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Phomaligols and Phomaligadiones: New Metabolites From The Blackleg Fungus

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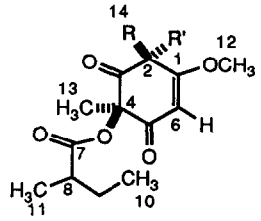
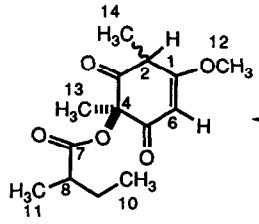
Abstract: The isolation and structure elucidation of four new metabolites (1-4) are described. The structures were determined employing 2D NMR spectroscopy. The isolation of these unusual metabolites from *Phoma lingam* may have chemotaxonomic implications.

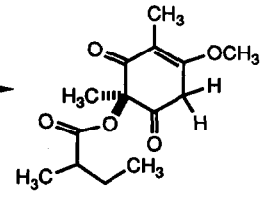
The blackleg fungus [*Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm.] causes widespread destruction among cruciferous crops and can be particularly devastating for canola (*Brassica napus* and *B. campestris*).[1] While virulent isolates of the fungus are readily recognized by their biosynthesis of epipolythiodioxopiperazines, no characteristic metabolites appear to have been isolated from weakly virulent isolates.[2,3] In continuation of our studies of the chemistry of *P. lingam*,[4-6] we have investigated a weakly virulent isolate that produces yellow pigments, both in liquid and in solid media. Now we describe the structure elucidation of phomaligols A (**1**) and A_1 (**2**), and phomaligadiones A (**3a/3b**) and B (**4**), new polyketides isolated from the blackleg fungus. The isolation of these metabolites for the first time from a weakly virulent isolate may have chemotaxonomic implications.



1 R=CH_3, R'=OH
1a R=CH_3, R'=OCH_3
2 R=OH, R'=CH_3



3a/3b



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RESULTS AND DISCUSSION

Phoma lingam, isolate NB-2 (weakly virulent strain) was grown in potato dextrose or Cove's media[7] in shake culture, for five to seven days. After filtration, the broth was concentrated and extracted with EtOAc. The crude EtOAc extract showed one major and several minor components in the ^1H NMR spectrum. A preliminary purification of the extract using flash column chromatography[8] (hexane-EtOAc), followed by preparative TLC, afforded phomaligols A (**1**) and A_1 (**2**), and phomaligadiones A (**3a/3b**) and B (**4**).

The ^1H NMR spectra of phomaligols A (**1**) and A_1 (**2**) were rather simple, each one revealing the spin systems of a *sec*-butyl group (9 protons), and five singlets for eleven protons: three methyl groups, an

Table 1. ^{1}H and ^{13}C NMR Data[a] for Phomaligols A (**1**), A_1 (**2**), and Phomaligadiones A (**3a** major epimer; **3b** minor epimer) and B (**4**) in $CDCl_3$.

C/H #	**1**		**2**		**3a** (major epimer)		**3b** (minor epimer)		**4**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	-------	172.93	-------	173.60	-------	174.01	-------	174.90	-------	163.95
2	-------	73.55	-------	74.35	3.40, dq (1.5, 7.0)	44.92	3.38, dq (1.5, 7.0)	46.17	-------	113.57
3	-------	202.60	-------	205.14	-------	201.77	-------	201.65	-------	198.58
4	-------	81.12	-------	81.69	-------	82.96	-------	82.76	-------	82.86
5	-------	191.58	-------	191.28	-------	192.51	-------	192.72	-------	204.23
6	5.53, s	99.96	5.53, s	99.40	5.55, d (1.5)	99.81	5.56, d (1.5)	99.69	3.62, dq (21.5, 1.5) 3.52, dq (21.5, 2.0)	38.13
7	-------	175.81	-------	175.61	-------	175.35	-------	175.70	-------	175.28
8	2.47, ddq (6.8, 7.0, 7.0)	39.90	2.47, ddq (6.8, 7.0, 7.0)	39.95	2.47, ddq (6.8, 7.0, 7.0)	40.01	2.47, ddq (6.8, 7.0, 7.0)	39.81	2.47, ddq (6.8, 7.0, 7.0)	39.97
9	1.71, m 1.47, m	26.57	1.71, m 1.47, m	26.95	1.68, m 1.47, m	26.60	1.68, m 1.47, m	26.60	1.68, m 1.47, m	26.60
10	0.94, t (7.5)	11.35	0.94, t (7.5)	11.39	0.94, t (7.5)	11.37	0.94, t (7.5)	11.37	0.93, t (7.5)	11.37
11	1.16, d (7.0)	16.29	1.16, d (7.0)	16.30	1.15, d (7.0)	16.30	1.13, d (7.0)	16.30	1.14, d (7.0)	16.30
12	3.86, s	56.83	3.86, s	57.05	3.78, s	56.65	3.77, s	56.46	3.83, s	55.66
13	1.66, s	24.17[b]	1.66, s	22.34	1.54, s	22.36	1.53, s	22.80	1.51 s	22.10
14	1.62, s	23.30[b]	1.62, s	26.57	1.43, d (7.0)	11.84	1.45, d (7.0)	17.23	1.77, dd (2.0, 1.5)	8.36
OH	2.62, br s	-----	3.44, br s	-----	-----	-----	-----	-----	------	-----

[a]Data recorded at 500 and 125.8 MHz, respectively. Values in brackets refer to J_{HH} in Hz.

[b]May be interchanged.

exchangeable proton, and a methine proton (Table 1). The δ_H values of both compounds were similar (within 0.01 ppm), except for the exchangeable signal at 2.62 ppm in **1** vs. 3.44 ppm in **2**. The molecular formula ($C_{14}H_{20}O_6$) of each compound, together with ^1H and ^{13}C NMR data indicated that they might be stereoisomers. Initially, the ^{13}C NMR data (14 carbons) seemed to be in contradiction with the number of oxygens indicated by the molecular formula. The apparent presence of two ketone C=O (203 and 192 ppm), two ester C=O (175 and 173 ppm), an OMe (60 ppm), and two quaternary carbons bound to oxygen (Table 1) could not be accommodated by six oxygens. Of the remaining seven carbons, four were assignable to a *sec*-butyl group, two to methyl groups, and one to an sp^2 methine.

For the elucidation of structures **1** and **2** analysis of the HMBC[9] and HMQC spectra of each compound and of the methyl derivative **1a** was crucial. The key correlations are summarized in Figure 1. For compound **1**, the carbon at δ_C 202.60 (C-3) showed correlations with the methyl groups at δ_H 1.66 (H_3-13) and 1.62 (H_3-14). The latter methyl group (δ_H 1.62) was attached to a carbon at δ_C 73.55 (C-2) and showed long range coupling with a carbon at δ_C 172.93 (C-1). The latter carbon (C-1) showed further correlations with a OMe group at δ_H 3.86 (H_3-12) and a methine proton at δ_H 5.53.The methine proton (H-6) was attached to a carbon at δ_C 99.96 (C-6) and displayed further correlations with carbons at δ_C 191.58 (C-5) and 81.12 (C-4) ppm. The latter carbon (C-4) was attached to the methyl group at δ_H 1.66 (H_3-13). Thus the assignment of an enol ether instead of an ester for the carbon at δ_C 172.93 (C-1) reconciled the HRMS and ^{13}C NMR data. In this way the cyclohexenedione structure in Figure 1 was deduced.

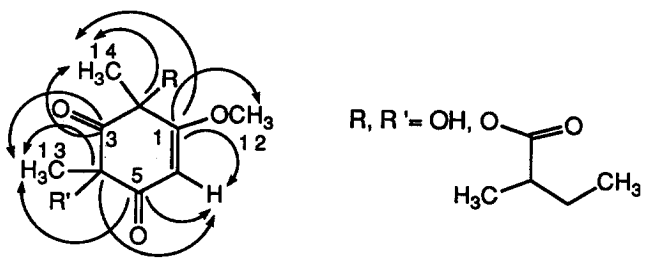

Figure 1. Selected HMBC Correlations for Phomaligols A (**1**) and A_1 (**2**).

At this point two substituents (OH and *sec*-butyl groups) remained to be assigned to quaternary carbons at δ_C 73.55 (C-2) and δ_C 81.12 (C-4). This problem was solved by analysis of the HMBC spectrum of the methyl ether derivative **1a**. In this derivative C-4 had a chemical shift (δ_C 81.73) similar to that in the parent compound **1** (δ_C 81.12), whereas C-2 was shifted to lower field relative to **1** (δ_C 78.34 vs. 73.55) and was correlated with the newly introduced methyl at δ_H 3.23. This correlation indicated that the OH group in **1** is bound to C-2 and allowed the unambiguous assignment of both structures **1** and **2**. The relative configurations of the C-2 and C-4 stereogenic centers of phomaligols could be assigned on the basis of NOE data. For compounds **1** and **2** no NOE enhancement was observed on irradiation of either one of the methyl groups H-13 or H-14. However, unlike compounds **1** and **2**, an NOE enhancement was observed for the C-2 MeO group (δ_H 3.23) of **1a** on irradiation of either one of the methyl groups (H-13 or H-14) and vice-versa (i.e. NOE enhancement for both methyl groups on irradiation at δ_H 3.23). Furthermore, when the C-1 MeO group at δ_H 3.83 was irradiated an NOE enhancement was observed for the methine proton at δ_H 5.59 and vice-versa. Based on these results the relative configuration of compound **1** is assigned as *trans* (Figure 2).[10]

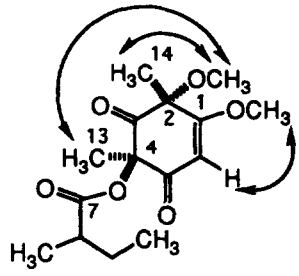

Figure 2. Selected NOE Data for Compound **1a**.

While TLC and HPLC of phomaligadiones (**3a/3b** and **4**) indicated a homogeneous material, the ^1H NMR spectrum (Table 1) was rather complex and indicated the presence of three structurally related components. Further purification attempts did not afford a single compound. However, detailed analysis of the ^1H and ^{13}C NMR spectra (Table 1) and proton decoupling experiments revealed that the components of that inseparable mixture were the epimers **3a** (major) and **3b** (minor) and the tautomer **4** (ca. 3:1:2). Diagnostic features of the ^1H NMR spectrum of the mixture included, for the major compound **3a**, a methyl group at δ_H 1.15 coupled to a methine proton at δ_H 3.40, which in turn coupled to another methine proton at δ_H 5.55, (identical spin system present in the minor compound **3b**, δ_H within 0.02), and, for compound **4**, a methylene group showing long range coupling to a methyl group at δ_H 1.78. Similar to phomaligols, each one of the three compounds contained a *sec*-butyl group, and a methyl group (singlet); unlike phomaligols, no readily exchangeable protons could be detected. Additionally, CIMS and EIMS suggested a molecular formula of $C_{14}H_{20}O_5$ for phomaligadiones (i.e. one oxygen less than the phomaligols). The ^{13}C NMR spectrum (proton decoupled, Table 1) of phomaligadiones A and B was complex, revealing different resonances for almost all (except the *sec*-butyl group) of the carbons of each compound. Nevertheless, HMQC and HMBC[9] spectra allowed the assignment of the carbon resonances for the three constituents **3a**, **3b**, and **4** of the mixture.[11] Compound **3a** was also the major component of the crude EtOAc extract. As expected, the protons at C-2 and C-6 in phomaligadiones were exchanged on treatment with deuterated methanol. The deuterium exchange was clearly demonstrated in the proton decoupled ^{13}C NMR spectrum: in **3a** and **3b** C-2 (44.92 and 46.17 ppm, respectively) and C-6 (99.81 and 99.69 ppm, respectively) became triplets, whereas in **4** only C-6 (38.13 ppm) changed multiplicity. Therefore it was unambiguously established that structures **3a/3b** and **4** represented the major tautomeric forms of phomaligadione.[12]

Phomaligols and phomaligadiones were present in broth extracts of other weakly virulent isolates of *P. lingam* grown in potato dextrose medium.[13] Our previous studies of weakly virulent isolates grown in minimal medium did not reveal characteristic metabolites.[6] However, this is not unusual as fungi may produce secondary metabolites only in very specific conditions. In contrast, virulent isolates of *P. lingam* produced their characteristic epipolythiodioxopiperazines in four different media, including potato dextrose medium.[14]

Biogenetically, phomaligols and phomaligadiones may derive from a pentaketide which is further methylated and oxidized. These metabolites are structurally unrelated with the epipolythiodioxopiperazines toxins synthesized by virulent isolates of the blackleg fungus.[15] The isolation of these new polyketides from a weakly virulent isolate of *P. lingam* supports the view that virulent and weakly virulent strains are in fact different species.[16] In order to substantiate this observation other weakly virulent isolates are presently being investigated.

EXPERIMENTAL

General Procedures

Fungal isolates were obtained from G. A. Petrie and R. K. Gugel, Agriculture Canada Research Station, Saskatoon, Saskatchewan. All chemicals were purchased from Aldrich Chemical Company, Inc., Madison, WI. Potato dextrose medium was purchased from Sigma Chemical Company, St. Louis, Missouri. All solvents were HPLC grade and used as such. Preparative TLC: (Merck, Kieselgel 60 F_{254}), 20 x 20 cm x 0.25 mm; analytical TLC (Merck, Kieselgel 60 F_{254}, aluminum sheets) 5 x 2 cm x 0.2 mm; compounds were visualized by exposure to UV and by dipping the plates in a 5% aqueous (w/v) phosphomolybdic acid solution containing a trace of ceric sulfate and 4% (v/v) H_2SO_4, followed by heating at 200° C. Flash column chromatography: Merck silica gel, grade 60, mesh size 230-400, 60 Å. NMR spectra were recorded on a Bruker AMX 500 spectrometer; for ^1H (500 MHz), δ values were referenced to $CHCl_3$ (7.24 ppm) and for ^{13}C (125.8 MHz) referenced to $CDCl_3$ (77.0 ppm). Mass spectra (MS) were obtained on a VG 70-250 SEQ hybrid mass spectrometer or on a Finnigan Mat Model 4500 mass spectrometer [high resolution (HR), electron impact (EI), fast atom bombardment (FAB), or chemical ionization (CI) with ammonia as carrier gas], employing a solids probe in both cases. Ultraviolet (UV) spectra were obtained on a Beckman DU®-65 spectrophotometer. Fourier transform infrared (FTIR) spectra were obtained on a Bio-Rad FTS-40 spectrometer using diffuse reflectance cell.

Fungal Cultures, Extraction, and Isolation of Metabolites

Phoma lingam, isolate NB-2 (Northbattleford-2, weakly virulent strain) was grown in Cove's liquid media[7] (containing urea, 750mg/L, glucose, 10 g/L, and 0.05% yeast extract) in shake culture (3 L), for five to seven days. After filtration (Whatman no. 2), the culture broth was freeze-dried, diluted with water (400 mL) and extracted with EtOAc (4x200 mL). The dried (Na_2SO_4) EtOAc extract was evaporated to dryness (180 mg), dissolved in hexane-EtOAc, (3:2), and subjected to flash column chromatography[8] (hexane-EtOAc, 3:2). The least polar fraction was further purified by preparative TLC (CH_2Cl_2-CH_3OH, 97:3, developed twice) to give an inseparable mixture (3.5 mg/L) of phomaligadiones A (**3a/3b**) and B (**4**), R_f 0.60, hexane-EtOAc (1:1). From the next fraction, after further fractionation by preparative TLC (hexane-EtOAc, 1:1, developed twice) phomaligols A (**1**, 2.5 mg/L), R_f 0.42, hexane-EtOAc (1:1), and A_1 (**2**, 1.0 mg/L), R_f 0.28, hexane-EtOAc (1:1) were obtained.

Phomaligol A (1). Colorless oil; $[\alpha]_D$ = -79° (*c* 1.13, $CHCl_3$, 24° C); HRMS-FAB m/z obsd. 285.1341 ($[M+1]^+$), calcd. for $C_{14}H_{21}O_6$ 285.1338; CIMS (NH_3) m/z (relative intensity) 302 ($[M+18]^+$ 74%), 285 ($[M+1]^+$ 100%); λ_{max} 250 (log ε 3.04);FTIR:υ_{max} 1730, 1677, 1614, 1457, 1371, 1246, 1140, 1118, 1012, 987 cm^{-1}.

Phomaligol A_1 (2). Colorless oil; $[\alpha]_D$ = -32° (*c* 0.54, $CHCl_3$, 24° C); HRMS-FAB m/z obsd. 285.1334 ($[M+1]^+$), calcd. for $C_{14}H_{21}O_6$ 285.1338; CIMS (NH_3) m/z (relative intensity) 302 ($[M+18]^+$ 75%), 285 ($[M+1]^+$ 100%); λ_{max} 250 (log ε 3.25);FTIR:υ_{max} 1736, 1673, 1612, 1454, 1375, 1244, 1139 1001 cm^{-1}.

Methyl Phomaligol A (1a). Compound **1a** was obtained by treatment of a MeI (2mL) solution of **1** (3.2 mg) with NaH (washed with hexane, ca. 10 mg), at 24° C. After five days the reaction mixture was diluted with EtOAc, filtered (cotton plug), and the solvent evaporated. Preparative TLC (hexane-EtOAc, 3:2) afforded compound **1a**. R_f 0.60, hexane-EtOAc (1:1). ^1H NMR ($CDCl_3$) δ: 5.59 (s, H-6), 3.83 (s, H-12), 3.23 (s, OMe), 2.47 (ddq, J = 6.8, 7.0, 7.0 Hz, H-8), 1.69 (m, H-9), 1.64 (s, H-13), 1.58 (s, H-14), 1.47 (m, H-9), 1.16 (d, J = 7.0 Hz, H-11), 0.94 (t, J = 9.5 Hz, H-10). ^{13}C NMR ($CDCl_3$) δ: 199.24 (C-3), 191.68 (C-5), 175.61 (C-7), 172.87 (C-1), 101.55 (C-6), 81.73 (C-4), 78.34 (C-2), 56.82 (C-12), 52.25 (OMe), 39.98 (C-8), 26.59 (C-9), 22.55 (C-13), 17.47 (C-14), 16.36 (C-11), 11.39 (C-10). CIMS (NH_3) m/z (relative intensity) 316 ($[M+18]^+$ 20%), 299 ($[M+1]^+$ 100%).

Phomaligadiones A (3a/3b) and B (4). Pale yelow oil; CIMS (NH_3) m/z (relative intensity) 286 ($[M+18]^+$ 93%), 269 ($[M+1]^+$ 100%). EIMS m/z (relative intensity) 268 ($[M]^+$, 1%), 184 ($[M-84]^+$, 26%); λ_{max} 250 (log ε 3.15), 360 (log ε 2.76).

ACKNOWLEDGEMENTS

We would like to thank Dr. G. A. Petrie and Mr. R. K. Gugel, Agriculture Canada Research Station, Saskatoon, Saskatchewan, for kindly providing isolates of *P. lingam*; Prof. D. E. Ward, University of Saskatchewan, for helpful discussions; Mr. B. Chatson, Mr. L. Hogge and Mr. D. Olson, Plant Biotechnology Institute, Saskatoon, for NMR and MS spectral determinations. N.R.C.C. no 36492.

REFERENCES AND NOTES

1. For a recent review on blackleg disease see, for example, Gugel, R. K.; Petrie, G. A. *Can. J. Plant Pathol.*, **1992**, 14, 36-45.
2. 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, 67-75.
3. Examination[6] of several of these isolates (grown in minimal medium) revealed only a group of lipids common to most living organisms: triacyl- and diacylglycerols, C_{16} and C_{18} fatty acids, and ergosterol.
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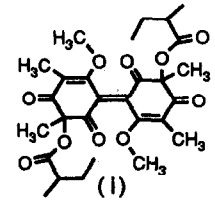
10. The methyl derivative of phomaligol A_1 (**2**, R=OMe, R'=Me) was also prepared; however the chemical shifts of the methyl groups H_3-13 and H_3-14 did not allow the unambiguous assignment of each resonance. ^1H NMR ($CDCl_3$) δ: 5.73 (s, H-6), 3.88 (s, H-12), 3.16 (s, OMe), 2.49 (ddq, J = 6.8, 7.0, 7.0 Hz, H-8), 1.74 (m, H-9), 1.60 (s, H-13/H-14), 1.59 (s, H-14/H-13), 1.50 (m, H-9), 1.18 (d, J = 7.0 Hz, H-11), 0.95 (t, J = 9.5 Hz, H-10). CIMS (NH_3) m/z (relative intensity) 316 ($[M+18]^+$ 15%), 299 ($[M+1]^+$ 100%).
11. Treatment of the phomaligadione mixture (9 mg) with MeI/NaH (26 h, 24° C), as described for **1a**, afforded compound (**i**) (1.2 mg) and several undetermined products. The structure of (**i**) was determined by analysis of the HMBC and HMQC spectra together with HREIMS (m/z obsd. 532.2328, calcd for $C_{28}H_{36}O_{10}$ 532.2308). Compound (**i**) appears to result from oxidative coupling of **3a/3b/4**. This reaction is being investigated.



(I)

12. As determined by ^1H NMR, the initial ratio of ca. 3:1:2 (**3a**:**3b**:**4**) over a 30-day period at 24° C ($CDCl_3$) changed to ca. 3:2:1. Slight decomposition of the mixture was indicated by the presence of several minor peaks in the ^1H NMR spectrum, and by the colour change of the solution (yellowish to reddish brown).
13. Twenty nine weakly virulent isolates of *P. lingam* were grown in liquid potato dextrose medium. Similarly to the isolate NB-2, fourteen of these isolates produced phomaligols, phomaligadiones, and yellow pigments. The structures of these yellow metabolites are being investigated.
14. Pedras, M. S. C., to be published elsewhere.
15. The broth extract from which these metabolites were isolated showed toxicity on a cotyledon bioassay[6] to *B. napus* and *B. juncea*. The phytotoxicity of the metabolites will be evaluated in due course.
16. See, for example, Taylor, J. L.; Borgmann, I; Séguin-Swartz, G. *Curr. Genet.*, **1991**, 19, 273-277.