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Isolation and Characterization of Phomodiol, a New Antifungal from *Phomopsis*

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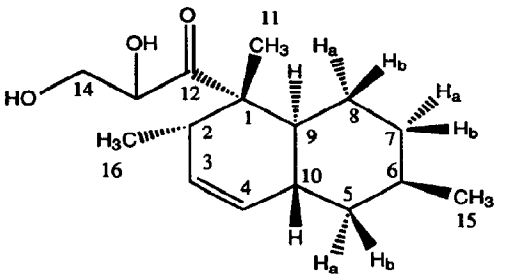
Abstract: Phomodiol, a novel antifungal, was isolated from *Phomopsis* sp. Its structure and relative stereochemistry were determined by MS and NMR. It was found to be inhibitory towards *Candida tropicalis*.

A number of toxins have been isolated from *Phomopsis* spp. in cases where these fungi were observed to occur as phytopathogens.[1] Very little work has been done on the secondary metabolites produced by *Phomopsis* spp. where they occur as endophytes of woody plants as opposed to pathogens; the work that has been done suggested that one species, *Phomopsis oblonga*, provided protection for elms against the bark beetles which act as vectors of dutch elm disease.[2] The novel compound phomodiol was discovered as part of an ongoing investigation into metabolites produced by fermentation of *Phomopsis* isolates growing endophytically in willows (*Salix* spp.). We report here the isolation, structural elucidation and antifungal activity of phomodiol (**1**).



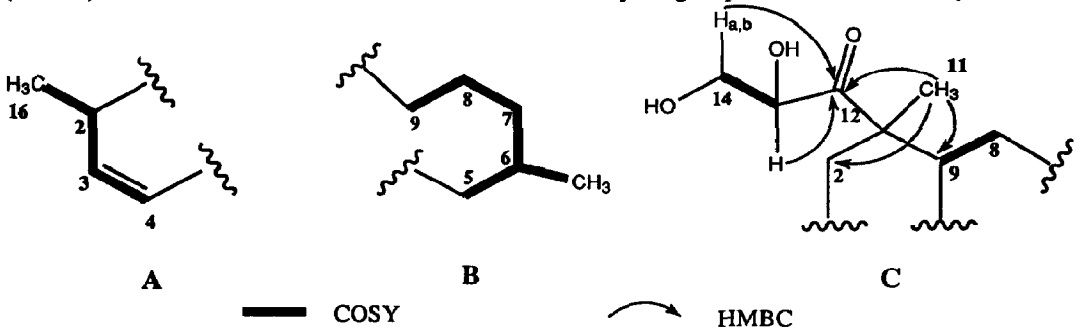
1

Fermentation and Isolation *Phomopsis* sp.(MF5862, Merck Microbial Resources Culture Collection), obtained from *Salix lasiolepis* (acquisition #087-73-06396, RBG, Kew, UK) was grown statically on millet medium[3] in twenty 250 ml shake flasks for 4 weeks (25°C, 12 hour light/dark cycle). The fermentation was extracted with H_2O/MeOH/CH_2Cl_2 (1/2/1), centrifuged and the lower, organic layer concentrated to dryness and reconstituted in 90% aq. MeOH. The 90% aq. MeOH sample was charged onto a Zorbax RX C8 preparative HPLC column (21.2 x 250 mm), using H_2O/CH_3CN (62/38) as the mobile phase with a flow rate of 10 ml/min, monitoring the UV absorbance of the effluent at 210 nm. Fractions containing phomodiol were combined and re-chromatographed on HPLC in a similar fashion, this time using a Zorbax RX C8 semi-preparative column (9.4 x 250 mm) with a mobile phase of

H_2O/CH_3CN (65/35) and a flow rate of 2 ml/min. A total of 1.5 mg of phomodiol was isolated as an amorphous solid from the twenty shake flask fermentation.

Structure Elucidation EI-MS[4] data indicated m/z $[M+H]^+$ 266.1870 (calculated m/z 266.1882) corresponding to $C_{16}H_{26}O_3$, a 177 base peak and a di-TMS derivative indicating two exchangeable protons. ^1H and ^{13}C NMR data are presented in Table 1.[4] It was possible to propose partial structures **A** and **B** based on proton correlations obtained from COSY and proton decoupling NMR experiments. The diol side chain moiety (**C**) was apparent from the chemical shift of the three attached protons (H-13, 2H-14), and the strong coupling between these protons found in the COSY and decoupled proton spectra. The carbon shifts of C-13 (76.3 ppm) and C-14 (64.3 ppm) suggested that they were carbons bearing hydroxyls, supported by the IR absorbances[4] at 3200-3500 cm^{-1}. Mass spectral data indicated a $C_2H_5O_2$ di-TMS fragment (found m/z 205.1093, calculated m/z 205.1081) which corresponded to the diol moiety postulated from the NMR results.

The absence of other proton correlations to the diol protons indicated that the diol moiety was attached to a quaternary center, of which there are two in the molecule, at 53 ppm (C-1) and 214 ppm (C-12). The only HMBC correlation (Table 1) to the diol protons came from the carbonyl (C-12); it was thus determined that the diol was adjacent to the carbonyl. An IR absorption at 1748 cm^{-1} was attributed to the carbonyl. The point of attachment of the carbonyl to the ring system was confirmed by HMBC data (Table 1) which showed a correlation between the C-11 methyl singlet protons and the carbonyl.



The C-11 methyl protons also showed HMBC correlations to C-2. In addition, C-1 correlated to H-2 by HMBC. Partial structure **A** was thus shown to be attached to the quaternary carbon C-1 at C-2. The double bond (C-3, C-4) was evidenced from the chemical shifts of H-3 (5.50 ppm), C-3 (131.1 ppm) and H-4 (5.35 ppm), C-4 (130.4 ppm), supported by the IR spectrum absorbance at 1635 cm^{-1}.

It appeared from HMBC and COSY data that C-9 and C-10, the only unassigned methines, were located at the points of attachment of the two ring system. HMBC data (**C**) indicated a key three-bond correlation between CH_3-11 and C-9, which placed C-9 at the ring juncture adjacent to C-1. Proton decoupling indicated that C-9 was coupled to the C-8 methylene protons (**B**). It was possible, through the HMBC and COSY data, to confirm that C-8 was also adjacent to the C-7 methylene protons, which were in turn adjacent to H-6 and CH_3-15. The C-10 methine showed an HMBC correlation to H-3, placing C-

10 as depicted in 1. C-5 clearly correlated to H-4, as seen in the HMBC data, as well as to H-10. HMBC correlations between H-$5_{a,b}$ and both C-6 and C-15 were observed. The high resolution mass spectral experiment showed a $C_{14}H_{21}O$ fragment (found m/z 205.1606, calculated m/z 205.1591), representing the molecule upon cleavage of the C-12 - C-13 bond, which was consistent with the trimethyl decalin ring system with a carbonyl attached. A UV spectrum indicated λ_{max}=205 nm (MeOH, ε=1511).

Table 1. NMR Data for Phomodiol (CD_3OD)

Carbon	$\delta^{13}C$	$\delta^{1}H$ *J Hz*	HMBC (H,C)	NOE
C-1	53.0		H2, H9, Me-11	
C-2	40.3	2.22 dq, *J*=4.9, 6.9	H3, Me-16, Me-11	
C-3	131.1	5.50 ddd, *J*=9.9, 4.9, 2.6	H10, H4, H2, Me-16	
C-4	130.4	5.35 br d, *J*=9.9	$H5_{a,b}$, H10, H3, H9	
C-5	43.5	H_a 0.80 br dd, *J*=11.9, 12.2 H_b 1.79 m, *J*=11.9	H6, H4, H10, H9, Me-15	
C-6	34.6	1.46 m	$H7_{a,b}$, $H5_{a,b}$, Me-15	
C-7	36.9	H_a 1.00 m H_b 1.70 m	H6, Me-15	
C-8	28.3	H_a 1.00 m H_b 1.46 m	H6	
C-9	41.5	1.63 dt, *J*=10.8, 2.3	$H8_{a,b}$, $H5_{a,b}$, H10, H4, Me-11	$H5_a$, $H7_a$, $H8_a$, Me-16
C-10	39.4	1.76 m, *J*=10.8, 12.2[1]	$H8_{a,b}$, $H5_{a,b}$, H4, H3, H9	
C-11-Me	16.9	1.30 s	H2, H9	H-2
C-12	214.7		H13, $H14_{a,b}$, Me-11	
C-13	76.3	4.41 dd, *J*=4.5, 5.9	$H14_{a,b}$	
C-14	64.3	3.62 dd, *J*=4.5, 11.5 3.81 dd, *J*=5.9, 11.5	H13	
C-15-Me	22.8	0.90 d, *J*=6.6	$H7_{a,b}$, H6, $H5_{a,b}$	$H7_b$, $H8_b$
C-16-Me	19.3	0.78 d, *J*=6.9	H2, Me-11	H9

[1]Doublets were seen within the multiplet, using proton decoupling experiments

Relative Stereochemistry The large coupling of 10.8 Hz between H-9 and H-10, obtained from proton decoupling experiments, confirmed the ring junction as *trans* with diaxial protons. The *trans* configuration was consistent with similar structures composed of *trans* decalin ring systems such as that found for dihydromevinolin, oblongolide and stemphyloxin I[5], for example. A model suggested that the ring conformation was a half chair in the case of ring A due to the endocyclic double bond, which was consistent with the 90° dihedral angle between H-10 and the plane of the double bond, as indicated by the zero and 2.6 Hz couplings to H-4 and H-3 respectively, and a chair in the case of ring B. The coupling constant of 4.9 Hz between H-2 and the olefinic proton H-3 indicated that the protons were not near 90° from one another, or the coupling constant would have been near zero, as would be expected if H-2 was pseudoaxial. This indicated that H-2 was in the pseudoequatorial position with the C-16 methyl in a pseudoaxial position. The H-5_a proton (0.8 ppm) exhibited a large coupling constant of 12.2 Hz, in

addition to the geminal coupling with H-5_b (J=11.9 Hz). The 12.2 Hz coupling represented the coupling to H-10, which revealed that H-5_a was an axial proton; the small coupling between H-5_a and H-6 placed H-6 in the equatorial position. This placed CH_3-15 in the axial position.

One dimensional NOE irradiations were done for H-9, CH_3-11, CH_3-15 and CH_3-16 (Table 1) . CH_3-16, H-5_a, H-7_a and H-8_a were enhanced when H-9 was irradiated, indicating that they were on the same face of phomodiol. Irradiation of CH_3-11 showed enhancement of H-2, indicating that CH_3-11 and H-2 are on the same face, with the diol side chain therefore on the opposite face of the molecule from H-2. H-7_b and H-8_b showed NOEs to CH_3-15, confirming the relative stereochemistry shown in **1**.

Phomodiol was tested in disk diffusion assays against species of *Bacillus*, *Streptomyces*, *Staphylococcus*, *Streptococcus*, *Aspergillus*, *Penicillium*, *Fusarium*, *Phoma*, *Trichoderma*, *Ustilago*, *Verticillium*, *Scopulariopsis*, *Rhizomucor*, *Candida*, *Brettanomyces*, *Cephalosporium*, *Ceratocystis*, *Cryptococcus*, *Kluyveromyces* and *Saccharomyces*. It was found to be active solely against *Candida tropicalis* with a minimum inhibitory concentration of 100 μg/ml.

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4. (a) NMR spectra were taken on a Varian Unity 400 spectrometer, with 1.5 mg phomodiol in 0.7 ml CD_3OD. Chemical shifts were referenced to CD_3OD at 3.30 ppm and 49.0 ppm for ^1H and ^{13}C, respectively, and reported relative to TMS. HR- and LR-EIMS data were obtained on a Finnigan MAT-212 at 90eV; (b) the IR spectrum was recorded as a neat sample on a ZnSe crystal using a Perkin-Elmer 1750 FT spectrophotometer.

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