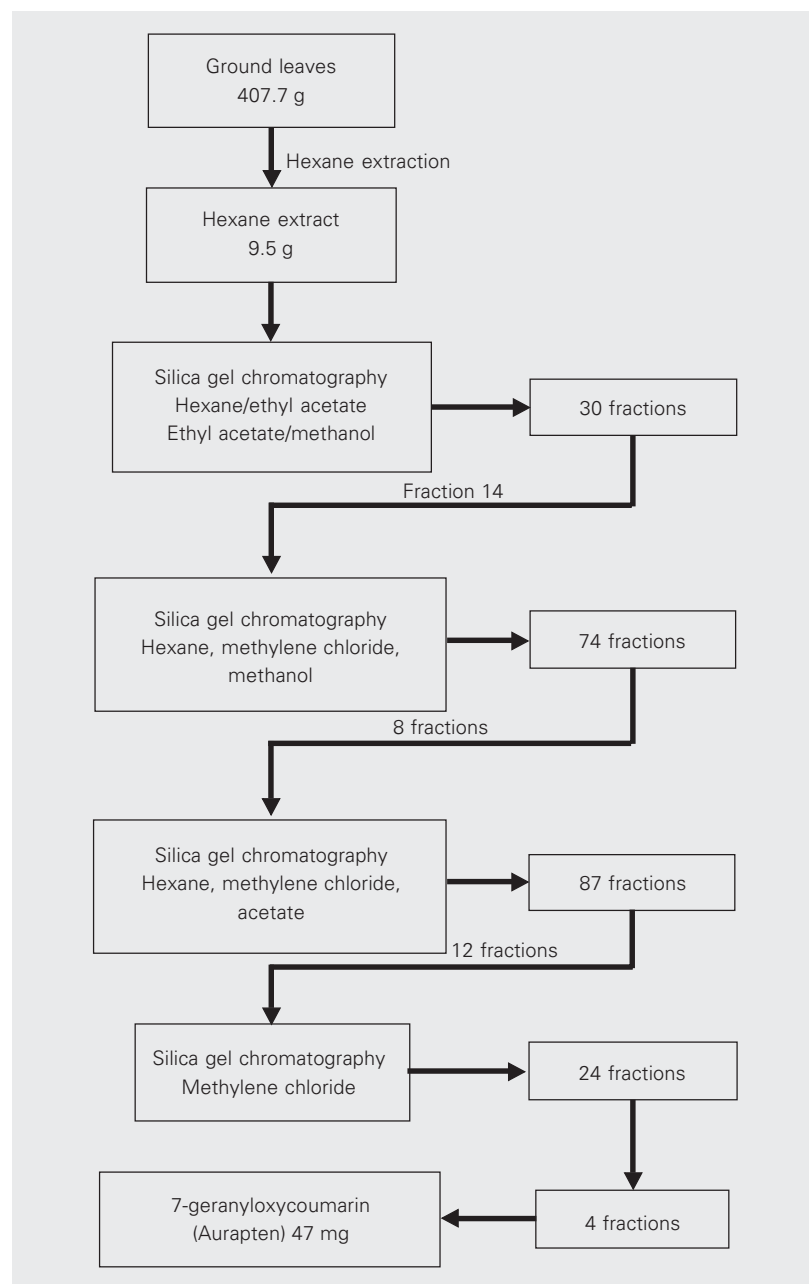


Figure 1. Flow diagram of the aurapten isolation procedure. Individual steps are described in Material and Methods. The solvents used for elution of the chromatography column are listed in the boxes on the left side. Silica gel column effluents were monitored by thin-layer chromatography to select fractions for further purification.

Figure 2. Antileishmanial activity of auraptin against *Leishmania major* Friedlin promastigotes. Cells were cultivated in the presence of different concentrations of auraptin and counted after 48 h. The height of the bars indicates the percentage of growth inhibition at each concentration compared to the control experiment containing only the auraptin solvent DMSO. The experiments were performed three times independently and each counting was performed in triplicate. Data are reported as means \pm SD.

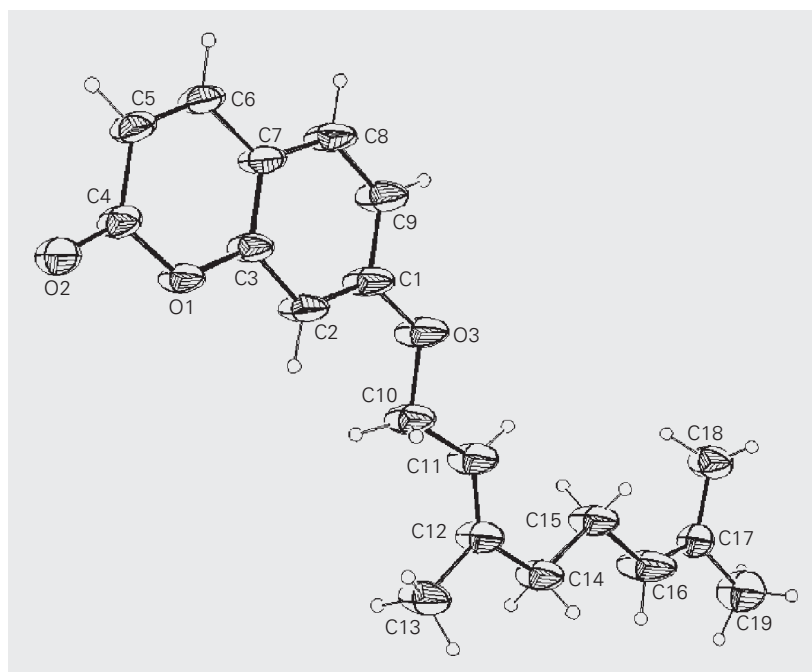
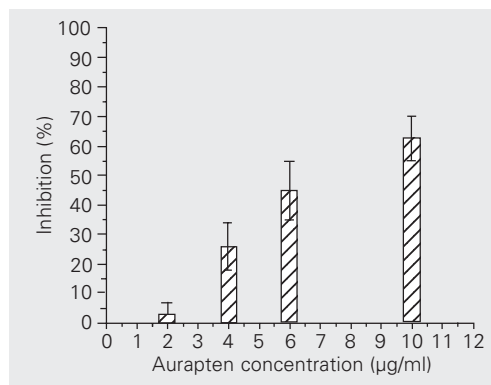
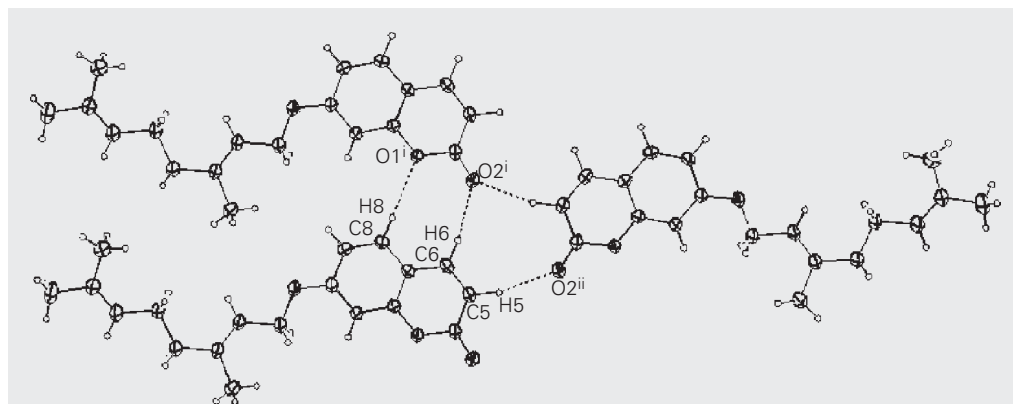


Figure 3. X-ray crystallographic structure of the auraptin. Displacement ellipsoids are shown at the 30% probability level. Carbon (C) and oxygen (O) atoms are labeled for clarity.

Figure 4. A view of auraptin's packing showing C-H...O interactions. Symmetry codes (i) and (ii) correspond to $[1 + x, y, z]$ and $[0.5 + x, y, 1.5 - z]$, respectively.



vent, showed no detectable inhibition of growth, alteration in cell morphology or motility at the concentrations tested.

Most studies directed at the detection of plant secondary metabolites with leishmanicidal activity have been done using the promastigote form of the parasite because of its easier maintenance under *in vitro* conditions. However, since the promastigote is not the infective form of the parasite in vertebrate hosts, these evaluations have only a suggestive value of the possible leishmanicidal activity of the compound tested.

There are several active compounds obtained from medicinal plants traditionally used worldwide for the treatment of leishmaniasis. Among these active molecules, described in the recent literature are quinoline alkaloids, isoquinoline alkaloids, indole alkaloids, terpenes, acetogenins, and lignans (9). Other natural compounds with antileishmanial activity are coumarins, chalcones, lactones, tetralones, and saponins (9). The compound described in the present study belongs to the class of the coumarins and its activity against *Leishmania* promastigotes is reported here for the first time.

To unambiguously assign the structure of this inhibitor and to gain insight into common structural motifs related to other coumarins and other leishmania inhibitors, its crystal structure was determined, and several structural features emerged. This new information will be valuable for the development of a new anti-

Leishmania drug since resistance to the currently available drugs such as antimonials and amphotericin B has emerged (3).

The refined molecular structure together with the atom-numbering scheme is shown in Figure 3. The molecule is a derivative of cinnamic acid that contains an aromatic system with electron delocalization. There is also a hydrophobic side chain consisting of ten carbon atoms with two double bonds with negative density. All bond distances and angles are close to normal values (10,11) and the molecule is planar, as can be seen in Figure 3. Three double weak intermolecular interactions of type C-H...O support the molecular packing (12) shown in Figure 4. They are the intermo-

lecular C8-H8...O1ⁱ and C6-H6...O2ⁱ [symmetry code: i) 1 + x, y, z] hydrogen bonds between two neighbor molecules. The distance and dihedral angles between donor and acceptor are 3.38 (1) Å, 180 (5)° and 3.27 (1) Å, 172 (5)°, respectively. The last of the intermolecular hydrogen bonds C5-H5...O2ⁱⁱ [symmetry code: ii) 0.5 + x, y, 1.5 - z] linking these two molecules shows 3.34 (1) Å of distance and 163 (3)° of dihedral angle between donor and acceptor. The planarity shown in the molecular configuration and the intermolecular interactions are consistent with typical structural features observed in anti-*Leishmania* inhibitors (13-15).

Figure 5 shows the *L. major* culture in the

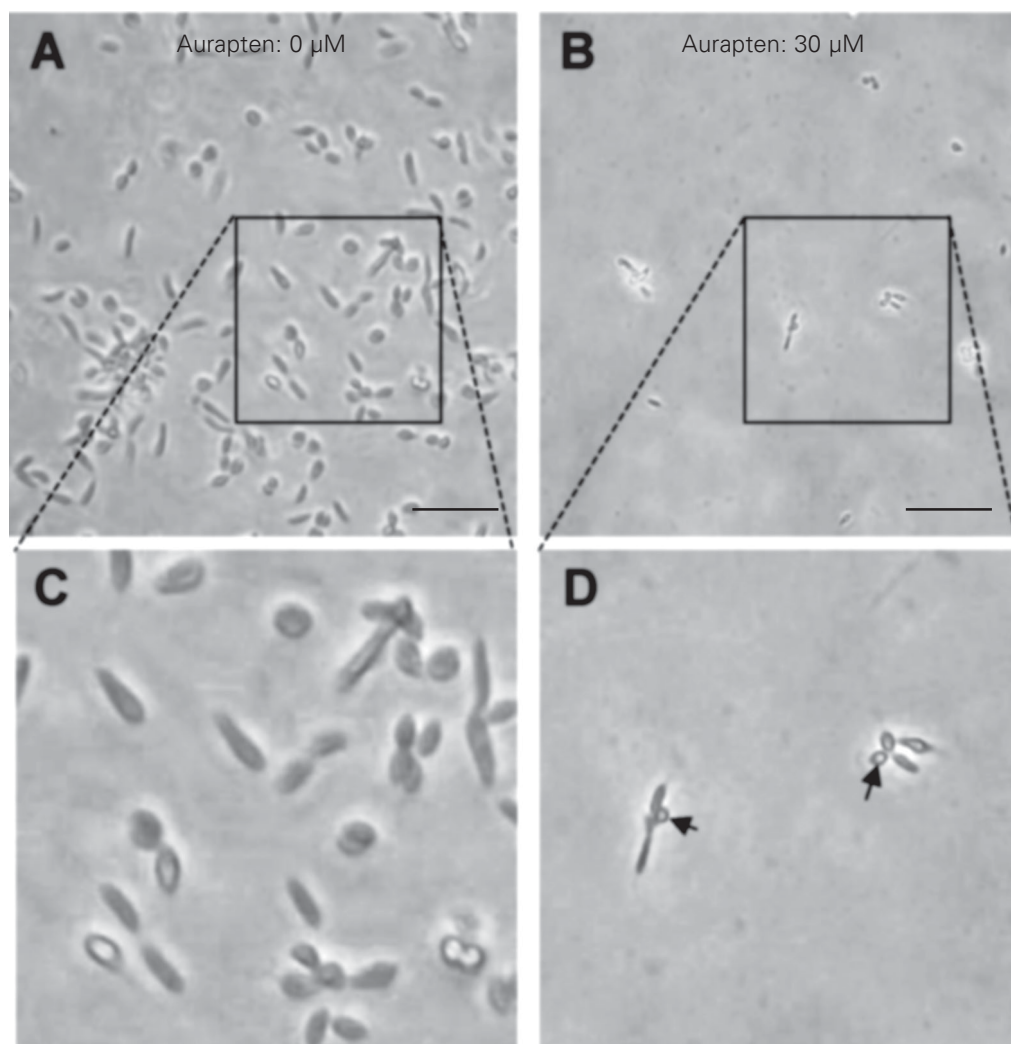


Figure 5. *Leishmania major* promastigote culture. Promastigotes were cultured in M199 medium supplemented with 10% fetal bovine serum at 26°C and cell viability was estimated by counting in a Neubauer chamber after 48 h of incubation in the presence or absence of the inhibitor. A, Negative control without auraptin in the presence of 0.5% DMSO. B, Condition containing 30 μM auraptin and 0.5% DMSO. C and D are expansions of Figures A and B, respectively (indicated by the squares in A and B), showing the different morphological characteristics of the parasite in the presence of auraptin. Vacuoles are indicated by arrows. Bar = 16.6 μm.

presence and absence of the inhibitor. Forty-eight hours after inoculation, a significant decrease in surviving promastigotes was observed in the presence of aurapten (Figure 5B) at a final concentration of 30 μ M. The surviving parasites showed abnormal morphology with the presence of large vacuoles and low flagellum motility. This preliminary evaluation of aurapten's activity using promastigotes yielded interesting results indicating that the compound should be investigated further as a potential anti-*Leishmania* compound.

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