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Isosteric probes provide structural requirements essential for detoxification of the phytoalexin brassinin by the fungal pathogen *Leptosphaeria maculans*

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Abstract—Brassinin is a plant defense metabolite with antimicrobial activity produced de novo by a variety of *Brassica* species in response to stress, that is, a phytoalexin. The inhibition of brassinin oxidase (BO), a brassinin-detoxifying enzyme produced by the phytopathogenic fungus *Leptosphaeria maculans*, is a target in our continuing search for novel crop protection agents. To probe the substrate specificity of BO, in particular the mechanism of the detoxification step, several analogues of brassinin, including functional group isosteres ((mono/dithio)carbamate, urea, and thiourea) and homologue methyl tryptaminedithiocarbamate, were investigated using fungal cultures and purified BO. It was concluded that the essential structural features of substrates of BO were: (i) an –NH at the (mono/dithio)carbamate, urea or thiourea group; (ii) a methylene bridge between indole and the functional group; (iii) a methyl or ethyl group attached to the thiol moiety of the (mono/di)thiocarbamate group. A general stepwise pathway for the oxidation of brassinin was proposed that accounts for the structural requirements of detoxification of brassinin analogues in *L. maculans*. All compounds that were BO substrates appeared to be oxidized in mycelial cultures to aldehydes, except for the two most polar compounds *N*′-(3-indolylmethyl)-*N*′′-methylurea and methyl *N*′-(3-indolylmethyl)carbamate. The substrate specificity of BO suggests that selective inhibitors can be designed for the potential control of *L. maculans*.

1. Introduction

Brassinin (**1**) is a plant defense metabolite with antimicrobial activity produced de novo by a variety of *Brassica* species in response to stress.[1] In general, such plant defense metabolites, known as phytoalexins, are crucial components of defense mechanisms that contribute to the overall plant fitness. In addition to this function, brassinin (**1**) has a fundamental significance due to its central position in the biosynthetic pathway of phytoalexins derived from tryptophan (Scheme 1).[1] Brassinin (**1**) is a precursor of a number of phytoalexins produced by *Brassica* spp. and other crucifer species (family Brassicaceae), as, for example, cyclobrassinin (**2**), brassilexin (**3**), spirobrassinin (**4**), dioxibrassinin (**5**), brassicanate A (**6**), and rutalexin (**7**) (Scheme 1).

Not surprisingly, some fungal plant pathogens are able to overcome phytoalexins using detoxification reactions that are carried out by detoxifying enzymes. That is, these fungal enzymes can convert phytoalexins to metabolites devoid of antifungal activity.[2] We have shown that brassinin (**1**) and related phytoalexins are detoxified by pathogens of crucifers via different pathways. Namely, *Leptosphaeria maculans* [(Desm.) Ces. et de Not., asexual stage of *Phoma lingam* (Tode ex Fr) Desm., virulent on canola] converted brassinin to indole-3-carboxylic acid (**8**), whereas *L. biglobosa* (previously known as *L. maculans*, avirulent on canola) converted brassinin to indolyl-3-methanamine (**9**), and *Sclerotinia sclerotiorum* (Lib.) de Bary glucosylated brassinin (**1**) to β-D-1-glucosylbrassinin (**10**, Scheme 2). These conversions were shown to be the main detoxification pathways of brassinin (**1**) that appeared to be carried out by somewhat specific enzymes.[2] To date, none of these brassinin-detoxifying enzymes have been purified, although enzymatic activities have been reported in cell-free homogenates of *L. maculans* (brassinin oxidase),[3] *L. biglobosa* (brassinin hydrolase),[4] and *S. sclerotiorum* (brassinin glucosyl transferase).[5]

Keywords: Brassinin; Brassinin oxidase; Phytoalexin detoxification inhibitor; Paldoxin; Phytoalexin; *Leptosphaeria maculans*; *Phoma lingam*.
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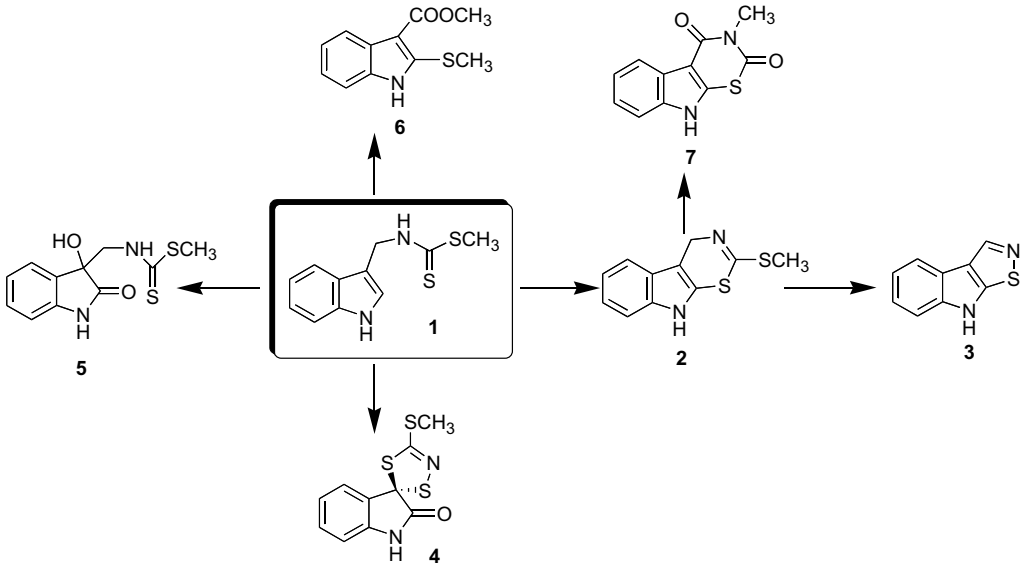
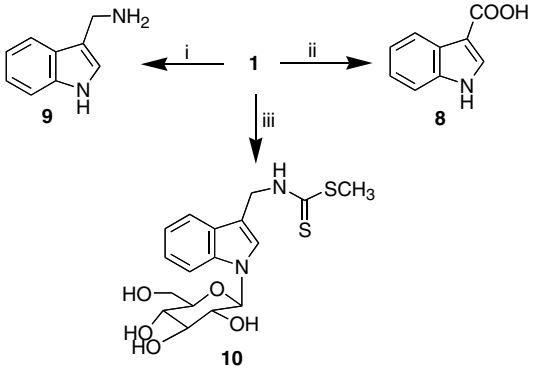
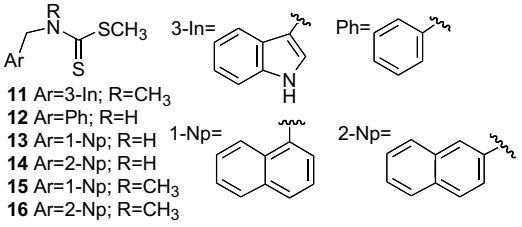


Scheme 1. Position of brassinin (**1**) in the biosynthetic pathway of crucifer phytoalexins.

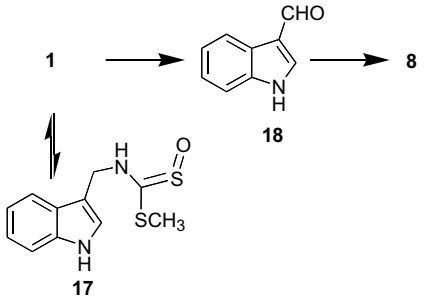


Scheme 2. Products of detoxification of brassinin (**1**) by fungal plant pathogens: (i) *Leptosphaeria biglobosa*, (ii) *L. maculans*, and (iii) *Sclerotinia sclerotiorum*.[2]

The inhibition of brassinin-detoxifying enzymes is a metabolic target in our continuing search for novel crop protection agents coined paldoxins (*p*hyto*al*exin *d*et*ox*ification *in*hibitor*s*).[2] Recently, we synthesized and evaluated over 60 brassinin analogues that were designed to act as inhibitors of brassinin detoxification in *L. maculans*.[6] Among the various functional groups tested, methyl dithiocarbamates having the hydrogen of H–(N–CS_2) free and a methylene bridge to the aromatic moiety (**12**–**14**) were metabolized to the corresponding carboxylic acids, regardless of the type of the aryl substituent (i.e., indolyl, phenyl or naphthyl). By contrast, *N*,*N*-disubstituted dithiocarbamates, for example, *N*′-methylbrassinin (**11**) and *N*-methyl dithiocarbamates **15** and **16**, were not metabolized. In addition, the rate of transformation of brassinin (**1**) in fungal cultures incubated with both brassinin (**1**) and *N*′-methylbrassinin (**11**) was substantially slower than that observed in cultures incubated with only brassinin (**1**), at identical concentrations. Nonetheless, it was not clear whether the decrease in the reaction rate was due to inhibition of the enzyme involved in brassinin transformation, or to the higher antifungal activity of *N*′-methylbrassinin (**11** caused 100% growth inhibition vs 45% for **1**, at 0.20 mM).[6]



The stepwise transformation of brassinin (**1**) incubated in cultures of *L. maculans* leads to indole-3-carboxylic acid (**8**) via indole-3-carboxaldehyde (**18**, Scheme 3). The presence of brassinin-*S*-oxide (**17**) during early stages of the incubation (4–8 h) raised the possibility that aldehyde **18** could arise directly from the enzymatic oxidation of **17** (Scheme 3).[7] However, because recent work[3] using cell-free homogenates of *L. maculans* afforded aldehyde **18** and not *S*-oxide **17**, it would appear more likely that aldehyde **18** derived directly from enzymatic transformation of brassinin (**1**). *S*-oxide **17** was



Scheme 3. Enzymatic stepwise transformation of brassinin (**1**) in cultures of *L. maculans*.[7]

spontaneously converted back to brassinin (**1**), however, indole-3-carboxaldehyde (**18**) was stable and established to be much less toxic to *L. maculans* than brassinin (**1**).[7] That is, the transformation of brassinin (**1**) to aldehyde (**18**) was an oxidative detoxification reaction catalyzed by a putative brassinin oxidase (BO) produced by *L. maculans*.

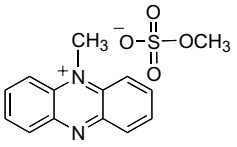
To probe the substrate specificity of BO, in particular the mechanism of oxidative detoxification, several isosteric[8] probes and analogues of brassinin (**1**) were investigated. Namely dithiocarbamates **11**, **20**, **25**, **26**, and **29**, monothiocarbamate **21**, carbamates **19** and **22**, urea **23**, thiourea **24**, dithiocarbonate **30**, amides **27** and **31**, and ester **28** were screened using fungal cultures and purified BO. Here we report results of this work and propose a general stepwise pathway for the oxidative detoxification of brassinin (**1**) and related BO substrates. This pathway accounts for the essential structural requirements that allow detoxification of these compounds by *L. maculans*.

2. Results and discussion

Compounds **1**, **11**, **19**–**24**, and **27**–**31** were synthesized as previously reported,[6] and new dithiocarbamates **25** and **26** as described in Section 4.

As shown in Table 1, antifungal assays using new dithiocarbamates **25** and **26** showed that their antifungal activity against *L. maculans* was substantially higher than that reported for brassinin (**1**). Interestingly, the propyl dithiocarbamate **26** was as potent as *N*′-methylbrassinin (**11**), to date the most inhibitory dithiocarbamate against *L. maculans*.[6]

Metabolism studies were carried out using mycelial cultures of *L. maculans*[6] incubated with brassinin (**1**) or synthetic compounds **11** and **19**–**31**. The substrate specificity of BO was determined using protein purified fractions as described in Section 4. Enzyme activities were determined at 24 °C using PMS (methyl 5-methylphenazinium methyl sulfate (**32**)) as the electron acceptor. The enzyme assays were carried out using brassinin (**1**) or synthetic compounds **11** and **19**–**31** as substrates. All compounds were found to be stable in buffer solutions for at least 60 min or in culture media (4 days). Enzymatic assays were initiated by adding each substrate (1.0 mM final concentration) to BO, followed by incubation of the reaction mixture; enzyme reaction mixtures were extracted and the extract was analyzed by HPLC for quantification of the product. No transformation was observed in the absence of BO. The chemical structure of reaction products was confirmed by comparison of UV spectra and HPLC retention times with those of authentic samples. The results of these experiments are summarized in Table 2.



PMS (**32**)

The results listed in Table 2 indicate that compounds **22** and **23** were oxidized to **18** in purified BO fractions but not in cell cultures, whereas compounds **20**, **21**, and **24** were oxidized to aldehydes in both cell cultures and in purified BO fractions. Importantly, compound **26**, which contains a $SCH_2CH_2CH_3$ instead of a SCH_3 group, was not transformed either in cultures or in BO fractions. Compounds **27**–**30** were transformed in cell cultures via hydrolysis but no oxidation products were detected in BO fractions, indicating that these compounds are not substrates of BO. Most interestingly, the specific activity of BO determined for compounds **21**–**25** was found to be rather different than that observed for brassinin (**1**, 835 ± 34 mU/mg, Table 2), whereas compound **20** showed a specific activity (**20**, 944 ± 184 mU/mg, Table 2) similar to that of brassinin (**1**). In general, the purified BO showed decreasing specific activities according to the substrate order **22** > **23** > **24** > **21** > **20** = **1** > **25** (1 mM, Table 2). The best substrate (**22**, 4058 ± 560 mU/mg, Table 2) showed a specific activity ca. fivefold higher than that of brassinin (**1**) while the poorest substrate (**25**, 434 ± 50 mU/mg, Table 2) showed a specific activity about half of that of brassinin (**1**). That is, the specific activity of BO decreased according to the functional group in the order: carbamate > urea > thiourea > monothiocarbamate > dithiocarbamate. Moreover, similar to brassinin (**1**), only compounds with these functional groups connected to indole by a methylene bridge were oxidized to the corresponding aldehydes (**18** and **18a**), that is, compounds **29** and **30** (containing an ethylene bridge) were not oxidized by BO.

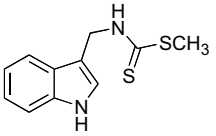
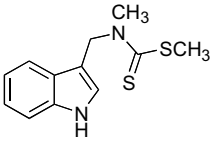
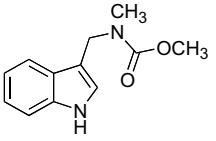
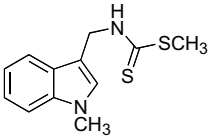
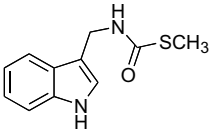
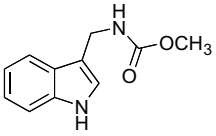
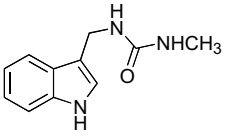
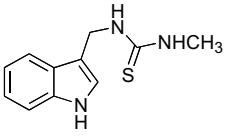
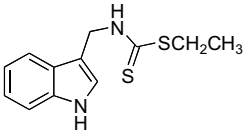
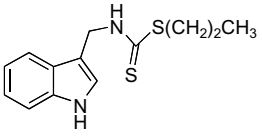
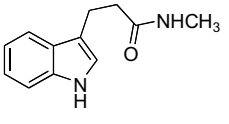
Overall, both the oxidative degradation of brassinin analogues **20**–**25** and the lack of oxidation of analogues **11**, **19**, **26**–**31** in BO purified fractions demonstrate that the following structural features are essential in BO substrates: (i) a free –NH at the functional ((mono/dithio)carbamate, urea, thiourea) group (neither **11**

Table 1. Antifungal activity of brassinins **1** and **11** and dithiocarbamates **25** and **26** against *Leptosphaeria maculans*

Compound	Percentage of inhibition[a]		
	0.50 mM	0.20 mM	0.10 mM
Brassinin (**1**)	100 ± 0	45 ± 6	No inhibition
N′-Methylbrassinin (**11**)	100 ± 0	100 ± 0	74 ± 2
Ethyl *N*′-(3-indolylmethyl)dithiocarbamate (**25**)	100 ± 0	76 ± 6	28 ± 2
Propyl *N*′-(3-indolylmethyl)dithiocarbamate (**26**)	100 ± 0	100 ± 0	78 ± 2

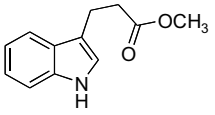
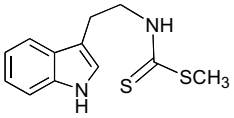
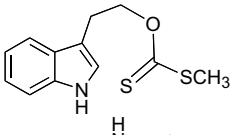
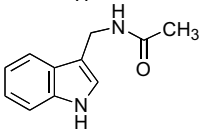
[a] Percentage of inhibition = 100 − [(growth on medium containing compound/growth on control medium) × 100] ± standard deviation.

Table 2. Transformation of brassinin (**1**) and analogues **11**, **19**–**31** (1.0 mM) by *Leptosphaeria maculans* and purified brassinin oxidase (BO)

Compound	Chemical structure	Product of *L. maculans*[a]	Product of BO (specific activity, mU/mg)[b]
Brassinin (**1**)		Indole-3-carboxaldehyde (**18**)	Indole-3-carboxaldehyde (**18**) (835 ± 34)
N′-Methylbrassinin (**11**)		No transformation	No transformation
Methyl *N*′-(3-indolylmethyl)-*N*′-methylcarbamate (**19**)		No transformation	No transformation
1-Methylbrassinin (**20**)		1-Methylindole-3-carboxaldehyde (**18a**)	1-Methylindole-3-carboxaldehyde (**18a**) (944 ± 184)
Brassitin (**21**)		Indole-3-carboxaldehyde (**18**)	Indole-3-carboxaldehyde (**18**) (2146 ± 59)
Methyl *N*′-(3-indolylmethyl)carbamate (**22**)		No transformation	Indole-3-carboxaldehyde (**18**) (4058 ± 560)
N′-(3-Indolylmethyl)-*N*′′-methylurea (**23**)		No transformation	Indole-3-carboxaldehyde (**18**) (3282 ± 501)
N′-(3-Indolylmethyl)-*N*′′-methylthiourea (**24**)		Indole-3-carboxaldehyde (**18**)	Indole-3-carboxaldehyde (**18**) (2564 ± 201)
Ethyl *N*′-(3-indolylmethyl)dithiocarbamate (**25**)		No transformation	Indole-3-carboxaldehyde (**18**) (434 ± 50)
Propyl *N*′-(3-indolylmethyl)dithiocarbamate (**26**)		No transformation	No transformation
N-Methyl-3-(3-indolylmethyl)propanamide (**27**)		3-(3-Indolyl)propanoic acid	No transformation

(*continued on next page*)

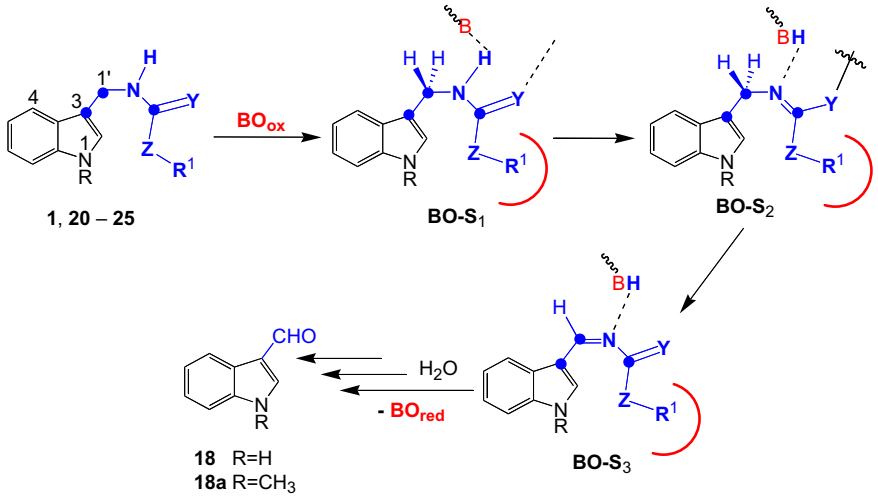
Table 2 (*continued*)

Compound	Chemical structure	Product of *L. maculans*[a]	Product of BO (specific activity, mU/mg)[b]
Methyl 3-(3-indolylmethyl)propanoate (**28**)		3-(3-Indolyl)propanoic acid	No transformation
Methyl tryptaminedithiocarbamate (**29**)		Various products[9]	No transformation
Methyl tryptopholdithiocarbamate (**30**)		Tryptophol	No transformation
N′-Acetyl-3-indolylmethanamine (**31**)		No transformation	No transformation

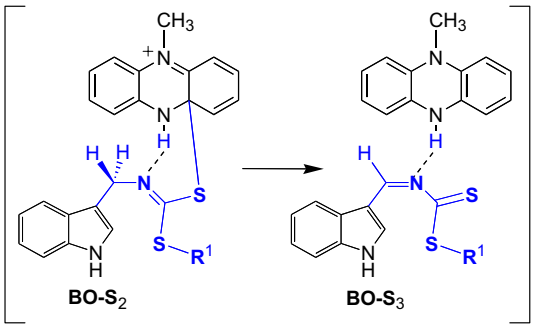
[a] Products detected by HPLC in liquid mycelial cultures incubated with compounds as described in Section 4.

[b] U = μmol/min; products of brassinin oxidase (BO) detected by HPLC using PMS (5-methylphenazinium methyl sulfate (**32**)) as the electron acceptor (0.1 mM) and substrates at 1.0 mM; standard deviations were determined from four independent experiments; other conditions as described in Section 4.

nor **19** was transformed); (ii) a methylene bridge between indole and the functional group (neither **29** nor **30**, which contain an ethylene bridge, was transformed); (iii) a methyl or ethyl group attached to the thiol moiety of the (mono/dithio)carbamate (**25** was transformed but **26** was not). Thus, based on these results and by analogy with reaction mechanisms catalyzed by quinoprotein[10] and flavoprotein[11] enzymes, a rationale is proposed in Scheme 4 for the stepwise enzymatic oxidative degradation of brassinin (**1**) and analogues by BO. The oxidative degradation of brassinin (**1**) and analogues **20**–**25** is proposed to be initiated with formation of hydrogen-bonding between a basic residue in the active site of BO and the (*N*′)-H as well as hydrophobic interactions of the Y = C–Z–R ((mono/dithio)carbamate, urea, thiourea) group to form the BO-substrate complex **BO-S**$_1$. An additional step involving nucleophilic attack by electron-rich Y on an electrophilic center of the coenzyme/BO complex, assisted by proton transfer from *N*′-H, could lead to the formation of an imido(dithio)carba-



Scheme 4. Proposed pathway for oxidative degradation of brassinin (**1**, R = H, R^1 = CH$_3$, Y = Z = S) and its isosteres **20**–**25** (R = H/CH$_3$, R^1 = CH$_3$/CH$_2$CH$_3$, Y, Z = S, O or NH) by brassinin oxidase (BO); atoms/bonds and full circles in blue represent essential structural features of BO substrates.



Scheme 5. Proposed intermediates (by analogy with flavoprotein enzymes[11]) for oxidative degradation of brassinin (**1**) by brassinin oxidase (BO) using artificial electron acceptor PMS (5-methylphenazinium methyl sulfate (**32**)).

mate complex **BO-S$_2$**. An elimination step, the key oxidative step, is proposed to involve concerted C–H bond cleavage and electron transfer from the substrate to the coenzyme/BO complex to give an imino(dithio)carbamate intermediate (**BO-S$_3$**). Subsequent hydrolysis of **BO-S$_3$** would release the corresponding aldehyde **18** or **18a** from the active site of BO. Furthermore, the fact that neither compounds **29** nor **30** are BO substrates suggests that the C-3 of the indolyl moiety is involved in the interaction of BO with the substrate. Consequently, a planar carbon (sp^2) adjacent to the CH_2 (C-1′) appears to be an essential structural feature of any BO substrate; however, additional structures need to be tested to verify this hypothesis. Following similar reasoning as that used for flavoprotein enzymes,[11] potential intermediates **BO-S$_3$** and **BO-S$_4$** formed between the artificial coenzyme PMS (**32**) and brassinin (**1**) are shown in Scheme 5. Clearly, enzyme kinetic studies are a must for the complete understanding of the mechanism of oxidation and complete catalytic cycle of BO.[12]

3. Conclusion

In this study, we compared the transformation of 14 brassinin analogues (compounds **11** and **19**–**31**) using BO fractions and in cell cultures of the fungus *L. maculans* and analyzed the substrate specificity of BO. To summarize, most of the tested compounds that were BO substrates were also oxidized in mycelial cultures to aldehydes **18**/**18a**, except carbamate **22** and methylurea **23**. Because both **22** and **23** are substantially more polar than brassinin (**1**) and compounds **20**, **21**, **24**, and **25**, these results suggest that the polarity of the substrate is an important factor contributing to its biotransformation in mycelial cultures. Although both methyl tryptaminedithiocarbamate (**29**) and methyl tryptopholdithiocarbamate (**30**) were biotransformed in cell cultures via oxidation[9] and hydrolysis,[6] respectively, no transformation occurred in BO fractions. These results indicate that, since neither **29** nor **30** are substrates of BO and **30** inhibited brassinin metabolism in cell cultures,[6] further studies need to be undertaken to establish the type of BO inhibition caused by **29**- and **30**. As well, similar studies need to be carried out with compounds **11** and **19**. Notwithstanding, our hypothetical oxidative pathway (Scheme 4) predicts that neither **11**, nor **19**, **29** and **30** will be competitive inhibitors of BO. Overall, the substrate selectivity of BO suggests that the design of a new generation of selective inhibitors can be optimized taking into consideration these new findings. It is expected that determination of the catalytic properties of BO and structure will greatly assist the development of BO inhibitors, that is, paldoxins for the selective control of *L. maculans*.

4. Experimental

4.1. General experimental procedures

Chemicals were purchased from Sigma–Aldrich Canada (Oakville, ON) and chromatography media and buffers from GE Healthcare. Solvents used in syntheses were dried over the following drying agents prior to use: THF and diethyl ether over sodium/benzophenone and CH_2Cl_2 over CaH_2. HPLC analysis was carried out with a high performance liquid chromatograph equipped with a quaternary pump, an automatic injector, a photodiode array detector (wavelength range 190–600 nm), a degasser, and Hypersil octadecylsilane (ODS) column (5 μM particle size silica, 200 mm × 4.6 mm internal diameter), equipped with an in-line filter. The retention times (t_R) are reported for a linear gradient elution of CH_3CN–H_2O, 25:75 to CH_3CN, 100%, for 35 min at a flow rate of 1.0 mL/min. NMR spectra were obtained on Bruker Avance 500 spectrometers; values were referenced to CD_3CN (CD_2HCN at 1.94 ppm). Fourier transform infrared (FTIR) spectra were measured by the diffuse reflectance method on samples dispersed in KBr. Mass spectra (MS) [high resolution (HR), electron impact (EI)] were obtained on a mass spectrometer using a solids probe.

4.2. Fungal cultures and antifungal bioassays

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

Antifungal bioassays using new dithiocarbamates **25** and **26** were carried out as previously reported.[6]

4.3. Syntheses

4.3.1. Ethyl *N*-(3-indolylmethyl)dithiocarbamate (25). Carbon disulfide (45.0 μL, 0.75 mmol) was added to a solution of 3-indolylmethanamine (100 mg, 0.68 mmol) and triethylamine (191 μL, 1.44 mmol) in pyridine (1 mL) at 0 °C. After stirring for 20 min, EtI (60.0 μL, 0.75 mmol) was added and the reaction mixture was stirred for an additional 30 min. The reaction mixture was acidified with H_2SO_4 (5.0 mL, 1.5 M), extracted with Et_2O, and the organic phase was dried (Na_2SO_4) and evaporated under reduced pressure. Fractionation by FCC (silica gel, EtOAc–hexane, 50:50) afforded **25** (116 mg, 68% yield) as colorless oil. HPLC

t_R = 20.3 min. ^{1}H NMR (500 MHz, CD_3CN): δ 9.26 (br s, 1H, D_2O exchangeable), 8.23 (br s, 1H, D_2O exchangeable), 7.64 (d, J = 8 Hz, 1H), 7.44 (d, J = 8 Hz, 1H), 7.32 (s, 1H), 7.18 (dd, J = 7.5, 7.5 Hz, 1H), 7.10 (dd, J = 7.5, 7.5 Hz, 1H), 5.05 (d, J = 5 Hz, 2H), 3.22 (q, J = 7.5 Hz, 2H), 1.29 (t, J = 7.5 Hz, 3H). ^{13}C NMR (125 MHz, CD_3CN) (two additional Cs are due to a minor rotamer): δ 197.3, 136.8, 127.2, 125.2, 122.4, 119.7, 119.1, 111.9, 111.0, 42.4, 30.6 (plus rotamer 29.1), 14.1 (plus rotamer 13.8). $FTIR_{max}$ (KBr): 3408, 1458, 745 cm^{-1}. HREIMS: *m/z* measured 250.0583 (250.0598 calculated for $C_{12}H_{14}N_2S$). EIMS *m/z* (% relative abundance) 250 (M^+, 81), 221 (17), 162 (33), 133 (100), 121 (30).

4.3.2. Propyl *N*-(3-indolylmethyl)dithiocarbamate (26). Preparation and separation as reported above for ethyl *N*-(3-indolylmethyl)dithiocarbamate (**25**) substituting iodopropane for iodoethane. The crude reaction mixture was subjected to flash column chromatography (silica gel, EtOAc–hexane, 50:50) to afford colorless oil of propyl *N*-(3-indolylmethyl)dithiocarbamate (**26**) in 72%. HPLC t_R = 23.5 min. ^{1}H NMR (500 MHz, CD_3CN): δ 9.26 (br s, 1H, D_2O exchangeable), 8.24 (br s, 1H, D_2O exchangeable), 7.64 (d, J = 8 Hz, 1H), 7.44 (d, J = 8 Hz, 1H), 7.32 (s, 1H), 7.18 (dd, J = 7.5, 7.5 Hz, 1H), 7.10 (dd, J = 7.5, 7.5 Hz, 1H), 5.05 (d, J = 5 Hz, 2H), 3.20 (t, J = 7.5 Hz, 2H), 1.96 (m, 2H), 0.98 (t, J = 7.5 Hz, 3H). ^{13}C NMR (125 MHz, CD_3CN): δ 197.1, 136.4, 127.8, 124.8, 121.8, 119.7, 119.3, 111.5, 110.5, 42.9, 36.3, 22.5, 12.6. $FTIR_{max}$ (KBr): 3410, 1457, 742 cm^{-1}. HREIMS: *m/z* measured 264.0789 (264.0755 calculated for $C_{13}H_{164}N_2S$). EIMS *m/z* (% relative abundance) 264 (M^+, 21), 222 (15), 162 (20), 133 (100).

4.4. Metabolism of brassinin (1), compounds 11 and 19–31 in mycelial cultures of *Leptosphaeria maculans*

Erlenmeyer flasks (125 mL) containing 50 mL of minimal media were employed. Each flask was inoculated with spores (10^8 spores/100 mL, spores) of *L. maculans* and incubated at 24 ± 1 °C on a shaker at 120 rpm under constant light. After 48 h a solution of compound to be screened in DMSO (50 μL) was added to the fungal culture (0.10 and 0.20 mM) and to uninoculated medium (control); DMSO was added to control cultures. Samples (2 mL) were withdrawn and either frozen or immediately extracted with EtOAc, the organic phases were concentrated and analyzed by HPLC. Experiments were performed in duplicate.

4.5. Brassinin oxidase activity

Leptosphaeria maculans was grown in liquid cultures as reported above (10^9 spores/100 mL); after 48 h, the inducer 3-phenylindole (0.02 mM solution in culture) was dissolved in DMSO and the cultures incubated for an additional 24 h. The fungal mycelia were filtered off, washed with water, the remaining water squeezed out between layers of cheesecloth and the mycelia pad frozen immediately. Frozen mycelia were mixed with ice-cold standard buffer (22 g of mycelia in 20 mL standard buffer, 20 mM diethanolamine (DEA), pH 8.3, containing 1.0 mM dithiothreitol) and ground using a mortar and pestle until a homogeneous mixture was obtained. The mixture was then centrifuged at 58,000*g* for 60 min and the supernatant was used for further purification of BO. Purification of BO was achieved in four steps: anion-exchange chromatography (DEAE Sephacel, Tris–HCl buffer, pH 8.0, eluted with NaCl, 0.0–0.3 M), chromatofocusing (PBE 94 resin in DEA buffer, pH 9.4, eluted with Polybuffer 96, pH 6.0), size exclusion chromatography (Superdex 200 HR 10/30 eluted with Tris–HCl buffer, 150 mM NaCl, pH 8.0), and anion-exchange chromatography (Q-Sepharose in DEA buffer, pH 8.4, eluted with NaCl, 0.0–0.3 M).[14]

Brassinin oxidase assays: The reaction mixture contained 20 mM DEA (pH 8.3), 1 mM DTT, 0.1% Triton X-100, substrate (brassinin (**1**) or compounds **11** and **19**–**31**, 1.0 mM), PMS (**32**, 0.1 mM), and 50 μL of purified BO 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 2 mL of EtOAc at t = 0. The products were extracted with EtOAc (2 mL), the extracts were concentrated using a rotary evaporator and were analyzed by HPLC. Quantification of products was carried out using standard calibration curves. One unit of activity (U) is defined as the amount of enzyme releasing 1 μmol of product (aldehyde **18** or **18a**) per min under the assay conditions. The specific activity of BO was expressed as milliunits (mU) per mg of protein.

Protein concentrations were determined by the Bradford method[15] using the Sigma prepared reagent and bovine serum albumin as the standard.

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