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The cruciferous phytoalexins rapalexin A, brussalexin A and erucalexin: Chemistry and metabolism in *Leptosphaeria maculans*

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ABSTRACT

The interactions of the cruciferous phytoalexins rapalexin A (**1**), brussalexin A (**2**) and erucalexin (**3**) with the fungal plant pathogen *Leptosphaeria maculans* were analyzed and their inhibitory activities against this pathogen were determined. The reaction of *L. maculans* to *N*-methyl *S*-(indolyl-3-methyl)carbamodithioate, an analogue of brussalexin A, was also investigated. Rapalexin A was resistant to metabolism and was the most inhibitory of all compounds tested, suggesting that increasing concentrations of rapalexin A in *Brassica* species would improve their disease resistance to *L. maculans*. By contrast, erucalexin was quickly detoxified by reduction to yield 3-dihydroerucalexins. The relative configurations of the diastereomeric mixture of dihydroerucalexins were established by 1D ^{1}H nuclear Overhauser enhancement spectroscopy (NOE). Brussalexin A was chemically unstable decomposing mainly to indolyl-3-methanol, a product with anti-cancer properties. For this reason, brussalexin A might be of interest to use as a prodrug.

1. Introduction

Rapalexin A (**1**), brussalexin A (**2**) and erucalexin (**3**) are cruciferous phytoalexins, that is, plant defense metabolites produced de novo in response to biotic or abiotic stress.[1,2] These plant metabolites have uncommon chemical structures: rapalexin A (**1**) is the first example of an indole isothiocyanate,[3] brussalexin A (**2**) contains the only known *N*-allyl thiolcarbamate,[4] and erucalexin (**3**) has a carbon substituent at C-2 of 3-oxoindole,[5] instead of the common C-3 substitution found in all cruciferous phytoalexins. Although erucalexin (**3**) has been isolated only from the wild crucifer dog mustard (*Erucastrum gallicum* L.), its biosynthetic precursor is 1-methoxybrassinin (**4a**), a phytoalexin commonly produced in *Brassica* species.[6] Despite the variety of phytoalexins produced in crucifers for protection against microbial pathogens, fungi are able to counter-attack by producing enzymes that metabolize and detoxify these plant defenses.[7] Recently, a few fungal enzymes that mediate these transformations were isolated from phytopathogenic fungi[2] and found to be specific targets that could stop plant fungal pathogens from invading crucifers. Toward this objective, paldoxins (phytoalexin detoxification inhibitors) have been designed and synthesized as an alternative to commercial fungicides.[1,8]

Among the many cruciferous phytoalexins reported to date, it is not certain which ones are resistant to fungal transformation, although several reports regarding metabolism of phytoalexins by plant pathogens have been published over the last few years.[1] Such work is of tremendous interest as it provides targets useful to stop pathogenic fungi from invading plants and identifies phytoalexins of interest to engineer plants with higher disease resistance levels. Considering the unusual structures and antifungal activities of rapalexin A (**1**), brussalexin A (**2**) and erucalexin (**3**), it was of great importance to establish their resistance to degradation by the blackleg fungus, one of the major pathogens of *Brassica* species [*Leptosphaeria maculans* (Desm.) Ces. et de Not. asexual stage *Phoma lingam* (Tode ex Fr.) Desm.].[9] Among more than 40 known cruciferous phytoalexins, *L. maculans* is known to metabolize and detoxify the phytoalexins brassinin (**4**), cyclobrassinin (**5**) and brassilexin (**6**), but not spirobrassinin (**7**) or camalexin (**8**).[1,2] Since such transformations appear to involve enzymes that are different in each fungal species, the structures of the detoxification products are not predictable. For example, in a case where bioinformatic comparison of two phylogenetically related fungal species suggested that the transformation of cruciferous phytoalexins would

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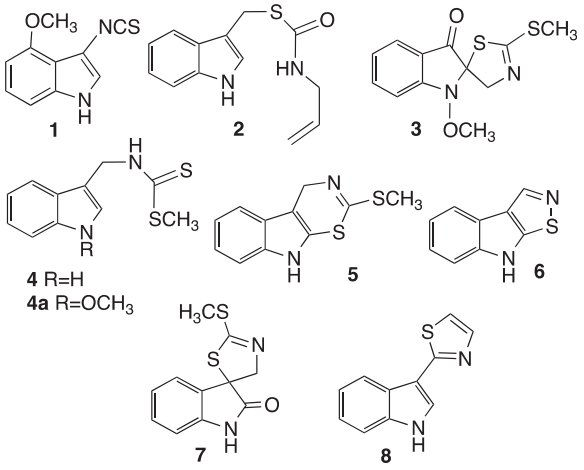
involve glucosyl transferases,[10] detoxification in one species occurred via oxidative degradation or hydrolysis, but not through glucosylation. Hence, for now, even with the availability of many genome sequences of plant pathogens and bioinformatic analysis, it is not feasible to predict metabolic reactions of fungal pathogens to cruciferous phytoalexins.

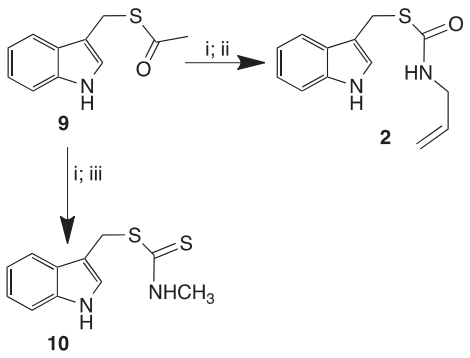


In continuation of previous work, we analyzed for the first time the chemistry involved in the interactions of rapalexin A (**1**), brussalexin A (**2**) and erucalexin (**3**) with *L. maculans* and determined their antifungal activities. In addition, the reaction of *L. maculans* to *N*-methyl *S*-(indolyl-3-methyl)carbamodithioate (**10**), an analogue of brassinin (**4**) and brussalexin A (**2**), was also investigated. Here we report that rapalexin A (**1**) was resistant to metabolism and was the most inhibitory of all compounds tested, suggesting that increasing concentrations of rapalexin A in *Brassica* species would improve disease resistance to *L. maculans*. By contrast, erucalexin (**2**) was detoxified by reduction, and brussalexin A (**2**) was chemically unstable decomposing to indolyl-3-methanol (**11**), which was slowly oxidized by *L. maculans* to the corresponding aldehyde.

2. Results and discussion

2.1. Chemical synthesis and antifungal activity of phytoalexins 1–3 and compound 10

The syntheses of rapalexin A (**1**),[3] and erucalexin (**3**)[5] were carried out as previously published, whereas the synthesis of brussalexin A (**2**)[4] was improved by omitting the *t*-Boc-protecting and deprotecting steps. In brief, 3-indolylmethylthioacetate (**9**) was hydrolyzed using hydrazine hydrate[11] followed immediately by condensation with allyl isocyanate. Similarly,



Scheme 1. Synthesis of brussalexin (**2**) and *N*-methyl indolyl-3-methylcarbamodithioate (**10**). Reagents and conditions: (i) $NH_2NH_2{\cdot}H_2O$, THF, 80 °C, 84%; (ii) Et_3N, allyl isocyanate, CH_2Cl_2, 86%; (iii) Et_3N, methyl isothiocyanate, CH_2Cl_2, 76%.

3-indolylmethylthioacetate (**9**) was used to prepare *N*-methyl *S*-(indolyl-3-methyl)carbamodithioate (**10**), a brassinin analogue havingindolyl-3-methyl attached to sulfur instead of nitrogen (Scheme 1).

The antifungal activity of each compound against *L. maculans* was determined using a mycelial growth assay reported in the experimental (Table 1). The activity of rapalexin A (**1**) was the highest, completely inhibiting mycelial growth at 0.50 mM, whereas brussalexin A (**2**) and erucalexin (**3**) displayed similar activities causing ca. 40% inhibition at 0.50 mM and *N*-methyl *S*-(indolyl-3-methyl)carbamodithioate (**10**) was slightly more inhibitory at similar concentration (ca. 55%).

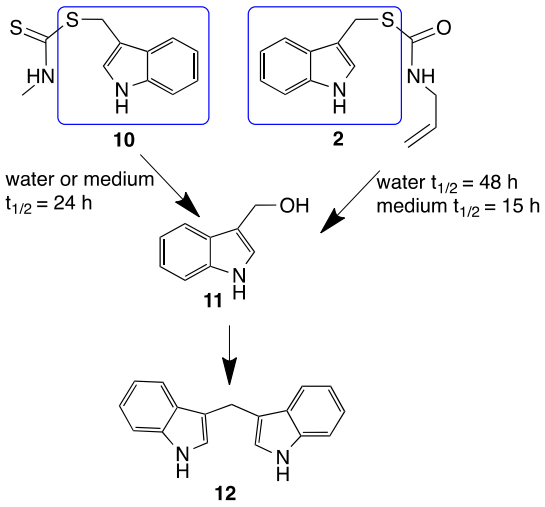
2.2. Metabolism, synthesis and antifungal activity of metabolites

Rapalexin A (**1**) dissolved in acetonitrile was added to cultures of *L. maculans* and also to non-inoculated media (control) to obtain a final concentration of 0.10 mM. HPLC analysis of neutral, basic and acidic extracts after different incubation times, up to 96 h, revealed that rapalexin A (**1**) was not metabolized (stable both in fungal cultures and in media, giving comparable recoveries). Unlike rapalexin A (**1**), brussalexin A (**2**) was not stable in either medium only (no fungal cells) or in water; HPLC analyses of solutions of brussalexin (**2**) in water established that upon standing it decomposed spontaneously to indolyl-3-methanol (**11**) over a period of 72 h ($t_{1/2}$ = 24 h) (Scheme 2). Although brussalexin (**2**) was not stable in culture medium or in water, some stabilizing factors appear to exist in plant tissues, since it has been consistently detected and isolated from Brussels sprouts.[4] In this connection, it is of interest to note that the major decomposition product of **2**, indolyl-3-methanol (**11**) has also been isolated from diverse crucifers, including *Brassica* species.[12] For this reason, compound **11**was tested for stability in fungal cultures, water, and minimal medium. In medium, indolyl-3-methanol (**11**) decomposed completely in 6 h to

Table 1
Antifungal activity of phytoalexins **1–3** and *N*-methyl *S*-(indolyl-3-methyl)carbamodithioate (**10**), against *Leptosphaeria maculans*

Compound	Inhibition ± SD (%)[a]		
	0.50 mM	0.20 mM	0.10 mM
Rapalexin A (**1**)	c.i.[b]	53 ± 6[b]	21 ± 6[b]
Brussalexin A (**2**)	43 ± 3[c]	25 ± 2[d]	7 ± 4[c]
Erucalexin (**3**)	40 ± 4[c]	17 ± 4[e]	6 ± 2[c]
N-methyl *S*-(indolyl-3-methyl)carbamodithioate (**10**)	61 ± 5[d]	35 ± 3[e]	17 ± 3[b]

[a] The percentage of inhibition was calculated using the formula:% inhibition = 100 − [(growth on amended/growth in control) × 100]; values are averages of three independent experiments conducted in triplicate; c.i. = complete inhibition; for statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; n = 3; different letters in the same column (b–d) indicate significant differences (P <0.05).



Scheme 2. Spontaneous transformation of brussalexin (**2**) and *N*-methyl *S*-(3-indolylmethyl) carbamodithioate (**10**) into indolyl-3-methanol (**11**) in medium and water solutions.

yield mainly 3,3′-diindolylmethane (**12**)[13] (ca. 45%) and undetermined minor products, while in water decomposed very slowly ($t_{1/2}$ >72 h).[14] Indolyl-3-methanol (**11**) was incubated with mycelia of *L. maculans* in water and extracts of cultures were analyzed by HPLC over a period of 48 h, only traces (<2%) of indole-3-carboxaldehyde were detected, indicating a relatively slow transformation. The antifungal activity of **11** against *L. maculans* was similar to that of **10** (52 ± 8 at 0.50 mM, 27 ± 5 at 0.20 mM, and19 ± 3 at 0.10 mM). Previously, indolyl-3-methanol (**11**) was found to be metabolized much faster (ca. 12 h) by the plant fungal pathogens *Rhizoctonia solani* and *Sclerotinia sclerotiorum*.[14]

To probe the structural component(s) contributing to the lower stability of brussalexin A (**2**) in aqueous solutions, *N*-methyl *S*-(3-indolylmethyl)carbamodithioate (**10**) was similarly incubated in water, samples were withdrawn at different times, extracted and the extracts analyzed by HPLC. As in the case of brussalexin A (**2**), compound **10** was not stable in water, decomposing to indolyl-3-methanol ($t_{1/2}$ = 20 h) (**11**, Scheme 2). Hence, considering that both compounds **2**and **10**give the same decomposition product in aqueous solutions and that brassinin (**4**), an *S*-methyl *N*-(3-indolylmethyl)carbamodithioate, is stable in both water and medium solutions, the spontaneous transformation is caused mainly by the *S*-(3-indolylmethyl)carbamothioate group and not by the allyl substituent of brussalexin A (**2**).

Erucalexin (**3**) (0.10 mM) was incubated with fungal cultures of *L. maculans* and medium only (control), samples were collected at different times, extracted and the extracts were analyzed by HPLC. After 24 h erucalexin (**3**, t_R = 14.6 min) was no longer detected in the cultures, but two new peaks were detected at t_R = 9.2 min (minor) and t_R = 10.0 min (major). Chromatograms of control samples showed only one peak corresponding to erucalexin (**3**), indicating it to be stable in the culture medium. Attempts to recover the compound with t_R = 9.2 min by extraction from cultures incubated in medium resulted in its transformation to the compound with t_R = 10.0 min. The chemical structure of this compound was determined to be demethoxyerucalexin (**14**) by comparison of its spectroscopic data with an authentic sample[5] available in our synthetic library. Subsequent biotransformation experiments to obtain the minor compound (t_R = 9.2 min) were carried out in water. Thus, mycelial mats of *L. maculans* grown in culture medium were transferred to sterilized water and incubated with erucalexin (**3**) (0.10 mM); samples were collected at different times, freeze-dried and the residues were dissolved in acetonitrile and analyzed by HPLC. These chromatograms showed a major peak with t_R = 9.2 min and minor peak with t_R = 11.9 min. HPLC-ESI-MS analysis indicated that the compound with t_R = 9.2 min corresponded to a compound two mass units higher than erucalexin (**3**). Attempts to purify this unknown metabolite by FCC on silica gel or alumina were unsuccessful, resulting in conversion of the compound with t_R = 9.2 min into demethoxyerucalexin (**14**). Eventually, the compound with t_R = 9.2 min was purified by FCC using reversed phase silica gel. HRMS-EI analysis of the purified material indicated a molecular formula of $C_{12}H_{14}N_2O_2S_2$, two mass units higher than erucalexin (**3**). The ^{1}H NMR spectrum of this compound showed SMe and OMe protons, four aromatic protons and two sets of methylene protons at δ_H 4.65 (d, *J* = 16 Hz, 1.2H), 4.26 (d, *J* = 16 Hz, 1.2H), 4.49 (d, *J* = 16.5 Hz, 0.6H), 4.45 (d, *J* = 16.5 Hz, 0.6H), plus signals at δ_H 5.24 (s, 0.3 H), 4.94 (s 0.6H), and 4.09(s, ca. 1H, D_2O exchangeable), indicating the presence of two diastereomers in a 2:1 ratio. These signals suggested that the likely structure was a diastereomeric mixture of dihydroerucalexin (**13**), the structures of which were further confirmed by synthesis, as follows. Racemic erucalexin (**3**) was reduced with $NaBH_4$ to yield a mixture of diastereomers in a 3:1 ratio. The major isomer could be purified while the minor isomer was obtained as 1:1 mixture of diastereomers. The ratio of diastereomers of dihydroerucalexin (**13**), formed by fungal transformation of erucalexin (**3**), was determined by ^{1}H NMR spectrum of freeze-dried fungal cultures incubated with erucalexin (**3**). The ^{1}H NMR spectral data indicated the presence of two sets of proton signals that corresponded to the major and minor diastereomers in a 2:1 ratio. That is, the major product was formed in both chemical and enzymatic reductions of racemic erucalexin (**3**), although the selectivity in chemical reduction was higher (3:1 vs 2:1). It is somewhat puzzling to find that the chemical reduction of erucalexin (**3**) with $NaBH_4$ affords a higher diastereomeric ratio (3:1) than the enzymatic process (2:1), however the reason for the difference is not understood at this point. Curves for the transformation of racemic erucalexin (**3**) by *L. maculans* and product formation are shown in Fig. 1.

Next, the relative stereochemistry at C-2 and C-3 of each diastereomer **13a** and **13b** was established based on the results of 1D ^{1}H nuclear Overhauser enhancement spectroscopy (NOE). NOE difference experiments were carried out using a synthetic mixture of diastereomers **13a** and **13b** (3:1) (Fig. 2A). When the methine proton (H-3) of the major diastereomer at δ_H 4.94 ppm was irradiated, the intensity (0.3%) of the methylene proton signal at δ_H 4.26 increased (Fig. 2C); similarly, when the methylene proton of the major diastereomer at 4.26 ppm was irradiated, the intensity (0.1%) of the methine proton signal at 4.95 ppm increased (Fig. 2D). On the other hand, when the methine proton at δ_H 5.24 ppm of the minor isomer was irradiated, no NOE difference was observed for any of the methylene protons (Fig. 2B). Consequently, the relative stereochemistry in the major diastereomer is assigned as (2*S*,3*S*)-3-dihydroerucalexin (**13a**) (and its enantiomer(2*R*,3*R*)-3-dihydroerucalexin), on which the OH and SCH_2 substituents have a *cis*

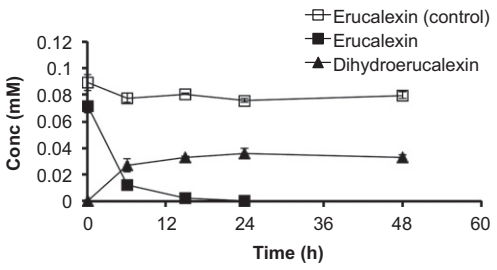


Figure 1. Progress curves of the transformation of erucalexin (**3**, 0.10 mM) and formation of dihydroerucalexin (**13**) in mycelial cultures of *Leptosphaeria maculans* in water.

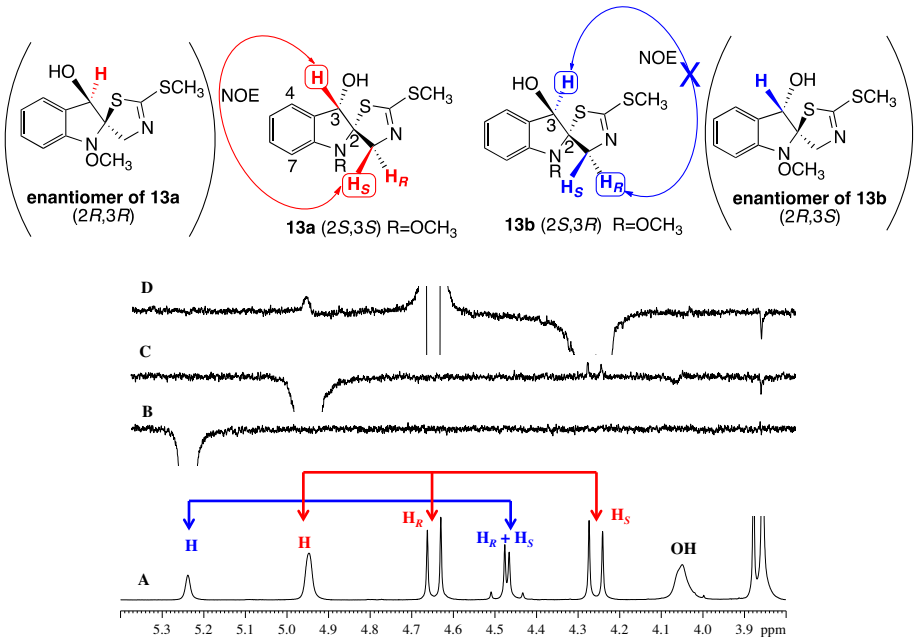


Figure 2. Assignment of the relative stereochemistry of dihydroerucalexin diastereomers based on 1-D NOE difference experiments:(2*S*,3*S*)-3-hydroxyerucalexin (**13a**) and (2*S*,3*R*)-3-dihydroerucalexin (**13b**). (A) section of ^{1}H NMR spectrum of mixture of **13a** and **13b** (3:1); (B) irradiation of H-3 of **13b**; (C) irradiation of H-3 of **13a**; (D) irradiation of H_S of **13a**.

relationship. In the minor isomer the relative stereochemistry is assigned as (2*S*,3*R*)-3-dihydroerucalexin (**13b**) (and its enantiomer(2*R*,3*S*)-3-dihydroerucalexin), with the OH and SCH_2 substituents having a *trans* relationship.

The faster decomposition of dihydroerucalexin (**13**) to demethoxyerucalexin (**14**) in culture medium than in water was investigated further using HPLC analysis, as follows. A diastereomeric mixture of dihydroerucalexin (**13a**/**13b**, 3:1) was dissolved in water (W) and in medium (M) (both containing 2% acetonitrile) at identical concentration (0.20 mM); solutions W and M were immediately injected in the HPLC and then on up to 48 h (triplicate samples). Similarly, solutions W and M (1.0 mL, 0.2 mM) were extracted with EtOAC, the solvent evaporated under reduced pressure (⩽30 °C), the residue was dissolved in acetonitrile (0.20 mM) and injected in the HPLC. The concentration of each sample was determined by HPLC analysis using calibration curves of dihydroerucalexin (**13**) and the conc. was plotted versus time, as shown in Figure 3. The concentration of dihydroerucalexin (**13a**/**13b**, 3:1) in non-extracted solutions W and M (direct injection) remained constant over a period of 48 h (Fig. 3).

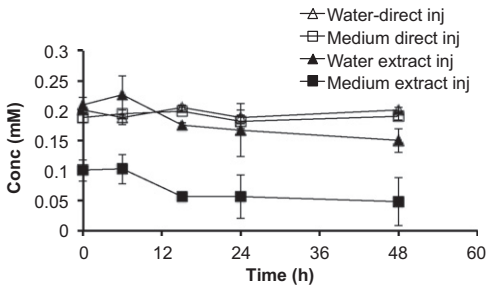
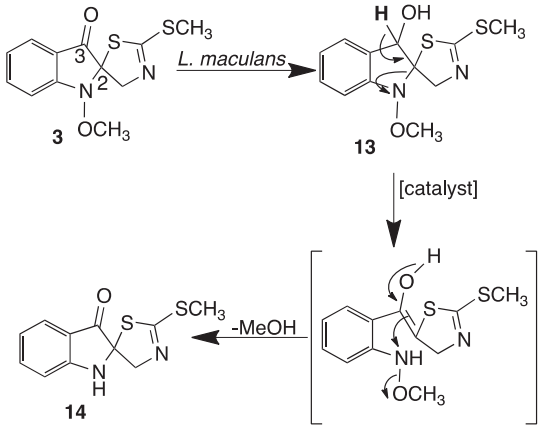


Figure 3. Progress curves of recovery of dihydroerucalexin (**13**, 0.20 mM) in water and medium solutions over a period of 48 h, obtained by HPLC analysis of solutions: △ direct injection of solution of compound incubated in water (0.20 mM); ◇ direct injection of solution of compound incubated in medium (0.20 mM); ▲ injection of extracted compound incubated in water (0.20 mM); ■ injection of extracted compound incubated in medium (0.20 mM).

Similarly, the concentrations obtained from EtOAc extracts of solutions W remained constant over 48 h; however, the concentrations of dihydroerucalexin (**13a**/**13 b**, 3:1) obtained from EtOAc extracts of solutions M were substantially lower, while the concentrations of demethoxyerucalexin (**14**) increased. These results indicated that decomposition of dihydroerucalexin (**13**) to demethoxyerucalexin (**14**) increased during extraction/concentration of samples, likely catalyzed by metal ions (Fe^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}) that were extracted as salts from the medium.

The antifungal activity of dihydroerucalexin was substantially lower than that of erucalexin (23 ± 4% vs 40 ± 4%, at 0.50 mM; 13 ± 2% at 0.20 mM and 7 ± 2 at 0.10 mM), suggesting that detoxification of erucalexin (**3**) in *L. maculans* is mediated by a dehydrogenase(s). The overall results demonstrate that dihydroerucalexin (**13**) is a detoxification product of erucalexin (**3**), and that demethoxyerucalexin (**14**) is a product of chemical degradation of dihydroerucalexin (**13**), but not a product of fungal metabolism (Scheme 3).



Scheme 3. Biotransformation of erucalexin (**3**) to dihydroerucalexin (**13**) by *Leptosphaeria maculans* and chemical transformation of **13** to demethoxyerucalexin (**14**).

3. Conclusion

This work established that rapalexin A (**1**) is a strongly antifungal phytoalexin that was resistant to metabolism in cultures of *L. maculans*, whereas erucalexin (**3**) was quickly metabolized by reduction at C-3 (ca. 24 h, $t_{1/2}$<6 h) to give a diastereomeric mixture (**13a** and **13b**). This mixture of dihydroerucalexins was less inhibitory to *L. maculans* than erucalexin (**3**), indicating that the enzymatic transformation was a detoxification reaction likely beneficial to the pathogen. Somewhat surprising, the chemical reduction of erucalexin (**3**) afforded a higher diastereomeric ratio of products (3:1) than the enzymatic process (2:1). The relative configurations of these products were established by 1D ^{1}H NOE differential spectroscopy. Brussalexin A (**2**) was not stable upon standing in medium or in water, converting spontaneously to indolyl-3-methanol (**11**). For this reason, it was not possible to establish if *L. maculans* was able to metabolize brussalexin A (**2**).

The overall results of this work suggest that rapalexin A (**1**) is a phytoalexin of great significance to improve the disease resistance of crucifers to *L. maculans*, one of the most damaging pathogens of oilseed crops.[9] However, considering that no genes in the biosynthetic pathway of rapalexin A (**1**) have been cloned, increasing its production in stressed plants is currently a difficult proposition. In addition, considering the spontaneous transformations of brussalexin A (**2**) and *N*-methyl *S*-(3-methylindolyl)carbamodithioate (**10**) to the anticarcinogenic compound indolyl-3-methanol (**11**), [15,16] their potential use as prodrugs might be of interest in medicinal chemistry.

4. Experimental

4.1. Materials and general procedures

Chemicals were purchased from Sigma–Aldrich Canada Ltd, Oakville, ON; solvents were HPLC grade and used as such. Flash column chromatography (FCC) was carried out using silica gel grade 60, mesh size 230–400 Å or WP C_{18} prepscale bulk packing 275 Å (J.T. Baker, NJ, USA)

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker 500 MHz Avance spectrometers, for ^{1}H, 500.3 MHz and for ^{13}C, 125.8 MHz; chemical shifts (δ) are reported in parts per million (ppm) relative to TMS; spectra were calibrated using the solvent peaks; spin coupling constants (J) are reported to the nearest 0.5 Hz. Fourier transform infrared (FT-IR) data were recorded on a spectrometer and spectra were measured by the diffuse reflectance method on samples dispersed in KBr. MS [high resolution (HR), electron impact (EI)] were obtained on a VG 70 SE mass spectrometer employing a solids probe.

HPLC analysis was carried out with Agilent high performance liquid chromatographs equipped with quaternary pump, automatic injector, and diode array detector (DAD, wavelength range 190–600 nm), degasser, and a column Eclipse XDB-C18 (5 μm particle size silica, 4.6 id × 150 mm), having an in-line filter, using mobile phase 50% H_2O–50% CH_3OH to 100% CH_3OH, for 25.0 min, linear gradient, and at a flow rate of 0.75 mL/min.

4.2. Synthesis and characterization of new compounds

4.2.1. Brussalexin A (2)

Hydrazine hydrate (109 mg, 2.19 mmol) was added to a solution of indolyl-3-methylthioacetate (**9**, 150 mg, 0.73 mmol) in THF (4 mL) and refluxed for 45 min.[11] The reaction mixture was concentrated, adsorbed onto silica gel, and fractionated (5% EtOAc in hexane) to afford indolyl-3-methanethiol (100 mg, 0.61 mmol) in 84% yield. Allyl isocyanate (28 mg, 0.34 mmol) was added to a solution of indolyl-3-methanethiol (50 mg, 0.31 mmol) and triethylamine (17 μL, 0.62 mmol) in CH_2Cl_2 (2 mL) and stirred at rt for 20 min. After complete conversion of the starting material, the reaction mixture was concentrated and separated by chromatography (silica gel, EtOAc–hexane, 20:80, v/v) to afford brussalexin (**2**, 65 mg, 0.26 mmol) in 86% yield. All spectroscopic data were identical to previously reported data.[4]

4.2.2. *N*-Methyl *S*-(3-methylindolyl)carbamodithioate (10)

Methyl isothiocyanate (49 mg, 0.67 mmol) was added to a solution of indolyl-3-methanethiol (100 mg, 0.613 mmol) and triethylamine (200 μL, 1.47 mmol) in CH_2Cl_2 (3 mL) and stirred at rt for 20 min. After complete conversion of the starting material, the reaction mixture was concentrated, and separated by column chromatography (silica gel, EtOAc–hexane, 20:80, v/v) to afford the product **10** (110 mg, 0.46 mmol) in 76% yield.

HPLC t_R = 11.2 min. ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.20 (br s, NH), 8.01 (br s, NH), 7.63 (d, J = 8 Hz, 1H), 7.41 (d, J = 8 Hz, 1H), 7.29 (d, J = 2 Hz, 1H), 7.17 (dd, J = 8, 8 Hz, 1H), 7.08 (dd, J = 7.5, 7.5 Hz, 1H), 4.69 (s, 2H), 3.12 (s, 3H), minor rotamer peaks were found at δ 4.78, and 2.93 (ca. 1.7: 0.3). ^{13}C NMR (125 MHz, $CDCl_3$): δ 199.4, 137.8, 128.0, 125.7, 123.3, 120.6, 120.0, 112.9, 110.8, 34.3, 32.1. FTIR (KBr) ν_{max} cm^{-1}: 3403, 3323, 2928, 1509, 1455, 1339, 1031, 940, 744. UV (HPLC, CH_3OH–H_2O) λ_{max} (nm): 219, 271. HRMS-EI *m/z*: measured 236.0435 ([M]$^+$, calcd 236.0442 for $C_{11}H_{12}N_2S_2$). MS (EI) *m/z* (% relative int.): 236 [M$^+$] (1), 163 (19), 130 (100).

4.2.3. Dihydroerucalexin (13)

$NaBH_4$ (1 mg, 0.03 mmol) was added to a solution of erucalexin (**3**, 15 mg, 0.053 mmol) in methanol (1 mL) at 0 °C and stirred at the same temperature. After 5 min, the reaction mixture was diluted with water (1 mL) and directly loaded onto a WP C_{18} silica gel column (H_2O–CH_3CN, 60:40, v:v) to afford the mixture of diastereomeric products **13a** and **13b** as a white solid in 3:1 ratio (9 mg, 0.03 mmol, 60% yield). Further chromatography (WP C_{18} silica gel column, H_2O–CH_3CN, 60:40, v:v) of the mixture yielded the major diastereomer **13a** containing <5% of the minor component.

HPLC t_R = 9.2 min (major, **13a**) and 11.9 min (minor, **13b**). Major isomer**13a** (containing <5% of the minor): ^{1}H NMR (500 MHz, CD_3CN): δ 7.31–7.23 (m, 2H), 7.00 (ddd, J = 8, 8, 1 Hz, 1H), 6.93 (d, J = 8 Hz, 1H), 4.94 (s, 1H), 4.65 (d, J = 16 Hz, 1H), 4.26 (d, J = 16 Hz, 1H), 4.09 (br s, OH), 3.86 (s, 3H), 2.51 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) (containing <5% of the minor): δ 164.9, 150.7, 130.4, 124.5, 123.9, 112.5, 74.3, 70.2, 66.8, 65.3, 15.5. Minor isomer **13b** (1:1 mixture): ^{1}H NMR (500 MHz, CD_3CN): δ 7.31–7.23 (m, 2H), 7.00 (ddd, J = 8, 8, 1 Hz, 1H), 6.93 (d, J = 8 Hz, 1H), 5.24 (s, 1H), 4.49 (d, J = 16.5 Hz, 1H), 4.45 (d, J = 16.5 Hz, 1H), 4.09 (br s, OH), 3.88 (s, 3H), 2.51 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) (1:1 mixture): δ 163.8, 152.0, 131.1, 130.5, 126.3, 124.1, 113.0, 103.9, 76.3, 66.7, 15.5. FTIR (KBr) ν_{max} cm^{-1} (1:1 mixture): 3317, 2929, 1686, 1614, 1566, 1463, 1193, 994, 954, 758. UV (HPLC, CH_3CN–H_2O) λ_{max} (nm): 208, 240, 285. HRMS-EI *m/z*: measured 282.0494 ([M]$^+$, calcd 282.0497 for $C_{12}H_{14}N_2O_2S_2$). MS (EI) *m/z* (% relative int.): 282 [M$^+$] (12), 250 (51), 191 (100), 177 (68), 149 (22), 132 (57), 117 (34).

4.3. Antifungal bioassays and metabolism by *Leptosphaeria maculans*

The antifungal activity of compounds was determined using a mycelial radial growth bioassay on potato dextrose agar (PDA) medium.[17] In brief, isolates of *L. maculans* (isolates BJ-125 or UAMH-9410) were grown on V8 agar plates for 14 days at 23 °C under constant light. Sterile tissue culture plates (6-well, 33 mm diameter) containing test (0.50, 0.20 and 0.10 mM) and control solutions (2 mL per well containing 1% CH_3CN) in PDA medium were inoculated with mycelium plugs 4 mm, cut from 7-day-old

V8 agar plates of *L. maculans* placed upside down on the center of each plate and incubated under constant light for 5 days. Mycelial growth in each treatment was measured and % inhibition values were calculated as previously reported. All bioassay experiments were carried out in triplicate, at least two times.

Metabolism studies of compounds in fungal cultures were carried out as previously reported. Erlenmeyer flasks (250 mL) each containing 100 mL minimal media were inoculated with spores of *L. maculans* (isolate BJ-125, 10^6/mL) and incubated at 23 °C on a shaker at 120 rpm under constant light. After two days, compounds (in CH_3CN) were added to the cultures (final conc. 0.10 mM) as well as uninoculated media (control). Samples (2 mL or 5 mL) were withdrawn immediately and at intervals between 6 and 72 h after incubation and were either frozen or immediately extracted with EtOAc, the organic extracts were concentrated and the residue dissolved in CH_3CN and analyzed by HPLC. Calibration curves with synthetic samples were built for quantitative analysis of each compound.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.05.020.

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