New Metabolites From the Endophytic Fungus *Cercophora samala* Associated With *Mitragyna inermis*

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**Abstract**
Two new natural products, mitrafungidione (1) elucidated as prototrop-isothers of (R)-3-acetyl-5-ethyl-4-hydroxy-5H-furan-2-one, and maristachone F (2a), elucidated as 5-(1-hydroxyethyl)-4-(hydroxymethyl)-3-methoxy-2-methylphenol, together with 5 known compounds have been isolated from the solid cultures of an endophytic fungus associated with *Mitragyna inermis* (Rubiaceae) and identified as *Cercophora samala*. The structures of these compounds were elucidated by detailed spectroscopic analysis and by comparison of their spectroscopic data with those reported in the literature. The absolute configuration of 1 and 2a were determined by extensive DFT calculations.

**Keywords**
*Cercophora samala*, endophytic fungus, mitrafungidione, maristachones, DFT calculations

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**Mitragyna inermis** (Willd.) O. Kuntze (Rubiaceae) is a small tree growing on low alluvial plains and swampy savanna in West Africa$^{1,2}$ In traditional medicine, this plant is used to treat diseases such as diabetes, fever, diarrhea, dysentery, cholera, malaria, rheumatic infections, and osteoarthritis diseases.$^{3,4}$ We have previously reported the isolation of a number of bioactive compounds from *M. inermis*. Studies of ethyl acetate extracts from the mycelia of the culture media of the endophytic fungus *Cercophora samala* Udagawa & T. Muroi (Lasiosphaeriaceae) associated with *M. inermis* afforded 2 new natural products, mitrafungidione (1) and maristachone F (2a), together with 5 known compounds. The structures of the new compounds were elucidated by detailed spectroscopic analysis, by comparison of their spectroscopic data with those reported in AntiBase,$^7$ and by DFT calculations. We report herein the isolation and structural elucidation of 1 and 2a. For the taxonomic identification, the endophytic fungus associated with *M. inermis* (Rubiaceae) was grown on potato-dextrose-agar (PDA) at 25 °C under near-ultraviolet light with 12 hours photoperiods. Genomic DNA was extracted from 6-day-old colonies, using Quiagen Plant Mini Kit (QUIAGEN) following the manufacturer’s protocol. The fungal isolate ED-I was identified as *C. samala*, based on the ITS sequence, as described in the experimental part.

After fermentation on solid rice medium at room temperature (28 °C) for 30 days under static conditions, the culture was extracted with ethyl acetate, and the resulting material was subjected to column chromatography using a CH$_2$Cl$_2$/MeOH solvent system with gradient elution. Further purification of subfractions on Sephadex LH-20 afforded 2 new natural products, mitrafungidione (1) and maristachone F (2a), together with 5 known compounds namely: versiconol,$^8$ cerebroside B,$^9$ and the trivial metabolites ergosterol, thymine, and uracil. Compound 1 (Figure 1) was obtained as reddish oil. The molecular formula C$_8$H$_{10}$O$_4$ was deduced from ESI HRMS with 4 double bond equivalents (DBE). The $^1$H NMR spectrum (Table 1) showed resonances of an A$_3$BCX-type spin...
system attributed to an oxymethine proton at δH 4.03 and 2 methylene protons at δH 1.72 (H6a) and 1.45 (H6b). The 1H,1H COSY spectrum (Figure 2) combined these fragments with a methyl triplet at δH 0.84 (H3-7) to an oxypropyl sequence CH3-CH2-CH-O-. The spectra showed additionally a methyl singlet at δH 2.16 (H-9) and a low-field 13C signal at 191.0, suggesting a carbonyl group (C-8). HMBC correlations further revealed the moiety as a Cγ-acetyl fragment. Additionally the D2O-exchangeable signal of a hydroxyl group at δH 3.30 was seen.

The 13C NMR, APT, COSY, HMBC and HSQC spectra confirmed these assignments. HMBC correlations indicated

Table 1. 1H (300 MHz), 13C (125 MHz), HMBC and COSY Data of Mitrafungidione (I) in DMSO-D6 (δ in ppm).

| C   | δC | δH (Mult., J in Hz) | HMBC          | COSY          |
|-----|----|---------------------|---------------|---------------|
| 2   | 174.7 |                     |               |               |
| 3   | 95.6  |                     |               |               |
| 4   | 195.0 |                     |               |               |
| 5   | 79.8  | 4.03 (1H, dd, 4.2 and 6.9) | C-4, C-2, C-6, C-7 | 1.45 (1H, m)  |
|     |       | 1.45 (1H, m)         | C-4, C-5, C-7  | 1.72 (1H, m)  |
| 6   | 24.5  | 1.72 (1H, m)         | C-4, C-5, C-7  | 0.84 (3H, t, 7.5) |
|     |       | 0.84 (3H, t, 7.5)    | C-5, C-6       | 4.03 (1H, dd, 4.2, 6.9) |
| 7   | 8.6   |                     | C-5, C-6       | 1.45 (1H, m)  |
|     |       | 1.72 (1H, m)         |                |               |
| 8   | 191.0 |                     |               |               |
| 9   | 28.0  | 2.16 (3H, s)         | C-8, C-3       |               |
| OH  |      | 3.30 (1H, s br)      |                |               |
additionally the connection of the oxypropyl fragment at C-5 with 2 carbonyl groups, forming a ketone (C-6, $\delta_C 195.0$) and, via the oxygen, of an ester carbonyl (C-2, $\delta_C 174.7$). The remaining C-acetyl unit must bridge these carbonyl groups, forming a furanedione derivative. The fact that no other methine proton was visible, suggested that one of the carbonyl groups is present in the enol form, resulting in 3-acetyl-5-ethyl-4-hydroxy-5H-furan-2-one (1a), or another prototropic isomeric form thereof (Figure 1).

Compound 1, trivially named mitrafungidione, is a further natural member of the tetrionic acid group and had been obtained previously by synthesis, but was published without NMR data. Although 6 isomers may co-exist in equilibrium, in the literature the hydroxy group of tetrionic acids is usually drawn as enol at C-4 (type 1a). However, our density-functional theory (DFT) calculations on the $\omega$B97XV/6-311 + G(2df,2p) level predicted that this isomer contributes only 7% to the equilibrium, which is dominated by 1b (67%); for 1c, a concentration of 26% was calculated, while the other isomers are neglectable. Nevertheless, for better comparison with the literature, we continued using structure 1a here. The shape of the experimental ECD spectrum was in agreement with DFT-data calculated for the Boltzmann-weighted (R)-1a-1c mixture (Figure 3). Mitrafungidione is therefore elucidated as the γ-butenolide (R)-1a, existing in methanol mainly as the isomers 1b and 1c (Figure 1 and Supplemental Figure S3).

Compound 2a (Figure 4) was obtained as yellowish oil. The molecular formula C$_{11}$H$_{16}$O$_4$, implying 4 DBEs, was deduced from the ESI HRMS spectrum. The 1H NMR spectrum of 2a in DMSO-$d_6$ showed 2 broad D$_2$O-exchangeable proton signals at $\delta_H$ 4.53, 4.88, and one at 9.17, suggesting the presence of a phenol. All further spectra were measured in deuteriomethanol. With the singlet of only one aromatic proton at $\delta_H$ 6.80 ($\delta_C$ 109.2) and 5 additional $^{13}$C signals of fully substituted aromatic carbons at $\delta_C$ 117.5, 122.5, 145.5, 157.5 and 159.4, a pentasubstituted benzene was expected, which is in agreement with the 4 DBEs. From the 1D and 2D NMR spectra, a 1-oxyethyl sequence [CH$_3$-CH(O)-], an oxymethylene singlet at $\delta_H$ 4.67, a methoxy signal at $\delta_H$ 3.72, and a methyl singlet at $\delta_H$ 2.10 were derived.

The oxyethyl group was connected with C-5 ($\delta_C$ 145.6), as the HMBC correlation of this atom with Me-11 indicated. In both $\alpha$-positions of C-5, the ring atoms C4- and CH-6 were assigned by correlations with CH-10. The oxymethylene group -O-CH$_2$-9 correlated with C-5, C-4, and the methoxy-carbon C-3, and was therefore connected with C-4. The methyl singlet Me-7 at $\delta_H$ 2.11 showed a cross signal with C-3 and is therefore a direct neighbor of the latter in position 2, so that the remaining phenolic hydroxy group should be placed at C-1 (Figure 5). The other hydroxy groups were attached to C-9 and

![Figure 2](image_url)  
*Figure 2.* Selected $^1$H-$^{13}$C HMBC and $^1$H-$^1$H COSY correlations of mitrafungidione tautomer 1a.

![Figure 3](image_url)  
*Figure 3.* Experimental (green, MeOH) and DFT-calculated ECD-spectrum (upper blue) of (R)-mitrafungidione (1, Boltzmann-weighted mixture of tautomers 1b-1d). For comparison, the calculated ECD spectra of the (S)-configured individual pure tautomers 1b-1c were drawn with the Boltzmann-intensities as used in the tautomer mixture of the (R)-enantiomer.

![Figure 4](image_url)  
*Figure 4.* Structure of (R)-maristachone F (2a, R = H) and maristachone A (2b, R = Me).
10 to agree with the empirical formula, resulting in 5-(1-hydroxyethyl)-4-(hydroxymethyl)-3-methoxy-2-methylphenol (2a) (Figure 4). Surprisingly, this rare phenol type is closely related to maristachone A (2b), a metabolite isolated from a sponge-derived *Stachylidium* sp.12; 2a was therefore named maristachone F. The absolute configuration of 2a was determined as (R) by comparison of experimental with DFT-calculated ECD data as for 1 (see Supporting Information).

### Experimental

#### General Experimental Procedures

Optical rotations: Polarimeter (Perkin-Elmer, model 343). ECD spectra were recorded on a JASCO J-810 spectrometer equipped with a JASCO etc.,-505S/PTC-423S temperature controller. The $^1$H NMR spectra were recorded on Varian Mercury-300 (300.141 MHz) and Varian VNMRS-300 (300.536 MHz) spectrometers equipped with 3 mm probes; $^{13}$C NMR spectra were measured at 125.707 MHz relatively to TMS as internal standard; shifts are reported as δ values. Electrospray-ionization mass spectrometry (ESIMS) and high-resolution mass spectra (ESIHRMS) were recorded on a micrOTOF time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany), as well as on an Apex IV 7 Tesla Fourier-transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Billerica, MA). Column chromatography (CC): silica gel (230, 400 mesh; Macherey-Nagel, Düren, Germany). Size exclusion chromatography was done on Sephadex LH-20 (Lipophilic Sephadex, Amersham Biosciences Ltd; purchased from Sigma-Aldrich Chemie, Steinheim, Germany). TLC was performed on pre-coated silica gel sheets of Polygram SIL G/UV$_{254}$ (Macherey-Nagel and Co., Düren, Germany), with mixtures of dichloromethane and methanol as eluents; spots were detected using UV light (254 and 365 nm) or by spraying with anisaldehyde/sulfuric acid staining reagent prepared by mixing of anisaldehyde (1 ml) and methanol (85 ml) with concentrated sulfuric acid (1 ml) and acetic acid (14 ml).

### Plant and Fungal Material

The endophytic fungus *C. samala* was obtained from the branches of *M. inermis* collected in December 2011 at Nkoteng in the Centre Region of the Republic of Cameroon. The plant was identified by Mr. Victor Nana, botanist at the National Herbarium (Yaoundé, Cameroon), where a voucher specimen (N° 8886/SRF/Cam.), has been deposited.

The endophytic fungus was isolated from a branch sample using techniques as described previously.13 A voucher specimen of the fungus is deposited in the culture collection at the Institute of Organic and Biomolecular Chemistry, Göttingen, Germany under the internal code ED-I.

### Taxonomy of the Fungus

The ITS rDNA of the fungal strain ED-I was amplified by PCR reaction, with the primer pairs ITS1f14 and ITS4.15 PCR products were checked on 1% agarose electrophoresis gel stained with ROTI®GelStain (Roth, Germany). Purified amplicons were sequenced in both directions with the PCR primers by GATC (actually Eurofins Genomics Germany GmbH). Raw nucleotide sequences were edited in MEGA version 7 and deposited in NCBI GenBank database (Accession number MW177563).

ITS sequence of isolate ED-I was used for BLAST searches in NCBI GenBank (accessed 12.08.2020). Higher scoring hits were sequences of *Cercophora samala* (AY999134, NG708364, MH861345) and *Podospora pauciseta* (MH864359, MH858484, MH858208) belonging to the family Lasiosphaeriaceae. Additional sequences of *Cercophora* and *Podospora* were retrieved from the GenBank, including those of ex-type isolates and aligned in MEGA 7. Phylogenetic analysis based on PhyML 3.1 was conducted with the GTR model using the web service Phylogeny.fr (http://www.phylogeny.fr/one_task.cgi?task_type=phyml). In the phylogenetic tree (Supplemental Material, Figure S1), the ITS sequence of ED-I clustered to *C. samala* 2HD71-5. The topology of this tree reflects the polyphyletic character of both genera *Cercophora* and *Podospora.*17

### Fermentation, Extraction, and Isolation of Metabolites

The endophytic fungus strain ED-I was cultured on slants of potato dextrose agar (PDA: cooking water from 200 g potatoes/L, enriched with glucose 40 g/L and agar-agar 20 g/L) at 28 °C for 6 days. Pieces of a well-grown agar subculture were used to inoculate 10 P-flasks, each containing sterilized rice medium (200 g of rice suspended in 200 ml of tap water and sterilized) and incubated at room temperature under static conditions for 30 days. The culture was extracted...
4 times with ethyl acetate (EtOAc) and the filtrate was concentrated to dryness in vacuo to afford 63 g of a yellow-brown extract. A portion of 60 g of the crude extract was fractionated by silica gel column chromatography using a CH$_2$Cl$_2$/MeOH gradient of increasing polarity (100:0-80:20). The resulting 106 subfractions of each 200 ml were combined on the basis of TLC analysis to yield 7 main fractions: F$_1$ (6.6 g), F$_2$ (3.8 g), F$_3$ (2.4 g), F$_4$ (3.2 g), F$_5$ (3.7 g), F$_6$ (3.1 g) and F$_7$ (7.7 g). Fractions F$_1$ (composed of subfractions 1-6) and F$_7$ (composed of subfractions 78-104) were complex mixtures containing mostly oils and polar compounds, respectively, and were not further studied. Fraction F$_2$ (composed of subfractions 7-21) was subjected to column chromatography over silica gel with CH$_2$Cl$_2$-MeOH (98:02), to yield ergosterol (76.7 mg). Fraction F$_3$ (composed of subfractions 22-34) was further separated by column chromatography over silica gel with CH$_2$Cl$_2$-MeOH (95:05); Sephadex LH-20 eluted with MeOH afforded finally maristachone F (2a, 8.6 mg). Fraction F$_4$ (composed of subfractions 35-47) was further separated by column chromatography over silica gel/CH$_2$Cl$_2$-MeOH (95:05) and on Sephadex LH-20/MeOH, affording mitrafungidione (1, 4 mg).

The remaining fractions F$_1$ and F$_7$ were not studied.

**Maristachone F (2a)**

Yellowish oil; OR: [α]$_{D}^{20}$ = –8.2° (MeOH, c = 30.2 mg/mL); ECD spectrum (MeOH) s. Supplemental Figure S5.

(+)-ESI HRMS: m/z 235.0946 [M + Na]$^+$ (calc. 235.0941 for C$_{11}$H$_{16}$O$_4$Na) and m/z 447.1988 [2M + Na]$^+$.

$^1$H (300 MHz, DMSO-$_d_6$): δ 9.17 (1H, s br, OH-1), 6.80 (1H, s, H-6), 2.01 (3H, s, Me-7), 3.65 (3H, s, Me-8), 4.44, 4.48 (2H, AB, 11.3 Hz, H$_2$-9), 4.53, 4.88 (2H, 2 s br, 9,10- OH), 5.15 (1H, q, 6.5, H-10), 1.44 (d, 3H), 1.46 (3H, d, 6.3).

**Mitrafungidione (1)**

Reddish oil; ECD spectrum s. Figure 3.

(-)-ESI HRMS: m/z 169.0512 [M-H]$^-$ (calc. 169.0506 for C$_{8}$H$_{9}$O$_4$) and m/z 361.0900 [2M-2H + Na]$^-$ (calc. 361.0904 for C$_{16}$H$_{18}$O$_8$Na).

$^1$H NMR, $^{13}$C NMR, $^1$H-$^{13}$C HMBC and $^1$H-$^1$H COSY data: Table 1 and Figure 2; see also Supplemental Material.

**Table 2.** $^1$H (300 MHz) and $^{13}$C (125 MHz) NMR Data of Maristachone F (2a) and $^1$H-$^{13}$C and $^1$H-$^1$H Correlations Exhibited in the 2D Spectra (125 MHz) in CD$_3$OD (δ in ppm).

| No. | δ$_C$ | δ$_H$ (mult., J in Hz) | $^1$H-$^{13}$C HMBC | $^1$H-$^1$H COSY |
|-----|-------|------------------------|---------------------|------------------|
| 1   | 157.6 | 6.82 (1H, s)           | C-1, C-4, C-2, C-10, C-5 |
| 2   | 117.6 | 2.11 (3H, s)           | C-3, C-1, C-2, C-6   |
| 3   | 159.5 | 3.73 (3H, s)           | C-3                 |
| 4   | 122.7 | 4.68 (2H, s br)        | C-3, C-5, C-4       |
| 5   | 145.6 | 5.17 (1H, q, 6.5)      | C-5, C-4, C-6, C-11  |
| 6   | 109.2 | 1.46 (3H, d, 6.3)      | C-5, C-10           |
| 7   | 9.4   |                        |                     |
| 8   | 62.3  |                        |                     |
| 9   | 56.1  |                        |                     |
| 10  | 67.1  |                        |                     |
| 11  | 24.8  |                        |                     |

| Supplementary Material | | | | | |
|------------------------|---|---|---|---|---|
| Table 2. | | | | | |

**Supplemental Material**

Supplemental material for this article is available online.
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