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Chemical defenses of crucifers: elicitation and metabolism of phytoalexins and indole-3-acetonitrile in brown mustard and turnip

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Abstract

The metabolism of the cruciferous phytoalexins brassinin and cyclobrassinin, and the related compounds indole-3-carboxaldehyde, glucobrassicin, and indole-3-acetaldoxime was investigated in various plant tissues of *Brassica juncea* and *B. rapa*. Metabolic studies with brassinin showed that stems of *B. juncea* metabolized radiolabeled brassinin to indole-3-acetic acid, via indole-3-carboxaldehyde, a detoxification pathway similar to that followed by the "blackleg" fungus (*Phoma lingam*/*Leptosphaeria maculans*). In addition, it was established that tetradeuterated brassinin was incorporated into the phytoalexin brassilexin in *B. juncea* and *B. rapa*. On the other hand, the tetradeuterated indole glucosinolate glucobrassicin was not incorporated into brassinin, although the chemical structures of brassinins and indole glucosinolates suggest an interconnected biogenesis. Importantly, tetradeuterated indole-3-acetaldoxime was an efficient precursor of phytoalexins brassinin, brassilexin, and spirobrassinin. Elicitation experiments in tissues of *Brassica juncea* and *B. rapa* showed that indole-3-acetonitrile was an inducible metabolite produced in leaves and stems of *B. juncea* but not in *B. rapa*. Indole-3-acetonitrile displayed antifungal activity similar to that of brassilexin, was metabolized by the blackleg fungus at slower rates than brassinin, cyclobrassinin, or brassilexin, and appeared to be involved in defense responses of *B. juncea*. © 2002 Published by Elsevier Science Ltd.

Keywords: *Brassica juncea*; *B. rapa*; Cruciferae; Brassinin; Cyclobrassinin; Glucobrassicin; Indole-3-acetaldoxime; Indole-3-carboxaldehyde; Indole glucosinolate; Blackleg disease

1. Introduction

Pathogen attack affects plant development and may have a strong negative impact on the quality and production of crops. Plants fight pathogens with an enormous and complex arsenal of defense mechanisms. A significant aspect of these defense mechanisms involves biosynthesis of secondary metabolites, which may be either constitutive such as phytoanticipins (VanEtten et al., 1994) or biosynthesized de novo, i.e. phytoalexins (Smith, 1996; Brooks and Watson, 1985). The important role of phytoalexins and phytoanticipins in plant defense responses is becoming better understood with the development of new transgenic and mutant organisms. Perhaps one of the most convincing arguments in favor of phytoalexins is the genetic engineering of the phytoalexin stilbene in tobacco plants which became more resistant to fungal infection than the wild-type plants (Hain et al., 1993). On the other hand, a phytoalexin-deficient mutant of *Arabidopsis thaliana* showed significantly higher susceptibility to the fungus *Alternaria brassicicola* than the wild-type parental plants (Thomma et al., 1999). Consequently, a large number of biological and chemical studies are directed to secondary metabolism of plants of economic importance. For example, metabolites from the plant family Cruciferae, which comprises a large number of economically important oilseed crops and vegetables, are being widely investigated (Bennett and Wallsgrove, 1994). Besides their economic importance, crucifers are also interesting plant model-systems, containing the first and only example to date of a completely sequenced plant genome (The *Arabidopsis* Genome Initiative, 2000).

Chemical characterization of secondary metabolites from crucifers has unraveled a remarkable array of phytoalexins (e.g. **1**–**4**) (Pedras et al., 2000), as well as a

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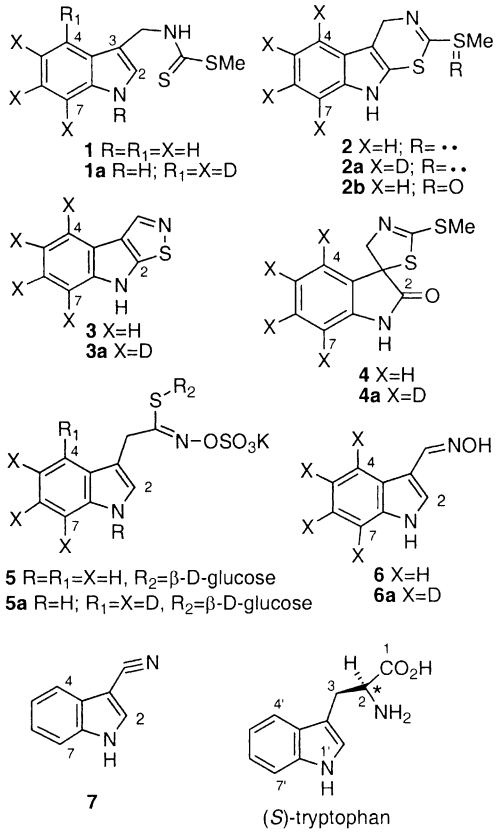


major group of secondary metabolites known as glucosinolates (e.g. **5**), (Fahey et al., 2001) both of which contain sulfur and nitrogen. In fact, the unique structure of the phytoalexin brassinin (**1**) resembles brassicin (**5**, R = H), the aglycone portion of indole glucosinolate **5** (i.e. glucobrassicin). This structural connection becomes more transparent considering that methoxy derivatives of brassinin (e.g. **1**, R = OMe, R_1 = H and **1**, R = H and R_1 = OMe) and glucobrassicin (e.g. **5**, R = OMe, R_1 = H, R_2 = β-D-glucosyl and **5**, R = H, R_1 = OMe and R_2 = β-D-glucosyl) are naturally occurring within the same plant species and that their concentration levels increase simultaneously in plants subjected to stress (Monde et al., 1991b). Furthermore, unambiguous biosynthetic studies have demonstrated that (*S*)-tryptophan is the precursor of both brassinin (**1**) and glucobrassicin (**5**). Most importantly, the C-2 of [2-^{13}C]-tryptophan was incorporated into the thiocarbonyl carbon of brassinin (**1**), demonstrating that potential intermediates must contain a 2-carbon unit at position C-3 of the indole ring (Monde et al., 1994). In this connection a number of suggestions and attempts to establish a biogenetic relationship between indole glucosinolates such as glucobrassicin (**5**) and cruciferous phytoalexins have been reported (Hanley et al., 1990). Furthermore, a number of studies have demonstrated that (*S*)-tryptophan is converted to **5** via indole-3-acetaldoxime (**6**) (Halkier and Du, 1997). Paradoxically, because glucosinolates are considered an undesirable group of metabolites in brassicas, a large number of oilseed breeding programs are directed at obtaining plants containing low amounts of glucosinolates. If, however, indole glucosinolates such as glucobrassicin (**5**) are precursors of cruciferous phytoalexins (brassinin is a precursor of **2**–**4**), from a plant defense perspective lowering glucosinolate contents in cruciferous crops may pose a substantial ecological risk. Nonetheless, despite a number of biosynthetic studies in crucifers, it is not ascertained whether or not this biogenetic relationship exists (Pedras et al., 2000). Thus we became interested in establishing the possible biogenetic relationship between indole glucosinolate **5** and brassinin (Pedras et al., 2001).

Moreover, the resistance of brown mustard (*Brassica juncea*) to the devastating blackleg disease [fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. et Not. asexual stage *Phoma lingam* (Tode ex Fr.) Desm] was reported to correlate with the accumulation of the phytoalexin brassilexin (**3**) (Rouxel et al., 1991). However, since *P. lingam* can rapidly metabolize and detoxify brassilexin (**3**) (Pedras and Okanga, 1999), it is suspected that additional antifungal metabolites are produced in brown mustard. We have now discovered that indole-3-acetonitrile (**7**) is an inducible metabolite likely involved in defense responses of brown mustard (*B. juncea*). Furthermore, we have also established the biosynthetic relationships among several secondary metabolites of *Brassica* species, namely indole-3-acetaldoxime (**6**) and phytoalexins **1**–**4**, as well as the metabolic degradation of [2-^{14}C]-brassinin in plant tissues. Here we report for the first time the syntheses of tetradeuterated aldoxime (**6a**), tetradeuterated glucobrassicin (**5a**), and radiolabeled brassinin (**1**), as well as results of the metabolic studies with these compounds and propose a new role for indole-3-acetonitrile (**7**).

2. Results and discussion

2.1. Phytoalexin elicitation in different plant tissues: cell suspension cultures, stems, leaves and roots

Previous experiments with leaves of *Brassica carinata* elicited with *P. lingam* demonstrated that both [4,5,6,7-D_4]-brassinin (**1a**) and [4,5,6,7-D_4]-cyclobrassinin (**2a**) were incorporated into brassilexin (**3a**) (Pedras et al., 1998). These results, however, indicated that experiments involving leaf uptake of solutions containing these phytoalexins were difficult to carry out due to the toxicity of the substrates to leaf petioles. Subsequently, we searched for a tissue to conduct biosynthetic studies in brassica oilseeds. Brown mustard (*B. juncea*) was chosen due to its resistance to blackleg disease and production of phytoalexins **1**–**4** and indole glucosinolate **5**. Cell suspension cultures of *B. juncea* can be produced

Table 1
Elicitation of phytoalexins in various plant tissues of *Brassica juncea* and *B. rapa*

Tissue (species)	Elicitor	Total amount of phytoalexins[a] per 100 g fresh tissue
Cell suspension cultures (*B. juncea*)	Fungal spores	Not detected
	Yeast extract	Not detected
	Jasmonic acid	Not detected
Stems (*B. juncea*)	Fungal spores	$8–10\times10^{-1}$ μmol of **2** and **3** after 3-day incubation
	$CuCl_2$ uptake	9–18 μmol of **1**–**4** after 5-day incubation
	Destruxin B	$<10^{-1}$ μmol of **4** after 24-h incubation
Leaves (*B. juncea*)	Fungal spores	$5–10\times10^{-1}$ μmol of **2**–**4** after 2-day incubation
	$CuCl_2$ uptake	$1–3\times10^{-1}$ μmol of **4** after 4-day incubation
Leaves (*B. rapa*)	$CuCl_2$ spray	1–2 μmol of **4** after 3-day incubation
Roots (*B. rapa*)	UV radiation	$1–5\times10^{-1}$ μmol of **2**–**4** after 3-day incubation

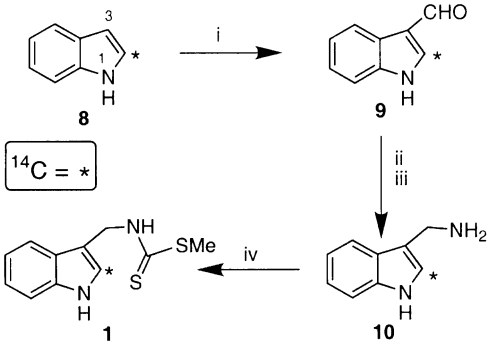
[a] Phytoalexins were extracted as described in Experimental and quantities were calculated from calibration curves ($R^2>0.999$).

in large quantities and compounds can be added to the cultures under sterile conditions (Pedras and Biesenthal, 2000), and thus constitute useful systems to study the phytoalexin pathway. In addition, because leaves represent a very complex matrix, cell suspension cultures are simpler systems to analyze and isolate metabolites. Despite these advantages, to date there are no published examples of phytoalexin production in cell suspension cultures of *Brassica* species. Thus, cell suspension cultures prepared from mesophyll tissue (apical meristem) of *B. juncea* were elicited with a range of common elicitors (Table 1). The cell suspension culture density, temperature and lighting were also varied (Zook, 1998), however, no phytoalexins were ever detected in our cell cultures (HPLC analysis with photodiode array detection).

Due to our inability to elicit phytoalexins in cell cultures, several elicitation experiments were conducted with stems, leaves and roots of *B. juncea* and *B. rapa* (turnip), as shown in Table 1. Stems were first investigated since phytoalexins were readily detected in their MeOH extracts, which were less complex than those of leaves. Stems of *B. juncea* were elicited (Table 1) as described in Experimental. Stem tissue was extracted after different incubation periods and the resulting extracts were analyzed by HPLC. In stems elicited with *P. lingam*, cyclobrassinin (**2**) was detected after 15 h of incubation whereas cyclobrassinin sulfoxide (**2b**) and brassilexin (**3**) were detected after 24 h. All three phytoalexins were detected in the stem extracts up to 3 days (duration of experiment). By contrast, phytoalexins were not detected in $CuCl_2$ elicited stems until after 3 days of incubation. In addition to cyclobrassinin (**2**), cyclobrassinin sulfoxide (**2b**), and brassilexin (**3**), the phytoalexins brassinin (**1**) and spirobrassinin (**4**) were also detected in $CuCl_2$ treated stems. In stems elicited with destruxin B only cyclobrassinin (**2**) was detected, but phytoalexins were not detected in stems treated with either salicylic acid, jasmonic acid or distilled H_2O (controls). Although slower, phytoalexin accumulation was relatively higher in stems elicited with $CuCl_2$ (Table 1). In stems elicited with *P. lingam* the highest quantity of brassilexin (**3**) was detected after 48 h of incubation ($4–5\times10^{-1}$ μmol/100 g fresh stem tissue), whereas elicitation with $CuCl_2$ (6–11 μmol/100 g fresh stem tissue) led to maximum phytoalexin production after 5 days.

Detached leaves from 3-week-old *B. juncea* were elicited by uptake of aq. $CuCl_2$ or with spore suspensions, as described in Experimental, and the extracts analyzed by HPLC (Table 1). Maximum phytoalexin production was observed 48 h after spore elicitation; brassilexin (**3**) was detected after 24 h of incubation and increased up to 48 h ($1–5\times10^{-1}$ μmol/100 g fresh leaf tissue), whereas spirobrassinin (**4**) amounts appeared to be constant after 24 h of incubation (1–5 μmol/100 g fresh leaf tissue). Leaves elicited with a $CuCl_2$ solution (1×10^{-3} M) were completely wilted after 12 h of incubation but lower concentrations induced production of spirobrassinin (**4**), the only phytoalexin detected ($0.5–3\times10^{-1}$ μmol/100 g fresh leaf tissue).

Root slices of *B. rapa* prepared as described in the Experimental were incubated, extracted and the resulting extracts were analyzed by HPLC (Table 1). Phytoalexins appeared to be present in larger amounts after



Scheme 1. Synthesis of [2-^{14}C]-brassinin (**1**): (i) $POCl_3$, DMF; (ii) $NH_2OH{\cdot}HCl$, NaOAc; (iii) $NaCNBH_3/TiCl_3$; (iv) pyridine/NEt_3, CS_2; MeI.

Table 2
Metabolism of brassinin (**1**/**1a**), cyclobrassinin (**2a**), indole-3-carboxaldehyde (**9**), glucobrassicin (**5a**), and indole-3-acetaldoxime (**6a**), in *Brassica juncea* and *B. rapa*

Precursor	Tissue, incubation time (*Brassica* species/elicitor)	Labeled metabolites (total amount of ^{14}C or D_4 incorporation[a])
[2-^{14}C]-Brassinin (**1**)	Stems, 4 days (*B. juncea*/fungal spores or $CuCl_2$ uptake)	**9** (1% of total ^{14}C-brassinin fed); **11** (1% of total ^{14}C-brassinin fed); **12** (1% of total ^{14}C-brassinin fed)
[4,5,6,7-D_4]-Brassinin (**1a**)	Leaves, 2 days, (*B. juncea*/fungal spores)	**3** (2.5% of D_4 incorporation); **4** (3.5% of D_4 incorporation)
[4,5,6,7-D_4]-Brassinin (**1a**)	Roots, 3 days (*B. rapa*/UV radiation)	**3** (9% of D_4 incorporation); **4** (15% of D_4 incorporation)
[4,5,6,7-D_4]-Cyclobrassinin (**2a**)	Leaves, 2 days (*B. juncea*/fungal spores)	**3** (0.5% of D_4 incorporation)
[2-^{14}C]-Indole-3-carboxaldehyde (**9**)	Stems, 4 days (*B. juncea*/$CuCl_2$ uptake)	**11** (1% of total ^{14}C- carboxaldehyde fed)
[4,5,6,7-D_4]-Glucobrassicin (**5a**)	Leaves, 2 days (*B. juncea*/fungal spores)	no incorporation into phytoalexins **2**, **3**, **4**[b]
[4,5,6,7-D_4]-Glucobrassicin (**5a**)	Leaves, 3 days (*B. juncea*/UV radiation)	no incorporation into phytoalexins **2** and **4**[b]
[4,5,6,7-D_4]-Glucobrassicin (**5a**)	Roots, 4 days (*B. rapa*/UV radiation)	no incorporation into phytoalexins **1**, **3** and **4**[b]; **9** (34% of D_4 incorporation); **12** (26% of D_4 incorporation)
[4,5,6,7-D_4]-Indole-3-acetaldoxime (**6a**)	Leaves, 4 days (*B. rapa*/$CuCl_2$ spray)	**4** (2.5% of D_4 incorporation); **7** (32% of D_4 incorporation); **9** (16% of D_4 incorporation)
[4,5,6,7-D_4]-Indole-3-acetaldoxime (**6a**)	Roots, 3 days (*B. rapa*/UV radiation)	**2** (10% of D_4 incorporation); **3** (2% of D_4 incorporation); **4** (14% of D_4 incorporation); **13**, **14**, **15** (98% of D_4 incorporation)

[a] The % of ^{14}C incorporation was established by HPLC (calibration curve $R^2 > 0.999$) according to the following equation: area of peak converted to dpm/total amount of label×100; the% of D_4 incorporation was established by HRMS–EI according to the following equation: % of $D_4 = [M+4]^+/([M]^+ + [M+4]^+) \times 100$ (HRMS data indicated that $[M+4]^+$ is not present in natural abundance samples).

[b] Only these phytoalexins were isolated in sufficient amounts for HRMS–EI analysis.

3 days of incubation: brassilexin (**3**, 0.5–1×10^{-1} μmol/100 g fresh root tissue), spirobrassinin (**4**, 0.4–2×10^{-1} μmol/100 g fresh root tissue) and cyclobrassinin (**2**, 0.5–2×10^{-1} μmol/100 g fresh root tissue); indole-3-acetonitrile (**7**) and indole-3-carboxaldehyde (**9**) were occasionally detected.

2.2. Administration of isotopically labeled brassinin (*1*), cyclobrassinin (*2*) and indole-3-carboxaldehyde (*9*)

[2-^{14}C]-Brassinin (**1**) was synthesized from commercially available [2-^{14}C]-indole as outlined in Scheme 1 and described in Experimental. The synthesis of [2-^{14}C]-brassinin (**1**) was performed on a mg scale and chromatographic separation was only performed on the final reaction mixture which was then analyzed by HPLC (photodiode array and radio detection) and LSC (liquid scintillation counter). [4,5,6,7-D_4]-Brassinin (**1a**) and [4,5,6,7-D_4]-cyclobrassinin (**2a**) were synthesized according to previously reported route but utilizing [4,5,6,7-D_4]-indole-3-carboxaldehyde as starting material (Pedras et al., 1998).

A summary of the metabolism studies with different compounds and tissues is shown in Table 2. To examine the metabolism of brassinin (**1**) in stems of *B. juncea*, [2-^{14}C]-brassinin (**1**) was administered to $CuCl_2$ elicited stems or to stems prior to elicitation with *P. lingam*, as described in the Experimental. Stems were extracted after incubation and analyzed by HPLC (photodiode array and radio detection). The results from HPLC analysis indicated that [2-^{14}C]-brassinin (**1**) was metabolized to several more polar compounds (elicitation with *P. lingam* or $CuCl_2$) with retention times below 5, and at 7.1 and 12.8 min (HPLC analysis, radio detector). In order to identify the radioactive metabolites, the extracts were separated by multiple chromatography and further analyzed by HPLC (photodiode array and radio detection). Based on the retention times and UV-spectra (compared with an authentic sample) the radioactive metabolite with t_R 7.1 min (6.7 min photodiode

Scheme 2. Metabolism of [2-^{14}C]-brassinin (**1**) in stems of *Brassica juncea*.

array detection) was identified as [2-^{14}C]-indole-3-carboxaldehyde (**9**) and the radioactive metabolite at t_R 12.8 min (12.3 min photodiode array detection) was identified as methyl [2-^{14}C]-indole-3-carboxylate (**12**) (a number of radioactive metabolites with retention times below 5 min were not identified). Further experiments involving administration of [2-^{14}C]-indole-3-carboxaldehyde (**9**) to stems of *B. juncea* elicited with $CuCl_2$ indicated that [2-^{14}C]-indole-3-carboxaldehyde (**9**) was metabolized to [2-^{14}C]-indole-3-carboxylic acid (**11**) and other polar metabolites with retention times below 5 min. [2-^{14}C]-Indole-3-carboxylic acid (**11**; $t_R = 6.1$ min, 5.6 min photodiode array detection) was identified by methylation with diazomethane and comparison of t_R and UV-spectra with those of authentic samples (Scheme 2).

Incorporation of radioactivity was estimated by HPLC (radio chromatograms) using a calibration curve (constructed from stock solutions prepared in serial dilution from an authentic sample of [2-^{14}C]-brassinin). After 60 h of incubation with *P. lingam* the radioactivity of each metabolite was ca. 1% of the total amount of brassinin fed (ca. 10^7 dpm), with 10% of brassinin remaining intact; however, after 3 days of incubation [2-^{14}C]-brassinin (**1**) was not detected. In whole stems elicited with $CuCl_2$, after 4 days of incubation 10% of [2-^{14}C]-brassinin (**1**) remained and the radioactivity of each metabolic metabolite was ca. 1% of the total amount of brassinin fed (10^7 dpm). Brassilexin (**3**) was isolated, purified, and analyzed by HPLC (photodiode array and radio detection) and LSC, however, no incorporation of [2-^{14}C]-brassinin (**1**) into brassilexin (**3**) was detected. The lack of incorporation suggests that brassinin was not transported through the plant tissue to the cell site where brassilexin (**3**) is produced and/or that [2-^{14}C]-brassinin (**1**) was metabolized to other metabolites before reaching the cell site. Overall, the results of these administration experiments (Scheme 2) indicated that [2-^{14}C]-brassinin (**1**) was metabolized to [2-^{14}C]-indole-3-carboxaldehyde (**9**), which was further metabolized to [2-^{14}C]-indole-3-carboxylic acid (**11**), and other polar metabolites ([2-^{14}C]-indole-3-carboxylate (**12**) is likely due to esterification of acid **11** during MeOH extraction). Interestingly, albeit an apparently minor transformation pathway, the metabolism of [2-^{14}C]-brassinin (**1**) in *B. juncea* is similar to the major brassinin detoxification pathway present in *P. lingam*.

[4,5,6,7-D_4]-Brassinin (**1a**) and [4,5,6,7-D_4]-cyclobrassinin (**2a**), synthesized as described in the Experimental, were separately administered to leaves of brown mustard; after solution uptake the leaves were elicited with a spore suspension of the fungus *P. lingam*, were incubated, and extracted as described in experimental. The extracts were fractionated and the percentage of incorporation of [4,5,6,7-D_4]-brassinin (**1a**) into brassilexin (**3**) and spirobrassinin (**4**) was determined (Table 2). As in previous work (Pedras et al, 1998), in uptake experiments cyclobrassinin was more phytotoxic to petiole and leaf tissue of *B. juncea* than brassinin. We suspect that this tissue damage may affect and account for the low deuterium incorporation from the exogenous precursor pool. Next, [4,5,6,7-D_4]-brassinin (**1a**) was incubated with turnip root slices and extracted; HPLC analysis of the extracts, after chromatographic fractionation yielded phytoalexins **3** and **4**. HRMS–EI analysis indicated substantial deuterium incorporation into both **3** and **4**), thus demonstrating that brassinin (1) is also a precursor of brassilexin (**3**) in *B. rapa* (Table 2). These results revealed for the first time that brassilexin (**3**) was produced in turnip roots (Pedras et al., 2001). The incorporation level of deuterium into brassilexin (**3**) was substantially higher than in the experiments with leaves of *B. juncea* or in our earlier work utilizing *B. carinata* (Pedras et al., 1998). Turnip root tissue appears to be an adequate system for biosynthetic studies since precursors can diffuse into the tissue with no need to uptake the substrate solution via the plant vascular system.

2.3. Feeding isotopically labeled glucobrassicin (*5*) and indole-3-acetaldoxime (*6*)

To investigate the possible biogenetic relationship between phytoalexins and indole glucosinolates, [4,5,6,7-D_4]-glucobrassicin (**5a**) was synthesized according to previously reported route (Viaud and Rollin, 1990; Chevolleau et al., 1993) but utilizing [4,5,6,7-D_4]-indole-3-carboxaldehyde as starting material. [4,5,6,7-D_4]-Glucobrassicin (**5a**) was administered to detached petiolated leaves of *B. juncea*; after solution uptake, elicitation (*P. lingam*), and a 48-h incubation, the H_2O droplets from each leaf were collected, combined, and extracted. Fractionation of the extract yielded cyclobrassinin (**2**), brassilexin (**3**), and spirobrassinin (**4**); measurement of the percentage of deuterium by HRMS–EI indicated no incorporation of deuterium ($\sim$0.1% relative to a natural abundance control sample). Similar results where obtained when leaves were elicited with UV light. This lack of deuterium incorporation was thought to be partly due to the substrate not reaching the appropriate cell site (Table 2).

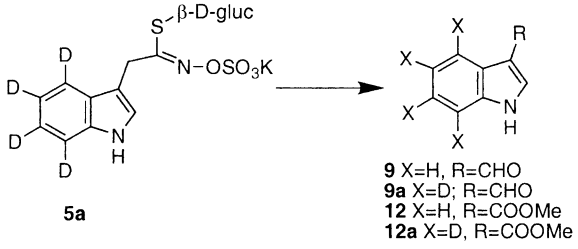
Subsequently, similar to the experiment described above for [4,5,6,7-D_4]-brassinin (**1a**), to obtain potentially higher deuterium incorporation, turnip roots were used. Thus, [4,5,6,7-D_4]-glucobrassicin (**5a**) was administered to UV-irradiated turnip root slices (*B. rapa*) and tissues incubated for 4 days; similar experiments were conducted with non-irradiated turnip roots. Following incubation, extraction and fractionation yielded phytoalexins **2**–**4**. Once again HRMS-EI analysis indicated that possible deuterium incorporation was too low to allow a reliable conclusion ($\sim$0.1%). However, two additional compounds containing deuterium were separated and identified unambiguously as indole-3-

carboxaldehyde (**9**) and methyl indole-3-carboxylate (**12**) (Table 2). These results indicated that metabolism of [4,5,6,7-D_4]-glucobrassicin (**5a**) to **9a** and **12a** occurred in turnip roots and that the metabolism was unrelated with phytoalexin biosynthesis (Scheme 3).

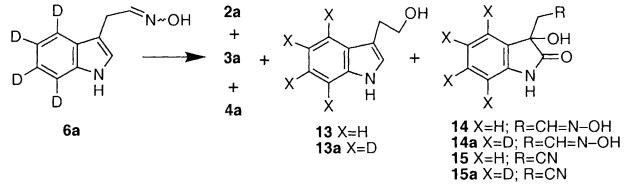
Next, we investigated the potential biogenetic relationship between indole-3-acetaldoxime (**6**) and brassinin (**1**). Thus, [4,5,6,7-D_4]-indole-3-acetaldoxime (**6a**) was administered to UV-irradiated roots of *B. rapa*; after incubation of the tissues, extraction, and fractionation of the extracts, phytoalexins **2–4** were obtained. As shown in Table 2, HRMS-EI analysis indicated significant deuterium incorporation into **2–4**. Furthermore, three additional compounds, subsequently established to be tryptophol (**13**/**13a**) and oxindoles **14**/**14a** and **15**/**15a**, were isolated and analyzed by HRMS–EI. Deuterium incorporation levels suggested that metabolites **13–15** were fully derived from metabolism of acetaldoxime **6a** (Scheme 4, incorporation of D_4 98%). This conclusion was consistent with the absence of compounds **13–15** in elicited control tissues. Additional studies indicated that oxime **14** dehydrated upon standing to yield nitrile **15**, suggesting that turnip tissue contained the enzyme system required to oxidize acetaldoxime **6** to oxoindole **14**, and that **15** might be an artifact of the isolation process. It is worthy to note that oxoindole **15** was previously isolated from *B. oleracea* (Monde et al., 1991a), however, its precursor oxindole oxime **14** was not described.

[4,5,6,7-D_4]-Indole-3-acetaldoxime (**6a**) was administered to leaves of *B. rapa* to investigate the potential biogenetic relationship with brassinin (**1**), as described in Experimental; after incubation of the tissues, extraction, and fractionation of the extracts, spirobrassinin (**4**), indole-3-acetonitrile (**7**), and indole-3-carboxaldehyde (**9**) were obtained, whereas compounds **13–15** were not detected. HRMS–EI analysis indicated the deuterium incorporations shown in Table 2. These results confirmed that [4,5,6,7-D_4]-indole-3-acetaldoxime (**6a**) is also a precursor of spirobrassinin in leaves of *B. rapa,* although the incorporation level was substantially lower than that observed in root slices (Table 2). These results indicate that leaf uptake of putative phytoalexin precursors result in lower deuterium incorporation and suggest that biosynthetic studies using petiolated leaves are less advantageous.

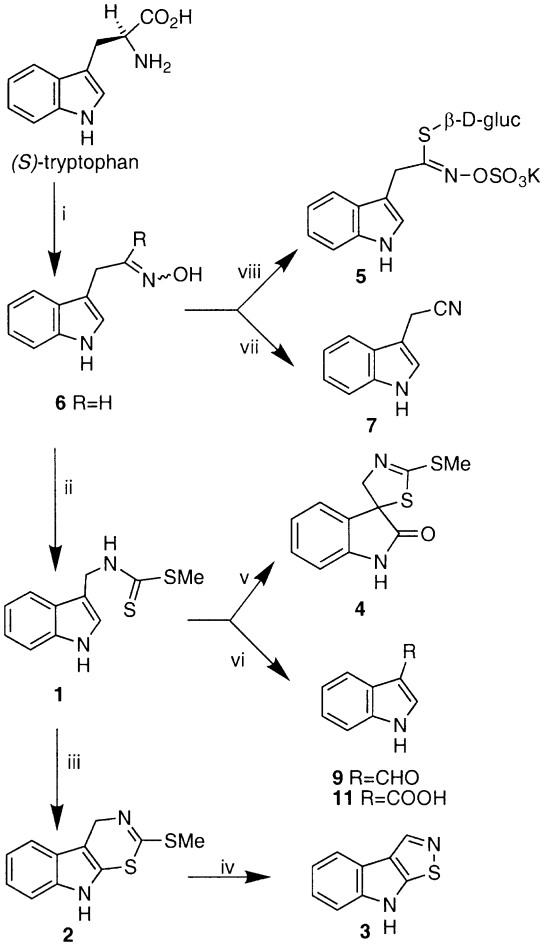
A summary of our results and published results from other groups (Pedras et al., 2000; Halkier and Du, 1997 and references therein) is shown in Scheme 5. Since indole-3-acetaldoxime (**6**) is a precursor of spirobrassinin (**4**) in both roots and leaves of *B. rapa* and we were unable to demonstrate that glucobrassicin (**5**) is a precursor of brassinin (**1**) or related phytoalexins **2–4**, it is likely that the pathway to phytoalexins **1**/**1a**–**4**/**4a** follows the tryptophan-aldoxime route and will branch out from the indole glucosinolate pathway a step earlier



Scheme 3. Metabolism of [4,5,6,7-D_4]-glucobrassicin (**5a**) in roots of *Brassica rapa*.



Scheme 4. Metabolism of [4,5,6,7-D_4]-indole-3-acetaldoxime (**6a**) in roots of *Brassica rapa*.



Scheme 5. Biogenetic relationship and metabolic pathway of tryptophan, phytoalexins **1–4** and related metabolites **5–7** from *Brassica* species; (i) in different crucifers (Halkier and Du, 1997); (ii) *B. rapa* (this work); (iii) *B. rapa* (Monde et al., 1994); (iv) *B. carinata* (Pedras et al., 1998); *B. rapa, B. juncea* (this work); (v) *B. rapa* (Monde et al., 1994); *B. carinata* (Pedras et al., 1998); (vi) *B. juncea* (this work); (vii) and (viii) in different crucifers (Halkier and Du, 1997).

than previously proposed (Scheme 5). Because the sulfur atoms in both **5** and **1** (only one of the sulfurs) are derived from cysteine (Monde et al., 1994; Halkier and Du, 1997 and references therein), thiohydroxymate **6** (R = SH) is a potential intermediate and branch point that could lead to **1** and **5** as previously proposed (i.e. derived from **6** R = S-cysteinyl, Halkier and Du, 1997 and references therein).

2.4. Indole-3-acetonitrile as a phytoalexin of B. juncea

2.4.1. Isolation, identification and antifungal activity

During the stem administration experiments with brown mustard (*B. juncea*), an unknown metabolite with t_R 11.7 min was detected by HPLC analysis of extracts from stems of *B. juncea* (elicited with either *P. lingam* or $CuCl_2$). This metabolite was not detected in stems treated with distilled H_2O, hence we were interested in its identification and in establishing its potential role in the plant's defense mechanisms. A scale-up experiment was conducted as described in the experimental; after multiple chromatography and spectroscopic analysis ($C_{10}H_8N_2$ by HRMS–EI in combination with NMR spectral data) this metabolite was established to be indole-3-acetonitrile (**7**). The chemical structure was confirmed by comparison of the physical data (HPLC, ^1H and ^{13}C NMR, HRMS) with those of an authentic commercial sample. Indole-3-acetonitrile was elicited in both stems and leaves of brown mustard, as shown in Table 3. The highest amounts of indole-3-acetonitrile (**7**) appeared to be produced in stems upon elicitation with $CuCl_2$ and a 5-day incubation.

Table 3
Elicitation of indole-3-acetonitrile (**7**) in stems and leaves of *Brassica juncea*

Tissue	Elicitor	Total amount of indole-3-acetonitrile (**7**)[a] per 100 g fresh tissue
Stems	Control	Not detected
	$CuCl_2$ uptake	$3–62\times10^{-1}$ μmol after 2-day incubation
		$37–110\times10^{-1}$ μmol after 5-day incubation
	Salicylic acid	$\sim1\times10^{-1}$ μmol after 5-day incubation
	Jasmonic acid	$\sim1\times10^{-1}$ μmol after 5-day incubation
	Destruxin B	$\sim1\times10^{-1}$ μmol after 5-day incubation
	Fungal spores	$\sim1\times10^{-1}$ μmol after 2-day incubation
Leaves	Control	Not detected
	$CuCl_2$ uptake	$1–6\times10^{-1}$ μmol after 5-day incubation
		$3–5\times10^{-1}$ μmol after 7-day incubation

[a] Extracted as described in Experimental; quantities were calculated from a calibration curve ($R^2 > 0.999$).

Table 4
Results of antifungal activity of indole-3-acetonitrile (**7**)[a]

Fungal species (incubation time[c])	Inhibition of fungal mycelial growth (%)[b]	
	1×10^{-4} M	5×10^{-4} M
Virulent *Phoma lingam* (100 h)	15±7	100±0
Rhizoctonia solani (30 h)	12±4	86±4
Sclerotinia sclerotiorum (24 h)	5±5	38±0
Alternaria brassicae (100 h)	No inhibition	No inhibition

[a] Antifungal bioassays were conducted in PDB media as described in Experimental; results are the means of at least three independent experiments conducted in triplicate.

[b] % Of inhibition was calculated according to the following formula: % inhibition = 100-[(growth in treated plate/growth in control)×100].

[c] Time required for control plates to be covered with mycelium to ca. 3/4 of plate diameter.

Indole-3-acetonitrile was tested against four of the major fungal pathogens of rapeseed (Pedras, 1998); the results in Table 4 indicate that indole-3-acetonitrile (**7**) was a strong inhibitor of virulent *P. lingam* and *Rhizoctonia solani*, but affected much less the growth of *Sclerotinia sclerotiorum* and did not appear to inhibit the growth of *Alternaria brassicae*.

2.4.2. Fungal metabolism

Because fungal pathogens of crucifers can detoxify effectively cruciferous phytoalexins (Pedras et al., 2000), time-course studies were carried out to determine if indole-3-acetonitrile (**7**) was metabolized by *P. lingam*. Indole-3-acetonitrile (**7**) (1×10^{-4} M) was incubated with virulent *P. lingam* and the percentage of transformation was determined by comparing the initial quantity of indole-3-acetonitrile (**7**) with that present at each time point, as described in Experimental. HPLC analysis of extracts of each sample indicated that after a 3-day incubation the quantity of indole-3-acetonitrile present was 87% of the initial amount, while after seven days was 45%. Since previous studies showed that both brassinin (**1**) and brassilexin (**3**) were completely metabolized by *P. lingam* in two days (Pedras et al., 2000), the metabolism of indole-3-acetonitrile appeared to be substantially slower than the metabolism of these cruciferous phytoalexins.

2.4.3. Biosynthesis

Indole-3-acetonitrile (**7**) is known to be an intermediate in the biosynthesis of indole-3-acetic acid via indole-3-acetaldoxime (**6**) (Normanly and Bartel, 1999) and an enzymatic degradation product of glucobrassicin (**5**) catalyzed by myrosinase (Halkier and Du, 1997). Therefore, these two pathways are possible sources of indole-3-acetonitrile (**7**) in elicited *B. juncea*. In order to determine the most likely source of elicited indole-3-acetonitrile (**7**), precursor studies using elicited leaves of *B. juncea* were conducted with synthetically prepared [4,5,6,7-D_4]-indole-3-acetaldoxime (**6a**), [4,5,6,7-D_4]-indole (**8a**) and commercially available (*S*)-[2′,4′,5′,6′,7′-D_5]-tryptophan, as described in the experimental. After a 4-day incubation the leaves were extracted, indole-3-acetonitrile (**7**) was isolated and was analyzed by HRMS–EI. The results indicated effective incorporation

of the D-label into indole-3-acetonitrile (**7**): 24% of D incorporation from acetaldoxime **6a**, whereas administration experiments with [4,5,6,7-D_4]-indole (**8a**) and (*S*)-[2′,4′,5′,6′,7′-D_5]-tryptophan led to incorporation of 7 and 25%, respectively. To avoid enzymatic degradation of glucobrassicin (**5**) the tissues of *B. juncea* were frozen in liquid N_2 prior to extraction with MeOH or extractions were immediately carried out in boiling MeOH. HPLC analysis of the extracts from the two groups of leaves showed that in both cases indole-3-acetonitrile (**7**) was present in the leaf extracts in similar amounts. These results indicate that in elicited *B. juncea* indole-3-acetonitrile (**7**) is likely produced directly from tryptophan via indole-3-acetaldoxime (**6**).

The antifungal activity of indole-3-acetonitrile (**7**), its production in elicited stems and leaves of *B. juncea* and its absence in controls suggest that it plays a role in the plant's defense mechanism. Our studies with *B. juncea* indicate that indole-3-acetonitrile is produced in response to stress, either biotic (fungal spores) or abiotic ($CuCl_2$) elicitation, is likely synthesized de novo from (*S*)-tryptophan, and possesses antifungal activity similar to those of other brassica phytoalexins (Pedras et al., 2000). Consequently, indole-3-acetonitrile (**7**) would be appropriately classified as a phytoalexin of *B. juncea*. Nonetheless, because indole-3-acetonitrile (**7**) can also result from degradation of glucobrassicin (**5**; Halkier and Du, 1997) this metabolite appears to have a dual role as phytoalexin and phytoanticipin of *B. juncea*. In *Brassica* species such as *B. napus* and *B. rapa*, where indole-3-acetonitrile (**7**) is partly constitutive, it would be more appropriately described as a phytoanticipin. Considering the stronger antifungal activity of indole-3-acetonitrile (**7**) against *P. lingam* and its potential role as a plant defense in *B. juncea*, it would be of interest to analyze its distribution among cultivars of *B. napus* with different levels of susceptibility to blackleg.

Although indole-3-acetonitrile (**7**) was shown to have antifungal activity (Seifert and Unger, 1994), it is somewhat surprising to find that such a well-known compound is here shown for the first time to have a role in plant defense, whether a phytoalexin or a phytoanticipin. Additional biological properties and detection of both **7** and **13** in a number of cruciferous species were previously reported (Wall et al., 1988).

3. Conclusions

Recent examples of phytoalexin detoxification by crucifer fungal pathogens suggest that enzymes involved in such processes may result from an intergeneric-genetic exchange between brassicas and their pathogens (Pedras et al., 2000). We have now demonstrated that brown mustard converts the phytoalexin brassinin (**1**) into nontoxic products via a pathway similar to that occurring in virulent *P. lingam*. Therefore, we suggest that this pathogen may have acquired a more effective mechanism for overcoming brassinin by adopting a phytoalexin degradation pathway operating *in planta*. Our results established an important biogenetic relationship, that is aldoxime **6**/**6a** is a precursor of phytoalexins **1**/**1a**–**4**/**4a**, whereas the postulated relationship between indole glucosinolate **5**/**5a** and these phytoalexins was not demonstrated. Nonetheless, our results have strong implications on plant breeding to produce low glucosinolate content oilseed brassicas, i.e. it is essential not to delete the indole acetaldoxime formation steps, as aldoxime **6** is a close precursor of important cruciferous phytoalexins. This knowledge is essential for an effective genetic manipulation of the phytoalexin pathway in cruciferous crops. Otherwise, ecologically unfit plants with higher disease susceptibility may be produced. Further work is necessary to find additional intermediates between aldoxime **6** and brassinin (**1**) as well as the branch point between the tryptophan pathway to indole glucosinolates and to phytoalexins. Furthermore, the results described here suggest that de novo production of indole-3-acetonitrile is a desirable biochemical trait that could lead to higher blackleg tolerance/resistance in crops where such a trait is essential (e.g. *B. rapa* and *B. napus*).

4. Experimental

4.1. General

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON unless otherwise stated. All solvents were analytical grade with the exception that HPLC grade solvents were used for HPLC analysis. [2-^{14}C]-Indole (sp. activity 50 mCi/mmol, 1.1×10^{11} dpm/mmol) was purchased from Moravek Biochemicals, Inc., Brea, CA. Phytoalexins were identified by comparison of retention times and UV-spectra (photodiode array detector) with authentic samples.

Analytical techniques were carried out as previously described (Pedras and Okanga, 1999); in addition HPLC system was equipped with a photodiode array detector (wavelength range 190–600 nm) and a Canberra Packard radiomatic flow scintillation analyzer. The radiodetector was equipped with a high performance flow cell (200 μl volume) and a Solar Scint® solid cartridge. HPLC: mobile phase A-linear gradient of H_2O–MeCN (75:25) to H_2O–MeCN (25:75), for 35 min, with a flow rate of 1 ml/min (Pedras and Khan, 1996); mobile phase B-linear gradient of H_2O–MeOH (both containing an ion-pairing reagent consisting of 0.15% triethylamine and 0.18% formic acid, 100:0 to 0:100) for 60 min with a flow rate of 1 ml/min (Zrybko et al., 1997). A Beckman LS 6500 multiple liquid scintillation

counter connected to a Wyse WY-370 data system was employed to analyze radioactive samples. Calibration curves were prepared from synthetic or commercially available products to quantify phytoalexins and related metabolites detected in the elicited tissue extracts.

4.2. Plant growth, and fungal cultures

Brassica rapa (turnip purple top, variety White Globe) and *B. juncea* cv. Cutlass (brown mustard) plants were grown in a growth chamber with a 16-h photoperiod (20 °C/18 °C) at 80% relative humidity. Seeds of *B. juncea* were obtained from Agriculture and Agri-Food Canada (Research Center, Saskatoon, SK). Cell suspension cultures of mesophyll tissue from *B. juncea* were obtained from Transgenic Plant Centre, PBI-NRC, Saskatoon SK. *B. rapa* seeds were purchased from Ferry Morse Seed Co., Fulton, KY USA 42041. Roots of *B. rapa* (turnip purple top) were purchased from a local store.

Phoma lingam virulent isolate BJ-125 and *Alternaria brassicae* isolate AB #11 were obtained from G. Séguin-Swartz, Agriculture and Agri-Food Canada Research Station, Saskatoon SK. *Rhizoctonia solani* isolate AG-2.1 was obtained from P. Verma, Agriculture and Agri-Food Canada Research Station, Saskatoon SK. *Sclerotinia sclerotiorum* isolate #33 was obtained from R. Morrall, Department of Biology, University of Saskatchewan, Saskatoon SK. Fungal culturing for the collection of mycelium or spores of *P. lingam* virulent isolate BJ-125 was performed following the procedure reported by Pedras and Khan (1996). Mycelium used in the antifungal bioassays was collected after 8 days growth. *R. solani*, *S. sclerotiorum* and *A. brassicae* were cultured on potato dextrose agar (PDA) media from older mycelia plugs.

4.3. Phytoalexin elicitation in different plant tissues: cell suspension cultures, stems, leaves and roots

4.3.1. Cell suspension cultures

Cell suspension cultures prepared from mesophyll tissue from *B. juncea* were maintained in MS basal salt mixture with 3% sucrose, 2 mg/ml 2,4-dichlorophenoxyacetic acid supplemented with Gamborg's B5 vitamins (Pedras and Biesenthal, 2000). Fresh maintenance media was added to cell suspension cultures 24 h prior to elicitation. The cell suspension cultures were then elicited and incubated. Parameters varied: cell suspension culture density (20, 40, 80, 120, 200 mg/ml), temperature (20, 22 and 25 °C) and lighting (constant light, constant dark or 16 h photoperiod). Elicitors tested: *P. lingam* virulent isolate BJ-125 (1×10^7 spores/ml) and avirulent isolate Unity (5×10^6 spores/ml) of *P. lingam*, yeast extract (1 mg/ml final concentration), jasmonic acid (1×10^{-3} M final concentration) and salicylic acid (1×10^{-3} M final concentration). After 24 and 48 h, a 10-ml sample volume was collected, the cells were filtered from the broth and then extracted with MeOH, the media was extracted with EtOAc. The MeOH and EtOAc extracts were concentrated to dryness and the resulting residues were analyzed by HPLC.

4.3.2. Stems

Stem elicitation with fungal spores: stems (10–15 cm length) from three-week-old *B. juncea,* stripped of all leaves and upper bud, were cut into 2-cm pieces, each piece was cut in half longitudinally and then placed in a Petri plate with the cut surface up and inoculated with a spore suspension (50 μl, 5×10^7 spores/ml). Control stems were treated with distilled H_2O. The stem pieces were incubated in the dark under constant temperature (25±0.5 °C). After various incubation periods, the stem tissue (17 g) was frozen in liquid N_2 and homogenized using a Polytron® homogenizer. The homogenized tissue was extracted with MeOH, and the MeOH was decanted and evaporated to dryness. The resulting residue was dissolved in H_2O and extracted with EtOAc. The EtOAc extract was dried over Na_2SO_4, concentrated to dryness under reduced pressure and analyzed by HPLC.

Stem elicitation with $CuCl_2$: stems (10–15 cm in length) from three-week-old *B. juncea* were stripped of all leaves except for the uppermost leaf and bud and standing upright were partially immersed (2 cm) in a $CuCl_2$ solution (2×10^{-3} M). Stems were incubated with a 16-h photoperiod (20±0.5 °C), and extracted at 24 h intervals for a total of 5 days. Stems were worked up and analyzed as described above.

Stem elicitation with destruxin B, jasmonic acid and salicylic acid: stems (10–15 cm length) from three-week-old *B. juncea* were prepared as described above. The base of each stem was placed in an aq. solution (1 ml) containing one of the following: destruxin B (final concentration 3×10^{-5} M) with MeCN (0.2%, v/v), jasmonic acid (final concentration 5×10^{-4} M) or salicylic acid (final concentration 1×10^{-3} M). After complete uptake of the elicitor solution, distilled H_2O was added and the stems incubated with a 16-h photoperiod (20±0.5 °C). Stems elicited with destruxin B were extracted at 24 h intervals for a total of 5 days. Stems elicited with jasmonic acid or salicylic acid were extracted at 24 h intervals for three days. Stems were worked up as described above and the extracts were analyzed by HPLC.

4.3.3. Leaves

Leaf elicitation with fungal spores: petiolated leaves from 3-week-old plants were cut and immediately placed in Falcon® tubes containing distilled H_2O or the control solution (10% MeOH and 0.1% Tween 80 in H_2O). After the H_2O was taken up through the petiole, the leaves were placed upper side down in a plastic washtub containing premoistened paper towels. The

petiole of each leaf was wrapped with premoistened cotton wool. A droplet suspension of spores of *P. lingam* (10 μl of 108 spores/ml) was applied to slightly bruised areas on the underside of each leaf (20–100 droplets per leaf). After allowing the H_2O droplets to evaporate, deionized H_2O was placed at the inoculation sites to replace the original spore inoculation droplets, the container was sealed, and the leaves were incubated at room temperature for 24 and 48 h. After incubation, the H_2O droplets were collected from each leaf, combined, and extracted with CH_2Cl_2. The leaf and petiole tissues were frozen in liquid N_2 and extracted with MeOH. The HPLC analysis of the CH_2Cl_2 extracts indicated the presence of phytoalexins and allowed quantification by comparison with a standard calibration curve.

Leaf elicitation with uptake of $CuCl_2$: leaves from 3-week-old *B. juncea* were elicited by uptake of an aq. $CuCl_2$ solution. The plants were at the 6–7-leaf stage and only the middle leaves were selected for elicitation. Leaves were cut from stems and immediately placed in one of the following $CuCl_2$ solutions: 1×10^{-3} M, 1×10^{-4} M, 8×10^{-5} M or 1×10^{-5} M. Control leaves were placed in distilled H_2O. Alternatively leaves were placed in distilled H_2O and sprayed with aq. $CuCl_2$ (1×10^{-3} M). All of the leaves were incubated with a 16-h photoperiod (20 ± 0.5 °C). After various incubation periods, leaves were worked up as described for stems and extracts were analyzed by HPLC.

4.3.4. Roots

Root elicitation with UV irradiation: similar to previous work by Monde et al. (1994), turnip roots (*B. rapa*) were cut horizontally in 10–15 mm thick discs and cylindrical holes (1.6 cm in diameter) were made on one surface with a cork-borer. After 6–24 h of incubation in moist covered plastic boxes (light or dark, room temperature or 20 °C), the roots were divided into two series: control and UV-irradiated slices. Root slices were irradiated on the surface with holes, with UV light in a laminar flow hood for 15 min. Each hole of irradiated and control discs was filled with distilled H_2O or with a solution of 0.2% of dimethylsulfoxide (DMSO) in 0.1% Tween 80 aq. solution. After 2–4 days of incubation the aq. phase was harvested and extracted with EtOAc. The combined organic phases were dried over Na_2SO_4, filtered, concentrated, and analyzed by HPLC. The aq. fraction was concentrated, dissolved in MeOH and analyzed by HPLC.

4.4. Synthesis of isotopically labeled compounds

4.4.1. [2-^{14}C]-Brassinin (**1**)

[2-^{14}C]-Brassinin (**1**) was prepared following conditions modified from Schallenberg and Meyer (1983) and Takasugi et al. (1988). Each reaction was monitored by TLC and all solutions were analyzed by LSC. [2-^{14}C]-Indole (**8**) (1.2×10^8 dpm, 9.7×10^{-4} mmol) in hexane-CH_2Cl_2 (3:2) was combined with indole (6.0 mg, 0.051 mmol), was concentrated to dryness and the homogenous mixture was dissolved in DMF (100 μl, 94 mg, 1.3 mmol). To this solution freshly distilled phosphorous oxychloride (30 μl, 49 mg, 0.32 mmol) was slowly added and the reaction was kept at room temperature. After 45 min the reaction mixture was slowly added to hot NaOH (5 M, 1 ml) (heated in a boiling H_2O bath) and kept at that temperature for 5 min. The reaction mixture was extracted with Et_2O (3×4 ml) and the combined Et_2O extracts were concentrated to dryness under reduced pressure. TLC (CH_2Cl_2-MeOH, 95:5, v/v) was used to monitor the reaction and identify the product as [2-^{14}C]-indole-3-carboxaldehyde (**9**; $R_f=0.45$, quantitative yield, LSC), which was used without further purification to prepare [2-^{14}C]-indole-3-carboxaldehyde oxime.

A solution of hydroxylamine hydrochloride (120 mg, 1.73 mmol) and sodium carbonate (85 mg, 0.80 mmol) in H_2O (1.0 ml) was added to [2-^{14}C]-indole-3-carboxaldehyde (**9**) dissolved in EtOH (1.0 ml). The reaction mixture was heated at 55 °C; after 30 min the reaction mixture was cooled to room temperature and extracted with Et_2O (3×4 ml). The combined Et_2O extracts were concentrated to dryness under reduced pressure. TLC (CH_2Cl_2–MeOH, 95:5, v/v) was used to monitor the reaction and identify the product as a mixture of isomers of [2-^{14}C]-indole-3-carboxaldehyde oxime ($R_f=0.38$, 0.51, quantitative yield, LSC). The product was used without purification to prepare [2-^{14}C]-indole-3-methanamine (**10**), by reduction either using Devarda's alloy or $TiCl_3$/$NaBH_3CN$. Thus, [2-^{14}C]-indole-3-carboxaldehyde oxime was dissolved in a 1:1 solution (v/v) of MeOH and 5 M NaOH. Devarda's alloy (1.0 g; 50% Cu, 45% Al, 5% Zn) was added and the reaction monitored by TLC (CH_2Cl_2–MeOH–NH_4OH, 80:20:1, v/v). After 25 min, the aq. phase was filtered and the alloy rinsed with distilled H_2O. The aq. layers were combined and extracted with Et_2O (4×8 ml). The Et_2O extracts were combined and concentrated to dryness under reduced pressure. Alternatively, [2-^{14}C]-indole-3-carboxaldehyde oxime and ammonium acetate (44 mg, 0.57 mmol) were dissolved in MeOH (0.5 ml). A neutralized aq. 20% $TiCl_3$ solution (320 μl, 63 mg, 0.41 mmol) and sodium cyanoborohydride (34 mg, 0.54 mmol) were then added. After 10 min the reaction was diluted with distilled H_2O (1 ml) and then extracted with Et_2O (3×10 ml). The Et_2O extracts were combined and concentrated to dryness under reduced pressure. Crude [2-^{14}C]-indole-3-methanamine (**10**) identified by TLC (CH_2Cl_2–MeOH–NH_4OH 80:20:1, v/v, $R_f=0.2$) was used to prepare [2-^{14}C]-brassinin (**1**).

Carbon disulfide (15 μl, 19 mg, 0.25 mmol) and triethylamine (25 μl, 18 mg, 0.18 mmol) were added to a

solution of crude [2-^{14}C]-indole-3-methanamine (**10**) in pyridine (0.5 ml) and the reaction was kept in an ice-H_2O bath. After 1 h methyl iodide (35 μl, 80 mg, 0.56 mmol) was added and the reaction kept at 4 °C. After 20 h H_2SO_4 (2 ml, 1.5 M) was added and the reaction mixture was extracted with Et_2O (10 ml). The Et_2O extract was concentrated to dryness. Purification by prep. TLC (CH_2Cl_2–hexane, 85:15, v/v) yielded [2-^{14}C]-brassinin (**1**). The purified product was analyzed by HPLC and LSC. The overall yield of brassinin from indole was determined using a calibration curve relating the UV-absorbance to moles of brassinin (15–20%). The specific activity of [2-^{14}C]-brassinin (**1**) was determined by relating the total moles to the radioactivity (dpm) of the sample.

4.4.2. [4,5,6,7-D_4]-Indole-3-carboxaldehyde (**9a**)

[4,5,6,7-D_4]-Indole-3-carboxaldehyde (**9a**) was synthesized from [4,5,6,7-D_4]-indole (**8a**) as previously reported starting from D_8-toluene (Pedras et al., 1998). The preparation of [4,5,6,7-D_4]-indole (**8**) is an adaptation taken from a synthesis of [1-^{15}N]-indole reported by Van den Berg et al. (1988).

[4,5,6,7-D_4]-Indole-3-carboxaldehyde (**9a**): HRMS–EI *m*/*z* (% relative abundance): measured 149.0780 (80), calculated for $[M]^+$ ($C_9H_2D_4NO_2$) 149.0779.

4.4.3. [4,5,6,7-D_4]-Brassinin (**1a**) and [4,5,6,7-D_4]-cyclobrassinin (**2a**)

[4,5,6,7-D_4]-Brassinin (**1a**) was synthesized from [4,5,6,7-D_4]-indole-3-carboxaldehyde (**9a**) as previously reported (Pedras et al., 1998) following an adaptation taken from a synthesis of brassinin (**1**) and cyclobrassinin (**2**) reported by Monde et al. (1994).

[4,5,6,7-D_4]-Brassinin (**1a**): HRMS–EI *m*/*z* (% relative abundance): measured 240.0693 (42), calculated for $[M]^+$ ($C_{11}H_8D_4N_2S_2$) 240.0693.

[4,5,6,7-D_4]-Cyclobrassinin (**2a**): HRMS–EI *m*/*z* (% relative abundance): measured 238.0536 (12), calculated for $[M]^+$ ($C_{11}H_6D_4N_2S_2$) 238.0536.

4.4.4. [4,5,6,7-D_4]-Indole -3-acetaldoxime (**6a**)

[4,5,6,7-D_4]-Indole-3-acetaldoxime (**6a**) was synthesized from [4,5,6,7-D_4]-indole-3-carboxaldehyde (**9a**), as follows. BuLi (0.47 ml, 0.46 mmol) was added dropwise by syringe to a stirred suspension of (methoxymethyl)-triphenylphosphonium chloride (158 mg, 0.46 mmol) in dry THF (1.5 ml) (argon atmosphere, room temperature). After 30 min, a solution of **7** (35 mg, 0.23 mmol) in THF (2 ml) was added at a rate such that the reaction temperature remained below 30 °C (Kozikowski et al., 1980). The reaction mixture was stirred for 2 h at room temperature, then H_2O (2 ml) was added dropwise. The reaction mixture was extracted with Et_2O, and the combined extracts were washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. After chromatography (alumina, 80–200 mesh, eluted with CH_2Cl_2) the reaction product [4,5,6,7-D_4]-indole-3-methoxyethylene (67 mg) was hydrolyzed (Me_2CO, 3 ml, 2 M HCl, 1.5 ml, 90 min, room temperature) to give the crude product [4,5,6,7-D_4]-indole-3-acetaldehyde. Next $NH_2OH{\cdot}HCl$ (37 mg, 0.46 mmol) and NaOAc (44 mg, 0.46 mmol) was added to a solution of crude [4,5,6,7-D_4]-indole-3-acetaldehyde (65 mg, EtOH, 2 ml). After 18 h the reaction mixture was concentrated to dryness, the resulting residue was diluted with H_2O (3 ml), extracted (EtOAc), and the extract fractionated by chromatography (silica gel, EtOAc-hexane, 25:75) to afford [4,5,6,7-D_4]-indole-3-acetaldoxime (16 mg, 38% over three steps).

[4,5,6,7-D_4]-Indole-3-acetaldoxime (**6a**): HRMS–EI *m*/*z* (% relative abundance): measured 178.1040 (30), calculated for $[M]^+$ ($C_{10}H_6D_4N_2O$) 178.1044.

4.4.5. [4,5,6,7-D_4]-Glucobrassicin (**5a**)

[4,5,6,7-D_4]-Glucobrassicin (**5a**) was synthesized as previously reported (Viaud and Rollin, 1990; Chevolleau et al., 1993) for the non-labeled compound, but replacing indole-3-carboxaldehyde (**9**) with [4,5,6,7-D_4]-indole-3-carboxaldehyde (**9a**).

[4,5,6,7-D_4]-Glucobrassicin (**5a**): HPLC t_R 15.2 min (mobile phase B); $[\alpha]_D = -22$ (*c* 0.25, H_2O; lit. $[\alpha]_D$ for **5**−9.5 *c* 2.0, H_2O, Viaud and Rollin, 1990). ^{13}C NMR (125.7 MHz, D_2O): δ 163.6, 136.4, 126.3, 124.3, 122.1 (*t*, $J_{C\text{-}D} = 24$ Hz), 119.4 (*t*, $J_{C\text{-}D} = 24$ Hz), 118.3 (*t*, $J_{C\text{-}D} = 19$ Hz), 111.9 (*t*, $J_{C\text{-}D} = 19$ Hz), 108.2, 81.5, 80.4, 77.1, 71.9, 68.7, 60.3, 49.1. FAB–MS *m*/*z* (% relative abundance): 451 $[M–K]^+$(65).

4.5. Precursor administration experiments and isolation of isotopically labeled metabolites

4.5.1. Administration of labeled compounds to stems

Administration of [2-^{14}C]-brassinin to stems of *B. juncea* elicited with spores of *P. lingam*: stems (10, 10–15 cm in length) from three-week-old *B. juncea* were stripped of all leaves except for the uppermost leaf and bud. The base of each stem was placed, first, into an aq. solution (0.5 ml) containing [2-^{14}C]-brassinin (**1**) (4×10^{-4} M, 1×10^6 dpm, 5×10^9 dpm/mmol) and phenosafranin dye (5% v/v of a 0.1% w/v solution) followed by complete uptake of distilled H_2O (0.5 ml). Stems were then treated as described above for stem elicitation experiment. After 68 h of incubation, the tissue was extracted as reported for the elicitation experiment and the EtOAc extract residue (330 mg) was analyzed by both HPLC and LSC. Fractionation of the EtOAc extract was performed using (C-18 reversed-phase silica gel 1.5×5 cm column, isocratic elution with H_2O–MeCN, (60:40, v/v, followed by MeCN 100%). The fraction containing brassilexin was further purified by prep. TLC (CH_2Cl_2).

Administration of [2-^{14}C]-brassinin and [2-^{14}C]-indole-3-carboxaldehyde to stems of *B. juncea* elicited

with $CuCl_2$: stems prepared as described above were placed in an aq. solution of $CuCl_2$ (2×10^{-3} M, continuous uptake for 48 h) and incubated at 20 ± 0.5 °C with a 16 h photoperiod. An aq. solution (1.0 ml) containing either [2-^{14}C]-brassinin (**1**) (2×10^{-4} M, 1×10^{6} dpm, 5×10^{9} dpm/mmol) or [2-^{14}C]-indole-3-carboxaldehyde (**9**) (2×10^{-4} M, 1×10^{6} dpm, 5×10^{9} dpm/mmol) was administered to each stem, followed by distilled H_2O (0.5 ml). After complete uptake, the stems were again elicited with an aq. $CuCl_2$ solution (2×10^{-3} M) and were incubated with a 16-h photoperiod (20 ± 0.5 °C). After 2 and 4 days the stems were extracted as described above for stem elicitation experiment. Fractionation of the EtOAc extract (C-18 reversed-phase silica gel 1.5×5 cm column, isocratic elution both H_2O–MeCN, 80:20, v/v, followed by H_2O–MeCN, 60:40, v/v, and then MeCN 100%) yielded with brassilexin and spirobrassinin which were then further purified (prep. TLC,CH_2Cl_2–MeOH 98:2, v/v, developed twice).

4.5.2. Administration of labeled compounds to leaves

Petiolated leaves were placed in Falcon® tubes containing the phytoalexin solution or the control solution (10% MeOH and 0.1% Tween 80 in H_2O). Initial uptake experiments with nonlabeled brassinin (**1**) and cyclobrassinin (**2**) indicated that both compounds were phytotoxic to *B. juncea* (wilting and necrotic tissue). Interestingly, cyclobrassinin was more phytotoxic than brassinin; that is cyclobrassinin at concentrations 3×10^{-4} M damaged the petiole to an extent that no solution uptake occurred, whereas brassinin caused a similar effect at a concentration 5×10^{-4} M. Leaves (ca. 2×500 g) were placed in Falcon® tubes containing either 1 ml of [4,5,6,7-D_4]-brassinin (3×10^{-4} M in 10% MeOH and 0.1% Tween 80 in H_2O) or [4,5,6,7-D_4]-cyclobrassinin (2×10^{-4} M in 10% MeOH and 0.1% Tween 80 in H_2O). Following uptake of the solution, 1 ml of deionized H_2O was added to each tube; after the H_2O was taken up through the petiole, the leaves were treated as described for the leaf elicitation experiment (ca. 150 leaves per compound). The CH_2Cl_2 extracts were fractionated by HPLC to give pure brassilexin (**3**/**3a**); 300 μg of brassilexin were isolated in the case of the administration with experiments with [4,5,6,7-D_4]-cyclobrassinin (**2a**) and 500 μg with [4,5,6,7-D_4]-brassinin (**1a**). Administration of [4,5,6,7-D_4]-indole-3-acetaldoxime (**6a**, 10^{-3} M in 5% MeOH and 0.1% Tween 80 in H_2O) to *B. rapa* leaves (ca. 60 g from 3-week-old turnip plants) was carried out as described above for [4,5,6,7-D_4]-brassinin but elicitation was carried out differently. After compound uptake, leaves were kept with the petiole immersed in H_2O, the top of each tube covered with aluminum foil, and the leaves sprayed with the solution of $CuCl_2$ 2×10^{-3} M (this is time = t_0). The leaves were incubated (cycle 16-h light/8-h dark at $20+0.5$ °C for 24 h), then sprayed twice with $CuCl_2$ (2×10^{-3} M), at 24 h intervals, and finally incubated for a further 24 h. After the incubation period (total 72 h), the leaves were worked up as described above. The EtOAc (172 mg) extract was fractionated (C_{18} reversed-phase silica gel, MeOH–H_2O, gradient elution 0:100 to 100:0) and analyzed by HPLC (F3, 5 mg, containing compounds **7** and **9** was analyzed without further separation; F4, 7 mg was separated by prep. TLC with CH_2Cl_2–MeOH, 98:2, v/v, developed twice, to yield compound **4**).

4.5.3. Administration of labeled compounds to roots

Administration of [2-^{14}C]-brassinin (**1**) and [2-^{14}C]-indole-3-carboxaldehyde (**9**) to UV-irradiated turnip root: turnip roots (*B. rapa*) were prepared as described above for the root elicitation experiment. After incubation each hole was filled with an aq. solution (0.5 ml) containing either [2-^{14}C]-brassinin (**1**) (1.02×10^{7} dpm, 4.67×10^{9} dpm/mmol) or [2-^{14}C]-indole-3-carboxaldehyde (**9**) (1.26×10^{7} dpm, 4.53×10^{9} dpm/mmol). After further incubation, the aq. broth from each hole was combined and extracted with EtOAc, with the extracts then combined and concentrated to dryness. The residue was dissolved in MeOH for LSC and HPLC analysis. The turnip tissue was frozen in liquid N_2, homogenized using the Polytron® homogenizer and extracted with MeOH for 24 h. The MeOH residue was dissolved in H_2O, further extracted with EtOAc, concentrated to dryness and dissolved in MeOH for HPLC and LSC analysis.

Administration of [4,5,6,7-D_4]-brassinin (**1a**) to UV-irradiated turnip root: slices were prepared as described above for elicitation experiments. The turnip slices were incubated with **1a** (ca. 1×10^{-3} M in 0.2% of DMSO and 0.1% Tween-80 aq. solution) for 3 days at 20 °C. The aq. solution was harvested, extracted with EtOAc, dried over Na_2SO_4, concentrated, and analyzed by HPLC. This extract was separated by multiple prep. TLC (CH_2Cl_2–MeOH, 98:2 v/v) to yield compounds **3** and **4**.

Administration of [4,5,6,7-D_4]-indole-3-acetaldoxime (**6a**) to UV-irradiated turnip root: slices were prepared as described above for elicitation experiments and each hole was filled with a solution of **6a** (ca. 1.2×10^{-3} M in 0.2% of DMSO and 0.1% Tween-80 aq. solution). The turnip slices were incubated for three days in the dark at 20 °C. The aq. solution was harvested, extracted with EtOAc, dried over Na_2SO_4, concentrated, and analyzed by HPLC. The aq. solution was also concentrated and analyzed by HPLC. The EtOAc extract (76 mg) was separated by FCC (gradient of CH_2Cl_2–MeOH, 95:5, 90:10, v/v, and MeOH 100%) to give eight fractions, which were concentrated and analyzed by HPLC. F1 (23 mg) and F2 (17 mg) were further fractionated by multiple prep. TLC (CH_2Cl_2–MeOH, 95:5 v/v) to yield cyclobrassinin (**2**), brassilexin (**3**), spirobrassinin (**4**),

tryptophol (**13**), 2,3-dihydro-3-hydroxy-2-oxo-1H-indole-3-acetaldoxime (**14**), and 2,3-dihydro-3-hydroxy-2-oxo-1H-indole-3-acetonitrile (**15**).

To obtain sufficient amounts of compounds **13**–**15**, additional precursor administration experiments with indole-3-acetaldoxime (**6**, 161 mg and 2.5 kg of turnip roots) were carried out; compounds **13** (0.5 mg), **14** (1 mg), and **15** (1 mg) were then isolated in sufficient amounts for chemical characterization. In the absence of turnip root tissues acetaldoxime **6**/**6a** was stable in solution for the duration of the experiments.

2,3-Dihydro-3-hydroxy-2-oxo-1*H*-indole-3-acetaldoxime (**14**): HPLC $t_R = 4.8$ min (mobile phase A); ^{1}H NMR (500 MHz; CD_2Cl_2): δ 7.37 (*m*, 3 H), 7.14 (*dd*, $J = 7.5$, 2 Hz, 1H), 6.95 (*dd*, $J = 7.5$, 2 Hz, 1H), 3.60 (*d*, $J = 18$ Hz, 1H), 3.37 (*d*, $J = 18$ Hz, 1H). UV λ_{max} (in MeCN): 210, 250 and 300 nm. HRMS–EI *m*/*z* (% relative abundance): measured 188.0586, calc. for $[M]^+$ ($C_{10}H_8N_2O_2$) 188.0584 (24), 148.0402 (100), 120.0447 (13).

Synthesis of 2,3-dihydro-3-hydroxy-2-oxo-1*H*-indole-3-acetonitrile (**15**): this compound was prepared as previously reported in one step from isatin and bromoacetonitrile (Hallmann, 1962, Monde et al., 1991a). HPLC $t_R = 3.7$ min (mobile phase A). ^{1}H NMR (300 MHz, Me_2CO-d_6) : δ 9.57 (*br s*, 1H), 7.59 (*d*, $J = 7.5$ Hz, 1H,), 7.32 (*ddd*, $J = 8$, 8, 1 Hz, 1H), 7.09 (*dd*, $J = 7.5$, 7.5 Hz, 1H), 6.97 (*d*, $J = 8$ Hz, 1H), 5.64 (*br s*, 1H), 3.14 (*d*, $J = 16.5$ Hz, 1H), 2.94 (*d*, $J = 16.5$ Hz, 1H). ^{13}C NMR (75.5 MHz, Me_2CO-d_6): δ 176.7, 141.9, 130.6, 129.7, 124.7, 122.7, 116.4, 110.5, 72.8, 26.6. HRMS–EI *m*/*z* (% relative abundance): measured 188.0587 (19), calc. for $[M]^+$ ($C_{10}H_8N_2O_2$) 188.0585, 148.0407 (100), 120.0447 (17). FTIR ν_{max}: 3258, 1720, 1665, 1623, 1469, 1393, 1348, 1221, 1175, 1114, 1094 cm^{-1}. UV (MeOH) λ_{max} (log ϵ) 209 (4.42), 250 (3.78) and 290 (3.22).

Isolated 2,3-dihydro-3-hydroxy-2-oxo-1*H*-indole-3-acetonitrile (**15**): $[\alpha]_D$ −0.5 (*c* 0.21 MeOH; lit. −37 *c* 0.21 MeOH, Monde et al., 1991a). HRMS–EI *m*/*z* (% relative abundance): measured 192.0839 (20), calc. for $[M+4]^+$ 192.0836 ($C_{10}H_4D_4N_2O_2$), 188.0587 (2), calc. for $[M]^+$ ($C_{10}H_8N_2O_2$) 188.0585, 152.0654 (100), 124.0701 (16), 96.0753 (10).

Administration of [4,5,6,7-D_4]-glucobrassicin (**5a**) to UV-irradiated turnip root: slices were prepared as described above for elicitation experiments and each hole was filled with a solution of **5a** (ca. 0.4×10^{-3} M in H_2O). The turnip slices were incubated for 4 days at room temperature, the aq. solution was harvested, extracted with EtOAc, dried over Na_2SO_4, concentrated, and analyzed by HPLC. The aq. extract was concentrated, dissolved in distilled H_2O and analyzed by HPLC. Brassinin (**1**), brassilexin (**3**), spirobrassinin (**4**), indole-3-acetonitrile (**7**), indole-3-carboxaldehyde (**9**) and methyl indole-3-carboxylate (**12**) were isolated from the EtOAc extract (prep. TLC, CH_2Cl_2–MeOH, 98 : 2, v/v). In the absence of turnip root tissues indole glucosinolate **5**/**5a** was stable in solution for the duration of the experiments.

Administration of [4,5,6,7-D_4]-glucobrassicin (**5a**) to UV-irradiated *B. juncea* leaves: leaves were cut and placed immediately in Falcon® tubes containing [4,5,6,7-D_4]-glucobrassicin (**5a**) at 1×10^{-3} M in distilled H_2O. During uptake, the top of each tube was covered with aluminum foil, and the leaves were irradiated for 45 min. After uptake, the petioles were immersed in H_2O and incubated in a growth chamber under fluorescent lighting (cycle 16-h light/8-h dark at 20 ± 0.5 °C) for 24 h. After incubation, the leaves were again irradiated for 30 min and all the leaves were incubated for further 24 h. After this period, the leaves were immediately frozen in liquid N_2, crushed and extracted with MeOH; the solvent was filtered, the leaf tissue rinsed with MeOH and the extracts combined. The MeOH extract was concentrated and extracted with hexane. The hexane extract was fractionated (C_{18} reversed-phase silica gel, MeOH–H_2O, gradient 0:100 to 100:0) and the resulting fraction F6 (7 mg) was further separated (prep. TLC, CH_2Cl_2–hexane, 9:1, v/v, developed twice) to yield compounds **2** and **4**.

4.6. Indole-3-acetonitrile (7) as a phytoalexin of B. juncea

4.6.1. Isolation

Mustard stems (43; 10–15 cm in length), stripped of all leaves except for the uppermost leaf and bud, were elicited with continuous uptake of an aq. $CuCl_2$ solution (2×10^{-3} M). After a 4-day incubation (16-h photoperiod, 20 ± 0.5 °C), the stems were worked up as described above for elicitation experiments. The extract residue (340 mg) was fractionated (C-18 reversed-phase silica gel 1.5×5 cm column, isocratic elution with H_2O–MeCN 8:2, followed by H_2O–MeCN 6:4, then 100% MeCN), and the fraction (19.6 mg) containing the HPLC peak with $t_R = 11.7$ min was further fractionated by prep. TLC (CH_2Cl_2–MeOH, 98:2, v/v, developed 3×) to yield 1.2 mg of metabolite (**7**). Spectroscopic data collected was identical in all respects to those of an authentic sample (Aldrich-Sigma Canada).

Indole-3-acetonitrile (**7**): HPLC $t_R = 11.7$ min (mobile phase A). HRMS–EI *m*/*z* measured 156.0683 (70), calculated for $[M]^+$ ($C_{10}H_8N_2$) 156.0687.

4.6.2. Bioassays of antifungal activity

The antifungal activity of indole-3-acetonitrile (**7**) was assayed against *P. lingam*, *R. solani*, *S. sclerotiorum* and *A. brassicae*. The bioassays were conducted using either potato dextrose broth (PDB) liquid media or minimal media as described by Pedras (1998). Indole-3-acetonitrile (**7**) dissolved in DMSO (final concentration 1%) was added to PDB and poured into 6-well Falcon® plates (2 ml per well); final concentration of indole-3-acetonitrile (**7**) was 5×10^{-4} M and 1.0×10^{-4} M. Con-

trol plates containing PDB and PDB with 1% DMSO were prepared. A plug (7 mm diameter) of mycelia was cut from the edge of an actively growing fungal culture using a cork borer and placed upside down at the centre of each well. The plates were incubated for at least one week under continuous light at 25±2 °C (three independent experiments performed in triplicate were conducted for each fungal species). The diameter of the mycelia growth was measured and the percentage of inhibition was calculated relative to control plates.

4.6.3. Fungal metabolism

P. lingam virulent isolate BJ-125 was cultured on V-8 agar plates (Pedras and Okanga, 1999). Liquid shake cultures (100 ml minimal media containing 5 g glucose) in 250 ml Erlenmeyer flasks (triplicate) were inoculated with 6 plugs of mycelium cut from the edge of actively growing plates. The flasks were incubated for 30 h under continuous light (25±2 °C) while shaking at 130 rpm. A solution of indole-3-acetonitrile (**7**) (final concentration 1×10^{-4} M) in DMSO (final concentration 1%) was added and the flasks were incubated for different periods. Samples (5 ml) were withdrawn and extracted with Et_2O (3×10 ml). Ether extracts were combined, dried over Na_2SO_4 and concentrated to dryness under reduced pressure. The residues were dissolved in MeOH and analyzed by HPLC. A calibration curve was used to quantify the amount of indole-3-acetonitrile (**7**) in each sample based on the peak areas in the UV chromatograms.

4.6.4. Biosynthesis

The biosynthetic origin of indole-3-acetonitrile (**7**) in *B. juncea* was studied by determining the deuterium incorporation of several possible D_4-precursors. Synthetically prepared [4,5,6,7-D_4]-indole-3-acetaldoxime (**6a**), [4,5,6,7-D_4]-indole (**8a**) and commercially available (*S*)-[2′,4′,5′,6′,7′-D_5]-tryptophan were separately fed to $CuCl_2$ elicited *B. juncea* leaves. A typical experiment was conducted as follows: leaves (20, ca. 50 g leaf tissue fr. wt) from 3-week-old *B. juncea* were excised and immediately placed in an aq. $CuCl_2$ solution (8×10^{-5} M). Leaves were incubated with a 16-h photoperiod (20±0.5 °C). Following 24 h of incubation, the leaves were fed one of the following solutions (1–2 ml per leaf) of [4,5,6,7-D_4]-indole (5.3×10^{-4} M), [4,5,6,7-D_4]-indole-3-acetaldoxime (**6a**) (5.8×10^{-4} M), or (*S*)-[2,4,5,6,7-D_5]-tryptophan (9.6×10^{-4} M). After uptake of the solutions, petioles were immersed in distilled H_2O; after an additional 3–4 days, leaf tissue was worked up as described above. The extract residue was dissolved in H_2O (25 ml) and extracted with EtOAc (3×50 ml). The combined EtOAc extracts were dried over Na_2SO_4 and concentrated to dryness, and analyzed by HPLC. The residue was fractionated (C-18 reversed-phase, isocratic elution with H_2O–MeCN 8:2 followed by H_2O–MeCN 6:4, then 100% MeCN) and the fraction containing indole-3-acetonitrile (**7**) was further separated by prep. TLC (CH_2Cl_2–MeOH 98:2, v/v, developed 3×).

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