Appl Microbiol Biotechnol (2008) 78:559–572
DOI 10.1007/s00253-008-1362-0

MINI-REVIEW

Diverse inhibitors of aflatoxin biosynthesis

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Received: 23 November 2007 / Revised: 9 January 2008 / Accepted: 10 January 2008 / Published online: 2 February 2008

Abstract Pre-harvest and post-harvest contamination of maize, peanuts, cotton, and tree nuts by members of the genus *Aspergillus* and subsequent contamination with the mycotoxin aflatoxin pose a widespread food safety problem for which effective and inexpensive control strategies are lacking. Since the discovery of aflatoxin as a potently carcinogenic food contaminant, extensive research has been focused on identifying compounds that inhibit its biosynthesis. Numerous diverse compounds and extracts containing activity inhibitory to aflatoxin biosynthesis have been reported. Only recently, however, have tools been available to investigate the molecular mechanisms by which these inhibitors affect aflatoxin biosynthesis. Many inhibitors are plant-derived and a few may be amenable to pathway engineering for tissue-specific expression in susceptible host plants as a defense against aflatoxin contamination. Other compounds show promise as protectants during crop storage. Finally, inhibitors with different modes of action could be used in comparative transcriptional and metabolomic profiling experiments to identify regulatory networks controlling aflatoxin biosynthesis.

Keywords Aspergillus · Secondary metabolism · Oxidative stress · Host resistance

Introduction

Aspergillus flavus and *A. parasiticus* are filamentous fungi that infect oil seeds and tree nuts and contaminate these commodities with toxic secondary metabolites known as aflatoxins (AFs; Richard and Payne 2003). AFs belong to the polyketide class of secondary metabolites and are synthesized by enzymes encoded within a large gene cluster (Yabe and Nakajima 2004; Yu et al. 2004). Since the discovery of AFs in the 1960s, researchers have screened numerous natural products and synthetic compounds, as well as extracts from diverse organisms for inhibitors of AF biosynthesis (Maggon et al. 1977; Dutton and Anderson 1980; Zaika and Buchanan 1987; D'Mello et al. 1998; Rusul and Marth 1988). Also, substantial effort has been dedicated to identifying organisms that inhibit AF biosynthesis during coculture with aflatoxigenic Aspergilli, with the goal of developing biocontrol organisms or finding new sources of inhibitory compounds (Mishra and Das 2003).

In this review, we examine inhibitors of AF biosynthesis and describe how their potential modes of action fit into emerging molecular genetic and genomic perspectives on this process. We define an inhibitor as a compound that inhibits AF biosynthesis during culture under conditions conducive to AF biosynthesis. Therefore, compounds that do not support AF biosynthesis when used as a sole carbon or nitrogen source, e.g., lactose, are excluded. For a review of nutritional factors influencing AF biosynthesis, please see Luchese and Harrigan (1993). We focus in this study on compounds that are specific inhibitors of AF biosynthesis rather than inhibitors that affect fungal growth. We also review other growth inhibitory compounds that inhibit AF accumulation at concentrations lower than those that inhibit growth. Many compounds that have been described as inhibitors of AF biosynthesis are fungistatic with no specificity for the AF biosynthetic pathway or its regulation. Thus, specificity of an inhibitor for AF biosynthesis can be determined only when AF production and growth are both reported, which is often not the case. Adding further complexity, some inhibitors of AF production (e.g., caffeine and trifluoperazine) are fungistatic when

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present throughout the duration of culture, but reduce AF production when added to static cultures.

Of particular interest from both agronomic and human health perspectives are those compounds that could be used to bolster resistance of host plants to AF contamination. Ideally, such a compound would be plant derived (preferably already present in the host crop), nutritionally positive or neutral, and synthesized through a well-described biosynthetic pathway. A number of such compounds have been identified for post-harvest control of AF, but fewer have been shown to be amenable to genetic engineering for pre-harvest resistance to AF contamination. In addition to applications in resistance engineering, we discuss shared properties of some inhibitors and how they might be used to further examine the regulation of AF biosynthesis. With a better understanding of the properties that are common to multiple inhibitors, we will have more tools to further examine the regulation of AF biosynthesis and design new applications in resistance engineering.

Comparison of inhibitors of AF biosynthesis

Most inhibitors of AF biosynthesis act at one of three levels: altering the physiological environment or other signaling inputs perceived by the fungus, interfering with signal transduction and gene expression regulatory networks upstream of AF biosynthesis, or blocking enzymatic activity of a biosynthetic enzyme (Fig. 1). In some studies, production of norsolorinic acid (NOR), the first stable intermediate in the AF biosynthetic pathway, was measured in addition to or in place of AF. These studies show that NOR production is inhibited in parallel to AF production, consistent with inhibition occurring at the level of AF pathway regulation rather than biosynthesis. Table 1 shows inhibitors with specificity for AF biosynthesis. If an IC_{50} value was not reported in the cited work, we calculated an approximate IC_{50} value using available data. Care must be taken when comparing IC_{50} values of inhibitors; however, because of the variety of culture conditions (e.g., static vs. shake culture; synthetic vs. rich medium). Other variables include differences in culture medium, temperature, age of culture, and the concentrations of test compounds. Additionally, inhibitors were tested against different strains of either *A. flavus* or *A. parasiticus*. All of these factors can influence AF production (Klich 2007). Table 1 presents the inhibitors discussed in this review. Inhibitors are grouped into biochemical classes. With such diverse chemistries, it seems unlikely that these compounds act in the same manner. We will discuss each class in more depth to form a framework for understanding how these inhibitors interact with the AF regulatory network.

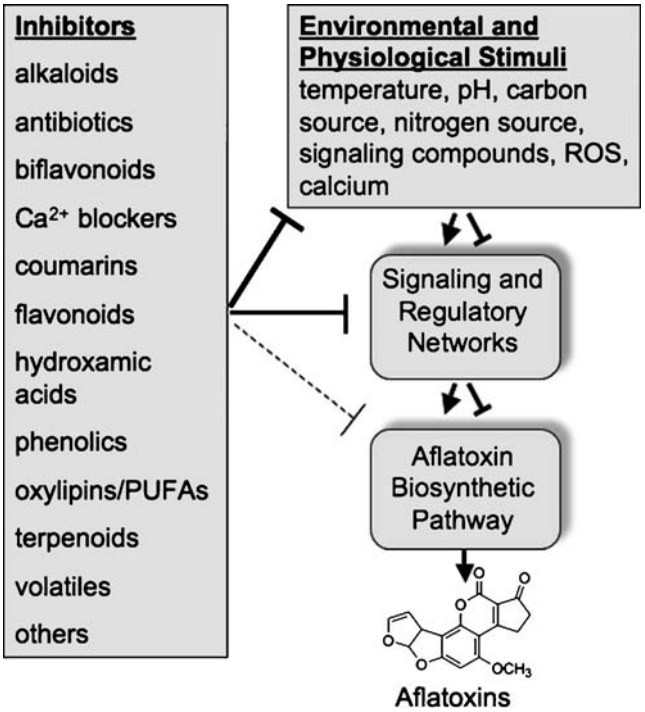

Fig. 1 Schematic representation of regulation and biosynthesis of AF with potential modes of action of major groups of inhibitory compounds. Inhibitors of AF biosynthesis may act at three levels: (1) modulate environmental and physiological factors affecting aflatoxin biosynthesis, (2) inhibit signaling circuits upstream of the biosynthetic pathway, or (3) directly inhibit gene expression or enzyme activity in the pathway. While the mode of action of most inhibitory compounds is unknown, studies using NOR accumulating mutants of the fungus suggest that most inhibitors act before the first dedicated step in the biosynthetic pathway. There is little evidence for the described compounds having an effect on gene transcription or enzyme activity of individual steps in the biosynthetic pathway. More likely, the known inhibitory compounds either alter known environmental and physiological modulators of AF biosynthesis (e.g., quenching of ROS), or they alter signaling transduction pathways in the upstream regulatory network

Phenylpropanoids

Inhibitory phenylpropanoids encompass a diverse assemblage of compounds, many with demonstrated antioxidant activity. The phenylpropanoids include simple phenolics and flavonoids such as flavones, flavonols, coumarins, chromones, and biflavonoids. Most of these compounds have antioxidant activity, but the strength of the oxygen quenching activity is not positively correlated with inhibition of AF biosynthesis. Consequently, while antioxidant activity may be important for the mode of action of this group of compounds, it seems likely that additional factors may be required for inhibition. These may include bioavailability and uptake, intracellular mobility, and interaction with specific enzymes, metabolite pools, or signaling pathways.

Table 1 Compounds inhibitory to aflatoxin (AF) production that lack a strong inhibition of growth

Inhibitor	IC_{50}[a]	AF[b]	CT[c]	Media[d]	Time[e]	Sp.[f]	Reference
Alkaloids							
Caffeine[g]	NA	<5	10	YES shake	7	p	Buchanan and Lewis 1984a, b
Piperine	<35 μM	35	35 μM	YES static	7	p	Madhyastha and Bhat 1984
	<25	25	24	PDA	7	f	Lee et al. 2002
Piperlongumine	<6	4	~6	PDA	7	f	Lee et al. 2002
Pipernonaline	<2	18	2	PDA	7	f	Lee et al. 2002
Piperoctadecalidine	<22	0	~22	PDA	7	f	Lee et al. 2002
Antibiotics							
Aflastatin A	<0.1 μM	0	0.4	PDB static	7	p	Ono et al. 1997
	<0.05 μM	0	0.4	PDA	7	p	Ono et al. 1997
Blasticidin A	04 μM			PDB static	7	p	Sakuda et al. 2000
Biflavonoids							
Amentoflavone	<0.02	10	0.02	YES	n.r.	f	Gonçalez et al. 2001
6,6″-Bigenkwanin	<0.02	15	0.02	YES	n.r.	f	Gonçalez et al. 2001
7,7″-Dimethoxyagastisflavone	<0.02	13	0.02	YES	n.r.	f	Gonçalez et al. 2001
Tetradimethoxybigenkwanin	<0.02	12	0.02	YES	n.r.	f	Gonçalez et al. 2001
Ca^{2+} channel blockers							
Diltiazem	~1.3			A&M static	3	p	Rao and Subramanyam 1999
Verapamil	<1	47	1	A&M static	3	p	Rao and Subramanyam 1999
Coumarins							
Bergapten	<0.1	23	0.1	Rice/corn steep static	6	f	Mabrouk and El-Shayeb 1992
p-Coumaric acid	1			YES static	11	f	Fajardo et al. 1995
	<0.1	23	0.1	GYS static	10	f	Aziz et al. 1998
	<0.1	12	0.1	GYS static	10	p	Aziz et al. 1998
Psoralene	<0.1	23	0.1	Rice/corn steep static	6	f	Mabrouk and El-Shayeb 1992
Xanthotoxin	<0.1	20	0.1	Rice/corn steep static	6	f	Mabrouk and El-Shayeb 1992
Flavonoids							
Cyanidin	~1.7			Suspended disc	5	f	Norton 1999
Cyanidin-3-galactoside	~4			Suspended disc	5	f	Norton 1999
Cyanidin-3-glucoside	~4			Suspended disc	5	f	Norton 1999
Delphinidin	~0.5			Suspended disc	5	f	Norton 1999
5,7-Dihydroxychromone	0.07			A&M static	14	p	DeLucca et al. 1987
Eriodictyol	<0.035	13	0.2	A&M static	14	p	DeLucca et al. 1987
Glyceollin	<0.2	5	0.2	Glucose salts	7	f	Song and Karr 1993
Kaempferol	~4			Suspended disc	5	f	Norton 1999
Khellin	<0.1	16	0.1	Rice/corn steep static	6	f	Mabrouk and El-Shayeb 1992
Luteolin	<0.035	0.4	0.2	A&M static	14	p	DeLucca et al. 1987
	~6			Suspended disc	5	f	Norton 1999
Malvidin	~2			Suspended disc	5	f	Norton 1999
Pelargonidin	~1			Suspended disc	5	f	Norton 1999
Pelargonidin-3-glucoside	~5			Suspended disc	5	f	Norton 1999
Peonidin	~2			Suspended disc	5	f	Norton 1999
Visnagin	<0.1	25.1	0.1	Rice/corn steep static	6	f	Mabrouk and El-Shayeb 1992
Hydroxamic acids							
4-Acetyl-benzoxazolin-2-one	0.12			Suspended disc	5	f	Miller et al. 1996
6-Methoxy-benzoxazolin-2-one	~0.1			Suspended disc	5	f	Miller et al. 1996
Others							
Cyclo(L-leucyl-l-prolyl)	1			YES static	4	p	Yan et al. 2004
Dioctatin A	4.0 μM			PDB static	4	p	Yoshinari et al. 2007
Gallic acid	~1.2			Walnut kernel	7	f	Mahoney and Molyneux 2004
Phytic acid	<10	13	10	SLS static	8	p	Gupta and Venkitasubramanian 1975
	NA	159	50 mg/g	Soybean meal	16	f	Hensarling et al. 1983
	~2.5			GV static	8	p	Ehrlich and Ciegler 1984
	NA	115	3.6	GV static, pH adjusted	8	p	Ehrlich and Ciegler 1984
	NA	250	3.6	S&M static	8	p	Ehrlich and Ciegler 1984

Table 1 (continued)

Inhibitor	IC_{50}[a]	AF[b]	CT[c]	Media[d]	Time[e]	Sp.[f]	Reference
	NA	~200	7	GV static	7	f	Ehrlich and Ciegler 1984
	NA	102	33 mg/g	Soybean meal	7	p	Ehrlich and Ciegler 1985
Trifluoperazine[g]	0.5			A&M	2	p	Praveen Rao et al. 1988
Phenolics							
Eugenol	<0.5	29	0.5	YES shake	10	p	Bullerman et al. 1977
	NA	160	0.5	YES shake	21	p	Bullerman et al. 1977
	<0.2	24	0.2	Low salt static	8	f	Hitokoto et al. 1980
	NA	360	0.61	YES static	10	p	Karapinar 1990
	NA	56	1.5	SMKY static	10	f	Bilgrami et al. 1992
	<1 μg/g	40	1 μg/g	Maize seeds	10	f	Bilgrami et al. 1992
	NA	137	6.1	YES static	15	f	Mahmoud 1994
	0.45			A&M static	3	p	Jayashree and Subramanyam 1999
Ferulic acid	1			A&M static	n.r.	f	Chipley and Uraih 1980
	~0.6			A&M static	n.r.	p	Chipley and Uraih 1980
	NA	84	1	YES static	11	f	Fajardo et al. 1995
	~11			Suspended disc	5	f	Norton and Dowd 1996
Vanillic acid	~1			YES static	11	f	Fajardo et al. 1995
Vanillylacetone	<5	11	5	PDA	7	f	Kim et al. 2004a, b
Polyunsaturated fatty acids (PUFAs)							
13*S*-HPODE	<1 μM	16	1 μM	A&M shake	2	p	Burow et al. 1997
13*S*-HPOTE	<0.1	0	0.1	A&M shake	2	p	Burow et al. 1997
Terpenoids							
Camphene	NA	67	0.025% *v/v*	PDA	3	p	Greene-McDowelle et al. 1999
Canthaxanthin	0.43			Suspended disc	5	f	Norton 1997
α-Carotene	6.1 μM			Suspended disc	5	f	Norton 1997
β-Carotene	0.12			Suspended disc	5	f	Norton 1997
β-Cryptoxanthin	0.012			Suspended disc	5	f	Norton 1997
α-Ionone	0.4 μM			Suspended disc	5	f	Norton 1997
β-Ionone	<0.5	26	~0.5	YES shake	7	p	Wilson et al. 1981
	400 μl/g			Peanuts	7	p	Wei et al. 1986
	37 μM			Suspended disc	5	f	Norton 1997
Limonene	NA	93	7.3	YES static	15	f	Mahmoud 1994
	NA	10	0.025% *v/v*	PDA	3	p	Greene-McDowelle et al. 1999
Lutein	1.1 μM			Suspended disc	5	f	Norton 1997
Lycopene	0.24			Suspended disc	5	f	Norton 1997
Zeaxanthin	0.06			Suspended disc	5	f	Norton 1997
Non-terpenoid volatiles							
Ethylene	<0.1 ppm	43	0.1 ppm	GMS agar	6	p	Roze et al. 2004a, b
Nonyl aldehyde	~7 μg			PDA	5	f	Zeringue et al. 1996

Compounds are grouped into classes under the heading *Inhibitor*.
[a] Given in millimolarity (mM) unless indicated otherwise
[b] AF production as a percentage of untreated control. Reported when IC_{50} was unavailable.
[c] Concentration of compound (mM, unless otherwise indicated) tested to give value in AF column.
[d] Media: *A&M* Adye and Mateles (1964), *GMS* glucose mineral salts, *GV* Gupta and Venkitasubramanian (1975), *GYS* glucose yeast extract salts, *PDA* potato dextrose agar, *PDB* potato dextrose broth, *S&M* Shih and Marth (1975), *SMKY* sucrose–magnesium sulfate–potassium nitrate–yeast extract (Diener and Davis 1967), *YES* yeast extract salts.
[e] Duration of treatment in days before AF was sampled.
[f] Species tested, *A. flavus* (f) and *A. parasiticus* (p)
[g] Inhibitory to growth when present throughout culture but not when added to mature cultures.

Simple phenolics and nonflavonoid phenylpropanoids

Many phenolic compounds that have antioxidant activity are abundant in plant tissues as precurors for lignin and other complex flavonoids. Eugenol is a major phenolic component of essential oils in cloves, cinnamon, and nutmeg. Studies have shown that extracts from the plants producing these spices are inhibitory to AF biosynthesis.

This inhibitory activity is presumably caused in part by eugenol, which has been shown to inhibit AF biosynthesis in multiple experiments (Hitokoto et al. 1980; Bilgrami et al. 1992; Jayashree and Subramanyam 1999). However, these findings conflict with other studies showing stimulation of AF biosynthesis by eugenol (Bullerman et al. 1977; Karapinar 1990; Mahmoud 1994). One factor that may be important in interpreting these differences is the time at which AF production was measured. For example, after 10 days of culture, 0.5 mM eugenol reduced AF to 29% of the control, but after 21 days, AF production was 160% of the control (Bullerman et al. 1977). Given sufficient time in culture, most inhibitors are eventually overcome.

Eugenol treatment influences markers for oxidative stress. Activities of enzymes involved in responding to oxidative stress (superoxide dismutase, xanthine oxidase, glutathione peroxidase, and two microsomal reductases) were reduced concurrent with a ~50% reduction of AF production on PDA plates. The antioxidant activity of eugenol may have lowered the physiological requirement for these enzyme activities. Glucose-6-phosphate dehydrogenase activity was also lower, consistent with reduced flux through the pentose phosphate pathway (PPP; Jayashree and Subramanyam 1999). The role of the PPP in AF biosynthesis is uncertain. On the one hand, it has been reported that glucose-6-phosphate dehydrogenase activity was reduced in an AF-conducive medium relative to a nonconducive medium (Buchanan and Lewis 1984b). On the other hand, transcripts of a glucose-6-phosphate dehydrogenase gene are induced during growth of *A. flavus* in AF-conducive culture conditions (Price et al. 2005). Also, gluconolactonase, another PPP enzyme, contains the consensus binding sequence for the AF regulator AflR (RAH, unpublished observation).

Ferulic acid is an abundant constituent of plant cell walls undoubtedly encountered by *A. flavus* during host infection. There are conflicting reports on its effect on AF production. Chipley and Uraih (1980) observed a 50% inhibition of AF at 0.2 mg/ml (1 mM) accompanied by a 30% reduction in growth. Fajardo et al. (1995) observed a complete inhibition of AF at the same concentration after 7 days in static YES culture, although inhibition was overcome by 11 days. In contrast, Norton and Dowd (1996) found a stimulation of AF production at 0.33 and 1 mg/ml. These conflicting results may be caused by different culture conditions. The first two studies were performed in liquid shake culture, whereas the latter study was performed using the suspended disc assay.

Vanillylacetone has more specificity for the AF pathway than the growth inhibitors vanillin and vanillic acid (Aziz et al. 1998; Kim et al. 2004b), although there is some evidence for AF inhibition by vanillic acid (Fajardo et al. 1995). Vanillylacetone, at a concentration of 5 mM, inhibited AF production to 11% of the control with little reduction in growth, whereas higher concentrations were increasingly inhibitory toward growth (Kim et al. 2004b). Kim et al. (2006) also found that vanillylacetone inhibited growth of *A. flavus* synergistically with strobilurin (a fungicide that inhibits complex III of the respiratory electron transport chain). They also found that yeast mutants with increased sensitivity to mitochondrial oxidative stress (*tsa1Δ*; thioredoxin peroxidase) were more susceptible to a combined H_2O_2 and vanillyl acetone treatment than a wild-type strain. This result indicates that the growth inhibitory activity of vanillylacetone may target mitochondrial function. Whether or not the anti-toxigenic activity may be attributed to altered mitochondrial function in Aspergilli needs to be tested.

Two intensively studied nonplant phenolics are the food preservatives butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Both exhibit strong antifungal as well as anti-toxigenic properties (Yousef and Marth 1983; Yousef and Marth 1984; Nesci et al. 2003). Readers are referred to Rusul and Marth (1988) for a more in-depth review of BHT and BHA.

Coumarins are phenylpropanoid toxins in plants. Three plant coumarins (xanthotoxin, bergapten, and psoralene) strongly inhibited AF production at concentrations below 0.1 mM, accompanied with moderate reductions in growth (Mabrouk and El-Shayeb 1992). How coumarins might influence AF biosynthesis is unknown. The authors suggest that structural similarities between coumarins and AF may result in competitive inhibition of biosynthetic enzymes. There are conflicting reports on the inhibitory activity of another coumarin, *p*-coumaric acid. Fajardo et al. (1995) found that *p*-coumaric acid had little effect on the growth of *A. flavus* and completely inhibited AF production. Likewise, Aziz et al. (1998) observed inhibition of AF production in broth cultures but reported growth inhibition as well on agar plates. Kim et al. (2004b) also reported growth inhibition on agar plates. It is unclear whether inhibition of AF production by *p*-coumaric acid is caused by a growth effect or a more specific influence on AF biosynthesis. Regardless, it is apparent that inhibitory compounds interact differently with *Aspergillus* spp. in liquid and solid cultures. These differences highlight the inherent difficulty of comparing studies on inhibitors of AF biosynthesis and extrapolating results obtained in vitro to conditions in host plants.

Flavonoids (polyphenols)

A variety of flavonoids are inhibitory to AF production, although many are active only at high concentrations. Norton (1999) tested several flavonoids for activity against AF production by *A. flavus*. These included cyanidin,

peonidin, delphinidin, pelargonidin, kaempferol, and luteolin. The IC_{50} values of these flavonoids ranged from ~0.5 mM (delphinidin) to ~6 mM (luteolin). Glycosylated forms of cyanidin and pelargonidin were inhibitory to AF biosynthesis but were less potent than the unglycosylated forms. In a study of flavonoids, abundant in peanut shells, luteolin was much more potent when tested against *A. parasiticus* (IC_{50} <0.35 mM vs. IC_{50} ~6 mM against *A. flavus*). Another flavonoid present in peanut shells, eriodictyol, was also inhibitory with minimal influence on growth (Delucca et al. 1987). The differences in the responses of *A. flavus* and *A. parasiticus* to luteolin may reflect differences between the two fungi or the culture conditions. *A. flavus* and *A. parasiticus* are known to respond differently to oxidative stress. For example, cumene hydroperoxide, an oxidizing agent, stimulates AF biosynthesis in *A. parasiticus* (De Luca et al. 1995) but is strongly inhibitory to growth in *A. flavus* at 1 mM and does not stimulate AF production at lower concentrations permissive to growth (CA Smith, unpublished data). Kaempferol had the opposite effect of luteolin, inhibiting AF production in *A. flavus* but not in *A. parasiticus* (Norton 1999).

A soybean isoflavonoid with promise for resistance engineering is the phytoalexin glyceollin. At concentrations as low as ~20 μM, glyceollin strongly inhibited AF production in a high-glucose liquid medium. Viable, glyceollin-producing seeds supported only one third the amount of AF production as heat-killed seeds. Whether this was because of the loss of phytoalexin biosynthesis, particularly glyceollin, or the loss of general defense responses is unclear, but the authors speculate that glyceollin contributes to the resistance to AF contamination in soybean (Song and Karr 1993). Glyceollin is particularly promising because it is a natural plant defense compound with a known biosynthetic pathway, although engineering of phytoalexin pathways has proved difficult (Welle and Grisebach 1988; Dixon 2005). How glyceollin might influence AF biosynthesis is uncertain and antioxidant activity for glyceollin has not been reported. There is some evidence that phytoalexins may play a role in resistance to AF contamination in peanut. Under drought stress, loss of stilbene phytoalexin production and a decrease in seed water activity correlated with the onset of AF production by *A. flavus* (Dorner et al. 1989).

One class of flavonoids meriting further investigation is the biflavonoids. Four structurally related biflavonoids (tetradimethoxybigenkwanin, 7,7″-dimethoxyagastisflavone, 6,6″-bigenkwanin and amentoflavone) were isolated from *Ouratea* species and tested against *A. flavus*. All four had excellent inhibitory activity at micromolar concentrations, reducing AFB_1 to <30% of the control at ~9 μM (Gonçalez et al. 2001). Biflavonoids make excellent candidates for resistance engineering because of their strong inhibitory activity and potential nutritional benefits. Unfortunately, the biosynthetic pathways responsible for biflavonoid synthesis in plants are not known.

Chromones are benzopyrans, and most are considered flavonoids. Khellin and visnagin, products from the plant *Ammi visnaga* were tested against *A. flavus*, and the related compound 5,7-dihydroxychromone was tested against *A. parasiticus* (Delucca et al. 1987; Mabrouk and El-Shayeb 1992). All three exhibited potent inhibitory activity (IC_{50}< 0.1 mM). Khellin and visnagin are pharmacological agents with cAMP–phosphodiesterase inhibitory activity (Bovalini et al. 1987; Duarte et al. 1998). cAMP has been shown to influence AF production (Roze et al. 2004a) but whether or not treatment with chromones influences intracellular cAMP concentrations in the Aspergilli is unknown.

Tannins are abundant components of walnut and peanut seed shells. Tannin extracts from peanut shells of some genotypes showed inhibitory activity toward AF production in *A. parasiticus* (Azaizeh et al. 1990). Gallic acid is one product of the breakdown of hydrolysable tannins. The hydrolysable tannin fraction of walnut seed coats from the resistant Tulare variety inhibited AF production by *A. flavus*. Although there was some initial growth inhibition, it was eventually overcome during the time course. Additional tests showed that gallic acid added to walnut endosperm media suppressed AFB_1 production, whereas ellagic acid, another hydrolysable tannin breakdown product, was stimulatory (Mahoney and Molyneux 2004). Gallic acid has antioxidant activity, and the authors propose this as the mechanism for inhibition of AF production.

Terpenoids

There are reports of eleven terpenoids inhibitory to AF biosynthesis (Table 1). Terpenoids are a major class of natural products synthesized in plants through the mevalonic acid pathway. With the exception of limonene, all of the terpenoids tested in solid or liquid media were potent inhibitors of AF biosynthesis with IC_{50} values less than 0.5 mM (Table 1). One inhibitory terpenoid, α-carotene, blocked synthesis of NOR, the first stable AF precursor, in an *A. parasiticus* mutant. This result indicates that inhibition may occur at the level of whole pathway regulation, preventing the accumulation of any pathway intermediates. Among the terpenoids, lutein was the most potent inhibitor at an IC_{50} value of 1.1 μM (Norton 1997). Limonene was inactive against AF production in broth but inhibitory as a volatile in a sealed Petri plate. Another volatile terpenoid, camphene, showed only a modest inhibition of AF production (Mahmoud 1994; Greene-McDowelle et al. 1999). β-carotene, lutein, and zeaxanthin are abundant constituents of maize kernels, and *A. flavus* likely encounters concentrations

of carotenoids in the kernel similar to those shown to inhibit AF production. The concentration of carotenoids in maize kernels varies widely among cultivars, ranging from <0.1 to 72 μg/g (Weber 1987), but the relationship between kernel carotenoid content and resistance to AF contamination is not known. Although several terpenoids have antioxidant/ROS quenching activity, Norton (1997) noted that the best inhibitors of AF production (e.g., lutein or α-carotene) were not the strongest ROS quenchers. Canthaxanthin and lycopene, terpenoids with high singlet oxygen-quenching coefficients (K_q), were much less potent AF inhibitors than other terpenoids with lower K_q values.

Alkaloids

Caffeine

Caffeine is a well-studied inhibitor of AF production. Caffeine inhibited AF production when added with replacement medium to *A. flavus* cultures (Buchanan and Lewis 1984a). This observation has been confirmed in a study showing that decaffeinated coffee beans and powder support higher AF production than normal beans and powder. Incorporation of coffee into Czapek's medium at concentrations of 1% and 3% *w*/*v* reduced total AF production by 25% and 52%, respectively, with no statistically significant reduction in fungal dry weight (Hasan 1999). The proposed mode of action for caffeine is interference with glucose uptake. Caffeine inhibited uptake of [^{14}C]-glucose in a dose-dependent manner with uptake reduced to ~50% at ~10 mM. Other potential modes of action for caffeine, such as inhibition of cAMP phosphodiesterase activity, were not correlated with inhibition of AF production (Buchanan et al. 1983).

Pepper alkaloids

Black pepper (*Piper nigrum*) spices have been shown to inhibit AF production by *A. flavus* (Mabrouk and El-Shayeb 1992). Piperine, an alkaloid constituent of many pepper species, inhibited AF production by *A. parasiticus* with a moderate reduction in growth (Madhyastha and Bhat 1984). Extracts of fruits of long pepper (*Piper longum*) were shown to have anti-toxigenic activity (Lee et al. 2002). Subsequent analysis of active components in the extract identified the alkaloids piperlongumine, piperine, pipernonaline, and piperoctadecalidine. Each of these compounds had some influence on fungal growth and AF production. Piperlongumine was the most active against AF production with the most limited influence on fungal growth (Lee et al. 2002). The mode of action of these alkaloids against AF production is not known.

Plant signaling molecules

Lipoxygenase-generated signals

Plant lipoxygenases (LOX) oxidize linoleic and linolenic acid to form hydroperoxy fatty acids, which can be modified further by allene oxide synthase to produce the signaling compound jasmonic acid (Feussner and Wasternack 2002). It has been proposed that hydroperoxy fatty acids can mimic endogenous fungal oxylipins or Psi factors shown to regulate sporulation and secondary metabolism in *A. nidulans*. Oxylipins could potentially interact with G-protein-coupled receptor complexes upstream of the heterotrimeric G-protein complex that has been shown to regulate AF/sterigmatocystin (ST) production (Hicks et al. 1997; Brodhagen and Keller 2006).

A mixture of linoleic acid (LA) peroxidation products stimulated AF production by *A. flavus* and *A. parasiticus* in synthetic medium, whereas LA alone had no significant effect (Fabbri et al. 1983; Passi et al. 1984). Also, sunflower seeds of increasing age, and therefore higher seed lipid peroxidation, supported greater AF biosynthesis (Passi et al. 1984). Some LOX products have antifungal activity (e.g., nonanol; Greene-McDowelle et al. 1999), whereas others influence AF production with little influence on fungal growth.

LOX pathway products that influence AF production without growth inhibition include methyl jasmonate (MeJA), 9*S*-hydroperoxy-trans-10,*cis*-12-octadecadienoic acid (9*S*-HPODE), 13*S*-hydroperoxy-*cis*-9,trans-11-octadecadienoic acid (13*S*-HPODE), and 13*S*-hydroperoxy-*cis*-9,trans-11,*cis*-15-octadecatrienoic acid (13*S*-HPOTE), although in one study, MeJA delayed spore germination (Goodrich-Tanrikulu et al. 1995; Burow et al. 1997). MeJA at a concentration of 100 μM stimulated AF production after 7 days in *A. parasiticus* cultures grown in static YES medium (Vergopoulou et al. 2001). The same concentration slightly inhibited AF and NOR production by *A. parasiticus* in A&M medium (Burow et al. 1997). In contrast, *A. flavus* was extremely susceptible to inhibition by MeJA with an IC_{50}< 10 nM on minimal medium agar plates (Goodrich-Tanrikulu et al. 1995). The IC_{50} value was much higher on a complex medium (~0.1 mM), although the inhibitory effect was still strong. MeJA was also effective as a vapor treatment on naturally inoculated pistachios. Storing 55 kg of pistachios with 100 μl of MeJA significantly reduced AF contamination (Goodrich-Tanrikulu et al. 1995).

The three hydroperoxy fatty acids varied in their effects on AF/ST production. At 100 μM in static A&M media, 13*S*-HPODE and 13*S*-HPOTE almost completely eliminated AF production in *A. parasiticus*. Treatment with 13*S*-HPOTE also suppressed NOR accumulation in the same *A. parasiticus* strain. When tested against *A. nidulans*, 13*S*-HPODE inhibited ST accumulation by 80%. Treatment with 9*S*-HPODE slightly increased or decreased AF

production, depending on the concentration tested, and also induced prolonged accumulation of transcripts for the AF/ST biosynthetic genes *ApaflM*(*ver-1*) and *AnstcU*. LA treatment also promoted the accumulation of these transcripts (Burow et al. 1997). Some hydroperoxy fatty acids may exert a stronger signaling influence on AF/ST biosynthesis than others. For example, AF biosynthesis by *A. parasiticus* was stimulated in synthetic medium containing a mixture of ~30% 9*S*-HPODE and ~70% 13*S*-HPODE, although 13*S*-HPODE has an inhibitory effect when tested alone (Fabbri et al. 1983).

Volatile alkanals and alkenals are also products of the LOX pathway (Zeringue et al. 1996; Feussner and Wasternack 2002). C_6, C_7, C_8, and C_9 alkenals each strongly inhibited AF production by *A. flavus* cultured on maize, cottonseed, and peanut. The C_9 alkenal, *trans*-2-nonenal, was the most potent inhibitor. Interestingly, C_7–C_9 alkenals stimulated AF production on maize kernels at the lowest concentration tested (1 μl/5 g dried kernels; Zeringue 1991). While growth data after alkenal treatment were not reported in this study, a subsequent study reported that the volatile C_6–C_9 alkenals and C_6–C_9 alkanals strongly inhibited radial growth of *A. flavus* on PDA in Petri plates, suggesting that most of these compounds are not specific inhibitors of AF biosynthesis (Zeringue et al. 1996). Wright et al. (2000) also found that C_6 and C_8 alkanals, as well as *n*-decyl aldehyde, were growth inhibitors. Nonyl aldehyde was the only volatile aldehyde that was inhibitory to AF production without a strong growth inhibition (Zeringue et al. 1996).

Volatiles released from silks of certain maize genotypes inhibited AF production more strongly than fungal growth. For example, *A. flavus* cultured in the presence of 1.5 g of maize silks from Pioneer 3136 (a susceptible line) grew normally, but AF production was only 28% of the control. The volatile(s) contributing to inhibition of AF production is not known (Zeringue 2000). Neem oil volatiles co-incubated with *A. flavus* or *A. parasiticus* in a sealed plate, inhibited AF production to 33% of the control while reducing growth to only 79%. The volatiles assayed included some active compounds identified in the studies already cited, e.g., trans-2-heptenal (Zeringue et al. 2001). Two other volatiles, 3-methyl-2-butanol and 3-methyl-1-butanol, had the unusual effect of inhibiting growth while stimulating AF production (Zeringue and McCormick 1990; Greene-McDowelle et al. 1999).

Ethylene

The volatile compound ethylene is an important plant signaling hormone. Many fungi can also produce ethylene, and fungi undoubtedly encounter ethylene in their environment—either in soil or host plants (Ilag and Curtis 1968). A potential relationship between ethylene and AF production was noted after the observation that non-toxigenic *A. flavus* and *A. oryzae* strains produce less ethylene than toxigenic *A. flavus* and *A. parasiticus* strains (Sharma et al. 1985). This relationship was supported by two subsequent observations. Firstly, addition of 2-chloroethyl phosphonic acid (CEPA), an ethylene-evolving compound, to static and shake cultures of *A. parasiticus* inhibited AF production. CEPA inhibited growth at the higher concentrations tested (1,400–1600 ppm) but not at the lower concentrations inhibitory to AF production. Second, enhanced endogenous ethylene biogenesis, because of the presence of the aldehyde trapping agent, dimedone, inhibited AF production (Sharma et al. 1988). More recently, Roze et al. (2004b) provided direct evidence that ethylene inhibits AF production in *A. parasiticus*. Application of ethylene in a flow-through system at concentrations as low as 0.1 ppm significantly reduced AF production by >50%. Treatment at 11.6 and 146 ppm further reduced AF production with no negative effects on growth or asexual sporulation. The activity of an *aflD* (*nor-1*) promoter::GUS fusion reporter was completely blocked at 0.6 ppm of ethylene, a result consistent with ethylene-regulating AF biosynthesis transcriptionally. Ethylene inhibition had a complex interaction with CO_2 concentration. CO_2 and ethylene inhibited AF production in an additive fashion at 0.1% and 2.0%, respectively (Roze et al. 2004b). In the presence of 3.0% CO_2, there was no added inhibition to the baseline inhibition of ethylene. The ethylene competitor 1-methylcyclopropene (1-MCP) stimulated AF production in control and cyclic-deoxyadenosine-monophosphate-treated *A. parasiticus* cultures, a finding consistent with ethylene regulation of AF production through an ethylene-responsive signal transduction pathway. Interestingly, ethylene also blocked the stimulatory effect of cAMP on AF production. In contrast to the results with *A. parasiticus*, ethylene treatment of *A. nidulans* did not inhibit ST production although it either suppressed or stimulated ascospore and asci formation, depending on the treatment method. In a follow-up study, ethylene inhibited AF production by *A. parasiticus* on peanuts, although the additive interaction with CO_2 was absent on this substrate (Gunterus et al. 2007). How ethylene inhibits AF production is unclear, but ethylene shows promise as a cheap and safe treatment for protection of stored grains and nuts. Ethylene and MeJA are both effective alone, so combination of these volatiles may be an even more effective treatment for grain storage.

Other plant-derived compounds

Phytic acid

Phytic acid (inositol hexakisphosphate) is an abundant component of seeds (typically representing >1% of dry seed

weight) and chelator of polyvalent cations, particularly zinc and iron (Raboy 2001). Because of the antinutritive effects of phytic acid on humans and animals, maize and soybean cultivars with reduced phytic acid are highly desirable. There are, however, conflicting reports on the influence of phytic acid on AF production. Autoclaved soybean seeds supported more AF production than unautoclaved seeds, possibly because of the breakdown of phytic acid and release of chelated zinc (Gupta and Venkitasubramanian 1975). To test whether increased zinc availability accounted for enhanced AF production on soybean seeds, zinc sulfate was added to soybean powder. Zinc sulfate stimulated AF production, and phytic acid suppressed this stimulation, presumably by chelation of zinc. Addition of phytic acid to a synthetic, high-sucrose medium at 10 mM (~6.6 mg/ml) inhibited AF accumulation in the culture medium by ~87%, although fungal growth was not reported (Gupta and Venkitasubramanian 1975). B AFs were more sensitive to inhibition than G AFs (inhibited to 8% and 46% of control, respectively).

Conflicting results were obtained in a later study, where addition of zinc sulfate to autoclaved soybean meal inhibited AF production, but simultaneous addition of phytic acid counteracted this inhibition. When added alone, phytic acid stimulated AF production (Hensarling et al. 1983). However, 33 μg/g phytic acid had no effect on AF production on autoclaved soybeans, but it did suppress AF production fivefold in autoclaved cottonseed (Ehrlich and Ciegler 1985). Ehrlich and Ciegler (1984) found that the effect of phytic acid on AF production by *A. parasiticus* varied in a synthetic medium depending on pH. Medium containing 14.3 mM phytic acid and in which the pH had not been adjusted (pH ~6.6) was strongly inhibitory to AF production, whereas there was no inhibition when the initial pH was lowered to 4.5. Phytic acid concentrations exceeding 20 mM were inhibitory to growth. These results suggest that the effect of phytic acid on AF production is strongly influenced by pH, and results should be interpreted with care.

Although regulation of AF production by phytic acid is attributed to chelation of zinc and other polyvalent cations, other properties may explain these results. Phytic acid is a natural antioxidant that can inhibit iron-catalyzed free radical production and lipid peroxidation (Graf et al. 1987), and this antioxidant activity may contribute to its anti-toxigenic properties. We have found that embryo extracts from *low phytic acid-1* maize (Raboy et al. 2000) support more AF production by *A. flavus* than normal kernels (RAH, unpublished data). It would be prudent to test low phytic acid varieties of crops susceptible to AF contamination for any increase in susceptibility.

Hydroxamic acids

Two hydroxamic acids, 4-acetyl-benzoxazolin-2-one (4-ABOA) and 6-methoxy-benzoxazolin-2-one (MBOA), were strong inhibitors of AF biosynthesis. MBOA was inhibitory toward growth at higher concentrations (>2.5 mM), whereas 4-ABOA did not significantly reduce growth until concentrations reached 50 mM. Benzoxazolin-2-one (BOA), another hydroxamic acid, had a strong growth inhibitory effect (Miller et al. 1996). Interestingly, MBOA has been shown to inhibit germination in cress (*Lepidium sativum* L.) seeds, presumably because of the inhibition of alpha-amylase induction (Kato-Noguchi and Macias 2006). How MBOA inhibits induction of alpha-amylase activity in seeds is uncertain, but perturbation of sugar utilization could conceivably interfere with AF biosynthesis.

Metabolites altering calcium signaling

There are multiple lines of evidence that support a role for calcium-dependent signaling in regulation of AF biosynthesis. Trifluoperazine (TFP), a calmodulin antagonist, inhibited fungal growth at concentrations >0.10 mM, and AF production was reduced to 18.4% of the control after 3 days growth in the presence of 0.14 mM TFP. Addition of TFP to 1-day-old cultures caused a modest reduction in growth (79% of the control), and an even greater reduction of AF accumulation to 42% of the control. Addition of 1 mM TFP with replacement medium to 7-day-old cultures led to a complete inhibition of AF production with no reduction in growth. Also, a set of calmodulin-dependent phosphorylated proteins were identified in 3-day-old TFP-treated cultures that were absent in untreated, toxigenic controls (Rao et al. 1998). However, whether these proteins are specifically associated with regulation of AF biosynthesis or are a response to TFP-mediated growth inhibition remains an open question. Also, TFP can antagonize other Ca^{2+}-binding proteins besides calmodulin, so it is also unclear if calmodulin is the TFP target associated with AF biosynthesis (Roufogalis et al. 1983). Another potential mode of action for TFP may be interference with acetyl-CoA carboxylase activity, one possible route for the production of malonyl-CoA precursors for AF biosynthesis. Acetyl-CoA carboxylase activity was reduced 3.8-fold in the presence of 0.14 mM TFP. Also, TFP at 0.14 mM reduced the incorporation of [14C]-acetate into AFB_1 to 55% of the control. Because 0.14 mM TFP is also the 50% growth inhibitory concentration of TFP, it is difficult to separate the growth inhibitory effect and the specific effect on AF biosynthesis (Rao and Subramanyam 2000).

Altered transport of calcium ions may also negatively affect AF production. Treatment of *A. parasiticus* with the calcium channel blockers verapamil and diltiazem strongly inhibited AF accumulation with only a negligible decrease in growth. Synthesis of G AFs was more sensitive to the channel blockers than was the synthesis of the B AFs. After treatment

with 1 mM verapamil, AFB_1 was still 78% of the control, whereas AFG_1 and AFG_2 concentrations were reduced below detectable levels (Rao and Subramanyam 1999).

Expression of calmodulin and calcineurin proteins was induced in a toxigenic *A. parasiticus* strain but not in a nontoxigenic strain. The toxigenic strain also contained higher levels of total phosphorylated protein. Intriguingly, AF was shown to inhibit calmodulin kinase activity in a dose-dependent manner (Jayashree et al. 2000). Although, these results suggest a relationship between AF production and the concentration of calcium signaling factors, the nature of the relationship remains unclear—particularly because the basis of atoxigenicity in the nontoxigenic strain is uncertain. Juvvadi and Chivukula (2006) identified calmodulin-binding domains in silico in the primary sequence of AF pathway transcriptional regulators AflR and AflJ, and three AF biosynthetic enzymes, presenting the possibility that calmodulin may influence transcriptional regulation of the AF biosynthetic gene cluster.

Antibiotics and cyclic dipeptides

Bacteria are a rich source of fungistatic and anti-aflatoxigenic compounds as evidenced by co-culturing experiments with *Aspergillus* spp. (e.g., Palumbo et al. 2006). Efforts in this area have yielded several highly active inhibitors of AF production. The bacterium *Achromobacter xylosoxidans* was found to inhibit accumulation of AF precurors (NOR and hydroxyversicolorone) in two biosynthetic mutant strains of *A. parasiticus*. The cyclic dipeptide cyclo(L-leucyl-L-prolyl) was purified biochemically and shown to be responsible for the observed inhibition. Single amino acids and mixtures of amino acids did not have the same effect. Two other cyclic dipeptides, cyclo(D-leucyl-D-prolyl) and cyclo(L-prolyl-L-valyl) were also inhibitory. The activity of cyclo(D-leucyl-D-prolyl) suggests that inhibition is not entantiomer specific (Yan et al. 2004).

Aflastatin A (AsA) isolated from *Streptomyces* sp. MRI142 (Sakuda et al. 1996) did not inhibit growth of *A. parasiticus* in broth cultures but did reduce the radial growth rate when dissolved in agar. It was very active against AF biosynthesis with an IC_{50} of <0.05 μM on agar plates and <0.1 μM in broth. AsA also had antimicrobial activity against Gram-positive bacteria and fungi. The minimum growth inhibitory concentration against *A. parasiticus* on PDA was >0.8 mM, much higher than the IC_{50} for inhibition of AF production (Ono et al. 1997). To investigate how AsA influences AF production, its effect on NOR production and AF biosynthetic gene expression were measured. NOR concentration in an AF biosynthetic mutant strain and transcript abundance of *aflR* and three biosynthetic genes in a wild-type strain were suppressed at ~0.2 μM AsA. The mode of action of AsA is not known, but it did enhance glucose utilization and accumulation of ethanol. Also, transcripts for aldehyde dehydrogenase (*aldA*) and acetyl-CoA synthetase (*facA*), involved in ethanol utilization, were suppressed by AsA (Kondo et al. 2001). These observations suggest that inhibition is probably a result of perturbations in primary metabolism as suggested by Yoshinari et al. (2007).

An elegant approach allowing dissection of anti-toxigenic and antimicrobial activities was used to investigate blasticidin A (BcA), an antibiotic from *S. griseochromognes* that is structurally related to AsA. Like AsA, BcA suppresses transcription of *aflR* and AF biosynthetic pathway genes. Synthetic derivatives of BcA lacking the tetramic acid moiety lost antimicrobial activity against *S. cerevisiae* but not AF inhibitory activity. Elimination of the tetrahydropyran ring increased the IC_{50} for AF inhibition greater than tenfold and further fragmentation abolished inhibition of AF biosynthesis (Sakuda et al. 2000).

More recently, dioctatin A (DotA), an inhibitor of human dipeptidyl aminopeptidase II from *Streptomyces* sp. SA-2581, was shown to inhibit AF production by *A. parasiticus* (IC_{50} 4 μM; Yoshinari et al. 2007). At concentrations up to 50 μM, DotA did not inhibit fungal growth. DotA also inhibited NOR production in an AF biosynthetic mutant. DotA treatment reduced expression of *AflR* and three AF biosynthetic genes. It also increased kojic acid production and induced a fluffy culture morphology with a drastic reduction in conidiation. Although DotA is an inhibitor of dipeptidyl aminopeptidase II in humans, the authors found no homologs present in the Aspergilli for which public genome sequences are available (*A. oryzae*, *A. nidulans*, *A. niger*, and *A. fumigatus*). DotA inhibition of both conidiation and AF biosynthesis indicates possible signaling through the FadA heterotrimeric G-protein signaling cascade (Shimizu et al. 2003;Yoshinari et al. 2007).

Oxidative stress and AF biosynthesis

This survey of inhibitors of AF biosynthesis has illustrated that many inhibitors have antioxidant activity. In contrast, strong inducers of AF biosynthesis such as cumene hydroperoxide, linoleic acid hydroperoxide and carbon tetrachloride are oxidants (De Luca et al. 1995). We have found only one report of an antioxidant, ascorbate, stimulating AF biosynthesis, and this phenomenon was attributed to the ability of ascorbate to form lipid peroxides in concert with ferrous ions (Patel et al. 1990). A large body of work has demonstrated a consistent correlation between markers of oxidative stress (lipid peroxidation, antioxidant enzyme activity, and ROS) and AF biosynthesis (Jayashree and Subramanyam 2000; Reverberi et al. 2005; Narasaiah et al. 2006). Furthermore, oxidative stress is also correlated with cellular development

and differentiation (Aguirre et al. 2005). For example, antioxidants inhibited formation of conidia in *Neurospora crassa* (Hansberg et al. 1993). This is intriguing considering the relationship between conidiation and AF/ST biosynthesis in some Aspergilli. What remains uncertain is the cause–effect relationship between AF biosynthesis, oxidative stress, and the signaling pathways whereby these processes interact. The stimulation and suppression of AF biosynthesis by oxidants and antioxidants, respectively, indicates that perturbations in the oxidative state of the fungal cell influence AF biosynthesis. However, AF biosynthesis and the concomitant reprogramming of primary metabolism may, in turn, contribute to changes in the oxidative state of the cell.

Redox reactions are fundamental to cellular catabolism and anabolism, and antioxidants may hinder vital processes. Maggio-Hall et al. (2005) found that β-oxidation was active during AF biosynthesis on lipid substrates. Inhibition of mitochondrial or peroxisomal β-oxidation by antioxidants may limit the availability of carbon skeletons for polyketide pathways during growth on lipid-rich seeds. The oxidative PPP is also up-regulated during AF biosynthesis, and antioxidants could interfere, thereby, reducing the pool of nicotinamide adenine dinucleotide phosphate (reduced form) available for the AF biosynthetic reactions. Brock and Buckel (2004) observed a substantial reduction (46% in the wild-type strain) of combined glucose-6-phosphate dehydrogenase and gluconate-6-phosphate dehydrogenase activities in *A. nidulans* when propionate and glucose were used as a carbon source instead of glucose alone. Comparison of an AF-producing strain of *A. parasiticus* and four AF biosynthetic mutants blocked at different steps in the AF biosynthetic pathway showed a consistent increase in multiple measures of oxidative stress with biosynthesis of progressively later pathway products (Narasaiah et al. 2006). Potentially, AF biosynthesis and oxidative stress are under positive feedback regulation as is common in processes under complex regulation.

Although antioxidants can inhibit AF biosynthesis, as mentioned earlier, the radical quenching activity of an antioxidant compound does not predict its effect on AF biosynthesis. Beyond ROS quenching, inhibition of specific oxidative enzymes may be important. Multiple plant flavonoids, including some biflavonoids, have been shown to inhibit LOX and cyclooxygenase activities in animal systems (Ferrándiz and Alcaraz 1991; Nijveldt et al. 2001; Kim et al. 2004a). Potentially, fungal LOX enzymes are also sensitive to inhibition by flavonoids, leading to reduced lipid peroxidation and, therefore, AF biosynthesis.

Application of inhibitors

The complexity of altering plant natural product pathways makes it critical to identify the most promising candidates before attempting to engineer a host crop species for resistance to AF contamination. One concern with the data on AF inhibitors is that the majority of experiments were conducted in vitro in media that do not approximate conditions on host plant seeds. Screening of inhibitors added to media that incorporate host seed extracts and with a pH closer to the seed pH may improve success in identifying efficacious inhibitors. Increasing the concentration of inhibitors *in planta* may be achieved by either up-regulating biosynthetic pathways already present in the host (e.g., luteolin in maize) or introducing transgenes encoding biosynthetic enzymes from other organisms. Other considerations include tissue-specific or inducible expression (Gurr and Rushton 2005; Munkvold 2003). In addition to production of inhibitors, the development of plants that can degrade mycotoxins is another promising approach (Duvick 2001; Igawa et al. 2007).

Although many inhibitors of AF biosynthesis are not amenable to genetic engineering or treatment of stored seeds, they still may have utility in studies investigating the complex regulation of AF production. With new systems biology approaches that allow researchers to combine datasets from profiling of transcripts, proteins, and metabolites, inhibitory compounds with different modes of action could provide useful probes for dissecting different facets of AF regulation. Global profiling data from inhibitors could also be compared with data sets generated using environmental conditions that influence AF production (temperature, pH, carbon, and nitrogen source) and regulatory gene mutants impaired in AF biosynthesis.

In summary, there are likely hundreds, if not thousands, of compounds that influence AF biosynthesis with IC_{50} values ranging from submicromolar to several millimolar. A common feature of many inhibitors is antioxidant activity, yet the modes of action of most inhibitors are still poorly understood. Some of these inhibitors block AF biosynthesis at lower concentrations but are also inhibitory toward growth at higher concentrations. This may indicate that secondary metabolism is sensitive to stress resulting from low concentrations of growth inhibitory compounds. In all cases, where inhibition of NOR was measured, it was inhibited in parallel with AF, consistent with inhibition of AF production occurring at the regulatory level of biosynthesis rather than at specific steps within the pathway. Elucidation of the regulatory targets of these inhibitors will greatly aid our understanding of how environmental and other signaling inputs can regulate AF biosynthesis.

Acknowledgment The authors gratefully acknowledge financial support from the Aflatoxin Multi-Crop Working Group, North Carolina Agricultural Research Service and Initiative for Future Agriculture and Food Systems grant no. 2001-52101-11507 from the USDA Cooperative State Research, Education, and Extension Service.

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