

# Inhibitory Effects of Gossypol, Gossypolone, and Apogossypolone on a Collection of Economically Important Filamentous Fungi

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**ABSTRACT:** Racemic gossypol and its related derivatives gossypolone and apogossypolone demonstrated significant growth inhibition against a diverse collection of filamentous fungi that included *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus alliaceus*, *Aspergillus fumigatus*, *Fusarium graminearum*, *Fusarium moniliforme*, *Penicillium chrysogenum*, *Penicillium corylophilum*, and *Stachybotrys atra*. The compounds were tested in a Czapek agar medium at a concentration of 100  $\mu\text{g}/\text{mL}$ . Racemic gossypol and apogossypolone inhibited growth by up to 95%, whereas gossypolone effected 100% growth inhibition in all fungal isolates tested except *A. flavus*. Growth inhibition was variable during the observed time period for all tested fungi capable of growth in these treatment conditions. Gossypolone demonstrated significant aflatoxin biosynthesis inhibition in *A. flavus* AF13 (B<sub>1</sub>, 76% inhibition). Apogossypolone was the most potent aflatoxin inhibitor, showing greater than 90% inhibition against *A. flavus* and greater than 65% inhibition against *A. parasiticus* (B<sub>1</sub>, 67%; G<sub>1</sub>, 68%). Gossypol was an ineffectual inhibitor of aflatoxin biosynthesis in both *A. flavus* and *A. parasiticus*. Both gossypol and apogossypolone demonstrated significant inhibition of ochratoxin A production (47%; 91%, respectively) in cultures of *A. alliaceus*.

**KEYWORDS:** filamentous fungi, mycotoxins, gossypol derivatives, inhibitory activities

## INTRODUCTION

Gossypol, an optically active disesquiterpene (C<sub>30</sub>) produced by the cotton plant (*Gossypium hirsutum*), is principally located in lysigenous glands found throughout aerial tissues, including cottonseed, and along the external surfaces of nonglandular root tissue. Cottonseed kernels contain, on average, about 1.3% gossypol by weight.<sup>1</sup> Gossypol is considered an antinutrient component of cottonseed and cottonseed meal,<sup>2</sup> which limits its use as an animal feed and practicably precludes its use as a human protein source. It is known to exhibit a wide range of bioactivity that includes anticancer, antimicrobial, and antiviral effects.<sup>3</sup> Gossypolone is a gossypol derivative formed by oxidation with ferric chloride.<sup>4</sup> Though less actively studied, gossypolone has been reported to exhibit some anticancer effects, although in general this activity is reduced in comparison with gossypol.<sup>5–7</sup> Apogossypolone is a related derivative that is formed by conversion of gossypol to apogossypol followed by oxidation to apogossypolone.<sup>8,9</sup> This compound has recently been reported to have stronger activity against some cancer cells and is of particular interest because it binds to and interferes with Bcl proteins that are associated with disrupting apoptosis mechanisms in mammalian cancer cells.<sup>9,10</sup>

Gossypol contributes to plant defenses through anti-insect activity,<sup>11</sup> and it may be involved in other plant defense functions that include fungal inhibition. The (–)-enantiomer of gossypol is four times as active as the (+)-enantiomer in inhibition of conidial germination, mycelial growth, and conidiophore development<sup>12</sup> in *Aspergillus flavus*. In more recent work, racemic gossypol, optical gossypol, and a number of related gossypol derivatives (Figure 1) were found to inhibit the growth of *A. flavus*.<sup>13</sup>

*A. flavus* is a ubiquitous saprophytic fungus commonly found in tropical and subtropical climes.<sup>14</sup> This organism is also an opportunistic pathogen of a number of oilseed crops (e.g., cotton, maize, peanuts, tree nuts) and has agronomic significance due to its production of the potent carcinogenic mycotoxin aflatoxin B<sub>1</sub>.<sup>15</sup>

Our initial investigations regarding the effects of a series of gossypol derivatives revealed that both gossypolone and apogossypolone were more effective than gossypol as growth inhibitors against an *A. flavus* strain.<sup>13</sup> To further assess the range of biological activity of these compounds, gossypol, gossypolone, and apogossypolone were evaluated for inhibition against a collection of economically significant filamentous fungi. In addition to *Aspergilli* that produce aflatoxins, other important fungal species selected for this study include the following: *Aspergillus fumigatus*, a major causative agent of human aspergillosis; *Aspergillus alliaceus*, which produces ochratoxin; *Fusarium graminearum*, a wheat pathogen; *Fusarium moniliforme*, a causal agent of ear rot in maize; *Penicillium chrysogenum*, a human pathogen (lung); *Penicillium corylophilum*, an animal pathogen, and *Stachybotrys atra*, which has become a fungal problem on cellulosic substrates under favorable conditions following flooding events in housing structures. Results of this investigation are presented here.

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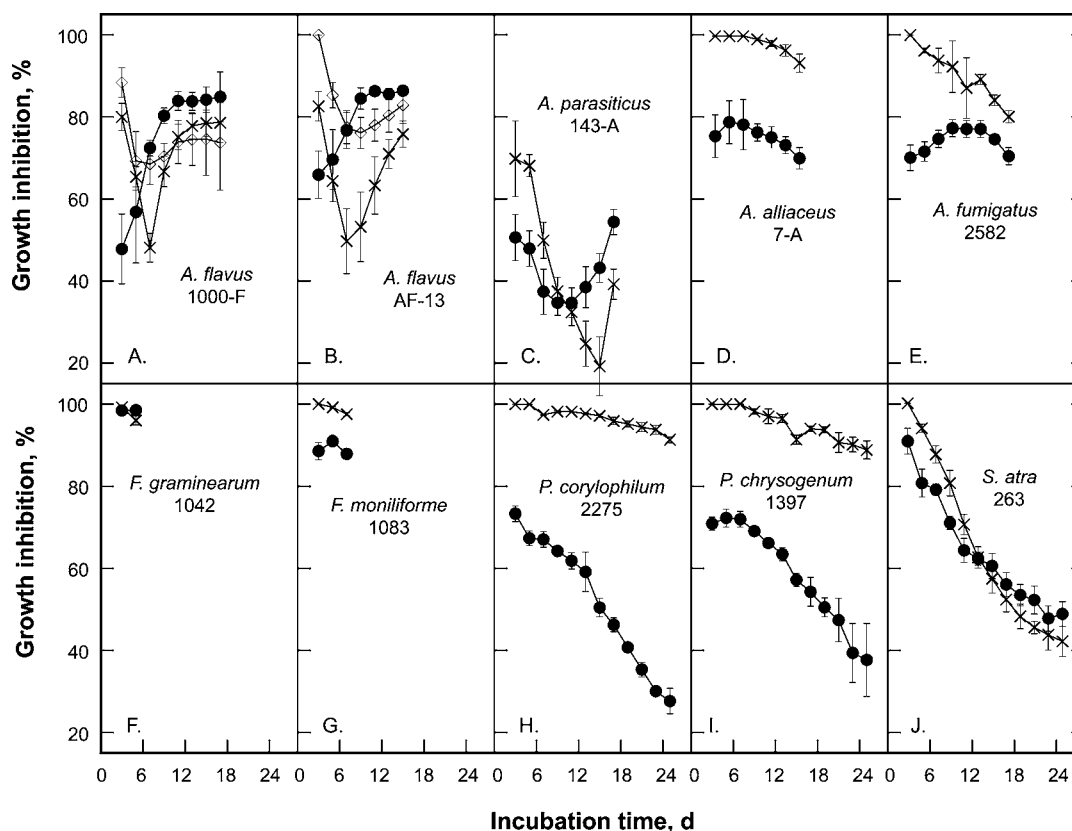
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**Table 2. Colony Areas of Fungal Species Grown in the Presence of Various Gossypol Derivatives at 100  $\mu\text{g}/\text{mL}$  Concentrations<sup>a</sup>**

fungus	growth period <sup>b</sup>	mean colony areas of plates at terminal reading ( $\text{cm}^2$ ) <sup>c</sup>				LSD <sup>d</sup>
		control	gossypol	gossypolone	apogossypolone	
<i>A. flavus</i> 1000-F	17	53.0 $\pm$ 11.3 a	8.02 $\pm$ 2.77 b	13.9 $\pm$ 5.3 b	11.4 $\pm$ 1.0 b	7.72
<i>A. flavus</i> AF13	15	50.2 $\pm$ 1.2 a	6.81 $\pm$ 0.63 c	8.56 $\pm$ 2.26 c	12.1 $\pm$ 1.6 b	1.84
<i>A. parasiticus</i> 143-A	17	55.9 $\pm$ 1.4 a	25.5 $\pm$ 1.6 c	0.0	34.0 $\pm$ 1.9 b	2.02
<i>A. alliaceus</i> 7-A	15	51.8 $\pm$ 1.0 a	15.4 $\pm$ 1.3 b	0.0	3.41 $\pm$ 1.1 c	1.41
<i>A. fumigatus</i> 2582	17	48.1 $\pm$ 1.7 a	14.2 $\pm$ 0.9 b	0.0	9.56 $\pm$ 0.65 c	1.66
<i>F. graminearum</i> 1042	5	43.8 $\pm$ 1.4 a	0.64 $\pm$ 0.2 c	0.0	1.76 $\pm$ 0.48 b	1.07
<i>F. moniliforme</i> 1083	7	40.7 $\pm$ 0.7 a	4.9 $\pm$ 0.5 b	0.0	1.01 $\pm$ 0.14 c	0.63
<i>P. corylophilum</i> 2275	25 <sup>e</sup>	18.1 $\pm$ 0.2 a	13.1 $\pm$ 0.5 b	0.0	1.56 $\pm$ 0.1 c	0.45
<i>P. chrysogenum</i> 1397	25 <sup>e</sup>	28.7 $\pm$ 3.4 a	17.9 $\pm$ 1.4 b	0.0	3.18 $\pm$ 0.5 c	2.64
<i>S. atra</i> 263	25 <sup>e</sup>	29.1 $\pm$ 1.1 a	14.9 $\pm$ 0.7 b	0.0	16.9 $\pm$ 0.8 c	1.10

<sup>a</sup>Six replicates at each condition; variances expressed as standard deviations. <sup>b</sup>Days required for fungal colony to almost fill control plate (i.e., one observation period prior to plates being filled). <sup>c</sup>Means within a row not followed by a common letter are significantly different based on LSD comparisons at  $P \leq 0.05$ . <sup>d</sup>Value shown is based on average number of observations per treatment. As the data was unbalanced for a few isolates, the actual LSD used for comparing means may have differed slightly. <sup>e</sup>Incubation period of 25 days; controls did not completely fill medium plates.



**Figure 2.** Variation in growth inhibition exhibited by gossypol related compounds in tested filamentous fungi during incubation periods. All terpenoid compounds were tested at a concentration of 100  $\mu\text{g}/\text{mL}$ . Gossypolone completely inhibited growth of all tested fungi except *A. flavus*; gossypolone data is shown only for *A. flavus* isolates. Symbol key: gossypol, (●); gossypolone, (◇); apogossypolone, (×). Error bars represent estimated standard deviations calculated by a propagation of error analysis.<sup>19</sup>

time of 25 days was given for colonies that failed to fill the plate. Each data point consisted of an average of two colony diameter measurements taken at 90° to each other. Colony areas were calculated from diameter measurements; inhibition was taken as the ratio of the treatment and control areas expressed as a percentage.

**Mycotoxin Analysis.** Plates from each *A. flavus*/*Aspergillus parasiticus* treatment were extracted for aflatoxin analysis. The entire contents from each plate were macerated, placed in 75 mL of 65% acetone (v/v, aqueous), and shaken in an orbital shaker (25 °C, 125 rpm) for at least 1 h. Solids were removed by filtration through qualitative filter paper (Whatman #4). Methylene chloride, 25 mL, was

added to the aqueous acetone filtrate. The biphasic mixture was shaken; phases were allowed to separate, and the organic (lower) phase was collected. Water was removed with anhydrous sodium sulfate, and the solvent was evaporated under ambient conditions. Each sample was resuspended in 5 mL of methylene chloride and transferred to a 1 dram vial. Contents were again allowed to dry by evaporation and resuspended in a small aliquot of acetone (volume depends on toxin level). Four microliters of each sample was spotted on silica gel G thin layer plates, which were developed in diethyl ether/methanol/water (96:3:1) mobile phase. Aflatoxins B<sub>1</sub> and G<sub>1</sub> were quantitated directly on thin layer plates by fluorescence densitometry

(Shimadzu 9301PC), comparing  $R_f$  values to standards (Sigma Chemical Co., St. Louis, MO).

Ochratoxin A was extracted from *A. alliaceus* cultures as described above. Ochratoxin A (OTA) analysis was performed according to the procedure described by Bonvehl.<sup>18</sup> Analyses were performed with a Waters 2695 HPLC combined with a Waters 2475 fluorescence detector; postcolumn derivatization was done with a photochemical reactor for enhanced detection (PHRED) (Aura Industries Inc., New York, NY) system. Detection of ochratoxin A was performed with an excitation wavelength of 333 nm and an emission wavelength of 460 nm. Sample extracts (10  $\mu$ L;  $n = 3$ ) were analyzed by injection onto a reverse phase column. The column used was a 150 mm  $\times$  3.9 mm i.d., 5  $\mu$ m, Nova-Pak C<sub>18</sub>, with a 20 mm  $\times$  3.9 mm i.d. guard column of the same material (Waters, Milford, MA). Column temperature was maintained at 38 °C. Elution flow rate was 0.8 mL/min with a mobile phase solvent consisting of acetonitrile/water/acetic acid (51:47:2, v/v/v). Retention time for ochratoxin A was about 6 min. A calibration curve with high linearity ( $r^2 = 0.998$ ) was constructed for ochratoxin A from a series of diluted standards (Sigma Chemical Co.).

**Calculations and Statistics.** The design of the experiment was completely randomized with six replicates per treatment for each isolate. At the completion of the growth cycle, three of the replicate plates for the *A. alliaceus*, *A. flavus*, and *A. parasiticus* isolates were then randomly chosen for toxin analysis. Least significant difference comparisons at a significance level of  $P \leq 0.05$  were performed on fungal colony areas and on total toxin levels. Standard deviations for % inhibition were estimated from an analysis of propagation of error based on the control and treatment colony areas and their standard deviations.<sup>19</sup>

## RESULTS

A fairly wide range of growth rates was displayed by the fungi under the growth conditions of the experiment (Table 2). The *Fusarium* isolates displayed the fastest growth rates, with the control plates being fully covered in 7 to 9 days. The *Aspergillus* isolates all showed relatively moderate growth rates, covering the plates in 15 to 19 days. The *Penicillium* and *Stachybotrys* isolates grew at significantly slower rates and had not fully covered the plates after 25 days of growth.

Table 2 shows that the three compounds of interest displayed variable growth inhibition against the fungal isolates. Racemic gossypol demonstrated a wide range of inhibition. It showed relatively poor activity against the slower growing *Penicillium* species, while it was moderately effective against *F. graminearum* and *A. flavus*. Apogossypolone was a more effective growth inhibitor, demonstrating good activity in all tested fungi except *A. parasiticus* and *S. atra*. Gossypolone was the most effective growth inhibitor tested, resulting in full inhibition in all tested fungi except *A. flavus*.

Analysis of the growth inhibition activity displayed during the course of the fungal incubations revealed a range of temporal trends. In some cases, relative inhibition remained somewhat nonvariable over the course of the incubation. For example, the measured gossypol growth inhibition of *A. fumigatus* was 70% at both 3 and 17 days, with little variation in the intermediate time points (Figure 2E). Likewise, gossypol growth inhibition of *A. alliaceus* varied from 76% at 3 days to 70% at 15 days (Figure 2D). In other cases, inhibition potency appeared to increase over the incubation period, e.g., gossypol inhibition of *A. flavus* 1000-F varied from about 48% at 3 days to 85% at 17 days (Figure 2A). Significant decreases in apparent inhibition over time were also observed. This trend was observed for gossypol inhibition of the *Penicillium* species (Figure 2H,I) and for apogossypolone inhibition of *A. parasiticus* and *S. atra* (Figure 2C,J). While almost all fungi studied were completely inhibited by gossypolone, both *A. flavus* isolates that exhibited

some growth in the presence of this compound appeared to lose a degree of potency over time (Figure 2A,B).

Aflatoxin analysis of the *A. flavus/A. parasiticus* cultures following incubation termination revealed apparent inhibition of aflatoxin biosynthesis by all of the tested compounds (Table 3). However, when the data were normalized to account for

**Table 3. Effect of Gossypol-Related Compounds on Mycotoxin Production in *A. flavus*, *A. parasiticus*, and *A. alliaceus*<sup>a</sup>**

treatment	toxin type <sup>b</sup>	toxin level $\pm$ SD, $\mu$ g	normalized toxin level <sup>c</sup>	inhibn (%)
<i>A. flavus</i> 1000-F				
control	B <sub>1</sub>	27.3 $\pm$ 0.94	n/a	n/a
gossypol	B <sub>1</sub>	9.64 $\pm$ 2.29	63.8	0
gossypolone	B <sub>1</sub>	7.55 $\pm$ 2.21	28.7	0
apogossypolone	B <sub>1</sub>	0.36 $\pm$ 0.12	1.66	94
LSD <sup>d</sup>		3.13		
<i>A. flavus</i> AF13				
control	B <sub>1</sub>	30.4 $\pm$ 1.32	n/a	n/a
gossypol	B <sub>1</sub>	2.87 $\pm$ 0.73	21.1	30.6
gossypolone	B <sub>1</sub>	1.25 $\pm$ 0.82	7.31	76
apogossypolone	B <sub>1</sub>	0.21 $\pm$ 0.1	0.83	97
LSD		1.62		
<i>A. parasiticus</i> 143-A				
control	B <sub>1</sub>	34.1 $\pm$ 1.87	n/a	n/a
gossypol	B <sub>1</sub>	13.8 $\pm$ 1.62	30.3	11.1
apogossypolone	B <sub>1</sub>	6.83 $\pm$ 0.56	11.2	67.2
LSD		2.92		
<i>A. parasiticus</i> 143-A				
control	G <sub>1</sub>	136 $\pm$ 10.2	n/a	n/a
gossypol	G <sub>1</sub>	102 $\pm$ 17.4	224	0
apogossypolone	G <sub>1</sub>	26.4 $\pm$ 0.7	43.4	68.1
LSD		23.3		
<i>A. alliaceus</i> 7-A				
control	OTA	928 $\pm$ 83	n/a	n/a
gossypol	OTA	147 $\pm$ 14	494	46.8
apogossypolone	OTA	5.42 $\pm$ 1.21	82.4	91.1
LSD		96.6		

<sup>a</sup>Data is reported as average weight of mycotoxin per plate ( $n = 3$ ).

<sup>b</sup>B<sub>1</sub> = aflatoxin B<sub>1</sub>; G<sub>1</sub> = aflatoxin G<sub>1</sub>; OTA = ochratoxin A.

<sup>c</sup>Mycotoxin data was transformed to normalize area differences between control and treated plates (n/a = not applicable). <sup>d</sup>LSD of treatments at  $P \leq 0.05$  for each isolate/toxin combination.

colony area differences between the control and compound-treated cultures, it became apparent that gossypol exhibited little aflatoxin inhibitory activity. In contrast, both gossypolone and apogossypolone appeared to inhibit aflatoxin production in *Aspergillus* species. Apogossypolone was the most effective agent with respect to aflatoxin inhibition, reducing toxin levels by 67–68% for *A. parasiticus* and 94–97% for *A. flavus*. Gossypolone also demonstrated significant aflatoxin inhibition activity in the *A. flavus* AF13 cultures (76% reduction in toxin) but not in the 1000-F cultures. Both gossypol and apogossypolone demonstrated significant inhibitory activity against ochratoxin A production in *A. alliaceus* cultures (Table 3).

## DISCUSSION

The considerable range in growth rates for the fungi used in this investigation was not unexpected. *A. flavus*, *A. parasiticus*,



and *A. fumigatus* isolates are adapted to more rapid growth at higher temperatures (30–37 °C).<sup>20</sup> The *Fusarium* isolates are adapted to optimal growth near 25 °C.<sup>21</sup> The *Stachybotrys atra* isolate is known to be a slow grower under most conditions. The Czapek Dox medium used in the study also may have contributed to the different rates of growth, as the fungi would likely have different abilities to cope with the limited available resources.

Racemic gossypol displayed a large variability in its ability to inhibit growth in the fungi included in this investigation. It was particularly effective (>80% inhibition) against *Fusarium* species, but much less effective (<30% inhibition) against the *Penicillium* species. This observation may be related to a differential effect of gossypol penetration through fungal cell walls, depending on the lipid composition of the fungal plasmalemma in question.<sup>22</sup> Overall, apogossypolone and gossypolone were more effective growth inhibitors, with gossypolone causing total inhibition in all tested fungi except *A. flavus*.

The variable inhibitions observed among species, and the changes in potency over time within a species, suggest that the compounds likely interact with the fungi through a variety of mechanisms. The literature of gossypol-affected metabolic enzymes is large and the current study of apogossypolone as a Bcl inhibitor also points to metabolic inhibition. In addition, the relatively biplanar structure of gossypol (i.e., essentially planar naphthalene rings with hydrophobic and hydrophilic sides oriented approximately perpendicular to each other) suggests that the molecule can at least partially intercalate into membranes and interfere with transport functions.<sup>22</sup> Other effects are also possible. Gossypol has been shown to be an uncoupler (inhibitor) of oxidative phosphorylation in mammalian mitochondrial membranes, presumably through perturbation of normal membrane function.<sup>23</sup> In addition, gossypol perturbation of membranes may negatively affect the function of membrane proteins (e.g., P<sub>450</sub> oxidases), contributing to some observed biological activities. For example, spore germination inhibition is distinct from mycelial growth inhibition. Gossypol is a known inhibitor of *A. flavus* conidial germination<sup>12</sup> and also appears to inhibit viral replication.<sup>3</sup> The almost complete inhibition of fungal growth by gossypolone suggests that the compound may act as a general spore germination inhibitor.

The mechanism of action for the inhibitory effects of gossypol and its derivatives is not well understood. Gossypolone differs structurally from gossypol in that its quinoid rings are more flexible (and able to pucker modestly into flattened boat-type conformations) compared with the essentially planar naphthalene rings of gossypol. In addition, the presence of the quinoid oxygen atom disrupts the side-to-side naphthalene ring polarity present within the gossypol molecule (Figure 1). The added flexibility might allow for stronger interactions with active sites, which may contribute to the improved inhibition of the compound compared with gossypol. Apogossypolone also would have the added flexibility of the backbone structure but differs in the loss of the formyl moieties. The aldehyde groups of gossypol are well-known to form Schiff's base derivatives with amines, including the terminal and lysine side chain amines of proteins. These groups allow gossypol to react in a nonspecific manner with amines of various moieties, including cellular proteins. This effect has been assumed to reduce the effective intracellular concentration of the compound and lower its activity. If this were the only

important factor, however, then apogossypolone would be expected to be more effective than gossypolone as a growth inhibitor. That it is not suggests that the aldehyde moieties play a more direct role in the inhibition, possibly allowing for gossypolone–Schiff's base interactions with proteins that contribute to critical functions.

Biological activity and animal toxicity effects of gossypol are well-known to be variable and subject to change with particular conditions used during study, which has made understanding the compound's various metabolic effects, or exploiting of the compound's range of activity, challenging. Nevertheless, the large variation in fungal growth inhibition observed over incubation time was unexpected. That some cultures were able to reduce the inhibitory effects over time suggests that the organism was able to adjust metabolically (e.g., by increasing an affected enzyme level) to correct for critical function, or was able to sequester or modify the compounds to reduce their effect. In some cases, fungal inhibition was relatively constant, suggesting that these organisms may have been less able to adjust or accommodate the inhibitory processes. Some fungi showed increased inhibition over time, suggesting that slowly increasing intracellular concentrations resulting from rate limiting penetration of the compounds through mycelial walls may have been a factor in the observed effects. In the case of reversals of growth inhibition (Figure 2A,B), reversibility of terpenoid binding to sites of action is consistent with this observation. Undoubtedly, all of these factors contribute to the net effect, with the apparent variation resulting from small differences in the capabilities of the various fungal species.

It is possible that gossypolone and apogossypolone show biological activities similar to those of plant-derived antimicrobial phytoalexins. A possible mechanism of action for this class of antimicrobial substances would be interference with membrane transport characteristics in cellular metabolism.<sup>24</sup> Thus, perhaps gossypolone and apogossypolone are disrupting the ability of the fungus to import needed carbon and nitrogen resources, limiting cell wall biosynthesis. This hypothesis is consistent with the ability of gossypol to interfere with transporter mechanisms. Gossypol is a known inhibitor of hexose transporter mechanisms in mammalian membranes.<sup>25,26</sup> The mechanism of aflatoxin inhibitory activity expressed by apogossypolone is currently unknown. There is good evidence to support the model that later stages of aflatoxin biosynthesis occur in vesicles termed "aflatoxisomes".<sup>27</sup> Further, the export of aflatoxin outside of the mycelium appears to occur through exocytosis by fusion of these vesicles with the fungal plasmalemma membrane.<sup>28</sup> Possibly, apogossypolone exerts its antiaflatoxic effects either by interfering with the formation of aflatoxisomes or by interfering with transport phenomena of aflatoxin substrates through vesicle membranes. In short, these gossypol-related compounds are probably exerting their inhibitory effects at multiple cellular locations through a variety of mechanisms.

Gossypol is generally understood to be a plant defensive compound that helps reduce insect predation. However, the compound is also found along the exterior root bark of cotton plants where insect damage is less likely; hence, it is possible that gossypol has additional biological roles to reduce or impede infection of root tissue. Nematode infection of cotton roots has not been found to elicit gossypol or its related compounds strongly,<sup>29</sup> but methyl jasmonate strongly elicits the production of gossypol in cotton hairy root tissue cultures.<sup>30</sup> The finding that gossypol has growth inhibitory

activity against a collection of fungal plant pathogens is consistent with this hypothesis.

Although gossypol and its derivatives have demonstrated a wide range of bioactivity, it has yet to find a commercial purpose that would justify the cost of its recovery from cotton plant tissues. This application deficit may be due to the lack of sufficient activity to compete with current products and unwanted side effects, i.e., insufficient therapeutic index, or complicating physical properties, e.g., instability to light and nonspecific reactivity with cellular components. The compounds evaluated in this report have not been well studied for antifungal activity. Given the ability of gossypol to inhibit fungal growth, the compound might be useful as a seed coating to impede fungal contamination of germinating seedlings. These compounds, as a class, tend to be sensitive to UV radiation, and this type of application might overcome this complication. Since gossypolone and apogossypolone exhibit some improved inhibitory activity compared with gossypol, testing these compounds for this purpose might also be warranted. Proof of the efficacy of these agents for this application, however, will require further investigation.

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### Notes

The authors declare no competing financial interest.

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