In planta sequential hydroxylation and glycosylation of a fungal phytotoxin: Avoiding cell death and overcoming the fungal invader

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To facilitate plant colonization, some pathogenic fungi produce phytotoxic metabolites that damage tissues; plants may be resistant to a particular pathogen if they produce an enzyme(s) that catalyzes detoxification of this metabolite(s). Alternaria blackspot is one of the most damaging and significant fungal diseases of brassica crops, with no source of resistance known within the *Brassica* species. Destruxin B is the major phytotoxin produced by the blackspot-causing fungus, *Alternaria brassicae* (Berkley) Saccardo. We have established that a blackspot-resistant species (*Sinapis alba*) metabolized ^{14}C-labeled destruxin B to a less toxic product substantially faster than any of the susceptible species. The first metabolite, hydroxydestruxin B (^{14}C-labeled), was further biotransformed to the β-D-glucosyl derivative at a slower rate. The structures of hydroxydestruxin B and β-D-glucosyl hydroxydestruxin B were deduced from their spectroscopic data [NMR, high resolution (HR)-MS, Fourier transform infrared (FTIR)] and confirmed by total chemical synthesis. Although these hydroxylation and glucosylation reactions occurred in both resistant (*S. alba*) and susceptible (*Brassica napus*, *Brassica juncea*, and *Brassica rapa*) species, hydroxylation was the rate limiting step in the susceptible species, whereas glucosylation was the rate limiting step in the resistant species. Remarkably, it was observed that the hydroxydestruxin B induced the biosynthesis of phytoalexins in blackspot-resistant species but not in susceptible species. This appears to be a unique example of phytotoxin detoxification and simultaneous phytoalexin elicitation by the detoxification product. Our studies suggest that *S. alba* can overcome the fungal invader through detoxification of destruxin B coupled with production of phytoalexins.

The interaction of plants with their pathogens involves an array of remarkably simple chemical reactions, some of which transform highly bioactive secondary metabolites (e.g., antimicrobial or phytotoxic produced by the plant or by the pathogen) into mutually harmless products (i.e., detoxification). For example, one of the most significant fungal pathogens [*Phoma lingam* (Tode ex Fries) Desmarzières, perfect stage *Leptosphaeria maculans* (Desmarzières) Ces. et de Notaris] of rapeseed (*Brassica napus* and *Brassica rapa*, Cruciferae family) is able to overcome the plant's natural chemical defenses, phytoalexins, through various detoxification steps (1). On the other hand, to facilitate tissue colonization, some pathogenic fungi produce phytotoxic metabolites which selectively damage host-plants [host-selective toxins (HSTs)], or a wider range of plants (nonselective toxins; ref. 2). Plants may be resistant to a particular pathogen if they produce an enzyme(s) that catalyzes detoxification of the pathogen's toxin(s) (3, 4). Consequently, such detoxification traits are desirable and of much importance in strategic plant breeding or in engineering disease resistance. Whereas a number of phytoalexin detoxifying reactions have been shown to occur in very diverse plant pathogenic fungi (1, 5, 6), detoxification of phytotoxins has been reported in only a few plant species (7).

Three remarkable examples help to illustrate the significance of phytotoxin detoxification in plants. The first plant disease resistance gene to be cloned encoded an enzyme able to detoxify HC-toxin, a host-selective toxin (HST) produced by a maize fungal pathogen (8). Detoxification of HC-toxin, a cyclic tetrapeptide, resulted from a simple reduction of a carbonyl on the side chain of one of the constituent amino acids (9, 10). In another example, the first transgenic tobacco plants resistant to wildfire (a bacterial disease of tobacco) were obtained by introducing the tabtoxin detoxifying gene (3). Tabtoxin is a nonselective toxin produced by the wildfire bacterium. Genetically engineered tobacco plants resistant to wildfire disease were generated by introducing the gene encoding an acetyltransferase, which catalyzed detoxification of tabtoxin. Interestingly, this gene was cloned from the phytopathogenic wildfire bacterium, which is self-resistant to its own toxin because of production of this detoxifying enzyme. A more recent example reported that transgenic sugar cane plants expressing a phytotoxin detoxifying gene were more resistant to leaf scald disease than wild-type plants (11). In this example, although the chemical structure of albicidin, the toxin produced by the leaf scald bacterium, has not been elucidated, the detoxifying gene appears to encode an esterase. The albicidin esterase gene was cloned from a biocontrol agent that provides protection against leaf scald disease (12). From these examples, it is apparent that phytotoxin detoxifying enzymes play an important role in protecting plants from the phytotoxin producing microorganism, whether bacterial or fungal.

Alternaria blackspot is one of the most damaging and widespread fungal diseases of rapeseed (*B. napus* and *B. rapa*), an oilseed of great economic importance worldwide (13). *Alternaria brassicae* (Berk.) Sacc., the blackspot causing fungus, produces the cyclic depsipeptide toxins destruxin B and homodestruxin B (1 and 2 in Fig. 1), both *in vitro* (14) and *in planta* (15, 16). Ultrastructural studies have shown that destruxin B causes tissue damage similar to that observed in plants naturally infected with *A. brassicae* (17). Nonetheless, the role of destruxins in the development of blackspot disease is not clearly established, partly because the sexual stage of *A. brassicae* is not known, and no pathotypes or non-destruxin-producing strains are available. A variety of assays to establish the phytotoxicity of destruxin B to whole plants (18), seedlings (19), excised leaves (14, 15, 18, 19), pollen grains (20), protoplast, and cell cultures (18, 19), suggest that destruxin B phytotoxicity is host-selective. This

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Abbreviations: LSC, liquid scintillation counting; R, resistant; S, susceptible.

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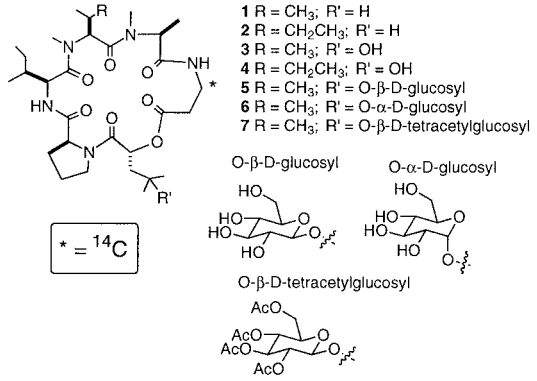

Fig. 1. Chemical structures of destruxin B (**1**), homodestruxin B (**2**), hydroxydestruxin B (**3**), hydroxyhomodestruxin B (**4**), β-D-glucosyl hydroxydestruxin B (**5**), α-D-glucosyl hydroxydestruxin B (**6**), and β-D-tetracetylglucosyl hydroxydestruxin B (**7**).

apparent selectivity might be due to detoxification reactions occurring in tissues of plant species resistant to *A. brassicae*. Considering that no sources of Alternaria blackspot resistance are known within *Brassica* species, such information is of enormous importance in generating blackspot-resistant plant lines.

Toward this end, we became interested in investigating the metabolism of destruxins in plants susceptible and resistant to *A. brassicae.* Sources of blackspot resistance within the family Cruciferae (syn. Brassicaceae) include *Sinapis alba,* a white mustard used as condiment and vegetable, and some weedy species. In a preliminary communication we reported that hydroxylation of destruxin B (**1**), and homodestruxin B (**2**), correlated with resistance and susceptibility to *A. brassicae* of two different species (21). Here we demonstrate that complete detoxification of destruxin B (**1**) in plants resistant to *A. brassicae* is a two-step process involving sequential hydroxylation and glucosylation. The chemical structure of the nontoxic destruxin B product, β-D-glucosyl hydroxydestruxin B (**5**, Fig. 1), was determined by a combination of spectroscopic analyses and chemical synthesis of both the β and α glucosides (**5** and **6**, Fig. 1) as well as the tetracetyl derivative **7** (Fig. 1). Results of the phytotoxicity assays of hydroxydestruxin B, hydroxyhomodestruxin B, and β-D-glucosyl hydroxydestruxin B (Fig. 1, compounds **3**-**5**) are described. Interestingly, although these hydroxylation and glucosylation reactions occur in both resistant and susceptible species, hydroxylation was the rate limiting step in the susceptible species, whereas glucosylation was the rate limiting step in the resistant species. Remarkably, it was established that hydroxydestruxin B (**3**) induced the biosynthesis of phytoalexins in resistant but not in susceptible species. Taken together, our studies suggest that *S. alba* can overcome the fungal invader through detoxification of destruxin B coupled with production of phytoalexins.

Materials and Methods

Materials. All chemicals were purchased from Sigma–Aldrich Canada (Oakville, ON). Destruxin B (**1**) (22), homodestruxin B (**2**), hydroxydestruxin B (**3**), hydroxyhomodestruxin B (**4**), [^{14}C]destruxin B, and [^{14}C]homodestruxin B (21), were prepared by total synthesis as previously described. Radiolabeled hydroxydestruxin B was synthesized as reported in the supplemental data, which is published on the PNAS web site, www.pnas.org (text and Fig. 5). Analytical TLC aluminum sheets were from EM Science (Kieselgel 60 F_{254}, 5 × 2 cm × 0.2 mm); compounds were visualized under UV light and by dipping the plates in a 5% aqueous (wt/vol) phosphomolybdic acid solution containing 1% (wt/vol) ceric sulfate and 4% (vol/vol) H_2SO_4, followed by heating. Preparative TLC glass plates were from EM Science (Kieselgel 60 F_{254}, or RP-8, 20 × 20 cm × 0.25 mm, or HPTLC-CN F_{254}, 10 × 10 cm × 0.2 mm); compounds were visualized under UV light. Flash column chromatography (FCC) silica gel was from EM Science (grade 60, mesh size 230–400, 60 Å, or JT Baker WP C-18 reversed phase silica gel 40 μm).

Seeds of *S. alba*, cultivars Ochre, Pennant, and Sabre, *Brassica juncea* cultivars Cutlass, Varuna, and SWP 083, *B. napus* cultivars Cresor, Quantum, Westar were obtained from Agriculture and Agri Food Canada (Saskatoon, SK); seeds of *B. rapa* CrGC 1–6 were obtained from the Crucifers Genetics Cooperative (Madison, WI). The seeds were sown in a commercial potting soil mixture, and plants were grown in a growth chamber, with 16 h light (fluorescent and incandescent)/8 h dark, at 24 ± 2°C.

Phytotoxicity Assays. Assays were conducted with whole plants, leaves, and cell suspension cultures of *S. alba,* Ochre, *B. juncea,* Cutlass, and *B. napus,* Westar, as described in ref. 18. Cell suspension cultures were obtained from protoplasts prepared from hypocotyls, as described in ref. 18. Experiments were carried out in 4-well plates, with each well containing 2-week-old cell suspension cultures and metabolite solutions at various concentrations (5×10^{-4} M, 5×10^{-5} M, and 1×10^{-5} M); the plates were incubated in complete darkness at 25 ± 0.5°C for 10 days. The cell viability was determined after adding phenosafranin to each well and counting cells directly in the 4-well plates by using an inverted microscope (18). The percent viability was determined by counting random fields of view for each replicate (18).

Analytical Procedures. HPLC analysis was carried out with a high-performance Hewlett–Packard liquid chromatograph equipped with quaternary pump, automatic injector, and photodiode array detector (wavelength range 190–600 nm) connected in series with a Canberra Packard (Mississauga, ON, Canada) Radiomatic 150TR flow scintillation analyzer (*ca.* 0.5 ± 0.1 min delay, fitted with a 210 μl high performance flow cell Solarscint), degasser, and a Hypersil (Agilent Technologies, Orangeville, ON, Canada) octadecylsilane (ODS) column (5 μm particle size silica, 4.6 i.d. × 200 mm), equipped with an in-line filter. Mobile phase: H_2O-CH_3CN, 75:25 to 100% CH_3CN, for 35 min, linear gradient, and 1.0 ml/min flow rate. Samples were dissolved either in CH_3CN or in methanol (MeOH). The ^{14}C counting window was 15–100 keV with an update time of 6 sec.

Introduction was performed on a Beckman Coulter LS-6500 connected to a Wyse (Mississauga, ON, Canada) WY-370 data system and results are quench corrected and reported in dpm.

NMR spectra were recorded on a Bruker (Billerica, MA) AMX 300 or 500 spectrometer; for ^{1}H (300 or 500 MHz), δ values were referenced to $CDCl_3$ ($CHCl_3$ at 7.26 ppm), and for ^{13}C (75.5 or 125.8 MHz) referenced to $CDCl_3$ (77.23 ppm).

MS spectra were obtained on a VG 70 SE mass spectrometer; high resolution (HR)-FAB was obtained at 70 eV employing a solids probe.

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

Optical Activity. Specific rotations (i.e., $[\alpha]_D$) were determined at ambient temperature on a Perkin–Elmer 141 polarimeter by using a 1 ml, 10 dm cell at the indicated concentration (*c*; g/100 ml); the units of $[\alpha]_D$ are 10^{-1} deg cm^2 g^{-1}.

Time Course of Metabolism of Destruxins. Time course studies were carried out with [^{14}C]destruxin B, [^{14}C]homodestruxin B, and [^{14}C]hydroxydestruxin B. Leaves of similar size and age were selected from 3-week-old plants resistant (R; *S. alba* cultivar Ochre) and susceptible (S; *B. napus* cultivar Westar and *B. juncea*

cultivar Cutlass) to *A. brassicae* and were cut at the base of their petiole and immediately placed in Eppendorf tubes containing the ^{14}C-labeled toxin (1 ml of toxin at 2×10^{-5} 77 dissolved in 2% aqueous CH_3CN per leaf). After the solution was taken up (this time = t_0), an additional 0.5 ml of H_2O was added to ensure the complete uptake of compound. Leaves (a minimum of three leaves per data point) were incubated in a growth chamber under fluorescent lighting (cycle 16-h light/8-h dark at 20 ± 0.5°C) for 12, 24, 48, 72, and 110 h keeping the petiole immersed in doubly distilled H_2O. After incubation, leaves were immediately frozen in liquid nitrogen, crushed into small pieces with a glass rod, and separately extracted with ethyl acetate (EtOAc; 12 h). The solvent was filtered, the leaf tissue rinsed with EtOAc and combined with the EtOAc extract, and the leaf residue extracted with MeOH (12 h). The extracts were concentrated to dryness on a rotary evaporator and dissolved in appropriate solvents for HPLC and liquid scintillation counting (LSC) analyses. Additional time course studies were carried out with [^{14}C]destruxin B and [^{14}C]hydroxydestruxin B and resistant *S. alba* cultivars Pennant and Sabre, and susceptible *B. napus* cultivars Quantum and Cresor, [^{14}C]destruxin B and susceptible *B. juncea* cultivars Varuna and SWP 083, and susceptible *B. rapa* cultivar CrGC 1–6. Percentage of transformation of each compound was calculated from LSC and HPLC data by using calibration curves to convert peak areas into dpm. The identification of metabolites **3** and **5** in leaves of the different plant species was based on chromatographic [high-performance thin-layer chromatography (HPTLC) and HPLC analysis in different solvent systems and co-elution with authentic synthetic samples] and spectroscopic data (comparison of UV spectra with authentic synthetic samples).

Analysis of Phytoalexins. The presence of phytoalexins known (1) to be produced in elicited leaves of *B. juncea*, *B napus*, *B. rap*a, and *S. alba* (brassilexin, brassinin, cyclobrassinin, cyclobrassinin sulfoxide, dioxibrassinin, sinalexin, spirobrassinin) was determined by HPLC analysis of leaf extracts of each plant species incubated with destruxin B, homodestruxin B, or hydroxydestruxin B, using the conditions described above. Chromatograms of leaf extracts of each plant species were compared with those of nonelicited leaves. Identification of potential phytoalexins was carried out through direct comparison of retention times and UV spectra of chromatographic peaks with those of authentic samples synthesized or isolated as reported in ref. 1 and amounts were determined from calibration curves.

Isolation of Hydroxydestruxin B (3) and Hydroxyhomodestruxin B (4). Experiments were conducted similarly to those reported above for time course studies. Leaves (*ca.* 250) of *S. alba*, cultivar Ochre, were incubated with destruxin B and homodestruxin B (total amount *ca.* 10 mg at 2×10^{-5} 77 dissolved in 2% aqueous CH_3CN) for 48 h. Leaves were frozen in liquid nitrogen and extracted with EtOAc followed by MeOH. The extracts were dried over Na_2SO_4, filtered, and the filtrate was concentrated to dryness in a rotary evaporator. The EtOAc residue (5.3 g) was fractionated by multiple column chromatography (C-18 reversed phase silica gel) eluting with CH_3CN-H_2O, gradient elution 30:70 to 100:0. The fractions were combined after HPLC analysis and further fractionated by preparative TLC (PTLC) on silica gel developed in acetone-hexane (65:35) to yield *ca.* 2 mg of chromatographically homogeneous material (TLC and HPLC).

Isolation of β-D-Glucosyl Hydroxydestruxin B (5). The fractionation steps are summarized in supplementary Fig. 6, which is published as supplemental data on the PNAS web site. Experiments were conducted similarly to those reported above for time course studies. The extraction and isolation protocol was designed and optimized following two small-scale experiments using radiolabeled material. After incubation of leaves (35 and 60 leaves) with [^{14}C]hydroxydestruxin B for 96 h, the leaves were extracted with hexane followed by MeOH, and each extract counted in an LSC. HPLC analysis with the Radiomatic detector indicated that the new metabolite with t_R = 6.8 min was mostly contained in the MeOH extract. This extract was diluted with water and extracted sequentially with EtOAc and butanol. The EtOAc extract contained recovered hydroxydestruxin B (*ca.* 30%) and a small amount of the new metabolite (<5%), whereas the butanol extract contained the new metabolite. Following the separation steps shown in the supplemental data (Fig. 6), a larger scale experiment was devised. Thus, leaves (*ca.* 580) of *B. napus*, cultivar Westar, were incubated with hydroxydestruxin B (total amount *ca.* 8 mg at 2.0×10^{-5} 77 dissolved in 2% aqueous CH_3CN) for 96 h. Leaves were frozen in liquid nitrogen and extracted with hexane followed by MeOH. The extracts were dried over Na_2SO_4, filtered, and the filtrate concentrated to dryness in a rotary evaporator. The MeOH residue (27 g) was dissolved in H_2O (100 ml), extracted with EtOAc, and the aqueous phase extracted with butanol. The butanol extract was dried over Na_2SO_4, filtered, and the filtrate concentrated to dryness on a rotary evaporator. The butanol residue (3 g) was fractionated by multiple column chromatography (C-18 reversed-phase silica gel) eluting with CH_3CN-H_2O (30:70). The fractions were combined after HPLC analysis (53 mg) and further fractionated by C-18 reversed-phase silica gel, eluting with CH_3CN-H_2O (25:75). The fractions containing the hydroxydestruxin B metabolite were combined (12 mg) and further fractionated on HPTLC-CN plates developed in CH_2Cl_2-MeOH (95:5) to yield *ca.* 500 μg of chromatographically homogeneous material (TLC and HPLC).

Results and Discussion

The metabolism of destruxin B (**1**) and homodestruxin B (**2**) by plant tissues was investigated by using radiolabeled compounds, prepared as described in ref. 21, and leaves of plants R (*S. alba* cultivar Ochre) and S (*B. napus* cultivar Westar and *B. juncea*

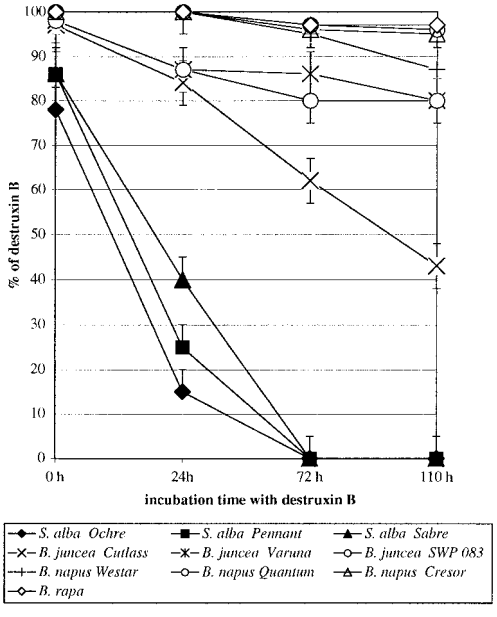

Fig. 2. Metabolism of [^{14}C]destruxin B (**1**) in *S. alba*, Ochre (R), Sabre (R), Pennant (R); *B. juncea*, Cutlass (S), Varuna (S), SWP 083 (S); *B. napus*, Westar (S), Cresor (S), Quantum (S); *B. rapa* (S) (R and S to *A. brassicae*); determined by HPLC analysis (Radiomatic detector) and LSC (data points represent the percentage of the total amount of [^{14}C]destruxin B (**1**) remaining in leaves—i.e., not metabolized—after incubation for different time periods).

Table 1. Metabolism of ^{14}C-homodestruxin B (1), in leaves of plants R and S to *A. brassicae*

Species	Cultivar	% of homodestruxin B converted to hydroxyhomodestruxin B* t_R = 21.5 min†
S. alba	Ochre (R)	>90% in 24 h
B. juncea	Cutlass (S)	10% in 24 h; 40% in 72 h
B. napus	Westar (S)	<10% in 72 h

*Results are averages of experiments conducted in triplicate; percentage of conversion determined by HPLC analysis as described in *Materials and Methods*; areas of peaks determined in counts and converted to dpm utilizing a calibration curve; the total HPLC peak areas were within 10–20% of the LSC total counting.

†t_R = retention time of compounds detected in Radiomatic detector.

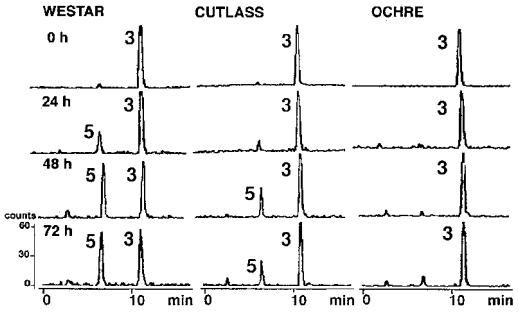

Fig. 3. HPLC chromatograms (Radiomatic detector) of extracts of leaves of *S. alba* cultivar Ochre (R), *B. juncea* cultivar Cutlass (S), and *B. napus* cultivar Westar (S) (R and S to *A. brassicae*) incubated with [^{14}C]hydroxydestruxin B (**3**) up to 72 h. For HPLC conditions see *Materials and Methods*.

cultivar Cutlass) to *A. brassicae*. Time course studies conducted with radiolabeled toxins administered to petiolated leaves and monitored by HPLC analysis of leaf extracts indicated that both toxins **1** and **2** were almost completely metabolized by *S. alba* (R), within 24 h (Fig. 2 and Table 1). Destruxin B (t_R = 19.3 min) was biotransformed to a metabolite with t_R = 10.4 min, and homodestruxin B (t_R = 21.5 min) was biotransformed to a metabolite with t_R = 12.8 min. *B. napus* (S) and *B. juncea* (S) also appeared to metabolize both destruxin B and homodestruxin B, but to a much lower extent; whereas 40% of destruxin B was transformed in 72 h by *B. juncea* (S), only about 10% was transformed in 72 h by *B. napus* (S; Fig. 2). These metabolites appeared to be identical to those produced in *S. alba*, as their UV spectra and t_R were identical in different solvent systems. Similar results were obtained for homodestruxin B (Table 1). These results indicated that the rate of transformation of destruxin B (**1**) and homodestruxin B (**2**) correlated with the blackspot resistance of *S. alba*.

To determine the chemical structure of the metabolites resulting from transformation of destruxins by plant material, isolation of chromatographically homogeneous material was required. Thus, large scale experiments were carried out with nonlabeled destruxins by using leaves of *S. alba*, as described in *Materials and Methods*. Following an incubation period of 48 h, the leaves were extracted with EtOAc and the extracts fractionated, while monitoring the presence of metabolites with t_R = 10.4 min and t_R = 12.8 min by HPLC. Multiple chromatographic fractionations yielded *ca.* 2 mg of each metabolite. High resolution (HR)-electron impact mass spectrometry (EIMS) analysis of the metabolite with t_R = 10.4 min ($C_{30}H_{51}N_5O_8$) indicated the presence of an additional oxygen as compared with destruxin B ($C_{30}H_{51}N_5O_7$). ^{1}H and ^{13}C NMR spectroscopic data of this metabolite (t_R = 10.4 min) were very similar to those of destruxin B except for the presence of two distinct methyl singlets at δ_H 1.33, δ_C 31.4 and δ_H 1.30, δ_C 29.5, and an additional quaternary carbon at δ_C 70.0. These spectroscopic data suggested that this product contained a tertiary hydroxyl group located either at the valyl or at the 2-hydroxy-3-methylpentanoyl residue. Further proton-decoupling experiments allowed the unambiguous assignment of structure **3** to the metabolite with t_R = 10.4 min (21). Similarly, the spectroscopic data of the metabolite with t_R = 12.8 min obtained from homodestruxin B indicated it to have structure **4**. Furthermore, based on chromatographic and spectroscopic data, it was established that metabolites **3** and **4** were also produced in *B. napus* and *B. juncea* incubated with destruxin B and homodestruxin B, respectively. As expected, metabolites **3** and **4** were not detected in control experiments carried out with leaves of *B. napus, B. juncea,* and *S. alba* incubated under similar conditions but without destruxins.

To establish that hydroxylation of destruxins was in fact a detoxification process, it was crucial to determine the phytotoxicity of hydroxydestruxins **3** and **4** to plants resistant and susceptible to *A. brassicae*. Because mg amounts were required for these bioassays, the total synthesis of **3** and **4** was carried out as previously reported (21). As expected, the synthetic hydroxydestruxins were identical in all respects to the metabolites obtained from plant biotransformation, thus corroborating the chemical structure assignments. The phytotoxicities of destruxins **1** and **2** and hydroxydestruxins **3** and **4** toward *S. alba,* Ochre, *B. napus,* Westar, and *B. juncea,* Cutlass were compared. A cell suspension culture assay allowed discrimination between the toxicity of destruxin B and hydroxydestruxin B (Table 2); the viability of cell cultures of the susceptible cultivars Westar and Cutlass treated with hydroxydestruxin B (**3**) for six days was close to those of untreated cell cultures, whereas the viability of cells treated with destruxin B (**1**) was substantially lower. Similarly, leaf puncture and leaf uptake bioassays carried out with hydroxydestruxin B (**3**) and hydroxyhomodestruxin B (**4**) indicated that the hydroxylated compounds were less phytotoxic than each of the destruxins (18). Altogether these bioassay results indicated that the metabolism of destruxins **1** and **2** to the respective hydroxylated products **3** and **4** were detoxifications occurring at a substantially faster rate in *S. alba* (R).

Table 2. Effect of destruxin B (1), hydroxydestruxin B (3), and β-D-glucosyl hydroxydestruxin B (5) on percent viability of 2-week-old cell cultures of *S. alba* cv. Ochre, *B. napus* cv. Westar, and *B. juncea* cv. Cutlass after 6 days of incubation

Toxin concentration	Destruxin B			Hydroxydestruxin B			β-D-glucosyl hydroxydestruxin B		
	Ochre	Westar	Cutlass	Ochre	Westar	Cutlass	Ochre	Westar	Cutlass
Control*	68 ± 2	64 ± 2	67 ± 3	68 ± 2	64 ± 2	67 ± 4	72 ± 3	65 ± 2	63 ± 4
1×10^{-5} M	67 ± 2	59 ± 2	65 ± 4	69 ± 1	62 ± 3	63 ± 2	69 ± 6	63 ± 4	61 ± 8
5×10^{-5} M	63 ± 2	52 ± 5	61 ± 4	65 ± 1	66 ± 2	63 ± 3	74 ± 3	64 ± 2	68 ± 1
5×10^{-4} M	36 ± 5	32 ± 3	53 ± 5	48 ± 2	61 ± 3	61 ± 1	72 ± 1	65 ± 3	61 ± 5

Results are the means of six independent experiments ± standard error.
*Control cell cultures containing 2% acetonitrile.

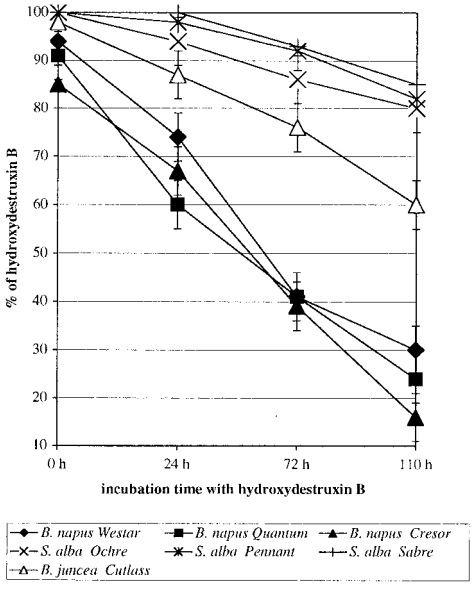

Fig. 4. Metabolism of [^{14}C]hydroxydestruxin B (**3**) in *S. alba*, Ochre (R), Sabre (R), Pennant (R); *B. napus*, Westar (S), Cresor (S), Quantum (S); *B. juncea*, Cutlass (S), (R and S to *A. brassicae*); determined by HPLC analysis (Radiomatic detector) and LSC (data points represent the percentage of the total amount of [^{14}C]hydroxydestruxin B (**3**) remaining in leaves, i.e., not metabolized, after incubation for different time periods).

Moreover, our time course studies with [^{14}C]destruxin B and *S. alba* indicated that hydroxydestruxin B was slowly (more than 4 days) metabolized to a more polar product, which was not detected in either *B. juncea* or *B. napus*. Subsequently, we investigated the metabolism of [^{14}C]hydroxydestruxin B, synthesized as reported in *Materials and Methods*, in leaves of *S. alba,* Ochre (R), *B. napus,* Westar (S), and *B. juncea,* Cutlass (S). Interestingly, these time course studies showed that hydroxydestruxin B was metabolized in all three species to a product with t_R = 6.8 min. Unexpectedly, however, *B. napus* (S) transformed 25% of hydroxydestruxin B in 24 h, whereas *B. juncea* (S) transformed about half of that amount (10%), and *S. alba* (R) transformed only 5%, as shown in the chromatograms in Fig. 3 and graph in Fig. 4. The results obtained over a period of 4 days indicated that hydroxydestruxin B was metabolized in *B. napus* (S) at a substantially faster rate than that observed in the other two species. Comparison of the HPLC chromatogram obtained with the photodiode array detector and that obtained with the Radiomatic detector, as shown in supplementary Fig. 7, which is published as supplemental data on the PNAS web site, indicated that the metabolite of hydroxydestruxin B coeluted with a complex mixture of polar compounds (t_R 6–8 min) and thus its isolation could be rather difficult. Subsequently, a protocol to isolate the new metabolite was devised and optimized in experiments conducted with [^{14}C]hydroxydestruxin B administered to *B. napus* Westar (S), as summarized in the supplemental data (Fig. 6). Multiple fractionation of the butanol extract eventually yielded a fraction of sufficient purity displaying a peak in the HPLC chromatogram (t_R = 6.8 min, Radiomatic detector; 6.4 min, diode array detector) with a UV spectrum resembling that of hydroxydestruxin B. A fractionation scheme similar to that summarized in the supplemental data (Fig. 6) was followed for larger scale experiments. These experiments led to detection and, after multiple fractionations, isolation of chromatographically homogeneous hydroxydestruxin B metabolite.

Analysis of the ^{1}H NMR spectrum of the hydroxydestruxin B metabolite (t_R = 6.4 min) showed resonances similar to those of hydroxydestruxin B and five additional signals (doublet at 4.53 ppm, J = 7 Hz; four multiplets at 4.0–3.2 ppm) suggesting the presence of a glycosyl group. The glycosyl moiety was corroborated by a fast atom bombardment (FAB) spectrum, which showed a likely molecular ion at m/z 772 ($M^+ + 1$) and a fragment ion at m/z 609 (corresponding to hydroxydestruxin B). It was suspected that the additional 162 mass units represented condensation of a hexose with the hydroxyl group of the hydroxydestruxin B (609 + 180 − 18 = 771). To determine the type of hexose, the metabolite was acetylated with Ac_2O in pyridine. Analysis of the ^{1}H NMR spectrum of the resulting tetracetyl derivative together with homonuclear decoupling experiments suggested a β-glucose unit, based on the magnitude of the vicinal coupling constants. However, the complete chemical structure of this metabolite was unambiguously assigned as **5**, based on direct comparison of its spectroscopic and HPLC data with those of synthetic α- (**6**) and β-D-glucosyl-hydroxydestruxin B (**5**) and their tetracetyl derivatives. In conclusion, the metabolite of hydroxydestruxin B (t_R = 6.4 min) with molecular formula $C_{36}H_{61}N_5O_{13}$ was β-D-glucosyl hydroxydestruxin B (**5**; Fig. 1). Although structures of at least 30 destruxins are known, neither compounds **5** or **6** appear to have been previously reported. Furthermore, chromatographic and spectroscopic data established that metabolite **5** was also produced in *S. alba* and *B. juncea* incubated with hydroxydestruxin B. Most importantly, bioassays to determine the phytotoxicity of **5** to leaves and cell suspension cultures of resistant and susceptible plants indicated that β-D-glucosyl hydroxydestruxin B had no toxic effect on either leaves or cell suspension cultures. The viability of cell suspension cultures incubated with β-D-glucosyl hydroxydestruxin B for 10 days was similar to those of control cultures (Table 2).

To further corroborate these results, we investigated the metabolism of [^{14}C]destruxin B in two additional disease-resistant cultivars of *S. alba* (Pennant and Sabre) and in susceptible cultivars of *B. juncea* (Varuna and SWP 083), *B. rapa* (CrGC 1–6), and *B. napus* (Quantum and Cresor) (Fig. 2). Both Pennant (R) and Sabre (R) showed time courses of biotransformation similar to that observed in Ochre (*S. alba*, R), whereas biotransformation in Varuna (S) and SWP 083 (S) was lower than that observed in Cutlass (*B. juncea*, S). Furthermore, no biotransformation of destruxin B was detected in CrGC 1–6 (S),

Table 3. Phytoalexins elicited by hydroxydestruxin B (3) in *S. alba* cv. Ochre (R), *B. napus* cv. Westar (S), and *B. juncea* cv. Cutlass (S)

		Phytoalexins detected (μg/g of fresh weight)		
Cultivar	24 h	48 h	72 h	110 h
Ochre	none	sinalbin A (150 ± 6) sinalexin (19 ± 3)	sinalbin A (240 ± 200) sinalexin (177 ± 94)	sinalbin A (790 ± 360) sinalexin (190 ± 95)
Cutlass		no phytoalexins detected for the duration of experiment		
Westar		no phytoalexins detected for the duration of experiment		

Results are the means of three independent experiments ± standard error.

Quantum (S), or Cresor (S) over a period of 120 h. Additional experiments to investigate the metabolism of [^{14}C]hydroxydestruxin B in Pennant, Sabre, Quantum, and Cresor showed that biotransformation of hydroxydestruxin B in Pennant (R) and Sabre (R) proceeded similar to that observed in Ochre (*S. alba*, R; Fig. 4), whereas transformation in Quantum (S) and Cresor (S) was similar to that observed in Westar (*B. napus*, S; Fig. 4). The identification of metabolites **3** and **5** was based on chromatographic and spectroscopic data, as well as direct comparison with synthetic samples.

An overview of the metabolism of [^{14}C]destruxin B (**1**) and [^{14}C]hydroxydestruxin B (**3**) in diverse cruciferous species can be obtained from graphs in Figs. 2 and 4. These data showed that the three cultivars of *S. alba* (R) metabolized destruxin B (**1**) to hydroxydestruxin B (**3**) substantially faster than cultivars of any other species. Among the *Brassica* species, Cutlass (*B. juncea*, S) appeared to metabolize destruxin B (**1**) to hydroxydestruxin B (**3**) faster, with *ca.* 43% of the initial amount of **1** detected after incubation for 110 h. On the other hand, a contrasting trend was observed for the metabolism of hydroxydestruxin B (**3**) to β-D-glucosyl hydroxydestruxin B (**5**); cultivars of *B. napus* (S) appeared to transform hydroxydestruxin B (**3**) to **5** substantially faster than cultivars of *S. alba* (R), whereas Cutlass (*B. juncea*, S) showed an intermediate rate. Most importantly, leaf and cell culture assays showed that hydroxydestruxin B (**3**) was substantially less toxic to any of the species tested than destruxin B (**1**), and β-D-glucosyl hydroxydestruxin B (**5**) showed no toxicity at all (Table 2).

Our studies indicated that the rate of hydroxylation of destruxin B in cruciferous plants correlated with species resistance to *A. brassica.* Furthermore, we observed that during this detoxification process the phytoalexins sinalexin (16) and sinalbin A (23) were produced in *S. alba* (R), however no phytoalexins were detected in *B. napus* (S) or *B. juncea* (S) (Table 3). It is well-known that phytoalexins can be biosynthesized in response to a variety of abiotic elicitors (1). However, it is remarkable that the detoxification product of destruxin B (**1**), hydroxydestruxin B (**3**), could also induce phytoalexin biosynthesis in the resistant species, whereas no phytoalexins were detected in susceptible species. Thus, the relatively slower glucosylation rate of hydroxydestruxin B in resistant *S. alba* may be beneficial because hydroxydestruxin B also elicits biosynthesis of phytoalexins in this species, but not in susceptible species. This appears to be the first report of selective phytoalexin elicitation with a toxin detoxification product, occurring only in the resistant species. These observations suggest that the blackspot resistance in *S. alba* due to detoxification of destruxin B may be amplified by well-synchronized phytoalexin elicitation.

Our hypothesis that in cruciferous species the hydroxylation of destruxins indicates the presence of a resistance trait to *A. brassicae* does not imply that all blackspot-resistant species have the same resistance mechanism. It has been proposed that the resistance to *A. brassicae* of certain destruxin B sensitive cruciferous weeds (*Camelina sativa*, *Capsella bursa-pastoris*) is because of production of the phytoalexin camalexin (19). In addition, we have observed that camalexin inhibits the production of destruxin B in *in vitro* cultures of *A. brassicae* (24). Thus, camalexin producing plants such as *C. sativa* and *C. bursa-pastoris* may prevent *A. brassicae* from biosynthesizing destruxin B. Consequently, destruxin B detoxification would be a redundant resistance mechanism in those plants.

In conclusion, our work has uncovered a detoxification mechanism present in *S. alba* (R) which is probably responsible for its blackspot resistance. That is, through hydroxylation of destruxins plants escape cell death and overcome the fungal invader. It is possible that a gene encoding destruxin B hydroxylase may also encode an Alternaria blackspot resistance trait.

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