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PHYTOALEXIN ACCUMULATION AND ANTIFUNGAL COMPOUNDS FROM THE CRUCIFER WASABI

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Key Word Index—*Eutrema wasabi*; *Wasabia japonica*; Cruciferae; wasabi; *Leptosphaeria maculans*; *Phoma lingam*; *Phoma wasabiae*; blackleg disease; antifungal; canola; mustard; phytoalexin.

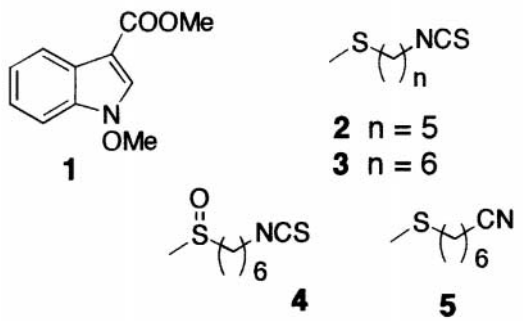
Abstract—The constitutive antifungal metabolites produced by wasabi (*Wasabia japonica*, syn. *Eutrema wasabi*), a plant resistant to virulent isolates of the blackleg fungus [*Leptosphaeria maculans* (Desm.) Ces. et de Not., asexual stage *Phoma lingam* (Tode ex Fr.) Desm.] were isolated and their chemical structures determined. In addition, the chemical structure and synthesis of the first wasabi phytoalexin, methyl 1-methoxyindole-3-carboxylate, as well as its antifungal activity towards isolates of *P. lingam* and *P. wasabiae* were established.

INTRODUCTION

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 (*Brassica napus* and *B. rapa*) and canola (*B. napus* and *B. rapa*) [1]. Two strains of the pathogen, classified as highly virulent (V) and weakly virulent (W) have different impacts on crop losses. The V strain isolates cause leaf spots and severe stem cankers on canola and rapeseed and are responsible for the largest yield losses [2], whereas W isolates, genetically closer to the *P. wasabiae* species than to the *P. lingam* species [3], cause little damage on these crops. Recent results established that wasabi (*Wasabia japonica*, syn. *Eutrema wasabi*) is resistant to the V strain of *P. lingam* and susceptible to the W strain [3, 4]. Therefore, it is of tremendous importance to determine the plant traits responsible for the resistance of wasabi to V blackleg isolates, as such agronomic traits are potentially transferable to canola. As in other plants [5], disease resistance traits of crucifers are related with both constitutive and induced defenses. For example, crucifers produce phytoalexins, i.e. induced chemical defenses (for a recent review see Ref. [6]), as well as multiple constitutive defenses, such as glucosinolates and isothiocyanates (for a recent review see Ref. [7]). To better understand blackleg disease resistance, we have been investigating both constitutive and induced antifungal secondary metabolites produced by wasabi. Here we wish to report the main phytoalexin produced by wasabi, methyl 1-methoxyindole-3-carboxylate (**1**), as well as constitutive antifungal metabolites and their activity towards isolates of the blackleg fungi *P. lingam* and *P. wasabiae*.

RESULTS AND DISCUSSION

Because wasabi is resistant to V isolates of *P. lingam*, it was of great interest to determine if



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phytoalexins or other antifungal metabolites were produced during fungal attack. This investigation allowed the isolation of methyl 1-methoxyindole-3-carboxylate (**1**), a new phytoalexin produced by foliar tissue of *W. japonica* under elicitation by *P. lingam*, *P. wasabiae* or $CuCl_2$. In addition, from non-elicited foliar tissue the following antifungal compounds were isolated: 5-(methylsulfamyl)pentyl-1-isothiocyanate (**2**), 6-(methylsulfamyl)hexyl-1-isothiocyanate (**3**), 6-(methylsulfinyl)hexyl-1-isothiocyanate (**4**) and 7-methylsulfamylheptanenitrile (**5**).

In preliminary experiments, a time-course response of wasabi 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 Section 3. Control (i.e. non-elicited) leaves were collected and treated in similar manner. Each extract was bioassayed for antifungal activity against *Cladosporium cucumerinum* (biodetection on TLC plates employing spores of *C. cucumericum*) [8]. Antifungal compounds were detected in the hexane and chloroform extracts of both elicited and non-elicited leaves, but no antifungal activity was detected in the methanol extracts. In addition, the leaf extracts were compared by high pressure liquid chromatographic (HPLC) analysis under the conditions described in Section 3. Under these conditions, one peak with $R_t = 18.0$ min was present on the chromatograms of hexane extracts of elicited leaf tissue (detected 48 h after elicitation), which was not detected on the chromatograms of extracts of control tissues. No apparent differences were detected in either the chloroform or methanol extracts. On the other hand, the TLC bioassay plate of the hexane extract of both elicited and non-elicited leaves showed four distinct areas of bioactivity (R_f of 0.77–0.83, 0.48–0.52, 0.29–0.31 and 0.09–0.12, CH_2Cl_2–hexane, 1:1, two elutions); the chloroform extract showed one distinct area of activity (R_f of 0.36–0.44, CH_2Cl_2–MeOH, 95:5, developed twice). Moreover, the extracts of both elicited and non-elicited leaves provided similar zones of bioactivity on the bioassay TLC plate. Importantly, no new antifungal zones were observed when comparing the elicited to the non-elicited leaf extracts.

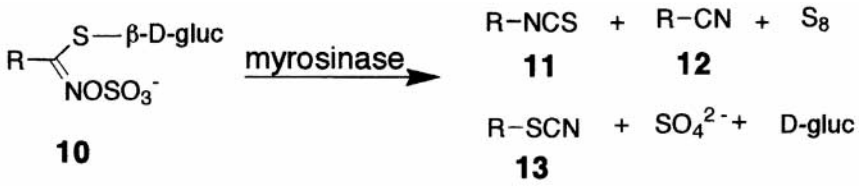
In order to obtain reasonable amounts of extracts of elicited leaves necessary for isolation and structure determination of the component with the $R_t = 18.0$ min (Table 1), $CuCl_2$ elicited wasabi leaves were processed in a manner similar to that used in the time course study. HPLC guided fractionation of the hexane extract led to the isolation and identification of compound **1**. The HRMS data indicated a molecular formula of $C_{11}H_{11}NO_3$, further corroborated by analysis of the NMR spectroscopic data. The NMR spectra of compound **1** displayed aromatic signals indicating the presence of an indole moiety, as well as signals likely due to two MeO groups (δ_H 4.14 and 3.90; δ_C 66.8 and 51.3) and a carbonyl group (δ_C 165.2). The assignment of structure **1** to this plant metabolite was confirmed by synthesis. Compound **1** was obtained from indoline in six steps, requiring only two chromatographic separations, a simplification of a previously published procedure [9]. Structure **1** represents a new phytoalexin produced by wasabi under elicitation by *P. lingam*, *P. wasabiae* or $CuCl_2$. Although this compound displays significant antifungal activity against *C. cucumerinum*, it was not detected during the time-course studies employing TLC biodetection method (i.e. elicited extracts did not appear different from non-elicited extracts) because **1** has an R_f (0.10, CH_2Cl_2–hexane, 1:1, developed twice) similar to that of constitutive antifungal metabolites and it is produced in relatively smaller amounts.

Table 1. HPLC retention times of antifungal compounds produced by wasabi (*Wasabia japonica*). Hypersil ODS column, mobile phase: 75% H_2O–25% CH_3CN to 100% CH_3CN, for 35 min, linear gradient and a flow rate 1.0 ml/min

Compound	Retention time (min)
1-Methoxyindole-3-carboxylate (**1**)	18.0
5-(Methylsulfamyl)pentyl-1-isothiocyanate (**2**)	26.3
6-(Methylsulfamyl)hexyl-1-isothiocyanate (**3**)	29.7
6-(Methylsulfinyl)hexyl-1-isothiocyanate (**4**)	10.1
7-Methylsulfamylheptanenitrile (**5**)	17.7
Sulfur[a]	13.3

[a]HPLC mobile phase: 25% H_2O–75% CH_3CN to 100% CH_3CN, for 15 min, linear gradient and a flow rate 1.0 ml/min.

Bioassay guided fractionation of the extracts obtained from non-elicited plant material led to the isolation and identification of elemental sulfur (S_8) and compounds **2**–**5**; while **3** was the major constituent of hexane extracts, **4** was the only constituent of chloroform extracts showing antifungal activity. The hexane extract was fractionated by a combination of flash column chromatography (FCC) and prep. TLC. The component responsible for the bioactive zone with $R_f = 0.77$–0.83 was identified as elemental sulfur, confirmed by comparison with an authentic sample (TLC, HPLC, MS). The components responsible for the bioactive zone with $R_f = 0.48$–0.52 were isolated and identified as a mixture of 5-(methylsulfamyl)pentyl-1-isothiocyanate (**2**) and 6-(methylsulfamyl)hexyl-1-isothiocyanate (**3**). Compounds **2** and **3** were separated by prep. TLC to yield colorless oils (purity confirmed by HPLC) whose structures were assigned by analysis of their spectroscopic data as follows. HRMS of **2** indicated a molecular formula of $C_7H_{13}NS_2$, consistent with the NMR spectroscopic data. The ^1H NMR spectrum of **2** was rather simple, displaying only five methylene signals (1.43, 1.61, 1.70, 2.50, 3.51) and a sharp methyl singlet (2.01). The ^{13}C NMR spectrum corroborated the presence of one methyl and five methylene carbons



Scheme 1.

and indicated the presence of an (iso)thiocyanate group (broad singlet at δ 126.6), which was confirmed by FTIR (strong absorption at 2098 cm^{-1}). Similarly, spectroscopic data indicated that compound **3** ($C_8H_{15}NS_2$) was the CH_2 higher homolog of **2**. The component responsible for the bioactive zone at $R_f = 0.29$–0.31 was isolated and identified as 7-methylsulfamylheptanenitrile (**5**); an additional component with an apparently higher bioactivity was detected and separated, but due to the extremely small amount present (less than 0.5 mg per 550 mg of extract) its structure could not be determined. The structure of nitrile **5** was readily assigned from analysis of its HRMS, NMR and FTIR spectra. Finally, the component responsible for the bioactive zone at $R_f = 0.09$–0.12 in the hexane extract was identified as 6-(methylsulfinyl)hexyl-1-isothiocyanate (**4**). The ^1H NMR spectrum of **4** showed five methylene (δ 1.48, 1.68, 1.79, 2.66, 3.50) and a methyl singlet (δ 2.56), similar to that of **3**, but with most of the signals resonating at *ca.* 0.5 ppm lower field. The remaining spectroscopic data allowed the assignment of structure **4**. The specific rotation of **4** ($[\alpha]_D$ −5.8, *c* 0.71, $CHCl_3$) was significantly lower than that previously reported ($[\alpha]_D$ −71) [10]; this low specific rotation suggests that **4** may be partially derived from non-enzymatic oxidation of for example isothiocyanate **3**. Compound **4** was present in larger amounts in the chloroform extract and corresponded to the bioactive zone with $R_f = 0.36$–0.44 on the TLC bioassay. Compounds **2**–**4** were previously isolated from wasabi but their ^{13}C NMR and IR spectroscopic data was not reported [10].

The presence of significant amounts of sulfur (*ca.* 10 mg/g of hexane extract) in leaf material was somewhat surprising, considering that plants do not appear to store elemental sulfur [11]. In this context it is worthy to note that elemental sulfur was recently reported to be a phytoalexin of cacao [12]. However, we have determined that elemental sulfur is present in both elicited and non-elicited wasabi leaves and therefore sulfur should not be classified as a wasabi phytoalexin. Although it is possible that elemental sulfur is stored in wasabi leaves, like in the case of pine needles [13], it is also possible that part of the sulfur present in the leaves results from decomposition of sulfur-containing metabolites (Scheme 1) produced by crucifers [7]. Nevertheless we have analyzed foliar tissue of the crucifer canola (cv. Westar) which also produces sulfur containing metabolites and found no detectable amounts of sulfur. Therefore, if the sulfur present in wasabi leaves results from sulfur-containing metabolites our results suggest that wasabi produces sulfur containing metabolites in higher amounts than canola (cv. Westar).

Preliminary results on the antifungal activity of isothiocyanates **2**–**4** towards blackleg isolates (Table 2) indicated that each compound inhibited spore germination of V isolates of *P. lingam* and also *P. wasabiae* at a concentration of 5×10^{-4} M, but had no significant effect on W isolates of *P. lingam*. Considering that control cultures of all isolates germinated consistently on solid medium containing DMSO (five independent experiments), these results are of great interest for their potential application. We have previously observed that other phytoalexins inhibited *P. lingam* at similar

Table 2. Germination and radial growth of spores of *Phoma lingam* virulent (V) isolate BJ 125, weakly virulent (W) isolate Unity, and *P. wasabiae* isolate CCRC 35135 incubated with dimethylsulfoxide (DMSO) or compounds **1**–**4**

Compound	Concentration (M)	*P. lingam*		*P. wasabiae*
		V isolate	W isolate	
Control containing DMSO[a]		white mycelium	white mycelium	white mycelium
Methyl 1-methoxyindole-3-carboxylate (**1**)	5×10^{-4}	no germination	similar to control	no germination
	1×10^{-4}	similar to control		similar to control
5-(Methylsulfamyl)-pentyl-1-isothiocyanate (**2**)	5×10^{-4}	no germination	similar to control	no germination
	1×10^{-4}	similar to control		similar to control
6-(Methylsulfamyl)hexyl-1-isothiocyanate (**3**)	5×10^{-4}	no germination	similar to control	no germination
	1×10^{-4}	similar to control		similar to control
6-(Methylsulfinyl)-hexyl-1-isothiocyanate (**4**)	5×10^{-4}	no germination	similar to control	no germination
	1×10^{-4}	similar to control		similar to control

[a]In five independent experiments fungal spores on control plates germinated and produced mycelium consistently in all the wells.

concentrations [6, 14, 15], but this is the first time we observed a selective inhibition of virulent isolates; however, it is not clear if isothiocyanates **2**–**4** affect both spore germination and mycelial growth of virulent isolates. Work is in progress to further determine the inhibitory levels of these compounds in both liquid and solid cultures of *Phoma* species.

Isothiocyanates (**11**), nitriles (**12**) and thiocyanates (**13**) in Cruciferae are derived from glucosinolates (**10**) (Fig. 1) [7]. Enzymatic degradation of glucosinolates (**10**) by myrosinases (thioglucoside glucohydrolase, EC 3.2.3.1) occurs after mechanical damage and microbial or pest attack [7]. Glucosinolates and their degradation products have been widely investigated for very diverse reasons. For example, the isothiocyanates **2**–**4** have been previously isolated as part of a study on the flavor components of wasabi paste [16]. In fact, the inhibitory effect on platelet aggregation of volatile components of wasabi oil were attributed to isothiocyanates such as **2** or **3** [17]. In addition, the biological activity of the components of wasabi extracts and homologous isothiocyanates was studied against the blue mussel (*Mytilus edulis*) where some repellent activity was observed [18]. Furthermore, there is a great number of studies on the activity of isothiocyanates towards fungi [19, 20], nematodes [21] and insects [7]. Nonetheless, to the best of our knowledge this appears to be the first report of the antifungal activity of the isothiocyanates present in the wasabi plant.

The phytoalexins from crucifers reported so far have an indole or indole related ring and at least one sulfur atom as common structural features [6, 22]. Most interestingly, crucifers appear to be the only plant family producing these sulfur metabolites. Several of these phytoalexins are produced by more than one *Brassica* spp. and can be elicited by diverse pathogens and/or abiotic factors. Brassinin (**6**), methoxybrassinin (**7**), [23] and brassilexin (**9**) [24] are among the first reported brassica phytoalexins, whereas sinalexin (**8**) was reported recently [25]. Methoxyindole **1** appears to be the first non-sulfur containing phytoalexin isolated from a crucifer. A compound similar to **1**, methyl 1-methylindole-3-carboxylate is a constitutive antifungal metabolite isolated from the crucifer *Camelina sativa* [26]. Phytoalexin **1** was previously prepared to characterize 1-methoxyindole-3-acetonitrile [10] but does not appear to have been isolated from natural sources. To the best of our knowledge, methyl 1-methoxyindole-3-carboxylate (**1**) represents the first phytoalexin reported from wasabi. The antifungal activity of **1** against the blackleg fungi (Table 2) suggests that **1** may be partly responsible for the resistance of wasabi to V isolates of *P. lingam*. If the blackleg resistance observed in wasabi is related to the presence of **1**, this resistance trait is potentially transferable to canola and other brassicas.

EXPERIMENTAL

General

All chemicals were purchased from Aldrich Chemical Company, Madison, WI, or Sigma Chemical Company, St. Louis, MO. All solvents were HPLC grade and used as such. Preparative TLC: (Merck, Kieselgel 60 F_{254}), 20×20 cm $\times$ 0.25 mm; analytical TLC (Merck, Kieselgel 60 F_{254}, aluminum sheets) 5×2 cm $\times$ 0.2 mm; compounds were visualized by exposure to UV and by dipping the plates in a 5% aqueous (w/v) phosphomolybdic acid solution containing a trace of ceric sulfate and 4% (v/v) H_2SO_4, followed by heating at 200°C. Flash column chromatography (FCC): silica gel Merck, grade 60, mesh size 230–400, 60 Å. 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 Hypersil ODS column (5 μm particle size silica, 4.6 i.d. $\times$ 200 mm) equipped with an in-line filter. HPLC mobile phase: 75% H_2O–25% CH_3CN to 100% CH_3CN, for 35 min, linear gradient and a flow rate 1.0 ml/min. Samples for HPLC analysis were dissolved in acetonitrile (*ca.* 0.5 mg/ml) and filtered through a cotton plug. NMR spectra were recorded on a Bruker AMX 500 or AMX 300 spectrometer; for ^1H (300 or 500 MHz), δ values were referenced to $CHCl_3$ (7.27 ppm), $CHDCl_2$ (5.32 ppm), or CD_2HCN (1.94 ppm) and for ^{13}C (75.5 or 125.8 MHz) referenced to $CDCl_3$ (77.2 ppm), CD_2Cl_2 (54.0 ppm) or CD_3CN (118.7 ppm). Fourier transform infrared (FTIR) spectra were obtained on a Bio-Rad FTS-40 spectrometer using diffuse reflectance cell. Mass spectra (MS) were obtained on a VG 70 SE mass spectrometer [high resolution (HR), electron impact (EI) or chemical ionization (CI) with ammonia as carrier gas], employing a solids probe.

Plant and fungal material

Wasabi (*Wasabia japonica*, *Eutrema wasabi*) plants were grown in a growth chamber with a 16 h photoperiod (20/18°C) at ambient humidity. Isolates of *P. lingam* and *P. wasabiae* were maintained in Petri dish cultures on V8 agar [20% (v/v) V8 juice, 0.75 g/l $CaCO_3$, 100 mg/l streptomycin sulfate, 40 mg/l Rose bengal and 15 g/l agar] [27].

Antifungal bioassays

Cladosporium cucumerinum bioassays were conducted by spotting samples on 1.5×20 cm TLC strips (aluminum backing) and developing strips in the appropriate solvent system followed by air dry-

ing (20 min) and then spraying (using a garden sprayer) with a 1.0×10^6 spores/ml suspension of *C. cucumerinum* in double strength potato dextrose broth (Difco). TLC strips were then incubated under high humidity in the dark for 36 h. Antifungal areas appeared white on a dark gray background due to fungal growth.

Blackleg fungi bioassays were conducted on agar plates. Compounds to be assayed were dissolved in DMSO (final concentration $< 2\%$), added to V8 agar at 50°C, quickly stirred and poured into 6-well tissue culture plates. Final concentration of each compound was 5.0×10^{-4} and 1.0×10^{-4} M. Plates (duplicates) were inoculated with either *P. lingam* virulent isolate BJ 125, *P. lingam* weakly virulent isolate Unity, or *P. wasabiae* and incubated at 23 ± 2°C. Control cultures (duplicates, five independent experiments) containing DMSO were incubated similarly. After 3 days of incubation the culture plates were examined for the presence or absence of mycelial growth.

Time course study for the production of antifungal compounds

Four wasabi plants were sprayed twice to the point of run-off with a $CuCl_2$ (10 mM) solution. Five leaves were excised at 24-h intervals for 12 days and stored at −20°C. Five control leaves were harvested from separate plants at the same time and treated in a similar manner throughout. Frozen leaves were crushed in liquid nitrogen (mortar and pestle), placed in a 250 ml Erlenmeyer flask and extracted with hexane (*ca.* 25 ml) by shaking at 200 rpm for 1 h. The hexane was decanted and the same amount of chloroform was added to leaf material, which was similarly extracted. The chloroform was decanted and the leaf material was similarly extracted with methanol (*ca.* 50 ml). All solvents were dried (Na_2SO_4) and evaporated under vacuum with a rotary evaporator. HPLC profiles of extracts from both elicited and control leaves were recorded. Antifungal compounds were visualized employing the *C. cucumerinum* bioassay described above. The TLC bioassay plate of the hexane extract showed four distinct areas of bioactivity having $R_f = 0.77$–0.83, 0.48–0.52, 0.29–0.31 and 0.09–0.12, CH_2Cl_2–hexane, 1:1, developed twice. Similarly, the TLC bioassay plate of the chloroform extract showed one distinct area of activity having $R_f = 0.36$–0.44, CH_2Cl_2–MeOH, 95:5, developed twice.

Elicitation of wasabi with Phoma lingam and P. wasabi

Leaves from wasabi plants (16 h photoperiod, 20/18°C at ambient humidity in growth chamber) were excised with a razor blade and immediately placed in a transparent plastic container lined with wet paper towels. The underside of each leaf was lightly wounded on several places with the tip of a glass rod and each wound was inoculated with a 10-μl-drop of a spore suspension of *P. lingam* isolates Unity and BJ 125 or *P. wasabi* (10^8 spores/ml, *ca.* 30 drops per leaf, three leaves per isolate). The water was allowed to evaporate, additional water was pipetted onto each inoculated site (100 μl) and the leaves incubated in the plastic container (sealed) in the laboratory (16 h photoperiod, 23 ± 2°C). After 72 h under conditions of high humidity, water droplets were collected, combined and extracted with CH_2Cl_2. The CH_2Cl_2 extract was concentrated to dryness, the residue was dissolved in CH_3CN and analyzed by HPLC as reported above.

Isolation and identification of antifungal compounds

Elicited wasabi leaves (1,700 g fresh weight) were crushed in liquid nitrogen, divided into 250 ml Erlenmeyer flasks (30) and extracted with hexane (2×100 ml per flask), chloroform (2×100 ml per flask) and methanol (2×100 ml per flask) in a manner similar to that followed for the time course. The crude hexane extract (2.1 g) was fractionated by dry flash chromatography (gradient elution with CH_2Cl_2–hexane, 1:1, to 100% CH_2Cl_2) [28]. The only fraction (249 mg) containing an HPLC peak with $R_t = 18.0$ min was further fractionated by a combination of FCC (CH_2Cl_2–hexane, 75:25) and prep. TLC (CH_2Cl_2–hexane, 9:1) to yield 1.3 mg of methyl 1-methoxyindole-3-carboxylate (**1**).

Non-elicited wasabi leaves (600 g fresh weight) were treated and extracted as described above. The crude hexane extract (205 mg) was fractionated by FCC (CH_2Cl_2–hexane 1:1) over silica gel and the presence of antifungal compounds detected by bioassay with *C. cucumerinum*. Further purification of those bioactive fractions by prep. TLC (hexane–EtOAc 9:1) provided 6-(methylsulfamyl)hexyl-1-isothiocyanate (**3**) (6.0 mg), 5-(methylsulfamyl)pentyl-1-isothiocyanate (**2**) (7.0 mg) and 7-methylsulfamylheptanenitrile (**5**) (2.5 mg).

The chloroform extract (78 mg) was fractionated by FCC ($CHCl_3$–MeOH, 95:5) and the bioactive components detected by bioassay with *C. cucumerinum*. Further purification by prep. TLC (CH_2Cl_2–MeOH, 95:5, two elutions) of the only fraction containing a bioactive component not previously identified in the hexane extract gave 6-(methylsulfinyl)hexyl-1-isothiocyanate (**4**) (1.3 mg).

Synthesis of methyl 1-methoxyindole-3-carboxylate

To a solution of $Na_2WO_4 \cdot 2H_2O$ (13.8 mg, 0.042 mmol) in MeOH–H_2O (10:1, 1 ml) and indoline (50 mg 0.42 mmol) at 0°C, was added 30% aqueous H_2O_2 (475 μl). The reaction mixture was stirred at 0°C for 15 min and excess CH_2N_2 (Et_2O soln) was added. After standing at room temperature for 1 h, the reaction mixture was concentrated to dryness, the residue rinsed first with H_2O

(3×2 ml) followed by CH_2Cl_2 (3×2 ml). The combined aqueous layers were extracted with CH_2Cl_2 (3×10 ml), the CH_2Cl_2 extracts combined with CH_2Cl_2 rinsings and the combined CH_2Cl_2 layers washed with H_2O (3×10 ml), dried over Na_2SO_4 and concentrated to dryness to yield crude 1-methoxyindole (56.3 mg). Purification by dry flash chromatography [28] (hexane, 100%, to hexane–EtOAc, 75:25) yielded pure 1-methoxyindole (22.7 mg, 37%). To a solution of 1-methoxyindole (22.7 mg, 0.15 mmol) in dry CH_2Cl_2 (1 ml) cooled to 0°C, a solution of chlorosulfonylisocyanate (32.5 mg, 0.22 mmol) in CH_2Cl_2 (600 μl) was added dropwise over 6 min. After stirring at 0°C for 15 min, MeOH was added (1 ml) and the solvent removed under vacuum, the residue dissolved in NaOH (5 M, 5 ml) and heated at 110°C overnight. The reaction mixture was allowed to cool to room temperature, acidified with conc. HCl, extracted with CH_2Cl_2 (3×10 ml), the combined organic layers dried over Na_2SO_4 and concentrated to dryness. The residue was methylated with excess CH_2N_2 (Et_2O soln) to yield crude product (14 mg). Purification by prep. TLC (hexane–EtOAc, 1:1, two elutions) yielded pure methyl 1-methoxyindole-3-carboxylate (**1**) (7.4 mg, 9% yield from indoline). This compound was identical in every respect to the isolated natural product.

Methyl 1-*methoxyindole*-3-*carboxylate* (**1**). R_f 0.52, hexane–EtOAc (1:1); ^{1}H NMR ($CDCl_3$): δ 3.90 (*s*, 3H), 4.14 (*s*, 3H), 7.29 (*m*, 2H), 7.46 (*d*, J = 7.7 Hz, 1H), 7.96 (*s*, 1H), 8.16 (*d*, J = 7.6 Hz, 1H); ^{13}C NMR ($CDCl_3$): δ 51.3 (*q*), 66.8 (*q*), 103.7 (*s*), 108.8 (*d*), 122.0 (*d*), 122.6 (*d*), 123.0 (*s*), 123.7 (*d*), 128.5 (*d*), 132.2 (*s*), 165.2 (*s*); HREIMS m/z measured: 205.0736 (205.0738 calcd. for $C_{11}H_{11}NO_3$); EIMS m/z (% relative abundance): 205 $[M]^+$ (100), 174 (31), 146 (68); FTIR ν_{max}: 2946, 1702, 1602, 1209, 1027, 743 cm^{-1}.

5-(*methylsulfamyl*)-*pentyl*-1-*isothiocyanate* (**2**). R_f 0.50, hexane–CH_2Cl_2 (1:1); ^{1}H NMR ($CDCl_3$): δ 1.43 (*m*, 2H), 1.61 (*m*, 2H), 1.70 (*m*, 2H), 2.01 (*s*, 3H), 2.50 (*t*, J = 7.1 Hz, 2H), 3.51 (*t*, J = 6.5 Hz, 2H); ^{13}C NMR ($CDCl_3$); δ 15.8 (*q*), 26.0 (*t*), 28.5 (*t*), 29.8 (*t*), 34.2 (*t*), 45.2 (*t*), 126.6 (*s*); HREIMS m/z measured: 175.0486 (175.0489 calcd. for $C_7H_{13}NS_2$): EIMS m/z (% relative abundance): 175 $[M]^+$ (100) 129 (42), 127 (26), 72 (27), 69 (34), 61 (72); FTIR ν_{max}: 2938, 2855, 2182, 2098, 1450, 1345 cm^{-1}.

6-(*methylsulfamyl*)*hexyl*-1-*isothiocyanate* (**3**). R_f 0.50, hexane–CH_2Cl_2 (1:1); ^{1}H NMR ($CDCl_3$): δ 1.41 (*m*, 4H), 1.60 (*m*, 2H), 1.68 (*m*, 2H), 2.08 (*s*, 3H), 2.48 (*t*, J = 7.3 Hz, 2H), 3.50 (*t*, J = 6.6 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 15.8 (*q*), 26.4 (*t*), 28.0 (*t*), 29.0 (*t*), 30.0 (*t*), 34.2 (*t*), 45.2 (*t*), 129.9 (*s*); HREIMS m/z measured: 189.0641 (189.0645 calcd. for $C_8H_{15}NS_2$); EIMS m/z (% relative abundance): 189 $[M]^+$ (89), 156 (36), 71 (36), 61 (100), 55 (60); FTIR ν_{max}: 2933, 2854, 2181, 2098, 1451, 1346 cm^{-1}.

6-(*methylsulfinyl*)-*hexyl*-1-*isothiocyanate* (**4**). R_f 0.40, CH_2Cl_2–MeOH (95:5); ^{1}H NMR ($CDCl_3$): δ 1.48 (*m*, 2H), 1.68 (*m*, 2H), 1.79 (*m*, 4H), 2.56 (*s*, 3H), 2.66 (*m*, 2H), 3.50 (*t*, J = 6.4 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 22.6 (*q*), 25.4 (*t*), 28.2 (*t*), 29.8 (*t*), 38.8 (*t*), 45.1 (*t*), 54.5 (*t*), 130.4 (*s*); HREIMS m/z measured: 205.0591(205.0595 calcd. for $C_8H_{15}NOS_2$): EIMS (% relative abundance): 205 $[M]^+$ (5), 188 (17), 142 (23), 71 (39), 55 (100); FTIR ν_{max}: 2931, 2856, 2103, 1714, 1458, 1039 cm^{-1}.

7-(*methylsulfamyl*)*heptanenitrile* (**5**). R_f 0.51, hexane-2-propanol (95:5); ^{1}H NMR ($CDCl_3$): δ 1.45 (*m*, 4H), 1.63 (*m*, 4H), 2.10 (*s*, 3H), 2.36 (*t*, J = 7.1 Hz, 2H), 2.51, (*t*, J = 7.1 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 15.8 (*q*), 17.3 (*t*), 25.5 (*t*), 28.1 (*t*), 28.5 (*t*), 28.9 (*t*), 34.3 (*t*), 119.9 (*s*); HREIMS m/z measured: 157.0924 (157.0925 calcd. for $C_8H_{15}NS$); EIMS m/z (% relative abundance): 157 $[M]^+$ (22), 110 (18), 83 (87), 69 (53), 61 (100); FTIR ν_{max}: 2928, 2856, 2244, 1461, 1426, 1041.

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