Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 3291–3294

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

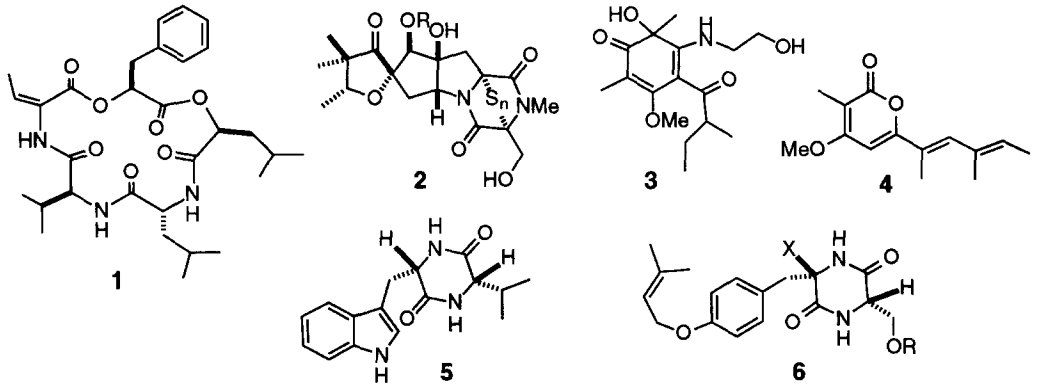
PHOMALAIRDENONE: A NEW HOST-SELECTIVE PHYTOTOXIN FROM A VIRULENT TYPE OF THE BLACKLEG FUNGUS *PHOMA LINGAM*

M. Soledade C. Pedras,*[a] Claudia C. Erosa-López,[a] J. Wilson Quail,[a] and Janet L. Taylor[b]
[a]*Department of Chemistry, University of Saskatchewan, 110 Science Place, SaskatoonSK S7N 5C9, Canada;*
[b]*Plant Biotechnology Institute, National Research Council of Canada, Saskatoon SK S7N 0W9, Canada*

Received 3 September 1999; accepted 15 October 1999

Abstract: The chemical structure and bioactivity of phomalairdenone (**7**), a new sesquiterpenic host-selective phytotoxin produced by an unusual virulent type isolate of the blackleg fungus [*Phoma lingam*, perfect stage *Leptosphaeria maculans* (Desm.) Ces. et de Not.] are reported.

The blackleg fungus [*Phoma lingam*, perfect stage *Leptosphaeria maculans* (Desm.) Ces. et de Not.] occurs worldwide causing significant devastation in important brassica crops such as the oilseeds rapeseed and canola (*Brassica napus, B. rapa*).[1] The traditional classification[2] of this phytopathogen includes a highly virulent strain,[3] responsible for most of the crop losses, and an avirulent (also known as weakly virulent) strain.[4] Fungal isolates belonging to each of these strains are chemically distinguishable through their phytotoxin profiles; while highly virulent isolates produce the phytotoxins phomalide (**1**) and sirodesmins (**2**), avirulent isolates produce metabolites with low phytotoxicity such as **3** and **4**.[5]



Because brown mustard (*B. juncea*) is resistant to the highly virulent blackleg isolates,[6] there is a great commercial interest in developing "canola quality lines"[7] of this mustard species. Recent results, however, place high constraint on this enterprise, as new blackleg isolates highly virulent towards brown mustard have been reported both in Canada (isolate Laird 2)[8] and Australia (isolate IBCN 18).[9] Consequently, we became most interested in examining the chemistry of these isolates to establish phytotoxin profiles and their structural relationships within the strains/groups of isolates of *P. lingam*. We have recently reported the production of a dioxopiperazine, L-valyl-L-tryptophan anhydride (**5**) by isolate Laird 2; other possibly phytotoxic metabolites appeared to decompose during the isolation process.[10] Importantly, none of the phytotoxins (e.g., **1** and **2**) or other secondary metabolites previously isolated from highly virulent (e.g., **6**) and avirulent (e.g., **3** and **4**)

0960-894X/99/$ - see front matter
PII: S0960-894X(99)00602-2

isolates of *P. lingam* appeared to be produced by isolate Laird 2. Now we wish to communicate that isolate Laird 2 produces a new host-selective phytotoxin,[11] which we named phomalairdenone A (**7**), as well as phomapyrone A (**4**), a metabolite previously isolated from avirulent isolates of *P. lingam*.[12]

Isolates Laird 2 (Canadian) and IBCN 18 (Australian), both virulent to *B. juncea*, and IBCN 14 and 17 (Australian), both avirulent to *B. juncea* were grown in liquid cultures,[10] the culture broths extracted with ethyl acetate, and the extracts analyzed by TLC, HPLC,[13] and ^{1}H NMR. The HPLC chromatograms of the three IBCN isolates were similar, all extracts contained two major compounds, sirodesmin PL (**2**, R = Ac, n = 2) and phomamide (**6**, X = R = H), similar to the isolates traditionally known as highly virulent.[5] On the other hand, the Laird 2 extract did not appear to contain peaks attributable to compounds available in our libraries; ^{1}H NMR spectra of each extract confirmed the HPLC results. Furthermore, bioassay of those extracts on leaves of canola, brown mustard, and white mustard (*Sinapis alba*) indicated that the extract of isolate Laird 2 was more phytotoxic to brown and white mustards than to canola.[10]

Bioassay-directed fractionation of the ethyl acetate extract of isolate Laird 2, followed by prep. TLC[14] led to isolation of compounds **4**, from a non-phytotoxic fraction, and **7** from a fraction selectively phytotoxic to brown and white mustards. Compound **4** was readily identified as phomapyrone A from its spectroscopic data and comparison with an authentic sample available in our library. Nonetheless, compound **7** did not resemble any of the previously isolated phytotoxins, despite our extensive library of "blackleg" metabolites. The HR-EIMS spectrum of **7** showed a likely molecular ion peak suggestive of the molecular formula $C_{15}H_{22}O_2$, whereas the ^{1}H NMR spectrum showed only 21 hydrogens, indicating the presence of one exchangeable proton, and the ^{13}C NMR spectrum confirmed the presence of 15 carbons.[15] Further analysis of HMQC and HMBC data indicated the presence of three isolated Me groups (singlets at δ_H/δ_C 0.79/34.0, 0.87/30.6, 1.01/14.8), and three additional proton spin systems, one of which contained a Me group (doublet at δ_H/δ_C 0.96/24.5). Thus, a tricyclic ring system containing an α,β-unsaturated ketone was established from the unsaturation number and NMR chemical shifts. Long-range correlations (Figure 1) suggested that this tricyclic structure was related to pentalenene (**8**).

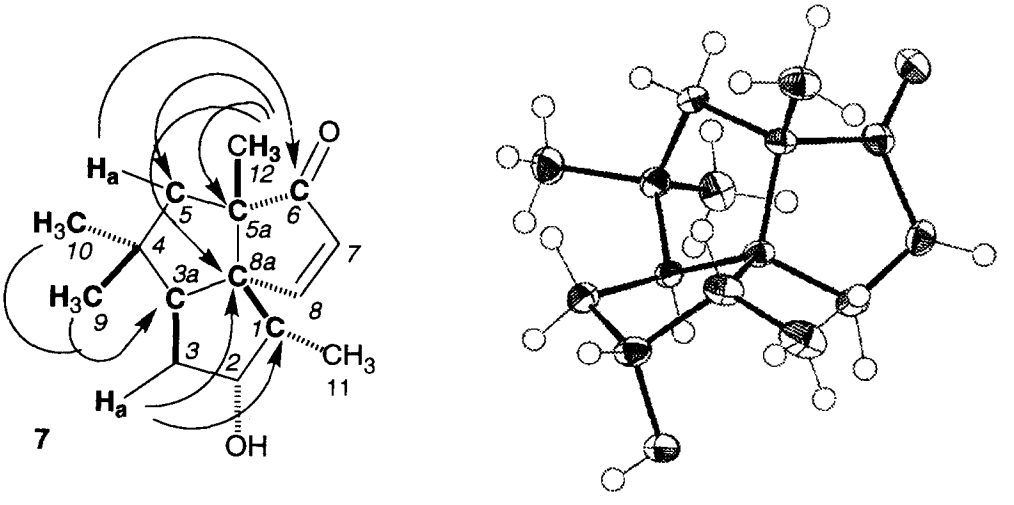
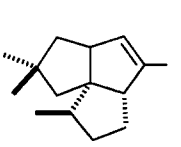
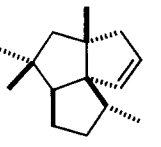


Figure 1. HMBC selected correlations and ORTEP diagram of phomalairdenone (**7**), crystal belonging to orthorhombic system and space group $P2_12_12_1$.

The complete structural assignment and relative stereochemistry of **7** was obtained by X-ray crystallographic analysis of a single crystal belonging to the orthorhombic system and space group $P2_12_12_1$ (ORTEP diagram shown in Figure 1).[16] To the best of our knowledge structure **7** has not been reported previously, although sesquiterpenes with tricyclic skeletons such as pentalenene (**8**)[17] are common in plants.[18] Phomalairdenone has the same tricyclic ring system as silphinene (**9**), a compound first isolated from roots of *Silphium perfoliatum*,[19] whose absolute configuration was established by correlation with modhephene.[20] Related sesquiterpenes are components of essential oils obtained from a variety of plant species, but appear to be less common in microorganisms, and have not been reported from phytopathogenic fungi.[17]



8



9

Finally, the selective phytotoxicity of phomalairdenone (**7**) to plants resistant and susceptible to isolate Laird 2, (canola, resistant; brown mustard, susceptible; white mustard, *S. alba*, susceptible), was evaluated as previously reported for other toxins.[21] Compound **7** caused necrotic, chlorotic, and reddish lesions on brown and white mustard leaves (5×10^{-4} M), whereas no damage was observed on canola leaves. Thus, the selective phytotoxicity of phomalairdenone (**7**) appears to mimic the pathogenicity range of isolate Laird 2. These results suggest that **7** may become a useful phytotoxin to select/screen brown mustard plants for resistance to the new types of blackleg isolates.[22] Furthermore, studies to establish the secondary metabolite profiles of blackleg fungal isolates are of great assistance in the chemotaxonomic classification of the "complex" species presently known as *Phoma lingam*. Thus, phomalairdenone (**7**) will be a useful chemotaxonomic marker for grouping new isolates in a "Laird 2 group". The metabolite profile obtained for isolate Laird 2 (**4**, **5**, **7**) suggests it to be distinct but related to the avirulent group, whereas the three Australian isolates appear to be closely related to the highly virulent group.

Acknowledgment: Support for the authors' work was obtained in part from: the Natural Sciences and Engineering Research Council of Canada and the University of Saskatchewan. We would like to thank G. Séguin-Swartz and R. K. Gugel, Agriculture and Agri-Food Canada, Saskatoon Research Station, SK, for providing IBCN isolates and Laird 2 isolate, respectively, I. L. Zaharia and K. Brown, Department of Chemistry, University of Saskatchewan, for optical rotation and FTIR, and 2D-NMR acquisitions, respectively.

References and Notes

1. Gugel, R. K.; Petrie, G. A. *Can. J. Plant Pathol.* **1992**, *14*, 36, and references therein.
2. Although no reclassification as occurred yet, overwhelming evidence suggests that blackleg disease may in fact be caused by a complex of *Phoma* species, including *P. lingam* and *P. wasabiae*. For an update on blackleg fungi see ref 5.
3. The so-called "highly virulent strain" causes leaf spots and severe stem cankers on rapeseed/canola (*Brassica napus*, *B. rapa*) and cabbage (*B. oleracea*).
4. The so-called "avirulent strain" causes only superficial leaf and stem lesions on rapeseed and cabbage.
5. For a recent review on blackleg metabolites see: Pedras, M. S. C. *Recent Res. Devel. Agricult. Food Chem.* **1998**, *2*, 513.
6. Keri, M.; Van den Berg, C. G. J.; McVetty, P. B. E.; Rimmer, S. R. *Phytopathology*, **1997**, *87*, 594.

7. "Canola- quality lines" refers to plants containing low levels of glucosinolates and erucic acid.
8. Taylor, J. L.; Pedras, M. S. C.; Morales, V. M. *Trends Microbiol.* **1995**, *3*, 202.
9. Chen, C. Y.; Howlett, B. J.; Plummer, K. M. *Eur. J. Plant Pathol.* **1996**, *102*, 349.
10. Pedras, M. S. C.; Smith, K. C.; Taylor, J. L. *Phytochemistry* **1998**, *49*, 1575.
11. Highly virulent isolates of *P. lingam* produce the host-selective toxin phomalide (**1**).
12. Pedras, M. S. C.; Morales, V. M.; Taylor, J. L. *Phytochemistry* **1994**, *36*, 1315.
13. HPLC analysis: HPLC analysis was carried out with a high performance liquid chromatograph equipped with quaternary pump, automatic injector, and photodiode-array detector (wavelength range 190-600 nm), degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 id × 200 mm), equipped with a low-dispersion column-inlet filter. The elution was isocratic for 10 min (80% H_2O-20% CH_3CN), followed by gradient elutions (10 min from 80% H_2O-20% CH_3CN to 60% H_2O-40% CH_3CN; 10 min from 60% H_2O-40% CH_3CN to 25% H_2O-75% CH_3CN) at a flow rate 1.0 mL/min.
14. From a 40 L culture, 360 mg of ethyl acetate extract were obtained; fractionation of this extract (reversed phase C-18 flash column chromatography, CH_3CN-H_2O, 1:1), followed by preparative TLC (CH_2Cl_2-MeOH, 95:5, multiple development) on fraction 2 (ca 40 mg) yielded 8 mg of compound **7** (HPLC t_r = 22.2 min), and on fractions 4–6 (ca 24 mg) yielded 7 mg of compound **4** (HPLC t_r = 29.6 min, conditions as described above).
15. Spectroscopic data of metabolite **7**: $[\alpha]_D$ -6.5 (c = 0.3, $CDCl_3$); ^1H NMR (500 MHz, CD_2Cl_2): δ 0.79 (s, 3H-9/10), 0.87 (s, 3H-10/9), 0.96 (*J* = 7.5 Hz, 3H-11), 1.01 (s, 3H-12), 1.54 (dd, *J* = 12.5, 3.0 Hz, $1H_b$-3), 1.55 (d, *J* = 13.5 Hz, $1H_a$-5), 1.78 (dd, *J* = 12.5, 7.5 Hz, $1H_a$-3), 1.96 (d, *J* = 13.5 Hz, $1H_b$-5), 2.17 (qd, *J* = 7.5, 3.5 Hz, 1H-1), 2.38 (dd, *J* = 12.5, 7.5 Hz, 1H-3a), 4.24 (dd, *J* = 3.5, 3.0 Hz, 1H-2), 5.97, (d, *J* = 5.5 Hz, 1H-7), 7.73 (d, *J* = 5.5 Hz. 1H-8); ^{13}C NMR (125 MHz, CD_2Cl_2): δ 14.8 (C-12), 24.5 (C-11), 30.6 (C-10/9), 34.0 (C-9/10), 41.1 (C-3), 43.6 (C-4), 46.7 (C-1), 54.8 (C-5), 60.9 (C-5a), 61.9 (C-3a), 71.3 (C-8a), 82.0 (C-2), 133.7 (C-7), 175.5 (C-8), 221.0 (C-6); HREIMS *m/z* measured: 234.1619 (234.1619 calcd. for $C_{15}H_{22}O_2$); EIMS *m/z* (% relative abundance): 234 $[M]^+$ (100), 216 (23), 201 (37), 161 (42); CIMS (NH_3) *m/z* (% relative abundance): 252 $[M+18]^+$ (60), 235 $[M+1]^+$ (100); FTIR ν_{max}: 2958, 2929, 1726,1272, 1122, 1072 cm^{-1}.
16. Colorless crystals were obtained from a concentrated solution of CH_2Cl_2-hexane kept at 5 °C (crystal size 0.45 × 0.18 × 0.15 mm^3). Data collected on a Nonius CAD-4 at 123(2) K using MoK_α radiation, λ = 0.71073 Å, ω scans, *a* = 6.867(2). *b* = 12.568. *c* = 15.802(2), Z = 4, psi-scan absorption correction, Θ_{max} = 27.88°, 2175 reflections collected giving 1899 unique reflections and 1665 > 2σ(I). Structure solution using NRCVAX; solution of structure by direct methods; hydrogens placed by geometry and not refined; refinement on F^2 with 156 parameters using SHELXL-97. Final R (all data) = 0.0463; R(F^2 > 2σ(F^2)) = .0377; wR (all data on F^2) = 0.0897, wR(F^2 >)) = 0.857; GoF = 1.024.
17. First isolation: Seto, H.; Yonehara, H. *J. Antibiot.* **1980**, *33*, 92. Biosynthetic studies, *inter alia*: (a) Cane, D. E. In *Comprehensive Natural Products*, Cane, D. E. Ed.; Pergamon: Oxford, 1999, Vol. 2, pp 155-200; (b) Cane, D. E.; Weiner, S. W. *Can. J. Chem.* **1994**, *72*, 118; (c) Cane, D. E.; Oliver, J. S.; Harrison, P. H. M.; Abell, C.; Hubbard, B. R.; Kane, C. T.; Lattman, R. *J. Am. Chem. Soc.*, **1990**, 4513.
18. Weirstahl, P.; Marschall, H.; Sellmann, I.; Jakupovic, J. *Eur. J. Org. Chem.* **1998**, 1205.
19. Bohlman, F.; Jakupovic, J. *Phytochemistry* **1980**, *19*, 259.
20. Fitjer, L.; Monzó-Oltra, H. *J. Org. Chem.* **1993**, *58*, 6171.
21. Pedras, M. S. C.; Séguin-Swartz, G.; Abrams, S. R. *Phytochemistry* **1990**, *29*, 777.
22. For a series of reviews on phytotoxins and applications, see, Graniti, A. *et al. Experientia* **1991**, *47*, 751.