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BINGERVONE, AN ANTIPROTOZOAL β-TRIKETONE DERIVATIVE FROM THE ROOTS OF *UVARIA AFZELII* (ANNONACEAE)

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ABSTRACT: *Uvaria afzelii* Scott Elliot (Annonaceae) is an African traditional medicinal plant. In Ivory Coast, roots of the plant are commonly used as antiparasitic and against others ailments. During our previous investigation on the roots one 1-indanone derivative was obtained together with other compounds that showing interesting antiprotozoal activities. The aim of this study was to isolate other antiprotozoal compounds. The chloromethylenic extract of the roots was fractionated on silica and Sephadex® LH-20 gels columns chromatography. The new syncarpic acid derivative, bingervone (**1**), was isolated together with the known compounds, demethoxymatteucinol (**2**) and emorydone (**3**). The structures of these compounds were established by NMR, IR, UV and HRESIMS spectroscopic data. Bingervone (**1**) displayed weak antitrypanosomal activity against *Trypanosoma brucei brucei* trypomastigotes, with a LC_{100} value of 114.8 µM, and moderated antileishmanial activities against *Leishmania donovani* and *L. major* promastigotes, with IC_{50} values of 38.9 and 44.4 µM, respectively. These antileishmanial activities were in the range of reference drug used.

INTRODUCTION: *Uvaria afzelii* Scott Elliot (Annonaceae) is a well-known traditional medicinal plant widely distributed throughout West African forest. It is an aromatic hairy scrambling shrub which grows in secondary bushes. It was used in folk herbal medicine for the treatment of fever, bronchitis, malaria and jaundice [1–4]. Previous phytochemical investigations of *U. afzelii* revealed the presence of C-methylated flavanones, chalcones, xanthones and miscellaneous compounds [5, 6]. Analysis of the essential oils obtained from its barks and roots showed that 2,5-dimethoxy-*para*-cymene, bornyl acetate, α-epi-cadinol and camphene were the main volatile terpenoids. Benzyl benzoate was found in relative important amounts [7]. Recently, we have reported the first 1-indanone derivative isolated

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from the roots of the species in addition to the two known compounds demethoxymatteucinol (**2**) and emoridone (**3**) [8]. In our continuing search for biologically active metabolites from *U. afzelii*, the chloromethylenic extract of the roots, possessing a strong antiprotozoal activity [3], was reexamined. In this paper, we report the isolation and the structure elucidation of a new β-triketone derivative, bingervone (**1**), and its antiprotozoal activity. Compounds **2** and **3** were obtained as known compounds.

MATERIALS AND METHODS:

General: For column chromatography, Merck Silica 60 (70–230 mesh) and Sephadex® LH-20 (Pharmacia) gels were used. TLC were carried out on aluminium plates coated with silica gel 60 F254 (Merck), and visualized with UV light, vanillin-H_2SO_4. Melting points were determined on a Stuart SMP10 melting point apparatus and were uncorrected. Optical rotations were measured on a PolAAr 32 polarimeter (Optical activity Ltd, Ramsey, UK). IR spectra were carried out using a Bruker Vector-22 spectrometer (Champs-sur-Marne, France). UV spectra were obtained in MeOH on a Philips PU 8720 spectrometer (Eindhoven, The Netherlands).

The ^1H and ^{13}C NMR spectra as well as 2D spectra (COSY, HSQC, HMBC and NOESY) were recorded in $CDCl_3$ on a Bruker AC-400 spectrometer (Champs-sur-Marne, France) operating at 400 MHz for ^1H and 100 MHz for ^{13}C. EIMS spectra were recorded on an Agilent Hewlett-Packard 6890 series apparatus equipped with an Agilent HP 5973 (Issy-les-Moulineaux, France) mass-selective detector (EI mode, 70 eV). HRESIMS spectra were registered with a Bruker Esquire LC00040 spectrometer (Champs-sur-Marne, France).

Plant material:

Roots of *Uvaria afzelii* Scot Elliot were collected in Bingerville (Ivory Coast) in April 2002 and identified by Professor Aké Assi of the Botanical Department. A voucher specimen, N° 343 CNF, was deposited at the herbarium of the Centre National de Floristique, Cocody University, Abidjan, Côte d'Ivoire.

Extraction and isolation: The air-dried and powdered roots (2kg) were extracted with methylene chloride (10L) in a Soxhlet apparatus during 18 hours and the solvent was removed under vacuum to give a brown extract (48 g). The extract was subjected to silica gel column chromatography (CC) eluting with Cyclohexane/EtOAc in a gradient from 9:1 to 0:10, v/v, to give fifteen fractions (F1 to F15) on the basis of TLC profile. Fraction F5 (5.5 g) essentially constituted of **2** (465.0 mg; Rf 0.50, n-Hexane/EtOAc 8:2) and **3** (2.93 g; Rf 0.27, n-Hexane/EtOAc 8:2) was subjected to three successive silica gel CC purification (60H, n-Hexane/CH_2Cl_2/MeOH 30:70:2; Cyclohexane/CH_2Cl_2 2:8 and n-Hexane/EtOAc 8:2), then to a Sephadex® LH-20 CC (CH_2Cl_2/MeOH 2:1). The residue obtained (Rf 0.46, n-Hexane/EtOAc 8:2) was recrystallized in MeOH to afford compound **1** (65.0 mg).

Bingervone (1):

Pale yellow needle crystals. $[\alpha]_D^{24}$ + 105.3 (c 0.19, MeOH); mp 82–83 °C; UV λ_{max} (MeOH) nm (log ε): 288 (2.96), 294 (3.00), 425 (2.56). IR ν_{max}: 2982, 2940, 1741, 1707, 1641, 1469, 1387, 1371, 1242, 1192, 1047 cm^{-1}. EIMS (%): *m/z* 270 $[M]^+$ (1), 242 (1), 210 (15), 195 (1), 183 (15), 168 (7), 151 (22), 140 (14), 135 (2), 123 (100), 115 (7), 107 (11), 98 (17), 91 (3), 81 (10), 69 (42). HRESIMS: *m/z* 293.1322 $[M+Na]^+$ (calc. for $C_{14}H_{22}O_5$ $[M+Na]^+$, 293.1365). ^1H NMR ($CDCl_3$, 400 MHz), ^{13}C NMR ($CDCl_3$, J_{mod}, 100 MHz): See **Table 1.**

Antiprotozoal assays:

All experiments were performed in triplicate, using 3 wells per condition. DMSO did not show toxicity at the maximum concentration used (0.1%).

Antileishmanial activity:

The antileishmanial activity of the isolated compounds was tested *in vitro* against *L. donovani* (WHO designation: MHOM/ET/1967/L82) and *L.* major (WHO designation: MHOM/BF/00/COU12/MON74), according to a method previously described [9]. Briefly, promastigotes were cultivated in HEPES (25 mM)-buffered RPMI 1640 medium enriched with 10% Fetal Calf Serum (FCS) and 50 µg/mL gentamicin at 27 °C in a dark environment. The screening was performed in flat-bottomed 96-well plastic tissue-

culture plates maintained at 27 °C. Promastigotes formed from a logarithmic phase culture were suspended to yield 10^6cells/mL after haemocytometer counting. Each well was filled with 100 µL of the parasite suspension, and the plates were incubated at 27 °C for 1 hour before addition of the samples dissolved in DMSO. The viability of promastigotes was assessed by the tetrazolium-dye (MTT) colorimetric method. The results were expressed as the concentrations inhibiting parasite growth by 50% after a 3-day incubation period. The starting concentration for screening was 100 µg/mL for extracts or 100 µM for pure compounds. Miltefosine and sitamaquine were used as reference compounds.

Trypanocidal activity: Compounds were tested for their activity against bloodstream forms of *T. brucei brucei* (*Glascow Veterany Research*, GVR 35, kindly supplied by Pr F.W. Jennings) as described earlier [10]. Briefly, the bloodstream parasites were maintained *in vitro* without the loss of their infectivity for 24 hours in the dark at 37 °C in a 5% CO_2 atmosphere. Screening was performed in 96-well tissue-culture plate in a final volume of 200 µL containing 2 x 10^5 parasites/mL, in supplemented Minimum Essential Medium (Gibco, BRL) and each sample to be tested at a starting concentration of 100 µg/mL (diluted in DMSO). The minimum lethal concentration (LC_{100}) was defined as the minimum concentration at which no motile parasites were observed microscopically. Confirmation of the LC_{100} was obtained by injecting naive mice intraperitoneally with 150 µL of the treated trypanosome suspension withdrawn from the well after 24 hours incubation period. The animals were aparasitemic 30 days post-infection. Pentamidine was used as reference compound.

RESULTS AND DISCUSSION: Bingervone (**1**) **Fig.1** was isolated from the chloromethylenic extract of the roots of *U. afzelii* as pale yellow needles. HRESIMS showed a $[M+Na]^+$ adduct at *m/z* 293.1322, corresponding to the molecular formula $C_{14}H_{22}O_5$ (calcd: 293.1365). No hydroxyl functionality was observed in the IR spectrum, although absorption bands at 1741 and 1707 cm^{-1} suggested the presence of an aliphatic ester and a cyclic carbonyl group, respectively. The EIMS spectrum showed peaks at *m/z* 242 $[M-28]^+$ and 123 $[M-147]^+$, due to the consecutive losses of one carbonyl and two acetate groups (**Fig.2**), supporting these observations.

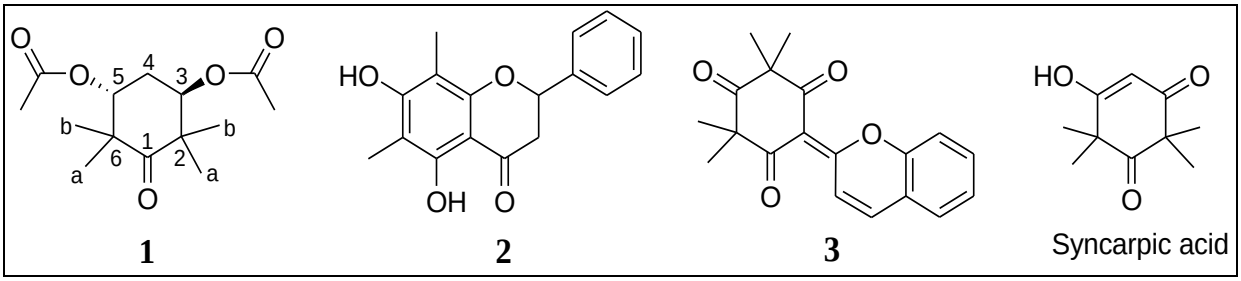

FIG. 1: CHEMICAL STRUCTURES OF COMPOUNDS 1–3 AND OF SYNCARPIC ACID.

The ^{1}H NMR spectrum of **1** exhibited five signals only, corresponding to three methyl, one oxymethine groups and one methylene group (**Table 1**). These data suggested a symmetric molecular structure for compound **1**. The oxymethine was observed at δ 5.11 ppm (2H, *t*, *J* = 5.8 Hz, H-3/H-5), coupled with the methylene at δ 2.20 ppm (2H, *t*, *J* = 5.8 Hz, H-4). Methyl groups of acetate appeared at δ 2.06 ppm (6H, s). The upfield singlet signals at δ 1.17 (6H) and 1.11 ppm (6H) were assigned to four methyl groups (2/6-CH_3a and 2/6-CH_3b, respectively). The ^{13}C NMR (Jmodulation spin-echo) spectrum (Table 1) of **1** revealed the presence of eight carbons corresponding to three methyls, one methylene, one oxymethine and three quaternary carbons. Among them, the acetate groups were observed through characteristic signals, δ 170.0 (C=O) and 20.9 ppm (CH_3).

The carbonyl signal at δ 215.6 ppm was assigned to C-1, and signals at δ 74.2 and 28.2 ppm appeared to be the oxymethine (C-3/C-5) and methylene (C-4) groups, respectively. The other methyl groups were observed at δ 24.8 (2/6-CH_3a) and 22.0 ppm (2/6-CH_3b), respectively. The quaternary carbon (C-

2/C-6) appeared at δ 48.4 ppm. These assignments of protons and carbons were confirmed by COSY and HMBC experiments (**Table 2**). Indeed, the COSY spectrum showed a single cross peak, between the methylene (δ 2.20 ppm, H-4) and the oxymethine protons (δ 5.11 ppm, H-3/H-5). The HMBC correlation between the oxymethine protons (H-3/H-5) and the ester carbonyls (δ 170.0 ppm) established the position of the acetate groups.

The structure of **1** as indicated in **Fig. 1** was deduced from the correlations observed in the NOESY spectrum (**Table 1**). Furthermore, the downfield resonance of the oxymethine protons suggested a *trans*-orientation of the acetate groups (meso-*cis* derivatives, δ c.a. 4.74 ppm) [11–13]. The absolute configuration at C-3 and C-5 were then determined as 3(*R*) and 5(*R*) by the positive value of optical activity compared to those of *trans*-diacetate-cyclohexanone derivatives [13]. On the basis of the above data, compound **1** was established to be (3*R*,5*R*)-3,5-diacetate-2,2,6,6-tetramethylcyclohexan-1-one, and was named bingervone according to the place where the plant was harvested. Compound **1** appeared as the bis-acetylated form of syncarpic acid (**Fig.1**). Syncarpic acid and a number of its derivatives such as **3** were previously obtained from *U. afzelii* [5]. To the best of our knowledge, in the Annonaceae family, these compounds have been characterized in two genera only. Indeed, syncarpic acid derivatives were reported from *Desmos* species [14, 15]. Nevertheless, β-triketones such as syncarpic acid derivatives have been mainly described in the Myrtaceae family, with interesting biological activities [16–21].

It is noteworthy that emorydone (**3**), an analogue bearing a benzopyranic ring, was found in two species of the genus *Psorothamnus* (syn. *Dalea*, Fabaceae) only [22, 23]. The limited occurrence of these compounds is remarkable with a distribution in taxonomically unrelated taxa.

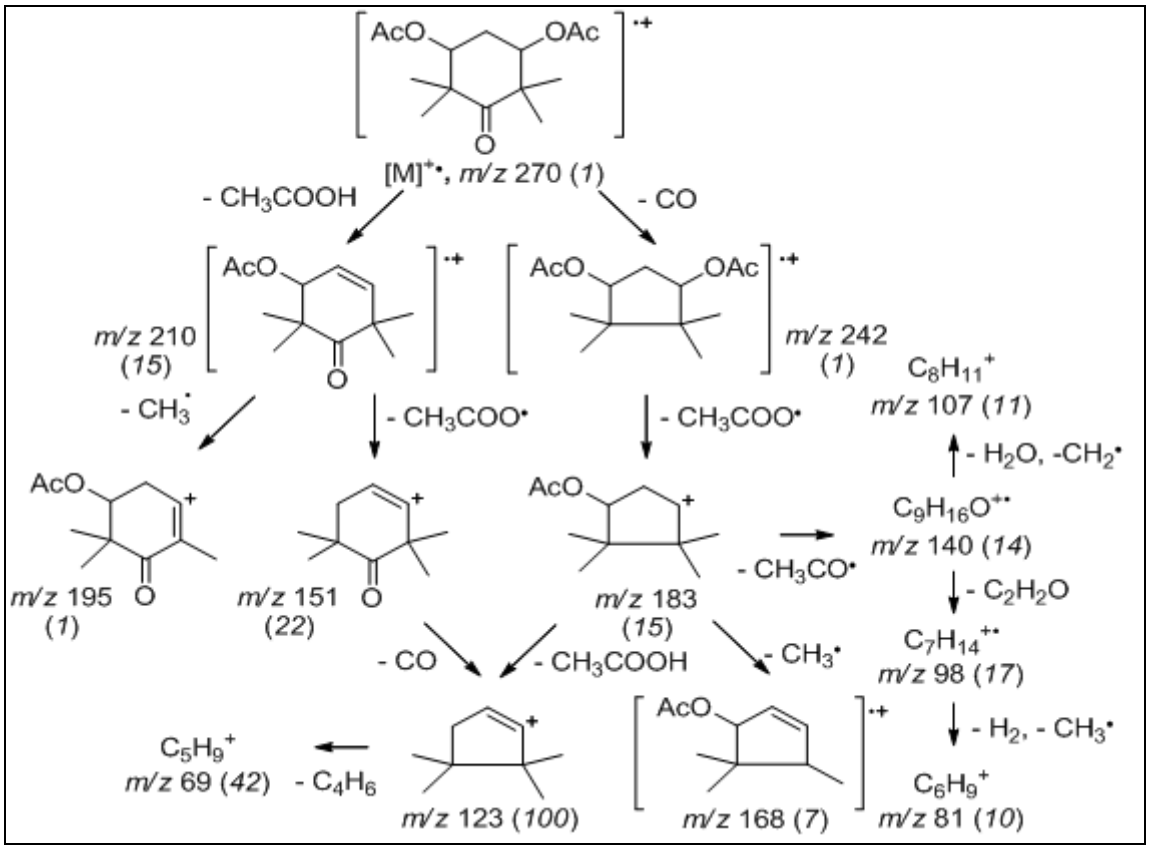

FIG.2: FILIATIONS FOR IMPORTANT FRAGMENTS OF COMPOUND 1 OBTAINED IN EIMS; (INTENSITY %).

Bingervone (**1**), tested for its antiprotozoal properties, showed significant *in vitro* antileishmanial activity against promastigote forms of *Leishmania donovani* and *L. major* promastigotes with IC_{50} (Inhibitory Concentrations 50%) values of 38.9 ± 4.1 and 44.4 ± 3.9 µM,

respectively. The antileishmanial activities of **1** were about 6 times weaker than those of miltefosine, the reference drug (IC_{50} = 7.1 µM and 8.4 µM, respectively). However, such activities were in the range of those of sitamaquine, also used as a control (IC_{50} = 35.4 µM), which was in the development process for the treatment of visceral leishmaniasis. The trypanocidal activity against *Trypanosoma brucei brucei* trypomastigotes was weak, with a LC_{100} (Lethal Concentration 100%) of 114.8 µM (pentamidine: IC_{50} = 12.5 µM). The antiprotozoal activities of **2** and **3** have been described earlier [8, 24].

TABLE 1: ^{1}H AND ^{13}C NMR DATA IN CDCL$_3$ OF BINGERVONE (1)

Position	δ_H	δ_C	(^{1}H-^{1}H) COSY	(^{1}H-^{13}C) HMBC	(^{1}H-^{1}H) NOESY
1		215.6		H-3/H-5, H_3a, H_3b	
2/6		48.4		H-3/H-5, H-4, H_3a, H_3b	
3/5	5.11 (*t*, 5.8)	74.2	H-4	H-3/H-5, H-4, H_3a, H_3b	H-4, H_3a
4	2.20 (*t*, 5.8)	28.2	H-3/H-5		H-3/H-5, H_3a, H_3b
2/6-CH_3a	1.17 (*s*)	24.8		H-3/H-5, H_3b	H-3/H-5, H-4, H_3
2/6-CH_3b	1.11 (*s*)	22.0		H-3/H-5	H-4, H_3
3/5-OCO<u>C</u>H$_3$	2.06 (*s*)	20.9			H_3a, H_3b
3/5-O<u>C</u>OCH$_3$		170.0		H-3/H-5, H_3	

CONCLUSION: Investigation of the chloromethylenic extract of the roots of *Uvaria afzelii* has led to the isolation of two β-triketone derivatives, bingervone (**1**) and emorydone (**3**). β-Triketone derivatives were found to have limited occurrence in plant kingdom. The third compound, demethoxymatteucinol (**2**), was a C-methylated flavanone previously obtained from *U. afzelii*. Their structures were determined thanks by NMR, UV, IR and HRESIMS spectroscopic data. The unusual β-triketone derivative, bingervone, showed antiprotozoal activities on *Leishmania donovani* and *L. major* similar to those of sitamaquine. Thus, it is worth to be studied in other *in vitro* and *in vivo* models for leishmaniasis experiments.

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