Biotransformation of the Brassica Phytoalexin Brassicanal A by the Blackleg Fungus

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The biotransformation of the brassica phytoalexin brassicanal A by the blackleg fungus [*Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm] was investigated. Three main biotransformation products were detected and isolated; their chemical structures were determined by spectroscopic methods and concomitant synthesis. Additionally, the antifungal activities of brassicanal A and its biotransformation products were compared. Overall, the biotransformation pathway suggests that the blackleg fungus has enzymes to carry out this biotransformation different from those involved in the biotransformation of the brassica phytoalexin brassinin.

Keywords: *Blackleg fungus; brassicanal A; brassinin; canola; Leptosphaeria maculans; Phoma lingam; phytoalexin; rapeseed*

INTRODUCTION

Brassica oilseeds are the third most important world source of edible vegetable oils, after soybean and palm (Luhs and Friedt, 1994). The oilseeds canola (*Brassica napus*, *Brassica rapa*), rapeseed (*Brassica napus, Brassica rapa*), and mustard (*Brassica juncea*) are among the most economically valuable brassica crops (Labana and Gupta, 1993). Canola, rapeseed, and other brassicas (Cruciferae family, syn. Brassicaceae) have defense mechanisms that can be associated with phytoalexin biosynthesis (Pedras and Séguin-Swartz, 1992; Pedras et al., 1996; Rouxel et al., 1995). Phytoalexins are secondary metabolites synthesized *de novo* by plants in response to diverse forms of stress, including pathogen attack (Brooks and Watson, 1985). The phytoalexins from brassicas have an indole ring and at least one sulfur atom as common structural features. Despite their close biogenetic relationship (L-tryptophan is the biogenetic precursor; Monde et al., 1994), brassica phytoalexins possess significantly different structures with various ring systems. These structural differences suggest that these phytoalexins have different biological activity; however, little is known about their antifungal or other antimicrobial activity.

We are studying the transformation of brassica phytoalexins by phytopathogenic fungi as part of an integrated research program aimed at understanding and controlling fungal diseases of canola and other economically important brassicas. Pathogenic attack by the blackleg fungus [*Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm.] on species of *Brassica* is a serious agricultural problem worldwide (Gugel and Petrie, 1992). Initially, we investigated the biotransformation of the phytoalexin brassinin (**1**) by the blackleg fungus and related species (Pedras and Taylor, 1991, 1993; Pedras et al., 1992). We determined that the virulence of the blackleg fungus correlated with its ability to rapidly metabolize and detoxify brassinin (**1**) (Pedras et al., 1992). Considering that brassinin (**1**) is an advanced precursor of

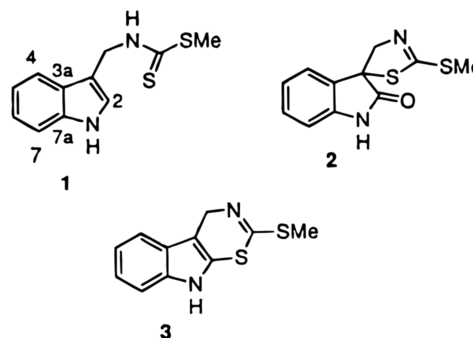

Figure 1. Structures of brassica phytoalexins.

spirobrassinin (**2**), cyclobrassinin (**3**) (Figure 1) (Monde et al., 1994), and possibly other related phytoalexins, the fast degradation of brassinin by the blackleg fungus may deprive the plant of important chemical defenses. Therefore, the overall result is a plant more susceptible to further fungal attack.

A possible strategy for controlling the blackleg fungus would be the inhibition of the enzymes involved in the detoxification of brassinin (**1**) and/or related phytoalexins. However, before such inhibitors can be rationally designed, it is important to determine whether the blackleg fungus metabolizes and detoxifies other brassica phytoalexins. Ultimately, a correlation between the bioactivity of the phytoalexins and of their biotransformation products will allow an understanding of the detoxification mechanisms utilized by the blackleg fungus to overcome the plant's defenses. It should then be possible to biorationally design antifungal agents selective against the blackleg fungus.

Herein we present results concerning the biotransformation of the brassica phytoalexin, brassicanal A (**4**) by the blackleg fungus. Additionally, the syntheses of brassicanal A and the products of biotransformation are described, the antifungal activities of these metabolites are compared, and the implications of these results are discussed.

MATERIALS AND METHODS

General. All chemicals were purchased from Aldrich Chemical Co., Inc., Madison, WI, or Sigma Chemical Co., St.

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Louis, MO. All solvents were of HPLC grade and used as such, unless indicated otherwise.

Preparative TLC: Merck, Kieselgel 60 F_{254}, 20 × 20 cm × 0.25 mm.

Analytical TLC: Merck, Kieselgel 60 F_{254}, aluminum sheets, 5 × 2 cm × 0.2 mm; compounds were visualized under UV light 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 (FCC): Silica gel Merck, grade 60, mesh size 230−400, 60 Å.

HPLC analysis was carried out with a high-performance Hewlett-Packard liquid chromatograph equipped with a quaternary pump, automatic injector, and diode array detector (wavelength range 190−600 nm), degasser, and Hypersil ODS column (5 μm particle size silica, 4.6 i.d. × 200 mm), equipped with a guard column filled with the same stationary phase. Mobile phase: 75% H_2O−25% CH_3CN to 100% CH_3CN, for 35 min, linear gradient, and a flow rate 1.0 mL/min. Organic extracts were dried over $MgSO_4$ and solvents removed under reduced pressure by rotary evaporator.

NMR spectra were recorded on a Bruker AM 300 or Bruker AMX 500 spectrometer; for ^{1}H (300 or 500 MHz), δ values were referenced to CD_3OD (3.31 ppm) and for ^{13}C (75.5 or 125.8 MHz) to CD_3OD (49.15 ppm).

Fourier transform infrared (FTIR) spectra were obtained on a Bio-Rad FTS-40 spectrometer using diffuse reflectance cell.

Mass spectra (MS) were obtained on a VG 70 SE mass spectrometer [high resolution (HR), electron impact (EI) or chemical ionization (CI) with ammonia as carrier gas], employing a solids probe.

Fungal Cultures. The virulent blackleg isolates (*P. lingam*) ENG-53 and BJ-125 and an avirulent isolate, Unity (now considered a different species; Pedras et al., 1995), were employed in this study. The fungal isolates were grown on V8 agar [20% (v/v) V8 juice, 0.75 g/L $CaCO_3$, 100 mg/L streptomycin sulfate, 40 mg/L Rose Bengal, 15 g/L agar] plates at 26 °C, under continuous light for 10 days. Spore suspensions of each isolate were prepared by overlaying the V8 agar plates with 10 mL of sterile distilled water, and the plate surfaces were rubbed with a flamed glass rod to dislodge the pycnidiospores. The suspension was filtered and transferred to centrifuge tubes and the spores were separated by centrifugation at 3000*g* for 10 min. After one washing with sterile distilled water, the spores were stored at −20 °C. Liquid cultures were grown in minimal medium supplemented with thiamin (MM) (Pedras et al., 1992). The cultures were incubated on a shaker at 150 rpm.

Fungal Spore Germination Assays. The antifungal activity (to isolate BJ-125) of brassicanal A (**4**), compounds **5**−**7**, and indole-3-carboxaldehyde was investigated using the following spore germination inhibition bioassay. Dimethyl sulfoxide (DMSO, control) or a DMSO solution (final concentration ≤1%) of the compound to be tested (final concentration of each compound 1 × 10^{-3}, 8 × 10^{-4}, and 5 × 10^{-4} M) was added to a spore suspension (2 × 10^6 spores/mL) in MM containing 5% Tween 80 (1.0 mL in a microcentrifuge tube) and incubated on a shaker at 150 rpm at room temperature. Aliquots (withdrawn at different times after vortex mixing of the microcentrifuge tubes containing the spore suspensions) of each suspension (10 μL) were pipetted to a hemacytometer, and the germinated and ungerminated spores were counted under the microscope at 40 × 10 magnification. Each assay was repeated at least twice.

Metabolism of Brassicanal A (4). Liquid shake cultures (100 mL of media in 250-mL Erlenmeyer flasks) inoculated with fungal spores (2 × 10^9) and incubated at 27 ± 2 °C were used for the metabolism studies. Control cultures of each isolate were grown separately. Solutions of brassicanal A (final concentrations 5 × 10^{-4} and 2 × 10^{-4} M) in DMSO (final concentration of DMSO in MM was 0.5% v/v) were administered to 35-h-old liquid cultures and to uninoculated media. Samples (10 mL) were withdrawn at 2−24-h intervals up to 6 days and were either immediately frozen or filtered; the broth and the mycelium were extracted with Et_2O. The extracted broth was acidified to pH 2 with HCl and extracted with Et_2O, followed by neutralization of the broth with NaOH and extraction with $CHCl_3$ [containing 1% NH_4OH (v/v)]. The extracts were analyzed by HPLC, as described below. Brassicanal A (**4**) was stable in uninoculated medium for at least 6 days.

Table 1. HPLC Retention Times of Brassinin, Brassicanal A, and Biotransformation Products

compound	retention time (min)
brassinin (**1**)	18.7
brassicanal A (**4**)	10.2
brassicanal A sulfoxide (**5**)	6.0
3-(hydroxymethyl)indole 2-methylsulfoxide (**6**)	3.4 (broad)
3-methylindole 2-methylsulfoxide (**7**)	8.7
indole-3-carboxaldehyde	6.3
indole-3-carboxylic acid	5.6

Metabolism of Compounds 5−7. Solutions of **5**−**7** (final concentration 2 × 10^{-4} M) in DMSO (final concentration of DMSO in MM was 0.5% v/v) were administered separately to 35-h-old liquid cultures and to uninoculated media and analyzed as described for brassicanal A. The compounds were stable in uninoculated medium for at least 6 days.

Analysis and Isolation of Metabolites. The analyses of the organic extracts and biotransformation products were performed with the HPLC system described above. The retention times shown in Table 1 were obtained with the mobile phase described under HPLC analysis. The samples to be analyzed by HPLC were dissolved in CH_3CN and filtered through a tight cotton wool plug. Only the chromatograms of the neutral organic extracts showed peaks not present in chromatograms of extracts of control cultures; acidic and basic extracts were similar to those of control cultures.

The isolation of products resulting from the fungal metabolism of brassicanal A (**4**) was carried out by FCC over silica gel (CH_2Cl_2−MeOH, 98:2 v/v), followed by preparative TLC. After TLC development with CH_2Cl_2−MeOH (95:5 v/v; brassicanal A, R_f 0.57; **5**, R_f 0.40; **6**, R_f 0.19; **7**, R_f 0.38), the fluorescent bands were scraped from the plate and eluted with CH_2Cl_2−MeOH (90:10 v/v).

The structures of the products were determined from analyses of the spectroscopic data (NMR, MS, FTIR, UV) of the purified metabolites and confirmed by synthesis, as described below.

Brassicanal A (4) Synthesis. Brassicanal A was prepared by formylation (Smith, 1954) of 2-(methylthio)indole (Hino et al., 1969) as follows. Phosphorus pentasulfide (0.47 g, 2.0 mmol) was added to a mixture of oxindole (1.33 g, 10.0 mmol), sea sand (1.5 g), and benzene (30 mL). The reaction mixture was heated under reflux with stirring for 80 min and then allowed to cool, and the benzene layer was decanted. The insoluble residue was extracted first with benzene and then with EtOAc. The benzene and EtOAc extracts were combined and evaporated to give crude 2-indolinethione (1.1 g, 7.4 mmol). A mixture of 2-indolinethione (1.1 g, 7.4 mmol), MeI (2.8 g, 20 mmol), Na_2CO_3 (1.2 g), and acetone (20 mL) was stirred at room temperature for 4 h and filtered. The filtrate was evaporated to leave a brown oily residue which was extracted with benzene. The benzene extract was purified (FCC, hexane−acetone, 9:1 v/v) to give 2-(methylthio)indole (0.8 g, 5 mmol). Phosphorus oxychloride (1.66 g, 10.0 mmol, freshly distilled) was added with stirring under argon atmosphere to DMF (3 mL, freshly distilled). A solution of 2-(methylthio)indole (0.17 g, 1.0 mmol) in DMF (1 mL) was added dropwise with continuous stirring. The reaction mixture was kept at room temperature for 45 min and then poured onto crushed ice. Aqueous NaOH (3.8 g in 20 mL) was added to the reaction mixture (slow addition, exothermic reaction!), and the product was filtered off, washed with water, and dried to yield brassicanal A (0.11 g): R_f 0.57 (CH_2Cl_2−MeOH, 95:5 v/v); ^{1}H NMR in Table 2; ^{13}C NMR in Table 3; FTIR ν_{max} 3151, 1626, 1581, 1448 cm^{-1}; EIMS, mass (relative intensity) = measured 191.0409 (M^+ $C_{10}H_9NOS$, calculated 191.0408) (100), 158 (68), 148 (17), 130 (9), 115 (7); CIMS, mass (relative intensity) = 192 (M^+ + 1) (100).

Table 2. ^{1}H NMR (300 MHz) Chemical Shifts (ppm) and Multiplicities (*J* in Hz) for Compounds 4–7 in CD_3OD

H no.	brassicanal A (**4**)	**5**[a]	**6**[a]	**7**[a]
4	8.06 m	8.10 dd (8.0, 1.5)	7.76 dd (8.0, 1.0)	7.65 dd (8.0, 1.0)
5/6	7.24 m	7.30 m	7.16 ddd (8.0, 7.0, 1.0)	7.00 ddd (8.0, 7.5, 1.0)
6/5	7.24 m	7.30 m	7.33 ddd (8.0, 7.0, 1.0)	7.30 ddd (8.0, 7.0, 1.0)
7	7.38 m	7.60 dd, (7.0, 1.0)	7.49 dd (8.0, 1.0)	7.42 dd (8.0, 1.0)
other	2.68 s, SCH_3	3.10 s, S(O)CH_3	3.00 s, S(O)CH_3	3.00 s, S(O)CH_3
	10.0 s, CHO	10.2 s, CHO	4.91 d (12.5), 4.89 d (12.5) CH_2OH	2.47 s, CH_3

[a] Assignments for H-4 and H-7 may be interchanged.

Table 3. ^{13}C NMR (75.5 MHz) Chemical Shifts (ppm) and Multiplicities for Compounds 4–7 in CD_3OD

C no.	brassicanal A (**4**)	**5**	**6**	**7**
2	148.76	147.70	134.47	132.52
3	117.63	116.98	120.68	118.43
3a	127.45	127.94	127.83	128.81
4	121.19	121.40	121.16	121.13
5	123.86	124.65	121.34	121.19
6	124.77	126.25	126.09	126.37
7	112.33	114.08	113.29	113.19
7a	138.91	138.60	139.30	139.49
other	185.4 CHO	185.94 CHO	55.06 CH_2OH	8.85 CH_3
	16.95 SCH_3	42.24 S(O)CH_3	41.08 S(O)CH_3	39.78 S(O)CH_3

Brassicanal A Sulfoxide (5) Synthesis. *m*-Chloroperbenzoic acid (5 mg in 0.5 mL of MeOH) was added to a stirred solution of brassicanal A (5 mg) in MeOH (1 mL) at 0 °C. After 30 min, the reaction mixture was treated with Me_2S (200 μL), concentrated, and separated by preparative TLC to yield quantitatively brassicanal A sulfoxide (**5**): R_f 0.40 (CH_2Cl_2–MeOH, 95:5 v/v); ^{1}H NMR in Table 2; ^{13}C NMR in Table 3; FTIR ν_{max} 3046, 1627, 1600, 1460 cm^{-1}; EIMS, mass (relative intensity) = measured 207.0359 (M^+, $C_{10}H_9NO_2S$, calculated 207.0354) (26), 190 (100), 175 (12), 144 (3), 115 (3); CIMS, mass (relative intensity) = 208 ($M^+ + 1$) (7), 192 (100).

3-(Hydroxymethyl)indole 2-Methylsulfoxide (6) Synthesis. $NaBH_4$ (5 mg) was added to stirred solution of brassicanal A sulfoxide (10 mg) in MeOH (2 mL) at room temperature. After 30 min, the mixture was quenched with water and the solvent was evaporated under reduced pressure. The crude product was purified by preparative TLC (CH_2Cl_2–MeOH, 93:7 v/v, developed twice) to yield 3-(hydroxymethyl)-indole 2-methylsulfoxide (**6**) (7.6 mg): R_f 0.19 (CH_2Cl_2–MeOH, 95:5 v/v); ^{1}H NMR in Table 2; ^{13}C NMR in Table 3; FTIR ν_{max} 3326, 2920, 1623, 1452, 1027 cm^{-1}; EIMS, mass (relative intensity) = measured 209.0499 ($C_{10}H_{11}NO_2S$, calculated 209.0510) (68), 192 (99), 176 (100), 117 (63); CIMS, mass (relative intensity) = 210 ($M^+ + 1$) (13), 192 (100).

3-Methylindole 2-Methylsulfoxide (7) Synthesis. Brassicanal A (10 mg), 10% palladium–charcoal (5 mg) and $NaBH_4$ (20 mg) in 2-propanol (3 mL) was refluxed with stirring; 60 min later a further quantity (10 mg) of $NaBH_4$ was added to the reaction mixture (Heacock and Hutzinger, 1964). After 2 h, the reaction mixture was filtered and treated as described for **6**. The residue was dissolved in MeOH and treated with *m*-chloroperbenzoic acid as described for **5**. The crude product was purified by preparative TLC (CH_2Cl_2–MeOH, 96:4) to yield 3-methylindole 2-methylsulfoxide (7.0 mg): R_f 0.38 (CH_2Cl_2–MeOH, 95:5 v/v); ^{1}H NMR in Table 2; ^{13}C NMR in Table 3; FTIR ν_{max} 3160, 1617, 1449, 1020 cm^{-1}; EIMS, mass (relative intensity) = measured 193.0562 (M^+, $C_{10}H_{11}NOS$, calculated 193.0561) (63), 178 (100), 160 (57), 117 (35); CIMS, mass (relative intensity) = 194 ($M^+ + 1$) (100).

RESULTS AND DISCUSSION

Virulent isolates of *P. lingam* were grown as described under Materials and Methods. After 35 h, brassicanal A (**4**), synthesized as described above, in DMSO was administered to fungal cultures and to uninoculated media (final concentrations 5×10^{-4} and 2×10^{-4} M). Control cultures of the fungus were grown separately. Cultures were incubated and samples were withdrawn at 2–24-h intervals (up to 6 days), extracted first with Et_2O, and then acidified and reextracted. Comparison of the HPLC chromatograms of extracts of fungal cultures containing brassicanal A and control cultures indicated that only the neutral extracts contained possible biotransformation products of brassicanal A. The optimum incubation time (in MM) for isolation of the putative products was determined by TLC and by HPLC analysis of each extract. Subsequently, the components of the neutral Et_2O extracts obtained from larger scale cultures were separated by chromatography, and each constituent was analyzed by ^{1}H and ^{13}C NMR, FTIR, and MS. Three main products were isolated after incubation of *P. lingam* with brassicanal A (**4**): brassicanal A sulfoxide (**5**), 3-(hydroxymethyl)-indole 2-methylsulfoxide (**6**), and 3-methylindole 2-methylsulfoxide (**7**). The structure of each compound was deduced from comparison of their spectroscopic data and those of brassicanal A as described below and confirmed by synthesis.

The ^{1}H NMR spectrum of each compound (CD_3OD) showed the four hydrogens characteristic of a 2,3-disubstituted indole nucleus. In addition, the sulfoxide **5** showed the aldehyde hydrogen, as well as the signal for the Me group, which was shifted downfield in both the ^{1}H (2.68 ppm in **4** vs 3.1 ppm in **5**) and the ^{13}C (16.9 ppm in **4** vs 42.2 ppm in **5**) NMR spectra. These changes in the chemical shifts suggested that the S–Me group present in brassicanal A (**4**) had been oxidized to the corresponding Me–S=O by the fungus. EIMS of **5** (molecular ion mass at 207 and base peak at 190) confirmed that oxygen addition had occurred. Further corroboration of the structure was provided by oxidation of brassicanal A (**4**) with *m*-chloroperbenzoic acid, as described under Materials and Methods.

Both compounds **6** and **7** showed, in addition to the indolyl hydrogens, a Me–(S=O) group, a methylene group in **6** (doublets at 4.89 and 4.91 ppm), and a methyl group in **7** (2.47 ppm); the aldehyde hydrogens were absent in both **6** and **7**. EIMS of **6** (molecular ion mass at 209 and base peak at 176) and of **7** (molecular ion mass at 193 and base peak at 178) confirmed that the aldehyde group of brassicanal A (**4**) had been reduced to the corresponding alcohol in **6** or to the corresponding methyl group in **7**. The structures of both **6** and **7** were also confirmed by synthesis. Metabolites **5**–**7** were not detected in uninoculated media containing brassicanal A (**4**) or in control fungal cultures.

Table 4. Products of Metabolism of Compounds 4−6 by *P. lingam* Isolates BJ-125, ENG-53, and Unity

fungal isolate (virulence on rapeseed)	compound added to fungal cultures[a]	products (%) of metabolism
BJ-125 (virulent)	**4**	**5** (10%), **6** (30%), **7** (40%) after 60 h; **5** (≤5%), **6** (30%), **7** (50%) after 6 days
ENG 53 (virulent)	**4**	**5** (10%), **6** (30%), **7** (40%) after 60 h; **5** (≤5%), **6** (30%), **7** (50%) after 6 days
BJ-125 (virulent)	**5**	**6** (>80%), **7** (≤5%) after 60 h
BJ-125 (virulent)	**6**	**7** (<10%) after 60 h; **7** (50%) after 6 days
BJ-125 (virulent)	**7**	no products of metabolism detected for 6 days
Unity (avirulent)	**4**	**5** (10%), **6** (≤5%), **7** (≤5%) after 6 days[b]

[a] Compounds at 5×10^{-4} M were dissolved in DMSO and added to germinated spores incubated in MM. [b] 80% of brassicanl A remaining in cultures.

Table 5. Results of Bioassays with Spores of *P. lingam*, Isolate BJ-125, Incubated with Compounds 4−7 and Indole-3-carboxaldehyde

compound added to ungerminated fungal spores	concentration[a] (μg/mL)	% of ungerminated spores
control		50% after 38 h; <1% after 72 h
brassicanal A (**4**)	1×10^{-3} M (200)	92% after 38 h; 25% after 72 h
	8×10^{-4} M (150)	75% after 38 h; <1% after 72 h
	5×10^{-4} M (100)	55% after 38 h; <1% after 72 h
5	1×10^{-3} M	75% after 38 h; <1% after 72 h
	8×10^{-4} M	65% after 38 h; <1% after 72 h
	5×10^{-4} M	50% after 38 h; <1% after 72 h
6	1×10^{-3} M	similar to control
7	1×10^{-3} M	similar to control
indole-3-carboxaldehyde	1×10^{-3} M	65% after 38 h; <1% after 72 h
	5×10^{-4} M	similar to control

[a] Compounds were dissolved in DMSO and added to ungerminated spores incubated in MM.

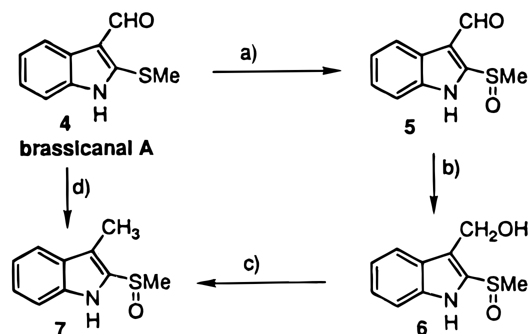

Figure 2. Biotransformation of brassicanal A by the blackleg fungus: (a) *Phoma lingam* or *m*-chloroperbenzoic acid; (b) *P. lingam* or $NaBH_4$; (c) *P. lingam*; (d) 1, $NaBH_4$/Pd-C; 2, *m*-chloroperbenzoic acid.

To ascertain the sequence of the biotransformation steps, compounds **5**−**7** (final concentration 2×10^{-4} M) were separately administered to cultures of *P. lingam* isolate BJ-125. The cultures were incubated and samples withdrawn at 2−24-h intervals, extracted, and analyzed by TLC and HPLC. The results of these experiments are summarized in Table 4. As predicted, **6** and **7** were detected in the cultures incubated with **5**, whereas only **7** was detected in the cultures incubated with either **6** or **7**. After incubation with **5** for 2 h, more than 50% of **5** had been converted to **6** (determined by HPLC analysis of the ethereal extract; traces of **7** present); after 60 h, **6** was the major metabolite (determined by HPLC analysis of the ethereal extract; traces of **5** and **7** present). On the other hand, the biotransformation of **6** to **7** was slower; after 6 days only 50% of **6** had been converted to **7**; compound **7** was stable in the cultures for at least 6 days.

The suggested sequence of reactions that brassicanal A (**4**) undergoes when incubated with virulent isolates of *P. lingam* is given in Figure 2. In the first biotransformation step, the SMe group of brassicanal A was oxidized to the corresponding sulfoxide **5**; this product was detected in the cultures 2 h after incubation with brassicanal A (**4**). Unexpectedly, however, the aldehyde group of **4** was reduced to the alcohol **6** and then further to the 3-methylindole **7**. The final product of the biotransformation of brassicanal A, metabolite **7**, was detected in the cultures 2 h after incubation and remained in the cultures for at least 6 days. Perhaps significantly, the biotransformation of brassicanal A (**4**) was different from that of brassinin (**1**) (Pedras and Taylor, 1993) and occurred much more slowly. While the biotransformation of brassinin (**1**) by virulent blackleg isolates rapidly yielded the corresponding oxidation products indole-3-carboxaldehyde and indole-3-carboxylic acid, the biotransformation of brassicanal A proceeded with reduction of the aldehyde. No traces of indole-3-carboxaldehyde or indole-3-carboxylic acid, the final metabolites of brassinin (**1**), were detected at any time during the biotransformation of **4**. It is also worthy to note that, in contrast with the inhibitory effect of brassinin (**1**) on the biosynthesis of the nonspecific blackleg phytotoxins, brassicanal A (**4**) did not have any similar effect.

The avirulent isolate Unity was also incubated with brassicanal A (**4**); in contrast with the results obtained for the two virulent isolates, the metabolism of **4** occurred much more slowly. As shown in Table 4, only 10% of **4** was metabolized to **5** (traces of **6** and **7** were detected) after 6 days.

The antifungal activities (to virulent blackleg isolates) of brassicanal A (**4**), its biotransformation products **5**, **6**, and **7**, and indole-3-carboxaldehyde were compared using a spore germination inhibition assay (Table 5). After 24 h of incubation, 20% of the fungal spores started to germinate in controls and in assays containing **6** or **7** (all three concentrations), whereas no germination was observed in spores incubated with either brassicanal A (**4**) or **5** (all three concentrations). After 38 h of incubation, 50% of the spores were found ungerminated in control assays and in assays with compounds **6** and **7**, whereas in assays with compounds **4**, **5**, and indole-3-carboxaldehyde (higher concentration, 1×10^{-3} M) most of the spores were ungerminated (92%, 75%, and 65%, respectively). After 72 h of incubation, ungerminated spores (ca. 25%) were only found in assays containing brassicanal A (1×10^{-3} M), whereas

mycelium appeared in the rest of the assays. These results (Table 5) indicate that brassicanal A (**4**) is significantly more inhibitory to blackleg spore germination than its biotransformation products or indole-3-carboxaldehyde.

It is now well documented that phytopathogens use phytoalexin metabolism as a detoxification mechanism to overcome plant's chemical defenses (Van Etten et al., 1989). We have previously determined that the blackleg fungus metabolizes (oxidative transformations) and detoxifies brassinin (**1**) effectively (Pedras et al., 1992; Pedras and Taylor, 1993). However, results of this study indicate that the virulent blackleg fungus cannot metabolize brassicanal A (**4**) as effectively. In fact, while brassinin (**1**) was completely metabolized in less than 24 h by virulent isolates, brassicanal A (**4**) was detected in mycelial extracts even after 60-h incubations with the same virulent isolates. On the other hand, but most importantly, we also determined in this study that the so-called "avirulent" isolates of *P. lingam* (Unity group, now considered a species related to the virulent group; Pedras et al., 1995) metabolized brassicanal A (**4**) at a much slower rate than the virulent isolates; after 6-day incubations, less than 20% of **4** was biotransformed to **5** (traces of **6** and **7** present). This result indicates that virulent blackleg isolates are significantly more effective in detoxifying brassicanal A than the related "avirulent" isolates.

Overall, our results suggest that the virulent blackleg fungus may utilize different enzymes to transform diverse phytoalexins; the enzyme(s) involved in the biotransformation of brassicanal A could be nonselective enzymes used in general detoxification processes. In any case, the process appears to be a detoxification beneficial to the virulent blackleg pathogen. Additionally, considering that preparation of chiral sulfoxides is of interest in synthetic methodology (Holland, 1988), the pathogenic blackleg fungus may be a useful bioagent for the oxidation of sulfides. Further work to understand mechanisms that allow the blackleg fungus to overcome the plant's chemical defenses is in progress.

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