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Detoxification of the cruciferous phytoalexin brassinin in *Sclerotinia sclerotiorum* requires an inducible glucosyltransferase

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Abstract

The phytoalexins, brassinin, 1-methoxybrassinin and cyclobrassinin, were metabolized by the stem rot fungus *Sclerotinia sclerotiorum* into their corresponding glucosyl derivatives displaying no detectable antifungal activity. Importantly, co-incubation of *S. sclerotiorum* with camalexins, various phytoalexin analogs, and brassinin indicated that a synthetic camalexin derivative could slow down substantially the rate of brassinin detoxification. Furthermore, inducible brassinin glucosyltransferase (BGT) activity was detected in crude cell-free extracts of *S. sclerotiorum*. BGT activity was induced by the phytoalexin camalexin, and the brassinin analogs methyl tryptamine dithiocarbamate and methyl 1-methyltryptamine dithiocarbamate. The overall results suggest that the fungus *S. sclerotiorum* in its continuous adaptation and co-evolution with brassinin producing plants, has acquired efficient glucosyltransferase(s) that can disarm some of the most active plant chemical defenses.

Keywords: Brassicaceae; Crucifer; Brassinin; Camalexin; Cyclobrassinin; Glucosyltransferase; Methoxybrassinin; Methyl 2-naphthylmethyl dithiocarbamate; *Sclerotinia sclerotiorum*

1. Introduction

A number of plant pathogens produce detoxifying enzymes that can effectively disarm phytoalexins (VanEtten et al., 2001). Such detoxification processes may be detrimental to the plant as phytoalexins are crucial chemical defenses produced de novo by plants to fight pathogens (Bailey and Mansfield, 1982; Essenberg, 2001). A better understanding of mechanisms of phytoalexin detoxification could lead to new approaches to control plant pathogens. For example, selective inhibitors of phytoalexin detoxifying enzymes produced by phytopathogenic fungi could be designed and exploited to protect the plant. Such inhibitors might allow the plant to build up the naturally occurring phytoalexins to a level at which the pathogen would not develop or spread. This control strategy would be environmentally advantageous if the inhibitors had minimal effect on other organisms. Towards this end, we have been investigating the metabolism and detoxification of cruciferous phytoalexins by economically important phytopathogenic fungi (Pedras et al., 2000). Crucifers (family Cruciferae or Brassicaceae), which comprise economically important oilseed and condiment crops and many vegetable species, produce unique phytoalexins such as brassinins **1**–**3**, containing a dithiocarbamate group (Pedras et al., 2000, 2003). Coincidentally, synthetic dithiocarbamates have been used extensively both as fungicides and herbicides (Leroux, 2003; Caldas et al., 2001). The phytopathogenic fungus *Sclerotinia sclerotiorum* (Lib.) de Bary causes stem rot disease in a vast range of plant species and families, making it one of the most economically important fungal pathogens (Boland and Hall, 1994; Purdy, 1979). The control of stem rot disease within *Brassica*

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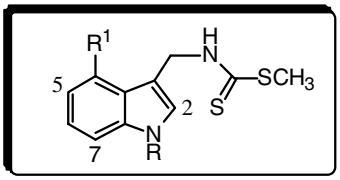
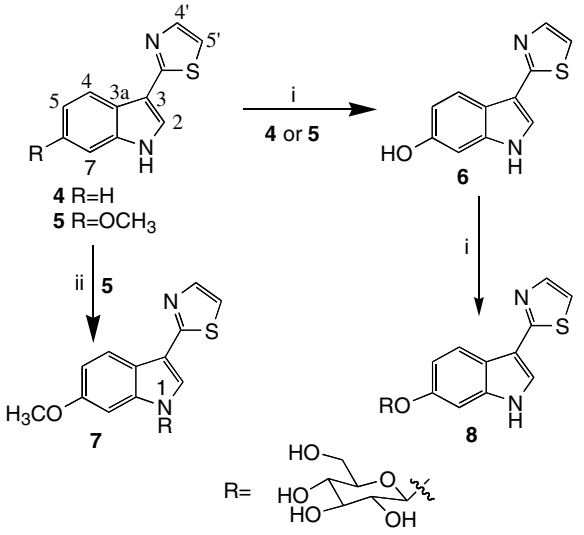
species is a worldwide problem as no sources of disease resistance are known currently (Kohli et al., 1995). Hence, it was of interest to investigate the reactions of *S. sclerotiorum* to the strongest antifungal crucifer phytoalexins.



1 R=R^1=H
2 R=OCH$_3$; R^1=H
3 R=H; R^1=OCH$_3$

An evaluation of the biological activity of diverse phytoalexins against *S. sclerotiorum* indicated that camalexin (**4**) and 6-methoxycamalexin (**5**) could inhibit completely the growth of the pathogen (Pedras and Ahiahonu, 2002). Furthermore, both camalexins **4** and **5** were detoxified via 6-hydroxycamalexin (**6**) to 6-*O*-(β-D-glucopyranosylcamalexin (**8**), a very unusual detoxification reaction in plant pathogenic fungi. In addition, *S. sclerotiorum* converted 6-methoxycamalexin (**5**) via a minor pathway to 1-β-D-glucopyranosylcamalexin (**7**), as summarized in Scheme 1. In continuation of this work, because both brassinin (**1**) and 1-methoxybrassinin (**2**) showed strong antifungal activity against *S. sclerotiorum*, we investigated their metabolism in cell cultures and compared it with that of the phytoalexin cyclobrassinin (**11**) and dithiocarbamate **14**.

Herein we report that both phytoalexins **1**, **2** and **11** were metabolized to yield products displaying no detectable antifungal activity. Importantly, co-incubation of *S. sclerotiorum* with various phytoalexin analogs and brassinin (**1**) indicated that a synthetic derivative of camalexin (**4**) could slow down substantially the rate of brassinin detoxification. Furthermore, inducible glucosyltransferase activity was detected in crude cell-free extracts of *S. sclerotiorum*; this activity was induced by camalexin (**4**), methyl tryptamine dithiocarbamate (**14**), methyl 1-methyltryptamine dithiocarbamate (**24**) and the phytoalexin spirobrassinin (**23**).



Scheme 1. Detoxification of camalexin (**4**) and 6-methoxycamalexin (**5**) by the plant pathogenic fungus *S. sclerotiorum*: (i) major pathway; (ii) minor pathway (Pedras and Ahiahonu, 2002).

2. Results

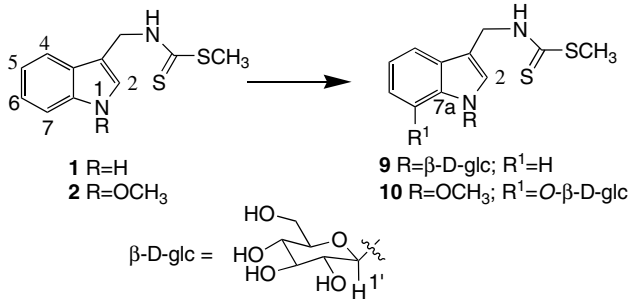
2.1. Metabolism, identification of metabolites and antifungal activity

Phytoalexins **1**, **2** and **11** were synthesized as previously reported (Pedras et al., 2003); the minimum inhibitory concentration (5.0×10^{-4} M) of brassinin (**1**) and 1-methoxybrassinin (**2**) to *S. sclerotiorum* was determined in liquid cultures. Due to lower solubility of cyclobrassinin (**11**) in aqueous solutions, the minimum inhibitory conc could not be determined; a slight inhibitory effect was observed at 5.0×10^{-5} M. Subsequently, brassinins **1** and **2** and cyclobrassinin (**11**) were separately incubated (at 1.0×10^{-4} M) with *S. sclerotiorum*, with culture samples withdrawn, extracted, and analyzed as described in the Experimental. Cultures incubated with DMSO or CH_3CN were used as controls. HPLC analysis of the broth extracts of fungal cultures incubated with brassinin (**1**) indicated it to be completely metabolized to a single product (HPLC R_t = 7.9 min) in ca. 24 h. To establish its structure, larger scale cultures of *S. sclerotiorum* were incubated with brassinin (**1**), were extracted, and the extract subjected to multiple chromatographic steps; a single biotransformation product substantially more polar than brassinin was further separated by prep. TLC. The structure of this polar metabolite (**9**) was determined by analyses of spectroscopic data as follows. Comparison of its ^{1}H NMR spectrum, obtained in CD_3CN, with that of brassinin (**1**) indicated the presence of an intact indole moiety (δ_H 7.64, *d* J = 8 Hz, 1H, 7.44, *d* J = 8 Hz, 1H; δ_H 7.24, *s*, 1H; δ_H 7.24, *ddd* J = 8, 8, 1, 1H, 7.14, *ddd* J = 8, 8, 1 Hz, 1H), as well as the intact methylene protons of the side-chain (δ_H 5.06) and the SCH_3 singlet (δ_H 2.59) of the dithiocarbamate group. Additional signals at δ_H 5.43 (*d*, J = 9 Hz, 1H) and several multiplets at δ_H 3.42–3.87 suggested the presence of a carbohydrate moiety.

As well, the molecular formula of $C_{17}H_{22}N_2O_5S_2$ obtained by HRMS-FAB and the ^{13}C NMR spectral data corroborated the presence of a carbohydrate residue. The identity of the monosaccharide unit was established through homonuclear ^{1}H–^{1}H decoupling experiments (upon addition of D_2O). The coupling constants (J = 7–9 Hz) indicated axial–axial proton couplings in

a pyranose ring, thus allowing the assignment of a β-glucopyranose substituent. 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 this metabolic product of brassinin (**1**) was assigned as 1-β-D-glucopyranosylbrassinin (**9**) (Scheme 2). Furthermore, cultures of *S. sclerotiorum* incubated with **9** indicated that this glucoside was further transformed but no biotransformation products were detected or isolated.

Similarly, HPLC analysis of the broth extract of fungal cultures incubated with 1-methoxybrassinin (**2**) indicated that **2** was completely metabolized a major product (HPLC R_t = 9.2 min) in ca. 12 h. To establish its structure, larger scale cultures of *S. sclerotiorum* were incubated with 1-methoxybrassinin (**2**) as above steps. The structure of the polar metabolite so obtained was determined by analyses of spectroscopic data, as described above for brassinin metabolite **9**. Additional signals suggested the presence of a carbohydrate moiety, which was corroborated by the ^{13}C NMR spectral data and HRMS-ESI. The identity of the monosaccharide unit was established through homonuclear ^1H–^1H decoupling experiments (upon addition of D_2O). In addition, as summarized in Fig. 1, NOE difference experiments (enhancement of the H-6 signal at δ_H 7.02 upon irradiation of H-1′ at δ_H 5.14 and vice-versa; enhancement of both CH_2 and H-5 at δ_H 5.00 and 7.06, respectively, upon irradiation of H-4 at δ_H 7.33; enhancement of H-2 at δ_H 7.41 upon irradiation of



Scheme 2. Transformation of brassinin (**1**) and 1-methoxybrassinin (**2**) by the phytopathogen *S. sclerotiorum*.

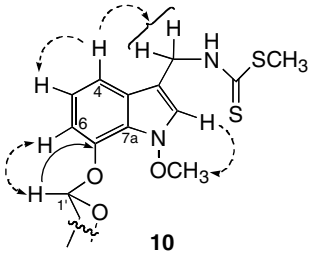
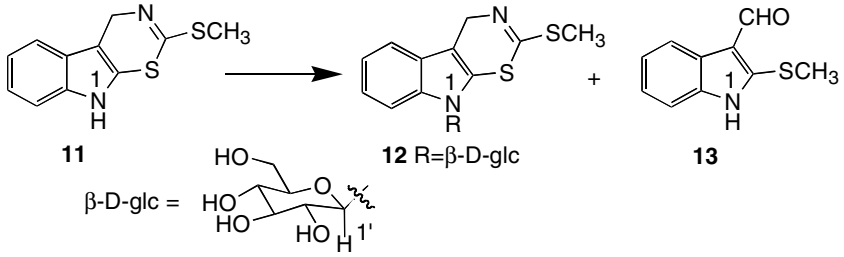


Fig. 1. Selected NOE (dashed lines) and HMBC (full line) correlations for 7-(*O*-β-D-glucopyranosyl)-1-methoxybrassinin (**10**).

CH_3-(O)) and HMBC (correlation between H-1′ and C-7 at δ_C 144.0) spectral data confirmed that the β-glucopyranose unit was located at C-7 and not at C-4. Thus, the structure of this metabolic product was assigned as 7-(*O*-β-D-glucopyranosyl)-1-methoxybrassinin (**10**) (Scheme 2).

Similar to the metabolism of brassinins **1** and **2**, HPLC analysis of the broth extract of fungal cultures incubated with cyclobrassinin (**11**) indicated it to be completely metabolized to a major product (HPLC R_t = min 8.6) in ca. 12 h. As described in the above examples, to establish the structure of this metabolic product, larger scale cultures of *S. sclerotiorum* incubated with cyclobrassinin (**11**) for 8 h, were filtered, extracted, and the broth extract fractionated by column chromatography followed by prep. TLC to yield a major metabolite (**12**) and also a minor metabolite (**13**). Spectroscopic analysis as described above for metabolites **9** and **10** indicated that cyclobrassinin was metabolized to the *N*-glucosyl derivative **12** (Scheme 3). The structure of the minor product was established to be brassicanal A (**13**) by comparison with a synthetic sample (Pedras and Khan, 1996).

In additional experiments, dithiocarbamates **14**, **16** and **17** were synthesized and administered to cultures of *S. sclerotiorum* to probe the specificity of the enzymes involved in the metabolism of brassinins **1** and **2**. Cultures were analyzed with metabolic products detected and isolated as described for brassinin (**1**). Similar to the brassinins, **14** was completely metabolized to a glucoside whose structure was deduced from spectroscopic data to be metabolite **15**, as described above for glucosides **9** and **10**. Furthermore, incubation of **16** and **17** with *S. sclerotiorum* showed that both compounds were metabolized in 48 h; however, the 1-naphthyl derivative **16** was transformed to several undetermined products, whereas the 2-naphthyl derivative **17** yielded one major product (**18**, HPLC R_t = 9.4 min). The HRMS-FAB mass measurement of **18** gave a molecular formula of $C_{19}H_{23}NO_6S_2$. The FTIR spectral data had a broad absorption band in the 3300 cm^{-1} region indicative of the presence of hydroxyl groups. The ^1H NMR spectrum showed a broad singlet at δ 8.54 (D_2O exchangeable), and resonances for a naphthalene ring system with substitutions at C-2 and C-5 or C-8 (δ 8.32, *d*, *J* = 9 Hz, 1H, 7.76, *s*, 1H, 7.53, *d*, *J* = 7.5 Hz, 1H, 7.46, *dd*, *J* = 9, 1 Hz, 1H, 7.42, *dd*, *J* = 7.5, 7.5 Hz, 1H, 7.15, *d*, 7 = 7.5 Hz, 1H), signals for an intact side chain (δ 5.05, CH_2, 2.59, SCH_3), and resonances for a β-D-glucopyranose unit. As in the previous examples, homonuclear ^1H–^1H decoupling experiments established the identity of the glucopyranosyl moiety. NOE difference experiments were used to determine the position of the glucosyl moiety either at C-5 or C-8, as shown in Fig. 2 (enhancement of H-6 at δ_H 7.15 upon irradiation of H-1′ at δ_H 5.09 of the *O*-β-D-



Scheme 3. Transformation of cyclobrassinin (**11**) by the phytopathogen *S. sclerotiorum*.

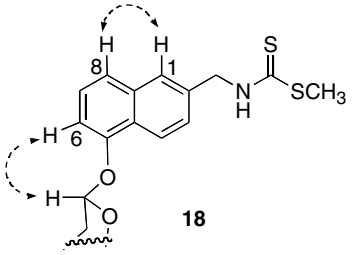
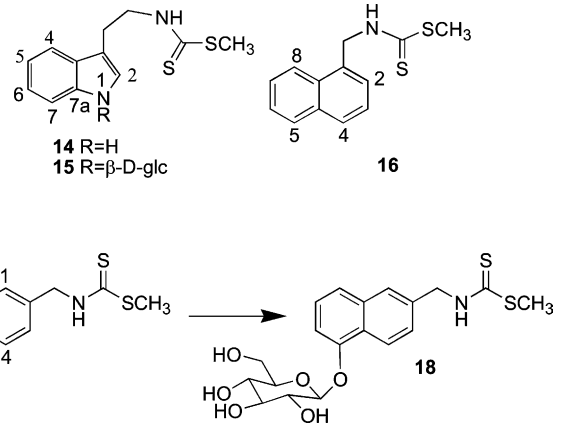


Fig. 2. Selected NOE (dashed lines) correlations for 5-(*O*-β-D-glucopyranosyl)-2-naphthylmethyldithiocarbamate (**18**).



Scheme 4. Transformation of methyl 2-naphthylmethyl dithiocarbamate (**17**) by the phytopathogen *S. sclerotiorum*.

glucopyranose unit, and vice-versa, and enhancement of H-8 at δ_H 7.53 upon irradiation of H-1′ at δ_H 7.76 and vice-versa). As expected, the ^{13}C NMR spectral data of metabolite **18** displayed 19 carbon signals, one of which indicated a deshielded sp^2 carbon atom at δ_C 154.0. Thus, the structure of this metabolic product was assigned as methyl 5-(*O*-β-D-glucopyranosyl)-2-naphthylmethyl dithiocarbamate (**18**) (Scheme 4).

The results of the biotransformations of metabolism of brassinin (**1**), 1-methoxybrassinin (**2**), cyclobrassinin (**11**) and analogs are summarized in Table 1; the percentages of the products obtained do not account for the total amount of compounds used in the incubation experiments since the respective glucosyl products are further transformed to unidentified substances.

The antifungal activity of **1**, **2**, **11**, **14, 16** and **17** against *S. sclerotiorum* was determined utilizing a radial mycelial growth inhibition assay (Table 2). After six days of incubation, the mycelium of control plates incubated with *S. sclerotiorum* covered the plates almost completely, while plates containing **1**, **2** and **4** (5×10^{-4} M) showed no mycelial growth. By contrast glucosides **9**, **10**, **12, 15** and **18** under similar conditions showed no inhibitory activity against *S. sclerotiorum*.

2.2. Co-metabolism of brassinin, camalexins and related indole derivatives

Previous work (Pedras and Ahiahonu, 2002) showed that camalexin (**4**) and related synthetic analogs were inhibitory against *S. sclerotiorum*. Subsequently, to evaluate a potential interaction between brassinins and camalexins, brassinin (**1**, at 5.0×10^{-5} M) and camalexin (**4**, at 1.0×10^{-4} and 5.0×10^{-5} M) were co-incubated with *S. sclerotiorum*; brassinin (**1**) was administered to each culture only after camalexin (**4**) was incubated for 10 min. Control cultures of *S. sclerotiorum* containing only brassinin (**1**) or camalexin (**4**) were incubated separately. The cultures were incubated for different periods and analyzed by HPLC. Additional co-incubation of brassinin (**1**) with either 6-fluorocamalexin (**19**), 6-fluoro-1-methylcamalexin (**20**), 6-fluoroindole-3-carboxaldehyde (**21**), 6-fluoro-l-methylindole-3-carboxaldehyde (**22**), or dithiocarbamates **14**, **16** and **17** were carried out. HPLC analysis of extracts of each culture over a 7-day period showed that only 6-fluorocamalexin (**19**) affected substantially the rate of metabolism of brassinin (**1**) (Table 3). For example, when brassinin (**1**) was co-incubated with 6-fluorocamalexin at 5.0×10^{-5} M, it was metabolized in ca. 96 h, whereas in cultures co-incubated with 6-fluorocamalexin at 1.0×10^{-4}, brassinin (**1**) was detected (2–5%) even after seven days.

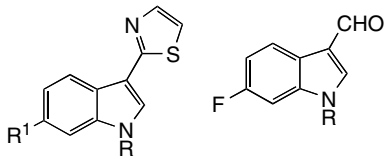


Table 1
Products of metabolism of brassinin (**1**), 1-methoxybrassinin (**2**), cyclobrassinin (**11**) and analogs **14**, **16** and **17** by *S. sclerotiorum*

Compound (1.0×10^{-4} M) added to fungal cultures[a]	Products of metabolism (% relative to compound added)
Brassinin (**1**)	Complete biotransformation to **9** (75%)[b] in 24 h
1-Methoxybrassinin (**2**)	95% Biotransformation to **10** (15%)[b] in 12 h
Cyclobrassinin (**11**)	90% Biotransformation to **12** (36%)[b] and **13** (6%) in 12 h
Methyl tryptamine dithiocarbamate (**14**)	Complete biotransformation to **15** (73%)[b] in 48 h
Methyl 1-naphthylmethyl dithiocarbamate (**16**)	Complete biotransformation to undetermined products in 48 h
Methyl 2-naphthylmethyl dithiocarbamate (**17**)	Biotransformation to **18** (41%)[c] in 48 h

[a] Compounds **1**, **14**, **16** and **17** were dissolved in DMSO, and **2** and **11** were dissolved in CH_3CN, added to six-day-old cultures and incubated at 24 ± 1 °C.
[b] Percentage yields of products determined by HPLC analyses using calibration curves; glucosyl derivatives **10**, **12** ,**15** and **18** were further transformed to undetermined products.
[c] Percentage yields of products represent isolated yields.

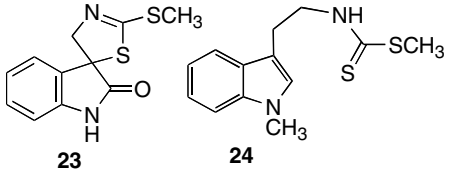
Table 2
Percentage of inhibition of *S. sclerotiorum* incubated with phytoalexins **1**, **2**, **4**, **11**, and analogs **14**, (2-day incubation, constant light), **16**, **17** and **19** (4 day incubation, constant light)

Compound	Concentration (M)	% Inhibition[a] ± SD
Brassinin (**1**)	5×10^{-4}	100
	3×10^{-4}	100
	1×10^{-4}	37 ± 8
1-Methoxybrassinin (**2**)	5×10^{-4}	100
	3×10^{-4}	100
	1×10^{-4}	56 ± 6
Camalexin (**4**)	5×10^{-4}	100
	3×10^{-4}	100
	5×10^{-5}	81 ± 6
Cyclobrassinin (**11**)	5×10^{-4}	Not soluble
	3×10^{-4}	Not soluble
	5×10^{-5}	<10
Methyl tryptamine dithiocarbamate (**14**)	5×10^{-4}	100
	3×10^{-4}	100
	1×10^{-4}	75 ± 6
Methyl 1-naphthylmethyl dithiocarbamate (**16**)	5×10^{-4}	80 ± 1
	5×10^{-5}	0
Methyl 2-naphthylmethyl dithiocarbamate (**17**)	5×10^{-4}	80 ± 1 0
	5×10^{-5}	0
6-Fluorocamalexin (**19**)	5×10^{-4}	70 ± 1
	5×10^{-5}	0

[a] % Inhibition = 100 − [(growth in treated/growth in control) × 100]; results are the mean of three independent experiments conducted in triplicate.

2.3. Transformation of brassinin (*1*) in crude cell-free extracts: evidence for brassinin glucosyltransferase activity

Glucosylation of brassinin (**1**) was obtained in crude cell-free extracts prepared from mycelia of *S. sclerotiorum*. Initially, no brassinin glucosyltransferase (BGT) activity was detected in crude cell-free extracts; however, when *S. sclerotiorum* was grown in the presence of compounds related to brassinin (**1**) such as camalexin (**4**), methyl tryptamine dithiocarbamate (**14**), methyl 1-methyltryptamine dithiocarbamate (**24**), or spirobrassinin (**23**), BGT activity was detected consistently. As shown in Table 4, camalexin (**4**), methyl tryptamine dithiocarbamate (**14**), and methyl 1-methyl tryptamine dithiocarbamate (**24**) were better inducers of BGT activity than spirobrassinin (**23**) (specific activity 0.07 vs. 0.02 nmol/min/mg of protein). Enzyme assays carried out with cell-free extracts of induced cultures of *S. sclerotiorum* in the absence of UDPG did not show BGT activity. Furthermore, the substrate affinity of BGT and/or related glucosyltransferase(s) produced by *S. sclerotiorum* was examined using the compounds shown in Table 5. These assays revealed that both brassinin (**1**) and methyl tryptamine dithiocarbamate (**14**) were glucosylated to a similar extent in the same amount of time. On the other hand, neither camalexin (**4**) nor 6-fluorocamalexin (**19**) were transformed in cell-free extracts containing BGT activity, but 6-hydroxycamalexin (**6**) was glucosylated to the expected glucoside **8**, though not as efficiently as brassinin (**1**). Subsequently, cell-free extracts were co-incubated with brassinin (**1**) and 6-fluorocamalexin (**19**), under standard assay conditions, and the product(s) of the enzymatic reaction(s) were analyzed by HPLC. These HPLC analyses indicated clearly that 1-(*O*-β-D-glucopyranosyl)brassinin (**9**) was the only product formed and that its rate of formation was affected substantially by the presence of 6-fluorocamalexin (**19**) (Table 4).



23 **24**

Table 3
Co-metabolism of brassinin (**1**) and compounds 4, and **19–22** by *S. sclerotiorum*

Compound added to fungal cultures[a]	Complete transformation of brassinin (**1**) to glucoside **9**
Brassinin (**1**) (1.0×10^{-4} M)	24 h
Brassinin (**1**) (5.0×10^{-5} M) + camalexin (**4**) (5.0×10^{5} M)	24 h
Brassinin (**1**) (5.0×10^{-5} M) + camalexin (**4**) (1.0×10^{-4} M)	24 h
Brassinin (**1**) (5.0×10^{-5} M) + 6-fluorocamalexin (**19**) (5.0×10^{-5} M)	96 h
Brassinin (**1**) (5.0×10^{-5} M) + 6-fluorocamalexin (**19**) (1.0×10^{-4} M)	Traces of brassinin (<5%)[b] remaining untransformed after 7 d
Brassinin (**1**) (5.0×10^{-5} M) + 6-fluoro-1-methylcamalexin (**20**) (5.0×10^{-5} M)	24 h
Brassinin (**1**) (5.0×10^{-5} M) + 6-fluoro-1-methylcamalexin (**20**) (1.0×10^{-4} M)	24 h
Brassinin (**1**) (5.0×10^{-5} M) + 6-fluoroindole-3-carboxaldehyde (**21**) (5.0×10^{-5} M)	24 h
Brassinin (**1**) (5.0×10^{-5} M) + 6-fluoroindole-3-carboxaldehyde (**21**) (1.0×10^{-4} M)	24 h
Brassinin (**1**) (5.0×10^{-5} M) + 6-fluoro-1-methylindole-3-carboxaldehyde (**22**) (5.0×10^{-5} M)	24 h
Brassinin (**1**) (5.0×10^{-5} M) + 6-fluoro-1-methylindole-3-carboxaldehyde (**22**) (1.0×10^{-4} M)	24 h

[a] Compounds were dissolved in DMSO, added to six-day-old cultures and brassinin was added 10 min after addition of each compound; incubation at 24 ± 1 °C.
[b] Percentage yields determined by HPLC analyses using calibration curves.

Table 4
Brassinin (**1**) glucosyltransferase (BGT) activity of cell-free extracts of *S. sclerotiorum* obtained from mycelia of liquid cultures incubated for 2 h with different inducers

Chemical inducer (5.0×10^{-5} M)	Specific activity[a] (nmol/min/mg of protein) ± SD
No inducer	No activity
Camalexin (**4**)	0.07 ± 0.04
Methyl tryptamine dithiocarbamate (**14**)	0.07 ± 0.01[b]
Methyl 1-methyltryptamine dithiocarbamate (**24**)	0.07 ± 0.01
Spirobrassinin (**23**)	0.02 ± 0.01

[a] Results are from three independent experiments carried out in triplicate; brassinin at 2.0×10^{-5} M was used in enzyme assays.
[b] Results are from ten independent experiments carried out in triplicate.

Table 5
Substrate affinity of glucosyltransferases in cell-free extracts of mycelia obtained from induced cultures of *S. sclerotiorum*

Substrate (3.0×10^{-5} M)	Specific activity[a] (nmol/min/mg) ± SD	Relative activity % (brassinin = 100)
Brassinin (**1**)	0.24 ± 0.03	100
Methyl tryptamine dithiocarbamate (**14**)	0.24 ± 0.03	100
Cyclobrassinin(**11**)	0.20 ± 0.03	83
Camalexin (**4**)	No detectable conversion	0
6-Fluorocamalexin (**19**)	No detectable conversion	0
6-Hydroxycamalexin (**6**)	0.10 ± 0.03	42

[a] Results are from three independent experiments carried out in triplicate.

3. Discussion

The results described above demonstrated that *S. sclerotiorum* produces enzymes able to detoxify phytoalexins **1**, **2** and **11** and dithiocarbamates **14** and **17** to glucosylated products having no detectable antifungal activity. While brassinin (**1**), cyclobrassinin (**11**) and analog **14** (no 1-*N*-substituents) were detoxified through direct *N*-glucosylation, 1-methoxybrassinin (**2**) and methyl 1-methyltryptamine dithiocarbamate (**24**) (Ahiahonu, 2003) were regioselectively oxidized at C-7 and then *O*-glucosylated. Thus, it appears that glucosylation occurs at *N*-1 of brassinin-like molecules when there is no *N*-substituent, otherwise glucosylation will occur after regioselective hydroxylation at C-7. Replacement of the indolyl moiety of brassinins with a naphthyl ring did not prevent the enzymatic detoxification of naphthylmethyl dithiocarbamates **16** and **17** (Table 1). This is not surprising as it was shown that replacing the naturally occurring plant hormone 3-indolylacetic acid with 2-naphthylacetic acid caused no substantial changes on the binding affinity to auxin binding sites of maize (Ray, 1977). Nonetheless, 2-naphthylmethyl dithiocarbamate **17** appears to mimic 1-substituted indoles better than 1-naphthylmethyl dithiocarbamate **16**, as **17** was detoxified mainly to the glucoside **18** whereas **16** yielded a number of undetermined products. Although *S. sclerotiorum* detoxifies dithiocarbamates through *N*-glucosylation, blackleg fungi *(Leptosphaeria maculans*, asexual stage *Phoma lingam)* detoxified brassinin (**1**) and related dithiocarbamates mainly via oxidation of the side chain at C-3 of the indole ring (Pedras et al., 2000; Pedras and Okanga, 2000). These different detoxification reactions suggest that these plant pathogenic fungi have evolved rather different but selective detoxifying enzymes and suggest that these fungal enzymes may be virulence traits, as in the case of other fungi (VanEtten et al., 2001).

The potential interaction between brassinin (**1**) and camalexin (**4**) was evaluated by co-incubation of both compounds in cell cultures; it was concluded that the

rate of co-metabolism of either **1** or **4** was not affected, relative to when each compound was incubated alone (Table 3). By contrast, when brassinin (**1**) was co-incubated with 6-fluorocamalexin (**19**) in cell cultures, brassinin (**1**) was metabolized at substantially slower rates (Table 3). In addition, the transformation of 6-fluorocamalexin (**19**) in cell cultures appeared to start only after brassinin (**1**) was completely transformed. This interaction between (**1**) and **19** suggests that 6-fluorocamalexin (**19**) has an inhibitory effect on the glucosylation of brassinin (**1**) and that this effect is not due to the antifungal activity of **19** (since **19** is less antifungal than camalexin (**4**), Table 2).

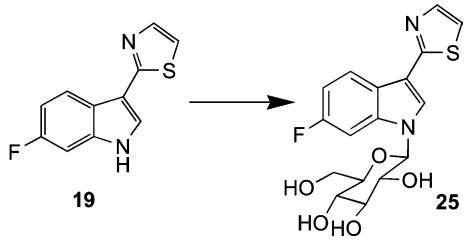
Enzyme assays carried out with crude cell-free mycelial extracts of non-induced cultures of *S. sclerotiorum* or induced cultures in the absence of UDPG did not show BGT activity. These results indicated that BGT is an inducible *N*-glucosyltransferase that requires the co-enzyme UDPG or equivalent (Table 4). The specific activity of crude cell-free extracts incubated separately with brassinin (**1**), camalexin (**4**), cyclobrassinin (**11**), dithiocarbamate **14**, 6-fluorocamalexin (**19**) or 6-hydroxycamalexin (**6**) indicated that the putative BGT is specific (Table 5). Furthermore, 6-fluorocamalexin (**19**) inhibited BGT and/or related glucosyltransferase(s) activity in cell-free extracts (Table 6). That is, 6-fluorocamalexin (**19**) is an inhibitor of brassinin (**1**) glucosylation both in cell cultures and in crude cell-free extracts (Tables 3 and 6, respectively). However, because **19** is *N*-glucosylated in cell cultures in 48 h (Scheme 5, Pedras and Ahiahonu, 2002), its lack of conversion to 6-fluoro-1-(β-D-glucopyranosyl)camalexin (**25**) in crude cell-free extracts is not completely understood. A possible explanation is that in cell cultures the unnatural 6-fluorocamalexin (**19**) may be transformed by non-specific glucosyltransferases which may be unstable under our extraction conditions and/or may require a different co-enzyme. Considering that neither camalexin (**4**) nor 6-fluorocamalexin (**19**) are glucosylated in cell-free extracts, it is likely that the thiazolyl ring prevents camalexins from fitting in the catalytic site of this putative BGT. Further conclusions will have to wait for detailed studies of fractions containing purified BGT and related enzymes.

In concluding, it is pertinent to point out that glucosylation is less usual in microorganisms, particularly in plant pathogens, but *O*-glucosylation and, to a lesser extent, *N*-glucosylation are common detoxification mechanisms among plants (Pedras and Ahiahonu, 2002; Hall et al., 2000). Interestingly, various fungi used as model systems for drug metabolism, transformed phenothiazine to the *N*-glucosylated derivative (Parshikov et al., 1999). The work reported here suggests that the fungus *S. sclerotiorum* in its continuous adaptation and co-evolution with brassinin producing plants, has acquired efficient glucosyltransferase(s) that can disarm the plant chemical defenses. Ultimately, it is anticipated that knowledge of the mechanisms of fungal detoxification can lead to the design of effective inhibitors that could prevent phytoalexin detoxification.

Table 6
Effect of 6-fluorocamalexin (**19**) on brassinin glucosyltransferase (BGT) in cell-free extracts of mycelia obtained from induced cultures of *S. sclerotiorum*

Substrate/concentration	Specific activity[a] (nmol/min/mg) ± SD	Relative activity % (brassinin = 100)
Brassinin (**1**)/3×10^{-5} M	0.24 ± 0.03	100
Brassinin (**1**) + 6-fluorocamalexin (**19**)/both at 3×10^{-5} M	0.083 ± 0.003	34
Brassinin (**1**)/3×10^{-5} M + 6-fluorocamalexin (**19**)/ 6×10^{-5} M	0.076 ± 0.003	32
Brassinin (**1**)/2×10^{-5} M	0.027 ± 0.004	100
Brassinin (**1**) + 6-fluorocamalexin (**19**)/both at 2×10^{-5} M	0.012 ± 0.002	44[b]

[a] Results are from three independent experiments carried out in triplicate.
[b] Relative to brassinin at 2×10^{-5} M.



Scheme 5. Transformation of 6-fluorocamalexin (**19**) by the phytopathogen *S. sclerotiorum* (Pedras and Ahiahonu, 2002).

4. Experimental

4.1. General procedures

All chemicals were purchased from Sigma–Aldrich Canada Ltd., Oakville, ON. All solvents were HPLC grade and used as such, except for CH_2Cl_2 and $CHCl_3$ which were redistilled. Organic extracts were dried (anhydr. Na_2SO_4) and solvents removed under reduced pressure in a rotary evaporator.

NMR spectra were recorded on Bruker Avance spectrometers; for ^1H (500 MHz), δ values were referenced to $CDCl_3$ (CCl_3H 7.27 ppm), CD_3CN (CD_2HCN 1.94 ppm), or $(CD_3)_2CO$ (CD_3COCD_2H 2.05 ppm) and for ^{13}C (75.5 or 125.8 MHz) referenced to $CDCl_3$ (77.23 ppm), CD_3CN (1.39, 118.69 ppm), or $(CD_3)_2CO$ (29.92 ppm). Fourier transform infrared (FTIR) spectra

were obtained on a Bio-Rad FTS-40 spectrometer using a diffuse reflectance cell. Mass spectra (MS) were obtained on a VG 70 SE mass spectrometer; HRMS-FAB was obtained at 70 eV employing a solids or continuous flow probe; HRMS-ESI was obtained on a QStar XL TOF mass spectrometer (MDS Sciex, Toronto, ON). Specific rotations, $[\alpha]_D$, were determined at ambient temperature on a Rudolph DigiPol DP781 polarimeter using a 1 ml, 10 cm path length cell; the units are 10^{-1} deg cm^2 g^{-1} and the concentrations (c) are reported in g/100 ml.

HPLC analyses were carried out with a high performance liquid chromatograph equipped with quaternary pump, automatic injector, and diode array detector (wavelength range 190–600 nm), degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 i.d. × 200 mm), with an in-line filter. Mobile phase: linear gradient H_2O–CH_3CN (3:1) to 100% CH_3CN in a 35 min period at a flow rate 1.0 ml/min.

4.2. Fungal cultures and antifungal assays

S. sclerotiorum (clone #33) was grown on potato dextrose agar (PDA) plates at 24 ± 1 °C, in the dark. Sclerotia were collected over a 4-week period and stored at room temperature in the dark. Erlenmeyer flasks (250 ml) containing 100 ml of minimal media (Pedras and Okanga, 2000) were inoculated with sclerotia of *S. sclerotiorum* and were incubated at 25 ± 1 °C on a shaker at 120 rpm under constant light. The antifungal activity of compounds was determined as previously reported (Pedras and Ahiahonu, 2002) but using either 12-well plates, 2-day incubation (22 mm diameter, 1 ml per well) or 6-well plates, 4-day incubation (35 mm diameter, 2 ml per well).

4.3. Metabolism

Six-day-old cultures of *S. sclerotiorum* were incubated with phytoalexins or compounds at : 25 ± 1 °C on a shaker at 120 rpm under constant light. Each compound dissolved in DMSO or CH_3CN was added to fungal cultures (final concentration 1×10^{-4} or 2×10^{-4} M) and to uninoculated medium (control). Samples were taken from the flasks up to seven days and extracted with EtOAc; the resulting aqueous layer was acidified (to pH 2 with dil HCl) and re-extracted with EtOAc, and finally the acidic aqueous layer was made alkaline and re-extracted with $CHCl_3$. After concentration of solvent, the extracts were analyzed by HPLC.

To obtain larger amounts of extract to isolate the products of metabolism of each compound, experiments were carried out with larger batches. The extracts were fractionated by FCC on silica gel with gradient elution: CH_2Cl_2–MeOH (100:0–85:15) or on RP C-18 silica gel, H_2O–CH_3CN (80:20–50:50). Each fraction was analyzed by HPLC. The metabolites were isolated by preparative TLC (silica gel, CH_2Cl_2–MeOH, 9:1, multiple development) and/or reversed phase preparative TLC (RP C-18 silica gel, H_2O–CH_3CN).

4.4. Preparation of crude cell-free extracts, protein and enzyme assays

S. sclerotiorum was grown in liquid cultures as reported above; after five days the inducer compound dissolved in DMSO (5×10^{-5} M in culture solution) was added to cultures and the cultures incubated for additional 24 h. The fungal mycelium was removed by the filteration, washed with H_2O, with the remaining H_2O squeezed out between filter paper and the mycelial pad frozen immediately. Frozen mycelia (12–20 g) was mixed with ice cold standard buffer (10–20 ml), 50 mM Tris–HCl, pH 8.0, containing 20% glycerol, 20 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyfluoride (PMSF) and 0.01% Triton X-100) and ground using a mortar and pestle until a homogenous mixture was obtained, similar to a previous report (Zenk et al., 2000). The mixture was then centrifuged (27,216g for 15 min) to obtain the cell homogenate (12–25 ml, 26–55 mg total protein), and the pellet was discarded.

The Bradford protein assay was used to quantify proteins in cell-free extracts using bovine serum albumin standard curves.

Enzyme assays were carried out at 27 °C, using brassinin (**1**) (or other compounds as reported) as substrate and UDPG as glucose donor (Orlean, 1982). In assays using brassinin (**1**) as substrate, product accumulation was linear for at least 3 h; the substrate was always present in large molar excess over the enzyme. The specific activity of cell-free extracts was defined as the amount (nmol) of glucopyranosyl product formed per min per mg of protein. The standard assay mixture contained 50 mM Tris–HCl buffer (98 μl, pH 8.0), UDPG (15 mM, 100 μl), and cell homogenate (400 μl, 0.88 μg protein). The reaction was started by adding brassinin (**1**) or potential substrate in DMSO (10 mM, final concentration in assay media 3×10^{-5} M) and incubated for 3 h with constant shaking at 27 °C. The reaction was stopped by extracting with EtOAc, the solvent was removed under reduced pressure, the extract dissolved in MeOH and analyzed by HPLC. Quantification of products was carried out using standard calibration curves.

4.5. Syntheses and spectral data

Brassinin (**1**), 1-methoxybrassinin (**2**), camalexin (**4**), cyclobrassinin (**11**), tryptamine dithiocarbamate (14), 6-fluorocamalexin (**19**), 6-fluoroindole-3-carboxaldehyde (**21**), and 6-fluoro-1-methylindole-3-carboxaldehyde (**22**) were synthesized as previously reported (Pedras et al., 2003; Pedras and Ahiahonu, 2002). 6-Hydroxycamalexin (**6**) was isolated from culture as previously reported

(Pedras and Ahiahonu, 2002). Satisfactory spectroscopic data were obtained for all known compounds.

*4.5.1. Methyl 1-naphthylmethyl dithiocarbamate (**16**)*

To a solution of 1-naphthaldehyde (129 mg, 0.83 mmol) in EtOH (2 ml) a solution of $NH_2OH \cdot HCl$ (115 mg, 1.65 mmol) and Na_2CO_3 (88 mg, 0.83 mmol) in EtOH–H_2O (0.5/1 ml) was added at room temperature. After stirring at room temperature for 2 h, the solvent was removed under reduced pressure, the residue dissolved in EtOAc (30 ml), washed with brine and dried (Na_2SO_4). Concentration under reduced pressure yielded 1-naphthaldehyde oxime (140 mg, 99%). To a solution of 1-naphthaldehyde oxime (50 mg, 0.29 mmol) in EtOH (10 ml), 10% Pd/C (10 mg) and glacial AcOH (2 ml) were added. The reaction mixture was shaken under 3-4 atmosphere of hydrogen at room temperature for 2 h. The catalyst was removed by filtration, conc. HCl (1 ml) was added to the filtrate, with the filtrate concentrated under reduced pressure. The residue was dissolved in H_2O (5 ml), then extracted with Et_2O, and the aqueous layer was basified with 5 M NaOH. The resulting mixture was extracted (CH_2Cl_2–MeOH, 9:1, v/v), the combined extracts washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure to yield crude 1-naphthylmethylamine (36 mg, 79%). This amine was used without further purification to prepare the dithiocarbamate. Separation of the crude dithiocarbamate by FCC (EtOAc: hexanes, 1:15, v/v) yielded methyl 1-naphthylmethyl dithiocarbamate (16) (49 mg, 89%). FTIR (KBr) (cm^{-1}) 3342, 3239, 1500, 1351; UV (CH_3CN) λ_{max} nm (log ε) 272.1 (4.57), 222.3 (5.30). ^1H NMR (300 MHz, $CDCl_3$) δ 7.91 (*m*, 3H), 7.58–7.42 (*m*, 4H), 7.03 (*s* br, 1H), 5.31 (*d* $J = 5$ Hz, 2H), 2.66 (*s*, 3H). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 189.9, 134.1, 131.8, 131.7, 129.6, 129.1, 127.9, 127.3, 126.5, 125.7, 123.6, 50.0, 18.5. HRMS-EI *m/z* calcd. for $C_{13}H_{13}NS_2$ 247.0489, found 247.0486.

*4.5.2. Methyl 2-naphthylmethyl dithiocarbamate (**17**)*

Prepared as described for methyl 1-naphthylmethyl dithiocarbamate (**16**) in similar yield but using as starting material 2-naphthaldehyde. FTIR (KBr) (cm^{-1}) 3203, 3006, 1520, 1353. UV (CH_3CN) λ_{max} nm (log ε) 249.9 (4.59), 220.8 (5.16). ^1H NMR (300 MHz, $CDCl_3$) 6 7.82 (*m*, 3H), 7.74 (*s*, 1H), 7.50 (*m*, 2H), 7.41 (*d*, $J = 8$ Hz, 1H), 7.27 (*s* br, 1H), 5.05 (*d*, $J = 5$ Hz, 2H), 2.66 (*s*, 3H). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 199.6, 133.9, 133.5, 133.2, 129.1, 128.1, 127.4, 127.3, 126.7, 126.5, 126.2, 51.5, 18.6. HRMS-EI *m/z* calcd. for $C_{13}H_{13}NS_2$ 247.0489, found 247.0485.

*4.5.3. 1-(β-D-glucopyranosyl)brassinin (**9**)*

HPLC $R_t = 7.9$ min.; $[\alpha]_D = -7.7$ (*c* 0.20, MeOH). ^1H NMR (500 MHz, CD_3CN) δ 8.34 (br *s*, 1H D_2O exchangeable), 7.64 (*d*, $J = 8$ Hz, 1H), 7.52 (*d*, $J = 8$ Hz, 1H), 7.44 (*s*, 1H), 7.24 (*ddd*, $J = 8, 8, 1$ Hz, 1H), 7.14 (*ddd*, $J = 8, 8, 1$ Hz, 1H), 5.46 (*d*, $J = 8$ Hz, 1H), 5.08 (*s*, 2H), 3.42–3.87 (*m*, 10H, 4H D_2O exchangeable), 2.59 (*s*, 3H) and a minor signal (ca. 1/8 intensity of the major one) due to a rotamer at 2.68 (*s*). ^{13}C NMR (125.8 M Hz, CD_3CN): δ 198.8 (*s*), 137.0 (*s*), 128.0 (*s*), 125.7 (*d*), 122.6 (*d*), 120.5 (*d*), 119.5 (*s*), 112.0 (*d*), 111.0 (*d*), 85.2 (*d*), 79.2 (*d*), 77.9 (*d*), 72.5 (*d*), 70.5 (*d*), 61.9 (*t*), 42.4 (*t*), 17.6 (q). HRMS-FAB *m/z*: measured 398.0965 (M^+, calcd. 398.0970 for $C_{17}H_{22}N_2O_5S_2$). MS-FAB *m/z* (relative intensity): 398 (M^+, 5), 154 (100), 137 (67), 136 (85), 107 (35) 77 (40), 57 (43), 55 (69). FTIR (cm^{-1}): 3317, 2952, 2922, 2852, 1740, 1581, 1462, 1377, 1115, 1076, 875, 743 cm^{-1}. UV (CH_3CN) λ_{max} (log ε): 218 (4.0), 270 (3.6).

*4.5.4. 7-(β-D-glucopyranosyl)-1-methoxybrassinin (**10**)*

HPLC $R_t = 9.2$ min; $[\alpha]_D = -55$ (*c* 0.54, MeOH). ^1H NMR (500 MHz, CD_3CN): δ 8.22 (br *s*, 1H D_2O exchangeable), 7.41 (*s*, 1H), 7.33 (*d*, $J = 7.7$ Hz, 1H), 7.06 (*dd*, $J = 7.7, 7.8$ Hz, 1H), 7.02 (*d*, $J = 7.7$ Hz, 1H), 5.14 (*d*, $J = 7.7$ Hz, 1H), 5.00 (*d*, $J = 4.4$ Hz, 2H), 4.14 (*s*, 3H), 3.42–3.84 (*m*, 10H, 4H D_2O exchangeable), 2.59 (*s*, 3H). ^{13}C NMR (125.8 MHz, CD_3CN): δ 198.5 (*s*), 144.0 (*s*), 126.8 (*s*), 125.5 (*d*), 123.6 (*s*), 121.2 (*d*), 113.7 (*d*), 108.7 (*d*), 107.7 (*s*), 101.6 (*d*), 77.1 (*d*), 76.9 (*d*), 74.1 (*d*), 70.5 (*d*), 67.2 (q), 61.9 (*d*), 42.1 (*t*), 17.6 (*q*). HRMS-ESI *m/z:* measured 445.1094 ($[M + H]^+$, calcd. 445.1097 for $C_{18}H_{25}N_2O_7S_2$). MS-ESI *m/z* (relative intensity): 445 ($[M + H]^+$, 58), 414 (72), 338 (100), 249 (25). FTIR (cm^{-1}): 3347, 2926, 2855, 1698, 1578, 1496, 1249, 1077 cm^{-1}. UV (CH_3CN) λ_{max} (log ε): 221 (4.5), 270 (4.0).

*4.5.5. 1-(β-D-glucopyranosyl)cyclobrassinin (**12**)*

HPLC $R_t = 8.6$ min; $[\alpha]_D = -14$ (*c* 0.23, MeOH). ^1H NMR (500 MHz, $(CD_3)_2CO$): δ 7.57 (*d*, $J = 8$ Hz, 1H), 7.50 (*d*, $J = 8$ Hz, 1H), 7.09–7.16 (*m*, 2H), 5.48 (br, s, 1H), 5.32 (*d*, $J = 17.7$ Hz, 1H), 4.77 (*d*, $J = 17.7$ Hz, 1H), 4.66 (br, s, 1H, D_2O exchangeable), 3.63–4.09 (*m*, 8H, 2H D_2O exchangeable), 2.54 (*s*, 3H). ^{13}C NMR (125.8 MHz, $(CD_3)_2CO$): δ 152.5 (*s*), 137.0 (*s*), 125.5 (*s*), 121.9 (*s*), 121.8 (*d*), 120.4 (*d*), 117.3 (*d*), 111.1 (*d*), 104.5 (*s*), 86.1 (*d*), 80.4 (*d*), 78.1 (*d*), 72.7 (*d*), 70.8 (*d*), 62.4 (*t*), 48.5 (*t*), 14.7 (*q*). HRMS-FAB *m/z:* measured 397.0881 ($[M + H]^+$, calcd. 397.0891 for $C_{17}H_{21}N_2O_5S_2$). MS-FAB *m/z* (relative intensity): 397 ($[M + H]^+$, 100), 396 (63), 395 (34), 329 (50). FTIR (cm^{-1}): 3380, 2923, 2852, 1617, 1452, 1346, 1249, 1079, 901, 734 cm^{-1}. UV (CH_3CN) λ_{max} (log ε): 231 (4.4), 286 (3.9).

*4.5.6. Methyl 1-(β-D-glucopyranosyl)tryptamine dithiocarbamate (**15**)*

HPLC $R_t = 8.9$ min.; $[\alpha]_D = -5$ (*c* 0.80, MeOH). ^1H NMR (500 MHz, CD_3CN): δ 8.18 (br *s*, 1H D_2O exchangeable), 7.68 (*d*, $J = 8$ Hz, 1H), 7.52 (*d*, $J = 8$ Hz, 1H), 7.29 (*s*, 1H), 7.22 (*ddd*, $J = 8, 8, 1$ Hz, 1H)

7.15 (*ddd*, $J = 8, 8, 1$ Hz, 1H), 5.44 (*d*, $J = 8$ Hz, 1H), 3.93 (*m*, 2H), 3.86 (*t*, $J = 8$ Hz, 1H), 3.45–3.76 (*m*, 8H, 3H exchangeable with D_2O), 3.08 (*m*, 2H), 2.76 (*s*, 1H exchangeable with D_2O), 2.53 (*s*, 3H), and a minor signal (ca. 1/8 intensity of the major one) due to a rotamer at 2.61 (*s*). ^{13}C NMR (125.8 MHz, CD_3CN): δ 198.8 (*s*), 137.0 (*s*), 128.9 (*s*), 123.7 (*d*), 122.4 (*d*), 119.3 (*d*), 119.1 (*d*), 113.3 (*s*), 110.8 (*d*), 85.2 (*d*), 79.1 (*d*), 77.9 (*d*), 72.6 (*d*), 70.6 (*d*), 62.0 (*t*), 47.4 (*t*), 23.8 (*t*), 17.5 (*q*). HRMS-FAB *m/z:* measured 413.1209 ($[M + H]^+$, calcd. 413.1205 for $C_{18}H_{25}N_2O_5S_2$). MS-FAB *m/z* (relative intensity): 413 ($[M + H]^+$, 41), 412 (25), 329 (42), 305 (30), 261 (100), 176 (98). FTIR (cm^{-1}): 3329, 2921, 2856, 1708, 1610, 1509, 1462, 1421, 1365, 1329, 1227, 1071, 1017, 930, 744, 620 cm^{-1}. UV (CH_3CN) λ_{max} (log ε): 223 (4.2), 273 (3.7).

4.5.7. *Methyl 5-(O-β-D-glucopyranosyl)-2-naphthylmethyl dithiocarbamate (**18**)*

HPLC $R_t = 9.4$ min.; $[\alpha]_D = -5$ (*c* 0.04, MeOH). ^1H NMR (300 MHz, CH_3CN) δ 8.54 (br *s*, 1H, D_2O exchangeable), 8.32 (*d*, $J = 9$ Hz, 1H), 7.76 (*s*, 1H), 7.53 (*d*, $J = 7.5$ Hz, 1H), 7.46 (*dd*, $J = 9, 1$ Hz, 1H) 7.42 (*dd*, $J = 7.5, 7.5$ Hz, 1H), 7.15 (*d*, $J = 7.5$ Hz, 1H), 5.09 (*d*, $J = 7.5$ Hz, 1H), 5.05 (*s*, 2H), 3.36–3.81 (*m*, 10H, 4H D_2O exchangeable), 2.59 (*s*, 3H). ^{13}C NMR (125.8 MHz, CD_3CN): δ 198.8 (*s*), 154.8 (*s*), 138.0 (*s*), 137.0 (*s*), 128.3 (*d*), 127.9 (*d*), 127.3 (*d*), 127.0 (*s*), 124.2 (*d*), 123.4 (*d*), 110.9 (*d*), 102.8 (*d*), 78.4 (*d*), 75.3 (*d*), 71.9 (*d*), 63.3 (*t*), 51.7 (*t*), 19.1 (q). HRMS-FAB *m/z:* measured 426.1048 ($[M + H]^+$, calcd. 426.1045 for $C_{19}H_{24}NO_6S_2$). FTIR (cm^{-1}): 3340, 3221, 2924, 2853, 1740, 1492, 1367, 1247, 1073, 929, 788 cm^{-1}. UV (CH_3CN) λ_{max} (log ε): 220 (4.6), 274 (3.7).

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