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OXIDATION OF THE PHYTOALEXIN MAACKIAIN TO 6,6a-DIHYDROXY-MAACKIAIN BY *COLLETOTRICHUM GLOEOSPORIOIDES*

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Key Word Index—*Glomerella cingulata*; isoflavonoid; pterocarpan; medicarpin; detoxification; *Stylosanthes* spp.; Leguminosae; anthracnose.

Abstract—Phytoalexins are low molecular weight antibiotic compounds produced by plants in response to infection by microbes. These antimicrobial compounds are thought to provide resistance to microbial invasion and colonization. (−)Maackiain and its pterocarpan relatives can be oxidized at a number of sites, including at the 6a carbon. A previously unknown metabolite was produced from (−)maackiain by the broad host-range pathogen *Colletotrichum gloeosporioides* (*Glomerella cingulata*). This unknown was identified by LC-MS-MS and NMR spectroscopy to be 6,6a-di-OH-maackiain (3,6,6a-trihydroxy-8,9-methylenedioxy-pterocarpan). It is produced by isolates that represent all four races and pathotypes of *C. gloeosporioides* isolated from the tropical forage legume *Stylosanthes* spp. We present evidence that the primary metabolite (−)6a-OH-maackiain is subsequently hydroxylated at carbon 6, a step resulting in a compound that is increased in polarity and decreased in toxicity relative to the parent compound and (−)6a-OH-maackiain. This further oxidation may be required for efficient excretion or carbon source scavenging.

INTRODUCTION

Many plants produce phytoalexins, which are fungitoxic, low molecular weight compounds synthesized *de novo* in response to microbial infection. It has been postulated that necrotrophic fungi that successfully attack these plants can do so because they have the ability to neutralize the toxicity of phytoalexins metabolically and/or by non-degradative tolerance [1–3]. A number of legume species produce pterocarpan derivatives as phytoalexins [8], and the ability to detoxify these phytoalexins has been characterized in detail among alfalfa pathogens [4] and a number of other pathogens of legumes [5–7].

Some fungal detoxification enzymes are able to metabolize more than one pterocarpan. For example, enzymes capable of acting on the phytoalexin (−)maackiain (**1**) are also capable of recognizing and metabolizing the closely related molecule (−)medicarpin (**4**) [4, 6]. Thus (−)maackiain (**1**), which can be readily and abundantly purified from red clover roots (*Trifolium pratense* L.), can be used as a model compound for detoxification of pterocarpans by pathogenic fungi. The hydroxylation of the 6a carbon of (−)maackiain (**1**) and (−)medicarpin (**4**) is a fungal detoxification reaction carried out by a large number of legume pathogens [4–6] and may be catalysed by a cytochrome P450 mono-oxygenase [9, 10].

Among the fungal parasites of legumes is the wide-host-range pathogen *Colletotrichum gloeosporioides* (*Glomerella cingulata*), the causal agent of anthracnose disease on the tropical forage legume *Stylosanthes* spp. [11]. *C. gloeosporioides* exists as two pathotypes with distinct disease phenotypes and host ranges. Type A is known to attack all species of *Stylosanthes* grown commercially, while type B is largely restricted to *S. guianensis* [11]. Differences in the complement of small chromosomes correlate with the pathotypes [12, 13].

We are interested in how pathogenic fungi attack plants, and specifically how they circumvent the toxic compounds produced by plants in response to invasion and infection. In the present work we describe a detoxification product of (−)maackiain (**1**) from cultures of *C. gloeosporioides*.

RESULTS AND DISCUSSION

Identification of (−)6,6a-*di*-OH-*maackiain*

C. gloeosporioides produced both (−)6a-OH-maackiain (**2**) and an unknown product when grown

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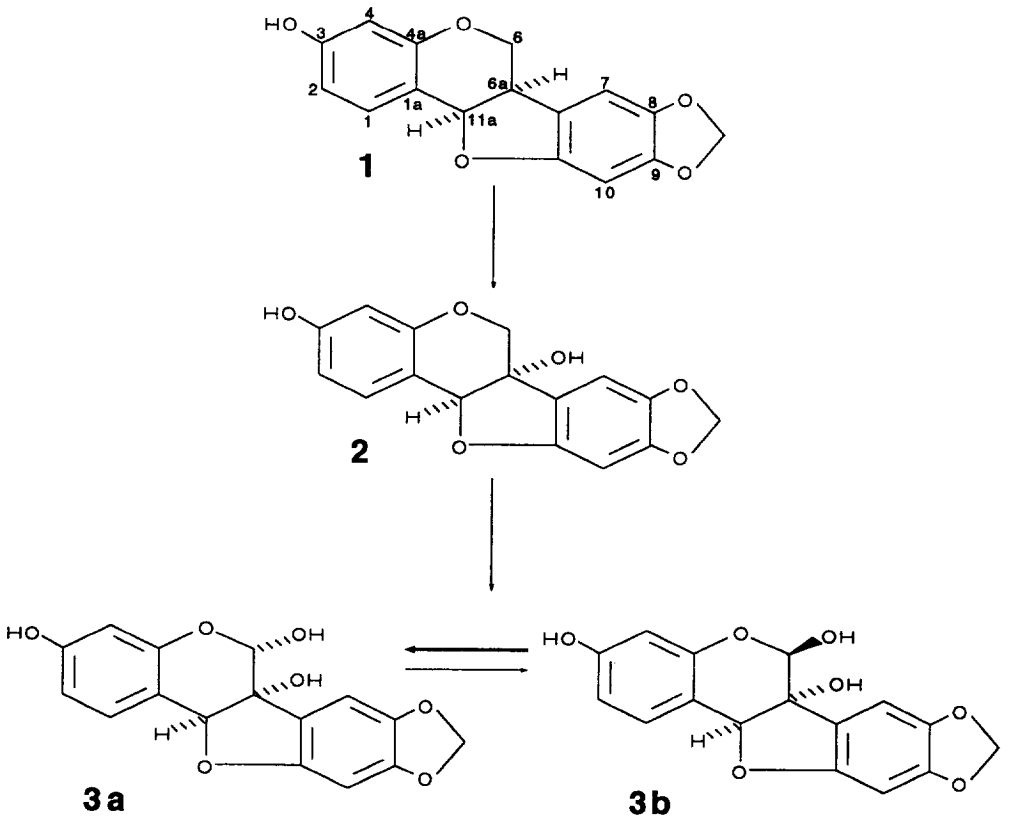


Fig. 1. Proposed pathway for detoxification of (−)maackiain in the *Stylosanthes* spp. pathogen *Colletotrichum gloeosporioides*. (−)Maackiain (**1**) is oxidized at the 6a position to form (−)6aOH-maackiain (**2**), which is then oxidized at the 6 position to form the stereoisomers of (−)6,6aOH-maackiain (**3a**) and (**3b**). **3a** and **3b** are predicted to exist in a 4:1 ratio by the PCMODEL molecular mechanics model with the hydroxyls *cis* at 6 and 6a (**3a**) being the more abundant form.

in the presence of sub-inhibitory concentrations of (−)maackiain (**1**). The unknown compound eluted from the reverse phase HPLC column before (−)6a-OH-maackiain (**2**) and (−)maackiain (**1**) suggesting an increase in polarity. The compound was purified by preparative reverse phase HPLC and found to have an UV spectrum identical to both (−)maackiain (**1**) and (−)6a-OH-maackiain (**2**) (see Experimental) [8].

The molecular weight and major fragmentation ions of the unknown compound were determined by direct inlet CI–mass spectrometry (see Experimental). A peak at *m/z* (rel. int.) 317 $[MH]^+$ (14) gave the molecular mass as 316, suggestive of the addition of two oxygens to (−)maackiain (**1**) (M_w 284). The base peak at 299 indicated the loss of water from M+1 species in which the ionizing proton had been attached to an alcoholic OH, and a peak at 315 (17) indicated the loss of H_2 from M+1 species in which the new hydrogen had been attached to any of the four ether oxygens. The other large peak, at 281 (42), suggested a loss of an additional water from 299.

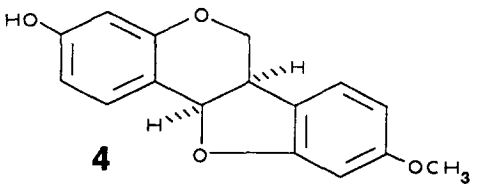
The ^1H NMR spectrum in $CDCl_3$ placed the two alcoholic hydroxyl groups at 6 and 6a, indicating that the structure of the unknown is (−)6,6a-di-OH-maackiain (**3**) (Fig. 1) ((−)3,6,6a-trihydroxy-8,9-methylenedioxy-pterocarpan) with a 4:1 ratio of tautomers **3a** and **3b** (**3a**, with *cis* hydroxyls, is presumed to predominate from molecular mechanics calculations as described below). The two alcoholic OH groups gave broad peaks at δ 2.85 and 3.85 in the predominant tautomer **3a**. Most of the NMR parameters of **3a** and **3b** (values in parentheses) were very close to those of (−)maackiain (**1**) [4] and (−)6a-OH-maackiain (**2**) [6]: H-1, δ 7.35 (7.32) *d*, J = 8.4 Hz; H-2, δ 6.59 (6.58) *dd*, J = 8.4, 2.5 Hz; H-4, δ 6.43 (6.39) *d*, J = 2.5 Hz; H-7, δ 6.83 (7.11) *s*; H-10, δ 6.41 (6.36) *s*; OCH_2O, δ 5.91 (5.88) and 5.95 (5.94) *d*, J = 1.4 Hz; and phenolic OH, δ 5.10 *br s*. The singlet at δ 5.40 (5.43) was like H-11a in **2**, and its lack of splitting showed an OH to be at 6a. The remaining OH could only be at 6, and accounted for the remaining peak, a broad doublet for H-6 at δ 5.30, J = 4.5 Hz, split by the attached OH but undergoing exchange broadening. The largest difference in shifts between **3a** and **3b** is for H-7, which is the hydrogen most spatially proximate to the configurational point of difference.

Molecular mechanics calculations using the program PCMODEL (Serena Software, Bloomington, IN) were used to predict which of **3a** or **3b** should predominate. The calculated minimum energy form had *cis*-hydroxyls (**3a**), with the 6-OH equatorial and the 6a-OH axial. This was 2.1 kcal mol^{-1} more stable than the lowest energy conformation of **3b**, with *trans*-hydroxyls and both OHs equatorial. This programme

did not take the anomeric effect into account, but since the alternate chair form **3a** was calculated to be more stable than the alternate chair form of **3b**, **3a** should also predominate over **3b** when the anomeric effect is included in calculations.

(−)6*a*-OH-*Maackiain and* (−)6,6*a*-*di*-OH-*maackiain as a pathway*

In time course experiments that follow (−)maackiain (**1**) detoxification *in vivo*, the four isolates of *C. gloeosporioides* representing both races and pathotypes we tested produced both (−)6a-OH-maackiain (**2**) and (−)6,6a-di-OH-maackiain (**3**). The ratios of the two metabolite peaks were determined in time course samples taken after all of the substrate (−)maackiain (**1**) had been metabolized. A shift in the ratios would either indicate conversion of one metabolite to the other or further metabolism or breakdown of the compounds at different rates. In all cases the change in the ratio of **2** to **3** decreased, indicating that there was an increase in (−)6,6a-di-OH-maackiain (**3**) relative to (−)6a-OH-maackiain (**2**) (data not shown), consistent with metabolism of **2** to **3** (Fig. 1). We propose that (−)maackiain (**1**) is first hydroxylated at the 6a position and subsequently hydroxylated at the 6 position. This is in contrast to the two successive reduction steps after the initial 1a-hydroxylation of (−)maackiain (**1**) and (−)medicarpin (**4**) by *Cercospora medicaginis* [4], but the end



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result is again the formation of more polar compounds. Since phytoalexins are thought to exert their toxicity at or on fungal membranes [14], increasing the polarity of the compound would tend to force it to partition into the cytosol and away from its presumed active site.

Hydroxylation of (−)3,6-dihydroxypterocarpan at the 6a position has been shown to be due to cytochrome P450 mono-oxygenases in elicitor-treated soybean tissue [15]. Preliminary experiments on microsomes of the pea pathogen *Ascochyta pisi* also suggest that the hydroxylation of (−)maackiain (**1**) at the 6a position is due to a cytochrome P450 monoxygenase [10]. Hydroxylation of a substrate at a difficultly oxidized position is commonly associated with the activity of cytochrome P450s [16]. Based on predictions of the energetics required for oxidation of aliphatic ring structures, the hydroxylation at carbon 6 may also involve the action of a cytochrome P450 (for a detailed review, see [16]).

The strains of *C. gloeosporioides* that we tested were isolated from *Stylosanthes* spp. and all were capable of producing both (−)6a-OH and (−)6,6a-di-OH-maackiain (**3**). However, production varied considerably from experiment to experiment. Production of **3** was sometimes concurrent with (−)6aOH maackiain (**2**), or one or the other of the metabolites was detected. Though we tried a number of growth conditions (media, carbon source, inoculum spore concentration, light and temperature), we could not determine the appropriate conditions to induce specific production of either metabolite. This variability is likely due to as yet unidentified metabolic factors that regulate the proposed second step in the pathway. That is, little or no expression of 6-hydroxylase activity would result in an apparent conversion to **2**, whereas high expression would result in an apparent conversion to **3**. Alternatively, both hydroxylations may be due to the same enzyme, as some cytochrome P450s are able to hydroxylate at multiple sites on a single substrate molecule [16]. Further genetic or biochemical analyses are required to test these models.

Relative toxicities of (−)*maackiain* (**1**), 6*a*-OH-*maackiain* (**2**) *and* (−)6,6*a*-*di*-OH-*maackiain* (**3**)

The relative toxicities of (−)maackiain (**1**) and the hydroxylated metabolites were determined by measuring the growth rate of an isolate of *Nectria haematococca* mating population I (MPI), a filamentous fungus that is not pathogenic on legumes, on media containing the compounds. The ID_{50} for (−)maackiain (**1**) was 67.6 μM (19.2 μg ml^{-1}). At the highest concentrations tested (80 mg ml^{-1}), (−)6a-OH-maackiain (**2**) (267 μM) reduced growth by 15% and (−)6,6a-di-OH-maackiain (**3**) (253 μM) reduced growth by 5% compared to unamended media. Re-extraction of the fungal mycelium and media after the growth rate assays with ethyl acetate and qualitative analysis by HPLC did not reveal any further metabolism of **1**, **2** or **3** (data not shown).

Stylosanthes spp., the host species from which the strains used in this study were isolated, produces the phytoalexin (−)medicarpin (**4**), a molecule closely resembling (−)maackiain (**1**) [8]. Previous results have shown that enzymes capable of metabolizing (−)maackiain (**1**) are also capable of performing the same reaction on (−)medicarpin (**4**) [4, 6]. We have shown that *C. gloeosporioides* can metabolize (−)maackiain (**1**) to (−)6a-OH-maackiain (**2**) and/or a previously unknown metabolite, (−)6,6a-di-OH-maackiain (**3**) *in vitro*. Though there is a close correlation between pathogenicity and the ability to detoxify phytoalexins, it remains to be shown whether these metabolic detoxification reactions are important in the pathogenesis of *C. gloeosporioides* or other pathogenic fungi.

EXPERIMENTAL

(−)Maackiain (**1**) was obtained from the roots of mature red clover plants as previously described [17],

and dissolved in dehydrated EtOH at a concn of 4 mg ml^{-1} for use as a stock for all assays. Samples for NMR spectroscopic analyses were obtained as follows: After incubating 4 mg of **1** overnight in *C. gloeosporioides* liquid culture, the culture medium was extracted twice with equal volumes of 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. The fr. containing the unknown compound was extracted twice with equal volumes of EtOAc, the solvent was removed under reduced pressure, and the residue was dissolved in $CDCl_3$ for NMR study.

Spectroscopy. HPLC absorbance peak fr. were collected and the samples scanned for UV absorbance (λ^{EtOH} = 240–360 nm). The presence of the previously identified compound **2** was confirmed by LC-gas ionization MS using a 34–100% acetonitrile/H_2O gradient as mobile phase. The samples were analysed on a Finnigan TSQ 7000 triple-quadruple mass spectrometer (Finnigan, San Jose, CA) equipped with an atmospheric pressure ionization source. Compound ionization was accomplished using an atmospheric pressure chemical ionization probe in which the mobile phase was used as the reactant gas. Product ions were generated via collision induced dissociation with neutral argon gas at 25 eV. **2**: *m/z* (rel. int.) 301$[M+H]^+$ (2), 299 (19), 283 (100), 281 (16). NMR spectroscopy was performed at 500 MHz in $CDCl_3$ with TMS as standard. The location and number of OH peaks in **3** were confirmed by exchange with D_2O.

3,6,6a-Trihydroxy-8,9-methylenedioxy-pterocarpan (**3**). UV λ_{max}^{EtOH} nm: 309, 287, 281 sh. CI-MS: *m/z* (rel. int.) 317 (14, $[M+H]^+$), 315 (17), 299 (100), 281 (42). ^1H NMR **3a**: δ 7.35 (*d*, J = 8.4 Hz, H-1); 6.83 (*s*, H-7); 6.59 (*dd*, J = 8.4, 2.5 Hz, H-2); 6.43 (*d*, J = 2.5 Hz, H-4); 6.41 (*s*, H-10); 5.95 and 5.91 (*d*, J = 1.4 Hz, OCl_2O); 5.40 (*s*, H-11a); 5.30 (*br*, *d*, J = 4.5 Hz, H-6); 5.10 (*br s*, phenolic OH); 3.85 (*br s*, alcoholic OH); 2.85 (*br s*, alcoholic OH). **3b**: δ 7.32 (*d*, J = 8.4 Hz, H-1); 7.11 (*s*, H-7); 6.58 (*dd*, J = 8.4, 2.5 Hz, H-2); 6.39 (*d*, J = 2.5 Hz, H-4); 6.36 (*s*, H-10); 5.94 and 5.88 (*d*, J = 1.4 Hz, OCH_2O); 5.43 (*s*, H-11a).

Fungal cultures. C. gloeosporioides (Penz.) and Sacc. isolates SR24, UQ62, UQ396 and 21808 from *Stylosanthes* spp. [11, 12] were maintained on V–8 juice agar slants [18]. *N. haematococca* MPI isolate T-488 was maintained on Martin's peptone–glucose agar (M-2) [19].

Metabolism assays. Fungal cultures for metabolism studies were inoculated with ground mycelium harvested from V-8 agar plates into glucose–asparagine liquid medium (GA) [19] and grown with shaking at 180 RPM at room temp. 2 mg **1** were added to 100 ml actively growing culture. Control cultures received a comparable amount of EtOH solvent. Thirty ml aliquots of the culture (fungal mycelium and media) were taken at 6, 12 and 22 hr and extracted twice with equal volumes EtOAc. The EtOAc extract was then evapd under red. press. and redissolved in EtOH for HPLC, UV or LC-MS. Samples were sepd 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. The ability of *N. haematococca* MPI isolate T-488 to metabolize **1**, **2** or **3** was tested by extracting the amended M-2 media with EtOAc, evaporating the extract to dryness and redissolving the extract in EtOH for HPLC analysis as above.

Toxicity studies. The relative toxicities of **1**, **2**, and **3** were determined as previously described [4]. Briefly, a 4 mm diameter 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 concns of the compounds were determined by absorption of UV light at 309 nm and the coefficients of extinction of **2** and **3** were assumed to be the same (coefficients of extinction; **1** [A_{309} = 0.1261 μmol ml^{-1} **2** A_{309} = 0.1333 μmol ml^{-1} (unpublished data)]. Daily measurements of the mycelial front were made and per cent inhibition determined relative to growth on unamended medium.

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