Metabolism of crucifer phytoalexins in *Sclerotinia sclerotiorum*: detoxification of strongly antifungal compounds involves glucosylation†

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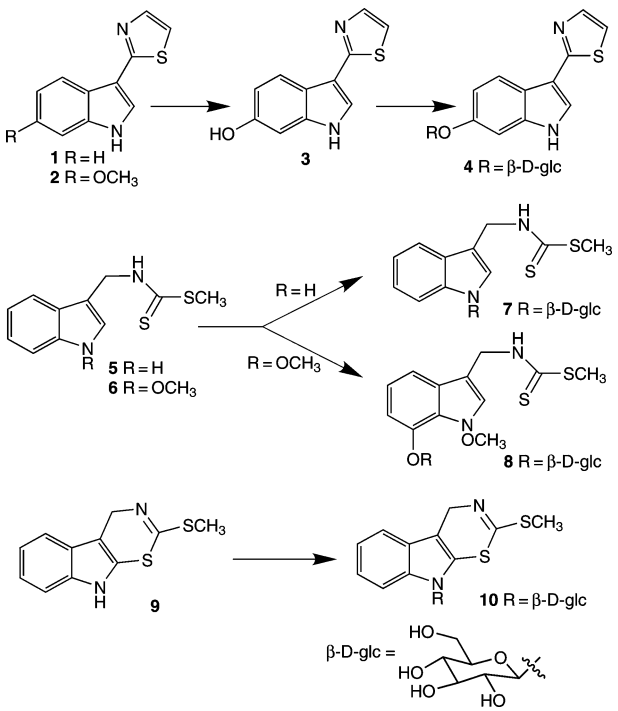
The strongly antifungal phytoalexins brassilexin and sinalexin were metabolized by the stem rot fungus *Sclerotinia sclerotiorum* to glucosyl derivatives, whereas the phytoalexins brassicanal A, spirobrassinin and 1-methoxyspirobrassinin, displaying lower antifungal activity, were transformed *via* non-glucosylating pathways. Significantly, these transformations led to metabolites displaying no detectable antifungal activity. The chemical characterization of all new metabolites as well as the chemistry of these processes and a facile chemical synthesis of 1-β-D-glucopyranosylbrassilexin are reported. Overall, our results indicate that phytoalexins, strongly antifungal against *S. sclerotiorum*, are detoxified *via* glucosylation, which in turn suggests that *S. sclerotiorum* has acquired efficient glucosyltransferase(s) that can disarm some of the most active plant chemical defenses. Consequently, we suggest that these glucosylation reactions are potential metabolic targets to control *S. sclerotiorum*.

Introduction

The fungus *Sclerotinia sclerotiorum* (Lib.) de Bary causes stem rot in crucifer plants, an economically important disease leading to severe yield losses around the world. Because no commercially useful stem rot resistant species are known, and the application of fungicides poses serious environmental concerns,[1,2] the discovery of metabolic targets that bring about the selective control of this pathogen is of enormous interest. However, the detection of such targets requires a reasonable understanding of the metabolic processes involved in the interaction of crucifers with *S. sclerotiorum*. It is well known that upon pathogen attack, crucifers produce *de novo* a complex mixture of defense metabolites that have antifungal activity, *i.e.* phytoalexins.[3] Furthermore, while some crucifer pathogens are able to detoxify phytoalexins effectively, others, although sensitive, are unable to transform these plant defenses.[4] For example, the pathogen *S. sclerotiorum* can detoxify camalexin (**1**) and 6-methoxycamalexin (**2**) *via* 6-hydroxycamalexin (**3**) to 6-oxy-(*O*-β-D-glucopyranosyl)camalexin (**4**) (Scheme 1), whereas *Leptosphaeria maculans* [Desm. Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr) Desm.] is unable to detoxify camalexin (**1**), albeit being sensitive to it.[4] As well, *S. sclerotiorum* can detoxify brassinin (**5**), 1-methoxybrassinin (**6**) and cyclobrassinin (**9**) to the glucosides **7**, **8**, and **10**, respectively (Scheme 1).[5] These glucosylation processes appear to be unusual reactions in plant pathogenic fungi, but are common transformations in plants.[6] To date, the significance of several phytoalexins in the crucifer–*S. sclerotiorum* interaction and the reaction of *S. sclerotiorum* to a number of seemingly important phytoalexins remains to be established.



Scheme 1 Biotransformation of camalexins **1** and **2**, brassinins **5** and **6** and cyclobrassinin **9** in *Sclerotinia sclerotiorum*.[5,6]

In continuation of our investigation of phytoalexin detoxification reactions occurring in *S. sclerotiorum*,[5,6] we have analyzed the metabolism of the phytoalexins brassilexin (**11**), sinalexin (**15**), brassicanal A (**14**), spirobrassinins **22** and **24**, as well as methyl derivatives **18** and **27**. Here we report for the first time the detoxification pathways and the characterization of various new metabolites as well as the chemistry involved in these processes and a facile chemical synthesis of β-D-glucopyranosylbrassilexin (**12**). Overall, our results indicate that phytoalexins, which are strongly antifungal against *S. sclerotiorum*, are detoxified *via* glucosylation.

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† Electronic supplementary information (ESI) available: ^{1}H NMR spectra of new compounds **12**, **16**, **17**, **19**, **23**, **25**–**28**, **31** and **32**. See DOI: 10.1039/b604400j

Consequently, we suggest that these glucosylation reactions emerge as potential metabolic targets to control *S. sclerotiorum*.

Results

Biotransformations

Phytoalexins **11**, **14**, **15**, **22**, and **24** and their derivatives **18** and **27** (1×10^{-4} M) were administered to fungal cultures of *S. sclerotiorum*, the cultures were incubated and analyzed over a period of several days to determine the best times to isolate potential metabolic products. HPLC chromatograms of extracts of cultures incubated with brassilexin (**11**) indicated that **11** was completely metabolized in *ca.* 48 h (Fig. 1). The metabolites were found to be an unknown compound with t_R = 4.5 min (**12**), the known phytoalexin brassicanal A (**14**), and 3-(amino)methylenindoline-2-thione (**13**), resulting from reduction of the isothiazole ring of brassilexin (**11**)[7] (Scheme 2). Enamine **13** was detected in culture immediately after adding brassilexin (**11**), while brassicanal A (**14**) was detected after 6 h of incubation and the unknown metabolite (**12**) was detected after 12 h. To determine the sequence of the biotransformation steps, enamine **13** was synthesized and administered to cultures of *S. sclerotiorum*. Culture samples were withdrawn at different times, these were extracted and the extracts were analyzed by HPLC;

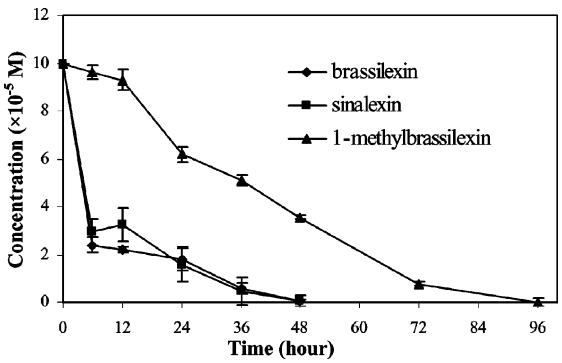
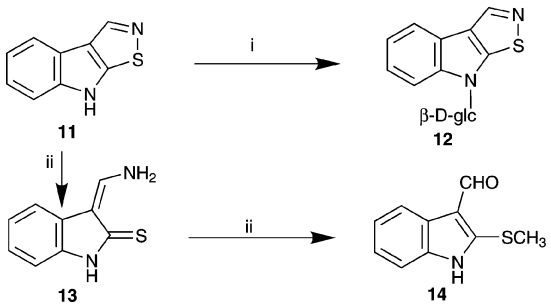


Fig. 1 Progress curves of the metabolism of brassilexin (**11**), sinalexin (**15**) and 1-methylbrassilexin (**18**) in *Sclerotinia sclerotiorum*. Cultures were extracted and extracts were analyzed by HPLC; concentrations were determined using calibration curves; each point is an average of experiments conducted in triplicate ± standard deviation.



Scheme 2 Biotransformation of brassilexin (**11**) in *Sclerotinia sclerotiorum*: (i) main pathway, (ii) minor pathway.

the chromatograms indicated that enamine **13** was completely metabolized to brassicanal A (**14**) in *ca.* 12 h. Subsequently, to isolate the unknown metabolite with t_R = 4.5 min (**12**), larger scale cultures of *S. sclerotiorum* were incubated with brassilexin (**11**). After 24 h, the extracts obtained from these cultures were fractionated by reverse phase silica gel chromatography, and each fraction was analyzed by HPLC. The fractions containing the unknown metabolite (**12**) were combined and further separated by preparative TLC to yield chromatographically homogeneous material. The ^{1}H NMR spectrum, obtained in CD_3OD, indicated the presence of the intact brassilexin (**1**) tricyclic system plus a doublet at δ_H 5.75 (J = 9 Hz, 1H) and several multiplets at δ_H 3.49–3.96, suggesting the presence of a carbohydrate moiety. Both the molecular formula ($C_{15}H_{16}N_2O_5S$ obtained by HRMS-ESI) and the ^{13}C NMR spectral data corroborated the presence of a carbohydrate residue. The identity of the carbohydrate moiety was assigned as a β-glucopyranosyl residue from ^{1}H–^{1}H homonuclear decoupling experiments (axial–axial couplings, J = 7–9 Hz, between the various protons). The β-glucopyranosyl unit was established to be located at N-1 from analysis of the HMBC data (correlations of the anomeric proton H-1′ with C-2 and C-7a of the indole moiety). Furthermore, the structure of this new metabolic product of brassilexin (**1**) was confirmed to be 1-β-D-glucopyranosylbrassilexin (**12**) by synthesis, as described below. Hence, the biotransformation of brassilexin (**11**) in *S. sclerotiorum* proceeded *via* two different pathways (Scheme 2): (i) glucosylation of brassilexin at N-1, and (ii) reductive ring opening of the isothiazole moiety. Although the yield of glucoside **12** was lower than that of brassicanal A (**14**) (Table 1), since **12** was further

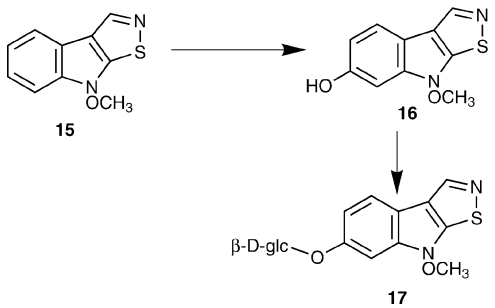
Table 1 Products of metabolism of phytoalexins **11**, **14**, **15**, **22**, and **24** and their analogues **18** and **27** (1×10^{-4} M) in cultures of *Sclerotinia sclerotiorum*

Compound added to cultures	Incubation time	Products of metabolism (%)[a]	Recovered starting material (%)[a]
Brassilexin (**11**)	24 h	**14** (18%); **12** (7%)	15
1-β-D-Glucopyranosylbrassilexin (**12**)	48 h	Complete transformation to undetermined products	None
3-(Amino)methylenindoline-2-thione (**13**)	6 h	**14** (10%)	None
Brassicanal A (**14**)	6 d	**20** (15%); **21** (13%)	28
Sinalexin (**15**)	30 h	**16** (2%); **17** (15%)	8
6-Oxy-(*O*-β-D-glucopyranosyl)sinalexin (**17**)	48 h	Complete transformation to undetermined products	None
1-Methylbrassilexin (**18**)	4 d	**19** (7%)	10
Spirobrassinin (**22**)	7 d	**23** (22%)	20
1-Methoxyspirobrassinin (**24**)	7 d	**25** (16%); **26** (7%)	20
1-Methylspirobrassinin (**27**)	7 d	**22** (7%); **28** (16%); **29** (5%)	16

[a] Percentage yields (molar) of products represent HPLC-determined yields.

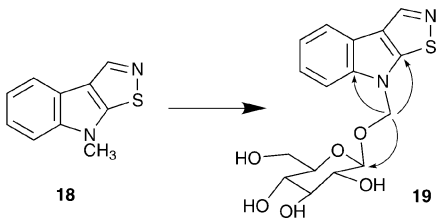
metabolized at a faster rate than brassicanal A (**14**) (48 h *vs.* 7 d), it becomes apparent that glucosylation represents the main metabolic pathway.

HPLC analysis of the broth extracts of cultures incubated with sinalexin (**15**) indicated it to be completely metabolized to two products with $t_R = 4.9$ and 12.0 min, in *ca.* 48 h (Fig. 1). To obtain sufficient quantities of each product for both chemical characterization and bioassay, larger scale mycelial cultures incubated with **15** were extracted, the extract was fractionated by reverse phase silica gel chromatography and each fraction was analyzed by HPLC. The fractions containing new metabolites were combined and further separated by reverse phase preparative TLC. The molecular formula of the less polar metabolite (**16**, $t_R = 12.0$ min) (obtained by HRMS-EI) indicated the presence of an additional oxygen atom relative to that of sinalexin (**15**) ($C_{10}H_8N_2O_2S$ *vs.* $C_{10}H_8N_2OS$) and the ^{1}H NMR spectrum indicated the presence of a substituted sinalexin, since only four protons were displayed in the aromatic region. Three of the signals were assigned to the spin system in the benzene ring and a singlet at δ_H 8.63 was assigned to the isothiazole ring. These spectroscopic data suggested that the less polar metabolite (**16**) contained an OH group located either at C-5 or C-6. That the OH group was attached to C-6 rather than C-5 was finally deduced from NOE experiments, as follows. Irradiation of the *N*-methoxy group at δ_H 4.14 caused an enhancement of the signal due to H-7 (δ_H 6.98) and *vice versa*. That is, assignment of the resonance of H-7 demonstrated that the HO group was located at H-6 and thus **16** was the structure of the less polar metabolite. The molecular formula of the more polar metabolite (**17**, $t_R = 4.9$ min, $C_{16}H_{18}N_2O_7$) 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 (^{1}H) decoupling experiments and X-ray crystallography (see ESI†). To establish the sequence of biotransformation steps of sinalexin (**15**), compound **16** was administered to cultures of *S. sclerotiorum*, samples were withdrawn at different times, and these were extracted and analyzed by HPLC. As expected, compound **16** was completely metabolized to **17** in *ca.* 12 h. This result indicated that sinalexin (**15**) was metabolized to 6-oxy-(*O*-β-D-glucopyranosyl)sinalexin (**17**) *via* 6-hydroxysinalexin (**16**) (Scheme 3).



Scheme 3 Biotransformation of sinalexin (**15**) in *Sclerotinia sclerotiorum*.

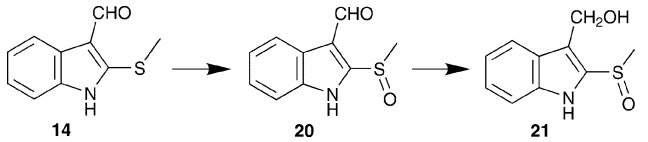
To probe the substrate specificity of the enzyme(s) involved in the metabolism of brassilexin (**11**) and sinalexin (**15**), 1-methylbrassilexin (**18**) was synthesized and incubated with cultures of *S. sclerotiorum* as described for brassilexin (**11**). HPLC analysis of extracts of fungal cultures incubated with 1-methylbrassilexin (**18**) showed that the rate of metabolism of **18** was slower than the transformation rates of brassilexin (**11**) and sinalexin (**15**) (Fig. 1). While the naturally-occurring **11** and **15** were completely metabolized in about two days, 1-methylbrassilexin (**18**) was completely metabolized to an unknown polar compound (**19**, $t_R = 4.4$ min) in about four days. To establish the structure of this polar metabolite (**19**), larger scale cultures of *S. sclerotiorum* incubated with 1-methylbrassilexin (**18**) were extracted and the extracts were fractionated by reverse phase silica gel column chromatography. Fractions containing the new metabolite were further separated by preparative TLC to yield a chromatographically homogeneous solid material. The ^{1}H NMR spectrum of this compound [**19**, $(CD_3)_2CO$)] showed five aromatic hydrogens, suggesting the presence of an intact brassilexin moiety, and a methylene group (δ_H: 6.13, d, $J = 11.5$ Hz, 1H; 5.90, d, $J = 11.5$ Hz) instead of the (*N*)Me group. The ^{13}C NMR spectrum of **19** confirmed the absence of the (*N*)Me group and the presence of the methylene at δ_C 73.4, which indicated that the (*N*)Me group had been oxidized to (*N*)CH_2O–R. Additional signals at δ_H 4.39 (d, $J = 8$ Hz, 1H) and several multiplets at δ_H 3.85–3.50 suggested the presence of a carbohydrate moiety. The molecular formula of $C_{16}H_{18}N_2O_6S$ (obtained by HRMS-ESI) and ^{13}C NMR spectral data also indicated the presence of a carbohydrate residue. The identity of the carbohydrate moiety was assigned as a β-glucopyranosyl residue from ^{1}H–^{1}H homonuclear decoupling experiments (axial–axial couplings, $J = 7$–9 Hz). HMBC spectral data showed correlations of (*N*)CH_2O protons with C-2 and C-7a of indole and also with the anomeric carbon (C-1′) as shown in Scheme 4, suggesting that the β-glucopyranose unit was attached to the oxygen atom of the (*N*)CH_2O group. From this reasoning the structure of the biotransformation product of 1-methylbrassilexin (**18**) was assigned as 1-methyl-(oxy-*O*-β-D-glucopyranosyl)brassilexin (**19**) (Scheme 4).



Scheme 4 Biotransformation of 1-methylbrassilexin (**18**) in *Sclerotinia sclerotiorum* and selected HMBC correlations of **19**.

HPLC analysis of extracts obtained from fungal cultures of *S. sclerotiorum* incubated with brassicanal A (**14**) showed it to be completely metabolized to 3-(hydroxymethyl)indole-2-methylsulfoxide (**21**) *via* brassicanal A sulfoxide (**20**) (Scheme 5) in *ca.* 7 d (Fig. 2). After isolation of metabolites **20** and **21** their structures were deduced from comparison of their spectroscopic data to those of brassicanal A and finally confirmed by synthesis.[8] To ascertain the sequence of the biotransformation steps, compound **20** was separately incubated with cultures of *S. sclerotiorum*, and extracts of the fungal cultures collected at different times were analyzed by HPLC. These experiments confirmed that the aldehyde group of brassicanal A (**14**) was enzymatically reduced to alcohol **21** (Scheme 5).

Compared to the transformation of brassilexin (**11**) and sinalexin (**15**), the biotransformation of the phytoalexin spirobrassinin (**22**) in *S. sclerotiorum* was a much slower process.



Scheme 5 Biotransformation of the phytoalexin brassicanal A (**14**) in *Sclerotinia sclerotiorum*.

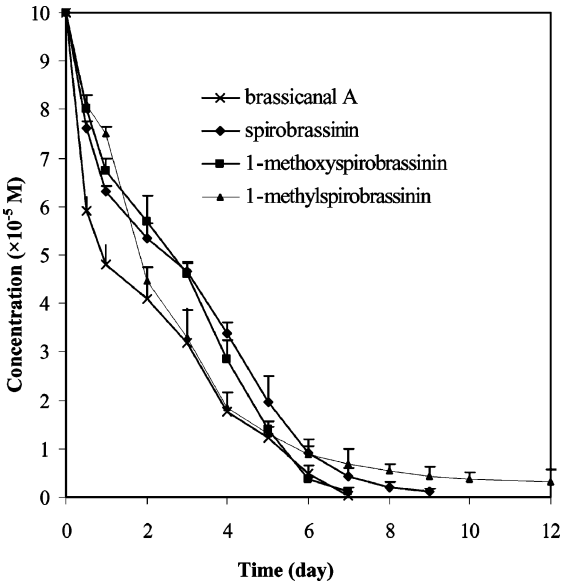
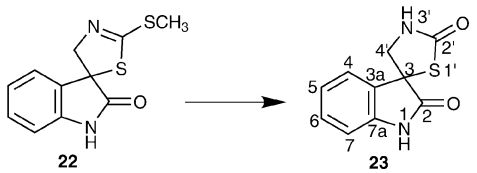


Fig. 2 Progress curves of the metabolism of brassicanal A (**14**), spirobrassinin (**22**), 1-methoxyspirobrassinin (**24**) and 1-methylspirobrassinin (**27**) in *Sclerotinia sclerotiorum*. Cultures were extracted and the extracts were analyzed by HPLC; concentrations were determined using calibration curves; each point is an average of experiments conducted in triplicate ± standard deviation.

Spirobrassinin (**22**) was detected in cultures up to nine days after incubation with *S. sclerotiorum* (Fig. 2); a single biotransformation product (**23**, HPLC t_R = 5.1 min) substantially more polar than spirobrassinin was detected. Similar to the experiments described above, to establish the structure of this metabolic product, larger scale cultures of *S. sclerotiorum* were incubated with spirobrassinin (**22**) for seven days, then filtered, extracted, and the broth extract fractionated by column chromatography followed by preparative TLC to yield a new metabolite (**23**). Standard spectroscopic analyses (^{1}H and ^{13}C NMR, HMQC, HMBC, and HRMS-EI) indicated the molecular formula $C_{10}H_8N_2O_2S$. Comparison of the ^{1}H NMR spectra of spirobrassinin (**22**) and that of the new metabolite (**23**) revealed the presence of an NH signal at δ_H 6.40 and the absence of the SCH_3 signal in the latter. A downfield shift for the C-2′ carbon (δ_H 163.2 in **22** to 171.9 in **23**) in the ^{13}C NMR spectrum suggested the presence of a carbonyl group (NHC=OSR). Hence, on the basis of these spectral data, the structure of the new metabolite **23** was assigned as a spirothiazolidinone attached to C-3 of the oxoindole ring (Scheme 6, **23**, $[a]_D = -35$). The enantiomeric excess (ee) of untransformed spirobrassinin (**22**) recovered from cultures after a seven day incubation period was determined to be 14% by ^{1}H NMR spectroscopy (integration of the SMe resonances) using the chiral solvating agent (*R*)-2,2,2-trifluoro-1-(9-anthryl)ethanol (TFAE, Table 2).[9] However, the enantiomeric excess of metabolite



Scheme 6 Biotransformation of spirobrassinin (**22**) in *Sclerotinia sclerotiorum*.

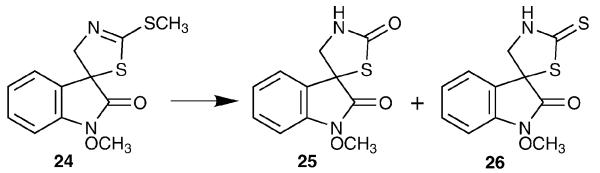
23 could not be determined (the diastereotopic methylene protons were not sufficiently resolved in the presence of the chiral solvating agent TFAE).

HPLC analysis of the broth extract of fungal cultures incubated with 1-methoxyspirobrassinin (**24**) indicated it to be completely metabolized to two products with t_R = 7.5 and 11.5 min in *ca.* 10 days (Fig. 2). The structure of each product was determined from comparison of their spectroscopic data and those of 1-methoxyspirobrassinin (**24**). The ^{1}H NMR spectra of both compounds (**25** and **26**) showed four aromatic hydrogens, characteristic of a 2-oxoindole nucleus and two additional hydrogens (H-4′) with geminal coupling. In addition, both compounds showed a signal for an exchangeable hydrogen and the absence of the SCH_3 signal. The ^{13}C NMR spectrum of the compound with t_R = 7.5 min (**25**) displayed a downfield shift attributable to C-2′ (δ_C 163.2 in **24** to 171.4 in **25**), suggesting the presence of a carbonyl group [NH(S)C=O], whereas the compound with the t_R = 11.5 min (**26**) showed a substantially higher chemical shift for C-2′ (δ_C 163.2 in **24** to 198.3 in **26**), suggesting the presence of a thiocarbonyl group [NH(S)C=S]. These data were consistent with the molecular formula of each compound determined by HRMS-EI (**25**, $C_{11}H_{10}N_2O_3S$, and **26**, $C_{11}H_{10}N_2O_2S_2$). That is, the SCH_3 group of 1-methoxyspirobrassinin (**24**) had been transformed to a carbonyl group in **25** and to a thiocarbonyl group in **26**. On the basis of these results, the structure of the major metabolite (t_R = 7.5 min) was established as the spirothiazolidinone **25** and the structure of the minor metabolite (t_R = 11.5 min) was established as the spirothiazolidinethione **26** (Scheme 7). As established for spirobrassinin (**22**), the ee of 1-methoxyspirobrassinin (**24**) isolated after incubation for seven days (33% ee) and of metabolites **25** (11% ee) and **26** (30% ee) were determined by ^{1}H NMR spectroscopy using the chiral solvating agent TFAE (Table 2).[9]

Table 2 Enantiomeric excess (ee) and optical rotation of spirobrassinins **22**, **24**, **27**, and metabolites **23**, **25**, **26**, and **28**

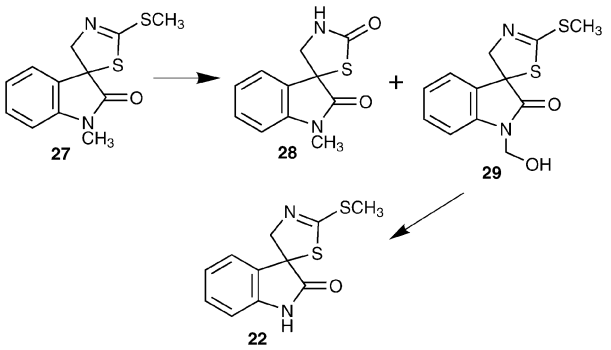
Compounds; amount recovered from cultures after incubation for 7 d	Ee (%)[a]	Optical rotation $[a]_D$
Spirobrassinin (**22**); 20%	14[b]	−15 (*c* 0.34, MeOH)
Spirooxathiazolidinone (**23**); 22%	nd[c]	−35 (*c* 0.33, MeOH)
1-Methoxyspirobrassinin (**24**); 20%	33[d]	+11 (*c* 0.21, MeOH)
Spirothiazolidinone (**25**); 16%	11[d]	−7 (*c* 0.34, MeOH)
Spirothiazolidinethione (**26**); 7%	30[d]	−31 (*c* 0.10, MeOH)
1-Methylspirobrassinin (**27**); 16%	26[e]	+7 (*c* 0.25, MeOH)
Spirothiazolidinone (**28**); 16%	33[e]	−5 (*c* 0.20, MeOH)

[a] Enantiomeric excess {ee = ([*R* − *S*]/[*R* + *S*]) × 100} was determined using chiral solvating reagent (*R*)-2,2,2-trifluoro-1-(9-anthryl)ethanol (TFAE) by ^{1}H NMR.[9] [b] Determined by integration of the ^{1}H NMR signals of SCH_3. [c] nd = not determined as ^{1}H NMR signals were not resolved. [d] Determined by integration of the ^{1}H NMR signals of OCH_3. [e] Determined by integration of the ^{1}H NMR signals of NCH_3.



Scheme 7 Biotransformation of 1-methoxyspirobrassinin (**24**) in *Sclerotinia sclerotiorum*.

Similar to the biotransformation of spirobrassinins **22** and **24**, the biotransformation of 1-methylspirobrassinin (**27**) – a synthetic analogue of spirobrassinin (**22**) – by *S. sclerotiorum* was a very slow process. Compound **27** was completely metabolized only after incubation for 12 days (Fig. 2). The metabolism of this compound by the fungus *S. sclerotiorum* led to the detection and isolation of three metabolites with t_R = 6.6, 11.1 and 13.2 min (**28**, **29**, and **22**, respectively, Scheme 8). The structure of each metabolite was determined from comparison of its spectroscopic data and those of 1-methylspirobrassinin (**27**). The ^{1}H NMR spectrum of the most polar compound (t_R = 6.6 min, **28**) showed the four aromatic hydrogens characteristic of a 2-oxoindole nucleus and two additional hydrogens (H-4′) showing geminal coupling. In addition, compound **28** showed a proton resonance attributable to the NH and the absence of the proton resonance due to SCH_3. The ^{13}C NMR of **28** showed a downfield shift for the C-2′ carbon (δ_C 163.2 in **27** to 171.9 in **28**) which suggested the presence of a carbonyl group, *i.e.* transformation of the N=C(SCH$_3$)S group to the NH–C=O(S) group. These assumptions were consistent with the molecular formula obtained by HRMS-EI ($C_{11}H_{10}N_2O_2S$,). Thus, on the basis of these results the structure of this metabolite was assigned as the spirothiazolidinone **28** (Scheme 8). The compound of intermediate polarity (t_R = 11.1 min, **29**), relative to 1-methylspirobrassinin (**27**) ($C_{12}H_{12}N_2OS_2$) contained an additional oxygen atom ($C_{12}H_{12}N_2O_2S_2$), as determined by HRMS-EI. Comparison of the ^{1}H NMR spectrum of the parent compound **27** with that of **29** indicated the presence of signals attributable to NCH_2OH (δ_H 5.21 and 5.35) and the absence of the NCH_3 signal. This reasoning was corroborated by the ^{13}C NMR spectrum [downfield shift for the (N)CH_2OH carbon δ_C 26.7 in **27** to 64.7 in **29**]. That is, the *N*–CH_3 group was oxidized enzymatically to the *N*–CH_2–OH group. Therefore, the structure of this metabolite was assigned as 1-hydroxymethylspirobrassinin (**29**, Scheme 8). The third metabolite was established as spirobrassinin (**22**) based on its spectroscopic data and comparison with an authentic sample.



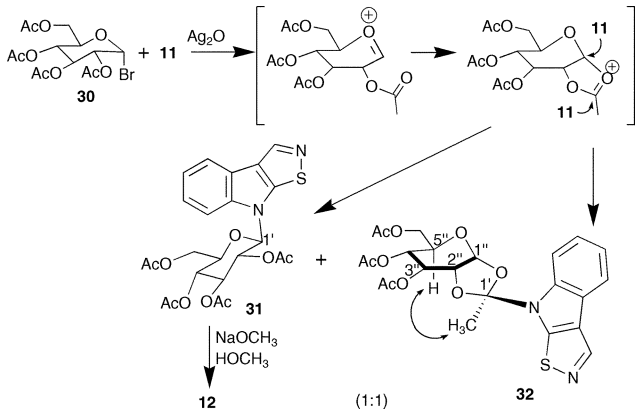
Scheme 8 Biotransformation of 1-methylspirobrassinin (**27**) in *Sclerotinia sclerotiorum*.

To establish the sequence of biotransformation steps, compound **29** was administered to cultures of *S. sclerotiorum*. As expected, spirobrassinin (**22**) was detected in the HPLC chromatogram of the broth extract of these cultures, demonstrating it to be a metabolite of **29** resulting from enzymatic oxidation followed by decarboxylation of **27** (Scheme 8). As described for 1-methoxyspirobrassinin (**24**), the ee values of untransformed 1-methylspirobrassinin (**27**) and metabolite **28** were determined using the chiral solvating agent TFAE (Table 2).[9]

Synthesis

The phytoalexins **11**, **14**, **15**, **22**, and **24** were synthesized following previously reported procedures,[3] whereas the 1-methyl derivatives of brassilexin (**11**) [1-methylbrassilexin (**18**)] and spirobrassinin (**22**) [1-methylspirobrassinin (**27**)] were synthesized from their parent compounds following treatment with NaH/MeI. The chemical synthesis of 1-β-D-glucopyranosylbrassilexin (**12**) was carried out to confirm the absolute stereochemistry of the biotransformation product of brassilexin (**11**) and to obtain sufficient amounts for bioassays.

N-Glucosylation of indolyl-containing molecules has been reported for a number of substrates,[10] including the syntheses of *N*-glucosylated brassinin (**5**), brassenins A and B, cyclobrassinin (**9**) and related compounds.[11] However, the indoline-indole methodology[12] or the various carbohydrate donors used in those preparations were not readily applicable to brassilexin (**11**). On the other hand, the reaction of 6-nitroindole with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**30**) in the presence of silver oxide, reported to yield a mixture of *O*-acetylated 1,2-*O*-[1-(6-nitroindol-1-yl)ethylidene]-α-D-glucose and 1,2-*O*-[1-(6-nitroindol-3-yl)ethylidene]-α-D-glucose, appeared promising.[13] Although in that synthesis no *N*-glucosylated product was observed, because brassilexin (**11**) had the C-2 and C-3 positions of the indole moiety blocked and no electron-withdrawing groups were present, it was expected to be substantially more reactive than 6-nitroindole. Thus, an approach similar to that used for 6-nitroindole was chosen to synthesize 1-β-D-glucopyranosylbrassilexin (**12**). Subsequently, coupling of brassilexin (**11**) with 1-bromo-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranose (**30**) in the presence of silver oxide yielded a mixture of D-glucopyranosylbrassilexins **31** and **32** in a 1 : 1 ratio (Scheme 9). Finally, deacetylation of **31** yielded 1-β-D-glucopyranosylbrassilexin (**12**), albeit in a rather poor yield (12%). Synthetic 1-β-D-glucopyranosylbrassilexin (**12**) was identical in all respects to the sample isolated from fungal cultures of *S. sclerotiorum* and was used to carry out all bioassays. It is likely that the yield of **31** could be improved by using other protecting groups in **30**, to prevent the neighboring group assistance effect depicted in Scheme 9.[14] The absolute stereochemistry of the stereogenic center C-1′ of compound **32** was established using NOESY data. The NOESY spectrum of **32** showed a correlation between the methyl group at C-1′ and the H-5″ of the glucosyl residue (Scheme 9). This correlation suggested that the new stereocenter C-1′ had the *S* configuration, which was consistent with that reported for tryptophan *N*-glucoside.[15] Furthermore, contrary to 1-β-D-glucopyranosylbrassilexin (**12**), the H–H coupling constants obtained for H-1″, H-2″ and H-3″ (*cf.* experimental data) suggest that the glucosyl moiety of **32** is not in a chair conformation.



Scheme 9 Synthesis of 1-β-D-glucopyranosylbrassilexin (**12**) and selected NOE of compound **32**.

Antifungal assays

The antifungal activity of brassilexin (**11**), brassicanal A (**14**), sinalexin (**15**), 1-methylbrassilexin (**18**), spirobrassinin (**22**), 1-methoxyspirobrassinin (**24**), and 1-methylspirobrassinin (**27**) against *S. sclerotiorum* was investigated using the mycelial radial growth bioassay reported in the Experimental section.[6] After incubation for three days, the mycelium of control plates incubated with *S. sclerotiorum* covered 100% of the plate surfaces, while plates containing **11** showed no mycelial growth at either 1×10^{-4} or 5×10^{-5} M and plates containing **15** showed growth at 1×10^{-4} M. By contrast plates containing metabolites **12**, **17**, **19**, **20**, **21**, **23**, **25**, **26** and **29** showed growth similar to controls, even at 5×10^{-4} M (Table 3).

Discussion and conclusion

Bioassays conducted with brassilexins **11** and **18**, sinalexin (**15**), spirobrassinins **22**, **24**, and **27** and brassicanal A (**14**) against *S. sclerotiorum* showed that the most antifungal compounds were brassilexins **11** and **18** and sinalexin (**15**), and that brassilexin (**11**) displayed the strongest activity (Table 3). On the other hand, the metabolites resulting from the fungal transformation of **11**, **14**, **15**, **18**, **22**, **24**, and **27** were not toxic to *S. sclerotiorum*, indicating that all these metabolic transformations were detoxification processes. Comparison of the inhibitory activity of brassilexin (**11**, 100% inhibition at 5×10^{-5} M, Table 3) and sinalexin (**15**, 100% inhibition at 1×10^{-4} M, 80 ± 4% inhibition at 5×10^{-5} M, Table 3) against *S. sclerotiorum* with the activities of camalexin (**1**, 100% inhibition at 1×10^{-4} M and 76 ± 5% at 5×10^{-5} M)[6] and brassinins **5** (100% inhibition at 3×10^{-4} M, 37 ± 8% inhibition

Table 3 Percentage of growth inhibition[a] of *Sclerotinia sclerotiorum* incubated with phytoalexins brassilexin (**11**), brassicanal A (**14**), sinalexin (**15**), spirobrassinin (**22**), 1-methoxyspirobrassinin (**24**), and derivatives 1-methylbrassilexin (**18**) and 1-methylspirobrassinin (**27**) (two days, constant light)

Compound assayed against *S. sclerotiorum*	Concentration/M	Inhibition ± SD (%)[a]
Control	—	0
Brassilexin (**11**)	1×10^{-4}	100 ± 0
	5×10^{-5}	100 ± 0
	2×10^{-5}	76 ± 5
1-β-D-Glucopyranosylbrassilexin (**12**)	5×10^{-4}	No inhibition
3-(Amino)methylenindoline-2-thione (**13**)	5×10^{-4}	64 ± 2
	3×10^{-4}	42 ± 2
	1×10^{-4}	18 ± 3
Brassicanal A (**14**)	5×10^{-4}	42 ± 5
	3×10^{-4}	17 ± 4
	1×10^{-4}	No inhibition
Sinalexin (**15**)	1×10^{-4}	100 ± 0
	5×10^{-5}	80 ± 4
	2×10^{-5}	60 ± 6
6-Oxy-(*O*-β-D-glucopyranosyl)sinalexin (**17**)	5×10^{-4}	No inhibition
1-Methylbrassilexin (**18**)	3×10^{-4}	100 ± 0
	1×10^{-4}	43 ± 3
	5×10^{-5}	24 ± 6
1-Methyl-(oxy-*O*-β-D-glucopyranosyl)brassilexin (**19**)	5×10^{-4}	No inhibition
Brassicanal A sulfoxide (**20**)	5×10^{-4}	No inhibition
3-(Hydroxymethyl)indol-2-methylsulfoxide (**21**)	5×10^{-4}	No inhibition
Spirobrassinin (**22**)	5×10^{-4}	58 ± 3
	3×10^{-4}	38 ± 8
	1×10^{-4}	26 ± 5
Spirothiazolidinone (**23**)	5×10^{-4}	No inhibition
1-Methoxyspirobrassinin (**24**)	5×10^{-4}	24 ± 4
	3×10^{-4}	10 ± 4
	1×10^{-4}	No inhibition
Spirothiazolidinone (**25**)	5×10^{-4}	No inhibition
Spirothiazolidinethione (**26**)	5×10^{-4}	No inhibition
1-Methylspirobrassinin (**27**)	5×10^{-4}	49 ± 2
	3×10^{-4}	36 ± 7
	1×10^{-4}	20 ± 7
1-Hydroxymethylspirobrassinin (**29**)	5×10^{-4}	No inhibition

[a] The percentage of inhibition was calculated using the formula: % inhibition = 100 − [(growth on treated/growth in control) × 100]; experiments were conducted in triplicate.

at 1×10^{-4} M) and **6** (100% inhibition at 3×10^{-4} M, 56 ± 6% inhibition at 1×10^{-4} M)[5] indicates that (i) **11** is a stronger inhibitor of mycelial growth than **1**, **5** or **6**, and (ii) **15** is a stronger inhibitor than **5** or **6** but shows similar activity to that of camalexin (**1**).

Results of the metabolism of brassilexin (**11**) suggested that the main pathway of brassilexin detoxification involved glucosylation at N-1 to yield the corresponding *N*-glucosylated compound **12**, whereas in the case of sinalexin (**15**), in which the N-1 position is blocked with a methoxy group, detoxification involved oxidation to 6-hydroxysinalexin (**16**) followed by glucosylation to 6-oxy-(*O*-β-D-glucopyranosyl)sinalexin (**17**). In addition, a minor pathway for detoxification of brassilexin (**11**) in *S. sclerotiorum* involved reductive ring opening of the isothiazole to the enamine **13**, followed by methylation and hydrolysis (or *vice versa*) to the known phytoalexin brassicanal A (**14**). The yield of metabolite **12** was lower than that of brassicanal A (**14**) (Table 1); however, since 1-β-D-glucopyranosylbrassilexin (**12**) was metabolized at a faster rate than brassicanal A (**14**) was transformed (48 h *vs.* 7 d), the main pathway for brassilexin (**11**) detoxification appears to be glucosylation (Scheme 2). This is the first time that a phytoalexin is found to be an intermediate of phytoalexin detoxification in *S. sclerotiorum*. However, in previous studies with other crucifer pathogens (*Rhizoctonia solani*) brassicanal A (**14**) was found to be an intermediate in the detoxification of cyclobrassinin (**9**).[4]

Perhaps not surprisingly, compared to brassilexin (**11**) and sinalexin (**15**), detoxification of 1-methylbrassilexin (**18**), an unnatural compound, occurred at a substantially slower rate (*ca.* 2 d *vs.* 4 d). Because oxidation of C-6 of the indole moiety was observed in the transformation of sinalexin (**15**), it was surprising to observe oxidation of 1-methylbrassilexin (**18**) at the (*N*)–CH_3 rather than at C-6. These differences are likely due to the substrate specificity of the enzymes involved in the transformations of the natural substrates **11** and **15**. The substrate specificity of such enzymes was previously formulated and probed using analogues of camalexins **1** and **2**[6] and brassinins **5** and **6**.[5]

The detoxification reactions of the less antifungal phytoalexins brassicanal A (**14**), spirobrassinins **22** and **24** and analogue **27** in *S. sclerotiorum* were slower and yielded no glucosylation products. The detoxification of brassicanal A (**14**) involved the oxidation of S(CH_3) to the corresponding sulfoxide and reduction of the aldehyde to the alcohol, a process similar to the detoxification of brassicanal A in *L. maculans*.[8] The detoxification of spirobrassinins **22**, **24**, and **27** involved the hydrolysis of the spirothiazolidine moiety to spirothiazolidinones **23**, **25**, and **28**, respectively. In addition, spirothiazolidinethione **26** was isolated as a minor metabolite of 1-methoxyspirobrassinin (**24**), and 1-hydroxymethylspirobrassinin (**29**) was isolated as a minor metabolite of 1-methylspirobrassinin (**27**). The optical rotation of metabolite **23** and the significant ee of metabolites **26** and **28** suggested that their enzymatic formation was somewhat stereoselective in *S. sclerotiorum*. Furthermore, the following suggest that two or more enzymes are involved in these processes (Table 2): (1) the significant ee of **24** and **27** (recovered from cultures, Table 2) and similarity to the ee of their biotransformation products **26** and **28**, (2) the high percentages of conversion of spirobrassinins **22**, **24** and **27** (*ca.* 80%), and (3) the similar rates of transformation of either (*R*)- or (*S*)-spirobrassinin in *S. sclerotiorum*. However, further studies with purified enzymes would be required to determine their potential substrate stereoselectivity and catalytic promiscuity.[16]

Overall, our results suggested that the plant pathogen *S. sclerotiorum* utilizes different enzymes to transform different phytoalexins. The enzymes involved in the biotransformation of brassicanal A (**14**) and spirobrassinins **22**, **24**, and **27** might be 'house-keeping' enzymes used in general detoxification processes. By contrast, the detoxification reactions of brassilexin (**11**) and sinalexin (**15**) might be catalyzed by substrate-selective glucosyltransferases, while sinalexin (**15**) might require a selective oxidase as well. It is worthy to note that replacement of the methoxy group (at N-1) of sinalexin (**15**) with a methyl group decreases the rate and the site of transformation of **18**. These results and our previous work[5,6] suggest that *S. sclerotiorum* has acquired or evolved efficient glucosyltransferase(s) that can disarm some of the most active plant chemical defenses. Especially because brassilexin (**11**) and sinalexin (**15**) display (hitherto) the strongest antifungal activity against *S. sclerotiorum* and are metabolized efficiently to inactive products, our results suggest that the detoxification of **11** and **15** are potential metabolic targets to control *S. sclerotiorum*. For example, application of potential brassilexin (**11**) and/or sinalexin (**15**) detoxification inhibitors to infected plants might prevent the pathogen from metabolizing these phytoalexins and thus the continuous depletion of these natural plant defenses. A concentration increase of strongly antifungal phytoalexins is expected to slow down if not stop growth of *S. sclerotiorum*. Nonetheless, before such inhibitors can be designed, a better understanding of the enzymes and enzymatic mechanisms involved in these fungal transformations is required.

Experimental

General experimental procedures

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON, Canada. All solvents were HPLC grade and used as such, except for CH_2Cl_2 and $CHCl_3$ which were redistilled. The remaining conditions are as previously reported.[5]

HPLC analysis was carried out with a high performance liquid chromatograph equipped with a quaternary pump, automatic injector, and diode array detector (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, for 35 min, linear gradient, and a flow rate of 1.0 ml min^{-1}. 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)$^{-1}$. 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: for ^1H (500 MHz) and for ^{13}C (125 MHz) the chemical shifts are referenced to a solvent peak, as previously reported;[7] spin coupling constants (*J*) are reported to the nearest 0.5 Hz. Mass spectra (MS) were obtained on a VG 70 SE mass spectrometer using a solids probe or on a Q Star XL, Applied Biosystems.

Fungal cultures

S. sclerotiorum clone #33 (obtained from C. Lefol, AAFC, Saskatoon, Canada) was grown on potato dextrose agar (PDA)

plates at 20 ± 1 °C, in the dark. Sclerotia were collected over a four-week period and stored at 20 °C in the dark. Erlenmeyer flasks (250 ml) containing 100 ml of minimal media were inoculated with sclerotia of *S. sclerotiorum* and were incubated at 22 ± 1 °C on a shaker at 120 rpm under constant light.

Time-course studies of metabolism. Six-day-old cultures of *S. sclerotiorum* were incubated with phytoalexins or analogues 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 1×10^{-4} M) 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.

Large-scale metabolism 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).

Antifungal bioassays

The antifungal activity of compounds was determined using the following mycelial radial growth bioassay. Solutions of each compound in DMSO (5×10^{-2} M) were used to prepare assay solutions in minimal media (5×10^{-4}, 3×10^{-4}, 1×10^{-4}, 5×10^{-5}, 2×10^{-5} M); control solutions contained 1% DMSO in minimal 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 three-day-old PDA plates of *S. sclerotiorum*, clone #33) placed upside down on the center of each plate and incubated under constant light for three days. All bioassay experiments were carried out in triplicate at least three times.

Syntheses and spectral data

Synthesis of 1-β-D-glucopyranosylbrassilexin (12). Condensation of brassilexin (**11**) with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**30**): a solution of glucosyl bromide **30** (142 mg, 0.35 mmol) in dry benzene (3 ml) was added dropwise during 30 min to a mixture of brassilexin (**11**) (20 mg, 0.11 mmol) and Ag_2O (31 mg, 0.13 mmol) in dry benzene (3 ml) under stirring. The reaction mixture was allowed to reflux for 20 h at 90 °C, was filtered through a tight cotton plug and the insoluble material was washed with benzene. The combined filtrate and washings were concentrated, the residue was subjected to column chromatography (silica gel, EtOAc–hexane, 3 : 7), followed by preparative TLC to afford 2,3,4,6-tetra-*O*-acetyl-1-β-D-glucopyranosyl brassilexin (**31**) [6 mg, 12% based on recovered brassilexin (**11**)] and 1-[1-(3,4,6-tri-*O*-acetyl-1,2-*O*-α-D-glucopyranosyl)ethylidene]brassilexin (**32**) [6 mg, 12% based on recovered brassilexin (**11**)]. **31**: $[\alpha]_D = -3$ (*c* 0.40, CH_3OH). δ_H (500 MHz, CD_2Cl_2): 8.71 (s, 1H), 7.90 (d, $J = 7.5$ Hz, 1H), 7.54 (d, $J = 8$ Hz, 1H), 7.41 (dd, $J = 7.5$, 8 Hz, 1H), 7.31 (dd, $J = 7.5$, 7.5 Hz, 1H), 5.90 (d, $J = 9$ Hz, 1H), 5.56 (dd, $J = 9.5$, 9.5 Hz, 1H), 5.43–5.37 (m, 2H), 4.33 (s, br, 2H), 4.18–4.16 (m, 1H), 2.17 (s, 3H), 2.12 (s, 3H), 2.02 (s, 3H), 1.52 (s, 3H); δ_C (125 MHz, CD_2Cl_2): 170.7 (s), 170.2 (s), 169.7 (s), 168.7 (s), 157.8 (s), 147.6 (d), 143.6 (s), 127.9 (s), 124.3 (d), 122.0 (d), 121.0 (d), 120.8 (d), 110.8 (d), 83.5 (d), 75.5 (d), 72.6 (d), 70.5 (d), 68.2 (d), 61.8 (d), 20.9 (q), 20.8 (q), 20.7 (q), 20.0 (q). HRMS-ESI *m/z*: measured 503.1110 ($[M - 1]^-$, calc. 503.1124 for $C_{23}H_{23}N_2O_9S$). MS-ESI *m/z* (relative intensity): 503 ($[M - 1]^-$, 100), 461 (10), 173 (6). FTIR ν_{max} (KBr): 3059, 2945, 1752, 1503, 1473, 1444, 1370, 1222, 1102, 1039 cm^{-1}. UV (CH_3OH) λ_{max} (log ε): 222 (4.7), 244 (4.1), 264 nm (4.0). **32**: $[\alpha]_D = -19$ (*c* 0.30, CH_2Cl_2). δ_H (500 MHz, CD_2Cl_2): 8.70 (s, H-3′), 7.90 (d, $J = 8$ Hz, H-4), 7.82 (d, $J = 8$ Hz, H-7), 7.41 (dd, $J = 7.5$, 8 Hz, H-6), 7.33 (dd, $J = 8$, 7.5 Hz, H-5), 5.88 (d, $J = 5$ Hz, H-1″), 5.37 (underneath the solvent peak, H-3″), 4.99 (d, $J = 9.5$, Hz, H-4″), 4.35–4.26 (m, H-6a″, H-6b″, H-2″), 4.19–4.17 (m, H-5″), 2.19 (s, 3H), 2.15 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H); δ_C (125 MHz, CD_2Cl_2): 170.8 (s), 170.0 (s), 169.3 (s), 158.6 (s), 147.5 (d), 142.0 (s), 126.7 (s), 126.4 (s) 124.5 (d), 122.0 (d), 120.5 (d), 113.9 (s), 113.1 (d), 98.3 (d), 73.7 (d), 69.5 (d), 68.4 (d), 67.8 (d), 63.5 (d), 22.2 (q), 21.1 (q), 20.9 (q), 20.8 (q). HRMS-ESI *m/z*: measured 505.1280 ($[M + 1]^+$, calc. 505.1275 for $C_{23}H_{25}N_2O_9S$). MS-ESI *m/z* (relative intensity): 505 ($[M + 1]^+$, 100), 331 (9). FTIR ν_{max} (KBr): 3057, 2932, 1746, 1469, 1439, 1370, 1225, 1175, 1131, 1093, 1042, 967 cm^{-1}. UV (CH_2Cl_2) λ_{max} (log ε): 229 (4.7), 245 (4.2), 264 nm (4.1). Deacetylation of 2,3,4,6-tetra-*O*-acetyl-1-β-D-glucopyranosyl brassilexin (**31**): sodium methoxide (0.1 M methanolic solution, 0.015 mmol) was added to a stirred solution of **31** (8.0 mg, 0.015 mmol) in dry methanol (0.3 ml) and the reaction mixture was allowed to stir at room temperature for 45 min. After concentration under reduced pressure, the crude residue was chromatographed using a small Pasteur pipette containing reverse phase silica to yield 1-β-D-glucopyranosylbrassilexin (**12**) (5 mg, 94% yield). HPLC t_R = 4.5 min; $[\alpha]_D = +19$ (*c* 0.22, CH_3OH). δ_H (500 MHz, CD_3OD): 8.75 (s, 1H), 7.92 (d, $J = 8$ Hz, 1H), 7.69 (d, $J = 8.5$ Hz, 1H), 7.39 (ddd, $J = 7.5$, 8, 1 Hz, 1H), 7.27 (dd, $J = 7.5$, 8 Hz, 1H), 5.75 (d, $J = 9$ Hz, 1H), 3.94 (dd, $J = 10$, 1 Hz, 1H), 3.86 (dd, $J = 9$, 9 Hz, 1H) 3.76–3.67 (m, 3H), 3.49 (dd, $J = 9$, 9 Hz, 2H); δ_C (125 MHz, CD_3OD): 157.9 (s), 147.4 (d), 145.0 (s), 127.4 (s), 124.1 (d), 121.3 (d), 120.7 (s), 120.0 (d), 111.3 (d), 85.1 (d), 80.0 (d), 77.6 (d), 72.6 (d), 70.6 (d), 61.9 (d). HRMS-ESI *m/z*: measured 337.0858 ($[M + 1]^+$, calc. 337.0858 for $C_{15}H_{17}N_2O_5S$). FTIR ν_{max} (KBr): 3349, 3069, 2910, 1510, 1475, 1446, 1376, 1256, 1075, 742 cm^{-1}. UV (CH_3OH) λ_{max} (log ε): 221 (4.6), 245 (4.1), 265 nm (4.0).

Synthesis of 1-methylspirobrassinin (27). Sodium hydride (60% suspension in mineral oil, 30.6 mg, 1.28 mmol) was added to a solution of spirobrassinin (**22**) (127 mg, 0.51 mmol) in THF (10 ml) at 0° C under an argon atmosphere. The reaction mixture was allowed to stir for 10 min, methyl iodide (49 µl, 0.77 mmol) was added, and stirring at room temperature was continued for 4 h. Ice-cold water was added to quench the reaction, the reaction mixture was extracted with EtOAc (3 × 30 ml) and the combined extracts were dried and concentrated. The crude reaction mixture was subjected to column chromatography on silica gel

(CH_2Cl_2–CH_3OH, 99 : 1) to yield 1-methylspirobrassinin (**27**) (125 mg, 93%). HPLC t_R = 15.9 min. δ_H (500 MHz, CD_3CN): 7.38–7.36 (m, 2H), 7.12 (ddd, J = 7.5, 7.5, 1 Hz, 1H), 6.95 (d, J = 8 Hz, 1H), 4.53 (d, J = 15.5 Hz, 1H), 4.44 (d, J = 15.5 Hz, 1H), 3.17 (s, 3H); δ_C (125 MHz, CD_3CN): 176.0 (s), 163.2 (s), 143.5 (s), 130.5 (s), 130.1 (d), 124.1 (d), 123.6 (d), 109.2 (d), 75.0 (t), 64.5 (s), 26.7 (q), 15.3 (q). HRMS-EI m/z: measured 264.0389 ([M]$^+$, calc. 264.0391 for $C_{12}H_{12}N_2OS_2$). MS-EI m/z (relative intensity): 264 ([M]$^+$, 67), 217 (82), 191 (100), 159 (22), 158 (21), 130 (41), 87 (41), 71 (32). FTIR ν_{max} (KBr): 2929, 1712, 1611, 1583, 1492, 1471, 1370, 1345, 1091, 940 cm^{-1}.

6-Hydroxysinalexin (16). HPLC t_R = 12.0 min. δ_H (500 MHz, CD_3CN): 8.63 (s, 1H), 7.74 (d, J = 8.5 Hz, 1H), 7.24 (br s, 1H D_2O exchangeable), 6.98 (d, J = 2 Hz, 1H), 6.83 (dd, J = 8.5, 2 Hz, 1H), 4.14 (s, 3H). HRMS-ESI m/z: measured 221.0377 ([M + H]$^+$, calc. 221.0379 for $C_{10}H_9N_2O_2S$). MS-ESI m/z (relative intensity): 221 (100), 190 (56), 114 (34). FTIR ν_{max} (KBr): 3353, 2928, 2857, 1611, 1460, 1248, 1203, 1075 cm^{-1}. UV (CH_3CN) λ_{max}(log ε): 228 (4.5), 266 nm (4.0).

6-Oxy-(*O*-β-D-glucopyranosyl)sinalexin (17). HPLC t_R = 4.9 min; $[\alpha]_D = -57$ (c 0.20, MeOH). δ_H [500 MHz, $(CD_3)_2CO$]: 8.73 (s, 1H), 7.86 (d, J = 8.5 Hz, 1H), 7.33 (d, J = 2 Hz, 1H), 7.06 (dd, J = 8.5, 2 Hz, 1H), 5.10 (d, J = 7.5 Hz, 1H), 4.23 (s, 3H), 3.94–3.47 (m, 8H, 2H D_2O exchangeable); δ_C (125 M Hz, CD_3OD): 156.3 (s) 155.7 (s), 147.9 (d), 142.5 (s), 123.9 (s), 121.1 (d), 112.8 (s), 112.6 (d), 102.1 (d), 97.7 (d), 77.4 (d), 77.1 (d), 74.0 (d), 70.6 (d), 63.9 (q), 61.7 (t). HRMS-ESI m/z: measured 383.0928 ([M + H]$^+$, calc. 383.0912 for $C_{16}H_{18}N_2O_7S$). MS-ESI m/z (relative intensity): 383 ([M + H]$^+$, 95), 185 (11), 114 (100). FTIR ν_{max} (KBr): 3359, 2926, 2854, 1611, 1459, 1248, 1205, 1073 cm^{-1}. UV (CH_3CN) λ_{max} (log ε): 228 (4.6), 267 nm (4.0).

X-Ray crystal data for **17**: $C_{16}H_{18}N_2O_7S$, M = 382.38, monoclinic, space group $P2_1$, a = 13.8821(3), b = 4.5502(2), c = 14.6589(4) Å, β = 109.8086(17)°, U = 871.16(5) Å^3, T = 173(2) K, Z = 2, μ(Mo-Kα) = 0.228 mm^{-1}, 10 196 reflections collected, 3438 independent reflections (R_{int} = 0.0632), final R values: R_1 = 0.0471, wR_2 = 0.1037 [$I > 2\sigma(I)$]; R_1 = 0.0559, wR_2 = 0.1090 (all data). CCDC reference number 603052. For crystallographic data in CIF or other electronic format see DOI: 10.1039/b604400j.

1-Methyl-(oxy-*O*-β-D-glucopyranosyl)brassilexin (19). HPLC t_R = 4.4 min; $[\alpha]_D = -109$ (c 0.060, MeOH). δ_H [500 MHz, $(CD_3)_2CO$, after adding D_2O]: 8.80 (s, 1H), 7.98 (d, J = 8 Hz, 1H), 7.76 (d, J = 8 Hz, 1H), 7.40 (ddd, J = 7, 7, 1 Hz, 1H), 7.29 (dd, J = 8, 7 Hz, 1H), 6.13 (d, J = 11.5 Hz, 1H), 5.90 (d, J = 11.5 Hz, 1H), 4.39 (d, J = 7.5 Hz, 1H), 3.86 (dd, J = 12, 3 Hz, 1H), 3.70–3.60 (m, 3H), 3.58–3.48 (m, 2H); δ_C [125 MHz, $(CD_3)_2CO$]: 161.6 (s), 148.3 (d), 144.6 (s), 124.5 (d), 121.7 (d), 121.2 (s), 120.8 (s), 120.5 (d), 111.1 (d), 100.0 (d), 76.5 (d), 73.5 (d), 73.4 (t), 70.1 (d), 63.4 (d), 61.4 (d). HRMS-FAB m/z: measured 367.0968 ([M + 1]$^+$, calc. 367.0963 for $C_{16}H_{19}N_2O_6S$). FTIR ν_{max} (KBr): 3350, 3068, 2910, 1509, 1476, 1446, 1375, 1257, 1073, 745 cm^{-1}. UV (CH_3OH) λ_{max} (log ε): 221 (4.4), 243 (3.9), 264 nm (3.8).

Brassicanal A sulfoxide (20). HPLC t_R = 6.3 min; $[\alpha]_D = -245$ (c 0.33, CH_3OH). δ_H (500 MHz, CD_3OD): 10.26 (s, 1H), 8.11 (d, J = 8 Hz, 1H), 7.59 (d, J = 8 Hz, 1H), 7.36 (ddd, J = 8, 8, 1 Hz, 1H), 7.31 (ddd, J = 8, 8, 1 Hz, 1H), 3.08 (s, 3H); δ_C (125 MHz, CD_3OD): 186.0 (s), 147.7 (s), 138.6 (s), 127.9 (s), 126.3 (d), 124.7 (d), 121.4 (d), 116.9 (s), 114.1 (d), 42.2 (q). HRMS-EI m/z: measured 207.0353 (M$^+$, calc. 207.0354 for $C_{10}H_9NO_2S$). MS-EI m/z (relative intensity): 207 (M$^+$, 21), 190 (100), 175 (14), 146 (16). FTIR ν_{max} (KBr): 3166, 2925, 2854, 1656, 1488, 1448, 1391, 1095, 1035, 747 cm^{-1}.

3-(Hydroxymethyl)indole-2-methylsulfoxide (21). HPLC t_R = 3.9 min. δ_H (500 MHz, CD_3CN): 10.40 (br s, 1H D_2O exchangeable), 7.69 (d, J = 8 Hz, 1H), 7.49 (d, J = 8 Hz, 1H), 7.28 (ddd, J = 7, 8, 1 Hz, 1H), 7.14 (ddd, J = 8, 7, 1 Hz, 1H), 4.88 (d, J = 13 Hz, 1H), 4.81 (d, J = 13 Hz, 1H), 2.9 (s, 3H); δ_C (125 MHz, CD_3OD): 139.3 (s), 134.5 (s), 127.8 (s), 126.1 (d), 121.3 (d), 121.1 (d), 120.7 (s), 113.3 (d), 55.1 (t), 41.1 (q). HRMS-EI m/z: measured 209.0508 (M$^+$, calc. 209.0511 for $C_{10}H_{11}NO_2S$). MS-EI m/z (relative intensity): 209 (M$^+$, 54), 192 (87), 176 (100), 147 (68), 117 (52), 91 (28). FTIR ν_{max} (KBr): 3268, 2929, 1711, 1667, 1450, 1212, 1023, 749 cm^{-1}.

Spirobrassinin (22) recovered from cultures (after incubation for 7 d). $[\alpha]_D = -15$ (c 0.34, MeOH); 14% ee (using chiral solvating agent TFAE, the ee was calculated by integration of the SMe signals observed in the ^{1}H NMR).

Spiro[3*H*-indole-3,5′-thiazolidin]-2(1*H*), 2′-dione (23). HPLC t_R = 5.1 min; $[\alpha]_D = -35$ (c 0.33, MeOH). δ_H (500 MHz, CD_3CN): 8.63 (br s, 1H D_2O exchangeable), 7.53 (d, J = 7.5 Hz, 1H), 7.31 (ddd, J = 7.5, 7.5, 1 Hz, 1H), 7.10 (ddd, J = 7.5, 8, 1.0 Hz, 1H), 6.95 (d, J = 8 Hz, 1H), 6.40 (br s, 1H D_2O exchangeable), 3.82 (d, J = 11 Hz, 1H), 3.77 (d, J = 11 Hz, 1H); δ_C (125 MHz, CD_3CN): 176.7 (s), 171.9 (s), 141.2 (s), 130.4 (d), 129.9 (s), 124.7 (d), 123.4 (d), 110.6 (d), 57.0 (s), 51.0 (t). HRMS-EI m/z: measured 220.0304 (M$^+$, calc. 220.0306 for $C_{10}H_8N_2O_2S$). MS-EI m/z (relative intensity): 220 (M$^+$, 48), 191 (59), 164 (36), 135 (27). FTIR ν_{max} (KBr): 3273, 2919, 2854, 1719, 1619, 1472, 1328, 1247, 1185, 1079, 748 cm^{-1}. UV (CH_3CN) λ_{max} (log ε): 212 (4.4), 250 (3.7), 297 nm (3.2).

1-Methoxyspirobrassinin (24) recovered from cultures (after incubation for 7 d). $[\alpha]_D = +11$ (c 0.21, MeOH); 33% ee (using chiral solvating agent TFAE, the ee was calculated by integration of the OMe signals observed in the ^{1}H NMR).

1-Methoxyspiro[3*H*-indole-3,5′-thiazolidin]-2(1*H*),2′-dione (25). HPLC t_R = 7.5 min; $[\alpha]_D = -7$ (c 0.34, MeOH); ee 11% (calculated using chiral solvating agent by ^{1}H NMR). δ_H (500 MHz, CD_3CN): 7.59 (d, J = 7.5 Hz, 1H), 7.43 (dd, J = 7.5, 7.5 Hz, 1H), 7.20 (dd, J = 7.5, 7.5 Hz, 1H), 7.08 (d, J = 7.5 Hz, 1H), 6.45 (br s, 1H D_2O exchangeable), 4.01 (s, 3H), 3.85 (d, J = 11 Hz, 1H), 3.80 (d, J = 11 Hz, 1H); δ_C (125 MHz, CD_3CN): 171.4 (s), 170.2 (s), 139.9 (s), 130.6 (d), 126.0 (s), 124.7 (d), 124.4 (d), 108.0 (d), 63.8 (q) 55.3 (s), 50.6 (t). HRMS-EI m/z: measured 250.0410 (M$^+$, calc. 250.0412 for $C_{11}H_{10}N_2O_3S$). MS-EI m/z (relative intensity): 250 (M$^+$, 100), 194 (24), 163 (23), 162 (39), 148 (53), 131 (32). FTIR ν_{max} (KBr): 3268, 2935, 2883, 1704, 1617, 1466, 1324, 1226, 1080, 750 cm^{-1}. UV (CH_3CN) λ_{max} (log ε): 212 (4.3), 256 nm (3.7).

1-Methoxy-2′-thioxospiro[3*H*-indole-3,5′-thiazolidin]-2(1*H*)-one (26). HPLC t_R = 11.5 min; $[\alpha]_D = -31$ (c 0.10, MeOH); ee 30% (calculated using chiral solvating agent by ^{1}H NMR). δ_H (500 MHz, CD_3CN): 8.18 (br s, 1H D_2O exchangeable), 7.57 (d, J = 7.5 Hz, 1H), 7.41 (dd, J = 7.5, 7.5 Hz, 1H), 7.19 (dd, J = 7.5, 7.5 Hz, 1H), 7.05 (d, J = 7.5 Hz, 1H), 4.23 (d, J = 13 Hz, 1H),

4.20 (d, J = 13 Hz, 1H), 3.98 (s, 3H); δ_C (125 MHz, CD_3CN): 198.3 (s), 169.6 (s), 139.9 (s), 130.8 (d), 124.8 (d), 124.5 (s), 124.3 (d), 108.2 (d), 63.9 (q) 59.5 (s), 58.7 (t). HRMS-EI m/z: measured 266.0189 (M^+, calc. 266.0184 for $C_{11}H_{10}N_2O_2S_2$). MS-EI m/z (relative intensity): 266 (M^+, 100), 194 (36), 175 (26), 162 (44), 148 (36), 144 (37), 116 (19). FTIR ν_{max} (KBr): 3220, 2935, 2859, 1731, 1617, 1503, 1463, 1291, 1058, 753 cm^{-1}. UV (CH_3CN) λ_{max} (log ε): 217 (4.4), 264 nm (4.2).

1-Methylspirobrassinin (27) recovered from cultures (after incubation for 7 d). $[\alpha]_D = +7$ (c 0.25, MeOH); 30% ee (using chiral solvating agent TFAE, the ee was calculated by integration of the NMe signals observed in the ^1H NMR).

1-Methylspiro[3*H*-indole-3,5′-thiazolidin]-2(1*H*),2′-dione (28). HPLC t_R = 6.6 min; $[\alpha]_D = -5$ (c 0.20, CH_3OH). δ_H (500 MHz, CD_3CN): 7.57 (dd, J = 7.5, 0.5 Hz, 1H), 7.39 (ddd, J = 8, 8, 1.1 Hz, 1H), 7.15 (ddd, J = 8, 8, 1 Hz, 1H), 6.98 (d, J = 8 Hz, 1H), 6.39 (br s, 1H D_2O exchangeable), 3.81 (d, J = 11 Hz, 1H), 3.75 (d, J = 11 Hz, 1H), 3.19 (s, 3H); δ_C (125 MHz, CD_3CN): 175.2 (s), 171.9 (s), 143.6 (s), 130.4 (d), 129.6 (s), 124.3 (d), 123.6 (d), 109.3 (d), 56.9 (s), 51.1 (t), 26.6 (q). HRMS-EI m/z: measured 234.0459 (M^+, calc. 234.0463 for $C_{11}H_{10}N_2O_2S$). MS-EI m/z (relative intensity): 234 (M^+, 44), 179 (11), 178 (100), 177 (17), 174 (18), 158 (11). FTIR ν_{max} (KBr): 3263, 3058, 2935, 2883, 1706, 1611, 1470, 1372, 1347, 1247, 1133, 1077, 754 cm^{-1}. UV (CH_3CN) λ_{max} (log ε): 214 (4.5), 257 (3.9), 299 nm (3.3).

1-Hydroxymethylspirobrassinin (29). HPLC t_R = 11.1 min. δ_H (500 MHz, $CDCl_3$): 7.45 (d, J = 8 Hz, 1H), 7.4 (dd, J = 8, 8 Hz, 1H), 7.20–7.14 (m, 2H), 5.35 (d, J = 11 Hz, 1H), 5.21 (d, J = 11 Hz, 1H), 4.76 (d, J = 14.5 Hz, 1H), 4.55 (d, J = 14.5 Hz, 1H), 2.82 (s, 3H); δ_C (125 MHz, $CDCl_3$): 176.9 (s), 164.7 (s), 140.8 (s), 130.6 (s), 130.3 (d), 124.7 (d), 124.6 (d), 110.0 (d), 75.4 (t), 64.8 (s), 64.7 (t), 16.1 (q). HRMS-EI m/z: measured 280.0348 (M^+, calc. 280.0340 for $C_{12}H_{12}N_2O_2S_2$). MS-EI m/z (relative intensity): 280 (M^+, 26), 250 (49), 203 (40), 177 (100), 149 (51), 117 (47), 87 (57). FTIR ν_{max} (KBr): 3311, 2935, 2854, 1739, 1620, 1583, 1464, 1086, 945, 743 cm^{-1}.

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