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# Design, synthesis, and evaluation of potential inhibitors of brassinin glucosyltransferase, a phytoalexin detoxifying enzyme from *Sclerotinia sclerotiorum*

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**Abstract**—*Sclerotinia sclerotiorum* is a fungal pathogen, which causes stem rot in crucifer crops and in several other plant families resulting in enormous yield losses all over the world. Brassinin is a phytoalexin produced by crucifer plants as part of a general defense mechanism against pathogens and other forms of stress. To the great detriment of crucifers, some fungal pathogens, as for example *S. sclerotiorum*, can detoxify brassinin. Detoxification of brassinin via glucosylation of the indole nitrogen is carried out by an inducible glucosyltransferase produced in *S. sclerotiorum*. Because brassinin is a precursor of several phytoalexins active against *S. sclerotiorum*, brassinin glucosyltransferase (BGT) is a potentially useful metabolic target to control *S. sclerotiorum*. Toward this end, we have designed, synthesized, and screened several brassinin analogues using both mycelial cultures and cell-free homogenates of *S. sclerotiorum*. A noticeable decrease in the rate of brassinin detoxification in cell cultures was observed in the presence of methyl (benzofuran-3-yl)methyldithiocarbamate, methyl (benzofuran-2-yl)methyldithiocarbamate, methyl (indol-2-yl)methyldithiocarbamate, 3-phenylindole, 6-fluoro-3-phenylindole, and 5-fluorocamalexin. In addition, these compounds caused substantial inhibition of BGT activity (ca. 80%) in cell-free homogenates of *S. sclerotiorum*, while only brassinin and 3-phenylindole were transformed to the corresponding β-D-1-glucopyranosyl products. These results indicate that, although many other glucosyltransferases appear to be produced by *S. sclerotiorum* in cell cultures, BGT is substrate specific. Overall these results show that selective and potent inhibitors of BGT can be developed.


## 1. Introduction

Metabolic transformations of exogenous organic molecules to generate less toxic products take place in virtually every living organism. Glucosylation of such molecules, naturally occurring or synthetic, is a common detoxification reaction in plants and is well documented to occur in the metabolism of pesticides and other xenobiotics.[1] The first report of a glucosylation occurring in a plant pathogenic fungus, *Sclerotinia sclerotiorum* (Lib.) de Bary, examined the detoxification of a crucifer phytoalexin.[2] Although the detoxifications of phytoalexins (plant antimicrobial metabolites produced de novo in response to stress) are common mechanisms of counterattack in plant pathogens, detoxifications via glucosylation are unusual in fungi.[3] The fungus *S. sclerotiorum* causes stem rot in crucifer crops [canola (*Brassica napus* and *Brassica rapa*), rapeseed (*B. napus* and *B. rapa*), and mustard (*Brassica juncea*)] and in several other plant families, causing enormous yield losses all over the world.[4] The lack of crucifer oilseeds resistant to stem rot disease and environmental concerns over the use of fungicides prompted our ongoing search for metabolic targets that could facilitate the selective control of this plant pathogen in crucifer crops.[5] Recent work suggests that *S. sclerotiorum* transforms crucifer phytoalexins via pathways that appear to correlate with the inhibitory activity of the phytoalexin.[6] For example, the phytoalexins brassicanal A (**1**) and spirobrassinins **4** and **5**, that display low inhibitory activity against *S. sclerotiorum*, were metabolized over a period of several days via oxidation and hydrolysis, respectively (Scheme 1). By contrast, the transformation of more potent phytoalexins like brassinin (**9**), brassilexin (**11**), sinalexin (**12**), and camalexin (**15**) was substantially faster (less than 2 days) and involved glucosylation (Scheme 2). Furthermore,

*Keywords*: Brassinin; *Sclerotinia sclerotiorum*; Detoxifying enzyme; Brassinin glucosyltransferase; Phytoalexin; Dithiocarbamate; Antifungal; Paldoxin.
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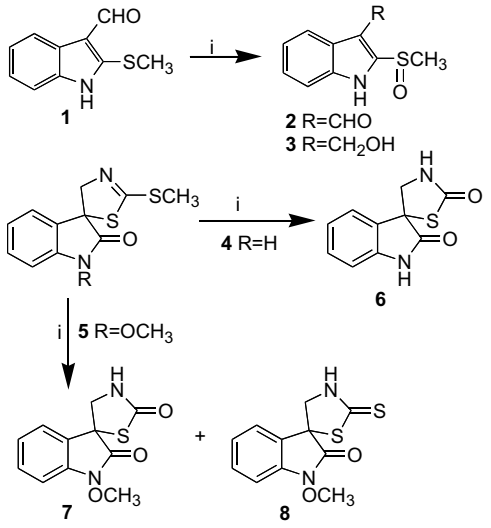
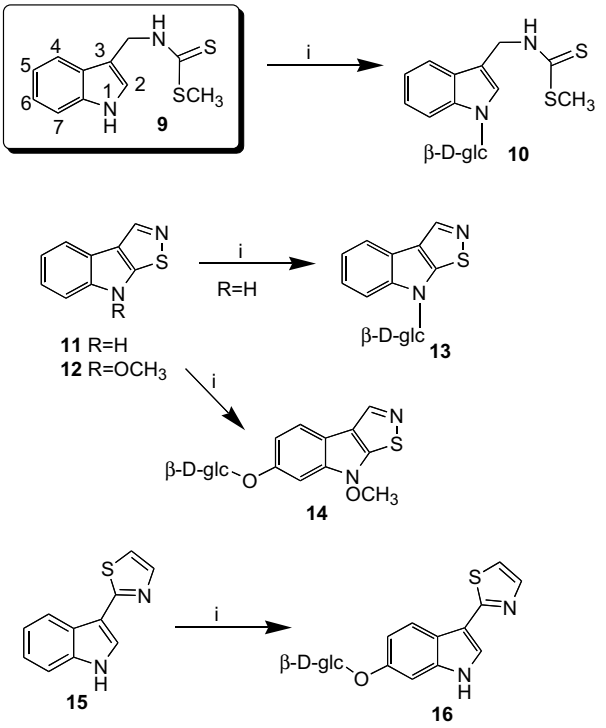



**Scheme 1.** Detoxification of the phytoalexins brassicanal A (**1**), spirobrassinin (**4**), and methoxyspirobrassinin (**5**); (i) *Sclerotinia sclerotiorum*.

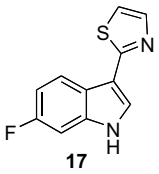



**Scheme 2.** Detoxification of the phytoalexins brassinin (**9**), brassilexin (**11**), sinalexin (**12**), and camalexin (**15**); (i) *Sclerotinia sclerotiorum*.

replacement of the methoxy group of sinalexin (**12**) with a methyl group (at *N*-1) decreased the rate and the site of transformation. Overall, these reports suggest that *S. sclerotiorum* may have acquired or evolved efficient glucosyltransferase(s) that can detoxify some of the most active plant chemical defenses.[6] In addition, the apparent substrate specificity of the glucosyltransferase(s) indicates that these glucosylation reactions may be potential metabolic targets to control *S. sclerotiorum*.[5] Toward this end, because brassinin (**9**) is a biosynthetic precursor of several other phytoalexins, the inhibition of brassinin glucosyltransferase(s) is our first target to generate crop protection agents against *S. sclerotiorum*. We have designed, synthesized, and screened potential inhibitors of brassinin glucosyltransferase using both mycelial cultures and cell-free homogenates of *S. sclerotiorum*, and herein we report the results of these studies.

## 2. Results and discussion

### 2.1. Design and synthesis of potential inhibitors




Previous work[5] established that brassinin glucosyltransferase (BGT) activity observed in cell-free homogenates of *S. sclerotiorum* was inducible by brassinin (**9**), camalexin (**15**) or related compounds. In addition, camalexin (**15**) and 6-fluorocamalexin (**17**) decreased the rate of transformation of brassinin (**9**) to the glucosyl product **10** in cell cultures as well as in cell-free homogenates. Neither **15** nor **17** were transformed in cell-free homogenates of *S. sclerotiorum*. Based on these results, two groups of compounds were designed to probe the structural requirements for BGT inhibition: (i) one group was based on the structure of the substrate brassinin (**9**) and (ii) another group was based on the structure of camalexin (**15**) (Fig. 1). As BGT appeared to be somewhat specific, it was anticipated that replacing the reactive *N*-1 of the indolyl moiety of brassinin (**9**) with oxygen or sulfur, compounds **18** and **19**, respectively, could generate competitive inhibitors of BGT. Changing the position of the side chain of brassinin (**9**) from C-3 to C-2 to yield compounds **22** and **23** would probe the substrate specificity of BGT since the bulkier substituent at C-2 in **22** or **23** could hinder glucosylation at *N*-1. Compounds **20** and **21** were designed to replace the C-7 or C-7a carbons in brassinin (**9**) with nitrogen to probe the effect of the indole ring. By analogy to **21**, compound **24** was designed to evaluate the contribution of the dithiocarbamate group. Compound **25** was devised to evaluate the quinoline inhibitory effect on BGT and its antifungal activity against *S. sclerotiorum*. Compounds **27**–**29** were designed to probe the effect of the thiazole ring of camalexins. Furthermore, because 3-phenylindole (**27**) was glucosylated at *N*-1, 3-phenylbenzofuran (**30**) was devised to prevent this transformation, whereas 2-phenylindole (**31**) was designed to evaluate the potential hindrance caused a bulky C-2 substituent in the indole nucleus. Thiabendazole (**32**) is a commercial fungicide that was used as a standard to compare with the antifungal activities of both brassinin (**9**) and camalexin (**15**). Among the designed inhibitors (Fig. 1), the synthesis of **22** and **24**–**27** is known[7–10] and compounds **31** and **32** are commercially available.

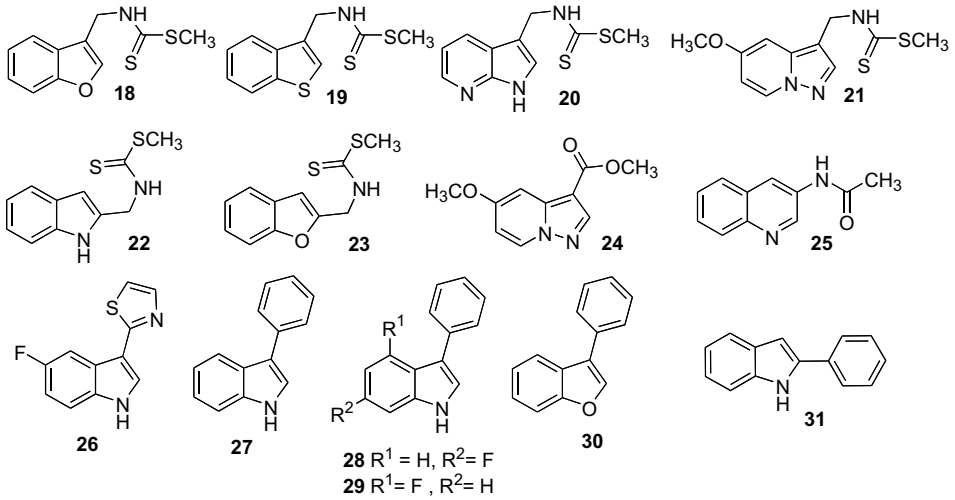
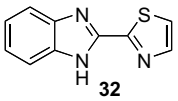



**Figure 1.** Potential brassinin detoxification inhibitors.

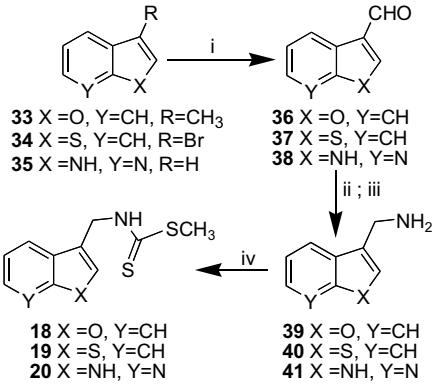
New conditions for the synthesis of compound **30** and the first synthesis of compounds **18**–**21**, **23**, **28**, and **29** are described below.



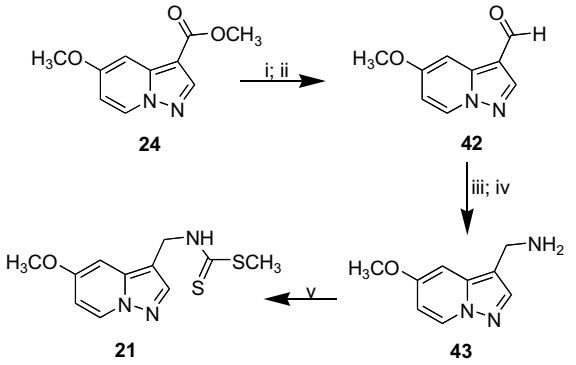

The synthesis of dithiocarbamates **18**–**21** was accomplished using aldehydes **36**–**38** and **42**, respectively, as reported earlier for brassinin.[11] Benzofuran-3-carboxaldehyde (**36**) was obtained by oxidation of 3-methylbenzofuran (**33**) with $SeO_2$ in a yield similar to that reported previously.[12] Thianaphthene-3-carboxaldehyde (**37**) was previously prepared from 3-bromothianaphthene (**34**) by reacting with $CO/H_2$ in the presence of palladium (II) acetate and di-1-adamantyl-*n*-butylphosphane.[13] We synthesized **37** by converting 3-bromothianaphthene (**34**) to thianaphthene-3-carboxaldehyde (**37**) by lithiation with *tert*-butyllithium followed by addition of dimethylformamide and water.[14] The preparation of aldehyde **38** was accomplished through Vilsmeier formylation of 7-azaindole (**35**) and benzofuran, respectively, in good yields.[15] Previous work reported that the reaction of 7-azaindole (**35**) with an equimolar amount of $POCl_3$/DMF at 80 °C yielded 7-azaindole-3-carboxaldehyde (**38**) in 50% yield.[15a] Application of this procedure yielded a mixture of 1-formyl-7-azaindole (yield 8%) and **38** (yield 10%) along with recovered starting material (yield 50%). Eventually, the preparation of aldehyde **38** was achieved in ca. 50% yield using 10 equivalents of $POCl_3$/DMF and refluxing the reaction mixture. Aldehyde **42** was obtained in 65% yield by reducing ester **24** to the corresponding alcohol with $LiAlH_4$, followed by oxidation with $MnO_2$ (Scheme 4). Aldehydes **36**–**38**, **42**, and **44** were converted to their corresponding dithiocarbamates **18**–**21** and **23** by reduction of the corresponding oximes to amines **39**–**41**, **43** (Schemes 3 and 4) and benzofuran-2-methanamine (Scheme 5).

6-Fluoro-3-phenylindole (**28**) and 4-fluoro-3-phenylindole (**29**) were synthesized using the Fischer indole synthesis, as reported earlier for 3-phenylindole (**27**).[10] Reaction of 3-fluorophenylhydrazine (**46**) with phenylacetaldehyde (**47**) at 100 °C afforded the corresponding phenylhydrazone, which upon treatment with $ZnCl_2$ in ethanol afforded a mixture of 6-fluoro-3-phenylindole (**28**) and 4-fluoro-3-phenylindole (**29**) (Scheme 6).

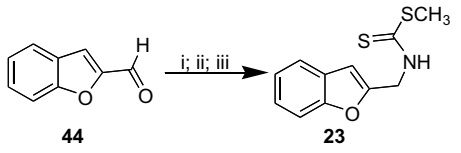
3-Phenylbenzofuran (**30**) was previously synthesized from α-phenoxyacetophenone by heating in polyphosphoric acid,[16] whereas a milder process[17] was reported for the synthesis of substituted 2-methylbenzofurans from 2-allylphenols. A similar method was applied to synthesize 3-phenylbenzofuran (**30**) from *o*-(1-phenylvinyl)phenol (**49**). Compound **49** was obtained from commercially available 22-hydroxyacetophenone (**48**), upon reaction with the Grignard reagent prepared from bromobenzene and magnesium followed by dehydration.[18] The product *o*-(1-phenylvinyl)phenol (**49**) was converted to 3-phenylbenzofuran (**30**), albeit in a rather



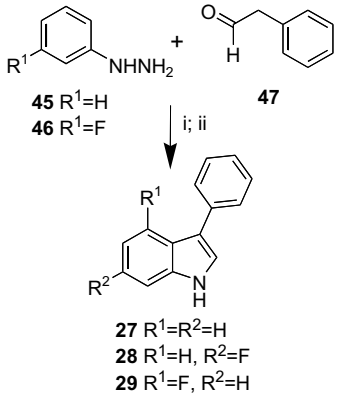

**Scheme 3.** Synthesis of dithiocarbamates **18**–**20**. Reagents and conditions: (i) for **33**, $SeO_2$, 1,4-dioxane, 105 °C; for **34**, *t*-BuLi, $Et_2O$, DMF; for **35**, $POCl_3$, DMF, 105 °C; (ii) $NH_2OH{\cdot}HCl$, $Na_2CO_3$, $EtOH/H_2O$, 90 °C; (iii) For **36** and **37**, $Na(CN)BH_3$, $NH_4OAc$, $TiCl_3$, MeOH; for **38**, Zn, HCl; (iv) Py, $Et_3N$, $CS_2$, $CH_3I$.



**Scheme 4.** Synthesis of methyl (5-methoxypyrazolo[1,5-*a*]pyridin-3-yl)methyldithiocarbamate (**21**). Reagents and conditions: (i) $LiAlH_4$, THF, 0 °C; (ii) $MnO_2$, $CH_2Cl_2$, 65%; (iii) $NH_2OH{\cdot}HCl$, $Na_2CO_3$, $EtOH/H_2O$, 90 °C, 91%; (iv) Zn, HCl; (v) Py, $Et_3N$, $CS_2$, $CH_3I$, 49%.



**Scheme 5.** Synthesis of dithiocarbamate **23**. Reagents and condition: (i) $NH_2OH{\cdot}HCl$, $Na_2CO_3$, $EtOH/H_2O$, 90 °C; (ii) $Na(CN)BH_3$, $NH_4OAc$, $TiCl_3$, MeOH; (iii) Py, $Et_3N$, $CS_2$, $CH_3I$.

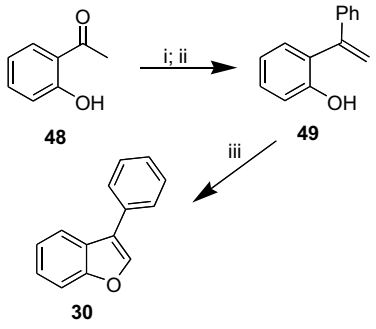


**Scheme 6.** Synthesis of 3-phenylindoles **27**–**29**. Reagents and conditions: (i) 100 °C, 1 h; (ii) $ZnCl_2$, EtOH, 100 °C.

poor yield (10%), using Pd (II)-catalyzed oxidative cyclization (Scheme 7).

## 2.2. Antifungal activity

The antifungal activity of all potential brassinin detoxification inhibitors against *S. sclerotiorum* was determined using a mycelial growth antifungal assay, as described in the experimental. As shown in Table 1, dithiocarbamates **22** and **23** showed antifungal activity (complete inhibition at 0.3 mM) similar to the naturally occurring brassinin (**9**), whereas dithiocarbamates **18**–**20** showed lower antifungal activity (45–89% inhibition at 0.50 mM). Dithiocarbamate **21** was not inhibitory at the concentration tested (not soluble in aqueous medium



**Scheme 7.** Synthesis of 3-phenylbenzofuran (**30**). Reagents and conditions: (i) Ph–Br, Mg, THF, 80 °C, 82%; (ii) $I_2$, benzene, 90 °C, 93%; (iii) $Cu(OAc)_2{\cdot}H_2O$, LiCl, $PdCl_2$, $DMF/H_2O$, 100 °C, 10%.

at concentrations $\geqslant$0.10 mM). The ester **24** displayed low antifungal activity (ca. 20% inhibition) and amide **25** displayed no antifungal activity. 5-Fluorocamalexin (**26**) was found to be less antifungal against *S. sclerotiorum* compared to camalexin (**15**) but more inhibitory than 6-fluorocamalexin (**17**). The highest antifungal activity was obtained with 3-phenylindole (**27**) and its analogues **28** and **29**. Although these three compounds were expected to have lower antifungal activity than camalexin (**15**), they were found to have the highest antifungal activity against *S. sclerotiorum* among all tested compounds (including the phytoalexins). Interestingly, 3-phenylindole (**27**) was found to inhibit a variety of fungi including spore germination in *Aspergillus niger* (at 0.26 mM) but this antifungal activity was blocked by phospholipids.[19] It was suggested that **27** binds to phospholipids in the cell membrane and that this interaction in turn affects all membrane-bound reactions.[20] This effect may explain the antifungal activity of compounds **27**–**29** on *S. sclerotiorum*. In addition, 3-phenylbenzofuran (**30**) showed lower toxicity (ca. 80% growth inhibition at 0.5 mM) and 2-phenylindole (**31**) did not show antifungal activity against *S. sclerotiorum* at the concentrations tested (not soluble in aqueous medium at concentrations $\geqslant$0.10 mM). The commercially available fungicide thiabendazole (**32**) was not very toxic to *S. sclerotiorum* (93% inhibition at 0.50 mM).

## 2.3. Metabolism of potential inhibitors of brassinin detoxification in cultures of *S. sclerotiorum*

Time course experiments were conducted with each of the designed compounds to investigate their metabolism in fungal cultures of *S. sclerotiorum*. Results of these experiments showed that all of the compounds were metabolized in fungal cultures within 12 to 72 h. The metabolic products of compounds **15**, **17**, **19**, **22**, and **27** (yielding one or two major metabolites) were isolated and their chemical structures were elucidated, as follows. HPLC analysis of the broth extracts of fungal cultures incubated with camalexins **15** and **17** showed them to be metabolized in ca. 24 and 72 h, respectively. The structures of products were determined as previously reported.[2] HPLC analysis of the broth extracts of fungal cultures incubated with dithiocarbamate **22**

**Table 1.** Percentage of growth inhibition[a] of *Sclerotinia sclerotiorum* incubated with potential brassinin detoxification inhibitors (48 h, constant light)

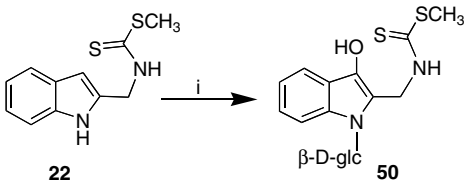
| Compound assayed against *S. sclerotiorum* | Concentration (mM) | Inhibition ± SD[a] (%) |
|---|---|---|
| Brassinin (**9**) | 0.30 | 100 ± 0 |
| | 0.10 | 37 ± 8 |
| Camalexin (**15**) | 0.10 | 100 ± 0 |
| | 0.05 | 81 ± 6 |
| 6-Fluorocamalexin (**17**)[b] | 0.50 | 70 ± 1 |
| | 0.05 | No inhibition |
| Methyl (benzofuran-3-yl)methyldithiocarbamate (**18**) | 0.50 | 45 ± 6 |
| | 0.30 | 23 ± 4 |
| | 0.10 | No inhibition |
| Methyl (thianaphthen-3-yl)methyldithiocarbamate (**19**) | 0.50 | 89 ± 4 |
| | 0.30 | 63 ± 3 |
| | 0.10 | 10 ± 5 |
| Methyl (7-azaindol-3-yl)methyldithiocarbamate (**20**) | 0.50 | 65 ± 5 |
| | 0.30 | 28 ± 4 |
| | 0.10 | No inhibition |
| Methyl (5-methoxypyrazolo[1,5-*a*]pyridin-3-yl)methyldithiocarbamate (**21**) | 0.10 | Not soluble |
| Methyl (indol-2-yl)methyldithiocarbamate (**22**) | 0.30 | 100 ± 0 |
| | 0.10 | 48 ± 2 |
| Methyl (benzofuran-2-yl)methyldithiocarbamate (**23**) | 0.30 | 100 ± 0 |
| | 0.10 | 32 ± 8 |
| Methyl 5-methoxypyrazolo[1,5-*a*]pyridine-3-carboxylate (**24**) | 0.50 | 20 ± 3 |
| | 0.30 | No inhibition |
| 3-(*N*-Acetylamino)quinoline (**25**) | 0.50 | No inhibition |
| 5-Fluorocamalexin (**26**) | 0.30 | 85 ± 3 |
| | 0.10 | 70 ± 4 |
| | 0.05 | 61 ± 2 |
| 3-Phenylindole (**27**) | 0.10 | 100 ± 0 |
| | 0.05 | 93 ± 1 |
| | 0.01 | 78 ± 4 |
| 6-Fluoro-3-phenylindole (**28**) | 0.10 | 93 ± 1 |
| | 0.05 | 87 ± 6 |
| | 0.01 | 65 ± 5 |
| 4-Fluoro-3-phenylindole (**29**) | 0.05 | 100 ± 0 |
| | 0.01 | 47 ± 3 |
| 3-Phenylbenzofuran (**30**) | 0.50 | 80 ± 1 |
| | 0.30 | 64 ± 3 |
| | 0.10 | 32 ± 5 |
| 2-Phenylindole (**31**) | 0.10 | Not soluble |
| Thiabendazole (**32**) | 0.50 | 93 ± 0 |
| | 0.10 | 86 ± 1 |

[a] The percentage of inhibition was calculated using the formula: % inhibition = 100−[(growth on amended/growth in control) × 100].
[b] Data from Ref. 5.

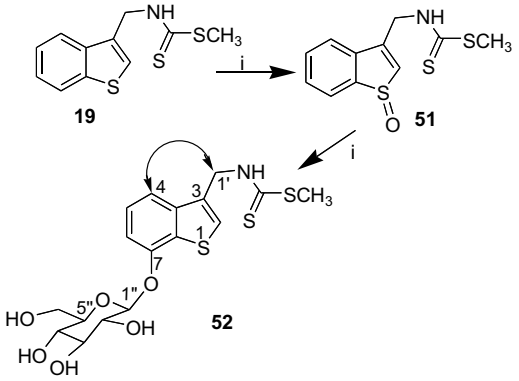
showed it to be metabolized to a major product with HPLC $t_R$ = 9.4 min in ca. 48 h. To establish the structure of this metabolite (**50**), larger scale cultures of *S. sclerotiorum* were incubated with dithiocarbamate **22**, were extracted, and the extract was fractionated by reverse phase silica gel chromatography. The fractions containing the metabolite with $t_R$ = 9.4 min (**50**) were combined and further separated by prep. TLC. The structure of this metabolite (**50**) was determined by analysis of its spectroscopic data as follows. The HRMS-ESI spectral data indicated a molecular formula of $C_{17}H_{22}N_2O_6S_2$. The $^1$H NMR spectrum showed two broad singlets at $\delta_H$ 9.58 and 9.25 ($D_2O$ exchangeable), resonances for an indole system with substitutions at C-2 and C-3 ($\delta_H$ 7.65, d, $J$ = 8 Hz, 1H, 7.37, d, $J$ = 8 Hz, 1H, 7.17, dd, $J$ = 8, 8 Hz, 1H, 7.08, dd, $J$ = 8, 8 Hz, 1H), an intact side chain ($\delta_H$ 5.34, dd, $J$ = 14.5, 6.5 Hz, 1H, 4.84, dd, $J$ = 14.5, 6.5 Hz, 1H, 2.62, $SCH_3$), and resonances for a hexose unit. The identity of the hexose unit was determined to be β-D-glucopyranose from homonuclear ($^1$H–$^1$H) decoupling experiments. The HMBC correlations of the anomeric proton with C-2 and C-7a of indole suggested that the β-D-glucopyranose unit was located at the *N*-1 position

of the indole ring, similar to 1-β-D-glucopyranosylbrassinin (**10**), the metabolite of brassinin (**9**).[5] A downfield shift for C-3 ($\delta_C$ 102.5 in **22** vs 125.7 in **50**) in the $^{13}C$ NMR spectrum suggested that a hydroxyl group was attached to C-3. Thus the structure of this metabolic product was assigned as methyl (1-β-D-glucopyranosyl-3-hydroxylindol-2-yl)methyldithiocarbamate (**50**, Scheme 8).

Dithiocarbamate **19** was administered to cultures of *S. sclerotiorum* and culture samples were withdrawn and analyzed over a period of time. The HPLC chromatograms of extracts of fungal cultures indicated that dithiocarbamate **19** was completely metabolized in ca. 6 h to two main products with $t_R$ = 9.6 and 11.0 min. Subsequently, larger scale cultures of *S. sclerotiorum* were incubated with the dithiocarbamate **19** for 6 h to isolate the metabolite with $t_R$ = 11.0 min (**51**) and for 48 h to isolate the metabolite with $t_R$ = 9.6 min (**52**). After isolation and purification, the structure of each compound was determined by standard spectroscopic methods, including $^1H$ and $^{13}C$ NMR spectroscopy, HMQC, HMBC, and HRMS-ESI. The molecular formula of the less polar metabolite (**51**, $t_R$ = 11.0 min, obtained by HRMS-ESI) indicated the presence of an additional oxygen atom relative to that of dithiocarbamate **19** ($C_{11}H_{11}NOS_3$ vs $C_{11}H_{11}NS_3$). The $^1H$ NMR spectrum of **51** ($CD_3OD$) indicated the presence of five aromatic hydrogens characteristic of a 3-substituted thianaphthene ring system, two additional hydrogens (H-12, AB quartet), and a singlet for a (S)$CH_3$ group. These spectroscopic data suggested that the additional oxygen atom of metabolite **51** was attached to a sulfur atom either at the thianaphthene ring or at the dithiocarbamate group. That the sulfoxide group was present in the thianaphthene ring rather than in the dithiocarbamate group was suggested by the up field chemical shift for H-2 ($\delta_H$ 7.54 in **19** vs 7.01 in **51**) in the $^1H$ NMR. Hence, on the basis of these spectral data, the structure of the less polar metabolite of methyl (thianaphthen-3-yl)methyldithiocarbamate (**19**) was assigned as methyl (thianaphthen-3-yl-1-*S*-oxide)methyldithiocarbamate (**51**, Scheme 9) and was confirmed by synthesis, as described below. The molecular formula of the polar metabolite (**52**, $t_R$ = 9.6 min, $C_{17}H_{21}NO_6S_3$) obtained by HRMS-ESI indicated the presence of a hexose unit, which was corroborated by NMR data. The identity of the hexose unit was determined as β-D-glucopyranose from homonuclear ($^1H$–$^1H$) decoupling experiments. In addition, the $^1H$ NMR spectrum suggested that the β-D-glucopyranose unit was located either at C-4 or C-7. That the β-D-glucopyranose unit was attached to C-7 rather than




**Scheme 8.** Transformation of methyl (indol-2-yl)methyldithiocarbamate (**22**); (i) *Sclerotinia sclerotiorum*.

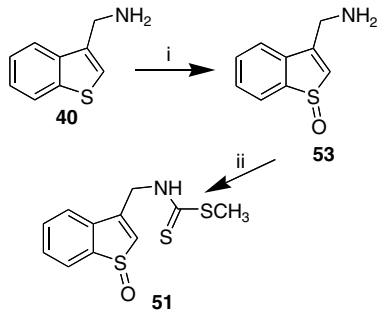



**Scheme 9.** Transformation of methyl (thianaphthen-3-yl)methyldithiocarbamate (**19**); (i) *Sclerotinia sclerotiorum*; double arrows represent selected NOE data.

C-4 was finally deduced from NOE experiments (upon addition of pyridine-$d_5$ to separate the signals due to H-1″ and H-1′), as follows. Irradiation of H-12 at $\delta_H$ 5.15 caused an enhancement of the signal due to H-4 ($\delta_H$ 7.53) and vice-versa (Scheme 9). Hence, on the basis of these spectral data, the structure of the polar metabolite of dithiocarbamate **19** was assigned as methyl (7-*O*-β-D-glucopyranosyl-7-oxythianaphthen-3-yl)methyldithiocarbamate (**52**, Scheme 9). To establish the sequence of biotransformation steps of dithiocarbamate **19**, compound **51** was administered to cultures of *S. sclerotiorum*, culture samples were withdrawn at different times and analyzed by HPLC. This result indicated that dithiocarbamate **19** was metabolized to methyl (7-*O*-β-D-glucopyranosyl-7-oxythianaphthen-3-yl)methyldithiocarbamate (**52**) via methyl (thianaphthen-3-yl-1-*S*-oxide)methyldithiocarbamate (**51**, Scheme 9).

The synthesis of methyl (thianaphthen-3-yl-1-*S*-oxide) methyldithiocarbamate (**51**) was carried out to confirm its structure and to obtain sufficient amounts for bioassay and biotransformation, as follows. Amine **40** was first oxidized to the corresponding sulfoxide **53** using $H_2O_2$ in TFA/$CH_2Cl_2$ (1:2).[21] After work-up and extraction, crude sulfoxide **53** was treated with carbon disulfide in pyridine and triethylamine, followed by iodomethane. This reaction mixture afforded multiple undetermined products, one of which was identified as methyl (thianaphthen-3-yl-1-*S*-oxide)methyldithiocarbamate (**51**) (overall yield 5%, based on **40**, Scheme 10).

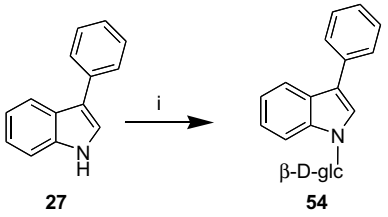
HPLC analysis of extracts of fungal cultures incubated with 3-phenylindole (**27**) indicated it to be completely metabolized to an unknown compound (HPLC $t_R$ = 10.9 min) in about 24 h. To establish the structure of this metabolic product, larger scale cultures of *S. sclerotiorum* incubated with 3-phenylindole (**27**) for 24 h were filtered, extracted, and the EtOAc extract fractionated by reverse phase column chromatography followed by prep. TLC to yield the unknown metabolite (**54**). The structure of this metabolite was determined by analysis of standard spectroscopic methods including $^1H$ and $^{13}C$ NMR spectroscopy, 2D NMR, and HRMS. Comparison of its $^1H$ NMR spectrum with that of 3-phenylindole (**27**) indicated the presence of an intact




**Scheme 10.** Synthesis of methyl (thianaphthen-3-yl-1-*S*-oxide)methyldithiocarbamate (**51**). Reagents: (i) $H_2O_2$, TFA/$CH_2Cl_2$ (1:2); (ii) Py, $Et_3N$, $CS_2$, $CH_3I$, 5%.

3-phenylindole. In addition, several multiplets at $\delta_H$ 3.51–3.98 suggested the presence of a carbohydrate moiety. The molecular formula of **54** ($C_{20}H_{21}NO_5$) determined by $^{13}C$ NMR and HRMS-ESI spectral data also corroborated the presence of a carbohydrate residue. As described above for metabolites **50** and **52**, the identity of the carbohydrate moiety was determined to be a β-glucopyranosyl residue. HMBC spectral data confirmed that the β-glucopyranose unit was located at *N*-1 (correlations of the anomeric proton H-1′ with C-2 and C-7a of indole) and thus the structure of **54** was assigned as 1-β-D-glucopyranosyl-3-phenylindole (Scheme 11).

Metabolites **50**–**52** and **54** were not toxic to *S. sclerotiorum*, that is the metabolism of the parent compounds led to detoxification. Importantly, compared to brassinin (**9**), methyl (indol-2-ylmethyl)dithiocarbamate (**22**) was detoxified at a substantially slower rate (ca. 12 h vs 48 h) in *S. sclerotiorum*. While detoxification of brassinin (**9**) involved *N*-1 glucosylation, the detoxification of its regioisomer **22** took place at two sites, glucosylation at *N*-1 along with oxidation at C-3 (Scheme 8). The detoxification of methyl (thianaphthen-3-ylmethyl)dithiocarbamate (**19**) was found to be faster than that of brassinin (**9**) (6 h vs 12 h). Although **19** was not expected to be transformed, it was quickly metabolized to sulfoxide **51** which was metabolized slowly to methyl (7-*O*-β-glucopyranosyl-7-oxythianaphthen-3-yl)-methyldithiocarbamate (**52**, Scheme 9). Since glucoside **52** was obtained from metabolism of **19** via **51**, it was surprising to observe the absence of the sulfoxide group in **52**. The metabolism of sulfoxide **51** in *S. sclerotiorum* could involve enzymatic O-glucosylation at C-7 followed by immediate reduction of the sulfoxide group (no other metabolic products were detected in cultures).




**Scheme 11.** Transformation of 3-phenylindole (**27**); (i) *Sclerotinia sclerotiorum*.

The detoxification of the strongest antifungal compound (among all the tested compounds), 3-phenylindole (**27**), involved direct N-glucosylation of the indolyl moiety, whereas the structurally related phytoalexin camalexin (**15**) was detoxified to 6-hydroxycamalexin followed by O-glucosylation to 6-*O*-β-D-glucopyranosyl-6-oxycamalexin (**16**) (Scheme 2). By contrast, 6-fluorocamalexin (**17**) was detoxified in *S. sclerotiorum* through direct N-glucosylation of the indole moiety.[2] Although the metabolic product of 5-fluorocamalexin (**26**) could not be characterized fully due to the small amount obtained, the LC–MS data suggested that, similar to 6-fluorocamalexin (**17**), metabolism of **26** occurred through direct N-glucosylation of the indolyl moiety. Previous work on the metabolism of 5-fluorocamalexin (**26**) in *Rhizoctonia solani* showed that the thiazole ring was metabolized to yield 5-fluoroindole-3-carbonitrile as a major product.[9]

The metabolism of other potential inhibitors such as dithiocarbamates **18**, **23** and 3-phenylbenzofuran (**30**) in *S. sclerotiorum* was analyzed by LC–MS as well. The LC–MS data suggested that the metabolism of **18**, **23**, and **30** was completed in about 12–24 h and involved oxidation at an undetermined position followed by O-glucosylation. That is, these results suggest that *S. sclerotiorum* employs different oxidases and/or glucosyltransferases to metabolize these exogenous compounds, regardless of their toxicity (Table 2).

### 2.4. Screening of potential inhibitors of brassinin detoxification in *S. sclerotiorum*

**2.4.1. Mycelial cultures.** Initial experiments using mycelial cultures of *S. sclerotiorum* were conducted to determine the rate of metabolism of brassinin (**9**) at two different concentrations (0.05 and 0.10 mM). It was established that brassinin at 0.05 mM was almost completely metabolized in 6 h whereas at 0.10 mM the complete metabolism of brassinin (**9**) to glucoside **10** occurred in 12 h (Table 2). Subsequently, in screening experiments for inhibitors of brassinin detoxification in mycelial cultures of *S. sclerotiorum*, brassinin (**9**) was used at 0.05 mM, whereas all potential inhibitors were used at two different concentrations (0.05 and 0.10 mM). In a typical screening experiment, brassinin (**9**) and the potential inhibitor were co-incubated in mycelial cultures of *S. sclerotiorum*, samples were withdrawn at different time intervals, extracted with ethyl acetate, and the ethyl acetate extracts were analyzed by HPLC-DAD to determine the amount of brassinin (**9**) transformed. Control cultures of *S. sclerotiorum* containing only brassinin (**9**, 0.05 and 0.10 mM) or each potential inhibitor (0.10 mM) were incubated separately. The stability of brassinin (**9**) and all potential inhibitors was determined by incubation in sterile media under similar conditions. Brassinin (**9**) and all potential inhibitors **18**–**31** were found to be stable in aqueous medium for at least 8 days.

**Table 2.** Biotransformation of brassinin (**9**) and compounds **15**, **17**–**28**, **30**, **31**, and **51** in cultures of *Sclerotinia sclerotiorum*

| Compounds at 0.10 mM | Biotransformation product (time required for complete transformation) |
|---|---|
| Brassinin (**9**) | 1-β-D-Glucopyranosylbrassinin (**10**) (12 h) |
| Camalexin (**15**) | 6-β-D-Glucopyranosyl-6-oxycamalexin (**16**) (24 h) |
| 6-Fluorocamalexin (**17**) | 1-β-D-Glucopyranosyl-6-fluorocamalexin (72 h) |
| Methyl (benzofuran-3-yl)methyldithiocarbamate (**18**) | *O*-Glucosylated product (12 h)[a] |
| Methyl (thianaphthen-3-yl)methyldithiocarbamate (**19**) | Methyl (thianaphthen-3-yl-1-*S*-oxide)methyldithiocarbamate (**51**) (6 h) |
| Methyl (7-azaindol-3-yl)methyldithiocarbamate (**20**) | Complete metabolism to undetermined products (4 d) |
| Methyl (5-methoxypyrazolo[1,5-*a*]pyridin-3-yl)methyldithiocarbamate(**21**) | Complete metabolism to undetermined products (4 d) |
| Methyl (indol-2-yl)methyldithiocarbamate (**22**) | Methyl (1-β-D-glucopyranosyl-3-hydroxylindol-2-yl)methyldithiocarbamate (**50**) (48 h) |
| Methyl (benzofuran-2-yl)methyldithiocarbamate (**23**) | *O*-Glucosylated products (24 h)[a] |
| Methyl-5-methoxypyrazolo[1,5-*a*]pyridine-3-carboxylate (**24**) | Complete metabolism to undetermined products (72 h) |
| 5-Fluorocamalexin (**26**) | 1-β-D-Glucopyranosyl-5-fluorocamalexin (72 h) |
| 3-Phenylindole (**27**) | 1-β-D-Glucopyranosyl-3-phenylindole (**54**) (24 h) |
| 6-Fluoro-3-phenylindole (**28**) | Complete metabolism to undetermined products (24 h) |
| 3-Phenylbenzofuran (**30**) | *O*-Glucosylated products (24 h)[a] |
| 2-Phenylindole (**31**) | Complete metabolism to undetermined products (6 h) |
| Methyl (thianaphthen-3-yl-1-*S*-oxide)methyldithiocarbamate (**51**) | (7-*O*-β-Glucopyranosyl-7-oxythianaphthen-3-yl)methyldithiocarbamate (**52**) (72 h) |

[a] Position of glucosylation undetermined, the structures of metabolites were determined by LC-DAD-MS.

The rate of disappearance of brassinin (**9**) in the presence of potential inhibitors **18**–**31** was compared with that observed in control cultures containing only brassinin at 0.05 and 0.10 mM (HPLC-DAD). Analysis of these results showed that five of the potential inhibitors were able to slow down substantially the rate of metabolism of brassinin (**9**, Figs. 2–6) in mycelial cultures. In the presence of methyl (benzofuran-3-yl)methyldithiocarbamate (**18**, 0.10 mM), brassinin (**9**) was found to be metabolized but at a substantially slower rate ($t_{1/2}$ = 9 h vs 3 h, Fig. 2). Dithiocarbamate **18** was itself metabolized to an undetermined O-glucosylated compound (detected by LC–MS) in the cultures of *S. sclerotiorum* in ca. 12 h. A similar effect was observed when brassinin (**9**) was co-incubated with methyl (benzofuran-2-yl)methyldithiocarbamate (**23**). As shown in Figures 3 and 4, dithiocarbamate **22** (at 0.10 mM) showed a less noticable effect on the rate of metabolism of brassinin than **23** (at 0.10 mM); the concentration of brassinin in cultures incubated with **22** was much lower (<0.01 mM, $t_{1/2}$ = 2 h, Fig. 3) than in cultures incubated

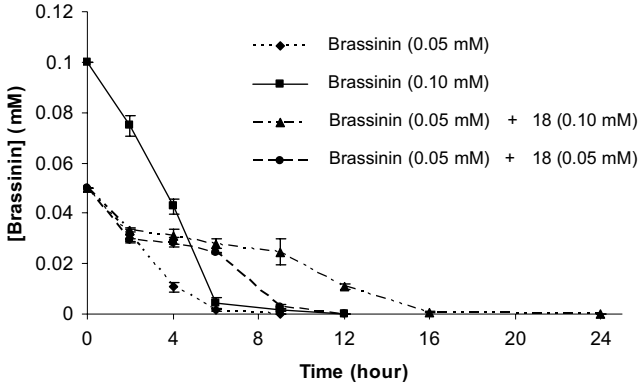



**Figure 2.** Transformation of brassinin (**9**, 0.05 mM) in the presence of methyl (benzofuran-3-yl)methyldithiocarbamate (**18**, 0.05 and 0.10 mM) in cultures of *Sclerotinia sclerotiorum*.

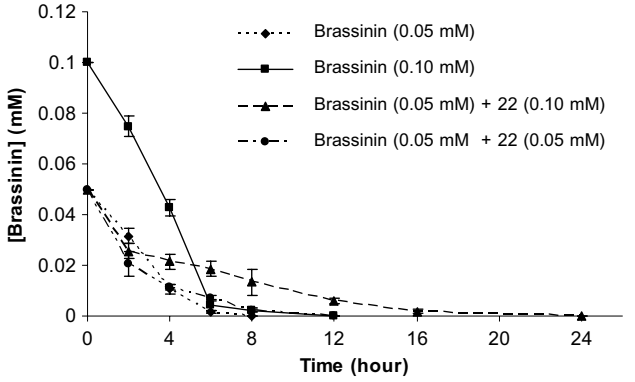



**Figure 3.** Transformation of brassinin (**9**, 0.05 mM) in the presence of methyl (indol-2-yl)methyldithiocarbamate (**22**, 0.05 and 0.10 mM) in cultures of *Sclerotinia sclerotiorum*.

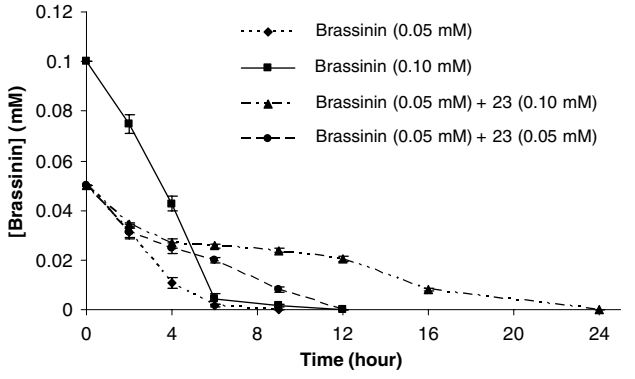



**Figure 4.** Transformation of brassinin (**9**, 0.05 mM) in the presence of methyl (benzofuran-2-yl)methyldithiocarbamate (**23**, 0.05 and 0.10 mM) in cultures of *Sclerotinia sclerotiorum*.

with **23** (0.03 mM, $t_{1/2}$ = 6 h, Fig. 4). Complete metabolism of brassinin (**9**) in the presence of **23** took place in ca. 20 h, a much slower rate than that observed in control cultures (cultures containing only brassinin,

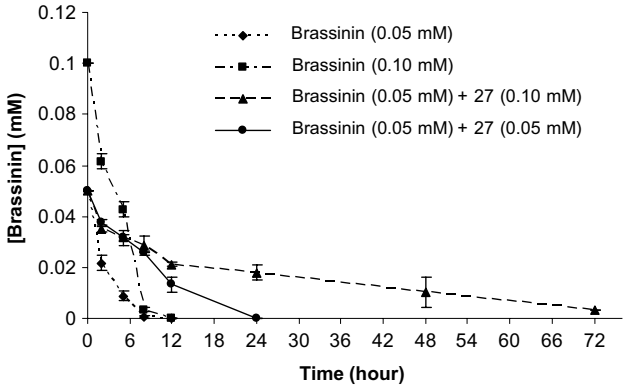



**Figure 5.** Transformation of brassinin (**9**, 0.05 mM) in the presence of 3-phenylindole (**27**, 0.05 and 0.10 mM) in cultures of *Sclerotinia sclerotiorum*.

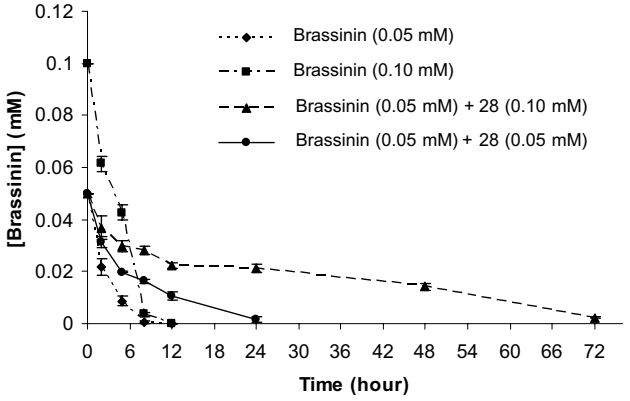



**Figure 6.** Transformation of brassinin (**9**, 0.05 mM) in the presence of 6-fluoro-3-phenylindole (**28**, 0.05 and 0.10 mM) in cultures of *Sclerotinia sclerotiorum*.

0.10 mM, 6 h) of *S. sclerotiorum*. Similar to dithiocarbamate **18**, compound **23** was completely metabolized in cultures of *S. sclerotiorum* to undetermined O-glucosylated products (detected by LC–MS) in 12 h.

Both 3-phenylindoles **27** and **28**, as well as 5-fluorocamalexin (**26**), were able to slow down the rate of metabolism of brassinin (**9**) from 12 to 72 h. The strongest effect was observed when brassinin (**9**) was co-incubated either with 3-phenylindole (**27**, 0.10 mM, $t_{1/2}$ = 10 h, Fig. 5) or with 6-fluoro-3-phenylindole (**28**, 0.10 mM, $t_{1/2}$ = 10 h, Fig. 6). As long as 3-phenylindoles **27** and **28** were present in the cultures, brassinin (**9**) was not metabolized; however, both **27** and **28** were completely metabolized in cultures of *S. sclerotiorum* to **54** and to an undetermined product, respectively, in about 24 h. When camalexin (**15**, 0.10 mM) was co-incubated with brassinin (**9**, 0.05 mM), brassinin was completely metabolized in about 24 h. 5-Fluorocamalexin (**26**) at 0.05 mM slowed down brassinin metabolism to 24 h and at 0.10 mM slowed down metabolism to 48 h (due to the broadness of the HPLC peaks obtained for camalexins, accurate $t_{1/2}$ of **26** could not be determined).

In summary, a noticeable decrease in the rate of brassinin glucosylation in mycelial cultures was observed in the presence of dithiocarbamates **18**, **22** and **23**, 3-phenylindoles **27** and **28**, as shown in Figures. 2–6 and 5-fluorocamalexin (**26**); however, these compounds were found to be metabolized in fungal cultures of *S. sclerotiorum*. The decrease in the rate of detoxification of brassinin (**9**) in the presence of compounds **18**, **22**, **23**, and **26**–**28** could be caused by the inhibition of BGT and/or by the strong inhibitory effect of these compounds on the mycelium growth of *S. sclerotiorum*. The remaining tested compounds **19**–**21**, **24**, **25**, and **30**–**32** did not show a detectable effect on the rate of brassinin metabolism. These results suggested that either compounds **19**–**21**, **24**, **25** or **30**–**32** did not affect BGT or were unable to reach the metabolic site inside the cell. These hypotheses were further investigated by co-incubating the potential inhibitors with mycelial cell-free homogenates containing BGT activity.

**2.4.2. Crude cell-free homogenates.** Brassinin glucosyltransferase (BGT) activity was determined using brassinin (**9**) as a substrate and UDPG as the glucose donor. After incubation of the assay mixture and extraction using ethyl acetate, HPLC analysis of the residue was used for the detection and quantification of the reaction product β-D-glucopyranosylbrassinin (**10**). Enzyme assays carried out using different concentrations of brassinin (**9**) indicated the relevant concentration range for inhibition assays (0.20–0.40 mM). In all inhibition assays brassinin (**9**) was used at 0.30 mM and the potential inhibitor was used at 0.30 and 0.60 mM. All compounds were found to be stable in the assay buffer.

Dithiocarbamates **18**–**23**, ester **24**, amide **25**, camalexins **15**, **17**, **26**, 3-phenylindoles **27**–**29**, 3-phenylbenzofuran (**30**), and 2-phenylindole (**31**) were tested as potential inhibitors of BGT. The percentage of inhibition caused by each compound was determined from the relative specific activity of BGT of cell-free homogenates (ratio of BGT specific activity of the cell-free homogenate containing brassinin plus the potential inhibitor and BGT specific activity of samples containing only brassinin, Table 3). Considerable inhibition of BGT activity in cell-free homogenates was caused by dithiocarbamates **18**, **22**, **23**, 3-phenylbenzofuran (**30**), 3-phenylindoles **27**–**29**, and camalexins **15**, **17**, **26**. Surprisingly, among all the tested compounds, the phenylindoles were the best inhibitors of BGT activity present in cell-free homogenates. 3-Phenylindole (**27**) and 6-fluoro-3-phenylindole (**28**) at 0.60 mM inhibited almost completely BGT activity (0.019 ± 0.003 nmol/mg/min, 13% relative activity, Table 3). Although in cell cultures both compounds **27** and **28** slowed down the transformation of brassinin substantially, this effect was attributed to their strong growth inhibitory activity. However, the relative specific activity of BGT obtained in cell-free homogenates (13%) indicates clearly that inhibition of brassinin detoxification by **27** and **28** in fungal cultures is due to inhibition of BGT activity as well. 3-Phenylindole (**27**) was glucosylated to 1-β-D-glucopyranosyl-3-phenylindole (**54**, specific activity 0.030 nmol/mg/min) slowly in cell-free homogenates, but no transformation

**Table 3.** Effect of potential inhibitors **15** and **17–31** on brassinin glucosyltransferase activity ($10^{-10} \times$ mol/mg/ min) in cell-free homogenates of mycelia of *Sclerotinia sclerotiorum*

| Substrate (0.30 mM) + potential inhibitor (0.30 and 0.60 mM) | Specific activity (0.1 nmol/ mg/ min) ± SD[a] | | % Relative activity | |
|---|---|---|---|---|
| | 0.30 mM | 0.60 mM | 0.30 mM | 0.60 mM |
| Brassinin (**9**) + solvent (control) | 1.50 ± 0.01 | 1.50 ± 0.01 | 100 ± 1 | 100 ± 1 |
| Brassinin (**9**) + camalexin (**15**) | 0.95 ± 0.02 | 0.84 ± 0.03 | 63 ± 1 | 56 ± 2 |
| Brassinin (**9**) + 6-fluorocamalexin (**17**) | 0.97 ± 0.01 | 1.00 ± 0.02 | 66 ± 1 | 63 ± 1 |
| Brassinin (**9**) + methyl (benzofuran-3-yl)methyl dithiocarbamate (**18**) | 0.75 ± 0.02 | 0.55 ± 0.03 | 50 ± 1 | 37 ± 2 |
| Brassinin (**9**) + methyl (thianaphthen-3-yl)methyl dithiocarbamate (**19**) | 1.22 ± 0.06 | 1.12 ± 0.04 | 81 ± 4 | 75 ± 3 |
| Brassinin (**9**) + methyl (7-azaindol-3-yl)methyl dithiocarbamate (**20**) | 1.02 ± 0.03 | 0.75 ± 0.07 | 68 ± 2 | 50 ± 5 |
| Brassinin (**9**) + methyl (5-methoxypyrazolo[1,5-*a*]pyridin-3-yl)methyl dithiocarbamate (**21**) | 1.47 ± 0.05 | 1.48 ± 0.05 | 98 ± 3 | 99 ± 3 |
| Brassinin (**9**) + methyl (indol-2-yl)methyl dithiocarbamate (**22**) | 0.63 ± 0.06 | 0.41 ± 0.05 | 42 ± 4 | 27 ± 3 |
| Brassinin (**9**) + methyl (benzofuran-2-yl)methyl dithiocarbamate (**23**) | 0.64 ± 0.01 | 0.43 ± 0.03 | 43 ± 1 | 29 ± 2 |
| Brassinin (**9**) + methyl 5-methoxypyrazolo[1,5-*a*]pyridine-3-carboxylate (**24**) | 1.51 ± 0.03 | 1.49 ± 0.08 | 100 ± 2 | 100 ± 5 |
| Brassinin (**9**) + 3-(*N*-acetylamino)quinoline (**25**) | 1.58 ± 0.06 | 1.53 ± 0.02 | 100 ± 4 | 100 ± 1 |
| Brassinin (**9**) + 5-fluorocamalexin (**26**) | 0.81 ± 0.06 | 0.72 ± 0.05 | 54 ± 4 | 48 ± 3 |
| Brassinin (**9**) + 3-phenylindole (**27**)[b] | 0.35 ± 0.02 | 0.19 ± 0.03 | 23 ± 1 | 13 ± 2 |
| Brassinin (**9**) + 6-fluoro-3-phenylindole (**28**) | 0.34 ± 0.06 | 0.20 ± 0.04 | 23 ± 4 | 13 ± 3 |
| Brassinin (**9**) + 4-fluoro-3-phenylindole (**29**) | 0.55 ± 0.08 | 0.43 ± 0.01 | 37 ± 5 | 29 ± 1 |
| Brassinin (**9**) + 3-phenylbenzofuran (**30**) | 0.79 ± 0.03 | 0.63 ± 0.01 | 53 ± 2 | 42 ± 1 |
| Brassinin (**9**) + 2-phenylindole (**31**) | 0.59 ± 0.02 | 0.59 ± 0.02 | 39 ± 1 | 39 ± 1 |

[a] Results are from experiments conducted in triplicate.
[b] 3-Phenylindole (**27**, 0.30 mM) was converted to 1-β-D-glucopyranosyl-3-phenylindole (**54**) with a specific activity of 0.03 nmol/mg/min.

of any of the other potential inhibitors was detected (Table 3).

## 3. Conclusion

*Sclerotinia sclerotiorum* is a plant pathogen that causes disease in a wide variety of plants, including several economically important crucifer species. In an effort to control this pathogen using environmentally 'neutral' molecules, potential inhibitors of detoxification of the phytoalexin brassinin (**9**) were synthesized and screened in cell cultures (**15**, **17–28**, **30**, **31**, and **51**, Table 2) and cell-free homogenates (**15** and **17–31**, Table 3). Such inhibitors, coined paldoxins, are designed to inhibit phytoalexin detoxifying enzymes and preferably have no antifungal activity or other deleterious effects on living organisms.[22] It was rather surprising to discover that, despite the structural variety of compounds tested, *S. sclerotiorum* was able to metabolize all of them, albeit at different rates (Table 2). Nonetheless, compounds **18**, **22**, **23**, and **26–28** were able to slow down substantially the metabolism of brassinin (**9**) in cell cultures (Figs. 2–6). A similar effect of compounds **18**, **22**, **23**, and **26–31** on brassinin metabolism was observed in cell-free homogenates, with the most potent effect observed for compounds **27** and **28** (Table 3). Importantly, in cell-free homogenates only 3-phenylindole (**27**) was transformed to the corresponding β-D-1-glucopyranosyl product (Table 3). Taken together these results indicate that: (i) although other glucosyltransferases appear to be produced by *S. sclerotiorum* in cell cultures, BGT obtained in cell-free homogenates appears to be substrate specific; (ii) compounds **27** and **28** were the most potent inhibitors of BGT in cell-free homogenates; (iii) **28** did not appear to be a substrate of BGT (Table 3). Hence, a high probability exists that additional BGT inhibitors can be developed to control *S. sclerotiorum* in crucifers. To facilitate this process, it would be important to isolate BGT and to determine the type of inhibition observed for structures as diverse as **22/23**, and **27/28**. Furthermore, to determine the structural requirements for both BGT activity and antifungal activity, the screening of new compounds is necessary. Finally, considering that compound **28** was metabolized in cultures at a slower rate than brassinin (**9**), it might be degraded at even slower rates in plants. Therefore, to determine its protective effect and that of any potent BGT inhibitors against stem rot disease, it is essential to carry out in vivo plant assays. These assays can establish whether BGT inhibitors devoid of antifungal activity are accessible and present a viable alternative to fungicides.

## 4. Experimental

### 4.1. General

All chemicals were purchased from Sigma–Aldrich Canada Ltd, Oakville, ON, Canada. All solvents were of HPLC grade and used as such, except for $CH_2Cl_2$ and $CHCl_3$, which were redistilled. HPLC analysis was carried out with a high performance liquid chromatograph equipped with a quaternary pump, automatic injector, and diode array detector (DAD, wavelength range 190–600 nm), degasser, and a Hypersil ODS column (5 mM particle size silica, 4.6 id × 200 mM), equipped with an in-line filter. Mobile phase: 75% $H_2O$–25% $CH_3CN$ to 100% $CH_3CN$ (v/v), for 35 min, linear gradient, and a flow rate of 1.0 mL $min^{-1}$. HPLC-mass spectrometry (HPLC-MS) data were obtained using an HPLC system equipped with an autosampler, binary pump, degasser, a DAD connected directly to a mass detector (MSD, ion trap mass spectrometer) with an

electrospray ionization (ESI) source. Chromatographic separation was carried out at room temperature using an Eclipse XSB C-18 column (5 μm particle size silica, 150 mM × 4.6 mM internal diameter). The mobile phase consisted of a gradient of 0.2% formic acid in water (A) and 0.2% formic acid in acetonitrile (B) (75% A to 75% B in 35 min, to 100% B in 5 min) and a flow rate of 1.0 mL $min^{-1}$. Additional conditions for the MSD were as previously reported.[23]

Specific rotations, $[\alpha]_D$, were determined at ambient temperature on a Rudolph DigiPol DP781 polarimeter using a 1 mL, 10.0 cm path length cell; the units are $10^{-1}$ deg $cm^2$ $g^{-1}$ and the concentrations ($c$) are reported in g $(100\ mL)^{-1}$. UV spectra were recorded on Varian-Cary spectrophotometer in MeOH or $CH_3CN$. Fourier transform IR spectra were obtained on a Bio-Rad FTS-40 spectrometer in KBr. NMR spectra were recorded on 500 MHz spectrometers: $\delta$ values were referenced as follows: for $^1H$ (500 MHz), $CDCl_3$ ($CHCl_3$ at 7.27 ppm), $CD_3CN$ ($CD_2HCN$ at 1.94 ppm), $(CD_3)_2CO$ ($CHD_2COCD_3$ at 2.05 ppm), and $CD_3OD$ ($CHD_2OD$ at 3.30 ppm); for $^{13}C$ (125.8 MHz), $CDCl_3$ (77.2 ppm), $CD_3CN$ (118.7 ppm), $(CD_3)_2CO$ (29.9 ppm), and $CD_3OD$ (49.2 ppm); spin coupling constants ($J$) are reported to the nearest 0.5 Hz. Mass spectra (MS) were obtained on a VG 70 SE mass spectrometer (electron impact, EI) using a solid probe or on a Q Star XL (electrospray ionization, ESI), Applied Biosystems. The Bradford protein assay was used to quantify proteins in cell-free extracts using bovine serum albumin standard curves. The optical densities (at 595 nm) were recorded on a Bio-Rad SmartSpec 3000 spectrophotometer.

### 4.2. Synthesis and spectroscopic characterization of compounds 18–21, 23, 29, 30, 50–52, and 54

**4.2.1. Methyl (benzofuran-3-yl)methyldithiocarbamate (18).** $SeO_2$ (488 mg, 4.4 mmol) was added to a solution of 3-methylbenzofuran, prepared as reported previously,[24] (**33**, 288 mg, 2.2 mmol) in 1,4-dioxane (3 mL) and the mixture was refluxed at 105 °C for 48 h. The black precipitate was filtered off, washed with $CH_2Cl_2$, and the filtrate was concentrated under reduced pressure. The residue was subjected to FCC on silica gel ($CH_2Cl_2$/hexane, 3:7, $CH_2Cl_2$, 100%) to yield benzofuran-3-carboxaldehyde (**36**, 280 mg, 88% yield) and benzofuran-3-methanol (28 mg, 8% yield), respectively.[12] To a solution of benzofuran-3-carboxaldehyde (**36**, 285 mg, 1.95 mmol) in EtOH (24 mL) was added a solution of $NH_2OH{\cdot}HCl$ (475 mg, 6.8 mmol) and $Na_2CO_3$ (371 mg, 3.5 mmol) in water (9 mL) and the mixture was refluxed for 2 h at 95 °C. After concentrating the reaction mixture under reduced pressure, water (10 mL) was added and the mixture was extracted with $Et_2O$ (2× 20 mL). The combined organic extracts were dried over $Na_2SO_4$, concentrated under reduced pressure to yield chromatographically pure benzofuran-3-carboxaldehyde oxime (265 mg, 84% yield). To a cold solution (0 °C) of benzofuran-3-carboxaldehyde oxime (202 mg, 1.25 mmol) in MeOH (1.5 mL) were added $Na(CN)BH_3$ (788 mg, 12.5 mmol) and $NH_4OAc$ (1.06 g, 13.7 mmol). To this mixture, a neutralized (neutralization was carried out using 5 N NaOH) solution of $TiCl_3$ (30% wt in 2 N HCl, 5 mL) was added. After stirring for 20 min at 0 °C, the reaction mixture was diluted with 1% aqueous $NH_4OH$ (50 mL) and extracted with EtOAc (2× 50 mL). The combined organic extracts were dried over $Na_2SO_4$ and concentrated under reduced pressure to yield 200 mg of crude benzofuran-3-methanamine (**39**). The crude amine (**39**, 200 mg, 1.4 mmol) was dissolved in pyridine (1 mL) and $Et_3N$ (390 μL, 2.8 mmol), and cooled to 0 °C. After adding $CS_2$ (336 μL, 5.6 mmol), the mixture was stirred for 1 h at 0 °C, $CH_3I$ (262 μL, 4.2 mmol) was added, and the mixture was kept at 3 °C for 15 h. The reaction mixture was poured into water (15 mL) and extracted with $Et_2O$ (2× 20 mL). The combined organic extracts were dried over $Na_2SO_4$ and concentrated under reduced pressure. Finally, the residue was subjected to FCC on silica gel ($CH_2Cl_2$/hexane, 3:7 and 5:5) to yield 145 mg of methyl (benzofuran-3-yl)-methyldithiocarbamate (**18**) in 48% yield from the oxime. Mp = 79–81 °C; HPLC $t_R$ = 24.3 min; $^1H$ NMR (500 MHz, $CD_3CN$): $\delta$ 8.39 (br s, 1H), 7.80 (s, 1H), 7.71 (d, $J$ = 7.5 Hz, 1H), 7.54 (d, $J$ = 7.5 Hz, 1H), 7.37 (dd, $J$ = 7.5, 7.5 Hz, 1H), 7.31 (dd, $J$ = 7.5, 7.5 Hz, 1H), 5.05 (d, $J$ = 5 Hz, 2H), 2.59 (s, 3H). $^{13}C$ NMR (125.8 MHz, $CD_3CN$): $\delta$ 199.5 (s), 155.6 (s), 144.5 (d), 127.3 (s), 125.1 (d), 123.3 (d), 120.4 (d), 116.9 (s), 111.8 (d), 40.4 (t), 17.7 (q). HRMS-EI $m/z$: measured 237.0275 ($[M]^+$, calcd 237.0282 for $C_{11}H_{11}NOS_2$). MS-EI $m/z$ (% relative intensity): 237 ($[M]^+$, 10), 189 (16), 131 (100). FTIR $\nu_{max}$ (KBr): 3339, 3232, 1498, 1451, 1379, 1322, 1305, 1185, 1101, 923, 856, 746 $cm^{-1}$.

**4.2.2. Methyl (thianaphthen-3-yl)methyldithiocarbamate (19).** To a solution of 3-bromothianaphthene (**34**, 218 mg, 1.01 mmol) in dry $Et_2O$ (4 mL), a solution of $t$-BuLi in pentane (1.30 M, 1.57 mL, 2.02 mmol) was added dropwise at −78 °C under argon atmosphere. After stirring the reaction mixture at −78 °C for 30 min, dry DMF (118 μL, 1.53 mmol) was added and the mixture was stirred for 1.5 h at room temperature. The reaction mixture was quenched with water (10 mL), extracted with $Et_2O$ (2× 15 mL), dried ($Na_2SO_4$), and concentrated. The residue was subjected to FCC on silica gel ($CH_2Cl_2$/hexane; 3:7) to yield thianaphthene-3-carboxaldehyde (**37**, 122 mg) in 73% yield as white solid. $^1H$ NMR (500 MHz, $CDCl_3$): $\delta$ 10.17 (s, 1H), 8.70 (d, $J$ = 8 Hz, 1H), 8.34 (s, 1H), 8.90 (d, $J$ = 8 Hz, 1H), 7.54 (dd, $J$ = 8, 8 Hz, 1H), 7.48 (dd, $J$ = 8, 8 Hz, 1H). $^{13}C$ NMR (125.8 MHz, $CD_3CN$): $\delta$ 185.7 (d), 143.4 (d), 140.9 (s), 136.9 (s), 135.6 (s), 126.6 (d), 126.5 (d), 125.2 (d), 122.8 (d). HRMS-EI $m/z$: measured 162.0137 ($[M]^+$, calcd 162.0139 for $C_9H_6OS$). MS-EI $m/z$ (% relative intensity): 162 ($[M]^+$, 100), 161 (99), 134 (16), 133 (22), 89 (26). FTIR $\nu_{max}$ (KBr): 3084, 2818, 2719, 1675, 1500, 1462, 1424, 1385, 1136, 1098, 857, 758 $cm^{-1}$. To a solution of thianaphthene-3-carboxaldehyde (**37**) (219 mg, 1.34 mmol) in EtOH (6 mL) was added an aqueous solution (2 mL) of $NH_2OH{\cdot}HCl$ (186 mg, 2.7 mmol) and $Na_2CO_3$ (170 mg, 1.6 mmol). After stirring at 90 °C for 2 h, the reaction mixture was concentrated, diluted with water (10 mL), and the mixture was extracted with $CH_2Cl_2$ (2× 15 mL). The organic phase was dried over $Na_2SO_4$ and concentrated to

dryness. The residue was subjected to FCC on silica gel ($CH_2Cl_2$/hexane; 3:7) to afford thianaphthene-3-carboxaldehyde oxime (mixture of *E* and *Z* isomer, 220 mg, 93% yield) as white solid. To a solution of thianaphthene-3-carboxaldehyde oxime (180 mg, 1.02 mmol) in MeOH (1.5 mL) at 0 °C were added $Na(CN)BH_3$ (637 mg, 10.1 mmol) and $NH_4OAc$ (856 mg, 11.1 mmol). To this mixture a neutralized (neutralization was carried out with 5 N NaOH, 1.64 mL) solution of $TiCl_3$ 30% wt in 2 N HCl (4.1 mL, 8.08 mmol) was added. After stirring for 10 min at 0 °C, the reaction mixture was diluted with 1% $NH_4OH$ (40 mL) and extracted with EtOAc (2× 50 mL). The organic phase was dried over $Na_2SO_4$ and concentrated to dryness to yield 264 mg of crude thianaphthene-3-methanamine (**40**) as colorless oil. $^1$H NMR (500 MHz, $CD_3CN$): $\delta$ 7.93 (d, $J$ = 7.5 Hz, 1H), 7.86 (d, $J$ = 8 Hz, 1H), 7.44–7.37 (m, 3H), 4.07 (s, 2H). $^{13}$C NMR (125.8 MHz, $CD_3CN$): $\delta$ 141.0 (s), 139.5 (s), 138.5 (s), 124.7 (d), 124.3 (d), 123.1 (d), 122.2 (d), 122.0 (d), 40.4 (t). HRMS-EI *m*/*z*: measured 163.0457 ([M]$^+$, calcd 163.0456 for $C_9H_9NS$). MS-EI *m*/*z* (%relative intensity): 163 ([M]$^+$, 100), 162 (80), 149 (30), 147 (93), 135 (57), 134 (28), 91 (26). FTIR $\nu_{max}$ (KBr): 3372, 3287, 3057, 2912, 2851, 1589, 1459, 1427, 1255, 825 cm$^{-1}$. To a solution of crude thianaphthene-3-methanamine (**40**, 264 mg, 1.6 mmol) in pyridine (1 mL) were added $Et_3N$ (448 μL, 3.2 mmol) and $CS_2$ (288 μL, 4.8 mmol) at 0 °C. After 1 h of stirring at 0 °C, $CH_3I$ (299 μL, 4.8 mmol) was added and the reaction mixture was kept at 3 °C for 16 h. The mixture was poured into cold 1.5 M $H_2SO_4$ (30 mL), extracted with $Et_2O$ (2× 30 mL). The combined organic extracts were dried over $Na_2SO_4$, and concentrated to dryness. Finally pure methyl (thianaphthen-3-yl)methyldithiocarbamate (**19**, 184 mg, 71% yield from oxime) was obtained after fractionation by FCC (silica gel, $CH_2Cl_2$/hexane, 40:60 and 50:50). $^1$H NMR (500 MHz, $CD_3CN$): $\delta$ 8.42 (br s, $D_2O$ exchangeable, 1H), 7.96 (d, $J$ = 7.5 Hz, 1H), 7.88 (d, $J$ = 7.5 Hz, 1H), 7.54 (s, 1H), 7.47–7.40 (m, 2H), 5.16 (d, $J$ = 5 Hz, 2H), 2.60 (s, 3H). $^{13}$C NMR (125.8 MHz, $CD_3CN$): $\delta$ 199.5 (s), 140.7 (s), 138.4 (s), 132.0 (s), 125.9 (d), 125.1 (d), 124.8 (d), 123.3 (d), 122.2 (d), 44.6 (t), 17.7 (q). HRMS-EI *m*/*z*: measured 253.0063 ([M]$^+$, calcd 253.0054 for $C_{11}H_{11}NS_3$). MS-EI *m*/*z* (relative intensity): 253 ([M]$^+$, 15), 205 (18), 163 (18), 147 (100). FTIR $\nu_{max}$ (KBr): 3335, 3228, 2916, 1495, 1427, 1376, 1301, 1074, 925, 757 cm$^{-1}$.

**4.2.3. Methyl (7-azaindol-3-yl)methyldithiocarbamate (20).** $POCl_3$ (3.2 mL, 34 mmol) was added to DMF (2.6 mL, 34 mmol) at 0 °C and the mixture was stirred until it was solidified. To this solid mixture, 7-azaindole (**35**, 400 mg, 3.4 mmol) was added and the mixture was refluxed at 105 °C for 14 h. The reaction mixture was diluted with ice-cold water (20 mL), basified with 5 N NaOH (30 mL) and extracted with $CH_2Cl_2$ (3× 45 mL). The combined organic extracts were washed with water and brine, dried over $Na_2SO_4$ and concentrated under reduced pressure. The resulting residue was subjected to FCC on silica gel (acetone/hexane, 1:3) to afford 7-azaindole-3-carboxaldehyde (**38**, 233 mg, 47% yield) as a white solid.[15a] To a solution of 7-azaindole-3-carboxaldehyde (**38**, 327 mg, 2.2 mmol) in EtOH (25 mL) was added a solution of $NH_2OH$·HCl (545 mg, 7.8 mmol) and $Na_2CO_3$ (427 mg, 4.0 mmol) in water (10 mL) and the mixture was refluxed for 2 h at 95 °C. After concentrating the reaction mixture under reduced pressure, the resulting precipitate was filtered off, washed with ice-cold water, and air-dried to yield 7-azaindole-3-carboxaldehyde oxime (341 mg) in 94% yield as white solid. To a stirred solution of 7-azaindole-3-carboxaldehyde oxime (100 mg, 0.6 mmol) in 17% HCl (20 mL) at room temperature was added zinc powder (1.2 g) in portions, after which stirring was continued for a further 45 min at room temperature. Excess 5 N NaOH was added to basify the reaction mixture, the precipitate was filtered off under vacuum, and the precipitate was washed with EtOAc. The filtrate was extracted with EtOAc (3× 50 mL), the combined organic extracts were dried over $Na_2SO_4$ and concentrated under reduced pressure. The residue was applied for FCC on silica gel ($CHCl_3$/MeOH/$NH_4OH$, 80:20:1) to yield 7-azaindole-3-methanamine (**41**, 32 mg, 35% yield) as a colorless oil. $^1$H NMR [500 MHz, $CD_3CN$]: $\delta$ 9.98 (br s, 1H), 8.26 (dd, $J$ = 4.5, 1 Hz, 1H), 8.03 (d, $J$ = 8, 1 Hz, 1H), 7.29 (s, 1H), 7.07 (dd, $J$ = 8, 4.5 Hz, 1H), 3.97 (s, 2H). $^{13}$C NMR [125.8 MHz, $CD_3CN$]: $\delta$ 149.5 (s), 143.3 (d), 127.5 (d), 122.6 (s), 119.3 (s), 117.5 (d), 115.5 (d), 37.6 (t). HRMS-EI *m*/*z*: measured 147.0799 ([M]$^+$, calcd 147.0796 for $C_8H_9N_3$). MS-EI *m*/*z* (% relative intensity): 147 ([M]$^+$, 100), 146 (70), 131 (72), 119 (53). FTIR $\nu_{max}$ (KBr): 3126, 3086, 2924, 2862, 1579, 1537, 1449, 1419, 1335, 1294, 1120, 769 cm$^{-1}$. 7-Azaindole-3-methanamine (**41**, 105 mg, 0.7 mmol) was dissolved in pyridine (3 mL) and $Et_3N$ (398 μL, 2.8 mmol), and cooled to 0 °C. After adding $CS_2$ (168 μL, 2.8 mmol), the mixture was stirred for 1 h at 0 °C, $CH_3I$ (175 μL, 2.8 mmol) was added, and the mixture was kept at 3 °C for 15 h. The reaction mixture was poured into water (15 mL) and extracted with EtOAc (2× 20 mL). The combined organic extracts were dried over $Na_2SO_4$ and concentrated under reduced pressure followed by addition of toluene (2× 2 mL) and concentration under reduced pressure. Finally, the residue was subjected to FCC on silica gel ($CH_2Cl_2$/methanol, 98:2) to obtain methyl [(7-azaindol-3-yl)methyl]dithiocarbamate (**20**, 140 mg, 83%) as white solid. Mp = 167–169 °C; HPLC $t_R$ = 15.3 (br) min; $^1$H NMR (500 MHz, $CD_3OD$): $\delta$ 8.19 (dd, $J$ = 5, 1 Hz, 1H), 8.12 (dd, $J$ = 8, 1 Hz, 1H), 7.44 (s, 1H), 7.11 (dd, $J$ = 8, 5 Hz, 1H), 5.07 (s, 2H), 2.60 (s, 3H). $^{13}$C NMR (125.8 MHz, $CD_3OD$): $\delta$ 198.9 (s), 148.4 (s), 142.5 (d), 128.3 (d), 125.4 (d), 120.2 (s), 115.6 (d), 110.5 (s), 42.2 (t), 16.9 (q). HRMS-EI *m*/*z*: measured 237.0396 ([M]$^+$, calcd 237.0394 for $C_{10}H_{11}N_3S_2$). MS-EI *m*/*z* (% relative intensity): 237 ([M]$^+$, 23), 163 (7), 132 (9), 131 (100), 104 (9), 103 (8). FTIR $\nu_{max}$ (KBr): 3252, 3147, 3030, 2985, 2921, 1581, 1492, 1420, 1380, 1326, 1068, 921, 764 cm$^{-1}$.

**4.2.4. Methyl (5-methoxypyrazolo[1,5-*a*]pyridin-3-yl)methyldithiocarbamate (21).** To a cold (0 °C) solution of methyl 5-methoxypyrazolo[1,5-*a*]pyridine-3-carboxylate[8] (**24**, 118 mg, 0.57 mmol) in dry THF (3.5 mL) was added $LiAlH_4$ (87 mg, 2.3 mmol) in small portions during 5 min. After stirring for 2 h at room temperature, the

reaction was quenched with 5 N NaOH (0.8 mL) and the precipitate was filtered off through a Celite pad. The pad was washed with THF and EtOAc, the filtrate was dried ($Na_2SO_4$) and concentrated under reduced pressure to yield 5-methoxypyrazolo[1,5-*a*]pyridine-3-methanol (118 mg). The crude 5-methoxypyrazolo[1,5-*a*]pyridine-3-methanol (118 mg, 0.58 mmol) was dissolved in $CH_2Cl_2$ (6 mL), $MnO_2$ (406 mg, 4.7 mmol) was added, and the mixture was stirred for 18 h at room temperature. $MnO_2$ was filtered off, the filter cake was washed with EtOAc, and the filtrate was concentrated under reduced pressure. The residue was applied to FCC on silica gel (EtOAc/hexane, 2:3) to afford 5-methoxypyrazolo[1,5-*a*]pyridine-3-carboxaldehyde (**42**, 66 mg, 65% yield from the ester **24**) as a white solid. Mp = 92–93 °C; $^1$H NMR (500 MHz, $CDCl_3$): $\delta$ 9.95 (s, 1H), 8.37 (d, $J$ = 7.5 Hz, 1H), 8.27 (s, 1H), 7.58 (s, 1H), 6.71 (dd, $J$ = 7.5, 2 Hz, 1H), 3.95 (s, 3H). $^{13}$C NMR (125.8 MHz, $CDCl_3$): $\delta$ 183.5 (d), 161.6 (s), 147.7 (d), 141.9 (s), 130.4 (d), 113.6 (s), 109.7 (d), 97.6 (d), 56.5 (q). HRMS-EI *m*/*z*: measured 176.0582 ([M]$^+$, calcd 176.0585 for $C_9H_8N_2O_2$). MS-EI *m*/*z* (% relative intensity): 176 ([M]$^+$, 83), 175 (100), 160 (9), 131 (11), 119 (16). FTIR $\nu_{max}$ (KBr): 3095, 1664, 1644, 1538, 1483, 1282, 1203, 1086, 1015, 831 cm$^{-1}$. To a solution of 5-methoxypyrazolo[1,5-*a*]pyridine-3-carboxaldehyde (**42**, 90 mg, 0.51 mmol) in EtOH (7.5 mL) was added a solution of $NH_2OH{\cdot}HCl$ (124.5 mg, 1.79 mmol) and $Na_2CO_3$ (97.6 mg, 0.92 mmol) in water (2.3 mL) and the mixture was refluxed at 95 °C for 3 h. After concentrating the reaction mixture under reduced pressure, the resulting precipitate was filtered, washed with water, and dried under vacuum to yield 5-methoxypyrazolo[1,5-*a*]pyridine-3-carboxaldehyde oxime (89 mg, 91%) as a white solid. To a stirred solution of 5-methoxypyrazolo[1,5-*a*]pyridine-3-carboxaldehyde oxime (120 mg, 0.63 mmol) in 17% HCl (20 mL), zinc powder (1.2 g) was added in portions at room temperature, after which the stirring was continued for a further 45 min at room temperature. Excess 5 N NaOH was added to basify the reaction mixture, the precipitate was filtered off under vacuum, and the filter cake was washed with EtOAc. The filtrate was extracted with EtOAc (3× 50 mL), the combined organic extracts were dried over $Na_2SO_4$ and concentrated under reduced pressure to yield crude 5-methoxypyrazolo[1,5-*a*]pyridine-3-methanamine (**43**, 82 mg) as a colorless oil. $CS_2$ (111 μL, 1.85 mmol) was added to a solution of crude amine **43** (82 mg, 0.46 mmol) and $Et_3N$ (258 μL, 1.85 mmol) in pyridine (1 mL) at 0 °C. After stirring the reaction mixture at 0 °C for an hour, $CH_3I$ (115 μL, 1.85 mmol) was added and the mixture was kept at 3 °C for 15 h. The reaction mixture was poured into water (20 mL) and extracted with EtOAc (3× 20 mL). The combined organic extracts were dried over $Na_2SO_4$and concentrated under reduced pressure followed by addition of toluene (2× 2 mL) and concentration. Finally, the residue was subjected to FCC on silica gel ($CH_2Cl_2$/MeOH, 99:1) to afford methyl (5-methoxypyrazolo[1,5-*a*]pyridin-3-yl)methyldithiocarbamate (**21**, 82 mg, 49% yield from the oxime) as a white solid. Mp = 138–139 °C; HPLC $t_R$ = 15.5 min; $^1$H NMR (500 MHz, $CD_3OD$): $\delta$ 8.30 (d, $J$ = 7.5 Hz, 1H), 7.88 (s, 1H), 7.14 (d, $J$ = 2 Hz, 1H), 6.57 (dd, $J$ = 7.5, 2 Hz, 1H), 5.03 (s, 2H), 3.87 (s, 3H), 2.60 (s, 3H). $^{13}$C NMR (125.8 MHz, $CD_3OD$): $\delta$ 199.1 (s), 157.3 (s), 142.8 (d), 140.3 (s), 129.2 (d), 107.4 (d), 106.2 (s), 94.5 (d), 55.2 (q), 40.3 (t), 16.9 (q). HRMS-ESI *m*/*z*: measured 266.0434 ([M-1]$^-$, calcd 266.0427 for $C_{11}H_{12}N_3OS_2$). MS-ESI *m*/*z* (% relative intensity): 266 ([M−1]$^-$, 100). FTIR $\nu_{max}$ (KBr): 3142, 2946, 1649, 1527, 1470, 1396, 1254, 1228, 1087, 922 cm$^{-1}$.

**4.2.5. Methyl (benzofuran-2-yl)methyldithiocarbamate (23).** To a solution of benzofuran-2-carboxaldehyde[15b] (**44**, 843 mg, 5.8 mmol) in EtOH (30 mL) was added a solution of $NH_2OH{\cdot}HCl$ (1.4 g, 20.2 mmol) and $Na_2CO_3$ (1.1 g, 10.4 mmol) in water (10 mL) and the mixture was refluxed for 2 h at 95 °C. After concentrating the reaction mixture under reduced pressure, the resulting precipitate was filtered off, washed with ice-cold water, and air-dried to yield chromatographically pure benzofuran-2-carboxaldehyde oxime (870 mg) in 93% yield. $Na(CN)BH_3$ (788 mg, 12.5 mmol) and $NH_4OAc$ (1.06 g, 13.7 mmol) were added to a cold solution (0 °C) of benzofuran-2-carboxaldehyde oxime (202 mg, 1.25 mmol) in MeOH (1.5 mL). To this mixture, a neutralized (neutralization was carried out using 2 mL of 5 N NaOH) solution of $TiCl_3$ (30% wt in 2 N HCl, 5 mL) was added. After stirring for 30 min at 0 °C, the reaction mixture was basified with 5 N NaOH (~3 mL), diluted with 1% aqueous $NH_4OH$ (50 mL), and extracted with EtOAc (2× 50 mL). The combined organic extracts were dried over $Na_2SO_4$ and concentrated under reduced pressure to yield 244 mg of crude benzofuran-2-methanamine as colorless oil. $^1$H NMR (500 MHz, $CD_3CN$):™ 7.57 (d, $J$ = 7.5 Hz, 1H), 7.47 (d, $J$ = 8 Hz, 1H), 7.28–7.21 (m, 2H), 6.62 (s, 1H), 3.91 (s, 2H). $^{13}$C NMR (125.8 MHz, $CD_3CN$): $\delta$ 161.4 (s), 155.1 (s), 129.2 (s), 123.9 (d), 123.0 (d), 121.1 (d), 111.0 (d), 101.8 (d), 39.7 (t). HRMS-EI *m*/*z*: measured 147.0682 ([M]$^+$, calcd 147.0684 for $C_9H_9NO$). MS-EI *m*/*z* (% relative intensity): 147 ([M]$^+$, 28), 146 (52), 132 (11), 131 (100), 130 (34). FTIR $\nu_{max}$ (KBr): 3379, 3286, 3055, 2912, 2849, 1602, 1453, 1252, 1175, 945, 876, 801 cm$^{-1}$. $CS_2$ (408 μL, 6.8 mmol) was added to a cold (0 °C) solution of crude benzofuran-2-methanamine (244 mg, 1.7 mmol) and $Et_3N$ (463 μL, 3.3 mmol) in pyridine (1 mL). After stirring for 1 h at 0 °C, $CH_3I$ (318 μL, 5.1 mmol) was added and the mixture was kept at 3 °C for 15 h. The reaction mixture was poured into water (20 mL) and extracted with $Et_2O$ (2× 20 mL). The combined organic extracts were dried over $Na_2SO_4$ and concentrated under reduced pressure followed by addition of toluene (2× 2 mL) and concentration. Finally, the residue was subjected to FCC on silica gel ($CH_2Cl_2$/hexane, 3:7 and 5:5) to afford methyl (benzofuran-2-yl)methyldithiocarbamate (**23**, 167 mg, 56% yield from oxime) as colorless oil. HPLC $t_R$ = 24.7 min; $^1$H NMR (500 MHz, $CD_3CN$):™ 8.48 (br s, 1H), 7.60 (d, $J$ = 7.5 Hz, 1H), 7.50 (d, $J$ = 7.5 Hz, 1H), 7.32 (dd, $J$ = 7.5, 7.5 Hz, 1H), 7.26 (dd, $J$ = 7.5, 7.5 Hz, 1H), 6.77 (s, 1H), 5.05 (d, $J$ = 5 Hz, 2H), 2.61 (s, 3H). $^{13}$C NMR (125.8 MHz, $CD_3CN$): $\delta$ 200.2 (s), 155.2 (s), 153.6 (s), 128.7 (s), 124.7 (d), 123.4 (d), 121.5 (d), 111.3 (d), 105.4 (d), 43.8 (t), 17.8 (q). HRMS-EI *m*/*z*: measured 237.0287 ([M]$^+$, calcd 237.0282 for $C_{11}H_{11}NOS_2$). MS-EI *m*/*z* (% relative intensity): 237 ([M]$^+$, 28), 189 (10), 132 (10), 131 (100), 77 (14). FTIR

$\nu_{max}$ (KBr): 3337, 3239, 2993, 2917, 1497, 1452, 1303, 1253, 1175, 1085, 932, 750 $cm^{-1}$.

**4.2.6. 6-Fluoro-3-phenylindole (28) and 4-fluoro-3-phenylindole (29).** To a solution of 3-fluorophenyl hydrazine hydrochloride (200 mg, 1.23 mmol) in water (5 mL), solid $Na_2CO_3$ (80 mg, 0.75 mmol) was added. When all the $Na_2CO_3$ was dissolved, the mixture was extracted with $CH_2Cl_2$ (2× 10 mL), the combined organic extracts were dried over $Na_2SO_4$ and concentrated under reduced pressure. To this residue, phenyl acetaldehyde (**47**, 108 μL, 0.97 mmol) was added and the mixture was stirred for 1 h at room temperature and then for 30 min at 100 °C. After that a solution of $ZnCl_2$ (376 mg, 2.91 mmol) in EtOH (3 mL) was added and the mixture was stirred at 100 °C for another 1 h. After cooling, the mixture was filtered, the solvent was removed under reduced pressure, and an aqueous solution of HCl (4%, 10 mL) was added. The mixture was extracted with $CH_2Cl_2$ (2× 15 mL), the combined organic extracts were dried over $Na_2SO_4$ and concentrated under reduced pressure. The residue was then subjected to FCC on silica gel ($CH_2Cl_2$/hexane, 1:4) to give a mixture (150 mg, 72% yield) of 6-fluoro-3-phenylindole (**28**) and 4-fluoro-3-phenylindole (**29**) in equal ratio. Finally, these two compounds were separated by reverse phase column chromatography using $H_2O/CH_3CN$ (55:45) as eluant. 6-Fluoro-3-phenylindole (**28**): HPLC $t_R$ = 27.4 min; $^1H$ NMR (500 MHz, $CD_3CN$): $\delta$ 9.54 (br s, $D_2O$ exchangeable, 1H), 7.87 (dd, $J$ = 5, 9 Hz, 1H), 7.69 (dd, $J$ = 8, 1 Hz, 2H), 7.52 (d, $J$ = 2.5 Hz, 1H), 7.47 (dd, $J$ = 8 Hz, 2H), 7.30 (dd, $J$ = 7.5, 7.5 Hz, 1H), 7.24 (dd, $J$ = 10, 2 Hz, 1H), 6.96 (ddd, $J$ = 10, 9, 2.5 Hz, 1H). $^{13}C$ NMR (125.8 MHz, $CDCl_3$): $\delta$ 160.5 (d, $^1J_{C-F}$ = 239 Hz), 137.0 (d, $^3J_{C-F}$ = 12.5 Hz), 135.5, 129.2, 127.9, 126.6, 122.9, 122.2 (d, $^4J_{C-F}$ = 3.5 Hz), 121.1 (d, $^3J_{C-F}$ = 10 Hz), 118.9, 109.4 (d, $^2J_{C-F}$ = 24 Hz), 98.0 (d, $^2J_{C-F}$ = 26 Hz). HRMS-EI *m/z*: measured 211.0797 ($M^+$, calcd 211.0797 for $C_{14}H_{10}NF$). MS-EI *m/z* (% relative intensity) 211 ($M^+$, 100), 183 (26). FTIR $\nu_{max}$ (KBr): 3419, 3054, 1626, 1601, 1503, 1419, 1222, 1035, 960, 758, 733 $cm^{-1}$. 4-Fluoro-3-phenylindole (**29**): HPLC $t_R$ = 25.8 min; $^1H$ NMR (500 MHz, $CD_3CN$): $\delta$ 9.69 (br s, $D_2O$ exchangeable, 1H), 7.64 (dd, $J$ = 8, 1.5 Hz, 2H), 7.43 (dd, $J$ = 7.5, 7.5 Hz, 2H), 7.41 (d, $J$ = 2.5 Hz, 1H), 7.34 (d, $J$ = 8 Hz, 1H), 7.31 (dd, $J$ = 7, 7 Hz, 1H), 7.18-7.16 (m, 1H), 6.83 (dd, $J$ = 8, 12 Hz, 1H). $^{13}C$ NMR (125.8 MHz, $CDCl_3$): $\delta$ 157.5 (d, $^1J_{C-F}$ = 248 Hz), 139.7 (d, $^3J_{C-F}$ = 12 Hz), 135.3, 129.2, 129.1, 128.6, 126.6, 123.3 (d, $^3J_{C-F}$ = 8 Hz), 122.9, 118.0 (d, $^4J_{C-F}$ = 3 Hz), 115.0 (d, $^2J_{C-F}$ = 19 Hz), 107.7 (d, $^4J_{C-F}$ = 3.5 Hz), 106.0 (d, $^2J_{C-F}$ = 21 Hz). HRMS-EI *m/z*: measured 211.0834 ($M^+$, calcd 211.0797 for $C_{14}H_{10}NF$). MS-EI *m/z* (% relative intensity): 211 ($M^+$, 100), 183 (21). FTIR $\nu_{max}$ (KBr): 3418, 3054, 1625, 1600, 1546, 1502, 1419, 1327, 1222, 1035, 758 $cm^{-1}$.

**4.2.7. 3-Phenylbenzofuran (30).** To a solution of *o*-(1-phenylvinyl)phenol[18] (**49**, 98 mg, 0.5 mmol) in DMF (1.25 mL) were added $Cu(OAc)_2 \cdot H_2O$ (300 mg, 1.5 mmol), aqueous LiCl (10 M, 150 μL, 1.5 mmol), and aqueous $PdCl_2$ (0.1 M, 100 μL, 0.01 mmol). After refluxing at 100 °C for 20 h, the reaction mixture was poured into water (25 mL) and extracted with $Et_2O$ (2× 25 mL). The combined organic extracts were dried over $Na_2SO_4$ and concentrated under reduced pressure. The residue was subjected to FCC on silica gel ($CH_2Cl_2$/hexane, 1:9) to afford 3-phenylbenzofuran (**30**, 8 mg, 10% yield based on recovery of starting material) as colorless oil. The spectroscopic data of **30** were identical with those of reported data.[16b]

**4.2.8. Methyl (1-β-D-glucopyranosyl-3-hydroxylindol-2-yl)methyldithiocarbamate (50).** HPLC $t_R$ = 9.5 min; $[\alpha]_D$ −211 (*c* 0.12, $CH_3OH$); $^1H$ NMR (500 MHz, $CD_3CN$/$D_2O$, 5.0:0.01, v/v): $\delta$ 9.57 (br s, $D_2O$ exchangeable, 1H), 9.25 (br s, $D_2O$ exchangeable, 1H), 7.66 (d, $J$ = 8 Hz, 1H), 7.37 (d, $J$ = 8 Hz, 1H), 7.16 (dd, $J$ = 8, 8 Hz, 1H), 7.08 (dd, $J$ = 8, 8 Hz, 1H), 5.33 (d, $J$ = 14.5 Hz, 1H), 4.86 (d, $J$ = 14.5 Hz, 1H), 4.56 (d, $J$ = 8 Hz, 1H), 3.93 (dd, $J$ = 12, 2 Hz, 1H), 3.64 (dd, $J$ = 12, 7 Hz, 1H), 3.45 (dd, $J$ = 8, 8 Hz, 1H), 3.38 (dd, $J$ = 8, 8 Hz, 1H), 3.31–3.24 (m, 2H), 2.62 (s, 3H). $^{13}C$ NMR (125.8 MHz, $CD_3CN$): $\delta$ 201.6 (s), 135.2 (s), 133.9 (s), 126.6 (s), 123.6 (d), 121.6 (s), 120.2 (d), 118.6 (d), 112.9 (d), 105.9 (d), 77.3 (d), 77.1 (d), 74.4 (d), 71.2 (d), 62.7 (t), 41.2 (t), 18.4 (q). HRMS-ESI *m/z*: measured 413.0840 ($(M-1)^-$, calcd 413.0846 for $C_{17}H_{21}N_2O_6S_2$). FTIR $\nu_{max}$ (KBr): 3359, 2922, 1471, 1384, 1256, 1072 $cm^{-1}$. UV ($CH_3CN$) $\lambda_{max}$ (log $\varepsilon$): 223 (4.5), 272 (4.2) nm.

**4.2.9. Methyl (thianaphthen-3-yl-1-*S*-oxide)methyldithiocarbamate (51).** To a solution of thianaphthene-3-methanamine (**40**, 19 mg, 0.12 mmol) in $CF_3COOH/CH_2Cl_2$ (1:2, 0.75 mL) was added $H_2O_2$ (30%, 53 μL, 0.47 mmol) at 0 °C and the reaction mixture was stirred at the same temperature. After 3 h, the mixture was neutralized with 10% $NaHCO_3$ and extracted with $CH_2Cl_2$ (2× 10 mL). The combined organic extracts were dried over $Na_2SO_4$ and concentrated under reduced pressure. The residue was immediately dissolved in $CH_2Cl_2$ (0.5 mL), cooled to 0 °C, $Et_3N$ (50 μL) and $CS_2$ (50 μL) were added, and the mixture was stirred at 0 °C. After 60 min, $CH_3I$ (50 μL) was added and the reaction mixture was stirred for 30 min at room temperature. The reaction mixture was poured into water (10 mL) and extracted with $CH_2Cl_2$ (2× 10 mL). The combined organic extracts were dried over $Na_2SO_4$, concentrated under reduced pressure, and the residue was applied for FCC on silica gel ($CH_2Cl_2$/MeOH, 99:1) to afford methyl (thianaphthen-3-yl-1-*S*-oxide)methyldithiocarbamate (**51**, 3 mg, 9%) as an off-white solid. HPLC $t_R$ = 10.8 min; $[\alpha]_D$ −252 (*c* 0.18, MeOH); $^1H$ NMR (500 MHz, $CD_3OD$): $\delta$ 7.97 (d, $J$ = 7.5 Hz, 1H), 7.72 (d, $J$ = 7.5 Hz, 1H), 7.67 (dd, $J$ = 7.5, 7.5 Hz, 1H), 7.61 (dd, $J$ = 7.5, 7.5 Hz, 1H), 7.01 (s, 1H) 5.05 (AB quartet, $J$ = 17, 17 Hz, 2H), 2.65 (s, 3H). $^{13}C$ NMR [125.8 MHz, $CD_3OD$]: $\delta$ 201.1 (s), 146.3 (s), 145.6 (s), 136.8 (s), 132.7 (d), 131.7 (d), 129.7 (d), 126.4 (d), 123.4 (d), 44.0 (t), 17.2 (q). HRMS-ESI *m/z*: measured 270.0079 ($[M+1]^+$, calcd 270.0075 for $C_{11}H_{12}NOS_3$). MS-EI *m/z* (% relative intensity): 270 ($[M+1]^+$, 100). FTIR $\nu_{max}$ (KBr): 3230, 3037, 2922, 1518, 1237, 1121, 1012, 935, 757 $cm^{-1}$. UV ($CH_3OH$) $\lambda_{max}$ (log $\varepsilon$): 222 (4.4), 246 (4.1), 270 nm (4.0).

**4.2.10. Methyl (7-*O*-β-D-glucopyranosyl-7-oxythianaphthen-3-yl)methyldithiocarbamate (52).** HPLC $t_R$ = 8.8 min; $[\alpha]_D$ −60 (*c* 0.26, $CH_3OH$); $^1H$ NMR [500 MHz, $(CD_3)_2CO$]: δ 9.33 (br s, 1H), 7.61 (s, 1H), 7.58 (d, *J* = 8 Hz, 1H), 7.36 (dd, *J* = 8, 8 Hz, 1H), 7.18 (d, *J* = 8 Hz, 2H), 5.23–5.21 (m, 3H), 3.90 (d, *J* = 10 Hz, 1H), 3.74–3.71 (m, 1H), 3.59–3.49 (m, 4H), 2.61 (s, 3H). $^{13}C$ NMR (125.8 MHz, $(CD_3)_2CO$: δ 199.3 (s), 153.2 (s), 140.5 (s), 132.4 (s), 130.3 (s), 126.2 (d), 126.1 (d) 116.1 (d), 109.5 (d), 101.6 (d), 77.6 (d), 77.5 (d), 74.1 (d), 70.8 (d), 62.1 (t), 44.8 (t), 17.5 (q). HRMS-ESI *m/z*: measured 430.0478 $(M-1)^-$, calcd 430.0458 for $C_{17}H_{20}NO_6S_3$). FTIR $\nu_{max}$ (KBr): 3335, 2925, 1710, 1602, 1552, 1462, 1378, 1216, 1078, 1032, 746 $cm^{-1}$. UV ($CH_3CN$) $\lambda_{max}$ (log ε): 224 (4.4), 254 (4.1), 304 (3.5) nm.

**4.2.11. 1-β-D-Glucopyranosyl-3-phenylindole (54).** HPLC $t_R$ = 11.8 min; $[\alpha]_D$ −21 (*c* 0.20, $CH_3OH$); $^1H$ NMR (500 MHz, $CD_3CN/D_2O$, 5.0:0.01, v/v): δ 7.93 (d, *J* = 8 Hz, 1H), 7.74 (d, *J* = 7.5 Hz, 2H), 7.69 (s, 1H), 7.61 (d, *J* = 8 Hz, 1H), 7.49 (dd, *J* = 7.5, 7.5 Hz, 2H), 7.34–7.29 (m, 2H), 7.23 (dd, *J* = 7.5, 7.5 Hz, 1H), 5.54 (d, *J* = 9 Hz, 1H), 3.96 (dd, *J* = 9, 9 Hz, 1H), 3.79 (dd, *J* = 10, 2 Hz, 1H), 3.68–3.50 (m, 4H). $^{13}C$ NMR (125.8 MHz, $CD_3CN$): δ 137.9 (s), 135.8 (s), 129.3 (d), 127.6 (d), 126.8 (s), 126.4 (d), 123.8 (d), 122.7 (d), 121.1 (d), 119.9 (d), 117.9 (s), 111.2 (d), 85.2 (d), 79.2 (d), 77.9 (d), 72.6 (d), 70.5 (d), 61.9 (t). HRMS-ESI *m/z*: measured 354.1345 ($(M-1)^-$, calcd 354.1346 for $C_{20}H_{20}NO_5$). FTIR $\nu_{max}$ (KBr): 3347, 2925, 1708, 1602, 1462, 1378, 1215, 1077, 1033, 746 $cm^{-1}$. UV ($CH_3CN$) $\lambda_{max}$ (log ε): 202 (4.2), 224 (4.2), 267 (3.8) nm.

### 4.3. Cultures and bioassays

*Sclerotinia sclerotiorum* clone #33 was grown on potato dextrose agar (PDA) plates at 20 ± 1 °C, in the dark. Sclerotia were collected over a 4-week period and stored at 20 °C in the dark. Erlenmeyer flasks (250 mL) containing 100 mL of minimal media[25] were inoculated with either sclerotia or mycelial plugs of *S. sclerotiorum* and were incubated at 22 ± 1 °C on a shaker at 120 rpm under constant light.

The antifungal activity of all compounds was determined using the following mycelial radial growth bioassay. Solutions of each compound in DMSO (50 mM) were used to prepare assay solutions in minimal media (0.50, 0.30, 0.10, 0.050, and 0.020 mM); control solutions contained 1% DMSO in media. Sterile tissue culture plates (12-well, 23 mM diameter) containing test solutions and control solution (1 mL per well) were inoculated with mycelium plugs (4 mM cut from 3-day-old PDA plates of *S. sclerotiorum*, clone #33) placed upside down on the center of each plate and incubated under constant light for 3 days. All bioassay experiments were carried out in triplicate at least three times.

### 4.4. Metabolism of potential inhibitors of brassinin detoxification in *S. sclerotiorum*

**4.4.1. Time-course studies.** Six-day-old cultures of *S. sclerotiorum* were incubated with compounds in Table 2 at 22 ± 2 °C on a shaker at 120 rpm under constant light. Each compound dissolved in $CH_3CN$ (200 μL) was added to fungal cultures (final concentration 0.10 mM) and to uninoculated medium (control); $CH_3CN$ (200 μL) was added to control cultures. Samples (5 mL each) were taken from the flasks at appropriate times, frozen or immediately extracted with EtOAc (2× 10 mL). Both organic and water phases were concentrated, dissolved in $CH_3CN$ (0.5 mL) or $CH_3OH$ (0.5 mL), and analyzed by HPLC.

**4.4.2. Large-scale experiments.** To obtain sufficient amounts of extracts to isolate the products of metabolism of each compound, experiments were carried out with one-liter batches, as described above for time-course studies. Only the chromatograms of the EtOAc extracts of fungal cultures showed peaks not present in chromatograms of extracts of control cultures. Thus, the EtOAc extracts were fractionated by FCC on reverse phase silica gel (gradient elution: $H_2O/CH_3CN$, 90:10, 80:20, 70:30, 50:50, 0:100), and each fraction was analyzed by HPLC. Finally, the metabolites were isolated by preparative TLC (silica gel, $CH_2Cl_2/CH_3OH$, 90:10, multiple development) and/or reverse phase preparative TLC (RP C-18 silica gel, $H_2O/CH_3CN$, 60:40).

### 4.5. Screening of potential inhibitors of brassinin detoxification

All cultures used in the screening experiments were initiated with mycelial plugs (inoculation using sclerotia resulted in less uniform mycelium growth due to the size variation of sclerotia).

**4.5.1. Mycelial cultures.** Six Erlenmeyer flasks (125 mL) each containing minimal media[15] (50 mL) were employed. Five of the flasks were each inoculated with three pieces of mycelial plugs (4-day-old, 6 mM) of *S. sclerotiorum* and incubated as described above. After 4 days of incubation potential inhibitors (final concentration 0.050 mM) in $CH_3CN$ [final concentration 0.5% (v/v)] were added to fungal cultures in two of the flasks (flasks 1 and 2). Similarly, the potential inhibitors (final concentration 0.10 mM) were added to fungal cultures in two other flasks (flasks 3 and 4). These four flasks (flasks 1, 2, 3, and 4) were incubated for 10 min and then brassinin (**9**, final concentration 0.05 mM) in $CH_3CN$ [final concentration 0.5% (v/v)] was added to each of the four flasks (flasks 1, 2, 3, and 4). To the flask 5, both brassinin (**9**) (dissolved in $CH_3CN$, final concentration 0.05 mM) and potential inhibitors (dissolved in $CH_3CN$, final concentration 0.05 mM) were added to uninoculated medium (control 1). To the fungal culture in flask 6 (control 2) was added $CH_3CN$ (150 μL). Samples were withdrawn and worked up as described above. The organic extracts were concentrated and the residues analyzed by HPLC-DAD and HPLC-MS.

**4.5.2. Crude cell-free homogenates**

**4.5.2.1. Preparation of crude cell-free extracts.** Erlenmeyer flasks (250× 5 mL) each containing 100 mL of PDB media were employed. All the flasks were

inoculated with sclerotia (five pieces) of *S. sclerotiorum* clone # 33. After 7 days, a solution of camalexin (50 mM, 100 μL) in DMSO was added as an inducer to each of the five flasks (final concentration 0.05 mM) and incubated for 24 h. The fungal mycelium was filtered off, washed with water, the remaining water squeezed out between filter paper, and the mycelial pad frozen immediately. Frozen mycelia were mixed with ice-cold Tris–HCl (50 mM, pH 8.0, containing 5% glycerol, 2 mM dithiothreitol, 2 mM PMSF, and 0.01% Triton X-100) buffer (ca. 15 mL) and ground at 4 °C using a mortar and pestle until a homogeneous mixture was obtained. The mixture was then centrifuged at 58,545*g* (22,000 rpm) for 40 min to obtain the cell homogenate and the pellet was discarded. The Bradford protein assay was used to estimate the quantities of proteins in the cell homogenate using a calibration curve prepared from bovine serum albumin (BSA).

**4.5.2.2. Enzyme assays.** Each potential inhibitor (final concentration 0.3 and 0.6 mM) dissolved in DMSO was added to a vial containing 2.0 mL of cell-free extracts and UDPG (final concentration 0.3 mM, dissolved in water), and the mixture was incubated at room temperature for 30 min. After that, brassinin (**9**, final concentration 0.3 mM) was added in each vial and the mixture was immediately divided into four samples in separate vials (0.5 mL each). Three samples were incubated for one more hour and the remaining sample was extracted immediately with EtOAc. After 60 min of incubation the three samples were extracted separately with EtOAc (2× 2 mL), the extracts were dissolved in $CH_3CN$ (100 μL) and analyzed by HPLC for the detection and quantification of the reaction product 1-β-D-glucopyranosylbrassinin (**10**). Control experiments containing only brassinin (**9**, 0.3 mM) and only potential inhibitors (0.3 mM) were performed similarly.

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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.2007.05.072.

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