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Brassinin oxidase mediated transformation of the phytoalexin brassinin: Structure of the elusive co-product, deuterium isotope effect and stereoselectivity

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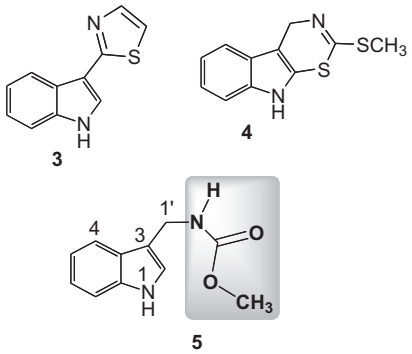
ABSTRACT

Brassinin oxidase, a fungal detoxifying enzyme that mediates the conversion of the phytoalexin brassinin into indole-3-carboxaldehyde, is the first enzyme described to date that catalyzes the transformation of a dithiocarbamate group into an aldehyde equivalent. Brassinin is an essential phytoalexin due to its antifungal activity and its role as biosynthetic precursor of other phytoalexins produced in plants of the family Brassicaceae (common name crucifer). In this report, the isolation, structure determination and synthesis of the elusive co-product of brassinin transformation by brassinin oxidase, *S*-methyl dithiocarbamate, the syntheses of dideuterated and (*R*) and (*S*) monodeuterated brassinins, kinetic analyses of isotope effects and chemical modifications of brassinin oxidase are described. The reaction of [1′-$^2H_2$]brassinin was found to be slowed by a kinetic isotope effect of 5.3 on the value of k_{cat}/K_m. This result indicates that the hydride/hydrogen transfer step preceding brassinin transformation is rate determining in the overall reaction. In addition, the use of (*R*) and (*S*)-[1′-^{2}H]brassinins as substrates indicated that the hydride/hydrogen transfer step is ca. 88% stereoselective for the pro-*R* hydrogen. A detailed chemical mechanism of the enzymatic transformation of brassinin is proposed.

1. Introduction

Brassinin oxidase (BOLm) is a fungal detoxifying enzyme that catalyzes the transformation of brassinin (**1**) into the non-toxic indole-3-carboxaldehyde (**2**).[1–3] BOLm appears to be the first and only enzyme described to date catalyzing the transformation of a dithiocarbamate group into an aldehyde (Scheme 1). BOLm is produced by the fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not. [asexual stage *Phoma lingam* (Tode ex Fr.) Desm.], one of the most economically important pathogens of cruciferous oilseeds.[4,5] Brassinin (**1**) is one of several crucifers' defensive products known as phytoalexins,[6,7] that is, antimicrobial metabolites produced de novo in response to stress caused by pathogens and abiotic sources. Phytoalexins are not produced in healthy plant tissues and are part of general defense mechanisms to ward off plant invaders. The fungal detoxification of brassinin (**1**) *in planta* represents a compounded loss for infected plants because brassinin (**1**) is both an antifungal metabolite and a biosynthetic precursor of additional antifungal phytoalexins. Hence, depletion of brassinin can lead to a fast decrease of several inducible plant defense metabolites, which facilitates pathogen invasion and subsequent plant death. Considering the biological importance of brassinin, inhibitors of brassinin detoxification by BOLm were devised as prospective crop protection agents against *L. maculans*.[3,8] Phytoalexin detoxification inhibitors were coined paldoxins and proposed as alternative crop protection agents against specific fungal pathogens.[9] Interestingly, the crucifer phytoalexins, camalexin (**3**) and cyclobrassinin (**4**) were shown to inhibit competitively BOLm activity,[2] indicating that the various constituents of phytoalexin blends produced by crucifers in response to stress have multiple physiological functions.



3 **4** **5**

To probe the substrate specificity of BOLm, various isosteric groups of dithiocarbamate (carbamate, thiolcarbamate, dithiocarbonate, ester, amide, urea and thiourea) were screened using puri-

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fied BOLm and phenazine methosulfate (PMS) as the electron acceptor.[1] Results of this study showed that the catalytic transformation of methyl *N*′-(3-indolylmethyl)carbamate (**5**) to indole-3-carboxaldehyde (**2**) was faster than that of any other functional group, including the dithiocarbamate (brassinin (**1**)). In addition, essential structural features of substrates of BOLm were a methyl or ethyl group attached to the thiol moiety of the (mono/di)thiocarbamate group, a free –NH at the side-chain functional group, and a methylene bridge (–CH_2–) between the C-3 of indole and the functional group. This last structural feature suggested that any BOLm substrate required a planar carbon (sp^2) adjacent to the CH_2 (C-1′). Based on those results, we proposed a chemical mechanism for the transformation of brassinin (**1**) to indole-3-carboxaldehyde (**2**) that suggested the formation of two imidodithiocarbamate intermediates I_1 and I_2, covalently bound to PMS (phenazine methosulfate).[1] However, the fate of the dithiocarbamate group of brassinin (**1**), the structure of the co-product(s) of the transformation and the origin of the oxygen atom of the aldehyde group were not identified (Scheme 2). BOLm accepted a wide range of cofactors (PMS, small quinones or flavin mononucleotide, FMN).

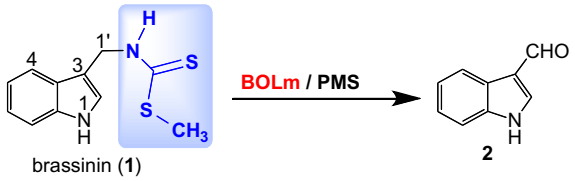
To design paldoxins effective against *L. maculans*, it is crucial to understand the chemical and catalytic mechanisms of the enzymatic transformation of brassinin (**1**).[10] Toward this end, we describe the isolation and structure determination of the previously unknown co-product of the BOLm mediated transformation of brassinin (**1**), the syntheses of dideuterated brassinin and corresponding carbamate, and kinetic analyses of deuterium isotope effects. A detailed chemical mechanism of the enzymatic transformation of brassinin (**1**) by BOLm that integrates these results with previous work is discussed.

2. Results

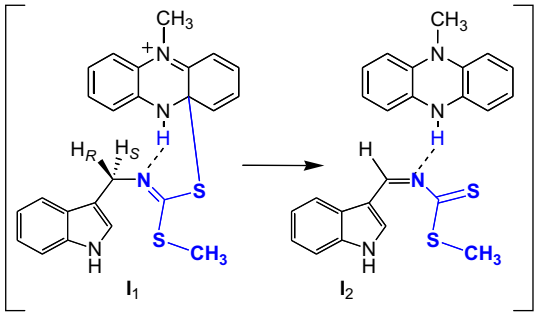
2.1. Chemistry

2.1.1. Chemical structure of the co-product of brassinin oxidation and origin of the aldehydic oxygen

The fate of the *S*-methyl dithiocarbamate group of brassinin (**1**) after transformation by BOLm has remained elusive until now.



Scheme 1. Transformation of brassinin (**1**) to indole-3-carboxaldehyde (**2**) mediated by BOLm from the plant fungal pathogen *Leptosphaeria maculans*.



Scheme 2. Proposed intermediates $\mathbf{I_1}$ and $\mathbf{I_2}$ of the catalytic transformation of brassinin (**1**) by BOLm using PMS as electron acceptor.[1]

HPLC chromatograms of extracts of the reaction mixture of brassinin and purified BOLm showed peaks due to the substrate (brassinin, **1**, t_R = 19.8 min) and indole-3-carboxaldehyde (**2**, t_R = 7.0 min) together with an additional peak due to an unknown component (t_R = 4.4 min). To find the chemical structure of this component, the extract of a BOLm-brassinin reaction mixture (2 mM brassinin with 2 mM PMS, 4 μg of purified enzyme) was separated and analyzed as described in Section 4. The ^{1}H NMR spectral data of the unknown compound at t_R = 4.4 min revealed the presence of a singlet at d 2.64 and its HRMS-ESI data suggested a molecular formula of $C_2H_5NS_2$. Altogether the spectroscopic data suggested that the second reaction product resulting from transformation of brassinin was *S*-methyl dithiocarbamate (**6**), the structure of which was confirmed by chemical synthesis (Scheme 3)[11] and direct comparison of spectroscopic data, including UV spectrum (Fig. S1). Unlike other dithiocarbamates, the antifungal activity of *S*-methyl dithiocarbamate (**6**) was substantially lower than that of brassinin (100% at 0.50 mM), causing only 18 ± 4% mycelial growth inhibition.

To establish the origin of the oxygen atom in indole-3-carboxaldehyde (**2**), the catalytic transformation of brassinin (**1**) by BOLm was carried out in $H_2{}^{18}O$–$H_2{}^{16}O$ (ca. 1:1). The HRMS-ESI (negative-ion) spectrum of indole-3-carboxaldehyde (**2**) obtained in this enzymatic transformation displayed peaks corresponding to natural abundance [^{16}O]indole-3-carboxaldehyde (**2**) (measured $[M-1]^-$ m/z = 144.0474; calculated for natural abundance C_9H_6NO m/z = 144.0455) and to labeled [^{18}O]indole-3-carboxaldehyde (measured $[M-1]^-$ m/z = 146.0503; calculated for $C_9H_7N^{18}O$ m/z = 146.0498). In control reactions (carried out in non-labeled H_2O), the [^{18}O]indole-3-carboxaldehyde peak was not detected (Table 1). These results strongly suggested that the oxygen of indole-3-carboxaldehyde (**2**) originated from water.

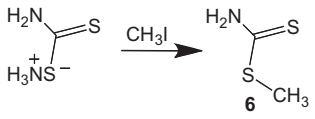
2.1.2. Synthesis of deuterated brassinins and methyl [1′,1′-$^2H_2$]-*N*′-(3-indolylmethyl)carbamate (5a)

[1′,1′-$^2H_2$]Brassinin (**1a**) and methyl [1′,1′-$^2H_2$]-*N*′-(3-indolylmethyl)carbamate (**5a**) were obtained from [1′,1′-$^2H_2$]indolyl-3-methanamine (**8a**), which was prepared from 3-indolecarbonitrile (**7**) after reduction with lithium aluminum deuteride, as summarized in Scheme 4 and described in Section 4. The syntheses of (*R*)-[1′-^{2}H] and (*S*)-[1′-^{2}H]brassinins followed a straightforward route previously used in other chiral deuterated amine preparations, where the amines were obtained from the corresponding alcohols.[12] The enantiomeric purity of these alcohols was determined to be >96%, after esterification with the Mosher's acid chloride[13] and analysis of the purified reaction products by ^{1}H NMR spectroscopy.[14]

2.2. Enzyme BOLm

2.2.1. Substrate kinetic isotope effects

To determine the deuterium isotope effects of the methylene group (–CH_2–) on the rate of conversion of brassinin (**1**) to indole-3-carboxaldehyde (**2**), dideuterated ([1′,1′-$^2H_2$])brassinin (**1a**) was used as substrate and specific activities and kinetic parameters were compared with those obtained for the transformation of non-labeled brassinin (**1**). The relative specific activity of BOLm was about fourfold higher for the non-labeled substrate than for the dideuterated substrate (Table 2). HRMS-ESI analyses

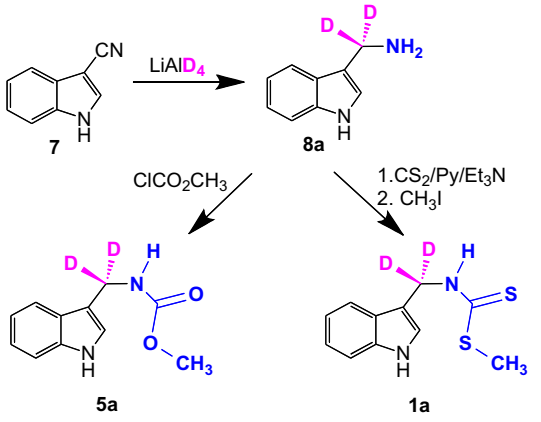


Scheme 3. Syntheses of *S*-methyl dithiocarbamate (**6**).

Table 1
Mass spectral data[a] of indole-3-carboxaldehyde (**2**) obtained from oxidation of brassinin (**1**) by BOLm in the presence of $H_2{}^{18}O$ (conditions as described in Section 4)

Substrate (solvent)	Indole-3-carboxaldehyde (**2**)	
	m/z 144.0455[b] (%)	*m/z* 146.0498[c] (%)
Indole-3-carboxaldehyde (**2**) (control)	144.0474 (100)	Not detected
Brassinin (H_2O)	144.0474 (100)	Not detected
Brassinin (H_2O + $H_2{}^{18}O$, ca. 1:1)	144.0474 (52)	146.0488 (48)

[a] HRMS-ESI negative mode.
[b] Calculated for natural abundance C_9H_6NO.
[c] Calculated for $C_9H_6N^{18}O$.



Scheme 4. Syntheses of [1′,1′-$^2H_2$]brassinin (**1a**) and methyl [1′,1′-$^2H_2$]-*N*′-(3-indolylmethyl)carbamate (**5a**).

of reaction extracts confirmed that indole-3-carboxaldehyde contained one deuterium atom (measured *m/z* = 145.0491; calculated for $C_9H_6{}^2HNO$ *m/z* = 145.0517) and that this peak was absent in control reactions of non-labeled brassinin. When this reaction was carried out in $H_2{}^{18}O$–$H_2{}^{16}O$ (ca. 1:1), the relative specific activity of BOLm was also about fourfold higher for non-labeled brassinin (**1**) in $H_2{}^{18}O$–$H_2{}^{16}O$ (ca. 1:1) than that obtained for the [1′,1′-$^2H_2$]brassinin under similar conditions. These results indicated that: (i) the transformation of non-labeled brassinin was substantially faster than that of [1′,1′-$^2H_2$]brassinin, (ii) $H_2{}^{18}O$ did not affect the rate of BOLm mediated transformation of brassinin.

To determine the effects of deuterium substitution on the steady-state kinetic parameters, various concentrations (0.05–2.8 mM) of brassinin and its deuterated analog **5** were used (Fig. 1). Values for the steady-state kinetic parameters, k_{cat} and K_m, determined for reaction at 20 °C are shown in Table 3. Dideuteration of brassinin decreased the k_{cat} by about 3.4-fold and increased the K_m about 1.6-fold (Fig. 1A and Table 3) causing an apparent deuterium isotope effect of 5.3 ± 0.9 on k_{cat}/K_m. This isotope effect is consistent with cleavage of the 1′-C–^2H bond of brassinin in the rate limiting step. The steady-state kinetic parameters for methyl *N*′-(3-indolylmethyl)carbamate were also determined (Fig. 1 B and Table 3). The results of these analyses showed that replacement of sulfur with oxygen (carbamate group) affected the kinetic parameters of the enzymatic reaction because, relative to brassinin, both the K_m and k_{cat} increased. As well, methyl [1′,1′-$^2H_2$]-*N*′-(3-indolylmethyl)carbamate (0.05–2.8 mM) was used to determine the effects of deuterium substitution on the steady-state kinetic parameters of BOLm (Fig. 1B and Table 3). Dideuteration of methyl *N*′-(3-indolylmethyl)carbamate decreased the k_{cat} about 4.9-fold and K_m decreased about 1.2-fold (Table 3) causing an apparent deuterium isotope effect of 3.9 ± 0.5 on k_{cat}/K_m.

To verify whether the hydride/hydrogen transfer from brassinin (**1**) to PMS catalyzed by BOLm was stereoselective, the enzymatic transformations of (*R*)-[1′-^2H]brassinin, (*S*)-[1′-^2H]brassinin, and non-labeled brassinin (**1**) were conducted over a period of 180 min (Fig. 2). The rates of formation of the aldehydes were similar for the transformations of (*S*)-[1′-^2H]brassinin and non-labeled brassinin (**1**), whereas the rate of transformation of (*R*)-[1′-^2H]brassinin was much slower (Fig. 2). These results demonstrated that the (*S*) enantiomer of [1′-^2H]brassinin was transformed at faster rate than its (*R*) antipode (Fig. 2). As well, the steady-state kinetic parameters determined for (*R*) and (*S*)

Table 2
Relative activity of BOLm (conditions as described in Section 4)[a]

Substrates (solvent)	Relative activity (%)
Brassinin (H_2O)	100 ± 4
Brassinin (H_2O + $H_2{}^{18}O$, 1/1)	96 ± 5
[1′,1′-$^2H_2$]Brassinin (H_2O)	26 ± 4
[1′,1′-$^2H_2$]Brassinin (H_2O + $H_2{}^{18}O$, 1/1)	23 ± 3

[a] Results are expressed as means and standard deviations of four independent experiments.

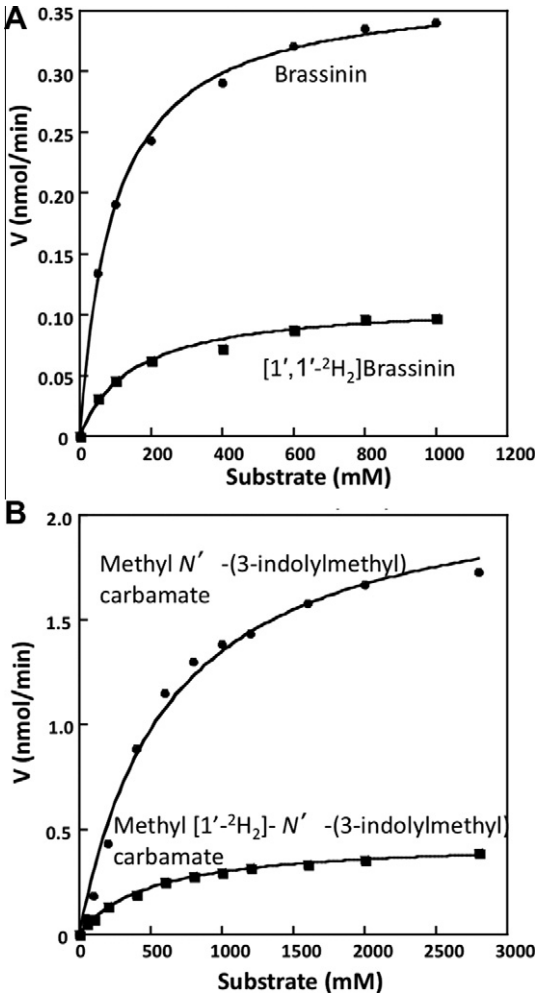


Figure 1. Saturation curves of brassinin (**1**) transformation by BOLm (purified). The reaction mixture contained substrate in the range of 0.05–2.8 mM and 1 mM of PMS. The mixture was incubated at 20 °C for 20 min in the presence of (A) increasing concentrations of brassinin (**1**) and [1′,1′-$^2H_2$]brassinin (**1a**); (B) methyl *N*′-(3-indolylmethyl)carbamate (**5**) and methyl [1′,1′-$^2H_2$]-*N*′-(3-indolylmethyl)carbamate (**5a**).

Table 3
Kinetic parameters k_{cat}, K_m and k_{cat}/K_m determined for BOLm at 20 °C using deuterated and natural abundance substrates

Substrate (concn range)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	KIE[a]
Brassinin (**1**) (0.05–1.0 mM)	0.95 ± 0.02	0.096 ± 0.006	9896 ± 652	—
[1′,1′-$^2H_2$]Brassinin (0.05–1.0 mM)	0.28 ± 0.02	0.151 ± 0.022	1854 ± 300	5.3 ± 0.9
Methyl *N*′-(3-indolylmethyl) carbamate (**5**) (0.05–2.8 mM)	5.62 ± 0.25	0.622 ± 0.078	9035 ± 1202	—
Methyl [1′,1′-$^2H_2$]-*N*′-(3- indolylmethyl)carbamate (0.05–2.8 mM)	1.15 ± 0.02	0.501 ± 0.027	2295 ± 130	3.9 ± 0.5
(*S*)-[1′-^{2}H]Brassinin	0.84 ± 0.02	0.094 ± 0.006	8936 ± 608	1.1 ± 0.1
(*R*)-[1′-^{2}H]Brassinin	0.41 ± 0.01	0.096 ± 0.010	4271 ± 457	2.3 ± 0.3

[a] KIE = $(k_{cat}/K_m)_H/(k_{cat}/K_m)_D$.

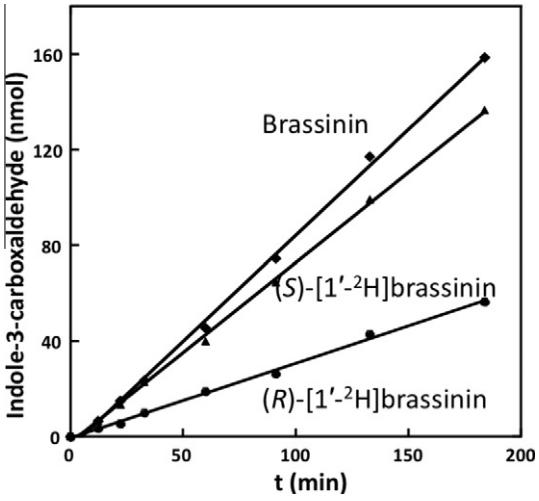


Figure 2. Reaction progress curves of brassinin (**1**), (*R*)-[1′-^{2}H]brassinin (*R*-**1**) and (*S*)-[1′-^{2}H]brassinin (*S*-**1**) transformations by BOLm (purified). Reaction assays were incubated at 20 °C for 0–180 min.

[1′-^{2}H]brassinins showed that, relative to non-labeled brassinin, the k_{cat} of (*R*)-[1′-^{2}H]brassinin decreased ca. 2.3-fold while the K_m remained similar. Therefore, the observed deuterium isotope effect of (*R*)-[1′-^{2}H]brassinin was 2.3 ± 0.3, while that of (*S*)-[1′-^{2}H]brassinin was negligible (Table 3).

To determine the degree of stereoselectivity of BOLm, the catalytic transformation of (*R*)-[1′-^{2}H]brassinin (*R*-**1**), (*S*)-[1′-^{2}H]brassinin (*S*-**1**), and non-labeled brassinin (**1**) by BOLm were allowed to proceed to completion (>97%) and the extracts of each reaction mixture analyzed by HRMS-EI. HRMS-EI spectra of [1′-^{2}H]indole-3-carboxaldehyde (>99% monodeuteration by ^{1}H NMR) and non-labeled aldehyde were used as references (Table 4). As expected, the HRMS-EI spectrum of aldehyde **2**, resulting from transformation of non-labeled brassinin (**1**), was identical to that of non-labeled **2** (Table 4); however, the spectra of aldehydes **2** and **2a** were different from the HRMS-EI spectra of aldehydes resulting from either *R*-**1** or *S*-**1**. Because the HRMS-EI instrument available to us cannot resolve the masses due to C_9H_7NO (*m/z* = 145.0528) and $C_8{}^{13}CH_6NO$ (*m/z* = 145.0483) or $C_8{}^{13}CH_7NO$ (*m/z* 146.0561) and $C_8{}^{13}CH_6NO$ (*m/z* 146.0590), quantification of the degree of stereoselectivity shown by BOLm was approximate. The isotopologue percentages could distinguish the [1′-^{2}H] aldehyde **2a** (145–11.3%, 146–77.4%, 147–8.2%) from the non-labeled aldehyde **2** (145–83.2%, 146–8.4%, 147—not detected) and indicate that about 88% (68.2/77.4 × 100) of deuterated aldehyde **2a** resulted from transformation of (*S*)-[1′-^{2}H]brassinin (*S*-**1**) (Table 4). Likewise, about 90% (75.3/83.2 × 100) of non-labeled aldehyde **2** resulted from transformation of (*R*)-[1′-^{2}H]brassinin (*R*-**1**) (Table 4). That is, these results suggest that BOLm is not highly stereoselective (<95%) since it exhibits a stereoselectivity of ca. 88% for abstraction of the pro-*R* hydrogen/hydride (considering the approximations used in the calculations, percentages were not corrected for enantiomeric purities of *S*-**1** or *R*-**1**).

2.2.2. Co-factor PMS and oxygen

Although PMS, the electron acceptor used in the enzymatic oxidation of brassinin, was expected to be reduced to PMSH, no confirmation has been obtained so far. During this work, it was observed that when PMS and brassinin (**1**) were used in equimolar amounts in BOLm assays, an additional peak at t_R = 12.5 min (broad) was detected in the HPLC chromatograms of extracts of these assay mixtures. The UV spectrum of this peak suggested it to be reduced PMS, that is, PMSH, which was confirmed by reduc-

Table 4
Mass spectral data[a] of indole-3-carboxaldehyde (**2**) and [1′-^{2}H]indole-3-carboxaldehyde (**2a**) synthetic samples (controls) and corresponding aldehydes obtained from oxidation of brassinin (*S*)-[1′-^{2}H]brassinin (*S*-**1**) and (*R*)-[1′-^{2}H]brassinin (*R*-**1**) by BOLm (conditions as described in Section 4)

Substrate	Indole-3-carboxaldehyde (**2**)			
	m/z 144.0449[b] (%)	*m/z* 145.0528[c] (%)	*m/z* 146.0561[d] (%)	*m/z* 147.0624[e] (%)
Non-labeled **2** (control)	144.0451 (100.0)	145.0524 (83.2)	146.0560 (8.4)	ND[f]
Deuterated **2a** (control)	144.0452 (100.0)	145.0487 (11.3)	146.0593 (77.4)	147.0624 (8.2)
Brassinin (**1**)	144.0451 (100.0)	145.0529 (83.5)	146.0565 (8.6)	ND[f]
(*R*)-[1′-^{2}H]Brassinin (*R*-**1**)	144.0452 (100.0)	145.0527 (75.3)	146.0585 (18.2)	147.0627 (1.7)
(*S*)-[1′-^{2}H]Brassinin (*S*-**1**)	144.0451 (100.0)	145.0507 (20.6)	146.0591 (68.2)	147.0625 (7.4)

[a] HRMS-EI.
[b] Calculated for natural abundance C_9H_6NO.
[c] Calculated for natural abundance C_9H_7NO; calculated for $C_8{}^{13}CH_6NO$ *m/z* 145.0483.
[d] Calculated for $C_8{}^{13}CH_7NO$; calculated for $C_9H_6{}^2HNO$ *m/z* 146.0590.
[e] Calculated for $C_8{}^{13}CH_6{}^2HNO$.
[f] ND = not detected (<1%).

ing PMS with sodium dithionite, and analyzing the ethyl acetate extract of the reaction mixture by HPLC (peak retention time and UV spectrum identical to those of HPLC peak obtained in enzyme assay mixture). It was further noted that PMSH oxidized spontaneously to PMS on exposure to air.

Next, to confirm that spontaneous oxidation of PMSH to PMS by molecular oxygen occurred in the enzymatic assays, the rate of transformation of brassinin under aerobic (oxygen) and anaerobic (argon) conditions was investigated. Parallel reactions were carried out under oxygen and under argon, as reported in Section 4. BOLm was incubated with various concentrations of PMS (1–1000 μM) using as substrates brassinin (1000 μM) (Fig. 3A) and its carbamate analog **5** (1000 μM) (Fig. 3B), separately. The reaction progress curves in Figure 3A and B show that there is low substrate conversion in the absence of PMS and that in the presence of oxygen or air, the concentration of PMS did not affect the rate of either reaction substantially. However, when BOLm was incubated with brassinin (1000 mM) and PMS (1 mM) under both anaerobic conditions (inert atmosphere of argon) and aerobic conditions, very substantial differences in the rates of transformation of brassinin were observed (shown in the Supplementary data, Fig. S2). That is, under anaerobic conditions the conversion of brassinin was much lower than under aerobic conditions (ca. 3% of brassinin transformation under argon relative to transformation under air, after 65 min). Overall, these results indicate an efficient in situ regeneration of PMS (oxidation of PMSH to PMS by oxygen), which explains why lower concentrations of PMS do not affect substantially the rates of brassinin transformation. The redox potentials of oxygen and PMS are entirely consistent with these findings.

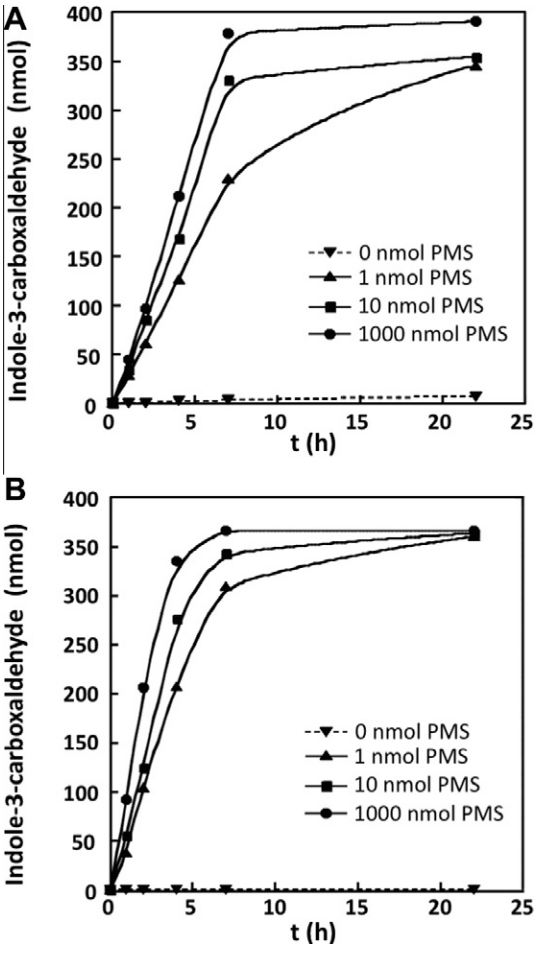


Figure 3. Reaction progress curves of brassinin (**1**) and methyl *N*′-(3-indolylmethyl)carbamate (**5**) transformations by BOLm (purified). Reaction assays were incubated at 20 °C for 0–22 h in the presence of increasing concentrations of PMS in the range of 1–1000 μM and brassinin 1.0 mM (A) or methyl *N*′-(3-indolylmethyl)carbamate 1.0 mM (B).

Considering that brassinin (**1**), indole-3-carboxaldehyde (**2**) and *S*-methyl dithiocarbamate (**6**) could occupy the same active site of BOLm, it was important to establish if compounds **2** or **6** had any inhibitory effect. Varying the concentration of brassinin (0.05–0.30 mM) at a saturated concentration of PMS (1.0 mM) and keeping the concentration of *S*-methyl dithiocarbamate (**6**) or indole-3-carboxaldehyde (**2**) constant (0.30 and 0.60 mM) did not affect BOLm activity. Likewise, varying the concentration of PMS (0.05–0.30 μM) and keeping the concentration of brassinin constant (0.05, 0.10 and 0.15 mM) did not affect the enzymatic activity. These results suggested that neither **2** nor **6** inhibit the transformation of brassinin catalyzed by BOLm.

2.2.3. Deglycosylation and other chemical modifications

Previously, it was shown that the molecular mass of the deglycosylated enzyme (deglycosylated using either PNGase F or *endo*-β-*N*-acetylglucosaminidase) was lower than that of the native BOLm by ca. 20% ([1]). Because PNGase F and *endo*-β-*N*-acetylglucosaminidase are known to release only N-linked glycans from glycoproteins,[15,16] BOLm was treated with an O-glycosidase, which cleaves O-glycans from glycoproteins, and with a mixture of O-glycosidase and *endo*-β-*N*-acetylglucosaminidase. SDS/PAGE analyses of these treated proteins showed a shift in the migration of BOLm sample treated with *endo*-β-*N*-acetylglucosaminidase (47 kDa), but no change was observed for the protein treated with O-glycosidase (57 kDa) (Fig. S3). These results indicated that BOLm is an N-glycosylated protein but not likely O-glycosylated. To further determine the effect of glycosylation of BOLm on its catalytic activity, purified BOLm was pretreated with PNGase F or endo-β-*N*-acetylglucosaminidase or O-glycosidase and then the specific enzymatic activity was examined. Since the specific activity of BOLm was not affected by any of the treatments, glycosylation is unlikely to contribute to its catalytic activity.

To obtain information on the nature of the amino acid residues occurring in the active site of BOLm, different protein modifying reagents were tested. The chemical modification of BOLm with selective reagents was carried out by incubating the enzyme with a large excess of various reagents. The reaction conditions for the modifications of cysteine, tyrosine, arginine, lysine, serine, histidine, aspartate, and glutamate residues are shown in Table 5. No significant difference in the activity of BOLm was observed upon reaction with BUT, EAM, NAI, DEPC, IAA and PMSF; however, modification of the enzyme with WRK (*N*-ethyl-5-phenylisoxazolium-3′-sulfonate, Woodward's reagent K) and EDC resulted in 60–80% loss of activity. The incubation of BOLm with WRK at pH 6.0 resulted in a time- and concentration-dependent loss of activity (Fig. 4A). Using the proposed model of Petra,[17] the apparent dissociation constant and the apparent first-order rate constant at saturation at 20 °C and pH 6.0 (K_I = 44 mM and k_2 = 0.035 min^{-1}, respectively) were calculated. Similarly, incubation with EDC at pH 6.0 resulted in a time and concentration-dependent loss of activity (Fig. 4B).

3. Discussion

Results of (i) co-product structure determination, (ii) origin of aldehydic oxygen, (iii) substrate isotope effects, and (iv) enzyme chemical modifications described above provide novel insights into the enzymatic transformation of brassinin by BOLm. The data obtained using dideuterated substrates suggests that C–H cleavage is the rate limiting step in the reaction, since [1′,1′-$^2H_2$]brassinin

Table 5
Effects of protein modifying reagents on BOLm activity

Reagent for chemical modification	Possible amino acid residues modified	Reaction buffer	Relative activity (100%)
BUT (10 mM)	Arg	A[a]	95 ± 3
EAM (2.5 mM)	Lys	A	97 ± 3
NAI (2.5 mM)	Tyr	A	111 ± 5
DEPC (2.5 mM)	His	A	103 ± 2
WRK (50 mM)	Asp, Glu	B[b]	17 ± 4
EDC (50 mM)	Asp, Glu	B	38 ± 2
IAA (2.5 mM)	Cys	B	106 ± 7
PMSF (2.5 mM)	Ser	B, C[c]	108 ± 5

[a] 100 mM phosphate buffer pH 7.5.
[b] 100 mM MES buffer pH 6.0.
[c] 100 mM phosphate buffer pH 8.1.

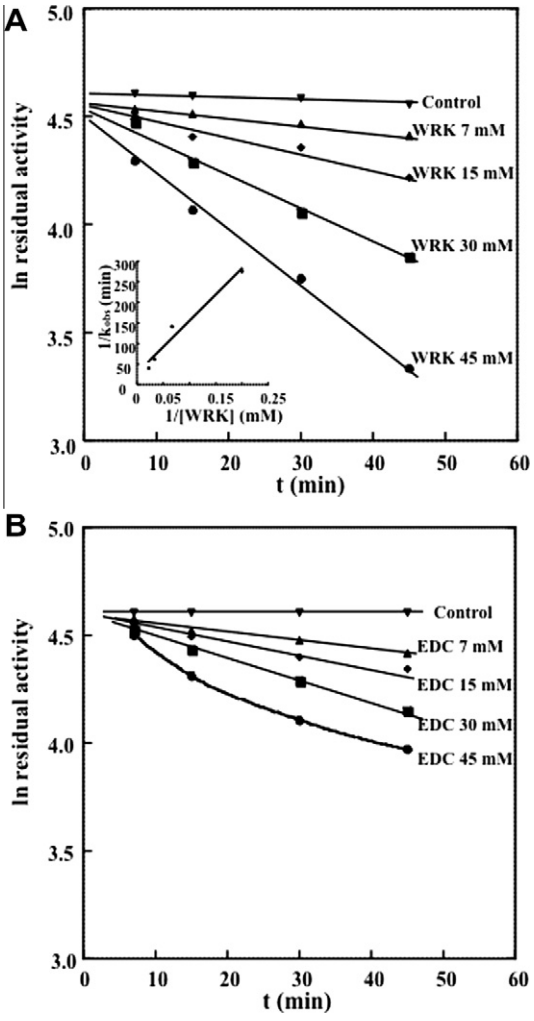
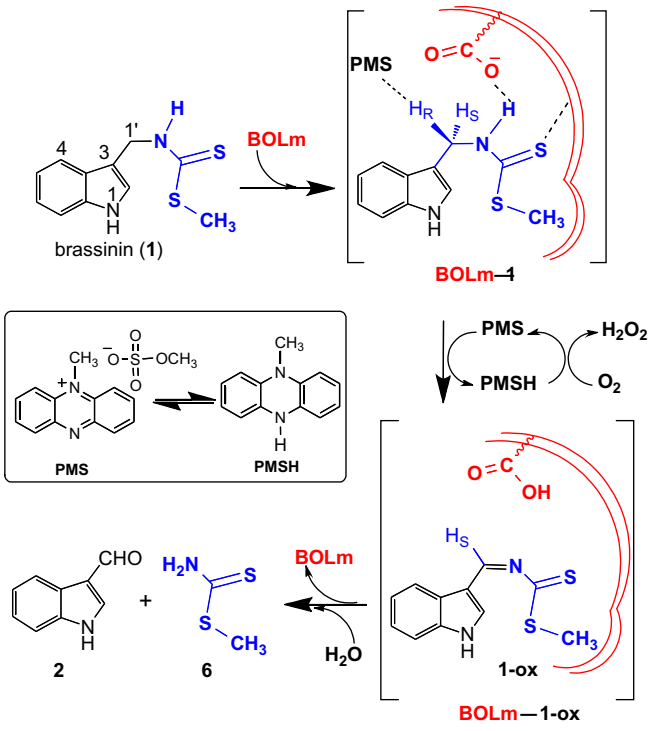


Figure 4. Inactivation of BOLm by carboxyl-specific reagents: (A) Woodward's reagent K and (B) *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide. BOLm was treated with 0, 5, 15, 30 and 45 mM of WRK or EDC as described in Section 4. At various time intervals, aliquots were withdrawn and assayed for residual activity. The inset shows $1/k_{obs}$ against the reciprocal of WRK concentration.

decreased the catalytic efficiency of BOLm relative to brassinin by ca. 5.3-fold. Similarly, methyl [1′,1′-$^2H_2$]-*N*′-(3-indolylmethyl)carbamate (**5**) decreased the catalytic efficiency of BOLm relative to the non-deuterated substrate **5** by ca. 3.9-fold. These observed isotope effects are within the range of the limiting classical value of $k_H/k_D \cong 7$ for a primary deuterium isotope effect.[18] Hence, these results suggest that direct hydride/hydrogen transfer from the enzyme–substrate complex [BOLm-**1**] to PMS to yield PMSH occurs during this BOLm mediated transformation. The alternative nucleophilic attack of the sulfur atom of C=S on PMS, associated with proton transfer from the N–H to PMS, as suggested previously[1] (by analogy with flavoprotein enzymes) appears unlikely. Furthermore, BOLm exhibits pro-*R* stereoselectivity (ca. 88%), consistent with both the observed KIE of (*R*)-[1′-^{2}H]brassinin (*R*-**1**) and the deuterium content of the aldehydes resulting from complete transformations (>97%) of *S*-**1** and *R*-**1**.

The overall results are consistent with the chemical mechanism proposed in Scheme 5. After formation of the enzyme–substrate complex [BOLm-**1**], the apparent rate determining step involves pro-*R* hydride/hydrogen transfer from brassinin (**1**) to PMS in concert with a carboxylate group (Asp or Glu) abstracting the proton of the substrate (CH_2)–**H**–N–(C=S), thus generating dehydrobrassinin (**1ox**) and PMSH. Our data suggest that PMSH is non-catalytically oxidized by atmospheric oxygen to PMS, however it remains unclear whether dehydrobrassinin (**1ox**) is hydrolyzed non-enzymatically to aldehyde **2** and *S*-methyl dithiocarbamate (**6**). It is possible that dehydrobrassinin (**1-ox**) is the enzymatic product of brassinin



Scheme 5. Proposed chemical mechanism for BOLm mediated transformation of brassinin (**1**) using PMS electron acceptor. Structures in brackets are proposed intermediates and red semicircles indicate active site with a tight hydrophobic pocket for SCH_3.

transformation by BOLm, but it may be rather labile in aqueous solutions and spontaneously hydrolyze to the more stable products **2** and **6**. This hydrolytic step is consistent with the incorporation of ^{18}O from labeled water ($H_2{}^{18}O$) into aldehyde **2**. Nonetheless, since the proposed product/intermediate **1-ox** was not detected in the enzymatic transformation of brassinin and PMS, further studies are required. In addition to *S*-methyl dithiocarbamate (**6**) and indole-3-carboxaldehyde (**2**), PMSH was the only compound detected by HPLC analysis. We attempted to synthesize dehydrobrassinin ($\mathbf{1_{ox}}$) by condensing *S*-methyl dithiocarbamate (**6**) with indole-3-carboxaldehyde (**2**) under various conditions but without success. Our results on the effects of chemical modifications of BOLm on kinetic parameters appear to be consistent with the involvement of one carboxyl group in the catalytic transformation of brassinin.

A similar chemical mechanism could be proposed for the catalytic transformation of carbamate **5**; since the k_{cat} for this substrate is higher than that of **1** (5.62 vs 0.95 s^{-1}), oxidation and/or hydrolysis of oxidized **5** might proceed at faster rate. This interpretation is consistent with the kinetic isotope effects observed for both **1** and **5**. Although the kinetic parameters k_{cat} and K_m obtained for BOLm using methyl *N*′-(3-indolylmethyl)carbamate (**5**) (k_{cat} = 5.62 s^{-1}; K_m = 0.622 mM) are different from those obtained using brassinin (**1**) (k_{cat} = 0.95 s^{-1}; K_m = 0.096 mM) as substrate, the catalytic efficiency of BOLm is similar for both carbamate and dithiocarbamate groups (9035 vs 9896 s^{-1} M^{-1}). These kinetic differences are not surprising, considering the relatively higher electronegativity of the oxygen atom and its smaller size relative to sulfur and the calculated pK_a of each group. Inhibition experiments showed that neither *S*-methyl dithiocarbamate (**6**) nor indole-3-carboxaldehyde (**2**) had inhibitory effect on BOLm activity. We interpret these results as an indication of the irreversibility of this enzymatic transformation.

In our previous work, kinetic experiments were carried out with BOLm, PMS (0.05–0.40 μM; K_m = 0.30 μM) and constant concentrations of a dead-end inhibitor in the presence of brassinin (0.60 mM).[2] Those results showed the characteristic plot of uncompetitive inhibition leading to the conclusion that substrate brassinin (**1**) would bind first to the enzyme followed by substrate PMS binding to the brassinin-enzyme binary complex. This mechanistic interpretation of those kinetic data did not consider the non-enzymatic re-oxidation of PMSH to PMS by oxygen. Consequently, our previous interpretation of an ordered mechanism for BOLm might be incorrect. Considering the additional information provided in the present work, BOLm exhibits a sequential kinetic mechanism that may be either ordered or random.

The reaction catalyzed by cytokinin oxidoreductases (CKXs) is somewhat similar to the brassinin (**1**) transformation catalyzed by BOLm. Plant CKXs (containing either a covalent or non-covalent FAD cofactor) catalyze the transformation of secondary amines to aldehydes in a single enzymatic step, which mediates the regulation of plant hormones known as cytokinins.[19] The enzymatic products of CKXs and BOLm, imine in the case of CKXs and imido in the case of BOLm, undergo non-enzymatic hydrolyses to aldehydes but oxygen is not required. For this reason CKXs are now considered dehydrogenases, not oxidases. In the case of BOLm, considering that the natural coenzyme catalyzing brassinin (**1**) transformation to dehydrobrassinin ($\mathbf{1_{ox}}$) remains unknown, we will continue using the term oxidase. Interestingly, structural analysis of complexes of a CKX of maize with a slowly reacting substrate revealed that the substrate displayed a 'plug-into-socket' binding mode that sealed the catalytic site and placed the carbon atom undergoing oxidation in contact with the reactive locus of the flavin.[20] As well, a hydrogen bond between the substrate amine group and an Asp-Glu pair was proposed to facilitate dehydrogenation. Despite these advances towards the understanding of CKXs, and the availability of mechanism based inhibitors,[21] the natural coenzymes of CKXs remain to be determined.[20] Similarly, the in vivo electron acceptor(s) remain(s) unknown in the catalytic dehydrogenation mediated by BOLm.

To screen for inhibitors of BOLm, that is, potential paldoxins against *L. maculans*, the availability of purified BOLm in sufficient quantities is essential. However, since relatively very small quantities of purified BOLm can be obtained using classic chromatographic techniques, heterologous expression of this enzyme is a foreseeable strategy to obtain sufficient quantities. As determined in this work, because deglycosylated BOLm has catalytic activity similar to that observed for the native enzyme, it appears that various expression systems, including bacterial, will be useful to generate crucial quantities of this enzyme.[22] Further work will use enantioselectively labeled substrates (e.g., **5** and related substrates)[1] to understand the relatively low stereoselectivity of BOLm. Nonetheless, the properties and chemical mechanism of BOLm reported in this study will greatly assist the design of inhibitors of BOLm.

4. Experimental

4.1. Materials and general procedures

Chemicals and deglycosylating enzymes were purchased from Sigma–Aldrich Canada (Oakville, ON) and chromatography media and buffers from GE Healthcare. All operations regarding protein extraction, purification and assays were carried out at 4 °C, except where noted otherwise. [1′,1′-$^2H_2$]Brassinin (**1a**), [1′-^{2}H]indole-3-carboxaldehyde (**2a**), methyl [1′,1′-$^2H_2$]-*N*′-(3-indolylmethyl)carbamate (**5a**), and *S*-methyl dithiocarbamate (**6**) were synthesized as described below. All new compounds were characterized using NMR spectral data (recorded on Bruker 500 MHz spectrometers) and HRMS data.

HPLC analysis was carried out with Agilent systems equipped with a quaternary pump, an automatic injector, a photodiode array detector (wavelength range 190–600 nm), a degasser, a Hypersil octadecylsilane (ODS) column (5 μm particle size silica, 200 mm × 4.6 mm internal diameter), and an in-line filter. The retention times (t_R) are reported using 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. High resolution mass spectral (HRMS) data were obtained using an Agilent HPLC 1100 series directly connected to a QSTAR XL Systems Mass Spectrometer (Hybrid Quadrupole-TOF LC/MS/MS) with turbo spray ESI source. Samples were dissolved in CH_3CN and analyzed using a Hypersil ODS C-18 column (5 μm particle size silica, 200 × 2.1 mm I.D. The mobile phase consisted of a linear gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (75:25–25:75 in 35 min to 0:100 in 5 min) and a flow rate of 1.0 mL/min. Data acquisition was carried out in either positive or negative polarity mode per LC run. Data processing was carried out by Analyst QS Software. HRMS-EI spectral data were obtained on a VG 70 SE mass spectrometer using a solids probe.

4.2. Isolation, spectroscopic analysis and antifungal activity of *S*-methyl dithiocarbamate (6)

For isolation of *S*-methyl dithiocarbamate (**6**), a BOLm assay was performed using 4 mg of purified BOLm, 2 mM brassinin and 2 mM PMS in a total volume of 4 mL. The reaction was carried out at 24 °C for 8 h and extracted with EtOAc (4 × 3 mL). The reaction extract was concentrated to dryness, dissolved in CH_3CN and separated by TLC on C_{18} silica gel plates (5 × 10 cm, layer thickness 0.2 mm). The mobile phase consisted of a mixture acetonitrile and water (1:1). Three TLC bands were visualized using a UV lamp

and the components eluted with CH_3CN after scraping each band. The components eluted from the plate were analyzed by LC-HRMS, HPLC-DAD (see Fig. S1), and ^1H NMR (CD_2Cl_2). *S*-Methyl dithiocarbamate (**6**) was stable in the solvents used for TLC separation and extraction at least for 24 h.

Mycelial growth inhibition assays of *L. maculans* isolate BJ 125 were carried out as previously described[23] using *S*-methyl dithiocarbamate (**6**) at three concentrations (0.50, 0.25, 0.12 mM). The mycelial growth inhibition was determined relative to control cultures grown in the absence of compound **6**, but otherwise identical conditions.

4.3. Synthesis

4.3.1. [1′,1′-$^2H_2$]Brassinin (1a)

A solution of 3-indolecarbonitrile (50 mg, 0.35 mmol) in THF (0.5 mL) was added to a suspension of lithium aluminum deuteride (30 mg, 0.70 mmol) in dry THF (0.5 mL) and stirred at 55–60 °C for 4 h. The reaction mixture was cooled to room temperature, diluted with NaOH (1 M, 0.5 mL) and filtered. The filtrate was diluted with water (10 mL) and extracted with EtOAc (3 × 20 mL). The combined organic extract was dried over Na_2SO_4 and concentrated. The residue was separated (SiO_2, CH_2Cl_2/MeOH/NH_4OH) to afford [1′,1′-$^2H_2$]indolyl-3-methanamine as a white solid (27 mg, 55% yield). Carbon disulfide (12 mg, 0.152 mmol) was added to a solution of [1′,1′-$^2H_2$]indolyl-3-methanamine (15 mg, 0.10 mmol) and Et_3N (21 mg, 0.202 mmol) in pyridine (1 mL) and stirred at rt for 15 min. Next, MeI (180 mg, 1.27 mmol) was added and stirring was continued for another 20 min. The reaction mixture was diluted with toluene (2 mL) and concentrated under reduced pressure. The crude product was separated (SiO_2, EtOAC/hexanes) to yield [1′,1′-$^2H_2$]brassinin (14 mg, 58%). ^1H NMR (500.3 MHz, CD_3CN): δ 9.26 (br s, NH), 8.24 (br s, NH), 7.63 (d, J = 8 Hz, 1H), 7.42 (d, J = 8 Hz, 1H), 7.31 (s, 1H), 7.16 (dd, J = 8, 8 Hz, 1H), 7.07 (dd, J = 8, 8 Hz, 1H), 2.55 (s, 3H). Minor peaks of a rotamer were observed at δ 2.67 and 8.55 (only dideuterated brassinin was detected). ^{13}C NMR (125.8 MHz, CD_3CN): δ 199.1 (s), 137.7 (s), 128.1 (s), 126.1 (s), 123.2 (d), 120.7 (d), 120.0 (d), 112.8 (d), 111.8 (s), 42.9 (quintet, J_{C-D} = 21.5 Hz), 18.5 (q). HRMS: *m/z* measured 238.0560 (238.0567 calcd for $C_{11}H_{10}{}^2H_2N_2S_2$).

4.3.2. [1′-^{2}H]Indole-3-carboxaldehyde

A solution of indole (200 mg, 1.71 mmol) in DMF-d_7 (0.6 mL) was added to a cooled solution of freshly distilled $POCl_3$ (288 mg, 1.88 mmol) in DMF-d_7 (0.4 mL) and stirred at 40 °C for 60 min. The reaction mixture was poured into ice-cold water and added dropwise to an ice-cold solution of NaOH (1%, w/v, 3 mL). The suspension was extracted with EtOAc (3 × 20 mL), the combined organic extract was dried over Na_2SO_4 and concentrated to dryness to yield [1′-^{2}H]indole-3-carboxaldehyde (221 mg, 89% yield).[24] ^1H NMR (CD_3CN) δ: ^1H NMR (500.3 MHz, CD_3CN): δ 10.07 (br s, NH), 8.17 (d, J = 7.6 Hz, 1H), 8.00 (s, 1H), 7.54 (d, J = 8 Hz, 1H), 7.31–7.24 (m, 2H). ^{13}C NMR (125.8 MHz, CD_3CN): δ 186.3 (t, J_{C-D} = 25.6 Hz), 138.7, 138.4 (s), 125.7 (s), 125.2 (d), 123.8 (d), 122.5 (d), 120.1 (t, $^2J_{C-D}$ = 3.7 Hz), 113.5 (d) HREIMS: *m/z* measured 144 (144. calcd for $C_9H_6{}^2HNO$).

4.3.3. Methyl [1′,1′-$^2H_2$]-*N*′-(3-indolylmethyl)carbamate (5a)

To a solution of [1′,1′-$^2H_2$]indolyl-3-methanamine (27 mg, 0.18 mmol) in CH_2Cl_2 (1 mL) was added methyl chloroformate (19 mg, 0.20 mmol) and triethylamine (39 mg, 0.39 mmol) and the reaction mixture was stirred at rt for 20 min. The reaction mixture was diluted with water (10 mL), extracted with EtOAc (3 × 20 mL) and the combined organic extract was dried over Na_2SO_4 and concentrated to dryness. Purification of the crude product (SiO_2, EtOAC/hexanes) afforded methyl [1′,1′-$^2H_2$]-*N*′-(3-indolylmethyl)carbamate **(5a)** as a white solid (27 mg, 73% yield). ^1H NMR (500.3 MHz, CD_3CN): δ 9.17 (br s, NH), 7.62 (d, J = 8 Hz, 1H), 7.40 (d, J = 8 Hz, 1H), 7.18 (s, 1H), 7.14 (dd, J = 8, 8 Hz, 1H), 7.06 (J = 8, 8 Hz, 1H), 5.78 (br s, NH), 3.6 (s, 3H) (only dideuterated **5a** was detected). ^{13}C NMR (125.8 MHz, CD3CN): δ 158.3 (s), 137.9 (s), 127.9 (s), 124.8 (d), 123.0 (d), 120.4 (d), 120.0 (s), 114.3 (s), 112.7 (d), 52.7 (quartet), 36.8 (quintet, J_{C-D} = 21.5 Hz). HRMS-EI: *m/z* measured 206.1028 (206.1024 calcd for $C_{11}H_{10}{}^2H_2N_2O_2$).

4.3.4. *S*-Methyl dithiocarbamate (6)

S-Methyl dithiocarbamate was prepared after modification of a previously published procedure.[25] MeI (321 mg, 2.26 mmol) was added dropwise to a cooled suspension of ammonium dithiocarbamate (250 mg, 1.97 mmol) in EtOH (3 mL) at 0 °C and the reaction mixture was stirred under argon for 4 h. The reaction mixture was concentrated (ca. 1/2 volume), diluted with water and the resulting suspension was filtered. The solid was dissolved in Et_2O (5 mL) and precipitated by adding *n*-pentane. Filtration of the precipitate afforded *S*-methyl dithiocarbamate (152 mg, 72% yield) with a mp 42–44 °C (lit value: 41 °C,[25]). HRMS-EI: *m/z* measured 106.9865 (106.9863 calcd for $C_2H_5NS_2$).

4.3.5. Co-factor PMSH

PMS (ca. 1 mmol in H_2O) was treated with an aqueous solution of sodium dithionite ($Na_2S_2O_4$, ca. 2 mmol) for 3 min, the reaction mixture was extracted with ethyl acetate and the extract analyzed by HPLC. PMSH oxidized spontaneously to PMS under exposure to aerobic conditions.

4.4. Fungal cultures and chromatographic purification of BOLm

Cultures of *L. maculans* (isolate BJ-125, IBCN collection, AAFC) were carried out as described previously.[2] For isolation and purification of BOLm, liquid cultures (600 mL) were induced with 3-phenylindole (0.05 mM), the cultures were incubated for an additional 24 h and then gravity filtered to separate mycelia from culture broth. The purification of BOLm was performed in four steps as previously described.[26] Protein concentrations were determined as described by Bradford[26] using the Coomassie Brilliant Blue method with bovine serum albumin (BSA) as a standard.

BOLm standard activity assay. The reaction mixture contained DEA (diethanol amine, 20 mM, pH 8.2), DTT (D,L-dithiothreitol, 1.0 mM), 0.1% (v/v) Triton X-100, brassinin (1.0 mM), PMS (1.0 mM) and protein extract (50–100 μL) in a total volume of 1000 μL. The reaction was carried out at 20 °C for 20 min. A control reaction was stopped by 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.[2] The amounts of brassinin (**1**), indole-3-carboxaldehyde (**2**), and *S*-methyl dithiocarbamate (**6**) in the reaction assay were determined using calibration curves built with pure compounds. Brassinin (**1**), indole-3-carboxaldehyde (**2**), and *S*-methyl dithiocarbamate (**6**) were stable in the buffer and solvents used for extraction and HPLC analysis at least for 24 h.

4.5. Origin of aldehydic oxygen

For the enzymatic experiments in $H_2{}^{18}O$, all the reaction components were dissolved in 50% of $H_2{}^{18}O$. The incubation was carried out in a saturated concentration of brassinin or dideuterated brassinin (1.0 mM). Incorporation of ^{18}O from $H_2{}^{18}O$ into indole-3-carboxaldehyde was determined by HRMS. A control sample was prepared using same experimental conditions but replacing $H_2{}^{18}O$ with natural abundance H_2O.

4.6. Substrate kinetic isotope effects and data analysis

Deuterium kinetic isotope effects (KIE) have been determined from the steady-state kinetic parameters: KIE = $(k_{cat}/K_m)_H/(k_{cat}/K_m)_D$. The isotope effects on the kinetic parameters were measured by varying the concentration of substrate in the range of 0.05–2.8 mM at pH 8.2 and at saturating levels of PMS (1.0 mM).

For reaction progress curves of (*R*) and (*S*)-[1′-^{2}H]brassinins, reaction assays were incubated at 20 °C for 0–3 h in the presence of *R*-**1** and *S*-**1** (0.20 mM), the reaction was stopped at various times (5–180 min) and analyzed as described above for the standard activity assay (Fig. 2).

The kinetic data were analyzed using the computer program KaleidaGraph. $K_{m(app)}$ and $V_{max(app)}$ were determined by measurement of the enzyme activity employing a concentration of brassinin (**1**) in the range of 0.05–1.0 mM and methyl [1′,1′-$^2H_2$]-*N*′-(3-indolylmethyl)carbamate (**5**) in the range of 0.05–2.8 mM. Data were fitted to the equation: $v = V_{max}S/(K_m + S)$, using a non-linear least-squares approach.[27] Kinetic constants possessed ±SE (standard errors) of 3% of the experimental measurement and R^2 values were between 0.980 and 0.998.

In order to analyze the mechanism of BOLm inactivation by WRK the following model was employed:[17]

$$E + I \overset{K_I}{\leftrightarrows} E \cdot I \overset{k_2}{\rightarrow} E{-}I$$

where E is free enzyme, I is WRK concentration, E·I is the enzyme–WRK reversible complex; E–I is the enzyme–WRK covalent complex, K_I is the apparent E·I dissociation constant, and k_2 is the intrinsic rate constant for covalent modification of BOLm. The inactivation rate (k_{obs}) was obtained by plotting the ln percentage of residual activity versus time (t). The observed inactivation rate constant (k_{obs}) can be expressed as:

$$1/k_{obs} = (K_I/k_2[I] + 1/k_2)$$

Plotting $1/k_{obs}$, measured at various WRK concentrations (range 5–45 mM), used for calculation of K_I and k_2.

4.7. Effect of PMS and oxygen

The progress curves for the transformation of brassinin (**1**) or methyl *N*′-(3-indolylmethyl)carbamate (**5**) by BOLm in the presence of various concentrations of PMS were obtained by measuring the time-dependent formation of indole-3-carboxaldehyde (**2**). The assay mixture consisted of 1 mM brassinin or 1 mM methyl *N*′-(3-indolylmethyl)carbamate (**5**), 20 mM DEA (pH 8.2), 1 mM DTT, 0.1% (v/v) Triton X-100, and 50 μL protein extract in a total volume of 1000 μL. The concentrations of the PMS were 0, 1, 10, 1000 μM, respectively. The samples were incubated at 20 °C, and samples (200 μl) were taken after incubation for 1, 2, 4, 7 and 22 h; the reaction was stopped by the addition of 2 mL of EtOAc. The products were extracted and quantified as described above for BOLm standard assay.

Oxygen and argon were purchased from Praxair (USA, argon contained traces of oxygen). The enzymatic assays were performed under conditions similar to those described above for the BOLm standard assay but in 5 mL vials containing 1 mM of brassinin, 1 μM of PMS and 100 μl of purified enzyme in a total volume of 2 mL. For enzymatic assays performed under argon or air, the reaction mixture stored in a capped and sealed vial was first purged with a stream of argon or air for 5 min and then kept under a steady stream of either gas for the duration of the experiment; 200 μL of the reaction mixture were collected every 15 min using a needle and syringe and the reaction was immediately quenched by adding to 2 mL of EtOAc. The products were extracted and quantified as described above for BOLm standard assay. Results obtained for enzymatic assays performed under an oxygen atmosphere were similar to those obtained under normal aerobic conditions (Fig. S2).

4.8. Inhibition

Inhibition experiments were carried out using one substrate at saturation level (brassinin or PMS) and varying the concentration of the second substrate in the region of its K_m (at different concentration of the product). Initial velocity experiments were conducted in the presence of *S*-methyl dithiocarbamate (**6**) or indole-3-carboxaldehyde (**2**) at 0.30 mM and 0.60 mM and varying the concentration of brassinin (0.05–0.30 mM) at a saturated concentration of PMS (1 mM). Next, experiments were carried out similarly but varying the concentration of PMS (0.05–0.30 μM) and keeping the concentration of brassinin constant (0.05, 0.10 and 0.15 mM).

4.9. Enzyme chemical modifications

The purified BOLm was incubated with 0.10 mL of corresponding reaction buffer for each chemical modification reagent and 0.010 mL of 2,3-butanedione (BUT, 10 mM), ethyl acetimidate (EAM, 2.5 mM), *N*-acetylimidazole (NAI, 2.5 mM), diethyl pyrocarbonate (DEPC, 2.5 mM), *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K, WRK, 5–50 mM), diazobenzenesulfonic acid (DBS, 10 mM), iodoacetamide (IAA, 2.5 mM), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC, 5–50 mM) and phenylmethanesulfonyl fluoride (PMSF, 2.5 mM). The enzyme activity was measured and expressed as percentage specific activity calculated as the ratio of specific activity of BOLm with chemical modification reagents and that without chemical modification reagents.

4.10. Analysis of deglycosylated BOLm

Purified BOLm (1 μg) was treated with PNGase F (Sigma, G5166), *endo*-β-*N*-acetylglucosaminidase (Sigma, A-0810) and O-glycosidase (Sigma, G1163), separately and altogether, following the manufacturer's protocols. Reactions were incubated at 37 °C for 3 h in non-denaturing conditions in the appropriate buffer (31 μL of total reaction volume). After incubation, enzymatic activities were tested under conditions described for BOLm activity assay or 3 μL of SDS/PAGE buffer was added to each reaction and samples were analyzed by SDS/PAGE (sodium dodecyl sulfate/polyacrylamide gel electrophoresis).

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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.2011.01.011.

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