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Transformation of the host-selective toxin destruxin B by wild crucifers: probing a detoxification pathway

M.S.C. Pedras*, S. Montaut, I.L. Zaharia, Y. Gai, D.E. Ward

Department of Chemistry, University of Saskatchewan, 110 Science Place, Saskatoon, SK, Canada S7N 5C9

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Abstract

The destruxin B detoxification pathway present in *Sinapis alba* is also present in three unrelated species, *Camelina sativa*, *Capsella bursa-pastoris*, and *Eruca sativa*, suggesting a conservation of this pathway across crucifers. The chemical structure of a destruxin B metabolite, (6′-*O*-malonyl)hydroxydestruxin B β-D-glucopyranoside, was also establised. Considering that *Camelina sativa* and *Capsella bursa-pastoris* detoxify destruxin B and produce the phytoalexins camalexins, these wild crucifers appear to represent unique and perhaps useful sources of blackleg resistance in strategic plant breeding.

Keywords: *Alternaria brassicae*; *Brassica* species; Destruxin B; Hydroxydestruxin B; Camalexin; Crucifer; *Camelina sativa*; *Capsella bursa-pastoris*; *Eruca sativa*

1. Introduction

Destruxin B (**1**) is a host-selective toxin produced both in vitro and in planta by the fungal pathogen *Alternaria brassicae* (Berk.) Sacc., the causative agent of Alternaria blackspot (Pedras et al., 2002). Blackspot is one of the most damaging and widespread fungal diseases of rapeseed (*Brassica napus* and *B. rapa*) and brown mustard (*B. juncea*). No *Brassica* species are known to be resistant to Alternaria blackspot. However, blackspot resistance is found within the family Cruciferae (syn. Brassicaceae), e.g. in white mustard (*Sinapis alba*), false flax (*Camelina sativa*) and Shepherd's purse (*Capsella bursa-pastoris*) (Conn et al., 1988; Conn and Tewari, 1986).

Plant detoxification of a host-selective toxin may impart resistance to the toxin producing pathogen, this trait is highly desirable and of enormous importance in strategic plant breeding or in engineering disease resistance (Karlovsky, 1999). However, detoxification of phytotoxins by plant tissues/cells has been studied in only a few species.

Recently, it was established that white mustard (blackspot resistant) metabolized ^{14}C-labeled destruxin B (**1**) to a less toxic product (Fig. 1) substantially faster than any of the susceptible *Brassica* species (Pedras et al., 2001). The first metabolite, hydroxydestruxin B (**2**) (^{14}C-labeled), was further biotransformed to the β-D-glucosyl derivative **3** at a slower rate. Although these hydroxylation and glucosylation reactions occurred in both resistant (*S. alba*) and susceptible (*Brassica napus*, *B. juncea*, and *B. rapa*) species, glucosylation (step ii) was the rate limiting step in the resistant species, whereas the hydroxylation (step i) was the rate limiting step in the susceptible species. Remarkably, it was observed that hydroxydestruxin B (**2**) induced the biosynthesis of phytoalexins in blackspot resistant species but not in susceptible species. This work appears to represent a unique example of phytotoxin detoxification and simultaneous phytoalexin elicitation by the detoxification product, as a mechanism to overcome the fungal invader. Although destruxin B (**1**) hydroxylation may impart *A. brassicae* resistance in some crucifers not all blackspot resistant species need to have this resistance mechanism (Pedras et al., 2001). For example, it has been suggested that in false flax and Shepherd's purse, Alternaria blackspot resistance is mostly due to production of the phytoalexin camalexin (Pedras et al., 2000), which inhibits the production of destruxin B in *A. brassicae* cultures (Pedras et al., 1998). Thus, camalexin producing plants such as *Camelina sativa* and

* Corresponding author. Tel.: +1-306-966-4772; fax: +1-306-966-4730.
E-mail address: soledade.pedras@usask.ca (M.S.C. Pedras).

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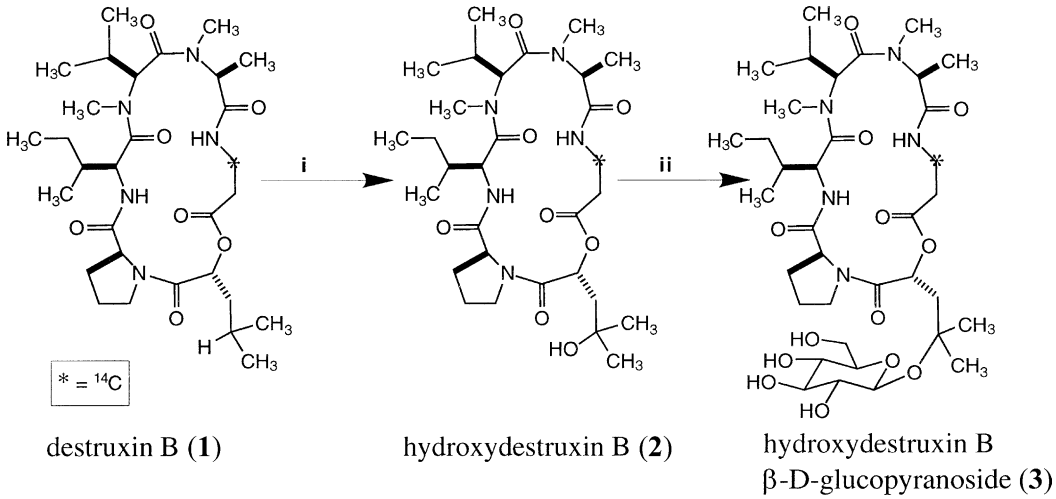

Fig. 1. Metabolism of the host-selective toxin destruxin B by crucifers: (i) hydroxylation; (ii) glucosylation (Pedras et al., 2001).

Capsella bursa-pastoris may prevent *A. brassicae* from biosynthesizing destruxin B (**1**). In this situation, destruxin B (**1**) detoxification could be a redundant resistance mechanism. Consequently, it is of great interest to establish the metabolism of destruxin B (**1**) in these wild crucifers to probe the correlation between destruxin B (**1**) detoxification and Alternaria blackspot resistance, as well as the potential conservation of this detoxification pathway across crucifers. In this paper we report that in *Camelina sativa*, *Capsella bursa-pastoris*, and *Eruca sativa* (rocket) (Conn et al., 1988; Conn and Tewari, 1986), destruxin B was metabolized via identical intermediates, although the rate-limiting steps were different. In addition, the chemical structure of a new product of destruxin B (**1**), metabolism i.e. (6′-*O*-malonyl)hydroxydestruxin B β-D-glucopyranoside is reported.

2. Results and discussion

Previous work (Pedras et al., 2001) established that hydroxydestruxin B (**2**) was converted to hydroxydestruxin B β-D-glucopyranoside (**3**) in four species: blackspot resistant *S. alba* and blackspot susceptible *B. napus*, *B. juncea*, and *B. rapa*. Hydroxydestruxin B β-D-glucopyranoside (**3**) was also slowly metabolized to a relatively more polar metabolite X (HPLC $t_R = 3.8$ min, Table 1). Subsequent studies using leaves of *E. sativa* indicated that hydroxydestruxin B (**2**) was directly converted to this polar metabolite. Thus, it became essential to establish the chemical structure of this additional destruxin B metabolite.

2.1. Isolation and structure determination of metabolite X

Radiolabeled metabolite X obtained from extracts of *B. napus* leaves incubated with ^{14}C-labeled hydroxydestruxin B (**2**) was isolated by HPLC, as described in the Section 3. Metabolite X gave hydroxydestruxin B β-D-glucopyranoside (**3**) on standing in MeOH at room temperature. A pure sample of ^{14}C-labeled metabolite X was completely converted into hydroxydestruxin B β-D-glucopyranoside (**3**) within 48 h at 5 °C. For structure determination large scale experiments were carried out with nonlabeled hydroxydestruxin B (**2**) utilizing leaves of *B. napus*, as described in Section 3. Both MS-FAB analysis and continuous flow FAB mass spectrum of this sample suggested a molecular ion of *m*/*z* 858 [$M^+ + 1$] mass units. The exact mass measured by static FAB (858.4349 Da). The difference of 86 (858–772) mass units between metabolite X and hydroxydestruxin B β-D-glucopyranoside (**2**) (772) suggested the presence of a $C_3H_3O_3$ unit attached to the glucosyl residue. Consequently we propose the structure shown on Fig. 2 for metabolite X, resulting from malonyl esterification at the 6′-*O*- position of glucose, as this is the most reactive hydroxyl group of glucose. However, alternate structures having the malonyl residue at either 2′-, 3′-, or 4′-*O* positions cannot be ruled out. The proposed structure is consistent with the observed lability of the

Table 1
HPLC retention times of ^{14}C-labeled destruxin B and metabolites derived from its biotransformation by cruciferous species

Compound	HPLC retention time ($t_R \pm 0.1$ min)[a]
^{14}C-labeled destruxin B	19.9
^{14}C-labeled hydroxydestruxin B	10.4
^{14}C-labeled hydroxydestruxin B β-D-glucopyranoside	6.8
^{14}C-labeled destruxin B metabolite X	3.8

[a] Detection carried out with a flow scintillation analyzer fitted with a 210 μl high performance flow cell Solarscint™; Hypersil ODS column (5 μm particle size silica, 4.6 i.d.×200 mm), mobile phase: H_2O–CH_3CN, 75:25–100% CH_3CN, for 35 min, linear gradient, and 1.0 ml/min flow rate; for other conditions see Section 3.

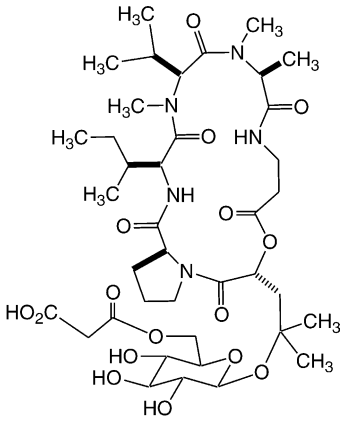

Fig. 2. Chemical structure of metabolite X: (6′-*O*-malonyl)hydroxydestruxin B β-D-glucopyranoside (**4**).

malonyl–glucosyl bond and the relative polarity of metabolite X. Malonylation of glucosides is not an uncommon detoxification process in plants (Withopf et al., 1997). For example, glucosyl- and malonyl-transferases can participate in selective herbicide detoxification by replacing -OH, -NH_2, -SH or -COOH groups. The herbicide-inactivating enzymes are often preferentially present in crop plants, but certain weeds and other wild plant species, in particular marine macroalgae, also appear to have high transferase activities (Sanderman et al., 1997).

2.2. Metabolism of destruxin B in Camelina sativa, Capsella bursa-pastoris, and Eruca sativa

Metabolism of destruxin B (**1**) in leaves of *C. sativa*, *C. bursa-pastoris*, and *E. sativa* was investigated using conditions similar to previous time-course experiments (Pedras et al., 2001). Radiolabeled compounds were synthesized as described previously (Pedras et al., 2001). A solution of ^{14}C-labeled destruxin B (**1**) was administered to leaves of *Camelina sativa* and, after incubation for different periods of time, leaves were individually extracted and the extracts analyzed by LSC and HPLC (Table 2). LSC results showed that 65–100% of the radioactivity used per leaf was recovered in the leaf extract. The HPLC chromatograms (radiodetection) showed that destruxin B (**1**) (t_R = 19.9 min), was slowly biotransformed (ca. 22% in 48 h, Table 2) to a metabolite with t_R = 6.8 min (chromatograms shown on Fig. 3 A). This metabolite was established to be hydroxydestruxin B β-D-glucopyranoside (**3**) upon purification and comparison of its UV spectrum and HPLC retention times with those of an authentic sample available in our laboratory (Pedras et al., 2001). These results suggested that *Camelina sativa* metabolized destruxin B to hydroxydestruxin B β-D-glucopyranoside (**3**); however, HPLC chromatograms of extracts from time-course experiments did not show the presence of the intermediate hydroxydestruxin B (**2**). A further administration experiment using ^{14}C-labeled hydroxydestruxin B (**2**) showed its rapid transformation (ca. 72% in 24 h, Table 3) to ^{14}C-labeled glucoside and to the malonylated derivative (chromatograms shown in Fig. 3B). These results confirmed that *Camelina sativa* had the enzymes responsible for complete transformation of destruxin B (**1**) (Tables 2 and 3). The phytoalexin camalexin (t_R = 16.5 min, broad peak) was not detected in the HPLC chromatograms (photodiode array detection) of extracts from time-course experiments and of fractions resulting from purification steps, suggesting that under these experimental conditions destruxin B (**1**) does not elicit production of phytoalexins in *Camelina sativa*.

Table 2
LSC and HPLC results[a] for leaf extracts of *Camelina sativa* incubated with ^{14}C-labeled destruxin B

Incubation time (h)	% Conversion of destruxin B (HPLC)[b]	% Radioactivity recovered in leaf extracts (LSC)[c]
0[d]	n. d.[e]	Complete recovery
24	<10	90±3
48	22±6	72±8
120	44±17	65±9
168	69±3	78±3

[a] Results are averages of experiments conducted in triplicate.
[b] Area of peaks is determined in counts and converted to dpm using a calibration curve. Results are presented as mean±standard deviation.
[c] LSC results are presented within 95% confidence limits.
[d] Time 0 is considered immediately after complete toxin uptake—ca. 12 h.
[e] n. d. = not detected.

Additional time-course experiments using leaves of *Capsella bursa-pastoris* (Fig. 4, Table 4) and *E. sativa* (Fig. 5A and B, Tables 5 and 6) were carried out as summarized above for *Camelina sativa* and described in Section 3. The metabolism of destruxin B (**1**) in these two species gave metabolites identical to those produced in *Camelina sativa*, as established from their UV spectra and t_R (HPLC) in different solvent systems. While *Capsella bursa-pastoris* (Fig. 4, Table 4) metabolized ca. 82% of destruxin B (**1**) in 24 h, *E. sativa* metabolized only ca. 63% of destruxin B (**1**) in 120 h (Fig. 5A, Table 5). Interestingly, *E. sativa* metabolized hydroxydestruxin B (**2**) with higher accumulation of its malonyl glucoside (**4**) (ca. 30% in 120 h, Table 6) than *Camelina sativa* or *Capsella bursa-pastoris* (Fig. 5B). These results suggest that in *E. sativa* the rate of malonylation is faster than glycosylation.

The overall results of the metabolism of ^{14}C-destruxin B in leaves of *Camelina sativa*, *Capsella bursa-pastoris* and *E. sativa* showed that *Capsella bursa-pastoris* metabolized destruxin B (**1**) to hydroxydestruxin B glucoside (**3**) substantially faster than the other two species, even though uptake of toxin solutions in *Capsella bursa-pastoris* was slower. We attribute this slower uptake to the

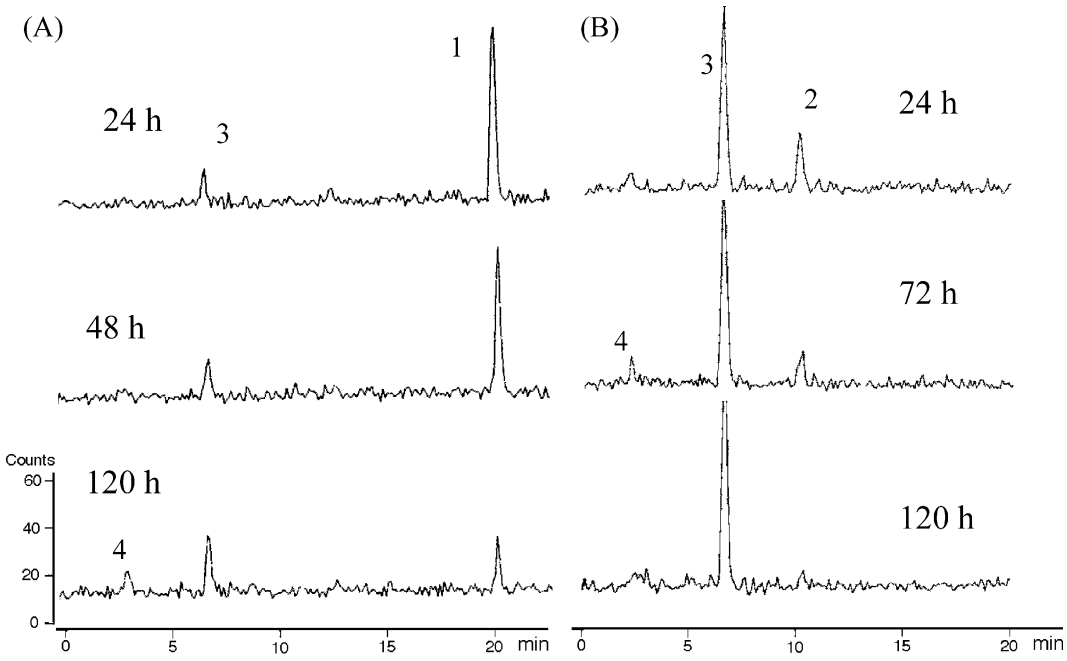

Fig. 3. HPLC chromatograms (Radiomatic detector) of leaf extracts of *Camelina sativa* incubated with: A—[^{14}C]destruxin B; B—[^{14}C]hydroxydestruxin B **2** (see Section 3); 0 h time immediately after complete uptake (12 h); **1** = destruxin B (t_R = 19.9 min), **2** = hydroxydestruxin B (t_R = 10.4 min); **3** = hydroxydestruxin B β-D-glucopyranoside (t_R = 6.8 min); **4** = (6′-*O*-malonyl)hydroxydestruxin B β-D-glucopyranoside (t_R = 3.8 min).

Table 3
LSC and HPLC results[a] for leaf extracts of *Camelina sativa* incubated with ^{14}C-labeled hydroxydestruxin B

Incubation time (h)	% Conversion of hydroxydestruxin B **2** to glucoside **3** (HPLC)[b]	% Conversion of hydroxydestruxin B **1** to malonylated glucoside **4** (HPLC)[b]	% Radioactivity recovered in leaf extracts (LSC)[c]
0[d]	47±9	n. d. [e]	Complete recovery
24	72±24	<10	94±3
72	74±6	<10	94±3
120	89±11	16±9	89±6

[a] Results are averages of experiments conducted in triplicate.
[b] Area of peaks is determined in counts and converted to dpm using a calibration curve. Results are presented as mean±standard deviation.
[c] LSC results are presented within 95% confidence limits.
[d] Time 0 is considered immediately after complete toxin uptake—ca. 12 h.
[e] n. d. = not detected.

absence of petioles and a smaller leaf area; leaves of *Capsella bursa-pastoris* showed no macroscopic signs of stress relative to controls. Furthermore, *E. sativa* transformed hydroxydestruxin B glucoside (**3**) to the malonylated derivative (**4**) substantially faster than *Camelina sativa* or *Capsella bursa-pastoris*. Comparison of these results with our previous work (Pedras et al., 2001) demonstrates that the transformation of destruxin B in *Camelina sativa* (69% in 168 h) was much slower than in *S. alba* (ca. 100% in 48 h). Although *Camelina sativa* and *Capsella bursa-pastoris* produce the phytoalexin camalexin, which is highly inhibitory to *A. brassicae*,

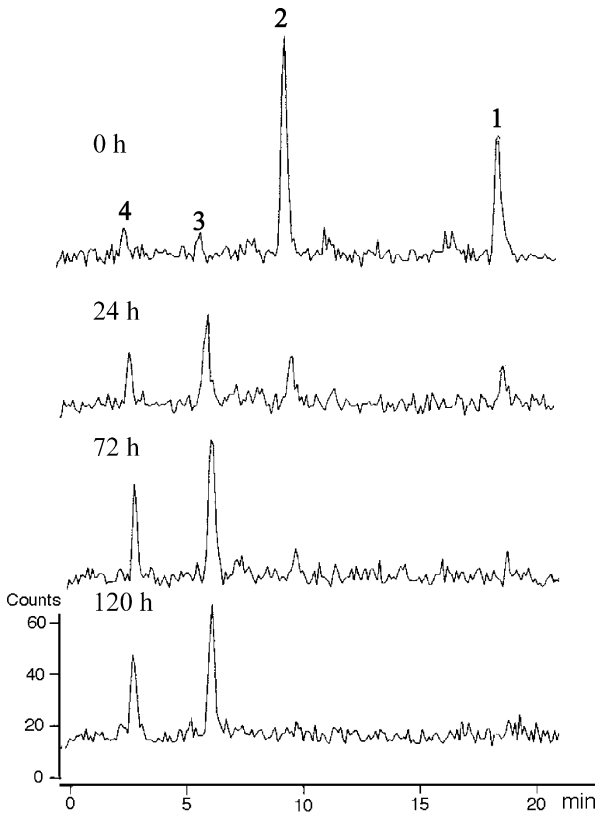

Fig. 4. HPLC chromatograms (Radiomatic detector) of leaf extracts of *Capsella bursa-pastoris* incubated with [^{14}C]destruxin B (see Section 3); 0 h time immediately after complete uptake (23 h); **1** = destruxin B (t_R = 19.9 min), **2** = hydroxydestruxin B (t_R = 10.4 min); **3** = hydroxydestruxin B β-D-glucopyranoside (t_R = 6.8 min); **4** = (6′-*O*-malonyl)-hydroxydestruxin B β-D-glucopyranoside (t_R = 3.8 min).

Table 4
LSC and HPLC results[a] for leaf extracts of *Capsella bursa-pastoris* incubated with ^{14}C-labeled destruxin B

Incubation time (h)	% Conversion of destruxin B (HPLC)[b]	% Radioactivity recovered in leaf extracts (LSC)[c]
0[d]	72±10	Complete recovery
24	82±4	80±5
72	87±3	89±8
120	c. t.[e]	93±4

[a] Results are averages of experiments conducted in duplicate.
[b] Area of peaks is determined in counts and converted to dpm using a calibration curve. Results are presented as mean±standard deviation.
[c] LSC results are presented within 95% confidence limits.
[d] Time 0 is considered immediately after complete toxin uptake—ca. 23 h.
[e] c. t. = complete transformation.

these wild species metabolize destruxin B (**1**) similar to cultivated species; moreover, phytoalexins did not appear to be elicited by destruxin B or hydroxydestruxin B (**2**). In conclusion, our work has shown that the destruxin B (**1**) detoxification pathway present in *S. alba* is also present in three other unrelated species which suggests a conservation of this pathway across crucifers. Considering that *Camelina sativa* and *Capsella bursa-pastoris* detoxify destruxin B (**1**) and produce the phytoalexins camalexins, these wild crucifers appear to represent unique and perhaps useful sources of Alternaria blackspot resistance in strategic plant breeding.

3. Experimental

3.1. General

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON. Destruxin B (Ward et al., 2001), hydroxydestruxin B (**2**), ^{14}C-destruxin B, and ^{14}C-hydroxydestruxin B (Pedras et al., 2001), were prepared by total synthesis as previously described. Analytical TLC aluminum sheets 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 (w/v) phosphomolybdic acid solution containing 1% (w/v) ceric sulfate and 4% (v/v) H_2SO_4, followed by heating. FCC C-18 reversed phase silica gel 40 μm (JT Baker WP).

HPLC analysis was carried out with a high performance Hewlett Packard liquid chromatograph equipped with a quaternary pump, automatic injector, and photodiode array detector (wavelength range 190–600 nm) connected in series with a Canberra Packard Radiomatic 150TR flow scintillation analyzer (ca. 0.5±0.1

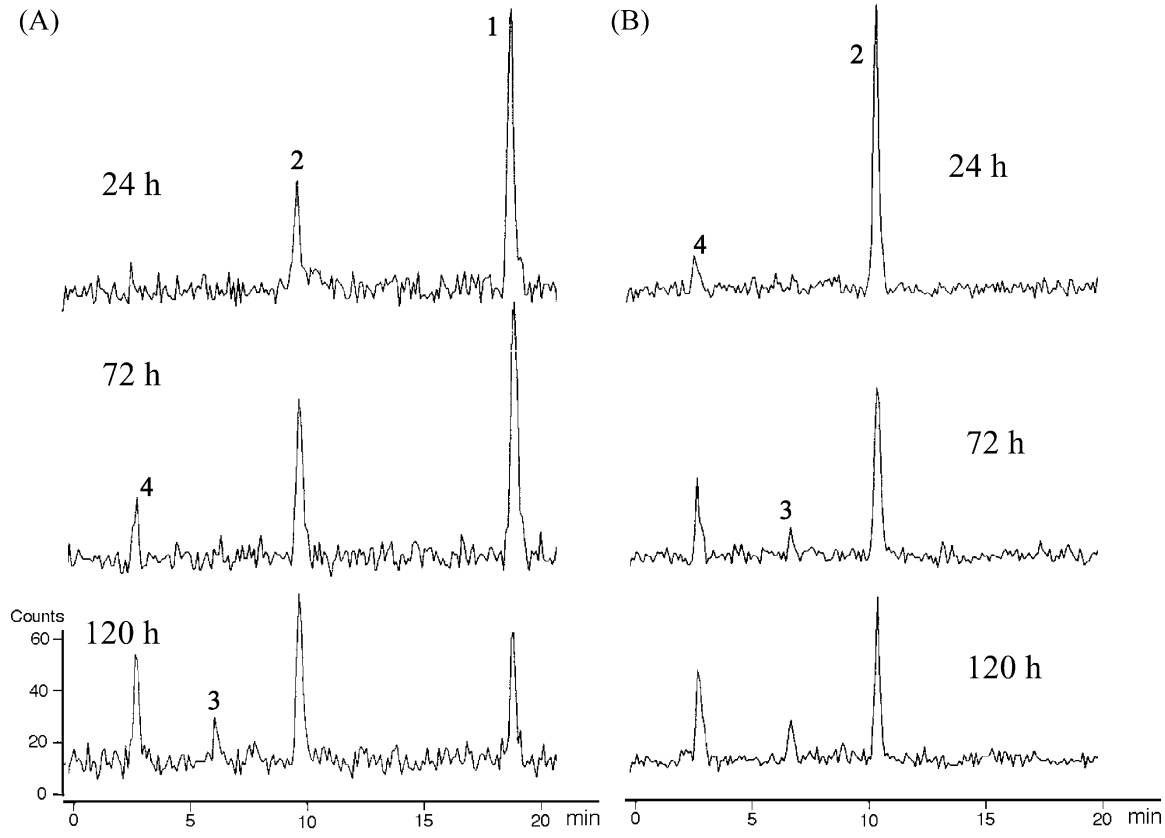

Fig. 5. HPLC chromatograms (Radiomatic detector) of leaf extracts of *Eruca sativa* incubated with: **A**—[^{14}C]destruxin B; **B**—[^{14}C]hydroxydestruxin B (see Section 3); 0 h time immediately after complete uptake (8 h); 1 = destruxin B (t_R = 19.9 min), 2 = hydroxydestruxin B (t_R = 10.4 min); 3 = hydroxydestruxin B β-D-glucopyranoside (t_R = 6.8 min); 4 = (6′-*O*-malonyl)hydroxydestruxin B β-D-glucopyranoside (t_R = 3.8 min).

Table 5
LSC and HPLC results[a] for leaf extracts of *Eruca sativa* incubated with ^{14}C-labeled destruxin B

Incubation time (h)	% Conversion of destruxin B (HPLC)[b]	% Radioactivity recovered in leaf extracts (LSC)[c]
0[d]	n. d.[e]	87±11
24	18±6	89±14
72	38±12	72±15
120	63±8	74±15

[a] Results are averages of experiments conducted in triplicate.
[b] Area of peaks is determined in counts and converted to dpm using a calibration curve. Results are presented as mean±standard deviation.
[c] LSC results are presented within 95% confidence limits.
[d] Time 0 is considered immediately after complete toxin uptake—ca. 8 h.
[e] n. d. = not detected.

Table 6
LSC and HPLC results[a] for leaf extracts of *Eruca sativa* incubated with ^{14}C-labeled hydroxydestruxin B

Incubation time (h)	% Conversion of hydroxydestruxin B **2** to destruxin B glucoside **3** (HPLC)[b]	% Conversion of hydroxydestruxin B **2** to malonylated glucoside **3** (HPLC)[b]	% Radioactivity recovered in leaf extracts (LSC)[c]
0[d]	n. c.[e]	n. c.[e]	Complete recovery
24	<10	12±4	Complete recovery
72	12±1	25±2	96±4
120	18±2	30±6	94±6

[a] Results are averages of experiments conducted in triplicate.
[b] Area of peaks is determined in counts and converted to dpm using a calibration curve. Results are presented as mean±standard deviation.
[c] LSC results are presented within 95% confidence limits.
[d] Time 0 is considered immediately after complete toxin uptake—ca. 8 h.
[e] n. c. = no conversion.

min delay, fitted with a 210 μl high performance flow cell Solarscint™), degasser, and a Hypersil 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, except where stated otherwise. Samples were dissolved either in CH_3CN or in MeOH. The ^{14}C counting window was 15–100 keV with an update time of 6 sec.

LSC was performed on a Beckman LS-6500 connected to a Wyse WY-370 data system and results are quench corrected and reported in dpm.

MS spectra were obtained on a VG 70 SE mass spectrometer; HR-FAB was obtained at 70 eV employing a solids or continuous flow probe.

3.2. Time-course of metabolism of destruxins

Seeds of *Camelina sativa* and *Capsella bursa-pastoris* were obtained from Plant Gene Resources, Agriculture and Agri Food Canada, Saskatoon, SK; seeds of *Eruca sativa* were purchased from a commercial seed company. 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.

Leaves (21-day old plants of *Eruca sativa* and *Camelina sativa*, 45-day old plants for *Capsella bursa-pastoris*) were cut at the base of the petiole and immediately placed in Eppendorf tubes containing ^{14}C-labeled destruxin B (8.4×10^4 dpm, 1 ml solution 2×10^{-5} M in 2% aqueous CH_3CN per leaf for *C. sativa;* 7.4×10^4 dpm, 1 ml solution 9.4×10^{-6} M in 2% aqueous CH_3CN per leaf for *E. sativa*; 8.3×10^4 dpm, 1 ml solution 1.0×10^{-5} M in 2% aqueous CH_3CN per leaf for *Capsella bursa-pastoris*) or ^{14}C-labeled hydroxydestruxin B (total amount 6.1×10^4 dpm, 1 ml solution 9.0×10^{-6} M in 2% aqueous CH_3CN per leaf for *E. sativa*; 9.2×10^4 dpm, 1 ml solution 1.3×10^{-5} M in 2% aqueous CH_3CN per leaf for *Camelina sativa)*. After the solution was taken up (i.e. no solution remaining in tube), additional water was added (0.5 ml), to ensure the complete uptake of compound (after uptake is complete = t_0: 12 h for *Camelina sativa,* 8 h for *E. sativa* and 23 h for *Capsella bursa-pastoris)*. Leaves were transferred to an incubator under fluorescent lighting (16 h light/8 h dark cycle, 20±0.5 °C) and incubated for different times, while keeping the base immersed in water. After incubation, leaves were individually frozen in liquid N_2, crushed with a glass rod and extracted with MeOH (50 ml). After shaking for 12 h, the solvent was filtered and leaf solids were rinsed with fresh MeOH (20 ml). Combined filtrate and rinsing was concentrated under reduced pressure.

3.3. Analysis of phytoalexins

The phytoalexins camalexin (t_R = 16.5 min, broad peak), 6-methoxycamalexin (t_R = 23.4 min, broad peak) and 1-methylcamalexin (t_R = 22.2 min), known to be produced in leaves of *Camelina sativa* and *Capsella bursa-pastoris* when elicited with *A. brassicae* (Pedras et al., 2000), were not detected by HPLC analysis (using the conditions described above) of leaf extracts of each plant incubated with destruxin B or hydroxydestruxin B. Comparison of HPLC chromatograms (direct comparison of retention times and UV spectra of each peak with those of authentic samples synthesized as previously reported, Pedras and Ahiahonu, 2002) of leaf extracts of each plant species with those of non-elicited leaves indicated the absence of peaks due to camalexins.

3.4. Isolation of metabolite X: (6′-O-malonyl)hydroxydestruxin B β-D-glucopyranoside

The extraction and isolation protocol was designed and optimized following two small scale experiments

utilizing radiolabeled material. After incubation of leaves (95 leaves) with ^{14}C-hydroxydestruxin B **2** for 96 h, the leaves were extracted with hexane followed by MeOH, and each extract counted in an LSC. HPLC analysis (Radiomatic detector) indicated that metabolite X ($t_R = 3.8$ min) was mostly contained in the MeOH extract. This extract was diluted with water, and extracted sequentially with EtOAc and butanol (contained metabolite X). The HPLC profile of the butanol extract indicated that the new metabolite co-eluted with a complex mixture of polar compounds, and thus, would present a difficult isolation. Subsequently, the purification protocol was developed and optimized on fraction F_2 (containing the metabolite X with $t_R = 3.8$ min) resulting from FCC fractionation (flash column chromatography, silica gel RP C-18, using gradient elution with H_2O–CH_3CN, fraction F_2 eluted with H_2O–CH_3CN, 80:20) of the butanol extract. Following the separation steps for a small scale separation of radioactive material, a larger scale experiment was devised as follows. Leaves (720) of 21-day old plants of *B. napus* (cv. Westar) were cut at the base of the petiole and immediately placed in a solution of hydroxydestruxin B (**2**) 2×10^{-5} M in 2% aqueous CH_3CN (ca. 8.8 mg hydroxydestruxin B (**2**) used). After the solution was taken up, additional water (1 ml) was added, to ensure complete uptake of compound. Leaves were transferred to an incubator under fluorescent lighting (16 h light/8 h dark cycle, 20 ± 0.5 °C) and incubated for 96 h, while keeping the base of the petiole in water. Leaves were frozen in liquid N_2, crushed with a glass rod and extracted with hexane (800 ml). After shaking for 1.5 h, the solvent was filtered and leaf solids were rinsed with fresh hexane (100 ml), the combined filtrate and rinsing being further concentrated under reduced pressure. The leaf solids were further extracted with MeOH (500 ml) for 16 h, then filtered and concentrated under reduced pressure. The residue (58.2 g) was dissolved in water (100 ml) and extracted with EtOAc (2×200 ml). After re-concentration under reduced pressure of remaining aqueous layer, the residue was redissolved in water (150 ml) and extracted with butanol (2×300 ml). The butanol extract (4.93 g) was divided in equal portions (ten) which were identically fractionated by FCC (silica gel RP C-18). The residue from combined fractions (345.5 mg, eluted with H_2O–CH_3CN, 80:20) was divided in equal portions (ten) which were identically fractionated by FCC (silica gel RP C-18). Combined fractions F_8 (121.1 mg, eluted with H_2O–CH_3CN, 80:20) were further fractionated by FCC (silica gel RP C-18) into 25 fractions. Fractions F_4–F_{14} (eluted with H_2O–CH_3CN, 85:15) were individually fractionated on the analytical HPLC column eluted with H_2O-CH_3CN (gradient of 100–70% water for 20 min, followed by linear elution with 70% H_2O–30% CH_3CN for 10 min); combined fractions collected between 14–19 min of elution were fractionated twice by HPLC to give (6′-*O*-malonyl)hydroxydestruxin B β-D-glucopyranoside **4** (ca. 100 μg).

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