Strategies of Cruciferous Pathogenic Fungi: Detoxification of the Phytoalexin Cyclobrassinin by Mimicry

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The remarkable metabolism of the cruciferous phytoalexin cyclobrassinin by the phytopathogenic root rot (*Rhizoctonia solani* Kuhn) and blackleg [*Phoma lingam* (Tode ex Fr.) Desm., asexual stage of *Leptosphaeria maculans* (Desm.) Ces. et de Not.] fungi is reported. It was established that *R. solani* metabolized and detoxified cyclobrassinin via the phytoalexin brassicanal A, which was further transformed into nontoxic products. Detoxification of cyclobrassinin in *P. lingam* avirulent isolate Unity occurred via the phytoalexin brassilexin, whereas the detoxification in *P. lingam* virulent isolate BJ 125 occurred via the phytoalexin dioxibrassinin. The chemistry involved in the structure determination of the intermediates of these three apparently different pathways and their antifungal activities are described. In addition, efficient syntheses of both phytoalexins brassicanal A and brassilexin by mimicry of the fungal biotransformation route are reported. Implications of these unprecedented transformations are discussed.

Keywords: *Brassica; cyclobrassinin; brassicanal A; brassilexin; Cruciferae*

INTRODUCTION

Enormous quantities of vegetable crucifers (Cruciferae, syn. Brassicaceae), such as broccoli (*Brassica oleracea* var. *botrytis*), cauliflower (*B. oleracea* var. *italica*), kale (*B. oleracea* var. *acephala*), radish (*Raphanus sativus*), and a variety of cabbages (*B. oleracea*), are consumed annually. Oilseed crucifers (*Brassica* spp.) constitute the third largest source of edible vegetable oils, and brown (*B. juncea*) and white (*Sinapis alba*) mustard seeds, as well as wasabi (*Wasabiae japonica*), are well-known condiments. In addition, the meal of oilseed brassicas is an important source of protein for animal feed. The worldwide impact of cruciferous crops is best assessed by the tremendous number and variety of scientific articles published annually.

Similar to other crops (Hammond-Kosac and Jones, 1996; Keen, 1993; Osbourn, 1996; Staskawicz et al., 1995), the disease resistance of crucifers is related with both constitutive and induced defenses. An important component of cruciferous induced defenses are the phytoalexins (Brooks and Watson, 1985), secondary metabolites having a broad antimicrobial activity. Interestingly, these metabolites were the first reported sulfur-containing phytoalexins (Figure 1) [for a recent review, e.g., Pedras et al. (1997)] produced by a plant family which, perhaps justifiably, has high sulfur requirements (Marquard and Walker, 1995). In general, the timing, rate of accumulation, and relative amounts of phytoalexins produced by plants play crucial roles in their resistance to pathogen invasion (Dixon, 1986; Dixon et al., 1994; Kuc, 1995; Smith, 1996). However, when pathogenic fungi can effectively disarm the plant by detoxifying phytoalexins, the outcome of the interaction can favor the pathogen and be detrimental to the plant. Although to date only a few examples demonstrate that brassica pathogens can detoxify phytoalexins efficiently (Pedras et al., 1997), multiple examples exist in other plant species (Daniel and Purkayastha, 1995; VanEtten et al., 1989). These enzymatic processes appear to reflect an adaptation of phytopathogens in their coevolution with plants. Nevertheless, because such transformations are usually simple chemical conversions, for example, oxidation, reduction, or one-carbon degradation mediated by P-450 enzymes, it is unclear whether pathogens acquire these enzymes from their host plants. In this regard, the unique chemical structures of brassica phytoalexins are providing a new insight into fungal strategies for degrading their hosts' defenses.

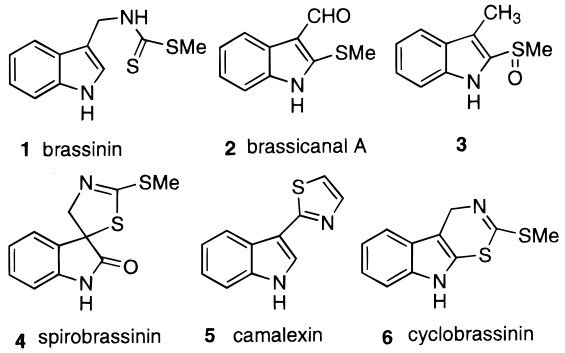

Figure 1. Chemical structures of cruciferous phytoalexins and a related metabolite (**3**).

An environmentally advantageous strategy for controlling pathogenic fungi of brassicas could include the inhibition of the fungal enzymes involved in the detoxification of phytoalexins. Toward this end, we have been investigating the intriguing effects of brassica phytoalexins on some of the most significant canola/rapeseed (*B. napus* and *B. rapa*) pathogens. We have demonstrated previously that the blackleg fungus [*Phoma*

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lingam (Tode ex Fr.) Desm., asexual stage of *Leptosphaeria maculans* (Desm.) Ces. et de Not.], one of the most successful pathogens of canola, can overcome the plant's chemical defenses by promptly transforming them into harmless metabolites. For example, the phytoalexin brassinin (**1**) was transformed into indole-3-carboxylic acid (Pedras et al., 1992; Pedras and Taylor, 1991, 1993), and brassicanal A (**2**) was transformed into 3-methylindole-2-methylsulfoxide (**3**) (Pedras and Khan, 1996); both metabolic products showed no antifungal activity against *P. lingam*. Nonetheless, this pathogen does not metabolize all brassica phytoalexins to a detectable level; we have established that the phytoalexins spirobrassinin (**5**) (Pedras and Khan, unpublished results) and camalexin (**6**) (Pedras et al., 1997) were not transformed by any of the blackleg fungal species, although the fungal spore germination appeared to be affected. Recently, we have examined the metabolism of the phytoalexin cyclobrassinin (**6**) by two different *P. lingam* species (herein called "virulent" and "avirulent" isolates as no formal reclassification of the *P. lingam* species has occurred; Pedras et al., 1995) and unraveled a very intriguing detoxification metabolism (Pedras and Okanga, 1998a). We have now examined the metabolism of cyclobrassinin (**6**) by the brassica fungal pathogen *Rhizoctonia solani* Kuhn, one of the fungi responsible for the root rot of crucifers. Here we describe for the first time the intermediates involved in this metabolic process, as well as their chemical synthesis and antifungal activity. In addition, we describe details of the transformation of cyclobrassinin by *P. lingam* and discuss implications of this discovery.

MATERIALS AND METHODS

General Procedures. All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON. All solvents were of HPLC grade and used as such except for CH_2Cl_2 and $CHCl_3$, which were redistilled. Solvents utilized in syntheses were dried over the following drying agents prior to use: benzene, THF, and EtO_2 over sodium/benzophenone and CH_2Cl_2 over CaH_2. Organic extracts were dried over Na_2SO_4 and solvents removed under reduced pressure in a rotary evaporator.

Analytical TLC. Merck, Kieselgel 60 F_{254}, aluminum sheets (5 × 2 cm × 0.2 mm) were used. Compounds were visualized under UV light and by dipping the plates in a 5% aqueous (w/v) phosphomolybdic acid solution containing 1% (w/v) ceric sulfate and 4% (v/v) H_2SO_4, followed by heating.

Preparative TLC. Merck, Kieselgel 60 F_{254} or RP-8 (20 × 20 cm × 0.25 mm) was used. Compounds were visualized under UV light.

Flash Column Chromatography (FCC). Silica gel from Merck, grade 60, mesh size 230−400, 60 Å, was used.

HPLC Analysis. HPLC was performed with a liquid chromatograph equipped with a quaternary pump, an automatic injector, a photodiode array detector, a degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 i.d. × 200 mm), equipped with an in-line filter. HPLC retention times given in the results and in Figures 2 and 3 were obtained under the following conditions: mobile phase, 75% H_2O/25% CH_3CN to 100% CH_3CN, for 35 min, linear gradient; flow rate = 1.0 mL/min.

NMR spectra were recorded on a Bruker AMX 300 or 500 spectrometer; for ^1H (300 or 500 MHz), δ values were referenced to CD_3OD (CD_2HOD at 3.31 ppm), CD_2Cl_2 ($CDHCl_2$ at 5.32 ppm), $CDCl_3$ ($CHCl_3$ at 7.26 ppm), or CD_3CN (CD_2HCN at 1.94 ppm) and for ^{13}C (75.5 or 125.8 MHz) δ values were referenced to CD_3OD (49.15 ppm), CD_2Cl_2 (54.00 ppm), $CDCl_3$ (77.23 ppm), or CD_3CN (118.69 ppm).

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

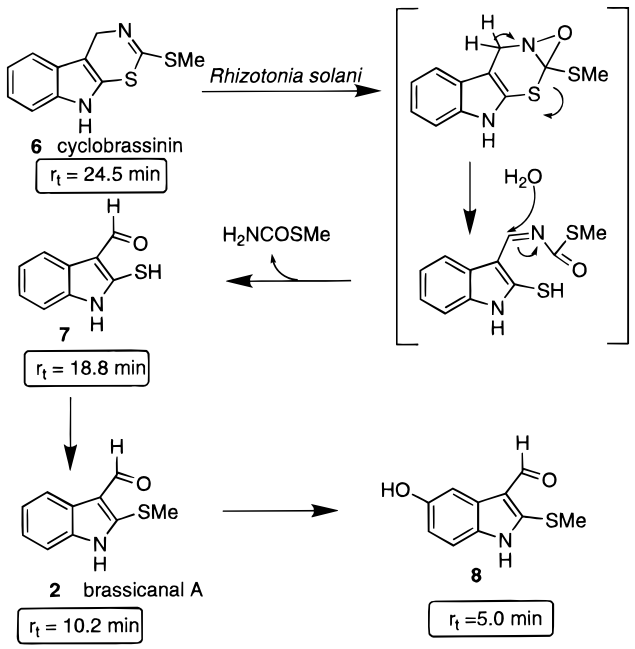

Figure 2. Transformation of cyclobrassinin (**6**) by the phytopathogenic fungus *R. solani* isolate AG 2-1; structures in square brackets represent proposed intermediates.

Mass spectra (MS) were obtained on a VG 70 SE mass spectrometer [high resolution (HR), electron impact (EI) was obtained at 70 eV, or chemical ionization (CI) was obtained at 50 eV with ammonia gas], employing a solids probe.

Optical rotation $[\alpha]_D$ was determined at ambient temperature on a Perkin-Elmer 141 polarimeter using a 1 mL, 10 dm cell; the units of $[\alpha]_D$ are 10^{-1} deg cm^2 g^{-1}.

Fungal Cultures. *Rhizoctonia solani*, virulent isolate AG 2-1, was grown on potato dextrose agar (PDA) plates at 24 ± 2 °C, under continuous light for 5−6 days. *Phoma lingam* virulent (V) isolate BJ 125 and avirulent (A) isolate Unity were grown on V8 agar under continuous light; after 15 days, the fungal spores were collected and stored at −20 °C (Pedras and Khan, 1996).

Fungal Radial Growth Assays. The antifungal activity to *R. solani* of compounds **2** and **6**−**8** was investigated using the following mycelial radial growth bioassay. A dimethyl sulfoxide (DMSO) solution (final concentration = 1%) of the compound to be tested (final concentration of each compound = 5×10^{-4} and 1×10^{-4} M) was added to PDA medium at ∼50 °C, mixed quickly, and poured onto Petri plates. An agar plug (5 mm diameter) cut from edges of 5-day-old solid cultures of *R. solani* was placed upside down on the center of each plate, and the plates were sealed with Parafilm and incubated under constant light at 24 ± 2 °C for 4 days. The diameter of the mycelial area was measured daily. Control plates containing only DMSO were prepared and incubated similarly. Each assay was repeated at least three times.

The antifungal activity to *P. lingam* A and V isolates of compounds **6** and **9**−**12** was investigated using the mycelial radial growth bioassay described above for *R. solani*, except that the agar plates were prepared with V8 juice agar medium (final concentration of each compound = 5×10^{-4} and 1×10^{-4} M). For assays with *P. lingam* a 5 μL drop of a spore suspension (2×10^8 spores/mL) of each isolate was placed in the center of each plate and the plates were incubated under constant light at 24 ± 2 °C for 7 days.

Metabolism of Cyclobrassinin (6). Liquid cultures of *R. solani* were initiated by inoculating potato dextrose liquid medium (100 mL of medium in a 250-mL Erlenmeyer flasks) with five agar plugs cut from the edges of 5-day-old solid cultures. Solutions of cyclobrassinin in DMSO were administered to 48-h-old liquid cultures (final concentration = 2.1×10^{-4} M) and to uninoculated media. Cultures were incubated on a shaker at 150 rpm, at 24 ± 2 °C. Samples (5−10 mL)

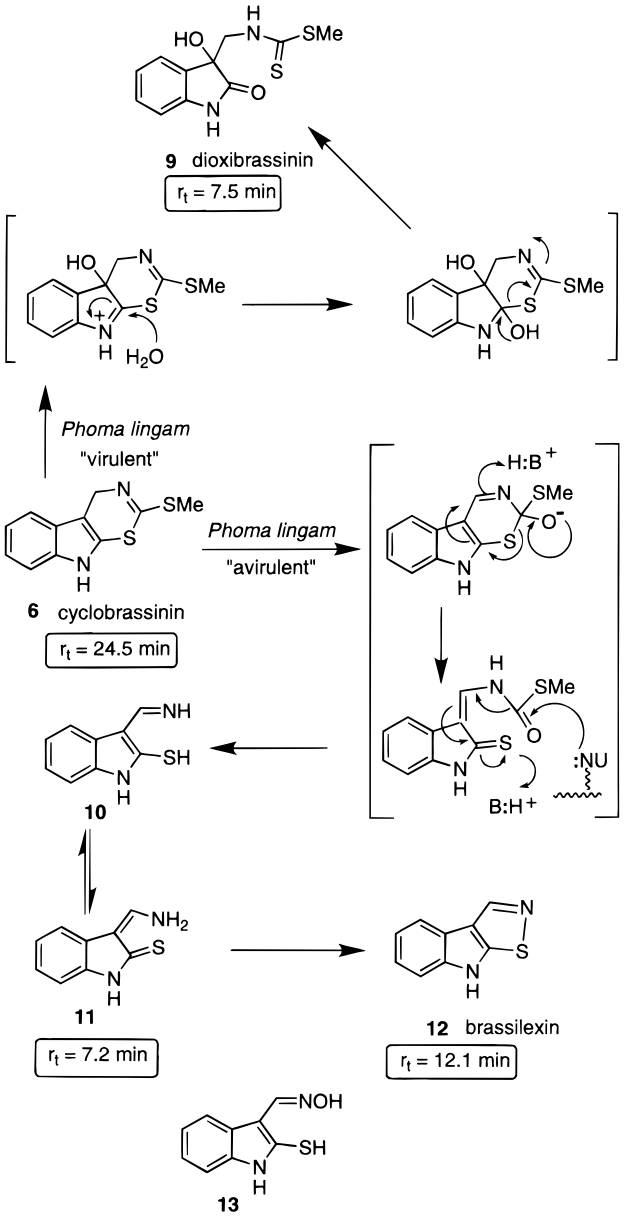

Figure 3. Transformation of cyclobrassinin (**6**) by the phytopathogenic fungus *P. lingam* A (avirulent) isolate (Unity) and V (virulent) isolate (BJ-125); structures in square brackets represent proposed intermediates.

were withdrawn at intervals up to 12 days and were either immediately frozen or filtered and extracted with Et_2O. The extracted broth was acidified to pH 2 with HCl and extracted with Et_2O, followed by neutralization with NaOH and extraction with $CHCl_3$ (containing 1% NH_4OH, v/v). Cyclobrassinin (**6**) was stable in uninoculated medium for at least 15 days.

Liquid shake cultures (100 mL of minimal medium in 250-mL Erlenmeyer flasks) inoculated with fungal spores (2×10^9) of *P. lingam* A and V isolates and incubated at 25 ± 2 °C were used for the metabolism studies. Control cultures of each isolate were grown separately. Solutions of cyclobrassinin (final concentrations = 4.3×10^{-4} M) in DMSO were administered separately to 48-h-old liquid cultures of each isolate and to uninoculated media (cyclobrassinin was stable in uninoculated medium for at least 2 weeks). Samples (5−10 mL) were withdrawn from each culture at intervals up to 6 days and extracted as described above.

Metabolism of Compounds 2 and 7−12. Solutions of compounds **2**, **7**, and **8** (final concentration = 2×10^{-4} M) in DMSO (final concentration of DMSO in medium = 0.5%, v/v) were administered separately to 48-h-old liquid cultures of *R. solani* and to uninoculated media and analyzed as described for cyclobrassinin. Solutions of compounds **10**/**11** and **12** (final concentration = 1.2×10^{-4} M) in DMSO (final concentration of DMSO in medium = 0.5%, v/v) were administered separately to 48-h-old liquid cultures of *P. lingam* A isolate and to uninoculated media and analyzed as described above for cyclobrassinin. A solution of compound **9** (final concentration = 1.2×10^{-4} M) in DMSO (final concentration of DMSO in medium = 0.5%, v/v) was administered to 48-h-old liquid cultures of *P. lingam* V isolate and to uninoculated media and analyzed as described above for cyclobrassinin. All of the compounds were stable in uninoculated medium for at least 10 days except for **10**/**11**, which on standing was slowly oxidized to **12** (=10% in 24 h).

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 Figures 2 and 3 were obtained with the mobile phase described under General Procedures. 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 cyclobrassinin (**6**) was carried out as follows. Erlenmeyer flasks (four) containing potato dextrose medium were inoculated with mycelia of *R. solani* AG 2-1. After 48 h, solutions of cyclobrassinin in DMSO (each 5 mg/500 μL) were added to fungal cultures in three flasks and to uninoculated medium. To the fungal culture in the fourth flask was added only DMSO (500 μL) (control). One test flask and the control flask were chosen for sampling. Samples (5 mL each) were taken immediately after the compounds had been added. Subsequently, 10-mL samples were taken after 6, 24, and 48 h, and final samples were taken after 4 days. The 10-mL samples were extracted and analyzed as described above. The final samples from the 4-day-old cultures were filtered, and the broth was extracted as described above. Preparative TLC [CH_2Cl_2/MeOH (97:3)] on the broth extract (11.6 mg) yielded 5-hydroxybrassicanal A (**8**, R_f = 0.15, 2.5 mg), 2-mercaptoindole-3-carboxaldehyde (**7**, R_f = 0.24, 1.1 mg), and brassicanal A (**2**, R_f = 0.38, 1.1 mg).

Erlenmeyer flasks (five) containing minimal medium were inoculated with spores of *P. lingam* A isolate. After 48 h, solutions of cyclobrassinin in DMSO (each 10 mg/500 μL) were added to fungal cultures in four flasks and to uninoculated medium. To the culture in the fifth flask was added only DMSO (500 μL)(control). One test flask and the control flask were chosen for sampling. Samples (10 mL each) were taken after 9 and 24 h, and final samples were taken after 28 h. The 10-mL samples were extracted and analyzed as described above. The final samples from the 28-h-old cultures were filtered, and the combined broth (380 mL) was extracted as described above. Preparative TLC [RP-8 plate, CH_3CN/H_2O (40:60)] on the broth extract (21.7 mg) yielded brassilexin (**12**, R_f = 0.20, 2.6 mg) and 3-methylenaminoindole-2-thione (**10**/**11**, R_f = 0.40, 2.4 mg).

Erlenmeyer flasks (five) containing minimal medium were inoculated with spores of *P. lingam* V isolate. After 48 h, solutions of cyclobrassinin in DMSO (each 10 mg/500 μL) were added to fungal cultures in four flasks and to uninoculated medium. To the culture in the fifth flask was added only DMSO (500 μL) (control). One test flask and the control flask were chosen for sampling. Samples (10 mL each) were taken after 6, 12, and 24 h, and final samples were taken after 30 h. The 10-mL samples were extracted and analyzed as described above. The final samples from the 30-h-old cultures were filtered, and the combined broth (370 mL) was extracted as described above. Preparative TLC [CH_2Cl_2/MeOH (95:5)] on the broth extract (39.6 mg) yielded dioxibrassinin (**9**, R_f = 0.30, 7.6 mg).

The structures of products and intermediates were determined from analyses of the spectroscopic data (NMR, MS,

FTIR, UV) of the purified metabolites and confirmed by synthesis, as described below.

Synthesis of Phytoalexins and Their Metabolites. Cyclobrassinin (Takasugi et al., 1988) and dioxibrassinin (Monde et al., 1991) were synthesized as previously described.

*Brassicanal A (**2**).* A solution of diazomethane (excess) in Et_2O was added to a solution of aldehyde **7** (20.3 mg, 0.11 mmol) in Et_2O (1 mL). The reaction mixture was kept at room temperature for 4 h. The solvent was then allowed to evaporate overnight in the fumehood. This afforded 21.9 mg of brassicanal A (100% yield): HPLC $t_r = 10.2$ min. Remaining data were identical to those previously reported (Pedras and Khan, 1996; Monde et al., 1990).

*2-Mercaptoindole-3-carboxaldehyde (**7**).* NaH (60%, 745 mg, 0.02 mol washed with hexane) was added slowly to a solution of 2-indolinethione [250.0 mg, 1.7 mmol, prepared according to the method of Hino et al. (1969)] in HCOOEt (5 mL, 0.06 mol), and the reaction mixture was kept at room temperature. After 12 h, the reaction mixture was diluted with water (10 mL, dropwise addition with cooling), the mixture was acidified with 2.5 M HCl, and the precipitate formed was filtered and was washed with water. The precipitate was dried to yield 106.0 mg of aldehyde **7**. The combined washings were acidified with 2.5 M HCl and combined with the filtrate. The filtrate was then extracted with EtOAc (2 × 120 mL), the combined extracts were dried over Na_2SO_4, and the solvent was evaporated under reduced pressure to yield 168 mg of **7**; total yield of aldehyde **7** was 274 mg (92%): HPLC $t_r = 18.8$ min; ^{1}H NMR (300 MHz, CD_3OD) δ 9.37 (s, 1H), 8.15 (d, $J = 8.0$ Hz, 1H), 7.48 (d, $J = 8.0$ Hz, 1H), 7.37 (dd $J = 8.0$, 8.0 Hz, 1H), 7.27 (dd, $J = 8.0$, 8.0 Hz, 1H); ^{1}H NMR (300 MHz, CD_3CN) δ 10.58 (br s, 1H, D_2O exchangeable), 9.65 (s, 1H), 8.12 (d, $J = 8.0$ Hz, 1H), 7.52 (d, $J = 8.0$ Hz, 1H), 7.37 (dd, $J = 8.0$, 8.0 Hz, 1H), 7.29 (dd, $J = 8.0$, 8.0 Hz, 1H); ^{13}C NMR (75.5 MHz, CD_3OD) δ 187.1, 139.1, 136.1, 126.9, 126.6, 124.5, 122.7, 122.3, 113.2; FTIR (cm^{-1}) 3229, 1621, 1454, 1247, 1216, 742; EIMS, *m/z* (%) 177 (M^+, 100), 149 (18), 148 (24), 121 (15); CIMS, *m/z* (%) 178 ($M^+ + 1$, 19), 164 (32), 150 (24), 146 (100), 132 (50); HREIMS, *m/z* calcd for C_9H_7NOS 177.0248, found 177.0249.

*3-Methylenaminoindole-2-thione (**11**).* Titanium trichloride (103 mg, 0.66 mmol) was added in small portions to a solution of oxime **13** (64 mg, 0.33 mmol), $NaBH_3CN$ (63 mg, 1 mmol), and NH_4OAc (32 mg, 0.4 mmol) in MeOH (3 mL). After stirring for 30 min at room temperature, the reaction mixture was diluted with water (20 mL) and extracted with Et_2O (3 × 20 mL). The combined Et_2O extracts were washed with water (120 mL) and dried over Na_2SO_4, and the solvent was evaporated under reduced pressure to yield 50.1 mg of thione **11** (85%): HPLC $t_r = 7.2$ min; ^{1}H NMR (500 MHz, CD_2Cl_2) δ 11.13 (br s, 1H, D_2O exchangeable), 8.98 (br s, 1H, D_2O exchangeable), 8.15 (dd, $J = 14.8$, 8.2 Hz, 1H), 7.41 (d, $J = 7.5$ Hz, 1H), 7.11 (m, 3H), 6.25 (br s, 1H, D_2O exchangeable); ^{1}H NMR (500 MHz, CD_3OD) δ 8.30 (s, 1H), 7.45 (d, $J = 6.9$ Hz, 1H), 7.05 (m, 3H); ^{13}C NMR (125.8 MHz, CD_2Cl_2) δ 179.0, 148.9, 138.0, 128.3, 124.1, 122.2, 115.3, 109.8, 108.4; ^{13}C NMR (125.8 MHz, CD_3OD) δ 177.6, 153.7, 151.7, 148.7, 140.4, 139.5, 129.9, 126.9, 125.0, 124.9, 124.1, 123.4, 122.5, 121.6, 120.9, 119.6, 115.8, 112.9, 111.7, 110.8, 110.5, 108.6; FTIR (cm^{-1}) 3238, 3128, 3067, 2924, 2852, 1641, 1623, 1549, 1446, 1398, 1303, 1233, 1210, 1014, 739; EIMS, *m/z* (%) 176 (M^+, 100), 149 (46), 117 (11; CIMS, *m/z* (%) 177 ($M^+ + 1$, 100), 164 (11), 145 (13); HREIMS, *m/z* calcd for $C_9H_8N_2S$ 176.0408, found 176.0412.

*Brassilexin (**12**).* Activated carbon (35 mg, 2.9 mmol) was added to a solution of 3-methylenaminoindole-2-thione (**11**) (51.0 mg, 0.29 mmol) in MeOH (2 mL), the reaction mixture was stirred at room temperature for 24 h and then filtered through a tight cotton plug, and the solvent was evaporated under reduced pressure to yield 41 mg of brassilexin (82% yield): HPLC $t_r = 12.1$ min; ^{1}H NMR (500 MHz, CD_3CN) δ 9.81 (br s, 1H, D_2O exchangeable), 8.68 (s, 1H), 7.90 (d, $J = 8.0$ Hz, 1H), 7.55 (d, $J = 8.0$ Hz, 1H), 7.32 (dd, $J = 8.0$, 8.0 Hz, 1H), 7.22 (dd, $J = 8.0$, 8.0 Hz, 1H); ^{13}C NMR (125.8 MHz, CD_3OD) δ 160.5, 148.7, 146.0, 128.4, 124.9, 121.5, 121.4, 120.9, 112.9; FTIR (cm^{-1}) 3371, 3274, 3070, 2958, 2924, 2853, 1645, 1619, 1504, 1465, 1368, 1240, 744; EIMS, *m/z* (%) 174 (M^+, 100), 146 (15), 142 (16); CIMS, *m/z* (%) 175 ($M^+ + 1$, 100), 145 (4); HREIMS, *m/z* calcd for $C_9H_6N_2S$ 174.0252, found 174.0253.

*2-Mercaptoindole-3-carboxaldehyde oxime (**13**).* To a solution of aldehyde **7** (100 mg, 0.56 mmol) in ethanol (2 mL) was added 1 M NH_2OH (1 mL, 1.1 mmol). The mixture was refluxed for 1 h and diluted with water (10 mL), and the resulting precipitate was filtered and dried to yield 47.6 mg of oxime **13**. The filtrate was extracted with EtOAc (2 × 20 mL). The combined EtOAc extracts were dried over Na_2SO_4, and the solvent was evaporated under reduced pressure to furnish another 65.8 mg of oxime **13**. Overall yield of the oxime was 113.4 mg (100%): HPLC $t_r = 17.4$ min; ^{1}H NMR (300 MHz, CD_3CN) δ 9.92 (br s, 1H, D_2O exchangeable), 8.52 (s, 1H, D_2O exchangeable), 8.04 (d, $J = 8.0$ Hz, 1H), 7.95 (s, 1H), 7.42 (d, $J = 8.0$ Hz, 1H), 7.31 (dd, $J = 8.0$, 7.0 Hz, 1H), 7.18 (dd, $J = 8.0$ Hz, 7.0 Hz, 1H); ^{13}C NMR (75.5 MHz, CD_3CN) δ 145.2 (d), 138.9 (s), 131.1 (s), 126.0 (d), 125.7 (s), 123.5 (d), 122.4 (d), 117.2 (s), 112.6 (d); FTIR (cm^{-1}) 3383, 3323, 1618, 1405, 1340, 844, 744; EIMS, *m/z* (%) 174 ($M^+ - 18$, 100), 149 (51); HREIMS, *m/z* calcd for $C_9H_6N_2S$ ($C_9H_8N_2OS - H_2O$) 174.0252, found 174.0251.

*5-Hydroxybrassicanal A (**8**):* HPLC $t_r = 5.0$ min; ^{1}H NMR (500 MHz, CD_3CN) δ 10.06 (s, 1H), 9.96 (br s, 1H, D_2O exchangeable), 7.45 (d, $J = 2.5$ Hz, 1H), 7.26 (d, $J = 9.0$ Hz, 1H), 6.74 (dd, $J = 9.0$, 2.5 Hz, 1H), 6.69 (s, 1H, D_2O exchangeable), 2.61 (s, 3H); ^{13}C NMR (125.77 MHz, CD_3OD) δ 185.7, 154.9, 148.1, 133.0, 128.3, 117.3, 114.1, 112.7, 106.0, 17.0; FTIR (cm^{-1}) 3171, 1612, 1585, 1444, 1365, 1224, 1083, 861; HRMS, *m/z* measured 207.0353 (207.0354 calcd for $C_{10}H_9NO_2S$); EIMS, *m/z* (intensity) 207 (M^+, 100), 192 (20), 174 (81), 164 (15); CIMS, *m/z* (intensity) 208 ($M^+ + 1$, 100), 148 (12).

*Dioxibrassinin (**9**):* $[\alpha]_D$ +21.3 (MeOH; *c* 0.0024); HPLC $t_r = 7.5$ min; ^{1}H NMR (300 MHz, CD_3OD) δ 7.36 (d, $J = 7.7$ Hz, 1H), 7.27 (dd, $J = 7.7$, 7.7 Hz, 1H), 7.01 (dd, $J = 7.7$, 7.7 Hz, 1H), 6.89 (d, $J = 7.7$ Hz, 1H), 4.32 (d, $J = 13.8$ Hz, 1H), 3.98 (d, $J = 13.8$ Hz, 1H), 2.53 (s, 3H); ^{13}C NMR (125.8 MHz, CD_3OD) δ 201.9, 180.6, 142.7, 131.1 (2×) 125.9, 123.9, 111.4, 76.3, 18.2; FTIR (cm^{-1}) 3273, 3223, 2918, 2850, 1720, 1714, 1622, 1470, 1378, 1189, 754; EIMS, *m/z* (%) 268 (M^+, 12), 220 (25), 191 (15), 164 (29), 149 (100); HREIMS, *m/z* calcd for $C_{11}H_{12}N_2O_2S_2$ 268.0340, found 268.0345.

RESULTS AND DISCUSSION

In initial experiments, we compared the bioactivity of cyclobrassinin (**6**) on the brassica pathogens utilized in this study, *R. solani* isolate AG 2-1, the nonaggressive or avirulent (A) *P. lingam*, and the aggressive or virulent (V) *P. lingam* (avirulent and virulent groups are now considered two different but related species; Pedras et al., 1995; Taylor et al., 1995), employing a mycelial growth inhibition assay (Pedras et al., 1997; Pedras and Khan, 1996). Cyclobrassinin (at a concentration of 5×10^{-4} M) inhibited the mycelial growth of the blackleg A isolate and the root rot isolate AG 2-1 (~30% inhibition relative to control cultures after incubation for 5 days); a slighter inhibitory effect (~15% inhibition relative to control cultures after incubation for 5 days) was observed on the blackleg V isolate at identical concentration.

In subsequent experiments, the phytoalexin cyclobrassinin (**6**) was incubated with cultures of the root rot pathogen *R. solani* to establish a time course for the biotransformation. Analysis of organic extracts of culture samples by HPLC indicated a rapid decrease in the concentration of cyclobrassinin (HPLC retention time, $t_r = 24.5$ min) and the sequential appearance of three constituents with $t_r = 18.8$, 10.2, and 5.0, min, respectively. Subsequently, to obtain sufficient quantities of each constituent for both chemical characterization and bioassay, larger scale fungal cultures incubated with

Table 1. Results of Bioassays of Cyclobrassinin (6), Brassicanal A (2), Dioxibrassinin (9), Brassilexin (12), and Related Metabolites against *P. lingam* A (Avirulent) Isolate (Unity), *P. lingam* V (Virulent) Isolate (BJ-125), and *R. solani* Isolate AG 2-1

		P. lingam		
compd added to agar medium	concn (M)[a]	*R. solani* isolate AG 2-1 % inhibition[b,c]	A isolate, % inhibition[b,d]	V isolate, % inhibition[b,d]
6, cyclobrassinin	5×10^{-4}	27	30	15
	1×10^{-4}	5	0	0
2, brassicanal A	5×10^{-4}	14		
	1×10^{-4}	0		
12, brassilexin	5×10^{-4}		100	100
	1×10^{-4}		100	100
9, dioxibrassinin	5×10^{-4}		0	0
	1×10^{-4}		0	0
7	5×10^{-4}	23		
	1×10^{-4}	5		
8	5×10^{-4}	5		
	1×10^{-4}	0		
11	5×10^{-4}		100	100
	1×10^{-4}		100	100

[a] Compounds dissolved in DMSO and added to agar medium, as described under Experimental Procedures. [b] The percentage of inhibition was calculated using the following formula: % inhibition = 100 − [(growth on treated/growth in control) × 100]; results are the mean of at least three separate experiments, SD ± 5. [c] Percentage of inhibition after 4-day incubations. [d] Percentage of inhibition after 5-day incubations.

cyclobrassinin were extracted with Et_2O, the extract was fractionated by chromatography, and each fraction was analyzed by HPLC. The fractions containing the aforementioned new constituents were analyzed by standard spectroscopic methods, including ^1H and ^{13}C NMR spectroscopy, a variety of 2D-NMR techniques, and HRMS, for structural elucidation. On the basis of these results the constituent of $t_r = 10.2$ min was established to be the known phytoalexin brassicanal A (**2**), the structure of which we confirmed by synthesis (Pedras et al., 1997). The structure of the constituent of $t_r = 5.0$ min was assigned as 5-hydroxybrassicanal A (**8**) on the basis of the analysis of the spectroscopic data, particularly HMBC and HMBC spectroscopic data, similar to 5-hydroxycamalexin (Pedras and Khan, 1997). The structure of the relatively less polar and less stable intermediate of $t_r = 18.8$ min was assigned as 2-mercaptoindole-3-carboxaldehyde (**7**) and confirmed by synthesis. To confirm the sequence of biotransformation steps, *R. solani* was incubated separately with metabolites **2** and **7**; these experiments indicated that aldehyde **7** was converted to brassicanal A (**2**), which in turn was transformed into aldehyde **8** (Figure 2). 5-Hydroxybrassicanal A (**8**) was further converted into polar undetermined products devoid of antifungal activity. The pathway for the biotransformation of cyclobrassinin (**6**) by *R. solani* is depicted in Figure 2; cyclobrassinin was completely transformed into metabolite **7** (∼96 h), which was further transformed into brassicanal A (**2**) and this into hydroxybrassicanal A (**8**) (=10 days). Eleven days after incubation of *R. solani* with cyclobrassinin, the mycelial mass of these cultures was similar to that of control cultures. Mycelial growth assays with *R. solani* indicated that **2**, **7**, and **8** had lower inhibitory effect at 5×10^{-4} M than cyclobrassinin (Table 1). As shown in Figure 2 (structures in square brackets are proposed intermediates), these puzzling results indicated that detoxification of cyclobrassinin (**6**) by *R. solani* occurred via the phytoalexin brassicanal A (**2**). Interestingly, despite the structural differences between brassicanal A (**2**) and camalexin (**5**), we have previously established that *R. solani* detoxified **5** also via the 5-hydroxy derivative (Pedras and Khan, 1997). Nonetheless, at this stage any conclusions regarding the similarity between the enzymes involved in the detoxification processes would be premature.

Next, *P. lingam* A isolate was incubated with cyclobrassinin (**6**). Analysis of organic extracts of culture samples by HPLC indicated a rapid decrease in the concentration of cyclobrassinin ($t_r = 24.5$ min) and the concurrent appearance of two additional constituents ($t_r = 7.2$ and 12.1 min) over a 12-h period (Figure 3). Subsequently, to obtain sufficient quantities of each constituent for chemical characterization and bioassays, larger scale fungal cultures were incubated with cyclobrassinin. Similar to the study conducted with *R. solani*, the fractions containing the aforementioned new constituents were analyzed by using standard spectroscopic methods. On the basis of those results the constituent of $t_r = 12.1$ min was established to be the known phytoalexin brassilexin (**12**) (Devys et al., 1988), the structure of which we confirmed by synthesis. The structure of the constituent of $t_r = 7.2$ min, a relatively much less stable metabolite, was assigned as a mixture of the related tautomers 3-(methylimino)indole-2-thiol (**10**) or 3-methylenaminoindole-2-thione (**11**) on the basis of spectroscopic data and synthesis. Cyclobrassinin was completely transformed into metabolite **10**/**11** in ∼12 h. Two days after incubation of the A isolate with cyclobrassinin, no brassilexin (**12**) or related metabolites were detected in the cultures, and the mycelial mass of cultures incubated with cyclobrassinin was similar to that of control cultures; that is, brassilexin was completely metabolized to nontoxic products. To confirm the sequence of biotransformation steps shown in Figure 3, the A isolate was incubated separately with metabolite **10**/**11** and brassilexin; these experiments indicated that metabolite **10**/**11** was converted to brassilexin (**12**) and that brassilexin was converted into polar undetermined products (∼24 h) devoid of antifungal activity. Bioassays of *P. lingam* A isolate incubated with brassilexin (**12**) at 5×10^{-4} and 1×10^{-4} M indicated complete inhibition of mycelial growth (Table 1). As summarized in Figure 3 (structures in square brackets are proposed intermediates), these results demonstrated that biotransformation of cyclobrassinin by *P. lingam* A isolate occurred via brassilexin (**12**), a phytoalexin significantly more toxic to the A isolate than cyclobrassinin (**6**); however, brassilexin was further metabolized to nontoxic undetermined product(s) in ∼24 h, as shown by mycelial mass (dry weight of mycelia) of liquid cultures. Consequently, detoxification of cyclobrassinin occurred via metabolites **10**/**11** and **12**, which were significantly more toxic to the A isolate than cyclobrassinin (Table 1). Cyclobrassinin appears to cause higher growth inhibition in solid cultures of the A isolate (∼30%, Table 1) than in liquid cultures (after 48 h, mycelial mass identical to controls); this apparently lower inhibitory effect is most likely due to the faster metabolism of cyclobrassinin in liquid cultures than in solid cultures.

Similar experiments carried out with *P. lingam* V isolate incubated with cyclobrassinin (**6**) afforded (∼24 h) yet another known phytoalexin, dioxibrassinin (**9**, $t_r = 7.5$ min, Figure 3), the chemical structure of which was determined from analysis of the spectroscopic data and confirmed by synthesis (Monde et al., 1991). It is worth noting that the optical rotation of dioxibrassinin isolated in this study has a positive value, whereas the

value previously reported is negative (cf. $[\alpha]_D$ +21.3 versus −7.6) (Monde et al., 1991). Two days after incubation of *P. lingam* V isolate with cyclobrassinin (**6**), no traces of the phytoalexin **6** or **9** or related metabolites were detected in the cultures. In addition, the mycelial mass of V isolate cultures incubated with cyclobrassinin was similar to that of control cultures. As suggested by these results, incubation of *P. lingam* V isolate with dioxibrassinin (**9**) indicated that the compound was metabolized to polar undetermined products devoid of antifungal activity against the V isolate. Bioassays of *P. lingam* V isolate incubated with dioxibrassinin at 5×10^{-4} M (Table 1) indicated no detectable inhibitory effect. As summarized in Figure 3 (structures in square brackets are proposed intermediates), these results indicated that detoxification of cyclobrassinin by *P. lingam* V isolate occurred via the phytoalexin dioxibrassinin (**9**) and that **9** was further metabolized to nontoxic product(s) in ∼24 h.

During the purification and characterization of compound **10**/**11** we observed that brassilexin (**12**) could be obtained from **10**/**11** upon standing in solution or on silica gel TLC plates. Interestingly, this transformation was catalyzed by activated charcoal to afford brassilexin almost quantitatively, demonstrating that metabolite **10**/**11** would be a synthetically useful brassilexin precursor if available in reasonable yields (Pedras and Okanga, 1998b). Thus, formylation of indolinethione (Hino et al., 1969) with EtOOCH afforded aldehyde **7**, which under standard conditions (Wenkert et al., 1958) yielded quantitatively oxime **13**. Subsequently, oxime **13** was reduced to the desired intermediate **10**/**11** with $NaBH_3CN$ in the presence of $TiCl_3$ (Leeds and Kirst, 1988). Finally, treatment of **10**/**11** with activated charcoal afforded brassilexin in excellent yield. In addition, treatment of aldehyde **7** with diazomethane yielded brassicanal A (**2**) quantitatively. Utilizing this route, brassilexin (**12**) was obtained from indolinethione (Hino et al., 1969) in four steps in ∼64% overall yield and brassicanal A (**2**) in ∼92% overall yield. Although previous syntheses of brassilexin (**12**) from indole-3-carboxaldehyde via cyclobrassinin (**6**) (Devys and Barbier, 1990a,b, 1992) or directly from indole-3-carboxaldehyde (Devys and Barbier, 1993) have been reported, our biomimetic route affords the best overall yield to date while following the simplest process in terms of purification and reaction conditions. This synthesis of brassicanal A (**2**) also presents a great improvement to our previously reported procedure, which employed methylation of indolinethione followed by standard Vilsmeier formylation of the corresponding 2-thiomethyl ether (Pedras and Khan, 1996).

Our overall results indicate that cyclobrassinin (**6**) is detoxified via brassicanal A (**2**), dioxibrassinin (**9**), or brassilexin (**12**), depending on the particular fungal species, and that each of these plant pathogens can detoxify **6** by adopting degradation pathways that may also operate in the plant. *In planta* direct biosynthetic relationships have been established between brassinin (**1**) and cyclobrassinin (**6**) and between brassinin (**1**) and spirobrassinin (**4**) (Monde et al., 1994). In addition, cyclobrassinin (**6**) has been proposed as a close precursor of both brassilexin (**12**) (Devys and Barbier, 1992) and brassicanal A (**2**) (Monde et al., 1996). Most importantly, we have recently established (Pedras et al., 1998) that cyclobrassinin (**6**) is also a precursor of brassilexin (**12**) *in planta*. Furthermore, in agreement with our results, compound **7** has also been proposed (Monde et al., 1996) as an intermediate in the biosynthesis of cyclobrassinin (**6**) to brassicanal A (**2**). Although a precursor of dioxibrassinin (**9**) has not been determined, the structural relationship suggests that cyclobrassinin is a likely precursor *in planta*. Thus, the detoxification of cyclobrassinin by each fungal species appears to occur by mimicry of biosynthetic pathways of plants. Because brassinin (**1**) is a biosynthetic precursor of cyclobrassinin (**6**) in plants, it would be useful to find a pathogen able to transform **1** into **6**. Paradoxically, it may be possible to determine part of the cruciferous phytoalexin biosynthetic pathway and to clone the corresponding genes by utilizing such pathogens. A clearer picture will eventually unfold when we are able to trace a complete map of phytoalexin transformation in both cruciferous and their pathogenic fungi.

CONCLUSION

Understanding phytoalexin detoxification pathways utilized by phytopathogenic fungi and co-occurring microorganisms is of great interest to a biorational approach to control plant pathogens. Specific inhibitors of phytoalexin-detoxifying enzymes could be designed and utilized to prevent fungal detoxification of those phytoalexins. Such inhibitors would allow the plant to build up the naturally occurring phytoalexins to a level at which the pathogen cannot survive. This strategy would be environmentally advantageous for controlling phytopathogenic fungi, as it is anticipated that the specific inhibitors would have no deleterious effects on other organisms.

In addition, perhaps due to their unique chemical structures, the fungal detoxification of cruciferous phytoalexins may have further interest. Our results provide remarkable examples of phytoalexin detoxification by phytopathogenic fungi, suggesting that enzymes involved in such processes may result from an intergeneric−genetic exchange between brassicas and their fungal pathogens. Here we demonstrated that in unforeseen interactions, three different canola/rapeseed pathogens can convert the phytoalexin cyclobrassinin into three different phytoalexins and, ultimately, each phytoalexin into nontoxic products. Therefore, we suggest that such pathogens may have acquired a more effective mechanism for overcoming this chemical by adopting phytoalexin biosynthetic pathways operating *in planta*. This strategy appears quite plausible, especially considering that most fungal pathogens have been coevolving with plants for innumerable generations.

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