A Novel Chemical Signal from the "Blackleg" Fungus: Beyond Phytotoxins and Phytoalexins

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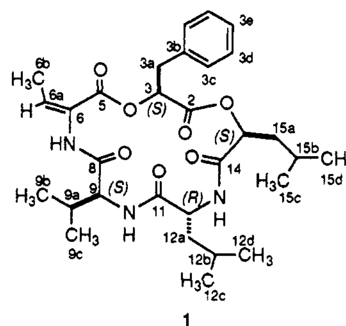
Summary: The structure elucidation of a novel pentadepsipeptide 1, which revealed unprecedented metabolic correlations and was isolated from *Phoma lingam*, is described.

Plant pathogens release chemical signals triggering specific responses on the host, which facilitate penetration and colonization of plant tissue. Host-specific phytotoxins are examples of such chemical signals produced by phytopathogenic fungi.[1] The blackleg fungus [*Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm], causative agent of the devastating blackleg disease of canola (*Brassica napus* and *Brassica campestris*) oilseed crops,[2] produces a complex array of nonspecific phytotoxins [epipolythiodioxopiperazines (EPTs)]; however, specific toxins have not been isolated.[3] It has been established that only virulent isolates of the blackleg fungus produce EPT toxins, but it remains to be determined whether these toxins are produced in infected plants.[3] Nevertheless, the symptomatic lesions on blackleg-infected canola leaves suggest that the fungus produces phytotoxins *in planta*.[4]

Host-selective toxins are valuable probes for investigating the molecular basis of plant fungal diseases and can be extremely useful tools for the rapid screening and selection of disease-resistant plants.[1] We have hypothesized for several years that *P. lingam* would produce host-selective toxins. However, a bioassay-directed search for host-selective toxins did not reveal the putative toxins.[5] Our research has finally confirmed that early speculation. We report here the complete chemical structure elucidation of a remarkable host-selective chemical signal produced by the blackleg fungus, which we named phomalide (1).



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During investigations on the biotransformation of the phytoalexin brassinin,[6] we discovered that the blackleg fungus synthesized a new metabolite, phomalide (1), which was structurally unrelated with the EPT phytotoxins. Unexpectedly, 1 was only produced by germinated spores of 24–60-h-old cultures; older cultures produced EPT toxins such as sirodesmin PL.[7] HPLC analyses of the culture broth extracts of 30 different isolates of *P. lingam* revealed that only the virulent isolates produced metabolite 1.[8] Although the culture broth of isolate Wainwright 1 (WW-1) appeared to have the highest concentration of phomalide, the process for obtaining it was not readily amenable to scale-up.[9] Only rapid extraction of unconcentrated culture broths with Et_2O gave acceptable isolation yields (1–4 mg/L). Subsequently, we found that the biosynthesis of phomalide was inhibited by sirodesmin PL. The HPLC profiles (Figure 1) of extracts of 52-h-old cultures of isolate WW-1 grown in minimal medium (MM) and MM containing sirodesmin PL clearly demonstrate the inhibitory effect. This inhibitory effect of sirodesmin PL may explain the unusually short production period of phomalide.

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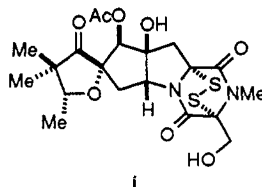
(1) For a recent multiauthor review on phytotoxins and their involvement in plant disease see: Graniti, A. *et al. Experientia* 1991, *47*, 751.

(2) For a recent review on blackleg disease history and control see, for example: Gugel, R. K.; Petrie, G. A. *Can. J. Plant Pathol.* 1992, *14*, 36.

(3) For a recent review on phytotoxins of the blackleg fungus see, for example: Pedras, M. S. C.; Séguin-Swartz, G. *Can. J. Plant Pathol.* 1992, *14*, 67. One of the major EPT toxins produced is sirodesmin PL (i).



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(4) Our recent work[6] has explained previously unsuccessful experiments to detect EPT toxins in canola leaves infected with blackleg. It appears that phytoalexin(s) present in leaf tissue inhibit sirodesmin biosynthesis.

(5) Pedras, M. S. C.; Abrams, S. R.; S éguin-Swartz, G. *Tetrahedron Lett.* 1988, *29*, 3471. Pedras, M. S. C.; Abrams, S. R.; Séguin-Swartz, G.; Quail, J. W.; Jia, Z. *J. Am. Chem. Soc.* 1989, *111*, 1904. Pedras, M. S. C.; Séguin-Swartz, G.; Abrams, S. R. *Phytochemistry* 1990, *29*, 777.

(6) Pedras, M. S. C.; Borgmann, I; Taylor, J. L. *Phytochemistry* (*Life Sci. Adv.*) 1992, *11*, 1. Pedras, M. S. C.; Taylor, J. L. *J. Org. Chem.* 1991, *56*, 2619. Pedras, M. S. C.; Taylor, J. L. *J. Nat. Prod.* 1993, *56*, 731.

(7) The age and concentration of spores used for initiating fungal cultures significantly affect the production of phomalide. Fungal mycelia did not produce phomalide but produced sirodesmins. The phytoalexin brassinin inhibited the biosynthesis of sirodesmin PL, but did not affect the biosynthesis of 1; addition of brassinin to the culture media did not appreciably increase the production of phomalide.

(8) Twenty-five of the 30 isolates were virulent. Liquid cultures were incubated in minimal media for 52 h at 25 ± 2 °C.[6] Phomalide was not detected in cultures more than 100 h old. HPLC analysis was carried out with an instrument system equipped with an RP-8 Spheri-5 column (5-μm particle size silica, 22-cm × 4.6-mm internal diameter) employing a gradient elution methanol–water and UV detection at 220 nm. Under these conditions the retention time of phomalide was 25.1 min and that of sirodesmin PL was 7.9 min. The detection limit of phomalide was 0.5 μg and that of sirodesmin PL was 0.1 μg.

(9) Concentrating the cultures by freeze –drying amounted to losses of 30–60%. HPLC analysis indicated that a 10^{-4} M phomalide solution (in minimal media) was 80% hydrolyzed after standing at room temperature for 28 h.

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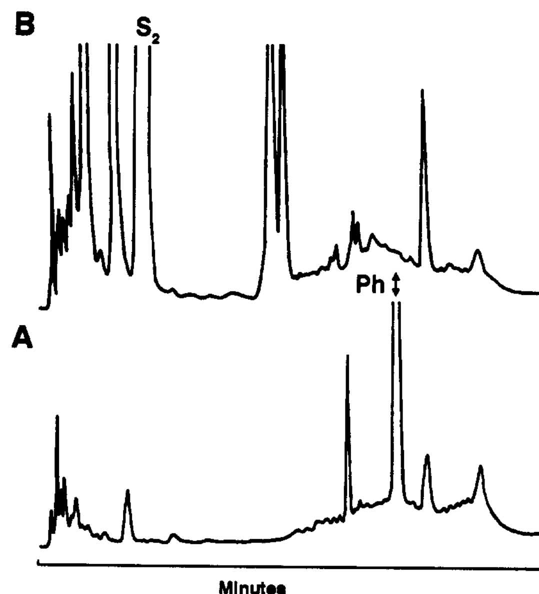


Figure 1. Chromatograms obtained by HPLC analysis of broth extracts of 52-h-old cultures of isolate WW-1 in MM, A, and in MM-containing sirodesmin PL (10^{-4} M), B. S_2 indicates the peak corresponding to sirodesmin PL, and Ph indicates peak corresponding to phomalide (1).

Purification of the concentrated Et_2O extract by preparative TLC (CH_2Cl_2–MeOH (95:5), R_f 0.56) yielded phomalide (1) as a chromatographically homogeneous solid: $[\alpha]_D$ −52.9° (*c* 0.14 $CHCl_3$); UV ($CHCl_3$) λ_{max} 257 (log ϵ 3.7) nm; FTIR ν_{max} (NaCl plate) 3276, 2959, 2934, 2870, 1748, 1726, 1693, 1680, 1659, 1543, 1384, 1277, 1216, 1155 cm^{-1}. The chemical structure of 1, including the absolute configurations of the four stereogenic centers and the configuration of the double bond, was established by a combination of spectroscopic methods and chemical degradation. The molecular formula of $C_{30}H_{43}N_3O_7$ was deduced for 1 based on results of HRMS-EI [*m/z* 557.3094, (M^+), calcd *m/z* 557.3101] and FAB-MS together with NMR data (Table I). The data suggested that phomalide had a cyclic depsipeptide structure.

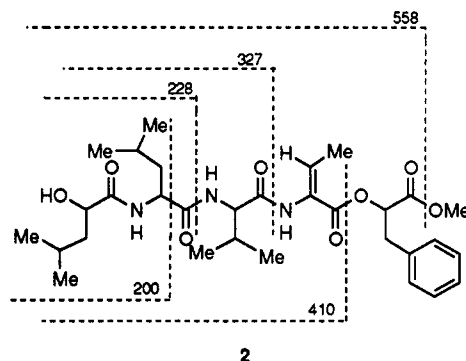
A combination of ^1H and ^{13}C NMR, $^1H,^1H$ and $^1H,^{13}C$ COSY, and TOCSY experiments revealed that the molecule had five discrete spin-coupled systems. Two of these spin systems could be attributed to the well-known amino acids valine (Val) and leucine (Leu) and another one to the rare amino acid dehydrothreonine (DhThr). The remaining two spin systems were assigned to 3-phenyllactic acid (O-Phe) and 2-hydroxyisovaleric acid (O-Leu) residues. The sequence of the five fragments composing the cyclic depsipeptide could be established by the HMBC experiment.[10] In phomalide the correlations between the carbon atoms of the five C=O groups and three-bond coupled protons (Table I) allowed the sequence DhThr-Val-Leu-O-Leu-O-Phe to be established unequivocally. This sequence was further confirmed by FAB-MS of phomalide methyl ester 2 (obtained upon standing in methanol at room temperature), as shown in Chart I. The configuration of the double bond of the DhThr residue was assigned as *E* based on NOE experiments. A positive NOE was observed for the proton at C-6a (6.80 ppm) and at C-9 (4.20 ppm) on irradiation of the proton at N-7 (8.1 ppm), whereas no NOE was observed for the protons at C-6b (1.80 ppm). Finally, the absolute configuration of the four stereogenic centers of phomalide was determined employing a methodology specifically developed for small amounts of depsipeptides.[11] The configurations of the residues Val, O-Phe, and O-Leu were established as *S* whereas the residue Leu was *R*.

Table I. ^1H and ^{13}C NMR Data and HMBC Correlations for Phomalide (1) in $CDCl_3$ as Solvent (Assignment of Resonances in the ^1H and ^{13}C NMR Spectra Was Made by Extensive Decoupling, by $^1H,^1H$ and $^1H,^{13}C$ COSY, and by TOCSY NMR Experiments)

residue	carbon	^{13}C;[a] δ	^1H;[b] δ, mult (*J* (Hz))	HMBC[c]
O-Phe	2	168.18		
	3	76.16	5.30, dd (9.0, 4.1)	2, 3a
	3a	37.03	3.30, dd (14.5, 4.1) and 3.18, dd (14.5, 9.0)	2, 3b, 3c
	3b	135.07		
	3c	128.68	7.26, m	
	3d	127.44	7.26, m	
	3e	129.32	7.26, m	
DhThr	5	166.05		
	6	124.57		
	6a	131.92	6.80, br q (7.7)	5, 6
	6b	14.41	1.80, d (7.7)	6, 6a
	7-NH		8.1, br s	5, 6a, 8
Val	8	170.02		
	9	59.07	4.20, dd (9.3, 8.2)	8, 9a, 9b, 9c, 11
	9a	27.27	2.23, m	8, 9, 9b, 9c
	9b	19.91	0.99, d (6.7)	
	9c	18.01	0.94, d (7.8)	
	10-NH		6.57, d (8.2)	9, 11
Leu	11	172.29		
	12	52.14	4.37, br q (7.5)	11, 12a, 12b, 14
	12a	39.27	1.75, m and 1.52, m	11, 12, 12b, 12c, 12d
	12b	24.92	1.54, m	12. 12a, 12c, 12d
	12c	22.39[d]	0.89, d (6.5)	
	12d	22.47[d]	0.92, d (6.7)	
	13-NH		6.52, d (9.6)	11, 12, 14
O-Leu	14	168.99		
	15	72.60	5.38, dd (8.2, 5.0)	2, 15a, 15b
	15a	39.34	1.68, m and 1.60, m	15, 15b, 15c, 15d
	15b	24.19	1.35, m	15, 15c, 15d
	15c	22.91	0.86, d (6.7)	
	15d	21.84	0.83, d (6.6)	

[a] Recorded at 95.5 MHz. [b] Recorded at 360 MHz. [c] Recorded at 500 MHz. Protons correlated to carbon resonances in ^{13}C column. Underlined numbers represent carbonyl carbons. [d] These assignments may be reversed.

Chart I Chemical Structure of Phomalide Methyl Ester (2) Showing Fragment Ions Obtained by FAB-MS (*m/z* 590 [M + 1]$^+$).



(10) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093.

(11) The analysis fundamentally consisted of acidic hydrolysis (6 M HCl, 20 h, 110 °C), Et_2O extraction, and derivatization of ether and aqueous phases. The amino acid derivatives [*N*-(trifluoroacetyl) methyl esters] were analyzed by chiral gas–liquid chromatography (Chirasil-Val, Alltech), and the hydroxy acid derivatives [esters of (*S*)-2-(trifluoromethyl)-2-methoxy-2-phenylacetic acid] were analyzed by ^1H NMR.

Phomalide is a highly unusual 15-membered ring depsipeptide composed of five residues. Depsipeptides (and cyclic peptides) typically incorporate either four or more than five residues.[12] The discovery of 1 reiterates the importance of investigating the secondary metabolism of fungi during their various developmental stages.[13]

The selective phytotoxicity of phomalide is noteworthy. Phomalide causes lesions on canola (blackleg susceptible cultivar) that closely resemble the symptomatic leaf lesions caused by blackleg disease. By contrast, mustard (*Brassica juncea*, blackleg resistant cultivar) is only slightly sensitive to 1.[14] These results expand greatly the understanding of the interaction between canola and the blackleg fungus. Phomalide is a host-selective chemical signal released by blackleg germinated spores, which can facilitate fungal penetration and colonization of canola tissue, similar to host-selective toxins.[1] However, unlike known selective toxins, this distinct chemical signal is produced only during initial fungal attack. Once phomalide is secreted by germinated spores, the plant cells are irreversibly damaged and colonization of the tissue by the fungal mycelia can occur more rapidly. Paradoxically, canola phytoalexin(s), by inhibiting the sirodesmin production,[6] may in fact be favoring fungal invasion. The ecological significance of these unprecedented results will become clearer as other host–pathogen systems are investigated.

The scarcity of phomalide coupled with its susceptibility to hydrolysis present obstacles to further study. Work is in progress to optimize conditions for producing phomalide and to obtain structural analogues so that an understanding of the mode of action at the molecular level may be obtained. Notwithstanding these obstacles, knowledge that phomalide differentiates resistant plant tissue from susceptible tissue opens new horizons for understanding and controlling the devastating blackleg disease.

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(12) See, for example: Turner W. B.; Aldridge, D. C. In *Fungal Metabolites II;* Academic: New York, 1983; pp 436–442. Kleinkauf, H.; von Doheren, H. *Eur. J. Biochem.* **1990**, *192*, 1.

(13) Phomalide was not detected in previous studies of *P. lingam*[3] presumably due to its limited production period (maximum of 36 h in liquid culture) and lability in aqueous solutions.

(14) The toxicity of phomalide (10^{-4} M, 5×10^{-5} M, 10^{-5} M, 5×10^{-6} M, 10^{-6} M, in 50% aqueous methanol) to canola and mustard plants was tested on cotyledons and leaves, as previously described for the sirodesmins.[5] While 5×10^{-6} M solutions caused lesions on canola, no obvious damage was observed on mustard at concentrations lower than 5×10^{-5} M.