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Ecological Roles of Tryptanthrin, Indirubin and *N*-Formylanthranilic Acid in *Isatis indigotica*: Phytoalexins or Phytoanticipins?

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Leaves of the plant species *Isatis indigotica* FORTUNE EX LINDL. (Chinese woad) produce the metabolites tryptanthrin, indirubin and *N*-formylanthranilic acid upon spraying with an aqueous solution of copper chloride but not after spraying with water. The antifungal activities of these metabolites against the phytopathogens *Alternaria brassicicola*, *Leptosphaeria maculans* and *Sclerotinia sclerotiorum* established that tryptanthrin is a much stronger growth inhibitor of *L. maculans* than the phytoalexin camalexin. The biosynthetic precursors of tryptanthrin and *N*-formylanthranilic acid are proposed based on the deuterium incorporations of isotopically labeled compounds. The overall results suggest that tryptanthrin is a phytoalexin and indirubin and *N*-formylanthranilic acid are phytoanticipins in the plant species *I. indigotica* and that chemical diversity and biodiversity are intimately connected.

Keywords: Brassicaceae, *Isatis indigotica*, alkaloid, antifungal activity, bioactive compounds, crucifer, isotopic labeling, phytoalexin, phytoanticipin.

Introduction

The genus *Isatis* L. (Brassicaceae family) comprises more than seventy species,[1] several of which have been investigated for their pharmacological properties. Indeed, numerous reports dealing with the isolation and structure elucidation of metabolites of *Isatis* species have been published, however, the ecological roles of such metabolites in their natural or cultivated environments remain unknown. For example, *Isatis indigotica* FORT. (Chinese woad), a biennial crucifer widely cultivated and used in traditional Chinese medicine,[2] and *I. tinctoria* L., an indigo dye plant, produce a number of metabolites with various biological activities.[2,3] Interestingly, air drying leaves of *I. tinctoria* and *I. indigotica* was shown to change their metabolite profiles causing the loss of glucosinolates and indigo precursors and the formation of tryptanthrin (**1**) and indirubin (**2**; *Figure 1*).[4] Both tryptanthrin (**1**) and indirubin (**2**) are compounds well-known for their pharmacological activities including antimicrobial, anti-inflammatory and anticancer.[3,5–8] Nonetheless, their roles in the producing organisms remain unknown to date.

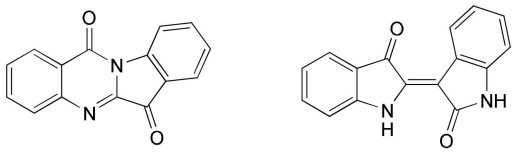

Figure 1. Chemical structures of tryptanthrin (**1**) and indirubin (**2**) isolated from dried roots and leaves of *Isatis indigotica* and *I. tinctoria*.

Phytoalexins are elicited antimicrobial metabolites produced *de novo* in plants under stress, but not produced under normal conditions.[9–11] By contrast, phytoanticipins are antimicrobial metabolites constitutively produced in amounts that usually increase under stress.[12,13] These two groups of metabolites have ecological roles that include involvement in molecular plant defense mechanisms against microbial pathogens.[10,11] Cruciferous phytoalexins encompass a

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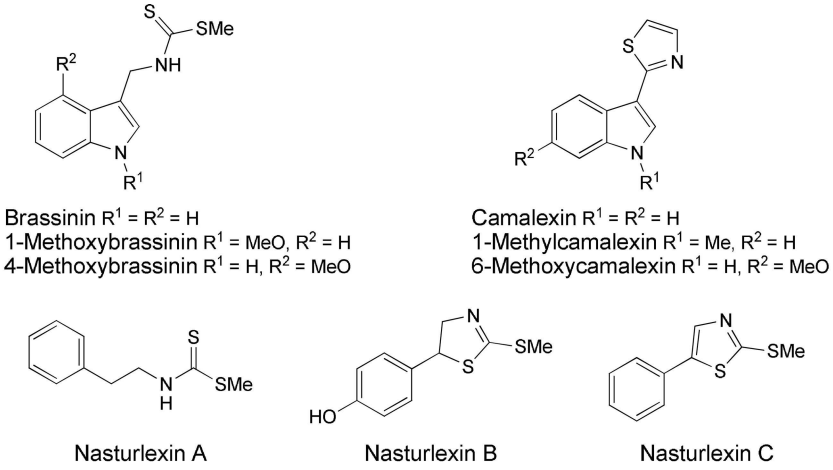

Figure 2. Chemical structures of the cruciferous phytoalexins brassinins and camalexins, derived from L-tryptophan, and nasturlexins, derived from L-phenylalanine.

group of alkaloids derived from L-tryptophan such as the brassinins and camalexins,[11] or from L-phenylalanine such as the nasturlexins (*Figure 2*).[14] To date, no phytoalexins or other elicited metabolites have been reported from *I. indigotica* or any other *Isatis* species.

In continuation of previous studies on the structure and biosynthesis of cruciferous phytoalexins,[14–18] we investigated and report herein the metabolites isolated from leaves of elicited Chinese woad (*I. indigotica*) and their antifungal activities against three fungal species pathogenic to crucifers. Furthermore, the biosynthetic precursors of tryptanthrin (**1**) and *N*-formylanthranilic acid (**3**) were investigated using isotopically labeled compounds.

Results and Discussion

Time-Course Analyses of Metabolite Production in Elicited Leaves of Isatis indigotica

Whole plants of *I. indigotica* were grown for three weeks in a growth chamber and elicited by spraying with an aqueous solution of $CuCl_2$. Leaves were excised after 1, 3 and 5 days of incubation, frozen in liquid nitrogen and ground, and the resulting leaf materials were extracted to yield non-polar (CH_2Cl_2) and polar (MeOH) extracts, as described in the *Experimental Section*. Control plants were sprayed with water and treated similarly. The HPLC-DAD chromatograms of the CH_2Cl_2 extracts of elicited leaves displayed two peaks at $t_R = 15.4$ and 20.7 min (HPLC-DAD method A; *Figure 3*) and another peak at $t_R =$ 2.1 min (HPLC-DAD method B; *Figure 4*) that were not observed in control leaves. The UV and ESI-MS spectra of these components were not available in our virtual libraries, suggesting that these compounds had not been detected in any of the crucifer species previously investigated.[15–19] After isolation and identification of the three elicited metabolites ($t_R = 2.1$, 15.4, 20.7 min), calibration curves were built for quantification by HPLC-DAD (amounts calculated based on peak areas), as summarized in *Table 1*.

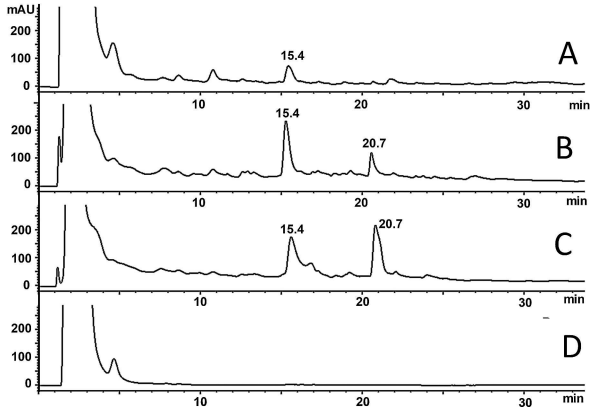

Figure 3. HPLC-DAD chromatograms (method A, detection at 220 nm) of extracts of leaves of *Isatis indigotica* after elicitation with $CuCl_2$: A) 1-day post-elicitation; B) 3-day post-elicitation; C) 5-day post-elicitation; D) control; $t_R = 15.4$ min, tryptanthrin (**1**); $t_R = 20.7$ min, indirubin (**2**).

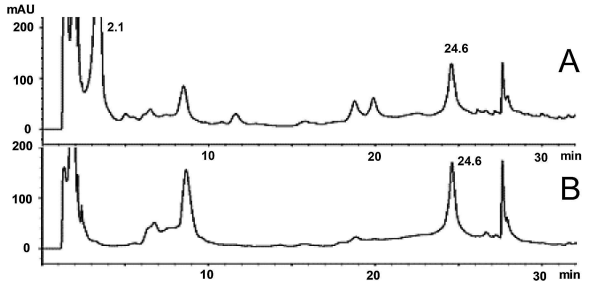

Figure 4. HPLC-DAD chromatograms (method B, detection at 220 nm) of extracts of leaves of *Isatis indigotica* after elicitation with $CuCl_2$: A) 1-day post-elicitation; B) control; $t_R = 2.1$ min, *N*-formylanthranilic acid (**3**); $t_R = 24.6$ min, isatan B (**4**).

Chemical Structure Determination of Unknown Metabolites with $t_R = 2.1$, *15.4 and 20.7 min*

To isolate the metabolites responsible for the HPLC peaks observed at $t_R = 15.4$ and 20.7 min (Method A) and at $t_R = 2.1$ min (method B), a larger scale experiment was carried out as described in the *Experimental Section*. In brief, leaves (ca. 100 g of elicited leaves, two days post elicitation) were extracted (MeOH) and filtered; the filtrate was concentrated and partitioned with AcOEt/H_2O. The AcOEt extract was concentrated and fractionated using multiple chromatographic columns to yield two elicited non-polar metabolites with $t_R = 15.4$ min (**1**) and 20.7 min (**2**), and the polar metabolite at $t_R = 2.1$ min (**3**). The chemical structures of these compounds (*Figure 1* and 5) were established

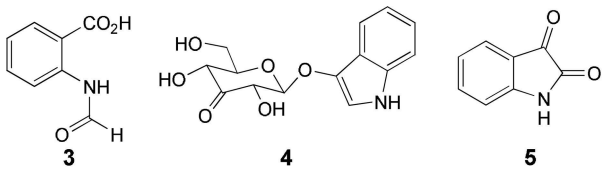

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Figure 5. Chemical structures of *N*-formylanthranilic acid (**3**), isatan B (**4**) and isatin (**5**) isolated from elicited leaves of *Isatis indigotica*.

by analyses of spectroscopic data and confirmed by comparison with authentic samples, as described below.

The HR-EI-MS spectrum of the elicited metabolite at $t_R = 15.4$ min suggested a molecular formula of $C_{15}H_8N_2O_2$ that implied 13 degrees of unsaturation (*m/z* M^+ 248.0581). The ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) displayed eight aromatic protons (δ(H) 8.60–7.41), whereas the ^{13}C-NMR data showed two low field signals (δ(C) 182.7, 158.3) and twelve additional sp^2 carbons. A literature search using these data suggested the structure of tryptanthrin (**1**).[20] This assignment was unambiguously confirmed by direct comparison of all spectroscopic data with those of an authentic standard of tryptanthrin (**1**; purchased from Accela ChemBio Inc., San Diego, CA).

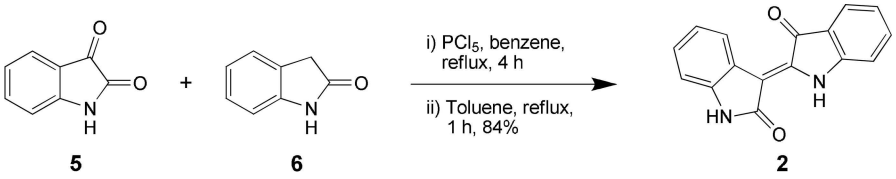
The HR-ESI-MS spectrum of the elicited metabolite at $t_R = 20.7$ min suggested a molecular formula of $C_{16}H_{10}N_2O_2$ that implied 13 degrees of unsaturation (*m/z* $[M+H]^+$ 263.0828). The ^{1}H-NMR spectrum (500 MHz, (D_6)DMSO) showed two broad singlets (δ(H) 11.0 and 10.9) and eight aromatic protons, suggesting that the compound contained two indolyl moieties. A literature search indicated that the spectroscopic data obtained for this compound were consistent with the structure of indirubin (**2**).[21] The structure was confirmed by direct comparison of all spectroscopic data with those of an authentic sample of indirubin (**2**) prepared as detailed in the *Supporting Information*.

The polar metabolite at $t_R = 2.1$ min (method B) displayed an HR-EI-MS spectrum consistent with the molecular formula $C_8H_7NO_3$ (five degrees of unsaturation, *m/z* M^+ 165.0412) and its structure was assigned as *N*-formylanthranilic acid (**3**) by direct comparison of its spectroscopic data with those of a synthetic sample prepared as previously reported.[22] Previously, both tryptanthrin (**1**) and indirubin (**2**) were reported to be formed spontaneously in post-harvested leaves and roots of *I. indigotica*, whereas *N*-formylanthranilic acid (**3**) was reported to be constitutive only in *I. tinctoria*.[4]

During the fractionation process, an additional compound with $t_R = 24.6$ min (method B) was obtained in a relatively larger amount (ca. 190 mg) than any of the elicited metabolites. This compound displayed an

Table 1. HPLC-DAD analyses of elicited metabolites in the leaves of *Isatis indigotica*.

Metabolites, t_R (HPLC method)	Amounts (μmole/100 g of fresh tissue ± SD[a])		
	1-day post-elicitation	3-day post-elicitation	5-day post-elicitation
Tryptanthrin (**1**), $t_R = 15.4$ min (method A)	12 ± 11	32 ± 14	33 ± 15
Indirubin (**2**), $t_R = 20.7$ min (method A)	12 ± 10	65 ± 17	149 ± 43
N-Formylanthranilic acid (**3**), $t_R = 2.1$ (method B)	32 ± 13	201 ± 74	165 ± 71



Scheme 1. Preparation of indirubin (**2**).

HR-FD-MS spectrum that suggested a molecular formula of $C_{14}H_{15}N_1O_6$, conveying eight degrees of unsaturation (*m/z* M^+ 293.0904). The ^{1}H-NMR spectrum ((D$_6$)DMSO) displayed an exchangeable singlet at δ(H) 10.65, three doublets at δ(H) 7.57 (1H, d, $J=8.0$), 7.30 (1H, d, $J=8.5$) and 7.14 (1H, d, $J=2.5$), and two triplets at δ(H) 7.07 (1H, t, $J=7.0$) and 6.96 (1H, t, $J=7.5$), suggesting an indole nucleus substituted at C-2 or C-3. In addition, nine protons observed at δ(H) 5.74 – 3.33, three of which were exchangeable, suggested the presence of a glycosyl moiety. The ^{13}C-NMR spectrum displayed a signal at δ(C) 206.0, suggesting a ketone group, plus nine signals at δ(C) 136.7–104.7 and four signals at δ(C) 76.7–60.8, which were consistent with the presence of a glycosyl moiety. Altogether these data suggested an indole attached to a ketohexose. A literature search indicated that the spectroscopic data of isatan B (**4**) were identical to those of the metabolite with $t_R=24.6$ min.[23] Therefore, the structure of metabolite at $t_R=24.6$ min was assigned as isatan B (**4**), previously reported to be a precursor of indigoids in *I. tinctoria*.[23] Isatin (**5**) was also obtained during the chromatographic separations and the structure was confirmed by comparison of its spectroscopic data with those of a commercially available sample.

Preparation of Indirubin (**2**)

A number of approaches have been developed to synthesize indirubin (**2**). In this work, a procedure similar to that used by Ichimaru et al. to prepare indirubin derivatives was used to prepare indirubin (**2**),[24] as detailed in the *Supporting Information*. In brief, isatin (**5**) was allowed to react with phosphorus pentachloride (PCl_5) in benzene at reflux, and the resulting precipitate was filtered and dissolved in toluene; oxindole (**6**) was added to the solution and the reaction mixture was heated at reflux for 1 h, followed by work-up and chromatography to yield indirubin (**2**) in reasonable yield (84%; *Scheme 1*).

Antifungal Activity of Elicited Metabolites: Tryptanthrin (**1**), *Indirubin* (**2**) *and* N-*Formylanthranilic Acid* (**3**)

The antifungal activities of the elicited metabolites tryptanthrin (**1**), indirubin (**2**) and *N*-formylanthranilic acid (**3**) were tested against three fungal species: *Alternaria brassicicola* (Schwein) Wiltshire and *Leptosphaeria maculans* (Desm.) Ces. et de Not. (asexual stage *Phoma lingam* (Tode ex Fr.) Desm.), both specific pathogens of crucifers and the generalist pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary. These species cause economically important losses in various crucifer species. The cruciferous phytoalexin camalexin was used for comparison as the positive control. As summarized in *Table 2*, tryptanthrin (**1**) was strongly antifungal against *L. maculans*, causing complete growth inhibition at 0.10 mM and 66% inhibition at the lowest tested concentration (0.010 mM). However, tryptanthrin (**1**) showed lower inhibitory activity against *A. brassicicola* (31% inhibition at 0.50 mM) and *S. sclerotiorum* (12% inhibition at 0.50 mM). Tryptanthrin appears to have a more selective inhibitory effect on fungal growth than camalexin, which completely inhibited the mycelial growth of the three species at 0.50 mM. Furthermore. indirubin (**2**) exhibited higher inhibitory activity than tryptanthrin (**1**) against *S. sclerotiorum* (20% inhibition at 0.50 mM), but substantially lower against *L. maculans*, whereas *N*-formylanthranilic acid (**3**) displayed inhibitory activity only against *A. brassicicola*.

Biosynthesis of Tryptanthrin (**1**) *and* N-*Formylanthranilic Acid* (**3**)

Tryptanthrin (**1**) was first isolated from a natural source in 1971.[25] Cultures of the yeast *Candida lipolytica* grown in a L-tryptophan (Trp) rich medium produced tryptanthrin (**1**),[25] substituted tryptanthrins if incubated with Trp and substituted anthranilic acids, or substituted Trps and anthranilic acid.[26] It was then proposed that tryptanthrin (**1**) was biosynthesized from anthranilic acid (**7**) and indolyl-3-pyruvic acid in *C. lipolytica*.[25] Since then, tryptanthrin (**1**) has been

Table 2. Antifungal activity of tryptanthrin (**1**), indirubin (**2**), *N*-formylanthranilic acid (**3**) and the phytoalexin camalexin against *Alternaria brassicicola*, *Leptosphaeria maculans* and *Sclerotinia sclerotiorum*

Concentration [mM]	% Inhibition ± S.D.[a]		
	A. brassicicola[b]	*L. maculans*[c]	*S. sclerotiorum*[d]
Tryptanthrin (**1**)			
0.50	31 ± 3[f]	c.i.[e]	12 ± 5[g]
0.20	22 ± 3[f]	c.i.[e]	n.i.[g]
0.10	20 ± 0[f]	c.i.[e]	n.i.[g]
0.050	n.i.[f]	88 ± 0[e]	n.i.[f]
0.020	n.i.[f]	78 ± 2[e]	n.i.[e]
0.010	n.i.[f]	66 ± 2[e]	n.i.[e]
Indirubin (**2**)			
0.50 M	13 ± 3[g]	33 ± 2[f]	20 ± 3[f]
0.20 M	7 ± 3[h]	20 ± 2[g]	14 ± 2[f]
0.10 M	n.i.[g]	8 ± 0[g]	12 ± 2[f]
N-Formylanthranilic acid (**3**)			
0.50	17 ± 4[g]	6 ± 3[g]	n.i.[h]
0.20	11 ± 3[g]	n.i.[h]	n.i.[g]
0.10	n.i.[g]	n.i.[h]	n.i.[g]
Camalexin – positive control			
0.50	c.i.[e]	c.i.[e]	c.i.[e]
0.20	c.i.[e]	41 ± 2[f]	c.i.[e]
0.10	76 ± 0[e]	24 ± 3[f]	c.i.[e]
0.050	25 ± 5[e]	n.i.[f]	81 ± 6[e]

[a] Percentage of inhibition = 100 – [(growth on amended/growth in control) × 100]; control plates contained PDA medium and 1% DMSO; c.i. = complete inhibition; n.i. = no inhibition. Values are averages of two independent experiments conducted in triplicate; for statistical analysis, one-way ANOVA tests were performed followed by Tukey's test with adjusted α set at 0.05; $n = 6$; different letters in the same row (e–h) indicate significant differences ($P < 0.05$). [b] Incubated for 60–70 h under continuous light. [c] Incubated for 4–5 days under continuous light. [d] Incubated for ca. 20 h in the dark.

isolated from different organisms including various plant species;[7] however, in plants no information regarding the biosynthesis of tryptanthrin (**1**) has been reported. Indirubin (**2**), a natural component of indigo dyes, was reported to derive from isatans A and B (**4**) and indican in *Isatis* species,[23,27] whereas *N*-formylanthranilic acid (**3**) was reported to derive from the degradation of gramine via indole-3-carboxylic acid in seedlings of *Hordeum vulgaris* L. (Poaceae).[28]

Current knowledge on the shikimate and L-Trp biosynthetic pathways in plants,[29,30] together with a retrobiosynthetic analysis of tryptanthrin (**1**), suggests that anthranilic acid (**7**) and *N*-formylanthranilic acid (**3**) are the primary biosynthetic precursors of tryptanthrin (**1**; *Figure 6*). To the best of our knowledge, no

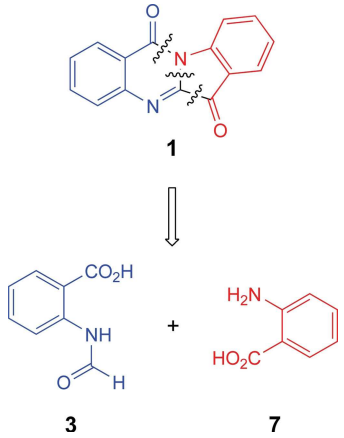


Figure 6. Retrobiosynthetic analysis: potential precursors of tryptanthrin (**1**) in the leaves of *Isatis indigotica*.

formal isotope labeling studies to uncover such precursors have been reported to date.

For this reason, to clarify the biosynthetic origin of tryptanthrin (**1**) in *I. indigotica*, the isotopically labeled compounds [3,5-D_2]anthranilic acid (**7a**), [$^{13}C_{11}$,$^{15}N_2$]Trp (**8a**) and [4,5,6,7-D_4]indole (**9a**; *Figure 7*) were administered separately to elicited leaves of *I. indigotica*. Tetradeuterated indole (**9a**; ca. 96% D_4) was synthesized as previously reported,[31] and dideuterated anthranilic acid (**7a**; ca. 96% D_2) was prepared by proton/deuteron exchange, as described in the *Supporting Information*. [$^{13}C_{11}$,$^{15}N_2$]Trp (**8a**; $^{13}C_{11}$,$^{15}N_2 \geq$ 98%) was commercially available. The whole plants were elicited, incubated, the leaves were excised, and the petioles were immediately immersed in aqueous solutions of the labeled compounds. After further incubation, leaves were frozen and then extracted, as described in the *Experimental Section*. Control samples (elicited leaves fed with non-labeled compounds or

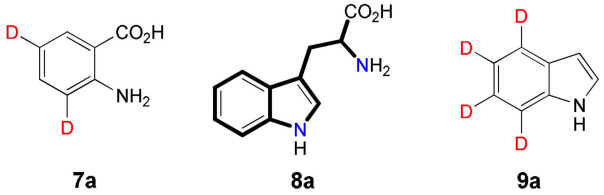


Figure 7. Structures of isotopically labeled compounds administered to the leaves of *Isatis indigotica*: [3,5-D_2]anthranilic acid (**7a**), L-[$^{13}C_{11}$,$^{15}N_2$]Trp (**8a**) and [4,5,6,7-D_4]indole (**9a**).

Table 3. Metabolism of [3,5-D_2]anthranilic acid (**7a**) in elicited leaves of *Isatis indigotica*

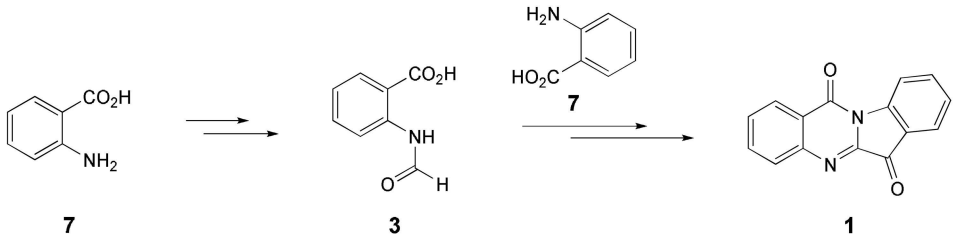
Deuterated metabolites detected in leaf extracts	% of Deuterium
[3,5-D_2]Anthranilic acid (**7a**)	ca. 95%
N-[3,5-D_2]Formylanthranilic acid (**3a**)	30 ± 18%
[D_2]Tryptanthrin (**1a**)	86 ± 3%[a]
[D_4]Tryptanthrin (**1b**)	2.3 ± 1.4%[a]

[a] Isotope incorporations calculated from HPLC-ESI-MS (peak intensities in positive and negative modes); % of incorporation = $\{[M\pm H+n]^+/([M\pm H]^+ + [M\pm H+2]^+ + [M\pm H+4]^+\}\times 100$, *n* represents D atoms, *n* = 2 for **1a**, *n* = 4 for **1b**; values represent the mean and standard deviation of triplicate samples.

solvent) were treated similarly. All leaf extracts were analyzed by HPLC-DAD-ESI-MS and the percentage of isotope incorporation into each metabolite was determined using the peak intensities of ESI-MS spectra (positive and negative ion modes, according to the equation reported in *Table 3*).

After administration of [3,5-D_2]anthranilic acid (**7a**) to elicited leaves of *I. indigotica* and incubation, tryptanthrin (**1**) was detected in leaf extracts and the deuterium incorporations were quantified. The ESI-MS spectrum of tryptanthrin showed a major ion peak at *m/z* 251.1 $[M+H+2]^+$ (100%), corresponding to [D_2]tryptanthrin (**1a**), and minor ion peaks at *m/z* 253.0 $[M+H+4]^+$ (4.7%), corresponding to [D_4]tryptanthrin (**1b**) and at *m/z* 249.1 $[M+H]^+$ (7.6%), corresponding to natural abundance tryptanthrin (**1**), which were further confirmed by HR-ESI-MS. Similarly, the ESI-MS spectrum of *N*-formylanthranilic acid (**3**) showed two major ion peaks at *m/z* 163.9 $[M-H]^+$ (100%) and *m/z* 165.9 $[M-H+2]^+$ (35%), corresponding to natural abundance *N*-formylanthranilic acid (**3**) and to *N*-[3,5-D_2]formylanthranilic acid (**3a**), respectively. By contrast, after feeding experiments using [$^{13}C_{11}$, $^{15}N_2$]Trp (**8a**) and [4,5,6,7-D_4]indole (**9a**), no incorporation of either ^{13}C, ^{15}N or D atoms were detected in tryptanthrin (**1**) or *N*-formylanthranilic acid (**3**). In addition, the amounts of labeled Trp present in the leaf extracts were substantially lower (55 ± 7%) than those present in the fed solutions (ca. 98%), whereas [4,5,6,7-D_4]indole (**9a**) was not detected in any of the leaf extracts. Indirubin (**2**) was detected only in trace amounts, therefore, no incorporations were detected (*Table 3*).

The overall results of the incorporation of isotopically labeled compounds into tryptanthrin (**1**) suggest that anthranilic acid (**7**) and *N*-formylanthranilic acid (**3**) are its precursors, as proposed in *Scheme 2*. [D_2]Tryptanthrin (**1a**) can be formed from two different precursor pools: either by i) coupling of [3,5-D_2]anthranilic acid (**7a**) and natural abundance **3** (highly abundant, higher probability event) or ii) coupling of [D_2]-**3a** and natural abundance **7**. However, [D_4]tryptanthrin (**1b**) can only be formed from one precursor pool by coupling of [D_2]-**7a** and [D_2]-**3a**, both very low abundance, hence the probability of forming [D_4]-**1b** is much lower than that of [D_2]-**1a**. Furthermore, the higher incorporation of deuterium into [D_2]tryptanthrin (**1a**) reflects the low dilution of both [D_2]tryptanthrin (**1a**; 86%) and [3,5-D_2]anthranilic acid (**7a**; 95%; *Table 3*) with their natural abundance counterparts **1** and **7**, respectively (because **1** and **7** are produced in very small amounts, *Table 1*). Similarly, these deuterium incorporation results are consistent with the relatively lower incorporation of deuterium into [D_4]tryptanthrin (**1b**; 2.3%; *Table 3*). By contrast, *N*-[3,5-D_2]formylanthranilic acid (**3a**; 30%; *Table 3*) is produced in larger amounts than **1a** and has a high dilution with the natural pool of **3**, which is produced in large amounts (*Table 1*). Because no isotope incorporations from either indole (**9a**) or Trp (**8a**) into tryptanthrin (**1**) were detected *in planta*, these compounds might have undergone enzyme catalyzed transformations unrelated to the biosynthesis of **1**. Furthermore, since substantial incorporation of deuterium from [3,5-D_2]anthranilic acid (**7a**) into *N*-[D_2]formylanthranilic acid (**3a**) was detected, anthra-



Scheme 2. Biosynthetic precursors of tryptanthrin (**1**) produced in *Isatis indigotica*.

nilic acid (**7**) appears to be the closest precursor of **3**. Alternatively, **3** could result from oxidation of indole (**9**) or catabolism of Trp (**8**), however, since no isotope incorporations from these compounds were detected, these hypotheses appear less likely.

We have shown that elicitation of whole plants of *I. indigotica* and isolation of elicited metabolites produced in leaves yielded tryptanthrin (**1**), indirubin (**2**) and *N*-formylanthranilic acid (**3**), which were not detected in non-elicited leaf extracts. Consequently, considering that metabolites **1**–**3** display antifungal activity against phytopathogenic fungi and are produced only in elicited leaves, the question arises whether their ecological role fits in the phytoalexin or phytoanticipin class. Because tryptanthrin (**1**) displays strong antifungal activity and is biosynthesized via intermediates derived *de novo* from anthranilic acid (**7**), we suggest that it is a phytoalexin in *I. indigotica*. However, indirubin (**2**) and *N*-formylanthranilic acid (**3**) might fit better in the phytoanticipin class due to their biosynthetic origin from very close precursors. This is because phytoalexins, by definition, are biosynthesized *'de novo'*, i.e., from remote precursors that may require several enzymatic transformations, whereas phytoanticipins either are constitutive (pre-formed) or result from single transformations such as hydrolysis, dimerization, or oxidation. Indeed, in spite of significant endeavors to clarify definitions,[9,10,12] many secondary or specialized plant metabolites that would be more appropriately considered phytoanticipins are reported as phytoalexins.[13] Not surprisingly, like for many other classifications, there are gray areas and cases where the distinction between phytoanticipins and phytoalexins is not so obvious. In such cases, examination of biosynthetic intermediates might be required to more accurately classify those plant metabolites as phytoanticipins or phytoalexins. Notwithstanding the caveat, in our view tryptanthrin (**1**) represents a new group of cruciferous phytoalexins in which anthranilic acid (**7**) is its primary biosynthetic precursor, whereas previously known phytoalexins derive from protein amino acids via the corresponding glucosinolates.[11] Importantly, because *Brassica* species do not appear to produce tryptanthrin (**1**), it would be worthwhile to transfer this pathway to *Brassica* species susceptible to *L. maculans*, to investigate its effect on plant disease resistance levels to this important pathogen.

Conclusions

In conclusion, our work on the elicited metabolites produced in leaves of *I. indigotica* has uncovered three elicited antifungal metabolites: tryptanthrin (**1**), indirubin (**2**) and *N*-formylanthranilic acid (**3**). These metabolites are likely to act as plant defenses in the interaction of *I. indigotica* with microbial pathogens and perhaps other stresses. Importantly, although these metabolites have been known for decades to display important pharmacological activities, this is the first time that an ecological defensive role can be assigned to these compounds. These findings substantiate what is currently indisputable, i.e., secondary metabolites are produced for the benefit of the producing organism, not for the benefit of human kind. As our investigation on cruciferous phytoalexins expands into different plant genera, the variety of chemical structures continues to increase, consistent with the current understanding that chemical diversity and biodiversity are intimately connected.

Experimental Section

General

All solvents were of HPLC grade and used as such, except for those used in chemical syntheses, as noted. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. Flash column chromatography (FCC): silica gel, grade 60, 230–400 μm. NMR spectra were recorded on Bruker Avance 500 MHz spectrometers. For ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125.8 MHz) spectra, the chemical shifts (δ) are reported in parts per million (ppm) relative to TMS. MS data (high resolution (HR)) were obtained on a Jeol AccuToF GCv 4G mass spectrometer (field desorption (FD)) or on a Qstar XL MS/MS System (ESI) by direct insertion.

HPLC Analysis

HPLC-DAD analyses were carried out with Agilent 1100 and 1200 series systems all equipped with quaternary pump, autosampler, diode array detector (DAD, wavelength range 190–600 nm) and degasser; used a Zorbax Eclipse XDB–C18 column (5 μm particle size silica gel, 150×4.6 mm i.d.), equipped with an in-line filter, with the mobile phase H_2O/MeCN from 75:25 to 25:75, linear gradient for 35 min and a flow rate of 1.0 mL/min (method A for phytoalexins and non-polar metabolites) or H_2O/MeCN from 100:0 to 10:90 in

20 min, with a flow rate of 1.0 mL/min (method B for polar metabolites).

HPLC-DAD-ESI-MS analysis was carried out with an Agilent 1100 series HPLC system equipped with an autosampler, binary pump, degasser and a diode array detector connected directly to a mass detector (Agilent G2440 A MSD-Trap-XCT ion trap mass spectrometer) with an electrospray ionization (ESI) source. Chromatographic separations were carried out at room temperature using an Eclipse XDB-C-18 column (5 μm particle size silica gel, 150 mm × 4.6 mm i.d.). The mobile phase (method C) consisted of a linear gradient of H_2O (with 0.2% HCO_2H)/MeCN (with 0.2% HCO_2H) from 75:25 to 25:75 in 25 min and a flow rate of 1.0 mL/min. Data acquisition was carried out in positive and negative polarity modes in a single LC run and data processing was carried out with Agilent Chemstation Software.

HPLC-ESI-HR-MS was performed on an Agilent HPLC 1100 series directly connected to QSTAR XL Systems mass spectrometer (Hybrid Quadrupole-TOF LC/MS/MS) with turbo spray ESI source. Chromatographic separations were carried out using a Hypersil ODS C-18 column (5 μL particle size silica gel, 200 mm × 2.1 mm i.d.) or a Hypersil ODS C-18 column (5 μL particle size silica gel, 100 mm × 2.1 mm i.d.). The mobile phase consisted of a linear gradient of H_2O (0.1% HCO_2H)/MeCN (0.1% HCO_2H) from 75:25 to 25:75 in 35 min, to 0:100 in 5 min and a flow rate of 0.25 mL/min. Data acquisition was carried out in either positive or negative polarity mode in a single LC run and data processing was carried out with Analyst QS Software.

Plant Material and Extractions

Seeds of *Isatis indigotica* Fort. (Chinese woad) were purchased from Sand Mountain Herbs (321 County Road 18 Fyffe, AL 35971, www.sandmountainherbs.com). The seeds were placed on a filter paper in a Petri dish and wet with distilled water. After germination occurred (6–7 days), seedlings were transplanted to perlite and nutrient free LG-3 soil (Sun Gro Horticulture Canada) in small pots (15 cm diameter) in a growth chamber at 16 h of light/8 h of dark, 20 °C day/18 °C night, 350–360 $\mu E\,m^{-2}\,s^{-1}$ and with ambient humidity.

For time-course HPLC analyses, 3-week-old plants were sprayed with an aqueous $CuCl_2$ solution (10 mM) and plants were kept in the growth chamber. Control plants were sprayed with H_2O. The leaves (ca. 0.8 g fresh weight per sample) were excised 1, 3 and 5 days after elicitation, frozen in liquid N_2 and ground, and the resulting leaf materials were individually extracted with MeOH (5 mL). The extracts were filtered, the filtrates were concentrated and rinsed with CH_2Cl_2. The CH_2Cl_2 extracts were concentrated, dissolved in MeCN/MeOH (1:1, 1 mL) and analyzed by HPLC-DAD using methods A and B, and by HPLC-DAD-ESI-MS using method C. For isolation of the unknown metabolites detected in elicited extracts, 3-week-old plants were sprayed with an aqueous $CuCl_2$ solution (10 mM) and plants were kept in growth chamber. After two days, elicited leaves (ca. 100 g) were frozen in liquid N_2, ground and rinsed with hexane (2× 300 mL). The leaf materials were shaken in MeOH for 1 h and filtered. The filtrate was concentrated and partitioned in AcOEt/H_2O. The organic extract was concentrated to dryness to yield the crude AcOEt extract (ca. 1.9 g), which was fractionated as described below.

Isolation of Metabolites

The AcOEt extract (ca. 1.9 g) was fractionated using flash column chromatography (FCC, gradient MeOH/CH_2Cl_2, 0:100–20:80) to give seven fractions, which were further fractionated by FCC and the eluting solvents were summarized in *Figure 8*.

Antifungal Bioassays

The antifungal activity of compounds against three fungal species was investigated using a mycelial radial growth bioassay (PDA media and DMSO solutions of each compound, 0.50, 0.25 and 0.10 mM; control solutions contained 1% DMSO in PDA media). *A. brassicicola* isolate UAMH 7474 and *L. maculans* isolate UAMH 9410 were obtained from the University of Alberta Microfungus Collection and Herbarium and *S. sclerotiorum* clone #33 was obtained from the AAFC Saskatoon Research Center. Spores of *A. brassicicola* and *L. maculans* were spotted onto potato dextrose agar plates (PDA) and allowed to grow for seven days under constant light at 23 ± 1 °C. Similarly, sclerotia of *S. sclerotiorum* were placed on PDA plates and allowed to germinate and grow mycelia for four days incubated in the dark. Plugs (3 mm) were cut from the edges of growing mycelia and placed inverted onto 12-well plates containing compounds in DMSO mixed into PDA. These 12-well culture plates containing compounds to be tested were allowed to grow under

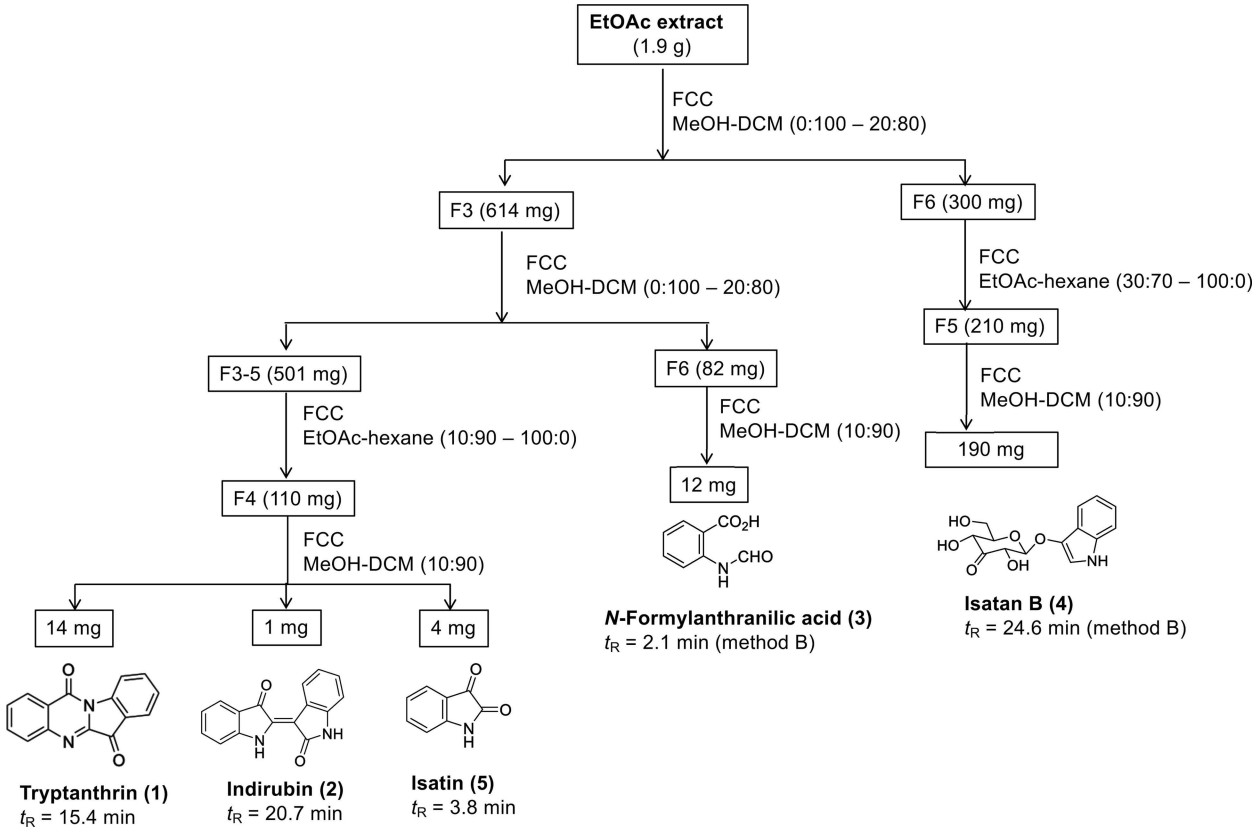


Figure 8. Isolation of metabolites from elicited leaves of *Isatis indigotica*.

constant light or dark (*Table 2*) at 23 ± 1 °C up to 96 h. The diameter of the mycelial mat was measured and compared to control mycelia grown on plates containing DMSO, using the formula in *Table 2* (the diameter of the mycelial plug was subtracted from the total mycelial growth).

Feeding Experiments Using [3,5-D_2]Anthranilic Acid (**7a**), *[$^{13}C_{11}$,$^{15}N_2$]Trp* (**8a**) *and [4,5,6,7-D_4]Indole* (**9a**)

For feeding experiments, three-week old plants were sprayed with an aqueous $CuCl_2$ solution (10 mM) and incubated under fluorescent light for 48 h. Leaves were cut and immediately immersed in an aqueous solution of [3,5-D_2]anthranilic acid (**7a**), [$^{13}C_{11}$,$^{15}N_2$]Trp (**8a**) and [4,5,6,7-D_4]indole (**9a**; 10^{-3} M dissolved in H_2O for **7a** and **8a**, or in H_2O/MeOH (1:9, v/v) for **9a**). Similar experiments were carried out with the corresponding natural abundance compounds (anthranilic acid (**7**), Trp (**8**) and indole (**9**)). When most of the solutions were uptaken, the tubes were refilled with distilled H_2O and leaves were further incubated under continuous fluorescent light. After 24 h, the leaves were frozen in liquid N_2, ground and extracted with MeOH. The MeOH extracts were filtered, concentrated and rinsed with CH_2Cl_2. The CH_2Cl_2 extracts were concentrated and analyzed by HPLC-DAD-ESI-MS using method C. Control samples included elicited leaves fed with solvent and, otherwise, treated similarly.

Supplementary Material

HPLC-DAD chromatograms (method B, detection at 220 nm) of extracts of leaves of *Isatis indigotica* after elicitation with $CuCl_2$; preparation of new compound [3,5-D_2]anthranilic acid (**7a**) and its ^{1}H- and ^{13}C-NMR spectra; preparation of indirubin (**2**).

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Author Contribution Statement

M. S. C. Pedras designed and supervised this study and wrote and revised the manuscript. A. Abdoli synthesized indirubin, carried out the antifungal assays with indirubin and tryptanthrin and performed all statistical analyses. Q. H. To carried out all elicitation and isotope-feeding experiments, isolated, identified and characterized all plant metabolites (**1**–**5**), prepared dideuterated anthranilic acid and carried out the antifungal assays with *N*-formylanthranilic acid. C. Thapa analyzed, purified and spectroscopically characterized dideuterated anthranilic acid. All authors read and reviewed the manuscript.

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