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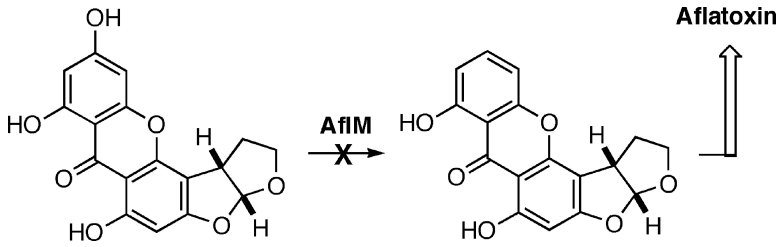


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# Ordering the Reductive and Cytochrome P450 Oxidative Steps in Demethylsterigmatocystin Formation Yields General Insights into the Biosynthesis of Aflatoxin and Related Fungal Metabolites

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***Abstract:*** The biosynthesis of the potent environmental carcinogen aflatoxin B1 involves ca. 15 steps beyond the first polyketide intermediate. Central among these is the rearrangement of the anthraqinone versicolorin A to the xanthone demethylsterigmatocystin. Genetic evidence strongly suggests that two enzymes are required for this process, a cytochrome P450, AflN, and a probable NADPH-dependent oxidoreductase, AflM. Given the overall redox change evident in this skeletal rearrangement, two rounds of oxidation and a reduction necessarily occur. Earlier experiments indicated that reductive deoxygenation of versicolorin A is not the first step. In the present report we consider a mechanistic alternative that AflM-mediated reduction is instead the last of these three reactions prior to formation of the xanthone intermediate. To this end, 9-hydroxydihydrodemethylsterigmatocystin was prepared by total synthesis as was its 9-deoxy analogue, an established aflatoxin precursor. During the final isolation of the "angular" synthetic xanthone targets it was found that acid catalysis promoted their isomerization to thermodynamically favored "linear" xanthones. Whole-cell and ground-cell incubations of the 9-hydroxy- and 9-deoxyxanthones were conducted with a mutant strain of *Aspergillus parasiticus* blocked at the first step of the pathway and examined for their ability to support aflatoxin production. The 9-deoxyxanthone gave dramatically enhanced levels of the mycotoxin. The 9-hydroxyxanthone, on the other hand, afforded no detectable increase in aflatoxins above controls, indicating that reductive deoxygenation at C-9 of a xanthone precursor does not take place in aflatoxin biosynthesis. Constraints imposed by earlier studies and the experiments in this paper serve to eliminate simple and intuitive conversions of versicolorin A to demethylsterigmatocystin and lead inescapably to a more subtle reaction sequence of oxidation−reduction−oxidation. Previous puzzling observations of extensive A-ring hydrogen exchange in the course of the rearrangement of versicolorin A to demethylsterigmatocystin have now been explained by a new mechanism that is consistent with all extant data. We propose that P450-mediated aryl epoxidation (AflN) initially disrupts the aromatic A-ring of versicolorin A. Oxirane opening enables A-ring proton exchange, as does the subsequent AflM-mediated reductive step. A second cycle of P450 oxidation (AflN), this time a Baeyer−Villiger cleavage, enables decarboxylation and the formation of demethylsterigmatocystin. Mechanistic and stereoelectronic principles that underlie this proposal are described and may prove general as illustrated in biogenetic hypotheses for four other fungal anthraquinone ⇒ xanthone transformations.

In the complex biosynthesis of the environmental carcinogen aflatoxin $B_1$ (**4**, AFB1), genetic evidence suggests two enzymes are required for the central skeletal rearrangement in the pathway of the anthraquinone versicolorin A (**2**, VA) to the xanthone demethylsterigmatocystin (**3**, DMST) (Scheme 1).[1] The first of these is AflN, which shows extensive primary sequence similarity to eukaryotic cytochromes P450.[1] The second, AflM, is an apparent NADPH-dependent oxidoreductase with notable similarities to the ActIII ketoreductase of the actinorhodin biosynthetic pathway in *Streptomyces coelicolor*[2,3] and to polyhydroxynaphthalene reductases, which have been demonstrated to carry out the aryl reductions/deoxygenations in melanin biosynthesis.[4] To account for the overall change in oxidation state in this transformation, the simplest explanation would invoke two cycles of P450-mediated oxidation (AflN) and one reductive step (AflM). Whole-cell,[5] ground-cell,[6] and cell-free studies described elsewhere[7] dictate that reduction cannot initiate the

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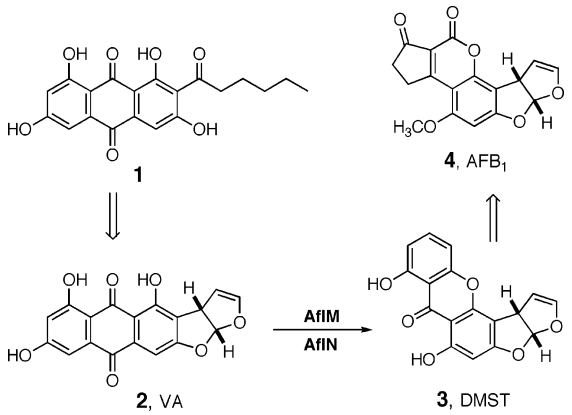
**10.1021/ja0455188 CCC: $30.25** 

***Scheme 1***




overall conversion. Guided by apparent biochemical precedent, we have argued[6] that Baeyer−Villiger oxidative cleavage of the anthraquinone could logically be the first step. We then synthesized the appropriate *o*-carboxybenzophenone substrates and tested their capacity to support aflatoxin biosynthesis. In whole-cell and ground-cell experiments, these potential intermediates failed to afford any detectable mycotoxin despite an acutely sensitive assay.

This was an unexpected outcome based upon our operating assumptions about other anthraquinone ⟹ xanthone transformations in fungal natural product biosynthesis. In this paper we take a closer look at this process constrained by the logic that the sequence of events in aflatoxin biosynthesis is either oxidation−reduction−oxidation or oxidation−oxidation−reduction. We now know the first step cannot be Baeyer−Villiger reaction and, therefore, must involve direct aryl oxidation. As a consequence, the first of these two alternatives is difficult to demonstrate, but the second is accessible to experimental test.

An important early step in the formation of other fungal xanthones is the reduction of emodin (**5**) to chrysophanol (**6**). In a cell-free system this reductive deoxygenation was clearly demonstrated to be NADPH-dependent with the hydrogen at C-6 derived from the reductant (Scheme 2A).[8,9] In contrast, the analogous reduced form of VA (**2**), 6-deoxyversicolorin A[10] (**7**, Scheme 2B) was not incorporated into AFB1 (**4**) under conditions where VA (**2**) was readily converted to the mycotoxin.[5,7] This result demonstrated that the AflM-mediated reduction does not initiate the overall conversion of **2** to **3**. Alternatively, however, reduction of a newly proposed biosynthetic intermediate, 9-hydroxyxanthone **8**, can be proposed to conclude the transformation. The structural similarity of VA (**2**) and emodin (**5**) to 9-hydroxy-DMST (**8**) is highlighted by boxes in Scheme 2. Thus, while AflM does not reduce VA (**2**) to 6-deoxy-VA (**7**), AflN-catalyzed oxidative cleavage and rearrangement can be invoked to give **8**, which can be visualized to undergo analogous reduction (AflM) in the third and final step to DMST (**3**). Circumstantial support for this idea can be found in the structures of related metabolites containing hydroxyl and methoxyl groups at this locus.[11]

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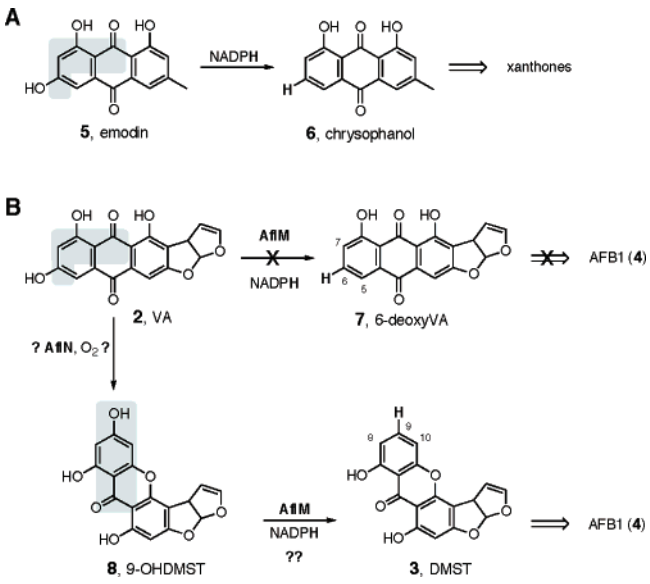
To test the deduction that AflM-mediated reduction is the last of the three-step skeletal rearrangement process, 9-hydroxydemethyldihydrosterigmatocystin (**25**, 9-OHDMDHST) and demethyldihydrosterigmatocystin (**22**, DMDHST), a known intermediate in the aflatoxin biosynthetic pathway,[7] were prepared by total synthesis. The tetrahydrobisfuran products were prepared for their comparative ease of synthesis and more sensitive detection relative to the corresponding dihydrobisfurans **8** and **3**. This was achieved by rapid extension of the methods described in ref 6.

***Scheme 2***




## Results

Addition of the aryllithium species derived from bromobenzofuran **9**[6] to each of the aldehydes **10** and **11** proceeded smoothly (80−90% yields) to the benzhydrol products **12** and **13** (Scheme 3). Pentane was found necessary for the halogen−metal exchange reactions to cleanly occur. The aldehydes, however, were insoluble in hydrocarbon solvents and, therefore, were added as concentrated tetrahydrofuran (THF) solutions. Oxidation to the corresponding benzophenones, though facile, proved low-yielding. Metal-based oxidants produced intractable mixtures likely arising from loss of methoxymethyl protecting groups. However, the desired benzophenones **14** and **15** were reliably generated, although only in moderate yields (50−60%), with either DDQ or the Dess−Martin periodinane.[12,13] The boron trichloride-mediated cleavage of each methoxymethyl ether in $CH_2Cl_2$, and the subsequent O-benzylation in *N*,*N*-dimethylformamide (DMF) with benzyl bromide and NaH proceeded without incident. A further round of methoxymethyl deprotection and O-benzylation produced benzyl-protected benzophenones **16** and **17** in good overall yields (50−60%, four steps) from benzophenones **14** and **15**. Fluoride-mediated cleavage of both silyl ethers was quenched with 3 M HCl, which cleanly furnished both the 15-deoxybenzophenone **18** and the 15-benzyloxybenzophenone **19** in excellent yield (86%).

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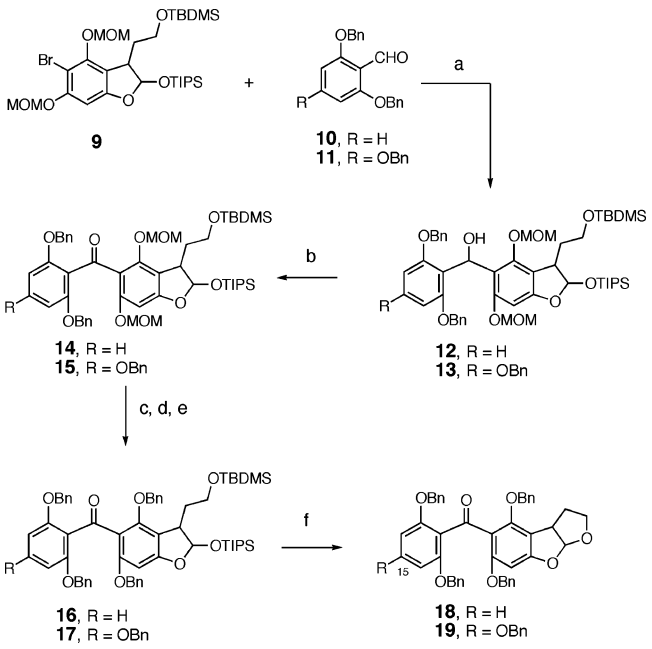
***Scheme 3*** [a]




[a] Reagents and conditions: (a) **9**, pentane, THF, −40 °C, *n*-BuLi, **10**, 92%. (b) Dess−Martin periodinane, $CH_2Cl_2$, 0 °C, 50%. (c) $BCl_3$, $CH_2Cl_2$, 0 → 25 °C. (d) NaH, BnBr, DMF, 0 → 25 °C, 74% over two steps. (e) repeat steps c and d, 78%. (f) Tetrabutylammonium fluoride (TBAF), THF, 0 → 60 °C, then HCl (3 M), 0 → 25 °C, 86%.

The 15-deoxybenzyl-protected precursor **18** was first subjected to hydrogenolysis with catalytic Pd black in EtOAc. As expected, a tetraphenolic benzophenone was evident upon $^1$H NMR analysis as were the "linear" and "angular" xanthone products (**20**:**21**:**22** in 10:3:1 ratio, respectively). Attempted recrystallization of the crude mixture from acetone and water failed to separate the isomeric components. Therefore, silica gel chromatography was employed. As anticipated, the major product tetraphenolic benzophenone **20** dehydrated upon exposure to silica gel.[14,15] Surprisingly, however, passage of the crude mixture through a column of silica gel yielded just the linear xanthone **21** in pure form (Figure 1C). It became apparent that the angular isomer **22** was gradually converting to the linear **21** upon exposure to silica gel. This meant that each attempt to separate the xanthone isomers resulted in further conversion of the angular to the linear and that the nonnatural linear xanthone was easily obtained in pure form. The desired angular isomer, dihydro-DMST (**22**), was only recovered in crude form and trace quantities (<0.5 mg) following preparative thin-layer chromatography (Figure 1A). Even this tiny amount of dihydro-DMST, however, isomerized over several days to the linear **21**, likely resulting from traces of silica gel contamination (Figure 1B). It became obvious that we would be compelled to incubate the angular dihydro-DMST xanthone **22** as a mixture with its linear isomer immediately following hydrogenolysis of **18**. The behavior of the angular and linear dihydro-DMST isomers is illustrated in Scheme 4.

Unambiguous characterization of the angular and linear forms of dihydro-DMST was achieved by correlation with a common product of known structure. Therefore, the linear, thermodynamically favored synthetic product **21** was O-methylated to afford **26** (Figure 2). Correspondingly, sterigmatocystin (**30**, Scheme 5) was hydrogenated to reduce the dihydrobisfuran double bond to the tetrahydrobisfuran ring system and O-methylated to give the angular *O*-methyldihydrosterigmatocystin (**27**, dihydro-OMST) isomer. $^1$H NMR spectra of these two

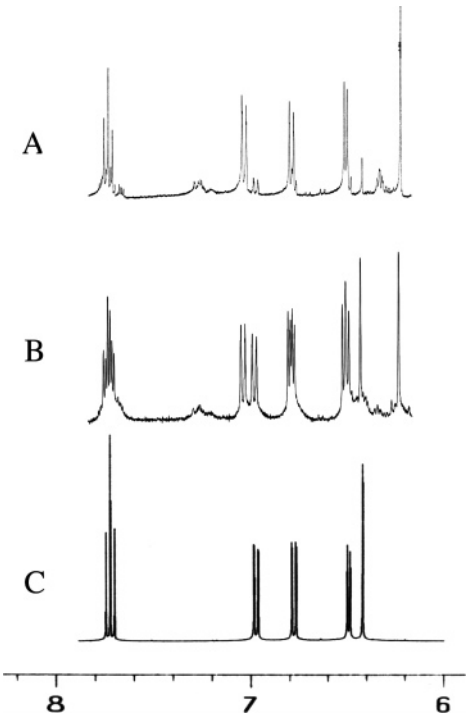
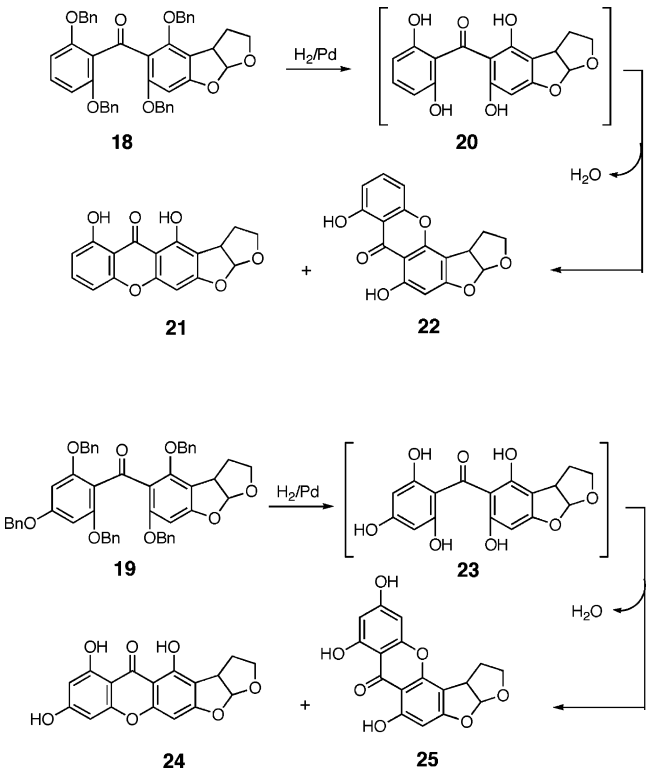



***Figure 1.*** Downfield region, $^1$H NMR spectra of (A) principally the "angular" xanthone **22** immediately after hydrogenolysis and isolation by preparative thin-layer chromatography; (B) same sample after 2 weeks, showing increased proportion of the "linear" isomer **21**; and (C) pure "linear" isomer **21**.

***Scheme 4***

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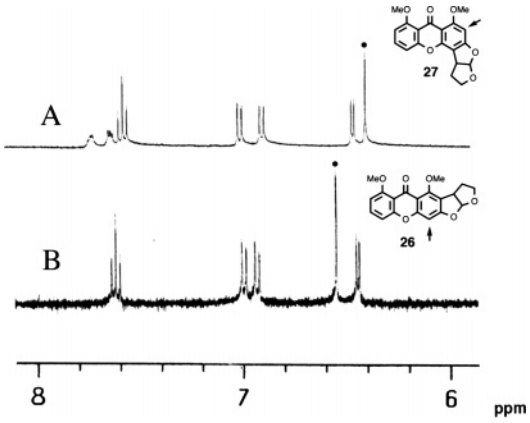
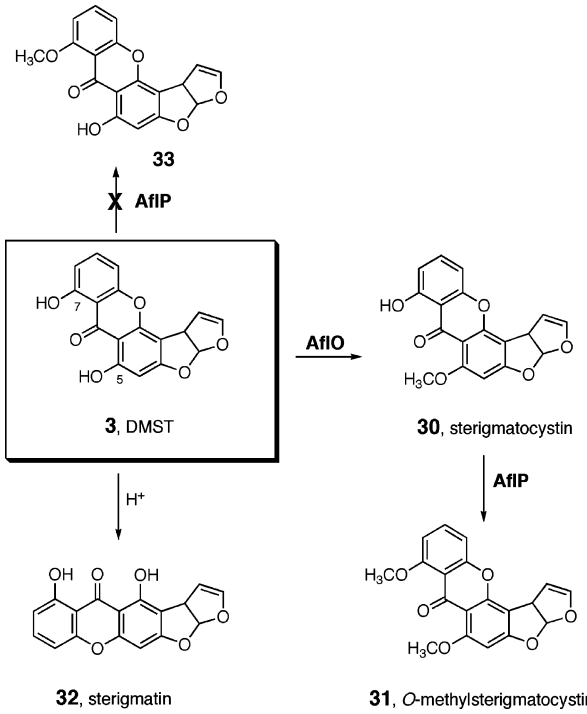



***Figure 2.*** Downfield region, $^1$H NMR spectra of (B) **26** and (A) **27**, the di-*O*-methyl derivatives of **21** and **22**, respectively. The C-ring aryl hydrogen (arrow) gives rise to an especially diagnostic singlet in the $^1$H NMR spectrum (●).

***Scheme 5***




products are shown in Figure 2 where the isolated C-ring hydrogen (●) can be seen to be especially diagnostic, appearing as a singlet nearly 0.2 ppm higher field in the angular isomer. An analogous chemical shift pattern can be seen in Figure 1 as well.

The behavior of the 15-hydroxybenzophenone **19** was similar to that of its 15-deoxy analogue after removal of the benzyl protecting groups (Scheme 4). Following hydrogenation, $^1$H NMR analysis of the crude product mixture revealed that two xanthones (ca. 8:1 ratio) were obtained while the pentahydroxy benzophenone **23** was *not* at all evident. The predominant product, **24**, was assumed to be linear by analogy with the deoxyxanthone series; the C-ring $^1$H NMR resonance (6.21 ppm) of angular **25** was nearly 0.2 ppm upfield of the corresponding peak in linear **24**. Attempted recrystallizations again failed to purify either xanthone isomer, and silica gel chromatography as before enabled the isolation of just the linear **24** in pure form. As a consequence, the angular 9-OH-dihydro-DMST (**25**), like

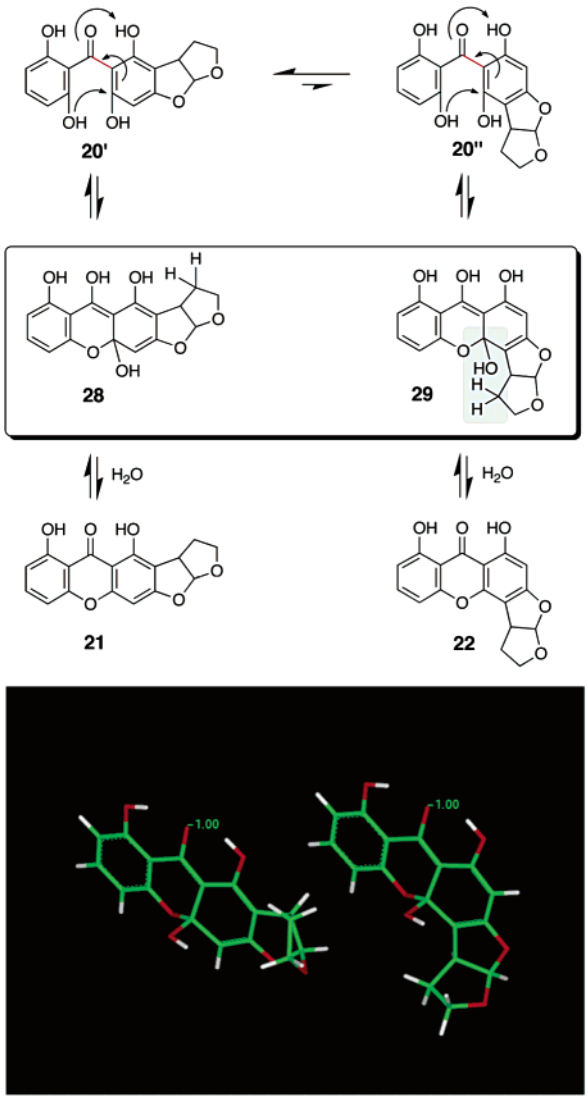



***Figure 3.*** Mechanism of isomerization of "angular" to "linear" xanthones **21** and **22** (top) and molecular modeling of proposed hemiacetal intermediates **28** (inset, left) and **29** (inset, right). Steric interactions indicated in **29** (gray box) are suggested to favor the "linear" form at equilibrium.

dihydro-DMST (**22**), would have to be used in biotransformation experiments as a mixture with its linear isomer.

The strong preference for the linear forms of xanthones **21** and **24** was surprising to us and deserves further comment. The tetrahydroxybenzophenone **20** is depicted in Figure 3 in its two possible rotamers prior to intramolecular addition and dehydration to the linear **21** and angular **22** dihydro-DMST isomers. Although it would seem there is only weak preference for the linear form over the angular, closure must proceed by way of hemiacetal intermediates such as **28** and **29**. While not obvious in flat projection, molecular modeling of these intermediates reveals in the angular isomer a steric interaction between the hemiacetal hydroxyl and one of the C-3′ methylene hydrogens (Figure 3, inset right) that is absent in the linear form (Figure 3, inset left). Such hindrance in the former may bias xanthone formation to the linear isomer.

Before proceeding to discussion of the biochemical experiments carried out with xanthones **21**/**22** and **24**/**25**, the unanticipated lability of the angular form of dihydro-DMST (**22**) on exposure to silica gel or mineral acids revealed an unappreciated element of "chemical logic" to the aflatoxin biosynthetic

pathway. DMST (**3**) and its tetrahydrobisfuran congener **22** undergo two separate rounds of O-methylation in vivo, which must occur prior to the final oxidative transformation to aflatoxin.[16] Each round is catalyzed by rigorously substrate-specific transferases with the capacity for neither cross-methylation of the C-5 or C-7 phenols nor methylation out of order (Scheme 5). The C-5 phenol is methylated first by AflO[17−19] and C-7 second by AflP.[20−22] The logic of this order, and rationale for O-methylation at C-5 particularly, is now apparent. The angular xanthones **3** and **22** formed biochemically are susceptible to acid-catalyzed isomerization to the thermodynamically favored linear forms, which would derail the aflatoxin pathway. The O-methylation to form sterigmatocystin (**30**) prevents this from occurring. As an aside, we suspect that the linear xanthone sterigmatin (**32**), isolated from the sterigmatocystin producer *Aspergillus versicolor*, may well be an artifact of its isolation by silica gel chromatography.[23−25]

Realizing now that the angular xanthone specifically formed in the biosynthetic reaction is thermodynamically disfavored gave added dimension and further impetus to find a mechanistic explanation for the anthraquinone ⟹ xanthone skeletal rearrangement in aflatoxin formation.

**Incorporation Experiments.** Whole-cell DIS1 fermentation studies and the subsequent thin-layer and high-performance liquid chromatographic (TLC and HPLC) analyses were carried out as described.[6] DIS1 is a deletion mutant of the aflatoxin producer *Aspergillus parasiticus* defective in the first biosynthetic step to norsolorinic acid (**1**, Scheme 1) and, thus, has very low background amounts of aflatoxins.[26] As noted above, the intended fermentation experiments were complicated by our inability to isolate either of the intended angular xanthones, **22** or **25**, in pure form. This was so owing to the facile isomerization of the desired angular xanthone to the linear, which could be readily isolated as a homogeneous isomer. Therefore, linear xanthones **21** and **24** were each incubated in pure form. The results of these experiments were then compared to parallel incubations containing mixtures of the linear and angular xanthones, each administered immediately following hydrogenolysis to maximize the proportion of the angular isomer. Any differences in the results of these two pairs of incubations were specifically attributed to the presence of either 9-hydroxyldihydro-DMST (**25**) or dihydro-DMST (**22**). As the results of the whole-cell and ground-cell experiments were nearly identical, only the whole-cell data will be presented. As expected, neither of the linear isomers **21** or **24** was incorporated (data not shown), although the deoxyxanthone **21** gave a trace of aflatoxin during the much longer whole-cell study. We regard this observation as an artifact that could owe to thermodynamically uphill equilibration of **21** with very small amounts of the angular isomer **22** during the time of the experiment. Fermentations were next conducted with mixtures of the linear and angular xanthones (**21** with **22** and **24** with **25,** characterized by $^1$H NMR spectroscopy immediately prior to the incubations). These experiments resulted in great increases of AFB2 and AFG2 from the deoxy series, **21** with **22**, attributable to the incorporation of dihydro-DMST (**22**; Figure 4, lane II, and Figure 5, chromatogram II). Significantly, no such increases were measured from the 9-hydroxyl series, **24** with **25**, establishing no incorporation of angular 9-hydroxyldihydro-DMST [**25**; Figure 4, lane III (identical to control lane I), and Figure 5, chromatogram III]. The overwhelming incorporation of dihydro-DMST, an established pathway intermediate, served as a positive control and gave us confidence in the nonincorporation of angular 9-hydroxyldihydro-DMST (**25**). We judged that **25** is not a precursor of DMST (**22**) or the aflatoxins.

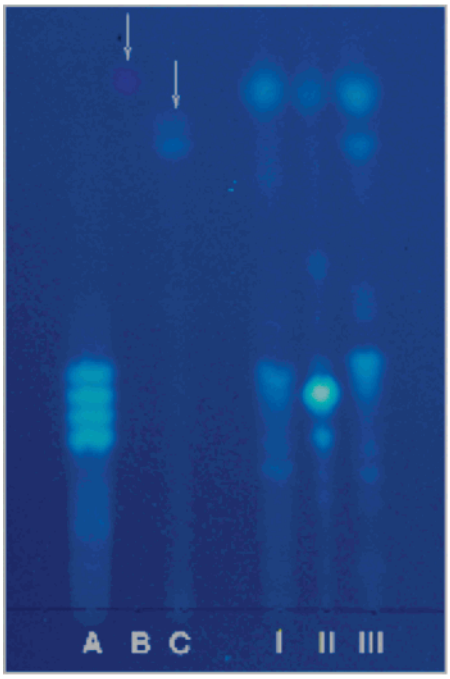



***Figure 4.*** Thin-layer chromatogram of DIS-1 whole-cell incubations developed in 60% $CHCl_3$, 30% EtOAc, and 10% $HCO_2H$, photographed under long-wave UV light. Lanes A−C are standards: (A) (top to bottom) AFB1 (**4**), AFB2, AFG1, andAFG2; (B) linear and angular 9-deoxyxanthones **21** (upper) and **22** (lower); and (C) linear and angular 9-hydroxyxanthones **24** (upper) and **25** (lower). Lanes I−III: (I) chloroform extract of DIS-1 control; (II) DIS-1 with **21**/**22** (**3**); and (III) DIS-1 with **24**/**25**.

## Discussion

An apparent cytochrome P450, AflN, and a probable NADPH-dependent reductase, AflM, are implicated by gene "knockout" experiments to be responsible for the rearrangement of the anthraquinone versicolorin A (**2**) to demethylsterigmatocystin (**3**). To account for both oxidative cleavage and reductive loss of the C-6 hydroxyl in this overall process, the simplest mechanism must invoke two rounds of oxidation and a single reductive step. Whole-cell[5] and cell-free[7] experiments with 6-deoxyversicolorin A (**7**, Scheme 6) gave no incorporation into aflatoxin under conditions where VA (**2**) itself was readily

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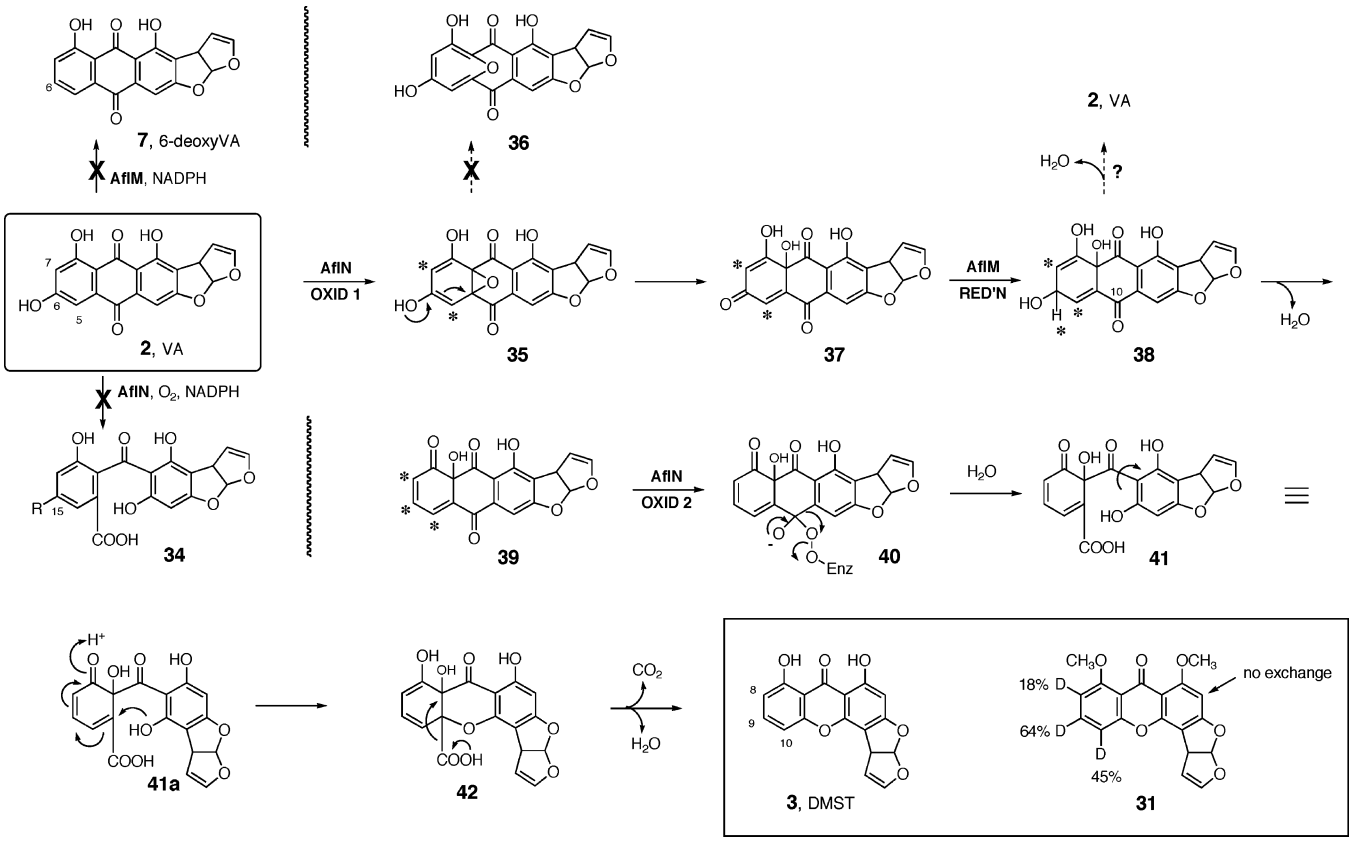
***Scheme 6***




incorporated. On the basis of these findings it is highly unlikely reduction (AflM) is the first step. As we have described,[6] possible biochemical precedent for initial Baeyer−Villiger cleavage in the biosynthesis of other fungal xanthones was considered and the 15-hydroxyl- (**34**, R = OH) and 15-deoxy-*o*-carboxybenzophenone (**34**, R = H) were prepared as their tetrahydrobisfurans and tested for their ability to support aflatoxin production. Contrary to expectation, neither of these proved an intermediate in the pathway under both whole-cell and ground-cell conditions where control experiments with known precursors were unambiguously successful.[6] These observations cast doubt on Baeyer−Villiger cleavage as the first step but constrain the possible order of steps to oxidation−reduction−oxidation or oxidation−oxidation−reduction. Here we treat the latter of these possibilities, that AflM-catalyzed reduction is the final step in this complex transformation. 9-Hydroxydihydro-DMST (**25**) and dihydro-DMST (**22**), a known intermediate in AFB2 biosynthesis, were prepared, and reductive deoxygenation of the former to the latter could not be experimentally demonstrated.

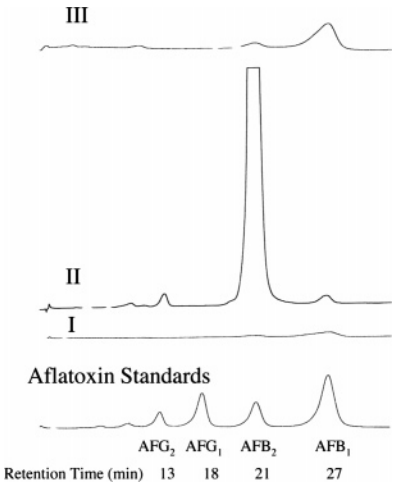



***Figure 5.*** HPLC chromatograms corresponding to lanes I−III in Figure 4: (I) chloroform extract of DIS-1 control; (II) DIS-1 with **21**/**22**; (III) DIS-1 with **24**/**25**.

While negative findings from incorporation experiments must be interpreted with caution, the usual caveats of permeability and distribution are unlikely to be of concern here as active ground-cell systems were available, control experiments with known precursors were invariably positive, and assay of the highly fluorescent aflatoxins is acutely sensitive. We are assured that the nonincorporation of potential intermediates in these experiments is meaningful, if disappointing.

Having been able to demonstrate neither reduction−oxidation−oxidation nor oxidation−oxidation−reduction as the order of reaction steps in the deep-seated rearrangement of VA (**2**) to DMST (**3**), we are forced to consider the remaining possibility of oxidation−reduction−oxidation, a reaction course far more difficult to test experimentally. An earlier, mystifying observation, however, becomes foveal to the present discussion. Despite their multiple hydroxyl groups, anthraquinones such as VA (**2**) are comparatively electron-deficient. For example, even in deuterated acidic media, aryl hydrogen exchange is slow.[27] Nonetheless, OMST (**31**, Scheme 6) isolated from fermentation in a $D_2O$-containing medium exhibited high extents of deuterium exchange in the A-ring but not the C-ring. The extensive

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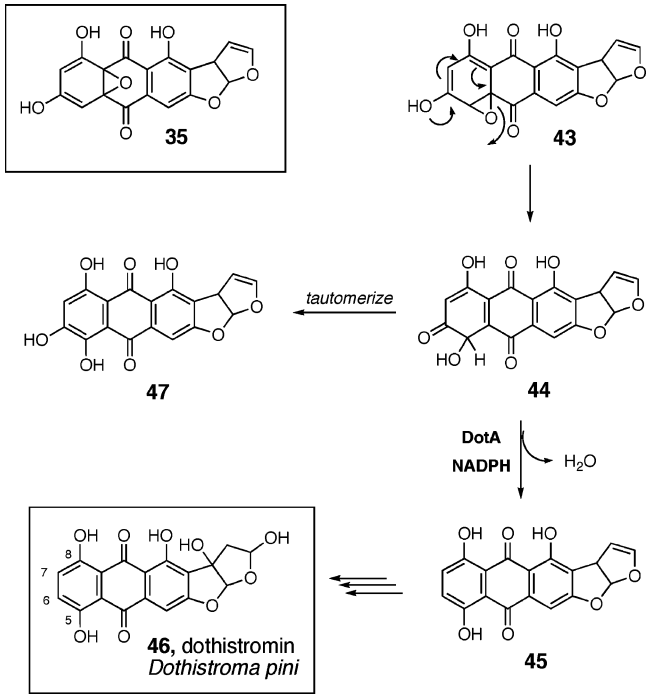
exchange at C-8 and C-10 suggest loss of aromaticity in the A-ring and transient keto/enol tautomerization. Remarkably, solvent exchange is most favorable at C-9 (64% ± 3%), originally the C-6 hydroxylated carbon in VA. Exchange at this site must occur *after* reduction (AflM). In an important comparison, cell-free experiments with NADPD gave only 7% ± 1% $d_1$-species (OMST), presumably through deuteride addition at this carbon followed by the same high extent of solvent exchange.[7] We propose the mechanism set out in Scheme 6 to account for the new and existing[16,28] experimental data for aflatoxin biosynthesis, including the incorporation of molecular oxygen (from $^{18}O_2$).[29] We reconsider the biogenesis of other fungal xanthones in light of this proposal.

Oxidation of VA (**2**) must logically be the first step, but not Baeyer−Villiger cleavage. We propose epoxidation uniquely across the ring A/B fusion to afford **35**. One is reminded of frenolicin,[30,31] the tetracyclines, and tetracenomycin C,[32,33] for example, where oxidation occurs across a fused ring in a polycyclic aromatic precursor. The possible electrocyclic opening of the benzene oxide to the oxepin **36** grossly violates Bredt's rule and is prohibited energetically. It does not play a role.[34] Exchange with the medium can be visualized to occur at C-5 and C-7 (*), and epoxide opening can be invoked to give the relatively stable dienone **37**. Reduction now mediated by AflM would install hydrogen (presumably deuterium from NADPD) at this site, but it would be vinylogous to the anthraquinone C-10 carbonyl and exchangeable with the medium. Dehydration of **38** can be seen to occur in the desired sense by $\beta$-elimination of the C-6 hydroxyl to give **39**. Alternatively, loss of the bridgehead hydroxyl trivially regenerates VA (**2**), which can re-enter the pathway. A second round of AflN-catalyzed oxidation, this time Baeyer−Villiger cleavage, would afford **41** following the migration of an electron-rich C-ring of the proposed adduct **40** and hydrolysis of the resulting lactone. Rotation of **41** in the active site to the presumably less stable conformation, followed by phenol addition to the electrophilic A-ring and subsequent decarboxylation and dehydration, gives DMST (**3**). Upon rearomatization of the A-ring, the bridgehead alcohol is necessarily lost. This feature of the mechanism might account in part for why no intermediates or hydroxylated shunt metabolites have been detected from this transformation (compare below).

Alternatively, a small change in geometry in the active site could result in epoxidation along the A-ring edge of VA (**2**) to yield **43** (Scheme 7). Both VA and the 5,8-dihydroxy anthraquinone **45** were isolated from dothistromin (**46**) producing cultures of *Dothistroma pini*.[35] By analogy to the process outlined in Scheme 6, we propose opening of the regioisomeric VA edge epoxide **43** to give the secondary alcohol **44**, whose reactivity would be tempered by intramolecular hydrogen

***Scheme 7***




bonding to the adjacent anthraquinone carbonyl. NADPH-dependent reduction and dehydration of **44** yield the 5,8-dihydroxyanthraquinone **45**. The tautomeric trihydroxy A-ring product **47** has not been observed. The gene responsible for this reduction, *dotA*, encodes a predicted protein that is 80% identical to the AflM ketoreductase of *A. parasiticus*. However, no such homologue of the *aflN* cytochrome P450 has yet been identified in *D. pini*, and none of the anthraquinones VA (**2**), **45**, or dothistromin (**46**) undergoes the analogous AflN-catalyzed oxidation to a xanthone product. Upon cross-incubation, dothistromin was not further converted by aflatoxin-producing cultures of *A. parasiticus*.[36] For these reasons, we propose that the A-ring of VA is differentially oxidized in *Dothistroma* and *Aspergillus* species. The presence of 5,8-dihydroxy anthraqunones in *D. pini* is suggestive of A-ring edge epoxide **43** and secondary alcohol **44** intermediates. Conversely, no such 5,8-dihydroxylated **45** or 5,6,8-trihydroxylated species **47** have ever been isolated from sterigmatocystin- or aflatoxin-producing strains of *Aspergillus*. Therefore, it seems most likely that the initial oxidation of VA (**2**) to DMS (**3**) occurs across the A/B ring junction, resulting in the proposed epoxide **35**, followed by the bridgehead alcohol **37** (Scheme 6). In our view, hydroxylation at the bridgehead position best accounts for the absence of any known intermediates between VA and DMS.

Perhaps the most significant deduction from our study of demethylsterigmatocystin (**3**) biosynthesis is that A-ring epoxidation primes the anthraquinone versicolorin for subsequent Baeyer−Villiger-type rearrangement. Otherwise, the Baeyer−Villiger oxidation of an unactivated′ anthraquinone may not be possible. The difficulty of this rearrangement is clear from determined efforts by Franck and co-workers some time ago to model this reaction chemically.[37,38] Synthetically and biosyn-

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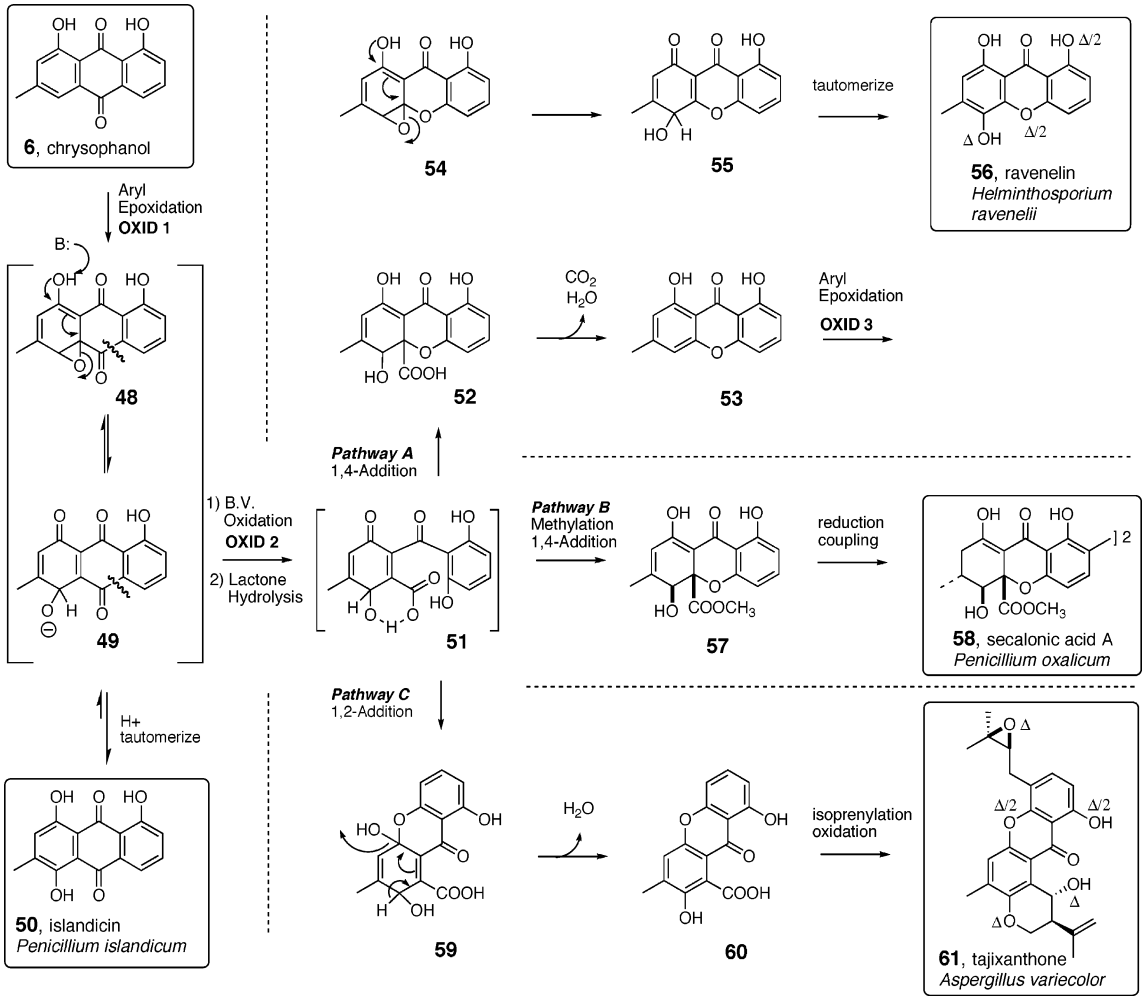
***Scheme 8***




thetically, the addition of an activated oxygen species to the carbonyl of a rigid, planar anthraquinone would generate a tetrahedral intermediate/transition state with such poor orbital alignment that aryl migration to oxygen is difficult, if not prohibitive. Alternatively, an initial A-ring epoxidation of the anthraquinone disrupts the planar nature of the substrate and introduces strain into the ring system that may be critical for Baeyer−Villiger rearrangement to occur.

At the outset of these studies, as described elsewhere,[6] we discussed the precedent of related biosynthetic pathways (ravenelin **56**, secalonic acid **58**, and geodin **70**). In accord with current thinking, we invoked Baeyer−Villiger rearrangements of chrysophanol-derived (**6**) anthraquinone precursors to these products. In view of the mechanism advanced in Scheme 6 for the conversion of VA (**2**) to DMST (**3**), and the experiments that support it, we have reevaluated these biogenetic proposals and suspect that these related pathways could likewise require epoxidative activation to prime precursor anthraquinones for subsequent Baeyer−Villiger rearrangement (Scheme 8).

Chrysophanol (**6**) is a common biosynthetic precursor to ravenelin (**56**), secalonic acid (**58**), and tajixanthone (**61**).[39,40] Whether islandicin[41,42] is a precursor of **56**, **58**, and **61** or a shunt product is not known. The only published evidence to bear on this question comes from Franck and co-workers, where pairwise competition incorporation experiments were carried out with radiolabeled emodin (**5**), chrysophanol (**6**), and islandicin (**50**) in secalonic acid-producing cultures of *Penicillium oxalicum*. Chrysophanol was found to be incorporated about 3.5 times more readily than emodin into secalonic acid, but islandicin, presumably the next most advanced intermediate, was utilized more than 3-fold less efficiently than emodin, or approximately 12 times less so than chrysophanol.[40] Therefore, while a very low incorporation of radiolabel was observed in the final product secalonic acid, the results were interpreted to mean that islandicin (**50**) was principally a shunt metabolite.

In view of Franck's results and the observed co-occurrence of VA (**2**), trihydroxyanthraquinone **45**, and dothistromin (**46**),[36] a unified proposal is put forward to interrelate chrysopanol (**6**) with ravenelin (**56**), secalonic acid (**58**), and tajixanthone (**61**) in Scheme 8. To initiate a common biogenetic path, epoxidation is invoked across an A-ring edge to form **48** and/or its open form **49**. These intermediates serve to disrupt both the aroma-

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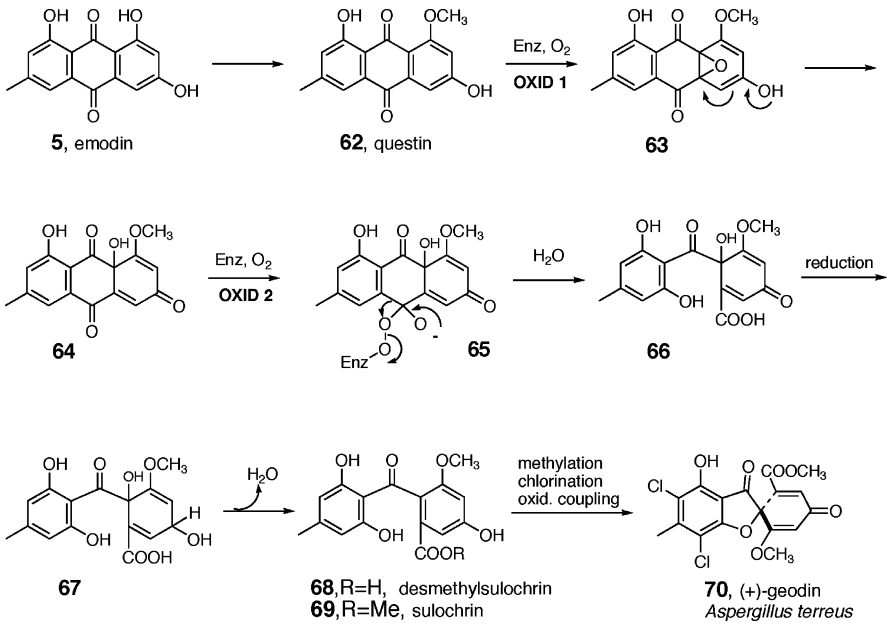
***Scheme 9***




ticity of ring A and the rigidity of the anthraquinone core. While hydrogen bonding of the newly formed *peri*-hydroxyl group in **49** may stabilize this labile species, deprotonation and tautomerization would leak this intermediate away to islandicin formation. Likewise, the inefficient uptake of islandicin into the P450 active site could account for Franck's low-level incorporation of **50** into secalonic acid. A rapid second oxidation by the same cytochrome P450 is then proposed to afford Baeyer−Villiger cleavage of **49** to *o*-carboxybenzophenone **51**, whose diverse chemical potential is illustrated in the three pathways A, B, and C in Scheme 8. We note that these biogenetic hypotheses are fully consistent with formation of a symmetrical B-ring in **51** required from [1,2-$^{13}C_2$]acetate incorporation experiments[43−48] and $^{18}O_2$ labeling (Δ) studies.[49,50]

Path A to ravenelin (**56**) envisions intramolecular addition of a B-ring phenol to the A-ring dienone of **51**, followed by a decarboxylative dehydration sequence to **53**. A further A-ring edge epoxidation and rearomatization gives ravenelin. Path B to secalonic acid (**58**) similarly involves addition of a B-ring phenol, but esterification prevents decarboxylative elimination to yield **57**. Rather than 1,4-addition to the dienone **51**, path C to tajixanthone (**61**) depicts the intramolecular 1,2-addition of a B-ring phenol directly to the A-ring carbonyl of **51**. Dehydration of the resulting **59** gives xanthone **60**, which through prenylation, aldehyde formation, and oxidation of **60** can be seen to yield tajixanthone.

Epoxidative activation is the central unifying theme of biogenetic proposals in Schemes 6−8. The conversion of questin (**62**) to desmethylsulochrin (**68**) during geodin (**70**) biosynthesis by what appears to be a straightforward Baeyer−Villiger-like oxidation (Scheme 9) would seem to be an exception to this view of anthraquinone cleavage.[51] In contrast to the examples considered in Scheme 8, emodin (**5**) is methylated to questin (**62**) rather than reduced to chrysophanol (**6**), and if Baeyer−Villiger cleavage were indeed the next step to **68**, it would have to overcome the intrinsically poor reactivity of an anthraquinone substrate and take place by migration of the *less* electron-rich aryl ring. While the occurrence of one of these events might be waved aside, that both must take place is disturbing on chemical grounds. If we reconsider this transformation in light of the proposal above and as set out in Scheme 9, however, these objections can be overcome. Emodin (**5**) is O-methylated to questin (**62**),[52] which we propose is oxidized to the epoxide **63** and, in turn, opens to the dienone **64**. Baeyer−Villiger cleavage is now invoked to **66** but, unlike the cases in Scheme 8, does not undergo xanthone formation. Reduction to **67** cannot undergo dehydration as suggested for **38** ⇒ **39** in Scheme 6 because a methoxyl group in **67** resides in the place of the hydroxyl in **38**. Dehydration in the only other possible sense rearomatizes this ring to form demethylsulochrin (**68**). This intermediate is known to be O-methylated, chlorinated, and oxidatively coupled by a characterized blue-copper enzyme to (+)-geodin (**70**).[53−55]

Variations in the possible order and the precise detail of the mechanistic events proposed in Schemes 6−9 are legion, but the essential ideas of our proposals are relatively few and generally applicable. Nucleophilic attack, or epoxidation, by activated oxygen species on a fundamentally electron-deficient anthraquinone is advanced to initiate ring cleavage. Elevation

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of the oxidation state of one of the anthraquinone rings serves to impart strain to an otherwise stable aromatic system and primes the resulting intermediate for Baeyer−Villiger oxidation, causing the migration to oxygen of the relatively electron-rich ring. The temporary loss of aromaticity in the nonmigrating ring should allow extensive deuterium exchange to occur in that ring prior to xanthone formation, as is known to occur during sterigmatocystin formation.[7] Unfortunately, most of the proposed intermediates of Schemes 6−9 are difficult to synthesize and stably administer to preparations of the biosynthetic enzymes, but we are led to these proposals by the stepwise elimination of testable hypotheses in the conversion of versicolorin to demethylsterigmatocystin and by what is known about the biosyntheses of other fungal metabolites. The validity of these ideas can be further examined by attempted incorporations of islandicin (**50**) into tajixanthone (**61**) and ravenelin (**56**), by the incorporation of xanthone **53** into ravenelin, from the observation of possible deuterium exchange, and from biomimetic model reactions.

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**Supporting Information Available:** Full synthetic details of xanthones **21**/**22** and **24**/**25**, whole-cell incorporation, and ground-cell incubation conditions (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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