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METABOLISM OF THE PHYTOALEXIN BRASSININ BY THE "BLACKLEG" FUNGUS

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ABSTRACT.—The metabolism of the phytoalexin brassinin [**1**] by the blackleg fungus (*Leptosphaeria maculans* = *Phoma lingam*, imperfect stage), a pathogen of *Brassica* species, was investigated in liquid cultures. The intermediates of the metabolism of brassinin by virulent and avirulent isolates were separated and the chemical structures determined by spectroscopic methods and/or concomitant synthesis. Virulent isolates of the fungus metabolized brassinin rapidly, whereas avirulent isolates revealed a much slower metabolism. The metabolism of putative intermediates was investigated as well. Additionally, metabolic routes are proposed for the biotransformation of brassinin by virulent and avirulent isolates, and possible implications are discussed.

The "blackleg" fungus [*Leptosphaeria maculans* (Desm.) Ces. et de Not. (Pleosporaceae) = *Phoma lingam* (Tode ex Fr.) Desm., imperfect stage] is the causative agent of the blackleg disease of cruciferous crops (comprising economically important *Brassica* species). A virulent strain of the fungus has caused significant rapeseed (*Brassica napus* L. and *Brassica campestris* L.) losses in recent years in Canada (1,2) and is considered a serious agricultural problem worldwide. Virulent isolates of the blackleg fungus synthesize in liquid culture a complex array of phytotoxins as, for example, the sirodesmins PL (3), H, J, and K (4,5), and phomalirazine (6). The production of such phytotoxins by the blackleg fungus in infected plant tissue has not yet been demonstrated (7).

Cruciferous plants, as many other plant families, react to pathogen attack and other forms of stress by synthesizing defense compounds having biocidal activity (i.e., phytoalexins) (8,9). Nevertheless, pathogens are capable of circumventing these plant defenses by metabolizing phytoalexins (10). Brassinin [**1**], one of the first phytoalexins reported from *Brassica* species (e.g., rapeseed, canola, broccoli, cabbage, turnip, etc.), contains a dithiocarbamate group attached to a 3-methylindolyl moiety (11). Although dithiocarbamates have long been known as very important pesticides and herbicides (12,13), so far crucifers appear to be the only plants producing these interesting compounds. Plants from diverse *Brassica* species show resistance to blackleg disease; however, there is no general understanding of the mechanism of resistance or of the chemistry involved in the plant-pathogen interaction.

Preliminary results on the biotransformation of the phytoalexin brassinin [**1**] by a virulent isolate of the blackleg fungus (14) indicated that this transformation is consistent with a detoxification process (15). Further investigation on the metabolism of brassinin indicated that avirulent isolates of *P. lingam* follow a metabolic pathway which is different from that of virulent isolates. Now we wish to report in detail the biotransformation of brassinin by both avirulent and virulent isolates of the blackleg fungus and discuss possible routes for these degradation processes.

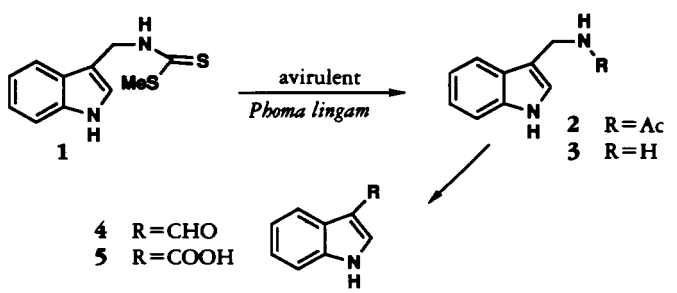
RESULTS AND DISCUSSION

P. lingam (avirulent isolate Unity, 10^8 spores per ml) was grown at 25 ± 2° in shake culture on minimal medium supplemented with thiamine (16). After 48 h, brassinin [**1**] [synthesized using a modification of a literature procedure (15,17)] solutions in Me_2SO were administered to fungal cultures and to uninoculated media (final concentration 5×10^{-4} M). Control cultures of the fungus were grown separately. Cultures were incubated and samples were withdrawn at 3–24 h intervals (up to 7 days) and extracted first with Et_2O and then with $CHCl_3/NH_4OH$. Extracts were analyzed by tlc

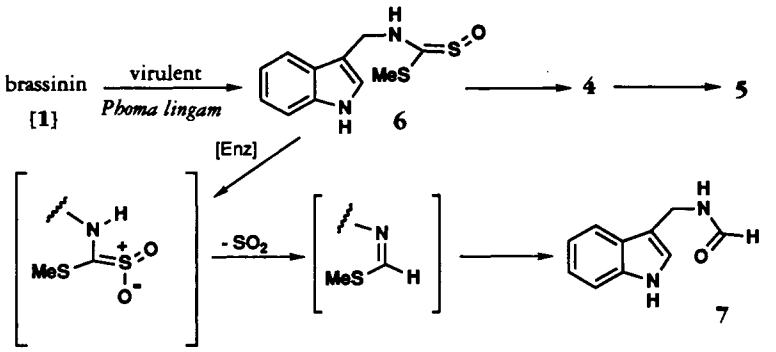
and by hplc to determine the optimum incubation time for isolation of the putative intermediates. Subsequently, the components of Et_2O extracts obtained from larger scale cultures were separated by preparative tlc and each constituent analyzed by cims, eims, and ^1H and ^{13}C nmr. Three products resulting from the metabolism of brassinin were identified: *N*-acetyl-3-indolylmethylamine [**2**], indole-3-carboxaldehyde [**4**], and indole-3-carboxylic acid [**5**] (Scheme 1). The structures of compounds **4** and **5** were readily determined by comparison of their spectroscopic data with those of authentic samples (obtained from Aldrich Chemical Company). The structure of intermediate **2** was deduced from spectroscopic data. Specifically, the ^1H-nmr spectrum of **2** showed resonances indicative of a 3-substituted indolyl system (δ 8.37 br s, 7.62 d, 7.40 d, 7.20 ddd, 7.18 br s, 7.12 ddd) and of an acetyl group (δ 1.94 s), and in the eims displayed strong ions at *m/z* 188 and 145. Synthesis of *N*-acetyl-3-indolylmethylamine [**2**] corroborated the assignment. The $CHCl_3$ extract contained *N*-acetyl-3-indolylmethylamine [**2**] and 3-indolylmethylamine [**3**]. Metabolite **3** was identified by tlc, eims, ^1H nmr, and direct comparison with an authentic sample. Metabolites **2–5** were not detected in uninoculated media containing brassinin [**1**].

To confirm that compounds **2–4** were intermediates in the metabolism of brassinin, solutions of each were added to cultures of isolate Unity. Samples were withdrawn at 3–24 h intervals, extracted (vide supra), and analyzed by tlc and hplc. Compound **2** (4×10^{-4} M) was partially converted to the aldehyde **4** and amine **3**. After incubation for 4 days the ratio of aldehyde **4** to acetylamine **2** was ca. 3:2 (determined by ^1H nmr of the Et_2O extract), whereas the amine **3** comprised less than 5% of the $CHCl_3$ extract. On the other hand, amine **3** (4×10^{-4} M) was converted to acetylamine **2** and, further, on to **4** and **5**. After incubation for seven days **4** was the major component of the Et_2O extract. Incubation of the aldehyde **4** (2×10^{-4} M) with Unity cultures resulted in partial conversion to the acid **5** (amine **3** was not detected in the $CHCl_3$ extract). After incubation for 4 days the ratio of aldehyde **4** to acid **5** was ca. 2:1 (determined by ^1H nmr of the Et_2O extract). Similar metabolic experiments using indole-3-carboxylic acid [**5**] were also conducted; however, further catabolites arising from acid **5** were not detected. Possible metabolic products might be very polar and more soluble in the aqueous media than in organic solvents (Et_2O, CH_2Cl_2, or EtOAc), thus precluding detection.

The metabolism of brassinin [**1**] by the avirulent Unity isolate of *P. lingam* was established as represented in Scheme 1. The biotransformation occurred in a series of steps in which the initial intermediate was either acetylamine **2** or amine **3**, and the last one the acid **5**. Analogous experiments showed that brassinin [**1**] was similarly metabolized by two other avirulent isolates (Peace 5 and Peace 3). In a similar study, we have previously established that virulent isolates convert **1** to intermediate **6** which is further metabolized to aldehyde **4** and acid **5**, as shown in Scheme 2 (14,15). Thus a striking



SCHEME 1



SCHEME 2

difference in the metabolism of brassinin [**1**] by avirulent and virulent isolates had emerged. While in virulent isolates the transformation of brassinin [**1**] rapidly yielded aldehyde **4** via intermediate **6** (14,15), in avirulent isolates **1** was slowly converted to aldehyde **4** via intermediates **2** and **3**.

In order to clarify this apparent difference, the metabolism of brassinin [**1**] and intermediates **2**, **3**, and **6** [synthesized according to Pedras *et al.* (15)] by avirulent isolate Unity and virulent isolate Leroy was reinvestigated in parallel (Table 1). The concentrations of the fungal spores and of each compound were similar for all cultures. Samples were withdrawn from each culture (3 h–5 days), extracted, and analyzed. The products resulting from the biotransformation of compounds **1**, **2**, **3**, and **6** after a 48-h incubation period are shown in Table 1.

As expected, the transformation of brassinin [**1**] was much slower with the avirulent isolate than with the virulent one (more than 5 days for Unity vs. 24 h for Leroy to yield the final product **5**). Analysis (by gc-eims) of the volatile components present in the head-space after incubation of **1** with both virulent and avirulent fungal cultures (4-h incubation period) indicated the presence of carbonyl sulfide and MeSH in each culture (not present in control cultures). Interestingly, a trace amount of the intermediate **6** was detected in the Et_2O extract of the avirulent isolate incubated with brassinin. Ad-

TABLE 1. Products of Metabolism[a] of Compounds **1**, **2**, **3**, and **6** by *Phoma lingam*, isolates Leroy and Unity.

Isolate	Compound[b]	Product[c]	
		Major product	Other products
Leroy[d]	**1**	metabolism completed in 24 h	no other products detected
Unity[e]	**1**	**2**	**4** + **3** (≤ 5% of total amount)
Leroy[d]	**2**	**2** (≤ 5% of initial amount)	no other products detected
Unity[e]	**2**	**2**	**4** + **3** (≤ 5% of total amount)
Leroy[d]	**3**	**4**	**5** + **3** (≤ 5% of initial amount)
Unity[e]	**3**	**2**	**4** + **3** [≤ 10% of total amount
Leroy[d]	**6**	metabolism completed in 24 h	no other products detected
Unity[e]	**6**	**2**	**4** + **3** (≤ 5% of total amount)

[a]After a 48-h incubation period.

[b]Concentration of each compound in media 4×10^{-4} M. Media and incubation conditions were identical for each culture.

[c]Identity of products determined by tlc, hplc, ^{1}H nmr, and/or eims.

[d]Virulent isolate.

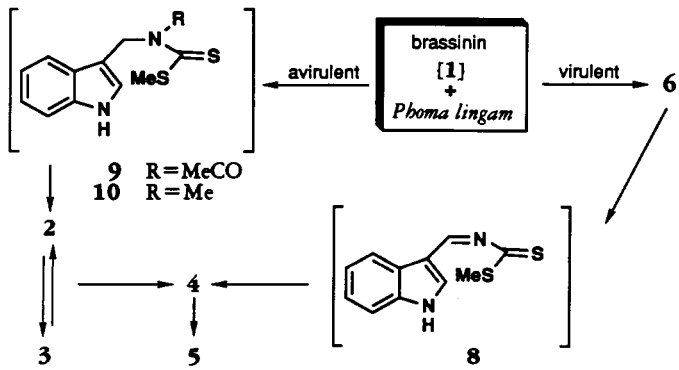
[e]Avirulent isolate.

ditionaly, a trace *N*-acetylamine **2** was detected in the Et_2O extract of the virulent isolate incubated with brassinin.

Not surprisingly, the biotransformation of compounds **2**, **3**, and **6** by the avirulent isolate was relatively slow; **2** was the major product even after a 5-day incubation period. By contrast, **2** was completely metabolized by isolate Leroy within 48 h. Except for traces of aldehyde **4**, which was present during the initial 24-hour incubation period, no other products were detected in the latter experiment. In the course of these studies, a minor metabolite (less than 0.1 mg from 12 mg of brassinin) resulting from transformation of brassinin by virulent isolate Leroy was detected and eventually isolated and identified as *N*-formyl-3-indolylmethylamine [**7**]. Compound **7** was not further metabolized by either virulent or avirulent isolates (up to five days incubation). Formamide **7** possibly results from oxidation of dithiocarbamate S-oxide **6** with concomitant loss of SO_2 (Scheme 2). In this connection, it has been reported that oxidation of alkyl dialkyldithiocarbamates by *m*-CPBA yielded dialkylformamides (18).

Also noteworthy was the effect of brassinin [**1**] on the biosynthesis of sirodesmins. Virulent isolates of *P. lingam* start synthesizing the sirodesmins after a 54-h to 60-h incubation period (15). Incubation in the presence of brassinin [**1**] inhibited the synthesis of those phytotoxins. By contrast, incubation with any of the intermediates **2**–**7** did not noticeably affect sirodesmin production. This observation suggests a possible role for brassinin and perhaps other phytoalexins, and may explain unsuccessful attempts to detect any of the sirodesmins in rapeseed foliar tissue infected with *P. lingam* (7).

The results described above are summarized in the pathway depicted in Scheme 3 (structures between brackets represent hypothetical intermediates). Two possible routes for the metabolism of brassinin [**1**] are proposed; each pathway could operate in both virulent and avirulent isolates. The major pathway in virulent isolates produces acid **5** via dithiocarbamate S-oxide **6** and the hypothetical intermediate **8** (or equivalent) (14). Alternatively, avirulent isolates produce the acid **5** via acetylamine **2** and amine **3**. This "avirulent route" would start with acetylation of **1**, followed by hydrolysis of the dithiocarbamate group to yield acetylamine **2** which would then be converted to aldehyde **4**, either directly or via the amine **3**. To test this hypothesis, acetylbrassinin [**9**] was prepared and administered to both virulent and avirulent fungal cultures. Unfortunately, extensive decomposition of **9** in the media during the initial 24-h incubation period precluded any inference. Similarly, *N*-methylbrassinin [**10**] was prepared, reasoning that the presence of an Me group on the nitrogen atom of the



SCHEME 3

dithiocarbamate group would inhibit the putative acetylation step. Addition of **10** to the fungal cultures also gave decomposition products, preventing further conclusions.

Alternatively, the apparent difference between the proposed pathways (Scheme 3) might result from each route utilizing identical intermediates but with different rate-determining steps. For example, the biotransformation of **2** to **4** may be much slower in avirulent than in virulent isolates, and/or **6** may be converted to **2** faster in avirulent than in virulent isolates. The overall result would be accumulation of **2** in cultures of avirulent isolates. However, considering that the transformation of **2** by virulent isolates did not give the expected products **4** and **5**, this hypothesis does not appear as plausible, although it cannot be ruled out at this stage. Currently we are exploring other possible inhibitors of brassinin degradation to probe the enzyme specificity in the "avirulent and virulent routes" operating in rapeseed pathogens.

In conclusion, the ability of plant pathogens to metabolize and detoxify phytoalexins has been correlated with the virulence of the pathogen (10). The dithiocarbamate group of the phytoalexin brassinin [**1**] can be converted to the significantly less toxic carboxylic acid group (15) by the blackleg fungus. This detoxification process may be an important factor for the virulent pathogen to overcome plant chemical defenses, as in other plant-pathogen interactions (10). In this context it should be noted that brassinin [**1**] is a biogenetic precursor of several other phytoalexins of *Brassica* species (19). Thus, the rapid metabolism of brassinin by virulent isolates of the blackleg fungus may deprive the plant of other important phytoalexins. The overall result would be a plant more susceptible to further fungal colonization. Furthermore, considering that dithiocarbamates, and thiocarbamates as well, have been used as pesticides and herbicides for more than two decades (13), their biotransformation may be a survival strategy used by diverse microorganisms. This hypothesis is currently being investigated.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All chemicals were purchased from Aldrich Chemical Company, Madison, WI. All solvents were hplc grade and used as such. Preparative tlc Merck, Kieselgel 60 F254), 20 × 20 cm × 0.25 mm; analytical tlc Merck, Kieselgel 60 F_{254}, aluminum sheets, 5 × 2 cm × 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°. Flash cc (20) Si gel Merck, grade 60, mesh size 230–400, 60 Å. Hplc analysis was carried out with the following Spectra Physics instrument system: liquid chromatograph model SP 8100, equipped with a dual pump and autosampler, uv/visible detector (variable wavelength) model SP 4200, and computing integrator model SP 4200. A reversed-phase RP-8 Spheri-5 column (5 mm particle size silica, 22 cm × 4.6 mm i.d., Brownlee Columns, Applied Biosystems, San Jose, CA) equipped with a NewGuard™ RP-8 guard column, was used with a gradient elution MeOH-H_2O (25:75, starting; 30:70, 10 min; 45:55, 12 min; 50:50, 23 min; 75:25, 27 min; 25:75, 30 to 33 min), a flow rate of 2.0 ml/min, and detection at 220 nm. The retention times under these conditions for compounds **1**, **2**, and **4–7** are reported in Table 2. Gc-ms analysis was performed using a Finnigan Model 4500 instrument, having the gas chromatograph equipped with an Ultra-2 (Hewlett Packard, Palo Alto, CA) fused silica capillary column (50 m × 0.32 mm i.d.), in splitless mode injection with splitter closed for 0.8 min, at 15° (isothermic), and He as carrier gas (flow 40 cm/sec, measured at 150°). Nmr spectra were recorded on a Bruker AM 360 or on a Bruker AMX 500 spectrometer; for ^1H (360 or 500 MHz), δ values were referenced to $CHCl_3$ (7.24 ppm) and for ^{13}C (90.5 or 125.8 MHz) referenced to $CDCl_3$ (77.0 ppm). Ms was obtained on a VG 70-250 SEQ hybrid mass spectrometer or on a Finnigan Mat Model 4500 mass spectrometer (hrms, eims or cims with NH_3 as carrier gas), employing a solids probe in all cases.

FUNGAL CULTURES.—*P. lingam* avirulent isolates Unity (DAOM 194207), Peace-3 (DAOM 194209), and Peace-5 (DAOM 194210), and virulent isolate Leroy (DAOM 194208), were obtained from G.A. Petrie and R.K. Gugel (Agriculture Canada Research Station, Saskatoon, Saskatchewan). Voucher specimens were deposited in the culture collection of the Center for Land and Biological Resources (Ottawa, ON, Canada) and were assigned the strain designation numbers reported in brackets.

The fungal isolates were grown on V8 agar [20% (v/v) V8 juice, 0.75 g/liter $CaCO_3$, 100 mg/liter streptomycin sulfate, 40 mg/liter Rose Bengal, 15 g/liter agar] plates at 21°, 12 h photoperiod for 10 days.

TABLE 2. *Retention Time of Compounds* **1**, **2**, and **4–7** by Hplc Analysis.[a]

Compound	Retention time
1	25.3 min
2	6.5 min
4	12.9 min
5	6.0 min
6	21.6 min
7	7.5 min

[a]Compound **3** is not eluted under identical conditions. For hplc conditions see Experimental.

Spore suspensions of each isolate were prepared by overlaying the V8 agar plates with 10 ml sterile distilled H_2O, and the plate surfaces were rubbed with a flamed glass rod to dislodge the pycnidiospores (herein called spores). The suspension was transferred to centrifuge tubes, and the spores were pelleted by centrifugation at 3000 × *g* for 10 min. After washing once, the spores were stored in sterile distilled H_2O at −20°. Liquid cultures were initiated by inoculating fungal spores (final concentration 10^8 spores per ml) in minimal media supplemented with thiamine (16). Cultures were incubated at 25 ± 2° in a shaker at 150 rpm.

BRASSININ METABOLISM.—Liquid shake cultures (100 ml media in 250-ml Erlenmeyer flasks) inoculated with fungal spores of the isolates listed above and incubated for 48 h were used throughout for the metabolism studies. Control cultures of each isolate were grown separately. Solutions of brassinin [**1**] (final concentration 2×10^{-4} M and 5×10^{-4} M) in DMSO [final concentration 1% (v/v)] were administered to 48-h-old liquid cultures and to uninoculated media and incubated at 25 ± 2°. The head-space volatiles of 3- to 5-h-old cultures were withdrawn from the gas phase with a gas-tight syringe (1200 μl), immediately injected into the gas chromatograph, and analyzed by gc-eims. Samples (10 ml) were withdrawn at 3–24 h intervals up to 7 days and were either immediately frozen or filtered (glass wool plug) and extracted three times with a similar volume of Et_2O. The aqueous layer was re-extracted twice with 1% NH_4OH in $CHCl_3$. The extracts were dried over Na_2SO_4, evaporated at reduced pressure, and analyzed by tlc and hplc. Brassinin was stable in uninoculated control cultures up to 48 h; slight decomposition was observed up to 7 days.

Similar experiments were carried out separately with the following compounds substituted for brassinin: *N*-acetyl-3-indolylmethylamine [**2**] (4×10^{-4} M), 3-indolylmethylamine [**3**] (4×10^{-4} M), indole-3-carboxaldehyde [**4**] (2×10^{-4} M and 4×10^{-4} M), indole-3-carboxylic acid [**5**] (2×10^{-4} M and 4×10^{-4} M), methyl (3-indolylmethyl)dithiocarbamate S-oxide [**6**] (4×10^{-4} M), *N*-formyl-3-indolylmethylamine [**7**] (4×10^{-4} M), *N*-acetylbrassinin [**9**] (4×10^{-4} M), or *N*-methylbrassinin [**10**] (4×10^{-4} M). In each case, after the compounds were added, cultures were incubated and samples (10–50 ml) withdrawn at 3–24 h intervals, up to 5 days. As above, the withdrawn samples were either immediately frozen or filtered (glass wool plug) and extracted with Et_2O, followed by reextraction with 1% NH_4OH in $CHCl_3$. The extracts were analyzed by tlc, hplc, and/or ^1H nmr.

ISOLATION OF METABOLITES.—The isolation of metabolites resulting from the fungal metabolism of brassinin [**1**] was carried out by preparative tlc. Typically, the organic extract residues were dissolved in CH_2Cl_2 and applied on tlc plates (a maximum of 15 mg per plate). After development with CH_2Cl_2-MeOH (96:4) twice (brassinin R_f 0.84; acetylamine **2** R_f 0.26; aldehyde **4** R_f 0.47; acid **5** R_f 0.16, metabolite **6** R_f 0.24, formylamine **7** R_f 0.26) the uv-active bands were scraped from the plate and eluted with CH_2Cl_2-MeOH (90:10 for all compounds except a ratio of 70:30 was used for the acid). The compounds were characterized by spectroscopy, as stated in each case.

SYNTHESIS OF 3-INDOLYLMETHYLAMINE [**3**].—3-Indolylmethylamine [**3**] was synthesized using a modification of a literature procedure (21). Indole-3-carboxaldehyde was treated with hydroxylamine hydrochloride (22) to give a mixture of *syn*- and *anti*-oximes in 98% yield. The oxime mixture (557 mg, 3.48 mmol) was dissolved in MeOH (20 ml) and 1 M NaOH (70 ml), treated with Devarda's alloy (7.4 g, exothermic reaction!), and stirred for 20 min. The reaction mixture was diluted with H_2O (100 ml), filtered, and immediately extracted with Et_2O (3 × 200 ml). After evaporation of the solvent, the crude amine (474 mg, 3.25 mmol) was purified by flash cc [CH_2Cl_2-MeOH-NH_4OH (80:20:1)] and crystallized (CH_2Cl_2/hexane) to yield amine **3** as white powdery crystals (86% yield from the aldehyde): R_f 0.28 [CH_2Cl_2-MeOH-NH_4OH (80:20:1)]; ^1H nmr (CD_3OD) δ 7.59 (dd, J = 8.0, 1 Hz, 1H), 7.34 (dd, J = 8.0 1 Hz, 1H), 7.20 (s, H-2), 7.10 (ddd, J = 8.0, 7.0, 1 Hz, 1H), 7.02 (ddd, J = 8.0, 7.0, 1 Hz,

1H), 4.01 (s, CH_2); ^{13}C nmr [$(CD_3)_2CO$] δ 137.68 (s), 128.15 (s), 123.08 (d), 121.98 (d), 119.79 (d), 119.24 (d), 115.66 (s), 111.95 (d), 47.67 (t); eims *m/z* (rel. int.) $[M]^+$ 146 (100), $[M-16]^+$ 130 (50), 118 (42).

SYNTHESIS OF *N*-ACETYL-3-INDOLYLMETHYLAMINE [**2**].—Ac_2O (100 μl, 1.1 mmol) was added to a stirred solution of 3-indolylmethylamine (130 mg, 0.89 mmol) in CH_2Cl_2 (4 ml), at room temperature. After 10 min the reaction mixture was diluted with toluene (5 ml), and the solvent was evaporated under reduced pressure. The crude product was crystallized (CH_2Cl_2) to give compound **2** as white needles (73%): R_f 0.21 [CH_2Cl_2-MeOH (96:4)]; ^1H nmr (CD_2Cl_2) δ 8.37 (br s, D_2O exchangeable), 7.62 (d, $J=7.8$ Hz, 1H), 7.40 (d, $J=7.2$ Hz, 1H), 7.20 (ddd, $J=7.2$, 7.2, 1 Hz, 1H), 7.18 (br s, H-2), 7.12 (ddd, $J=7.0$, 7.0, 1 Hz, 1H), 5.73 (br s, D_2O exchangeable), 4.57 (d, $J=4.7$ Hz, CH_2), 1.94 (s, Me); ^{13}C nmr ($CDCl_3$) δ 169.79 (s), 136.39 (s), 126.49 (s), 123.14 (d), 122.46 (d), 119.88 (d), 118.72 (d), 112.70 (s), 111.29 (d), 35.20 (t), 23.15 (q); eims *m/z* (rel. int.) $[M]^+$ 188 (60), $[M-Ac]^+$ 145 (51), 130 (100); cims *m/z* (rel. int.) $[M+NH_4]^+$ 206 (40), $[M+H]^+$ 189 (100), 130 (84).,

SYNTHESIS OF *N*-FORMYL-3-INDOLYLMETHYLAMINE [**7**].—Methyl formate (200 μl) was added to a stirred solution of 3-indolylmethylamine (20 mg, 0.14 mmol) in CH_2Cl_2 (500 μl), at 50°. After 2 h the solvent of the reaction mixture was evaporated under reduced pressure to yield a brown residue which was purified by preparative tlc [CH_2Cl_2-MeOH (96:4)] and crystallized (CH_2Cl_2) to give compound **7** as white powdery crystals in 70% yield: R_f 0.21 [CH_2Cl_2-MeOH (96:4)]; ^1H nmr ($CDCl_3$) δ 8.22 (br s, HCO), 8.11 (br s, D_2O exchangeable), 7.62 (dd, $J=7.8$, 1 Hz, 1H), 7.37 (dd, $J=7.9$, 1 Hz, 1H), 7.22 (ddd, $J=8.1$, 7.9, 1 Hz, 1H), 7.16 (br s, H-2), 7.14 (ddd, $J=8.0$, 7.9, 1 Hz, 1H), 5.65 (br s, D_2O exchangeable), 4.66 (d, $J=1$ Hz, CH_2), resonances of a minor conformer at δ 8.29 (br s, HCO), 7.55 (d, $J=7.9$ Hz), 7.30 (d, $J=8.0$ Hz), 4.57 (d, $J=1$ Hz, CH_2); ^{13}C nmr ($CDCl_3$) δ 160.83 (s), 123.22 (d), 122.81 (s), 122.65 (d), 120.16 (s), 120.05 (d), 118.73 (d), 118.37 (s), 111.36 (d), 33.72 (t); cims *m/z* (rel. int.) $[M+NH_4]^+$ 192 (68), $[M+H]^+$ 175 (38), 130 (100).

SYNTHESIS OF *N*-ACETYLBRASSININ [**9**].—Acetyl chloride (30 μl in 200 μl CH_2Cl_2) was added to a stirred solution of brassinin (37 mg, 0.16 mmol) in CH_2Cl_2 (2 ml), at 0°. After 5 min the reaction mixture was diluted with toluene (1 ml), and the solvent was evaporated under reduced pressure. The crude product was purified by flash cc (CH_2Cl_2) to give compound **9** in 89% yield: R_f 0.30 (CH_2Cl_2); ^1H nmr (CD_2Cl_2) δ 8.23 (br s, D_2O exchangeable), 7.63 (d, $J=7.8$ Hz, 1H), 7.38 (d, $J=8.1$ Hz, 1H), 7.20 (dd, $J=8.1$, 7.1 Hz, 1H), 7.14 (br s, H-2), 7.12 (dd, $J=8.1$, 7.0 Hz, 1H), 5.68 (br s, CH_2), 2.62 (s, SMe), 2.41 (s, Ac); ^{13}C nmr ($CDCl_3$) δ 210.43 (s), 171.69 (s), 136.10 (s), 126.36 (s), 123.33 (d), 122.46 (d), 120.00 (d), 116.90 (d), 116.74 (s), 111.26 (d), 44.44 (t), 25.25 (q), 22.17 (q); cims *m/z* (rel. int.) $[M+NH_4]^+$ 296 (4), $[M+H]^+$ 279 (10), 237 (7), 130 (100).

SYNTHESIS OF *N*-METHYLBRASSININ [**10**].—*N*-Methylbrassinin was prepared similarly to brassinin (17) but using *N*-methyl-3-indolylmethylamine (23) instead of amine **3**. After purification by flash cc (CH_2Cl_2), *N*-methylbrassinin was obtained in 20% yield: R_f 0.60 (CH_2Cl_2); ^1H nmr ($CDCl_3$) δ 8.14 (br s, D_2O exchangeable), 7.62 (br m, 1H), 7.36 (d, $J=8.1$ Hz, 1H), 7.28 (br s, 1H), 7.21 (br m, 1H), 7.13 (br m, 1H), 5.52 (br s, CH_2), 3.23 (br s, Me), 2.70 (s, SMe), signals of a minor conformer at δ 5.13 (br s, CH_2), 3.47 (br s, Me); eims *m/z* (rel. int.) $[M]^+$ 250 (14), 130 (100); cims *m/z* (rel. int.) $[M+H]^+$ 251 (100), 130 (70).

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