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Discovery of inhibitors and substrates of brassinin hydrolase: Probing selectivity with dithiocarbamate bioisosteres

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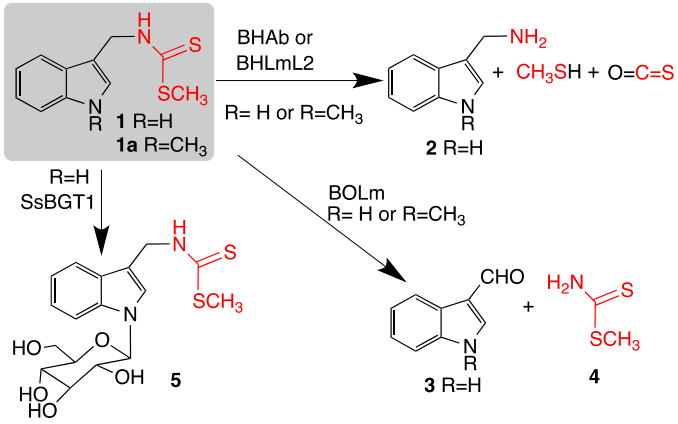
ABSTRACT

Brassinin hydrolase (BHAb), an inducible enzyme produced by the plant pathogen *Alternaria brassicicola* under stress conditions, catalyzes the hydrolysis of the methyl dithiocarbamate group of the phytoalexin brassinin, to indolyl-3-methanamine, methane thiol and carbonyl sulfide. Thirty four substrate inspired compounds, bioisosteres of brassinin and a range of related compounds, were evaluated as potential substrates and inhibitors of BHAb for the first time. While six compounds containing thiocarbamate, carbamate and carbonate groups displayed inhibitory activity against BHAb, only two were found to be substrates (thionecarbamate and dithiocarbamate). Methyl naphthalen-1-yl-methyl carbamate, the most potent inhibitor of the six, and methyl *N*′-(1-methyl-3-indolylmethyl)carbamate inhibited BHAb through a reversible noncompetitive mechanism (K_i = 89 ± 9 and 695 ± 60 μM, respectively). Importantly, these carbamate inhibitors were resistant to degradation by *A. brassicicola*. Carbonates were also inhibitory of BHAb, but a quick degradation by *A. brassicicola* makes their potential use as crop protectants less likely. Overall, these results indicate that indolyl and naphthalenyl carbamates are excellent lead structures to design new paldoxins that could inhibit the detoxification of brassinin by *A. brassicicola*.

1. Introduction

Detoxification of the phytoalexin brassinin (**1**) by fungal plant pathogens is mediated by different enzymes with catalytic activities that depend on the fungal species. Brassinin hydrolases (BHs) catalyze the hydrolysis of the methyl dithiocarbamate of brassinin (**1**), to indolyl-3-methanamine (**2**), methane thiol and carbonyl sulfide (Scheme 1).[1–3] Brassinin (**1**) is an antimicrobial plant metabolite produced de novo by crucifers in response to fungal attack and other forms of stress.[4–6] BHAb is produced by *Alternaria brassicicola* (Schwein.) Wiltshire, whilst BHLmL2 is produced by *Leptosphaeria maculans* (Desm.) [Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm., isolate Laird 2 (LmL2), mustard virulent isolates]. BHs were characterized using LC-ESI-MS/MS of tryptic digests followed by sequence alignment analyses and chemical modifications, which suggested that both enzymes belonged to the family of amidases having the catalytic Ser/Ser/Lys triad.[1] To date, no enzymes involved in the catalytic hydrolysis of dithiocarbamate groups have been reported, that is, BHs have unique substrate specificity and catalytic activity designated as dithiocarbamate hydrolase. While both BHAb and BHLmL2 catalyze the hydrolysis of brassinin (**1**),[1] brassinin oxidase (BOLm) from *L. maculans* (canola virulent isolates)[7] and brassinin glucosyl transferase (SsBGT1) from *Sclerotinia sclerotiorum* (Lib.) de Bary[8] catalyze oxidation and glucosylation detoxifications, respectively (Scheme 1). Importantly, in every example investigated hitherto, brassinin detoxifying enzymes appear to be inducible by compounds that cause some sort of cell stress.

Native BHAb is a dimeric protein of 120 kDa, whereas native BHLmL2 is a tetrameric protein with a molecular mass of 220 kDa.



Scheme 1. Enzyme mediated transformations of brassinin (**1**): BOLm, brassinin oxidase from *Leptosphaeria maculans* (isolates virulent on canola)[7]; BHLmL2, brassinin hydrolase from *L. maculans* (isolates virulent on brown mustard); BHAb, brassinin hydrolase from *Alternaria brassicicola*[1]; SsBGT1, brassinin glucosyl transferase from *Sclerotinia sclerotiorum*.[8]

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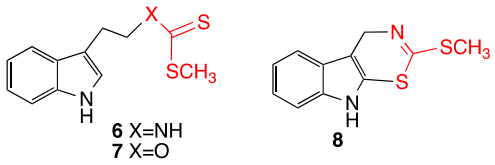
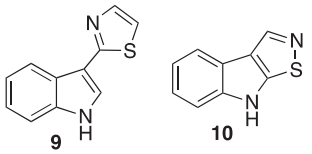
The purified enzymes showed hydrolytic activity toward brassinin (**1**), 1-methylbrassinin (**1a**, R = CH_3), methyl tryptaminedithiocarbamate (**6**) and methyl tryptopholdithiocarbonate (**7**). Although brassinin (**1**) was the best substrate for both BHLmL2 and BHAb, BHAb exhibited relatively higher activity (about twofold) with 1-methylbrassinin (**1a**) than BHLmL2. Conversely, the rates of hydrolysis of methyl tryptaminedithiocarbamate (**6**) and methyl tryptopholdithiocarbonate (**7**) catalysed by BHLmL2 were substantially higher than those catalysed by BHAb.[1] Investigation of the effect of various cruciferous phytoalexins on the activities of BHAb and BHLmL2 indicated that only the phytoalexin cyclobrassinin (**8**) was a competitive inhibitor of both enzymes.



Because phytoalexins are plant metabolites with defensive roles against pathogens, metabolic transformations that lead to a decrease of their concentrations in disease stressed tissues are detrimental to plants. Within crucifers (family Brassicaceae), brassinin (**1**) is of additional significance because it is inhibitory to many fungal pathogens and is a biosynthetic precursor of several other phytoalexins equally potent.[6] For this reason, paldoxins (*phyto**a**lexin **d**etoxification **in**hibitors*) are being developed and evaluated as potential crop protectants against pathogens that detoxify brassinin.[4] Recently, paldoxins for BOLm were designed based on different phytoalexin scaffolds and evaluated for inhibition of brassinin oxidation. The best inhibitors of BOLm were based on the structures of camalexin (**9**)[9] and brassilexin (**10**)[10]; however, to date no compounds other than cyclobrassinin (**8**) were found to inhibit BHAb or BHLmL2 mediated hydrolysis of brassinin (**1**). Considering that inhibitors of these hydrolytic enzymes are potential protectants of cruciferous crops against some of the most damaging fungal pathogens, it is of great interest to develop such compounds. Toward this end, bioisosteres of brassinin (**1**) and a range of related compounds were evaluated as potential substrates and inhibitors of BHAb and herein we describe results of this work.



2. Results and discussion

2.1. Design and synthesis of potential substrates and inhibitors of BHAb

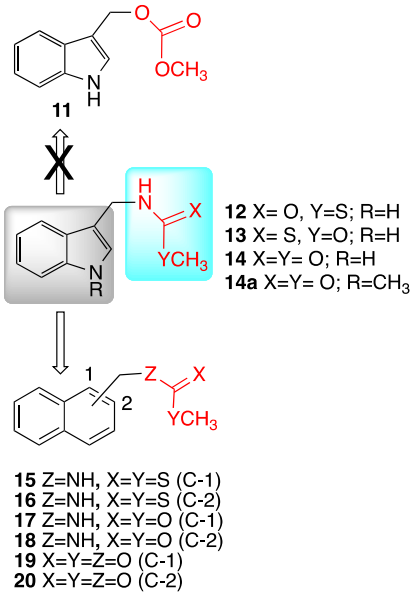
Obviously, the chemical mechanism of transformation of brassinin (**1**) mediated by BHAb[1] is fundamentally different from that mediated by BOLm (hydrolysis vs oxidation, Scheme 1).[7,11] Nonetheless, because both BHAb and BOLm mediate transformations that occur at the dithiocarbamate group, the design of bioisosteres[12] of brassinin (**1**) necessary to probe the substrate specificities of each enzyme is based on similar principles. For example, replacement of the sulfur atoms of the dithiocarbamate of **1** with oxygen atoms generates three different isosteres, thiolcarbamate **12** (brassitin, a known cruciferous phytoalexin),[13] thionecarbamate **13**, and carbamate **14**. These compounds can provide insights into the effect of toxophore size and polarity on the rate of enzymatic hydrolysis. Similarly, replacement of the indolyl ring of brassinin with 1- and 2-naphthalenyl moieties and dithiocarbamate, carbamate and carbonate groups creates additional compounds (**15**–**20**) that probe size and hydrophobic interactions in the active site of BHAb (Scheme 2) (new syntheses and spectroscopic characterization described in Section 4.3.3). The synthesis of 3-indolyl carbonate **11** could not be achieved,[13] likely due to its high reactivity. For similar purposes, other functional groups such as ureas, thioureas, sulfamides, sulfonamides, amides, and esters were also incorporated, amounting to a total of 23 compounds (Table S2, prepared according to references cited in Supplementary data).

2.1.1. Synthesis of naphthalenylmethyl carbamates 17 and 18 and carbonates 19 and 20

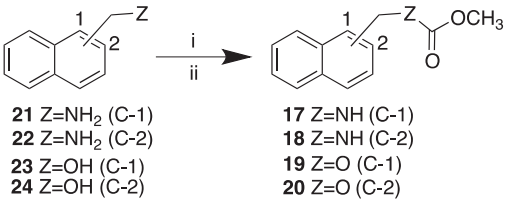
A variety of methods are available for the preparation of carbamates, as for example the Hofmann rearrangement of carboxamides mediated by hypervalent iodine species,[14] Curtius rearrangement of acyl azides,[15] trapping of carbamic acid species with (trimethylsilyl)diazomethane,[16] or coupling of primary aliphatic amines and dialkyl carbonates in supercritical carbon dioxide.[17] Similarly, the corresponding carbonates can be prepared by direct condensation of carbon dioxide with alcohols using trisubstituted phosphine-carbon tetrabromide[18] or silver carbonate and alkyl halides.[19] In this work, we used a simpler procedure for preparation of both carbamates and carbonates; treatment of the corresponding amines and alcohols with methyl chloroformate in the presence of TEA,[11] or sodium hydride, respectively, afforded the desired carbamates **17** and **18** and carbonates **19** and **20** (Scheme 3). The preparation of naphthalenylmethyl carbonates does not appear to have been previously reported.

2.2. Antifungal bioassays and biotransformations by *Alternaria brassicicola*

The antifungal activity of compounds **12**–**20** against *A. brassicicola* was determined employing the mycelial radial growth assay described in the Section 4, using DMSO to dissolve each compound.



Scheme 2. Bioisosteres of brassinin (**1**) and corresponding naphthalenyl derivatives designed to probe the substrate specificity of BHAb.



Scheme 3. Synthesis of 1- and 2-naphthalenylmethyl carbamates **17** and **18** and carbonates **19** and **20**. Reagents and conditions: (i) $ClCO_2Me$, Et_3N, CH_2Cl_2, rt, **17** (97%), **18** (96%); (ii) $ClCO_2Et$, NaH, THF, 0 °C, **19** (80%), **20** (51%).

Assay control plates contained solvent without compound. After 4 days of incubation, the mycelial growth in each plate was measured and the results were statistically analyzed (one-way ANOVA, Table 1). Methyl naththalen-2-ylmethyl carbamate **18** was the most inhibitory of all compounds tested, causing complete mycelial growth inhibition of *A. brassicicola* at 0.50 mM, whereas 3-indolylmethyl carbamate **14** displayed the lowest activity. In addition, the 2-substituted naphthalenyl derivatives were significantly more inhibitory than the 1-substituted counterparts. Previously, compounds **15** and **16** were shown to display similar inhibitory activities against *L. maculans*[13] and *S. sclerotiorum*.[20]

Cultures of *A. brassicicola* were incubated with each compound and their transformations were monitored by HPLC (photodiode array and ESI-MS detection). Samples were withdrawn from cultures immediately after addition of each compound and then as described for each case, up to five days. The samples were subjected to neutral, acidic and basic extractions, and the extracts were analyzed by HPLC, as reported in the Section 4. Media incubated with each compound (control solutions) were analyzed similarly to determine the chemical stability of each compound during the incubation experiments. All compounds were stable in the control solutions for the duration of the experiment (up to 120 h).

As shown in Tables 1 and S1, except for 3-indolylmethyl carbamate **14**, all compounds were hydrolyzed to the corresponding amines or alcohols, however the transformation rates were quite different. Specifically, it is of interest to compare the group of four indolyl isosteres (dithio/thiocarbamates/carbamates) **1** and **12–14**, where substantial differences in transformation rates were found (Table 1). For example, while dithiocarbamate **1** and thiolcarbamate **12** showed $t_{1/2}$ = 6 and 72 h, respectively, the transformation rate of thionecarbamate **13** was in between, with a $t_{1/2}$ = 18 h, and carbamate **14** was not transformed. Purified BHAb (Table 2) mediated the transformation of **1** and **13**, but did not transform **12** or **14**. These results indicated that the transformations of **1** and **13** in cultures of *A. brassicicola* were mediated by BHAb, and that the fungus did not produce enzymes able to transform **14**. Furthermore, since naphthalenyl carbamates **17** and **18** were transformed in culture, it was clear that the presence of a carbamate group was not sufficient to prevent its enzyme-mediated hydrolysis.

2.3. Substrates and inhibitors of BHAb

In preliminary assays, the hydrolase activity of BHAb was screened using a range of indolyl and naphthalenyl synthetic compounds to evaluate the substrate specificity (Tables 2 and S2). In addition to 1-methylbrassinin (**1a**), only compound **13** was found to be a reasonable substrate, although the specific activity of BHAb was three-fold lower relative to brassinin (**1**). As summarized in Table 2, among the naphthalenyl containing compounds, only dithiocarbamate **15** was enzymatically transformed to the corresponding amine (ca. 5% transformation relative to 100% of brassinin), whereas neither indolyl carbamates **14**, as previously established,[1] nor **14a** were hydrolyzed by BHAb. Of the 23 compounds listed in Table S2, only dithiocarbamate **26** was transformed (ca. 5% transformation relative to 100% of brassinin) to the corresponding amine.

Next, the potential inhibitory effects of compounds **12**, **14**, **14a**, **16–20** on BHAb (Table 2), together with those compounds **21–43** (Table S2), were tested at 0.10 and 0.30 mM (concentrations close to the concentration of substrate required for half-maximal activity, $S_{0.5}$ = 0.24 mM) in the presence of brassinin (0.10 mM), as reported in Section 4.5. Results of these assays are presented in Tables 2 (compounds **12–20**) and S2 (compounds **21–43**). Although most of the compounds had no effect on BHAb (**16**, **18** and **21–43**), 3-indolylmethyl carbamates **14** and **14a**, 1-naththalenylmethyl carbamate **17**, and naphthalenylmethyl carbonates **19** and **20** displayed inhibitory activity. Methylation of the nitrogen of the indole ring increased the inhibitory activity of **14a** about twofold relative to the non-methylated analogue **14**; however, the inhibitory effect observed with **17** was the strongest among the compounds tested (K_i = 89 μM).

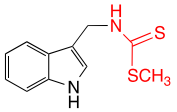
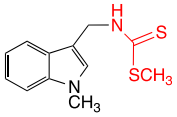
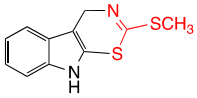
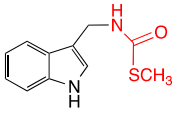
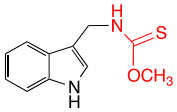
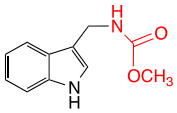
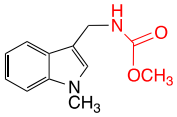
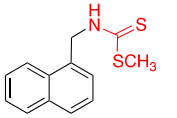
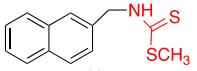
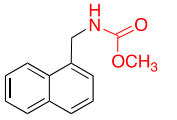
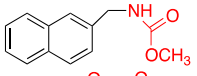
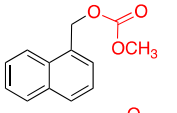
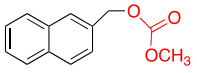
Additional experiments were carried out with compounds known to covalently modify the active sites of Ser/Ser/Lys hydrolases. Since our previous work revealed that chemical modification of the BHAb with PMSF (5.0 mM) resulted in loss of activity (51%), to get additional information on the inhibitory effects of serine modifying compounds, Pefabloc and phenylphosphorodiamidate (0.50 and 1.0 mM) were incubated with BHAb followed by incubation with brassinin (0.10 mM). These experiments indicated that Pefabloc (41 ± 4% at 0.50 mM; 55 ± 8% at 1.0 mM) was inhibitory, but phenylphosphorodiamidate did not affect BHAb activity.

Table 1
Antifungal activity of brassinin (**1**) and compounds **12–20** against *Alternaria brassicicola* and $t_{1/2}$ for biotransformation of each compound in liquid cultures

Compound name/$t_{1/2}$ h/r_t (HPLC method A)	% Inhibition[a]		
	0.50 mM	0.20 mM	0.10 mM
Brassinin (**1**)/6 h[b]/12.1 min	59 (±0)^m	40 (±1)^m	30 (±2)^m
Brassitin (**12**)/72 h/7.5 min	54 (±3)^m	19 (±1)^n	7 (±2)^n
Methyl *N*′-(3-indolylmethyl) thionecarbamate (**13**)/18 h/10.1 min	54 (±3)^m	30 (±1)^o	18 (±1)^n
Methyl *N*′-(3-indolylmethyl) carbamate (**14**)/no biotransformation/6.2 min	28 (±2)^n	15 (±1)^n	8 (±1)^n
Methyl *N*-(1-naphthalenylmethyl) dithiocarbamate (**15**)/12 h/17.8 min	49 (±1)^m	42 (±1)^m	33 (±1)^m
Methyl *N*-(2-naphthalenylmethyl) dithiocarbamate (**16**)/12 h/17.8 min	67 (±4)^o	61 (±3)^p	50 (±3)^o
Methyl *N*-(1-naphthalenylmethyl) carbamate (**17**)/60 h/11.8 min	66 (±1)^o	16 (±4)^n	2 (±1)^n
Methyl *N*-(2-naphthalenylmethyl) carbamate (**18**)/60 h/11.9 min	100 (±0)^p	71 (±1)^p	17 (±1)^n
Methyl *N*-(1-naphthalenylmethyl) carbonate (**19**)/12 h/16.2 min	38 (±3)^q	14 (±1)^n	3 (±1)^n
Methyl *N*-(2-naphthalenylmethyl) carbonate (**20**)/12 h/ 16.7 min	55 (±3)^m	21 (±3)^n	12 (±2)^n

[a] Percentage of growth inhibition calculated using the formula: % inhibition = 100 − [(growth on amended medium/growth in control medium) × 100]. Data are the means ± SE; for statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; n = 3; different letters in the same column (m–q) indicate significant differences (P <0.05).
[b] Data from Ref. 3.

Table 2
Substrates and inhibitors of BHAb

Compound (#)/r_t (HPLC method B)	Structure	Relative specific activity (%)	Inhibition (%) 0.10/0.30 mM	Type of inhibition/ K_i (μM)
Brassinin (**1**)/2.7 min		100	—	—
1-Methylbrassinin (**1a**)[a]		50 ± 4[b]	—	—
Cyclobrassinin (**8**)[a]/2.7 min		No transformation	16 ± 2/26 ± 4	competitive/410 ± 80
Brassitin (**12**)/1.6 min		No transformation	9 ± 3/18 ± 2	Not determined
Methyl *N*′-(3-indolylmethyl) thionecarbamate (**13**)/2.1 min		32 ± 2	—	—
Methyl *N*′-(3-indolylmethyl) carbamate (**14**)/1.5 min		No transformation	10 ± 4/21 ± 4	Not determined
Methyl 1-methyl-*N*′-(3-indolylmethyl) carbamate (**14a**)/2.2 min		No transformation	28 ± 9/46 ± 3	Non-competitive/695 ± 60
Methyl *N*-(1-naphthalenylmethyl) dithiocarbamate (**15**)/5.7 min		5 ± 1	—	—
Methyl *N*-(2-naphthalenylmethyl) dithiocarbamate (**16**)/5.7 min		No transformation	No inhibition	—
Methyl *N*-(1-naphthalenylmethyl) carbamate (**17**)/3.1 min		No transformation	70 ± 3/89 ± 2	Non-competitive/89 ± 9
Methyl *N*-(2-naphthalenylmethyl) carbamate (**18**)/3.1 min		No transformation	No inhibition	—
Methyl *N*-(1-naphthalenylmethyl) carbonate (**19**)/5.1 min		No transformation	38 ± 4/62 ± 4	Mixed/142 ± 14
Methyl *N*-(2-naphthalenylmethyl) carbonate (**20**)/5.2 min		No transformation	29 ± 3/52 ± 3	Competitive/97 ± 22

[a] Compounds previously tested as substrates.[1]
[b] Data from Ref. 1.

2.4. Determination of the types of inhibition of BHAb by com pounds 14a, 17, 19 and 20

To examine the kinetic mechanism by which the strongest inhibitors (Table 2, compounds **14a**, **17**, **19** and **20**) affected BHAb activity, different concentrations of brassinin (**1**) were incubated with various concentrations of each inhibitor, as describe in Section 4.5. The kinetics of inhibition of BHAb is shown in the form of Lineweaver–Burk double reciprocal plots ($1/S$ vs $1/V$) (Fig. 1). These experiments revealed that incubation of brassinin (**1**) with methyl *N*′-(1-methyl-3-indolylmethyl)carbamate (**14a**) and methylnaphthalen-1-yl-methylcarbamate (**17**) resulted in an increase of $1/V_{max}$ values (decrease of V_{max} of BHAb) in a dose-dependent manner (Fig. 1A and B). The results showed that the intersection points of all curves were on the $1/S$ axis indicating that the apparent K_m values were essentially unchanged relative to the untreated control. That is, these results indicated that inhibition by **14a** and **17** could be attributable to either a reversible noncompetitive or an irreversible mechanism.

To determine whether methyl *N*′-(1-methyl-3-indolylmethyl) carbamate (**14a**) and methylnaphthalen-1-yl-methylcarbamate (**17**) inhibited BHAb reversibly, the purified enzyme was incubated with brassinin (**1**) in the presence of methyl *N*′-(1-methyl-3-indolylmethyl)carbamate (**14a**) (0.30 mM) and methylnaphthalen-1-yl-methylc-

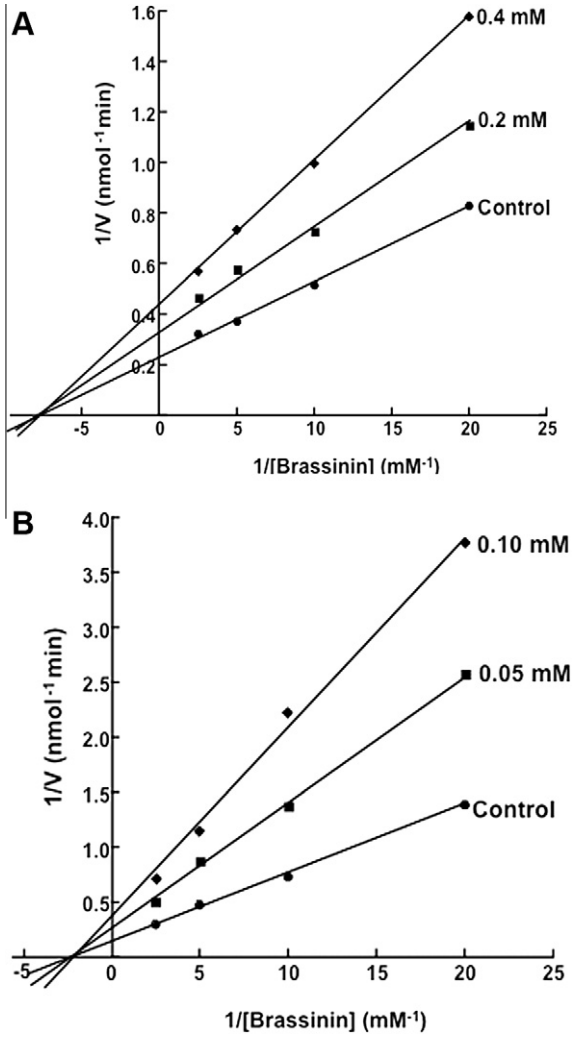


Figure 1. Lineweaver–Burk plots of BHAb activity. In the presence of (A) methyl *N*′-(1-methyl-3-indolylmethyl)carbamate (**14a**) and (B) methylnaphthalen-1-yl-methylcarbamate (**17**).

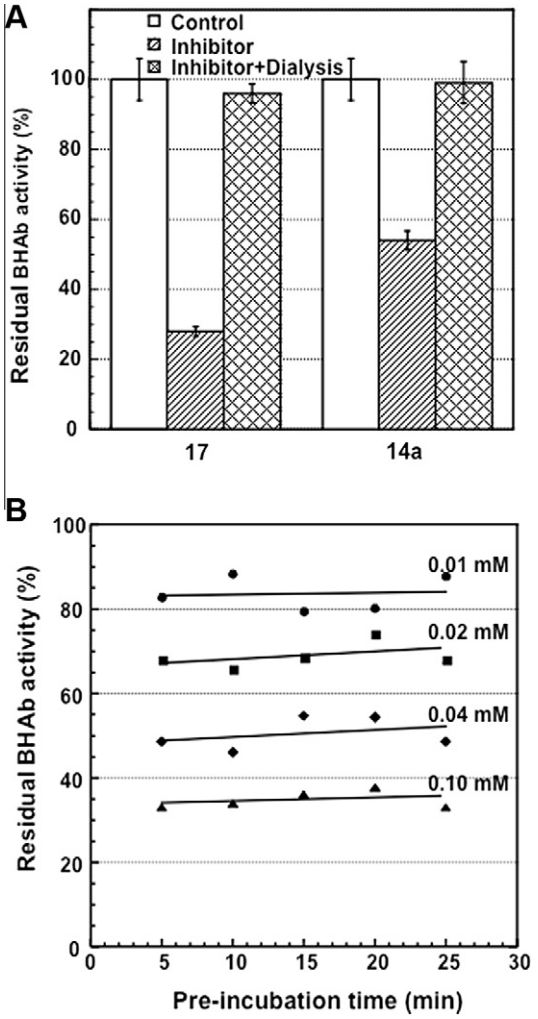


Figure 2. Characterization of the type of BHAb inhibition by methyl *N*′-(1-methyl-3-indolylmethyl)carbamate (**14a**) (0.30 mM) and methyl naphthalen-1-yl-methylcarbamate (**17**). (A) Effects of methyl *N*′-(1-methyl-3-indolylmethyl)carbamate (**14a**) (0.30 mM) or methyl naphthalen-1-yl-methylcarbamate (**17**) (0.1 mM) on BHAb activity after dialysis. (B) Time- and concentration-dependent activity of BHAb under increasing concentrations of methyl naphthalen-1-yl-methylcarbamate (**17**). Control experiments were performed in the absence of compounds (dialysis of the purified enzyme). Results are expressed as mean ± standard deviation of three separate experiments.

arbamate (**17**) (0.10 mM) and then subjected each mixture to extensive dialysis (3 × 2 h , 4–8 °C). Dialysis restored the activity of BHAb in the enzyme solutions treated with either compound **14a** or **17** (Fig. 2A), indicating that these two compounds inhibited BHAb through a reversible noncompetitive mechanism. The inhibitory constant for methyl *N*′-(1-methyl-3-indolylmethyl)carbamate (**14a**) and methylnaphthalen-1-yl-methylcarbamate (**17**) were $K_i = 695 \pm 60$ and 89 ± 9 μM, respectively (Table 2). Further confirmation that the inhibition was reversible was obtained as follows. Since one of the characteristics of irreversible inhibitors is the time dependency of the extent of inhibition, a set of experiments was carried out in the presence of brassinin at 0.50 mM and various concentrations of methyl naphthalen-1-yl-methylcarbamate (**17**). Aliquots were withdrawn at different times (5–25 min) to measure the remaining residual activity. It was apparent that methyl naphthalen-1-yl-methylcarbamate (**17**) did not inhibit BHAb in a time-dependent manner indicating that the reaction was a reversible process (Fig. 2B). These results indicated that both **14a** and **17** inhibited brassinin hydrolysis through a reversible noncompetitive process. That is, both **14a** and **17** can bind to the enzyme and to the enzyme-substrate complex, which suggests the presence of an additional site of interaction with BHAb.

Next, the kinetics of inhibition of BHAb by methyl naphthalen-1-yl-methylcarbonate (**19**) and methyl naphthalen-2-yl-methylcarbonate (**20**) was investigated using different concentrations of brassinin (**1**) incubated with various concentrations of each inhibitor, as described in Section 4.5. Results of these experiments are shown in the form of Lineweaver–Burk double reciprocal plots (1/*S* vs 1/*V*) (Fig. 3A and B). These results indicated that methyl naphthalen-1-yl-methylcarbonate (**19**) exhibited mixed inhibition, as shown by the lines intersecting at a common point. Kinetic analysis of methyl naphthalen-2-yl-methylcarbonate (**20**) based on Lineweaver–Burk plots revealed that the intersection points of all curves were on the 1/*V* axis, indicating that this compound was a competitive inhibitor of BHAb. The K_i values were determined to be 97 ± 22 μM for **20** and 142 ± 14 μM for **19** (Table 2).

Overall, these results suggested that substitution at positions 1 or 2 of the naphthalenyl ring affected the interaction of carbamates and dithiocarbamates with BHAb. Specifically, substitution at C-1 yielded more potent inhibitors, for example **17** versus **18**. By contrast, naphthalenyl carbonates **19** and **20** were similarly potent inhibitors, albeit through different kinetic mechanisms, mixed and competitive, respectively.

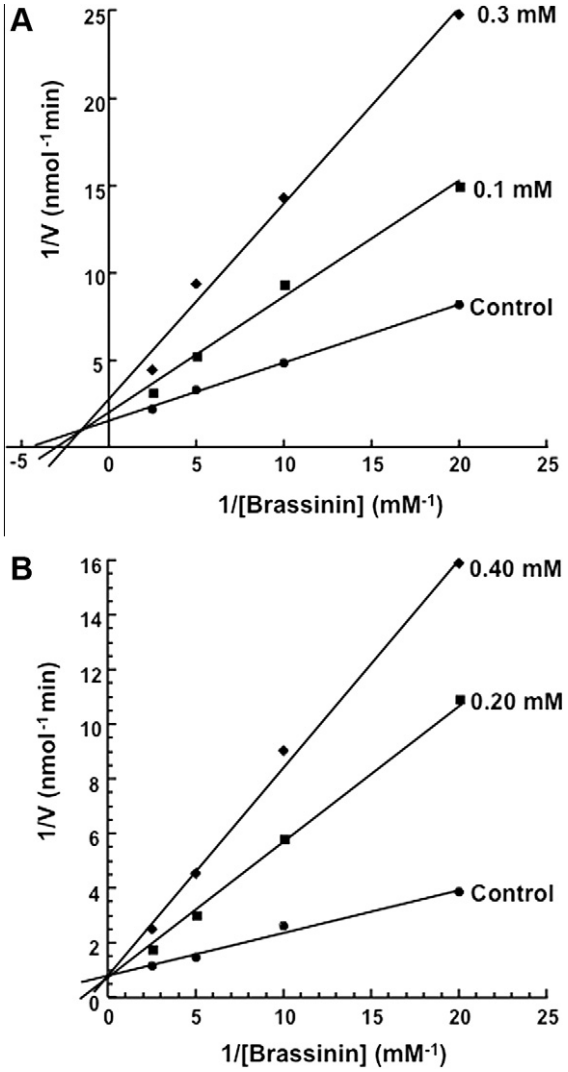


Figure 3. Lineweaver–Burk plots of BHAb activity. In the presence of (A) methyl naphthalen-1-yl-methylcarbonate (**19**) and (B) methyl naphthalen-2-yl-methylcarbonate (**20**).

2.5. Enzymatic hydrolysis of naphthalenylcarbonates 19 and 20

During purification of BHAb and substrate specificity assays, it was observed that semi-purified enzymatic fractions of BHAb hydrolyzed methyl naphthalen-1-yl-methylcarbonate (**19**) and methylnaphthalen-2-yl-methylcarbonate (**20**), but did not hydrolyze the corresponding dithiocarbamates **15** and **16**, nor carbamates **17** or **18**. By contrast, mycelial cultures of *A. brassicicola* biotransformed compounds **15**–**20** to the corresponding amines or alcohols (Table S1). These results suggested the presence of enzymes other than BHAb in protein fractions obtained from mycelia. To determine the presence of enzyme(s) with carbonate or carbamate specificities, the cell-free extracts of the *A. brassicicola* mycelia were fractionated by size exclusion chromatography on a Superdex 200 column and protein fractions were analyzed for hydrolytic activity using carbonates **19** and **20** and brassinin (**1**). The results obtained are given in Figure S1, which showed a single peak of enzymatic activity using brassinin (**1**) as substrate (ca. 120 kDa) and at least two peaks with activity (ca. 150 and 350 kDa) obtained for methyl naphthalen-1-yl-methylcarbonate (**19**) (Fig. S1). That is, these results indicated the presence of different isoforms of hydrolytic enzymes with activity against naphthalenylcarbonates, but not against carbamates or dithiocarbamates.

3. Conclusion

In previous work, among six natural plant defense products, only cyclobrassinin (**8**) was found to inhibit competitively BHAb[1]; in the work reported in this manuscript, among the 34 substrate inspired compounds screened for the first time for substrate and inhibitory activity of BHAb, six displayed inhibitory activity (**12**, **14**, **14a**, **17**, **19** and **20**), but only two were found to be substrates (**13** and **15**) of BHAb. Methyl naphthalen-1-yl-methylcarbamate (**17**), the strongest inhibitor of the six, and methyl *N*′-(1-methyl-3-indolylmethyl)carbamate (**14a**) inhibited BHAb through a reversible noncompetitive mechanism. Most importantly, these carbamate inhibitors **14a** and **17** were resistant to degradation by *A. brassicicola* (Table S1). Hence, these compounds are excellent leads to design new paldoxins that inhibit the detoxification of brassinin (**1**) by *A. brassicicola*. By contrast, although both carbonates **19** and **20** inhibited BHAb, since these were quickly degraded (Table 1 and S1) in cultures of *A. brassicicola*, their potential use as lead structures to design paldoxins is of less interest. Further work with additional fungal species needs to be carried out to determine if the presence of carbonate hydrolases is common among plant pathogens.

Fungicides containing dithiocarbamate and carbamate groups have been used for decades to protect crops.[21] While dithiocarbamates have multisite targets, carbamates like methyl benzimida zol-2-yl-carbamate and other benzimidazole carbamates have been shown to bind to β-tubulin assembly in mitosis.[22] Carboxylesterases (CES, EC 3.1.1.1) are multifunctional enzymes that catalyze the hydrolysis of substrates containing ester, amide, thioester, or carbamate groups and have a broad substrate specificity in mammalian systems, partly attributed to a large active site that allows entry of structurally diverse substrates including endobiotics and xenobiotics.[23] However, some carbamate hydrolases appear to have high substrate specificity.[24,25] Considering that compounds **15**–**20** were transformed in cultures of *A. brassicicola*, it is likely that, in addition to brassinin hydrolase, CES with broad substrate range were also produced. For this reason, it was puzzling to find that carbamates **14**/**14a** were not transformed in culture. Further work with other plant pathogens will be carried out to establish the scope of this finding.

Due to sequence homology and presence of the Ser/Ser/Lys triad, BHAb is placed in the amidase signature superfamily of serine hydrolases. These serine hydrolases include the well-studied fatty acid amide hydrolase (FAAH) from mammalian systems. Synthetic FAAH inhibitors that use a covalent irreversible mechanism, as well as reversible FAAH inhibitors have been reported, including *N*-aryl carbamates that act as reversible noncompetitive inhibitors of FAAH.[26–28] However, despite the number of studies using FAAH, the role of the carbamate group remains unclear in several cases. Similarly, although our carbamates **14a** and **17** acted as reversible noncompetitive inhibitors of BHAb, since carbamate **18** displayed no effect, it is not clear which additional structural motifs of **14a** and **17** contributed to their inhibitory effect.

Overall, our results indicated that the catalytic and potential inhibitory sites of BHAb are highly selective in the interactions with different compounds. This is a very desirable feature from the perspective of designing selective inhibitors of BHAb. However, to carry out BHAb structure-based design of inhibitors other than substrate inspired inhibitors, it is important to determine protein–substrate and protein–inhibitor structures. Because BHAb is produced in very small amounts, is somewhat unstable, and no heterologous expression systems are available to obtain recombinant protein, crystallization and X-ray diffractometry studies using BHAb are virtually impossible. Hence, the development of effective expression systems for BHAb and other phytoalexin detoxifying enzymes will be essential to design paldoxins that protect crucifers

against black spot and other major fungal diseases of economically important crops.

4. Experimental

4.1. Materials and general procedures

Chemicals 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. All compounds were characterized using NMR spectral data (recorded on Bruker 500 MHz spectrometers) and HRMS-EI data.

HPLC analyses were carried out with Agilent systems equipped with a quaternary pump, an automatic injector, a photodiode array detector (wavelength range 190–600 nm) and a degasser. Elution method A: an Eclipse XDB-C18 column (5 μm particle size silica, 150 mm × 4.6 i.d.), mobile phase linear gradient of H_2O–CH_3OH (50:50 to 0:100) for 25.0 min and a flow rate 0.75 mL/min; elution method B (for detection of amines) a Zorbax SB C18 column (3.5 μm particle size silica, 100 × 3 mm i.d.), a linear gradient of H_2O–CH_3OH (each solvent containing 0.01% *n*-propylamine, 40:60 to 0:100), for 10 min and a flow rate of 0.5 mL/min.

High-resolution mass spectral (HRMS) data were obtained using an Agilent HPLC 1100 series directly connected to a QSTAR XL 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 H_2O–CH_3CN (each solvent containing 0.1% formic acid, 75:25 to 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 using a VG 70 SE mass spectrometer using a solids probe.

4.2. Synthesis

Compounds **1**,[13] **1a**, **12**,[6] **13**,[6] and **14**–**16**[13] were prepared as previously reported, and their purity determined to be ⩾98% by HPLC and ^{1}H NMR.

4.2.1. Methyl *N*-[(1-naphthalenyl)methyl]carbamate (17)

Methyl chloroformate (58 μL, 0.73 mmol) and triethylamine (183 μL, 1.3 mmol) were added to a solution of 1-naphthalenylmethanamine (**21**, 100 mg, 0.64 mmol) in CH_2Cl_2 (5 mL), and the reaction mixture was stirred at rt for 2 h. The reaction mixture was diluted with water, extracted with EtOAc, 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-naphthalenylmethyl)carbamate (**17**) as a white solid (134 mg, 0.62 mmol, 97% yield; mp 74–75 °C). ^{1}H NMR (500.3 MHz, CD_3OD): (two rotamers 0.2:0.8) δ 8.09 (d, J = 8.2 Hz, 0.2H), 8.04 (d, J = 8.2 Hz, 0.8H), 7.84 (d, J = 7.5 Hz, 1H), 7.81–7.75 (m, 1H), 7.53–7.38 (m, 4H), 5.06 (s, 0.4H), 4.72 (s, 1.6), 3.65 (br s, 3H).[14] ^{13}C NMR (125.8 MHz, CD_3OD): (two rotamers) δ 159.6, 138.1, 135.6, 135.4, 132.8, 132.7, 129.8, 129.7, 129.2, 129.1, 127.4, 127.2, 126.9, 126.8, 126.5, 126.4, 126.2, 124.8, 124.4, 63.5, 52.7, 43.6. HRMS-EI: *m/z* (%) 215.0950 (15) (215.0946 calcd for $C_{13}H_{13}NO_2$), 158 (60), 141 (21), 129 (100).

4.2.2. Methyl *N*-[(2-naphthalenyl)methyl]carbamate (18)

As reported above for **17**, but using 2-naphthalenylmethanamine (**22**, 91 mg, 0.58 mmol). Purification of the crude product (SiO_2, EtOAc–hexanes) afforded methyl 2-naphthalenylmethyl)carbamate (**18**) as a white solid (120 mg, 0.56 mmol, 96% yield; mp 110–111 °C). ^{1}H NMR (500.3 MHz, $CDCl_3$): δ 7.83 (m, 3H), 7.72 (s, 1H), 7.50 (m, 2H), 7.41 (m, 1H), 5.16 (br s, NH), 3.74 (s, 3H). ^{13}C NMR (125.8 MHz, CD_3CN): δ 157.3, 136.1, 133.5, 133.0, 128.7, 127.9, 127.8, 126.4, 126.2, 126.1, 125.8, 52.4, 45.4. HRMS-EI: *m/z* (%) 215.0943 (100) (215.0946 calcd for $C_{13}H_{13}NO_2$), 200 (62), 156 (40), 141 (41), 129 (46).

4.2.3. Methyl (1-naphthalenylmethyl)carbonate (19)

1-Naphthalenylmethanol (**23**, 100 mg, 0.63 mmol) in THF (1 mL) was added to a cold solution of NaH (75 mg suspension in oil, 1.9 mmol) in THF (3 mL), the reaction mixture was stirred at 0 °C for 30 min, and then methyl chloroformate (94.5 μL, 0.82 mmol) was added. After stirring for 5 h at rt, the reaction mixture was cooled to 0 °C, diluted with water, the solvent layer separated and the aqueous reextracted with EtOAc, 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-naphthalenylmethyl)carbamate (**19**) as a white semi-solid (109 mg, 0.50 mmol, 80% yield). ^{1}H NMR (500.3 MHz, $CDCl_3$): δ 8.10 (m, 1.6H), 7.90 (m, 3.4H), 7.63–7.45 (m, 6.4H), 5.70 (s, 0.6H), 5.68 (s, 2H), 3.86 (s, 0.6H), 3.84 (s, 3H). ^{13}C NMR (125.8 MHz, $CDCl_3$): δ 155.8, 133.7, 131.6, 130.8, 129.6, 128.7, 127.7, 126.8, 126.0, 125.2, 123.5, 68.1, 54.9. HRMS-EI: *m/z* measured 216.0792 (216.0786 calcd for $C_{13}H_{12}O_3$).

4.2.4. Methyl (2-naphthalenylmethyl)carbonate (20)

As reported above for **19**, but using 2-naphthalenylmethanol (**24**, 100 mg, 0.63 mmol). Purification of the crude product (SiO_2, EtOAc–hexanes) afforded methyl 2-naphthalenylmethyl)carbamate (**20**) as a white solid (34 mg, 0.16 mmol, 51% yield based on recovered starting material, 50 mg; mp 51–52 °C). ^{1}H NMR (500.3 MHz, CD_3CN): δ 7.86 (m, 4H), 7.50 (m, 3H), 5.35 (s, 2H), 3.83 (s, 3H). ^{13}C NMR (125.8 MHz, CD_3CN): δ 156.0, 133.4, 133.3, 132.8, 128.6, 128.2, 127.9, 127.7, 126.6, 126.5, 125.9, 70.0, 55.1. HRMS-EI: *m/z* measured 216.0781 (216.0786 calcd for $C_{13}H_{12}O_3$).

4.3. Antifungal bioassays and biotransformations of compounds 12–20 by *Alternaria brassicicola*

Compounds **12**–**20** were used in antifungal bioassays, carried out as previously described.[3] In brief, 7-day-old cultures of *A. brassicicola* on PDA under constant light at 23 ± 1 °C were used for mycelial radial growth assays. Plugs (4 mm) were cut from the edges of mycelia and placed inverted onto 12-well agar plates amended with test compounds (dissolved in DMSO). The final concentrations of each compound in agar varied from 0.10 to 0.50 mM, with a DMSO concentration of 1%. The plates were allowed to grow under constant light at 23 ± 1 °C; the diameter of the mycelial mat was measured after 96 h and compared to control cultures grown on plates containing DMSO only.

Liquid cultures of each pathogen were grown in 250 ml Erlenmeyer flasks containing 100 mL of minimal medium inoculated fungal spores for a final concentration of 1×10^6/100 mL. After 48 h at 23 ± 1 °C, under constant light a solution of each compound (**12**–**20**) in CH_3CN (100–250 μL depending on solubility) was added to the cultures, for a final concentration of 0.10 mM. Similar control solutions containing compounds only or mycelia were prepared. The flasks were returned to the shaker, samples (5–10 mL) were withdrawn at various times and immediately frozen or extracted with EtOAc. The extracts were analyzed by HPLC using elution method A for compounds **12**–**20**.

4.4. Isolation, chromatographic purification and activity assay of BHAb

A. brassicicola (isolate ATCC 96866) spores and liquid cultures were obtained under the conditions described previously.[1] For

purification of BHAb, 0.6 L of liquid cultures were induced with 3-phenylindole (0.20 mM). The cultures were incubated for an additional 24 h and then gravity filtered to separate mycelia from culture broth. Frozen mycelia (5 g) obtained from *A. brassicicola* (0.6 L) were suspended in ice-cold extraction buffer (10 mL) and ground (mortar) for 5 min. The extraction buffer consisted of 25 mM diethanolamine (DEA), pH 8.3, 5% (v/v) glycerol, 1 mM EDTA and 1:200 (v/v) protease inhibitor cocktail (P-8215, Sigma). The suspension was centrifuged for 60 min at 58,000*g* and the resulting supernatant (10 mL) was used for chromatographic separation, as previously described.[1] Protein concentrations were determined as described by Bradford using the Coomassie Brilliant Blue method with bovine serum albumin (BSA) as a standard.

The reaction mixture contained 20 mM DEA (pH 8.3), 0.1% Triton X-100, 0.05–1.0 mM brassinin, (in 5 μL acetonitrile), and 50–100 μL of purified protein in a total volume of 1.0 mL. The reaction was carried out at 23 °C for 30 min and was stopped by the addition of 0.1 mL solution of 30% ammonium sulfate (w/v) and 15% ammonium hydroxide (w/v) as previously described.[1] The reaction assays were extracted with 2 mL of chloroform–methanol (95:5, v/v) and concentrated to dryness in a rotary evaporator. Extracts were dissolved in methanol (200 μL) and analyzed by HPLC using elution method B. The quantification was carried out using a calibration curve of indolyl-3-methanamine.

4.5. Inhibitory effects of compounds 12–20

To determine the inhibitors of BHAb, experiments were carried out using brassinin at 0.10 mM (substrate) in the presence of 0.10 and 0.30 mM of each test compound. The reaction was initiated by addition of purified BHAb. The standard deviations for each assay were determined from four independent experiments. To determine the type of inhibition, experiments were carried using brassinin at 0.10–0.40 mM in the presence of 0.050–0.40 mM of each inhibitor (methyl *N*′-(1-methyl-3-indolylmethyl)carbamate (**14a**) and methyl naphthalen-1-yl-methylcarbamate (**17**)). The kinetics of inhibition was transformed into Lineweaver–Burk double reciprocal plots ($1/S$ vs $1/V$). To determine K_i for a noncompetitive inhibitor, a secondary plot was constructed using the apparent $1/V$ values obtained in presence and absence inhibitors versus inhibitor concentration. K_i for a competitive inhibitor was calculated on the basis of apparent K_m values obtained in presence and absence of inhibitors versus inhibitor concentration. For both types of inhibition, noncompetitive and competitive, linear regression was used to determine *x*-axis intercept value which is equal to negative K_i. The K_i value for the mixed type inhibitor was determined from Dixon plots of $1/V$ versus $[I]$.

4.6. Inhibitory effects of serine modifying reagents

Inhibitory studies of purified enzyme were done in 100 mM phosphate buffer (pH 7.5) using brassinin 0.10 mM (substrate) in presence of 0.50–1.0 mM of compound as phenylmethanesulfonyl fluoride (PMSF), 4-(2-aminoethyl)benzene sulfonyl fluoride (Pefabloc) and phenylphosphorodiamide.

4.7. Mechanism-based inactivation studies

The inactivation of BHAb was studied at 23 °C in buffer solutions containing 20 mM DEA (pH 8.3), 0.1% Triton X-100, 0.50 mM brassinin, 100 μL of purified protein and various concentrations of methyl *N*′-(1-methyl-3-indolylmethyl)carbamate (**14a**) or methyl naphthalen-1-yl-methylcarbamate (**17**) (3.0 mL final volume). Portions (400 μL) were removed at suitable time intervals (5, 10, 15, 20 and 25 min), the reaction was stopped and the residual enzymatic activities were determined. Additionally, inactivation studies were performed by preincubation of the purified enzyme with 0.30 mM methyl (1-methyl-3-indolylmethyl)carbamate (**14a**) or 0.10 mM methylnaphthalen-1-yl-methylcarbamate (**17**) at room temperature for 20 min (Fig. 2, **17** is a stronger inhibitor than **14a**). Samples were then placed in the Slide-A-Lyzer membranes (molecular weight cutoff, 3500, Pierce Chemical) and dialyzed tree times for 2 h at 4 °C against 1 L of a buffer containing 20 mM DEA (pH 8.3), 0.1% Triton X-100, and 2% glycerol. The dialyzed samples were collected and BHAb activity was determined as described above. Control incubations lacked inhibitors and the samples were compared with the controls. In separate experiments, BHAb activities were determined in the presence of inhibitor before and after dialysis.

4.8. Gel filtration on Superdex 200

The soluble protein extract from mycelia (0.3 ml, ca. 1 mg) was subjected to gel filtration on Superdex 200 (1 × 40 cm column dimension). Proteins were eluted with 25 mM DEA buffer pH 8.3 containing 0.015% Triton X-100, 1% glycerol (v/v), and 50 mM NaCl. The column was calibrated with protein markers consisting of blue dextran (2000 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa) and carbonic anhydrase (29 kDa). Fractions of 0.4 ml were collected at a flow rate of 0.4 mL/min and used for testing the enzyme activity of different substrates.

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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.2011.11.009.

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