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Klaivanolide, an antiprotozoal lactone from *Uvaria klaineana*

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Abstract

Bioguided-fractionation of a CH_2Cl_2 extract of the stems of *Uvaria klaineana* (Annonaceae) led to isolation of klaivanolide, a novel bisunsaturated 7-membered lactone (5-acetoxy-7-benzoyloxymethyl-7*H*-oxepin-2-one), together with benzyl benzoate. Klaivanolide showed potent in vitro antileishmanial activity against both sensitive and amphotericin B-resistant promastigote forms of *Leishmania donovani* with IC_{50} values of 1.75 and 3.12 μM, respectively. The compound also showed in vitro trypanocidal activity against trypomastigote forms of *Trypanosoma brucei brucei* GVR 35. Its structure was established by 1D and 2D NMR and other spectroscopic techniques.

Keywords: *Uvaria klaineana*; Klaivanolide; Lactone; *Leishmania donovani*; *Trypanosoma brucei brucei*; Antileishmanial; Trypanocidal

1. Introduction

Leishmaniasis and trypanosomiasis are major parasitic diseases widely distributed throughout the world. Clinical resistance to pentavalent antimonial drugs is increasing and pentamidine and amphotericin have toxic side effects in therapeutic doses used to treat leishmaniasis. Trivalent arsenical agents (melarsopol, Arsobal®) used in stage II of African trypanosomiasis have side effects including encephalopathy which can be lethal in 10% of cases. The resistance of *Trypanosoma brucei gambiense*, one of the African species, to melarsopol is also increasing (Cohen, 2000), leading to the need of new antiprotozoal compounds.

Uvaria species are said to possess antiparasitic activities but extracts and pure compounds have generally been tested for antiplasmodial activity (Nkunya et al., 1991). In the course of our search for antiparasitic activities of Gabonese Annonaceae, extracts of *Uvaria klaineana* stems were tested in vitro against *Leishmania donovani* promastigote forms and *Trypanosoma brucei brucei* trypomastigote forms.

Uvaria klaineana Engl. & Diels is an endemic liana of Gabon and Congo belonging to Annonaceae (Le Thomas, 1969). No previous report on scientific investigations of this species is in the literature.

2. Results and discussion

Crude MeOH and CH_2Cl_2 extracts showed in vitro antileishmanial and trypanocidal activity against *Leishmania donovani* and *Trypanosoma brucei brucei*. Bioguided fractionation of the CH_2Cl_2 extract led to isolation of two compounds. The first was an oil identified on the basis of spectral analysis as benzyl benzoate (**1**). The second (**2**) was a colourless powder. Its molecular formula was $C_{16}H_{14}O_6$ from elemental analysis and by ESIMS with two peaks at m/z 341 $[M+K]^+$ and m/z 325 $[M+Na]^+$. The IR spectrum showed a strong lactone absorption at 1779 cm^{-1} and two ester carbonyls at 1736 and 1723 cm^{-1}. The ^1H NMR spectrum showed five aromatic protons at δ 8.02 (2H), δ 7.58 (2 H) and δ 7.45 (1H) suggesting a benzoyl group. A base peak at m/z 105 C_6H_5–CO in the EIMS followed by the usual losses of CO and acetylene, observed at m/z 77 and 51, confirmed the presence of a benzoyl ester. The ^1H NMR spectrum also exhibited two deshielded vinylic

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protons at δ 7.37 and δ 6.27 and an ABMX system involving two methylene protons bearing an oxygen observed at δ 4.57 and δ 4.52, one downfield oxymethine proton at δ 6.14 and one olefinic proton at δ 5.32, as confirmed by COSY. These data indicated that the oxymethine proton at δ 6.14 was located between a methylene and an olefinic group. A methyl singlet was also observed at δ 2.10, suggesting the presence of an acetate, confirmed in the EIMS by the acetyl fragment ion at m/z 43 and a Mac Lafferty transposition ion (M–CH_3COOH) observed at m/z 242. The EIMS also showed a significant ion at m/z 180 (M–C_6H_5COOH) resulting from Mac Lafferty elimination of the benzoic acid. Subsequent elimination of ketene (ion at m/z 138) followed by significant ions at m/z 110 (138-CO), and m/z 54 (138-CH $\equiv$ C–OR) confirmed the presence of an exomethylene group linked at C-7 on the oxepin-2-one system.

In the ^{13}C NMR spectrum, the three expected carbonyl groups, deduced from the IR data, were observed at δ 169.7, 168.4 and 165.9, and located by HMBC (Table 1). The signal at δ 169.7, was assigned to the acetate by ^2J correlation with the methyl group at δ 2.10. The benzoyl ester was located at δ 165.9 by ^3J correlations with the two aromatic protons at δ 8.02. Further correlations with the signals at δ 4.57 and 4.52 indicated that the benzoyloxy group was attached to the single methylene group of the molecule. The signal at δ 168.4 was therefore attributed to the lactone function. Its ^2J correlation with the olefinic proton at δ 6.27 indicated the conjugation of the lactone group with the first double bond. Further correlations observed (Table 1) between the remaining ethylenic protons (δ 5.32 and 7.37) and carbons at δ 143.3 and 150.7 evidenced the conjugation of the two double bonds in a 7-*H* oxepin-2-one system.

All NMR and MS data were consistent with the 5-acetoxy-7-benzoyloxymethyl-7*H*-oxepin-2-one structure for compound **2** which was named klaivanolide (Fig. 1).

Table 1
^1H, ^{13}C and HMBC NMR data for klaivanolide (**2**)

Position	δ_H (J, Hz)	δ_C	HMBC
2		168.4	
3	6.27 (*d*, 5.5)	121.5	C-2, C-4
4	7.37 (*d*, 5.5)	143.3	C-3, C-5
5		150.7	
6	5.32 (*d*, 8.0)	108.8	C-4, C-5
7	6.14 (*ddd*, 8.0, 6.0, 4.2)	67.2	
8a	4.57 (*dd*, 11.7, 4.2)	64.6	Ar-CO, C-7
8b	4.52 (*dd*, 11.7, 6.0)		Ar-CO, C-7
CH_3-CO	2.10 (*s*)	20.8	CH_3-CO
CH_3-CO		169.7	
Ar-CO		165.9	
1′		129.5	
2′, 6′	8.02 (*dd*, 8.4, 1.3)	129.6	Ar-CO, C-3′, C-4′, C-5′
3′, 5′	7.45 (*brt*, = 8)	128.4	C-1′
4′	7.58 (*tt*, 7.4, 1.3)	133.2	C-2′, C-6′

Subsequent in vitro studies examined the activity of klaivanolide against *Leishmania donovani* promastigote forms and *Trypanosoma brucei brucei* trypomastigote forms. Klaivanolide showed strong antileishmanial activity with an IC_{50} of 1.75 μM against the sensitive strain as well as against the amphotericin B-resistant strain of *L. donovani* with an IC_{50} of 3.12 μM. Pentamidine used as a standard exhibited in the same conditions an IC_{50} of 8.52 μM (Table 2). After a 48 h incubation period, klaivanolide showed a trypanocidal activity against *Trypanosoma brucei brucei* GVR 35 with a MEC (minimum effective concentration) of 33.24 μM. Klaivanolide was about three times more active than pentamidine on *L. donovani* and *L. donovani* Amb-R in vitro systems. Benzyl benzoate was inactive on these models, nevertheless it posseses a strong acaricidal activity against *Dermatophagoides pteronyssinus* strain, the European house dust mite ($EC_{50} = 0.04$ g/m^2). Thus, klaivanolide exhibited potent in vitro antileishmanial and trypanocidal activities and could be an interesting lead in the fight against leishmaniasis and trypanosomiasis which justifies further pharmacomodulation studies to optimize this activity.

3. Experimental

3.1. General experimental procedures

EIMS were registered on an Automass Thermoquest with EI source (70 eV) at 150 °C and ESIMS on a Navigator Aqa Thermoquest with an ES source (MeOH, flow rate: 5 μl/min). UV spectra were recorded on a Philips PU 8700 series UV/vis spectrophotometer. IR spectra were recorded on a Bruker Vector 22. Optical rotations were determined using a Schmidt–Haensch Polartronic E polarimeter. ^1H NMR spectra and the ^1H–^1H (COSY) and ^1H–^{13}C (HMQC and HMBC) correlation spectra were registered at 400 MHz, on a Bruker ARX-400, and ^{13}C NMR spectra at 50 MHz on a Bruker AC-200 P spectrometer.

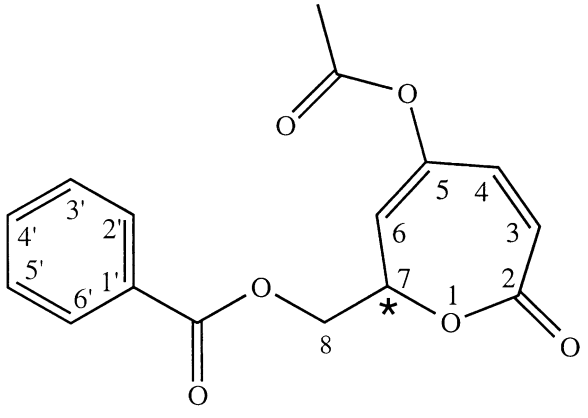

Fig. 1. Structure of klaivanolide (**2**).

Table 2
In vitro antileishmanial (IC_{50} μM), and trypanocidal (MEC[a] μM) activities of klaivanolide (**2**)

	Klaivanolide	Pentamidine
L. donovani WT[b]	1.75±0.22	7.34±0.83
L. donovani AmB-R[c]	3.12±0.42	8.52±0.73
T. brucei brucei	33.24±4.81	2.27±0.34

[a] MEC: minimum effective concentration.
[b] WT: wild type.
[c] AmB-R: amphotericin B-resistant.

3.2. Plant material

Plant material was collected from "Forêt des Abeilles" in October 1999 in the Lope region, Gabon and identified by Dr. H. Bourobou-Bourobou of the National Herbarium of Gabon, where voucher specimens were deposited.

3.3. Extraction and isolation

The air dried and pulverized stems were extracted with MeOH at room temperature. After removal of the solvent under reduced pressure at 40 °C, the MeOH extract afforded a residue (18%) which was dissolved in 80% aqueous MeOH and extracted with CH_2Cl_2 to lead CH_2Cl_2 extract (3.51 g). This extract was chromatographed over a column of silica gel (Merck 70–230 mesh) and eluted with CH_2Cl_2–hexane mixtures followed by CH_2Cl_2–EtOAc mixtures. Fractions eluted with CH_2Cl_2–hexane (70:30) yielded benzyl benzoate (**1**). Fractions eluted with CH_2Cl_2–EtOAc (80:20) afforded a colourless powder which was crystallized from hexane, affording 708 mg of **2** as colourless crystals.

Klaivanolide (**2**); colourless crystals, mp 73–74 °C; $[\alpha]_D^{20}$ +32° (MeOH, *c* 1); UV λ_{max}^{MeOH} nm (log ϵ): 230 (4.17), 268 (4.24); IR ν_{max} cm^{-1}; 1779, 1736, 1723, 1230, 709; ^1H and ^{13}C NMR spectral data: see Table 1; ESIMS *m*/*z* 341 $[M+K]^+$ (51), 325 $[M+Na]^+$ (100); EIMS 70 eV, *m*/*z* (rel. int.): 302 M^+ (1), 301 $M\text{-}1^+$ (3), 272 M-30 (32), 271 (4), 242 $[M–CH_3COOH]^+$ (32), 180 $M–C_6H_5COOH^+$ (11), 138 (27), 110 (18), 105 (100), 77 (97), 54 (22), 51 (61), 43 (98). (Found: C, 63.47; H, 4.73. $C_{16}H_{14}O_6$ requires C, 63.57; H, 4.67%).

3.4. Biological evaluation

3.4.1. Antileishmanial screening

Leishmania donovani (MHOM/IN/80/DD8) promastigotes originating from the WHO strain collection at the London School of Hygiene and Tropical Medicine were used for in vitro screening. The antileishmanial screening was performed according to the method described by Mbongo et al. (1997). Briefly, promastigotes were cultivated in HEPES (25 mM)-buffered RPMI 1640 medium enriched with 10% fetal calf serum (FCS) and 50 μg/ml gentamycin at 27 °C in a dark environment. The screening was performed in flat-bottomed 96-well plastic tissue-culture trays maintained at 27 °C in an atmosphere of 95% air/5% CO_2. Promastigote forms from a logarithmic phase culture were suspended to yield 10^6 cells/ml after hemocytometer counting. Each well was filled with 100 μl of the parasite suspension, and plates were incubated at 26 °C for 1 h before drug addition. The compounds to be tested were dissolved in DMSO and then added to each well. At up to 2% (v/v), DMSO had no effect on parasite growth. Each concentration was screened in triplicate. The viability of promastigotes was checked using the tetrazolium-dye (MTT) colorimetric method. The results are expressed as the concentrations inhibiting parasite growth by 50% (IC_{50}) after a 3-day incubation period.

3.4.2. Trypanocidal screening

Trypanosoma brucei brucei GVR 35, kindly supplied by F.W. Jennings (Glasgow, United Kingdom) was used for in vitro screening.

In vitro evaluation: the test was conducted according to the method described by Loiseau et al. (2000). The drug incubation infectivity test (DIIT) was used for compound evaluation. Briefly, the bloodstream forms of *T. brucei brucei* GVR were maintained in vitro without loss of infectivity for 48 h in the dark at 37 °C in a 5% CO_2 atmosphere. Screening was performed in a 96 well tissue culture plates in a volume of 200 μl containing 2×10^4 parasites, in minimum essential medium (Gibco BRL), and the compounds to be tested (diluted in H_2O–DMSO). Drug concentrations were evaluated in triplicate. The minimum effective concentration (MEC) was defined as the minimum concentration at which no viable parasite was observed microscopically. Confirmation of the MEC was performed by injecting naive mice intraperitoneally (i.p.) with 150 μl of treated trypanosome suspension withdrawn from the wells after a 48 h period. The animals were aparasitemic 30 days postinfection. Thus, the MEC was assessed both visually, using an optical microscope after a 48 h incubation period, and in vivo since the mice were checked for parasitemia weekly for 30 days.

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