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Discovery of inhibitors of brassinin oxidase based on the scaffolds of the phytoalexins brassilexin and wasalexin

M. Soledade C. Pedras *, Zoran Minic, Vijay K. Sarma-Mamillapalle, Mojmir Suchy

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

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ABSTRACT

Inhibitors of brassinin oxidase (BOLm), a unique phytoalexin detoxifying enzyme produced by the plant pathogenic fungus *Leptosphaeria maculans* (asexual stage *Phoma lingam*), were designed based on scaffolds of the phytoalexins brassilexin and wasalexin. Evaluation of these compounds using purified BOLm established that the inhibitory effect of brassilexin and derivatives decreased as follows: 6-chlorobrassilexin ≈ 6-bromobrassilexin > 5-bromobrassilexin ≈ 5-chlorobrassilexin ≈ 6-fluorobrassilexin > 8-methylbrassilexin > brassilexin ≈ 5-fluorobrassilexin. 6-Chlorobrassilexin was determined to be the best competitive inhibitor of BOLm discovered to date, with a K_i = 31 μM. Importantly, brassilexin and derivatives did not appear to induce BOLm in fungal cultures. Overall, these results suggest that the brassilexin scaffold is a good lead for further development of paldoxins against *L. maculans*, as it inhibits competitively BOLm without apparent induction.

1. Introduction

Brassinin oxidase (BOLm) is a unique enzyme produced by the plant fungal pathogen *Leptosphaeria maculans* (Desm.) Ces. et de Not. [asexual stage *Phoma lingam* (Tode ex Fr.) Desm.]. BOLm catalyzes the detoxification of the phytoalexin brassinin (**1**) to indole-3-carboxaldehyde (**2**), a metabolite devoid of antifungal activity against *L. maculans*.[1] The detoxification of phytoalexins by plant pathogenic fungi is documented in a number of plant families, including the family that produces brassinin (**1**), the Brassicaceae (common name crucifers).[2] Phytoalexins are antimicrobial metabolites produced de novo by plants as a defense response to stress caused by pathogen attack, heat, UV-radiation, metal salts, etc.[3] Although each cruciferous species produces phytoalexin blends with a composition that depends on the type of stress, a common set of related structures is usually found within the genus *Brassica*.[4] The detoxification of brassinin (**1**) by either degradation or conjugation is catalyzed by enzymes that vary according to the particular fungal species. For example, degradation of brassinin (**1**) to indole-3-carboxaldehyde (**2**) is mediated by BOLm, which is only produced by isolates of *L. maculans* virulent on canola,[1] whereas hydrolysis of brassinin (**1**) to indolyl-3-methanamine (**3**) is mediated by brassinin hydrolase,[5] an enzyme produced by isolates of *L. maculans* virulent on brown mustard. By contrast, detoxification of brassinin via conjugation with β-D-glucopyranose to β-D-glucobrassinin (**4**) is mediated by brassinin glucosyl transferase, an enzyme produced by *Sclerotinia sclerotiorum* (Lib.) de Bary, a plant pathogen with broad host range (Scheme 1).[2,6,7]

Due to their enormous economic significance, *Brassica* species (e.g., the oilseeds canola and rapeseed, and the vegetables broccoli, cauliflower, cabbage, turnip and rutabaga) have been widely investigated[8,9] and are known to produce the phytoalexins brassinin (**1**), 1-methoxybrassinin (**5**), cyclobrassinin (**6**), rutalexin (**7**), brassilexin (**8**), spirobrassinin (**9**), and rapalexins A (**10**) and B (**11**) (Fig. 1).[4] It is pertinent to emphasize that brassinin (**1**) is a precursor of several of these phytoalexins (**6**–**8**) and thus a key metabolite within the *Brassica* species.[4] For this reason, its detoxification by plant pathogens is highly detrimental to the plant and thus is of current interest to find methods for inhibiting such enzymatic reactions. Paldoxin is a term coined to define inhibitors of phytoalexin detoxifying enzymes, that is, **p**hyto**al**exin **d**et**ox**ification **in**hibitors; paldoxins are a new generation of selective chemicals designed for sustainable treatments of agricultural crops.[2,10] Toward this end, potential inhibitors of brassinin detoxification by BOLm from *L. maculans* were designed by replacement of the dithiocarbamate with isosteric groups that included carbamate, dithiocarbonate, urea and thiourea.[11] It was established that strongly antifungal

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

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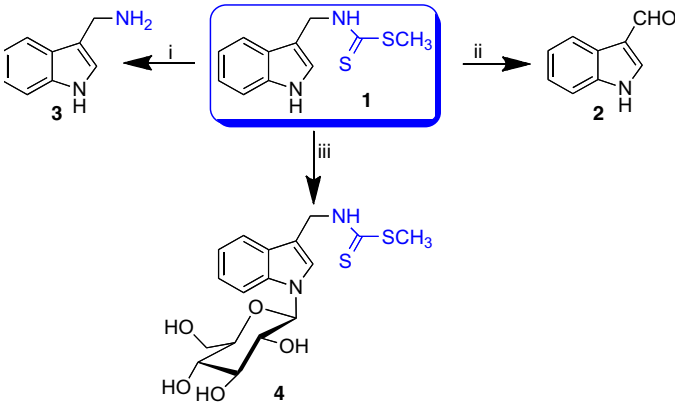


Scheme 1. Detoxification of the phytoalexin brassinin (**1**) by: (i) *Leptosphaeria maculans*, isolates virulent on brown mustard; (ii) *L. maculans*, isolates virulent on canola; (iii) *Sclerotinia sclerotiorum*, all isolates have broad host range.

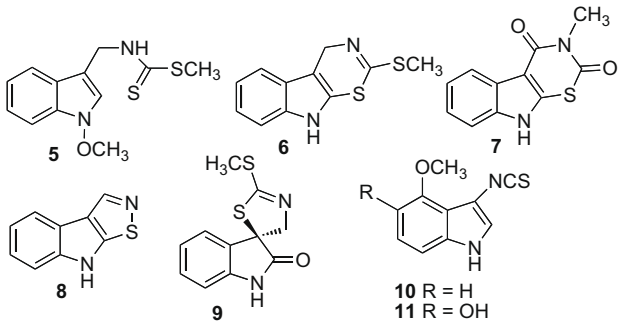
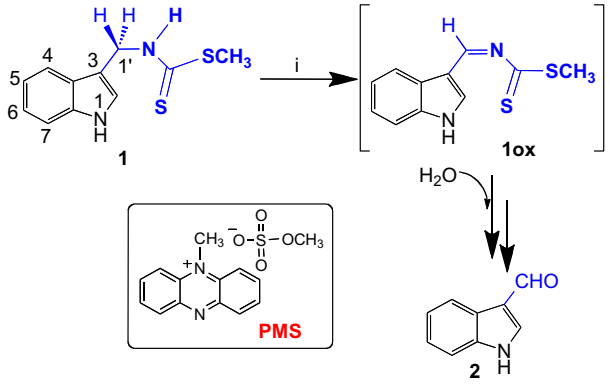


Figure 1. Chemical structures of phytoalexins produced by *Brassica* species.

compounds against *L. maculans* decreased the rate of brassinin (**1**) detoxification in fungal cultures; however, because BOLm was not available at that time, whether the rate decrease was due to the antifungal activity (mycelial growth inhibition) of the compound or a direct inhibitory effect on BOLm could not be established. Subsequently, several dithiocarbamate isosteres were used to probe the mechanism of brassinin detoxification using purified BOLm and determine the substrate specificity.[12] It was concluded that BOLm substrates required a free –NH at the (mono/dithio)carbamate (urea or thiourea group), a methylene bridge between indole and the functional group, and a methyl or ethyl group attached to the thiol moiety of the (mono/di)thiocarbamate group.

In previous work, purified BOLm was used to screen about 80 potential brassinin (**1**) detoxification inhibitors.[1] Surprisingly, among those 80 compounds, the phytoalexin camalexin (**12**) emerged as the strongest inhibitor (53% inhibition at 0.30 mM), followed by cyclobrassinin (**6**, 37% inhibition at 0.30 mM). The competitive inhibitory effects of cyclobrassinin (**6**) and camalexin (**12**) on BOLm activity were likely due to their structural similarity to the putative intermediate(s) involved in the enzymatic reaction (Scheme 2).[1,12] More recently, cyclobrassinin (**6**) was also found to be a competitive inhibitor of brassinin hydrolases.[5] These landmark discoveries suggested that phytoalexins have multiple functions, including inhibition of pathogen growth and inhibition of pathogens' detoxifying enzymes. Consequently, the concentrations of phytoalexin blends that a plant produces in response to fungal infection need to be maintained (or increased) to stop pathogen proliferation.

Cyclobrassinin (**6**) is a phytoalexin produced by *Brassica* species and is metabolized by *L. maculans* whereas camalexin (**12**) is not



Scheme 2. Proposed intermediate for detoxification of the phytoalexin brassinin (**1**) by: (i) BOLm (modified from Ref. 12; PMS = artificial electron acceptor).

produced by *Brassica* species and is not metabolized by *L. maculans*. For these reasons, the camalexin scaffold was used to design BOLm inhibitors. 5-Methoxycamalexin (**12a**) and 6-methoxycamalexin (**12b**) were found to be potent inhibitors of BOLm based on a structure–activity investigation of camalexin (**12**) related structures.[13] Nevertheless, because it is of great importance to discover additional structures having inhibitory activity against BOLm, we screened a number of additional cruciferous phytoalexins. Here we report a structure–activity investigation of brassilexin (**8**), wasalexins A (**13**) and B (**14**), and related structures and the discovery of new and potent competitive inhibitors of BOLm (Fig. 2).

2. Results and discussion

2.1. Chemistry: design and synthesis

In a preliminary screening of various cruciferous phytoalexins (e.g., **5**–**9**, **12**–**14**), brassilexin (**8**) and wasalexins A (**13**) and B (**14**), showed promising inhibitory activity of brassinin degradation by BOLm. Although both brassilexin (**8**)[14] and wasalexins (**13**, **14**)[15] are metabolized by *L. maculans*, we have shown that the metabolism of their synthetic derivatives is substantially slower than the phytoalexins themselves. Structural modifications were made on each phytoalexin scaffold as summarized in Fig. 3 (group A for brassilexin derived structures and group B for wasalexin derived structures), to correlate structures with inhibition of BOLm activity. Because previous studies with camalexin (**12**) suggested that replacement of indolyl hydrogens (H-5 and H-6) with halogens led to stronger inhibitory activity,[13] 5/6-halobrassilexins **8a**–**8h** were synthesized as previously described[16] and evaluated. In addition, syntheses[16] and evaluation of 8-methylbrassilexin (**15a**) and 8-acetylbrassilexin (**16**) (resulting from replacement of the H-1 of **8** with bulkier groups), isothiazolo[5,4-*b*]benzo[*b*]thiophene

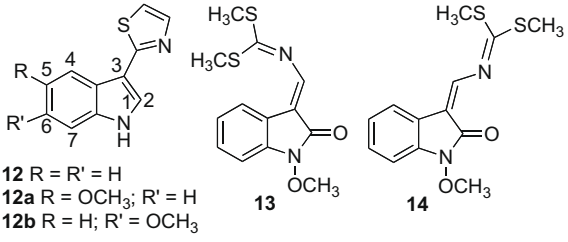


Figure 2. Chemical structures of phytoalexins of crucifers found in cruciferous species (e.g., *Arabidopsis thaliana*, *Wasabiae japonica*) but not found in *Brassica* species.

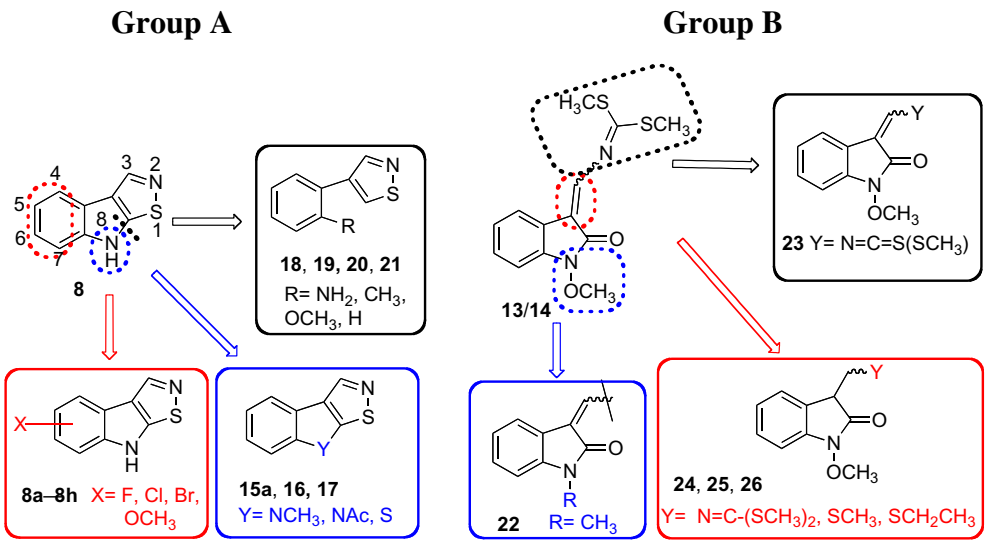
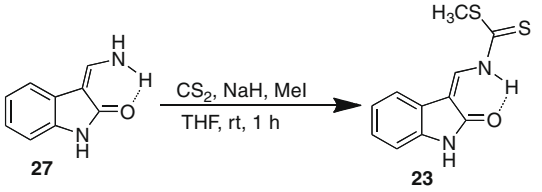


Figure 3. Chemical structures of potential paldoxins for BOLm based on: group A—brassilexin scaffold, group B—wasalexin scaffold.

(**17**) (resulting from replacement of the indole-isothiazole fused-ring system of **8** with benzothiophene) and phenylisothiazoles **18**–**21** (resulting from cleavage of the *a* bond of the indole ring of **8** and replacement of the NH group *ortho* to the isothiazole with various substituents) were carried out. Compounds **15**–**21** were previously shown to display some antifungal activity, but had no inhibitory effect on brassilexin detoxification in fungal cultures.[17] Although the isothiazole ring is a structural component of a variety of compounds with pharmacological activity, including antibacterial, antiviral, and insecticidal,[18] antifungal activity appears to have been reported only in our earlier work.

Wasalexins A (**13**) and B (**14**) are naturally occurring phytoalexins that interconvert to a mixture of *E*/*Z* isomers in solution,[15] and are not produced by *Brassica* species, contrary to brassilexin (**8**). The importance of the methoxy group of wasalexins A/B at *N*-1 and the imidodithiocarbamate group was evaluated using compounds **22** and **23**. Due to the extended conjugation of the wasalexin scaffold, namely that between 2-oxoindole and the substituent at C-3, it was also of interest to evaluate skeletons having a saturated C-3, as for example compounds **24**–**26**.[15] Compounds **24**–**26** were obtained as racemic mixture[15] and were evaluated as such (group B, Fig. 3). The chemical structures of all tested potential brassinin detoxification inhibitors are presented in Tables 1 and 2.

Syntheses of wasalexins A (**13**) and B (**14**) and compound **22** were reported previously.[16] *S*-methyl (2-oxoindolen-3-ylidine)methylcarbamodithioate (**23**) was prepared similarly, as described in Section 4, from 3-aminomethyleneoxindole (**27**) upon treatment with carbon disulfide and iodomethane; the formation of the single *Z*-isomer is attributed to the stabilizing effect of the intramolecular hydrogen bond between the (N)–H and O═(C) atoms (Scheme 3).



Scheme 3. Synthesis of *S*-methyl (2-oxoindolen-3-ylidine)methylcarbamodithioate (**23**).

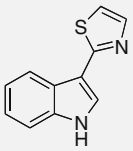
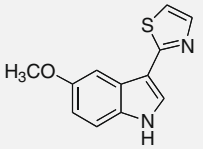
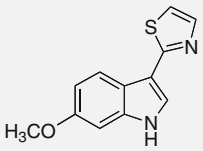
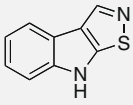
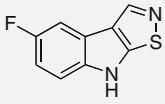
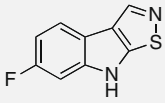
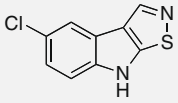
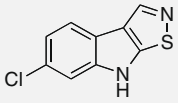
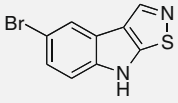
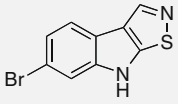
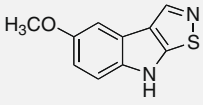
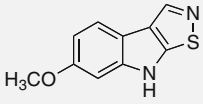
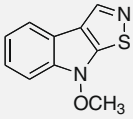
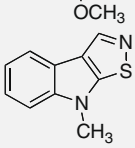
2.2. Enzyme: inhibition of BOLm and type of inhibition

The inhibitory effects of the compounds shown in Table 1 (**8**, **8a**–**8f**, **15**, **15a**, **15b** and **16**–**21**) and 2 (**13**, **14** and **22**–**26**) on BOLm activity were tested at 0.10 mM and 0.30 mM using brassinin (**1**, 0.10 mM) as substrate and purified enzyme, as described in Section 4. For comparison with our previous results, camalexins **12**, **12a** and **12b** were also used in the evaluation. The concentrations of inhibitors were based on the K_m of BOLm for the substrate brassinin (**1**, 0.15 mM under the reported enzyme assay conditions).[1] Results of these enzymatic assays are summarized in Tables 1 and 2. Compounds in group A, with structures based on the brassilexin (**8**) scaffold were more potent inhibitors than those in group B. It is particularly noteworthy that 6-substituted brassilexins were substantially more potent inhibitors than 5-substituted brassilexins, with chlorobrassilexins and bromobrassilexins displaying higher inhibitory activities than their fluorinated counterparts. Methylation of the indolyl nitrogen of brassilexin (**8**) contributed to a slight increase of the inhibitory activity of the resulting derivative (**15a**), while an acetyl group in the same position (derivative **16**) abolished the inhibitory effect. On the other hand, sinalexin (**15**), a natural derivative of brassilexin, did not display inhibitory activity. Interestingly, 5-fluoro-8-methylbrassilexin (**15b**) showed no inhibitory activity against BOLm, that is, fluorine at C-5 of 8-methylbrassilexin abolished the inhibitory effect observed for **15a**. Consistently, these results suggest that the size of the substituents placed on brassilexin (**8**) play an important role in inhibiting BOLm activity. No inhibitory effects were observed using phenylisothiazoles **18**–**21**. Furthermore, although none of the modifications carried out on the wasalexin scaffolds **13**/**14** contributed to an increase of the inhibitory effect of the natural products, it was interesting to observe that replacement of the methoxy substituent with methyl did not affect the inhibitory activity of **22** relative to **13**/**14**.

Next, the IC_{50} values of the best inhibitors of BOLm discovered to date were determined from the logarithmic concentration–response plots for each inhibitor (0.05–0.30 mM), at a constant brassinin concentration (0.10 mM), as reported in Section 4. These results indicated that the best inhibitor of BOLm was 6-chlorobrassilexin (**8d**), which showed a K_i value about twofold lower than that of 5-methoxycamalexin (**12a**) (Table 3).

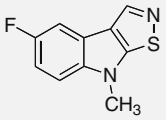
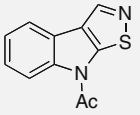
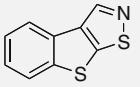
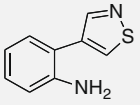
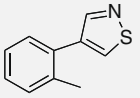
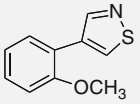
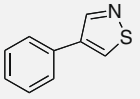
Considering that 6-chlorobrassilexin (**8d**) had substantial inhibitory activity on BOLm and a structure different from those of camalexin (**12**) or cyclobrassinin (**6**), it was important to determine its type of inhibition. The kinetics of inhibition of BOLm, shown in the

Table 1
Effect of brassilexin (**8**) and compounds of group A (**8a**–**8f** and **15**–**21**) on BOLm activity

Compound (#)	Structure	Inhibition[a] (%)	
		0.1 mM	0.3 mM
Camalexin (**12**)[b]		30 ± 4	53 ± 4
5-Methoxycamalexin (**12a**)[b]		51 ± 4	72 ± 1
6-Methoxycamalexin (**12b**)[b]		41 ± 6	63 ± 5
Brassilexin (**8**)[16]		8 ± 2	16 ± 2
5-Fluorobrassilexin (**8a**)		7 ± 4	14 ± 6
6-Fluorobrassilexin (**8b**)[16]		22 ± 3	40 ± 5
5-Chlorobrassilexin (**8c**)[16]		21 ± 6	40 ± 2
6-Chlorobrassilexin (**8d**)[16]		56 ± 5	66 ± 7
5-Bromobrassilexin (**8e**)[16]		30 ± 5	45 ± 2
6-Bromobrassilexin (**8f**)[16]		42 ± 4	63 ± 4
5-Methoxybrassilexin (**8g**)[16]		5 ± 1	18 ± 4
6-Methoxybrassilexin (**8h**)[16]		15 ± 2	38 ± 4
Sinalexin (**15**)[16]		n.d.	n.d.
8-Methylbrassilexin (**15a**)[16]		14 ± 6	24 ± 4

(*continued on next page*)

Table 1 (*continued*)

Compound (#)	Structure	Inhibition[a] (%)	
		0.1 mM	0.3 mM
5-Fluoro-8-methylbrassilexin (**15b**)[16]		n.d.	n.d.
8-Acetylbrassilexin (**16**)[16]		n.d.	n.d.
Isothiazolo[5,4-*b*]benzo[*b*]thiophene (**17**)[17]		n.d.	n.d.
2-(Isothiazol-4-yl)aniline (**18**)[17]		n.d.	n.d.
4-(*o*-Tolyl)isothiazole (**19**)[17]		n.d.	n.d.
4-(*o*-Methoxyphenyl)isothiazole (**20**)[17]		n.d.	n.d.
4-Phenylisothiazole (**21**)[17]		n.d.	n.d.

[a] BOLm activity was determined as reported in Section 4; inhibition is expressed as a percentage of activity of control (100%); results are expressed as means and standard deviations of four independent experiments; n.d. = not detected.
[b] Data from Ref. 13.

form of Lineweaver–Burk double reciprocal plots (1/S vs 1/V) at 0.10 and 0.30 mM concentration (Fig. 4), indicates that inhibition of BOLm by 6-chlorobrassilexin (**8d**) is competitive. The structure similarity between chloro and bromobrassilexins suggests that these brassilexin derivatives are also competitive inhibitors of BOLm.

Because induction of BOLm could be detrimental to plants infected by *L. maculans*, it was of great importance to establish if brassilexin (**8**) or any of its synthetic derivatives affected BOLm production in culture. Hence, to evaluate the induction of BOLm activity, mycelial cultures of *L. maculans* were incubated with compounds **8**, **8c**–**8h**, **28** and **29** (0.10 and 0.20 mM) for 24 h. The cultures were filtered, the mycelia were extracted with extraction buffer (Section 4), and the resulting cell-free extracts were analyzed for BOLm activity using brassinin (**1**) as substrate; the total protein content of each cell-free extract was determined using a calibration curve built using BSA. Importantly, as summarized in Table 4, the results of these experiments established that, relative to control cultures, most brassilexin derivatives (0.10 or 0.20 mM) inhibited production of BOLm activity in fungal cultures. For example, the total BOLm activity observed for mycelia of cultures incubated with 0.10 mM of brassilexin (**8**) was ca. 25% of that observed for control cultures. Similarly, 6-chlorobrassilexin (**8d**) also inhibited BOLm production in fungal cultures (ca. 38%). Previously, we had found that 3-phenylindole (**28**) induced production of BOLm activity in much larger amount (ca. 558% at 0.10 mM)[13] than any other compound tested, including camalexin (**12**) and the commercial fungicide thiabendazole (**29**).[13] For this reason, 3-phenylindole (**28**) has been added to fungal cultures to increase the production of BOLm, which was necessary for characterization studies.[1] Although puzzling, the induction effect of **28** suggests that its mechanism of action is different from that of compounds shown in Table 4. All tested compounds shown in Table 4 appeared to inhibit protein synthesis since the total protein content of mycelia was substantially lower than that in control cultures.

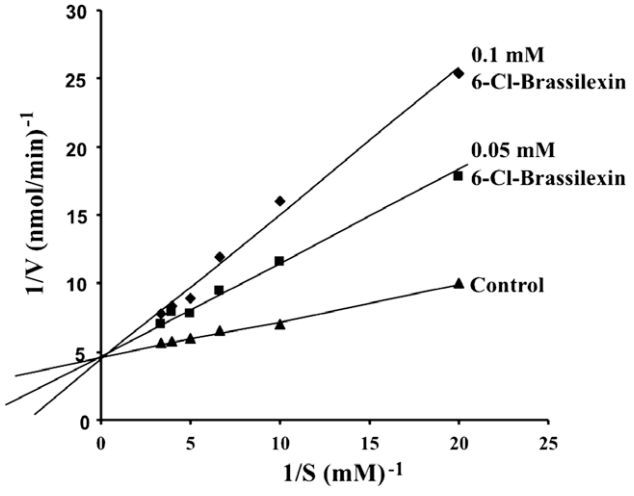
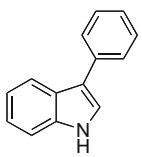


Figure 4. Lineweaver-Burk plots of BOLm activities in the presence of 6-chlorobrassilexin (**8d**) (purified enzyme was used for BOLm activity measurements carried out as described in Section 4).


28

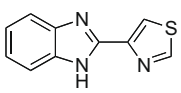
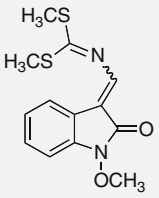
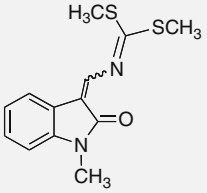
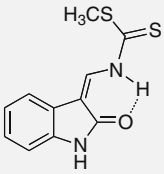
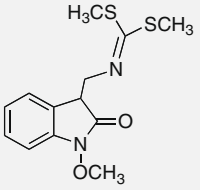
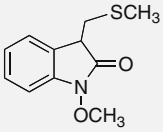
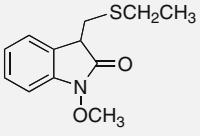

29

Table 2
Effect of wasalexins A (**13**) and B (**13**) and compounds of group B (**22–26)** on BOLm activity[a]

Compound (#)	Structure	Inhibition[a] (%)	
		0.1 mM	0.3 mM
Wasalexins A (**13**) and B (**14**)[15]		8 ± 5	14 ± 4
(E/Z)-S,S-Dimethyl (1-methyl-2-oxoindolen-3-ylidine)methylcarbonimidodithioate (**22**)[15]		8 ± 3	18 ± 2
S-Methyl (2-oxoindolen-3-ylidine)methylcarbamodithioate (**23**)		8 ± 2	14 ± 7
Dihydrowasalexin (**24**)[15]		n.d.	n.d.
1-Methoxy-3-(methylthiomethyl)indolin-2-one(**25**)[15]		n.d.	n.d.
1-Methoxy-3-(ethylthiomethyl)indolin-2-one (**26**)[15]		n.d.	n.d.

[a] BOLm activity was determined as reported in Section 4; inhibition is expressed as a percentage of activity of control (100%); results are expressed as means and standard deviations of four independent experiments; n.d. = not detected.

Table 3
Inhibition constants, type of inhibition and IC_{50} of selected BOLm inhibitors

Compound (#)	K_i (mM)	Inhibition type	IC_{50} (mM)
Cyclobrassinin (**6**)	253	Competitive	422
6-Chlorobrassilexin (**8d**)	31	Competitive	52
6-Bromobrassilexin (**8f**)	91	Not determined	152
Camalexin (**12**)	155	Competitive	260
5-Methoxycamalexin (**12a**)	57	Not determined	95

The antifungal activities of the tested compounds shown in Table 4 (previously reported and summarized in Table S1 in Supplementary data) showed no obvious correlation with their inhibition or induction of BOLm activity, but was clearly related with the total protein amounts. Altogether, our results indicate that the brassilexin scaffold is a very good lead to design paldoxins, since brassilexins **8c–8h** are inhibitors of BOLm activity and do not induce substantially BOLm production in fungal cultures.

3. Conclusion

Our current results emphasize that different phytoalexins have multiple inhibitory effects against the important fungal pathogen *L. maculans*, including fungal growth inhibition and inhibition of detoxifying enzymes. Considering that the phytoalexin brassilexin (**8**) is a strong inhibitor of brassinin (**1**) degradation and mycelial growth of *L. maculans*, and does not induce BOLm production in culture, it appears to be a very promising scaffold for development of paldoxins against *L. maculans*. Nonetheless, since paldoxins are supposed to have no effect on living systems, and some of the current structures still display antifungal activities, additional scaffolds need to be designed and screened.

4. Experimental

4.1. General experimental procedures and compound characterization

All solvents were HPLC grade and used as such. Organic extracts were dried over anhydrous Na_2SO_4 and solvents removed under re-

Table 4
Relative total and specific activities of BOLm[a] and protein content[b] of cell-free extracts of mycelia obtained from cultures incubated with inhibitors **12**–**12b**, **8**, **8c**–**8h**, **28** and **29**

Compound added to mycelial cultures (#)	Concd (mM)	Total activity% of BOLm (specific activity)[a,b]	Relative total protein[b] (%)
Control culture	n.a.[c]	100 (1.0 ± 0.1)	100
Camalexin (**12**)	0.10	126 (4.5 ± 0.5)	28
	0.20	166 (6.9 ± 0.3)	24
5-Methoxycamalexin (**12a**)	0.10	48 (1.7 ± 0.3)	28
	0.20	139 (5.8 ± 0.6)	24
6-Methoxycamalexin (**12b**)	0.10	62 (2.3 ± 0.1)	27
	0.20	59 (3.7 ± 0.2)	16
Brassilexin (**8**)	0.10	25 (0.8 ± 0.1)	30
	0.20	35 (1.2 ± 0.1)	29
5-Chlorobrassilexin (**8c**)	0.10	61 (1.8 ± 0.6)	34
	0.20	146 (5.6 ± 0.5)	26
6-Chlorobrassilexin (**8d**)	0.10	38 (1.4 ± 0.1)	27
	0.20	29 (1.3 ± 0.2)	22
5-Bromobrassilexin (**8e**)	0.10	34 (1.2 ± 0.1)	28
	0.20	51 (1.9 ± 0.2)	27
6-Bromobrassilexin (**8f**)	0.10	95 (2.7 ± 0.4)	35
	0.20	171 (6.9 ± 1.0)	25
5-Methoxybrassilexin (**8g**)	0.10	46 (1.3 ± 0.2)	34
	0.20	59 (2.3 ± 0.4)	25
6-Methoxybrassilexin (**8h**)	0.10	81 (2.7 ± 0.4)	30
	0.20	77 (6.9 ± 1.0)	24
3-Phenylindole (**28**)	0.10	558 (30.9 ± 3.0)	18
	0.20	549 (39.2 ± 6.8)	14
Thiabendazole (**29**)	0.10	67 (1.9 ± 0.2)	35
	0.20	115 (4.1 ± 0.1)	28

[a] Activity of BOLm in control cultures = 0.986 nmol/min; specific activity of BOLm in control cultures = 0.064 ± 0.003 nmol/min/mg; results are expressed as means and standard deviations of four independent experiments.
[b] BOLm specific activities and protein concentrations were obtained from protein extracts of mycelia cultures incubated with each compound, as described in Section 4; results are expressed as means and standard deviations of three independent experiments.
[c] n.a. = no amendment.

duced pressure in a rotary evaporator. Compounds were characterized using NMR spectral data (recorded on Bruker 500 MHz spectrometers) and HRMS data (obtained on a VG 70 SE mass spectrometer using a solids probe or on a Q Star XL, Applied Biosystems). Satisfactory spectroscopic data identical to those previously reported were obtained for all compounds.

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 id × 200 mm), equipped with an in-line filter. Mobile phase: 75% H_2O–25% CH_3CN to 100% CH_3CN, for 35 min, linear gradient, and a flow rate 1.0 mL/min.

4.2. Synthesis of *S*-methyl (2-oxoindolen-3-ylidine)methylcarbamodithioate (23)

To a stirred solution of enamine **27** (116 mg, 0.73 mmol) in dry THF (3 mL, cooled to 0 °C), NaH (60% suspension in mineral oil, 24 mg, 0.73 mmol) and CS_2 (50 μL, 0.87 mmol) were added, followed by CH_3I (after 10 min, 45 μL, 0.73 mmol). The mixture was stirred for 1 h at 0 °C, was diluted with brine (30 mL) and was extracted (EtOAc). The combined organic extract was dried and was concentrated. The residue was fractionated by flash column chromatography (25 g SiO_2, hexane/acetone, 2:1). The less polar fraction after concentration yielded dithiocarbamate **23** (28 mg, 15%) as a yellow solid, mp. 220–222 °C.

S-Methyl (2-oxoindolen-3-ylidine)methylcarbamodithioate (**23**): HPLC: t_R 23.7 min; ^{1}H NMR (500 MHz, $CDCl_3$): δ 12.30 (d, J = 10 Hz, D_2O exch., 1H), 8.68 (d, J = 10 Hz, 1H), 7.70 (br s, D_2O exch., 1H), 7.50 (d, J = 7.5 Hz, 1H), 7.21 (dd, J = 7.5, 7.5 Hz, 1H), 7.08 (dd, J = 7.5, 7.5 Hz, 1H), 6.93 (d, J = 7.5 Hz, 1H), 2.78 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 200.1 (s), 170.2 (s), 138.3 (s), 132.8 (s), 128.0 (d), 122.7 (s), 122.7 (d), 119.5 (d), 110.3 (d), 108.8 (s), 19.0 (q). HREIMS *m/z*: measured 252.0393 (calculated for $C_{11}H_{12}N_2OS_2$: 252.0391). FTIR ν_{max} (cm^{-1}) 3154, 2920, 1677, 1620, 1461, 1359, 1261, 1101, 988, 813, 748.

4.3. Fungal cultures and preparation of protein extracts

Liquid cultures of *L. maculans* (virulent isolate BJ-125, IBCN collection, AAFC) were handled as described previously.[17] In brief, fungal spores were subcultured on V8 agar under continuous light at 23 ± 1 °C; after 15 days, fungal spores were collected aseptically and stored at −20 °C. Liquid cultures were initiated by inoculating minimal media (100 mL)[19] with fungal spores at 10^7/mL in Erlenmeyer flasks, followed by incubation on a shaker (100 rpm/min) under constant light at 23 ± 1 °C. After 48 h of incubation, 3-phenylindole was added to cultures (0.05 mM) to induce BOLm, as previously reported, and cultures were further incubated for 24 h. The mycelial mat was gravity filtered and stored at −20 °C.

Frozen mycelia (0.3–1.4 g) from *L. maculans* were suspended in ice-cold extraction buffer (1–2 mL) and ground (mortar) for 5 min. The extraction buffer consisted of diethanolamine (DEA, 25 mM, pH 8.3), 5% (v/v) glycerol, D,L-dithiothreitol (DTT, 1 mM) and 1/200 (v/v) protease inhibitor cocktail (P-8215, Sigma–Aldrich Canada). The homogenate was centrifuged at 4 °C for 30 min at 50,000*g*. The resulting supernatant was used for determination of specific activity of BOLm; protein concentrations were determined as described by Bradford[20] using the Coomassie Brilliant Blue method with BSA as a standard.

4.4. Enzyme assays and chromatographic purification of BOLm activity

The reaction mixture contained DEA (20 mM, pH 8.3), DTT (1 mM), 0.1% (v/v) Triton X-100, brassinin (**1**, 1.0 mM), phenazine (0.50 mM) and protein extract (50–100 μL) in a total volume of 500 μL. The reaction was carried out at 24 °C for 20 min. A control reaction was stopped by the addition of EtOAc (2 mL) at t = 0. The product was extracted with EtOAc (2 mL) and concentrated to dryness. The extract was dissolved in CH_3CN (200 μL) and analyzed by HPLC-DAD. The amounts of brassinin (**1**) and in-

dole-3-carboxaldehyde (**2**) in the reaction assay were determined using calibration curves built with pure compounds. One enzyme unit (U) is defined as the amount of the enzyme that catalyzes the conversion of one micromole of substrate per minute ($\mu mol \cdot min^{-1}$ = U).

For purification of BOLm, the mycelia of liquid cultures (600 mL) prepared as described above and stored at −20 °C up to 72 h was used. The purification of BOLm was performed in four steps as previously described.[1]

4.5. Inhibitory effect of potential paldoxins on BOLm and type of inhibition

Purified BOLm was used to screen all compounds shown in Tables 1 and 2 for inhibitory activity. Inhibition experiments were carried out using brassinin (**1**, 0.10 mM final concentration) and test compounds at 0.10 and 0.30 mM final concentrations. Standard deviation values for assays were determined from four independent experiments. Furthermore, the type of inhibition of selected compounds was assessed using different concentration of inhibitors (0.05–0.30 mM), at a fixed brassinin concentration of 0.10 mM. The IC_{50} values were determined from logarithmic concentration–response plots for BOLm inhibitor and these values were converted into inhibitor dissociation constants (K_i) by use of the Cheng–Prusoff expression: $K_i = IC_{50}/(1 + [S]/K_m)$,[21] where $[S]$ = 0.10 mM and K_m = 0.150 mM.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.02.054.

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