Phomopsidone, a Novel Depsidone from an Endophyte of the Medicinal Plant *Eupatorium arnottianum*

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The medicinal plant *Eupatorium arnottianum* can be found in the Northeast and center of Argentina and the South of Bolivia. From plant material collected in Argentina an endophytic *Phomopsis* was isolated. The fungus was identified by microscopic features and analysis of its ITS sequence. Cultures yielded, besides mellein and nectriapyrone, a novel depsidone derivative for which we propose the name phomopsidone (**1**). The structure of **1** was determined from its spectroscopic data.

Key words: Eupatorium arnottianum, Endophytic *Phomopsis*, Phomopsidone

Introduction

The genus *Eupatorium* (Asteraceae) consists of 45 different species (Herz, 2001). In the literature pharmacological and insecticidal activities have been ascribed to preparations derived from a variety of *Eupatorium* spp. (Woerdenbag, 1993). In Argentina and neighboring countries about fifteen species of *Eupatorium* are used in folk medicine by the native population (Clavin *et al.*, 1999). *Eupatorium arnottianum* (common names "clavel" or "uoué") occurs in the Northeast and center of Argentina and the South of Bolivia and is used for the treatment of gastric pains (Iharlegui and Hurel, 1992), asthma, bronchitis, and colds (Girault, 1987). Infusions of *E. arnottianum* showed analgesic (Clavin *et al.*, 2000a), antiviral (against Herpes Simplex Virus type 1; Clavin *et al.*, 2000b), and antimicrobial activities (Penna *et al.*, 1997).

In some cases unusual and valuable drugs are produced by endophytic fungi (Strobel, 2002) and in a few cases these fungi even produce compounds exhibiting the pharmacological activities reported for the plant (Weber *et al.*, 2004). In the course of our ongoing investigation of endophytic fungi from medicinal plants, *Phomopsis* sp. strain E02091 was isolated from *E. arnottianum* collected in Argentina. *Phomopsis* species are widely distributed as pathogens, endophytes or even symbionts of plants (Uecker, 1988). Some of them produce highly active mycotoxins. Here we describe the taxonomy of the producing organism, its fermentation, as well as the isolation and structure elucidation of its metabolites and their biological activities.

Materials and Methods

Producing organism

Phomopsis strain E02091 was isolated from leaves and stems of *Eupatorium arnottianum* collected in Argentina. Plant material was cut, surface-sterilized and plated onto 2% malt agar with penicillin G and streptomycin sulfate (200 mg/l each) as described earlier (Weber *et al.*, 2004). The mycelial culture is deposited in the culture collection of the Institute of Biotechnology and Drug Research (IBWF e. V.), Kaiserslautern, Germany.

Taxonomy

The fungal isolate shows all characteristics of the genus *Phomopsis*. The species, however, could not be unequivocally determined. On YMG medium (4 g/l yeast extract, 10 g/l glucose, 10 g malt extract/l; for solid media agar 2%; pH 5.5) the strain forms dark pycnidial conidiomata, in which

both α- and β-conidia are produced. The α-conidia are hyaline, nonseptate, and elliptic while the β-conidia are hyaline, nonseptate, filiform, and curved. The methods for DNA extraction and ITS amplification have been described by Köpcke *et al.* (2002). The primers used for amplification were ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG) and ITS4 (5′-TCCTCCGCTTATTGATATGC) according to White *et al.* (1990). The method was slightly modified: A GeneAmp PCR System 9700 was employed (Applied Biosystem, Foster City, CA, USA). The PCR amplification cycle consisted of 30 s at 94 °C, 1 min at 50 °C, and 1 min at 72 °C. PCR products were sequenced by MWG Biotech (Ebersberg, Germany) using the same primers as for the amplification. Database search was performed with the FASTA function of the GCG Wisconsin Package.

*Fermentation of phomopsis sp. E02091 and isolation of phomopsidone (**1**)*

The fungus was grown at room temperature in double malt medium (40 g malt extract/l; pH 5) in 2 l Erlenmeyer flasks containing 1 l of medium on a rotary shaker (120 rpm). 5–10 pieces of mycelium from well-grown agar plates were used as inoculum. When the glucose was completely used up (14 d), the culture fluid was separated from the mycelium by filtration. The culture broth was extracted with an equal volume of EtOAc, the organic phase dried with Na_2SO_4, concentrated *in vacuo* and the residue dissolved in MeOH. The crude extract (219 mg) was applied onto a column (2.5 × 10 cm) containing silica gel (Merck 60, 0.063–0.2 mm) and washed with cyclohexane/ethylacetate (1:1 v/v). An enriched product (40 mg) was obtained after elution with 100% EtOAc and applied onto a Chromabond column (C18 ec, 1000 mg; Macherey-Nagel). After washing with H_2O/MeOH (1:1) pure phomopsidone (**1**, Fig. 1) was obtained by elution with 100% MeOH; yield: 7.8 mg.

*Mellein (**2**) and nectriapyrone (**3**)*

2 and **3** (Fig. 2) were identified by HPLC-DAD-MS (HP-LC/MSD-System Series 1100, Hewlett Packard, Waldbronn, Germany) with a LiChroCART Supersphere 100 RP-18 column (125 × 2 mm; 4 μm particle size). A gradient H_2O/acetonitrile 0–100% at a flow rate of 0.8 ml/min was applied within 20 min. Retention times (min): Mellein, 11.5; nectriapyrone, 11.6.

Spectroscopic characterization of phomopsidone

^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probe head equipped with a shielded gradient coil. The spectra were recorded in $SO(CD_3)_2$, and the solvent signals (2.50 and 39.51 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine-shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimized for $^1J_{CH}$ = 145 Hz and $^nJ_{CH}$ = 10 Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). Mass spectra were recorded with a LC-MS (HP 1100; APCI, positive/negative mode) and a Micromass Q-TOF MICRO instrument (HR electrospray spectra), while the UV and IR spectra were recorded with a Perkin-Elmer λ 16 and a Bruker IFS 48 spectrometer.

Phomopsidone (**1**): UV (MeOH): λ_{max} (log ε) = 332 nm (2.91). – IR (KBr): ν = 3440, 1740, 1610,

Table I. ^{1}H (500 MHz) (δ; multiplicity; J) and ^{13}C (125 MHz) (δ; multiplicity) NMR data for phomopsidone (**1**). The spectra were recorded in $SO(CD_3)_2$, and the solvent signals (2.50 and 39.51 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm and the coupling constants J are given in Hz. The multiplicities of the carbon signals were determined indirectly from HMQC experiments.

Position	^{1}H	^{13}C
1	–	144.6; s
2	6.69; s; 1H	115.7; d
3	–	161.9; s
4	–	115.2; s
4a	–	159.7; s
5a	–	147.2; s
6	–	138.6; s
7	–	109.2; s
8	–	144.6; s
9	–	113.5; s
9a	–	148.0; s
11	–	161.0, s
12	2.37; s; 3H	20.9; q
13	4.77; s; 2H	52.1; t
14	–	168.0; s
15	5.23; s; 2H	67.8; t
16	2.14; s; 3H	10.8; q
6-OH	10.98; brs; 1H	–

1495, 1450, 1410, 1285, 1150, 1120, 1015, 865 and 785 cm^{-1}. – ^1H and ^{13}C NMR: see Table I. – HRMS (ESI, $M+H^+$) found: *m/z* 359.0788, $C_{18}H_{14}O_8$ requires 359.0767.

Biological assays

Antimicrobial activities were determined using the agar diffusion assay. Inhibition of growth of germinated seeds of *Setaria italica* and *Lepidium sativum* was tested according to Anke *et al.* (1989). Nematicidal activity was measured as described by Stadler *et al.* (1994).

Cytotoxic activity was assayed as described previously (Zapf *et al.*, 1995) with slight modifications. Jurkat (ATCC TIB 152) and Colo-320 cells (DSMZ ACC144) were grown in RPMI 1640 medium (GIBCO, BRL) MDA-MB-231 (ATCC HTB26) and MCF-7 (ATCC HTB 22) cells in D-MEM (GIBCO, BRL), supplemented with 10% fetal calf serum (FCS) (GIBCO, BRL), 65 μg/ml of penicillin G and 100 μg/ml of streptomycin sulfate. The assays contained 1×10^5 cells/ml medium.

The NF$\varkappa$B reporter gene assay was performed in Jurkat cells using the luciferase reporter plasmid pNF$\varkappa$B-Luc (Stratagene) as described earlier (Weber *et al.*, 2004).

Results and Discussion

Phomopsis sp. E02091

The *Phomopsis* strain was isolated and characterized as described in the experimental section. The morphological features are characteristic for the genus. E02091 has an ITS sequence identity of 98.3% (10 differences) with *Phomopsis* sp. GJS83–377 (AF 102999) and 98.4% with *Diaporthe helianthi* (AJ 312351). *Phomopsis* is the asexual stage of *Diaporthe.*

Isolation and structure determination

Phomopsidone (**1**)

Phomopsidone (**1**, Fig. 1) was isolated from the culture fluid of the *Phomopsis* strain E02091 cultivated in 1 l of double malt medium, as described above. **1** is a new compound.

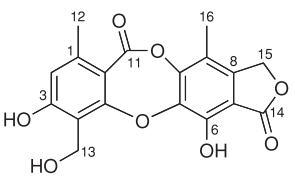

Fig. 1. The structure of phomopsidone (**1**).

The structure of **1** was elucidated based on NMR and MS data. HRESIMS data of the ions $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ revealed that the elemental composition of the compound is $C_{18}H_{14}O_8$. This means that **1** has 12 unsaturations. Strong HMBC correlations from 12-H_3 to C-1, C-2 and C-11a, from 2-H to C-4, C-11a and C-12, and from 13-H_2 to C-3, C-4 and C-4a, reveal that the western part of the molecule is a penta-substituted benzene ring, accounting for 4 unsaturations (the atom numbering is adopted from that normally used for depsidones). The down-field chemical shift of C-3 together with the weak HMBC correlations from 12-H_3 as well as 2-H to C-3 indicate that C-3 is oxygenated. A weak HMBC correlation from 12-H_3 to C-11 shows that C-11 is attached to C-11a, and its chemical shift suggests that it is a carbonyl group (acid, ester or lactone) or an oxygenated double bond carbon atom. The chemical shift of C-4a reveals that it is an oxygenated aromatic carbon atom. In the eastern part of the molecule, strong HMBC correlations from 16-H_3 to C-8, C-9 and C-9a, and from 15-H_2 to C-7, C-8 and C-9 show that the two remaining protonated carbon atoms are vicinal. 15-H_2 also gives a strong HMBC correlation to C-14, which obviously is a carbonyl carbon atom, and a weak to C-6 and C-9a. The chemical shift of C-15/15-H_2 indicates that it is part of a $-CH_2-O-CO-$ group, which in light of the remaining atoms only can form a five-membered lactone ring including also C-7 and C-8. It is now reasonable to assume that the remaining carbon atom, C-5a, closes a second benzene ring between C-6 and C-9a, and altogether 11 of the 12 unsaturations have thereby been accounted for. The remaining unsaturation must be in the form of a ring. As the 13-H_2 do not give HMBC correlations to anything except the benzene carbon atoms indicated above, this is a benzyl alcohol that is not part of a ring. The same goes for the C-3 oxygen atom, it can not possibly be part of a ring and must therefore also be protonated. The last ring therefore involves the C-4a oxygen atom and C-11, which must be the carbonyl carbon atom of a lactone, and two vicinal carbon atoms in the second aromatic group. Accounting for the remaining atoms, as well as comparing the chemical shifts, it is obvious that C-5a, C-6 and C-9a all are oxygenated, and that one of the oxygen atoms is protonated while the other

two are part of the last ring. Now one can imagine four different possibilities; a) C-11 linked to C-9a and C-4a to C-5a; b) C-11 linked to C-5a and C-4a to C-6; c) C-11 linked to C-6 and C-4a to C-5a; and d) C-11 linked to C-5a and C-4a to C-9a, and there are no HMBC correlations that indicate which one is at hand. However, in the ^{1}H NMR spectrum there is a signal for a bound proton at δ 10.98, showing that the molecule has a phenolic hydrogen atom interacting with a carbonyl oxygen atom. This is only possible in option a) and d) above, and the complete lack of any correlation between 13-H_2 and 16-H_3 in the NOESY spectrum rules out alternative d). This leaves us with structure **1** for phomopsidone.

Mellein (**2**) and nectriapyrone (**3**)

2 and **3** (Fig. 2) were detected and identified by HPLC-DAD-MS (see Materials and Methods). Mellein (**2**) has been described from *Aspergillus melleu*s (Nishikawa, 1933), *Fusarium larvarum* (Grove and Pople, 1979), *Cercospora taiwanensis* (Camarda *et al*., 1976), *Phomopsis oblonga* (Claydon *et al.*, 1985), and endophytes of medicinal plants (Weber *et al.*, 2005). Mellein and nectriapyrone were isolated by Claydon *et al.* (1985) from *Phomopsis oblonga*, commonly found on the bark of trees of the genus *Ulmus.* Trees infected by *P. oblonga* are protected from the attack of insects of the genus *Scolytus* (bark beetle) (Webber, 1981).

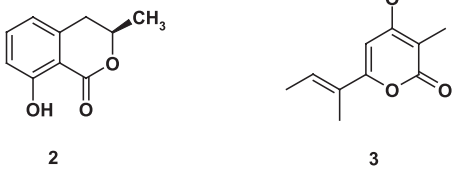

Fig. 2. The stuctures of mellein (**2**) and nectriapyrone (**3**).

Biological properties

In the plate diffusion assay phomopsidone did not show antimicrobial activity against *Bacillus brevis*, *B. subtilis*, *Micrococcus luteus*, *Enterobacter dissolvens*, *Penicillium notatum*, *Paecilomyces variotii*, *Mucor miehei*, and *Nematospora coryli* up to a content of 100 μg per filter disc.

No phytotoxic activity towards *Setaria italica* or *Lepidium sativum* was detected up to concentrations of 667 μg/ml. No nematicidal or cytotoxic activies were observed at concentrations up to 100 μg/ml against *Meloidogyne incognita* and *Caenorhabditis elegans* or Jurkat, Colo-320, MDA-MB-231, or MCF-7 cells (data not shown). Mellein and nectriapyrone exhibit antibacterial and antifungal activities (Takeuchi *et al*., 1992; Wenke, 1993; Nair and Carey, 1975).

The transcription factor NF$\varkappa$B is a key player in inflammation. NF$\varkappa$B-dependent transcription was assayed in Jurkat cells as described above. At concentrations of 20 and 50 μg/ml no effects were observed for **1**.

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