Phytochemistry, Vol. 29, No. 3, pp. 777–782, 1990.
Printed in Great Britain.

0031–9422/90 $3.00+0.00

MINOR PHYTOTOXINS FROM THE BLACKLEG FUNGUS *PHOMA LINGAM*

M. SOLEDADE C. PEDRAS,* GINETTE SÉGUIN-SWARTZ† and SUZANNE R. ABRAMS

Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Sask. S7N OW9, Canada; †Agriculture Canada Research Station, Saskatoon, Sask. S7N OX2, Canada

(*Received in revised form* 26 July 1989)

Key Word Index—*Phoma lingam*; *Leptosphaeria maculans*; blackleg fungus; phytotoxins; epipolythiodioxopiperazines; sirodesmins; phomalirazine; 3-(methylthio)phomamide.

Abstract—The isolation, structure determination and phytotoxicity of metabolites produced by a virulent isolate of the blackleg fungus *Phoma lingam* are reported. The metabolite profiles of several fungal isolates are compared and the biogenetic significance of those metabolites containing an epipolythiodioxopiperazine group is discussed.

INTRODUCTION

The fungus *Phoma lingam* (Tode ex Fr.) Desm., the asexual stage of *Leptosphaeria maculans* (Desm.) Ces. et de Not. causes blackleg in rapeseed (*Brassica napus* and *B. campestris*) and other crucifers. Blackleg affects crops in various parts of the world. A virulent strain of the fungus has caused significant crop losses in recent years in Canada [1]. Three strains of the pathogen have been characterized on the basis of virulence, host range, and cultural tests: (i) a highly virulent strain causing leaf spots and severe stem cankers on rapeseed and cabbage (*B. oleracea*); (ii) a weakly virulent strain causing superficial leaf and stem lesions on rapeseed and cabbage; (iii) a strain causing disease only on stinkweed (*Thlaspi arvense*) [1, 2].

Considering the wide variability in virulence, it was of interest to examine the metabolite profiles of various strains of *P. lingam*, particularly with regard to the phytotoxic substances produced. A systematic screening for phytotoxins produced by different isolates and srains of *P. lingam* was initiated. The immediate goal was to use possible phytotoxins in a plant breeding programme aimed at screening/selecting for blackleg resistance [3]. In subsequent studies phytotoxins could be used to study plant–pathogen interactions at the molecular level [4], and also could be used to differentiate virulent and non-virulent isolates of *P. lingam* [5].

Phoma lingam produces a major phytotoxin, sirodesmin PL (**1**), and the corresponding deacetyl derivative (**5**) [6]. Our studies on active metabolites produced in liquid culture by a virulent isolate of *P. lingam* have led to the isolation of two novel phytotoxins, sirodesmin H (**4**) [7] and phomalirazine (**6**) [8].

Now we wish to report, for the first time, the isolation and identification of two additional phytotoxins, sirodesmins J (**2**) and K (**3**) and a very intriguing metabolite, 3-(methylthio)phomamide (**8**). In addition we describe the metabolite profile of other isolates of *P. lingam* and discuss their possible biogenetic significance.

RESULTS AND DISCUSSION

Phoma lingam isolate Leroy (highly virulent strain, Table 1) was grown in still culture for three weeks. Broth and mycelium were separated and extracted with different solvents. The crude organic extracts and the extracted aqueous broth were tested on punctured cotyledons or leaves of rapeseed (susceptible host) and mustard (*B. juncea*, non-host) seedlings. Host and non-host plants were used in every bioassay to assure toxin detection and to differentiate specific toxins from non-specific ones. The mycelial extracts had no effect on the seedlings of either species. Only the broth extracts caused cotyledon or leaf lesions on both plant species, similar to those caused by the pathogen. In addition a 0.05% solution ($MeOH–H_2O$, 1:1) of the broth ethyl acetate extract caused larger lesions than any other solvent extract of the broth at equivalent concentration. For this reason ethyl acetate was used in subsequent extractions.

The isolation of phytotoxins was guided by the above mentioned cotyledon or leaf puncture assay. Flash column chromatography [9] of the toxic ethyl acetate extract gave active fractions containing the following components, in order of increasing polarity: sirodesmin

Table 1. *Phoma lingam*: isolates, strain, and host range

Isolate	Strain	Host
Leroy	Highly virulent	Rapeseed
ENG 88	Highly virulent	Rapeseed
FRA 88	Highly virulent	Rapeseed
Unity	Weakly virulent	Rapeseed
Thl SASK 1	Thlaspi	Stinkweed
Thl SASK 2	Thlaspi	Stinkweed
Thl SASK 3	Thlaspi	Stinkweed
Thl SASK 4	Thlaspi	Stinkweed

*Author to whom correspondence should be addressed.
N.R.C.C. No. 30713.

PL (**1**), sirodesmin J (**2**), sirodesmin K (**3**), sirodesmin H (**4**), deacetylsirodesmin PL (**5**), and phomalirazine (**6**). The two most polar fractions contained phomamide (**7**) and the structurally related metabolite (3-methylthio)phomamide (**8**). Compounds **2**, **3** and **8** are now reported for the first time.

Sirodesmin PL (**1**), the major component of the toxic extract (50–70%, w/w), was obtained after repeated crystallization of the least polar chromatographic fraction. It was identified by comparison of physical and spectroscopic data (^{1}H NMR in Table 2) with literature values [6, 10]. Deacetylsirodesmin PL (**5**) and sirodesmin H (**4**) were obtained in the same fraction together with minor components. Further fractionation by flash column chromatography, followed by prep. TLC yielded **4** and **5**. Compound **5** was readily identified as deacetylsirodesmin PL by comparison of its ^{1}H NMR spectrum with that of **1**. Direct comparison of physical and spectroscpic data of **5** and the methanolysis product of **1** confirmed the identity of the sample. Compound **4** was identified as a dethiosirodesmin. The assignment of its structure was based on spectroscopic data (^{1}H NMR in Table 2) and confirmed by desulphurization of sirodesmin PL (**1**) with triphenylphosphine [7]. Sirodesmin H (**4**) has a unique structure, it is the first reported naturally occurring monosulphur bridged dioxopiperazine.

Sirodesmins J (**2**) and K (**3**) were obtained as a mixture; neither preparative TLC (CH_2Cl_2–MeOH) nor reverse phase HPLC (H_2O–MeOH) afforded chromatographically homogeneous material. The ^{1}H NMR spectrum showed signals attributable to a mixture of at least three compounds related to sirodesmin PL. Separation of this material was finally achieved by normal phase HPLC (CH_2Cl_2–*iso*-PrOH). Later it became obvious that the use of methanol in the separation process was promoting the conversion of each one of **2** and **3** into a mixture of di- (**1**), tri- (**2**) and tetrasulphur (**3**) compounds. The ^{1}H NMR spectrum of **3** (Table 2) showed spin systems similar to **1** which accounted for 26 hydrogens. The ^{1}H NMR of **2** (Table 2) showed doubled signals for each spin system, although TLC and HPLC analysis indicated a single compound. The CI mass spectrum of both compounds showed the highest mass peak at *m/z* 519 ($C_{20}H_{26}N_2O_8S_3+1$) which suggested that they were structural isomers. It appeared likely that one of those isomers might differ from sirodesmin PL (**1**) ($C_{20}H_{26}N_2O_8S_2$) only in the size of the sulphur bridge and thus could be prepared from **1**. The sulphurization of compounds containing an epidithiodioxopiperazine group gives tri- and tetrasulphur bridged isomers [11]. Treatment of sirodesmin PL (**1**) with sulphur in pyridine [10] afforded a mixture of two products which had

Table 2. ^{1}H NMR chemical shifts (multiplicity, coupling constant in Hz) of compounds **1**–**4**

H	1	2*	3	4
7	5.53	5.33 and 5.11	5.39	5.29
	(*s*)	(*s*) (*s*)	(*s*)	(*s*)
13	4.31	4.53 and 4.34	4.61	3.91
	(*m*)	(*m*) (*m*)	(*t*, 9.3)	(*dd*, 5.7, 8.5)
14	4.29	4.55 and 3.75	4.26	4.30
	(*m*)	(*d*†, 12.9) (*d*†, 12.9)	(*dd*, 8.0, 11.9)	(*m*)
		4.20 and 3.98	4.06	
		(*d*†, 12.7) (*d*†, 12.7)	(*dd*, 6.4, 11.9)	
11	3.92	3.93	3.91	3.87
	(*q*, 6.3)	(*q*, 6.4)	(*q*, 6.3)	(*q*, 6.3)
5	3.24	3.34 and 3.24	3.43	2.95
	(*s*)	(*d*, 15.9) (*d*, 15.9)	(*d*, 15.8)	(*d*, 15.6)
		3.08 and 2.91	2.76	2.80
		(*d*, 16.0) (*d*, 16.0)	(*d*, 15.8)	(*d*, 15.6)
15	3.13	3.23 and 3.05	3.07	3.06
	(*s*)	(*s*) (*s*)	(*s*)	(*s*)
12	2.74	2.86 and 2.11	2.68	2.53
	(*dd*, 8.8, 14.1)	(*dd*, 9.0, 14.4) (*dd*, 8.6, 14.4)	(*dd*, 9.3, 13.9)	(*dd*, 8.5, 14.8)
	1.72	2.76 and 1.99	1.71	2.13
	(*dd*, 8.2, 14.1)	(*dd*, 9.3, 14.2) (*dd*, 8.1, 14.2)	(*dd*, 9.3, 13.9)	(*dd*, 5.7, 14.8)
18	1.24	1.25 and 1.23	1.22	1.22
	(*d*, 6.3)	(*d*, 6.4) (*d*, 6.4)	(*d*, 6.3)	(*d*, 6.3)
16/17	1.09/1.01	1.10/1.08/0.99	1.09/0.99	1.05/0.98
	(*s*) (*s*)	(*s*) (*s*) (*s*)	(*s*) (*s*)	(*s*) (*s*)
Ac	2.08	2.05 and 2.06	2.03	2.07
	(*s*)	(*s*) (*s*)	(*s*)	(*s*)
OH	4.18	4.18 and 3.90	4.03	4.21
	(*s*)	(*s*) (*s*)	(*s*)	(*s*)
OH	3.38	2.92 and 2.66	3.25	3.02
	(*m*)	(*m*) (*m*)	(*m*)	(*dd*, 5.7, 7.8)

*See text for explanation.
†After D_2O addition.

chromatographic properties (TLC and HPLC) identical with **2** and **3**. In addition the ^{1}H NMR spectrum of each product was identical with **2** and **3**, respectively. Therefore, one of the compounds isolated from the broth extract had to have a tetrasulphur bridge and the other one a trisulphur bridge. The presence of a tetrasulphur bridge in **3** was confirmed later by CI mass spectrometry using a probe tip made of fused-silica (capillary column material) and *iso*-butane as reagent gas. Employing this technique a weak $[M]^+$ at *m/z* 551 ($[M+1]^+$) was obtained for compound **3**, whereas compound **2** showed a $[M]^+$ at *m/z* 519 ($[M+1]^+$). The doubled signals observed in the ^{1}H and ^{13}C NMR spectra of **2** are explained by two stable conformations which are due to the presence of a trisulphur bridge [10, 12, 13].

The most polar active fraction, after repeated crystallization, gave phomalirazine (**6**) which has already been the subject of a preliminary communication [8]. Compound **6** possesses a novel ring system and a noteworthy structural feature is that one of the nitrogen atoms of the epidithiodioxopiperazine ring is not alkylated. The structure of **6** was assigned from spectroscopic data and biogenetic consideration, and confirmed by X-ray analysis.

The cyclic dipeptide phomamide (**7**) and the structurally related compound **8** were isolated from fractions which showed no biological activity. Compound **7** was purified by preparative TLC and crystallized from ethyl acetate. Comparison of spectroscopic data (CI mass spectrum, ^{1}H and ^{13}C NMR) with literature values [14], and high resolution FAB mass spectrometry of the monoacetyl derivative **9**, confirmed the identity of the sample.

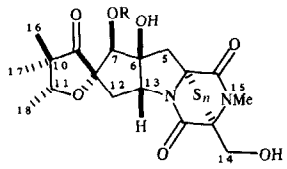
3-(methylthio)Phomamide (**8**) was obtained in pure form in small quantities after selective precipitation from a solution of methanol–methylene chloride. Any attempt to recover the compound from the mother liquors by prep. TLC led to decomposition. The structure that we propose is consistent with the ^{1}H NMR spectrum [obtained in $CDCl_3$ and in $(CD_3)_2CO$], high resolution FAB and CI mass spectrometry. Thus, high resolution FAB indicated that 3-(methylthio)phomamide (**8**) had a molecular formula of $C_{18}H_{24}O_4N_2S$. CI (solid probe, *iso*-butane) gave a $[M]^+$ at *m/z* 365 ($[M+1]^+$) and two major fragment ions which resulted from loss of MeSH (base peak) and loss of C_5H_8, respectively. In agreement with the molecular formula, the ^{1}H NMR spectrum showed 24 hydrogens, three of which were D_2O-exchangeable. The chemical shifts of the dimethylallyl ether group (nine hydrogens), and of the *para*-disubstituted phenyl group (four hydrogens) were virtually identical with the corresponding hydrogens of acetylphomamide (**9**) (both spectra obtained in $CDCl_3$). The remaining hydrogen signals were typical of these cyclic dipeptides. Thus, the benzylic methylene group showed an AB system with the usual $^2J_{HH}$ (13.8 Hz) and no further coupling. The hydroxymethyl group, after D_2O exchange, showed an ABX system ($J_{7A,6}=5.0$, $J_{7B,6}=6.4$, $J_{7A,7B}=11$ Hz), similar to the corresponding hydrogens of phomamide (**7**). Evidence for the presence of the thiomethyl group was provided by the sharp singlet at δ2.20 in the ^{1}H NMR spectrum, and by the fragmentation of the $[M]^+$ in the CI mass spectrum. The stereochemistry of 3-(methylthio)phomamide was assigned by analogy with that of its co-metabolites. Compound **8**, to the best of our knowledge, has a new structure and possibly carries an interesting biogenetic significance.

Following the isolation and identification of phytotoxic metabolites from the highly virulent isolate Leroy, we screened the isolates of *P. lingam* listed in Table 1. All the isolates were grown in conditions similar to the isolate Leroy. Likewise, the broth and the mycelium of each isolate were separated, extracted and tested on cotyledons of rapeseed and mustard seedlings.

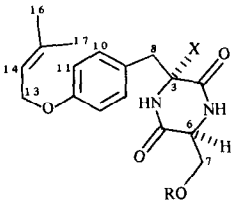
Similarly to the isolate Leroy, the virulent isolates ENG 88 and FRA 88 (Table 1), produced phytotoxic metabolites. These metabolites could only be detected in the broth extracts (TLC and HPLC). The major components could be readily identified in the ^{1}H NMR spectra of the corresponding extracts. Thus, the major component of the isolate FRA 88 was deacetylsirodesmin PL (**5**), followed by sirodesmin PL (**1**) and phomamide (**7**) (3:2:2, molar ratio). The major component of the isolate ENG 88 was **1**, followed by **7** (5:1, molar ratio). In addition sirodesmins H (**4**), J (**2**) and K (**3**) were also detected in both broth extracts. No acetylated derivatives of **1** (e.g. compounds **10–12**) were detected in any of the extracts. Phomalirazine (**6**) was not detected in any of the extracts, but this may reflect its low concentration. In isolate Leroy detection of phomalirazine in the crude extract was possible only after fractionation.

Although the extracts of the weakly virulent isolate Unity (Table 1) showed no biological activity, phytotoxic components could be present in amounts undetectable either by TLC or HPLC. To determine whether this weakly virulent isolate produced phytotoxins, the broth extract was fractionated by flash chromatography (as described for isolate Leroy) and the fractions were analysed. None of the previously isolated phytotoxins could be detected either by TLC or HPLC. The major component of the extract was a mixture of fatty acids (mainly oleic acid) which was characterized by ^{1}H NMR and GC-MS.

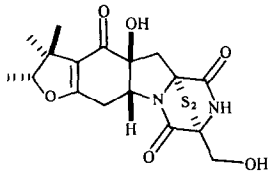
Next we screened four isolates of the Thlaspi strain which causes disease only on stinkweed (Table 1). The



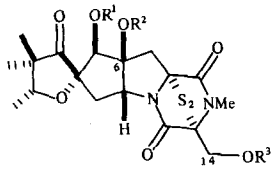
1 R = Ac $n=2$
2 R = Ac $n=3$
3 R = Ac $n=4$
4 R = Ac $n=1$
5 R = H $n=2$



7 R = X = H
8 R = H, X = SMe
9 R = Ac, X = H



6



10 R^1 = R^2 = Ac, R^3 = H
11 R^1 = R^3 = Ac, R^2 = H
12 R^1 = R^2 = R^3 = Ac

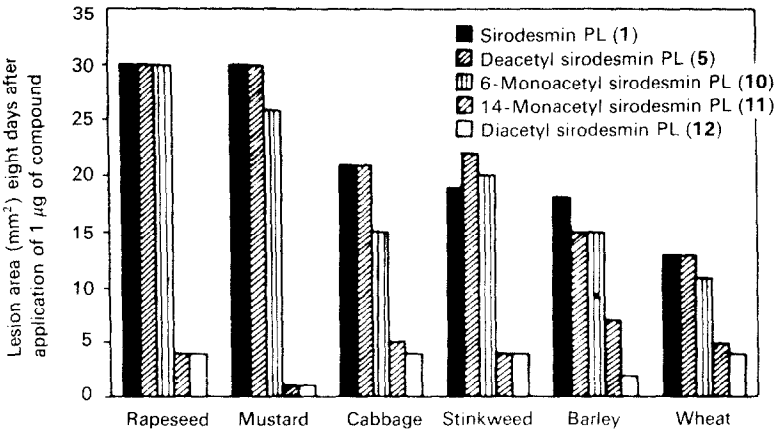


Fig. 1 Phytotoxicity data for compounds **1**, **5**, **10**–**12**. The values are the means of three independent experiments. For each compound tested there were three replicate pots with five seedlings per pot and per genotype.

extracts of the four isolates, besides being tested on rapeseed and mustard, were also tested on stinkweed. Surprisingly, no phytotoxic activity was detected in those extracts. As in the case of the weakly virulent isolate Unity, no indication of phytotoxic metabolite production was found.

Difficulties encountered during the isolation of **2** and **3** indicated that they were relatively unstable. When each one of those compounds was dissolved in methanol there was over 50% decomposition in less than 24 hr. The major products resulted from interconversion and consisted in each case of a mixture of **1**, **2** and **3**. Thus, **2** and **3** were tested on cotyledons or leaves as a mixture and therefore the minimal active concentration could not be established for each compound. Likewise, the lability of the monosulphur bridge present in sirodesmin H (**4**) [7] prevents a reliable determination of its active concentration. However, it has been reported that sporidesmin E and dehydrothiogliotoxin (epitrithiodioxopiperazines) are more active than the corresponding mono-, di-, and tetrathio compounds [15]. Sirodesmin PL is stable under identical conditions.

We have studied the effect of acetylation on the biological activity of sirodesmin PL (**1**). Three acetylated derivatives, **10**–**12** were prepared and tested on cotelydons of rapeseed, mustard, cabbage, and stinkweed, and leaves of barley (*Hordeum vulgare*), and wheat (*Triticum aestivum*). The phytotoxicity data are shown in Fig. 1. The size of the lesions caused by sirodesmin PL (**1**), deacetylsirodesmin PL (**5**), and 6-monoacetylsirodesmin PL (**10**) was similar (at *ca* 200 μM). Diacetylsirodesmin PL (**12**) and 14-monoacetylsirodesmin PL (**11**) caused comparable lesions but at 10 times higher concentration. The difference in the activity of these derivatives suggests that acetylation of **1** at the 14-OH (**11** and **12**) considerably reduces the phytotoxicity of the molecule, whereas the phytotoxicity is not considerably affected by acetylation at the 6-OH (**10**). Thus, the 14-OH may be involved in the mechanism of action of this toxin.

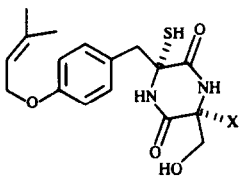
*Biological studies to determine the potential use of those phytotoxins are underway.

Considering that isolates Leroy, ENG 88, and FRA 88 are virulent, it is not surprising to find that they produce identical phytotoxic metabolites. There are quantitative differences, but qualitative similarities suggest that other virulent isolates of *P. lingam* will produce similar phytotoxins. In this regard a simple and useful method for helping to differentiate strains of *P. lingam* [5] could be developed.* On the other hand, the four isolates of the Thlaspi strain did not produce phytotoxic metabolites. This may be a genetic characteristic of these isolates or may be due to the culture conditions.

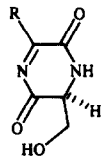
The sirodesmins contain an epipolythiodioxopiperazine ring which is also present in other biologically active metabolites produced by diverse fungal species [15, 16]. Interestingly, sirodesmin PL (**1**) is a minor metabolite of the fungus *Sirodesmium diversum*, whereas its C-8 epimer is a major one [10]. The C-8 epimers of **2**, **3** have also been isolated from *S. diversum* [10]. However, sirodesmin H (**4**) is the first example of a naturally occurring monosulphur bridged compound. Dioxopiperazines having a disulphur bridge are the most widely distributed [16].

The biosynthesis of epipolythiodioxopiperazines in general and of sirodesmins in particular, represents a challenging problem [17]. While the primary precursors are known to be cyclodipeptides (e.g. **7** [18]), the pathway and intermediates leading to the final products are not understood. For example, it is not known at which stages *N*-methylation and oxidative cyclization of the dioxopiperazine onto the phenyl ring occur. Also, the sulphur-acceptor intermediate is not known, although proposals have been advanced and experimental work has been done [17]. The isolation of metabolites **1**–**4** and **6**–**8** from the same fungus provides clues which allow a better understanding of the biosynthesis of the epipolythiodioxopiperazines.

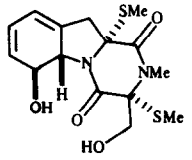
The novel methylthio derivative **8** may be one of the first-formed sulphur derivatives, the corresponding thiol **13** being perhaps the direct precursor of **8**. This suggests that the biosynthetic pathway may involve stepwise addition of sulphur to a possible dehydrodioxopiperazine (e.g. **14**), previously proposed as a sulphur-acceptor intermediate [17]. The resulting monothiol **13** could either be methylated to give **8**, or accept another thiol group to form dithiol **15**. Naturally occurring dioxopiperazines



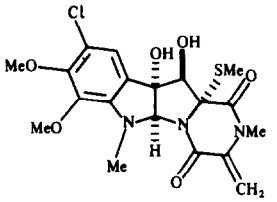
13 X = H
15 X = SH



14



16



17

containing dithiol groups have not been reported, but the corresponding di(methylthio) compounds are well known [16]. These di(methylthio) metabolites may be formed by reduction and methylation of the corresponding epidisulphide, similar to the formation of **16** [19]. Considering that account [19], it seems likely that the only monomethylthio metabolite reported (**17**) [20] may be a degradation product of the corresponding epidisulphide (sporidesmin). However, the monomethylthio compound **8** is the first example of a dioxopiperazine having a monosulphur substituent and no other changes elsewhere in the molecule. Thus, in the pathway to epipolythiodioxopiperazines, in *P. lingam*, the introduction of sulphur can occur immediately following the cyclodipeptide formation, as it has been shown in other fungi [13, 21]. On the other hand inspection of the chemical structure **6** seems to indicate that *N*-methylation of the dioxopiperazine would occur after the oxidative cyclization onto the phenyl ring [8]. Whether the sulphur bridge is formed prior to the oxidative cyclization, or not, is not apparent. Ultimately only incorporation experiments of those plausible precursors will allow definitive conclusions.

EXPERIMENTAL

Mps: uncorr. Prep. TLC: silica gel (Merck, Kieselgel 60 F_{254}), 0.25 mm; spots visualized by exposure to UV and by dipping the plates in a 5% aq (w/v) phospomolybdic acid soln containing a trace of ceric sulphate and 4% (v/v) H_2SO_4, followed by heating at 200°. NMR: for ^1H, δ values referenced to $CHCl_3$ (7.24 ppm) and for ^{13}C referenced to $CDCl_3$ (77.0 ppm).

Phoma lingam isolates and culture. Isolates listed in Table 1 were obtained from G. A. Petrie (Agriculture Canada Research Station, Saskatoon). The fungal isolates were grown in liquid minimal medium supplemented with thiamine as described in ref. [3]. Three weeks after inoculation the liquid cultures were harvested (filtered through cheesecloth), the mycelium frozen and the broth freeze-dried immediately.

Phytotoxin bioassay. Rapeseed, mustard, stinkweed, cabbage, wheat, and barley plants were grown under controlled environmental conditions [3]. Cotyledons of 10-day-old plants, or leaves of 20-day-old plants were punctured with a needle and a 10 μl droplet of the test solute dissolved in 50% aq MeOH was placed on each wound. There were three replicate pots with five seedlings per pot. The phytotoxic activity of culture filtrates, filtrate extracts, fractions and pure substances was always tested on rapeseed and mustard seedlings, except in the cases mentioned in the results and discussion section. Samples of culture filtrates were assayed after filter-sterilization; crude exts were tested at concns of 2, 1, and 0.5 mg/ml. Frs collected from CC and pure compounds were tested at concns of 1, 0.5, 0.4, 02, 0.1, 0.01, and 0.001 mg/ml. The symptoms that developed within 2–10 days varied from little or no reaction to brownish-yellow lesions. In no case were lesions observed with aq MeOH.

Extraction and isolation of toxins. Freeze-dried broth was diluted with H_2O to one-tenth of the initial vol. and extracted (×4) with different solvents (hexane, $CHCl_3$, CH_2Cl_2, Et_2O, EtOAc). EtOAc was the most efficient solvent (*ca* 50 mg of crude ext per l of culture). The extracted broth was acidified to pH 2 with conc HCl and reextracted with Et_2O. The exts were dried (Na_2SO_4) and concd to dryness at *ca* 30°. The broth extracts and the extracted broth were bioassayed. The broth extracts of the virulent isolates (Leroy, ENG 88, and FRA 88) were active, whereas those of the acidified broths were not. The mycelium was extracted with MeOH in a blender. The MeOH extract was concd and partitioned between EtOAc and H_2O. The EtOAc extract was dried (Na_2SO_4) and concd to dryness at *ca* 30°. None of the mycelial extracts showed biological activity.

The EtOAc ext (0.6 g) of the isolate Leroy was fractionated by flash CC [9] eluted with CH_2Cl_2–MeOH (24:1; 2 l) followed by elution with MeOH (200 ml). Eleven frs (200 ml) were collected and bioassayed. The active frs (1–7) contained the following components, in order of increasing polarity: sirodesmin PL (**1**), sirodesmin J (**2**), sirodesmin K (**3**), sirodesmin H (**4**), deacetylsirodesmin PL (**5**), and phomalirazine (**6**). Frs 8–11 showed no activity. Fr. 8 contained 3-(methylthio)phomamide (**8**) and fr. 11 phomamide (**7**).

Sirodesmin PL (**1**, 300 mg) was obtained after repeated crystallization (CH_2Cl_2–hexane) of the first fr. (R_f 0.5; CH_2Cl_2–MeOH, 24:1). A second purification of fr 2 by flash CC (CH_2Cl_2–MeOH, 49:1) gave a fr containing a mixt. (14 mg) of **2**, **3**. Similarly, further sepn of fr. 4 gave a fr containing a mixt. (12 mg) of deacetylsirodesmin PL (**5**, R_f 0.35; CH_2Cl_2–MeOH, 24:1) and sirodesmin H (**4**, R_f 0.37; CH_2Cl_2–MeOH, 24:1). The last two compounds (**4** and **5**) were sepd by prep. TLC (CH_2Cl_2–MeOH, 97:3, two developments and crystallized from (CH_2Cl_2–MeOH, 97:3, two developments) and crystallized from (CH_2Cl_2–hexane). Compounds **2**, **3** were sepd by prep. HPLC, with UV detection at 235 nm, using a Partisil 10 M 9/50 column, eluted with CH_2Cl_2–*iso*-PrOH (24:1) at a flow rate of 5 ml/min. Under these conditions **2** (R_f 0.43; CH_2Cl_2–MeOH, 24:1) elutes at *ca* 8 min and **3** (R_f 0.42) at *ca* 12 min in the same solvent system.

Phomalirazine (**6**, 1 mg) was obtained after selective pptn from $CHCl_3$, followed by repeated crystallization (EtOAc) of fr. 6 (R_f 0.15; $CHCl_2$–MeOH, 19:1). To obtain enough sample for chemical and biological studies, additional crude broth extract (*ca* 6 g) was further processed.

Phomamide (**7**, 10 mg) was recovered from fr. 11 (R_f 0.55; EtOAc–MeOH, 4:1), after additional flash CC (CH_2Cl_2–MeOH, 3:2) and crystallization from EtOAc. Although **7** was present in non-active frs, its identification was necessary to assign the main signals present in the ^1H NMR spectra of crude broth extracts.

As frs 8–11 exhibited no activity they were not analysed initially. Later, it was noticed that the ^1H NMR spectrum of phomalirazine (**6**) recovered from mother-liquors showed a small percentage of an 'impurity' clearly related to phomamide (**7**). Further analysis showed that the 'impurity' was present in fr. 8 (R_f 0.12; CH_2Cl_2–MeOH, 19:1). Equivalent frs obtained from additional crude extract (6 g) were combined; selective pptn from

a soln of CH_2Cl_2–MeOH gave a very small amount of **8**. The ^1H NMR spectrum of **8** showed signals identical with the 'impurity' present in recovered phomalirazine (**6**).

Sirodesmin J (**2**). Powdery material, mp 129–131°. UV $\lambda_{max}^{CHCl_3}$ nm: 277; $[\alpha]_D^{25}$ −323° ($CHCl_3$; *c* 0.13); FTIR λ_{max}^{film} cm^{-1}: 3445, 1743, 1683, 1371, 1231, 1083. ^1H NMR (360 MHz, $CDCl_3$): see Table 2. ^{13}C NMR (90.5 MHz, $CDCl_3$): δ (see text for explanation) 14.24, 17.31, 20.44, 20.49, 27.77, 28.78, 34.52, 35.35, 47.52, 47.55, 49.27, 62.37, 62.56, 67.40, 69.22, 75.52, 75.65, 77.69, 78.82, 79.07, 79.18, 79.39, 80.14, 80.56, 80.75, 88.97, 89.67, 163.13, 164.34, 167.38, 168.09, 169.56, 169.63, 217.32, 218.12. CIMS (methane) 100 eV, *m/z* (rel. int.): 519 $[M+1]^+$ (6), 487 $[M+1-S]^+$ (20), 423 $[M+1-S_3]^+$ (100).

Sirodesmin K (**3**). Powdery material, mp 140–143°. UV $\lambda_{max}^{CHCl_3}$ nm: 289; $[\alpha]_D^{25}$ −253° ($CHCl_3$; *c* 0.11); FTIR λ_{max}^{film} cm^{-1}: 3456, 1744, 1678, 1374, 1230, 1083, 1048. ^1H NMR (360 MHz, $CDCl_3$): see Table 2. ^{13}C NMR (90.5 MHz, $CDCl_3$): δ 14.25 (*q*), 17.33 (*q*), 20.40 (*q*), 20.55 (*q*), 28.92 (*q*), 33.12 (*t*), 47.53 (*s*), 48.71 (*t*), 63.58 (*t*), 69.06 (*d*), 76.05 (*s*), 77.94 (*s*), 78.91 (*d*), 79.91 (*s*), 80.27 (*d*), 89.62 (*s*), 166.94 (*s*), 168.41 (*s*), 169.65 (*s*), 217.50 (*s*). CIMS (*iso*-butane) 100 eV, *m/z* (rel. int.): 551 $[M+1]^+$ (1), 519 $[M+1-S]^+$ (16), 487 $[M+1-S_2]^+$ (92), 423 $[M+1-S_4]^+$ (100).

Preparation of **2** *and* **3**. Sirodesmin PL (**1**, 150 mg) was dissolved in pyridine (23 ml) and rhombic S (34 mg) added. After stirring for 24 hr at room temp pyridine was evapd under red pres. The residue was dissolved in CH_2Cl_2, poured onto a silica column, and excess S eluted with CH_2Cl_2. Further elution with CH_2Cl_2–EtOAc (1:1) gave a fr. containing starting material (*ca* 38 mg) and another fr. containing two products. Prep. HPLC, as described above, gave sirodesmins J (70 mg) and K (25 mg). The yield of sirodesmin K increased when the reaction was allowed to proceed for a longer time.

3-(*methylthio*)*Phomamide* (**8**). ^1H NMR (360 MHz, $CDCl_3$): δ 7.13 (2H, *d*, *J* = 8.6 Hz), 6.84 (2H, *d*, *J* = 8.6 Hz), 5.87 and 5.85 (2H, 2 *br s*, NH), 5.45 (1H, *m*, H-14), 4.47 (2H, *d*, *J* = 6.8 Hz, H-13), 3.78 and 3.71 (2H, 2 *m*, H-7; after D_2O exchange become 2 *dd*, *J* = 5.0, 11 Hz and *J* = 6.4, 11 Hz, respectively), 3.45 and 2.91 (2H, 2 *d*, *J* = 13.8 Hz, H-8), 3.24 (1H, *m*, H-6; after D_2O exchange becomes *dd*, *J* = 5.0, 6.4 Hz), 2.20 (3H, *s*, SMe), 2.18 (1H, *br s*, OH), 1.78 and 1.72 (6H, 2 *br s*, H-16 and H-17). HRFABS $C_{18}H_{25}O_4N_2S$ obsd 365.1523, calcd 365.1535. CIMS (*iso*-butane, solid probe) *m/z* (relative intensity): 365 $[M+1]^+$ (26), 317 $[M+1-MeSH]^+$ (100), 249 $[M+1-MeSH-C_5H_8]^+$ (63).

Acetylphomamide (**9**). ^1H NMR (360 MHz, $CDCl_3$): δ 7.10 (2H, *d*, *J* = 8.5 Hz), 6.87 (2H, *d*, *J* = 8.5 Hz), 6.10 and 5.82 (2H, 2 *br s*, NH), 5.47 (1H, *m*, H-14), 4.48 (2H, *d*, *J* = 6.7 Hz, H-13), 4.40 and 3.76 (2H, 2 *dd*, *J* = 3.2, 11.3 Hz and *J* = 7.0 11.3 Hz, respectively, H-7), 4.18 (2H, *m*, H-3 and H-6), 3.31 and 2.90 (2H, 2 *dd*, *J* = 3.4, 14 Hz and *J* = 9.0, 14 Hz, respectively, H-8), 2.08 (3H, *s*, MeCO), 1.78 and 1.73 (6H, 2 *br s*, H-16 and H-17). HRFABS $C_{19}H_{25}O_5N_2$ obsd 361.1734, calcd 361.1764. CIMS (*iso*-butane, solid probe) *m/z* (relative intensity): 361 $[M+1]^+$ (20), 293 $[M+1-C_5H_8]^+$ (100), 233 $[M+1-C_5H_8-HCO_2Me]^+$ (23).

Sirodesmin PL derivatives (**10**–**12**). Acetylated derivatives of sirodesmin PL (**1**) were prepd to use as standards for screening the fungal exts and for biological testing. Diacetylsirodesmin PL (**12**) and 14-monoacetylsirodesmin PL (**11**) were prepd by acetylation of sirodesmin PL (**1**) with Ac_2O–pyridine, followed by the usual work up. Prep. TLC (CH_2Cl_2–MeOH, 24:1) of the reaction mixt. gave diacetylsirodesmin PL (**12**, R_f 0.65) and 14-monoacetylsirodesmin PL (**11**, R_f 0.63). 6-Monoacetylsirodesmin PL (**10**) was prepd by partial hydrolysis of **12** (30 mg) dissolved in MeOH–36.5% HCl (100:1). After standing for 30 hr at room temp. solvent was evapd and the residue purified by prep. TLC (EtOAc–hexane, 1:1, two developments) to give compound **10** (20 mg, R_f 0.54; CH_2Cl_2–MeOH, 24:1).

Acknowledgements—We thank Dr G. A. Petrie (Agriculture Canada Research Station, Saskatoon) for providing the fungal isolates, Mr M. Mazurek (Plant Biotechnology Institute, Saskatoon) for NMR determinations and Mr L. Hogge and Mr D. Olson (Plant Biotechnology Institute, Saskatoon) for MS determinations.

REFERENCES

1. Petrie, G. A. and Lewis, P. A. (1985) *Can. J. Plant Pathol.* **7**, 253.
2. Mc Gee, D. C. and Petrie, G. A. (1978) *Phytopathology* **68**, 625.
3. De March, G., Séguin-Swartz, G. and Petrie, G. A. (1986) *Can. J. Plant Pathol.* **8**, 422.
4. Durbin, R. D. (ed.) (1981) in *Toxins in Plant Disease*, pp. 16–17. Academic Press, New York.
5. Petrie, G. A. (1988) *Can. J. Plant Pathol.* **10**, 188.
6. Férézou, J. P., Riche, C., Quesneau-Thierry, A., Pascard-Billy, C., Barbier, M., Bousquet, J. F. and Boudart, G. (1977) *Nouv. J. Chim.* **1**, 327.
7. Pedras, M. S. C., Abrams, S. R. and Séguin-Swartz, G. (1988) *Tetrahedron Letters* **29**, 3471.
8. Pedras, M. S. C., Abrams, S. R., Séguin-Swartz, G., Quail, J. W. and Jia, Z. (1989) *J. Am. Chem. Soc.* **111**, 1904.
9. Still, W. C., Kahn, M. and Mitra, A. (1978) *J. Org. Chem.* **43**, 2923.
10. Curtis, P. J., Greatbanks, D., Hesp, B., Cameron, A. F. and Freer, A. A. (1977) *J. Chem. Soc. Perkin Trans. 1* 180.
11. Safe, S. and Taylor, A. (1970) *J. Chem. Soc. (C)* 432.
12. Rahman, R., Safe, S. and Taylor, A. (1969) *J. Chem. Soc. (C)* 1665.
13. Kirby, G. W., Rao, G. V. and Robins, D. J. (1988) *J. Chem. Soc. Perkin Trans. 1* 301.
14. Férézou, J. P., Quesneau-Thierry, A., Barbier, M., Kollmann, A. and Bousquet, J. F. (1980) *J. Chem. Soc. Perkin Trans. 1* 113.
15. Taylor, A. (1971) in *Microbial Toxins* Vol VII, (Kadis, S., Ciegler, A., and Ajl, S. J., eds. p. 363. Academic Press, New York.
16. Turner, W. B. and Aldridge, D. C. (1983) in *Fungal Metabolites II*, pp. 417–420. Academic Press, London.
17. Kirby, G. W. and Robins, D. J. (1980) in *The Biosynthsis of Mycotoxins* (Steyn, P. S., ed.) pp. 301–326. Academic Press, New York.
18. Férézou, J. P., Quesneau-Thierry, A., Servy, A., Zissmann, E. and Barbier, M. (1980) *J. Chem. Soc. Perkin Trans. 1* 1746.
19. Kirby, G. W., Robins, D. J., Sefton, M. A. and Talekar, R. R. (1980) *J. Chem. Soc. Perkin Trans. 1* 119.
20. Jamieson, W. D., Rahman, R. and Taylor, A. (1969) *J. Chem. Soc. (C)* 1564.
21. Kirby, G. W., Robins, D. J. and Stark, W. M. (1983) *J. Chem. Soc. Chem. Commun.* 812.