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Detoxification pathways of the phytoalexins brassilexin and sinalexin in *Leptosphaeria maculans*: isolation and synthesis of the elusive intermediate 3-formylindolyl-2-sulfonic acid†

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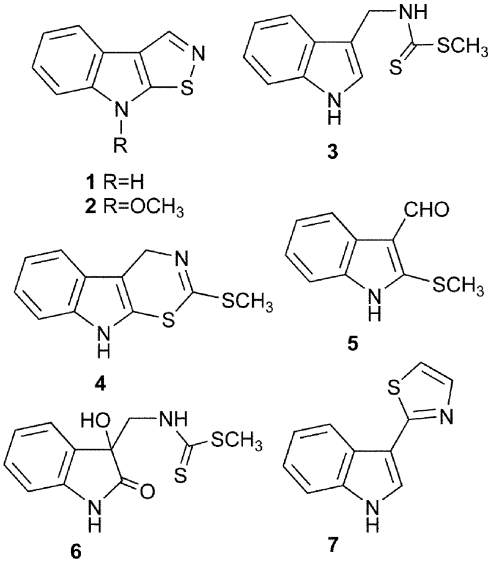
Brassilexin and sinalexin are among the most potent antifungal phytoalexins produced by crucifer plants. Nonetheless, the crucifer pathogen *Leptosphaeria maculans* (Desm.) Ces. et de Not. (asexual stage *Phoma lingam* (Tode ex Fr.) Desm.) is able to detoxify these phytoalexins. To understand and control these reactions, the elusive brassilexin and sinalexin detoxification pathways in *L. maculans* were investigated and compared to those of the synthetic derivatives 8-methylbrassilexin and 8-acetylbrassilexin. The chemical structures, syntheses and antifungal activities of the metabolic intermediates, including the most polar metabolite identified thus far, 3-formylindolyl-2-sulfonic acid, were established. Our overall findings suggest that the first detoxification step of brassilexin involves its reductive bioconversion to 3-aminomethyleneindole-2-thione, followed by hydrolysis and oxidation to the water soluble metabolite 3-formylindolyl-2-sulfonic acid.

Introduction

Brassilexin (**1**) and sinalexin (**2**) are among the most potent antifungal phytoalexins produced by crucifer plants. While brassilexin (**1**) is produced by several *Brassica* species, sinalexin (**2**) has been isolated only from *Sinapis alba*.[1,2] Phytoalexins are secondary metabolites produced *de novo* by plants in response to diverse forms of stress, including fungal invasion. To counterattack, many plant fungal pathogens, including those of crucifers, have evolved enzymatic systems able to detoxify phytoalexins.[3] In particular, the crucifer pathogen *Leptosphaeria maculans* (Desm.) Ces. et de Not. (asexual stage *Phoma lingam* (Tode ex Fr.) Desm.) is able to detoxify various phytoalexins including brassinin (**3**), cyclobrassinin (**4**), brassicanal A (**5**) and dioxibrassinin (**6**); however, *L. maculans* could not metabolize camalexin (**7**). Interestingly, cyclobrassinin (**4**) detoxification in avirulent isolates of *L. maculans*‡[4] to undetermined products occurred *via* brassilexin (**1**). However, the metabolic product(s) of brassilexin detoxification could not be determined, likely due a much lower solubility in organic solvents than in aqueous solution.[5]

Considering the antifungal activity of brassilexin (**1**) against *L. maculans*, it appears likely that an increase of its concentration in the plant, through, for example, inhibition of its degradation or detoxification, could prevent the spread of fungal infection. To apply this concept, selective detoxification inhibitors, paldoxins,[2] can be designed based on the structures of brassilexin (**1**) and putative detoxification intermediates. Hence, the elusive brassilexin detoxification pathway in *L. maculans* has to be understood and the chemical structures of the detoxification intermediate(s) established. Toward this end, we have investigated the detoxification of brassilexin (**1**) and sinalexin (**2**) in *L. maculans* and report here the chemical structures, syntheses and antifungal activities of these intermediates. In addition, these metabolic pathways were compared to those of the synthetic derivatives 8-methylbrassilexin (**13a**) and 8-acetylbrassilexin (**13b**). Our overall findings suggest that effective inhibitors of brassilexin (**1**) detoxification must prevent the reductive bioconversion of brassilexin (**1**) into 3-aminomethyleneindole-2-thione (**8**).



Results and discussion

Biotransformations

Brassilexin (**1**) was synthesized as described recently,[6] and its biotransformation in cultures of *L. maculans* was analyzed over a period of several days. HPLC analyses of the EtOAc extracts of cultures incubated with brassilexin (**1**, final concentration 1.0×10^{-4} M) either in water (seven-day-old mycelia) or in minimal medium (48-h-old mycelia) showed a decrease in the area of the peak ($R_t = 13.0$ min) corresponding to brassilexin (**1**) and the presence of a new peak (HPLC $R_t = 8.4$ min). This peak corresponded to a new metabolite determined to be 3-aminomethyleneindole-2-thione (**8**), resulting from reduction of the isothiazole ring of brassilexin (**1**). Metabolite **8** was detected in EtOAc extracts of cultures containing brassilexin (**1**) after 4 h of incubation and was metabolized to undetermined products

† Electronic supplementary information (ESI) available: Preparation of compounds **13a** and **13b**, spectroscopic data for compounds **1**, **2**, **8**, **13a**, **13b**, **14** and **15**, Scheme 6. See http://www.rsc.org/suppdata/ob/b5/b501907a/

‡ Avirulent isolates of *L. maculans*/*P. lingam* are now considered a different species, see for example, ref. 4.

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within 48 h to several days (faster in cultures incubated in minimal media, 48 h, than in cultures incubated in water, over 120 h, Fig. 1). Brassilexin (**1**) was stable in control solutions for at least 10 days. Feeding experiments carried out with synthetic enamine **8** (final concentration 1.0×10^{-4} M) showed it to be completely metabolized in 48 h. Control aqueous solutions containing enamine **8** indicated its slow oxidation to brassilexin (**1**) (<5% in 24 h). Furthermore, HPLC analysis of the aqueous layers of cultures incubated with brassilexin (obtained from mycelial cultures incubated in water and extracted with EtOAc) showed a new peak in 12-h-old cultures (increasing for the duration of the experiment, 120 h) not detected in control cultures. To obtain a sufficient amount of this polar metabolite for structure determination, 7-day-old mycelia was incubated with brassilexin (**1**) in sterile water, as described in the experimental. After 72 h, the mycelia was filtered off, the filtrate was concentrated and was extracted with EtOAc to remove less polar compounds (brassilexin (**1**) and enamine **8**). The remaining aqueous layer was concentrated further and the residue was fractionated using reverse phase column chromatography to afford a solid material (1 mg). Analysis of the spectroscopic data including HRMS-ESI (negative ion) and NMR suggested the molecular formula $C_9H_7NO_4S$, indicative of an indole ring. ^{1}H NMR (500 MHz, DMSO-d_6) spectra showed the presence of a broad singlet at δ_H 12.18 (NH), a sharp singlet at δ_H 10.46, indicating the presence of an aldehyde group, and four aromatic protons. Similar to brassilexin (**1**), the presence of an indole nucleus substituted at positions 2 and 3 was confirmed by analysis of the ^{13}C NMR spectral data. Based on the spectral data and molecular formula, the substituent at C-2 was thought to be a sulfonic acid, a hypothesis which was further corroborated by the absorption bands at 1419 and 1215 cm^{-1} in the FTIR spectrum. Thus, the structure of the new metabolite was assigned as 3-formylindolyl-2-sulfonic acid (**12**). This assignment was confirmed by synthesis, as described below.

The fungal biotransformation of 3-aminomethyleneindole-2-thione (**8**) to 3-formylindolyl-2-sulfonic acid (**12**) suggested that **8** was first hydrolyzed to 2-sulfanylindolyl-3-carbaldehyde (**9**) which was subsequently oxidized to sulfonic acid **12**, probably *via* 3-formylindolyl-2-sulfenic acid (**10**) and 3-formylindolyl-2-sulfinic acid (**11**). Sulfenic acids are known to be intermediates in the oxidations of RSH to sulfur oxides (RSO_xH) and disulfides (RSSR) in living systems.[7] Although aldehyde **9** was not detected in either the EtOAc extract or in the aqueous layer of cultures incubated with brassilexin (**1**) or 3-aminomethyleneindole-2-thione (**8**), an incubation experiment with **9** showed its complete metabolism to sulfonic acid **12** in 24 h (Table 1). Furthermore, incubation of fungal cultures with sulfonic acid **12** (final concentration 1.0×10^{-4} M) showed it to be biotransformed slowly to undetermined products (present in the cultures even after 120 h). Based on these results, the proposed biotransformation pathway of brassilexin in *L. maculans* is depicted in Scheme 1.

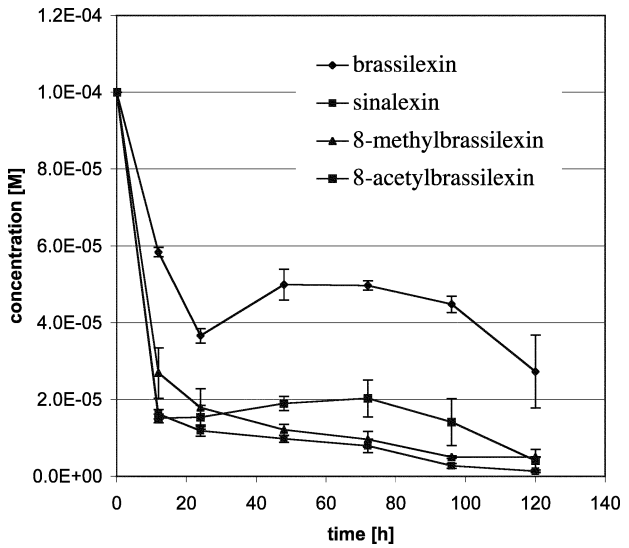
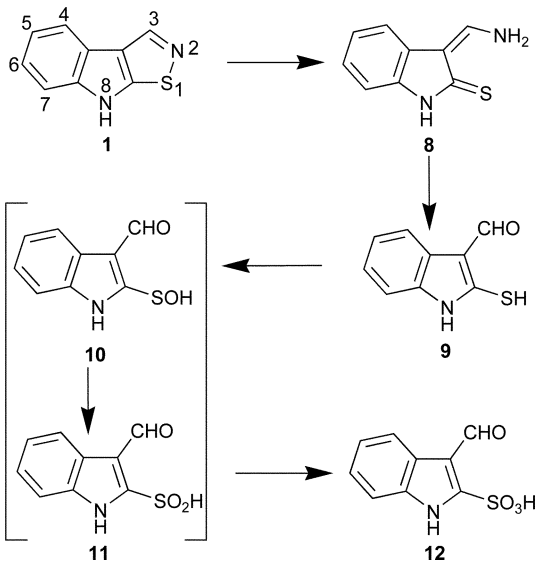


Fig. 1 Metabolism of brassilexin (**1**), sinalexin (**2**), 8-methylbrassilexin (**13a**) and 8-acetylbrassilexin (**13b**) in cultures of *Leptosphaeria maculans*/*Phoma lingam* (in water). Cultures were extracted and extracts analyzed by HPLC; concentrations were determined using calibration curves, each point is an average of triplicate experiments ± standard deviation. 8-Acetylbrassilexin (**13b**) is hydrolyzed slowly to brassilexin (**1**) in aqueous control solutions (*ca.* 5% in 24 h).



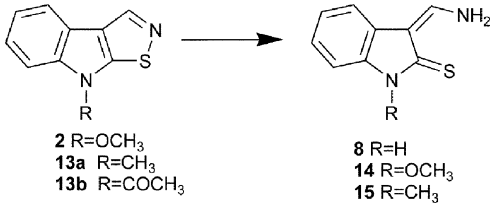
Scheme 1 Detoxification pathway of brassilexin (**1**) in *Leptosphaeria maculans*/*Phoma lingam*; structures in parentheses are proposed intermediates.

Table 1 Transformation of 3-aminomethyleneindole-2-thione (**8**), 2-sulfanylindolyl-3-carbaldehyde (**9**), 3-formylindolyl-2-sulfonic acid (**12**) and 3-aminomethylene-1-metylindole-2-thione (**15**) by *Leptosphaeria maculans*/*Phoma lingam* (in water)

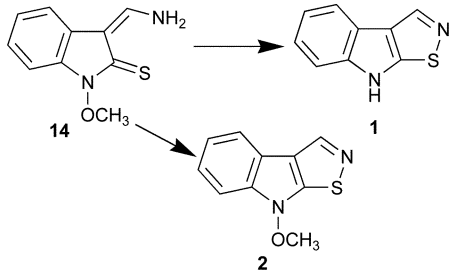
Compound added to fungal cultures[a]	Products (%)[b] of metabolism after incubation for 12–96 h; (% total recovery)
3-Aminomethylene-indole-2-thione (**8**)	12 h: **1** (13 ± 5); **8** (43 ± 2); **12** (<5); (56 ± 5) 24 h: **1** (6 ± 3); **8** (11 ± 5); **12** (52 ± 6); (69 ± 6) 48 h: **1** (<5); **8** (<5); **12** (68 ± 8); (68 ± 8)
2-Sulfanylindolyl-3-carbaldehyde (**9**)	12 h: **9** (24 ± 2); **12** (50 ± 5); (74 ± 5) 24 h: **9** (<5); **12** (57 ± 3); (57 ± 5)
3-Formylindolyl-2-sulfonic acid (**10**)	No metabolism in 72 h
3-Aminomethylene-1-methylindole-2-thione (**15**)	12 h: **13a** (15 ± 6); **15** (47 ± 4); (62 ± 6) 24 h: **13a** (15 ± 5); **15** (42 ± 3); (57 ± 5) 96 h: **13a** (11 ± 5); **15** (45 ± 5); (56 ± 5)

[a] Compounds (concentration in culture 1.0×10^{-4} M) were dissolved in acetonitrile, added to 7-days-old cultures and incubated at 24 ± 2 °C. [b] Average percentage yields (% ± standard deviations; triplicate samples) were calculated by HPLC analysis using calibration curves constructed for each compound.

The biotransformation of sinalexin (**2**) by *L. maculans* was investigated as reported above for brassilexin (**1**). HPLC analyses of the EtOAc extracts of cultures incubated with sinalexin (**2**) either in water (7-day-old mycelia) or in minimal medium (48-h-old mycelia) over a period of several days showed a decrease in the area of the peak (R_t = 20.1 min) corresponding to sinalexin (**2**, Fig. 1) and the presence of a new peak (R_t = 13.4 min) detectable after 4 h of incubation. The new metabolite corresponding to this peak was determined to be 3-aminomethylene-1-methoxyindole-2-thione (**14**, Scheme 2), resulting from reduction of the isothiazole moiety, similar to the first biotransformation step of brassilexin (**1**, Scheme 1). Cultures of *L. maculans* incubated with 3-aminomethylene-1-methoxyindole-2-thione (**14**)[6] (shaker, 24 h) yielded brassilexin (**1**), 3-aminomethyleneindole-2-thione (**8**) and sulfonic acid **12** (<5%). Furthermore, HPLC analysis of aqueous solutions of 3-aminomethylene-1-methoxyindole-2-thione (**14**) under similar conditions (shaker, 24 h) indicated its decomposition mainly to brassilexin (**1**, *ca.* 20% in 24 h, 50% in 96 h; a small amount of sinalexin (**2**) was detected, <5%, Scheme 3). These results suggested that sinalexin (**2**) was biotransformed to 3-aminomethylene-1-methoxyindole-2-thione (**14**), although conclusions about further biotransformation intermediates were precluded by the decomposition of 3-aminomethylene-1-methoxyindole-2-thione (**14**) in aqueous solution (Scheme 3).



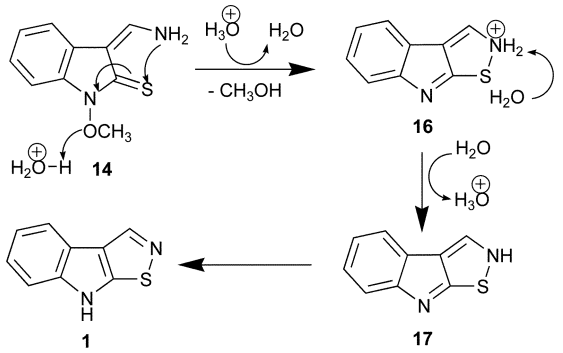
Scheme 2 Detoxification pathway of sinalexin (**2**), 8-methylbrassilexin (**13a**) and 8-acetylbrassilexin (**13b**) in *Leptosphaeria maculans*/*Phoma lingam*.



Scheme 3 Conversion of 3-aminomethylene-1-methoxyindole-2-thione (**14**) to brassilexin (**1**) (*ca.* 50% formed in 96 h) and sinalexin (**2**) (<5% formed in 96 h) in aqueous solution.

Subsequently, to obtain further insight into the biotransformation pathway of sinalexin (**2**) in *L. maculans*, 8-methylbrassilexin (**13a**) was synthesized and incubated with fungal cultures as described for brassilexin (**1**). It was hypothesized that if the fungus reduced 8-methylbrassilexin (**13a**) to 3-aminomethylene-1-methylindole-2-thione (**15**, Scheme 2), this enamine would be more stable than the 1-methoxyenamine **14** and thus would facilitate the determination of the intermediates of this biotransformation pathway. HPLC analysis of cultures incubated with 8-methylbrassilexin (**13a**) indicated that only enamine **15** was produced, and that no other new peaks were detected by HPLC (either in the EtOAc extract and aqueous layer). A synthetic sample of 3-formyl-1-methylindolyl-2-sulfonic acid (**23**) confirmed that no identical compound was formed in the cultures (Table 1). These results demonstrated that under the experimental conditions described, 1-methylenamine **15** was not metabolized to 1-methyl-3-formylindolyl-2-sulfonic acid (**23**) but was biotransformed slowly to undetermined products. In addition, the biotransformation of 8-acetylbrassilexin (**13b**) was also examined and found to occur after hydrolysis of the acetyl group to yield brassilexin (**1**), whose biotransformation followed the pathway depicted in Scheme 1. Although chemical hydrolysis of the acetyl group also occurred in control solutions containing 8-acetylbrassilexin (**13b**, <5% in 24 h), it was substantially slower than the enzymatic process occurring in *L. maculans* (Fig. 1).

The lower stability of 3-aminomethylene-1-methoxyindole-2-thione (**14**), in aqueous solution, relative to 3-aminomethyleneindole-2-thione (**8**) and 3-aminomethylene-1-methylindole-2-thione (**15**) and its unexpected conversion to brassilexin (**1**) may be rationalized as shown in Scheme 4. The sequential elimination of CH_3OH promoted by protonation followed by nucleophilic attack of the enamine nitrogen on the thione sulfur, and subsequent proton shift might be driven by formation of the thermodynamically more stable aromatic 8*H*-isothiazolo[5,4-*b*]indole ring (Scheme 4). In agreement with this hypothesis, it was established that 3-aminomethylene-1-methoxyindole-2-thione (**14**) was stable in aprotic solvents (CH_2Cl_2, $CHCl_3$, EtOAc, CH_3CN) since no other products were detected by TLC or ^{1}H NMR. The percentages of conversion of 1-methoxyenamine **14** to brassilexin (**1**) in a solution of CD_3CN–D_2O could be determined by integration of the signals due to H-3 of brassilexin (**1**) at δ_H 8.70, s, and the corresponding signal at δ_H 8.30, s, of enamine **14** (*cf.* ESI†).

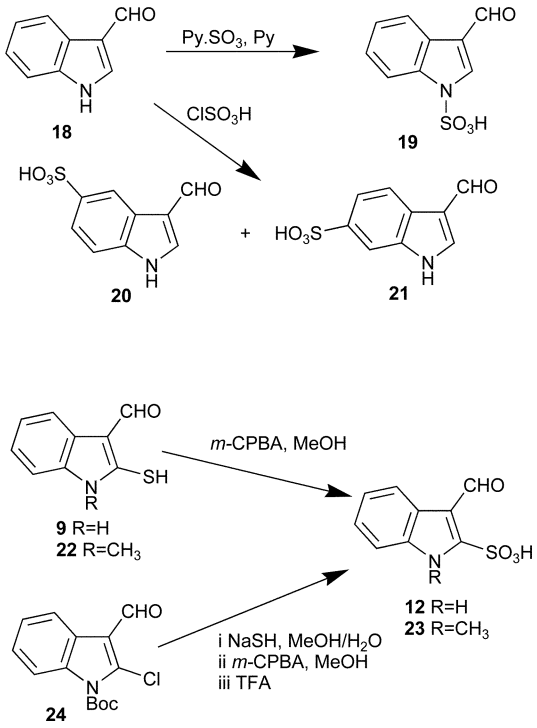


Scheme 4 Proposed pathway for the conversion of 3-aminomethylene-1-methoxyindole-2-thione (**14**) to brassilexin (**1**) in aqueous solution.

In conclusion, these metabolic transformations of brassilexin (**1**), sinalexin (**2**) and analogues **13a** and **13b** in cultures of *L. maculans* showed that the rate of metabolism of brassilexin (**1**) was slower than the rates of biotransformation of **2**, **13a** and **13b** (Fig. 1).

Syntheses

The chemical synthesis of 3-formylindolyl-2-sulfonic acid (**12**) was carried out to confirm the chemical structure of the final biotransformation product of brassilexin (**1**) and to obtain sufficient amounts for bioassays. It has been reported,[8] that sulfonation of 3-alkylindoles using Py·SO_3 in refluxing pyridine yielded exclusively the corresponding 3-alkylindolyl-2-sulfonic acids and that *N*-sulfonation was not observed. However, sulfonation of indole-3-carbaldehyde (**18**) using this method yielded only the product of *N*-sulfonation (**19**, Scheme 5). The electron withdrawing character of the formyl group can deactivate the position 2 of the indole nucleus towards electrophilic substitution and promote *N*-sulfonation. Subsequent sulfonation of position 2 was not observed despite large excess (up to 10 equivalents) of Py.SO_3 and prolonged reflux (48 h) in pyridine. In addition, sulfonation of indole-3-carbaldehyde using $ClSO_3H$[9] led to a mixture of 3-formylindolyl-5-sulfonic acid (**20**) and 3-formylindolyl-6-sulfonic acid (**21**, Scheme 5). Another method to prepare sulfonic acid **12** was attempted through oxidation of 2-sulfanylindolyl-3-carbaldehyde (**9**) with *m*-CPBA or H_2O_2; however these conditions led to multiple products including the



Scheme 5 Synthesis of 3-formylindolylsulfonic acids **12**, **19–21** and **23**.

sulfonic acid **12** in very low yield (Scheme 5). Next, *N*-Boc-2-chloroindolyl-3-carbaldehyde (**24**)[10] was treated with NaSH in MeOH/water,[11] followed by extraction and *m*-CPBA mediated oxidation. The Boc-protected 3-formylindolyl-2-sulfonic acid was separated by column chromatography and deprotected by treatment with trifluoroacetic acid (Scheme 5). 3-Formylindolyl-2-sulfonic acid (**12**) was obtained in 48% yield based on aldehyde **24**. Furthermore, as mentioned above, to prove that 1-methyl-3-formylindolyl-2-sulfonic acid (**23**) was not formed during the biotransformation of 8-methylbrassilexin (**13a**), 1-methyl-2-sulfanylindole-3-carbaldehyde (**22**), prepared by Vilsmeyer formylation of 1-methylindoline-2-thione[6] was oxidized with *m*-CPBA (Scheme 5). This transformation afforded 1-methyl-3-formylindolyl-2-sulfonic acid (**23**) in 25% yield based on 1-metylindole-2-thione.

Antifungal bioassays

The antifungal bioassays against *L. maculans* were carried out as described in the Experimental section (see also Table 2). Due to the lower solubility of 8-acetylbrassilexin (**13b**) in the bioassay medium, its antifungal activity could not be compared to that of **1**, **2** and **13a** at similar concentrations. Nonetheless, it could be determined that the antifungal activities of the enamines **8**, **14** and **15** are lower than those of brassilexin (**1**), sinalexin (**2**) and 8-methylbrassilexin (**13a**). Furthermore, the antifungal activity of 2-sulfanylindolyl-3-carbaldehyde (**9**) was lower than that of enamine **8** whereas 3-formylindolyl-2-sulfonic acid (**12**) did not exhibit antifungal activity. As discussed above, enamines **8**, **14** and **15** undergo slow oxidation on standing with the formation of brassilexin (**1**), sinalexin (**2**) and 8-methylbrassilexin (**13a**), respectively. Thus the results of these bioassays do not reflect accurately the antifungal activity of each enamine, which is likely lower than the percentages reported in Table 2. Consequently, the biotransformations of brassilexin (**1**) and analogues in *L. maculans* can be considered as detoxification processes.

Conclusion

The antifungal activity displayed by the phytoalexins brassilexin (**1**) and sinalexin (**2**) indicated that these compounds could be of interest to inhibit the growth of some plant pathogens. However, previous results on the metabolism of brassilexin (**1**) by isolates of *L. maculans* suggested it to be detoxified to very polar compounds that could not be extracted with organic solvents.[5] To establish the detoxification pathway of brassilexin (**1**), we have now overcome the extraction problems caused by the much higher polarity of the metabolic product. By incubating fungal spores of *L. maculans* in a defined medium for 7 days, followed by filtration and washing, the resulting mycelial mat could be transferred into sterile water and incubated directly with the compounds under investigation. The resulting aqueous broth was sufficiently free of salts to be concentrated (freeze-dried) and the residues analyzed by HPLC and ^{1}H NMR. Using this culture method, it was discovered that *L. maculans* transformed brassilexin (**1**) to the very polar metabolite 3-formylindolyl-2-sulfonic acid (**12**), which was devoid of antifungal activity. To the best of our knowledge this is the first time that 3-formylindolyl-2-sulfonic acid (**12**) is described. Indolyl-3-acetic acid was reported to yield (2-sulfoindolyl)-3-acetic acid in the presence of Mn^{2+} and $NaHSO_3$;[12] the structure of (2-sulfoindolyl)-3-acetic acid was then corroborated by sulfonation of indolyl-3-acetic acid using $Py{\cdot}SO_3$ complex.[8,12]

Interestingly, the rate of metabolism of brassilexin (**1**) in *L. maculans* was slower than the rates of transformation of sinalexin (**2**), 8-methylbrassilexin (**13a**) and 8-acetylbrassilexin (**13b**). Because the range of antifungal activity displayed by these compounds is quite similar, it is apparent that these rate differences are not correlated with their antifungal activity but perhaps related to their different solubility in aqueous solutions. Furthermore, considering that the first step in the detoxification of brassilexin (**1**) involves reduction of its isothiazole ring, potential inhibitors of this detoxification pathway need to slow down or inhibit this step. It is expected that the isolation of fungal enzyme(s) involved in such a crucial detoxification reaction will

Table 2 Results[a] of antifungal bioassay against *Leptosphaeria maculans*/*Phoma lingam*

Compound	% Inhibition		
	1×10^{-4}	2×10^{-4}	5×10^{-4}
Brassilexin (**1**)[b]	8 ± 1	62 ± 1	C. I.
Sinalexin (**2**)[b]	24 ± 7	69 ± 4	C. I.
3-Aminomethyleneindole-2-thione (**8**)	25 ± 7	32 ± 6	53 ± 13
2-Sulfanylindolyl-3-carbaldehyde (**9**)	N. I.	21 ± 2	32 ± 3
3-Formylindolyl-2-sulfonic acid (**12**)	N. I.	N. I.	N. I.
8-Methylbrassilexin (**13a**)[b]	38 ± 1	72 ± 4	C. I.
8-Acetylbrassilexin (**13b**)	11 ± 3	Not soluble	Not soluble
3-Aminomethylene-1-methoxyindole-2-thione (**14**)	N. I.	27 ± 6	57 ± 6
3-Aminomethylene-1-methylindole-2-thione (**15**)	25 ± 7	53 ± 6	79 ± 14

[a] % inhibition = 100 − [(growth on treated/growth on control) × 100] ± SD; results are the means of at least three independent experiments; C. I. = complete inhibition; N. I. = no inhibition. [b] Results as reported previously.[6]

assist the biorational design of crop protection agents selective against *L. maculans*, *i.e.* paldoxins for blackleg disease.

Experimental

General experimental procedures

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON. All solvents were HPLC grade and used as such, except for CH_2Cl_2 and $CHCl_3$ that were redistilled. Organic extracts were dried over anhydrous Na_2SO_4 and solvents removed under reduced pressure in a rotary evaporator.

HPLC analysis was carried out with a high performance liquid chromatograph equipped with quaternary pump, automatic injector, and diode array detector (wavelength range 190–600 nm), 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 A: 75% H_2O–25% CH_3CN to 100% CH_3CN, for 35 min, linear gradient, and a flow rate 1.0 ml min^{-1}; B: 100% H_2O for 10 min, then linear gradient to 50% H_2O–50% CH_3CN for 15 min, flow rate 1 ml min^{-1}. UV spectra were recorded on Varian-Cary spectrophotometer in MeOH. Fourier transform IR spectra were obtained on a Bio-Rad FTS-40 spectrometer in KBr. NMR spectra were recorded on Bruker Avance 500 spectrometers; for ^1H (500 MHz), δ values were referenced as follows: CD_3SOCD_3 (CD_3SOCHD_2 2.50 ppm); for ^{13}C (125 MHz) CD_3SOCD_3 (39.51 ppm). Mass spectra (MS) were obtained on a VG 70 SE mass spectrometer using a solid probe or on a Q Star XL, Applied Biosystems.

Fungal cultures

Fungal cultures of *Leptosphaeria maculans*/*Phoma lingam* virulent isolate BJ 125 were obtained from the IBCN collection, Agriculture and Agri-Food Canada Research Station, Saskatoon SK. Cultures were handled as described previously.[13]

Fungal metabolism

Time-course studies of metabolism. Solutions of brassilexin (**1**), sinalexin (**2**), 8-methylbrassilexin (**13a**), 8-acetylbrassilexin (**13b**), 3-aminomethyleneindole-2-thione (**8**), 3-aminomethylene-1-methoxyindole-2-thione (**14**), 3-aminomethylene-1-metylindole-2-thione (**15**), 2-sulfanylindolyl-3-carbaldehyde (**9**) in acetonitrile (0.3 ml) and 3-formylindolyl-2-sulfonic acid (**12**) in water (0.3 ml) were added to liquid shake cultures (100 ml of minimal media in 250 ml Erlenmeyer flasks, final concentration 1.0×10^{-4} M) of isolate BJ-125 (44 h old, 10^8/100 ml spores). Cultures were incubated on a shaker at 130 rpm, at 24 ± 2 °C. Samples (2.5 ml) were withdrawn at appropriate times, frozen or immediately extracted with EtOAc (2 × 5 ml). Both, organic and water phases were concentrated, dissolved in CH_3CN (1.0 ml) or MeOH–H_2O (1 : 1, 1.0 ml) and analyzed by HPLC. Experiments were performed in triplicate and control flasks, containing the mycelia in minimal media or compounds in minimal media were incubated under similar conditions. For biotransformations in water the 7-days-old mycelia of *L. maculans* (BJ-125, 10^8/100 ml spores) was filtered off and transferred to 100 ml of sterile water in 250 ml Erlenmeyer flasks. The cultures were then incubated with solutions of **1**, **2**, **8**, **9**, **12–15** on a shaker at 130 rpm, at 24 ± 2 °C. Samples (2.5 ml) were withdrawn and worked up as reported above.

Large-scale metabolism experiments. To obtain larger amounts of extracts to isolate the products of metabolism experiments were carried out as follows. Seven days old mycelia of *L. maculans* (BJ-125, 10^8/100 ml spores) was filtered off and transferred to 100 ml of sterile water in 250 ml Erlenmeyer flask. Brassilexin (**1**, 25 mg, five flasks), sinalexin (**2**, 20 mg, five flasks), 8-acetylbrassilexin (**13b**, 20 mg, five flasks) and 8-methylbrassilexin (**13a**, 10 mg, two flasks) were administered to cultures and incubated as above. After 72 h the mycelia was filtered off, the broth was concentrated to a small volume (*ca.* 30 ml) and extracted with EtOAc. The layers were separated and the water layer was freeze-dried. In the case of brassilexin (**1**), the residue from the aqueous layer was subjected for reverse-phase column chromatography on pre-packed columns, eluting first with 100% H_2O followed by 10% CH_3CN in H_2O to yield two fractions. The later fraction was concentrated to afford 1 mg of sulfonic acid **12**. In all other cases, the EtOAc extracts were concentrated and subjected to preparative TLC on silica gel plates to afford the following metabolites listed in order of increasing polarity. Metabolites obtained from sinalexin (**2**) metabolism: sinalexin (**2**, 1 mg), brassilexin (**1**, 1 mg), 3-aminomethylene-1-methoxyindole-2-thione (**14**, 5 mg) 3-aminomethyleneindole-2-thione (**8**, 8 mg) (hexane–acetone (2 : 1)). Metabolites obtained from 8-acetylbrassilexin (**13b**): 8-acetylbrassilexin (**13b**, 1 mg), brassilexin (**1**, 1 mg), 3-aminomethyleneindole-2-thione (**8**, 10 mg) (hexane–acetone (2 : 1)). Metabolites obtained from 8-methylbrassilexin (**13a**): 8-methylbrassilexin (**13a**, 2 mg), 3-aminomethylene-1-metylindole-2-thione (**15**, 4 mg) (hexane–EtOAc (2 : 1)).

Antifungal bioassays

Antifungal bioassays against *L. maculans* were carried out as follows: a DMSO solution of the compound to be tested (final concentration 5.0×10^{-4}, 2.0×10^{-4} and 1.0×10^{-4} M, final DMSO concentration 1%) was added to potato dextrose agar medium at *ca.* 50 °C, mixed quickly and poured onto 6-well plates (2.5 ml). An agar plug (8 mm diameter) cut from edges of 7-day-old solid cultures was placed upside down on the center of each plate and the plates were incubated at 24 ± 2 °C under constant light for 5 days. The diameter of the mycelia (in mm) was then measured and compared with control plates containing only DMSO. Each assay was conducted in triplicate and repeated at least three times.

Synthesis

Sulfonation of indole-3-carbaldehyde with sulfur trioxide pyridine complex. A mixture of indole-3-carbaldehyde (**18**, 145 mg, 1.0 mmol) and Py·SO_3 (159 mg, 1.0 mmol) in pyridine (0.6 ml) was refluxed for 2 h. After being cooled to room temperature, the mixture was diluted with water (50 ml), was neutralized with 1 M HCl, and was concentrated. The aqueous phase was freeze-dried and the residue was subjected for FCC (SiO_2, CH_2Cl_2–MeOH, 4 : 1). Concentration of the eluate afforded 200 mg of the solid residue, which was subjected to repeated FCC (SiO_2, CH_2Cl_2–MeOH, 9 : 1 and 4 : 1) to afford an orange solid, 3-formylindolyl-1-sulfonic acid (**19**, 130 mg, 58%). Mp 135–137 °C (crystallization from MeOH–Et_2O); HPLC: $R_t = 1.9$ min; λ_{max}(MeOH)/nm 211 (ε/dm^3 mol^{-1} cm^{-1} 18900), 239 (7800), 289 (8100). ν_{max}(KBr)/cm^{-1}: 3498, 3121, 1657, 1530, 1243 and 1059. δ_H (500 MHz; DMSO-d_6): 7.24 (dd, $J = 8$, 8 Hz, 1H), 7.30 (dd, $J = 8$, 8 Hz, 1H), 7.87 (d, $J = 8$ Hz, 1H), 8.07 (d, $J = 8$ Hz, 1H), 8.35 (s, 1H), 9.93 (s, 1H). δ_C (125 MHz; DMSO-d_6): 114.2 (d), 116.6 (s), 120.6 (d), 122.5 (d), 123.6 (d), 124.8 (s), 135.5 (s), 139.1 (d), 185.8 (d). HRMS (ESI): calc. for $C_9H_6NO_4S$ ($M - H^+$) m/z 224.0027, found 224.0023.

Sulfonation of indole-3-carbaldehyde with chlorosulfonic acid. Chlorosulfonic acid (70 μl, 1.0 mmol) was added to a suspension of indole-3-carbaldehyde (**18**, 73 mg, 0.50 mmol) in dichloromethane (3 ml) and the mixture was allowed to stir at room temperature for 3 h. The precipitate was filtered off, dried and subjected to FCC (SiO_2, CH_2Cl_2–MeOH, 4 : 1). Concentration of the eluate afforded a mixture of 3-formylindolyl-5-sulfonic acid (**20**) and 3-formylindolyl-6-sulfonic acid (**21**) as a red solid (64 mg, 57%, in a 7 : 3 ratio, not determined which isomer is major). HPLC: $R_t = 1.8$ min; δ_H (500 MHz; DMSO-d_6): 7.45 (d, $J = 8$ Hz, 0.7H), 7.50 (d, $J = 8$ Hz, 0.3H), 7.55 (d, $J = 8$ Hz, 0.7H), 7.77 (s, 0.3H), 8.01 (d, $J = 8$ Hz, 0.3H), 8.31 (d,

J = 3 Hz, 0.7H), 8.33 (d, J = 3 Hz, 0.3H), 8.41 (s, 0.7H), 9.92 (s, 0.3H), 9.93 (s, 0.7H), 12.22 (br s, D_2O exchangeable, 1H). δ_C (125 MHz; DMSO-d_6): 109.7 (d, minor), 111.5 (d, major), 118.1 (s, major), 118.5 (d, major), 118.6 (d, minor), 119.8 (d, minor), 120.2 (s, major), 121.7 (d, major), 123.1 (d, minor), 124.2 (s, minor), 136.1 (s, minor), 137.0 (s, major), 139.3 (d, major), 139.4 (s, minor), 142.0 (s, major), 143.6 (s, minor), 185.0 (d, major + minor).

Oxidation of 2-sulfanylindolyl-3-carbaldehyde (9). To a solution of aldehyde **9**[5] (18 mg, 0.1 mmol) in MeOH (1 ml) at 0 °C, *m*-CPBA (52 mg, 0.3 mmol) was added and stirring was allowed to continue for 10 min at 0 °C. The reaction mixture was quenched with dimethyl sulfide (50 μl), was concentrated and the residue was chromatographed (SiO_2, CH_2Cl_2–MeOH, 4:1). Concentration of the eluate afforded a residue (10 mg), which was subjected to repeated chromatography (SiO_2, CH_2Cl_2–MeOH, 4 : 1) to yield sulfonic acid **12** (2 mg, 9%).

Synthesis of 3-formylindolyl-2-sulfonic acid (12). To a solution of *N*-Boc-2-chloroindolyl-3-carbaldehyde[10] (**24**, 70 mg, 0.25 mmol) in MeOH (2 ml) a solution of NaSH (42 mg, 0.75 mmol) in water (0.25 ml) was added dropwise. After being stirred at room temperature for 30 min the mixture was diluted with brine (20 ml) and extracted with EtOAc. The combined extract was dried and the residue was dissolved in MeOH (3 ml). This solution was cooled to 0 °C, *m*-CPBA (129 mg, 0.75 mmol) was added and the stirring was allowed to continue for 15 min at 0 °C. The reaction was stopped by addition of dimethyl sulfide (0.1 ml), the solvent was evaporated and the residue subjected to FCC (SiO_2, CH_2Cl_2–MeOH, 9 : 1). The most polar fraction was concentrated to dryness, the residue was dissolved in trifluoroacetic acid (TFA, 2 ml) and allowed to stir for 15 min at room temperature. The reaction mixture was concentrated to dryness and the residue was subjected to FCC (SiO_2, CH_2Cl_2–MeOH, 4 : 1) to afford 3-formylindolyl-2-sulfonic acid (**12**, 27 mg, 48%, based on aldehyde **24**) as a colorless solid. Mp 300–305 °C (decomposition); HPLC: R_t = 1.6 min; λ_{max}(MeOH)/nm 212 (ε/dm^3 mol^{-1} cm^{-1} 17400), 244 (9800), 304 (7100). ν_{max}(KBr)/cm^{-1}: 3451, 3181, 1645, 1419, 1215 and 1060. δ_H (500 MHz; DMSO-d_6): 7.18 (dd, J = 8, 8 Hz, 1H), 7.22 (dd, J = 8, 8 Hz, 1H), 7.44 (d, J = 8 Hz, 1H), 8.10 (d, J = 8 Hz, 1H), 10.46 (s, 1H), 12.18 (br s, D_2O exch., 1H). δ_C (125 MHz; DMSO-d_6): 111.9 (s), 112.6 (d), 121.5 (d), 122.4 (d), 123.5 (d), 124.6 (s), 134.0 (s), 152.4 (s), 187.3 (d). HRMS (ESI): calc. for $C_9H_6NO_4S$ (M–H$^+$) m/z 224.0023, found 224.0023.

Synthesis of 3-formyl-1-methylindolyl-2-sulfonic acid (23). A solution of 1-methylindole-2-thione[6] (33 mg, 0.20 mmol) in dry DMF (0.5 ml) was cooled to 0 °C and $POCl_3$ (40 μl, 0.4 mmol) was added dropwise. The cooling bath was removed and the mixture was allowed to stir at room temperature for 40 min. After being cooled to 0 °C, the mixture was diluted with 10% aqueous K_2CO_3 (10 ml) and neutralized with 1 M HCl. The crude 1-methyl-2-sulfanylindolyl-3-carbaldehyde (**22**) was extracted with EtOAc, the combined organic extract was washed with brine, was dried and was concentrated. To this residue (MeOH, 3 ml) cooled to 0 °C, *m*-CPBA (104 mg, 0.6 mmol) was added and the stirring was allowed to continue for 15 min at 0 °C. The reaction was stopped by addition of dimethyl sulfide (0.1 ml), the solvent was evaporated and the residue chromatographed (SiO_2, CH_2Cl_2–MeOH, 4 : 1) to afford 3-formyl-1-methylindolyl-2-sulfonic acid (**23**, 12 mg, 25%, based on 1-methylindole-2-thione) as a colorless solid. Mp 299–300 °C (decomposition); HPLC: R_t = 1.5 min; λ_{max}(MeOH)/nm 214 (ε/dm^3 mol^{-1} cm^{-1} 26100), 250 (15000), 303 (11300). ν_{max}(KBr)/cm^{-1}: 3412, 2933, 1630, 1464, 1211 and 1033. δ_H (500 MHz; DMSO-d_6): 3.99 (s, 3H), 7.25 (dd, J = 8, 8 Hz, 1H), 7.33 (dd, J = 8, 8 Hz, 1H), 7.57 (d, J = 8 Hz, 1H), 8.25 (d, J = 8 Hz, 1H), 10.68 (s, 1H). δ_C (125 MHz, DMSO-d_6): 31.2 (q), 110.6 (d), 112.9 (s), 122.0 (d), 122.7 (d), 123.7 (s), 123.9 (d), 136.3 (s), 151.4 (s), 189.2 (d). HRMS (ESI): calc. for $C_{10}H_8NO_4S$ (M − H$^+$) m/z 238.0178, found 238.0179.

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