Depsidones from an Endophytic Fungus *Chaetomium* sp. Associated with *Zanthoxylum leprieurii*

Ferdinand Mouafo Talontsi[a], Clovis Douanla-Meli[b] and Hartmut Laatsch[a]

[a] Institute of Organic and Biomolecular Chemistry, Georg-August University, Tammannstr. 2, D-37077 Göttingen, Germany

[b] Institute of Biology, University of Kassel, Heinrich-Plett-Strasse 40, D-34132 Kassel, Germany

Reprint requests to Prof. Dr. Hartmut Laatsch. Tel.: +49/551/3933211. Fax: +49/551/399660.
E-mail: hlaatsc@gwdg.de

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A new depsidone, namely chaetosidone A (**1**), together with six known fungal metabolites were obtained from a culture of *Chaetomium* sp., an endophytic fungus isolated from *Zanthoxylum leprieurii* leaves. The structure of the new compound **1** was elucidated using MS and NMR experiments, and by biosynthetic considerations. Chaetosidone A (**1**) is the missing link in the series of orsellinic acid-derived depsidones. Compound **1**, corynesidone B (**2**) and corynether (**3**) displayed inhibitory effects on two Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, at concentrations of 40 μg per paper disk and moderate toxicity towards brine shrimp larvae (*Artemia salina*).

Key words: Endophyte, *Chaetomium* sp., Depsidones, Toxicity, Antimicrobial Activity

Introduction

Plant endophytes are among the most promising sources of bioactive natural products with interesting biological potential [1 – 4]. *Chaetomium* species are widely distributed in soil, organic compost and plants [5]. Chemical studies of some *Chaetomium* spp. have afforded more than 250 bioactive metabolites, among them the cytotoxic alkaloid chaetominine [6], antimalarial depsidones [7], azaphilones [8], and the cytotoxic chaetoconvosins [9], which have drawn the attention of both synthetic chemists and pharmacologists.

In the course of our screening program for new antimicrobial and cytotoxic metabolites from endophytic fungi inhabiting Cameroonian medicinal plants [10, 11], an endophytic fungus *Chaetomium* sp. strain CAFTM23, isolated from *Zanthoxylum leprieurii*, attracted our attention because of the interesting antimicrobial and cytotoxic activities of its crude ethyl acetate extract. Bioassay-guided fractionation led to the isolation of a highly substituted new depsidone, namely chaetosidone A (**1**), together with further six known compounds. Herein we report on their isolation, structure elucidation, and biological activities.

Results

The fungus *C.* sp. CAFTM23 was obtained from fresh leaves of *Z. leprieurii* and fermented in biomalt agar (10 L) at room temperature for 40 days. The culture was successively extracted with ethyl acetate to afford a yellowish crude extract. The latter was repeatedly chromatographed on silica gel, Sephadex LH-20, and by preparative HPLC to yield seven compounds, chaetosidone A (**1**) together with the known corynesidone B (**2**) [12], corynether (**3**) [12], kojic acid (**4**), ergosterol, ergosterol peroxide, and adenosine (Fig. 1).

Chaetosidone A (**1**) was isolated as a colorless powder with the molecular weight of 316 Dalton. HRESIMS fixed its molecular formula as $C_{16}H_{12}O_7$, with eleven double bond equivalents. The IR spectrum showed a strong absorption band at 3417 cm^{-1}, indicating a hydroxyl group and two bands at 1765 and 1740 cm^{-1} assigned to a lactone ring and a carboxylic

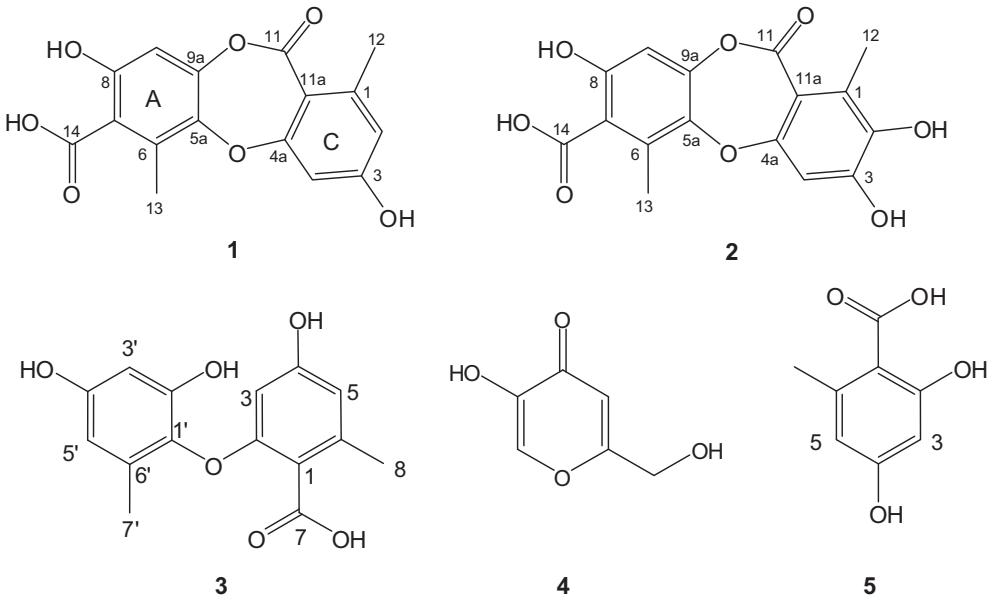

Fig. 1. Structure of compounds **1**–**5**.

acid group [13, 14]. The ^{1}H NMR spectrum (Table 1, Fig. S2 in the Supporting Information available online; see note at the end of the paper for availability) showed one exchangeable proton at $\delta_H = 10.65$ (s, br), three aromatic protons appearing as singlets at $\delta_H = 6.61$ (H-9) and $\delta_H = 6.60$ ppm (H-2 and H-4), and two methyl singlets at $\delta_H = 2.40$ (H_3-12) and 2.35 ppm (H_3-14). The ^{13}C NMR/APT and HSQC data displayed 16 carbon signals, according to two methyl groups [$\delta_C = 13.1$ (C-12) and 20.5 ppm (C-14)], three sp^2 methine carbon atoms ($\delta_C = 104.6$, 105.5, 115.8 ppm), one ester/lactone at $\delta_C = 162.4$ ppm (C-11), one carboxylic acid carbon ($\delta_C = 168.8$ ppm), and nine quaternary carbons, of which five were oxygenated (Fig. S3, Supporting Information).

The aromatic methyl protons H_3-13 showed strong HMBC correlations to the quaternary carbon signals at $\delta_C = 140.9$ (C-5a), 129.1 (C-6) and 118.4 ppm (C-7), while the aromatic proton at $\delta_H = 6.61$ ppm (H-9; $\delta_C = 105.3$ ppm) showed HMBC cross signals with C-5a, C-7, C-8, and C-9a. The second methyl group at $\delta_H = 2.40$ ppm (C-12) correlated with the carbon signals at $\delta_C = 144.8$ (C-1), 115.8 (C-2) and 111.3 ppm (C-11a), while the two aromatic protons H-2 and H-4 at $\delta_H = 6.60$ ppm correlated with C-2, C-3, C-4, C-4a, and C-11a of the aromatic ring C (Fig. 2). One of these protons must be in *ortho*-position to CH_3-12, as their

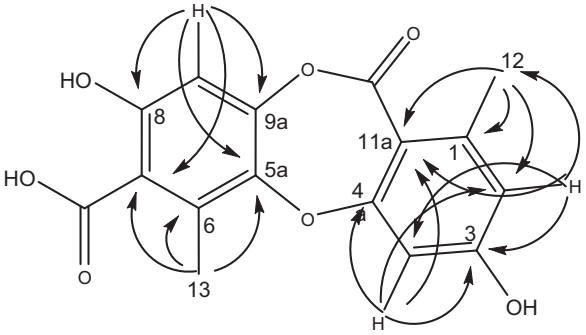

Fig. 2. Selected HMBC (→) correlations observed for **1**.

HMBC and long-range COSY correlation has demonstrated.

These data were fitting best on two orsellinic acid (**5**) residues, which are cyclodimerized under the loss of two molecules of water, forming depsidones or a bis-lactone. As there were no correlations between rings A and C, the final structure of **1** had to be derived from biosynthetic considerations [15] (Fig. 3): In a first step, a depside is formed from two molecules of orsellinic acid (**5**) by esterification of the more reactive OH-4 under formation of lecanoric acid (**6**). The subsequent oxidative ring closure occurs sometimes in *o*-position of OH-2 (*i. e.* at C-3), yielding *e. g.* a variolaric acid precursor **7**, but preferably at C-5, yielding **1**. Both

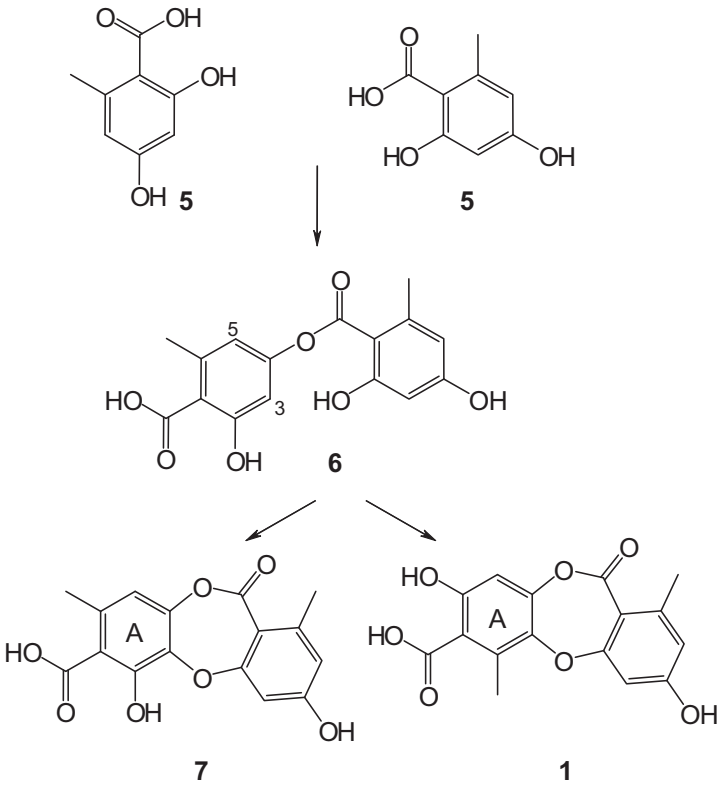

Fig. 3. Putative formation of depsidones by dimerization of orsellinic acid (**5**) *via* esterification and subsequent oxidative biaryl ether formation.

isomers can be clearly distinguished by their HMBC pattern of the methyl in rings A, and it follows that the new depsidone has structure **1**. The respective *ortho*-connected depsides (like isolecanoric acid (**8**), see Fig. S1, Supporting Information) [16] are extremely rare, and only two bis-lactones of type **9** derived thereof have been described from products in nature. This latter structure was easily excluded, as it should have very similar shifts for both lactone-carbonyls, but no signal of a chelated hydroxy group as found for **1**. In addition, a depsidone **10** derived from **8** was excluded by the HMBC pattern.

For biosynthetic reasons, also an isomer of **1** with opposite annellation in ring A (**11** in Fig. S1, Supporting Information) is not valid, as the biaryl ether bond should be formed by ring closure *via* phenol oxidation, which is possible for **1**, but not plausible for **11**.

Many metabolites with a structural similarity to **1**, such as corynesidones [12], excelsional [14], and stictic acid [17], or the phomopsides [18] have been isolated previously from lichens and fungi. However, compound **1** is the parent compound in the series of more than 60 orsellinic acid-derived depsidones and had not been described so far. The highly oxygenated compounds **1** and **2** show an increase in the structural diversity of fungal metabolites from the medicinal plant *Zanthoxylum leprieurii* and demonstrate that tropical fungi such as those from Cameroonian flora deserve increased attention from mycologists and natural products researchers.

The known fungal metabolites corynesidone B (**2**) [12], corynether A (**3**) [12], kojic acid (**4**), ergosterol, ergosterol peroxide, and adenosine were identified on the basis of their spectroscopic data (1D, 2D NMR and HRMS; Fig. S4, S5, Supporting Information) and a subsequent search in AntiBase [19].

The antibacterial activities of compounds **1**–**3** were evaluated against three bacterial strains, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis*, using the agar diffusion method as described earlier [20].

Table 1. ^{1}H (300 MHz) and ^{13}C NMR (125 MHz) data for compounds **1** and **2** in [D_6]DMSO (TMS, δ values)[a]. The spectra are shown in Figs. S2–S5, Supporting Information.

No	**1**		**2**		Lit.[b]
	δ_H	δ_C	δ_H	δ_C	δ_C
1		144.8		141.6[d]	128.1
2	6.60 (br s)	115.8		127.9[d]	141.6
3		162.0[c]		150.2[c]	149.3
4	6.60 (br s)	104.6	6.65 (s br)	104.0	104.1
4a		161.9[c]		153.4[c]	155.0
5a		140.9		141.8	142.8
6		129.2		128.9	133.5
7		118.4		118.1	110.0
8		153.6		154.4	160.3
9	6.61 (br s)	105.5	6.62 (br s)	105.5	106.4
9a		145.3		145.5	149.4
11		162.4		162.2	161.6
11a		111.3		111.4	112.9
12	2.40 (br s)	13.2	2.21 (br s)	13.3	12.5
13	2.35 (br s)	20.5	2.40 (br s)	13.2	14.1
14		168.8		168.9	172.0
OH	10.65 (br s)		10.60 (br s)		
OH			8.60 (br s)		

[a] Assigned by DEPT, COSY, HSQC, and HMBC experiments; [b] measured in [D_6]acetone, Chomcheon *et al.*; ref. [12]; [c] assignments may be exchanged; [d] assigned on the basis of predictions with ACD CNMR Predictor.

Table 2. Toxicity and antimicrobial activities of compounds **1**–**3**. Values are inhibition diameters (in mm) or mortality rates (*A. salina*), respectively.

	B. subtilis[a]	*E. coli*[a]	*S. aureus*[a]	*A. salina* (%)[b]
1	12	n. d.	10	40
2	10	n. d.	11	45
3	12	n. d.	10	30
c. e.	25	11	21	80

[a] 40 μg mL^{-1} per paper disk ($d = 9$ mm); [b] 10 μg mL^{-1}, c. e.: crude extract; n. d.: not determined.

All the tested compounds exhibited moderate inhibitory activity against *B. subtilis* and *S. aureus* at concentrations of 40 μg per paper disk, while moderate cytotoxicity towards brine shrimp larvae (*Artemia salina*) at 10 μg mL^{-1} (Table 2) was also observed.

In summary, plant endophytes are a source of new bioactive metabolites with interesting biological properties. The current study was focused on *Chaetomium* sp., an endophyte occurring in *Zanthoxylum leprieurii* leaves, which produced two highly oxygenated depsidones and other compounds. Chaetosidone A (**1**), corynesidone B (**2**) and corynether (**3**) showed inhibitory effects on two Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, at concentrations of 40 μg per paper disk and moderate cytotoxicity towards brine shrimp larvae (*Artemia salina*).

Experimental Section

General procedures

UV/Vis spectra were recorded on a Perkin-Elmer Lambda 15 UV/Vis spectrometer. IR spectra were recorded on a Perkin-Elmer (Model 1600) FTIR spectrometer. The NMR spectra were measured on a Bruker (Bruker Daltonics, Bremen, Germany) AMX 300 (300.135 MHz) and a Varian (Agilent Technologies Inc., Santa Clara, CA, USA) Inova 600 (599.740 MHz) spectrometer. ESI-HRMS spectra were recorded on a Fourier-transform ion cyclotron resonance mass spectrometer APEX IV (Bruker Daltonics). Preparative HPLC was performed with a Jasco Pu-1587 pump and a Jasco UV-1575 UV detector with a Nucleodur 100 C18 (5 μm, 250 × 8 mm, endcapped, Macherey-Nagel, Düren, Germany) column and a mobile phase of H_2O-CH_3OH with 0.1 % TFA (gradient 15 – 100 % CH_3OH in 16 min); for isolation of **1** and **2**, detection was achieved at 220 nm. Flash chromatography was carried out on silica gel (230 – 400 mesh). Zones were detected under UV at 254 and 365 nm; R_f values were measured on Polygram SIL G/UV254 (Macherey-Nagel & Co.). Anisaldehyde-sulfuric acid staining reagent was prepared by mixing anisaldehyde (1.0 g) and methanol (85 mL) with concentrated sulfuric acid (5 mL) and acetic acid (10 mL). Size exclusion chromatography was done on Sephadex LH-20 (Lipophilic Sephadex; Amersham Biosciences, Ltd., purchased from Sigma-Aldrich Chemie, Steinheim, Germany). Biomalt was obtained from a local health food shop.

Fungal material

Fresh healthy leaves of *Z. leprieurii* were collected from Mount Kalla, Yaoundé, Republic of Cameroon in September 2007 and identified by Mr. Victor Nana, botanist at the National Herbarium, Yaoundé, Cameroon. For isolation of endophytes, the samples were first washed with tap water to remove dust and debris, and then air-dried on sterile filter paper. The cleaned material was cut into small pieces using a blade. Sterile conditions were maintained for the isolation of endophytes, and all the work was performed in a laminar flow hood to avoid contamination. Surface sterilization of the samples was achieved with 95 % EtOH for 30 s, 10 % sodium hypochlorite for 10 min, 70 % EtOH for 2 min, and the material then dried aseptically as described previously [3]. The inner tissue was placed on isolation medium (water agar; WA) in Petri dishes supplemented with 100 mg L^{-1} chloramphenicol to suppress bacterial growth, and incubated at 25 °C

until the outgrowth of endophytes was discerned. Individual fungal colonies were removed and transferred onto sterile potato dextrose agar (PDA) and periodically checked for purity. Each isolate was kept in a slant agar tube for future investigations. The endophytic fungus isolate CAFTM23 was identified as *Chaetomium* sp. by one of the authors (C. D-M.) on basis of its 16S rRNA gene sequence and is deposited in the microbial collection at the Institute of Organic and Biomolecular Chemistry, Georg-August University of Göttingen, Göttingen, Germany.

Fermentation and isolation of compounds

The producing strain CAFTM23 was cultured on PDA agar at 25 °C for six days. Agar plugs were used to inoculate ten P-flasks, each containing sterilized biomalt medium (20 g L^{-1} in tap water, agar: 5 g L^{-1}) and incubated at room temperature under static conditions for 40 days. The culture was extracted three times with ethyl acetate (EtOAc), and the filtrate was concentrated to dryness *in vacuo* to afford a yellowish crude extract (27.1 g). The extract was fractionated by silica gel column chromatography using CH_2Cl_2-MeOH gradient elution to provide three major fractions according to TLC. The first fraction contained only fatty acids, ergosterol, and ergosterol peroxide, while the second fraction was purified on silica gel CC and Sephadex LH-20 using MeOH to afford corynether A (**3**). The third fraction was purified on Sephadex LH-20 using CH_2Cl_2-MeOH (1 : 1), and subfractions were further separated by preparative HPLC (H_2O-CH_3OH with 0.1 % TFA; gradient 15 – 100 % CH_3OH in 26 min) to afford chaetosidone A (**1**, 4 mg, t_R = 13.22 min), corynesidone B (**2**, 10 mg, t_R = 13.20 min), kojic acid (**4**), and adenosine.

*Chaetosidone A (**1**)*

Colorless powder, m. p. 280 – 282 °C; $R_f = 0.12$, CH_2Cl_2-MeOH (95 : 5). – IR (KBr): ν_{max} = 3417, 1765, 1740, 1562, 1430 cm^{-1}. – ^1H and ^{13}C NMR ([D_6]DMSO, 300 MHz) see Table 1. – MS ((+)-ESI): m/z = 339 (100) $[M+Na]^+$, 655 (30) ($[2M+Na]^+$). – HRMS ((+)-ESI): m/z = 315.0514 (calcd. 315.0510 for $C_{16}H_{11}O_7$, $[M+H]^+$).

Brine shrimp microwell cytotoxicity and biological activities

The isolated compounds **1**–**3** were tested for cytotoxicity and antimicrobial activity according to earlier descriptions [3, 19].

Supporting information

^1H and ^{13}C spectra of chaetosidone A (**1**) and corynesidone B (**2**) as well as a pathway of the formation of depsidones by dimerization of orsellinic acid (**5**) are given as Supporting Information available online (DOI: 10.5560/ZNB.2013-3168).

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