Tenualexin, Other Phytoalexins and Indole Glucosinolates from Wild Cruciferous Species

by **M. Soledade C. Pedras*** and **Estifanos E. Yaya**

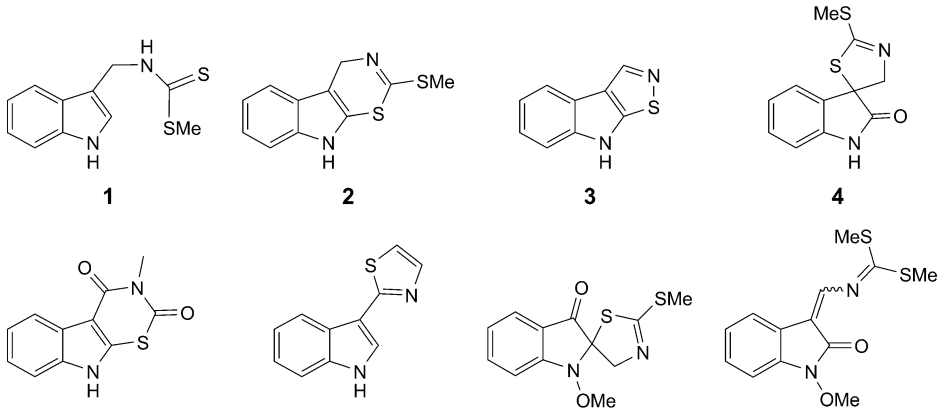
Department of Chemistry, University of Saskatchewan, 110 Science Place, Saskatoon, SK, S7N 5C9, Canada

In general, the chemodiversity of phytoalexins, elicited metabolites involved in plant defense mechanisms against microbial pathogens, correlates with the biodiversity of their sources. In this work, the phytoalexins produced by four wild cruciferous species (*Brassica tournefortii*, *Crambe abyssinica* (crambe), *Diplotaxis tenuifolia* (sand rocket), and *Diplotaxis tenuisiliqua* (wall rocket)) were identified and quantified by HPLC with photodioarray and electrospray mass detectors. In addition, the production of indole glucosinolates, biosynthetic precursors of cruciferous phytoalexins, was evaluated. Tenualexin, (=2-(1,4-dimethoxy-1*H*-indol-3-yl)acetonitrile), the first cruciferous phytoalexin containing two MeO substituents in the indole ring, was isolated from *D. tenuisiliqua*, synthesized, and evaluated for antifungal activity. The phytoalexins cyclobrassinin and spirobrassinin were detected in *B. tournefortii* and *C. abyssinica*, whereas rutalexin and 4-methoxybrassinin were only found in *B. tournefortii*. *D. tenuifolia*, and *D. tenuisiliqua* produced 2-(1*H*-indol-3-yl)acetonitriles as phytoalexins. Because tenualexin appears to be one of the broad-range antifungals occurring in crucifers, it is suggested that *D. tenuisiliqua* may have disease resistance traits important to be incorporated in commercial breeding programs.

Introduction. – The plant family Brassicaceae, common name crucifer, contains more than 3,700 species of enormous diversity, including economically important cruciferous oilseeds, vegetables, condiments, as well as numerous wild species. *Brassica* species are among the most economically valuable cruciferous species, representing sources of edible and industrial oils, food, and fodder, while wild crucifers are of great interest as sources of agronomic traits potentially transferable to cultivated *Brassica* species [1]. Among these agronomic traits, resistance to pests and diseases are much sought after, as they facilitate production of disease-resistant species through intercrossing or other breeding methods. Due to the susceptibility of cultivated *Brassica* species to fungal diseases, it is of particular interest to uncover the chemical defenses produced by wild cruciferous species and to evaluate their effects in the most prevalent fungal pathogens of brassica crops.

Phytoalexins are among the ecologically relevant natural products produced by crucifers to defend themselves against microbial pathogens [2]. These secondary metabolites are elicited and produced *de novo* in plants exposed to stress, whether biotic or abiotic. Importantly, in general, the chemodiversity of phytoalexins correlates with the biodiversity of their sources. For example, within crucifers, cultivated *Brassica* species produce the phytoalexins brassinin (**1**), cyclobrassinin (**2**), brassilexin (**3**), spirobrassinin (**4**), and rutalexin (**5**), whereas wild species such as *Arabidopsis thaliana* L. and *Camelina sativa* L. produce camalexin (**6**), *Erucastrum gallicum* (WILLD.)

O.E.SHULZ produces erucalexin (**7**), and *Thlaspi arvensis* L. and *Thellungiella salsuginea* (PALLAS) O.E.SHULZ produce wasalexins (**8**). This chemodiversity suggests that different wild crucifers produce different phytoalexins, although the number of species investigated to date is still rather small as to allow reasonable projections. Toward this end, we have investigated four wild species native of various habitats: *Brassica tournefortii* GOUAN, an invasive species known as African mustard, Saharan mustard, or Asian mustard, *Crambe abyssinica* R.E.Fr. (crambe), cultivated for its high content of erucic acid, a non-edible but important industrial oil, and *Diplotaxis tenuifolia* (L.) DC. (sand rocket) and *Diplotaxis tenuisiliqua* DELILE (wall rocket) both used in salads in several Mediterranean countries [3]. Herein, we report the phytoalexins produced by these species and the discovery of tenualexin (**13**), the first phytoalexin containing two MeO substituents in the indole ring. In addition, the production of indole glucosinolates, the biosynthetic precursors of cruciferous phytoalexins, was also evaluated over a five-day period.



Results and Discussion. – Elicitation of plant secondary metabolites can be achieved by biotic (*e.g.*, pathogens) or abiotic (*e.g.*, UV radiation, metal salts) factors. The structures and amounts of elicited metabolites depend on the plant species and their tissues, the elicitor type, and the environmental conditions. Within the same plant species and tissue, metal salts, and UV irradiation cause leaf damage, and subsequent elicitation depends on salt concentrations and intensity/exposure to UV light, respectively.

Elicitation and HPLC Analysis of Plant Metabolites. To establish conditions that induce the biosynthesis of plant metabolites, initial experiments were carried out using various concentrations of $CuCl_2$ (2–10 mM) sprayed on whole potted plants. While *B. tournefortii*, *C. abyssinica*, and *D. tenuifolia* showed severe foliar damage when sprayed with 5 mM or higher concentrations of $CuCl_2$, concentrations lower than 10 mM did not cause obvious damage on leaves of *D. tenuisiliqua.* Subsequent experiments to quantify elicited metabolites produced by *B. tournefortii*, *C. abyssinica*, and *D. tenuifolia* were carried out spraying with 2 mM solution of $CuCl_2$, while *D. tenuisiliqua* was sprayed

with 10 mM solution of $CuCl_2$. Leaves of each plant species were excised after different incubation periods and extracted as described in the *Exper. Part* to give nonpolar (NP) and polar extracts (P). Control plants (sprayed with H_2O) were treated similarly to yield NP control and P control extracts. The NP and P extracts were analyzed by HPLC-DAD/ESI-MS (DAD, diode array detector), and chromatograms were compared with those of NP and P extracts of control leaves, respectively. All the NP extracts showed the presence of elicited metabolites (*i.e.*, either not detected in controls or detected in trace amounts). All metabolites present in the NP extracts of *D. tenuisiliqua*, except for a peak with t_R 19.7 min, were available in our metabolite libraries and were identified by comparison of their HPLC retention times (t_R values), UV, and MS data with those of authentic samples. Quantification of each metabolite was carried out using calibration curves built for HPLC-DAD analysis with authentic synthetic compounds (*Tables 1* and *2*). Similarly, the metabolites present in P extracts were analyzed by HPLC-DAD/ESI-MS, and the chromatograms were compared with those of controls, but no elicited metabolites could be detected under the conditions used.

Given the biosynthetic relationship of indolic phytoalexins and indole glucosinolates, the indole glucosinolate production over the same time period and quantity were

Table 1. *HPLC-DAD Analysis of Phytoalexins and Indole Glucosinolates of* Brassica tournefortii (21-day-old) *and* Crambe abyssinica (17-day-old)

Metabolites (HPLC method, t_R min)	Incubation period [h]	Total amount of metabolites (nmol/g of fresh tissue ± S.D.)[a]	
		B. tournefortii	*C. abyssinica*
Cyclobrassinin (**2**)	24	79 ± 9	40 ± 8
(*Method A*; 25.2)	72	16 ± 1	18 ± 2
	120	11 ± 0	ND[b])
Spirobrassinin (**4**)	24	139 ± 24	10 ± 3
(*Method A*; 12.0)	72	114 ± 8	84 ± 11
	120	115 ± 6	72 ± 23
Rutalexin (**5**)	24	25 ± 2	ND[b])
(*Method A*; 14.5)	72	24 ± 2	ND[b])
	120	26 ± 4	ND[b])
4-Methoxybrassinin (**9**)	24	26 ± 3	ND[b])
(*Method A*; 19.9)	72	2.8 ± 0.3	ND[b])
	120	ND[b])	ND[b])
Arvelexin (**12**)	24	13 ± 3	15 ± 1
(*Method A*; 13.8)	72	30 ± 13	183 ± 73
	120	18 ± 8	237 ± 118
Glucobrassicin (**14**)	24	1847 ± 442	365 ± 164
(*Method B*; 19.6)	72	897 ± 540	612 ± 296
	120	575 ± 102	248 ± 41
4-Methoxyglucobrassicin (**16**)	24	386 ± 108	ND[b])
(*Method A*; 26.2)	72	204 ± 119	ND[b])
	120	101 ± 30	ND[b])

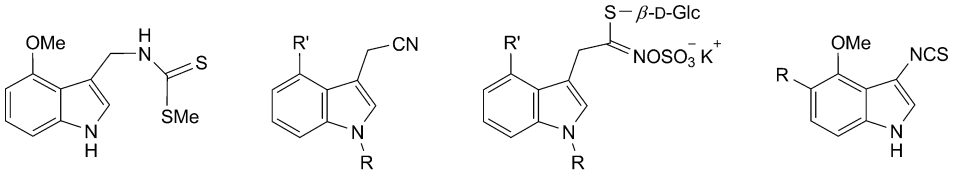
[a]) Amounts of each metabolite were determined by HPLC-DAD using calibration curves. Values are averages (triplicate) ± standard deviation (S.D.). [b]) ND, Not detected.

Table 2. *HPLC-DAD Analysis of Phytoalexins and Indole Glucosinolates of* Diplotaxis tenuifolia (24-day-old) *and* D. tenuisiliqua (28-day-old)

Metabolites (HPLC method, t_R min)	Incubation period [h]	Total amount of metabolites (nmol/g of fresh tissue ± S.D.)[a]	
		D. tenuifolia	*D. tenuisiliqua*
Arvelexin (**12**)	24	40 ± 7	8 ± 1
(*Method A*; 13.6)	72	204 ± 51	33 ± 4
	120	350 ± 25	31 ± 16
2-(1,4-Dimethoxy-1*H*-indol-3-yl)acetonitrile (tenualexin, **13**)	24	ND[b])	4 ± 1
(*Method A*; 19.7)	72	ND[b])	5 ± 1
	120	ND[b])	2 ± 1
Glucobrassicin (**14**)	24	716 ± 48	ND[b])
(*Method B*; 19.6)	72	263 ± 86	ND[b])
	120	137 ± 46	ND[b])
4-Methoxyglucobrassicin (**16**)	24	54 ± 7	ND[b])
(*Method B*; 26.2)	72	59 ± 9	ND[b])
	120	34 ± 6	ND[b])

[a]) Amounts of **12**, **13**, **14**, and **16** determined by HPLC-DAD using calibration curves. Values are averages (triplicate) ± standard deviation (S.D.). [b]) ND, Not detected.

determined as well. HPLC-DAD/ESI-MS Data indicated that glucobrassicin (**14**) and 4-methoxyglucobrassicin (**16**) were produced by *B. tournefortii* and *D. tenuifolia*, while only glucobrassicin (**14**) was detected in extracts of *C. abyssinica*; no indole glucosinolates were detected in P extracts of *D. tenuisiliqua*. 1-Methoxyglucobrassicin (**15**) was not detected in any of the four species (*Tables 1* and *2*).



9

10 R = R' = H
11 R = MeO, R' = H
12 R = H, R' = MeO
13 R = R' = MeO

14 R = R' = H
15 R = MeO, R' = H
16 R = H, R' = MeO
17 R = R' = MeO

18 R = H
19 R = OH

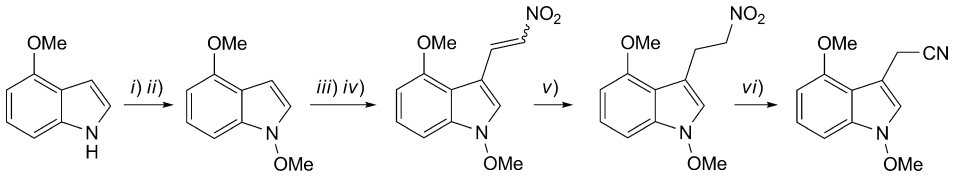
Prior to this work, *C. abyssinica* was investigated and reported to produce the elicited metabolites arvelexin (**12**) and rapalexin B (**19**) [4]; however, rapalexins A (**18**) and B (**19**) were not detected in the current work. To the best of our knowledge, no elicited metabolites or phytoalexins have been reported from *B. tournefortii*, *D. tenuifolia*, and *D. tenuisiliqua.* It was particularly puzzling to find that no indole glucosinolates were detected in the extracts of *D. tenuisiliqua*, because 2-(1*H*-indol-3-yl)acetonitriles are known to be derived from glucobrassicins [5]. Previously, glucobrassinin (**14**), together with its 1-methoxy and 4-methoxy derivatives, **15** and **16**, respectively, were identified in several accessions of *D. tenuifolia*, while only aliphatic glucosinolates were detected in *D. tenuisiliqua* [6].

Isolation, Identification, Synthesis, and Antifungal Activity of New Metabolite **13** *from* Diplotaxis tenuisiliqua. To isolate the new metabolite with t_R of 19.7 min present in NP extracts of *D. tenuisiliqua*, a larger-scale extraction of elicited leaves (300 g) was carried out. The NP extract was fractionated using multiple chromatographic methods to yield the desired metabolite with t_R of 19.7 min in sufficient amount (0.8 mg) for spectroscopic characterization. The HR-EI-MS of this sample showed a molecular-ion peak consistent with the molecular formula $C_{12}H_{12}N_2O_2$ (*m/z* 216.0897 (M^+; calc. 216.0899)). Consistent with the degree of unsaturation of eight and the molecular formula, the ^{1}H-NMR spectrum exhibited signals of twelve H-atoms: a *singlet* at δ(H) 7.20 (1 H), a *doublet* of *doublet* at δ(H) 7.18 (1 H), two *doublet*s at δ(H) 7.03 (1 H) and δ(H) 6.51 (1 H), two Me *singlet*s at δ(H) 4.06 and 3.92, and a CH_2 *singlet* at δ(H) 4.02. The presence of four H-atom signals in the aromatic region (δ(H) 7.20–6.51), their multiplicity, and the degree of unsaturation suggested the presence of an indole ring substituted at N(1), C(3), and either C(4) or C(7). Furthermore, by biosynthetic analogy to other cruciferous indolic structures, the two MeO groups (δ(H) 4.06 and 3.92) were assumed to be attached to the indole ring at N(1) and C(4), and the CH_2 substituent located at C(3). Finally, considering that the indole ring counted for six unsaturations, the two remaining unsaturations should be due to the functional group that was assigned as a nitrile. Based on this reasoning, the structure of the new metabolite with t_R of 19.7 min was assigned as 2-(1,4-dimethoxy-1*H*-indol-3-yl)acetonitrile (**13**). This structure was confirmed by synthesis, as outlined in *Scheme 1* and described in the *Exper. Part*.

In short, commercially available 4-methoxy-1*H*-indole (**20**) was reduced to the corresponding 4-methoxyindoline [7], followed by oxidation and methylation [8] to yield 1,4-dimethoxyindole (**21**). Formylation of **21** yielded 1,4-dimethoxy-1*H*-indole-3-carboxaldehyde that was condensed with excess of $MeNO_2$ [9] to yield 1,4-dimethoxy-3-(2-nitroethenyl)-1*H*-indole (**22**). Compound **22** was reduced with $NaBH_4$ [10] to yield **23** that was then dehydrated to yield 2-(1,4-dimethoxy-1*H*-indol-3-yl)acetonitrile (**13**; *Scheme 1*).

The antifungal activity of 2-(1,4-dimethoxy-1*H*-indol-3-yl)acetonitrile (**13**) was assayed against four economically important plant pathogenic fungi (*Alternaria brassicicola*, *Leptosphaeria maculans*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*) as described in the *Exper. Part.* At the highest concentration (0.50 mM), **13** inhibited completely the growth of mycelia of *A. brassicicola*, *R. solani*, and *S. sclerotiorum*,

Scheme 1. *Synthesis of 2-(1,4-Dimethoxy-1*H*-indol-3-yl)acetonitrile* (**13**)



i) $NaBH_3CN$, AcOH. *ii*) Na_2WO_4, H_2O_2, K_2CO_3, $(Me)_2SO_4$; 41%. *iii*) $POCl_3$, DMF. *iv*) $MeNO_2$, $AcONH_4$, 105°. *v*) $NaBH_4$, THF, MeOH. *vi*) CS_2, Et_3N, 40°; 77%.

Table 3. *Antifungal Activity*[a]) *of Tenualexin* (**13**) *against the Plant Pathogenic Fungi* Alternaria brassicicola, Leptosphaeria maculans, Rhizoctonia solani, *and* Sclerotinia sclerotiorum

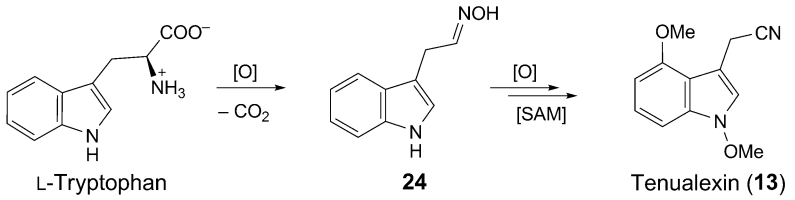
Tenualexin (**13**)[b])	*A. brassicicola* [%]	*L. maculans* [%]	*R. solani* [%]	*S. sclerotiorum* [%]
0.50 mM	100±0	69±4	100±0	100±0
0.20 mM	47±4	31±4	86±0	57±2
0.10 mM	31±4	18±4	67±4	23±2

[a]) Percentage inhibition = 100 – [(diameter of mycelium on medium containing compound – plug)[c])/(diameter of mycelium on control medium – plug)[c]) × 100)] ± standard deviation. Results are given averages (triplicate) ± standard deviations. [b]) Dissolved in DMSO and added to potato dextrose agar medium. [c]) Plug diameter, 4 mm.

whereas inhibition of *L. maculans* was not complete (69%; *Table 3*). Therefore, considering that 2-(1,4-dimethoxy-1*H*-indol-3-yl)acetonitrile is an elicited metabolite (not present in control plants) and displays antifungal activity, it should be considered a phytoalexin, for which the name tenualexin (**13**) is proposed. Tenualexin (**13**) is a phytoalexin in *D. tenuisiliqua* and a new plant metabolite that does not appear to have been synthesized previously.

2-(1*H*-Indol-3-yl)acetonitriles **10**–**12** were found as phytoalexins in some crucifers, including caulilexin C (**11**) produced by *B. oleraceae* var. *botrytis* [11] and arvelexin (**12**) produced by *T. arvensis* [12]. Compound **10** was identified in various species, but sometimes it is also produced in controls, although in much smaller amounts; in such cases, it is more appropriate to consider them as phytoanticipins. Interestingly, although **13** has not been reported to date, its potential biosynthetic precursor 1,4-dimethoxyglucobrassicin (**17**) was isolated as the desulfo derivative from roots of *Barbarea vulgaris* ssp. *arcuata* [13][14]. Since **17** was not detected in any of the extracts of leaves of *D. tenuisiliqua*, we assume that 2-(1,4-dimethoxy-1*H*-indol-3-yl)acetaldehyde oxime (**24**) is a more likely immediate precursor of tenualexin (**13**; *Scheme 2*). Nonetheless, the precursor role of **17** cannot be ruled out. Due to its strong antifungal activity, the biosynthetic pathway of **13** is of great interest and warrants further investigation.

Scheme 2. *Proposed Biosynthetic Pathway of Tenualexin* (**13**) *in* Diplotaxis tenuisiliqua



L-Tryptophan **24** Tenualexin (**13**)

Conclusions. – Elicitation of *B. tournefortii*, *C. abyssinica*, *D. tenuifolia*, and D. *tenuisiliqua* with $CuCl_2$ solutions, followed by incubation and HPLC analyses of extracts of their elicited and control leaves showed that all species produced phytoalexins. *B. tournefortii* and *C. abyssinica* produced arvelexin (**12**) together with phytoalexins common to *Brassica* species (cyclobrassinin (**2**) and spirobrassinin (**4**)),

whereas no *Brassica* phytoalexins were detected in *D. tenuifolia* and *D. tenuisiliqua* under identical experimental conditions. It is pertinent to point out that all cruciferous phytoalexins reported to date are derived from the same basic building block, L-tryptophan, and tenualexin (**13**) is no exception.

Based on previous antifungal assays using caulilexin C (**11**) and arvelexin (**12**) [15], tenualexin (**13**) appears to be the most potent antifungal agent among these three metabolites. These results suggest that *D. tenuisiliqua* could have disease resistance traits of interest in commercial breeding programs.

Financial support of this work was obtained from the *Natural Sciences and Engineering Research Council of Canada* (Discovery Grant to *M. S. C. P.*), the *Canada Research Chairs Program*, the *Canada Foundation for Innovation*, the *Saskatchewan Government*, and the University of Saskatchewan (teaching assistantship to *E. E. Y.*).

Experimental Part

General. Chemicals were purchased from *Sigma–Aldrich Canada Ltd.*, Oakville, ON, Canada, or *Alpha Aeser*, Ward Hill, MA, USA. Org. extracts were dried (Na_2SO_4), and solvents were removed under reduced pressure in a rotary evaporator. Flash column chromatography (FC): silica gel, grade *60*; mesh size, 230–400 Å. Prep. TLC: silica-gel plates, *Kieselgel 60* F_{254}, visualization under UV light. HPLC: *Agilent* chromatographs equipped with quaternary pump, automatic injector, and diode array detector (DAD; wavelength range 190–600 nm), degasser, and a *Hypersil ODS* column (5-μm particle size silica, 4.6 i.d. × 200 mm), having an in-line filter; mobile phase, *Method A* (for NP extracts): H_2O/MeCN linear gradient (75 : 25 to 25 : 75 in 35 min, to 0 : 100 in 5 min), and at a flow rate 1.0 ml/min; *Method B* (for P extracts): H_2O/MeCN (100 : 0 to 25 : 75 in 35 min, to 0 : 100 in 5 min) and a flow rate of 1 ml/min. Samples were dissolved in MeCN for *Method A*, and in H_2O/MeOH 1 : 1 for *Method B*. HPLC-DAD-ESI-MS: *Agilent 1100* series HPLC system equipped with an autosampler, binary pump, degasser, and a DAD connected directly to a mass detector (*Agilent G2440A MSD-Trap-XCT* ion trap mass spectrometer) with an electrospray ionization (ESI) source. Chromatographic separation was carried out at r.t. using an *Eclipse XSB C-18* column (5-μm particle size silica, 150 × 4.6 mm i.d.). The mobile phase consisted of a linear gradient of 0.2% HCOOH in H_2O and 0.2% HCOOH in MeCN (75 : 25 to 25 : 75 in 35 min, to 0 : 100 in 5 min) and a flow rate of 1 ml/min. Data acquisition was carried out in positive and negative polarity modes in a single LC run. Data processing was carried out using *Agilent Chemstation* software. ^{1}H- and ^{13}C-NMR spectra: *Bruker Avance 500* MHz spectrometers; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. HR-EI-MS: *VG 70 SE* mass spectrometer, employing a solids probe; in *m*/*z* (rel. %).

Plant Material, Elicitation, and Extraction. Seeds of *Brassica tournefortii*, *Crambe abyssinica*, *Diplotaxis tenuisiliqua*, and *Diplotaxis tenuifolia* were obtained from *Plant Gene Resources*, *AAFC*, Saskatoon, Canada. The seeds were sown in a commercial potting soil mixture, and plants were grown in a growth chamber under controlled environmental conditions (at 25°/16 h, light, 16°/8 h, dark) for 2–4 weeks (*B. tournefortii*, 21 d; *C. abyssinica*, 17 d; *D. tenuifolia*, 24 d; and *D. tenuisiliqua*, 28 d).

Plants were sprayed with $CuCl_2$ solns. (2–10 mM) to the point of run-off and incubated in a growth chamber for different periods. Leaves from elicited and control plants were harvested at 24, 72, and 120 h, weighed, separately frozen in liquid N_2, crushed, and extracted with MeOH (2 × 20 ml) on a shaker for 1 h. MeOH Extracts were filtered and concentrated under reduced pressure. The residue was rinsed with CH_2Cl_2 (6 ml) and decanted to yield the nonpolar (NP) extract and a residue (polar (P) extract). The NP extract was concentrated, dissolved in MeCN (500 μl), and analyzed by HPLC-DAD-ESI-MS. The P extract was dissolved in MeOH/H_2O 1 : 1 (2 ml) and analyzed by HPLC-DAD-ESI-MS.

Isolation of New Metabolite **13**. A larger-scale experiment was conducted to isolate and characterize the new metabolite (t_R 19.7 min) from 21-day-old leaves of *D. tenuisiliqua*. Plants were elicited with $CuCl_2$ soln. (10 mM), leaves were harvested, weighed (300 g), and crushed in liquid N_2. The crushed leaves were divided into two parts and extracted with MeOH overnight. The plant tissue was filtered, and

the filtrate was concentrated. The crude MeOH extract was washed with CH_2Cl_2 and concentrated. The NP extract was subjected to FC (hexane/AcOEt 80:20 to 0:100 and CH_2Cl_2/MeOH 95:5). The fractions were analyzed by HPLC-DAD, and the fraction containing a peak at t_R 19.7 min was further fractionated by FC (hexane/AcOEt 90:20 to 70:30). Final fractionation of the fraction containing the peak at t_R 19.7 min was carried out by prep. TLC (hexane/AcOEt 85:15) to yield *2-(1,4-dimethoxy-1*H*-indol-3-yl)acetonitrile* (**13**) as an oil (0.8 mg).

Synthesis of Tenualexin (**13**). $NaBH_3CN$ (382 mg, 6.06 mmol) was added during 10 min to a soln. of *4-methoxy-1*H*-indole* (**20**; 300 mg, 2.04 mmol) in AcOH (6 ml), the mixture was stirred at r.t. for 1 h, diluted with H_2O, basified with 2M NaOH aq. soln., and extracted with Et_2O. The combined org. phase was dried and concentrated to yield crude 4-methoxyindoline (303 mg, 97%). $Na_2WO_4 \cdot 2\ H_2O$ (126 mg, 0.38 mmol) in H_2O (1 ml) was added to a stirred soln. of 4-methoxyindoline (333 mg, 2.25 mmol) in MeOH (8 ml) and cooled to $-20°$. Next, a soln. of H_2O_2 (1.5 ml, 19.6 mmol) in MeOH (2 ml) was added dropwise to the cooled suspension, the mixture was stirred at r.t. for 10 min, and K_2CO_3 (2.5 g, 18 mmol) and $(MeO)_2SO_2$ (341 µl, 3.60 mmol) were added under vigorous stirring. After stirring for 10 min, the mixture was diluted with H_2O and extracted with Et_2O (3×40 ml). The org. extract was dried, concentrated, and subjected to FC (CH_2Cl_2 100%) to yield **21** (163 mg, 0.92 mmol) in 41% yield.

$POCl_3$ (127 µl, 1.36 mmol) was added dropwise to a soln. of **21** (160 mg, 0.91 mmol) in DMF (2 ml) at 0°. The mixture was stirred for 1 h at r.t., basified with $NH_3 \cdot H_2O$ (28%), diluted with H_2O, and extracted with Et_2O (3×30 ml). The org. layer was dried, concentrated, and subjected to FC (CH_2Cl_2/MeOH 98:2) to yield 1,4-dimethoxyindole-3-carboxaldehyde (149 mg, 0.73 mmol) in 81% yield. $AcONH_4$ (26 mg, 0.34 mmol) was added to a soln. of 1,4-dimethoxy-1*H*-indole-3-carboxaldehyde (139 mg, 0.68 mmol) in $MeNO_2$ (1.5 ml) and refluxed for 90 min. The mixture was allowed to cool, diluted with H_2O, extracted with CH_2Cl_2, and concentrated to yield crude **22**, which was used in the next step without further purification. $NaBH_4$ (78 mg, 2.04 mmol) was added to a soln. of crude **22** (169 mg, 0.68 mmol) in THF (4 ml) and MeOH (500 µl). The mixture was stirred for 3 h at r.t. and diluted with H_2O. The mixture was extracted by CH_2Cl_2, concentrated, and subjected to FC (CH_2Cl_2 100%) to yield **23** (65 mg, 0.38 mmol) in 26%. Et_3N (500 µl, 3.60 mmol) and CS_2 (217 µl, 3.60 mmol) were added to a soln. of **23** (45 mg, 0.18 mmol) in MeCN (4 ml). The mixture was stirred at 40° for 20 h in an air-tight reaction vial, the solvent was evaporated, and the residue was diluted with H_2O and extracted (CH_2Cl_2, 3×20 ml). The org. layer was dried, concentrated, and the crude mixture was fractionated by FC (CH_2Cl_2 100%) to yield (**13**; 30 mg, 0.14 mmol, 77%). Brown oil. t_R 19.7 min (*Method A*). UV (HPLC, H_2O/MeCN): 220, 265, 295. FT-IR (KBr): 2935, 2359, 1502, 1456, 1303, 1260, 1060, 1033. ^{1}H-NMR (500 MHz, $CDCl_3$): 7.20 (*s*, 1 H); 7.18 (*dd*, *J* = 8, 8, 1 H); 7.03 (*d*, *J* = 8, 1 H); 6.51 (*d*, *J* = 8, 1 H); 4.06 (*s*, 3 H); 4.02 (*s*, 2 H); 3.92 (*s*, 3 H). ^{13}C-NMR (125 MHz, $CDCl_3$): 154.6; 134.0; 124.3; 120.3; 118.9; 112.8; 101.9; 101.1; 100.2; 66.1; 55.4; 16.1.l. MS-ESI: 217.0 (100, $[M+1]^+$), 186.0 (20). HR-EI-MS: 216.0897 (87, M^+, $C_{12}H_{12}N_2O_2^+$; calc. 216.0897), 185.0 (100), 170.0 (34).

Antifungal Bioassays. Leptosphaeria maculans, *Rhizoctonia solani* AG 2–1, *Sclerotinia sclerotiorum*, and *Alternaria brassicicola* were obtained from *AAFC Research Centre*, Saskatoon, Canada. Solid cultures were initiated with spores of *A. brassicicola* and *L. maculans*, sclerotia of *S. sclerotiorum*, and mycelia of *R. solani.* Spores of *A. brassicicola* and *L. maculans*, sclerotia of *S. sclerotiorum*, mycelia of *R. solani* were grown on potato dextrose agar (PDA) plates at $23 \pm 2°$ under constant light. Solns. of **13** in DMSO were used to prepare sterile assay plates (6 wells per plate, 36-mm diameter, 2 ml per well) in PDA media (0.50, 0.20, and 0.10 mM; final DMSO concentration, 1%); control plates contained 1% DMSO in PDA. Plates containing test solns. and the solvent were inoculated with mycelial plugs (4-mm diameter) cut from the edge of actively growing cultures and placed upside down on the center of each plate. The plates were sealed with parafilm and incubated at $23 \pm 2°$ under constant light for 24 h for *S. sclerotiorum*, 72 h for *R. solani*, and 120 h for *L. maculans* and *A. brassicicola*. The diameter of the mycelia in control media and in tenualexin-containing media was measured every 24 h. The antifungal activity was calculated using the equation given in *Table 3*. The experiment was conducted in triplicate and repeated three times.

REFERENCES

[1] S. I. Warwick, 'Genetics and Genomics of the Brassicaceae, Plant Genetics and Genomics: Crops and Models 9', Eds. R. Schmidt, I. Bancroft, 2011, Vol. 9, pp. 33–65.
[2] M. S. C. Pedras, E. E. Yaya, E. Glawischnig, *Nat. Prod. Rep.* **2011**, *28*, 1381.
[3] A. Protap, S. K. Gupta, 'Biology and Breeding of Crucifers', Ed. S. K. Gupta, CRC Press, 2009, pp. 37–67.
[4] M. G. Sarwar, M.Sc. Thesis, University of Saskatchewan, Canada, 2007.
[5] N. Agerbirk, M. De Vos, J. H. Kim, G. Jander, *Phytochem. Rev.* **2009**, *8*, 101.
[6] L. F. D'Antuono, S. Elementi, R. Neri, *Phytochemistry* **2008**, *69*, 187.
[7] X. Wang, Y. Gao, Y. M. Xu, L. Li, Z. Zhang, J. Liu, *Synth. Commun.* **2009**, *39*, 4030.
[8] M. Somei, T. Kawasaki, *Heterocycles* **1989**, *29*, 1251.
[9] L. Canoira, J. G. Rodriguez, J. B. Subirats, J.-A. Escario, I. Jimenez, A. R. Martinez-Fernandez, *Eur. J. Med. Chem.* **1989**, *24*, 39.
[10] A. B. Robertson, N. P. Botting, *Tetrahedron* **1999**, *55*, 13269.
[11] M. S. C. Pedras, M. G. Sarwar, M. Suchy, A. M. Adio, *Phytochemistry* **2006**, *67*, 1503.
[12] M. S. C. Pedras, P. B. Chumala, M. Suchy, *Phytochemistry* **2003**, *64*, 949.
[13] N. Agerbirk, B. L. Petersen, C. E. Olsen, B. A. Halkier, J. K. Nielsen, *J. Agric. Food Chem.* **2001**, *49*, 1502.
[14] N. Agerbirk, C. E. Olsen, *Phytochemistry* **2012**, *77*, 16.
[15] M. S. C. Pedras, S. Hossain, *Phytochemistry* **2011**, *72*, 2308.

Received August 1, 2013