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Purification, identification and activity of phomodione, a furandione from an endophytic *Phoma* species

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Abstract

Phomodione, [(4a*S**,9b*R**)-2,6-diacetyl-7-hydroxy-4a,9-dimethoxy-8,9b-dimethyl-4a.9b-dihydrodibenzo[*b*,*d*]furan-1,3(2*H*,4*H*)-dione], an usnic acid derivative, was isolated from culture broth of a *Phoma* species, discovered as an endophyte on a Guinea plant (*Saurauia scaberrinae*). It was identified using NMR, X-ray crystallography, high resolution mass spectrometry, as well as infrared and Raman spectroscopy. In addition to phomodione, usnic acid and cercosporamide, known compounds with antibiotic activity, were also found in the culture medium. Phomodione exhibited a minimum inhibitory concentration of 1.6 μg/mL against *Staphylococcus aureus* using the disk diffusion assay, and was active against a representative oomycete, ascomycete and basidiomycete at between three and eight micrograms per mL.

Keywords: *Phoma*; Furandione; Phomodione; Usnic acid; Cercosporamide; Antibacterial; Antifungal; NMR; Mass spectrometry; X-ray crystallography

1. Introduction

Natural products from plants, lichens, fungi and marine organisms provide an important source of compounds with potential as therapeutic agents (Newman et al., 2000; Strobel et al., 2004). Bacterial and viral drug resistance, and the spread of fungal and parasitic diseases necessitate the search for additional antibiotic compounds with activity at low concentrations and with reasonably low toxicity to humans. It has proven effective to search for these compounds among endophytic bacteria and lichens, since they represent biological associations that provide protection against competing organisms (Francolini et al., 2004). There appears to be a higher probability of discovering bioactive compounds from endophytic species (Strobel et al., 2004).

Usnic acid **1** (Fig. 1), a dibenzofuran originally isolated from lichens (Knopp, 1844; Stark et al., 1950), has been shown to act as a growth regulator in higher plants (Huneck and Schreiber, 1972). In humans, it can act as an anti-inflammatory (Kumar and Müller, 1999), antimitotic (Cardarelli et al., 1997), antineoplastic (Takai et al., 1979), antibacterial (Ingolfsdottir et al., 1998) and antimycotic (Yamamoto et al., 1995) agent.

Numerous derivatives of usnic acid **1** have been isolated from a variety of different lichens and fungi (Huneck and Schmidt, 1980). Like usnic acid, these dibenzofuran

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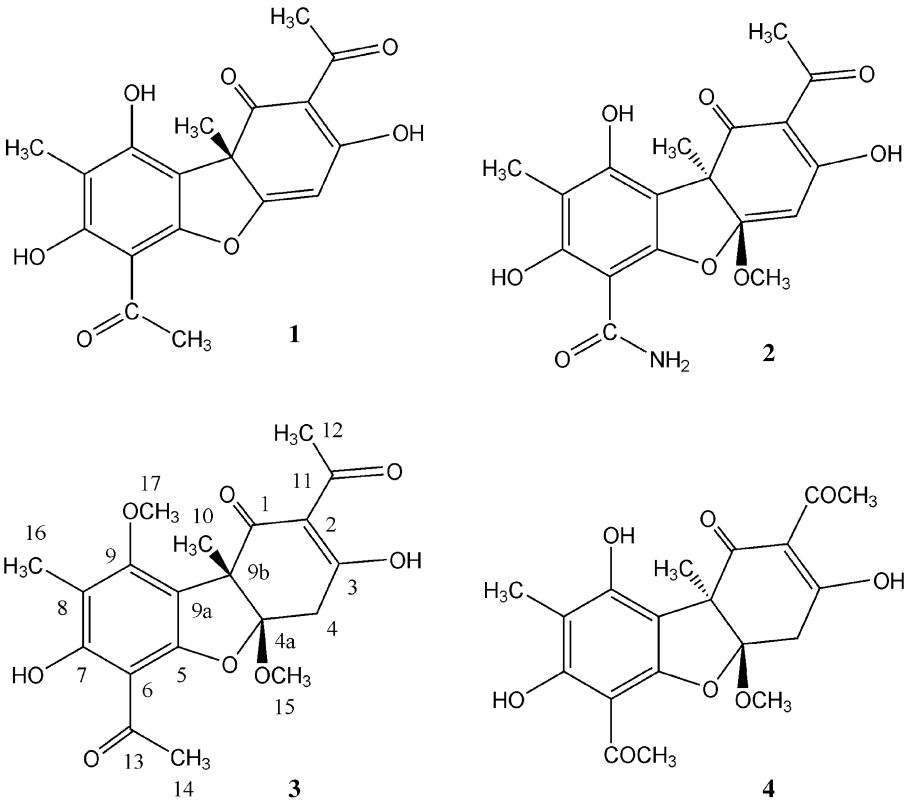

Fig. 1. Structures of usnic acid 1, cercosporamide 2, phomodione 3, showing numbering used), and (−)-pseudoplacodiolic acid 4.

derivatives often show useful biological activities. For example, cercosporamide **2** (Fig. 1), an usnic amide from *Cercosporidium henningsii* (Sugawara et al., 1991; Conover et al., 1992) has phytotoxic and antifungal effects and has selective activity as a Pkc1 kinase inhibitor (Sussman et al., 2004).

The *Phoma* sp. products described here include usnic acid **1**, the previously characterized cercosporamide **2**, and the newly discovered phomodione **3**, a related compound. Its structure and preliminary biological activity are investigated.

2. Results and discussion

2.1. Isolation and characterization of the fungus

The isolate of the fungus (NG-25) used in this study was obtained from the lower crown of *Saurauia scaberrinae* growing in the central highlands of Papua New Guinea (PNG). The organism was isolated from the inner tissues obtained from surface sterilized small stems of this tree, and therefore is considered to be an endophyte (Strobel et al., 2000). This fungal isolate was one of many endophytes recovered from this tree located near Goroka, PNG. Products from this fungus possessed antifungal activity and thus its biology, taxonomy and biochemistry were further pursued. When grown on gamma-irradiated carnation leaves placed on water agar to provide sterile plant substrate, the organism produced dark ostiolate pycnidia (ca. 100 μm in dia) that were immersed in the mycelium of the fungus as well as the agar or leaf base (Fig. 2). The hyphae were felty and olivaceous-brown when grown on potato dextrose agar. Each pycnidium possessed an ostiole, having little or no neck, through which conidiospores emerged. The single celled conidia average 3.03×1.72 μm and were cylindrical and straight as observed by ESEM and were smaller under regular SEM after critical point drying, i.e. 2.36×1.42 μm (Fig. 2). The organism answers the morphological description of *Phoma* sp. (Sutton, 1980). Additionally, this was confirmed by ITS-5.8S rDNA analysis followed by a BLAST search which revealed that the 5 closest relatives of this fungus are *Phoma pinodella* at the 99% level (517/522/bases) (Sutton, 1980). The sequences are deposited in GenBank as EU180709. The fungus itself is deposited as isolate No. 2323 in the living mycological culture collection of Montana State University. It is also deposited in the Agricultural Research Service Culture Collection as NRRL accession 46751.

2.2. Isolation and activity of products

The fungus was cultured on potato dextrose broth with 6 g of sucrose per liter for 2–3 weeks at room temperature with slow agitation. Biological activity and results from

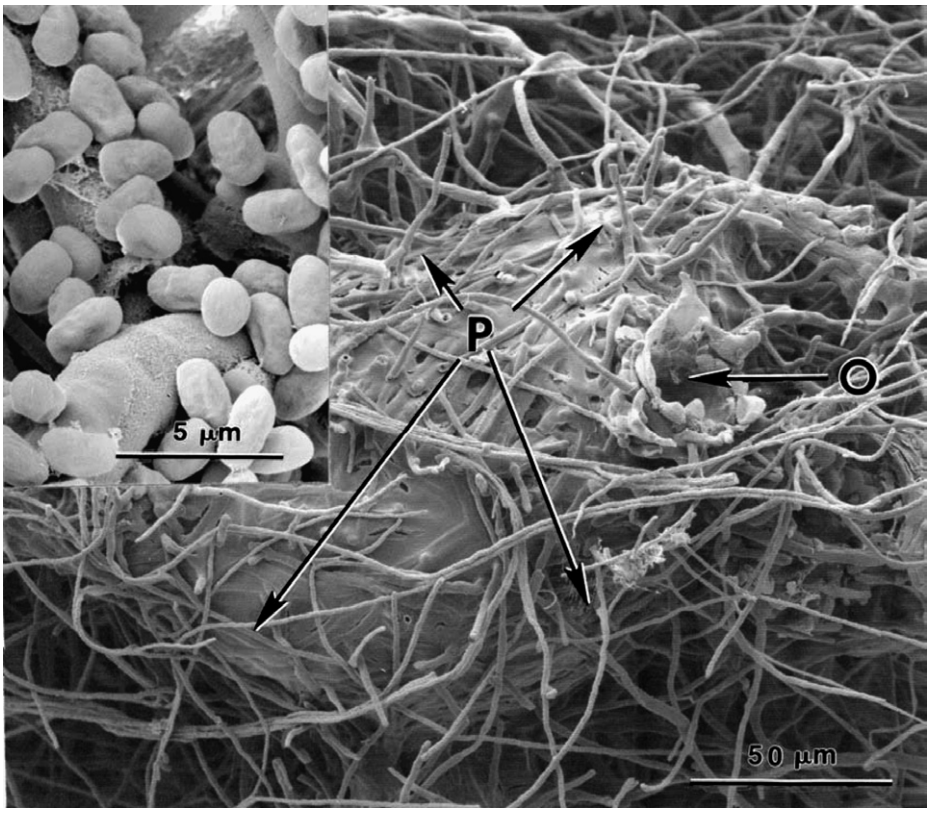

Fig. 2. Scanning electron micrograph of a pycnidium (P) of the fungal species showing the ostiole (O). Insert shows the conidiospores.

TLC were used to guide the purification of the three major products. An effort was made to find the best culture conditions for production of phomodione **3**, including the type of culture medium, the amount of sugar and the length of time in culture. When the fungus was cultured in potato dextrose broth with six grams per liter of sucrose, the biomass increased greatly during the first week or two, but did not increase significantly after that. If the culture medium was harvested after 12 days, the reducing sugars had decreased from about 20 μg/μL to about 15.5 μg/μL, and the sugar concentration dropped to less than half that amount by the third week.

As the concentration of reducing sugars decreased, the relative amounts of three products changed. Table 1 shows that the amount of usnic acid **1** decreased from 8% of the final extract mass to about 5%, while the cercosporamide **2** and phomodione **3** increased from 2% to 9% and from undetectable to 3%, respectively. The delayed production of cercosporamide **2** and phomodione **3** coupled with a simultaneous decrease in usnic acid **1** suggests that these compounds may be biotransformation products of the latter.

Table 1
Major products extracted from culture medium as percentage[a] of pooled extract (mg) from multiple extracts

Days in culture	Phomodione	Cercosporamide	Usnic acid
12	ND[b]	2% (3.3 mg)	8% (12.6 mg)
15	0.2% (0.36 mg)	8% (14.4 mg)	7% (12.6 mg)
21	3% (5.4 mg)	9% (16.3 mg)	5% (9.0 mg)

[a] % of peak area measured by HPLC at 250 nm.
[b] Not detected.

The biological activities of the three major components of the fungal extract were compared. None of the compounds was effective against *Escherichia coli* at 500 μg or lower (Table 2), but minimum inhibitory concentrations (MICs) of all three compounds were approximately the same on *Staphylococcus aureus*, indicating that these compounds may be much more effective against gram positive bacteria. The antimycotic activity was tested using a representative species from three classes of fungi. The representative oomycete, ascomycete and basidiomycete were *Pythium ultimum*, *Sclerotinia sclerotiorum*, and *Rhizoctonium solani*, respectively. In each case, usnic acid **1** was less effective than either cercosporamide **2** or phomodione **3**. MICs for phomodione **3** were comparable to cercosporamide **2** against representatives of all three classes of fungi. The relative toxicities of these compounds are being evaluated.

2.3. Structural determination

High resolution mass spectrometry was used to define the composition of phomodione **3** and its principal fragments. These were determined from the direct insertion

Table 2
Average MIC from two trials each of antibacterial and antifungal activity by usnic acid, cercosporamide and phomodione

Compound	*E. coli*[a] (μg) gram negative	*S. aureus*[a] (μg) gram positive	*P. ultimum*[b] (μg/mL) oomycete	*S. sclerotiorum*[b] (μg/mL) ascomycete	*R. solani*[b] (μg/mL) basidiomycete
Usnic acid	>500	2.0	10–15	>10	>10
Cercosporamide	>500	2.0	3–4	5–8	8–10
Phomodione	>500	1.6	4–5	3–5	5–8

[a] Approximately 0.5 mm clear zone of inhibition.
[b] Complete inhibition of growth for 3 days.

probe, using the three exact mass, two-isotope ratio technique (Grange et al., 1996; Grange and Brumley, 1997) to distinguish between compositions of similar exact mass. The composition of the compound was determined to be $C_{20}H_{22}O_8$. The relative abundances of the ions and neutral losses, by mass differences, in the mass spectrum, suggested a relationship to usnic acid **1** (Table 3).

Major structural features of phomodione **3**, including relative stereochemical configuration, were established from X-ray diffraction of a crystal grown from methanol (Fig. 1). The structure, however, displayed disorder in the C2, C3 and C11 positions and high quality coordinates were not obtained ($R(f) > 0.18$). Final structural details were therefore established from NMR spectroscopic data using the gradient-selected heteronuclear single quantum correlation (HSQC) (Palmer et al., 1991) and heteronuclear multiple bond correlation (HMBC) (Willker et al., 1993) experiments. All shift assignments and the 14 HMBC correlations observed are given in Table 4 and Fig. 3, respectively. The NMR data, however, does not allow for the determination of whether phomodione **3** contains a C2 anion or a tautomeric structure in the solvent used (d_4-methanol). The structures of the six tautomers possible for phomodione **3** as well as the C2 anion (Fig. 3) were thus constructed on a computer and then energy minimized at the B3LYP/D95* level of theory. The NMR chemical shifts (^{13}C) were computed for all structures (B3PW91/D95**) and compared to experimental data using a previously described procedure (Harper et al., 2001, 2003). The best correlation of computed shielding to shift was obtained for the structure **3E**, the anion (Fig. 4). However, tautomer **3A** was statistically indistinguishable from **3E** and must therefore also be considered. All other tautomers were rejected with a high statistical probability that they were incorrect structures. Prior X-ray diffraction of the closely related derivative (−)-pseudoplacodiolic acid **4** (Fig. 1) has demonstrated that form **A** is preferred in the solid-state (Huneck et al., 1981), and suggests that phomodione exists as this tautomer (Fig. 3). Of course, the tautomeric form within an organism depends on physiological conditions such as pH. The analysis presented here serves to identify energetically favorable forms of phomodione. Complete coordinates for the final computer optimized structure **3A** are included as supporting information.

Table 4
NMR shift assignments and important HMBC ^1H–^{13}C correlations in phomodione

Position	δ ^{13}C	δ ^1H	HMBC
1	194.5		
2	138.3		
3	147.7		
4	29.42	1.21	C2, C3
4a	112.5		
5	157.8		
6	104.0		
7	162.0		
8	111.7		
9	163.8		
9a	116.0		
9b	58.0		
10	15.01	1.60	C1, C4a, C9a, C9b
11	178.1		
12	23.10	1.8	C11
13	202.7		
14	30.48	2.54	C6, C13
15	49.14	3.30	C4a
16	7.72	1.96	C7, C8, C9
17	61.08	3.84	C9

Table 3
Molecular ion (M^+) and fragment ion compositions for significant ions in the mass spectrum of phomodione and relationship to usnic acid[a]

Composition	Theoretical mass	Experimental mass	Error (millimass units)	Comment
$C_{20}H_{22}O_8$ M^+	390.1315	390.1324	+0.9	Usnic acid + C_2H_6O [CH_3 + CH_3O]
$C_{19}H_{18}O_7$	358.1053	358.1067	+1.4	CH_3OH (32) loss from above
$C_{15}H_{14}O_5$	274.0841	274.0845	−0.4	$C_4H_4O_2$ (84) loss from 358, like usnic acid
$C_{14}H_{16}O_5$	264.0998	264.1002	−0.4	Unique to this compound
$C_{13}H_{13}O_5$	249.0763	249.0771	+0.8	Base Peak (100%) usnic acid base peak + 1(O)
$C_{14}H_{15}O_4$	247.0970	247.0975	+0.5	$C_5H_3O_3$ (111) loss from 358, like usnic acid
$C_{13}H_{13}O_4$	233.0814	233.0818	+0.4	CH_3O (31) loss from 264
$C_{13}H_{11}O_4$	231.0657	231.0659	+0.2	C_2H_3O (43) loss from 274, like usnic acid

[a] The mass spectrum of usnic acid from the NIST library was used as reference.

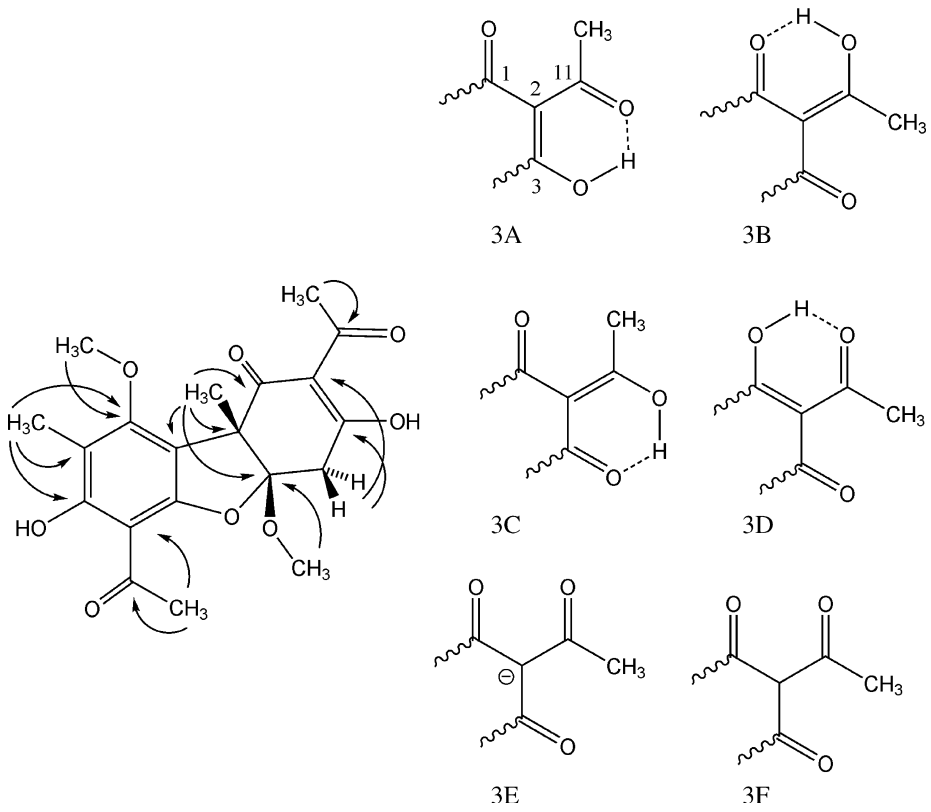

Fig. 3. HMBC correlations observed in phomodione **3** in CD_3OD (left structure). Disorder at C2, C3 and C11 and the inability to accurately locate hydrogen atoms by X-ray diffraction prevent an unambiguous identification of the specific tautomer present in the ring containing C2. The six tautomers, A–F, shown at the right were therefore constructed on a computer, energy minimized and a statistical comparison performed between experimental shifts and shift computed for the six tautomers. This evaluation eliminated structures 3B, 3C, 3D, and 3F with high probability. Tautomer 3A has been previously observed in the closely related compound (−)-pseudoplacodiolic acid **4** and was thus selected as the most probable tautomeric form.

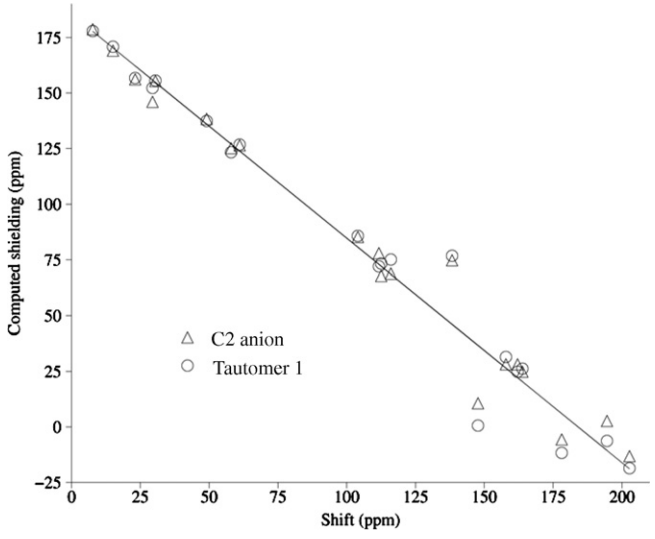

Fig. 4. A plot of the correlation of experimental ^{13}C NMR shift versus computed shielding for the two best fit structures (**3A** and **3F**) of phomodione **3**. The four alternative tautomeric structures considered (see Fig. 3) gave significantly worse fits, statistically, and were therefore rejected as probable structures.

Several features in the vibrational spectra lend support to the proposed structure of phomodione **3**. In the IR spectrum, the peak at 720 cm^{-1} is due to the CH_2 rocking vibration and the peak at 1317 cm^{-1} is due to the CH_2 wag/twist at C4. The peak at 1521 cm^{-1} is indicative of a *para*-substituted aromatic hydrocarbon. The peaks from about 3000 to 2800 cm^{-1} are due to CH stretching vibrations. The Raman spectrum shows six distinct peaks in this region that are most likely due to CH stretching vibrations. The broad absorbance centered at 3336 cm^{-1} in the IR spectrum is due to the OH stretch and the peak at 1385 cm^{-1} in the Raman spectrum is due to the OH bend. The peaks at 1644 and 1731 cm^{-1} in the IR spectrum are most likely due to the C=O stretch, the former to the stretch at C11/C13 and the latter to the stretch at C1(C3). Both IR and Raman spectra are supplied in the supporting information.

2.4. Concluding remarks

In summary, phomodione **3**, a furandione with similarities in structure and activity to cercosporamide **2** is produced in submerged cultures of a *Phoma* species isolated from *Saurania scaber*, a New Guinea plant. Structural data obtained from phomodione **3** by high resolution NMR and crystallography is best represented by the tautomer depicted in Fig. 3, and is corroborated by IR and Raman

spectroscopy. Additional biological activities of this compound are being explored.

3. Experimental

3.1. General

Unless otherwise specified, chemicals were reagent grade. Mps. were uncorr. TLC was on silica gel 60 F_{254} (EMD), eluted with $CHCl_3$:MeOH, 9:1 and visualized with UV at 254 and 366 nm and with vanillin–sulfuric acid spray. Prep HPLC: Varian Dynamax SD200, UV-1 on a Varian Pursuit diphenyl, 10 μm column 250 × 10 mm. Method: isocratic, 10 mM NH_4OAc buffer, pH 4 to CH_3CN (HPLC grade), 50% B, at 2 mL/min and 254 nm (UV). APCI-MS (pos): Shimadzu 2010EV, single quadrupole instrument at 1.5 kV, Phenomenex Synergi 4 μm Fusion RP 80, 150 × 3 mm, using the same program as the prep HPLC with flow rate of 1 mL/min. High resolution MS: Finnigan MAT 900S double focusing mass spectrometer, EI, 70 eV, 100 m, 50–600*m/z* from a rapidly heated direct insertion probe. Accurate masses were measured at 10,000 resolution. The infrared absorption spectra were collected using a Thermo Nicolet 6700 FT-IR spectrometer equipped with a Thermo Smart Orbit ATR accessory with a diamond crystal at a resolution of 4 cm^{-1}. The Raman spectra were collected using a Thermo Nicolet NXR FT-Raman module with a 1064 nm Nd:YVO_4 laser, a liquid nitrogen cooled Ge detector with a 180° backscattering geometry at a resolution of 8 cm^{-1}.

3.2. Fungal isolation and identification

The fungal isolate (NG-25) was one of many endophytes obtained from the inner tissues of a small section of surface sterilized tree stem using techniques described in Strobel et al. (2000). The stem was taken from a cutting of *S. scaberrinae* growing in the central highlands of Papua, New Guinea near Goroka (PNG at 04°23′37″ S and 143°14′49″ E). After growth on gamma-irradiated carnation leaves placed on water agar, the isolate was identified as a *Phoma* sp. based on both morphological and ITS-5.8S rDNA analyses (Sutton, 1980).

Freshly prepared wet specimens of the fungus were examined by environmental scanning electron microscopy (ESEM) and images were recorded with an FEI XL30 ESEM FEG in the environmental mode as described by Castillo et al. (2005). In addition, for regular SEM, the fungal specimens were critical point dried, gold sputter-coated, and images were recorded with an XL30ESEM FEG (FEI Company, Hillsboro Ore) in the high vacuum mode using the Everhart-Thornley detector (Castillo et al., 2005). Conidia were measured using Image J software (available online: http://rsb.info.nih.gov/ij/).

The fungus was grown on potato dextrose broth for 7 days and the mycelium was harvested and the nucleic acid (DNA) was extracted using DNeasy Plant and Fungi Mini Kit (Qiagen) according to the manufacturer's directions. The ITS regions of the fungus were amplified using PCR and the universal ITS primers ITS1 (5′ TCC GTA GGT GAA CCT GCG G 3′) and ITS4 (5′ TCC TCC GCT TAT TGA TAT GC 3′). All other procedures were carried out as previously described by Ezra et al. (2004). The DNA was sequenced at the W.M. Keck Facility at Yale University. The sequence data of this fungus are deposited in GenBank.

The electron micrograph was prepared according to Castillo et al. (2005). The microbial material was critical point dried, gold sputter-coated and images were recorded with an XL30ESEM FEG (FEI Company, Hillsboro Ore) in the high vacuum mode using the Everhart-Thornley detector.

3.3. Extraction, isolation and identification of fungal products

The best medium for producing products from *Phoma* sp. was potato dextrose broth (Difco) with 6 g/L of sucrose. It was cultured at room temp. with shaking at ca. 80 RPM. The medium was harvested at specified times. Triplicate samples were taken and assayed for reducing sugars using a Sigma Glucose (HK) assay kit according to the manufacturer's directions with 1, 5 and 10 μL of filtered broth per culture. Mycelium was filtered under vacuum through weighed filter papers, and the filtrate extracted x3 with equal volumes of $CHCl_3$ or CH_2Cl_2, dried with anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure. The weighed residue was dissolved in the minimum CH_2Cl_2, placed on a silica gel (60–200 mesh) column and eluted with hexane to afford usnic acid **1**, with hexane:EtOAc 99:1 to give phomodione **3**, and hexane:EtOAc 98:2 to yield cercosporamide **2**. The separations were monitored by TLC. These crude fractions were purified by prep HPLC and crystallization.

Usnic acid **1**: recrystallized from hexane/EtOAc (1:10, v/v) yielding yellow solid, m.p. 203–203.5 °C (lit. m.p. 201–203 °C, Huneck and Schreiber, 1972), TLC R_f 0.98, HPLC elution time, 9.0 min, compared with authentic standard (Aldrich, St. Louis, MO), Cercosporamide **2**: recrystallized from EtOAc, yielding reddish crystals, m.p. 187.5–188.5 (lit. m.p 188–189, Sugawara et al., 1991), TLC R_f 0.54–0.57 (blue-gray with vanillin-sulfuric acid), HPLC elution time 7.5 min. Usnic acid **1** was identified by its mass spectrum (compared to reference in NIST library), and cercosporamide **2** was identified by its melting point (Sugawara et al., 1991) and EI-MS: *m/z* (% rel. int.) 331 (80) $[M]^{+\cdot}$, 314 (9), 247 (30), 230 (96), 220 (54), 203 (100). A theoretical mass of 331.0692 and an experimental mass of 331.0683 verified the formula $C_{16}H_{13}NO_7$.

Phomodione **3**: recrystallized from EtOAc:MeOH, 1:9, yielding light tan crystals, m.p. 209–215 dec. $[a]_D^{26.3}$ −0.006° (MeOH, 0.08 g/100 mL). R_f on TLC, 0.90–0.88 (forest green with vanillin–sulfuric acid), HPLC elution

time 7.6–7.7 min. UV: λ_{max} MeOH (log e): 282 (4.8), 240 (3.7), 204 (5.3); Raman ν^{solid} (cm^{-1}): 3302 (0.101), 2960 (0.181), 2917 (0.336), 2905 (0.335), 2872 (0.251), 2860 (0.124), 2851 (0.191), 2746 (0.092), 1636 (0.198), 1478 (0.142), 1443 (0.270), 1385 (0.098), 1299 (0.163), 1130 (0.178), 1096 (0.163), 955 (0.076), 549 (0.223); IR $\nu^{solid\ ATR}$ (cm^{-1}): 3336 (0.068), 2952 (0.150), 2921 (0.246), 2852 (0.186), 1731 (0.082), 1644 (0.123), 1616 (0.130), 1585 (0.117), 1521 (0.090), 1456 (0.111), 1410 (0.081), 1376 (0.146), 1347 (0.108), 1317 (0.107), 1279 (0.123), 1191 (0.024), 1167 (0.021), 1080 (0.142), 996 (0.125), 954 (0.115), 923 (0.079), 871 (0.092), 807 (0.077), 748 (0.107), 720 (0.100).

EI-MS: m/z (% rel. int.) 390 (26). $[M]^{+\cdot}$, 358 (12), 274 (11), 264 (53), 249 (100), 247 (51), 233 (15), 231 (11); MF $C_{20}H_{22}O_8$ with a theoretical mass of 390.1315 and experimental mass of 390.1324.

3.4. Structure determination

Gauge invariant (Ditchfield, 1974) chemical shift computations were performed for all structures using the B3PW91 method and the D95** basis set (Becke, 1993; Perdew and Wang, 1992). Structures considered were geometry optimized before the computation of chemical shift at the B3LYP/D95* level of theory (Lee et al., 1988; Becke, 1993). All computations were performed using the Gaussian 03 program (Frisch et al., 2003) using parallel processing.

NMR spectra were acquired at 26 °C on a Varian iNOVA 500 MHz spectrometer in methanol-d_4. Spectra were referenced to the center line of the methanol ^1H and ^{13}C multiplets at 3.31 and 49.15 ppm, respectively. One bond $^1H/^{13}C$ correlations were observed with the gradient HSQC sequence. Multiple bond $^1H/^{13}C$ correlations were observed with the phase-cycled and gradient selected HMBC sequence.

3.5. Antibacterial and antifungal assays

Antifungal tests were done in triplicate with the oomycete *P. ultimum*, ascomycete, *S. sclerotiorum*, and basidiomycete, *R. solani.* One mL of potato dextrose broth (DIFCO) was pipetted into each well of a sterile 24-well plate. Test samples were dissolved in MeOH at known concentrations. One to 20 μg of test sample was added to each well in no more than 10 μL of MeOH. The three fungi were cultured on potato dextrose agar (DIFCO) on a petri dish for ca. 5 days. Uniformly cut plugs of ca. 1.5 mm in diameter were removed from the fungal plate, placed into each well, and incubated at room temperature for 3 days. Fungal growth was observed daily under a dissecting microscope and scored for growth compared with MeOH controls. MIC was defined as the lowest concentration at which fungal growth was inhibited.

Antibacterial inhibition assays were performed using the disk diffusion method (Wilkinson et al., 2003). A thin layer of LB broth containing *S. aureus* (ATCC 25923) or *E. coli* (Life Technology 18290-015) was spread over the entire surface of a petri dish containing nutrient agar. Various amounts of test compound dissolved in MEOH were placed on sterile disks Whatman 3MM filter paper. MeOH was used as control. The dried disks were placed on the surface of the bacteria plates and incubated at 37 °C. Zones of inhibition around the disks were measured in mm at 8, 24 and 48 h. MIC was defined as the concentration at which a 0.5 mm clear zone appeared around a disk.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2007.10.031.

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