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Synthetic Inhibitors of the Fungal Detoxifying Enzyme Brassinin Oxidase Based on the Phytoalexin Camalexin Scaffold

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Brassinin (**1**) is an essential phytoalexin produced in plants of the family Brassicaceae (common name crucifer) due to its role as a biosynthetic precursor of other phytoalexins and antimicrobial activity. The dithiocarbamate group of brassinin (**1**) is the toxophore responsible for its fairly broad antifungal activity. To the detriment of many agriculturally important crops, several pathogenic fungi of crucifers are able to overcome brassinin by detoxification. In this work, inhibitors of brassinin oxidase, a phytoalexin detoxifying enzyme produced by the plant pathogenic fungus *Leptosphaeria maculans* (asexual stage *Phoma lingam*), were synthesized and evaluated. The camalexin scaffold was used for the design of brassinin oxidase inhibitors (i.e., paldoxins, phytoalexin detoxification inhibitors) because camalexin is a phytoalexin not produced by the *Brassica* species and *L. maculans* is unable to metabolize it. The inhibitory effect of camalexin and derivatives decreased as follows: 5-methoxycamalexin > 5-fluorocamalexin = 6-methoxycamalexin > camalexin > 6-fluorocamalexin; 5-methoxycamalexin was determined to be the best inhibitor of brassinin oxidase discovered to date. In addition, the results suggested that camalexin might induce fungal pathways protecting *L. maculans* against oxidative stress (induction of superoxide dismutase) as well as brassinin toxicity (induction of brassinin oxidase). Overall, these results revealed additional biological effects of camalexin and its natural derivatives and emphasized that different phytoalexins could have positive or negative impacts on plant resistance to different fungal pathogens.

KEYWORDS: Brassinin; camalexin; *Leptosphaeria maculans*; *Phoma lingam*; paldoxin; phytoalexin; superoxide dismutase

INTRODUCTION

Phytoalexins are antimicrobial secondary metabolites produced de novo by plants in response to stress, including bacterial and fungal infection, heat, heavy metal salts, and UV radiation (*1*). In general, cruciferous phytoalexins, produced by plants of the family Brassicaceae (common name crucifer), are biosynthesized from tryptophan and are produced as blends whose composition depends on the plant species and on the particular elicitor (stress factor) (*2*). Brassinin (**1**) is an essential phytoalexin due to its role as biosynthetic precursor of other cruciferous phytoalexins and its antimicrobial activity. The dithiocarbamate group of brassinin (**1**) is the toxophore responsible for its fairly broad antifungal activity (*3*). To the detriment of many agriculturally important crops, several pathogenic fungi of crucifers are able to overcome phytoalexins, such as brassinin (**1**) by detoxification (*4*). These detoxification reactions can rather quickly deprive the plant of its defense chemicals and facilitate an outcome favoring the fungal pathogen.

Cruciferous species include a wide variety of crops cultivated worldwide, for example, the oilseeds canola (*Brassica napus* and *B. rapa* L.) and rapeseed (*B. napus* and *B. rapa*) and many vegetables, such as rutabaga (*B. napus* ssp. *napobrassica* L.), turnip (*B. rapa* ssp. *rapa* L.), and cauliflower (*B. oleraceae* var. *botrytis*). Economically significant diseases of the oilseeds canola and rapeseed caused by fungi such as the "blackleg" fungi [*Leptosphaeria maculans* (asexual stage *Phoma lingam*) and *L. biglobosa*] are a global issue. *L. maculans* is a pathogen with well-established stratagems to invade crucifers, including production of specific enzymes that detoxify essential plant defenses, such as the phytoalexin brassinin (**1**) (*4*). The enzyme involved in the oxidative detoxification of brassinin (**1**) by *L. maculans*, brassinin oxidase (BO), was recently purified and shown to catalyze the transformation of brassinin (**1**) to indole-3-carboxaldehyde (**3**, **Scheme 1**), a nonantifungal metabolite (*5*). This transformation appears to have no counterpart in the microbial metabolism of dithiocarbamates, despite the wide use of dithiocarbamates as fungicides for many decades (*6*). BO was stable and appeared to exhibit substrate specificity (*7*), suggesting that selective inhibitors could be designed. Such selective BO inhibitors could be developed to prevent fungal

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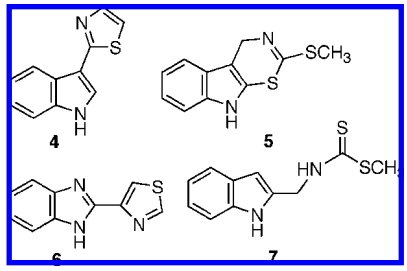
detoxification of brassinin (**1**) in infected plants and, hence, act as crop protection agents against *L. maculans* (*8*). Paldoxin is a term coined to address this new class of fungal enzyme inhibitors, that is, **p**hyto**al**exin **d**et**ox**ification **in**hibitors, that conceptualizes a new generation of chemicals designed for sustainable treatments of agricultural crops (*5, 9*). Paldoxins are envisioned to inhibit unique metabolic reactions in fungal phytopathogens and, therefore, are less likely to affect nontargeted organisms and, thus, are expected to have minimal impact on cultivated ecosystems. The successful "design" of paldoxins presupposes a reasonable mechanistic understanding of the particular phytoalexin detoxification reaction, which must include the chemical structures of specific intermediate(s)/product(s) of the enzymatic transformation. After design and chemical syntheses, potential paldoxins have to be screened using both fungal cultures and detoxifying enzyme(s) assays. However, the unavailability of phytoalexin detoxifying enzymes (*4*) poses a substantial limitation to the discovery of paldoxins. Except for BO, no other phytoalexin detoxifying enzymes appear to have been purified to homogeneity (*5*).

Toward the discovery of paldoxins against *L. maculans*, an evaluation of about 80 potential brassinin (**1**) detoxification inhibitors using purified BO uncovered two phytoalexins and two synthetic compounds displaying inhibitory effect on BO activity (*5*). The phytoalexin camalexin (**4**) was the strongest inhibitor (53% inhibition at 0.30 mM), followed by cyclobrassinin (**5**, 37% inhibition at 0.30 mM), the commercial fungicide thiabendazole (**6**, 25% at 0.30 mM), and a synthetic brassinin isomer (**7**, 23% inhibition at 0.30 mM). The competitive inhibitory effect of both camalexin (**4**) and cyclobrassinin (**5**) on BO activity was thought to be due to their structural similarity to the putative intermediates involved in the enzymatic reaction (*5*). Notwithstanding its inhibitory effect on BO activity, camalexin (**4**) induced BO activity in mycelial cultures of *L. maculans* (*10*). These apparently contradictory effects (induction and inhibition of BO) suggest that camalexin (**4**) exhibits a mechanism(s) of action that is likely to involve more than one molecular target within the fungal cell. Because camalexin (**4**) is a phytoalexin not produced by *Brassica* species and *L. maculans* is unable to metabolize it, the camalexin scaffold is a good lead for the development of potential paldoxins against *L. maculans*. Herein is reported a structure−activity investigation of compounds based on the camalexin (**4**) scaffold and the discovery of potent inhibitors of BO with interest for paldoxin development to treat canola crops.

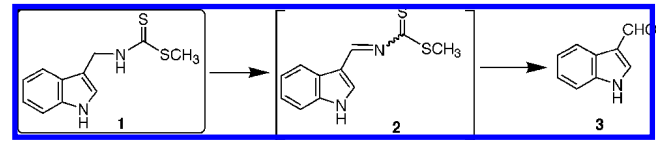


MATERIALS AND METHODS

Chemicals and General Experimental Procedures. All solvents were HPLC grade and used as such. 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: 75% H_2O−25% CH_3CN to 100% CH_3CN, for 35 min, linear gradient, and a flow rate 1.0 mL/min. Fourier transform IR spectra were obtained on a Bio-Rad FTS-40 spectrometer in KBr. NMR spectra were recorded on 500 MHz spectrometers; δ values were referenced as follows: for ^{1}H (500 MHz), $CDCl_3$, 7.27 ppm; for ^{13}C (125 MHz), $CDCl_3$, 77.23 ppm. Mass spectra (MS) were obtained on a VG 70 SE mass spectrometer using a solids probe or on a Q Star XL, Applied Biosystems.

Scheme 1. Detoxification of the Phytoalexin Brassinin (**1**) by the Virulent Canola Pathogen *Leptosphaeria maculans*



Syntheses of Compounds. All synthetic compounds were purified using flash column chromatography (FCC) on silica gel; satisfactory spectroscopic data identical to those previously reported were obtained for all previously reported compounds. Syntheses of brassinin (**1**) (*2*), camalexin (**4**) (*11*), and compounds **14** (*12*), **15** (*13*), **16** (*11*), **17** (*11*), **18** (*12*), **19** (*14*), **20** (*14*), **21** (*15*), **22** (*16*), **23** (*16*), and **24** (*17*) were carried out as reported in the respective publications. Compounds **10**, **11**, and **13** were prepared as follows.

Synthesis of 2-(3-Indolyl)-1,3,4-thiadiazole (10). Lawesson's reagent (118 mg, 0.295 mmol) was added to a solution of 1-(3-carboxyindolyl)-2-formylhydrazide (**9**, 50 mg, 0.246 mmol) (*18*) in 1,4-dioxane (2.0 mL) and refluxed for 30 min. The reaction mixture was diluted with water (20 mL) and extracted with EtOAc (3 × 20 mL). The combined organic extract was dried over Na_2SO_4 and concentrated to dryness. The residue was purified by FCC on silica gel (CH_2Cl_2/Et_2O) to afford compound **10** as an off-white solid (20 mg, 41% yield), mp 162−164 °C. ^{1}H NMR (500.3 MHz, CD_3CN): δ 9.91 (br s, NH), 9.11 (s, 1H), 8.27 (d, J = 7.0 Hz, 1H), 7.98 (d, J = 3.0 Hz, 1H), 7.55 (dd, J = 5.1, 1.4 Hz, 1H), 7.31−7.26 (m, 2H). ^{13}C NMR (125.8 MHz, CD_3CN): δ 164.1 (s), 150.6 (d), 138.1 (s), 129.4 (d), 125.9 (s), 124.6 (d), 122.9 (d), 122.0 (d), 113.5 (d), 108.3 (s). HR-EI-MS: found, 201.0362; calcd, 201.0362 for $C_{10}H_7N_3S$. FTIR (KBr) υ_{max} (cm^{-1}): 3176, 1617, 1552, 1450, 1344, 1244, 1125, 1013, 743.

Synthesis of 2-(3-Indolyl)-1,3,4-oxadiazole (11). Compound **11** was prepared by adaptation of a previous procedure (*18*). A solution of indole-3-carboxyhydrazide (**8**, 50 mg, 0.285 mmol) in 98% formic acid (300 μL) was stirred at rt for 4 h. The reaction mixture was poured into ice, was extracted in EtOAc (3 × 20 mL), the combined organic extract was washed with sat. $NaHCO_3$, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The crude reaction mixture was purified by crystallization (hexane/EtOAc) to afford the product 1-(3-carboxyindolyl)-2-formylhydrazide (**9**) as an off-white solid (52 mg, 90%). A solution of 1-(3-carboxyindolyl)-2-formylhydrazide (**9**, 20 mg, 0.098 mmol) in $POCl_3$ (500 μL) was stirred at rt for 12 h. The reaction mixture was poured into ice, was basified with 5% NaOH (5 mL), and extracted with EtOAc (3 × 20 mL). The combined organic extract was washed with water, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was separated by FCC on silica gel (EtOAC/hexane) to yield compound **11** as an off-white solid (13 mg, 72% yield). Complete ^{1}H and ^{13}C NMR spectral data is reported here for the first time, partial data similar to a literature report (*18*). ^{1}H NMR (500.3 MHz, CD_3CN): δ 9.98 (br s, NH), 8.58 (s, 1H), 8.21 (d, J = 7.0 Hz, 1H), 7.98 (d, J = 3.0 Hz, 1H), 7.56 (d, J = 7.4 Hz, 1H), 7.32−7.27 (m, 2H). ^{13}C NMR (125.8 MHz, CD_3CN): δ 163.3 (s), 153.0 (d), 137.9 (s), 128.9 (d), 125.8 (s), 124.6 (d), 122.9 (d), 121.9 (d), 113.6 (d), 101.8 (s). HR-EI-MS: found, 185.0590; calcd, 185.0590 for $C_{10}H_7N_3O$. FTIR (KBr) υ_{max} (cm^{-1}): 3180, 1627, 1524, 1376, 1245, 1133, 1105, 1010, 743.

Synthesis of 1-Naphthalenylthiazole (13). Compound **13** was prepared as reported for phenylthiazole (*19*). 1-Iodonaphthalene (**12**, 100 mg, 0.394 mmol) and 2-(trimethylsilyl)thiazole (123 mg, 0.788 mmol) were added to a mixture of copper iodide (150 mg, 0.788 mmol) and sodium pentafluorophenoxide (190 μL, 4 M in 1,3-dimethyl-2-imidazolidinone, 0.788 mmol) in 1,3-dimethyl-2-imidazolidinone (500 μL) under an argon atmosphere, and the reaction mixture was heated

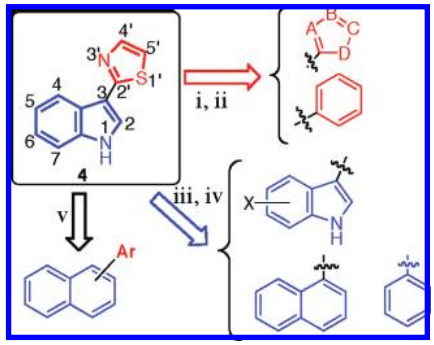


Figure 1. Design of paldoxins based on the camalexin (**4**) scaffold (thiazolyl, red; indolyl, blue substructures) for treatment of canola infected with *Leptosphaeria maculans*: (i) atom replacement of the thiazolyl moiety (bioisosteres), (ii) ring replacement of the thiazolyl moiety, (iii) modification of the indolyl moiety, (iv) replacement of indolyl with naphthyl and phenyl substituents, and (v) replacement of both indolyl and thiazolyl moieties.

at 130 °C for 12 h. The reaction mixture was cooled to room temperature, was diluted with $CHCl_3$, and passed through a small pad of silica gel. The residue was separated by FCC on silica gel (EtOAC/hexane) to afford compound **13** (51 mg, 60% yield), whose spectral data matched literature reports (*20, 21*).

Fungal Cultures. Liquid cultures of *L. maculans* (virulent isolate BJ-125, IBCN collection, AAFC) were handled as described previously (*22*). 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 (*23*). Liquid cultures were initiated by inoculating minimal media (100 mL) (*24*) with fungal spores at 10^7/mL in Erlenmeyer flasks, followed by incubation on a shaker under constant light at 23 ± 1 °C.

For purification of BO, 600 mL of 48 h old liquid cultures prepared as described above, were incubated with 3-phenylindole (**19**, 0.05 mM final concentration in cultures to induce BO) for an additional 24 h and then gravity filtered to separate mycelia from culture broth. The mycelia was stored at −20 °C up to 72 h and used to obtain protein extracts containing BO activity.

For analysis of BO induction, 72 h old liquid cultures (20 mL) were coincubated with compounds **4**, **6**, **14**−**17**, **19**, and **23** (final concentrations in culture 0.10, 0.20, and 0.50 mM), and after an additional incubation for 24 h, the mycelia were separated from the culture broth by filtration.

Preparation of Protein Extracts for Analysis of BO and SOD Activity. Frozen mycelia (0.3−1.4 g) from *L. maculans* were suspended in ice-cold extraction buffer (1 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 50000 g. The resulting supernatant was used for determination of specific activity of BO and superoxide dismutase (SOD). Protein concentrations were determined as described by Bradford (*25*) using the Coomassie Brilliant Blue method with BSA as a standard.

BO Activity Assay. 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 indole-3-carboxaldehyde (**3**) in the reaction assay were determined using calibration curves built with pure compounds (*5*). One enzyme unit (U) is defined as the amount of the enzyme that catalyzes the conversion of one micromole of substrate per minute ($\mu mol \cdot min^{-1} = U$).

SOD Activity Assay. SOD activity (total) was determined as previously reported (*26, 27*). The reaction mixture (1 mL total volume) consisted of potassium phosphate (100 mM, pH 7.4), EDTA (0.1 mM), cytochrome C, (0.01 mM), xanthine, (0.05 mM), xanthine oxidase (0.005 units), and protein extract (50 μL). One enzyme unit will inhibit the rate of reduction of cytochrome C by 50% in a coupled system using xanthine and xanthine oxidase (pH 7.4, 23 °C, in 1.0 mL reaction volume).

Chromatographic Purification of BO and Inhibitory Effect of Potential Paldoxins. The purification of BO was performed in four steps as previously described (*5*). The purified enzyme was used for screening compounds **4**, **6**, **10**, **11**, and **13**−**24** for inhibitory activity. To determine potential inhibitors of BO, inhibition experiments were carried out using brassinin (**1**, 0.10 mM final concentration) and test compounds **4**, **6**, **10**, **11**, and **13**−**24** (0.10 and 0.30 mM final concentrations). Standard deviation values for assays were determined from four independent experiments.

Antifungal Activity. The antifungal activity of compounds was determined using a mycelial radial growth bioassay, as described previously (*22*). All bioassays were carried out in triplicate.

RESULTS

Design and Syntheses. To develop specific inhibitors of BO activity by structural modification of the camalexin (**4**) scaffold, molecular design focused on structures obtained by (i) atom replacement of the thiazolyl moiety (bioisosteres), (ii) ring replacement of the thiazolyl moiety, (iii) modification of the indolyl moiety, (iv) replacement of indolyl with naphthyl and phenyl substituents, and (v) replacement of both indolyl and thiazolyl moieties (**Figure 1**).

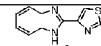
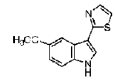
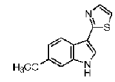
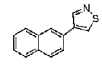
Brassinin (**1**), camalexin (**4**), and other compounds shown in **Table 1** were prepared as previously described (references for synthesis are shown in **Table 1**; compound **6** was commercially available). Modified conditions for the synthesis of 2-(3-indolyl)-1,3,4-oxadiazole (**11**) (*18*), and first time preparations of 2-(3-indolyl)-1,3,4-thiadiazole (**10**) and 1-naphthalenylthiazole (**13**) were carried out as summarized in **Schemes 2** and **3**, respectively.

Inhibition of Brassinin Oxidase Activity. The inhibitory effects of compounds **4**, **6**, **10**, **11**, and **13**−**24** on BO activity were tested at 0.10 mM and 0.30 mM using brassinin (**1**) as substrate (0.10 mM) and purified BO, as described in Materials and Methods. Thiabendazole (**6**), a common fungicide, was used as the reference compound due to its commercial availability and BO inhibitory activity (ca. 25% at 0.30 mM) (*5*). The concentrations of inhibitors were based on the K_m of BO for brassinin (**1**, 0.15 mM under the enzyme assay conditions), the natural substrate. Results of these enzymatic assays are summarized in **Table 1**.

Relative to camalexin (**4**), 5-methoxycamalexin (**16**) was the most potent inhibitor of BO activity (ca. 72% at 0.30 mM), followed by 5-fluorocamalexin (**14**) and 6-methoxycamalexin (**15**), a natural phytoalexin (*2*) (ca. 63% at 0.30 mM). The inhibitory effect of the 6-fluoro derivative **15** (46%, 0.30 mM) on BO activity was similar to that of camalexin (**4**; 53%, 0.30 mM). By contrast, *N*-1 methylation of camalexin (**4**) led to a derivative (**18**) devoid of inhibitory activity. Interestingly, while 2-naphthalenylisothiazole (**23**) displayed a significant inhibitory effect (42%, 0.30 mM), similar to that of camalexin (**4**), 1-naphthalenylisothiazole (**22**) showed substantially lower inhibitory effect (21%, 0.30 mM). In addition, 3-phenylindole (**19**) did not affect BO activity, but 6-fluoro-3-phenylindole (**20**) showed some inhibitory effect (17%, 0.30 mM). Unexpectedly, compounds **10**, **11**, and **21**, the isosteres of camalexin (**4**), showed no inhibitory effect.

Induction of Brassinin Oxidase Activity. An increase of BO activity in fungal mycelia implies a faster detoxification of brassinin, which is beneficial to the pathogen *L. maculans* but detrimental to the plant. Previously (*10*), it was shown that both

Table 1. Inhibitory Effect of Camalexin (**4**) and Compounds **6**, **10**, **11**, and **13**−**24** on BO Activity

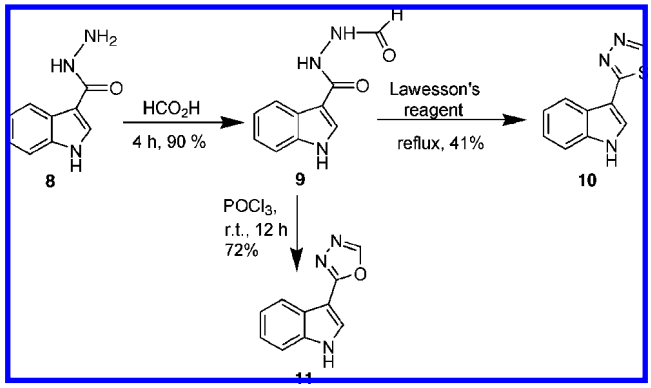
Compound (#) / (reference for synthesis)	Structure	Inhibition (%)[a] 0.10 mM	0.30 mM
Thiabendazole (**6**) (commercial fungicide)[b]		16 ± 3	25 ± 7
Camalexin (4)[b] / (*11*)		30 ± 4	53 ± 4
5-Fluorocamalexin (**14**) / (*12*)		47 ± 5	63 ± 2
6-Fluorocamalexin (**15**) / (*13*)		29 ± 10	46 ± 2
5-Methoxycamalexin (**16**) / (*11*)		51 ± 4	72 ± 1
6-Methoxycamalexin (**17**) / (*11*)		41 ± 6	63 ± 5
1-Methylcamalexin (**18**) / (*12*)		n. d.	n. d.
3-Phenylindole (**19**) / (*14*)		n. d.	n. d.
6-Fluoro-3-phenylindole (**20**) / (*14*)		11 ± 4	17 ± 6
1-(Thiazol-2-yl)-1H-benzimidazole (**21**) / (*15*)		n. d.	n. d.
2-(3-Indolyl)-1,2,3-thiadiazole (**10**) / (this work)		n. d.	n. d.
2-(3-Indolyl)-1,3,4-oxadiazole (**11**) / (*18* and this work)		n. d.	n. d.
1-Naphthalenylisothiazole (**22**) / (*16*)		16 ± 4	21 ± 6
2-Naphthalenylisothiazole (**23**) / (*16*)		29 ± 2	42 ± 3
1-Naphthalenylthiazole (**13**) / (this work)		n. d.	n. d.
2-Phenylimidazole (**24**) / (*17*)		n. d.	n. d.

[a] BO activity was determined as reported in Materials and Methods; 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 5.

camalexin (**4**) and 3-phenylindole (**19**) were strong inducers of BO activity in mycelial cultures as well as mycelial growth inhibitors. Hence, it was of interest to determine if any of the most potent BO inhibitors shown in **Table 1** displayed BO inducing activity; to this end, compounds displaying more than 25% inhibition of BO activity were further tested, as follows.

Mycelial cultures of *L. maculans* were incubated with compounds **4**, **6**, **14**−**17**, **19**, and **23** (0.10, 0.20, and 0.50 mM) for 24 h to evaluate potential induction of BO activity. The cultures were filtered, the mycelia were extracted with extraction buffer (Materials and Methods), and the resulting cell-free extracts were analyzed for BO activity using brassinin (**1**) as

Scheme 2. Synthesis of 2-(3-Indolyl)-1,3,4-thiadiazole (**10**) and 2-(3-Indolyl)-1,3,4-oxadiazole (**11**)



Scheme 3. Synthesis of 1-Naphthalenylthiazole (**13**)

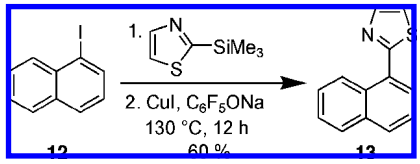


Table 2. Relative Specific Activity of Brassinin Oxidase (BO)[a] and Protein Content[b] of Cell-Free Extracts of Mycelia Obtained from Cultures Incubated with Inhibitors **4**, **6**,**14**−**17**,**19**, and **23** and Antifungal Activity[c] of Inhibitors

cmpd added to mycelial cultures (#)	concn (mM)	relative specific activity of BO[a,b]	relative protein[b]	antifungal activity[c] (%)
control culture		1.0	1.0	
camalexin (**4**)	0.10	4.5 ± 0.5	0.28	35 ± 5
	0.20	6.9 + 0.3	0.24	65 ± 5
	0.50	2.5 ± 0.1	0.10	100 ± 0
5-fluorocamalexin (**14**)	0.10	5.0 ± 0.2	0.30	76 ± 7
	0.20	4.9 ± 0.4	0.23	81 ± 7
	0.50	7.9 ± 0.6	0.19	100 ± 0
6-fluorocamalexin (**15**)	0.10	3.9 ± 0.1	0.36	29 ± 9
	0.20	4.0 ± 0.2	0.28	47 ± 5
	0.50	1.8 ± 0.3	0.24	83 ± 8
5-methoxycamalexin (**16**)	0.10	1.7 ± 0.3	0.28	60 ± 7
	0.20	5.8 ± 0.6	0.24	84 ± 4
	0.50	1.6 ± 0.3	0.13	100 ± 0
6-methoxycamalexin (**17**)	0.10	2.3 ± 0.1	0.27	17 ± 4
	0.20	3.7 ± 0.2	0.16	78 ± 7
	0.50	4.0 ± 0.1	0.11	100 ± 0
2-naphthalenyl-isothiazole (**23**)	0.10	2.6 ± 0.2	0.26	41 ± 7
	0.20	8.5 ± 0.1	0.17	53 ± 4
	0.50	16.3 ± 3.3	0.16	not soluble
3-phenylindole (**19**)	0.10	30.9 ± 3.0	0.18	100 ± 0
	0.20	39.2 ± 6.8	0.14	
	0.50	5.7 ± 0.2	0.10	
thiabendazole (**6**)	0.10	1.9 ± 0.2	0.35	100 ± 0
	0.20	4.1 ± 0.2	0.28	
	0.50	4.9 ± 0.3	0.06	

[a] Specific activity of BO relative to BO in control culture (0.064 ± 0.003 nmol/min/mg); results are expressed as means and standard deviations of four independent experiments. [b] BO specific activities and protein concentrations were obtained from protein extracts of mycelia, as described in Materials and Methods. [c] Percentage of inhibition = 100 − [(growth on medium containing compound/growth on control medium) × 100)] ± standard deviation.

substrate. The total protein content of each cell-free extract was determined using a calibration curve built using BSA. The results of these analyses are summarized in **Table 2**. In general, relative to control cultures all tested compounds induced BO activity, although there were substantial differences in the percentage of induction. For example, relative to controls, camalexin (**4**) induced the highest amount of BO activity at 0.20 mM (6.9 ± 0.3) whereas its 5-fluoro derivative **14** did so at a substantially higher concentration (0.50 mM, 7.9 ± 0.6). Of all compounds tested, 3-phenylindole (**19**) induced the highest amount of BO activity (ca. 39-fold at 0.20 mM). Previously, 3-phenylindole (**19**) was used to obtain reasonable amounts of BO for purification from mycelial cultures (*5*, *10*). All tested compounds appeared to inhibit protein synthesis since the total protein content of mycelia was substantially lower than that in control cultures (**Table 2**).

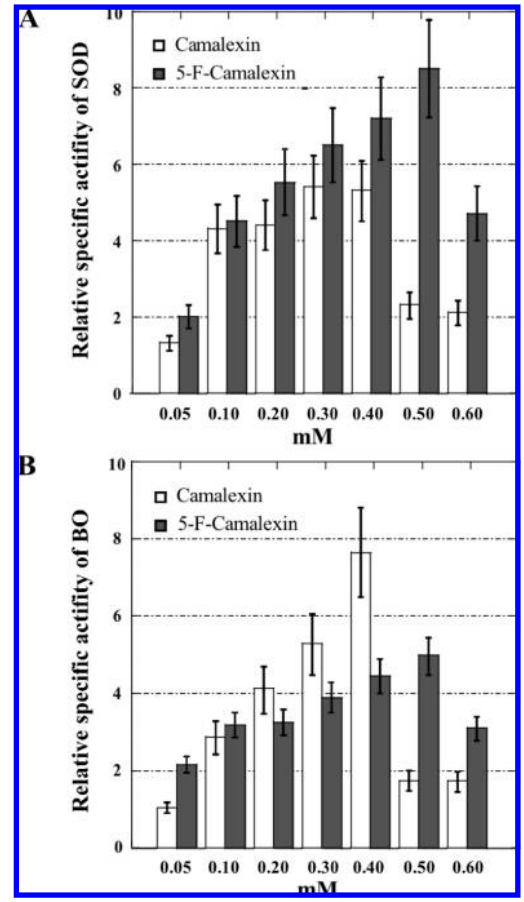


Figure 2. Effect of camalexin (**4**) and 5-fluorocamalexin (**14**) on the activity of superoxide dismutase (SOD) and brassinin oxidase (BO). Results are expressed as means and standard deviations of three independent experiments. C = control.

Induction of Superoxide Dismutase Activity. To further understand the role of camalexins in inducing stress related changes in cells of *L. maculans*, a potential correlation between BO activity and superoxide dismutase (SOD) activity was investigated for compounds **4** and **14**. SOD activity is known to increase with an increase in the production of reactive oxygen species observed in cellular responses to oxidative stress. That is, SOD is a component of the antioxidant defense response in eukaryotic organisms, including fungi (*28*, *29*). Thus, total SOD activity was determined using protein extracts of mycelial cultures obtained after treatment with camalexins at different concentrations. In addition, to correlate the effect of camalexins on the induction of BO and SOD activities, BO activity was determined in parallel experiments. As shown in **Figure 2**A, SOD activity increased progressively in mycelia of cultures incubated with camalexin (**4**, 0.05−0.40 mM) and with 5-fluorocamalexin (**14**, 0.05−0.50 mM, after 24 h of incubation), followed by a decrease at higher concentrations. Similarly, BO activity increased progressively in mycelia of cultures incubated with camalexin (**4**, 0.05−0.40 mM) and with 5-fluorocamalexin (**14**, 0.05−0.50 mM, after 24 h of incubation), followed by a

decrease at higher concentrations (**Figure 2**B). Camalexin (**4**) appeared to be a slightly stronger inducer of BO activity, whereas 5-fluorocamalexin (**14**) was a stronger inducer of SOD activity.

Antifungal Activity. The antifungal activity of compounds **4**, **6**, **14**−**17**, **19**, and **23** was determined using the mycelial growth inhibition assay described in Materials and Methods. The results of growth inhibition, summarized in **Table 2**, are expressed as percent of growth in untreated controls, after five days of inoculation. In general, for all tested compounds, mycelial growth decreased with increasing concentration of compounds (**Table 2**, column 5). Camalexin (**4**) and its derivatives **14**−**17** had similar mycelial growth inhibition activity. Both thiabendazole (**6**) and 3-phenylindole (**19**) displayed potent growth inhibition activity. Due to the low solubility of 2-naphthalenylisothiazole (**23**) in the assay medium, the antifungal activity at 0.50 mM could not be determined.

DISCUSSION

The camalexin (**4**) scaffold guided the design of potential paldoxins for the brassinin detoxifying enzyme produced by *L. maculans* both in planta and in vitro. A total of 16 potential inhibitors were tested, of which nine showed significant inhibitory effect on BO activity. Among these, 5-methoxycamalexin (**16**) revealed to be the best inhibitor of BO activity. Perhaps significantly, both 5-substituted camalexins **14** and **16** showed higher inhibitory activities than the corresponding 6-substituted **15** and **17**. This is particularly interesting because fluorine is smaller and a much stronger electron withdrawing substituent than the methoxy group, although both substituents are electron donating by the resonance effect. Yet, considering the small number of compounds evaluated, no general conclusion regarding substituent effects can be drawn at this stage. Because 1-methylcamalexin (**18**) did not show inhibitory activity on BO, this result might indicate that the indolyl N-H is involved in hydrogen bonding in the active site of the enzyme. However, because 2-naphthalenylisothiazole (**23**) displayed an inhibitory effect (42%, 0.30 mM) similar to that of camalexin (**4**), such a conclusion would be incorrect. Considering that 1-naphthalenylisothiazole (**22**) caused inhibition of BO activity and 1-naphthalenylthiazole (**13**) did not, the thiazole moiety does not appear to be sufficient to cause inhibition of BO activity. Among the structures displaying no inhibitory effects, it was particularly surprising to find that the bioisosteres of camalexin **10** and **11** showed no inhibitory effect on BO activity. Altogether the results indicated that the camalexin (**4**) scaffold is a reasonable model to design potential paldoxins, particularly because none of these compounds were metabolized by *L. maculans*. Nevertheless, a much larger number of structures need to be synthesized and must be assayed before the paldoxin activity can be predicted. To this end, the availability of a crystal structure of BO would be of great assistance, though the small amounts of BO produced by *L. maculans* suggest that this is highly unlikely to occur for the wild-type enzyme.

No direct correlation was found between BO inhibitory activity and BO inducing activity among all tested compounds; for instance, the strongest inducer of BO activity, 3-phenylindole (**19**), showed no inhibitory effect on BO activity. Furthermore, the ability to induce BO activity did not directly correlate with antifungal activity (*10*). Here was demonstrated that 3-phenylindole (**19**) induced BO activity to a much larger extent than thiabendazole (**6**), but the latter displayed a much stronger inhibition of mycelial growth of *L. maculans* (ca. 100% for **6** vs 40% for **19**, at 0.01 mM (*10*)). Because camalexins **4** and **14**−**17** are both inducers and inhibitors of BO activity, they appear to target multiple cell sites of *L. maculans*, with opposite outcomes for the plant and the pathogen. For example, inhibition of BO activity is positive to the plant and negative to the pathogen, whereas induction of BO activity is detrimental to the plant. Hence, it is not desirable for the plant to be exposed to BO inhibitors displaying inducer activity. Consequently, future evaluation of potential paldoxins will require determination of their BO inducing ability.

To better understand the effect of camalexins on *L. maculans*, SOD activity was investigated in mycelia incubated with camalexin (**4**) and 5-fluorocamalexin (**14**). An increase in SOD activity relative to control cultures suggested an increase in reactive oxygen species, which in turn could indicate that camalexins cause oxidative stress in fungal cells, up to a certain concentration. In addition, because the relative activities of SOD and BO are of similar magnitude relative to controls, a correlation between the regulatory mechanism(s) involved in the biosynthesis of BO and SOD appears to exist.

In principle, cruciferous phytoalexins have ecological roles of protecting plants against fungi and other pathogenic organisms; however, the current understanding of such roles and mechanisms of action are still incomplete. To date, the functions of camalexin (**4**) have been investigated more thoroughly than brassinin (**1**) due to its occurrence in the model plant *Arabidopsis thaliana* (*2*). Camalexin (**4**) appears to be an important factor in the disease resistance of *A. thaliana* against *Alternaria brassicicola* (*30*) and *Botrytis cinerea* (teleomorph *Botryotinia fuckeliana*) (*31*), as well as *L. maculans*, albeit less significant (*32*). Recently, transcriptomic analysis of *A. brassicicola* exposed to camalexin (**4**) indicated activation of mechanisms to preserve cell membrane integrity and limit intracellular accumulation of camalexin (*33*). In addition, treatment of *A. brassicicola* with allylisothiocyanate, a metabolite of crucifers, induced genes related to cell protection against oxidative cell damage. Our results suggest that camalexin (**4**) might induce pathways protecting *L. maculans* against oxidative stress as well as brassinin toxicity. Camalexin may also have similar roles in other crucifer pathogenic fungi, as demonstrated by our work, which showed that camalexin could induce a brassinin detoxifying enzyme in the fungal plant pathogen *Sclerotinia sclerotiorum* (*14*).

Overall, our current results point to additional roles for camalexin (**4**) and emphasize that different phytoalexins can have positive or negative impacts on plant resistance to different fungal pathogens. Until now, no plant species have been reported to coproduce both brassinin (**1**) and camalexin (**4**); however, it would be very helpful to introduce the camalexin pathway in *Brassica* species and compare their fungal resistance to wild type plants. Considering that indolyl-3-acetaldoxime is a precursor of both brassinin (**1**) and camalexin (**4**) and that a few genes of the camalexin pathway downstream from the aldoxime have been cloned (*31*, *34*), such a possibility may soon become reality.

Finally, while the camalexin (**4**) scaffold allowed the development of BO inhibitors, since potential paldoxins are desirable to have no other significant biological activities, additional structures ought to be designed and screened. In the meantime, the protection level in planta provided by camalexins **4** and **14**−**17** against *L. maculans* should be determined.

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