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Phytochemistry 64 (2003) 949–956
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Phytoalexins from *Thlaspi arvense*, a wild crucifer resistant to virulent *Leptosphaeria maculans*: structures, syntheses and antifungal activity

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Received 18 April 2003; received in revised form 30 May 2003

Abstract

Phytoalexins are inducible chemical defenses produced by plants in response to diverse forms of stress, including microbial attack. Our search for phytoalexins from cruciferous plants resistant to economically important fungal diseases led us to examine stinkweed or pennycress (*Thlaspi arvense*), a potential source of disease resistance to blackleg. We have investigated phytoalexin production in leaves of *T. arvense* under abiotic (copper chloride) and biotic elicitation by *Leptosphaeria maculans* (Desm.) Ces. et de Not. [asexual stage *Phoma lingam* (Tode ex Fr.) Desm.], and report here two phytoalexins, wasalexin A and arvelexin (4-methoxyindolyl-3-acetonitrile), their syntheses and antifungal activity against isolates of *P. lingam*/*L. maculans*, as well as the isolation of isovitexin, a constitutive glycosyl flavonoid of stinkweed, having antioxidant properties but devoid of antifungal activity.

Keywords: *Thlaspi arvense*; Stinkweed; *Brassicaceae*; Crucifer; G *Leptosphaeria maculans*; 4-Methoxyindolyl-3-acetonitrile; *Phoma lingam*; Phytoalexin; Wasalexin A; Wasalexin synthesis

1. Introduction

Phytoalexins are inducible secondary metabolites produced de novo by plants in response to diverse forms of stress, including microbial attack (Bailey, 1982). Until now the majority of the cruciferous phytoalexins have been isolated from widely cultivated *Brassica* species. However, because the production of phytoalexins can have a significant impact on plant disease resistance (Pedras et al., 2000), it is of great interest to screen a wider range of cruciferous species for production of these antimicrobial metabolites. Crucifers (family Cruciferae or Brassicaceae) comprise a number of economically important oilseed and condiment crops such as canola (*Brassica napus* and *B. rapa*), rapeseed (*B. napus Sinapis alba*), many vegetable species including turnip (*B. rapa*), broccoli (*Brassica oleracea* var. *botrytis*), cauliflower (*B. oleracea* var. *italica*), kale (*B. oleracea* var. *acephala*), radish (*Raphanus sativus*), and cabbage (*B. oleracea*). In addition, the wild crucifer *Arabidopsis thaliana* is an important model plant and the first flowering plant to have its genome sequenced (The Arabidopsis Genome Initiative, 2000). A number of cruciferous weeds are also known for their negative impact on cultivated systems.

Our search for phytoalexins from cruciferous plants resistant to economically important fungal diseases (Pedras et al., 2000), led us to examine stinkweed or pennycress (*Thlaspi arvense*), a potential source of disease resistance to blackleg (Gugel et al., 1990). Blackleg of crucifers, caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not. [asexual stage *Phoma lingam* (Tode ex Fr.) Desm.], occurs worldwide and can be particularly devastating for the oilseed crops rapeseed and canola. Most importantly, stinkweed is resistant to the virulent strain of *L. maculans*/*P. lingam* but susceptible to the Thlaspi strain (Petrie et al., 1995; Gugel et al., 1990). Interestingly the resistance range of stinkweed is similar to that of the crucifer wasabi (*Wasabia japonica*, syn. *Eutrema wasabi*), a perennial plant widely cultivated in Japan (Pedras and Sorensen, 1998). Accordingly, it is of enormous importance to determine the chemical traits responsible for the resistance of

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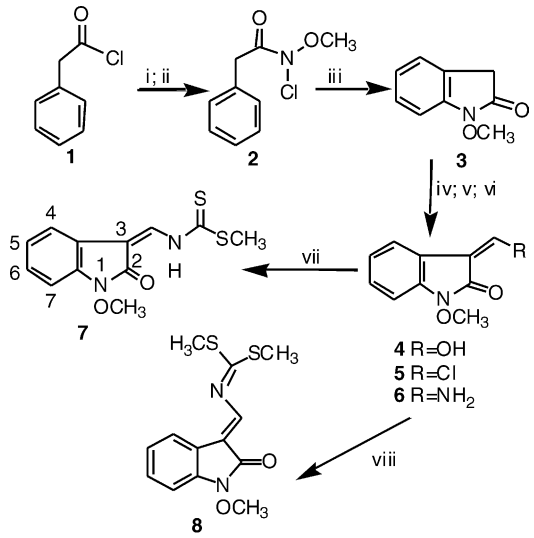
doi:10.1016/S0031-9422(03)00441-2

stinkweed to blackleg isolates, as such agronomically important traits are transferable to canola. We have investigated phytoalexin production in leaves of stinkweed under elicitation by $CuCl_2$ and *P. lingam*/*L. maculans* and report here two phytoalexins, wasalexin A (**8**) (Scheme 1) and arvelexin (**10**), their syntheses and antifungal activity against isolates of *P. lingam*/*L. maculans*, as well as the isolation of isovitexin (**11**), a constitutive glycosyl flavonoid of stinkweed, having antioxidant properties but devoid of antifungal activity.

2. Results and discussion

In preliminary experiments, a time-course response of stinkweed plants to abiotic elicitation with $CuCl_2$ was investigated. After spraying and incubating plants, leaves were excised at 24 h intervals, crushed in liquid N_2 and extracted as described in the Experimental. Control (i.e. non-elicited) leaves were collected and treated in a similar manner. Analysis of these extracts by high performance liquid chromatography (HPLC) under the conditions described in the Experimental indicated the presence of two peaks at $t_R = 13.9$ and 24.4 min in the extracts of elicited leaves, but not detectable on the chromatograms of extracts of control tissues. The results of this time-course experiment indicated that the production of these two elicited metabolites increased as shown in Table 1. Furthermore, biotic elicitation of stinkweed with fungal isolates Mayfair 2 (weakly virulent on canola) or Thlaspi 9 (virulent on stinkweed) indicated the presence of similar peaks in the HPLC chromatograms ($t_R = 13.9$ and 24.4 min) of extracts of infected leaves. Thus, in order to isolate these phytoalexins, larger scale experiments were carried out with plants treated with $CuCl_2$ solution. Elicited leaves were extracted and the residue was separated by flash column chromatography (CH_2Cl_2–MeOH) as described in the experimental. Fractions F2–F4 were combined and further subjected to multiple chromatography to yield the elicited compound with retention time $t_R = 13.9$ min, whereas fractions F9–F12 were combined and further subjected to multiple chromatography to yield a bright yellow compound with $t_R = 24.4$ min. This yellow compound was identified as the phytoalexin wasalexin A (**8**) by comparison of its NMR, HRMS, FTIR, UV, and HPLC data, with that of an authentic sample available in our laboratory. Interestingly, wasalexin A (**8**) was previously isolated from wasabi leaves (*E. wasabi*) after elicitation with $CuCl_2$ or *P. lingam*/*L. maculans* (Pedras et al., 1999). The synthesis of wasalexin A (**8**) and desmethyl analog **7** was carried out as outlined on Scheme 1 and described in the Experimental, in seven steps and 27% overall yield. The key intermediate 1-methoxy-2-oxoindole (**3**) was prepared from phenylacetyl chloride, and methoxyl amine hydrochloride, followed by chlorination using *t*-butyl hypochlorite, to yield the corresponding *N*-chloro-*N*-methoxyamide **2** (Kawase et al., 1989). Ag_2CO_3 promoted electrophilic cyclization afforded 1-methoxy-2-oxoindole (3) in 79% yield (Kikugawa and Kawase, 1984). Following formylation of **3** with ethyl formate and NaH (Pedras and Okanga, 1999), the resulting enol was converted to the chloromethylene intermediate **4** using thionyl chloride (Behringer and Weissauer, 1952), followed by amination to afford 1-methoxy-2-oxo-3-aminomethylene indole (**6**) in 83% yield. Treatment of enamine **6** with carbon disulfide under basic conditions provided the dithiocarbamate salt, which was then methylated to afford a mixture of wasalexins A (**8**) and B and/or methyl dithiocarbamate **7**, depending on the reaction conditions. Thus, when one equivalent of each NaH and MeI was used, dithiocarbamate **7** was the main product, two equivalents of each NaH and MeI led to the exclusive formation of wasalexins A (**8**) and B in 68% yield from 1-methoxy-2-oxoindole (**3**). As previously reported (Pedras et al., 1999), the ratio of wasalexins A (**8**) and B was determined to be 2:1 by ^1H NMR spectroscopy and HPLC analysis. The naturally occurring wasalexin A (**8**) can be separated from the isomeric mixture by slow crystallization using CH_2Cl_2–hexane. The *Z* configuration of the double bond of methyl dithiocarbamate **7** was established on the basis of NOE experiments. Thus, irradiation of the aromatic proton H-4 resulted in the enhancement of the intensity (9%) of the peak corresponding to the side chain proton. The *Z* stereochemistry can be explained by the presence of intramolecular hydrogen bonding between



Scheme 1. Synthesis of wasalexin A (**8**); reagents and conditions: (i) CH_3ONH_2·HCl, Na_2CO_3, C_6H_6–H_2O (1:1), r.t., 88%; (ii) *t*-BuOCl, CH_2Cl_2, r.t., 99%; (iii) Ag_2CO_3, TFA, 0 °C, 79%; (iv) EtOCHO, NaH, r.t., 99%; (v) $SOCl_2$, r.t., 99%; (vi) NH_4OH, MeOH, 83%; (vii) NaH (1.1 eq), CS_2, THF, r.t.; CH_3I (1.1 eq), yields are reported in the experimental; (viii) NaH (2.1 eq), CS_2, THF, r.t.; CH_3I (2.1 eq), 68%.

Table 1
Production of phytoalexins[a] by *Thlaspi arvense* upon elicitation with $CuCl_2$

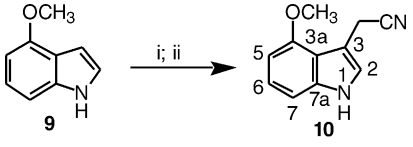
Phytoalexin	24 h (μmol/100 g fresh weight)	48 h (μmol/100 g fresh weight)	72 h (μmol/100 g fresh weight)	96 h (μmol/100 g fresh weight)
Arvelexin (**10**)	0.65±0.05	1.5±0.3	1.8±0.2	2.6±0.2
Wasalexin A (**8**)	3.7±0.5	7.1±0.3	10.3±0.8	9.1±0.6

[a] Results are presented as means±standard deviation.

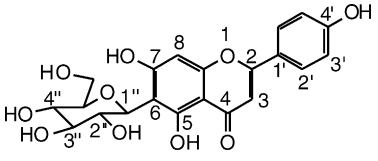
the oxygen of the carbonyl group (C-2) and the hydrogen on the nitrogen of the side chain (N–H).

The molecular formula of the elicited compound with $t_R = 13.9$ min was determined to be $C_{11}H_{10}ON_2$ by HRMS-EI, which suggested eight degrees of unsaturation. The ^{1}H NMR spectrum of this metabolite showed five aromatic protons, as well as two methylene and three methoxy protons. The spin systems of the aromatic protons showed that the proton at δ 7.14 (H-6, *dd*, $J = 7.5$, 7.5 Hz) was coupled to two protons at δ 7.04 (H-7, *d*, $J = 7.5$ Hz) and δ 6.57 (H-5, *d*, $J = 7.5$ Hz), the coupling constants further suggesting that the orientation of H-6–H-7 and to H-5 is *ortho*. An exchangeable broad proton at δ 8.26 and a broad singlet at δ 7.14 (H-2) suggested the aromatic protons to be part of an indole ring substituted at either position 4 or 6. Analysis of the HMBC spectrum showed correlations of the most downfield carbon at δ 154.6 (C-4) with methoxy protons, and the methylene protons at δ 4.07 with carbons at δ 120.2 (-CN), δ 112.6 (C-2), δ 105.0 (C-3), and δ 117.7 (C-4a). FTIR data showed a weak absorption at 2251 cm^{-1} which was attributed to a nitrile group, also consistent with the ^{13}C NMR chemical shift at δ 120.2. Therefore, structure **10** was assigned to this elicited compound with $t_R = 13.9$ min. This structural assignment was confirmed by synthesis through coupling of 4-methoxyindole (**9**) with bromoacetonitrile under Grignard conditions, in 52% overall yield (Scheme 2), as described in the Experimental. Although our synthetic preparation of **10** is very simple, a more economic two-step conversion of indole-3-carboxaldehyde to 4-methoxyindolyl-3-acetonitrile (**3**) was recently reported (Yamada et al., 1998). Compound **10** was first isolated from roots of Chinese cabbage (*B. pekinensis*) infected with *Plasmodiophora brassicae* (Nomoto and Tamura, 1970); however, no antifungal activity has been reported to date.

Together with the elicited compounds **8** and **10**, another bright yellow compound with $t_R = 3.5$ min, also present in control tissues, was isolated from more polar fractions. The HRMS-FAB spectrum of this compound showed a molecular ion at m/z 433.1134 [$M^+ + 1$] which together with the NMR spectroscopic data indicated a molecular formula of $C_{21}H_{20}O_{10}$. The ^{1}H NMR spectrum of this compound (DMSO-d_6) displayed seven protons at sp^3 carbons and six aromatic protons. Four of these aromatic proton signals at δ 6.91 (2H, *d*, $J = 8$ Hz, H-3′ and H-5′) and at δ 7.90 (2H, *d*, $J = 8$ Hz, H-2′ and H-6′) showed an ortho relationship. These data as well as 2D NMR spectra suggested the presence of a para-hydroxyphenyl moiety. The long range correlation of the aromatic proton at δ 6.46 (H-3, *s*) with a carbon at δ 121.0 (C-1) as well as protons H-2′ and H-6′ with a carbon at δ 164.7 (C-2) indicated the connectivity of the *para*-hydroxyphenyl moiety to a flavonoid type ring. Furthermore, the ^{1}H NMR and ^{13}C NMR spectra also revealed the existence of a glucose moiety. The anomeric proton at δ 4.59 (*d*, $J = 9$ Hz, H-1) and the remaining $^2J_{H-H}$ suggested that the glycoside moiety was a β-glucopyranoside. Since the anomeric proton (δ 4.59) showed long range correlations with carbons at δ 160.7 (C-5), δ 163.2 (C-7), and δ 109.0 (C-6) its connectivity with an aromatic system was evident. The unusually high field anomeric carbon signal (δ 73.1) of this compound, as well as its resistance to acid hydrolysis was suggestive of a C–C linked β-glucopyranoside moiety. Final analysis of the HMBC data of this compound led to its identification as isovitexin (**11**). Comparison of the ^{1}H and ^{13}C NMR (Ramarathnam et al., 1989, NMR data was reported in MeOH-d_4), UV, and $[\alpha]_D$ (17.2, *c* 0.37, EtOH, lit. 16.21, Briggs and Cambie, 1958) spectroscopic data of compound **11** with those published showed good agreement. Isovitexin (**11**, apigenin 6-C-β-glucopyranoside) was first isolated from *Vitex lucens* (Briggs and Cambie, 1958; Horowitz and Gentili, 1964), and later on established to show potent anti-oxidant activity (Ramarathnam et al., 1989). To the best of our knowledge this is the first report of isovitexin (**11**) production by *T. arvense*.



Scheme 2. Synthesis of arvelexin; reagents and conditions: (i) CH_3I/Mg, Et_2O, r.t.; (ii) $BrCH_2CN$, r.t., 52%.



Antifungal activity assays were carried out with metabolites **7**, **8**, **10**, **11** and indolyl-3-acetonitrile (introduced for comparison with **10**), against three standard isolates of *L. maculans*/*P. lingam*: BJ 125 (virulent on canola), Mayfair 2 (weakly virulent on canola), and Thlaspi 9 (virulent on stinkweed). Results of these assays indicate that compound 11 displays no antifungal activity whereas **7**, **8**, **10** and indolyl-3-acetonitrile are inhibitory to the three isolates (5×10^{-4} M, Table 2), though indolyl-3-acetonitrile appears to more inhibitory to virulent isolate BJ 125 than any of the other three compounds.

Compound **10** displays antifungal activity, and its formation is induced by stinkweed under biotic (*L. maculans*/*P. lingam*) or abiotic ($CuCl_2$) elicitation but it is not produced in control plants. Thus metabolite **10** satisfies the conditions to be a phytoalexin for which we propose the name arvelexin. Arvelexin accumulates in the leaves of stinkweed to levels comparable with those of other phytoalexins (Pedras et al., 2002). Interestingly, we found that the structurally related indolyl-3-acetonitrile is also a phytoalexin in *B. juncea* (resistant to BJ 125) but not in *B. rapa* or *B. napus* (Pedras et al., 2002). It is noteworthy that two unrelated plant species, wasabi and stinkweed, apparently resistant to the same fungal isolates of *L. maculans*/*P. lingam* produce the phytoalexin wasalexin A (**8**). Wasalexin production could suggest it has a role in resistance/defense of these two species against virulent blackleg isolates. It is anticipated that a better insight into defense mechanisms of crucifers will be obtained with the investigation of phytoalexin profiles of unrelated species. Eventually, as phytoalexins from unrelated cruciferous species are discovered and biological activities established, the "design" of species producing a phytoalexin blend active against a range of pathogens may become a feasible strategy.

3. Experimental

3.1. General

All chemicals were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON. Analytical HPLC analysis was carried out with a high performance liquid chromatograph equipped with quaternary pump, automatic injector, and photodiode array detector (wavelength range 190–600 nm), degasser, and a Hypersil ODS column (5 μm particle size silica, 4.6 i.d. × 200 mm), equipped with an in-line filter. Mobile phase: H_2O-CH_3CN, 75:25 to 100% CH_3CN, for 35 min, linear gradient, and 1.0 ml/min flow rate. Samples were dissolved either in CH_3CN or in MeOH. Other conditions as previously reported (Pedras and Okanga, 1999).

3.1.1. Plant material

Seeds of *Thlaspi arvense* (stinkweed or pennycress) were obtained from Plant Gene Resources, Agriculture

Table 2
Antifungal activity of compounds **7**, **8**, **10**, and **11** against *Leptosphaeria maculans* (*Phoma lingam*) isolates BJ 125 (virulent on canola), Mayfair 2 (virulent on brown mustard), and Thlaspi 9 (virulent on stinkweed)

Compound concentration (M)	BJ 125 % inhibition (mycelium diameter)[a]	Mayfair 2 % inhibition (mycelium diameter)[a]	Thlaspi 9 % inhibition (mycelium diameter)[a]
Desmethylwasalexin (**7**)			
1×10^{-4}	No inhibition	No inhibition	No inhibition
3×10^{-4}	15 (17 ±1)	No inhibition	No inhibition
5×10^{-4}	26 (15 ±1)	20 (13 ±1)	14 (13 ±1)
Wasalexin A (**8**)			
1×10^{-4}	No inhibition	No inhibition	No inhibition
3×10^{-4}	17 (15 ±1)	20 (11 ±1)	No inhibition
5×10^{-4}	42 (12 ±1)	28 (10 ±1)	30 (11 ±1)
Arvelexin (**10**)			
1×10^{-4}	No inhibition	No inhibition	No inhibition
3×10^{-4}	11 (18 ±1)	33 (15 ±1)	No inhibition
5×10^{-4}	73 (8 ±1)	53 (12 ±1)	59 (11 ±1)
Isovitexin (**11**)			
5×10^{-4}	No inhibition	No inhibition	No inhibition
Indolyl-3-acetonitrile			
1×10^{-4}	No inhibition	No inhibition	No inhibition
3×10^{-4}	77 (8 ±1)	13 (18 ±1)	11 (18 ±1)
5×10^{-4}	complete inhibition	25 (16 ±1)	57 (11 ±1)

[a] The percentage of inhibition was calculated using the formula: % inhibition = 100− [(growth on treated/growth in control) × 100]; results are the mean of at least three independent experiments (mm ± standard deviation); control plates contained potato dextrose agar and 1% DMSO.

and Agri Food Canada, Saskatoon Research Center, SK. The seeds were sown in a commercial potting soil mixture, and plants were grown in a growth chamber, with 16 h light (fluorescent and incandescent)/8 h dark, at 24±2 °C.

3.2. Time-course study for the production of phytoalexins and biotic elicitation of phytoalexins

Elicitation with $CuCl_2$: 4-week-old plants were sprayed with a $CuCl_2$ (2 mmol) solution at 0, 24, and 48 h, and incubated for 2 additional days. Leaves were excised at the base, were weighed, frozen in liquid nitrogen, crushed, and then soaked in EtOAc for 24 h. The EtOAc was decanted, dried (anhyd. Na_2SO_4), and concentrated under reduced pressure. The residues from both elicited and control leaves were analyzed by HPLC.

Elicitation with fungal isolates: leaves of 4-week-old plants were cut, the petioles covered with moist cotton wool, leaves were damaged slightly by scraping with pipette tip and dipped into a spore suspension (10^6 spores/ml, isolates Mayfair 2 or Thlaspi 9). Leaves were placed into a Petri dish, the plate was sealed with parafilm and incubated (16 h photoperiod, 20±0.5 °C) for 7 days. After incubation, the leaves were frozen, and treated as described above. The EtOAc extracts of treated and control leaves were analyzed by HPLC.

3.3. Antifungal bioassays

3.3.1. Mycelial radial growth

Bioassays to determine antifungal activity were carried out with *P. lingam*/*L. maculans* isolates BJ 125, Mayfair 2, and Thlaspi 9. Each isolate was grown on V8 agar under continuous light; after 15 days the fungal spores were collected and stored at −20 °C. A DMSO solution of the compound to be tested (final concentration of each compound 5.0×10^{-4}, 2.5×10^{-4} and 10^{-4} M; final DMSO concentration 1%) was added to agar medium at ca. 50 °C, mixed quickly and poured onto 12-well plates (1 ml). An agar plug (4 mm diameter) cut from edges of 7-day-old solid cultures was placed upside down on the center of each plate, the plates were sealed with parafilm, and incubated at 23±2 °C under constant light for 96 h. 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.

3.4. Isolation of phytoalexins

Four-week-old plants (*T. arvense*) were sprayed with $CuCl_2$ solution (2×10^{-3} M) every 24 h for 3 consecutive days. All leaves were collected (182 g) 48 h after the last spray, frozen in liquid N_2, crushed, and extracted with EtOAc (12 h at r.t.). The EtOAc extract was dried (Na_2SO_4), filtered, and concentrated to dryness under reduced pressure; the crude extract residue (0.74 g) was separated by FCC (i.d. 2 cm, silica gel, 100 ml fractions, CH_2Cl_2 400 ml; CH_2Cl_2–MeOH 97:3, 400 ml, CH_2Cl_2–MeOH 94:6, 400 ml).

Fractions F2–F4 from FCC on EtOAc extracts were combined (39 mg) and subjected to FCC (i.d. 1 cm, 50 ml silica gel, CH_2Cl_2–hexane 30:70, 200 ml, 50:50, 200 ml, CH_2Cl_2 200 ml). Twelve fractions collected were concentrated to dryness under reduced pressure. Fractions F10–F12 were combined (18 mg) and further subjected to prep. TLC (CH_2Cl_2–MeOH, 98:2) to yield arvelexin (**10**, 1 mg, HPLC $t_R = 13.9$ min). Fractions F7 and F8 from FCC of EtOAc extracts were combined (60.5 mg) and separated by FCC (i.d. 2 cm, CH_2Cl_2–MeOH 99:1, 200 ml, 97:3, 200 ml). Fractions F4 and F5 were combined and further subjected to RP-FCC (CH_3CN–H_2O, 30:70, 70 ml) to yield wasalexin A (**8**, 1.5 mg, HPLC $t_R = 24.4$ min).

3.5. Wasalexin A (*8*)

3.5.1. 1-Methoxy-2-oxoindole (*3*)

A solution of crude *N*-chloro-*N*-methoxy-phenylacetamide (**2**, 1.1 g, 5.5 mmol, Kawase et al., 1989) in TFA (5 ml) was added to a stirred suspension of Ag_2CO_3 (3.04 g, 11 mmol) in TFA (10 ml) at 0 °C. After stirring for 15 min at 0 °C, the pH of the reaction mixture was adjusted to 7 using 20% solution of Na_2CO_3. The mixture was extracted with CH_2Cl_2, the combined organic extracts dried (over Na_2SO_4) and concentrated under reduced pressure. The residue was separated by FCC (CH_2Cl_2) to yield a slightly yellow oil which was crystallized from CH_2Cl_2–hexane to afford compound **3** as colorless needles (0.712 g, 79%), m.p. 84–86 °C (lit. 84.5–86 °C, Et_2O, Somei et al., 2001). The spectral data are in agreement with those previously reported (Somei et al., 2001).

3.5.2. 1-Methoxy-3-formyl-2-oxoindole (*4*)

NaH (60% suspension in mineral oil, washed with hexane, 185 mg, 5.5 mmol) was added to a stirred solution of 1-methoxy-2-oxoindole (**3**, 82 mg, 0.5 mmol) in ethyl formate (1.5 ml). After stirring for further 2 min the reaction mixture became a paste which was cooled to 0 °C, diluted with water (10 ml) and acidified to pH 5 with 1 M HCl. The product was extracted with Et_2O, the extracts were combined, dried (over Na_2SO_4), and concentrated under reduced pressure to afford a brownish solid (95 mg, 99%), of sufficient purity to use in the next step. An analytical sample was obtained by crystallization from diethyl ether–hexane solution, m.p. 183–185 °C, (lit. 190 °C, EtOH, Sakan et al., 1964).

HPLC $t_R = 8.8$ min; ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.63 (*br s*, 1H, D_2O exchangeable), 7.87 (*s*, 1H), 7.58

(*d*, *J* = 8 Hz, 1H), 7.20 (*dd*, *J* = 8, 8 Hz, 1H), 7.03 (*dd*, *J* = 8, 8 Hz, 1H), 6.98 (*d*, *J* = 8 Hz, 1H), 3.90 (*s*, 3H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 164.7 (*s*), 156.6 (*d*), 136.7 (*s*), 127.1 (*d*), 122.9 (2× *d*), 119.1 (*s*), 107.1 (*d*), 103.6 (*s*), 64.3 (*q*); HREIMS *m*/*z* (% relative abundance) measured: 191.0582 (191.0582 calc. for $C_{10}H_9NO_3$); EIMS *m*/*z* (% relative abundance): 191 [M^+] (10), 162 (100), 148 (11), 132 (7), 104 (7), 119 (31), 77 (8); FTIR ν_{max} 3051, 2630, 1684, 1614, 1461, 1421, 1394, 1206, 1104 cm^{-1}.

3.5.3. 1-Methoxy-3-chloromethylene-2- oxoindole (**5**)

Crude 1-methoxy-3-formyl-2-oxoindole (**4**, 95 mg, 0.5 mmol) was dissolved in thionyl chloride (2.5 ml, 34.3 mmol) and allowed to stand for 1 h at r.t. The thionyl chloride was evaporated to yield a yellow residue (104 mg, 99%), of sufficient purity to use in the next step. An analytical sample was obtained by crystallization from CH_2Cl_2–hexane solution, m.p. 98–100 °C.

HPLC t_R = 18.1 min; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.97 (*d*, *J* = 8 Hz, 1H), 7.50 (*s*, 1H), 7.37 (*dd*, *J* = 8, 8 Hz, 1H), 7.10 (*dd J* = 8, 8 Hz, 1H), 6.98 (*d*, *J* = 8 Hz, 1H), 3.90 (*s*, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 161.5 (*s*), 139.8 (*s*), 130.8 (*d*), 130.3 (*d*), 127.2 (*s*), 125.0 (*d*), 123.4 (*d*), 117.4 (*s*), 107.6 (*d*), 64.2 (*q*); HREIMS *m*/*z* (% relative abundance) measured: 209.0240 (209.0244 calc. for $C_{10}H_8ClNO_2$); EIMS *m*/*z* (% relative abundance): 209 [M^+] (100), 194 (14), 174 (49), 159 (30), 146 (24), 129 (6), 115 (16), 102 (10), 89 (7), 59 (6); FTIR ν_{max}: 3059, 2943, 1721, 1616, 1461, 1421, 1320, 1220, 1073, 769, 741, 690 cm^{-1}.

3.5.4. 1-Methoxy-3-aminomethylene-2-oxoindole (**6**)

Ammonium hydroxide (28% solution, 20 ml, 340 mmol) was added to a solution of crude 1-methoxy-3-chloromethylene-2-oxoindole (104 mg, 0.5 mmol) in MeOH (2.5 ml). After stirring for 90 min at r.t., the reaction mixture was diluted with brine (20 ml), extracted with CH_2Cl_2, the extracts were combined, dried over Na_2SO_4, and concentrated under reduced pressure to yield a yellow solid (83 mg, 83%), of sufficient purity to use in the next step. An analytical sample was obtained by crystallization from CH_2Cl_2–hexane solution, m.p. 162–164 °C.

HPLC t_R = 3.3 min; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.26 (*br s*, 1H, D_2O exchangeable), 7.59 (*m*, 1H), 7.28 (*d*, *J* = 8 Hz, 1H), 7.15 (*t*, *J* = 8 Hz, 1H), 7.03 (*m*, 2H), 5.40 (*br s*, 1H, D_2O exchangeable), 4.08 (*s*, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 164.6 (*s*), 144.1 (*d*), 134.4 (*s*), 124.4 (*d*), 121.9 (*d*), 120.6 (*s*), 116.0 (*d*), 107.1 (*d*), 95.7 (*s*), 64.3 (*q*); HREIMS *m*/*z* (% relative abundance) measured: 190.0743 (190.0743 calc. for $C_{10}H_{10}N_2O_2$); EIMS *m*/*z* (% relative abundance): 190 [M^+] (95), 175 (11), 159 (48), 144 (8), 132 (100), 104 (17), 89 (6), 77 (15); FTIR ν_{max}: 3171, 2934, 1672, 1561, 1466, 1306, 1199, 1100, 1033, 953, 740, 604 cm^{-1}.

3.5.5. Methyl 1-methoxy-3-aminomethylene-2-oxoindole dithiocarbamate (**7**)

NaH, (60% suspension in mineral oil, washed with hexane, 15 mg, 0.43 mmol) and CS_2 (30 μl, 0.52 mmol) were added to a solution of crude 1-methoxy-3-aminomethylene-2-oxoindole (83 mg, 0.43 mmol) in dry THF (1 ml) at 0 °C. After stirring the mixture for 5 min at 0 °C, methyl iodide (30 μL, 0.48 mmol) was added and the stirring was continued for further 1 h at 0 °C. The solvent was evaporated and the residue was chromatographed on silica gel (hexane–acetone, 5:1). Concentration of the less polar fraction afforded **7** as a yellow residue (50 mg, 36%, m.p. 140–141 °C, acetone–hexane); the more polar fraction contained an oily yellow material containing wasalexins A and B (13 mg, 9%).

HPLC t_R = 29.4 min; ^{1}H NMR (500 MHz, $CDCl_3$): δ 12.17 (*d*, *J* = 10 Hz, 1H, D_2O exchangeable), 8.68 (*d*, *J* = 10 Hz, 1H), 7.50 (*d*, *J* = 8 Hz, 1H), 7.30 (*dd*, *J* = 8, 8 Hz, 1H), 7.12 (*dd*, *J* = 8, 8 Hz, 1H), 7.05 (*d*, *J* = 8 Hz, 1H), 4.10 (*s*, 3H), 2.79 (*s*, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 200.4 (*s*), 164.1 (*s*), 137.2 (*d*), 133.3 (*s*), 128.2 (*d*), 123.3 (*d*), 119.5 (*d*), 118.7 (*s*), 108.0 (*d*), 106.3 (*s*), 64.5 (*q*), 19.2 (*q*); HREIMS *m*/*z* (% relative abundance) measured: 280.0340 (280.0340 calc. for $C_{12}H_{12}N_2O_2S_2$); EIMS *m*/*z* (% relative abundance): 280 [M^+] (61), 249 (78), 232 (75), 201 (19), 173 (100), 159 (24), 144 (30), 129 (12), 115 (25), 90 (56); FTIR ν_{max}: 3059, 2946, 1683, 1645, 1455, 1321, 1230, 1101, 1058, 998, 740 cm^{-1}.

3.5.6. Wasalexin A (**8**)

NaH, (60% suspension in mineral oil, washed with hexane, 37 mg, 1.1 mmol) and CS_2 (30 μl, 0.52 mmol) were added to a solution of crude 1-methoxy-3-aminomethylene oxoindole (83 mg, 0.43 mmol) in dry THF (1 ml) at 0 °C. After stirring the mixture for 5 min at 0 °C, methyl iodide (70 μl, 1.1 mmol) was added and the stirring was continued for further 30 min at 0 °C and then for 2 h at r.t. The mixture was then cooled to 0 °C, diluted with water (20 ml), extracted with EtOAc, dried over Na_2SO_4, concentrated under reduced pressure and the residue chromatographed on silica gel (hexane–acetone, 2:1). Evaporation of the solvent afforded 100 mg (68%) of yellow oily material, which contained wasalexins A and B in the ratio 2:1. The naturally occurring wasalexin A separated as follows: 50 mg of the mixture of both isomers was dissolved in CH_2Cl_2 (2 ml) and hexane (5 ml) and the mixture allowed to stand for 78 h at 0 °C and then for 5 h at −10 °C. This process afforded wasalexin A as orange cubes, m.p. 139–140 °C (24 mg, 48%). HPLC t_R = 24.4 min; spectroscopic data were consistent with those previously reported (Pedras et al., 1999).

3.6. Arvelexin (**10**)

Magnesium turnings (20 mg, 0.82 mmol) in dry Et_2O (under argon) were allowed to react with iodomethane

(70 μl, 1.1 mmol) at r.t. until all magnesium was consumed (30 min). Next, after distilling off Et_2O and MeI and the residue dissolved in Et_2O, a solution of 4-methoxyindole (25 mg, 0.17 mmol) in Et_2O (500 μl) was added dropwise to the reaction mixture under stirring at r.t. After stirring for 15 min, bromoacetonitrile (90 μl, 1.3 mmol) was added dropwise at r.t., and the mixture stirred for further 60 min at r.t. (Filler et al., 1989). The solvent was evaporated under reduced pressure, the residue was suspended in distilled water (40 ml), and was neutralized with 2 M HCl (0.5 ml). The aqueous phase was extracted with CH_2Cl_2, the organic layer dried over $MgSO_4$, filtered, and concentrated to dryness to yield the crude product (54 mg). After FCC (CH_2Cl_2–hexane, 40:60) arvelexin (**8**) was obtained in 52% yield (16.5 mg).

HPLC $t_R = 13.9$ min; ^{1}H NMR (CD_2Cl_2): δ 8.26 (*bs*, 1H, D_2O exchangeable), 7.14 (*bs*, 1H), 7.11 (*dd*, $J = 8$, 8 Hz, 1H), 7.04 (*d*, $J = 8$ Hz, 1H), 6.57 (*d*, $J = 8$ Hz, 1H), 4.07 (*s*, 2H), 3.93 (*s*, 3H); ^{13}C NMR (CD_2Cl_2): δ 154.6 (*s*), 138.5 (*s*), 123.7 (*d*), 120.2 (*s*), 117.7 (*s*), 112.6 (*d*), 105.3 (*d*), 105.0 (*s*), 100.0 (*d*), 55.3 (*q*), 15.6 (*t*); FTIR ν_{max} 3396, 2955, 2850, 2251,1619, 1590, 1259, 1089 cm^{-1}; HRMS-EI: *m*/*z* 186.0788 (186.0793 calc. for $C_{14}H_{18}O_3$), EI-MS: *m*/*z* (relative intensity) 186.07 [M$^+$] (99%), 171.05 (100%).

3.7. Isovitexin (*11*)

HPLC $t_R = 3.5$ min; $[\alpha]_D = +17.2$ (*c* 0.37, EtOH, lit. +16.21; Briggs and Cambie, 1958); ^{1}H NMR (DMSO-d_6): δ 13.72 (*s*, 1H, D_2O exchangeable), 7.90 (*d*, $J = 8$ Hz, 2H), 6.91 (*d*, $J = 8$ Hz, 2H), 6.74 (*s*, 1H), 6.46 (*s*, 1H), 4.59 (*d*, $J = 9$ Hz, 1H), 4.08 (*dd*, $J = 9$, 9 Hz, 1H), 3.68 (*d*, $J = 11$ Hz, 1H), 3.41 (*dd*, $J = 11$, 5 Hz, 1H), 3.22–3.13 (*m*, 3H); ^{13}C NMR (DMSO-d_6): δ 181.7 (*s*), 164.7 (*s*), 163.3 (*s*), 161.4 (*s*), 160.7 (*s*), 156.4 (*s*), 128.4 (*d*), 121.0 (*s*), 116.0 (*d*), 109.0 (*s*), 102.8 (*s*), 102.6 (*d*), 93.9 (*d*), 81.5 (*d*), 79.0 (*d*), 73.1 (*d*), 70.6 (*d*), 70.1 (*d*), 61.4 (t) (previous NMR data was reported in MeOH-d_4, Ramarathnam et al., 1989). HR-FAB *m*/*z*: 433.1134 [M + 1]$^+$.

Acknowledgements

We thank Dallas Kessler and Richard Gugel, Plant Gene Resources, Agriculture and Agri Food Canada, Saskatoon, SK, for a generous gift of seeds of *T. arvense*. Financial support from the Natural Sciences and Engineering Research Council of Canada (NSERC Discovery Grant to M.S.C.P), NATO (postdoctoral fellowship to M.S.) and the University of Saskatchewan (graduate teaching assistantship to P.B.C.) is gratefully acknowledged.

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