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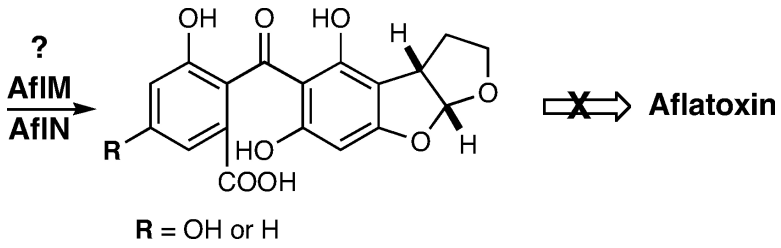

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Synthesis and Fate of *o*-Carboxybenzophenones in the Biosynthesis of Aflatoxin

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Abstract: *o*-Carboxybenzophenones have long been postulated to be intermediates in the oxidative rearrangement of anthraquinone natural products to xanthones in vivo. Many of these Baeyer−Villiger-like cleavages are believed to be carried out by cytochrome P450 enzymes. In the biosynthesis of the fungal carcinogen, aflatoxin, six cytochromes P450 are encoded by the biosynthetic gene cluster. One of these, AflN, is known to be involved in the conversion of the anthraquinone versicolorin A (**3**) to the xanthone demethylsterigmatocystin (**5**) en route to the mycotoxin. An aryl deoxygenation, however, also takes place in this overall transformation and is proposed to be due to the requirement that an NADPH-dependent oxidoreductase, AflM, be active for this process to take place. What is known about other fungal anthraquinone → xanthone conversions is reviewed, notably, the role of the *o*-carboxybenzophenone sulochrin (**25**) in geodin (**26**) biosynthesis. On the basis of mutagenesis experiments in the aflatoxin pathway and these biochemical precedents, total syntheses of a tetrahydroxy-*o*-carboxybenzophenone bearing a fused tetrahydrobisfuran and its 15-deoxy homologue are described. The key steps of the syntheses entail rearrangement of a 1,2-disubstituted alkene bearing an electron-rich benzene ring under Kikuchi conditions to give the 2-aryl aldehyde **43** followed by silyltriflate closure to a differentially protected dihydrobenzofuran **44**. Regiospecific bromination, conversion to the substituted benzoic acid, and condensation with an *o*-bromobenzyl alcohol gave esters **47** and **50**. The latter could be rearranged with strong base, oxidized, and deprotected to the desired *o*-carboxybenzophenones. These potential biosynthetic intermediates were examined in whole-cell and ground-cell experiments for their ability to support aflatoxin formation in the blocked mutant DIS-1, defective in its ability to synthesize the first intermediate in the pathway, norsolorinic acid. Against expectation, neither of these compounds was converted into aflatoxin under conditions where the anthraquinones versicolorin A and B readily afforded aflatoxins B1 and B2. This outcome is evaluated further in a companion paper appearing later in this journal.

Both heme and non-heme oxygenases play prominent roles in creating the structural diversity of every natural product class. While these enzymes typically catalyze hydroxylation reactions at C−H bonds, they also can mediate pivotal oxidative cleavage, insertion, desaturation, and rearrangement chemistry that is much less well understood. In the biosynthesis of the potent environmental carcinogen aflatoxin B1 (**11**, AFB1) from *Aspergillus parasiticus* and *A. flavus*, at least four of six cytochromes P450 are thought to play critical roles among the ca. 15 transformations that convert the earliest polyketide-derived intermediate, the anthraquinone norsolorinic acid (**1**, NA), to the highly modified coumarin product **11** (Scheme 1).[1,2]

One such P450 (AflG)[3] contributes to the multistep conversion of the hexanone side chain of NA to the tetrahydrobisfuran of versicolorin B (**2**, VB; Scheme 1).[4−10] A second monooxygenase (AflL) catalyzes the desaturation of VB to versicolorin A (**3**, VA).[11−13] Not all of the available VB is consumed by AflL in vivo, and the resulting substrate/product mixture constitutes the first branch point of the biosynthetic pathway.[12,14] Gene disruption experiments suggest that the anthraquinones VB and VA convert to xanthone bisfurans **4** and **5**, respectively, through the joint action of a cytochrome P450 (AflN)[15] and an NADPH-dependent ketoreductase (AflM).[13,16,17] This complex transformation is the focus of the experiments that follow.

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(3) The genetic nomenclature of the aflatoxin biosynthetic pathway is designated throughout this and the accompanying paper according to the recently published ordering of Yu et al. (Yu, J.; Chang, P. K.; Ehrlich, K. C.; Cary, J. W.; Bhatnagar, D.; Cleveland, T. E.; Payne, G. A.; Linz, J. E.; Woloshuk, C. P.; Bennett, J. W. *Appl. Environ. Microbiol.* **2004**, *70*, 1253−1262). In this review, the aflatoxin genes, originally named according to their respective substrate or enzymatic function, were standardized with the three-letter code "*afl*" and arranged alphabetically according to their ordering in vivo: *aflG = avnA, aflL = verB, aflM = ver1, aflN = aflS, aflO = omt1, aflP = omtII, and aflQ = ordA.*
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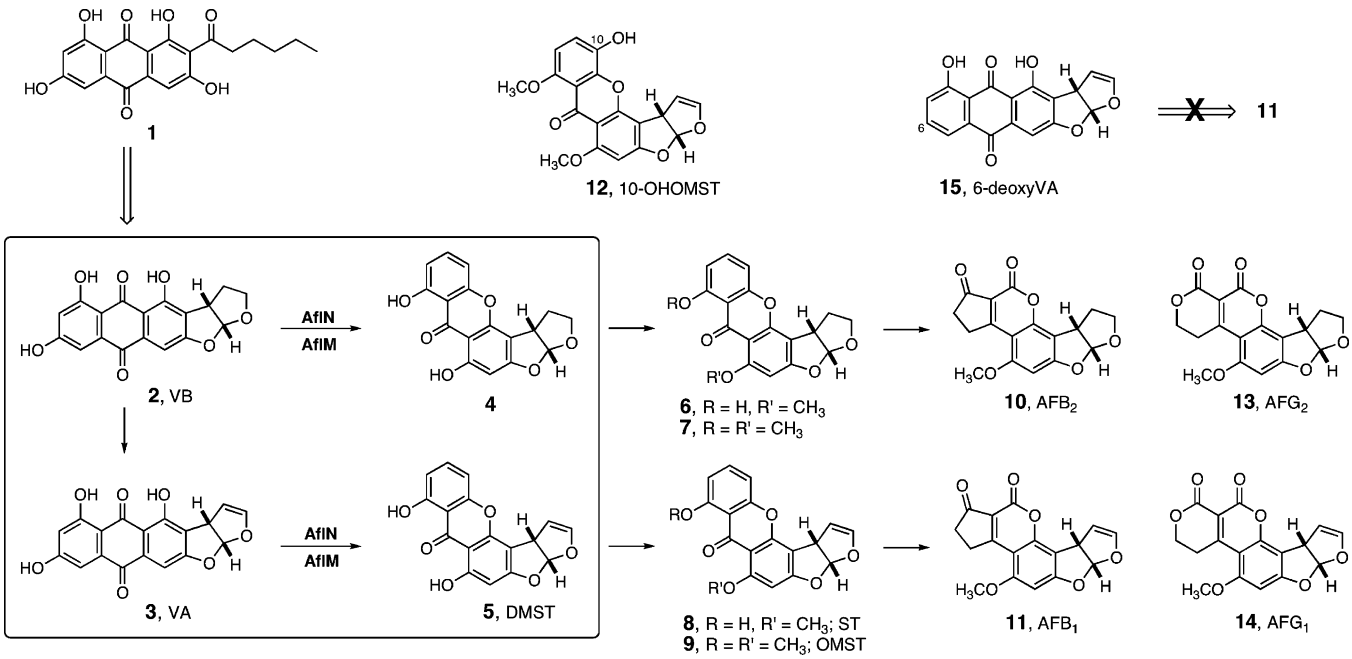
10.1021/ja045520z CCC: $30.25

Scheme 1



Successive O-methylations then are catalyzed by substrate-specific SAM-dependent O-methyltransferases (AflO followed by AflP) during the conversion of demethylsterigmatocystins **4** and **5** to the *O*-methylsterigmatocystins **7** and **9**.[18−23] Recently, we confirmed and extended the observation of Wolochuk et al. that the coumarins aflatoxin B2 and B1 (**10** and **11**, respectively) are formed through the action of a *single* P450 (AflQ) from the *O*-methylsterigmatocystins (**7** and **9**, respectively; OMST).[24] We showed that this remarkable sequence takes place through two oxidative cycles of this enzyme to first hydroxylate **9** at C-10 to 10-hydroxyOMST (**12**, Scheme 1) and then to cleave the xanthone A-ring followed by reclosure, decarboxylation, and demethylation.[25,26] The loss of C-10 uniquely at the oxidation state of carbon dioxide had been established earlier in a sensitive radiochemical assay[27] and provides important support to the idea that two cycles of P450-catalyzed oxidation are required.[26]

Additionally, the G-series (**13**/**14**) and B-series (**10**/**11**) aflatoxins arise in parallel from the AflQ-catalyzed oxidation of OMSTs (**7** and **9**) during a final branching step of the biosynthetic pathway. Although the mechanism for lactone formation has not been established, Yabe conclusively demonstrated that the B-series coumarins are not precursors of the G-series.[28] Thus, the biosynthetic pathway concludes with aflatoxins B2 (**10**) and G2 (**13**) containing the fully saturated tetrahydrobisfuran moiety and aflatoxins B1 (**11**) and G1 (**14**, Scheme 1) containing the electron-rich dihydrobisfuran, the lethal seat of its activation as a carcinogen.[29−31]

Although of unknown mechanism, the conversion of anthraquinones **2**/**3** to xanthones **4**/**5** has been characterized by genetic and biochemical experiments. Genetic disruptions of *either* AflN[15] *or* AflM[13,16] led to the accumulation of VA and blocked the formation of sterigmatocystin (**8**, ST). Pathway intermediates were not detected from these experiments, and notably, separate disruptions of either gene yielded the same phenotype. The corresponding in trans complementation of each gene successfully restored xanthone formation.

Insights into the anthraquinone → xanthone conversion have been gained in isotope labeling experiments. For example, a single labeling pattern was observed from the incorporation of [1,2-$^{13}C_2$]acetate into sterigmatocystin (**8a**, ST; Scheme 2). Therefore, rings A and C from VA must never become C_2-symmetric during the course of the rearrangement of the anthraquinone to the xanthone framework.[32−34] Incorporation of oxygen (*) from $^{18}O_2$ into ring C of ST suggested a Baeyer−

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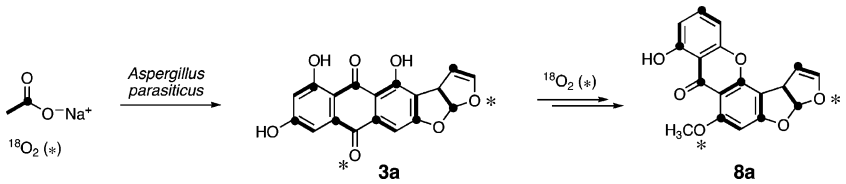
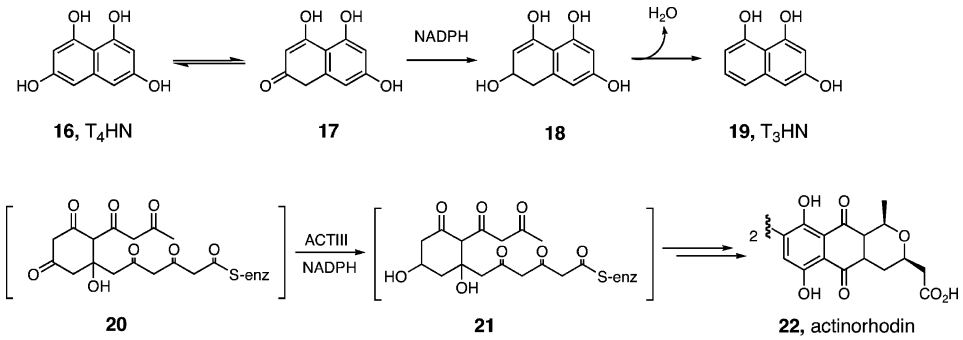
Scheme 2



Scheme 3



Villiger-like addition to the anthraquinone carbonyl and regiospecific insertion of molecular oxygen following migration of ring C.[35,36]

A key mechanistic question is whether AflN-mediated oxidation takes place before or after AflM-catalyzed reduction. Important precedent for anthraquinone reduction as the first step of xanthone formation can be found in the well-documented NADPH-dependent conversion of emodin (**23**) to chrysophanol (**27**, Scheme 4).[37,38] One can visualize these reactions as occurring directly on the keto-tautomer of the C-6 phenol or by conjugate addition at C-6 stabilized by the anthraquinone C-9 carbonyl and elimination of hydroxide. Similarly, the NADPH-dependent reductases (99% identical to each other)[39] that function on 1,3,6,8-tetrahydroxy (**16**, T_4HN)- and 1,3,8-trihydroxynaphthalene (**19**, T_3HN) early in melanin biosynthesis are 64% identical to those of AflM in a BLASTP primary sequence alignment (Scheme 3).[40] Interestingly, the ^1H NMR signals of T_4HN (**16**) in acetone corresponded to ca. 30% of the keto-tautomer **17** in equilibrium with the phenolic form, while no such signals were evident in the corresponding spectrum of T_3HN.[41] These observations argue that reduction of the keto-tautomer takes place in these reactions. Consistent with this view, the biosynthesis of actinorhodin (**22**) in *Streptomyces coelicolor* includes an ActIII ketoreductase-mediated deoxygenation of a seemingly pre-aromatic cyclohexadienone intermediate **20**.[42] The predicted amino acid sequence of ActIII is 33% identical and 53% similar to that of AflM. Against expectation, however, the corresponding deoxygenated analogue of VA, 6-deoxyVA[43] (**15**, Scheme 1), was not converted to AFB1 in either whole-cell[44] or cell-free experiments[45] despite its structural similarity to **19**, **22**, and **27**. If reduction is not the first step in the anthraquinone → xanthone conversion, oxidation must initiate the overall process. Potential roles for the P450 AflN can be visualized by considering the fate of other anthraquinones in related biosynthetic pathways.

Three cases become germane, and each is thought to involve a Baeyer−Villiger oxidation (Scheme 4). In the first, the spirodienone (+)-geodin (**26**)[46] arises from oxidative phenolic coupling of sulochrin (**25**), a reaction known to be catalyzed by a blue copper protein.[47−49] Sulochrin would appear to arise by Baeyer−Villiger cleavage of questin (**24**) and obvious methylation.[50−52] Next, secalonic acid A (**29**)[53−55] and ravenelin (**31**)[56] are known to be derived from emodin (**23**) and chrysophanol (**27**). The biosyntheses of these fungal metabolites are thought to proceed by way of Baeyer−Villiger oxidation of chrysophanol to **28**. By analogy to sulochrin (**25**) formation, one and two such Baeyer−Villiger oxidations of **27** are proposed to yield intermediates **28** and **30**, respectively. The free carboxylate of **28** may be lost as carbon dioxide in the formation of **30**. In each case, aryl epoxidation of these benzophenone

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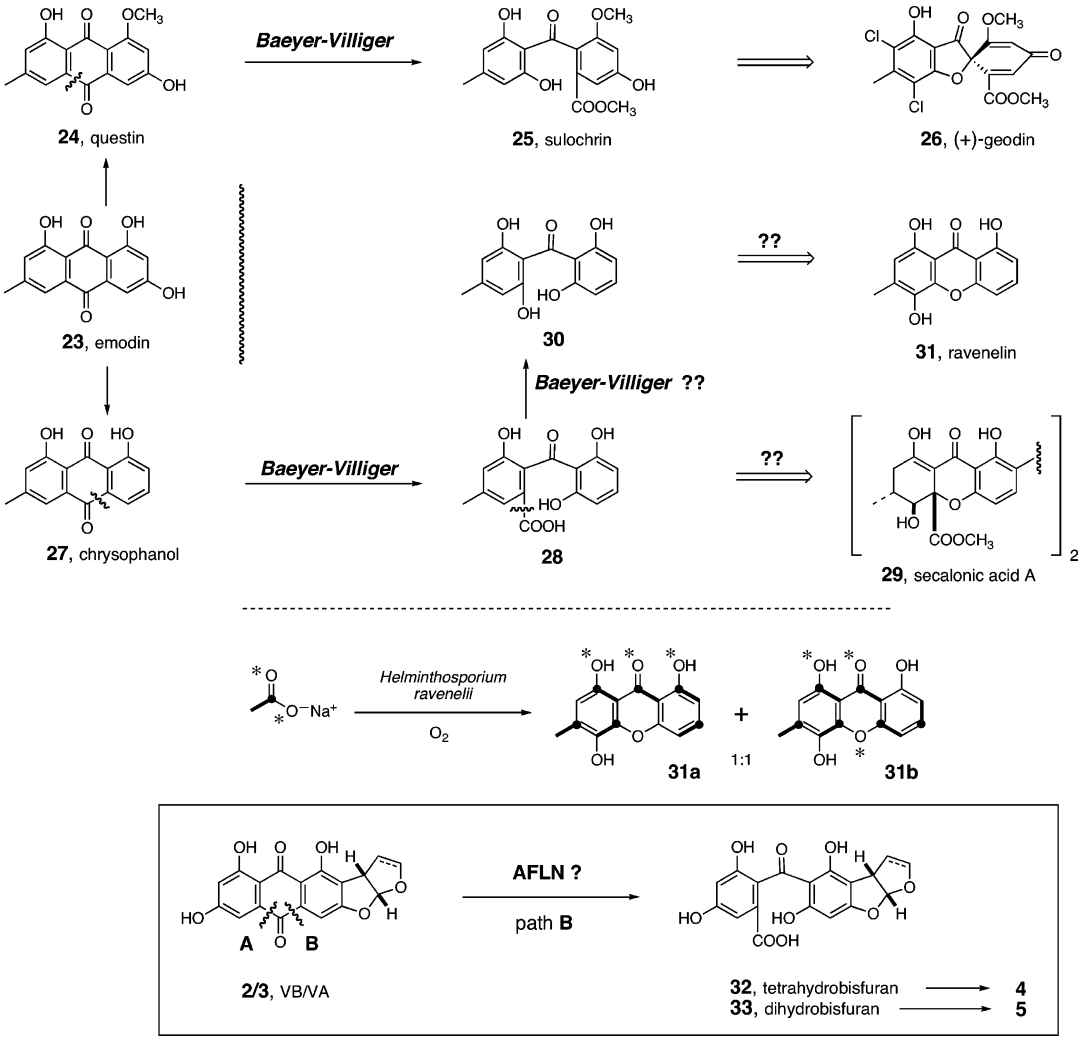
Scheme 4



intermediates can be invoked (among a limited number of possible mechanisms) to achieve xanthone closure. All three metabolites **26**, **29**, and **31** arise from benzophenone precursors comprising one symmetrical aromatic ring. In contrast to the single labeling pattern observed from [1,2-$^{13}C_2$]acetate in sterigmatocystin (**8a**, Scheme 2),[32] incorporation of this tracer into geodin, secalonic acid,[57] and ravenelin[58−60] (**31a**/**31b**, Scheme 4) yielded two orientations in a 1:1 ratio, doubtlessly arising through reaction intermediates with local C_2 symmetry.

In the formation of all three of these metabolites, a mechanistic pattern can be seen that requires multiple oxidative cycles. The geodin (**26**) spirodienone results from Baeyer−Villiger and oxidative phenolic coupling reactions, whereas genesis of the xanthone-like structure of secalonic acid (**29**) and of ravenelin (**31**) can be invoked from Baeyer−Villiger oxidation and aryl epoxidation of chrysophanol (**27**, Scheme 4). The biosynthesis of ST from VA also requires two rounds of oxidation and a reduction to account for the overall redox changes that take place in the course of the rearrangement. In considering the possible Baeyer−Villiger oxidations of VA, cleavage **B** in **2**/**3** (Scheme 4) would produce asymmetric A-ring *o*-carboxybenzophenone intermediates **32**/**33**, whereas the alternative reaction (cleavage **A**) would afford a symmetric 2,4,6-trihydroxyphenyl intermediate from the anthraquinone A-ring and can be excluded. Neither *o*-carboxybenzophenone **28** nor **30** has been tested as an intermediate in the formation of ravenelin or the secalonic acids, respectively. The necessary intervention of symmetrical intermediates and the precedent of sulochrin (**25**) in geodin biosynthesis, however, logically support the intermediacy of these cleaved anthraquinone products in the pathways to **29** and **31**. Analogously, for the case of aflatoxin, no intermediates are known between the anthraquinones **2**/**3** and the xanthones **4**/**5**. We deduce from these biochemical precedents that *o*-carboxybenzophenone **33** (Scheme 4) could be the first intermediate en route to demethylsterigmatocystin (**5**).[33,34,36,45,61]

We have chosen to prepare the reduced tetrahydrobisfuran **32** on two accounts to test the potential intermediacy of this hypothetical anthraquinone cleavage product in the biosynthesis of AFB2. First, the tetrahydrobisfuran-containing products are significantly more stable and readily synthesized than their dihydrobisfuran counterparts. Second, in the aflatoxin-producing organism, *A. parasiticus*, AFB2 (**10**) appears in much smaller

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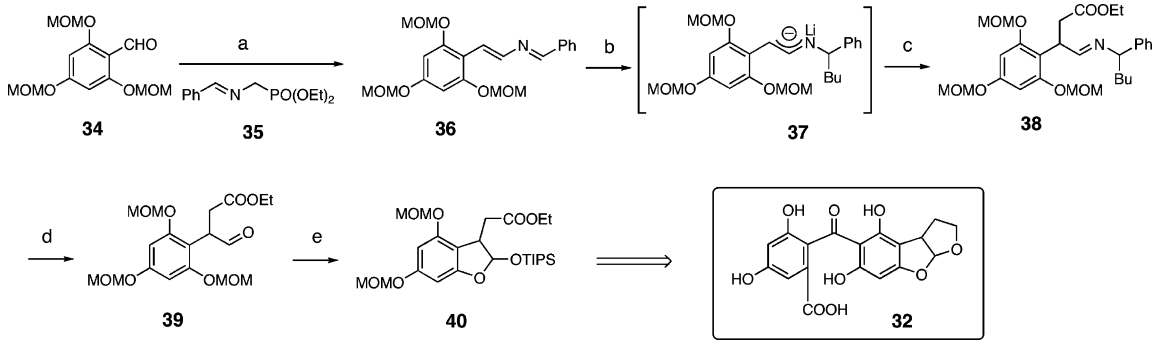
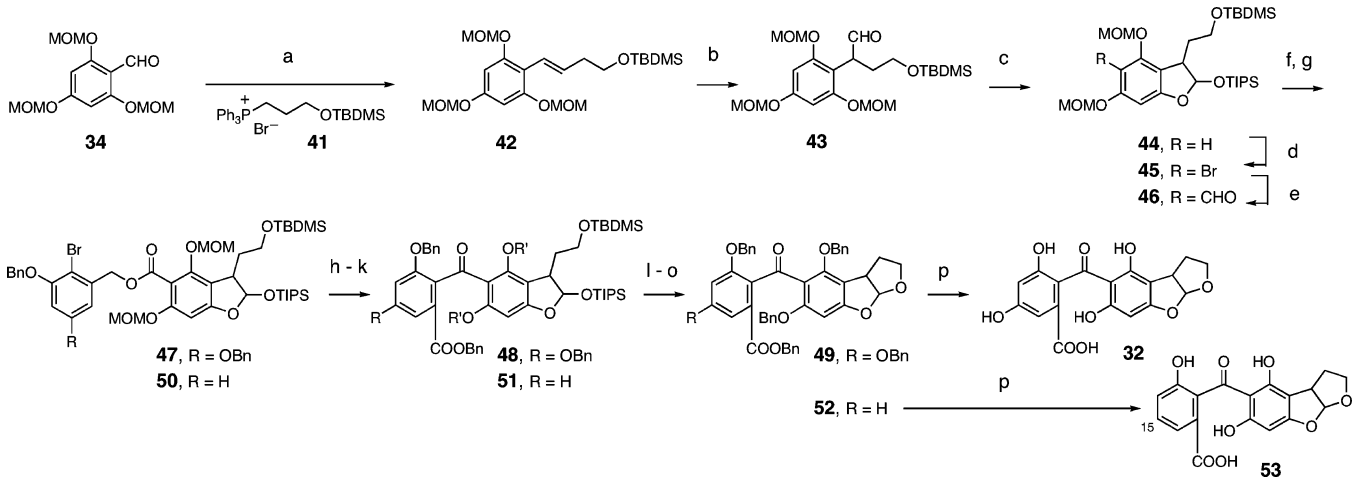
Scheme 5 [a]



[a] Reagents and conditions: (a) **35**, THF, *n*-BuLi, −78 → −60 → −78 °C, then add **34**, −78 → 25 → −78 °C; (b) *n*-BuLi, −78 °C, 2 h; (c) ethyl bromoacetate, −78 → 25 °C; (d) tartaric acid (1 M), 24% from **34**; (e) TIPSTfl, DIPEA, THF, 0 °C, 78%. MOM = methoxymethyl, TIPSTfl = triisopropylsilyl trifluoromethanesulfonate, DIPEA = diisopropylethylamine.

Scheme 6 [a]



[a] Reagents and conditions: (a) **41**, *n*-BuLi, THF, −78 → −25 → −78 °C, then add **34**, −78 → 0 °C, 80%; (b) Ag_2O, I_2, dioxane, water, −78 → 25 °C, 4 h; (c) TIPSTfl, DIPEA, THF, 0 °C, 69% over two steps; (d) NBS, $CHCl_3$, 0 °C, 93%; (e) pentane, −45 °C, *n*-BuLi, 2 min, then add DMF, AcOH, 83%; (f) $NaClO_2$, THF, water, *t*-BuOH, NaH_2PO_4, 25 °C; (g) PPh_3, DEAD, THF, benzyl alcohols **55** *or* **57**, 25 °C, 76% from **46**; (h) *n*-BuLi, THF, −100 °C; (i) TPAP, NMO, CH_2Cl_2, 0 → 25 °C; (j) $NaClO_2$, THF, water, *t*-BuOH, NaH_2PO_4, 2-methyl-2-butene, 0 → 25 °C; (k) PPh_3, DEAD, THF, BnOH, 25 °C, 62% over four steps; (l) BCl_3, CH_2Cl_2, 0 → 25 °C, 93%; (m) NaH, BnBr, DMF, 0 → 25 °C, 75%; (n) repeat steps l and m, 72%; (o) TBAF, THF, 0 → 60 °C, then HCl (3 M), 25 °C, 86%; (p) H_2 (30 psi), Pd black, EtOAc, C18 chromatography, 67%. TBDMS = *tert*-butyldimethylsilyl, NBS = *N*-bromosuccinimide, DEAD = diethyl azodicarboxylate, TPAP = tetrapropylammonium perruthenate, NMO = 4-methylmorpholine *N*-oxide, TBAF = tetrabutylammonium fluoride.

amounts than the unsaturated AFB1 (**11**). That is, incorporation experiments to detect the formation of AFB2 will be more sensitive by enhancing the amount of the *minor* metabolite rather than the major (AFB1) and, as so, confer an analytical advantage without recourse to radiolabeled substrates.

Results

A multistep synthesis of the tetrahydrobisfuran-containing *o*-carboxybenzophenone **32** was undertaken from 2,4,6-trihydroxybenzoic acid. Following esterification and methoxymethyl ether formation, LAH reduction and oxidation with TPAP/NMO readily afforded the protected aldehyde **34** (Scheme 5). Initial elaboration of the fused tetrahydrobisfuran was attempted by modification of a method originally developed by Martin et al.[62] and extended for dihydro- and tetrahydrobisfuran syntheses.[44,61] Benzaldehyde **34** was converted to azadiene **36** following Horner−Emmons condensation with iminophosphonate **35**. Addition of *n*-butyllithium gave the lithioenamine **37**, which was quenched with ethyl bromoacetate to alkylate specifically on carbon.[63,64] Unfortunately, hydrolysis of the resulting imine **38** gave poor yields of the desired phenylacetaldehyde **39** under even mild conditions, presumably owing to the intrinsic instability of the electron-rich product in acid.

The strong electron donor properties of the tri-*O*-methoxymethyl phenyl ring were turned to advantage by preparing the branched phenylacetaldehyde **43** (Scheme 6) in a Pinacol-like rearrangement of a linear precursor under functionally neutral conditions. Wittig reaction of benzaldehyde **34** with the readily prepared propylphosphonium bromide **41**[65] smoothly afforded the styrene **42** in 80% yield (5:2 trans:cis). When treated with I_2/Ag_2O in dioxane/water,[8,66] the initially formed iodohydrin rearranged to the phenylacetaldehyde **43** in excellent yield and was readily scalable. The desired product was found to be prone to polymerization and decomposition. As a consequence, **43** was

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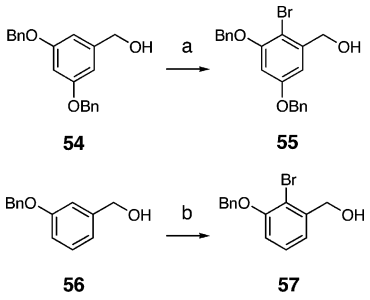
Scheme 7. Preparation of 2-Bromobenzyl Alcohols **55** and **57**[a]



[a] Reagents and conditions: (a) NBS, $CHCl_3$, 0 → 25 °C, 92%; (b) *n*-BuLi, toluene, −78 → −5 °C, 1 h, −78 °C, then 1,2-dibromotetrachloroethane, −78 → 25 °C, 12 h, 52%.

partially purified by rapid filtration through a plug of silica gel and reacted with triisopropylsilyl triflate (TIPSTfl) to induce selective methoxymethyl deprotective cyclization[8] yielding the stable siloxyacetal **44**.

Electrophilic bromination of **44** with NBS was regiospecific and high-yielding. Metal−halogen exchange and reaction with DMF generated the aldehyde **46** in superior yield to direct carbonylation with CO_2. Pinnick oxidation[67] gave the corresponding labile carboxylic acid, which was coupled with 2-bromo-3,5-bis(*O*-benzyl)benzyl alcohol under Mitsunobu conditions to give the ester **47**. The *o*-bromobenzyl alcohol **55** used in this reaction was easily prepared by reaction of the symmetrically substituted precursor alcohol **54** (Scheme 7) with NBS. Treatment of the ester **47** at −100 °C with *n*-BuLi according to the protocol of Lampe and others in their syntheses of balinol[68−70] proceeded well to the unstable *o*-hydroxymethyl benzophenone. The latter was, therefore, oxidized in two steps to the *o*-carboxylic acid and converted to the stable benzyl ester **48** (R′ = MOM). At this point, the *O*-methoxymethyl ethers on the B-ring were exchanged for benzyl groups (**48**, R′ = Bn) to allow final deprotection by hydrogenolysis. Parallel reactions in which the MOM groups were retained to the final deprotection gave low yields of the fused tetrahydrobisfuran corresponding to **49**, presumably owing to side reactions with the formaldehyde released on hydrolysis. Attempts to cleave both MOM ethers simultaneously under a variety of protic and Lewis acidic conditions led to extensive decomposition and traces of singly cleaved phenols. However, a single ether was reliably cleaved with just 1 equiv of BCl_3. In this way, we obtained a 50/50 mixture of regioisomeric phenols, which co-eluted on silica gel. The phenolic pair was converted to the corresponding benzyl ethers with NaH and benzyl bromide in DMF. We then subjected the resulting mixture to a second round of BCl_3-mediated methoxymethyl cleavage followed by O-benzylation and obtained the fully benzyl-protected benzophenone **48**. Complete benzyl protection having been achieved, the TBDMS and TIPS groups were removed with TBAF,[61] and acidic workup was accompanied by intramolecular ether formation to afford **49** in excellent overall yield. Hydrogenolysis over palladium black, reverse-phase chromatography on C_{18} silica gel and lyophilization of the aqueous fractions furnished the pure acid **32**.

With *o*-carboxybenzophenone **32** in hand, whole-cell fermentation experiments were conducted with the *A. parasiticus* DIS-1 mutant.[71] The DIS-1 strain is characterized by a 2.6 kb insertion into *hexA*, one of two genes encoding specialized fatty acid synthase subunits associated with the first step of the aflatoxin biosynthetic pathway.[72] Therefore, aflatoxin biosynthesis is disrupted, and only trace amounts of aflatoxins are synthesized relative to wild-type producers. The availability in DIS-1, however, of all the other required biosynthetic enzymes, coupled with only background levels of aflatoxins, enabled any increases to be readily detected.[45] Owing to their coumarin nuclei, **10**, **11**, **13**, and **14** are highly fluorescent, and their formation is easily visualized. Furthermore, the aflatoxins **10**, **11**, **13**, and **14** are readily separated by HPLC, and their relative amounts can be easily quantified.

The successful conversions of known, earlier precursors versicolorin B (**2**) and A (**3**) to aflatoxin products were implemented as positive controls. The use of both of these substrates ensured that the relevant biosynthetic enzymes were present and, indeed, compatible with both tetrahydro- and dihydrobisfuran-containing substrates. The fermentations were conducted according to the method of Bhatnagar et al.[73] Adye and Mateles (AM) medium was inoculated with a spore suspension of DIS-1 and shaken for 48 h in the dark at 30 °C. The resulting mycelia were collected on cheesecloth, washed with water, and resuspended in minimal Replacement Medium (RM).[74] One gram portions of cells were alternatively incubated with acetone solutions of VA, VB, *o*-carboxybenzophenone **32**, and acetone in the absence of added substrate. DIS-1 incubations of VA and VB resulted in markedly increased aflatoxin production relative to the incubations of the pure acetone negative control. Much to our disappointment, however, repeated incubation of *o*-carboxybenzophenone **32** resulted in no detectable increase in aflatoxin production (data not shown).

Once convinced of the nonincorporation of *o*-carboxybenzophenone **32** into aflatoxin, we chose to synthesize and incubate the corresponding 15-deoxybenzophenone **53** (Scheme 6). This experiment was conceived to confirm the earlier result that 6-deoxyversicolorin A (**15**, Scheme 1) was not converted to aflatoxin under conditions where versicolorin A was readily transformed to the mycotoxin.[44] That is, this experiment was intended to independently verify the previous interpretation that C-6 reduction is *not* the initial step of the anthraquinone to xanthone conversion.

The synthesis of 15-deoxybenzophenone **53** was easily adapted from the route described above following substitution of the A-ring precursor, 2-bromo-3-benzyloxybenzyl alcohol (**57**, Scheme 7), for the analogous 2-bromo-3,5-dibenzyloxybenzyl alcohol (**55**). The two bromides were prepared by different means. 3,5-Dibenzyloxybenzyl alcohol was sufficiently electron-rich to undergo bromination with NBS. In contrast, the comparatively electron-deficient 3-benzyloxybenzyl alcohol (**56**) was *o*-metalated with *n*-BuLi in toluene, and the resulting

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lithio−anion was quenched with 1,2-dibromotetrachloroethane in modest yield.[68] 2-Bromo-3-benzyloxybenzyl alcohol (**57**) and the benzoic acid derived from **46** were then reacted under Mitsunobu conditions. The resulting ester **50** was smoothly converted to 15-deoxy-*o*-carboxybenzophenone **53** in comparable overall yield in the manner described for the preparation of the 15-hydroxy analogue **32** (Scheme 6).

Acetone solutions of VA, VB, and 15-deoxybenzophenone **53** were administered to *A. parasiticus* DIS-1 whole-cell fermentations, as described above. Again, VA and VB were converted to aflatoxins, while the 15-deoxybenzophenone **53** was clearly not (data not shown).

Neither of the benzophenones **32** or **53** was converted to aflatoxins upon whole-cell fungal incubation. We turned our attention, therefore, to the solubility of our benzophenone substrates relative to those of the versicolorin anthraquinones. Versicolorins A and B are planar, neutral, and relatively hydrophobic. As such, they seem comparatively well suited to the hydrophobic environment of membrane-bound eukaryotic P450 monooxygenases.[75] By contrast, the *o*-carboxybenzophenones **32** and **53** are water-soluble, charged at physiological pH, and rotationally flexible. As a consequence, we were concerned that the hydrophilic benzophenones might be incapable of penetrating the fungal cell wall and/or reaching the membrane-anchored sites thought to house the AflS monooxygenase.

We explored this possibility in the following manner. DIS-1 cells were grown, as described, in 48 h in AM medium, collected, ground with a mortar and pestle, suspended in buffer, and centrifuged. The resulting filtrate was separated from the cellular pellet, and the pellet was resuspended in fresh buffer. The extract and pellet suspensions were independently inoculated with acetone solutions of VA and pure acetone controls. A measurable increase in aflatoxin production from VA was obtained upon incubation with the pellet suspension, but no comparable increase in AFB1 (**11**) was detected upon incubation of VA with the membrane-free preparation. Therefore, a simple "ground-cell" procedure was employed in a final attempt to convert either of the benzophenone substrates **32** and **53** to aflatoxin. Cells were collected, ground with sand in a mortar and pestle, and suspended with buffer. The sandy suspension was used directly for substrate incubations.

The results of the ground-cell incubations are depicted by the representative TLC plate in Figure 1. Lanes A−E correspond to small molecule standards as indicated, and the lanes I−V represent chloroform extracts of the ground-cell incubations. The experiments demonstrate that the known aflatoxin intermediates versicolorin A (**3**) and B (**2**) were successfully incorporated into aflatoxins AFB1 (**11**) and AFB2 (**10**), respectively (blue spots, lanes II and III), while the benzophenone substrates **32** and **53** were not (lanes IV and V). Lane I is the negative control, an extract of the fungal cells incubated with acetone in the absence of added substrate. The absence of incorporation of benzophenones **32** and **53** was confirmed in additional trials and by HPLC analysis.

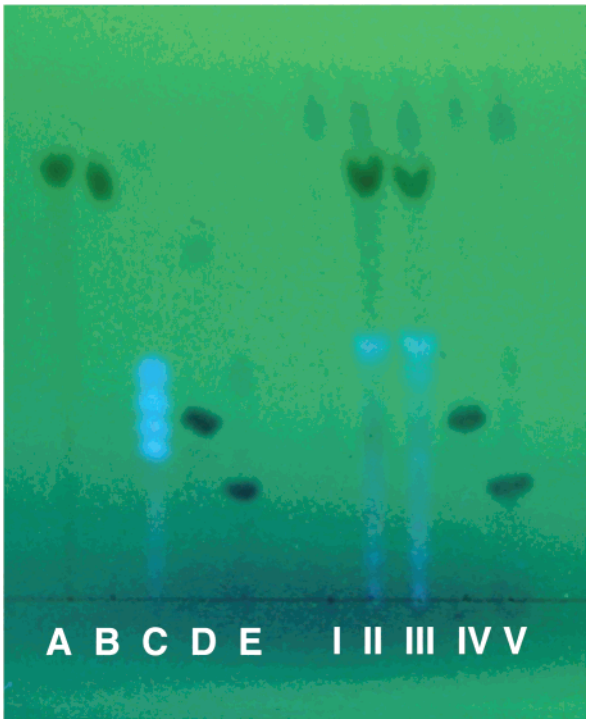

Figure 1. Thin-layer chromatogram of DIS-1 ground-cell incubations developed in 60% $CHCl_3$, 30% EtOAc, and 10% HCO_2H, photographed under short-wave UV light. Lanes A−E standards: A, versicolorin A (**3**); B, versicolorin B (**2**); C (top to bottom), AFB1 (**11**), AFB2 (**10**), AFG1 (**14**), AFG2 (**13**); D, 15-deoxybenzophenone **53**; E, 15-hydroxybenzophenone **32**. Lanes I−V: I, chloroform extract of DIS-1 control; II, DIS-1 with versicolorin A (**3**); III, DIS-1 with versicolorin B (**2**); IV, DIS-1 with 15-deoxybenzophenone **53**; V, DIS-1 with 15-hydroxybenzophenone **32**.

Discussion

Separate disruptions of the aflatoxin pathway genes, *aflM* and *aflN*, led to the accumulation of VA and VB, suggesting essential roles for both encoded proteins in the cryptic oxidative cleavage, rearrangement, and reduction to demethylsterigmatocystins **4** and **5**. The failure of 6-deoxyversicolorin A (**15**) to incorporate into AFB1 (**11**) under conditions where the closely related versicolorin A (**3**) is readily transformed to the mycotoxin, and the inability of the reduced, that is, 15-deoxy-*o*-carboxybenzophenone **53**, to support its synthesis all indicate that AflM-catalyzed reduction is not the first step in the conversion of **2**/**3** to **4**/**5**. An AflN-mediated oxidation must, therefore, initiate this process. Consideration of other known anthraquinone → xanthone conversions led us to explore the possibility that the first chemical step was a Baeyer−Villiger oxidation and ring opening to yield *o*-carboxybenzophenone **32**. If cleavage was to occur in sense **A** shown in Scheme 4, a symmetrical 2,4,6-trihydroxybenzophenone would be generated and the asymmetric labeling pattern from [1,2-$^{13}C_2$]acetate incorporation would be lost in subsequent biosynthetic steps. This outcome is contrary to experiment, and therefore, path **A** can be omitted from further consideration. Analogous cleavage in sense **B**, however, opens the anthraquinone to an asymmetrically substituted A-ring in keeping with skeletal mapping experiments using doubly ^{13}C-labeled acetate.[32−34] Notwithstanding apparent biochemical precedent and chemical logic pointing to *o*-carboxybenzophenone **32** as the next probable intermediate beyond versicolorin A/B, experimental evidence could not be obtained to support this hypothesis despite the

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ability to detect aflatoxin production with exquisite sensitivity. Moreover, positive controls with known intermediates readily afforded aflatoxins under the assay conditions.

The evidence leads inescapably to oxidation of VA and VB as the first step toward xanthone formation. The experiments described here, however, exclude addition of the cytochrome P450-activated oxygen of AflN to the anthraquinone carbonyl followed by Baeyer−Villiger rearrangement. The only alternative becomes attack of the activated oxygen species directly on the aryl rings themselves. Deeper consideration of the order, identity, and effects of two oxidative and one reductive step carried out by AflN and AflM is taken up in a companion paper later in this journal. An additional set of experiments has led to a new hypothesis of xanthone biosynthesis from anthraquinones that could prove general for related acetogenin natural products.

Experimental Section

Only the key synthetic steps are described below. The remaining full synthetic details are available in Supporting Information.

General Materials and Methods: Melting points were determined with a Thomas−Hoover oil bath apparatus in open capillaries and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity^{Plus} 400 MHz spectrometer and are referenced to $CDCl_3$ (7.27 and 77.0 ppm) and d_6-acetone (2.04 and 29.9 ppm) as indicated. High- and low-resolution mass spectra were recorded by Dr. Joseph Kachinski of the Department of Chemistry, The Johns Hopkins University, using a VG Instruments 70-S 250 GC/MS at 70 eV in EI$^+$, CI$^+$, or FAB operating modes. Additional high-resolution mass spectra were performed by Dr. Christopher Hadad of The Ohio State University and by Dr. Ronald Cerny of The University of Nebraska. Infrared spectra were recorded on a Perkin-Elmer 1600 series FTIR spectrophotometer. Combustion analyses were performed by Atlantic Microlab, Inc. (Norcross, GA). Flash chromatography was performed using EM silica gel 60 (230−400 mesh). Thin-layer chromatography (TLC) was performed on Analtech Uniplate glass plates containing fluorescent indicator. TLC plates were photographed by James Van Rensselaer, The Johns Hopkins University. Reagents and aflatoxin standards were purchased from Aldrich, Inc. Media components were purchased from Difco (Detroit, MI) or Fisher (Pittsburgh, PA). Nonaqueous reactions were performed in flame-dried glassware under an atmosphere of N_2 or Ar. Solvents were distilled immediately prior to use (THF and Et_2O from sodium/benzophenone ketyl, and CH_2Cl_2 and CH_3CN from CaH_2). During workup, organic solutions were dried over anhydrous Na_2SO_4. HPLC was performed with a Perkin-Elmer 235C diode array detector and series 410 LC pump using a Phenomenex (Torrance, CA) reverse-phase column (Prodigy 5μ ODS(3) 100Å, 250 × 4.60 mm, 5 micron). Mini-prep columns were purchased from Qiagen (Valencia, CA).

Synthesis of 5-Keto-[(2′,4′-dihydroxy-6′-carboxy)phenyl)]-4,6-dihydroxy-[2,3-b]-benzo-3a,8a-dihydrobisfuran (32). [4-(2,4,6-Tris-(*O*-methoxymethyl)phenyl)but-3-enyloxy]-*tert*-butyldimethylsilyl Ether (42). A slurry of 3-trimethylsiloxypropyltriphenylphosphonium bromide (**41**) (12.0 g, 23.3 mmol) in THF (100 mL) was cooled to −78 °C. A hexane solution of 2.43 M *n*-BuLi (25.6 mmol) was gradually added. The resulting yellow solution turned red following 40 min of stirring at −78 °C, then 40 min at −25 °C. The ylide solution was then cooled to −78 °C, and benzaldehyde **34** (6.2 g, 21.7 mmol) in THF (50 mL) was slowly cannulated under Ar. The solution was stirred 30 min at −78 °C and 30 min at 0 °C. The mixture was then diluted with hexanes and filtered through a silica plug. The resulting filtrate was concentrated and eluted through a column of silica gel with 10−20% EtOAc in hexanes. Styrene **42** (7.6 g, 17.4 mmol, 80%, trans:cis isomers = 2.4:1) was isolated as a clear oil. TLC R_f 0.50 (20% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.53 (m, 2H), 6.21, 6.18 (t, J = 1.6 Hz, 1H), 5.81, 5.78 (t, J = 6.8 Hz, 1H), 5.14, 5.12, 5.10 (s, 6H), 3.70, 3.61 (t, J = 7.2 Hz, 2H), 3.45 (m, 9H), 2.42, 2.21 (dq, J = 2.0, 6.8 Hz, 2H), 0.88, 0.85 (s, 9H), 0.05, 0.00 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 157.8, 157.0, 156.5, 156.0, 130.5, 130.1, 121.9, 121.5, 111.5, 97.7, 97.6, 95.0, 94.9, 94.7, 94.7, 63.5, 62.8, 56.2, 56.2, 56.1, 38.6, 33.8, 26.1, 26.0, 18.4, 18.4, −5.1, −5.2. IR ($CHCl_3$): ν 2955, 1607, 1473, 1393, 1156, 1048, 925, 836 cm^{-1}. MS-EI *m*/*z* (rel inten): 442 (M$^+$, 13), 297 (14), 265 (12), 89 (12), 73 (18), 45 (100). HRMS−FAB (*m*/*z*): M$^+$Na calcd for $C_{22}H_{38}O_7Si$, 465.2279; found, 465.2314.

{3-[2-(*tert*-Butyldimethylsilanyloxy)ethyl]-4,6-bis-(*O*-methoxymethyl)-2,3-dihydrobenzofuran-2-yloxy}triisopropylsilane (44). Styrene **42** (9.4 g, 21.2 mmol), silver(I) oxide (5.2 g, 22.3 mmol), dioxane (100 mL), and water (30 mL) were cooled to 0 °C. A dioxane (80 mL) solution of iodine (5.4 g, 21.2 mmol) was gradually added, and the mixture was stirred 1 h at 0 °C and 2 h at room temperature. The mixture was passed through a plug of silica. The filtrate was diluted with Et_2O and washed with 10% aqueous sodium bisulfite. The etherial layer was dried and concentrated. The crude aldehyde **43** was eluted through a short column of silica gel with 20% EtOAc in hexanes. The resulting clear oil was prone to decomposition and taken immediately to the next reaction. TLC R_f 0.37 (20% EtOAc in hexanes).

Crude aldehyde **43**, DIPEA (5.8 mL, 33.5 mmol), and THF (250 mL) were cooled to 0 °C under Ar. Triisopropylsilyl triflate (7.7 mL, 28.7 mmol) was added dropwise over 5 min. The mixture was stirred 1.5 h and warmed to room temperature. Following quenching with *N*,*N*-dimethylethanolamine (2.5 mL, 25 mmol), the mixture was diluted with Et_2O and washed with cold aqueous 5% HCl, aqueous $NaHCO_3$, and brine. The Et_2O layer was dried and concentrated, and the resulting residue was eluted through a column of silica gel with 5% EtOAc in hexanes. The dihydrobenzofuran **44** was isolated (8.4 g, 14.7 mmol, 69% yield) as a clear oil. TLC R_f 0.63 (10% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.32 (d, J = 2.1 Hz, 1H), 6.24 (d, J = 2.1 Hz, 1H), 5.83 (d, J = 1.6 Hz, 1H), 5.10 (m, 4H), 3.68 (m, 2H), 3.45 (s, 6H), 3.29 (dq, J = 0.8, 4.4 Hz, 1H), 1.98 (m, 1H), 1.70 (m, 1H), 1.13 (m, 21H), 0.88 (s, 9H), 0.02, 0.00 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 160.2, 159.0, 154.4, 111.1, 106.8, 96.0, 95.0, 94.4, 92.9, 61.1, 56.3, 56.2, 47.1, 34.7, 26.1, 18.3, 18.0, 17.9, 12.3, −5.2. IR ($CHCl_3$): ν 2949, 2867, 1609, 1498, 1465, 1389, 1255, 1217, 1156, 1132, 1052, 1021, 949, 882, 836 cm^{-1}. MS-CI *m*/*z* (rel inten): 571 (M + 1, 17), 397 (23), 363 (9), 339 (10), 73 (9), 45 (100). Anal. Calcd for $C_{29}H_{54}O_7Si_2$: C, 61.01; H, 9.53. Found: C, 61.27; H, 9.54.

5-Bromo-{3-[2-(*tert*-butyldimethylsilanyloxy)ethyl]-4,6-bis-(*O*-methoxymethyl)-2,3-dihydrobenzofuran-2-yloxy}triisopropylsilane (45). Acetal **44** (6.0 g, 10.5 mmol) and $CHCl_3$ (150 mL) were cooled to 0 °C under Ar. NBS (2.1 g, 11.5 mmol) was added, and the ice bath was removed. One hour later, the mixture was washed with water and brine, concentrated, and passed through a column of silica with 5−10% EtOAc in hexanes. Pure bromide **45** (6.4 g, 9.8 mmol, 93% yield) was isolated as a faintly yellow oil. TLC R_f 0.67 (10% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.45 (s, 1H), 5.90 (d, J = 0.8 Hz, 1H), 5.2 (m, 4H), 3.8 (m, 2H), 3.58 (s, 3H), 3.45 (s, 3H), 3.40 (dq, J = 0.8, 4.4 Hz, 1H), 1.96 (m, 1H), 1.68 (m, 1H), 1.1 (m, 21H), 0.87 (s, 9H), 0.01, 0.00 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 159.3, 155.1, 151.6, 116.6, 106.4, 98.8, 98.4, 95.5, 95.4, 61.7, 60.7, 58.2, 57.9, 56.5, 55.4, 48.3, 34.6, 26.1, 18.3, 18.0, 18.0, 17.9, 12.3, −5.3. IR ($CHCl_3$): ν 2949, 2867, 2360, 1616, 1588, 1464, 1388, 1256, 1212, 1156, 1041, 926, 882, 836, 777 cm^{-1}. MS-CI *m*/*z* (rel inten): 649 (M + 1, 82), 591 (20), 475 (100), 132 (18), 90 (23), 45 (43). HRMS-EI (*m*/*z*): M$^+$ calcd for $C_{29}H_{53}BrO_7Si_2$, 648.2513; found, 648.2500. Anal. Calcd for $C_{29}H_{53}BrO_7Si_2$: C, 53.60; H, 8.22. Found: C, 53.99; H, 8.32.

5-Formyl-{3-[2-(*tert*-butyldimethylsilanyloxy)ethyl]-4,6-bis-(*O*-methoxymethyl)-2,3-dihydrobenzofuran-2-yloxy}triisopropylsilane (46). Arylbromide **45** (7.3 g, 11.3 mmol) in pentane (125 mL) was cooled to −45 °C under Ar. *n*-BuLi (16.1 mL, 1.4 M in hexanes) was added over 90 s, and the resulting slurry was stirred an additional

30 s. Dry DMF (4.4 mL, 57 mmol) was added in one portion. Stirring was continued for 1 h at −45 °C. An Et_2O solution of acetic acid (24 mL, 1 M) was added at −45 °C. The mixture was diluted with Et_2O (300 mL) and washed with aqueous $NaHCO_3$, water, and brine, then dried, concentrated, and passed through a column of silica gel with 10% EtOAc in hexanes. Benzaldehyde **46** (5.6 g, 9.4 mmol, 83% yield) was isolated as a yellow oil. TLC R_f 0.28 (10% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$): δ 10.32 (s, 1H), 6.46 (s, 1H), 5.95 (s, 1H), 5.21, 5.07 (m, 4H), 3.70 (m, 1H), 3.60 (m, 1H), 3.50 (s, 3H), 3.47 (s, 3H), 3.42 (m, 1H), 2.01 (m, 1H), 1.71 (m, 1H), 1.07 (m, 21H), 0.85 (s, 9H), −0.01, −0.03 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 187.9, 165.3, 163.1, 156.4, 116.2, 112.9, 107.4, 100.5, 95.2, 93.9, 60.7, 57.7, 56.6, 47.4, 34.2, 26.0, 18.3, 17.9, 17.8, 12.2, −5.3. IR ($CHCl_3$): ν 2949, 2870, 1680, 1613, 1467, 1390, 1236, 1118, 1080, 1042, 926, 834, 778 cm^{-1}. MS-EI *m*/*z* (rel inten): 599 (M + 1, 100), 541 (8), 45 (20). HRMS-EI (*m*/*z*): M^+ calcd for $C_{30}H_{54}O_8Si_2$, 598.3357; found, 598.3341. Anal. Calcd for $C_{30}H_{54}O_8Si_2$: C, 60.16; H, 9.09. Found: C, 60.67; H, 9.15.

5-[3′,5′-Bis-(*O*-benzyl)-2′-bromobenzyl]benzoyl-{3-[2-(*tert*-butyldimethylsilanyloxy)ethyl]-4,6-bis-(*O*-methoxymethyl)-2,3-dihydrobenzofuran-2-yloxy}triisopropylsilane (47). (*Procedure A*) Benzaldehyde **46** (3.0 g, 5.0 mmol), THF (25 mL), *t*-BuOH (25 mL), H_2O (10 mL), 2-methyl-2-butene (9 mL, 2 M in THF), and NaH_2PO_4 (20 mL, 1 M in H_2O) were cooled to 0 °C. Sodium chlorite (80% tech. grade, 2.5 g, 22.1 mmol) was added, and the ice bath was removed. After 40 min, the mixture was diluted with EtOAc and water. The organic layer was washed with water and brine, dried, and concentrated. The resulting oily residue was combined with 3,5-dibenzyloxy-2-bromobenzyl alcohol (**55**) (2.6 g, 6.4 mmol), PPh_3 (3.1 g, 11.6 mmol), and THF (100 mL) and cooled to 0 °C under Ar. DEAD (1.84 mL, 11.6 mmol) was gradually added; the ice bath was removed, and the mixture was stirred 30 min, and then concentrated and passed through a column of silica gel with 10% EtOAc in hexanes. Ester **47** was isolated as a clear oil (3.8 g, 3.8 mmol, 76% yield, over two steps). TLC R_f 0.80 (20% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.35 (m, 10H), 6.96 (d, J = 2.8 Hz, 1H), 6.58 (d, J = 2.8 Hz, 1H), 6.51 (s, 1H), 5.89 (s, 1H), 5.47 (dd, J = 9.4, 24.2 Hz, 2H), 5.15 (m, 6H), 5.02 (s, 2H), 3.74 (m, 2H), 3.67 (m, 1H), 3.48 (s, 3H), 3.40 (s, 3H), 2.0 (m, 1H), 1.65 (m, 1H), 1.1 (m, 21H), 0.92 (s, 9H), 0.05 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.2, 161.5, 158.9, 156.3, 155.8, 152.0, 137.6, 136.5, 136.4, 128.7, 128.7, 128.3, 128.1, 127.7, 127.1, 114.4, 111.2, 107.6, 106.3, 103.8, 101.1, 98.6, 95.0, 93.9, 71.0, 70.4, 66.5, 60.7, 57.4, 56.2, 47.7, 34.5, 26.0, 18.4, 18.0, 17.8, 12.2, −5.4. IR ($CHCl_3$): ν 2949, 2866, 1734, 1596, 1465, 1327, 1259, 1158, 1045, 834 cm^{-1}. MS−FAB *m*/*z* (rel inten): 995 (M + 1, 3), 597 (33), 553 (17), 495 (31), 383 (23), 157 (32), 115 (100). HRMS-EI (*m*/*z*): M^+Na calcd for $C_{51}H_{71}BrO_{11}Si_2$, 1017.3610; found, 1017.3613. Anal. Calcd for $C_{51}H_{71}BrO_{11}Si_2$: C, 61.49; H, 7.18. Found: C, 61.48; H, 7.22.

5-Keto-[2′,4′-bis-(*O*-benzyl)-6′-carboxybenzyl]-{3-[2-(*tert*-butyldimethylsilanyloxy)ethyl]-4,6-bis-(*O*-methoxymethyl)-2,3-dihydrobenzofuran-2-yloxy}triisopropylsilane (48, R′ = OMOM). (*Procedure B*) Bromide **47** (3.6 g, 3.7 mmol) in THF (30 mL) was cooled to −100 °C under Ar, and *n*-BuLi (3.0 mL, 1.3 M in hexanes) was added. The bath (N_2/pentane) temperature was then warmed to −78 °C and stirring continued for 30 min. Saturated aqueous $NaHCO_3$, then Et_2O (150 mL), and H_2O (50 mL) were added. The etherial layer was washed with water and brine, then dried, concentrated, and passed through a plug of silica gel with 20% EtOAc in hexanes. The filtrate was concentrated, and the resulting crude alcohol product was immediately combined with CH_2Cl_2 (100 mL), NMO (0.48 g, 4.1 mmol), and 4 Å sieves under Ar. TPAP (0.08 g, 0.24 mmol) was added and stirring continued for 30 min. The green mixture was concentrated, then diluted with EtOAc, and passed through a plug of silica gel. The filtrate was concentrated, combined with THF (24 mL), H_2O (6 mL), *t*-BuOH (24 mL), 2-methyl-2-butene (12 mL, 2 M in THF), and aqueous NaH_2PO_4 (9 mL, 1 M), and stirred at room temperature. Sodium chlorite (80% tech. grade, 3.0 g, 26.5 mmol) was added and stirring continued until the aldehyde was consumed. The mixture was diluted with water (100 mL) and extracted with EtOAc. The organic layer was washed with brine, and then dried, concentrated, and combined with THF (25 mL), PPh_3 (1.18 g, 4.5 mmol), and benzyl alcohol (0.6 mL, 6.0 mmol) at room temperature. DEAD (0.75 g, 4.5 mmol) was gradually added, and the mixture was stirred for 10 min. The mixture was then concentrated and passed through a column of silica gel with 10−20% EtOAc in hexanes. Benzophenone **48** (R′ = MOM; 2.2 g, 2.3 mmol, 62% yield from bromide **47**) was isolated as a clear oil. TLC R_f 0.45 (20% EtOAc in hexanes). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.3 (m, 13H), 6.99 (d, J = 2.1 Hz, 1H), 6.95 (d, J = 6.7 Hz, 2H), 6.62 (d, J = 2.4 Hz, 1H), 6.35 (s, 1H), 5.94 (s, 1H), 5.20 (dd, J = 8.2, 21.0 Hz, 2H), 5.07 (s, 2H), 4.88 (s, 4H), 4.49 (m, 2H), 3.66 (m, 2H), 3.42 (m, 1H), 3.27 (b, 3H), 2.95 (s, 3H), 2.04 (b, 1H), 1.60 (b, 1H), 1.10 (m, 21H), 0.88 (s, 9H), 0.01 (d, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 191.1, 167.8, 162.3, 160.0, 159.0, 157.1, 154.9, 136.2, 135.9, 135.7, 133.7, 128.7, 128.5, 128.3, 128.3, 128.1, 127.8, 127.6, 127.0, 117.8, 115.7, 107.1, 106.7, 103.1, 99.9, 94.8, 93.6, 70.5, 70.4, 67.4, 65.9, 60.9, 57.0, 55.6, 47.8, 34.3, 26.0, 18.3, 17.9, 17.8, 12.2, −5.4. IR ($CHCl_3$): ν 2948, 2867, 1730, 1599, 1465, 1389, 1326, 1258, 1215, 1143, 1044, 924, 836 cm^{-1}. MS−FAB *m*/*z* (rel inten): 1043 (M + Na, 100), 953 (8), 181 (13), 115 (33). HRMS-EI (*m*/*z*): M^+Na calcd for $C_{58}H_{76}O_{12}Si_2$, 1043.4768; found, 1043.4766. Anal. Calcd for $C_{58}H_{76}O_{12}Si_2$: C, 68.20; H, 7.50. Found: C, 68.22; H, 7.62.

Microbiological Procedures

Organisms. The wild-type aflatoxin producing strain of *A. parasiticus* (SU-1, ATCC 56775) was purchased from the American Type Culture Collection (ATCC) of Manassas, VA. The averufin-accumulating strain (AVR-1, SRRC-165) of *A. parasiticus* used for whole-cell incubations was generously provided by Shannon Beltz and Maren Klich of the USDA-ARS-SRRC. The HexA disruption mutant (DIS-1) was provided by Professor John Linz of Michigan State University. Versicolorin A biosynthesis was achieved with the accumulating strain (Wh-1, ATCC 36537) of *A. parasiticus* as described.[45] Sterigmatocystin was isolated from the accumulating strain (ATCC 28286) of *A. versicolor* as described.[26]

AVR-1 and DIS-1 stock cultures were maintained on plates of potato−dextrose agar (PDA) containing potatoes (300 g) diced, boiled, strained through cheesecloth, and combined with glucose (20 g) and agar (15 g). The mixture was diluted to 1 L with distilled water and autoclaved.

Media. Adye and Mateles (AM) medium[74] contained (per liter) 50 g of sucrose, 10 g of KH_2PO_4, 3 g of $(NH_4)_2SO_4$, 1 g of anhydrous $MgSO_4$, and 2 mL of trace metals solution. The trace metals solution contained (per liter) 0.35 g of $Na_2B_4O_7 \times 10\ H_2O$, 0.25 g of $(NH_4)_6Mo_7O_{24} \times 4\ H_2O$, 5.0 g of $Fe_2(SO_4)_3 \times 6\ H_2O$, 0.15 g of $CuSO_4 \times 5\ H_2O$, 0.055 g of $MnSO_4 \times H_2O$, and 8.8 g of $ZnSO_4 \times 7\ H_2O$.

Replacement Medium (RM) contained (per liter) 1.62 g of glucose, 5 g of KH_2PO_4, 0.5 g of KCl, 0.25 g of anhydrous $MgSO_4$, and 2 mL of trace metals solution. The medium was autoclaved at 140 °C, 20 psi, for 20 min.

30% Glycerol Buffer (0.5 L). Glycerol (150 mL), potassium phosphate (pH 7.5, 0.05 M), EDTA (1 mM), benzamidine HCl (100 μM, 8 mg), phenylmethylsulfonyl fluoride (100 μM, 8 mg), and mercaptoethanol (2 mM, 0.07 mL) were diluted to 500 mL with distilled water and stored at 4 °C.

Whole-Cell Feeding Experiments. AM medium (500 mL) in a 2 L Erlenmeyer flask was inoculated with a 20% glycerol spore suspension (50 μL). After incubation in the dark for 48 h at 175 rpm and 28 °C, the mycelial spheres were collected on cheesecloth, washed with RM, and separated into portions of approximately 1.0−1.2 g of wet cells. Each portion was transferred to a 50 mL Erlenmeyer flask containing RM medium (10 mL). All substrates (1 mg/mL) were administered as acetone solutions (20 μL). Incubation continued in the

dark for an additional 48 h at 175 rpm and 28 °C. The medium was then decanted from the mycelial spheres, and the cells were steeped in acetone. Acetone washings were combined with the medium solution, diluted with water, and extracted with chloroform. The extract was dried over Na_2SO_4, concentrated, and examined by TLC (6:3:1 chloroform/ethyl acetate/formic acid). Subsequent HPLC analysis was performed with a Phenomenex reverse-phase column (Prodigy 5μ ODS-(3) 100 Å, 250 × 4.60 mm, 5 micron) eluting at 0.8 mL/min of water: methanol (55:45) and monitoring at 360 nm.

Ground-Cell Feeding Experiments. AM medium (100 mL) in a 500 mL Erlenmeyer flask was inoculated with a 20% glycerol spore suspension (50 μL) of the *A. parasiticus* DIS-1 mutant. After incubation in the dark for 48 h at 175 rpm and 28 °C, the mycelial spheres were collected on cheesecloth and washed with distilled water. Approximately 5 g of wet cells, 5 g of sand, and 3−5 mL of 30% glycerol buffer were ground in a mortar for 5 min at room temperature.

To each incubation tube (Eppendorf, 1.5 mL) were added the sandy ground-cell mixture (1 mL), aqueous SAM (Cl) and NADPH (30 μL of an approximately 1 mg/mL stock solution), and each of the designated aflatoxin precursors (10 μL, in acetone, of an approximately 1 mg/mL stock solution).

Incubations were shaken in the dark for 8 h at 175 rpm and 28 °C. The contents of each Eppendorf tube were loaded onto a QIAprep spin column (2 mL, QIAGEN, Inc.) and centrifuged at 14 000 rpm for 1 min to filter sand and cellular debris. Chloroform (50 μL) was then added to the spin column filter, and the centrifugation step was repeated. The filter was discarded, and the filtrate was transferred to a fresh Eppendorf tube (1.5 mL). Following brief vortexing and centrifugation, the chloroform layer was analyzed by TLC or HPLC (as described above).

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Supporting Information Available: Complete synthetic details of *o*-carboxybenzophenones **32** and **53**. This material is available free of charge via the Internet at http://pubs.acs.org.

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