Xanthepinone, an Antimicrobial Polyketide from a Soil Fungus Closely Related to *Phoma medicaginis*

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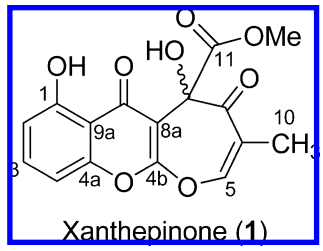
The isolation, biological characterization, and structure elucidation of xanthepinone, a novel antifungal metabolite isolated from the broth of submerged cultures of a soil fungus, are described. Xanthepinone inhibits the conidial germination of *Magnaporthe grisea* (2 μg/mL), *Phytophthora infestans* (5 μg/mL), and *Botrytis cinerea* (10 μg/mL) while showing only weak antibacterial activity; cytotoxicity was not observed up to 50 μg/mL. Molecular taxonomy revealed that the producing strain is close to species in the genus *Phoma* as well as to uncultured soil fungi and endophytes.

In the course of our screening for novel plant protectants, fungal strain 132-98, isolated from a soil sample collected in a high mountain region of the French Alps, was found to produce a compound that strongly inhibited germination of conidia of *Magnaporthe grisea*, *Botrytis cinerea*, and *Phytophthora infestans*. The effects on mycelial growth of filamentous fungi were less pronounced, and cytotoxic activities were absent. Therefore, this fungus was selected for the isolation and characterization of the active principle.

The producing organism was isolated from the soil sample. On agar, the strain grew better at 18 °C than at 27 °C. According to its 18S r RNA gene sequence the strain was closely related to members of the genus *Phoma*, especially *P. medicaginis* (CBS 533.66), and other members of the same genus. On common agar media, no formation of conidia was observed. Biological evaluation identified the active compound **1** in extracts from the culture broth. Subsequent purification by column chromatography and preparative HPLC yielded 718 mg of **1** from a fermentation on a 15 L scale.



Xanthepinone (**1**)

The elemental composition of **1** was determined to be $C_{16}H_{12}O_8$ by high-resolution MS, requiring 11 double-bond equivalents. Subsequent ^{13}C NMR experiments revealed the presence of 10 quaternary carbons. The ^1H NMR spectrum indicated a 2,3-disubstituted phenol unit, and the phenolic proton was found to exhibit a sharp resonance at δ_H 11.71. This gave evidence for the presence of a hydrogen-bonded acyl group in an *ortho* position to the hydroxy group, which could be confirmed by HMBC correlations. The *meta* position of the aromatic system was substituted with oxygen, as judged by the chemical shift of the respective quaternary carbon (δ_C 153.0). Together with the quaternary carbons at δ_C 98.4 and 162.5, a chromone scaffold was likely, the 2-position

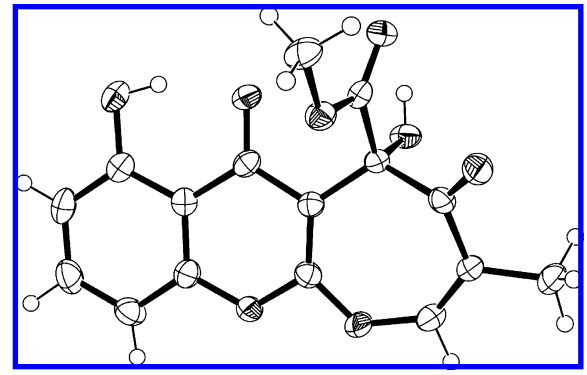


Figure 1. Crystal structure of **1** (ORTEP, ellipsoids drawn at 50% probability).

of which carried an additional oxygen substituent. The carbon resonating at δ_C 162.5 showed an HMBC correlation with an olefinic proton at δ_H 7.00, with allylic coupling to a methyl group, thus forming an α-methyl β-keto enol ether. Taking into account the additional quaternary carbon at δ_C 80.3 and the methyl ester moiety also found in the NMR spectra, the structure of **1** could be established, while some doubts remained about the keto enol ether moiety due to the very unusual chemical shift for its enolic C-5 of δ_C 142.6. However, suitable crystals could be grown, which allowed the structure to be unambiguously determined by X-ray crystallography (Figure 1). However, the absolute configuration of the C-8 stereogenic center could not be deduced from the obtained diffraction data.[1]

It appears reasonable to assume that xanthepinone (**1**) is biogenetically related to xanthones such as blennolides A and B,[2] the scaffold of which is found in the dimeric secalonic acids.[3] The diversonolic esters in their structure revised by Nicolaou and Li[4] contain a similar α,β-oxygenated ester motif to that found in the seven-membered ring of **1**. However, only a few related compounds with anellated oxepine rings have been described so far. An isomeric compound is brocaenol C,[5] while fusidienol,[6] fusidienol A,[7] and the isofusidienols[8] bear resemblance to xanthepinone (**1**) as well (Figure 2). Remarkably, fusidienol A originates from an unidentified *Phoma* species.

The antibacterial activities of compound **1** were very weak. Only *Proteus vulgaris* and *Staphylococcus aureus* were affected at 50 μg/mL. In contrast, its antifungal activity was more pronounced. The growth of yeasts was inhibited at 10 μg/mL or higher, e.g., *Candida albicans* with an MIC of 50 μg/mL (Table 2). Germination of conidia of the tested ascomycota, zygomycota, and oomycota

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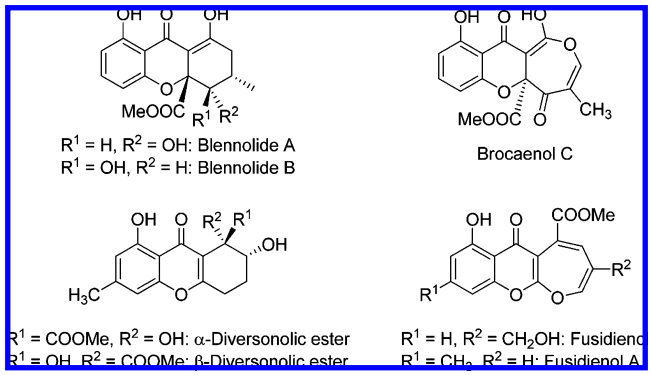

Figure 2. Natural compounds related to xanthepinone (**1**).

Table 1. ^{1}H (500 MHz) and ^{13}C (126 MHz) NMR Data for **1** in $CDCl_3$

position	δ_H (*J* in Hz)	δ_C, mult.
1		160.6, qC
2	6.86, dd (8.4, 0.6)	113.0, CH
3	7.58, t (8.4)	136.5, CH
4	6.89, dd (8.4, 0.6)	106.7, CH
4a		153.0, qC
4b		162.5, qC
5	7.00, q (1.3)	142.6, CH
6		121.3, qC
7		188.8, qC
8		80.3, qC
8a		98.4, qC
9		184.8, qC
9a		108.6, qC
10	1.97, d (1.3)	15.3, CH_3
11		169.9, qC
12	3.84, s	53.9, CH_3
OH-1	11.71, s	

Table 2. Antimicrobial Activity of Compound **1** in the Serial Dilution Test

organism	MIC (μg/mL)
Yeasts	
Candida albicans[a]	50
Nematospora coryli[b]	10
Saccharomyces cerevisiae	25
Bacteria	
Bacillus brevis ATCC 9999	>50
B. subtilis ATCC 6633	>50
Escherichia coli K12	>50
Enterobacter disolvens	>50
Micrococcus luteus	>50
Proteus vulgaris[c]	50
Pseudomonas fluorescens	>50
Staphylococcus aureus[d]	50

[a] Positive control: amphotericin B, MIC 10 μg/mL. [b] Positive control: amphotericin B, MIC 15 μg/mL. [c] Positive control: streptomycin sulfate MIC 5 μg/mL. [d] Positive control: vancomycin MIC 20 μg/mL.

Table 3. Inhibition of Conidial Germination by Compound **1**

organism	IC_{100} (μg/mL)
Botrytis cinerea	10
Magnaporthe grisea	2
Mucor miehei[a]	25
Paecilomyces variotii[a]	50
Penicillium notatum	25
Phytophthora infestans	5

[a] Positive control: amphotericin B, MIC 5 μg/mL.

was completely inhibited by 2 μg/mL for *Magnaporthe grisea*, 5 μg/mL for *Phytophthora infestans*, 10 μg/mL for *Botrytis cinerea*, and up to 50 μg/mL for *Paecilomyces variotii* (Table 3). No cytotoxic activity up to 50 μg/mL was observed with HeLa S3 or Hep G2 cells.

Experimental Section

General Experimental Procedures. The melting point was determined with a Dr. Tottoli apparatus and is uncorrected. The optical rotation was measured with a Krüss P8000 polarimeter at 589 nm. UV and IR spectra were measured with a Perkin-Elmer Lambda-16 spectrophotometer and a Bruker IFS48 FTIR spectrometer, respectively. NMR spectra were recorded with a Bruker DRX-500 spectrometer at 500 MHz and 126 MHz, respectively. The spectra were measured in $CDCl_3$, and the chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H 7.26, δ_C 77.16).[9] ESIMS and HRESIMS spectra were recorded on a Finnigan Mat95 spectrometer using polyethylene glycols as internal reference.

Microorganisms. Strain IBWF 132-98 was isolated from a soil sample. It was grown and kept on YMG-medium consisting of glucose 1%, malt extract 1% (Difco Laboratories, Detroit, MI), and yeast extract 0.4% (Hartge Ingredients, Hamburg, Germany), in tap water. For solid media, 1.5% agar was added. A sample is deposited in the strain collection of the IBWF (Institute of Biotechnology and Drug Research, Kaiserslautern, Germany).

The strain could not be identified by microscopic characteristics since the formation of conidial structures was not observed on common agar media. ITS sequencing of the 5.8S rRNA gene (GenBank accession number EU167575) revealed that the strain was closely related to *Phoma medicaginis* CBS 533.66 with a similarity of >99%. The two strains differed only in a single base pair (length of the sequence: 564 bp). In addition, high similarity to uncultured endophytic fungi as well as several soil fungi was found.

Methods and reagents for DNA extraction and PCR amplification of the ITS region (primers ITS4 and ITS5) and the 5′ end of the 18S rRNA gene (primers NS1 and SR4) have previously been described.[10]

Magnaporthe grisea strain 70-15 was obtained from the Fungal Genetics Stock Centre, Kansas. The strain was grown on CM medium as described by Talbot et al.[11] Conidia were harvested from plates incubated at 28 °C in a 16 h light and 8 h dark cycle. During the dark period the temperature was lowered to 24 °C. *B. cinerea* and *P. infestans* were obtained from BASF SE.

Fermentation. Fermentations in 500 mL Erlenmeyer flasks containing 200 mL of YMG medium were inoculated with four pieces (1 cm × 1 cm) cut from agar slants. The flasks were incubated on a rotary shaker at 120 rpm and 20 °C.

Fermentations on a larger scale were carried out in a Biostat (Braun, Melsungen, Germany) containing 20 L of YMG medium with stirring (120 rpm) and aeration (3 L of air per minute) at 20 °C. To prevent foaming, silicone antifoam (Merck, Darmstadt, Germany) was added. The fermentor was inoculated with 200 mL of a well-grown culture in the same medium. Daily samples were withdrawn and assayed for pH, glucose and maltose content, and biological activity toward germination of *M. grisea* conidia. After 11 days, the biological activity had reached a plateau and the fermentation was stopped. The culture broth was separated by filtration. Mycelia contained no active compounds and were discarded. The culture broth of daily samples was extracted with EtOAc, and the organic phase was dried over Na_2SO_4 and concentrated *in vacuo*. The residue was dissolved in MeOH to a concentration of 10 mg/mL and used for biological assays as well as for HPLC analysis.

Isolation of the Compound. The broth from one fermentation (15 L) was passed through a column of Diaion HP21 resin in H_2O (Mitsubishi Chemical Industry Ltd, Düsseldorf, column size: 350 × 55 mm). The eluate was discarded. After washing with H_2O (2 L), the compound was eluted with MeOH (1.5 L), followed by acetone (1.5 L). The extracts were combined and concentrated. The crude product (4.3 g) was further fractionated by chromatography on silica gel in cyclohexane−EtOAc (column size: 220 × 45 mm; silica gel 60, 63−200 μm particle size; Merck, Darmstadt, Germany). Elution with cyclohexane−EtOAc with increasing amounts of EtOAc (60:40, 55:45, 50:50, 2.5 L each) yielded two fractions with antifungal activity. Fraction 1 (342 mg) eluted with 40% EtOAc and fraction 2 (588 mg) with 50% EtOAc. Final purification was achieved by preparative HPLC with a Jasco modular HPLC system (Groß-Umstadt, Germany) consisting of two binary pumps (PU-1586) and a UV-1570 M multiwavelength detector with a LiChroSpher 100 RP-18 column (250 × 25 mm, 5 μm particle size; Merck) and elution with 45% MeCN in water at a flow

rate of 7.5 mL/min. Each run was performed with 60 mg aliquots of the active mixtures. The total yield from 15 L was 718 mg of compound **1**.

Biological Assays. Germination assay: Conidia from 10-day-old cultures were harvested. After centrifugation at 1000*g* for 10 min, the conidia were resuspended in distilled H_2O to a concentration of 5×10^5 per mL. The test was carried out in 96-well microtiter plates (Sarstedt, Nürnberg, Germany) with 0.5×10^4 conidia/well in 0.2 mL of distilled H_2O and incubation for 16 h at 28 °C. Germinated conidia were counted with an inverted microscope (Leica DM IRB). Tests were conducted in triplicates and 3×100 conidia were counted. The test was conducted and evaluated as described before.[12]

Antimicrobial activity was determined in the serial dilution test. Bacteria were tested in nutrient broth (Difco),; yeasts and fungi, in YMG medium.

Cytotoxic activity was assayed as described previously[13] with slight modifications. HeLa S3 (ATCC CCL 2.2) and Hep G2 (DSMZ ACC 180) cells were grown in D-MEM (Gibco, BRL), supplemented with 10% fetal calf serum (Gibco, BRL), 65 μg/mL of penicillin G, and 100 μg/mL of streptomycin sulfate. The assays were conducted with 1×10^5 cells/mL medium.

Xanthepinone (1): yellow crystals; mp (MeOH) 146−147 °C; $[\alpha]_D^{25}$ −31.7 (*c* 0.74, $CDCl_3$); UV λ_{max}^{MeOH} (log ϵ) 206 (4.18), 221 (4.13), 247 (4.18), 284 (sh, 3.65), 327 (3.59) nm; IR (KBr) ν_{max} 3436, 2957, 1753, 1655, 1607, 1472, 1414, 1244, 1167, 814, 707 cm^{-1}; NMR data see Table 1; ESIMS *m/z* 333 $(M + H)^+$, 355 $(M + Na)^+$, 371 $(M + K)^+$, 687 $(2\,M + Na)^+$, 703 $(2\,M + K)^+$; HRESIMS *m/z* 355.0438 (calcd for $C_{16}H_{12}O_8 + Na^+$, 355.0424).

X-ray Crystallographic Data of 1. Crystals of **1** were grown by slow evaporation of an MeOH solution of **1**. Data were obtained at 153 K on a Bruker APEX diffractometer with graphite-monochromated Mo Kα radiation. Formula $C_{16}H_{12}O_8$, crystal size $0.38 \times 0.14 \times 0.05$ mm^3, monoclinic, space group $P2_1$, $a = 7.9214(10)$ Å, $b = 10.7896(14)$ Å, $c = 8.7901(11)$ Å, $\beta = 104.961(2)°$, $V = 725.81(16)$ $Å^3$, $Z = 2$, $D = 1.520$ g cm^{-3}, $R = 0.0449$, $R_w = 0.0604$. CCDC-745500 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html [or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: (internat.) +44-1223/336-033; e-mail: deposit@ccdc.cam.ac.uk]. Attempts to determine the absolute configuration by anomalous dispersion using Cu Kα radiation were unsuccessful.

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Supporting Information Available: NMR spectra and CIF of xanthepinone (**1**). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Note added in proof: after submission of the revised version of the present manuscript, a paper by Krohn et al. was published, in which the partial synthesis of xanthepinone by chemical oxidation of microsphaeropsone A is reported. The authors determined the absolute configuration of levorotatory xanthepinone to be (*R*) by comparison of simulated and measured solid state CD spectra of microsphaeropsone A using the solid state CD/TDDFT method. See: Krohn, K.; Kouam, S. F.; Kuigoua, G. M.; Hussain, H.; Cludius-Brandt, S.; Flörke, U.; Kurtán, T.; Pescitelli, G.; Di Bari, L.; Draeger, S.; Schulz, B. *Chem.-Eur. J.* **2009**, DOI: 10.1002/chem.200900749.
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