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DETOXIFICATION OF THE PHYTOALEXINS MAACKIAIN AND MEDICARPIN BY FUNGAL PATHOGENS OF ALFALFA

SCOTT SOBY,* SRIYANI CALDERA,† ROBERT BATES† and HANS VANETTEN

Departments of Plant Pathology and †Chemistry, University of Arizona, Tucson, AZ 85721, U.S.A.

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Key Word Index—*Cercospora medicaginis*; *Colletotrichum* spp; *Medicago sativa*; *Nectria haematococca*; alfalfa; pterocarpan; phytoalexin detoxification.

Abstract—Nine fungal pathogens of alfalfa (*Medicago sativa*) (lucerne) were assayed for their ability to metabolize the pterocarpanoid phytoalexins (−)maackiain and (−)medicarpin. All of the alfalfa fungal isolates were able to metabolize both (−)maackiain and (−)medicarpin. Six different initial reaction products were observed, and often a single isolate produced multiple metabolic products. All the products have been previously described, except for those produced by *Cercospora medicaginis*. This fungus hydroxylated the pterocarpans at the 1a carbon to form a 1a[*R*]OH-dienone, in which the hydroxyl group is *trans* to the bridgehead protons, and is the 1a epimer of the previously described *cis* form of the compound. (−)Maackiain and both of the 1a hydroxylated epimers were tested for toxicity on isolates of *Nectria haematococca* mating population I and *Saccharomyces cerevisiae* that do not degrade maackiain or its 1aOH-dienone products. Both epimers were less toxic than maackiain, and the *trans* epimer was less toxic than the *cis* form.

INTRODUCTION

Many plants produce low molecular weight antibiotic compounds, known collectively as phytoalexins, in response to invasion and infection by microbes [1]. It has been proposed that the production of these compounds provides the plant with an active mechanism of broad-spectrum defence against both fungi and bacteria [1, 2]. Many phytopathogenic fungi are able to metabolize phytoalexins to less toxic compounds, suggesting that detoxification provides one way to overcome this chemical defence [3].

Red clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L.) produce the pterocarpan phytoalexins (−)maackiain (**1a**) and (−)medicarpin (**1b**), respectively (Fig. 1). These compounds are structurally similar, are often produced in the same plant species, and have been proposed to be substrates for identical fungal metabolic detoxification pathways [3]. Previous work has shown that (−)maackiain (**1a**) and (−)medicarpin (**1b**) are toxic to several genera of fungal pathogens of legume and non legume hosts [4–7]. A number of species of fungi are able to metabolize (−)maackiain and (−)medicarpin by hydroxylation of the 6a [8, 9] or 1a carbons [8, 10], or opening of the dihydrofuran ring ('C') to form isoflavans [8, 10–13] (Fig. 1). In the present study we examined a group of alfalfa foliar pathogens, including three species of *Colletotrichum* that are involved in the alfalfa anthracnose disease complex [14], and the root and crown pathogens *Verticillium albo-atrum* and *Fusarium oxysporum* f.sp. *medicaginis* for their ability to metabolize (−)medicarpin and (−)maackiain. We also describe the novel detoxification of these phytoalexins to a previously unreported epimer of the 1a-hydroxy-dienone by the Summer Black Stem pathogen *Cercospora medicaginis*.

*Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

Metabolism of (−)*maackiain* **1a** *and* (−)*medicarpin* **1b** *by alfalfa-pathogenic fungi*

(−)Maackiain (**1a**) can be readily and abundantly purified from the roots of mature red clover plants, and was thus used in initial studies to determine the parameters for fungal metabolism. (−)Medicarpin (**1b**) was subsequently used as substrate to test if it is metabolized in the same way as **1a**. With the exception of a tomato isolate of *V. albo-atrum*, which did not metabolize **1a**, and *Cercospora medicaginis*, which made unknown products from both **1a** and **1b**, all the alfalfa pathogens were able to metabolize **1a** and **1b** to metabolic products that could be identified by comparison with spectra of known compounds or with published spectral data [8]. A summary of the fungal metabolites produced is presented in Table 1.

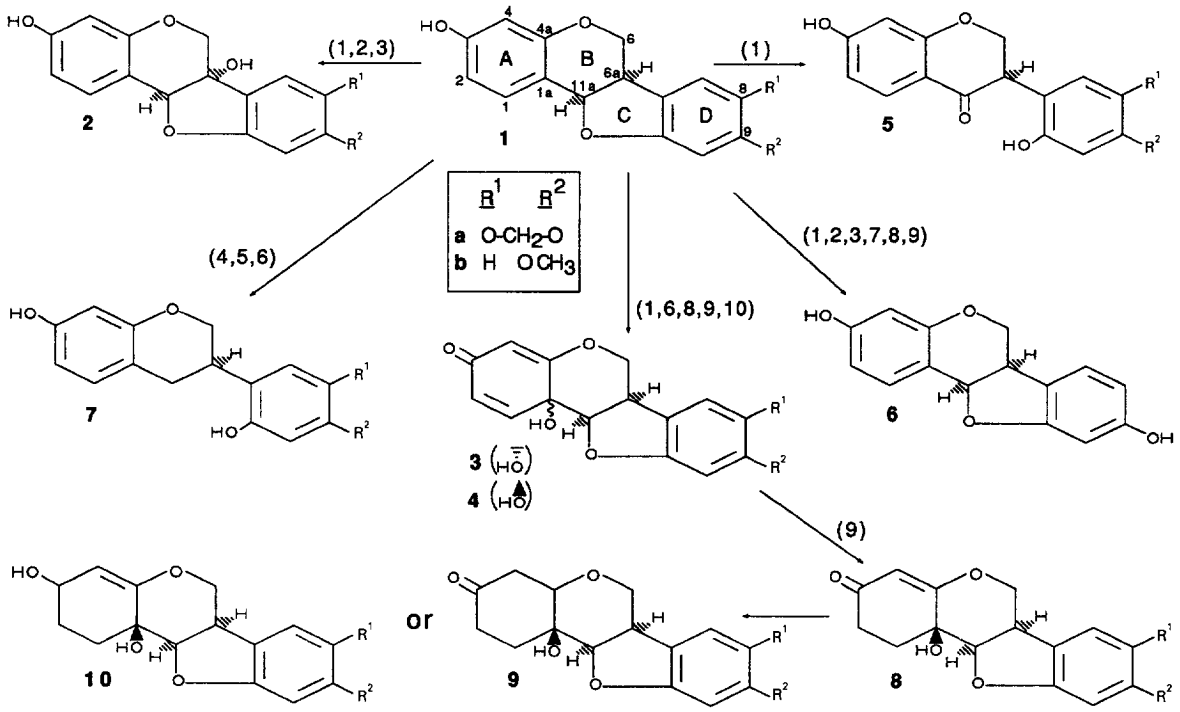


Fig. 1. Fungal metabolic products of (−)maackiain and (−)medicarpin. **a**, $R^1 + R^2 = OCH_2O$; **b**, $R^1 = H$, $R^2 = OCH_3$. **1a**, maackiain; **1b**, medicarpin; **2a**, 6a-OH-maackiain; **2b**, 6a-OH-medicarpin; **3a**, 1a[*S*]OH-maackiain; **3b**, 1a[*S*]OH-medicarpin; **4a**, 1a[*R*]OH-maackiain; **4b**, 1a [*R*]OH-medicarpin; **5a**, sophorol; **5b**, vestitone; **6**, demethylmedicarpin; **7a**, dihydromaackiain; **7b**, vestitol. Numbers beside the arrows indicate the fungal isolates in Table 1 that use the indicated pathway. The proposed pathway for *Cercospora medicaginis* (pathway 9) detoxification of maackiain (**1a**) is shown (**1a** → **4a** → **8a** → **9a** or **10a**).

Three species of *Colletotrichum* are associated with the anthracnose disease complex of alfalfa; *C. trifolii* is the primary pathogen in the formation of leaf and stem lesions, and can be readily isolated from lesions early in the course of disease. There are three races of *C. trifolii*, which correspond to genetic resistance in cultivars of the host plant. *C. dematium* f. *truncatum* and *C. destructivum* do not appear to be involved in primary pathogenesis, but can be isolated from lesions late in the course of the disease [14]. All three species hydroxylated **1a** and **1b** at the 6a position (Fig. 1, **2**), but only *C. trifolii* was additionally able to cause C-ring fission to produce the isoflavanones sophorol (**5a**) or vestitone (**5b**) and/or hydroxylation at the 1a position, resulting in the dienones **3a** and **3b** (Fig. 1). All these reactions result in less toxic compounds [7]. An additional compound produced by *C. destructivum* was detected by the conversion of 4-[^{3}H]-(−)maackiain to an unknown product with an absorbance ratio (λ_{max}^{EtOH} = 278:309) of 3:2 and a polarity greater than any of the known products (data not shown), and thus is unlikely to be the result of one enzymatic modification. Since the only first-step metabolic products of **1a** and **1b** in *C. destructivum* are the 6a-OH products **2**, this unknown product may be the result of further metabolism of the 6a-OH compounds **2**. While not all isolates of *C. trifolii* produced all possible metabolic products, it is apparent that it employs multiple metabolic pathways and that the ability to metabolize pterocarpans is independent of its race structure. These three species of *Colletotrichum* are also able to degrade medicarpin (**1b**) by demethylation of the methoxy group at carbon 9. The resulting product, demethylmedicarpin **6**, has been shown by a number of different assays to be less toxic than **1b** [15]. Demethylmedicarpin **6** has been reported to be a metabolic product of other species of *Colletotrichum* [9, 15]. Thus, species of *Colletotrichum* employ several independent methods of pterocarpan detoxification.

Stemphylium alfalfae, *Phoma medicaginis* and *Leptosphaerulina briosiana* cause disease in the stems and leaves of the host. They produce the isoflavan dihydromaackiain (**7a**) from (−)maackiain (**1a**) and *S. alfalfae* produces vestitol (**7b**) from (−)medicarpin (**1b**). These isoflavans have been shown to be almost as fungitoxic as the parent compounds [16, 17], but **7** may be converted to other compounds *in planta* either by these fungi, other associated organisms, or by the host, as interconversion of isoflavans and pterocarpans has been reported in plants [3]. In the *in vitro* assays employed here, there did not appear to be a rapid degradation of **7**. Unlike *S. alfalfae* and *P. medicaginis*, which produce only the isoflavans (**7**), *L. briosiana* was also able to produce the 1a hydroxydienone derivative **3a**, which is less toxic than the parent compound.

Table 1. Initial metabolites of (−)maackiain (**1a**) and (−)medicarpin (**1b**) produced by fungal alfalfa pathogens

Species	Isolate	Metabolic products			
		Maackiain	Medicarpin	Host/Source	
(1)	*Colletotrichum trifolii*				
	T-456	**2a,3a,5a**	**2b,3b,5b,6**	Red clover	K. L. Leath
	ARNLW(race 3)	**2a,3a,5a**	**2b,3b,5b,6**	Alfalfa	M. Dickman
	SB-2(race 2)	**2a,3a,5a**	n.t.	Alfalfa	N. O'Neill
	3-5(race 1)	**2a,3a,5a**	n.t.	Alfalfa	N. O'Neill
	2sp2(race 1)	**3a,5a**	n.t.	Alfalfa	N. O'Neill
	SB-1(race 1)	**3a,5a**	n.t.	Alfalfa	N. O'Neill
(2)	*C. dematium*				
	T-545	**2a**	**2b,6**	Alfalfa	M. Dickman
(3)	*C. destructivum*				
	385	**2a**	**2b,6**	Alfalfa	M. Dickman
(4)	*Stemphylium alfalfae*				
	KS1	**7a**	**7b**	Alfalfa	D. Studeville
(5)	*Phoma medicaginis*				
	T-430	**7a**	n.t.	Alfalfa	K. L. Leath
(6)	*Leptosphaerulina briosiana*				
	T-452	**3a**	**3b**	T-451	Cornell U.
	T-451	**3a,7a**	n.t.	Alfalfa	Cornell U.
(7)	*Verticillium albo-atrum*				
	T-513	N.D.	**6**	Tomato	V. Higgins
(8)	PLY-4	**3a**	**3b,6**	Alfalfa	F. Gray
(9)	*Cercospora medicaginis*				
	SS1	**4a**	**4b,6**	Alfalfa	This work
	DS1	**4a**	n.t.	Alfalfa	D. Studeville
(10)	*Fusarium oxysporum* f.sp. *medicaginis*				
	P/2	**3a**	**3b**	Alfalfa	ATCC

n.t., not tested.
Cornell U., teaching collection, sector from T-451.
N.D., none detected.

Isolates of the root and crown pathogens *F. oxysporum* f.sp. *medicaginis* and *V. albo-atrum* obtained from alfalfa were able to detoxify **1a** and **1b** by formation of the 1a hydroxydienones **3a** and **3b** (Fig. 1), but a *V. albo-atrum* isolate from tomato was unable to metabolize these phytoalexins. Both alfalfa and tomato isolates of *V. albo-atrum* were able to demethylate medicarpin to form **6**, but *F. oxysporum* f.sp. *medicaginis* was not.

Identification of metabolites of (−)*maackiain* (**1a**) *and* (−)*medicarpin* (**1b**)*produced by* Cercospora medicaginis

Cercospora medicaginis, a leaf-spotting fungus, grows in liquid medium as discrete mycelial spheres. (−)Maackiain (**1a**) added to 2-week old (mature) spheres (*ca* 0.5 cm diameter), was not metabolized. However, after the mycelial spheres mature (*ca* 0.5–1.0 cm dia.), they produce a large number of propagules that rapidly germinate to form individual hyphae. When these 'germlings' were decanted away from the mature spheres and **1a** added to the decantate, the substrate was readily metabolized (Fig. 2). Recovery of **1a** but no metabolites after 3 days incubation from cultures containing mycelial spheres and germlings, and lack of metabolic activity of growth medium from which actively growing mycelium had been removed by filtration, suggest that **1a** is sequestered from the detoxifying enzymes present either in the germlings or sphere mycelium by the mature spheres. In a culture containing an equal number of germlings in the absence of mycelial spheres, 2 mg of (−)maackiain is completely metabolized by 22 hr (data not shown). *C. medicaginis* produced three metabolic products that did not have HPLC retention times characteristic of previously identified compounds. Studies using tritium-labelled **1a** verified that these compounds are derived from (−)maackiain (**1a**) (Fig. 2). GC-mass spectra of the least polar compound indicated that the metabolite had an increase of 16 M_r an EI-mass spectrometry fragmentation spectrum identical to the previously identified 1aOH-dienone **3a**, but a slightly longer GC retention time (see experimental, [8]). The UV spectra and decomposition in base of **3a** and this unknown were also identical (data not shown). However, the consistent differences in GC and HPLC retention times indicated that the product was not **3a**, being slightly less polar than **3a**.

Comparison of the NMR spectral data (Table 2) indicated that **3a** and the least polar unknown, whose structure we propose to be **4a**, Fig. 1, are stereoisomers

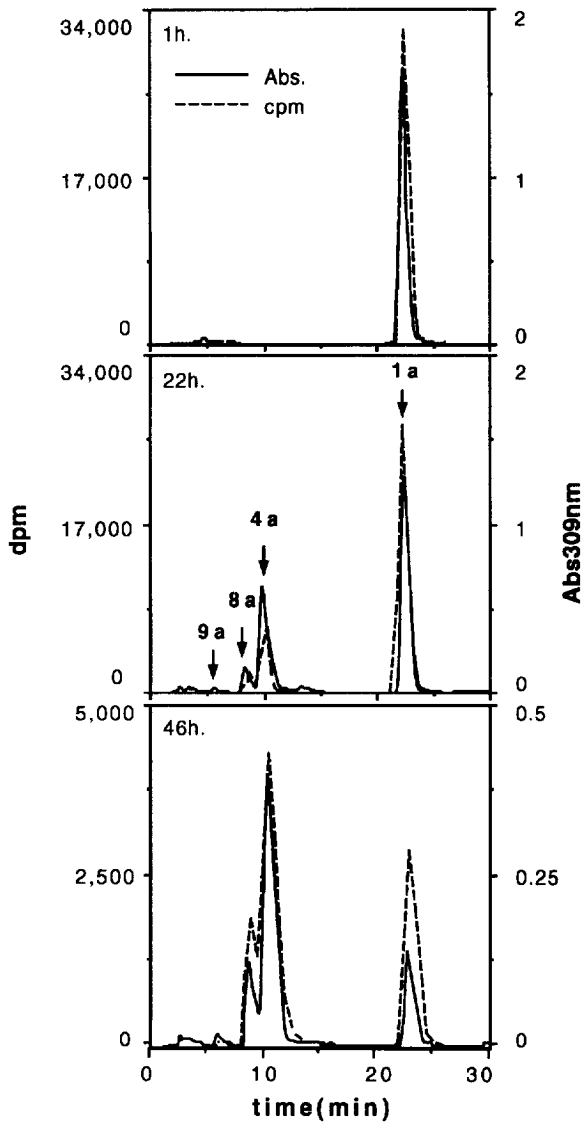
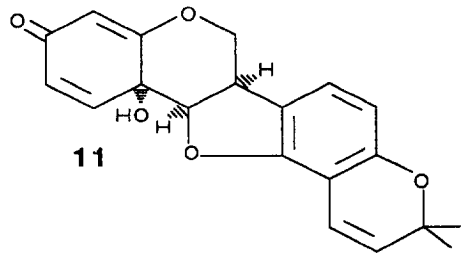


Fig. 2. HPLC separation of metabolic products of *C. medicaginis*. Metabolic products of 4-[^{3}H]-maackiain produced by incubation in liquid culture of *C. medicaginis* were collected and extracted at 1, 22 and 46 hr and separated by reversed-phase HPLC. A series of 30-s fractions were collected, the cpm were determined for each fraction and plotted with absorbance at 309 nm (λ_{max} for (−)maackiain (**1a**) and its metabolic products) against time of elution.

differing in configuration at C-1a, with **3a** being stereochemically related to 1a-hydroxyphaseollone **11** [18] (note the similarity of chemical shifts for C-1, C-2, C-4, C-6α, C-6β, and C-11a and $J_{6\beta,6a}$ in **3a** and **11**). The 9.8–10.0 Hz values for $J_{1,2}$ in **3a** [8], **4a** and **11** are indicative of dienone rather than aromatic A-rings. The configurations at C-1a were assigned from the widely differing $J_{6\beta,6a}$ values (11.0 Hz in **4a** and **1a**; *ca* 0 Hz in **3a** and **11**) combined with molecular mechanics calculations which suggested a flatter maackiain-like conformation for **4a** and a more bent conformation (in which ring B has



flipped to the other chair conformation) for **3a** and **11**. Thus $J_{6\beta,6a}$ is large in **1a** and **4a** because it is axial–axial, and small in **3a** and **11** because it is not. This necessitates revising the stereochemistry tentatively assigned to 1a-hydroxyphaseollone [18] to that shown in **11**. We propose that **4a** produced by *C. medicaginis* be known as 1a[*R*]OH-(−)maackiain, and **3a** [8] be known as 1a[*S*]OH-(−)maackiain, in accordance with accepted pterocarpan nomenclature [19]. While several fungi produce the 1a hydroxydienones of pterocarpans [3, 8, 10], *C. medicaginis* is the first fungus known to produce the **4a** epimer. In the absence of NMR data or comparative HPLC studies we could not determine if the 1aOH dienone produced by *Ascochyta rabiei* [10] is an *S* or *R* epimer.

The proposed *C. medicaginis* metabolic pathway for (−)maackiain (**1a**) and its further detoxification products (**1a** → **4a** → **8a** → **9a** or **10a**) is presented in Fig. 1. Structures were assigned to **4a** and **8a** based on HPLC, GC-mass spectrometry, NMR (summarized in Table 2) and UV spectral data, and **9a** and **10a** were assigned based on HPLC and GC-mass spectral data. The similarity of B-ring NMR parameters for **4a** and **8a** shows that **8a** has the same B-ring conformation as **1a** and **4a** and is consistent with **8a** being the 1,2-double bond reduction product of **4a**. The most polar metabolic product has not been purified. However, GC-mass spectral data indicate that this compound has a molecular weight of 304 and that the C and D rings have not been altered, thus it may have one of the two proposed structures **9a** or **10a** in Fig. 1. GC-mass spectral data also indicate that *C. trifolii* further metabolized 1a[*S*]OH-medicarpin **3b** in the same way—that is, reduction of the 1–2 double bond, followed by reduction of the 4–4a double bond. However, our time-course studies suggest that other fungi producing the 1a-hydroxydienone are apparently incapable of further metabolism of this product. Incubation of *C. medicaginis* with (−)medicarpin (**1b**) apparently results in metabolism to **4b** → **8b** → **9b** or **10b**, as indicated by GC-mass spectrometry. *C. medicaginis* was also able to demethylate **1b** to form demethylmedicarpin (**6**).

All of the fungal pathogens isolated from alfalfa were able to metabolize **1a** and **1b**, usually by multiple routes. The pterocarpan phaseollin is likewise subject to multiple routes of fungal metabolism [3]. This contrasts markedly with the single metabolic path used by fungi to detoxify the pterocarpanoid phytoalexin pisatin, which has only been observed to be detoxified by C-3 *O*-demethylation (→ **2a**).

Table 2. ^{1}H NMR ($CDCl_3$/TMS, 500 MHz) chemical shifts (δ) and coupling constants (J, in Hz) for (−)maackiain **1a** and some pterocarpan metabolites

δ	**1a**	**4a**	**8a**	**3a**	**11***
1	7.37	6.84	2.07, 2.31	6.61	6.69
2	6.55	6.17	2.31, 2.75	6.03	6.09
4	6.41	5.82	5.58	5.44	5.48
7	6.72	6.58	6.50	6.56	—
10	6.44	6.51	6.41	6.17	—
OCH_2O	5.89, 5.92	5.94, 5.96	5.86, 5.88	5.82, 5.83	—
6α	4.22	4.47	4.33	5.04	5.08
6β	3.64	4.75	4.33	4.23	4.33
6a	3.47	3.87	3.87	3.86	3.91
11a	5.47	4.78	4.71	5.06	5.14
Coupling constants					
$J_{1,2}$	8.3	9.9	†	9.8	10
$J_{2,4}$	2.4	1.8	†	1.8	2
$J_{6\alpha,6\beta}$	11.1	11.0	†	11.0	10
$J_{6\alpha,6a}$	5.1 (3.4)‡	7.2 (6.0)	7.8	3.2 (4.5)	4
$J_{6\beta,6a}$	11.0 (10.9)	11.0 (8.7)	10.9	*ca.* (1.3)	*ca.* 0
$J_{6a,11a}$	6.9 (6.9)	10.8 (9.1)	10.1	10.1 (9.1)	10
J_{OCH_2O}	1.0	1.1	1.2	1.3	—

*Data are from [18].

†Not clear in spectrum.

‡Values (in parentheses) for minimum energy conformation were determined by the Karplus equation using the PCMODEL software package.

Toxicity of 1a[*S*]*OH-maackiain* (**3a**) *and* 1a[*R*]*OH-maackiain* (**4a**)

The antimicrobial activities of **1a**, **3a** and **4a** were determined using an isolate of *Nectria haematococca* mating population I (MPI) and a haploid strain of *S. cerevisiae*. Neither isolate was able to metabolize maackiain. Over four experiments, the mean concentration required to inhibit colony growth of *N. haematococca* MPI by 50% (ED_{50}) for **1a** was 16.7 μg/ml (s.d. = 4.1), for 1a[*S*]OH-maackiain (**3a**) it was 44 μg ml^{-1} (s.d. = 1.4), and for 1a[*R*]OH-maackiain **4a** it was 79.3 μg ml^{-1} (s.d. = 4.7). Thus the *S* epimer **3a** was about one-third as toxic as the parent compound **1a**, and the toxicity of the *R* epimer **4a** was about one-fifth that of **1a**.

S. cerevisiae was completely inhibited on YEPD agar media supplemented with 40 μg ml^{-1} of **1a**. There was no apparent inhibition at up to 86 μg ml^{-1} of **3a** and 150 μg ml^{-1} of **4a**, the maximum concentrations tested. Metabolites **3a** and **4a** are considerably less toxic to both *N. haematococca* MPI and *S. cerevisiae* than the parent compound. The reduced toxicity of **4a** relative to **3a** may also be due to the B-ring conformation of **4a** which results in a more planar molecule than either its 1aOH epimer **3a** or the parent compound **1a**.

EXPERIMENTAL

Chemicals. **1a** was obtained from the roots of mature red clover plants as previously described [4, 20], and dissolved in dehydrated EtOH at a concn of 4 mg ml^{-1} for use as a stock for all assays. **1b** was kindly provided by the Samuel Roberts Noble Foundation, Ardmore, OK, U.S.A. 4-[^{3}H]-Maackiain (4.1 Ci mol^{-1}) was prepared by solvent exchange (G. DiCenzo, method to be published elsewhere). Samples for NMR were obtained by incubating 4 mg of **1a** overnight in *C. medicaginis* liquid culture. The culture medium was extracted 2 × with equal volumes EtOAc, evapd and redissolved in 2 ml EtOH for HPLC purification using a C-18 reversed phase preparative column (250 × 22.5 mm) with a 30–90% acetonitrile gradient as the mobile phase. Frs containing the unknown compounds were extracted 2 × with equal volumes EtOAc, EtOAc was removed under reduced pressure, and resuspended in ^{2}H-$CHCl_3$. Approximately 75% of **1a** was converted to **4a**, 20% to **8a** and the remainder to **9a**/**10a** (see Fig. 2 for example).

Fungal cultures. Colletotrichum trifolii, Colletotrichum dematium, f. *truncatum, Colletotrichum destructivum, Stemphylium alfalfae, Phoma medicaginis*, var. *medicaginis, Cercospora medicaginis, Nectria haematococca*, mating population (MP) VI isolate 156-30-6, *Leptosphaerulina briosiana, Verticillium albo-atrum*, and *Fusarium oxysporum*, f.sp. *medicaginis*, were maintained on V-8 juice agar slants [21]. *Nectria haematococca* MPI isolate T-488 was maintained on Martin's peptone-glucose agar (M-2) [4] *S. cerevisiae* haploid strain AAY1048 was maintained on YEPD agar (1% yeast extract, 2% bacto-peptone, 2% dextrose, 2% agar). *Cercospora*

medicaginis was isolated from foliar lesions from alfalfa plants collected near Manhattan, Kansas.

Metabolism assays. Fungal cultures for metabolic studies were inoculated into glucose–asparagine liquid medium (GA) from fresh V-8 agar slant cultures and grown with shaking at 180 RPM at room temp. Propagules from non-sporulating cultures were produced by grinding mycelium harvested from V-8 agar plates. 2 mg (−)Maackiain (**1a**) from a 4 mg ml^{-1} EtOH stock solution was added to 100 ml actively growing cultures. Control cultures received a comparable amount of EtOH solvent. Aliquots, 30 ml, of the culture were taken at 4, 12 and 22 hr and extracted 2 × with equal volumes EtOAc. For (−)medicarpin assays, **1b** was added to a 10 ml aliquot of culture prepared as described above at a concentration of 10 μg ml^{-1} and the entire culture extracted as above at the time point indicated by the **1a** experiments as the optimal time for detection of metabolic products (typically 12 or 22 hr). The EtOAc extract was then evapd under reduced pressure and redissolved in EtOH for HPLC, UV or GC-MS. In order to confirm that previously unidentified compounds were products of pterocarpan metabolism, 4-[^{3}H]-maackiain diluted to a final specific activity of 280 mCi mol^{-1} (10 μg ml^{-1} final concentration (−)maackiain) was added to 5 ml culture and extracted as above. Cpm were determined by collecting 30 s HPLC fractions and then correlated to time of HPLC elution.

Chromatography. Samples were applied to silica gel TLC and resolved with toluene–EtOAc 3 : 2 or separated by HPLC reversed-phase chromatography on a C-18 column with a 34–100% acetonitrile gradient as the mobile phase. Absorbance was measured at 309 and 278 nm for **1a** and its metabolites, and at 304 and 287 nm for **1b** and its metabolites.

Spectroscopy. Individual fluorescent bands from TLC or HPLC absorbance peak fractions were collected and the samples scanned for UV asorbance (λ = 240–360 nm). The presence of previously identified compounds [8] was confirmed and the molecular ion and fragmentation patterns of unknown compounds were determined by GC-EI mass spectroscopy using split injection into a crosslinked 5% phenylmethyl silicone column with helium carrier gas at 70 to 300° (2° min^{-1}) (70 eV). **1a**, ret. time 15.02 min: *m/z* (rel. int.) 284 [M]$^+$ (100), 283 (29), 267 (14), 197 (6), 175 (8), 162 (16), 151 (10), 147 (9), 134 (16), 115 (6). **1b**, ret. time 14.16 min: *m/z* (rel. int.) 270 [M]$^+$ (100), 255 (40), 253 (7.9), 148 (21), 147 (12), 137 (7.5), 135 (7.3), 134 (5.4), 133 (5.3), 91 (3.3). **3a**, ret. time 13.65 min.; **3b**, ret. time 15.00 min., EIMS see [8]. **4a**, ret. time 13.79 min: *m/z* (rel. int.) 300 [M]$^+$ (100), 218 (22), 176 (30), 175 (68), 163 (16), 162 (21), 161 (8), 133 (13), 69 (12), 54 (17), 53 (20). **4b**, ret. time 15.08 min: *m/z* (rel. int..) 286 [M]$^+$ (17), 270 (8), 163 (11), 162 (100), 161 (77), 148 (20), 147 (10), 137 (11), 133 (18), 108 (18), 91 (7). **8a**, ret. time 16.53 min: *m/z* (rel. int.) 302 [M]$^+$ (74), 176 (52), 175 (100), 164 (17), 163 (27), 162 (17), 151 (12), 133 (23), 93 (9), 69 (24), 53 (23), **8b**, ret. time 15.32 min: *m/z* (rel. int.) 288 [M]$^+$ (47), 164 (13), 163 (7), 162 (33), 161 (100), 149 (16), 133 (17), 121 (12), 91 (9). **9a** or **10a**, ret. time 15.18 min: *m/z* (rel. int.) 304 [M]$^+$ (100), 258 (7), 218 (7), 188 (16), 176 (16), 175 (32). 163 (46), 162 (9), 167 (6), 147 (6), 133 (12). **9b** or **10b**, ret. time 13.77 *m. m/z* (rel. int.) 290 [M]$^+$ (17), 163 (15), 162 (100), 148 (59), 121 (28), 91 (8).

Toxicity studies. The relative toxicities of maackiain (**1a**), 1a[*S*]OH-maackiain (**3a**) and 1a[*R*]OH-maackiain (**4a**) were determined using a previously described assay [4]. Briefly, a 4 mm dia. plug of actively growing *N. haematococca* MPI was placed on the surface of 1 ml M2 medium in a 35 mm Petri plate containing various concns of the above compounds. The concn of the 1a[*R*]OH-maackiain epimer (**4a**) and the metabolite **8a** were assumed to be the same as **3a** (coefficient of extinction (Abs_{309}) = 0.1261 μmol ml^{-1}, unpublished data). Daily measurements of the mycelial front were made and per cent inhibition determined relative to growth on unamended medium. Toxicity of the three compounds was determined in *S. cerevisiae* by suspending a colony in water and spotting 10 μl (*ca* 10^4 cfu) of the suspension into the centre of a 35 mm Petri plate containing various concentrations of the test compounds. The plates were incubated at 32° for 24 hr and the treatments were scored as either allowing growth or not.

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