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Phomopsichalasin, a Novel Antimicrobial Agent from an Endophytic *Phomopsis* sp.

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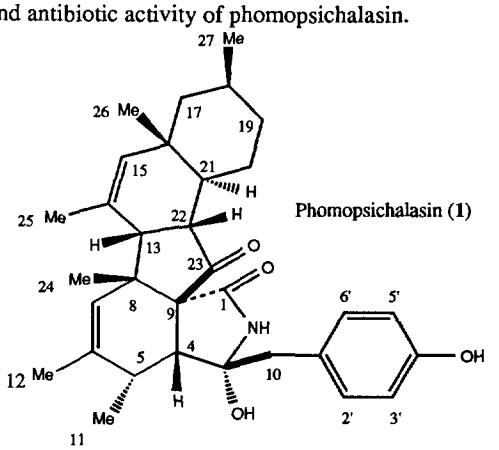
Abstract: The antimicrobial agent phomopsichalasin (1) was isolated from an endophytic *Phomopsis* sp. fermented on shredded wheat. The structure was determined by UV, IR, NMR and mass spectroscopy. Phomopsichalasin represents the first cytochalasin-type compound with a three ring system replacing the cytochalasin macrolide ring.

A large number of cytochalasins have been isolated from a variety of filamentous fungi[1]. The well-documented cell-level biological effects of the cytochalasins have been attributed largely to their ability to bind with actin filaments[1a]. Two cytochalasins have also been found to act as HIV protease inhibitors[2]. The cytochalasins are structurally comprised of an isoindolone unit fused to an 11-, 13- or 14- carbon macrocyclic ring[1,3]. Cytochalasins are generally differentiated from one another according to whether they possess an isopropyl, an indolyl or a phenyl group attached at C-10 (Figure 1)[1a]. The novel compound phomopsichalasin (**1**) has been discovered which, while partially comprised of an isoindolone and a C-10 substituent, is strikingly different from the cytochalasins in having a 13-membered tricyclic system rather than a macrolide, a phenol substituent at C-10 rather than a phenyl group and an -OH moiety at C-3. It also represents a novel fused ring system. This paper describes the fermentation, isolation, structural elucidation and antibiotic activity of phomopsichalasin.



Phomopsichalasin (**1**)

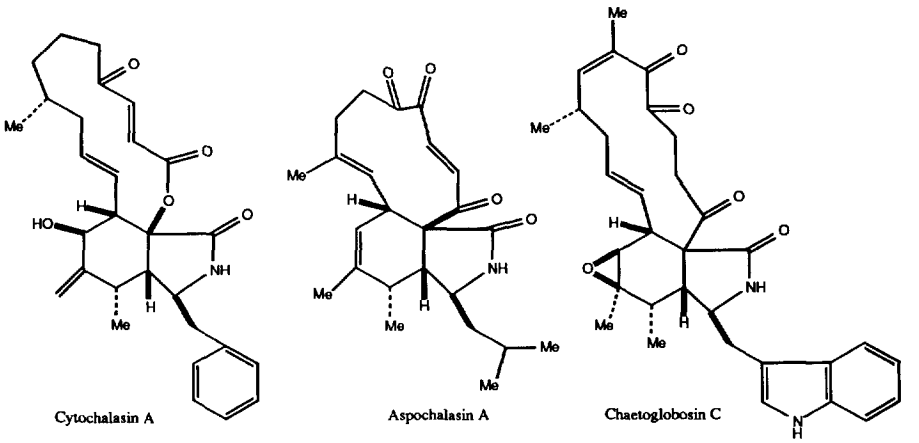


Figure 1. Three structural types of known cytochalasins.

RESULTS AND DISCUSSION

Biological Activity

Phomopsichalasin showed mainly antibacterial activity in disk diffusion assays. At 4 μg/disk phomopsichalasin exhibited zones of 12 mm against *Bacillus subtilis*, 11 mm against *Salmonella gallinarum* and 8 mm against *Staphylococcus aureus*. It also exhibited an 8 mm zone against the yeast *Candida tropicalis*.

Structure Determination

Mass spectral data (HR-EIMS) for phomopsichalasin indicated a molecular formula of $C_{32}H_{41}O_4N$ which was in agreement with the ^1H NMR and ^{13}C NMR proton and carbon counts, and the DEPT spectrum. The presence of three exchangeable protons, which were later attributed to the two -OH groups and one -NH moiety, was consistent with the carbon and carbon-bound proton counts. This was corroborated by the formation of a tri-TMS derivative. Thirteen degrees of unsaturation were indicated by the molecular formula. The ^{13}C NMR spectrum indicated the presence of two carbonyl groups, two double bonds and an aromatic ring which accounted for eight degrees of unsaturation, thus the remaining degrees of unsaturation must be due to five additional rings.

^1H and ^{13}C NMR data are presented in Table 1. One bond carbon-proton connectivities were ascertained from HMQC NMR data. The large number of contiguous quaternary carbons, as well as poor dispersion of protons in the ^1H NMR spectrum, caused ambiguity in the interpretation of the proton correlation data, minimizing the occurrence of ^1H-^1H connectivities.

It was, however, possible to correlate the partial structure extending from H-13 to H-17 (Figure 2) using COSY and long range COSY to ascertain proton connectivities, and HMBC data to ascertain two and three bond ^{1}H-^{13}C connectivities.

Table 1. Phomopsichalasin: ^{1}H and ^{13}C chemical shift assignments (CD_3OD).

C asst.	^{13}C ppm	^{1}H ppm	HMBC (H,C)	NOE (H,H)
C-1	178.2	--		
C-3	89.7	--	H-4, H-10	
C-4	52.4	2.73 d (1H, J=1.7)	H-5, H-10, CH_3-11	H-5, H-10, H-13, CH_3-24, CH_3-26, CH_3-27
C-5	30.7	2.09 br q (1H, J=7.3)	H-7, CH_3-11, CH_3-12	H-4, H-13, CH_3-24, CH_3-25
C-6	136.6	--	H-4, H-5, H-7, $CH_3$12	
C-7	127.5	5.20 br s (1H)	H-12, CH_3-24	
C-8	45.2	--	H-4, H-7, H-13, H-22	
C-9	66.1	--	H-4, H-7	
C-10	46.3	2.99 br s (2H) (d, J=13.9)*	H-4, H-2'/6'	H-4
CH_3-11	21.0	0.81 obsc (3H) (d, J=7.4)*	H-4	
CH_3-12	22.7	1.64 br s (3H)	H-7	
C-13	52.0	2.85 br d (1H, J=8.0)	CH_3-24	H-5, H-22, CH_3-24
C-14	129.5	--	H-15, CH_3-25	
C-15	139.7	5.36 br s (1H)	H-13, H-17, CH_3-25, CH_3-26	
C-16	37.0	--	H-15, H-17, CH_3-26	
C-17	49.4	0.67 dd (1H, J=12.2) 1.45 m (1H)	CH_3-26, CH_3-27	H-20, CH_3- 26, CH_3-27
C-18	28.4	1.62 obsc (1H)	H-17, CH_3-27	
C-19	36.9	0.56 m (1H) (dq, J=3.7, 12.7)* 1.64 obsc (1H)	CH_3-27	
C-20	24.5	1.1 (1H, dddd, J=12.7, 2.2, 2.4) 1.5 obsc (1H)		H-17, H-22
C-21	42.2	1.42 m (1H)	H-15, CH_3-26	
C-22	50.8	2.24 dd (1H, J=8.0,12.7)	H-13	H-13, H-20, CH_3-26, CH_3-27
C-23	221.3	--	H-4, H-22	
CH_3-24	26.3	1.52 br s (3H)	H-7, H-13	H-5, H-13
CH_3-25	25.5	1.88 br s (3H)	H-15	H-5
CH_3-26	19.9	0.80 s (3H)	H-17	H-4, H-17, H-20, H-22, CH_3-27
CH_3-27	23.1	0.78 d (3H, J=2.2)		H-4, H-17, H-20, H-22, CH_3-26
C-1'	128.0	--	H-3'/5', H-10	
C-2', C-6'	132.8x2	7.14 d (2H, J=8.5)	H-2'/6', H-10	
C-3', C-5'	116.1x2	6.74 d (2H, J=8.5)	H-2'/6', H-3'/5'	
C-4'	157.6	--	H-2'/6', H-3'/5'	

*Spectrum taken in CD_3CN, in which compound is partially soluble, to obtain J values.

H-17a exhibited HMBC correlations to the quaternary carbon, C-16, as well as to C-18. Long range COSY data showed correlations from H-17a,b to H-21. The downfield chemical shift of C-17 (49.4 ppm) as compared to C-18 (28.4 ppm), agreed with a computer generated chemical shift prediction database which was used, as did the other carbons in this ring[4]. H-19a,b and H-20a,b showed clear proton connectivities to each other as did H-20a,b and H-21. H-21, H-22 and H-13 correlated with each other in the COSY and long range COSY experiments. C-21 showed HMBC correlations to H-15, as did C-13 and C-16. C-13 also gave a three-bond HMBC correlation to CH_3-25. The olefinic carbons C-14 and C-15 were clearly adjacent, as evidenced by HMBC correlations between C-14 and H-15, as well as between H-15 and CH_3-25, the methyl singlet shown to be adjacent to C-14 by HMBC.

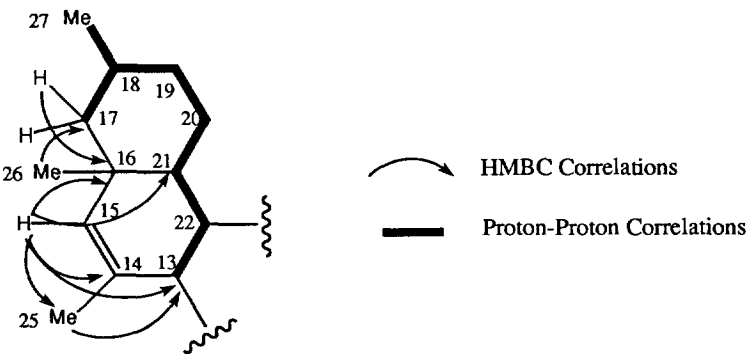


Figure 2. Selected correlations for phomopsichalasin.

Few proton-proton connectivities were observed in the rings formed by C-1 to C-9, but numerous HMBC correlations were used (Table 1, Figure 3). H-4 showed correlations to C-3, C-6, C-8, C-9, C-10 and CH_3-11. The chemical shift of C-3 at 89.7 ppm was consistent with that of a carbon bearing a hydroxyl group, and adjacent to an amide nitrogen. The very large shift of C-3 in the ^{13}C NMR deuterium isotope shift experiments was a strong indication that C-3 was β to an -OH and an -NH while C-4 and C-10 were adjacent to C-3, as evidenced by their γ shifts (Table 2). C-5 exhibited HMBC correlations from CH_3-12, CH_3-11 and H-7, while H-5 exhibited a correlation to C-6. This placed C-5 adjacent to C-6, which in turn was adjacent to the other olefinic carbon, C-7. H-4 and H-7 showed correlations to the quaternaries C-8 and C-9, while H-7 showed a three-bond correlation to CH_3-24, the methyl singlet adjacent to C-8, which indicated the positions of these two quaternary carbons. Their respective chemical shifts, 45.2 and 66.1

ppm, confirmed the position of C-9 between the two carbonyls, a finding supported by the predicted ^{13}C shifts[4].

HMBC correlations were observed from H-22 and H-4 to the C-23 carbonyl (Figure 3), placing C-23 between the isoindolone and tricyclic ring systems as shown (**1**). The methyl protons CH_3-24 showed a three bond HMBC correlation to C-13 while C-8 showed correlations with H-13 and H-22, indicating that C-8 and C-13 were adjacent. HMBC correlations were also seen from C-9 to H-22. These correlations corroborated the position of the C-23 carbonyl as assigned.

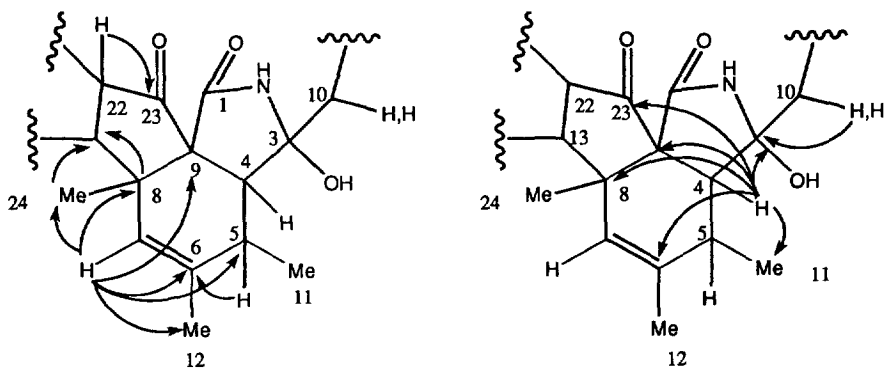


Figure 3. Selected HMBC correlations observed for phomopsichalasin.

The presence and relative position of the amide (C-1 and N-2) were established using a variety of spectral techniques since the SIMBA experiment[5], a selective HMBC which reveals proton connectivities to a single carbon, failed to show any proton correlations to C-1. The IR absorption at 1685 cm^{-1} was consistent with the presence of an amide, which was also indicated by the mass spectral data by virtue of the molecular formula and the number of exchangeable protons. The compound did not react with ninhydrin, indicating the absence of a free amine. A ^{13}C deuterium isotope shift experiment indicated that C-1 experienced a shift of 0.069 ppm which would be explained by its proximity to the -NH moiety. The chemical shift of C-1 is consistent with that of an amide carbonyl, and there has been much precedent for this type of ring system in compounds produced by *Phomopsis* spp[6].

Table 2. Deuterium isotope shift experiment* (CD_3OD/CD_3OH, 125 MHz).

^{13}C Assignment	CD_3OD	CD_3OH	Δppm
C-10	46.335	46.439	0.104
C-4	52.392	52.457	0.065
C-3	89.638	89.823	0.185
C-3', C-5'	116.265	116.377	0.112
C-4'	157.950	158.147	0.197
C-1	178.196	178.265	0.069

*Shifts not shown were all less than 0.017 ppm

The final evidence for the C-1, N-2, C-3 partial structure came from a sample of phomopsichalasin which was modified by exposure to DMSO-d_6 during the course of solvent changes done to elucidate the exchangeable protons. Specifically, the protons at 2.99 ppm (H-10) disappeared and an olefinic proton was seen to appear at 5.5 ppm. Mass spectral data, in conjunction with ^1H-NMR data, suggested that a double bond had formed between C-3 and C-10: phomopsichalasin was found to lose H_2O (HR-EIMS m/z 485.2914 observed), with no loss of nitrogen. The presence of a free -OH group was therefore confirmed, ruling out the possibility of a γ-lactone ring in phomopsichalasin, instead of the γ-lactam ring.

The ^{13}C and ^1H NMR chemical shifts of C-1' - C-6' and H-2', 3', 5' and 6' indicated the presence of an aromatic ring. The shift of C-4' (157.6 ppm) suggested that it was a phenol ring. This was corroborated by the deuterium isotope shift experiment (Table 2), where C-4' was observed to undergo a substantial β shift of 0.197 ppm. H-10a,b were the only protons which showed long range COSY correlations to the aromatic protons, as well as HMBC correlations to C-1', C-2' and C-6'. This placed the phenol ring as shown in **1**.

Relative Stereochemistry

The stereochemistry of the cytochalasin-like compounds tends to be consistent for all of the compounds, especially the absolute configuration of the isoindolone ring system[7]. The six membered ring is generally *cis*-fused to the five membered ring in the isoindolone, and *trans*-fused to the macrocyclic ring[1a]. Further published studies have shown that the six membered ring is in a twist-boat conformation, which is corroborated by an NOE correlation in phomopsichalasin between H-5 and CH_3-24[1a]. The macrocyclic ring carbons are in chair conformations and the substituents at C-10 are in variable, compound-specific conformations, in compounds with stereochemically relevant C-10 substituents[1a]. A NOESY experiment was carried out for phomopsichalasin in order to understand the relationships between the moieties on the isoindolone system and those on the fused three ring system (Table 1). Spectral crowding in

the upfield region of the ^1H NMR spectrum, and the high degree of overlap, made 1D NOE irradiations across the molecule impractical.

The NOE between H-4 and H-5 concurred with the relative stereochemistry established for the cytochalasins. The NOE observed between CH_3-24, H-5 and H-13 showed that they were on the same face of phomopsichalasin. This was a crucial correlation which indicated the relative stereochemistry of the isoindolone system relative to the three ring system to which it is fused. Further, the NOE observed between H-13 and H-22, confirmed by the observed J=8 coupling constant, as well as H-22 and CH_3-26/CH_3-27 led to the resolution of the overlapping methyl problem as follows. NOEs were found between both CH_3-26/CH_3-27 and H-17 (1.45 ppm), H-20 (1.10 ppm) and H-22. Finally, H-20 (1.10 ppm) showed a 1D NOE to H-22, establishing CH_3-26, CH_3-27, H-17 (1.45 ppm) and H-22 as having the same relative stereochemistry as H-4, H-5 and CH_3-24. In fact, NOEs were observed between CH_3-26/CH_3-27 and H-4. A molecular model of phomopsichalasin, assembled using the relative stereochemistry above, necessitated the orientation of the C-1 and C-23 carbonyl substituents as shown in **1**. This orientations of C-23 and C-1 are consistent with that indicated for the cytochalasins[1a,6c,6d,8].

EXPERIMENTAL

Spectroscopic Methods

UV spectra were recorded using a Beckman DU-70 spectrophotometer. The sample was dissolved in CD_3OD. IR spectra were recorded as neat samples on a ZnSe multiple internal reflectance (MIR) crystal using a Perkin-Elmer 1750 FT-IR spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Varian XL-300, a Varian Unity 400 or a Varian Unity 500 spectrometer. DEPT, HMQC and HMBC data were obtained on a Varian Unity 400 spectrometer. The HMBC spectra were optimized for J_{CH} of 4 and 7 Hz. Chemical shifts were reported in ppm downfield from TMS at 0 ppm. Spectra were referenced using the solvent peak as the internal standard. The reference position was that for CD_3OD (δ_H=3.30 ppm, δ_C=49.0 ppm). Coupling constants were reported in Hz, multiplicities were reported as follows: s = singlet, d = doublet, t = triplet, m = multiplet, obsc = obscured and br = broad. Deuterium isotope shift experiments were carried out in CD_3OD and CD_3OH on a Varian Unity 500 spectrometer, using a 3 mm carbon detected microcarbon probe (np=83968, sw=27991.6, fn=262144). A limited number of experiments were carried out in CD_3CN, in which the compound was not fully soluble, and DMSO-d_6, which caused the compound to undergo modification. HR-EI mass spectral data were obtained on a Jeol SX-102 at 90eV, using

perfluorokerosene as the internal standard. Trimethylsilyl derivatives were prepared with a 1/1 mixture of BSTFA-pyridine, at room temperature.

Description of the Producing Organism

The *Phomopsis* isolate (MF6031, Merck Microbial Resources Culture Collection) was isolated from surface sterilized twigs of *Salix gracilostyla* var. *melanostachys* (acquisition #237-71-5282, Wakehurst Place, UK). Colonies were grown on malt agar attaining 35 mm in diameter after 7 d at 28°C or 50 mm in 7 d at 25 °C. They were slightly raised, floccose, silky, with the margin at first white but developing buff to gray tones when a thin layer of stromatic tissue develops at the agar surface, with scattered conidiomata developing beneath aerial hyphae after 1 wk, *reverse* hyaline to grayish buff when young, becoming mottled dark brownish gray to fuscous black with age as stromatic tissues develop. Odors and exudates were absent. Conidiomata were eustromatic, hemispherical to pulvinate 0.5 to 1.5 mm diameter, dull, dark grayish brown to black, dehiscing by or through preformed ostioles, with 1-4 ostioles per conidioma, exuding a cream-colored spore mass when mature. Conidia were of the α-type hyaline, smooth, bigutulate, ellipsoidal to narrowly ovate, often pointed at the proximal end, sometimes with a slight median constriction, 6.4-8.0 μm length, 2.4-3.2 μm width. β-type conidia were absent.

Fermentation

A conidial suspension of this isolate was prepared by swabbing conidia from the actively growing culture onto a sterile cotton swab and placing the cotton tip into 2 ml of sterile water. This inoculum was used to seed a 250 ml shake flask containing potato dextrose broth (Difco). These seed cultures were incubated at 25 °C for four days at 98% relative humidity under fluorescent lights on a 12 hour light/dark schedule. The flasks were placed on a rotary shaker and shaken at 220 rpm. Two ml portions of the seed culture were pipetted aseptically into twenty 250 ml flasks containing a shredded wheat medium. Each flask of shredded wheat medium contained: 11.8 g of Nabisco Original Shredded Wheat and 5 ml of distilled water. The flasks were autoclaved for 20 minutes at 121 °C and 15 psi prior to use. The medium was inoculated within 24 hours of preparation. The inoculated flasks were incubated at 25 °C for 4 weeks at 98% relative humidity under fluorescent lights on a 12 hour light/dark schedule.

Isolation and Characterization

After 28 days a 50 ml aliquot of CH_2Cl_2 was added to each fermentation flask, and the flasks were placed on a rotary shaker for 3 hours (220 rpm, 25 °C). The mixture was filtered through Whatman filter paper under vacuum and the solid portion re-extracted with CH_2Cl_2 for 2.5 hours, with stirring. This mixture was filtered, the two extracts combined, concentrated to dryness, reconstituted in 10 ml of CH_2Cl_2 and charged onto 100 ml of silica in a scintered glass funnel. This flash silica column was eluted using a stepwise gradient from CH_2Cl_2 to EtOAc. Phomopsichalasin eluted with CH_2Cl_2 /EtOAc (1/1). This fraction was concentrated to dryness, reconstituted in 1 ml of hexane/EtOAc (4/1) and charged onto a 22 ml silica column. The column was eluted using a stepwise gradient of hexane/EtOAc. Phomopsichalasin eluted with hexane/EtOAc (6/4); the eluent was concentrated to dryness and reconstituted in CH_3CN/H_2O (9/1). Seven injections were made onto a Zorbax RX C8 semi-preparative HPLC column (9.4 mm i.d. x 25 cm), heated to 45°C, using a mobile phase of CH_3CN/H_2O (45/55), flowing at 2 ml/min, monitoring UV at 230 nm and collecting 2 ml fractions. Fractions were analyzed on a Zorbax RX C8 analytical column (4.6 mm i.d. x 25 cm) using CH_3CN/H_2O (65/35) as the mobile phase, heated to 45 °C, with a flow rate of 1 ml/min. Phomopsichalasin had a retention time of 12.8 min. A total of 5.65 mg of phomopsichalasin was isolated as an oil. Its physical characteristics were as follows: UV: MeOH, λ_{max} 245 nm, ε=2782; λ 225 nm, ε=1152); $[\alpha]_D^{25}$=-7.16°; IR: 3828, 2922, 1724, 1685, 1516, 1445, 1376, 1227, 1109 cm^{-1}; Mass Spec: $C_{32}H_{41}O_4N$, m/z found: 503.3040; calculated: 503.3033, fragment ions: 485, 470 (base peak), 282, 266, 105.

Antibiotic Assay

Phomopsichalasin was tested for antibiotic activity using disk diffusion assays by placing 1/4" disks containing test material on agar plates seeded with bacteria or fungi. Fungi tested included: *Alternaria solani, Aspergillus niger, Botrytis allii, Candida tropicalis, Ophiostoma ulmi, Cryptococcus laurentii, Penicillium* sp., *Phoma* sp. and *Ustilago zeae.* Bacteria tested included: *Bacillus subtilis, Enterococcus faecium, Erwinia atroseptica, Pseudomonas aeruginosa, Salmonella gallinarum, Staphylococcus aureus* and *Vibrio percolans.*

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