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Phomapyrones from blackleg causing phytopathogenic fungi: isolation, structure determination, biosyntheses and biological activity

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Abstract

The isolation and structure determination of phomapyrones D–G, three 2-pyrones and a coumarin, from a group of isolates of the fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm, is reported. As well, phomenin B, infectopyrone, and polanrazines B and C were also obtained for the first time from these isolates. In addition, based on results of incorporations of ^{13}C-labeled acetate and malonate, and deuterated methionine, a polyketide pathway is proposed for the biosyntheses of phomapyrones.

Keywords: *Brassica napus*; Canola; *Sinapis alba*; White mustard; *Brassica juncea*; Brown mustard; Cruciferae; *Leptosphaeria maculans*; *Phoma lingam*; Phytotoxin; 2-pyrones; Polyketide biosynthesis

1. Introduction

Blackleg of crucifers is a plant disease caused by the fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm (Gugel and Petrie, 1992). Recent reclassification of this species introduced the new name *Leptosphaeria biglobosa* to enclose the isolates traditionally known as avirulent, weakly virulent or B group (*L. maculans*/*P. lingam* isolates least damaging), while the virulent group (most damaging) remains *L. maculans*/*P. lingam* (for a recent review see Hewlett et al., 2001). We have analyzed a large number of blackleg causing fungi and reported the secondary metabolites produced by a new group of isolates able to infect brown mustard (*Brassica juncea* L.), a crop previously known to be resistant to blackleg disease. These new isolates were found to produce as major metabolites polanrazine A (**1**) (Pedras et al., 1998), phomalairdenone A (**2**) and phomapyrone A (**3**) (Pedras et al., 1999). In continuation of that work we have isolated from cultures of isolates Laird 2 and Mayfair 2, four new metabolites which we named phomapyrones D–G (**4**–**7**). As well, phomenin B (**8**), infectopyrone (**9**), and polanrazines B (**10**) and C (**11**) were isolated for the first time from these isolates. Here we report the structure determination of metabolites **4**–**7**, and propose a biosynthetic pathway for phomapyrones based on results of incorporations of ^{13}C-labeled acetate and malonate, and deuterated methionine, as well as lack of incorporation of deuterated propanoic acid.

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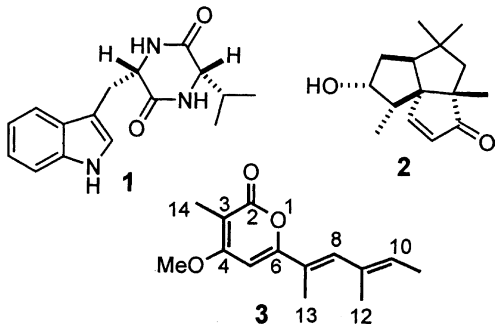
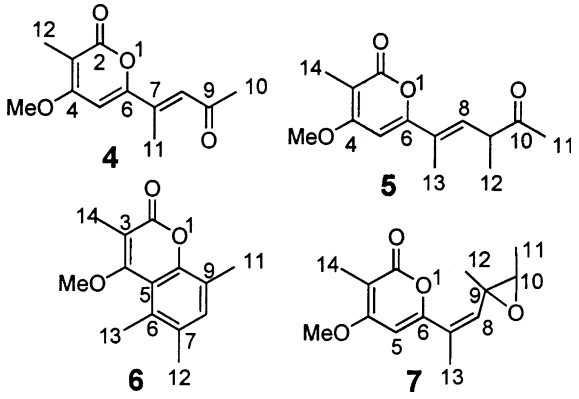


2. Results

2.1. Isolation and structure determination

Extracts (2.3 g) of cultures of isolate Mayfair 2 (*L. maculans*/*P. lingam*) were fractionated by flash column chromatography to yield the new phomapyrones D (**4**, 10 mg), E (**5**, 3.5 mg), F (**6**, 4 mg), and G (**7**, 6.5 mg), as well as phomapyrone A (**4**, 36 mg), phomenin B (**8**, 28 mg), infectopyrone (**9**, 4 mg), and polanrazines B (**10**, 4.5 mg) and C (**11**, 21 mg).



The molecular formula of phomapyrone D (**4**) was determined to be $C_{12}H_{14}O_4$ (HREI-MS), indicating six degrees of unsaturation. Analysis of the ^1H and ^{13}C NMR spectroscopic data indicated the presence of three methyl groups, one methoxy group, and eight sp^2 hybridized carbons (Tables 1 and 2). Analysis of the HMQC and HMBC data was instrumental in the assignment of the structure. Among the three carbonyl-like carbons, the carbon at δ_C 165.2 was assigned to C-2 as it displayed only a correlation with the methyl protons at δ_H 2.05 (H_3-12). Likewise, the carbon at δ_C 164.5 displayed correlations with methyl protons at δ_H 2.05, methoxy protons at δ_H 3.95 and methine proton at δ_H 6.52 (H-5), allowing its assignment as C-4. An additional correlation of the methyl group at δ_H 2.05 (H_3-12) with a tetrasubstituted olefinic carbon at δ_C 106.3 allowed the assignment of C-3. Further correlations of the proton at δ_H 6.52 with the carbon at δ_C 158.2 (C-6), and of this carbon with a methyl group at δ_H 2.36 (H_3-11) allowed the complete assignment of a 2-pyrone unit and established that a substituent was located at C-6. The partial structure of this substituent was deduced similarly. The long-range correlation of an olefinic proton at δ_H 7.15 (H-8) with both a carbonyl carbon at δ_C 199.7 (C-9) and an sp^2 carbon at δ_C 139.8 (C-7) suggested the presence of an α,β-unsaturated ketone in the side chain. Further, the correlation of the carbonyl carbon at δ_C 199.7 (C-9) with a methyl group at δ_H 2.36 (δ_C 33.0) allowed the complete assignment of the side chain. Finally, the configuration of the double bond was assigned as 7-*E* on the basis of NOE data, after an NOE enhancement observed on the signal of H-5 upon irradiation of the proton at δ_H 2.36 (H-11), and vice versa (no effect on signal of H-8, δ_H 7.15). Hence, the structure **4** was unambiguously assigned to phomapyrone D.

Phomapyrone E (**5**) had a molecular formula of $C_{14}H_{18}O_4$ (HREI-MS) indicating six degrees of unsaturation. The ^1H and ^{13}C NMR spectra showed the presence of four methyl groups, one methoxy group and

Table 1
^1H NMR (500 MHz) chemical shifts (ppm), multiplicity, *J* (Hz) of phomapyrones D (**4**), E (**5**), F (**6**), and G (**7**) in $CDCl_3$

Hydrogens	4	5	6	7
5	6.52, s	6.21, s	–	6.19, s
8	7.15, s	6.51, d, 10	7.17, s	6.02, s
9	–	3.53, m	–	–
10	2.36, s	–	–	3.05, q, 6
11	2.36, s	2.17, s	2.41, s	1.46, d, 6
12	2.05, s	1.30, d, 7	2.33, s	1.50, s
13		2.00, s	2.61, s	1.99, s
14		1.97, s	2.19, s	1.96, s
OMe	3.95, s	3.94, s	3.82, s	3.88, s

Table 2
^{13}C NMR (125 MHz) chemical shifts (ppm) of phomapyrones D (**4**), E (**5**), F (**6**), and G (**7**) (in $CDCl_3$)

Carbons	4	5	6	7
2	165.2	165.2	164.5	164.8
3	106.3	103.4	114.1	103.5
4	164.5	166.0	167.1	165.5
5	97.5	93.3	116.1	95.4
6	158.2	159.4	133.3	158.9
7	139.8	128.8	131.1	127.1
8	126.5	132.8	134.6	137.7
9	199.7	48.2	123.5	60.3
10	33.0	208.0	150.6	62.4
11	13.9	28.4	16.1	14.5
12	9.4	16.9	20.9	17.9
13	–	13.3	16.7	21.0
14	–	9.2	10.9	9.1
Ome	56.7	56.6	60.7	56.6

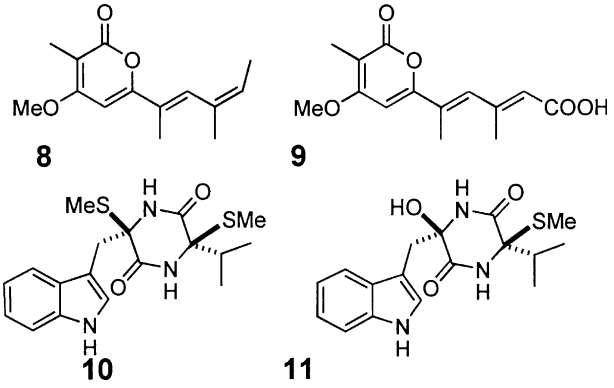
eight sp^2 hybridized carbons. The ^1H NMR spectrum displayed a spin system containing a proton at δ_H 3.53 (H-9) coupled to an olefinic proton at δ 6.51 (H-8, d, $J = 10$ Hz) and methyl protons at δ_H 1.30 (H_3-12, d, $J = 7$ Hz). As for phomapyrone D (**4**), the HMBC spectrum displayed the typical correlations of a 3,4,6-trisubstituted-2-pyrone. For instance, long-range correlations were observed between the proton at δ_H 6.21 (H-5) and carbons at δ_C 103.3 (C-3), δ_C 166.0 (C-4), and δ_C 159.4 (C-6). The long-range correlation of protons at δ_H 6.51 (H-8) and at δ_H 2.00 (H_3-13) with C-6 (δ_C 159.4) and the proton at δ_H 6.21 (H-5) with a carbon at δ_C 128.8 (C-7) suggested the connection of C-6 of 2-pyrone moiety to C-7 of the side chain. Additional long-range correlations were also observed between the methyl protons at δ_H 1.97 (H_3-14) and carbons at δ_C 165.2 (C-2) and δ_C 103.3 (C-3). Moreover, the proton at δ_H 3.53 (H-9) showed a long-range correlation with a carbonyl carbon at δ_C 208.0, which was assigned to C-10. The methyl protons at δ_H 2.17 (δ_C 28.3) showing the only long-range correlation with carbon C-10 (δ 208.0) were assigned to H_3-11. The *E* stereochemistry of the double bond at C-7 was deduced on the basis of NOE difference data; irradiation of proton at δ_H 2.00 (H_3-13) caused an NOE enhancement on signal at δ_H 6.21 (H-5), and vice versa, but it had no effect on the proton at C-8 (δ_H 6.51). Hence, the structure of phomapyrone E was assigned unambiguously as **5**.

The molecular formula of phomapyrone F (**6**) was established as $C_{14}H_{16}O_3$ on the basis of HREI-MS and NMR spectroscopic data. The ^1H NMR spectrum displayed signals for four methyl groups, one methoxy group, and one sp^2 hybridized methine, accounting for the total number of protons. The proton decoupled ^{13}C NMR spectrum displayed 14 carbon resonances, of which three had chemical shift above δ_C 150 suggestive of oxygen-bearing sp^2 carbons. The HMBC spectrum displayed all characteristic correlations indicating the presence of a fully substituted 2-pyrone moiety. The carbon signal at δ_C 164.5 correlating with methyl group at δ_H 2.19 was assigned to C-2. As for phomapyrones **4** and **5**, the methyl group C-14 (δ_H 2.19, H_3-14) showed further long-range correlations with carbons at δ_C 114.1 and 167.1, which were assigned to C-3 and C-4, respectively. C-4 also exhibited a long-range correlation with the methoxy group at δ_H 3.82. Additionally, analysis of HMBC data indicated the presence of an aromatic nucleus fused to the 2-pyrone moiety along the C-5–C-10 bond, to form a coumarin ring. The positions of substitution of the remaining methyl groups at δ_H 2.61, 2.33 and 2.41 were determined by analysis of NOE difference data. Thus, irradiation of methyl protons at δ_H 2.61 caused an NOE enhancement on the methoxy group (δ_H 3.82) and methyl group at δ_H 2.33 (H_3-12), indicating the location of these methyl groups on C-6 (δ_H 2.61, H_3-13) and C-7 (δ_H 2.33, H_3-12), respectively. Likewise, irradiation of proton at δ_H 7.17 caused an NOE enhancement on the signals of methyl protons at δ_H 2.41 (H_3-11) and 2.33 (H_3-12) indicating the methyl group at δ_H 2.41 is located at C-9. Hence, phomapyrone F has structure **6**.

The molecular formula of phomapyrone G (**7**) was established on the basis of HRMS and NMR data as $C_{14}H_{18}O_4$ indicating 6° of unsaturation. The ^1H NMR spectrum displayed signals of two olefinic, one methine, and five methyl proton which accounted for the total number of hydrogens. The ^{13}C NMR spectrum displayed fourteen carbon signals, three of which were sp^2 hybridized attached to oxygen, on the basis of their chemical shifts. As for the structural assignments of phomapyrones **4**–**6**, HMBC data allowed the assignment of the signal at δ_C 164.8 to C-2 based on the only long-range correlation with the methyl group at δ_H 1.96. Similarly, another low field carbon at δ_C 165.5, showing a long-range correlation with the methoxy group at δ_H 3.88, was assigned to C-4. The latter also exhibited long-range correlations with the methyl group at δ_H 1.96 (H_3-14) and the proton at δ_H 6.19 (H-5). The long-range correlation of the proton H-5 with the carbon at δ_C 127.1 (C-7), together with the long-range correlation of the proton at δ_H 6.02 (H-8) with the carbon at δ_C 158.9 (C-6), indicated a substituent at C-6. The chemical shifts of two sp^3 hybridized carbons at δ_C 60.3 (C-9) and δ_C 62.4 (C-10) suggested that these carbons were attached to oxygen. Consequently, to satisfy the chemical formula an epoxide was assigned to C-9/C-10. The *Z* stereochemistry of side chain was determined on the basis of NOE data. Irradiation of the proton signal at δ_H 1.99 (H_3-13) caused an NOE enhancement on the signal at δ_H 6.19 (H-5) and δ 6.02 (H-8). Furthermore, irradiation of proton signal at δ_H 6.02 (H-8) caused an NOE enhancement on the signal at δ_H 1.99 (H_3-13) and δ 3.06 (H-10), but had no effect on the signals at δ_H 1.96 (H_3-12) and δ_H 1.47 (H_3-11). Additionally, irradiation of H-10 (δ_H 3.06) caused an NOE enhancement on the signals of H-8 (δ_H 6.02) and H_3-11 (δ 1.47). Therefore, the structure of phomapyrone G was assigned as **7**, however the configurations of the stereogenic centers C-9 and C-10 remain undetermined.

Additional pyrones **8** and **9** were isolated for the first time from isolates Mayfair 2 and Laird 2. After spectroscopic analyses their structures were determined to be identical to the known metabolites phomenin B (**8**) and infectopyrone (**9**), isolated previously from *Phoma tracheiphila* (Tringali et al., 1993) and from *Alternaria infectoria*, respectively (Larsen et al., 2003). Furthermore, dioxopiperazines **10** and **11** were also isolated from isolates Mayfair 2 and Laird 2. These metabolites were shown to be identical to polanrazines B (**10**) and C (**11**) by comparison of spectroscopic data with those of authentic samples (Pedras and Biesenthal, 2001).

Results of phytotoxicity assays using phomapyrones A (**3**), D (**4**), and phomenin B (**8**) indicated no activity even at relatively high concentration (10^{-3} M).



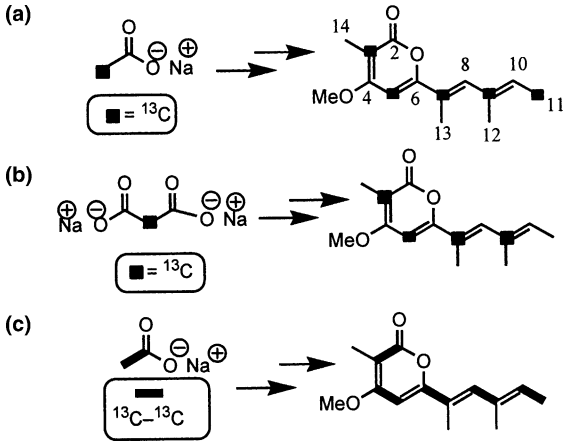


Fig. 1. Labelling pattern of phomapyrone A (**3**) isolated from cultures containing: (a), sodium [2-^{13}C]acetate; (b), sodium [2-^{13}C]malonate; and (c), sodium [1,2-$^{13}C_2$]acetate.

2.2. Biosynthetic origin of phomapyrones

Sodium [2-^{13}C]acetate, sodium [1,2-^{13}C]acetate, sodium [2-^{13}C]malonate, [3,3,3-$^{2}H_3$]propanoic acid, and L-[Me-$^{2}H_3$]methionine were separately administered to cultures of isolate Mayfair 2. After extraction of the broth, and separation of the extract, phomapyrone A (**3**) was obtained and analyzed by both NMR spectroscopy and HRMS; on the other hand, phomapyrones **4**–**7** were obtained in insufficient amount for NMR studies. The results obtained from these experiments are summarized in Fig. 1 and Table 3. The distribution of ^{13}C labelling in phomapyrone A (**3**) was determined by comparison of the integrated and normalized intensities of carbon signals of enriched **3** with those of the unenriched (i.e. natural abundance) sample. The ^{13}C NMR spectrum of phomapyrone A (**3**) isolated from administration of sodium [2-^{13}C]acetate displayed 14 signals, which were assigned by comparison with the ^{13}C NMR data of the natural abundance metabolite. The signal intensities of C-3, C-5, C-7, C-9, and C-11 were enhanced relative to the other carbons (Table 3, Fig. 1(a)). Completable experiments using sodium [2-^{13}C]malonate, showed that the ^{13}C NMR signals of C-3, C-5, C-7, and C-9 were more intense, whereas the ^{13}C NMR signal of C-11 was not as enhanced (Table 3, Fig. 1(b)). In addition, the ^{13}C NMR. spectrum of phomapyrone A (**3**) resulting from incorporation of so-

Table 3
Incorporation results of feeding experiments with sodium [2-^{13}C]acetate and sodium [2-^{13}C]malonate based on the ^{13}C NMR of phomapyrone A (**3**)

Carbon	Phomapyrone A natural sample		Phomapyrone A enriched, [2-^{13}C]acetate			Phomapyrone A enriched, [2-^{13}C]malonate		
	SI[a]	NSI[b]	SI[a]	NSI[b]	R[c]	SI[a]	NSI[b]	R[c]
C-2	2.09	0.22	1.89	0.31	1.40	0.47	0.18	0.82
C-3	2.54	0.26	3.25	0.54	2.08[d]	1.49	0.56	2.15[d]
C-4	3.10	0.32	2.34	0.39	1.22	0.77	0.29	0.91
C-5	8.94	0.92	8.14	1.34	1.46[d]	5.46	2.05	2.23[d]
C-6	3.88	0.40	2.52	0.42	1.05	1.01	0.38	0.95
C-7	3.58	0.37	3.29	0.54	1.46[d]	2.25	0.85	2.29[d]
C-8	8.28	0.86	5.76	0.95	1.10	2.47	0.93	1.08
C-9	4.53	0.47	4.39	0.72	1.53[d]	3.66	1.38	2.94[d]
C-10	6.92	0.71	4.96	0.82	1.15	1.78	0.67	0.94
C-11	7.21	0.74	9.26	1.53	2.06[d]	3.06	1.15	1.55[d]
C-12	7.12	0.74	5.47	0.90	1.22	2.34	0.88	1.19
C-13	10.14	1.04	6.40	1.05	1.00	2.65	1.00	0.96
C-14	8.63	0.89	5.85	0.96	1.08	2.30	0.86	0.97
OCH_3	9.68	**1.00**	6.07	**1.00**	**1.00**	2.66	**1.00**	**1.00**

[a] SI = ^{13}C signal intensity of sample.
[b] NSI = ^{13}C normalized signal intensity of sample relative to OCH_3.
[c] R = [^{13}C normalized signal intensity of enriched sample]/[^{13}C normalized signal intensity of natural sample].
[d] Enriched carbons.

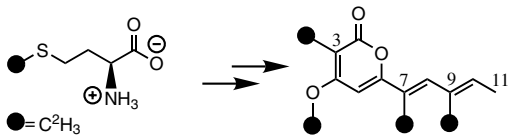


Fig. 2. Labelling pattern of phomapyrone A (**3**) isolated from cultures containing L-[C^2H_3]methionine.

dium [1,2-^{13}C]acetate displayed fourteen signals due to natural abundance ^{13}C plus 10 doublets flanking carbons C-2 to C-11. The doublets due ^{13}C–^{13}C coupling indicated the incorporation of five intact C_2 units; however, the carbon signals of methyl groups C-12, C-13, C-14 and OMe did not show satellite peaks (Fig. 1(c)).

After the polyketide origin of phomapyrone A (**3**) was established, the origin of its four methyl groups, i.e. C-12–C-14 and (O)CH_3 was investigated. Thus, HREI-MS analysis of phomapyrone A (**3**) isolated from cultures incubated with L-[Me-$^2H_3$]methionine showed incorporation of several deuterium atoms: $M^+ + 12$ (4%), $M^+ + 9$ (11%), $M^+ + 6$ (30%), $M^+ + 3$ (70%), relative to naturally occurring compound. However, phomapyrone A (**3**) isolated from cultures incubated separately with [3,3,3-$^2H_3$]propanoic acid showed no deuterium incorporation by HREI-MS analysis. Consequently, the most likely source of the four C_1 units present in phomapyrone A (**3**) is L-methionine, as summarized in Fig. 2.

3. Discussion

Metabolites containing a 2-pyrone moiety have been isolated from both terrestrial and marine organisms. Phomapyrones A (**3**), B, and C, were obtained from cultures of weakly virulent Canadian isolates of L *maculans*/*P. lingam* (Pedras et al., 1994), whereas phomapyrone A (**3**) was also reported from Polish isolates (Pedras and Biesenthal, 2001). Phomapyrone A (**3**) was isolated from *P. tracheiphila* by an independent group and named phomenin A (**3**), which was reported along with phomenin B (**8**) (Tringali et al., 1993). Infectopyrone (**9**) was reported from the phytopathogenic fungi *Alternaria infectoria* and *Stemphylium* sp. as a potential mycotoxin (Larsen et al., 2003). The chemical study of *Ercolania funereal*, a marine mollusc, led to the isolation of several cyercenes, one of which is identical to phomapyrone A (**3**) (Vardaro et al., 1992). Additional 2- and 4-pyrone polypropionates were also reported from other marine molluscs such as *Siphonaria diemenensis* (Hochlowski and Faulkner, 1983) and *Placida dendritica* (Cutignano et al., 2003). Interestingly, some pyrone metabolites from molluscs were suggested to have a defensive role (Di Marzo et al., 1993; Vardaro et al., 1992).

This investigation on the biosynthetic origin of phomapyrone A (**3**) produced by the fungus *L. maculans*/*P. lingam* indicates its polyketide origin. Namely, the labeling pattern obtained for phomapyrone A (**3**) after incorporation of [2-^{13}C]malonate, [2-^{13}C]acetate, and [1,2-^{13}C]acetate and the lack of incorporation of propanoic acid. Furthermore, the incorporation of the four C^2H_3 groups (HRMS, M + 12, 4%) derived from methionine, suggests the origin of the methyl groups at C-3, C-7, and C-9 and OMe. In conclusion, phomapyrone A (**3**) was found to be an acetate/malonate derived pentaketide with four methyl groups derived from methionine, as summarized in Fig. 3. These results further suggest that the new phomapyrones D–G (**4–7**) are of polyketide origin, resulting from a pentaketide chain as well, but phomapyrone D might result from methylation of a tetraketide or decarboxylation of a pentaketide. Previous studies of the biosynthetic origin of 2-pyrones from the fungus *Gliocladium vermoesenii* indicated a polyketide origin (Avent et al., 1992a,b), whereas another study of the biosynthetic origin of 2-pyrone and 4-pyrone metabolites from molluscs indicated a polypropionate origin (Vardaro et al., 1992). The polyketide origin of pyrones from *G. vermoesenii* is consistent with the results reported above. It might be that pyrones isolated from molluscs are polypropionates and those from fungi are polyketides.

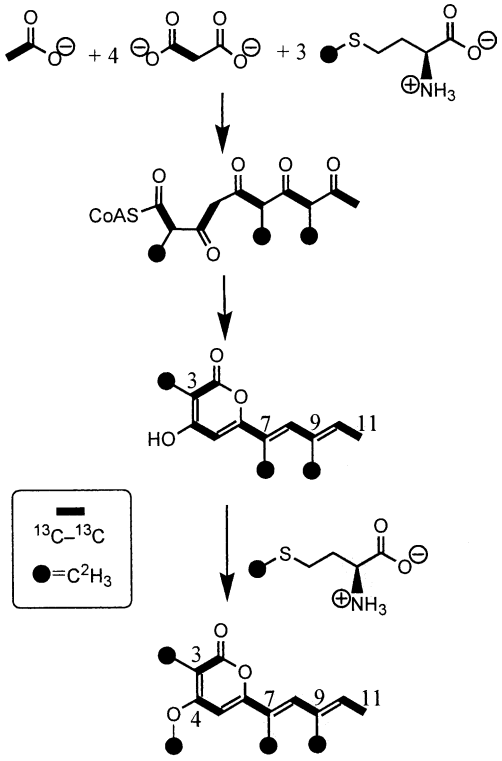


Fig. 3. Biosynthetic pathway of phomapyrone A (**3**) produced by *Leptosphaeria maculans*/*Phoma lingam*.

4. Experimental

4.1. General

Sodium [2-^{13}C]acetate, sodium [l,2-^{13}C]acetate, sodium [2-^{13}C]malonate, [3,3,3-$^{2}H_3$]propanoic acid, and L-[Me-$^{2}H_3$]methionine were purchased from Cambridge Isotope Laboratories, Inc., Andover, MA. Analytical 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 i.d. × 200 mm), equipped with an in-line filter. Mobile phase: H_2O–CH_3CN A:B ,v:v, isocratic A:B(1:4), 10 min, gradient 1:4 to 3:2, 10 min, 3:2 to 3:1, 10 min, 3:1(A:B) to B , 10 min, at a flow rate 1.0 mL/min. Samples were dissolved either in CH_3CN or in MeOH. Other conditions are as previously reported (Pedras and Okanga, 1999).

Fungal cultures were grown as reported previously (Pedras and Biesenthal, 2001). Isolates Mayfair 2 and Laird 2 were obtained from Agriculture and Agri-Food Canada, Saskatoon, Saskatchewan, IBCN collection.

4.2. Isolation of metabolites

A total of 122 L cultures were grown in minimal medium (Pedras et al., 1997) for 6 days; the cultures were filtered, and the broth was combined. The broth obtained (110 L) was concentrated (22 L) by freeze drying, and was extracted with EtOAc (800 mL × 3/1 L broth). The combined extracts were dried (anhyd Na_2So_4) and concentrated to dryness under reduced pressure (2.3 g). The EtOAc extract was subjected to flash Si gel cc and eluted first with CH_2Cl_2 followed by CH_2Cl_2–MeOH (97:3, 200 mL) and then with CH_2Cl_2–MeOH (90:10). All fractions collected were concentrated to dryness under reduced pressure. Fractions F_3 to F_6 were combined, subjected to prep. Si gel TLC, and developed with CH_2Cl_2–MeOH (98:2, for three times) to yield phomapyrone F (**6**) (4.0 mg). Fraction F_7 from FCC of EtOAc extracts was further subjected to FCC (2 cm × 15 cm, silica gel, 50 mL fractions), eluted first with CH_2Cl_2–EtOAc (95: 5, 200 mL) followed by CH_2Cl_2–EtOAc (90:10, 200 mL) and then CH_2Cl_2–EtOAc (80:20, 200 mL). Twelve fractions were collected and concentrated to dryness under reduced pressure. Fractions F_2 and F_3 were combined (39.5 mg), subjected to prep. TLC (hexane–EtOAc, 9:1, multiple development) to yield phomapyrone A (**3**) (36 mg) and phomenin B (**8**) (28 mg). Fractions F_6 to F_9 were combined, further subjected to prep. Si gel TLC (CH_2Cl_2–MeOH, 98:2) to obtain phomapyrone D (**4**) (10 mg). This material was further applied to prep. TLC (hexane–Et_2O, 50:50, multiple elution) to yield phomapyrone E (**5**) (5 mg), phomapyrone G (**7**) (6.5 mg), and polanrazine B (**10**, 4.5 mg), respectively. Fractions F_{10}–F_{12} were combined and subjected to prep. TLC (CH_2Cl_2–MeOH, 97:3, develaped three times) to yield infectopyrone (**9**) (4 mg) and polanrazine C (**11**, 21 mg).

4.3. Phytotoxicity assays

4.3.1. Phytotoxicity

Seeds of canola *(Brassica napus)* cv. Westar, white mustard (*Sinapis alba*) cv. Ochre, and brown mustard (*Brassica juncea*) were obtained from Plant Gene Resources, Agriculture and Agric-Food Canada Research Station, Saskatoon, SK. The seeds were sown in commercial potting soil mixture, and plants were grown in a growth chamber, under controlled environmental conditions, as previously reported (Pedras and Biesenthal, 2001). Phytotoxicity assays were conducted on leaves of 2-week-old plants with phomapyrones A (**3**), D (**4**) and phomenin B (**8**) (5×10^{-4} and 1×10^{-3} M in H_2O–MeOH, 1:1, v/v) as previously described (Pedras and Biesenthal, 2001). Plants were incubated in a growth chamber for a week.

4.4. Pressure administration experiments

Cultures of isolate Mayfair 2 (250 mL flasks with 100 mL per flask, total 2 L, 5×10^8 spores per 100 mL) were incubated on a shaker at 120 rpm under fluorescent light. Each flask was administration ,of 48 and 72 h with [2-^{13}C]acetate at a concentration of 25 mg of sodium [2-^{13}C]acetate per 100 mL of culture. After 6 days of incubation the culture was filtered, and worked up as described above. The EtOAc extract (32 mg) was separated by RP-FCC (1 cm × 10 cm, RP-C18, 20 mL fractions) and eluted first with CH_3CN–H_2O (2:3, 1 mL) followed by CH_3CN (40 mL). Ten fractions were collected and concentrated to dryness under reduced pressure. Fractions F_5 and F_6 were combined (5.0 mg) and further subjected to prep. TLC (hexane–EtOAc, 9:1, multiple elution) to yield phomapyrone A (**3**, 2.8 mg).

Similar experiments were carried out with sodium [2-^{13}C]malonate (10 L of culture to yield phomapyrone A, 6.5 mg), [1,2-^{13}C]acetate (5 L of culture to yield phomapyrone A (**3**), 4 mg), [3-$^{2}H_3$]propanoic acid (5 L of culture to yield phomapyrone A (**3**), 4.5 mg), and L-[$C^{2}H_3$]–methionine (5 L of culture to yield phomapyrone A (**3**), (3.5 mg).

4.5. Characterization data

4.5.1. Phomapyrone D (4)

HPLC R_t = 18.9 min for ^{1}H and ^{13}C NMR spectra, see Tables 1 and 2; UV λ_{max} 210, 250, 350 nm; FTIR (cm^{-1}) 2996, 1681, 1357, 1013, 746; HREI-MS: *m/z*

222.0893, calc. for $C_{12}H_{14}O_4$ 222.0892, EI-MS: *m/z* (relative intensity) 222 (100%), 207 (17.9%), 194 (17%), 179.06 (20%), 159 (49%), 139 (24%), 57 (56%).

4.5.2. Phomapyrone E (**5**)

HPLC R_t = 22.2 min for ^1H and ^{13}C NMR spectra, see Tables 1 and 2; UV λ_{max} 230, 335 nm; FTIR (cm^{-1}) 2970, 2927, 1692, 1554, 1355, 1170, 1013, 751; HREI-MS: *m/z* 250.1206, calc. for $C_{14}H_{18}O_4$ 250.1205, EI-MS: *m/z* (relative intensity) 250 (13%), 208 (100%), 165 (11%).

4.5.3. Phomapyrone F (**6**)

HPLC R_t = 30.4 min. for ^1H and ^{13}C NMR spectra, see Tables 1 and 2; UV λ_{max} 210, 290 nm; FTIR (cm^{-1}) 2925, 1711, 1590, 1202, 1093; HREI-MS: *m/z* 232.1095, calc. for $C_{14}H_{16}O_3$ 232.1099, EI-MS: *m/z* (relative intensity) 232 (100%), 217 (56%), 189.09 (63%).

4.5.4. Phomapyrone G (**7**)

HPLC R_t = 23.0 min. for ^1H and ^{13}C NMR spectra, see Tables 1 and 2; UV λ_{max} 230, 330 nm; FTIR (cm^{-1}) 2974, 2929, 1700, 1554, 1247, 1166, 1025; HREI-MS: *m/z* 250.1202, calc. for $C_{14}H_{18}O_4$ 250.1205, EI-MS: *m/z* (relative intensity) 250 (20%), 208 (66%), 139(49%), 83(28%).

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