Metabolism of the crucifer phytoalexins wasalexin A and B in the plant pathogenic fungus *Leptosphaeria maculans*†

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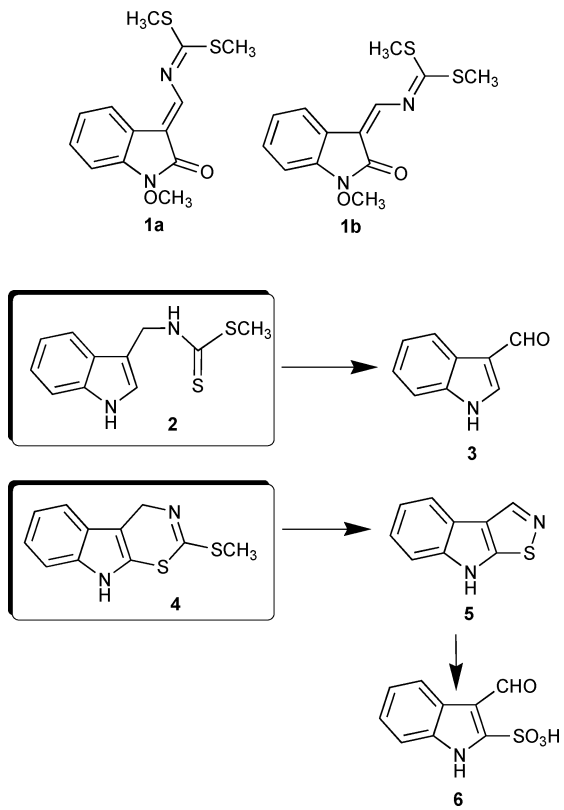
Wasalexins A and B are crucifer phytoalexins produced by two substantially different plant species, a wild species abundant in the Canadian prairies and a condiment plant widely cultivated in Japan. Interestingly, both plant species are resistant to an economically important fungal plant pathogen, the blackleg fungus [*Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm.]. The transformation of wasalexins A and B in cultures of isolates of *L. maculans*, an isolate highly virulent towards canola (BJ 125) and a less common isolate which is virulent towards wasabi (Laird 2/Mayfair 2) was investigated. It was established that both fungal isolates are able to efficiently metabolize and detoxify wasalexins A and B through reduction in the case of wasalexin A or through hydrolysis followed by reduction in the case of wasalexin B. Moreover, a close structural analogue of wasalexins, which does not occur naturally, was also found to be reduced in cultures of *L. maculans.* The structures of the new metabolic products were elucidated using spectroscopic methods and were confirmed by synthesis. Bioassays indicated that the biotransformation of wasalexins is a detoxification process that may contribute to the aggressive nature of these fungal isolates towards plants that produce wasalexins.

Introduction

The phytoalexins wasalexins A (**1a**) and B (**1b**) are produced in response to biotic and abiotic stress in wasabi (*Wasabia japonica* syn. *Eutrema wasabi*), a condiment plant widely cultivated in Japan,[1] and in pennycress or stinkweed (*Thlaspi arvense*), a weed common in the Canadian prairies.[2] Phytoalexins are chemical defenses biosynthesized *de novo* by plants in response to pathogen attack and other forms of stress. Because wasalexins A (**1a**) and B (**1b**) were found to be produced in two rather different plant species that are known to be resistant to the fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not. [asexual stage *Phoma lingam* (Tode ex Fr.) Desm.], it was of great interest to investigate their effect on this pathogen. *L. maculans* is the causative agent of "blackleg", an economically important disease of crucifer crops worldwide. This plant pathogen is able to transform enzymatically plant defense metabolites, phytoalexins and related metabolites, to products with significantly lower antifungal activity.[3] For example, *L. maculans* detoxified the phytoalexins brassinin (**2**),[3] cyclobrassinin (**4**)[3] and brassilexin (**5**)[4] to products **3** and **6**, respectively (Scheme 1). Detoxification reactions of phytoalexins in plant pathogenic fungi are detrimental to the plant and favourable to the pathogen. Consequently, inhibition of these enzymatic processes could be used to prevent fungal colonization of plant tissues. Toward this goal, we have been evaluating detoxification pathways of crucifer phytoalexins and designing phytoalexin detoxification inhibitors, *i.e.* paldoxins, to protect the plant against fungal colonization.[3]



Scheme 1 Metabolism of brassinin (**2**),[3] cyclobrassinin (**4**)[3] and brassilexin (**5**)[4] in *Leptosphaeria maculans.*

In a continuation of those studies, we investigated the transformation of wasalexins A (**1a**) and B (**1b**) in two different isolates of *L. maculans*, an isolate that is highly virulent towards canola

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Table 1 Metabolism of wasalexin A (**1a**) and its metabolites **7** and **8** in cultures of *Leptosphaeria maculans* isolates BJ 125 and Laird 2/Mayfair 2

Compound added to fungal cultures	Products of metabolism (%)[a] after incubation for 6–96 h	
	Isolate BJ 125	Isolate Mayfair 2
Wasalexin A (**1a**)[b]	6 h: **7** (47 ± 3); **8** (46 ± 1)	6 h: **7** (28 ± 8); **8** (18 ± 2)
Dihydrowasalexin (**7**)[b]	6 h: **8** (49 ± 3) 12 h: **8** (66 ± 1) 24 h: **8** (78 ± 3)	6 h: **7** (18 ± 2); **8** (24 ± 4); 12 h: **7** (<5); **8** (21 ± 7) 24 h: **8** (31 ± 6)
1-Methoxy-3-methylindol-2-one (**8**)[c]	Slow transformation to undetermined products (79 ± 6 after 96 h)	Slow transformation to undetermined products (38 ± 5 after 96 h)

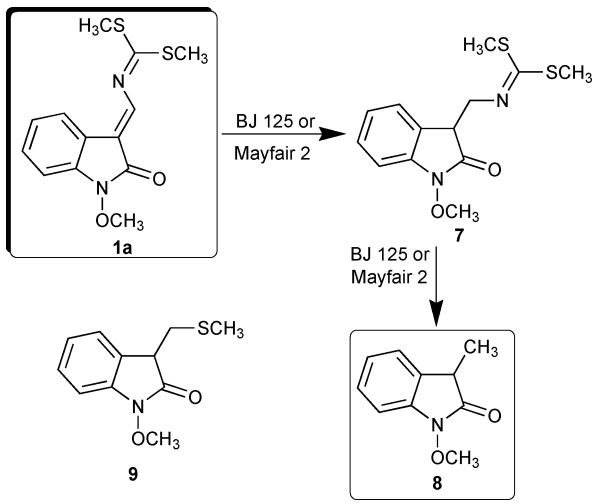
[a] Average percentage yield relative to total amount of compound added to culture (% ± standard deviations; triplicate samples) calculated by HPLC analysis using calibration curves constructed for each compound. [b] Compound (5×10^{-5} M concentration in the culture) dissolved in DMSO was added to 7-d-old mycelia in water and was incubated at 24 ± 2 °C. [c] Compound (5×10^{-5} M concentration in the culture) dissolved in DMSO was added to 48-h-old mycelia in minimal media and was incubated at 24 ± 2 °C.

(BJ 125) and a less common isolate which is virulent towards wasabi (Laird 2/Mayfair 2).[5] It was established that both isolates are able to efficiently metabolize and detoxify wasalexins using either two sequential reductive steps in the case of wasalexin A (**1a**) or hydrolysis followed by reduction in the case of wasalexin B (**1b**). Moreover, a close structural analogue of wasalexins, which does not occur naturally, was also found to be reduced in both isolates of *L. maculans*. The structures of the new products were determined by spectroscopic methods and confirmed by total synthesis. The results of these studies are described here for the first time.

Results and discussion

Fungal metabolism

Wasalexins A (**1a**) and B (**1b**) were synthesized and obtained as a mixture of *E* (wasalexin A) and *Z* (wasalexin B) stereoisomers in a 2 : 1 ratio, as described previously.[6] The more stable *E* isomer (**1a**) could be obtained by crystallization of the mixture using a dichloromethane–hexane solution.[2] The less stable *Z* isomer (**1b**) could not be obtained as a single compound.[1] The metabolism of wasalexins A and B (5×10^{-5} M) in cultures of *L. maculans* was analyzed over a period of several days by HPLC. The HPLC chromatograms of EtOAc extracts of cultures of *L. maculans*, isolates BJ 125 and Mayfair 2 (mycelia in water) incubated with wasalexin A (**1a**) (t_R 25.2 min) showed two new peaks, at t_R 10.3 min and t_R 23.7 min, not present in control cultures (no wasalexins). Furthermore, wasalexin A (**1a**) was not detected in extracts of cultures of isolates BJ 125 or Mayfair 2 after incubation for six hours; that is **1a** appeared to be completely metabolized. Subsequently, from larger scale cultures, the metabolites corresponding to the new peaks at t_R 10.3 min and t_R 23.7 min were isolated and purified (Table 1). The metabolite with t_R 10.3 min (**8**) was found to have the molecular formula $C_{10}H_{11}NO_2$ (HRMS-EI). The ^{1}H NMR spectrum (see the ESI†) displayed signals due to four aromatic protons and signals at δ 3.45 (J = 8 Hz, q, 2H) and δ 1.52 (J = 8 Hz, d, 3H) indicating the presence of a methine–methyl spin system. This metabolite was proposed to be 1-methoxy-3-methylindol-2-one (**8**), the structure of which was confirmed by synthesis as described in the Experimental section. Similarly, analysis of the spectroscopic data obtained for the metabolite with t_R 23.7 min and comparison with those of wasalexin A (**1a**) suggested it to be dihydrowasalexin (**7**). Synthesis of this metabolite confirmed its structure unambiguously. Subsequent incubation of dihydrowasalexin (**7**) in cultures of *L. maculans* isolates BJ 125 and Mayfair 2 indicated it to be the precursor of oxindole **8** (Scheme 2, Table 1). Oxindole **8** was slowly biotransformed (in more than 96 h) to undetermined products. As shown in Scheme 2, wasalexin A was first reduced at the exocyclic double bond to yield dihydrowasalexin (**7**), followed by further reduction of the methylene to a methyl to yield **8**. These metabolism experiments (Scheme 2, Table 1) were carried out with mycelia incubated in water because one of the metabolites of wasalexin A, dihydrowasalexin (**7**), was found to decompose non-enzymatically in minimal media (<5% of **7** remaining in solution after 12 h) with the formation of 1-methoxy-3-methylsulfanylmethylindol-2-one (**9**). Further studies to understand the formation of **9** were carried out and are described below.



Scheme 2 Metabolism of wasalexin A (**1a**) in *Leptosphaeria maculans* isolates BJ 125 and Laird 2/Mayfair 2.

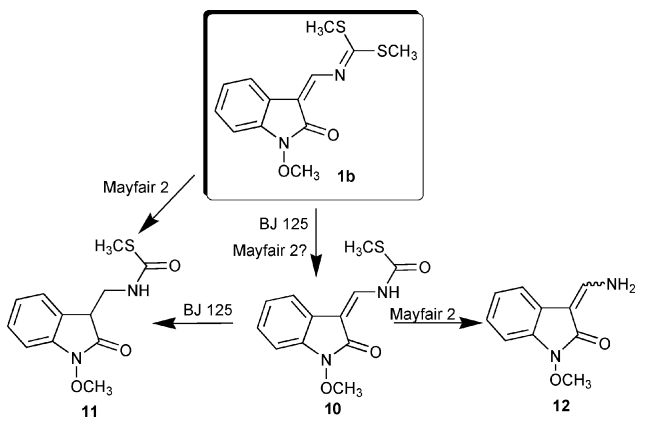
Next, because wasalexin B (**1b**) could not be obtained as a single compound (on standing in solution it is converted to wasalexin A (**1a**)), a mixture of wasalexins A and B (**1a** + **1b**, 2 : 1) was incubated with cultures of *L. maculans* and their metabolism analyzed over a period of several days. HPLC analyses of the EtOAc extracts of cultures of *L. maculans* isolate BJ 125 (48-h-old mycelia in minimal media) incubated with wasalexins A and B (**1a** + **1b**, 2 : 1) showed the disappearance of both wasalexins peaks (t_R 22.6 and 25.2 min) within 12 h. Dihydrowasalexin (**7**, t_R 23.7 min) and

Table 2 Metabolism of wasalexins A + B (**1a** + **1b**, 2 : 1) and metabolites **10**–**12** (metabolism of compounds **7** and **8** shown in Table 1) in cultures of *Leptosphaeria maculans* isolates BJ 125 and Laird 2/Mayfair 2

	Products of metabolism (%)[a] after incubation for 6–96 h	
Compound added to fungal cultures	Isolate BJ 125	Isolate Mayfair 2
Wasalexins A + B (**1a** + **1b**, 2 : 1)[b]	6 h: **7** (15 ± 1); **8** (28 ± 4) **10** (9 ± 1); **11** (< 5)	6 h: **7** (28 ± 8); **8** (18 ± 2) 12 h: **7** (15 ± 1); **8** (31 ± 1); **11** (8 ± 4)
S-Methyl 1-methoxy-3-aminomethylene-2-oxindole thiocarbamate (**10**)[c]	12 h: **11** (16 ± 3) 24 h: **11** (62 ± 7) 48 h: **11** (64 ± 5)	12 h: **11** (<5); **12** (<5) 24 h: **11** (<5); **12** (5 ± 2) 48 h: **11** (7 ± 1); **12** (18 ± 4) 96 h: **11** (9 ± 1); **12** (42 ± 7)
S-Methyl 1-methoxy-3-aminomethyl-2-oxindole thiocarbamate (**11**)[c]	Slow transformation to undetermined products (31 ± 3 recovered after 96 h)	Slow transformation to undetermined products (12 ± 2 recovered after 96 h)
1-Methoxy-3-aminomethyleneindol-2-one (**12**)[b]	Not formed	Slow transformation to undetermined products (57 ± 5 after 96 h)

[a] Average percentage yield (% ± standard deviations; triplicate samples) was calculated by HPLC analysis using calibration curves constructed for each compound. [b] Compounds (5×10^{-5} M concentration in the culture) dissolved in DMSO were added to 7-d-old cultures in water and were incubated at 24 ± 2 °C. [c] Compounds (5×10^{-5} M concentration in the culture) dissolved in DMSO were added to 48-h-old cultures in minimal media and were incubated at 24 ± 2 °C.

1-methoxyindol-2-one (**8**, t_R 10.3 min) together with two additional metabolites with t_R 9.0 and 20.6 min were detected in the HPLC chromatograms of extracts of cultures. The presence of additional metabolites in cultures of isolate BJ 125 incubated with the mixture of wasalexins A and B suggested that the metabolism of wasalexin B (**1b**) was different from the metabolism of wasalexin A (**1a**). Subsequently, larger scale experiments allowed the isolation of the metabolites responsible for peaks with t_R 9.0 and 20.6 min in the HPLC chromatograms. The HRMS-EI data of the metabolite with t_R 20.6 min (**10**) suggested the molecular formula $C_{12}H_{12}N_2O_3S$, *i.e.* one less SCH_3 and an additional oxygen relative to wasalexin. Comparison of the ^1H and ^{13}C NMR spectral data of this metabolite with those of wasalexins indicated this metabolite to be thiocarbamate **10** (Scheme 3), the structure of which was confirmed by synthesis. Thiocarbamate **10** was found to be biotransformed efficiently to **11** in cultures of BJ 125 (Table 2, Scheme 3). The HRMS-EI spectral data of the metabolite with t_R 9.0 min (**11**) suggested the molecular formula $C_{12}H_{14}N_2O_3S$. The ^1H NMR spectrum displayed signals due to four aromatic protons, singlets at δ 4.05 ppm (3H) and δ 2.37 (3H) indicating the presence of OCH_3 and SCH_3 groups, respectively, and multiplets at δ 4.19 (1H), 3.62 (1H) and 3.46 (1H) ppm. Furthermore, the two lowest field signals in the ^{13}C NMR spectrum suggested the presence of two carbonyls likely due to amide and (thio)carbamate groups. Thus, the NMR data of the metabolite with t_R 9.0 min (**11**) indicated that the double bond present in the side chain of both wasalexin B and metabolite **10** had been reduced whereas the thiocarbamate remained intact. The compound was proposed to be *S*-methyl 1-methoxy-3-aminomethyl-2-oxindole thiocarbamate (**11**). This structure was confirmed by synthesis as described in the Experimental section.



Scheme 3 Metabolism of wasalexin B (**1b**) in *Leptosphaeria maculans* isolates BJ 125 and Laird 2/Mayfair 2.

HPLC analysis of the EtOAc extracts obtained from cultures of *L. maculans* isolate Mayfair 2 (48-h-old mycelia in minimal media) incubated with wasalexins A and B (**1a** + **1b**, 2 : 1) (Table 2) indicated the presence of dihydrowasalexin (**7**), oxindole **8** and thiocarbamate **11** but metabolite **10** was not detected. The absence of **10** in these chromatograms suggested that either wasalexin B (**1b**) was transformed directly to thiocarbamate **11** or that **10** did not accumulate because it was immediately transformed to **11**, *i.e.* the rate of transformation of **10** is similar to its rate of formation (Scheme 3). Furthermore, dihydrowasalexin (**7**) was not a likely intermediate in the formation of **11** because this metabolite was not detected when **7** was incubated in cultures of isolate Mayfair 2. Considering that in cultures of isolate Mayfair 2 metabolite **10** was found to be metabolized to thiocarbamate **11** and to 1-methoxy-3-aminomethyleneindol-2-one (**12**)[2] (Table 2), **10** is a likely precursor of **11**.

As summarized in Scheme 3, the metabolism of wasalexin B in isolates BJ 125 and Laird 2/Mayfair 2 yields **11** likely *via* intermediate **10**. However, in cultures of isolate Laird 2/Mayfair 2 metabolite **10** appears to be transformed mainly to enamine **12**, whereas in BJ 125 **10** yields mainly metabolite **11**. Such differences are not unusual considering that these two isolates are part of different pathogenicity groups (currently classified as belonging to the same species).[5]

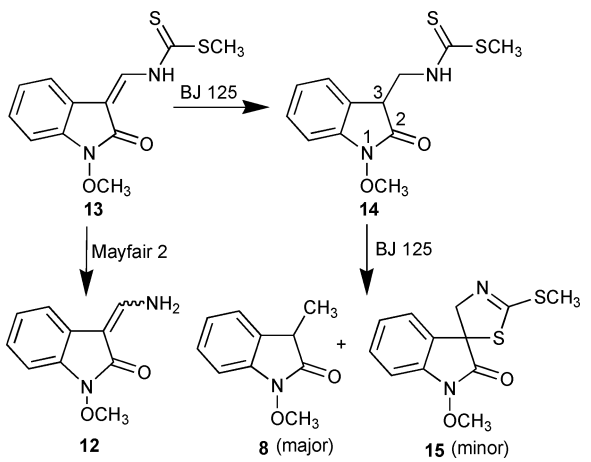
Next, to further understand the enzymatic reactions leading to the transformation of wasalexins A (**1a**) and B (**1b**), 1-methoxy-3-aminomethylene-2-oxindole dithiocarbamate (**13**) was synthesized[2] and its transformation in cultures of *L. maculans* was investigated. When methyl 1-methoxy-3-aminomethylene-2-oxindole dithiocarbamate (**13**) was incubated with isolate BJ 125

Table 3 Metabolism of methyl 1-methoxy-3-aminomethylene-2-oxindole dithiocarbamate (**13**) and its metabolites **14** and **15** (metabolism of **12** in Table 2) in cultures of *Leptosphaeria maculans* isolates BJ 125 and Mayfair 2

Compound added to fungal cultures	Products of metabolism (%)[a] after incubation for 6–96 h	
	Isolate BJ 125	Isolate Mayfair 2
Methyl 1-methoxy-3-aminomethylene-2-oxindole dithiocarbamate (**13**)[b]	12 h: **14** (12 ± 1); **15** (<5) 24 h: **14** (29 ± 2); **15** (<5) 48 h: **12** (<5); **14** (29 ± 1); **15** (<5)	12 h: **15** (<5) 24 h: **12** (<5); **15** (<5) 48 h: **8** (<5); **12** (9 ± 1); **14** (5 ± 1); **15** (<5) 72 h: **8** (<5); **12** (14 ± 1); **14** (<5); **15** (<5)
Methyl 1-methoxy-3-aminomethyl-2-oxindole dithiocarbamate (**14**)[b]	12 h: **15** (<5) 24 h: **8** (<5); **15** (<5) 48 h: **8** (7 ± 2); **15** (6 ± 1) 96 h: **8** (29 ± 9); **15** (6 ± 1)	12 h:**15** (<5) 24 h: **15** (<5) 48 h: **8** (11 ± 1); **15** (<5) 96 h: **8** (13 ± 1); **15** (<5)
1-Methoxyspirobrassinin (**15**)[b]	Slow transformation to undetermined products (96 h, 49 ± 8)	Slow transformation to undetermined products (96 h, 34 ± 5)

[a] Average percentage yield (% ± standard deviations; triplicate samples) was calculated by HPLC analysis using calibration curves constructed for each compound. [b] Compounds (5×10^{-5} M in the culture) dissolved in DMSO were added to 48-h-old cultures in minimal media and were incubated at 24 ± 2 °C.

a peak corresponding to a likely new metabolite with t_R 18.5 min (**14**) was observed in the HPLC chromatograms of EtOAc extracts (12–48 h incubations), in addition to metabolite **8** (major, Table 3) and **15** (by comparison with our HPLC-UV library database). Subsequently, a larger scale biotransformation experiment was carried out to isolate the metabolites. HRMS-EI spectral data indicated that the new metabolite with t_R 18.5 min (**14**) had the molecular formula $C_{12}H_{14}N_2O_2S_2$. The ^{1}H NMR spectrum of this new metabolite was similar to that of thiocarbamate **11** but a signal at δ 200.2 ppm in the ^{13}C NMR spectra (see the ESI†) suggested the presence of a thiocarbonyl carbon. Hence the structure of this compound was proposed to be methyl 1-methoxy-3-aminomethyl-2-oxindole dithiocarbamate (**14**) (Scheme 4). This structure was confirmed by synthesis as described in the Experimental section. In addition, the structure of metabolite **15** was confirmed to be 1-methoxyspirobrassinin. These results showed that the metabolism of dithiocarbamate **13** in cultures of *L. maculans* isolate BJ 125 was much slower than that of wasalexin A (**1a**) (*ca.* 20% of **13** remaining after 96 h *vs* complete metabolism of **1a** in 6 h) yielding two products, the major product appeared to be 1-methoxy-3-methylindol-2-one (**8**) together with small amount of



Scheme 4 Metabolism of methyl 1-methoxy-3-aminomethylene-2-oxindole dithiocarbamate (**13**) in *Leptosphaeria maculans* isolates BJ 125 and Laird 2/Mayfair 2.

1-methoxyspirobrassinin (**15**) (Table 3, Scheme 4). Slow oxidation of dithiocarbamate **14** (*ca.* 3% in 96 h) to 1-methoxyspirobrassinin (**15**) was also observed in control flasks containing dithiocarbamate **14** in minimal medium. 1-Methoxyspirobrassinin (**15**)[8] was found to be slowly metabolized in cultures of *L. maculans* isolate BJ 125 to undetermined product(s) (Table 3, *ca.* 49% of **15** remaining after 96 h).

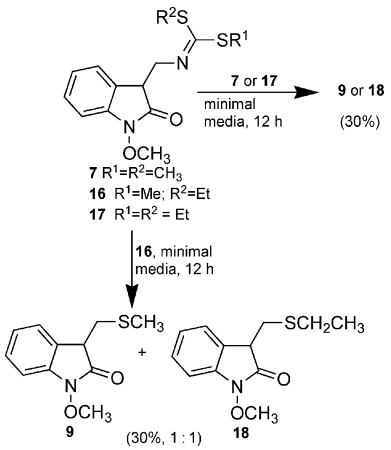
Similarly, the metabolism of dithiocarbamate **13** in cultures of *L. maculans* isolate Mayfair 2 was slower than that of wasalexin A (**1a**) (*ca.* 72 h *vs.* complete metabolism of **1a** in 6 h). Enamine **12** was found to be the major product while dithiocarbamate **14** was a minor metabolite (Table 3, Scheme 4). As in the case of isolate BJ 125, dithiocarbamate **14** was found to be slowly transformed to 1-methoxy-3-methylindol-2-one (**8**) and 1-methoxyspirobrassinin (**15**) (Table 3, Scheme 4). 1-Methoxyspirobrassinin (**15**) was found to be slowly metabolized to undetermined product(s) (*ca.* 34% of **15** remaining after 96 h). Once again, it is worthy to note that in cultures of isolate Mayfair 2 compound **13** appears to be transformed mainly to enamine **12**, whereas in cultures of BJ 125 **13** yields mainly metabolite **8** (Scheme 4, Table 3). As mentioned in the case of metabolite **10**, these differences are not unusual and are perhaps reflecting the substantial differences between these isolates.[5]

Metabolites **7**, **8**, **11** and **14** containing a stereogenic center were found to be readily epimerizable in an acidic solution of CD_3CN–D_2O (1 : 2, 1 mL containing 10 µl of 0.1 M DCl). Hence, the low values of the optical rotations of these metabolites are likely due to their racemization in liquid culture (Experimental section, $[\alpha]_D$ *ca.* −5–(+6)). However, to the best of our knowledge, compounds **7**, **8**, **11** and **14** are not known, thus the possibility that enantiomerically pure metabolites with low values of $[\alpha]_D$ are products of the fungal metabolism cannot be excluded. Conversely, it cannot be ruled out that the enzyme(s) involved in these conversions are not stereoselective.

Decomposition of dihydrowasalexin (7)

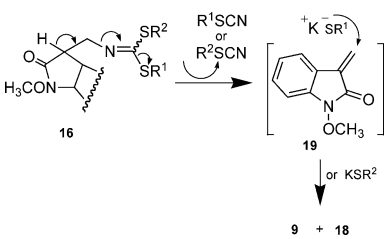
As stated above, dihydrowasalexin (**7**) was found to decompose in minimal media (<5% of **7** remaining after standing in solution for 12 h) with the formation of 1-methoxy-3-methylsulfanylmethylindol-2-one (**9**, *ca.* 30% yield) and additional undetermined

products. The decomposition of dihydrowasalexin (**7**) in water yielded the same product **9** but was found to be significantly slower (*ca.* 80% of **7** remaining after standing in solution for 24 h). For this reason, additional studies were carried out to understand this non-enzymatic reaction. It was surmised that the SCH_3 group of **9** was likely derived from one of the SCH_3 groups of **7**, thus analogues **16** and **17** were thought to confirm this hypothesis. Compound **16** was prepared, added to minimal medium (5×10^{-5} M) and incubated under similar conditions used for **7** (room temperature on a shaker). HPLC analysis of extracts of the solution containing **16** (<10% of **16** after 12 h) indicated the presence of oxindoles **9** and **18** in an 1 : 1 ratio (*ca.* 30% overall yield, Scheme 5). In addition, compound **17** was prepared and treated similarly to yield **18** and undetermined products (*ca.* 30% overall yield, Scheme 5).



Scheme 5 Decomposition of dihydrowasalexin (**7**), *S*-ethyl,*S*-methyl 1-methoxy-3-aminomethyl-2-oxindole iminodithioate (**16**) and *S*,*S*-diethyl 1-methoxy-3-aminomethyl-2-oxindole iminodithioate (**17**) in minimal media.

A rationale for the formation of both compounds **9** and **18** from **16** is proposed in Scheme 6. Elimination of volatiles R^1SCN and R^2SCN from **16** could lead to unstable 1-methoxy-3-methyleneindol-2-one (**19**) plus KSR^2 and KSR^1. Next, since **19** is an α,β-unsaturated amide, Michael addition of sulfides KSR^1 or KSR^2 (K^+ from KNO_3 and K_2HPO_4 present in minimal media) could yield stable thioethers **9** and **18**. In fact, the reaction of 3-methyleneindol-2-one,[8] presumably less reactive than the 1-methoxy analogue **19**, with various thiols was reported.[9] Nonetheless, the amount of sulfides **9** and **18** formed in these



Scheme 6 Rationale for formation of compounds **9** and **18** from *S*-ethyl,*S*-methyl 1-methoxy-3-aminomethyl-2-oxindole iminodithioate (**16**) in minimal media.

reactions is low (*ca.* 30%, Fig. 1) which suggests that intermediate alkene **19** undergoes additional transformation to polar products insoluble in EtOAc and thus not detected or isolated.

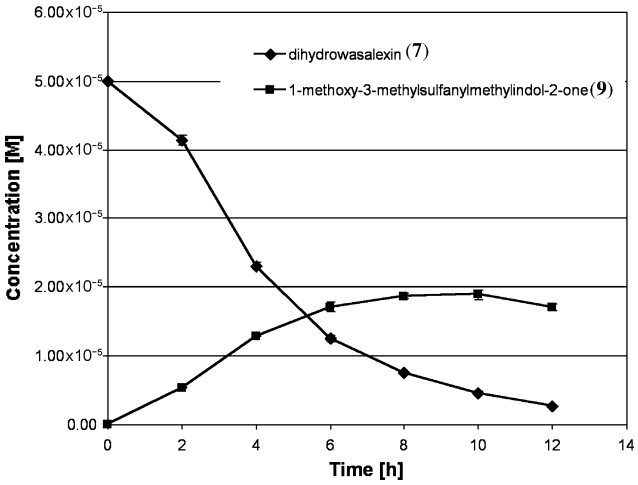
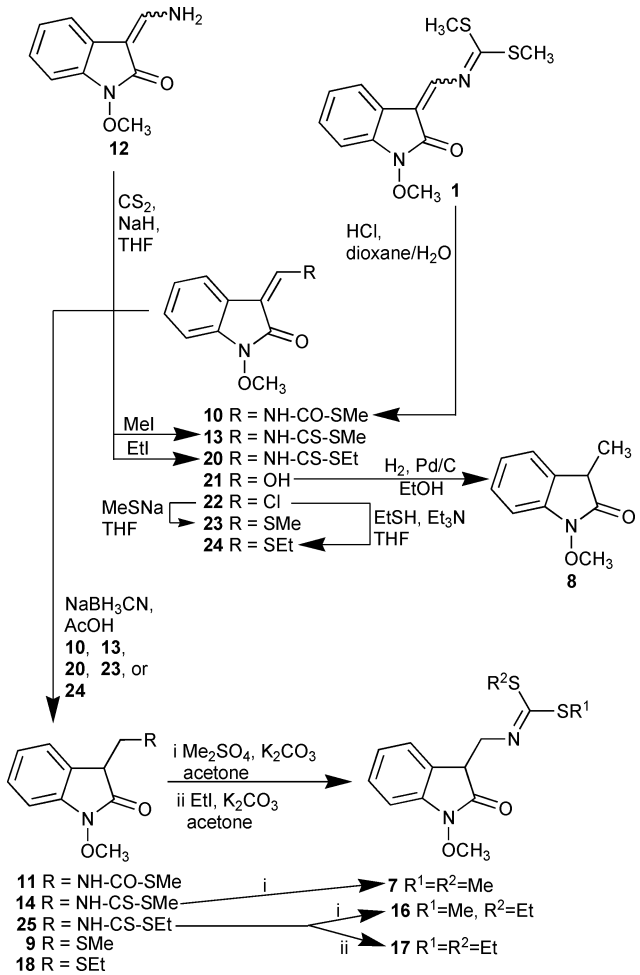


Fig. 1 Formation of 1-methoxy-3-methylsulfanylmethylindol-2-one (**9**) from dihydrowasalexin (**7**) in minimal media. Concentrations were determined using calibration curves; each point is an average of triplicate samples ± standard deviation.

Synthesis of new metabolites

The chemical synthesis of the metabolites involved in the biotransformation pathways of wasalexins A (**1a**) and B (**1b**) and dithiocarbamate **13** was carried out in order to confirm their structures and to obtain sufficient amounts to test their antifungal activities. 1-Methoxy-3-methylindol-2-one (**8**) was prepared (44% yield) by catalytic hydrogenation[10] of 1-methoxy-2-oxoindole-3-carboxaldehyde (**21**) (Scheme 7). *S*-Methyl 1-methoxy-3-aminomethylene-2-oxindole thiocarbamate (**10**) was obtained upon acidic hydrolysis (dioxane–HCl) of a wasalexin mixture (**1a**, **1b**) (65% yield, Scheme 7). Ethyl 1-methoxy-3-aminomethylene-2-oxindole dithiocarbamate (**20**) was prepared from enamine **12** in 33% yield (based on 1-methoxyindol-2-one)[2] using EtI as an alkylating agent (Scheme 7). *S*-Methyl 1-methoxy-3-aminomethyl-2-oxindole thiocarbamate (**11**), methyl 1-methoxy-3-aminomethyl-2-oxindole dithiocarbamate (**14**) and ethyl 1-methoxy-3-aminomethyl-2-oxindole dithiocarbamate (**25**) were synthesized in moderate yields (**11**, 56%; **14**, 63%; **25**, 67%) using $NaBH_3CN$–AcOH reduction[11] of the corresponding compounds **10**, **13** and **20** (Scheme 7). Subsequent methylation of dithiocarbamates **14** and **25** with $(CH_3)_2SO_4$–K_2CO_3 in acetone afforded dihydrowasalexin (**7**) in 51% yield and *S*-ethyl,*S*-methyl 1-methoxy-3-aminomethyl-2-oxindole iminodithioate (**16**) in 50% yield (Scheme 7). When dithiocarbamate **25** was treated with EtI–K_2CO_3, *S*,*S*-diethyl 1-methoxy-3-aminomethyl-2-oxindole iminodithioate (**17**) was obtained in 40% yield (Scheme 7). 1-Methoxy-3-chloromethyleneindol-2-one (**22**)[2] was used as a starting material for the synthesis of 1-methoxy-3-methylsulfanylmethylindol-2-one (**9**) and 1-methoxy-3-ethylsulfanylmethylindol-2-one (**18**). Treatment of **22** with MeSNa or EtSH–Et_3N in THF afforded sulfides **23** and **24** which were subjected to $NaBH_3CN$–AcOH-mediated reduction[11] (Scheme 7). 1-Methoxy-3-methylsulfanylmethylindol-2-one (**9**) and 1-methoxy-3-ethylsulfanylmethylindol-2-one (**18**)



Scheme 7 Synthesis of compounds **7**–**11**, **13**, **14**, **16**–**18**, **20** and **23**–**25**.

were obtained in 26 and 23% yield, respectively, based on chloromethylene indol-2-one **22** (Scheme 7).

Antifungal bioassays

Antifungal bioassays of wasalexins **1a** and **1b**, methyl 1-methoxy-3-aminomethylene-2-oxindole dithiocarbamate (**13**) and their metabolites against *L. maculans* isolates BJ 125 and Mayfair 2 were carried out as summarized in Table 4 and described in the Experimental section. Wasalexins **1a** and **1b** and dithiocarbamate **13** were used at 2×10^{-4} M, a concentration lower than that used routinely (5×10^{-4} M)[4] due to their low solubility in potato dextrose agar media. Wasalexin A (**1a**) appeared to be slightly more antifungal against isolate Mayfair 2 than to isolate BJ 125 (Table 4), whereas the antifungal activity of dihydrowasalexin (**7**) was higher against isolate BJ 125 than to isolate Mayfair 2. Furthermore, the antifungal activity of 1-methoxy-3-methylindol-2-one (**8**), the final biotransformation product of wasalexin A (**1a**) in isolate BJ 125 was significantly lower than wasalexin A. Interestingly, among all compounds tested for antifungal activity against isolates BJ 125 and Mayfair 2, 1-methoxy-3-methylsulfanylmethylindol-2-one (**9**), that results from decomposition of dihydrowasalexin (**7**), appeared to be the most active, completely inhibiting the growth of *L. maculans* isolate BJ 125 at 5×10^{-4} M.

Conclusion

Wasalexins A and B are phytoalexins produced by plants of the same family but rather different species that are resistant to the fungal pathogen *L. maculans.* To better understand the effect of these phytoalexins on the pathogen and the reactions of the pathogen to the phytoalexins, metabolic studies were carried out. Similar to other crucifer phytoalexins,[3] wasalexins A (**1a**) and B (**1b**) were found to be efficiently metabolized by *L. maculans* isolates BJ 125 (6 h) and Mayfair 2 (<12 h). The key step in this biotransformation appeared to be the reduction of double bond in the side chain of the oxoindole ring. To compare the reactions of the iminodithioate group of wasalexins with those of dithiocarbamate groups, *e.g.* **2**, the biotransformation of **13** in *L. maculans* isolates BJ 125 and Mayfair 2 was investigated as well. The metabolism of **13** in isolates BJ 125 and Mayfair 2 yielded metabolites **8** and **12**, respectively. The antifungal activities of the various metabolites indicate that the metabolism of wasalexins A (**1a**), B (**1b**) and **13** in *L. maculans* isolates BJ 125 and Mayfair 2 are all detoxification processes, since metabolites with lower antifungal activity are obtained. Interestingly, methyl 1-methoxy-3-aminomethylene-2-oxindole dithiocarbamate (**13**) was reduced faster in isolate BJ 125 (major pathway) than in isolate Mayfair 2. Furthermore, perhaps significantly, this reductive transformation was substantially slower (48 h in BJ 125 and 72 h in Mayfair 2) than those of wasalexins, suggesting that the enzyme(s) is (are) somewhat specific. Although further work needs to be carried out, based on the structures of the metabolic products it is tempting to propose that similar enzymes are involved in the reduction reactions occurring in both isolates BJ 125 and Mayfair 2. On the other hand, hydrolysis of thiocarbamate **10** and dithiocarbamate **13** seems to occur only in isolate Laird 2/Mayfair 2. Since detoxification of the dithiocarbamate group of brassinin (**2**) *via* hydrolysis was previously reported in isolates of *L. maculans* that were weakly virulent towards canola,[3] this putative hydrolase might be an enzyme characteristic of such group.

Finally it is pertinent to compare the metabolism of wasalexins A and B and dithiocarbamate **13** with that of brassinin (**2**) in isolates that are virulent towards canola (BJ 125 and similar group); however, no similar studies have been reported for isolate Mayfair 2. Enzymatic transformation of brassinin (**2**) in cultures of isolate BJ 125 yielded aldehyde **3** (Scheme 1), which is also the first product of enzymatic detoxification, apparently carried out by brassinin oxidase.[12] In addition, screening a large number of compounds related to brassinin (**2**) suggested that the –CH_2–NH–C=S moiety at the C-3 of indole is required for the compound to be enzymatically oxidized by brassinin oxidase.[13] The work reported here is consistent with those findings and further suggests that replacement of the indole nucleus with an indol-2-one can prevent oxidation of the CH_2 attached to C-3. Considering that the transformation of dithiocarbamate **14** in cultures of isolate BJ 125 is not an oxidation and that its biotransformation is rather slow (20% remaining after 96 h), it would be important to evaluate its effect on the metabolism of brassinin (**2**). If reversible (or irreversible) inhibition of brassinin oxidase is observed, compound

Table 4 Results of antifungal bioassays against *Leptosphaeria maculans* isolates BJ 125 and Laird 2/Mayfair 2

Compound	Concentration/M	BJ 125% inhibition[a]	Laird 2/Mayfair 2% inhibition[a]
Wasalexin A (**1a**)	1×10^{-4}	36 ± 5	51 ± 6
	2×10^{-4}	43 ± 3	59 ± 5
	5×10^{-4}	NS[b]	NS[b]
Dihydrowasalexin (**7**)	1×10^{-4}	41 ± 8	NI[c]
	2×10^{-4}	51 ± 5	23 ± 2
	5×10^{-4}	63 ± 4	34 ± 4
1-Methoxy-3-methylindol-2-one (**8**)	1×10^{-4}	NI[c]	NI[c]
	2×10^{-4}	NI[c]	NI[c]
	5×10^{-4}	21 ± 4	31 ± 9
1-Methoxy-3-methylsulfanylmethylindol-2-one (**9**)	1×10^{-4}	50 ± 5	36 ± 2
	2×10^{-4}	54 ± 4	42 ± 6
	5×10^{-4}	100 ± 0	50 ± 4
S-Methyl 1-methoxy-3-aminomethylene-2-oxindole thiocarbamate (**10**)	1×10^{-4}	NI[c]	NI[c]
	2×10^{-4}	NI[c]	39 ± 4
	5×10^{-4}	NS[b]	NS[b]
S-Methyl 1-methoxy-3-aminomethyl-2-oxindole thiocarbamate (**11**)	1×10^{-4}	NI[c]	NI[c]
	2×10^{-4}	19 ± 3	NI[c]
	5×10^{-4}	25 ± 5	29 ± 5
1-Methoxy-3-aminomethyleneindol-2-one (**12**)	1×10^{-4}	NI[c]	NI[c]
	2×10^{-4}	NI[c]	NI[c]
	5×10^{-4}	31 ± 4	30 ± 8
Methyl 1-methoxy-3-aminomethylene-2-oxindole dithiocarbamate (**13**)	1×10^{-4}	32 ± 5	35 ± 6
	2×10^{-4}	37 ± 3	44 ± 5
	5×10^{-4}	NS[b]	NS[b]
Methyl 1-methoxy-3-aminomethyl-2-oxindole dithiocarbamate (**14**)	1×10^{-4}	35 ± 3	33 ± 9
	2×10^{-4}	49 ± 4	50 ± 5
	5×10^{-4}	69 ± 4	56 ± 2
1-Methoxyspirobrassinin (**15**)	1×10^{-4}	28 ± 5	38 ± 8
	2×10^{-4}	35 ± 7	42 ± 10
	5×10^{-4}	55 ± 4	54 ± 2

[a] % inhibition = 100 − [(growth on treated/growth on control) × 100] ± SD; results are the means of at least three independent experiments conducted in triplicate. [b] NS = not soluble. [c] NI = no inhibition.

14 might be a good lead structure for development of a second generation of brassinin detoxification inhibitors.

Experimental

General experimental procedures

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON. All solvents were HPLC grade and used as such, except for THF (dried over Na/benzophenone). Organic extracts were dried with Na_2SO_4 and solvents removed under reduced pressure in a rotary evaporator. Flash column chromatography (FCC) was carried out using silica gel grade 60, mesh size 230–400 Å. Preparative thin layer chromatography (PTLC) was carried out on silica gel plates, Kieselgel 60 F_{254} (20 × 20 cm × 0.25 mm), compounds were visualized under UV light. Melting points were obtained on a melting point apparatus and are uncorrected. Specific rotations $[\alpha]_D$ were determined at ambient temperature on a polarimeter using a 1 ml, 10 cm path length cell; the units are 10^{-1} deg cm^2 g^{-1} and the concentrations are reported in g per 100 ml. HPLC analysis was 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 reverse phase C-18 column (5 μm particle size silica, 4.6 i.d. × 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, flow rate 1 ml min^{-1}. UV spectra were recorded in CH_3CN. Fourier transform IR spectra were obtained in KBr. NMR spectra were recorded on 500 series spectrometers (see the ESI†); for ^{1}H (500 MHz), δ values were referenced as follows $CDCl_3$ (7.23 ppm); for ^{13}C (125 MHz) $CDCl_3$ (77.23 ppm). Mass spectra (MS) were obtained on a mass spectrometer using a solids probe.

Fungal cultures

Fungal cultures of *L. maculans* isolates BJ 125 and Mayfair 2 were obtained from the IBCN collection, Agriculture and Agri-Food Canada Research Station, Saskatoon SK. Cultures were handled as described previously.[14]

Fungal metabolism

Time course study. Compounds to be investigated (**1a**, **1b** and **7**–**15**) were dissolved in DMSO and were added to cultures of *L. maculans* isolates BJ 125 or Mayfair 2 (10^8 spores per 100 ml) in minimal media (48-h-old, 100 ml in 250 ml Erlenmeyer flasks, final concentration 5×10^{-5} M). Cultures were incubated on a shaker at 130 rpm, 24 ± 2 °C. Samples (5 ml) were withdrawn at appropriate times, frozen or immediately extracted with EtOAc (2 × 5 ml). Organic and water phases were concentrated, residues were dissolved in CH_3CN (1 ml) and were subjected for HPLC analysis. Experiments were performed in triplicate, control flasks containing the mycelia in minimal media as well as tested compounds in minimal media were incubated under similar conditions. For biotransformations in water, 7-d-old cultures of isolates BJ 125

and Mayfair 2 (10^8 per 100 ml spores) were filtered off, the mycelia was washed with sterile water and was transferred to 100 ml of sterile water in 250 ml Erlenmeyer flasks. The compounds were added to the mycelial cultures and worked up and analyzed as described above.

Large-scale metabolism experiments. Compounds **1a**, **1a** + **1b**, **10** and **13** were dissolved in DMSO (1.2 ml) and were added to cultures of isolates BJ 125 or Mayfair 2 (10^8 spores per 100 ml) in minimal media (48-h-old mycelia) or in water (7-d-old mycelia). Cultures were incubated on a shaker at 130 rpm, 24 ± 2 °C as follows: **1a**, **1a** + **1b**, (24 h); **10** (BJ 125 48 h, Mayfair 2 96 h); **13** (72 h). The mycelia were filtered off and the filtrates were extracted with EtOAc. The combined organic extracts were dried, were concentrated and were subjected for PTLC as follows: **1a**, **1a** + **1b** and **13** (hexane–EtOAc (2 : 1)); **10** (hexane–acetone (2 : 1)). Metabolites (listed in order of decreasing polarity) obtained from wasalexin A (**1a**) metabolism: in BJ 125, *S*-methyl 1-methoxy-3-aminomethyl-2-oxindole thiocarbamate (**11**, 2 mg, $[a]_D$ −1.5 (*c* 0.13, CH_2Cl_2)); 1-methoxy-3-methylindol-2-one (**8**, 3 mg, $[a]_D$ 0.10 (*c* 0.13, CH_2Cl_2)); in Mayfair 2, *S*-methyl 1-methoxy-3-aminomethyl-2-oxindole thiocarbamate (**11**, 2 mg, $[a]_D$ −0.50 (*c* 0.10, CH_2Cl_2)); 1-methoxy-3-methyl oxindole (**8**, 3 mg, $[a]_D$ − 4.6 (*c* 0.12, CH_2Cl_2)). Metabolites obtained from methyl 1-methoxy-3-aminomethylene-2-oxindole dithiocarbamate (**13**) metabolism: in BJ 125, methyl 1-methoxy-3-aminomethyl-2-oxindole dithiocarbamate (**14**, 3 mg, $[a]_D$ 5.7 (*c* 0.13, CH_2Cl_2)); methyl 1-methoxy-3-aminomethylene-2-oxindole dithiocarbamate (**13**, 5 mg recovered, 30%); in Mayfair 2, 1-methoxy-3-aminomethyleneindol-2-one (**12**, 2 mg); methyl 1-methoxy-3-aminomethyl-2-oxindole dithiocarbamate (**14**, 1 mg, $[a]_D$ 0.9 (*c* 0.09, CH_2Cl_2)); methyl 1-methoxy-3-aminomethylene-2-oxindole dithiocarbamate (**13**, 2 mg recovered, 12%). Metabolites obtained from *S*-methyl 1-methoxy-3-aminomethylene-2-oxindole thiocarbamate (**10**) metabolism: in BJ 125, *S*-methyl 1-methoxy-3-aminomethyl-2-oxindole thiocarbamate (**11**, 2 mg, $[a]_D$ 6.0 (*c* 0.12, CH_2Cl_2)); *S*-methyl 1-methoxy-3-aminomethylene-2-oxindole thiocarbamate (**10**, 3 mg recovered, 19%); in Mayfair 2, 1-methoxy-3-aminomethyleneindol-2-one (**12**, 5 mg); *S*-methyl 1-methoxy-3-aminomethylene-2-oxindole thiocarbamate (**10**, 1 mg recovered, 6%).

Antifungal bioassays

Antifungal bioassays against *L. maculans* isolates BJ 125 and Mayfair 2 were performed as follows: a DMSO solution of the compound to be tested (final concentration 5×10^{-4}, 2×10^{-4}, 1×10^{-4} M, 1% final DMSO concentration) was added to potato dextrose agar medium at *ca.* 50 °C, was mixed quickly and was poured onto 6-well plates (2.5 ml). An agar plug (8 mm diameter) cut from edges of 7-d-old solid cultures was placed upside down on the center of each plate and the plates were incubated at 24 ± 2 °C under constant light for 5 d (BJ 125) or 6 d (Mayfair 2). The diameter of the mycelia (in mm) was then measured and compared with control plates containing only DMSO. Each assay was conducted in triplicate and repeated at least three times.

Time course study of decomposition of dihydrowasalexin (7) and *S*-ethyl,*S*-methyl 1-methoxy-3-aminomethyl-2-oxindole iminodithioate (16) in minimal media. Compounds **7** and **16** were dissolved in DMSO (0.5 ml) and were added to minimal media (100 ml in 250 ml Erlenmeyer flasks, final concentration 5×10^{-5} M) or water. Samples (5 ml) were withdrawn at different times and were immediately extracted with EtOAc (2 × 5 ml). The organic extracts were worked up as described above and analyzed by HPLC. Experiments were performed in triplicate.

Synthesis

Dihydrowasalexin (7) and *S*-ethyl,*S*-methyl 1-methoxy-3-aminomethyl-2-oxindole iminodithioate (16). Dimethyl sulfate (14 μl, 0.15 mmol) and K_2CO_3 (21 mg, 0.15 mmol) were added to separate solutions of methyl 1-methoxy-3-aminomethyl-2-oxindole dithiocarbamate (**14**, 32 mg, 0.11 mmol) or ethyl 1-methoxy-3-aminomethyl-2-oxindole dithiocarbamate (**25**, 37 mg, 0.12 mmol) in acetone (500 μl) and the mixtures were stirred for 20 h at rt. The reaction mixtures were diluted with brine (20 ml) and were extracted with EtOAc. The combined organic extracts were dried, were concentrated and the residues were subjected for FCC as follows: **14**, hexane–acetone (5 : 1); **25**, hexane–EtOAc (4 : 1) to afford dihydrowasalexin (**7**, 17 mg, 51%) and *S*-ethyl,*S*-methyl 1-methoxy-3-aminomethyl-2-oxindole iminodithioate (**16**, 19 mg, 50%, mixture of *E* : *Z* isomers, 1 : 1).

Dihydrowasalexin (7). Slightly yellow solid, mp 79–81 °C; HPLC: t_R = 23.7 min; λ_{max} (CH_3CN)/nm 232 (log ε, 3.9); ν_{max} (KBr)/cm^{-1}: 2924, 1730, 1613, 1578, 1466, 1318, 1232, 1034, 749. δ_H (500 MHz; $CDCl_3$): 2.18 (s, 3H), 2.53 (s, 3H), 3.75 (m, 1H), 3.86 (dd, *J* = 6.5, 6.5 Hz, 1H), 4.02 (m, 1H), 4.04 (s, 3H), 6.98 (d, *J* = 7.5 Hz, 1H), 7.07 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.31 (m, 2H). δ_C (125 MHz; $CDCl_3$): 14.9 (2 × q), 45.6 (d), 52.8 (t), 63.6 (q), 107.0 (d), 123.0 (d), 125.0 (d), 125.1 (s), 128.3 (d), 141.1 (s), 154.7 (s), 171.3 (s). HRMS (EI): calc. for $C_{13}H_{16}N_2O_2S_2$ (M^+) *m*/*z* 296.0653, found 296.0655. MS (EI) *m*/*z* (% relative abundance): 296 [M^+] (22), 249 (68), 176 (68), 134 (100), 117 (48).

S-*Ethyl,*S-*methyl 1-methoxy-3-aminomethyl-2-oxindole iminodithioate (16).* Slightly yellow oil, mixture of *E* and *Z* isomers; HPLC: t_R = 26.4 min; λ_{max} (CH_3CN)/nm 232 (log ε, 3.7); ν_{max} (KBr)/cm^{-1}: 2926, 1727, 1618, 1580, 1464, 1322, 1228, 1034, 749. δ_H (500 MHz; $CDCl_3$): 1.00 (t, *J* = 7.5 Hz, 3H), 1.34 (t, *J* = 7.5 Hz, 3H), 2.19 (s, 3H), 2.51 (s, 3H), 2.70 (m, 1H), 2.82 (m, 1H), 3.07 (q, *J* = 7.5 Hz, 2H), 3.74 (m, 1H), 3.90 (m, 1H), 4.04 (m, 1H), 4.04 (s, 3H), 6.98 (d, *J* = 8 Hz, 1H), 7.06 (dd, *J* = 8, 8 Hz, 1H), 7.30 (m, 2H). δ_C (125 MHz; $CDCl_3$): 14.1 (q), 14.7 (q), 14.9 (q), 15.4 (q), 25.9 (t), 26.7 (t), 45.6 (2 × d), 52.7 (t), 53.0 (t), 63.6 (2 × q), 107.0 (2 × d), 123.0 (2 × d), 124.8 (d), 125.0 (d), 125.1 (s), 125.2 (s), 128.3 (d), 141.1 (s), 141.2 (s), 160.2 (br s), 171.3 (s), 171.4 (s). HRMS (EI): calc. for $C_{14}H_{18}N_2O_2S_2$ (M^+) *m*/*z* 310.0810, found 310.0812. MS (EI) *m*/*z* (% relative abundance): 310 [M^+] (24), 263 (62), 176 (100), 148 (79), 117 (91).

1-Methoxy-3-methylindol-2-one (8). 10% Pd/C (45 mg) was added to a solution of 1-methoxy-2-oxoindole-3-carboxaldehyde[2] (**21**, 96 mg, 0.5 mmol) in EtOH (7 ml). The mixture was shaken at 3 atm of H_2 for 5 min at rt. The catalyst was filtered off, the filter was washed with CH_2Cl_2 and the filtrate was concentrated. The residue was subjected to FCC, hexane–acetone (5 : 1), to afford 1-methoxy-3-methylindol-2-one (**8**, 39 mg, 44%) as colorless solid, mp 62–64 °C; HPLC: t_R = 10.3 min; λ_{max} (CH_3CN)/nm 238 (log ε, 3.6); ν_{max} (KBr)/cm^{-1}: 2979, 2938, 1727, 1618, 1466, 1323, 1239, 1101, 1051, 963, 748. δ_H (500 MHz; $CDCl_3$): 1.52 (d, *J* = 7.5 Hz, 3H), 3.45 (q, *J* = 7.5 Hz, 1H), 4.05 (s, 3H), 7.00 (d, *J* = 7.5 Hz,

1H), 7.10 (dd, $J = 7.5$, 7.5 Hz, 1H), 7.26 (d, $J = 7.5$ Hz, 1H), 7.32 (dd, $J = 7.5$, 7.5 Hz, 1H). δ_C (125 MHz; $CDCl_3$): 15.2 (q), 39.2 (d), 63.7 (q), 107.4 (d), 123.2 (d), 124.1 (d), 127.1 (s), 128.3 (d), 140.4 (s), 173.3 (s). HRMS (EI): calc. for $C_{10}H_{11}NO_2$ (M^+) m/z 177.0790, found 177.0789. MS (EI) m/z (% relative abundance): 177 [M^+] (47), 149 (100), 118 (52), 91 (19), 77 (11).

***S*-Methyl 1-methoxy-3-aminomethylene-2-oxindole thiocarbamate (10).** HCl (0.5 M, 3 ml) was added to a solution of wasalexins[6] (**1a**, **1b**, 144 mg, 0.49 mmol) in dioxane (5 ml). The mixture was refluxed with stirring for 3 h, was allowed to cool (rt) and was diluted with brine (50 ml). The mixture was extracted (EtOAc), the combined extract was washed with 10% K_2CO_3 solution (20 ml), was dried and was concentrated. The residue was subjected to FCC, hexane–acetone (2 : 1), to yield *S*-methyl 1-methoxy-3-aminomethylene-2-oxindole thiocarbamate (**10**, 84 mg, 65%) as a slightly yellow solid. Mp 123–125 °C; HPLC: $t_R = 20.6$ min; λ_{max} (CH_3CN)/nm 261 (log ε, 4.4), 306 (4.4); ν_{max} (KBr)/cm^{-1}: 3266, 3050, 2942, 1687, 1630, 1458, 1236, 1116, 1052, 945, 735. δ_H (500 MHz; $CDCl_3$): 2.51 (s, 3H), 4.08 (s, 3H), 7.04 (d, $J = 8$ Hz, 1H), 7.10 (dd, $J = 8$, 8 Hz, 1H), 7.28 (dd, $J = 8$, 8 Hz, 1H), 7.42 (d, $J = 8$ Hz, 1H), 8.07 (d, $J = 11$ Hz, 1H), 10.88 (br d, D_2O exch., $J = 11$ Hz, 1H). δ_C (125 MHz; $CDCl_3$): 12.9 (q), 64.3 (q), 104.1 (s), 107.6 (d), 118.7 (s), 118.7 (d), 122.9 (d), 127.6 (d), 131.2 (d), 136.7 (s), 163.9 (s), 168.4 (s). HRMS (EI): calc. for $C_{12}H_{12}N_2O_3S$ (M^+) m/z 264.0569, found 264.0562. MS (EI) m/z (% relative abundance): 264 [M^+] (100), 233 (16), 217 (45), 185 (21), 157 (63), 130 (16), 103 (11).

Preparation of compounds 9, 11, 14, 18 and 25. $NaBH_3CN$ (415 mg, 6.6 mmol) was added to separate solutions of compounds **10**, **13**, **20**, **23** and **24** (0.33 mmol) in AcOH (4 ml). The mixtures were stirred at 55 °C for 18 h, were diluted with brine (30 ml) and were extracted (EtOAc). The combined organic extracts were washed with 10% K_2CO_3 solution (30 ml), were dried, were concentrated and the residues were subjected to FCC as follows: **10** and **13**, hexane–acetone (2 : 1); **20**, hexane–acetone (5 : 1); **23** and **24**, hexane–EtOAc (4 : 1) to yield the following products.

1-Methoxy-3-methylsulfanylmethylindol-2-one (**9**, 29 mg, 26%, based on **22**), slightly yellow oil. HPLC: $t_R = 14.1$ min; λ_{max} (CH_3CN)/nm 238 (log ε, 3.7); ν_{max} (KBr)/cm^{-1}: 2924, 1726, 1618, 1465, 1325, 1225, 1076, 750. δ_H (500 MHz; $CDCl_3$): 2.97 (dd, $J = 7.5$, 7.5 Hz, 1H), 3.28 (dd, $J = 4$, 4 Hz, 1H), 3.67 (dd, $J = 7.5$, 4 Hz, 1H), 4.05 (s, 3H), 7.02 (d, $J = 8$ Hz, 1H), 7.11 (dd, $J = 8$, 8 Hz, 1H), 7.36 (dd, $J = 8$, 8 Hz, 1H), 7.49 (d, $J = 8$ Hz, 1H). δ_C (125 MHz; $CDCl_3$): 16.9 (q), 35.2 (d), 44.2 (t), 63.7 (q), 107.5 (d), 123.3 (d), 124.6 (s), 125.2 (d), 128.8 (d), 140.9 (s), 170.9 (s). HRMS (EI): calc. for $C_{11}H_{13}NO_2S$ (M^+) m/z 223.0667, found 223.0661. MS (EI) m/z (% relative abundance): 223 [M^+] (79), 192 (38), 164 (47), 148 (67), 133 (16), 117 (100), 90 (33).

S-Methyl 1-methoxy-3-aminomethyl-2-oxindole thiocarbamate (**11**, 50 mg, 56%), colorless solid. Mp 115–118 °C; HPLC: $t_R = 9.0$ min; λ_{max} (CH_3CN)/nm 238 (log ε, 3.7); ν_{max} (KBr)/cm^{-1}: 3300, 2939, 1714, 1615, 1525, 1466, 1321, 1226, 1082, 747. δ_H (500 MHz; $CDCl_3$): 2.37 (s, 3H), 3.46 (m, 1H), 3.62 (m, 1H), 4.05 (s, 3H), 4.19 (m, 1H), 6.27 (br s, D_2O exch., 1H), 7.02 (d, $J = 8$ Hz, 1H), 7.13 (dd, $J = 8$, 8 Hz, 1H), 7.33 (d, $J = 8$ Hz, 1H), 7.36 (dd, $J = 8$, 8 Hz, 1H). δ_C (125 MHz; $CDCl_3$): 12.6 (q), 41.0 (d), 43.9 (t), 63.9 (q), 107.8 (d), 122.3 (s), 123.7 (d), 124.8 (d), 129.2 (d), 140.8 (s), 168.7 (s), 171.6 (s). HRMS (EI): calc. for $C_{12}H_{14}N_2O_3S$ (M^+) m/z 266.0725, found 296.0727. MS (EI) m/z (% relative abundance): 266 [M^+] (12), 175 (100), 144 (31), 117 (19).

Methyl 1-methoxy-3-aminomethyl-2-oxindole dithiocarbamate (**14**, 59 mg, 63%), slightly yellow solid. Mp 112–114 °C; HPLC: $t_R = 18.5$ min; λ_{max} (CH_3CN)/nm 238 (log ε, 4.2); ν_{max} (KBr)/cm^{-1}: 3246, 2917, 1699, 1617, 1509, 1465, 1319, 1103, 951, 748. δ_H (500 MHz; $CDCl_3$): 2.67 (s, 3H), 3.57 (m, 1H), 3.85 (m, 1H), 4.06 (s, 3H), 4.88 (m, 1H), 7.04 (d, $J = 7.5$ Hz, 1H), 7.15 (dd, $J = 7.5$, 7.5 Hz, 1H), 7.38 (m, 2H), 8.22 (br s, D_2O exch., 1H). δ_C (125 MHz; $CDCl_3$): 18.3 (q), 42.4 (d), 46.5 (t), 64.0 (q), 107.9 (d), 121.9 (s), 123.9 (d), 124.9 (d), 129.3 (d), 140.6 (s), 172.0 (s), 200.2 (s). HRMS (EI): calc. for $C_{12}H_{14}N_2O_2S_2$ (M^+) m/z 282.0497, found 282.0505. MS (EI) m/z (% relative abundance): 282 [M^+] (10), 251 (61), 234 (83), 175 (100), 144 (75), 117 (57), 63 (11).

1-Methoxy-3-ethylsulfanylmethylindol-2-one (**18**, 28 mg, 23%, based on **22**), slightly yellow oil. HPLC: $t_R = 17.4$ min; λ_{max} (CH_3CN)/nm 238 (log ε, 3.8); ν_{max} (KBr)/cm^{-1}: 2927, 1726, 1617, 1462, 1324, 1217, 1068, 750. δ_H (500 MHz; $CDCl_3$): 1.25 (t, $J = 7.5$ Hz, 3H), 2.58 (q, $J = 7.5$ Hz, 2H), 2.97 (dd, $J = 7.5$, 7.5 Hz, 1H), 3.33 (dd, $J = 3.5$, 3.5 Hz, 1H), 3.65 (m, 1H), 4.05 (s, 3H), 7.02 (d, $J = 7.5$ Hz, 1H), 7.11 (dd, $J = 7.5$, 7.5 Hz, 1H), 7.35 (dd, $J = 7.5$, 7.5 Hz, 1H), 7.49 (d, $J = 7.5$ Hz, 1H). δ_C (125 MHz; $CDCl_3$): 15.0 (q), 27.3 (t), 32.6 (d), 44.4 (t), 63.7 (q), 107.5 (d), 123.3 (d), 124.6 (s), 125.2 (d), 128.8 (d), 140.9 (s), 170.9 (s). HRMS (EI): calc. for $C_{12}H_{15}NO_2S$ (M^+) m/z 237.0824, found 237.0823. MS (EI) m/z (% relative abundance): 237 [M^+] (45), 206 (16), 175 (11), 148 (24), 117 (33), 90 (12).

Ethyl 1-methoxy-3-aminomethyl-2-oxindole dithiocarbamate (**25**, 66 mg, 67%), slightly yellow solid. Mp 134–136 °C; HPLC: $t_R = 21.1$ min; λ_{max} (CH_3CN)/nm 238 (log ε, 4.1); ν_{max} (KBr)/cm^{-1}: 3263, 2927, 1712, 1619, 1507, 1462, 1323, 1091, 948, 749. δ_H (500 MHz; $CDCl_3$): 1.38 (t, $J = 7.5$ Hz, 3H), 3.28 (q, $J = 7.5$ Hz, 2H), 3.57 (m, 1H), 3.85 (m, 1H), 4.06 (s, 3H), 4.87 (m, 1H), 7.04 (d, $J = 8$ Hz, 1H), 7.15 (dd, $J = 8$, 8 Hz, 1H), 7.38 (m, 2H), 8.15 (br s, D_2O exch., 1H). δ_C (125 MHz; $CDCl_3$): 14.2 (q), 29.8 (t), 42.4 (d), 46.3 (t), 64.0 (q), 107.9 (d), 122.0 (s), 123.9 (d), 124.9 (d), 129.3 (d), 140.7 (s), 172.0 (s), 199.5 (s). HRMS (EI): calc. for $C_{13}H_{16}N_2O_2S_2$ (M^+) m/z 296.0653, found 296.0659. MS (EI) m/z (% relative abundance): 296 [M^+] (10), 265 (100), 234 (43), 203 (13), 175 (92), 145 (21), 117 (65), 77 (19).

1-Methoxy-3-aminomethyleneindol-2-one (12). For the synthesis and spectral data (IR, NMR, MS) see reference.[2] λ_{max}(CH_3CN)/nm 258 (log ε, 4.3), 294 (4.2).

1-Methoxyspirobrassinin (15). For synthesis see reference;[7] for spectral data see reference.[15]

***S*,*S*-Diethyl 1-methoxy-3-aminomethylene-2-oxindole iminodithioate (17).** K_2CO_3 (26 mg, 0.19 mmol) and EtI (30 µl, 0.37 mmol) were added to a stirred solution of ethyl 1-methoxy-3-aminomethylene-2-oxindole dithiocarbamate (**25**, 46 mg, 0.16 mmol) in acetone. The mixture was stirred for 18 h at rt, was diluted with brine (25 ml) and was extracted with EtOAc. The combined organic extract was dried, was concentrated and the residue was subjected to FCC, hexane–EtOAc (4 : 1), to afford *S*,*S*-diethyl 1-methoxy-3-aminomethylene-2-oxindole iminodithioate (**17**, 20 mg, 40%) as a slightly yellow oil. HPLC: $t_R = 29.4$ min; λ_{max} (CH_3CN)/nm 232 (log ε, 3.7); ν_{max} (KBr)/cm^{-1}: 2928, 1727, 1618, 1579, 1463, 1322, 1228, 1084, 749. δ_H (500 MHz;

$CDCl_3$): 1.03 (t, $J = 7$ Hz, 3H), 1.35 (t, $J = 7$ Hz, 3H), 2.75 (m, 1H), 2.88 (m, 1H), 3.06 (q, $J = 7$ Hz, 2H), 3.77 (m, 1H), 3.94 (dd, $J = 5.5$, 5.5 Hz, 1H), 4.04 (s, 3H), 4.07 (m, 1H), 6.98 (d, $J = 8$ Hz, 1H), 7.07 (dd, $J = 8$, 8 Hz, 1H), 7.30 (m, 2H). δ_C (125 MHz; $CDCl_3$): 14.1 (q), 15.3 (q), 26.1 (t), 26.8 (t), 45.5 (d), 52.6 (t), 63.6 (q), 107.1 (d), 123.1 (d), 124.9 (d), 125.0 (s), 128.3 (d), 141.2 (s), 152.4 (s), 171.4 (s). HRMS (EI): calc. for $C_{15}H_{20}N_2O_2S_2$ (M^+) m/z 324.0966, found 324.0973. MS (EI) m/z (% relative abundance): 324 [M^+] (15), 263 (93), 176 (100), 148 (64), 117 (69).

Ethyl 1-methoxy-3-aminomethylen-2-oxindole dithiocarbamate (20). CS_2 (72 µl, 1.2 mmol) and NaH (60% suspension in mineral oil, 33 mg, 1 mmol) were added to a stirred, cooled (0 °C) solution of 1-methoxy-3-aminomethylene oxindole (**12**, prepared from 1 mmol of 1-methoxyoxindole[6]) in dry THF (2 ml). After 5 min at 0 °C, EtI (88 µl, 1.1 mmol) was added and the stirring continued for further 1 h at 0 °C. The mixture was diluted with 1 M HCl (30 ml) and was extracted with EtOAc. The combined organic extract was dried, was concentrated and the residue was subjected for FCC, hexane–acetone (5 : 1). Ethyl 1-methoxy-3-aminomethylene-2-oxindole dithiocarbamate (**20**, 97 mg, 33%, based on 1-methoxyoxindole) was obtained as a bright yellow solid, mp. 123–125 °C; HPLC: $t_R = 34.3$ min; λ_{max} (CH_3CN)/nm 269 (log ε, 4.2), 348 (4.3); ν_{max} (KBr)/cm^{-1}: 3178, 2932, 1684, 1641, 1496, 1454, 1217, 1101, 1056, 996, 915, 778, 742. δ_H (500 MHz; $CDCl_3$): 1.44 (t, $J = 7.5$ Hz, 3H), 3.39 (q, $J = 7.5$ Hz, 2H), 4.10 (s, 3H), 7.05 (d, $J = 8$ Hz, 1H), 7.12 (dd, $J = 8$, 8 Hz, 1H), 7.30 (dd, $J = 8$, 8 Hz, 1H), 7.50 (d, $J = 8$ Hz, 1H), 8.68 (d, $J = 10$ Hz, 1H), 12.10 (br d, D_2O exch., $J = 10$ Hz, 1H). δ_C (125 MHz; $CDCl_3$): 13.6 (q), 30.5 (t), 64.4 (q), 106.1 (s), 107.8 (d), 118.6 (s), 119.3 (d), 123.1 (d), 128.0 (d), 133.0 (s), 137.0 (d), 163.9 (s), 199.4 (s). HRMS (EI): calc. for $C_{13}H_{14}N_2O_2S_2$ (M^+) m/z 294.0497, found 294.0498. MS (EI) m/z (% relative abundance): 294 [M^+] (65), 263 (100), 232 (27), 201 (19), 173 (79), 144 (23).

1-Methoxy-3-methylsulfanylmethyleneindol-2-one (23). MeSNa (88 mg, 1.25 mmol) was added to a solution of 1-methoxy-3-chloromethyleneindol-2-one[2] (**22**, 105 mg, 0.5 mmol) in THF (3 ml). The mixture was stirred for 2 h at rt, was concentrated, the residue was suspended in CH_2Cl_2 (10 ml) and SiO_2 was added. The residue, obtained after concentration was subjected to FCC, hexane–acetone (2 : 1), to afford 1-methoxy-3-methylsulfanylmethyleneindol-2-one (**23**, 74 mg, 67%) as yellow oil, which was immediately used in the next step.

1-Methoxy-3-ethylsulfanylmethyleneindol-2-one (24). EtSH (92 µl, 1.25 mmol) and Et_3N (170 µl, 1.25 mmol) were added to a solution of 1-methoxy-3-chloromethyleneindol-2-one[2] (**22**, 105 mg, 0.5 mmol) in THF (3 ml). The mixture was stirred for 15 min at rt, was diluted with brine (20 ml) and was extracted with EtOAc. The combined extract was dried, was concentrated and the residue was subjected to FCC, hexane–acetone (5 : 1), to afford 1-methoxy-3-methylsulfanylmethyleneindol-2-one (**24**, 87 mg, 74%) as yellow oil, which was immediately used in the next step.

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