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# Inhibition of Aflatoxin Biosynthesis in *Aspergillus flavus* by Diferuloylputrescine and *p*-Coumaroylferuloylputrescine

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A mixture of diferuloylputrescine/*p*-coumaroylferuloylputrescine (85:15, w/w) demonstrated inhibitory activity against aflatoxin $B_1$ biosynthesis in *Aspergillus flavus* isolate AF13. Inhibition was concentration dependent, with a 50% effective dose of 30 $\mu$g of diferuloylputrescine/*p*-coumaroylferuloylputrescine per milliliter of medium. Aflatoxin inhibition levels of up to 93% were achieved using this conjugated polyamine material. This diconjugated polyamine mixture did not display inhibitory effects on *A. flavus* growth (mycelial weight) at any of the concentrations tested. A survey of hand-dissected corn (*Zea mays*) kernel tissues, including endosperm, germ, pericarp, and wax, revealed that the highest concentrations of these conjugated polyamines were localized in the pericarp of the seed. Analysis of a number of corn accessions did not reveal a correlation between diferuloylputrescine/*p*-coumaroylferuloylputrescine concentration and resistance/susceptibility to *A. flavus* infection. The localization of these diconjugated polyamine components in the pericarp, which functions as a physical barrier and surrounds the internal food storage reserves, suggests a defensive role for these materials.

**KEYWORDS: Aflatoxin; *Aspergillus flavus*; corn; diferuloylputrescine; *p*-coumaroylferuloylputrescine; pericarp; *Zea mays***

## INTRODUCTION

*Aspergillus flavus* is a common saprophytic fungus capable of opportunistic seed pathogenesis in a number of oilseed crops (e.g., cotton, corn, peanuts, and tree nuts) in favorable environmental conditions. Serious economic impacts, resulting from regulatory constraints, occur in such commodities when infected by *A. flavus* due to contamination by aflatoxin, a potent carcinogenic mycotoxin. Corn (*Zea mays*) has been subject to a number of investigations regarding susceptibility to aflatoxin contamination due to its economic importance as a food/feed commodity worldwide.

Maize seed contains a number of components that help to resist fungal invasion, including physical and chemical barriers. The cyclic hydroxamate family of compounds has been well characterized (*1*−*5*). Normally present in tissues in a glycosylated form, the aglycon is released by the action of a glycosidase following induction by mechanical or biological stress (*2*). The aglycon, a cyclic hydroxamate, possesses antimicrobial (*3*, *4*) as well as insect feeding deterrent activities (*3*, *5*). In addition, a member of this class of compounds (4-acetylbenzoxazolin-2-one) is a known inhibitor of mycotoxin biosynthesis (*6*). More recently, a class of polyamine conjugates has been isolated and characterized from maize kernels (*7*). The primary components present were diferuloylputrescine (**1**) and *p*-coumaroylferuloylputrescine (**2**) (**Figure 1**). An improved extraction technique revealed that relatively high levels of these compounds appear to be localized in the seed pericarp (*7*). The localization of these components suggests a defensive role against potential pathogens. Indeed, polyamine conjugates have been reported to display fungicidal (*8*) and insecticidal (*9*) characteristics. To explore possible biological activities of **1** and **2**, an investigation was initiated to determine their effects on growth and aflatoxin $B_1$ production by *A. flavus*.

## MATERIALS AND METHODS

**Biological Materials.** *A. flavus* isolate AF13 (ATCC 96044) was isolated from soil samples collected in southwestern Arizona (near Yuma) and maintained on a 5% vegetable juice (Campbell Soup Co., Camden, NJ) agar medium (pH 5.2) at 30 °C (*10*). Conidial suspensions of the fungus were constructed with sterile deionized water and contained $1-2 \times 10^6$ spores/mL.

Accessions of corn were obtained from the following sources: Ames 3124, Ames 3125, and PI 561859 from the North Central Regional Introduction Station, USDA, ARS, Ames, IA; GRIN, PI 614819 (MP 715) from Dr. W. Paul Williams, Mississippi State University; and PI 619430 (TX 807), PI 619431 (TX 811), PI 633839 (TX 114), PI 644840 (TX 714), and PI 633842 (TX 745) from Dr. Javier Betran, Texas A&M University, College Station, TX. Yellow dent no. 2 corn kernels 33A14 were kindly provided by Pioneer Hi-Bred International (Johnston, IA).

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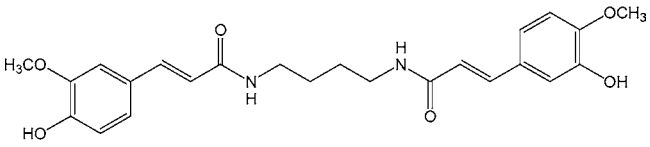
Diferuloylputrescine (**1**)



*p*-Coumaroylferuloylputrescine (**2**)

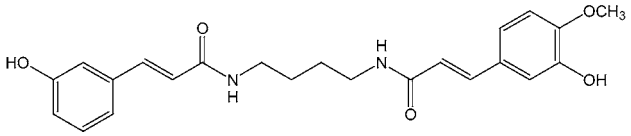


**Figure 1.** Structures of diferuloylputrescine (**1**) and *p*-coumaroylferuloylputrescine (**2**).

**Diferuloylputrescine/*p*-Coumaroylferuloylputrescine Preparation/Purification.** Corn bran (Dietfiber corn bran, Ultrafine, NU 20085, obtained by dry milling corn from Bunge Milling, Danville, IL) was extracted using a model ASE 200 Accelerated Solvent Extractor (Dionex, Inc., Sunnyvale, CA) with methylene chloride, first at 40 °C, followed by extraction at 100 °C (*7*). The 100 °C methylene chloride extract was further purified to a final preparation that contained **1**/**2** as an 85:15 (w/w) mixture using a previously described silica solid-phase extraction method (*7*). Methylene chloride was removed under a stream of nitrogen. Samples were resolvated with dimethyl sulfoxide (DMSO) to yield stock solution concentrations of 30−40 mg/mL.

**Fungal Incubations.** A chemically defined culture medium (*11*), containing sucrose as a carbon source (50 g/L) and sodium nitrate as a nitrogen source (3 g/L) (*12*), was used as a fungal growth medium. The medium was adjusted to pH 5.0 before heat sterilization. Incubations were conducted in 20 mL of medium in 50-mL flasks. Aliquots of **1**/**2** preparation were added after the medium had been allowed to cool. All treatment incubation flasks, including controls, contained 0.35% (v/v) DMSO, which was necessary as a carrier solvent for **1**/**2**. An additional set of control cultures did not contain DMSO. Each incubation flask was seeded with 100 $\mu$L of a conidial suspension. Fungal fermentations were carried out in a shaking incubator (200 rpm) in the dark at 31 °C for 6 days. Each experiment had four replicates and was repeated a total of four times. The results reported herein are representative of those experiments.

**Aflatoxin Analysis.** In the conditions used in these experiments, *A. flavus* isolate AF13 produced only aflatoxin $B_1$. Cultures were stopped and aflatoxin was solubilized by adding acetone to yield a 50% (v/v) solution. An aliquot of this solution was spotted beside aflatoxin standards ($B_1$, $B_2$, $G_1$, and $G_2$) on silica gel G thin-layer chromatography plates. After development in diethyl ether/methanol/water (96:3:1), aflatoxin $B_1$ was quantified directly by fluorescence densitometry (*13*) with a model CS-9301PC densitometer (Shimadzu, Kyoto, Japan). The limit of detection for this analysis method is 1 ng/g (1 ppb); analysis range is from 1 ng/g to 1 mg/g; toxin recovery is >95%.

**Biomass Estimation.** Following aflatoxin analysis, the fungal mycelia of each culture were collected by filtration in vacuo. The residue was collected on filter paper, dried at 50 °C for 24−48 h, and weighed.

**Kernel Dissections.** The intact yellow dent kernels (33A14, 100 g) were first extracted with chloroform at 25 °C by vigorously stirring for 5 min to remove surface wax from the kernels. Solvent was removed by means of rotoevaporation and under a stream of nitrogen. The wax extract was stored at 5 °C under nitrogen until analysis was conducted. The dewaxed kernels were then air-dried and soaked in excess deionized water at 25 °C for 24 h before dissection. Hand dissection was conducted to separate endosperm, germ, and pericarp tissues. After dissection, tissue fractions were dried at 55 °C for 48 h and stored in a low-humidity environment (desiccator) prior to grinding. The following dry tissue yields were obtained from 100 g of seed: endosperm, 59.2 g; germ, 6.38 g; pericarp, 2.71 g; wax, 0.087 g. Not all seeds used for wax extraction were subjected to dissection due to small seed size. Dry kernel tissues were ground to 20 mesh (pericarp and endosperm in a Wiley mill; germ in an electric coffee grinder). After grinding, the fractions were extracted as described below.

**Table 1.** Effect of Polyamine Conjugates **1**/**2** on *A. flavus* Biomass Production

| concn **1**/**2**[a] ($\mu$g/mL) | biomass[b] (g) |
|---|---|
| 0 | 0.195 ± 0.007 |
| 10 | 0.199 ± 0.009 |
| 20 | 0.206 ± 0.016 |
| 30 | 0.230 ± 0.016 |
| 40 | 0.217 ± 0.022 |
| 50 | 0.220 ± 0.018 |
| 75 | 0.227 ± 0.007 |
| 100 | 0.206 ± 0.007 |

[a] Concentration of **1**/**2** (85:15) is expressed as $\mu$g/mL of medium. [b] Mean *A. flavus* biomass is expressed as g of dry weight ($n = 4$).

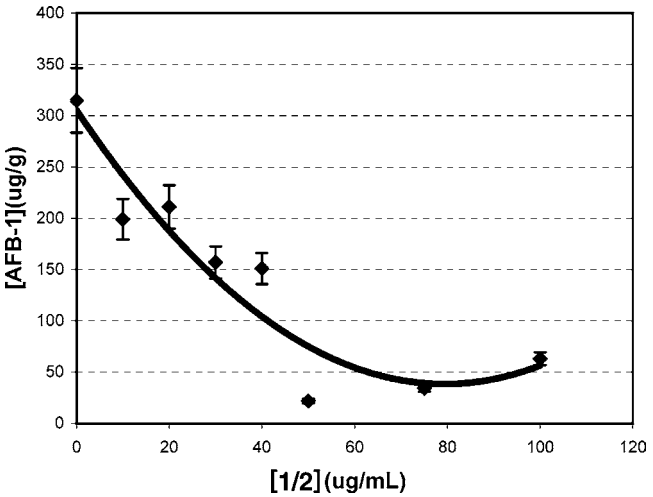



**Figure 2.** Effect of polyamine conjugates **1**/**2** on aflatoxin $B_1$ production in *A. flavus*. Concentration of **1**/**2** (85:15) is expressed as $\mu$g/mL of medium. Mean aflatoxin $B_1$ concentration ($n = 4$) is expressed as $\mu$g/g of dry weight (fungal biomass) (polynomial fit, $r^2 = 0.89$, $P < 0.05$).

**Tissue Extractions.** Extractions were performed with a model ASE 200 Accelerated Solvent Extractor (Dionex Inc.), using ethanol. The sample (2−4 g) was placed in an 11-mL stainless steel extraction vessel, and the remaining volume was filled with washed sea sand. The extractor was programmed to extract at a pressure of 1000 psi (69 bar) and a temperature of 100 °C, extracting each sample with a total of 22 mL of solvent, delivered in three 10-min extractions. These ethanol extracts were evaporated, weighed, and solvated in chloroform/methanol (85:15, v/v) to a concentration of 1 mg/mL. Four separate extractions were performed for each tissue sample ($n = 4$), and one HPLC injection was made from each extract.

**HPLC Analysis of Diconjugated Polyamines.** Analyses were performed on a Hewlett-Packard model 1100 HPLC (Agilent Technologies, Palo Alto, CA) with an autosampler and detection via two detectors in series, with the effluent first entering a Hewlett-Packard model 1100 diode array UV−vis detector and then entering a Sedex model 55 evaporative light scattering detector (ELSD) (Richard Scientific, Novato, CA) operated at 40 °C, with nitrogen as a nebulizing gas, at a pressure of 2.0 bar. The HPLC column was a 100 mm × 3 mm i.d., 5 $\mu$m, LiChrosorb DIOL (Varian/Chrompack, Walnut Creek, CA), and the mobile phase was a ternary gradient with A = 1000 hexane/1 acetic acid, B = 2-propanol, and C = water, with the following linear gradient timetable: 0 min, 90/10/0; 30 min, 58/40/2; 40 min, 45/50/5; 50 min, 45/50/5; 51 min, 50/50/0; 52 min, 90/10/0; 60 min, 90/10/0, at a flow rate of 0.5 mL/min. The retention times of **2** and **1** were about 24 and 25 min, respectively. In this HPLC method, the common phospholipids phosphatidylethanolamine and phosphatidylcholine were also detected with the ELSD in all of the extracts with retention times of 23 and 31 min, respectively. A sample of the purified **1**/**2** mixture was prepared as described under Diferuloylputrescine/*p*-Coumaroylferuloylputrescine Preparation/Purification and as previously described (*7*); it was used

**Table 2. 1/2** Concentrations in Dissected Tissues of Corn Kernels

| fraction | extract yield (g/100 g of fraction) | μg of **2**/g of extract | μg of **2**/g of fraction | μg of **1**/g of extract | μg of **1**/g of fraction |
|---|---|---|---|---|---|
| wax | 0.087[a] | 84 ± 10 | 0.073 ± 0.008[a] | 581 ± 30 | 0.505 ± 0.022[a] |
| germ | 20.83 ± 0.18 | 26 ± 10 | 5.42 ± 0.70 | 100 ± 30 | 20.80 ± 6.45 |
| endosperm | 2.78 ± 0.03 | 111 ± 30 | 3.09 ± 0.75 | 358 ± 60 | 9.95 ± 1.53 |
| pericarp | 2.88 ± 0.42 | 5313 ± 940 | 153 ± 26.1 | 14150 ± 2910 | 407.6 ± 83.9 |

[a] Units expressed as per g or per 100 g of kernels, because the wax fraction was obtained by extraction of intact kernels ($n = 4$).

**Table 3. 1/2** Concentrations in Corn Accessions with Various Degrees of Resistance to *A. flavus*

| accession | % extract | μg of **2**/g of extract | μg of **2**/g of kernel | μg of **1**/g of extract | μg of **1**/g of kernel |
|---|---|---|---|---|---|
| Ames3124(R)[a] | 7.89 ± 0.15 | 430 ± 20 | 33.9 ± 1.6 | 2650 ± 120 | 209.1 ± 9.5 |
| Ames 3125(R) | 9.36 ± 0.01 | 610 ± 10 | 57.1 ± 0.9 | 2760 ± 10 | 258.3 ± 0.9 |
| PI 561859(R)[b] | 8.29 ± 0.66 | 1730 ± 60 | 143.4 ± 5.0 | 5380 ± 110 | 446.0 ± 9.1 |
| PI 614819(R)[c] | 9.04 ± 0.23 | 1470 ± 140 | 132.9 ± 12.7 | 5590 ± 460 | 505.3 ± 41.6 |
| PI 619430(R) | 8.89 ± 0.11 | 1840 ± 30 | 163.6 ± 2.7 | 3400 ± 120 | 302.2 ± 10.7 |
| PI 619431(S) | 8.03 ± 0.13 | 4820 ± 110 | 387.0 ± 8.8 | 18400 ± 620 | 1478 ± 49.8 |
| PI 633839(S) | 8.80 ± 0.21 | 1450 ± 60 | 127.6 ± 5.3 | 5740 ± 240 | 505.1 ± 21.1 |
| PI 633840(S) | 7.53 ± 0.09 | 130 ± 20 | 10.2 ± 1.6 | 580 ± 70 | 43.7 ± 5.3 |
| PI 633842(S) | 5.85 ± 0.07 | 800 ± 0 | 46.8 ± 0.0 | 2220 ± 50 | 129.9 ± 2.9 |
| 33A14[d] | 5.93 ± 0.03 | 1450 ± 20 | 85.9 ± 1.2 | 5570 ± 110 | 330.3 ± 6.5 |

[a] Accession is reported to be R = resistant to *A. flavus* or S = susceptible to *A. flavus*. [b] Resistance is believed to have a chemical basis. [c] Selected for reduced aflatoxin accumulation in the grain following inoculation of developing ears with *A. flavus*. [d] Resistance/susceptibility to *A. flavus* infection is unknown.

to construct a calibration curve that exhibited a linear relationship between peak area at $A_{320}$ and mass in the range of 1−30 μg of **1** and 1−10 μg of **2**.

## RESULTS AND DISCUSSION

No growth inhibition of *A. flavus* was observed with the **1**/**2** material. Biomass production was not significantly different in the various treatments over the range of concentrations tested (**Table 1**). In addition, the level of carrier solvent (0.35% DMSO) included in fungal fermentations had no effect on fungal growth, as there was no difference in biomass production between control cultures either with or without DMSO.

The **1**/**2** combination tested demonstrated moderate inhibition of aflatoxin biosynthesis in *A. flavus*. Aflatoxin production was inversely related to the concentration of **1**/**2** in the fermentation medium (polynomial fit, $r^2 = 0.89$; $P < 0.05$) (**Figure 2**). On the basis of interpolation, the $ED_{50}$ of the **1**/**2** material was 30 μg/mL (∼70 μM). A maximum inhibition level of 93% was observed with this **1**/**2** mixture at a concentration of 50 μg/mL.

Extraction of dried kernel tissues by means of accelerated solvent extraction revealed the greatest concentrations of **1**/**2** were localized in the pericarp tissue (**Table 2**). Germ tissue contained the next highest concentrations, although at levels ∼30 times lower than in pericarp. The wax fraction contained the lowest **1**/**2** levels.

Accelerated solvent extraction of whole corn kernels (ground) from a number of seed lines did not reveal any correlations between **1**/**2** concentrations and resistance/susceptibility to *A. flavus* infection (**Table 3**). Both the highest and lowest **1**/**2** concentrations were registered by susceptible varieties in this survey. The lowest concentrations of conjugated polyamines were grouped in the susceptible lines.

The combination of **1**/**2** used in this study had no effect on growth of *A. flavus*, but did cause a significant reduction in the quantity of aflatoxin $B_1$ produced by the fungus. **1** has previously demonstrated a lack of growth inhibition against *Fusarium culmorum* and *A. flavus* (*6*). The **1**/**2** preparations used in this investigation were comprised by weight of 85% **1** and 15% **2**. This ratio was obtained from the direct extraction of corn bran. A similar ratio of these conjugated polyamines is assumed to exist *in plantae*. It would appear to be valid to test these materials in combination, as the model liquid fermentations employed in this study would simulate the levels of **1** and **2** experienced by an invading fungal pathogen.

Other work has shown that the material that yielded the highest concentrations of **1** and **2** was corn bran (*7*). Corn bran is obtained from a dry milling process and is composed primarily of pericarp tissue (*14*). Furthermore, comparison of extractable **1**/**2** from corn pericarp and aleurone tissues revealed 25-fold higher levels in the pericarp (*7*). In addition, a complete survey of corn kernel tissues definitively localized the highest **1**/**2** concentrations to the pericarp of the seed (**Table 2**). The endosperm preparation probably had low levels (a few percent) of pericarp contamination and, thus, actual endosperm **1**/**2** levels are probably lower than the reported values. The wax layer of the seed lies in close proximity to the pericarp and yet displayed the lowest **1**/**2** concentrations. Thus, it would appear that these conjugated polyamines are primarily localized in the pericarp tissue of corn seed. Although these results indicate that polyamine conjugates are localized in the pericarp, the finding that very low **1**/**2** levels were extracted into the wax fraction suggests that either they are not in the outer region of the pericarp or they are tightly bound into the tissue matrix of the pericarp and are extractable only when the pericarp is ground.

A survey of a number of corn accessions did not demonstrate a correlation between resistance/susceptibility to *A. flavus* infection and **1**/**2** concentrations (**Table 3**). It should be noted, however, that many corn varieties displaying aflatoxin resistance do so because of their "tight husk" characteristics. These characteristics reduce the effects of insect predation that is one of the primary routes for fungal entry of *A. flavus* into the cob via exit holes of burrowing insect larvae. The seed accession purported to have a chemical basis for resistance (PI 561859) did demonstrate fairly high concentrations of **1**/**2**. It would be interesting to test variety PI 619431, with the highest **1**/**2** levels, for insect feeding deterrence activity. Another trend to note is that the lowest **1**/**2** concentrations in this survey were grouped in the *A. flavus*-susceptible lines.

The localization of **1**/**2** in corn kernels suggests a possible defensive role. The high levels of these compounds in the pericarp tissue surrounding kernel reserves would allow for maximum exposure to potential invading pathogens or foraging insects. Starch and protein reserves are located in the endosperm, whereas lipid and free sugar reserves are localized in the germ. These reserves represent potential nutrient sources for fungi and insects. Plants have evolved a number of defensive strategies to protect these storage components. **1**/**2** may well represent part of a chemical deterrent situated in tissues surrounding the internal kernel tissues.

The physiological function of these conjugated polyamines in corn is not known. Polyamine conjugates have been reported to have fungicidal (*8*), insecticidal (*9*), and viricidal (*15*) activities. Diconjugated polyamines may have biological activities of their own. **2** and **1** have been reported to display inhibitory activity against yeast α-glucosidase (*16*), although at fairly high concentrations (2 mM). The data reported herein confirm such potential biological activity. Whereas the **1**/**2** material did not display inhibitory activity against the growth of *A. flavus*, it was a fairly potent inhibitor of aflatoxin biosynthesis, with an $ED_{50}$ of 30 μg/mL (30 ppm). Assuming the levels of these materials extracted from corn bran (3280 μg of **1**/g or 3280 ppm) (*7*) are representative of tissue levels *in plantae*, actual concentrations of these conjugated polyamines (**1**) in pericarp tissue are ∼100-fold higher than those levels displaying antiaflatoxigenic activity in this in vitro study. Thus, results from this study strengthen the suggestion of a defensive role for these naturally occurring compounds in maize pericarp.

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